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# THE ROLE OF CtIP (RBBP8) IN TAMOXIFEN RESISTANCE AND HUMAN BREAST CANCER

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# THE ROLE OF CtIP (RBBP8) IN TAMOXIFEN RESISTANCE AND HUMAN BREAST CANCER

**by**

**Minhao Wu, M.D., M.S.**

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## Dedication

To my wife Lanlan and my daughter Mikayla,

and my parents Jintai Bo and Duojia Wu

## Acknowledgements

I would like to take this opportunity to first express my deep gratitude to my mentor, Dr. C. Marcelo Aldaz, for his guidance, advice, encouragement and support during my graduate studies in his laboratory. I want to thank my supervisory committee, Drs. John Richburg (also for serving as my co-supervisor and committee chair), Shawn Bratton, Carla Van Den Berg and Bob Sanders, for their help and suggestions. I sincerely thank Dr. David Ramos Soler, Dr. Martin C. Abba, Dr. Maria I. Nunez, and Dr. John H. Ludes-Meyers for their encouragement and input on my research. Many thanks to our collaborators, Dr. Richard Baer and Dr. Christos Hatzis for their great help on this work. Special thank to my previous laboratory colleague and good friend, Dr. Yuhui Hu, for her long-term encouragement, friendship, and support from the first day I came to the laboratory. I also thank my laboratory and departmental colleagues and friends, in particular Hyunsuk Kil, Jeff A. Drake, Dr. Hongxia Sun, Kathleen A. Hawkins, and Dr. Elangovan Thavathiru, for their friendship and support. I also appreciate the funding support from Susan G. Komen Breast Cancer Foundation and DoD Breast Cancer Research Program. Finally, I want to thank my wife, Lanlan Gu, for her love and unconditional support and my parents for their love and encouragement.

# THE ROLE OF CtIP (RBBP8) IN TAMOXIFEN RESISTANCE AND HUMAN BREAST CANCER

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Acquired resistance to the antiestrogen tamoxifen constitutes a major clinical challenge in breast cancer therapy. However, the mechanisms involved are still poorly understood. Therefore, the overall goal and focus of this dissertation are to better understand the phenomenon of tamoxifen resistance. In preliminary studies, we generated two independently derived isogenic MCF-7 breast cancer cell line variants (TAMR1 and TAMR2) that were resistant to the inhibitory growth effects of tamoxifen. Using serial analysis of gene expression (SAGE), we identified CtIP (CtBP-interacting protein), a BRCA1 (Breast cancer 1)- and CtBP (C-terminal binding protein)-interacting protein, as one of the most significantly down-regulated transcripts in the aforementioned tamoxifen resistant cells. We hypothesized that CtIP silencing constitutes a critical event for the development of tamoxifen resistance in breast cancer. We found that silencing endogenous CtIP in tamoxifen sensitive cells confers tamoxifen resistance and estrogen independence. On the other hand, re-expression of CtIP in tamoxifen resistant cells

restores sensitivity to the inhibitory growth effects of tamoxifen. Importantly, poor clinical response to neo-adjuvant endocrine therapy is associated with CtIP deficiency in primary breast carcinomas. Meta-analysis of seven publicly available gene expression microarray data sets shows that CtIP expression is significantly associated with estrogen receptor (ER), disease free survival and breast cancer metastasis status. Furthermore, we found CtIP protein expression in a majority of ER positive breast cancer cell lines, but none or very little CtIP expression in ER negative lines. These findings indicate that CtIP silencing may be a novel mechanism for the development of tamoxifen resistance in breast cancer, and suggest that CtIP is likely associated with ER function and that CtIP gene and protein expression may be useful biomarkers for breast cancer prognosis and clinical management. Subsequent studies found a BRCA1-CtIP-CtBP complex in tamoxifen sensitive but not resistant cells, whereas BRCA1 is associated with ER in both cell lines. We also observed different patterns of occupancy by BRCA1, CtIP and CtBP on ERE (Estrogen Response Element) region of the pS2 promoter after E2 or tamoxifen treatment. These results support the potential involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance.

# Table of Contents

# List of Tables

# List of Figures

# List of Abbreviations

4-OH-TAM: 4-hydroxytamoxifen

ANG1: Angiopoieting-1

BRCA1: Breast cancer 1, early onset

CtBP: C-terminal binding protein

CtIP: CtBP-interacting protein

CCNA2: Cyclin A2

CCNF: Cyclin F

CDC45L: CDC45 cell division cycle 45-like

E1: Estrone

E2: 17β-Estradiol

E3: Estriol

EBAG9: Estrogen receptor binding site associated, antigen, 9

ER: Estrogen receptor

FAT: FAT tumor suppressor homolog 1

Ikaros: zinc finger protein, subfamily 1A, 1

LMO4: LIM domain only 4

PgR: Progesterone receptor

REA: Prohibitin 2

SAGE: Serial analysis of gene expression

SEM: Standard error mean

SERM: Selective estrogen receptor modulator

TSSC3: Pleckstrin homology-like domain, family A, member 2

ZBRK1: Zinc finger protein 350

# Chapter 1: Introduction

## 1.1. Overview of breast cancer

Breast cancer is the most frequently diagnosed cancer and ranks second among cancer death in women in the United States (American Cancer Society, 2007). It is estimated, by the American Cancer Society, that about 240,510 American women will be diagnosed with breast cancer in 2007, and approximately 40,460 women diagnosed with breast cancer will die of the disease. Figure 1.1. shows the recent incidence and mortality rate trends of breast cancer in the United States. In effort to eradicate this disease, intensive epidemiologic, clinical and genetic studies have been conducted and led to the identification of a number of risk factors associated with breast cancer (Bernstein and Ross, 1993; Mettlin, 1999; Pike et al., 1993). Besides being female and getting older, women with inherited genetic mutations in the BRCA1 and BRCA2 genes, family history of breast cancer, high breast tissue density, biopsy-confirmed hyperplasia, and high-dose radiation are at higher risk of developing breast cancer. Additional factors that are also believed to contribute to increased risk include early onset of menstruation, nulliparity or delayed first childbirth (i.e. never having children or a first pregnancy after age 30), late menopause, long-term estrogen replacement therapy, extended use of oral contraceptives, postmenopausal obesity, and alcohol consumption (Bernstein and Ross, 1993; Mettlin, 1999; Pike et al., 1993). Breast carcinoma arises from the epithelium of the mammary gland, which includes the breast milk-producing lobules and the ducts that carry milk to the nipple. However, it is still not yet clear about the pathway to breast cancer development. There is some evidence from animal models and molecular genetic analysis to indicate that the breast epithelium undergoes a transformation from normal to

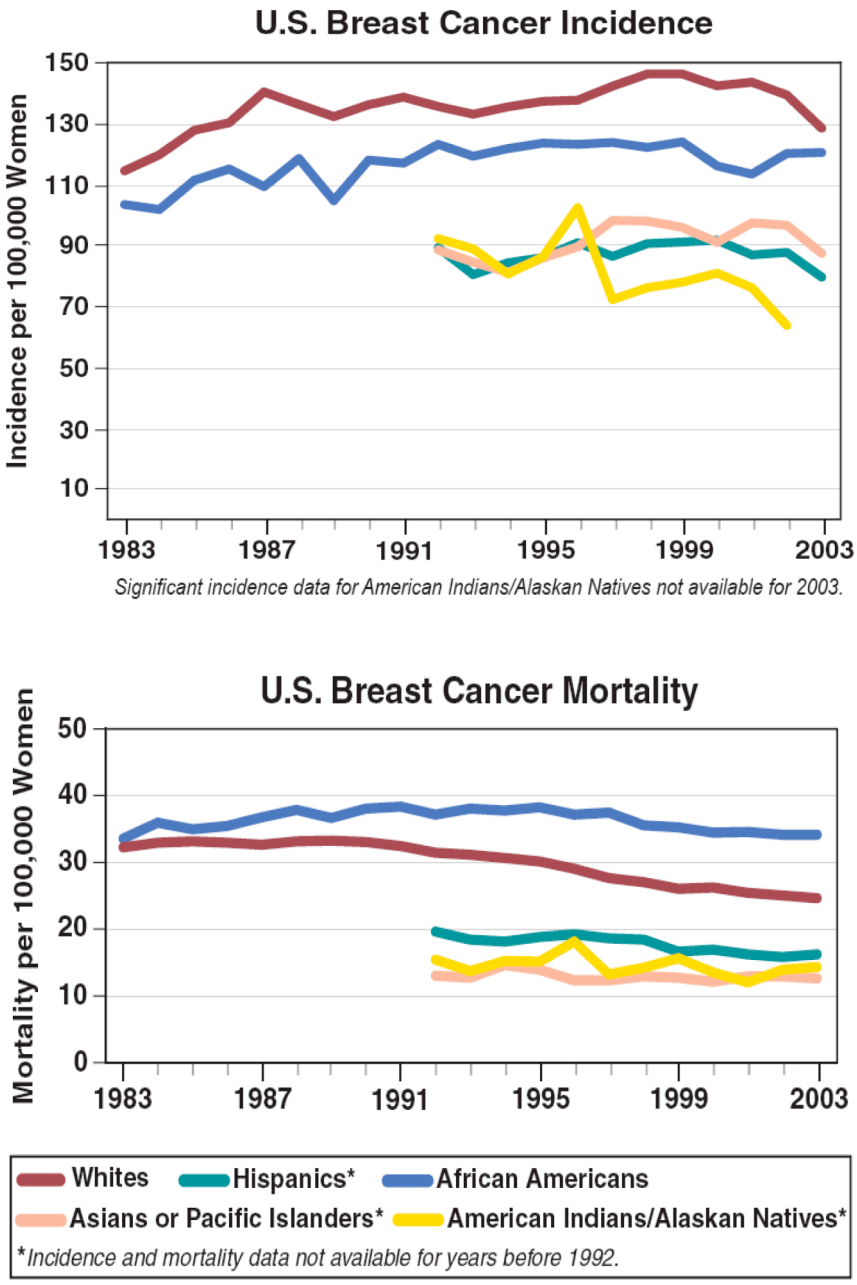



Figure 1.1. U.S. breast cancer incidence and mortality rate trends**.**

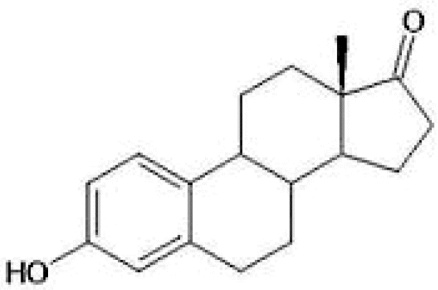
This figure was obtained from the National Cancer Institute (http://planning.cancer.gov/disease/Breast-Snapshot.pdf).

hyperplastic, followed by the appearance of atypia, progressing through a noninvasive preneoplastic phase called ductal carcinoma in situ (DCIS) to invasive carcinoma, in which the basement membrane has been breached (Lakhani, 1999). Histologically, invasive ductal carcinomas (IDCA) are the most frequently observed invasive type, representing about 80 percent of total invasive breast cancer cases, while only 5 to 10 percent of breast cancers are infiltrating lobular carcinomas (ILCA) (van de Vijver, 1993). The treatment and prognosis of breast cancer are influenced in part according to staging categories at the time of diagnosis. Currently, the most commonly used staging system that has designated staging by TNM (primary tumor, regional lymph nodes and distant metastasis) classification is provided by the American Joint Committee on Cancer (AJCC) (Singletary et al., 2002; Woodward et al., 2003). In the clinic, treatment options may include surgery (lumpectomy or mastectomy with removal of some of the axillary lymph nodes), radiation therapy, chemotherapy, endocrine therapy and targeted biological therapy, usually used in combination.

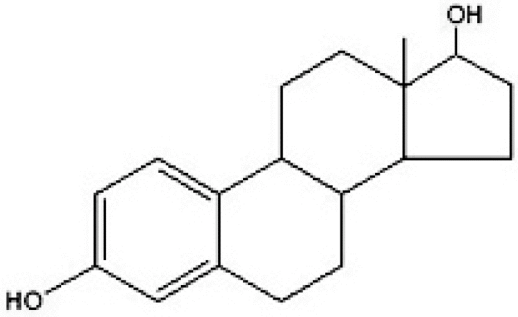
## 1.2. ESTROGEN

### 1.2.1. Biosynthesis, endogenous sources and physiologic functions

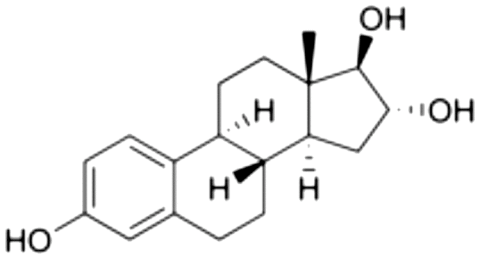
Sex steroids, including the naturally occurring estrogens Estrone (E1), 17β-estradiol (E2), and estriol (E3) (Figure 1.2.), are derivatives of cholesterol. The steroid production process involves many enzymatic steps, most of which use cytochrome P450 heme-containing enzymes (Figure 1.3.). The rate-limiting step in steroidogenesis is the transfer of cholesterol from the cytosol to the inner membrane of the mitochondrion,




**Estrone ($E_1$)**




**17β-Estradiol ($E_2$)**




**Estriol ($E_3$)**

Figure 1.2. Chemical structures of common estrogens.

where the cleavage of the side chain of cholesterol occurs by the cytochrome P450 enzymes. The last step in estrogen synthesis is aromatization that converts androgens to estrogens. This reaction is catalyzed by the P450 aromatase monooxygenase enzyme complex present in the smooth endoplasmic reticulum. The principal sites of aromatase expression in women are the ovarian granulosa cells (premenopausal), the placental syncytiotrophoblast (pregnant), and the adipose and skin fibroblasts (postmenopausal) (Simpson et al., 1994). Aromatase activity has also been detected in muscle (Matsumine et al., 1986), brain (Balthazart and Absil, 1997; Balthazart et al., 1990; Garcia-Segura et al., 1999; Naftolin et al., 1975), bone (Sasano et al., 1997; Shozu and Simpson, 1998) and the testes (Brodie and Inkster, 1993). Another group of enzymes that also plays important roles in steroidogenesis is the family of 17β-hydroxysteroid dehydrogenases (17β-HSDs). In fact, the more potent 17β-estradiol is converted from estrone by type 1 of 17β-HSD (Figure 1.3.). In women, the theca and granulosa cells of the ovaries are the primary sources of estradiol, while estrone and estriol are synthesized primarily in the liver from estradiol. At menopause, estrogen production from ovaries significantly decreases. Therefore, in postmenopausal women, estrogen is primarily formed by aromatization of C19 steroids in peripheral tissues (Grodin et al., 1973). Ovarian function and estrogen synthesis are regulated by the pituitary gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). For instance, genes responsive to FSH control the expression of steroidogenic enzymes (Richards, 1994). In men, the major sources of estrogen are the peripheral tissues, like adipose and skin (MacDonald et al., 1979). It is estimated that only about 15% of the circulating level of estrogen is synthesized in the testes (Hemsell et al., 1974). Estrogen has widespread physiologic functions. In women, estrogen regulates the menstrual cycle, development of secondary sexual characteristics (including normal breast development) and reproduction. In addition, it is involved in the

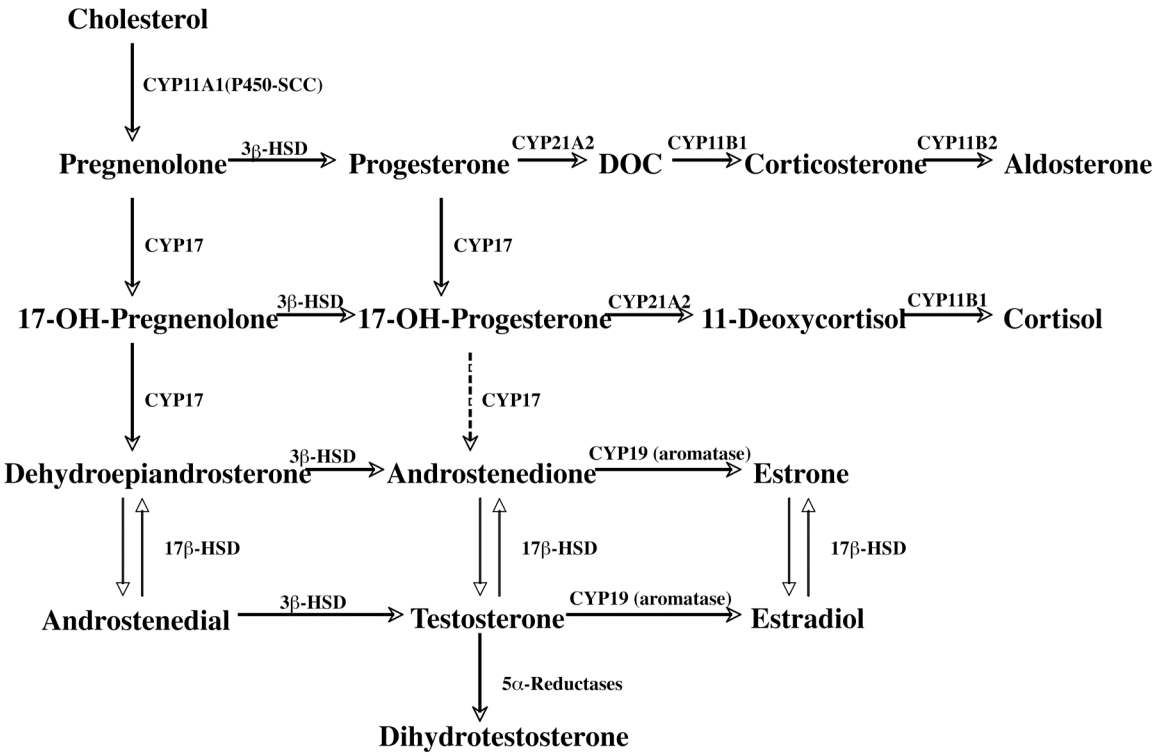



Figure 1.3. Steroid biosynthesis pathways.

modulation of bone density, regulation of lipoprotein synthesis and cholesterol transport (Christiansen et al., 1981; Eriksen et al., 1988; Oursler et al., 1991; Paganini-Hill et al., 1996). Estrogen is also thought to have neuroprotective (Green et al., 1997; Henderson, 1997) and vasoprotective actions (Kim et al., 1999; White et al., 1995). In young adult men, estrogen is essential for fusion of epiphyses and maintenance of bone mass (Carani et al., 1997; Morishima et al., 1995; Smith et al., 1994).

### 1.2.2. Metabolism

Estrogens are metabolized through the catechol pathway. Phase I metabolism is catalyzed by several cytochrome P450 enzymes and yields predominantly 2-hydroxycatechol estrogens (Jefcoate et al., 2000; Liehr, 2000) or 4-hydroxycatechol estrogens (Hayes et al., 1996). Catechol estrogens can further undergo continuous metabolic redox cycling, a process that can yield estrogen quinones and hydroquinones. Phase II detoxication pathways include sulfation, methylation and reaction with glutathione (GST) (Cavalieri et al., 2000). The catechol estrogens, for instance, can be methylated by catechol O-methyltransferase to form methoxylated estrogen metabolites (Gruber and Huber, 2001). The conjugates are excreted into the bile or urine.

Note: Although estrogens occur naturally in several structurally related forms as described above, the predominant intracellular estrogen is17β-estradiol (E2). Therefore, in the following dissertation, the term estrogen refers to17β-estradiol (E2).

### 1.2.3. Estrogen's role in normal breast development and breast cancer

Although mammary development begins during embryogenensis, major breast development in women is not initiated until the onset of puberty. Normal breast

development at puberty and during sexual maturity is stimulated by and dependent on the high levels of estrogen produced by the ovary. In young women, the lobular units of the terminal ducts of the breast tissue are highly responsive to estrogen. Mammary epithelial cells seem to be the main site of estrogen action. Estrogen stimulates the growth and differentiation of the ductal epithelium, as well as the growth of connective tissue (Porter, 1974).

The role of estrogen as a fundamental factor in the etiology and progression of human breast cancer has been well documented. The first evidence suggesting a relationship between estrogen and breast cancer growth was reported by George Beatson more than 100 years ago who discovered that ovariectomy could lead to breast cancer regression in premenopausal women (Beatson, 1896). The importance of ovarian function and ovarian hormones in breast cancer development was subsequently confirmed by work on mice (Allen and Doisy, 1983; Lathrop and Loeb, 1916). Since then, there has been considerable evidence from numerous studies that associates increased risk of breast cancer with prolonged exposure to estrogens. The extent of exposure to ovulary cycles, including early menarche, late first full-term pregnancy and late menopause, is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer (Pike et al., 1993). In postmenopausal women, in spite of the dramatic decrease of serum estrogen levels at menopause, the concentration of estrogen in breast cancer tissues is more than 20-fold higher than that present in the plasma and does not differ significantly from that in premenopausal patients, indicating a more significant role of local biosynthesis of estrogen in postmenopausal breast cancer patients which is further supported by the observation of high levels of aromatase in their breast tumors (Castagnetta et al., 1996; Miller and O'Neill, 1987; Pasqualini et al., 1996). Long-

term replacement therapy (estrogen replacement therapy, with estrogen alone or hormone replacement therapy, with estrogen plus progestin), which also elevates blood levels of estrogen, can significantly increase breast cancer risk (Collaborative Group on Hormonal Factors in Breast Cancer, 1997), and the greatest increase in breast cancer risk appears to be associated with hormone replacement therapies that combine estrogen and progestin (Schairer et al., 2000).

How estrogen contributes to the development of breast cancer remains complex. Current evidence has suggested that estrogen may promote breast cancer by acting either as an initiator or as a promoter. Recent animal studies have demonstrated that estrogen and its catechol metabolites are carcinogens in various tissues including mammary glands (Harvell et al., 2000; Nandi et al., 1995; Shull et al., 1997; Turan et al., 2004; Yager, 2000; Yager and Liehr, 1996; Yue et al., 2003). Catechol estrogens are capable of redox cycling, a process that produces quinone intermediates that can form unstable DNA adducts (Cavalieri et al., 1997) and reactive oxygen species that can oxidatively damage lipids and DNA (Cavalieri et al., 2000; Liehr, 2000; Mobley et al., 1999; Nutter et al., 1994; Yager and Liehr, 1996). Thus, estrogen has been proposed to have genotoxic activity (Liehr, 2000). On the other hand, the role of estrogen as a tumor promoter in breast cancer is well established from both experimental and clinical observations. For instance, the growth of several human breast cancer cell lines, such as the MCF-7 cell line, require estrogen *in vitro* and *in vivo*. It is now clear that estrogen promotes breast cancer progression by stimulating malignant cell proliferation. The proliferative effects of estrogen are mediated by estrogen receptors (See ESTROGEN RECEPTORS section for detail). Antiestrogens, aromatase inhibitors and ovariectomy, all of which block or limit the promotional effects of estrogen, are effective in treating some breast cancer patients.

## 1.3. ESTROGEN RECEPTORS

### 1.3.1. Two estrogen receptors

Estrogen's biological and physiological effects are mediated by estrogen receptors (ERs). Estrogen receptors are members of the nuclear hormone receptor superfamily that function as transcription factors when bound by their respective ligands. The estrogen receptor was first described in the uterus of rats (Jensen and Jacobson, 1962; Jensen et al., 1968; Toft and Gorski, 1966). It is now known that there are two subtypes of ER, ERα and ERβ, encoded by different genes (Green et al., 1986a; Kuiper et al., 1996) (Figure 1.4.).

### 1.3.2. Estrogen receptor α

ERα was the first cloned estrogen receptor and it was isolated and sequenced from MCF-7 human breast cancer cells in the late 1980s (Green et al., 1986b; Greene et al., 1986; Walter et al., 1985). The human ERα gene is located on chromosome 6q (Menasce et al., 1993) and its protein contains 595 amino acids with a molecular weight of 66 kDa (Green et al., 1986b). A 46 kDa isoform and several splicing variants of ERα have also been described, although their biological function remains unclear (Flouriot et al., 2000; Murphy et al., 1997; Poola et al., 2000). ERα consists of six functional domains (A-F) transcribed by eight exons (Figure 1.4.) (Kumar et al., 1987; Kumar et al., 1986). The N-terminal A/B domain contains a hormone-independent transcriptional activation function 1 (AF1), a constitutive activation function contributing to ERα transcriptional activity. The C domain corresponds to the DNA binding domain (DBD), which is highly conserved in the primary sequence of members of the nuclear hormone

receptor superfamily. It contains two functional zinc fingers and is involved in specific binding of the receptor to target DNA sites. The D domain is the hinge region and contains the nuclear localization signal. The ligand-binding domain (LBD) in the C-terminal (the E/F region) harbors an activation function 2 (AF2), which is a ligand-dependent activation function. This domain also interacts with coregulatory proteins. As described above, estrogen stimulates breast cancer cell proliferation through its interaction with and activation of the estrogen receptor. About two-thirds of breast cancers are ERα positive, while only 15-25% of normal breast epithelial cells express ERα (Ali and Coombes, 2002). Thus, endocrine manipulation is developed to block the effect of estrogen on malignant cells. The positivity of ERα, along with PgR (Progesterone receptor), is a historically proved prognostic marker in predicting response to endocrine therapy in breast cancer patients. In the clinic, patients with ERα (+) breast cancer show a 53% objective response rate to endocrine therapy. Among these patients, 69% are ERα (+) and PgR (+) and 32% are ERα (+) and PgR (-) (Horwitz, 1988). In contrast, only 13% ERα (-) patients respond to endocrine therapy (Horwitz, 1988).

### 1.3.3. Estrogen receptor β

ERβ was cloned, ten years later after ERα, from a rat prostate cDNA library (Kuiper et al., 1996), and the gene encoding ERβ is located on chromosome 14 in human (Enmark et al., 1997). The wild type full-length human ERβ protein generally consists of 530 amino acids (Ogawa et al., 1998), although a number of ERβ isoforms have been described (Lewandowski et al., 2002). ERβ is structurally similar to ERα (Figure 1.4.). They are highly homologous in their DBDs (96%) and have moderate sequence identity in the LBD (53%) (Kuiper et al., 1997; Ogawa et al., 1998). But the N-terminal region (AF1) between ERα and ERβ has only about a 30% identity (Ogawa et al., 1998).

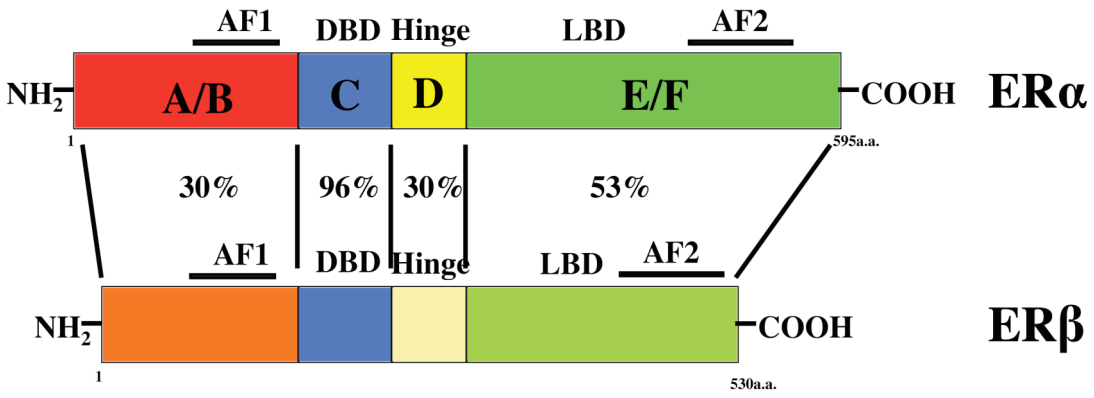



Figure 1.4. Structure and functional domains of ERα and ERβ.

Functionally, the transcriptional activity of ERα is mediated by AF1 and AF2 (Berry et al., 1990; Kumar et al., 1987; Tora et al., 1989), whereas ERβ activity is mediated by AF2 only, due to lack of AF1 activity (Hall and McDonnell, 1999). In addition to the domain difference, the tissue abundance and distribution of ERα and ERβ also differ. Although overlapped expression of ERα and ERβ has been found in breast, brain, cardiovascular system, urogenital tract and bone (Enmark et al., 1997; Gustafsson, 1999; Kuiper et al., 1997; Taylor and Al-Azzawi, 2000), ERα is predominantly expressed in breast, ovarian stroma, uterus and vagina, and is the main subtype of ER in the liver (Kuiper et al., 1997). In contrast, ERβ is present in several nonclassic target tissues such as the kidney, intestinal mucosa, lung, bone, brain, immune system and prostate (Kuiper et al., 1997). Moreover, the role of ERβ in breast cancer growth and development is not as clear as the role of ERα and is still controversial (Speirs, 2002). Large studies are needed to determine whether ERβ may play a critical role in either breast carcinogenesis or disease progression.

### 1.3.4. Estrogen receptor knock out mice

To gain further insights into the physiological roles of ERα and ERβ in estrogen target tissues, mutant mice that lack ERα (αERKO), ERβ (βERKO) or ERα and ERβ (αβERKO) have been generated (Couse et al., 1999; Krege et al., 1998; Lubahn et al., 1993). Interestingly, a loss of either of ERα and/or ERβ is not lethal, and the mice survive to adulthood (Couse et al., 1999; Krege et al., 1998; Lubahn et al., 1993). The female αERKO mice are infertile and have hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea (Lubahn et al., 1993). αERKO males appear overtly normal, but have decreased fertility (Lubahn et al., 1993). Mice lacking of ERβ (βERKO) developed normally, and females are fertile and exhibit normal sexual behavior, but have

fewer and smaller litters than wild-type mice, a result of reduced ovarian efficiency (Krege et al., 1998). In addition, the βERKO females have normal breast development and lactate normally (Krege et al., 1998). Fertility of male βERKO mice are not affected, but older males display signs of prostate and bladder hyperplasia (Krege et al., 1998). In αβERKO mice, both sexes exhibit normal reproductive tract development but are infertile (Couse et al., 1999). Significantly, ovaries of adult αβERKO females contain seminiferous tubule-like structures, an ovarian phenotype that is distinct from that of the individual ERKO mutants, which indicates that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary (Couse et al., 1999).

While the crucial role of ERα in the etiology and progression of breast cancer have been repeatedly and firmly established, the role of ERβ in breast cancer is still controversial (Speirs, 2002). Therefore, in the following dissertation, we limit our discussion to ERα that will simply be referred as ER unless otherwise indicated.

## 1.4. MECHANISMS OF ER ACTION

### 1.4.1. Classical genomic actions of ER

In the classical model of ER action (Figure 1.5.), estrogen diffuses into the cell and binds to ER in the nucleus. This binding induces a conformational change of the receptor, which leads to dissociation from heat shock proteins and formation of receptor dimers. The activated ER then binds as a dimer to small palindromic DNA motifs known as estrogen response elements (EREs) that are present in the promoter regions of estrogen-responsive genes. The consensus estrogen response element sequence is defined as 5'GGTCAnnnTGACC3' where n denotes a random nucleotide (Walker et al., 1984). However, only a small number of the most estrogen inducible genes contain these

consensus EREs. In most cases, variant EREs or even partial EREs that are often separated by many base pairs mediate the inducibility of estrogen, either alone or in combination (El-Ashry et al., 1996; Pike, 1999). Promoter-bound ER recruits and forms a complex with coregulatory proteins, which function as intermediates between the receptor and the general transcriptional machinery and subsequently modulate transcriptional activity of estrogen-responsive genes. Transcription of many genes is increased by estrogen, while transcription of some others is repressed (Dobrzycka et al., 2003). Two different domains, AF1 and AF2, mediate transcriptional activity of ER. AF1 is constitutively active and its activity is hormone-independent and regulated by phosphorylation, whereas AF2 is integral to the LBD and its activation hormone-dependent (Smith, 1998; Tsai and O'Malley, 1994; Webster et al., 1989). The two activating domains can act synergistically and/or independently in a cell and promoter context specific manner (Gronemeyer, 1991). ER coregulatory proteins include coactivators, such as AIB1 and SRC3, that enhance ER transcriptional activity and corepressors, such as NCoR-1 and SMRT, that decrease ER transcriptional activity (Horwitz et al., 1996; McKenna et al., 1999). Typically, coactivators bind ER when the receptor is bound by estrogen. Coactivators enhance ER-driven transcription through different mechanisms including the recruitment of histone acetyltransferase (HAT) complex which help unwind the DNA, allowing gene transcription to occur (Horwitz et al., 1996; McKenna et al., 1999). Furthermore, some of the coactivators themselves possess intrinsic histone acetyltransferase activity (Chen et al., 1997; Spencer et al., 1997). In contrast, corepressor proteins influence ER transcriptional activity at least in part through the recruitment of histonedeacetylase complexes (HDAC) which allow DNA to wrap more tightly around the core histone proteins (Chen and Evans, 1995; Horlein et al., 1995). In fact, these coregulatory proteins may be indeed as important as ER itself in

mediating transcriptional activity as well as influencing tumor growth. For example, AIB1, an ER coactivator, is overexpressed in 65% of breast cancers and is gene-amplified in about 5-10% (Anzick et al., 1997; Bouras et al., 2001; List et al., 2001b; Murphy et al., 2000), strongly suggesting its important role in breast cancer. In addition, reducing the level of AIB1 has been shown to impede ER-mediated gene transcription as well as tumor growth in cell line models (List et al., 2001a). Moreover, high levels of AIB1 were reported to enhance the agonistic activity of tamoxifen in breast cancer cells, which may also contribute to tamoxifen resistance (Shou et al., 2004; Smith et al., 1997; Takimoto et al., 1999). In summary, the classical genomic action of ER involves ligand binding to the receptor, dissociation of heat shock proteins from the receptor and receptor dimerization. The receptor dimer then binds to EREs located in the promoter regions of estrogen responsive genes, recruits coregulatory proteins, and activates transcription. In this dissertation, we will focus on the classical genomic mode of action of ER via interaction with DNA recognition motifs such as ERE elements.

### 1.4.2. Nonclassical genomic actions of ER

Besides the classical genomic mechanism of direct DNA binding (EREs), ER has also been shown to regulate gene expression at alternative regulatory DNA sequences such as AP-1, SP-1, and other poorly defined non-ERE sites (Kushner et al., 2000; Ray et al., 1997; Safe, 2001) (Figure 1.5.). For instance, ER can indirectly modulate AP-1 response elements through its interaction with AP-1 transcription factors such as c-fos and c-jun (Webb et al., 1995), which regulate genes involved in many cellular processes, including proliferation, differentiation, cell motility and apoptosis (Altucci et al., 1996; Dong et al., 1999; Geum et al., 1997; Kushner et al., 2000). In this circumstance, ER mediates transcription via tethered interaction to specific promoter complexes through

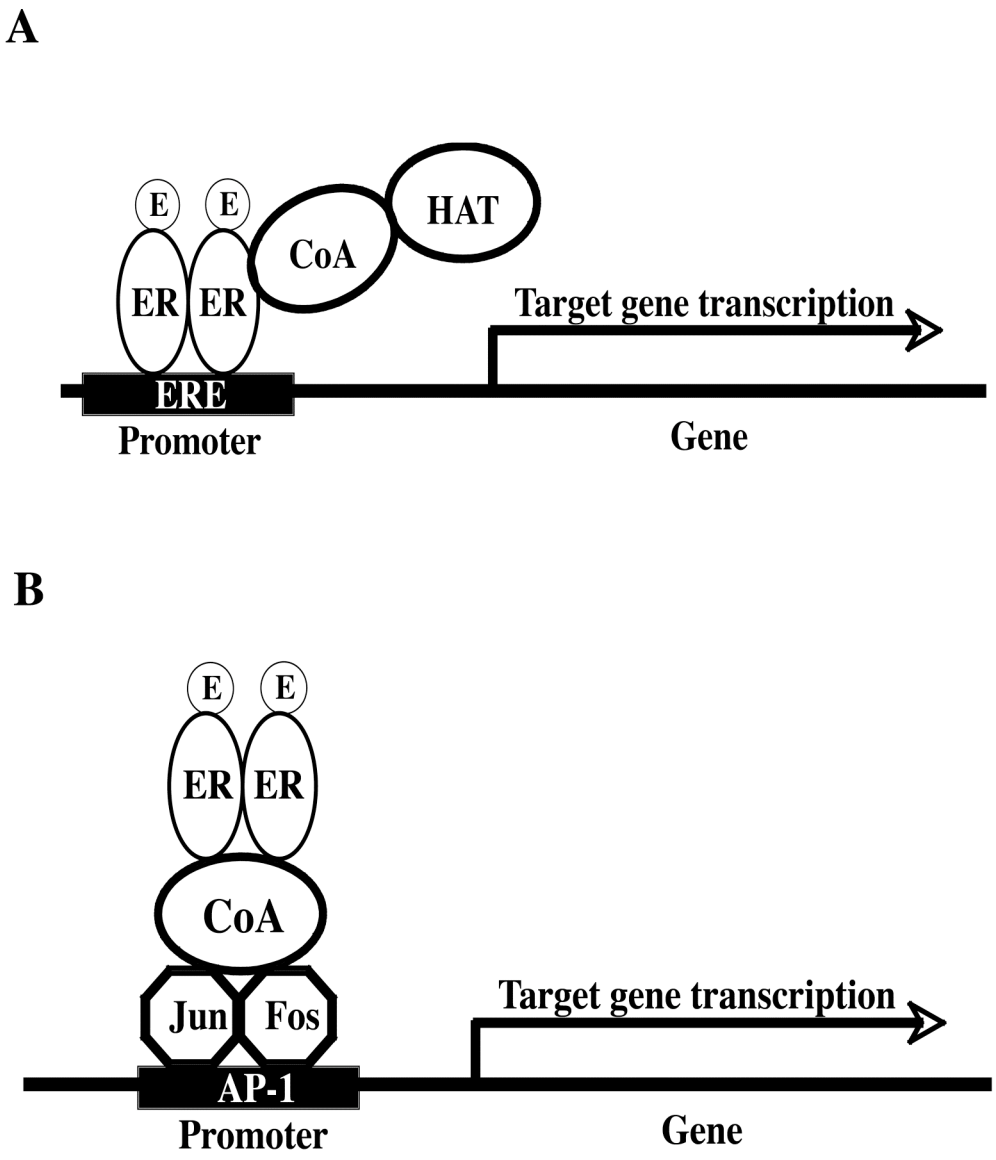



Figure 1.5. Genomic action of ER.

