The Role of Sp1 and Sp3 in the Regulation of Estrogen Inducible Genes in Human Breast Cancer Cells

by

Lin Li

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

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DEDICATION

I would like to dedicate this thesis to my beloved parents, Jiying Wang and Guangquan Li, for instilling in me the drive and determination to follow my dreams and pursue my goals.

Abstract

Estrogen responsive genes often have Sp1/3 binding site (s) in their regulatory regions. Sp1 and Sp3 transcription factors recognize and bind to Sp1/3 sites with similar affinity. Most studies suggested that Sp1 is an activator and Sp3 is more likely a repressor or weak activator.

Our lab first predicted and identified one Sp1/3 site (-420) in the proximal promoter of estrogen responsive trefoil factor 1 (TFF1) gene. Using chromatin immunoprecipitation (ChIP) assays, we find that mutation of the Sp1/3 site reduced estrogen responsiveness and prevented binding of Sp1 and Sp3, but not estrogen receptor α (ER) to the episomal promoter. Following estrogen treatment, the level of ER bound to the native promoter increases. There is clearance of Sp1, but not of Sp3, from the promoter, while histone deacetylase (HDAC) 1 and HDAC2 remain bound. These data are consistent with a model in which Sp1 or Sp3 aid in recruitment of HDACs and lysine acetyltransferases (KATs) to mediate dynamic acetylation of histones associated with the TFF1 promoter.

Using re-ChIP assays, we demonstrate that either Sp1 or Sp3 but not both bind to a TFF1 promoter in both MCF-7 and ZR-75 breast cancer cells. The co-occupancy of Sp3 with ER and with serine 5 phosphorylated RNA polymerase II (pS5-pol II) on the TFF1 promoter increased following estrogen treatment. These results are first to demonstrate that Sp3 and ER are involved in the activation of estrogen induced transcription of the TFF1 gene. This observation is found also to be true for the *c-myc* gene, which has only two possible Sp1 binding sites in its estrogen responsive promoter 2. In contrast, Sp1 is preferentially bound to the non-estrogen responsive in full DPYD gene promoter in Hela and MCF-7 cells.

ChIP assays were applied to several other estrogen responsive genes (TGF-α, cyclin D1, cathepsin D and RARα). A common feature of these gene promoters was that Sp3 occupancy was lagged behind ER and Sp3 occupancy became more dynamic in the presence of estrogen.

Taken together, Sp3, instead of Sp1, is preferentially associated with the active TFF1 and *c-myc* promoter.

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Thanks to my children for changing my life completely and for being a constant source of joy and surprises.

And finally, a special thanks to my dear husband, Jixian, for all his unconditional love and support.

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List of Abbreviation

aa	amino acid
ABR	Affinity Bioreagents Inc
AF-1	Activation Function-1
AF-2	Activation-Function-2
AP-1	activator protein 1
apM-1	adipose most abundant gene transcript-1
AIs	Aromatase inhibitors
BTD	Buttonhead
CTD	carboxyl-terminal domain
ChIP	Chromatin immunopresipitation
CAM	chorioallantoic membrane
Cdk	cyclin-dependent kinase
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified essential medium
DBD	DNA-binding domain
DCIS	Ductal carcinoma *in situ*
DHs	DNase I hypersensitive sites
DPD	dihydropyrimidine dehydrogenase
EMSA	Electrophoretic mobility shift assay
ERDs	Estrogen-receptor downregulators
ERE	estrogen responsive element
ERR	estrogen responsive region
E1	estrone
E2	estradiol
E3	estriol
ER	estrogen receptor
ERα+	estrogen receptor α positive
ERα-	estrogen receptor α negative
ERK	extracellular regulated kinase
FRAP	fluorescence recovery after photobleaching
5-FU	5-fluorouracil
FBS	fetal bovine serum
HAT	histone acetyltransferase
HDAC	histone deacetylase
HLH	helix-loop-helix
h	hours
HD	Huntington's disease
HUVEC	human umbilical vein endothelial cells
IBC	Inflammatory breast cancer
IDC	Invasive ductal carsinoma
IgG	immunoglobulin
ILC	Invasive lobular carsinoma

Inr	initiator element
IGF-1	Insulin-like growth factor 1
KATs	K-Acetyltransferases
KLF	Krüppel-like Factor
LBD	ligand-binding domain
LCIS	Lobular carcinoma *in situ*
β-ME	β-mercaptoethanol
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
Min	minute
MISS	membrane initiated steroid signaling
MSK1/2	mitogen and stress activated kinase 1 and 2
mRNA	message RNA
miRNA	microRNAs
NucE	nucleosome E
Nuc T	nucleosome T
NMR	nuclear magnetic resonance
NuRD	nucleosome remodelling and histone deacetylation
NMPs	Nuclear matrix proteins
NLSs	nuclear localization sequences
ORF	open reading frame
O-GlcNAc	O-linked N-acetylglucosamine
OGT	O-β-N-acetylglucosaminyltransferase
PMSF	phenylmethanesulfonyl fluoride
pol	polymerases
PML	promyelocytic leukemia
PIAS1	protein inhibitor of activated STAT1
PKA	protein kinase A
PP2A	protein serine/threonine phosphatase 2A
Pl3K	phosphoinositide 3-kinase
RT-PCR	Reverse-transcription PCR
rRNA	ribosome RNA
RNA	ribonucleic acids
SAGE	serial analysis of gene expression
Ser2	Serine 2
Ser5	serines 5
pS5-pol II	serine 5 phosphorylated RNA Polymerase II
Ser7	Serin 7
SERMs	Selective Estrogen Receptor Modulators
snRNA	small nuclear RNA
SUMO	Small ubiquitin-related modifier
SDS	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SKY	spectral karyotyping
SDGA	sucrose density gradient analysis
TAF110	TATA binding- protein-associated factor
TF	transcription factor

TFSEARCH	transcription factor search
TGFBR	transforming growth factor β (TGFβ)/TGFβR receptor
TFF1	trefoil factor 1
TSA	trichostatin A
TS	thymidylate synthase
TGFα	Transforming growth factor or Tumor growth factor α
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor-2 promoter

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I. INTRODUCTION

1 . Gene Expression and Transcriptional Regulation in Eukaryotes

Genetics is the science that is studying heredity and variation in living organisms. Modern genetics initiated from the study by garden peas of an Austrian monk, Gregor Mendel. The genetic material, deoxyribonucleic acid, or DNA was first isolated by the Swiss physician Friedrich Miescher in 1869 (Dahm, 2005). DNA carries the genetic instructions used in the development and functioning of all living organisms. The DNA sequence containing the instruction, genetic codes, necessary to produce a functional ribonucleic acids (RNA) in a regulatory manner, has been defined as a gene (Pearson, 2006;Pennisi, 2007). Genes can be regulated and expressed into functional proteins or RNA. The first major step in gene expression, transcription, is for RNA polymerases (pol) to read and transcribe the DNA into RNA. Depending on the RNA synthesized, several types of RNA polymerases have been characterized in eukaryotes. RNA pol I synthesizes ribosomal RNA (rRNA) i.e. 18S and 28S rRNA (Grummt, 1999). RNA pol II synthesizes precursors of message RNA (mRNA) and most small nuclear RNA (snRNA) and microRNAs (miRNA) (Lee *et al.*, 2004b). RNA pol III synthesizes tRNAs, 5S rRNA and other small RNAs found in the nucleus and cytosol (Willis, 1993). In eukaryotes, the largest, catalytic subunits of all three eukaryotic polymerases are highly homologous (Allison *et al.*, 1985). However, the CTD domain of the largest subunit of pol II (Rpb1), which is essential for viability, makes it unique when compared it to the other two eukaryotic RNA polymerases (Corden, 1990;Laybourn and Dahmus, 1990). In human cells, this CTD contains 52 tandemly repeated heptapeptides with the consensus sequence Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7 ($Y^1S^2P^3T^4S^5P^6S^7$). The phosphorylation of CTD plays

important roles in the transition from assembling the preinitiation complex to elongation during transcription (Laybourn and Dahmus, 1990). Of all three polymerases, pol II has been the one extensively studied. Due to the high level of controls required over pol II-driven transcription, binding of a large number of transcription factors is required leading to further initiating the transcription of a gene.

1.1. Polymerase II and α-Amanitin. RNA polymerases such as pol I, pol II and pol III can be distinguished by their sensitivities to the mushroom amanita phalloide toxin α-amanitin. The level of sensitivity goes as follows: pol II > pol III > pol I. α-Amanitin is a well-known specific inhibitor of pol II *in vitro* and *in vivo*. α-Amanitin was reported to block both transcription initiation and elongation (Wieland and Faulstich, 1991;Chafin *et al.*, 1995;Rudd and Luse, 1996). Early studies found that α-amanitin binds to the Rpb1 subunit of pol II with high affinity (*K*d 10^{-9} M) (Cochet-Meilhac and Chambon, 1974;Lutter, 1982). Using mouse fibroblasts exposed to α-amanitin, one study found that the endogenous pol II largest subunit (mRpb1) is degraded, and this degradation is not mediated through activating a proteolytic system. This degradation parallels decreased transcriptional activity in nuclei from α-amanitin-treated cells. However, a caveat in this was the extended time of treated time 2 to 24 hrs (Gong *et al.*, 2004). Later on, one co-crystalline structure of α-amanitin-pol II study (at 2.8 Å resolution) gave a better explaination for the mechanism of α-amanitin inhibition of pol II activity (Bushnell *et al.*, 2002). This study demonstrated that the key player is the bridge helix extending across the cleft in between the two large subunits of pol II, Rbp1 and Rbp2. The pol II residues interacting with α-amanitin are located almost entirely in the bridge helix, defined as the cleft region of Rbp1. The conformation change of this bridge helix recognized and bound

by α- amanitin is required for the translocation of DNA and RNA, a step needed to empty the site for the next round of RNA synthesis. This α-amanitin binding does not influence the affinity of nucleoside triphosphates for pol II (Cochet-Meilhac and Chambon, 1974;Chafin *et al.*, 1995) and the formation of phosphodiester bonds (Vaisius and Wieland, 1982;Gu *et al.*, 1993). Instead, it is the translocation rate of DNA and RNA that is seriously impaired. Taken together, the results suggest that the inhibition caused by α-amanitin is caused by blocking the translocation or the degradation of pol II. Because of its inhibiting transcription property, α-amanitin has been used in many studies to synchronize gene transcription (Shang *et al.*, 2000;Burakov *et al.*, 2002;Metivier *et al.*, 2003a). In this thesis work, α-amanitin was used to treat MCF-7 cells to synchronize the

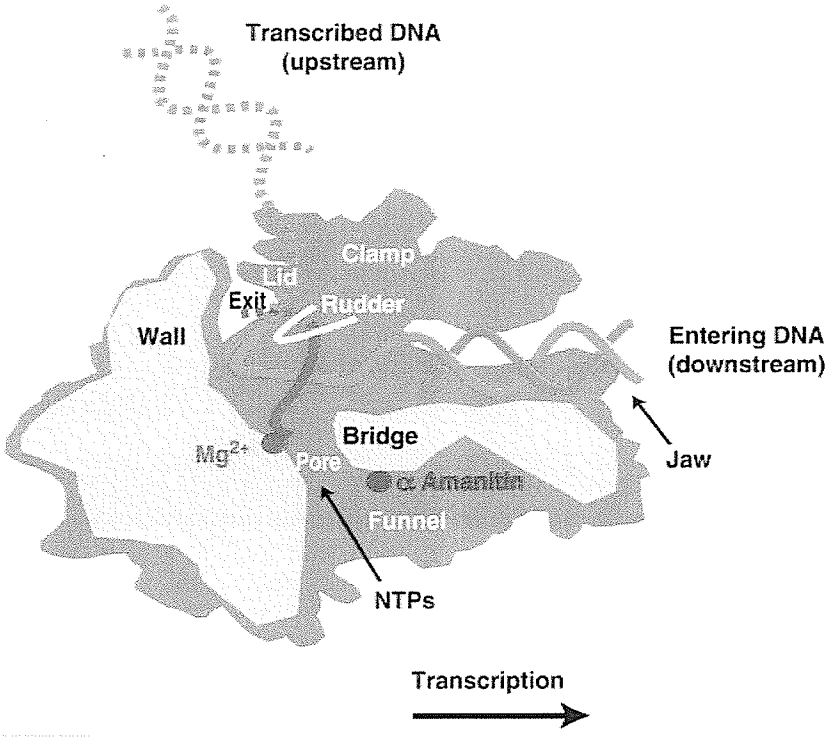


Figure 1. The location of α-amanitin binding (red dot) relative to the nucleic acids and functional elements of the enzyme (Bushnell *et al.*, 2002).

transcription of estrogen- responsive genes studied (e.g. TFF1 and *c-myc* gene) following estrogen treatment to investigate the role of Sp1 and Sp3 in the regulation of estrogen-responsive genes.

1.2. Polymerase II and transcription. Pol II is the polymerase responsible for transcription of protein-coding genes which comprise the largest group genes in the eukaryotic genome. As described above, the CTD domain contains 52 heptad repeats of Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7 ($Y^1S^2P^3T^4S^5P^6S^7$) in human cells. This CTD of pol II presents a docking platform for factors involved in diverse co-transcriptional events. Changing post-translational modification patterns of CTD repeats is correlated with the recruitment of suitable factors at different stages of the transcription cycle. Phosphorylation of serine residues within the heptapeptide of CTD has been well-studied (Phatnani and Greenleaf, 2006). The modification of the Pol II CTD heptapeptide during transcription was reviewed and summarized in figure 2 (Egloff and Murphy, 2008). The hypophosphorylated CTD preferentially associates with the preinitiation complex at the promoter. The serines at 5 (Ser5) of the repeats become phosphorylated by cyclin-dependent kinase (Cdk)7, a component of the general transcription factor TFIIH, upon initiation. Chromatin immunoprecipitation (ChIP) is a powerful technique used to determine the location of the *in situ* protein-DNA interactions on the genome that occur inside the nucleus of living cells or tissues. ChIP assays demonstrated that phosphorylated Ser5 predominantly loaded on the 5' end promoter-proximal region (Zhang and Corden, 1991;Komarnitsky *et al.*, 2000;Schroeder *et al.*, 2000;Cheng and Sharp, 2003;Gomes *et al.*, 2006). This phosphorylation event is crucial in recruiting the

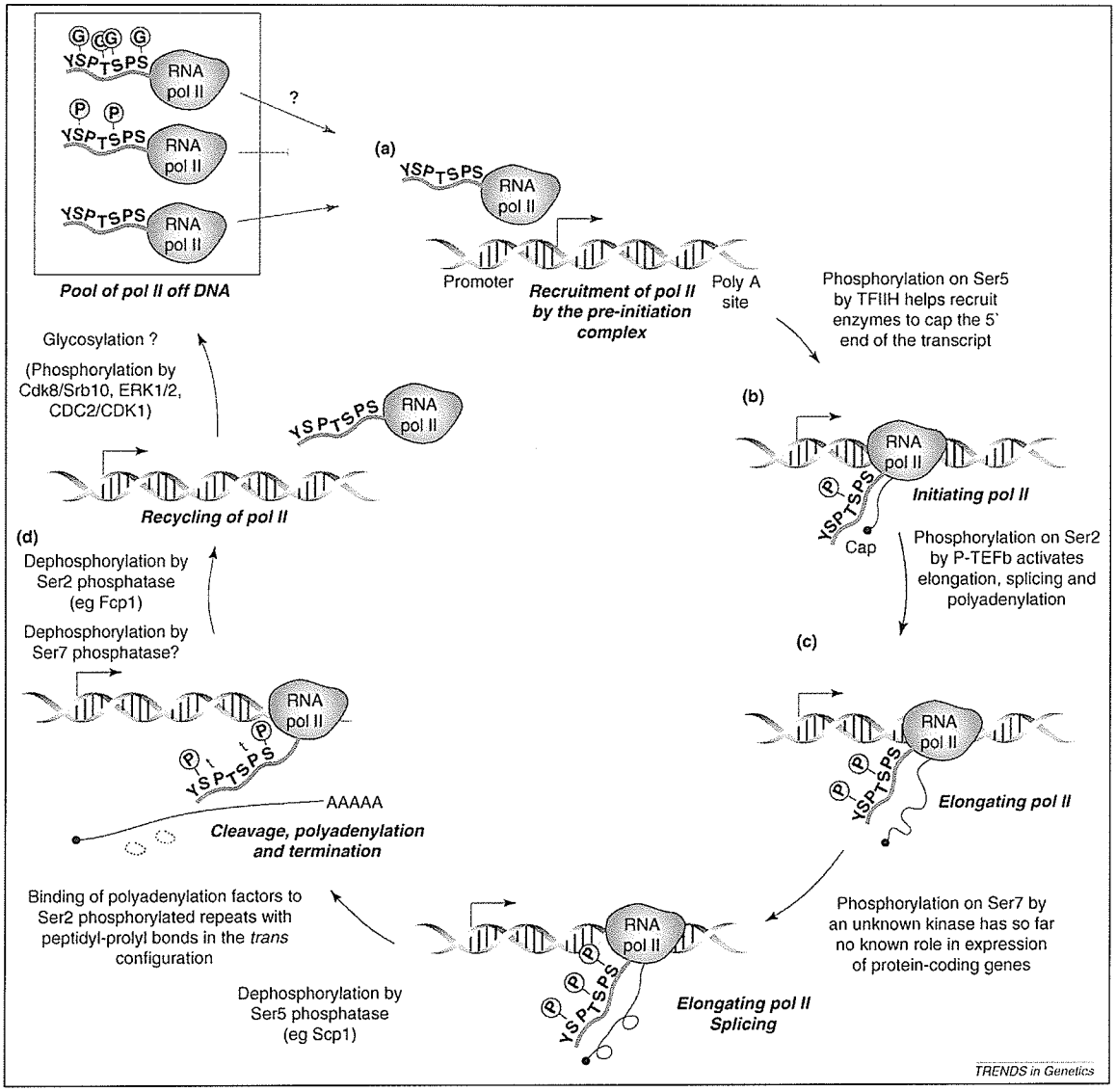


Figure 2. Modification of the pol II CTD heptapeptide during transcription of protein-coding genes (Egloff and Murphy, 2008).

enzymes to modify the nascent RNAs to sites of transcription *in vivo* (Komarnitsky *et al.*, 2000;Schroeder *et al.*, 2000). The Serine 2 (Ser2) residue is phosphorylated by T-Cdk9, a component of p-TEFb (Peterlin and Price, 2006). This phosphorylation step is involoved in overcoming an early elongation block and facilitating pol II elongation (Peterlin and Price, 2006). ChIP assays demonstrated that it is the pol II Ser-2 phosphorylation form that is detected throughout the gene and increases toward the 3' end of genes (Komarnitsky *et al.*, 2000;Cheng and Sharp, 2003;Gomes *et al.*, 2006). ChIP assays indicate that Serine 7 (Ser7) phosphorylation happens in a manner similar to phosphorylation of Ser2, predominantly peaks at the 3' end of the transcribed gene. This Ser7 phosphorylation mark pattern suggests its possible roles in processing the 3' end of the transcripts and/or transcription termination. Further research found that Ser7 phosphorylation plays a gene-specific role in recruiting the Integrator complex for snRNA gene expression. Although more work needs to be done to precisely determine its roles, Ser7 phosphorylation also plays important roles in the expression of protein-coding genes (Chapman *et al.*, 2007;Egloff *et al.*, 2007). The kinase responsible for Ser7 phosphorylation is not identified yet. All the combinations of these serine residue phosphorylations form a set of CTD codes and become part of a gene specific signal (Egloff and Murphy, 2008).

Pol II driven eukaryotic gene expression starts from unpacking DNA to make the target genes available for transcription. The available genes then get transcribed to primary transcript and further processed into mRNA. RNA processing includes splicing the primary RNA (to remove introns), adding RNA caps and poly A tails. The mature mRNA will be then transported from the nucleus to cytoplasm and translated into

proteins, which are transported to their cellular destination(s) with proper post-translational modifications. Gene expression can be regulated for all the expression steps from the transcription to post-translational modification of a protein. This doctoral thesis is focused on studying the role of the transcription factors Sp1 and Sp3 in the regulation of estrogen responsive genes driven by pol II in breast cancer cells.

1.3. The Regulation of Gene Transcription.

1.3.1. Transcriptional cis-elements. Transcriptional activation is through the loading of the basal transcription complex to the transcription start site of a gene promoter. The regulatory regions of genes contain a series of cis-elements, which are DNA sequences recruiting DNA-binding proteins or interacting with proteins brought to promoter regions. Cis-acting elements are typically upstream of the start site and are spread throughout the gene. Cis-acting elements include the core promoter, upstream proximal elements, inducible elements and enhancer elements. The core promoter includes the minimal DNA elements required for assembling the transcription initiation complex to initiate transcription. Upstream proximal elements (e.g. Sp1 sites and AP-1 sites) are short elements (6-20 nt) typically affecting the efficiency of transcription initiation but not the specificity of the initiation site. Inducible elements are specific activator elements such as hormone responsive elements (e.g., ERE). Enhancer elements can be distal (~10 kb or greater) and can be functional in both orientations either upstream or downstream within one gene. Enhancer elements may affect transcription efficiency but not initiation and cannot direct transcription themselves. However, these elements may be required for full gene expression.

1.3.2. Transcription factors. Transcription factors are proteins interacting with cis-acting elements and with other proteins at the promoter. The association of transcription factors with cis-acting elements facilitates the recruitment and assembly of the pol II transcription initiation complex as well as promoter clearance for transcription initiation. Transcription factors are necessary for basal transcription activity that can be obtained in the absence of enhancers or regulatory upstream elements. Combinations of different cis-elements and factors (e.g. coactivators) that recognize them provide specificity and coordinate regulation of pol II transcription. Transcription factors can work as either activators or repressors acting from both distal (enhancers or silencers) and proximal sites. Many transcription factors do not bind DNA directly, but interact with DNA bound proteins through protein/protein interactions and assist regulating gene transcription. One current model suggests that looping of DNA can facilitate interaction of factors bound at distant sites with proteins bound to proximal sites and/or basal transcription factors including the TBP-associated factors (TAFs) and further influence transcription (Su *et al.*, 1991).

Several types of conserved domains or motifs have been identified are present in eukaryotic gene transcription factors. They are typically composed of an activation domain, a DNA binding domain and sometimes a ligand binding domain. The organization of activation domains is not as well understood, but these domains often contain negatively charged rich regions. Activation domain DNA typically interacts with other trans-acting factors or directly with pol II to affect various aspects of transcription. Ligand binding domains can bind hormone, heavy metals or other ligands (e.g. estrogen receptor binds estrogen). In general, binding of a ligand alters protein conformation and

changes either the DNA binding activity or potential to interact with other proteins (Shang and Brown, 2002;Riggs and Hartmann, 2003). There are several kinds of DNA-binding domains identified. The zinc finger motif, coded by small stretches of DNA, fold into loops or fingers in a Zn++ ion dependent manner. ER, Sp1 and Sp3 all have zinc finger DNA-binding domain, although ER has two Cys4 and Sp1/Sp3 have 3 Cys2His2 zinc fingers. A Helix-turn-helix motif (HTH) is a structure with two α-helixes connected by a beta-turn. A Leucine zipper motif contains two leucine-rich polypeptides that form α-helices with leucines on the same side of the helix every other 7 amino acid (AA) two turns. The purpose of these background materials is to facilitate understanding of the subsequent sections of the role of Sp1 and Sp3 in the regulation of estrogen responsive genes in ER positive breast cancer MCF-7 and ZR-75 cells. The next section of the introduction provides a general overview of how DNA is packed and remodeled to make genes available for gene transcription.

1.4. DNA organization in mammalian cells. Nuclear DNA is highly packed and gene expression starts by remodeling the highly packed DNA to make target genes accessible to transcription factors and the transcription machinery. Nuclear DNA folding is crucial for systematic packing of long DNA molecules into the nucleus. In one human cell, there are about 3 billion DNA base pairs and the linear length of these DNA molecules is about two meters long (Human Genome Project Information of the DOE, http://www.allaboutpopularissues.org/human-genome-project-information-faq.htm). It takes about 10^4-fold compaction for these DNA molecules to fit into about 6 μm area of the nucleus (Fig.3). The packaging order of eukaryotic DNA into chromosomes includes 11 nm nucleosome fibers, 30 nm chromatin fibers, looped DNA domain and mitotic

chromosome. The 11 nm primary DNA nucleosome packing ratio is about 6:1 and it shows beads on string appearance by wrapping DNA around histones. The nucleosome consists of a histone octamer, arranged as a $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers, around which DNA is wrapped. Histone H1 binds to the linker DNA, which joins

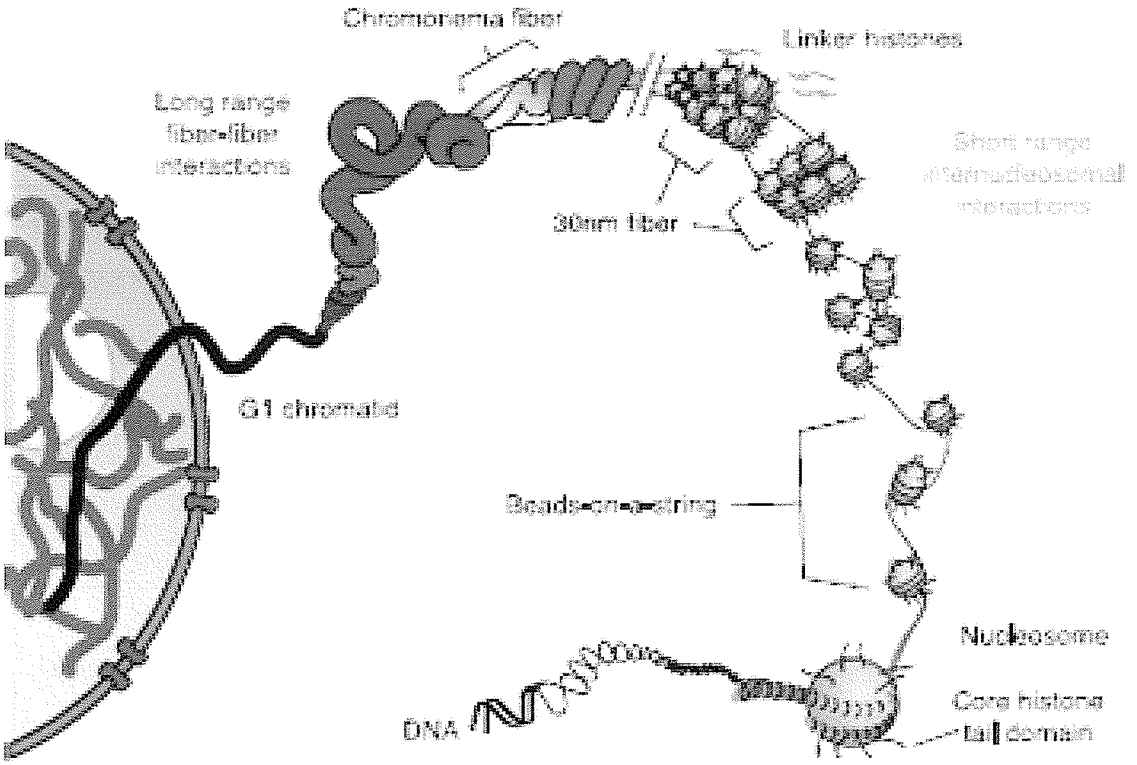


Figure 3. The packaging of eukaryotic DNA into chromosomes (Horn and Peterson, 2002)

nucleosome shows that the N terminal tails emanate from the nucleosome in all directions and would be available to interact with linker DNA, nearby nucleosomes or with other proteins (Luger *et al.*, 1997;Luger and Richmond, 1998). The extended beads on string form of nucleosomes is rarely adopted in nature since the chromatin must be kept in a highly compacted state due to the spatial limitation. The higher order nucleosomal

organization is the helically coiled 30 nm chromatin fibers consisting of nucleosome arrays with a packing ratio about 40:1. H1 is important for stabilizing the 30 nm fiber nucleosomal structure. The further supercoiling and compaction create a final packing ratio of about 1000:1 in interphase chromosomes and about 10,000:1 in mitotic chromosomes.

1.5. Histone modifications and chromatin remodeling. Since eukaryotic DNA is highly packed, the chromatin structure of the promoter needs to be remodeled for better accessibility to the basal transcriptional machinery. Transcriptionally active regions in chromatin are characterized by nuclease accessibility. Transcriptionally active genes and genes with the potential or competence for transcription have sensitive domains through the gene and hypersensitive sites near the promoter. A hypersensitive site is a region of chromatin which is super-sensitive to cleavage by DNAase I and other nucleases (DNase II and micrococcal nuclease). Hypersensitive sites are hallmarks for active genes, and many of these genes often have more than one hypersensitive site. Most hypersensitive sites are found in chromatin of expressed genes, but not inactive genes. Hypersensitive sites happen before gene promoters are activated as the binding of transcription factors displaces histone octamers. DNAase sensitivity occurs not only in promoters but also through whole transcription units. In a hypersensitive site, the nucleosomal structure is organized in a way causing a 100 fold increase in sensitivity to enzyme attack than in bulk chromatin.

Histone modifications are found to play important roles in remodeling the chromatin structure of the promoters. The long tails of H3 and H4 histones and the core of the histones (H2A and H3) can be modified. Modifications of histones include but not

limited to: acetylation, phosphoryaltion, methylation, ADP-ribosylation and ubiquitination. These modifications typically change the net charge distribution on histones and further affect the interactions of histone-histone and histone-DNA interaction as well. More discussion will be given to histone modifications, especially acetylation, in the next section.

1.5.1. Histones and histone modification. Histones are small, basic proteins found in all eukaryotic nuclei. They are very rich in basic amino acids such as lysine and arginine, which facilitate interactions with the negative charge of phosphate groups from DNA and also contribute to the water solubility of histones. Core histones undergo post-translational modifications at many sites, including acetylation, methylation, ubiquitination and phosphorylation. Most core histone modifications are found within the N-terminal and C-terminal tails. However, with the advent of sensitive mass spectrometry methods, we have learned that several modifications reside in the histone fold (Cosgrove *et al.*, 2004;Cosgrove and Wolberger, 2005;Cosgrove, 2007). Some modifications (active marks) are generally associated with transcriptionally active chromatin regions while others (repressive marks) correlate with silent regions. Histone acetylation usually marks active genes, and di- or trimethylation of K4 of H3 is also an active mark whereas di- or trimethylation of H3 at K9 constitutes a repressive mark (Peterson and Laniel, 2004;Sims, III and Reinberg, 2006). The role of a given chromatin mark or group of marks may vary with the cellular context and the gene under study.

1.5.2. Dynamic histone acetylation. At physiological ionic strength chromatin is folded into higher order structures. H1 and core histone N-terminal tails stabilize the folding of the chromatin fiber. Transcriptionally active and poised genes are found in DNAase I-

sensitive, decondensed chromatin domains that are accessible to transcription factors and the transcription machinery, while transcriptionally repressed genes are in condensed chromatin regions. Transcribed/poised chromatin domains are associated with acetylated histones (Calestagne-Morelli and Ausio, 2006a;Calestagne-Morelli and Ausio, 2006b). The core histones are reversibly modified by acetylation of lysines located in their basic N-terminal domains. These sites of acetylation are evolutionarily highly conserved. Core histone acetylation and deacetylation are catalyzed by K-Acetyltransferases (KATs; formerly acetyltransferases, EC number 2.3.1.48) (Allis *et al.*, 2007) and histone deacetylases (HDACs, EC number 3.5.1), with the level of acetylation being decided by the net activities of these two enzymes (Spencer and Davie, 1999). In mammalian cells, transcriptionally active chromatin regions have core histones undergoing high rates of acetylation and deacetylation, while in repressed chromatin regions the rate of reversible acetylation is slow. In MCF-7 (ERα positive, ERα+) and MDA MB 231 (ERα negative, ERα-) cells, 11-14% of the histones are engaged in rapid acetylation-deacetylation, while 50- 60% of the histones are involved in a slow rate of acetylation (Sun *et al.*, 2001). In ERα+ human breast cancer cells, estrogen slightly increased the steady state level of acetylated histones. An analysis of the kinetics of histone acetylation/deacetylation revealed that estrogen decreased the rate of deacetylation, with no effect on the acetylation rate (Sun *et al.*, 2001).

1.5.3. Function of histone acetylation. For chromatin remodeling, acetylation may cause subtle changes in the nucleosome core and promote interaction of non-histone proteins with the chromatin fiber, or control the formation of nucleosome arrays (Struhl, 1998). Acetylation of the histone tails is required for nucleosome remodeling (Lomvardas and

Thanos, 2001;Reinke and Horz, 2003;Nourani *et al.*, 2004). Histone acetylation destabilizes higher order chromatin structure, enhances solubility of chromatin at physiological ionic strength, alters histone tail conformation, alters the capacity of H1 to condense the chromatin fiber, and maintains the unfolded structure of the transcribed nucleosome (Spencer and Davie, 1999;Wang *et al.*, 2001). One study shows that H4 acetylation at K16 has a pivotal role in the decondensation of chromatin (Shogren-Knaak *et al.*, 2006). Acetylation at specific sites on a core histone may also be "read" by other proteins. For example, the bromodomain recognizes an acetylated lysine, and proteins with this motif may bind to a specific acetylated lysine residue (Lee and Workman, 2007).

1.5.4. Lysine acetyltransferases and histone deacetylases. KATs, HDACs, histone kinases, histone phosphatases, lysine methyltransferases, lysine demethylases and ATP-dependent chromatin remodeling complexes mediate chromatin remodeling and are components of a complex epigenetic network regulating gene expression during development and differentiation. Since the first purification and cloning of a KAT (Gcn5) (Brownell *et al.*, 1996) and HDAC (HDAC1) (Tauton *et al.*, 1996), multiple KATs and HDACs have been identified (reviewed in (Spencer and Davie, 1999;Davie and Moniwa, 2000;Lee and Workman, 2007;Yang and Seto, 2007). KATs often have transcriptional coactivator activity and when recruited to a gene promoter by a transcription factor will increase the level of acetylated histones and enhance transcriptional activity of the promoter (Lee and Workman, 2007). Both acetyltransferases and deacetylases were found to form complexes with certain transcription factors e.g. Sp1 and Sp3 (Won *et al.*, 2002a;Won *et al.*, 2002b).

Breast cancer cell lines such as MCF-7 cells have elevated expression of HDAC 1 and 2 relative to normal breast epithelial cells (Feng *et al.*, 2007). Our lab has found that most of HDAC2 is in complex with HDAC1 in MCF-7 human breast cancer cells. However, HDAC1 is in excess compared to HDAC2. HDAC1 and HDAC2 account for at least 50% of the HDAC activity in MCF-7 cells. Several transcription factors repress gene expression by recruiting HDAC1/2 corepressor complexes to the promoters that they affect. Further, pending the promoter context, transcription factors recruit HDAC1 and 2 corepressor complexes to mediate dynamic deacetylation of histones and non-histone chromosomal proteins associated with or close to the promoter. Our laboretory demonstrated the dynamic loading of HDAC1/2 on TFF1 gene promoter in response to estrogen stimulation in MCF-7 breast cancer cells (Sun *et al.*, 2005b). Sun *et. al.* from our laboretory also reported that the Sp1 and Sp3 transcription factors are preferentially associated with phosphorylated HDAC2 in breast cancer cells, although most of the HDAC2 is not phosphorylated in these cells (Cai *et al.*, 2001;Pflum *et al.*, 2001;Tsai and Seto, 2002;Sun *et al.*, 2002a). Others and Sun *et. al.* from our laboretory demonstrated that mammalian HDAC1 and HDAC2 are phosphorylated by the protein kinase CK2 (Cai *et al.*, 2001;Pflum *et al.*, 2001;Tsai and Seto, 2002;Sun *et al.*, 2002a). HDAC1 is phosphorylated at serines 421 and 423, while HDAC2 is phosphorylated at serines 394, 422 and 424. HDAC2 is primarily, if not solely, phosphorylated by CK2 (Tsai and Seto, 2002).

1.6. Nuclear matrix and chromatin remodeling. The nuclear matrix consists of both nuclear proteins and RNA. The nuclear matrix proteins may be analyzed by isolating the nuclear matrices typically by DNase I digestion and extraction with 0.25 M ammonium

salt (Samuel *et al.*, 1997). Milder methods for isolating nuclear matrices are also available (Jackson and Cook, 1985). Nuclear matrix proteins (NMPs) represent about 30% of the nuclear protein. This subset of the cellular proteome includes proteins with roles in the organization and function of nuclear DNA. NMPs are involved in establishing chromatin loop domains and in the organization of chromosome territories (Coffey, 2002). The nuclear matrix has a pivotal role in the processing of genetic information (Stein *et al.*, 2004). DNA replication, transcription and DNA repair occur at defined nuclear matrix sites (Dimitrova and Berezney, 2002;Jackson, 2003). Transcription factors including tumor suppressors (e.g. Rb, p53) and hormone receptors (e.g. ER) dynamically associate with specific nuclear matrix sites. The cancer cell nuclear matrix proteome has proteins involved in the aberrant processing of genetic information, the disorganization of the genome, and altered nuclear structure. As such, these NMPs are potential biomarkers of the disease. Enzymes involved in chromatin remodeling such as SWI/SNF, KAT and HDACs (HDAC1, HDAC2) are associated with the nuclear matrix. Similarly, HDAC1 and 2, but not HDAC4, were associated with the nuclear matrix of breast cancer cells (Sun *et al.*, 2001). ER, Sp1 and Sp3 are NMPs.

2. Breast cancer

2.1. Incidence of breast cancer in North America. Breast cancer is the most common cancer in women. It is also one of the leading causes of death in women worldwide. It is a rare disease in men representing approximately 1% of the total breast cancer cases globally (Fentiman *et al.*, 2006;Leinung *et al.*, 2007). According to the statistics of American Cancer society, 192,370 new cases of invasive breast cancer in women and 1910 new cases in male will be diagnosed in the United States in 2009. Among these

cases, 40,170 female and 440 men are expected to die of breast cancer (Jemal *et al.*, 2009).

2.2. Types of breast cancer. According to the World Health Organization, more than 100 types and subtypes of breast tumors have been reported. The two most common types of non-invasive breast cancer are ductal carcinoma *in situ* (DCIS; also known as intraductal carcinoma) and lobular carcinoma *in situ* (LCIS; also called lobular neoplasia) (Lakhani *et al.*, 2006). They are named after the parts of the breast in which they originate. Fortunately, nearly all women diagnosed at this early stage of breast cancer can be successfully cured.

Invasive (also called infiltrating) breast cancer is diagnosed when cancer cells that started *in situ* (the milk ducts or lobes) invade to healthy surrounding tissue. Invasive breast cancer has the potential to travel to other parts of human body through bloodstream and lymph system and become metastatic (Turashvili *et al.*, 2005). Invasive ductal carcinoma (IDC) is the most common type of invasive breast cancer representing about 78% of all malignancies (Shackney and Silverman, 2003). Several common subtypes of IDC are: 1) Inflammatory breast cancer (IBC) is an advanced, aggressive form of cancer, which starts out in the milk ducts and proceeds to invade the skin and lymph system (Ferrara, 2008;Molckovsky *et al.*, 2009); 2) Medullary carcinoma is named for its resemblance to brain (medulla) tissue and is generally a subtype with favourable prognosis (Malyuchik and Kiyamova, 2008); 3) Metaplastic breast cancer is a rare form of breast cancer with uncertain prognosis (Reis-Filho *et al.*, 2005); 4) Paget's disease of the nipple shows up in and around the nipple and usually signals the presence of breast cancer beneath the skin (Lev-Schelouch *et al.*, 2003;Kanitakis, 2007); and 5) Tubular

carcinoma takes its name from its microscopic appearance. It is a rare type of breast cancer and it has a better prognosis than most forms of invasive breast cancer (Sullivan *et al.*, 2005).

Invasive lobular carcinoma (ILC) represents about 5% of all breast cancer diagnosis. ILC is diagnosed when the cancer cells leave the lobes and infiltrates fatty tissue and other breast tissues close to the lobes (Rakha *et al.*, 2008). There are other less common invasive breast cancer types, each of these types of breast cancer occur in less than 5% of all cases that are diagnosed (Tumours of the breast and female genital organs, World Health Organization classification of tumours, 2003, ISBN 92 832 2412 4).

2.3. Treatment of Breast Cancer. Breast cancer can be treated by two methods, local and systemic (Kurtz, 2006;Gonzalez-Angulo *et al.*, 2007;Pruthi *et al.*, 2007). Surgery and radiation therapy are local treatments that are used to remove or destroy the cancer cells in a specific area. Chemotherapy and hormonal therapy are examples of systemic treatments that will destroy or control cancer cells systemically over the body.

2.3.1. Local treatments.

2.3.1.1. Surgery. The purpose of surgery is to remove the tumor from the breast and to assess the stage of disease. Surgery is often combined with other treatments such as radiation therapy, chemotherapy, hormone therapy, and/or biologic therapy.

2.3.1.2. Radiation therapy. Radiation is usually used to destroy cancer cells remaining after surgery or to reduce the size of a tumor before surgery.

2.3.2. Systemic therapy. Systemic therapy includes biologic therapy, chemotherapy, and hormone therapy(Pruthi *et al.*, 2007). Systemic treatment is often used to shrink the

tumor enough to make surgical removal possible or to allow for less extensive surgery. However, removal of most of the cancer by surgery is not possible, and therefore systemic therapies are the main treatment option after the surgery. Systemic therapy is also used in treating women with metastatic breast cancer (Untch and von Minckwitz, 2009).

