MECHANISMS OF COACTIVATION OF ESTROGEN RECEPTOR α (ERα)- AND ERα/Sp-MEDIATED TRANSACTIVATION BY VITAMIN D RECEPTOR INTERACTING PROTEIN 205 (DRIP205) IN BREAST CANCER CELLS

A Dissertation

by

QIAN WU

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 2005

Major Subject: Biochemistry

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Approved by:

Chair of Committee, Stephen Safe
Committee Members, Gary Kunkel
David Peterson
James Sacchettini
Head of Department, Gregory Reinhart

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ABSTRACT

Mechanisms of Coactivation of Estrogen Receptor α (ERα)- and ERα/Sp-mediated Transactivation by Vitamin D Receptor Interacting Protein 205 (DRIP205) in Breast Cancer Cells. (December 2005)

Qian Wu, B.S., Peking University

Chair of Advisory Committee: Dr. Stephen Safe

Vitamin D interacting protein 205 (DRIP205) is a mediator complex protein that anchors the complex to the estrogen receptor (ER) and other nuclear receptors (NRs). In ZR-75 breast cancer cells treated with 17β-estradiol (E2) and transfected with a construct containing three tandem estrogen responsive elements ($pERE_3$), DRIP205 coactivates ERα-mediated transactivation. DRIP205Δ587-636 is a DRIP205 mutant in which both NR boxes within amino acids 587-636 have been deleted and, in parallel transfection studies, DRIP205Δ587-636 also coactivates ERα. Moreover, both wild-type and variant DRIP205 also colocalize with ERα in the nuclei of transfected cells. AF1 and AF2 of ERα are both required for DRIP205 coactivation. Extensive deletion analysis of DRIP205 shows that multiple domains of this protein play a role in coactivation of ERα and in interactions with ERα. On the other hand, both DRIP205 and DRIP205Δ587-636 coactivate E2-induced transactivation of

ERα/Sp1 in cells transfected with a construct containing three GC-rich sites ($pSp1_3$). Coactivation of ERα/Sp1 by DRIP205 is dependent on AF1 of ERα. Enhancement of ERα and ERα/Sp1 by DRIP205 does not require NR boxes of DRIP205, and deletion mutants DRIP205 (1-714) and DRIP205 (516-1566) significantly coactivate ERα and ERα/Sp1. RNA interference study showed that DRIP205 coactivation of ERα/Sp was abolished in cells transfected with iSp3 and iSp4, suggesting that Sp3 and Sp4 are required for coactivation of ERα/Sp by DRIP205 in ZR-75 cells.

DEDICATION

To my parents, Xianghua Li and Baokang Wu,

and my husband, Zhijun Cai.

ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor, Dr. Stephen Safe, for being my example of a brilliant hardworking scientist and for providing me the great opportunity to learn from him and to work in his laboratory. I'm so grateful to him for his guidance, understanding and support throughout my graduate studies. I would also like to thank my committee members, Dr. David Peterson, Dr. Gary Kunkel and Dr. James Sacchettini, for their instruction, discussions, recommendations and contributions to my education and research. I also wish to thank Dr. Robert Burghardt for all the training and guidance in imaging studies and for his kindly advice as well.

I would like to thank my colleagues, Dr. Thu Nguyen, Dr. Mark Wormke, Dr. Brad Saville, Dr. Matt Stoner, Dr. Kyoung-hyun Kim, Dr. Chunhua Qin, Dr. Xiangrong Li, Dr. Sharon Ngwenya, and also Wanru Lee, Chien-Cheng Chen, Shaheen Khan, Fei Wu for the training I received over the years and for their collaborative efforts and scientific discussions. I also want to express my appreciation to Lorna Safe for all her help and kindness and to Kim Daniel and Kathy Mooney for their administrative assistance.

Finally, I want to deeply thank my parents for their everlasting and unconditional support. They always believed in me and I couldn't have achieved this without their love and encouragement throughout my life. I'm so grateful to them and I wish they could have been here to see me at graduation. I also appreciate all the love and support from my husband Cai. He has so much faith

in me and always encourages me to pursue my career, and that means a lot to me which cannot be put into words. I would not be where I am without him.

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INTRODUCTION

1.1 Breast Cancer

1.1.1 Cancer

A complex mammal is composed of individual cells that cooperatively form tissues and organs that display age-, sex- and species-specific phenotypes. Normal cells are derived from parental cells through cell division, and serve their destiny by performing tissue-specific function. Cells also undergo apoptosis or programmed cell death in order to maintain tissue or whole body homeostasis. Thus, in response to signals sent by either the environment or other cells, cells divide, rest, differentiate, and die as required for maintenance of the organism (1).

The harmony of a complex living organism can be potentially disrupted by DNA damage that results in mutations. The initiation of a tumor cell requires only a single mutation in a parental cell that somehow survives in a daughter cell. Successive rounds of mutations give rise to progenitor cells with the ability to rapidly proliferate and at the same time, escape from apoptosis (2). Oncogenes and tumor-suppressive genes are targets for enhancing tumor progression. The

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

multiple-step development of cancer is illustrated by colorectal cancer, which typically develops over decades and appears to require at least seven genetic changes (Fig. 1) (3). The neoplastic process is initiated by mutation of adenomatous polyposis coli (*APC*) gene, which acts as a gatekeeper for epithelial cell proliferation. In humans, complete inactivation of APC has been found in the earliest neoplastic lesions, called dysplastic aberrant crypt loci (ACF), which are precursors of adenomas (4). K-RAS is an oncogene, and only one genetic event is required for its activation (5,6). *DCC*, *DPC4* and *JV18-1* genes are candidate tumor suppressor genes involved in colorectal neoplasia, and these genes require two genetic events (one in each allele) for their inactivation (7). Another tumor suppressor gene, p53, is involved in later events in colorectal tumorigenesis (8). This process of colorectal tumor progression is also accelerated by mutations in mismatch repair (MMR) genes, such as *mutS* and *mutL* (6,9,10).

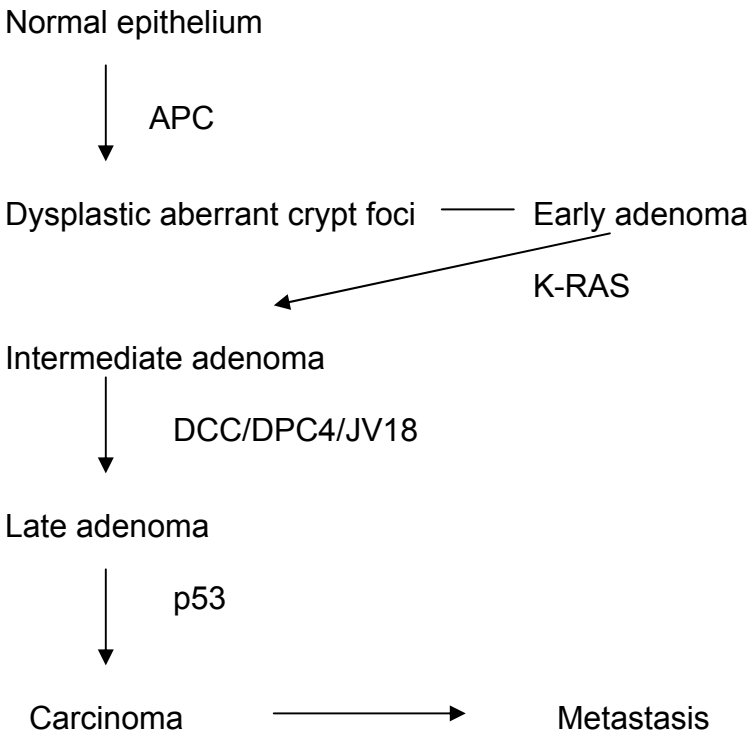

Fig. 1 Colorectal tumorigenesis and associated genetic events (3).

Cancer development is more complex than a simple linear accumulation of oncogenic mutations. Regulation of cell growth and apoptosis are strictly controlled. Normally, cells proliferate when required, and at the same time, the development of mutated cells leading to deregulated growth are suppressed by induction of apoptosis. Potential oncogenic alterations call for abnormality, clonal expansion of cells and development of neoplastic phenotypes and these pathways are opposed by growth-inhibitory processes, such as apoptosis, differentiation or senescence. Tumor progression only occurs in the very rare instances when these growth-inhibitory processes are compromised by mutations (Fig. 2) (11).

Development of efficient diagnosis and treatment of cancer requires an understanding of the molecular mechanisms of cancer biology, which include cancer cell initiation, growth and metastasis, and identification of critical genes that lead to differences between cancer cells and normal cells.

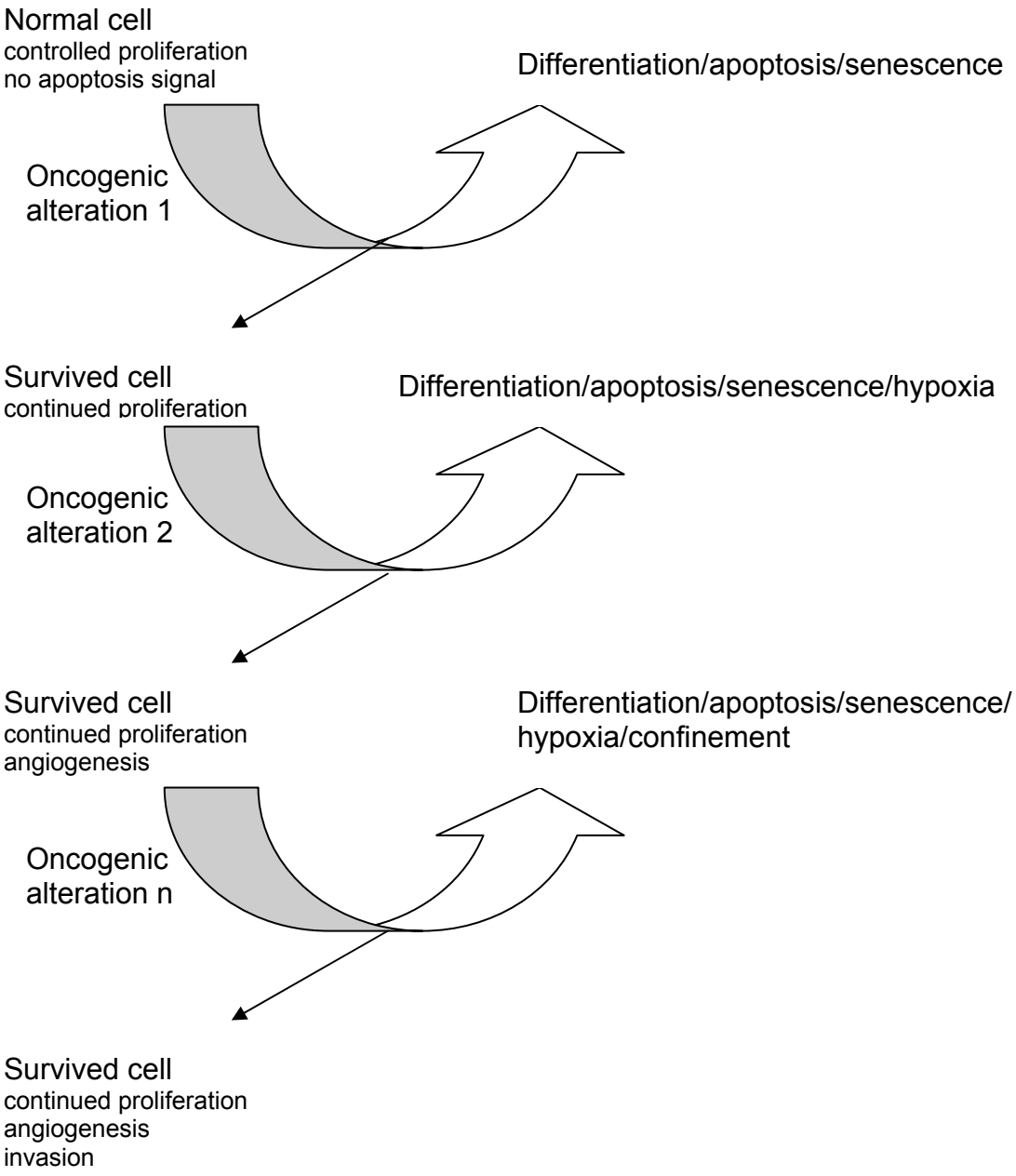

Fig. 2 Cancer development is more complex than the simple linear accumulation of genetic changes (11).

1.1.2 Breast Cancer

Breast cancer is the most common malignancy in women and the second leading cause of cancer deaths. The incidence of breast cancer has been increasing steadily from 1:20 in 1960 to 1:7 today. In 2005, it is estimated that around 211,240 women will be diagnosed with invasive breast cancer in the United States and about 40,410 women will die from this disease. Breast cancer is very rare in younger women, sharply increases with age and plateaus at the age of forty-five (Fig. 3) (12).

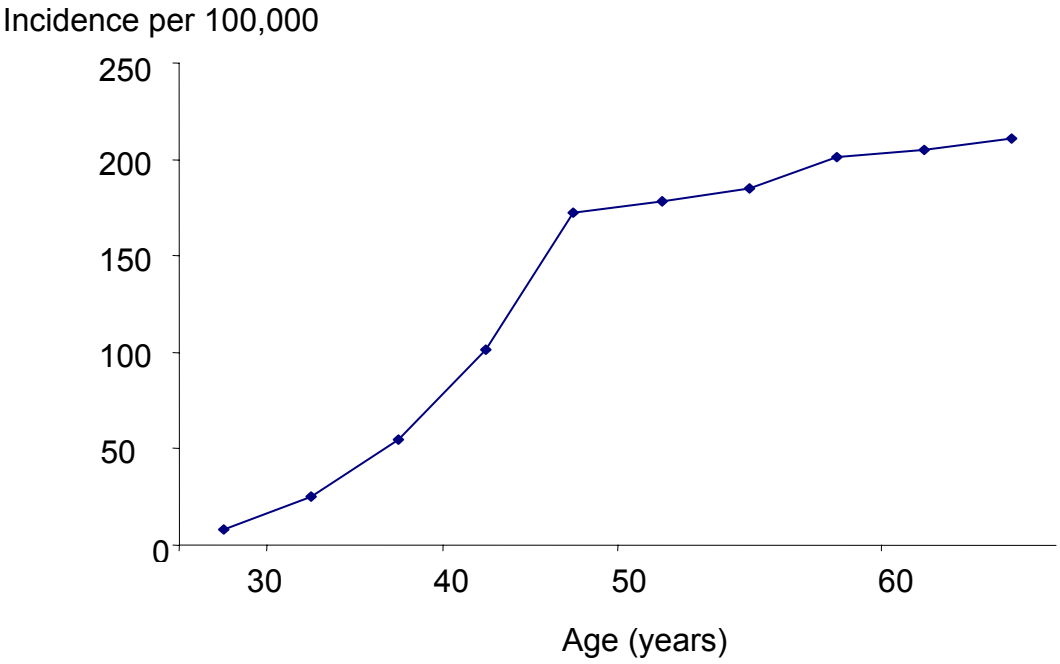

Fig. 3 Age-specific incidence rates for breast cancer in US white females (12).

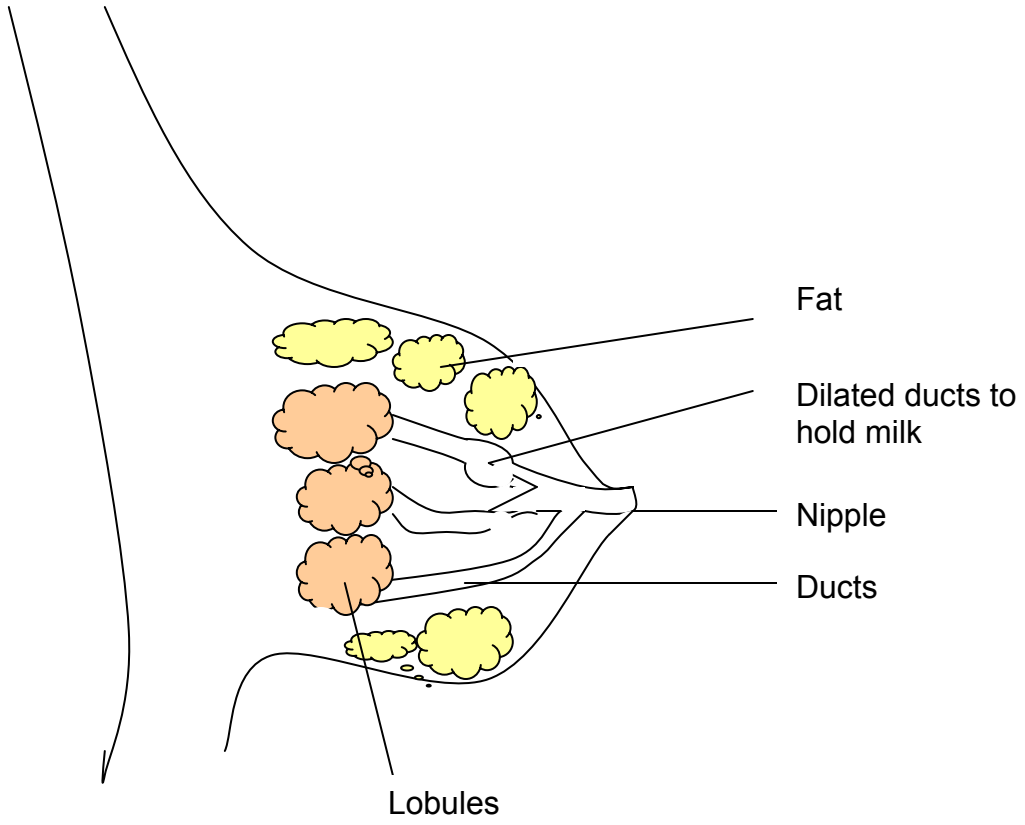

Fig. 4 Basic structure of the breast (13).

. Fig. 4 shows the basic structure of the mammary gland that includes ducts, lobules, dilated ducts, nipple and fat. In order to understand the prognosis and treatment of breast cancer, stages have been developed for describing the extent of breast cancer (13). Stage 0 is used to describe non-invasive breast cancer. Stage 1 describes tumors that are becoming invasive and measure up to 2 cm in diameter but lymph nodes are not involved. Tumors in stage 2 are up to 5 cm in diameter and have spread to the lymph nodes under the arm of the same side as the cancer. These stages are early stages for this disease. Stage 3A describes invasive breast cancer with tumors spreading to the lymph nodes that are clumping or sticking to one another. Stage 3B describes invasive breast cancer with cancer spreading to breast skin, chest wall, or internal lymph nodes. Stage 4 is advanced stage with invasive breast cancer spreading beyond breast, under the arm and to internal mammary lymph nodes.

Breast cancer is a heterogeneous disease. The molecular evolution of breast cancer has been studied, and the results suggest that this is a complex and heterogeneous event. Some scientists propose that breast cancer progression is a multi-step process, which is similar to Volgenstein's model for colon carcinogenesis (14). Support for this model includes: 1. The presence of E-cadherin mutations that inactivate the gene in lobular breast cancers, and this represents a major difference between lobular breast cancers and ductal carcinomas; 2. The development of p53 mutations are observed in high-grade ductal cancers but not in low-grade tumors; 3. Moreover, a subset of aggressive

high-grade tumors is defined by sequential Her2/neu and Ras mutations, and Rb dysfunction that define a separate subset of aggressive ductal carcinomas.

Other scientists believe that breast cancer progression is a complex series of stochastic genetic events leading to distinct and divergent pathways in development of invasive breast cancer (15). The “lobular” or “ductal” type in these pathways is no longer distinctive. Nuclear grade (degree of differentiation) is strongly associated with the number and pattern of molecular genetic abnormalities in breast cancer cells (16-18). Grade I tubular breast cancers show less genomic changes and highly recurrent losses of chromosome 16q, while grade III breast carcinomas show complex genotypes with multiple losses and high level gains at multiple sites. The distinctive genetic profiles of grade I and grade III carcinomas suggest that progression from low- to high-grade is rare. Studies using expression microarrays suggest that breast carcinomas can be classified into four categories according to their transcriptome: estrogen receptor (ER)-positive, HER-2-positive, basal and normal breast-like cancers (19). The prognostic significance of the different gene expression patterns was reported in other studies indicating that patients with HER-2-positive or basal carcinomas (high-grade) do worse than patients with ER-positive or normal breast-like cancers (low-grade) (20,21).

1.1.3 Risk Factors for Development of Breast Cancer

1.1.3.1 Endogenous Hormones

Estrogen plays an important role in the etiology of human breast cancer. Animal studies have shown that estrogens can induce and promote mammary tumor formation and growth in rodents while ovariectomy or antiestrogenic drugs have the opposite effects (22). The hormone-dependence of breast cancer is reflected in the age-dependent breast cancer incidence (Fig. 3). Breast cancer can be detected in young women in their 20s, and the incidence rises sharply with age until menopause and this corresponds to increased overall lifetime exposure to estrogens.

Additional evidence linking estrogens and breast cancer is the worldwide variation in breast cancer risk with urinary and blood estrogen levels in women samples. Higher breast cancer incidence occurs in North America and Western European women, and lower rates are observed in Asia. Total urinary estrogen levels were 36% higher in the North American women compared with Asian women of the same age (23). Serum estrogen levels were 20% higher in pre-menopausal women in Los Angeles compared to women in Shanghai (24); in post-menopausal women, estrogen levels were 36% higher in women in Los Angeles than in Japan (25).

The effects of cumulative lifetime exposure of breast epithelium to estrogen can be observed in other widely acknowledged risk factors for breast

cancer, including early menarche (26,27), older age at menopause (27,28), age at first pregnancy or childlessness (27,29) and postmenopausal obesity (30).

1.1.3.2 Exogenous Hormones

1.1.3.2.1 Hormone Replacement Therapy (HRT)

Hormone replacement therapy (HRT) increases the risk of breast cancer (31,32). A recent meta-analysis of studies that included over 160,000 women showed that for current or recent use of HRT, the risk of breast cancer increases in relation to the increasing duration of HRT use. Breast cancer risk increased by 2.3% ($p=0.0002$) for each year of use for women whose last use of HRT was less than five years before diagnosis. On the other hand, women who stopped using HRT five or more years before diagnosis had no increased risk, and this was independent of the duration of use (33). Multiple studies of combination hormone replacement therapy (CHRT), in which a progestin is given either sequentially or continuously with estrogen, have shown increased breast cancer risk to a greater extent than observed for estrogen alone (31,34-36).

1.1.3.2.2 Oral Contraceptives (OCP)

The data on oral contraceptives (OCP) use and breast cancer risk are controversial. Results from a meta-analysis of over 150,000 women suggest that a modest increase in breast cancer risk with current and recent combined contraceptive (COC) use. Risk was greatest for those who began COCs before the age of 20, and tended to decline with increasing age at diagnosis (37). Two

studies did not find any correlation between OCP use and increased risk of breast cancer (38,39).

1.1.3.3 Family History and Inherited Susceptibility

A family history of breast cancer, especially from a first-degree family member diagnosed at an early age or had bilateral disease, is associated with an increased risk of breast cancer. *BRCA1* and *BRCA2* are two major breast cancer genes identified as genetic risk factors for this disease. However, studies have shown that *BRCA1* and *BRCA2* together account for only about 5% of all breast cancers (40). In addition to breast cancer, *BRCA1* has been associated with increased risk of ovarian cancer, while *BRCA2* may play a role in male breast cancer and possibly other cancers such as pancreatic and prostate cancers. More than 200 different mutations have been described for each *BRCA* gene. There are two BRCA1 mutations that are observed in individuals of Ashkenazi Jewish descent: 185delAG occurs in about 0.9% and 5382insC occurs in 0.13% of those individuals (41,42). For *BRCA2*, 6174delT mutation appears in about 1.5% of the Ashkenazi population and 999del5 appears in about 0.6% of the Icelandic population (43). In family-based studies, the lifetime risk for breast cancer from BRCA1 mutations are 80-90%, and the lifetime risk for ovarian cancer is 64% by age 70 (44). In another non-family-based study of Jewish women, the risk for breast cancer was 56% and the risk for ovarian cancer was 16% among women with BRCA1 and BRCA2 mutations, respectively (45).

Other candidate genes have smaller effects on cancer outcomes (46). Candidate genes include those that encode enzymes involved in estrogen synthesis and metabolism, and receptors and coregulators that are critical for estrogen function (Fig. 5) (47). For example, the A2 allele, which is the minor allele of the *CYP17* gene that encodes for the P450c17α enzyme, involves a single nucleotide polymorphism (SNP) thymine-to-cytosine change in the promoter region. This mutation is associated with higher serum levels of E2 (48,49), and with an increased risk of breast cancer (50,51). However, results from other studies of this gene mutation have been inconsistent. For example, one study showed an inverse association for premenopausal women (52). Similar inconsistencies of cancer outcome were also observed for CYP19 gene. This gene encodes aromatase which is a critical enzyme that catalyzes the rate-limiting step in estrogen biosynthesis (53-56).

Another candidate gene in association with breast cancer incidence is *HSD17B1*, which encodes 17HSD (17-β hydroxysteroid dehydrogenase) type 1 enzyme catalyzing the final step in E2 synthesis by converting estrone (E1) into the more active E2. Increased risk of breast cancer was originally found in individuals who were homozygous for the serine allele at position 312 (polymorphism serine-to-glycine) of 17HSD (57). Individuals with both *CYP17* and *HSD17B1* high-risk alleles were shown to have increased risk of breast cancer in a dose-dependent manner (58,59). Other estrogen-metabolizing genes, *CYP1A1*, *CYP1B1*, *CYP2D6*, *SULT1A1* and *COMT*, have been studied

for association with breast cancer risks, and results have been inconsistent (60-62).

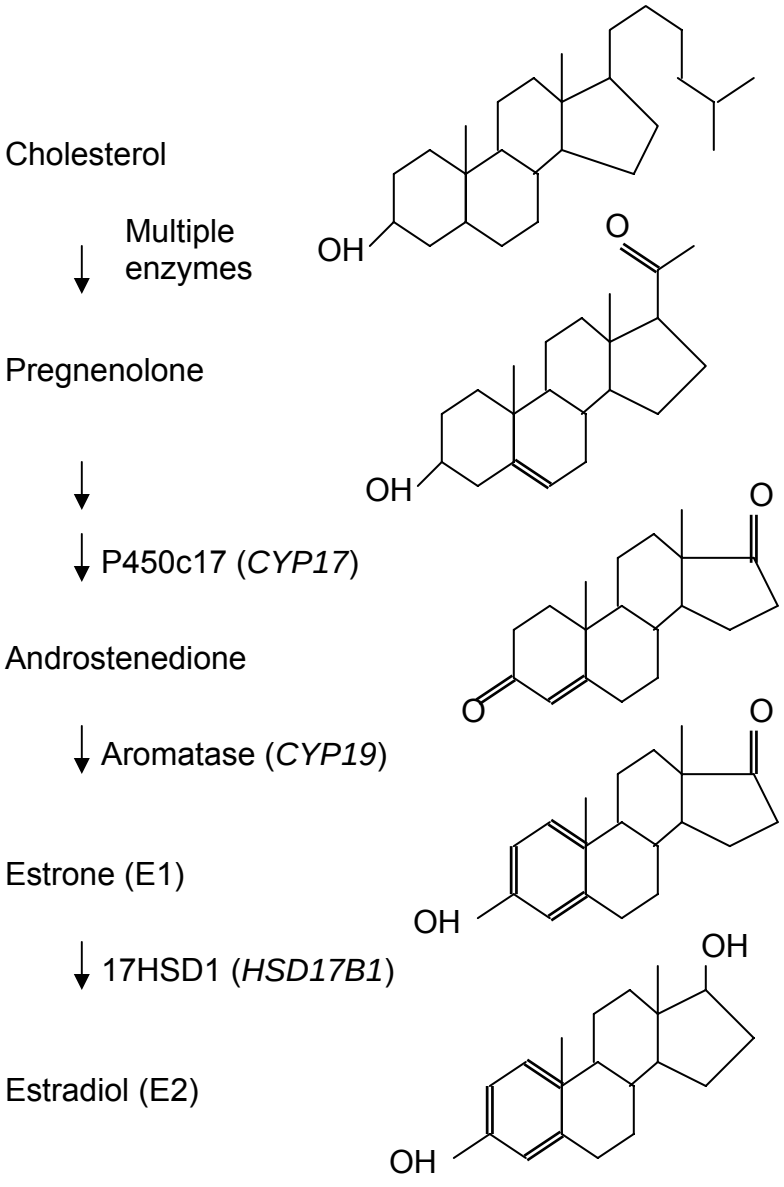

Fig. 5 Ovarian estrogen biosynthesis from cholesterol and candidate genes that may play a role in breast cancer etiology (47).

1.1.3.4 Diet

Diet plays an important role in breast cancer risk as shown in the international patterns of breast cancer occurrence and changes in rates of breast cancer following migration to high-risk from low-risk countries (63). Howe and coworkers found that the breast cancer risk of postmenopausal women was positively associated with both total fat (RR=1.46, p=0.0002) and saturated fat (RR=1.57, p<0.0001) (64). Alcohol consumption increases breast cancer incidence for women who consumed up to 60 g of alcohol per day (two to five drinks) (65), and another study hypothesized that alcohol may increase plasma estrogen and insulin-like growth factor levels (66). High fiber intake may protect against breast cancer, because fiber may decrease the intestinal reabsorption of estrogens excreted via the billiary system (67). It has been shown that a high-fiber diet was associated with a reduced incidence of mammary cancer (68). The effects of phytoestrogens have been controversial. Several studies showed that soy intake may reduce the breast cancer risk of premenopausal (69,70) and postmenopausal women (70), while others found no association between dietary soy intake and breast cancer (71).

1.1.3.5 Environmental Compounds

Both natural and synthetic environmental estrogens have been shown to mimic the estrogenic activity of steroid hormones. Phytoestrogens in the diet, as discussed above, antagonize estrogen action and protect individuals from tumor formation. On the other hand, it has been suggested that environmental

estrogens, such as organochlorine pesticides, PCBs, alkylbisphenols, alkylphenols and phthalates, act as estrogen agonists and increase the risk of breast cancer (47). However, the results are still controversial. Soto and coworkers first showed that several organochlorine compounds including PCB congeners, endosulfan, toxaphene, dieldrin, phthalates and various phenolic compounds were oestrogenic in breast cancer cells (72-74). An earlier study showed that aldrin and dieldrin were not estrogenic in a uterotrophic assay (75). Some of these weakly estrogenic pesticides even showed anti-estrogenic activities in some in vivo assays. When rats were cotreated with estrone plus chlordane, *p,p'*-DDD, *p,p'*-DDE, toxaphene, dieldrin, heptachlor and lindane, estrone-induced uterine wet weight increase was significantly inhibited (76). A number of studies reported associations between environmental estrogens and increased risks of human breast cancer. Elevated levels of polychlorinated biphenyls (PCBs) were found in fat samples from women with cancer, compared with those who had benign breast disease, and this suggested a role for environmentally derived estrogens in the genesis of mammary carcinoma (77). In another study, after adjustment for first-degree family history of breast cancer,

lifetime lactation, and age at first full-term pregnancy, conditional logistic regression analysis showed a four-fold increase in relative risk of breast cancer for an elevation of serum DDE concentrations from 2.0 ng/mL (10th percentile) to 19.1 ng/mL (90th percentile), suggesting a strong association of DDE with breast cancer. The estrogenic activities of most environmental compounds are at least 1000 times less than E2. However, it was uncertain if combinations of various compounds found in the environment could act synergistically. Synergistic interactions initially reported by Arnold and coworkers (78) were not observed (79,80). Most recent studies showed no association of PCBs/DDE exposure with breast cancer risk (81). A nested case-control study examined serum concentrations of DDE, PCBs and the development of breast cancer up to 20 years later. Results showed that exposure to relatively high levels of DDE or PCBs did not result in an increased risk of breast cancer (82). Other independent case-control studies also showed that there is no direct association of environmental DDE or PCBs exposure with female breast cancer risk (83-85).

