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COMPLEX SYSTEMS IN POST-GENOMIC BIOLOGY

CYCLE XX

TITLE:

GENOME WIDE ANALYSIS OF ESTROGEN RESPONSE ELEMENT (ERE) DISTRIBUTION: FUNCTIONAL ANALYSIS OF ESTROGEN-INDUCED GENE REPRESSION

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To my family for their constant love and support

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TABLE OF CONTENTS

ABSTRACT

Estrogens play an essential role in both physiological development and breast cancer progression, but the gene networks and pathways by which estrogenic hormones regulate these events are only partially understood.

In the last few years several approaches to computational prediction of functional binding sites have been developed. They are all based on one pattern matching that usually is the representation as a matrix of acceptable nucleotides at each position of the known binding sites for a given protein. Following this criteria and using the data generated from chromatin immunoprecipitation (ChIP) on chip experiment (Kwon et al., 2007) (Carroll et al., 2006) we built a new ERE weighted alignment matrix (ERE-m). We used this matrix in a pattern discovery algorithm to perform a genome-wide scanning for putative estrogen responsive genes. To eliminate false positive motif we use the phylogenetic sequence conservation. We focused our attention on down-regulated ERE-containing genes and we validated the *in silico* analysis by ChIP and expression studies.

An interesting gene in this group is CDH-1, because it encodes for E-Cadherin, a trans-membrane protein important for cell-cell adhesion and involved in Epithelial-Mesenchimal Transition (EMT), a natural event during development that plays a key role in tumor progression. We demonstrated that ERα is recruited at the E-cadherin promoter even in the absence of estrogen stimulation, in breast cancer cells. Moreover we demonstrated that in absence of estrogen stimulation ERα is required to maintain the basal level of CDH-1 expression, while in presence of the ligand it becomes a repressor. Our data suggest a possible new role for ERα as ligand-independent activator that can be essential for the determination of epithelia morphology.

Our results show that the same factor (ER) bound to the same sequence (ERE) can evoke either activation or repression at different gene contexts. This may be explained by the hypothesis that transcriptional complexes with distinct composition exist in the nuclei, taking care of the transcription of distinct subsets of genes, in response to the same stimulus. For this reason, I joined the laboratory of M.G. Rosenfeld, who was examining the possibility that genes with a common mode of regulation in response to stimuli can share the same transcriptional machinery. Results of this study demonstrated that ligand induces rapid interchromosomal interactions among subsets of

estrogen receptor α-bound transcription units, with a dramatic reorganization of nuclear territories requiring nuclear actin/myosin-1 transport machinery, dynein light chain 1, and a specific subset of transcriptional coactivators and chromatin remodeling complexes. We establish a molecular mechanism by which the hormone-induced interchromosomal interactions serving to achieve enhanced, coordinated transcription and RNA splicing for nuclear receptor target genes.

INTRODUCTION

The importance of estrogenic hormones and their receptors (ER) as a target of therapy in breast cancer has been the stimulus for understanding both the factors involved in assisting ER in regulating transcription and for identifying the specific gene targets and the DNA elements responsible for activation or inhibition. Surprisingly little is known of the actual *cis*-regulatory elements involved. The completion of the human genome sequence and the advent of technologies such as tiling arrays for the whole human genome for the first time makes a comprehensive analysis of the genomic targets of ER action possible. The full understanding of the *trans*-acting factors and *cis*-regulatory targets of ER action in various estrogen-responsive cell types will support the development of improved selective ER modulators useful for the prevention and treatment of breast cancer and other diseases.

ESTROGENIC HORMONES AND MECHANISMS OF ACTION

Estrogens are a group of steroid hormones family, named for their importance in the estrous cycle, and functioning as the primary female sex hormone. They are produced from a molecule of cholesterol and they have as basic structure an aromatic ring with eighteen carbons. The major naturally occurring estrogens in women are three: 17 β–estradiol, estriol and estrone.

The most active one is the 17 β–estradiol that is converted into estriol after oxidation of C17 that became estrone after hydroxylation of C16.

Estrogens are produced primarily into developing follicles in the ovaries, the corpus luteum, and the placenta under the Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) control. Some estrogens are also produced in smaller amounts by other tissues such as the liver, adrenal glands, and the breasts.

Estrogens are potent mitogens for different target tissues especially for the mammary gland where they play a physiological role in the development and a pathological one during breast cancer progression. Moreover, estrogen are involved in other human disease states, including cardiovascular, osteoporosis, and Alzheimer.

Although several studies have been developed, the gene networks and pathways by which estrogenic hormones regulate these events are only partially understood.

The effects of estrogen are mediated via a specific nuclear receptor (NR) call estrogen receptor (ER) a ligand-activated enhancer protein that is a member of the

steroid/nuclear receptor superfamily that includes 60 different 'classical' members of the nuclear hormone receptor family (Nilsson et al., 2001).

Until 1995, it was assumed that there was only one ER and that it was responsible for mediating all of the physiological and pharmacological effects of natural and synthetic estrogens. However, in 1995, a second ER, ER beta, was cloned from a rat prostate cDNA library (Kuiper et al., 1996). The former ER is now called ER alpha. The discovery of ER beta has forced a reevaluation of the biology of estrogen and, because of the abundance of ER beta in the male urogenital tract, has refocused attention on the role of estrogen in males.

THE STRUCTURE OF ESTROGEN RESEPTORS

ER alpha and ER beta belong to the steroid/thyroid hormone superfamily of nuclear receptors, members of which share a common structural architecture (Mangelsdorf et al., 1995) that is composed of three independent but interacting functional domains: the NH2-terminal or A/B domain, the C or DNA-binding domain, and the D/E/F or ligand-binding domain (Fig. 1).

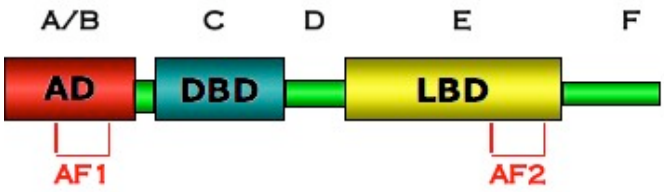


Figure 1 Diagrammatic representation of the domain structure of nuclear receptors. The A/B domain at the NH2 terminus contains the AF-1 site where other transcription factors interact. The C/D domain contains the two-zinc finger structure that binds to DNA, and the C/F domain contains the ligand binding pocket as well as the AF-2 domain that directly contacts coactivator peptides. DNA binding domain (DBD); ligand binding domain (LBD); activation domain (AD).

The N-terminal domain of nuclear receptors encodes a ligand-independent activation function (AF1), a region of the receptor involved in protein-protein interactions (Webb et al., 1998) and transcriptional activation of target-gene expression. Comparison of the AF1 domains of the two estrogen receptors has revealed that, in ER alpha, this domain is very active in stimulation of reporter-gene expression from a variety of estrogen

response element (ERE)-reporter constructs, in different cell lines but the activity of the AF1 domain of ER beta under the same conditions is negligible (Cowley and Parker, 1999). Dissimilarity in the NH2-terminal regions of ER alpha and ER beta is one possible explanation for the difference between the two receptors in their response to various ligands. In ER alpha, two distinct parts of AF1 are required for the agonism of E2 and the partial agonism of tamoxifen, respectively (McDonnell et al., 1995). In ER beta, this dual function of AF1 is missing (McInerney et al., 1998). The importance of ER beta AF1 in transcriptional activity therefore remains to be clarified.

The COOH-terminal or ligand-binding domain (LBD) mediates ligand binding, receptor dimerization, nuclear translocation, and transactivation of target gene expression (Brzozowski et al., 1997). Amino acid residues that line the surface of the ligand-binding cavity, or that interact directly with bound ligands, span the LBD from helix 3 to helix 12. The LBD also harbors activation function 2 (AF2), which is a complex region whose structure and function are governed by the binding of ligands. Crystallographic studies with the LBDs of ER alpha and ER beta revealed that the AF2 interaction surface is composed of amino acids in helix 3, 4, 5, and 12 and that the position of helix 12 is altered by binding of ligands. When the ER alpha LBD is complexed with the agonists, E2 or diethylstilbestrol (DES), helix 12 is positioned over the ligand-binding pocket and forms the surface for recruitment and interaction of coactivators. In contrast, in the ER alpha- and ER beta -LBD complexes with raloxifene or the ER alpha -LBD 4-OH-tamoxifen complex, helix 12 is displaced from its agonist position over the ligand-binding cavity and instead occupies the hydrophobic groove formed by helix 3, 4, and 5. In this position, helix 12 foils the coactivator interaction surface. It is evident that, different ligands induce different receptor conformations and that the positioning of helix 12 is the key event that permits discrimination between estrogen agonists (E2 and DES) and antagonists (raloxifene and 4-OH-tamoxifen) (Shiau et al., 1998). The LBDs of ER alpha and ER beta share a high degree of homology in their primary amino acid sequence and are also very similar in their tertiary architecture. It is, therefore, not surprising that the majority of compounds tested so far bind to ER alpha and ER beta with similar affinities (Kuiper et al., 1998) or have similar potencies in activation of ERE-mediated reporter gene expression (Barkhem et al., 1998).

The DNA binding domain (DBD) contains a two zinc finger structure, which plays an important role in receptor dimerization and in binding of receptors to specific DNA sequences call estrogen response element (ERE). The DBDs of ER alpha and ER beta are highly homologous (Enmark et al., 1997). Thus ER alpha and ER beta can be expected to bind to various EREs with similar specificity and affinity.

ESTROGEN RESPONSE ELEMENTS (ERE)

The estrogen response elements were first observed in the 5'-flanking region of the Xenopus vitellogenin A2 gene. The minimal ERE core sequence is a 13 bp palindromic inverted repeat: 5'-GGTCAnnnTGACC-3'.

This ERE sequence was shown to function in an orientation and distance-independent manner that are also properties of an enhancer (Klein-Hitpass et al., 1986).

Specific contacts between the ER dimer and the sugar–phosphate backbone of the ERE are important in sequence recognition and high affinity binding. Each ER monomer is bound to DNA in the major groove with the ER dimer located predominantly on one face of the DNA helix (Koszewski and Notides, 1991). The fourth base pair of the ERE half site (AGGTCA) provides a positive contact for the P-box, whereas the third base pair (AGGTCA) provides binding energy (Schwabe et al., 1993). The CII zinc finger is involved in half-site-ERE spacing recognition and ER dimerization (Martinez and Wahli, 1989).

Controversy still exists concerning ER DNA binding via ERE half sites, although a number of examples exist (Klinge et al., 1997). Since the identification of a canonical ERE, several computational approaches have been undertaken to identify target genes based on the presence of EREs within promoter proximal regions (Bourdeau et al., 2004; Vega et al., 2006). In the first comprehensive studies, Bourdeau and co-workers screened for all EREs in the human and mouse genomes and identified in excess of 70,000 EREs within the human genome, over 17,000 of which were within 15 kb of mRNA start sites. Elimination of EREs that were not conserved between the human and mouse genomes reduced the number of gene proximal EREs to 660. A number of these sites were validated as genuine ER interaction sites, supporting to some degree the use of computational models to predict putative ER target genes.

ESTROGEN AND GENE REGULATION

The biological effects of estrogen are mediated through at least four ER pathways: the classical ligand-dependent, the ligand-independent, the DNA binding-independent and the nongenomic pathways.

The classical pathway states that in the absence of hormone the receptor is sequestered in a multiprotein inhibitory complex within the nuclei of target cells. The binding of ligand induces an activating conformational change within the ER and promotes homodimerization and high affinity binding to specific DNA response elements (EREs), which are cis-acting enhancers located within the regulatory regions of target genes (Fig. 2). The DNA-bound receptors contact the general transcription apparatus either directly or indirectly via cofactor proteins, of which several have been identified, including SRC-1, GRIP1, AIB1, CBP/p300, TRAP220, PGC-1, p68 RNA helicase, and SRA. It is generally accepted that the ER-coactivator interactions stabilize the formation of a transcription preinitiation complex and facilitate the necessary disruption of chromatin at the ERE. Depending on the cell and promoter context, the DNA-bound receptor exerts either a positive or negative effect on expression of the downstream target gene (Rosenfeld and Glass, 2001).

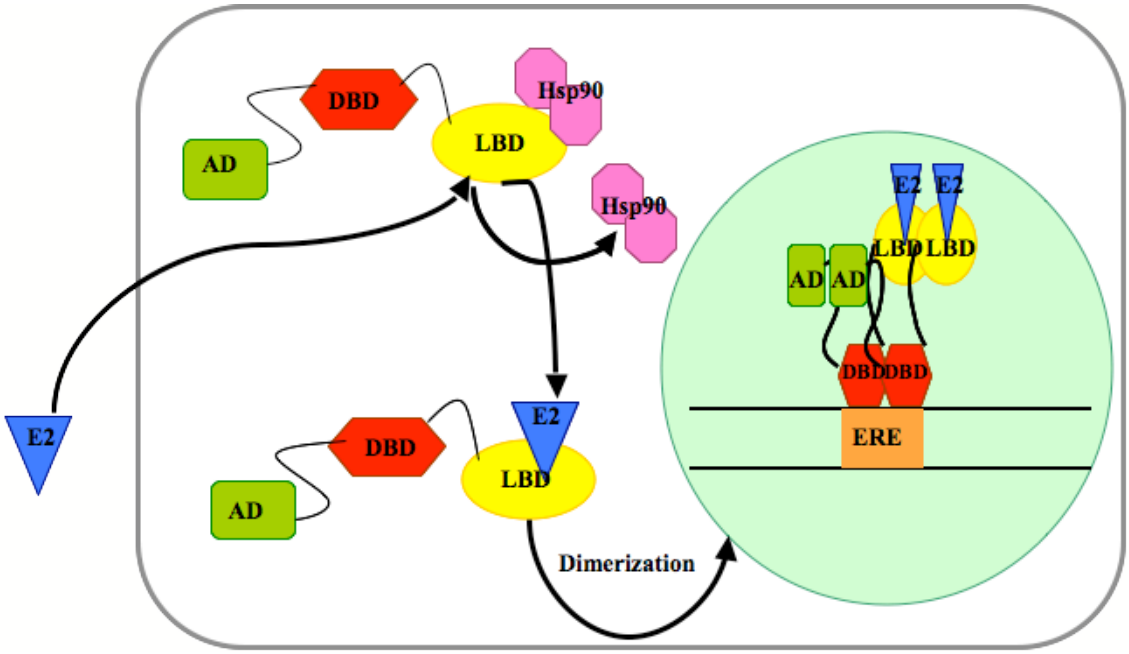


Figure 2 Diagrammatic representation of the ligand-dependent mechanism of estrogen action. The binding of estrogen (E2) induces the dissociation of Hsp90 proteins that hide the signal of nuclear

localization, than ERs can dimerize and translocate into nucleus and bind to specific DNA respons elements (EREs).

The ER can modulate also in absence of estrogen by extracellular signaling such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) that can activate ER and increase the expression of ER target genes (Smith, 1998).

Specific receptor domains of the ER are critical to E2-independent activation. Specifically, the effects of elevated intracellular cAMP are mediated through AF-2, whereas growth factor activation of ER requires the N-terminal AF-1 domain of the receptor (El-Tanani and Green, 1997). The majority of evidence indicates that modification of the phosphorylation state of the ER by cellular kinases may serve as an important mechanism of ligand-independent activation. The serine 118 residue of the human ERalpha AF-1 is phosphorylated by the mitogen-activated protein kinase (MAPK) pathways following treatment with EGF or IGF, enabling the receptor to interact with the ERalpha-specific coactivator p68 RNA helicase and activate target gene transcription (Kato, 2001).

These mechanisms provide an explanation for the regulation of genes in which a functional ERE-like sequence into the promoter. However ER activated by estrogen can induced genes without ERE-like sequence. The agonist-bound ER can indeed lead to gene regulation in the absence of direct DNA binding. One example is the interaction between ER alpha and the c-rel subunit of the NFkappa B complex. This interaction prevents NFkappa B from binding to and stimulating expression from the interleukin-6 (IL-6) promoter. In this way, E2 inhibits expression of the cytokine IL-6 (Galien and Garcia, 1997). Another example of indirect action on DNA is the physical interaction of ER alpha with the Sp1 transcription factor. ER alpha enhancement of Sp1 DNA binding is hormone independent (Porter et al., 1997), and both ER alpha and ER beta can activate transcription of the retinoic acid receptor alpha 1 (RAR-1) gene, presumably by the formation of an ER-Sp1 complex on GC-rich Sp1 sites in the RAR1 promoter (Sun et al., 1998; Zou et al., 1999). Moreover, both ER alpha and ER beta can interact with the fos/jun transcription factor complex on AP1 sites to stimulate gene expression, however, with opposite effects in the presence of E2 (Paech et al., 1997).

Finally, other proposed mechanism of estrogen-regulated transcription involved an indirect nongenomic function of ER through cell-surface ER forms that are linked to

intracellular signal transduction proteins. It is now clear that ER and membrane-coupled tyrosine kinase pathways are integrally linked, as E2 has been shown to activate the MAPK signaling pathway in a variety of cell types. Importantly, there is increasing evidence that some of the vascular protective effects of E2 through ER alpha are mediated by a nongenomic mechanism involving a biphasic activation of endothelial nitric oxide synthase by estrogen through the MAPK and phosphatidylinositol 3-kinase/Akt pathways (Simoncini et al., 2000). It is still controversial whether the putative membrane ER is similar to one or both of the intracellular forms. The extensive data gathered on the structures of the two known nuclear ER forms clearly indicate that neither is a transmembrane protein. However, Razandi et al. reported that the membrane and nuclear forms of each ER originate from the same transcript and exhibit similar affinities for E2. These studies further demonstrated that the membrane-bound ERs were G protein-linked and able to elicit a variety of signal transduction events, including the induction of cell proliferation (Razandi et al., 2000). Recently, however, work has suggested that estrogen can function through the G protein-coupled receptor, GPR30 (Revankar et al., 2005). These studies suggest that signaling through GPR30 may play a role in the cellular response to estrogen.

DIFFERENT STRATEGIES TO IDENTIFY NEW ER TARGETS

The advent of expression microarrays afforded the ability to investigate global gene changes after ligand treatment. A significant number of studies have been published detailing microarray-based gene changes after nuclear receptor activation. These studies led to identification of a significant number of new estrogen targets, such as GATA3 (Hoch et al., 1999), in addition to a number of previously identified targets including pS2/TFF-1, Cathepsin D, RIP140/NRIP-1, and c-myb (Soulez and Parker, 2001). One important study of detailed set of microarrays experiments over a time course of estrogen treatment was conduced by Katzenellenbogen and co-workers. Interesting of genes regulated, the highest proportion of estrogen-induced genes were those involved in transcriptional regulation and cell proliferation and approximately 70% of the changes after estrogen treatment were down-regulated genes, including a number of proapoptotic genes, fitting with a model of estrogen-induced cell survival (Frasor et al., 2003).

Additional studies using gene expression profiling with microarrays has been used to obtain a genomic view of gene regulation by estrogen and antiestrogen in breast cancer. These studies led to identification of different clusters of genes showing specific coregulation patterns: cluster of genes displaying temporal-specific up- or down-regulation with different time kinetics, clusters of genes responding to different antiestrogenic drugs in either antagonstic or agonistic fashion, genes responding specifically to antiestrogens, but not to estrogen (Cicatiello et al., 2004; Scafoglio et al., 2006).

The mechanism of negative regulation by estrogen was not revealed by these studies and did not distinguish between direct transcriptional inhibitions, physiologic squelching by sequestration of limiting factors away from these genes, or induction of inhibitory factors. It is possible that all of these mechanisms may play a role.

A different technique that has also been used to identify differentially regulated genes on a scale comparable to microarray analysis is SAGE libraries. These studies, for exemple, have clarified the role of WISP-2 as a differentially regulated estrogen gene, as well as validation of previously identified ER targets (Inadera et al., 2002). Moreover, using a similar approach, a number of other targets have been identified sac as the pro-proliferative gene cyclin D1, the antiapoptotic factor TIT-5, and EIT-6. Interestingly, EIT-6 was estrogen induced in more than one breast cancer cell line and was shown to promote colony growth in vitro, supporting its role as a mediator of cell division. A total of 61 tags were observed to change after estrogen treatment, including 22 that were down-regulated. However, approximately 45,000 sequencing events from each library, untreated and estrogen treated were required to identify these 61 tags, highlighting the large-scale sequencing required to adequately cover transcript changes on a genome-wide scale.

The application of ChIP to clarify protein-DNA binding dynamics has provided significant information about a cyclic pattern of ER association with estrogen target promoter regions, with maximal recruitment at 45 min after estrogen stimulation. A number of additional proteins subsequently associate with the promoter regions including p300, p160 cofactors, CBP, pCAF, CARM1, and RNA PolII, all of which then cycle off the promoter (Shang et al., 2000). Recently, the application of ChIP combined with sequencing has been used to identify ER binding sites to define new target genes as well as cis-regulatory regions. The benefit of this method is that it

allows for identification of cis-regulatory regions without bias toward promoter regions or known gene targets. This method has successfully been applied to identify the known target, TFF-1, as well as 11 other targets, including RARA (Laganiere et al., 2005a).
More recently, genome-wide ChIP couple with microarry, known as ChIP-on chip, have been undertaken to identify ER binding sites in an unbiased manner. Surprisingly, recent promoter and tiling array analyses suggest that ER alpha binds relatively rarely to gene promoters compared with intergenic regions, suggesting a critical role of longe-distance enhancers in regulated gene expression in mammalian cells (Carroll et al., 2005; Carroll et al., 2006). However, a new technology based on ChIP couple with a DNA selection and ligation (DSL) strategy and a full genome promoter array (ChIP-DSL platform) reveled that ER alpha bound to > 3% of human genes in promoter-proximal regions in MCF7 cells, reinforcing the importance of direct binding events in the promoter-proximal regions during regulated gene expression (Kwon et al., 2007).