(A) Classical pathway. (B) Non-classical pathway. See text for detail.

protein-protein interaction rather than functions as the major transcription factor. These nonclassical genomic ER signaling pathways, particularly at the AP-1 sites, could be important clinically. Its been shown that tamoxifen can act as an agonist on genes under the control of an AP-1 response element (Paech et al., 1997; Webb et al., 1995). Moreover, elevated levels of activated jun N-terminal kinase and phosphorylated c-jun together with increased levels of AP-1 transcriptional activity have been reported in both preclinical models of tamoxifen resistance and in human tumor samples (Johnston et al., 1999; Schiff et al., 2000), suggesting that ER signaling through AP-1 sites may play important roles in the development of tamoxifen resistance in breast cancer.

### 1.4.3. Nongenomic actions of ER

The traditional ligand-induced ER signaling pathway involving nuclear interaction as described above usually takes hours or days for gene transcription and protein synthesis to occur. However, estrogen has also been shown to be able to exert stimulatory effects that cannot be explained by transcriptional mechanisms because of its rapid onset (within seconds or minutes of the addition of estrogen) (Pietras and Szego, 1977). This rapid and nongenomic activity is also called MISS (membrane initiated steroid signaling) (Nemere et al., 2003). However, the receptors responsible for this rapid estrogen activity and their precise cellular localization still remain controversial. Several studies suggest this activity is mediated by a small portion of traditional ER, an alternatively spliced truncated form of ER, or other membrane receptors distinct from classic ER (Figtree et al., 2003; Filardo, 2002; Levin, 2002; Li et al., 2003). Some of these studies using various techniques also suggest that a small pool of ER is located in the plasma membrane and cytoplasm (Figtree et al., 2003; Levin, 2002; Li et al., 2003). Nevertheless, the nongenomic activity has been found to be the predominant type of

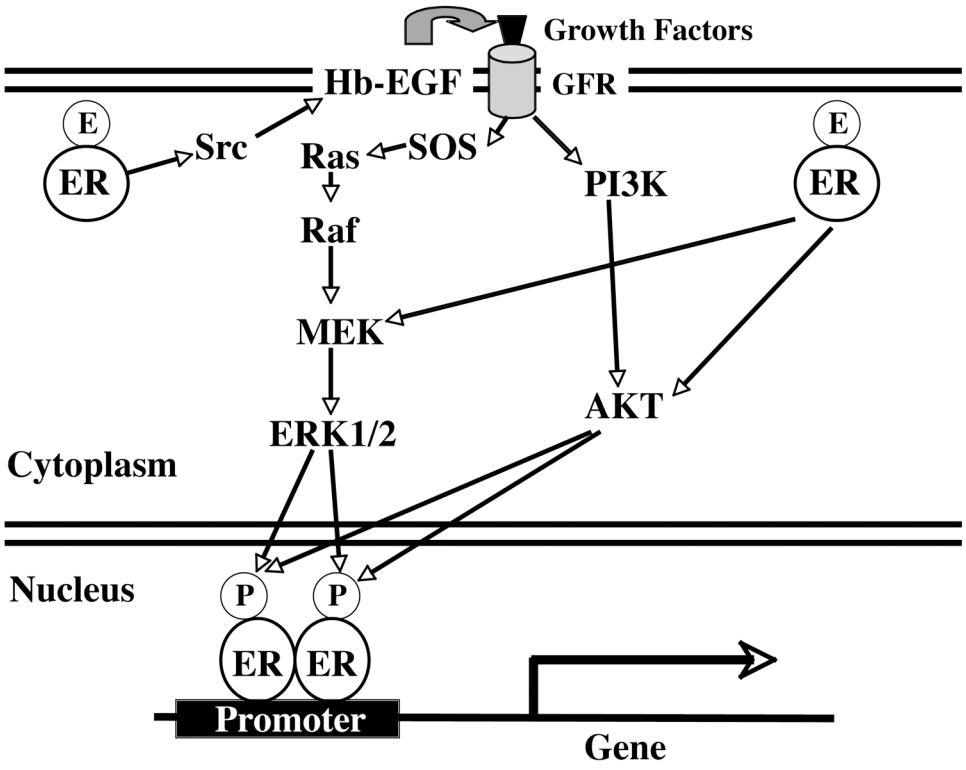



Figure 1.6. Non-genomic ER signaling and its crosstalk with growth factor receptor pathways involving the MAPK and PI3K-AKT pathways.

estrogen signaling at least in some tissues such as bone and endothelial cells (Kousteni et al., 2002; Li et al., 2003). Other examples of effects mediated by this alternative pathway include the rapid insulinotropic effect of estrogen on pancreatic β cells (Nadal et al., 1998), the short-term vasodilation of coronary arteries (Kim et al., 1999), and rapid activation of growth signaling pathways in neuronal cells (Watters et al., 1997). The nongenomic activity of ER results in elevated cell proliferative and survival signals through the activation of proliferation-related factors, such as growth factor receptors, MAPKs, PI3K, and Akt, in target cells. However, mechanisms by which estrogen activates membrane ER function are still not clear. One postulated mechanism involves activation of growth factor signaling through direct interaction between activated membrane ER and growth factors. ER has been reported to bind to membrane signaling molecules including insulin-like growth factor receptor 1 (IGFR1), the p85 regulatory subunit of PI3K, Src and Shc (Castoria et al., 2001; Kahlert et al., 2000; Simoncini et al., 2000; Song et al., 2002). In this case, estrogen activates growth signaling just like a growth factor binding to its membrane receptor, which leads to the activation of key molecules such as MAPK or Akt. Another well-studied potential mechanism for MISS activity of ER involves indirect interaction of the EGFR and activation of its downstream kinases like ERK, MAPK and Akt (Levin, 2002; Levin, 2003). In both circumstances, these kinases, once activated by nongenomic ER activity, can in turn phosphorylate ER as well as its coregulators or other components of the transcriptional machinery to enhance nuclear ER transcriptional activity (Shou et al., 2004; Stoica et al., 2003; Sun et al., 2001) (Figure 1.6.).

### 1.4.4. Ligand-independent activation of ER

Most nuclear receptors are phosphoproteins (Weigel, 1996). As we discuss above, ER can be phosphorylated by enzymes such as mitogen-activated protein kinases, which are activated in response to various growth signalings and transduce extracellular signals to intracellular targets by way of membrane receptors (Figure 1.6.). Phosphorylation of ER by these protein kinases usually results in ligand-independent activation of the receptor and creates crosstalk between the ER signaling pathways and other growth signaling pathways (Kato, 2001; Lannigan, 2003). Phosphorylation of the ER occurs predominantly on specific serine residues in the AF1 region of ER. Of particular interest is serine 118, which is phosphorylated by MAP kinases ERK1 and ERK2 (Kato et al., 1995), resulting in ligand-independent activation of ER (Bunone et al., 1996). In addition to S118 phosphorylation, upon EGF stimulation, serine 167 in AF1 is also phosphorylated by ribosomal S6 kinase RSK (Joel et al., 1998), which is itself activated by ERK1/2. Furthermore, phosphorylation of S167 has also been implicated in the PI3K/Akt pathway. The serine/threonine protein kinase Akt (also known as protein kinase B) is one of the downstream targets of PI3K (Datta et al., 1999). Akt can phosphorylate ER at S167, resulting in ligand-independent activation of ER (Campbell et al., 2001; Martin et al., 2000). Besides S118 and S167, phosphorylation of S104 and S106, which is mediated by cyclin A/CDK2 complex, has also been reported in U-2 OS human osteosarcoma cells (Rogatsky et al., 1999).

## 1.5. OVERVIEW OF ENDOCRINE THERAPIES

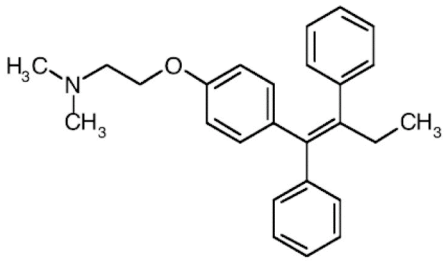
Endocrine therapy is currently the most important systemic treatment of ER-positive breast cancer at all stages. As adjuvant therapy for patients with early stage ER-positive breast cancer, recent data from long-term follow-up of patients treated by

endocrine therapy suggests this therapy is curative in many patients (Early Breast Cancer Trialists' Collaborative Group, 2005). In addition to these benefits in early stage disease, endocrine therapy is also very effective in the treatment of advanced metastatic breast cancer (Moy and Goss, 2006). Current endocrine therapies are based on targeting the ER signaling pathway by blocking the action of estrogen on its receptor or reducing circulating estrogen levels.

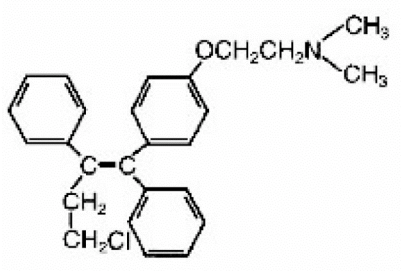
The non-steroidal tamoxifen, a selective estrogen receptor modulator (SERM), has been used for the treatment of all stages of ER (+) breast cancer for more than 25 years (Jordan, 2003). It is also the first approved drug by the FDA as a cancer chemopreventive for reducing breast cancer incidence in both pre- and post-menopausal women at high risk of developing breast cancer (Jordan, 2003). It acts by blocking the action of estrogen at the ER within breast tumors. In the adjuvant setting, tamoxifen treatment in women with operable breast cancer for five years show a maximal benefit with a 51% reduction in recurrence and about 28% reduction in overall mortality (Ali and Coombes, 2002). Thus, the widespread use of tamoxifen during the last 30 years certainly contributed to the significant decrease of national breast cancer mortality rates over the past 15 years (Early Breast Cancer Trialists' Collaborative Group, 2005). Because of the success of tamoxifen, several other SERMs such as toremifene, idoxifene, arzoxifene and raloxifene, have been developed and are in clinical test or use (Coombes et al., 1995; Milla-Santos et al., 2001; Miller, 2002; Pyrhonen et al., 1999).

**SERMs** are a new category of agents that represent a major therapeutic advance for clinical practice. Unlike estrogens, which are uniformly agonists, and pure antiestrogen, which are uniformly antagonists, SERMs exert selective agonist or

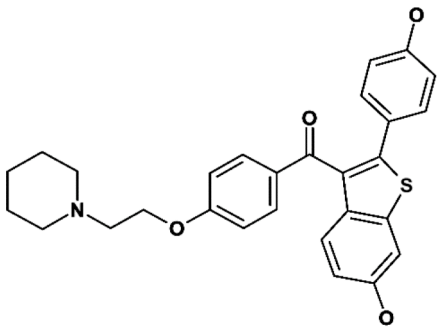
antagonist effects on various estrogen target tissues. For instance, in addition to its ER antagonism in the breast and brain, tamoxifen acts as an ER agonist in bone, liver and uterus (MacGregor and Jordan, 1998). These tissue selective effects of tamoxifen have been associated with an increased incidence of endometrial cancer, cerebrovascular and venous thromboembolic events, as reported by the ATAC study group (Barker, 2006). Besides tamoxifen, other well-known SERMs include toremifene, raloxifene and arzoxifene (Figure 1.7.). These agents are also mixed agonists/antagonists of the ER. Toremifene, which is a triphenylethylene derivative and structurally similar to tamoxifen, has very similar preclinical and clinical activities as well as side effects to those of tamoxifen (Howell et al., 2004). However, unlike tamoxifen, toremifene does not produce detectable DNA adducts in rats (Hard et al., 1993). To date, the use of teromifene is restricted by the FDA to postmenopausal women with metastatic breast cancer (Lewis and Jordan, 2005). Raloxifene, which is a benzothiophene derivative, is the most widely studied newer SERM. Raloxifene binds to ER with an affinity equal to estradiol (Black et al., 1983). Like tamoxifen, it acts as an antagonist in breast tissue and inhibits estrogen-induced cell proliferation (Liu et al., 2003). Raloxifene possesses less estrogenic effects in endometrium than tamoxifen, but has similar effects in bone as estradiol (Balfour and Goa, 1998). Therefore, raloxifene is approved for treatment and prevention of osteoporosis in postmenopausal women, because it was shown to maintain bone density in this population (Ettinger et al., 1999; Rossouw et al., 2002). In the multiple outcomes of raloxifene evaluation (MORE) trial, raloxifene therapy compared with placebo was associated with a reduced incidence of invasive, ER (+) breast cancer, without the increased risk of endometrial cancer (Cauley et al., 2001; Cummings et al., 1999). Additionally, clinical trials to evaluate raloxifene as a breast cancer chemopreventive and its ability to reduce the incidence of coronary heart disease are currently ongoing



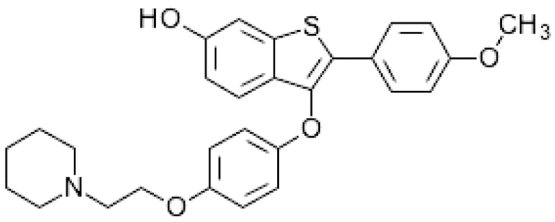
**Tamoxifen**



**Toremifene**



**Raloxifene**



**arzoxifene**

Figure 1.7. Chemical structures of common SERMs.

(Mosca, 2001; Vogel et al., 2002). Arzoxifene (old name LY 353381) is a long-acting raloxifene analog, which inhibits breast cancer cell growth more potently than raloxifene or tamoxifen *in vitro* (Dhingra, 1999). Arzoxifene is also shown to protect against bone loss and reduce serum cholesterol levels in ovariectomized rats (Ma et al., 2002; Sato et al., 1998). Moreover, it is also devoid of the uterotrophic effects of tamoxifen (Suh et al., 2001). Nonetheless, with the hope for ideal SERMs that will mimic estrogen's benefits in the bone, brain and heart and yet will block the harmful effects of estrogen in the breast and uterus, research efforts are currently under way to develop more tissue selective SERMs which will ultimately provide a new paradigm for improving the health of women.

In addition to SERMs, fulvestrant (Faslodex, ICI 182, 780), a pure estrogen antagonist that provides complete ER antagonism without any agonist effects, is currently in clinical use for the treatment of advanced breast cancer in postmenopausal women with recurrence or progression following antiestrogens such as tamoxifen. Fulvestrant targets and degrades the ER thereby inhibiting estrogen signaling through the ER (Morris and Wakeling, 2002; Robertson et al., 2005).

In postmenopausal women, local estrogen synthesis relies on the cytochrome P450 enzyme aromatase. Aromatase inhibitors (AIs), especially the third-generation non-steroidal AIs, which highly selectively reduce peripheral estrogen synthesis by suppressing aromatase activity have been developed and licensed for clinical use. Results from the ATAC (Arimidex, Tamoxifen, Alone or in combination) clinical trial of post menopausal women showed that anastrozole, a third generation AI, compared favorably with tamoxifen (Howell et al., 2005). However, anastrozole demonstrated only a

marginal five-year survival advantage over tamoxifen in large clinical trials (Barker, 2006). Moreover, it is still too early to judge the long-term adverse effects of AI treatment associated with prolonged estrogen restriction. At least, a longer-term view of patterns of adverse effects of anastrozole have emerged, as reported in an update from the ATAC study, in which more frequent bone fractures together with increased alkaline phosphotase and hypercalcaemia, and an increase in hypercholesterolemia were observed (Buzdar et al., 2006). Although these concerns warrant further longer-term follow-up to fully assess its long-term effects, Anastrozole has now been accepted as an alternative first-line treatment to tamoxifen (Barker, 2006).

## 1.6. TAMOXIFEN

### 1.6.1. Pharmacology

Tamoxifen belongs to the category of triphenylethylene derivatives. It is administered to patients as trans-tamoxifen and primarily metabolized to N-desmethyltamoxifen and trans-4-hydroxytamoxifen (4-OH-TAM) by members of the cytochrome P450 family, CYP3A and CYP2D6, respectively (Marsh and McLeod, 2007; Osborne, 1998). 4-OH-TAM, the active metabolite of tamoxifen, has an affinity for ER similar to that of 17β-estradiol (Buckley and Goa, 1989). The metabolites of tamoxifen are subject to conjugation leading to excretion by sulfation and glucuronidation (Nishiyama et al., 2002) predominantly in the feces. Tamoxifen is usually referred to as a selective estrogen receptor modulator (SERM), which has both estrogenic and antiestrogenic effects, depending on different tissues. In breast tissue, tamoxifen's antagonistic activity accounts for its tumor inhibitory ability, while it is estrogenic in the bone and stimulates endometrial proliferation. The dimethylaminoethoxy side chain and the trans configuration are essential for the antiestrogenic activity of tamoxifen (Buckley

and Goa, 1989). The usual dosage is 20 mg/day by mouth (Sunderland and Osborne, 1991), and it will take several weeks for tamoxifen to reach its therapeutic blood levels, although its serum concentration varies from patient to patient (Buckley and Goa, 1989). The serum half-life of tamoxifen and its metabolites ranges from 7 to 14 days (Buckley and Goa, 1989). Indeed, tamoxifen can even be detected in serum for several weeks and in tumor tissue for several months after treatment is stopped (Lien et al., 1991).

### 1.6.2. Clinical aspects

Tamoxifen was originally developed as an oral contraceptive, but later the potential of its antiestrogenic action was recognized (Jordan, 2003). It was approved for the treatment of advanced breast cancer by the FDA in 1977 (Osborne, 1998). In 1985, tamoxifen was approved by the FDA as an adjuvant therapy with chemotherapy in postmenopausal women with lymph node-positive breast cancer, and one year later, the use of adjuvant tamoxifen alone was further approved in the same group of patients (MacGregor and Jordan, 1998). In 1989 and 1994, the FDA approved the use of tamoxifen in the treatment of premenopausal women with ER positive advanced breast cancer and the claim that tamoxifen prolonged the overall survival of patients with breast cancer, respectively (MacGregor and Jordan, 1998). Thus, with beneficial effects in both pre- and post-menopausal women with ER positive breast cancer either in an adjuvant setting or for the treatment of advanced metastatic disease, tamoxifen has become the first-line endocrine agent for treating breast cancer. Furthermore, tamoxifen became the first drug approved by the FDA in 1999 to prevent breast cancer after data from clinical trials showed it reduced the chance of developing breast cancer by 50% in high-risk pre- and post-menopausal women (Cuzick et al., 2003; Fisher et al., 1998). In addition, results from adjuvant breast cancer trials showed 5-year tamoxifen therapy reduces the incidence

of contralateral second primary breast tumor by 50% (Early Breast Cancer Trialists' Collaborative Group, 1998). In postmenopausal women, long-term tamoxifen treatment slightly increases the bone density of the axial skeleton (Love et al., 1992). However, tamoxifen may decrease bone mineral density in premenopausal women (Powles et al., 1996). In spite of its benefits in treating breast cancer patients, there have been many concerns with the side effects of tamoxifen. The most serious side effect of tamoxifen is its potential tumor-promoting activity. Long-term usage of tamoxifen has been associated with an increased incidence of endometrial cancer in breast cancer patients, mostly due to its estrogenic activity in the uterus (Early Breast Cancer Trialists' Collaborative Group, 1998). In addition, tamoxifen has also been reported to be a potent hepatocarcinogen in rats (Williams et al., 1993) and its electrophilic metabolites can form covalent DNA adducts (Osborne, 1998). But the mechanisms by which tamoxifen induces liver carcinogenesis in rats are still not known. Other common side effects of tamoxifen include menopausal symptoms and thromboemolic events (Osborne, 1998).

### 1.6.3. Mechanism of action

Tamoxifen basically functions through its ability to bind the ER thereby competing with available estrogens and blocking their effects. Since tamoxifen has tissue-specific agonist-antagonist activity, the most important biological consequence of occupying ER with tamoxifen becomes whether the activated receptor complex induces an antiestrogenic or estrogenic response. However, it is still not clear about the mechanisms of the tissue selective, mixed estrogenic-antiestrogenic activity of tamoxifen. As discussed previously, the transcription activity of the ER is mediated by AF1 and AF2. The activity of AF1 and AF2 differs depending on the cellular environment and promoter context (Tzukerman et al., 1994). Unlike estrogen which is an pure agonist and

ICI 182,780 which blocks both AF1 and AF2 activity (Metzger et al., 1995), tamoxifen, as well as other SERMs such as raloxifene, inhibits AF2 but not AF1 activation of ER and thereby ER activity (Ali and Coombes, 2002). In the breast, ER activity is mainly due to the activation of AF2. Thus, tamoxifen acts largely as an antagonist in breast cells. In contrast, the activation of AF1 of ER is more significant in other tissues like the endometrium, in which tamoxifen is an ER agonist (Ali and Coombes, 2002). Moreover, using protein crystallography and techniques evaluating surface changes, recent research progress in understanding the conformational changes of ER when bound to different ligands suggests that the unique change of ER confirmation induced by tamoxifen, as well as other SERMs, may explain the particular pharmacological properties of tamoxifen in target tissues. Upon binding to estrogen, helix 12 of ER is positioned over the hydrophobic ligand binding pocket, which is essential for the recruitment of coactivators to the AF2 site and subsequent transcription initiation (Brzozowski et al., 1997; Pike et al., 2000). In contrast, although tamoxifen bind at the same site within ER, which is the hydrophobic ligand binding pocket, as estrogen, its side chain is too large to fit within the pocket, preventing the reorientation of helix 12 and thereby the formation of a competent AF2 region (i.e. disrupting AF2) (Shiau et al., 1998). Therefore, the conformational changes of ER induced by estrogen and tamoxifen are structurally different, and these events resulting from different ligands binding subsequently translate into agonism or antagonism at the receptor. In addition, differential expression and recruitment to the ER of coregulator proteins may also be a mechanism to explain the action as well as the mixed agonistic-antagonistic activity and tissue selectivity of tamoxifen. To date, more than 20 coregulatory proteins have been discovered that bind to ERs and modulate their function, acting either as coactivators or a corepressors (Hall et al., 2001). It has been shown that tamoxifen-bound ER interacts with NCoRs, resulting in repression of its

agonist activity. Moreover, a recent study (Shang and Brown, 2002) found that both tamoxifen and raloxifene induce the recruitment of corepressors to target gene promoters in mammary cells. In contrast, in endometrial cells, tamoxifen acts like estrogen by stimulating the recruitment of coactivators to a subset of genes, whereas this recruitment does not occur with raloxifene, which lacks estrogen agonistic effect in the endometrium. Furthermore, this estrogen-like activity of tamoxifen in the uterus was dependent on a high level of steroid receptor coactivator 1 (SRC-1) expression (Shang and Brown, 2002). Therefore, variable local concentration of different coregulatory proteins may contribute to the unique pharmacology of tamoxifen. Alternatively, tamoxifen-ER complex may also activate transcription by tethering to promoters that do not contain the classical estrogen response elements, in this case, through the interaction between the receptor complex and other DNA-bound transcription factors (Shang and Brown, 2002).

### 1.7. TAMOXIFEN RESISTANCE

Since the recognition of the role of estrogen as a fundamental factor in the etiology and progression of human breast cancer, treatment of breast cancer has, for a long time, been directed towards inhibiting the tumor promoting effects of estrogen. Tamoxifen is the most commonly used antiestrogen for the treatment of patients with ER positive breast cancer. It has been shown to be effective in halting breast cancer progression and has also been recently approved as a chemopreventive agent for reducing breast cancer incidence in both pre- and post-menopausal women at high risk. Despite the clear beneficial effects of tamoxifen in treating breast cancer patients, most initially tamoxifen responsive breast tumors acquire resistance. This constitutes a major clinical challenge in breast cancer therapy. Indeed, almost all patients with advanced metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse

and die from their disease. However, the mechanisms underlying tamoxifen resistance are still poorly understood. Better understanding the mechanisms involved may suggest novel strategies to overcome tamoxifen resistance and further improve breast cancer survival. Thus, clarification of the mechanisms for resistance would have important clinical implications. Several different mechanisms have been proposed to contribute to the development of tamoxifen resistance. We will discuss some most commonly hypothesized mechanisms in the following paragraphs.

**Postulated mechanisms of tamoxifen resistance**

### 1.7.1. Loss of ER expression /function

Since expression of ER is the main predictor of response to tamoxifen therapy, it is clear that lack of ER expression is the main mechanism of de novo resistance to tamoxifen, with the majority (90%) of ER/PgR negative tumors not responding to antiestrogens (Clarke et al., 2001). It is also known that the effects of tamoxifen are primarily mediated through the ER. In this respect, loss of ER expression has been hypothesized to be responsible for acquired resistance to tamoxifen. However, most studies demonstrated that ER expression is maintained following the development of tamoxifen resistance in most tumors that were initially ER positive, tamoxifen sensitive (Gutierrez et al., 2005; Johnston et al., 1995). In fact, only 17-28% of patients with acquired resistance to tamoxifen lose ER expression (Gutierrez et al., 2005; Johnston et al., 1995). Additionally, studies have shown that about two-thirds of patients who had relapsed on tamoxifen still responded to the pure ER antagonist faslodex (Howell et al., 1996), and similar clinical benefit was also provided by aromatase inhibitors (Osborne et al., 2002). These observations suggest that ER still expresses and continues to regulate tumor growth in many tamoxifen resistant patients. Mutations of ER that can alter the function of the receptor might also affect the response to antiestrogens. However, such

mutations are relatively uncommon in human primary breast carcinomas (Karnik et al., 1994; Roodi et al., 1995). Thus, they appear not to contribute significantly to resistance to tamoxifen.

### 1.7.2. Altered expression of ERβ

As we discussed in previous sections of this chapter, a second ER was cloned from a rat prostate cDNA library in 1996 (Kuiper et al., 1996) and subsequently named as ERβ to distinguish it from the classical ER, which was later renamed ERα. The role of ERβ in tamoxifen resistance is still not clear. Recent studies showed that transcription of AP-1 dependent genes is increased when ERβ is bound to tamoxifen, raloxifene or ICI 164, 384 (Paech et al., 1997). Interestingly, another study found that the levels of ERβ mRNA is about 2-fold higher that ERα levels in tamoxifen resistant tumors when compared to tamoxifen sensitive tumors (Speirs et al., 1999). These observations might suggest a role of ERβ in the resistance to tamoxifen. However, there are also other studies reporting that ERβ has a negative effect on ERα-promoted transcription (Hall and McDonnell, 1999; Pettersson et al., 2000). Therefore, more work is still needed to elucidate the role of ERβ in the development of tamoxifen resistance.

### 1.7.3. Pharmacological mechanisms

It has been proposed that reduced intra-tumoral concentration of tamoxifen and increased metabolism of tamoxifen to agonistic metabolites could be potential mechanisms of resistance. In a nude mice xenograft model used to study tamoxifen resistance *in vivo*, resistant tumors were characterized by markedly lower intracellular tamoxifen levels and by isomerization of the potent antiestrogenic metabolite trans-4-hydroxy-tamoxifen to the less potent cis isomer (Osborne et al., 1991). Moreover, in a

later human study (Johnston et al., 1993) that tested tamoxifen concentration in 51 patients with locally recurrent breast cancer, serum tamoxifen concentrations were found similar in patients with acquired and de novo resistance. But intra-tumoral concentrations were significantly lower in patients with acquired resistance. The authors concluded that reduced intra-tumoral tamoxifen levels during prolonged therapy may be an important mechanism for acquired resistance in breast cancer (Johnston et al., 1993). In addition, tamoxifen is primarily metabolized to N-desmethyltamoxifen and 4-hydroxytamoxifen. N-desmethyltamoxifen can be converted to a further active metabolite 4-hydroxy-N-desmethyltamoxifen by cytochrome P450 enzyme CYP2D6 (Desta et al., 2004). This metabolite was found present in the blood at higher concentrations than 4-hydroxytamoxifen in patients that received adjuvant tamoxifen (Stearns et al., 2003). Interestingly, baseline plasma 4-hydroxy-N-desmethyltamoxifen concentrations were lower in women who carried a variant CYP2D6 allele than in those who carried the wild-type genotype (Stearns et al., 2003). In addition, patients with a wild-type CYP2D6 allele had significantly decreased plasma levels of 4-hydroxy-N-desmethyltamoxifen when co-treated with paroxetine, a selective serotonin reuptake inhibitor (SSRI) which is often prescribed to alleviate tamoxifen-associated hot flashes and can inhibit CYPs (Stearns et al., 2003). Therefore, these results indicate that pharmacogenomic effects as well as drug interactions may alter the metabolism and efficacy of tamoxifen, which may further influence therapeutic outcomes from tamoxifen treatment.

### 1.7.4. Altered expression of coregulatory proteins

Since coactivators and corepressors have important function in mediating the transcriptional activation by the ER, altered expression patterns of coregulatory proteins could contribute to tamoxifen resistance.

AIB1 (also known as NCOA3, SRC3, RAC3 and ACTR) is an ER coactivator which was identified on the basis of its frequent amplification in breast cancer (Anzick et al., 1997). Cell line studies showed that AIB1 enhances the agonistic activity of tamoxifen (Webb et al., 1998). Consistent with the *in vitro* results, a recent study measuring AIB1 expression by western blot analysis in 187 patients who had received tamoxifen adjuvant therapy and 119 patients who had received no adjuvant therapy showed that, in patients receiving adjuvant tamoxifen therapy, high AIB1 expression was associated with worse disease free survival (DFS), which is indicative of tamoxifen resistance (Osborne et al., 2003). In contrast, high AIB1 expression in patients not receiving adjuvant tamoxifen therapy was associated with better prognosis and longer DFS (Osborne et al., 2003). In addition to AIB1, experimental data also showed that overexpression of SRC1 (NCOA1) enhances estrogen-stimulated expression of target genes and increases tamoxifen's agonist activity (Smith et al., 1997; Tzukerman et al., 1994). However, no evidence to date regarding the overexpression of SRC1 in samples from patients with tamoxifen resistant tumors is available, and therefore it requires further validation in clinical settings.

In contrast to estrogen-bound ER which recruits coactivators, tamoxifen-bound ER usually recruits corepressors, which, in turn, recruit HDACs, facilitating chromatin condensation through histone deacetylation and inhibition of ER-regulated genes (Lavinsky et al., 1998; McKenna et al., 1999). N-CoR1 was shown to be strongly associated with endogenous ER in the presence of the mixed anti-estrogen, trans-hydroxytamoxifen, while only weak association was observed in the absence of ligand (Lavinsky et al., 1998). In addition, blocking the activity of N-CoR1 by anti-N-CoR1 IgG

reversed repression by trans-hydroxytamoxifen-bound ER, therefore converting trans-hydroxytamoxifen into agonist in MCF-7 cells (Lavinsky et al., 1998). Moreover, subsequent studies on a mouse model of tamoxifen resistance revealed that N-CoR1 levels declined in many of the tumors (MCF-7 xenografts) that acquired resistance to the antiproliferative effects of tamoxifen, relative to tumors retaining a response to the drug (Lavinsky et al., 1998). Together, these data raise the possibility that a decrease in levels of N-CoR1 could cause a shift in tamoxifen from antagonist to agonist, which further contributes to resistance. However, no clinical data are available to date to further support this hypothesis. A report from another study also showed no difference in levels of SMRT (NCoR2, another ER corepressor) mRNA in a cohort of 19 tamoxifen-resistant human breast tumor samples compared with tamoxifen-treated or untreated tumors (Chan et al., 1999). Thus, the role of corepressors in the development of tamoxifen resistance still remains to be elucidated, requiring large clinical studies.

### 1.7.5. Crosstalk between ER and growth factor receptor signaling pathways

As we discussed in the previous sections of this chapter, while non-genomic activity of ER can activate growth factor signaling pathways, the downstream kinase cascade can in turn phosphorylate ER and its coregulatory proteins to enhance ER nuclear transcriptional activity, which creates a bidirectional molecular crosstalk between ER and growth factor pathways. The ER is known to be activated through phosphorylation at multiple sites, mostly within AF1, by various signaling kinases in the growth factor pathways, including MAPKs ERK1 and ERK2, Akt, c-Src, pp90rsk1 and CDK2 (for detail, see Nongenomic actions of ER and Ligand-independent activation of ER sections of this chapter). As a result, stimuli such as growth factors that increase intracellular growth signaling kinase activities can induce ER transactivation even in the

absence of ligand or in the presence of tamoxifen (Ali and Coombes, 2002; Ali et al., 1993; Shou et al., 2004), which could play a potential role in tamoxifen resistance. ERK1/2 expression and activity are increased in several cell line models of endocrine resistance (Coutts and Murphy, 1998; Shim et al., 2000) and elevated ERK1/2 activity has been shown to correlate with shorter duration of response to endocrine therapy in clinical breast cancer (Gee et al., 2001). Recently IGF-II was also reported to induce an increase of both IGF-1R and EGFR activation in tamoxifen resistant cells (Hutcheson et al., 2003) (Knowlden et al., 2003). In addition to directly activating ER, growth factor signaling may also indirectly modulate ER activity by regulating its coregulatory proteins mostly through phosphorylation. Phosphorylation of coactivators enhances ER-dependent transcription even in the absence of ligand or in the presence of antiestrogens (Ali et al., 1993; Hong and Privalsky, 2000; Shou et al., 2004), probably resulting from their increased nuclear localization (Wu et al., 2002), their enhanced binding ability with ER (Font de Mora and Brown, 2000) and direct activation of their intrinsic enzymatic activities (Lopez et al., 2001). An example would be the ER coactivator AIB1 (SRC3), which can be phosphorylated and activated by a variety of signaling kinases (Font de Mora and Brown, 2000; Wu et al., 2002). Formation of a potent transcriptional coactivator complex as a result of phosphorylation has been shown to convert tamoxifen-bound ER into an estrogen agonist rather than an antagonist in some experimental systems (Shou et al., 2004). On the other hand, phosphorylation of the corepressor SMRT was found to cause its nuclear export, preventing its access to and repression of ER transcriptional activity in the nucleus (Hong and Privalsky, 2000). Thus, increased growth factor receptor signaling pathways and their downstream kinase activities during tamoxifen treatment may lead to tamoxifen resistance in breast cancer via direct or indirect modulation of ER function.

In addition, considerable experimental and clinical evidence suggests that overexpression or activation of the EGFR/HER2 signaling pathway and its crosstalk with ER are associated with a poor response to tamoxifen and may contribute to resistance to tamoxifen. It has long been proposed that HER2 is involved in resistance to tamoxifen. HER2-overexpressing MCF-7 cells were shown to be estrogen dependent but tamoxifen resistant *in vivo* (Benz et al., 1993) and the inhibitory effect of tamoxifen on cell proliferation can be restored by treating these cells with the MAPK inhibitor U0126 (Kurokawa and Arteaga, 2001). In addition, increases in EGFR and HER2 protein expression have also been observed in tamoxifen resistant cells (Knowlden et al., 2003). Furthermore, a recent study demonstrated that *in vivo* growth of HER2-overexpressing MCF-7 cells was stimulated by tamoxifen, and molecular cross-talk between the ER and HER2 pathways was increased in these cells, with cross-phosphorylation and activation of both the ER and the EGFR/HER2 receptors, the signaling molecules Akt and ERK 1/2, and AIB1 with both estrogen and tamoxifen treatment (Shou et al., 2004). Interestingly, tamoxifen acts as an agonist on endogenous estrogen responsive genes in these HER2 overexpressing cells, which is due to the ability of tamoxifen-bound ER complex to recruit coactivators such as AIB1 rather than corepressors as observed in parental MCF-7 cells (Shou et al., 2004). Moreover, all these phenomena were blocked by gefitinib, an EGFR-tyrosine kinase inhibitor (Shou et al., 2004). These pre-clinical findings suggest that tamoxifen-stimulated growth of HER2-overexpressing cells, both *in vitro* and *in vivo*, is highly dependent on bidirectional crosstalk between ER and HER2. Consistent with experimental data, clinical studies also have shown that patients with tumors that overexpressed HER2 or EGFR are relatively resistant to tamoxifen but still responded well to aromatase inhibitors (Dowsett et al., 2001; Ellis et al., 2001). Moreover, as we

discussed above, the ER coactivator AIB1 can be phosphorylated by kinases in the HER2 signaling pathway. Interestingly, a more recent study demonstrated that patients whose tumors expressed high levels of both AIB1 and HER-2 had poor outcomes (such as shortened disease free survival) with tamoxifen therapy, suggesting that the antitumor activity of tamoxifen in patients with breast cancer may be determined, in part, by tumor levels of AIB1 and HER-2 (Osborne et al., 2003). Taken together, this evidence supports an important role of EGFR/HER2 growth factor receptor pathway in both de novo and acquired resistance to tamoxifen.

### 1.8. OVERVIEW OF SAGE AND ITS USE IN BREAST CANCER RESEARCH

With the completion of the Human Genome Project in 2003, we now have access to the complete sequence information on all the genes encoded by the human genome. However, to fully understand the complex pathophysiology of diseases such as cancer, this information may still not be sufficient. Therefore, comprehensive approaches that will enhance our understanding of how the different genes are altered in the various cancer processes and how complex gene interactions produce particular outcomes are of much importance so that the foundation for the next level of complexity can be created. In the past few years, numerous techniques, including the cDNA library analysis known as Expressed Sequence Tags (ESTs) and cDNA microarray analysis, have been developed for the analysis of global gene expression changes in which thousands of genes can be assayed simultaneously. In parallel with such developments, Velculescu et al. in 1995 (Velculescu et al., 1995) described a completely different technical approach for the analysis of global gene expression, serial analysis of gene expression (SAGE). It is a novel, extremely powerful, efficient and comprehensive approach for analyzing global gene expression profiles.

SAGE technique is based on the principle that a short nucleotide sequence (i.e. 14 base-pair tag) contains sufficient information to uniquely identify a transcript provided that the tag is obtained from a defined position within the transcript (Velculescu et al., 1995; Velculescu et al., 1997). Using this short sequence and ligating series of "tags" back to back (concatemers) allows for the efficient analysis, both qualitative and quantitative, of all mRNA transcripts. Thus, the expression level of a transcript is directly proportional to the number of times a specific tag is observed in the final count. Unlike chip-based gene expression assays, SAGE provides a statistical description of the mRNA population present in a cell without prior selection of the genes to be studied. In addition to the digital format information generated by SAGE, the SAGE data obtained can be directly compared with data generated from any other laboratory or with data in public databases. Furthermore, SAGE databases can be constantly updated and subjected to re-interpretation. An additional key feature of the SAGE technique, which makes it potentially much more powerful than other technologies, is that in the initial step one obtains both quantitative information on the abundance of each mRNA and a partial sequence. Other techniques for the analysis of global gene expression changes such as cDNA microarray rely on microchips using arrays of oligonucleotides or cDNA on solid supports, and most require very expensive hardware for the analysis of the data. All microarray approaches available so far require the previous knowledge of sequence information from specific genes or EST sequences. In contrast, SAGE provides qualitative and quantitative information on known as well as unknown transcripts. All of these constitute the major advantages of the SAGE method. One disadvantage of SAGE is that this methodology is not ideal for the comparison of multiple samples, for example hundreds of samples, in a relatively short time compared to microarray approaches.