2.3.2.1. Biologic therapy. Approximately 15% to 30% of breast cancers overproduce the growth-promoting protein HER2/neu. These tumors are likely to grow faster and are generally tend to return than HER2 negative tumors. Herceptin® (tratuzumab) is a monoclonal antibody which directly targets the HER2 protein of breast tumors and offers a real survival benefit for some women with metastatic breast cancer, although tratuzumab has also been shown to be effective in early-stage breast cancer that overexpresses HER2. In 2006, the FDA approved tratuzumab treatment for all HER2 positive breast cancers (Laudadio *et al.*, 2007;Dean-Colomb and Esteva, 2008;Haq and Geyer, 2009).

2.3.2.2. Chemotherapy. Adjuvant chemotherapy refers to the use of chemotherapy after the tumor has been removed for the purpose of increasing the cure rate of the patient. The advantage of chemotherapy is dependent upon multiple factors including the size of the cancer, the number of lymph nodes involved, the presence of estrogen or progesterone receptors, and the amount of HER2/neu protein made by the cancer cells (Herceg and Vrbanec, 2009).

2.3.2.3. Hormone therapy. Hormonal therapy is also called "anti-estrogen therapy". The goal of this therapy is to starve the breast cancer cells of the hormone they prosper on, which is estrogen. Estrogen, a hormone produced by the ovaries, promotes the growth of

many breast cancers. Estrogens exert these wide range of actions by binding to the estrogen receptor (ER) in target cells (Jensen and Jordan, 2003). About 75% of breast cancers are ER-positive ("ER-positive" or "ER+") (Briest and Wolff, 2007). The ER status remains the best predictor of response, although other factors exist and need to be identified (Cheung, 2007). Women whose breast cancers test positive for estrogen receptors can be given hormone therapy to block the effects of estrogen on the growth of breast cancer cells. If either estrogen or progesterone receptors are present, a response to hormonal therapy is usually observed. ILC tumors are often positive for estrogen and progesterone receptors and respond well to hormone therapy (Bentrem and Craig, V, 2002;Ma *et al.*, 2009).

There are mainly four kinds of hormonal therapy working by decreasing the amount of estrogen in the body, blocking the interaction between estrogen and the estrogen receptor and eliminating the estrogen production. 1) Aromatase inhibitors (AIs) reduce the estrogen level in postmenopausal women by blocking an enzyme responsible for producing estrogen (Santen *et al.*, 2009;Samphao *et al.*, 2009). AIs have been approved for use in treating both early and advanced breast cancer. 2) Selective estrogen-receptor modulators (SERMs) such as tamoxifen block the estrogen receptor. SERMs can act as estrogens in certain tissues and as anti-estrogens or be inactive in other tissues. These special properties make significant therapeutic advances for SERMs to treat estrogen-modulated diseases in a tissue-specific manner without risking of systemic estrogen activity (Nath and Sitruk-Ware, 2009). 3) Estrogen-receptor downregulators (ERDs) destroy the estrogen receptor. With no receptor in the cell, estrogen signaling is

blocked (Johnston, 2005). 4) Ovarian shutdown or removal eliminates estrogen in the body because the ovaries are the major source of estrogen in women before menopause.

3. Estrogen Receptor

3.1. Estrogen receptor α and β. Estrogens (also oestrogens or œstrogens) are a group of female steroid sex hormones produced primarily by the ovaries, and in smaller amounts by the adrenal cortex, synthesized mainly by the ovarian follicular cells in non-pregnant women. There are three endogenous estrogens: estrone (E1), estradiol (E2) and estriol (E3). E2 is the predominant form and E1 and E3 are less powerful estrogens. Estrogen is transported in the blood by complexing with sex hormone-binding globulin or albumin. The concentration of free estrogen represents the true hormone concentration available to cause a response. Estrogens are not only involved in the reproductive process but also in the growth, differentiation, and function of diverse target tissues throughout the body. As described above (section 2.3.2.3. Hormone therapy), estrogens act through interacting with the estrogen receptor (ER) in target cells. Before the identification of the ER, steroid hormone action was thought to be working through enzymatic pathways. The ERs are encoded by two independent genes: alpha and beta (ERα and ERβ) to regulate target gene expression (Couse and Korach, 1999). Epidemiological and animal studies identified estrogen exposure as a high risk factor for breast cancer (Pike *et al.*, 1993). Elevated levels of ER α in benign breast epithelium were found highly related to an increased risk of breast cancer (Ali and Coombes, 2000). ERα is found overexpressed in around 70% of breast cancers, referred as "ER positive" (ER+) (McGuire *et al.*, 1986;Dickson and Lippman, 1988). ERα-positive tumors have been found to be more differentiated and less

metastatic than ERα-negative tumors (Garcia *et al.*, 1992). ERα has been therefore taken as a good prognostic factor and is targeted in anti-estrogen therapies. The null mice of ERs suggested that ERα, instead of ERβ is important in the postnatal development of the uterus and mammary glands (Couse and Korach, 1999). The expression of ERβ declines during breast tumorigenesis and is found less in invasive breast tumors than normal tissues (Leygue *et al.*, 1998;Bardin *et al.*, 2004) suggesting the role of ERβ as a tumor suppressor (Skliris *et al.*, 2003). ERβ is found expressed alone or together with ERα. Emerging evidence suggests that these two ERβ expression cohorts function differentially (Murphy and Watson, 2006). ERβ is found to attenuate ERα activity (Korach *et al.*, 2003). However, attempts to correlate ERβ with various biomarkers in breast cancer are not conclusive (Speirs, 2002) and the specific functions of ERβ in breast cancers are of great interest for breast cancer reseachers (Marino *et al.*, 2005;Murphy and Watson, 2006;Fox *et al.*, 2008). ERα has been the major ER studied for a long period (Carroll and Brown, 2006). In this thesis, ERα is the ER studied throughout the whole project unless otherwise specified.

3.2. The regulation of estrogen responsive genes. Estrogen is well known to exert many normal physiological as well as pathological effects. However, the exact mechanisms are not well defined and estrogen responsive genes are believed to be strong contributors to these effects. In a serial analysis of gene expression (SAGE) study including 12,550 genes of breast cancer cells, about 0.4% of the genes were up-regulated after estrogen treatment (Charpentier *et al.*, 2000). The mitogenic effects of estrogen were found largely due to its ability to increase the expression of key cell cycle regulatory genes (Prall *et al.*,

1997;Prall *et al.*, 1998). The classical pathway for estrogen to initiate gene expression is that ligand binding triggers ER to disassociate from the chaperone proteins (e.g. heat shock proteins) through conformational changes to form an 'activated' ER. The activated ER then dimerize and bind to specific DNA sequences, EREs harbouring ER binding site(s) and further recruiting coactivators to activate transcription. This mechanism was first proposed in the late 1960s based on studying the subtle differences of ER sizes by sucrose density gradient analysis (SDGA) technique (Jensen *et al.*, 1968). SDGA demonstrated the transformation of the 4S ER-estradiol complex to a 5S complex active form of the receptor. The molecular events were then further identified and validated by X-ray crystallography (Brzozowski *et al.*, 1997;Shiau *et al.*, 1998). A palindromic inverted repeat DNA sequence (GGTCAnnnTGACC) identified from the *Xenopus vitellogenin* gene has been considered the minimal canonical ERE sequence (Klein-Hitpass *et al.*, 1986;Klein-Hitpass *et al.*, 1988). This ERE sequence possesses an enhancer's properties and functions as well as in an orientation and distance-independent manner (Klinge, 2001). EREs are found in both the proximal and distal promoter regions (Carroll *et al.*, 2005;Carroll and Brown, 2006). One enhancer ERE site was located at a region 10.5 kb upstream of the TFF1 transcription initiation site (Giamarchi *et al.*, 1999;Carroll *et al.*, 2005). Interestingly, more estrogen responsive genes have been found containing imperfect, even non-palindromic EREs (Anolik *et al.*, 1995;Driscoll *et al.*, 1998).

Except for the classical pathway, several other molecular mechanisms were reported to regulate estrogen responsive genes. For estrogen responsive genes without ERE in their proximal promoters (Klinge, 2001), ER regulates gene expression through "non-

classical" pathways. One non-classical pathway, referred as 'transcription factor crosstalk', modulates gene expression by interactions of the liganded ER with other DNA bound transcription factors such as Sp1 or AP-1 (Kushner *et al.*, 2000a;Kushner *et al.*, 2000b). In this case, ER does not bind DNA and functions more like a transcriptional co-activator and further activates other transcription factors. The mechanism for this pathway is that ER modulates the activities of these transcription factors by stabilizing their interaction with DNA and/or recruiting coactivators to the transcriptional complex (Stein and Yang, 1995;Ray *et al.*, 1997;Kushner *et al.*, 2000a;Kushner *et al.*, 2000b;Safe, 2001). This is supported by one study showing that ER without a functional DNA-binding domain are still able to modulate the activity of the AP-1 transcription factor (Jakacka *et al.*, 2001). Another non-classical pathway is referred to as "non-genomic"

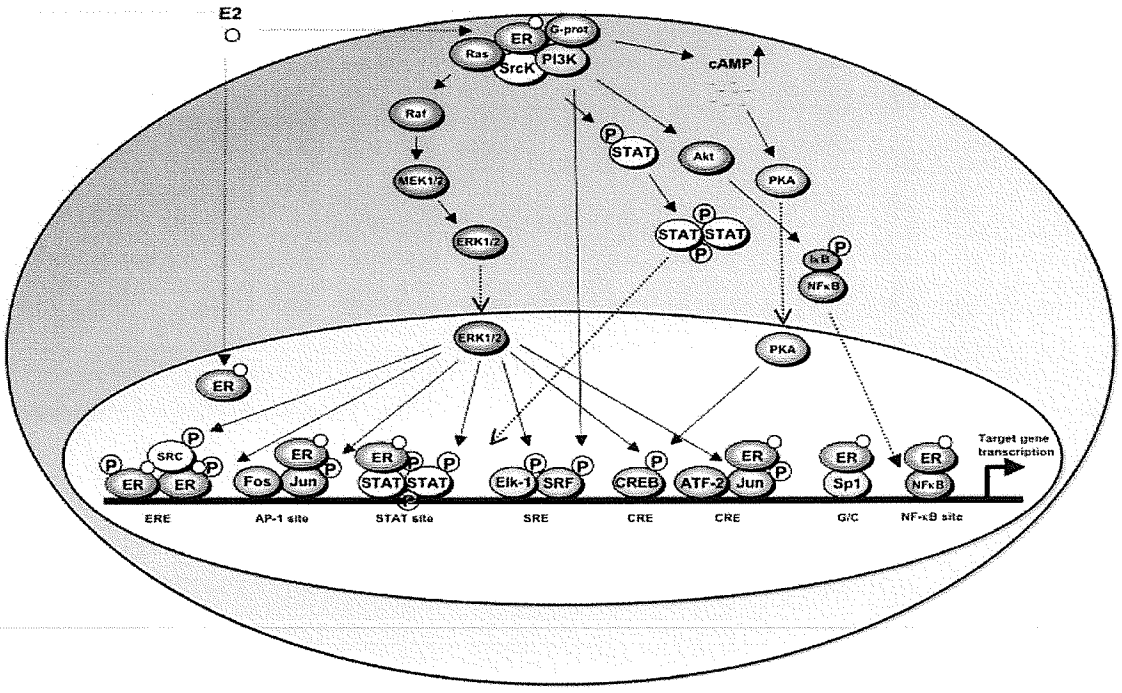


Figure 4. Molecular mechanisms for estrogen to regulate estrogen responsive genes (Bjornstrom and Sjoberg, 2005).

pathway or "membrane initiated steroid signaling" (MISS). In this "nongenomic" pathway, estrogen binds to membrane ER localized outside of the cell nucleus and activates signal transduction pathways in the cytosol. Nevertheless, although it is called non-genomic, this pathway still ends up with regulating certain estrogen responsive genes through activating extracellular regulated kinase (ERK) and protein kinase B (AKT), as well as signal transduction pathways, (Aronica *et al.*, 1994;Tesarik and Mendoza, 1995;Migliaccio *et al.*, 1996;Le, V *et al.*, 1997;Kelly *et al.*, 1999;Levin, 1999). In addition, the unliganded ER is able to function through the mitogen-activated protein kinase (MAPK/ERK) pathway (Kato *et al.*, 1995) and phosphoinositide 3-kinase (PI3K/AKT) pathway (Levin, 2005), which is usually referred to ligand-independent activation. These molecular mechanisms for estrogen to start gene transcription have been well reviewed and summarized in figure 4 (Bjornstrom and Sjoberg, 2005).

3.3. Structural domains of ER. ER belongs to the steroid/nuclear receptor superfamily that share highly conserved structures and common mechanisms affecting gene transcription (Escriva *et al.*, 2004). ER has six domains named A through F from the N- to C terminus, which are organized into three major functional domains (Kuiper *et al.*, 1998). N-terminal Activation Function-1 (AF-1) (domains A and B) modulate transcription in a gene and cell-specific manner. The region between aa residues 41 through 150 of AF-1 is required for its activity (Metzger *et al.*, 1995;McInerney and Katzenellenbogen, 1996). The region between aa residues 91 through 121 is necessary for synergy with the LBD to generate maximum transcriptional activity (Danielian *et al.*, 1992). Ser118 in AF-1 is found as the target for phosphorylation by the MAPK pathway in response to growth factors (Ali *et al.*, 1993;Joel *et al.*, 1998;Weitsman *et al.*, 2006).

This suggests an important role of phosphorylation for AF-1 function. The central DNA-binding domain (DBD, C domain) is composed of two functionally distinct zinc fingers. ER interacts directly with the DNA helix through its DBD domain. Analysis of deletion mutants of ERα demonstrated that amino acids 179–215 of the N-terminal zinc finger of the DNA-binding domain were required for ligand-independent receptor action (Platet *et al.*, 2000). The hinge region or D domain is a 40–50 aa sequence between the DBD and ligand-binding domain (LBD). This region contains sequences for receptor dimerization (Kumar and Chambon, 1988;Ogawa *et al.*, 1998) and nuclear localization sequences (NLSs) (Picard *et al.*, 1990;Ylikomi *et al.*, 1992). Additionally, this hinge region is also able to interact with nuclear co-repressor proteins (Safer *et al.*, 1998). The LBD (domains E) contains Activation-Function-2 (AF-2). AF-2 is localized at the C-terminal end of the E domain, which is comprised of aa numbers 530 through 553 in ER (Seielstad *et al.*, 1995). This domain is highly conserved within the nuclear receptor superfamily, and is recognized by various transcriptional co-activators (Beato *et al.*, 1996;Horwitz *et al.*, 1996;McKenna *et al.*, 1999). The F domain located at the C-terminus helps to distinguish estrogen agonists versus antagonists (Montano *et al.*, 1995). A third transactivation domain named AF-2a or tau2 was localized to the N-terminal region of the LBD of ER (Danielian *et al.*, 1992).

3.4. Human ERα isoforms. Two human ERα (hERα) isoforms have been characterized within ERα positive breast cancer cell lines such as MCF-7: the full-length 66 kD ERα 66 and 46 kD ERα46. The ERα46 form has N-terminal deletion and doesn't have the AF-1 domain of ERα66. ERα46 was found to antagonize the proliferative action of ERα66 in MCF-7 cells partially through inhibiting hERα66 AF-1 activity (Penot *et al.*, 2005). Most

studies, including this thesis project, were done with antibodies only recognizing the ERα 66 form. All the descriptions for ERα in this thesis refer to the ERα 66 form unless otherwise specified.

3.5. ER, Anti-estrogen therapy and SERMs. Anti-estrogen therapy (also endocrine therapy) started from the 1940s and clinical using of tamoxifen was a milestone for breast cancer treatment. Anti-estrogen therapy is much less toxic and has fewer side-effects than chemotherapy. One study showed that 60% of ER+ patients responded well to endocrine therapy (Jensen and Jordan, 2003). Clinical studies showed that about one third of patients with metastatic breast cancer respond to endocrine therapy (Glauber and Kiang, 1992). Estrogen and ER as targets of therapy in breast cancer supports the importance of studying mechanisms for estrogen and ER involvement in the incidence and progression of breast cancer. Many recent studies have been focusing on factors assisting estrogen receptors in regulating gene transcription.

The discovery of ER lead to a major therapeutic advance for clinical practice treating breast cancer in a tissue specific manner, which is the use of SERMs. SERMs are a class of drugs that act on the ER. The SERMs lack the steroid structure of estrogens but possess a tertiary structure that recognizes and binds to the ER, and thereby selectively inhibit or stimulate estrogen-like action in various tissues. The X-ray crystalline structures of ER showed that the LBD consists 12 α-helices with a 'pocket' into which the ligand fits (Brzozowski *et al.*, 1997). The fitting of the ligand alters the conformation of the LBD with helix 12 forming a 'lid' over the pocket. The ligands are trapped in a hydrophobic environment and a surface is formed on the LBD with which coactivator

proteins interact. SERMs (e.g. tamoxifen) act on ER through relocating helix 12 by their bulky side chain and preventing coactivator molecules from binding to the right site to form the active ER complex form (Berry *et al.*, 1990;McInerney and Katzenellenbogen, 1996). Thereby, estradiol and the SERMs (e.g. raloxifene) form different amino acid (aa) contacts in the ligand binding pocket of ER, which suggests that different positioning of helix 12 allows ER to interact with co-activators or co-repressors (Brzozowski *et al.*, 1997). Tamoxifen, the most commonly used SERM, is effective in patients whose cancers are positive for hormone receptors no matter of postmenopausal or premenopausal. As a non-patented drug, tamoxifen has a significant advantage over newer drugs in being low-cost and well-tolerated with a well defined side-effect profile. It was speculated that a multi-functional SERM such as tamoxifen may be the aspirin of the 21(st) century (Singh *et al.*, 2008).

3.6. Current opinions about estrogen regulated gene transcription. Several wonderful studies exploring how estrogen starts transcription contributed greatly to developing the concept of dynamic interactions between a receptor and the chromatin template of hormone-regulated gene (Shang *et al.*, 2000;McNally *et al.*, 2000;Reid *et al.*, 2003;Metivier *et al.*, 2003a). Using ChIP assay, Brown's lab studied the loading pattern of ERα and a group of coactivators on several estrogen responsive gene promoters after MCF-7 cells were treated with estrogen for a series of time points. For the first time they demonstrated that ERα and a number of coactivators rapidly cycle on and off estrogen responsive promoters in a cyclic fashion followed by transcription after estrogen treatment. Combining ChIP and genetic approaches, they further showed that recruitment of the p160 class of coactivators is sufficient for gene activation and for the growth

stimulatory actions of estrogen in breast cancer. This study supports a model that ERα cofactors play unique roles in estrogen signaling (Fig.5)(Shang *et al.*, 2000). Later on Gannon's lab elegantly illustrated a comprehensive picture of events resulting in transcriptional activation of an estrogen responsive gene, TFF1 in MCF-7 ERα positive breast cancer cells (Reid *et al.*, 2003;Metivier *et al.*, 2003a) (reviewed by (Mellor, 2006)). In their studies, the cyclical association of 46 transcription factors with TFF1 promoter was determined by ChIP assays and re-ChIP after removal of α-amanitin block followed with estrogen treatments. This study again showed that ERα association with TFF1 promoter is cyclical. However, the cyclical association exists no matter the cells were

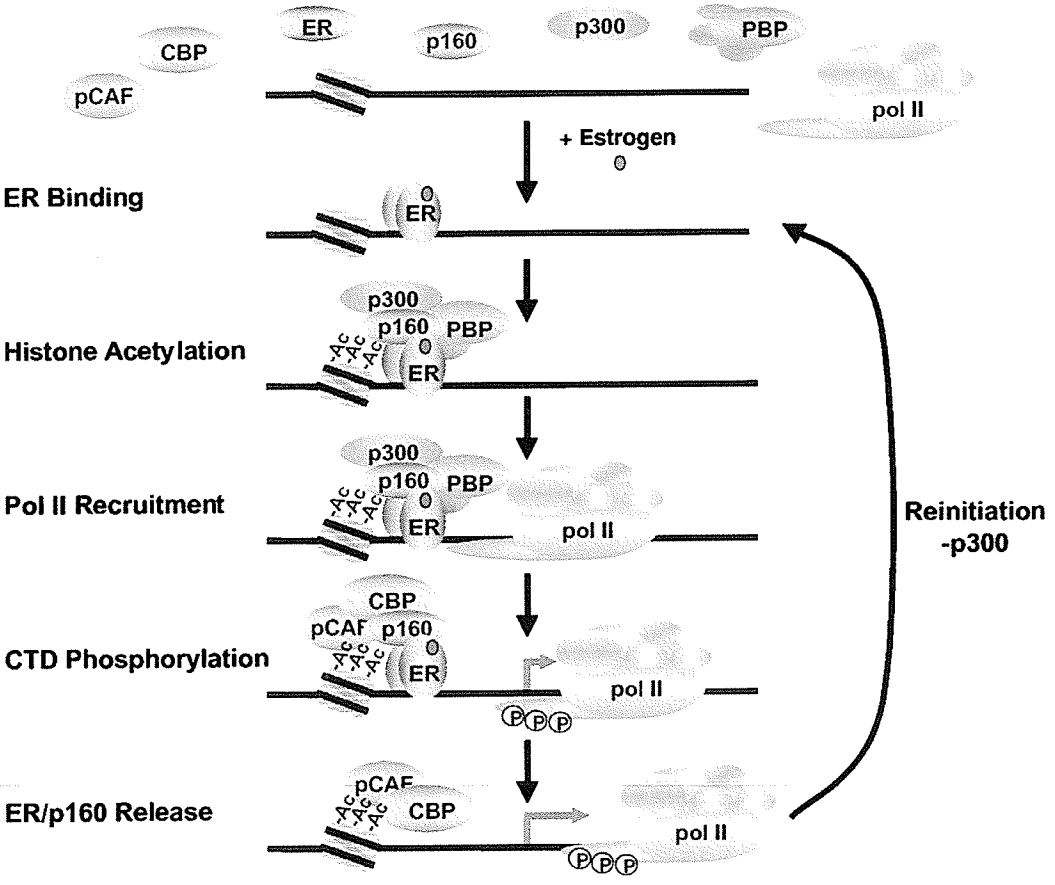


Figure 5. Cyclic model of ER transcription complex assembly (Shang *et al.*, 2000).

treated with estrogen or not. This study further defined the concept of a "transcriptional clock" from Brown's study, which directs and achieves the sequential and combinatorial assembly of a transcriptionally productive complex on a promoter. These studies start a new paradigm for understanding of gene regulation on chromatin templates. This was the first demonstration that both unliganded and liganded ERs cycle on estrogen responsive promoters with a series of coactivators and pol II in a well-organized order to start gene transcription (Shang *et al.*, 2000;Metivier *et al.*, 2003a). The cyclical association of unliganded ERα is believed to help the poising of promoters for transcription. ERα was found going through three different but linked cycles in response to estrogen stimulation. The first ERα cycle is unproductive and only prepares the promoter for transcription, which is followed by two alternative productive cycles, each leading to transcription. The cyclic turnover of ERα by proteasome-mediated degradation explains how estrogen responsive genes respond to estradiol continuously (Reid *et al.*, 2003). The silencing components, such as the nucleosome remodelling and histone deacetylation (NuRD) complex, containing a Mi2-type ATPase, and HDAC complexes, were also found to play important roles in regulating a productive transcription cycle. The variable epigenetic modifications (e.g. methylation and/or acetylation status) of histones and the position occupied by the two key nucleosomes (see section 4.1 TFF1 gene organization) were also key factors in recording the cyclic nature and in directing the progress through each type of cycle. The best part of these important studies is that they integrated chromatin remodeling with the kinetic association of transcription factors with the TFF1 promoter (Metivier *et al.*, 2003a). Despite of all these achievements, some studies, mostly from fluorescence recovery after photobleaching (FRAP) experiments, challenged the

residence time of the receptor on the promoter. These studies demonstrated a highly dynamic turnover of transcription complexes at the promoter and suggested the residence time of the receptors is very short, which fits better to a 'hit and run' model instead of the 'cycling on the promoter' model (McNally *et al.*, 2000;Stenoien *et al.*, 2001;Becker *et al.*, 2002). Finally, it is important to mention a new revised model for ER-mediated

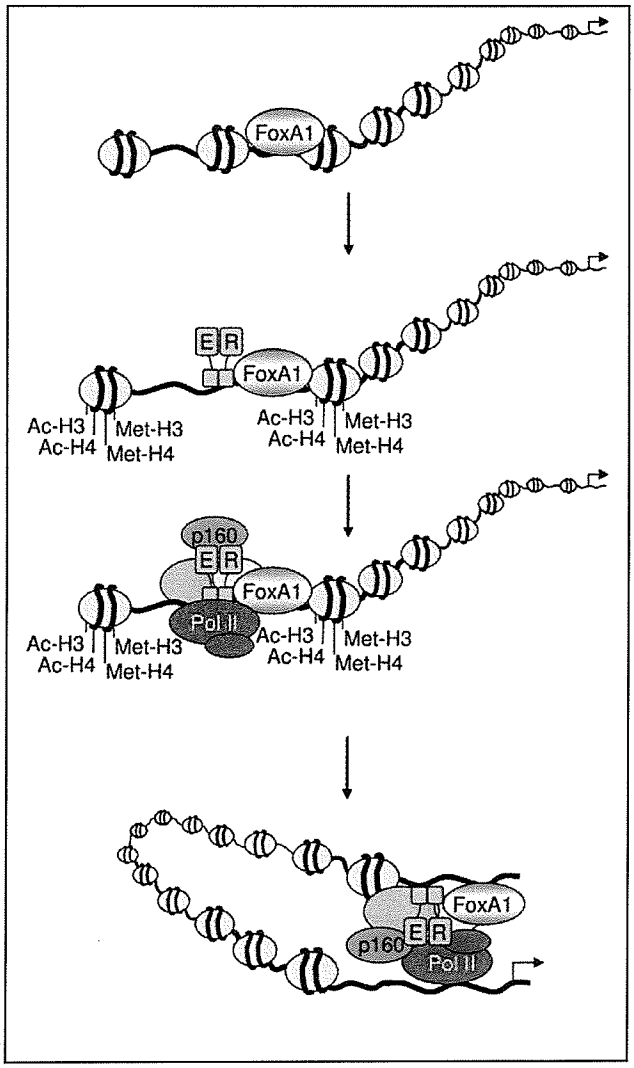


Figure 6. A revised model of ER-mediated transcription (Carroll and Brown, 2006).

transcription proposed by Brown's lab through chromosome-wide mapping of ER binding sites and ChIP-chip analysis. This model proposed that a pioneer factor such as FoxA1 on chromatin together with a nearby ERE (usually distal enhancer) facilitates ER association followed by chromatin looping (Fig. 6). The chromatin looping promotes the physical contact between the distal enhancer and the proximal promoter and further initiates gene transcription (Carroll *et al.*, 2005;Carroll and Brown, 2006).

3.7. ER, estrogen responsive genes and Sp factors. As described above (Section 2.2 The regulation of estrogen responsive genes), the loading of ERα onto E2 responsive promoters may be direct or indirect. ERα can enhance gene expression through interacting with another transcription factor which may stabilize the DNA binding of that transcription factor. For promoters with a consensus or near consensus ERE, ERα binding is likely direct. However, for other estrogen responsive promoters ERα may bind indirectly through another transcription factor (e.g. Sp1, FoxA1) (Carroll *et al.*, 2005;Lin *et al.*, 2007). Both ERα and ERβ were found to interact with AP-1 (Webb *et al.*, 1995;Paech *et al.*, 1997b). It is well-established that ERα can interact with Sp1 to promote transcription (Paech *et al.*, 1997a;Safe, 2001;Levin, 2005). Increasing evidence showed that DNA element Sp1 protein binding site(s) (Sp1 site) play important roles in estrogen action (Xie *et al.*, 1999;Safe, 2001). Sp1 sites can be recognized and associated with Sp1 and Sp3 transcription factors with similar affinity. This section will focus on discussing how Sp1 and are involved in the regulation of estrogen responsive genes.

Estrogen responsive promoters of genes expressed in human breast cancer cells often have an ERE or half-site ERE positioned next to an Sp1 binding site (ERE (1/2)

(N)x Sp1) (Bouwman *et al.*, 2000;Abdelrahim *et al.*, 2002). Cathepsin D, RARα and tumor growth factor α (TGFα) have a half-site ERE located near one or more Sp1 sites, and these Sp1 sites are required for the E2 response (Vyhlidal *et al.*, 2000). However, some estrogen responsive genes have only Sp1 site(s), but not ERE(s), in the regulatory region and ERα is found to function through interactions with Sp1 (Krishnan *et al.*, 1994;Porter *et al.*, 1996;Porter *et al.*, 1997;Wang *et al.*, 1998;Qin *et al.*, 1999). Genes like adenosine deaminase (ADA) and cyclin D1 do not have palindromic ERE or ERE half-sites but have Sp1 sites in their respective estrogen responsive regions and thus ERα acts through the Sp1 (Xie *et al.*, 1999;Castro-Rivera *et al.*, 2001a). This maybe also true for the *c-myc* gene as neither ERE nor half ERE but two possible Sp1 binding sites were found in the estrogen responsive promoter 2 of *c-myc* (Dubik and Shiu, 1992;Miller *et al.*, 1996). ChIP assays have validated that E2 addition to MCF-7 breast cancer cells resulted in the association of Sp1 and ERα with many estrogen responsive promoters including cathepsin D and cyclin D1 promoters (Castro-Rivera *et al.*, 2001a). Combining promoter analysis and ChIP assays, Safe's lab reported that quite a few estrogen responsive gene promoters contain Sp1 sites which are important for a response to estrogen treatment. Using episomal promoter models, they found that Sp1 is the transcription factor interacting and assisting with ERα to respond to estrogen stimulation. However, using siRNA assays, they demonstrated that Sp3 plays roles similar to Sp1 for vascular endothelial growth factor (VEGF) gene to respond to estrogen stimulation and Sp3 can also exert its function through interacting with ERα (Stoner *et al.*, 2000;Higgins *et al.*, 2006d). The next section of the introduction provides a general overview of the Sp1 and Sp3 transcription factors.

4. Sp1 and Sp3

The consensus Sp1 binding DNA element sequence (CCCCGCCCC, GC box) was first identified from the SV40 promoter (Dynan and Tjian, 1983a;Dynan and Tjian, 1983b). The general transcription factor Sp1 binds and acts through GC boxes and/or GT boxes to regulate gene expression (Kadonaga *et al.*, 1987a;Kadonaga *et al.*, 1988;Courey and Tjian, 1988;Courey *et al.*, 1989). For a long time Sp1 was recognized as a constitutive transcription activator of housekeeping genes and other TATA-less genes which are usually not highly regulated. However, increasing evidence suggests that these GC/GT boxes are not only required for the transcriptional regulation of many housekeeping, but also tissue-specific, viral, and inducible genes (Suske, 1999;Philipsen and Suske, 1999;Bouwman and Philipsen, 2002;Wierstra, 2008). One ChIP-on-chip study analyzing the distribution of Sp1 binding along human chromosomes 21 and 22 demonstrated a large number of Sp 1/3 binding sites (Cawley *et al.*, 2004). This study estimated at least 12,000 Sp1 binding sites in the whole genome which are located on genes involved in almost all the cellular processes (Cawley *et al.*, 2004). Interestingly, only about 20% of the Sp1/3 sites were those at the 5' end of protein coding genes, with about 40% of the sites located at the 3' end of noncoding RNA genes.

4.1. Sp and Krüppel-like Factor (KLF) factors. Sp1 was initially identified as a factor, expressed in HeLa cells, which selectively activated *in vitro* transcription from the SV40 promoter. It was named Sp1 after its purification on Sephacryl and phosphocellulose columns (Dynan and Tjian, 1983a;Dynan and Tjian, 1983b). Following the purification and cloning of Sp1 (Briggs *et al.*, 1986;Kadonaga *et al.*, 1987a), Sp3 and several other members of the Sp family of transcription factors were characterized based on their

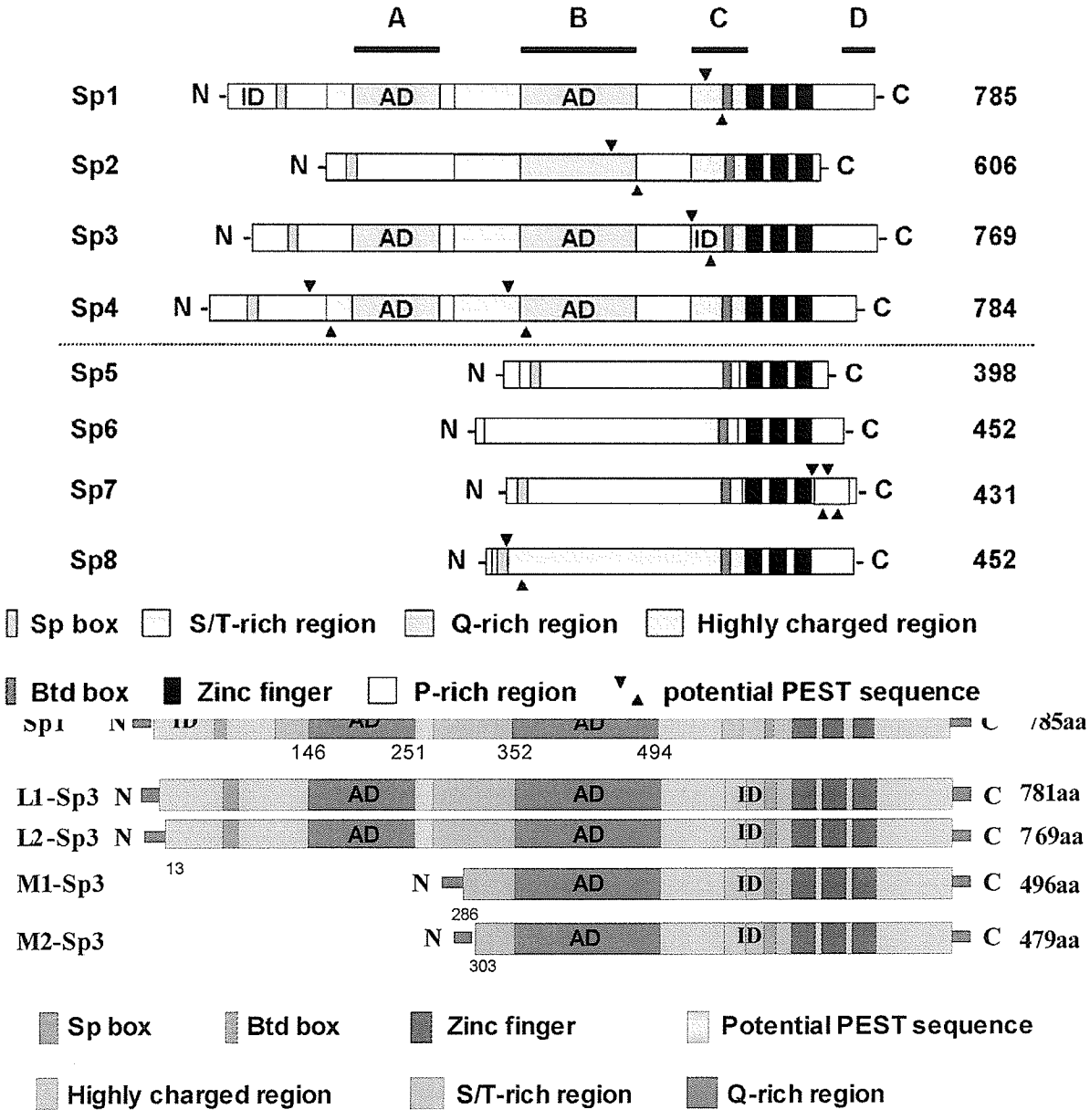


Figure 7. Sp family and Sp3 isoforms. Adapted from (Bouwman and Philipsen, 2002)

homology to Sp1 (Fig.7) (Kadonaga *et al.*, 1987b). Sp1 and Sp3 belong to the Specificity Protein/Krüppel-like Factor (SP/KLF) transcription factor family characterized by the highly conserved (sequence identity more than 65%) DNA binding domain, three adjacent Cys_2His_2-type zinc fingers (Philipsen and Suske, 1999;Suske *et al.*, 2005).Within this family, the nine Sp members are united by the conserved zinc finger DNA-binding domain (Suske, 1999) but distinguished from the KLF members by the presence of a Buttonhead (BTD) domain on the N-terminal side of the DNA binding domain. Sp1, Sp2, Sp3 and Sp4 have been classified as a subgroup as they share a similar modular structure. Sp1 and Sp3 are ubiquitously expressed in mammalian cells. However, Sp2 and Sp4 have a restricted expression pattern. Sp4 expression is high in the central nervous system and in retinal neurons (Lerner *et al.*, 2005). Sp2 is expressed in several cell lines and at higher levels in cancer cells (Phan *et al.*, 2004). Further studies showed that Sp2 has a different consensus DNA binding site and evolutionary history than the other Sp family members. Therefore it is the opinion of some investigators that Sp2 should not be considered a member of the Sp family (Kingsley and Winoto, 1992;Bouwman and Philipsen, 2002;Kolell and Crawford, 2002).

4.2. Similarity and distinction of Sp1 and Sp3. Sp1 and Sp3 share more than 90% sequence homology in the DNA-binding domain and they bind to the same cognate DNA-element (Sp1-binding site) with similar affinity. Sp1 and Sp3 regulate the transcriptional activity of many genes involved in a wide range of biological processes, including differentiation, cell cycle progression, and oncogenesis (Li *et al.*, 2004;Sapetschnig *et al.*, 2004;Jinawath *et al.*, 2005). Both Sp1 and Sp3 are autoregulated genes. Sp1/3 sites were characterized in the proximal promoters of both Sp1 and Sp3

genes (Nicolas *et al.*, 2001;Tapias *et al.*, 2004). Knocking down one of these factors will decrease the levels of both factors (Nicolas *et al.*, 2001;Lou *et al.*, 2005). $Sp1^{-/-}$ mice were growth retarded and died after day 11 of embryonic development (Marin *et al.*, 1997). Similar to $Sp1^{-/-}$ mice, knocking out Sp3 expression resulted in growth retardation and the mice died at birth due to respiratory failure and cell-autonomous hematopoietic defects were also observed (Bouwman *et al.*, 2000;Van Loo *et al.*, 2003). The compound heterozygous Sp1/Sp3 mice are not viable, which provides evidence that the levels of both transcription factors are important in maintaining appropriate gene expression programs (Kruger *et al.*, 2007). A comparison of the phenotypes of the $Sp1^{-/-}$ and $Sp3^{-/-}$ mice suggested redundant functions in early development and distinct functions of Sp1 and Sp3 in later developmental stages. Sp1 and Sp3 levels changed in a different pattern in mouse germ cells during spermatogenesis (Ma *et al.*, 2008). Our lab demonstrated that Sp1 and Sp3 have different intranuclear distribution patterns and Sp3 comes back to the daughter cells before Sp1 during mitosis (He *et al.*, 2005). All these studies strongly support that Sp1 and Sp3 have different functional roles in the regulation of gene expression. Most of the previous studies have focused primarily on Sp1. This thesis studied the role of both Sp1 and Sp3 in the regulation of estrogen induced genes in breast cancer cells. Sp1 sites will be refered to Sp1/3 binding sites since Sp1 and Sp3 both recognize and bind to the sites with similar affinity.

4.3. Sp1, Sp3 and cancer.

4.3.1. Sp1 target genes and cancer hallmarks. There are six cancer hallmarks: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis.

Sp1 target genes play important roles in all of these six hallmarks of cancer (Hanahan and Weinberg, 2000;Hahn and Weinberg, 2002).

As described above, Sp1 and Sp3 are involved in the regulation of thousands of genes involved in diverse biological processes (Liang *et al.*, 2004;Jinawath *et al.*, 2005). Hence, dysregulation of Sp1 and Sp3 has been found in various cancers and diseases. Sp1 was initially considered to be a constitutive activator since a large number of Sp1 target genes encode proteins for intermediary metabolism. However, increasing evidence suggests that Sp1 is also intimately involved in cell growth control and tumorigenesis (Black *et al.*, 2001;Safe and Abdelrahim, 2005). Many Sp1 target genes are key players in cell proliferation and oncogenesis. Sp1 target genes include factors involved in cell cycle progression and arrest (e.g. cyclins) (Sherr and Roberts, 1999;Feng *et al.*, 2000;Abdelrahim *et al.*, 2002;Lagger *et al.*, 2003), both pro- and anti-angiogenic factors involved in invasion and metastasis (Yuan *et al.*, 2007), pro- and anti-apoptotic factors involved in genomic stability (Kavurma *et al.*, 2001;Kavurma and Khachigian, 2003;Olofsson *et al.*, 2007), proto-oncogene (e.g. *c-myc*) and tumor suppressors (e.g. p53 gene) stimulating cell proliferation and oncogenesis (DesJardins and Hay, 1993;Wierstra and Alves, 2007). Inhibition or knocking down Sp1 usually decreases the tumor formation, growth and metastasis (Jiang *et al.*, 2004;Lou *et al.*, 2005;Yuan *et al.*, 2007).

