MOLECULAR MECHANISMS OF ESTROGEN-DEPENDENT TRANSCRIPTIONAL REGULATION THROUGH AP-1

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MOLECULAR MECHANISMS OF ESTROGEN-DEPENDENT TRANSCRIPTIONAL REGULATION THROUGH AP-1

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Gene regulation by estrogen (E2) plays an important role in mediating physiological responses in normal and disease states. Estrogen receptors (ERs) facilitate these responses by at least two genomic modes of action: (1) binding directly to estrogen response elements (EREs) or (2) binding indirectly to DNA through transcription factors (like AP-1). Although the ERE pathway is well studied, little is known about the mechanism of E2-dependent actions through this indirect pathway (*e.g.,* ER/AP-1 pathway).

Using an unbiased proteomic approach, that utilizes affinity purification and iTRAQ labeling, I examine the composition of AP-1 complexes in order to better understand the foundation of ER tethering. The results from this analysis identify several AP-1 and non-AP-1 transcription factors associated with E2-responsive AP-1 sites. My results also identify putative coregulators that may play a role in mediating ER/AP-1 responses *in vivo*.

In further studies, I characterize the genomic interplay between E2-signaling and the AP-1 regulator, Jun N-terminal Kinase 1 (JNK1). Interestingly, I show that JNK1 binds to discrete regions of the genome in an E2-regulated manner and correlate these binding events with ERα occupancy. I also define the transcription factors responsible for tethering JNK1 to promoter regions. These results reveal the emerging theme that MAP kinases (like JNK1) can form stable, chromatin-associated complexes. Furthermore, I describe the necessity of JNK1 activity in mediating E2-

dependent transcriptional outcomes in breast cancer cells and demonstrate the importance of JNK1 in E2-dependent tumor cell growth. Finally, I show that JNK1 can phosphorylate coactivators involved in E2-dependent complexes, as well as histone H3. Modification of these factors may play a role in facilitating E2-dependent transcriptional responses *in vivo*. My results establish a new paradigm for estrogen signaling which now includes JNK1 as an E2-dependent coregulator.

BIOGRAPHICAL SKETCH

Gary Isaacs was born and raised in Lincoln, Delaware. In 1995, he started his undergraduate studies at Liberty University, where he gained a strong background in general biology, zoology, botany, genetics, parasitology and biochemistry. Gary completed his course work in 1999, graduating Magna Cum Laude in the Honor's Program. He was awarded "Biology Student of the Year" and received the "Outstanding Senior Honor's Thesis" award for his independent research project. After graduating, Gary taught high school biology and chemistry at Christian Tabernacle Academy, a private institution located in his hometown. It was during this time that he learned how rewarding teaching can be. Three years after graduating college, Gary began his graduate career at Cornell University in the field of Biochemistry, Molecular and Cellular Biology (BMCB). In 2003 he joined the lab of Dr. W. Lee Kraus to study the molecular mechanisms of estrogen-dependent transcriptional responses. He received multiple awards, including the Breast Cancer Pre-doctoral Fellowship by the U.S. Department of Defense and the Outstanding Graduate Teaching Assistant by the BMCB faculty. In December 2008, he successfully defended his Ph.D. thesis.

To my parents and wife, for teaching me what truly matters.

ACKNOWLEDGMENTS

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I feel that I am unable to convey adequately the appreciation and love I feel for my parents, Gary and Ann Isaacs. Their lives are a testimony to the rewards available to those who live faithful lives dedicated to Truth. It is still my hope that one day I will achieve that which they have already attained. Their love and encouragement have steadied me throughout my life. It is an honor to be their son.

To my wife, Jami, I owe so much. She gave up things she held dear to support me as I pursued my studies at Cornell. She has proven herself as a wonderful mother to our children (Andrew, Mia, and Rachel) and a loving wife (my "perfect fit"). I love you!

I am also grateful to my lab-mates for making the day-to-day routine of research such a pleasant experience. To the earliest members (Dr. Edwin Cheung, Dr. Mi Young Kim, and Dr. Mari Acevedo) I am thankful for all their direction as they instructed me in the basics of being a successful graduate student. Dr. Miltiadis Kininis, my former contemporary, was a great "thinker" and I too feel fortunate that we walked this road together. I have enjoyed many interactions with Dr. Dave Wacker, Raga Krishnakumar, Nasun Hah, Dr. Tong Zhang, Joanna Berrocal, Molly Shook, Miao Sun, Xin Luo, and Elizabeth Fogarty. It has been an honor to work with them all. I would like to thank Kris Frizzell for the numerous times we've carpooled

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I would also like to thank my family at West Groton Bible Church for their constant encouragement. It has been an honor serving with of you. God be will all of you until we meet again.

After finishing the biggest challenge I have thus far faced, I realize that it is not my efforts alone that have brought me through. It is true: *The work that was begun in me will be completed…because He is faithful.*

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LIST OF ABBREVIATIONS

E2	17b-estradiol
AF	Activation function
AP-1	Activating protein-1
AR	Androgen receptor
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CARM-1	Coactivator-associated arginine methyltransferase 1
CBP	CREB binding protein
ChIP	Chromatin immunoprecipitation
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
DBD	DNA binding domain
DRIP	Vitamin D receptor interacting protein
EDC	Estrogen-dendrimer conjugate
EP400	E1A binding protein 400
ER	Estrogen receptor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
Fos	Finkel-Biskis-Jinkins osteosarcoma virus oncogene
Fra	Fos-related antigen
FRK	Fos-regulating kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFP	Green fluorescent protein
GR	Glucocorticoid receptor
HAT	Histone acetyltransferase
HAT	Histone acetyltransferase
HME	Histone modifying enzyme
iTRAQ	isobaric tag for relative and absolute quantitation
JIP	JNK interacting protein
JNK	Jun N-terminal kinase
Jun	Avian sarcoma virus 17 oncogene
kD	KiloDalton
LBD	Ligand binding domain
MADS	MCM1, AGAMOUS, DEFICIENS, SRF
Maf	Avian musculoaponeurotic fibrosarcoma virus oncogene
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitrogen activated protein kinase kinase kinase
MARE	Maf recognition element
MAST	Motif alignment and search tool
MEF	Myocyte enhancer factor
MEME	Multiple Em for motif elicitation
MS/MS	Tandem mass spectrometry
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NR	Nuclear receptor
OHT	Tamoxifen
PARP	Poly (ADP-ribose) polymerase
PCAF	p300/CBP associated factor

Pdb	Protein data bank
Pol II	RNA polymerase II
POU	PIT1, OCT1, Unc-86
PPAR	Peroxisome proliferator-activated receptor
qPCR	Quantitative polymerase chain reaction
Ral	Raloxifene
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SCX	Strong cation exchange
SEM	Standard error of the mean
SERMs	Selective estrogen receptor modulators
SMARC	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
Sp1	Simian-virus-40-protein-1
SRC	Steroid receptor coactivator
SRF	Serum response factor
SWI/SNF	SWItch/sucrose non-fermentable
TAF	TBP-associated factor
Tam	Tamoxifen
TESS	Transcription element search system
TF	Transcription factor
Thr	Threonine
TPA	12-0-tetradecanoylphorbol-13-acetate
TRAP	Thyroid receptor associated protein
TRE	TPA response element
TSS	Transcription start site

Tyr	Tyrosine
UBA	Ubiquitin-activating enzyme
UV	Ultraviolet

CHAPTER 1

Transcriptional Regulation by Estrogens Through the AP-1 Pathway: An Overview

Physiology of Estrogens

It is well-known that estrogens, such as 17β-estradiol (E2), play pivotal roles in the regulation of sexual development and fertility in both males and females (Couse and Korach, 1999a; Couse and Korach, 1999b; Nef and Parada, 2000; O'Donnell et al., 2001). Estrogens also regulate metabolic processes in fat, liver, and bone tissues (DeCherney, 1993; Vaananen and Harkonen, 1996). In addition to these roles in normal physiological processes, estrogens also play pivotal roles in many disease states. For example, estrogens can act as potent mitogens in some cancers (*e.g.*, breast, uterine) causing hormone-dependent growth and proliferation (Foster et al., 2001; Prall et al., 1998; Sommer and Fuqua, 2001). A variety of synthetic estrogen antagonists ("antiestrogens") have been developed and are used clinically to reverse the mitogenic action of estrogens in estrogen-dependent cancers (*e.g.*, Tamoxifen, Tam; Raloxifene, Ral). Interestingly, these same compounds may have estrogen-like agonistic activities in some tissues (*e.g.*, bone, endometrium), functioning more like tissue- or cell type-specific "selective estrogen receptor modulators" (SERMs) than pure antagonists (Harper and Walpole, 1967; McDonnell et al., 2002; Paech et al., 1997; Webb et al., 1995). Gaining a greater understanding of the molecular actions of estrogens and SERMs will aid in the development of new compounds that are even more effective in the treatment of breast cancers.

Estrogen Receptors

The molecular actions of estrogens are mediated through estrogen receptor (ER) proteins which bind the hormones, dimerize, and regulate the transcription of estrogen-responsive genes. ERs exist as two isoforms, ERα and ERβ (Warner et al., 1999), which are members of a conserved superfamily of nuclear receptors that function as transcription factors (Mangelsdorf et al., 1995). These isoforms have

unique, but overlapping, patterns of expression in a variety of tissues (Couse and Korach, 1999a; Nilsson et al., 2001; Pettersson and Gustafsson, 2001). (From now on, the use of the term "ERs" will refer to both ERα and ERβ, unless otherwise stated.) Although their activities are functionally distinct, both isoforms share a similar structure. ERs contain an N-terminal activation function (AF-1), a DNA-binding domain (DBD), and a C-terminal activation function (AF-2) which contains the ligand-binding domain (LBD) (Enmark and Gustafsson, 1999) (Figure 1.1). The expression of ERs in cells is a well-known prognostic indicator for some estrogen-dependent breast cancers (Jensen et al., 2001) and serves as the protein target for SERMs (McDonnell et al., 2002; Sommer and Fuqua, 2001). While several nuclear and non-nuclear mechanisms of ER-dependent transcription have been described (Figure 1.2) [reviewed in (Barnes et al., 2004)], only the ligand-dependent nuclear actions of ER are addressed in this work.

ER-dependent Transcriptional Regulation in the Nucleus: Direct DNA Binding

Upon hormone stimulation, ERs dissociate from nuclear chaperone proteins, dimerize, and bind to DNA sequences known as estrogen response elements (EREs). Estrogen-dependent transcriptional regulation through the ERE pathway involves a variety of cofactors that function with liganded ERs to modify histones, alter chromatin structure, and recruit the RNA polymerase II (Pol II) transcriptional machinery (Kraus and Wong, 2002). Many coactivators (*i.e.*, stimulatory cofactors) bind directly to agonist-activated AF-2 of ER through short α-helical "LXXLL" motifs called NR boxes (Glass and Rosenfeld, 2000; Leo and Chen, 2000; Robyr et al., 2000). In general, antagonists fail to induce the proper AF-2 conformation and thus block receptor-coactivator interactions (Nichols et al., 1998). Coactivators include the following: (1) histone-modifying enzyme (HME) complexes that contain

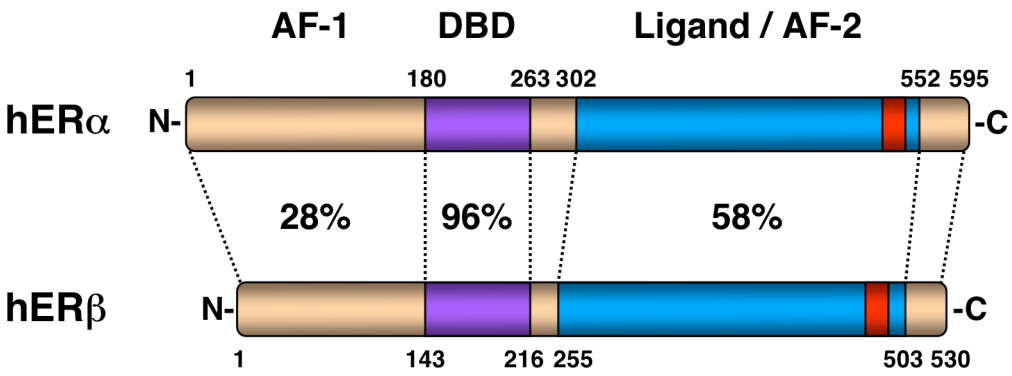


Figure 1.1 Schematic of ERα and ERβ domains.

ERs share a conserved functional domain structure. This includes an amino-terminal activation function 1 domain (AF-1), a DNA-binding domain (DBD), and an activation function 2 domain (AF-2) that contains the ligand-binding pocket. Sequence homology is represented as a percentage for each functional domain.

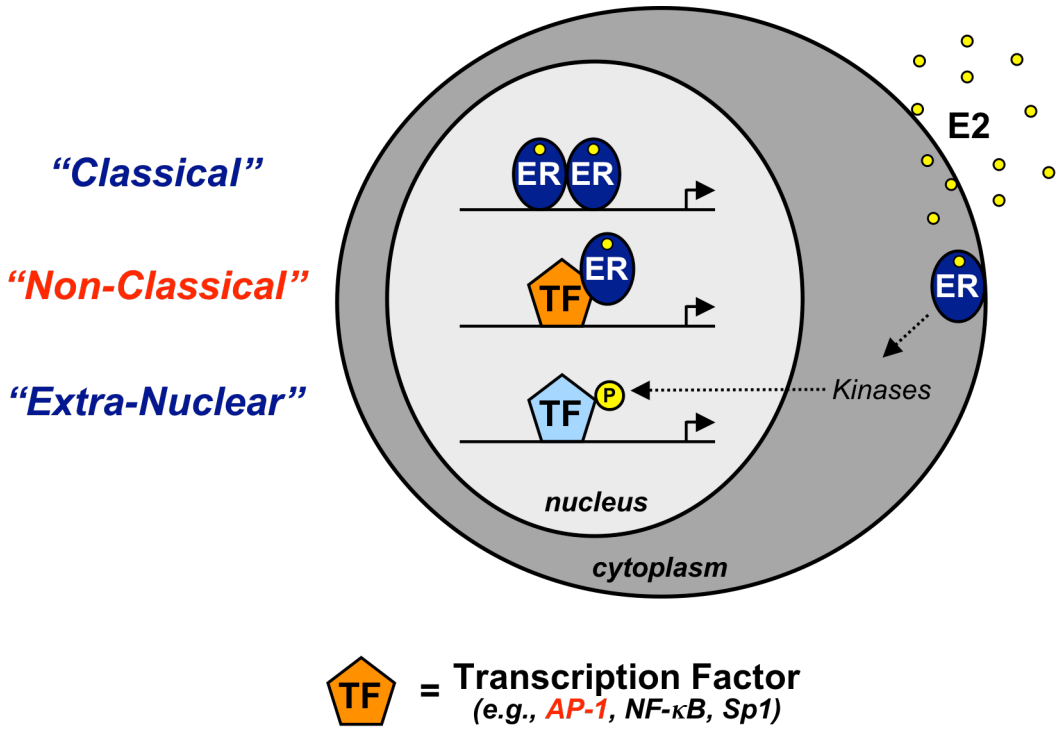


Figure. 1.2 Estrogen-dependent signaling pathways.

Estrogen (E2) signaling pathways include: (1) the ligand-dependent binding of the estrogen receptor (ER) directly to EREs ("Classical"), (2) the ligand-dependent binding of ER to DNA-bound transcription factors (TF) ("Non-Classical"), and (3) the activation of kinase cascades by membrane-associated ER ("Extra-Nuclear"). Figure modified from (McDevitt et al., 2008).

members of the steroid receptor coactivator (SRC) family of proteins as the receptor-binding subunit (Leo and Chen, 2000), (2) chromatin remodeling complexes such as SWI/SNF (Kingston and Narlikar, 1999; Robyr et al., 2000), and (3) Mediator complexes (*e.g.*, TRAP and DRIP) which contain Med220/ TRAP220 as the primary receptor-binding subunit (Malik and Roeder, 2000; Rachez and Freedman, 2001) (Figure 1.3). HMEs include the histone acetyltransferases (HATs) p300/CBP and the histone methyltransferase (HMT) CARM-1, which covalently modify histones to change the structure and function of chromatin (Davie and Chadee, 1998; Narlikar et al., 2002). Chromatin remodeling complexes alter local nucleosomal structure to relieve chromatin-mediated transcriptional repression (Hebbar and Archer, 2003; Kingston and Narlikar, 1999; Varga-Weisz, 2001). Mediator functions to stabilize the formation of a stable Pol II-dependent transcription preinitiation complex (Malik and Roeder, 2000; Rachez and Freedman, 2001). These coactivators are recruited by steroid receptor proteins to promoter regions containing hormone-responsive elements and ultimately facilitate transcriptional activation (Kinyamu and Archer, 2004).

ER-dependent Transcriptional Regulation: Tethering Pathway Overview

Cellular signaling by estrogens is not limited to ERE-dependent transcription. In fact, multiple lines of evidence point to the interaction of ER with several transcription factors via their recognition elements (*e.g.,* NF-κB, Sp1, AP-1). Indeed, the direct interaction of ER with NF-κB (Vandel et al., 1995) has been described with respect to E2-mediated regulation of the interleukin-6 promoter (Ray et al., 1997; Stein and Yang, 1995). Interactions with AP-1 have also been described (Teyssier et al., 2001; Webb et al., 1995), as well as the E2-dependent regulation of genes harboring AP-1 binding elements (such as collagenase, human insulin growth factor 1, chicken ovalbumin, ovine follicle-stimulating hormone β, human choline

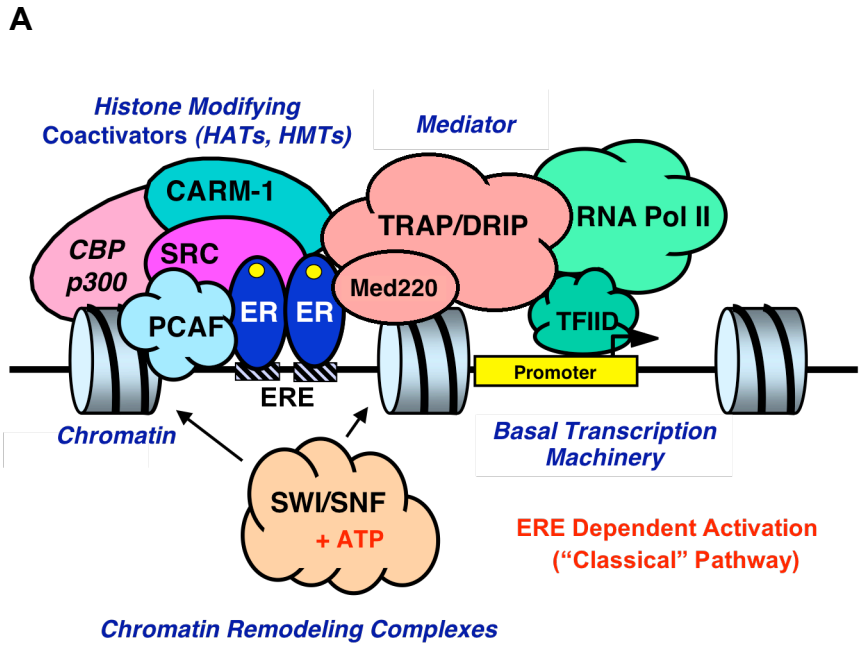


Figure 1.3 ER activation through an estrogen response element.

(A) After binding estrogen, ER dimerizes and binds to estrogen response elements where it then recruits a cohort of factors such as histone modifying proteins, chromatin remodeling proteins, and proteins associated with the basal transcription machinery (**B**). The canonical estrogen response element is shown. N = any DNA base. Figure modified from (Acevedo and Kraus, 2004).

acetyltransferase gene) (Gaub et al., 1990; Miller and Miller, 1996; Paech et al., 1997; Schmitt et al., 1995; Umayahara et al., 1994; Webb et al., 1995; Webb et al., 1999) or those requiring AP-1 factors (DeNardo et al., 2005).

An interesting aspect of the ER/AP-1 pathway is that, under certain cell type and promoter contexts, some classical ER antagonists can function as agonists (Harper and Walpole, 1967; McDonnell et al., 2002; Paech et al., 1997; Webb et al., 1995; Webb et al., 1999). Indeed, using an *in vitro* chromatin assembly and transcription system, our lab has reconstituted ER-dependent activation through AP-1 binding elements by both E2 and SERMs (Cheung et al., 2005) (Figure 1.4). Although the molecular details of the ERE pathway are well characterized, our understanding of ligand-dependent activation of the tethered pathway is quite limited, especially with regard to the mechanisms of altered SERM pharmacology.

AP-1 Family of Transcription Factors

The AP-1 transcription factor is a dimeric complex composed of members of the Fos (c-Fos, FosB, Fra1, and Fra2) and Jun (c-Jun, JunB, and JunD) bZIP protein families (Foletta et al., 1998). Initially, AP-1 complexes were found to mediate gene induction by the tumor promoting phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA). Because of this, the specific DNA sequence which AP-1 bound was called a TRE (for TPA response element) (Figure 1.5). Fos and Jun, which were named after the viral oncoproteins in the Finkel-Biskis-Jinkins osteosarcoma virus and avian sarcoma virus 17, respectively (Vogt, 2002), were later identified as the proteins that bound TREs. The stability and activity of AP-1 directly results from the composition of its dimers [reviewed in (Eferl and Wagner, 2003; Shaulian and Karin, 2001; Shaulian and Karin, 2002)]. For instance, Fos proteins do not form stable homodimers, but rather, form heterodimers with Jun proteins that are more stable than

Figure 1.4 Comparison of direct and tethered ER complexes.

(A) Using an *in vitro* chromatin assembly and transcription system the Kraus lab has demonstrated ligand-dependent transcriptional activation of ERE and AP-1 driven reporters (Cheung et al., 2005). ERα can activate transcription at both reporters in the presence of 17β-estradiol (E2) but only the antiestrogens, Tamoxifen (OHT), ICI, and Raloxifene can activate transcription through AP-1 sites. AdE4 = adenovirus E4 promoter products.

(B) This altered pharmacology whereby E2 agonists and antagonist can activate transcription through AP-1 is one of the defining elements of ER activation through AP-1 *in vivo*.

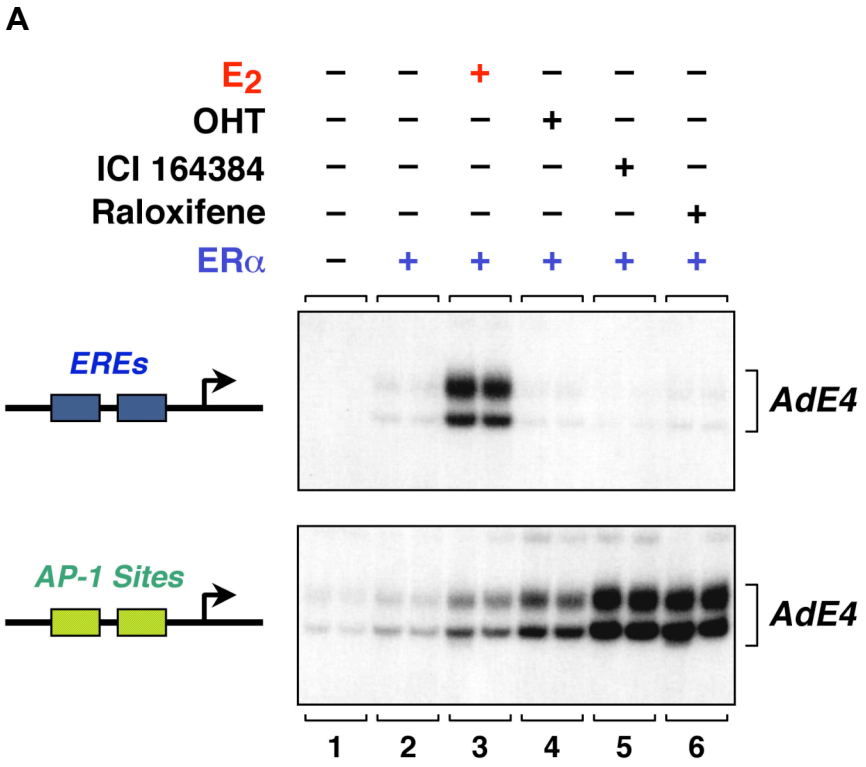
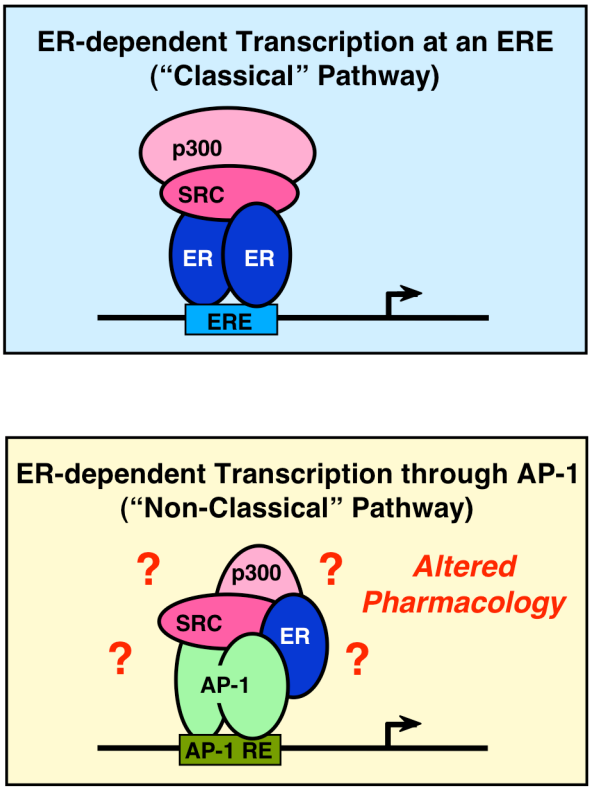

A
E2 – – + – – –
OHT – – – + – –
ICI 164384 – – – – + –
Raloxifene – – – – – +
ERα – + + + + +
EREs
AdE4
AP-1 Sites
AdE4
1 2 3 4 5 6

Figure 1.4 (Continued)

B



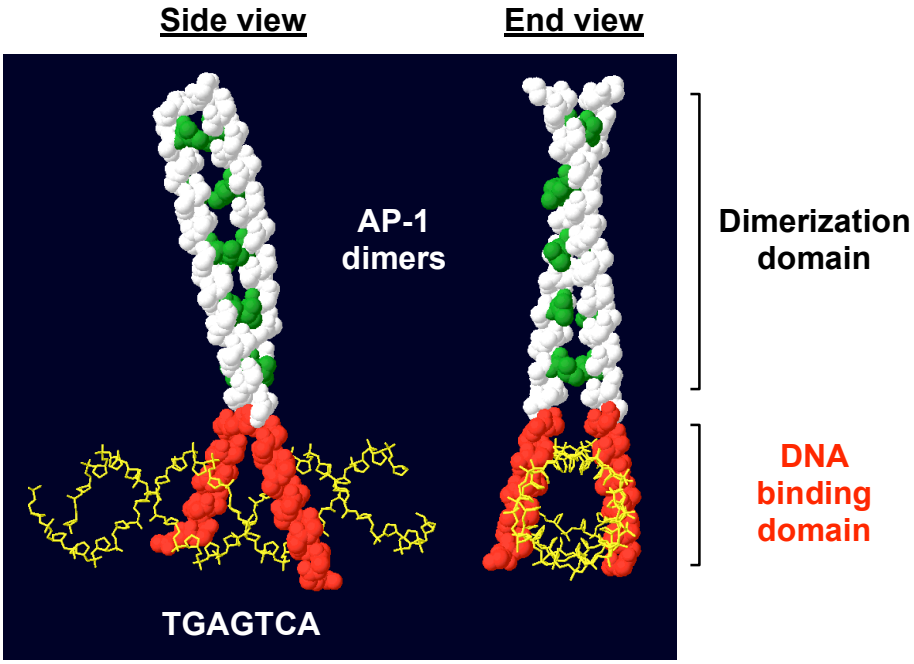


Figure 1.5 The AP-1 transcription factors. AP-1 proteins (members of the Jun and Fos protein families) are basic-leucine zipper proteins. The leucine zipper domain (shown in white with the leucine residues colored green) allows the dimerization of Jun-Jun and Jun-Fos members. Once dimerized, their basic regions (shown in red) interact with specific DNA sequences (shown in yellow) known as TPA-response elements (TREs; TGAGTCA sequence). The model above only shows the bZIP regions of c-Fos and c-Jun. The picture was generated with Pdb viewer using the published crystal structure (Glover and Harrison, 1995).