ESTROGEN RECEPTOR AND THE MAMMARY GLAND

The female mammary gland undergoes a surge of cell division during puberty, and throughout adult life there is cyclical proliferation and involution during estrous cycles (Russo et al., 1999). Estrogen is obligatory for normal development as well as for induction and progression of mammary carcinoma. During pubertal growth and during the estrous cycle the majority of proliferating cells both in terminal end buds and ducts are ER alpha negative (Zeps et al., 1998). Induction of the progesterone receptor (PR) by estrogen does occur in ER alpha -containing cells, and this induction occurs at much lower plasma levels of estrogen than are required for epithelial cell proliferation. These observations have led to the concept of two distinct types of responses to estrogen in the breast: 1) an indirect action in the mammary epithelium which occurs via ER-containing stromal cells and 2) a direct effect on ER alpha -containing cells that occurs at low estrogen concentrations and results in induction of PR and differentiation of the epithelium (Wiesen et al., 1999). The stroma, upon estrogen stimulation, produces growth factors that cause replication of epithelial cells. From studies involving ERKO mice it is clear that ER beta, in the absence of ER alpha, cannot mediate estrogen-dependent growth and development of the mammary gland (Couse and Korach, 1999). In addition, with the use of reconstitution experiments with ERKO mouse breasts it has

been shown that the presence of ER alpha in breast stroma, but not in the epithelium, is sufficient for estrogen-dependent ductal growth (Cunha et al., 1997).

Estrogen plays also an important role to promote breast cancer development and progression. Moreover, ER is used as a marker of prognosis for patients with breast cancer indeed it has been shown that presence of estrogen receptor is associated with a more favorable outcome in breast cancer prognosis. The ER-positive breast carcinoma is associated with well-differentiated tumor histology, low cell proliferation rate and negatively lymph node status. ER-negative tumors often correlate with aggressive diseases, amplification of oncogenes and upregulation of metastasis associated growth factors and proteases (Osborne, 1998). However, the motivation of cancer cells invasiveness is not very clear and most likely includes multiple factors, such as activation of hormone-related signaling pathways, changes of levels and activities of estrogen receptor (ER) cofactors as well as aberrant expression of multiple cell surface protein, particularly E-cadherin.

E-cadherin is a cell surface molecule responsible for cell-cell junction, which is suppressed in majority types of cancer via a number of signaling pathways during EMT process. The expression level of E-cadherin is gradually used as diagnostic marker for certain types of breast carcinoma, especially infiltrating pleomorphic lobular carcinoma (Siitonen et al., 1996). Since expression of estrogen receptor protects against cancer cell invasion and proliferation (Rochefort et al., 1998) and estrogen stimulation has been suggested to induce E-cadherin suppression directly (Oesterreich et al., 2003) or indirectly (Fujita et al., 2003) may the protective role of ER against breast cancer probably mediated by supporting the transcription of E-cadherin gene.

MATERIALS AND METHODS

BIONFORMATIC TOOLS

PATSER (Hertz and Stormo, 1999; Stormo and Hartzell, 1989)

This program scores the L-mers (subsequences of length L) of the indicated sequences against the indicated alignment or weight matrix. The elements of an alignment matrix are simply the number of times that the indicated letter is observed at the indicated position of a sequence alignment. Such elements must be processed before the matrix can be used to score an L-mer. A weight matrix is a matrix whose elements are in a form considered appropriate for scoring an L-mer.

Each element of an alignment matrix is converted to an element of a weight matrix by first adding pseudo-counts in proportion to the a priori probability of the corresponding letter (see option "-b" in section 1 below) and dividing by the total number of sequences plus the total number of pseudo-counts. The resulting frequency is normalized by the a priori probability for the corresponding letter.

The final quotient is converted to an element of a weight matrix by taking its natural logarithm. The use of pseudo-counts here differs from previous versions of this program by being proportional to the a priori probability.

Version 3 of this program differs from previous versions by also numerically estimating the p-value of the scores. The p-value calculated here is the probability of observing a particular score or higher at a particular sequence position and does NOT account for the total amount of sequence being scored.

The p-value is calculated for each possible integer score and the values are stored. The actual scores for the sequences are determined from the true weight matrix. The true scores are converted to their corresponding integer values and their p-values are looked up.

MAKEMATRIX

This algorithm builds an alignment matrix from a list of aligned sequences with a same length. This algorithm aligns the sequences without searching for specific pattern.

SLOGOS (Schneider and Stephens, 1990)

This software is used to make graphical representation of a nucleotide sequence using to displaying the patterns in a set of aligned sequences. The characters representing the sequence are stacked on top of each other for each position in the aligned sequences. The height of each letter is made proportional to its frequency, and the letters are sorted so the most common one is on top. The height of the entire stack is then adjusted to signify the information content of the sequences at that position. From these 'sequence logos', one can determine not only the consensus sequence but also the relative frequency of bases and the information content (measured in bits) at every position in a site or sequence. The logo displays both significant residues and subtle sequence patterns.

INGENUITY PATHWAYS KNOWLEDGE BASE (IPA 3.1) (www.ingenuity.com)

The Ingenuity Pathways Knowledge Base is currently the world's largest database of knoledge on biological network. The IPA 3.1 searches for the presence of biological functions that are enriched in a set of genes under analysis.

CELL CULTURE, TREATMENT AND RNA EXTRACTION

MCF-7 human breast cancer cells, Hela cervical cancer cells were cultured in MEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS). The T47D human breast cancer cells and T47D sfRON cell line, derived from stable transduction of T47D epithelia breast cancer cells by lentivirus vector expressing a truncated form of a Tyrosine Kinase receptor called RON (Bardella et al., 2004), were cultured RPMI-1640 with 10% (v/v) heat-inactivated foetal bovine serum (FBS). For estrogen treatment, cells were first transferred into ‘stripped’ medium (SM), devoid of estrogenic activity [Phenol-Red-free medium supplemented with 10% (v/v) dextran-coated charcoal-stripped FCS] for 4 days. Treatments were performed with 10 nM 17β-oestradiol (Sigma).

The total RNA was extracted using RNeasy mini kit (Qiagen) following the manufacturer’s instructions. One μg of RNA was treated with DNase I (Invitrogen) to

remove any trace of DNA then the RNA was reverse transcribed to cDNA with RETROscript (Ambion) following the manufacturer's instruction.

REAL-TIME QUANTITATIVE PCR

Quantitative real-time PCR monitored with SYBRGreen was performed using the Mcx3005P (Stratagene) using the following condition: 95°C for 10 min, 95°C for 15 sec, 60°C for 25 sec and 75°C for 30 sec for 40 cycles. Negative cDNA control was cycled in parallel with each run. Specific primer pairs were designed with Primer3 software. As reference gene, 18 S rRNA was used. Fluorescence data were analyzed with McxPro-Mcx3005P (Statagene) software and expressed as Ct, the number of cycle needed to generate a fluorescent signal above a predefined threshold. Target gene mRNA level were normalized to the reference gene according to Livak and Schmittgen (Livak and Schmittgen, 2001).

TRANSIENT CELL TRANSFECTION AND SITE-DIRECTED MUTAGENESIS

Cells were transfected following Invitrogen's protocol available online using Lipofectamine 2000.

The luciferase assay system used was purchased from Promega Corp. (Madison, WI). Luciferase values were normalized using a β−galactosidase gene-expressing plasmid (pCMV, Clontech, Palo Alto, CA) as an internal control for transfection efficiency, as described previously (Harrington et al., 2003).

The amounts of plasmids used were: 1 μg of the reporter constructs, 100 ng of β−galactosidase, and 100 ng of estrogen receptor constructs. Every experiment was performed at least three independent times.

Site-directed mutagenesis was performed on the E-cadherin promoter using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The mutagenesis sense and antisense primers were designed using the Stratagene web site.

The plasmids were then sequenced on both strands to confirm mutation of the desired site. All the siRNA used was provided by QIAGEN.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ASSAY

Protein–DNA cross-linking was performed by adding 1% (w/v) formaldehyde to cells culture medium for 10 min at 37 °C. Cells were washed with ice-cold PBS containing protease inhibitors (1 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml pepstatin A) and lysed with lysis buffer [1% (w/v) SDS, 10 mM EDTA and 50 mM Tris/HCl, pH 8.1] for 10 min on ice. The lysate was sonicated to reduce DNA length to 200–1000 bp and debris was removed by centrifugation for 10 min at 9750 g at 4 °C. The supernatant was diluted 10-fold in dilution buffer [0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.1, and 167 mM NaCl) containing protease inhibitors (as above). Soluble chromatin was pre-cleared by incubation with 45 ml of Protein A–agarose beads (Sigma) for 2 h at 4 °C with agitation. Immunoprecipitation of 1 ml of soluble chromatin was performed overnight at 4 °C using specific antibodies or rabbit IgG control. Immune complexes were then precipitated with Protein A–agarose beads (Sigma) for 1 h at 4 °C with rotation. Beads were pelleted and were washed sequentially with the buffers: 0.1% (w/v) SDS/1% (v/v) Triton X-100/2 mM EDTA/20 mM Tris/HCl (pH 8.1)/150 mM NaCl; 0.1% (w/v) SDS/1% (v/v) Triton X-100/2 mM EDTA/20 mM Tris/HCl (pH 8.1)/500 mM NaCl; 0.25 M LiCl/1% (v/v)/Nonidet P-40/1% (w/v) sodium deoxycholate/1 mM EDTA/10 mM Tris/HCl (pH 8.1) and twice with Tris-EDTA (pH 8.0). Immune complexes were eluted by adding 250 ml of 1% (w/v) SDS in 0.1 M NaHCO3 to pelleted beads, before incubation at room temperature for 15 min with rotation. Elution was repeated and eluates were combined. Cross-linking was reversed at 65 °C overnight and DNA was purified on Qiaquick spin columns (Qiagen) and eluted in 50 ml of water.

Specific sequences from immunoprecipitated and input DNA were detected by quantitative real time PCR and SYBR Green-detection (Stratagene) on a Mcx300P System (Stratagene) using specific primers designed surrounding ERE sequence. Fold enrichment ratios were calculated from experimental Ct values, previously normalized

against Ct values from IgG control, and then input percentages were calculated compared to serial diluted input samples.

ChIP-DSL, 3D AND 3C ASSAYS

Genomic tiling by ChIP-DSL (Fig. M1) was previously described (Garcia-Bassets et al., 2007; Kwon et al., 2007). Two anti-ERα antibodies (HC-20 and H-184, Santa Cruz Biotechnology) were combined for ChIP analyses. The 3D assay began with the conventional 3C assay after restriction digestion with Bam H1 and Bgl II using the procedure identical to that previously described for mammalian cells (Vakoc et al., 2005). Ligated and unligated DNA after 3C was sonicated as in standard ChIP experiments. In order to detect loci associated to the TFF1 enhancer, the DNA was annealed to a specific biotinylated capture oligonucleotides corresponding to the *TFF1* enhancer (5'-Bio-GACAGAGACGACATGTGGTGAGGTCATCTTGGCTGAGGG) together with the oligonucleotide pool corresponding to the tiled paths. After capture, oligonucleotide ligation, selection, amplification, and hybridization were as previously described in the ChIP-DSL assay (Kwon et al., 2007) (Fig.M2). Doubled blank intensity was first added to raw data to reduce low intensity bias when computing ratios. The percentile rank for each probe was determined within individual experiments and the medianpercentile-rank (MPR) was calculated for each probe across 4 replicates (Buck and Lieb, 2004). The data was then smoothed using a sliding window of 10kb and steps of 500bp, taking the median MPR value of the probes in each window. A window was assigned a value of zero if it had <5 probes above the background to further minimize stochastic signals. Obviously, this method as designed will miss genuine, highly localized signals in favor of clusters of signals. A p-value was calculated for each window by randomly assigning MPR values from a pool of all probes above background 1000 times and counting the number of times the median value of the randomized window exceeded the experimental value. The negative log p-value was plotted at each window position when the p-value is ≤0.05. 3C validation was carried out with 0.25, 0.5, and 1μl of processed DNA under fixed PCR conditions of 34 cycles for short-range interactions, 36 cycles for long-range interactions, and 30 cycles for BAC controls using a 32P-labeled primer for the *TFF1* enhancer in

combination with primers targeting individual genomic loci (see primer sequences, their genomic coordinates, and expected sizes in Fig. S10 in Attachement 1, Nunez et al, submitted). Four BAC DNA clones covering the genomic regions around the *TFF1* locus were purchased from Invitrogen, amplified, purified, and quantified by qPCR. Equal amount of each BAC DNA was mixed, digested with Bam HI and Bgl II, and ligated in a high concentration (~200ng/µl in a 20µl reaction) to promote inter-molecular ligation. The processed BAC DNA was tested by qPCR to determine the linear range and then used to produce reference PCR signals for each primer pair. The products were resolved in a 10% native polyacrylamide gel, and quantified with a PhosphoImager (Molecular Dynamics).

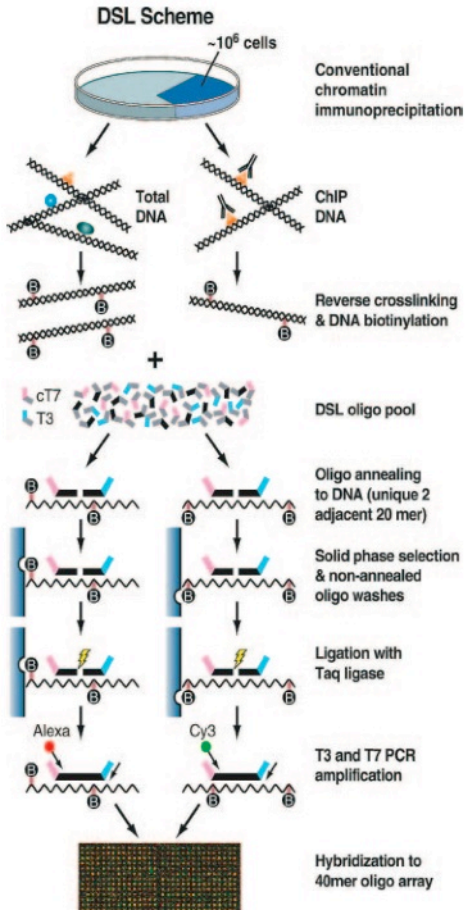


Figure M1. The ChIP-DSL scheme (Know et al. 2007).

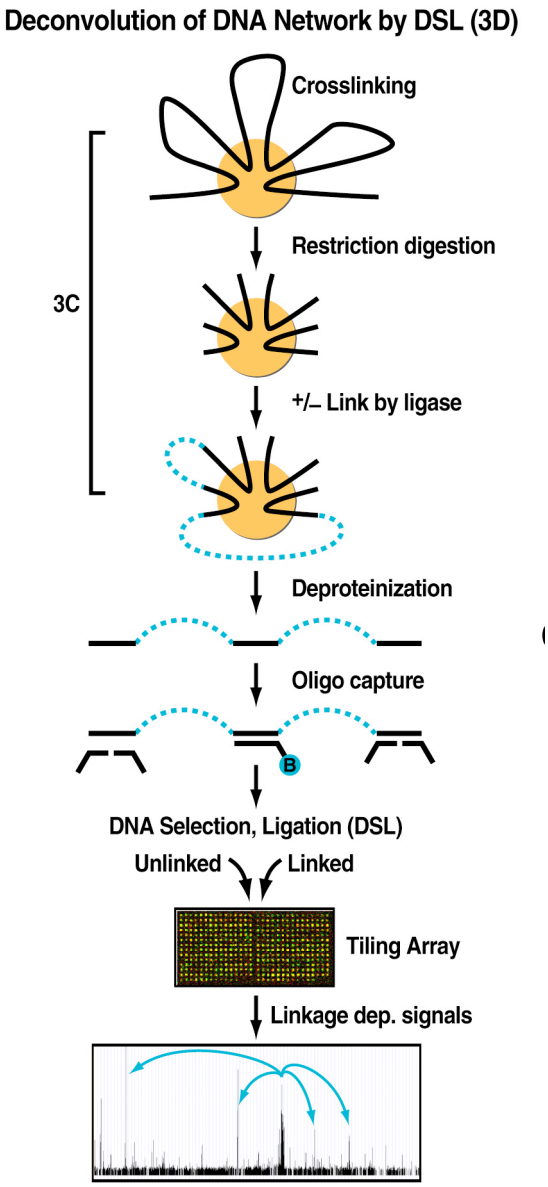


Figure M2. Diagramme of the 3D technology (Nunez et al. 2007).

DNA-ImmunoFISH

The cells were processed for FISH essentially as described (Cai and Kohwi-Shigematsu, 1999) except that specific oligonucleotide probes labeled with specific haptens were used as listed in Fig. S11 (Attachement 1, Nunez et al, submitted). For triple-labeled FISH, probes to promoter regions were labeled at the 5' position with

digoxigenin (DIG) and probes to enhancer regions were labeled with either Biotin (Bio) or Fluorescein (FITC). For double-labeled FISH, promoters were labeled with Bio and enhancers with FITC. After hybridization, specific probes were detected by using a mix of quantum dot (Qdot)-conjugated antibodies in 1:200 dilution (sheep anti-DIG Fab fragment primary antibody-conjugated with Qdot 655, streptavidin-conjugated with Qdot 605, and goat anti-FITC whole IgG primary antibody-conjugated with Qdot 525, all from Invitrogen). Single chromosome paint probes were commercially acquired from Applied Spectral Imaging (Vista). Each probe was custom-labeled with different fluorophores: Chr1 (1-585-605), Chr2 (1-585-606) and Chr21 (1-585-649) in aqua, red and green, respectively. Hybridization and detection protocols were performed as recommended by the manufacturer.

IMAGING ACQUISITION AND PROCESSING

2D FISH images were acquired with a Zeiss Axioplan 2e microscope (Carl Zeiss, Inc) and 3D images were obtained with a Nikon TE-200 DeltaVision deconvolution microscope at the UCSD Moores Cancer Center Digital Imaging Facility. The commercial Huygens software package (Scientific Volume Imaging) and the NIH Image J package (http://rsb.info.nih.gov/ij/) were used to deconvolve optical sections, which were then merged to produce 2D or 3D pictures. For colocalization analysis, individual cells were cropped and a region of interest (ROI) was defined using the software's object analyzer tool and a precise definition of the ROI was obtained for each cell. Co-localization of signals from different channels was determined using the colocalization analyzer tool of Huygens. In each cell, a single value of Pearson's coefficient in the refined ROI was determined after imposing a threshold value for all channels, each of which was calculated using the automatic thresholding function of the Imaris algorithm in the Huygens package. Nonspecific colocalization was identified from apparently co-localized 2D images by determining Pearson's coefficients of deconvolved stacks after subtracting background signal outside the ROI. Statistical comparison of Pearson's coefficients obtained with individual cells from multiple independent experiments was performed with a one-tailed two-sample ttest using the software SSPS 14.0 for Windows. Multiple data set comparison was carried out by

ANOVA using nonparametric methods, which provides box plots with data in quartiles, and error bars at the 5th and 95th percentile and outliers plotted.

SINGLE-CELL MICROINJECTION

Single-cell antibody microinjection experiments were performed as described (Perissi et al., 2004). The antibodies used are listed in Fig. S11 (Attachement 1, Nunez et al, submitted). These siRNAs were purchased from Qiagen (Valencia, CA), each of which was custom-designed and validated.

QUANTIFICATION AND DEPLETION OF CELLULAR ATP

ATP depletion and quantification were performed in mock-treated and hormone induced cells (103-104 cells per assay) using the ApoSensor ATP depletion Assay kit (Axxora). A calibration curve was generated with 5, 10, 25, 50, 100, 250, and 500 nM of ATP, which was used to calculate the ATP concentration in experimental cells. Rotenone (Sigma) was used at a final concentration of 10μM to enhancer ATP depletion as previously reported (White et al., 2002).

PHARMACOLOGICAL TREATMENT OF CELLS

Transcription was inhibited by treat the cells for 1 or 6 hrs with 100 nM α-amanitin (Sigma) to block transcription initiation, or with 5,6-dichlorobenzimidazole riboside (Sigma) to interfere with transcriptional elongation. Actin depolymerization was induced with latrunculin A (LA), which is known to specifically cap actin monomers, whereas actin stabilization was stimulated by jaspaklinolide (JP), which binds F-actin and prevents depolymerization. These drugs (gift of J. Durán and V. Malhotra) were suspended in DMSO as a 1000X stock and applied to cultured cells at the final concentration of 1μM as described (Bubb and Spector, 1998). Nuclear actin was detected by using a monoclonal antibody (2G2) (Progen).

WESTER BLOT

Total protein extracts were obtained by direct on-plate lysis of the cells, previously washed three times with cold PBS, with a boiling buffer containing 0.125 M Tris/HCl, pH 6.8, and 2.5% SDS; the lysates were then homogenized by ten passages through an insulin needle and cleared at 9000 g for 10 min. Proteins (50 mg) were separated by SDS-PAGE on pre-cast NuPAGE Novex 10% Bis-Tris gels (Invitrogen) and transferred to PVDF membranes (Bio-Rad) by electrotransfer. Membranes were blocked with phosphate buffered saline containing 0.5% Tween 20 (PBS-T) and 5% dried nonfat dry milk powder for 1h at room temperature. Blots were probed with primary antibodies (list below) in PBS-T containing 1% nonfat dry milk powder overnight at 4°C. After washing, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and visualized by enhanced chemiluminescence (ECL®; Amersham Biosciences).

RESULTS

The research presented here is focused on different aspect of estrogen mediated genes regulation in order to identify general models operating at different gene sets in response to the same stimulus.

The first part of this study has been focused on the prediction of functional estrogen receptor binding sites on genome scale by computational approaches in order to build a new ERE weighted alignment matrix (ERE-m) and perform a genome-wide scanning for putative estrogen responsive genes using that matrix in a pattern discovery algorithm that finds statistically overrepresented motif. We focused our attention on a very interesting group of genes that present a high-score ERE while showing negative regulation by estrogen and involved in cell cycle and proliferation. Putative targets identified in *silico* were then validated by ChIP and expression studies.