4.3.2. Sp1 and Sp3 protein levels. Sp1 and Sp3 protein levels are often greater in cancer cells than in normal cells (Lou *et al.*, 2005) and the Sp1 level correlated with tumor grade/stage and poor prognosis (Wang *et al.*, 2003b;Yao *et al.*, 2004;Safe and Abdelrahim, 2005). Most of the current studies have focused on Sp1 protein/mRNA expression. Sp1 protein levels were found to be abnormal in some cancers (Kitadai *et al.*,

1992;Zannetti *et al.*, 2000;Shi *et al.*, 2001;Chiefari *et al.*, 2002). Sp1 levels were greater in breast carcinomas compared to benign breast lesions (Zannetti *et al.*, 2000); Sp1 was expressed at higher levels in human hepatocellular carcinomas compared to control livers (Lietard *et al.*, 1997); Sp1 levels were greater in human thyroid tumors than in normal thyroid tissues (Chiefari *et al.*, 2002); and Sp1 levels were greater in human gastric cancer tissue than in normal adjacent gastric mucosal tissue (Kitadai *et al.*, 1992;Jiang *et al.*, 2004). Of note, Sp1 expression is a predictor of survival of gastric cancer (Wang *et al.*, 2003b). Acknowledging that Sp1 overexpression had a role in gastric cancer, it was proposed that reducing the level of Sp1 would reduce the metastatic potential of the gastric cancer cells. Indeed, decreasing expression of Sp1 with Sp1 specific siRNA in gastric cancer cells reduced their growth and metastatic potential when injected into the stomach wall of mice (Jiang *et al.*, 2004). Further, knocking down Sp1 and Sp3 levels to those of normal cells reduced the potential of fibrosarcoma cells to form tumors in mice (Lou *et al.*, 2005). The loss of an important growth inhibitory pathway of transforming growth factor β (TGFβ)/TGFβR receptor (TGFBR) signalling is critical for oncogenesis in some tumours. Sp3 was reported to act as a repressor of this pathway in breast cancer cells and Sp3 exerts a similar function suppressing p21 in pancreatic cancer cells (Safe and Abdelrahim, 2005).

Considering that (1) Sp1/3 binding sites are distributed widely in chromosomes, (2) Sp1/Sp3 overexpression is seen in transformed cells and (3) reduction of Sp1/Sp3 overexpression prevents transformation (invasion), it is possible that overexpression of Sp1/Sp3 leads to changes in gene expression and chromatin structure. Taken together, Sp1 and Sp3 at least potentially support tumorigenesis. Furthermore, Sp1 is also

implicated in the pathology of many human diseases including Huntington's disease (HD) (Freiman and Tjian, 2002) and Human Burkitts' lymphoma (Hu *et al.*, 2002).

4.4. Structure of Sp1 and Sp3. Sp1 and Sp3 proteins have several sub-domains with respective functions (Kadonaga *et al.*, 1988;Courey and Tjian, 1988;Courey *et al.*, 1989;Pascal and Tjian, 1991;Li *et al.*, 2004) (Fig.7). The *trans*-activation domain consists of two subdomains (A and B), each of which can stimulate transcription when tethered to DNA through a DNA-binding domain. Subdomains A and B are composed of glutamine-rich regions, which are required for *trans*-activation. The serine/threonine-rich subregions located next to the glutamine-rich regions are believed to be involved in post-translational modification (Roos *et al.*, 1997;Yang *et al.*, 2001). Sp1 and Sp3 undergo a number of post-synthetic modifications which add to their complexity (Sapetschnig *et al.*, 2004). Domain C is a region with highly charged amino acids. The Buttonhead element within domain C is important in the synergistic activation by Sp1 or Sp3 with sterol-regulatory element-binding proteins and may also contribute to the factor's transactivation potential (Yieh *et al.*, 1995;Athanikar *et al.*, 1997;Harrison *et al.*, 2000) (Fig. 7). The carboxyl-terminal of domain C has the family marker region, featuring three Cys2His2 zinc "fingers", which are required for sequence-specific DNA binding to GC-rich promoter elements. A carboxyl-terminal domain, termed D, is required for Sp1's synergistic activation along with subdomains A and B (Pascal and Tjian, 1991). Harrison et al. (Harrison *et al.*, 2000) identified the highly conserved Sp box (SPLALLAATCSR/KI) at the N-terminus of the Sp proteins. It contains an endoproteolytic cleavage site that is close to a proteasome-dependent degradation target region of Sp1 *in vitro* (Su *et al.*, 1999). The putative PEST sequence of both Sp1 and Sp3 is located in the highly charged

domain C region. This sequence is a potential target motif for inducible proteolysis (Mortensen *et al.*, 1997). Interestingly, the inhibitory domain of Sp1 is located at the N-terminus, whereas that of Sp3 is immediately in front of the zinc-finger domain. This difference in the positioning of the inhibitory domain in the two proteins is believed to be a major reason for the distinct functions of Sp1 and Sp3 (Dennig *et al.*, 1996;Suske, 1999).

The human Sp1 amino acid sequence was deduced from the cDNA sequence (Takahara *et al.*, 2000). Sp3 was cloned later based on the sequence similarity to Sp1. However, the earlier reported Sp3 had an incomplete N-terminal DNA sequence. For a while, all Sp3 studies were performed with N-terminally truncated versions of Sp3. Oleksiak and Crawford (Oleksiak and Crawford, 2002) determined the amino acid sequence of one long form Sp3 sequence by combining genome walking and database mining to identify the 5' end of the human Sp3 gene. Later on, this long form Sp3 open reading frame was cloned that starts with a classic AUG initiation codon instead of the non-AUG codon reported previously (Kennett *et al.*, 1997;Sapetschnig *et al.*, 2004). This long form Sp3 sequence has 781 amino acids, whereas the previously used truncated forms of Sp3 have 769 (Bouwman and Philipsen, 2002) or 697 amino acids (Suske, 1999). Sp1 and Sp3 genes encode approximately 105- and 115-kDa proteins (long form Sp3), respectively. One interesting feature of the Sp3 protein family is that Sp3 has four isoforms, two long (L1-Sp3, L2-Sp3) and two short isoforms (M1-Sp3,M2-Sp3) (Sapetschnig *et al.*, 2004) (Fig. 7). These isoforms do not arise from alternatively spliced Sp3 RNA but are products of alternative translational initiation sites at postions 1, 37, 856 and 907 (Sapetschnig *et al.*, 2004). So the internal translational initiations of the Sp3

mRNA result in the genesis of the short isoforms (Kennett *et al.*, 1997). The factors regulating the translational initiation of Sp3 mRNA are poorly understood. The structure of long Sp3 isoforms is very similar to that of Sp1, except for the position of the inhibition domain (Suske, 1999). The short and long Sp3 isoforms are expressed in all mammalian cells. The two Sp3 short isoforms do not have the transactivation A domain, but do retain the B domain and the inhibitory domain, which is believed to be responsible for their repression function (Fig. 7). The short Sp3 isoforms are possibly responsible for Sp3-mediated transcriptional repression (Kennett *et al.*, 2002). It has been suggested that increased levels of the short Sp3 isoforms are the reason for the decrease or loss of human reduced folate carrier gene expression in multiple tumor cell lines (Rothem *et al.*, 2003).

4.5. Sp1 and Sp3 nuclear location, foci and nuclear matrix. The structural organization and compartmentalization of the nucleus play an important role in the regulation of gene expression (Zaidi *et al.*, 2005). Many transcription factors are located throughout the nucleus excluding the nucleoli, showing a punctate pattern under high-resolution fluorescence microscopy (Grande *et al.*, 1997). Quite a few of these regulatory factors are associated with the nuclear matrix (Stenoien *et al.*, 2000;Zaidi *et al.*, 2005). Furthermore, visualization studies of nuclear matrix preparations have revealed a punctate distribution of transcription factors (Stenoien *et al.*, 2000;Zaidi *et al.*, 2005), suggesting their targeting to specific sites in the nuclear matrix. Although the function of such a subnuclear compartmentalization of transcription factors is not yet understood, its biological significance has been demonstrated by the occurrence of disease and cancer often linked with mislocalization (Mancini *et al.*, 1999;Choi *et al.*, 2002;Zaidi *et al.*,

2005). Sp1 is found in both non-matrix nuclear fractions and nuclear matrix preparations (van Wijnen *et al.*, 1993), but it was not known whether Sp3 is associated with the nuclear matrix until our lab reported this finding (He *et al.*, 2005).

There are significant differences between Sp1 and Sp3 as illustrated in knockout studies and studies investigating their transcriptional roles (Kruger *et al.*, 2007). Our lab's discoveries add to the list of features that distinguish Sp1 from Sp3. Through application of high resolution fluorescence deconvolution microscopy and concurrent indirect immunolocalization of Sp1 and Sp3, our lab demonstrated that Sp1 and Sp3 are localized in distinct non-overlapping foci in the nucleus (He *et al.*, 2005). The subnuclear foci containing Sp1 or Sp3 were infrequently associated with sites of transcription. Sp1 and Sp3 were present in distinct foci associated with the nuclear matrix. It is possible that the nuclear matrix binding sites, which transiently retain these transcription factors, regulate the level of Sp1 and Sp3 available to associate with Sp-binding sites in chromatin. Alterations in either Sp1 or Sp3 targeting to nuclear matrix sites or changes in the concentrations of these factors could result in aberrant remodeling of chromatin leading to dysfunction of the genome, including genomic instability. We found that throughout the mitotic process; while being displaced from the condensed chromosomes and dispersed through the cell, Sp1 and Sp3 maintained their separate punctuate distributions. In metaphase, Sp1 and Sp3 foci show a high degree of colocalization with actin filaments, suggesting that F-actin is involved in the organization of Sp1 and Sp3 domains during mitosis. In late telophase, Sp1 and Sp3 were equally segregated between daughter cells, and their subnuclear organization as distinct foci is restored. Thus, the maintenance of

Sp1 and Sp3 nuclear levels and their correct functionality are assured after cell division (He and Davie, 2006).

4.6. Mechanisms regulating Sp1 and Sp3 transcriptional activities. Sp1 and Sp3 are ubiquitously expressed in mammalian cells to regulate various genes. Single or multiple Sp1 binding sites have been mapped in promoters and enhancers of genes involved in almost all cellular processes. Sp1 and Sp3 may act as negative or positive regulators of gene expression (Shou *et al.*, 1998;Suske, 1999;Bouwman and Philipsen, 2002;Barth *et al.*, 2002). Multiple mechanisms have been reported to account for the different activities of Sp1 and Sp3, with many of these occurring post synthetically.

4.6.1. Alterations in relative levels of Sp1 and Sp3. It had been believed that Sp1 was mostly involved in activity of TATA-less promoters through interacting with the general transcription machinery. Sp3 often antagonizes Sp1-mediated transactivation through the Sp1/Sp3 ratio, which allows tight control of promoter activity (Suske, 1999). The number of Sp1 molecules in a mammalian cell has been estimated to be in the order of 5000 – 10 000, corresponding to an intranuclear concentration of 1.25 × 10–7 to 2.5 × 10–7 mol/L (Letovsky and Dynan, 1989). Our lab showed that the Sp3 protein level is about one third of Sp1 in MCF-7 breast cancer cells. There are numerous reports showing that the abundance of the Sp1/Sp3 transcription factors and their relative levels regulate expression of genes. There is evidence that the level of Sp1 varies throughout the cell cycle; in the human breast cancer cell line HBL-100, levels of Sp1 are greatest in the G1 phase of the cell cycle (Grinstein *et al.*, 2002). However, our analyses of the human breast cancer cell line MCF-7 found that Sp1 was uniformly present throughout the cell

cycle phases (unpublished results). Further investigations will be required to determine whether cell-cycle-dependent regulation of Sp1 abundance is cell-type specific. In human vascular smooth muscle cells, down regulation of Sp1 levels is thought to be a key event in estrogen-inhibited mechanical strain induced mitogenesis (Ling *et al.*, 2001). The relative levels of Sp1 and Sp3 also appear to regulate the expression of monoamine oxidase (MAO) B during Caco-2 cell differentiation. Sp3 is a repressor of the MAO B promoter. During Caco-2 cell differentiation, Sp3 levels decline and MAO B gene expression increases (Wong *et al.*, 2003). Similarly, interleukin-1β downregulated type-II-collagen gene expression in proliferating rabbit articular chondrocytes by increasing the expression of Sp3 (a repressor of this gene's promoter), while decreasing protein levels of Sp1 (Chadjichristos *et al.*, 2002;Chadjichristos *et al.*, 2003). In cardiomyocytes, insulin-like growth factor 1 (IGF-1) increased the abundance of Sp1 and Sp3 proteins, and the expression of IGF-1-responsive cyclin D3 and Glut1 genes (Li *et al.*, 2003). The IGF-1 response of these genes was abrogated when Sp1 levels were lowered with siRNA for Sp1. The relative levels of Sp1 and Sp3 are key to the regulation human secretin receptor gene expression in pancreas-derived PANC-1 and BPD-1 cells (Pang *et al.*, 2004). A high ratio of Sp1 to Sp3 supported human secretin receptor expression, while a high Sp3 to Sp1 ratio repressed the gene (Pang *et al.*, 2004). This is also true for the regulation of E2F-associated phosphoprotein promoters (Schwarzmayr et al., 2008). In the regulation of the cholesterol ester transfer protein promoter, which has three Sp1-binding sites, transient transfection studies in HepG2 cells have shown that the Sp1 to Sp3 ratio could be a key factor in determining the relative contribution of these sites to total promoter activity (Le Goff *et al.*, 2003). There are also reports that the relative

levels of Sp3 isoforms change with differentiation, with the differentiated Caco-2 cells expressing more long than short isoforms (Gartel *et al.*, 2000). Furthermore, alterations in the relative levels of Sp1 to Sp3 have been reported, with Sp3 levels being greater than Sp1 in primary keratinocytes or higher Sp3 short forms in tumor cell lines (Chadjichristos *et al.*, 2002;Rothem *et al.*, 2004).

4.6.2. Sp1 is capable of synergistic activation; Sp3 is not. For promoters containing multiple Sp-binding sites, it has been shown that Sp1 exerts its transcriptional synergism through direct protein–protein interactions that loop the intervening DNA (Mastrangelo *et al.*, 1991;Su *et al.*, 1991). Synergistic transcriptional activation is mediated through the capacity of Sp1's D domain to form multimers (Pascal and Tjian, 1991). Scanning transmission electron microscopy provided evidence that Sp1 first forms a tetramer and then assembles multiple stacked tetramers at the DNA binding site (Mastrangelo *et al.*, 1991;Heinloth *et al.*, 2003). The interesting feature of this structure is that a Sp1 multimer would present several interacting surfaces to proteins associating with Sp1 (e.g. p300/CBP, HDAC1, TAF_{II} subunits of TFIID, CRSP, E2F1 and ER) (Porter *et al.*, 1997;Doetzlhofer *et al.*, 1999b;Xiao *et al.*, 2000;Li *et al.*, 2004). The net activity of these factors to promote or hinder transcription would depend on the abundance, affinity and residence time of these factors on the Sp1 multimer (Porter *et al.*, 1997). This may be why Sp1 has been described either as a coactivator or a corepressor. However, Sp3 can not form multimers (Yu *et al.*, 2003). An *in vitro* study has shown that Sp3 cannot synergistically activate transcription of promoters containing multiple Sp- binding sites (Yu *et al.*, 2003). Further, this study demonstrated that, on an individual Sp1 binding site, purified Sp1 bound as a multimer, whereas Sp3 bound as a monomer. Several studies

have reported that Sp3 efficiently represses the Sp1-dependent transcription of promoters containing adjacent multiple Sp1-binding sites. For these promoters, Sp3 competes with Sp1, binding to the GC boxes and thereby blocking the synergistic transactivation function of Sp1 (Birnbaum *et al.*, 1995;Dennig *et al.*, 1996;Majello *et al.*, 1997;Yu *et al.*, 2003). However, there are promoters for which Sp3 activates rather than represses. In the regulation of the p21 promoter, which has six Sp1-binding sites, Sp3 appears to be a stronger transactivator of the p21 promoter than Sp1 (Sowa *et al.*, 1999;Gartel *et al.*, 2000). Using small interfering RNAs, one later study found that HDAC4 repress the expression of p21 through interacting with Sp1 at the Sp1/3 binding sites of the p21 proximal promoter (Mottet *et al.*, 2008). Thus, the repressive action of Sp3 is promoter- and cellular-context dependent. The studies exploring the role of Sp1 and Sp3 in the regulation of the p21 promoter are typical of many of the studies investigating the transcriptional role of these transcription factors. However, a criticism of these studies is that the evidence suggesting that Sp1 and Sp3 bind to the promoter's Sp site(s) is based on the *in vitro* electrophoretic mobility shift assay (EMSA), which uses naked DNA and nuclear extracts. This assay may not reflect the *in situ* situation, where Sp1 or Sp3 binds to a promoter in a chromatin context. To obtain the *in situ* information, the ChIP assay must be used (Stoner *et al.*, 2004). Furthermore, a popular approach to distinguish the activity of Sp1 versus Sp3 on a specific promoter is to introduce a promoter–reporter construct and Sp1 or Sp3 expression vectors into *Drosophila* SL2 cells, which do not express either Sp1 or Sp3 (Gartel *et al.*, 2000). Investigators of such studies are rarely concerned with which Sp3 isoform is being expressed. Vectors with the sequence coding for the short Sp3 isoforms (e.g. pPacSp3) will have a different functional consequence

than vectors expressing the full-length Sp3 isoform (e.g. pPacUSp3) (Ge *et al.*, 2001). However, because the synthesis of the short versus long Sp3 isoforms is regulated at the level of translation, it should be noted that the use of an expression vector coding for the long isoform sequence will yield long and short isoforms of Sp3, with the relative levels of the isoforms dictated by the translation machinery of the cell-type employed. Nevertheless, the existence of both activity domain and inhibitory domains suggest that Sp1 and Sp3 long forms can be either activators or repressors. The two N-term truncated Sp3 short forms have only one activity domain and the intact DNA binding zinc-finger domain. Hence they are able to bind DNA and exert functions more likely as transcriptional repressors or weak activators.

4.6.3. Affinity of Sp1 for Sp-binding sites. The consensus Sp1-binding sequence is GGGGCGGGG. Crystal structure and nuclear magnetic resonance (NMR) studies demonstrated that each of the three zinc fingers of the Sp protein DNA binding domain recognizes three bases in one DNA strand and a single base in the complementary strand of the Sp1-binding DNA sequence (Pavletich and Pabo, 1991;Narayan *et al.*, 1997). The last c-terminal finger has lower specificity and can bind only two bases, which helps explain the diversity of Sp1/3 loading on promoter sequences (Oka *et al.*, 2004). Most reported Sp1/3 regulated promoters contain non-consensus Sp1/3 binding sites instead of the consensus DNA sequences. Several variants of the consensus Sp1 recognition site have been analyzed. The central A- or T-substituted variant bound 3-fold and 6-fold less strongly, respectively, than the consensus site. A central G-substitution reduced Sp1 binding by at least 30-fold in a study done with naked DNA (Letovsky and Dynan, 1989). Sp1 will also bind to a Sp site in a nucleosome. Further, a ternary Sp1-nucleosome

complex can still be formed by the binding of Sp1 to nucleosome cores (Li *et al.*, 1994;Utley *et al.*, 1997;Steger and Workman, 1997). However, the affinity of Sp1 for a site in nucleosomal DNA is considerably less than that in naked DNA. The affinity of Sp1 for the constructed nucleosomal SV40 early promoter DNA was approximately 10–20-fold less than for naked DNA. The Sp1 affinity for a nucleosomal-binding site diminishes further as the site is placed closer to the center of the nucleosomal DNA (Li *et al.*, 1994). Sp1- and Sp3-associated DNA-binding activities increased during the maturation of primary human monocytes into macrophages in cell culture (Wu *et al.*, 2003). Sp1-binding activity was also enhanced during adipocyte differentiation (Barth *et al.*, 2002). In differentiated adipocytes, the TNF-β induced inhibition of the adipose most abundant gene transcript-1 (apM-1) gene expression is believed to be due to a decrease of Sp1-binding activity (Barth *et al.*, 2002). Interleukin-1β repressed COL2A1 gene expression by decreasing the binding activity of both Sp1 and Sp3 (Chadjichristos *et al.*, 2003). In the study of *Helicobacter pylori* triggered vascular endothelial growth factor-A (VEGF-A) gene expression, enhanced expression of this gene by this bacterium was mediated by enhanced recruitment of Sp1 and Sp3 to two proximal GC-rich VEGF-A promoter elements (Strowski *et al.*, 2004). Two other studies have shown that the Sp1/3-DNA binding activity and the nature of the Sp1/3-binding site influenced activity of specific promoters (Russell *et al.*, 2003). The DNA-binding activity of Sp1 and Sp3 is also regulated by alterations in protein–protein interactions and by post-translational modification, as discussed below.

4.6.4. Proteins interacting with Sp1 and Sp3. Sp1 and Sp3 associate with many other proteins. Protein-binding sites have been identified throughout Sp1 (Table 1). When

Table 1. Factors physically interacting with Sp1/Sp3.

Factors and domain	Sp1/Sp3 domain	Reference
$hTAF_{II}130$ central four glutamine-rich regions	Sp1 activation domains A and B	(Saluja *et al.*, 1998)Saluja D., 1998
$dTAF_{II}110$	Sp1/Sp3 activation domains A and B	(Gill *et al.*, 1994)
TAFII55 glutamine-rich regions	Sp1 DNA binding domain	(Chiang and Roeder, 1995)
TBP C-terminal	Sp1/Sp3 activation domains A and B	(Torigoe *et al.*, 2003)
IE2	Sp1 C-terminal	(Kim *et al.*, 2000c)
P53	Sp1	(Englund *et al.*, 2001)
E2F N-termimal	Sp1 C-terminal	(Karlseder *et al.*, 1996) 1996
KLF6	Sp1 C-terminal	(Botella *et al.*, 2002)
YY1 C-terminal 83 aa	Sp1 C-terminal	(Lee *et al.*, 1993)
AR DNA-binding domain	Sp1	(Lu *et al.*, 2000;Curtin *et al.*, 2001)
NF-kappaB	Sp1 DNA-binding domain	(Chapman and Perkins, 2000)
ER F domain	Sp1 DNA-binding domain	(Kim *et al.*, 2003a)
P300 acetyltransferase region	Sp1 DNA binding domain	(Suzuki *et al.*, 2000b;Jang and Steinert, 2002)
Smad	Sp1	(Datta *et al.*, 2000;Naso *et al.*, 2003)
TAF1	Sp1 DNA-binding domain	(Suzuki *et al.*, 2003)
HDAC1	Sp1 C-terminal	(Doetzlhofer *et al.*, 1999a)
Stat 3	Sp1	(Loeffler *et al.*, 2005;Lin *et al.*, 2006)
HDAC4	Sp1	(Mottet *et al.*, 2008)
cyclin A	Sp1	(Fojas *et al.*, 2001)
PP2A	Sp1	(Lacroix *et al.*, 2002)
proteasome subunit rpt6	Sp1	(Wang *et al.*, 2008)6642

organized as a multimer, Sp1 presents multiple docking sites for interacting proteins. Current evidence suggests that Sp3 does not form multimers, and hence does not establish the same higher-order protein complexes that Sp1 is capable of forming (Yu *et al.*, 2003).

Both Sp1 and Sp3 have a D domain, which may allow Sp1 to form higher-order complexes with Sp3. Our low-stringency immunoprecipitation experiments with cellular extracts from breast cancer MCF-7 cells showed that Sp1 does not form complexes with Sp3 (Sun *et al.*, 2002b). Co-immunoprecipitation and gel mobility shift assays have shown that Sp1 does not form heteromers with Sp3 in mouse L cells (Yu *et al.*, 2003). However, several studies have shown that they do appear in the same complex in other cell types (Choi *et al.*, 2002;Raab *et al.*, 2002;Chamboredon *et al.*, 2003;Chadjichristos *et al.*, 2003;Zhang *et al.*, 2003b;Canaff *et al.*, 2008). This difference may be caused by different experimental methods (that is, nuclear extraction buffers with differing nonionic detergent concentrations). The other possibility is that the Sp1/Sp3 complex may be cell specific or cellular-stage-dependent.

Sp1 and Sp3 interact directly or indirectly with transcription factors, transcriptional regulators and chromatin remodeling factors (e.g. ERα, HDAC1, p300/CBP, SWI/SNF, an ATP-dependent chromatin remodeling complex) to regulate gene expression (Li *et al.*, 2004) (Table 1). Sp1 interacts with transcription-associated proteins to activate or repress gene expression. Sp1 and Sp3 may act as repressors by recruiting the Sin3A HDAC1/HDAC2 complex by binding to RbAp48 and/or Sin3A (Zhang and Dufau, 2003;Clem and Clark, 2006). Alternatively, Sp1 and Sp3 may act as a transcriptional activator by recruiting p300 or CBP, which are coactivators with potent

KAT activity (Ammanamanchi *et al.*, 2003). The recruitment of chromatin remodeling complexes by Sp1, such as SWI/SNF family proteins, remodels chromatin so that it is more accessible to other factors (Chen *et al.*, 1994;Lu *et al.*, 2003). In regards to regulating estrogen responsive genes, co-immunoprecipitation and indirect immunofluorescence studies demonstrated that Sp1 and Sp3 associate with HDAC1 and HDAC2 and with ERα, albeit at low frequencies in MCF-7 cells (He *et al.*, 2005). Estrogen can induce the expression of estrogen responsive genes by forming complexes between the ERα and Sp1 (Kim *et al.*, 2003b). ERα and Sp1 recruit coactivators that interact with the transcription initiation complex to start transcription (Sriraman *et al.*, 2003;Kim *et al.*, 2003b;Stoner *et al.*, 2004). Each of these two factors (ERα and Sp1) may enhance the binding of the other. For example, ERα increased the affinity of Sp1 for the enhancer of the SK3 gene in L6 cells (Jacobson *et al.*, 2003).

Transcriptional repression can be achieved by perturbing communication between Sp1/Sp3 and the basal transcription initiator complex (Zhang and Dufau, 2003;Tan *et al.*, 2003). In the regulation of human luteinizing hormone receptor gene transcription, orphan receptors EAR3/COUP-TFI repressed promoter activity by binding to Sp1 and perturbing the interaction of Sp1 and TFIIB. Sp1 may also act as a repressor by recruiting HDAC1 or HDAC4, a corepressor (Sun *et al.*, 2002b;Mottet *et al.*, 2008). HDAC1 and HDAC2 were shown to potently repress the luteinizing hormone receptor gene transcription; mSin3A, a component of the Sin3 HDAC complex, potentiated the inhibition mediated by HDAC1 (Zhang and Dufau, 2002;Zhang and Dufau, 2003). Chang *et al* (Chang *et al.*, 2001) provided evidence that Sp1 recruits HDAC1 to the dihydrofolate reductase promoter and that HDAC1 may bridge an interaction between

Sp1 and hypophosphorylated pRb, resulting in the repression of the dihydrofolate reductase gene during withdrawal of cells from the cell cycle. HDAC inhibitors (e.g. sodium butyrate) have been shown to enhance the expression of several genes by acting through the Sp1/3 site (sometimes referred to as the butyrate-response element), with the HDAC inhibitor inhibiting the HDAC activity recruited by Sp1 and Sp3 (Davie, 2003). In addition, proteins binding to Sp1 can block its transactivation activity. c-Myc binds to the C-terminal zinc finger domain of Sp1 and, independent of HDAC, represses the activity of the p21 promoter (Gartel *et al.*, 2001;Gartel and Shchors, 2003). Furthermore, displacement of Sp1/Sp3 binding to promoters is another mechanism by which the activities of these factors are regulated. For example, induction of platelet-derived growth-factor-A-chain expression is correlated with the displacement of Sp1 and Sp3 by Egr-1 (Silverman *et al.*, 1997).

It is conceivable that Sp1 multimers simultaneously recruit histone lysine acetyltransferase and HDAC activities to establish dynamic histone lysine acetylation of promoter-bound histones (Spencer and Davie, 1999;Davie et al., 2008). The net effect in promoting or hindering transcription depends on the abundance, affinity, and residence time of these factors on the Sp1 higher-order complex. Taken together, Sp1 and Sp3 can dynamically recruit and form complexes with many other factors, which can cause region-specific changes in histone lysine acetylation and pol II recruitment within the gene promoters and activate or repress gene expression.

4.6.5. Sp1 and Sp3 modifications. Sp1 is modified by phosphorylation, glycosylation and sumoylation (Jackson and Tjian, 1988;Jackson *et al.*, 1990;Verger *et al.*, 2003).

Acetylation of Sp1 has also been reported. Sp3 modifications include phosphorylation, sumoylation, and acetylation.

4.6.5.1. Phosphorylation. Phosphorylation of Sp1 occurs under a variety of circumstances with a variety of kinases at different sites throughout the Sp1 protein (Samson and Wong, 2002). It has been shown that altered levels of phosphorylation of Sp1 and Sp3 result in changed DNA-binding activity (Ye and Liu, 2002;Lacroix *et al.*, 2002;Arinze and Kawai, 2003;Chu *et al.*, 2003). The MAPK pathway, but not the protein kinase A (PKA) pathway, is involved in the phosphorylation of both Sp1 and Sp3 (Merchant *et al.*, 1999;Tang *et al.*, 2002;Ko *et al.*, 2003). p42/p44 MAPK directly phosphorylates Sp1 on threonines 453 and 739, both *in vitro* and *in vivo* (Milanini-Mongiat *et al.*, 2002). In the nuclear extracts of Ha-*Ras*-transformed fibroblasts cells, tyrosine phosphorylation of the long Sp3 isoform decreased, whereas phosphorylation of the two short isoforms of Sp3 increased. They were also phosphorylated by p42/p44 MAPK (Bakovic *et al.*, 2003). Co-immunoprecipitation experiments have shown that cyclin A and Sp1 can interact physically; the zinc-finger region of Sp1 and the amino-terminal domain of cyclin A interact. *In vitro* and *in vivo* phosphorylation studies indicate that cyclin A–CDK complexes phosphorylate Sp1. The phosphorylation site is located in the N-terminal region of Sp1. Cyclin A–CDK-mediated phosphorylation appears to augment Sp1 DNA-binding activities (Fojas *et al.*, 2001;Haidweger *et al.*, 2001). Protein kinase CK2 has also been implicated in the phosphorylation of Sp1 at the C terminus. This phosphorylation event reduced DNA-binding and transactivation activity of Sp1 (Zhang and Kim, 1997;Armstrong *et al.*, 1997). Protein phosphorylation/dephosphorylation is typically dynamic. Several Sp1 and Sp3

phosphatases have been reported. Protein phosphatase 1 dephosphorylated Sp1 and Sp3 in lung epithelial cells (Chu *et al.*, 2003). In primary human T lymphocytes, protein serine/threonine phosphatase 2A (PP2A) dephosphorylated Sp1 *in vitro* and strongly interacted with Sp1 *in vivo* (Lacroix *et al.*, 2002). Cieslik *et al.* (Cieslik *et al.*, 1999) demonstrated that the endothelial nitric-oxide synthase gene could be regulated by Sp1 phosphorylation and dephosphorylation, through a dynamic interaction between protein kinase CK2 and protein serine/threonine phosphatase 2A. Sp1 of resting T lymphocytes was phosphorylated and, following stimulation of the cells to enter the cell cycle, Sp1 was dephosphorylated. No changes in Sp1 glycosylation were observed. Similarly, serum-stimulated cycling NIH-3T3 cells had lower levels of phosphorylated Sp1, which migrates more slowly on a SDS–PAGE than unmodified protein, than G0-phase cells (Lacroix *et al.*, 2002). There is evidence that phosphorylation of Sp1 in the G1 phase Balb/c 3T3 is greater than that of G0-phase cells (Black *et al.*, 1999). In contrast to most cell-cycle phases, Sp1 of mitotic cells was phosphorylated, and mitotically phosphorylated Sp1 binds DNA poorly (Martinez-Balbas *et al.*, 1995;Dovat *et al.*, 2002;Lacroix *et al.*, 2002). Taken together, phosphorylation of Sp1 and Sp3 has been one way to up- or down regulate gene transcription (Ge *et al.*, 2003;Lam *et al.*, 2003;Ko *et al.*, 2003).

4.6.5.2. Glycosylation. *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) is a ubiquitous and abundant post-translational modification found on nuclear and cytoplasmic proteins, and is thought to be a dynamically regulated modification much like phosphorylation. Nucleocytoplasmic enzymes, UDP-G1cNAc: polypeptide *O*-β-*N*-acetylglucosaminyltransferase (OGT) – *O*-β-*N*-acetylglucosaminidase, catalyze this

highly dynamic glycosylation/ deglycosylation of proteins (Kamemura and Hart, 2003). *O*-GlcNAcylation of Sp1 at multiple sites occurs by covalent linkage of the monosaccharide *O*-GlcNAc to serine and threonine residues (Jackson and Tjian, 1988;Han *et al.*, 1997;Brasse-Lagnel *et al.*, 2003). Modification of Sp1 by *O*-GlcNAc may stimulate or repress transcription (Du *et al.*, 2000;Kang *et al.*, 2003). As a mechanism to reduce the transactivation activity of Sp1, *O*-GlcNAcylation of Sp1 prevents its interaction with TATA binding- protein-associated factor (TAF110) (Roos *et al.*, 1997;Yang *et al.*, 2001). *O*-GlcNAcylation of Sp1 may also interfere with the access of Sp1 to Sp1 modifying enzymes (e.g. kinases), affecting other Sp1 modifications. Inhibition of HeLa peptide *O*-GlcNAc-β-*N*-acetylglucosaminidase, the enzyme that removes *O*-GlcNAc from proteins, by *O*-(2- acetamido-2-deoxy-D-glucopyranosylidene) amino-*N*-phenylcarbamate, results in an increase in Sp1 *O*-GlcNAc levels, which is accompanied by a decrease in Sp1 phosphorylation (Haltiwanger *et al.*, 1998). *O*-GlcNAcylation of Sp1 may play a role as a nutritional checkpoint. When glucose levels are elevated, *O*-GlcNAcylation of Sp1 is increased. In contrast, under glucose starvation conditions, decreased *O*-glycosylation of Sp1 is accompanied by increased proteolysis by the proteasome. The reduced levels of Sp1 have an impact on the expression of several genes (Han *et al.*, 1997;Su *et al.*, 1999;Walgren *et al.*, 2003).

Several studies have demonstrated that both increased *O*-glycosylation and phosphorylation of Sp1 lead to its translocation into the nucleus. Inhibition of *O*-glycosylation did result in a slight increase in cytoplasmic Sp1 (Black *et al.*, 1999;Brasse-Lagnel *et al.*, 2003;Majumdar *et al.*, 2003). However, immunochemical staining revealed that, regardless of whether hepatoma cells were treated with insulin, glucagon, or

deoxynorleucine (an inhibitor of *O*glycosylation), the majority of Sp1 was located in the nucleus. One study demonstrated that Sp1 is associated with the nuclear matrix (van Wijnen *et al.*, 1993). Interestingly, in our studies with breast-cancer cells, we found that Sp1 and Sp3 are nuclear proteins, although they may or may not associate with the nuclear matrix. There is a subpopulation of nuclear Sp1 that can be readily extracted with nonionic detergents (e.g., 0.25% Nonidet-P40) and the remainder is harder to extract (He *et al.*, 2005). Thus, lysis of cells with nonionic detergents will result in the extraction of nuclear Sp1 into the cytoplasmic fraction. Little is known about the glycosylation of Sp3. One study demonstrated that methylglyoxal modification of mSin3A would increase modification of Sp3 by O-linked N-acetylglucosamine. This modification decreases binding affinity of the repressor complex (mSin3A-Sp3) to the angiopoietin-2 promoter containing a glucose-responsive GC box, which further results in increased Ang-2 expression (Yao *et al.*, 2007).

4.6.5.3. Sumoylation and acetylation. It is well-known that conjugating ubiquitins to lysine residues is a mark of proteins degradated by the 26S proteasome. Ubiquitination is involved in gene activation and many other cellular functions (Weissman, 2001;Salghetti *et al.*, 2001). Small ubiquitin-related modifiers (SUMOs) are proteins structurally related to ubiquitin and also with similar size (their molecular masses are 11 and 9 kDa, respectively). However, SUMOs usually don't serve as a signal for protein degradation. Sumoylation is more likely implicated in diverse regulation functions as subcellular compartmentalization, protein stability, chromatin structure regulation, transcription factor activity (Verger *et al.*, 2003;Gill, 2003). Sumo is covalently ligated to proteins

through lysine residues generally within the tetrapeptide consensus motif, ψ-Lys-X-Glu (ψ is a large hydrophobic amino acid, mostly isoleucine or valine, and X is any residue). Three mammalian SUMOs have been reported as SUMO-1/Smt3C, SUMO-2/Smt3A and SUMO-3/Smt3B. These SUMOs exert distinct functional consequences (Saitoh and Hinchey, 2000;Muller *et al.*, 2001). Sumo-1 shares 18% sequence identity to ubiquitin but does not serve as a signal for protein degradation (Seeler and Dejean, 2003). Sumoylation plays important roles in many biological processes (Verger *et al.*, 2003). Sumoylation of transcription factors has typically negative effects on their activity, although the exact mechanisms are not yet clear (Verger *et al.*, 2003). Both endogenous Sp1 and Sp3 can be sumoylated by SUMO-1 (Ross *et al.*, 2002;Sapetschnig *et al.*, 2002;Spengler and Brattain, 2006;Wang *et al.*, 2008).

The sumoylation of Sp1 was found much later than other modifications. Western blot analysis revealed that the molecular mass of sumoylated Sp1 is about 125 kDa. The SUMO-1 modification of Sp1 was mapped at residue lysine 16 located in the N-terminal negative regulatory domain of Sp1 (Spengler and Brattain, 2006). The same residue regulates the N-terminal cleavage of Sp1 *in vivo*, which can relieve the constitutively repressed Sp1 activity caused by sumoylation of the same Lysine-16 residue. Through studying the properties of constructs as sumoylation-deficient Sp1, Sp1(E18A), and a constitutively SUMO-1-modified Sp1, further combining GAL4-Sp1 fusion assay, Spengler *et. al.* (2006) demonstrated that sumoylated Sp1 was deficient in proteolytic processing and repressed Sp1 transcriptional activity. They proposed that sumoylation preserves the integrity of a negative regulatory domain so that Sp1-dependent transcription would be inhibited, which suggests that posttranslational competition at

lysine 16 controls Sp-dependent transcription (Spengler and Brattain, 2006). However, another study demonstrated that sumoylation of Sp1 can also facilitate Sp1 degradation through altering its subcellular location and further interacting with proteasome subunit rpt6, which leads to the final degradation of Sp1 (Wang *et al.*, 2008). Furthermore, one study exploring the mechanism of Sp1 processing demonstrated that cyclinA/cdk2 phosphorylation of Serine-59 can induce Sp1 proteolytic processing, which further results in a desumoylated, derepressed and unstable Sp1 product (Spengler *et al.*, 2008).

In vitro transcription studies with a reporter construct with two adjacent Sp-binding sites demonstrated that Sp1 and Sp3, expressed and isolated from insect cells, are strong activators (Braun *et al.*, 2001). However, in transient transfection studies in human or *Drosophila* Schneider SL2 cells, Sp3 is a weak activator or a repressor. Post-synthetic modification, which is one way to regulate the transcriptional activity of Sp1 and Sp3, could be one explanation of this phenomenon. Within the inhibitory domain of Sp3 is the sequence IKEE, which is crucial to the inhibitory activity of this domain and is also a site of post-synthetic modifications. Sumoylation of Sp3 by the protein inhibitor of activated STAT1 (PIAS1), an E3 ligase, occurs at a single lysine (aa551) located in its inhibitory domain (Sapetschnig *et al.*, 2002) (Fig. 7). Another minor sumoylation site is K120 which only exists in the N-terminal of two long forms of Sp3. All Sp3 forms can be sumoylated at K551 (Ross *et al.*, 2002). This modification silenced or significantly decreased Sp3 activity (Ross *et al.*, 2002;Sapetschnig *et al.*, 2002;Hasleton *et al.*, 2003). Further evidence that sumoylation has a role in Sp3 inhibitory domain function is the demonstration that the coexpression of a SUMO-1 protease, which removed SUMO from Sp3, stimulated the transcription activity of Sp3 in transient transfection studies in murine

cells (Ross *et al.*, 2002). In accord, Ellis *et al.* found that blocking sumoylation by mutation converted the short Sp3 isoforms into potent transactivators of the SRC1A gene promoter, although sumoylation had little effects on the transcriptional properties of the Sp3 long forms (Ellis *et al.*, 2006). This result further supports the model that Sp3 mediated transcription is highly dependent on the isoform bound, SUMOylation status and the promoter context (Ellis *et al.*, 2006). In P19 cells, sumoylated repressive forms of Sp3 localize to the nuclear periphery and nuclear dots, which colocalize with promyelocytic leukemia (PML) bodies, whereas the nonsumoylated Sp3, a mutant in which the sumoylation site (K539, K551 for the complete sequence) is mutated, had a diffuse nuclear location (Ross *et al.*, 2002). In contrast, the diffuse nuclear distribution of Sp3 was not altered when SUMO-1 was overexpressed in Ishikawa cells, suggesting that the nuclear location of Sp3 is not altered when it is sumoylated (Sapetschnig *et al.*, 2002). The reasons for these discordant results are unclear, although cell-type or anti-Sp3-antibody differences may be involved. One study with persuasive evidence indicated that sumo-modified Sp3 repressed transcription through provoking the establishment of compacted repressive chromatin. Using ChIP-qPCR, this study was able to demonstrate that one series of chromatin remodellers (e.g. Mi-2, MBT-domain proteins) were recruited to both Gal4 transgene and mouse Dhfr (mDhfr) gene promoters in a sumoylation dependent manner, which leads to the establishment of repressive chromatin possessing characteristics of heterochromatin. This event was also accompanied with establishment of histone modifications associated with repressed genes (e.g. H3K9 and H4K20 trimethylation). All these chromatin changes render DNA inaccessible to the

transcription machinery (Stielow *et al.*, 2008a;Stielow *et al.*, 2008b). Up to now, this is the first study connecting Sp3 sumoylation directly to chromatin packing status.