However, this can be overcome by increasing the sequencing power of the performing laboratory.

In breast cancer research, SAGE has been used to analyze and compare epithelial populations from normal breast epithelium and ductal carcinoma in situ (DCIS) lesions. As a result, various chemokines and cytokines such as HIN1, LIF, IL-8 and GRO were observed downregulated in DCIS when compared to normal tissue (Porter et al., 2001). The Aldaz lab is one of a few experts in the country at utilizing SAGE. The effects of estrogen on gene expression in estrogen-dependent breast cancer cells were first investigated by the Aldaz lab using SAGE (Charpentier et al., 2000). This study demonstrated that a discrete number of genes were found to be upregulated by estradiol treatment, among which five novel genes were identified and cloned (E2IG1-5). The Aldaz lab has also used SAGE to generate a high resolution transcriptome analysis for the identification of breast cancer biomarkers and molecular signatures of relevance in diagnosis and prognosis of human breast cancer (Abba et al., 2004; Abba et al., 2005; Hu et al., 2004). In addition, similar approaches were being used to study hormonal regulation of mammary epithelial cells from genetically modified mice (Aldaz et al., 2002; Hu et al., 2004). Summaries of searchable SAGE library databases can be viewed at a specially created web site: http://sciencepark.mdanderson.org/ggeg. Novel statistical methods for SAGE analysis have also been developed (Baggerly et al., 2003). SAGE libraries generated by Dr. Aldaz's lab were also donated to the to the CGAP's SAGE database http://www.ncbi.nlm.nih.gov/SAGE.

## 1.9. CtIP, A PROTEIN WITH MULTIPLE PARTNERS AND PUTATIVE FUNCTIONS INCLUDING TUMOR SUPPRESSION

The human CtIP gene (CtBP interacting protein and also known as RBBP8) encodes an 897 amino acid nuclear protein that is widely expressed in various human tissues (Fusco et al., 1998; Schaeper et al., 1998; Wong et al., 1998; Yu and Baer, 2000). CtIP was originally discovered as a binding partner to the co-repressor CtBP (Figure 1.8.). The interaction between these two proteins is disrupted by the adenoviral oncoprotein E1A (Schaeper et al., 1998).

CtIP is known to interact with various tumor suppressor proteins (Figure 1.8.). CtIP's LECEE motif was found to bind Rb family (Rb and p130) and from this derives its alternative name RBBP8 for Rb binding protein 8 (Fusco et al., 1998; Meloni et al., 1999). CtIP was also found to bind the tumor suppressor BRCA1 (Li et al., 1999; Sum et al., 2002; Wong et al., 1998; Yu et al., 1998). Ikaros (zinc finger protein, subfamily 1A, 1) family members, proteins of importance in lymphoid development and differentiation, also bind CtIP. The Ikaros proteins are also known to behave as tumor suppressors and are found dysregulated in human leukemias (Koipally and Georgopoulos, 2002; Rebollo and Schmitt, 2003). CtIP was also reported to bind the LIM-domain protein LMO4 (LIM domain only 4) (Sum et al., 2002). LMO4 plays a role in mammary differentiation and it is known to be overexpressed in breast cancer (Visvader et al., 2001). This protein also appears to play a role in transcriptional repression and interacts also with the tumor suppressor BRCA1 (Breast cancer 1, tumor suppressor).

Recent studies suggest that CtIP plays an important role in cell cycle regulation and DNA damage response (Foray et al., 2003; Li et al., 2000; Liu and Lee, 2006; Wu-

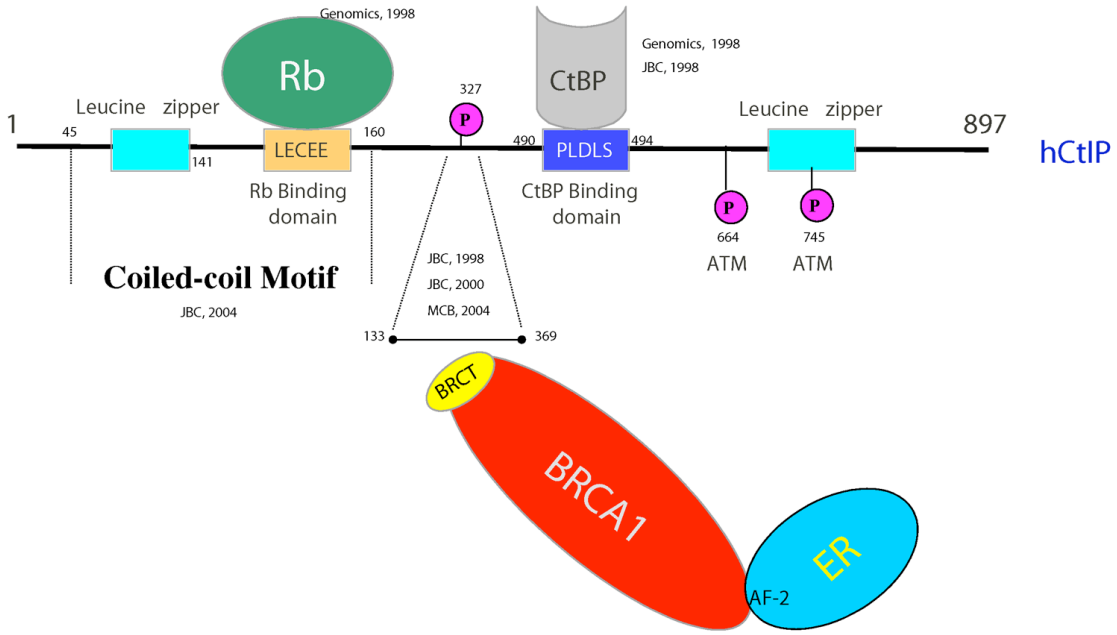



Figure 1.8. Domain structure of human CtIP (hCtIP).

The hCtIP protein contains a CtBP-binding motif, an Rb-binding motif and a phosphorylation site that corresponds to Ser327. The region between amino acid positions 45-160 is a coiled-coil that mediates homodimerization of CtIP. The ATM target sites that correspond to Ser664 and Ser 745 are shown. The hCtIP-interacting proteins, BRCA1, Rb and CtBP, are shown. BRCA1 also interacts with AF-2 domain on ER through its N-terminal region.

Baer and Baer, 2001; Yu and Chen, 2004). CtIP was reported to be hyperphosphorylated in cells exposed to ionizing radiation (Li et al., 2000; Wu-Baer and Baer, 2001). As shown in Figure 1.8. two sites have been identified as targets of phosphorylation by the ATM kinase. It has also been suggested that the interaction between CtIP and BRCA1 during the G2 phase of the cell cycle is required for activation of the checkpoint kinase Chk1 [reviewed by (Chinnadurai, 2006)]. Thus, highlighting the importance of CtIP as a potential regulator of the G2/M transition via Chk1 during the DNA damage response. The role of CtIP in normal cell cycle regulation, in particular at the G1/S transition, is a bit more controversial or complex, as reported by Wen-Hwa Lee's laboratory (Chen et al., 2005). On the one hand it has been reported that depletion of CtIP in Rb +/+ MEFs or in NIH3T3 cells leads to Rb hypophosphorylation and cell cycle arrest. Such cell cycle arrest was not evident in CtIP depleted Rb -/- MEFs or Saos-2 cells suggesting that the CtIP mediated arrest is Rb dependent. This would point to CtIP as a promoter of cell cycle progression and cell proliferation (oncogenic?). On the other hand reported evidence has also been provided that CtIP clearly behaves as a co-repressor of gene transcription and possibly as a tumor suppressor gene (as will be discussed in the following paragraphs) (Chinnadurai, 2006; Liu and Lee, 2006; Wu and Lee, 2006). However, it has also been reported that cell cycle regulation by the CtIP/Rb complex may not be uniform in different cell types, since MCF10A immortalized mammary epithelial cells continue to proliferate in spite of being depleted of CtIP (Liu and Lee, 2006).

It was shown that inactivation of CtIP in mice leads to early embryonic lethality, and the life span of *Ctip*+/- heterozygotes, which have haploid insufficiency for *Ctip*, was shortened due to the development of multiple types of tumors. This finding clearly

demonstrates that CtIP is a critical protein in early embryogenesis and implicates CtIP in tumorigenesis (Chen et al., 2005).

The CtIP-BRCA1 interaction takes place via the BRCT domains of BRCA1 where most mutations occur in BRCA1 breast cancer patients. Furthermore, such protein-protein interaction is abolished by tumor-associated mutations in the BRCT domains (Li et al., 1999; Wong et al., 1998; Yu et al., 1998), suggesting that interaction between CtIP and BRCA1 is of functional relevance in breast cancer suppressor activity. It has been shown that amino acid residues 299-345 of CtIP mediate the interaction with the BRCT domains (Wong et al., 1998; Yu and Baer, 2000; Yu and Chen, 2004). Recently, it was also reported that phosphorylation at CtIP's Ser327, possibly via a Cdk, appeared to be critical for the aforementioned interaction (Varma et al., 2005; Yu and Chen, 2004). (Figure 1.8.) Available evidence also suggests that CtIP is involved in transcriptional repression [reviewed in (Chinnadurai, 2006; Wu and Lee, 2006)]. As mentioned, CtIP interacts with the co-repressor CtBP. The CtIP-LMO4-BRCA1 complex acts as a transcriptional repressor on specific gene targets (Sum et al., 2002). CtIP may also participate in transcriptional repression by its interaction with Ikaros proteins in an HDAC independent manner. Significantly, recent studies demonstrated that BRCA1, CtIP and ZBRK1 (zinc finger protein 350, transcriptional repressor) form a repressor complex exerting its action on various genes. Specifically it was demonstrated that a recognition site for ZBRK1 at the promoter of the Angiopoieting-1 (ANG1) is responsible for repression in expression exerted by the BRCA1-CtIP-ZBRK1 complex (Furuta et al., 2006). Furthermore, disruption in complex formation leads to ANG1 up-regulation that in turn promotes endothelial cell survival and vascular enlargement, of much relevance in tumorigenesis.

## 1.10. DISSERTATION AIMS

The role of estrogen as a fundamental factor in the etiology and progression of human breast cancer has been well documented. The extent of exposure to ovulatory cycles is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer (Pike et al., 1993). Thus, treatment of breast cancer has, for a long time, been directed towards inhibiting the tumor promoting effects of estrogen. Tamoxifen is the most commonly used antiestrogen for the treatment of breast cancer and has been approved by the FDA as a preventive agent (Jordan, 2003; Osborne, 1998).

Although tamoxifen has been shown to be effective in halting breast cancer progression, a very important clinical problem is the development of tamoxifen resistance in patients chronically exposed to this antiestrogen. This constitutes a major clinical challenge in breast cancer therapy. For many women, resistance develops after the first phase of tamoxifen treatment (Katzenellenbogen et al., 1997). Furthermore, a very significant portion of patients with localized breast cancer, and all of the patients with metastatic disease become resistant to antiestrogen therapies (Ali and Coombes, 2002). In most cases of resistance, the ER is still present and apparently continues regulating tumor growth. Unfortunately, the mechanisms involved in the development of tamoxifen resistance are still poorly understood. Although, as we discussed in the previous sections of this chapter, numerous mechanisms have been proposed to contribute to the development of tamoxifen resistance, much work is still needed to learn whether some of the postulated mechanisms so far can explain resistance to tamoxifen therapy in a majority of patients, or simply each of the enumerated possibilities account for minor

portions of resistant cases. Thus, studies geared at better understanding the precise and most common mechanisms involved in tamoxifen resistance are of considerable clinical significance.

The studies described in this dissertation are designed with the overall goal of better understanding the phenomenon of tamoxifen resistance. In preliminary studies, we generated MCF-7 breast cancer cell line variants that are resistant to the inhibitory growth effects of tamoxifen. These new isogenic breast cancer cell lines represent a unique model that closely resembles the *in vivo* scenario. Based on the fact that most of the enumerated putative causes for resistance to tamoxifen will be reflected by changes in gene expression of key players representing directly or indirectly the involved pathways, we **hypothesize** that most cases of tamoxifen resistance are the result of a cellular adaptation phenomenon which will have a direct reflection in the patterns of global gene expression. Therefore, the first aim of this dissertation was to identify key genes involved in the development and manifestation of tamoxifen resistance in breast cancer, as well as direct or indirect bio-markers of tamoxifen resistance with promise for potential use in the clinical management of breast cancer patients, by defining the global gene expression profiles of above-mentioned tamoxifen resistant isogenic MCF-7 breast cancer cell lines and comparing them with their tamoxifen sensitive parental MCF-7 counterpart through the use of the combination of a comprehensive and unbiased powerful global gene expression approach SAGE with state-of-the-art bioinformatic and statistical approaches. As a result of the SAGE databases mining, we identified that the transcript encoding for CtIP (also known as Retinoblastoma binding protein 8, RBBP8) was significantly down-regulated (15-fold) in both tamoxifen resistant cell lines when compared to their tamoxifen sensitive parental MCF-7 counterpart. CtIP has been shown to be a binding

partner of tumor suppressors, BRCA1 (Li et al., 1999; Sum et al., 2002; Wong et al., 1998; Yu et al., 1998) and Rb (Fusco et al., 1998; Meloni et al., 1999), and also forms heterodimers with the transcriptional co-repressor CtBP (Schaeper et al., 1998). Recent studies with mutant mice have suggested that CtIP itself may be a tumor susceptibility gene (Chen et al., 2005). Interestingly, other studies demonstrated that BRCA1 physically interacts with ER and inhibits transcriptional activity of the receptor (Fan et al., 2001; Zheng et al., 2001). Moreover, CtIP was also shown to form a complex with BRCA1 and the transcriptional co-repressor CtBP, which is important for the repression of p21 promoter activity (Li et al., 1999). Based on the significant level of CtIP downregulation and the importance of its direct links with important tumor suppressors and regulators of gene expression, we **hypothesize** that CtIP silencing constitutes a critical event for the development of tamoxifen resistance in breast cancer. We further **speculate** that CtIP could bridge BRCA1 and CtBP to form a transcriptional repressor complex, which in turn may modulate ER signaling pathways through the interaction between BRCA1 and ER and account for the inhibitory growth effects of tamoxifen. In order to circumvent the transcriptional inhibitory effects of tamoxifen, tamoxifen resistant cells could silence CtIP expression, which in turn, disrupts the repressor complex and allows breast cancer cells to resume proliferation. Thus, we focused our studies on CtIP and functionally characterized its mechanistic role in the development of tamoxifen resistance. Results are presented in subsequent chapters.

# Chapter 2: Materials and Methods

## 2.1. HUMAN BREAST CANCER CELL LINES

The MCF-7 cell line batch used in these studies is derived directly from the original MCF-7 cell line. MCF-7 cells were maintained in IMEM (phenol red free) medium (Invitrogen, CA) supplemented with 5% fetal bovine serum (FBS) (Hyclone, UT), glutamine (2 mM) and gentamicin (50 μg/ml). The tamoxifen resistant MCF-7 isogenic cell line variants (termed TAMR1 and TAMR2) were generated by culturing MCF-7 cells under continuous 4-OH-TAM (1 μM) exposure for approximately two years. TAMR1 cells were maintained in phenol red free IMEM plus 5% fetal bovine serum, glutamine (2 mM), gentamicin (50 μg/ml) and 4-OH-TAM (1 μM). TAMR2 cells were maintained in phenol red free IMEM plus 5% charcoal stripped fetal bovine serum (CSS, Hyclone, UT), glutamine (2 mM), gentamicin (50 μg/ml) and 4-OH-TAM (1 μM). Other breast cancer cell lines used, including T-47D, SUM-44-PE, ZR-75-1, MDA-MB-231, MDA-MB-435, SKBR3 and BT-474, were maintained in DMEM (Cambrex Bio Science, MD) supplemented with 10% FBS. The BT-483 cell line was maintained in RPMI (Cambrex Bio Science, MD) supplemented with 10% FBS. The UACC-812 breast cancer cell line was grown in L-15 medium (Invitrogen, CA) supplemented with 10% FBS. All cells were maintained in a 37°C, 5% $CO_2$ humidified incubator. Cell culture media was changed every 2 to 3 days.

## 2.2. CHEMICALS

We purchased 4-OH-TAM and 17-β-Estradiol (E2) from Sigma-Aldrich, MO.

## 2.3. SAGE

Serial analysis of gene expression (SAGE) was performed on tamoxifen sensitive parental MCF-7 line and tamoxifen resistant TAMR1 and TAMR2 lines. All analyses were performed while cells were exponentially growing. Total RNA was isolated using TRIzol reagent (Invitrogen, CA) following the manufacturer's protocol. SAGE libraries were generated following standard procedures as originally described by Velculescu et al. (Velculescu et al., 1995) (Figure 2.1.) and mostly using commercially available reagents (I-SAGE kit, Invitrogen, CA). In brief, total RNA was first bound to oligo (dT) magnetic beads that capture poly A+ RNA directly from the sample. Double stranded cDNA was then synthesized on the beads containing the mRNA and cleaved by an anchoring enzyme, Nla III. The cleaved cDNA was divided into two tubes and ligated with LS Adapters A and B that contain unique primer binding sites, the recognition sequence (5'-GGGAC) for a tagging enzyme. The cDNA was further cut by a tagging enzyme, BsmFI and blunt-end-filled in. The enzyme BsmFI cuts 10 and 14 bases in the 3' direction from its recognition site, thus adding the "Tag" sequence to the linkers. Ditags (~102 bp) were formed by ligation, amplified using PCR, digested with anchoring enzyme, isolated and concatenated to form concatemers (~500 to ~800 bp). Concatemers were finally cloned into the pZErO vector to obtain a SAGE library. Sequencing was performed using an ABI 3700 DNA Analyzer (Applied Biosystems, CA). SAGE libraries were generated at an approximate resolution of 60,000 SAGE tags per library.

## 2.4. SAGE LIBRARY ANALYSIS

SAGE tag extraction from sequencing files was performed using the SAGE2000 software v.4.0 (a kind gift of Dr. Kinzler, John Hopkins University, Baltimore, MD). Prior to analysis, all three SAGE libraries generated from parental MCF-7, TAMR1 or

TAMR2 cells were processed to correct abundances for spurious contributions due to sequencing errors. Sequencing errors could contaminate a library with spurious tags and also decrease the real abundance of actual tags. To correct for sequencing errors, we used a dynamic approach similar to that proposed by Colonge and Feger (Colinge and Feger, 2001), which accounts for potential contribution from sequencing errors by considering all neighborhoods of similar tags throughout the entire library. For tag-to-gene mapping, a refined version of NCBI's SAGEmap map was used (ftp://ftp.ncbi.nih.gov/pub/sage/map/Hs/NlaIII). Differential Expression Analysis: To extract the biologically relevant genes, an ANOVA-based multivariate approach called multiple linear contrast analysis (MLCA) was employed. Using combinations of appropriately defined contrasts, any expression pattern across the libraries can be defined and genes matching that pattern at a desired confidence level extracted. The MLCA is to our knowledge unique to the i-Sight platform and very flexible allowing to identify genes based on very specific expression patterns at the required level of statistical stringency. The MLCA was applied to gene-level data to extract individual genes with the desired expression profiles. Alternatively, iterative k-means clustering was used to first group the genes in groups with similar expression patterns and then apply the MLCA to the cluster centroids to extract clusters that match the desired expression signatures. Although the primary analysis is gene-centered, the original tag-based data after correction and normalization were also analyzed to provide an independent analysis path that is free of potential biases introduced in the tag-to-gene mapping and aggregation steps. Expression Patterns and Contrasts: Two contrasts were defined in order to identify differentially expressed genes in the tamoxifen resistant cells, one comparing the average expression level in the two resistant cell lines to that of the parental strain and the second one comparing the expression levels between the two resistant strains. The null hypothesis for

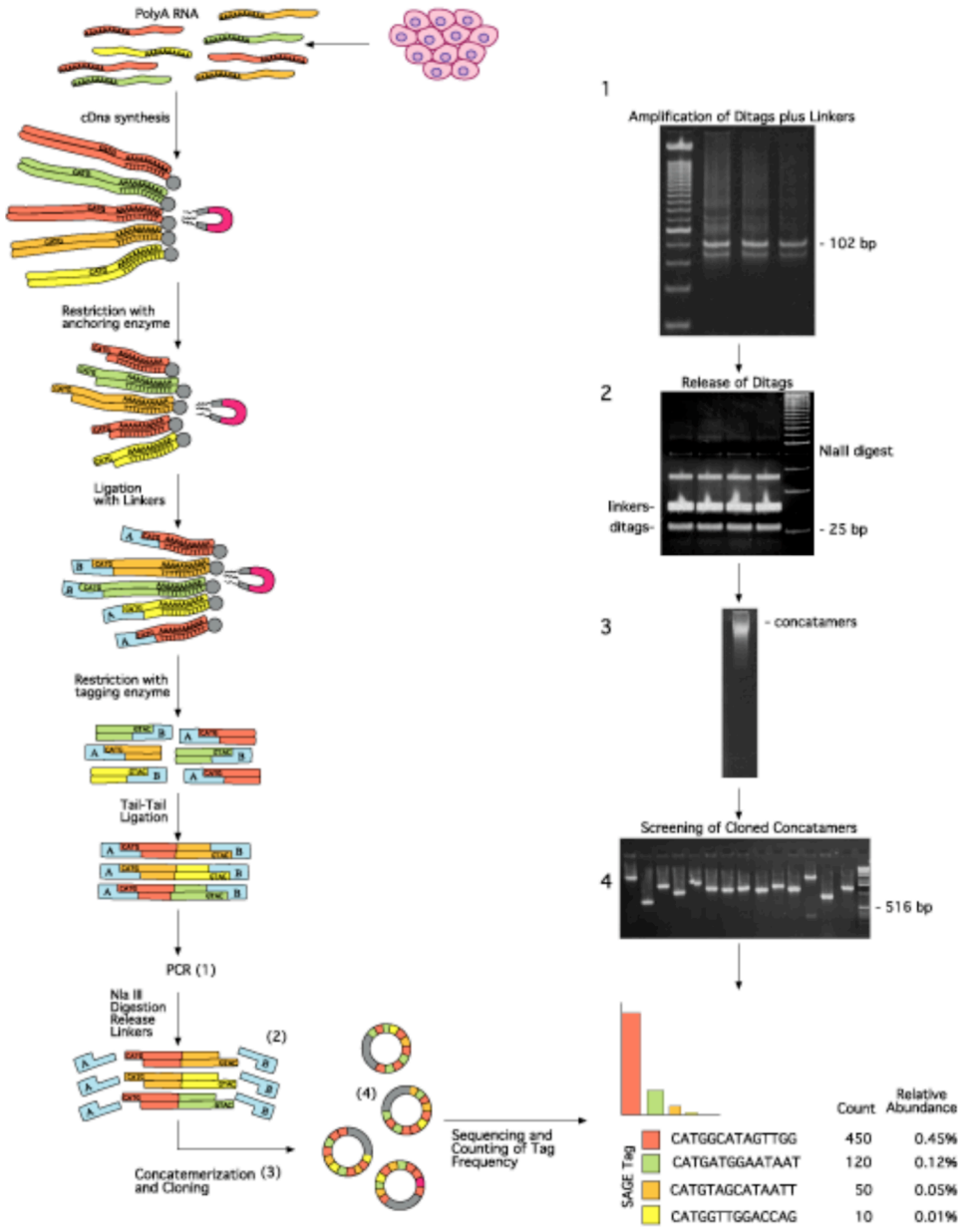



Figure 2.1. Schematic representation of the SAGE protocol.

Adapted from (Velculescu et al., 1995).

first contrast tests for lack of differential expression between resistant and parental strains and the second tests for consistent expression between the two resistant cell lines. Significance of the null hypotheses for these tests was set at the 95% level after a Bonferoni-type adjustment for the multiplicity of comparisons.

### 2.5. REAL-TIME QUANTITATIVE RT-PCR

RNA samples for real-time PCR analyses were the same as those used in SAGE. Primers for amplification of CtIP were designed through Primer3 web-based software (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The forward and reverse primers used were 5'GAAGAGGAGGAATTGTCTACTGCC3' and 5'CCACAAACGCTTTCTGCTTG3'. SYBR green PCR master mix was purchased from Applied Biosystems, CA. All of the real-time RT-PCR reactions were performed using the ABI Prism 7700 Sequence Detection system (Applied Biosystems, CA). Cycling conditions were 94 °C for 1 minute, followed by 40 cycles at 94 °C 12 seconds and 60 °C for 1 minute. We used 18S rRNA as the control gene for normalization. For each sample, PCR reactions were performed in triplicate for CtIP gene and control 18S rRNA gene. Statistical significance was determined by a student t-test.

### 2.6. WESTERN BLOT ANALYSIS

Cells were washed twice with ice-cold 1× PBS and then lysed with RIPA buffer [10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, PH 7.2, 1× protease inhibitor cooktail (Roche Applied Science, IN)]. Cell lysates were passed 10 times through 21-gauge needle and microcentrifuged at 21,000×g for 10 minutes at 4°C. Supernatants were collected and protein concentration was measured with Pierce Protein Assay Kit (Pierce, Rockford, IL), according to the

manufacturer's instruction. Equal amount (30-50 μg) of protein from each sample was separated on 6-10% SDS-PAGE and transferred to PVDF membranes by electroblotting. Blots were first blocked with blocking buffer [5% milk in 1× Tris-buffered saline containing 0.1% Tween-20 (TBS-T)] for 1 hour and then incubated with primary antibodies for 1-2 hours at room temperature or overnight at 4 °C. After washing 3 times in TBS-T, blots were incubated with horseradish peroxidase-labeled secondary antibodies for another 1 hour. Labeled proteins were detected using KPL Protein DetectorTM chemiluminescence detection reagents and exposed to X-ray films. Antibodies used for western analysis were: CtIP (14-1; T-16, Santa Cruz, CA; 19E8, GeneTex, TX), ER (HC-20, Santa Cruz, CA), anti-Flag (M2, Sigma, MO), β-actin (AC-15, Sigma, MO), BRCA1 (Ab-1, Calbiochem, CA), CtBP (MS mAb, BD Biosciences Pharmingen, CA), NCoR (rabbit Ab, Upstate, CA), Phospho-Estrogen Receptor alpha (Ser118) (16J4, Mouse mAb, Cell Signaling, MA).

### 2.7. ELECTROPORATION

Cells were seeded into 150-mm plates ($1.5\times10^6$ cells/plate) and allowed to reach a logarithmic growth phase the day before electroporation. On day of electroporation, cells were harvested, washed twice in 1× PBS, and resuspended in basal growth IMEM media (no supplements). Plasmid DNA (10 μg) was first added to the bottom of a 0.4 cm electroporation cuvet (Bio-Rad Laboratories, CA), followed by the addition of $4\times10^6$ resuspended cells. The cuvet was then placed into the cuvet holder of the Gene Pulser II system (Bio-Rad Laboratories, CA) and pulsed (270 V, 1050 μF). Electroporated cells were immediately transferred to normal growth medium and maintained in a 37°C/5% $CO_2$ humidified incubator until further experiments.

## 2.8. RNA INTERFERENCE

Potential siRNA target sites on CtIP mRNA were selected using the siRNA target finder algorithm from Ambion (http://www.ambion.com/techlib/misc/siRNA_finder). siRNA Expression Cassette (SEC), which encodes siRNA targeting the selected siRNA target site on CtIP mRNA (from 2492 to 2512, NM_002894; AATGATAGCTTGGAAGATATG), were generated downstream of the human H1 polymerase III promoter by PCR using the Ambion Silencer Express siRNA Expression Cassette Kits and following manufacturer's protocol. A negative control SEC expressing siRNA with no significant homology to human, mouse or rat genome sequences was also generated by the same method. The SECs were cloned into the mammalian expression pSEC-puro vector (Ambion) and sequenced to confirm that the clones were the desired sequences. To obtain cell clones that stably expressed siRNAs targeting CtIP, MCF-7 cells were transfected with either pSEC-CtIP-puro or pSEC-Control-puro by electroporation. One day later, puromycin (0.6 μg/ml) was added into the culture medium. After 3 weeks of selection, puromycin resistant clones were picked up, expended and analyzed for CtIP expression levels by immunoblotting. The stable clone that gave the lowest level of CtIP expression was frozen at –80°C for future use.

## 2.9. GENERATION OF DOUBLE-STABLY TRANSFECTED TET-OFF TAMR1 CELLS WITH DOXYCYCLINE INDUCIBLE RESTORATION OF CTIP

The Tet-off gene expression system was purchased from BD Biosciences Clontech, CA. Full-length human CtIP cDNA with three N-terminal Flag epitope tags was cloned into pTRE2hyg response vector (pTRE2hyg-FLAG-CtIP). To generate double-stable Tet-off TAMR1 cell clones, cells were co-transfected with pTet-Off and pTRE2hyg-FLAG-CtIP vectors. After electroporation, cells were plated in 10-cm dishes

and allowed to grow in regular medium containing 4-OH-TAM (1 μM) plus DOX (1 μg/ml) for 48 hours. Cells were then selected for resistance to G418 (800 μg/ml) and hygromycin B (200 μg/ml). Fresh DOX (1 μg/ml) was added to tamoxifen-containing medium (regular culture medium for TAMR1 cells) every 2 days to maintain a constant suppression of CtIP expression during the selection process. Hygromycin and G418 double-resistant colonies began to appear after 3-4 weeks of selection. Thirty-seven large and healthy colonies were isolated using cloning cylinders and transferred to individual wells for expansion. Each clone was first screened by immunoblotting using anti-Flag M2 antibody for DOX-responsive CtIP expression in the presence or absence of 1 μg/ml DOX. We then used anti-CtIP (14-1) antibody to assess the total CtIP protein in the positive clones. Clone #32, in which the level of total CtIP protein expressed upon the withdrawal of DOX was similar to that produced in the parental MCF-7 cells, was chosen for further studies.

### 2.10. IN VITRO CELL PROLIFERATION ASSAYS

Prior to treatment, cells were cultured in estrogen-free medium for 48 hours. On day 0, $1\times10^4$ cells in estrogen-free medium were plated in triplicate in 12-well plates. E2 (10 nM), 4-OH-TAM (10 nM) or ethanol (vehicle control, 1 μl/ml) was added directly into the medium at the same time. Fresh medium with the adequate treatment was changed every 2 days. Cell counts were performed at various time-points as indicated in figures.

### 2.11. DNA METHYLATION ANALYSES

#### Genomic DNA isolation

Genomic DNA from MCF-7 and TAMR1 cells was isolated by standard methods using phenol and Proteinase K.

**Bisulfite modification of genomic DNA**

Bisulfite treatment of genomic DNA converts non-methylated cytosines (C) to uracils (U), which will be ultimately detected as thymidines (T). The bisulfite modification reaction was carried out using CpGenome DNA modification kit (Chemicon, CA) following manufacturer's protocol.

**PCR amplification and genomic sequencing**

About 150 ng of bisulfite-treated genomic DNA was used as template for PCR reaction. Primers amplifying a CpG-rich region found on CtIP gene promoter (-384 to -102) were designed as following: forward primer: 5'TTTTTTTATAGTTTTAGAAAGTGTT3; reverse primer: 5'ACCCAAAAATAATACTAAAATAAC3'. PCR amplification was carried out with a hot-start at 94 °C for 10 minutes, followed by 36 cycles of amplification (30 sec at 94 °C, 30 sec at 52 °C and 40 sec at 70 °C) and a final 7 minutes extension at 70 °C. Each PCR product was resolved on a 1.2% agarose gel and purified with QIAquick gel extraction kit (Qiagen, CA). Purified PCR products were subject to direct sequencing using an ABI 3700 DNA Analyzer (Applied Biosystems, CA). The same forward primer used for PCR amplification was used as sequencing primer. We also treated TAMR1 cells with 5-aza-2'-deoxycytidine, a DNA methylation inhibitor, at different concentration (1, 2, and 3 μM) for 72 hours.

### 2.12. IMMUNOPRECIPITATION

To prepare cell lysates, cells were washed twice in ice-cold 1× PBS and lysed in 0.5% NP-40 buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 10% glycerol, and protease inhibitor cooktail). For immunoprecipitation, approximately 500 μl

cell lysates (from ~$1\times10^7$ cells) were co-incubated with each indicated antibody for 2 hours at 4°C. After adding 50 µl protein A/G beads (Santa Cruz, CA), the mixture was rocked at 4°C for 2 hours. Beads were then extensively washed three times with 0.5% NP-40 buffer and finally boiled for 10 minutes in 2× SDS loading buffer (0.1M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% β-mercaptoethanol and 0.004% bromphenol blue). Supernatants were loaded onto 6-10% SDS-PAGE, followed by western blot analysis using primary antibodies as indicated. Antibodies used for IP are as following: CtIP (H-300, Santa Cruz, CA), BRCA1 (C-20, Santa Cruz, CA), ER (HC-20, Santa Cruz, CA), and CtBP (H-440, Santa Cruz, CA).

### 2.13. CHROMATIN IMMUNOPRECIPITATION ASSAYS

ChIP assays were carried out with the ChIP assay kit (Upstate Biotechnology, IL) as previously described (Shang et al., 2000). All reagents used were provided by the kit. MCF-7 cells were grown in 150 mm plates to 90% confluence in hormone-free media supplemented with 5% charcoal-dextran stripped serum for 7 days. Cells were then treated with $10^{-8}$ M estradiol, $10^{-6}$ M 4-OH-TAM or control vehicle for 1 hour. After being washed once with PBS (room temperature), cells were subsequently fixed with 1% formaldehyde in PBS at $37^0$C for 10 min. At the end of fixation, cells were quickly rinsed with ice-cold PBS twice, scraped into 1 ml of ice-cold PBS and spinned down at 3000 rpm for 2 min at 4 $^0$C. Cell pellets were lysed with 300 µl of lysis buffer and incubated on ice for 10 min. Lysates were sonicated three times for 15 seconds each at 50% duty cycle using an Out Control intensity of 3 followed by microcentrifugation at 4 °C, 14000×g for 10 minutes. Soluble chromatin was diluted 1:10 in dilution buffer and immunocleared by incubating with sheared salmon sperm DNA–Protein A/G agarose (50 µl/1 ml chromatin preparation) for 2 h at 4°C. For immunoprecipitation of protein-DNA complex, specific

antibodies were added and incubated overnight at 4°C, followed by addition of 50 µl salmon sperm DNA–Protein A/G agarose for another 1 h at 4°C. Precipitates were washed sequentially with 1 ml of low-salt wash buffer, high-salt wash buffer, and LiCl wash buffer and were washed twice with 1 ml of TE buffer. The DNA-protein complex was eluted with 100 $\mu$L of freshly made elution buffer (1% SDS, 0.1 M $NaHCO_3$) and heated at 65 °C overnight to reverse the formaldehyde cross-linking. DNA fragments were then purified with a Qiagen Gel Extraction kit (Qiagen, CA) and subject to PCR amplification with the following primers for the promoter region of the pS2: –430F: 5'-ATTAGCTTAGGCCTAGAC-3'; –245R: 5'-TACTCATATCTGAGAGGCCCT-3'.

### 2.14. HUMAN BREAST CARCINOMA SAMPLES

Primary breast cancer formalin-fixed, paraffin-embedded tissue samples were collected retrospectively from 59 postmenopausal patients with stage II-III ER (+) breast carcinomas (median age 78 years; range 60 to 92). The 59 patients were treated at a single institution (Instituto Valenciano Oncología, Valencia, Spain) between 1999 and 2002 with four months neoadjuvant endocrine therapy consisting of tamoxifen (23 patients) or letrozole (36 patients) for large non-operable or locally advanced ER (+) breast cancers. All patients gave written informed consent before the submission of tumor samples for CtIP analyses, and the local ethic committee approved the study protocol and informed consent form. Determination of response to the referred endocrine adjuvant therapy was made after 4 months of patient follow-up.

### 2.15. IMMUNOHISTOCHEMISTRY AND STATISTICAL ANALYSES

Samples were obtained from the described primary breast cancer tissues prior to endocrine therapy. Tissue sections were obtained from formalin fixed paraffin embedded

samples. Tissue sections were deparaffinized with xylene and rehydrated by graded alcohols. Endogenous peroxidase activity was blocked with 3% $H_2O_2$ in water for 10 minutes. The sections were then boiled in 10mM Citrate Buffer (pH 6.0) for 15 minutes in a microwave oven followed by a 20-minute cool down at room temperature to retrieve antigenic epitopes. The tissue samples were exposed to 10% goat serum in PBS, in order to block unspecific antibody binding, for 30 minutes and incubated with an anti-CtIP antibody (H-300, rabbit polyclonal, 1:100 dilution) for 1 hour at room temperature. The sections were then washed, incubated with a goat-anti-rabbit secondary antibody, developed with DAB, and counter stained with Harris´ hematoxylin for microscopic evaluation. CtIP expression levels were then scored blindly using the immunoreactive score (IRS) method as previously described (Chui et al., 1996; Friedrichs et al., 1993). In brief, the IRS was calculated by multiplying the percentage of CtIP-positive cells (scored 0 to 4: 0, 0%; 1, 0-25%; 2, 26-50%; 3, 51-75%; 4, >75%) with the CtIP staining intensity (scored 0 to 3: 0, none; 1, weak; 2, moderate; 3, strong). We used One-way ANOVA followed by Tukey's test post hoc comparisons, and Pearson's correlation test to assess the association between CtIP protein status (raw IRS scores) and clinical response to endocrine therapy. All statistical analyses were two side, and $p<0.05$ was considered as statistically significant. Analyses were conducted using SPSS version 11.5 software (SPSS Inc., Chicago, Illinois).

### 2.16. META-ANALYSIS OF BREAST CANCER MICROARRAY DATA SETS

CtIP gene expression profiles and clinicopathological data of 828 breast carcinomas were obtained from seven published and publicly available breast cancer microarray data sets (Gruvberger et al., 2001; Perou et al., 1999; Sorlie et al., 2003; van de Vijver et al., 2002; Wang et al., 2005; West et al., 2001; Zhao et al., 2004). The

Oncomine cancer microarray database (http://ww.oncomine.org) was employed for data collection, processing and visualization (Rhodes et al., 2004). CtIP gene expression was log-transformed, median centered for each gene expression dataset, and SD normalized to 1 per array. The gene module application was employed for differential expression analysis (two side t-test). We used a meta-analysis approach to determine and summarize the CtIP mRNA expression pattern from the seven independent studies. Meta-analysis is a set of statistical techniques or procedures for combining information from different studies. In our studies, we combined CtIP gene expression information from the above-mentioned seven publicly available microarray data sets, which contain gene expression information all from ER positive or negative primary breast carcinomas. However, the topics of these published studies were neither related to CtIP itself nor to response to endocrine therapy, and none of these studies has examined CtIP gene expression levels in their individual microarray data set. In fact, we collected only CtIP gene expression information that has already been included in the microarray data sets. We computed summary estimates (effect sizes) of CtIP expression changes by the Standardized Mean Difference (SMD) method using the exact t values and sample size for each groups. To calculate the pooled effects of CtIP profile each study was weighed by the inverse of the individual and between-study variance according to a random effects model. Meta-analysis was carried out using Comprehensive Meta-Analysis software (Biostat Inc., NJ). All effect sizes were presented with 95% CI based on the estimated variances.