Jun:Jun homodimers (Angel and Karin, 1991). The AP-1 members that compose these dimers can be quite different. Some AP-1 proteins, such as c-Fos, FosB, and c-Jun, contain potent activation domains which allow them to promote cell transformation (Jochum et al., 2001). In contrast, Fra1 and Fra2 have weak activation domains and weak transforming activity (Bergers et al., 1995; Foletta et al., 1994), while JunB and JunD have no transforming ability (Vandel et al., 1995).

Regulation of AP-1 proteins

Before exploring ER-dependent activation through the AP-1 pathway, it is important to have a basic understanding of AP-1 activation. Induction of AP-1 activity can occur by two different mechanisms: (1) by increasing the transactivation ability of the AP-1 factors and (2) increasing the overall level of the AP-1 factors. Both of these mechanisms are modulated by MAPK cascades explained in more detail below.

MAP kinase cascades

AP-1 proteins are regulated by MAP kinase cascades which convey a response from various cell surface stimuli to intracellular targets by signal transduction pathways. Signal transduction occurs by a series of three kinases that form a phosphorylation-relay [reviewed in (Davis, 2000; Hagemann and Blank, 2001; Johnson and Lapadat, 2002; Vlahopoulos and Zoumpourlis, 2004)]. The first kinase, known as a MAP kinase-kinase-kinase (MAPKKK) is activated by phosphorylation of serine and threonine residues (Davis, 2000; Kyriakis and Avruch, 2001). The activated MAPKKK phosphorylates a MAPK-kinase (MAPKK) in a similar fashion, which then becomes active and phosphorylates the final kinase, a MAP kinase (MAPK). MAP kinases differ from their upstream enzyme activators in that their

phosphorylation is on a Thr-x-Tyr motif, where x is any given amino acid (Davis, 2000; Kyriakis and Avruch, 2001) (Figure 1.6A). This phosphorylation enables the MAP kinases to translocate to the nucleus and phosphorylate their target protein(s) (Cavigelli et al., 1995). There are three subfamilies of MAP kinases which include the extracellular signal-regulated kinases, ERKs; the c-Jun N-terminal kinases, JNKs; and the p38s (Johnson and Lapadat, 2002).

AP-1 regulation by post-translational modification

Phosphorylation of c-Jun on serines 63 and 73 (Ser63/73) in its amino-terminal region enhance its ability to activate transcription (Hibi et al., 1993; Pulverer et al., 1991; Smeal et al., 1992). This activation is most likely due to the phosphorylation-dependent recruitment of the transcriptional coactivator CBP (CREB binding protein) to c-Jun (Bannister et al., 1995). c-Jun is phosphorylated by specific MAPK members, JNKs, following cell stimulation by various conditions (*e.g.,* TPA, UV treatment, protein synthesis inhibitors) [reviewed in (Ip and Davis, 1998)]. Because c-Jun is activated by phosphorylation, many genes which are regulated by AP-1 behave as "immediate-early" genes. These genes are rapidly induced upon cell stimulation independently of *de novo* protein synthesis.

Three JNK proteins are known, JNK1, JNK2 and JNK3, with overlapping and distinct functions. These kinases can all bind to a docking site in c-Jun known as the δ (delta) domain (Dai et al., 1995) and phosphorylate Ser63/73 when activated (Hibi et al., 1993) (Figure 1.6 B and C). JNK1 and JNK2 are more ubiquitously expressed while JNK3 is mainly expressed in the brain (Mohit et al., 1995). Due to splice variations, ten JNK isoforms exist (4 for JNK1, 4 for JNK2, 2 for JNK3) with JNK1 expressed predominantly as a 46 kD protein and JNK2 as a 54 kD protein (Derijard et al., 1994; Kallunki et al., 1994; Mohit et al., 1995). Although JNK1 and JNK2 have

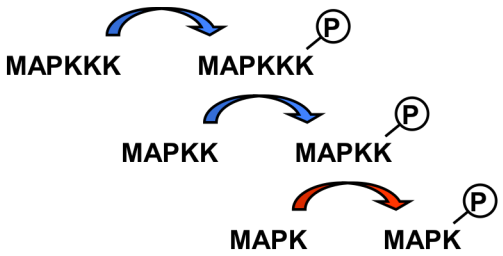
Figure 1.6 The JNK MAP kinases: activation, recognition and modification.

(A) MAP kinases (like JNKs) are activated as the result of a phosphorylation relay system. Blue arrows represent phosphorylation of serine and threonine residues, red arrow represents phosphorylation of threonine and tyrosine residues.

(B) JNK substrates, like c-Jun and JIP-1, contain a JNK binding domain composed of basic and hydrophobic amino acids (shown as red and green letters). Once bound, JNK can phosphorylate proline-directed serines. Serines 63 and 73 in c-Jun (shown in magenta) are well-known targets of JNK modification.

(C) The picture of JNK1 was generated with Pdb viewer using the published crystal structure of JNK1 (Heo et al., 2004). SP600125 (structure shown in the white box), a competitive ATP inhibitor of JNK MAP kinases, is bound in the ATP-binding pocket. The JNK binding domain of JIP (sequence from B) is also shown bound to JNK1. Red and green amino acids are the same as those illustrated in B.

A



B

JNK binding domain

c-Jun: KILKQSMTLNL

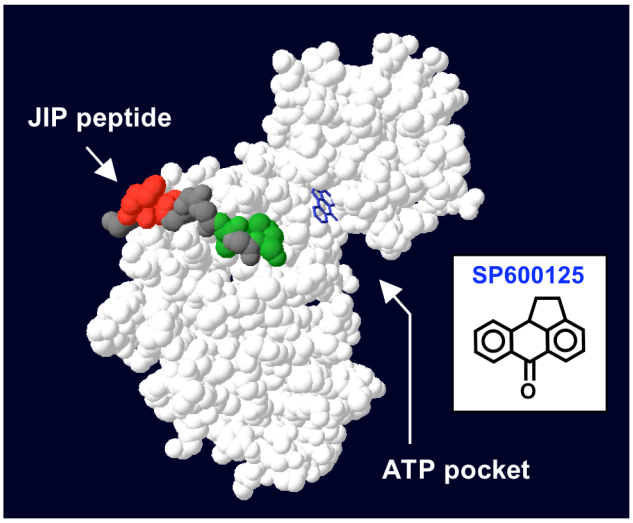
JIP-1: KRPTTLNL

JNK enzymatic target

c-Jun: LTSPDVGLLKLASP

C

JNK1 Crystal Structure



redundant functions, recent studies using JNK knockout mice have highlighted specific roles for JNK1 and JNK2 [reviewed in (Sabapathy et al., 2004)]. JNK1 seems to be the main kinase which phosphorylates c-Jun in response to cell stimulation (Sabapathy et al., 2004) , while JNK2 is thought to mediate ubiquitin-mediated c-Jun turnover in unstimulated cells (Fuchs et al., 1998; Sabapathy et al., 2004). Although c-Jun can also be phosphorylated by other MAPK members (*e.g.,* ERK1 and ERK2), the phosphorylated residues are in the C-terminal region of the protein and are not associated with transactivation, but rather, the inhibition of homodimers to bind DNA (Chou et al., 1992; Minden et al., 1994).

c-Fos can also be regulated by phosphorylation. Its C-terminal region contains a sequence similar to the phosphorylated sequence of c-Jun (Sutherland et al., 1992). Threonine-232, the c-Fos equivalent of serine-73 in c-Jun, is located in this region and is phosphorylated by the Fos-regulating kinase (FRK) but not by JNKs (Deng and Karin, 1994). I have found that JNK1 can phosphorylate c-Fos *in vitro* when bound to c-Jun. Although this may be the result of artificially altering JNK1 specificity, it also demonstrates the role of c-Jun as a scaffold protein between JNK and Jun interacting proteins, like c-Fos. The mechanism of transcriptional activation through c-Fos phosphorylation is still unclear. I have also conducted *in vitro* acetylation assays that also indicate that both c-Fos and c-Jun are targets of acetylation by p300 (data not shown) revealing the possibility of another post-translational modification that could regulate AP-1 activity. To date, it is unknown if the modification state of c-Fos and c-Jun plays a role in the ability of ER to mediate transcription through AP-1.

Activation of MAP kinase cascades can also result in the upregulation of transcription at the *c-FOS* and *c-JUN* genes [for review see (Karin, 1995)] increasing the overall levels of AP-1 components. Both genes are poised for activation through the MAP kinase-dependent phosphorylation of the transcription factors that

constitutively occupy their promoters [*e.g.,* Elk-1 at *c-FOS* (Treisman, 1992); c-Jun at *c-JUN* (Rozek and Pfeifer, 1993)]. So, MAP kinase activation can not only affect AP-1 activity by post-translational modification, but it can also indirectly affect AP-1 activity by altering the relative AP-1 abundance and composition, as well.

Molecular Crosstalk in the ER/AP-1 Pathway

Although ERs do not bind directly to TREs (Jakacka et al., 2001), they can be recruited by protein-protein interactions with c-Jun and can convey E2 responsiveness to genes lacking EREs. AP-1 activity can be induced by E2 treatment and reduced by antiestrogens without an increase in c-Fos and c-Jun expression (Philips et al., 1993). E2 can also inhibit AP-1 activity most likely by a mechanism involving ERβ and JNK (Srivastava et al., 1999). How E2 effects AP-1-induced gene transcription has been shown to depend on the ER isoform involved (Paech et al., 1997; Watanabe et al., 1997; Webb et al., 1995). Although ERα plays a role in E2-activating effects through AP-1, ERβ mediates the E2-inhibiting effects through AP-1 (Paech et al., 1997). The AF-1 region of ERα and ERβ are quite different suggesting this domain may be responsible for the differential regulation of ER-responsive genes (Couse et al., 1997). These phenomena may be facilitated by the differential recruitment of AF-1 coactivators to TREs and their subsequent phosphorylation by JNK (Feng et al., 2001), though, this still needs to be determined.

As noted above, ERs interact with the coactivators SRC-1 and CBP/p300. c-Jun and c-Fos also directly interact with SRC-1 and CBP/p300 and these transcriptional activators regulate AP-1 dependent transcriptional outcomes (Bannister et al., 1995; Lee et al., 1998). Because of this, it has been proposed that the mutual inhibition between some nuclear receptors and AP-1 is due to competition for the same coactivator (Kamei et al., 1996). It is also believed that certain coactivators may

also facilitate the positive interference between ERα and AP-1 as deletion of ERα helix 12 (Webb et al., 1999) or mutations in ERα AF-2 that prevent the binding of p160 coactivators dramatically inhibits estrogen-mediated transcriptional activation through AP-1 (Teyssier et al., 2001). The Kraus lab has used a biochemical approach, involving an *in vitro* chromatin assembly and transcription system, to compare estrogen signaling through the ER/AP-1 pathway to estrogen signaling through the ER/ERE pathway. Interestingly, these studies have shown that even though a similar set of transcriptional coactivators (*e.g.,* SRCs, CBP/p300) are utilized by both pathways, their interactions, activities, and requirements in the two pathways are distinct (Cheung et al., 2005). Taken together, these results suggest that although the ER-mediated activities at EREs and TREs may be similar, they do represent distinct mechanisms of ER action. It has also been shown that estrogen treatment causes the recruitment of c-Fos, as well as, ERα to endogenous promoters containing TRE sites (Kininis et al., 2007). This illustrates that ER-mediated activities at TREs are not limited to coactivator-dependent mechanisms, but may also affect the core AP-1 component. A more complete understanding of the proteins used by both pathways is needed to fully comprehend the coactivator crosstalk between ER and AP-1.

Association of ER with AP-1

Although previous studies have shown that ERα directly interacts with c-Jun (Qi et al., 2004; Teyssier et al., 2001; Webb et al., 1995), the nature of the interaction (*i.e.*, the protein domains involved) is unclear, as contradictory results have been published. For instance, in 1995 a group using GST-tagged ERα constructs demonstrated an interaction between *in vitro* translated c-Jun and an amino-terminal region (amino acids 1-185) of ERα, while the ligand binding domain of ERα could not facilitate c-Jun binding (Webb et al., 1995). Later, in 2001, another group (using a

similar *in vitro* binding assay) demonstrated an interaction between the hinge region of ERα (specifically amino acids 259-302) and the carboxy-terminal region of c-Jun (Teyssier et al., 2001). They went on to further contradict the previous study by showing the interaction of c-Jun with an ERα construct lacking the first 250 amino acids. They also stated that the phosphorylation status of c-Jun was not a factor in ERα binding. Finally, in 2004, it was shown that ERα interacts with the amino-terminal region of c-Jun and that this interaction was strongest when c-Jun was phosphorylated on Ser63 and Ser73 (Qi et al., 2004). What can be drawn from these contrasting studies is that ERα can directly interact with c-Jun and that this interaction does not absolutely require the phosphorylation of c-Jun. ERα can also interact with JunB and JunD but not members of the Fos family (c-Fos, FosB, Fra2, Fra1. The protein domains that facilitate these interactions are still unclear.

Mouse model system for studying non-classical ERα action

The understanding that estrogen-dependent actions were mediated through ER lead to the development of model systems designed to tease apart the molecular actions of the receptor. In 1993, a ERα knock-out mouse was made by insertional disruption of the ERα gene (Lubahn et al., 1993). This gene was not referred to as ERα since ERβ was not discovered until 1996 (Kuiper et al., 1996; Mosselman et al., 1996). Similar methods were used to generate the ERβ knock-out mouse in 1998 (Krege et al., 1998). These knock-out models have provided an invaluable resource for determining the biological functions of ERα and ERβ [summarized in (Couse and Korach, 1999a)] although they provide little to no information concerning the differences between the different molecular mechanisms of ER action. In order to provide a more useful tool for non-classical ER studies, Jakacka and colleagues determined the specific amino acids in the DNA-binding domain of mouse ERα that

facilitated ERE binding but did not affect AP-1 binding (Jakacka et al., 2001) (Figure 1.7A). Indeed, the E207A/G208A mutant ("AA") eliminated ERE binding and activation of ERE-containing reporter genes while still able to activate a TRE-containing reporter and interact with c-Jun. This non-classical ER was introduced into mouse by targeted insertion ("knock-in") to distinguish between classical and non-classical ERα actions *in vivo* (Jakacka et al., 2002).

The knock-in model provided several useful findings. First, while the ERα heterozygous knock-out females ($ER\alpha^{+/-}$) were fertile (Dupont et al., 2000; Lubahn et al., 1993), the heterozygous knock-in females were not. This phenotypic difference is either the result of antagonism between the wild-type allele and the AA mutation, or the net imbalance of contributions from the classical and non-classical pathways. Whatever the mechanism, it underscores the importance of the non-classical pathway in the development and function of the female reproductive system. Because the heterozygous AA males had normal fertility (Jakacka et al., 2002), a cross with $ER\alpha^{+/-}$ females produced hemizygous AA progeny ($ER\alpha^{AA/-}$) (Figure 1.7B). These mice have been used successfully in at least three studies to date to identify a physiological role for non-classical ERα signaling in uterus (O'Brien et al., 2006), bone (Syed et al., 2005) and testes (Weiss et al., 2008). Future studies using the hemizygous AA mice will most likely define the role of ERE-independent hormone signaling in other tissues.

Genomic analyses: ChIP and bioinformatics

Recent scientific advancements, like the sequencing of the human genome and the development of large-scale analytical techniques using microarray technology (*e.g.,* ChIP-chip), have facilitated the examination of ER-containing complexes on a genomic scale. Indeed, several reports (9 to be exact) have described the genomic

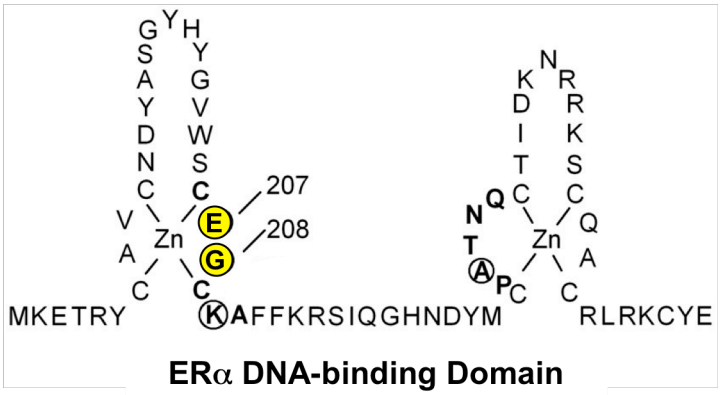
Figure 1.7 Mouse models for studying ER-dependent processes.

Although ERα and ERβ knock-out mice are invaluable tools in our search to understand the role of the ER in mediating physiological responses, these models do not discern between the different mechanistic pathways of ER-dependent actions.

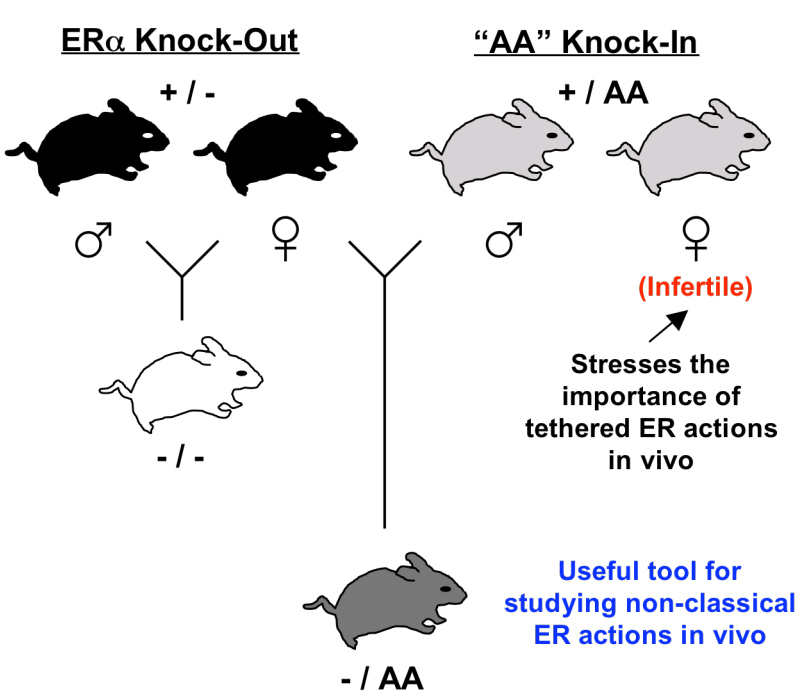
(A) Jakacka and colleagues described a double mutation in the DNA binding domain of ERα (E207A / G208A, shown in yellow) that abolished its ability to interact with an ERE while retaining the ability to activate transcriptional responses through an AP-1 reporter (Jakacka et al., 2001).

(B) Knock-in mice were made with this double alanine ("AA") ERα to determine the role of non-classical signaling *in vivo* (Jakacka et al., 2002). Since the knock-in heterozygous females were infertile, the heterozygous males had to be crossed with heterozygous knock-out females in order to produce mice expressing only the non-classical ERα. A diagram of the crossing scheme is shown.

A


MKETRY
KAFFKRSIQGHNDYM
RLRKCYE
207
208
Zn
Zn
ERα DNA-binding Domain

B


ERα Knock-Out
+ / -
"AA" Knock-In
+ / AA
(Infertile)
Stresses the importance of tethered ER actions in vivo
- / -
- / AA
Useful tool for studying non-classical ER actions in vivo

localization of ERα using ChIP-based assays [ERα localization conclusions and methods reviewed in (Kininis and Kraus, 2008)]. Bioinformatic analyses of the sequences composing ERα bound regions indicated an enrichment in AP-1 motifs providing genomic support for non-classical ERα complexes *in vivo* (Carroll et al., 2006; Kininis et al., 2007). The determination of ERα/AP-1 complexes has also been accomplished using an expression microarray approach (DeNardo et al., 2005). This study identified E2-regulated genes that were affected by a blockade in the AP-1 pathway (accomplished by the inducible expression of a c-Jun dominant negative). These genes, not surprisingly, were enriched for AP-1 sites demonstrating that even expression microarrays can be used to predict ERα/AP-1 complexes *in vivo*.

Unfortunately, our understanding of non-classical E2 signaling from the perspective of AP-1 is greatly lacking. Only two "genomic scale" data sets (c-Fos and c-Jun) are available for AP-1 factors (Bruce et al., 2005a; Bruce et al., 2005b). The data from these studies are not particularly useful with respect to ER signaling since the ChIP-chip analysis was not conducted in the presence of hormone and the cell-line used did not even express ER. Examination of AP-1 factors, in a manner similar to that of ERα, is greatly needed to further the understanding of hormone-dependent tethering through these proteins.

Remaining questions

Previous genomic studies (mentioned above) have demonstrated that AP-1 sites are associated with ERα binding. Knowing this only reminds us that non-classical ERα complexes exist instead of furthering our understanding. What needs to be determined, besides the cataloging of specific promoters containing ERα/AP-1 complexes (a feat which still needs to be accomplished), is the contribution of AP-1 (or other tethering components) to these tethered complexes. What are the AP-1

members that mediate E2 signaling? For years it seems we have viewed the non-classical ER pathway as a modified version of ERE-dependent signaling when, in fact, it most likely represents an entirely different mode of E2 (and even SERM) signaling. Future studies should focus on mapping all the AP-1 family members by ChIP using estrogen-responsive models, instead of just relying on bioinformatic analyses to determine possible AP-1 sequences, which may not represent functional AP-1 sites. Understanding the hormone-dependent binding of AP-1 and its related factors, I believe, will truly allow a greater understanding of the ERα/AP-1 pathway.

Besides the known interaction between ER and c-JUN (which remains ill-defined), there may also be a physical interaction between ER and AP-1 coregulators such as JNK. It is possible that JNK could associate with liganded ERs since a recent study has shown that JNK1 directly interacts with another steroid receptor, the glucocorticoid receptor (GR). This interaction occurs in a ligand-dependent manner through a "delta-like" docking domain in GR (Bruna et al., 2003). Furthermore, E2 with ERβ can repress AP-1-dependent transcription by the inhibition of JNK activity (Srivastava et al., 1999) suggesting a possible functional interaction between ER and JNK. Further analysis of the molecular crosstalk between these two pathways will help to elucidate the mechanisms of ER-dependent activities through AP-1.

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CHAPTER 2

Estrogen Regulation Through the Tethered Pathway is Mediated By Multiple AP-1 and AP-1 Like Proteins*

***Dr. Nina Heldring contributed to this work by assisting with the proteomic confirmations and performing the ChIP experiments.**

Summary

In this study, I set out to define the AP-1 components involved in mediating the ERα/AP-1 pathway. I discovered various AP-1 family members were recruited to an estrogen responsive TRE site, thereby expanding the view that other AP-1 factors, besides just c-Jun, can mediate E2-dependent responses. I also found that "AP-1 like" transcription factor family members (those belonging to the CREB and Maf protein families) might also play a role in the recruitment of ER to E2-regulated promoter regions. Finally, I identified several putative AP-1 coregulators that may mediate transcriptional outcomes through TREs. Together, my results expand the limited understanding of E2-regulated events mediated through TRE and TRE-like motifs and provide new avenues for future research.

Introduction

Estrogen (E2) signaling can occur through at least two distinct pathways. The first pathway is mediated by the direct binding of estrogen receptors (ERs) to estrogen-response elements (EREs). The second pathway is mediated by the indirect "tethering" of ER to DNA through protein-protein interactions with other transcription factors like AP-1. Although it is known that ER can interact with c-Jun (an AP-1 member) at TPA-response elements (TREs) (discussed at length in Chapter 1), it remains to be determined what other AP-1 members might facilitate the ERα tethering pathway. It is also unclear if AP-1 specific coregulators play a role in mediating hormone responses through TREs. Although previous work has recapitulated ERα/AP-1 dependent transcription *in vitro* (Cheung et al., 2005), the composition of these TRE-dependent complexes has yet to be determined.

In this study, I determined the compostition of TRE-specific complexes in order to have a better understanding of the factors involved in ERα/AP-1 complexes.