Interestingly, the sumoylated lysine of Sp3 is also subject to acetylation. Sp3 is acetylated by the KAT p300 at K539 (Braun *et al.*, 2001;Ammanamanchi *et al.*, 2003). Mutation of this lysine (K539) (K551 for the complete sequence) converted the activity of Sp3 from a weak activator to a stronger one in transient transfection studies in *Drosophila* Schneider SL2 cells. Clearly, this lysine is critical to the repressive activity of Sp3. It was first thought that acetylation of this site may have a role in the repressive activity of Sp3. However, since finding that this site is also sumoylated, the role of acetylation in the repressor activity of Sp3 is now unclear. Unfortunately, Braun *et al.* (Braun *et al.*, 2001) did not test a mutant Sp3 in which K539 was changed to glutamine; such a change may mimic the acetylated state. However, mutation of the IKEE to IKEA or IKED rendered Sp3 a strong activator (Sapetschnig *et al.*, 2002). This mutation prevents the sumoylation of Sp3 but retains the acetylation site, suggesting that sumoylation rather than acetylation has a role in silencing the activity of Sp3. However, it has not been determined whether these mutants can be acetylated. Treatment of MCF-7L breast-cancer cells with the HDAC inhibitor trichostatin A (TSA) resulted in the activation of the transforming growth-factor-β receptor type II (RII) gene. This study demonstrated that Sp3 is a key factor in the regulation of the RII promoter and that TSA inhibits the HDAC activity recruited by Sp3. However, it is not clear whether it is the absence of HDAC activity or the acetylation of Sp3 that is responsible for the TSA-mediated stimulation of the RII promoter. It is possible that the role of Sp3 acetylation is to prevent sumoylation of the inhibitory domain, and hence to prevent the silencing

function of sumoylation. Nonetheless, one study showed that acetylated Sp3 is a transcriptional activator (Ammanamanchi *et al.*, 2003). In addition to interacting with Sp3, p300 also binds to Sp1; the physical and functional interactions occur between the acetyltransferase region of p300 and the DNA-binding domain of Sp1 (Suzuki *et al.*, 2000c;Song *et al.*, 2003;Ryu *et al.*, 2003). Once bound to DNA, Sp1 cannot bind to p300 or be acetylated (Suzuki *et al.*, 2000c). Treatment of mammalian cells with HDAC inhibitors or with the induction of oxidative stress resulted in the acetylation of Sp1 *in situ* (Ryu *et al.*, 2003;Dempsey *et al.*, 2003;Ferrante *et al.*, 2003). *In vitro* studies suggest that acetylation enhances Sp1 DNA-binding activity. Further, transient transfection studies indicate that acetylation, which increased with HDAC inhibitors, enhanced the activation activity of Sp1. However, the HDAC inhibitors may have also inhibited the HDACs recruited to the reporter construct by Sp1; thus the role of Sp1 acetylation remains to be determined.

4.6.5.4. Methylation of CpG sequence. DNA methylation plays a major role in the regulation of gene expression in mammalian wild-type and cancer cells, often resulting in transcriptional silencing. GC/GT boxes are commonly found in promoters, embedded in CpG-rich methylation-free islands. GC/GT motifs, Sp1/3 binding sites, are required to maintain the methylation-free active status of the adenine phosphoribosyltransferase gene (Macleod *et al.*, 1994;Brandeis *et al.*, 1994). The EMSA studies showed that methylation within the consensus Sp1/3 binding site did not reduce Sp1/Sp3 binding, whereas methylation outside induced a significant decrease in Sp1/Sp3 binding and further regulated gene expression (Zhu *et al.*, 2003).

4.7. Functional roles of Sp1 and Sp3. Although Sp1 and Sp3 recognize and bind to the same DNA sites, they have different functional roles. Unlike Sp1, which is usually an activator of transcription, Sp3 functions as an activator or a repressor, with its function being dependent upon several parameters, including promoter context and the cellular background (Majello *et al.*, 1997;Sowa *et al.*, 1999;Gartel *et al.*, 2000;Yu *et al.*, 2003), state of modification and chromatin structure (Suske, 1999;Bouwman and Philipsen, 2002;Li *et al.*, 2004;Sapetschnig *et al.*, 2004). Sp3 does not have the ability of Sp1 to bind to the DNA as multimers or to activate transcription in a synergistic manner by binding to multiple sites (Yu *et al.*, 2003), but it can antagonize Sp1, blocking its synergistic transactivation function (Yu *et al.*, 2003). Several studies suggested that Sp1 is responsible for basal transcription and Sp3 appeared in the induced active complex (Ward and Samuel, 2003;Ye *et al.*, 2005;Jaiswal *et al.*, 2006;Zeissig *et al.*, 2007). In a study of the protein kinase regulated by RNA (PKR) promoter, which binds Sp1 and Sp3, Sp3 but not Sp1 was a component of an inducible complex (Ward and Samuel, 2003). ChIP assays revealed that the binding of Sp3 at the PKR promoter *in vivo* was interferon dependent, whereas the binding of Sp1 was constitutive. A 15-bp element kinase conserved sequence in the PKR promoter is required for both basal and interferon-inducible transcription. Interferon treatment results in two new complexes: iKIBP1 and iKIBP2, which contain STAT1, STAT2, IRF-9, and Sp3, but not Sp1. This study suggested different roles for Sp1 and Sp3, with Sp1 being involved in basal transcription and Sp3 having a role in the inducible transcription of the PKR gene (Ward and Samuel, 2003). Higgins *et al.* found that in E2-responsive ZR-75 cells that Sp3 and Sp1 was bound to the E2-responsive VEGF receptor-2 (VEGFR2) promoter before and after the

cells were treated with E2, and through knock down studies found that ERα/Sp3 played a critical role, while ERα/Sp1 had a minimal role in transactivation of the VEGFR2 promoter (Higgins *et al.*, 2006d). However, one study for estrogen to induce the expression of the human prolactin receptor gene found that the loading of Sp1 and Sp3 to the gene promoter did not change in the presence of estrogen (Dong *et al.*, 2006). Most current publications demonstrated the involvement of both Sp1 and Sp3 in the regulation of gene transcription but didn't further elucidate their differential functional roles regarding gene regulation. There is not sufficient evidence to functionally discriminate Sp1 and Sp3.

In summary, Sp1 and Sp3 act through promoter GC boxes to up or down regulate numerous genes (Suske, 1999). In mammalian cells, Sp1 mostly acts as an activator, while Sp3 serves as a repressor or an activator (Philipsen and Suske, 1999). The activity of Sp1 and Sp3 at any specific promoter is governed by several variables, including the relative levels of Sp1 and Sp3, the level and modification state of interacting proteins with which Sp1 or Sp3 forms a complex, and the binding activities and post-translational modifications. Furthermore, the methylation of Sp1 binding GC boxes did not influence the binding of Sp1 to DNA (Harrington *et al.*, 1988;Zhu *et al.*, 2003). However, the methylation around GC boxes can be one way to regulate Sp1 and Sp3 mediated gene expression (Zhu *et al.*, 2003;Douet *et al.*, 2007). Sp1/3 binding sites were characterized in the promoters of both Sp1 and Sp3 (Nicolas *et al.*, 2001;Tapias *et al.*, 2004). Nevertheless, up to now, not much work has been done on the transcriptional regulation of Sp1 and Sp3 (Nicolas *et al.*, 2003). As described above (section 3.6.2), a functionally significant distinction between Sp1 and Sp3 is that Sp3 lacks the capacity of Sp1 to form

multimers (Yu *et al.*, 2003). The Sp1-multimer configuration allows Sp1 to synergistically activate transcription with promoters that have two or more adjacent Sp-binding sites, and to juxtapose Sp sites that are separated by distances of 2 kb or greater, resulting in the looping out of the intervening DNA. Further, the Sp1 multimer offers multiple binding sites for interacting proteins. The inability of Sp3 to form multimers suggests that there is a dynamic interplay between Sp1 multimers and Sp3 long and short isoforms for binding to Sp1/3 binding sites. The lack of an activation domain in both of the short Sp3 isoforms indicates that, at best, the promoter will recruits a weak activator. However, there is mounting evidence that transcription factor binding to any given promoter is cyclical, with the promoter loading and releasing the transcription factor (Reid *et al.*, 2003;Metivier *et al.*, 2003a;Metivier *et al.*, 2006). Thus, the promoter will have the opportunity to recruit the stronger activator and initiate a productive round of transcription. Factors that dictate the loading of Sp1 or Sp3 isoforms will be determined by their availability, promoter context (that is, other factor binding sites), and nuclear location of the gene relative to nuclear sites harboring Sp1 and Sp3. Current evidence suggests that certain modifications of Sp3, such as sumoylation, will determine nuclear location, positioning Sp3 at or near PML bodies. Promoters with Sp-binding sites positioned next to PML bodies may be more susceptible to recruit sumoylated Sp3, which will prevent full activation or repress promoter activity. Considering the impact of modifications of Sp1 and Sp3 on their transactivation activities, it is critical that Sp1 and Sp3 modification-specific antibodies be developed so that the loading of these factors onto promoters can be evaluated using ChIP. This section about Sp1 and Sp3 has highlighted the diverse roles of Sp1 and Sp3 in regulating gene expression. However, the

function of these factors is more complex. Sp1 has been shown to have boundary activity, which blocks the spread of heterochromatin (Ishii and Laemmli, 2003). Sp1 is bound to the locus control region of the human β-globin locus (Pasceri *et al.*, 1998). Position effect variegation is observed when a crippled β-globin locus control region is placed in a condensed chromatin region; however, Sp1 reduces the position effect variegation (McMorrow *et al.*, 2000). It will be interesting to determine whether Sp3 also has boundary activity and whether modifications of Sp1 or Sp3 influence the functions of these proteins in chromatin architecture.

4.8. Breast cancer cell lines used for estrogen responsive gene studies. For research purposes, there are many established breast cancer cell lines with different genetic backgrounds. Most cell lines were obtained from pleural effusions from breast cancer patients. MCF-7 cell line express ER and are the most popular cell model of estrogen responsive breast cancer cells. These cells were used in many studies for the estrogen responsive model gene TFF1 (Shang *et al.*, 2000;Burakov *et al.*, 2002;Reid *et al.*, 2003). However, cell lines from different laboratories may possess distinct karyotypes and biological properties, and it is not rare to see conflicting research results from the claimed MCF-7 cell lines used in different laboratories (Osborne *et al.*, 1987). The MCF-7 (T5) cell line used in this project was cloned from MCF-7 cells. ZR-75 ER positive breast cancer cells are another popular cell line. The MDA- MB-231 cell line was also obtained from a pleural effusion. These cells are estrogen receptor negative and highly invasive in *in vitro* assays. These cells were mostly used as a negative control cell line in TFF1 gene studies since the TFF1 gene is repressed in this cell line. TFF1 has been a popular model gene to study the regulatory mechanism of estrogen induced gene expression, as its gene

structure is well studied and it contains all the elements required for a classical estrogen responsive gene (Jeltsch *et al.*, 1987;Berry *et al.*, 1989;Nunez *et al.*, 1989b). *C-myc* is also a well established estrogen responsive gene without identified ERE sites but only Sp1 sites in its estrogen responsive promoter 2 region (Dubik and Shiu, 1988;Nunez *et al.*, 1989a;Dubik and Shiu, 1992). In addition to TFF1 and *c-myc*, a number of other estrogen responsive genes were examined in this study in breast cancer cell lines to assess the involvement of Sp1 and Sp3 in their gene regulation.

5. TFF1 gene

5.1 TFF1 gene organization. The human TFF1 gene was previous named as pS2. TFF1 gene is located at the q22.3 locus of human chromosome 21 (Hattori et al., 2000). The gene consists of three exons. A schematic diagram of the TFF1 gene organization is shown in figure 8. Two nucleosomes , nucleosome E (NucE) and nucleosome T (NucT), were mapped in between -440 and +5 of the human TFF1 promoter regions (Fig 8, TFF1 gene organization). These two nucleosomes cover the ERE and TATAA box with ERE (-405) at the edge of NucE and TATAA box (-35) at the edge of Nuc T (Sewack and Hansen, 1997). TFF1's NucE remained in place in MCF-7 cells cultured in the absence and presence of E2. Its position is preferred rather than a fixed one (Metivier *et al.*, 2003a). In MCF-7 cell lines, two strong DNase I hypersensitive sites were induced by estrogen upstream of the TFF1 gene. The site in the proximal promoter region spans up to the transcription start site including ERE. The other one is in a distal upstream region, -10.5 Kb from the CAP site, that also correlates with TFF1 expression (Giamarchi *et al.*, 1999). Later studies indicated that this distal site is recognized and associated with ER

and acts as an enhancer to regulate gene transcription (Carroll and Brown, 2006). The ERE of the human TFF1 gene studied in the present project is the one in the proximal promoter region. For this ERE, one half site has the consensus sequence while the other half site deviates from the consensus sequence [5'-GGTCAnnnTGGCC-3' for TFF1 promoter and 5'-GGTCAnnnTGACC-3' for consensus sequence] (Berry et al., 1989). *In vivo* footprinting demonstrated that the perfect half ERE (GGTCA) site of the endogenous TFF1 was protected without estrogen treatment and both half ERE sites were occupied in the presence of estrogen (Kim *et al.*, 2000b). The same study found that the footprinting patterns in and around the TATA and CAAT sequences were identical with and without estrogen treatments. This observation suggests that the basal promoter of TFF1 is accessible and poised for transcription even in the absence of hormone (Kim *et al.*, 2000b).

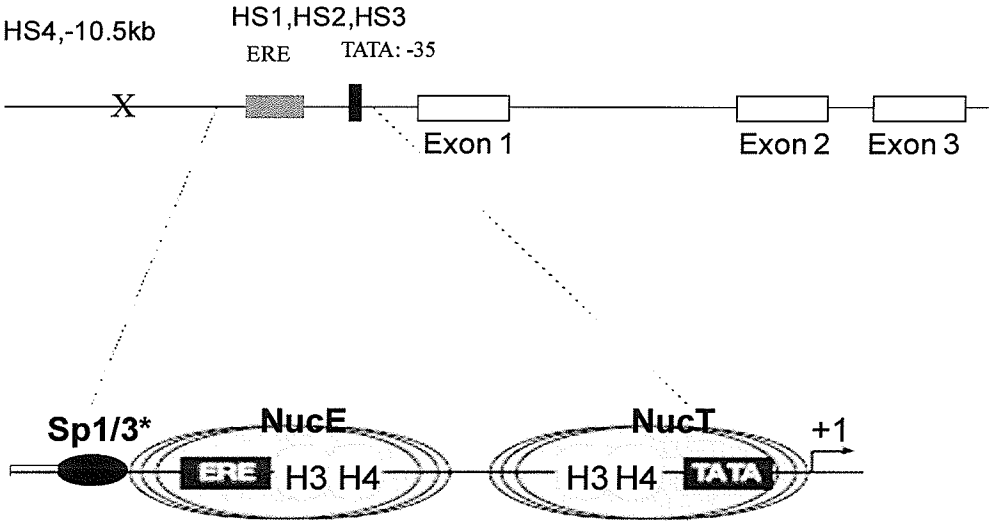


Figure 8. TFF1 gene organization. Exons 1,2, and 3 are represented by open rectangles. A more detailed diagram of the promoter region is shown in the lower portion of the figure. Positions of several regulatory regions in respect to the start site for TFF1 promoter are shown in black rectangles. TATA = TATA boxes, Sp1= Sp1 and Sp3 cognate element, ERE = estrogen responsive element. HS=DNase I hypersensitive sites. * The Sp1/3 was partially identified by this project. Adapted from (Metivier *et al.*, 2006).

5.2. TFF1 gene protein product. The TFF1 gene product is a cysteine-rich secreted protein consisting of 60 amino acids. TFF1 protein is normally located in the mucosa of the gastrointestinal tract, mainly stomachand is also widely expressed at high levels in malignant breast epithelial cells (Prest *et al.*, 2002;Rodrigues *et al.*, 2003). Nonetheless, TFF1 is only expressed in breast cancer cells, but not in normal mammary cells (Rio *et al.*, 1987). TFF1-null mice are devoid of mucus in the gastric antrum and severe hyperplasia and dysplasia is exhibited (Lefebvre *et al.*, 1996). All mTFF1-/- mice developed antropyloric adenoma indicating that mouse TFF1 is essential for normal differentiation of the antral and pyloric gastric mucosa. 30 percent of the mTFF1-/- developed carcinomas, which suggests that mTFF1 may function as a gastric-specific tumor suppressor gene working together with some other factors to inhibit malignancy. Abnormalties were also observed in small intestine and lymphoid cells of the mTFF1-/- mice (Lefebvre *et al.*, 1996). Differential expression analyses of TFF1 null antropyloric tumors suggested that TFF1 is important in protein folding and /or secretion (Torres *et al.*, 2002). *In vitro* wounding and migration assays showed that TFF1 was able to stimulate the migration of breast tumor cells (Prest *et al.*, 2002). Through plating human umbilical vein endothelial cells (HUVEC) on the Matrigel matrix substratum to form tube-like structures and combining with chorioallantoic membrane (CAM) assay, Rodrigues *et. al* demonstrated that TFF1 protein has angiogenic activity and is important in the formation of new blood vessels during normal and pathophysiological processes (Prest *et al.*, 2002;Rodrigues *et al.*, 2003).

5.3. Sp1, Sp3, ERα, histone lysine acetylation and the estrogen responsive TFF1 gene. The TFF1 gene promoter has an imperfect ERE and two AP-1 sites with the upstream site

being imperfect (Metivier *et al.*, 2003b). TFF1 gene is responsive to estrogen and TPA in ERα positive breast cancer cells (Berry *et al.*, 1989;Espino *et al.*, 2006). The two nucleosomes mapped on TFF1 promoter, NucE and NucT, undergo extensive remodeling during transcription (Sewack and Hansen, 1997;Metivier *et al.*, 2003a;Mellor, 2006) (Fig. 7). The ligand bound ERα recruits coactivators with KAT activity to the TFF1 promoter (Chen *et al.*, 1999;Shang *et al.*, 2000). Estrogen responsive genes such as cathepsin D, TFF1 and cyclin D1 associate with coactivators/KATs such as CBP, p300, SRC- 1, following the loading of the estradiol (E2)-bound ER onto an ERE (Chen *et al.*, 1999;Shang *et al.*, 2000;Castro-Rivera *et al.*, 2001a;Burakov *et al.*, 2002;Reid *et al.*, 2003;Metivier *et al.*, 2003a). Recruitment of these KATs results in chromatin remodeling and an increase in the steady state level of acetylated histones in that region. As a low level of KATs (CBP, p300) are associated with the TFF1 promoter in cells grown under estrogen deplete conditions (Chen *et al.*, 1999;Shang *et al.*, 2000), it was reasoned that the promoter would be engaged in dynamic histone lysine acetylation. By inhibiting HDACs with butyrate or TSA, ChIP assays done by our laboratory showed that acetylation of H3 and H4 at the promoter and at exon 2 and 3 was increased (Espino *et al.*, 2006). Similar to the results of Thomson et al. (Thomson *et al.*, 2001) with the uninduced c-fos gene, the TFF1 gene is engaged in dynamic acetylation before induction. Interestingly, we found that the HDAC inhibitors had a differential effect on HDAC1 association with the promoter versus coding region. HDAC inhibitors resulted in the partial dissociation of HDAC1 from the promoter, but at the coding region there was either no change or an increased association. Other investigators have noted that exposure of cells to TSA results in displacement of HDAC1 from a promoter (Mishra *et al.*,

2001;Ghoshal *et al.*, 2002;He and Margolis, 2002). ChIP assays demonstrated that E2 addition to MCF-7 breast cancer cells resulted in the association of Sp1 and ERα with the cathepsin D and cyclin D1 promoters (Castro-Rivera *et al.*, 2001a). One recent microarray and RNA interference study suggest that about 60% of estrogen response genes (induced or repressed) are ERα and Sp protein dependent (Wu *et al.*, 2008a). The TFF1 gene is expressed in estrogen responsive breast cancer cells. As described above, Sp1 or Sp3 may recruit coactivators as p300/CBP or corepressors like HDACs (Xiao *et al.*, 2000). We speculate that Sp1 or Sp3 recruit HDACs and KATs to the TFF1 promoter to catalyze dynamic histone acetylation, placing the TFF1 promoter in a poised state to respond to events initiated upon estrogen administration to the cell.

Despite the different roles of Sp1 and Sp3, they are generally thought to compete for the same binding sites. Some studies support the existence of a Sp1/Sp3 complex (Choi *et al.*, 2002;Zhang *et al.*, 2003b). However, we and others found that Sp1 does not form complexes with Sp3 in human breast cancer MCF-7 or mouse L cells (Sun *et al.*, 2002a;Yu *et al.*, 2003). It is conceivable that although not in a complex together, Sp1 and Sp3 are colocalized in the nucleus. A number of imaging studies of Sp1 or Sp3 have been published, most of them showing a diffuse or punctate localization of Sp1 and Sp3 in the nucleus of various cell types (Birnbaum *et al.*, 1995;Pombo *et al.*, 1998;Ross *et al.*, 2002;Spann *et al.*, 2002;Sapetschnig *et al.*, 2002;Zhang *et al.*, 2003b;Sapetschnig *et al.*, 2004;Spengler *et al.*, 2005). In one of our studies, using fluorescence microscopy followed by deconvolution analysis in conjunction with biochemical partitioning assays, He *et al.* were able to demonstrate that Sp1 and Sp3 are nuclear matrix proteins organized into different domains, which had minor overlap with transcription sites and interacting

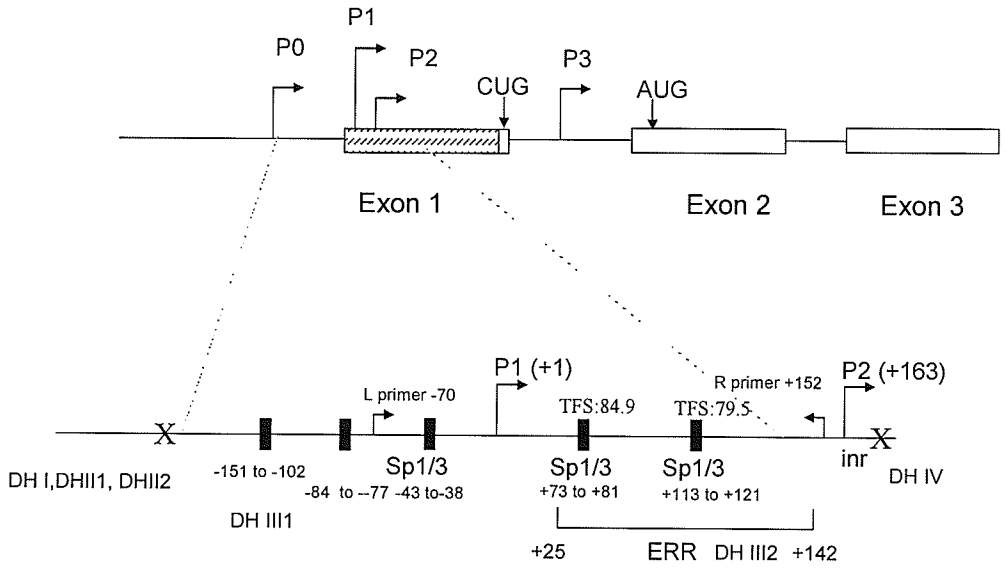
proteins like ERα and HDAC1 and HDAC2 (He *et al.*, 2005). A number of ChIP studies have shown that both Sp1 and Sp3 are associated *in situ* with the GC box(es) of a variety of promoters (Stoner *et al.*, 2004;Chan *et al.*, 2004;Zelko and Folz, 2004;Lee *et al.*, 2004a;Liu *et al.*, 2004b;de Leon *et al.*, 2005;Sun *et al.*, 2005b;Khalil *et al.*, 2008). Nevertheless, whether Sp1 and Sp3 occupy these promoters simultaneously is not known. Sp1 and Sp3 could associate with the Sp1 element separately or as a multi-protein Sp1/Sp3 complex.

Taken together, most studies have established the importance of the cognate DNA element of Sp1/3 in the regulation of estrogen responsive genes by *in vitro* studies *e.g.* EMSA or promoter deletion analysis (Safe, 2001). In this thesis project, we employed *in situ* ChIP and reChIP techniques to study the association of Sp1 and Sp3 with TFF1 promoter in the absence or presence of estrogen in MCF-7 cells. Furthermore, ChIPs were also performed to study the sumoylation and lysine acetylation status of the TFF1 promoter. All these studies help to elucidate the mechanism for estrogen to regulate estrogen responsive genes. The following section is about the background review and my studies of the estrogen responsive gene *c-myc*.

6. *C-myc* Gene

6.1. *C-myc* gene and c-myc gene organization. The human *c-myc* gene has been mapped to the 8q24 locus of the chromosome 8 (Taub *et al.*, 1982). *C-myc* consists of three exons: an untranslated first exon (exon I) and two protein coding exons (exons II and III) (Fig.9). Studies of *c-myc* chromatin have revealed multiple DNase I hypersensitive sites (DHs), some of which overlap with cis-acting *c-myc* regulatory elements (Siebenlist *et al.*, 1984;Spencer and Groudine, 1991;Miller *et al.*, 1993). DH I

locates at -1851 and it is a negative repressor binding site. DH II_1 and DH II_2 locate at -1380 and -751 individually and both have unknown functions. DH III_1 locates at -124 and it is associated with P1 promoter activity. DH III_2 locates at +91 and it is associated with P2 promoter activity. DH IV locates at +800 which correlates with the appearance of the block of transcriptional elongation in differentiated HL 60 cells. The DH V was mapped at +1800 with an unknown function. All the locations are given with respect to the P1 promoter.



Note: +25 to +142 is the estrogen responsive region

Figure 9. *c-myc* gene organization. Exons 1,2, and 3 are represented by open rectangles. Shaded region shows the untranslated portion of the exon 1. Transcription start sites, P0, P1, P2, and P3 are shown by arrows. A more detailed diagram of the P1 and P2 promoter regions is shown in the lower portion of the figure. Positions of several regulatory regions in respect to the start site for P1 promoter are shown in black rectangles. CUG, AUG-translation initiation site, ERR, estrogen responsive region, Sp1 =Sp1/3 binding sites, Inr = initiator element. DH=DNase I hypersensitive sites.

6.2. *C-myc* gene protein product. The c-*myc* gene has 2 major promoters P1 and P2 located 161 nucleotides apart in exon I. Transcription initiated at P1 and P2 produces 2.4

and 2.2 kb *c-myc* transcripts, respectively. These transcripts give rise to 95% of total *c-myc* mRNA. Two minor promoters: P0 and P3, located in intron I account for less than 5% of *c-myc* mRNA. Both P1 and P2 contain a consensus TATA box element. A strong consensus initiator element (Inr) occurs only at the P2 transcription start site. A number of different regulatory sequences have been described in the 5' upstream promoter region, in the 5' and 3' untranslated regions, and within intron I (Fig. 9). There is only one open reading frame (ORF) in all *c-myc* mRNA species.

Three c-Myc proteins were observed *in vivo*. The major one is 64 kDa. One minor protein species of 67 kDa contains an additional 14 amino acids at the N-terminus of the p64 sequence (Hann and Eisenman, 1984;Ramsay *et al.*, 1984). The third isoform of c-Myc protein originates from the translation initiation site approximately 100 amino acids downstream of the N-terminus and lacks Myc transcription activation (Spotts *et al.*, 1997).

6.3. C-Myc functions. C-Myc protein is a member of Myc family of transcription factors, which also includes N-Myc and L-Myc genes. As a transcription factor, c-Myc regulates the expression of about 15% of all genes involved in the regulation of the cell cycle, cellular differentiation, and apoptosis (Ruf *et al.*, 2001). Myc-family transcription factors contain the bHLH/LZ Leucine Zipper in their DNA binding domains. The *c-myc* gene products bind as dimers to the specific DNA sequences on the target genes. All the dimerized complexes bind to the same E-box sequences, CACGTG and CACATG, as well as to several low affinity, noncanonical DNA sequences (Blackwell *et al.*, 1990;Prendergast *et al.*, 1991;Blackwell *et al.*, 1993). The expression of *c-myc* is correlated with the proliferative potential of the cell. *C-myc* is an immediate early response gene directly downstream of mitogenic signaling cascades. Mitogenic

stimulation cause rapid and transient bursts in both *c-myc* mRNA and c-Myc protein expression as cells enter the G1 phase followed by declining to low steady-state levels in proliferating cells (Kelly *et al.*, 1983;Campisi *et al.*, 1984;Moore *et al.*, 1987). c-Myc also plays an important role in cellular differentiation. It has been shown in many cells that down-regulation of *c-myc* expression accompanies terminal differentiation and permanent withdrawal from the cell cycle (Griep and Westphal, 1988;Holt *et al.*, 1988;Prochownik *et al.*, 1988;Canelles *et al.*, 1997). However, a paradoxical discovery from the work on *c-myc* was the finding that under certain circumstances c-Myc is able to induce apoptosis or programmed cell death (Askew *et al.*, 1991;Evan *et al.*, 1992). In summary, *c-myc* gene plays critical roles in the regulation of the cell cycle progression, cellular differentiation and apoptosis.

6.4. *C-myc* genetic alterations and the role of *c-myc* in breast tumorigenesis. *C-myc* is a protooncogene. Protooncogenes are genes that are normally involved in the regulation of cell proliferation but lead to tumorigenesis when abnormally expressed in cells. Genetic alterations leading to oncogenic activation of *c-myc* include gene amplification (Dalla-Favera *et al.*, 1982), chromosomal translocation (Magrath, 1990), chromosomal rearrangement (Louis *et al.*, 2005), proviral insertion (Payne *et al.*, 1982), and retroviral transduction (Fulton *et al.*, 1987). In addition, abnormal c-Myc protein levels caused by a point mutation in the *c-myc* coding sequence have been reported in several tumors (Bhatia *et al.*, 1993).

Dysregulated expression of *c-myc* plays an important role in tumorigenesis. Alterations in *c-myc* expression have been demonstrated in many types of cancer including breast carcinoma (Cole, 1986;Spencer and Groudine, 1991;DePinho *et al.*,

1991). *c-myc* is one of the most common oncogene aberrations in breast cancer (Escot *et al.*, 1986;Garcia *et al.*, 1989;Berns *et al.*, 1992;Spaventi *et al.*, 1994). In human breast cancers, about 16% have amplified *c-myc*, 5% have rearranged *c-myc* and about 70% have over-expressed *c-myc*. All these results together strongly suggest the importance of c-myc in the genesis and/or progression of breast cancer (Nass and Dickson, 1997;Deming *et al.*, 2000). In ER+, hormone-dependent breast cancer cells (MCF-7 and T47D), the *c-myc* gene is directly regulated by estrogen (Dubik and Shiu, 1988;Dubik and Shiu, 1992). The maximum *c-myc* mRNA 12-fold accumulation, was achieved one hour after addition of estradiol to MCF-7 cells (Dubik and Shiu, 1988). In the ER- MDA-MB-231 human breast cancer cell line, *c-myc* is expressed at high constitutive levels and its expression is independent of estrogens (Dubik *et al.*, 1987). Antisense *c-myc* oligonucleotides were found to inhibit both estrogen-dependent and estrogen-independent growth of MCF-7 and MDA-231 cell lines, respectively (Watson *et al.*, 1991b). These studies in human breast cancer cell lines indicate that c-Myc is important for cancer cell growth (Watson *et al.*, 1991a).

6.5. Regulation of transcriptional initiation. A large body of work has been done to identify cis-acting elements of the *c-myc* promoter that are involved in the regulation of transcriptional initiation. The nature of protein-DNA interactions in a putative responsive region were well studied with EMSA or episomal gene expression assays. The region from -60 to -37 is found to be essential for P1 transcription (Nishikura, 1986). It contains a GC-rich sequence CCGCCC (-38 to -43) that binds Sp1 and Sp3 transcription factors (Majello *et al.*, 1995). Five tandem CT-boxes (CCCTCCCC) located 101 bp upstream of P1 are required to promote transcription from P1 and for maximal activity of P2

(DesJardins and Hay, 1993). Mutations of these CT-boxes decreases the absolute and relative activities of P1 and P2 promoters. A single copy of the CT-box (CCTCCCTCCCT) in an inverted orientation is located 53 bp upstream of P2. This element has an inhibitory effect on P1 and is required for P2 transcription, respectively. It can bind both Sp1/Sp3 and MAZ transcription factors (DesJardins and Hay, 1993). The locations of the elements are given relative to the P1 promoter. In a search for the mechanism of estrogen-mediated activation of *c-myc*, a 116-bp estrogen responsive region (ERR) was identified within the *c-myc* promoter (Dubik and Shiu, 1992). There is neither a ERE nor a half ERE identified and confirmed within the promoter 2 for direct ERα binding site. However, two possible Sp1/3 binding sites are present which may bind the ERα indirectly (Miller *et al.*, 1996), although some article listed a possible half ERE (Safe and Kim, 2008). However, the presence of the GC-rich element was believed to be important to estrogen-mediated regulation of the *c-myc* promoter, since activation of several estrogen responsive genes by the Sp1/ERα complex has been reported (Miller *et al.*, 1996;Duan *et al.*, 1998a;Duan *et al.*, 1998b;Qin *et al.*, 1999). Promoter 2 is the preferred promoter out of its four promoters in ER+ breast cancer MCF-7 cells (Miller *et al.*, 1996).

7. Other estrogen responsive genes studied in this project

As described above, TGFα, cyclin D1, cathepsin D and RARα are all well-established estrogen responsive genes, although they have different promoter contexts in regard to the estrogen responsive element and Sp1/3 protein loading sites (Fig. 10). In this project, ChIP assays were also applied to study the loading patterns of ERα, Sp1 and Sp3 on these gene promoters in the absence and presence of estrogen.

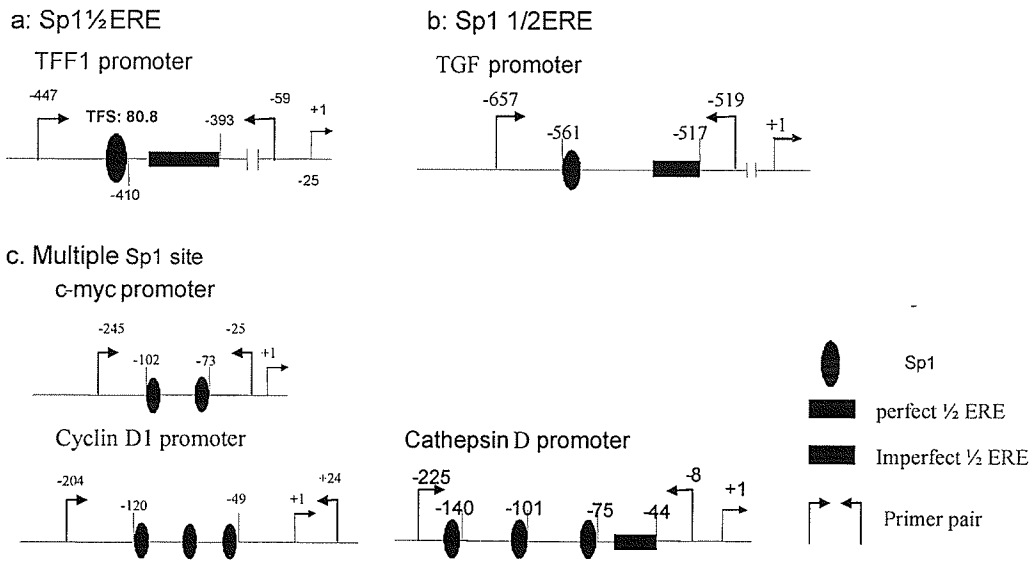


Figure 10. Multiple promoter context.

8. Preliminary analysis for some other not well established 'estrogen responsive genes'

Except for the well-established estrogen responsive genes, pilot experiments were done with several not as well studies, controversial or speculated estrogen induced genes in this thesis project. ERα is a good prognostic marker of breast cancers. The *ERα* gene has been found to be auto-regulated and three half EREs in its P1 promoter are responsible for estrogen-induced transcription (Treilleux *et al.*, 1997;Castles *et al.*,

1997;Donaghue *et al.*, 1999). However, ERα was also reported to be in the protein complex interacting with *ERα* gene minimal promoter through its interactions with Sp1 and USF-1 factors, but not directly with DNA (deGraffenried *et al.*, 2002). *P21* (*p21Waf1*) gene encodes a potent cyclin-dependent kinase inhibitor which functions as a regulator of cell cycle progression at G1. Estrogen and ERα are involved in the regulation of *p21* expression (Thomas *et al.*, 1998;Sasakawa *et al.*, 2002). High doses of estrogen (1μg/ml, about 3 μM) were found to induce the expression of *p21*, which suggests that *p21* may be a target for transcriptional activity by ERα or other transcription factors induced by estrogen (Wright *et al.*, 2005). However, it was also demonstrated that low dose estrogen (10 nM) can repress the expression of *p21,* and c-Myc is required for this estrogen mediated decrease of *p21* (Mukherjee and Conrad, 2005). In addition, it was demonstrated that ERα and HDAC1 or HDAC4 are recruited to the proximal Sp1 sites of the promoter to repress *p21* expression in cycling cells (Varshochi *et al.*, 2005;Mottet *et al.*, 2008). When treated with ICI 182,780, ERα and HDACs are dissociated from Sp1, and release the repression of the *p21* promoter and further induce the expression of *p21* (Varshochi *et al.*, 2005). It should be noted that unliganded ERα was able to exert its antiproliferative function through physically interacting with p21, which helps to explain the favorable prognosis of ERα breast cancers (Maynadier *et al.*, 2008). The *p53* gene encodes a transcription factor which functions as a tumor suppressor that is involved in preventing tumorgenesis. *p53* has been described as "the guardian of the genome" referring to its role in conserving stability by preventing genome mutation. Some studies have shown that estrogen can induce the expression of *p53* (Hurd *et al.*, 1995;Hurd *et al.*,

1997;Hurd *et al.*, 1999;Wright *et al.*, 2005). However, it was speculated that increased *p53* level may contribute to the *p21* induction by estrogen (Wright *et al.*, 2005).

9. Sp1, Sp3 and the constitutive expressed DPYD gene

In addition to estrogen induced genes, we also studied how Sp1 and Sp3 regulate the constitutive expressed DPYD gene. 5-fluorouracil (5-FU) is one of the most widely prescribed cancer chemotherapy drugs for the treatment of several malignancies including carcinomas of the colon, breast, skin and head and neck (Johnston *et al.*, 1999;Johnson and Diasio, 2001). 5-FU blocks DNA synthesis through inhibiting thymidylate synthase (TS), which disrupts the intracellular nucleotide pools (Mattison *et al.*, 2002). The initial and rate-limiting enzyme in the 5-FU catabolic pathway is dihydropyrimidine dehydrogenase (DPD) (Johnson and Diasio, 2001). It is now well established that the variation in DPD enzyme activity is responsible for much of the observed interpatient and intrapatient variability in the clinical pharmacokinetics of 5-FU (Diasio and Johnson, 1999). Elevated DPD activity has been suggested as a determinant of decreased sensitivity to 5-FU (McLeod *et al.*, 1998), while DPD deficiency is often accompanied by severe and life-threatening toxicity (Van Kuilenburg *et al.*, 1999). Hence, an understanding of the mechanisms controlling the expression of DPD has become important in improving 5-FU-based chemotherapy (Dobritzsch *et al.*, 2001). Great efforts have been made to identify mechanisms regulating DPD enzyme levels. A correlation between *DPYD* mRNA level and enzyme activity has been reported, and suggests that the transcriptional regulation is an important mechanism leading to variability in DPD protein levels (Johnston *et al.*, 1999;Takabayashi *et al.*, 2000;Johnson *et al.*, 2000;Fujiwara *et al.*, 2002).

Methylation of the cytosine residue in the 5'-CpG-3' sequence is a recognized epigenetic mechanism of gene silencing. It may result in inhibition of transcription factor binding to the methylated promoter or modulation of the chromatin structure, which in turn suppresses gene expression. DNA methylation is involved in cell-specific and developmental stage-specific gene expression (Wang *et al.*, 2003a). Since it has been widely recognized that DNA methylation of promoter regions containing Sp1 binding sites results in the decreased expression of many genes (Wang *et al.*, 2003a;Mulero-Navarro *et al.*, 2006), it is reasonable to propose DNA methylation as a regulatory mechanism of the *DPYD* gene. In the follow-up study, we studied how CpG methylation of the regulatory region of *DPYD* promoter correlates to *DPYD* transcription in RKO cells

10. Research Outline

The goal of my thesis project was to understand the role of Sp1 and Sp3 in the regulation of estrogen induced gene expression in ER+ human breast cancer cell lines. The association of transcription factors Sp1 and Sp3 with TFF1 and *c-myc* gene promoters was studied in the presence and absence of estrogen in human breast cancer cell lines. Further, the association of Sp1 and Sp3 with the estrogen responsive genes cyclin D1, cathepsin D, TGFα and RAR was also explored. Lastly, the role of Sp1 and Sp3 in the regulation of the constitutive expressed dihydropyrimidine dehydrogenase (DPYD) gene was studied.

Through the application of the chromatin immunoprecipitation (ChIP) assay, the loading of Sp1 and/or Sp3 onto the promoters of estrogen-responsive genes was determined *in situ* in ER+ MCF-7 breast cancer cells cultured under estrogen deplete and

complete conditions. The estrogen responsive genes studied were TFF1 and *c-myc*. Although both genes are estrogen responsive, their promoter context is different. The TFF1 promoter has an imperfect ERE which is positioned next to a Sp1/3 binding site. However, the c-*myc* promoter has several Sp1 binding sites but does not have a ERE or half-site ERE. To achieve promoter clearance and synchronize the promoters, estrogen starved MCF-7 cells were incubated with the transcriptional inhibitor α-amanitin for two hours. Following removal of the inhibitor, either ethanol or estradiol was added to the MCF-7 cells, and the association of ERα, Sp1, Sp3, and RNA polymerase II were followed as a function of time using ChIP and re-ChIP assays.