Interesting, in the group of genes that have a high-score ERE while showing negative regulation by estrogen in our previous analysis there is CDH-1 encoding for E-Cadherin a trans-membrane protein important for cell-cell adhesion. The expression level of E-cadherin has been gradually used as diagnostic marker for certain types of breast carcinoma. Then we focus our attention on the effect of estrogen receptor on E-Cadherin expression and using as experimental model ERα negative cell lines, we demonstrated that in absence of estrogen stimulation ERα is required to maintain the basal level of CDH-1 expression, while in presence of the ligand it becomes a repressor.

In the last part we focused our attention on a new mechanism of liganded nuclear receptors regulated gene expression. We report that ligand induces rapid interchromosomal interactions among subsets of estrogen receptor α-bound transcription units, with a dramatic reorganization of nuclear territories requiring nuclear actin/myosin-1 transport machinery, dynein light chain 1, and a specific subset of transcriptional coactivators and chromatin remodeling complexes. We establish a molecular mechanism by which the hormone-induced network of interactive hubs become co-associated with distinct interchromatin granules, long thought to be "storage" sites for the splicing machinery and various transcription elongation factors, thereby serving to achieve enhanced, coordinated transcription and RNA splicing for nuclear receptor target genes.

CHAPTER I

The first part of our study has been focused on the prediction of functional estrogen receptor binding sites on genome scale by computational approaches.

The computational discovery of regulatory elements is possible manly because they occur several times in the genome and because they may be evolutionary conserved among different species. This means that new regulatory elements can be discovered searching for overrepresented motifs across regulatory regions (Sandve and Drablos, 2006). This apparently simple approach is complicated by the fact that most transcription factor binding sites (TFBSs) are short, and they can have some variation without loss of function. Therefore most motifs are also found as random hits throughout the genome, and it is a challenging problem to distinguish between false positive hits and true positive binding site. Motif finding is essentially a signal-to-noise problem. It has been estimated that in human DNA about 3% of intergenic regions are regulatory elements (Kellis et al., 2003).

For this reason most algorithms to identify the genomic regulatory elements use orthogonal data. Several algorithms include additional prior knowledge about gene regulation; regulatory elements are not randomly distributed, but tend to form clusters of regulatory modules, (Kreiman, 2004), and the presence of co-occurring motifs can be used to identify putative regulatory modules.

Functional sequences are preferentially conserved over the course of evolution by selective pressure: this is another characteristic, with the over-representation, that Corà and coworker applied to determine transcription factor binding sites in the human genome (Cora et al., 2007). The hypothesis that many orthologous genes expressed similarly in a tissue-specific manner in human and mouse, are likely to be co-regulated by orthologous transcriptional factors is the base of the cis-regulatory regions search (Huber and Bulyk, 2006).

Usually, the TFBSs are represented with a “consensus sequence”: this method has been widely used to represent the specificity of transcription factors (TF). However, the consensus sequence is not flexible enough to account for all variations: in general, it refers to a sequence that matches all of a site closely, but not necessarily exactly (Stormo, 2000).

An alternative to consensus sequence is a position weight matrix (PWM) or profile. The PWM summarizes the statistical properties of a collection of TF binding sites and represents the DNA sequences. The PWM is the formalism to represent DNA motifs bound to a particular TF because it contains two kinds of knowledge: the thermodynamics interactions between TF and DNA and the evolutionary selection (Berg and von Hippel, 1987). The underlying assumptions are that natural selection gave rise to a certain level of sequence specificity for each TF and that sequences that gave rise to the same physically binding affinity are equally likely to be selected (Bussemaker et al., 2007).

The discovery of motifs in sequence data was an early problem to be addresses in computational biology. The DNA motif discovery algorithms that have been developed can be divided into three main groups:

1. **Complete ab initio methodologies**: parameter-free algorithms for de novo identification of potential TFBS. This group contains all methodologies that implement a simple search for the most probable sub-sequence in a set of sequences. In this case, there are no assumptions about the biological features of the sequences.
2. **Partial ab initio methodologies**: algorithms that assume some biological knowledge. There are two categories of algorithms: the first contains algorithms that use "complementary information" (see below), while the second contains algorithms which assume that the found subsequences are possible TFBS, and describe a sequence motif by means of a position-specific scoring matrix.
3. **Matrix-based methodologies**: algorithms detect potential TFBS by sliding window search, with one specific PWM, of match subsequences.

An example of a Complete ab initio methodology is Weeder (Pavesi et al., 2001). This algorithm allows extending exhaustive enumeration of signals without giving as input the exact length of the patterns to be found. Each motif is evaluated according to the number of sequences in which it appears and how well conserved it is in each sequence with respect to expected values derived from the oligo frequencies analysis of upstream sequences in the same organism. Then, the algorithm compares the top-scoring motifs of each run with a clustering method to detect which ones could be more likely to correspond to a TFBS. The consensus for a set of TFBSs can be seen as a perfect form recognized by a TF. Then, the algorithm enumerates all the possible oligos of the same

length of the motif to be found. For each one, it counts how many times it appears in the sequences. The sequences that are overrepresented form a new set of sequences. Then, it ranks the motifs found according to some statistical measure and gives as output the highest-ranking motifs.

Another algorithm in this category is YMF (Yeast Motif Finder) written by Sinha and coworker (Sinha and Tompa, 2003). YMF uses an exhaustive search algorithm to find motifs with the greatest z-score. The z-score of a motif is the number of standard deviations by which its observed number of instances in the actual input sequences exceeds its expected number of instances.

Both algorithms do not need any input parameter. With many parameters to set, the user explores the parameters space and makes arbitrary judgment calls on which output to trust. Different studies have showed that the programs are often quite sensitive to parameters (Hu et al., 2005).

However, the algorithms that used "complementary information", like overrepresented in evolutionarily conserved upstream regions or infer about co-regulation (Gene Ontology and results of a set of microarray experiments), improve the signal/noise ratio by selecting for analysis those portions of the upstream regions that are more likely to be functionally relevant (Cora et al., 2005). These methodologies are grouped in the "Partial ab initio" set. An example is the algorithm by Caselle and coworker (Caselle et al., 2002), where the genome is grouped in sets based on words that are overrepresented in the upstream region, and then their frequencies in the reference sample are compared to the whole genome. For each of these sets they compare the average expression in microarray experiments with the genome-wide average. If the difference is statistically significant, the set is a putative TFBS.

Other examples in the "Partial ab initio" set are algorithms that used a different type of "complementary information". One example is Consensus. This algorithm employs a greedy heuristic (Stormo and Hartzell, 1989) and builds up an entire alignment of the sites by adding in a new one at each iteration. As best alignment of a potential sites is the one with highest information content. Then, the goal of Consensus is to determine a sequence alignment that maximizes a log-likelihood statistics describe in a PWM.

An expectation-maximization (EM) method was implemented in the MEME program (Bailey and Gribskov, 1998). MEME method allows for the simultaneous identification of multiple patterns, the starting point derived each subsequence occurring in the input

sequences. For every subsequence the algorithm evaluated the quality and the accuracy of the statistical significance by a product of the P-value of column information contents.

In the latter two algorithms, the basic assumption is that the sequences that are overrepresented in the genome are putative TFBSs; then, they consider the alignments for every motif like a start point to build a PWM.

The third group is a set of methodologies that search for the presence of a PWM in all sequence positions using a sliding window approach. One example is MatInspector (Quandt et al., 1995): this algorithm detects potential sequence matches by automatic searches with a library of pre-compiled matrices. The search method includes position weighting of the matrices based on the information content of individual positions and calculates a relative matrix similarity.

Another example is Patser (Hertz and Stormo, 1999). This algorithm computes the numerically estimation of the p-value of the match score between a subsequence and a specific matrix. The p-value is the probability of observing a particular score at a particular sequence position. The motif with the highest p-value is a putative TFBS.

A statistic comparing the accuracy of the main tools to discovery TFBSs is found in Tompa et. al, but it is very difficult to compare the performance of methods, in particular on complex genomes like the human (Tompa et al., 2005).

GENOME-WIDE ANALYSIS OF ERE MATRIX M00191 (TRANSFAC)

Currently, there are two comprehensive and annotated databases that contain information on TFs binding site profiles, JASPAR (Sandelin et al., 2004) that contains a smaller non-redundant set of TFs binding site (each TF has only one profile) and TRANSFAC (Matys et al., 2003) that contains multiple profile models for some TFs. Since TRANSFAC contains the most representative matrix (M00191) for the ER alpha responsive element we performed a genome-wide analysis for this matrix using the Patser algorithm (Stormo and Hartzell, 1989) in order to define a set of putative estrogen responsive genes.

We performed our analysis on the region -2000/+500 of each gene present in the human genome.

In order to select the most representative ERE we used two different approaches. First of all we chose the genes with a significant score for ERE (OS: observed score) lower than the value calculated by:

Average CS + 1 Standard Deviation CS

into the distribution of the scores (CS: Calculated Score) generated from 1000 permutation of each analyzed sequence.

The second approach was to arrange the CS in a raising order than we performed 1000 permutation of each analyzed sequence and we chose the genes just if them OS for ERE was into the first lower 50 CS. This means that we had no more than 5% ($\alpha^1 = 0.05$) of false positive because the significant OS are localized into 100%α.

Using these approaches we obtained 14713 and 14420 genes respectively from the about 2700 annotated genes. This means that about 57-59% of human genes have an ERE, this suggest that both approaches are inaccurate.

Moreover the matrix M00191 is not the best alignment matrix for the using of Patser algorithm because this matrix has a lower quality (NNAR**G**NN**A**NNN**TGACC**YNN) compare to the ERE consensus (G**GT**C**A**NNN**TGACC**). For this reason we decided to improve the quality of the ERE matrix before performing the computational genome screening.

A NEW ERE WEIGTHED ALIGNMENT MATRIX (ERE-m)

In order to build a new ERE weight matrix we implemented the data generated from chromatin immunoprecipitation (ChIP) on chip experiment (Kwon et al., 2007) (Lin et al., 2007) using the algorithm MakeMatrix. Both data sets are generated by genome-wide analysis of differential ER alpha promoter occupancies after estradiol treatment in MCF7 cells. The major difference between these two studies is on the performed ChIP on chip technique.

The new matrix was compared to the two matrices for the ERE into the TRANSFAC database using the information content method that is the measure of significance for the PWM (IC, also called relative entropy (Schneider et al., 1986):

[1] Probability to have a false signal

$$I(p) = \sum_{j=1}^{L} \sum_{i=A}^{T} f_{i,j} \log \frac{f_{i,j}}{P_i}$$

where p is a pattern, L is the pattern length, i is the index of a base at position j of the PWM, $f_{i,j}$ is the frequency of the base i at position j of the PWM, and P_i is the probability of observing that base in the data. The IC is the weighted average for the binding energies form each of the sites represented in the matrix, the lower IC is, the higher the variability in the site (GuhaThakurta, 2006).

Moreover, a position in the motif at which all nucleotides occur with equal probability has information content f 0 bits, while a position at which any single nucleotide can occur has information content of 2 bits. The information content at a given position can therefore be thought as giving a measure of the tolerance for substitutions in that positions: position that are highly conserved and thus have a low tolerance for substitutions correspond to high information content, while positions with a high tolerance for substitutions correspond to low information content.

As shown in Tab. 1 our matrix has an IC higher than the matrixes present in TRANSFAC.

Table 1 The IC Value for the ERE matrix analyse

MATRIX	IC
OUR MATRIX (ERE-m)	12,72
ERE TRANSFAC M00191	11,68
ERE TRANSFAC M00511	11,78

GENOME-WIDE ANALYSIS OF ERE-m

We scanned the sequences for all promoter regions of all human genes (-2000/+500) with our matrix ERE-m using Partser program. The weight matrix is successively aligned to each position of the sequence, and the score is the sum of weights for the letters aligned at each position (Hertz & Stormo 1999).

The number of genes that we found using this new matrix was lower than the number of genes found with M00191 matrix but still too high to be significant. Since the selection of regulatory elements can be improved if they are conserved over the course of evolution, we decided to perform our analysis using both mouse and rat genomes.
Using these approaches we obtained 14641 and 11179 genes from mouse and rat respectively.
Moreover we identified for each annotated human genes their respective murine or rat's orthologous. Then, we crossed the orthologous genes with the genes identified as ERE containing into murine of rat's genome. This subset of gene was then crossed with human ERE containing genes. Using this approach we obtained 6984 genes that have an ERE conserved in both human and mouse or human and rat.

ESTROGEN-MEDIATED GENE REPRESSION

The number of genes obtained from our analysis is biologically significant but it is still hard to know is these genes are really modulated by ER alpha. Moreover, most of work that has been done in the last 5 years on investigation of estrogen-regulated transcription has been focused on up-regulated genes, although down-regulated genes constitute a significant fraction of all estrogen dependent expression changes in cell lines (Frasor et al., 2003) and tumor samples (Nishidate et al., 2004). For theses reasons, we focused our attention on down-regulate genes. In order to identify a set of biologically significant genes we crossed the ERE containing genes with the set of genes identified as modulated by estrogen by Scafoglio and coworker and we found, over all, 134 genes that have a putative ERE and that are down-regulated by estrogen (Tab 2).

Table 2. The List of Down-Regulated Genes

GENE	Fold Change	GENE	Fold Change	GENE	Fold Change
PGM5	-3.4	PSCD3	-1.6	DLGAP4	-1.4
EGR1	-2.9	MXD4	-1.6	SLC13A2	-1.4
MUC1	-2.9	FXYD3	-1.6	LOC51149	-1.4
ARNT2	-2.4	EPHB1	-1.6	SBF1	-1.4
FLNC	-2.3	FKBP8	-1.6	ILF3	-1.4
BCL2L1	-2.2	SEC24B	-1.5	PLD2	-1.4
PTCRA	-2.1	DSC2	-1.5	RARG	-1.4
CRIP2	-2.1	PNOC	-1.5	ENDOG	-1.4
CTSH	-2.1	SYNGR3	-1.5	SMARCA2	-1.4
GPR30	-2	SMAD3	-1.5	CORO1A	-1.4
CSRP1	-2	HSD17B1	-1.5	GATA2	-1.4
GYS1	-2	FOXI1	-1.5	PYGM	-1.4
ENO1	-2	PITPNM1	-1.5	ITGA3	-1.4
NR4A1	-2	RXRB	-1.5	OTUB1	-1.4
CRABP2	-2	MGMT	-1.5	TREH	-1.4
ITGB4	-2	CYFIP1	-1.5	RGS14	-1.4
ENO3	-1.9	PPP5C	-1.5	CYP1A1	-1.4
CLDN4	-1.8	ASAHL	-1.5	PMP22	-1.4
CLN3	-1.8	LPHN1	-1.5	TJP2	-1.4
ABCC3	-1.8	LGALS3	-1.5	TUBGCP2	-1.4
PTPRF	-1.8	BRD3	-1.5	TCEA2	-1.4
RHOBTB2	-1.8	DNM2	-1.5	GLG1	-1.3
COMT	-1.8	PTPN9	-1.5	CXCL16	-1.3
FBP1	-1.8	ETHE1	-1.5	CHP	-1.3
NEDD4L	-1.8	ECH1	-1.5	IER3	-1.3
TLE1	-1.8	FSTL3	-1.5	PTMS	-1.2
SPINT1	-1.7	LLGL2	-1.5	PITRM1	-1.1
MYH11	-1.7	PPP2R4	-1.5	AP2B1	-1.1
HIP1R	-1.7	PYGB	-1.5	STAC3	-1.1
CAPN1	-1.7	ACTR1A	-1.5		
PPAP2C	-1.7	FLI1	-1.5		
MYH9	-1.7	RUTBC1	-1.5		
NFIC	-1.7	CACNB3	-1.5		
PLXND1	-1.7	TP53I11	-1.5		
RXRA	-1.7	PTPRT	-1.5		
PTTG1IP	-1.7	UCP2	-1.5		
SLC9A3R2	-1.7	NUDT14	-1.5		
PPP1R10	-1.7	LOC196463	-1.5		
TOM1L2	-1.7	ELF4	-1.5		

ALDH3A1	-1.6	PRSS8	-1.5		
TACSTD2	-1.6	S100A13	-1.5		
CIB1	-1.6	PRKCZ	-1.5		
FGFR4	-1.6	NDST1	-1.5		
CAPNS1	-1.6	SCAMP2	-1.5		
EPHA4	-1.6	GNB2	-1.5		
IDH2	-1.6	GUK1	-1.5		
P2RX4	-1.6	LY6E	-1.5		
CTSB	-1.6	MAPK3	-1.5		
AP3D1	-1.6	ATP2A3	-1.5		
SCN1B	-1.6	VAV2	-1.5		
ZBED4	-1.6	GNAL	-1.4		

Since the estrogen receptor is critical in determining the phenotype of human breast cancer and is the most important therapeutic target, we checked the functional classes in which these genes are involved.

As shown in Fig. 4 most of these genes are linked to the regulation of cellular growth and proliferation such as gene expression, cell death and cell signaling.

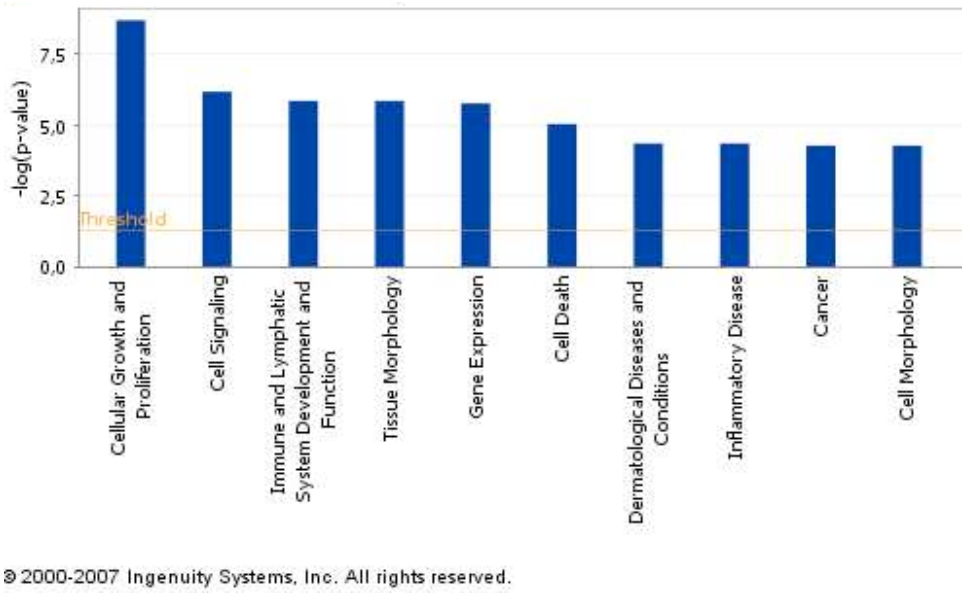


Figure 3 The Graphical Representation of Functional Classes. Picture obtained from Ingenuity Systems.

In order to perform a biological validation of our computational analysis we chose 7 genes from the set of down-regulated by estrogen. These are linked to control of apoptosis, cell signaling, and cellular migration and invasion (Tab. 3).

Table 3. The List of Analyzed Genes

GENE	FUNCTION	ERE
BCL2L1	The protein encoded by this gene belongs to the BCL-2 protein family. The longer isoform acts as an apoptotic inhibitor and the shorter form acts as an apoptotic activator.	GGTCGCATGATCC
CLDN4	This gene encodes an integral membrane protein, which belongs to the claudin family. The protein is a component of tight junction strands.	CTTCAGCCTGTCC
GATA2	The GATA family of transcription factors, which contain zinc fingers in their DNA binding domain.	AGTGAGGGCGTCC
GPR30	This gene is a member of the G-protein coupled receptor 1 family and encodes a multi-pass membrane protein that localizes to the endoplasmic reticulum. The protein binds estrogen, resulting in intracellular calcium mobilization and synthesis of phosphatidylinositol 3,4,5-trisphosphate in the nucleus. This protein therefore plays a role in the rapid nongenomic signaling events widely observed following stimulation of cells and tissues with estrogen.	GGTCTCTATGCCT

ITGA3	ITGA3 encodes the integrin alpha 3 chain. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain.	GCCCGGCTGGCCT
ITGB4	Integrins mediate cell-matrix or cell-cell adhesion, and transduced signals that regulate gene expression and cell growth. This gene encodes the integrin beta 4 subunit, a receptor for the laminins.	GGTCTGACTCACC
SMAD3	TGF-beta signaling mediator	GGCCGAGCTCCCC

As first step we checked whether the chosen genes were really down regulated by estrogen at the mRNA level, by qRT-PCR. As shown in Fig. 3 all the genes analyzed were repressed after estrogen treatment.