Several techniques have recently been developed to identity protein-protein interactions on a large scale. These studies, conducted in yeast, used the yeast 2-hybrid method (Uetz and Hughes, 2000), an affinity chromatography approach coupled with tandem mass spectrometry (MS/MS) (Gavin et al., 2002; Ho et al., 2002), or a quantitative MS/MS approach (Ranish et al., 2003) to define protein-protein interactions. This latest approach identified the composition of *in vitro* assembled promoter complexes using DNA templates to "fish out" promoter-binding factors from yeast nuclear extract. It also utilized isotopically-labeled tags which allowed the quantitative comparison of similarly purified complexes. I decided to use this proteomic approach to identify components associated with TRE-dependent complexes.

Results

Immobilized templates can capture enhancer specific complexes

In order to better understand how liganded ER activates transcriptional responses through AP-1, I decided to take a step back and define the AP-1 components that may play a role in the tethering of ERα. To this end, I developed an immobilized template assay to isolate enhancer-specific complexes. Three templates were generated by PCR using a biotinylated forward primer. Each template had a unique PstI site located upstream of the adenovirus E4 promoter. Five tandem TREs or 4 tandem EREs were inserted between the PstI site and the promoter region to isolate AP-1 or ER complexes respectively (Figure 2.1A). A random sequence was inserted to serve as a negative control. My plan was to immobilize the various DNA templates to streptavidin coated beads through the 5' biotin moiety, incubate the DNA with HeLa nuclear extract to assemble enhancer-specific complexes, wash the DNA to

remove nonspecific proteins, and elute the DNA-bound proteins by PstI digestion. I would then detect the proteins by Western blotting.

I used the ERE template to isolate ER-containing complexes as a proof of principle. The HeLa nuclear extract was supplemented with recombinant ERα, since HeLa cells do not express either ERα or ERβ. Western analysis demonstrated that I could purify ERα in an enhancer-specific manner using the immobilized templates (Figure 2.1B). Moreover, when I added E2 during the assembly step, I noticed that I could then purify two known ER coactivators, SRC1 and Med1 (also known as Med220). This demonstrated that I could isolate both proteins that directly and indirectly bind enhancer DNA elements. Furthermore, using the TRE (but not the ERE) template I found that I could purify two AP-1 factors, c-Fos and c-Jun. Taken together, the immobilized template assay could isolate enhancer-specific complexes from HeLa nuclear extract.

Unbiased proteomic screen identifies TRE-binding proteins

Since the TRE-containing promoter template mediates E2-dependent transcription *in vitro* (Cheung et al., 2005), I hypothesized that some of the factors facilitating this response may be associated with the TRE sequence even in the absence of ERα. With the immobilized template assay working in my hands, I then sought to identify the repertoire of factors that associated with the template in a TRE-dependent fashion. To this end I utilized an unbiased proteomic screen, previously used to identify the protein components of affinity-purified RNA polymerase II pre-initiation complex in yeast (Ranish et al., 2003). Briefly, this screen is based on the use of isotopically labeled tags and tandem mass spectrometry to compare the relative abundance of tryptic peptides between two isolated complexes. The power of this method is that it can distinguish specific components of affinity-

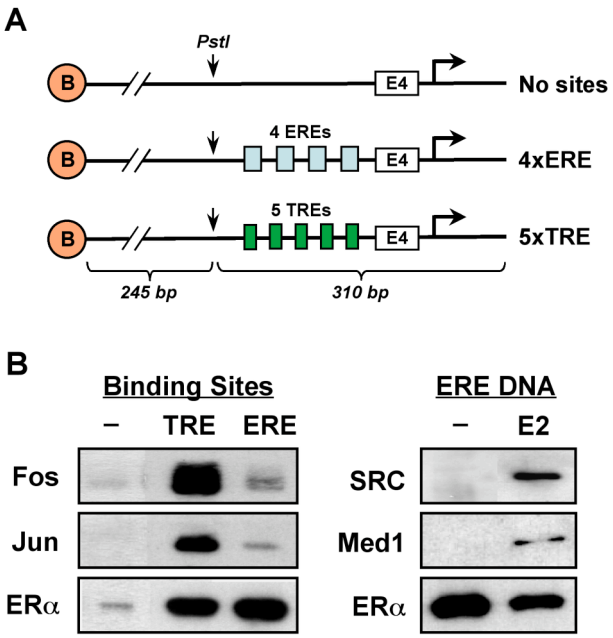


Figure 2.1 Immobilized DNA templates can isolate enhancer-specific complexes. **(A)** A schematic of the immobilized DNA templates used to purify complexes from HeLa nuclear extract is shown.

(B) Western blotting of the *Pst1* eluted material demonstrates the specificity of AP-1 (Fos and Jun) recruitment to the TRE template (*left*) and the ligand-dependent coactivator recruitment to the ERE template (*right*).

purified complexes from a high background of co-purifying proteins eliminating the need for stringent purification procedures.

To identify the components of TRE-associated complexes, I purified TRE complexes from HeLa nuclear extract samples using the TRE immobilized template. The non-TRE template was used as a control for factors associating with the TRE template in an enhancer independent fashion. The control and TRE samples were digested with trypsin and differentially labeled with either isotopically light tags (114 Daltons) or isotopically heavy tags (117 Daltons). Once labeled, the samples were mixed together and subjected to further purification using strong cation exchange (SCX) fractionation. This reduced the complexity of the sample allowing for a more complete identification of the individual peptides by MS/MS (Figure 2.2). The SCX fractions were analyzed by MS/MS by my collaborator, Jeff Ranish, at the Institute for Systems Biology in Seattle, WA.

Using Protein Pilot software and the MS/MS spectral data, I determined both the identity and relative abundance of the purified peptides. The relative abundance of each peptide was expressed as the ratio of 117 signal to 114 signal as detected by MS/MS. The Protein Pilot program normalizes the 117:114 ratios so that the average ratio is equal to 1. This is based on the assumption that the majority of purified proteins are "co-purified contaminants" and largely represent non-specific template binding. Silver-staining of the isolated complexes revealed vastly complex mixtures of proteins with no apparent difference in the banding pattern, justifying the assumption for normalization (data not shown). Ratios greater than 1 represented an enrichment of a peptide in the TRE template compared to the control template. The peptide ratios from a given protein were averaged to determine the protein enrichment. Peptides that mapped to more than one protein were not used in the protein enrichment

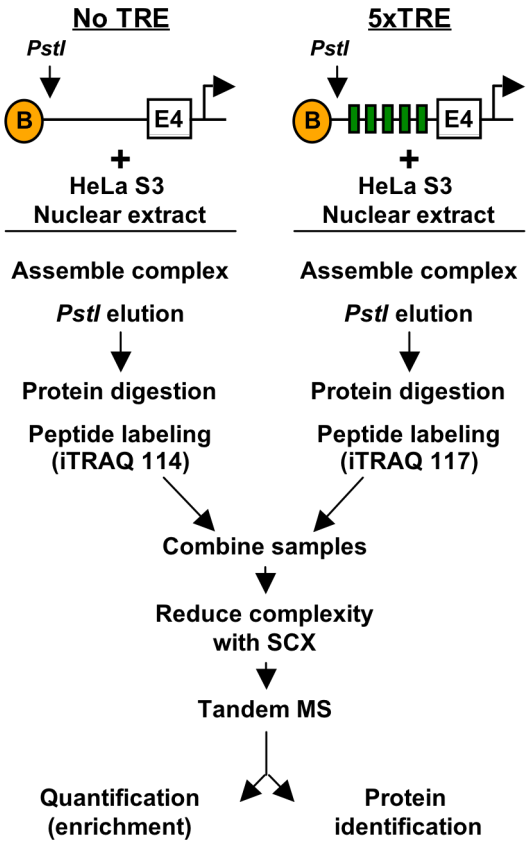


Figure 2.2 Schematic of proteomic method to identify TRE-associated factors. Control and TRE complexes were purified from HeLa nuclear extract using immobilized templates. Peptides from each sample were isotopically labeled, combined for further processing, and analyzed by tandem mass spectrometry (MS) to determine the identity and relative abundance of the proteins in each complex. B = bead, E4 = adenoviral E4 promoter.

calculation. Using this method, I identified 1,063 proteins and their relative abundance between the TRE and control templates.

This analysis revealed several transcription factors enriched in the TRE purified sample (Figure 2.3A). Besides c-Fos and c-Jun, I identified three other AP-1 factors (Fra2, JunD, JunB) that bound the DNA template in a TRE-dependent manner. The binding of these factors to the TRE template was confirmed by Western blotting (Figure 2.4). Surprisingly, I also identified transcription factors belonging to the CREB and MAF protein families, which classically bind cyclic AMP response elements (CREs) and Maf recognition elements (MAREs), respectively. The proteins comprising these families are similar to AP-1 proteins in that they bind DNA sequences closely resembling TREs (Figure 2.3B). It is also known that AP-1 and CREB members can dimerize and bind their respective DNA elements (Eferl and Wagner, 2003). These findings not only implicate AP-1 proteins other than c-Fos and c-Jun in the ER tethering pathway, but they also suggest that AP-1-like proteins (like those belonging to CREB and Maf families) may also be playing a role in ER tethering. This could be accomplished by members of these families binding to TREs or by enabling the recruitment of ER to TRE-like enhancer sequences (like CREs or MAREs).

My analysis also identified other TRE-specific components that may act as regulators of TRE-dependent transcriptional outcomes (Figure 2.5). These factors were not as enriched as the direct TRE-binding proteins which is consistent with the idea that these proteins are indirectly recruited to DNA, possibly through the transcription factors mentioned above. Although, I can not rule out a weak but direct interaction between these potential regulators and the TRE-containing DNA template. These factors included MAPKK7b (an upstream activator for the AP-1 MAP kinase, JNK), SMARCA6 (an ATPase-containing protein associated with chromatin

A

Protein	Fold	p-value	Family
JunB	6.9	2.2×10^{-6}	AP-1
JunD	6.8	1.9×10^{-1}	
c-Jun	4.9	1.7×10^{-2}	
Fra2	3.4	5.0×10^{-2}	
c-Fos	3.4	*	
ATF2	3.7	*	CREB
ATF7	2.9	1.9×10^{-2}	
CREB1	2.1	5.1×10^{-2}	
BACH1	7.5	4.4×10^{-3}	MAF
MafK	5.9	9.0×10^{-4}	
MafG	3.7	1.7×10^{-3}	
MafF	2.6	*	

B

Protein Family	Recognition Element	Consensus Sequence
AP-1	TRE	TGACTCA
CREB	CRE	TGACGTCA
MAF	MARE	TGCTGACTCAGCA

Figure 2.3 Proteomic approach identifies factors enriched in TRE-containing template.

(A) The direct binding DNA factors enriched in the TRE-containing immobilized template are shown. Fold and p-values determined by Protein Pilot software. Fold equals the average 117:114 ratio for the given protein and represents the TRE specificity (TRE:control ratio). * = p-value not determined due to the limited number of peptides.

(B) The similarity between the DNA sequences classically bound by the protein families mentioned in A are shown.

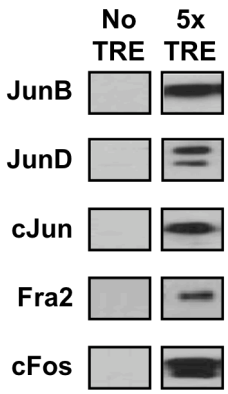


Figure 2.4 Confirmation of AP-1 proteins enriched by proteomic approach.
The material purified using the TRE and control immobilized templates was analyzed by immunoblotting. The AP-1 factors identified from the proteomic screen as enriched for TRE-binding were confirmed, demonstrating not only their specificity but also adding credibility to the proteomic results.

Protein	Fold	p-value	Related Process
MAPKK7b	2.2	7.3×10^{-3}	AP-1 MAP kinase
Med8	1.9	*	Transcriptional coactivator
TFIIAα	1.7	*	Transcription machinery
SMARCA6	1.6	3.3×10^{-4}	Chromatin remodeling
RPB1	1.6	1.7×10^{-1}	Transcription machinery
ZSCAN20	1.6	1.2×10^{-1}	Unknown
UBA5	1.6	*	Ubiquitin conjugation
ZIC2	1.5	*	Unknown
TAF1	1.5	6.7×10^{-2}	Transcription machinery
EP400	1.5	1.2×10^{-2}	Histone acetylation

Figure 2.5 Coregulator proteins are also enriched in the TRE-containing template.

Several factors were identified by our proteomic analysis as enriched in TRE-specific complexes. The factors (minus the direct TRE-binding factors shown in Figure 3.4) with at least 1.5 fold enrichment are shown. Fold and p-values determined by Protein Pilot software. Fold equals the average 117:114 ratio for the given protein and represents the TRE specificity (TRE:control ratio). * = p-value not determined due to the limited number of peptides.

remodeling), and UBA5 (a ubiquitin-activating enzyme). Taken together, my approach identified several putative coregulators of TRE-associated complexes. Future studies will establish the role of these factors not only in TRE-dependent transcriptional outcomes, but determine if these factors play a role in mediating E2-dependent transcriptional outcomes through AP-1 complexes.

Proteins identified by proteomic approach are modulated by E2 signaling in cells.

Armed with an understanding of the cohort of proteins able to bind TREs *in vitro*, and knowing that these enhancers facilitate E2 dependent transcriptional activation in the presence of ERα, I then wondered if I could detect the association of these factors with ERα in cells using chromatin immunoprecipitation (ChIP). Because the proteomic studies were done with nuclear extracts from HeLa cells, ChIP studies were conducted in a HeLa cell-line that stably expressed ERα (HeLa-ER cells). Candidate regions for ChIP analysis were chosen by overlaying ERα-bound regions with regions containing AP-1 binding sequences (*i.e.,* TREs or CREs). I defined ERα-bound regions by ChIP-chip analysis using Nimblegen promoter arrays (ChIP procedure, analysis, and arrays described in Chapter 3). TRE and CRE motifs (obtained from TRANSFAC) were mapped to genomic locations using MAST (same method as described in Chapter 3). Candidate regions that also contained an ERE motif (mapped by MAST) within the ERα-bound region were omitted to avoid ambiguity concerning ERα recruitment.

ChIP-qPCR analysis of the candidate genes revealed the ligand-dependent association of ERα with TRE and CRE-containing promoter regions (Figure 2.6 A and B). The expression of these genes was transcriptionally regulated by E2 (unpublished data from Dr. Nina Heldring) demonstrating that the recruitment of ERα correlated with the transcriptional activation of these genes. Examination of AP-1 members by

ChIP demonstrated the occupancy of these factors at endogenous TREs (Figure 2.6C). The binding of JunD, Fra2, and c-Fos was enhanced by E2 treatment suggesting that the presence of ERα at these regions either increases the affinity of AP-1 for DNA or plays a role in the recruitment of these AP-1 factors. JunB occupancy was not E2 regulated demonstrating selectivity in the liganded ER modulation of AP-1. It is also important to note that AP-1 factors can be found at CREs due to the high sequence similarity between these motifs and the dimerization between members of these families (Figure 2.6C, see c-Fos ChIP *bottom panel*). Interestingly, the CREB family members ATF2 and CREB1 showed E2 dependent recruitment to CREs containing ERα (Figure 2.6D). Together, these examples demonstrate that E2 regulated outcomes at TRE and TRE-like motifs may be mediated by more than just c-Fos and c-Jun. Other factors, like Fra2, JunD, ATF2, and CREB1, may be more directly responsible for conveying the hormone responsiveness at these promoters.

Discussion

This work describes the use of immobilized DNA templates coupled with a quantitative proteomic approach to identify enhancer-specific complexes. Many of the studies that have focused on understanding the mechanism ERα activation through AP-1 sites have focused on the mapping and manipulation of ER-c-Jun interaction surfaces, the role of various ligands, or the perturbation of ERα-coativator associations (Cheung et al., 2005; Jakacka et al., 2001; Qi et al., 2004; Teyssier et al., 2001; Webb et al., 1995; Webb et al., 1999). In this study, I attempted to identify the repertoire of AP-1-associated factors that would be present on a known E2-responsive TRE. I wanted to understand what ERα would "see" when it viewed an assembled AP-1 complex.

Using an immobilized template to isolate TRE-complexes, I found that several AP-1 members were able to bind the AP-1 consensus (Figure 2.3). This demonstrates how complex the AP-1 composition can be, further complicating our understanding of tethered ERα complexes. Interestingly, I did identify several AP-1-like proteins belonging to the CREB and Maf transcription factor families. The presence of these other factors suggests at least two conclusions. First, a canonical TRE can allow the binding of other AP-1-like transcription factors *in vitro*. Although overlap between TRE- and CRE-binding proteins has been demonstrated before [reviewed in (Eferl and Wagner, 2003)], it reminds us that "TRE", "CRE", and "MARE" sequences, found *in vivo*, are not limited to the just the protein families to which they immediately refer. Indeed, ChIP analysis of c-Fos localization demonstrated the presence of this AP-1 protein at a CRE-containing promoter (Figure 2.6C). This also argues for the confirmation of specific AP-1 binding factors when bioinformatic approaches are used to explain ERα recruitment or hormone responsiveness, since the motif, by itself, is mechanistically ambiguous. Secondly, the association of these AP-1-like factors opens the door to other "tethering" proteins besides c-Jun. Indeed, work by Sabbah et al. nearly a decade ago demonstrated the interaction of ERα with ATF2 (Sabbah et al., 1999), yet little more is known about how this interaction mediates ERα tethering in cells. More recent work by the Katzenellenbogen lab reported evidence for an interaction between ERα and CREB1 (Lazennec et al., 2001). Although this weak interaction was most likely mediated by an indirect association, it underscores the idea that factors other than c-Fos/c-Jun dimers are associated with tethered ERα complexes.

I examined tethered ERα complexes *in vivo* to determine if the TRE-bound factors from the proteomic study could be linked to E2-regulated complexes in cells. To this end, I examined promoters in HeLa-ERα cells that had the following

characteristics: (1) they displayed E2-dependent transcriptional activity, (2) ligand-dependent binding of ERα, (3) did not contain an ERE in the ERα-associated region, and (4) contained an AP-1-like element (TRE or CRE) under the ERα-associated region. The presence of several AP-1 factors was confirmed at these genes (Figure 2.6). It is interesting to note in these examples that E2 treatment causes the increase in AP-1 factors and not simply the recruitment of ERα. Indeed, previous work from our lab has shown the E2-dependent recruitment of c-Fos to the TRE-containing *UGT2B15* promoter (Kininis et al., 2007). A model that defines AP-1 proteins as DNA-bound transcription factors that act as a "landing pad" for ERα does not seem to fit the description seen *in vivo*. Perhaps these tethered complexes are formed in solution in the nucleoplasm before they actually associate with the DNA. An alternate model would be that AP-1 proteins are loosely associated with their DNA elements and liganded ERα stabilizes these AP-1 factors on DNA. ER may stabilize some AP-1 members (c-Fos, JunD) but not others (JunB) (Figure 2.6C).

The presence of ATF2 and CREB1 at a CRE was also confirmed by ChIP (Figure 2.6D). As was the case for TRE, the factor recruitment was ligand-dependent. To my knowledge this was the first description of the binding of ERα to a confirmed CRE. Even if this interaction is indirect (as the evidence mentioned above suggests), it still demonstrates that ERα can associate with a wide variety of AP-1-like proteins in cells. Future work, using reporter assays or *in vitro* transcription systems, will need to be conducted to determine the role of each tethering factor in E2-mediated transcriptional activation.

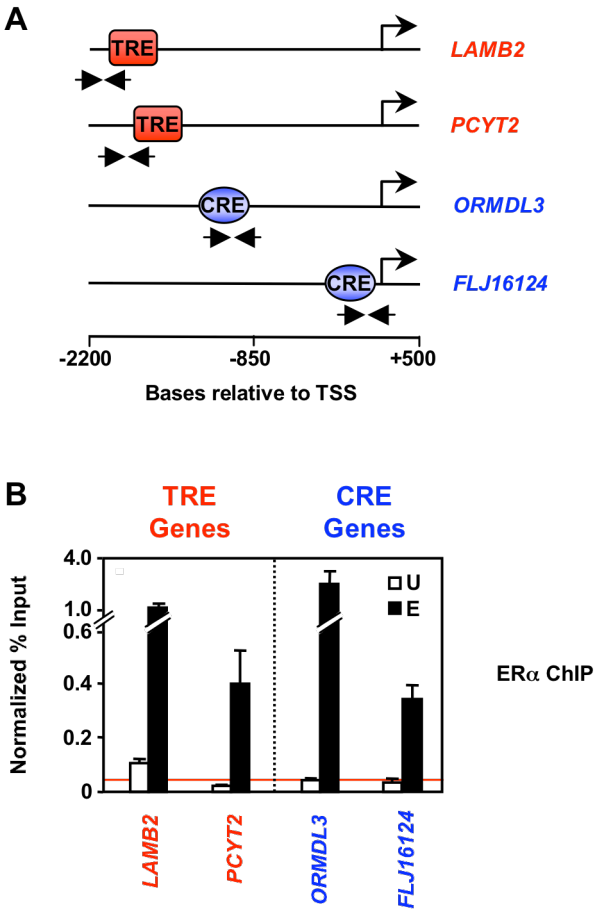
My analysis also identified several putative AP-1 coregulators (Figure 2.5). Although I did not focus on these factors due to the lack of available reagents, future studies should determine if they are indeed bona fide coregulators of AP-1 transcription. It is interesting that four of the factors are associated with the basal

Figure 2.6 Identification of tethered ERa complexes in HeLa-ER cells.

(A) A schematic of the promoter region for two TRE and two CRE genes is shown. The arrows represent the location of the primer sequences used for PCR (below).

(B) Quantitative PCR of ERα ChIP material demonstrated the hormone-induced occupancy of ERα at these regions.

(C, D) ChIP material for AP-1 (C) and CREB (D) family members are also shown for the same genes. TSS = transcription start site, U = untreated, E = E2-treated. Red line represents the average signal from "no antibody" immunoprecipitations.


A
TRE
TRE
CRE
CRE
LAMB2
PCYT2
ORMDL3
FLJ16124
-2200
-850
+500
Bases relative to TSS
B
TRE Genes
CRE Genes
Normalized % Input
4.0
1.0
0.6
0.4
0.2
0
U
E
LAMB2
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ORMDL3
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ERα ChIP

Figure 2.6 (Continued)

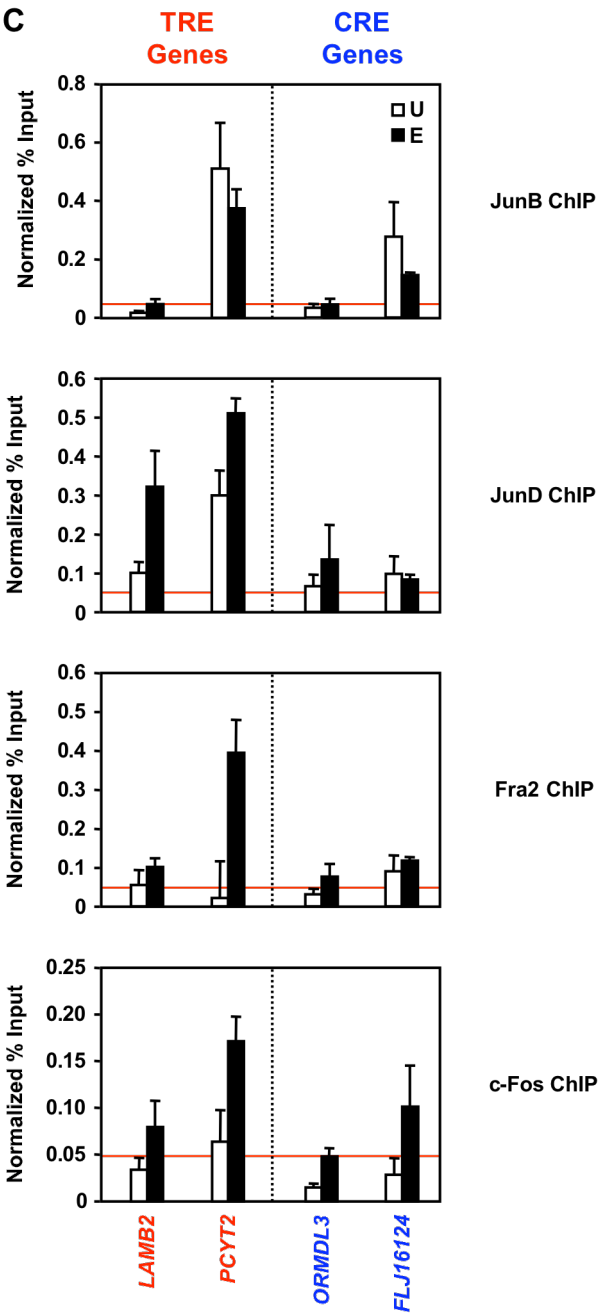
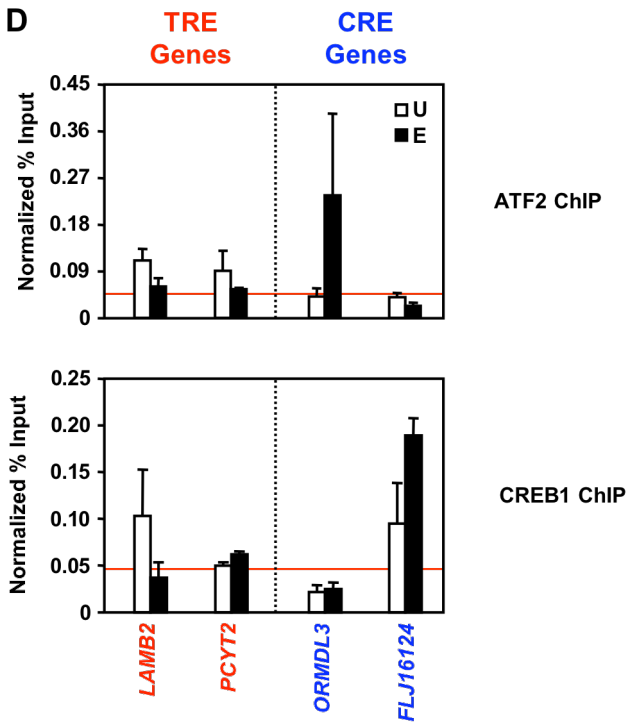


Figure 2.6 (Continued)



transcription machinery. An interaction between TBP (a component of TFIID) and the bZIP domains of c-Fos and c-Jun has already been reported adding validity to the proteomic enrichment for the TFIID component TAF1 (*i.e.,* TAF250) (Ransone et al., 1993). Moreover, I identified UBA5, an E1 activating enzyme in the ubiquitin conjugation pathway. Previous work reported another enzyme in the ubiquitin conjugation pathway, Ubc9, as an AP-1interacting protein suggesting that it plays a functional role in the association between c-Jun and the glucocorticoid receptor (Gottlicher et al., 1996). Other putative coregulators (like Med8, SMARCA6, and EP400) may also play a role in mediating E2-dependent outcomes, similar to the coregulators, SRC-1 (Cheung et al., 2005; DeNardo et al., 2005) and p300 (Cheung et al., 2005; DeNardo et al., 2005; Kamei et al., 1996).