# Chapter 3: Global gene expression analysis identifies CtIP (RbBP8) as one of the most significantly downregulated transcripts in tamoxifen resistant human breast cancer cells

## 3.1. INTRODUCTION

The role of estrogen as a fundamental factor in the etiology and progression of human breast cancer has been well documented. It was already observed, more than 100 years ago, that ovariectomy could lead to breast cancer regression in pre-menopausal patients (Beatson, 1896). The extent of exposure to ovulary cycles is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer (Pike et al., 1993). Estrogen sustains the growth of breast cancer cells that express the receptor for this hormone. Indeed, approximately 70% of breast cancer patients are positive for estrogen receptor (ER) or progesterone receptor (PgR) expression at diagnosis. Therefore, for a long time, treatment of breast cancer has been directed towards inhibiting the tumor promoting effects of estrogen. Tamoxifen, a non-steroidal antiestrogen, has been used as standard first-line endocrine therapy for patients with estrogen receptor positive breast cancers since 1970s, during which time over 400,000 lives have been saved (Jordan, 2003). Although other drugs such as aromatase inhibitors may be slightly more effective than tamoxifen, tamoxifen is still the most used antiestrogen and will remain so for several years to come because of its well-documented benefits (Wong and Ellis, 2004). Tamoxifen is also the first approved drug by the FDA as a cancer chemopreventive for reducing breast cancer incidence in both pre- and post-menopausal women at high risk (Jordan, 2003). As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival in early breast cancer (Osborne,

1998). It is also effective for patients with untreated metastatic breast cancer (Osborne, 1998).

Despite the benefits of tamoxifen in treating breast cancer, unfortunately, many tumors that initially respond to tamoxifen therapy develop resistance. This phenomenon has become a serious obstacle in breast cancer treatment. For many women, resistance develops after the first phase of tamoxifen treatment (Katzenellenbogen et al., 1997). Interestingly, in most cases tamoxifen resistance is reversible, suggesting a cellular adaptation mechanism rather than a permanent genetic alteration (Katzenellenbogen et al., 1997). Moreover, almost all patients with advanced metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse and die from their disease (Normanno et al., 2005). The mechanisms involved in the development of tamoxifen resistance are still poorly understood. Numerous mechanisms have been proposed to contribute to the development of tamoxifen resistance, including altered drug metabolism, loss of expression or mutation of ER (This is a rare occurrence in the development of resistance. In fact, loss of ER expression has been demonstrated only in 17-28% of patients with acquired resistance to tamoxifen (Gutierrez et al., 2005; Johnston et al., 1995) and mutations of ER have rarely been found in human primary breast carcinomas.), lack of expression of progesterone receptor, increased expression of estrogen receptor β, post-translational modifications of ER (These modifications could be the result of oncogenic activation of other growth factor signaling pathways.), altered expression of co-regulators and increased growth factor signaling (See Chapter 1 for details). Interestingly, a recent study demonstrated that DIBA, an ER zinc finger inhibitor, restores the antagonistic action of tamoxifen in tamoxifen resistant breast cancer cells through targeted disruption of the ER DNA-binding domain and its

interaction with the proximal N-terminal domain to suppress ligand-dependent and -independent ER transcription and influence the recruitment of cofactor to the ER (Wang et al., 2006). These findings strengthen the important role of ER in the development of tamoxifen resistance and suggest a possible new approach in modifying tamoxifen resistance (Wang et al., 2006). However, whether these postulated mechanisms so far could explain resistance to tamoxifen therapy in a majority of patients is still unclear. Thus, studies geared at better understanding the precise and most common mechanisms involved in tamoxifen resistance are of considerable clinical significance. Better understanding the precise mechanisms involved in tamoxifen resistance and tumor recurrence may suggest novel strategies to circumvent tamoxifen resistance and improve survival rate in breast cancer.

We hypothesize that most cases of anti-estrogen resistance are the result of a cellular adaptation phenomenon that will have a direct reflection in the patterns of global gene expression. With the aim of identifying key genes involved in the development of tamoxifen resistance, we defined the global gene expression profiles of recently developed tamoxifen resistant MCF-7 breast cancer cell lines and compared them with their tamoxifen sensitive parental MCF-7 counterpart by using serial analysis of gene expression (SAGE). We observed that the mRNA expression of CtIP, a BRCA1- and CtBP-interacting protein, is 15-fold downregulated in the tamoxifen resistant cells when compared to their tamoxifen sensitive counterparts.

## 3.2. RESULTS

### 3.2.1. Generation and characterization of tamoxifen resistant MCF-7 cell line variants

To better understand the phenomenon of tamoxifen resistance, we generated two independently derived isogenic MCF-7 breast cancer cell line variants (TAMR1 and TAMR2) that are resistant to the inhibitory growth effects of tamoxifen. The TAMR1 and TAMR2 variants were developed by growing parental MCF-7 cells under chronic exposure to 4-OH-TAM (1 μM) for approximately two years (Figure 3.1.). These cells were maintained in phenol red-free IMEM medium containing 5% fetal bovine serum (TAMR1) or 5% charcoal-stripped fetal bovine serum (TAMR2) plus 4-OH-TAM (1 μM). As shown in Figure 3.2.A, in contrast to their parental MCF-7 cells, both TAMR1 and TAMR2 cells cultured in estrogen-depleted medium proliferated in the presence of tamoxifen. Additionally, despite continuous exposure to 4-OH-TAM, both tamoxifen resistant variants are still estrogen responsive (Figure 3.2.B) as compared to control vehicle treatment (Figure 3.2.C) and express equivalent levels of ER protein as their parental MCF-7 cells (Figure 3.4.C). Therefore, both TAMR1 and TAMR2 cells express functional ER, as do their parental MCF-7 cells. The tamoxifen resistant phenotypes of these cells appear not to be a consequence of changes in ER expression or function. These data are also consistent with the clinical findings that the majority of patients with acquired resistance to tamoxifen still express functional estrogen receptors (Gutierrez et al., 2005; Johnston et al., 1995).

### 3.2.2. SAGE libraries generation

To identify key changes in gene expression that are associated with, and may cause, the development of tamoxifen resistance, we performed extensive Serial Analysis

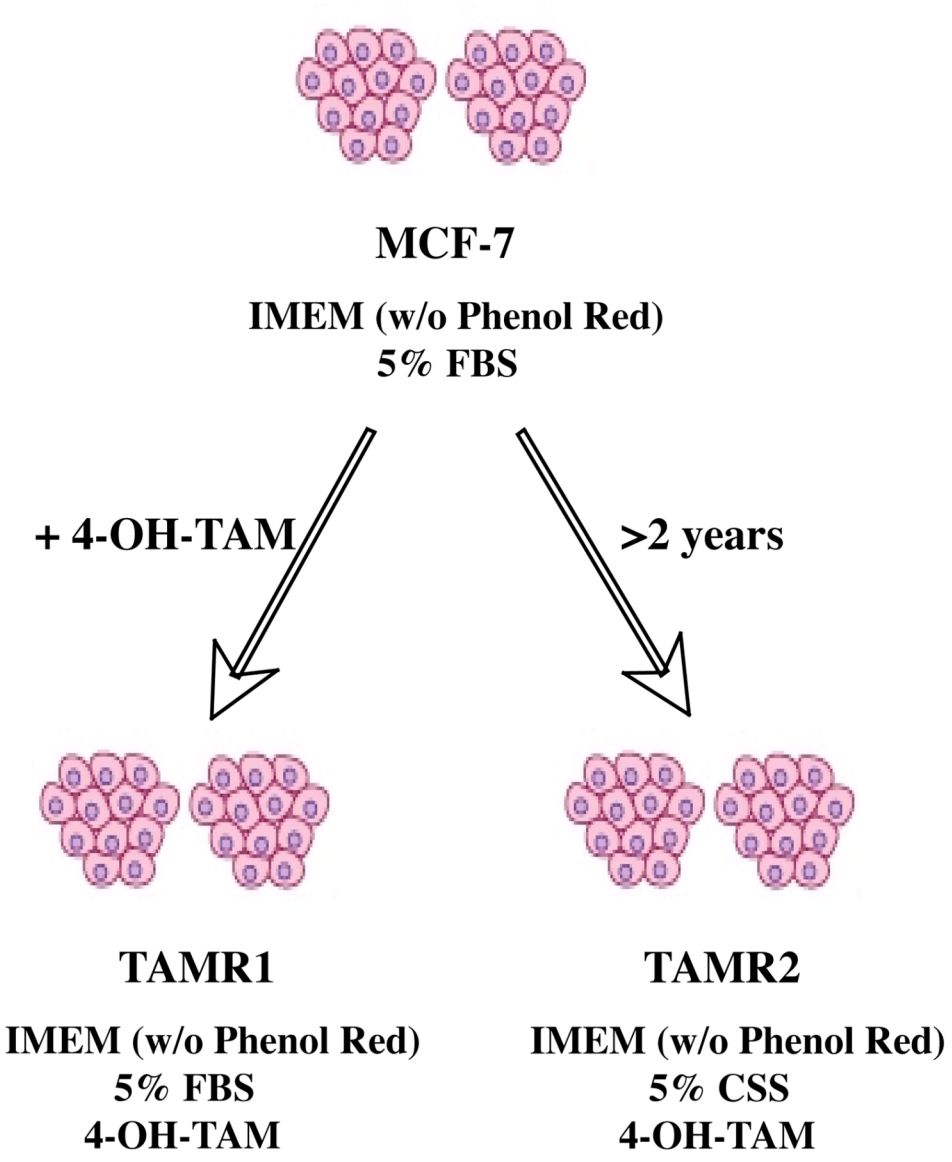



Figure 3.1. Development of tamoxifen resistant MCF-7 cell line variants.

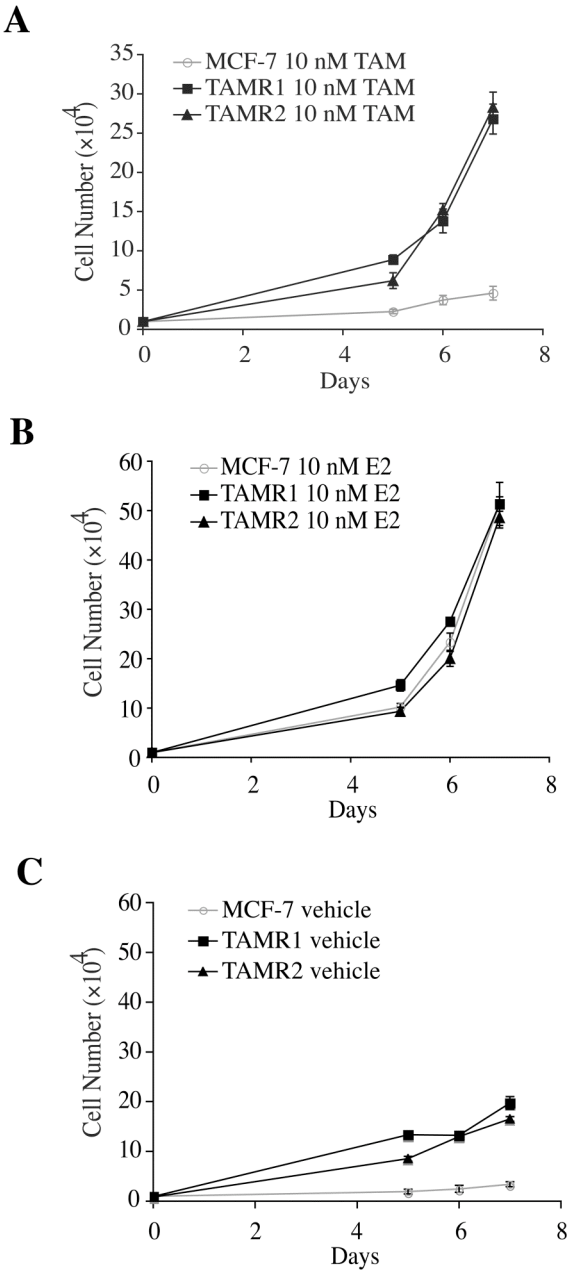



Figure 3.2. *In vitro* characterization of parental MCF-7, TAMR1 and TAMR2 cells.

Growth curves of parental MCF-7, TAMR1 or TAMR2 cells cultured in the presence of 10 nM 4-OH-TAM (A), 10 nM E2 (B) or vehicle (C). The results are presented as mean ± SEM of triplicate determinations.

of Gene Expression (SAGE) analyses on the TAMR1 and TAMR2 variants described above and their parental MCF-7 cell line (all analyses were performed while at exponentially growing). SAGE is a novel and powerful approach to analyze global gene expression. The SAGE technique is based on the principle that a short nucleotide sequence (i.e. 14 base pairs tag) contains sufficient information to uniquely identify a transcript, provided it is isolated from a defined position within the transcript (Velculescu et al., 1995; Velculescu et al., 1997). Using this short sequence and ligating series of "tags" back to back (concatemers), allows for the efficient analysis, both qualitative and quantitative, of all mRNA transcripts. We sequenced a minimum of 60,000 tags per cell line library providing virtually a complete picture of the entire transcriptome. This allowed us to identify key changes of gene expression involved in tamoxifen resistance.

### 3.2.3. Differential expression analysis of SAGE libraries

The SAGE libraries obtained from the parental MCF-7, TAMR1 and TAMR2 cells were analyzed using a unique and state-of-the-art approach, the i-Sight discovery platform. A summary of i-Sight Discovery platform is shown in figure 3.3. This platform is a statistical advanced platform, which provides numerous web based tools specially developed for SAGE analyses. It allows one to organize the genes of any analysis or comparison by functional categories (based on gene Ontology annotations) and evaluate the statistical contribution of each functional category, as representatively shown in Table 3.1.

The MLCA analysis at the gene-level data identified 243 genes that were upregulated and 247 downregulated by at least a fold of 2 at a statistical significance level of 95% in the tamoxifen resistant relatively to tamoxifen sensitive parental MCF-7

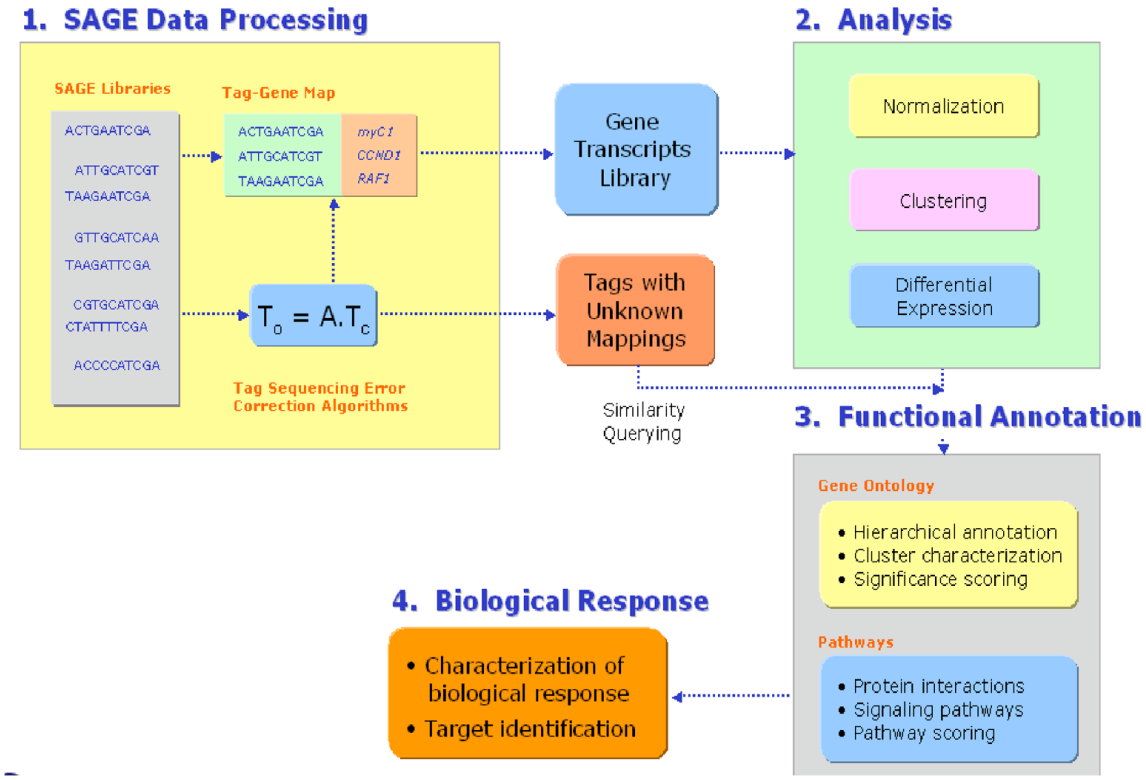



Figure 3.3. Summary of i-sight Discovery platform.

| Gene Ontology: biological_process | Selected Genes | Total Genes | Score* |
|---|---|---|---|
| cell communication | 14 | 539 | |
| cell-cell signaling | 3 | 68 | |
| transmission of nerve impulse | 3 | 18 | 4.97 |
| synaptic transmission | 3 | 18 | 4.97 |
| signal transduction | 10 | 381 | |
| cell surface receptor linked signal transduction | 3 | 83 | |
| intracellular signaling cascade | 5 | 91 | |
| protein kinase cascade | 3 | 31 | 2.89 |
| cell growth and/or maintenance | 63 | 1717 | |
| cell cycle | 13 | 183 | 2.12 |
| mitotic cell cycle | 3 | 43 | |
| regulation of cell cycle | 10 | 126 | 2.37 |
| cell cycle checkpoint | 3 | 17 | 5.27 |
| cell organization and biogenesis | 7 | 73 | 2.86 |
| cytoplasm organization and biogenesis | 5 | 55 | 2.71 |
| organelle organization and biogenesis | 4 | 44 | 2.71 |
| cytoskeleton organization and biogenesis | 3 | 36 | |
| cell proliferation | 7 | 157 | |
| regulation of cell proliferation | 6 | 75 | 2.39 |
| negative regulation of cell proliferation | 4 | 43 | 2.78 |
| metabolism | 35 | 1194 | |
| carbohydrate metabolism | 4 | 41 | 2.91 |
| lipid metabolism | 3 | 71 | |
| nucleobase, nucleoside, nucleotide and nucleic acid | 15 | 531 | |
| transcription | 10 | 244 | |
| transcription, DNA-dependent | 10 | 235 | |
| regulation of transcription, DNA- | 3 | 36 | |
| transcription from Pol II promoter | 7 | 177 | |
| regulation of transcription from Pol II | 3 | 103 | |
| protein metabolism | 12 | 426 | |
| protein catabolism | 4 | 67 | |
| proteolysis and peptidolysis | 4 | 67 | |
| protein-ligand dependent protein | 3 | 31 | 2.89 |
| ubiquitin-dependent protein | 3 | 31 | 2.89 |
| protein modification | 5 | 165 | |
| response to stress | 3 | 115 | |
| transport | 8 | 239 | |
| protein transport | 3 | 70 | |
| intracellular protein transport | 3 | 58 | |

Table 3.1. Representative partial profile and display format of genes identified by the i-Sight platform and functional categorization (unrelated experiment).

(*) Scores significant at the 95.0% level according to a chi-square test are shown. Categories highlighted in blue are those at the most detailed level of the given annotation hierarchy that have a significant score. All categories are active links that lead to the specific genes ID.

| Gene | Fold | Role |
|---|---|---|
| Upregulated | | |
| CDC45L | 9 | Promotes initiation of S-phase |
| CCNA2 | 8 | Promotes G1/S and G2/M transitions |
| WEE1 | 4 | Inhibits G2/M transition |
| CCNF | 4 | Promotes G2/M transition (?) |
| EBAG9 | 3.5 | Estrogen receptor activated |
| MPHOSPH6 | 2.6 | Overexpressed in M-phase |
| CDC20 | 2 | Promotes exit from M-phase |
| Downregulated | | |
| CtIP(RBBP8) | 15 | Tumor suppressor; Regulates G1/S checkpoint |
| PLK | 10 | Involved in M-phase activities; induces mitosis |
| FAT | 7 | Tumor suppressor (cadherin family); control of cell proliferation |
| TSSC3 | 6 | Tumor suppressor |
| FKB1 | 6 | Activation induces anti-apoptotic genes |
| CDC5L | 6 | Promotes G2/M transition |
| CDC25B | 6 | Promotes G2/M transition |
| REA | 2 | Repressor of Estrogen Receptor activity |

Table 3.2. Cell-cycle and cell proliferation related genes differentially expressed in tamoxifen resistant MCF-7 cells.

cells. Some of the key genes identified using this approach and their roles in cell cycle regulation are summarized in Table 3.2. The differential pattern seems to indicate that in tamoxifen resistant cells, most upregulated, such as CDC45L, CCNA2, CCNF and CDC20, are those related to transition through cell cycle and on the other hand many anti-proliferative genes, including PLK, FAT, TSSC3 and REA, are downregulated as are several mitosis-related genes. Among the key genes identified, one gene named CtIP (also known as Retinoblastoma binding protein 8, RBBP8) was particularly interesting to us, because it was significantly down-regulated (15-fold) in both tamoxifen resistant cell lines when compared to their tamoxifen sensitive parental MCF-7 counterpart (Figure 3.4.A).

### 3.2.4. Validation of SAGE Findings

In order to validate the SAGE findings, we performed real-time RT-PCR using the same RNA samples for SAGE analysis and confirmed significantly lower CtIP mRNA expression in the tamoxifen resistant cells than in the tamoxifen sensitive parental MCF-7 cells (Figure 3.4.B). Next, we determined whether the differential expression of CtIP detected at the mRNA level could also be observed at the protein level. By western analysis, we detected high levels of CtIP protein in the tamoxifen sensitive parental MCF-7 line, but not in the two tamoxifen resistant lines (Figure 3.4.C). In fact, both TAMR1 and TAMR2 cells appear to express little or no CtIP protein. Together, the above data confirm that the expression of CtIP is significantly decreased in tamoxifen resistant cells at both mRNA and protein levels and raise the possibility that CtIP silencing could be a novel mechanism for the development of tamoxifen resistance.

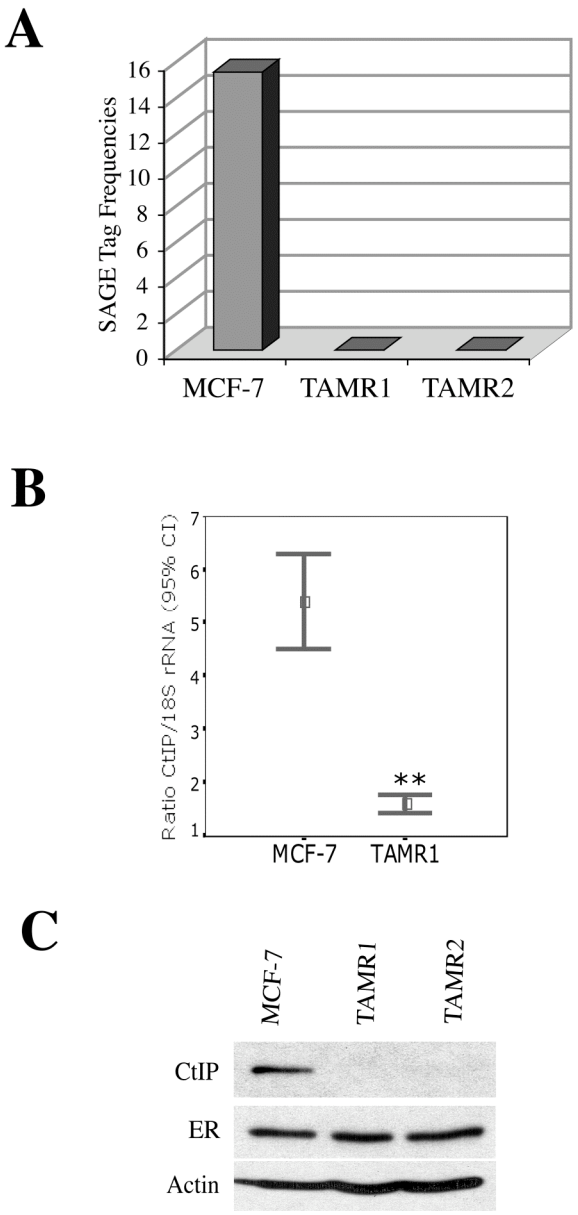



Figure 3.4. CtIP expression levels in parental MCF-7, TAMR1 and TAMR2 cells

as determined by SAGE (A), real-time PCR (B) and western analysis (C). ER protein levels in these cells were also determined (C).

### 3.2.5. CtIP gene promoter methylation study

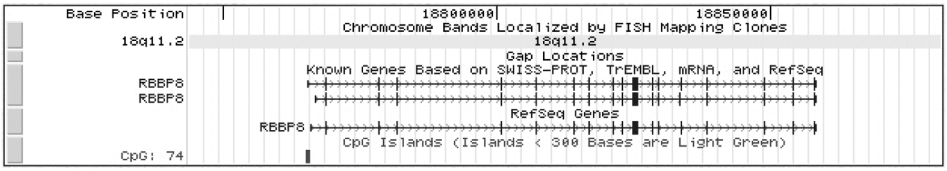
It is well known that methylation of cytosine residues at cytosine-guanine sites (CpG islands) usually located in the promoter region of genes, can be a specific mechanism of transcriptional repression (Nan et al., 1997). Our search through the UCSC Genome Browser has identified typical CpG islands in the CtIP promoter region (Figure 3.5.A). To determine whether silencing of CtIP expression in tamoxifen resistant cells (TAMR1 cells) is the result of promoter methylation, we performed bisulfite genomic sequencing of a CpG-rich island found on the CtIP gene promoter region (from -384 to –102). We did not find any evidence of CpG methylation at the investigated region. In addition, we also did not observe any significant restoration of CtIP expression after treating TAMR1 cells with 5-aza-2'-deoxycytidine, a CpG methylation inhibitor (Figure 3.5.B). Thus, in the tamoxifen resistant cells, methylation appears not to be the major mechanism for CtIP silencing.

Together, the above data identify and confirm that the expression of CtIP is significantly decreased in tamoxifen resistant cells at both mRNA and protein levels and raise the possibility that CtIP silencing could be a novel mechanism for the development of tamoxifen resistance. We also ruled out promoter methylation as the mechanism for Ctip silencing.

## 3.3. DISCUSSION

The clinical use of antiestrogens such as tamoxifen has been shown to be extremely effective in inhibiting ER-positive breast cancer progression. However, acquired resistance to tamoxifen therapy remains one of the major clinical challenges to

**A**




**B**

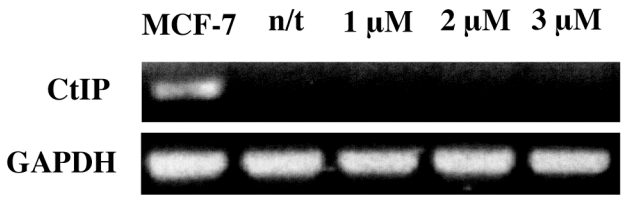



Figure 3.5. CtIP promoter methylation study.

(A) Notice a CpG Island 5' of CtIP coding region (from UCSC Genome Browser). (B) TAMR1 cells were treated without (n/t) or with 5-Aza-2'-deoxycytidine (1, 2, or 3 μM) for 72 hours. CtIP expression was determined by RT-PCR. Untreated parental MCF-7 cells were used as a positive control (left lane). GAPDH was used as loading control. In addition, bisulfite genomic sequencing of a CpG-rich island found on the CtIP gene promoter region (from -384 to –102) didn't find any evidence of CpG methylation.

overcome in the treatment of breast cancer. Unfortunately, the precise mechanisms of tamoxifen resistance are still poorly understood. Indeed, to date genes that confer an acquired resistance to tamoxifen have not been identified yet. Although numerous postulated mechanisms as previously described have been proposed to contribute to the development of tamoxifen resistance, none of them so far has been validated to explain resistance to tamoxifen therapy in a majority of patients. Interestingly, most of the enumerated putative causes for resistance to tamoxifen will be reflected by changes in gene expression of key players representing directly or indirectly the involved pathways. Therefore, we hypothesized that most cases of tamoxifen resistance are the result of a cellular adaptation phenomenon, which will have a direct reflection in the patterns of global gene expression.

In the current study, we developed and *in vitro* characterized MCF-7 human breast cancer cell line variants that are resistant to the growth inhibitory effects of tamoxifen. Based on the results that a functional ER is still present in these cells and apparently regulates cell proliferation, the development of tamoxifen resistance in these new isogenic cells appears not to result from loss or changes of ER expression/function. To test our hypothesis, we have used a powerful global gene expression methodology SAGE to identify key players implicated in resistance to tamoxifen by defining global gene expression profiles of these two independently derived isogenic tamoxifen resistant MCF-7 breast cancer cell line variants and their parental tamoxifen sensitive MCF-7 line. Through mining the SAGE databases using state-of-the-art bioinformatic and statistical approaches, we observed different gene expression patterns between tamoxifen resistant cells and their parental tamoxifen sensitive cells. As a result, our studies also led to the

identification of a number of highly differentially expressed genes in tamoxifen resistant MCF-7 cells.

Among key transcripts with increased expression in tamoxifen resistant cells, we identified several important regulators of cell cycle transition, including CDC45L, CCNA2 (cyclin A2) and CCNF (cyclin F). CDC45L is an essential component of the replication fork involved in DNA unwinding during replication elongation (Pacek et al., 2006). Besides its role in the initiation of DNA replication, CDC45L has also been implicated in initiation of S-phase (Saha et al., 1998). Thus, the significant upregulation of this gene in tamoxifen resistant cells may indicate a novel link between enhanced DNA replication process and tamoxifen unresponsiveness. Cyclin A2 (CCNA2) binds and activates cdk2 kinase and promotes DNA synthesis in S phase and G2/M transition (Pagano et al., 1992). Deregulation of the expression of this cyclinA2 was shown to be associated with oncogenesis in some cancers (Dutta et al., 1995; Masaki et al., 2003). More interestingly, it was reported that the cyclin A2/cdk2 complex can phosphorylate and enhance the transcriptional activity of ER (Trowbridge et al., 1997). Moreover, our recent paper has shown that tamoxifen, as well as E2, induce expression of cyclin A2 in MCF- 7 cells (Hodges et al., 2003). Interestingly, in our analysis from the isogenic MCF-7 variants, we observed that only in the tamoxifen resistant variants cyclinA2 is overexpressed about 8 fold compared to parental MCF-7 cells. On the other hand we did not find major differences in other critical cyclins such as cyclin D1 and E in all these exponentially growing cells. This raises the possibility that cyclinA2 might be directly induced by tamoxifen, which in turn, coupled with cdk2 kinase, increases the transcriptional activity of ER and then induces the expression of several growth related genes, constituting a positive regulatory loop in tamoxifen resistant cells. Therefore, the

role of cyclin A2 in the development of tamoxifen resistance deserves additional investigation. In addition to cyclin A2, we also observed a significant increase in CCNF (cyclin F) expression in tamoxifen resistant cells compared with parental cells. Cyclin F is a new member of the cyclin family and related to cyclin A and B by sequence (Kraus et al., 1994). This member also belongs to the F-box protein family which is characterized by an approximately 40 amino acid motif, the F-box (Bai et al., 1996). However, the function of cyclin F is still unknown. Thus far, no association of cyclin F expression with breast cancer and tamoxifen resistance has been reported. Whether this cyclin plays a role dependent or independent of other cyclins in breast cancer progression or the development of resistance to tamoxifen requires further functional characterization.

Interestingly, SAGE also identified upregulation of EBAG9 (estrogen receptor binding site associated antigen 9), an estrogen-responsive gene, in tamoxifen resistant cells. EBAG9 has been shown to be upregulated after estrogen treatment, which is mediated through ER which binds to the estrogen-responsive element (ERE) found in the 5'-flanking region of this gene (Ikeda et al., 2000; Watanabe et al., 1998). EBAG9 gene is located at chromosome 8q23, a region that is frequently amplified in tumors (Ikeda et al., 2000). Moreover, its protein product is a tumor-associated antigen that is expressed at high frequency in a variety of cancers including breast cancer (Ikeda et al., 2000; Watanabe et al., 1998). Based on these observations together with SAGE findings, we can speculate that EBAG9 might play a role in breast cancer progression, perhaps through its modulation of hormonal regulation and/or response of breast cancer cells.

In contrast to the upregulated genes found in tamoxifen resistant cells, most downregulated key genes have anti-proliferative function, some of which also serve as

tumor suppressors. One of these downregulated genes, CtIP (RbBP8), appeared to be particularly interesting due to the significant level of its deregulation (15-fold) and the importance of its direct links with important tumor suppressors (BRCA1 and Rb) and regulators of gene expression (CtBP). Therefore, we functionally characterized its mechanistic role in the development of tamoxifen in further detail and will describe and discuss the role of CtIP in resistance to tamoxifen in detail in next chapters of this dissertation.

The transcript of the gene FAT, which an ortholog of the Drosophila fat gene, was found significantly decreased (7-fold) in tamoxifen resistant cells. The Drosophila fat locus encodes a tumor suppressor gene, and recessive (loss-of-function) mutations lead to hyperplastic overgrowth of the imaginal discs (Bryant et al., 1988). The gene product belongs to the cadherin superfamily, a group of integral membrane proteins characterized by the presence of cadherin-type repeats (Mahoney et al., 1991) . It encodes a transmembrane protein containing 34 cadherin repeats in association with a number of other motifs (Mahoney et al., 1991) . Analysis of the expression of human FAT homolog in fetal and adult tissues revealed that FAT mRNA is present in many epithelial and some endothelial and smooth muscle cells (Dunne et al., 1995). Thus, FAT probably functions as an adhesion molecule and signaling receptor, and is likely to be important in developmental processes and cell communication. Currently, whether FAT is involved in mammary development or modulates breast epithelium functions is unknown. In addition to the FAT tumor suppressor, we also observed that TSSC3 (tumor suppressing subtransferable candidate 3) was downregulated 6-fold in tamoxifen resistant cells compared to sensitive parental cells. The TSSC3 gene is one of several genes in the imprinted gene domain of chromosome 11p15.5, which is considered to be an important

tumor suppressor gene region (Qian et al., 1997). TSSC3 is also the first apoptosis-related gene found to be imprinted in placenta, liver, and fetal tissues, where it is expressed from the maternal allele during normal human development (Qian et al., 1997). Moreover, TSSC3 appears to be a potential growth inhibitory gene, since in a study that examined the imprinting status of TSSC3 in normal human adult brain, neuroblastomas, medulloblastomas, and glioblastomas, strong allelic bias resembling imprinting could only be detected in most tumors but not in normal human adult brain or blood (Muller et al., 2000).

We have discussed in chapter one the importance of the ER coregulatory proteins in mediating transcriptional activity by the ER and therefore altered expression of coregulators may contribute to the tamoxifen resistance phenotype. Interestingly, in our SAGE studies, we observed a significant decrease in the expression of a gene named REA (repressor of estrogen receptor activity). REA encodes a 37-kDa protein that is an ER-selective coregulator (Montano et al., 1999). It interacts directly with ER and that this interaction is ligand-dependent and is observed preferentially with the dominant negative ER and with the antiestrogen-bound ER (Montano et al., 1999). REA markedly potentiates the inhibitory effectiveness of dominant negative ERs as well as the inhibitory activity of antiestrogens. In addition, REA competes with the coactivator SRC1 for modulation of ER transcriptional activity, while the protein itself lacks intrinsic transcription-repression activity (Montano et al., 1999). These *in vitro* findings suggest that REA may play an important role in determining the sensitivity of estrogen target cells, including breast cancer cells, to antiestrogens and estrogens. Animal studies (Mussi et al., 2006) showed that REA deletion in mice was embryonic lethal. REA heterozygous mutant mice exhibit specific morphological phenotypes such as faster mammary ductal

elongation in virgin animals, increased lobuloalveolar development during pregnancy, and delayed mammary gland involution after weaning, which are associated with significantly increased cell proliferation and ER transcriptional activities (Mussi et al., 2006). These *in vivo* observations indicate that REA is a physiological modulator of ER function in the mammary gland and consequently a reduction or loss of REA function may cause overactivation of ER and increase breast cancer risk in humans (Mussi et al., 2006). On the basis of its role as an ER-selective coregulator in modulating ER activity and sensitivity to antiestrogens, REA, whose downregulation was found by SAGE in tamoxifen resistant cells, appears to be another very relevant gene associated with the development of resistance to antiestrogens. It is possible that in order to overcome the inhibitory growth effects of tamoxifen and survive during prolonged antiestrogen exposure, tamoxifen resistant cells chronically enhance ER signaling and reduce antiestrogen sensitivity by downregulating REA. Therefore, our SAGE results together with previous known function of REA make it another good candidate gene for tamoxifen resistance in breast cancer and thereby deserves further detailed investigations.

To summarize our SAGE studies, we provide a detailed analysis of gene expression at a global level in our recently developed *in vitro* cell line models of tamoxifen resistance. As a result, more than 400 transcripts were found differentially expressed in tamoxifen resistant cells compared to their sensitive counterparts, with about 243 genes upregulated and 247 genes downregulated by at least a fold of 2 at a statistical significance level of 95%. We observed that most key genes identified overexpressing in tamoxifen resistant cells are related to the control of cell proliferation and cell cycle progression (including CCNA, CCNF, CDC45 and EBAG9) and on the other hand the expression of many anti-proliferative genes including some tumor suppressors and ER

coregulators (including CtIP, FAT, TSSC3 and REA) are significantly decreased. It appears that tamoxifen exposed cells might bypass the inhibitory growth effects of tamoxifen and continue proliferating in spite of the presence of antiestrogen by on one hand enhancing cell survival and proliferative signal pathways to confront tamoxifen's inhibitory effects, on the other hand decreasing growth inhibitory signaling pathways directly or indirectly activated by tamoxifen.