II. HYPOTHESIS AND RESEARCH OBJECTIVES

My overall hypothesis is that Sp1 and Sp3 play different roles in the regulation of estrogen responsive genes and that Sp1 is an activator in the regulation of estrogen induced TFF1 and *c-myc* gene transcription in breast cancer cells. This hypothesis was tested by the following five specific aims.

Specific aim 1. To identify the Sp1/3 site in the TFF1 gene proximal promoter.

Specific aim 2. To determine whether Sp1 and/or Sp3 associate with the estrogen responsive region in TFF1 and c-myc promoters in the absence and presence of estrogen in MCF-7 cells

Specific aim 3. To determine whether ERα co-occupied the promoter with Sp1 and/or Sp3 to activate gene transcription in response to estrogen treatment in MCF-7 cells.

Specific aim 4. To determine whether estrogen responsive promoters with more than one Sp1 site associate with Sp3 and/or Sp1.

Specific aim 5. To determine whether estrogen responsive promoters bound to Sp1and/or Sp3 are associated with RNA polymerase II.

III. MATERIALS AND METHODS

1. Reagents. α-Amanitin and 17 β-estradiol (E2) were purchased from Sigma Chemical Co (St. Louis, MO). DNase and RNase-free H_2O were purchased from Invitrogen (Burlington, ON, CA). 5-Aza-2'-deoxycytidine (AzaC) was purchased from Sigma (St. Louis, MO, USA).

2. Cell culture and treatments. Human breast cancer cell lines MCF-7 (ATCC). T5 cell line was cloned from MCF-7 cell line by Dr. Leigh Murphy's lab. ZR-75 (ER+ and estrogen dependent, ATCC) and MDA-MB-231 (ER- and estrogen independent, ATCC) were cultured in DMEM (GIBCO, Burlington, ON, CA) medium containing 8% fetal bovine serum (FBS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 0.3% glucose. Under estrogen deplete conditions, cells were grown in estrogen-depleted medium, consisting of phenol-red-free DMEM (GIBCO, Burlington, ON, CA), 2% charcoal stripped FBS, penicillin (100 units/ml), streptomycin (100 mg/ml), and 0.3% glucose as described above. Cells were grown in a 37^0C humidified incubator with 5% CO2. In some cases, MCF-7 (T5) cells were incubated with 10 nM E2 for different time periods as indicated. The human cervical cancer cell line HeLa, the human embryonic kidney epithelium cell line HEK293 and the human colon carcinoma RKO cell line were obtained from ATCC and maintained in DMEM and with 10% FBS (HyClone) and without antibiotics. The cells were incubated at 37°C in an atmosphere of 5% CO_2.

3. Reverse-transcription PCR (RT-PCR) for analyses of mRNA levels.

3.1. Genomic RNA isolation. RNA from MCF-7 (T5) cells treated with or without 10^{-8}M E2 was isolated using the Qiagen RNeasy Kit (Qiagen, CA). Briefly, one 10 cm diameter dish of MCF-7 (T5) cells were harvested by a rubber scraper in cold PBS and

the cells were used fresh or stored in -70 ^{0}C. Then the cells were homogenized (fresh or frozen) and centrifuged for 10 minutes at the maximum speed of the microcentrifuge (14,000 rpm). The supernatant fluid was then mixed with 70% ethanol and further taken to run through a supplied mini-column. This step was repeated until all of the solution was run through the column. Then the supplied binding buffer was added to the mini column followed by twice washing with supplied washing buffer. At last the RNA was eluted with RNase-free H_2O and stored in -80^{0}C. The quantification of total RNA was done by spectrophotometric measurement. It is important to adjust the pH of water used for spectrophotometric measurement of RNA higher than 7.5. Acidic pH affects the UV absorption spectrum of RNA and significantly decreases its A260/A280 ratio. Typically, distilled water has pH < 6. Water was adjusted to a slightly alkaline pH by adding concentrated Na2HPO4 solution to a final concentration of 1 mM. Pure RNA has an A260/A280 ratio of 1.8-2.1 in pH 7.5. An absorbance of 1 unit at 260 nm corresponds to 40 μg of RNA per ml.

3.2. cDNA Synthesis and analysis. cDNA was synthesized using MMLV Reverse Transcriptase (Invitrogen,CA). The PCR was done with Platinum Taq polymerase (Invitrogen,CA) and the appropriate primers to detect TFF1, *c-myc*, cathepsin D, cyclin D1, RAR,TGFα, ERα, p21, DPYD, GAPDH, and cyclophilin 33. The primers to TFF1 were 5'-TGG GGC ACC TTG CAT TTT CC -3' and 5'- CGG GGG GCC ACT GTA CAC GTC -3'; to *c-myc* were 5'- AAA AGA TCC TCT CTC GCT AAT CTT -3'and 5'- CCG AAA ACC GGC TTT TAT ACT CAG CG -3'; to cathepsin D were 5'- TGC ACA AGT TCA CGT CCA TC -3' and 5'-TGT AGT TCT TGA GCA CCT CG -3'; to cyclin D1 were 5'- GGA GCA CAT TTT CAG ACC TTC G-3' and 5'- AAA ACC CTG AAA

ATG ACC CTC G-3'; to RAR were 5'-CTG CGA GGT AAT TCC TCC CCT-3' and AGG CAG ATC ACA GAC GGG TTC-3'; to TGFα were 5'- CCT AAG CTT ATT TTC CCA ACG T-3' and CGC TGA TTT CTT CTC TAG GTC AC-3'; to ERα were 5'-ACC CTC CAC ACC AAA GCA TCT-3' and 3'- CCC TCG GGG TAG TTG TAC ACG-5', to p21 were 5'-GAG CCG CGA CTG TGA TGC GCT AAT G-3' and 5'-TTA CAA AGT CCT TCC GTG CAC ATG TCC G-3'; to DPYD were 5'-TTT GAT CCA GAA ACC CAC CTG-3' and 5'-AGG GTA CGC CTC TCT TTG GTT C-3'; to GAPDH were 5'- CCA GGA AAT GAG CTT GAC AAA GTG -3' and 5'- AAG GTC ATC CCT GAG CTG AAC GGG-3' and to cyclophilin 33 were 5'- GCT GCG TTC ATT CCT TTT G- 3'and 5'- CTC CTG GGT CTC TGC TTT G- 3'. The PCR products were resolved electrophoretically on a 1.5 % (w/v) agarose gel and stained with ethidium bromide. The ethidium bromide stock (dissolved in water) concentration is 10 mg/ml. The final ethidium bromide concentration used in agrose gel is usually 0.5 μg/ml. However, the gel background is much lower and the image is improved at lower ethidium bromide concentrations such as 0.1μg/ml when compared to higher ethidium bromide concentration. The gel was analyzed by scanning densitometry on a Kodak Image Station and the density of the amplified PCR product from each treatment was plotted. The fold induction of RNA levels in response to treatment was determined by dividing the net density value of PCR products from treated cells by that of products from untreated cells.

4. Electrophoretic mobility shift assay (EMSA). Potential Sp1 binding sites in the TFF1 promoter were predicted by the TFSEARCH program (Parallel Application TRC Laboratory, Japan). The oligonucleotides were synthesized as follows: P1, For-5'-GAG CTC CTT CCC TTC CCC CTG CA-3', Rev-5'-TCG CAG GGG GAA GGG AAG GAG

CT-3'; and oligonucleotides with consensus Sp1 sites, For-5'-GAT GCC TGC GGG GCG GGG CAG AGG GG- 3', Rev-5'-TCC CCT CTG CCC CGC CCC CAG GC AT-3'. The synthesized oligonucleotides were end labeled and incubated with 10 μg of MCF-7 (T5) cell nuclear extract. DNA was radiolabeled with ^{32}P by incorporating an [α-^{32}P]dNTP during a 3′ fill-in reaction using Klenow fragment and T4 polynucleotide kinase. Nonspecific competitor DNA such as poly(dI•dC) or poly(dA•dT) is included in the binding reaction to minimize the binding of nonspecific proteins to the labeled target DNA. These repetitive polymers provide an excess of nonspecific sites to adsorb proteins in crude lysates that will bind to any general DNA sequence. The order of addition of reagents to the binding reaction is important in that, to maximize its effectiveness, the competitor DNA must be added to the reaction along with the extract prior to the labeled DNA target. Besides poly(dI•dC) or other nonspecific competitor DNA, a specific unlabeled competitor sequence can be added to the binding reaction. A 200-fold molar excess of unlabeled target is usually sufficient to out-compete any specific interactions. Antibody competition analysis was carried out as an EMSA assay except that the 2 μg of antibodies was first incubated with nuclear extract for 10 min, then labeled DNA was added and incubated for another 30 min on ice.

5. Chromatin immunoprecipitation (ChIP) assay.

5.1. Formaldehyde crosslinking. MCF-7 (T5) or other cells grown on a 150×20-mm tissue culture dish to approximately 80–100% confluence are incubated with 10 ml of 1% formaldehyde in 1× PBS for 8–10 min at room temperature. Then glycine is added to final concentration of 0.125 M to quench the cross-linking reaction of formaldehyde. The cells are washed twice with 1× ice-cold PBS and harvested with a rubber scraper. Then

cells are pelleted with at least 700 x g for 1 to 5 mins depending on the centrifuge used. Then either the cells were stored at −80 °C or resuspended in lysis buffer containing protease inhibitors (1mM PMSF, 1μg/ml aprotinin, 10μg/ml leupeptin, 50μM iodoacetamide). Depending on the nature of the target protein to be investigated, phosphatase and kinase inhibitors (eg. 1 mM NaF, 1 mM NaVO3) were included in the lysis buffer. Two cell dishes with or without estrogen treatment were harvested with a rubber scraper in cold PBS. The cells were pelleted with 700 x g for 1 min and stored at -70^{0}C. Cells from one 10 cm dish without crosslinking were collected fresh or frozen for RT-PCR analysis to confirm that the cells responded properly to estrogen treatment.

5.2. Optimization of the ChIP protocol. In the ChIP assay, an antibody is used to capture a specific DNA-associated protein. The success of this protocol is entirely dependent on the quality of the antibody used. An antibody of good quality should be specific and efficient in the immunoprecipitation of a specific protein (Spencer and Davie, 2002).

5.2.1. Determining the efficiency of an antibody to immunoprecipitate its target antigen. After the cells were crosslinked with formaldehyde, the cell lysate was sonicated and diluted to 2A260 units/ml with ChIP dilution buffer. One milliliter of the diluted lysate was aliquoted into each of several microcentrifuge tubes, and different amounts of test antibody were added to each tube so that different dilutions (i.e. 1/100, 1/250, 1/400, 1/1000) of the antibody were tested. The tubes were then incubated for 30 min to overnight at 4 ^{0}C. The length of time for incubation with the primary antibody will vary with each type of antibody and the propensity for the antibody to associate with non target proteins. Following primary antibody incubation, 60 μl of a 50:50 (v/v) Protein

A/G slurry (Santa Cruz) was added to each tube for every milliliter of cell lysate. The tubes were incubated for an additional 2 h and then centrifuged for 3 min at x700g or 0.5 to 1 min for maximum speed in a microcentrifuge. Low-speed or quick high-speed centrifugation is used to protect the Protein A/G resin from damage. The supernatant is then transferred to a fresh microcentrifuge tube, and the protein A/G–Sepharose was wahsed sequentially with ChIP washing buffers and saved for determining the antibody specificity. The crosslinked antigen with its antibody complex can be eluted from the protein A/G beads and reverse crosslinked through boiling for one h or incubating at 65^0C overnight. An equal volume of elution supernatant (i.e. 20 μl) is then withdrawn from each tube, and placed into a fresh microcentrifuge tube containing SDS loading buffer (i.e. 5 μl of 5x). The samples were boiled for 3 min., electrophoresed onto a SDS gel, and transferred to nitrocellulose membrane. If the protein in question migrates at a molecular weight close to that of the primary antibody's heavy (51-60 kDa for IgG) and light chains (IgG 22-25 kDa), β-mercaptoethanol (β-ME) should be excluded from the loading buffer as this will prevent the dissociation of the primary antibody and prevent the antibody (146-170 kDa for IgG) from entering the gel during electrophoresis. The nitrocellulose membrane was then immunoblotted with the test antibody to detect for the presence or absence of the target protein from the supernatant. If the antibody was efficient at removing its target antigen from the supernatant, the level of antigen should decrease as the amount of antibody present is increased. In formaldehyde-crosslinked cells, the charge of the target protein may become altered, causing the target protein to migrate to a slightly different position on a SDS gel. In addition, the formaldehyde may crosslink the target protein to another protein and this may also alter its migration. Hence,

when working with formaldehyde crosslinked proteins to test the efficiency of an antibody, the crosslinked antigen should be throughly reverse crosslinked as described above.

5.2.2. Determining the specificity of an antibody for its target antigen. Specificity is extremely important to determine since many commercially available antibodies can nonspecifically immunoprecipitate proteins. In fact some commercial antibodies immunoprecipitate nontarget protein substrates more efficiently than their target proteins. To determine the specificity and functionality of an antibody, the Protein A/G–Sepharose saved in Section 5.1.1 from each ChIP sample is washed with various high-salt and/or high-detergent buffers as described in the next section to remove nonspecifically bound material. Following the washes, the associated proteins were isolated by resuspending the Sepharose in TE buffer (pH 8) containing SDS loading buffer and boiling the sample for 3–5 min. The extracted proteins were then loaded onto a SDS gel and transferred to nitrocellulose membrane, and the membrane was immunoblotted with the test antibody. A functional antibody will show an increase in antigen associated with protein A/G as the amount of antibody is increased. The absence of antigen from the Sepharose may indicate that the antibody is nonfunctional for immunoprecipitation assays or that the immunoprecipitation buffer and/or wash buffers are too stringent to allow binding of the antibody to its target antigen. In addition, antibody purification (Con A Sepharose 4B) was also tried to minimize noise background for the ChIP assay.

5.2.3. Determining the type and amount of protein–Sepharose to use. Whether one chooses Protein A or G to isolate antibody–antigen complexes depends on the nature of the primary antibody. The antibodies produced in rabbits have an equal binding affinity

for Protein A and G while goat antibodies, which bind both Protein A and Protein G, display a preference for Protein G. When an antibody is capable of binding to both Proteins A and G, one can use a 50:50 (v/v) resin slurry containing both Protein A– and ProteinG–Sepharose in an equal molar ratio to improve the yield of immunoprecipitated antibody–antigen complexes. However, in most cases, a 50:50 (v/v) slurry containing only one type of resin is added. The quality of Protein A– and Protein G–Sepharoses also varies between companies. Thus, different company brands should always be tested before starting the ChIP protocol. Once the quality of the antibody and the crosslinking conditions have been determined, the amount of Protein A/G necessary for capturing the primary antibody must be determined as smaller amounts of Protein A/G results in less background. This is accomplished by aliquoting the buffer (1x RIPA or dilution buffer) used to dilute the cell lysate to 2 A260 units/ml into several microcentrifuge tubes and adding the predetermined optimal amount of primary antibody to this buffer (see Section 5.1.1). Different amounts of 50:50 Protein A/G slurry (i.e. 20, 40, 50, and 60 µl) are added to each tube and the sample is incubated for 2 h. The samples are then centrifuged at maximum speed in a microcentrifuge tube for 0.5 to 1 min and the supernatant is transferred to a fresh tube. An aliquot of the supernatant from each tube is collected, transferred to its respective tube, supplemented with SDS loading buffer, and boiled for 3 min. The sample is then electrophoresed through a SDS gel and transferred to a nitrocellulose membrane. The primary antibody has already been added to the samples and, therefore, is immobilized on the membrane. Because of this, the membrane is immunoblotted only with secondary antibody to ultimately visualize the progressive

decrease in unbound primary antibody with increasing amounts of Protein A/G–Sepharose.

5.2.4. Sonication efficiency. The extent to which one can fine-map the location of a specific protein along a gene depends on the extent of DNA fragmentation. For example, when studying the association of a specific protein with a 200-bp region of DNA, and the genomic DNA is sonicated to an average size of 500 bp, one should consider that a total of 300 bp of DNA sequence adjacent to the target DNA region will be immunoprecipitated along with the target DNA sequence in the ChIP assay. Thus, the amplification of this 200-bp region from ChIP DNA would suggest that the target immunoprecipitated protein is associated either within this 200-bp region or within a region up to 300 bp away from this target sequence (Fig. 11). For further explanation of this concept, refer to (Kadosh and Struhl, 1998).

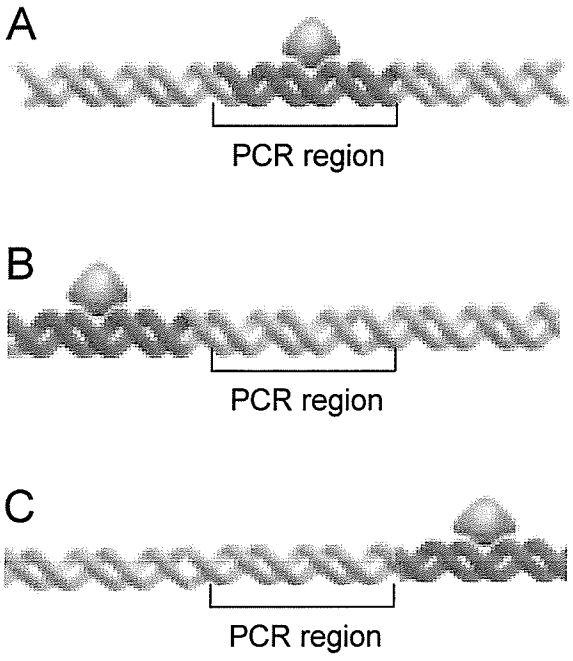


Figure 11. The final ChIP DNA PCR fragment may not contain the actual domain with targeted chromatin protein.

When using the same sonicator probes, the volume of a sample and the depth to which the sonication probe is placed in the sample can affect the efficiency of sonication (Orlando, 2000). So depending on the extent of crosslinking, these factors should always be adjusted to obtain the proper size of the genomic DNA. During sonication, generally the sonicator probe should be placed at certain depth (can be found in manual) into the sample for the best efficiency and also to avoid foaming. Foaming can greatly decrease the efficiency of sonication. In most ChIP assays, smaller sample volumes of 400–500 µl are more ideal to use. However, as the sample volume decreases it becomes more difficult to place the sonicator probe deep enough into the sample to avoid foaming. To increase the depth of a sample, the cell lysate sample is transferred to a microcentrifuge tube prior to sonication. In the ChIP assay, the cell lysate contains SDS. Therefore, positioning the sonicator probe too close to the sample surface tends to cause foaming. Cooling the sample between sonication pulses and placing the sample in an ice water bath during sonication decrease the incidence of foaming. As well, the chance of sample foaming is greatly reduced when the output energy emitted from the sonicator probe is decreased. In addition, longer crosslinking time (> 30 min) may result in difficulty to sonicating samples to smaller DNA fragment sizes.

5.3. Chromatin immunoprecipitation (ChIP). Fresh crosslinked or stored cells were treated with lysis buffer (5 mM PIPES, 85 mM KCl, 0.5 % NP-40, pH 8.0) to isolate nuclei for 20 min (x2) at 4^0C. The chromatin was sonicated to an average fragment size of 500 bp or 400 bp depending on the size of PCR products and microcentrifuged at 13,500 rpm for 10 min at 4^0C. After centrifugation, 0.2 A260 units of supernatant were

used as inputs, and the remaining volume was diluted 5-fold in dilution buffer (1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl (pH 8.1), 1.1 % Triton X-100, and 0.01% SDS). A portion of the diluted fraction (18 A260 units) was subjected to immunoprecipitation overnight with antibodies against estrogen receptor α (Novo Castra, UK), Sp1 (Upstate, MA), Sp3 (Santa Cruz, CA) and RNA polymerase II (Upstate), which recognizes the CTD serine 5 phosphorylated RNA polymerase II. Preimmune IgG served as a negative control for these antibodies (control immunoprecipitation): anti-HDAC1 and anti-HDAC2 polyclonal antibodies (Affinity Bioreagents Inc. (ABR), CO) and anti-ERα mouse monoclonal antibody (Novo Castra, UK). The immunoprecipitated complexes were recovered by Protein A/G plus-Agarose (Santa Cruz) pre-coated with BSA and salmon sperm DNA (Invitrogen,CA). Beads were then consecutively washed with 1 ml Washing Buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), Washing Buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl), Washing Buffer III (0.5 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0), and finally two times with TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). RNase A (20 μg) was added to each immunoprecipitate and incubated for 30 min at 37^0C. Precipitated complexes were eluted from the beads by incubation with 100 μl of 1% SDS, 0.1 M NaHCO3. Proteinase K digestion and reversal of cross-links were done by overnight incubation at 65^0C. ChIP DNA was purified using QIAquick PCR Purification Kit (Qiagen, CA). ChIP and input DNA were analyzed by PCR using specific primer sets to each promoter. The TFF1 promoter primers used were 5′-GAC GGA ATG GGC TTC ATG AGC-3′ (forward) and 5′-GAT AAC ATT TGC CTA AGG AGG-3′ (reverse) respectively to amplify a 385-bp

fragment. The intron-exon 2 primer pair for TFF1 was 5′ CTG GGG CAC CTT GCA TTT TCC 3′ (forward) and 5′ CGG GGG GCC ACT GTA CAC GTC 3′ (reverse) to amplify a 229-bp fragment, which served as control for checking the fidelity of the ChIP assay. The c-*myc* P2 promoter primer pair was 5′-GAA AAA AGA TCC TCT CTC GCT AAT CTC-3′ (forward) and 5′- CCG AAA ACC GGC TTT TAT ACT CAG CG -3′ (reverse) to amplify a 221-bp fragment. Cathepsin D promoter primers were 5′- GGA ATC CTC CAG ACC CCA GAA GCT GG -3' (forward) and 5′- GAT CAG CTG ACC CGC GTG TTT GCA C -3′ (reverse) to amplify a 220- bp fragment. The TGFα promoter primer pair used was 5′- TGA TTC AGG GGA CCA CTT TCC -3′ (forward) and 5′- GCA CCC GTT ACC TAC ACC GAG -3′ (reverse) to amplify a 148-bp fragment. RAR promoter primers were For-, 5′- CGG TCC AGT CTT CAA CTA GGA GTG -3′; Rev-, 5′- GAG GCT GAA ACT TTA CCC GGA G -3′ to amplify a 217-bp fragment. Cyclin D1 promoter primers were 5′- AGC GGG GCG ATT TGC ATT TCT ATG A -3' (forward) and 5′- AGC AAA GAT CAA AGC CCG GCA GAG A T -3′ (reverse) to amplify a 194-bp fragment. The p21 promoter primer pair was 5′- AAA GCC AGA TTT GTG GCT CAC TTC GTG G -3′ (forward) and 5′- GGC TCC ACA AGG AAC TGA CTT CGG CAG- 3′ (reverse) to amplify a 227-bp fragment. The p53 promoter primer pair was 5'-CCC AGC ACT GAT ATA GGC ACT-3′ (forward) and 5′ -TTT TGG GGT GGA AAA TTC TG-3′ (reverse) to amplify a 380 bp fragment; The ERα promoter primer pair was 5'-ATA ATT GCC AAA GCT TTG GTT CG-3' (forward) and 5'-CTG CAA AAT GCT CCC AAA GTA GAT AG-3' (reverse) to amplify a 278 bp fragment. The AP2γ promoter primer pair was 5'-GTC CAG TGA CCC GGA CAG-3' (forward) and 5'- ACC TCG CAG TCC TCT TCG TA-3' (reverse) to amplify a 300 bp fragment.

The DPYD promoter primer pair was 5'-TCT ACT CCC TCC CTC CCT TC-3' (forward) and 5'-CTC GAG TCT GCC AGT GAC AA-3' (reverse) to amplify a 266 bp fragment.

5.4. ChIP DNA analysis. The DNA isolated from a ChIP assay can be analyzed by either Southern slot blot analysis or PCR. If analyzed by Southern slot-blotting, the DNA can be applied to a nylon membrane and hybridized to radiolabeled or digoxygenin (DIG)-labeled DNA probes (Spencer and Davie, 2001). Alternatively, the ChIP DNA can be radiolabeled or DIG-labeled and used as a probe to hybridize to specific genomic DNA sequences blotted on a nylon membrane (Chadee *et al.*, 1999). In this present study, the method of choice for ChIP DNA analysis has been PCR. We applied two methods to quantify the PCR products.

5.4.1. Method 1 to quantify the PCR products. Method 1 was as we described previously using PCR to analyze ChIP DNA (Spencer *et al.*, 2003). For studying the association of transcription factors or histones with a specific DNA region, we typically use 2 μl of input DNA in one PCR reaction and 4 μl of ChIP DNA in another identical PCR, with both reactions containing primers to the target DNA region. However, different antibodies display different efficiencies in their ability to immunoprecipitate their target antigen. Therefore, the volume of ChIP DNA in each PCR is ultimately dependent on the quality of the antibody. The PCR is carried out until the reaction is in the linear stage of amplification. This typically takes between 27 and 31 cycles depending on the primer set. The rate of amplification can be determined by conducting the PCR amplification procedure in an iCycler (Bio-Rad, CA). Alternatively, a classic PCR approach can be used whereby identical PCRs are carried out for different cycle numbers and the level of PCR product in each reaction is measured by agarose gel electrophoresis

combined with scanning densitometry. The level of PCR product in each PCR reaction can then be plotted against the cycle number to determine the number of cycles required to reach a linear stage of amplification. For each promoter studied, the conditions (e.g. template amount and cycle numbers) and the linear range of PCR product amplification for both the inputs and ChIP DNA samples were optimized. Following optimization, PCR was performed and the products were electrophoresed on a 1.5 % agarose gel containing 0.5% ethidium bromide, and quantified with a Kodak Image Station™.

To determine whether a specific DNA sequence becomes associated with a transcription factor (TF) or a modified histone after a treatment, we measure the levels of PCR product from ChIP and input DNA in control and treated cells by agarose gel electrophoresis using ethidium bromide to stain DNA and scanning densitometry for quantification. Then we determine the level of DNA sequence enrichment in ChIP DNA for both control and treated cells by dividing the net density of PCR product from ChIP DNA by the net density of product from the respective input DNA. The fold enrichment of DNA sequence in the treated sample is then determined by dividing the level of sequence enrichment for the treated sample by that for the control sample. Although the ChIP protocol is useful for studying the binding of proteins to DNA, it is usually more of a qualitative than a quantitative approach. One can determine if a specific protein is associated with a specific DNA sequence; however, whether this association is true for every cell in the cell lysate is difficult to determine.

5.4.2. Method 2 to quantify the PCR products. Method 2 was quantitative real-time PCR using SYBR green with the primers listed below and the same input and ChIP DNAs that were used with method 1. TFF1 promoter primers were 5′- GTG AGC CAC

TGT TGT CAG GCC AAG C -3' (forward) and 5'- CCC ATG GGA GTC TCC TCC AAC CTG A -3' (reverse) to amplify a 150-bp fragment. C-*myc* promoter primers were 5'- ATA ATG CGA GGG TCT GGA CGG CTG A -3' (forward) and 5'- GCG ATC CCT CCC TCC GTT CTT TTT C -3' (reverse) to amplify a 153-bp fragment. TGFα promoter primers were 5'- CGG CGA AAC TCA CAG GTC CCT TTC C -3' (forward) and 5'- TGA CTT CAG ACA CCA CCC CCG ACT G -3' (reverse) to amplify a 244-bp fragment. RAR α promoter primers were 5'- CCC ACG GTC CAG TCT TCA AC -3' (forward) and 5'- GGAGGGTACGGAGCAGAGGT -3' (reverse) to amplify a 182-bp fragment. Cyclin D1 and cathepsin D promoter primers were the same as described in method 1. The amount of ChIP DNA PCR product per A260 was divided by that of the input to calculate % of input. All the PCR products were sequenced for sequence confirmation.

6. Re-ChIP. Re-ChIP is one powerful technique to study if two factors occupy the same DNA fragment. In re-ChIP experiments, after the elution for the first ChIP, the supernatant was diluted 10 times with lower pH dilution buffer (the same as described above except the lower pH is 7.2) and subjected again to the ChIP procedure. First ChIP was performed using one antibody. Then, before reversal of protein–DNA cross-linking, the first protein antibody immunoprecipitated chromatin fragments were subjected to re-precipitation using the second primary antibody. During the subsequent PCR experiment only those DNA fragments that are simultaneously bound to both proteins should be amplified. ChIP and input DNA were analyzed by PCR using trefoil factor 1 (TFF1) and *c-myc* promoter primers as described above. (TFF1 primers as 5'-GAC GGA ATG GGC TTC ATG AGC-3' (forward); and 5'-GAT AAC ATT TGC CTA AGG AGG-3'; c-myc

primers as: 5'- ATA ATG CGA GGG TCT GGA CGG CTG A -3' (forward) and 5'-GCG ATC CCT CCC TCC GTT CTT TTT C -3' (reverse). The PCR products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and quantified using a Kodak Image Station. The linear range of PCR product amplification was determined, and the amount of ChIP-DNA template was optimized. For some of the re-ChIP DNA, PCR quantification method 2 described above was also applied for the DNA analysis when it is necessary. All the PCR products were sequenced for sequence confirmation.

7. ChIP assay for engineered episomal TFF1 promoter.

7.1. Cloning and transfection. The engineered episomal TFF1 promoter was constructed by my lab co-worker Ms Jenny Yu. Briefly, pGL3 luciferase reporter plasmid (Promega Corp.) was modified with the insertion of the TFF1 promoter sequence into its polylinker site immediately upstream of the luciferase expression gene. The promoter fragment of TFF1 (-528 to +40) was synthesized from MCF-7 (T5) genomic DNA using the following primers: 5'- GGG TAC CGA TTA CAG GCG TGA GCC ACT G- 3' (forward) and 5'-CAA GCT TGC CTC CTC TCT GCT CCA AGG- 3' (reverse). The fragment was inserted into pGL3 vector (Promega) between KpnI and HindIII and transferred into Escherichia coli to obtain a luciferase reporter plasmid (pTFF1-Luc) (fig of the map). The DNA sequence of the P1 (Sp1/Sp3) binding site was mutated using ExSite PCR-Based Site-Directed Mutagenesis Kit (Stragagene) following the manufacturer's protocol. Briefly, pTFF1-Luc double-stranded DNA was mutated by PCR using the primers, 5'-TTC <u>A</u>CT T<u>AA</u> CCC TGC AAG GTC- 3' (forward; mutations underlined) and 5'-GGA GCT CAT GAA GCC CAT TCC- 3' (reverse) to obtain P1-

mutated plasmid (pTFF1DSp1-Luc). All plasmid constructs were confirmed by restriction enzyme mapping and DNA sequencing. Plasmids for transfection were prepared using Qiagen Plasmid Mega Kit.

Transient transfection and reporter assays MCF-7 (clone 11) cells, which express an inducible GFP-ER, were cultured and induced to express GFP-ER as previously described (Zhao *et al.*, 2002). Briefly, the cells were grown in estrogen-depleted medium for 6 days. For transfection, cells were grown in phenol-red-free DMEM (GIBCO) with 7% charcoal dextran-treated FBS with doxycycline (1 μg/ml) for 24 hr before transfection. At 90% cell confluence, cells were transfected using Lipofectamine 2000 transfection reagent (Invitrogen, CA) according to the manufacturer's instruction, typically with 1 μg of reporter plasmid and 0.3 μg of control plasmid pCMXbgal. After 24 h, the cells were washed and given fresh medium that contained 7% charcoal dextran treated FBS with 10 nM E2 and doxycycline (1 μg/ml) for 24 h.

7.2. ChIP assays for mutated and non-mutated episomal TFF1 promoter. Formaldehyde cross-linked MCF-7 (clone 11) cells were prepared as described above (section 5.1). The ChIP assay was performed as described above (section 5.3 and 5.4.) with transfected cells with the following minor modifications. The DNA was purified by QIAquick PCR purification kit (Qiagen) and the primer set was flanking the TFF1 promoter region of the pTFF1-Luc (5'-GGG TAA ATA CTG TAC TCA CTG CAG-3') and the pGL3 vector region (5'-CTA GCA AAA TAG GCT GTC CC-3'). Amplified fragments (284 bp) were analyzed on a 1.2% agarose gel. The PCR product was sequenced to confirm that transient vector and not the endogenous TFF1 gene was amplified. Anti–actin monoclonal antibody (Sigma) served as an antibody control.

8. Western blots. Western blot was used routinely to check the protein level or antibody specificity and efficiency in this project. Cell extracts were prepared as described below. Cells were seeded in 6 or 10 cm Petri dishes. After treatment, cells were washed with ice-cold PBS and harvested in 500 μl SDS lysis buffer [100 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 4.0% SDS], boiled for 3-5 min, and placed on ice for 10 min. Following protein concentration determination using the Bradford assay, 15 μl of 0.2% bromophenol blue (BPB) in β-ME was added to each sample. To extract proteins from the beads after immunoprecipitation, 60 μl of the SDS buffer containing β-ME was added and samples were boiled for 3-5 minutes. The extracts were subjected to SDS-PAGE (20 μg protein/lane or 30 μl/lane for immunoprecipitated samples). For the Western blot, different amounts of protein extract were resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electro-blotted onto nitrocellulose membrane at 100V for 1.5 h. The same amount of protein extract from none-treated cells was used as the positive control. Proteins resolved by SDS-PAGE were visualized either by Coomassie blue staining or by transfering to a nitrocellulose membrane and immunochemical staining with various antibodies as per manufacturers' instructions (i.e. developed with a 1:1000 dilution of ERα, 1:1000 Sp1 antibody, 1:1000 of Sp3 antibody and 1: 5000 of the second antibody). Enhanced chemiluminescence kits were purchased from Perkin-Elmer (Boston, MA) or from Amersham Biosciences (Piscataway, NJ) for quantitative analysis using the Storm phosphorimager.

9. **Analysis of DNA bound to Sp1 and Sp3.** Cycling MCF-7 cells were crosslinked with 1% formaldehyde. The fixed chromatin was sonicated to about 500 bp DNA fragments. The DNA (free DNA and DNA with crosslinked protein) was purified with QIAquick

PCR Purification Kit (Qiagen, CA) and the final columns product is in DNase free H2O (Invitrogen,CA). The DNA bound proteins were reverse crosslinked by boiling in 1x SDS loading buffer for one h and then analyzed by Western blot with Sp1 and Sp3 antibody.

10. 5-Aza-2'-deoxycytidine (AzaC) Treatment. One mM stock solution 5-Aza-2'-deoxycytidine (AzaC) was prepared in 1X PBS, filter-sterilized, aliquoted and then frozen at -80°C until used. Cells were seeded at low density ($1x10^5$ cells in each well of a 6-well tissue culture plate or $10x10^5$ cells in a T75 flask), incubated for 24 h and then treated with AzaC or mock-treated with PBS. The media containing fresh AzaC was changed every 24 h until the cells were collected.

IV. RESULTS

1. Analysis of DNA bound Sp1 and Sp3. Our lab demonstrated that phosphorylated HDAC2 is preferentially cross-linked to DNA with formaldehyde in MCF-7 cells (Sun *et al.*, 2002a;Sun *et al.*, 2005a). Analysis of DNA bound Sp1 and Sp3 were done to determine whether Sp1 and all the Sp3 isoforms are cross-linked to DNA. Cycling MCF-7 cells were crosslinked with 1% formaldehyde. The fixed chromatin was sonicated and purified with QIAquick PCR Purification Kit. The DNA bound proteins were reverse crosslinked by boiling in 1x SDS loading buffer for one h and then analyzed by Western blot with Sp1 and Sp3 antibody. Figure 12 shows that Sp1 and all the Sp3 isoforms were cross-linked to DNA with 1% formaldehyde (Fig. 12). Furthermore, to study the proteins binding to a specific DNA sequence, it is important to check the chromatin fragment size. In this study, the chromating fragment size is usually under 500 bp (Fig. 13).

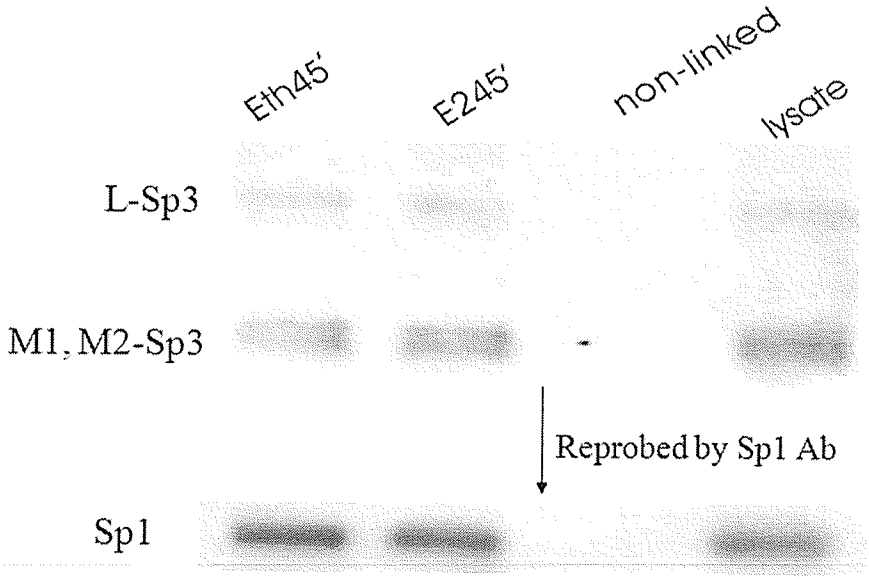


Figure 12. All Sp1 and Sp3 isoforms are cross-linked to DNA.

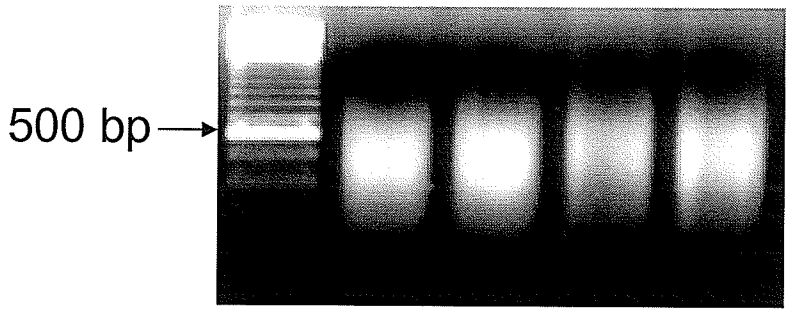


Figure 13. Chromatin fragment size for ChIP assay

2. Identification of the Sp1 element in the TFF1 proximal promoter.

2.1. E2 activates the TFF1 gene in MCF-7 cells. TFF1 is a well established estrogen-responsive gene and the TFF1 promoter has an imperfect ERE (Fig. 8) (Sewack and Hansen, 1997;Kim *et al.*, 2000b;Lu *et al.*, 2001). To synchronize the TFF1 promoter, estrogen-dependent MCF-7 cells were cultured in the absence of E2 for 3 days followed by incubation with 2.5 mM α-amanitin for two hours to synchronize the promoters (Shang *et al.*, 2000;Metivier *et al.*, 2003a). Following rapid removal of α-amanitin, the cells were exposed to 10 nM E2 for up to 45 min (Fig. 14). Immunoblot analyses of Sp1, Sp3 and ERα revealed that there was no change with the levels of these proteins during these treatments (Fig. 15). To monitor the expression of the TFF1 gene following the addition of estrogens, RT-PCR was conducted using the housekeeping gene GAPDH as a reference. Increased levels of TFF1 mRNA were detected after 15 min of E2 addition (Fig. 16A). Following the recruitment RNA pol II to a promoter, the enzyme goes through a series of phosphorylation events, with phosphorylation at serine 5 showing that the pol II has engaged in initiation (Xie *et al.*, 2006). The occupancy of the initiated form of pol II on the TFF1 promoter was determined using the ChIP assay with antibodies

against pS5-pol II. Figure 16B shows that following the addition of E2, pS5-pol II, was associated with the TFF1 promoter. No association of pol II with the TFF1 promoter was detected in MDA-MB-231 cells in which the TFF1 gene is transcriptionally inactive (Fig. 16B). Interestingly, pS2-pol II loaded on the TFF1 promoter after E2 treated the cells for 45 mins, suggesting that transcription elongation had begun (Fig. 16C).

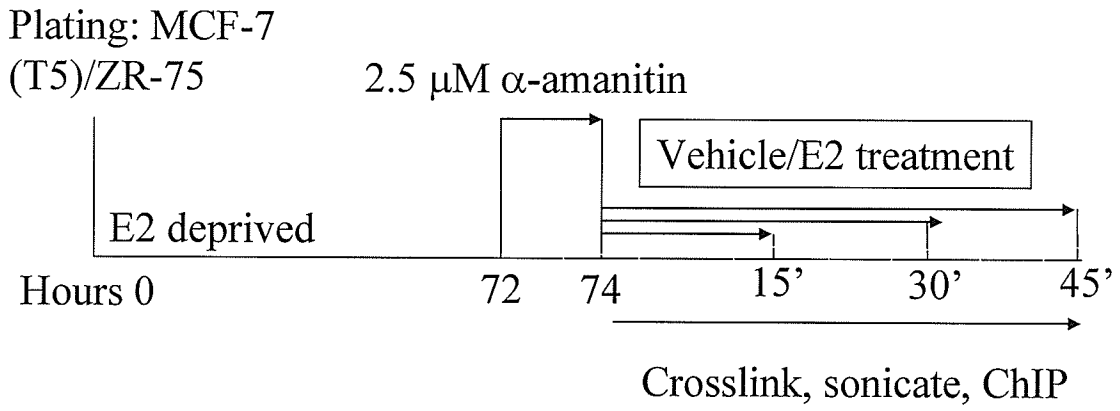


Figure 14. Transcription from estrogen responsive gene promoters was synchronized by treatment for 2 h with α-amanitin following 45 min treatment with E2 or vehicle control.