Finally, this study determined four new genes regulated, I believe, through tethered ERα complexes. Further testing with more candidate promoters may lead to the identification of more E2-regulated, ERE-independent genes.

Although initially I wanted to extend the proteomic analysis to include the identification of ERα-containing TRE complexes, the efficiency of ERα-binding to the TRE-containing template was not sufficient enough to allow the identification of ER-dependent factors. Future modifications of the methodology described in this work may enable the elucidation of these factors and an even greater understanding of the mechanisms involved in ERα/AP-1 complex formation. Additionally, I hope to determine the genomic localization of AP-1 components before and after E2 treatment using ChIP-chip. It would be interesting to see if the E2-regulated AP-1 occupancy, described for selected genes in this work, represents a global theme for ER-associated AP-1 complexes.

Materials and Methods

Nuclear extract preparation. Nuclear extracts were prepared as described previously (Dignam et al., 1983; Kraus and Kadonaga, 1998). Briefly, HeLa S3 cells were maintained in MEM Eagle medium (Sigma M0518) pH 7.4 and supplemented with 5% calf serum, $NaHCO_3$, Penstrep, and MEM non-essential amino acids (Sigma M7145). Cells were grown in suspension up to 8L and collected when the culture reached a density of 0.5-1.0 x 10^6 cells per mL. Cells were harvested by centrifugation (J6-B rotor, 10 minutes at 3K rpm) and resuspended in cold PBS. The cells were collected (GSA rotor, 10 minutes at 3K rpm), washed a second time with cold PBS, and collected by GSA centrifugation. Cells were resuspended in 5 cell pellet volumes of hypotonic buffer (20mM Hepes pH 7.9, 10mM KCl, 1.5mM $MgCl_2$, 2mM DTT, 1mM Benzamidine, 2ug/mL Aprotonin, 2ug/mL Leupeptin, 0.2ug/mL Pepstatin, 0.2mM PMSF) and incubated on ice for 15 minutes. Cells were then pelleted using an IEC Clinical Centrifuge (10 minutes at setting 5), resuspended with 2 cell pellet volumes of hypotonic buffer, and dounced 15 times using a tight glass pestle. The intact nuclei were collected after the cytoplasmic lysate was removed by centrifugation (clinical centrifuge, 15 minutes at setting 6). Nuclei were resuspended in 0.5 nuclei volumes of hypotonic buffer plus 20% glycerol. While stirring, 0.4 nuclei volumes of hypertonic buffer (20mM Hepes pH 7.9, 1M KCl, 1mM Benzamidine, 2ug/mL Aprotonin, 2ug/mL Leupeptin, 0.2ug/mL Pepstatin, 0.2mM PMSF) were added and the extraction proceeded for 45 minutes. The extracted nuclei and lipid fraction were removed after centrifugation (SS34 rotor, 30 minutes at 16K rpm) and the remaining HeLa nuclear extract (HNE) was dialyzed for 4hrs in dialysis buffer (20mM Hepes pH 7.9, 100mM KCl, 0.1mM EDTA, 20% glycerol, 1mM DTT, 1mM Benzamidine). After dialysis, precipitates were pelleted by centrifugation (SS34

rotor, 20 minutes at 15K rpm) and the extract was aliquoted and stored at -80C. The protein concentration was determined by Bradford assay.

Plasmids. The templates used are derived from pIE0-E4 [described previously (Cheung et al., 2005)]. TREx5 is the pIE0-E4 plasmid with an insertion of 5 tandem TPA-response elements (TREs) upstream of the E4 promoter sequence. The unique PstI site in both plasmids was moved so it was in the same location relative to the transcription start site (TSS). Sequences for the modified plasmids are available upon request.

Immobilized pull-down assay. Templates used for pull-down assays were generated from the plasmids described above by PCR using a biotinylated forward primer (5'-GATTGGTTCGCTGACCATTTCCGG-3') located ~460 bases upstream of the TSS and a reverse primer (5'-CAGCCTAACAGTCAGCCTTACCAG-3') located ~85 downstream of the TSS. For each pull-down, approximately 360ng of amplified template was incubated with 5ul of Dynabeads M-280 Streptavidin beads (10ug/ul) in binding buffer (10mM Tris pH 7.5, 1mM EDTA, 1M NaCl, 0.003% IGEPAL) for 15 minutes at room temperature. (Since these beads are paramagnetic, all washes were carried out by sequestering the beads to the side of the tube using a magnet, removing the buffer, and then resuspending the beads in new buffer.) The DNA-bound beads were rinsed with binding buffer and then resuspended in blocking buffer (20mM Hepes pH 7.6, 100mM KOAc, 5mM MgOAc, 1mM EDTA, 3.5% glycerol, 60mg/ml casein (Sigma C5890), 5mg/ml polyvinylpyrrolidone (USB 20611), 2.5mM DTT) for 30 minutes at room temperature. The blocked beads were then washed 3 times with blocking buffer that lacked casein and polyvinylpyrrolidone and resuspended to their original concentration (10ug/ul) in HNE binding buffer (20mM Hepes pH 7.9, 100mM

KCl, 6mM $MgCl_2$, 0.2mM EDTA). The beads were then incubated with 385ug of HeLa nuclear extract diluted 1:1 with HNE binding buffer. Protein complexes were allowed to form on the DNA templates for 1 hr at room temperature before the beads were washed with HNE binding buffer and resuspended in PstI buffer (50mM Tris pH 7.9, 100mM NaCl, 10mM $MgCl_2$) with 60 units PstI (Roche 10798991001). After 30 minutes at 37C, the beads were pelleted and the released DNA-bound proteins were collected and analyzed by SDS-PAGE / Immunoblotting. For proteomic studies using tandem mass spectrometry (MS/MS), the pull-down assay was scaled up 300 times to provide enough eluted material for further processing. Only 680 units of PstI was used (~11x) in the final elution so as not to interfere with subsequent protein identifications by MS/MS.

Peptide preparation/Isotope labeling. The DNA-bound proteins, eluted from the immobilized template assay, were concentrated to 70ul using Microcon spin columns and SDS was added to 0.3%. The samples were boiled for 5 minutes to dissociate DNA-protein interactions, cooled to room temperature, and reduced with 10mM TCEP. The spin columns were rinsed with 300ul of 7.2M urea (made fresh) and this mixture was added to the reduced proteins. After a 45 minute incubation at room temperature, cysteine residues were blocked using 8mM MMTS for 10 minutes. Samples were examined by SDS-PAGE/ silver staining and the protein concentrations were calculated using dilution standards of HeLa nuclear extract. Approximately 800ug of each sample was diluted with TE (10mM Tris pH 8.3, 1mM EDTA) to reduce the urea concentration to <0.9M and the SDS concentration to <0.01%. Samples were then digested with 40ug of Trypsin and 8ug of Endoprotease LysC overnight at 37C. After 1:1 dilution with Buffer A (5mM KH_2PO_4 pH 2.7, 25% Acetonitrile), each sample was individually loaded onto a PolySULFOETHYL A

(PolyLC, 202SE0503) HPLC column, washed with Buffer A, and eluted with Buffer B (5mM KH_2PO_4 pH 6.0, 25% Acetonitrile, 0.5M TEAB). The eluates were dried, resuspended in water and loaded onto a reverse phase C18 columns [Nest Group, 218SPE1000]. The columns were washed with 2% Acetonitrile, 0.1%TFA and eluted with 80% Acetonitrile, 0.1%TFA. After the peptide samples were dried, 600ug (75%) were labeled with either iTRAQ-114 or iTRAQ-117 (Applied Biosystems) according to the manufacturer's specifications. Isotopically labeled samples were combined and the excess ethanol from labeling was removed by evaporation. The peptide mixture was diluted 20 fold with Buffer A, loaded onto the PolySULFOETHYLA A column, and fractionated by running the following gradient at 0.2 mL/min: 0-15% Buffer C (5mM KH_2PO_4 pH 2.7, 25% Acetonitrile, 600mM KCl) for 30 min, 15-60% Buffer C for 20 min, and 60-100% Buffer C for 15 min. I collected 32 fractions of 0.4 ml. Each fraction was dried under reduced pressure and desalted using reverse phase C18 columns as described above.

Protein identification. Peptide fractions were resuspended in 2% acetonitrile, 0.1% TFA. Approximately 40% of the sample was loaded onto an HPLC C-18 column using an Agilent 1100 Binary pump in a split-flow configuration coupled to a LC Packings Famos autosampler. Peptides were resolved by running the following acetonitrile gradient at 0.3mL/min: 2-10% for 5 min, 10-25% for 75 min, 25-35% for 15 min, and 35-80% for 5 min. Masses were detected using a QSTAR Pulsar i with 0.75s scan time for each MS read followed by 3 MS/MS reads using 2s scan time. Only the most intense ions for charge states 2-4 were analyzed. Data files from the individual fraction runs were collectively analyzed using Protein Pilot software.

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CHAPTER 3

Estrogen Regulates the JNK1 Genomic Localization Program to Control Gene Expression and Cell Growth*

***Dr. Nina Heldring, Dr. Matt Gamble, and Adam Diehl contributed to this work by assisting with the expression, ChIP-chip, and bioinformatic analysis, respectively.**

Summary

Steroid hormone and MAPK signaling pathways functionally intersect, but the molecular mechanisms of this crosstalk are unclear. Here I demonstrate an extensive and unexpected convergence of the estrogen and JNK1 signaling pathways at the genomic level in breast cancer cells. Estrogen signaling promotes a nearly complete redistribution of JNK1 to estrogen receptor alpha (ERα)-bound promoters, primarily through an ERα tethering pathway. JNK1 functions as a transcriptional coregulator of ERα at many of these promoters in a manner dependent on its kinase activity. The convergence of ERα and JNK1 at target gene promoters regulates estrogen-dependent gene expression outcomes, as well as downstream estrogen-dependent cell growth responses. Analysis of existing gene expression profiles from breast cancer biopsies suggests a role for functional interplay between ERα and JNK1 in the progression and clinical outcome of breast cancers.

Introduction

Diverse signaling pathways regulate a wide variety of cellular processes, including global transcription programs, in normal and disease states. For example, steroid hormones, such as estrogens, act through nuclear receptors to directly regulate the expression of a defined set of target genes (Acevedo and Kraus, 2004; Kininis and Kraus, 2008). In contrast, growth factors act through cytoplasmic membrane receptors to stimulate intracellular signaling pathways, including mitogen activated protein kinase (MAPK) cascades, that indirectly regulate gene expression through a variety of target transcription factors (Turjanski et al., 2007). Although functional crosstalk between steroid hormone and growth factor/MAPK signaling pathways was demonstrated nearly two decades ago in models of steroid hormone-dependent cancer

(Lange, 2004; Smith, 1998), our understanding of how these pathways converge at the genomic level to regulate gene expression remains rudimentary.

To explore the convergence of these signaling pathways at target gene promoters, I considered the possibility that Jun N-terminal kinase (JNK1), a MAPK whose expression is upregulated in breast cancers (Figure 3.1), might function as a coregulator of estrogen receptor alpha (ERα) at the promoters of estrogen-regulated genes. Although the traditional view has been that MAPK-mediated phosphorylation events (*e.g.,* phosphorylation of transcription factors) do not occur at the genes that they ultimately regulate, the terminal kinases of various signaling pathways are found in the nucleus under activating conditions (Edmunds and Mahadevan, 2004; Turjanski et al., 2007). In addition, genomic analyses in yeast (Pascual-Ahuir et al., 2006; Pokholok et al., 2006) and gene specific analyses in cultured mammalian cells (Edmunds and Mahadevan, 2004) have shown that some signaling kinases bind to the promoters of genes whose expression they regulate. The aim of this study is to characterize the genomic relationship between ERα and JNK1 in regard to their genomic occupancy and transcriptional outcomes.

Results

JNK1 is recruited to genomic regions after estrogen treatment.

In order to determine if JNK could be modulated by estrogen (E2) *in vivo*, I performed chromatin immunoprecipitation (ChIP) in MCF-7 cells treated with and without E2 using antibodies for JNK1. I examined the JNK1 occupancy at several genomic regions previously reported by ChIP to bind the ERα (Kininis et al., 2007). Since it was already known that E2 could modulate protein-DNA interactions at these regions, they seemed to be the best candidates for JNK1 ChIP. Indeed, I could detect the presence of JNK1 by ChIP-qPCR at these loci (Figure 3.2A). Interestingly, I noted

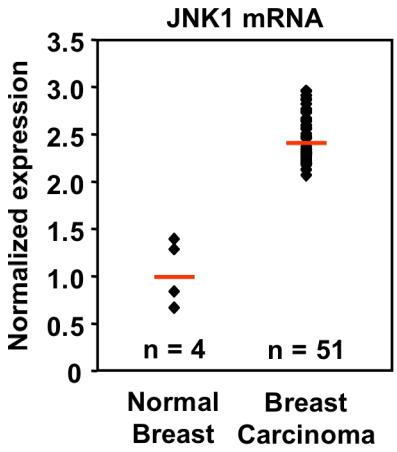


Figure 3.1 JNK1 expression is elevated in breast carcinomas.

The relative expression of MAPK8 (*i.e.,* JNK1) from 4 normal breast stroma samples and 51 breast tumor samples is shown. The data were obtained from a larger gene expression analysis (Finak et al., 2008) through the Oncomine database (Rhodes et al., 2004). The Oncomine-reported p-value was $<3.0 \times 10^{-4}$. The values were normalized to an average expression level of 1 for the normal breast samples. Red lines represent the average signal in each category.

that JNK1 occupancy was induced by E2 treatment. The hormone-dependent binding of ERα was also demonstrated for these regions, confirming the previous report (Figure 3.2B). This novel JNK1 result was quite amazing since it implies that the MAP kinase might be an estrogen-dependent cofactor in DNA-bound complexes.

Estrogen treatment does not affect global JNK1 localization.

It is known that mitogen activated protein kinases (MAPKs), like JNK1, are regulated by the dual phosphoryation of their Thr-Pro-Tyr motif by upstream MAPK kinases, and that this modification results in the translocation of the MAPK into the nucleus and the activation of its enzymatic activity [for a review, see (Davis, 2000)]. Because glucocorticoids and other steroid hormones have been shown to alter the enzymatic activity and cellular distribution of JNK (Bruna et al., 2003), I wondered if E2 treatment altered the localization or activation status of JNK1 in this system.

To this end, cytoplasmic and nuclear extracts were prepared from MCF-7 cells with and without E2 treatment. Western blotting showed that the nuclear retention of ERα was increased upon hormone treatment demonstrating the effectiveness of estrogen signaling, while GAPDH served as a cytoplasmic control illustrating proper fractionation. Blotting for JNK1 revealed that although it was present in both the cytoplasmic and nuclear extracts, only the phosphorylated form of JNK1 was detected in the nuclear extract (Figure 3.3A), consistent with previous findings (Gupta et al., 1995). The constitutive JNK1 phosphorylation may be the result of HER-2 dependent MAP kinase hyperactivation [described for ERK (Keshamouni et al., 2002; Kurokawa et al., 2000)] or related to the elevated kinase activity associated with breast cancer cell-lines [*e.g.,* AKT (Lin et al., 2005)]. Surprisingly, E2 treatment did not alter the localization of JNK1 or the fraction of activated (phosphorylated) protein. Analysis of MCF-7 cells by immunofluorescence also demonstrated similar levels of JNK1 in the

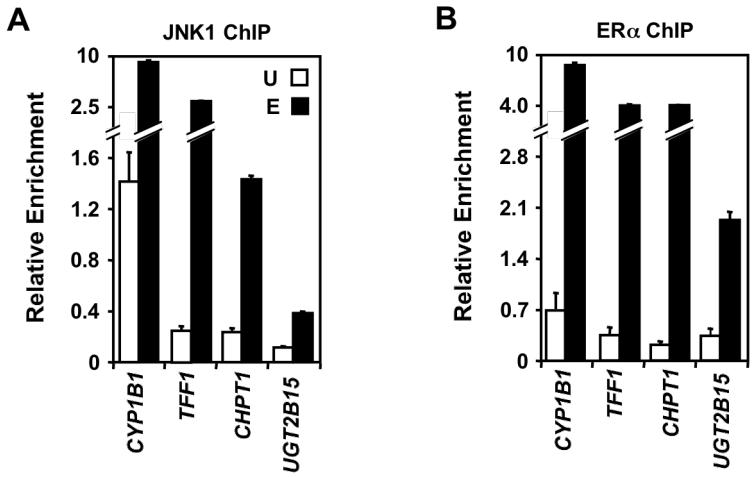


Figure 3.2 ChIP analysis of JNK1 at ERα-occupied regions.

ChIP-qPCR results demonstrating JNK1 **(A)** and ERα occupancy **(B)** at promoters in MCF-7 cells treated with ethanol (U) or E2 (E). Each bar = mean + SEM, $n \geq 3$.

cytoplasmic and nuclear compartments (Figure 3.3B).

Although the antibodies used for ChIP analysis were not phosphorylation specific, I believe that the JNK1 complexes identified contain the phosphorylated form of the protein since this is the predominant JNK form in the nucleus. Taken together, I conclude that E2 treatment alters the occupancy of activated JNK1 on DNA without altering the overall nuclear pool of JNK1.

ChIP-chip identifies global patterns of E2-dependent JNK1 occupancy at promoter regions.

To obtain a broader understanding of our JNK1 ChIP results I turned to DNA microarrays that allow a more global analysis of ChIP material rather than a gene by gene approach. I used commercially available arrays from Nimblegen that contained approximately 19,000 promoter regions tiled from -2200 to +500 base pairs (bp) relative to each transcription start site. Three biologically independent ChIP samples (representing two different JNK1 antibodies) were hybridized to the arrays and analyzed using strict peak finding criteria. The comparison of JNK1 occupancy before and after E2 treatment revealed 801 promoters that contained significantly higher levels of JNK1 occupancy after E2 treatment ("JNK1-recruited") and 235 promoters that demonstrated a significant loss of JNK1 occupancy after E2 treatment ("JNK1-released") (Figure 3.4A). Averaging of peak centered ChIP-chip data across these classes illustrates the distinct patterns JNK1 promoter localization in response to E2 (Figure 3.4B). Gene-specific ChIP-qPCR revealed both a high confirmation rate (~93%) for JNK1 peaks on the array (Figure 3.5) and local features consistent with our ChIP-chip tiling (Figure 3.6). I also noted that the JNK1 peaks were found throughout the promoter regions with no apparent preference for the TSS, as is the case for other DNA-associated factors (*i.e.*, PARP-1) (Krishnakumar et al., 2008).

Figure 3.3 Estrogen treatment does not affect global JNK1 localization.

(A) MCF-7 cells were treated with ethanol (U) or E2 (E) for 45 min., and fractionated into cytoplasmic (Cyto) and nuclear (Nuc) extracts. The extracts were analyzed by Western blotting to determine the subcellular localization of JNK1. Arrows indicate the slower migration of phosphorylated JNK1. ERα and GAPDH were used as fractionation controls.

(B) MCF-7 cells treated as above were subjected to immunostaining with a JNK1 antibody, and visualized by confocal microscopy. A single nucleus in each panel is denoted by a dotted circle. This data is consistent with my subcellular fractionation analysis, which shows no gross change in JNK1 localization upon estrogen treatment.

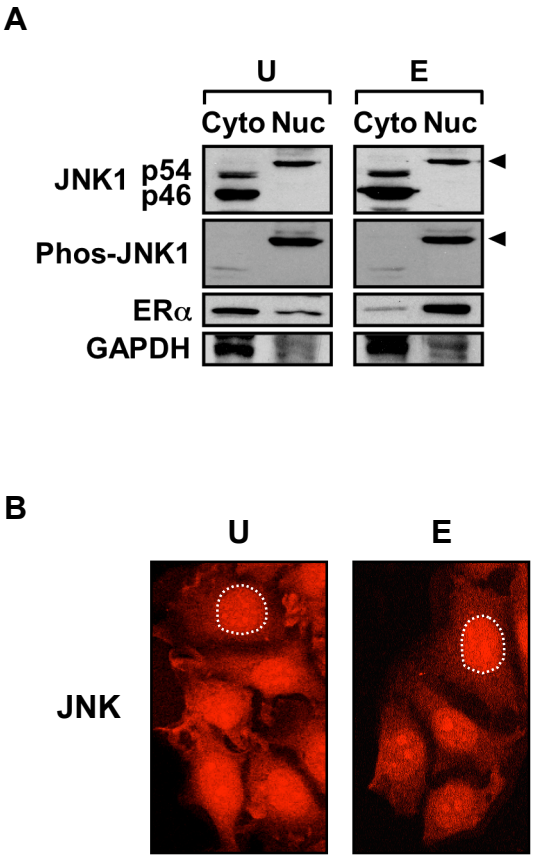

A
U
E
Cyto Nuc
Cyto Nuc
JNK1 p54
p46
Phos-JNK1
ERα
GAPDH
B
U
E
JNK

Figure 3.4 ChIP-chip reveals JNK1 localization patterns at promoter regions.

(A) A heatmap showing JNK1 ChIP-chip signals from MCF-7 cells across target promoters before (U) and after (E) a 45 min. treatment with E2 is shown. The promoters were arrayed from -2200 to +500 bp relative to each TSS (x-axis). Analysis of JNK1 occupancy revealed promoters with more JNK1 after E2 treatment ("Recruited"), less JNK1 after E2 treatment ("Released"), no change in JNK1 after E2 treatment ("Constitutive"), and no JNK1 ("Absent"). The genes in each category (y-axis) are ordered from those with the 5'-most JNK1 peak to those with the 3'-most JNK1 peak. Only 2% of the 17,297 "Absent" genes are represented.

(B) The genes in each category were aligned to the maximal JNK1 ChIP-chip signal. The peak-centered data was then averaged to demonstrate the overall pattern for each category shown in (A).

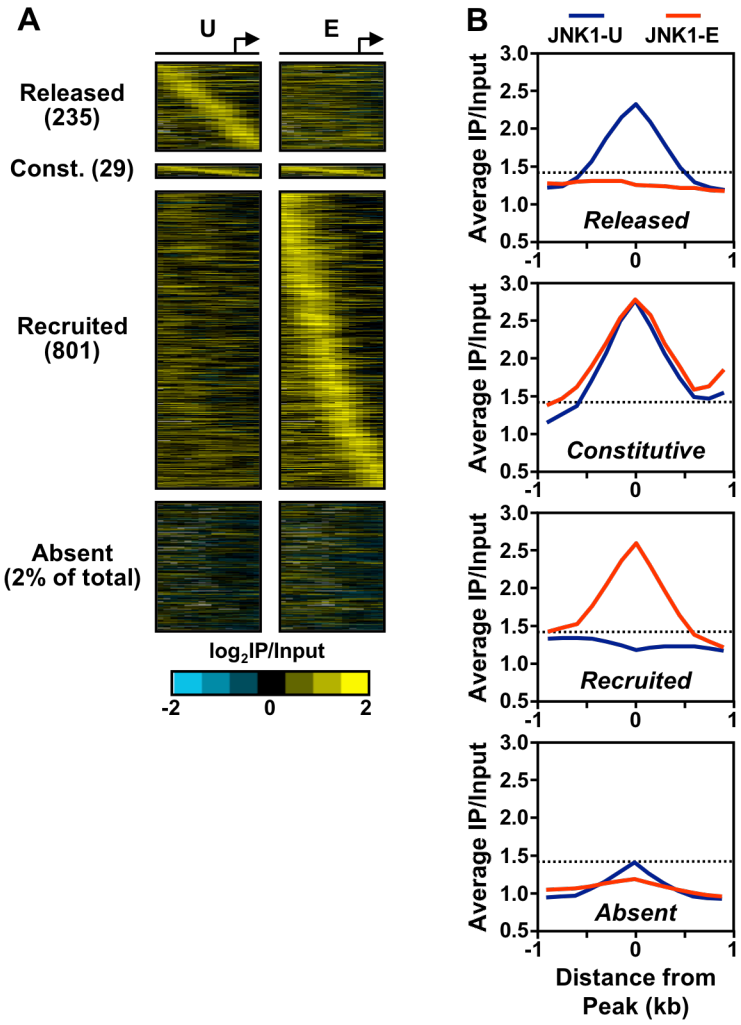
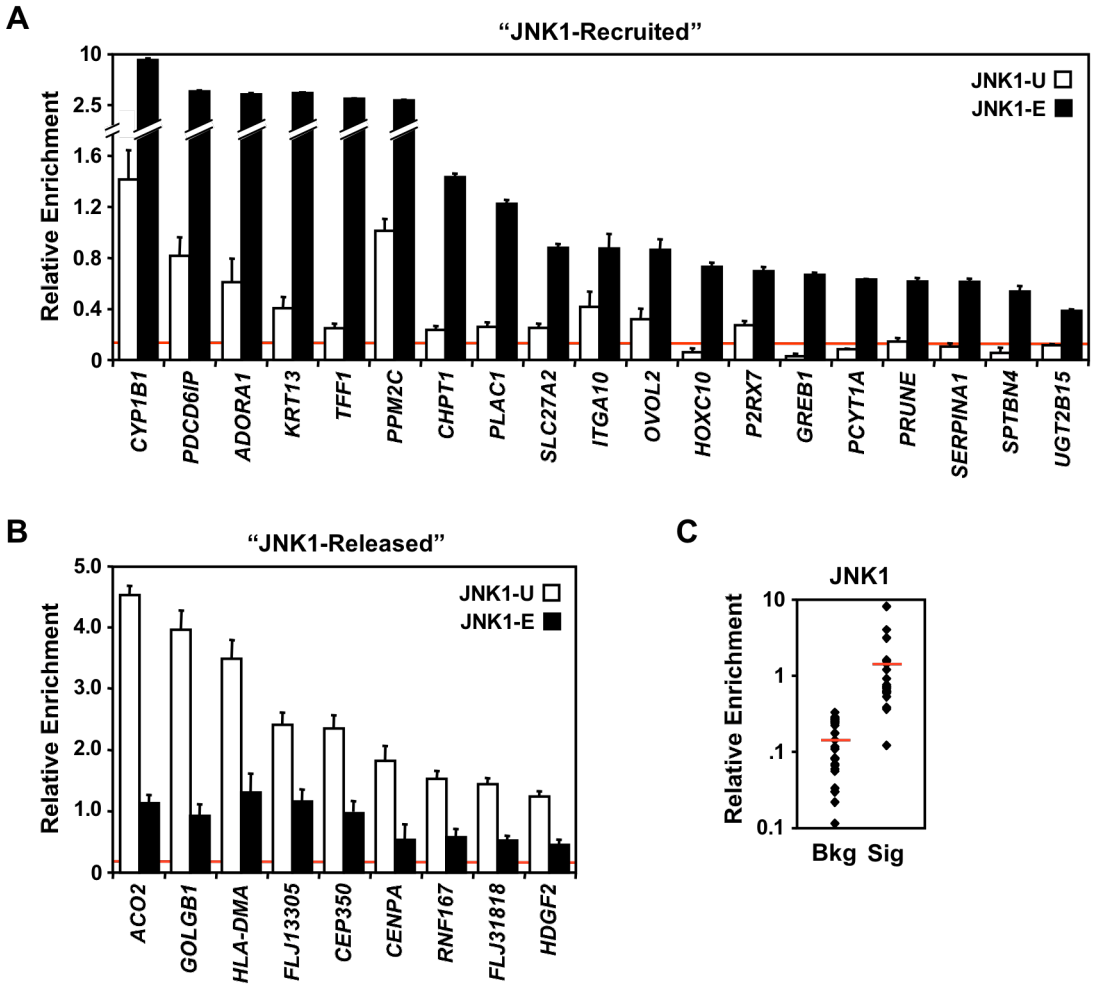

A
U
E
Released
(235)
Const. (29)
Recruited
(801)
Absent
(2% of total)
log2IP/Input
-2
0
2
B
JNK1-U
JNK1-E
Average IP/Input
3.0
2.5
2.0
1.5
1.0
0.5
-1
0
1
Released
Constitutive
Recruited
Absent
Distance from
Peak (kb)

Figure 3.5 ChIP-qPCR confirms JNK1 ChIP-chip categories.