1.1.4 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) (86). For EBC, the primary goal for treatment is to prevent recurrence and prolong overall survival; while for ABC, the major treatment aim is to achieve a sustained duration of response to treatment, alleviate symptoms and maintain the quality of life with minimal toxicity (87).

In 1896, Beatson first reported that ovarian function was always associated with the growth of human breast cancer (88). Subsequently, hormonal manipulation has played an important role in the treatment of breast cancer and more targeted therapies have been developed in recent years. Since the 1940s, high-dose estrogens (e.g. diethylstilbestrol) have been used in the breast cancer patients (89). Hormone therapies have shown efficacy. However, they are associated with side-effects, including cardiac toxicity, edema, vomiting, and vaginal bleeding (90).

The ovaries are the major source of estrogen in premenopausal women. After menopause, ovarian function is reduced and residual estrogen is mainly produced in peripheral tissues, through conversion of androgens to estrogens via aromatase. These are the foundations of distinct strategies developed for treatment of premenopausal and postmenopausal women with breast cancer. In premenopausal women, ovarian ablation is the most effective way of decreasing

estrogen levels, while in postmenopausal women, aromatase inhibition and estrogen receptor (ER) antagonism are the major targets for treatments.

Antiestrogens bind to the ER, and block the binding of endogenous estrogen, and thus block expression of estrogen-responsive genes involved in tumor growth and progression. Tamoxifen was the first selective ER modulator (SERM) and was introduced in the 1970s. This antiestrogen showed similar anti-cancer efficacy and an improved safety profile compared to high-dose estrogen therapies (91). Research has shown that tamoxifen reduced the risk of breast cancer recurrence in women with EBC (92) and it also slowed the progression of the disease in women with ABC (93). Tamoxifen treatment is also associated with side-effects, including an increased risk of endometrial cancer and thromboembolic events (92,94). Patients that initially respond to tamoxifen may eventually develop resistance.

In the last decade, aromatase inhibitors (AIs) have been used in the treatment of breast cancer in postmenopausal women. Aminoglutethimide was one of the earliest AIs to be tested in patients with breast cancer. However, it may cause serious side effects such as sedation (95). Steroidal injectable AI, fomestane was a second-generation AI, which was tested in phase II trials, but its usage was limited due to its toxicity (96). The third-generation AIs include non-steroidal agents, such as anastozole and letrozole, and also the steroidal exemestane. Anastozole and letrozole are at least as effective as tamoxifen for treatment of postmenopausal women with ABC (97) (98), and anastrozole was better tolerated than tamoxifen. These drugs were also shown to be effective for treatment of tamoxifen-resistant disease (99,100). The most common side effects associated with anastozole and letrozle are bone pain, dyspnoea, nausea, vomiting and abdominal pain (100).

1.2 Transcription

The genome has all the instructions or genes that define an organism. The hereditary information stored in an organism's genome produces the simplest, unicellular bacterium with about 500 genes, while the development of a human requires information encoded by approximately 25,000-30,000 genes (101). Each gene encodes the information to synthesize a particular protein (via mRNA) or tRNA or rRNAs. Both constitutive and inducible gene expression (by a variety of signaling pathways) are controlled by transcriptional regulators, activators and repressors. Protein-DNA interactions are the basis for recognition of DNA elements by transcription activators or repressors. The initiation of transcription is facilitated by the recruitment of the transcription machinery by the DNA-bound activator. Binding sites for transcription regulators are not necessarily confined to precise locations near transcriptional start sites, and they can be thousands of bases upstream or downstream of the transcriptional start site as long as they are physically close to the start site due to the compaction of chromatin (102). The transcription cycle can be divided into a number of distinct but interacting steps (Fig. 6). Early steps in transcription initiation have been studied in great detail, since they are important targets for transcription regulation in response to various signals (103,104).

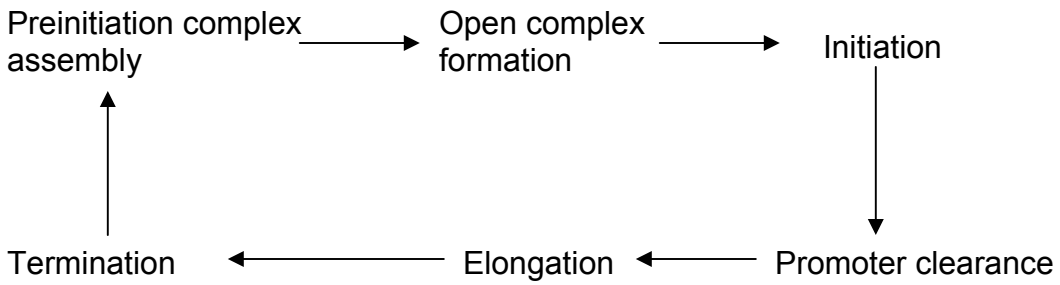

Fig. 6 The transcription cycle (103).

1.2.1 Chromatin Structure

DNA in eukaryotes is highly condensed and tightly bound to an equal mass of histone proteins. Nucleosomes are the fundamental repeating units of chromatin. A nucleosome is comprised of 146 bp of nucleosome core DNA that wraps 1.65 turns around an histone octamer, the linker DNA between adjacent histone octamers, and histone proteins that bind the linker DNA and nucleosome core (Fig. 7) (105). Crystal structure data indicates that histones form a cylinder consisting of two heterodimers of histones H3 and H4 and two heterodimers of histones H2A and H2B (106).

DNA packaging has been regarded as a general deterrent to transcription (107,108). In vitro experiments with bacterial and eukaryotic polymerases and

chromatin templates showed that nucleosomes inhibited transcription initiation (109,110). Depletion of histone subunits in yeast resulted in increased transcription of genes (111,112).

Nucleosomes can also potentiate gene expression. Genome-wide expression analysis in yeast showed that reduction of nucleosome density leads to increased expression of some genes and decreased expression of some others (113). Estrogen-regulated transcription from the Xenopus vitellogenin B1 promoter was potentiated by generation of a nucleosome-dependent static loop (114).

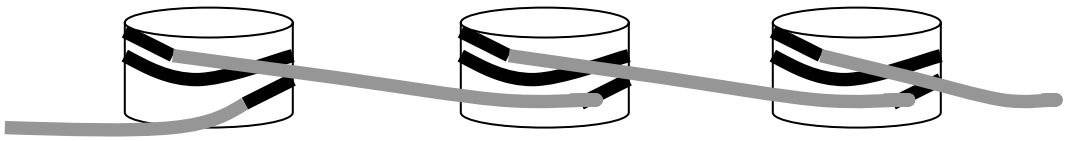

Fig. 7 Schematic of nucleosome structure (105). Histone octamer is shown as a disk, nucleosome core DNA as a black ribbon and linker DNA as a grey ribbon.

1.2.2 Promoter

The promoters of genes transcribed by RNA polymerase II have been traditionally described as consisting of two distinct regions (Fig. 8). The minimum promoter region is comprised of essential core promoter elements where the transcriptional machinery is recruited and transcription is initiated. The more distal promoter regions are highly variable and play an important role in regulation of gene expression by binding gene-specific transcription factors (115).

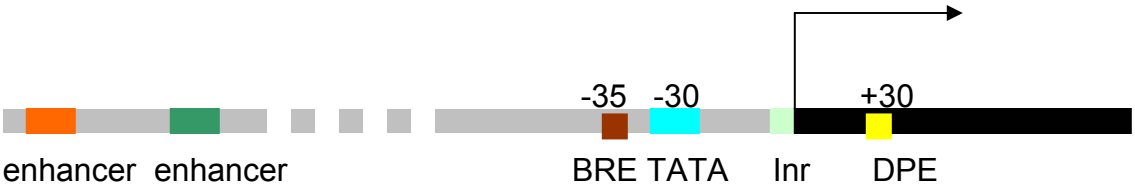

Fig. 8. Promoter structure.

The core promoter elements usually cover approximately 100 bp and contain the transcription start site. A TATA box is a critical feature of many core promoters, and it is recognized by the TATA-binding protein (TBP) subunit of transcription factor, TFIID (116). A typical strong TATA box sequence is TATAAAA and is located 25-30 bp (~40-120 bp in yeast) upstream of the transcription start site (117). A weak TATA box might have one or more nucleotide substitutions in the DNA element. Another core element is the initiator (Inr). The initiator was originally characterized as a sequence with a pyrimidine (Py) rich consensus: PyPyANA/TpyPy, with the 'A' nucleotide located at position +1 (118). More complex initiators include additional downstream recognition sequences for gene-specific regulators such as YY1 (yin yang I) (119), TFII-I (120), E2F (121), upstream stimulatory factor (USF) (122), and TAFII150 subunit of TFIID (123). Both TATA box and the initiator may work independently or cooperatively (124). Composite promoters, which contain both TATA and Inr elements, are found primarily in viral genes, while most class II genes contain TATA-directed promoters and some contain Inr-directed promoters(125). There are also core promoters that do not contain TATA or Inr elements. These are called null promoters (126) and often have multiple transcription start sites (127,128). BRE (TFIIB-recognition element) was first characterized as a sequence recognized by TFIIB (129), and TFIIB functions together with TBP to direct transcription by RNA polymerase (130). Another core elements, DPE

(downstream promoter element), probably serves as a binding site for TAF (TBP-associated factor) subunits of the general transcription factor TFIID (131).

Transcriptional regulators bind to DNA sequences in the promoter that have been called UAS (upstream activating sequences), URSs (upstream repressing sequences), enhancers and silencers. Enhancers were originally identified as cis-acting elements whose functions were to enhance transcription independent of their orientation and distance to the transcription start site (132). Enhancer activities have been associated with multiple transcription factors. One model suggests that transcriptional activation through enhancers is a multi-step process. First, activator binds to a specific DNA sequence at the enhancer. Then, DNA-bound activator binds and recruits numerous co-regulatory proteins, including coactivators and chromatin remodeling factors. The protein-DNA complex is stabilized and promoter access by the general transcription machinery is increased (133) (Fig. 9).

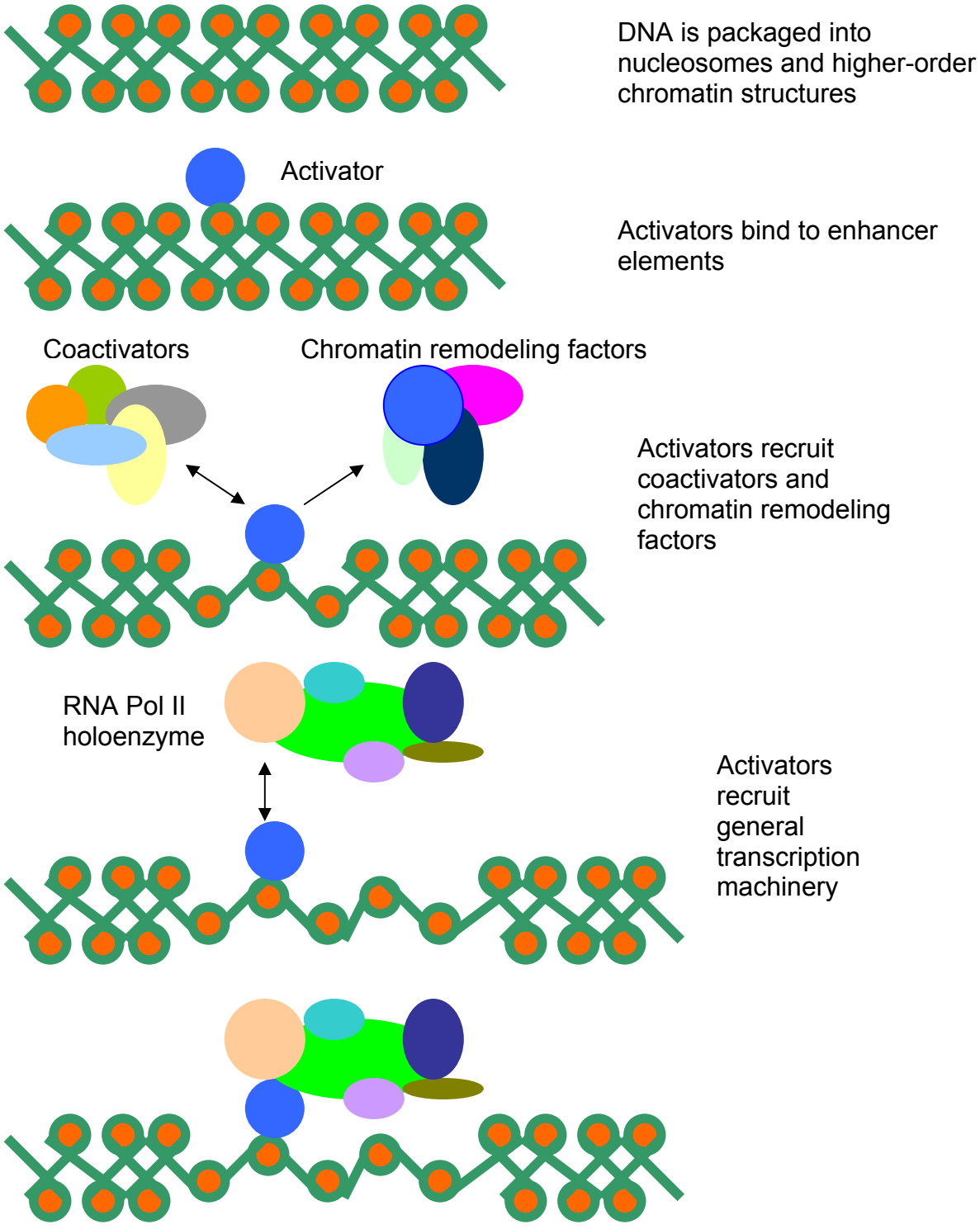

Fig. 9 A model for activators in transcription initiation(125).

1.2.3 RNA Polymerase II-directed Transcription

1.2.2.1 RNA Polymerase II Structure

RNA polymerase II has a molecular weight of >0.5 MDa and consists of 12 subunits that are highly conserved in eukaryotes. Rpb4 and Rpb7 are dissociable from the other 10 subunits and deletion of these two subunits leaves the holoenzyme incapable of activating promoter-directed transcription initiation in vitro (134).

X-ray diffraction data suggested a backbone model of a 10-subunit yeast Pol II with a pair of jaws, a clamp and a pore. Subunits Rpb1, Rpb5 and Rpb9 form the pair of jaws, and they can grip DNA downstream of the active center. The clamp on the DNA near the active center is formed by subunits Rpb1, Rpb2 and Rpb6, and it allows entry of straight promoter DNA for transcription initiation and contributes to the stability of the transcribing complex. Three loops outside the clamp may play roles in RNA unwinding and DNA rewinding. The pore is beneath the active center, and it allows entry of the substrates and exit of transcripts. The rim of the pore includes a loop of Rpb1 that binds a Mg^{2+} ion with three aspartate residues. A flexible 100-residue linker protrudes out of the clamp base and connects to the C-terminal domain of Rpb1, where RNA exits (135,136).

The crystal structure of elongating complex revealed that differences between the transcribing complex and free enzyme include closure of the clamp over the DNA and RNA and creation of a binding site complementary to the

DNA-RNA hybrid at the base of the clamp (137). The structure of the 12-subunit yeast Pol II in solution calculated from electron microscopic images of a single molecule found that the Rpb4/Rpb7 subunit complex was ideally positioned to determine the path of the nascent RNA transcript (138). The crystal structure of the complete 12-subunit RNA Pol II further explained the role of Rpb4 and Rpb7 in maintaining the conformation and regulating RNA binding and transcription through interactions with general transcription factors, mediators and CTD phosphatase (139,140).

The largest subunit of RNA polymerase II (Rpb1) has a unique extension of the carboxyl-terminal domain (CTD) that contains tandem repeats of the seven-amino acid sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (141). The CTD consensus sequence is a highly conserved structure in eukaryotes and the number of repeats varies from 26 in yeast to 52 in mice (142-144). CTD phosphorylation has been related to the transition from transcription initiation to elongation. RNA polymerase lacking CTD phosphorylation is commonly detected in preinitiation complexes (PIC) (119,145-147), while heavily phosphorylated CTDs were primarily found in elongating polymerase (148-150).

Two cyclin-dependent kinases, Cdk7 and Cdk8, are components of the PIC and catalyze CTD phosphorylation (151-154). Phosphorylation by Cdk7, a subunit of the general transcription factor TFIIH, has been shown to be important for enhancing elongation (155,156). Conditional mutations in the yeast homologue of Cdk7 (Kin28) caused a complete loss of transcription (157). The

yeast homologue of Cdk8 and Srb10, inhibits transcription after uniquely phosphorylating the CTD prior to the initiation of complex formation (158). Another study in yeast showed that both homologues of the two kinases, Kin28 and Srb10, could promote transcription in vitro and in vivo, and inhibition of both kinases resulted in a 70% loss of transcription (152).

The structural basis for CTD action is unclear. The X-ray crystal structure of a Pin1-bound single copy of doubly phosphorylated CTD sequence (Tyr-P.Ser-Pro-Thr-P.Ser-Pro-Ser) was solved. The CTD was shown to bind as an extended coil (159). The structure of a four heptad Ser-5 phosphorylated CTD with a RNA guanylytransferase Cgt1 has also been reported. A distinct docking site was localized to the Cgt1 N-terminal domain and multiple residues in the sequence contribute to the extensive contact between the two peptides (160). Comparison of the two studies suggested that the CTD form markedly different conformations depending on the specific binding partner. The flexibility of CTD structure, combined with covalent phosphorylation, enables the CTD to interact with multiple structurally different protein partners (104).

1.2.2.2 General Transcription Factors

TATA binding protein (TBP), a subunit of TFIID, is the central component of the eukaryotic transcription machinery. X-ray crystallography showed a highly symmetric alpha/beta structure containing a DNA-binding fold. This fold resembles a molecular saddle (161,162) that is bound to the widened minor groove of an 8 bp TATA element, unwinding about a third of a helical turn and

bending the DNA toward the major groove (163,164). TBP does not bind to promoter elements with high orientation specificity, suggesting that TBP alone cannot define the orientation of general transcription factor assembly on a promoter (165).

TBP-associated factors (TAFs), copurified with TBP in the TFIID fraction, were originally described as coactivators (166,167). TAFs are required for transcription from TATA-less promoters in vitro (168-170). TAFs specifically bind the Inr (171), and if together with other initiator-dependent cofactors (TICs), can mediate core promoter selectivity (172). Core promoter selective effects were also observed in several studies, showing that TAFs can both inhibit and enhance TBP function, depending on the context of the core promoter elements (173-175).

TFIIA binds TBP and stabilizes TBP-DNA interactions (176,177). Crystal structure analysis showed that TFIIA is heterodimer composed of two domains, the beta-barrel interacts with TBP-DNA complex and the four-helix bundle motif projects away from TBP-TATA and provides a binding surface for other factors (178,179). Interaction between TFIIA and TBP is critical for transcriptional activation of some genes, since either TBP or TFIIA mutants with compromised ability to bind each other, results in significantly impaired transcription in response to activators (180,181). TFIIA mediates transcriptional activation by multiple activators, including Zta, Sp1, VP16 and NTF-1 (182,183). TFIIA may

also function as a derepressor by blocking effects of several negative transcriptional regulators through TBP (184-187).

TFIIB was originally divided into two functional domains, with the relatively stable C-terminal domain interacting with TBP on DNA, and the unstable N-terminal domain responsible for recruitment of RNA polymerase to the promoter (188,189). Mutations in TFIIB shift the transcription start site (190) and markedly diminish TFIIB binding to the polymerase (191). Crystal structure data showed that the C-terminal core domain of TFIIB recognizes the preformed TBP-DNA complex through protein-protein and protein-DNA interactions (192). The N-terminal region contains a zinc ribbon fold where TFIIB interacts with the polymerase and a highly conserved homology block that forms a finger loop inserted into the polymerase active center to determine the transcription start site (193-195).

TFIIF binds tightly to RNA polymerase II and stabilizes the preinitiation complex, like bacterial σ factor (196,197). TFIIF can induce isomerization of the preinitiation complex, including TFIIE, by tightly wrapping the promoter DNA for almost a full turn around Pol II, and TFII H can further tighten the wrap around Pol II (198,199).

TFIIH can be divided into two subcomplexes, core TFIIH and a kinase/cyclin subcomplex (125). Core TFIIH plays dual roles in DNA excision repair and in transcription by Pol II (200-203). TFIIH is required for promoter opening (204). The TFIIH kinase/cyclin pairs are Cdk7/cyclin H in humans and Kin28/Ccl1 in yeast that phosphorylate serines in the RNA polymerase II CTD (154,205).

TFIIE functions are closely related to TFIIH (125), and TFIIE might be important for TFIIH recruitment (206), serving as a checkpoint for preinitiation complex formation and stimulating TFIIH-dependent kinase and helicase activity (207-210).

1.3. Sp-family Proteins

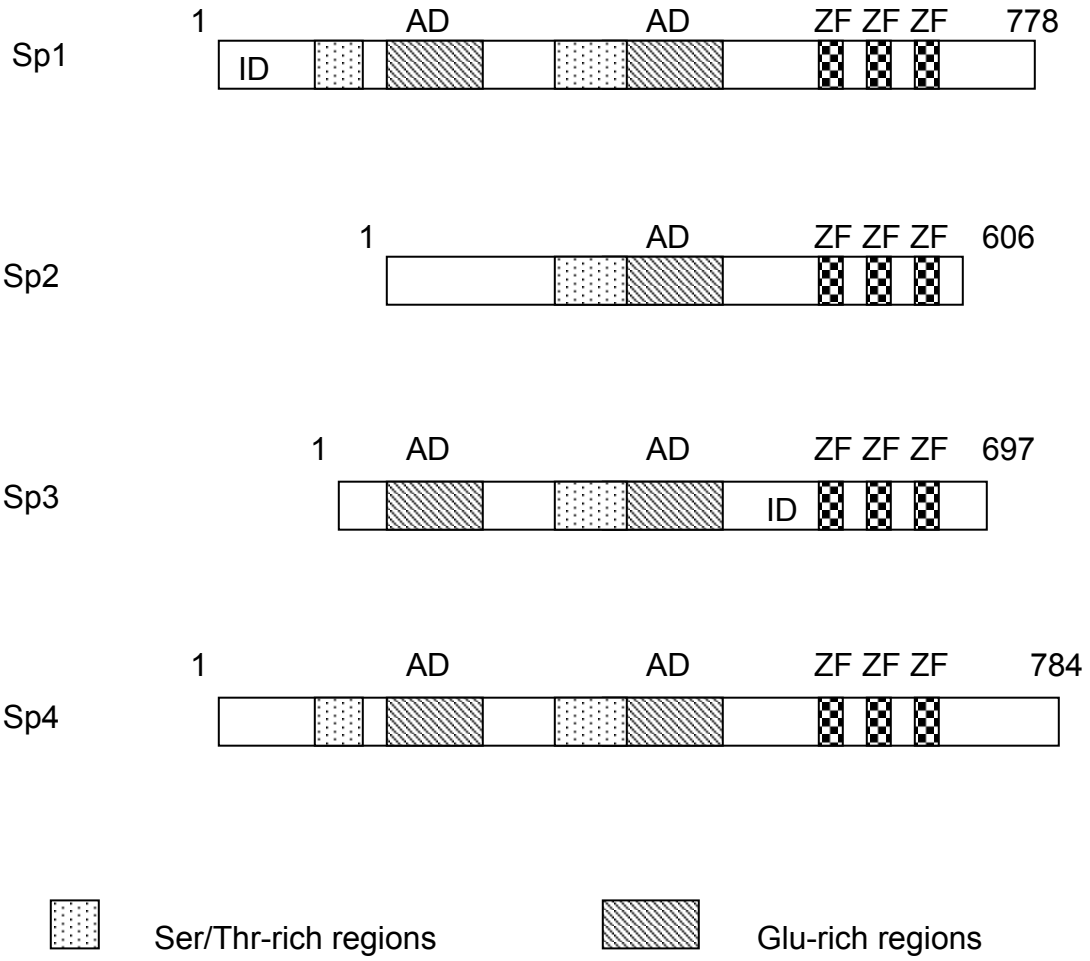

Fig. 10 Structural features of Sp-family proteins (211,212). AD, activation domain; ID, inhibitory domain; ZF, zinc finger.

1.3.1 Sp1

The first identified specificity protein (Sp) family member, Sp1, is a transcriptional activator that binds to a GC-rich promoter sequence, known as a "GC box", in the simian virus 40 (SV40) early promoter and selectively activates transcription at functional recognition sites (213,214). Subsequent studies showed that coordination of Sp1 and other transcription factors binding to CCAAT sequences in the promoter is required for optimal expression of the thymidine kinase (TK) gene (215).

Sp1 was cloned using HeLa cells, and the DNA binding activity of Sp1 was localized in the C-terminal 168 amino acids, which consisted of 3 zinc finger motifs (Fig. 10) (216). Mutations of Sp1 revealed multiple regions for transcriptional activation, and glutamine content was high in the two most active domains (217). The synergism of transactivation associated with multiple Sp1 binding sites was examined, and it was shown that the glutamine-rich activation domains A and B and the C-terminal domain D are required (218,219). Local self-interaction of Sp1 proteins binding proximal and distal GC boxes was confirmed by observation of a DNA-loop by electron microscopy (220,221). Post-translational modifications of Sp1, including glycosylation, acetylation and phosphorylation, may play a role in mediating Sp1 regulated transcription (222,223).

Sp1 interacts with various nuclear transcription factors. The two glutamine-rich activation domains can bind the C-terminal conserved domain of TBP (224),

the glutamine-rich activation domain of TAF110 (225), and the DNA-binding domain of TAFII55 (226). Association of the retinoblastoma-related protein p107 and Sp1 represses Sp1-dependent transcription (227). Physical interaction of Inr-binding protein YY1 and Sp1 may result in synergistic activation of YY1-directed transcription (228,229). Interaction of Sp1 and the cell cycle regulator E2F1 has also been reported (230,231). In order to identify other cofactors for Sp1-directed transcriptional activation, a large complex CRSP (cofactors required for Sp1) was characterized and the complex contained at least nine subunits ranging from 33 to 200 KDa (232). Sp1 binding to nucleosomal SV40 early promoter DNA was shown to be 10-20-fold greater than naked DNA, suggesting that it is important for assembly of active chromatin structures (233).

Sp1 is ubiquitously expressed in mice with highest levels in developing hematopoietic cells, fetal cells, and spermatids, indicating the association of elevated Sp1 levels with differentiation processes (234). Sp1 plays an important role in controlling cell cycle regulated genes such as thymidine kinase (215), B-myb (235) and dihydrofolate reductase (231). The activity of Sp1 may be regulated by retinobastoma protein p107 (227) and G1-specific cyclins (236). In addition, Sp1 binding sites are required to prevent methylation of CpG islands (237,238).

Inactivation of the Sp1 gene in mice showed that $Sp1^{-/-}$ embryos are developmentally retarded, exhibit a broad range of abnormalities and die around day 11 of gestation. However, Sp1 knockdown did not affect expression of many

putative target genes, including cell cycle-regulated genes, the methylation-free state of CpG islands and the active chromatin structure formation. The only clear genomic difference in the Sp1-null embryos was the greatly reduced expression of the methyl-CpG-binding protein MeCP2. These observations suggest that Sp1 is dispensable for growth and differentiation of primitive cells, and it is critical in the maintenance of differentiated cells probably through transcriptional regulation of genes like MeCP2 (239).

1.3.2 Sp2 and Sp3

Sp2 and Sp3 were identified for their binding to a GT box during analysis of the T-cell antigen receptor alpha promoter (240). Both Sp2 and Sp3 contain zinc finger motifs and transactivation domains similar to those of Sp1 (Fig. 10), and they are also expressed ubiquitously. Sp3 also binds to GC boxes. No functional analysis of Sp2 has been reported and little is known about this Sp-family member (211).

Like Sp1, Sp3 activates transcription in cotransfection experiments in Drosophila SL-2 cells, which lack Sp1 and Sp3 proteins (241-244). Other studies reported that Sp3 can repress Sp1-mediated transactivation (245-247). It is possible that the inhibitory effects of Sp3 on Sp1 may be due to its competitive binding to the same promoter element (211,212). Another hypothesis is that Sp3 may actively repress Sp1-mediated transcription activation through an active repressor domain (212). Domain analysis showed that glutamine-rich domains of Sp3 have a strong activation function and interact with TAFII110, but are

silenced by an inhibitory region with a stretch of highly charged amino acids (248). Gene fusion experiments showed that Sp3 repressor activity was mapped to a 72-amino acid region located to the amino-terminal side of the zinc-finger DNA-binding domain (249). It was shown that the Sp3 repressor domain is independent of the DNA-binding domain. Fusion proteins containing this repressor domain and a heterologous DNA-binding domain repress transcriptional activation by various activators, suggesting that Sp3 may act as a transcriptional repressor via protein-protein interactions with components of the general transcription machinery (250).

It was postulated that Sp3 is a dual-function regulator and its role as a transcriptional activator or repressor may be dependent on both promoter- and cell-context (211,212). In a study of a promoter with long terminal repeats (LTR) of the HERV-H family retrovirus-like elements in the endogenous human genome, it was reported that Sp1 and Sp3 both bind to the GC/GT box required for promoter activity and stimulate transcription in the teratocarcinoma cell line Ntera2D-1, while in HeLa and Drosophila SL-2 cells, Sp1 activates transcription, but Sp3 represses Sp1-mediated transcriptional activation (251).

Since Sp1 and Sp3 are both ubiquitously expressed, transcriptional regulation of Sp1- and Sp3-mediated genes are also dependent on the relative abundance of these proteins (211). It was shown that the kinase domain receptor (KDR) promoter activity was 10-30-fold higher in endothelial cells (EC) than non-endothelial cells and Sp1/Sp3 ratios in EC were 2-10-fold higher. The

attenuation of Sp1-mediated KDR promoter activation by Sp3 overexpression suggested that the Sp1/Sp3 ratio might play an important role in KDR gene regulation (252). Regulation of epithelial-specific human papillomavirus type 16 (HPV-16) expression involves Sp1 activation and Sp3 antagonism. During epithelial differentiation and cellular transformation, high Sp1/Sp3 ratios in epithelial cells was thought to correlate with activation of the HPV-16 promoter (253). Another study showed that in keratinocyte differentiation, the induction of the p21 promoter was enhanced by Sp3 overexpression, and Sp1 and Sp3 both contributed to the basal activity of the p21 promoter (254). Transcriptional activation of the muscle-specific pyruvate kinase-M and β-enolase promoter in response to hypoxia was also shown to involve Sp1 and Sp3. Hypoxia causes the progressive depletion of Sp3, whereas Sp1 protein levels remain unchanged, and this resulted in decreased Sp3-mediated repression (255).