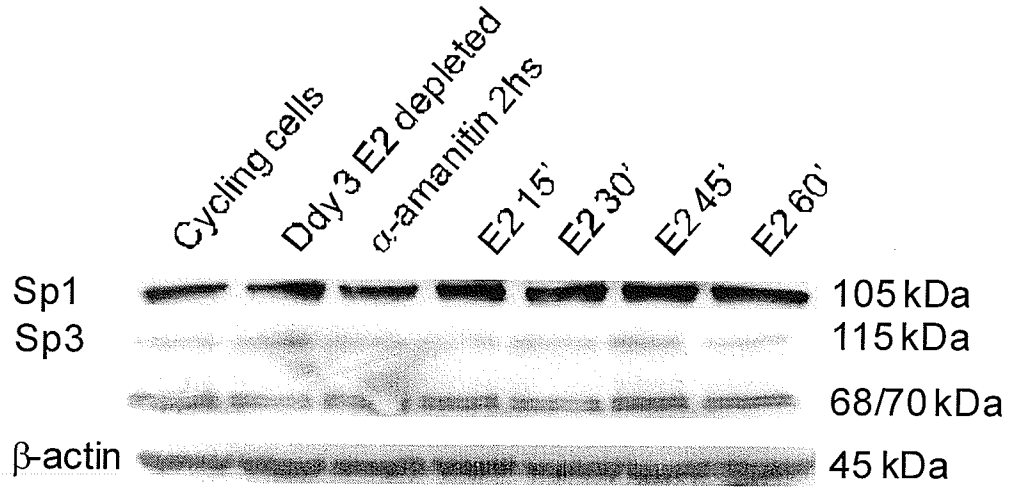


Figure 15. Sp1 and Sp3 protein level in α-amanitin and E2 treated MCF-7 (T5) cells.

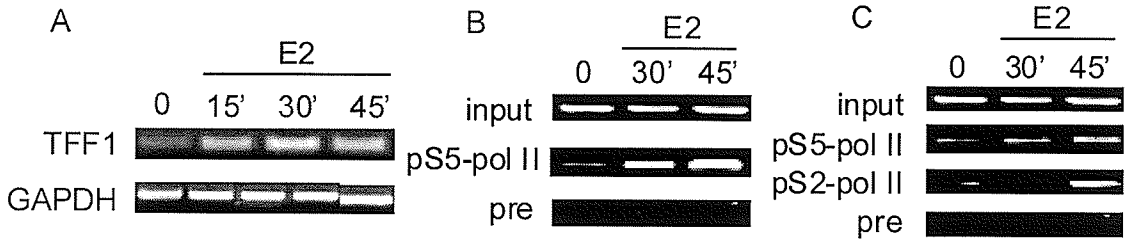


Figure 16. Estrogen induced expression of TFF1 gene. A. MCF-7 cells cultured under estrogen- and serum-deprived conditions were treated for 15, 30, 45 min with 10 nmol/L E2. Cells were harvested at the indicated time and RNA was extracted for further RT-PCR analysis with primers corresponding to TFF1 intron A–exon 2 and GAPDH exon VII as loading control. B. Occupancy of the TFF1 promoter by pS5-pol II and rabbit preimmune antibodies (pre) (negative control) at different times as measured by the ChIP assay. Input and ChIP DNA were analyzed by primers to the TFF1 promoter. C. Occupancy of the TFF1 promoter by pS2-pol II and rabbit preimmune antibodies (pre) (negative control) at different times as measured by the ChIP assay. Input and ChIP DNA were analyzed by primers to the TFF1 promoter (Li and Davie, 2008).

2.2. Using EMSA to identify the Sp1/3 binding site in the estrogen responsive TFF1 promoter in MCF-7 cells. Since Sp1 binding sites are often found adjacent to ERα binding sites in the promoter of estrogen responsive genes in human breast cancer cells (Abdelrahim *et al.*, 2002;Schultz *et al.*, 2003), we used the transcription factor search (TFSEARCH) program to identify possible Sp1 binding sites in the TFF1 promoter (Heinemeyer *et al.*, 1998). In TFSEARCH program, the positional weight matrix (PWM) is used to search for putative transcription factor binding sites. PWMs are constructed by collecting and aligning the nucleotide sequence of experimentally validated transcription factor binding sites. The frequency of each nucleotide (A, C, G, or T) at each column of the alignment is calculated in the matrix. The scoring system is a measurement of how well a string matches with the pattern specified by the PWM. Increasing the threshold will direct the program to find the binding site that favours high score at each position in the matrix (Zhang *et al.*, 2002). At a threshold score setting of 85, no Sp1 sites were

found next to the ERE. However, when the threshold setting was reduced to 80, one Sp1 site (-420) 5' to the ERE (-406) was identified (Fig. 17A). The sequence of this putative Sp1 binding site, named P1 (Fig. 17B), suggested that its affinity for Sp1 would be weak (Sun *et al.*, 1992).

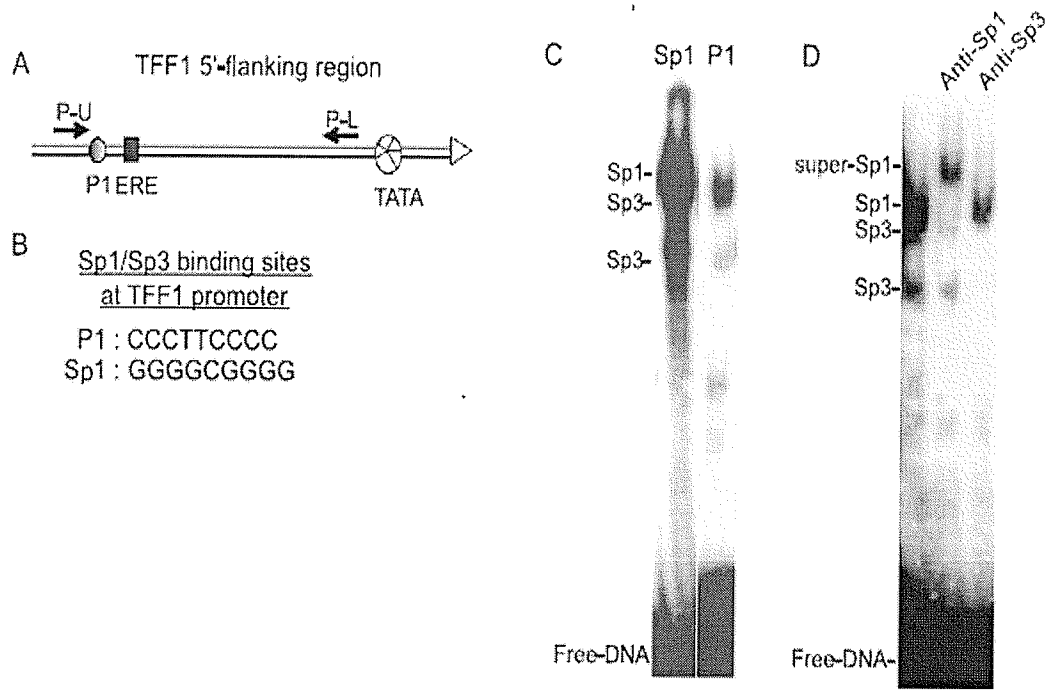


Figure 17. Analysis of Sp1 binding sites in the TFF1 promoter. A. Map of 5' flanking region of TFF1 gene. The potential Sp1 binding site is indicated as P1. The arrowheads indicate the location of primers (set 1) used in PCR to identify TFF1 promoter region (P-U and P-L). B. Sequences of P1 and consensus Sp1 binding site. C. EMSA of the Sp1 binding site in the TFF1 promoter. The synthesized oligonucleotides with P1 sequence and Sp1 consensus sequence were end labeled and incubated with 10 μg of nuclear extract from MCF-7 (T5) cells. D. EMSA antibody competition assay. MCF-7 (T5) nuclear extract was incubated with anti-Sp1 or anti-Sp3 antibodies for 10 min before adding ^{32}P end-labeled P1 oligonucleotides and then incubating for another 30 min. Sp1, Sp3, and supershift Sp1 complexes bands are indicated (Sun *et al.*, 2005b).

Oligonucleotides with the P1 sequence were end labeled and incubated with a nuclear extract from MCF-7 cells grown in serum complete medium. Fig. 17C shows that P1 oligonucleotides formed protein complexes with mobilities similar to those generated

with the consensus Sp1 binding sequence. The pattern of protein–oligonucleotide complexes was indicative of Sp1 and Sp3 association (Sowa *et al.*, 1999;Won *et al.*, 2002a). Dr. Sun's supershift study further verified this EMSA result. The addition of an anti-Sp1 antibody supershifted the more abundant slowest migrating complex, while anti-Sp3 antibodies removed the two less abundant faster migrating complexes (Fig. 17D). The EMSA data suggested that Sp1 was more abundant than Sp3, which is in agreement with our previous immunoblot results showing that the levels of Sp1 were greater (about threefold) than that of Sp3 in MCF- 7 (T5) cells (Sun *et al.*, 2002a). The intensities of the P1-protein complexes relative to those forming with the consensus Sp1 oligonucleotide were consistent with P1 having a relatively weak Sp1/Sp3 binding site (Fig. 17C). It should be acknoledged that this work was done by Dr. Jan-min Sun. I repeated and reproduced the result of EMSA assay (data not shown).

2.3 Mutation analysis of the Sp1/Sp3 P1 site in the TFF1 promoter.

2.3.1. Luciferase assay to study the contribution of the Sp1 binding site to TFF1 promoter activity. Previous studies have demonstrated that the imperfect ERE is required for the estrogen response of the TFF1 promoter, with the TFF1 promoter failing to respond to estrogen stimulation when the ERE is mutated (Lu *et al.*, 2001). To determine the contribution of the Sp1/Sp3 site, the P1 site was mutated to prevent Sp1/Sp3 binding. The estrogen response of the pTFF1DSp1-Luc was significantly reduced, illustrating a response approximately two-thirds that of the pTFF1 promoter (Fig. 18A). Repetitions of this assay yielded a mean reduction of 32% (± 3 SEM; n = 5 separate experiments done in triplicate) for the E2 induction of the pTFF1DSp1-Luc relative to that of the pTFF1 promoter. This work was done by Ms. Jenny Yu.

2.3.2. ChIP assay on episomal TFF1 promoter. The mutation of the Sp1 site is relatively close to the ERE (see Fig. 17A). To determine whether the mutation in the Sp1 site adverse affected ER binding, ChIP assays were performed on the transfected clone 11 cells. The primer set, with one primer belonging to the reporter plasmid sequence, was designed so that only the episomal TFF1 promoter was detected. ERα binding to the episomal TFF1 promoter was detected in the absence and presence of E2 (24 h) for both the native and Sp1-mutated reporter constructs (Fig. 18B). At first glance, it appears that

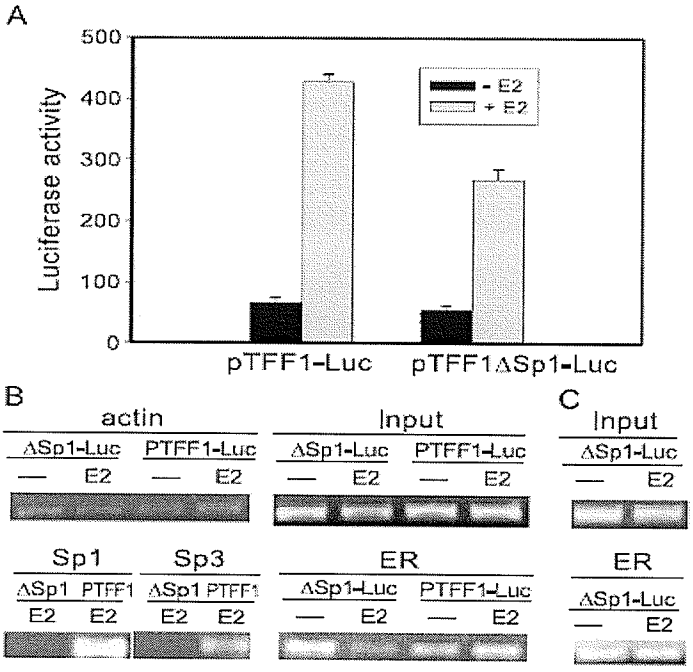


Figure 18. Effect of Sp1 and Sp3 binding on the TFF1 promoter activity. MCF-7 (clone 11) cells were cultured in estrogen-depleted medium for 6 days and induced with doxycycline (1 μg/ml) to express GFP-ER 24 h before transfection. The plasmids pTFF1-Luc and pTFF1ΔSp1-Luc were transfected with pCMXbgal as described in the Materials and Methods section. The transient-transfected cells were incubated with or without E2 (10 nM) in the presence of doxycycline for 24 h. A. Luciferase and β-galactosidase assays were performed and the luciferase activities were normalized to β -galactosidase activities. B.ChIP assays were performed with transfected cells using anti-Sp1, anti-Sp3, anti-ER, and anti-actin (negative control) antibodies. C. ChIP assays were repeated with transfected cells using anti-ER antibodies. Input and ChIP DNA were analyzed by PCR using TFF1 promoter and pGL3 vector specific primers (Sun *et al.*, 2005b).

the loading of the ER onto the pTFF1DSp1-Luc was less than that of ER bound to pTFF1-Luc in E2-treated cells. However, the input of this sample [see input pTFF1DSp1-Luc (E2)] was lower than the other analyzed samples, which accounts for the apparent reduction in abundance of the PCR fragment immunoprecipitated with anti-ER antibodies. In repeats of the ChIP assay, I confirmed that ER loading to the pTFF1DSp1-Luc in clone 11 was similar in cells cultured in the absence and presence of E2 (Fig. 18C). The ChIP assay was also applied to investigate whether the mutation in the Sp1 site prevented Sp1 or Sp3 binding. The same primer set designed for the episomal TFF1 promoter was used in this study. Fig. 18B shows that the mutated Sp1 site did not bind Sp1 or Sp3 in the E2-treated (24 h) clone 11 cells. In contrast, Sp1 and Sp3 did bind to the episomal TFF1 promoter (pTFF1-Luc), with Sp1 binding appearing to be greater than that of Sp3.

2.3.3. ChIP assays demonstrate that Sp1 and Sp3 bind to the endogenous TFF1 promoter *in situ*. To directly determine whether Sp1 and Sp3 are bound to the endogenous TFF1 promoter *in situ*, we applied the ChIP assay with the culture conditions used previously to study the loading of ER, coactivators, and corepressors onto estrogen responsive promoters in human breast cancer cells (Chen *et al.*, 1999;Shang *et al.*, 2000;Shang and Brown, 2002;Burakov *et al.*, 2002). For all of our ChIP assays, we monitored the lengths of the DNA fragments following sonication by agarose gel electrophoresis to ensure that the average DNA fragment length was 400–500 bp. The precipitated DNA was subjected to PCR with the use of specific primers for the TFF1 promoter region. In order to investigate the temporal occupancy of ER, Sp1, and Sp3 onto the endogenous estrogen induced TFF1 gene, we carried out the ChIP assay with antibodies against ER, Sp1, Sp3 and rabbit preimmune IgG (as a negative control) with

MCF-7 cells treated with E2 for 45 mins with 15 mins intervals. The input and ChIP DNA was analyzed with primer sets to the promoter and to the intron A–exon 2 region of the TFF1 gene (Fig. 8). In accordance with the results of others, few TFF1 promoter fragments were associated with ER in MCF-7 cells cultured in the absence of E2 (Fig. 19A) (Shang *et al.*, 2000;Metivier *et al.*, 2003a;Stoner *et al.*, 2004). As shown in Figure 19A, E2 induced a dramatic increase in the occupancy by ER on the TFF1 promoter following 15 min E2 addition. A decline in ER occupancy was consistently noted at 45 min. The occupancy of Sp1 on the TFF1 promoter remained unaltered before and after E2 addition, with a slight but reproducible increase observed at 30 min. Sp3 association was also observed with the TFF1 promoter before and after E2 addition. However, a more pronounced increase in TFF1 promoter occupancy was observed for Sp3 compared to Sp1 at 30 min. As a control, we demonstrated that the TFF1 intron A–exon 2 region was not associated with Sp1, Sp3, or ERα (Fig. 19A). Further, in control ChIP assays with rabbit preimmune IgG, minimal or no TFF1 promoter or intron A–exon 2 sequences were detected.

The amount of the TFF1 promoter immunoprecipitated with anti-Sp1, Sp3 and ER antibodies was quantified and presented as % of input DNA (Fig. 19B). In the presence of estradiol, ER loading steadily increased, peaking at 30 min and then slightly decreasing at 45 min. Following the addition of estradiol, Sp3 association with the TFF1 promoter varied, reaching a maximum at 30 min. In contrast, Sp1 association with the TFF1 promoter was marginally altered.

We also applied ChIP assays to human breast cancer MDA MB 231 (ER-) cells and MCF-7 (ER+) cells grown under estrogen depleted conditions and then treated with 10

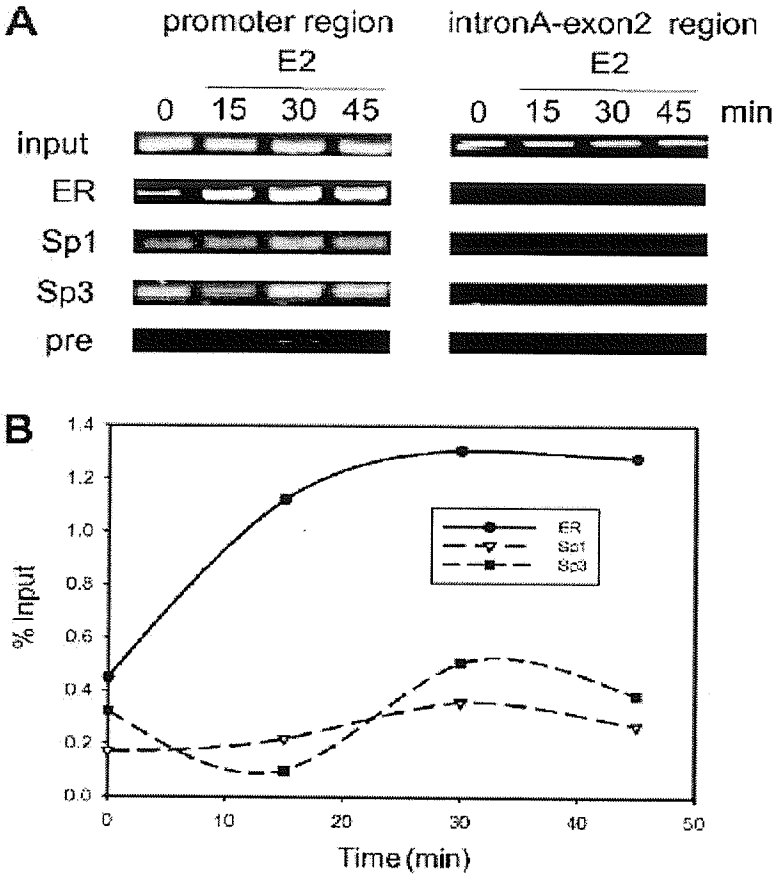


Figure 19. Temporal loading of ERα, Sp1, and Sp3 following addition of estradiol to MCF-7 cells. A. After 2 h treatment with 2.5 mM α-amanitin, cells were washed and placed in media supplemented with 2.5% dextran charcoal treated FCS including 10 nM E2. Cells were cross-linked with formaldehyde at 15 min intervals up to 45 min. ChIP assays were performed with anti-ERα, anti-Sp1, anti-Sp3 and rabbit preimmune (negative control) antibodies. Input and ChIP DNA were analyzed by primers to the TFF1 promoter and to the intron A-exon 2 (see Fig. 17A). B. The amount of ChIP TFF1 sequence was quantified by realtime PCR as indicated in Materials and Methods Section and presented as % of input. The result is representative of an experiment repeated with three separate preparations (Li and Davie, 2008).

nM E2 for 30 min and a longer time course treatment. Estrogen increases the transcriptional activity of the TFF1 gene in MCF-7 cells; but in MDA MB 231 cells, the TFF1 gene is transcriptionally inactive (Kim *et al.*, 2000b;Zhao *et al.*, 2002). After

formaldehyde cross-linking of the MCF-7 (T5) and MDA MB 231 cells, ChIP assays were performed with antibodies directed against ER, Sp1, Sp3, and integrin β (control). Following the addition of E2 to the MCF-7 cells for 30 min, the association of ER with the TFF1 promoters was markedly enhanced. ChIP assays with anti-ER antibodies using MDA MB 231 cells failed to detect TFF1 promoter sequences in the immunoprecipitated DNA, demonstrating the specificity of the ChIP assay with anti-ER antibodies (Fig. 20A). Under estrogen depleted conditions, the TFF1 promoter was bound to Sp1 and Sp3 in MCF-7 cells (Fig. 20B). Following the addition of E2, the TFF1 promoter was bound to Sp3, but there was a marked decrease in the association of the TFF1 promoter with Sp1.

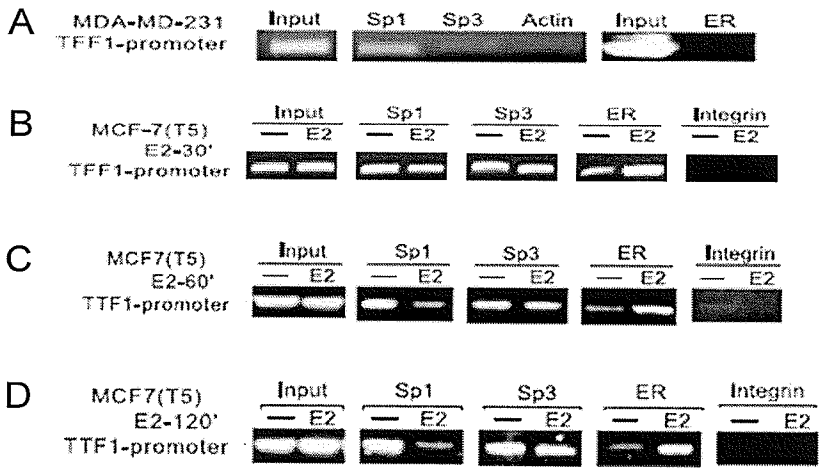


Figure 20. Effect of E2 on the association of Sp1 and Sp3 with the estrogen responsive TFF1 promoter *in situ*. (A) MDA MB 231 cells grown in E2 complete medium were lysed, sonicated, and the complexes were immunoprecipitated by anti-Sp1, anti-Sp3, anti-ER, and anti-actin (control) antibodies. The input and ChIP DNA were analyzed by PCR with primers to the TFF1 promoter (set 1). Panel (B,C,D) MCF-7 (T5) cells, grown with or without (-) 10 nM E2, were lysed and sonicated. The proteins of interest in the lysate were immunoprecipitated by anti-Sp1, anti-Sp3, anti-ER, and anti-integrin (negative control) antibodies. Input and ChIP DNAwere analyzed by PCR with primers to the TFF1 promoter (set 1) E2 incubation for 30 min; panel C, E2 incubation for 60 min; panel D, E2 incubation for 120 min (Sun *et al.*, 2005b).

Analyses of the TFF1 promoter in MDA-MB-231 cells revealed that Sp1, but not Sp3, was associated with the transcriptionally repressed promoter (Fig. 20A). Control experiments (anti-integrin antibodies and MDA-MB-231) were done to check the validity of the ChIP assay data with the antibodies against ER, Sp1, and Sp3. We used anti-integrin antibodies (Fig. 20B) to demonstrate that these antibodies would not immunoprecipitate the cross-linked TFF1 promoter.

The association of ER, Sp1, and Sp3 with the TFF1 promoter at 60 and 120 min post-E2 addition was also monitored by the ChIP assay. Fig. 20C and D show that at both times there was a reduction in the TFF1 promoter fragments associated with Sp1, while the level of TFF1 promoter fragments bound to Sp3 remained unaltered or slightly increased. These results demonstrate that Sp1 and Sp3 are bound to the TFF1 promoter in MCF-7 cells cultured in estrogen depleted conditions, and that following the addition of E2 (30, 60, and 120 min), ER binding is enhanced, Sp3 remains unaltered, and the binding of Sp1 is reduced.

2.3.4. ChIP assays to study the association of KATs and HDACs with the TFF1 promoter. The level of the acetylated H3 and H4 bound to the TFF1 promoter in MCF-7 cells grown in estrogen depleted conditions and then incubated with E2 for 1 h was investigated in ChIP assays with antibodies against acetylated H3 and acetylated H4. Fig. 21A shows that there was an increase in the TFF1 promoter fragments associated with acetylated H3 and H4 following the addition of E2. As a control, anti-integrin antibodies failed to immunoprecipitate TFF1 promoter sequences. The HDACs associated with Sp1 and Sp3 were identified as HDAC1 and phosphorylated HDAC2 (Sun *et al.*, 2002a). As Sp3 is bound to the TFF1 promoter in MCF-7 cells grown in the absence and presence of

E2, we used the ChIP assay to determine whether HDAC1 and HDAC2 were also associated with the TFF1 promoter. Further, our cell culture conditions were comparable to those used by Burakov et al. (Burakov *et al.*, 2002) to study the loading of the KAT CBP and p300 onto the TFF1 promoter following addition of E2. We performed a time course experiment shown in Fig. 21B and observed that HDAC1 and HDAC2 remained bound to the TFF1 promoter at 45, 60, 90, and 120 min post-E2. In contrast to HDACs, ER loading onto the TFF1 promoter cycles on at 45, 60, and 120 min and off at 90 min (Fig. 21B). Sp3 was also released at 90 min but remained loaded at other times (Fig. 21B). Sp1 did not load onto the TFF1 promoter at 90 min and remained low (data not shown).

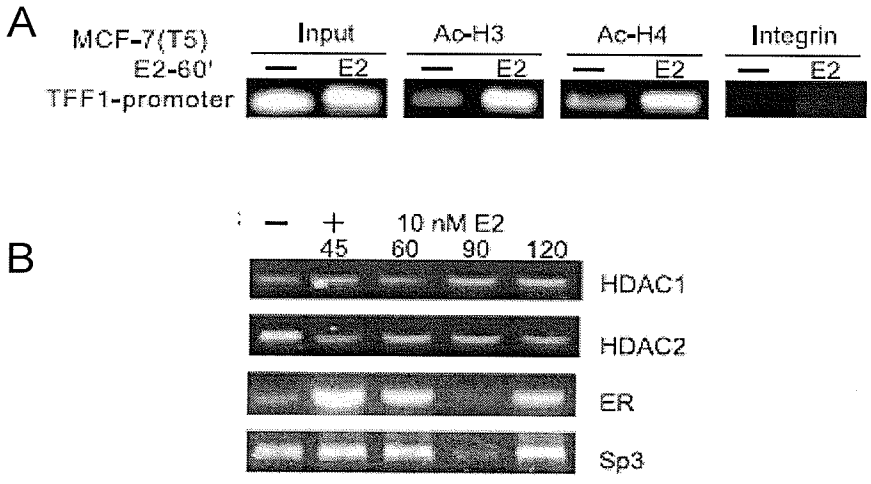


Figure 21. Effect of E2 on the association of acetylated histones, HDAC1 and HDAC2 with the TFF1 promoter. (A) MCF-7 (T5) cells grown in estrogen depleted medium and incubated with or without (-) 10 nM E2 for 1 h, were lysed, and complexes were immunoprecipitated with anti-acetylated H3, anti-acetylated H4, and anti-integrin antibodies. Input and ChIP DNA were analyzed by PCR with primers to the TFF1 promoter (set 2). (B) MCF-7 (T5) cells grown in estrogen-depleted medium with (+) or without (-) 10 nM E2 for a time course of 45–120 min were lysed and sonicated. The proteins of interest in the lysate were immunoprecipitated with anti-HDAC1, anti-HDAC2, anti-Sp3, and anti-ER antibodies. TFF1 promoter sequences in total DNA (input) and ChIP DNA were detected by PCR with set 1 TFF1 promoter primers (Sun *et al.*, 2005b).

However, at 90 min post-E2 addition, Sp3 and ER were both released from the TFF1 promoter (Fig. 21B). The association of both HDACs with the TFF1 promoter throughout the post-E2 time course is in contrast to the KATs (e.g., p300, CBP, SRC-1, SRC-3), which move on and off of the TFF1 promoter during the 120-min time period following the administration of E2 to the cells (Burakov *et al.*, 2002;Metivier *et al.*, 2003a). The anti-HDAC2 antibody detects both unmodified and phosphorylated HDAC2. Our lab demonstrated that phosphorylated HDAC2 is preferentially cross-linked to DNA with formaldehyde in MCF-7 cells (Sun *et al.*, 2002a;Sun *et al.*, 2005a). Although not providing direct evidence, these observations are consistent with a model in which Sp1 and Sp3 recruit HDAC1 and phosphorylated HDAC2 to the TFF1 promoter in the MCF-7 cells grown in the absence and presence of E2.

2.3.5. ERα co-occupies the TFF1 promoter with Sp3 after estrogen addition to cells.

Our previous studies demonstrated that Sp1 and Sp3 have distinct nuclear locations and do not form complexes together (Sun *et al.*, 2002a;He *et al.*, 2005). Since the TFF1 promoter has one Sp1 binding site, we expected that either Sp1 or Sp3 but not both would be associated with the TFF1 promoter. To test this idea we used a re-ChIP assay with antibodies against Sp1 and Sp3. First, the ChIP assay was performed using anti-Sp1 or anti-Sp3 antibody. Then, before reversal of protein–DNA cross-linking, the anti-Sp1 ChIP DNA was subjected to re-ChIP using the anti-Sp3 antibody or *vice versa*. During subsequent PCR only those TFF1 DNA fragments that were simultaneously bound to Sp1 and Sp3 proteins should be amplified. As a control in the re-ChIP assay, the re-ChIP was done with rabbit preimmune IgG. Figure 22A shows that Sp1 and Sp3 do not associate with the same TFF1 promoter in MCF-7 breast cancer cells in the presence or absence of

E2. These results demonstrate a TFF1 promoter is bound to either Sp1 or Sp3. This finding was also established in ZR-75 cells (He *et al.*, 2005). Next we determined whether TFF1 promoters bound to Sp1 or Sp3 were associated with ER following the addition of E2 using the re-ChIP assay. First, the ChIP assay was performed using anti-ER antibody. Then, before reversal of protein–DNA cross-linking, the anti-ER ChIP DNA was subjected to re-ChIP using anti-Sp1 or anti- Sp3 antibodies. As a control, we did not add a second antibody, only beads. During the subsequent PCR only those TFF1

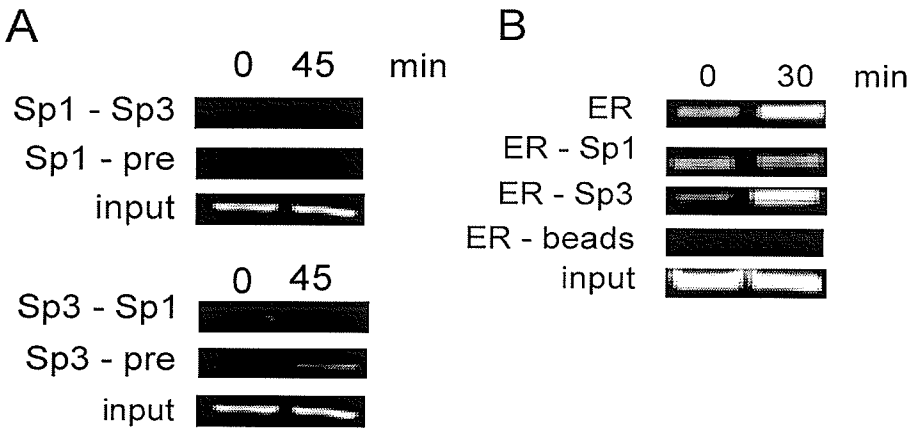


Figure 22. Co-occupancy of ER with Sp1 or Sp3 on the TFF1 promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 mM α-amanitin for 2 h and then incubated with E2 for 30 or 45 min and were further subjected to a re-ChIP assay. A. For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-Sp1 or anti-Sp3 antibodies followed by a second round of ChIP with anti-Sp3 or anti-Sp1 antibodies, respectively. As a control, the second round of ChIP assays was also done with rabbit preimmune antibodies (pre). Input and re-ChIP DNA were analyzed by primers to the TFF1 promoter. B. For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-ERα antibodies followed by a second round of ChIP with anti-Sp1, anti-Sp3 antibodies or no antibodies (negative control). Input and ChIP DNA were analyzed by primers to the TFF1 promoter (Li and Davie, 2008).

DNA fragments that are simultaneously bound to both ER and Sp1 or Sp3 proteins should be amplified. Figure 22B shows that the co-occupancy of Sp1 and ER on TFF1 promoter remains at a similar level regardless of the presence of E2. However, a much

higher level of Sp3 and ER co-occupancy on the TFF1 promoter was observed in the presence of E2. These results suggest that the TFF1 promoter bound to ER is associated with Sp1 or Sp3, with ER bound TFF1 promoters tending to favor co-occupancy with Sp3.

2.3.6. Sp3 and pS5-pol II co-occupy the TFF1 promoter after E2. Since Sp3 may function as an activator or a repressor, we determined whether a Sp3 bound TFF1 promoter could support transcription. To address this question we applied the re-ChIP assay to find out if a TFF1 promoter bound to Sp3 was also associated with the pS5-pol II, the initiating form of the enzyme. The assay was performed with anti-Sp1 or anti-Sp3 antibody for the first ChIP and anti-pS5-pol II for the re-ChIP. As a control, rabbit preimmune IgG was used in the re-ChIP. Figure 23 shows that a TFF1 promoter bound to Sp1 was associated with pS5-pol II in cells treated with E2. Similarly a promoter associated with Sp3 also had pS5-pol II present. This result suggests that a TFF1 promoter associated with Sp1 or Sp3 is transcriptionally active, and provides evidence that Sp3 is acting as an activator in the E2-induced expression of the TFF1 promoter.

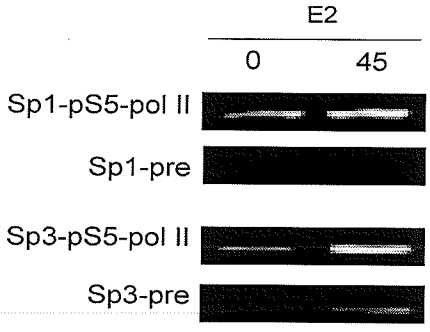


Figure 23. Co-occupancy of pS5-pol II and Sp1 or Sp3 in the TFF1 promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 mM α-amanitin for 2 h and then were treated with E2 for 45 min. The formaldehyde cross-linked chromatin was first ChIPed with antibodies against Sp1 or Sp3. The ChIPed DNA isolated with the Sp1 or the Sp3 antibody were submitted to a second round of ChIP with antibodies against pS5-pol II or rabbit preimmune (pre) antibodies. Input and ChIP DNA were analyzed by primers to the TFF1 promoter (Li and Davie, 2008).

3. Using ChIP and re-ChIP to study the association of ER, Sp1, Sp3 with *c-myc* gene promoter.

3.1. *C-myc* response to estrogen stimulation rapidly in MCF-7 (T5) cells. It has been well-known that *c-myc* is an estrogen responsive gene. To explore the mechanisms for estrogen to regulate the *c-myc* gene, we performed ChIP and re-ChIP on human ER+ MCF-7 breast cancer cells. We initially analyzed the effects of estrogen on the expression of c-Myc in MCF-7 (T5) cells. The cells were starved for estrogen and serum for three days and then treated with α-amanitin for two hours followed by a time course of estrogen treatment. Cells from each time point were collected and one dish was used for RT-PCR to check if *c-myc* responds to estrogen treatment in MCF-7 (T5) cells. In the

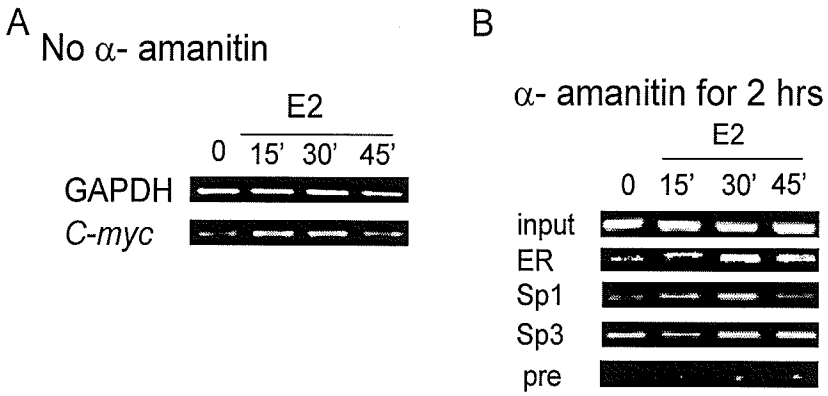


Figure 24. ER, Sp1 and Sp3 associate with the *c-myc* promoter in MCF-7 (T5) cells with and without estrogen treatment. A. MCF-7 cells cultured under estrogen- and serum-deprived conditions were treated for 15, 30, 45 min with 10 nmol/L E2. Cells were harvested at the indicated time and RNA was extracted for further RT-PCR analysis with primers corresponding to c-myc exon 1 and GAPDH exon VII as a loading control. B. After 2 h treatment with 2.5 mM α-amanitin, cells were washed and placed in media supplemented with 2.5% dextran charcoal treated FCS including 10 nM E2. Cells were cross-linked with formaldehyde at 15 min intervals up to 45 min. ChIP assays were performed with anti-ER, anti-Sp1, anti-Sp3 and rabbit preimmune (negative control) antibodies. Input and ChIP DNA were analyzed by primers to the c-myc promoter.

experiment described in Fig. 24A, E2 was added to MCF-7 cells grown in the absence of estrogen, and the expression of *c-myc* mRNA and a housekeeping gene GAPDH (negative control) were tested in the absence and presence of estrogen. Upon E2 (10^{-8} M) treatment, *c-myc* mRNA was detected starting at 15 min. The mRNA levels increased further and remained elevated through the last time point at 45 min.

3.2. Sp1 and Sp3 associate with the *c-myc* promoter differentially in response to estrogen treatment. Analysis of the *c-myc* promoter identified a 116 bp region that is responsible for its estrogen responses (Dubik and Shiu, 1992). This estrogen responsive region (+25 to +141) of c-*myc* promoter harbors two Sp1/3 sites involved in its promoter activity and Sp1 was found to be the principle protein bound to it (Dubik and Shiu, 1992;Miller *et al.*, 1996). However, this *c-myc* promoter lacks an ERE or a half site ERE (Dubik and Shiu, 1992;Vaquero and Portugal, 1998;Albert *et al.*, 2001). Using the ChIP assay, several labs have verified the binding of ER to the *c-myc* promoter *in situ* (Shang *et al.*, 2000;Liu and Bagchi, 2004;Zou *et al.*, 2007). We next determined whether E2 induced the expression of *c-myc* through the recruitment of ER, Sp1 and Sp3 to the estrogen responsive region of the c-*myc* promoter by employing the ChIP assay (Fig. 24B). MCF-7 cells grown under estrogen depleted conditions were treated with or without 2.5 mM α-amanitin for 2 h and then were exposed to E2 (10^{-8} M) for 45 min. The cells were harvested and chromatin was isolated following 1% formaldehyde cross-linking. The crosslinked chromatin was fragmented and immunoprecipitated with antibodies against ER, Sp1 and Sp3. The DNA obtained from immunoprecipitated chromatin was amplified using primers flanking the estrogen responsive region of the P2 promoter. The preimmuno serum was used as antibody negative control (Fig. 24B). Our

results indicated that in the absence of E2, ER, Sp1 and Sp3 all associate with the c-*myc* (Fig. 24B) promoter. In the presence of E2, ER binding to the estrogen responsive region of c-*myc* increased in the presence of estrogen (Fig. 24B). Both Sp1 and Sp3 associate with the promoter in a dynamic pattern, although Sp3 loading increased more with estrogen treatment than the loading of Sp1. The negative preimmune-serum control indicated that the observed receptor and cofactor interactions were specific. Collectively, these results indicated that E2-mediated expression of c-*myc* genes involves the interaction of ER, Sp1 and Sp3 with the estrogen responsive promoter region.

3.3. ER co-occupies the *c-myc* P2 promoter with Sp1 or Sp3 after estrogen addition to cells. Increasing evidence indicates that the promoters of a number of estrogen responsive genes without consensus EREs or lacking *bona fide* EREs use Sp1 to recruit ER proteins in response to estrogen. It was postulated that ER is recruited to the P2 promoter through Sp1 (Dubik and Shiu, 1992), which is true for several reported genes (Krishnan *et al.*, 1994;Porter *et al.*, 1996;Bruning *et al.*, 2003;Salvatori *et al.*, 2003). Both Sp1 and Sp3 physically interact with the ER (Gadbois *et al.*, 1992). Using re-ChIP assays, we examined whether Sp1 or Sp3 coexist with ER on the estrogen responsive region of the same *c-myc* promoter. The MCF-7 cells used in this study were treated with α-amanitin for 2 h before E2 treatment. First, the ChIP assay was performed using an anti-ER antibody. Then, before reversal of protein–DNA cross-linking, the anti-ER ChIP DNA was subjected to re-ChIP using anti-Sp1 or anti- Sp3 antibodies. As a control, we did not add a second antibody, only beads. During the subsequent PCR only those *c-myc* DNA fragments that were simultaneously bound to both ER and Sp1 or Sp3 proteins should be amplified. Figure 25A shows that the co-occupancy of Sp1 and ER on the *c-*

myc promoter remains at a similar level regardless of the presence of E2. However, much higher level of Sp3 and ER co-occupancy on the *c-myc* promoter were observed in the presence of E2. These results suggest that the *c-myc* promoter bound to ER associates with Sp1 or Sp3, with ER bound *c-myc* promoters tending to favor co-occupancy with Sp3. The anti-Sp3 antibodies used in this experiment were directed against the C-terminal peptide epitopes and these antibodies specifically recognized all Sp3 isoforms.