(A, B) ChIP-qPCR confirmation of "JNK1-recruited" (A) and "JNK1-released" (B) promoters in MCF-7 cells treated with ethanol (U) or E2 (E). Greater than 93% (28/30) of the regions tested by ChIP-qPCR confirmed the ChIP-chip results. *CHPT1* and *UGT2B15* are false negatives due to the limited number of probes present in their respective peak regions on the ChIP-chip array (due to repeat masking). ChIP-qPCR and visual inspection of their ChIP-chip tiling profiles confirms that they are true "JNK1-recruited genes." Each bar = mean + SEM, $n \geq 3$. The red line in each graph represents background JNK1 signal.

(C) ChIP-qPCR to determine JNK1 occupancy at bound (Significant; Sig) and unbound (Background; Bkg) regions as defined by the ChIP-chip experiments demonstrates the validity of the peak calls**.** Red bars represent the average signal in each category.


A
"JNK1-Recruited"
Relative Enrichment
JNK1-U
JNK1-E
10
2.5
1.6
1.2
0.8
0.4
0
CYP1B1
PDCD6IP
ADORA1
KRT13
TFF1
PPM2C
CHPT1
PLAC1
SLC27A2
ITGA10
OVOL2
HOXC10
P2RX7
GREB1
PCYT1A
PRUNE
SERPINA1
SPTBN4
UGT2B15
B
"JNK1-Released"
Relative Enrichment
JNK1-U
JNK1-E
5.0
4.0
3.0
2.0
1.0
0
ACO2
GOLGB1
HLA-DMA
FLJ13305
CEP350
CENPA
RNF167
FLJ31818
HDGF2
C
JNK1
Relative Enrichment
10
1
.1
0.1
Bkg
Sig

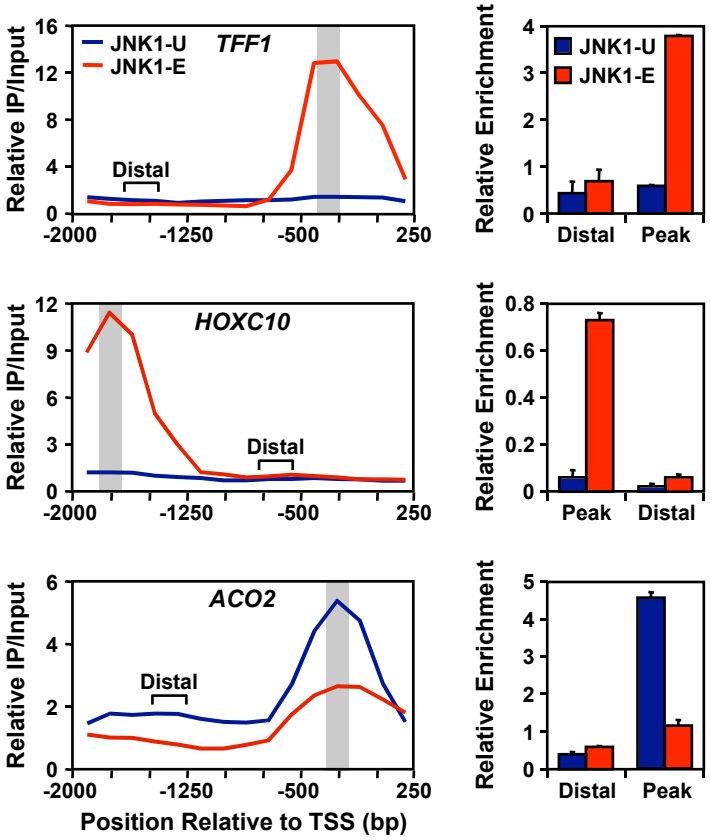


Figure 3.6 ChIP-qPCR analysis is consistent with ChIP-chip tiling features.

ChIP-chip tiling (*left*) and ChIP-qPCR (*right*) analyses of JNK1 at three promoter regions from MCF-7 cells treated with ethanol (U) or E2 (E) is shown. The average JNK1 ChIP-qPCR signals from peak (gray box) and non-peak (bracket) regions defined by the ChIP-chip tiling are consistent with the array profiles. For the ChIP-qPCR, each bar = mean + SEM, $n \geq 2$.

These results confirmed the previous finding that JNK1 required E2 treatment for occupancy at some loci, but also revealed that E2 treatment could contribute to the removal of JNK1 from other loci. Interestingly, a relatively small number of promoters (29 genes) contained similar levels of JNK1 before and after hormone treatment demonstrating that the majority of identified JNK1 complexes are regulated by E2.

JNK1-bound promoters are enriched for specific cellular functions.

I next wanted to determine if the JNK1-recruited and JNK1-released genes were enriched for particular cellular functions. Using Genecodis (Carmona-Saez et al., 2007), I determined that both the "JNK1 recruited" and "JNK1 released" gene sets showed enrichment for specific ontological categories (Table 3.1). For example, the "JNK1 recruited" gene set is enriched in genes encoding components of G-protein-coupled receptor signaling pathways and enzymes that metabolize steroid hormones. The proteins encoded by both of these ontological categories of genes would be expected to affect estrogen signaling responses, either by modulating growth factor signaling (Smith, 1998) or by metabolizing estrogens into less active or alternately active forms (Zhu and Conney, 1998). The "JNK1 released" gene set is enriched in genes encoding several mRNA-binding proteins associated with nuclear splicing. These include SF3B5 [a component of the splicesome complex (Zhou et al., 2002)], DHX38 [an RNA helicase (Schwer and Guthrie, 1991)], and RBM8A [a component of the exon junction core complex (Ballut et al., 2005)]. These genes reinforce the recent link between JNK signaling and the regulation of alternative splicing (Pelisch et al., 2005).

Table 3.1 Gene ontology of JNK1 bound promoters.

Gene set	Ontology[a]	p-value[b]
"JNK1-recruited" promoters *(801 genes)*	• GPCR signaling pathway	9.3×10^{-4}
	• Glucuronosyltransferase activity	2.5×10^{-5}
	• Metabolism of androgens and estrogens	6.0×10^{-6}
"JNK1-released" promoters *(235 genes)*	• Nuclear mRNA splicing via the spliceosome	1.3×10^{-5}
Five random gene sets[c]	• None	<0.001

[a] Ontologies were obtained using Genecodis for the JNK1-recruited and JNK1-released genes. The entire gene list represented on the ChIP-chip array was used as the background reference. Ontological assignments representing less than 5 genes were not considered.

[b] p-values were determined by Genecodis using Chi-square tests. Randomized gene lists (of equal size to each gene set analyzed) were generated from the genes present on the ChIP-chip array to determine a significance threshold and demonstrate the specificity of ontology assignments.

[c] Five random gene sets were generated using the programming language R from the total number of genes present on the ChIP-chip array. No gene ontologies were enriched (*i.e.,* all p-values were >0.001) in the random lists using the criteria described above.

Recruited JNK1 peaks correlate with ERα occupancy.

Given the estrogen-dependent alterations in the JNK1 genomic localization program, I tested the possibility that the JNK1 peaks might correspond to sites of ERα binding. My initial investigation of JNK1 localization already demonstrated that JNK1 was recruited to regions where ERα was also recruited. To explore this more globally, I performed ERα ChIP-chip using the same array platform that I used for JNK1. These results show a strong correlation between JNK1 and ERα binding sites in the promoter regions, with about 85% of the JNK1 recruited peaks overlapping an ERα peak (Figure 3.7). ChIP-qPCR assays confirmed that the JNK1 recruited peaks correlated with an E2-induced occupancy of ERα at promoter (Figure 3.8A) and distal enhancer regions (Figure 3.8C). To my surprise, the JNK1 released peaks were not associated with ERα occupancy (Figure 3.8B). These patterns of E2-dependent regulation of JNK1 and ERα binding are clearly evident for specific target promoters where the overlay of JNK1 and ERα peak regions using ChIP-chip tiling illustrates the specificity of their association (Figure 3.9). This suggests that JNK1 and ERα are co-recruited at JNK1 recruited regions. JNK1 seems to be modulated by a different mechanism at JNK1-released regions.

Nuclear E2 signaling is required for JNK1 recruitment.

The induced binding of JNK1 and ERα at promoters suggests that E2 signaling causes the convergence of ERα and MAP kinase pathways at these particular regions. Although it is well-known that E2 is a lipophilic compound, able to diffuse directly into the nucleus to facilitate ER activation, it is also known that E2 can activate cellular processes (*e.g.,* kinase cascades) in the cytoplasm ("extra-nuclear" signaling) mediated by membrane-associated ERs and other E2-activated proteins [reviewed in

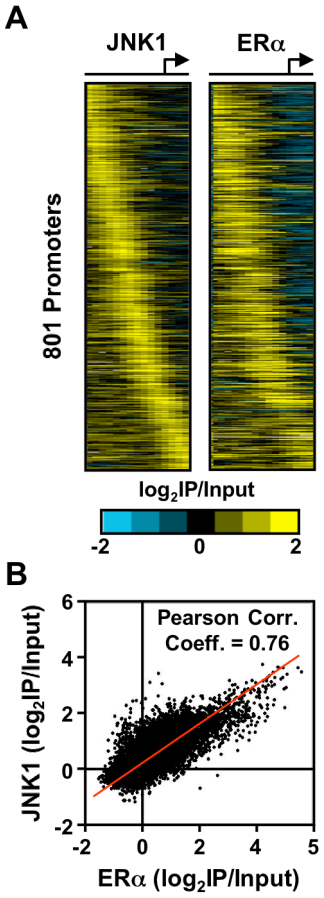


Figure 3.7 JNK1 binding correlates with ERα occupancy at target promoters.

(A) Heatmaps showing JNK1 and ERα ChIP-chip signals for the "JNK1 recruited" genes from MCF-7 cells after a 45 min. treatment with E2. The promoters were arrayed from -2200 to +500 bp relative to each TSS (x-axis). The genes in each category (y-axis) are ordered from those with the 5'-most JNK1 peak to those with the 3'-most JNK1 peak. ERα heatmap is ordered according to JNK1 heatmap.

(B) Pearson correlation analysis of the JNK1 and ERα peak data from (A) shown as a scatter-plot.

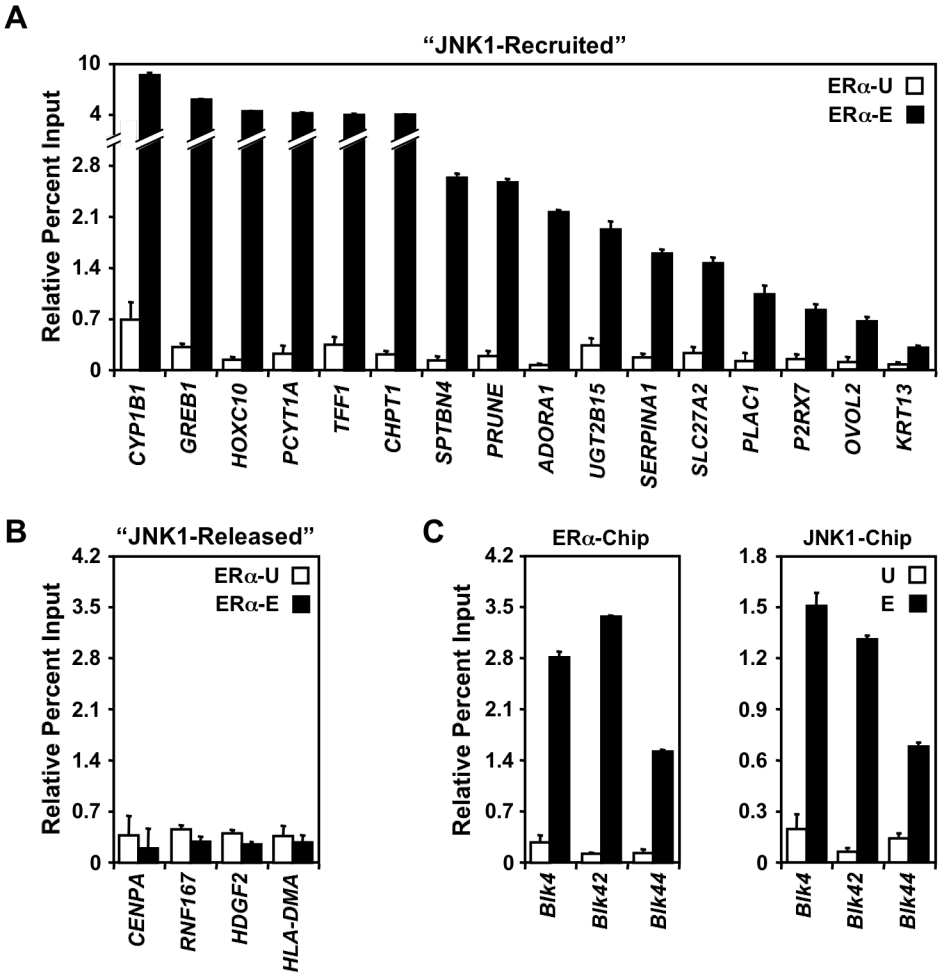


Figure 3.8 ERα is bound at "JNK1-recruited" regions.

(A, B) ChIP-qPCR analyses of ERα binding at "JNK1-recruited" (A) and "JNK1-released" (B) promoters in MCF-7 cells treated with ethanol (U) or E2 (E) revealed the association of ERα with JNK1 recruited but not JNK1 released peaks. Each bar = mean + SEM, $n \geq 2$.

(C) ChIP-qPCR analysis of JNK1 and ERα for three previously identified ERα-bound enhancer regions (Carroll et al., 2005) demonstrating that ERα / JNK1 complexes do not just occur at promoter regions. Each bar = mean + SEM, $n \geq 3$.

Figure 3.9 JNK1 recruited peaks have a peak profile similar to ERα.

(A) The average JNK1 and ERα ChIP signals from ChIP-chip (*left*) and ChIP-qPCR (*right*) are shown for three "JNK1-recruited" promoters (*GREB1*, *HOXC10*, *CYP1B1*). **(B)** Similar analysis as in (A) but for three "JNK1-released" promoters (*CEP350, CENPA, RNF167*). ChIPs from MCF-7 cells treated with ethanol (U) or E2 (E). The gray box indicates the regions of ChIP-qPCR; x-axis represents number of bp from the TSS. Each bar = mean + SEM, $n \geq 2$.

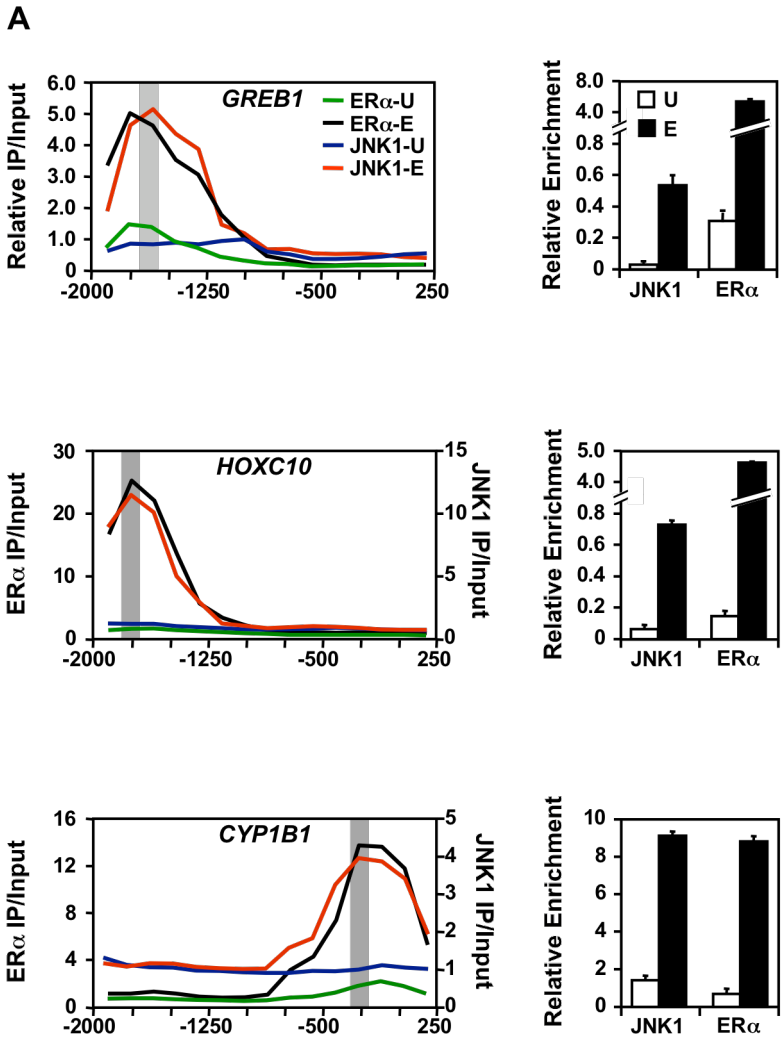
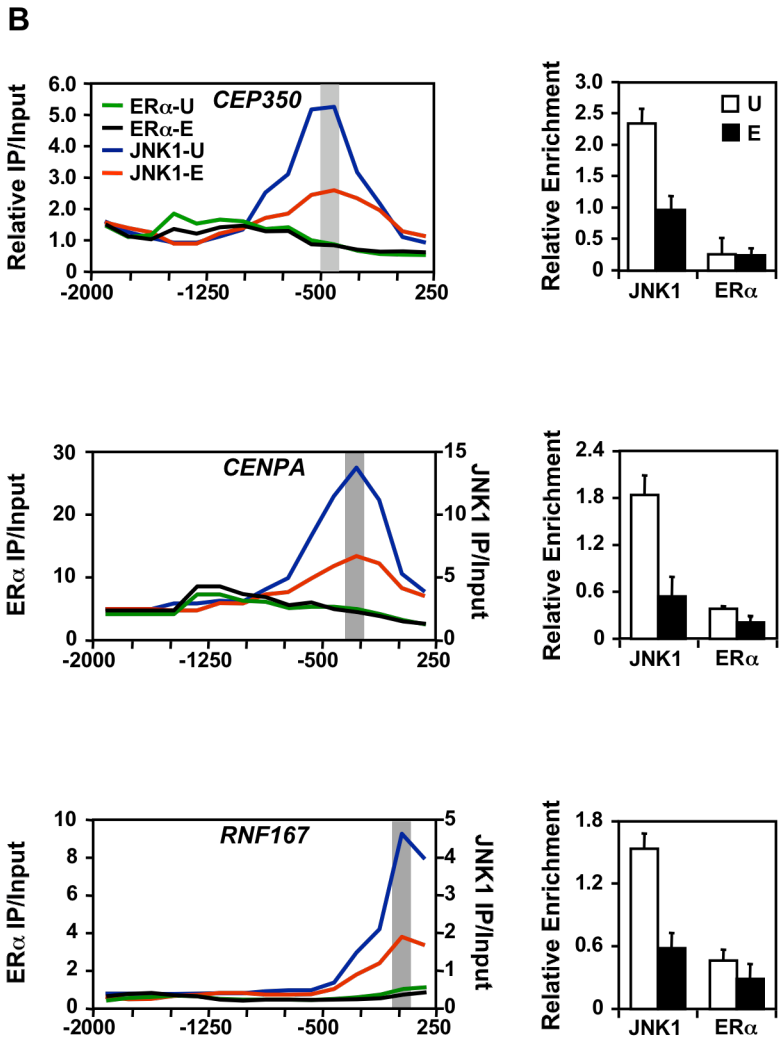

A
GREB1
ERα-U
ERα-E
JNK1-U
JNK1-E
Relative IP/Input
6.0
5.0
4.0
3.0
2.0
1.0
0
-2000
-1250
-500
250
Relative Enrichment
8.0
4.0
0.8
0.6
0.4
0.2
0
U
E
JNK1
ERα
HOXC10
ERα IP/Input
JNK1 IP/Input
30
20
10
0
15
10
5
0
5.0
4.0
0.8
0.6
0.4
0.2
CYP1B1
16
12
8
4
5
4
3
2
1
10
8
6
4
2

Figure 3.9 (Continued)



(Fu and Simoncini, 2008)]. These cascades could ultimately be responsible for the JNK1 recruitment observed in the ChIP assays.

In order to determine the requirement of E2 signaling on JNK1 recruitment, I performed ChIP assays using conjugated E2 derivatives, which can initiate cytoplasmic but not nuclear estrogen signaling pathways (Harrington et al., 2006). Indeed, treatment of MCF-7 cells with these compounds resulted in the rapid phosphorylation of ERK demonstrating that the E2-conjugates, like E2, could initiate a cytoplasmic response (Figure 3.10A). These reagents failed to promote the binding of ERα, as expected, since they can not enter the nucleus (Figure 3.10B). Examination of JNK1 occupancy revealed that, like ERα, the E2-conjugates could not induce the binding of the MAP kinase to these regions (Figure 3.10C). These results suggest a direct role for ERα at the sites of E2-dependent JNK1 recruitment. Although membrane-initiated estrogen signaling may contribute to the hormone-induced binding of ERα and JNK1 to promoters, it is not sufficient to elicit the response alone.

Bioinformatics links specific transcription factors with JNK1 occupancy.

Because JNK1 does not bind to DNA directly, its association with DNA must be facilitated by DNA-bound transcription factors (TF). I used a series of bioinformatic analyses to determine which TFs might be facilitating the binding of JNK1 to the promoter regions. First, I used MEME (Multiple Em for Motif Elicitation) and MAST (Motif Alignment and Search Tool) (Bailey et al., 2006) in an unbiased search for DNA sequence motifs enriched in JNK1-bound regions. These results yielded a number of high confidence motifs for both the "JNK1 recruited" and "JNK1 released" peaks, but notably did not include estrogen response elements (EREs) (Figure 3.11). I then used TESS (Transcription Element Search System) to predict the TFs that might bind to these enriched sequences. The TF whose binding site had the highest

Figure 3.10 Nuclear estrogen signaling is required for JNK1 recruitment to target promoters.

(A) MCF-7 cells were treated with ethanol (U), 10 nM E2 (E), 10 nM E2-conjugated BSA (E2-BSA), or 10 nM E2-dendrimer conjugate (EDC) for 10 min., followed by Western blotting for ERK and phosphorylated ERK (P-ERK). The concentrations of the estrogen derivatives were based on the amount of E2 in the conjugates, as previously described (Harrington et al., 2006).

(B,C) The binding of ERα (A) and JNK1 (B) was determined by ChIP-qPCR at three selected promoters after a 45 min. treatment as described in (A). Bars represent the mean ChIP signal relative to the maximal E2 signal for each experiment, $n \geq 3$. Error bars = SEM for U, E2-BSA, and EDC.