Among key changes identified, CtIP is of particular interest to us, since this gene, which is directly linked with important tumor suppressors and the transcriptional corepressor, is downregulated approximately 15-fold in tamoxifen resistant cells. In addition, recent studies with mutant mice have suggested that CtIP itself may be a tumor susceptibility gene (Chen et al., 2005). Therefore, our follow-up studies were focused on CtIP and its role in endocrine resistance. In the present study, we also determined that in the tamoxifen resistant cells CtIP silencing appears not to result from promoter CpG methylation. A previous screening of 89 human tumor cell lines derived from various tissues failed to detect any homozygous deletions of the CtIP locus (Wong et al., 1998). However, analysis of CtIP cDNA from these 89 cell lines revealed five missense and eleven silent mutations (Wong et al., 1998). It remains to be determined whether genetic alterations play a role in silencing CtIP expression in the tamoxifen resistant cells. It has also been shown that E2F6, a member of the E2F family of transcriptional regulators, can be recruited to the CtIP promoter and repress transcription of the CtIP gene (Oberley et al., 2003). Moreover, two consensus E2F-binding sites that are functionally repressed by Rb were found on the CtIP promoter (Liu and Lee, 2006). Interestingly, CtIP was shown to activate its own promoter (Liu and Lee, 2006). Therefore, it is also possible that the

lack of CtIP expression in the tamoxifen resistant cells is the result of transcriptional regulatory events.

# Chapter 4: CtIP silencing as a novel mechanism of tamoxifen resistance in human breast cancer

## 4.1. INTRODUCTION

It has long been established that estrogen is involved in breast carcinogenesis. Treatment of breast cancer has, for a long time, been directed towards inhibiting the tumor promoting effects of estrogen. In fact, the concept that changing the hormone milieu of the patient with breast cancer could lead to tumor regression was recognized even before anti-hormones and synthetic endocrine agents were available. As mentioned earlier removal of the ovaries was prescribed in advanced breast cancer cases with the hope that this would cause tumor regression and control its growth (Beatson, 1896). The clinical use of an antiestrogen for the treatment of breast cancer was first reported by Cole et al. in 1971, who described the potential use of tamoxifen in the management of breast cancer (Cole et al., 1971). Today, tamoxifen is the endocrine therapy of choice for all stages of estrogen receptor-positive breast cancer (Osborne, 1998) and is currently prescribed for the prevention of breast cancer in both pre- and post-menopausal women at high risk (Fisher et al., 1998; Jordan, 2003). Almost all patients with estrogen receptor-positive tumors in western countries have been treated with this drug either as adjuvant therapy following surgery or as first-line treatment for advanced disease. The level of response to this therapy usually correlates with the expression of ER and possibly PgR, in the tumor. Generally, the highest response rates are seen in tumors expressing both ER and PgR (70%), with lower response rates in ER (-) but PgR (+) tumors (45%), and in ER (+) but PgR (-) tumors (34%), and lowest response rates in ER and PgR negative tumors (<10%) (Honig, 1996). Unlike the widely used cytotoxic chemotherapies, tamoxifen treatment is well tolerated (Osborne, 1998). In addition, tamoxifen also demonstrates the

ability to increase both disease free and overall survival (Early Breast Cancer Trialists' Collaborative Group, 1992). Although this approach to breast cancer therapy is effective, much is still unknown about how tamoxifen inhibits breast cancer development. Tamoxifen belongs to a family of drugs named selective estrogen receptor modulator (SERM). In other words, tamoxifen effects are tissue specific, acting as an antagonist of estrogen in some tissues like the breast and an agonist in others such as bone and endometrium. As a consequence, the higher risk for endometrial carcinoma in women exposed to long-term tamoxifen treatment constitutes more than an undesirable side effect.

Another important clinical problem is that, of those tumors that initially respond to tamoxifen therapy, a significant fraction eventually becomes resistant to such therapies. Furthermore, almost all patients with advanced breast cancer eventually develop tamoxifen resistance and acquire estrogen independent growth. Nevertheless, some of these patients still respond at least temporarily to second and third line endocrine therapy. For example, many tumors which have developed tamoxifen resistance are likely to still respond to pure antiestrogens such as ICI 182,780 (Howell et al., 1996). This indicates that some of the mechanisms involved in tamoxifen resistance appear to be specific for this compound or type of compounds. In addition, it has been observed that the specific resistance to endocrine therapy is in many instances reversible, which suggest a cellular adaptation phenomenon rather than a genetic based alteration. Nevertheless, the issue of resistance to tamoxifen constitutes a major clinical challenge. Although numerous mechanisms have been proposed to contribute to the resistance to tamoxifen therapy, we still understand very little about the precise and most common mechanisms by which resistance to tamoxifen develop.

Since our research interest is to better understand the phenomenon of tamoxifen resistance, we developed two isogenic tamoxifen resistant *in vitro* models based on MCF-7 breast cancer cell line, which expresses functional wide-type ER and grows maximally in estrogen-containing media but exhibits dramatic growth inhibition when treated with antiestrogens (Soule et al., 1973). These resistant lines were derived by maintaining MCF-7 breast cancer cells under continuous 4-OH-TAM exposure for approximately two years. After an initial period of growth arrest the breast cancer cell line variants regained active exponential growth in spite of exposure to a high 4-OH-Tam concentration (see Chapter 3, Results section). In order to identify genes implicated in the development of tamoxifen resistance, we obtained and compared global gene expression profiles of the mentioned tamoxifen resistant cell variants and their parental tamoxifen sensitive cells by using serial analysis of gene expression (SAGE). SAGE is a powerful, comprehensive and unbiased global gene expression method that allows one to obtain a snap-shot of all genes expressed (i.e. transcriptome) (Velculescu et al., 1995). Our analysis of the gene expression data identified various dysregulated genes in tamoxifen resistant cells when compared with their tamoxifen sensitive counterparts. We first focused our analysis specifically on genes that play critical roles in cell cycle progression and cell proliferation. Among these we identified, the CtIP (RBBP8) transcript was downregulated (practically silent) approximately 15-fold in tamoxifen resistant cells. These results were validated by multiple approaches at the gene and protein expression level. CtIP is a binding partner of tumor suppressors, BRCA1 (Li et al., 1999; Sum et al., 2002; Wong et al., 1998; Yu et al., 1998) and Rb (Fusco et al., 1998; Meloni et al., 1999), and also forms heterodimers with the transcriptional corepressor CtBP (Schaeper et al., 1998). Emerging evidence also suggests that CtIP may itself act as a tumor suppressor

gene (Chen et al., 2005). Based on the significant level of CtIP downregulation and the importance of its direct links with important tumor suppressors and regulators of gene expression, we hypothesize that downregulation of CtIP constitutes a critical event for the development of tamoxifen resistance in breast cancer cells. Therefore, the present study was designed to further characterize the functional role of CtIP in the development of tamoxifen resistance and evaluate the clinical relevance of CtIP as a potential biomarker of the tamoxifen resistant phenotype as well as for breast cancer prognosis.

## 4.2. RESULTS

### 4.2.1. Silencing endogenous CtIP in tamoxifen sensitive MCF-7 cells confers tamoxifen resistance and estrogen independence

To further explore the putative role of CtIP in the development of tamoxifen resistance, we first examined whether silencing the expression of endogenous CtIP in tamoxifen sensitive cells can induce a tamoxifen resistant phenotype. Knockdown of CtIP protein levels in tamoxifen sensitive parental MCF-7 cells was achieved using RNA interference techniques. As shown in Fig. 4.1.A, in the resulting clone (MCF-7/CtIP siRNA) stably transfected with a vector expressing siRNA targeting CtIP mRNA, the silencing of CtIP protein levels reproduces quite closely the expression difference observed between the tamoxifen sensitive (parental MCF-7) and tamoxifen resistant (TAMR1 and TAMR2) cells. The negative control siRNA clone (MCF-7/(-) control siRNA) showed unchanged CtIP protein levels when compared to parental MCF-7 cells (Fig. 4.1.A). In addition, we observed an equal level of ER expression in all three clones, indicating that ER protein expression was not affected by the siRNA intervention (Fig. 4.1.A).

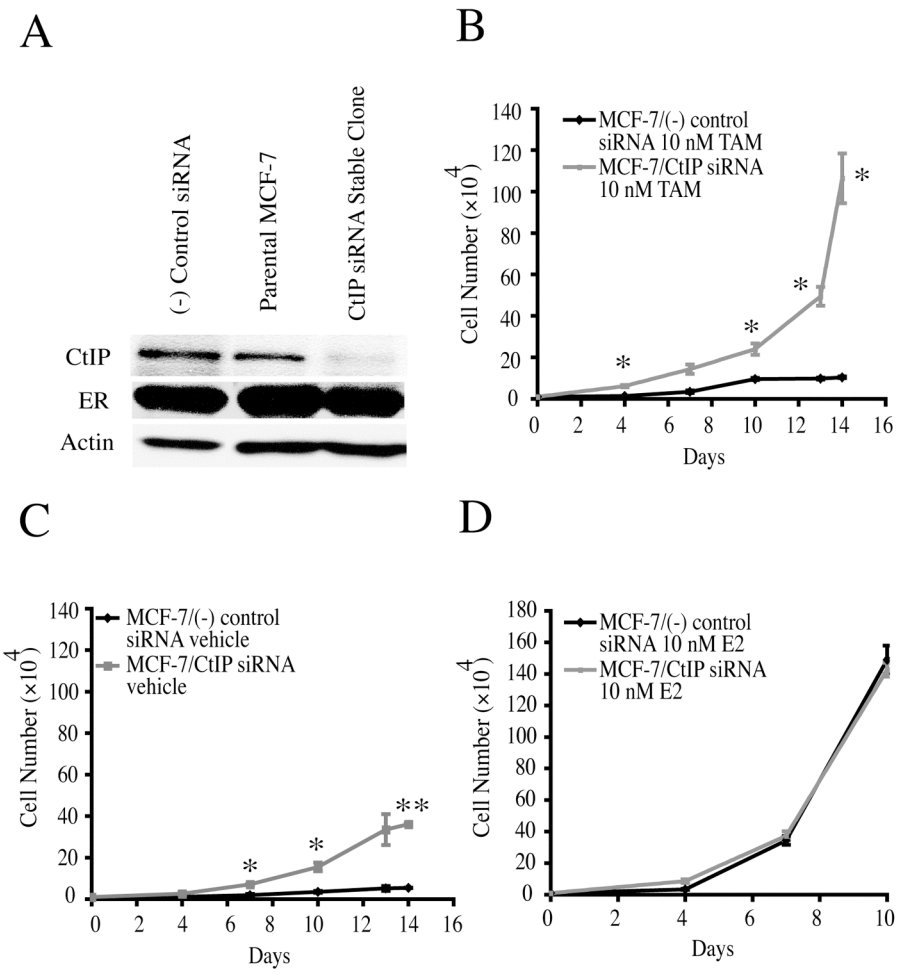



Figure 4.1. Silencing of CtIP protein expression in tamoxifen sensitive MCF-7 cells confers tamoxifen resistance and estrogen independence *in vitro*.

(A) Western blot analysis of CtIP and ER proteins in parental MCF-7 cells, MCF-7/(-) control siRNA and MCF-7/CtIP siRNA stable clones. Growth curves of control and MCF-7/CtIP siRNA cells treated with 4-OH-TAM (B) or vehicle control (C) for 2 weeks. The results are presented as mean ± SEM of triplicate determinations. * and ** indicate $p<0.05$ and $p<0.01$ by t-test, respectively. (D) Growth curves of control and MCF-7/CtIP siRNA cells exposed to E2 for 10 days. Each data point and error bar show the mean ± SEM. Note that Y-axis scales for cell number in B and C are different from D.

To test whether silenced CtIP expression in parental MCF-7 cells leads to the tamoxifen sensitive-to-tamoxifen resistant transition, we next compared cell proliferation between MCF-7/CtIP siRNA and MCF-7/(-) control siRNA cells under various stimuli. Hormone-starved experimental and control siRNA cells were exposed to 4-OH-TAM for 2 weeks and cell numbers were determined at various time points as indicated (Fig. 4.1.B). Growth of MCF-7/(-) control siRNA cells was inhibited by exposure to tamoxifen, whereas growth of MCF-7/CtIP siRNA cells was not inhibited by tamoxifen, indicating acquired resistance to tamoxifen ($p<0.05$) (Fig. 4.1.B). Interestingly, when cultured in conditions completely devoid of estrogen, inhibition of the growth of MCF-7/(-) control siRNA cells was observed, indicating estrogen dependence. On the other hand, CtIP silenced MCF-7/CtIP siRNA cells grew continuously even in the absence of estrogen, suggesting the acquisition of an additional relevant phenotypic characteristic, i.e. estrogen independent growth ($p<0.001$) (Fig. 4.1.C). Interestingly, the MCF-7/CtIP siRNA cells still retain response to estrogen-stimulated cell proliferation when exposed to estradiol (Fig. 4.1.D), indicating that ER is still functional and capable of regulating cell growth. In fact, after 10 days in culture in the presence of estrogen, the cell number of both control and MCF-7/CtIP siRNA cells increased about 145-fold (Fig. 4.1.D), which is 5 to 8 times more than that of the MCF-7/CtIP siRNA cells cultured in the presence of tamoxifen (Fig. 4.1.B) or in the absence of estrogen (Fig. 4.1.C) respectively (over the same time period), suggesting that estrogen still appears to stimulate cell proliferation in MCF-7 cells regardless of CtIP status. Taken together, these data indicated that CtIP silencing leads to resistance to the inhibitory growth effects of tamoxifen and estrogen independent growth *in vitro*.

### 4.2.2. Re-expression of CtIP in tamoxifen resistant cells restores sensitivity to the inhibitory growth effects of tamoxifen

Next, we addressed the reciprocal question of whether re-expression of CtIP in tamoxifen resistant cells abrogates resistance to tamoxifen. Since both tamoxifen resistant lines have similar proliferation profiles in the presence of tamoxifen, we selected the TAMR1 cell line for further functional studies. To this end, a Tet-off inducible gene expression system was employed to re-express CtIP in the tamoxifen resistant TAMR1 cells. TAMR1 cells were transiently co-transfected with Tet-off inducible vectors containing N-terminal Flag-tagged full-length human CtIP cDNA. After transfection, cells were immediately divided equally into two batches. The first batch was treated with 4-OH-TAM or vehicle, and cultured in the presence of doxycycline (DOX). The second batch was treated with 4-OH-TAM or vehicle, but cultured in the absence of DOX. Expression of FLAG-CtIP was analyzed by immunoblotting after 72 hours of transfection. FLAG-CtIP was only detected in TAMR1 cells cultured without DOX (Fig. 4.2.A, bottom panel: left), indicating the restoration of CtIP is tightly controlled by DOX. Cell proliferation was determined in TAMR1 cells with or without CtIP re-expression. This experiment showed that cells with restored CtIP protein expression displayed a significant growth inhibition by tamoxifen in comparison with control cells having no CtIP restoration (Fig. 4.2.A, bottom panel: middle). Without tamoxifen treatment (vehicle control), transient CtIP restoration had no significant effect on TAMR1 cell proliferation (Fig. 4.2.A, bottom panel: right). Thus, transient restoration of CtIP appeared to abrogate resistance to tamoxifen in tamoxifen resistant cells.

To further confirm the observations derived from the transient transfection experiments, we developed double-stably transfected TAMR1 Tet-off FLAG-CtIP cells.

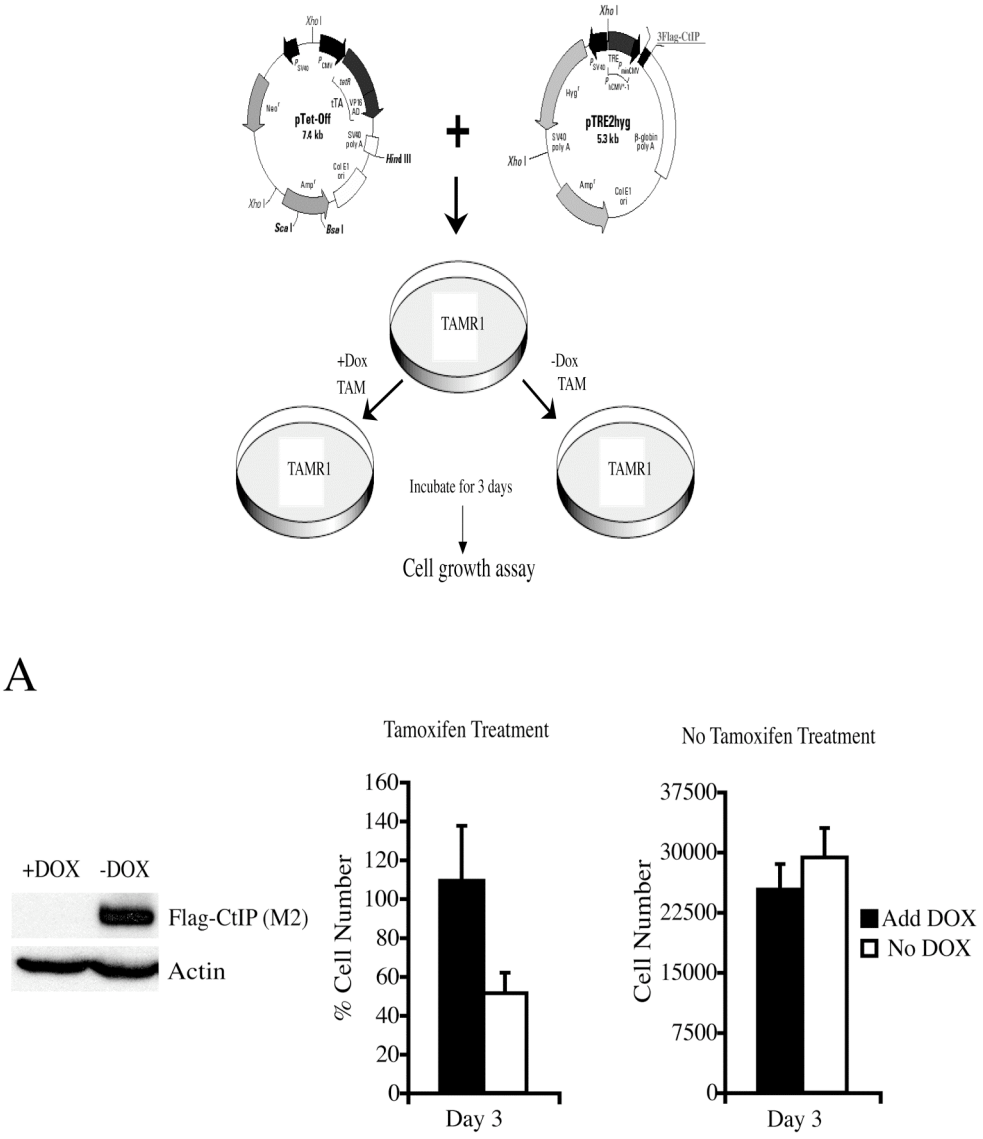



Figure 4.2. Tamoxifen resistant cells regain sensitivity to the inhibitory growth effects of tamoxifen upon restoration of CtIP protein expression.

(A) (up panel) TAMR1 cells were transiently co-transfected with the pTet-off regulatory vector and the pTRE2hyg-FLAG-CtIP inducible expression vector containing N-terminal Flag-tagged full-length human CtIP cDNA. After transfection, cells were immediately divided equally into two batches. The first batch was treated with tamoxifen or vehicle, and cultured in the presence of doxycycline (DOX). The second batch was treated with tamoxifen or vehicle, but cultured in the absence of DOX. Cell growth assays were performed 72 hours after transfection. (bottom panel: left) Expression of FLAG-CtIP

protein in TAMR1 cells transiently co-transfected with inducible FLAG-CtIP expression vectors and cultured in the presence or absence of doxycycline (DOX) for 3 days. (bottom panel: middle and right) Transient CtIP restoration partially abrogates resistance to tamoxifen in TAMR1 cells. TAMR1 cells were transiently co-transfected with inducible FLAG-CtIP expression vectors and treated with 4-OH-TAM (bottom panel: middle) or vehicle control (bottom panel: right) in the presence or absence of DOX for 3 days. Cell proliferation was determined as shown. Percent cell number (bottom panel: middle) represents cell numbers relative to vehicle control treated cells. The results are presented as mean ± SEM of triplicate determinations.

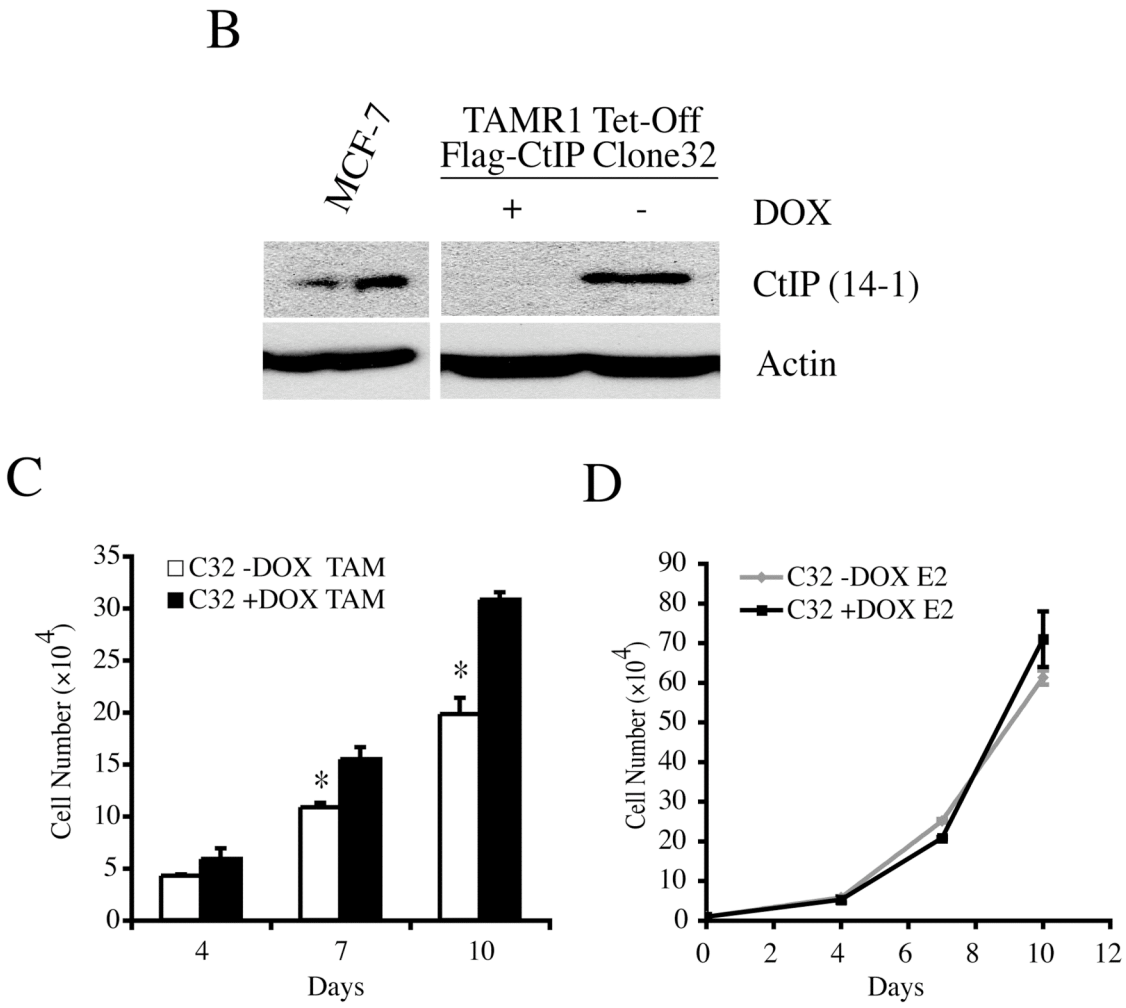



Figure 4.2. (B) Total CtIP protein expression in the double-stably transfected TAMR1 Tet-off FLAG-CtIP clone 32 cells in the presence or absence of DOX. CtIP expression in parental MCF-7 cells is shown for comparative purpose. (C) CtIP re-expression upon DOX withdrawal restores sensitivity to the inhibitory growth effects of tamoxifen in TAMR1 Tet-off FLAG-CtIP clone 32 cells. The proliferation of clone 32 cells under the treatment of 4-OH-TAM was determined in the presence (black bars) or absence (white bars) of DOX. *, $p<0.05$ by t-test. (D) Effect of E2 on the proliferation of clone 32 cells cultured with or without DOX. Note that Y-axis scales for cell number in C and D are different from each other.

Among the various stably transfected clones obtained, clone 32 was selected for further study. This clone showed no CtIP expression in the presence of DOX but similar CtIP protein levels to those produced in parental MCF-7 cells upon DOX withdrawal, as determined by western blot analysis (Fig. 4.2.B). Next, we measured the growth of the double-stably transfected TAMR1 Tet-off FLAG-CtIP cells under conditions in which CtIP restoration was either induced or repressed. Cells from clone 32 were cultured in two different conditions. Half of the cells were grown in hormone-free medium containing DOX while the other half was cultured in the same medium but devoid of DOX. After 3 days of incubation, cells were treated with either E2, 4-OH-TAM or vehicle control. As shown in Fig. 4.2.C, the growth of TAMR1 Tet-off FLAG-CtIP cells was significantly inhibited by tamoxifen when DOX was removed from the medium as compared to cells from the same clone but cultured in the presence of DOX ($p<0.05$). Cells still responded well to estrogen regardless of DOX status (Fig. 4.2.D). Taken together, these results demonstrate that sensitivity to the inhibitory growth effects of tamoxifen in previously tamoxifen-resistant cells is restored, at least partially by CtIP re-expression.

### 4.2.3. Poor clinical response to endocrine therapy is associated with CtIP deficiency in breast cancer patients

To determine whether there is a relationship between CtIP status and endocrine therapy response *in vivo*, we evaluated CtIP expression by immunohistochemistry (IHC) in 59 ER+, non-operable primary breast carcinomas (i.e. prior to treatment) from patients who received endocrine therapy as single neo-adjuvant therapy. Based on the clinical response to the therapy after four months of follow-up, patients were classified into 4 groups: complete response (CR), 4 cases (7%); partial response (PR), 32 cases (54%); stable disease (SD), 17 cases (29%), and progressive disease (PD), 6 cases (10%). These

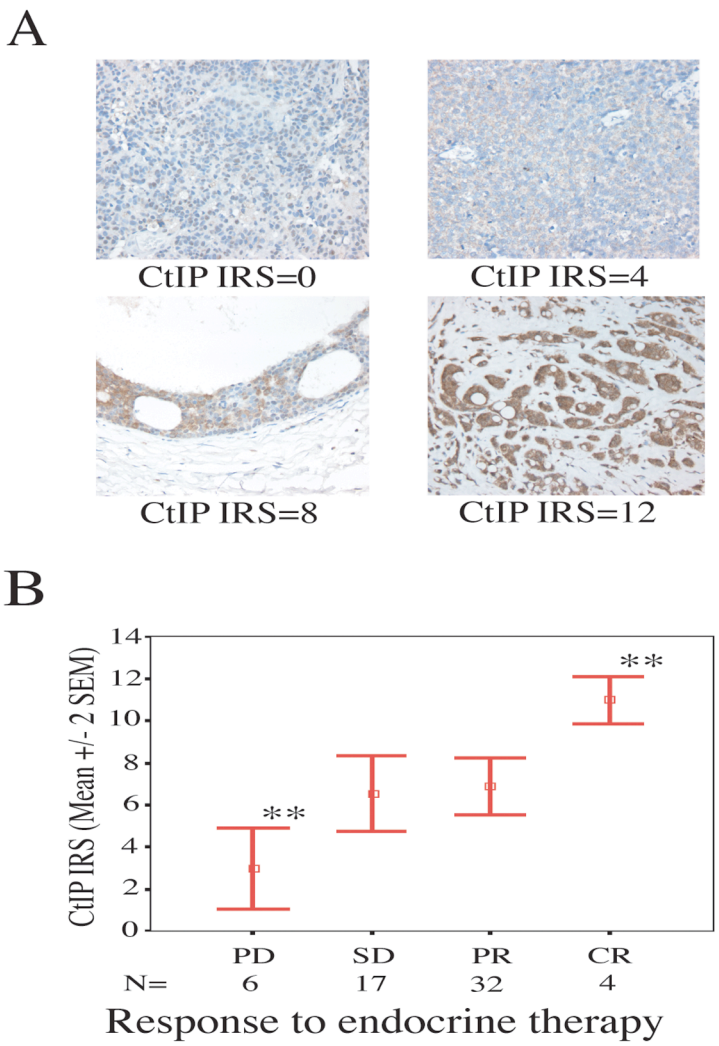



Figure 4.3. Poor clinical response to endocrine therapy is significantly associated with CtIP deficiency in primary breast carcinomas.

(A) CtIP expression patterns in human breast carcinomas. CtIP expression was evaluated by immunohistochemistry and semi-quantified using immunoreactive scores (IRS) in 59 inoperable hormone receptor-positive primary breast carcinomas (prior to treatment) from patients who received endocrine therapy as single neoadjuvant therapy. Representative tumor CtIP immunoreactivity (IRS scores 0, 4, 8 and 12) is shown. (B) CtIP status strongly correlates with clinical response to endocrine therapy (Pearson Correlation, p=0.004), and patients with progressive disease (PD) have significantly lower CtIP IRS than those who completely respond to endocrine therapy (CR). **, p=0.006 by ANOVA and Tukey's post tests. Complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD).

cases are representative of a larger cohort previously reported in which CR was reported to be between 4% and 10% (Eiermann et al., 2001). Immunoreactive scores (IRS) for CtIP were used to semiquantify IHC staining intensity and percentage of positive cells (Chui et al., 1996; Friedrichs et al., 1993). IRS ranging from 0 to 12 represents CtIP protein staining from undetectable to the highest expression level respectively (Fig. 4.3.A). One-way ANOVA analysis of CtIP IRS revealed significantly different CtIP expression in the 4 response groups (p=0.01). Remarkably, we observed that patients who had the worst response to endocrine therapy (defined as progressive disease, PD) had significantly lower CtIP expression than those who had the best response to endocrine therapy (defined as complete response, CR) (p=0.006) (Fig. 4.3.B). Moreover, Pearson's correlation analysis showed a significant correlation between CtIP status and clinical response to endocrine therapy (p=0.004). These data indicate that poor response to endocrine therapy is associated with CtIP deficiency in breast cancer patients.

### 4.2.4. CtIP expression is associated with ER, disease free survival and breast cancer metastasis status

To further explore the clinical relevance of CtIP expression in breast cancer, we evaluated information of seven publicly available breast cancer gene expression microarray data sets (Gruvberger et al., 2001; Perou et al., 1999; Sorlie et al., 2003; van de Vijver et al., 2002; Wang et al., 2005; West et al., 2001; Zhao et al., 2004) through the use of the web-based Oncomine cancer microarray database (http://www.oncomine.org) (Rhodes et al., 2004). Clinico-pathological and gene expression data from a total of 828 breast carcinomas was obtained using this publicly available resource. Since ER plays a critical role in the clinical management of breast cancer patients, we first analyzed levels of CtIP mRNA expression in the mentioned microarray sets according to ER status of the tumors (Fig. 4.4.A). By using a meta-analysis approach, we directly compared CtIP

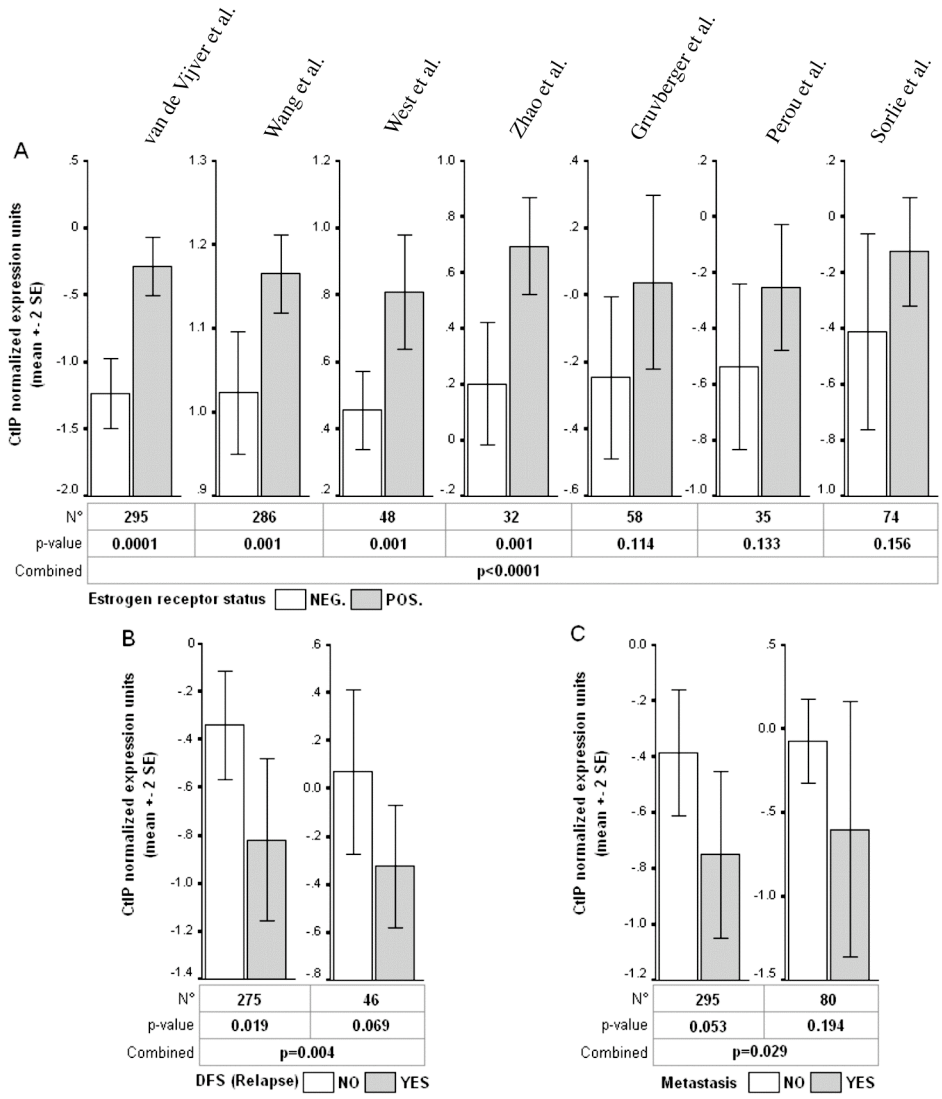



Figure 4.4. CtIP expression is associated with ER status and prognosis in breast cancer.

CtIP gene expression profiles and clinico-pathological data of 828 breast carcinomas were obtained from seven published and publicly available breast cancer microarray data sets as described in Materials and Methods. Data were collected and visualized using the Oncomine cancer microarray resource. (A) Oncomine's database output and meta-analysis showing CtIP expression patterns relative to ER status across seven breast cancer microarray studies. CtIP transcripts are represented as normalized expression units ±

standard error (95% CI). (B) Oncomine's database output of CtIP expression patterns relative to Disease Free Survival (DFS) in two of the seven data sets that have 5 years follow-up information available. The van de Vijver et al. dataset (left) shows a significant association between loss of CtIP expression and relapse (p=0.019). The Sorlie et al. study (right) shows a trend which does not reach statistical significance (p=0.069). Meta-analysis (pooling both studies together) shows an excellent statistical inverse correlation between decreased CtIP expression and breast cancer relapse (p=0.004). (C) Oncomine's database output of CtIP expression patterns related to metastasis status (left, the van de Vijver et al. study; right, the Sorlie et al. study). The analysis shows a statistical significant decrease of CtIP expression in association with metastatic breast carcinomas (p=0.029).

expression profiles between 590 ER positive (+) and 238 ER negative (-) breast carcinomas by combining all seven microarray data sets. We found a dramatically significant association between high CtIP expression and ER (+) status in breast carcinomas ($p<0.0001$) (Fig. 4.4.A). Next, we analyzed CtIP expression profiles versus disease free survival (DFS) in two microarray data sets that had at least 5 years of follow-up clinical information available. Analysis from the study of Van de Vijver et al. (van de Vijver et al., 2002) showed a statistical significant association between loss of CtIP and disease relapse ($p=0.019$). A trend was also found in the Sorlie et al. study (Sorlie et al., 2003) but did not reach statistical significance ($p=0.069$), possibly due to the low number of samples (Fig. 4.4.B). However, by using the meta-analysis approach and pooling both studies together, we found a highly significant association between decreased CtIP expression and disease relapse ($p=0.004$) (Fig. 4.4.B). Furthermore, CtIP mRNA expression levels were significantly lower in invasive breast carcinomas that had distant metastasis when compared with breast cancer counterparts that did not metastasize ($p=0.029$) (Fig. 4.4.C).

### 4.2.5. CtIP expression in human breast cancer cell lines

To perform a comparative analysis of CtIP expression levels, we determined CtIP protein expression by western blot analysis in 10 human breast cancer lines. As can be observed in Figure 4.5., 3 out of 6 ER positive (+) breast cancer lines express abundant CtIP (MCF-7, T-47-D and ZR-75-1) and one of these lines (BT-483) expresses some CtIP, i.e. in total 4/6 express detectable CtIP. In contrast, 4 out of 4 ER negative (-) breast cancer lines either do not express CtIP at all or very little (UAC 812, MDA-MB-231, MDA-MB-435, SKBR3).

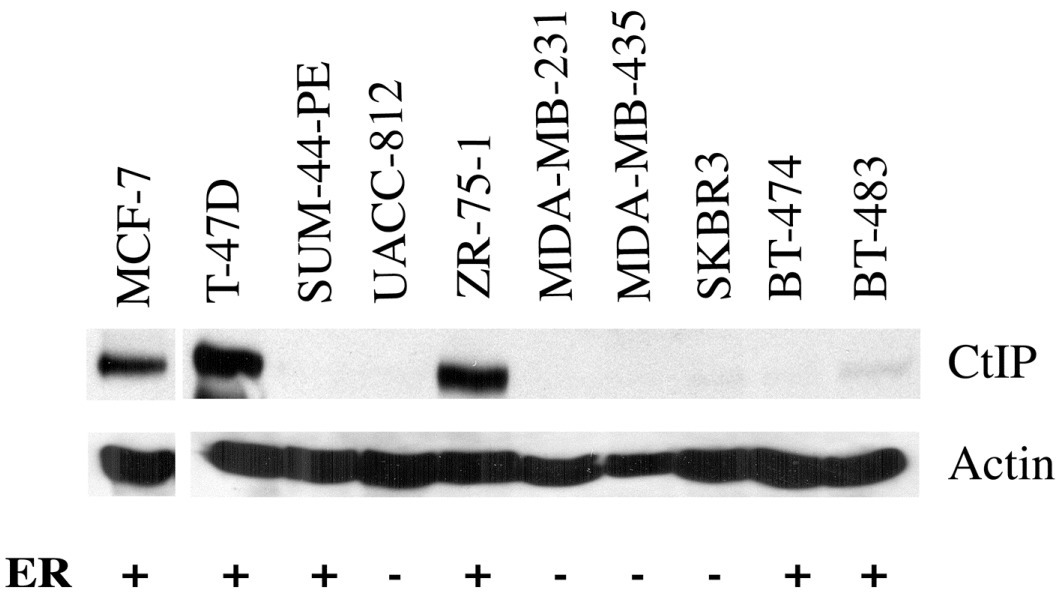



Figure 4.5. CtIP protein expression in breast cancer cell lines as determined by western blot analysis.

