MECHANISMS OF HORMONAL ACTIVATION OF CDC25A AND COACTIVATION OF ESTROGEN RECEPTOR α BY PROTEIN INHIBITOR OF ACTIVATED STAT3 (PIAS3)

A Dissertation

by

WAN-RU LEE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Toxicology

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ABSTRACT

Mechanisms of Hormonal Activation of Cdc25A and Coactivation of Estrogen Receptor α by Protein Inhibitor of Activated STAT3 (PIAS3). (December 2006)
Wan-Ru Lee, B.S., National Taiwan University;
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The estrogen receptor (ER) is a ligand-activated transcription factor that regulates gene expression. The classical mechanisms of nuclear ER action include ligand-induced dimerization of ER which binds estrogen responsive elements (EREs) in promoters of target genes. In addition, non-genomic pathways of ER action have also been identified in breast cancer cells.

Cdc25A is a tyrosine phosphatase that catalyzes dephosphorylation of cyclin/cyclin-dependent kinase complexes to regulate G1- to S-phase cell cycle progression. Cdc25A mRNA levels are induced by 17β-estradiol (E2) in ZR-75 breast cancer cells, and deletion analysis of the Cdc25A promoter identified the -151 to -12 region as the minimal E2-responsive sequence. Subsequent mutation/deletion analysis showed that at least three different cis-elements were involved in activation of Cdc25A by E2, namely, GC-rich Sp1 binding sites, CCAAT motifs, and E2F sites. Studies with inhibitors and dominant negative expression plasmids show that E2 activates Cdc25A expression through activation of genomic ERα/Sp1 and E2F1 and cAMP-dependent activation of NF-YA. Thus, both genomic and non-genomic pathways of estrogen action are involved in induction of Cdc25A in breast cancer cells.

The PIAS family was initially identified as cytokine-induced inhibitors of STATs which contain several conserved domains involved in binding to other nuclear coactivators. In this study we have investigated coactivation of ERα by PIAS3 in breast cancer cell lines transiently cotransfected with the $pERE_3$ constructs which contain three tandem EREs linked to a luciferase reporter gene. PIAS3 coactivated ERα-mediated

transactivation in cells cotransfected with $pERE_3$ and wild-type ERα. In contrast to many other coactivators, PIAS3 also enhanced transactivation of ERα when cells were cotransfected with the TAF1 ERα mutant. In addition, PIAS3 does not interact with activation function 2 (AF2) domain of ERα in a mammalian two-hybrid assay. These data indicate that coactivation of ERα by PIAS3 was AF2-domain independent. Analysis of several PIAS3 deletion mutants showed that the region containing amino acids 274 to 416 of PIAS3 are required for coactivation suggesting that the RING finger domain and acidic region of PIAS3 are important for interactions with wild-type ERα. These results demonstrate that PIAS3 coactivated ERα and this represents a non-classical LXXLL-independent coactivation pathway.

DEDICATION

To my husband, my son and my daughter

My parents,

My parents-in-law,

My grandmother and my aunts,

For their love, support, patience, and friendship

ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor, Dr.Stephen Safe, for giving me the opportunity to do meaningful research, and for his guidance throughout my graduate career. I also want to thank the other members of my committee: Dr. Burghardt, Dr. Phillips, Dr. Porter, and also Dr. Donnelly for his willingness to substitute for one of my committee members at the last minute. I appreciate all members of the Safe lab for their friendship and collaboration. I also thank Lorna Safe, Kim Daniel, and Kathy Mooney for their administrative help.

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INTRODUCTION

1.1 Cancer

1.1.1 What is cancer?

Normal cells grow, divide, and die in an orderly fashion. During the early years of a person's life, cells in many tissues divide more rapidly until the individual becomes an adult. After that, cells in most parts of the body divide only to replace worn-out or dying cells and to repair cell damage. Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. Cancer develops when cells in specific tissues exhibit uncontrolled or dysregulaated growth. Even though cancer is often regarded as a single condition, it consists of more than 100 different diseases depending on its tissue origin. Compared to the physiology of normal cells, cancer cells exhibit deregulated homeostasis, uncontrolled growth, and invasiveness that are caused by cellular genetic or epigenetic alterations.

Cancer is the second leading cause of death in the United States. Half of all men and one third of all women in the United States will develop cancer during their lifetimes. About 1.4 million new cases of cancer will be diagnosed in 2006 and approximately 0.56 million people will die from this disease. Approximately, 1 out of 4 deaths are due to cancer. The 5-year survival rate from all cancers combined after first diagnosis is approximately 65%, whether in remission, under treatment, or disease-free (1).

1.1.2 Breast cancer

Breast cancer is the leading cancer among white and African American women and an estimated 211,240 new cases of invasive breast cancer will be diagnosed in women in the United States during 2005. In addition to invasive breast cancer, 58,490 new cases of

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This dissertation follows the style of *Journal of Biological Chemistry*.

in situ breast cancer are expected to occur among women during 2005 (2). While its incidence continues to rise, the mortality rate from breast cancer has remained almost unchanged in the past 5 decades, occupying first place as a cause of cancer-related deaths in nonsmoking women (3).

1.1.2.1 Development of the mammary gland

The mammary gland comprises stromal and epithelial cells that communicate with each other through the extracellular matrix (ECM). The major functional units of the mammary gland are the lobular structures comprising several small blind-ended ductules situated at the end of the terminal ducts and known as terminal ductal lobular units (TDLUs). The entire ductal system is lined by a continuous layer of luminal epithelial cells that are, in turn, surrounded by a layer of myoepithelial cells as shown in Figure 1-1. These myoepithelial cells are in direct contact with the basement membrane. The TDLUs are surrounded by delimiting fibroblasts and embedded in a specialized intralobular stroma. The luminal epithelial cells are the major proliferating cell type, whereas cell division or expression of antigens associated with proliferation is exceedingly rare in the myoepithelial cell type (4).

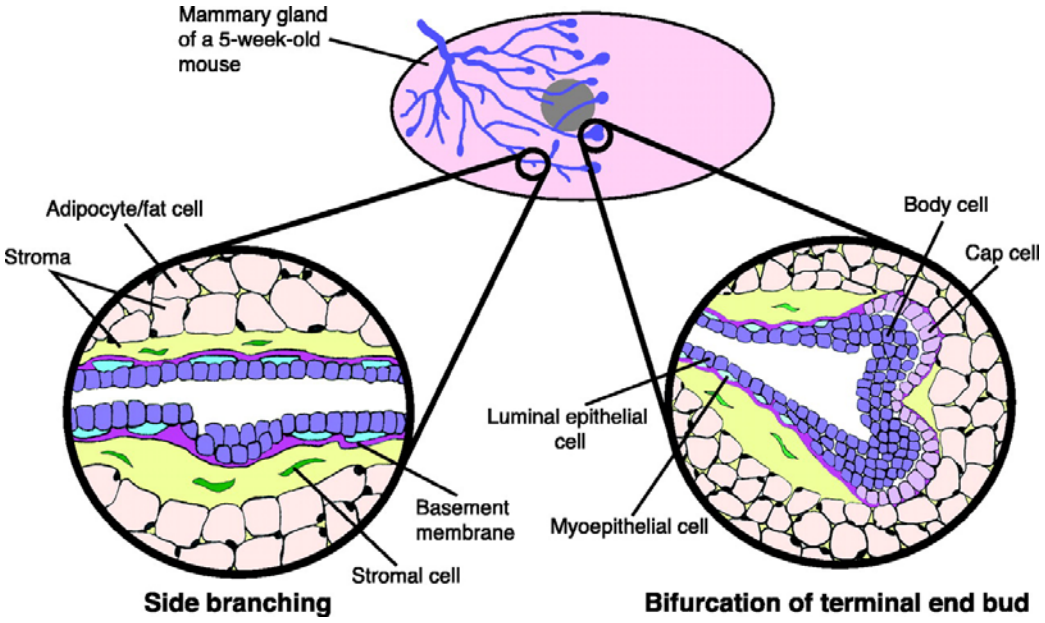

Figure 1-1 Two distinct mechanisms of branching morphogenesis in the pubertal mouse mammary gland (5).

Unlike most vertebrate organs, breast tissue continually changes in structure throughout the lifetime of reproductively-active females. Development of the mammary gland can be divided into 5 distinct stages which include the embryonic and prepubertal stage, puberty, pregnancy, lactation, and involution. Between birth and puberty, the growth of this structure is isometric in relation to the rest of the body, but at puberty, under the influence of ovarian and pituitary hormones, the gland undergoes the first phase of allometric growth. In early puberty, the primitive ductal structures begin to rapidly divide and multiply to form a treelike structure composed of many ducts. Once ovulatory menstrual cycles have begun, there is a cyclical increase in proliferation associated with the luteal phase, and the TDLUs become more elaborate in terms of the number of alveoli they contain during each successive ovulatory cycle (6).

This progressive development of the epithelium continues until approximately 35

years of age. The second phase of allometric growth in the mammary gland occurs during pregnancy. During early pregnancy, there is another burst of activity in which the ductal trees expand further and the number of ductules within the TDLUs greatly increase. These ductules differentiate to synthesise and secrete milk and lactate in late pregnancy, and in the postnatal period. Once weaning has occurred, the mammary gland involutes; the secretory luminal epithelial cells apoptose, the alveoli collapse and both epithelial and stromal components are remodeled to resemble the prepregnant state.

Interestingly, the developing mammary gland displays many of the properties associated with tumor progression. For example, the terminal end bud (TEB) is a rapidly proliferating mass of epithelial cells that invades into stromal tissue, much like a solid tumor. Moreover, many of the vital factors required for mammary development are also involved in breast cancer.

1.1.2.2 Risk factors for development of breast cancer

A risk factor is anything that increases your probability of developing a disease, such as cancer. Different cancers have different risk factors and these risk factors can be divided into several categories. Based on epidemiological studies conducted in different populations, several well-established risk factors for breast cancer have been identified and these include: age, geographic location and socioeconomic status, reproductive events (menarche, menopause, pregnancy, breastfeeding), exogenous hormones (hormone replacement therapy and oral contraceptives), lifestyle risk factors (alcohol, diet, obesity and physical activity), mammographic density, history of benign breast disease, ionizing radiation, bone density, height, IGF-1 and prolactin levels, exposure to chemopreventive agents, as well as genetic factors (high- and low-penetrance breast cancer susceptibility genes) (Table 1-1).

Table 1-1 Summary of breast cancer risk factors (7).

	Breast Cancer Risk Factors	Magnitude of Risk
	Factors that increase breast cancer risk	
Well-confirmed factors	Increasing age	++
	Geographical region (USA and western countries)	++
	Family history of breast cancer	++
	Mutations in BRCA1 and BRCA2 genes	++
	Mutations in other high-penetrance genes (p53, ATM, NBS1, LKB1)	++
	Ionizing radiation exposure (in childhood)	++
	History of benign breast disease	++
	Late age of menopause (>54)	++
	Early age of menarche (<12)	++
	Nulliparity and older age at first birth	++
	High mammography breast density	++
	Hormonal replacement therapy	+
	Oral contraceptives recent use	+
	Obesity in postmenopausal women	+
	Tall stature	+
	Alcohol consumption (~1 drink/day)	+
Probable factors	High insulin-like growth factor I (IGF-I) levels	++
	High prolactin levels	+
	High saturated fat and well-done meat intake	+
	Polymorphisms in low-penetrance genes	+
	High socioeconomic status	+
	Factors that decrease breast cancer risk	
Well-confirmed factors	Geographical region (Asia and Africa)	--
	Early age of first full-term pregnancy	--
	Higher parity	--
	Breast feeding (longer duration)	--
	Obesity in premenopausal women	-
	Fruit and vegetables consumption	-
	Physical activity	-
	Chemopreventive agents	-
Probable factors	Non-steroidal anti-inflammatory drugs	-
	Polymorphisms in low-penetrance genes	-

++ (moderate to high increase in risk) -- (moderate to high decrease in risk);

+ (low to moderate increase in risk) - (low to moderate decrease in risk)

Reproductive risk factors

Several reproductive factors such as nulliparity (having no children), late age at first birth, early age at menarche, and late age at menopause all have been associated with an increase in breast cancer risk.(8-11). For each of these factors, breast cancer risk tends to increase throughout the range of the variables. For example, risk is enhanced with increasing age at menopause from before 40 years of age to after 50 years of age. Women who have a first birth after age 30 years have a 50% to 100% higher risk of breast cancer relative to those who had their first child by age 20. Women who reached menarche by age 12 have only a 20 to 30 percent higher breast cancer risk compared with women who reached menarche at age 14 years.

The relationship between parity and breast cancer risk is more complex. Relative to nulliparous women, breast cancer risk actually is increased for one to two decades after giving birth, perhaps because of the increased exposure to circulating steroid hormones during pregnancy (12). After this time, however, breast cancer risk is lower in parous women compared with nulliparous women. This delayed (but long-lasting) reduction in risk may be related to hormone-induced changes in the cells of the breast, which result in their decreased susceptibility to carcinogens (12). Overall, the reduction in risk associated with parity outweighs the initial increase in risk, as the reduction occurs later in life when a woman's absolute breast cancer risk is much higher.

The duration of lifetime exposure to ovarian hormones is closely related to breast cancer risk. Early age at menarche (less than 12 years of age versus more than 14 years of age) has been associated with a 10~20% increase in breast cancer risk (13,14) and a 1-year delay in the onset of menarche is associated with a 5% reduction in risk for developing breast cancer in later life (15). Similarly, delayed menopause maximizing the number of ovulatory cycles leads to an increased breast cancer risk and each 1-year delay in the onset of menopause is associated with a 3% increase in risk (16). In contrast, surgically induced menopause before the age of 35 results in a decreased breast cancer risk. These women have only 40% of the risk of women experiencing natural menopause. It has been demonstrated that mammary epithelial cell proliferation, which is linked to

breast cancer development, can be correlated with serum ovarian hormonal levels. Proliferation rates are low in the follicular phase of the menstrual cycle, when estradiol and progesterone levels are also low, whereas during the luteal phase proliferation rates are two-fold higher and correlate with the significantly increased ovarian hormone levels (17). The higher cellular proliferative activity confers a higher susceptibility of the mammary gland to be transformed by chemical carcinogens (18). After menopause, ovarian hormone levels drop and this correlates with a substantial decrease in mammary epithelial cell proliferation (19). Numerous prospective epidemiological studies also provide strong evidence for this mechanism. Accordingly, postmenopausal women who develop breast cancer have on average 15% higher levels of circulating estradiol than other postmenopausal women (19).

Prolonged lactation has been demonstrated to be protective (20). There is a 4.3% decrease in the relative risk of breast cancer for every 12 months of breastfeeding, in addition to a decrease of 7.0% for each birth (21). The decrease of breast cancer risk due to prolonged lactation may be explained in part by the reduction of total number of ovulatory menstrual cycles and consequently cumulative ovarian hormone exposure (19).

Genetic risk factors

The most important risk factor for breast cancer is age. After controlling for age, the greatest increase in risk has been associated with a family history of breast cancer, with the number, type and age at onset of affected relatives being important determinants of risk. Family history of breast cancer especially combined with mutations in high-penetrance breast cancer susceptibility genes, such as BRCA1 and BRCA2, p53, PTEN, ATM, NBS1 or LKB1, which are responsible for a large proportion of the hereditary breast cancer greatly increases risk for this disease.

Over the last decade, two breast cancer susceptibility genes have been identified: BRCA 1 and BRCA 2. Women who carry deleterious mutations in BRCA1 or BRCA2 have a considerably increased lifetime risk of breast cancer (~ 80%), that is roughly ten

times greater than that of the general population (22). BRCA1 is a tumor suppressor gene whose primary function is maintaining genomic integrity (23). Germline mutations in BRCA1 are associated with approximatively 42% of breast cancer families and 81% of families with both ovarian and breast cancer (24). Loss of heterozygosity (LOH) in BRCA1 gene is frequently observed in hereditary breast cancers and it is one of the most common mechanisms by which the normal allele is inactivated (25). Germline mutations in BRCA2 are linked with approximately 76% of breast cancer families in which both females and males are affected. This percentage decreases to 32% in families where only the women have breast cancer, and this is further increased to 14% in breast-ovarian cancer families (24). Similar to BRCA1, LOH plays an important role in the development of BRCA2-induced breast cancers (26).

p53 is found mutated in all of the major histogenetic groups, including cancers of the colon, stomach, breast, ovary, lung, brain, and esophagus (27). Among high-penetrance genes, p53 was the first tumor suppressor gene linked to hereditary breast cancer (28). Women with germline mutation in p53 have an 18-fold higher risk for developing breast cancer before age of 45 compared to the general population, and the risk declines with age (maximum is before the age of 20) (29).

There are also low penetrance genes (but present in a high percentage of individuals) that enhance breast cancer risk in combination with exogenous (e.g. diet, pollution) and endogenous (e.g. hormones) factors (30). These genes include phase I metabolic enzyme which metabolically activate carcinogens (e.g. the cytochrome P450 family proteins) and phase II enzymes which inactivate carcinogens (e.g. N-acetyl transferase and GST family proteins). Polymorphisms in both phase I and II enzymes involved in xenobiotic and endobiotic metabolism therefore may modulate the relative risk of breast cancer for an individual (31).

Lifestyle risk factors

Other than aging and genetic risk factors, lifestyle-related risk factors, such as alcohol, obesity and high-fat diet, breast-feeding and pregnancy as well as oral

contraceptive use, are risk factors by choice.

Several major reviews of epidemiologic data confirm a statistically significant association between moderate to heavy alcohol intake and subsequent risk of developing breast cancer (32,33). There is evidence of a dose-response relationship. One combined analysis of data from 53 studies around the world estimated that the risk factor for breast cancer increased 7% for each additional 10 g of alcohol consumed daily (34). The association between alcohol intake and greater breast cancer risk has been observed regardless of the type of alcohol consumed, and alcohol intake is associated with a higher risk for both premenopausal and postmenopausal breast cancer (33). Furthermore, alcohol causes alterations of the immune system and nutritional deficiencies, including but not limited to folate, pyridoxal phosphate, vitamin B12, vitamin D, vitamin A and retinoids, vitamin E, zinc and selenium, all of which impair the ability of the human body to repair or in inhibit tumor development (35).

Adult weight gain has been consistently associated with a greater risk for postmenopausal breast cancer (36,37). Findings from two of the largest cohort studies suggest that there is a doubling of risk associated with a weight gain >20 kg and this was limited to women who had never used postmenopausal hormone replacement therapy (36,37). In those studies, a 20% greater risk was observed for weight gains of 2-20 kg.

The human diet contains a great variety of natural and chemical carcinogens and anti-carcinogens (38). Some of these compounds may act through the generation of oxygen free radicals, which can lead to DNA damage, or other deleterious components. Well-done meat consumption has been associated with increased breast cancer risk (39), probably due to production of heterocyclic aromatic amines (HAAs) and other harmful compounds in the process of cooking. A high intake of fat, especially unsaturated fatty acids, may be weakly associated with an increased breast cancer risk (40), while a particular type of polyunsaturated fatty acids (PUFAs), omega-3 PUFAs, seem to be protective (41,42).

1.1.2.3 Treatment of breast cancer

Breast cancer prognosis is dependent on the stage of disease at diagnosis. Five-year survival rates range from 84% for early breast cancer (EBC) to 18% for advanced breast cancers (ABC) (43). The chance of recovery and choice of treatment depend on the stage of the patient's cancer, the type of breast cancer, certain characteristics of the cancer cells, the patient's age and general state of health.

There are treatments for all patients with breast cancer. Four types of treatment are used: surgery, radiation therapy, chemotherapy and endocrine therapy. In addition, biological therapy, using the patient's immune system to fight cancer, and bone marrow transplantation are also being investigated in clinical trials. Among these treatments, surgery has been applied to most patients with breast cancer.

Surgery and radiation therapy

Surgical treatment of breast cancer in the early days of radical mastectomy was aimed at curing the disease by cutting it out. This included surgically removing a very large surrounding area of normal tissue in an attempt to remove microscopic traces of the cancer spreading beyond a more obvious mass. For women with early-stage invasive breast cancer, surgery is usually followed by radiation therapy (RT) (44,45). Radiotherapy was used to destroy any cancer that could not be removed by surgery. In patients with early breast cancer who undergo breast-conserving surgery and receive 50 Gy of radiation to the whole breast, an additional dose of 16 Gy of radiation to the tumor bed reduces the risk of local recurrence, especially in patients younger than 50 years of age (46).

Chemotherapy

Chemotherapy is generally reserved for patients with hormone-sensitive disease who have failed one or more hormonal treatments or those with who have symptomatic disease that requires prompt symptom relief. Chemotherapy is called a systemic treatment because the drugs enter the bloodstream, travel through the body, and can kill

cancer cells outside the breast area. Population- and hospital-based studies evaluating survival during the pre- and postchemotherapy era suggests that cytotoxic chemotherapy prolongs survival by an average of about 9 to 12 months (47). The cytotoxic agents most commonly used for the treatment of metastatic breast cancer and having substantial single-agent activity include combinations of alkylating agents and antimetabolites such as cyclophosphamide, methotrexate, and 5-fluorouracil (CMF) used in the 1970s. Anthracyclin-based combinations including 5-fluorouracil, doxorubicin and cyclophosphamide (FAC); 5-fluorouracil, epirubicin and cyclophosphamide (FEC) were extensively used in the 1980s-1990s (48).

For patients with estrogen receptor (ER) - positive and progesterone receptors (PR) - positive breast cancer are normally treated with hormone therapy using drugs that change the way hormones work or by surgery that removes organs that make hormones, such as the ovaries.

Hormone therapy

In 1896, Beatson found that some premenopausal women with breast cancer benefited from removal of their ovaries (49). In 1936, Professor Antoine Lacassagne showed that the agent in the ovaries that caused mammary cancer was estrogen (50). After ER α was discovered, the link between ERα expression and response to hormone or endocrine therapy was established.

Selective estrogen receptor modulators (SERM) including both steroidal and nonsteroidal antiestrogens (51) were initially evaluated in rodent models and shown to exhibit antiestrogenic activity in mammary tumors. The compounds were then successfully translated into the clinic (52). Although targeting the ER with the nonsteroidal antiestrogen tamoxifen has increased survival of breast cancer patients (53), the strategic application of long-term antihormonal treatments (54) has created an important increase in disease-free and overall survival (55). However, tamoxifen is not a pure antiestrogen, and this drug exhibits partial estrogen-like actions that produce a suboptimal blockade of estrogen-stimulated breast tumor growth. In addition, 30% of

women treated with tamoxifen complain of side-effects including hot flashes, vaginal discharge, and vaginal bleeding (56,57). Tamoxifen treatment is also associated with a higher risk of less common long-term side-effects; studies have found a two to four times higher relative risk of developing endometrial cancer in women taking tamoxifen than in agematched populations (58,59). Due to the imitations of tamoxifen, other hormone therapies have been developed for treatment of hormone-sensitive breast cancer. Currently, aromatase inhibitors are used to produce an estrogen-free environment and these compounds are effective inhibitors of ER-positive breast cancer growth (60). Aromatase inhibitors inhibit the enzymatic conversion of adrenal androgens into estrogens in peripheral tissues, the major source of estrogens in postmenopausal women. Most importantly, the use of aromatase inhibitors for treatment of breast cancer avoids some of the estrogen-like side effects observed in patients treated with tamoxifen. One of the improved new SERMs is raloxifene, which was originally a discarded breast cancer drug named keoxifene. Raloxifene maintains bone density in overiectomized rats (61) and prevents carcinogen-induced rat mammary carcinogenesis (62). Raloxifene is currently available for the prevention of osteoporosis but with breast and endometrial safety.

1.1.3 The role of estrogen in breast cancer

Estrogens are ovarian steroid hormones required for establishment and maintenance of the female reproductive tract. They also play important roles in development of the male reproductive tract, in bone formation, lipid metabolism and maintenance of the cardiovascular and nervous systems (63,64). The naturally occurring estrogens 17β-estradiol (E2), estrone (E1) and estriol (E3) are C18 steroids derived from cholesterol (Figure 1-2). The most biologically active estrogen in breast tissue is E2. Circulating estrogens primarily originate from ovarian steroidogenesis in premenopausal women and peripheral aromatization of ovarian and adrenal androgens in postmenopausal women (65). Estrogens play a major role in promoting the proliferation of both the normal and neoplastic breast epithelium (66,67). Estradiol acts locally in the

mammary gland, stimulating DNA synthesis and promoting bud formation, through an ER-mediated mechanism.

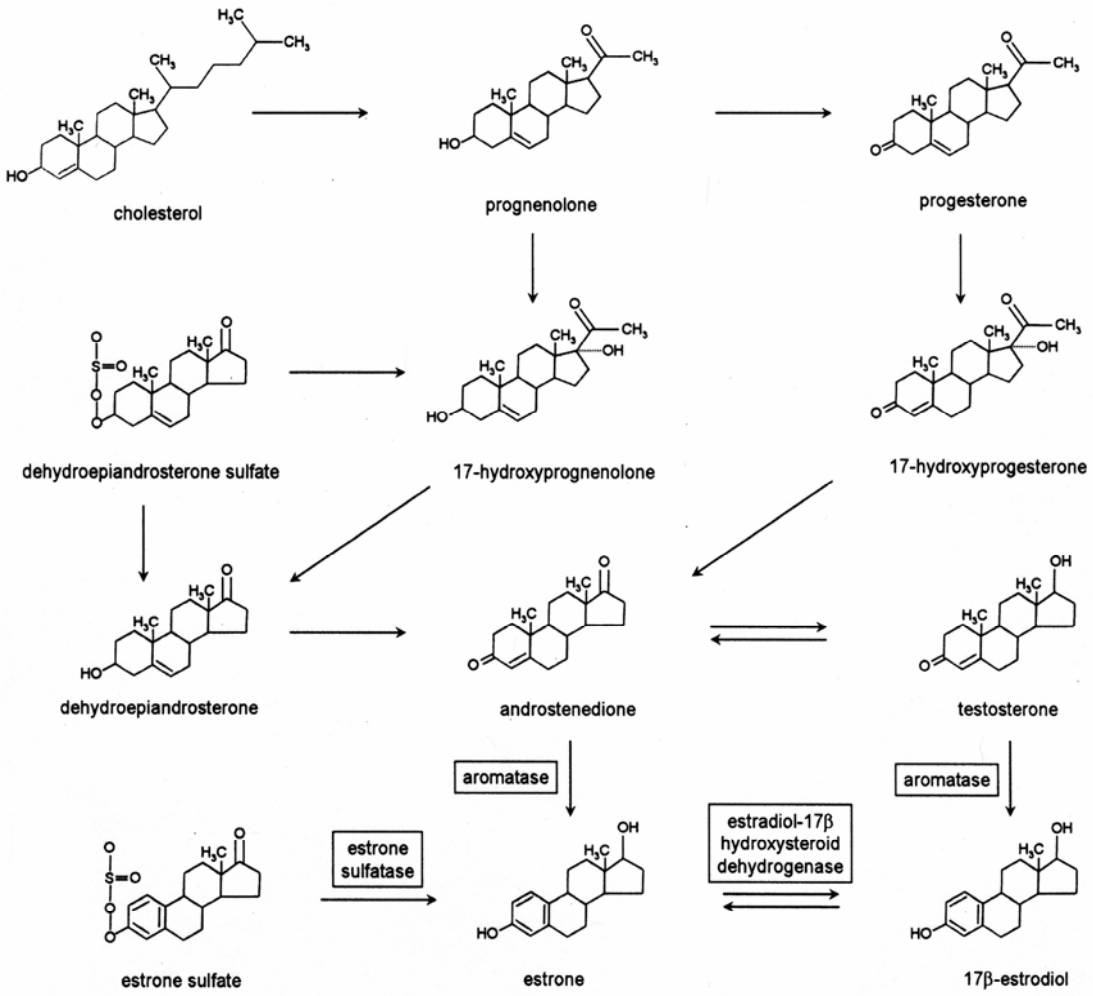

Figure 1-2 Steroidogenic pathways leading to the biosynthesis of estrogens.

1.1.3.1 Estrogens in human breast carcinogenesis

As described earlier, an association between the risk of breast cancer and

persistently elevated blood levels of estrogen has been consistently observed in many studies. There are three mechanisms that are responsible for the carcinogenicity of estrogens: receptor-mediated hormonal activity (68), a cytochrome P450-mediated metabolic activation and redoxcycling (69) and the induction of aneuploidy by estrogen (70,71). There is also evidence that estrogen may compromise the DNA repair system and allows accumulation of lesions in the genome essential for estrogen-induced tumorigenesis (72).

Receptor-mediated pathway

The receptor-mediated activity of estrogen is generally related to induction of expression of the genes involved in the control of cell cycle progression and growth of human breast epithelium. The classical mechanism of estrogen action involves binding of the hormone to nuclear ER, which then forms a dimer that interacts with estrogen-response elements (EREs) in regulatory regions of estrogen-responsive genes. The DNA-bound ER complex associates with basal transcription factors, coactivators and corepressors to alter gene expression. The presence of ERα-positive and ERα-negative cells with different proliferative activity in the normal human breast may help to elucidate the genesis of ERα-positive and ERα-negative breast cancers. It has been postulated that either ERα-negative breast cancers result from the loss of the ability of the cells to synthesize ERα during clinical evolution of ERα-positive cancers, or that ERα-positive and ERα-negative cancers are different entities (73). The newly discovered ERβ opens another possibility that those cells traditionally considered negative for ERα might be positive for ERβ (74). ERα and ERβ are encoded by separate genes. The DNA binding domains (DBDs) of ERα and ERβ are highly homologous (75) and thus they bind to the same EREs. The ligand-binding domain of these two ERs also share a high degree of homology and it is not surprising that many compounds tested so far bind to both receptors with similar affinities (76). However, estrogen responses mediated by ERα and ERβ may vary with tissue-specific expression of their coactivators that transmit the effect of ER-ligand complex to the transcription factor complex at the promoter of

target genes.

Estrogen metabolism

Studies in rodents have demonstrated that estrogens or their catechol metabolites are carcinogens in various tissues, including the kidney, liver, uterus, and mammary glands (77-80). E2 and estrone are the two major endogenous estrogens and these compounds undergo cytochrome p450-dependent oxidation of catecholestrogen to give 2-hydroxycatechol or 4-hydroxycatechol estrogen (81,82). Further oxidation gives the estrogen 3,4-quinone which can form unstable adducts with adenine and guanine in DNA, leading to depurination and mutations in vitro and in vivo (77,83). Reduction of estrogen quinones back to hydroquinones (catechols) activates redox cycling and produces reactive oxygen species. This may account for the oxidative damage to lipids and DNA that is associated with estrogen treatment (84,85). In addition to preventing metabolism of catechol estrogen to quinones, the 2-methoxy catechol may be a protective metabolite (86,87).