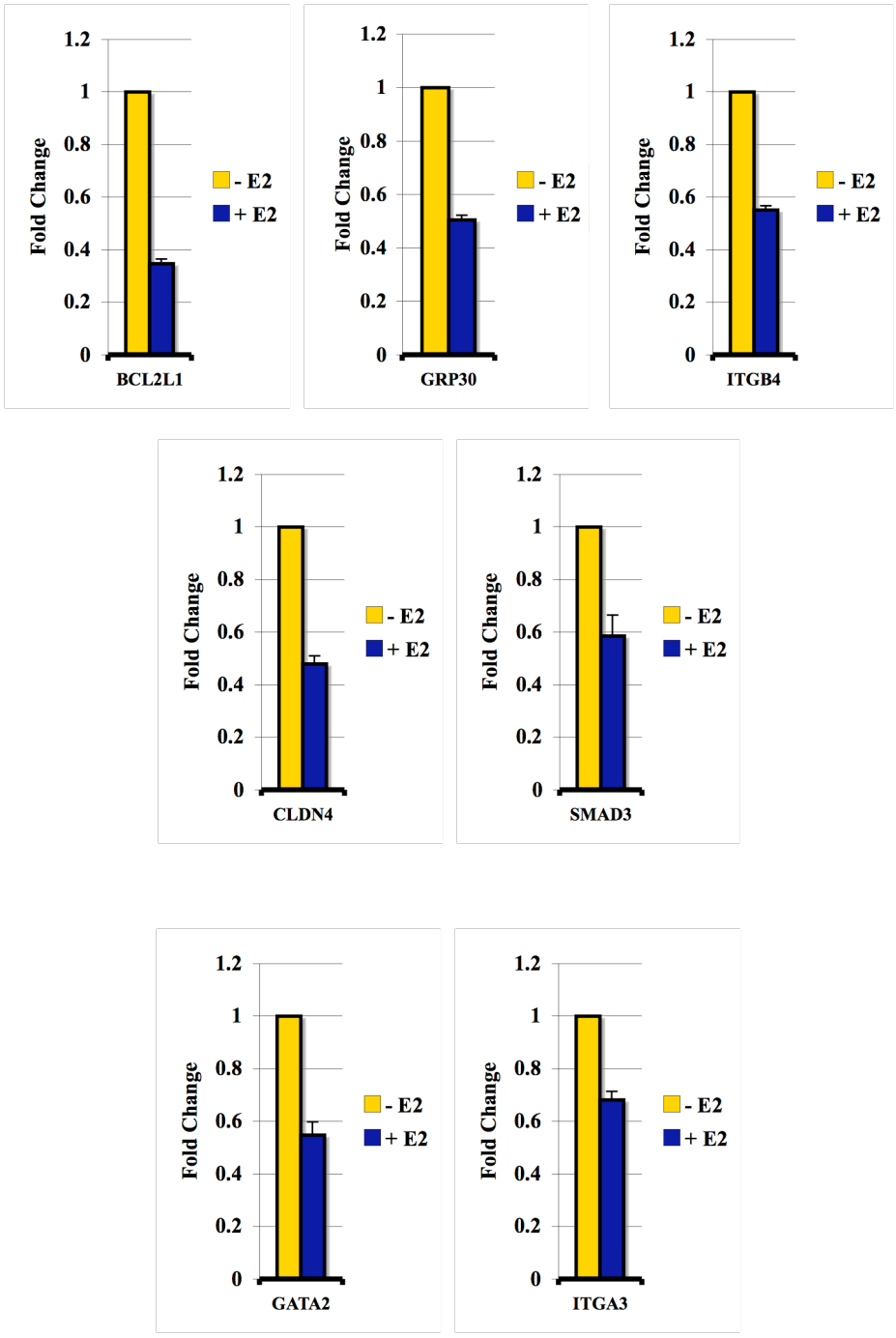
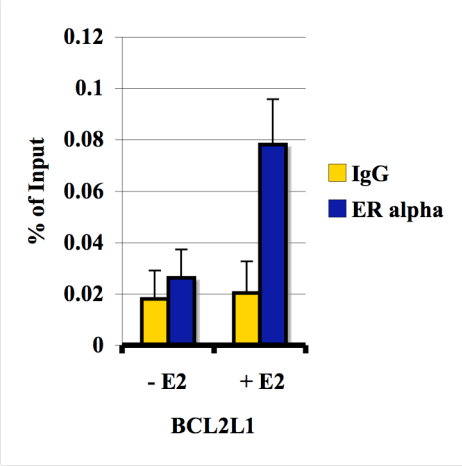
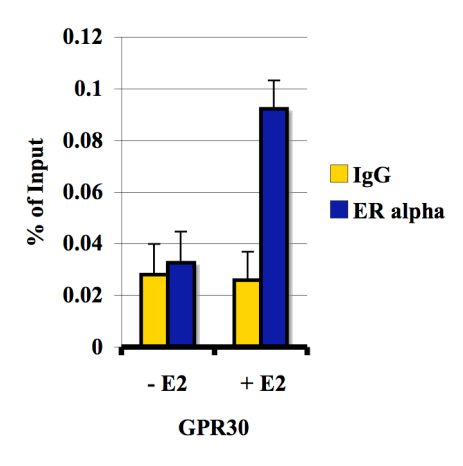
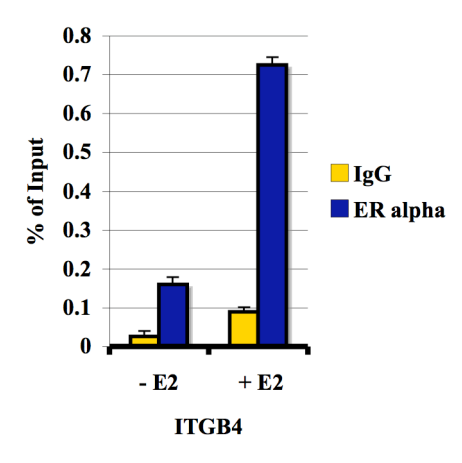


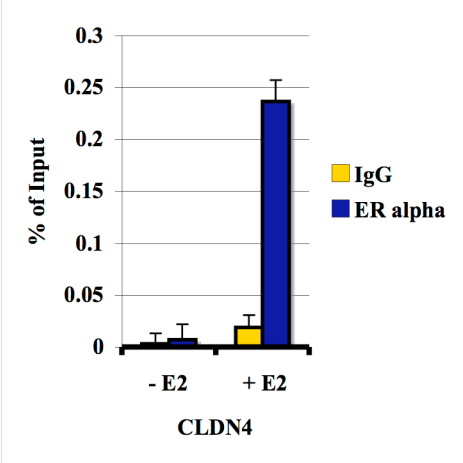
Figure 4 E2 Down-Regulates Genes Expression in MCF-7 Human Breast Cancer Cells. Quantitative real-time PCR was used to evaluate changes in mRNA level of selected genes in MCF-7 cells in after 2h of treatment with 10nM 17β-estradiol.

Then to determine whether ER alpha is recruited on the identified ERE after estrogen treatment we performed a chromatin immunoprecipitation analysis (Fig. 4). We demonstrated that ER alpha is indeed recruited on ERE of BCL2L1, GRP30, ITGB4, CLDN4 and GATA2 promoter region analyzed. These results, albeit limited to a small number of genes, provide evidence that our computational approach can be used to improve the discovery of both known and new regulatory element across the genome.









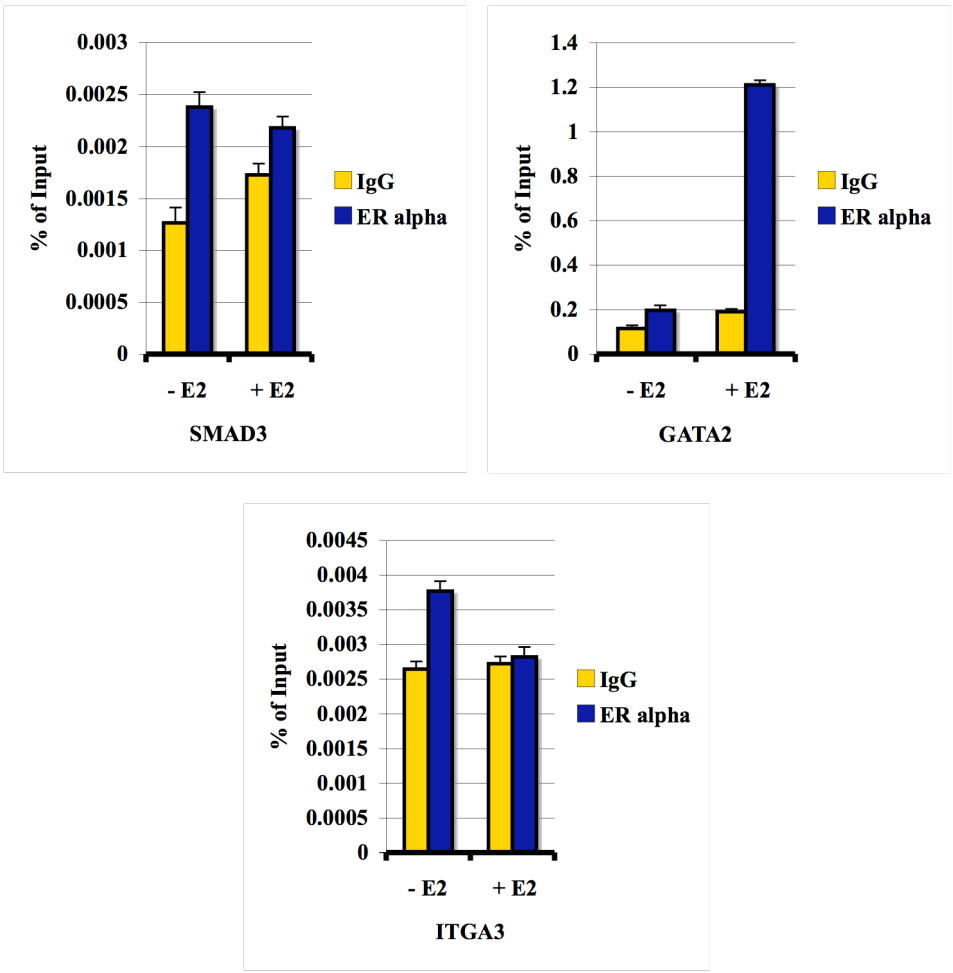


Figure 5 ER alpha was recruited on most of the gene promoters selected in MCF7 Human Breast Cancer Cells. Chromatin Immunoprecipitation (ChIP)/ Quantitative real-time PCR show occupancy analysis of ER alpha on the ERE of gene promoters selected. The cells were treated for 1h with either 10nM E2 or control vehicle.

CHAPTER II

Since using the computational approach described before, we found a putative estrogen response element in the E-Cadherin promoter and E-cadherin was found to be an estrogen down-regulated gene in breast cancer cells in a microarray gene expression profiling study published by Scafoglio and coworker, we focused this part of our study to demonstrate that this sequence is a functional ERE.

Moreover, it is well established that estrogen play an important role in development and progression of breast cancer. This process required both stromal invasion and acquisition of cell motility. Cancer cell motility can take place by more than one migration strategy. They can move as single cells, by using either mesenchymal or amoeboid migration, or as cell clusters, known as collective migration (Friedl and Wolf, 2003). The first strategy that has been shown required for efficient invasion and motility of cancer cells is known as epithelial-to-mesenchymal transition (EMT). This process is usually associated with down-regulation of E-cadherin expression (Berx and Van Roy, 2001; Thiery, 2002). For this reason we also focused our attention on the role of ER alpha in regulation of E-Cadherin expression during EMT in breast cancer progression.

E-CADHERIN IS A TARGET GENE DOWN-REGULATED BY ESTROGEN

To investigate further the mechanistic basis for the estrogen-dependent regulation of E-Cadherin we first analyzed whether this gene is truly a primary estrogen down-regulated gene. For this reason we decided to analyzed the effect of estrogen treatment using short time stimuli.

As shown in Fig. 6, E-Cadherin mRNA was indeed rapidly reduced to 20% of the initial level by 1h and 30 min of treatment with E2.

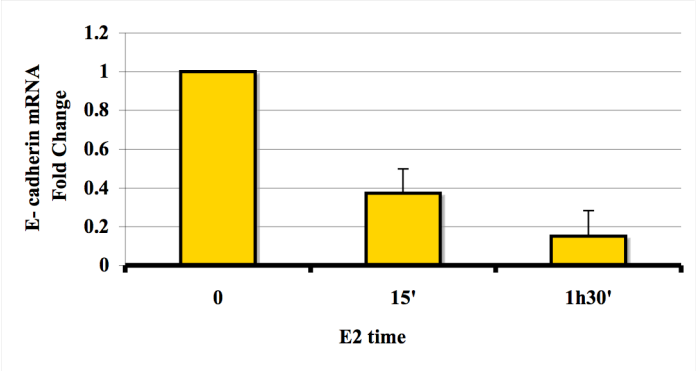


Figure 6 E2 Down-Regulates E-Cadherin Gene Expression in MCF-7 Human Breast Cancer Cells. Quantitative real-time PCR was used to evaluate changes in E-Cadherin mRNA level in MCF-7 cells in a time course analysis after treatment with 10nM 17β-estradiol.

ANALYSIS OF THE HUMAN E-CADHERIN PROMOTER

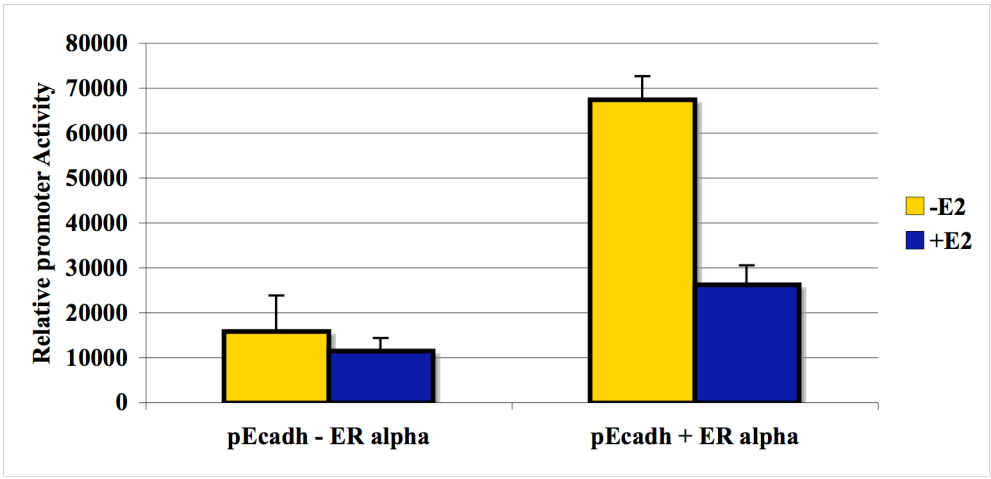
The human E-Cadherin promoter is about 1.5 Kb but it has been well demonstrated that the basic regulatory region is localized between nucleotide -300 and +100 (Liu et al., 2005). Within this region three clusters of transcription factor binding sites are localized, called E-boxes that are consensus sites for the E12/E47 basic helix-loop-helix and Zn-fingers transcription factors (Peinado et al., 2004; Perez-Moreno et al., 2001). Using the computational approach described before, we found a putative half-ERE (-164/-152).

To evaluate whether this half-ERE was necessary for E2-mediated down-regulation the basic promoter was cloned in a luciferase reporter vector and was transiently transfected along with ER alpha into the ER-negative Hela cell line.

As shown in Fig. 7 A, a 1h and 30 min treatment with E2 was able to significantly reduce the activity of the fragment tested. Moreover, as shown in Fig. 7 A (yellow bars) the expression of ER alpha in the absence of ligand was able to increase the basal activity of E-Cadherin promoter. To evaluate the role of the half-ERE site present in the human E-Cadherin promoter, we used site-directed mutagenesis to alter this site. We deleted the first three bases of half-ERE (pDEcadh) to ensure that the binding site would not be recognizable by the ER. Transient transfections were performed in HeLa cells with the clone containing the desired deletion. This deletion was able to

completely block the repressive E2 effect, as well as the increase of the basal promoter activity observed in absence of ligand (Fig. 7 B). This result indicates that the half-ERE element in the E-Cadherin promoter is necessary for repression by the E2-ER.

A



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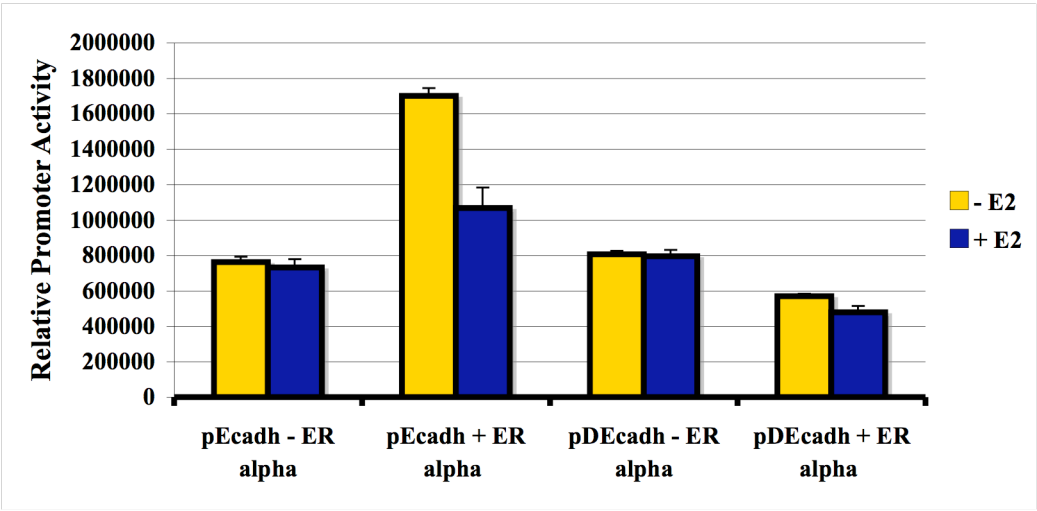
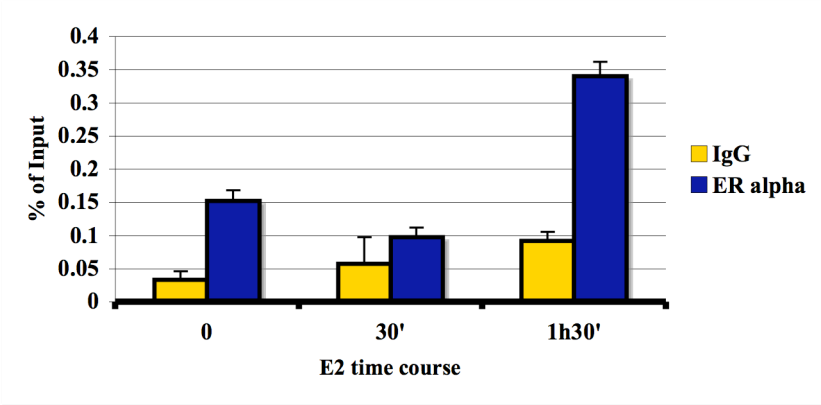


Figure 7 E-Cadherin Promoter Activity is Down-Regulated by ER alpha. A. The human E-Cadherin promoter or empty vector (pGL3basic) were transfected into HeLa cells along with or without ERα and β-galactosidase used as an internal control, the promoter activity was assessed in the absence (control vehicle) or presence of 10 nM E2. B. The first three bases of half-ERE in the E-Cadherin promoter were deleted using site-directed mutagenesis. The wt and deleted promoter constructs were transfected into HeLa cells, and promoter activity was assessed in the absence (control vehicle) or presence of 10 nM E2. Luciferase assay was performed after 1h and 30 min of E2 (10 nM) or vehicle treatment.

To examine ER alpha recruitment to the E-Cadherin promoter in MCF7 cells in vivo, we used chromatin immunoprecipitation (ChIP) assay. The cells were treated for 30 min or 1h and 30 min with either 10nM E2 or control vehicle, chromatin was cross-linked with formaldehyde, and DNA-protein complexes were immunoprecipitated with antibodies to ER alpha and dimethyl-lysine 9 of histone H3 (diMeH3K9) that is a marker of repressed promoters. ER alpha was recruited to the E-Cadherin promoter in a ligand-dependent manner (Fig. 8 A) and H3 became more methylated on lysine 9 indicating that the chromatin in this region became a less permissive environment for transcription (Fig. 8 B).

A



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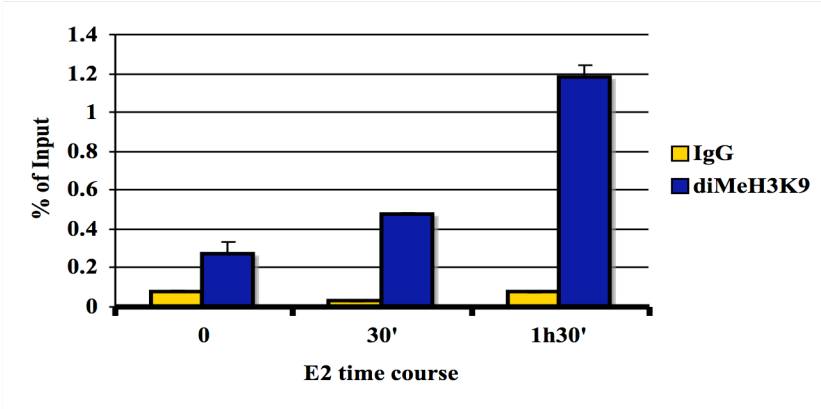
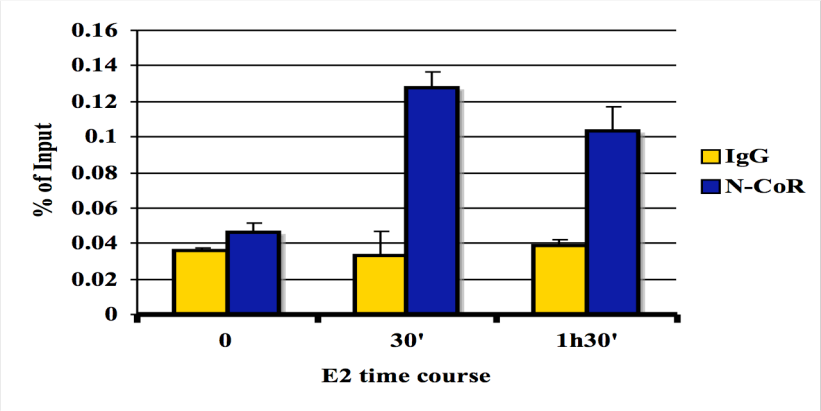


Figure 8 ER alpha is Recruited to the E-Cadherin Promoter. Chromatin Immunoprecipitation (ChIP)/ Quantitative real-time PCR show occupancy analysis of ER alpha (A) and diMeH3K9 (B) on the ERE of E-Cadherin promoter. The cells were treated for 30 min or 1h and 30 min with either 10nM E2 or control vehicle.