Estrogen receptor (ER) expression status is also indicated as (+) positive or (-) negative.

## 4.3. DISCUSSION

Previous SAGE studies identified CtIP as one of the most significantly downregulated transcripts in two independently developed tamoxifen resistant cell lines when compared to their tamoxifen sensitive parental MCF-7 line. Immunoblotting analyses not only validated the SAGE observations but also demonstrated a dramatic difference in CtIP protein expression levels; with high levels of protein expression in parental MCF 7 breast cancer cells and practically undetectable levels of CtIP protein product in the tamoxifen resistant derivative isogenic cell lines. In the present study, we describe the functional involvement of CtIP in the development of tamoxifen resistance.

Based on RNAi studies, we showed that silencing the expression of CtIP in tamoxifen-sensitive parental MCF-7 cells confers tamoxifen resistance *in vitro*. Thus, we were able to reproduce the tamoxifen resistant phenotype simply by shutting down the expression of one gene i.e. CtIP. Notably, CtIP silencing also leads to estrogen independent growth *in vitro*, suggesting that the development of tamoxifen resistance and estrogen independence might share common mechanisms in breast cancer cells. Moreover, we showed in the reciprocal experiment that re-expression of CtIP in tamoxifen resistant cells restores sensitivity to the inhibitory growth effects of tamoxifen. It is worth mentioning here that we employed an inducible mammalian expression system rather than a conventional constitutive gene expression system to re-express CtIP in the resistant cells. The strength of this approach is that it avoids dealing with the effects of CtIP expression in the resistant cells during the selection process which was conducted under the presence of tamoxifen. In other words, we can re-express CtIP expression at will once the double-stable transfectants have been established. Taken together, these

results demonstrate that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer cells, and suggest that CtIP silencing may be a novel mechanism by which cells can circumvent the inhibitory effects of tamoxifen to resume proliferation and ultimately acquire resistance to tamoxifen.

Consistent with the *in vitro* observations, immunohistochemical evaluation of CtIP protein expression in primary breast carcinomas prior to adjuvant treatment revealed a significant relationship between CtIP status and patients' response to endocrine therapy. We observed that patients with progressive disease and resistance to endocrine therapy had significantly lower CtIP levels in their primary breast cancers than those who completely respond. These data suggest that CtIP status in breast tumors may be a potential prognostic biomarker to predict response to endocrine therapy in the clinic. To evaluate the prognostic value of CtIP determination as a biomarker of endocrine response would require further studies in larger patient cohorts. Additionally, to date no information is available regarding the role of CtIP in breast cancer tumorigenesis or prognosis. In the present study, our meta-analysis from seven publicly available breast cancer gene expression data sets provided very interesting correlations. Analysis of the only two gene expression data sets with patients' follow-up information revealed that decreased CtIP expression was significantly associated with relapse-free survival and development of breast cancer metastasis. Thus, tumors with low levels of CtIP expression appear to behave more aggressively than tumors with high levels of CtIP expression. These results suggest that decrease or loss of CtIP may play a significant role in breast cancer progression. These data also raise the possibility that determination of CtIP expression may be of value as a prognostic marker in breast cancer.

In addition, we found a significant association of CtIP expression with ER status. Furthermore, expression studies from 10 breast cancer cell lines found abundant CtIP protein expression in ER (+) breast cancer cell lines, such as MCF-7, T-47-D and ZR-75-1, but not in any of the ER (-) lines. Even though the sample is small, these observations strengthen the possible reverse association between ER expression and CtIP expression in breast cancer, as also observed in primary breast carcinomas. These findings also suggest that CtIP may function in a pathway or pathways associated with ER signaling and perhaps regulate ER-mediated cell proliferation. Interestingly, while ER (-) cells that are intrinsically resistant to tamoxifen treatment express either none or very little CtIP protein, BT-474, an ER (+) but tamoxifen resistant breast cancer cell line (Wang et al., 2006), also expresses nearly undetectable CtIP protein when compared to ER (+) and tamoxifen sensitive lines such as MCF-7 and T-47-D, which further supports the important role of CtIP in the development of tamoxifen resistance.

The human CtIP (also known as RBBP8) encodes an 897 amino acid nuclear protein that is widely expressed in various human tissues (Fusco et al., 1998; Schaeper et al., 1998; Wong et al., 1998; Yu and Baer, 2000). It was initially identified as a co-factor of transcriptional co-repressor CtBP (Schaeper et al., 1998). CtIP is also known to interact with tumor suppressors, Rb family proteins (Rb and p130) (Fusco et al., 1998; Meloni et al., 1999) and BRCA1 (Li et al., 1999; Sum et al., 2002; Wong et al., 1998; Yu et al., 1998), as well as the transcriptional repressors such as LIM-only protein LMO4 (Sum et al., 2002) and Ikaros family members (Koipally and Georgopoulos, 2002). Recent studies suggest that CtIP plays an important role in cell cycle regulation and DNA damage response (Foray et al., 2003; Li et al., 2000; Liu and Lee, 2006; Wu-Baer and Baer, 2001; Yu and Chen, 2004). Emerging evidence also suggests that CtIP may itself

be a tumor susceptibility gene. Analysis of CtIP cDNA from 89 human tumor cell lines revealed five missense and eleven silent mutations (Wong et al., 1998). In a more recent screening study of 109 colon cancers, CtIP was found to be a frequent target for microsatellite instability (MSI) (Vilkki et al., 2002). More importantly, it has been shown that inactivation of CtIP in mice leads to early embryonic lethality, and the life span of Ctip+/- heterozygotes, which have Ctip haploid insufficiency, was shortened due to the development of multiple types of tumors. These findings demonstrate that CtIP is a critical protein in early embryogenesis and implicates an important role of CtIP in tumorigenesis (Chen et al., 2005). In addition, CtIP interacts with the BRCT domains of BRCA1 where most mutations occur in BRCA1 breast cancer patients, and such protein-protein interaction is abolished by tumor-associated mutations in the BRCT domains (Li et al., 1999; Wong et al., 1998; Yu et al., 1998), suggesting that interaction between CtIP and BRCA1 is of functional relevance in the breast cancer suppressor activity. It has been shown that amino acid residues 299-345 of CtIP mediate its interaction with the BRCT domains of BRCA1 (Wong et al., 1998; Yu and Baer, 2000; Yu and Chen, 2004). Recently, it was also reported that phosphorylation at Ser327 in CtIP appeared to be critical for its interaction with BRAC1 BRCT domains (Varma et al., 2005; Yu and Chen, 2004). Available evidence suggests that CtIP is involved in transcriptional repression [reviewed in (Chinnadurai, 2006; Wu and Lee, 2006)]. Significantly, recent studies demonstrated that BRCA1, CtIP and ZBRK1 form a repressor complex at a recognition site of ZBRK1 in ANG1 promoter and a defect of this complex formation de-represses ANG1 transcription, promoting endothelial cell survival and vascular enlargement (Furuta et al., 2006). Interestingly, other studies demonstrated that BRCA1 physically interacts with ER and inhibits transcriptional activity of the receptor (Fan et al., 2001; Zheng et al., 2001). In this study, we found that high CtIP expression is significantly

associated with ER (+) status. Thus, it is possible that CtIP may functionally be linked with ER signaling via its interaction with BRCA1. Moreover, CtIP was also shown to form a complex with BRCA1 and the transcriptional co-repressor CtBP, which is important for the repression of p21 promoter activity (Li et al., 1999). Together, it raises the possibility that in physiological conditions, CtIP could bridge BRCA1 and CtBP to form a transcriptional repressor complex, which in turn may modulate ER signaling pathways through the interaction between BRCA1 and ER. Therefore, we hypothesize that, in tamoxifen sensitive cells, BRCA1, CtIP and CtBP form a transcriptional repressor complex that leads to inhibition of a full ER positive transcriptional response, accounting for the inhibitory growth effects of tamoxifen. In order to circumvent the transcriptional inhibitory effects of tamoxifen, tamoxifen resistant cells silence CtIP expression, which in turn, disrupts the repressor complex and allows breast cancer cells to resume proliferation.

To summarize, results from the present study clearly demonstrate that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer cells, and indicate that CtIP silencing may be a novel mechanism by which cells can circumvent the inhibitory effects of tamoxifen to resume proliferation and ultimately acquire resistance to this widely used antiestrogen. Furthermore, the association of CtIP deficiency with poor clinical response to endocrine therapy, disease free survival and breast cancer metastasis status suggests that CtIP gene and protein expression may be useful biomarkers for breast cancer prognosis and clinical management. Finally, the findings that CtIP expression is significantly associated with estrogen receptor positive status and CtIP protein expression was found in a majority of ER positive breast cancer cell lines but not in ER negative lines suggest that that CtIP is likely associated with ER function.

# Chapter 5: Involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance

## 5.1. INTRODUCTION

Tamoxifen, an antiestrogen, has been used for endocrine treatment of all stages of estrogen receptor (ER) positive breast cancer for almost three decades and was the first approved drug by the FDA as a cancer chemopreventive for reducing breast cancer incidence in both pre- and post-menopausal women at high risk (Jordan, 2003). As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival in early breast cancer. It is also effective for patients with untreated metastatic breast cancer (Osborne, 1998). Despite the benefits of tamoxifen in treating breast cancer, unfortunately, almost all the breast cancers that initially respond to tamoxifen therapy develop resistance. The mechanisms involved in the development of tamoxifen resistance are still poorly understood. For many women, resistance develops after the first phase of tamoxifen treatment (Katzenellenbogen et al., 1997). Moreover, all patients with advanced metastatic disease ultimately become resistant to antiestrogen therapies. In most cases of resistance, the ER is still present and apparently continues regulating tumor growth.

In order to better understand the phenomenon of tamoxifen resistance, we developed MCF-7 breast cancer cell line variants that are resistant to the inhibitory growth effects of tamoxifen. These new isogenic breast cancer cell lines represent a unique model that closely resembles the *in vivo* scenario. Using serial analysis of gene expression (SAGE), we defined the global gene expression profiles of the mentioned tamoxifen resistant MCF-7 breast cancer cell lines and compared them with their

tamoxifen sensitive parental MCF-7 counterpart. By mining the SAGE databases using a novel suite of bioinformatic tools, we identified CtIP, a BRCA1- and CtBP-interacting protein, as one of the most significantly down-regulated transcripts in tamoxifen resistant breast cancer cells. This result was independently confirmed by quantitative real-time RT-PCR and western blot analyses. In previous studies, we found that silencing endogenous CtIP in tamoxifen sensitive breast cancer cells confers tamoxifen resistance and estrogen independence. On the other hand, re-expression of CtIP in tamoxifen resistant breast cancer cells restores sensitivity to the inhibitory growth effects of tamoxifen. Importantly, CtIP protein expression status strongly correlates with clinical response to neo-adjuvant endocrine therapy and patients with progressive disease express significantly lower CtIP protein in their primary breast carcinomas than those who respond. Meta-analysis of seven publicly available gene expression microarray data sets shows that CtIP expression is significantly associated with estrogen receptor (ER), disease free survival and breast cancer metastasis status. Furthermore, in expression studies we found CtIP protein expression in a majority of ER positive breast cancer cell lines that we tested, but none or very little CtIP expression in ER negative lines. Together, these findings indicate that CtIP silencing might be a novel mechanism for the development of tamoxifen resistance in breast cancer, suggest that CtIP is likely associated with ER function and that CtIP gene and protein expression may be useful biomarkers for breast cancer prognosis and clinical management.

CtIP is an 897-amino-acid nuclear protein that was initially identified as a co-factor of transcriptional corepressor CtBP (Schaeper et al., 1998). Subsequently, it was demonstrated that CtIP also interacts with two important tumor suppressors, Rb family proteins (Rb and p130) (Fusco et al., 1998; Meloni et al., 1999) and BRCA1 (Wong et al.,

1998; Yu et al., 1998; Li et al., 1999; Sum et al., 2002). CtIP binds CtBP via its PLDLS motif. CtBP can repress gene transcription in a histone deacytlase (HDAC)-dependent or -independent manner (Chinnadurai, 2002). Rb family proteins bind CtIP at the LECEE motif. Mutation on this motif disrupts their interaction (Meloni et al., 1999). BRCA1 interacts with CtIP through its C-terminal BRCT repeats. This interaction is mediated via the N-terminal 133 to 369 amino acids of CtIP (Wong et al., 1998; Yu and Baer, 2000) in a phosphorylation-dependent manner (Yu and Chen, 2004). More importantly, tumor-associated mutations in the BRCT domain (common in BRCA patients) abolish this interaction (Wong et al., 1998; Yu et al., 1998; Li et al., 1999), suggesting that tumor-suppressive functions of BRCA1 could be modulated by CtIP. The CtIP-BRCA1 complex also plays a critical role in cell cycle checkpoint control and transcriptional activation of genes, such as p21 and GADD45, after DNA damage (Li et al., 2000; Yu and Chen, 2004). In addition, BRCA1 also physically interacts with ER and inhibits ligand-dependent and -independent transactivation of the receptor (Fan et al., 2001; Zheng et al., 2001). Interestingly, a study on the BRCA1- and BRCA2-associated familial breast cancer has suggested that the majority of such tumors are less likely to respond to antiestrogen than sporadic tumors (Osin et al., 1998). Furthermore, a recent report also demonstrated that loss of full-length Brca1 could alter the agonist/antagonist activity of tamoxifen in mammary epithelial cells, suggesting a potential role of BRCA1 in the cellular response to tamoxifen (Jones et al., 2005). More significantly, very recently it was shown that inactivation of CtIP in mice leads to early embryonic lethality, and the life span of Ctip+/- heterozygotes, which have haploid insufficiency for Ctip, was shortened due to the development of multiple types of tumors. This finding clearly demonstrates that CtIP is a critical protein in early embryogenesis and implicates an important role of CtIP in tumorigenesis (Chen et al., 2005). Since CtIP binds BRCA1 and

CtBP using different motif, it has been shown that CtIP can bridge BRCA1 and CtBP to form a complex, which is important for the regulation of the transactivation of the p21 promoter (Li et al., 1999). In summary, abundant evidence has accumulated in recent years suggesting that CtIP likely plays critical roles in tumor suppression as well as in transcriptional corepression. Our observations indicating that loss of CtIP expression is involved in the development of tamoxifen resistance and CtIP is likely associated with ER function. Together, all evidence raises the possibility that in physiological conditions, CtIP could bridge BRCA1 and CtBP to form a transcriptional repressor complex, which in turn may modulate ER signaling pathways through the interaction between BRCA1 and ER, and that downregulated CtIP expression could lead to the disruption of a functional BRCA1-CtIP-CtBP complex that may contribute to the development of tamoxifen resistance. Therefore, in this chapter, we test the potential involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance.

## 5.2. RESULTS

### 5.2.1 Expression of other corepressors and ER phosphorylation in tamoxifen resistant cells

Since decreased expression of ER corepressors may contribute to tamoxifen resistance, we examine the protein expression level of NCoR in tamoxifen resistant cell lines. As shown in figure 5.1., compared to tamoxifen sensitive parental MCF-7 cell line, there is no significant change in NCoR protein expression in both tamoxifen resistant cell lines (TAMR1 and TAMR2). Besides, we also determined the level of the transcriptional corepressor CtBP and found no difference in protein expression between tamoxifen sensitive and resistant cells (Figure 5.1.). These data indicate that the tamoxifen resistant phenotype of these isogenic cells is unlike the result of altered expression corepressors. In

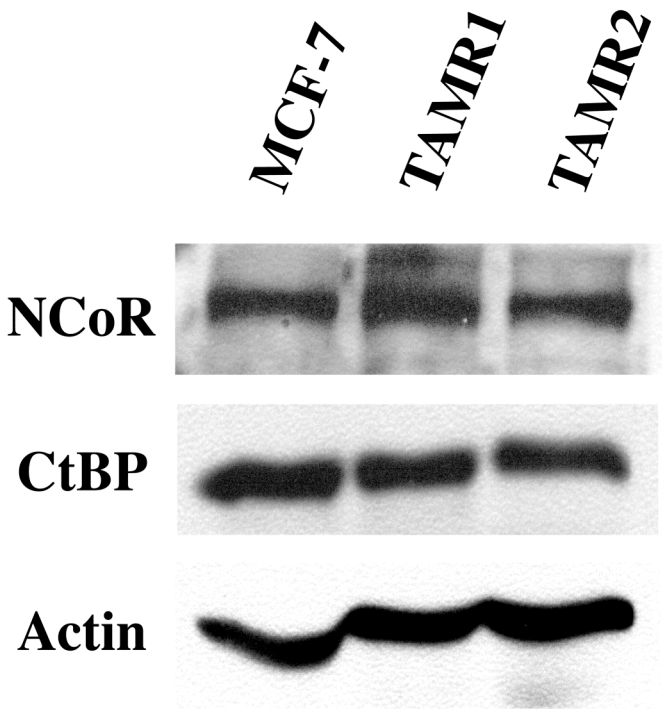



Figure 5.1. Western blot analysis of NCoR and CtBP in MCF-7, TAMR1 and TAMR2 cells.

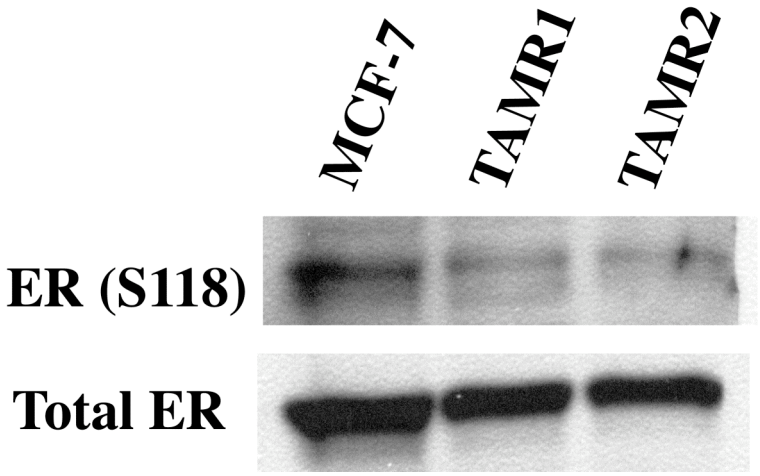



Figure 5.2. Western blot analysis of phospho-ER at serine 118 in MCF-7, TAMR1 and TAMR2 cells.

Total ER expression is also shown as a control.

addition, it is known that the ER is subject to phosphorylation by a variety of signaling kinases, resulting in its activation in the absence of ligand or the presence of tamoxifen. Of particular interest, serine 118 of ER can be phosphorylated by MAP kinases ERK1 and ERK2 (Kato et al., 1995), resulting in ligand-independent activation of ER (Bunone et al., 1996). Therefore, we tested the level of phosphorylated ER at serine 118 using a phospho-specific antibody. In figure 5.2., interestingly we observed a relatively decreased level of phospho-serine 118 of ER in tamoxifen resistant cells compared to parental cells. These findings suggest that ligand-independent activation of ER, at least the phosphorylation at serine 118 by elevated growth signalings, does not play a significant role in the development of resistance to tamoxifen in our experimental cell line models.

### 5.2.2. BRCA1, CtIP and CtBP forms a complex *in vivo* in tamoxifen sensitive but not resistant cells

To test whether BRCA1, CtIP and CtBP can form a complex *in vivo*, we carried out Co-immunoprecipitation assays in sensitive parental MCF-7 cells. MCF-7 cell lysates were immunoprecipitated with anti-BRCA1, anti-CtIP or a control antibody. As can be observed (Figure 5.3.A), the BRCA1 antibody can co-immunoprecipitate CtBP, and the CtIP antibody can also pull down both BRCA1 and CtBP. Since BRCA1 binds CtIP via its BRCT domains and CtIP in turn binds CtBP, these results suggest that the three proteins may be part of a common complex. Unlike tamoxifen sensitive MCF-7 cells, when TAMR1 cell lysates were immonuprecipitated with anti-BRCA1, anti-CtIP and anti-CtBP antibodies, CtBP protein was detected, except in its own immunoprecipitates, neither in BRCA1 nor in CtIP immunoprecipitates (Figure 5.3.B). These findings indicate that the BRCA1, CtIP and CtBP complex is disrupted in tamoxifen resistant cell possibly due to decreased availability of CtIP caused by its silencing.

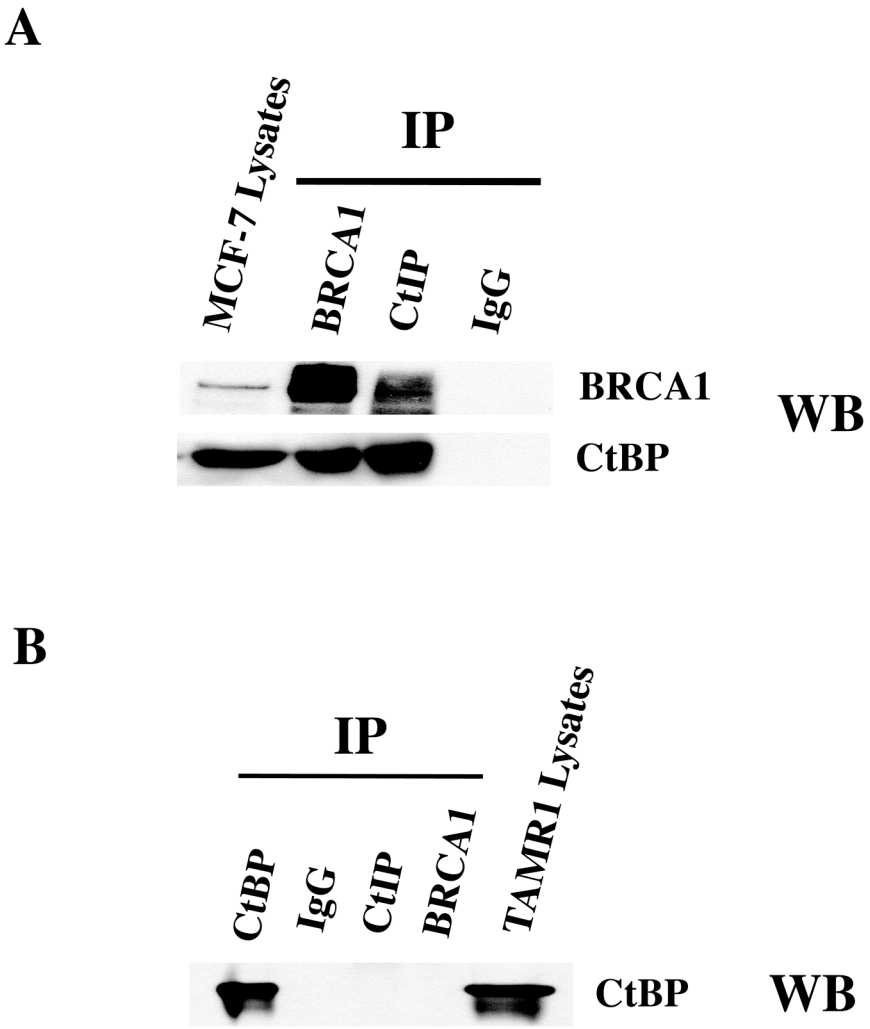



Figure 5.3. BRCA1, CtIP and CtBP form a complex *in vivo* in tamoxifen sensitive MCF-7 cells but not in tamoxifen resistant TAMR1 cells.

(A) MCF-7 cell lysates were prepared in IP buffer and immunoprecipitated with anti-BRCA1 antibody, anti-CtIP antibody or control IgG antibody. The immunoprecipitates were resolved on SDS-PAGE and followed by western blot using anti-BRCA1 or anti-CtBP antibodies, as indicated. (B) TAMR1 lysates were immunoprecipitated with anti-BRCA1, anti-CtIP, anti-CtBP or control IgG antibodies, followed by immunoblotting with anti-CtBP antibody. IP: immunoprecipitation; WB: western blot.

### 5.2.3. BRCA1 interacts with ER *in vivo* in both tamoxifen sensitive and resistant cells

Previous studies reported that BRCA1 is associated with ER directly *in vivo* (Fan et al., 2001). To confirm these findings and determine whether endogenous interaction between BRCA1 and ER is present in our experimental models, we carried out coimmunoprecipitation assays in both tamoxifen sensitive parental MCF-7 and tamoxifen resistant TAMR1 cells. As a result, strong association of BRCA1 and ER was found in both cell lines (Figure 5.4.A&B). To analyze the effect of estrogen and tamoxifen on the endogenous interaction between BRCA1 and ER, we treated MCF-7 cells with estrogen, tamoxifen or vehicle control for 30 minutes. Cells were harvested and lysed. Immunoprecipitation were performed using BRCA1 antibody or control IgG, followed by western analysis with ER and BRCA1 antibodies as indicated. An endogenous interaction of ER with BRCA1 was observed in these cells in the absence of ligand, and this interaction was significantly reduced after estrogen treatment but enhanced in the presence of tamoxifen (Figure 5.4.C).

### 5.2.4. Occupancy by ER, BRCA1, CtIP and CtBP on E2-responsive gene (pS2) promoter in MCF-7 cells

To determine whether BRCA1, CtIP and CtBP can regulate ER function in a physiological manner, we performed a ChIP (Chromatin Immnunoprecipitation) assay on parental MCF-7 cells on the promoter of a well-characterized estrogen-responsive gene, pS2. As can be observed (Figure 5.5.), when cells were treated with vehicle control (first lane), all proteins were detected associated with the ERE containing region of the pS2 promoter. After estrogen treatment (middle lane), the amount of ER protein appeared to increase, but the other 3 proteins BRCA1, CtIP and CtBP were not detected or barely so.

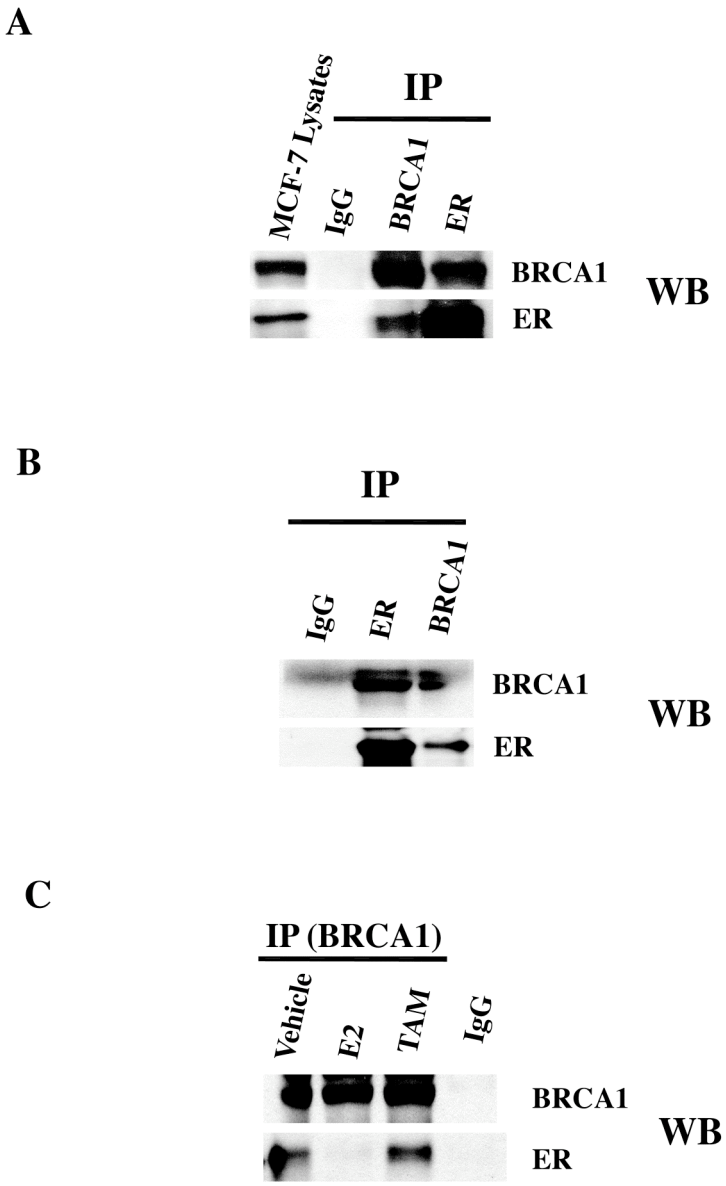



Figure 5.4. Endogenous interaction of BRCA1 with ER in MCF-7 and TAMR1 cells.

MCF-7 (A) or TAMR1 (B) cells were lysed in IP buffer and immunoprecipitated with anti-BRCA1 or anti-ER antibodies. The immunoprecipitates were separated on SDS-PAGE followed by western blot using anti-ER or anti-BRCA1 antibodies, respectively. (C) MCF-7 cells were treated with estrogen, 4-OH-TAM or vehicle control for 30 minutes. Cell lysates were prepared and immunoprecipitated with anti-BRCA1 or control IgG antibody. The immunoprecipitates were resolved on SDS-PAGE and immunoblotted using anti-BRCA1 or anti-ER antibodies, as indicated. IP: immunoprecipitation; WB: western blot.

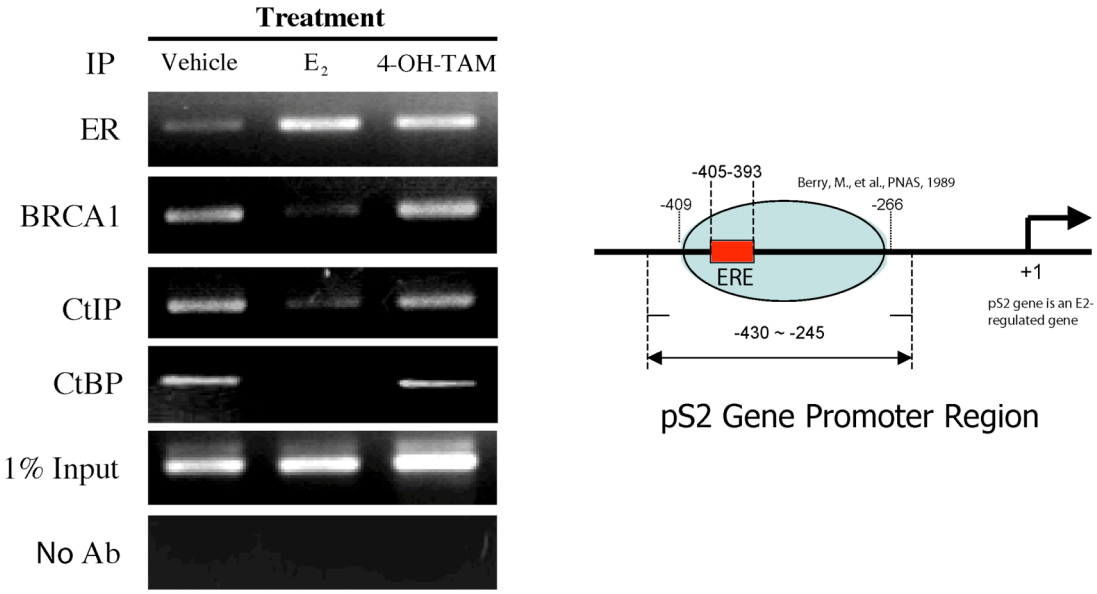



Figure 5.5. ChIP analysis of ER, BRCA1, CtIP and CtBP on pS2 gene promoter in MCF-7 cells.

On the other hand, after treatment with the antiestrogen 4-OH-TAM, ER also increased in abundance but the rest of the proteins BRCA1, CtIP and CtBP were retained in the ERE containing region of the pS2 promoter.

### 5.3. DISCUSSION

Previous studies indicated that decreased level of ER corepressor might contribute to tamoxifen resistance (Lavinsky et al., 1998). In the present study, we show that the protein expression level of ER corepressor NCoR, as well as transcriptional corepressor CtBP, are not significantly changed in our tamoxifen resistant experimental cell line models. In addition, phosphorylation of ER by growth factors-activated protein kinases usually results in activation of the receptor and creates crosstalk between the ER signaling pathways and other growth signaling pathways (Kato, 2001; Lannigan, 2003), even in the absence of ligand or in the presence of tamoxifen (Ali and Coombes, 2002; Ali et al., 1993; Shou et al., 2004), which could play a potential role in tamoxifen resistance. Our results showed ER phosphorylation at least at serine 118 is not elevated in tamoxifen resistant cells. Together, these data indicate that CtIP silencing observed in our experimental models appears to be rather an independent mechanism than integrated with either downregulation of corepressors or with ligand-independent activation of ER by growth factor receptors pathways, although integration with other possibility such as increased coactivators expression still needs to be ruled out.

As mentioned, BRCA1, CtIP and CtBP have been shown to be able to form a complex *in vivo* (Li et al., 1999). In current studies, we determined that BRCA1, CtIP and CtBP form a complex *in vivo* but only observed in tamoxifen sensitive but not resistant cells. This is possibly due to the lower availability of CtIP caused by its silencing in the

resistant cells thereby disrupting the complex formation. Nonetheless, these findings support the potential involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance. In addition, studies also unveiled that BRCA1 physically interacts with ER (Fan et al., 2001) and inhibits transcriptional activity of the receptor (Fan et al., 2001; Zheng et al., 2001). It has also been shown that BRCA1 along with ER is associated with estrogen-responsive gene promoters in the absence of estrogen and promoter occupancy by BRCA1 is reduced upon estrogen treatment (Zheng et al., 2001). Here, we observed a strong endogenous interaction of ER with BRCA1 in both tamoxifen sensitive and resistant cells. Furthermore, in parental MCF-7 cells this interaction is downregulated when cells were treated with estrogen, whereas tamoxifen enhances this interaction. In consistent with the observation of our Co-IP results and those previous findings (Zheng et al., 2001), we observed in ChIP assays that, in the absence of ligand, ER and BRCA1 are associated with EREs at the pS2 promoter. After challenging cells with E2, more ER is recruited to the promoter region, while BRCA1 dissociates from the promoter. In addition, we also observed that CtIP and CtBP behave in the same fashion as BRCA1 on the pS2 promoter when responding to E2. Interestingly, tamoxifen appeared to stabilize BRCA1, CtIP and CtBP on the pS2 gene promoter. Taken all these evidence together, the present studies strongly support the potential involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance and also raises the possibility that in tamoxifen sensitive cells, BRCA1, CtIP and CtBP form a repressor complex on estrogen-responsive gene promoter with unliganded ER and repress target gene transcription. Upon estrogen stimulation, ER undergoes a conformational change that leads to the release of the BRCA1-CtIP-CtBP repressor complex from the promoter and facilitates the recruitment of co-activators to promote target gene transcription. Unlike estrogen, the antiestrogen tamoxifen stabilize

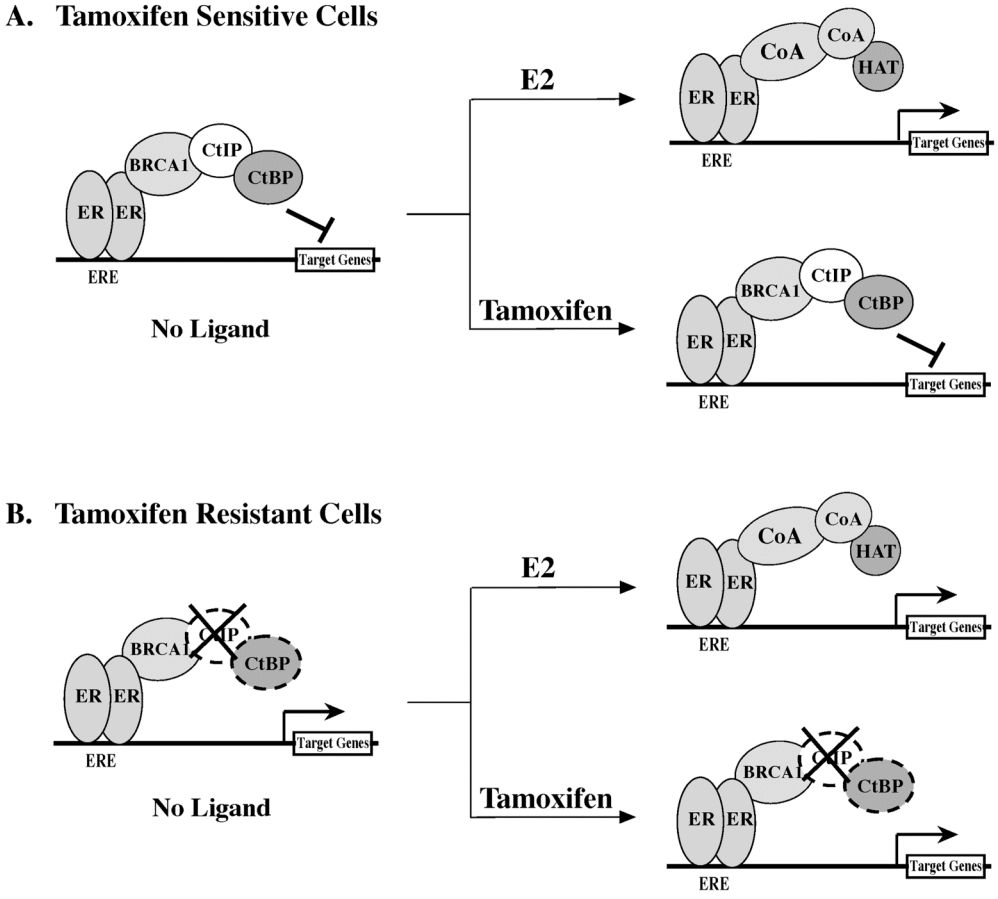



Figure 5.6. Schematic hypothetical model of the proposed BRCA1-CtIP-CtBP repressor complex in tamoxifen sensitive and resistant breast cancer cells.