There are two lines of evidence that support a role for estrogen metabolites as causative factors for human breast cancer. In postmenopausal women, estrogen levels in breast tissue are 10 to 50 times the levels in blood (88), and concentrations of estradiol are higher in malignant tissues compared to nonmalignant tissues (81). Furthermore, levels of estrogen metabolites and conjugates detected in breast tissue range from 3 to 13 pmol per gram of tissue (89), demonstrating that oxidative pathways are active in the breast. The second line of evidence supporting a role for estrogen metabolites in human breast cancer comes from studies of associations of breast-cancer risk and polymorphisms in genes encoding enzymes involved in estrogen synthesis and metabolism. For example, breast cancer patients have a higher percentage of CYP19 tetranucleotide repeat polymorphisms than controls and this correlates with the decreased activity of phase II enzymes in breast cancer patients and a possible increase in the more genotoxic phase I catecholestrogen metabolites (90).

1.2 Cancer and the cell cycle

1.2.1 An overview of cell cycle regulation

Early embryonic cells can proceed through continuous cycles of DNA replication and nuclear division (Figure 1-3). However, as embryogenesis continues, a new regulatory system is introduced to regulate cell cycle progression. A gap called G1 phase is incorporated between nuclear division (M phase) and DNA synthesis (S phase); and another gap called G2 phase occurs between S and M (Figure 1-3). These gaps allow for

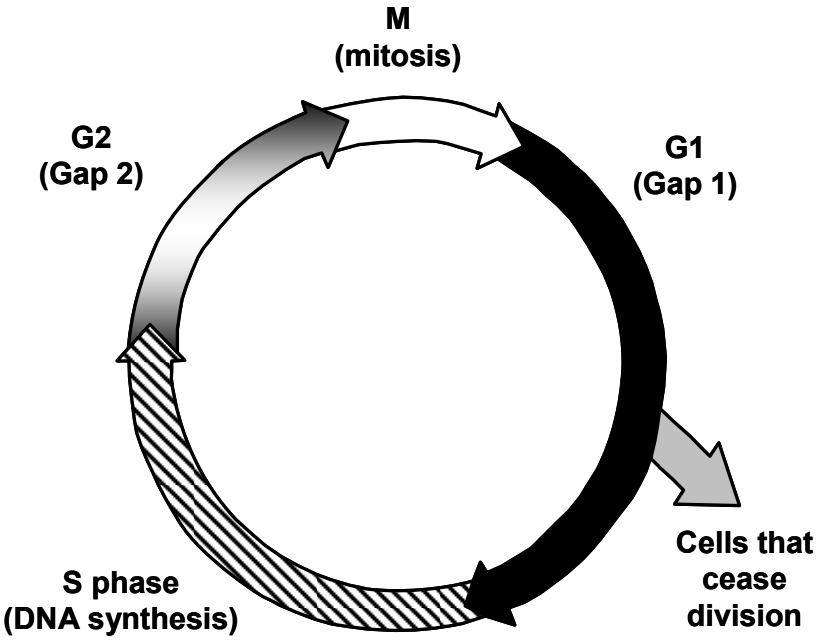

Figure 1-3 Stages of the cell cycle.

the repair of DNA damage and replication error.

The transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins. Key regulatory proteins are the

cyclin-dependent kinases (CDK), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. Nine CDKs have been identified and five of them are active during the cell cycle (Table 1-2). When activated, CDKs induce downstream processes by phosphorylation of selected proteins (91,92). CDK protein levels remain stable during the cell cycle, in contrast to their activating proteins, the cyclins. The cyclins perform multiple regulatory functions. In addition to the appropriate phosphorylation state of CDKs, binding to a cyclin is necessary for CDK activation. Cyclins not only interact with CDKs but also target these complexes into the nucleus; since CDKs lack nuclear localization signals (93). Some CDKs bind more than one cyclin (92,94). The cyclin bound by a given CDK probably provides some substrate specificity. The cyclins were so named because of their cyclic expression during the cell cycle (Figure 1-2) (92,95). Because of this cyclic expression, CDK complexes are activated only at specific times during the cell cycle. Part of the cyclic expression of cyclin proteins is due to their regulated degradation. Cyclins contain protein motifs rich in proline (P), glutamate (E), serine (S), and threonine (T) (PEST sequences), which target them for degradation by ubiquitination at specific times (95-98). Thus, a cycling cell enters and exits cell cycle phases in parallel with the synthesis and degradation of specific cyclins (Figure 1-4, Table 1-2). In general, before a cell can enter the next phase of the cell cycle, the appropriate cyclin from the previous phase is degraded, and the cyclin for the next phase is synthesized.

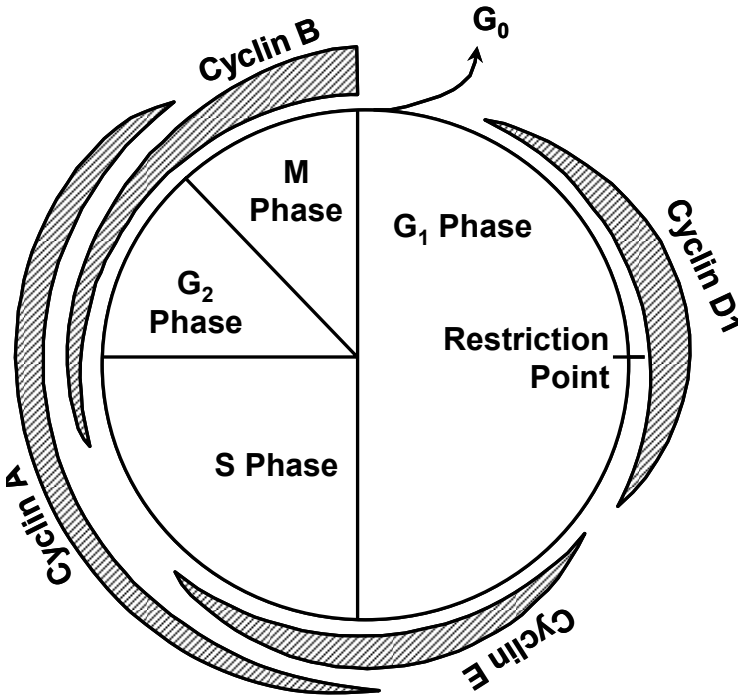

Figure 1-4 Schematic drawing of cell cycle-dependent levels of cyclins. Thickness of hashed bars indicates relative intracellular cyclin concentration.

Table 1-2 Cyclin-CDK complexes are activated at specific points of the cell cycle.

CDK	Cyclin	Cell cycle phase activity
CDK4	Cyclin D1, D2, D3	G1 phase
CDK6	Cyclin D1, D2, D3	G1 phase
CDK2	Cyclin E	G1/S phase transition
CDK2	Cyclin A	S pahse
CDK1	Cyclin A	G2/M phase transition
CDK1	Cyclin B	Mitosis
CDK7	Cyclin H	All cell cycle phases

1.2.1.1 G1/S phase transition and regulation

G1 is a period when many signals intervene to influence cell division and differentiation. Diverse metabolic stress and environmental signals are integrated and

interpreted during this period. On the basis of these inputs, the cells decide whether to enter S phase or arrest at G1 phase. In higher eukaryotes, Cdk2 combines with E-type cyclines (E1, E2) and cyclin A (99,100). Cyclin E levels are constantly high in early embryonic cells, allowing Cdk2 to intiate S phase as soon as M phase is complete (100). There are various mechanisms to enforce the existence of G1 phase by keeping Cdk2 inactive until mitogenic signals intervene. One of these mechanisms is based on limiting the supply of cyclin E expression which is dependent on E2F transcription factors (101,102). In mitotically resting cells, and in cells that have just emerged from M phase, E2F factors are bound to the retinoblastoma protein (Rb) or its family members, p107 and p130 (103). Rb binding turns E2Fs into repressor complexes (like E2F4 and E2F5) or inactive transactivators (like E2F1, -2 and -3) (102). The hypophosphorylated form of the pRB prevents premature entry into S phase by binding to E2Fs which regulate expression of S phase genes. Phosphorylation of pRB at serine and threonine residues in mid-to-late G1 phase by mitogen-induced cyclin D-dependent kinases removes the growth-inhibitory effects of pRB thereby allowing activation of cell cycle regulatory genes including cyclins E and A.

The c-myc protooncogene is a transcription factor and member of the helix-loop-helix/leucine zipper protein family, whose endogeneous expression is rapidly induced by mitogens and whose ectopic expression can induce entry of quiescent cells into S phase (104). c-Myc activates several genes important for G1/S control and these include cyclins D1, D2 and E, and the Cdc25A phosphatase, an essential regulator of S phase entry which catalyzes removal of the inhibitory phosphates (Tyr15/Tyr14) on Cdk2 (105-107). In terms of the key activities, c-Myc has been implicated at least three distinct programs: regulation of cyclinE-Cdk2 activity, E2F-dependent transcription, and cell growth (104,108,109). Both RB-E2F and Myc-regulated programs convergently control the abundance and activity of cyclinE-Cdk2 (110,111). Cdk2 activity appears to complete and maintain the neutralizing phosphorylation of pRB, a process initiated by cyclinD-dependent kinase in G1, thereby preventing unscheduled activation of pRB in S phase, and feeding a regulatory loop which amplifies both E2F and cyclinE-Cdk2 ativity.

$p27^{kip1}$ is another target of the cyclinE-cdk2 kinase. The phosphorylation and subsequent degradation of $p27^{kip1}$ allows the timely elevation of Cdk2 activity necessary for S phase initiation and progression.

1.2.1.2 G2/M phase transition and regulation

A number of different cyclin/CDK complexes involved in cell cycle regulation have been identified. Cyclin A is induced shortly after cyclin E and binds Cdk2 in S phase and Cdk1 (cdc2) in G2 and M phases. The entry into mitosis is under the control of B-type cyclins, which also associate with Cdk1 (112,113). Regulation of cyclin B/Cdk1 complexes at multiple levels ensures the precise timing of mitotic entry. In human cells, cyclin B is synthesized from the end of S phase, mainly due to cell cycle-regulated transcription (114,115). In addition, cyclin B1 mRNA is thought to be more stable in G2 phase as compared to G1 phase, and becomes more unstable after DNA damage (116). These data suggest that cyclin B levels are also controlled by regulation of mRNA stability. After association of a specific cyclin with its appropriate CDK, the cyclin/CDK complex is regulated by a number of phosphorylation events. Phosphorylation of the conserved threonine (Thr161) in the T-loop of Cdk1 is required for activation of the cyclin B/Cdk1 complex and is mediated by the CDK activating kinase (CAK). CAK was found to be a cyclin/CDK complex itself, composed of cyclin H and Cdk7. A third component, MAT1, is thought to function in stabilizing the cyclin H/Cdk7 interactions (117-119). CDK regulation occurs through distinct mechanisms in different species. During G2, mammalian cyclin B/Cdk1 complexes are held in an inactive state by phosphorylation of Cdk1 at the two negative regulatory sites, Thr 14 and Tyr15. Inactivation of Cdk1 via Tyr15 phosphorylation plays a very important role in the control of initiation of mitosis in both fission yeast and animal cells (120,121). Phosphorylation of Cdk1-Tyr15 is carried out by the Wee1/Mik1 family of protein kinases (122,123). The distinct locations of these Cdk1 inhibitory kinases may co-operate to guarantee inactivation of multiple Cdk1 subpopulations before the onset of mitosis. Genetic studies identified Cdc25 as a positive regulator of Cdk1, by

counteracting Wee1 activity (124). Dephosphorylation of Thr14 and Tyr15 by Cdc25C in late G2 activates the cyclin B/Cdk1 complex and triggers the initiation of mitosis (125). Cyclin B/cdc2 complexes in turn are thought to phosphorylate Cdc25C, which further activates Cdc25C, including the full activation of cyclin B/Cdk1 by forming an autocatalytic feedback loop (126,127)

Another mechanism for regulating cyclin/CDK complexes is to localize these complexes and their regulators into particular subcellular compartments. Like most cyclins, cyclin A appears in the nucleus upon its synthesis (S phase) and remains nuclear until its degradation (M phase). In contrast, cyclin B1 is initially localized in the cytoplasm during S phase and G2 phase, and is translocated into the nucleus at the beginning of mitosis (128). Deletion studies revealed the existence of a cytoplasmic retention signal (CRS) in the N-terminal part of cyclin B1, since deletion of this sequence caused cyclin B1 to become nuclear (129). Phosphorylation of cyclin B1 within the CRS is required to allow nuclear import of the protein. Thereafter, it was shown that the cytoplasmic localization of cyclin B1 is the result of continuous export from the nucleus during interphase. The nuclear export was shown to be mediated by a hydrophobic nuclear export signal (NES) within the CRS of cyclin B and was blocked by leptomycin B, a specific inhibitor of the nuclear export factor CRM1 (130-132). Phosphorylation of cyclin B1 functions to promote nuclear import as well as to inhibit nuclear export (130). Therefore, it is thought that phosphorylation of cyclin B1 at the G2/M transition blocks complex formation with CRM1 and prevents its return to the cytoplasm. Induction of p21 expression resulted in an accumulation of cells in both G1 and G2 phases of the cell cycle and in inhibition of cyclin E-, cyclin A- and cyclin B-associated kinase activity (133,134). p21 inhibits cyclin/CDK complexes by blocking CAK-mediated activation (134-136). As cells enter mitosis, phosphorylation of key components of the subcellular structures results in complete reorganization of the architecture of the cell. This phosphorylation is mainly due to the activation of the cyclinB/Cdk1 complexes. Besides regulating the activity of itself by phosphorylating Cdc25C, cyclin B/Cdk1-mediated phosphorylation also induces changes in the

microtubule network, the actin microfilaments and the nuclear lamina (137-139). Finally, downregulation of transcription during mitosis is thought to be mediated by cyclin B/Cdk1, since cyclin B/Cdk1 directly inhibits pol III-mediated transcription by TFIIIB (140).

1.2.1.3 The role of Cdc25 in cell cycle

The mammalian Cdc25 family consists of three members, Cdc25A, Cdc25B and Cdc25C, which appear to have specificity for different cyclin/CDK complexes. Whereas Cdc25A promotes entry into S phase by acting on cyclinA/Cdk2 and cyclinE/Cdk2 (141), both Cdc25B and Cdc25C play a role in the onset of mitosis. Cdc25B is thought to regulate centrosomal microtubule nucleation during mitosis (142). Cdc25C is the phosphatase responsible for triggering activation of cyclinB/cdc2 complexes by dephosphorylating the inhibitory Cdk1 sites Thr14 and Tyr15 (143-148). Cdc25C is localized in the cytoplasm during interphase, and enters the nucleus just prior to mitosis (149,150). The cytoplasmic localization was shown to require a 58 amino acid region in Cdc25C, which contain a 14-3-3 binding site. Ogg et al showed that Ser216 within this region is the major phosphorylation site of Cdc25C during interphase, and this serine is not phosphorylated during mitosis (151). Subcellular localization may contribute to negative regulation of Cdc25A activity during interphase. Cdc25B is localized in both the cytoplasm and the nucleus and this localization is thought to be dependent on the combined effects of an NLS, an NES and on the interaction with 14-3-3 proteins (152). A positive feedback loop between cdc2 and Cdc25C in necessary for the full activation of cyclin B/cdc2 complexes at the onset of mitosis. There are indications that kinases other than cyclinB/cdc2 may provide the initial trigger for activating Cdc25C, which was found to be phosporylated and activated by cyclinE/Cdk2 and cyclinA/Cdk2 in vitro (153). In conclusion, although cyclinB/cdc2 complexes contribute to activation of Cdc25C, an upstream kinase is probably required for the initial Cdc25C activation.

1.2.2 Checkpoints in cell cycle

When the genetic material is damaged, a delay in cell cycle progression facilitates DNA repair, thereby avoiding the replication and subsequent propagation of potentially hazardous mutations. Highly conserved DNA-repair and cell-cycle checkpoint pathways allow cells to deal with both endogenous and exogenous sources of DNA damage. How much an individual is exposed to these agents and how their cells respond to DNA damage are critical determinants of whether that individual will develop cancer.

1.2.2.1 G1 and G1/S checkpoint responses

The ability of the cell cycle checkpoints, signaling pathways which monitor the integrity and replication status of the genome, to inhibit entry into S phase is associated with the function of the p53 tumor suppressor (154). The p53 protein is a transcription factor which becomes stabilized and active upon DNA damage, and in turn regulates transcription of a large number of genes, among them the $p21^{WAF1/CIP1}$ Cdk inhibitor (CKI) capable of silencing the Cdks which are essential for S phase entry (111,154). Recent research on the molecular mechanisms of p53 pathways indicate an early activation of ataxia telangiectasia mutated (ATM) or ataxia telangiectasia related (ATR), two large kinases from the PI-3 kinase superfamily (155,156). ATM/ATR directly phosphorylate the p53 transcription factor in its amino-terminal transactivation domain, particularly on Ser15. Thr 18 and Ser 20 are also targeted by CHK1/CHK2 (157-161). In addition, the ubiquitin ligase MDM2 that normally binds p53 and ensures rapid p53 turnover, is targeted after DNA damage by ATM/ATR (162), as well as by CHK2/CHK1. These modifications of p53 and MDM2 contribute to the stabilization and acculmulation of the p53 protein and the subsequent induction of $p21^{CIP1/WAF1}$, the key transcriptional target of p53, which silences the cyclinE/Cdk2 kinase and casues G1 arrest. Cyclins E and A, and the activator of the cyclinE/Cdk2 kinase, the Cdc25A phosphatase, are also induced in late G1. In response to genotoxic stress, this physiologically operating mechanism is enhanced through increased activity of CHK1 and CHk2, leading to downregulation of Cdc25A and consequently to inhibition of the cyclinE/Cdk2

complexes (159,163,164). The CHK1/CHK2-Cdc25A checkpoint is implemented rapidly, independent of p53, and this delays the G1/S transition for only a few hours, unless the sustained p53-dependent mechanism prolongs the G1 arrest.

1.2.2.2 S-phase checkpoint pathways

There are at least two parallel branches of this checkpoint that decrease ongoing DNA synthesis, both of which are controlled by the ATM/ATR signaling machinery. One of these effector mechanisms operates through the Cdc25A-degradation cascade. The inhibition of Cdk2 activity downstream of this pathway blocks the loading of Cdc45 onto chromatin. Cdc45 is a protein required for the recruitment of DNA polymerase α into assembled pre-replication complexes, so the inhibition of Cdk2 activity prevents the initiation of new origin firing (159,163). The other branch of the intra-S-phase checkpoint reflects the impact of ATM-mediated phosphorylation of NBS1 on several sites. The concept of the two parallel effector branches of the intra-S-phase checkpoint has been documented for responses to both ionizing radiation (165) and to ultraviolet light (166). Apart from the inhibition of replication-origin firing, another critical function provided by S-phase checkpoints is to protect the integrity of the stalled replication forks. It helps prevent the conversion of primary lesions into DNA breaks and facilitates the subsequent recovery of DNA replication (163,167).

1.2.2.3 The G2 checkpoint

The G2 checkpoint prevents cells from initiating mitosis when they experience DNA damage during G2, or when they progress into G2 with some unrepaired damage inflicted during previous S or G1 phases (168,169). The critical target of the G2 checkpoint is the mitosis-promoting activity of the cyclinB/Cdk1 kinase, whose activation after various stresses is inhibited by ATM/ATR, CHK1/CHK2 and/or p38-kinase-mediated subcellular sequestration, degradation and /or inhibition of the Cdc25 family of phosphatases that normally activate Cdk1 at the G2/M boundary (164,169-172). p53 activates several genes that interfere with the Cdk1 activity (173). It

strongly induces p21 (174), the CDK inhibitor, which inhibits the Cdk1 kinase activity. p53 also activates the GADD45 gene, which dissociates the cyclinB/Cdk2 complex (175,176), and the 14-3-3 sigma gene (177). The 14-3-3 sigma protein binds Cdk1 and anchors the Cdk1/cyclinB complex in the cytoplasm (173,178), preventing its nuclear translocation and arresting the cell in G2. An additional mechanism through which p53 induce G2 arrest is by direct repression of cyclin B1 and Cdk1 gene transcription.

1.2.3 Cell cycle and cancer

Aberrant activation of the cell cycle can be achieved by upregulation of cell-cycle activators (often encoded by protooncogenes) or downregulation of cell-cycle inhibitors (often encoded by tumor suppressor genes). Abnormal regulation of these genes is involved in promoting the transformation of a normal cell into a continuously proliferating cell, which is independent of growth-promoting and resistant to growth-inhibiting signals. This cell transformation is supported by other mechanisms, such as angiogenesis as well as evasion of apoptosis and immune surveillance, and will create the clonogenic malignant cell.

1.2.3.1 Oncogene

The well-studied upregulated cell-cycle activators in cancer include cyclin D, cyclin E, cyclin A, Cdc25B, c-Myc. Overexpression of cyclin D1 caused by gene amplification or aberrant activation of protein synthesis is frequently found in several human tumors (179,180). Aberrant activation of the cyclin D1 gene can be induced by chromosomal rearrangement which brings this gene under the influence of a strong promoter or enhancer (179,181). Cyclin E overexpression has also been associated with different tumors and elevated cyclin E levels in a tumor predict decreased survival of breast cancer patients (182). Deregulated expression of cyclin E also induces chromosomal instability and thereby contributes to tumorigenesis. (183). Increased expression of cyclin A has also been detected in many types of human cancers. For example, , higher expression of cyclin A has been found in up to 80% of hepatocellular carcinomas (184).

Elevated c-Myc expression due to translocation juxtaposing the c-myc gene with the immunoglobulin gene enhancer results in B-cell tumors (181). Overexpression of the c-Myc protein can also be achieved by gene amplification, an alteration frequently seen in breast cancer (185).

1.2.3.2 Tumor suppressor gene

Similar to the activation of cell-cycle, defects in cell-cycle checkpoints lead to uncontrolled proliferation and can result in malignancy. Genes involved in the negative regulation of the cell cycle are called tumor suppressor genes. In general, two major pathways are involved in the negative regulation of the cell cycle: namely the "Rb pathway" and the "p53 pathway". Rb is mutated in several human tumors (186) and mutated or deleted Rb is unable to repress the function of E2F. The Rb gene is most often implicated in adult cancers, such as small cell carcinomas and inherited allelic loss of Rb confers increased susceptibility to tumor formation. (187). Inactivation of the Rb pathway is also accomplished by mutations of other regulatory factors. Loss-of-function mutations in the INK4a family members, particularly $p16^{INK4a}$, occur frequently in human cancers (188). For example, in melanomas, one copy of mutant $p16^{INK4a}$ is inherited and the second copy is lost in the tumor cell. Unlike regulators of the Rb pathway, p53 is not required for cell cycle progression. Its role is to arrest the cycle only when the cell is damaged, by either G1 arrest or by inducing apoptosis (189). The p53 gene is mutated in more than 50% of human cancers and is the most frequent genetic alteration associated with malignancy (190). Furthermore, inactivation of the p53 pathway can occur via defects in upstream or downstream regulators which have been observed in several human cancers. In general, tumors with an intact wild-type p53 have a better prognosis and a better response to therapy compared to tumors with defective p53 (191). Overexpression of MDM2, the negative regulatgor of p53, by gene amplification or other mechanisms has been reported in leukemia and lymphoma, breast carcinoma, sarcoma and glioma and may represent an alternative mechanism for escaping p53-mediated growth arrest (192-194).

1.3 Gene regulation

Control of gene expression is achieved through the coordinated action of various cis- and trans-acting factors including matrix attachment regions (MARs), locus control regions (LCR), nucleosome remodeling, histone modifications, gene methylation, transcription factors, enhancers and silencers, and promoters (195-197).

1.3.1 Promoter organization

In general, the promoter is an integral part of the gene and often makes sense only in the context of its own gene. Promoter regions comprise the genomic DNA sequences found upstream from the transcribed sequence but often overlap with, or include, the first exon of a gene. Promoters are the central processors of transcriptional control, as the regulatory information contributed by the other elements must be integrated within the context of a promoter in order to influence gene expression (195).

1.3.1.1 Architecture of the core promoter

Recognition of the core promoter by the transcription machinery is essential for correct positioning and assembly of RNA Pol II and the general transcription factors. Sequence elements found in core promoters include the TATA element (TBP-binding site), BRE (TFIIB-recognition element), Inr (initiator element) and DPE (downstream promoter element) (198). Most promoters contain one or more of these elements, but no one element is absolutely essential for promoter function. The TATA box was the first identified core promoter element. In most RNA polymerase II-transcribed genes examined, the TATAAA sequence was present 25 to 30 bp upstream of the transcription start site (199). At TATA-containing promoters, formation of the TBP/TATA complex is the initial step in assembly of the transcription machinery. The TFIIB recognition element (BRE) is the only well-characterized element in the core promoters. Protein-DNA crosslinking studies confirmed that TFIIB is in close proximity to the upstream sequences (200). A study with human TFIIB established the existence of a

eukaryotic BRE that prefers a 7-bp sequence: G/C G/C G/ACGCC (201). Recognition of the BRE was found to be mediated by a helix-turn-helix motif at the C-terminus of TFIIB (202). Interestingly, this motif is missing in yeast and plants, suggesting that the BRE may not contribute to gene regulation in these organisms. The TFIIB and BRE interaction was originally reported to stimulate RNA polymerase II transcription in an in vitro assay reconstituted with purified basal factors (201). However, it was also observed that the BRE is a repressor of basal transcription in vitro with crude nuclear extracts as well as in vivo in transfection assays (203). Repression associated with TFIIB-BRE interactions can be relieved when transcriptional activators were bound to distal sites, which resulted in an increased amplitude of transcriptional activation. The Inr was defined as a discrete core promoter element that is functionally similar to the TATA box and can function independently of a TATA box in an analysis of the lymphocyte-specific terminal transferase (TdT) promoter (204). TATA box and Inr function synergistically with one another when separated by 25-30 bp but act independently when separated by more than 30 bp. The DPE was identified as a downstream core promoter motif that is required for the binding of purified TFIID to a subset of TATA-less promoters (205). The DPE is conserved from *Drosophila* to humans and is typically but not exclusively found in TATA-less promoters. The DPE functions cooperatively with the Inr, and the core sequence of the DPE is located at precisely +28 to +32 relative to the A+1 nucleotide in the Inr motif (206).

1.3.1.2 RNA polymerase II transcription machinery

Regulation of transcription is one of the most important steps in control of cell growth and differentiation. Transcription is carried out by the enzyme RNA polymerase (Pol) along with other factors termed general transcription factors (GTFs). The GTFs include TBP, TFIIB, TFIIE, TFIIF, and TFIIH and were identified biochemically as factors required for accurate transcription initiation by RNA pol II from doublestranded DNA templates in vitro (207). The GTFs are involved in recognition of promoter sequences, the response to regulatory factors and conformational changes essential to the

activity of Pol II during the transcription cycle (Table 1-3) (208). Pol II transcription typically begins with the binding of gene-specific regulatory factors near the site of transcription initiation. These factors can act indirectly on the transcription machinery by recruiting factors that modify chromatin structure, or directly by interacting with components of the transcription machinery. Both the direct and indirect mechanisms result in recruitment of the transcription machinery to a core promoter (described in the previous section) (209). Pol II and the general factors are all bound to the promoter forming the preinitiation complex (PIC) (Figure 1-5). Order-of-addition experiments demonstrated that PIC assembly is nucleated in vitro by TBP binding to the TATA element followed by binding of TFIIB, RNA pol II-TFIIF, TFIIE, and TFIIH (210). Next, a conformational change occurs in which 11-15 bp of DNA surrounding the transcription site are melted and the template strand of the promoter is positioned within the active site cleft of Pol II to form the open complex (211). Initiation of transcription begins with synthesis of the first phosphodiester bond of RNA. After synthesis of about 30 bases of RNA, Pol II releases its contacts with the core promoter and the rest of the transcription machinery and enter the stage of transcription elongation (212).

Table 1-3 Yeast general transcription factors (213).

Factor[a]	Mass (kDa)	Gene(s)	Essential	Charateristics
TBP (factor d)	27	*SPT15*	Yes	Binds TATA element; nucleates PIC assembly; recruits TFIIB
TFIIB (factor e)	38	*SUA7*	Yes	Stabilizes TATA-TBP interaction; recruits RNA pol II-TFIIF; affects start site selection; zinc ribbon
TFIIF (factor g)	82	*TFG1*, *SSU71*	Yes	Facilitates RNA pol II promoter targeting; stimulates elongation; functional interaction with TFIIB
	47	*TFG2*	Yes	σ factor homology; destabilizes nonspecific RNA pol II-DNA interactions
	27	*TFG3*, *ANC1*, *SWP29*, *TAF30*	No	Common subunit of TFIID, TFIIF, and the SWI/SNF complex
TFIIE (factor a)	66	*TFA1*	Yes	Recruits TFIIH; stimulates TFIIH catalytic activities; functions in promoter melting and clearance; zinc binding domain
	43	*TFA2*	Yes	
TFIIH[b] (factor b)	95	*SSL2*, *RAD25*	Yes	Functions in promoter melting and clearance; ATPdependent DNA helicase (39 3 59); DNA-dependent ATPase; ATPase/helicase required for both transcription and NER
	85	*RAD3*	Yes	ATP-dependent DNA helicase (59 3 39); DNA-dependent ATPase; ATPase/helicase required for NER but not transcription
	73	TFB1	Yes	Required for NER
	59	TFB2	Yes	Required for NER
	50	SSL1	Yes	Required for NER; zinc binding domain
	47, 45	CCL1	Yes	TFIIK subcomplex with Kin28
	37	TFB3	Yes	Zinc RING finger; links core-TFIIH with TFIIK; unlike Mat1, not a subunit of kinase/cyclin subcomplex
	33	KIN28	Yes	TFIIK subcomplex with Ccl1

[a] The initial designations of the yeast general transcription factors by Kornberg's laboratory are denoted in arentheses.
[b] TFIIH is composed of core-TFIIH (Rad3, Ssl1, Tfb1 to Tfb4), plus Ssl2/Rad25 and the TFIIK kinase/cyclin ubcomplex (Kin28, Ccl1).