1.3.3 Sp4

Sp4 was identified along with Sp3, by binding to a GT1 element in the uteroglobin promoter (256). Comparison of Sp1, Sp3 and Sp4 as activators on the LTR of the HIV-1 promoter showed that they perform differently in transcriptional activation. Interaction of Sp1 with DNA-bound NF-κB is required

for HIV-1 gene expression. Sp4 is an activator, and Sp3 represses basal expression (247). Sp4-directed transactivation is distinct from Sp1, because Sp4 is unable to synergistically activate transcription through interaction with other proteins at adjacent DNA binding sites (247,257). Mapping of the synergistic activation of Sp1 showed that the glutamine-rich activation domains A and B and a C-terminal domain D are all required (219). The absence of D-domain in Sp4 may account for the lack of synergistic activation (212).

Northern analysis showed that Sp4 is abundant in the brain but barely detectable in other organs (256). In situ hybridization showed that Sp4 is highly expressed in mouse embryos in the developing central nervous system (CNS). As early as day 9 of early development, Sp4 expression was observed in the posterior neuropore. In later embryos, Sp4 expression was detected throughout the CNS, as well as in other structures. In Sp4 knockout mice, it was shown that Sp4 is required for male reproductive behavior. Two-thirds of homozygous mutants die within the first few days, and homozygous male mutants do not breed (258).

1.4. Estrogen Receptor (ER)

1.4.1 Nuclear Receptor Superfamily

The nuclear receptor superfamily is the largest group of eukaryotic transcription factors and the 48 family members have been organized into three groups based on the chemical similarity of their ligands (259). The steroid hormone receptors consist of the androgen receptor (AR), mineralcorticoid receptor (MR), ER, glucocorticoid receptor (GR) and progesterone receptor (PR). Receptors that form heterodimers with RXR include the thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic-acid receptor (RAR), 9-cis-retinoic-acid receptor (RXR) and ecdysone receptor (EcR). The third group, orphan receptors, is composed of a large number of genes whose physiological ligands are unknown, such as peroxisome proliferator activation receptor (PPAR), steriodogenic factor 1 (SF-1), nerve growth factor-induced receptor (NGFI), chicken-ovalbumin-upstream-promoter transcription factor (COUP-TF).

The nuclear receptors regulate development and metabolism through control of gene expression in response to ligands (260). The adrenal steroids that act through GR and MR, regulate body homeostasis, control glycogen and mineral metabolism and also mediate the stress response. The sex steroids activate ER and AR and stimulate development and determination of the embryonic reproductive system, control reproduction and reproductive behavior in the adult and regulate development of secondary sexual characteristics. Vitamin D activates the VDR and is important for normal bone development,

calcium metabolism and bone differentiation (261). In order to understand how small and simple molecules can induce these diverse and complex responses, steroid and thyroid hormone receptors were the first identified in the early 1970s through the use of radiolabeled ligands (262-264). In the 1980s, cDNAs encoding steroid hormone receptors were first cloned (260,265).

Hormone-specific responses are mediated by interactions of nuclear receptors with their cognate hormone-responsive elements (HRE) (266). The first HRE was identified for the GR by mutational analysis of mouse mammary tumor virus (MMTV) promoter (260). The GRE consists of two short, imperfect inverted repeats separated by three nucleotides (267). Response elements for progesterone, mineralocorticoid, and androgen receptors were identified later, and shown to be similar to that of GR (267-273).

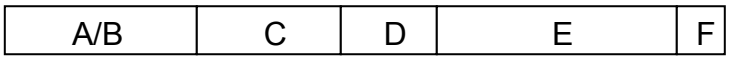

Fig. 11 Functional domains of NRs (274).

The letter names A/B, C, D, E and F designate the different regions of NRs which define specific structural domains (Fig. 11) (259,274). The N-terminal A/B domain is the least conserved domain across the superfamily, and this domain contains a transactivation function (AF1) (275-278). The C region is the most conserved region for the NRs, and contains the DNA-binding domain (DBD), and is also responsible for dimerization (279,280). The DBD consists of two zinc finger motifs that allow for the specific recognition of DNA. NMR and crystal structure analysis have shown that the DBD is structurally defined both in the presence and in the absence of DNA and can bind to DNA as a dimer (279-284). The D region is a variable hinge region. It contains a nuclear localization domain for GR and PR, and/or a transactivation domain for TR and GR (275,278,285-287). The E/F region is relatively large for most NRs and contains the ligand-binding domain (LBD). Other functions include protein-protein association, dimerization, nuclear localization and transactivation (276-278,286-300).

1.4.2 Estrogen Receptor Types and Functional Domains

The first clue that the estrogen receptor mediated the biological effects of estrogen came from observations that 17β-estradiol (E2) exhibited highly specific binding in the uterus (301). Two groups reported the cloning of ERα (302,303) in 1986 and ERβ was cloned in 1995 (304). The cloning of the receptor cDNAs enabled expression of ERα and ERβ proteins in in vitro mammalian (305) or yeast (306) cell systems. These techniques, as well as cell-free in vitro transcription assays (307), lead to characterization of the functional

domains of ER (308), which are similar to other members of the nuclear receptor superfamily (Fig. 11).

The N-terminal A/B domain is the least conserved region and there is only 17% identity between human ERα and ERβ (309). The A/B domain contains a ligand-independent constitutive activation function (AF1) (310-313). AF1 is the region where ER interacts with multiple proteins, including the p160 steroid receptor coactivator-1 (SRC1) and p300 (314-317), general transcription factor TBP (318) and other coactivator proteins such as p68 RNA helicase (319), MMS19 (320) and RNA coactivator SRA (321,322). The first 40 amino acids at the N-terminal region are not required for ER activity (313,323). AF1 is also responsible for ligand-independent activation of ER through several different kinase signaling pathways (324). Serine 118 is the target for phosphorylation by the MAPK pathway in response to growth factors (325-327). Serines 104 and 106 can be phosphorylated by the cyclin A-CDK2 complex (328).

The DBD of ER contains two zinc finger motifs, which play an important role in receptor dimerization and DNA recognition (329-331). The estrogen response element (ERE) was first identified from the Xenopus Vitellogenin A2 gene promoter, and contains a palindromic 5'-GGTCAnnnTGACC-3' sequence (332). The specific recognition between the DBD and the ERE is dependent on three amino acids in the so-called P-box (EGckA) located at the C-terminal side of the ER zinc finger 1 (333). The second zinc finger has the nuclear localization signal (334,335). The crystal structure of the ER DBD-DNA complex showed

that the protein binds as a symmetrical dimer to its palindromic binding site (336).

The hinge region or D domain separating the DBD and LBD contains sequences for receptor dimerization (305,337) and nuclear localization signals (334,335).

The E domain, or the LBD confers ligand specificity to the ER and also mediates receptor dimerization, nuclear localization and ligand-dependent gene transactivation (261,274,338). Crystal structure data showed that agonist and antagonist bind at the same site within the core of the LBD but exhibit different models of binding and induce distinct conformations of the AF2 of the LBD (339,340). The interaction of ligands with the LBD is dependent on the amino acid residues lining the surface of the ligand binding cavity from helix 3 to helix 12 (310,329,339,340). The structure and function of AF2 is dependent on the binding of ligands and subsequent interaction with coactivators or corepressors (341-346). The AF2 interaction surface consists of amino acids in helix 3, 4, 5, and 12. With agonist binding to the LBD, helix 12 of ERα is positioned over the ligand-binding pocket and forms the coactivator-binding surface. In contrast, with antagonist binding, helix 12 of ERα is removed from its agonist position and instead occupies the hydrophobic groove formed by helices 3, 4 and 5 (339,340,346,347).

The C-terminal F domain is unique to the ER among NRs. It was reported that the F domain of ER has a specific modulatory function that affects the

antagonist effects and the transcriptional activity of liganded ER (348). The F domain also contains an inhibitory sequence to the dimerization signal in the E domain (349). A recent study also reported that mutations in the F domain alter ligand-activated responses (350).

1.4.3 Estrogen Receptor-mediated Transactivation

1.4.3.1 Classical EREs

The first ERE was observed in the 5' flanking region of the Xenopus Vitellogenin A2 gene (332). However, most other EREs differ from the consensus sequence by one or more bases and these are designated as non-palindromic EREs (351-353). Other gene promoters containing functional EREs include Xenopus Vitellogenin A1 and B1 (354,355); chicken very low density apolipoprotein II (apo-VLDII) (356); and human genes encoding pS2 (357), the neuropeptide oxytocin (358), protooncogenes c-fos and c-myc (359,360), transforming growth factor-α (hTGF-α) (361), lactoferrin (362), prolactin (363) and PR (364).

ERα binding to an ERE induces DNA bending toward the major groove (365-370), which is important for facilitating interactions between multi-protein complexes bound to different sites and for promoting DNA looping.

1.4.3.2 ERα/Sp

1.4.3.2.1 ERE1/2(n)$_x$Sp1

Regulation of estrogen-mediated gene expression does not always rely on the functional consensus or nonconsensus EREs on gene promoters (371).

Analysis of the c-myc promoter identified a 116-bp E2-responsive region containing a GGGCA$(n)_{16}$GGCGGG sequence with a non-consensus ERE half site and a Sp1 binding GC-box (372). The DBD of ER was essential for E2-regulated transactivation of c-myc. In the promoter region of creatine kinase B (CKB), a similar motif was identified (GGTCA$(n)_{21}$GGCGGG) and shown to be involved in the E2-responsiveness (373). ER can bind to the ERE half-site (ERE1/2) and Sp1 binds to the GC box. Another ERE1/2$(n)_{23}$Sp1 motif was identified in the cathepsin D gene promoter, and gel mobility shift and transient transfection assays showed that both ERα and Sp1 binding were required for estrogen responsiveness (374). The E2 responsive ERE1/2$(n)_x$Sp1 motifs were also identified in heat shock protein 27 (Hsp27) (375) and TGFα (376) promoters. In gel mobility assays, a broad retarded band was observed with breast cancer cell nuclear extracts incubated with oligonucleotides containing ERE1/2(n)xSp1 motifs from either the cathepsin D, TGFα or Hsp27 gene promoters. However, incubation of the ERE1/2(n)xSp1 motif with recombinant ERα and Sp1 proteins did not give the retarded band. It was suggested that other nuclear proteins were also required to stabilize protein complex formation and facilitate interactions with basal transcription factors (377).

1.4.3.2.2 ERE Independent Action

Mutation of the ERE1/2 in the Hsp27 promoter did not result in the loss of E2 responsiveness, suggesting that the GC-rich site alone was sufficient for E2-responsiveness (378). In transient transfection studies, E2 induced reporter

gene activity in cells transfected with a DBD deletion mutant of ERα, suggesting that ERα binding to DNA was not required for ERα/Sp1 action through GC-rich sites. Kinetic analysis showed that ERα enhanced the on-rate of Sp1 DNA binding, but did not affect the off-rate (dissociation) of the Sp1-DNA complex.

Promoter analysis indicated similar mechanisms of ER/Sp1 action for several E2-induced genes including c-fos protooncogene (379), RARα1 (380), insulin-like growth factor binding protein 4 (IGFBP4) (381), bcl-2 (382), E2F1 (383,384), adenosine deaminase (ADA) (385), cathepsin D (386), thymidylate synthase (TS) (387), DNA polymerase α (DNAPα) (388), cad (389), cyclin D1 (390), epidermal growth factor receptor (EGFR) (391) and low density lipoprotein receptor (LDLR) genes (392). For vascular endothelial growth factor (VEGF), E2-decreased VEGF mRNA transcription was also mapped to GC-rich sites. Gel mobility shift and DNA footprinting assays showed that both Sp1 and Sp3 bound to this region of the VEGF promoter. Using the dominant negative form of Sp3, it was confirmed that Sp3 was required for this response (393). In ZR-75 cells, E2-induced activation of the same GC-rich region of the VEGF promoter was inhibited by transfection of small inhibitory RNAs for Sp1 and Sp3 (394).

Other transcription factors may also play important roles in ER/Sp1-mediated transactivation, and the mechanisms of regulation are dependent on promoter- and cell-context (371,377). For example, the c-fos, CKB, RARα, cathepsin D and TGFα promoters all contain Sp1$(n)_x$ERE/ERE1/2 motifs, but E2-induced transactivation is dependent on ERα/Sp1 interaction with GC rich

elements in breast cancer cells for the former 3 promoters, while for the latter two promoters, both ERα and Sp1 bind to their recognition elements. It was reported that E2-induced expression of E2F1 is dependent on different mechanisms in MCF-7 and ZR-75 breast cancer cells (384). In MCF-7 cells, induction of E2F1 by E2 is regulated by ERα/Sp1/NFY cooperation with both GC-rich and NFY sites. However, in ZR-75 cells, E2F1 is activated by genomic action of ERα/Sp1 with GC-rich sites and nongenomic cAMP-dependent phosphorylation of NFYA.

1.4.3.2.3 Domain Requirement of ERα for ERα/Sp1

Domain swapping experiments showed that the AF1 domain of ERα is responsible for ERα/Sp1 activation by E2 in cells transfected with pSp1 promoter and it was shown that amino acids 79-117 were important for this (395). On the LDLR promoter (ER/Sp1), mutations of AF2 or DBD of ERα have no effect on transactivation by ERα, but the deletion of AF1 completely abolished the transactivation, and amino acids 67-139 were shown to be important (392). The DBD of ERα was required for ERα/Sp1 activation of GC-rich promoters by antiestrogens 4-OH tamoxifen or ICI 182780, but not for ERα/Sp1 activation by E2 (377,396). ERαTAF1 carrying three point mutations in

helix 12 of the E domain of ERα (D538N, E542Q and D545N) has been reported to abolish E2-dependent activation of ERE promoters by disrupting the interacting interface of p160/SRC coactivators (274,397-400). However, ERαTAF1 still activated ERα/Sp1 by estrogens and antiestrogens (396). Further studies indicated that activation of ERα/Sp1 by E2 required the C-terminal F domain of ERα, which was not required for antiestrogen activation of ERα/Sp1. Overexpression of a C-terminal F domain peptide (aa. 575-595) specifically blocked E2-mediated ERα/Sp1 transactivation, suggesting that other nuclear cofactors interacting with F domain may be important for ERα/Sp1 action (396).

1.4.3.3 ERα and Coactivators

1.4.3.3.1 p160/SRC Coactivators

The p160/SRC coactivator family consists of three related members: SRC-1/NcoA-1, SRC-2/GRIP1/NcoA-2/TIF2 and SRC-3/ACTR/RAC3/AIB1/TRAM1 /p/CIP (401,402) (Fig. 12).

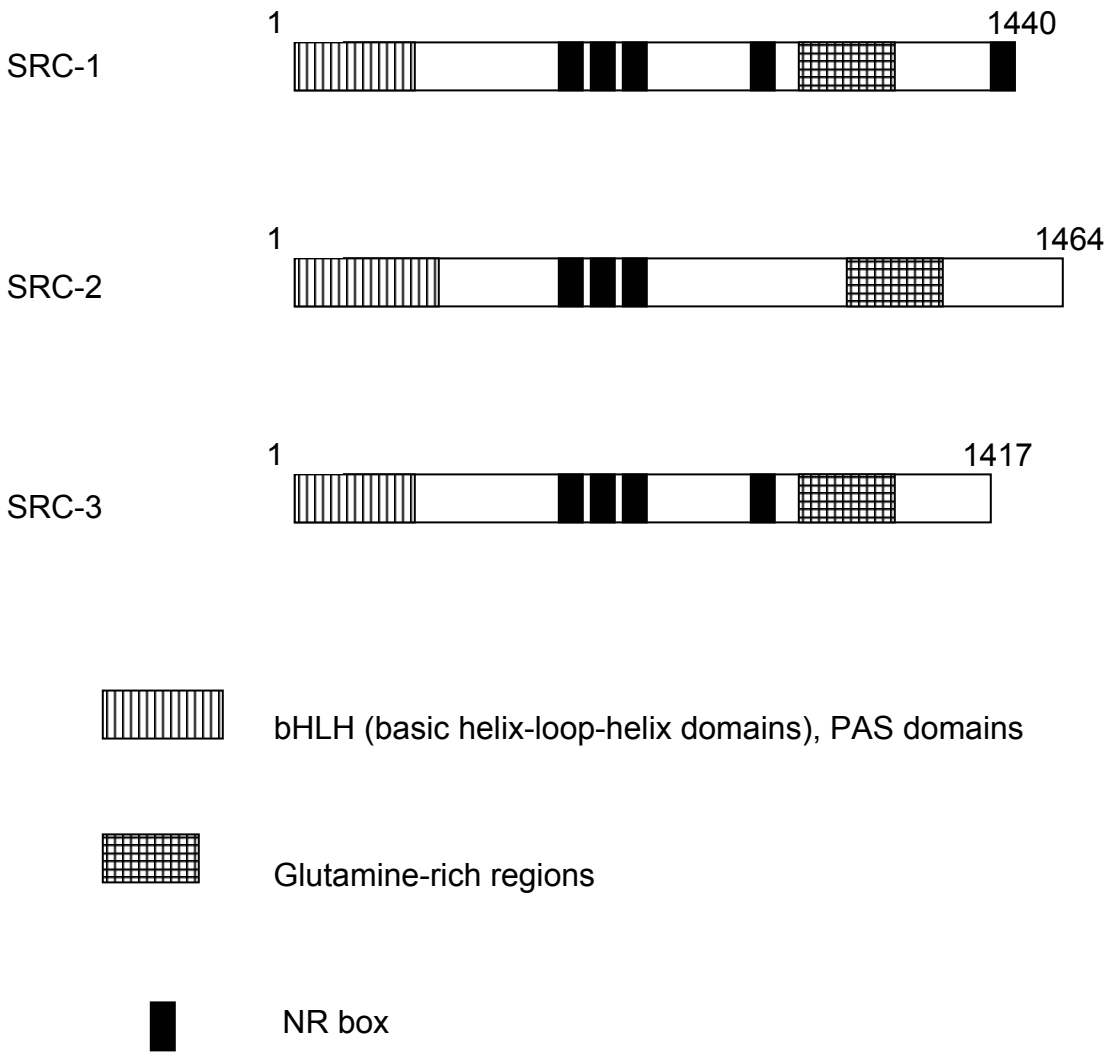

Fig. 12 Structural features of p160/SRC coactivators (324,401,402).

SRC-1 was cloned using a yeast two-hybrid system as a protein that interacts with and enhances hormone-induced transactivation using the PR LBD as bait. SRC-1 coactivates ligand-dependent transactivation of a broad range of nuclear receptors, including PR, GR, ER, TR, RXR, and coexpression of SRC-1 reversed the squelching ability of ER to PR (403). SRC-1 also coactivates PPARγ (404). SRC-1 facilitates interactions of AF1 and AF2 of ER (314). It was also shown that SRC-1 binding and enhancement of ER-mediated transactivation is dependent on the integrity of leucine-rich motifs LXXLL or NR boxes in SRC-1 and other coactivators which are required for interactions with key hydrophobic amino acids in helix 12 of the ER (405). ER AF1 activity also requires binding to SRC-1. Unlike AF2, which binds NR boxes in the central region of SRC-1, AF1 binds at the C-terminus of p160s (316). SRC-1 has intrinsic histone acetyltransferase activity (HAT) (406). The HAT activity of SRC-1 maps to its C-terminal region and is specific for histones H3 and H4. SRC-1 also interacts with other transcription factors, such as TBP and TFIIB, indicating that SRC-1 may act as a bridging factor between ER and the RNA polymerase II initiation complex (407).

SRC-1 and ERα were co-expressed in stromal cells. However, in the epithelium of the estrogen-responsive rat mammary gland, they are expressed in distinct subsets of cells. Moreover, treatments of these cells with E2 induced PR gene expression and suggested that SRC-1 was not necessary for ERα-

mediated induction of PR in mammary epithelial cells and was not sufficient for PR induction in stromal cells (408). Homozygous mutants from SRC-1 gene disruption were viable and fertile, but exhibited slightly decreased growth and development in response to E2 in estrogen target organs. SRC-1 knock out mice showed increased TIF2 expression, probably compensating for the loss of SRC-1 function in target tissues, providing in vivo evidence of partial functional redundancy between mSRC-1 and mSRC-2/GRIP1/mTIF2 (409). These results suggested that ERα and SRC-1 have distinct patterns of expression and function within the mammary gland and SRC-1 plays a cell-type specific role in ER action (324).

SRC-2 interacts with the ERα LBD in the presence of E2, but not 4-hydroxy tamoxifen. In two-hybrid assays, SRC-2 coactivated ERα in HeLa cells transfected with a construct containing a classical ERE and treated with E2 (410,411). In mammalian two-hybrid and GST pull-down assays, SRC-2 interacted with AF1 of ERα (316). Like SRC-1, SRC-2 also enhances transactivation by AR, ERα, PR, GR, TR, RXR, RAR, and VDR (410). Cocrystallization data showed that SRC2/GRIP1 interacts with the hydrophobic groove formed by amino acids from helices 3, 4, 5 and 12 of the ERα LBD. Antagonist binding to LBD resulted in helix 12 occluding the coactivator recognition groove by mimicking the interactions of the NR box peptide with the LBD (346).

SRC-3 represents AIB1 (412), ACTR (413), RAC3 (414,415), TRAM1 (416) and p/CIP, which were identified in independent studies (417,418). SRC-3 has intrinsic HAT activity, and it forms complexes with PCAF (p300/CBP-associated factor) and CBP/p300 (413). SRC-3 enhanced E2-induced reporter activity in CV-1 cells transfected with ER and SRC-3 (412), but did not stimulate ERβ-mediated transcription (419). SRC-3 can be acetylated by p300/CBP in vitro and in MCF-7 cells (420). Three lysine residues located immediately upstream of the LXXLL motifs of SRC-3 were acetylated and acetylation decreased SRC-3-ERα interactions in vitro. Interestingly, SRC-3 amplification and overexpression has been observed in four of the five ER-positive breast and ovarian cancer cell lines (BT-474, MCF-7, ZR-75-1 and BG-1). Higher expression of SRC-3 has also been detected in 64% of the primary breast tumors (412).

Three NR boxes are conserved in SRC coactivators, and an additional NR box is present in the C-terminus of hSRC-1 (401). The NR boxes in the central part of all three SRC coactivators are important for mediating interactions with nuclear receptors (403,411,414,417), and the nonconserved NR box motif in the C-terminus of hSRC-1 mediates the hormone-dependent interactions of hSRC-1 with PR (403). The mutation of key residues in the four NR boxes of hSRC-1 abolished interactions with AF2 of ER and this mutant failed to coactivate ligand-dependent activity of ER (405). It has been shown that distinct NR box motifs exhibit differential binding to different receptors (421). It was also reported that

residues immediately adjacent to the LXXLL motif regulate the affinity of the interaction with receptors, suggesting that flanking sequences at the interacting interface impart specificity of LXXLL motifs and their interactions with different receptors (342). It has been shown that other motifs are also involved in coactivator-receptor interactions. An FXXLL sequence was required for NSD-1 (nuclear receptor-binding SET domain-containing protein 1) to mediate interaction with NR LBDs (422); an FXXLF in several AR coactivators including ARA70/RFG, ARA55/Hic-5 and ARA54, was required for coactivation of AR (423), suggesting that other short sequence repeats may also be important for receptor-coactivator interactions.

1.4.3.3.2 CBP/p300

CREB-binding protein (CBP) was originally characterized as a coactivator required for efficient activation of the phosphorylated transcription factor cAMP-response element-binding protein (CREB) (424). CBP is a coactivator for multiple transcription factors, including p53 (425), NF-κB (426) and nuclear receptors (427-429). Interactions between CBP and nuclear receptors (RXR, TR or ER) are mediated by the N-terminal domain of CBP, which contains an NR-box (Fig. 13). CBP and p300 are considered to be “cointegrators” (401). CBP also interacts with SRC-1 (430,431), SRC-2 (432), and SRC-3 (417). CBP and SRC-1 synergistically enhance ERα-induced reporter gene activity in transient transfection studies (430,433) and CBP enhances E2-dependent target gene transcription through interaction directly with ERα at the N-terminus of CBP.

CBP interacts with NR LBDs in a hormone-dependent manner (427) and CBP also interacts with the basal transcription factor TBP (424) and RNA polymerase II (434), suggesting that CBP may serving as a bridging protein to recruit the general transcription machinery to NR-coactivator complexes.

CBP has HAT activity and it acetylates all four histones in nucleosomes (435,436). CBP also acetylates multiple nuclear proteins, including p53 (437,438), GATA-1 (439), HMG-I (Y) (440) and SRC-3 (420).

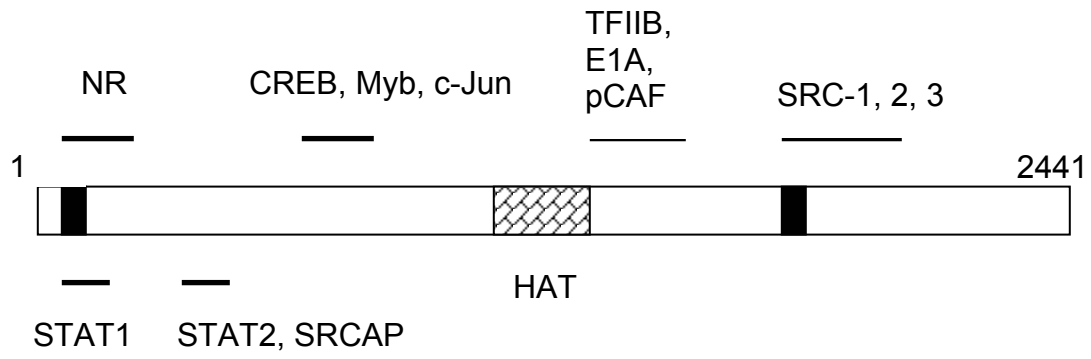

Fig. 13 Regions of CBP required for interactions with NRs, coactivators and other transcription factors (324).

1.5. DRIP205

1.5.1 Mediator Complexes

1.5.1.1 Yeast Mediator

Activators bind to specific distal enhancer element in the gene promoter to initiate transcription. It was proposed that other protein factors, termed mediators, were required for transcriptional activation in addition to the general transcription factors (441). SRB proteins were first characterized from genetic screens for suppressors of partial truncations in the CTD of Pol II (442,443). Addition of SRB proteins to the Pol II core enzymes and the general transcription factors form the RNA polymerase II holoenzyme. The RNA Pol II holoenzyme stimulated activator-regulated transcription, while polymerase and general transcription factors alone did not have this function, suggesting that the holoenzyme is the complex recruited to the promoter in vivo (444).

Mediator complex isolated from yeast contains about 20 proteins, including SRBs (SRB2, SRB4, SRB5, SRB6, SRB7), other genetically identified proteins and novel Mediator proteins (MEDs) (MED1, MED2, MED4, MED6, MED7,

MED8, MED11). Mediator complex enabled activated transcription and enhanced basal transcription and CTD phosphorylation efficiency (445). Analysis of free and elongating forms of RNA Pol II found none of the 15 subunits of the Mediator complex in the elongating fraction, suggesting that Mediator enters and leaves initiation complex in the transcription cycle (446).

Mediator consists of two functionally distinct complexes, Rgr1 (composed of two to three modules) and Srb4, each of which interacts with a distinct sets of Pol II and general transcription factors (447). Electron microscopic images of a single yeast holoenzyme-Mediator complex showed three domains (head, middle and tail) with Mediator contacts centered on the Pol II Rpb3/Rpb11 heterodimer, on the side opposite to the active site cleft (448). The modular organization (Fig. 14) of the yeast Mediator was proven in several studies, showing that SRB8, SRB9, SRB10, SRB11 formed one subcomplex (151,449). Genetically defined polypeptides GAL11, SIN4 and HRS1/PGD1 constituted another subcomplex anchored through Rgr1 (450), while the Mediator core is composed of the remaining polypeptides (451).

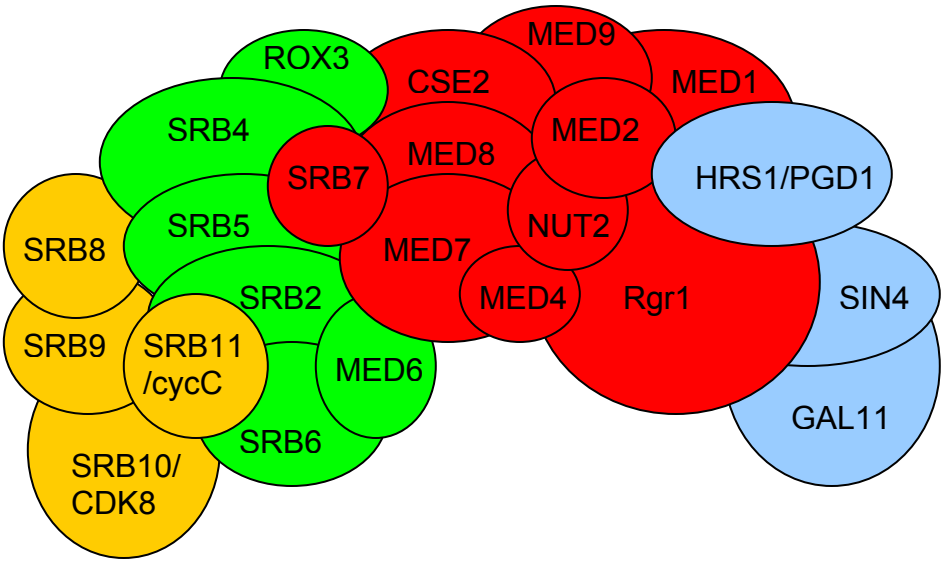

Fig 14 Modular organization of yeast Mediator complex (125,452).

1.5.1.2 Metazoan Mediator

The metazoan SRB/Mediator-like complexes that have been characterized include SMCC (SRB and MED cofactor complex), NAT (negative regulator of activated transcription), TRAP (thyroid hormone receptor associated proteins), ARC (activator-recruited cofactors), DRIP (vitamin D receptor interacting proteins) and CRSP (cofactors required for Sp1 activation).

The best characterized of these is the SMCC, which was isolated from human cell lines using epitope-tagged SRB7, SRB10 or SRB11 on the basis of their ability to mediate activation of GAL4 chimeras (453). SMCC is essentially identical to the coactivator complex TRAP (454), which was characterized several years earlier on the basis of its association and co-purification with ligand-bound thyroid hormone receptor (455). ARC (activator-recruited cofactors) was isolated through its ability to interact with the specific activator SREBP-1 (sterol-response-element-binding protein), and it synergistically potentiated gene activation by SREBP-1 and Sp1 (456). DRIP complex was characterized on the basis of its interaction with the ligand-bound vitamin D receptor ligand binding domain (457). CRSP contains the fewest subunits, and was first observed as a coactivator complex required for transcription activation by Sp1 (232). NAT was isolated by affinity purification of the human homologue of yeast SRB10 and it negatively regulates activated transcription, as observed for SMCC (153). The negative effect may be related to the repressive function of SRB10/CDK8 (157,158). Repression may also be related to the methods used for isolating NAT and SMCC, where the distinct SRB10/CDK8-SRB11/cyclin C complex was used as purification bait and could contribute to negative regulation (452).