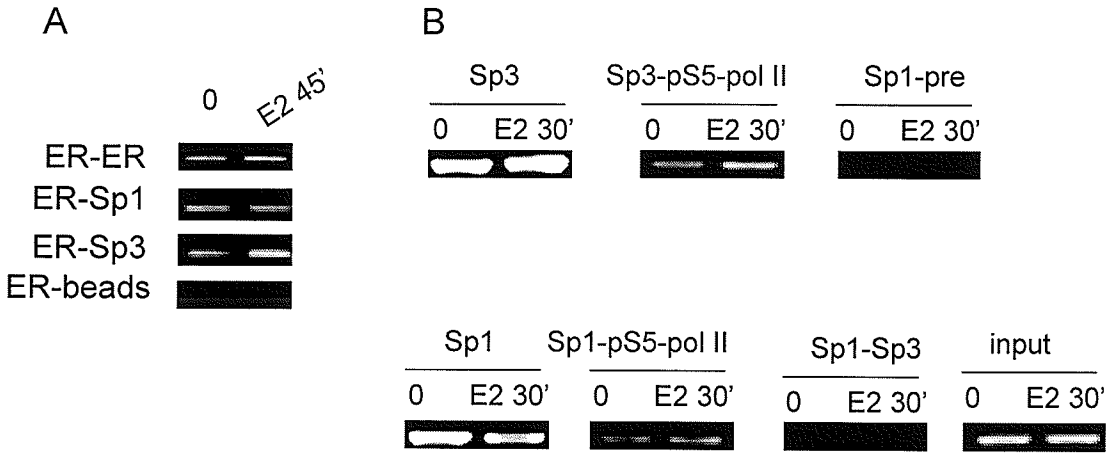


Figure 25. Analysis of co-occupancy of ER, Sp1, Sp3 and pS5-pol II on the *c-myc* promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 mM α-amanitin for 2 h and then incubated with E2 for 30 or 45 min and were further subjected to a re-ChIP assay. A. For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-ER antibody followed by a second round of ChIP with anti-Sp1 or anti-Sp3 antibodies, respectively. As a control, the second round of ChIP assays was also done with only beads. Input and re-ChIP DNA were analyzed by primers to the *c-myc* promoter. B. For the first round of ChIP, the formaldehyde cross-linked chromatin was first ChIPed with anti-Sp3 or anti-Sp1 antibody followed by a second round of ChIP with anti-pS5-pol II, anti-Sp3 antibodies or rabbit preimmune IgG (negative control). Input and ChIP DNA were analyzed by primers to the *c-myc* promoter.

3.4. Co-occupancy of Sp1 and Sp3 on *c-myc* promoter. As described above, the TFF1 promoter has one Sp1/3 binding site and we demonstrated that a TFF1 promoter is bound

to either Sp1 or Sp3. *C-myc* has two Sp1/3 binding sites in its estrogen responsive region of the P2 promoter and both Sp1 and Sp3 associate with this region. It is possible that both Sp1 and Sp3 reside on the same P2 promoter of *c-myc* gene. To test this idea we used a re-ChIP assay with antibodies against Sp1 and Sp3. First, the ChIP assay was performed using anti-Sp1 or anti-Sp3 antibody. Then, before reversal of protein–DNA cross-linking, the anti-Sp1 ChIP DNA was subjected to re-ChIP using the anti-Sp3 antibody or *vice versa*. During the subsequent PCR only those *c-myc* DNA fragments that were simultaneously bound to Sp1 and Sp3 proteins should be amplified. As a control in the re-ChIP assay, the re-ChIP was done with rabbit preimmune IgG. Figure 25B shows that Sp1 and Sp3 do not associate with the same P2 promoter of the *c-myc* gene in MCF-7 breast cancer cells in the presence or absence of E2. Again, these results demonstrate that the P2 promoter is bound to either Sp1 or Sp3 even though there are two Sp1/3 sites in the P2 promoter of the *c-myc* gene.

3.5. Sp3 and pS5-pol II co-occupy the *c-myc* promoter after E2 treatment. *C-myc* is an immediate early gene and it has totally different promoter context than that of TFF1. We expected to see a different way for Sp proteins to support *c-myc* gene transcription. Hence, the re-ChIP assay was performed to find out how Sp proteins co-occupy the *c-myc* promoter with pS5-pol II, the initiating form of the enzyme. The assay was done with anti-Sp1 or anti-Sp3 antibody for the first ChIP and anti-pS5-pol II for the re-ChIP. As a control, rabbit preimmune IgG was used in the re-ChIP. Figure 22B shows that a *c-myc* P2 promoter bound to Sp1 was associated with pS5-pol II in cells treated with E2. Similarly a promoter associated with Sp3 also had pS5-pol II present (Fig. 25B), although the co-occupancy increased in the presence of estrogen. This result suggests that a P2

promoter associated with Sp1 or Sp3 is transcriptionally active, and provides evidence that Sp3 is acting more like an activator in the E2-induced expression of the *c-myc* promoter.

4. ER, Sp1 and Sp3 associate with the estrogen responsive genes promoters as of cyclin D1, cathepsin D, TGFα and RAR. There is a long list of estrogen responsive genes with Sp1/3 binding sites in their estrogen responsive regions. The estrogen responsive genes studied here have different promoter contexts (Fig. 10) with either a half site ERE (cathepsin D, TGFα, RAR) or no ERE (cyclin D1, *c-myc*). Cathepsin D, RARα and TGFα have half site EREs located near one or more Sp binding sites, and these Sp binding sites are required for the estrogen response (Krishnan *et al.*, 1994;Wang *et al.*, 1997;Sun *et al.*, 1998;Vyhlidal *et al.*, 2000;Castro-Rivera *et al.*, 2001a). These genes were studied with ChIPs to directly determine whether Sp1 and Sp3 are bound to these endogenous promoters. The temporal association of ER with these different α-amanitin synchronized promoters in cells incubated with and without estradiol was determined in kinetic ChIP assays and quantified as described for the TFF1 promoter. Fig. 26 shows that the bimodal cycling of ER in the absence of estradiol was observed for all tested estrogen responsive promoters regardless of the nature or presence of an ERE. The cyclic loading of ER onto the RARα and TGFα promoters with half site EREs was similar to that of ER's temporal association with the c-*myc* promoter, which may lack an ERE. The amplitude of ER loading throughout the time course varied among the various promoters. In the presence of estradiol, ER loading onto the promoters generally increased, reaching a maximum at 30 min. ER association at 45 min following estradiol

addition declined markedly for cathepsin D, cyclin D1, RARα and TGFα promoters, but only a minor decline was observed for the TFF1 and c-*myc* promoters.

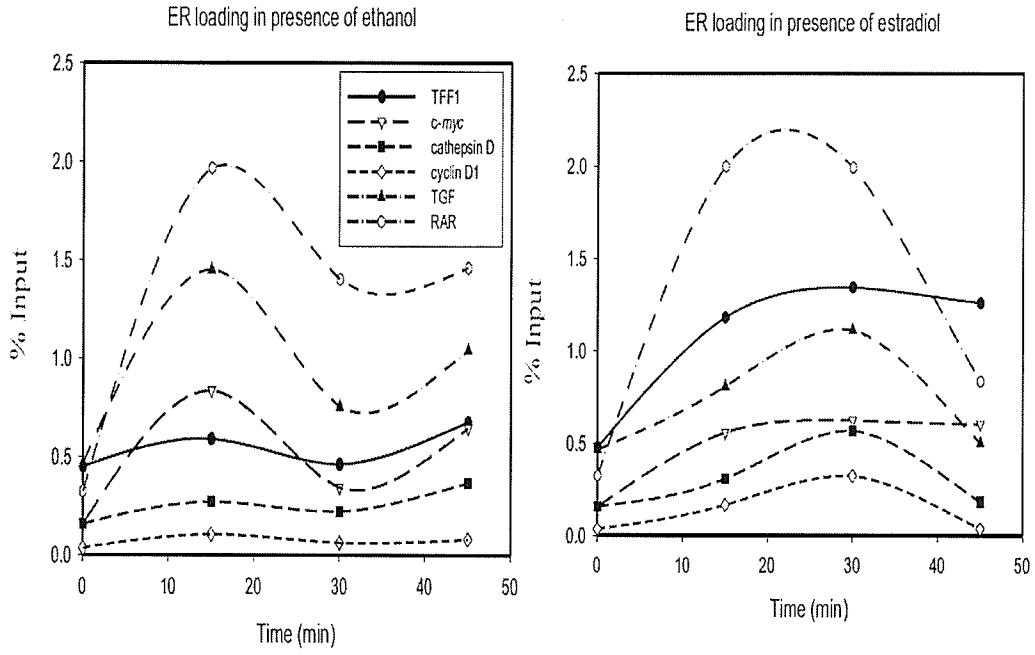


Figure 26. Dynamics of unliganded and liganded ER binding to estrogen responsive promoters. After 2 h incubation with 2.5 μM α-amanitin, MCF-7 cells were washed with PBS and exposed to either 10^{-8} M estradiol or ethanol for the times indicated. Cells from each time point were subjected to the ChIP assay with the antibodies against Sp1, Sp3 or ER. Cells from each time point were subjected to the ChIP assay with anti-ER antibodies, and the input and ChIP DNA were subjected to PCR with primers sets to the promoters indicated in the inset. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

4.1. Dynamics of Sp1 and Sp3 association with estrogen responsive promoters. As demonstrated with the TFF1 promoter (Fig. 8), Sp1 temporal association with the TGFα, RARα, cathepsin D, cyclin D1 and c-*myc* promoters was different from that of Sp3. The loading of Sp1 and Sp3, respectively, onto the TGFα and RARα promoters in cells treated with and without estradiol was similar (Fig. 27). In the absence of estradiol, Sp1 loading onto these promoters increased, reaching a maximum at 30 min and then declined,

more so for the RARα promoter. In comparison, Sp3 loading reached a maximum at 15 min and then declined. In the presence of estradiol, the loading of Sp1 on the TGFα promoter varied slightly with time, reaching a maximum at 30 min. The loading dynamics of Sp1 onto the RARα promoter was somewhat different from that of TGFα in that loading increased at 45 min. Sp3 loading onto the TGFα and RARα promoters following the addition of estradiol exhibited the greatest amplitudes, with decreased loading at 15 min, increased loading at 30 min, followed by a decreased loading at 45 min. For the cathepsin D promoter, Sp1 loading remained relatively constant following release of the transcription block in cells incubated with and without estradiol (Fig. 27). In the absence of estradiol, loading of Sp3 decreased as function of time. However, in the presence of estradiol, Sp3 showed the cyclical loading pattern observed with the other promoters. The loading patterns of Sp1 and Sp3 onto promoters lacking EREs (c-*myc* and cyclin D1) were distinct from the ERE containing promoters and distinct from each other (Fig. 28). In the presence of ethanol, Sp1 loading onto the c-*myc* promoter had a cyclical pattern being greater at 15 and 45 min and lower at 30 min. With the cyclin D1 promoter, Sp1 loading increased throughout the time course. Sp3 loading onto the c-*myc* promoter remained relatively constant throughout the time course in the absence of estradiol, while Sp3 loading onto the cyclin D1 promoter initially decreased and then increased. In the presence of estradiol, loading of Sp1 on the c-*myc* and cyclin D1 promoters remained relatively constant, although Sp1 loading declined at 30 min with the c-*myc* promoter. Sp3 loading on the c-*myc* promoter initially decreased and then increased for the remainder of the time course with estradiol, while Sp3 loading on the cyclin D1 promoter

had a cyclical loading pattern somewhat similar to that observed with the ERE containing promoters, but with a lesser amplitude.

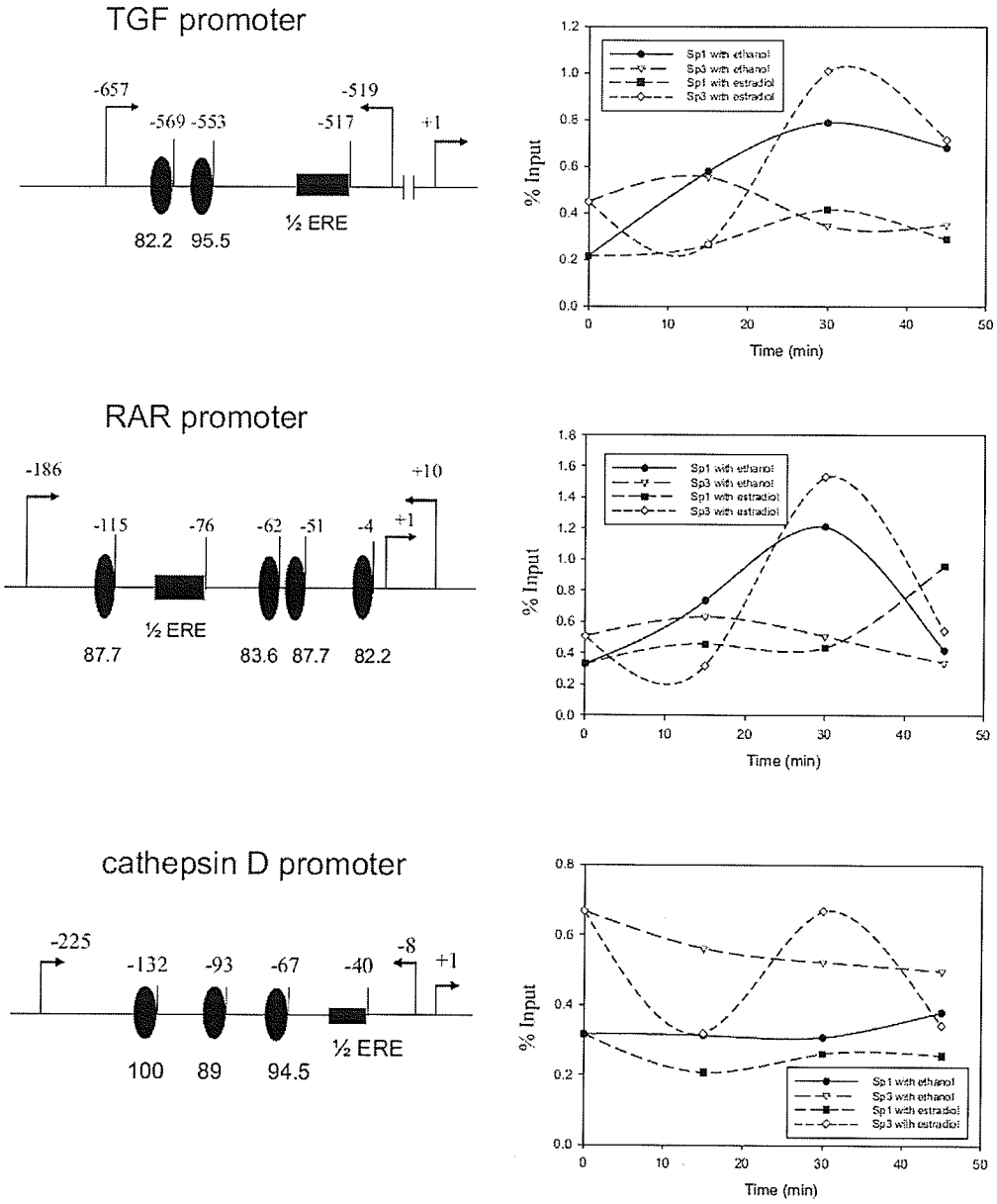


Figure 27. Dynamics of Sp1 and Sp3 association with TGFα, RARα and cathepsin D promoters. The left panels show schematics of the TGFα, RARα and cathepsin D promoters with the ovals showing the position of the Sp binding sites relative to the half site ERE. The TRANSFAC scores of the Sp binding sites are shown. The right panels show the kinetic ChIP assays with antibodies against Sp1 and Sp3 with cells treated with α-amanitin followed by incubations with and without E2. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

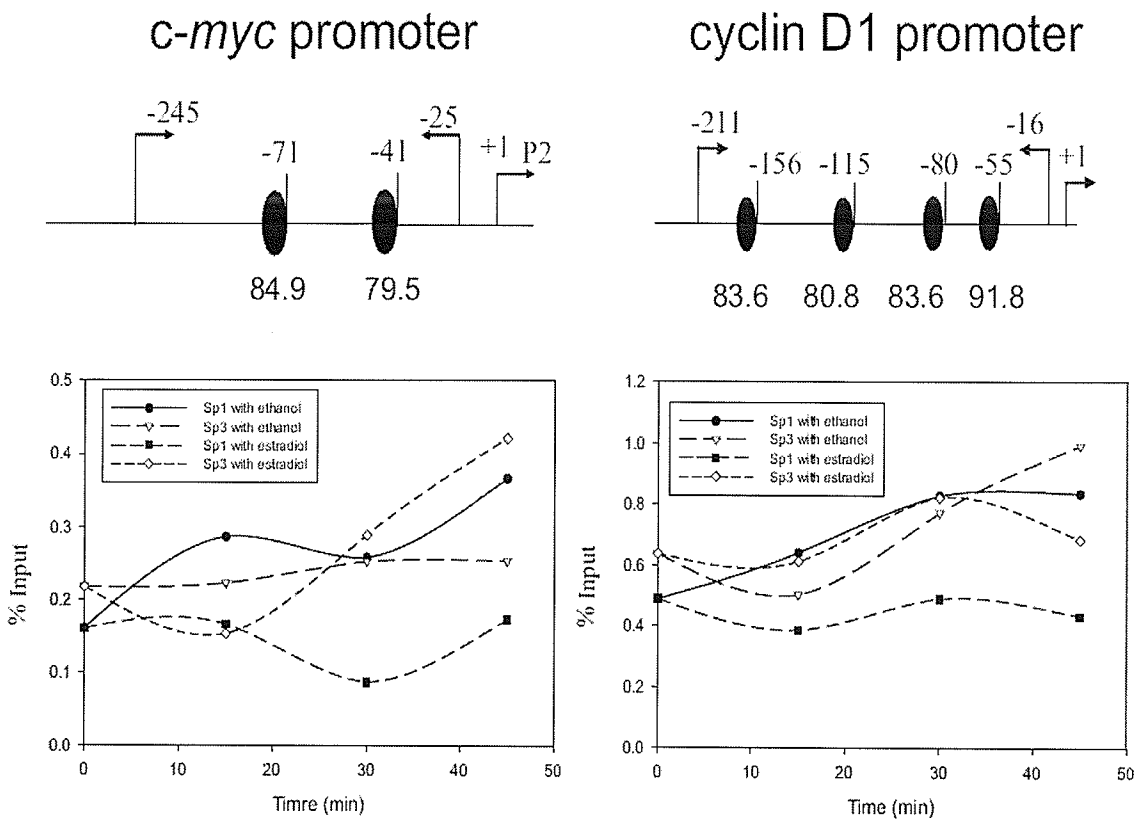


Figure 28. Dynamics of Sp1 and Sp3 association with the c-*myc* and cyclin D1 promoters. The upper illustrations show schematics of the c-*myc* and cyclin D1 promoters with the ovals showing the position of the Sp binding sites. The TRANSFAC scores of the Sp binding sites are shown. The lower panels show the kinetic ChIP assays with antibodies against Sp1 and Sp3 with cells treated with α-amanitin followed by incubations with and without estradiol. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

4.2. Relative loading of ER and Sp factors on estrogen responsive promoters. The relationship between the Sp factors and ER were compared. For RARα and TGFα promoters, Sp1 association with these promoters was maximal at 30 min in the absence of estradiol when ER binding had declined. Sp3 association declined throughout the time course (Fig. 29). In the presence of estradiol, Sp3 binding to these promoters was maximal at 30 min when the ER association was increased. For both promoters the

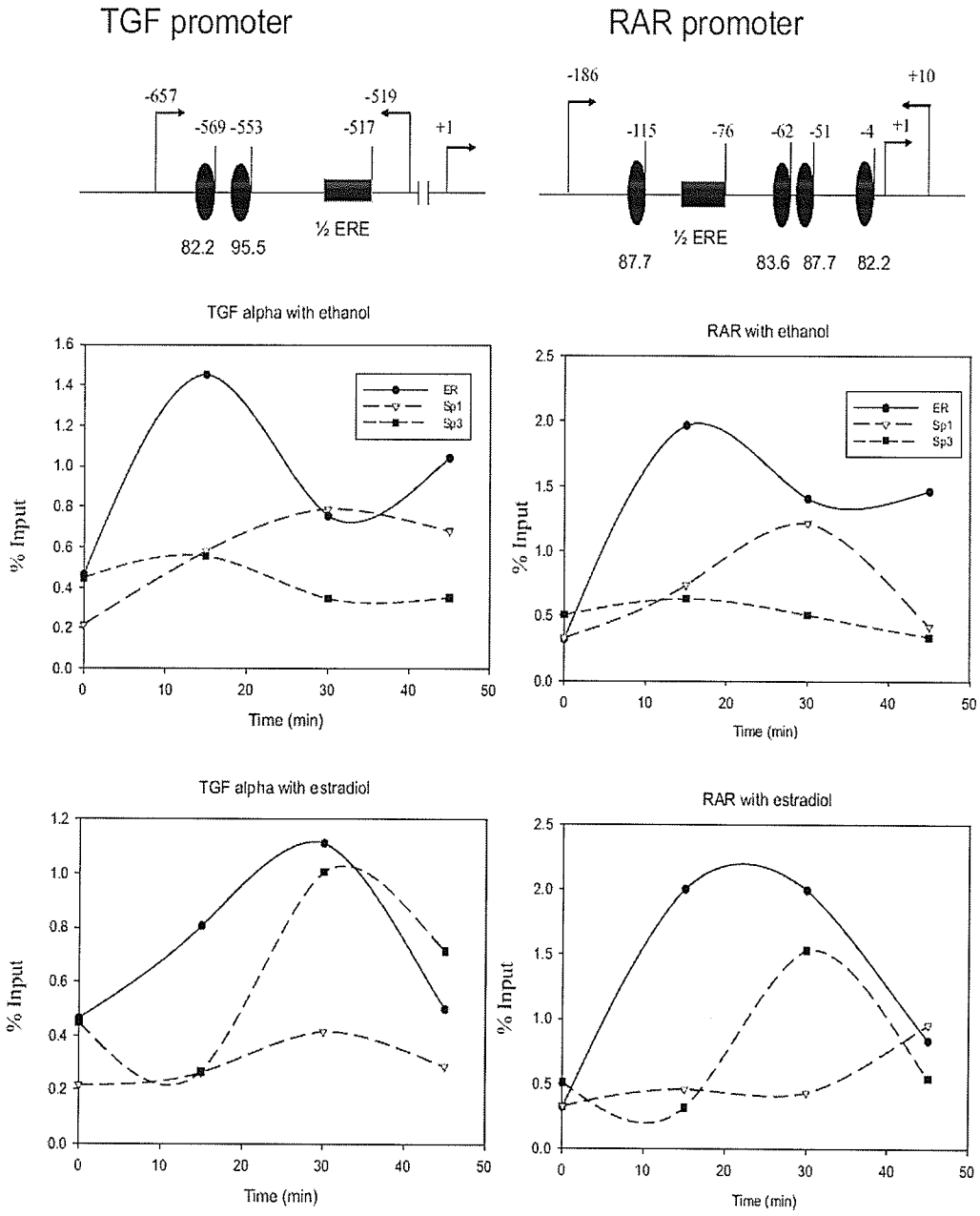


Figure 29. Comparative dynamics of Sp1, Sp3 and ER association with the TGFα and RARα promoters. The upper panels show schematics of the TGFα and RARα promoters with the ovals showing the position of the Sp binding sites relative to the half site ERE. The TRANSFAC scores of the Sp binding sites are shown. The lower panels show the kinetic ChIP assays with antibodies against Sp1, Sp3 and ER with cells treated with α-amanitin followed by incubations with and without estradiol. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

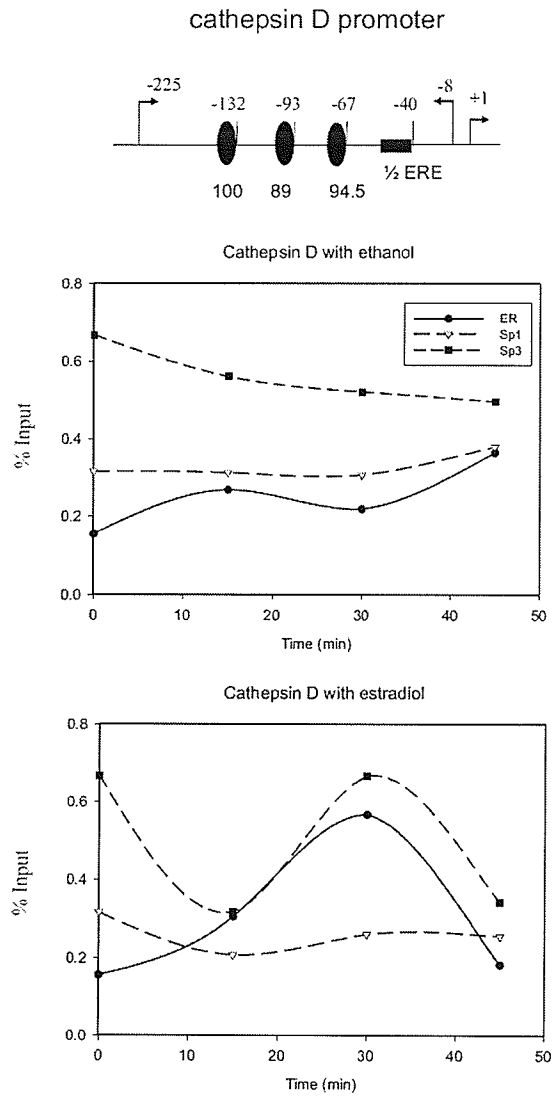


Figure 30. Comparative dynamics of Sp1, Sp3 and ER association with the cathepsin D promoter. The upper panel shows a schematic of the cathepsin D promoter with the ovals showing the position of the Sp binding sites relative to the half site ERE. The TRANSFAC scores of the Sp binding sites are shown. The lower panels show the kinetic ChIP assays with antibodies against Sp1, Sp3 and ER with cells treated with α-amanitin followed by incubations with and without estradiol. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

increased loading of Sp3 was delayed when compared to that of ER. For the cathepsin D promoter, Sp1 remained unaltered while the Sp3 association declined in cells not treated with estradiol and ER cycled with minor changes in amplitude (Fig. 30). The association of Sp3 with the cathepsin D promoter was similar to that of RARα and TGFα promoters

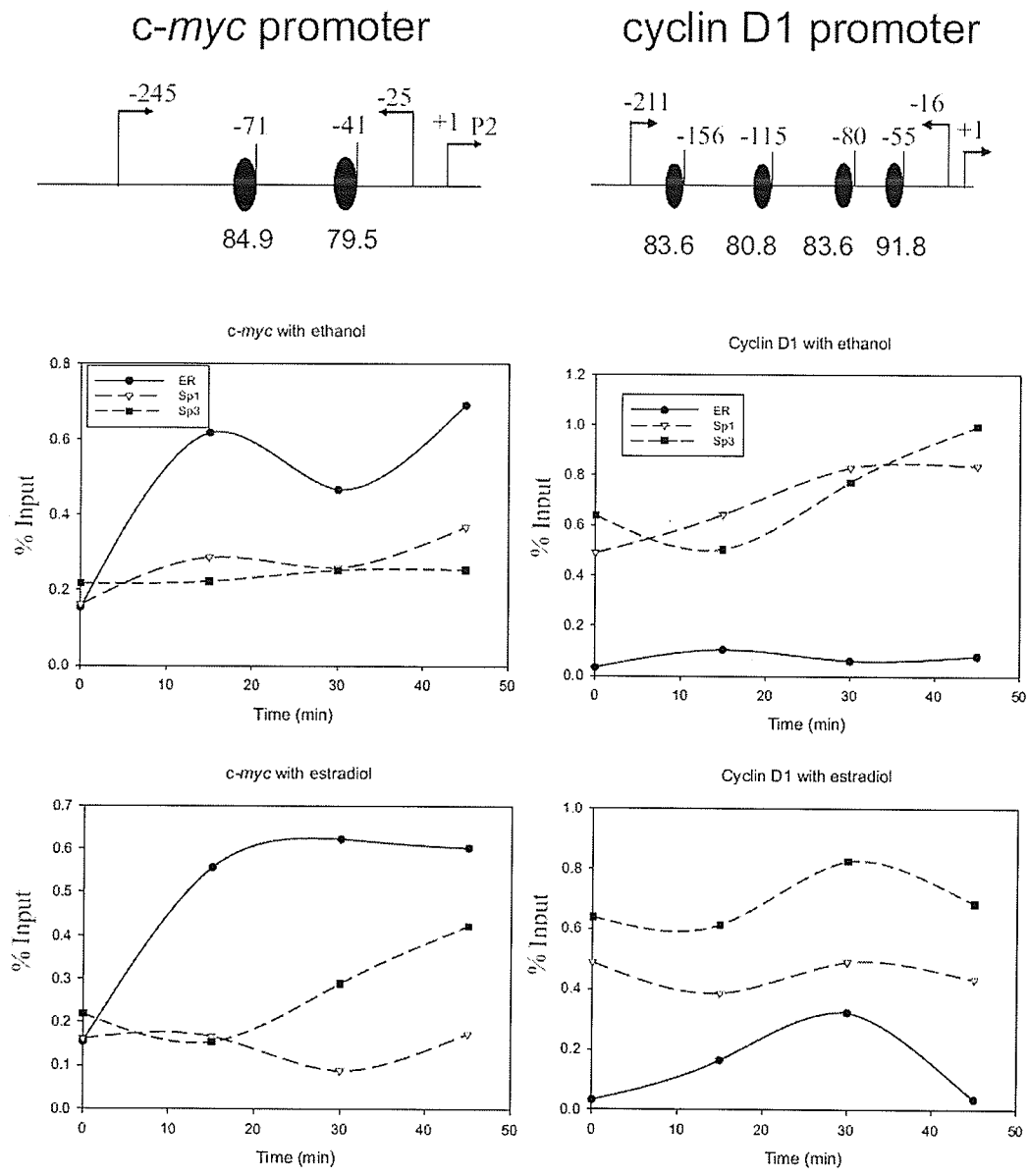


Figure 31. Comparative dynamics of Sp1, Sp3 and ER association with the c-*myc* and cyclin D1 promoters. The upper illustrations show schematics of the c-*myc* and cyclin D1 promoters with the ovals showing the position of the Sp binding sites. The TRANSFAC scores of the Sp binding sites are shown. The lower panels show the kinetic ChIP assays with antibodies against Sp1, Sp3 and ER with cells treated with α-amanitin followed by incubations with and without estradiol. The amount of immunoprecipitated promoter was quantified as indicated in the Materials and Methods section and presented as % of input.

being greatest at 30 min with estradiol which was the time that ER binding was maximal. The association of Sp1 with the cathepsin D promoter remained essentially the same throughout the time course. C-*myc* and cyclin D1 promoters lack an ERE. In the absence of estradiol, Sp3 association with the c-*myc* promoter remained unaltered throughout the time course, while Sp1 cycling on the promoter paralleled that of ER; although the amplitude of the Sp1 cycle was considerably less than that of ER (Fig. 31). With the cyclin D1 promoter, both Sp3 and Sp1 association increased following 15 min, while ER cycled with low amplitude. In the presence of estradiol, Sp3's association with the c-*myc* promoter steadily increased following 15 min and this increase in Sp3 association was delayed compared to the increase in liganded ER. Sp3 and Sp1 association with the cyclin D1 promoter was maximal at 30 min post-estradiol, with the increase in Sp3 loading being delayed compared to that of liganded ER. A common feature of the RARα, TGFα, c-*myc* and cyclin D1 promoters was Sp3 increased association was delayed compared to that of liganded ER.

5. Characterization of Sp family transcription factors binding to the DPYD promoter region in vitro and *in situ*. EMSA and super shift assays suggested that Sp1 and Sp3 may be involved in the regulation of the DPYD gene (Zhang et al., 2006). To directly determine whether ER, Sp1 and Sp3 are bound to the endogenous DPYD promoter *in situ*, ChIP assays were applied to study the loading of Sp1 and Sp3 onto the DPYD promoter in HeLa cells harvested at about 95% confluency. In accordance with the EMSA result, Sp1, and to a much lesser extent, Sp3 binds to the DPYD promoter *in situ* in Hela cells (Fig. 32).

The *DPYD* gene is one estrogen non-responsive house-keeping gene constitutively expressed in MCF-7 cells. ChIP assays were also performed to study the association of ER, Sp1 and Sp3 with the DPYD promoter in MCF-7 cells. Similar to the observed results for Hela cells, ChIP assays demonstrated the binding of Sp1 and to a much lesser extent of Sp3 on the DPYD promoter in MCF-7 cells. However, ChIP assays were unable to detect ER association with DPYD promoter in MCF-7 cells grown under estrogen depleted conditions and then treated with estradiol for 30 min.

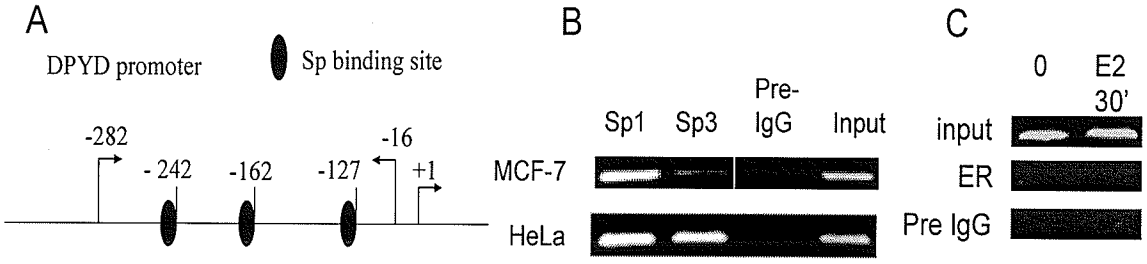


Figure 32. The association of ERα, Sp1, and Sp3 with the DPYD gene promoter in MCF-7 cells. A.DPYD promoter map shows Sp binding sites (-282 and -16 are the position of primers for the ChIP assay shown in B and C. B. The association of Sp1 and Sp3 on DPYD promoter. C. The loading of ER on DPYD promoter in MCF-7 cells grown under estrogen depleted conditions and then 30 min with estradiol. Data shown are representative of three independent experiments.

Methylation of the cytosine residue in the 5'-CpG-3' sequence is a recognized epigenetic mechanism of gene silencing. CpG methylation of the regulatory region of the DPYD promoter suppresses DPYD transcription in RKO cells. 5-Aza-2'-deoxycytidine treatments induced demethylation of the promoter and markedly increased DPYD mRNA levels in RKO cells (Zhang et al., 2007). The same cells treated with 5-Aza-2'-

deoxycytidine treatments were used for the ChIP assay to compare the Sp1 and Sp3 binding pattern on DPYD promoters. Fig. 33A shows that Sp1 and Sp3 load on the DPYD promoter in DPYD repressing RKO cells and DPYD expressing Hela cells. Fig. 30B suggests much more Sp1 binds to demethylated DPYD promoters and the Sp3 loading diminished in DPYD depressed RKO cell lines (Fig. 33B).

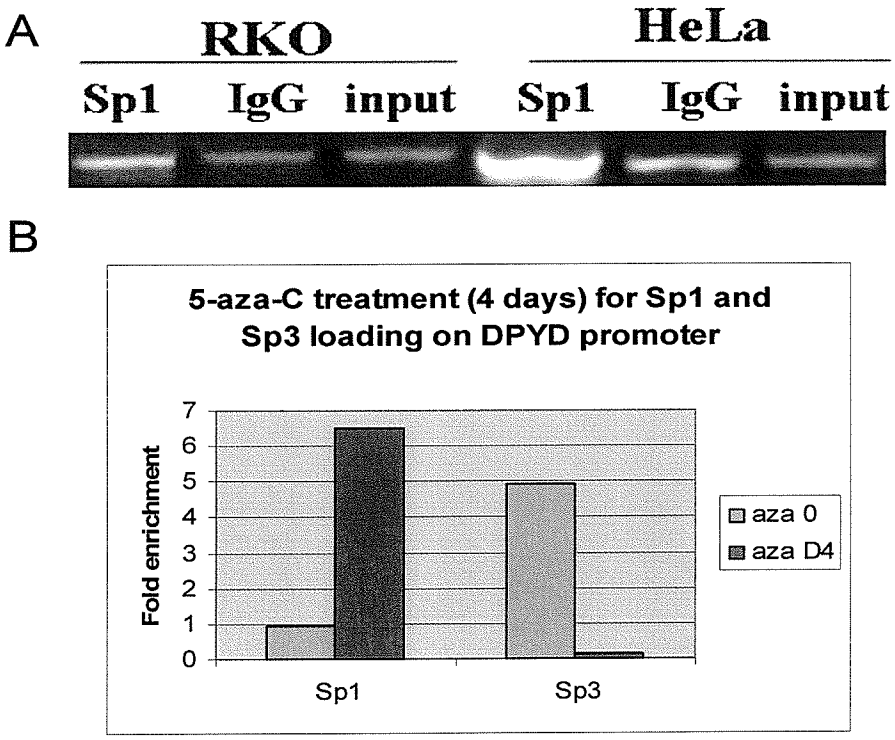


Figure 33. Sp1 and Sp3 bind to the DPYD promoter region in RKO cells. A. ChIP assay to show Sp1 and Sp3 loading on the DPYD promoter in Hela and T5 cells. Antibodies used in the immunoprecipitation are shown above the picture. B. ChIP assay to show Sp1 and Sp3 loading on DPYD promoter in the absence and presence of 5-aza-C in RKO cells. Data shown are representative of three independent experiments (Zhang *et al.*, 2006).

6 . Some pilot studies of this project.

6.1. Preliminary analysis of relatively unstudied estrogen responsive genes. Pilot experiments were done with several not as well-studied estrogen responsive genes such as p21, p53 and ERα. To study the loading pattern of ER, Sp1 and Sp3 on these gene promoters, RT-PCR and ChIP were applied to MCF-7 (T5) cells grown in estrogen depleted DMEM in the presence and absence of estrogen. When treated with estrogen (10nM), p21 expression decreased (Fig. 34A) and the ERα loading on the tandem Sp1 region also decreased (Fig. 34B). However, the Sp1 and Sp3 loading did not change.

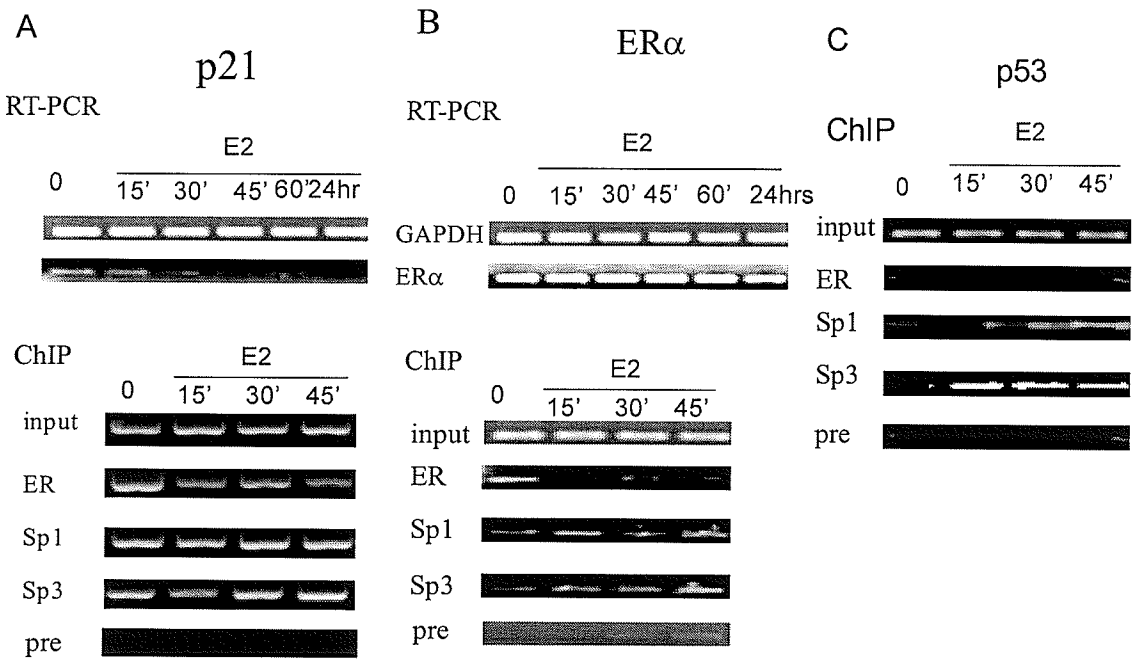


Figure 34. ER, Sp1, Sp3 load on to ERα, p21 and p53 gene promoters. RT-PCR and ChIP assays were performed as described in the Materials and Methods section.

Surprisingly, ERα expression levels did not change in the presence of estrogen (Fig. 34B), although the loading pattern of ERα, Sp1 and Sp3 to the *ERα* gene promoter is very similar to that of the p21 promoter (Fig. 34B). The loading of ERα, Sp1 and Sp3 on the p53 promoter did not change in the presence of estrogen (RT-PCR data not shown Fig. 34C), although it was interesting to know that Sp3 became the major Sp protein loaded its promoter in the presence of estrogen. This result is consistent with the observation that high dose estrogen, but not low dose, induced p53 expression due to p21 induction (Wright *et al.*, 2005). It is important to note that these studies were just pilot non-published observation.