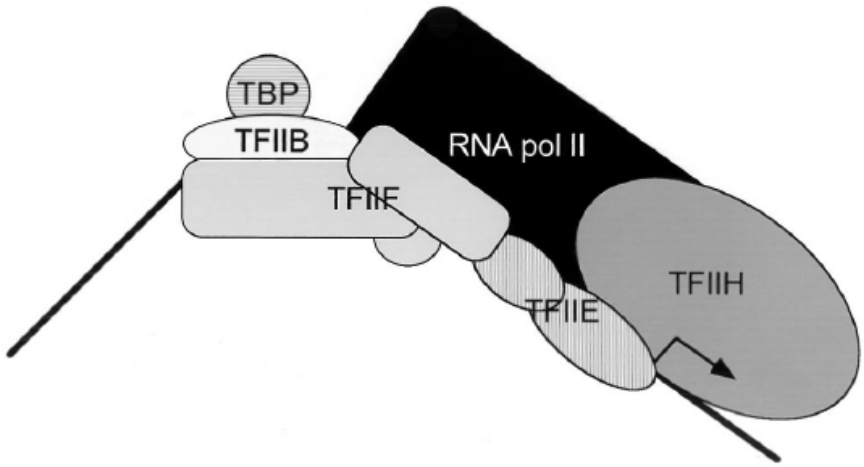

Figure 1-5 Schematic depiction of the transcription PIC. PIC assembly is nucleated by TBP binding to the TATA box, inducing a sharp bend in the DNA template, followed by association of TFIIB, RNA pol II/TFIIF, TFIIE, and TFIIH. Each pattern denotes a distinct general transcription factor. Subunit composition is indicated, except for TFIIH (9 subunits) and RNA pol II (12 subunits). Although PIC assembly can occur by stepwise addition of the general transcription factors (GTFs) in vitro, the discovery of RNA pol II holoenzyme complexes that include GTFs suggests that stepwise assembly might not occur in vivo (213).

Pol II is especially equipped to cooperate with processing factors and other nuclear proteins, mostly through interactions with a unique domain from the large subunit of the enzyme (214). This carboxy-terminal domain (CTD) of Pol II is composed of tandem repeats of a heptad with the conserved consensus sequence YSPTSPS. The CTD can allosterically regulate capping enzymes and regulate transcriptional elongation and termination (215).

1.3.2 Transcriptional coregulators

Transcriptional regulation is dependent not only on transcription factor activation and chromatin remodeling, but also on a group of transcription factor coregulators – coactivators and corepressors. In addition to transcription factor activation and chromatin changes, there is an expanding array of additional modifications involved in transcriptional regulation.

1.3.2.1 Transcriptional coactivators

In general, coactivators do not bind to DNA, but interact indirectly through association with other DNA-binding proteins (e.g., nuclear receptors). Once recruited to the promoter, coactivators enhance transcriptional activity through a combination of mechanisms, including efficient recruitment of basal transcription factors such as template-activating factors and TATA-binding protein. In addition, coactivators possess themselves, or recruit other nuclear proteins that possess, enzymatic activities crucial for efficient gene expression including the histone acetyltransferase (HAT) (e.g., CBP/p300, p160s), methyltransferases (e.g., CARM1), ubiquitin ligases (e.g., E6-AP) and ATPase (e.g., SWI/SNF).

The p160/steroid receptor coactivator (SRC) family is a well-studied group of transcriptional coregulatory proteins that function through histone tail modifications, altering chromatin structure, and facilitating transcription initiation. The members include SRC1, glucocorticoid receptor interacting protein (GRIP1) and P/CIP (SRC3). The p160/SRC family share a common structure that includes an N-terminal basic

helix-loop-helix domain, a PAS domain, a C-terminal transcriptional activation domain and a central region containing three nuclear receptor interacting LXXLL motifs (216). SRC1 and SRC3 exhibit HAT activity, which is necessary for the formation of an open chromatin structure (217). SRC coactivators can also interact with general coactivators such as the CREB binding protein (CBP) and p300 (218).

In addition to HAT activity, coactivator-mediated methylation of proteins in the transcription machinery may also contribute to transcriptional regulation by NRs . For example, coactivator-associated arginine methyltransferase 1 (CARM1) binds to the C-terminal domain of GRIP1, methylates histone H3, and enhances transcriptional activation by NRs (219).

Another coactivator related to chromatin modification is SWI/SNF, a complex with ATPase activity, which alters nucleosomal structure and is involved in the transcriptional regulation of NRs (220). The ATP-dependent chromatin-remodeling complexes use energy from ATP hydrolysis to increase the mobility of nucleosomal DNA, thereby regulating a variety of cellular processes, including transcription, DNA replication, and DNA repair and recombination. The targeting of SWI/SNF is thought to be achieved through the interaction of DNA-binding transcription factors, coactivators, or general transcription machinery. Different SWI/SNF components have been shown to mediate critical interactions between ER and mammalian SWI/SNF (221,222). In the context of NR-coactivator complexes, multiple interactions are probably involved in the recruiting and stabilization of SWI/SNF on NR target-gene promoters.

Another NR binding protein, the steroid receptor activator (SRA), is unique among coactivators. It functions as an RNA transcript rather than as a protein (223). SRA is selective for steroid hormone receptors and mediates transactivation via their N-terminal activation function. In addition, the E6-associated-protein (E6-AP), an ubiquitin ligase, has been identified as a coactivator of progesterone receptor (PR) (224). E6-AP also coactivates the hormone-dependent transcriptional activities of other nuclear hormone receptors.

1.3.2.2 General transcriptional repressors

In general, corepressor proteins coordinate the inactivation of transcriptionally active complexes through their direct interactions with DNA-binding transcription factors and the coordinate rcruitment of chromatin modifying enzymes that may return the nucleosome to an inactive state. The first corepressors identified for nuclear receptors were SMRT (Silencing mediator of retinoid and tyroid hormone receptors) and NCoR (nuclear hormone receptor corepressor) (225). These two proteins share a common molecular architecture and approximately 45% amino acid homology (226). Both SMRT and NCoR can be divided into a N-terminal portion having three to four distinct transcriptional repression (or silencing) domains (RDs), and a C-terminal portion composed of two or three nuclear receptor interaction domains (NIDs) (227,228) Both proteins interact with NRs through a C-terminal region, and nucleate the multiprotein repressor complexes through N-terminal repression domains, which interact with chromatin remodeling enzymes such as histone deacetylase (HDAC). HDACs inhibit gene transcription by remove the acetyl group from histons, which allows histons to bind DNA. Nuclear receptor-SMRT/NCoR complexes can also be regulated by phosphorylation of the corepressor, which can occur even in the absence of receptor ligand. Phosphorylation of corepressors can either enhance or inhibit the interaction between receptors and corepressors. For example, phosphorylation of the C terminus of SMRT by casein kinase/CK2 stabilizes corepressor binding to T3Rs (229). In contrast, negative regulation of SMRT occurs in response to growth factor receptor-mediated phosphorylation through a Ras-MEKK1-MEK1 pathway (230).

A new aspect of transcriptional regulation is related to oxidant signals, reactive oxygen intermediates, and cellular redox state on cell physiology, function, and viability. Elements of this complex system can directly impact gene transcription. One example of this type of regulation involve nicotinamide adenine dinucleotide (NAD), a widespread small biological molecule that participates in numerous cellular reactions including transcriptional control (231). One of the NAD-dependent coregulatory proteins is the C-terminal binding protein (CtBP), an ubiquitious corepressor with numerous interacting

proteins (232). CtBP may mediate repression in an HDAC-dependent or -independent manner. NADH binding changes the CtBP three-dimensional confirmation, resulting in a shift in protein-protein interactions and increased corepressor activity (233). Another transcriptional regulator influenced by NAD is Sir2p, which is required for nucleolar silencing (234). Sir2p is critical for silencing of the telomere and rDNA loci and this activity requires NAD as a cofactor for its HDAC activity (234,235).

1.4 Nuclear hormone receptor superfamily

Members of the nuclear hormone receptor superfamily are ligand dependent transcription factors which modulate a large number of essential cellular activities. The superfamily consists of receptors for steroid hormones (e.g. estrogen, progestin and androgen), steroid derivatives (e.g. dihydroxyvitamin D3) and non-steroids (e.g. retinoids and thyroid hormone). In addition, there are members of this superfamily for which endogenous ligands have not yet been identified, namely the “orphan receptors”.

Nuclear hormone receptors can be divided into two groups: type I and Type II receptors. Type I receptors include the classic steroid hormone receptors, such as glucocorticoid receptor (GR), which undergo nuclear translocation upon hormone binding and associate with their consensus sequences on DNA as homodimers. Type II receptors include retinoic acid receptor (RAR), retinoid X receptor (RXR), thyroid hormone receptor, and vitamin D3 receptor, which reside in the nucleus, regardless of the presence of ligand, and heterodimerize with RXR on their DNA binding sites.

1.4.1 Structure and function of nuclear receptors

Members of the nuclear hormone receptor superfamily were first cloned during the 1980s and comparison of their cDNAs and protein sequences revealed common structural motifs (236). These common structural motifs between members of the nuclear hormone receptor superfamily suggest that they are evolutionarily linked.

Based on the sequence homologues between nuclear hormone receptors, their general structure can be divided into six subregions, including the N-terminal half of the

receptor or A/B region and region C which includes the conserved DNA-binding motif. The C-terminus is subdivided into a short region D, called the “hinge-region”, region E which corresponds to the ligand binding domain and region F which is only present in some receptors (Figure 1-6).

1.4.1.1 The A/B domain

The A/B region is the most poorly conserved region within the NR family in terms of length and amino acid sequence. The A/B domain contains a transcription activation function-1 (AF1) that synergistically interacts with ligand dependent AF2 located at the C-terminal region of the receptor. AF1 is responsible for promoter context and cell type receptor activity. For example, glucocorticoid receptor (GR) and other steroid hormone receptor mutants that only express their A/B and C domains (DNA binding domain) were constitutively active and stimulated transcription from simple promoters containing their cognate binding sites (237,238). The A/B domain appears to directly or indirectly contact a variety of coactivator and corepressor proteins as well as other transcription factors (239-241) suggesting that A/B domains interact with tissue-specific cofactors. The functions of many proteins depend upon their structures, however, the AFs in A/B domains when expressed as peptides exhibit a random coil configuration (242,243). The N-terminal AFs of some nuclear hormone receptors may contain helical structures (242). There are two hypothesized models for AF1 folding. The first hypothesis is that the AF1 does not have to be well-structured, but must present a cluster of charges, sufficient to activate the transactivation function (244,245). The second hypothesis states that the act of binding one or more of its cognate partners induces the appropriate folding of the A/B domain. Two models are consistent with the second hypothesis, namely, the induced-fit and selected-subset models. The induced-fit model assumes that nonspecific initial binding due to random interactions between the binding partner (BP) and the AF domain induces a rapid shift in structure of the AF, leading to collapse of the molecule into the correct functional shape resulting in enhanced, specific AF1-BP binding. When sufficient quantities of BP are in proximity to the properly folded subpopulation of AF, they bind

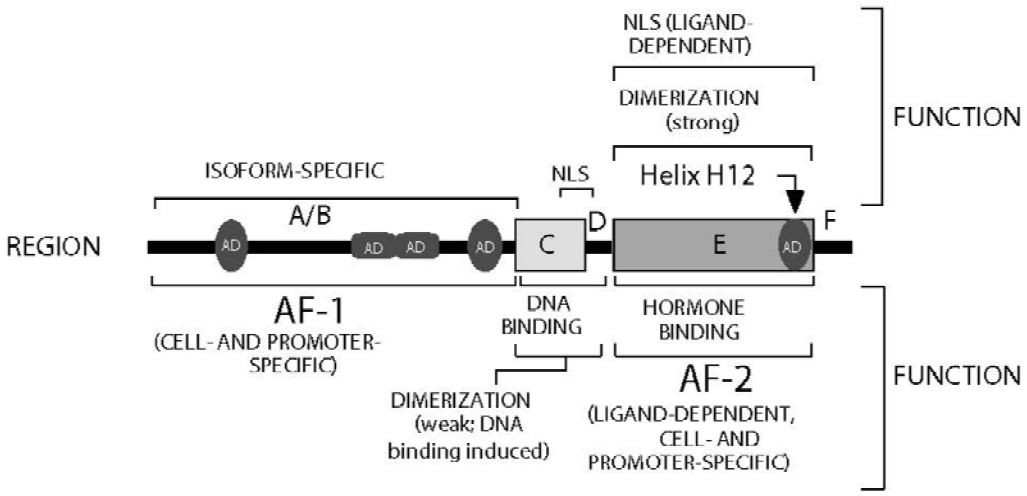

Figure 1-6 Schematic illustration of the structural and functional organization of NRs. The evolutionary conserved regions C and E are indicated as boxes and a black bar represents the divergent regions A/B, D, and F. Note that region F may be absent in some receptors. Domain functions are depicted below and above the scheme. Two transcription activation functions (AFs) have been described in several nuclear receptors, a constitutively active AF1 in region A/B and a ligand-inducible AF2 in region E. Within these activation functions, autonomous transactivation domains (ADs) have been defined in the estrogen (ER) and progesterone receptor (PR) N-terminal regions. In the case of the estrogen, retinoid and thyroid hormone receptors an autonomous activation domain (AF2 AD) encompassing helix H12 has been detected at the C-terminal end of the ligand binding domain E (246).

with high affinity and shift the remainder of the AF1 into the fully formed functional AF-BP structure. In addition to the binding of coactivators and corepressors, posttranslational modifications of receptors may contribute to the structure of the N-terminal AFs. Phosphorylation is well known to affect activity of certain steroid hormone receptors, and many of the phosphorylated amino acids are in the A/B domain (247). A recent study proposed that binding of the ER to its response element can regulate the structure and biological activity of the receptor and influence recruitment of coactivators to the ER at target gene promoters (248). This suggests that the DBD-RE binding represents an active event in which intramolecular forces induce folding to give functional receptors.

1.4.1.2 The DNA-binding domain (DBD)

The most conserved domains in nuclear receptors is within 66 amino acids located in region C. Deletion analysis and single amino acid mutations indicates that this region contains the DNA-binding motif which is required for sequence specific recognition and binding of the receptor to hormone responsive elements (HREs). The DNA-binding motif contains highly conserved cysteine residues which are required for coordinating Zn^{2+} ion. Each of the two Zn^{2+} ions is bound by four cysteines forming a C2-C2 zinc-finger (Figure 1-7). Although the 66 amino acid fragment is necessary for the receptor to bind DNA, the flanking amino acid sequences are also important for ERα (249). DNA binding of the C domain of ERα was weak and was only observed on perfect ERE palindromic sequences, however the addition of amino acids from region D greatly enhanced the receptor binding to consensus and nonconsensus EREs (249).

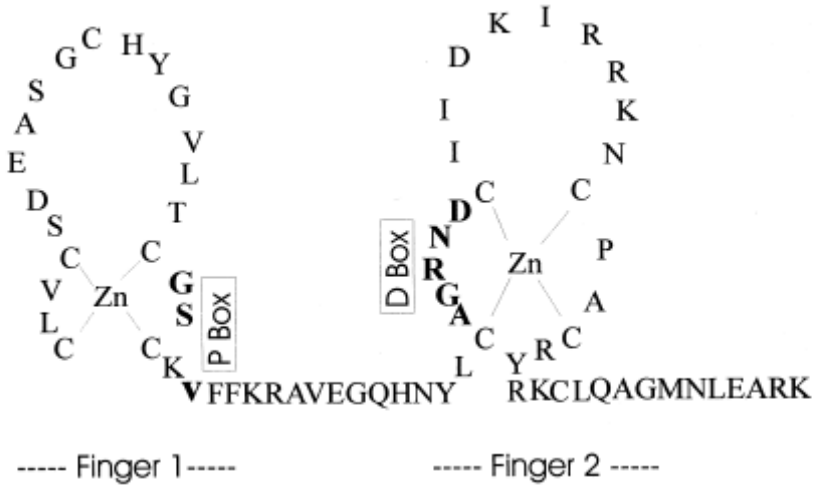

Figure 1-7 Sequence of amino acid residues of the human glucocorticoid receptor, showing two zinc finger motifs. The three highlighted amino acids around the first zinc finger (P-box) are those essential for discrimination between GRE and ERE, whereas the highlighted amino acids around the second zinc finger, known as D-box, are important for protein:protein interactions in the dimeric DBD:GRE complex (250).

The two zinc-fingers have different structures and functions. The helix of the first zinc-finger is primarily involved in site-specific recognition based on its interaction with certain bases in the cognate response element hexamer. Also within this helix are the amino acids responsible for site-specific discrimination of binding. These 3-4 amino acids have been termed the P box (250,251). A loop formed in the second zinc-finger provides the DBD homodimerization interface and the helical region, and some non-specific DNA interactions.

In general, nuclear hormone receptors bind to DNA as dimers. Type I steroid hormone receptors bind as homodimers, whereas type II receptors bind preferentially as heterodimers. For type I receptors, it was shown that two receptors cooperatively bind to their palindromic response element, and the DBD is sufficient to provide this cooperative binding. Replacement of four amino acids in region C (the D-box) of ER with the homologous amino acids of the retinoic acid receptor (RAR) abolished cooperative binding (252). Thus, the DBD of a receptor contains information which specifies dimerization and cooperative DNA-binding functions as well as the DNA sequence to specificity.

In order to further understand the DNA-binding properties of nuclear hormone receptors, x-ray crystal structures of the DBD of GR, ER, and RXR bound to their response elements have been solved (250,251,253). The central feature of the secondary structure elements within the GR DBD is found in three helical regions. Helices I and III are oriented perpendicular to each other and form the base of a hydrophobic core. NMR studies indicate that helices I and III are both regular α-helices, whereas helix II is somewhat distorted (254). The crystal structures show that helix I fits into the major groove of the DNA helix and provides critical contacts between three amino acids in the protein helix and certain bases in the major groove (250). The dimer interface, a loop of five amino acids that is also called the D box, lies between the first two cysteines of the second zinc finger.

The relative orientation of the two DBDs in the homodimeric complex is determined not only by the response element sites, but also by monomer interactions

critical for recognition of spacing and orientation of hexameric half-sites (250). The crystal structures of the DBD homodimer: DNA complex of the GR and ER shows that each DBD exposes an α-helix to the bases in the major groove of the DNA, and the recognition surfaces of these complexes are supported in the major groove. Each monomer contacts the sugar phosphate backbone on either side of the major groove. Interestingly, the structure of the RXR DBD has an additional helix immediately after the second zinc finger. This additional helix presumably facilitates both protein:DNA and protein:protein interactions required for high affinity binding of the RXR DBD to its cognate response element. The amino acids in this third helix are conserved in the isoforms of RXR found in different species; suggesting that the third helix may be a general feature of the receptor. There are indications that the third helix in the RXR DBD functions not only in RXR homodimerization, but may also function in the well known interactions of RXR with other members of the nuclear receptor superfamily (251,255).

Functional analysis in gene expression assays showed that the DBD of PR and GR can activate gene transcription in vitro and in vivo (256-258) and deletion analysis defined one nuclear localization signal within the DBD of PR (259).

In summary, The DBD of nuclear hormone receptors is multifunctional. It contains sequence specific DNA-binding activity, information for homo- and hetero-dimerization, cooperative binding to other receptor molecules, a weak transcriptional activation function and a nuclear localization signal.

1.4.1.3 Functional domains in the C-terminus

The ligand binding domain (LBD) localized in the C-terminal E region is the second most conserved domain of the nuclear receptors (NRs). All LBDs are composed of a series of 11~12 α-helices (H1~H12) closely folded in a similar manner. The unliganded LBD of RXR-α was the first of these structures to be solved (260). Although the secondary structure in the unliganded and liganded RXR-α and RAR-γ are similar, the latter structure is more compact suggesting that ligand binding may function to stabilize the conformation of a large portion of the LBD.

The ERα LBD is the first structure of Type I NRs which compare the binding of an agonist and an antagonist (261). The agonist (E2) and antagonist (raloxifene) bind at the same site but induce different conformations due to major alterations in the position of helix 12. When antagonist is bound, H12 appears to be in a position that blocks the LBD-binding site for coactivators via their cognate LXXLL motif.

The C-terminal part of the LBDs of the RAR, the TR, and the ER have a ligand-inducible activation function, termed AF2 (262,263). In the unliganded RXR-α LBD structure, the region is essential for AF2 function and adopts a helical structure, which corresponds to the C-terminal helix H11. The helix is often known as the AF2 activation helix. Both deletion and mutation studies have shown that AF2 is essential for ligand induced transcriptional activation. It appears that activation of AF2 upon ligand binding corresponds to major conformational changes, which create the proper surface required for efficient interaction with transcriptional coregulatory factors, which are the putative mediators of AF2 function (264,265). The amphipathic C-terminal activation helix, even in the absence of ligand, constitutes an active conformation, which has been observed in the structures of the ligand-bound RAR-α and TR (266). This may explain ligand-independent activation of this receptor. An approach in defining the LBD suggests that at least part of the D-region is required for hormone binding (267). Also, deletion of eight amino acids from the C-terminus of TR abolishes hormone binding completely. Thus, the minimal hormone binding domain comprises the entire E-region and part of the D-region.

Steroid hormone receptors bind to their response elements as dimers and the existence of two dimerization signals within the coding sequence of nuclear hormone receptors has been described (268). One is localized in the DBD and is considered to be weak; the other is stronger and dependent on hormone (269). The sequences in the dimerization domain reveal a heptate repeat of hydrophobic residues which are conserved in all members of the nuclear hormone receptor superfamily. This suggests that nuclear hormone receptors have a leucine-zipper type dimerization interface (270). Taken together, the C-terminus of nuclear receptor contains sites for ligand binding,

homo- and/or hetero-dimerization, and transcriptional activation.

1.4.2 Nuclear receptor-mediated gene regulation

Transcriptional regulation by NRs is a complex process. It requires recruitment of specific classes of coactivators and other transcription-related factors being recruited to the target promoter by the DNA-bound receptor in the chromatin environment of the nucleus. Each factor in the collection contributes one or more distinct activities, such as chromatin remodeling, histone modification, cofactor complex assembly and recruitment of the basal transcription machinery. The ultimate goal is to stimulate the transcription of target genes by RNA polymerase II (RNA pol II).

1.4.2.1 DNA recognition by nuclear receptors

The initial step in transactivation is the binding of receptor dimers to their HRE within the regulatory region of hormone responsive genes. Several in vitro studies have demonstrated that tight binding of both type I and II NRs to their cognate HREs occurs in a ligand-independent manner (271) although under certain cellular conditions, NR occupancy of HREs is ligand-dependent (272). However, in the case of the ER, ligand binding does not change the affinity of the receptor homodimer for its specific RE (273) indicating that binding of the receptor to the template may be influenced by other factors within the cell.

The identity of HREs is due to three features: the nucleotide sequence of the two core motif half-sites, the number of base pairs separating them and the relative orientation of the motifs. According to the type of interaction with HREs, the nuclear receptor superfamily has been divided into four classes. The DNA-binding sites for nuclear receptors exist as one or two copies of hexamer sequences. The class I receptors all recognize a 5’-AGAACA-3’ core while the class II receptors recognize a 5’-AGGTCA-3’ core (274). Steroid hormone receptors only bind inverted repeats with a three-nucleotide spacer, except for the androgen receptor which also binds direct repeats (Figure 1-8). Among the best-characterized non-palindromic arrangements are the direct

repeats of 5'-AGGTCA-3', which are targets for the nuclear receptors that form heterodimers with RXR (275). The repeats vary in the length of the spacer that separates the two hexameric half-sites. The number of spacing nucleotides restricts the species of receptor dimers that can activate these HREs (276).

Analysis of all natural steroid-response elements revealed that one half-site is more likely a consensus sequence while the other can show divergence from the consensus sequence (274). The consensus half-site is initially recognized bound with high affinity and binding of the first DBD results in a conformational change in the protein that supports the formation of the proper dimerization interface, that enables a second monomer to bind co-operatively to the second half-site in the HRE.

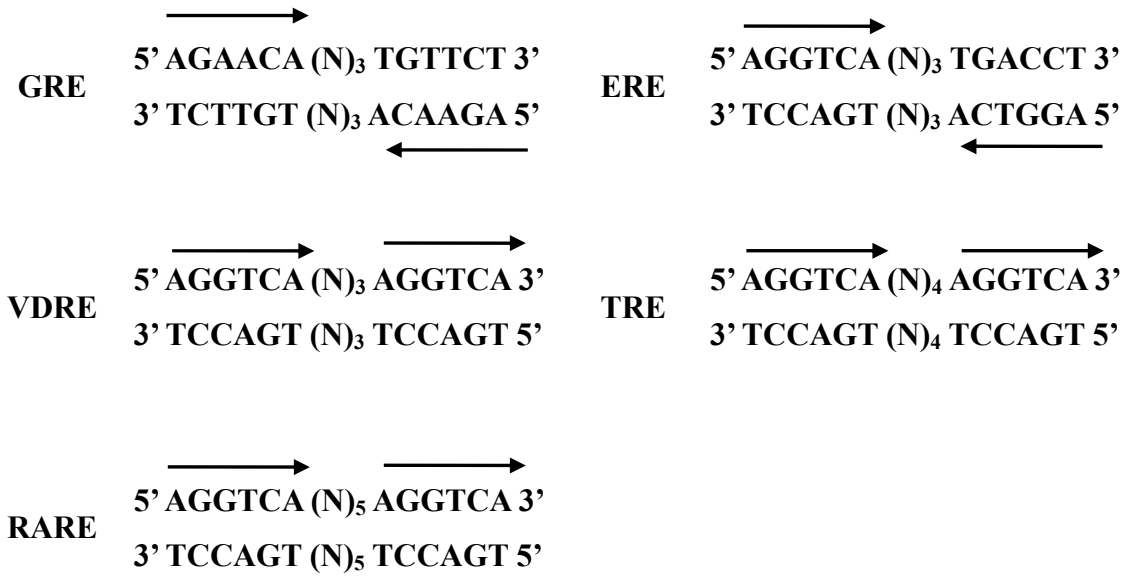

Figure 1-8 Consensus sequences of hormone response elements. Consensus sequences of DNA sites that bind the glucocorticoid receptor (GRE), estrogen receptor (ERE), vitamin D3 receptor (VDRE), thyroid hormone receptor (TRE), and retinoic acid receptor (RARE).

1.4.2.2 Nuclear receptor-directed initiation of gene expression

The initiation of mRNA synthesis by RNA polII involves the direct binding of core promoter DNA elements by a collection of "basal" transcription factors. Direct interactions between the p160 proteins and AF2 domains of some NRs have been clearly demonstrated (277,278). Once bound to the NR, p160 proteins facilitate recruitment of p300/CBP to the regulatory region. After p300/CBP is bound to DNA via the NR (Figure 1-9), it may increase the number of productive preinitiation complexes present within the promoter (279). Furthermore, p300/CBP can interact with Pol II through the intermediate of RNA helicase A and recruit the Pol II holoenzyme complex to the promoter region (280). The acetyltransferase activity of p300/CBP is also essential for mediating transactivation by both ER (281) and TR/RXR (282) from repressive chromatin. The arigine methyltransferase CARM1 coactivator can synergize with p160 and p300 proteins to enhance ligand-dependent transcriptional activation by NRs, possibly through to methylation of histone H3 (283). The next step involved is recruitment of TRAP220/SMCC/mediator complex. The DNA tethered SMCC complex can also recruit the Pol II holoenyzme to the proximal promoter. NRs also facilitate recruitment of SWI/SNF to the promoter region. SWI/SNF can induce remodeling of the proximal promoter region and facilitates binding of the basal transcriptional machinery to the DNA template and association between TBP and TATA box.

In general, the binding of ligand-activated NRs to DNA-response elements in the promoter region of a hormone-responsive gene stimulates the assembly of a stable basal transcription factor/RNA pol II transcription PIC at the promoter, with recognition of the TATA box and other core promoter elements by a complex called TFIID being a critical initial step of NR-mediated transcriptional activation.

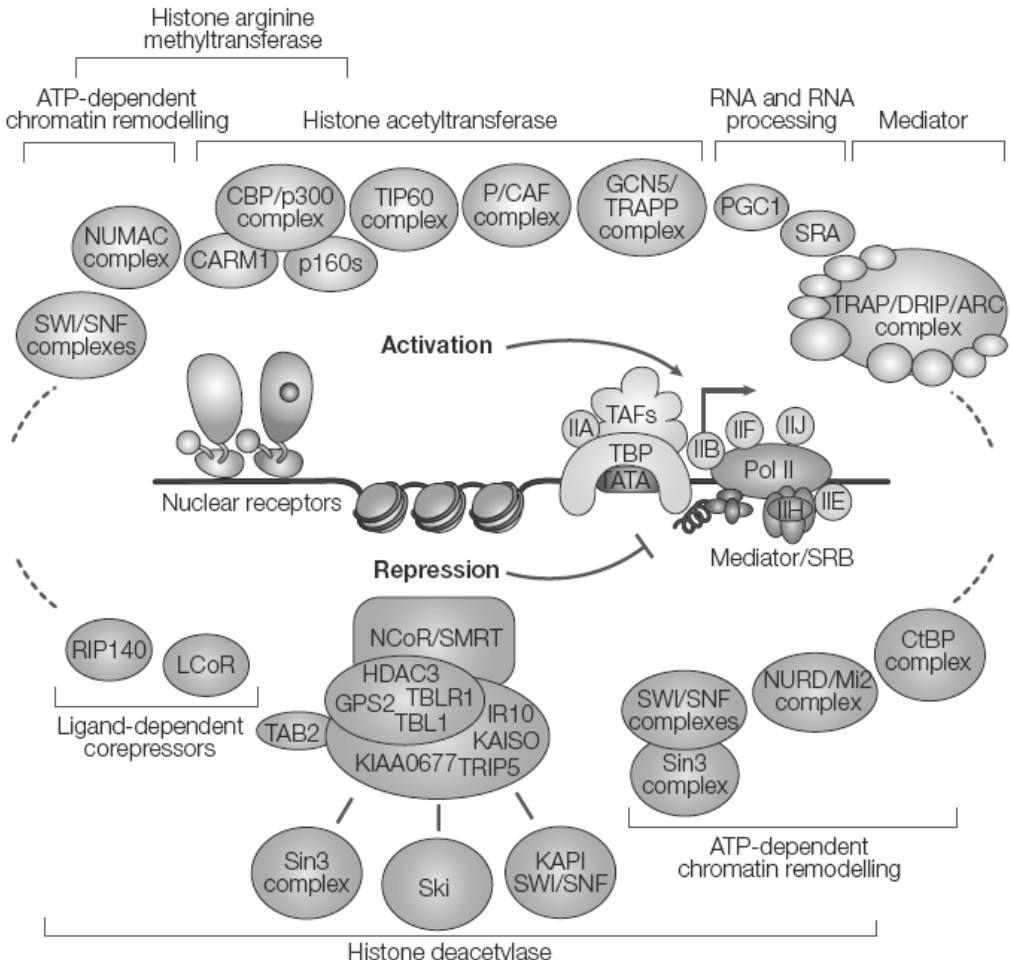

Figure 1-9 Coactivator and corepressor complexes for regulation of nuclear receptor-mediated transcription (284).