The components of these complexes are compared in Table 1 (125,452,458). The shaded boxes represent common subunits in different complexes. The functions of the metazoan Mediator complexes and the

common components in different Mediator complexes indicate that these are highly related. This phenomenon suggests that these complexes may be also derived from a single metazoan Mediator-like complex in vivo, and the different isolation and purification steps may result in the characterization of complexes with different individual components. Another explanation for the variation of subunit composition is that metazoan Mediator complex exists as different forms, composed of some common protein factors plus specific proteins required for transcription in different cells and species (125). Recent proteomic analysis using multidimensional protein identification technology (MudPIT) suggests that most of the Mediator-related proteins identified in different laboratories are subunits of the Mediator-like complex (459).

The heterogeneity of the various Mediator complexes may reflect the modular organization of metazoan Mediator complex. The smallest complex CRSP does not contain the kinase-cyclin pair SRB10/SRB11, which is a component of the DRIP and ARC complexes, and CRSP also lacks other polypeptides like TRAP240 and TRAP230. It was hypothesized that CRSP might represent a relatively stable coactivator core of the larger Mediator-like complex (452).

Table 1 Subunit composition of mediator complexes (125,452,458).

Unified designation	TRAP/SMCC	DRIP	ARC	CRSP	NAT	Yeast SRB/Mediator
MED240	TRAP240	DRIP250	ARC250			SRB8
MED230	TRAP230	DRIP240	ARC240		p230	SRB9
MED220	TRAP220	DRIP205	ARC205	CRSP200		NUT1
MED150	TRAP170	DRIP150	ARC150	CRSP150	p150	Rgr1
	TRAP150α					GAL11
	TRAP150β	DRIP130	ARC130	CRSP130	p140	
			ARC105			
MED100	TRAP100	DRIP100	ARC100	CRSP100		SIN4
	TRAP97	DRIP97			p95	
MED95	TRAP95	DRIP92	ARC92			
	TRAP93			CRSP85	p90	SRB4
MED78	TRAP80	DRIP77	ARC77	CRSP77		
	TRAP78	DRIP70	ARC70	CRSP70	p70	Med1
CDK8	CDK8				p56	SRB10
					p45	Med2
			ARC42		p37	
	TRAP37			CRSP34		
MED36	TRAP36/28	DRIP36	ARC36			SRB5
MED34	hMed7	DRIP34	ARC34	CRSP33		Med7
cyclinC	cyclinC				cyclinC	SRB11
MED33	hMed6	DRIP33	ARC33			Med6
			ARC32		p30	Med4
	TRAP24				p23	Med8
	TRAP22				p22	Rox3
					p21	SRB2
MED17	hSRB7				hSRB7	SRB7
MED10	hNut2	hNut2			hNut2	Nut2
						Med11
						SRB6

1.5.2. DRIP205/ARC205/RB18A/TRAP220/PBP/Med220

Vitamin D receptor interacting protein 205 (DRIP205) was characterized as a component of the whole DRIP complex, including at least 10 subunit proteins ranging from 65 to 250 kDa (460). DRIPs from Namalwa B-cell nuclear extracts bind to immobilized vitamin D receptor (VDR) ligand binding domain (LBD) in a ligand-dependent manner. Functional analysis of the VDR-DRIP complex in cell-free transcription assays revealed that the DRIP complex enhanced ligand-dependent transcription by VDR/RXR. VDR-mediated activation was specifically depleted from the nuclear extract by liganded, but not unliganded, VDR LBD, and the complex stimulated cell-free transactivation by VDR RXR when added back to a transcription extract.

When the components of the DRIP complex were further identified, it was found that the DRIPs are almost indistinguishable from components of the ARC complex (457). The ARC complex consists of 16 or more subunits, and functions in a chromatin-selective manner to potentiate gene activation by the transcriptional activators SREBP-1a and Sp1. (456). Direct interaction of ARC and activators were demonstrated for several different activators, including SREBP-1a, VP16 and the p65 subunit of NF-κB, whereas direct interactions of ARC and Sp1 were not detected. The DRIP/ARC complex also shares several subunits with the smaller CRSP complex (232).

DRIP205, DRIP150 and DRIP77 bound to VDR LBD in GST pull-down assays, while only one subunit, DRIP205, bound the VDR LBD in a ligand-

dependent manner, suggesting that DRIP205 is the anchor protein for interactions with VDR (457). DRIP205 is a 205 KDa protein (1566 aa.). Sequence analysis showed that DRIP205 has two LXXLL motifs (known as nuclear receptor interacting region, also called NR box), at 588-592 and 631-635.

It was reported that in yeast and mammalian cells, DRIP150 enhances AF1-mediated transactivation of the glucocorticoid receptor (GR), whereas DRIP205 interacts with the GR ligand binding domain in a hormone-dependent manner and facilitates GR transactivation in concert with DRIP150. In vitro GST pull down assays were carried out using fragments of DRIP150 or DRIP205 (GST-DRIP150 1345-1493 or GST-DRIP205 527-774), and therefore the conclusions of this study were not based on results obtained from full length DRIPs (461).

It was also shown that the ER interacts with the DRIP complex and DRIP205 was the only DRIP protein that bound to AF2 domain of ERα in a ligand-dependent manner in GST pull-down assays. However, it should be noted that some of the key experiments were performed using small fragments of DRIP205 (527-714). In a mammalian two-hybrid interaction in COS-7 cells transfected with the Gal4DBD-DRIP205 527-714 chimera and VP16-ER LBD (ligand binding domain/AF2), E2 induced a 3 and 2.5-fold increase in reporter gene activity using the LBD of ERα and ERβ, respectively. The DRIP205 527-714 fragment selectively inhibits ER-mediated transactivation in U2OS cells stably overexpressing ERα (462).

DRIP205 was also identified as a coactivator for the farnesoid X receptor (FXR). FXR is a bile acid sensor that regulates expression of a number of genes, the products of which control bile acid and cholesterol homeostasis (463). GST pull-down assays showed that interaction between FXR and DRIP205 is induced by bile acids when DRIP205 527-774 was used. In transient transfection assays, DRIP205 efficiently enhanced a bile acid-activated FXRE-driven reporter gene in a dose-dependent manner in COS-1 cells cotransfected with FXR/RXR, demonstrating that DRIP205 enhances FXR-mediated transactivation. In HepG2 cells, overexpression or inhibition of DRIP205 expression levels by RNA interference modulated the ligand-dependent induction of endogeneous FXR target gene mRNA expression levels (464).

DRIP205 is identical to RB18A (Recognized by PAb1801 moAntibody). RB18A was identified in the process of screening for p53 interacting proteins from a cDNA expression library of human B lymphoma cells. Western blot analysis and immunoprecipitation studies demonstrated that RB18A was recognized by several anti-p53 moAb reacting with N- or C-terminal domains of p53. The full-length sequence of RB18A cDNA and computer analysis demonstrated no significant homologies between RB18A and p53. Amino Acid sequence analysis showed that RB18A protein contained 23.8% residues with hydroxyl side chains (17.7% serine and 6.1% threonine) and was highly charged (13.7% basic and 17.8% acidic). The molecular weight (MW) of the protein was predicted as 166 kDa, however, western blot analysis showed a MW of 205 kDa.

The difference is more likely due to high glycosylation and phosphorylation sites present in the protein core. RB18A regulated p53 specific binding to its cognate DNA binding site through the C-terminal domain of RB18A (465). Further studies showed that RB18A regulated p53-dependent transactivation on several promoters, including Bax, $p21^{Waf1}$ and IGFBP3 genes. In addition, chromosome localization by fluorescence in situ hybridization showed that the RB18A gene was localized on chromosome 17q12-q21.1. The labeling of two loci on the same chromosome suggested the presence of a family of genes (and the corresponding proteins) sharing sequence homologies. Data bank analysis showed that BRCA1 was already mapped on these two loci (466). RB18A was reported to down-regulate p53-dependent apoptosis, due to a specific diminution of p53wt protein levels. This diminution was achieved by RB18A-dependent up-regulation of MDM2 (a p53-regulating protein) expression at the promoter level, and this resulted in increased proteasome-dependent degradation of p53. However, the exact mechanism by which RB18A regulates the MDM2 promoter remains unknown. A gel-shift assay did not detect any specific binding of RB18A recombinant protein on the MDM2 promoter (467).

DRIP205 is also identical to TRAP220, thyroid hormone receptor-associated protein 220, which is one protein from a group of distinct nuclear proteins associated with human thyroid hormone receptor α and immunopurified from HeLa cells grown in the presence of thyroid hormone (T3). In the absence of ligand, thyroid hormone receptor (TR) did not form a complex with TRAPs, indicating that ligand induces the formation of TR/TRAP complex. TR/RXR heterodimers may not be necessary for formation of TR/TRAP since the RXR was not detected in the purified complex. However, RXR was required for in vitro transactivation using the TR/TRAP complex. In gel shift assays, TRAPs can supershift both TR/DNA and TR/RXR/DNA bands, indicating a higher order complex formation (455). Ligand-dependent interactions were shown for TRAP220 and TRα via several methods. Functional assays showed that TRAP220 moderately enhanced TRα-mediated transcription in transfected cells. TRAP220 also interacted with other nuclear receptors, including the vitamin D receptor, retinoic acid receptor α, retinoid X receptor α, peroxisome proliferation-activated receptor (PPAR)α, PPARγ and the ER, suggesting that TRAPs may broadly coactivate members of the nuclear receptor superfamily (468). The two NR boxes display different preferences for specific nuclear receptors. RXR has a weak yet specific activation function 2 (AF2)-dependent preference for NR1, while TR, VDR, and PPAR all show a strong AF2-dependent preference for NR2 (469).

Disruption of the *Trap220* gene revealed that TRAP220 mutations are recessive embryonic lethal, and null mutants die in an early gestational stage (E11.5) with heart failure and show impaired neuronal development with extensive apoptosis, suggesting it is essential for embryonic survival. *Trap220*$^{-/-}$ mouse embryonic fibroblasts show impaired cell growth and impaired TR-driven transcription. RAR/RXR function was not affected and p53- and Gal4-VP16-driven transcription was also normal, indicating that TRAP220 has gene- and activator-selective functions (470). A later report showed that the embryonic lethality of null embryos at E11.5 is due to placental insufficiency. When embryonic development was partially rescued until E13.5, the embryonic loss was due to hepatic necrosis coupled with poor myocardial development as observed in hypomorphs (471).

DRIP205 is also highly homologous to PBP. PBP was identified as a protein that interacts with the PPAR (472). The interaction is dependent on the C- terminus of PPARγ since the deletion of the last 12 amino acids from the C-terminus abolished the interaction. PBP interacts with ER in a GST pull-down assay and PBP enhances transcriptional activity of ER in transfected CV-1 cells. When expression levels of PBP were examined in breast tumors, high levels were detected in 8 out of 15 primary breast cancers and 3 (MDA-MB-361, MDA-MB-453, MDA-MB-468) out of 6 breast cancer cells lines. Overexpression was not detected in MCF-7, MDA-MB-134-VI, and MDA-MB-157 breast cancer cells. In situ hybridization revealed the *PBP* gene localized to chromosome 17q12, a

region that is amplified in some breast cancers. *PBP* gene amplification occurred in 6 out of 25 breast tumor samples and 2 (MDA-MB-361 and MDA-MB-453) out of 6 breast cancer cell lines (473). The PBP null mutation is embryonic lethal at embryonic day 11.5, suggesting that there is no functional redundancy between PBP and other coactivators. It was also shown that the lethality is attributed, at least in part, to defects in the development of placental vasculature, similar to those found in PPARγ mutants (474).

The DRIP complex appears to be different from p160/CBP coactivators in several aspects, although they both bind nuclear receptors and facilitate transactivation. Firstly, DRIPs and p160 coactivators do not co-purify; secondly, the DRIP complex does not have intrinsic histone acetyl-transferase (HAT) activity, which is a common feature for p160/CBP coactivators; thirdly, the DRIP complex coactivation on NRs is markedly dependent on chromatin templates (457).

It has been suggested that these two groups of coactivators act separately but possibly in a coordinate manner and this action may be ligand- and NR-dependent. RXR-specific ligands only induced p160 binding to RXR, and PPARγ-specific ligands exclusively recruited DRIP205 but not p160 coactivators to PPARγ (475). Another study suggested an exchange between the two coactivator complexes at the target promoter based on the observation of a cyclic association and dissociation of coactivators with the pS2 promoter and recruitment of p160s and DRIPs occurring in opposite phases in chromatin

immunoprecipitation studies (476). These two groups of coactivators may also function in different development process. During the differentiation process, DRIP/Mediator complex is the major VDR binding complex in proliferating keratinocytes, while members of the SRC/p160 family are predominant after differentiation. Both DRIP205 and SRC-3 enhanced vitamin D-induced transcription in proliferating cells, but DRIP205 was no longer effective after differentiation (477).

Synergism between Mediator and p300/CBP-SRC was demonstrated in ERα-dependent transcription with chromatin templates, but not with naked DNA. This synergism is important for preinitiation complex formation (478). Another report suggested that the sequential recruitment of SRC complexes and Mediator complexes by nuclear receptors is driven by facilitated recruitment, rather than competition, and this was determined using an ERα AF2 point mutant (L540Q), which selectively binds and recruits Med220, but not SRCs. The recruitment of Med220 to the pS2 promoter was delayed in cells expressing this mutant, and activation of most estrogen-responsive target genes was impaired (479).

The mechanisms of ERα- and ERα/Sp1-mediated gene expression in breast cancer cells have been studied in this laboratory, and we have shown that coactivation of these responses are highly variable in different cell lines (396). This study investigates ligand-dependent coactivation of ERα by DRIP205 in breast cancer cell lines transfected with $pERE_3$. The results demonstrate that coactivation of ERα by DRIP205 in ZR-75 breast cancer cells is complex and involves multiple regions of DRIP205 and ERα. In mammalian two-hybrid and coimmunoprecipitation assays, interactions of DRIP205 and ERα also involved multiple domains of both proteins. Moreover, coactivation of ERα by DRIP205 and interaction of these proteins do not require the NR box motifs of DRIP205. We also examined ligand-dependent coactivation of ERα/Sp by DRIP205 in various breast cancer cell lines transfected with pSp3. The results show that coactivation of ERα/Sp by DRIP205 requires AF1 domain of ERα and multiple regions of DRIP205 in ZR-75 cells. However, the two NR boxes of DRIP205 are not necessary for coactivation of ERα/Sp by DRIP205. RNA interference assay showed that decrease of Sp3 or Sp4 levels in cells transfected with pSp_3 abolished the DRIP205 coactivation of ERα/Sp, suggesting that Sp3 and Sp4 are important for DRIP205 coactivation of ERα/Sp.

CHAPTER II

MATERIALS AND METHODS *

2.1 Cell Lines, Chemicals and Biochemicals

Fetal Bovine Serum (FBS) was obtained from JRH Biosciences, Lenexa, KS or Atlanta Biologicals, Inc., Norcross, GA. Antibiotic Antimycotic Solution (AAS) (100×) was obtained from Sigma, St. Louis, MO. COS-7 cells and MCF-7, ZR-75, MDA-MB-231 breast cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). COS7 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Gibco Invitrogen Corporation, Carlsbad, CA) supplemented with 2.2 g/L sodium bicarbonate, 5% FBS and 10 ml/L AAS. 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, 10% FBS and 10 ml/L AAS. MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM/F12) (Sigma) supplemented with 2.2 g/L sodium bicarbonate, 5% FBS and 10 ml/L AAS. Cells were cultured and grown in a 37°C incubator with humidified 5% CO_2 – 95% air. DMSO, E2 and 4-hydroxy tamoxifen were

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Table 2 Summary of primers and restriction enzymes for generating pcDNA3, GAL4 chimera and Xpress-tagged (X) constructs.

	Primers[1]	Templates	Restriction Enzymes
pcDNA3-DRIP205mB	Fragment A: Upper, TCT TCT GGA TCT AGC CAG TCC AAA AAT TC Lower, **ACA ACG AGC CTG AGG AAC CTA ACC CTG AAG** Fragment B: Upper, AT**C TTC AGG GTT AGG TTC CTC AGG CTC GTT GT** Lower, TAG CAT TTA GGT GAC ACT ATA GAA TAG G Two-step PCR: Upper, TCT TCT GGA TCT AGC CAG TCC AAA AAT TC Lower, TAG CAT TTA GGT GAC ACT ATA GAA TAG G	pcDNA3-DRIP205 pcDNA3-DRIP205 Annealed Fragments A+B	NdeI, Not I
pcDNA3-Dm3 (DRIP205 Δ587-636)	Fragment A: Upper, AAC CAT TCA AGC CGA CAC CCC AGC ACT GT Lower, **CTG GGC AGG ATT ATC TGG GTT CTG AGA** CAC Fragment B: Upper, **TCT CAG AAC CCA GAT AAT CCT GCC CAG** GAT TT Lower, TTG CTG TCT AAT CCG GGC CCC GAG AGA GTA Two Step PCR: Upper, AAC CAT TCA AGC CGA CAC CCC AGC ACT GT Lower, TTG CTG TCT AAT CCG GGC CCC GAG AGA GTA	pcDNA3-DRIP205 pcDNA3-DRIP205 Annealed Fragments A+B	NheI, SspI
pMD	Upper, TAG GAT CCA AAG TTC TCT GGA ACG GCT CCA TGC Lower, TAC GAC GCG TAC TAA TTC CCA ATC AGG GCC ACA T	pcDNA3-DRIP205mB	BamHI, MluI
pMDm3	Upper, same as pMD Lower, same as pMD	pcDNA3-Dm3	BamHI, MluI
pMDm4	Upper, GCT AGA ATT CCC GGC TAG CAG Lower, CGT CAC GCG TCT ATG TCC TGT T	pcDNA3-DRIP205mB	EcoRI, MluI
pMDm1	Upper, same as pMD Lower, TAC GAC GCG TAC TAT GTC CTG TTG ACA TCA AAG	pcDNA3-DRIP205mB	BamHI, MluI
pMDm5	Upper, same as pMD Lower, TAC GAC GCG TCA CCG ACT CAT GCC GAT CT	pcDNA3-DRIP205mB	BamHI, MluI
pMDm6	Upper, GCT AGG ATC CAA GAT TTC TCA ACC CTT TAT Lower, same as pMD	pMD	BamHI, MluI
pMDm7	Upper, GCT AGG ATC CAA TGT ATG TCC ATC CCT GTG ACG A Lower, TAC GAC GCG TCT GAA TAG TGA TTT TGG GAA T	pMD	BamHI, MluI
pMDm7Δ	Upper, same as pMDm7 Lower, same as pMDm7	pcDNA3-Dm3	BamHI, MluI
pMDm13	Upper, same as pMDm7 Lower, TAC GAC GCG TTG GGT TCT GAG ACA CCT TGC T	pcDNA3-DRIP205mB	BamHI, MluI
pMDm12	Upper, same as pMDm6 Lower, same as pMDm7	pMD	BamHI, MluI
pMDm8	Upper, GCT AGG ATC CAA ATT CCT AAG GGA ACA GTG ATG GT Lower, same as pMD	pMD	BamHI, MluI
XDm5	Upper, GGT AGG ATC CAA GAT GAG TTC TCT GGA ACG Lower, GGT AGA TAT CCT ACA CCG ACT CAT GCC GAT	pcDNA3-DRIP205	BamHI, EcoRV
XDm7	Upper, same as pMDm7 Lower, GAC TGA TAT CCT ACT GAA TAG TGA TTT TGG GAA TG	pMD	BamHI, EcoRV
XDm7Δ	Upper, same as pMDm7 Lower, same as XDm7	pMD	BamHI, EcoRV
XDm12	Upper, same as pMDm6 Lower, same as XDm7	pMD	BamHI, EcoRV

1 Overlapping sequence used to anneal fragment A and B are indicated in bold type.

purchased from Sigma, ICI 182,780 was kindly provided by Dr. Alan Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK) and all other biochemicals were the highest quality available from commercial source.

2.2 Plasmids, Cloning and Oligonuleotides

pcDNA3-DRIP205 expression plasmid was kindly provided by Dr. Leonard P. Freedman, Merck Research Laboratories (West Point, PA). For the convenience of further cloning, the GGATCC sequence (which is the BamHI site, and translates to Gly-Ser) in the coding region of DRIP205 was mutated to GGTTCC (which also translates to Gly-Ser) using two-step PCR (Table 2). The first step was to amplify several cycles of the annealed fragments A and B. Then, the primers were added when the reactions were paused, and the products of the first step would serve as a template for the second step. The final products were digested with NdeI and NotI, and ligated back to pcDNA3-DRIP205, which was digested with NotI and then partially digested with NdeI. This construct was called pcDNA3-DRIP205mB and used as a template for generating other constructs by PCR. The DRIP205 expression plasmid with deletion of both NR boxes, namely pcDNA3-Dm3 (DRIP205 Δ587-636) was generated using two-step PCR (Table 2). The final PCR products and plasmid pcDNA3-DRIP205mB were separately digested with NheI and SspI, followed by ligation.

pM and pVP16 vectors were purchased from Clontech, Palo Alto, CA. pcDNA3.1/His A, B, C was purchased from Invitrogen, Carlsbad, CA. pMD,

pMDm3, pMDm4, pMDm1, pMDm5, pMDm6, pMDm7, pMDm7Δ, pMDm13, pMDm12, pMDm8 expression plasmids were generated by PCR. PCR products were digested with restriction enzymes described in Table 1, and ligated back into the pM vector, which was digested with the same set of restriction enzymes.

XDm5 expression plamids were constructed by PCR. PCR products were digested with BamHI and EcoRV and ligated to pcDNA 3.1/His C. XDm7, XDm7Δ, XDm12, XDm8 expression plasmids were generated by PCR. PCR products were digested with BamHI, EcoRV and ligated to pcDNA3.1/His A. VP16-hER, VP16-TAF1 VP16-HE19 expression plasmids were generated by digestion of pM-hER, pM-TAF1 expression plasmids with EcoRI, SalI, and ligated to pVP vector digested with EcoRI and SalI.

*pfu*Turbo DNA polymerase from Statagene (La Jolla, CA) was used in PCR reactions. All the primers were ordered from IDT, Coralville, IA. All constructs were sequenced afterward to confirm the cloning.

2.3 Transient Transfection Assays

ZR-75, MCF-7 or MDA-MB-231 cells were seeded in 12-well plates in DMEM/F12 medium without phenol red (Gibco Invitrogen Corporation, Carlsbad, CA) supplemented with 2.2 g/L sodium bicarbonate, 10 ml/L AAS and 2.5% charcoal stripped FBS. After incubation for 12 hr. at 37°C in 5% CO_2-95% air, cells were co-transfected with DNA using the calcium phosphate method. In coactivation experiments, cells were co-transfected with 250 ng pcDNA3.1-β-gal (used as an internal control), 1 μg pERE$_3$-LUC or pSp1$_3$-LUC, pcDNA3-hERα,

various amount of DRIP 205 or deletion mutants constructs. The pcDNA3 empty vector was used to maintain DNA mass balance among different treatment groups. In the mammalian two-hybrid assay, cells were co-transfected with 250 ng pcDNA3.1-β-gal (used as an internal control), 500 ng pGAL4$_5$-LUC, 500 ng pM or pM-DRIP wild type or deletion mutants, 500 ng pVP, or VP-hERα, or VP-HE19, or VP-TAF1. Six hours after transfection, cells were shocked with 25% glycerol/PBS for 1 min, washed with PBS (2$\times$), and then treated with DMSO or 10 nM E2 for another 30-48 hr. In small inhibitory RNA interference assay, cells were transfected with 20 nM siRNAs using Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA), and 24 hr later, cells were cotransfected with 100 ng pcDNA3.1-β-gal (used as an internal control), 300 ng pSp1$_3$-LUC and 100 ng hERα expression plasmid with Genejuice transfection reagent (Novagen). Cells were then treated with DMSO or 10 nM E2 for another 12-20 hr. Each treatment was replicated three or four times. Cells were then washed twice in PBS and harvested with 100 µl reporter lysis buffer (Promega Corporation, Madison, MI). After one freeze-thaw cycle, cell lysates were centrifuged for 30 sec and the supernatant was used to determine protein activity. Luciferase (Promega Corporation, Madison, MI) and β-galactosidase (Applied Biosystems, Foster City, CA) activities were read by a Packard Luminometer. Relative luciferase activity was calculated by dividing luciferase activity by β-galactosidase activity for each well. In coactivation experiments, fold induction was calculated by dividing relative luciferase activity of E2 treated

groups by relative luciferase activity in controls (DMSO-treated). In the mammalian two-hybrid assay, fold induction was calculated by dividing fold induction of E2/DMSO of VP-hERα or VP-hERα deletion mutants by fold induction of E2/DMSO obtained using the empty vector pVP.

2.4 Western Blot Analysis

For determination of ERα protein levels, ZR-75 cells were seeded, transfected and harvested as described above. After luciferase and β-gal activity were read, 6 µl of 5 M NaCl was added to the remaining ~60 µl lysates to obtain maximal protein yield. Lysates were incubated on ice for 1 hr with occasional vortexing followed by centrifugation (16,000 g, 10 min, 4°C). Equal amounts of total protein from each treatment group were separated by SDS-PAGE and transferred to a PVDF membrane (transfer buffer: 48 mM Tris, 39 mM glycine, 0.025% SDS).

For GAL4-DRIP fusion protein confirmation, COS-7 cells were seeded in 6-well plates with DMEM/F12 medium without phenol red supplemented with 2.5 % charcoal stripped FBS. After overnight incubation, cells were transfected with 2 µg of each fusion protein expression plasmids using Lipofectamine 2000 reagent (Invitrogen Corporation, Carlsbad, CA). Medium was changed 12 hr after the transfection, and after 48 hr, cells were trypsinized, transferred and washed with PBS (3×). Cellular and nuclear extracts were obtained using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology,

Rockford, IL). Cellular and nuclear lysates were separated by 7.5% SDS-PAGE, and transferred to a PVDF membrane.

For Sp protein interference by siRNAs, ZR-75 cells were seeded in 60 mm plates and transfected as described in transient transfection assay. After treatment of 12 hr, cells were lysed in RIPA buffer (PBS + 1% IGEPAL + 0.5% sodium deoxycholate + 0.1% SDS) supplemented with protease inhibitor cocktail (Sigma) and 1 mM PMSF for 2 hr on ice with occasional vortexing. Supernatants were then separated by centrifuging at 16, 000 rpm at 4°C. Equal amounts of protein were loaded for each well, separated by SDS-PAGE and transferred to a PVDF membrane.

Membranes were blocked with 0.05% Tween 20/PBS supplemented with 5% non-fat dry milk for 1 hr. Membranes were probed with primary antibody overnight, washed with 0.05% Tween 20/PBS (3×), probed with HRP-conjugated secondary antibody for 1 hr, washed with 0.05% Tween 20/PBS (3×) and finally rinsed with deionized water. ERα HC20, Sp1 PEP2, Sp3 D-20, Sp4 V-20 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution for detecting ERα, Sp1, Sp3 and Sp4, respectively and mouse monoclonal GAL4DBD RK5C1 antibody (Santa Cruz Biotechnology) was used at 1:500 dilution for detecting GAL4 fusion proteins. Membranes were visualized using the ECL detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). After autoradiography, band intensities were determined by a scanning laser

densitometer (Sharp Electronics Corporation, Mahwah, NJ) using Zero-D Scanalytics software (Scanalytics Corporation, Fairfax, VA).

2.5 Coimmunoprecipitation Assay

[^{35}S]hERα, [^{35}S]DRIP205, [^{35}S]DRIP205 Δ587-636, [^{35}S]XDm5, [^{35}S]XDm7, [35S]XDm7Δ, [^{35}S]XDm12 were *in vitro* translated using the T7 Quickcoupled Transcription Translation Systems (Promega Corporation, Madison, MI). [^{35}S]hERα (0.2 µl) and ^{35}S-labeled DRIP205 wild-type or variant protein (0.5 µl) were incubated in 0.5 ml coimmunoprecipitation buffer (PBS + 0.001% IGEPAL CA630). The coimmunoprecipitation buffer was freshly supplemented with 1 µM E2, 1:100 dilution of protease inhibitor cocktail (Sigma) and 1mM PMSF (Sigma). After incubation for 1 hr on a rocker at 4°C, 400 ng antibody was added. DRIP205 antibody c-19 was purchased from Santa Cruz Biotechnology. This antibody was raised against the C-terminus of TRAP220 of human origin, and was used to pull down DRIP205 Δ587-636. ERα HC 20 antibody was used for ERα immunoprecipitation. For Xpress-tagged DRIP205 deletion mutants, anti-Xpress antibody from Invitrogen was used. After incubation for 1 hr at 4°C, 20 µl of 50% slurry of protein G sepharose beads (Amersham Biosciences, Piscataway, NJ) were added to the reaction solution, followed by incubation for 2 hr on a rocker at 4°C. Samples were then centrifuged at 12,000 rpm, 4°C for 1 min. The supernatant was carefully removed, and the pellet was washed with PBS + 1% IGEPAL CA630 (3×), and

finally washed with PBS. The final pellet was boiled in 30 µl of 2×SDS sample buffer, and proteins were separated on a 6% SDS-PAGE and visualized by autoradiography.

2.6 Immunocytochemistry

MCF-7 or COS7 cells were seeded onto 2-well glass chamber slides at 100,000 cells per well in DMEM/F12 medium supplemented with 5% charcoal stripped FBS. COS-7 cells were cotransfected with 250 ng pMD (GAL4-DRIP205), or pMDm3 (GAL4-DRIP205Δ587-636), and 250 ng ERα expression plasmids for DRIP205 and ERα colocalization. After 24 hr incubation in a 37°C incubator with 5% CO_2, cells were treated with DMSO vehicle or 10 nM E2 for 1 hr. Slides were then washed with PBS, fixed with -20°C methanol, air dried and washed with PBS + 0.3% Tween 20 (PBS/Tween). Slides were blocked for 1 hr with 5% donkey serum in antibody dilution buffer (1% BSA in PBS/Tween), washed with PBS/Tween briefly, incubated with anti-DRIP 205 c-19 antibody (Santa Cruz) at 1:100 dilution in antibody dilution buffer at 4°C for 12 hr. Slides were washed (goat serum for controls), with PBS/Tween (3 × 10 min), incubated with donkey anti-goat IgG FITC (Santa Cruz) at 1:200 dilution in antibody dilution buffer for 1 hr and washed with PBS/Tween (3 × 10 min). Slides were subsequently blocked with 5% donkey serum in antibody dilution buffer for 1 hr, washed with PBS/Tween briefly, incubated with anti-ERα H-184 antibody (Santa Cruz) at 1:100 dilution in antibody dilution buffer at 4°C for 12 hr (rabbit serum for controls), washed with PBS/Tween (3 × 10 min), incubated with

donkey anti-rabbit IgG Alexa Fluor 594 (Molecular Probes) at 1:500 dilution in antibody dilution buffer for 1 hr and washed with PBS/Tween (3 × 10 min). Slides were finally washed in deionized water, and coverglass mounted using Prolong Gold antifade reagent with DAPI (Molecular Probes). Immunofluorescence images of DRIP 205 and ERα were examined using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam high-resolution digital camera. Digital images were captured using Axiovision 3.0 software.