(A) In tamoxifen sensitive cells, BRCA1, CtIP and CtBP form a repressor complex on estrogen-responsive gene promoter with unliganded ER and repress target gene transcription. Upon estrogen stimulation, ER will undergo a conformational change that leads to the release of the BRCA1-CtIP-CtBP repressor complex from the promoter and facilitates the recruitment of co-activators to promote target gene transcription. Unlike estrogen, the antiestrogen tamoxifen stabilize the repressor complex on the promoter, resulting in the repression of target gene transcription. (B) In tamoxifen resistant cells, in order to circumvent the inhibitory effects of being under high tamoxifen concentrations, cells chronically silence CtIP expression, which, in turn, disrupts the BRCA1-CtIP-CtBP repressor complex, lifting the repressive barrier and allowing target gene transcription and ultimately cell proliferation even in the presence of tamoxifen.

the repressor complex on the promoter, resulting in the repression of target gene transcription. On the other hand, in tamoxifen resistant cells, in order to circumvent the inhibitory effects of being under high tamoxifen concentrations, cells chronically silence CtIP expression, which, in turn, disrupts the BRCA1-CtIP-CtBP repressor complex, lifting the repressive barrier and allowing target gene transcription and ultimately cell proliferation even in the presence of tamoxifen (Figure 5.6.).

In summary, the studies presented in this chapter exclude decreased expression of corepressors and ligand-independent activation of ER as possible causes responsible for the tamoxifen resistance phenotype of our experimental cell line models. In addition, our findings strongly indicate the potential involvement of a BRCA1-CtIP-CtBP complex in the development of resistance to tamoxifen in breast cancer cells.

# Chapter 6: Concluding remarks

## 6.1. SUMMARY

Breast cancer is by far the most common cancer in women in the Western world. Estrogen plays a pivotal role in the etiology and progression of human breast cancer. Therefore, for a long time, treatment of breast cancer has been directed towards inhibiting the tumor promoting effects of estrogen. Tamoxifen treatment is the first line of endocrine therapy for patients with estrogen receptor positive breast cancers. Tamoxifen belongs to a family of drugs named SERM (Selective Estrogen Receptor Modulator). Although other drugs such as aromatase inhibitors may be slightly more effective than tamoxifen, it is still the most used antiestrogen and will remain so for several years to come because of its well-documented benefits. As adjuvant therapy, tamoxifen reduces the risk of recurrence and improves overall survival in early breast cancer. It is also effective for patients with untreated metastatic breast cancer (Osborne, 1998). Furthermore, tamoxifen is also being used as a cancer chemopreventive for reducing breast cancer incidence in both pre- and post-menopausal women at high risk (Jordan, 2003). Despite the benefits of tamoxifen in treating breast cancer, unfortunately, many tumors that initially respond to tamoxifen therapy develop resistance. This phenomenon has become a serious obstacle in breast cancer treatment. In the clinic, almost all patients with advanced metastatic disease and as many as 40% of patients receiving adjuvant tamoxifen eventually relapse and die from their disease (Normanno et al., 2005). The mechanisms involved in the development of tamoxifen resistance are still poorly understood. Although numerous mechanisms have been proposed to contribute to the development of tamoxifen resistance, much work is still needed to learn whether some of the postulated mechanisms so far can explain resistance to tamoxifen therapy in a

majority of patients, or simply each of the enumerated possibilities account for minor portions of resistant cases. Therefore, the overall goal of this dissertation is to better understand the phenomenon of tamoxifen resistance.

Working towards our overall goal, we first developed ER expressing, isogenic breast cancer cells that are resistant to the inhibitory effects of tamoxifen. These new isogenic breast cancer cell lines represent a unique model that closely resembles the *in vivo* scenario. Based on the fact that most of the enumerated putative causes for resistance to tamoxifen will be reflected by changes in gene expression of key players representing directly or indirectly the involved pathways, we hypothesized that most cases of tamoxifen resistance are the result of a cellular adaptation phenomenon which will have a direct reflection in the patterns of global gene expression. Therefore, to test our hypothesis, in chapter 3 we aimed to identify key genes involved in the development and manifestation of tamoxifen resistance in breast cancer, as well as direct or indirect biomarkers of tamoxifen resistance with promise for potential use in the clinical management of breast cancer patients. We defined and compared global gene expression profiles of the mentioned tamoxifen resistant cell variants and their parental tamoxifen sensitive breast cancer cells by using serial analysis of gene expression (SAGE). SAGE is a powerful, comprehensive and unbiased global gene expression method that allows one to obtain a snap-shot of all genes expressed (Velculescu et al., 1995). We provided a detailed analysis of gene expression at a global level in above-mentioned cell line models of tamoxifen resistance. By mining the SAGE databases using state-of-the-art bioinformatic and statistical approaches, we observed different gene expression patterns between tamoxifen resistant cells and their parental tamoxifen sensitive cells. As a result, more than 400 transcripts were found differentially expressed in tamoxifen resistant cells

compared to their sensitive counterparts, with about 243 genes upregulated and 247 genes downregulated by at least a fold of 2 at a statistical significance level of 95%. We observed that most key genes identified overexpressing in tamoxifen resistant cells are related to the control of cell proliferation and cell cycle progression (including CCNA, CCNF, CDC45 and EBAG9) and on the other hand the expression of many anti-proliferative genes including some tumor suppressors and ER coregulators (including CtIP, FAT, TSSC3 and REA) are significantly decreased. Our results appear to suggest that tamoxifen exposed cells might bypass the inhibitory growth effects of tamoxifen and continue proliferating in spite of the presence of antiestrogen by on one hand enhancing cell survival and proliferative signal pathways to confront tamoxifen's inhibitory effects, on the other hand decreasing growth inhibitory signaling pathways directly or indirectly activated by tamoxifen.

Among these key changes identified, one gene named CtIP (also known as Retinoblastoma binding protein 8, RBBP8) was particularly interesting to us, because we observed that the expression of the transcript for CtIP is downregulated 15-fold in tamoxifen resistant cells when compared to their tamoxifen sensitive counterparts. This result was independently confirmed by quantitative real-time RT-PCR and western blot analyses. By reviewing the literature of CtIP, we found that the biological function of CtIP is still poorly understood. Neither known enzymatic activity nor functional domains are found on the CtIP protein. Thus, studies geared at better understanding the function of CtIP have been focused on the biological significance derived from the interactions with its binding partners. CtIP was initially identified as a co-factor of transcriptional co-repressor CtBP (Schaeper et al., 1998). CtIP is also known to interact with tumor suppressors, Rb family proteins (Rb and p130) (Fusco et al., 1998; Meloni et al., 1999)

and BRCA1 (Li et al., 1999; Sum et al., 2002; Wong et al., 1998; Yu et al., 1998), as well as the transcriptional repressors such as LIM-only protein LMO4 (Sum et al., 2002) and Ikaros family members (Koipally and Georgopoulos, 2002). Recent studies suggest that CtIP plays an important role in cell cycle regulation and DNA damage response (Foray et al., 2003; Li et al., 2000; Liu and Lee, 2006; Wu-Baer and Baer, 2001; Yu and Chen, 2004). Emerging evidence also suggests that CtIP may itself be a tumor susceptibility gene (Wong et al., 1998) (Vilkki et al., 2002). More importantly, it has been shown that inactivation of CtIP in mice leads to early embryonic lethality, and the life span of Ctip+/- heterozygotes, which have Ctip haploid insufficiency, was shortened due to the development of multiple types of tumors. These findings demonstrate that CtIP is a critical protein in early embryogenesis and implicates an important role of CtIP in tumorigenesis (Chen et al., 2005). Based on the significant level of CtIP downregulation and the importance of its direct links with important tumor suppressors and regulators of gene expression, we hypothesized that CtIP silencing constitutes a critical event for the development of tamoxifen resistance in breast cancer.

In chapter 3, we also investigated the mechanism causing CtIP downregulation in tamoxifen resistant cells. We focused on one of the most common causes of gene silencing, which is the promoter CpGs methylation. However, we found no evidence of CpG methylation on the CtIP gene promoter region in tamoxifen resistant cells, ruling out promoter methylation as the mechanism for CtIP silencing. Therefore, the mechanism associated with CtIP downregulation in resistant cells deserves future studies. One possible start point in the future could be the investigation of the involvement of a transcriptional regulatory mechanism.

In chapter 4, we aimed to focus on the functionally characterization of the role of CtIP in the development of tamoxifen resistance. We demonstrated that silencing endogenous CtIP by means of siRNA in tamoxifen sensitive breast cancer cells confers tamoxifen resistance and estrogen independence. On the other hand, re-expression of CtIP in tamoxifen resistant breast cancer cells restores sensitivity to the inhibitory growth effects of tamoxifen. Importantly, poor clinical response to endocrine therapy was found to be associated with CtIP deficiency in primary breast carcinomas. Additionally, meta-analysis of publicly available gene expression microarray data sets shows that CtIP expression is significantly associated with ER, disease free survival and breast cancer metastasis status. Furthermore, in expression studies we found CtIP protein expression in a majority of ER positive breast cancer cell lines that we tested, but none or very little CtIP expression in ER negative lines. Our results demonstrate that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer cells, and indicate that CtIP silencing may be a novel mechanism by which cells can circumvent the inhibitory effects of tamoxifen to resume proliferation and ultimately acquire resistance to this widely used antiestrogen. In addition, the association of CtIP deficiency with poor clinical response to endocrine therapy, disease free survival and breast cancer metastasis status suggests a potential use of CtIP gene and protein expression in the clinic as biomarkers for breast cancer prognosis and clinical management. Thus, future validation of these ideas in large patients cohort is warranted. Furthermore, the possible reverse association identified between ER expression and CtIP expression in breast cancer suggest that CtIP is likely associated with ER function.

Available evidence suggests that CtIP is involved in transcriptional repression [reviewed in (Chinnadurai, 2006; Wu and Lee, 2006)]. Interestingly, other studies

demonstrated that BRCA1 physically interacts with ER and inhibits transcriptional activity of the receptor (Fan et al., 2001; Zheng et al., 2001). Moreover, CtIP was also shown to form a complex with BRCA1 and the transcriptional co-repressor CtBP, which is important for the repression of p21 promoter activity (Li et al., 1999). Based on these known CtIP function and the findings from chapter 4, we speculated that CtIP may bridge BRCA1 and CtBP to form a transcriptional repressor complex, which in turn may modulate ER signaling pathways through the interaction between BRCA1 and ER, and that downregulated CtIP expression could lead to the disruption of a functional BRCA1-CtIP-CtBP complex that may contribute to the development of tamoxifen resistance.

In chapter 5, by showing similar expression of the ER corepressor NCoR as well as the transcriptional corepressor CtBP between tamoxifen sensitive and resistant cells, we first ruled out the possibility of decreased corepressors expression as a cause of the tamoxifen resistant phenotype of our cell line models. Moreover, we also determined that ligand independent activation of ER by enhanced growth signaling at least at the site of serine 118 is also unlikely to be another alternative explanation for our resistant models. Based on these observations, it seems to suggest that CtIP silencing observed in our experimental models could be rather an independent than an integrated mechanism. However, this still needs future studies to exclude whether CtIP silencing is integrated with other known causes such as increased coactivator expression. We next further tested the potential involvement of a BRCA1-CtIP-CtBP complex in the development of tamoxifen resistance. Our results demonstrated the formation of a BRCA1-CtIP-CtBP complex in tamoxifen sensitive but not tamoxifen resistant cells, supporting a potential role of such protein complex in the development of tamoxifen resistance. In addition, we observed a strong endogenous association between ER and BRCA1 in both sensitive and

resistant cells. Interestingly, this *in vivo* interaction is disrupted upon estrogen treatment but enhanced by addition of tamoxifen. Moreover, we demonstrated that, under physiological context, ER, BRCA1, CtIP and CtBP are associated with ERE elements of the promoter region of the endogenous estrogen responsive gene pS2 in the absence of ligand. Upon treating cells with estrogen, the BRCA1-CtIP-CtBP complex appears to dissociate with the promoter. On the other hand, tamoxifen seems to stabilize this complex on the pS2 promoter. All these data strongly support the potential involvement of a BRCA1-CtIP-CtBP complex in modulating ER signaling and the development of tamoxifen resistance.

### 6.2. FUTURE DIRECTIONS

In this dissertation, we demonstrated that CtIP expression is significantly decreased both at mRNA and protein levels in tamoxifen resistant cells when compared to their parental tamoxifen sensitive counterparts. It is known that methylation of cytosine residues at cytosine-guanine sites (CpG islands) located in the promoter region of genes is a common mechanism of transcriptional repression. However, we found no evidence of CpG methylation on the CtIP gene promoter region in tamoxifen resistant cells, ruling out promoter methylation as the mechanism for CtIP silencing. Therefore, future work is needed to investigate the mechanism associated with CtIP downregulation in tamoxifen resistant cells. It has been shown that the human CtIP promoter contains two consensus E2F-binding sites, TCTCCCGC and TTCGCCGC only 19bp from each other and very close to the transcription start site, which behave as repressive elements for CtIP expression (Liu and Lee, 2006). Interestingly, it has also been shown that E2F6, a member of the E2F family of transcriptional regulators, can be recruited to the CtIP promoter and repress transcription of the CtIP gene (Oberley et al., 2003). It is known

that E2F1-3 function as transcriptional activators while E2F4, E2F5 and E2F6 act predominantly as repressors. However, E2F6 is significantly different to the other E2F family members in that it lacks the C-terminal trans-activation domain while retaining the DNA binding domain and E2F6 has been shown to act as a potent transcriptional repressor in general. Based on this evidence, it is possible that CtIP downregulation in tamoxifen resistant cells may be the consequence of increased transcriptional repression by E2F6 on the CtIP promoter via the aforementioned E2F-binding sites. Thus, one start point in the future could be the investigation of whether E2F6 plays a role in CtIP downregulation in tamoxifen resistant cells through a transcriptional regulatory mechanism.

Our studies described in chapter 4 and 5 demonstrate that CtIP silencing is critical for the development of tamoxifen resistance in breast cancer cells and strongly support the potential involvement of a BRCA1-CtIP-CtBP complex in modulating ER signaling and the development of tamoxifen resistance. These findings also led us to propose a hypothetical model of the BRCA1-CtIP-CtBP repressor complex in tamoxifen sensitive and resistant breast cancer cells (Figure 5.6.). Knowing the potential involvement of this complex in the development of resistance to tamoxifen, future work should aim to dissect detailed roles of such complex in modulating ER function as well as response to antiestrogens, which will provide us with novel insights on how CtIP silencing could ultimately lead to resistance to tamoxifen. Our hypothesis would be that CtIP may play a role as a corepressor of ER activity and such repressive activity of CtIP may be linked to the inhibitory growth effects exerted by tamoxifen. We further speculate that CtIP may exert repression on ER transcriptional activity through the interaction with BRCA1 and CtBP to form a transcriptional repressor complex. Therefore, future investigation could

focus on determining whether CtIP is a repressor of ER activity, whether the effect of CtIP accounts for the antagonist activity of tamoxifen, and whether CtIP exerts repression on ER transcriptional activity through the formation of a transcriptional repressor complex with BRCA1 and CtBP. The effect of CtIP (depletion or overexpression) on the ER transcriptional activity and the antagonist activity of tamoxifen could be tested by means of the ERE-TK luciferase reporter assays and monitoring expression of endogenous $E_2$-responsive genes. In addition, the CtIP mutants that are deficient in their ability to bind either BRCA1 (S327A mutant) or CtBP (D-PLDLS mutant) could be used to determine whether the putative repressive effect of CtIP on ER transcriptional activity requires the interaction between CtIP and BRCA1 or CtIP and CtBP.

In addition, another potential line of future research would be to characterize roles of other important key genes identified by SAGE in the development of tamoxifen resistance. In this dissertation, we extensively characterized the functional role of one of the most interesting genes (CtIP) in the development of tamoxifen resistance. In fact, by defining and comparing global gene expression profiles of tamoxifen sensitive and resistant cell lines, we observed several other highly differentially expressed genes as discussed in chapter 3 that could also play important roles in the development of resistance. Therefore, future studies should also aim to clarify roles of these other important genes in tamoxifen resistance by using similar strategies described in this dissertation. One very interesting candidate gene could be REA, which is an ER selective coregulator and has been shown to be significantly downregulated in tamoxifen resistant cells. Previous *in vitro* and *in vivo* studies suggest that REA may play an important role in determining the sensitivity of estrogen target cells, including breast cancer cells, to antiestrogens and estrogens and that REA is a physiological modulator of ER function in

the mammary gland (Montano et al., 1999; Mussi et al., 2006). Therefore, it is possible that besides silencing CtIP, tamoxifen resistant cells could also downregulate REA to desensitize them to the inhibitory effects of tamoxifen and thereby achieve maximal growth capability in the presence of tamoxifen.

Overall, the research findings described and discussed in this dissertation contribute to our understanding of mechanisms of tamoxifen resistance in breast cancer. The *in vitro* tamoxifen resistant breast cancer cell line models we developed over years have been proven to be a unique and excellent source to study antiestrogen resistance. Indeed, global gene expression profiles of these experimental models generated by SAGE, which will be ultimately available in public domains, provide us, as well as other investigators in this field, a valuable resource for the identification of novel targets implicated in the development of tamoxifen resistance. I hope the identification of CtIP silencing as a novel mechanism for the development of tamoxifen resistance in breast cancer and its potential use as a biomarker for breast cancer prognosis and clinical management will open-up a myriad of novel possibilities both for the diagnostic/prognostic fields as well as for therapeutic areas of breast cancer.

## References:


Abba, M. C., Drake, J. A., Hawkins, K. A., Hu, Y., Sun, H., Notcovich, C., Gaddis, S., Sahin, A., Baggerly, K., and Aldaz, C. M. (2004). Transcriptomic changes in human breast cancer progression as determined by serial analysis of gene expression. Breast Cancer Res 6, R499-513.

Abba, M. C., Hu, Y., Sun, H., Drake, J. A., Gaddis, S., Baggerly, K., Sahin, A., and Aldaz, C. M. (2005). Gene expression signature of estrogen receptor alpha status in breast cancer. BMC Genomics 6, 37.

Aldaz, C. M., Hu, Y., Daniel, R., Gaddis, S., Kittrell, F., and Medina, D. (2002). Serial analysis of gene expression in normal p53 null mammary epithelium. Oncogene 21, 6366-6376.

Ali, S., and Coombes, R. C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2, 101-112.

Ali, S., Metzger, D., Bornert, J. M., and Chambon, P. (1993). Modulation of transcriptional activation by ligand-dependent phosphorylation of the human oestrogen receptor A/B region. Embo J 12, 1153-1160.

Allen, E., and Doisy, E. A. (1983). Landmark article Sept 8, 1923. An ovarian hormone. Preliminary report on its localization, extraction and partial purification, and action in test animals. By Edgar Allen and Edward A. Doisy. Jama 250, 2681-2683.

Altucci, L., Addeo, R., Cicatiello, L., Dauvois, S., Parker, M. G., Truss, M., Beato, M., Sica, V., Bresciani, F., and Weisz, A. (1996). 17beta-Estradiol induces cyclin D1 gene transcription, p36D1-p34cdk4 complex activation and p105Rb phosphorylation during mitogenic stimulation of G(1)-arrested human breast cancer cells. Oncogene 12, 2315-2324.

American Cancer Society (2007). Cancer Facts & Figures 2007. Atlanta: American Cancer Society.

Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. Science 277, 965-968.

Baggerly, K. A., Deng, L., Morris, J. S., and Aldaz, C. M. (2003). Differential expression in SAGE: accounting for normal between-library variation. Bioinformatics 19, 1477-1483.

Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell 86, 263-274.

Balfour, J. A., and Goa, K. L. (1998). Raloxifene. Drugs Aging 12, 335-341; discussion 342.

Balthazart, J., and Absil, P. (1997). Identification of catecholaminergic inputs to and outputs from aromatase-containing brain areas of the Japanese quail by tract tracing combined with tyrosine hydroxylase immunocytochemistry. J Comp Neurol 382, 401-428.

Balthazart, J., Foidart, A., and Hendrick, J. C. (1990). The induction by testosterone of aromatase activity in the preoptic area and activation of copulatory behavior. Physiol Behav 47, 83-94.

Barker, S. (2006). Non-steroidal anti-estrogens in the treatment of breast cancer. Curr Opin Investig Drugs 7, 1085-1091.

Beatson, G.T. (1896). On the treatment of inoperable cases of carcinogen of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet 2: 104-107, 162-165.

Benz, C. C., Scott, G. K,. Sarup, J. C., Johnson, R. M., Tripathy, D., Coronado, E., Shepard, H. M. and Osborne, C. K. (1993) Estrogendependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Research and Treatment 24 85–95.

Bernstein, L., and Ross, R. K. (1993). Endogenous hormones and breast cancer risk. Epidemiol Rev 15, 48-65.

Berry, M., Metzger, D., and Chambon, P. (1990). Role of the two activating domains of the oestrogen receptor in the cell-type and promoter-context dependent agonistic activity of the anti-oestrogen 4-hydroxytamoxifen. Embo J 9, 2811-2818.

Black, L. J., Jones, C. D., and Falcone, J. F. (1983). Antagonism of estrogen action with a new benzothiophene derived antiestrogen. Life Sci 32, 1031-1036.

Bouras, T., Southey, M. C., and Venter, D. J. (2001). Overexpression of the steroid receptor coactivator AIB1 in breast cancer correlates with the absence of estrogen

and progesterone receptors and positivity for p53 and HER2/neu. Cancer Res 61, 903-907.

Breast. In: American Joint Committee on Cancer.: AJCC Cancer Staging Manual. 6th ed. New York, NY: Springer, 2002, pp 171-180.

Brodie, A., and Inkster, S. (1993). Aromatase in the human testis. J Steroid Biochem Mol Biol 44, 549-555.

Bryant, P. J., Huettner, B., Held, L. I., Jr., Ryerse, J., and Szidonya, J. (1988). Mutations at the fat locus interfere with cell proliferation control and epithelial morphogenesis in Drosophila. Dev Biol 129, 541-554.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389, 753-758.

Buckley, M. M., and Goa, K. L. (1989). Tamoxifen. A reappraisal of its pharmacodynamic and pharmacokinetic properties, and therapeutic use. Drugs 37, 451-490.

Bunone, G., Briand, P. A., Miksicek, R. J., and Picard, D. (1996). Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. Embo J 15, 2174-2183.

Buzdar, A., Howell, A., Cuzick, J., Wale, C., Distler, W., Hoctin-Boes, G., Houghton, J., Locker, G. Y., and Nabholtz, J. M. (2006). Comprehensive side-effect profile of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: long-term safety analysis of the ATAC trial. Lancet Oncol 7, 633-643.

Campbell, R. A., Bhat-Nakshatri, P., Patel, N. M., Constantinidou, D., Ali, S., and Nakshatri, H. (2001). Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. J Biol Chem 276, 9817-9824.

Carani, C., Qin, K., Simoni, M., Faustini-Fustini, M., Serpente, S., Boyd, J., Korach, K. S., and Simpson, E. R. (1997). Effect of testosterone and estradiol in a man with aromatase deficiency. N Engl J Med 337, 91-95.

Castagnetta, L. A., Lo Casto, M., Granata, O. M., Polito, L., Calabro, M., Lo Bue, A., Bellavia, V., and Carruba, G. (1996). Estrogen content and metabolism in human breast tumor tissues and cells. Ann N Y Acad Sci 784, 314-324.

Castoria, G., Migliaccio, A., Bilancio, A., Di Domenico, M., de Falco, A., Lombardi, M., Fiorentino, R., Varricchio, L., Barone, M. V., and Auricchio, F. (2001). PI3-kinase in concert with Src promotes the S-phase entry of oestradiol-stimulated MCF-7 cells. Embo J 20, 6050-6059.

Cauley, J. A., Norton, L., Lippman, M. E., Eckert, S., Krueger, K. A., Purdie, D. W., Farrerons, J., Karasik, A., Mellstrom, D., Ng, K. W., et al. (2001). Continued breast cancer risk reduction in postmenopausal women treated with raloxifene: 4-year results from the MORE trial. Multiple outcomes of raloxifene evaluation. Breast Cancer Res Treat 65, 125-134.

Cavalieri, E., Frenkel, K., Liehr, J. G., Rogan, E., and Roy, D. (2000). Estrogens as endogenous genotoxic agents--DNA adducts and mutations. J Natl Cancer Inst Monogr, 75-93.

Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K., et al. (1997). Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci U S A 94, 10937-10942.

Chan, C. M., Lykkesfeldt, A. E., Parker, M. G., and Dowsett, M. (1999). Expression of nuclear receptor interacting proteins TIF-1, SUG-1, receptor interacting protein 140, and corepressor SMRT in tamoxifen-resistant breast cancer. Clin Cancer Res 5, 3460-3467.

Charpentier, A. H., Bednarek, A. K., Daniel, R. L., Hawkins, K. A., Laflin, K. J., Gaddis, S., MacLeod, M. C., and Aldaz, C. M. (2000). Effects of estrogen on global gene expression: identification of novel targets of estrogen action. Cancer Res 60, 5977-5983.

Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90, 569-580.

Chen, J. D., and Evans, R. M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454-457.

Chen, P. L., Liu, F., Cai, S., Lin, X., Li, A., Chen, Y., Gu, B., Lee, E. Y., and Lee, W. H. (2005). Inactivation of CtIP leads to early embryonic lethality mediated by G1 restraint and to tumorigenesis by haploid insufficiency. Mol Cell Biol 25, 3535-3542.

Chinnadurai, G. (2002). CtBP, an unconventional transcriptional corepressor in development and oncogenesis. Mol Cell 9, 213-224.

Chinnadurai, G. (2006). CtIP, a candidate tumor susceptibility gene is a team player with luminaries. Biochim Biophys Acta 1765, 67-73.

Christiansen, C., Christensen, M. S., and Transbol, I. (1981). Bone mass in postmenopausal women after withdrawal of oestrogen/gestagen replacement therapy. Lancet 1, 459-461.

Chui, X., Egami, H., Yamashita, J., Kurizaki, T., Ohmachi, H., Yamamoto, S., and Ogawa, M. (1996). Immunohistochemical expression of the c-kit proto-oncogene product in human malignant and non-malignant breast tissues. Br J Cancer 73, 1233-1236.

Clarke, R., Skaar, T. C., Bouker, K. B., Davis, N., Lee, Y. R., Welch, J. N., and Leonessa, F. (2001). Molecular and pharmacological aspects of antiestrogen resistance. J Steroid Biochem Mol Biol 76, 71-84.

Cole, M. P., Jones, C. T., and Todd, I. D. (1971). A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer 25, 270-275.

Colinge, J., and Feger, G. (2001). Detecting the impact of sequencing errors on SAGE data. Bioinformatics 17, 840-842.

Collaborative Group on Hormonal Factors in Breast Cancer (1997). Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Lancet 350, 1047-1059.

Coombes, R. C., Haynes, B. P., Dowsett, M., Quigley, M., English, J., Judson, I. R., Griggs, L. J., Potter, G. A., McCague, R., and Jarman, M. (1995). Idoxifene: report of a phase I study in patients with metastatic breast cancer. Cancer Res 55, 1070-1074.

Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker, V. R., Davis, B. J., and Korach, K. S. (1999). Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. Science 286, 2328-2331.

Coutts, A. S., and Murphy, L. C. (1998). Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. Cancer Res 58, 4071-4074.

Cummings, S. R., Eckert, S., Krueger, K. A., Grady, D., Powles, T. J., Cauley, J. A., Norton, L., Nickelsen, T., Bjarnason, N. H., Morrow, M., et al. (1999). The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. Jama 281, 2189-2197.

Cuzick, J., Powles, T., Veronesi, U., Forbes, J., Edwards, R., Ashley, S., and Boyle, P. (2003). Overview of the main outcomes in breast-cancer prevention trials. Lancet 361, 296-300.

Datta, S. R., Brunet, A., and Greenberg, M. E. (1999). Cellular survival: a play in three Akts. Genes Dev 13, 2905-2927.

Desta, Z., Ward, B. A., Soukhova, N. V., and Flockhart, D. A. (2004). Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Ther 310, 1062-1075.

Dhingra, K. (1999). Antiestrogens--tamoxifen, SERMs and beyond. Invest New Drugs 17, 285-311.

Dobrzycka, K. M., Townson, S. M., Jiang, S., and Oesterreich, S. (2003). Estrogen receptor corepressors -- a role in human breast cancer? Endocr Relat Cancer 10, 517-536.

Dong, L., Wang, W., Wang, F., Stoner, M., Reed, J. C., Harigai, M., Samudio, I., Kladde, M. P., Vyhlidal, C., and Safe, S. (1999). Mechanisms of transcriptional activation of bcl-2 gene expression by 17beta-estradiol in breast cancer cells. J Biol Chem 274, 32099-32107.

Dowsett, M., Harper-Wynne, C., Boeddinghaus, I., Salter, J., Hills, M., Dixon, M., Ebbs, S., Gui, G., Sacks, N., and Smith, I. (2001). HER-2 amplification impedes the antiproliferative effects of hormone therapy in estrogen receptor-positive primary breast cancer. Cancer Res 61, 8452-8458.

Dunne, J., Hanby, A. M., Poulsom, R., Jones, T. A., Sheer, D., Chin, W. G., Da, S. M., Zhao, Q., Beverley, P. C., and Owen, M. J. (1995). Molecular cloning and tissue expression of FAT, the human homologue of the Drosophila fat gene that is located on chromosome 4q34-q35 and encodes a putative adhesion molecule. Genomics 30, 207-223.

Dutta, A., Chandra, R., Leiter, L. M., and Lester, S. (1995). Cyclins as markers of tumor proliferation: immunocytochemical studies in breast cancer. Proc Natl Acad Sci U S A 92, 5386-5390.

Early Breast Cancer Trialists' Collaborative Group (1992). Systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. 133 randomised trials involving 31,000 recurrences and 24,000 deaths among 75,000 women. Lancet 339, 71-85.

Early Breast Cancer Trialists' Collaborative Group (1998). Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet 351, 1451-1467.

Early Breast Cancer Trialists' Collaborative Group (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet 365, 1687-1717.

Eiermann, W., Paepke, S., Appfelstaedt, J., Llombart-Cussac, A., Eremin, J., Vinholes, J., Mauriac, L., Ellis, M., Lassus, M., Chaudri-Ross, H. A., et al. (2001). Preoperative treatment of postmenopausal breast cancer patients with letrozole: A randomized double-blind multicenter study. Ann Oncol 12, 1527-1532.

El-Ashry, D., Chrysogelos, S. A., Lippman, M. E., and Kern, F. G. (1996). Estrogen induction of TGF-alpha is mediated by an estrogen response element composed of two imperfect palindromes. J Steroid Biochem Mol Biol 59, 261-269.

Ellis, M. J., Coop, A., Singh, B., Mauriac, L., Llombert-Cussac, A., Janicke, F., Miller, W. R., Evans, D. B., Dugan, M., Brady, C., et al. (2001). Letrozole is more effective neoadjuvant endocrine therapy than tamoxifen for ErbB-1- and/or ErbB-2-positive, estrogen receptor-positive primary breast cancer: evidence from a phase III randomized trial. J Clin Oncol 19, 3808-3816.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J. A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. J Clin Endocrinol Metab 82, 4258-4265.

Eriksen, E. F., Colvard, D. S., Berg, N. J., Graham, M. L., Mann, K. G., Spelsberg, T. C., and Riggs, B. L. (1988). Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241, 84-86.

Ettinger, B., Black, D. M., Mitlak, B. H., Knickerbocker, R. K., Nickelsen, T., Genant, H. K., Christiansen, C., Delmas, P. D., Zanchetta, J. R., Stakkestad, J., et al. (1999). Reduction of vertebral fracture risk in postmenopausal women with osteoporosis treated with raloxifene: results from a 3-year randomized clinical trial. Multiple Outcomes of Raloxifene Evaluation (MORE) Investigators. Jama 282, 637-645.

Fan, S., Ma, Y. X., Wang, C., Yuan, R. Q., Meng, Q., Wang, J. A., Erdos, M., Goldberg, I. D., Webb, P., Kushner, P. J., et al. (2001). Role of direct interaction in BRCA1 inhibition of estrogen receptor activity. Oncogene 20, 77-87.

Figtree, G. A., McDonald, D., Watkins, H., and Channon, K. M. (2003). Truncated estrogen receptor alpha 46-kDa isoform in human endothelial cells: relationship to acute activation of nitric oxide synthase. Circulation 107, 120-126.

Filardo, E. J. (2002). Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol 80, 231-238.

Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., et al. (1998). Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90, 1371-1388.

Flouriot, G., Brand, H., Denger, S., Metivier, R., Kos, M., Reid, G., Sonntag-Buck, V., and Gannon, F. (2000). Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. Embo J 19, 4688-4700.

Font de Mora, J., and Brown, M. (2000). AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 20, 5041-5047.

Foray, N., Marot, D., Gabriel, A., Randrianarison, V., Carr, A. M., Perricaudet, M., Ashworth, A., and Jeggo, P. (2003). A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein. Embo J 22, 2860-2871.

Friedrichs, K., Gluba, S., Eidtmann, H., and Jonat, W. (1993). Overexpression of p53 and prognosis in breast cancer. Cancer 72, 3641-3647.

Furuta, S., Wang, J. M., Wei, S., Jeng, Y. M., Jiang, X., Gu, B., Chen, P. L., Lee, E. Y., and Lee, W. H. (2006). Removal of BRCA1/CtIP/ZBRK1 repressor complex on ANG1 promoter leads to accelerated mammary tumor growth contributed by prominent vasculature. Cancer Cell 10, 13-24.

Fusco, C., Reymond, A., and Zervos, A. S. (1998). Molecular cloning and characterization of a novel retinoblastoma-binding protein. Genomics 51, 351-358.

Garcia-Segura, L. M., Wozniak, A., Azcoitia, I., Rodriguez, J. R., Hutchison, R. E., and Hutchison, J. B. (1999). Aromatase expression by astrocytes after brain injury:

implications for local estrogen formation in brain repair. Neuroscience 89, 567-578.

Gee, J. M., Robertson, J. F., Ellis, I. O., and Nicholson, R. I. (2001). Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. Int J Cancer 95, 247-254.

Geum, D., Sun, W., Paik, S. K., Lee, C. C., and Kim, K. (1997). Estrogen-induced cyclin D1 and D3 gene expressions during mouse uterine cell proliferation in vivo: differential induction mechanism of cyclin D1 and D3. Mol Reprod Dev 46, 450-458.

Green, P. S., Bishop, J., and Simpkins, J. W. (1997). 17 alpha-estradiol exerts neuroprotective effects on SK-N-SH cells. J Neurosci 17, 511-515.

Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M., and Chambon, P. (1986a). Cloning of the human oestrogen receptor cDNA. J Steroid Biochem 24, 77-83.

Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., and Chambon, P. (1986b). Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. Nature 320, 134-139.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y., and Shine, J. (1986). Sequence and expression of human estrogen receptor complementary DNA. Science 231, 1150-1154.

Grodin, J. M., Siiteri, P. K., and MacDonald, P. C. (1973). Source of estrogen production in postmenopausal women. J Clin Endocrinol Metab 36, 207-214.

Gronemeyer, H. (1991). Transcription activation by estrogen and progesterone receptors. Annu Rev Genet 25, 89-123.

Gruber, D. M., and Huber, J. C. (2001). Tissue specificity: the clinical importance of steroid metabolites in hormone replacement therapy. Maturitas 37, 151-157.

Gruvberger, S., Ringner, M., Chen, Y., Panavally, S., Saal, L. H., Borg, A., Ferno, M., Peterson, C., and Meltzer, P. S. (2001). Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 61, 5979-5984.

Gustafsson, J. A. (1999). Estrogen receptor beta--a new dimension in estrogen mechanism of action. J Endocrinol 163, 379-383.

Gutierrez, M. C., Detre, S., Johnston, S., Mohsin, S. K., Shou, J., Allred, D. C., Schiff, R., Osborne, C. K., and Dowsett, M. (2005). Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J Clin Oncol 23, 2469-2476.

Hall, J. M., Couse, J. F., and Korach, K. S. (2001). The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276, 36869-36872.

Hall, J. M., and McDonnell, D. P. (1999). The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140, 5566-5578.

Hard, G. C., Iatropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R., and Williams, G. M. (1993). Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD(BR) rats. Cancer Res 53, 4534-4541.

Harvell, D. M., Strecker, T. E., Tochacek, M., Xie, B., Pennington, K. L., McComb, R. D., Roy, S. K., and Shull, J. D. (2000). Rat strain-specific actions of 17beta-estradiol in the mammary gland: correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. Proc Natl Acad Sci U S A 97, 2779-2784.

Hayes, C. L., Spink, D. C., Spink, B. C., Cao, J. Q., Walker, N. J., and Sutter, T. R. (1996). 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1B1. Proc Natl Acad Sci U S A 93, 9776-9781.

Hemsell, D. L., Grodin, J. M., Brenner, P. F., Siiteri, P. K., and MacDonald, P. C. (1974). Plasma precursors of estrogen. II. Correlation of the extent of conversion of plasma androstenedione to estrone with age. J Clin Endocrinol Metab 38, 476-479.

Henderson, V. W. (1997). The epidemiology of estrogen replacement therapy and Alzheimer's disease. Neurology 48, S27-35.

Hodges, L. C., Cook, J. D., Lobenhofer, E. K., Li, L., Bennett, L., Bushel, P. R., Aldaz, C. M., Afshari, C. A., and Walker, C. L. (2003). Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res 1, 300-311.

Hong, S. H., and Privalsky, M. L. (2000). The SMRT corepressor is regulated by a MEK-1 kinase pathway: inhibition of corepressor function is associated with SMRT phosphorylation and nuclear export. Mol Cell Biol 20, 6612-6625.

Honig, S. F. Treatment of metastatic disease: hormonal therapy and chemotherapy. In: J.R. Harris, M.E. Lippman, M. Morrow and S. Hellman, Editors, Diseases of the Breast, Lippincott-Raven, Philadelphia, PA (1996), pp. 669–734.

Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C. K., and et al. (1995). Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377, 397-404.

Horwitz, K. B. (1988). The central role of progesterone receptors and progestational agents in the management and treatment of breast cancer. Semin Oncol 15, 14-19.

Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996). Nuclear receptor coactivators and corepressors. Mol Endocrinol 10, 1167-1177.

Howell, A., Cuzick, J., Baum, M., Buzdar, A., Dowsett, M., Forbes, J. F., Hoctin-Boes, G., Houghton, J., Locker, G. Y., and Tobias, J. S. (2005). Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years' adjuvant treatment for breast cancer. Lancet 365, 60-62.

Howell, A., DeFriend, D. J., Robertson, J. F., Blamey, R. W., Anderson, L., Anderson, E., Sutcliffe, F. A., and Walton, P. (1996). Pharmacokinetics, pharmacological and anti-tumour effects of the specific anti-oestrogen ICI 182780 in women with advanced breast cancer. Br J Cancer 74, 300-308.

Howell, S. J., Johnston, S. R., and Howell, A. (2004). The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. Best Pract Res Clin Endocrinol Metab 18, 47-66.