1.4.2.3 Role of coregulators in NR-mediated transactivation

Nuclear receptors carry out many different transcriptional functions through the recruitment of a group of positive and negative regulatory proteins, referred to as coactivators or corepressors, respectively (216). Alternatively, coregulators can also be classified into two main groups according to their functions. The first group contains

factors that covalently modify histones (ie. acetylation, methylation, phosphorylation, ubiquitylation). The second group includes ATP-dependent chromatin remodeling factors that modulate promoter accessibility to transcription factors and to the basal transcriptional machinery. These coregulators are not exclusive to NRs, and are used in a similar way by numerous other DNA-bound transcription factors. For a detailed description of coregulators please refers to Section 1.3.2.

Ligand binding is the crucial molecular event that switches the function of nuclear receptors from active repression to transcriptional activation. Structural and molecular studies of the interactions between nuclear receptors and coregulators has provided evidence that hormone binding induces a conformational change in the ligand-binding domain of the receptor, which results in reduced affinity for corepressors and enhanced affinity for coactivators. Similarly, agonist binding to steroid hormone receptors also induces specific conformations that favors coactivator binding, whereas antagonist binding promotes interactions with corepressors. However, in some cases, recruitment of ubiquitylation machinery and proteasome-dependent degradation of the repressors are required to fully promote the release of the corepressors in response to ligand binding (285).

Recent studies using chromatin immunoprecipitation (ChiP) analysis have revealed detailed and coordinated patterns of cofactor recruitment and preferential selectivity for factors that have similar enzymatic activities (286,287). In the case of the E2-responsive pS2 promoter, recruitment of different classes of cofactors occurs in a precise temporal and sequential fashion (209,286). Interestingly, some coregulators seemed to be redundant, and different family members were equally capable of being recruited alternatively to the promoter (286). By contrast, preferential interactions between a nuclear receptor and a specific HAT-containing enzyme or a specific member of the NCoA family of coactivators have also been observed (288,289).

1.5 Estrogen receptor (ER)

The estrogen receptors (ERα and ERβ) are Class I members of the nuclear receptor superfamily of ligand-inducible transcription factors (290). ERα and ERβ possess the hallmark modular structure characteristic of other NRs (Figure 1-10). Because ERα and ERβ display a high degree of sequence similarity in the DNA- and ligand-binding domains (DBD, 96% and LBD, 53%, respectively), it is not surprising that these receptors interact with identical response elements and exhibit similar ligand binding affinity profiles (291). ERα is widely expressed and is the predominant ER subtype in the breast, uterus, and bone. On the other hand, ERβ is expressed primarily in the ovary, prostate, testis, lung, thymus, spleen and in localized areas of the brain (292).

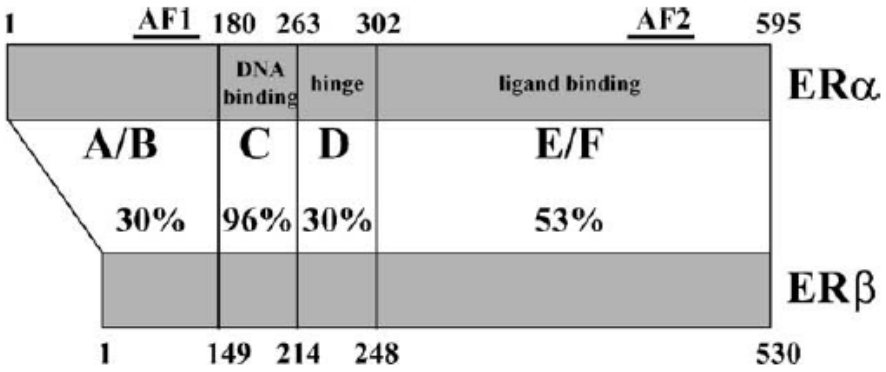

Figure 1-10 Structure and homology between human ERα and the long form of ERβ (293).

1.5.1 Biological roles of estrogen receptors

In the late 1960s and early 1970s, the ER was initially used as a predictor of breast cancer response to endocrine ablation. Tumors that were ER rich were more likely to respond to endocrine therapy than if the tumor was ER poor (294). From the 1970s to the present day, the ER has evolved to be the most effective target for breast cancer therapy. The present challenge is to dissect the individual roles of ERα and ERβ as transcription factors that participate in normal and aberrant physiological processes.

Estrogen has multiple physiological functions and is a central modulator at the molecular, cellular, and behavioral level. It regulates the growth, differentiation, and physiology of the reproductive process through the ER. Estrogen plays a critical role in sexual differentiation and maturation as well as growth of female secondary sexual organs. Estrogen has multiple effects on the gene expression in both the vagina and the uterus and in the mammary gland where estrogen together with progesterone regulate lactogenesis. E2 also affects other tissues, such as bone, liver, brain and the cardiovascular system. Because of the functional diversity displayed by estrogens which act through the ER, understanding the basis of ER actions at the molecular level can identify molecular targets that are the basis for therapeutic intervention and treatment of hormone-related disease such as breast cancer (295).

1.5.1.1 The role of ERα and ERβ in breast cancer

The ERα is an important target for development of drugs for treatment and prevention of breast cancer (53). The interaction of estrogen with the ERα can result in increased proliferation of breast cancer cells and some forms of endocrine therapy block the interactions of estrogen with ERα. This can be accomplished by inhibiting the production of estrogen by ovariectomy, or be inhibiting conversion of steroidal precursors to estrogen using aromatase inhibitors. The ERα can also be targeted directly using SERMs such as tamoxifen and raloxifene as competitive inhibitors of ERα-E2 interactions (296), or by the removal and degradation of ERα by “pure” antiestrogens such as ICI 182,780 (297). Endocrine manipulation is one of the least toxic and most

effective therapies for treatment of hormone responsive breast cancers. Current studies have shown that 5 years of adjuvant tamoxifen treatment is beneficial in pre- and postmenopausal women with ER-positive tumors (59). In addition, tamoxifen can be used for the prevention of breast cancer in high risk patients (298).

The role of ERβ in breast cancer growth and development is not as clear as the role of ERα (299). ERβ modulate estrogenic activity in breast cancer because it is expressed in normal and malignant breast tissue, binds E2 and can heterodimerize with ERα (300,301). One problem is that although ERβ mRNA level expression has been determined using PCR based techniques, ERβ antibodies are not well characterized or effective for detecting this protein and this has led to inconsistencies in the literature. For example, one study reported that ERβ is a good prognostic indicator for breast cancer and expression of ERβ was associated with better survival in patients receiving adjuvant tamoxifen (302). Another study showed that ERβ is associated with negative axillary node status and low grade tumors (303). In addition, ERβ patients had a better disease free survival rate (304) and levels of ERβ were decreased in proliferative preinvasive tumors (305). These studies suggest a protective role for ERβ in breast cancer. In contrast, there is evidence suggesting that ERβ is a poor prognostic indicator. For example, tumors that expressed both ERα and ERβ were node positive and of a higher grade (306) and ERβ mRNA levels were also elevated in tumors that displayed tamoxifen resistance (307).

Overall, the majority of studies suggest that the presence of ERβ is a good prognostic marker for breast cancer. However, the relative amounts of ERα and ERβ must be considered. As normal breast tissue becomes tumorigenic, the amount of ERα increases whereas the amount of ERβ decreases (308). The majority of ER present in breast tumors is ERα and therefore the biological relevance of ERβ expression in breast cancer remains a topic of debate.

1.5.2 Molecular mechanisms of ER actions

The biological effects of estrogens are mediated through ERα and ERβ. There are

two models for the ER actions namely the genomic and non-genomic pathways.

1.5.2.1 Gene regulation through genomic pathway

Estrogen diffuse passively in and out of cells (309) and the classical mechanism of ER action involves binding of E2 to ER in the nucleus; the receptors form dimers and bind to specific response elements known as estrogen response elements (EREs) located in promoters of target genes (Figure 1-11) (64). Hormone binding also induces a conformational change within the ligand binding domain of the receptors, and this allows coactivator proteins to be recruited (310). This leads to alteration of chromatin, histone unwinding, interactions with components of the basal transcription machinery complex, and subsequent mRNA expression.

There are several human genes that are regulated by ERs and do not contain ERE-like sequences (Figure 1-11) (311). The receptors in such cases are tethered through protein-protein interactions to a transcription factor complex that contacts the DNA (312). Several genes are activated by E2 through the interaction of ERs with Fos and Jun proteins at AP-1 binding sites (313). An E2-responsive AP-1 element was initially identified in the proximal promoter of the ovalbumin gene (314). Fos and Jun family proteins bind AP-1 elements as homo- or heterodimers. These proteins contain leucine zipper domain that mediates DNA binding and are typically associated with genes that rapidly respond to various extracellular stimuli (315).

Mechanistic studies of ER/AP-1 actions have shown that the requirement for ER structural domains is dependent on the receptor subtype and on ligand structure. For example, E2-dependent activation of ERα/AP-1 complexes require the AF2 domain of the receptor, which binds p160 coactivators and stabilizes the formation of a multiprotein complex containing c-Jun, ERα, and transcriptional coactivators at the promoter. However, the ER DBD is required for tamoxifen-activated ERα/AP-1 dependent activity (316). Furthermore, ICI, 182,780, an antiestrogen that inhibits ER dimerization and ER DNA (ERE) binding, activates an AP-1 reporter construct (317). Interestingly, full length ERα containing mutations in AF1 also compromised E2-mediated AP-1 activity,

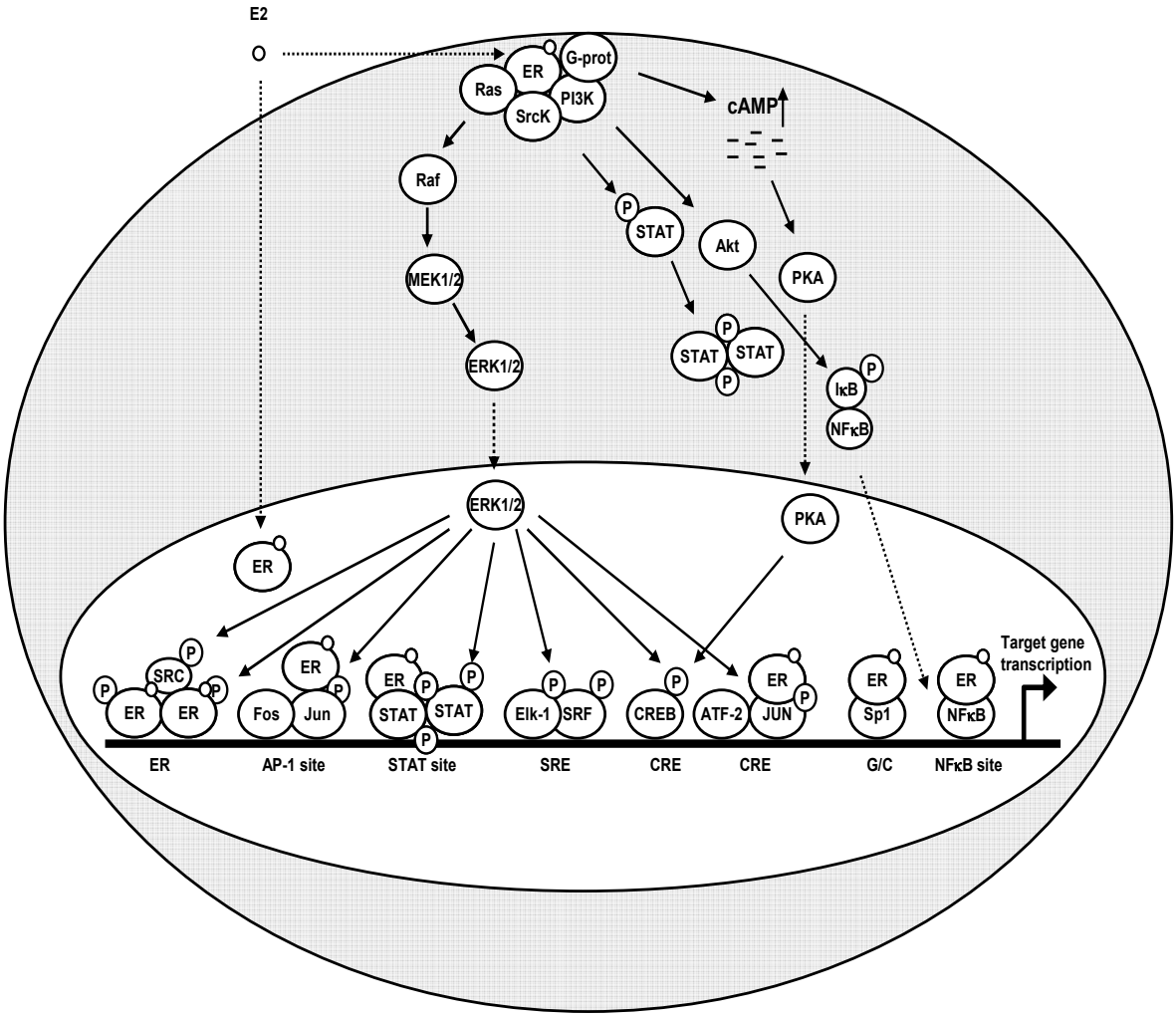

Figure 1-11 Genomic and nongenomic actions of ER on a target gene promoter (359).

indicating that ERα/AP-1 action requires both AF1 and AF2 (318).

ERβ also activates transcription from an AP-1 element. However, the effects of estrogen and antiestrogens on ERβ/AP-1 contrasts to those observations for ERα/AP-1. E2, ICI, 182,780, tamoxifen, and raloxifene all activate AP-1 reporter construct in cells cotransfected with ERα whereas, in the presence of ERβ, E2 not only acts as antagonist but also inhibits the activity of tamoxifen and raloxifene dependent induction of ERβ/AP-1. Moreover, tamoxifen or raloxifene alone behave as full ERβ/AP-1 agonists (319).

Genes that contain GC-rich promoter sequences are regulated in a similar manner through the interaction of ERs with the Sp transcription factor (316,320-323). E2-responsive GC-rich elements were initially identified in the c-myc gene promoter (324). This site contains a nonconsensus ERE-half site (ERE½) and a Sp1 binding site that was required for estrogen-mediated induction. Similar ERE½/Sp1 elements have been subsequently characterized in the cathepsin D (325), heat shock protein 27 (Hsp27) (326) and TGF α gene promoters. However, mutation of the ERE1/2 in the Hsp27 promoter did not result in the loss of E2 responsiveness, and the E2-dependent ERα/Sp1 action is also observed in cells transfected with a DBD deletion ERα mutant. The data suggested that GC-rich site alone was sufficient for E2-responsiveness and ERα binding to DNA was not required for activation of genes through GC-rich sites. The ERE-independent ERα/Sp1 action has also been observed for several genes including retinoic acid receptor α (327), c-Fos (328), insulin-like growth factor-binding protein-4 (329), bcl-2 (330), adenosine deaminase (331), thymidylate synthase (332), cyclin D1 (333), cad (334), E2F-1 (335).

Although both ERα and ERβ forms complexes with Sp1 protein, only ERα induces consensus Sp1 element-linked reporter gene activity whereas ERβ, exhibits minimal or decreased the basal reporter gene activity and these responses are ligand- and cell type-specific. Interestingly, it was recently reported that both ERα and ERβ regulate EGF receptor gene expression through GC-rich elements and, depending on the ligand, ERβ exerts full agonist activity on this promoter, indicating that promoter context is also

an important factor in ERβ/Sp1 action (336).

ERα/Sp1 protein-protein interactions were investigated in vitro using GST pull-down assays, which showed interactions between the C-terminal end of Sp1 with multiple regions of ERα (337). Additionally, it has been shown using a series of ER α deletion mutants and ERα/ERβ chimeric mutants that the AF1 domain of ERα is critical for ERα/Sp1-mediated transactivation. Recent studies also indicated that E2-dependent activation of ERα/Sp1 required the C-terminal F domain of ERα, which was not required for antiestrogen activation of ERα/Sp1 (338).

ERα not only interacts with Sp1 but also with Sp3 protein, another member of Sp protein family. It was found that vascular endothelial growth factor (VEGF) gene expression is regulated by ERα/Sp1 or ERα/Sp3 either positively or negatively and the Sp1/Sp3 ratio is critical for VEGF gene regulation. By using Sp protein deficient SL2 cells, upregulation of the VEGF promoter activity with E2 treatment was observed in cells cotransfected with ERα and Sp1 expression plasmid whereas downregulation of the same promoter activity was observed when cells cotransfected with ERα and Sp3 expression plasmid (339,340).

Even though the ERE-independent genomic actions are not involved with direct binding of ER to DNA, the DBD of the receptors is frequently required (316,320-323). Mutation analysis has revealed specific residues within the second zinc finger structure of the ERβ DBD that discriminate between the classical mechanism of ER action and the modulation of AP-1 and STAT5 activities through tethering (341). The data suggests that the DBD may be required for proper protein-protein interactions or it may be involved in recruiting additional coregulator proteins to the promoter region.

1.5.2.2 Non-transcriptional mechanisms of signal transduction through ER

The genomic pathway is complex and tissue-specific and many induced genes and estrogenic responses are only observed several hours after treatment with E2. This pathway fails to explain certain rapid estrogen-induced actions, such as those occurring in the vasculature and nerve cells, which are often observed within seconds after

exposure to E2. The rapid estrogen signaling is linked to non-genomic pathways where estrogen activates cell membrane or intracellular (cytosolic) forms of the ER or other proteins to alter the transmembranous flux of the sodium, potassium and calcium ions that rapidly modulate the internal state of cells (342,343). Alternative hypotheses related to plasma membrane-ER have also been reported and involve regulation of cell membrane-ion channels (344), G-protein-coupled receptors (345), and tyrosine kinases - many of these are related to membrane/cytosolic ER or GPR30.

Activation of various protein-kinase cascades had been associated with the nongenomic actions of the steroid hormones. Hormone-induced non-genomic pathways in various cells include the mobilization of intracellular calcium (346), stimulation of adenylate cyclase activity and cAMP production (347,348). E2 activates MAPK signaling in several cell types, including breast cancer (349), endothelial (350), bone (351), and neuroblastoma (345) cells. E2 also activates the phosphatidylinositol-3-kinase (PI3-K) signaling pathway in endothelial (350), breast cancer (352), and liver (353) cells. ER-activated nongenomic pathways modify ER and its coactivators by phosphorylation, resulting in an altered topology of these proteins that can result in ligand-independent activation of the ER or differential responsiveness to selective ER modulators (Figure 1-11) (354). Specific binding sites for estrogen at the outer surface of isolated endometrial cells were first reported during the 1970s (355). Membrane localization of ERα has been reported by several groups (356,357) and a serine in the E domain of ERα is required for the localization and function of membrane ERα (358). Mutation of S522 to alanine (S522A) results in a form of ERα that is poorly localized in the membrane after expression in CHO cells and membrane ER decrease by 62% compared to cells transfected with wild-type ERα. In addition, there is decreased co-localization of ERα S522A with caveolin-1, a protein that facilitates ER transport to the membrane. Furthermore, expression ERα S522A in MCF-7 significantly decreased membrane ERα since this ERα mutant binds and sequesters endogenous membrane ERα (358). In contrast, S522A did not affect nuclear localization or function of endogenous ERα.

1.5.3 Regulation of estrogen receptor activity

Cellular responses to E2 are highly controlled, involving regulation of the ERα levels through transcriptional, posttranscriptional, and posttranslational mechanisms (360). Binding of E2 to ERα induced proteasome-dependent degradation of the receptor and the half-life of bound ERα protein decreased from around 5 days to 3–4 h (361). ER activity is also regulated through the posttranslational modification and interactions with different coregulators which mediate transcriptional activation or repression by multiple mechanisms. Interactions of ERα with coregulators results in recruitment of the transcriptional machinery to the promoter by remodeling chromatin, or through activation of coregulatory protein dependent enzymatic activities, including histone acetyltransferase, deacetylase, ATPase, protease, kinase, ubiquitin ligase, and histone methyl transferaseactivities (219,220,224). The activity of the ER can involve ligand-independent or non-genomic effects.

1.5.3.1 Phosphorylation of ERα

The ligand-independent activity of the ER is a result of kinase-dependent phosphorylation of primarily serine residues in the AF1 domain of ERα. E2, 4-OHT and ICI 164,384 induce ERα phosphorylation at S118 in ERα-positive MCF-7 cells and both E2 and phorbol ester (TPA) induced S118 phosphorylation of ERα in COS-1 cells transfected with ERα (362). Treatment of SK-Br-3 cells with EGF also induced MAPK-dependent phosphorylation of S118 and that S118 is required for epidermal growth factor (EGF) activation of the ER via MAP kinase (363) . In response to E2, S167 is another phosphorylation site in MCF-7 cells transfected with recombinant ER and phosphorylation was casein kinase II-dependent (364). PI3-K-dependent phosphorylation of S167 has also been observed (365) and phosphorylation of S104 and S106 is mediated by the cyclinA-CDK2 complex in U-2 OS human osteosarcoma cells (366). S236 in the DNA binding domain, is phosphorylated by protein kinase A and phosphorylation of this site plays a role in ERα dimerization (367).

In general, phosphorylation of Ser residues in the AF1 domain influences the recruitment of coactivators, resulting in enhanced ER-mediated transcription (368,369).

The importance of Ser-118 phosphorylation has been studied by a number of different groups and there are discrepancies in the literature concerning the transcriptional activity of the mutant ERα, in which Ser-118 has been mutated to Ala(ERα S118A), compared to wild type ERα. In cell lines of either fibroblast or epithelial origin ERα S118 exhibited decreased E2-dependent transactivation compared to wild type ERα (363,370,371), whereas mutation of Ser-118 to an acidic residue enhanced the transcriptional response (370). In contrast, Le Goff et al. (372) found that ERα S118 and ERα exhibit similar activies in mediating hormone-induced gene expression, however, an ERα mutant in which Ser-104, Ser-106 and Ser-118 were all mutated to Ala exhibited decreased activity.

Tyrosine phosphorylation of ERα has been detected at Y537 in MCF-7 cells (373) and $p60^{c\text{-}src}$ and $p56^{lck}$ mediate this phosphorylation E2 does not induce. Y537 phosphorylation indicating that it is associated with basal activity of ERα. The AF2 region plays an important role in mediating transcriptional activation by cAMP (374), however, mutagenesis of putative PKA phosphorylation sites did not prevent activation of ERα-mediated transcription by cAMP (375). Thus PKA may regulate coactivator function rather than the phosphorylation of ERα.

1.5.3.2 Ubiquitination of ERα

Treatment of cultured breast epithelial cells with E2 significantly decreases levels of ERα in cultured breast epithelial cells (376) and this is due to a activation of ubiquitin-proteasome pathway (224,377-379). In addition to E2, the pure ER antagonist ICI 182,780 inhibits ERα-dependent activity by inducing rapid downregulation of the receptor (380) and the selective ER modulator (SERM) GW5638 also induces ERα degradation (379).

The Ub-proteasome system consists of the 26S proteasome complex which is composed of a 20S catalytic core for protein proteolysis and two ATPase-containing 19S

regulatory particles that recognize polyubiquitin- tagged substrates (381). Like many other transcription factors, stimulation of ERα transcriptional activation appears to be associated with receptor ubiquitination and proteasomal degradation (379,382). Several proteins possessing Ub ligase activity (e.g. E6AP, p300, BRCA1, and MDM2), as well as SUG1, a component of the 19S proteasome, associate with ERα and modulate receptor signaling (224,383-386). These observations suggest that proteasome- mediated receptor degradation is important for ER function.

Blocking ERα turnover by a proteasome- specific inhibitor such as MG132, decreased expression of an ERα-responsive luciferase reporter, suggesting that proteasomal degradation of ERα is required for its transactivation function (287,387). However, it has recently been shown that MG132, and other proteasome inhibitors decrease production of a functional firefly luciferase enzyme (388), and this may also contribute to decreased activity of ERα-responsive constructs expressing luciferase. This complements other reports shown that proteasomal degradation is not essential for ERα transcriptional activity but functions to limit E2-induced transcriptional output (389).In the absence of estrogen, ERα is also ubiquitinated and degraded via an ubiquitin-proteasome pathway (390). However, estrogen-dependent ubiquitination of the receptor required the E domain within the ERα LBD, whereas this domain was not required for ubiquitination of the unliganded receptor. Tateishi et al (390) reported that ERα is regulated by two independent ubiquitin-proteasome pathways, which are switched by ligand binding to ERα. One pathway is necessary for the transactivation of the receptor and the other is involved in quality control of the receptor.

1.6 Research objectives

1.6.1 Objective 1

Cdc25A phosphatase is expressed in all eukaryotes and, mammals and the Cdc25A, Cdc25B, and Cdc25c forms are encoded by distinct genes (391-393). Cdc25 phosphatases play a critical role in cell cycle progression by regulating phosphorylation of cyclin-dependent kinase (cdk)/cyclin complexes at specific phases of the cell cycle

(141,148,391-400). Cdc25 phosphatases are dual-specific protein tyrosine phosphatases and catalyze rapid dephosphorylation of cyclin/cdk complexes on threonine14 and tyrosine15 (144,148,392,401). Regulation of G1 to S-phase progression in the cell cycle is primarily by Cdc25A associated with dephosphorylation and activation of the cdk2/cyclin A and cdk2/cyclin E complexes (402-405). Cdc25 phosphatases are overexpressed in a wide variety of tumors, and overexpression of Cdc25A and Cdc25B in cooperation with ras immortalizes mouse embryo fibroblasts (402-405).

Cdc25A plays an important role in cell cycle progression and in malignant transformation in breast cancer and this protein is also over-expressed in human mammary tumors (406). Mitogen activation of cells results in increased expression of Cdc25A in G1 for subsequent activation of cdk2 and progression of cells through S-phase. E2 induces Cdc25A protein and mRNA levels in MCF-7 breast cancer cells (407,408). Foster et al. (408) studied interactions of Cdc25A with multiple G1 to S-phase cell cycle regulators in breast cancer cells and showed that cdk2 activity is required for activation of Cdc25A which is a critical protein in hormone-dependent proliferation of MCF-7 breast cancer cells. The first objective of this study was to investigate the molecular mechanism of E2-dependent activation of Cdc25A in ZR75 breast cancer cells and identify which cis-acting elements and trans-acting factors in the Cdc25A promoter are required for E2-induced transactivation.

1.6.2 Objective 2

The PIAS family, originally identified as cytokine-induced inhibitors of STATs, consists of five structurally related mammalian proteins, PIAS1/GBP, PIAS3, PIASxα, PIASxβ, and PIASy. These proteins contain several conserved domains. The conserved N-terminal region of PIAS proteins contains several well characterized domains. The SAF-A/B, Acinus, PIAS (SAP) domain binds A/T-rich DNA and may be involved in targeting PIAS proteins to the nuclear scaffold (409). The SAP domain encompasses an LXXLL motif that is required for transcriptional repression (410). The RING-finger-like zinc-binding domain (RLD) mediates the SUMO-E3-ligase activity of PIAS proteins and

binds directly to Ubc9, the SUMO E2 enzyme (411). Most PIAS proteins also contain a PINIT motif, which plays a role in nuclear retention (411). The C termini of PIAS proteins are more diverse; however, all contain an acidic domain preceded by several serines (Ser/Ac). Within the acidic domain, a SUMO-1 Interaction Motif (SIM) exists (412). Also, a serine- and threonine-rich region (S/T) is present in the C termini of all PIAS proteins except for PIASγ. The function of this region is unknown.

Other than being the negative regulators of cytokine signaling which inhibit the activity of STAT-transcription factors, PIAS proteins also function as transcriptional coregulators in various important cellular pathways. It has been reported that PIAS proteins modulate the ligand-dependent transactivation potential of the steroid hormone receptors (413). Depending on the receptor type, the cell lines and promoters used in transactivation assays, both activating and repressing effects on transcription were observed upon expression of a distinct PIAS family member, indicating that PIAS proteins play a cell context-dependent dual role as activators or repressors in steroid hormone signaling. Jimenez-Lara and co-workers (412) identified PIAS3 as a binding partner of GRIP1/TIF2. PIAS3 also interacts with TBP in a yeast two-hybrid screening assay (414). The localization of SUMO E2-ligase activity and TBP-binding activity to opposite ends of PIAS proteins suggests that these proteins might “dock” at TBP and sumoylate transcription factors at the promoter.

An important mechanism for controlling the activity of transcriptional regulators appears to be their targeting to specific subnuclear sites. Increasing evidence indicates that the PIAS/SUMO system is involved in this process. Sapetschnig and co-workers (415) demonstrated that the repressor function of Sp3 resides in a small inhibitory domain that serves as an interface for binding to PIAS1 and harbors a SUMO attachment site . PIAS1 promotes the modification of Sp3 by SUMO, which further enhances the interaction of PIAS1 and Sp3. SUMO triggers the targeting of Sp3 to PML (Promyelocytic leukemia) nuclear bodies, acting as a major regulatory switch that converts Sp3 from a transcriptional activator to a repressor (415).

The SUMO E3-ligase activity of PIAS proteins often results in sumoylation of

transcription factors which interact with PIAS proteins. Unlike the ubiquitination process, the E3 ligase is dispensable and plays a role by enhancing sumoylation of target proteins. In addition to sumoylation, PIAS proteins can have SUMO-independent effects. Lee and co-workers (416) showed that PIAS1 binding to Msx1 homeodomain protein is required for localizing Msx1 to the nuclear periphery and the SUMO sites in Msx1 are not required for this localization to occur. It was concluded that the localization of Msx1 to the nuclear periphery by PIAS1 binding is the important determinant for the repressive activity of Msx1. In contrast, the SUMO ligase activity of PIAS1 toward Msx1 is apparently not important for this process.

Several studies have reported that SUMO-1 regulates the hormone-induced transactivation of some nuclear receptors. This regulation can be achieved by sumoylation of either receptors or coregulators, indicating that sumoylation can be an integral part of nuclear hormone receptor function. In particular, a recent report showed that ERα-mediated transcription is stimulated by SUMO-1 expression. It has been speculated that enhanced ERα-dependent transcription by SUMO-1 may be due to sumoylation of the coactivator steroid receptor coactivator 1 (SRC-1) (417).