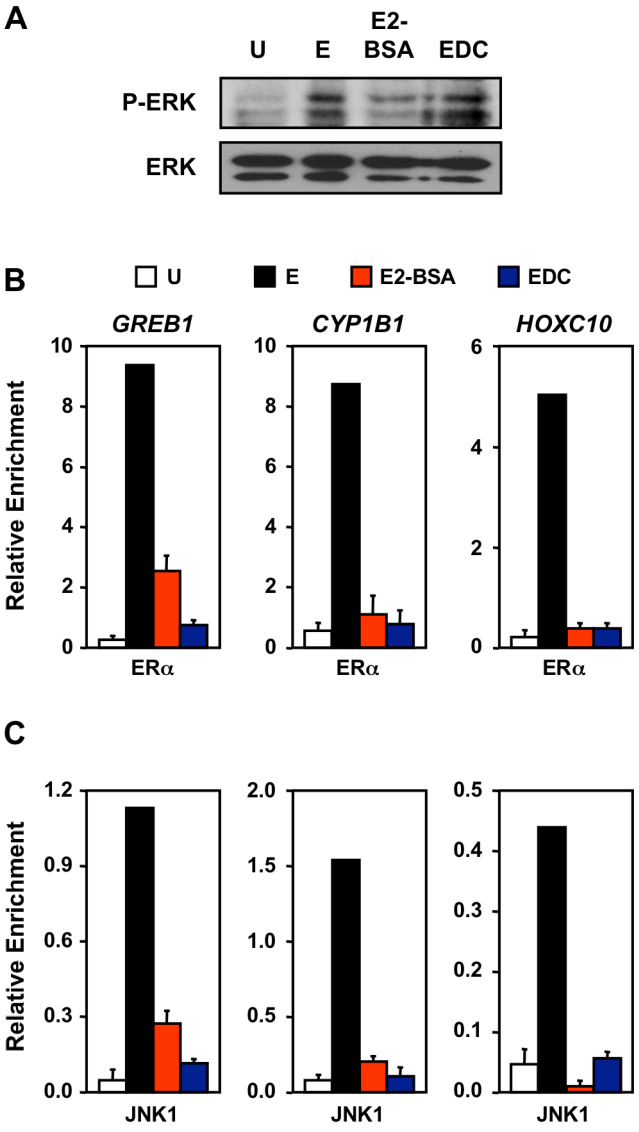
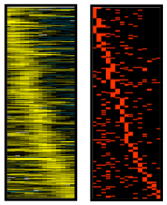
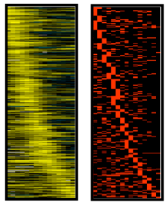
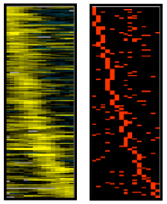
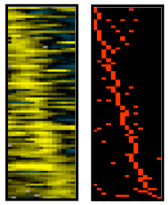
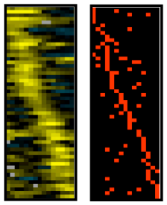
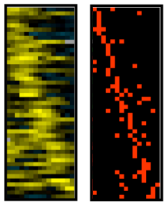
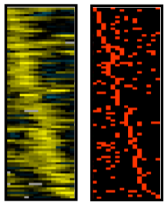

A
U
E
E2-BSA
EDC
P-ERK
ERK
B
U
E
E2-BSA
EDC
GREB1
CYP1B1
HOXC10
Relative Enrichment
ERα
C
Relative Enrichment
JNK1

Figure 3.11 Unbiased bioinformatic analysis using MEME/MAST.

The DNA sequences from JNK1-bound regions (± 250 bp from the center of each JNK1 peak) were analyzed using Multiple Em for Motif Elicitation (MEME) and Motif Alignment and Search Tool (MAST) software (Bailey et al., 2006), as described in the Materials and Methods. The results are divided into "JNK1-recruited" and "JNK1-released" peaks. The p-values for enriched sequences were determined by a Fisher exact test using the base count of motifs within peak regions versus those in JNK1-negative regions. Motif predictions were examined by Transcription Element Search System (TESS) (Schug, 2008) to determine the transcription factor most likely to bind that sequence. "Genes" represents the number of promoters where the enriched sequence falls within 225 bp of the JNK1 peak. The JNK1 ChIP-chip heatmap (JNK1) and the corresponding heatmap for the enriched sequence (Motif) is shown for these promoters. The number of genes and the motif mapping refer to the enriched sequences from MEME (not from mapping TRANSFAC weight matrices; shown in subsequent figures).

	"JNK1 Recruited"				"JNK1 Released"		
Motif No.	1	2	3	4	5	6	7
p-value	7.0×10^{-14}	1.2×10^{-21}	1.1×10^{-13}	5.5×10^{-5}	8.8×10^{-6}	7.1×10^{-4}	2.9×10^{-4}
TESS	AP-1	MEF-2	POU3F2 (OCT7)	SRF	AP1	GATA-1	POU1F1 (PIT1)
Type	Fos/Jun Family bZIP	MADS Box; SRF-related Protein	POU Homeo-domain	MADS Box	Fos/Jun Family bZIP	Zinc Finger	POU Homeo-domain
Genes	204	315	157	89	53	47	84
Map	 JNK1 Motif	 JNK1 Motif	 JNK1 Motif	 JNK1 Motif	 JNK1 Motif	 JNK1 Motif	 JNK1 Motif

alignment score with the MEME motif was chosen as the best TF candidate. This analysis identified AP-1, as well as other TFs not previously associated with JNK1 (Figure 3.11). These included the MADS box transcription factors MEF2 and SRF; the POU homeodomain transcription factors (PIT-1) and POU3F3 (OCT7); and the zinc finger transcription factor GATA-1.

Next, I mapped motif probability weight matrices for each of these transcription factors [obtained from TRANSFAC (Wingender et al., 2001)] (Figure 3.12) to the JNK1 peaks. Selected TRANSFAC motifs were mapped across the promoter regions present on the ChIP-chip array using MAST. Alignments with a p-value $< 1.5 \times 10^{-4}$ were considered true motif calls, a threshold previously reported (Kininis et al., 2007). Motifs that fell within 375 bp of a JNK1 peak were counted as peak motifs and compared to the number of motifs outside of the peak regions. Although EREs were not identified in the unbiased search, I also included an ERE probability weight matrix in this directed search since I had already determined that ERα was associated with JNK1 recruited peaks. This analysis yielded high confidence sites for all of the matrices searched (Table 3.2 and Figure 3.13A).

To test the validity of the bioinformatics analyses, I determined if the results could be used to make accurate predictions about factor binding. For this analysis, I focused on genomic regions containing high confidence AP-1 motifs (Figure 3.13 B and C). Although "JNK1 recruited" regions showed E2-induced binding of JNK1 and ERα, as expected (Figure 3.13D), only the regions with a high confidence AP-1 motif showed binding of c-Fos, a component of the AP-1 heterodimer. Surprisingly, the binding of c-Fos was also induced by E2 treatment (Figure 3.13D, *bottom*). Together, these results support the validity of my bioinformatic analyses by demonstrating the recruitment of JNK1 and c-Fos to regions containing predicted AP-1 sites.

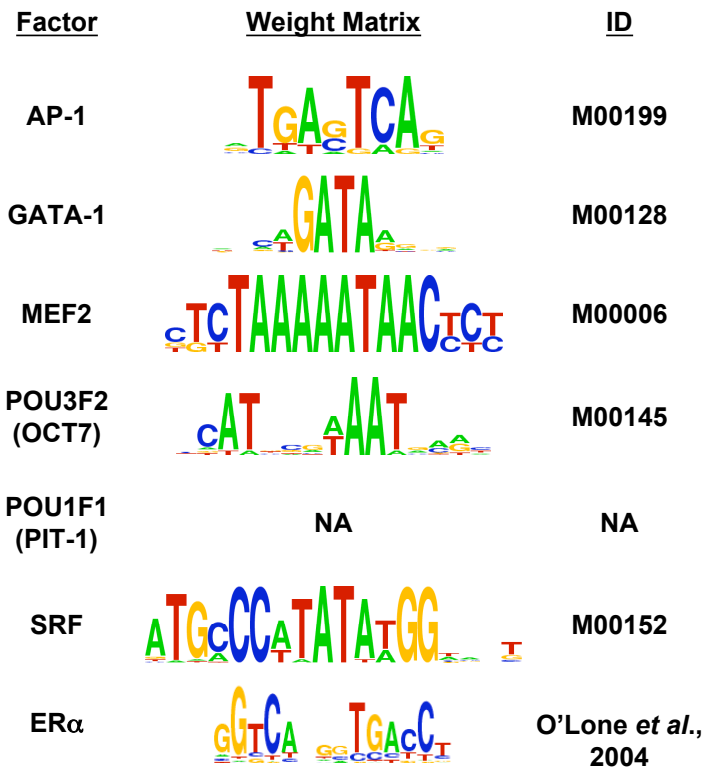


Figure 3.12 TRANSFAC motifs used for motif mapping.

An unbiased bioinformatic analysis using MEME and TESS (Figure 3.11) identified several putative transcription factors (TFs) as potential mediators of JNK1 binding. The weight matrices for these factors (obtained from TRANSFAC) are shown with the corresponding TRANSFAC ID or reference [the ERE sequence was previously reported (O'Lone et al., 2004)]. The mapping of these matrices was used to define TF binding sites used in subsequent analysis. Note, no weight matrix was available for PIT-1.

Table 3.2 Directed bioinformatic analysis of JNK1 Peak Regions

Factor	Matrix ID[a]	Genes[b]		p-value[c]
AP-1	V$AP1_C	Recruited	120	4.94×10^{-47}
		Released	36	2.33×10^{-13}
GATA-1	V$GATA1_04	Recruited	188	$<1.00 \times 10^{-300}$
		Released	42	1.93×10^{-66}
MEF2	V$MEF2_01	Recruited	325	$<1.00 \times 10^{-300}$
		Released	77	3.94×10^{-141}
POU3F2 (OCT7)	V$BRN2_01	Recruited	209	$<1.00 \times 10^{-300}$
		Released	53	3.60×10^{-138}
SRF	V$SRF_01	Recruited	149	$<1.00 \times 10^{-300}$
		Released	31	3.23×10^{-48}
ER	(O'Lone et al., 2004)	Recruited	100	1.84×10^{-7}
		Released	35	2.78×10^{-5}

Selected TRANSFAC motifs were mapped across the promoter regions present on the ChIP-chip array using MAST. Motifs that fell within 375 bp of a JNK1 peak were counted as peak motifs and compared to the number of motifs outside of the peak regions.

[a] The TRANSFAC IDs (or reference) for the weight matrices used in the analysis are listed. The ERE sequence was previously reported (O'Lone et al., 2004).

[b] "Genes" = the number of promoters that have the given motif within 375 bp of the center of the JNK1 peak.

[c] p-values were determined by Fisher's Exact Tests using the programming language R, They were based on the probability of finding bases that compose the given motif in a JNK1-bound region divided by the probability of finding bases that compose the given motif in JNK1-absent regions.

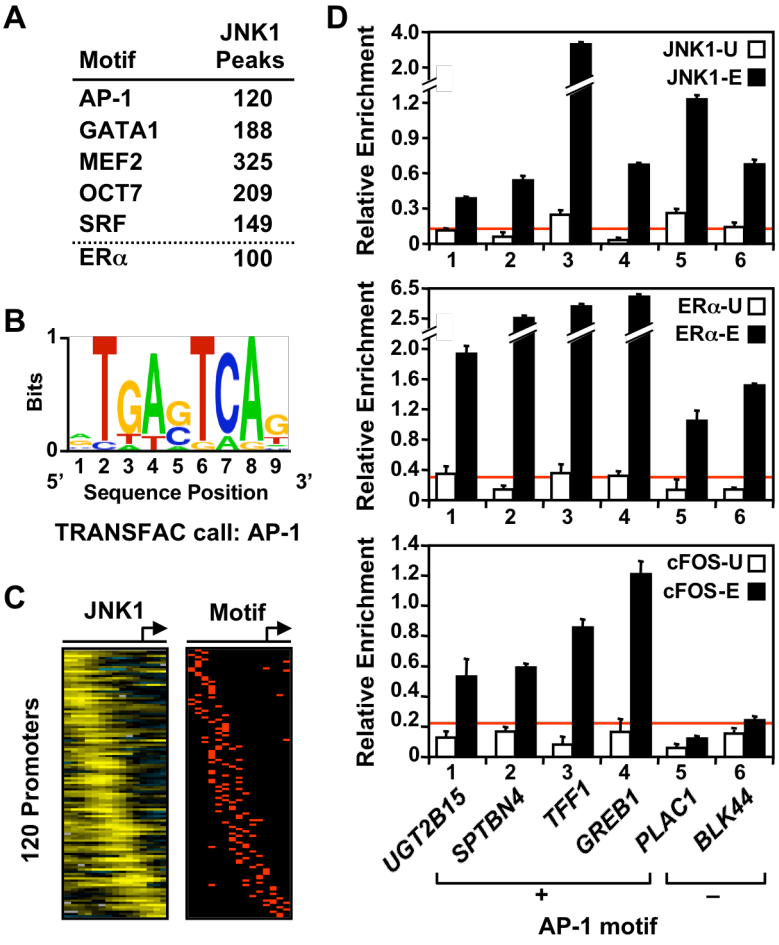
Figure 3.13 JNK1 peaks contain likely ERα tethering factor motifs.

(A) The results of the targeted search for transcription factor binding sites under the JNK1-recruited regions is summarized (shown in Table 3.2). Motifs and their likely associated binding factors were identified based on an initial unbiased search (Figure 3.11) and were mapped with MAST using position weight matrices from TRANSFAC (Figure 3.12).

(B) The AP-1 motif from TRANSFAC is shown as a position weight matrix.

(C) Heatmaps showing the location of JNK1 binding (by ChIP-chip) and predicted AP-1 binding sites (by MAST) for promoters containing an AP-1 motif within 225 bp of a JNK1-recruited peak.

(D) ChIP-qPCR analyses of c-Fos binding at JNK1- and ERα-recruited regions before (U) and after estrogen (E) treatment. *UGT2B15*, *SPTBN4, TFF1* and *GREB1* contain at least one predicted AP-1 motif under the JNK1 peak. *PLAC1* contains an ERE sequence and is included (along with *BLK44*) as a negative control for c-Fos binding. Mean ± SEM, n ≥ 3. Red lines represent background binding levels.


A
Motif
JNK1 Peaks
AP-1 120
GATA1 188
MEF2 325
OCT7 209
SRF 149
ERα 100
B
Bits
Sequence Position
5'
3'
TRANSFAC call: AP-1
C
JNK1
Motif
120 Promoters
D
Relative Enrichment
JNK1-U
JNK1-E
ERα-U
ERα-E
cFOS-U
cFOS-E
UGT2B15
SPTBN4
TFF1
GREB1
PLAC1
BLK44
+
–
AP-1 motif

JNK1 acts as a coregulator at E2-responsive genes.

Since JNK1 occupancy at genomic loci is affected by E2 signaling I wondered if JNK1 might have a direct role in mediating E2-dependent transcriptional responses. To this end, JNK1-depleted MCF-7 cells were generated by retroviral- mediated gene transfer of a short hairpin RNA (shRNA) sequence targeting JNK1 mRNA. Two different shRNA sequences gave similar levels of JNK1 mRNA and protein depletion (results for one are shown, Figure 3.14A). Control cells harboring an shRNA sequence directed against GFP [described previously (Kim and Rossi, 2003)] were generated in parallel. I examined the E2-dependent expression of target genes in MCF-7 cells using reverse transcription-qPCR (RT-qPCR). Stable short hairpin RNA (shRNA)-mediated knockdown of JNK1 or chemical inhibition of JNK catalytic activity using a JNK-specific ATP competitor, SP600125 (Bennett et al., 2001), inhibited the E2-stimulated (or E2-repressed) expression of some, but not all, estrogen target genes (Figure 3.14 B and C). Although "off-target" effects can occur when using chemical inhibitors *in vivo*, I believe that the SP600125 effects (Figure 3.14 B and C) represent specific JNK inhibition since they agree so strongly with the JNK1 knock-down data for the same genes. Thus, JNK1 and its kinase activity are required for full E2-dependent regulation of estrogen target genes in MCF-7 cells implicating JNK1 as a hormone-dependent transcriptional coregulator of ERα.

E2-dependent growth of breast cancer cells requires JNK1.

As stated above, JNK1 expression is elevated in breast cancer carcinomas relative to healthy breast tissues. One implication from this correlation would be that elevated JNK1 levels provide a proliferative advantage to hormone-dependent tissues like the breast. JNK1 is required for full E2-responsiveness at target genes, but does it affect other E2-regulated outcomes, such as cell growth? Along these lines, I tested

Figure 3.14 JNK1 activity is required for full estrogen-dependent transcriptional responses.

(A) Analysis of JNK1 mRNA and protein levels in MCF-7 cells stably expressing control (GFP) and JNK1 shRNA.

(B) The transcriptional response of E2-regulated genes from MCF-7 cells treated ± E2 for 3 hrs was determined by RT-qPCR. The effect of JNK1 knockdown (*Top*) or the effect of the JNK inhibitor SP600125 (SP) (*Bottom*) on E2-regulated, JNK1 recruited genes is shown. Asterisks represent p-values <0.05 (*) or <0.01 (**) (Student's t-test) versus E2 control (E; black bars). The E2-regulation of *HOXC10* is not affected by JNK1 knock-down or chemical inhibition. Mean ± SEM, n ≥ 3.

(C) E2-dependent down-regulation of gene expression also requires JNK1 activity as the JNK inhibitor impairs E2-dependent repression of target genes. MCF-7 cells treated as in (B) Expression data for *GOLGB1* was from 6 hrs E2 treatment. Each bar = mean ± SEM, n ≥ 2. Raw expression data from independent experiments were normalized to *ACTB* expression; the untreated control condition was set to 100.

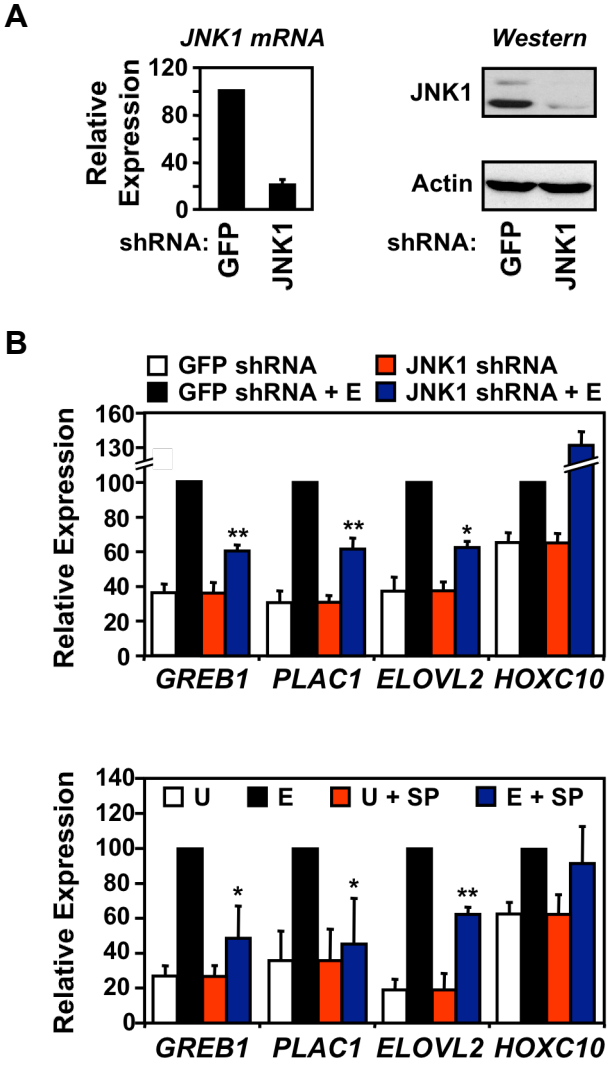
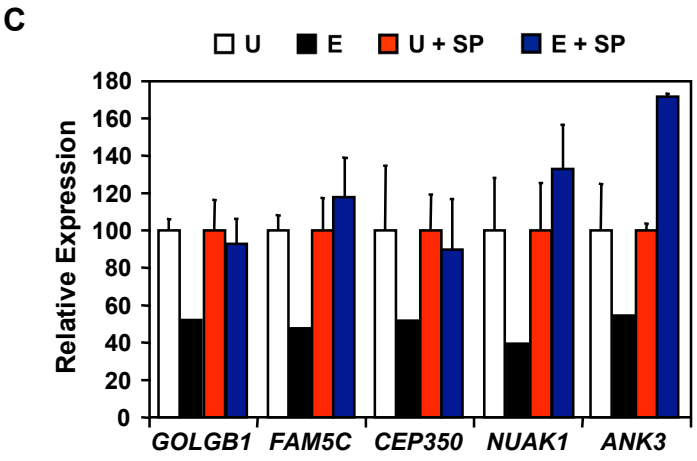

A
JNK1 mRNA
Relative Expression
120
80
40
0
shRNA:
GFP
JNK1
Western
JNK1
Actin
shRNA:
GFP
JNK1
B
GFP shRNA
JNK1 shRNA
GFP shRNA + E
JNK1 shRNA + E
Relative Expression
160
130
100
80
60
40
20
0
**
**
*
GREB1
PLAC1
ELOVL2
HOXC10
U
E
U + SP
E + SP
Relative Expression
140
120
100
80
60
40
20
0
*
*
**
GREB1
PLAC1
ELOVL2
HOXC10

Figure 3.14 (Continued)



my JNK1 knock-down line to determine if the loss of JNK1 affected the growth of these hormone stimulated breast cancer cells. Indeed, JNK1 knock-down resulted in the loss of E2-stimulated growth while the GFP knock-down responded to hormone as expected (Figure 3.15). This evidence, along with the role of JNK1 as a hormone-dependent transcriptional coregulator of ERα, suggests a physiological link between estrogen and JNK1 signaling at the genomic level with cell growth outcomes. This link may have relevance for the growth and clinical outcomes of estrogen-dependent breast cancers.

Discussion

Collectively, my results characterize the functional interplay between the estrogen and MAPK signaling pathways that has been observed previously (Lange, 2004; Smith, 1998). This association is manifested in an extensive and unexpected molecular crosstalk at the genomic level.

I demonstrated that JNK1 binds to specific sites in the genome. This illustrates the fact that signaling molecules, like MAP kinases, associate with chromatin-bound complexes broadening the understanding of how and where these kinases phosphorylate their substrates. An even more amazing finding was that E2 treatment caused a nearly complete redistribution of the JNK1 promoter localization pattern (97% of the peaks changed) (Figure 3.4). This redistribution was not due to the net movement of JNK1 to or from the nuclear compartment, nor was it due to a net change in the phosphorylation status of JNK1 (Figure 3.3). These facts are in agreement with each other since the phosphorylation of JNK is tightly linked to its nuclear translocation (Gonzalez et al., 1993; Lenormand et al., 1998). Cytoplasmic E2 signaling was not sufficient for the recruitment of JNK1 to E2-induced promoters (Figure 3.10). Together, these data highlight the fact that the E2-dependent, genomic

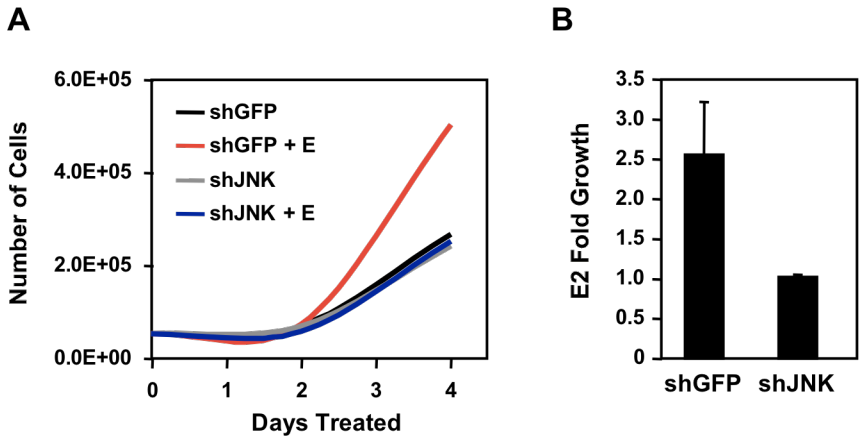


Figure 3.15 JNK1 is required for E2-induced proliferation in MCF-7 cells.

Cells expressing a shRNA construct for GFP or JNK1 were grown for 2 days in E2-free media before being plated at equal densities and treated with ethanol or E2 (100 nM; E).

(A) Cells were counted 2 and 4 days after E2 addition.

(B) The average E2-dependent fold in proliferation for day 4 is shown. Bars equal the mean ± SEM, $n \geq 2$.

changes of JNK1 occupancy involve the activated, nuclear pool of JNK1. The E2-independent activation (and thus translocation) of JNK has no doubt played a role in keeping the hormone-dependent crosstalk between these pathways enigmatic to date.

My investigation into the E2-modulated binding of JNK1 at target genes led to the discovery that JNK recruited but not JNK released peaks correlated with ERα binding (Figure 3.7-9). This suggests that at least two modes of hormone signaling influence the genomic occupancy of JNK1. Bioinformatic analyses determined several transcription factors as likely JNK1-tethering factors. It is interesting to note that due to the strong correlation between JNK1 and ERα at JNK1 recruited regions, the TFs associated with these JNK1 complexes are also implicated in mediating ERα complexes. Indeed, ERα exhibits two distinct modes of genomic binding: direct binding to DNA containing ERE sequences and indirect binding or tethering through other DNA-binding transcription factors, such as activator protein-1 (AP-1, a heterodimer of c-Fos and c-Jun or related proteins) (Kushner et al., 2000). My analysis identified E2 recruited JNK1 and ERα complexes at promoters containing EREs, but implied that this was not the major mode of JNK (and ERα) recruitment. EREs represented only ~10 percent of all the sites identified (100 out of 1091 sites for the JNK1-recruited peaks (Table 3.2 and Figure 3.13A). These bioinformatic results, together with the ChIP-chip results described above, suggest that the E2-dependent recruitment of JNK1 occurs primarily, but not exclusively, through an ERα tethering mechanism mediated by diverse types of DNA-binding transcription factors.

I further demonstrated that JNK1 can act as a coregulator of ERα-dependent transcriptional outcomes in a manner that requires its catalytic activity. The gene-specific impairment of E2-dependent transcriptional responses by JNK1 knock-down was mirrored in the loss of E2-stimulated growth in the JNK1 shRNA expressing line. It is quite interesting to note that these effects are mediated specifically by JNK1

despite the presence of the highly redundant MAPK, JNK2 (note that JNK2 expression actually increased with JNK1 knock-down) (Figure 3.16). Together, these data suggest that the specific E2-regulated genomic activities of JNK1 can ultimately affect hormone-dependent cellular processes. Indeed, JNK activity is important in tumor growth and development as several studies have demonstrated the role of JNK in Ras-mediated tumoricity [reviewed in (Davis, 2000)] and in oncogene activation [reviewed in (Ip and Davis, 1998)]. Estrogen signaling may play a large role in these JNK-dependent processes since JNK1 expression is elevated in breast carcinomas (Figure 3.1). In addition, the expression of the JNK1 phosphatase, MPK-1, a negative regulator of JNK1 activity, is reduced in high grade malignant breast cancers (Figure 3.17).

