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-
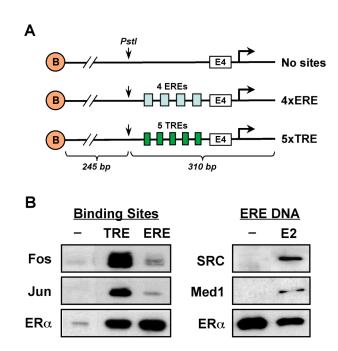
Figure 2.1 Immobilized DNA templates can isolate enhancer-specific complexes.

(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
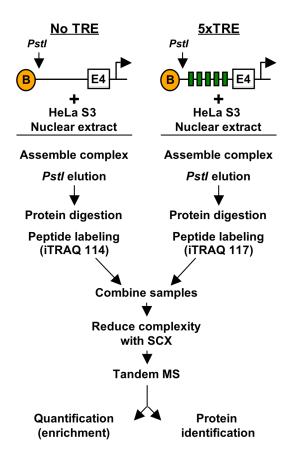
Figure 2.2 Schematic of proteomic method to identify TRE-associated factors. Control and TRE complexes were purified from HeLa nuclear extract using immobilized templates. Peptides from each sample were isotopically labeled, combined for further processing, and analyzed by tandem mass spectrometry (MS) 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
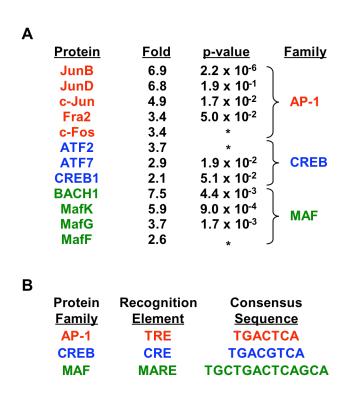
A

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

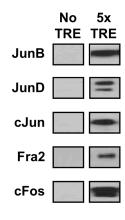**(B)** The similarity between the DNA sequences classically bound by the protein families mentioned in A are shown.

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
TFIIα	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
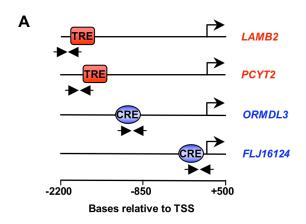
characteristics: (1) they displayed E2-dependent transcriptional activity, (2) ligand-dependent binding of ERα, (3) did not contain an ERE in the ERα-associated region, and (4) contained an AP-1-like element (TRE or CRE) under the ERα-associated region. The presence of several AP-1 factors was confirmed 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.
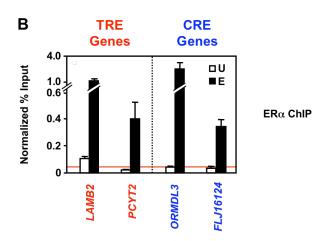
A

TRE — LAMB2

TRE — PCYT2

CRE — ORMDL3

CRE — FLJ16124

-2200   -850   +500

Bases relative to TSS

B

TRE Genes   CRE Genes

4.0
1.0
0.6

Normalized % Input

0.4

0.2

☐ U
■ E

LAMB2   PCYT2   ORMDL3   FLJ16124
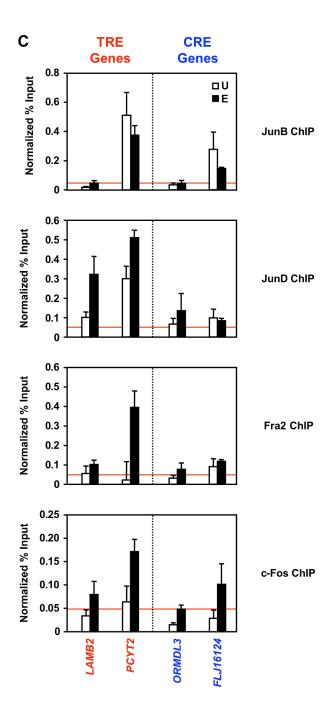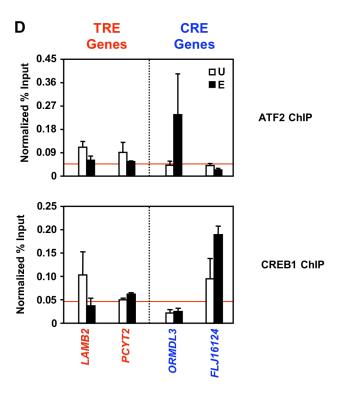
ERα ChIP

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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$_2$PO$_4$ 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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