Over-expression of PIAS3 can induce apoptosis in prostate cancer cell lines both in vitro and in vivo. Wang and co-workers (418) checked 100 human cancer samples including 13 breast cancer samples and showed increased nuclear staining of PIAS3 in approximately 97% of these sample compared to staining in the corresponding normal tissues. The second objective of this study was to investigate coactivation of ER by PIAS3 and also identify the functional domains of PIAS3 required for this enhanced activity, and for interactions with ERα.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, cells, and antibodies

MCF-7 and ZR-75 breast cancer cells, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 cells were routinely maintained in DME/F12 medium with phenol red and supplemented with 5% fetal bovine serum (FBS) plus antibiotic antimycotic solution (Sigma, St. Louis, MO). COS-7 and HeLa cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Gibco Invitrogen Corporation, Carlsbad, CA) medium with phenol red and supplemented with 5% FBS plus antibiotic antimycotic solution. ZR-75 cells were maintained in RPMI 1640 media (Sigma) supplemented with 2.2 g/L sodium bicarbonate, 2.38 g/L HEPES, 0.11 g/L sodium pyruvate, 4.5 g/L glucose, and 7.5 % FBS plus antibiotic antimycotic solution. Cells were cultured and grown in an air-carbon dioxide (95:5) atmosphere at 37°C. For transient transfection studies, cells were grown for 1 day in DME/F12 medium without phenol red and 2.5% FBS stripped with dextran-coated charcoal. ICI 182780 was kindly provided by Dr. Alan Wakeling (AstraZenaca Pharmaceuticals, Macclesfield, UK). The kinase inhibitors H8 and SQ22536 were purchased from Cal-Biochem (La Jolla, CA). Cdc25A, PIAS3, ERα and Sp1 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Myc-tag antibodies was obtained from Cell Signaling Technology (Beverly, MA). GAPDH antibody was obtained from Ambion, Inc. (Austin, TX). SYBR® Green PCR master mix and dNTP mix were obtained from Applied Biosystems (Foster City, CA). SuperScript™ III reverse transcriptase and oligo $(dT)_{20}$ were obtained from Invitrogen (Carlsbad, CA). All other chemicals and biochemicals were the highest quality available from commercial sources.

2.2 Cloning and plasmids

2.2.1 Cdc25A experiment

The pcdc25A-1 was kindly provided by Joan Massagué (Cell Biology Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY). Wild-type human estrogen receptor α (hERα) expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). The ER deletion mutant ERα11C was provided by Professor Pierre Chambon (Institut de Genitique et de Biologie Moleculaire et Cellulaire, Illkirch, France). The DP1 mutants DP1 Δ103-126 and DP1 Δ 127-411 bind E2F-1 but not DNA (419) and were kindly provided by Dr. Harlow (Harvard Medical School, Boston, MA). E132 is an E2F-1 mutant that also does not bind DNA (420) and was provided by Dr. Nevins (Duke University, Durham, NC). The dominant negative Sp1 construct (pEBG-Sp) expresses the DNA binding domain (amino acids 592-758) but not the activation domain of Sp1 (421) and was provided by Dr. Thiel (University of Cologne, Cologne, Germany). The dominant negative NF-YA construct (Δ 4YA13m29) encodes for a dominant negative NF-YA (421) and was provided by Dr. Mantovani (University of Milan, Milan, Italy Cdc25A promoter variants, pcdc25A-2 (-209/+129), pcdc25A-3 (-184/+129), pcdc25A-4 (-31/+129), pcdc25A-5 (-151/-12), and pcdc25A-6 (-184/-65), were made by PCR amplification using pcdc25A-1 as template (Table 2-1). The PCR products were purified and ligated into pGL2 basic vector (Promega Corp.) between XhoI and HindIII polylinker sites. Site-directed mutagenesis was performed using the two-step overlap extension PCR method (Table 2-1). PCR primers were synthesized by Genosys/Sigma (The Woodlands, TX). All ligation products were transformed into competent Escherichia coli cells. Plasmids were isolated, and clones were confirmed by DNA sequencing (Gene Technologies Laboratory, Texas A&M University).

Table 2-1 Summary of primers for generating variant constructs of pcdc25A

	Primers[a]	Template
pcdc25A-2	Forward: 5′-GTA TCT CGA GCT CTT CTG CTC TGG GCT-3′	pcdc25A-1
	Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	
pcdc25A-3	Forward: 5′-GTA GCT CGA GTT CTG AFGA GCC GAT GAC CTG-3′	pcdc25A-1
	Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	
pcdc25A-4	Forward: 5′-GTA TCT CGA GAG CAG CTG GCC CCA CTG A-3′	pcdc25A-1
	Reverse: 5′-CTT TAT GTT TTT GGC GTC TTC CA-3′	
pcdc25A-5	Forward: 5′-GCT CGA GAG CCG CTT TCT TCT TCC CCT CT-3′	pcdc25A-1
	Reverse: 5′-GAT AAG CTT CTC AGT GGG GCC AGC TGC T-3′	
pcdc25A-6	Forward: 5′-GTA GCT CGA GTT CTG AFGA GCC GAT GAC CTGm3′	pcdc25A-1
	Reverse: 5′-CCG CAA GCT TGA ATC CAC CAA TCA GTA AGC-3′	
pcdc25A-1m1	5′-CGC CCG GCT GGG **TT**C GAG GTA-3′	pcdc25A-1
pcdc25A-1m2	5′-CTG CTC TGG GCT C**TT** CCC CCT TC-3′	pcdc25A-1
pcdc25A-1m3	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1
pcdc25A-1m12	5′-CTG CTC TGG GCT C**TT** CCC CCT TC-3′	pcdc25A-1m1
pcdc25A-1m13	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1m2
pcdc25A-1m23	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-1m2
pcdc25A-1m123	5′-CGC CCG GCT GGG **TT**C GAG GTA-3′	pcdc25A-1m23
pcdc25A-5m1	5′-CTA GGA AAG GGG **TT**C GGG GCA G-3′	pcdc25A-5
pcdc25A-5m2	5′-GAT TCC GT**A A**GG CGC CAA C-3′	pcdc25A-5
pcdc25A-5m3	5′-GAT TCC GT**A A**GG CGC CAA C-3′	pcdc25A-5m1
pcdc25A-5m4	5′-GTT GCT T**AC** TGA TAC GTG GAT TCC-3′	pcdc25A-5
	5′-CCT CTC ATT G**TA** CCA GCC TAG CTG-3′	

[a] Mutations are underlined and substituted bases are indicated in bold.

2.2.2 PIAS3 experiment

TAF1-ERα and Null-ERα expression plasmids were provided by Dr. D. McDonnell (Duke University, Durham, NC). The human ER deletion construct 19c-ERα was provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France). The $pERE_3$ reporter containing three consensus ERE sites linked to a luciferase gene was created by cloning an oligonucleotide with three ERE elements into BamHI-HindIII cut pXP-2 plasmid (408). The wild-type mouse PIAS3 expression vector was originally provided by Dr. Olli A. Jänne (Institute of Biomedicine University of Helsinki FIN-00014 Helsinki, Finland) and used as the PCR template for further cloning. The PIAS3 expression plasmids used in the transfection assay including WT and deletion mutants (PAIS3#2~6) were generated by PCR amplification and ligated into pCDNA3.1/His/Myc vector (Invitrogen). For mammalian two-hybrid assay, the expression plasmids of GAL4-DBD-ERα chimeras including pM-ER, pM-ER (A/B) and pM-ER (C/F) were made by Dr. B. Saville in this lab as previously described (422). The VP-16-PIAS3 chimera expression plasmid was generated by PCR amplification and ligted into pACT vector (Promega). Oligonucleotide primers used for in this study are listed in Table 2-2.

Table 2-2 Summary of primers used for cloning the PIAS3 constructs

Clones	Primers
WT (PIAS3#1)	F, 5’-GTA CTG GTC GAC TCT AGA GTG ATG AGT TTC CGA GTG TCT-3’ R, 5'-GTA CTG GCG GCC GCG TCC AAG GAA ATG ACG TCT-3'
PIAS3#2	F, 5’-GTA CTG GTC GAC TCT AGA GTG ATG AGT TTC CGA GTG TCT-3’ R, 5'-GTA CTA CTG GCG GCC GCA GCC ACT TCA CTG TCG GGG T-3'
PIAS3#3	F, 5'-GTA CTG GTC GAC TCT AGA ACT ACA AGT CTC CGG GTG T-3' R, 5'-GTA CTG GCG GCC GCG TCC AAG GAA ATG ACG TCT-3'
PIAS3#4	F, 5'-GTA CTG GTC GAC TCT AGA ACT ACA AGT CTC CGG GTG T-3' R, 5’-GTA CTG GCG GCC GCT CCC TCC TGG ACT GCG CTG TAC T-3’
PIAS3#5	F, 5’-GTA CTG GTC GAC TCT AGA ATT CAG CCA GAG AGT AAG AA-3’ R, 5'-GTA CTG GCG GCC GCG TCC AAG GAA ATG ACG TCT-3'
PIAS3#6	F’ 5’-GTA CTG GTC GAC TCT AGA TTG CCC CCC ACC AAG AA-3’ R, 5'-GTA CTG GCG GCC GCG TCC AAG GAA ATG ACG TCT-3'
PIAS3#1 Δ393-416	F, 5’-AGC GCA GTC CAG GAG GGA GTC GAC CCC AC-3’ R, 5’-GCA GTG CTT CTT GGT GGG GTC GAC TCC C-3’

2.3 Transient transfection and luciferase assay

2.3.1 Cdc25A experiment

For transfection experiments, 2255,000 ZR-75 cells were initially seeded in 12-well plates. Twenty-four h after seeding, ZR-75 cells were transfected by the calcium phosphate method with Cdc25A promoter-luciferase reporter constructs, ERα expression vector and pCDNA3/*His*/*lacZ* (Invitrogen) that was used as a standard reference control plasmid for determining transfection efficiencies. After 5 h, cells were shocked with 25% glycerol and washed with PBS. Fresh DME/F12 without phenol red and charcoal-stripped FBS containing DMSO or 10 nM E2 in DMSO were added to the cells and incubated for 24 h.

2.3.2 PIAS3 experiment

MCF-7, HeLa, COS-7 and ZR-75 cells were seeded in DME/F-12 medium without phenol red containing 2.5 % dextran/charcoal-stripped FBS. After 24 h cells were transfected with GeneJuice transfection reagent (Novagen, Madison, WI) according to manufacture's recommendation. Five hours after transfection, cells were replaced with fresh DME/F12 without phenol red and treated with DMSO or 10 nM E2 for 36 h.

For transient transfection of siRNA, MCF-7 cells were seeded in DME/F-12 medium without phenol red containing 2.5 % dextran/charcoal-stripped FBS. At the same time, cells were transfected with siRNA of PIAS3 (5nM, final concentration) using siPORT^{TM} NeoFx^{TM} transfection reagent (Ambion).

Cells from each experiment were then harvested in 100 µl of 1X Reporter lysis buffer (Promega). Luciferase assays were performed on 30 µl of the cell extract using the Luciferase assay system (Promega). Light emission was detected on a Lumicount luminometer (Packard, Meriden, CT). β-Galactosidase assays were performed on 20 µl of cell extract using the luminescent Galacton-Plus assay kit (Tropix, Bedford, MA). The luciferase activity observed in each treatment group was normalized to β-gal activity obtained from the same sample to correct for transfection efficiencies. Data are

expressed as fold induction (by E2 or other chemicals) compared to the solvent (DMSO) control.

2.4 Western blot assay

Cells were seeded into 60 mm tissue culture plates in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS. After 24 h, cells were treated with 10 nM E2 and harvested at designated time points and lysed in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 10% [vol/vol] glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein from each treatment group were boiled in 1x Laemmli buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 175 mM β-mercaptoethenol), separated by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with Blotto (5% milk, Tris-buffered saline [10 mM Tris-HCl, pH 8.0, 150 mM NaCl], and 0.05% Tween 20) and probed with primary antibodies. Following incubation with peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Perkin Elmer Foster City, CA).

2.5 Nuclear extract preparation and EMSA

Cells were seeded in 100 mm tissue culture plates using DME/F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were treated for 1 h with DMSO or 10 nM E2. Nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. Nuclear extracts obtained from different treatment groups were incubated for 20 min in HEGD buffer with poly-(dI-dC), unlabeled oligonucleotides or antibodies for supershift assays. The mixture was then incubated for additional 20 min after addition of ^{32}P-labeled oligonucleotide. Reaction mixtures were separated on 5% polyacrylamide gels (acrylamide:bis-acrylamide 30:0.8) at 140 V in 1X TBE (0.09 M Tris-HCl, 0.09 M boric acid and 2 mM EDTA, pH 8.3). Gels were dried and protein-DNA complexes were

visualized using a Storm 860 instrument (Amersham Biosciences, Piscataway, NJ). Oligonucleotides used for EMSA in this study are summarized as follows (mutations are underlined and substituted bases are indicated in bold).

Sp1	5′-ATT CGA TCG GGG CGG GGC GAG C-3′
Cdc25A	5′-ACT AGG AAA GGG GGG CGG GGC AGC A-3′
Cdc25A mutant	′-CTA GGA AAG GGG **TT**C GGG GCA G-3′

2.6 RT-PCR assay

Total RNA was extracted using Nucleospin RNA purification kit (BD Biosciences Clontech), following the manufacturer's instructions. An aliquot of 750 ng RNA was used as the template for cDNA synthesis by incubating with oligo-d(T) primer and multiscribe reverse transcriptase (Perkin Elmer) at 48°C for 40 min. PCR amplification was performed with Taq PCR Master Mix (Promega, Madison, WI). The following conditions were used for the PCR assays: one cycle of 2 min at 95°C; 34 cycles of 30 sec at 95°C; 30 sec at 57.5°C; 1 min at 72°C; one cycle of 5 min at 72°C. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Oligonucleotide primers used for PCR in this study include the following:

Cdc25A	F' 5'-AGC CCC AAA GAG TCA ACT AAT CCA GA-3'
	R' 5'-CCG GTA GCT AGG GGG CTC ACA-3'
GSα	F' 5'-GTG ATC AAG CAG GCT GAC TAT-3'
	R' 5'-GCT GCT GGC CAC CAC GAA GAT GAT-3'

2.7 Coimmunoprecipitation assay

COS-7 cells were seeded into 60 mm tissue culture plates in phenol red-free DME/F-12 medium containing 2.5% charcoal-stripped FBS. After 24 h., transient transfections were performed by using GeneJuice transfection reagent (Novagen) according to the manufacturer's protocol. After 4-6 h., transfected cells were treated with 10 nM E2 for 24h. Cells were harvested and lysed by using 1 ml of RIPA buffer (1x PBS, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF in isopropanol, aprotinin, 100 mM sodium orthovanadate), and cellular debris was removed by centrifugation at 10,000xg for 10 min at 4ºC. The supernatant was transferred to a fresh microcentrifuge tube and precleared by adding 20 µl of protein A-agarose conjugate slurry (Sigma) and incubated at 4ºC for 1h. After centrifugation for 1 min, the supernatant was transferred to another new microcentrifuge tube, and 2.5 µg of rabbit polyclonal anti-ERα antibody (Santa Cruz) was added and incubated at 4 ºC for 1 hr. After incubation, 20 µl of protein A-agarose conjugate slurry (Sigma) was added and incubated at 4ºC for another 1 h. The immunoprecipitate was collected by centrifugation, gently washed with 500 µl RIPA buffer (3X), and resuspended and denatured in 50 µl of 2x Laemmli buffer. The immunoprecipitated sample was analyzed in a western blot assay.

2.8 Chromatin immunoprecipitation assay (ChIP)

ZR-75 cells (2×10^7 cells) were treated with DMSO (time 0) or 10 nM 17β -estradiol (E2) for varying times. Cells were then fixed with 1.5% formaldehyde, and the crosslinking reaction was stopped by addition of 0.125 M glycine. Nuclei were collected, and sonicated to desired length (500~1,000 bp) of chromatin. The chromatin was pre-cleared by addition of protein A-conjugated beads (Upstate), and incubation at 4°C for 1 h with gentle agitation. The pre-cleared chromatin was immunoprecipitated with antibodies (Santa Cruz Biotechnology) to Sp1, ERα, NF-Y, and E2F1 at 4°C overnight, together with protein A-conjugated beads. The beads were then extensively washed, and protein-DNA crosslinks were reversed. PCR was performed with the purified DNA and

the following primers: (1) Cdc25A forward primers, 5′-CTT CTG AGA GCC GAT GAC CT-3′; reverse primer, 5′-CAC CTC TTA CCC AGG CTG TC-3′; amplifying a 225 bp region of the human Cdc25A promoter from -186 to +39; (2) CNAP1 forward primers (Activemotif), 5′-ATG GTT GCC ACT GGG GAT CT-3′; reverse primer, 5′ -TGC CAA AGC CTA GGG GAA GA-3′; amplifying a 174 bp region of the CNAP1 exon; (3) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primers (Activemotif), 5′-TAC TAG CGG TTT TAC GGG CG-3′; reverse primer, 5′-TCG AAC AGG AG GAG CAG AGA GCG A-3′; amplifying a 167 bp region of human GAPDH promoter. PCR products were resolved on a 2% agarose gel.

2.9 Real-time PCR.

For experiments involving siRNA, pancreatic cancer cells were transfected as described previously. Total RNA was isolated using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was eluted with 30 μL RNase-free water and stored at −80 °C. RNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's protocol. PCR was carried out using SYBR Green PCR Master Mix from PE Applied Biosystems (Warrington, UK) on an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The 20-μL final volume contained 0.5 μM of each primer and 2 μL cDNA template. GAPDH was used as an exogenous control to compare the relative amount of target gene in different samples. The PCR profile was as follows: 1 cycle of 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The comparative CT method was used for relative quantitation of samples. Primers (QuantiTect assays) were purchased from Qiagen (Valencia, CA). The following primers were used:

pS2 (F): 5′- TTG GAG AAG GAA GCT GGA TGG -3′
pS2 (R): 5′- ACC ACA ATT CTG TCT TTC ACG G -3′

2.10 Statistical analysis

Statistical significance was determined by ANOVA and Student's t-test, and the levels of probability are noted. The results are expressed as means ± SD for at least three separate (replicate) experiments for each treatment.

CHAPTER III
RESULTS

3.1 Cdc25A is activated by E2 in ZR-75 cells

3.1.1 Deletion and mutational analysis of the Cdc25A gene promoter

Previous studies show that E2 induced Cdc25A mRNA and protein levels in MCF-7 cells and the antiestrogen ICI182780 inhibited the hormone-induced response (423,424). Results in Figure 3-1 show that E2 also induced Cdc25A mRNA levels in ZR-75 cells, and a two-fold or greater increase was observed from 6 to 24 h after treatment. The -460 to +129 region of the Cdc25A promoter contains multiple GC-rich motifs, two CCAAT motifs, and two E2F-1 binding sites (Fig. 3-2A) (424). ZR-75 cells were transfected with pcdc25A-1 which contains the -460 to +129 promoter insert and E2 induced luciferase activity. The deletion constructs pcdc25A-2, pcdc25A-3, and pcdc25A-4 containing the -209 to +129, -184 to +129, and -31 to +129 region, respectively, of the Cdc25A promoter were also transfected into ZR-75 cells, and E2 induced activity in cells transfected with the former two constructs. The results show that basal activity was decreased approximately 40-50% after deletion of the upstream GC-rich site (#1), whereas deletion of GC-rich site #2 did not affect activity. Subsequent deletion of the -184 to -31 region of the promoter resulted in almost complete loss of basal and hormone-induced activity, suggesting that E2-responsiveness was associated with the GC-rich, CCAAT, and E2F-1 binding sites within this region of the Cdc25A promoter. pcdc25A-5 was also highly E2-responsive in transient transfection assays confirming that the 3′ +129 to -11 region was not required for E2-induced transactivation. Thus, the -151 to -12 region of the Cdc25A promoter was the minimal sequence required for E2-responsiveness, but this does not exclude hormone-responsiveness of other upstream (5′) cis-elements. Results in Figure 3-2B show that E2 induces transactivation in cells transfected with pcdc25A-1 and pcdc25A-5, and the hormone-induced response was significantly inhibited by the antiestrogen ICI 182780. These data confirm the role of E2/ER in mediating activation of Cdc25A.

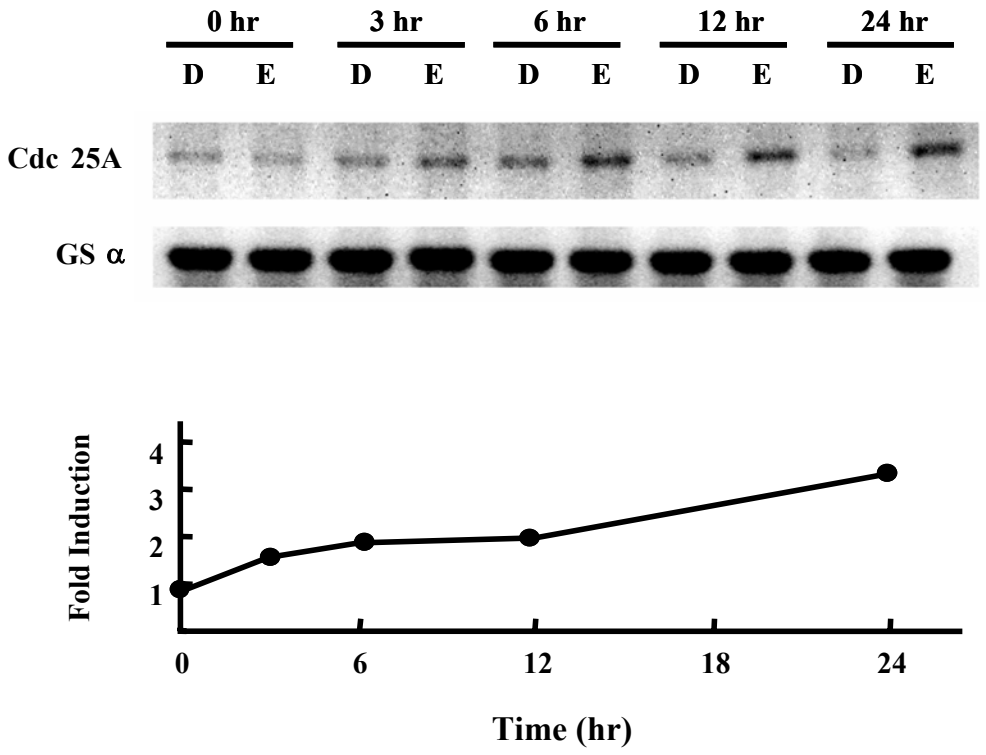

Figure 3-1 Hormone inducibility of Cdc25A in ZR-75 cells. Induction of mRNA levels. ZR-75 cells were treated with DMSO (solvent) or 10 nM E2 for different times, and mRNA levels were determined by RT-PCR analysis.

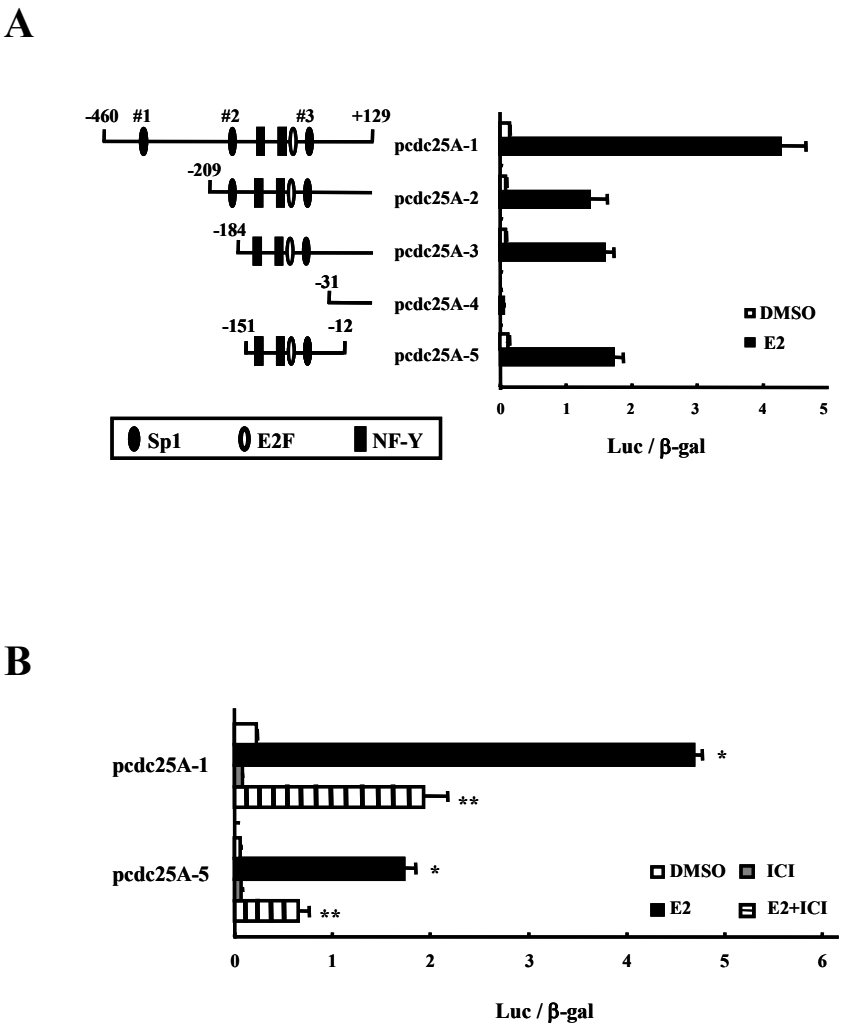

Figure 3-2 Deletion analysis of Cdc25A promoter-reporter (luciferase) constructs. (A) ZR-75 cells were transfected with the various constructs, cells were treated with DMSO or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. (B) Inhibition by ICI 182780. Cells were treated as described in (A); however, ICI 182780 and ICI 182780 plus E2 treatment groups were also added. Results in (A) and (B) are expressed as mean ± SE for at least three replicate determinations for each treatment group and significant ($P < 0.05$) induction by E2 (*) or inhibition by E2 plus ICI 182780 (**) are indicated.

Previous studies in the laboratory have characterized activation of E2-responsive genes through interactions of ERα/Sp1 with GC-rich promoter sequences (420), and the Cdc25A promoter contains three consensus Sp1 binding sites. Results in Figure 3-3A illustrate that mutation of one or more of the three GC-rich motifs at -384, -191, and -39 decreases hormone-responsiveness of several constructs compared to that observed in cells transfected with pcdc25A-1. These results suggest that hormone-dependent activation of ERα/Sp1 plays a role in mediating induction of Cdc25A by E2, but other pathways also contribute to this response. Previous studies have demonstrated that ERα /Sp1-mediated transactivation, through interaction with GC-rich *cis*-elements, can also be observed for ERα11C/Sp1 in which the DNA binding domain of ERα has been deleted (335). Figure 3-3B compares hormone-induced transactivation in ZR-75 cells cotransfected with pcdc25A-1 plus wild-type human ERα or ERα11C, and the induction of luciferase activity by E2 in cells cotransfected with the ERα deletion constructs confirms that the ERα/Sp1 pathway plays a role in hormonal regulation Cdc25A. Gel mobility shift assays comparing the binding of nuclear extracts from ZR-75 cells to ^{32}P-labeled consensus Sp1 and Cdc25A-Sp1 (-52 to -28) oligonucleotides show a similar pattern of protein-DNA interactions (Fig. 3-4). Radiolabeled GC-rich (Sp1) and Cdc25A oligonucleotides alone did not give retarded bands (lanes 1 and 8); incubation with nuclear extracts gave one major retarded band (lanes 2 and 9) which was supershifted with Sp1 antibody (lanes 3 and 10) and unaffected by non-specific IgG (lanes 4 and 11). Both retarded bands were decreased after competition with 100-fold excess unlabeled Sp1 (lanes 5 and 12) and Cdc25A (lanes 6 and 13) oligonucleotides but not by mutant Cdc25A oligonucleotide (lanes 7 and 14). The role of Sp1 in mediating hormone-induced luciferase activity in cells transfected with pcdc25A-5 was also confirmed by decreased inducibility after cotransfection with dominant negative Sp1 expression plasmid (Fig. 3-5). Approximately 40% of hormone-induced transactivation was observed in replicate studies, thus confirming a role for ERα/Sp1 in mediating activation of Cdc25A.

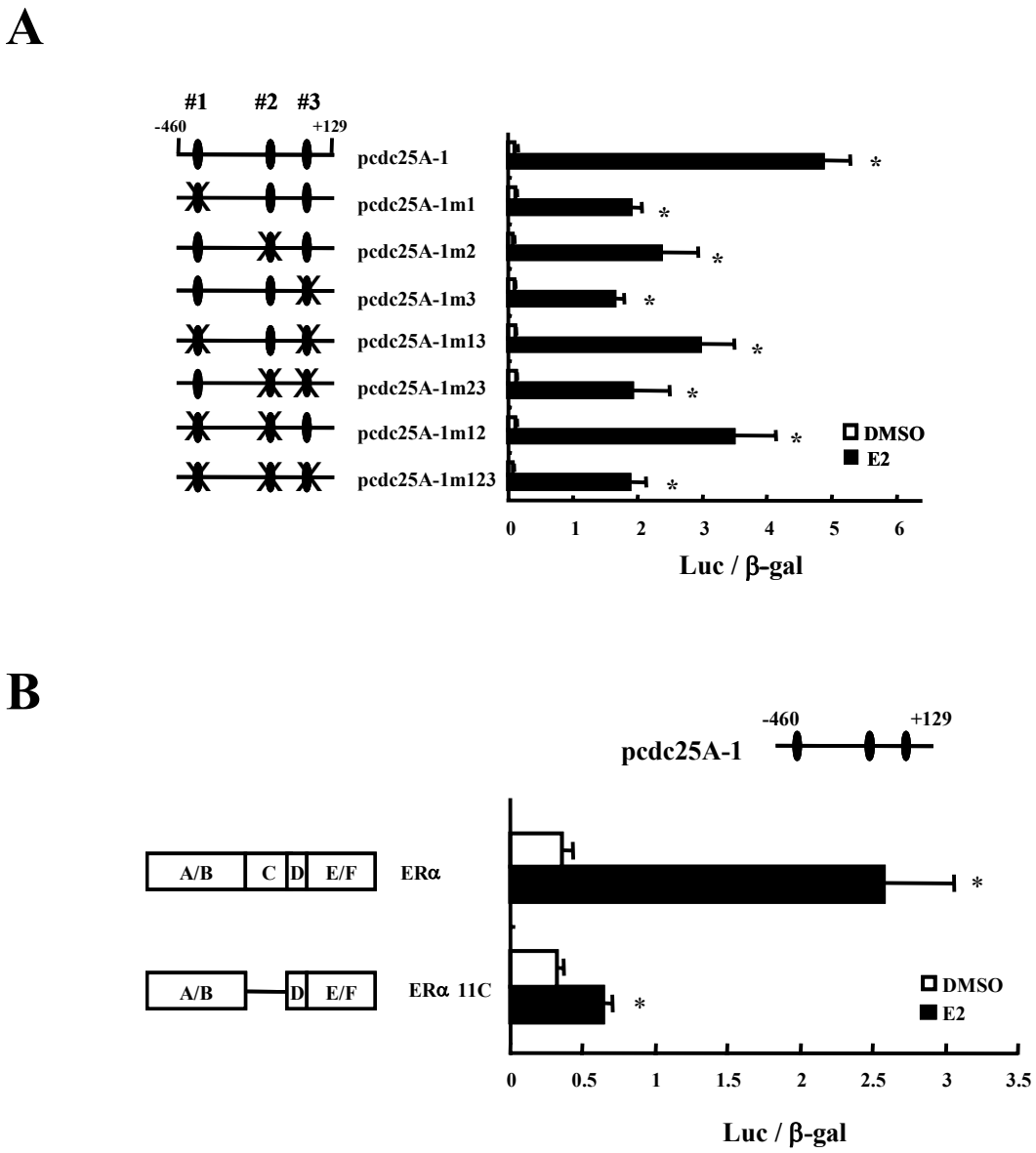

Figure 3-3 Role of ER/Sp1 in mediating activation of Cdc25A. (A) Mutational analysis of GC-rich sites. ZR-75 cells were transfected with pcdc25A-1 or a series of mutant constructs, treated with DMSO or 10 nM E2, and luciferase activity determined as described in the Materials and Methods. (B) Inducibility by ER11C. Cells were treated as described in (A); however, both wild-type ER and ER11C were cotransfected. Results in (A) and (B) are expressed as mean ± SE for three replicate determinations for each treatment group. Significant ($P < 0.05$) induction by E2 (*) is indicated.