These results support a model for the estrogen- and ERα-dependent recruitment of pre-activated JNK1 from the nuclear compartment (i.e., nucleoplasm or chromatin) to the promoters of estrogen target genes. JNK1, in turn, serves a coregulator function required for efficient estrogen-dependent transcription of these genes. This role of JNK1 in the genomic estrogen signaling pathway is supported by JNK1's kinase activity, which likely targets histones or other proteins in the promoter-assembled transcription complexes (claims supported by the work presented in Chapter 4) (Figure 3.18). In sum, my studies have identified a genomic nexus between the estrogen and JNK1 signaling pathways that regulates target gene expression and downstream cell growth responses. Similar genomic systems are likely to integrate the signaling pathways for other steroid hormones and signal-regulated nuclear kinases. Future studies will aim to define the E2-regulated targets of JNK1 and determine the molecular mechanisms of JNK-dependent phosphorylation in mediating E2-regulated outcomes.

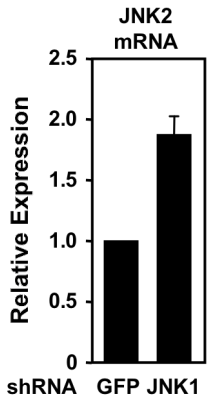


Figure 3.16 JNK2 expression is regulated by JNK1.

JNK2 expression in MCF-7 cells stably expressing an shRNA to GFP or JNK1 was determined by RT-qPCR. Raw expression data from independent experiments were normalized to *ACTB* expression and expressed relative to the GFP shRNA sample. Each bar = mean + SEM, n = 4.

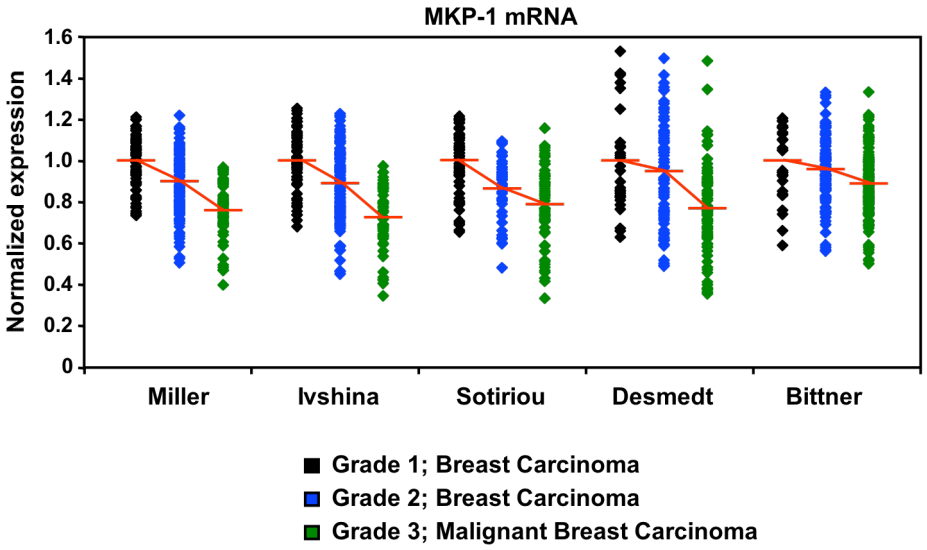


Figure 3.17 Expression of the JNK phosphatase, MKP-1, decreases with breast cancer progression.

The relative expression of MKP-1 across three breast carcinoma grades is shown from five independent studies (Bittner, 2005; Desmedt et al., 2007; Ivshina et al., 2006; Miller et al., 2005; Sotiriou and Desmedt, 2006). The p-values for negative correlation was <0.001 for all five studies. The values were normalized so that the average expression level for the Grade 1 sample from each study was 1. Red bars represent the average signal in each category.

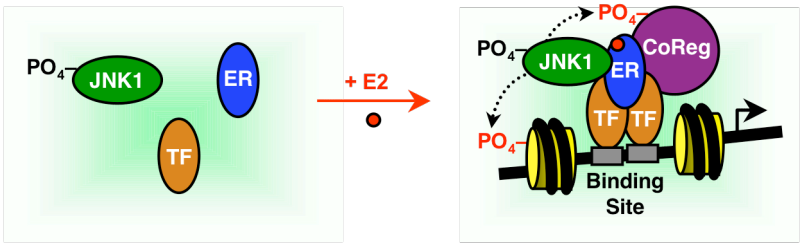


Figure 3.18 JNK1 is an estrogen-dependent coregulator.

A model illustrating the estrogen-dependent regulation of JNK1 localization at promoters, including features described in Chapter 4 (i.e., JNK1 phosphorylation targets), is shown. Activated (phosphorylated) JNK1 is co-recruited with ERα to promoters containing certain transcription factors, such as AP-1. JNK1 then phosphorylates transcriptionally relevant targets, such as H3 or coactivators of ERα, allowing full E2-responsiveness at the given promoter.

Materials and Methods

Cell culture. MCF-7 cells were maintained in MEM with Hank's salts (Sigma; M1018) supplemented with 5% calf serum, sodium bicarbonate, penicillin/streptomycin, and gentamicin. Prior to all experimental procedures and treatment with control vehicle (ethanol) or E2 (100 nM), the cells were grown for at least 3 days in phenol red-free MEM Eagle modified, with Earle's salts (Sigma; M3024) supplemented with 5% charcoal-dextran calf serum, L-glutamine, sodium bicarbonate, penicillin/streptomycin, and gentamicin. For the JNK inhibition experiments, the cells were treated with 20 μM SP600125 (SP) (BIOMOL) for 10 hrs before treatment with E2.

Antibodies. The antibodies used are as follows: JNK1 (Santa Cruz, sc-474), phosphorylated JNK (Santa Cruz, sc-6254), JNK (Santa Cruz, sc-7345), c-Fos (rabbit polyclonal generated in the Kraus lab), ERα (rabbit polyclonal generated in the Kraus lab), ERK (Santa Cruz, sc-154), phosphorylated ERK (Cell Signaling, 9106L), GAPDH (kindly provided by Eric Alani, Cornell University).

Preparation of cell extracts.

• ***JNK1 localization:*** Estrogen-starved MCF-7 cells were treated with ethanol or 100 nM E2 for 45 min.., washed with ice-cold PBS, released by scraping, and collected by centrifugation. The cell pellets were resuspended in hypotonic buffer (10 mM Tris•HCl pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium vanadate, 4 μg/ml aprotonin, 4 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF), incubated on ice for 10 min., and homogenized by Dounce 40 times with a tight glass pestle. The lysate was centrifuged at 8,000 rpm in a microfuge at 4°C and

the supernatant was collected as the cytoplasmic fraction. The nuclei were washed twice with hypotonic buffer + 0.1% NP-40 and resuspended in hypertonic buffer (10 mM Tris•HCl pH 7.9, 420 mM KCl, 10% glycerol, 10 mM NaCl, 3 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 1 mM sodium vanadate, 4 μg/ml aprotonin, 4 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF). After a 10 minute incubation on ice, the extracted nuclei were pelleted by centrifugation as above and the supernatant was collected as the nuclear fraction. The protein concentration for both fractions was determined by Bradford assays.

• ***Detection of Activated ERK:*** Estrogen-starved MCF-7 cells were grown for 24 hrs in serum-free medium, followed by a 10 min. treatment with ethanol, 10 nM E2, 10 nM 17β-estradiol 17-hemisuccinate:BSA (E2-BSA) (Steraloids), or 10 nM estrogen-dendrimer conjugate (EDC) (Harrington et al., 2006) (kindly provided by John Katzenellenbogen, University of Illinois, Urbana-Champaign). The cells were washed with ice-cold PBS, released by scraping, and collected by centrifugation. The cell pellets were resuspended in lysis buffer (50 mM Tris•HCl pH 7.9, 500 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 5 mM NaF, 1 mM sodium vanadate, 4 μg/ml aprotonin, 4 μg/ml leupeptin, 1 mM DTT, 1 mM PMSF) and subjected to three freeze-thaw cycles using liquid nitrogen. Lysates were collected after maximum centrifugation in a microfuge at 4°C. Protein concentrations were determined by Bradford assays.

Immunofluorescence. Estrogen-starved MCF-7 cells were grown on coverslips and treated with ethanol or 100 nM E2 for 45 min. After a wash with PBS, the cells were crosslinked on the coverslips for 10 min. at room temperature with a formaldehyde solution (3% formaldehyde, 5% sucrose in PBS) and the reaction was stopped by

addition of 125 mM glycine. The cells were rinsed twice with PBS, permeablized for 15 min. with 0.1% Triton X-100 made in PBS, and blocked for 20 min. with 5% BSA made in PBS. The cells were washed two more times with PBS and incubated for 30 min. with a JNK1 antibody (1:250 dilution with PBS). Afterwards, the cells were washed 3 times with TBST (10 mM Tris•HCl pH 7.9, 150 mM NaCl, 0.05% Tween-20) and incubated with a fluorescein-conjugated secondary antibody (Jackson; 115-095-146) (1:1000 dilution with PBS) for 30 min.. The coverslips were then washed 5 times with TBST, mounted to slides using Vectashield (Vector Laboratories; H-1000), and visualized using a Leica Confocal Microscope System.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed as described previously (Kininis et al., 2007), with minor modifications . The cells were grown to ~80% confluence and treated with ethanol or 100 nM E2 for 45 min. The cells were then crosslinked with 10 mM dimethyl suberimidate•HCl (DMS; Pierce, 20700) for 10 min. at room temperature, followed by 1% formaldehyde for 10 min at 37°C, with subsequent quenching by 125 mM glycine for 5 min. The crosslinked cells were collected by centrifugation, resuspended in lysis buffer [0.5% SDS, 10 mM EDTA, 50 mM Tris•HCl pH 7.9, 1x protease inhibitors (Roche; 1836153)], and sonicated three times for 10 seconds using a Branson Digital Sonifier at 27% power. This resulted in DNA fragments of ~500 bp as determined by agarose gel electrophoresis. Cell debris was removed by centrifugation and the remaining lysate was diluted 10-fold using dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 17 mM Tris•HCl pH 8.0, 167 mM NaCl, 1x protease inhibitors). After a 1 hr pre-clearing step using protein G-agarose beads (Invitrogen; 15920-010), a portion of the lysate was collected as "input" material, while the remaining lysate was incubated overnight with antibodies against JNK1, ERα, c-Fos, or without antibodies as a control. The lysates were then

incubated with protein G-agarose beads for 4 hours to capture the immunoprecipiated complexes. The beads were then washed three times with wash buffer (0.25% NP-40, 0.05% SDS, 2 mM EDTA, 20 mM Tris•HCl pH 8.0, 250 mM NaCl, 2 μg/ml leupeptin and 2 μg/ml aprotinin) and once with TE. The immunoprecipitates were resuspended in elution buffer (1%SDS, 100 mM $NaHCO_3$) and incubated overnight at 65°C to reverse the crosslinks. The proteins were digested for 45 min. at 37°C with 12.5 μg proteinase K and the DNA was precipitated with ethanol/sodium acetate following an extraction with phenol:chloroform:isoamyl alcohol. The DNA pellets were dissolved in water and analyzed by qPCR. Before averaging, the ChIP values for each region were normalized. Each ChIP experiment was conducted with at least three independent chromatin isolates to ensure reproducibility.

Ligation-mediated PCR (LM-PCR). For ChIP-chip analysis, immunoprecipitated genomic DNA was blunted and amplified by LM-PCR as described previously (Krishnakumar et al., 2008). The material was purified following digestion with RNase (Roche) using QIAquick columns (QIAGEN). qPCR on selected regions was used to confirm that the LM-PCR procedure preserved the binding patterns of the initial immunoprecipitated material. The LM-PCR for the ChIP-chip experiments was done using three independent ChIP experiments from cells treated with or without E2.

ChIP-chip. After LM-PCR, the immunoprecipitated material was labeled with Cy5 and the reference ("input") material was labeled with Cy3. The labeled samples were combined and hybridized to human HG18 RefSeq Promoter Arrays (Nimblegen; C4226-00-01). Briefly, this array contains ~19,000 well-characterized RefSeq promoters tiled with 50-mer to 75-mer probes every 100 bp. The tiled regions cover ~2200 bp upstream and ~500 bp downstream of each TSS.

ChIP-chip data analysis.

• ***Moving window analysis:*** Data processing was done essentially as described previously (Krishnakumar et al., 2008) using the statistical programming language R (Team, 2006). All R scripts are available upon request. The pairwise data files supplied by Nimblegen were used to calculate the log2 ratio data for each array. The ratio values were subjected to lowess normalization and the arrays were normalized to each other using equivalent sum of squares scaling. An error model was generated using a 600 bp moving window with 150 bp steps in which both the mean probe $\log_2$ ratio and p-value were calculated for each window. The moving window analysis was also performed on a composite fold array that represented the average JNK1 ratio in the presence of E2 divided by the average JNK1 ratio in the absence of E2. All p-values were calculated using a nonparametric Wilcoxon signed-rank test.

• ***Definition of significant bound regions:*** JNK1-bound regions were defined as the windows containing: (1) positive means in all three biological replicates, (2) at least 5 probes, and (3) p-values <0.05. Constitutive regions were defined as JNK1-bound regions (present in the E2-treated and untreated samples) that did not have a significant p-value (≥0.032) from the composite fold analysis. Recruited regions were defined as JNK1-bound regions (present in the E2-treated samples) that had both a significant p-value (<0.032) and a fold ratio >1. Released regions were defined as JNK1-bound regions (present in the untreated samples) that had both a significant p-value (<0.032) and a fold ratio <1. Of the defined regions, 98% of the recruited regions and 95% of the released regions had an absolute fold change of ≥1.3.

• ***Visual representation of the data:*** The TSS-anchored heat maps used to visualize the ChIP-chip data were generated with Java Treeview (Saldanha, 2004).

For genes with multiple TSSs, the most 5' TSS in a given tiled region was used for alignment as +1.

Bioinformatic analyses.

• ***De novo motif prediction:*** Three gene lists were generated for *de novo* motif predictions: (1) JNK1-recruited, (2) JNK1-released, and (3) JNK1-negative. The recruited and released lists report the 500 bp surrounding the location of the maximum fold change (positive or negative) for the JNK1-recruited and JNK1-released genes, respectively. The JNK1-negative list reports the regions on the array with no significant JNK1 signal. These lists were formulated using the tools on the Galaxy browser (Elnitski et al., 2006) so genomic locations from JNK1-bound regions would not be present in the background regions. Genomic sequences for all regions were obtained from a local mirror of the UCSC genome browser, release HG18. JNK1-negative sequences were extracted in the same manner and used to compute background nucleotide frequencies and 1st- through 3rd-order Markov background models.

De novo motif detection was carried out using MEME (Multiple Em for Motif Elicitation) (Bailey et al., 2006) on repeat masked sequences, using the 3rd order background model. A width range of 6 to 15 nucleotides was specified and any number of sequence occurrences was allowed within peak regions. The top 20 motifs in each peak class were retained for further analysis. Motifs with a Pearson's correlation coefficient ≥ 0.6 were grouped as similar motifs and were represented by the motif with the greatest MEME score. MAST (Motif Alignment and Search Tool) (Bailey et al., 2006) was used to scan for the locations of all motif instances within both bound and unbound sequences, using a p-value threshold of 1.5×10^{-4} as previously reported (Kininis et al., 2007). Motifs were accepted as having a potential

association with JNK1 binding only if they were significantly enriched within bound peaks relative to background sequences. Fisher's exact tests were used to determine enrichments relative to background (heretofore generically referred to as "foreground" and "background" classes) with p-values corrected for multiple testing using the Holm method in R. Contingency tables were constructed based on the number of observed motifs and total number of *k*-tuples in foreground and background sequences, where *k* is the width of the motif.

• ***Assigning transcription factors to the predicted motifs:*** TESS (Transcription Element Search Software) (Schug, 2008) was used to predict the transcription factors that might bind to the enriched sequences from MEME. Position weight matrices for the predicted transcription factors (listed below with their TRANSFAC identification tags) were obtained from the TRANSFAC database (Wingender et al., 2001) and were converted to probability models. Pseudocounts were introduced to avoid over-fitting the motif models, which were based on relatively limited training datasets. The adjusted matrices for the predicted transcription factors were mapped to the JNK1-bound and JNK1-negative regions with MAST using a 6^{th} order Markov model. Fisher's exact tests were used to determine the enrichments for each motif, as described above. In addition, promoters were scanned for the presence of EREs in the same manner and the enrichment calculated. The TRANSFAC transcription factor motifs used for mapping are as follows: AP-1 (M00199), MEF2 (M00006), SRF (M00152), POU3F2/OCT7 (M00145), and GATA1 (M00128). The ER motif was a consensus defined by O'Lone *et al.*, 2004 (O'Lone et al., 2004).

Gene ontology. Gene ontology (GO) analyses were performed using Genecodis (Carmona-Saez et al., 2007). JNK1 gene sets (i.e., "JNK1-recruited", "JNK1-

released") were uploaded and compared to the total gene list represented on the ChIP-chip array. Search parameters included the following: (1) lowest GO level, (2) a minimum of 5 genes per category, (3) each category represented by a single GO identifier, false discovery rate (FDR) <0.01. P-values were calculated by Genecodis using Chi-square tests. Ten randomly selected gene lists (5 of similar size to the JNK1-recruited list, 5 of similar size to the JNK1-released list) were analyzed in a similar manner to empirically determine the FDR. No GO terms were reported for these random lists using the criteria above. Uninformative gene categories were not recorded.

JNK1 knockdown. JNK1-depleted MCF-7 cells were generated by stable retroviral-mediated gene transfer of a short hairpin RNA (shRNA) sequence specifically targeting the JNK1 mRNA using the pSUPER.retro system under appropriate drug selection (Oligoengine). Two different shRNA sequences, obtained from SuperArray and cloned into the pSUPER vectors using unique EcoRI/XhoI sites, gave similar levels of JNK1 mRNA depletion. The JNK1 target sequences are as follows: 5'-CAGAGAGCTAGTTCTTATGAA-3' and 5'-CCTACAGAGAGCTAGTTCTTA-3'. Control cells harboring a shRNA sequence directed against GFP were generated in parallel The GFP target sequence used (5'-GAAGCTGACCCTGAAGTTCATC-3') was based on previous work (Kang et al., 2001).

Gene-specific expression analyses. The expression of endogenous target genes was determined as described previously (Kininis et al., 2007), with minor modifications. MCF-7 cells were grown to ~80% confluence and treated with ethanol or 100 nM E2 for 3 or 6 hours. Cells were washed with cold PBS and the total RNA was collected using TRIZOL (Invitrogen) according to the manufacturer's specifications. First

strand cDNA synthesis was performed using 2 μg of total RNA, 2 μg oligo(dT), and 600 units of MMLV reverse transcriptase (Promega). The resulting cDNA from each sample was treated with 3 units of RNAse H (Ambion) for 30 min. at 37°C and then diluted 1:5 with water. E2-treated samples were further diluted 1:10 and analyzed by q PCR using a 96-well DNA Engine Opticon (MJ Research) or a 384-well Prism 7700 (ABI) real-time PCR thermocycler for 45 cycles (95°C for 15 sec, 60°C for 1 min) following an initial 10 min. incubation at 95°C. The fold change in expression of each gene was calculated using a standard curve of diluted cDNA from untreated samples (1:1, 1:10, 1:100) and normalized against the fold change of β-actin, a well-characterized housekeeping gene that I used as an internal control. Independent experiments were scaled in relation to E2 expression levels with error bars representing the SEM.

Primers for quantitative real-time PCR (qPCR).

The qPCR primers used for ChIP analyses are as follows:

ACO2 forward	5'- CTTGCACCAGGCCCGTCT -3'
ACO2 reverse	5'- AAGATGTTTTACCCAAGAACAAAT -3'
ACO2distal forward	5'- CTTCAGTCCTCTGCTATCTCCTG -3'
ACO2distal reverse	5'- CCAAGTTTTGTGATGCCAAG -3'
ADORA1 forward	5'- GCCTTGTGTCTGGATGATGTT -3'
ADORA1 reverse	5'- TCCCCAAACCACTGTACTCA -3'
Blk4 forward	5'-ATCCTTGATTTGGGGCAAT -3'
Blk4 reverse	5'- CTTGCAGGCCTCTCCTTCTA -3'
Blk42 forward	5'- GGCAGGCCAAACACACATG -3'
Blk42 reverse	5'- GCCCTGGACACAAACTGCAT -3'

Blk44 forward	5’- GGGAAAATATGCAGAAGAAAACGA -3’
Blk44 reverse	5’- CATTTATTCAACACCTCTGATGTCCTA -3’
CENPA forward	5’- CCATCTCTGCGTTGCTAAGG -3’
CENPA reverse	5’- GTGCCCTCCAGTCAAAACAC -3’
CEP350 forward	5’- AGTGACAGCAGTGGGTAACG -3’
CEP350 reverse	5’- GGGGATTCGACAAGAATGAA -3’
CHPT1 forward	5’- TCTCTGAATCCGCAGTGATG -3’
CHPT1 reverse	5’- TCCCTTTCTGTACGGAGGAA -3’
CYP1B1 forward	5’- CGTGCGGCCTCGATTG -3’
CYP1B1 reverse	5’- AGGTGCCCACGTTTCCATT -3’
FLJ13305 forward	5’- GAAGGAGGGCGGTACATTCT -3’
FLJ13305 reverse	5’- CCAACTCTGGGCTTTTATTGG -3’
FLJ31818 forward	5’- ACAGCAGATGCCCTCAAGAA -3’
FLJ31818 reverse	5’- TCCAAATTAAAGGACAGGAGGT -3’
GOLGB1 forward	5’- ATGCTCCGCTTCCTCAAAG -3’
GOLGB1 reverse	5’- CCACTCGACACTTCCTGTCC -3’
GREB1 forward	5’- AGTGTGGCAACTGGGTCATTCTGA -3’
GREB1 reverse	5’- GGTATGATTCATCATTGTCTGCTGCG -3’
HDGF2 forward	5’- CCCCTTCACTCCCTTAGAGC -3’
HDGF2 reverse	5’- GAGGTTGGAGCACAGCAGTT -3’
HLA-DMA forward	5’- TTGCACATATACACACCACTCCT -3’
HLA-DMA reverse	5’- CTATCTCCTCCGCCTCCTCT -3’
HOXC10 forward	5’- AACGGTTTCGATCAAACTGGTGGG -3’
HOXC10 reverse	5’- AGCAGTCAATCCAGGGAGCCATTT -3’
HOXC10distal forward	5’- CCCTCCACCCCTCTACCTC -3’
HOXC10distal reverse	5’- AGTAACAGCGCCATCTAGCA -3’

ITGA10 forward	5’- TCGTGTCCTCCATCCTGTCT -3’
ITGA10 reverse	5’- TCAGGTCCCCTCCTTATCCT -3’
KRT13 forward	5’- ACCCAGTATTAGAACGGGACCTGA -3’
KRT13 reverse	5’- TCCAGGACATCCCAGTCAGAAGTT -3’
OVOL2 forward	5’- TTGCCTCTCCTAACCACCCGAT -3’
OVOL2 reverse	5’- GCGGCTAGAAGATGTAGCCAATGT -3’
P2RX7 forward	5’- TGGAAGCTCCCAGTCTTGTGA -3’
P2RX7 reverse	5’- CACTTTTTTGGTCTCATGTCTCTTG -3’
PCYT1A forward	5’- CCCTCGCTGTCACTTACCA -3’
PCYT1A reverse	5’- GTTGCAGGTGTGTGCCTATC -3’
PDCD6IP forward	5’- TTCCTGATACTTTTTCCGTTTACC -3’
PDCD6IP reverse	5’- ACTACTGTTGACGGGCTGCT -3’
PLAC1 forward	5’- TGACAGAACTCATTCACAGGAAG -3’
PLAC1 reverse	5’- GGCAACAGCAAGCACTACAA -3’
PPM2C forward	5’- TTGGTGAACACTAGGGAAGATAAG -3’
PPM2C reverse	5’- GGCATTGGTATTGTCTGTGG -3’
PRUNE forward	5’- ACATACACATTTGTTTACCGAACGA -3’
PRUNE reverse	5’- TCCGCAATGTCCCTAGCAA -3’
RNF167 forward	5’- CCAGAGGGAGGAGAGGTTTG -3’
RNF167 reverse	5’- AGGTTAGCGATGGAGGGACT -3’
SERPINA1 forward	5’- TGGAGGAGGAATGAAGAAAGCA -3’
SERPINA1 reverse	5’- AGCAGGACCCCAAATTCTGA -3’
SLC27A2 forward	5’- CACGCCTGCAATATCTCCTTTAAT -3’
SLC27A2 reverse	5’- CACGGTTTCTTTAAATGGTGATGA -3’
SPTBN4 forward	5’- GACTACACGTGCGTGACACC -3’
SPTBN4 reverse	5’- ACGTCCCACACCCTATCGTA -3’

TFF1 forward	5’- ATAACATTTGCCTAAGGAGGCCCG -3’
TFF1 reverse	5’- TCAGCCAAGATGACCTCACCACAT -3’
TFF1distal forward	5’- GGCCTGGTGTCCTCTGTG -3’
TFF1distal reverse	5’- CCCCATTTTGATCCGAGAA -3’
UGT2B15 forward	5’- TGAACTGTACACACTAATTGGTGAGTCA -3’
UGT2B15 reverse	5’- TCGTGGTGCAAGTAATGTCTTCTAA -3’

The qPCR primers used for expression analyses are as follows:

ACTB forward	5’- AGCTACGAGCTGCCTGAC -3’
ACTB reverse	5’- AAGGTAGTTTCGTGGATGC -3’
ANK3 forward	5’- CGCTCCTTCAGTTCGGATAG -3’
ANK3 reverse	5’- TTCCCTTGTGAATGTTAGATGCT -3’
CEP350 forward	5’- AAAGTGGCCTTAGCTTTTTGC -3’
CEP350 reverse	5’- GAAGATGTAAGTTTGTATTTCTTGCAG -3’
ELOVL2 forward	5’- AGAGGGTGGTTCATGTTGGA -3’
ELOVL2 reverse	5’- CAAGGTGAGGATACCCCTGA -3’
FAM5C forward	5’- TTTACAGTGCTTTTGTGGAACAG -3’
FAM5C reverse	5’- TTGTCAGCAAGTTCATGTGTG -3’
GOLGB1 forward	5’- CATGGGAGGACAGCATCTTC -3’
GOLGB1 reverse	5’- GATCAAGGGCAAAAGCAAAG -3’
GREB1 forward	5’- GCCGTTGACAAGAGGTTC -3’
GREB1 reverse	5’- GGGTTGAGTGGTCAGTTTC -3’
HOXC10 forward	5’- GACACCTCGGATAACGAAGC -3’
HOXC10 reverse	5’- TTTCTCCAATTCCAGCGTCT -3’
MAPK8 forward	5’- CATCATGAGCAGAAGCAAGC -3’

MAPK8 reverse	5’- GCTGCGCATACTATTCCTTG -3’
MAPK9 forward	5’- TCATCCTGGGTATGGGCTAC -3’
MAPK9 reverse	5’- CAATATGGTCAGTGCCTTGG -3’
NUAK1 forward	5’- CAGTCACACACGCTGCTCTT -3’
PLAC1 forward	5’- CAGTGAGCACAAAGCCACAT -3’
PLAC1 reverse	5’- AACCACAGGAAACAGGAAGC -3’

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CHAPTER 4

Determination of JNK1 Substrates Using an *In vitro* Kinase System

Summary

JNK family members, like other MAP kinases, transduce cellular signals by phosphorylating target proteins. Identifying JNK substrates is critical to understanding the mechanisms of JNK-mediated processes (*i.e.,* transcriptional responses, cell-growth outcomes). Using a candidate approach, I determined that JNK1 can phosphorylate proteins associated with estrogen-dependent signaling. This modification can occur in *cis* (*e.g.,* SRC-1, p300) or in *trans* (*e.g.,* Histone H3). These novel JNK targets may be responsible for mediating JNK-dependent outcomes at hormone-responsive genes.