6.2. Lysine sumoylation and acetylation on TFF1 promoter. Both Sp1 and Sp3 can be sumoylated. Acetylation is a mark of activated gene transcription and sumoylation is a mark of gene repression. We expected to see higher levels of lysine acetylation and lower

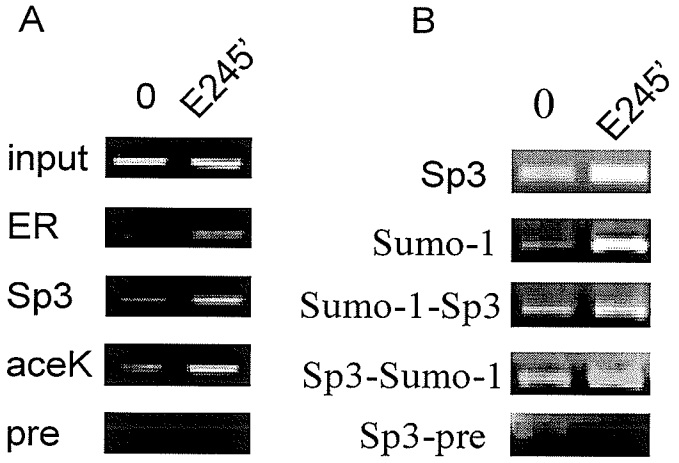


Figure 35. Lysine acetylation and sumoylation of the TFF1 promoter. MCF-7 cells grown in E2-deprived medium were first treated with 2.5 mM α-amanitin for 2 h and then were treated with E2 for ChIP and re-ChIP assays.

lysine sumoylation on the TFF1 promoter in the presence of estrogen. ChIPs were applied using the conditions described above. To our surprise, these pilot experiments suggest that both lysine acetylation and sumoylation level on TFF1 promoter increased after estrogen treatment (Fig. 35A,B). After the first ChIP with a sumo-1 antibody, re-ChIP was performed to test whether Sp3 co-occupies the same DNA fragment with the sumoylated lysine. Interestingly, re-ChIPs suggest that the co-ocupancy of Sp3 and sumo-1 increased on the TFF1 promoter in response to estrogen treatment (Fig. 35B).

V. MAJOR FINDINGS

As well-established estrogen responsive genes, TFF1 and *c-myc* have been extensively studied. We were the first to identify the Sp1/3 site upstream of ERE site as estrogen responsive element on the TFF1 promoter in MCF-7 breast cancer cells. We were also the first to show that Sp1 and Sp3 associate with *c-myc* P2 promoter *in situ*.

TFF1 gene has one Sp1/3 site and one ERE site in its estrogen responsive region and *c-myc* has only two possible Sp1/3 sites in its estrogen responsive P2 promoter. Using *in situ* ChIP and re-ChIP, the present study demonstrated for the first time that Sp3, instead of Sp1, is the Sp protein preferentially associated with estrogen initiated TFF1 and *c-myc* gene promoters. In contrast, Sp1 was found engaged on the constitutively expressed DPYD gene promoter in cycling Hela and MCF-7 cells.

Our previous laboratory studies showed that Sp1 and Sp3 do not coexist in the same cellular complex. Using re-ChIP assay, this present study was also the first to demonstrate that Sp1 and Sp3 do not co-occupy the same TFF1 or *c-myc* p2 promoters in MCF-7 breast cancer cells, although these two gene promoters have different promoter contexts.

Dynamic ChIP assays were performed on six estrogen responsive genes in MCF-7 cells. It was found that the Sp3 occupancy on all the promoters became more dynamic after estrogen treatment. In addition, the occupancy of Sp3 is always lagged behind the occupancy of ER.

VI. DISCUSSION

The goal of this study is to evaluate the roles of Sp1 and Sp3 in the regulation of estrogen responsive genes in response to estrogen activity in breast cancer cells. Sp1 has been mostly reported as a transcriptional activator, and the functional role of Sp3 is a repressors or weak activator in gene regulation (Ammanamanchi and Brattain, 2001;Zhang *et al.*, 2003a;Ou *et al.*, 2004;Clem and Clark, 2006;Yao *et al.*, 2007;Hua *et al.*, 2007). Both Sp1 and Sp3 can exert their functions through interactions with the core promoter complexes (Ge *et al.*, 2003). The mixed results reported in the literature due to the complicated modifications of these proteins, their interacting proteins, promoter and the cellular contexts (Ibanez-Tallon *et al.*, 2002;Li *et al.*, 2004). In the study of Dnmt1 gene regulation, Sp1 and Sp3 were found recruited to the promoter differentially in a cell-cycle dependent manner (Kishikawa *et al.*, 2002). Another study for the NR1 promoter suggests that Sp1 and Sp3 take turns as active regulator depending on whether the cells are differentiated or not (Liu *et al.*, 2004a). Regarding the role of Sp1 and Sp3 in the regulation of estrogen responsive genes, Sp1 was reported mostly as the one factor regulating estrogen induced gene transcription (Safe, 2001). However, many of these works were done *in vitro*. Using ChIP assays, Sp1 and Sp3 were found to recognize and bind to Sp1/3 sites in many gene promoters and the binding of Sp1 and Sp3 can be tissue and gene promoter-specific (Baek *et al.*, 2001;Magee *et al.*, 2005;Goto *et al.*, 2006;Amin *et al.*, 2006). Several studies suggested that Sp1 was responsible for basal transcription and Sp3 appeared in the induced active complex (Ward and Samuel, 2003;Jaiswal *et al.*, 2006;Kiela *et al.*, 2007). Nontheless, most reports did not further elucidate the functional roles of Sp1 and Sp3 regarding gene regulation. There is insufficient evidence to

functionally discriminate between Sp1 and Sp3. As well-established estrogen responsive genes, TFF1 and *c-myc* have been extensively studied. Using *in situ* ChIP and re-ChIP assays, the present study shows for the first time that Sp3, instead of Sp1, is the Sp protein preferentially associated with estrogen initiated TFF1 and *c-myc* gene promoter activation, although these two gene promoters have different promoter contexts.

1. The chromatin immunoprecipitation assay and genomic instability.

The ChIP assay in which formaldehyde is used to cross-link proteins to DNA is routinely used to determine which transcription factor, cofactor or chromatin remodeling factor is associated with the gene of interest. Although the ChIP assay is a powerful technique to study the *in situ* interactions of proteins and DNA elements, this assay has several potential limitations. Our results demonstrated that the ChIP assay using anti-HDAC2 antibodies immunoprecipitated primarily DNA fragments associated with the phosphorylated form of HDAC2. Although the unmodified form of HDAC2 is more abundant than the modified form, the unmodified form of HDAC2 cross-links poorly to DNA with formaldehyde (Sun *et al.*, 2002a). This is not a novel observation. Solomon and Varshavsky (Solomon and Varshavsky, 1985) reported that, while the cross-linking of histones and nucleosomal DNA by formaldehyde was successful, the cross-linking of DNA to the DNA binding protein and lac repressor was a failure, demonstrating that the cross-linking efficiency of formaldehyde was unpredictable. It is advisable to determine which DNA cross-linked protein your antibody is recognizing in the ChIP assay. In the analyses of histone modifications it must be kept in mind that the histone modifications are dynamic. Thus the results of the ChIP assay for a specific promoter will depend upon

the steady state of the modification at the time of cross-linking with formaldehyde (Clayton *et al.*, 2006).

We have analyzed the chromosomes of cancer cells in culture by spectral karyotyping (SKY). Our studies have revealed that breast cancer cells (MCF-7) are aneuploid (He *et al.*, 2008). We were surprised to learn that the MCF-7 cell's karyotype was quite variable, providing evidence that each cell in culture had a different chromosomal composition. Thus the copy number of any particular chromosome may vary from cell to cell. For example, chromosome 21, which harbors the TFF1 gene (21q22.3), varied from 1 to 5 copies in MCF-7 cells. It is unclear whether all the TFF1 genes in the MCF-7 cells with multiple copies of chromosome 21 are estrogen responsive. Realizing that genomic instability is rampant in these cells, this presents issues with procedures such as the ChIP assay and chromosome genomic hybridization as both will average the events occurring in the cell population. Clearly, the analyses of transcription factor dynamic loading onto estrogen responsive genes, for example, have been performed are being done in a background of genomic instability when using cancer cell lines as a model system.

2. Sp1 and Sp3 associate with TFF1 promoters throught the P1 site. Previous reports have demonstrated that the ERE is required for the TFF1 promoter to elicit a response to E2 (Lu *et al.*, 2001). Our results demonstrate that Sp1 and Sp3 are associated with the promoter of the TFF1 gene in human MCF-7 breast cancer cells and that the Sp1/3 binding site located upstream of the ERE has a role in the estrogen responsiveness of the TFF1 promoter. These observations suggest that Sp1/Sp3 contribute to the estrogen-

regulated response of the TFF1 gene, as is the case with many other estrogen responsive genes expressed in breast cancer cells (Stoner *et al.*, 2004).

Our results suggest that the Sp1 (P1) site is occupied by Sp1 or Sp3 in MCF-7 cells cultured in E2 depleted conditions. However, following the addition of E2, Sp3 is usually the factor loaded onto the P1 site. Analyses of transcription factor occupancy within the TFF1 promoter by *in situ* DNase I footprinting supports that the P1 Sp1/Sp3 binding site is occupied in MCF-7 cells grown under E2 depleted and complete (24 h) conditions (see Figure 2 in (Kim *et al.*, 2000b)). In *in situ* dimethylsulfate footprinting analyses, there was increased sensitivity of guanine residues in the P1 Sp1/ 3 binding site in MCF-7 cells grown in the absence of E2. Two hours following the addition of E2, the P1 site was protected from cleavage, suggesting an alteration in Sp1/ Sp3 interaction with this site following exposure to E2.

The TFF1 promoter has two positioned nucleosomes (Bates and Hurst, 1997;Metivier *et al.*, 2003a). NucE, which harbors the ERE, is positioned between -270 and -410, with the ERE being positioned at -405 to -393. This nucleosome remained in place in MCF-7 cells cultured in the absence and presence of E2. One study indicates that nucE's position is a preferred rather than a fixed one (Metivier *et al.*, 2003a). Relative to the preferred position of nucE, the P1 Sp1/Sp3 site is located in the linker DNA region upstream of the nucleosome. It is possible that the location of the Sp1/Sp3 binding site may contribute to the preferred positioning of Nuc E. Sp1 will also bind to sites within nucleosomes, but the affinity of Sp1 for a consensus Sp1 binding site in a nucleosome is reduced 10- to 20-fold relative to that of naked DNA (Li *et al.*, 1994). As the binding affinity of Sp1/Sp3 for the P1 site is markedly lower than that of a consensus Sp1 binding

site, it is unlikely that Sp1 and Sp3 would bind to the P1 site when placed in a nucleosome.

In addition to the P1 Sp1 site, we identified two potential Sp1 sites 3' to the ERE at -370 and at -304, which were referred to as P2 and P3, respectively. Both sites were detected by the TRANSFAC program when the threshold setting was set to 70, indicating both sites had weak affinities for Sp1. In the *in vitro* EMSA, both P2 and P3 oligonucleotides formed complexes with Sp1 and Sp3 (unpublished results). However, *in situ* footprinting assays failed to detect protein occupancy at these sites (Kim *et al.*, 2000b). Both P2 and P3 Sp binding sites are located in a positioned nucleosome (Sewack and Hansen, 1997;Reid *et al.*, 2003). Hence, the binding of Sp1/Sp3 to these weak sites positioned close to the dyad axis of symmetry in a positioned nucleosome would be highly disfavored. To further explore the potential role of the P2 and P3 Sp sites in the activity of the promoter, we observed that the estrogen response of transiently transfected reporter constructs with a mutation in the P1 and P2 sites or mutation of the P1, P2, and P3 sites was no different from the response of reporter construct with only the P1 mutation (unpublished results). Thus, by this *in vitro* functional assay, the P2 and P3 sites were not involved in the estrogen activation of the TFF1 promoter. Our laboratory studies demonstrated that DNA probes without Sp binding site(s) did not form complexes with Sp1 and Sp3 (Sun *et al.*, 1994).

We addressed the concern that mutation of the Sp1 (P1) site, which is adjacent to the ERE, would compromise ER binding to the episomal TFF1 promoter. ChIP assay results show that this is not the case. ER loading onto the episomal wild-type and mutant TFF1 promoters was observed at comparable levels in clone 11 cells in the absence or presence

of E2. This is in contrast to the results with the endogenous promoter where ER loads rarely onto the promoter in the absence of E2. There are two considerations to explain this observation. First, the promoter is episomal and may not be in the same chromatin configuration as the endogenous promoter which has a positioned nucleosome covering the ERE. The difference between factor loading onto an episomal versus endogenous promoter is analogous to that observed by Smith and Hager (Smith and Hager, 1997) for the MMTV promoter in that NF1 loaded onto the MMTV promoter when it was episomal but not when it was endogenous. The second explaination is that GFP-ER levels are high in clone 11 cells in the absence of E2 and may drive the loading of ER onto the episomal TFF1 promoters (Zhao *et al.*, 2002).

3. Chromatin context is an important factor in the differential loading of Sp1 and Sp3 on TFF1 promoters. Our results with the ChIP assay applied to the transfected clone 11 cells demonstrated that Sp1 and Sp3 did not load onto the mutant episomal TFF1 promoter. Further, it appeared that Sp1 was loading preferentially to Sp3 onto the episomal wild-type TFF1 promoter in clone 11 cells with E2. Thus, in the episomal state, the TFF1 promoter loads both Sp1 and Sp3 in proportions similar to those found in the nucleus. This is in marked contrast with our results showing the loading of Sp3 more than Sp1 onto the endogenous promoter in cells cultured with E2. This result suggests that the chromatin context is an important factor in the differential loading of Sp1 and Sp3 following the addition of E2 to breast cancer cells. Further, our analyses of the episomal TFF1 promoter illustrates the limitations of the transient transfection assay in deciphering the function of transcription factor binding sites in promoters. Although the transient transfection assay is used widely to analyze the role of Sp1 and ER binding sites in

estrogen responsive promoters (Gadbois *et al.*, 1992;Lu *et al.*, 2001;Castro-Rivera *et al.*, 2001a), our data demonstrate the problems in extrapolating observations with episomal constructs to the native promoter. For example, Sp1 and Sp3 differentially affect transcriptional activity of promoters (Li *et al.*, 2004). Thus, the transcriptional response observed in the transient transfection assay in which Sp1 is primarily loaded onto the episomal TFF1 promoter would not be the same as the transcriptional influence of Sp3 loaded onto the native promoter in E2-treated cells.

DNase I hypersensitive site (DHS) mapping of the TFF1 promoter identified a constitutive DHS (HS2) that was present in MCF-7 cells cultured under E2 complete and depleted conditions and in MDA-MB-231 cells (Giamarchi *et al.*, 1999). HS2, which is located immediately upstream of the estrogen inducible DHS (HS1) that spans the ERE, corresponds to the position of the Sp1 (P1) binding site. Our results suggest that in MCF-7 cells, HS2 corresponds to the disruption of chromatin imposed by the binding of Sp1 or Sp3, while in MDA MB 231, the HS2 site identifies the occupancy of Sp1.

4. Sp1 and Sp3 regulate the TFF1 gene through interacting with KATS and HDACs. Although some reports have found that Sp1 may form complexes with Sp3, we demonstrated that Sp1 and Sp3 are in separate complexes in human MCF-7 breast cancer cells. This is in agreement with an analysis of Sp1 and Sp3 complexes in mouse L cells (Sun *et al.*, 2002a;Yu *et al.*, 2003). Sp1 differs from Sp3 in its capacity to form multimers (Yu *et al.*, 2003). When organized as a multimer, Sp1 would present multiple docking sites for interacting proteins such as KATs and HDACs. Indeed, we observed that Sp1 immunoprecipitates had HDAC and KAT activities ((Sun *et al.*, 2002a) and unpublished observations). Sp3, which does not form multimers, would not establish the same higher

order protein complexes as Sp1. In analyses of Sp3 immunoprecipitates, we found that Sp3 was associated with HDAC activity and to a lesser extent KAT activity ((Sun *et al.*, 2002a) and unpublished observations). These results suggest that Sp3 associates with KATs and HDACs. In the absence of E2, Sp1/Sp3 is bound to the P1 site in the TFF1 promoter. Under these conditions, ChIP assays show that the native TFF1 promoter ERE is infrequently occupied with ER (data herein and (Chen *et al.*, 1999;Shang *et al.*, 2000;Burakov *et al.*, 2002;Reid *et al.*, 2003;Metivier *et al.*, 2003a)). Later reports demonstrate that the unliganded ER will cycle on and off of the TFF1 promoter (Reid *et al.*, 2003;Metivier *et al.*, 2003a). Further, it is noted that *in situ* footprinting has provided evidence that unliganded ER binds to the consensus ERE half site (Kim *et al.*, 2000b). Our previous studies showed that Sp1 and Sp3 were associated with HDAC complexes containing HDAC1 and phosphorylated HDAC2 in MCF-7 breast cancer cells (Sun *et al.*, 2002a). As both HDACs are associated with the TFF1 promoter in cells grown in the absence of E2, it is conceivable that Sp1 and Sp3 are the transcription factors responsible for their recruitment. Sp1 and Sp3 may also have a role in the recruitment of p300 to the TFF1 promoter (Xiao *et al.*, 2000;Suzuki *et al.*, 2000c). However, the TFF1 promoter also has binding sites for AP-1, which has an important function in the estrogen response of this gene (Suzuki *et al.*, 2000a;Espino *et al.*, 2006). Transcription factors c-Fos and c-Jun binding to the AP-1 site may also have a role in recruiting KATs to the TFF1 promoter. Our ChIP assay analyses of the acetylation status of H3 and H4 bound to the TFF1 promoter suggest that the steady state levels of these acetylated histones are low in cells cultured in the absence of E2. This observation suggests that the HDACs are more enzymatically active than the KATs associated with the transcriptionally poised TFF1

promoter, resulting in a low steady state level of acetylated histones. However, we found that TFF1 promoter- associated H3 and H4 are dynamically acetylated and would be constantly cycling between high and low acetylation levels (Sun *et al.*, 2001). Increasing the steady state level of acetylation by inhibiting histone deacetylases is not sufficient to activate the transcriptionally poised TFF1 promoter. This result is analogous to TSA not increasing the transcriptional activity of the poised *c-jun* promoter in mouse fibroblasts or the basal expression of collagenase in human glioblastoma cells although TSA increased the level of acetylated histones bound to these promoters (Thomson *et al.*, 2001;Martens *et al.*, 2003). The addition of E2 and the enhanced loading of ER onto the TFF1 promoter result in the increased steady state level of acetylated H3 and H4. The recruitment by ER of multiple coactivators/KATs CBP and p300 results in acetylation of the promoter-bound histones and transcriptional activation of the TFF1. Thus, in the absence of E2, Sp1 and Sp3, through their recruitment of chromatin modifying enzymes may help in maintaining the TFF1 promoter in a state of readiness to respond to the molecular events that occur following the loading of E2-bound ER. One recent study demonstrated that Sp1/3 binding sites can bind ER and Sp proteins to either activate or repress the E2-dependent expression of genes (Wu *et al.*, 2008c). It is conceivable that the coactivators (e.g. KATS) and corepressors (e.g. HDACs) recruited by Sp proteins on the promoters decide how the promoters respond to the estrogen treatment.

One surprising observation of this study is that our pilot experiments suggest that levels of both lysine acetylation and sumoylation are increased on the TFF1 promoter after estrogen treatment. Usually sumoylation is considered as a repressive modification. More detailed work is required to further explore the role of these two modifications in

the regulation of estrogen responsive genes. As all Sp3 isoforms can be sumoylated and the long and short form Sp3 usually have different functions, antibodies against individual Sp3 isoforms, especially sumoylated isoforms, would be helpful to study the role of Sp3 in the regulation of gene expression.

5. The episomal TFF1 promoter is responsive to E2 in the presence of a transfected ER. Initial transfection studies with pTFF1-Luc in MCF-7 (T5) breast cancer cells demonstrated a poor response of the TFF1 promoter following the addition of 10 nM E2. The poor response to E2 in the MCF-7 cell line is consistent with previous studies showing that an episomal TFF1 promoter is unresponsive to endogenous ER (Lu *et al.*, 2001;Castro-Rivera *et al.*, 2001a). However, the episomal TFF1 promoter is responsive to E2 in the presence of a transfected ER (Lu *et al.*, 2001). It has been proposed that the high copy numbers of plasmids in transfected cells and limiting levels of endogenous ER prevent a response to endogenous ER (Castro-Rivera *et al.*, 2001a). To fulfill the need for increased ER expression, we used the MCF-7 clone 11 cell line, which has a stably expressed, doxycycline-inducible green fluorescent protein fused to ER (GFP-ER) (Zhao *et al.*, 2002). GFP-ER is fully functional as a ligand activated transcription factor (Htun *et al.*, 1999;Zhao *et al.*, 2002). Addition of 10 nM E2 to the clone 11 cells expressing GFP-ER resulted in a six- to seven fold induction of the TFF1 luciferase reporter (Fig. 15A). The 10 nM E2 concentration used in the present study has been shown to fully occupy the receptor and maximally stimulate TFF1 gene expression (Weaver *et al.*, 1988;Kim *et al.*, 2000a).

6. Sp3 is preferentially associated with the active TFF1 promoter. In addition to our work, others have also demonstrated that Sp1 and Sp3 are associated with the TFF1

promoter in E2 responsive breast cancer cells before and after E2 addition (Sun *et al.*, 2005b;Higgins *et al.*, 2006c;Higgins *et al.*, 2006d). The association of Sp1 and Sp3 with the promoters of E2 responsive genes in breast cancer cells appears to be a common feature, regardless of whether the promoter has an ERE (Higgins *et al.*, 2006a;Higgins *et al.*, 2006d). However, in re-ChIP assays, we show that it is either Sp1 or Sp3 but not both binding to the TFF1 promoter in MCF-7 and also ZR-75 cells (He *et al.*, 2005). As Sp1 and Sp3 have different nuclear locations in MCF-7 cells, it is possible that a TFF1 promoter occupancy with Sp1 or Sp3 may be dependent upon the promoter's nuclear position next to Sp1 or Sp3 foci. Our study provides evidence that Sp3 preferentially co-occupied the TTF1 promoter with ERα. Further we demonstrated that the Sp3 occupied promoter supported transcription demonstrating that in the context of this E2 regulated promoter, Sp3 was acting as an activator rather than a repressor. Consistent with our results, several studies have shown that Sp3 has a prominent role in E2-induced expression of genes in breast cancer cells. Higgins *et al.* (Higgins *et al.*, 2006d) found that in E2-responsive ZR-75 cells that Sp3 and Sp1 were bound to the E2-responsive VEGFR2 before and after the cells were treated with E2, and through knock down studies found that ERα/Sp3 played a critical role, whereas ERα/Sp1 had a minimal role in transactivation of the VEGFR2 promoter. In contrast to the TFF1 promoter, the VEGFR2 promoter lacks an ERE and has multiple Sp binding sites (Higgins *et al.*, 2006d). Sp3 was also found to be required in the E2-induced expression of the RARα and carbamoylphosphate synthetase/aspartate transcarbamylase/dihydroorotase promoters (Higgins *et al.*, 2006b). We and others have demonstrated that Sp1 or Sp3 associate with ER and that this interaction was ligand independent (He *et al.*, 2005;Higgins *et al.*,

2006d;Khan *et al.*, 2007). However, our observations about the localization of ER, Sp1 and Sp3 in the interphase MCF-7 nucleus show that a minor population of ERα binds to Sp1 or Sp3 (He *et al.*, 2005). Our re-ChIP assays provide evidence that ligand bound ER tended to be associated with Sp3 rather than Sp1 on the TFF1 promoter. There are several properties of Sp1 that distinguish this transcription factor from Sp3 that may explain our observations. In addition to having different locations in the nucleus and nuclear matrix, Sp1 but not Sp3 can form multimers (Li *et al.*, 2004;He *et al.*, 2005). As the Sp binding site is positioned close to the ERE in the TFF1 promoter, a Sp1 multimeric complex may sterically interfere with the binding of ER, providing a possible explaination for the preferred co-occupancy of E2-bound ER and Sp3 on the promoter. There are several E2 responsive promoters that have multiple Sp binding sites but are lacking an ERE. In this promoter context, ER is acting as a coactivator in that it is not binding to the promoter DNA directly but is being recruited to the promoter by Sp1 and/or Sp3 (Castro-Rivera *et al.*, 2001a;Castro-Rivera *et al.*, 2001b;Higgins *et al.*, 2006d). In such promoter contexts, the Sp1 multimer may associate with one or more ER factors, and this arrangement may be preferred over a Sp3 association with a single ER. Re-ChIP studies will be required to sort out the preferred associations of Sp1 family members with E2 bound ERα in different promoter contexts found in E2 responsive genes in breast cancer cells.

7. **Sp1 and Sp3 regulate *c-myc* in response to estrogen treatment**. *C-myc* is a *bona fide* estrogen responsive gene. There is no ERE or half ERE identified and confirmed but two Sp1/3 sites are predicted in its estrogen responsive region of the P2 promoter (Dubik and Shiu, 1992;Miller *et al.*, 1996). To the best of our knowledge this is the first report that Sp1 and Sp3 are associated with the c-*myc* promoter *in situ*. Using ChIP and reChIP

techniques, the studies presented here demonstrate that ERα, Sp1 and Sp3 associate with *c-myc* promoter 2. To our surprise, the loading pattern of these factors is about the same as that of TFF1 promoter although *c-myc* has a different promoter context than the TFF1 promoter. Interestingly, Sp1 and Sp3 also don't co-occupy the same *c-myc* promoter 2, although there are two Sp1/3 sites in the P2 promoter and even more Sp1/3 sites in promoter 1 which are immediately upstream of promoter 2. Additionally, higher ERα-Sp3 co-occupancy was also observed after estrogen treatment and this is also true for the co-ocupancy of Sp3 and pS5-pol II. The reChIP assays for ERα and Sp1/Sp3 support the hypothesis that ER is recruited by Sp1 to the *c-myc* promoter. Possibly Sp3 became the one recruiting ER after estrogen treatment. More ER/Sp protein dependent estrogen responsive genes should be studied to decide which Sp protein is the major player. There was one study showing that individual ERE sequences can induce different conformational changes of ER and further lead to association of the receptor with different transcription factors and then assist in the differential modulation of estrogen responsive genes in the target (Wood *et al.*, 1998;Wood *et al.*, 2001). One newly proposed theory suggests that the ligand structure classes of ER selectively use different Sp proteins to regulate genes responsive to estrogen treatment (Wu *et al.*, 2008b). It would be helpful to perform a similar study with some other ER ligands and different promoter context to elucidate the mechanisms by which Sp proteins regulate estrogen responsive genes.

8. Sp1 and Sp3 dynamically associate with estrogen regulated promoters. Except for TFF1 and *c-myc* genes, we also studied the association of ER, Sp1 and Sp3 with several other well-studied estrogen responsive genes using dynamic ChIP assays. We observed

that ERα cycles on α-amanitin synchronized estrogen responsive promoters that have different promoter contexts with an imperfect ERE (TFF1), half-site ERE (RARα, TGFα, cathepsin D) or no ERE (cyclin D1, c-*myc*). The frequency of cycling was faster for the unliganded ERα than for the liganded ERα. Gannon and colleagues reported that unliganded ER cycled on the TFF1 promoter in MCF-7 cells with a frequency of 20 min (Metivier *et al.*, 2003a). In the presence of estradiol, ER loading was initially similar to that of unliganded ER but for the subsequent cycle the extent and duration (frequency of 45 min) of ER association increased. Our results for unliganded ER loading onto the TFF1 promoter are similar to their report. However, our results with the liganded ER are more similar to the second cycle of ER loading onto the TFF1 promoter reported by Gannon's group. Gannon's study observed that the first cycle of ER in the presence of ligand was not transcriptionally productive as indicated by the lack of Ser5 phosphorylated pol II association with the TFF1 promoter (Metivier *et al.*, 2003a). In contrast we found that pS5-pol II was associated with the TFF1 promoter 30 min following the addition of ligand, which is consistent with the increased loading of ER. Thus, in contrast with the Gannon's study, we observe that the first cycle is transcriptionally productive. Our results further highlight the distinct characteristics of Sp1 and Sp3 transcription factors. Regardless of the promoter context, Sp1 association with these promoters in MCF-7 cells treated with and without estradiol was distinct from that of Sp3. One of our lab study demonstrated that Sp1 and Sp3 locate in distinct subnuclear foci and that these distinct foci are maintained on the nuclear matrix (He *et al.*, 2005). Possibly the positioning of a promoter next to foci rich in either Sp1 or Sp3 will favor which of these two factors the promoter will preferentially associate with. Our

previous study regarding the TFF1 promoter demonstrated that Sp1 and Sp3 were associated with the TFF1 promoter in MCF-7 cells cultured under estrogen depleted conditions. However, following the addition of α-amanitin which clears the promoter of pol II (Reid *et al.*, 2003), the levels of association of Sp1, Sp3 and ER with the TFF1 promoter are reduced, but these factors remain bound. Nevertheless, we observe that at 30 min following the addition of estradiol to cells treated with α-amanitin or cells grown under estrogen depleted conditions, the TFF1 promoter is associated with Sp3 and to a lesser extent with Sp1 (Sun *et al.*, 2005b). In a re-ChIP assay we demonstrated that Sp1 and Sp3 are not associated on the same TFF1 promoter (He *et al.*, 2005). Thus we conclude from both studies that following the addition of estradiol the TFF1 promoter tends to be bound to Sp3 rather than Sp1. With regards to ER and the Sp factors, we demonstrated that ER can form a complex with either Sp1 or Sp3 and in re-ChIP assays we found that the TFF1 promoter bound to Sp1 or Sp3 is associated with ER (He *et al.*, 2005). Thus the binding of either Sp factor does not prevent the ER from binding to the TFF1 promoter and *vice versa* ER does not prevent the association of Sp1 or Sp3. The temporal association of ER and Sp1 with the cyclin D1 and cathepsin D promoters has been analyzed by Safe and colleagues (Krishnan *et al.*, 1994). ER loading onto the cyclin D1 promoter reached a maximum at 15 min and was absent at 30 min, while ER loading onto the cathepsin D promoter was maximal at 30 min. Sp1 loading onto the cyclin D1 promoter was not observed until 15 min following the addition of estradiol and remained bound at 30 and 45 min. In contrast, they observed that Sp1 was associated with the cathepsin D promoter at 15 min and then was gone at 30 min. Although these authors did not use α-amanitin to synchronize these promoters, our results with estradiol-ER are

similar in that liganded ER association with these promoters reached a maximum at 30 min followed by a marked decrease in ER association. Our results with Sp1 loading differed from their observations in that Sp1 loading onto these promoters remained fairly constant at 15, 30 and 45 min post estradiol administration. The extent of association of Sp1 and Sp3 with the various promoters in cells cultured with and without estradiol appeared to vary with the promoter context. With the exception of TFF1, the estrogen responsive promoters analyzed have several Sp binding sites. Hence the results obtained in our ChIP assays would be an average of Sp factors associating with these sites. However, regardless of the promoter context, we observed that Sp3 cycled on the promoters in cells treated with estradiol. With the exception of the TFF1 promoter, this cycle of Sp3 association with the promoter was not observed in cells cultured in the absence of estradiol. Interestingly, the loading of Sp3 typically lagged that of liganded ER. The delayed loading may be a consequence of ER altering the structure of the promoter, favoring the binding of Sp3. Sp3 binding to these promoters may be involved in events resulting in ER removal from the promoter. We have demonstrated that Sp3 is associated with histone deacetylase 1 and 2 (Sun *et al.*, 2002a;Sun *et al.*, 2005b) and possibly, the recruitment of histone deacetylases and subsequent deacetylation of histones are events involved in the removal of ER from the promoter.

9. Spatial organization of genes next to Sp1- or Sp3-rich domains may influence gene regulation. How Sp1 and Sp3 isoforms regulate gene expression (as transcriptional activators or repressors) is dependent upon several parameters, including promoter context, state of modification, and chromatin structure (Sapetschnig et al., 2004). One of our lab studies demonstrated that Sp3 regroups into daughter nuclei before Sp1. Thus Sp3

has the priority to bind to Sp1/3 sites before Sp1 and, depending on the promoter context, can act as an activator or a repressor (He and Davie, 2006). Our lab work also demonstrated that Sp1 and Sp3 occupy different subnuclear sites and exhibit different associations with the nuclear matrix (He *et al.*, 2005). The lack of colocalization of Sp1 and Sp3 in four different cell lines and the mutually exclusive binding of Sp1 and Sp3 to the TFF1 promoter argue strongly against the existence of complexes containing both Sp1 and Sp3 (He *et al.*, 2005). The subnuclear foci containing Sp1 or Sp3 were infrequently associated with sites of transcription (He *et al.*, 2005). This observation is consistent with reports for other transcription factors (e.g., ERα and Oct1) (Grande *et al.*, 1997;Stenoien *et al.*, 2000). However, there are exceptions with Runx2 subnuclear foci colocalizing with sites of transcription (Zaidi *et al.*, 2002). It is possible that the Sp1 and Sp3 foci represent storage sites, allowing the sequestration of Sp1 and Sp3 and regulating their concentration in the nucleus. The distribution of transcription factors into foci may play a role in the regulation of nucleoplasmic concentrations of factors and/or the assembly of multiprotein complexes (Hendzel *et al.*, 2001). Because Sp1 can bind its DNA binding site in nucleosomes, it is possible that without high-affinity nuclear matrix binding sites to temporally retain these transcription factors that Sp1 and Sp3 would associate with Sp binding sites in chromatin, leading to the aberrant remodeling of chromatin and dysfunction of the genome. One of our lab studies show that Sp3 and a subpopulation of Sp1 are bound to the nuclear matrix (He *et al.*, 2005). The fact that Sp1 and Sp3 have different solubility properties may contribute to their difference in functionality. Furthermore, even though Sp1 and Sp3 have a strong affinity for the nuclear matrix, they do not colocalize. This indicates that Sp1 and Sp3 are associated

with different nuclear matrix proteins. The same study suggested that high-affinity nuclear matrix proteins that are involved in the nuclear organization of HDAC1 and HDAC2 are different from those binding to Sp1, Sp3, and ER. Our re-ChIP assay demonstrated that Sp1 and Sp3 did not occupy the same TFF1 or *c-myc* promoter at the same time. This result demonstrates that it is either Sp1 or Sp3 that resides at any particular time on a TFF1 or *c-myc* promoter. If both alleles are active, it is possible that Sp1 is located to the promoters of one allele, whereas Sp3 is on the promoters of the other, but this could vary from cell to cell. ChIP assays will present an average of these interactions. Pending the Sp3 isoform and its modification state associated with one promoter, the transcriptional activity of a Sp3 charged promoter may be very different from that of a Sp1 occupied promoter. We propose that the temporal and dynamic occupancy of Sp1 and Sp3 onto a promoter and the activation capacity of the Sp family member will confer heterogeneity in the activation state of that promoter. Possibly the position of a promoter next to Sp1 or Sp3 foci will influence which of the two factors occupies the Sp binding site. The relative position of a promoter with Sp1/Sp3 binding sites next to nuclear matrix sites enriched in Sp1 or Sp3 in three-dimensional nuclear space may decide which transcription factor will reside on the promoter. As with splicing factors, which dynamically move from a nuclear speckle storage site to nearby transcripts engaged in the splicing process (Lamond and Spector, 2003;Moen, Jr. *et al.*, 2004), we envisage that a promoter positioned next to a nuclear matrix site rich in Sp3 will tend to have Sp3 and not Sp1 dynamically residing on that promoter. Thus, spatial organization of genes next to Sp1- or Sp3-rich domains would influence gene regulation. Future

studies will need to address the nuclear positioning of Sp1/Sp3-regulated genes next to nuclear matrix sites storing Sp1 or Sp3.

In conclusion, my results do not support my original hypothesis that Sp1 is the activator. Sp3, instead of Sp1, is preferentially associated with the active TFF1 and *c-myc* promoter (Fig. 36). Figure 36 shows the proposed model for the association of Sp1 and Sp3 to estrogen regulated genes in breast cancer cells. In the absence of estrogen, Sp1 and Sp3 scans the gene region with their collaborating factors (CFs) in a 'hit and run' manner, although they would not stay at the same Sp1/3 site simultaneously. After estrogen added, the estrogen induced genes may use the same nuclear matrix proteins (e.g. ER) and Sp3 becomes the major Sp protein associated with the activated genes.

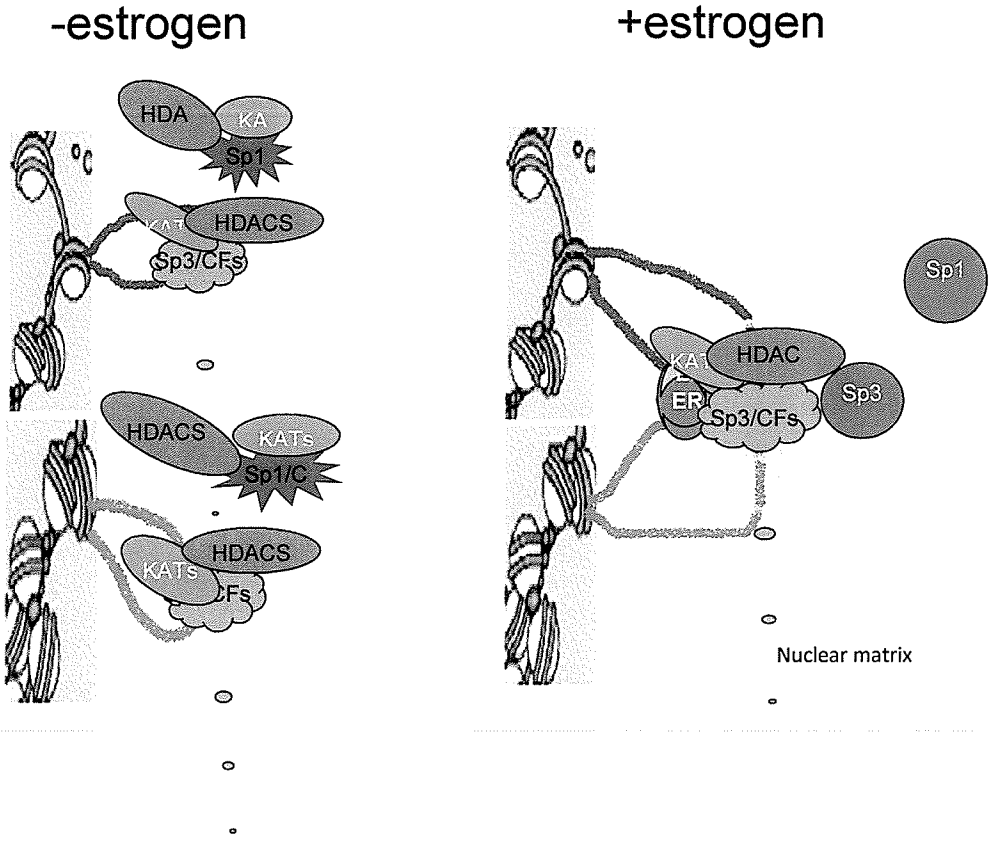


Figure 36. Proposed model.

VII. FUTURE DIRECTIONS

This present study was the first to show that Sp3 is the major Sp protein driving estrogen induced expression of TFF1 and c-myc genes in MCF-7 breast cancer cells. Future work will be aimed at addressing how and why certain Sp protein is chosen to drive the estrogen induced gene expression.

More ER/Sp protein dependent estrogen responsive genes should be studied to decide which Sp protein is the major player. This can be done with the same approaches used in this thesis project. We expect to get two distinct gene groups as Sp1 driven and Sp3 driven group in the same MCF-7 breast cancer cell lines. However, these two groups can be tissue and cell line dependent. We also expect that all estrogen responsive genes are driven by Sp3 protein.

The mechanism(s) involved in the selection of which Sp protein will be used for the regulation of genes in response to estrogen treatment should be explored. Chromatin conformational preference could be one reason for estrogen responsive genes to choose certain Sp protein complex. DNase digestion following with a series of PCR will be performed to determine nucleosome position sliding in response to estrogen treatment. This will help to explain whether the estrogen stimulated chromatin conformational changes are in favor of the chosen Sp protein complex.

RNA/DNA FISH and fluorescence microscope will be used to monitor which Sp protein is located in the ongoing estrogen responsive gene transcription site after estrogen treatment. This study will help to elucidate that if the estrogen responsive genes choose their Sp proteins because of the convenience for storage sites of Sp1, Sp3 and ERs. The same approach will also be used to monitor which modification(s) of Sp protein(s) is

located in the ongoing estrogen responsive gene transcription site after estrogen treatment. This study will relate the modification(s) of Sp protein(s) to the estrogen induced gene transcription.

One of the more important future extensions of the work will be addressing the individual function of the four Sp3 isoforms. The structural difference between the two short and long forms of Sp3 suggests their functional differences. It has been difficult to develop antibodies against individual Sp3 isoforms. However, the expression of Sp3 isoforms was found tissue and cellular specific. RT-PCR can be used to study the expression of the same gene (e.g. TFF1) in cell lines or tissues expressing different levels of Sp3 isoforms (Gartel *et al.*, 2000; Rothem *et al.*, 2004). Then the DNA cross-linked Sp3 isoforms can be studied by western blot to find the preferred cross-linked Sp3 isoform(s). The preferred DNA crosslinked Sp3 isoform(s) will be correlated to the expression of the estrogen responsive genes. Furthermore, ChIP and reChIP can be used to correlate the enriched modification of the Sp3 isoforms to the expression of the estrogen responsive genes.

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