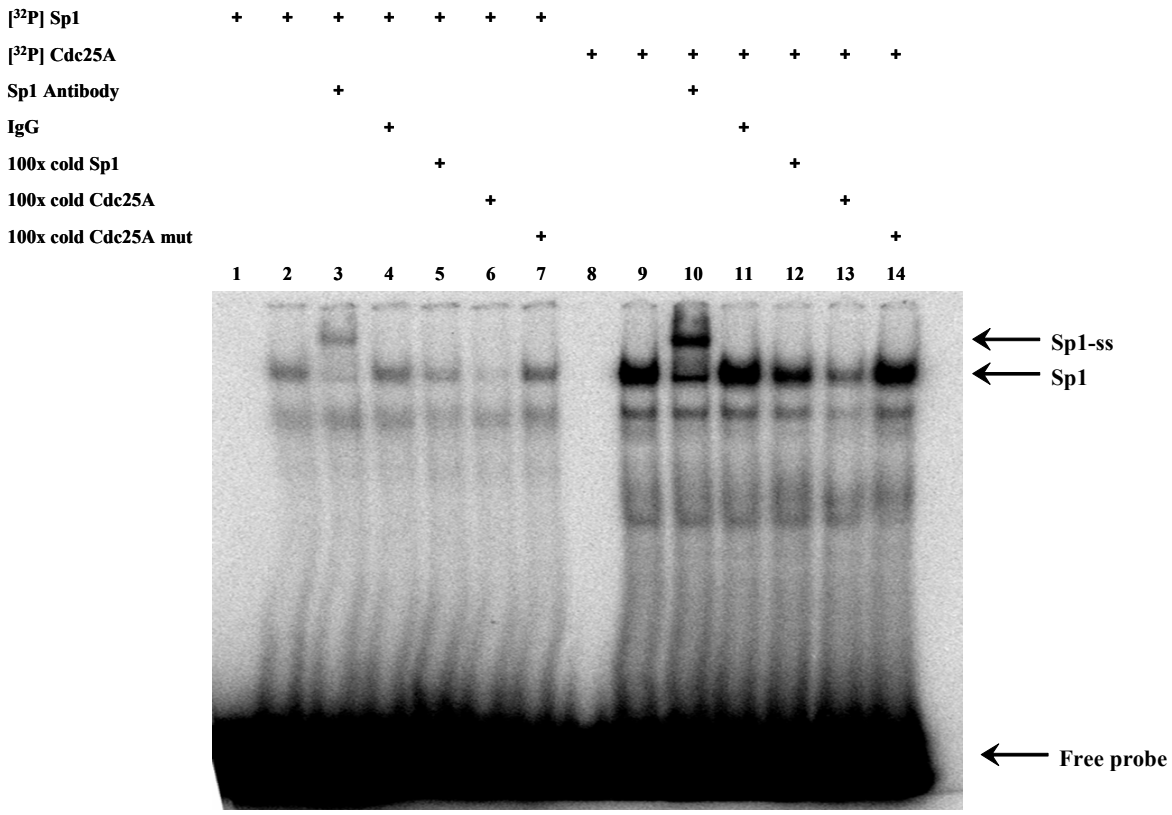

Figure 3-4 Gel mobility shift assay. Nuclear extracts from ZR-75 cells were incubated with 32P-labeled oligonucleotides and other antibodies/ oligonucleotides, and a gel mobility shift assay was carried out as described in the Materials and Methods. Sp1-DNA binding and antibody supershifted complexes are indicated by arrows. Lanes 1 and 8 represent incubation of the free probe alone.

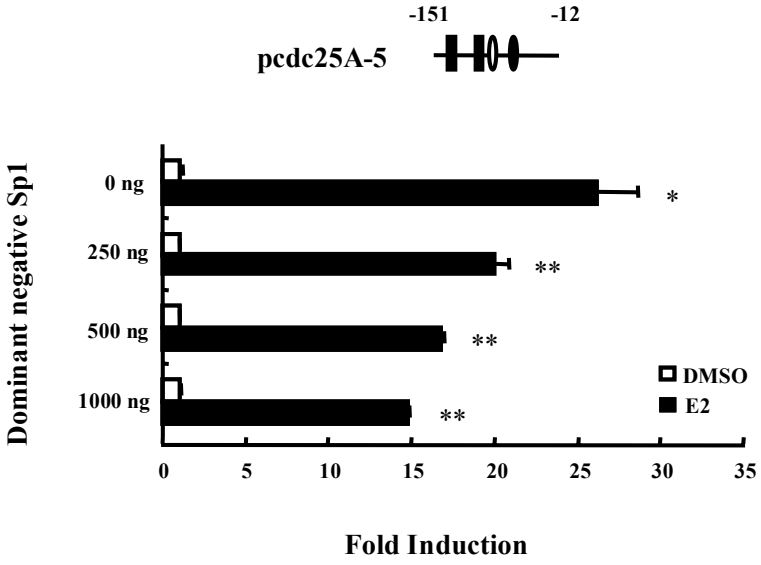

Figure 3-5 Effect of dominant negative Sp1 in ZR-75 cells. Cells were transfected with pcdc25A-5 (containing promoter region from -151 to -12), treated with DMSO or 10 nM E2, and cotransfected with various amount of dominant-negative Sp1 expression plasmid as described in Materials and Methods. dnSp1 (250~1000ng) resulted in significantly (**$p < 0.05$) decreased E2-induced luciferase activity.

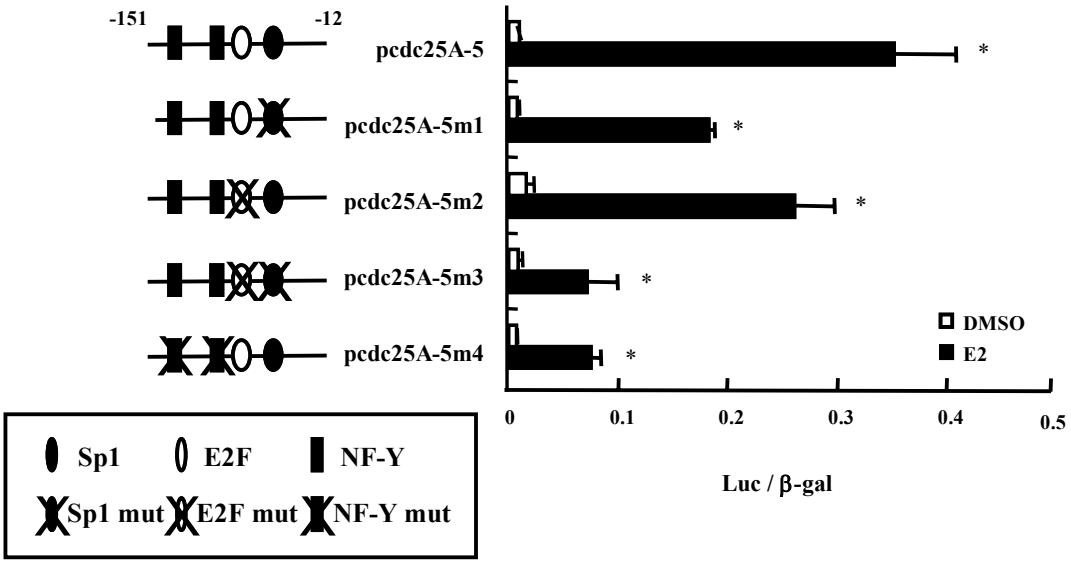

Figure 3-6 Role of CCAAT sites in hormonal activation of Cdc25A. (A) Mutational analysis of -151 to -12 region of the Cdc25A promoter. ZR-75 cells were transfected with the various constructs, treated with DMSO or E2, and luciferase activity determined as described in the Materials and Methods. Results are mean ± SE for three replicate determinations for each treatment group, and significant induction by E2 (*) is indicated.

Mutation analysis of the -151 to -12 region of the promoter was also determined in ZR-75 cells transfected with pcdc25A-5 and four constructs containing mutations of the GC-rich (pcdc25A-5m1), E2F-1 (pcdc25A-5m2), GC-rich and E2F-1 (pcdc25A-5m3), and NFY (pcdc25A-5m4) motifs. E2 induced activity in cells transfected with wild-type and mutant constructs (Fig. 3-6); however, the fold-induction was lower in cells transfected with the mutant plasmids. These results demonstrate that multiple sites in the Cdc25A promoter region contribute to the E2-responsiveness of this gene.

3.1.2 Role of NF-Y and E2F1 in activation of Cdc25A gene expression

The role of NF-YA in mediating activation of Cdc25A by E2 was further investigated (Fig. 3-7) in cells transfected with constructs containing only the CCAAT sites (pcdc25A-6 and pcdc25A-5m3) and a dominant negative expression plasmid for NF-YA (Δ4YA13m29) (335,425). The results showed that dominant negative NF-YA significantly inhibited hormonal activation of both pcdc25A-6 and pcdc25A-5m3. Previous studies in this laboratory confirmed expression of NF-YA in ZR-75 cells and showed that hormonal activation of CCAAT motifs in the E2F-1 gene promoter were due to cAMP/PKA-dependent activation of NF-YA through non-genomic pathways (417,426). Results in Figure 3-8A show that the PKA inhibitor SQ22536 inhibited induction of luciferase activity by E2 in cells transfected with pcdc25A-5m3, and both H8 (adenylcyclase inhibitor) and SQ22356 significantly inhibited hormonal activation of chimeric GAL4-NF-YA in ZR-75 cells transfected with expression plasmids for GAL4-NF-YA and a pGAL4-luc reporter plasmid (Fig. 3-8B). These results suggest that the CCAAT sites within the Cdc25A promoter that bind NF-Y proteins are activated through non-genomic pathways of estrogen action. However, in cells transfected with pcdc25A-1 and pcdc25A-5, E2-induced activity was inhibited only12-15% in cells cotreated with 100 μM SQ22536.

The E2F1 binding site at -63 is another potential E2-responsive motif in the Cdc25A promoter since E2F1 is induced by E2 in MCF-7 and ZR-75 cells (427), and E2 also induces Rb protein phosphorylation which results in derepression of E2F1. E2 induces transactivation in ZR-75 cells transfected with pcdc25A-5, and cotransfection with dominant negative expression plasmids for the E2F1 binding partner DP1 (DPΔ103-126

and DRΔ127-411) or E2F1 (E132) (427) significantly decreased transactivation (Fig. 3-9A). A second E2F1 motif at -3 in the Cdc25A promoter is present in pcdc25A-4 which exhibited low activity (Fig. 3-2A) but is hormone inducible (approximately 2.5-fold). Results in Figure 3-9B show that the fold-induction of luciferase activity by E2 in cells transfected with pcdc25A-4 was also significantly inhibited after cotransfection of the dominant negative DPΔ103-126, DPΔ127-411, and E132 expression plasmids. However, these plasmids also significantly altered basal activity in solvent (DMSO)-treated cells. ChIP assay with primers targeted to the proximal region of the Cdc25A promoter (Fig. 3-10A) confirmed that E2F1 and NF-YA were constitutively bound to the promoter and ERα binding is increased after treatment with E2. This is consistent with association of ERα which interacts with promoter bound Sp1. Results obtained using immunoprecipitation with TFIIB antibodies show that TFIIB binds to the GAPDH gene promoter but not exon 1 of CNAP1 as previously described (428), and this serves as a positive control for the ChIP assay. These results demonstrate that hormone-dependent induction of Cdc25A gene expression in ZR-75 cells requires activation of both genomic and non-genomic pathways of estrogen action. The multiple E2-responsive cis-elements identified in this study demonstrate the complexity of hormonal regulation of Cdc25A, and it is possible that other promoter regions and interactions between DNA-bound transcription factors may also be important.

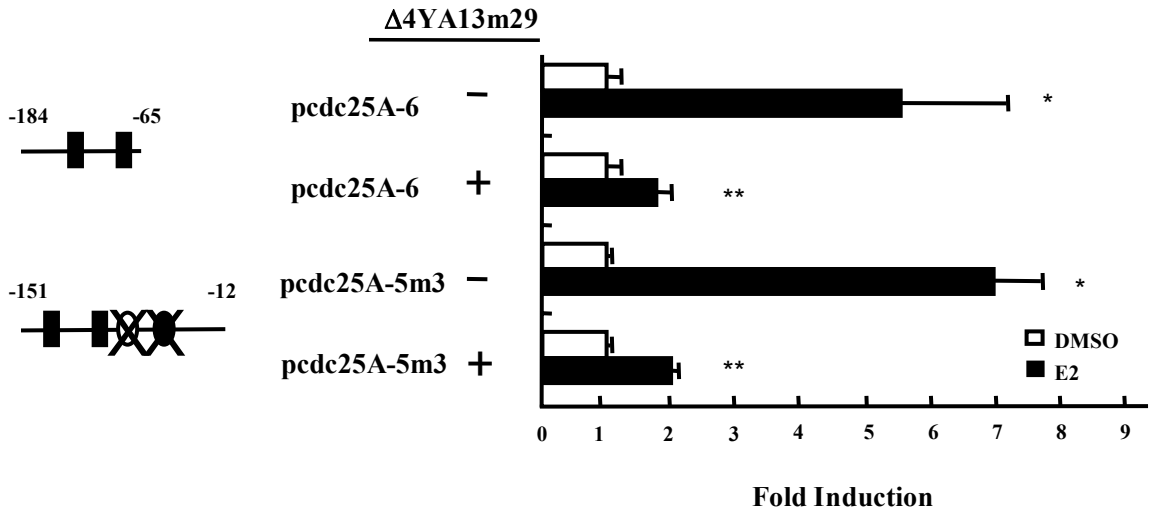

Figure 3-7 Effects of dominant negative 4YA13m29 expression. Cells were transfected with the various constructs, treated with DMSO or E2, and luciferase activity determined as described in the Materials and Methods and the effects of 4YA13m29 (dominant negative NF-YA) expression were also determined. Results are expressed as mean ± SE for three replicate determinations for each treatment group, and significant induction by E2 (*) or inhibition of the induced response (**) are indicated.

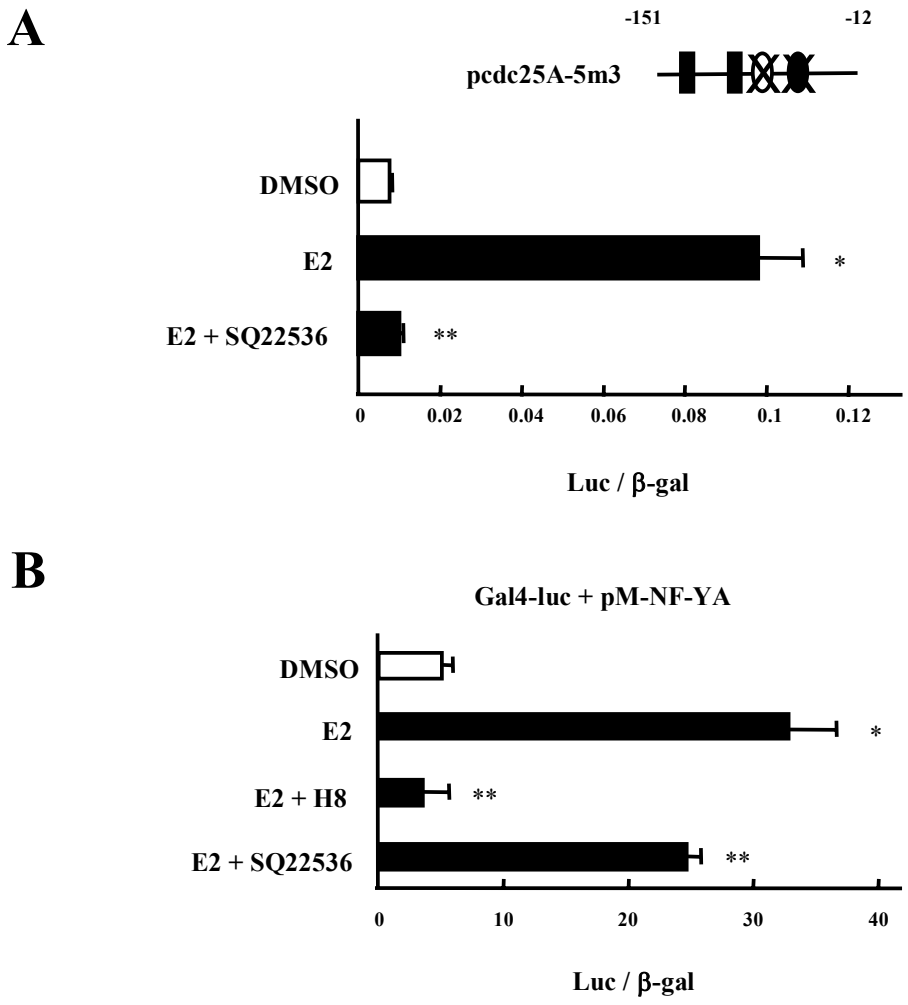

Figure 3-8 Effect of kinase inhibitors. (A) Inhibition of transactivation by SQ22536. ZR-75 cells were transfected with pcdc25A-5m3 as described in Material and Methods and treated with DMSO, E2, and E2 plus SQ22536. SQ22536 alone did not affect activity (data not shown). (B) Inhibition by H8 and SQ22536. ZR-75 cells were transfected with GAL4-luc/pM-NF-YA as described in Material and Methods and treated with DMSO, E2, E2 plus H8, and E2 plus SQ22536. H8 plus SQ22536 alone did not affect activity (data not shown). Results in (A-B) are mean ± SE for three replicate determinations for each treatment group, and significant induction by E2 (*) or inhibition of the induced response (**) are indicated.

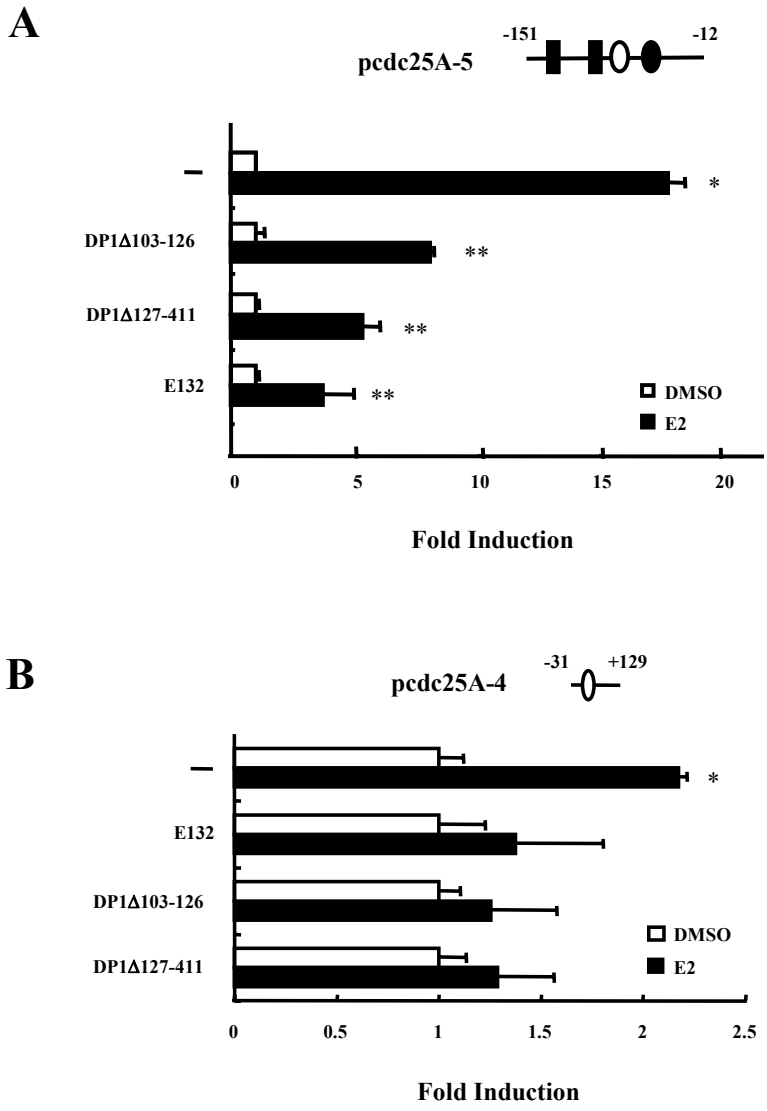

Figure 3-9 Role of E2F1 in hormone activation of Cdc25A. (A) pcdc25A-5 and (B) pcdc25A-4 were transfected in ZR-75 cells, treated with DMSO or 10 nM E2, cotransfected with dominant negative expression plasmids for E2F1 (E132) or DP1 (DP1103-126; DP1127-411), and luciferase activity determined as described in the Materials and Methods. Results are expressed as mean ± SE for three replicate determinations for each treatment group, and significant ($P < 0.05$) induction by E2 (*) and inhibition of this response (**) are indicated.

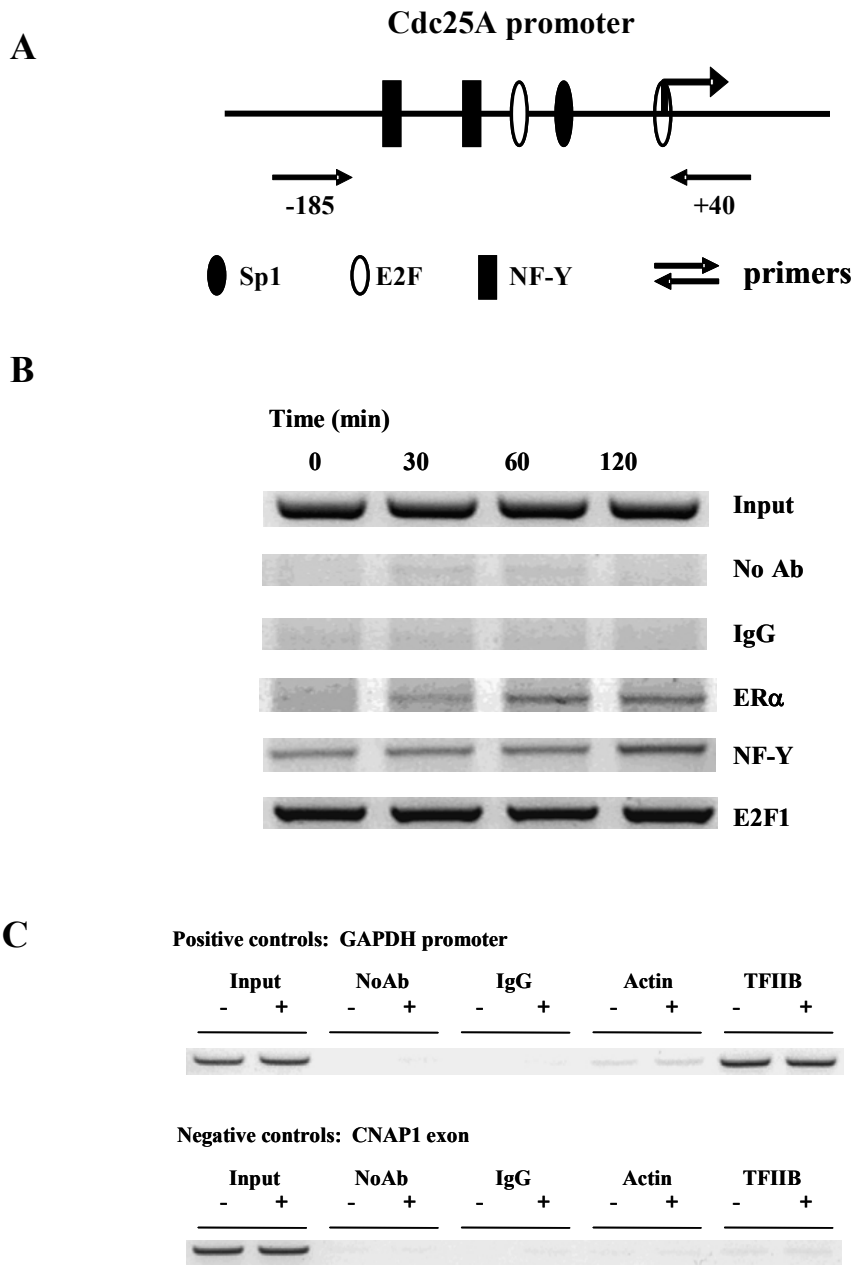

Figure 3-10 Transcription factor binding to the Cdc25A promoter. (A) ZR-75 cells were treated with DMSO (0 time) or 10 nM E2 for 30, 60, or 120 min, and the ChIP assay was carried out essentially as described in the Materials and Methods using antibodies to ERα, NF-YA, and E2F1, and non-specific IgG. Sp1 antibodies also showed consistent binding to the Cdc25A promoter over the 0-120 min time period (data not shown). The primers amplified a 225 bp sequence from -186 to +39 in the Cdc25A promoter. (B) TFIIB promoter interactions. (C) Control ChIP analysis showed that TFIIB specifically interacts with the GAPDH but not CNAP1 promoters as previously described (429).

3.2 PIAS3 coactivates ERα-mediated transactivation

3.2.1 Coactivation of ERα by PIAS3

Coactivation of ERα-dependent transactivation by PIAS3 was initially examined in HeLa cells cotransfected with ERα (414). Cells were transfected with $pERE_3$, which contained three tandem EREs in a minimal TATA-luciferase construct, and ERα expression plasmid and cotransfection with PIAS3 expression plasmid induced a 2 to 3-fold enhancement (i.e coactivation) of E2-induced activity. In my study, coactivation of ERα by PIAS3 was investigated in MCF-7 cells since this will determine coactivation of ERα by PIAS3 in a breast cancer cell context and also facilitate studies on the role endogenous PIAS3 using RNA interference. The results in Figure 3-11 show that E2 induces a 2.3-fold incease in reporter gene activity in MCF-7 cells transfected with 2.5 ng ERα expression plasmid, and cotransfection with 25, 50 and 100 ng PIAS3 expression plasmid resulted in a 3.1-, 4.8- and 24.3-fold enhancement of E2-induced luciferase activity.

We also investigated the coactivation of ERα by endogenous PIAS3 in this cell line. Small inhibitory RNA of PIAS3 was transfected into MCF-7 cells and Western blot analysis of PIAS3 expression in MCF-7 showed that the endogenous PIAS3 protein was decreased after transfection of different amounts of iPIAS3. The effect of iPIAS3 on E2-induced pS2 expression was determined by Real-Time PCR. MCF-7 cells were transfected with iNS (nontargeting siRNAs as a negative control) or iPIAS3 and treated with 10 nM E2. Six h after treatment with E2, the cells were harvested and purifed RNA was analysed by Real-Time PCR. The results show that when MCF-7 cells were transfected with iPIAS3, the E2 induced pS2 gene expression was significantly repressed (Fig. 3-12). These results indicate that PIAS3 is involved in the E2-dependent transactivation of pS2 gene and confirms a role for PIAS3 as an endogenous coactivator of ERα-dependent transactivation.

3.2.2 Coactivation of ERα by PIAS3 deletion mutants

Previous studies showed that PIAS3 can activate Smad-dependent transcription (264). It was also shown that PIAS3 activates Smad-dependent transactivation through its interaction with Smads and p300/CBP and the RING domain in PIAS3 is important for this coactivation and interaction. Another group showed that in yeast two-hybrid screening assay, PIAS3 interacts with TBP (TATA-binding protein) (325-335,430-432). Domains of PIAS3 required

for coactivation of ERα were investigated in MCF-7 and COS-7 cells cotransfected with ERα, $pERE_3$, and wild-type or deletion mutants of PIAS3 (Fig. 3-13). Results show that multiple regions of PIAS3 are required for coactivation of ERα.

The pattern of coactivation of ERα by PIAS3 deletion variants was similar but not identical in MCF-7 (Figure 3-13B) and COS-7 (Figure 3-13C) cells. Deletion of the C-terminal amino acids 274-584 (PIAS3#2, which lose the SAP domain with a LXXLL motif inside) resulted in loss of coactivation in both cell lines whereas the N-terminal deletion mutant (loss of amino acids 1-273) significantly coactivated ERα. Thus the LXXLL motif within the N-terminal SAP domain is not required for coactivation of ERα. PIAS3#4, which only contains the ring finger domain of PIAS3 coactivated ERα and PIAS3#5 containing the acidic and serine-rich regions in the N-terminal region (amino acids 393-584) also coactivated ERα in both cell lines. In contrast, PIAS3#6 which contains only the N-terminal serine-rich domain (amino acids 416-584) coactivate ERα in COS-7 but not MCF-7 cells. Thus PIAS3 coactivates ERα through multiple domains and exhibits some cell context-dependent effects. The role of the acidic region (393-416) of PIAS3 on coactivation of ERα was determined in ZR-75 breast cancer and HeLa cells (Figure 3-14) transfected with $pERE_3$, ERα, wild-type PIAS3#1 or the deletion mutant PIAS3#1Δ393-416. Coactivation was observed in cells transfected with both PIAS3#1 and PIAS3#1Δ393-416 and although lower coactivation was observed in cells transfected with the latter expression plasmid, the results showed that this acidic region was not required for coactivation by PIAS3.