For DRIP205 and Sp1 colocalization experiments, COS-7 cells were seeded as described above, cotransfected with 250 ng of pMD and 250 ng of Sp1 expression plasmids. The experiments were performed as described above, and anti-Sp1 PEP2 (Santa Cruz) antibody was used. For Sp1 and ERα colocalization, MCF-7 cells were seeded and incubated for 24 hr and treated with DMSO or 10 nM E2 for 1 h, and immunostaining experiment were performed as described above. Anti-Sp1 PEP2 (goat) (Santa Cruz) was used in this experiment. For ERα and Sp4 colocalization, COS-7 cells were seeded, transfected with 250 ng of ERα and 250 ng of Sp4 expression plasmid, treated with DMSO or 10 nM E2 for 1 h, and immunostaining experiments were carried out using Sp4 V-20 and ERα D-12 antibodies (Santa Cruz).

CHAPTER III

RESULTS *

3.1 DRIP205 Coactivation of ERα Involves Multiple Domains of Both Proteins

3.1.1 Coactivation of Wild-type and Variant ERα by DRIP205

Coactivation of ERα-dependent transactivation by DRIP205 was initially examined in ERα-positive ZR-75 and ERα-negative MDA-MB-231 cells. The full-length DRIP205 expression plasmid (Fig. 15A) encodes for 1566 amino acids, which is identical to amino acids 16-1581 of the TRAP220 coding sequence. Both cell lines were transfected with $pERE_3$, which contains three tandem EREs in a minimal TATA-luciferase construct, and an hERα expression plasmid. The transfected $pERE_3$ construct is overexpressed in the transfected cells and minimal E2-inducibility is observed in ZR-75 cells in the absence of co-transfected ERα. This system is ideal for investigating coactivation of ERα and determining receptor and coactivation domain requirements for transactivation in breast cancer cell context. The results illustrated in Figures 15B and 15C show

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* Part of this chapter is reprinted with permission from " Vitamin D-interacting protein 205 (DRIP205) coactivation of estrogen receptor α (ERα) involves multiple domains of both proteins" by Wu, Q., Burghardt, R. and Safe, S., (2004), *J. Biol. Chem*., 279(51), 3602-53612.

that E2 induces transactivation 6- and 4-fold in ER-positive ZR-75 cells and ER-negative MDA-MB-231 cells, respectively, and this was enhanced >3- and >5-fold by DRIP205. Fig. 15D shows that transfected DRIP205 has no significant effect on ERα protein expression levels in the presence or absence of E2. ERα levels were lower in cells treated with E2, which is consistent with previous results and is due to degradation of ERα through the proteasome pathway (480).

E2 also induced transactivation in ZR-75 cells transfected with $pERE_3$ and wild-type or variant ERα expression plasmids with mutations in helix 12 (TAF1) (Fig. 16A) or deletion of AF1 (HE19) (Fig. 16B). However, studies with DRIP205 showed that coactivation was observed only with wild-type ERα, suggesting that AF1 and wild-type AF2 were both necessary for functional DRIP205-ERα interactions. The requirements for both AFs of ERα for coactivation by DRIP205 were further investigated in competition experiments by transfecting GRIP1-NR box peptide and ERα-AF1 peptide expression plasmids. Previous studies show that these peptides competitively squelch AF2 and AF1 respectively (396,481-483). The results show that both peptides inhibit DRIP205 coactivation of ERα (Fig. 16C and 16D), indicating that coactivation by DRIP205 is associated with both AF1 and AF2 of ERα.

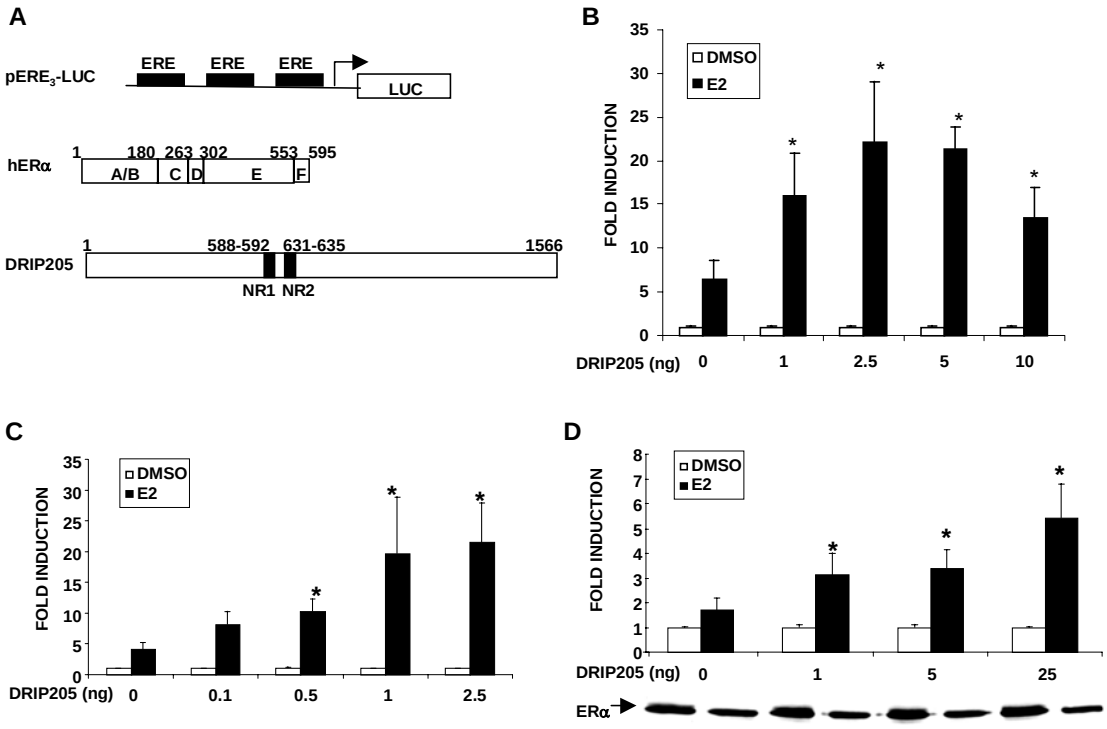


Fig. 15 Coactivation of wild-type ERα by DRIP205. A, Constructs used for transfection assays. B, Coactivation of ERα by DRIP205 in ZR-75 cells. Cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal as the internal control, 5 ng hERα and various amounts of DRIP205 expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity determined as described in Materials and Methods. Results are expressed as fold induction (compared to DMSO) and shown as means ± S.E. for three different experiments for each treatment. Significant ($p<0.05$) enhancement is indicated by an asterisk (*). C, Coactivation of ERα by DRIP205 in MDA-MB-231 cells. Cells were cotransfected and analyzed as described in B. D, ERα protein expression. The same cotransfection assays were performed as outlined in B and Western blot analysis of whole cell lysates was carried out as described in Materials and Methods.

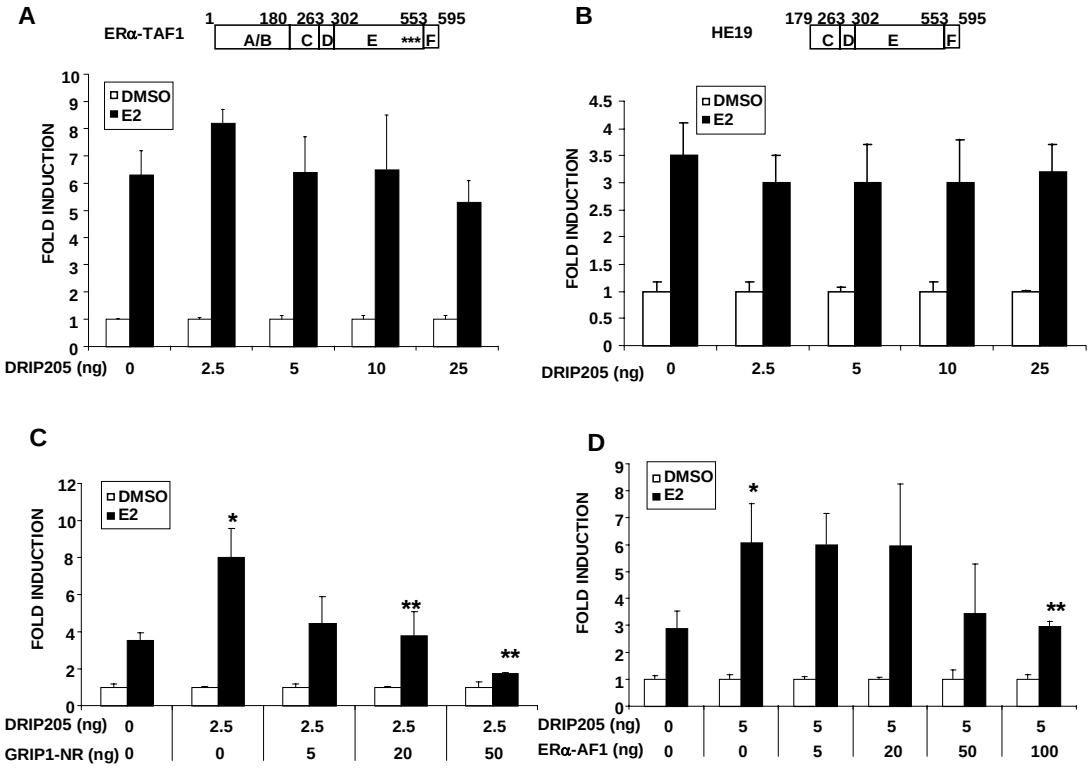


Fig. 16 AF1 and AF2 of ERα are both required for coactivation by DRIP205 in ZR-75 cells. Cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 25 ng ERα-TAF1 (A) or HE19 (B) and various amounts of DRIP205 expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. No significant enhancement of the fold induction was observed for DRIP205. C, D, Coactivation of ERα by DRIP205 was decreased by both GRIP1-NR and ERα-AF1 peptides. Cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 5 ng hERα, 0 or 2.5 ng DRIP205 and increasing amounts of GRIP1-NR (C) or ERα-AF1 (D) peptide expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Significant induction ($p<0.05$) (*) and decreased activity (**) by competing peptides are indicated.

3.1.2 Coactivation of ERα by DRIP205 Deletion Mutants

Previous studies have shown that the NR boxes in DRIP205/TRAP220 contribute to the physical and functional interactions of these coactivators with ER and other NRs (396,462,481,484,485). Their role in coactivation of ERα was further investigated in ZR-75 cells transfected with $pERE_3$ and DRIP205 Δ587-636, in which both NR boxes were deleted. The results (Fig. 17A) show that like wild-type DRIP205, DRIP205 Δ587-636 also coactivates ERα. These studies demonstrate that coactivation of ERα in ZR-75 cells by DRIP205 does not require NR1 or NR2. Moreover, coactivation of ERα by DRIP205 Δ587-636 was also decreased by overexpression of GRIP1-NR and ERα-AF1 (Fig. 17B and 17C), and this complements their inhibition of wild-type DRIP205 coactivation of ERα (Fig. 16B and 16C). These results suggest that coactivation of ERα is dependent on both C- and N-terminal sequences of DRIP205 and/or their interactions between these domains of DRIP205.

Coimmunoprecipitation of hERα with DRIP205 or DRIP205 Δ587-636 was investigated by incubation of *in vitro* translated Xpress-tagged wild-type and variant [^{35}S] DRIP205, [^{35}S] ERα, and DRIP205 and ERα antibodies followed by SDS-PAGE (Fig. 17D). Lanes 1, 3, 6 show [^{35}S]-labeled ERα, DRIP205 and DRIP205 Δ587-636, and the latter two bands were indistinguishable on the gel due to only small differences in molecular mass. DRIP205 antibodies did not pull down ERα/E2 alone (lane 2), but immunoprecipitated radiolabeled DRIP205/E2 (lane 4), and coimmunoprecipitated DRIP205 and ERα/E2 (lane 5). DRIP205 antibodies also immunoprecipitated radiolabeled DRIP205 Δ587-636/E2 alone (lane 7), and coimmunoprecipitated DRIP205 Δ587-636 and ERα/E2 (lane 8). Complementary experiments were also carried out using ERα antibodies and [^{35}S]–labeled ERα, wild-type and variant DRIP205 (lane 9-16). ERα antibodies immunoprecipitated ERα (lanes 12) and coimmunoprecipitated ERα and wild-type (lane 14) or mutant (land 16) DRIP205. These coimmunoprecipitation experiments confirm interactions between ERα with DRIP205 and DRIP205 Δ587-636 and complement their functional interactions in transactivation assays.

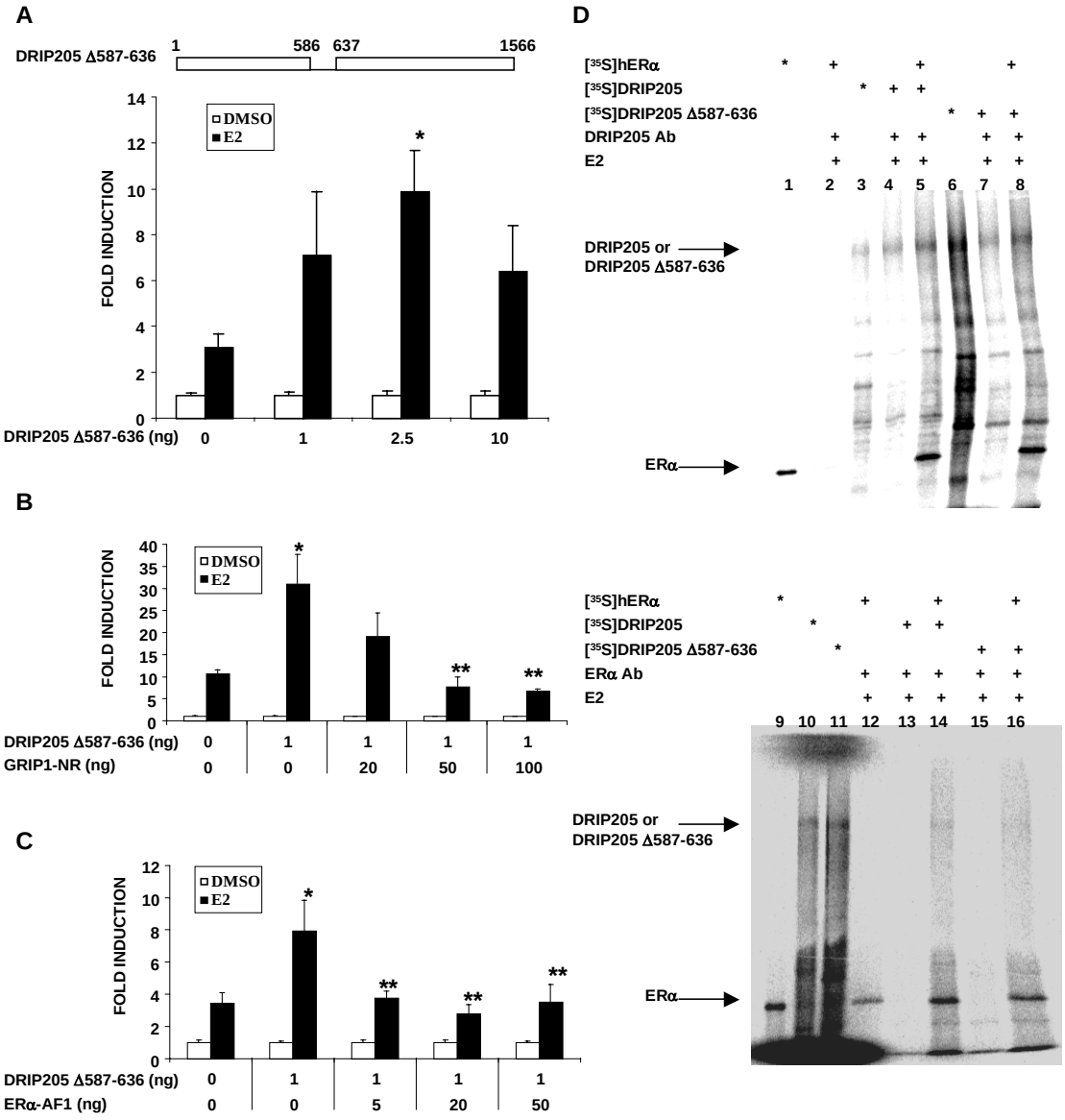


Fig. 17 NR boxes deletion mutant DRIP205 Δ587-636 coactivates ERα. A, Coactivation of ERα by DRIP205 Δ587-636 in ZR-75 cells. Cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 5 ng hERα and various amount of DRIP205 Δ587-636 expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Results are expressed as means ± S.E. for three seperate experiments for each treatment group and significant ($p<0.05$) enhancement is indicated (*). AF2 (B) and AF1 (C) are required for DRIP205 Δ587-636 coactivation of ERα. Fig. 17 (continued) Cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 5 ng hERα, 0 or 1 ng DRIP205 and increasing amounts of GRIP1-NR (B) or ERα-AF1 (C) peptide expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Significant coactivation ($p<0.05$) (*) and significant inhibition by competing peptides are indicated (**). D, Coimmunoprecipitation of ERα and DRIP205 or DRIP205 Δ587-636. *In vitro* translated proteins (lanes 1, 3, 6, 9, 10, 11; 30% of input) were incubated, immunoprecipitated with specific antibodies and analyzed by SDS-PAGE as described in Materials and Methods. Protein bands were identified by comparison with *in vitro* translated proteins and molecular markers (250 KDa, 150 KDa, 100 KDa, 75 KDa, 50 KDa).

Colocalization of ERα with DRIP205 (Fig. 18A) and DRIP205Δ587-636 (Fig. 18B) was also investigated by transfection studies in COS7 cells using the GAL4-DRIP205 expression plasmids. This cell line is ERα-negative and only expresses low levels of DRIP205. In cells transfected with ERα and DRIP205/DRIP205Δ587-636 expression plasmids, their corresponding expressed proteins were primarily localized in the nucleus; however, some cytoplasmic staining was observed for ERα. In cells transfected with DRIP205/ERα and treated with DMSO (solvent control) or 10 nM E2 for 1 h, immunostaining showed that ERα and DRIP205 were extensively colocalized (Fig. 18A, right panels). Estrogen treatment induced a punctate pattern of DRIP205 nuclear staining, whereas this was not observed for ERα. In the corresponding experiment with DRIP205Δ587-636 (Fig. 18B), punctate staining of the deletion mutant was observed in solvent-treated cells and this was enhanced in E2-treated cells (Fig. 18B, left panel). DRIP205Δ587-636 also colocalized with ERα (Fig. 18B, right panel), confirming that interaction of DRIP205 with ERα did not require the NR boxes.

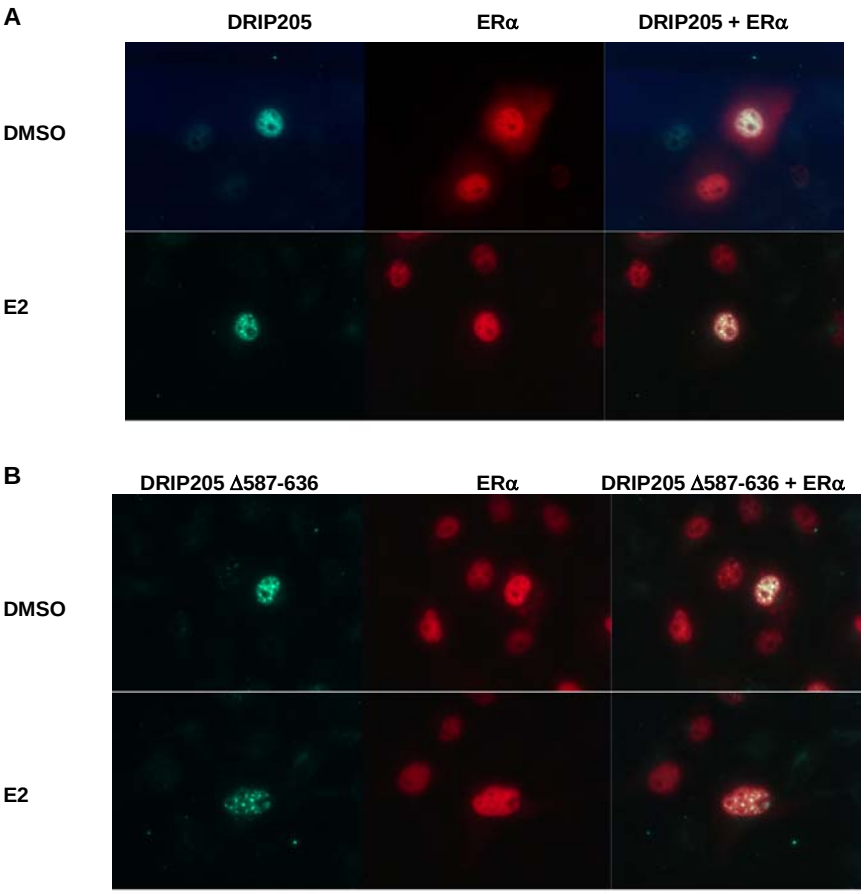
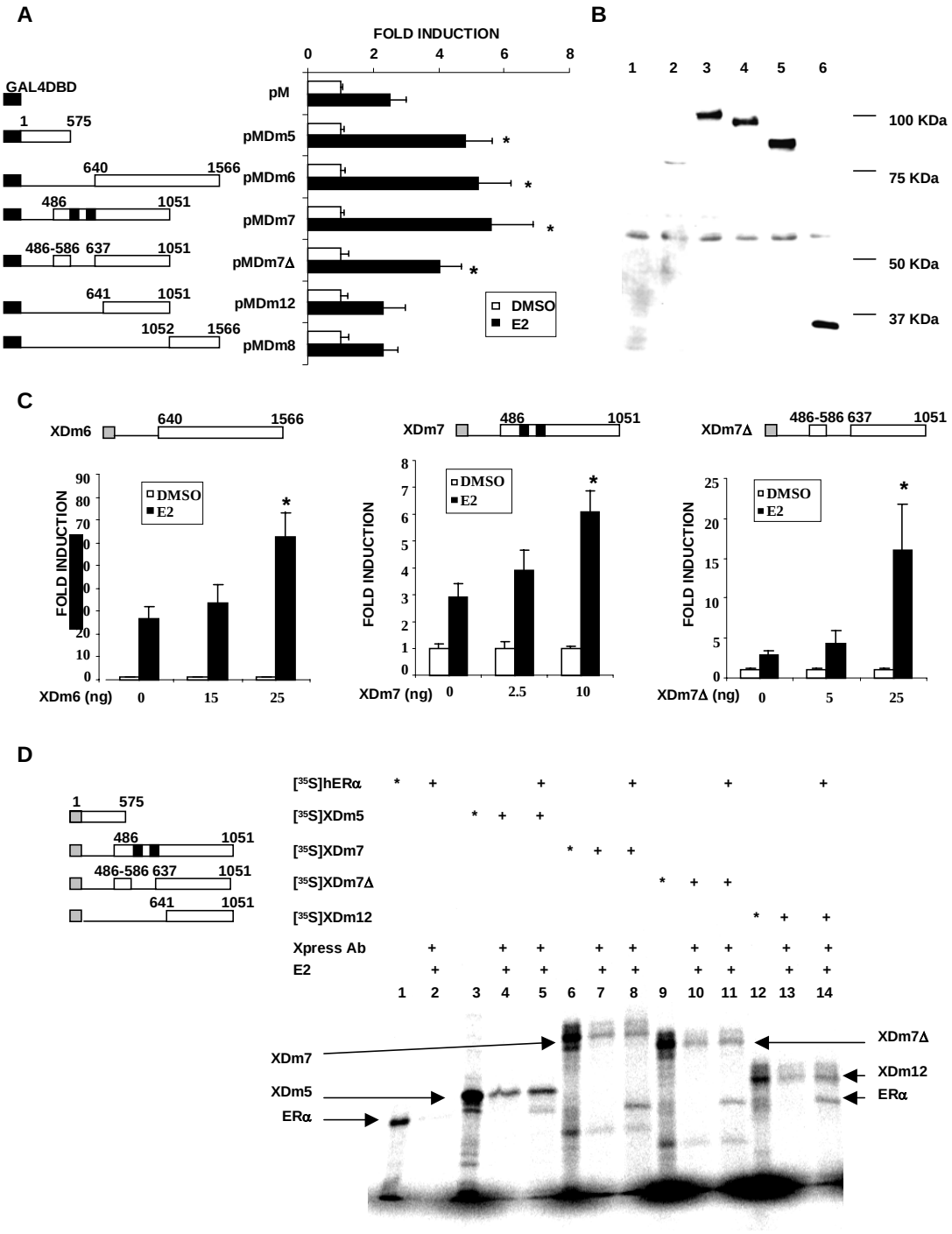


Fig. 18 Colocalization of DRIP205 wild type or NR box deletion mutants DRIP205 Δ587-636 with ERα. COS7 cells were co-transfected with 500 ng pMD (A, for DRIP205) or pMDm3 (B, for DRIP205 Δ587-636) and 500 ng ERα expression plasmids, treated with DMSO or 10 nM E2 for 1hr, and the Immunostaining experiments were performed as described in Materials and Methods. The same cells were analysed for each treatment.

Initial coactivation studies with N- and C-terminal deletion mutants of DRIP205 were determined in ZR-75 cells transfected with a series of GAL4-DRIP205 deletion constructs, which are readily expressed in the nucleus due to the nuclear localization signal in the GAL4 DNA binding domain of the fusion protein. The results in Fig. 19A summarize coactivation studies with a series of constructs that express amino acids 1-575 (pMDm5), amino acids 486-1051 (pMDm7), amino acids 486-586 + 637-1051 (pMDm7Δ), amino acids 640-1566 (pMDm6), amino acids 641-1051 (pMDm12) and amino acids 1052-1566 (pMDm8). Significant coactivation was observed for several GAL4-DRIP205 chimeras (pMDm5, pMDm7, pMDm7Δ and pMDm6), two of which express N-terminal (pMDm5) or C-terminal (pMDm6) regions of DRIP205 and do not contain the central NR box sequences. These results confirm that the NR boxes of DRIP205 are not required for coactivation of ERα and that the coactivation activities of DRIP205 include sequences within both N- and C-terminal regions of this protein. Results in Figure 19B summarize Western blot analysis of nuclear extracts from cells transfected with several of the GAL4-DRIP205 fusion proteins and the results show that all of the proteins were expressed in the nucleus. pMDm5, which contains N-terminal amino acids 1-575, gave a less intense band than the other proteins but could be detected in nuclear extracts. Coactivations of ERα by Xpress-tagged DRIP205 variants were also investigated in ZR-75 cells. Transfection with XDm7, XDm7Δ and XDm6 coactivated ERα (Fig. 19C) and these data were consistent with results obtained

with the corresponding GAL4-DRIP205 chimeras. XDm5 does not contain a nuclear localization signal and transfection with XDm5 did not coactivate ERα (data not shown). Interactions of Xpress-tagged DRIP205 deletion proteins with ERα were also investigated using in vitro translated proteins and Xpress antibody (Fig. 19D). Expression of [^{35}S]-labeled ERα and Xpress-tagged DRIP205 proteins alone (lanes 1, 3, 6, 9 and 12) are shown and the Xpress antibody immunoprecipitates [^{35}S]-labeled DRIP205 chimeras (lanes 4, 7, 10 and 13) but not ERα (lane 2). Moreover, the Xpress antibody immunoprecipitates ERα only after coincubation with Xpress-tagged DRIP205 chimeras (lanes 5, 8, 11, and 14). The coimmunoprecipitation studies show that ERα interacts with multiple domains of DRIP205 and the interactions do not require NR boxes.

Fig. 19 Coactivation of ERα by DRIP205 deletion mutants. A, Coactivation of ERα by GAL4-DRIP fusion proteins. ZR-75 cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 5 ng hERα, 100 ng pM empty or pM DRIP deletion mutants, treated with DMSO and 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Results are showed as means ± S.E. for three separate experiments for each treatment group and significant ($p<0.05$) coactivation is indicated (*). B. GAL4-DRIP fusion proteins expression in nuclear extracts. COS-7 cells were transfected with 2 μg pMDm5 (lane 2), pMDm7 (lane 3), pMDm7Δ (lane 4), pMDm12 (lane 5) and pMDm4 (lane 6) and nuclear lysates were analyzed by Western blot analysis as described in Materials and Methods. GAL4 DBD monoclonal antibody RK5C1 was used. C, Coactivation of ERα by Xpress-tagged DRIP205 deletion mutants. ZR-75 cells were cotransfected with 1000 ng pERE$_3$-LUC, 250 ng β-gal, 5 ng hERα and various amounts of XDm6, XDm7 or XDm7Δ expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Significant ($p<0.05$) coactivation is indicated (*). D, Coimmunoprecipitation of ERα and various Xpress-tagged DRIP205 deletion mutants. *In vitro* translated proteins (lane 1, 3, 6, 9 and 12; 30% of input) were incubated, immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. Protein bands were identified by comparing them to in vitro translated proteins and molecular markers as indicated in the legend to Fig. 17.


A
FOLD INDUCTION
0 2 4 6 8
GAL4DBD
pM
1 575
pMDm5
640 1566
pMDm6
486 1051
pMDm7
486-586 637 1051
pMDm7Δ
641 1051
pMDm12
1052 1566
pMDm8
DMSO
E2
B
1 2 3 4 5 6
100 KDa
75 KDa
50 KDa
37 KDa
C
XDm6
640 1566
XDm7
486 1051
XDm7Δ
486-586 637 1051
FOLD INDUCTION
DMSO
E2
XDm6 (ng) 0 15 25
XDm7 (ng) 0 2.5 10
XDm7Δ (ng) 0 5 25
D
1 575
486 1051
486-586 637 1051
641 1051
[35S]hERα
[35S]XDm5
[35S]XDm7
[35S]XDm7Δ
[35S]XDm12
Xpress Ab
E2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
XDm7
XDm5
ERα
XDm7Δ
XDm12
ERα

3.1.3 DRIP205 Activation Function

Previous studies showed that DRIP205 not only interacts with ER and other NRs, but also is a component of the mediator complex of proteins and related nuclear factors (153,452,454,456-458,460,468,486). A series of GAL4-DRIP205 constructs containing multiple deletions in DRIP205 were used to investigate their activation function (AF) activity (i.e. interactions with other coregulatory proteins and the basal transcription machinery) in a mammalian one-hybrid assay and interactions with VP16-ERα (wild-type and variants) in a mammalian two-hybrid assay. ZR-75 cells were transfected with chimeric GAL4-DRIP205 constructs, pGal4$_5$ and transactivation was determined. The highest activity was observed for the following constructs where pMDm7Δ >> pMDm7 > pMDm12 > pMDm4 (Fig. 20A). Maximal AF activity was observed for pMDm7Δ, in which the NR boxes (amino acids 587-636), N-terminal amino acids 1-485 and C-terminal amino acids 1052-1566 were deleted. Insertion of the NR boxes (to give pMDm7) resulted in significant loss of AF activity. The NR boxes of DRIP205 also exhibited activity (pMDm4), regions flanking the NR boxes (i.e. amino acids 486-586 and amino acids 640-1051) cooperatively contribute to the AF of DRIP205. In MCF-7 cells, maximal AF activity was also observed for pMDm7Δ and significant AF activities were detected for the following constructs where pMDm7Δ > pMDm7 > pMDm12 > pMDm1 > pMDm4 (Fig. 21A), suggesting that in both cell lines, similar regions in DRIP205 are important for interacting with other coregulatory factors or the basal transcription machinery.