Hu, Y., Sun, H., Drake, J., Kittrell, F., Abba, M. C., Deng, L., Gaddis, S., Sahin, A., Baggerly, K., Medina, D., and Aldaz, C. M. (2004). From mice to humans: identification of commonly deregulated genes in mammary cancer via comparative SAGE studies. Cancer Res 64, 7748-7755.

Hutcheson, I. R., Knowlden, J. M., Madden, T. A., Barrow, D., Gee, J. M., Wakeling, A. E., and Nicholson, R. I. (2003). Oestrogen receptor-mediated modulation of the EGFR/MAPK pathway in tamoxifen-resistant MCF-7 cells. Breast Cancer Res Treat 81, 81-93.

Ikeda, K., Sato, M., Tsutsumi, O., Tsuchiya, F., Tsuneizumi, M., Emi, M., Imoto, I., Inazawa, J., Muramatsu, M., and Inoue, S. (2000). Promoter analysis and chromosomal mapping of human EBAG9 gene. Biochem Biophys Res Commun 273, 654-660.

Jefcoate, C. R., Liehr, J. G., Santen, R. J., Sutter, T. R., Yager, J. D., Yue, W., Santner, S. J., Tekmal, R., Demers, L., Pauley, R., et al. (2000). Tissue-specific synthesis and oxidative metabolism of estrogens. J Natl Cancer Inst Monogr, 95-112.

Jensen E. V., Jacobson H. I. Basic guides to the mechanism of estrogen action. Recent Prog. Horm. Res., 18: 318-414, 1962.Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., and DeSombre, E. R. (1968). A two-step mechanism for the interaction of estradiol with rat uterus. Proc Natl Acad Sci U S A 59, 632-638.

Joel, P. B., Smith, J., Sturgill, T. W., Fisher, T. L., Blenis, J., and Lannigan, D. A. (1998). pp90rsk1 regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. Mol Cell Biol 18, 1978-1984.

Johnston, S. R., Haynes, B. P., Smith, I. E., Jarman, M., Sacks, N. P., Ebbs, S. R., and Dowsett, M. (1993). Acquired tamoxifen resistance in human breast cancer and reduced intra-tumoral drug concentration. Lancet 342, 1521-1522.

Johnston, S. R., Lu, B., Scott, G. K., Kushner, P. J., Smith, I. E., Dowsett, M., and Benz, C. C. (1999). Increased activator protein-1 DNA binding and c-Jun NH2-terminal kinase activity in human breast tumors with acquired tamoxifen resistance. Clin Cancer Res 5, 251-256.

Johnston, S. R., Saccani-Jotti, G., Smith, I. E., Salter, J., Newby, J., Coppen, M., Ebbs, S. R., and Dowsett, M. (1995). Changes in estrogen receptor, progesterone receptor, and pS2 expression in tamoxifen-resistant human breast cancer. Cancer Res 55, 3331-3338.

Jones, L. P., Li, M., Halama, E. D., Ma, Y., Lubet, R., Grubbs, C. J., Deng, C. X., Rosen, E. M., and Furth, P. A. (2005). Promotion of mammary cancer development by tamoxifen in a mouse model of Brca1-mutation-related breast cancer. Oncogene 24, 3554-3562.

Jordan, V. C. (2003). Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov 2, 205-213.

Kahlert, S., Nuedling, S., van Eickels, M., Vetter, H., Meyer, R., and Grohe, C. (2000). Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem 275, 18447-18453.

Karnik, P. S., Kulkarni, S., Liu, X. P., Budd, G. T., and Bukowski, R. M. (1994). Estrogen receptor mutations in tamoxifen-resistant breast cancer. Cancer Res 54, 349-353.

Kato, S. (2001). Estrogen receptor-mediated cross-talk with growth factor signaling pathways. Breast Cancer 8, 3-9.

Kato, S., Endoh, H., Masuhiro, Y., Kitamoto, T., Uchiyama, S., Sasaki, H., Masushige, S., Gotoh, Y., Nishida, E., Kawashima, H., et al. (1995). Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. Science 270, 1491-1494.

Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. (1997). William L. McGuire Memorial Lecture. Antiestrogens: mechanisms of action and resistance in breast cancer. Breast Cancer Res Treat 44, 23-38.

Kim, H. P., Lee, J. Y., Jeong, J. K., Bae, S. W., Lee, H. K., and Jo, I. (1999). Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor alpha localized in caveolae. Biochem Biophys Res Commun 263, 257-262.

Knowlden JM, Hutcheson IR, Barrow D & Nicholson RI (2003b) IGF-1R and EGFR crosstalk in tamoxifen resistant breast cancer cells. Breast Cancer Research and Treatment 82 S171.

Knowlden, J. M., Hutcheson, I. R., Jones, H. E., Madden, T., Gee, J. M., Harper, M. E., Barrow, D., Wakeling, A. E., and Nicholson, R. I. (2003). Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. Endocrinology 144, 1032-1044.

Koipally, J., and Georgopoulos, K. (2002). Ikaros-CtIP interactions do not require C-terminal binding protein and participate in a deacetylase-independent mode of repression. J Biol Chem 277, 23143-23149.

Kousteni, S., Chen, J. R., Bellido, T., Han, L., Ali, A. A., O'Brien, C. A., Plotkin, L., Fu, Q., Mancino, A. T., Wen, Y., et al. (2002). Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 298, 843-846.

Kraus, B., Pohlschmidt, M., Leung, A. L., Germino, G. G., Snarey, A., Schneider, M. C., Reeders, S. T., and Frischauf, A. M. (1994). A novel cyclin gene (CCNF) in the region of the polycystic kidney disease gene (PKD1). Genomics 24, 27-33.

Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., Sar, M., Korach, K. S., Gustafsson, J. A., and Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A 95, 15677-15682.

Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J. A. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138, 863-870.

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 93, 5925-5930.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R., and Chambon, P. (1987). Functional domains of the human estrogen receptor. Cell 51, 941-951.

Kumar, V., Green, S., Staub, A., and Chambon, P. (1986). Localisation of the oestradiol-binding and putative DNA-binding domains of the human oestrogen receptor. Embo J 5, 2231-2236.

Kurokawa, H., and Arteaga, C. L. (2001). Inhibition of erbB receptor (HER) tyrosine kinases as a strategy to abrogate antiestrogen resistance in human breast cancer. Clin Cancer Res 7, 4436s-4442s; discussion 4411s-4412s.

Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K., Uht, R. M., and Webb, P. (2000). Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74, 311-317.

Lakhani, S. R. (1999). The transition from hyperplasia to invasive carcinoma of the breast. J Pathol 187, 272-278.

Lannigan, D. A. (2003). Estrogen receptor phosphorylation. Steroids 68, 1-9.

Lathrop, A. E., and Loeb, L. (1916). Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors. J Cancer Res 1, 1-19.

Lavinsky, R. M., Jepsen, K., Heinzel, T., Torchia, J., Mullen, T. M., Schiff, R., Del-Rio, A. L., Ricote, M., Ngo, S., Gemsch, J., et al. (1998). Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci U S A 95, 2920-2925.

Levin, E. R. (2002). Cellular functions of plasma membrane estrogen receptors. Steroids 67, 471-475.

Levin, E. R. (2003). Bidirectional signaling between the estrogen receptor and the epidermal growth factor receptor. Mol Endocrinol 17, 309-317.

Lewandowski, S., Kalita, K., and Kaczmarek, L. (2002). Estrogen receptor beta. Potential functional significance of a variety of mRNA isoforms. FEBS Lett 524, 1-5.

Lewis, J. S., and Jordan, V. C. (2005). Selective estrogen receptor modulators (SERMs): mechanisms of anticarcinogenesis and drug resistance. Mutat Res 591, 247-263.

Li, L., Haynes, M. P., and Bender, J. R. (2003). Plasma membrane localization and function of the estrogen receptor alpha variant (ER46) in human endothelial cells. Proc Natl Acad Sci U S A 100, 4807-4812.

Li, S., Chen, P. L., Subramanian, T., Chinnadurai, G., Tomlinson, G., Osborne, C. K., Sharp, Z. D., and Lee, W. H. (1999). Binding of CtIP to the BRCT repeats of BRCA1 involved in the transcription regulation of p21 is disrupted upon DNA damage. J Biol Chem 274, 11334-11338.

Li, S., Ting, N. S., Zheng, L., Chen, P. L., Ziv, Y., Shiloh, Y., Lee, E. Y., and Lee, W. H. (2000). Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature 406, 210-215.

Liehr, J. G. (2000). Is estradiol a genotoxic mutagenic carcinogen? Endocr Rev 21, 40-54.

Lien, E. A., Wester, K., Lonning, P. E., Solheim, E., and Ueland, P. M. (1991). Distribution of tamoxifen and metabolites into brain tissue and brain metastases in breast cancer patients. Br J Cancer 63, 641-645.

List, H. J., Lauritsen, K. J., Reiter, R., Powers, C., Wellstein, A., and Riegel, A. T. (2001a). Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 276, 23763-23768.

List, H. J., Reiter, R., Singh, B., Wellstein, A., and Riegel, A. T. (2001b). Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. Breast Cancer Res Treat 68, 21-28.

Liu, F., and Lee, W. H. (2006). CtIP activates its own and cyclin D1 promoters via the E2F/RB pathway during G1/S progression. Mol Cell Biol 26, 3124-3134.

Liu, H., Lee, E. S., Gajdos, C., Pearce, S. T., Chen, B., Osipo, C., Loweth, J., McKian, K., De Los Reyes, A., Wing, L., and Jordan, V. C. (2003). Apoptotic action of 17beta-estradiol in raloxifene-resistant MCF-7 cells in vitro and in vivo. J Natl Cancer Inst 95, 1586-1597.

Lopez, G. N., Turck, C. W., Schaufele, F., Stallcup, M. R., and Kushner, P. J. (2001). Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 276, 22177-22182.

Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., Carbone, P. P., and DeMets, D. L. (1992). Effects of tamoxifen on bone mineral density in postmenopausal women with breast cancer. N Engl J Med 326, 852-856.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., and Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci U S A 90, 11162-11166.

Ma, Y. L., Bryant, H. U., Zeng, Q., Palkowitz, A., Jee, W. S., Turner, C. H., and Sato, M. (2002). Long-term dosing of arzoxifene lowers cholesterol, reduces bone turnover, and preserves bone quality in ovariectomized rats. J Bone Miner Res 17, 2256-2264.

MacDonald, P. C., Madden, J. D., Brenner, P. F., Wilson, J. D., and Siiteri, P. K. (1979). Origin of estrogen in normal men and in women with testicular feminization. J Clin Endocrinol Metab 49, 905-916.

MacGregor, J. I., and Jordan, V. C. (1998). Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev 50, 151-196.

Mahoney, P. A., Weber, U., Onofrechuk, P., Biessmann, H., Bryant, P. J., and Goodman, C. S. (1991). The fat tumor suppressor gene in Drosophila encodes a novel member of the cadherin gene superfamily. Cell 67, 853-868.

Marsh, S., and McLeod, H. L. (2007). Pharmacogenetics and oncology treatment for breast cancer. Expert Opin Pharmacother 8, 119-127.

Martin, M. B., Franke, T. F., Stoica, G. E., Chambon, P., Katzenellenbogen, B. S., Stoica, B. A., McLemore, M. S., Olivo, S. E., and Stoica, A. (2000). A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinology 141, 4503-4511.

Masaki, T., Shiratori, Y., Rengifo, W., Igarashi, K., Yamagata, M., Kurokohchi, K., Uchida, N., Miyauchi, Y., Yoshiji, H., Watanabe, S., et al. (2003). Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 37, 534-543.

Matsumine, H., Hirato, K., Yanaihara, T., Tamada, T., and Yoshida, M. (1986). Aromatization by skeletal muscle. J Clin Endocrinol Metab 63, 717-720.

McKenna, N. J., Lanz, R. B., and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20, 321-344.

Meloni, A. R., Smith, E. J., and Nevins, J. R. (1999). A mechanism for Rb/p130-mediated transcription repression involving recruitment of the CtBP corepressor. Proc Natl Acad Sci U S A 96, 9574-9579.

Menasce, L. P., White, G. R., Harrison, C. J., and Boyle, J. M. (1993). Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. Genomics 17, 263-265.

Mettlin, C. (1999). Global breast cancer mortality statistics. CA Cancer J Clin 49, 138-144.

Metzger, D., Berry, M., Ali, S., and Chambon, P. (1995). Effect of antagonists on DNA binding properties of the human estrogen receptor in vitro and in vivo. Mol Endocrinol 9, 579-591.

Milla-Santos, A., Milla, L., Rallo, L., and Solano, V. (2001). Phase III randomized trial of toremifene vs tamoxifen in hormonodependant advanced breast cancer. Breast Cancer Res Treat 65, 119-124.

Miller, C. P. (2002). SERMs: evolutionary chemistry, revolutionary biology. Curr Pharm Des 8, 2089-2111.

Miller, W. R., and O'Neill, J. (1987). The importance of local synthesis of estrogen within the breast. Steroids 50, 537-548.

Mobley, J. A., Bhat, A. S., and Brueggemeier, R. W. (1999). Measurement of oxidative DNA damage by catechol estrogens and analogues in vitro. Chem Res Toxicol 12, 270-277.

Montano, M. M., Ekena, K., Delage-Mourroux, R., Chang, W., Martini, P., and Katzenellenbogen, B. S. (1999). An estrogen receptor-selective coregulator that potentiates the effectiveness of antiestrogens and represses the activity of estrogens. Proc Natl Acad Sci U S A 96, 6947-6952.

Morishima, A., Grumbach, M. M., Simpson, E. R., Fisher, C., and Qin, K. (1995). Aromatase deficiency in male and female siblings caused by a novel mutation and the physiological role of estrogens. J Clin Endocrinol Metab 80, 3689-3698.

Morris, C., and Wakeling, A. (2002). Fulvestrant ('Faslodex')--a new treatment option for patients progressing on prior endocrine therapy. Endocr Relat Cancer 9, 267-276.

Mosca, L. (2001). Rationale and overview of the Raloxifene Use for the Heart (RUTH) trial. Ann N Y Acad Sci 949, 181-185.

Moy, B., and Goss, P. E. (2006). Estrogen receptor pathway: resistance to endocrine therapy and new therapeutic approaches. Clin Cancer Res 12, 4790-4793.

Muller, S., van den Boom, D., Zirkel, D., Koster, H., Berthold, F., Schwab, M., Westphal, M., and Zumkeller, W. (2000). Retention of imprinting of the human apoptosis-related gene TSSC3 in human brain tumors. Hum Mol Genet 9, 757-763.

Murphy, L. C., Dotzlaw, H., Leygue, E., Douglas, D., Coutts, A., and Watson, P. H. (1997). Estrogen receptor variants and mutations. J Steroid Biochem Mol Biol 62, 363-372.

Murphy, L. C., Simon, S. L., Parkes, A., Leygue, E., Dotzlaw, H., Snell, L., Troup, S., Adeyinka, A., and Watson, P. H. (2000). Altered expression of estrogen receptor coregulators during human breast tumorigenesis. Cancer Res 60, 6266-6271.

Mussi, P., Liao, L., Park, S. E., Ciana, P., Maggi, A., Katzenellenbogen, B. S., Xu, J., and O'Malley, B. W. (2006). Haploinsufficiency of the corepressor of estrogen receptor activity (REA) enhances estrogen receptor function in the mammary gland. Proc Natl Acad Sci U S A 103, 16716-16721.

Nadal, A., Rovira, J. M., Laribi, O., Leon-quinto, T., Andreu, E., Ripoll, C., and Soria, B. (1998). Rapid insulinotropic effect of 17beta-estradiol via a plasma membrane receptor. Faseb J 12, 1341-1348.

Naftolin, F., Ryan, K. J., Davies, I. J., Reddy, V. V., Flores, F., Petro, Z., Kuhn, M., White, R. J., Takaoka, Y., and Wolin, L. (1975). The formation of estrogens by central neuroendocrine tissues. Recent Prog Horm Res 31, 295-319.

Nan, X., Campoy, F. J., and Bird, A. (1997). MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. Cell 88, 471-481.

Nandi, S., Guzman, R. C., and Yang, J. (1995). Hormones and mammary carcinogenesis in mice, rats, and humans: a unifying hypothesis. Proc Natl Acad Sci U S A 92, 3650-3657.

Nemere, I., Pietras, R. J., and Blackmore, P. F. (2003). Membrane receptors for steroid hormones: signal transduction and physiological significance. J Cell Biochem 88, 438-445.

Nishiyama, T., Ogura, K., Nakano, H., Ohnuma, T., Kaku, T., Hiratsuka, A., Muro, K., and Watabe, T. (2002). Reverse geometrical selectivity in glucuronidation and sulfation of cis- and trans-4-hydroxytamoxifens by human liver UDP-glucuronosyltransferases and sulfotransferases. Biochem Pharmacol 63, 1817-1830.

Normanno, N., Di Maio, M., De Maio, E., De Luca, A., de Matteis, A., Giordano, A., and Perrone, F. (2005). Mechanisms of endocrine resistance and novel therapeutic strategies in breast cancer. Endocr Relat Cancer 12, 721-747.

Nutter, L. M., Wu, Y. Y., Ngo, E. O., Sierra, E. E., Gutierrez, P. L., and Abul-Hajj, Y. J. (1994). An o-quinone form of estrogen produces free radicals in human breast cancer cells: correlation with DNA damage. Chem Res Toxicol 7, 23-28.

Oberley, M. J., Inman, D. R., and Farnham, P. J. (2003). E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. J Biol Chem 278, 42466-42476.

Ogawa, S., Inoue, S., Watanabe, T., Hiroi, H., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998). The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem Biophys Res Commun 243, 122-126.

Osborne, C. K. (1998). Tamoxifen in the treatment of breast cancer. N Engl J Med 339, 1609-1618.

Osborne, C. K., Bardou, V., Hopp, T. A., Chamness, G. C., Hilsenbeck, S. G., Fuqua, S. A., Wong, J., Allred, D. C., Clark, G. M., and Schiff, R. (2003). Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. J Natl Cancer Inst 95, 353-361.

Osborne, C. K., Coronado, E., Allred, D. C., Wiebe, V., and DeGregorio, M. (1991). Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. J Natl Cancer Inst 83, 1477-1482.

Osborne, C. K., Pippen, J., Jones, S. E., Parker, L. M., Ellis, M., Come, S., Gertler, S. Z., May, J. T., Burton, G., Dimery, I., et al. (2002). Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in

postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. J Clin Oncol 20, 3386-3395.

Osin, P., Gusterson, B. A., Philp, E., Waller, J., Bartek, J., Peto, J., and Crook, T. (1998). Predicted anti-oestrogen resistance in BRCA-associated familial breast cancers. Eur J Cancer 34, 1683-1686.

Oursler, M. J., Osdoby, P., Pyfferoen, J., Riggs, B. L., and Spelsberg, T. C. (1991). Avian osteoclasts as estrogen target cells. Proc Natl Acad Sci U S A 88, 6613-6617.

Pacek, M., Tutter, A. V., Kubota, Y., Takisawa, H., and Walter, J. C. (2006). Localization of MCM2-7, Cdc45, and GINS to the site of DNA unwinding during eukaryotic DNA replication. Mol Cell 21, 581-587.

Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science 277, 1508-1510.

Paganini-Hill, A., Dworsky, R., and Krauss, R. M. (1996). Hormone replacement therapy, hormone levels, and lipoprotein cholesterol concentrations in elderly women. Am J Obstet Gynecol 174, 897-902.

Pagano, M., Pepperkok, R., Verde, F., Ansorge, W., and Draetta, G. (1992). Cyclin A is required at two points in the human cell cycle. Embo J 11, 961-971.

Pasqualini, J. R., Chetrite, G., Blacker, C., Feinstein, M. C., Delalonde, L., Talbi, M., and Maloche, C. (1996). Concentrations of estrone, estradiol, and estrone sulfate and evaluation of sulfatase and aromatase activities in pre- and postmenopausal breast cancer patients. J Clin Endocrinol Metab 81, 1460-1464.

Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., et al. (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc Natl Acad Sci U S A 96, 9212-9217.

Pettersson, K., Delaunay, F., and Gustafsson, J. A. (2000). Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 19, 4970-4978.

Pietras, R. J., and Szego, C. M. (1977). Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265, 69-72.

Pike, A. C., Brzozowski, A. M., and Hubbard, R. E. (2000). A structural biologist's view of the oestrogen receptor. J Steroid Biochem Mol Biol 74, 261-268.

Pike, C. J. (1999). Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. J Neurochem 72, 1552-1563.

Pike, M. C., Spicer, D. V., Dahmoush, L., and Press, M. F. (1993). Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol Rev 15, 17-35.

Poola, I., Koduri, S., Chatra, S., and Clarke, R. (2000). Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach. J Steroid Biochem Mol Biol 72, 249-258.

Porter, D. A., Krop, I. E., Nasser, S., Sgroi, D., Kaelin, C. M., Marks, J. R., Riggins, G., and Polyak, K. (2001). A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 61, 5697-5702.

Porter, J. C. (1974). Proceedings: Hormonal regulation of breast development and activity. J Invest Dermatol 63, 85-92.

Powles, T. J., Hickish, T., Kanis, J. A., Tidy, A., and Ashley, S. (1996). Effect of tamoxifen on bone mineral density measured by dual-energy x-ray absorptiometry in healthy premenopausal and postmenopausal women. J Clin Oncol 14, 78-84.

Pyrhonen, S., Ellmen, J., Vuorinen, J., Gershanovich, M., Tominaga, T., Kaufmann, M., and Hayes, D. F. (1999). Meta-analysis of trials comparing toremifene with tamoxifen and factors predicting outcome of antiestrogen therapy in postmenopausal women with breast cancer. Breast Cancer Res Treat 56, 133-143.

Qian, N., Frank, D., O'Keefe, D., Dao, D., Zhao, L., Yuan, L., Wang, Q., Keating, M., Walsh, C., and Tycko, B. (1997). The IPL gene on chromosome 11p15.5 is imprinted in humans and mice and is similar to TDAG51, implicated in Fas expression and apoptosis. Hum Mol Genet 6, 2021-2029.

Ray, P., Ghosh, S. K., Zhang, D. H., and Ray, A. (1997). Repression of interleukin-6 gene expression by 17 beta-estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF-kappa B by the estrogen receptor. FEBS Lett 409, 79-85.

Rebollo, A., and Schmitt, C. (2003). Ikaros, Aiolos and Helios: transcription regulators and lymphoid malignancies. Immunol Cell Biol 81, 171-175.

Rhodes, D. R., Yu, J., Shanker, K., Deshpande, N., Varambally, R., Ghosh, D., Barrette, T., Pandey, A., and Chinnaiyan, A. M. (2004). ONCOMINE: a cancer microarray database and integrated data-mining platform. Neoplasia 6, 1-6.

Richards, J. S. (1994). Hormonal control of gene expression in the ovary. Endocr Rev 15, 725-751.

Robertson, J. F., Come, S. E., Jones, S. E., Beex, L., Kaufmann, M., Makris, A., Nortier, J. W., Possinger, K., and Rutqvist, L. E. (2005). Endocrine treatment options for advanced breast cancer--the role of fulvestrant. Eur J Cancer 41, 346-356.

Rogatsky, I., Trowbridge, J. M., and Garabedian, M. J. (1999). Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. J Biol Chem 274, 22296-22302.

Roodi, N., Bailey, L. R., Kao, W. Y., Verrier, C. S., Yee, C. J., Dupont, W. D., and Parl, F. F. (1995). Estrogen receptor gene analysis in estrogen receptor-positive and receptor-negative primary breast cancer. J Natl Cancer Inst 87, 446-451.

Rossouw, J. E., Anderson, G. L., Prentice, R. L., LaCroix, A. Z., Kooperberg, C., Stefanick, M. L., Jackson, R. D., Beresford, S. A., Howard, B. V., Johnson, K. C., et al. (2002). Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. Jama 288, 321-333.

Safe, S. (2001). Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam Horm 62, 231-252.

Saha, P., Thome, K. C., Yamaguchi, R., Hou, Z., Weremowicz, S., and Dutta, A. (1998). The human homolog of Saccharomyces cerevisiae CDC45. J Biol Chem 273, 18205-18209.

Sasano, H., Uzuki, M., Sawai, T., Nagura, H., Matsunaga, G., Kashimoto, O., and Harada, N. (1997). Aromatase in human bone tissue. J Bone Miner Res 12, 1416-1423.

Sato, M., Zeng, G. Q., Rowley, E., and Turner, C. H. (1998). LY353381 x HCl: an improved benzothiophene analog with bone efficacy complementary to parathyroid hormone-(1-34). Endocrinology 139, 4642-4651.

Schaeper, U., Subramanian, T., Lim, L., Boyd, J. M., and Chinnadurai, G. (1998). Interaction between a cellular protein that binds to the C-terminal region of adenovirus E1A (CtBP) and a novel cellular protein is disrupted by E1A through a conserved PLDLS motif. J Biol Chem 273, 8549-8552.

Schairer, C., Lubin, J., Troisi, R., Sturgeon, S., Brinton, L., and Hoover, R. (2000). Menopausal estrogen and estrogen-progestin replacement therapy and breast cancer risk. Jama 283, 485-491.

Schiff, R., Reddy, P., Ahotupa, M., Coronado-Heinsohn, E., Grim, M., Hilsenbeck, S. G., Lawrence, R., Deneke, S., Herrera, R., Chamness, G. C., et al. (2000). Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors in vivo. J Natl Cancer Inst 92, 1926-1934.

Shang, Y., and Brown, M. (2002). Molecular determinants for the tissue specificity of SERMs. Science 295, 2465-2468.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M. A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103, 843-852.

Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95, 927-937.

Shim, W. S., Conaway, M., Masamura, S., Yue, W., Wang, J. P., Kmar, R., and Santen, R. J. (2000). Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 141, 396-405.

Shou, J., Massarweh, S., Osborne, C. K., Wakeling, A. E., Ali, S., Weiss, H., and Schiff, R. (2004). Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96, 926-935.

Shozu, M., and Simpson, E. R. (1998). Aromatase expression of human osteoblast-like cells. Mol Cell Endocrinol 139, 117-129.

Shull, J. D., Spady, T. J., Snyder, M. C., Johansson, S. L., and Pennington, K. L. (1997). Ovary-intact, but not ovariectomized female ACI rats treated with 17beta-estradiol rapidly develop mammary carcinoma. Carcinogenesis 18, 1595-1601.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D. P., Ley, K., Chin, W. W., and Liao, J. K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407, 538-541.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., Michael, M. D., and et al. (1994). Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr Rev 15, 342-355.

Singletary, S. E., Allred, C., Ashley, P., Bassett, L. W., Berry, D., Bland, K. I., Borgen, P. I., Clark, G., Edge, S. B., Hayes, D. F., et al. (2002). Revision of the American Joint Committee on Cancer staging system for breast cancer. J Clin Oncol 20, 3628-3636.

Smith, C. L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biol Reprod 58, 627-632.

Smith, C. L., Nawaz, Z., and O'Malley, B. W. (1997). Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11, 657-666.

Smith, E. P., Boyd, J., Frank, G. R., Takahashi, H., Cohen, R. M., Specker, B., Williams, T. C., Lubahn, D. B., and Korach, K. S. (1994). Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. N Engl J Med 331, 1056-1061.

Song, R. X., McPherson, R. A., Adam, L., Bao, Y., Shupnik, M., Kumar, R., and Santen, R. J. (2002). Linkage of rapid estrogen action to MAPK activation by ERalpha-Shc association and Shc pathway activation. Mol Endocrinol 16, 116-127.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., et al. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A 100, 8418-8423.

Soule, H. D., Vazguez, J., Long, A., Albert, S., and Brennan, M. (1973). A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51, 1409-1416.

Speirs, V. (2002). Oestrogen receptor beta in breast cancer: good, bad or still too early to tell? J Pathol 197, 143-147.

Speirs, V., Malone, C., Walton, D. S., Kerin, M. J., and Atkin, S. L. (1999). Increased expression of estrogen receptor beta mRNA in tamoxifen-resistant breast cancer patients. Cancer Res 59, 5421-5424.

Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature 389, 194-198.

Stearns, V., Johnson, M. D., Rae, J. M., Morocho, A., Novielli, A., Bhargava, P., Hayes, D. F., Desta, Z., and Flockhart, D. A. (2003). Active tamoxifen metabolite plasma

concentrations after coadministration of tamoxifen and the selective serotonin reuptake inhibitor paroxetine. J Natl Cancer Inst 95, 1758-1764.

Stoica, G. E., Franke, T. F., Wellstein, A., Morgan, E., Czubayko, F., List, H. J., Reiter, R., Martin, M. B., and Stoica, A. (2003). Heregulin-beta1 regulates the estrogen receptor-alpha gene expression and activity via the ErbB2/PI 3-K/Akt pathway. Oncogene 22, 2073-2087.

Suh, N., Glasebrook, A. L., Palkowitz, A. D., Bryant, H. U., Burris, L. L., Starling, J. J., Pearce, H. L., Williams, C., Peer, C., Wang, Y., and Sporn, M. B. (2001). Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. Cancer Res 61, 8412-8415.

Sum, E. Y., Peng, B., Yu, X., Chen, J., Byrne, J., Lindeman, G. J., and Visvader, J. E. (2002). The LIM domain protein LMO4 interacts with the cofactor CtIP and the tumor suppressor BRCA1 and inhibits BRCA1 activity. J Biol Chem 277, 7849-7856.

Sun, M., Paciga, J. E., Feldman, R. I., Yuan, Z., Coppola, D., Lu, Y. Y., Shelley, S. A., Nicosia, S. V., and Cheng, J. Q. (2001). Phosphatidylinositol-3-OH Kinase (PI3K)/AKT2, activated in breast cancer, regulates and is induced by estrogen receptor alpha (ERalpha) via interaction between ERalpha and PI3K. Cancer Res 61, 5985-5991.

Sunderland, M. C., and Osborne, C. K. (1991). Tamoxifen in premenopausal patients with metastatic breast cancer: a review. J Clin Oncol 9, 1283-1297.

Takimoto, G. S., Graham, J. D., Jackson, T. A., Tung, L., Powell, R. L., Horwitz, L. D., and Horwitz, K. B. (1999). Tamoxifen resistant breast cancer: coregulators determine the direction of transcription by antagonist-occupied steroid receptors. J Steroid Biochem Mol Biol 69, 45-50.

Taylor, A. H., and Al-Azzawi, F. (2000). Immunolocalisation of oestrogen receptor beta in human tissues. J Mol Endocrinol 24, 145-155.

Toft, D., and Gorski, J. (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci U S A 55, 1574-1581.

Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989). The human estrogen receptor has two independent nonacidic transcriptional activation functions. Cell 59, 477-487.

Trowbridge, J. M., Rogatsky, I., and Garabedian, M. J. (1997). Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex. Proc Natl Acad Sci U S A 94, 10132-10137.

Tsai, M. J., and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 63, 451-486.

Turan, V. K., Sanchez, R. I., Li, J. J., Li, S. A., Reuhl, K. R., Thomas, P. E., Conney, A. H., Gallo, M. A., Kauffman, F. C., and Mesia-Vela, S. (2004). The effects of steroidal estrogens in ACI rat mammary carcinogenesis: 17beta-estradiol, 2-hydroxyestradiol, 4-hydroxyestradiol, 16alpha-hydroxyestradiol, and 4-hydroxyestrone. J Endocrinol 183, 91-99.

Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W., and McDonnell, D. P. (1994). Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8, 21-30.

van de Vijver, M. J. (1993). Molecular genetic changes in human breast cancer. Adv Cancer Res 61, 25-56.

van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., et al. (2002). A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347, 1999-2009.

Varma, A. K., Brown, R. S., Birrane, G., and Ladias, J. A. (2005). Structural basis for cell cycle checkpoint control by the BRCA1-CtIP complex. Biochemistry 44, 10941-10946.

Velculescu, V. E., Zhang, L., Vogelstein, B., and Kinzler, K. W. (1995). Serial analysis of gene expression. Science 270, 484-487.

Velculescu, V. E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M. A., Bassett, D. E., Jr., Hieter, P., Vogelstein, B., and Kinzler, K. W. (1997). Characterization of the yeast transcriptome. Cell 88, 243-251.

Vilkki, S., Launonen, V., Karhu, A., Sistonen, P., Vastrik, I., and Aaltonen, L. A. (2002). Screening for microsatellite instability target genes in colorectal cancers. J Med Genet 39, 785-789.

Visvader, J. E., Venter, D., Hahm, K., Santamaria, M., Sum, E. Y., O'Reilly, L., White, D., Williams, R., Armes, J., and Lindeman, G. J. (2001). The LIM domain gene

LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. Proc Natl Acad Sci U S A 98, 14452-14457.

Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., and Wolmark, N. (2002). The study of tamoxifen and raloxifene: preliminary enrollment data from a randomized breast cancer risk reduction trial. Clin Breast Cancer 3, 153-159.

Walker, P., Germond, J. E., Brown-Luedi, M., Givel, F., and Wahli, W. (1984). Sequence homologies in the region preceding the transcription initiation site of the liver estrogen-responsive vitellogenin and apo-VLDLII genes. Nucleic Acids Res 12, 8611-8626.

Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., and et al. (1985). Cloning of the human estrogen receptor cDNA. Proc Natl Acad Sci U S A 82, 7889-7893.

Wang, L. H., Yang, X. Y., Zhang, X., An, P., Kim, H. J., Huang, J., Clarke, R., Osborne, C. K., Inman, J. K., Appella, E., and Farrar, W. L. (2006). Disruption of estrogen receptor DNA-binding domain and related intramolecular communication restores tamoxifen sensitivity in resistant breast cancer. Cancer Cell 10, 487-499.

Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., et al. (2005). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 365, 671-679.

Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., Kawashima, H., and Muramatsu, M. (1998). Isolation of estrogen-responsive genes with a CpG island library. Mol Cell Biol 18, 442-449.

Watters, J. J., Campbell, J. S., Cunningham, M. J., Krebs, E. G., and Dorsa, D. M. (1997). Rapid membrane effects of steroids in neuroblastoma cells: effects of estrogen on mitogen activated protein kinase signalling cascade and c-fos immediate early gene transcription. Endocrinology 138, 4030-4033.

Webb, P., Lopez, G. N., Uht, R. M., and Kushner, P. J. (1995). Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. Mol Endocrinol 9, 443-456.

Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M. P., Chen, D., Huang, S. M., Subramanian, S., McKinerney, E., et al. (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 12, 1605-1618.

Webster, N. J., Green, S., Tasset, D., Ponglikitmongkol, M., and Chambon, P. (1989). The transcriptional activation function located in the hormone-binding domain of the human oestrogen receptor is not encoded in a single exon. Embo J 8, 1441-1446.

Weigel, N. L. (1996). Steroid hormone receptors and their regulation by phosphorylation. Biochem J 319 ( Pt 3), 657-667.

West, M., Blanchette, C., Dressman, H., Huang, E., Ishida, S., Spang, R., Zuzan, H., Olson, J. A., Jr., Marks, J. R., and Nevins, J. R. (2001). Predicting the clinical status of human breast cancer by using gene expression profiles. Proc Natl Acad Sci U S A 98, 11462-11467.

White, R. E., Darkow, D. J., and Lang, J. L. (1995). Estrogen relaxes coronary arteries by opening BKCa channels through a cGMP-dependent mechanism. Circ Res 77, 936-942.

Williams, G. M., Iatropoulos, M. J., Djordjevic, M. V., and Kaltenberg, O. P. (1993). The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. Carcinogenesis 14, 315-317.

Wong, A. K., Ormonde, P. A., Pero, R., Chen, Y., Lian, L., Salada, G., Berry, S., Lawrence, Q., Dayananth, P., Ha, P., et al. (1998). Characterization of a carboxy-terminal BRCA1 interacting protein. Oncogene 17, 2279-2285.

Wong, Z. W., and Ellis, M. J. (2004). First-line endocrine treatment of breast cancer: aromatase inhibitor or antioestrogen? Br J Cancer 90, 20-25.

Woodward, W. A., Strom, E. A., Tucker, S. L., McNeese, M. D., Perkins, G. H., Schechter, N. R., Singletary, S. E., Theriault, R. L., Hortobagyi, G. N., Hunt, K. K., and Buchholz, T. A. (2003). Changes in the 2003 American Joint Committee on Cancer staging for breast cancer dramatically affect stage-specific survival. J Clin Oncol 21, 3244-3248.

Wu, G., and Lee, W. H. (2006). CtIP, a multivalent adaptor connecting transcriptional regulation, checkpoint control and tumor suppression. Cell Cycle 5, 1592-1596.

Wu, R. C., Qin, J., Hashimoto, Y., Wong, J., Xu, J., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (2002). Regulation of SRC-3 (pCIP/ACTR/AIB-1/RAC-3/TRAM-1) Coactivator activity by I kappa B kinase. Mol Cell Biol 22, 3549-3561.

Wu-Baer, F., and Baer, R. (2001). Effect of DNA damage on a BRCA1 complex. Nature 414, 36.

Yager, J. D. (2000). Endogenous estrogens as carcinogens through metabolic activation. J Natl Cancer Inst Monogr, 67-73.

Yager, J. D., and Liehr, J. G. (1996). Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 36, 203-232.

Yu, X., and Baer, R. (2000). Nuclear localization and cell cycle-specific expression of CtIP, a protein that associates with the BRCA1 tumor suppressor. J Biol Chem 275, 18541-18549.

Yu, X., and Chen, J. (2004). DNA damage-induced cell cycle checkpoint control requires CtIP, a phosphorylation-dependent binding partner of BRCA1 C-terminal domains. Mol Cell Biol 24, 9478-9486.

Yu, X., Wu, L. C., Bowcock, A. M., Aronheim, A., and Baer, R. (1998). The C-terminal (BRCT) domains of BRCA1 interact in vivo with CtIP, a protein implicated in the CtBP pathway of transcriptional repression. J Biol Chem 273, 25388-25392.

Yue, W., Santen, R. J., Wang, J. P., Li, Y., Verderame, M. F., Bocchinfuso, W. P., Korach, K. S., Devanesan, P., Todorovic, R., Rogan, E. G., and Cavalieri, E. L. (2003). Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. J Steroid Biochem Mol Biol 86, 477-486.

Zhao, H., Langerod, A., Ji, Y., Nowels, K. W., Nesland, J. M., Tibshirani, R., Bukholm, I. K., Karesen, R., Botstein, D., Borresen-Dale, A. L., and Jeffrey, S. S. (2004). Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. Mol Biol Cell 15, 2523-2536.

Zheng, L., Annab, L. A., Afshari, C. A., Lee, W. H., and Boyer, T. G. (2001). BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. Proc Natl Acad Sci U S A 98, 9587-9592.

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