N-CoR AND CtBP COMPLEX ARE RECRUITED AT THE E-CADHERIN PROMOTER

To examine possible corepressor complex recruitment by E2-ER alpha at the E-Cadherin promoter, we tested the presence of N-CoR that is one of the major corepressor complexes for ER alpha. Moreover, it is known that Slug can repress E-Cadherin expression by recruiting of CtBP complex. Hence, we tested the recruitment of both Slug and CtBP after E2 treatment. As shown in Fig. 9 A, N-CoR was recruited at the E-Cadherin promoter already 30 min after E2 treatment, as compared to CtBP and Slug that were recruited after 1h 30 min (Fig. 9 B).

A



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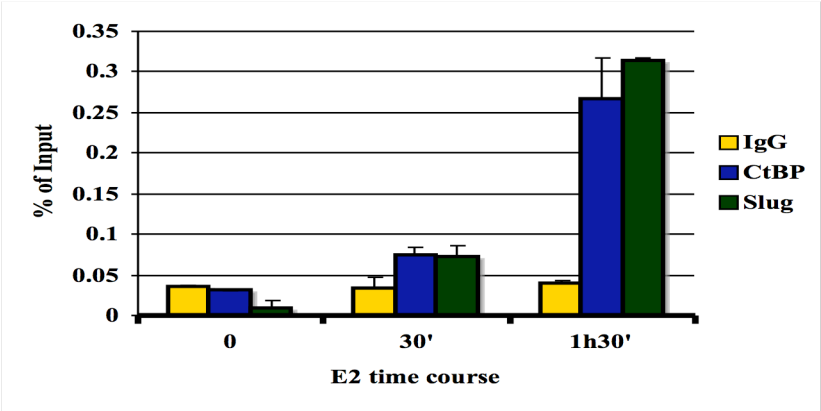


Figure 9 N-CoR, CtBP and Slug are Sequentially Recruited at the E-Cadherin Promoter. Chromatin Immunoprecipitation (ChIP)/ Quantitative real-time PCR show occupancy analysis of N-CoR (A), CtBP and Slug (B) on E-Cadherin promoter. The cells were treated for 30 min or 1h and 30 min with either 10nM E2 or control vehicle.

Functional analysis of human E-cadherin promoter indicated that Sp1 play important roles in promoting E-cadherin transcription (Liu et al., 2005). Therefore, we examined at the recruitment of Sp1 at the E-Cadherin promoter after E2 treatment. Indeed, as shown in Fig. 10, Sp1 is dismissed from the E-Cadherin promoter after E2 treatment, indicating that when E2-bound ER is present, the E-Cadherin promoter loses an important transactivator.

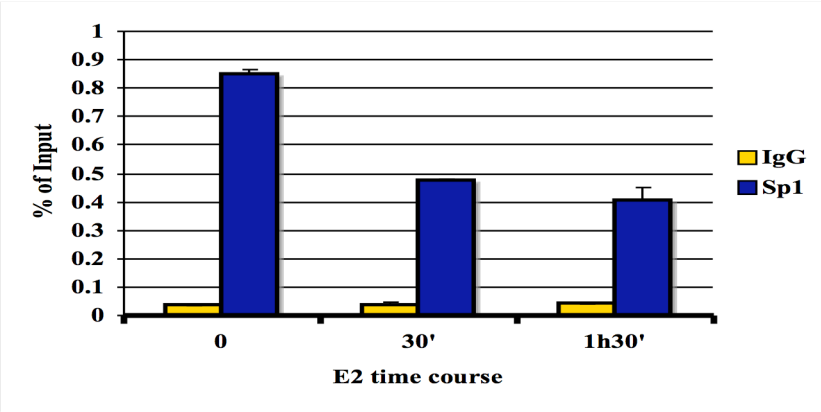


Figure 10 Sp1 is dismissed from the E-Cadherin promoter after E2 treatment. Chromatin Immunoprecipitation (ChIP)/ Quantitative real-time PCR show occupancy analysis of Sp1 on E-Cadherin promoter. The cells were treated for 30 min or 1h and 30 min with either 10nM E2 or control vehicle.

ROLE OF UNLIGAND ER ALPHA ON E-CADHERIN EXPRESSION

Our experiment showed that E-Cadherin is down-regulated by E2 and this repression corresponds to recruitment of ER alpha at the E-Cadherin promoter. Moreover, the luciferase assay showed that unligand ER alpha could play a role in the regulation of the basal E-Cadherin transcription. Therefore, to evaluate this hypotesis we transiently transfected ER alpha wt or ER alpha with a triple point mutation in the DNA binding domain (mutDBD), that greatly reducing the affinity of the receptor for EREs, into the ER-negative Hela cell line and we checked the expression of endogenus E-Cadherin. As shown if Fig. 11, expression of ER alpha was able to increase the basal transcription of E-Cadherin in absence of ligand, while ER alpha mutDBD did not, thus demonstrating that the DBD domain is required for activation.

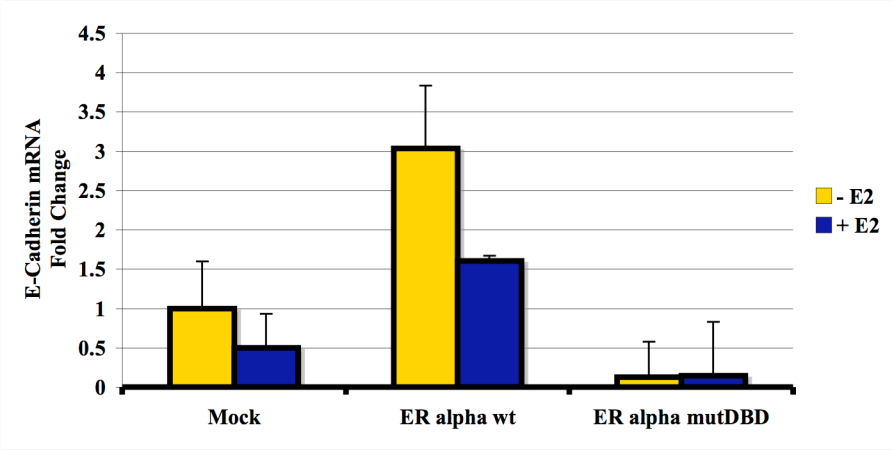
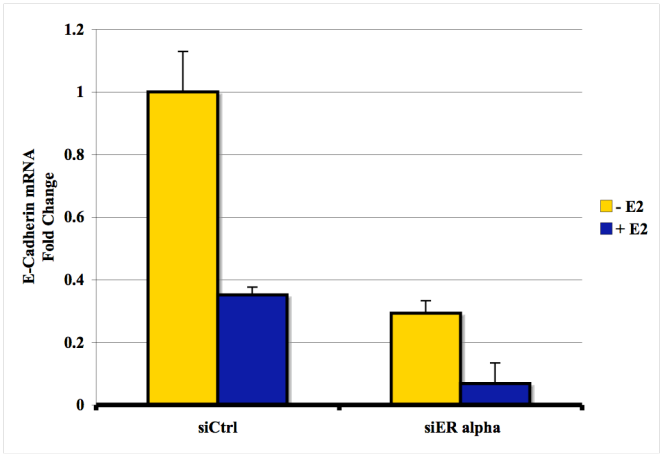


Figure 11 Basal Expression of E-Cadherin is increase by ER alpha in absence of ligand. Quantitative real-time PCR was used to evaluate changes in E-Cadherin mRNA level in Hela cells in the absence (control vehicle) or presence of 10 nM E2 after transfection of ER alpha wt or ER alpha mutDBD.

To determine if unliganded ER alpha is involved in the basal expression of E-Cadherin in a cell type that naturally expresses ER alpha, we treated the MCF-7 cells with short interfering RNA (siRNA) to ER alpha. The siRNA treatment, which resulted in evident down-regulation of ER alpha mRNA (figure 12B), produced a marked reduction in E-Cadherin level, whereas no decline in E-Cadherin mRNA occurred with control siRNA (Fig. 12). This result provide further evidence that unliganded ER alpha is required for basal E-Cadherin transcription.

A



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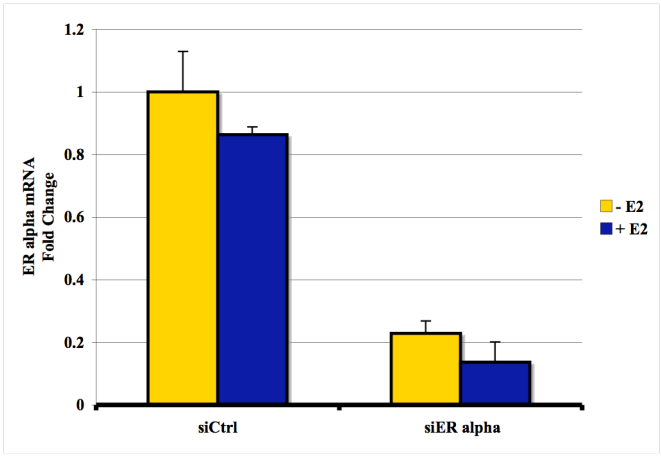


Figure 12 Unliganded ER alpha Increased the Basal Expression of E-Cadherin gene. A. Quantitative real-time PCR was used to evaluate changes in E-Cadherin mRNA level in MCF-7 cells in the absence (control vehicle) or presence of 10 nM E2 after transfection of siRNA to ER alpha. B. Quantitative real-time PCR was used to evaluate changes in ER alpha mRNA level in MCF-7 cells in the absence (control vehicle) or presence of 10 nM E2 after transfection of siRNA to ER alpha.

ER ALPHA AND BREAST TUMOR PROGRESSION

In many types of epithelial cancers, the ability to undergo metastasis has been associated with a loss of epithelial features and acquisition of mesenchymal properties leading to migration of individual cells, a process known as epithelial-to-mesenchymal transition (EMT), one of the genes involved in this process is E-Cadherin. In 2004 Bardella and coworker published an interesting model for EMT; they used T47D ER alpha positive epithelia breast cancer cells to generate the T47D sfRON cell line through stable transduction of a lentivirus vector expressing a truncated form of a Tyrosine Kinase receptor called RON (MST1R). These cells lost expression of E-Cadherin and epithelial morphology and acquired motility (Fig. 13). Moreover, ChIP analysis of H3 methylation at the E-Cadherin promoter in these cells demonstrated that the gene is heterochromatic in T47DsfRON cells (Fig. 13 D).

Since we have been shown that ER alpha is required for basal E-Cadherin expression we tested the expression of ER alpha in this cells line. As shown in Fig.14 the T47D sfRON lost completely the expression of ER alpha, as well.

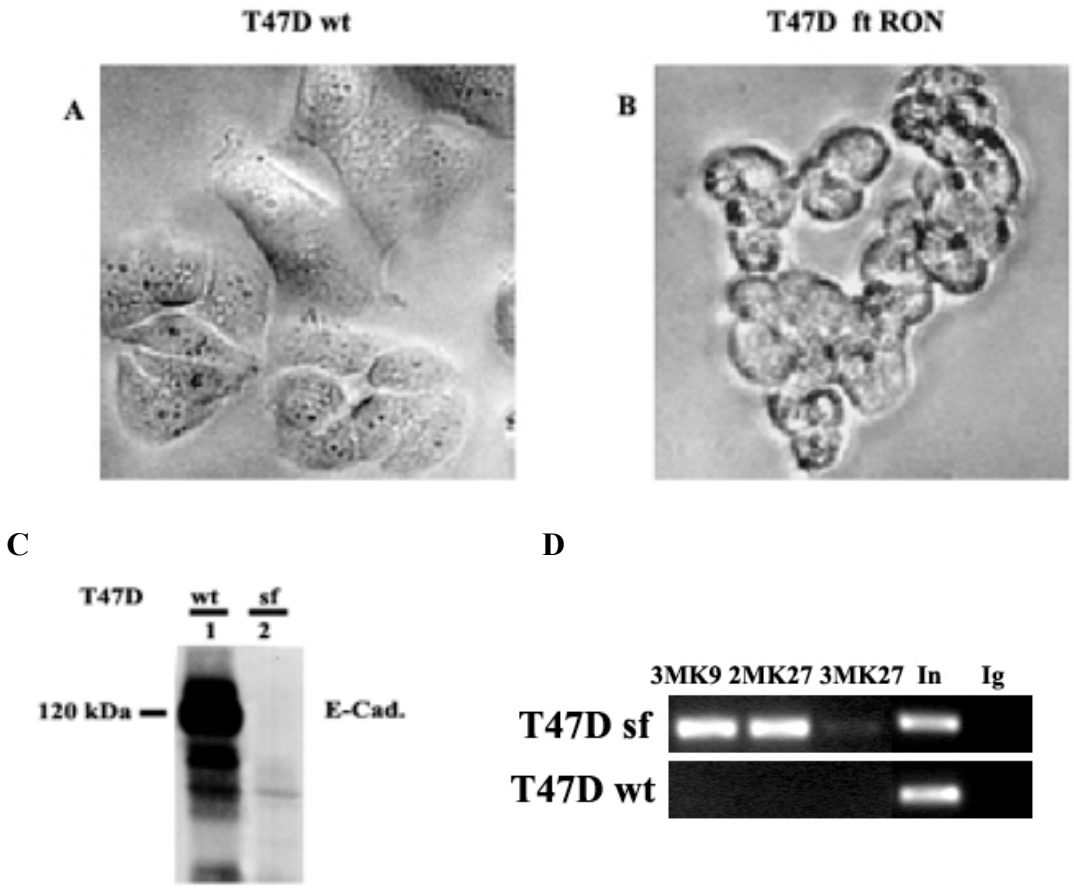


Figure 13 T47D sfRON lose the E-Cadherin expression and change morphology. A. Picture of T47D epithelial breast cancer cells wt. B. Picture of T47D after stable transduction of a lentivirus vector expressing a truncated form of a Tyrosine Kinase receptor called RON C. Immunoblotting of cell extracts from T47D wt (1) and T47D sfRON (2) (Pannels A, B and C are reproduced from Bardella et al., 2004). D. Chromatin Immunoprecipitation (ChIP) shows presence of specific eterochromatin markers on E-Cadherin promoter in T47D wt and sfRON cells (3MK9: threemethyl- lysine 9 of histone H3; 2MK27: dimethyl- lysine 27 of histone H3; 3MK27 threemethyl- lysine 27 of histone H3).

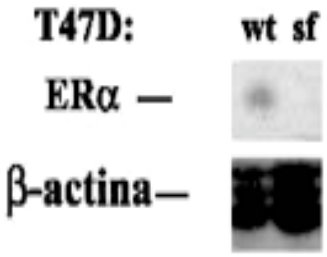


Figure 14 T47D sfRON lose the ER alpha expression. Immunoblotting of cell extracts from T47D wt and T47D sfRON was done with ER alpha antibody (Santa Cruz).

It has been shown that activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells induces an in vivo molecular phenotype of ER alpha-negative human breast tumors (Creighton et al., 2006). We asked whether inhibition of kinase activity of sfRON could revert the molecular epithelial phenotype. As shown in Fig. 15 A the cells treated with the kinase inhibitor K252a reacquired an epithelial phenotype. This morphological change is accompanied by expression of both E-Cadherin and ER alpha (Fig. 16 A). Moreover ER alpha in this condition was recruited at the E-Cadherin promoter (Fig. 16 B). These data further support a possible new role for ERα as ligand-independent activator that can be essential for the determination of epithelial morphology.

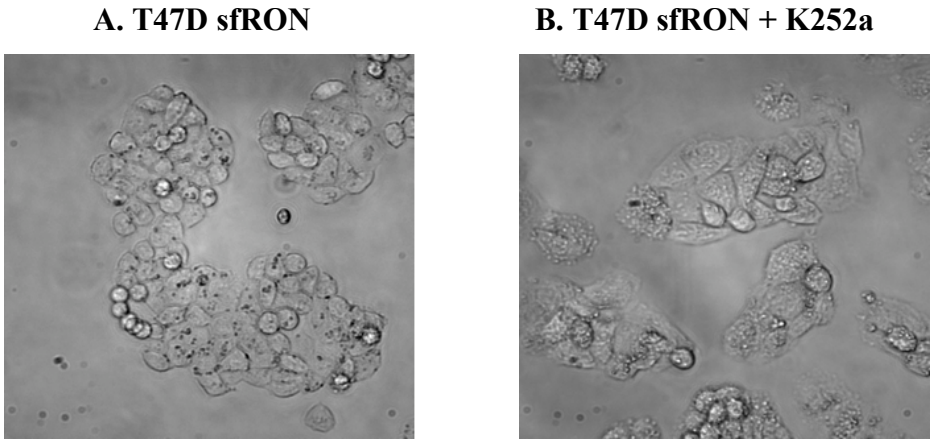
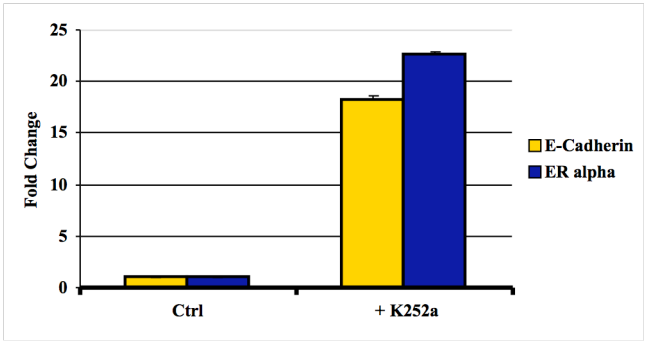


Figure 15 T47D sfRON change morphology by treatment with K252a. Cells ware treated with 300nM of K252a (B) or control vehicle (A) for 6h.

A



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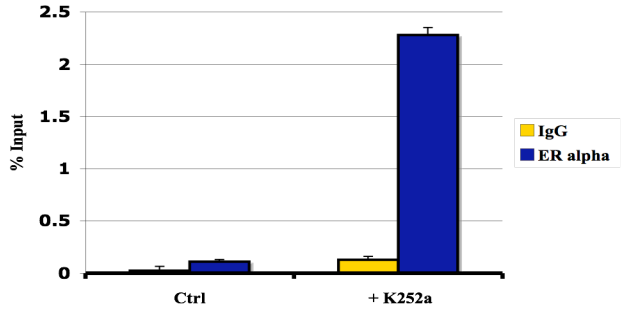

Figure 16 T47D sfRON express E-Cadherin and ER alpha after K252a treatment. A. Quantitative real-time PCR was used to evaluate changes in E-Cadherin and ER alpha mRNA level in T47D sfRON cells in the absence (control vehicle) or presence of 300 nM K252a. B. Chromatin Immunoprecipitation (ChIP)/ Quantitative real-time PCR show occupancy analysis of ER alpha on E-Cadherin promoter in level in T47D sfRON cells in the absence (control vehicle) or presence of 300 nM K252a.

CHAPTER III

The nuclear receptor (NR) superfamily of transcriptional regulators play a central role in many developmental and disease processes, and the system has been extensively studied as a model to learn the mechanism for spatial and temporal control of gene expression (Dennis and O'Malley, 2005; Dilworth and Chambon, 2001; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Perissi and Rosenfeld, 2005).

The work described in Chapter I and II clearly indicates that the same factor (ER) bound to the same sequence (ERE) can evoke either activation or repression at different gene contexts. Individual NRs have consensus binding sites in promoters and enhancers, which have been characterized in detail, but only in a limited number of NR-regulated genes. In the case of the pS2 gene (also known as TFF-1), for example, binding by estrogen receptor-α (ERα) initiates sequential recruitment of a large number of transcription factors onto the promoter to start transcription (Metivier et al., 2003). Both Carroll et al, 2005 and Bassets et al, 2007, describe genome-wide occupancy studies of ERα to determine the genetic program governed by its target genes. Such exhaustive studies have provided a wealth of information and candidates of regulated proximal and distal (putative long distance enhancer) regions. Ultimately, these observations raise general questions, as to whether and how those remote binding sites may communicate with appropriate target genes via long-distance intrachromosomal or interchromosomal interactions and as to whether genes showing a common mode of regulation in response to stimuli share the same transcriptional machinery. For this reason, the piece of work described in this Chapter was carried out in the laboratory of M.G. Rosenfeld at UCSD, who was examining the functional relationships between nuclear structure and gene expression, using as a model the well-characterized regulation of Estrogen receptor α (ERα) target genes in response to hormone and studying the dynamic responses of the cell to different signals, resulting in changes in chromatin compaction levels and movement of genomic loci.

Results of this study, which are exposed in the manuscript that follows, demonstrated that ligand induces rapid interchromosomal interactions among subsets of estrogen receptor α-bound transcription units, with a dramatic reorganization of nuclear territories requiring nuclear actin/myosin-1 transport machinery, dynein light chain 1, and a specific subset of transcriptional coactivators and chromatin remodeling

complexes.

Specifically, we were able to demonstrate that ligand induced rapid interchromosomal interactions is required to ER alpha trans-activation activity. Indeed, the knocking down of components of the transport machinery by specific siRNA impairs both the interchromosomal interaction and PS2 trans-activation even if does not impair the recruitment of ER alpha and its coactivator complex on the PS2 promoter. Colocalization of target genes and coactivators with RNA-processing factors in nuclear spikes was also demonstrated. This data suggest a molecular mechanism by which the hormone-induced interchromosomal interactions serve to achieve enhanced, coordinated transcription and RNA splicing for nuclear receptor target genes.

MANUSCRIPT SUBMITTED

Nuclear Receptor-Activated Transcription Requires Nuclear Actin/Myosin-mediated Interchromosomal Gene Networking in Interchromatin Granules

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Abstract

While the role of liganded nuclear receptors in mediating the coactivator/corepressor exchange in regulated gene expression is well established, a key unanswered question is whether previously unrecognized, induced interchromosomal interactions serve to achieve an integrated, ligand-dependent transcriptional response. Here, we report that ligand induces rapid interchromosomal interactions among subsets of estrogen receptor α-bound transcription units, with a dramatic reorganization of nuclear territories requiring nuclear actin/myosin-1 transport machinery, dynein light chain 1, and a specific subset of transcriptional coactivators and chromatin remodeling complexes. We establish a molecular mechanism by which the hormone-induced network of interactive hubs become co-associated with distinct interchromatin granules, long thought to be "storage" sites for the splicing machinery and various transcription elongation factors, and with other transcription–associated complexes, thereby serving to achieve enhanced, coordinated transcription and RNA splicing for nuclear receptor target genes.