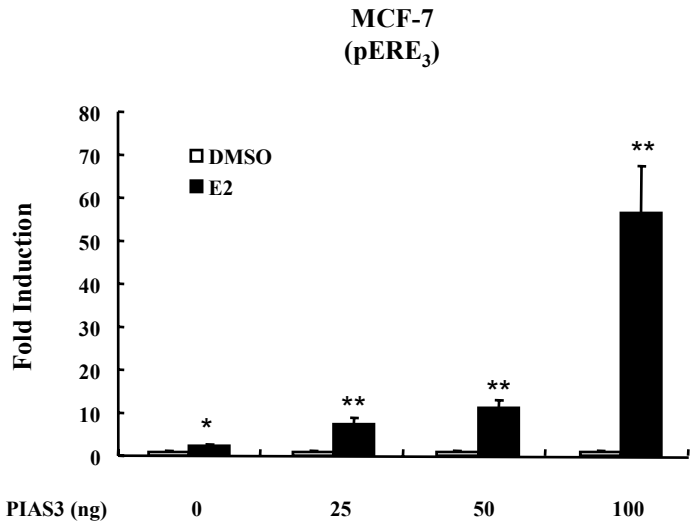

Figure 3-11 Enhancement of ERα-mediated transactivation by PIAS3. MCF-7 cells were transfected with $pERE_3$, ERα, β-galactosidase and increasing amonts of pcDNA3.1-PIAS3 expression plasmid. After transfection, cells were treated with DMSO or 10 nM E2 for 36 h. Luciferase activity was normalized with β-galactosidase activity and results are expressed as fold induction and compared to that observed for DMSO alone. Significant ($p<0.05$) induction by E2 (*) or coactivation by PIAS3 expression plasmids (**) is indicated.

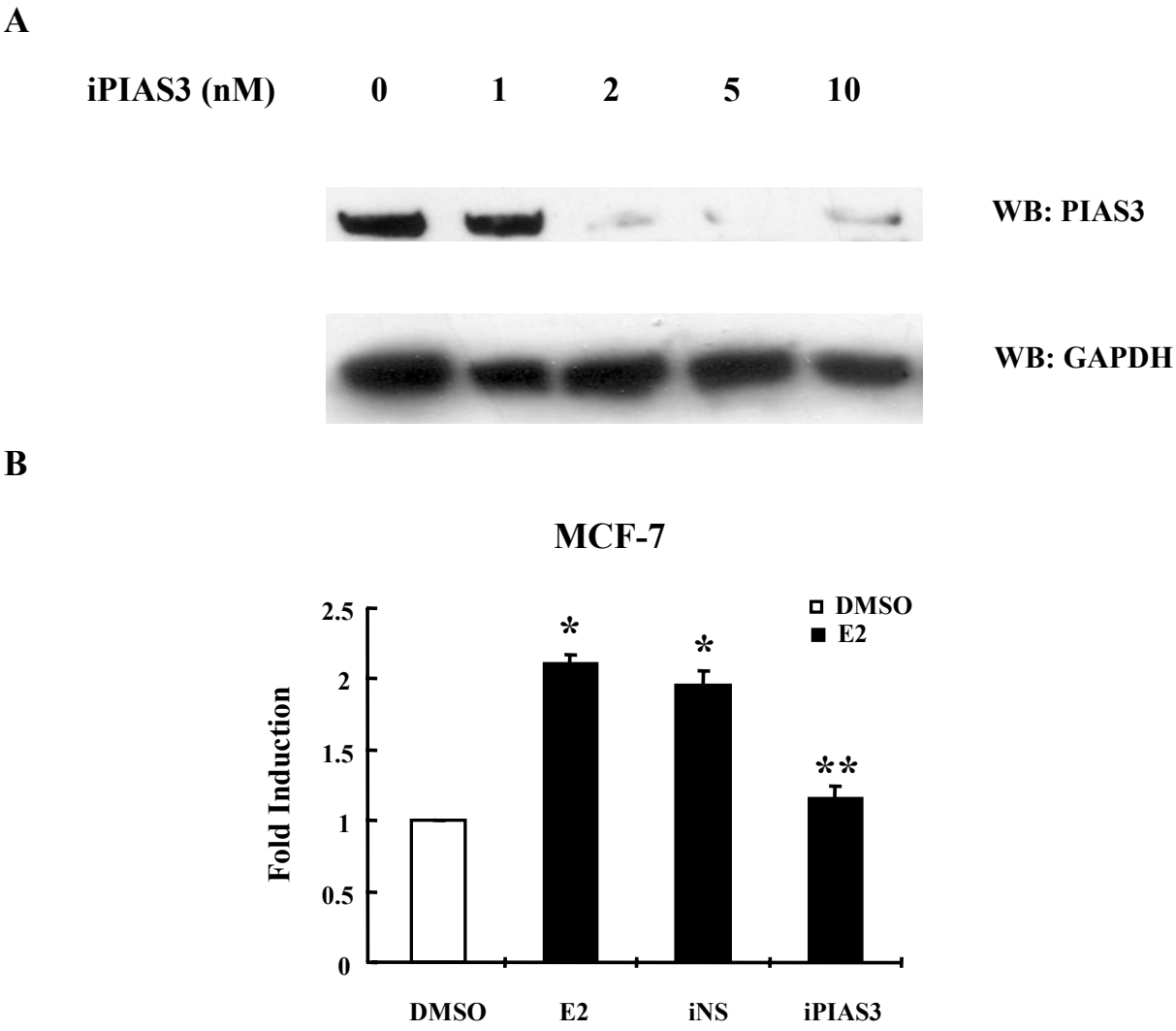

Figure 3-12 Inhibition of PIAS3 expression abolishes the E2-dependent transactivation of the pS2 gene. (A) MCF-7 cells were tranfected with 0, 1, 2, 5, 10 nM (final concentration) of PIAS3 specific RNAi (iPIAS3). After 48 h transfection, cells were harvested and the total protein was extracted and analysed by Western blot using anti-PIAS3 antibody as described in the Material and Methods. (B) MCF-7 cells were transfected with 5 nM of non-specific (control) or PIAS3 specific RNAi. After 48 h cells were then treated with DMSO or E2 for 3 h. Cells were harvested and the total RNA was extracted as described in the Material and Methods. Expression levels of pS2 mRNA was analyzed by Real-Time PCR. Results are expressed as fold-induction compared to that observed for DMSO alone. Significant ($p<0.05$) induction by E2 (*) or repression by inhibitory RNA (**) is indicated.

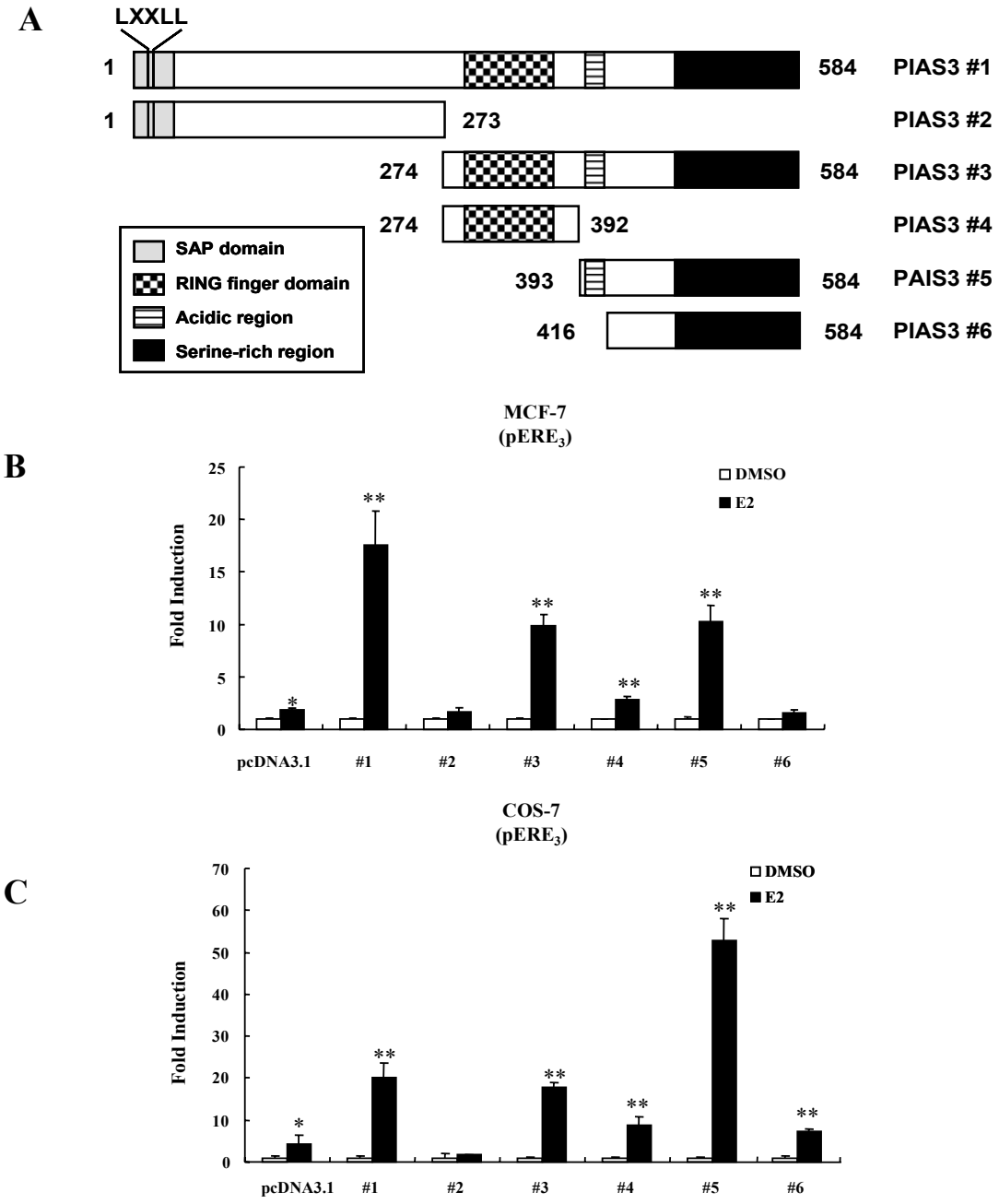

Figure 3-13 Multiple regions of PIAS3 are required for coactivation of ERα. (A) Truncation mutants of PIAS3. Coactivation of ERα by PIAS3 mutants in MCF-7 (B) and COS-7 (C) cells. MCF-7 or COS-7 cells were transfected with $pERE_3$, ERα, β-galactocidase and various truncation mutants of PIAS3 in pCDNA3.1; wild-type PIAS3 expression plasmid was designated PIAS3#1. After transfection cells were treated with DMSO or 10 nM E2 for 36 h. Results are expressed as fold induction of luciferase activity by E2 compared to that observed for DMSO alone. Significant ($p<0.05$) induction by E2 (*) and coactivation by PIAS3 expression plasmids (**) is indicated.

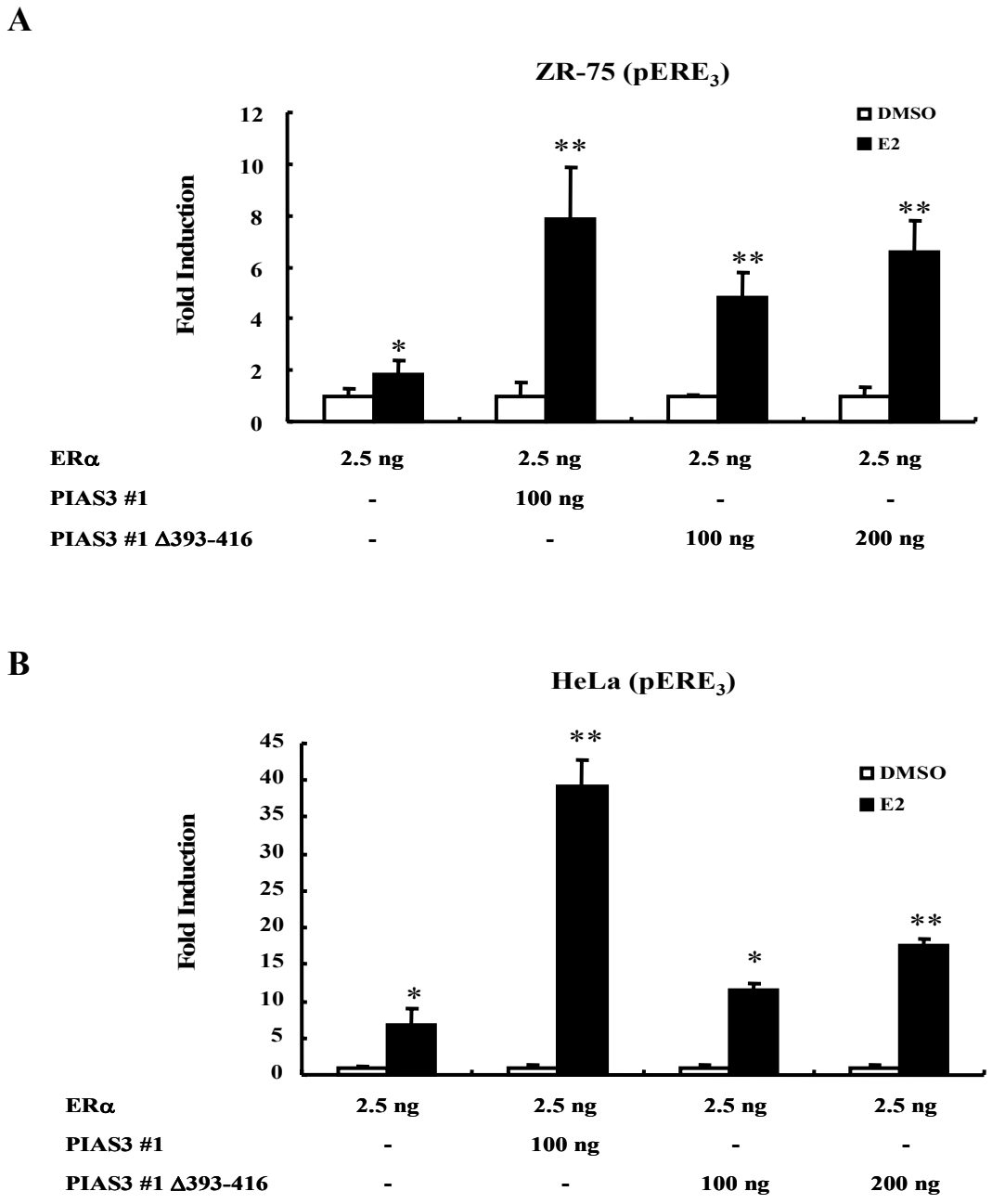

Figure 3-14 Role of the acidic region of PIAS3 for coactivation of ERα. ZR-75 (A) HeLa (B) cells were transfected with pERE$_3$-luc, ERα, β-galactosidase and a truncation mutant (deletion aa 393-416) of PIAS3 (PIAS3#1 Δ393-416) in pCDNA3.1. After transfection cells were treated with DMSO or 10 nM E2 for 36 h. Results are expressed as the fold-induction of luciferase activity by E2 compared to that observed for DMSO alone. Significant ($p<0.05$) induction by E2 (*) or coactivation by PIAS3 expression plasmids (**) is indicated.

3.2.3 Coactivation of variant ERα by PIAS3

ERα contains two major activation domains and we therefore investigated the coactivation activity of PIAS3 with three ERα variants and the results are summarized in Figure 3-15. The TAF1-ERα mutant contains three mutations in helix 12 (D538N, E542Q, and D545N) that block AF2-dependent interaction with coactivators and inactivates AF2-dependent transcriptional activation. 19C-ERα is an A/B domain deletion mutant which lacks AF1. The null-ERα contains mutations on AF2 and deletion of AF1 exhibits minimal hormone responsiveness (291,349,352,423,424).

When HeLa cells were transfected with wild type ERα and $pERE_3$, E2 induced a 43-fold increase in reporter gene activity and cotransfection with 100 ng PAIS3 expression plasmid significantly enhanced E2-induced luciferase activity and coactivated this response by 530-fold. In cells transfected with 19C-ERα and $pERE_3$, E2 induced a 3.4-fold increase in reporter gene activity and after cotransfection with 100 ng PAIS3 expression plasmid the E2-induced luciferase activity waas enhanced 3.3-fold (Fig. 3-15). E2 also induced luciferase activity in HeLa cells transfected with $pERE_3$ and TAF1-ERα; cotransfection with PAIS3 also significantly enhanced E2-induced luciferase activity as shown in Figure 3-15. However, in cells cotransfected $pERE_3$, PIASA3 and null-ERα, only minimal induction by E2 and coactivation by PIAS3 was observed (Fig.3-15). These data suggest that helix 12 on AF2 is not required for coactivation by PIAS3 when the AF1 domain of ERα is intact. However, when the AF1 domain of ERα is deleted (i.e. 19c-ERα), the intact AF2 function is required for coactivation by PIAS3, and mutations of helix 12 amino acids (i.e. null-ERα) resulted in loss of coactivation activity. Thus coactivation of ERα by PIAS3 is associated with multiple domains of ERα but is not dependent on the helix 12 region of ERα which interacts with LXXLL boxes present in many nuclear receptor coactivators including PIAS3. These results are consistent with the coactivation of ERα by PIAS3 deletion mutants in which indicate that the LXXLL box in PIAS3 is not essential for coactivation of ERα (Figure 3-13).

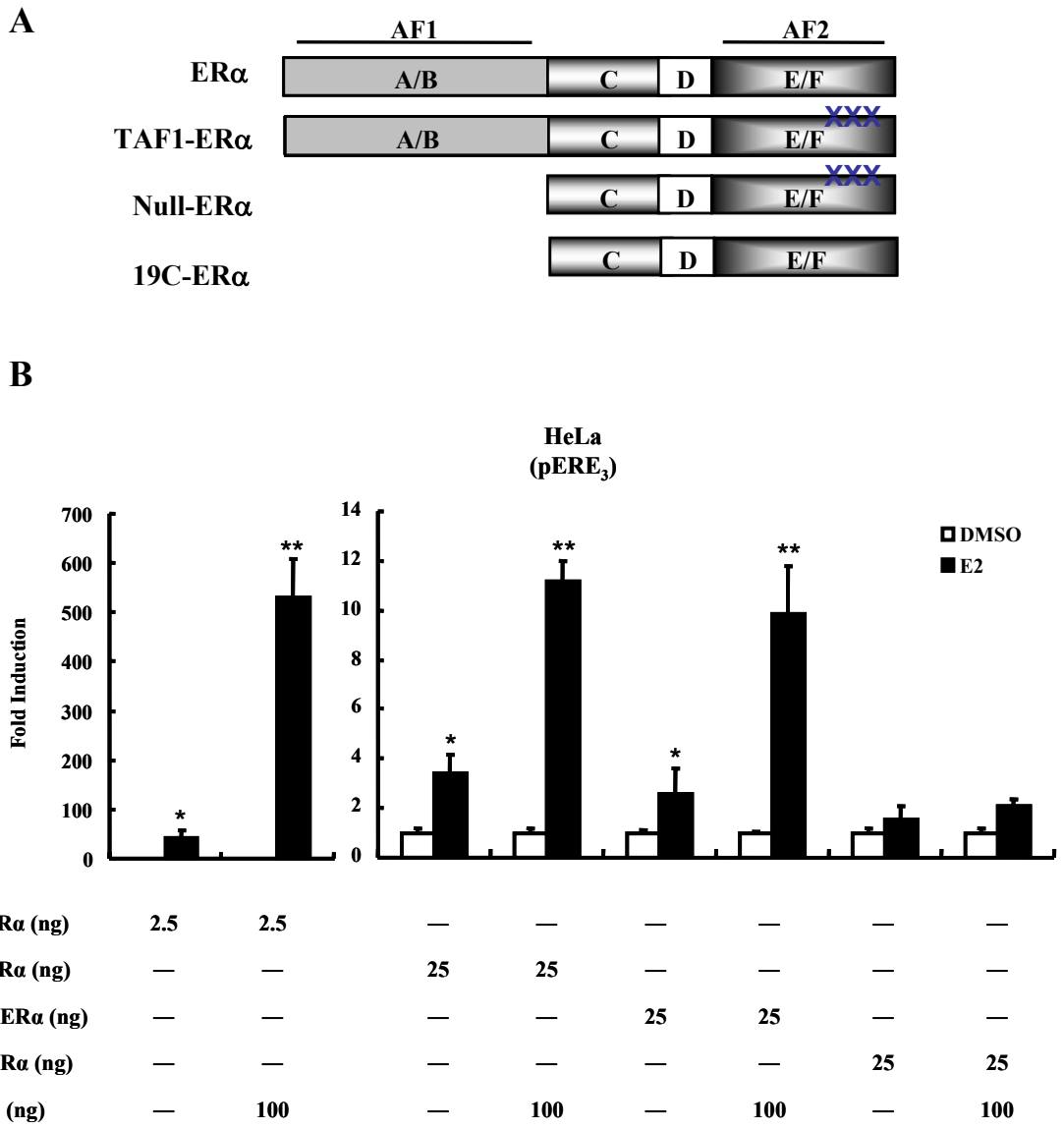

Figure 3-15 Coactivation of wild-type and mutant ERα by PIAS3. A. ERα mutants. The TAF1-ERα mutant contains three mutations on helix 12 of ERα (D542N, E546Q, and D549N). The null-ERα mutant is an A/B domain deletion mutant and also contains three mutations on helix 12. B. Transfection studies. HeLa cells were transfected with $pERE_3$, various ERα mutants, β-galactosidase and PIAS3 expression plasmid. After transfection, cells were treated with DMSO or 10 nM E2 for 36 h. Luciferase activity was normalized with β-galactosidase activity and results are expressed as fold induction of luciferase activity by E2 and compared to that oberseved for DMSO alone. Significant ($p<0.05$) induction by E2 (*) or coactivation by PIAS3 expression plasmids (**) is indicated.

3.2.4 Interactions of ERα with PIAS3

Interactions between PIAS3 and ERα were investigated in a mammalian two-hybrid assay. HeLa cells were transfected with expression vectors for the GAL4 DBD (pM) or the chimeras of DBD fused to ERα (pM-ERα) in the presence of the VP16 activation domain alone (VP16) or VP16 fused to the PIAS3 (VP16–PIAS3), and pGAL45 (five tandem GAL4 response elements linked to a luciferase reporter gene). The results (Fig.3-16 A) show that after transfection with pM-ERα and VP16-PIAS3, E2 significantly induced luciferase activity compared to the control luciferase values obtained in cells transfected with pM in the presence of pM-ERα and VP16. The results show that in the absence of ligand VP16-PIAS3 does not interact with pM-ER and transactivation is E2-dependent. These data confirm interactions of PIAS3 and ERα in a mammalian two-hybrid assay and this interaction is hormone-dependent.

In this study, we have shown that wild-type and some variant PIAS3 constructs coactivate ERα-dependent transactivation. Therefore we also used the mammalian two-hybrid assay to investigate interactions of ERα with wild-type and variant PIAS3 in HeLa cells transfected with VP16-PIAS3 variants and pM-ERα. The results show that luciferase activity was significantly increased by E2 in cells transfected with pM-ERα and VP16-PIAS3 #1 and VP16-PIAS3#3 (Fig. 3-16B), but not with VP16-PIAS3#2 or VP16-PIAS3#4. Thus in the two-hybrid assay, interactions of ERα with PIAS3 may require RING finger domain of PIAS3.

Interactions between ERα and PIAS3 were also investigated in coimmunoprecipitation studies. Myc-tagged full-length and deletion mutants of PIAS3 and ERα were cotransfected in COS-7 cells and treated with 10 nM E2. After 24 h, the cells were lysed and immunoprecipitated with an ERα antibody, and the presence of PAIS3 in the immunoprecipitate was determined by Western blotting with a monoclonal antibody against His-tag. The results (Fig 3-16C) show that wild type PIAS3 is coimmunoprecipitated by ERα antibodies whereas the acidic region - deleted PIAS3 (PIAS3 Δ393-416) was not coimmunoprecipitated with ERα. PIAS3 was not immunoprecipitated by ERα antibodies in cells transfected with either ERα or PIAS3 alone or using control rabbit IgG Ab for

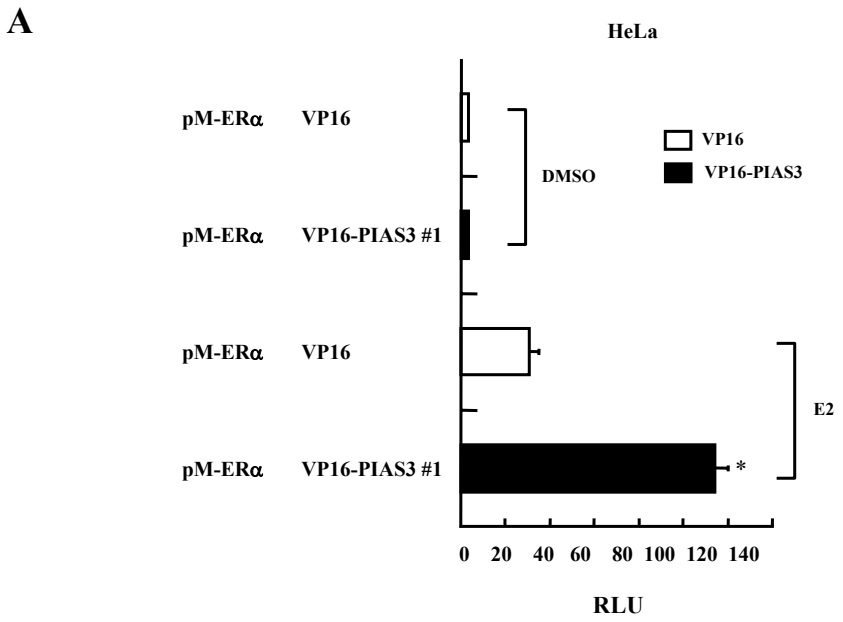

Figure 3-16 Interaction of various PIAS3 deletion mutants with ERα in a mammalian two-hybrid assay. A, B. Mammalian two-hybrid assays in HeLa cells. Hela cells were transfected with 50 ng of β-galactosidase, 125 ng of pGAL4-LUC, 150 ng pVP16 or VP16-PIAS3 wild-type or deletion mutants, and 25 ng of pM-ERα. After transfection cells were treated with DMSO or 10 nM E2 for 36 h. Luciferase activity was normalized to β-galactosidase activity, and expressed as relative luciferase units (RLU). Significant ($p<0.05$) interactions (*) are indicated. C. Interactions of PIAS3 and ERα in a co-immunoprecipitation assay. Myc-tagged PAIS3 was transfected into COS-7 cells with or without ERα. After transfection, cells were treated with 10 nM E2 for 24h, and cell extracts were immunoprecipitated (IP) with anti-ERα antibody; and precipitates were then analyzed by Western blot (WB) using anti-Myc tag and anti-ERα antibodies as described in the Materials and Methods.

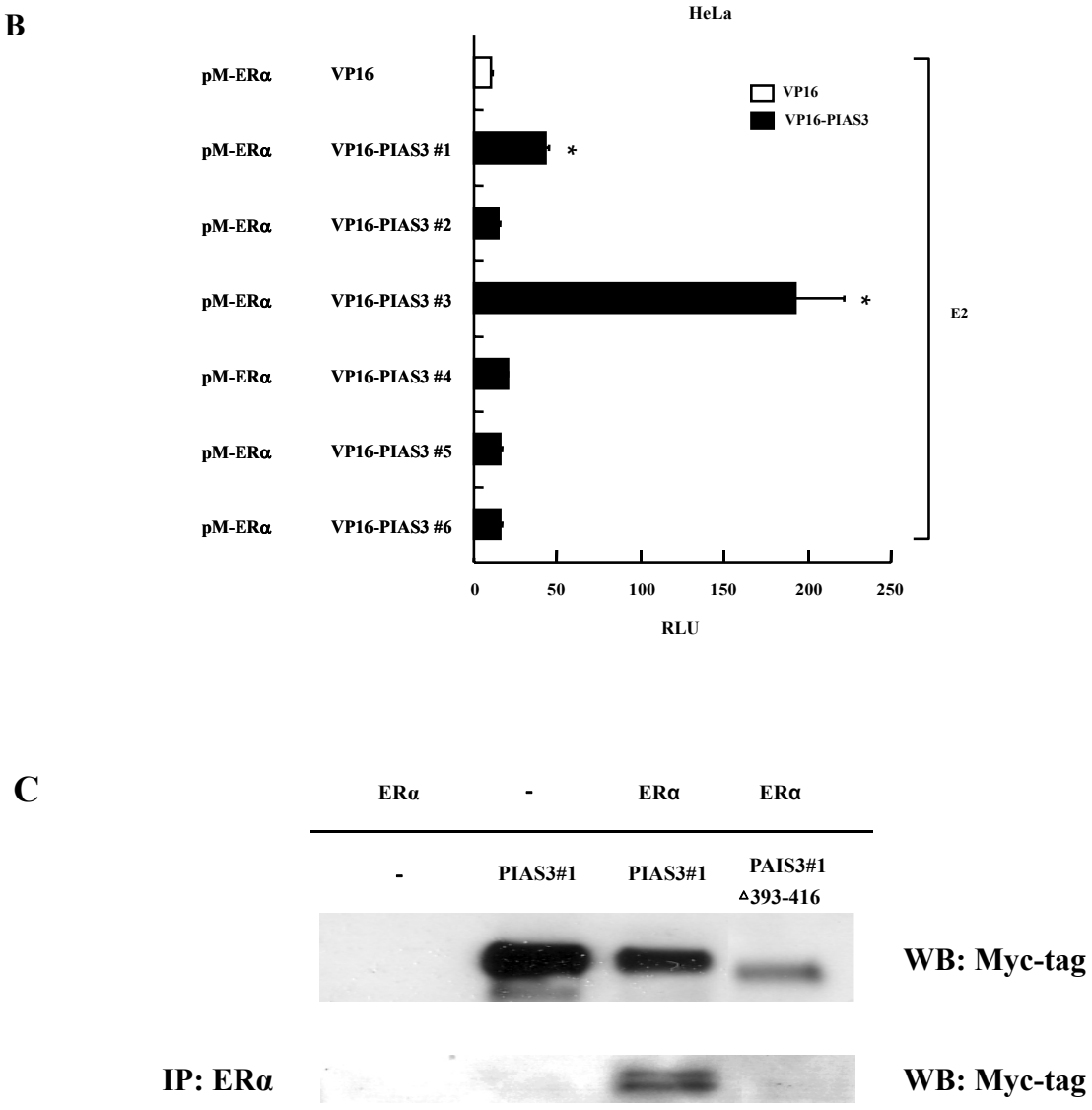

Figure 3-16 continued.