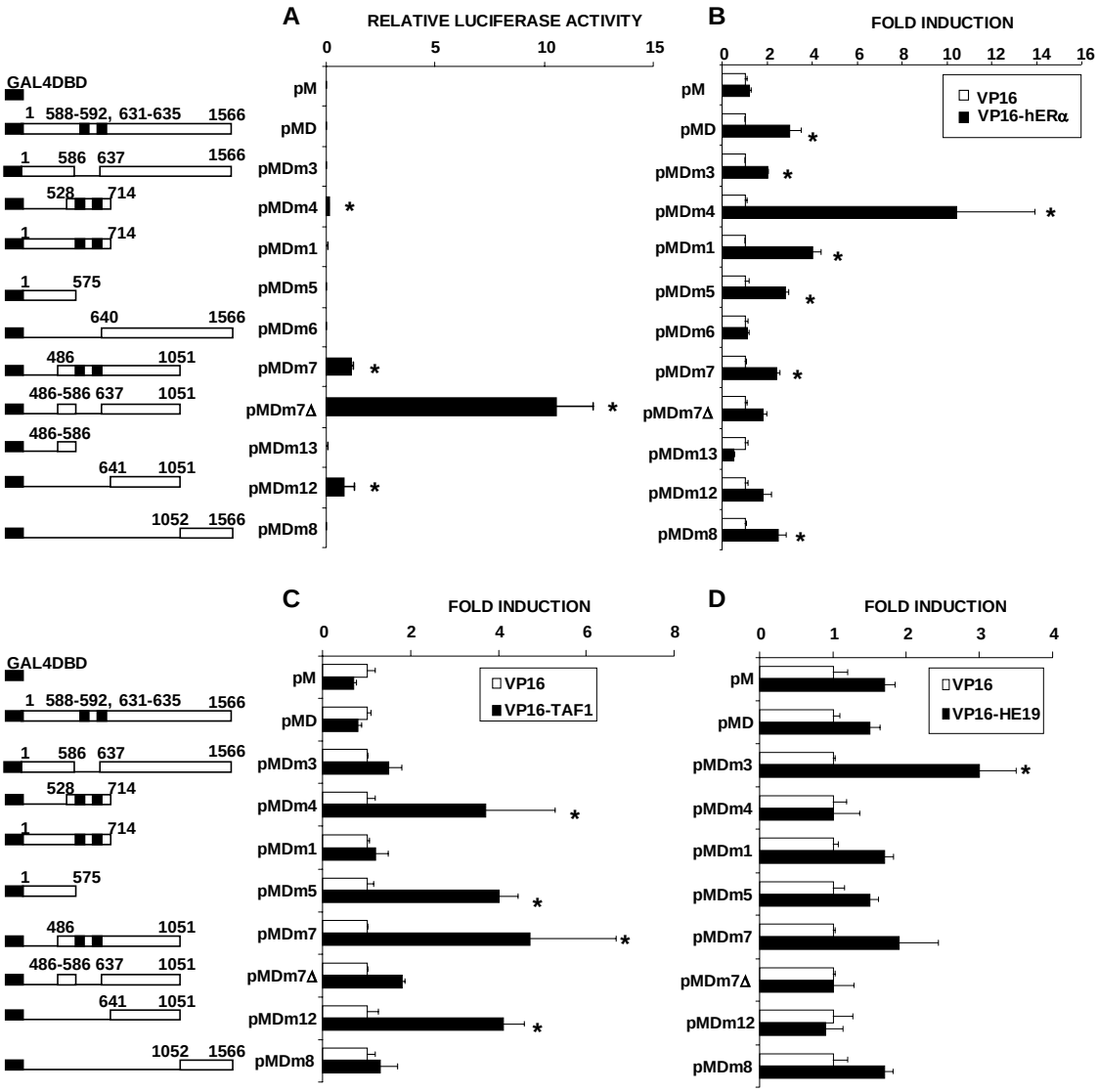


Fig. 20 Mapping of DRIP205 activation function and interactions with ERα in ZR-75 cells. A, DRIP205 activation. Cells were transfected with 250 ng β-gal, 500 ng pGAL4$_5$-LUC, 500 ng pM or pM DRIP wild-type or deletion mutants and luciferase activity was determined as described in Materials and Methods. B, C, D, Interactions of wild-type and variant pM-DRIP205 with VP16-ERα (B), VP16-TAF1 (C) and VP16-HE19 (D). Cells were transfected with 250 ng β-gal, 500 ng pGAL4$_5$-LUC, 500 ng pM or pM DRIP wild-type or deletion mutants, and 500 ng pVP16 empty or VP16-hERα (B), VP16-TAF1 (C) and VP16-HE19 (D), treated with DMSO or 10 nM E2 and luciferase activity was determined as described in the Materials and Methods. Fold induction by E2 for each treatment group was calculated by dividing the fold induction observed for E2/DMSO in cells transfected with VP16-hERα (wild-type or variant) by the fold induction (E2/DMSO) observed in cells transfected with pVP16 (empty vector) alone.

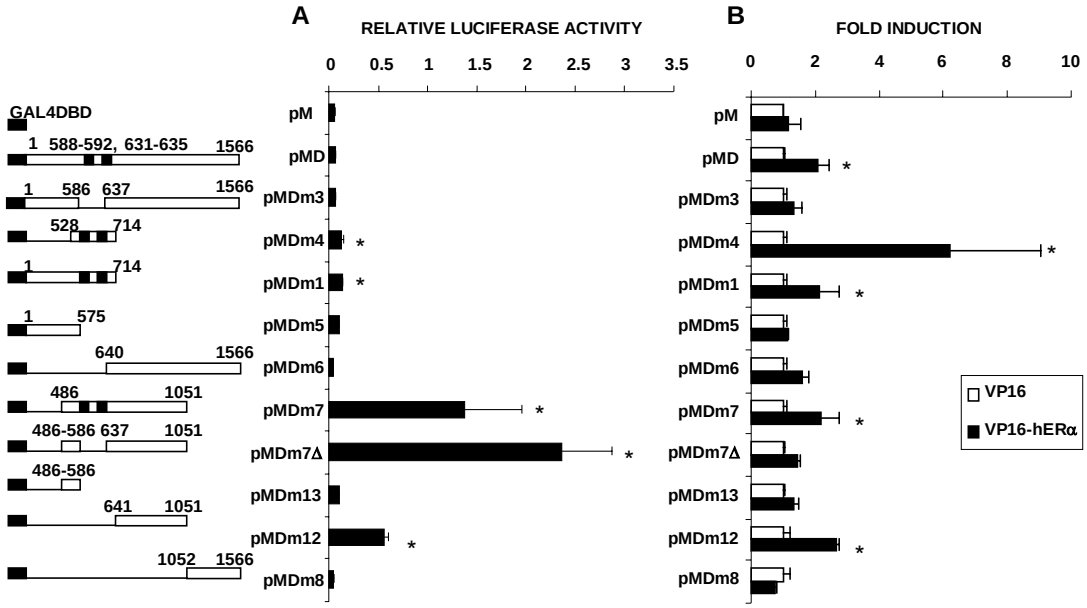


Fig. 21 Mapping of DRIP205 activation function and interactions with ERα in MCF-7 cells. A, DRIP205 activation. Cells were transfected with 250 ng β-gal, 500 ng pGAL4$_5$-LUC, 500 ng pM or pM DRIP wild-type or deletion mutants and luciferase activity was determined as described in Materials and Methods. B, Interactions of wild-type and variant pM-DRIP205 with VP16-ERα. Cells were transfected with 250 ng β-gal, 500 ng pGAL4$_5$-LUC, 500 ng pM or pM DRIP wild-type or deletion mutants, and 500 ng pVP16 empty or VP16-hERα, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in the Materials and Methods. Fold induction by E2 for each treatment group was calculated by dividing the fold induction observed for E2/DMSO in cells transfected with VP16-hERα by the fold induction (E2/DMSO) observed in cells transfected with pVP16 (empty vector) alone.

3.1.4 DRIP205 Interactions with ERα in Mammalian Two-hybrid Experiments

E2-dependent interactions of the same GAL4-DRIP205 constructs with ERα were determined in ZR-75 cells, transfected with VP16-ERα, where wild-type ERα is fused to the VP16 activation domain. The results (Fig. 20B) show that VP16-ERα interacts with multiple regions of DRIP205. E2 significantly induced activity in cells transfected with VP16-ERα and GAL4-DRIP205 (wild-type) and seven other constructs including pMDm4, pMDm1, pMD, pMDm5, pMDm8, pMDm7 and pMDm3. pMDm4, which expresses the NR box sequences (amino acids 528-714) exhibited the highest E2-dependent activity, suggesting that this sequence facilitates but is not required for ERα-DRIP205 interaction. All GAL4-DRIP205 chimeras that contain the NR boxes exhibit E2-induced interactions with ERα and addition of flanking sequences decreased hormone-induced activity. However, constructs such as pMDm8, pMDm5, and pMDm3, which do not contain the NR boxes also exhibit E2-dependent interactions with VP16-ERα, confirming that multiple domains of DRIP205 are involved in interactions with ERα. In MCF-7 cells, highest E2-dependent activity was also observed for pMDm4, like in ZR-75 cells, and significant E2-induced activity was observed for other constructs including GAL4-DRIP205 wild type chimera pMD, and pMDm1, pMDm7 and pMDm12. Among these constructs, pMDm12 does not contain NR boxes, but exhibits E2-dependent interactions with VP16-ERα (Fig. 21B).

Helix 12 of ERα is a major site for recruitment of LXXLL-box coactivators (324,487-492). However, E2-dependent activation of TAF1 (Fig. 16A), which contains helix 12 mutations, is observed in several cell lines, suggesting that other sites within ERα are required for interaction with coactivators. We therefore used VP16-TAF1 to investigate ligand-dependent interactions with GAL4-DRIP205 constructs (Fig. 20C). The results showed that > 2-fold induced activity was only observed for 4 chimeric proteins, namely pMDm4, pMDm7, which contain NR boxes and pMDm5, pMDm12, which express N- and C-terminal regions flanking the NR boxes. These results confirm that multiple regions of DRIP205 (± NR boxes) interact with ERα in regions outside helix 12 of ERα. A comparison of results in Figures 20B and 20C suggest that mutations of helix 12 did not affect ligand-dependent interactions of ERα with pMDm4, pMDm5, pMDm7 or pMDm12. In contrast, E2 did not induce activity in cells transfected with pMD (full length DRIP205), pMDm3, pMDm1, pMDm8 plus VP16-TAF1, suggesting that some regions of DRIP205 required an intact helix 12 for E2-dependent interactions with ERα; however, these interactions were not necessarily LXXLL box-dependent (eg. pMDm8 and pMDm3). These results suggest that DRIP205 interactions with ERα in breast cancer cells are highly complex and are dependent on multiple domains of both proteins. This was further illustrated by comparing interactions of the GAL4-DRIP205 constructs with VP16-HE19 (C-F domains) (Fig. 20D). E2-dependent GAL4-DRIP205 interactions with VP16-HE19 in a mammalian two-hybrid assay gave significant

induction in ZR-75 cells transfected with pM and pVP-HE19 (compared to pM plus pVP). Therefore, a comparison of transactivation results obtained for VP16-HE19 plus GAL4-DRIP205 constructs, with VP16-HE19 plus GAL4 (pM) alone, shows that enhanced activity was observed only for pMDm3, which does not contain the NR boxes. A comparison of these data with DRIP205 interactions observed using VP16-ERα suggests that DRIP205 interactions are primarily AF1-dependent or require both AF regions of ERα. The results confirm that DRIP205 interaction with and coactivation of ERα are complex and dependent on multiple regions of both proteins.

These results clearly show that DRIP205 coactivates ERα in various breast cancer cells. The coactivation of ERα requires AF1 and AF2 domains of ERα, and multiple regions of DRIP205. However, the two LXXLL motifs in the central region of DRIP205 are not required. Immunostaining confirmed that DRIP205 and ERα colocalize in the nucleus and deletion of two NR boxes in DRIP205 did not affect the colocalization of the two proteins. Interaction studies using coimmunoprecipitation assay and mammalian two-hybrid assay both showed that DRIP205 and ERα interact at multiple regions of both proteins.

3.2. DRIP205 Coactivation of ERα/Sp1 in Breast Cancer Cells

3.2.1 Coactivation of ERα/Sp1 by DRIP205

Coactivation of ERα/Sp1-dependent transactivation by DRIP205 was examined in ERα-positive MCF-7, ZR-75 and ERα-negative MDA-MB-231 cells. The three cell lines were transfected with ERα and wild type DRIP205 expression plasmid and $pSp1_3$-LUC, which contains three GC-rich sites in a minimal TATA-luciferase construct. The results showed that E2 induces transactivation 3-5-fold in all three cell lines, and the induction was enhanced ~2-3-fold by DRIP205 (Fig. 22B, C, D).

Colocalization of ERα, Sp1 and DRIP205 was also examined. ERα and DRIP205 colocalization has already been reported (Fig. 18) (493). In MCF-7 cells, endogenous Sp1 and ERα both exhibit nuclear staining (Fig. 23A, left and middle panels), and they were perfectly colocalized (Fig. 23A, right panels); however, some cells express different levels of Sp1 and ERα, and exhibited different staining intensities associated with the colocalized proteins. No significant differences were observed for DMSO (solvent control) and E2 treatments. Since endogenous DRIP205 staining is very low in breast cancer cells, COS-7 cells were used to examine colocalization of DRIP205 and Sp1. Cells were cotransfected with pMD (GAL4-DRIP205) and Sp1 expression plasmids. DRIP205 alone exhibits nuclear staining, and E2 induced a punctate pattern of nuclear staining as previously shown in Fig. 23B (left panels). DRIP205 and Sp1 are also colocalized, as shown in Fig. 23B (right panels).

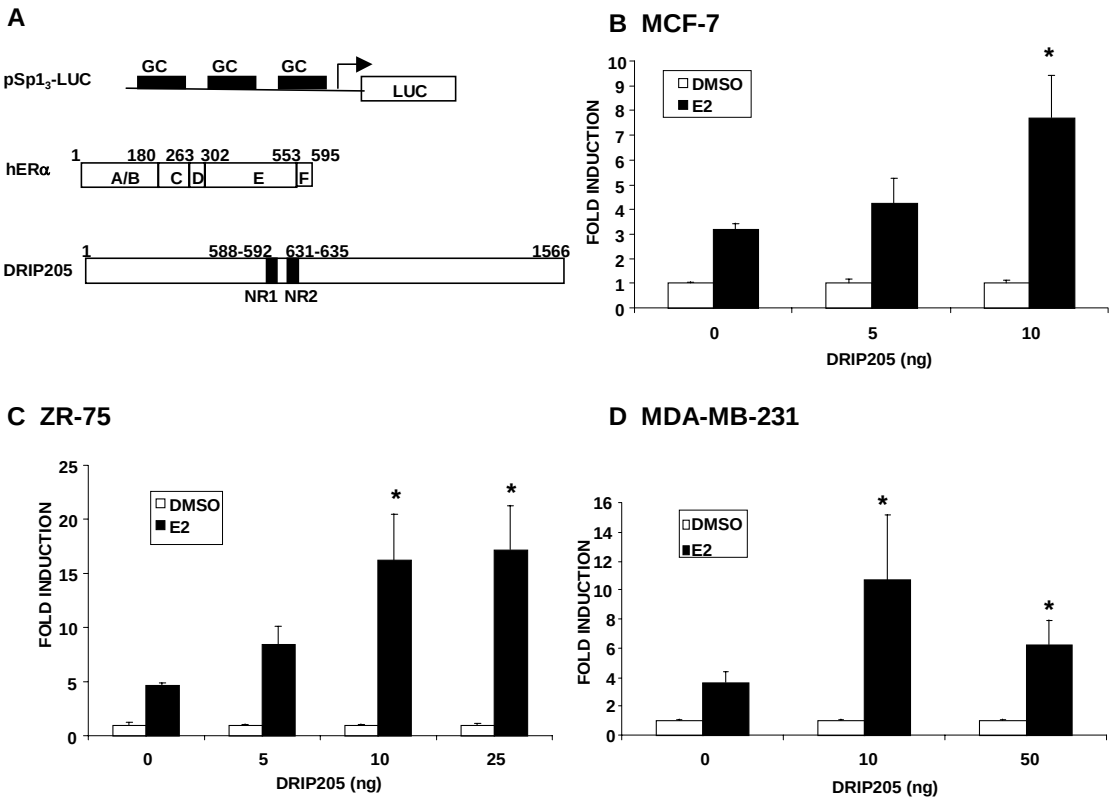


Fig. 22 Coactivation of ERα/Sp1 by DRIP205. A. Constructs for transfection assays. Coactivation of ERα/Sp1 by DRIP205 in MCF-7 (B), ZR-75 (C) and MDA-MB-231 (D) breast cancer cells. Cells were transfected with 1000 ng of pSp1$_3$-Luc, 250 ng of β-galactosidase as internal control, hERα (300 ng in B, 50 ng in C and 250 ng in D) and various amount of DRIP205 expression plasmids, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in Materials and Methods. Results are expressed as fold induction (compared with DMSO) and shown as mean ± S.E for three different experiments for each treatement group. Significant ($p<0.05$) coactivation by DRIP205 is indicated by an asterisk (*).

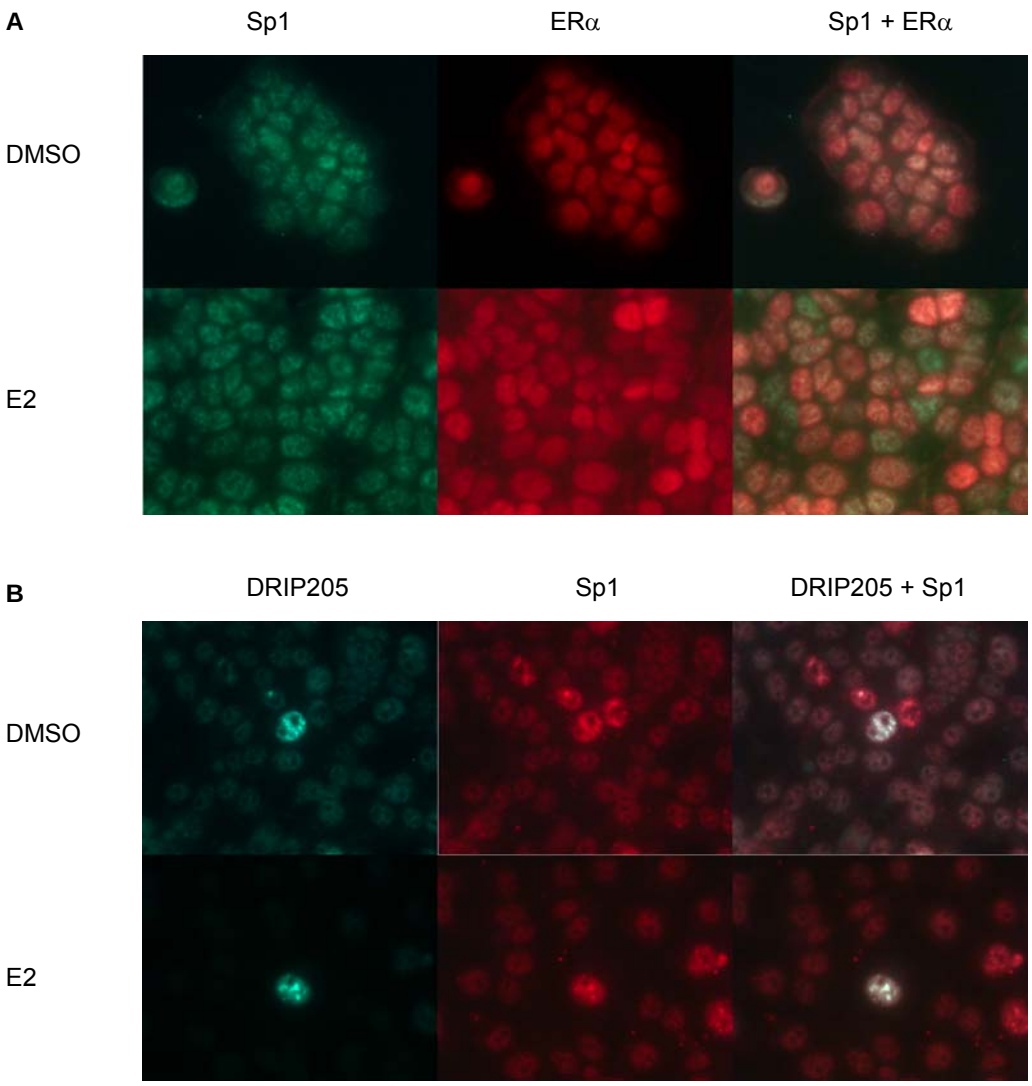


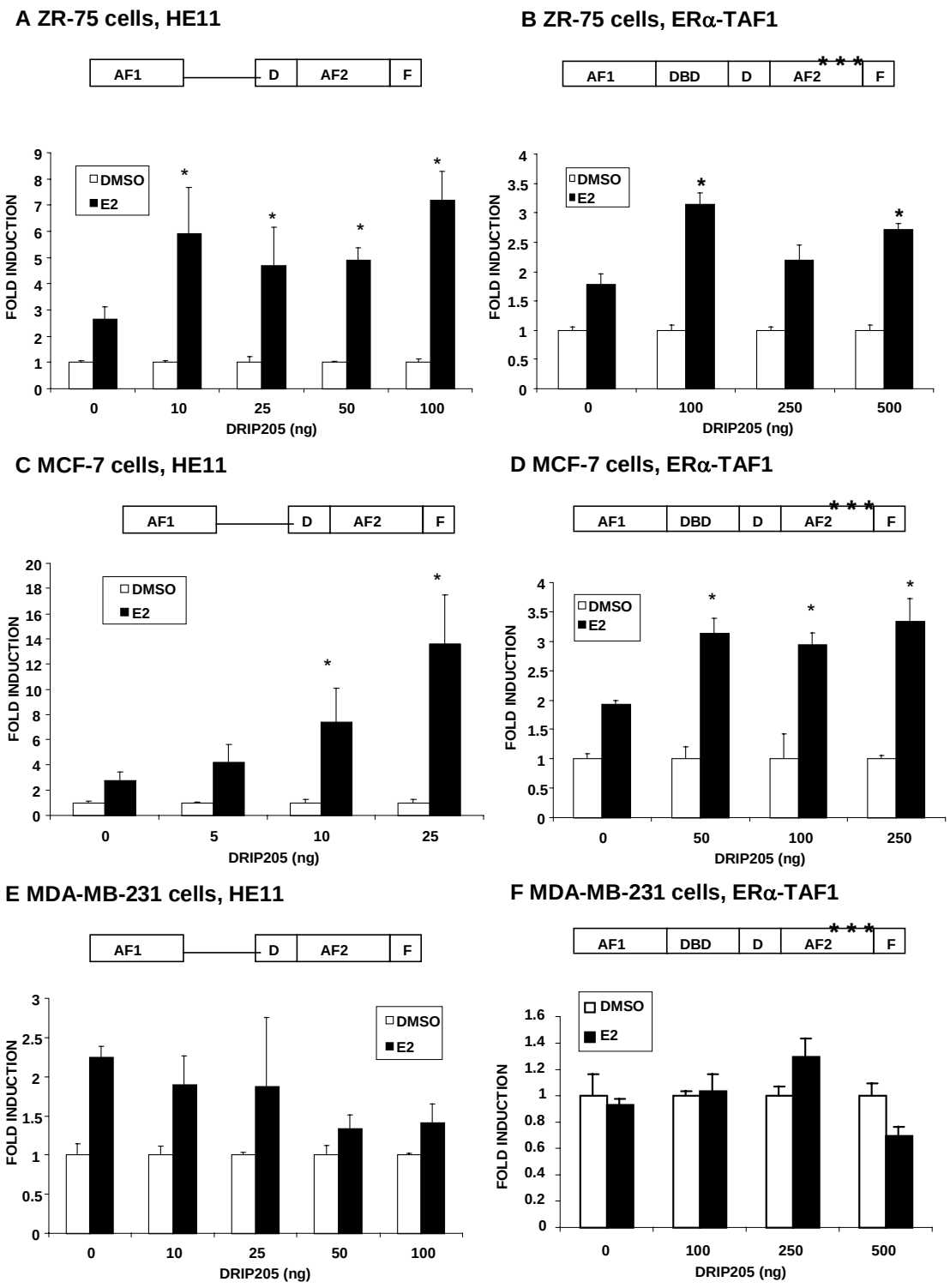
Fig. 23 Colocalization of Sp1 with ERα and DRIP205. A, Colocalization of Sp1 with ERα. MCF-7 cells were seeded in 2-well chamber slides for 24 hrs, treated with DMSO or 10 nM E2 for 1 h, and the immunostaining experiments were performed as described in the Materials and Methods. The same cells were analysed for each treatment. B. Colocalization of Sp1 with DRIP205. COS-7 cells were cotransfected with 250 ng of pMD (GAL4-DRIP205) and 250 ng of Sp1 expression plasmids, treated with DMSO or E2 for 1 h, and immunostaining experiments were performed as described in A.

3.2.2 Coactivation of Variant ERα/Sp1 by DRIP205

Coactivation of E2-induced transactivation was also investigated in ZR-75, MCF-7, and MDA-MB-231 cells cotransfected with $pSp1_3$-LUC and ERα variants HE11 (DBD deletion mutant) or ERα-TAF1 (with mutations in helix 12) and DRIP205 expression plasmid (Fig. 24). In ZR-75 cells transfected with $pSp1_3$-LUC, HE11 or ERα-TAF1, E2 induced approximately a 2-3-fold increase in luciferase activity (Figs. 24 A and B) and DRIP205 expression significantly enhanced transactivation of both HE11/Sp1 and ERα-TAF1/Sp1, by ~2.7-fold and ~1.7-fold, respectively. Coactivation of variant ERα/Sp1 by DRIP205 was also investigated in MCF-7 cells transfected with $pSp1_3$-LUC and HE11 or ERα-TAF1 and DRIP205 expression plasmid. Similar to the observations in ZR-75 cells, activation of HE11/Sp1 and ERα-TAF1/Sp1 by DRIP205 in MCF-7 cells was enhanced by 4.7- and 1.7-fold, respectively (Figs. 24 C and D). These results suggest that DBD and intact helix 12 (coactivator interacting surface) of ERα were not required for coactivation of ERα/Sp1-activated transcription in ER-positive ZR-75 and MCF-7 breast cancer cells.

Interestingly, in ER-negative MDA-MB-231 cells, E2 induced reporter gene activity by ~2-fold in cells transfected with $pSp1_3$-LUC and HE11; however, enhancement of HE11/Sp1-mediated transactivation by DRIP205 was not observed (Fig. 24E), suggesting that DBD of ERα was required for coactivation of ERα/Sp1 by DRIP205. E2 failed to induce transcription in MDA-MB-231 cells transfected with $pSp1_3$-LUC and ERα-TAF1, and DRIP205 did not coactivate ERα-TAF1/Sp1 (Fig. 24F), suggesting that the integrity of helix 12 coactivator binding core of ERα is required for ERα/Sp1-mediated transactivation and is necessary for coactivation of E2-induced transcription of GC-rich promoter constructs by DRIP205 in ER-negative MDA-MB-231 cells. Compared with results in ER-positive ZR-75 and MCF-7 cells, these results indicate that the domains of ERα that are critical for coactivation of ERα/Sp1 by DRIP205 are cell-context dependent. This controversy may be due to differences in mechanisms of DRIP205 interactions with ERα in different cell lines, which may

Fig. 24 Coactivation of variant ERα/Sp1 by DRIP205 is cell-context dependent. ZR-75 (A, B), MCF-7 (C, D), MDA-MB-231 (E, F) breast cancer cells were transfected with 1000 ng $pSp1_3$-Luc, 250 ng HE11 (A, C, E) or 1000 ng ERα-TAF1 (B, D, F), and various amount of DRIP205 expression plasmid, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in the Materials and Methods. Significant enhancement by DRIP205 is indicated by an asterisk (*) in ZR-75 (A, B) and MCF-7 cells (C, D). Coactivation of variant ERα/Sp1 was not observed in MDA-MB-231 cells (E, F).


A ZR-75 cells, HE11
AF1
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
10
25
50
100
B ZR-75 cells, ERα-TAF1
AF1
DBD
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
100
250
500
C MCF-7 cells, HE11
AF1
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
5
10
25
D MCF-7 cells, ERα-TAF1
AF1
DBD
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
50
100
250
E MDA-MB-231 cells, HE11
AF1
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
10
25
50
100
F MDA-MB-231 cells, ERα-TAF1
AF1
DBD
D
AF2
F
DMSO
E2
FOLD INDUCTION
DRIP205 (ng)
0
100
250
500

involve other transcription factors and depend on individual expression patterns of nuclear factors in different cell lines. It is possible that expression of other nuclear proteins that may interact and/or facilitate DRIP205-ERα/Sp1 complex formation are changed in ER-negative MDA-MB-231 cells compared to ER-positive cell lines.

Previous studies in this laboratory have shown that the AF1 domain of ERα was important for activation of ERα/Sp1-mediated transcription by E2. Deletion of the AF1 domain of ERα totally abolished ERα/Sp1-activated transcription and deletion of amino acids 50-117 of ERα caused significant loss of E2-induced activity (395). Coactivation studies by DRIP205 were carried out using partial AF1 deletion mutants of ERα. Enhancement by DRIP205 was observed in cells cotransfected with the AF1 C-terminal deletion mutant ERαΔ122-178 of ERα (Fig. 25B); however, coactivation was not detected in cells transfected with the AF1 N-terminal deletion mutant ERαΔ3-50 of ERα (Fig. 25A), suggesting that coactivation of ERα/Sp1-mediated transactivation by DRIP205 requires an intact N-terminal AF1 domain of ERα.

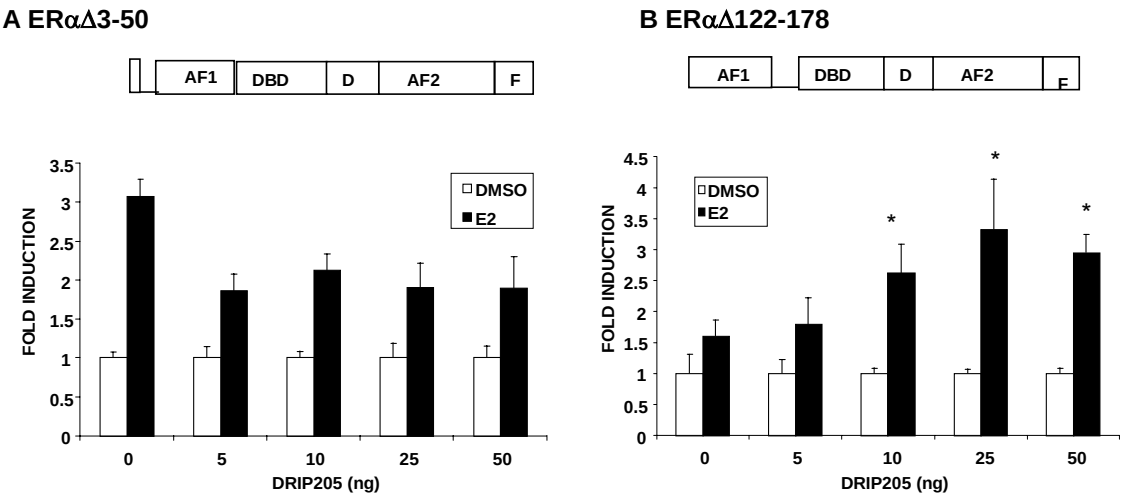


Fig. 25 The N-terminal AF1 region of ERα is required for coactivation of ERα/Sp1 by DRIP205 in ZR-75 cells. Cells were cotransfected with 1000 ng pSp1$_3$-Luc, 250 ng β-galactosidase as an internal control, 500 ng of ERαΔ3-50 (C) or ERαΔ122-178 (D) and various amount of DRIP205 expression plasmids (as indicated), treated with DMSO or 10 nM E2 and luciferase activity was determined as described under Meterials and Methods. Significant enhancement ($p<0.05$) by DRIP205 is indicated by an asterisk (*) in A. Coactivation of ERαΔ3-50/Sp1 by DRIP205 (B) was not observed.