Introduction

The nuclear receptor (NR) superfamily of transcriptional regulators plays a central role in developmental homeostasis and disease processes, and has been extensively studied as a model to identify the molecular mechanism for precise spatial and temporal control of gene expressions (Dennis and O'Malley, 2005; Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Perissi and Rosenfeld, 2005;

Rosenfeld et al., 2006; Spiegelman and Heinrich, 2004). Intensive investigation in this area has established a clear role of coactivator/corepressor exchange in gene activation. However, whether the exchange of cofactors is based solely on diffusion, or involves a more active mechanism(s), has remained an unsolved issue. The genome-wide identification of DNA binding sites for nuclear receptors, such as estrogen receptor (ERα), has provided further insights into the molecular basis of ligand-dependent gene activation events. In common with other transcription factors, ERα was found to bind to both gene promoters and many other remote intergenic sites, only a few of which have clearly been established to function as enhancers *in vivo* (Carroll et al., 2005; Carroll et al., 2006; Cawley et al., 2004). These data raise the general question as to whether and how some of these remote binding sites might communicate with their putative target genes via long-distance intra-chromosomal, or even interchromosomal interactions, and whether the underlying mechanisms might account for some specific aspects of the cofactor exchange program during gene activation by nuclear hormones.

While the architectural organization of the nucleus is still poorly understood, several nuclear structures have been characterized, including nuclear lamina, nucleoli, PML bodies, Cajal bodies, nuclear speckles, etc. (Gruenbaum et al., 2005; Handwerger and Gall, 2006; Hernandez-Verdun, 2006; Lamond and Spector, 2003; Spector, 1993). These morphologically and compositionally distinct nuclear structures coexist with individual chromosomes, which are known to occupy distinct regions in the nucleus, often referred to as chromosomal territories (Croft et al., 1999; Edelmann et al., 2001; Foster and Bridger, 2005; Zink et al., 1998). However, except for the nucleolus, little is known of how various nuclear domains arise and influence gene expression.

Repartitioning of active genes has been suggested for *Hox* genes in ES cells (Chambeyron and Bickmore, 2004), *IgH* in β lymphocytes (Kosak et al., 2002), *c-maf*

in T cells (Hewitt et al., 2004), *Mash1* in neuronal cells (Williams et al., 2006), *Cftr* in adenocarcinoma cells (Zink et al., 2004), etc. A key issue here is whether these active gene loci are looped out of their nuclear territories to engage in long-distance interactions with other active genes, regulatory loci or some sort of factories. Many recent studies have now documented interchromosomal interactions to provide novel control mechanisms for regulated gene expression in interphase nuclei: Interchromosomal interactions were discovered for the *IFNγ* gene in chr. 10 with the regulatory regions of the T_H2 cytokine locus in chr. 11 in developing T cells (Spilianakis et al., 2005). Promoters for specific olfactory receptors were shown to interact with a proposed enhancer in a mutually exclusive manner, providing a model for selective activation of a single odorant receptor-encoding gene (Lomvardas et al., 2006). Shifts in looping and long-distance intra- and interchromosomal interactions were also found to explain the coordinated regulation of the two imprinted genes *Igf2* and *H19* (Murrell et al., 2004; Zhao et al., 2006b), which involves, at lease in part, the action of CTCF (Ling et al., 2006). Thus, a major issue is whether a potentially dynamic system, including interchromosomal interactions, operates to provide coordinated control for regulated gene expression, such as those mediated by liganded nuclear receptors in mammalian cells.

Here, we report that a network involving multiple ERα binding sites within the same chromosome and between those located in different chromosomes is induced in response to 17β-estradiol (E_2) in mammalian cells, which has permitted the elucidation of a previously unrecognized, actin motor-dependent reorganization of nuclear territories. This network interaction is rapid and depends upon the recruitment of a subset of ERα coactivators and specific components of chromatin remodeling complexes, including nuclear dynein light chain-1 and actin polymerization. Dynamic

assembly of nuclear motors involving g-actin, nuclear myosin-1, and actin-associated proteins that cause branching, permits facilitated assembly of ERα-bound loci with interchromatin granules, also referred to as nuclear speckles, that harbor key factors for transcriptional elongation and essentially all factors required for pre-mRNA splicing. Our findings provide a general organizational principle of nuclear subdomains and a model for coordinated regulation of specific gene transcription the nucleus.

Results

Unbiased identification of long-range, estrogen-induced chromosomal interactions

We began an investigation of long-distance chromosomal interactions in response to nuclear hormones in an open-ended fashion by coupling the Chromosome Conformation Capture (3C) assay (Dekker et al., 2002) with the ChIP-DSL strategy that we recently developed for large-scale promoter array and tiling array analyses (Kwon et al., 2007), a technology we refer to as Deconvolution of DNA interaction by DSL or the 3D assay. As diagrammed in **Fig. 1A**, we prepared E_2-stimulated MCF-7 breast cancer cells according to the established 3C protocol by *in situ* restriction digestion followed by DNA ligation under an extreme dilution condition, with a parallel reaction without DNA ligase as a negative control. We used individual biotinylated oligonucleotides to capture specific DNA fragments under investigation (the estrogen receptorα binding region located in the *TFF1* enhancer in this case). During the annealing step, all DSL oligonucleotide pairs targeting individual genomic blocks in a ~1.4 Mb tiled path surrounding the ERα-regulated *TFF1* gene were included to detect potential co-captured genomic DNA as a result of DNA ligation during 3C (**Fig. 1B**). The paired oligonucleotides were next selected, ligated, amplified, and hybridized to the corresponding tiling array.

We repeated the *TFF1* enhancer capture experiments several times and used the median percentage ranking statistics to identify consistently high ranking signals (Buck and Lieb, 2004). After data smoothing to emphasize signal clusters, we determined the probability that a given signal might be detected by chance via permutating the dataset 1000 times. The resulting statistically significant signals (**Fig. 1B**) were then validated by individual 3C assays in both mock-treated and E_2-induced MCF-7 cells after titrating ligated DNA to ensure that the PCR was operating in a quantitative range (see examples in **Fig. S1**) and normalizing the 3C signals using randomly ligated BAC controls (**Fig. 1C**). 3C validation generally matched the 3D results: Within the ~150kb region near the *TFF1* gene (3C probe **a** to **h** in **Fig. 1B**), we detected both E_2-independent background interactions due to random collisions as previously suggested (Dekker, 2006) and the expected, E_2-dependent DNA-DNA interaction (probe **d** as highlighted by a blue box in **Fig. 1C**), the latter of which is consistent with the proposed looping event between the *TFF1* promoter and enhancer (Carroll et al., 2005).

Interestingly, we also detected the long-distance interaction of the *TFF1* enhancer with multiple discrete loci, several of which corresponded to the intergenic ERα binding sites mapped by ChIP-DSL (e.g. 3C probe A, B, H and J in **Fig. 1B** and **1C**). Multiple 3D-negative regions, included as controls (C, D, E, I, K), gave no 3C signals, whereas the interaction of the *TFF1* enhancer with locus G and H was either modestly induced by E_2 or "constitutive", which might guide other hormone-dependent long-range interactions (e.g. A, B, and J) (**Fig. 1C**). The ERα binding site **F** showed strong E_2-inducible interaction with the *TFF1* enhancer as determined by 3C, but was undetected by 3D, which we note is due to multiple restriction sites surrounding the locus, resulting in an isolated high-ranking peak in the raw 3D data that was eliminated during data smoothing designed to emphasize clusters of signals. Therefore, despite the

possibility that some interactions, such as that with locus F, might have been missed by 3D, the data have clearly established the engagement of the *TFF1* enhancer in both E_2-dependent and independent long-distance interactions with multiple genomic loci that are beyond the traditional gene boundaries.

We next performed quantum dot (Q-dot)-based fluorescence *in situ* hybridization (FISH) (Cai and Kohwi-Shigematsu, 1999), confirming the series of E_2-dependent interactions predicted by 3D, showing E_2-treated cells (45 min) in which both alleles converge (**Fig. 1D** and **Fig. S2)**. The *TFF1* locus resides in chr. 21, and by chr. 21 painting, we found that all FISH foci were confined in the expected chromosomal territories (blue patches in **Fig. 1D**). We note in these studies that the basal distance between loci is not fixed, but rather exhibits wide variance between cells, consistent with the fluidity of basal and E_2-induced changes in chromatin structure in these large intervals, which is in contrast to the notion that this distance is fixed. These findings provided independent evidence for E_2-induced long-range chromosomal interactions at the single cell level, motivating an extensive study of possible interchromosomal interactions.

Hormone-induced interchromosomal interactions

To investigate potential E_2-dependent interchromosomal interactions, we included in the 3D experiment a set of 20 multiple different chromosomes, one of which, *GREB1*, is another well-characterized ERα-inducible gene located in chr. 2 (Ghosh et al., 2000) and recent ChIP-DSL mapping confirmed direct ERα binding to multiple promoters and enhancers of the *GREB1* gene (Kwon et al., 2007). When the 3D TFF1 capture experiments were analyzed, we detected two clusters of significant signals coincident with an enhancer and promoter in the *GREB1* gene (**Fig. 2A**), while

the other 19 tiled regions, representing 7 distinct chromosomes, showed no signal, two of which are illustrated (**Fig. 2B** and **2C**). This finding suggests that the *TFF1* gene may be also engaged in inter-chromosomal interactions with other, but not all, ERα target gene regions in a hormone-dependent manner.

Because the *TFF1* and *GREB1* genes reside in two separate chromosomes, chr. 21 and chr. 2, respectively, FISH analysis was used to provide an independent approach to visualize these putative hormone-dependent interchromosomal interactions. The two genes were independently localized in the nuclei of mock-treated MCF-7 cells, but after 45 min of E_2-stimulation, the two genes became colocalized as revealed by FISH (**Fig. 2D)**. In our analysis, about half of the cells exhibited mono-allelic interactions, while the other half exhibited bi-allelic interactions, suggesting that many ERα-bound genes may be functionally mono-allelic in a subset of cells (**Fig. 2D**). Indeed, mono-allelic interactions have been observed in other studies of interchromosomal interactions (Cook, 1998; Lomvardas et al., 2006; Paixao et al., 2007) and have been proposed to share the property of asynchronous DNA replication (Ensminger and Chess, 2004). ANOVA analysis of variance is shown; the program automatically divides data into quartiles; cells in which E_2 cause both, or only one allele to interact, are independently plotted. In the subsequent figures in this manuscript, we measured the distance between *TFF1* and *GREB1* loci in both control vehicle-treated and E_2-treated cells, but display only the bi-allelic interaction data for clarification; in each case, the percent of E_2-induced interactions in cells in which only one allele pair interacts, was similar in each case to those in which both alleles interact, of course, gave the bimodal plots analogous to that in **Fig. 2E**.

With simultaneous chromosomal painting, we observed the two chromosomes "kissing" in a large percentage of E_2-induced cells, instead of occupying four well-

separated nuclear territories before E_2-induction; in this figure **(Fig. 2D)**, 3D FISH in cells in which both alleles interact are shown as a deconvoluted Z-stack of control versus E_2-treated cells **(Fig. 2E)**. Similar data were obtained using two-dimensional FISH (here chr. 21 and chr. 2 paints were included) **(Fig. 2D, left)**.

Interestingly, we did not detect chr. 2:chr. 2 or chr. 21: chr. 21 pairing in response to E_2, suggesting a specificity in chromosomal repartitioning as part of the nuclear reorganization program in response to a hormone signal. To establish the generality of hormone-induced interchromosomal interaction events, we performed a similar analysis on androgen receptor (AR)-regulated genes in LNCaP prostate cancer cells. Based on the identification of AR target gene promoters by ChIP-DSL (our unpublished results), we tested a panel of AR target genes and found another example of hormone-induced interchromosomal interaction, which, in this case, took place between the *KLK2* gene in chr. 19 and the *TMPRSS2* gene in chr. 21 in response to the androgen agonist, DHT (45 min) (**Fig.2F** and **2G)**. Together, these data establish a generality of ligand-induced interchromosomal interaction events.

Rapid, nuclear receptor-dependent movement of nuclear territories

Because of the large extent of chromosomal abnormalities in MCF-7 breast cancer cells, we evaluated the possible hormone-induced nuclear reorganization in primary human primary mammary epithelia cells (**HMECs)** to ascertain that the detected interchromosomal interactions were not a cancer cell-specific phenomenon. We observed the expected *TFF1:GREB1* interaction in one or both alleles in ~90% of E_2-treated cells with ~50% of the cells showing bi-allelic interaction **(Fig. 3A** and **Fig. S3E)**.

Since it was unexpected to observe the specific, E_2-induced interchromosomal pairing as early as 45 minutes, we determined the kinetics of the hormonal response by FISH analysis at 2, 5, and 60 min after E_2 stimulation in the primary breast epithelial cells. Assuming immediate fixation, we found that, even at 2 min, the earliest time point evaluated, chr.2 and chr.21 were induced to "kiss", with the interactions between *TFF1* and *GREB1* reaching high levels at 5 min **(Fig. 3B)**. We also observed similar kinetics for other chromosomal long-distance interactants in both HMEC **(Fig. S3)** and MCF-7 breast cancer cells (data not shown).

To determine whether all E_2-regulated genes "converged" to interact with one another in a single domain of the nucleus, we simultaneously assayed a collection corresponding to selected ERα-targeted promoter/enhancer pairs from different chromosomes (chr. 1, 2, 6, 14, 20, 21), including a series of interactants on chr. 21 by FISH in normal breast epithelial cells, and a non-ERα binding control, which corresponds to an intronic sequence in the *PDE9A1* transcription unit (see **Fig. 10**). While each region was recorded in distinct nuclear locations in unstimulated cells, treatment with E_2 caused a convergence of these ERα target genes to 7 to 8 foci (**Fig. 3D**), with the PDE9A1 detected as the two remaining green signals, indicating that this non E_2-responsive gene remained independently localized from the rest of interacting clusters (**Fig. 3C**). These findings suggest that different E_2-regulated gene sets are engaging in distinct network interactions in the nucleus.

Requirement for ERα-dependent interchromosomal interactions

Having established the rapid, signal-dependent gene repartitioning in the nucleus, we next determined whether the observed nuclear organization is a requirement for or a consequence of hormone-regulated gene expression. Treatment of

the normal breast epithelial cells with α-amanitin or DRB (5,6-dichloro-1β-d-ribofuranosylbenzimidazole) at concentrations sufficient to inhibit gene transcription (see Methods) prevented all E_2-dependent interchromosomal interactions **(Fig. 3E; Fig. S4).**

We next evaluated whether the observed chromosome pairing was dependent on ERα binding. By using a specific siRNA that has been proven to effectively knockdown ERα (Garcia-Bassets et al., 2007; Perissi et al., 2004), we showed that that the E_2-induced *GREB1*:*TFF1* interactions were lost in the presence of this siRNA (**Fig. 3F**). As an independent test for the requirement of ERα binding in these events, we took advantage of the observation that siRNA against *FoxA1* eradicates ERα binding and E_2-dependent gene activation (Carroll et al., 2005; Laganiere et al., 2005b). After two days of the *FoxA1* siRNA treatment, we found a complete loss of E_2-dependent interactions between *GREB1* and *TFF1* (**Fig. 3F**). These results therefore establish the requirement for nuclear receptor binding in mediating the observed interchromosomal interactions.

To further investigate whether specific exchange of ERα corepressors for coactivators was required, we performed single cell nuclear microinjection using specific siRNAs or blocking antibodies against several specific coactivators as previously established (Perissi et al., 2004). Inactivation of CBP/p300 (**Fig. 4A**) or the p160 coactivators SRC1/pCIP (**Fig. 4B**) with siRNAs or with short periods of intranuclear antibody injection abolished the E_2-dependent *TFF1:GREB1* interactions. Injection of blocking antibody against the P220/PBP component of the mediator complex (Perissi et al., 2004), or siRNA against *PBP* also effectively inhibited *TFF1:GREB1* interactions in E_2-treated cell (**Fig. 4C**). Finally, we examined the histone lysine demethylase 1 (LSD1), which has recently been shown to be essential for

E_2-dependent gene activation (Garcia-Bassets et al., 2007). Unexpectedly, we observed that the *LSD1* siRNA that effectively deplete *LSD1* and blocked E_2-dependent induction of *GREB1* and *TFF1,* had little effect on the E_2-dependent interchromosomal interactions between the two genes (**Fig. 4D**). Together, these findings suggest that the E_2-dependent interchromosomal interactions among specific ERα-regulated genes require both the liganded estrogen receptor and a subset of the serially-recruited coactivators to the bound receptor, but not all associated factors, such as LSD1, that are required for E_2/ERα-induced transcriptional activation. Therefore, the network of interactions is likely established as the process of, rather than the consequence of, regulated gene transcription.

Actin cables and nuclear motor-directed chromosomal movements

The observed interchromosomal interactions between the *TFF1* and *GREB1* genes are clearly non-random events because we did not detect chr. 2:chr. 2 and chr. 21:chr. 21 pairings, consistent with the current concept of chromosomal territories. From our results we can estimate a movement of 0.1- 0.9μm/min. over a distance of 1-5μm, in excess of the smaller, salutatory movements of <0.2μm that occur every 1-2s, an average Brownian motion value (Gunawardena and Rykowski, 2000). Further, the observed chromosomal movement was clearly energy-dependent because inhibiting ATP regeneration by rotenone treatment **(Fig. S5)** caused a block of E_2/ERα-induced interchromosomal interactions (**Fig. 5A**). Given the relatively rapid kinetics of the E_2-induced chromosomal movement, we therefore investigated whether an actin-dependent mechanism might be involved. Nuclear actin has been shown to associate with many transcriptional complexes and reported to play an important role in transcriptional activation, particularly in yeast (de Lanerolle et al., 2005; Percipalle and Visa, 2006).

We approached this questions by using both treatment with pharmacological agents reported to specifically block the actions of actin/motor-linked events and by single cell nuclear microinjection of either specific IgGs to provide a rapid inhibition in the nuclear compartment, or by injecting siRNAs against mRNAs encoding these specific structural proteins. While there is no filamentous actin in the nucleus, there is an oligomerized g-actin, which can be specifically detected by a monoclonal antibody (Gonsior et al., 1999). As shown in **Fig. 5B**, this antibody stained a network of actin fibers in the normal breast epithelial cell nucleus, consistent with the description of specificity of the antibody, with E_2-treatment arguably causing apparent structural alterations in the stainable nuclear actin pattern.

Treatment of E_2-stimulated breast epithelial cells with latrunculin, a well-characterized drug that blocks actin polymerization (Rizk and Walczak, 2005) caused a complete loss of E_2-induced interchromosomal interactions **(Fig. 5C; Fig. S6**). Jasplakinolide, another agent known to inhibit depolymerization of actin network (Holzinger, 2001), which would prevent new actin polymerization for establishment of new connections, similarly abolished the E_2-induced *TFF1:GREB1* interactions **(Fig. 5C and Fig. S6)**. Together these data reveal that interchromosomal interactions require dynamic actin reorganization.

We next used the single cell nuclear microinjection assay to determine the potential requirement for nuclear Myosin-1, which has been identified to be present in the nucleus (Percipalle and Farrants, 2006), finding that the antibodies against myosin-1 blocked E_2-induced *TFF1:GREB1* interactions **(Fig. 5D**). Immunohistochemical analysis confirmed nuclear localization of the injected IgG, strongly suggesting the functional requirement of myosin-1 in the nucleus, rather than as an indirect effect of disrupted cytoskeleton. Similarly, we found that a specific *Myosin-1* siRNA abolished

the E_2-induced *TFF1:GREB1* interactions **(Fig. 5D)**. Single cell nuclear microinjection of neutralizing antibodies against the actin-related proteins, ARP2/3, which are instrumental in the formation of actin branches (Higgs and Pollard, 2001), or inactivation of these transcribed by specific siRNAs all caused a complete loss of the E_2-induced *TFF1:GREB1* interchromosomal interactions (**Fig. 5E**). These observations strongly implicate the nuclear actin and myosin-based motor in mediating E_2-dependent chromosomal movements and regional interactions.

The dynein motor is required for chromosomal segregation events (McGrath, 2005), and a recent report indicates that a component of the dynein motor, the Dynein Light Chain-1 (DLC1) directly interacts with the liganded ERα (Rayala et al., 2005). We therefore examined whether DLC1 might be required for the E_2-induced interchromosomal interactions using the single cell nuclear microinjection assay. Indeed, we found that depletion of *DLC1* by siRNA (**Fig. S7**) effectively abolished the *GREB1:TFF1* interchromosomal interactions in the E_2-treated primary breast epithelial cells (**Fig. 6A**). These observations strongly indicate that a factor that is often a component of the dynein complex is probably serving as a link between DNA-bound ERα and the actin-based motor to mediate E_2-dependent chromosomal movements. Finally, we tested a number of actin-fold proteins, finding that BAF53, and BAF170, but not BAF155, were also required for the E_2-dependent interchromosomal interactions between *TFF1* and *GREB1* (**Fig. 6B-E**), and g-actin has been noted to associate with a number of chromatin remodeling complexes (Olave et al., 2002).

In concert with the potential functional importance of hormone-induced movement and interchromosomal interactions, latrunculin and specific siRNA against *ARP2/3, DLC1*, and *BAF53*, all of which effectively decreased their specific targets

transcripts **(Fig. S8),** block E_2-induced activation of *TFF1* and *GREB1* gene expression **(Fig. 6F;** data not shown**)**.

Interchromosomal Granules: Hubs for interchromosomal interactions?