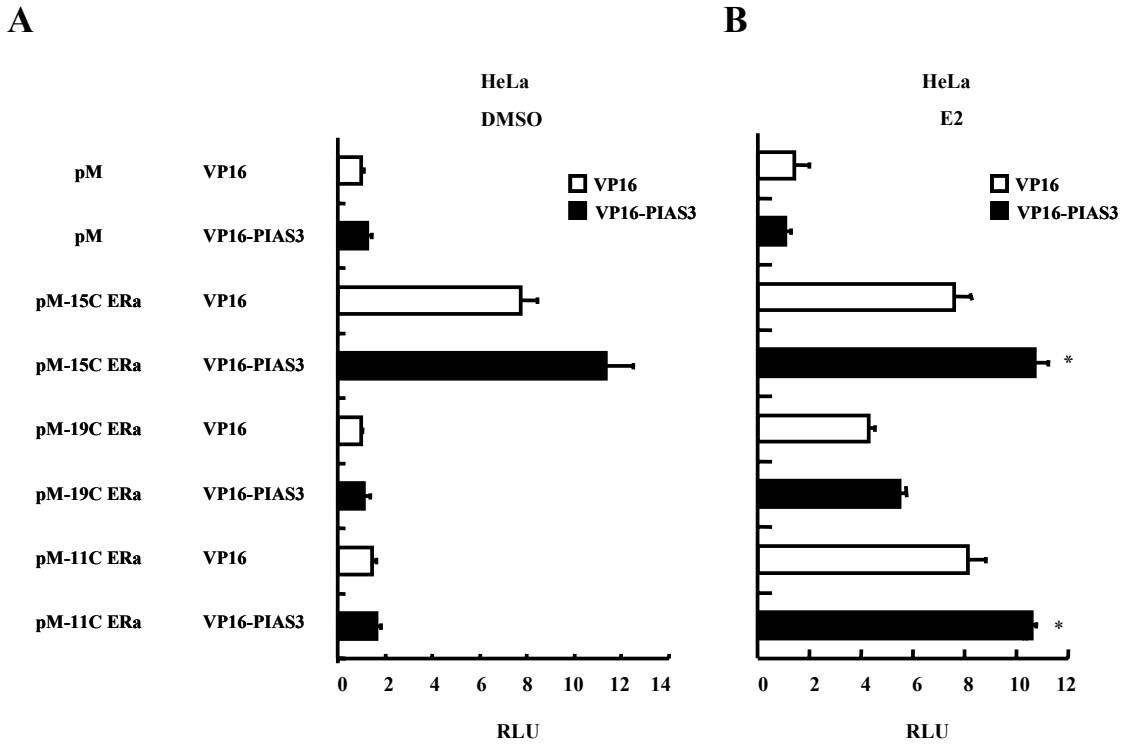

Figure 3-17 Interaction of various ERα deletion mutants with PIAS3. Hela cells were transfected with 50 ng of β-galactosidase, 125 ng of pGAL4-LUC, 150 ng pVP16-PIAS3 wild-type and 25ng pM-ER deletion mutants or pM empty vector. After transfection cells were treated with DMSO or 10 nM E2 for 36 h. Luciferase activity was normalized to β-galactosidase activity, and expressed as relative luciferase units (RLU). Significant ($p<0.05$) interactions (*) induced by E2 are indicated.

immunoprecipitation. These data further confirm that PIAS3 interacts with ERα and in the coimmunoprecipitation studies, deletion of the acidic region of PIAS3 decreased interactions with ERα.

We also investigated the interaction between wild type PAIS3 and ERα deletion mutants using a mammalian two-hybrid assay in HeLa cells. The results (Fig. 3-17) show that in the absence of E2 stimulation, luciferase activity was highly variable among the pM-ERα (variants) with high luciferase activity associated with cells transfected with pM-15C ERα plus VP16 and pM-15C ERα pluc VP16-PAIS3. After treatment with E2, the interaction associated with pM-15C ERα and VP16-PIAS3 was similar in the E2 and DMSO treatment groups. However, significant induction by E2 using the ERα deletion mutants was observed only in cells transfected with pM-11C ERα and VP16-PIAS3. These results suggest that in the mammalian two-hybrid assay system, E2-induced interactions between pM-ERα (variants) and VP-PIAS3 were minimal.

3.2.5 Coactivation of ERα/Sp1 by PIAS3

Several hormone-responsive genes in breast cancer cells are regulated through interactions of ERα/Sp1 with GC-rich promoter elements (333,335,407,408). We therefore investigated coactivation of ERα/Sp1-dependent transactivation by PIAS3. ZR-75 cells were transfected with ERα and PIAS3 expression plasmids as well as $pSp1_3$, which contains three GC-rich sites in a minimal TATA-luciferase construct. Results in Figure 3-18 shows that E2 caused a 2.4-fold increase in reporter gene activity in ZR75 cells transfected with 5 ng ERα expression plasmid, and cotransfection with 25, 50 and 75 ng PIAS3 expression plasmid resulted in a 1.3-, 1.7- and 2.1-fold enhancement of E2-induced luciferase activity. These data also show that PIAS3 enhanced activity in cells treated with DMSO indicating that PIAS3 also modulated Sp-dependent transactivation. Current studies are further investigating the molecular mechanism of ERα/Sp coactivation by PIAS3.

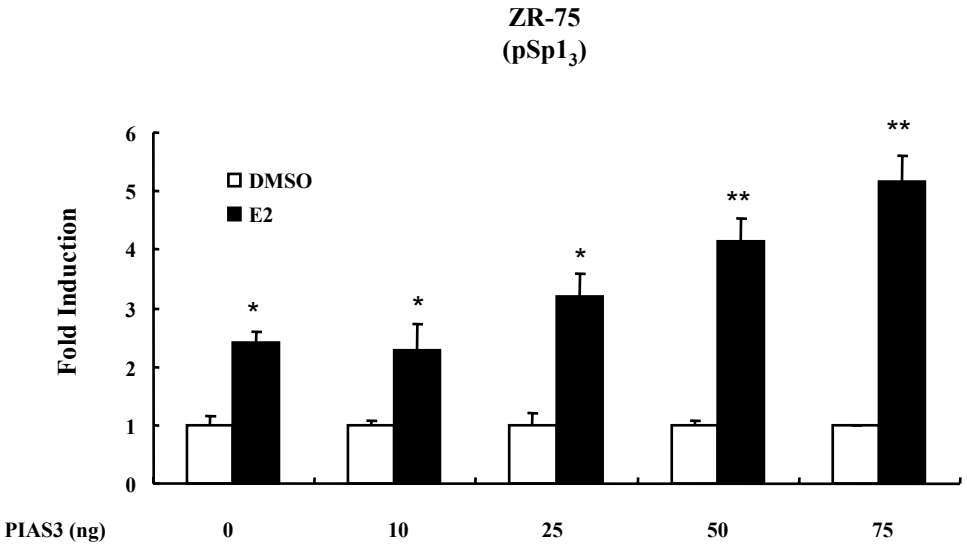

Figure 3-18 Coactivation of ERα/Sp1 by PIAS3. ZR-75 cells were transfected with pSp1$_3$, ERα, β-galactosidase and increasing amounts of pCDNA3-PIAS3 (0, 10, 25, 50 and 75 ng) expression plasmid. After transfection cells were treated with DMSO or 10 nM E2 for 36 h. Luciferase activity was normalized with β-galactosidase activity and results are expressed as fold induction and compared to that observed for DMSO alone. Significant ($p<0.05$) induction by E2 (*) or coactivation of ERα/Sp1 by PAIS3 (**) is indicated

CHAPTER IV
DISCUSSION AND SUMMARY

4.1 Mechanism of induction of Cdc25A by E2 in ZR-75 cells

E2 is a mitogen in ER-positive breast cancer cell lines and induces cell proliferation which is accompanied by induction of multiple functional classes of genes and protooncogenes including those required for cell cycle progression and nucleotide biosynthesis (407,408). For example, treatment of MCF-7 cells with E2 significantly enhances G0/G1 to S-phase progression and is accompanied by induction of cyclin D1, E2F1, cdk activities, Rb phosphorylation, downregulation of the cdk inhibitors p21 and p27 (422). Previous studies report that E2 also induces Cdc25A (423,424), and this is consistent with the observed G0/G1 to S-phase progression. This study shows that Cdc25A gene expression is also induced by E2 in ZR-75 cells and pcdc25A-1 and related constructs are hormone-responsive and inhibited by the antiestrogen ICI 182780 (Figure 3-2B). Figure 4-1 illustrates some of the important genes/proteins involved in the E2-dependent G0/G1 → S phase progression and highlights those genes that are E2-responsive. The molecular mechanisms associated with regulation of these genes in breast cancer cells is complex and variable and in some case may be dependent on cell context. For example, cyclin D1 is regulated in MCF-7 cells through proximal GC-rich motifs which interact with ERα/Sp1 and a CRE site which is activated through a non-genomic pathway of estrogen action involving activation of cAMP/PKA. In MCF-7 cells, estrogen induces an unusual Sp1-ERα-NFY complex bound to proximal GC-rich and CCAAT sites. Thus both Sp1 and NFY transcription factors are activated by ERα (ligand bound). However, the hormone receptor acts through protein-protein and not protein-DNA interactions. In contrast, E2F1 is activated by E2 in ZR-75 breast cancer cells through independent interactions of ERα/Sp1 with GC-rich motifs and activation of NFY through non-genomic activation of cAMP/PKA dependent phosphorylation of NF-YA bound to CCAAT sites. These results illustrate that activation of E2F1 by E2 involves non-classical genomic and non-genomic pathways which are cell context-dependent. This study has focused on investigating the molecular

mechanisms of hormone-dependent activation of Cdc25A by extensive promoter analysis in ZR-75 cells.

The Cdc25A promoter does not contain a consensus or non-consensus estrogen responsive element (ERE); however, several GC-rich sites that bind Sp1 protein are present in the E2-responsive proximal region of the promoter (Figure 4-2) (433). Previous studies in this laboratory have demonstrated that ERα/Sp1 interactions with GC-rich motifs in several gene promoters including cyclin D1 were required for hormone-induced transactivation (434). Sp1 knockdown in MCF-7 cells (335,425) inhibited E2-induced G0/G1 to S-phase progression in MCF-7 cells, suggesting a possible role for ERα/Sp1 activation of Cdc25A through interaction with one or more of the proximal GC-rich sites. The results (Figure 3-3, 3-4, 3-5) demonstrate that this pathway contributes to hormonal activation of Cdc25A; however, the induction response is still observed even with constructs in which all three GC-rich sites are mutated (Fig. 3-3A) suggesting that other hormone-responsive pathways are involved in activation of Cdc25A by E2.

The minimal E2-responsive region of the Cdc25A promoter (-151 to -12) contains GC-rich, CCAAT and E2F1 motifs, and a second E2F1 site at -3 is also present outside this minimal promoter. Previous studies showed that E2F1-Rb complexes at the -3 site were important for inhibition of Cdc25A by the bovine papillomavirus E2 protein in cervical adenocarcinoma cells (335,425). In ZR-75 cells, only minimal basal activity was observed in cells transfected with a construct (pcdc25A-4) containing the -3 but not -62 E2F1 site (Fig. 3-2A); however, pcdc25A-4 was E2-responsive (Fig. 3-9B). Both dominant negative DP-1/E2F1 expression plasmids decreased hormone-induced transactivation in ZR-75 cells transfected with pcdc25A-4 (Fig. 3-9B) and pcdc25A-5 (Fig. 3-9A) confirming a role for E2F1 in mediating E2-dependent induction of Cdc25A. ChIP assays confirm interaction of E2F1 with the Cdc25A promoter (Fig. 3-10A), suggesting that induction of Cdc25A is due, in part, to E2-dependent Rb phosphorylation and subsequent derepression of E2F1. Both NF-YA and E2F1 are constitutively bound to the Cdc25A promoter and their band intensities are not significantly increased after treatment with hormone. This observation is consistent with hormonal activation of both transcription factors through phosphorylation of

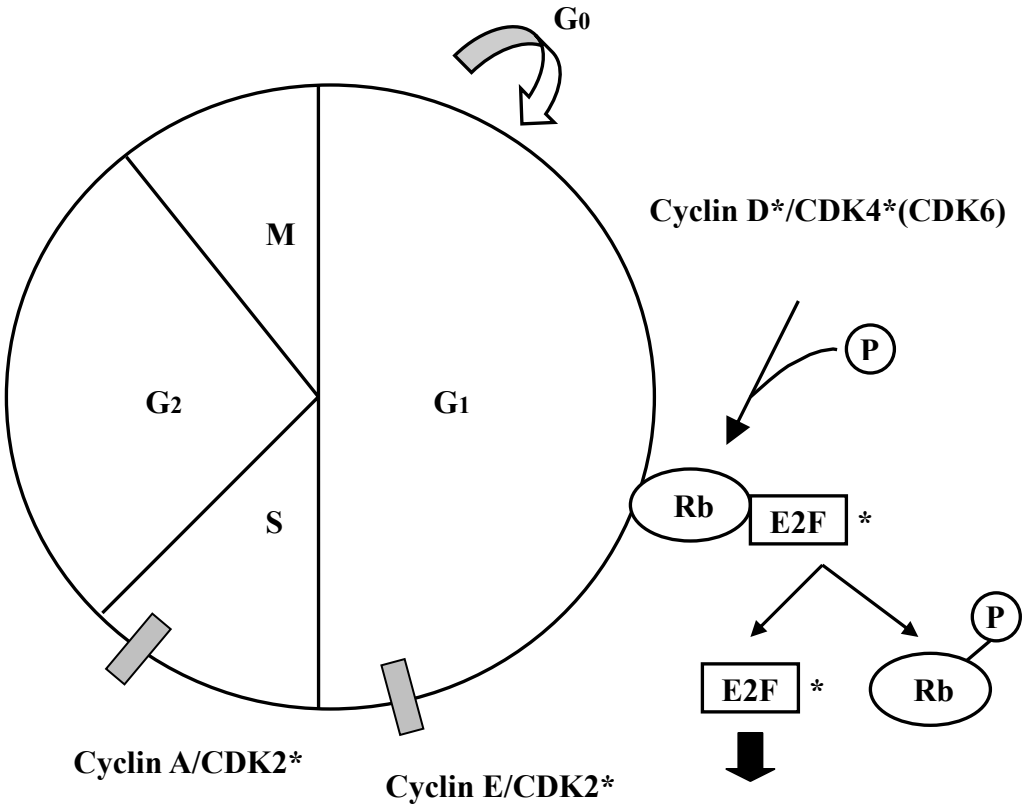

Figure 4-1 The genes/proteins involved in the E2-dependent G0/G1 to S pahse progression.

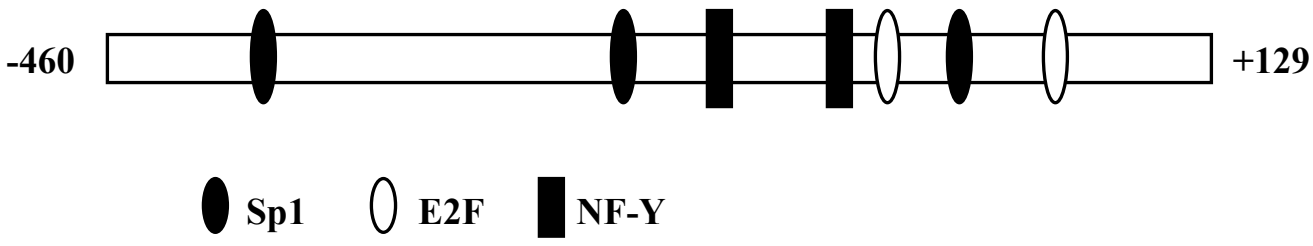

Figure 4-2 Promoter region of Cdc25A gene.

Rb and NF-YA. Moreover, since E2 also induces E2F1 mRNA/protein expression in ZR-75 cells (335), this pathway may also contribute to the induction response at later time points, but would not be apparent in this study due to the shorter duration (2 h) of the ChIP experiment.

Constructs containing the two CCAAT sites were also E2-responsive, and expression of dominant negative NF-YA significantly blocked hormone-dependent activation (Fig. 3-6, 3-7, 3-8). Previous studies show that NF-YA bound to CCAAT sequences in the E2F1 gene promoter (347,374,435,436) was also activated through non-genomic pathways of estrogen action that involved activation of cAMP/PKA (437). Hormonal activation of cAMP/PKA has previously been observed (335) and involves phosphorylation of downstream transcription factors including NF-YA. Results in Figure 3-6 show that E2 induced transactivation in cells transfected with pcdc25A-6 and pcdc25A-5m3 or GAL4-NF-YA/pGAL4-luc. These data coupled with the effects of cAMP/PKA inhibitors and dominant negative NF-YA expression, confirm that induction of Cdc25A by E2 also involves activation of cAMP/PKA and subsequent kinase-dependent activation of NF-YA. Previous studies have shown that activation of human TIMP (330,333,335,425,438,439) and hormone-dependent activation of E2F1 (286,287,440-442) were dependent on cAMP/PKA phosphorylation of NF-YA bound to CCAAT motifs. However, based on PKA inhibitor studies in cells transfected with pcdc25A1 and pcdc25A-5 and treated with E2, we conclude that the contribution of the non-genomic (cAMP/PKA) pathway of estrogen action was relatively small (<15%). The activation of Cdc25A by genomic and non-genomic pathways of estrogen action is not unprecedented since c-fos, cyclin D1, bcl-2, and E2F1 are also induced by E2 through both nuclear and extranuclear ER pathways in breast cancer cells (277,278). These genes are important for cell growth/survival and clearly highlight the importance of both pathways for ER-positive breast cancer cell proliferation.

4.2 Coactivation of E2-induced transactivation by PIAS3

Transcriptional regulation by ligand-activated NRs is a complex process that is dependent on multiple factors including the structure of the receptor agonist. For example, NR-mediated transactivation requires specific classes of coactivators and other transcription-related factors that are recruited to the target gene promoter by the DNA-bound receptor in the chromatin environment of the nucleus. Each nuclear factor contributes one or more distinct activities that are required for gene activation. Coregulators can be classified into two main groups according to their functions. The first group contains factors that covalently modify histones (ie. acetylation, methylation, phosphorylation, ubiquitylation) and modulate promoter DNA accessibility. The second group includes ATP-dependent chromatin remodeling factors that also modulate promoter accessibility to transcription factors and to the basal transcriptional machinery. Steroid hormone receptors such as ERα have been extensively used as models for determining the mechanisms of ligand-dependent receptor-mediated transactivation, which requires the assembly and recruitment of a complex of nuclear coregulatory proteins (279).

The initial step in transactivation is the ligand-induced binding of receptor dimers to their cognate HREs within promoter regions of hormone-responsive genes and this is accompanied by interactions with coregulatory proteins and the basal transcription complex. Direct interactions between the p160 coactivator proteins and AF2 domains of NRs through LXXLL motifs in the coactivators have been demonstrated in several studies (280). Once bound to the NR, p160 proteins facilitate recruitment of p300/CBP coregulators which exhibit intrinsic HAT activity that mediates acetylation of histones and there is also enhanced histone acetylation by SRCs which also possess HAT activity. After p300/CBP binding to the NR complex, it has been suggested that this increases the number of productive preinitiation complexes on the promoter (283). Furthermore, p300/CBP can interact with Pol II through the RNA helicase A and recruit the Pol II holoenzyme complex to the promoter region (443). In addition, the arginine methyltransferase CARM1 synergizes with p160 proteins and p300 to enhance

ligand-dependent transcriptional activation by NRs, possibly through its ability to methylate histone H3 (444,445). The next step involved is the recruitment of the TRAP220/SMCC/mediator complex. . The multiprotein Mediator complexes also known as the TRAP and DRIP complexes, are a class of NR coactivators that enhance transactivation through interactions with DNA-bound NRs and the RNA pol II transcriptional machinery and stabilize the formation of transcription PICs at the promoter (223). DRIP205 and DRIP150 which are two components of the DRIP/TRAP complex coactivate ERα-mediated transactivation in ZR-75 breast cancer cells (224). However, in contrast to many coactivators that act through their LXXLL motifs, coactivation of ERα by DRIP205 and DRIP150 does not require their LXXLL motifs suggesting a more complex mechanism of coactivation. NRs also facilitate recruitment of SWI/SNF to the promoter region. SWI/SNF catalyzes remodeling of the proximal promoter region in order to permit binding of the basal transcriptional machinery to the DNA template and facilitate the association between TBP and the TATA box. The steroid receptor activator (SRA), is unique among coactivators which functions as an RNA transcript rather than as a protein (409). SRA is selective for steroid hormone receptors and mediates transactivation via their N-terminal activation function. In addition, the E6-associated-protein (E6-AP), an ubiquitin ligase that has been identified as a coactivator of the progesterone receptor (PR) (409). E6-AP also coactivates the hormone-dependent transcriptional activities of other members of the nuclear hormone receptor superfamily. A large number of coactivators have been identified and these proteins (and mRNA) differ in their structure and function and they act through both LXXLL box-dependent and independent pathways. There is increasing evidence that coactivator function is highly varable and dependent on ligand structure, nuclear receptor and cell context.

The PIAS family was originally identified as cytokine-induced inhibitors of STATs and they consist of five structurally related mammalian proteins, PIAS1/GBP, PIAS3, PIASxα, PIASxβ, and PIASy. These proteins contain several conserved domains. The conserved N-terminal region of PIAS proteins contains several well characterized

domains (Figure 3-13 A). The SAF-A/B, Acinus, PIAS (SAP) domain binds A/T-rich DNA and may be involved in targeting PIAS proteins to the nuclear scaffold (411). The SAP domain encompasses an LXXLL motif that is required for transcriptional repression of STAT1 by PIAS3y (411). The RING-finger-like zinc-binding domain (RLD) mediates the SUMO-E3-ligase activity of PIAS proteins and binds directly to Ubc9, the SUMO E2 enzyme (412). Most PIAS proteins also contain a PINIT motif, which plays a role in nuclear retention (428). The C termini of PIAS proteins are more diverse; however all contain an acidic domain preceded by several serines (Ser/Ac). Within the acidic domain, a SUMO-1 Interaction Motif (SIM) exists (410) and a serine- and threonine-rich region (S/T) is present in the C termini of all PIAS proteins except for PIASγ and the function of this region is unknown.

PIAS proteins are not only negative regulators of cytokine signaling that inhibit the activity of STAT-transcription factors, these proteins also function as transcriptional coregulators of various important cellular pathways. It has been reported that PIAS proteins modulate the ligand-dependent steroid hormone-mediated transactivation (446-448). Depending on the receptor type, cell line and gene promoter PIAS proteins both enhanced and repressed steroid hormone receptor-mediated transaction (413). These results clearly demonstrate the highly flexible activity of PIAS proteins which stimulated our interest in these proteins as coactivators of ERα and ERα/Sp1 in breast cancer and other cancer cell lines. Both activating and repressing effects on transcription were observed upon expression of a distinct PIAS family member, indicating that PIAS proteins play a cell context-dependent dual role as activators or repressors of steroid hormone signaling.

In this study, the results show that PIAS3 coactivated ERα in MCF-7 cells transfected with $pERE_3$ (Fig. 3-11). Moreover, decreased endogenous PIAS3 levels by specific inhibitory RNA significantly suppressed the induction of pS2 gene expression by E2 (Fig. 3-12). This further confirms that PIAS3 functions as an endogenous coactivator of E2-mediated transactivation in MCF-7 cells and therefor we futher investigated the mechanism of this coactivation response using a series of PIAS3

mutant constructs in order to identify domains required for coactivation. Deletion analysis of PIAS3 showed that the N-terminal region of PIAS3 is not required for coactivation of ERα (Fig. 3-13). This indicates that coactivation of ERα by PIAS3 does not require the LXXLL motif suggesting that "classical" interaction with helix 12 of ERα is not necessary for the coactivation activity of PIAS3. The C-terminal region of PIAS3 includes the RING finger-like domain, the acidic region and the serine-rich sequences. Previous studies showed that the RING finger-like domain is required for the SUMO-E3 ligase activity (413) and ERα is sumoylated in the presence of SUMO-1(412). When PIAS3 acts as E3 ligase for ERα sumoylation it stimulates SUMO-1 conjugation to ERα and it has been reported that extensive sumoylation of ER represses hormone-induced transactivation (412). Our results indicate that the RING finger-like domain of PIAS3 is not required for the ERα coactivation suggesting that PIAS3-dependent sumoylation of ERα does not play a role in coactivation of ERα in these studies (Fig. 3-13). However, deletion of the acidic regionof PIAS3 resulted in a significant loss of coactivator activity (Fig. 3-14). Although the PIAS3#5 coactivates ERα-mediated transactivation, this variant does not interact with ERα in mammalian two-hybrid assay (Fig. 3-16B) suggesting that coactivation of ERα-mediated transactivation by PIAS3#5 may require recruitment of other coregulatory protein(s). Jimenez-Lara and coworkers (449) showed that PIAS3 modulates the transcriptional activation of androgen receptor through cooperative interactions with the nuclear receptor coactivator TIF2 . The interaction between TIF2 and PIAS3 occurs through the acidic region of PIAS3, which is conserved through out the PIAS family of proteins (450,451). TIF2 coactivates ERα through interactions with the ERα AF2 domain in a ligand-dependent fashion (413). Taken together, these data support the hypothesis that PIAS3 may coactivate ERα-mediated transactivation through TIF2.

Unlike most coactivators which are recruited by the AF2 of ERα, PIAS3 did not require the critical helix 12 region of ERα AF2 for coactivation of ERα when HeLa cells were transfected with Taf-1ERα (Figure 3-15). PIAS3 exhibited minimal coactivation activity in HeLa cells transfected with $pERE_3$ and the null-ERα mutant which is an AF1

deletion mutant that also contains three point mutations on helix 12 (Fig. 3-15). These data suggest that the AF1 of ERα is also involved in coactivation of ERα by PIAS3. However, when HeLa cells were transfected with $pERE_3$ and 19C-ERα, an AF1 deletion mutant, PIAS3 still coactivated 19C-ER (Fig 3-15) suggesting that the AF2 of ERα is involved in coactivation by PIAS3 but only when AF1 of ERα is deleted. These results suggest that coactivation of ERα-mediated transactivation by PIAS3 is complex and may involve interactions with more than one region of ERα and these results are comparable to recents studies in this laboratory on the coactivation of ERα by DRIP205 and DRIP 150. Coactivation of ERα by DRIP205 and DRIP150 in ZR75 breast cancer cells was independent of the LXXLL motifs in both proteins. DRIP150 enhanced transactivation in cells transfected with ERα and 19C-ERα but not TAF1-ERα and this profile for coactivation of wild-type/ variant ERα by DRIP150 differed from PIAS3. DRIP205 coactivated ERα-mediated transactivation, however, coactivation was not observed in cells transfected with 19C-ERα or TAF1-ERα. Thus the coactivation activities of PIAS3, DRIP150 and DRIP205 differed with respect to their requirements for different domains of ERα, however, coactivation of ERα by all three proteins was independent of their LXXLL motifs.

The results from mammalian two-hybrid studies showed that the PIAS3 interacts with AF1 of ERα when cells treated with DMSO and E2, suggesting that the interactions of PIAS3 and AF1 of ERα are ligand-independent (Fig. 3-17). However, PIAS3 also interacts with AF2 of ERα but only when cells are stimulated with E2 (Fig. 3-17B), suggesting that the interactions between PIAS3 and AF2 of ERα are ligand-dependent. Thus both AF1 and AF2 of ERα are involved in coactivation by PIAS3 and these regions of ERα are also required for physical interactions with PAIS3.

Several studies have reported that SUMO-1 regulates hormone-induced transactivation of some nuclear receptors (412). This regulation can be achieved by sumoylation of either receptors or coregulators indicating that sumoylation can be an integral part of nuclear hormone receptor function. Moreover, a recent report showed that ERα-mediated transcription is stimulated by SUMO-1 expression. It has been

speculated that the enhanced ERα-dependent transcription by SUMO-1 may be due to sumoylation of the coactivator steroid receptor coactivator 1 (SRC-1) (414).

Jimenez-Lara and co-workers identified PIAS3 as a binding partner of GRIP1/TIF2 (333,335,452-454) and it was also reported that PIAS3 interacts with TBP in a yeast two-hybrid (455). Their data suggest that the TBP interaction domain of PIAS1 requires the 39 amino acids from aa 453 to aa 491, which includes the acidic region. The localization of SUMO E3-ligase activity and TBP-binding activity to opposite ends of PIAS proteins suggests that these proteins might "dock" at TBP and sumoylate transcription factors at the promoter. Based on these studies, PIAS3 may have cooperative coactivation with TIF2 and/or TBP.

ERα/Sp1-mediated transactivation has been linked to hormone activation of several genes involved in cell cycle progression, DNA synthesis and metabolism of purines and pyrimidines (456). In vitro studies show that ERα interacts with both Sp1 and Sp3, and the C-terminal DBD of Sp1 is the major interaction site for ERα. Recently Kim et al used the FRET technique to investigate the interactions between ERα and Sp1 in living MCF-7 breast cancer cells. Results from FRET analysis showed that ERα interacts with Sp1 in living breast cancer cells and the interactions are ligand-dependent. Only a few coactivators of ERα such as DRIP205, and DRIP150 have been reported as coactivators for ERα/Sp1 in ZR-75 breast cancer cells and research on identificaon of ERα/Sp1 coactivaors is in progress. The results of transfection assays in ZR-75 cells showed that PIAS3 enhanced E2-induced luciferase activity of $pSp1_3$ (Fig. 3-18) suggesting that coactivation of ERα/Sp1 by PIAS3 was also observed in ZR-75 cells transfected with $pSp1_3$ (Fig. 3-11). The molecular mechanisms of this response are currently being investigated.

In conclusion, we have shown here that PIAS3 interacts with ERα and functions as a coactivator for ERα-mediated transacivation. The RING finger-like domain of PIAS3 is important for interactions with ERα and the acidic region is critical for its coactivation activity. PIAS3 also functions as a coactivator for ERα/Sp1 pathway in ZR-75 breast cancer cells. Moreover, we have also shown that knockdown of PAIS3 in

MCF-7 cells results in decreased induction of pS2 by E2 demonstrating an endogenous role for PIAS3 in hormone-induced transactivation. The role of PIAS3 and its interactions with other coactivators in the induction response and the potential temporal effects of E2-dependent recruitment of coactivators may also be important and is currently being investigated.

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