3.2.3 Coactivation of ERα/Sp1 by DRIP250 Deletion Mutants

Several laboratories have shown that the NR boxes in DRIP205 are responsible for interactions and coactivation with NRs (396,462,481,484,485); however, previous studies in this laboratory have shown that in breast cancer cells, multiple regions of DRIP205 are involved in coactivation and interaction of ERα besides the two NR boxes (Fig. 17, 19) (493). In ZR-75 cells, DRIP205 deletion mutants were examined for their coactivation of ERα/Sp1-mediated transactivation. Cells were cotransfected with $pSp1_3$-LUC and hERα, and the C-terminal 852 amino acids deletion mutant of DRIP205. DRIP205 (1-714) enhanced E2-induced transactivation by ~2-fold (Fig. 26A). The N-terminal 515 amino acids deletion mutant DRIP205 (516-1566) also coactivated ERα/Sp1, and the fold induction was enhanced 3.5-fold (Fig. 26B). However, both N-terminal 515 amino acids and C-terminal 852 amino acids deletion mutant DRIP205 (528-714), which contain the two LXXLL motifs, did not coactivate ERα/Sp1-activated transcription (Fig. 26C). In contrast, the NR box deletion mutant DRIP205Δ587-636, enhanced ERα/Sp1-mediated transactivation by approximately 2-fold (Fig. 26D). These results suggest that the two NR boxes are neither necessary nor required for coactivation of ERα/Sp1 by DRIP205 in ZR-75 breast cancer cells; however, both N-terminal and C-terminal regions of DRIP205 are involved in the coactivation function.

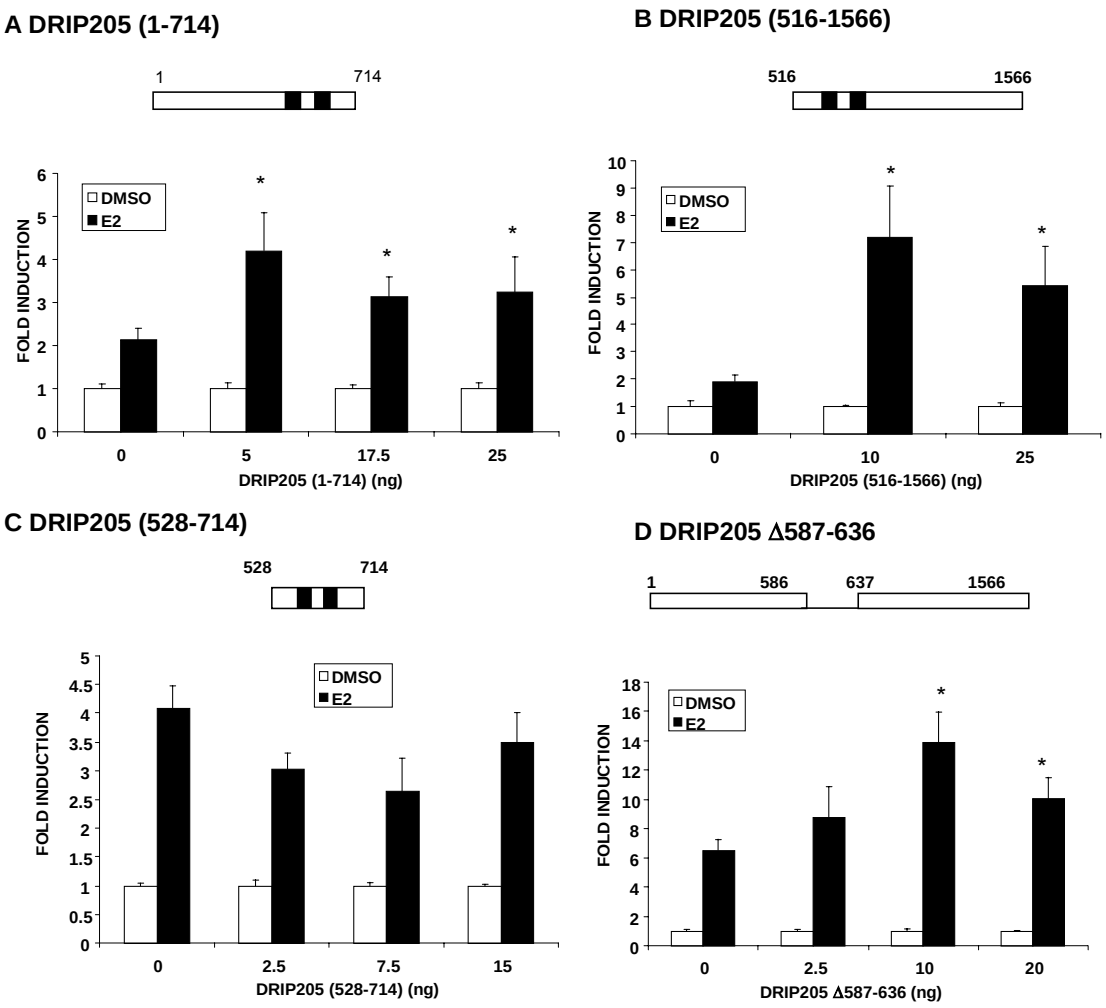


Fig. 26 Coactivation of ERα/Sp1 by DRIP205 deletion mutants. ZR75 cells were cotransfected with 1000 ng pSp1$_3$-Luc, 50 ng of hERα and various amount of DRIP205 deletion mutants DRIP205 (1-714) (A), DRIP205 (516-1566) (B), DRIP205 (528-714) (C) and DRIP205Δ587-636 (D), treated with DMSO or 10 nM E2, and luciferase activity was determined as described in Materials and Methods. Significant coactivation ($p<0.05$) of ERα/Sp1 by DRIP205 variants is indicated by an asterisk (*).

3.2.4 Role of Sp Family Proteins in DRIP205 Coactivation of ERα/Sp

Previous studies suggested that both ERα/Sp1 and ERα/Sp3 were required for hormonal activation of GC-rich promoter constructs (393,394). Sp4 has also been detected in many cancer cell lines including ZR-75 cells (494). To determine the role of Sp1, Sp3 and Sp4 proteins in hormonal activation of ERα/Sp, we used small inhibitory RNAs (siRNAs) to knock down the endogenous Sp proteins. ZR-75 cells were transfected with siRNAs (NS: nonspecific; iSp1, inhibitory Sp1; iSp3, inhibitory Sp3; iSp4, inhibitory Sp4; iGL2, inhibitory luciferase), cotransfected with $pSp1_3$-LUC and hERα expression plasmid and treated with DMSO or 10 nM E2. Results showed that basal activities were decreased in cells transfected with iSp1 (70% loss), iSp3 (78% loss), iSp4 (88% loss) (DMSO treatment, Fig. 27A, white columns), indicating that all of the three Sp proteins bind GC-rich sites and activate basal transcription. E2-induced activities were also decreased by all three Sp siRNAs

(Fig. 27A, black columns). However, fold-inducibility by E2 was not significantly changed. This suggests that Sp1, Sp3 and Sp4 are all involved in ERα/Sp action in ZR-75 cells. Results in Figs. 27 B and C confirmed that transient transfection of siRNAs for Sp1, Sp3, and Sp4 knocked down their corresponding proteins in ZR-75 cells without affecting expression of the other Sp proteins or β-actin which was used as a loading control. For example, iSp1 down-regulated Sp1 protein expression, while Sp3 and Sp4 protein levels were not significantly changed. In the RNA interference studies, the decreased protein (Sp1, Sp3 or Sp4) levels were similar in cells treated with DMSO or 10 nM E2. These data were obtained from whole cell lysates of transfected cells indicating that both transfection efficiencies (>70%) and siRNA-dependent protein down-regulation were highly effective. In cells transfected with iGL2, a greater than 90% decrease in luciferase activity was observed (Fig. 27A).

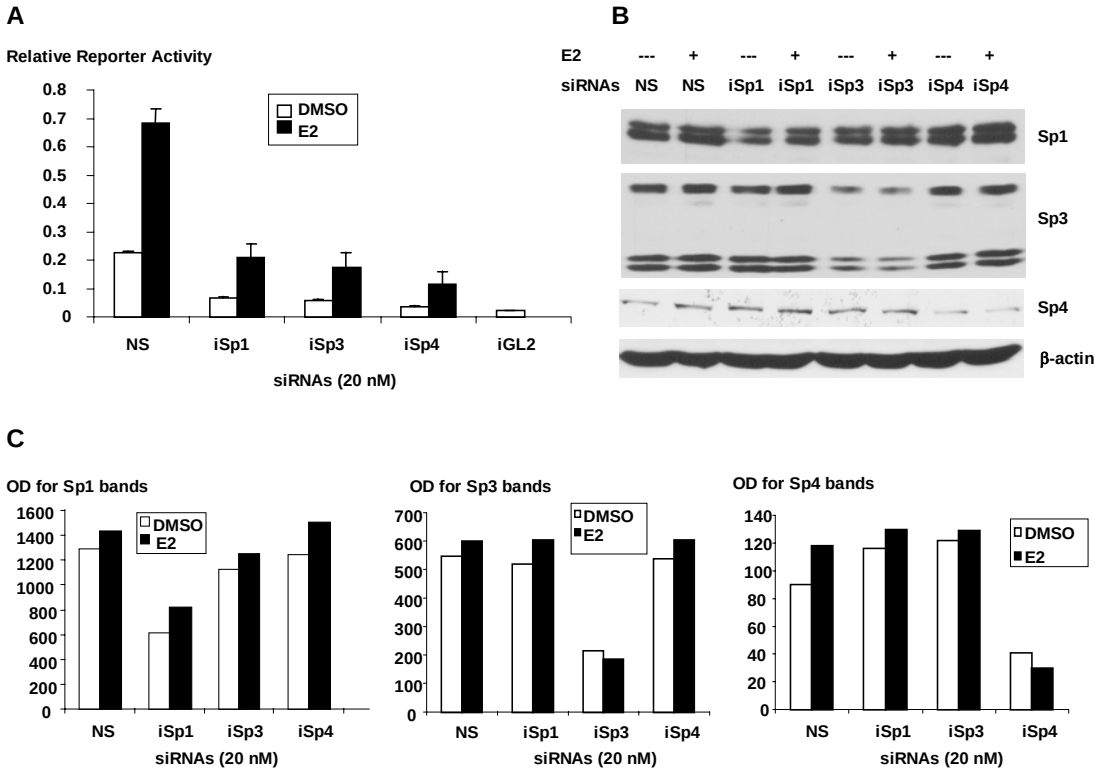


Fig. 27 Role of Sp family proteins in hormonal activation of ERα/Sp in ZR-75 cells. A, siRNAs for Sp1, Sp3 and Sp4 decreased ERα/Sp-mediated transactivation. Cells were transfected with 20 nM siRNAs (NS: nonspecific; iSp1, inhibitory Sp1; iSp3, inhibitory Sp3; iSp4, inhibitory Sp4; iGL2, inhibitory luciferase), cotransfected with 100 ng β-gal, 300 ng $pSp1_3$-LUC and 200 ng ERα, treated with DMSO or 10 nM E2 and luciferase activity was determined as described in the Materials and Methods. B and C, siRNAs for Sp1, Sp3 and Sp4 down-regulate their corresponding protein levels. Cells were transfected with siRNAs, treated with DMSO or 10 nM E2. Protein levels were analysed by Western blot and protein band intensities were determined by scanning laser densitometry as described in Materials and Methods.

Previous studies have shown that antiestrogens also activate ERα/Sp-mediated transactivation in cells transfected with $pSp1_3$-LUC (395,396). Therefore we examined the role of three Sp family proteins in antiestrogen-activated ERα/Sp. ZR-75 cells were transfected with 20 nM siRNAs, cotransfected with $pSp1_3$-LUC and ERα expression plasmid, treated with DMSO or 1 μM ICI 182,780 or 1 μM 4-OH tamoxifen. Basal activities were decreased in cells transfected with iSp1, iSp3 and iSp4 (Fig. 28, white columns). ICI 182,780 induced luciferase activity by 9-fold and an 11-fold increase in activity was observed using 4-OH tamoxifen in ZR-75 cells transfected with nonspecific siRNA. Transfection with siRNAs for all three Sp proteins (iSp1, iSp3, iSp4) decreased ICI 182,780 treated and 4-OH tamoxifen treated luciferase activity. However, the fold induction was not changed (Fig. 28). These results were similar to that observed for E2 (Fig. 27A), suggesting that all three Sp family proteins are involved in antiestrogen-activated ERα/Sp.

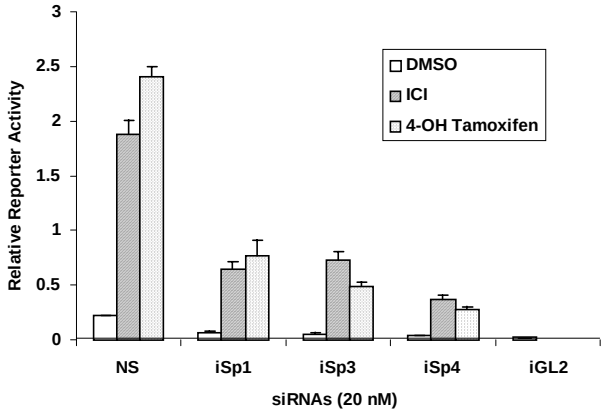


Fig. 28 Role of Sp family proteins in antiestrogen-activated ERα/Sp1 in ZR-75 cells. Cells were transfected with 20 nM siRNAs, cotransfected with 100 ng β-gal, 300 ng pSp1$_3$-LUC and 200 ng hERα, treated with DMSO or 1 μM ICI 182,780 or 1 μM 4-OH tamoxifen and luciferase activity was determined as described in Materials and Methods.

The DBD of ERα is not required for activation of ERα/Sp by E2, whereas this region of ERα was important for antiestrogen-activated ERα/Sp (396). It is possible that the requirement of the DBD for antiestrogen activation may not involve all three Sp proteins. Therefore, we used the same siRNA approach to further investigate the role of Sp proteins in mediating estrogen/antiestrogen activation of HE11/Sp. ZR-75 cells were transfected with siRNAs, cotransfected with $pSp1_3$-LUC and HE11 expression plasmid, treated with DMSO, 10 nM E2, 1 μM ICI 182,780 and 1 μM 4-OH tamoxifen. Results (Fig. 29) indicated that in cells transfected with NS (nonspecific siRNA), E2 induced an ~4-fold increase in relative luciferase activity whereas induction by ICI 182,780 or 4-OH tamoxifen was minimal compared to the response observed for wild type ERα (Fig. 28). Basal activities were decreased in cells transfected with iSp1 (80% loss), iSp3 (73% loss) and iSp4 (69% loss). E2-induced luciferase activity was significantly decreased by transfection with iSp1 (87% loss) compared to NS, with the fold induction (E2/DMSO) decreased from 3.7-fold to 2.4-fold, respectively. Transfection with iSp3 or iSp4 also decreased relative luciferase activity from E2-treated cells to 33% and 39%, respectively; however, the changes in the fold induction were minimal. Relative luciferase activities were also significantly decreased in cells transfected with iSp1, iSp3, or iSp4 and treated with 1 μM ICI 182,780 or 1 μM 4-OH tamoxifen, and the largest decrease in activity was observed in cells transfected with iSp1. Fold induction was increased in cells transfected with iSp3 and treated with 1 μM ICI 182,780 from 1.3-fold (NS) to

3.2-fold. In cells transfected with iSp3 or iSp4 and treated with 1 μM 4-OH tamoxifen, fold induction was also increased from 1.5-fold (NS) to 2.7- (iSp3) and 2.7-fold (iSp4), respectively. These results suggest that Sp1, Sp3 and Sp4 proteins are all involved in HE11/Sp-mediated transactivation in response to estrogen and antiestrogens and Sp1 protein is the most significant contributor to basal and induced activity in response to E2. Interestingly, the loss of Sp1, Sp3 or Sp4 protein differentially affected the fold induction of activity by E2 and the antiestrogens, suggesting a possible ligand preference for individual Sp proteins in activation of HE11/Sp.

We further investigated the role of Sp family proteins in coactivation of ERα/Sp by DRIP205. ZR-75 cells were transfected with siRNAs for nonspecific Sp1, Sp3 or Sp4 protein, cotransfected with $pSp1_3$-LUC, ERα and DRIP205, treated with DMSO or 10 nM E2. Results in Fig. 30A show that transfection of iSp1, iSp3 and iSp4 decreased both basal and E2-induced luciferase activities in two transfection groups transfected with 0 or 2.5 ng DRIP205 expression plasmid. Changes in fold inducibility in the coactivation experiments (Fig. 30B) show that wild-type DRIP205 enhances transactivation of ERα/Sp by ~2 fold in cells transfected with NS (nonspecific siRNA). In cells transfected with iSp1, ERα/Sp-mediated transactivation was enhanced approximately 3.5-fold by DRIP205, whereas DRIP205 did not coactivate ERα/Sp in cells transfected with iSp3 or iSp4. Similar results were observed in two additional experiments suggesting that DRIP205 preferentially coactivates ERα/Sp3 and ERα/Sp4 and

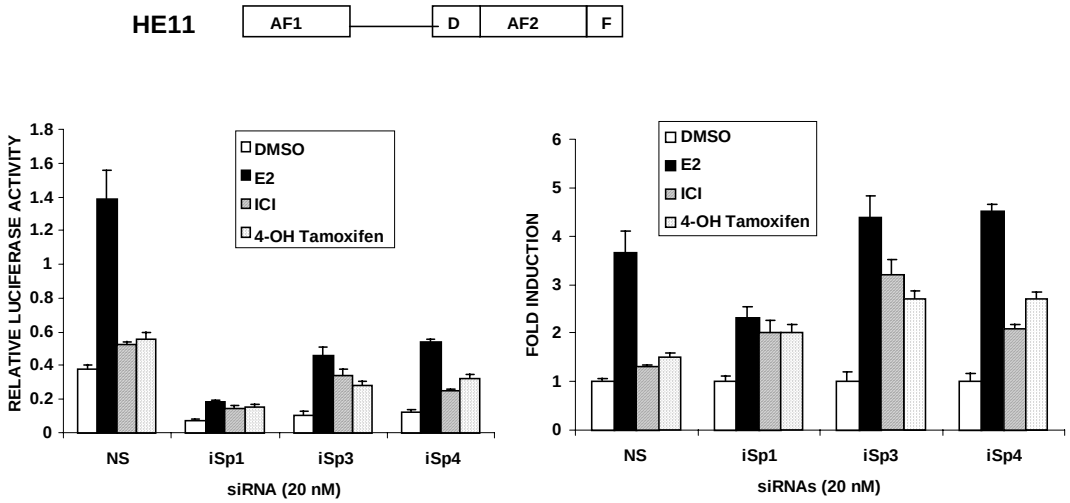


Fig. 29 Role of Sp family proteins in HE11/Sp action in ZR-75 cells. Cells were transfected with 20 nM siRNAs, cotransfected with 100 ng β-galactosidase, 200 ng $pSp1_3$-LUC and 200 ng HE11 expression plasmid, treated with DMSO or 10 nM E2 or 1 μM ICI 182,780 or 1 μM 4-OH tamoxifen and luciferase activity was determined as described in Materials and Methods.

this may involve some cooperativity between Sp3 and Sp4.

Colocalization of ERα and Sp4 was examined in COS-7 cells. Cells were transfected with hERα and Sp4 expression plasmid, treated with DMSO or 10 nM E2 for 1 h. Results in Fig. 31 showed that both ERα and Sp4 exhibit nuclear staining. Sp4 staining in the nucleus is not as uniform as ERα staining. Sp4 seems to show more intense staining in the middle of the nucleus, with some lower staining in surrounding areas and E2 treated cells showed more punctate staining than cells treated with DMSO. ERα and Sp4 are colocalized in the nucleus.

These results showed that DRIP205 coactivates ERα/Sp-mediated gene expression in various breast cancer cell lines. The coactivation of ERα/Sp by DRIP205 requires AF1 domain of ERα and multiple regions of DRIP205, but does not require the two NR boxes of DRIP205. In RNA interference assays, DRIP205 coactivates ERα/Sp in cells transfected with siRNA for Sp1, but does not enhance ERα/Sp-mediated transactivation in cells transfected with siRNA for Sp3 or Sp4, indicating that Sp3 and Sp4 are both required for coactivation of ERα/Sp by DRIP205.

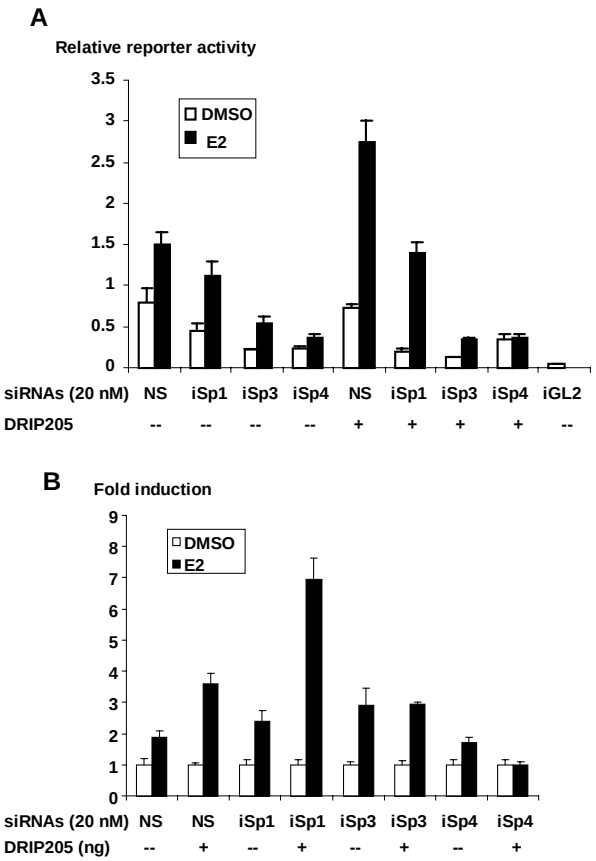


Fig. 30 Role of Sp family proteins in DRIP205 coactivation of ERα/Sp. Data presented as relative luciferase activities (A) or fold induction (B). ZR-75 cells were transfected with 20 nM siRNAs, cotransfected with 100 ng β-galactosidase, 300 ng pSp1$_3$-LUC, 200 ng hERα and 0 or 2.5 ng of DRIP205 expression plasmid, treated with DMSO or 10 nM E2 and luciferase activity was determined in Materials and Methods.

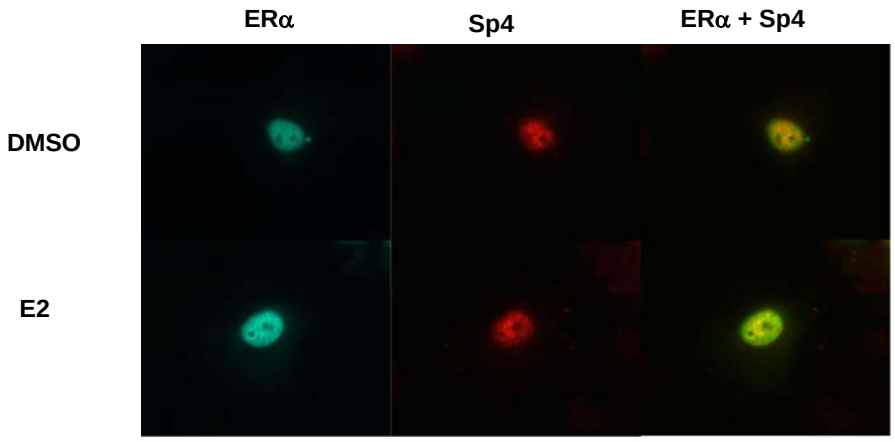


Fig. 31 Colocalization of ERα and Sp4. COS-7 cells were transfected with hERα and Sp4 expression plasmid, treated with DMSO or 10 nM E2 for 1 h and the Immunostaining experiments were performed as described in Materials and Methods. The same cells were analysed for each treatment.

CHAPTER IV

DISCUSSION AND CONCLUSIONS*

4.1 Coactivation of Nuclear Receptors

The nuclear receptor superfamily of transcription factors contains ligand-activated and orphan receptors that interact with genomic cis-elements in target gene promoters to induce gene expression (274,397-399,495). 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 nuclear complex of coactivator/coregulatory proteins (399,478,479,496-498). The p160/SRC coactivators were first discovered as nuclear proteins that specifically interact with ligand-bound NRs (403,499). Other coregulatory proteins such as p300/CBP and pCAF are also components of the receptor-coregulatory complex, and these proteins, in part, modify chromatin structure and promote accessibility through their HAT activities (435,436).

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* Part of this chapter is reprinted with permission from " Vitamin D-interacting protein 205 (DRIP205) coactivation of estrogen receptor α (ERα) involves multiple domains of both proteins" by Wu, Q., Burghardt, R. and Safe, S., (2004), *J. Biol. Chem*., 279(51), 3602-53612.

SRC-1/NcoA-1 was cloned using the PR LBD as bait in a yeast two-hybrid screen of a human B-cell cDNA library. SRC-1 enhances ligand-dependent transactivation of a broad range of nuclear receptors, including PR, GR, ER, TR, RXR, and PPARγ. AF2 of ER binds to the central region of SRC-1 (405). AF1 activity of ER also requires SRC-1 (316), and SRC-1 facilitates interactions of AF1 and AF2 of ER (314). SRC-1 also interacts with general transcription factors, such as TBP and TFIIB, suggesting that SRC-1 may serve as a bridging factor between NRs and general transcription machinery (407). SRC-2/GRIP1/NcoA-2/TIF2 coactivates ERα in HeLa cells and interacts with both AF1 and AF2 of ER (316,410,411). Cocrystallization data showed that GRIP1 interacts with the hydrophobic groove formed by helices 3, 4, 5, and 12 of the ERα LBD. The coactivator recognition groove was occluded with antagonist binding to LBD by mimicking the interactions of the NR box with the LBD (346). SRC-3/ACTR/AIB1/TRAM1/RAC3/p/CIP specifically enhanced E2-induced transactivation of ERα, but does not stimulate ERβ-mediated transcription (412,419).

Functional redundancy of SRC proteins has been suggested, because SRC-1 knock-out mice showed increased SRC-2/TIF2 expression. Targeted disruption of the SRC-1 gene caused slightly decreased growth and development in homozygous mutants in response to E2 in estrogen target organs, but these mutant transgenic animals were viable and fertile (324). Amplification and overexpression of AIB1/SRC-3 was observed in four of the five

ER-positive breast and ovarian cancer cell lines (BT-474, MCF-7, ZR-75-1 and BG-1). Higher expression of SRC-3 has also been detected in 64% of the primary breast tumors examined (412). Both SRC-1 and SRC-3 exhibit intrinsic HAT activity and recruit other coregulatory proteins such as p300/CBP and pCAF (406,413).

The conserved NR boxes in the central region of all three SRC proteins are important for mediating interactions with NRs (315,411,414,417), and the nonconserved LXXLL motif in the C-terminus of hSRC-1 mediates the hormone-dependent interactions of hSRC-1 with PR (315). The enhancement of ER-mediated transactivation by SRC-1 is dependent on the integrity of LXXLL motifs (NR boxes) in the central region of SRC-1 that binds the AF2 region of ER. Mutation of key residues in the four NR boxes of hSRC-1 disrupted interactions of hSRC-1 with AF2 of ER, and this hSRC-1 mutant did not coactivate ligand-dependent transactivation of ER (405). Amino acids immediately adjacent to the LXXLL motif regulate the affinity of the interaction with receptors and impart specificity of LXXLL motifs (342).

Mediator complex proteins also enhance transactivation through recruitment of RNA polymerase II to target gene promoters (153,452,454,456-458,460,468,486), and several studies report ligand-dependent interactions of DRIP205 and/or related mediator proteins with NRs. DRIP205 appears to be the critical linkage protein between NRs and other protein components of the mediator complex (462,469,476,484,485,500-506). In GST pull-down assays, three proteins from the mediator complex, DRIP205, DRIP150 and DRIP77 bound to the VDR LBD. However, DRIP205 was the only protein that bound the VDR LBD in a ligand-dependent manner, suggesting that DRIP205 is the anchor protein for interactions with VDR (457). The mediator complex of proteins binds ERα and ERβ (ligand-dependent), whereas extracts from TRAP220$^{-/-}$ embryo fibroblast do not bind ER, demonstrating the critical role of TRAP220 in ER-mediator complex interactions and transactivation (506). The central region of DRIP205 (amino acids 527-714) that contains two NR boxes interacts with NRs, including GR (461), ERα (462), FXR (464), TR (468) and PPARγ (472) in GST pull-down assays, confirming the interactions between LXXLL motifs in DRIP205 with NRs.

Previous reports have investigated coactivation of ERα by DRIP205 and interactions of these proteins and the results are variable and dependent on the assay and cell context. TRAP220 interacted preferentially with ERβ compared with ERα and interactions were dependent on the two NR boxes in TRAP220 (505). TRAP220 did not coactivate ERα-mediated transactivation in HeLa cells

and exhibited weak interactions with ERα that were dependent on extended LXXLL motifs (485). In contrast, PBP (PPARγ binding protein) potentiated E2-dependent transactivation in CV-1 cells (473). In a mammalian two-hybrid assay in COS-7 cells, E2 induced GAL4-TRAP220 (549-718) interactions with VP16-ER (DEF) (ERα and ERβ); and in GST pull-down assays, TRAP220 (549-718) preferentially bound ERβ and their interactions were dependent on both NR boxes. However, interactions of TRAP220 (549-718) with ERα were not sufficient (505). Burakov and coworkers reported that in GST pull down assays, DRIP205 (527-714) did not bind the N-terminal ABCD domain of ERα, and interactions with the LBD (E/F) were dependent on intact DRIP205 NR boxes, and L539A/L540A mutation in helix 12 of ERα abrogated interactions (462,476).