Introduction

In my previous work (discussed at length in Chapter 3), I described a hormone-dependent correlation between ERα and JNK1 occupancy at various promoter regions. I further demonstrated at certain E2-regulated genes occupied by JNK1 that these promoters require the catalytic activity of JNK1 to attain maximal hormone responsiveness. The question then arises: "What are the targets of JNK1 activity that might play a role in mediating E2-dependent transcriptional outcomes?" One way to start addressing this question would be to show the *in vivo* association of known JNK1 targets at E2-regulated promoters containing JNK1. This method would most likely be expensive and technically challenging (*e.g.,* purchasing many antibodies and working out the appropriate chromatin immunoprecipitation conditions). I decided to use an *in vitro* kinase assay to determine if JNK1 could phosphorylate known coactivators of ERα.

In order to conduct these kinase assays I needed purified JNK1 in its active form and candidate substrates to test. A previous study reconstituted a MAP kinase pathway in bacteria as a means to purify rat JNK2 (rJNK2) in its active form

(Khokhlatchev et al., 1997). They used a two-plasmid system that enabled the expression of three kinases involved in the JNK pathway. A phosphorylation relay is established whereby the first kinase, a MAP kinase kinase kinase (MAPKKK), phosphorylates the second kinase, a MAP kinase kinase (MAPKK), which phosphorylates the third kinase, a MAP kinase (MAPK) (Figure 4.1A). This signaling cascade is initiated by the expression of a constitutively active MAPKKK (referred to as MEKK-C since it is a carboxy-terminal fragment of MEKK1) expressed from a low-copy plasmid. The remaining two kinases are expressed from a high-copy plasmid containing a different selectable marker. Isolation of the terminal MAPK is facilitated by the use of a 6x Histidine tag (Figure 4.1B). The two-plasmid system allows the removal of the upstream kinase (MEKK-C) if purification of inactive MAPK is desired. In this study, I modify this system to purify recombinant human JNK1 for use in my *in vitro* kinase assays.

I wanted to determine what factors, known to associate with ERα in a ligand-dependent manner, could serve as potential substrates of JNK1 activity. Coactivators, like p300 and SRC-1 have already been implicated in the ER/AP-1 pathway and are very good candidates as possible substrates for JNK1 activity (Cheung et al., 2005; Feng et al., 2001; Teyssier et al., 2001). Having a better understanding of the JNK1 substrates involved in E2-dependent outcomes will help to further elucidate the mechanisms surrounding hormone-dependent actions *in vivo*.

Results

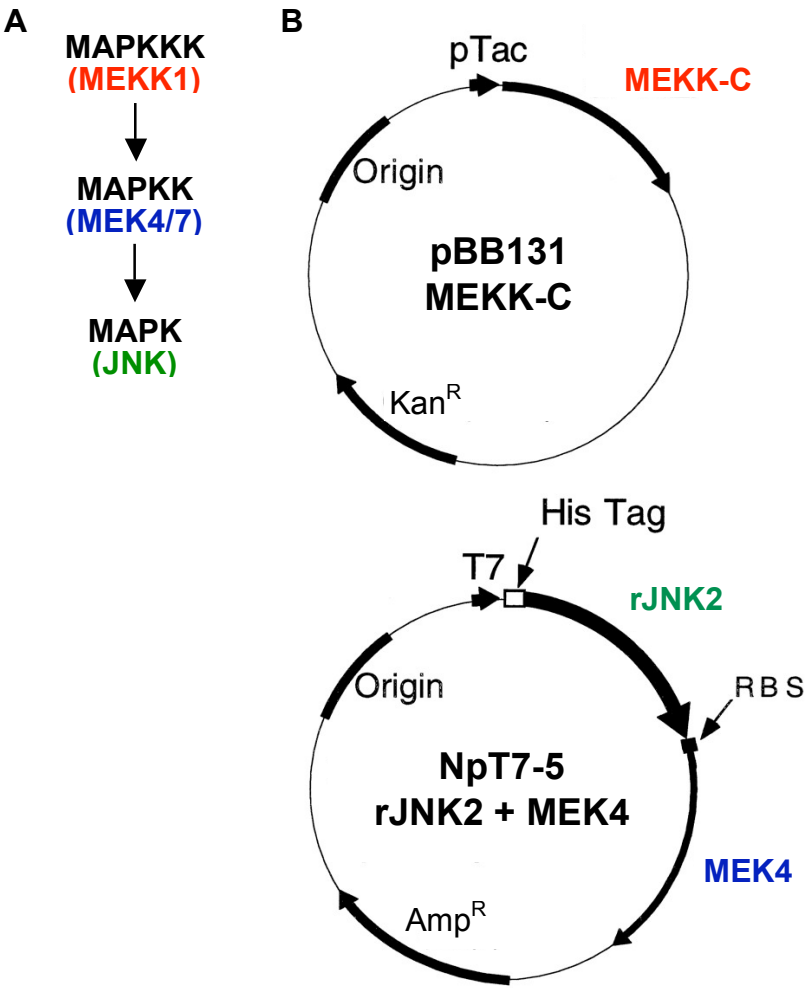
JNKs can be purified in their active and inactive forms from bacteria.

Using the MAP kinase system previously established in Dr. Melanie Cobb's lab, I purified JNK2 in its active and inactive forms. After I replaced the rJNK2 sequence with that of hJNK1α1 (details described in Methods and Materials), I

Figure 4.1 Schematic of plasmid system used to reconstitute MAP kinase phosphorylation relay in E. coli.

(A) The phosphorylation cascade for MAP kinases involves at least three enzymes. When activated, the MAP kinase kinase kinase (MAPKKK) phosphorylates a MAP kinase kinase (MAPKK) which, in turn, phosphorylates a MAP kinase (MAPK). The proteins representing these kinase tiers are shown in parentheses.

(B) By introducing these three factors into bacteria using a two-plasmid system, activated (phosphorylated) MAP kinases can be purified. Note that only the final MAPK is expressed as a 6xHis fusion protein.


A
MAPKKK
(MEKK1)
MAPKK
(MEK4/7)
MAPK
(JNK)
B
pTac
MEKK-C
Origin
pBB131
MEKK-C
Kan^R
His Tag
T7
rJNK2
Origin
RBS
NpT7-5
rJNK2 + MEK4
MEK4
Amp^R

purified the active form of this enzyme as well (Figure 4.2A). As a test, I compared the ability of my active and inactive rJNK2 to phosphorylate c-Jun, a well-known substrate of JNK. As expected, only the rJNK2 that was purified using the complete phosphorylation relay system resulted in the phosphorylation of c-Jun (Figure 4.2B). Comparison of the active rJNK2 and active hJNK1α1 demonstrated that they both phosphorylated c-Jun with similar efficiencies (data not shown).

JNK1 phosphorylates coactivators of ERα.

Now that I had an *in vitro* kinase assay established, I wanted to determine if JNK1 could phosphorylate proteins likely to play a role in mediating E2-dependent transcriptional outcomes *in vivo*. SRC-1 and p300 seemed to be good candidates for analysis since they both associate with liganded ER (Davie and Chadee, 1998; Leo and Chen, 2000; Narlikar et al., 2002). They also, like JNK1, associate with AP-1 proteins (Bannister et al., 1995; Lee et al., 1998). These coactivators have also been implicated in the formation of E2-dependent complexes tethered to DNA through AP-1 sites (Cheung et al., 2005; Feng et al., 2001).

I purified ERα, SRC-1 and p300 and determined if they were, indeed, substrates of JNK1 phosphorylation. I noticed that p300 and SRC-1 were efficiently modified by JNK1 while ERα was phosphorylated less efficiently by comparison (note the film exposure times for autoradiography) (Figure 4.3A). While all three proteins contain proline-directed serines or threonines, it is interesting to note that both p300 and SRC-1 contain a JNK binding motif similar to the docking motif found in the JNK-interacting proteins, glucocorticoid receptor (GR) (Bruna et al., 2003) and c-Jun (Dai et al., 1995) (Figure 4.3B). This further implicates these coactivators as likely substrates of JNK1 *in vivo*. No JNK interaction motif was found in the ERα sequence that most likely explains the lower efficiency of phosphorylation.

Figure 4.2 Active, recombinant JNK can be purified from bacteria.

(A) Rat JNK2 was purified from *E. coli* using the two-plasmid system as previously reported (Khokhlatchev et al., 1997). The rat JNK2 sequence was cloned out and replaced with the human JNK1 sequence and then purified in a similar manner.

(B) An *in vitro* kinase assay, using purified JNK2, demonstrated that the active enzyme (P-JNK2) could phosphorylate recombinant c-Jun. Purification of JNK2 from bacteria lacking the upstream activator plasmid resulted in an inactive enzyme (unphosphorylated JNK2) that was unable to phosphorylate the c-Jun substrate.

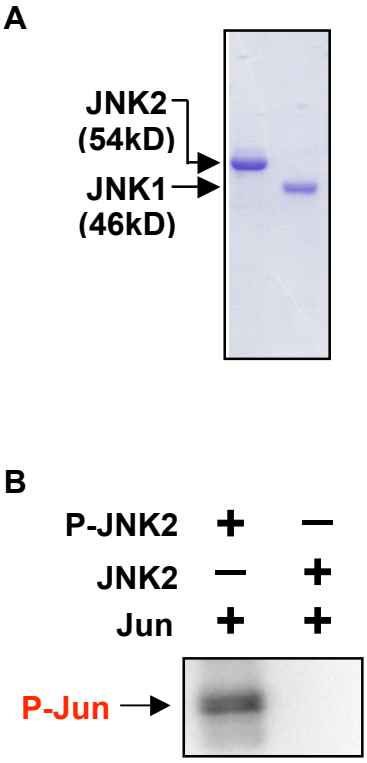
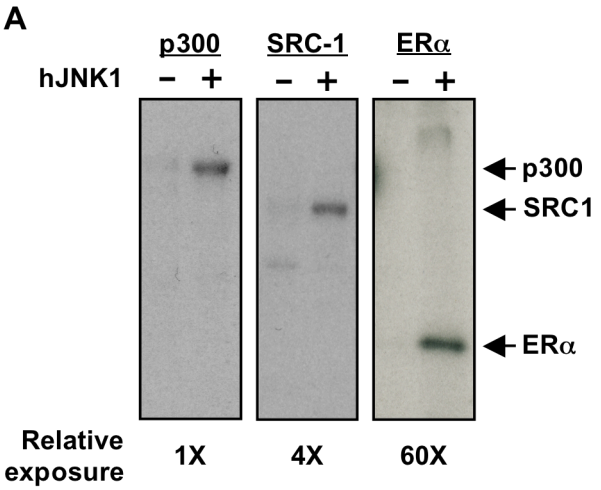

A
JNK2
(54kD)
JNK1
(46kD)
B
P-JNK2 + −
JNK2 − +
Jun + +
P-Jun

Figure 4.3 JNK1 phosphorylates coactivators.

(A) Kinase reactions were conducted using recombinant JNK1 incubated with ERα (160 nM), p300 (160 nM), or SRC1 (140 nM). Although phosphorylation of ERα was detected, the coactivators SRC1 and p300 were substantially modified in comparison (note the relative exposure times for each autoradiogram)

(B) The amino acid sequence of p300, SRC-1 and ERα were examined for a putative JNK interaction domain (Dai et al., 1995; Gupta et al., 1996; Kallunki et al., 1996; Yasuda et al., 1999). SRC-1 and p300 but not ERα had a motif similar to the JNK-interacting sequence in c-Jun. Taken together, this data points to SRC1 and p300 as probable *in vivo* targets of JNK1 enzymatic activity. Key: + = basic amino acids; x = any amino acid; T = Threonine; Φ = hydrophobic amino acids; R = Arginine; K = Lysine; M = Methionine; V = Valine. Numbers represent the first amino acid position in the motif.



B

JNK Binding Motif ++xxxxTΦxΦ		
1635	RRxxxxTMxM	p300
1013	RxKxxxxTMxV	SRC-1
	None	ERα

JNK1 phosphorylates H3.

Because recent evidence has implicated histones (Clayton and Mahadevan, 2003; Thomson et al., 1999) as well as co-regulatory proteins (Yang et al., 2003) as targets of MAPK cascades, I wondered if histones might serve as targets for JNK1 phosphorylation. Using the *in vitro* kinase assay, I also determined that H3 was phosphorylated by JNK1 (Figure 4.4). This modification was prevented when the histone was incorporated into the chromatin context of a mononucleosome. The DNA template used for the nucleosome assembly contained an AP-1 binding site flanking the nucleosome positioning sequence. Interestingly, JNK1 was able to phosphorylate nucleosomal H3 when recombinant AP-1 dimers (Figure 4.4; Figure 4.5A) were added to the reaction. This mechanism of AP-1-dependent phosphorylation of H3 by JNK1 is most likely facilitated by the recruitment of JNK1 to the mononucleosome by DNA-bound AP-1. Indeed, *in vitro* DNase I footprinting analysis demonstrated that the recombinant AP-1 dimers could bind AP-1 sites even when they were assembled into a nucleosomal array (Figure 4.5B). This strengthens the notion that the AP-1 factors were most likely recruiting JNK1 activity to the mononucleosome template rather than just altering JNK1 specificity by interactions in solution. Together, I demonstrated that H3 phosphorylation by JNK1, though inhibited by nucleosomal structure, can occur when JNK1 activity is targeted to the nucleosome by DNA-binding transcription factors.

Discussion

JNK1 has recently been shown to play a role in E2-mediated transcriptional outcomes. Not only is the enzyme recruited to E2-regulated promoter regions in a hormone-dependent manner, but JNK enzymatic activity is also required for full E2 responsiveness at certain genes (discussed at length in Chapter 3). The goal of this

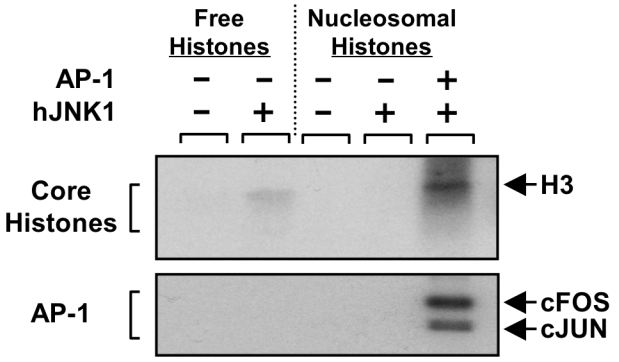


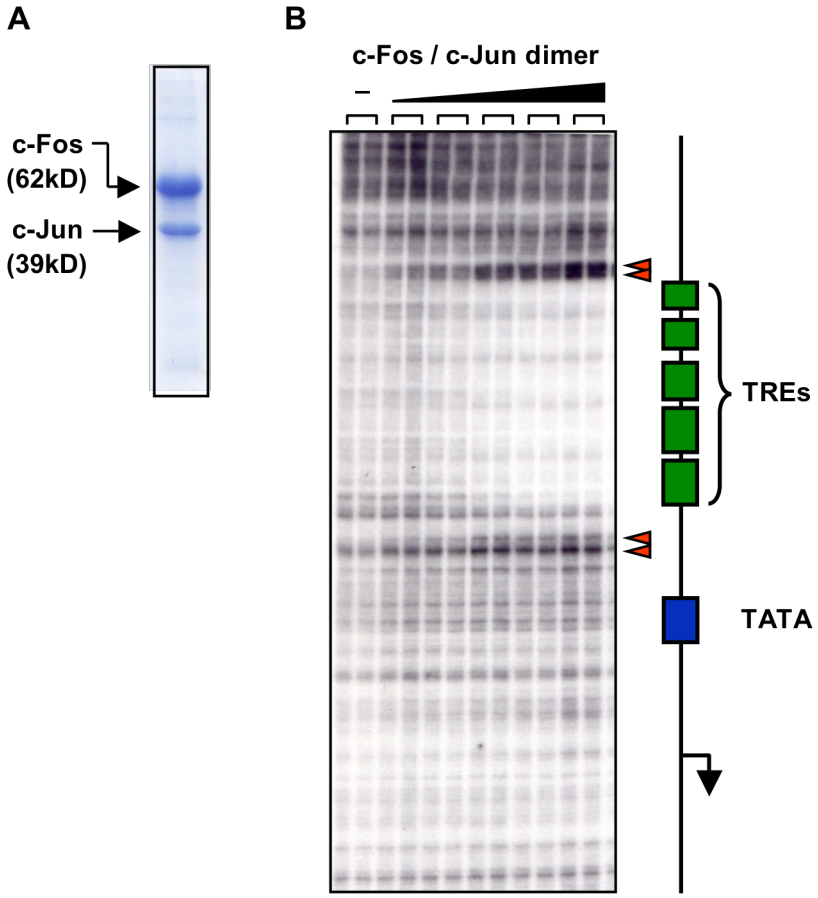
Figure 4.4 JNK1 phosphorylates the nucleosomal histone H3.

Kinase reactions were conducted using recombinant JNK1 incubated with core histones (140 ng) or core histones assembled into mononucleosomes by salt dialysis. Although phosphorylation of H3 was detected, incorporation of H3 into the context of a nucleosome prevented this modification. Because the DNA template used for mononucleosome assembly contained a flanking AP-1 binding site, we added recombinant AP-1 (c-FOS/c-JUN heterodimers, 300 nM) to "target" JNK1 specifically to the assembled template. As expected, JNK1 phosphorylates the AP-1 proteins quite well (exposure length is 5% that for the H3 autoradiogram). In the presence of AP-1, H3 phosphorylation was restored. Taken together, this data suggests H3 as a possible *in vivo* target of JNK1 enzymatic activity, and demonstrates that JNK1 can phosphorylate H3 incorporated into nucleosomes.

Figure 4.5 Purified c-Fos/c-Jun dimers can bind TREs in a chromatin context.

(A) c-Fos and c-Jun heterodimers were purified as described previously (Ferguson and Goodrich, 2001).

(B) DNase I footprinting analysis of a chromatinized template [assembled as previously described (Kraus and Kadonaga, 1999)] containing 5 TREs demonstrated specific protection of TRE regions with the addition of the purified AP-1 dimers. Red arrows point to regions of DNaseI hypersensitivity. Together, these features indicate that the purified AP-1 heterdimers can bind TREs even when they are assembled in the context of chromatin.


A
c-Fos
(62kD)
c-Jun
(39kD)
B
c-Fos / c-Jun dimer
–
TREs
TATA

study was to further define how the enzymatic activity of JNK1 might contribute to E2-dependent outcomes at these promoters. I established an *in vitro* kinase assay using activated JNK1 purified from bacteria.

Using a candidate approach, I focused my attention on the co-regulators SRC-1 and p300, wondering if these E2-dependent coactivators might serve as substrates for JNK1. Indeed these proteins, as well as ERα, were phosphorylated by JNK1 *in vitro*. Since SRC-1 and p300 have a putative JNK binding domain, similar to that found in c-Jun and other JNK substrates [reviewed in (Bogoyevitch and Kobe, 2006)], it is likely that these proteins serve as bona fide substrates of JNK *in vivo*, although this has yet to be determined. I believe that SRC-1 and p300 may require phosphorylation by JNK to fully potentiate E2-dependent transcriptional responses. This could be mediated through the stabilization / formation of phosphorylation-dependent protein-protein interactions, as proposed for CBP/p300 with AP-1 complexes (Arias et al., 1994). JNK phosphorylation could also alter the coactivator complex activity (note that p300 has acetyltransferase activity), a mechanism previously described for the histone acetyltransferase, ATF2 (Kawasaki et al., 2000). Although weakly phosphorylated, it is possible that ERα, under certain conditions, would be an *in vivo* target for JNK. Recent findings have demonstrated that other nuclear receptors [specifically GR, androgen receptor (AR), retinoic acid receptor α (RARα), retinoid X receptor α (RXRα), peroxisome proliferator-activated receptor γ1 (PPARγ1), and Nur77] are targets of JNK suggesting that ERα may also be a bona fide substrate *in vivo* (Adam-Stitah et al., 1999; Bruna et al., 2003; Camp et al., 1999; Gioeli et al., 2006; Han et al., 2006; Srinivas et al., 2005). These studies show that phosphorylation by JNK can effect the nuclear export (*e.g.,* AR, Nur77), degradation (*e.g.,* RARα), or transcriptional activity (*e.g.,* PPARγ1) of nuclear receptors suggesting a similar mechanism of action for ERs.

It is also possible that none of these factors are modified by JNK1 *in vivo*, but their interaction with the MAP kinase facilitates the phosphorylation of other local JNK1 substrates. Indeed, recruitment of JNK by c-Jun facilitates the phosphorylation and activation of JunD, which is unable to bind JNK by itself (Kallunki et al., 1996). This demonstrates that JNK binding can target phosphorylation sites in *trans*, as well as in *cis*. In this regard, I have shown that JNK1 can phosphorylate H3 in a nucleosome when locally recruited by AP-1 transcription factors. This is quite interesting since recent work has demonstrated the importance of H3 serine 10 phosphorylation in mediating transcriptional responses (Huang et al., 2006). Taken together, my work has identified several new targets of JNK1 phosphorylation and suggests that the local modification of these factors might play a role in E2-dependent transcriptional outcomes. Future work will focus on the identification of the specific amino acids in SRC-1, p300, and H3 that are phosphorylated by JNK1, and demonstration of the significance of these modifications with regard to transcriptional regulation in cells.

Methods and Materials

Plasmids. Plasmids for the MAP kinase purification system were obtained from Dr. Melanie Cobb, University of Texas Southwestern Medical Center. The NpT7-5 plasmid, containing rat JNK2 (rJNK2) and MEK4 was modified to replace the JNK2 sequence with that of human JNK1α1 (hJNK1α1). Briefly, the hJNK1α1 sequence (a kind gift from Dr. Roger Davis, University of Massachusetts Medical School) was amplified by PCR inserting an NcoI site and 6x histidine sequence upstream of the JNK1 sequence and a ribosomal binding site (RBS) and NcoI site downstream of the JNK1 sequence. The PCR primer sequences are as follows: 5'- CCAGCCATGGGCC

ATCACCATCACCACATAGCAGAAGCAAGCGTGACAA-3' and 5'-CCAGCCATGGTCTCCTTTCACAGACAAGTGCGCCATCTGCGAGGTTTTCACTGCTGCACCTGTGCTA-3'. The NpT7-5 plasmid was digested with NcoI removing the rJNK2 and RBS and ligated to the NcoI digested PCR product. The replacement of rJNK2 with hJNK1α1 was confirmed by sequencing.

Purification of JNK. The rJNK2 was purified from bacteria as previously reported (Khokhlatchev et al., 1997). The inactive enzyme was purified in the same manner from bacteria lacking the plasmid containing the constitutively active MAPKKK (pBB131 with MEKK-C). The purification of hJNK1α1 was similar to that of rJNK2 with two major modifications: (1) the final cation exchange chromatography was not necessary since the initial purification procedure yielded pure protein, and (2) the final dialysis buffer contained 150 mM NaCl instead of 50 mM NaCl.

***In vitro* kinase assay.** Kinase reactions were conducted with recombinant rJNK2 (active and inactive) and hJNK1α1 using recombinant c-Jun as a substrate positive control. Briefly, the purified JNK (25-300 nM) was incubated with various substrates for 30 minutes at 30C in kinase buffer (25 mM HEPES pH 7.5, 10 mM magnesium acetate, 50 μM ATP, 2 μCi γ-^{32}P ATP). After the labeled proteins were resolved using SDS polyacrylamide gel electrophoresis, the gels were dried on filter paper and the ^{32}P signal was detected using a phosphoimager system. The substrates tested are as follows: core histones from HeLa cells (140 ng), salt-dialyzed mononucleosomes (containing approximately 140 ng HeLa core histones) purified as described (Kim et al., 2001), flag-tagged SRC1 (140 nM) purified as described (Thackray and Nordeen, 2002), flag-tagged ERα (160 nM) and His-tagged p300 (160 nM) were purified as

described (Kraus and Kadonaga, 1998), and c-FOS/c-JUN dimers (300 nm, purified as described (Ferguson and Goodrich, 2001)).

DNase I footprinting. DNase I primer extension footprinting was performed as described previously (Cheung et al., 2002; Pazin and Kadonaga, 1998). Purified AP-1 proteins (c-Fos/c-Jun heterodimers;12-200 nM) were added after chromatin assembly of the 5xTRE-containing DNA template was complete, followed by a 15 minute incubation at 27C to allow interaction of the factors with the chromatin template.

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