The actin-based, motor-driven interchromosomal interactions suggest that the interaction zones may be non-randomly distributed in the nucleus. The observation that many activated genes are looped out from the interior to the periphery of their nuclear territories (see Introduction) and the establishment of these interaction "centers" prior to gene activation suggest a possible spatial relationship with interchromatin granules, commonly known as nuclear speckles, which are enriched with several key transcriptional elongation factors, chromatin remodeling complexes, and essentially all factors required for pre-mRNA splicing (see details in Discussion). To test this hypothesis, we colocalized the FISH probes with the splicing factor SC35, a marker for nuclear speckles (Fu and Maniatis, 1990), one hour after the release of cells from α-amanitin block. In mock-treated primary breast epithelial cells, the positions of *TFF1* and *GREB1* foci were entirely distinct from SC35-positive speckles (**Fig. 7A**). In contrast, upon the E_2 treatment, the two colocalized *TFF1*/*GREB1* foci became intimately associated with two of the SC35-positive speckles in nearly all cell nuclei **(Fig. 7A**). The colocalization was confirmed with a three-dimensional immuno-FISH analysis (**Fig. S9**).

The next issue was to examine whether different ERα gene targets, which were suggested to localize in multiple chromosomal interacting hubs (**Fig. 3C**) would be present in distinct nuclear speckles. We mixed either 6 (or 20) probes from 6 distinct chromosomes (chr. 1, 2, 6, 14, 20, 21) for FISH analysis in conjunction with staining with anti-SC35, finding that all ERα targets genes became colocalized with distinct

nuclear speckles in the presence of E_2, except for the two alleles of a non–regulated intronic region for which a probe was included as a control (**Fig. 7B**). These observations are consistent with the hypotheses that the E_2-induced gene network may trigger the formation of nuclear speckles or the induced ERα target genes may join pre-existing nuclear speckles.

Because the interchromosomal interactions between *TFF1* and *GREB1* depend on actin cables, we further tested the hypothesis by blocking actin oligomerization with latrunculin, which prevented E_2-induced interchromosomal interactions and their association with nuclear speckles (**Fig. 7C**). A similar effect was also observed with Jasplakinolide **(Fig. 7C)**. Furthermore, siRNAs against *DLC1, BAF53, ARP2, nuclear myosin-1* or *g-actin* all similarly blocked the colocalization of the FISH probes with nuclear speckles and destroyed the morphology of nuclear speckles in general (**Fig. 7D, Fig. S10,** and data not shown). In contrast, siRNA against *LSD1*, which prevented E_2-induced gene expression (Garcia-Bassets et al., 2007), but not the specific interchromosomal interactions between *TFF1* and *GREB1* (**Fig. 4F**), had no effect on the speckle morphology (see below). Considered together, the data support the hypothesis that nuclear speckles might be hubs for long-distance intra- and inter-chromosomal interactions for some specific gene sets in the nucleus.

The model that nuclear speckles are hubs for gene network may initially appear to be contradictory to a long-standing view that they are "storage" sites for splicing factors and subsets of transcriptional factors because nascent transcripts were not coincident with such nuclear domains (Fakan and Bernhard, 1971), active transcription and splicing could be detected outside nuclear speckles or in the early G1 phase before the formation of any nuclear speckles (Ferreira et al., 1994; Zhang et al., 1994), and inhibition of transcription for 6 hrs induced further "build-up" of nuclear speckles

(Spector et al., 1991). We found that treatment with α-amanitin or DRB for 45 min, both of which prevent interchromosomal interactions (**Fig. 3D**), actually caused complete loss of the nuclear speckles, with a broad nuclear dispersion of SC35 staining (**Fig. 7E**); however, a prolonged treatment (6hrs) with these transcription inhibitors induced an apparent aggregation of all splicing factors into round structures. Thus, those "built-up" aggregates are distinct from normal nuclear speckles. Taken together, the evidence presented here suggests that nuclear speckles colocalize with the interchromosomal hubs for a subset of active genes, subserving coordinated transcription and splicing, which in this case are signal-dependent.

Finally, because *LSD1* siRNA, which blocked E_2-dependent transcription of the *TFF1* and *GREB1* transcription units, but not their interchromosomal interactions, we investigated whether there might be an effect on coalescence with the nuclear speckles. Intriguingly, depletion of *LSD1* by specific siRNA prevented the *TFF1/GREB1* hub to interact with nuclear speckles (**Fig. 7F** and **7G**), consistent with a role for this histone demethylase in transcriptional and/or co-transcriptional RNA processing events, and suggesting the role of specific co-activator "cargo" in initiating hub:nuclear speckle interactions.

Discussion

Our findings reveal a previously unappreciated role of liganded nuclear receptors in initiating interchromosomal interactions, causing interactive hubs in multiple distinct territories, that we suggest are functionally important for ligand-, or other signal-, dependent enhancement of gene transcription. Receptor-mediated recruitment of coactivators and other molecules, including dynein light chain 1, initiates facilitated movement of chromosomal regions, based on transient, dynamic interactions

with and assembly of nuclear actin/motor machinery. Ligand-induced physical connections between interacting interchromosomal hubs and the interchromatin granules (nuclear speckles), the functional meaning of which in gene expression has been debated in the past two decades, provides evidence of a dynamic nuclear architecture that is required for integration of regulated gene transcriptional and RNA processing programs (**Fig. 7H**). These findings provide a new perspective for the organization of the nucleus in general, and are in accord with the presence of actin in Pol II and coactivator complexes (Bettinger et al., 2004; Percipalle and Visa, 2006).

Hormone-regulated long-distance chromosomal interactions

Taking advantage of the ability of ligand for nuclear receptors to rapidly institute transcriptional activation events, and the identification of target genes for specific nuclear receptors genome-wide (Carroll et al., 2006; Garcia-Bassets et al., 2007), we have now documented a surprising network of ligand-dependent intra- and inter-chromosomal interactions, which is in contrast to linear, pair-wise promoter/enhancer interactions often envisioned for regulated transcriptional activation. While nuclear territories have been suggested to be largely immobile in interphase nuclei, chromosome motility has been observed in yeast, flies and mammalian cells (Gasser, 2002). Our current observations have clearly documented selective, signal-dependent chromosomal movements, which argues for a surprising plasticity and rapid regulation of chromosome location in the mammalian nucleus.

We have established that the observed interchromosomal interactions are ATP-dependent and occur rapidly (2 to 5 min) following the addition of ligand. These events are nucleated by the binding of liganded receptors to their cognate DNA sites, as no effects are observed in the absence of ERα, and events that cause failure in ERα

binding, for example, when *FoxA1* is inactivated (Carroll et al., 2005; Laganiere et al., 2005b), abolish the E_2-induced interchromosomal interactions. However, other cofactors necessary for effective ligand-induced gene activation, including *LSD1* (Garcia-Bassets et al., 2007; Metzger et al., 2005; Shi et al., 2005), were surprisingly not required. Thus, hormonal treatment can cause rapid changes in nuclear architecture prior to gene activation, which may be similar to the events described in the development of the olfactory system (Lomvardas et al., 2006) and T cells (Spilianakis et al., 2005).

Nucleoskeletal requirements for E_2-induced nuclear territory movement

The rapidity of the observed interaction events and the physical distances involved suggests the involvement of an active motor rather than simple Brownian motion. While the existence of a putative motor system in the nucleus has remained controversial for many years, increasing evidence suggests that nuclear actin, and myosin1, are present in the nucleus and hence might be actively involved in gene transcription (Hofmann et al., 2006; Percipalle and Visa, 2006). Indeed, we found that pharmacological inhibition of actin dynamics effectively blocked E_2-induced interchromosomal interactions.

As diagrammed in a proposed model (**Fig.7G**), liganded ERα appears to be connected to the actin motor via its direct interaction with coactivators and the dynein light chain 1 (Rayala et al., 2005), which we would suggest reflects its interactions with the actin-based motor. It is thus tempting to speculate that DLC-1, capable of direct interactions with ERα, serve as a bridge through which the nuclear receptor-bound DNA may be then connected to the putative nuclear actin/myosin motor machinery. This proposed role of nuclear actin is consistent with its proposed role in regulation of

cofactor transport (Vartiainen et al, 2007). Components in the machinery, including g-actin and actin-fold components, such as BAF53 and BAF170 of the Brg1 complex, which is known to interact with ERα (Belandia et al., 2002) may mediate specific interchromosomal interactions in an ATP-dependent manner. Interestingly, while multiple target genes may come into a close contact to achieve a coordinated response to hormone stimulation, they clearly do not "collapse" into a single nuclear domain; rather, there appear to form networks in multiple interactive hubs, suggesting an architectural determinant for regulated gene expression in the nucleus.

Transient interchromosomal interaction hubs in nuclear speckles

While simple organisms, such as yeast, may organize their nucleus to facilitate a series of gene expression events (Blobel, 1985), higher eukaryotic cells seem to have partitioned their nucleus into various subdomains (Belandia et al., 2002; Spector, 1993). Many hubs may become established in the early G1 phase, perhaps helping to define the general nuclear architecture of the nucleus, while others may transiently form in response to specific signals. Strikingly, the dynamic E_2-dependent, ERα-mediated interchromosomal interactions have proven to coincide with a previously described nuclear substructure, called interchromatin granules or nuclear speckles (Fakan and Bernhard, 1971; Misteli et al., 1997). Nuclear speckles have been long considered to be "storage" sites for splicing factors and a subset of the transcriptional machinery (Singer and Green, 1997) because they do not correspond to sites where nascent transcripts are localized (Cmarko et al., 1999) and pre-mRNA splicing could certainly take place outside nuclear speckles (Zhang et al., 1994). However, these nuclear domains are enriched with phosphorylated Pol II (Bregman et al., 1995), several transcriptional elongation factors, such as P-TEFb (Herrmann and Mancini, 2001), key chromatin

remodeling complexes, such as SWI/SNF (Reyes et al., 1997), and essentially all components of the splicing machinery (Lamond and Spector, 2003). Consistent with an active role of nuclear speckles in gene expression, it has been reported earlier that active genes seem more likely to be associated with nuclear speckles than silent genes (Smith et al., 1999).

The data we report in this manuscript suggest that interchomatin granules are dynamic hubs for transient chromosomal interactions in the nucleus for specific, regulated gene transcriptional programs; in a sense fulfilling the criteria of a "nuclear factory". In support of this hypothesis, we find that nuclear speckles rapidly disappear on general inhibition of gene transcription, suggesting that they actually require active interactions with transcription units for their formation/maintenance. Thus, for hormone-induced genes, the detected interchromosomal interactions in interchromatin granules may play an important role in coordinated regulation of gene expression by permitting efficient coupling of transcriptional initiation, elongation, and RNA processing events. While raising many fundamental questions with regard to the nuclear architectural basis for many other coordinated transcriptional events, our data, which have revealed the requirement of an actin-based nuclear motor for hormone-induced interchromosomal interactions, establish a framework towards further understanding of regulated gene expression.

Experimental Procedures

Cell culture

MCF-7 cells were cultured in MEM supplemented with 10% FBS in a 7% CO2 humidified incubator. Primary normal human epithelial cells (HMEC) were from Lonza Bioproducts (CC-2651) and cultured using the media and protocol provided by

the supplier. Prior to induction, cells of 60% confluency were hormone-deprived for 4 days in phenol-free media plus charcoal-depleted FBS, synchronized for 2 hrs by treating cells with 2.5 nM α-amanitin, and then induced with 100 nM 17β-estradiol (E_2) (Sigma) for 60 min.

ChIP-DSL, 3D and 3C assays

Genomic tiling by ChIP-DSL was previously described (Garcia-Bassets et al., 2007; Kwon et al., 2007). Two anti-ERα antibodies (HC-20 and H-184, Santa Cruz Biotechnology) were combined for ChIP analyses.

The 3D assay began with the conventional 3C assay after restriction digestion with Bam H1 and Bgl II using the procedure identical to that previously described for mammalian cells (Vakoc et al., 2005). Ligated and unligated DNA after 3C was sonicated as in standard ChIP experiments. The DNA was annealed to a specific biotinylated capture oligonucleotides corresponding to the *TFF1* enhancer (5'-Bio-GAC, AGA, GAC, GAC, ATG, TGG, TGA, GGT, CAT, CTT, GGC, TGA, GGG) together with the oligonucleotide pool corresponding to the tiled paths. After capture, oligonucleotide ligation, selection, amplification, and hybridization were as previously described in the ChIP-DSL assay (Kwon et al., 2007). Doubled blank intensity was first added to raw data to reduce low intensity bias when computing ratios. The percentile rank for each probe was determined within individual experiments and the median-percentile-rank (MPR) was calculated for each probe across 4 replicates (Buck and Lieb, 2004). The data was then smoothed using a sliding window of 10kb and steps of 500bp, taking the median MPR value of the probes in each window. A window was assigned a value of zero if it had <5 probes above the background to further minimize stochastic signals. Obviously, this method as designed will miss genuine,

highly localized signals in favor of clusters of signals. A p-value was calculated for each window by randomly assigning MPR values from a pool of all probes above background 1000 times and counting the number of times the median value of the randomized window exceeded the experimental value. The negative log p-value was plotted at each window position when the p-value is ≤0.05.

3C validation was carried out with 0.25, 0.5, and 1μl of processed DNA under fixed PCR conditions of 34 cycles for short-range interactions, 36 cycles for long-range interactions, and 30 cycles for BAC controls using a ^{32}P-labeled primer for the *TFF1* enhancer in combination with primers targeting individual genomic loci (see primer sequences, their genomic coordinates, and expected sizes in **Fig. S10**). Four BAC DNA clones covering the genomic regions around the *TFF1* locus were purchased from Invitrogen, amplified, purified, and quantified by qPCR. Equal amount of each BAC DNA was mixed, digested with Bam HI and Bgl II, and ligated in a high concentration (~200ng/μl in a 20μl reaction) to promote inter-molecular ligation. The processed BAC DNA was tested by qPCR to determine the linear range and then used to produce reference PCR signals for each primer pair. The products were resolved in a 10% native polyacrylamide gel, and quantified with a PhosphoImager (Molecular Dynamics).

DNA-ImmunoFISH

The cells were processed for FISH essentially as described (Cai and Kohwi-Shigematsu, 1999) except that specific oligonucleotide probes labeled with specific haptens were used as listed in **Fig. S11**. For triple-labeled FISH, probes to promoter regions were labeled at the 5' position with digoxigenin (DIG) and probes to enhancer regions were labeled with either Biotin (Bio) or Fluorescein (FITC). For double-labeled

FISH, promoters were labeled with Bio and enhancers with FITC. After hybridization, specific probes were detected by using a mix of quantum dot (Qdot)-conjugated antibodies in 1:200 dilution (sheep anti-DIG Fab fragment primary antibody-conjugated with Qdot 655, streptavidin-conjugated with Qdot 605, and goat anti-FITC whole IgG primary antibody-conjugated with Qdot 525, all from Invitrogen).

Single chromosome paint probes were commercially acquired from Applied Spectral Imaging (Vista). Each probe was custom-labeled with different fluorophores: Chr1 (1-585-605), Chr2 (1-585-606) and Chr21 (1-585-649) in aqua, red and green, respectively. Hybridization and detection protocols were performed as recommended by the manufacturer.

Imaging acquisition and processing

2D FISH images were acquired with a Zeiss Axioplan 2e microscope (Carl Zeiss, Inc) and 3D images were obtained with a Nikon TE-200 DeltaVision deconvolution microscope at the UCSD Moores Cancer Center Digital Imaging Facility. The commercial Huygens software package (Scientific Volume Imaging) and the NIH Image J package (http://rsb.info.nih.gov/ij/) were used to deconvolve optical sections, which were then merged to produce 2D or 3D pictures.

For colocalization analysis, individual cells were cropped and a region of interest (ROI) was defined using the software's object analyzer tool and a precise definition of the ROI was obtained for each cell. Co-localization of signals from different channels was determined using the colocalization analyzer tool of Huygens. In each cell, a single value of Pearson's coefficient in the refined ROI was determined after imposing a threshold value for all channels, each of which was calculated using the automatic thresholding function of the Imaris algorithm in the Huygens package.

Nonspecific co-localization was identified from apparently co-localized 2D images by determining Pearson's coefficients of deconvolved stacks after subtracting background signal outside the ROI. Statistical comparison of Pearson's coefficients obtained with individual cells from multiple independent experiments was performed with a one-tailed two-sample t-test using the software SSPS 14.0 for Windows. Multiple data set comparison was carried out by ANOVA using nonparametric methods, which provides box plots with data in quartiles, and error bars at the 5^{th} and 95^{th} percentile and outliers plotted.

Single-cell microinjection

Single-cell antibody microinjection experiments were performed as described (Perissi et al., 2004). The antibodies used are listed in **Fig. S11**. These siRNAs were purchased from Qiagen (Valencia, CA), each of which was custom-designed and validated.

Quantification and depletion of cellular ATP

ATP depletion and quantification were performed in mock-treated and hormone-induced cells (10^3-10^4 cells per assay) using the ApoSensor ATP depletion Assay kit (Axxora). A calibration curve was generated with 5, 10, 25, 50, 100, 250, and 500 nM of ATP, which was used to calculate the ATP concentration in experimental cells. Rotenone (Sigma) was used at a final concentration of 10μM to enhancer ATP depletion as previously reported (White et al., 2002).

Pharmacological treatment of cells

Transcription was inhibited by treating cells for 1 or 6hrs with 100 nM α-amanitin (Sigma) to block transcription initiation, or with DRB (Sigma) to interfere with transcriptional elongation. Actin depolymerization was induced with latrunculin

A (LA), which is known to specifically cap actin monomers, whereas actin stabilization was stimulated by jaspaklinolide (JP), which binds F-actin and prevents depolymerization. These drugs (gift of J. Durán and V. Malhotra) were suspended in DMSO as a 1000X stock and applied to cultured cells at the final concentration of 1μM as described (Bubb and Spector, 1998). Nuclear actin was detected by using a monoclonal antibody (2G2) (Progen).

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References

Bailey, T.L., and Gribskov, M. (1998). Methods and statistics for combining motif match scores. J Comput Biol *5*, 211-221.

Bardella, C., Costa, B., Maggiora, P., Patane, S., Olivero, M., Ranzani, G.N., De Bortoli, M., Comoglio, P.M., and Di Renzo, M.F. (2004). Truncated RON tyrosine kinase drives tumor cell progression and abrogates cell-cell adhesion through E-cadherin transcriptional repression. Cancer research *64*, 5154-5161.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., and Nilsson, S. (1998). Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Molecular pharmacology *54*, 105-112.

Belandia, B., Orford, R.L., Hurst, H.C., and Parker, M.G. (2002). Targeting of SWI/SNF chromatin remodelling complexes to estrogen-responsive genes. Embo J *21*, 4094-4103.

Berg, O.G., and von Hippel, P.H. (1987). Selection of DNA binding sites by regulatory proteins. Statistical-mechanical theory and application to operators and promoters. Journal of molecular biology *193*, 723-750.

Berx, G., and Van Roy, F. (2001). The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res *3*, 289-293.

Bettinger, B.T., Gilbert, D.M., and Amberg, D.C. (2004). Actin up in the nucleus. Nat Rev Mol Cell Biol *5*, 410-415.

Blobel, G. (1985). Gene gating: a hypothesis. Proc Natl Acad Sci U S A *82*, 8527-8529.

Bourdeau, V., Deschenes, J., Metivier, R., Nagai, Y., Nguyen, D., Bretschneider, N., Gannon, F., White, J.H., and Mader, S. (2004). Genome-wide identification of high-affinity estrogen response elements in human and mouse. Molecular endocrinology (Baltimore, Md *18*, 1411-1427.

Bregman, D.B., Du, L., van der Zee, S., and Warren, S.L. (1995). Transcription-dependent redistribution of the large subunit of RNA polymerase II to discrete nuclear domains. J Cell Biol *129*, 287-298.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Buck, M.J., and Lieb, J.D. (2004). ChIP-chip: considerations for the design, analysis, and application of genome-wide chromatin immunoprecipitation experiments. Genomics *83*, 349-360.

Bussemaker, H.J., Foat, B.C., and Ward, L.D. (2007). Predictive modeling of genome-wide mRNA expression: from modules to molecules. Annual review of biophysics and biomolecular structure *36*, 329-347.

Cai, S., and Kohwi-Shigematsu, T. (1999). Intranuclear relocalization of matrix binding sites during T cell activation detected by amplified fluorescence in situ hybridization. Methods *19*, 394-402.

Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoute, J., Shao, W., Hestermann, E.V., Geistlinger, T.R*., et al.* (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell *122*, 33-43.

Carroll, J.S., Meyer, C.A., Song, J., Li, W., Geistlinger, T.R., Eeckhoute, J., Brodsky, A.S., Keeton, E.K., Fertuck, K.C., Hall, G.F*., et al.* (2006). Genome-wide analysis of estrogen receptor binding sites. Nat Genet *38*, 1289-1297.