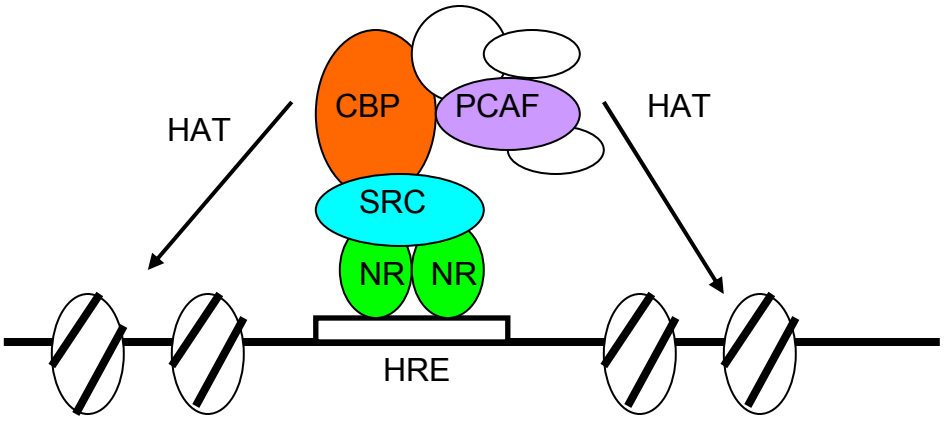
Chromosome localization showed that the RB18A/DRIP205/PBP gene was localized on chromosome 17q12-q21.1 by fluorescence in situ hybridization. This chromosomal region is amplified in some breast cancers and this region also contains the breast cancer gene BRCA1 and the oncogene HER-2, indicating that overexpression of RB18A/PBP may play a role in breast cancer (465,472). TRAP220$^{-/-}$ mice are recessive embryonic lethal, and die in an early gestational stage with heart failure and show impaired neuronal development with extensive apoptosis (470). Similarly, a PBP null mutation is embryonic lethal at E11.5, indicating that there is no functional redundancy between DRIP205/PBP and other coactivators (474).

p160/SRC coactivators and DRIP/TRAP mediators share some characteristics. They both bind NRs and facilitate ligand-activated transcription and contain functional and signature LXXLL motifs that play a role in interacting with AF2 of NRs. They also interact with general transcriptional factors and may serve as bridging factors between NRs and RNA polymerase II to activate transcription. However, these two groups of proteins differ in many aspects. DRIP mediators do not have HAT activity, which is a common feature for p160/SRC coactivators and p300/CBP coregulators. Intrinsic HAT activities enable SRC coactivators to remodel chromatin and stabilize the preinitiation complex. DRIPs and p160 coactivators do not exist in the same nuclear complexes, and have never been co-purified biochemically (484). SRC coactivators also exhibit functional redundancy; SRC-1 knock out mice are viable and fertile and show increased SRC-2 expression. However, TRAP220/PBP mutations are recessive embryonic lethal.

It has been suggested that p160/SRC coactivators and DRIP/TRAP mediators function separately but coordinately (Fig. 32), and their actions are ligand- and NR-dependent. In PPARγ/RXR-mediated transactivation, RXR-specific ligands induced p160 binding to RXR, while PPARγ-specific ligands exclusively recruited DRIP205 but not p160 coactivators to PPARγ (457). Using a chromatin immunoprecipitation assay, a cyclic association and dissociation of coactivators was observed in the E2-responsive region of the pS2 promoter and recruitment of p160s and DRIPs occurred in opposite phases, suggesting an

exchange between the two coactivator complexes at the target promoter (476). These two groups of coactivators also function in different development processes. During differentiation, DRIP/Mediator complex is the major VDR binding complex in proliferating keratinocytes, while members of p160/SRC family coactivators are predominant after differentiation. Moreover, both DRIP205 and SRC-3 enhanced vitamin D-induced transcription in proliferating cells. However, after differentiation, DRIP205 was no longer functional (477). It was also suggested that in these experiments the SRC coactivators and DRIP/TRAP mediators were recruited sequentially rather than competitively. In cells expressing an ERα AF2 point mutant (L540Q), which selectively binds and recruits Med220 but not SRCs, the recruitment of Med220 to the pS2 promoter was delayed and activation of most E2-responsive genes were impaired (479).

A. p160/CBP/PCAF complex



B. DRIP/TRAP/ARC complex

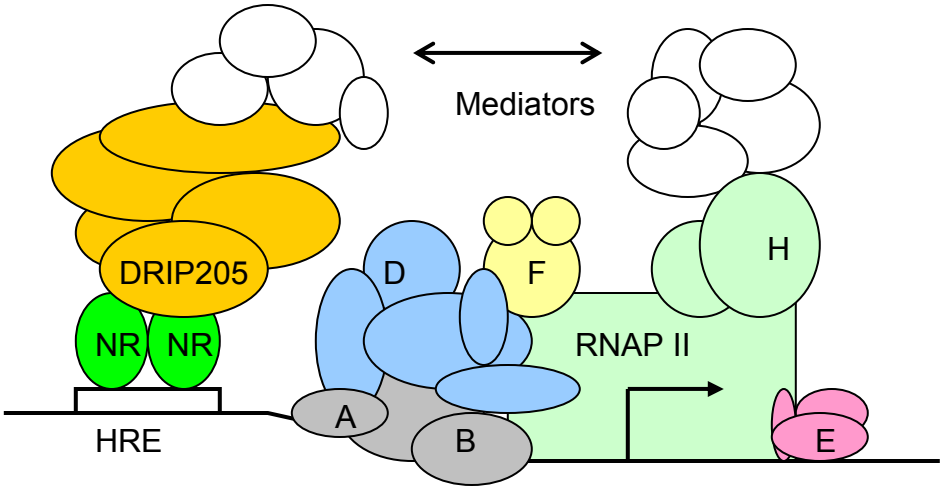
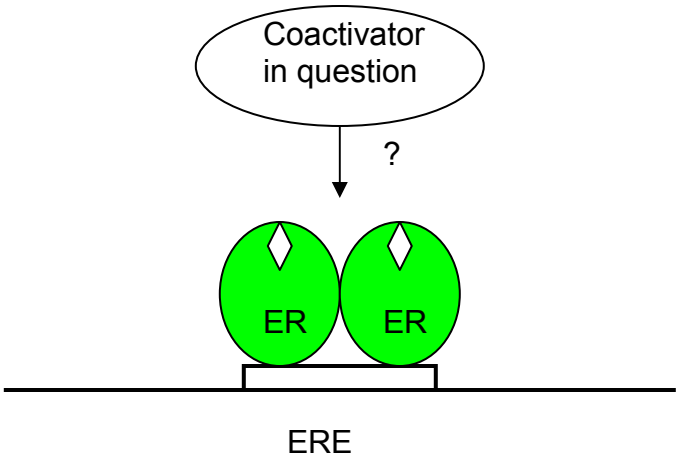


Fig. 32 Models of nuclear receptor-coactivator complex interactions (491,507).

A. Classical ERE-dependent



B. GC-rich sites-dependent

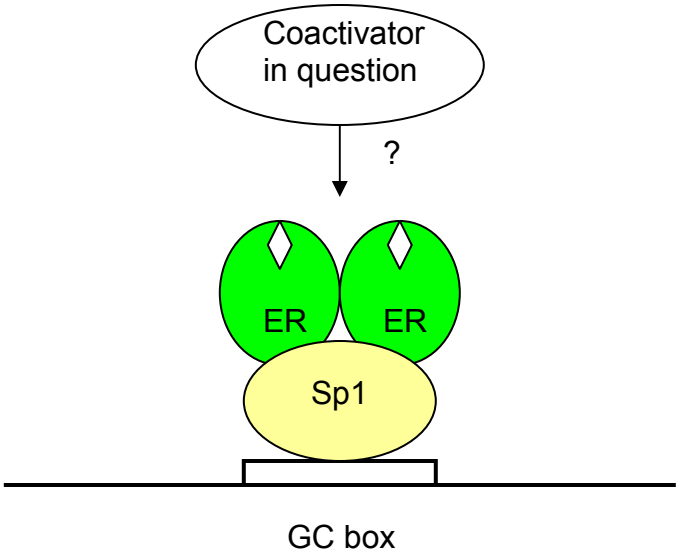


Fig. 33 Estrogen activation of gene transcription through genomic pathways in which ERα directly binds to an ERE (A) or ERα interacts with DNA bound Sp1 (B).

4.2 Coactivation of ERα and ERα/Sp1 by DRIP205

Previous studies in this laboratory have investigated coactivation of both ERα- and ERα/Sp1-mediated transactivation by SRC coactivators (Fig. 33). Coactivation was observed for SRC coactivators in CHO (Chinese hamster ovary) cells transfected with $pERE_3$-LUC, hERα and SRC-1 or SRC-2 or SRC-3 and in COS-1 (monkey kidney) cells transfected with $pERE_3$-LUC, hERα and SRC-1 or SRC-2. Results were consistent with previous reports by other groups showing that SRCs enhanced ER-mediated transactivation in COS-1, CHO, CV-1 and HeLa cells transfected with ERE-dependent promoter constructs (316,403,412,419). However, in both ER-positive MCF-7 and ER-negative MDA-MB-231 cells, coactivation was only observed for SRC-2, and SRC-1 or SRC-3 expression actually inhibited ERα-mediated transactivation in MDA-MB-231 cells exhibiting corepressor-like activity. Also, cotransfection of SRC-1, SRC-2 or SRC-3 did not enhance ERα/Sp1-mediated transactivation in either MCF-7 or MDA-MB-231 cells transfected with $pSp1_3$-LUC and hERα. Moreover, most of these coactivators significantly inhibited the induction response (Table 3). Coimmuno-precipitation studies showed that SRC-1, SRC-2 and SRC-3 physically interacts with ERα/Sp1 complex. However, in gel mobility shift assays, SRC-1 and SRC-3 decreased ERα/Sp1 interaction with GC-rich sites on DNA (396,508). These results suggest that coactivation of ERα- or ERα/Sp1-mediated transactivation by SRC coactivators is cell context-dependent. With

few exceptions, SRC coactivators do not coactivate ERα although some of p160 coactivators are amplified and overexpressed in breast cancer cells. The inhibition of ERα/Sp1 by SRCs may be due to competition with other functional coregulatory proteins and destabilization of preinitiation complex.

Table 3 Effects of SRC coactivators on ERα- and ERα/Sp1-mediated transactivation (396,508).

Cells	Coactivators	$pERE_3$-LUC	$pSp1_3$-LUC
CHO	SRC-1	Coactivation	No effect
	SRC-2	Coactivation	No effect
	SRC-3	Coactivation	No effect
COS-1	SRC-1	Coactivation	Inhibition
	SRC-2	Coactivation	Inhibition
	SRC-3	No effect	Inhibition
MDA-MB-231	SRC-1	Inhibition	Inhibition
	SRC-2	Coactivation	Inhibition
	SRC-3	Inhibition	Inhibition
MCF-7	SRC-1	No effect	No effect
	SRC-2	Coactivation	Inhibition
	SRC-3	Inhibition	Inhibition

The studies reported in this work have used a similar approach to investigate coactivation of both ERα- and ERα/Sp1-mediated transactivation by DRIP205 in a breast cancer cell context and to determine regions of ERα and DRIP205 required for coactivation and interactions (Fig. 33).

The results consistently show that DRIP205 coactivates ERα in ZR-75 and MDA-MB-231 breast cancer cells transfected with $pERE_3$ (Fig. 15), and although the fold-enhancement of induction is variable, coactivation by DRIP205 was consistently observed in both cell lines. Western blot analysis of ERα clearly shows that DRIP205 does not alter ERα protein expression, so that the enhancement of luciferase activity by DRIP205 is not due to changes in ERα levels. Wild-type DRIP205 did not coactivate HE19 (an AF1 domain deletion mutant of ERα) or ERα-TAF1 (an ERα mutant with point mutations in helix 12) (Fig. 16A and 16B), suggesting that both AF1 and AF2 of ERα are required for coactivation of ERα-mediated transactivation by DRIP205. Moreover, overexpression of ERα AF1 and GRIP1-NR boxes peptides, which competitively squelch AF1 and AF2 of ERα, respectively, decreased luciferase activity enhanced by DRIP205 (Fig. 16C and 16D), confirming that coactivation of ERα by DRIP205 involves both AF1 and AF2 of ERα. These results are consistent with previous reports showing that both AF1 and AF2 play a role in coactivation of ER by SRC coactivators (316,509,510).

DRIP205 coactivates ER and other NRs and also anchors interactions of the DRIP complex with NRs and other transcription cofactors. Therefore, we examined deletion variants of DRIP205 to determine domains required for ERα coactivation and interactions with ERα and other nuclear cofactors. The deletion mutant DRIP205Δ587-636 does not contain the NR boxes, but like wild-type DRIP205, this variant coactivates ERα (Fig. 17A) and coimmunoprecipitates with ERα (Fig. 17D). Moreover, coactivation of ERα by DRIP205Δ587-636 is decreased in cells transfected with ERα-AF1 and GRIP1-NR box peptides (Fig. 17B and 17C), indicating that AF1 and AF2 of ERα are both required for coactivation of ERα by NR box deletion mutant DRIP205Δ587-636.

Previous studies using cellular imaging techniques have demonstrated that E2 induces colocalization of ERα and SRCs in cells treated with E2, and ligand-induced ERα-SRC interactions were dependent on LXXLL box motifs in SRCs (511,512). We observed colocalization of ERα with both DRIP205 and DRIP205Δ587-636 in COS-7 cells treated with DMSO or E2 (Fig. 18); however, treatment with E2 enhanced the punctate pattern of wild-type/variant DRIP205 staining alone and in colocalization with ERα. These results complement the functional coactivation studies (Fig. 16 and 17) and show that in contrast to SRCs, coactivation of ERα by DRIP205 does not require the NR boxes. We also examined coactivation of ERα by other GAL4- or Xpress-tagged DRIP205 deletion mutants, and show that coactivation can be observed with both N- and

C-terminal domains of DRIP205 and coactivation did not require the NR boxes. Coimmunoprecipitation assay also showed that besides the LXXLL motifs, other regions of DRIP205 interact with liganded-ERα (Fig. 19).

Because multiple domains within DRIP205 are sufficient for coactivation and interaction with ERα, we also examined interactions of GAL4-DRIP205 (wild-type and variants) with VP16-ERα (wild-type and variants) in a mammalian two-hybrid assay (Fig. 20B and 21B). The results were compared to the AF activity of the GAL4-DRIP205 constructs, which would reflect their interactions with other nuclear cofactors or basal transcriptional machinery required for transactivation (Fig. 20A and 21A). pMDm12, which contains amino acids 641-1051, exhibits significant activity in ZR-75 and MCF-7 cells. Addition of a small fragment of amino acids 486-586, which do not have any activity on its own, generates pMDm7Δ. Maximal AF activity was observed for pMDm7Δ in both ZR-75 and MCF-7 cells, suggesting that two regions of amino acids 486-586 and 637-1051 have synergistic effects on transcription activation. The NR box sequence (amino acids 587-636) is deleted from pMDm7Δ and inclusion of this region (pMDm7) decreased transactivation. Similar patterns from one-hybrid assays of DRIP205 in ZR-75 and MCF-7 cells showed that interaction regions of DRIP205 with other transcription factors or coregulators are almost identical in the two cell lines.

A comparison between the AF activity of GAL4-DRIP constructs and their ligand-dependent interactions with VP16-ERα (wild-type and variant) in

mammalian two-hybrid assays provides some insights on regions within DRIP205 that interact with ERα and/or other nuclear cofactors. pMDm7Δ exhibited highest AF activity, but minimal interactions with ERα, ERα-TAF1 or HE19 in mammalian two-hybrid assays (Fig. 20 and 21). However, pMDm7Δ coimmunoprecipitates with ERα (Fig. 19D) and coactivates ERα (Fig. 19A), suggesting that this variant DRIP205 protein facilitates interactions with ERα and other nuclear cofactors.

In two-hybrid assays in ZR-75 cells (Fig. 20 B, C and D), pMD interacts with VP16-ER but neither VP16-ER-TAF1 nor VP16-HE19, indicating and confirming that AF1 and AF2 are both required for interaction of DRIP205 and ERα. Interestingly, pMDm4, which contains both NR boxes, interacts with VP16-ERα and VP16-ER-TAF1 (with AF2 mutations to disrupt coactivator binding surface) but not VP16-HE19 (AF1 deletion mutant but has an intact AF2), suggesting that interactions of DRIP205 NR boxes with ERα may be dependent on AF1, but not helix 12 of ERα. Moreover, pMDm3, which includes all the other regions of wild-type DRIP205 except two NR boxes, interacts with VP16-ERα and VP16-HE19 but not VP16-ER-TAF1, suggesting that other regions but not the two NR boxes of DRIP205, interact with helix 12 of ERα.

Results of the mammalian two-hybrid studies indicate that multiple domains of ERα and DRIP205 are involved in protein-protein interactions and in coactivation of ERα by DRIP205. The lack of specificity for DRIP205-ERα

interactions in the two-hybrid assay is similar to the complex interactions of p160 coactivators with ER and other NRs, which showed that AF1 and AF2 of ERα and the central region (containing NR boxes) and C-terminal regions of SRC coactivators were all required for interaction of SRCs with ERα (316,509,510). Our data suggest that p160 coactivators and DRIP205 also differ with respect to the role of their respective NR boxes, which play an important role in p160-ERα interactions and AF2-dependent coactivation (324,487-492). In contrast, NR boxes are not required for DRIP205-ERα interactions and AF1- and AF2-dependent coactivation. However, these characteristics of DRIP205 may be consistent with the critical role for this protein as a component of several nuclear cofactor complexes involved in transcriptional activation of genes and interactions with multiple transcription factors.

Previous studies in this laboratory showed that E2 induces expression of several genes via ER-Sp1 protein interactions with GC-rich promoter elements in which Sp1 but not ER binds DNA (374,379-385,387-390) in breast cancer cells. ERα physically interacts with Sp1 and preferentially binds to the C-terminal region of Sp1 in biochemical studies (395). In living MCF-7 cells, FRET (fluorescence resonance energy transfer) analysis showed that ERα interacts with Sp1 and the interaction is dependent on the C-terminal DBD of Sp1. Stronger FRET signals were detected in cells treated with E2 over time up to 480 sec., suggesting that the interaction of ERα and Sp1 increases in response to estrogen over time (513). Coactivation of ERα/Sp1 by DRIP205 was

consistently observed in MCF-7, ZR-75 and MDA-MB-231 breast cancer cells transfected with $pSp1_3$-LUC, although the enhancement of fold induction was variable (Fig. 22). The three proteins colocalized in the nucleus (Fig. 23), indicating the interactions of three proteins at transcriptionally active sites.

Previous reports showed that the AF1 domain of ERα is responsible for estrogen-activated ERα/Sp1, and neither the DBD nor helix 12 of AF2 of ERα is required (378,395,396). Gel mobility shift assays showed that both wild-type ERα and HE11 (DBD deletion mutant of ERα) enhanced interactions of Sp1-DNA complex, while HE19 (AF1 deletion mutant of ERα) did not (378). Deletion of the AF1 domain (amino acids 3-180) totally abolished transactivation of ERα/Sp1 (94% loss). Cells transfected with ERαΔ3-50 retained ability to activate ERα/Sp1; however, cells transfected with ERαΔ3-79 and ERα (79-117) only induced approximately 50% of the reporter gene activity compared to that observed for wild-type ERα. Deletion mutants ERαΔ3-101 and ERαΔ3-117 are almost inactive in transactivation in cells transfected with constructs containing GC-rich promoters (Fig. 34) (395). These results indicate that amino acids 51-117 of ERα is an important region for AF1 function in activation of ERα/Sp1. DBD deletion mutants of ERα, HE11, ΔZF1 and ΔZF2, induced luciferase activity in cells transfected with E2 responsive GC-rich promoter constructs pSp1, $pSp1_3$, pADA (adenosine deaminase promoter –86 to -65), pRARα1 (retinoic receptor α1 promoter –79 to -49) (378,396). ERα-TAF1, which has a

disrupted SRC coactivator binding surface in helix 12, activates ERα/Sp1, and SRC coactivators do not enhance but rather inhibit ERα/Sp1-dependent transactivation, suggesting that helix 12 coactivator interacting sites and SRC coactivators are not important in transactivation of ERα/Sp1. Transfection assays using various deletion mutants of ERα showed that the hinge region, N-terminus and C-terminus of LBD and F domain of ERα are required for estrogen-activated ERα/Sp1 (396).

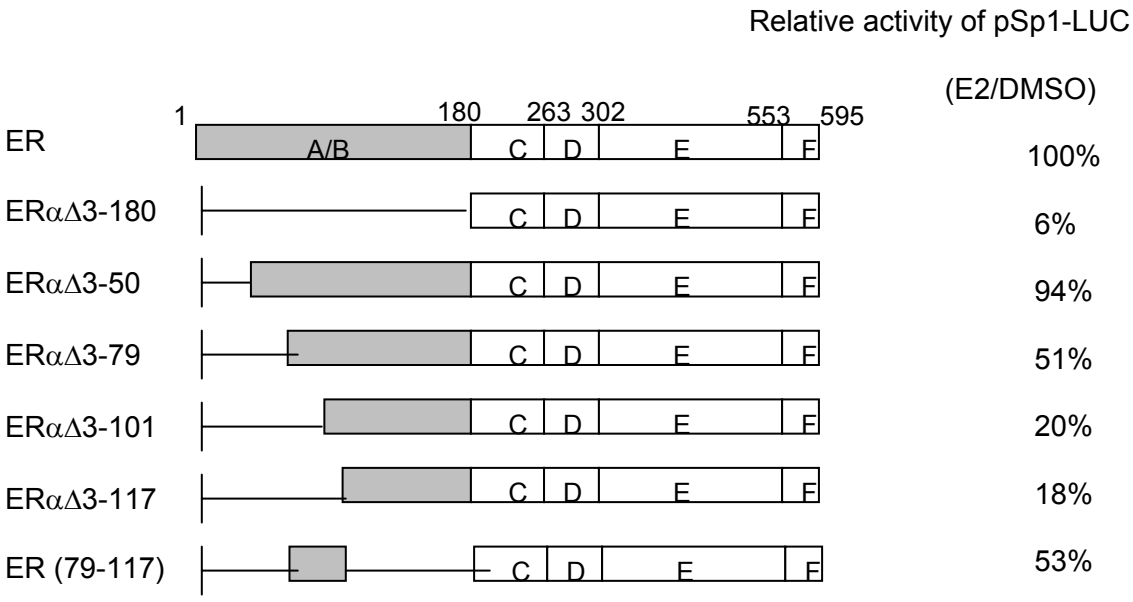


Fig. 34 AF1 of ERα is important for estrogen-dependent activation of ERα/Sp1 (395).

Estrogen-activated HE11/Sp1 and ERα-TAF1/Sp1 were both enhanced by DRIP205 in two ER-positive breast cancer cell lines (ZR-75 and MCF-7) (Fig. 24), suggesting that DBD and helix 12 of ERα are not required for coactivation of ERα/Sp1 by DRIP205. Interestingly, DRIP205 did not coactivate either HE11/Sp1 or ERα-TAF1/Sp1 in ER-negative MDA-MB-231 cells, indicating that coactivation of ERα/Sp1 by DRIP205 in MDA-MB-231 cells is dependent on the DBD and helix 12 of ERα. The controversial results of domain requirement of ERα for coactivation of ERα/Sp1 by DRIP205 reflect cell-context differences in coactivator interactions with ERα and other coregulators, which may depend on individual expression patterns of nuclear factors in different cell lines. It is possible that expression of other nuclear proteins that may interact and/or facilitate DRIP205-ERα/Sp1 complex formation are changed in ER-negative MDA-MB-231 cells compared to ER-positive cell lines.

AF1 deletion mutants of ERα, ERαΔ3-50 and ERαΔ122-178 were used to further define the region within this domain required for coactivation of ERα/Sp1 by DRIP205 in ZR-75 cells. ERαΔ3-50 is E2-inducible in cells transfected with $pSp1_3$-LUC, which is consistent with previous results in this laboratory (395); however, DRIP205 expression did not coactivate ERαΔ3-50/Sp1 (Fig. 25A), suggesting that the amino acids 3-50 of ERα are important in coactivation of ERα/Sp1 by DRIP205. ERαΔ122-178 is also active in cells transfected with $pSp1_3$-LUC (Fig. 25B), indicating that amino acids 122-178, which localize at the

C-terminal of AF1 of ERα are not required for activation of ERα/Sp1. Coactivation by DRIP205 was observed in cells transfected with $pSp1_3$-LUC and ERαΔ122-178, suggesting that the C-terminal region of ERα (amino acids 122-178) is not necessary for coactivation of ERα/Sp1 by DRIP205. These results indicate that DRIP205 coactivation on ERα/Sp1 in ZR-75 cells requires the N-terminal 50 amino acids of ERα.

We also examined deletion mutants of DRIP205 to determine domains required for ERα/Sp1 coactivation in ZR-75 cells. Previous reports showed that the NR box fragment DRIP205 (528-714) interacts with a broad range of NRs in GST pull-down assays, including GR, ERα, FXR, TR and PPARγ (461,462,464,468,472). However, our results showed that DRIP205 (528-714) did not enhance ERα/Sp1 transactivation (Fig. 26C), indicating that other regions of DRIP205 are required for coactivation. In contrast, the NR box deletion mutant, DRIP205Δ587-636 significantly coactivated ERα/Sp1 (Fig. 26D), which is consistent with previous results showing that ERα-mediated transactivation was enhanced by this mutant (Fig. 17A). This indicates that the two LXXLL motifs in the central region of DRIP205 are not required for DRIP205 coactivation of both ERα and ERα/Sp1. Coactivation was also observed with cells transfected with $pSp1_3$-LUC, ERα and variant DRIP205 (1-714) or DRIP205 (516-1566) expression plasmids (Figs. 26A and B), confirming that

both N-terminal and C-terminal regions of DRIP205 are involved in coactivation of ERα/Sp1.

Therefore, DRIP205 serves as a coactivator of both ERα- and ERα/Sp1-mediated transactivation in breast cancer cells, unlike SRCs, which do not function as coactivators in breast cancer cells. DRIP205 coactivation of ERα and ERα/Sp1 is not dependent on NR boxes in the central region of DRIP205, but requires multiple regions from both C-terminal and N-terminal regions of the protein. Enhancement of ERα-mediated transactivation by DRIP205 requires both AF1 and AF2 of ERα; however, DRIP205 coactivation of ERα/Sp1 is primarily AF1-dependent, but DBD- and helix 12-independent in ER-positive ZR-75 cells.

4.3 Differential Roles of Sp Proteins in ERα/Sp-dependent Transactivation and Coactivation by DRIP205

Previous studies in this laboratory showed that in the VEGF (vascular endothelial growth factor) promoter, both Sp1 and Sp3 proteins bound the GC-rich region and were required for E2-induced transactivation and interactions with ER in ZR-75 cells. RNA interference studies with small inhibitory RNAs for Sp1 or Sp3 showed that E2-induced reporter gene activity in cells tranfected with VEGF promoter construct containing the GC-rich sequence was decreased by down-regulation of Sp1 or Sp3, suggesting that E2-induced transactivation of VEGF in ZR-75 cells requires both ERα/Sp1 and ERα/Sp3 (394). Sp4 is also an activator which binds to the same GC-rich motifs as Sp1 or Sp3 (247,256). Sp4

has been detected in many cancer cells lines including ZR-75 cells (494). Colocalization of ERα and Sp4 in ZR-75 cells was examined by immunostaining (Fig. 31). Sp4 is localized in the nucleus. However, the nuclear staining of Sp4 is not as uniform as ERα and Sp4 shows more intense staining in the central region of the nucleus rather than the area around the nuclear membrane. Sp4 and ERα colocalized in the nucleus, with a ring of ERα staining at the area around the nuclear membrane. We next investigated the role of Sp family proteins (Sp1, Sp3 and Sp4) and coactivation of ERα/Sp-mediated transactivation by DRIP205.

Initial experiments were carried out to examine the role of Sp family proteins in activation of ERα/Sp by estrogen and antiestrogens. Previous studies showed that ERα/Sp-mediated transcription through GC-rich sites were activated by both estrogen and antiestrogens in breast cancer cells (395). In living MCF-7 cells, significantly more intense FRET signals were detected after treatment with estrogen or antiestrogens in cells transfected with CFP-Sp1 and YFP-ERα (513). Our results showed that reporter gene activities were decreased in DMSO-treated ZR-75 cells transfected with siRNAs for Sp1, Sp3 or Sp4 and $pSp1_3$-LUC, indicating that all three Sp proteins bound GC-rich sites on the promoter and activated basal transcription (Fig. 27A). Western blot analysis of whole cell lysates confirmed down-regulation (>50%) of Sp family proteins in cells transfected with corresponding siRNAs (Figs. 27B and C). Treatment with E2, ICI 182,780 or 4-OH tamoxifen induced reporter gene activity, and induced

activities were decreased in cells transfected with iSp1, iSp3 or iSp4 (Figs. 27A and 28). However, fold-inducibility by E2 or ICI 182,780 or 4-OH tamoxifen was not significantly changed in cells transfected with siRNAs. These results indicate that in estrogen- or antiestrogen-activated ERα/Sp, all three Sp family proteins are involved and may cooperate in activation of GC-rich promoters in ZR-75 cells.

Previous studies in this laboratory showed that the DBD of ERα was not required for estrogen-activated ERα/Sp action (378), but was required for antiestrogen-activated ERα/Sp-mediated transactivation (396). We used RNA interference to further determine the role of Sp proteins in estrogen-activated HE11/Sp. E2-induced reporter gene activity was decreased in ZR-75 cells transfected with iSp1, iSp3 or iSp4. However, the fold induction (E2/DMSO) was decreased only in cells transfected with iSp1 (Fig. 29), suggesting that Sp1 is the primary player in mediating estrogen-activated HE11/Sp. Consistent with previous reports, ICI or 4-OH tamoxifen did not significantly induce reporter gene activity in cells transfected with $pSp1_3$-LUC and HE11. Interestingly, transfection of iSp3 significantly increased the fold induction of luciferase activity (from 1.3- to 3.2-fold) in ICI-treated cells, suggesting that Sp3 alone or in combination with corepressors may inhibit activation of HE11/Sp by ICI 182,780. Fold induction was also increased in 4-OH tamoxifen-treated cells transfected with iSp3 or iSp4 compared to NS (from 1.5- to 2.7- and 2.7-fold), suggesting that both Sp3 and Sp4 may repress HE11/Sp in tamoxifen-treated cells. However, these increases

were not comparable to ICI- or tamoxifen-activated wild-type ERα/Sp, where the fold induction was 9- and 11-fold, respectively.

Finally, we investigated the role of Sp family proteins in coactivation of ERα/Sp by DRIP205. Results consistently showed that DRIP205 enhanced ERα/Sp in ZR-75 cells transfected with nonspecific siRNA or iSp1, but did not coactivate ERα/Sp in cells transfected with iSp3 or iSp4 (Fig.30), indicating that Sp3 and Sp4 are indispensable in coactivation of ERα/Sp by DRIP205. These results suggests that DRIP205 preferentially coactivates ERα/Sp3 and ERα/Sp4 and this may also involve cooperative interactions between the Sp proteins. The RNA interference approach demonstrated that Sp1, Sp3 and Sp4 play a role in ERα/Sp-mediated transactivation, and coactivation by DRIP205 primarily involves ERα/Sp3 and ERα/Sp4.

These studies clearly demonstrate that hormonal activation of genes in breast cancer cells is complex and involves ERα alone or in combination with Sp family proteins or other factors, different promoters, coactivators, coregulator proteins and ligands. This complexity forms the basis for the tissue-selective action of ERα and ERα ligands in maintaining homeostasis and also facilitates the development of SERMs (selective estrogen receptor modulators) for treatment of breast cancer.

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