Caselle, M., Di Cunto, F., and Provero, P. (2002). Correlating overrepresented upstream motifs to gene expression: a computational approach to regulatory element discovery in eukaryotes. BMC bioinformatics *3*, 7.
Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J.*, et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell *116*, 499-509.
Chambeyron, S., and Bickmore, W.A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. Genes Dev *18*, 1119-1130.
Chen, J.D., and Evans, R.M. (1995). A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature *377*, 454-457.
Cicatiello, L., Scafoglio, C., Altucci, L., Cancemi, M., Natoli, G., Facchiano, A., Iazzetti, G., Calogero, R., Biglia, N., De Bortoli, M.*, et al.* (2004). A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. Journal of molecular endocrinology *32*, 719-775.
Cmarko, D., Verschure, P.J., Martin, T.E., Dahmus, M.E., Krause, S., Fu, X.D., van Driel, R., and Fakan, S. (1999). Ultrastructural analysis of transcription and splicing in the cell nucleus after bromo-UTP microinjection. Mol Biol Cell *10*, 211-223.
Cook, P. (1998). Duplicating a tangled genome. Science (New York, NY *281*, 1466-1467.
Cora, D., Di Cunto, F., Caselle, M., and Provero, P. (2007). Identification of candidate regulatory sequences in mammalian 3' UTRs by statistical analysis of oligonucleotide distributions. BMC bioinformatics *8*, 174.
Cora, D., Herrmann, C., Dieterich, C., Di Cunto, F., Provero, P., and Caselle, M. (2005). Ab initio identification of putative human transcription factor binding sites by comparative genomics. BMC bioinformatics *6*, 110.
Couse, J.F., and Korach, K.S. (1999). Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine reviews *20*, 358-417.
Cowley, S.M., and Parker, M.G. (1999). A comparison of transcriptional activation by ER alpha and ER beta. The Journal of steroid biochemistry and molecular biology *69*, 165-175.
Creighton, C.J., Hilger, A.M., Murthy, S., Rae, J.M., Chinnaiyan, A.M., and El-Ashry, D. (2006). Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors. Cancer research *66*, 3903-3911.
Croft, J.A., Bridger, J.M., Boyle, S., Perry, P., Teague, P., and Bickmore, W.A. (1999). Differences in the localization and morphology of chromosomes in the human nucleus. J Cell Biol *145*, 1119-1131.
Cunha, G.R., Young, P., Hom, Y.K., Cooke, P.S., Taylor, J.A., and Lubahn, D.B. (1997). Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. Journal of mammary gland biology and neoplasia *2*, 393-402.
de Lanerolle, P., Johnson, T., and Hofmann, W.A. (2005). Actin and myosin I in the nucleus: what next? Nat Struct Mol Biol *12*, 742-746.
Dekker, J. (2006). The three 'C' s of chromosome conformation capture: controls, controls, controls. Nat Methods *3*, 17-21.
Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. Science (New York, NY *295*, 1306-1311.

Dennis, A.P., and O'Malley, B.W. (2005). Rush hour at the promoter: how the ubiquitin-proteasome pathway polices the traffic flow of nuclear receptor-dependent transcription. The Journal of steroid biochemistry and molecular biology *93*, 139-151.
Edelmann, P., Bornfleth, H., Zink, D., Cremer, T., and Cremer, C. (2001). Morphology and dynamics of chromosome territories in living cells. Biochim Biophys Acta *1551*, M29-39.
El-Tanani, M.K., and Green, C.D. (1997). Two separate mechanisms for ligand-independent activation of the estrogen receptor. Molecular endocrinology (Baltimore, Md *11*, 928-937.
Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. The Journal of clinical endocrinology and metabolism *82*, 4258-4265.
Ensminger, A.W., and Chess, A. (2004). Coordinated replication timing of monoallelically expressed genes along human autosomes. Hum Mol Genet *13*, 651-658.
Fakan, S., and Bernhard, W. (1971). Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. Exp Cell Res *67*, 129-141.
Foster, H.A., and Bridger, J.M. (2005). The genome and the nucleus: a marriage made by evolution. Genome organisation and nuclear architecture. Chromosoma *114*, 212-229.
Frasor, J., Danes, J.M., Komm, B., Chang, K.C., Lyttle, C.R., and Katzenellenbogen, B.S. (2003). Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology *144*, 4562-4574.
Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nature reviews *3*, 362-374.
Fu, X.D., and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature *343*, 437-441.
Fujita, N., Jaye, D.L., Kajita, M., Geigerman, C., Moreno, C.S., and Wade, P.A. (2003). MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell *113*, 207-219.
Galien, R., and Garcia, T. (1997). Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. Nucleic acids research *25*, 2424-2429.
Garcia-Bassets, I., Kwon, Y.S., Telese, F., Prefontaine, G.G., Hutt, K.R., Cheng, C.S., Ju, B.G., Ohgi, K.A., Wang, J., Escoubet-Lozach, L.*, et al.* (2007). Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell *128*, 505-518.
Gasser, S.M. (2002). Visualizing chromatin dynamics in interphase nuclei. Science *296*, 1412-1416.
Ghosh, M.G., Thompson, D.A., and Weigel, R.J. (2000). PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res *60*, 6367-6375.
Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes Dev *14*, 121-141.
Gonsior, S.M., Platz, S., Buchmeier, S., Scheer, U., Jockusch, B.M., and Hinssen, H. (1999). Conformational difference between nuclear and cytoplasmic actin as detected by a monoclonal antibody. Journal of cell science *112 (Pt 6)*, 797-809.

Gruenbaum, Y., Margalit, A., Goldman, R.D., Shumaker, D.K., and Wilson, K.L. (2005). The nuclear lamina comes of age. Nat Rev Mol Cell Biol *6*, 21-31.
GuhaThakurta, D. (2006). Computational identification of transcriptional regulatory elements in DNA sequence. Nucleic acids research *34*, 3585-3598.
Gunawardena, S., and Rykowski, M.C. (2000). Direct evidence for interphase chromosome movement during the mid-blastula transition in Drosophila. Curr Biol *10*, 285-288.
Handwerger, K.E., and Gall, J.G. (2006). Subnuclear organelles: new insights into form and function. Trends Cell Biol *16*, 19-26.
Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. (2003). Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Molecular and cellular endocrinology *206*, 13-22.
Hernandez-Verdun, D. (2006). The nucleolus: a model for the organization of nuclear functions. Histochem Cell Biol *126*, 135-148.
Herrmann, C.H., and Mancini, M.A. (2001). The Cdk9 and cyclin T subunits of TAK/P-TEFb localize to splicing factor-rich nuclear speckle regions. J Cell Sci *114*, 1491-1503.
Hertz, G.Z., and Stormo, G.D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. Bioinformatics (Oxford, England) *15*, 563-577.
Hewitt, S.L., High, F.A., Reiner, S.L., Fisher, A.G., and Merkenschlager, M. (2004). Nuclear repositioning marks the selective exclusion of lineage-inappropriate transcription factor loci during T helper cell differentiation. Eur J Immunol *34*, 3604-3613.
Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annual review of biochemistry *70*, 649-676.
Hoch, R.V., Thompson, D.A., Baker, R.J., and Weigel, R.J. (1999). GATA-3 is expressed in association with estrogen receptor in breast cancer. International journal of cancer *84*, 122-128.
Hofmann, W.A., Johnson, T., Klapczynski, M., Fan, J.L., and de Lanerolle, P. (2006). From transcription to transport: emerging roles for nuclear myosin I. Biochem Cell Biol *84*, 418-426.
Holzinger, A. (2001). Jasplakinolide. An actin-specific reagent that promotes actin polymerization. Methods Mol Biol *161*, 109-120.
Hu, J., Li, B., and Kihara, D. (2005). Limitations and potentials of current motif discovery algorithms. Nucleic acids research *33*, 4899-4913.
Huber, B.R., and Bulyk, M.L. (2006). Meta-analysis discovery of tissue-specific DNA sequence motifs from mammalian gene expression data. BMC bioinformatics *7*, 229.
Huber, M.A., Kraut, N., and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current opinion in cell biology *17*, 548-558.
Inadera, H., Dong, H.Y., and Matsushima, K. (2002). WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochemical and biophysical research communications *294*, 602-608.
Jepsen, K., and Rosenfeld, M.G. (2002). Biological roles and mechanistic actions of co-repressor complexes. Journal of cell science *115*, 689-698.

Kato, S. (2001). Estrogen receptor-mediated cross-talk with growth factor signaling pathways. Breast cancer (Tokyo, Japan) *8*, 3-9.
Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E.S. (2003). Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature *423*, 241-254.
Klein-Hitpass, L., Schorpp, M., Wagner, U., and Ryffel, G.U. (1986). An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. Cell *46*, 1053-1061.
Klinge, C.M., Bodenner, D.L., Desai, D., Niles, R.M., and Traish, A.M. (1997). Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements in vitro. Nucleic acids research *25*, 1903-1912.
Kosak, S.T., Skok, J.A., Medina, K.L., Riblet, R., Le Beau, M.M., Fisher, A.G., and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. Science *296*, 158-162.
Koszewski, N.J., and Notides, A.C. (1991). Phosphate-sensitive binding of the estrogen receptor to its response elements. Molecular endocrinology (Baltimore, Md *5*, 1129-1136.
Kreiman, G. (2004). Identification of sparsely distributed clusters of cis-regulatory elements in sets of co-expressed genes. Nucleic acids research *32*, 2889-2900.
Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences of the United States of America *93*, 5925-5930.
Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., and Gustafsson, J.A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology *139*, 4252-4263.
Kwon, Y.S., Garcia-Bassets, I., Hutt, K.R., Cheng, C.S., Jin, M., Liu, D., Benner, C., Wang, D., Ye, Z., Bibikova, M.*, et al.* (2007). Sensitive ChIP-DSL technology reveals an extensive estrogen receptor alpha-binding program on human gene promoters. Proc Natl Acad Sci U S A *104*, 4852-4857.
Laganiere, J., Deblois, G., and Giguere, V. (2005a). Functional genomics identifies a mechanism for estrogen activation of the retinoic acid receptor alpha1 gene in breast cancer cells. Molecular endocrinology (Baltimore, Md *19*, 1584-1592.
Laganiere, J., Deblois, G., Lefebvre, C., Bataille, A.R., Robert, F., and Giguere, V. (2005b). From the Cover: Location analysis of estrogen receptor alpha target promoters reveals that FOXA1 defines a domain of the estrogen response. Proc Natl Acad Sci U S A *102*, 11651-11656.
Lamond, A.I., and Spector, D.L. (2003). Nuclear speckles: a model for nuclear organelles. Nat Rev Mol Cell Biol *4*, 605-612.
Lin, C.Y., Vega, V.B., Thomsen, J.S., Zhang, T., Kong, S.L., Xie, M., Chiu, K.P., Lipovich, L., Barnett, D.H., Stossi, F.*, et al.* (2007). Whole-Genome Cartography of Estrogen Receptor alpha Binding Sites. PLoS Genet *3*, e87.
Ling, J.Q., Li, T., Hu, J.F., Vu, T.H., Chen, H.L., Qiu, X.W., Cherry, A.M., and Hoffman, A.R. (2006). CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. Science (New York, NY *312*, 269-272.
Liu, Y.N., Lee, W.W., Wang, C.Y., Chao, T.H., Chen, Y., and Chen, J.H. (2005). Regulatory mechanisms controlling human E-cadherin gene expression. Oncogene *24*, 8277-8290.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif *25*, 402-408.
Lomvardas, S., Barnea, G., Pisapia, D.J., Mendelsohn, M., Kirkland, J., and Axel, R. (2006). Interchromosomal interactions and olfactory receptor choice. Cell *126*, 403-413.
Loots, G.G., Locksley, R.M., Blankespoor, C.M., Wang, Z.E., Miller, W., Rubin, E.M., and Frazer, K.A. (2000). Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. Science (New York, NY *288*, 136-140.
Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P*., et al.* (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.
Martinez, E., and Wahli, W. (1989). Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. The EMBO journal *8*, 3781-3791.
Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A.E., Kel-Margoulis, O.V*., et al.* (2003). TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic acids research *31*, 374-378.
McDonnell, D.P., Clemm, D.L., Hermann, T., Goldman, M.E., and Pike, J.W. (1995). Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Molecular endocrinology (Baltimore, Md *9*, 659-669.
McGrath, J.L. (2005). Dynein motility: four heads are better than two. Curr Biol *15*, R970-972.
McInerney, E.M., Weis, K.E., Sun, J., Mosselman, S., and Katzenellenbogen, B.S. (1998). Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology *139*, 4513-4522.
McKenna, N.J., and O'Malley, B.W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465-474.
Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R., and Schule, R. (2005). LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. Nature *437*, 436-439.
Misteli, T., Caceres, J.F., and Spector, D.L. (1997). The dynamics of a pre-mRNA splicing factor in living cells. Nature *387*, 523-527.
Murrell, A., Heeson, S., and Reik, W. (2004). Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nature genetics *36*, 889-893.
Nass, S.J., Herman, J.G., Gabrielson, E., Iversen, P.W., Parl, F.F., Davidson, N.E., and Graff, J.R. (2000). Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer research *60*, 4346-4348.
Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. (2001). Mechanisms of estrogen action. Physiological reviews *81*, 1535-1565.
Nishidate, T., Katagiri, T., Lin, M.L., Mano, Y., Miki, Y., Kasumi, F., Yoshimoto, M., Tsunoda, T., Hirata, K., and Nakamura, Y. (2004). Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. International journal of oncology *25*, 797-819.

Oesterreich, S., Deng, W., Jiang, S., Cui, X., Ivanova, M., Schiff, R., Kang, K., Hadsell, D.L., Behrens, J., and Lee, A.V. (2003). Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. Cancer research *63*, 5203-5208.
Olave, I.A., Reck-Peterson, S.L., and Crabtree, G.R. (2002). Nuclear actin and actin-related proteins in chromatin remodeling. Annual review of biochemistry *71*, 755-781.
Osborne, C.K. (1998). Steroid hormone receptors in breast cancer management. Breast cancer research and treatment *51*, 227-238.
Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J., Kushner, P.J., and Scanlan, T.S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science (New York, NY *277*, 1508-1510.
Paixao, T., Carvalho, T.P., Calado, D.P., and Carneiro, J. (2007). Quantitative insights into stochastic monoallelic expression of cytokine genes. Immunology and cell biology *85*, 315-322.
Pavesi, G., Mauri, G., and Pesole, G. (2001). An algorithm for finding signals of unknown length in DNA sequences. Bioinformatics (Oxford, England) *17 Suppl 1*, S207-214.
Peinado, H., Ballestar, E., Esteller, M., and Cano, A. (2004). Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. Molecular and cellular biology *24*, 306-319.
Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nature reviews *7*, 415-428.
Percipalle, P., and Farrants, A.K. (2006). Chromatin remodelling and transcription: be-WICHed by nuclear myosin 1. Current opinion in cell biology *18*, 267-274.
Percipalle, P., and Visa, N. (2006). Molecular functions of nuclear actin in transcription. J Cell Biol *172*, 967-971.
Perez-Moreno, M.A., Locascio, A., Rodrigo, I., Dhondt, G., Portillo, F., Nieto, M.A., and Cano, A. (2001). A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. The Journal of biological chemistry *276*, 27424-27431.
Perissi, V., Aggarwal, A., Glass, C.K., Rose, D.W., and Rosenfeld, M.G. (2004). A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell *116*, 511-526.
Perissi, V., and Rosenfeld, M.G. (2005). Controlling nuclear receptors: the circular logic of cofactor cycles. Nat Rev Mol Cell Biol *6*, 542-554.
Planas-Silva, M.D., and Waltz, P.K. (2007). Estrogen promotes reversible epithelial-to-mesenchymal-like transition and collective motility in MCF-7 breast cancer cells. The Journal of steroid biochemistry and molecular biology *104*, 11-21.
Porter, W., Saville, B., Hoivik, D., and Safe, S. (1997). Functional synergy between the transcription factor Sp1 and the estrogen receptor. Molecular endocrinology (Baltimore, Md *11*, 1569-1580.
Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic acids research *23*, 4878-4884.
Rayala, S.K., den Hollander, P., Balasenthil, S., Yang, Z., Broaddus, R.R., and Kumar, R. (2005). Functional regulation of oestrogen receptor pathway by the dynein light chain 1. EMBO reports *6*, 538-544.
Razandi, M., Pedram, A., and Levin, E.R. (2000). Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Molecular endocrinology (Baltimore, Md *14*, 1434-1447.

Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., and Prossnitz, E.R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science (New York, NY *307*, 1625-1630.
Reyes, J.C., Muchardt, C., and Yaniv, M. (1997). Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. J Cell Biol *137*, 263-274.
Rizk, R.S., and Walczak, C.E. (2005). Chromosome dynamics: actin's gone fishing. Curr Biol *15*, R841-842.
Rochefort, H., Platet, N., Hayashido, Y., Derocq, D., Lucas, A., Cunat, S., and Garcia, M. (1998). Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview. The Journal of steroid biochemistry and molecular biology *65*, 163-168.
Rosenfeld, M.G., and Glass, C.K. (2001). Coregulator codes of transcriptional regulation by nuclear receptors. The Journal of biological chemistry *276*, 36865-36868.
Rosenfeld, M.G., Lunyak, V.V., and Glass, C.K. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes & development *20*, 1405-1428.
Russo, J., Ao, X., Grill, C., and Russo, I.H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast cancer research and treatment *53*, 217-227.
Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W.W., and Lenhard, B. (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic acids research *32*, D91-94.
Sandve, G.K., and Drablos, F. (2006). A survey of motif discovery methods in an integrated framework. Biology direct *1*, 11.
Scafoglio, C., Ambrosino, C., Cicatiello, L., Altucci, L., Ardovino, M., Bontempo, P., Medici, N., Molinari, A.M., Nebbioso, A., Facchiano, A.*, et al.* (2006). Comparative gene expression profiling reveals partially overlapping but distinct genomic actions of different antiestrogens in human breast cancer cells. Journal of cellular biochemistry *98*, 1163-1184.
Schneider, T.D., and Stephens, R.M. (1990). Sequence logos: a new way to display consensus sequences. Nucleic acids research *18*, 6097-6100.
Schneider, T.D., Stormo, G.D., Gold, L., and Ehrenfeucht, A. (1986). Information content of binding sites on nucleotide sequences. Journal of molecular biology *188*, 415-431.
Schwabe, J.W., Chapman, L., Finch, J.T., and Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell *75*, 567-578.
Shang, Y., Hu, X., DiRenzo, J., Lazar, M.A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell *103*, 843-852.
Shi, Y.J., Matson, C., Lan, F., Iwase, S., Baba, T., and Shi, Y. (2005). Regulation of LSD1 histone demethylase activity by its associated factors. Mol Cell *19*, 857-864.
Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927-937.
Siitonen, S.M., Kononen, J.T., Helin, H.J., Rantala, I.S., Holli, K.A., and Isola, J.J. (1996). Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. American journal of clinical pathology *105*, 394-402.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature *407*, 538-541.
Singer, R.H., and Green, M.R. (1997). Compartmentalization of eukaryotic gene expression: causes and effects. Cell *91*, 291-294.
Sinha, S., and Tompa, M. (2003). YMF: A program for discovery of novel transcription factor binding sites by statistical overrepresentation. Nucleic acids research *31*, 3586-3588.
Smith, C.L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biology of reproduction *58*, 627-632.
Smith, K.P., Moen, P.T., Wydner, K.L., Coleman, J.R., and Lawrence, J.B. (1999). Processing of endogenous pre-mRNAs in association with SC-35 domains is gene specific. J Cell Biol *144*, 617-629.
Soulez, M., and Parker, M.G. (2001). Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. Journal of molecular endocrinology *27*, 259-274.
Spector, D.L. (1993). Macromolecular domains within the cell nucleus. Annu Rev Cell Biol *9*, 265-315.
Spector, D.L., Fu, X.D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. Embo J *10*, 3467-3481.
Spiegelman, B.M., and Heinrich, R. (2004). Biological control through regulated transcriptional coactivators. Cell *119*, 157-167.
Spilianakis, C.G., Lalioti, M.D., Town, T., Lee, G.R., and Flavell, R.A. (2005). Interchromosomal associations between alternatively expressed loci. Nature *435*, 637-645.
Stormo, G.D. (2000). DNA binding sites: representation and discovery. Bioinformatics (Oxford, England) *16*, 16-23.
Stormo, G.D., and Hartzell, G.W., 3rd (1989). Identifying protein-binding sites from unaligned DNA fragments. Proceedings of the National Academy of Sciences of the United States of America *86*, 1183-1187.
Sun, G., Porter, W., and Safe, S. (1998). Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex. Molecular endocrinology (Baltimore, Md *12*, 882-890.
Thiery, J.P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nature reviews *2*, 442-454.
Tompa, M., Li, N., Bailey, T.L., Church, G.M., De Moor, B., Eskin, E., Favorov, A.V., Frith, M.C., Fu, Y., Kent, W.J.*, et al.* (2005). Assessing computational tools for the discovery of transcription factor binding sites. Nature biotechnology *23*, 137-144.
Vakoc, C.R., Letting, D.L., Gheldof, N., Sawado, T., Bender, M.A., Groudine, M., Weiss, M.J., Dekker, J., and Blobel, G.A. (2005). Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. Mol Cell *17*, 453-462.
Varshochi, R., Halim, F., Sunters, A., Alao, J.P., Madureira, P.A., Hart, S.M., Ali, S., Vigushin, D.M., Coombes, R.C., and Lam, E.W. (2005). ICI182,780 induces p21Waf1 gene transcription through releasing histone deacetylase 1 and estrogen receptor alpha from Sp1 sites to induce cell cycle arrest in MCF-7 breast cancer cell line. The Journal of biological chemistry *280*, 3185-3196.
Vega, V.B., Lin, C.Y., Lai, K.S., Kong, S.L., Xie, M., Su, X., Teh, H.F., Thomsen, J.S., Yeo, A.L., Sung, W.K.*, et al.* (2006). Multiplatform genome-wide identification and modeling of functional human estrogen receptor binding sites. Genome biology *7*, R82.

Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E.*, et al.* (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Molecular endocrinology (Baltimore, Md *12*, 1605-1618.
Wiesen, J.F., Young, P., Werb, Z., and Cunha, G.R. (1999). Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. Development (Cambridge, England) *126*, 335-344.
Williams, R.R., Azuara, V., Perry, P., Sauer, S., Dvorkina, M., Jorgensen, H., Roix, J., McQueen, P., Misteli, T., Merkenschlager, M.*, et al.* (2006). Neural induction promotes large-scale chromatin reorganisation of the Mash1 locus. J Cell Sci *119*, 132-140.
Zeps, N., Bentel, J.M., Papadimitriou, J.M., D'Antuono, M.F., and Dawkins, H.J. (1998). Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. Differentiation; research in biological diversity *62*, 221-226.
Zhang, G., Taneja, K.L., Singer, R.H., and Green, M.R. (1994). Localization of pre-mRNA splicing in mammalian nuclei. Nature *372*, 809-812.
Zhao, Z., Tavoosidana, G., Sjolinder, M., Gondor, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Sandhu, K.S., Singh, U.*, et al.* (2006a). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nature genetics *38*, 1341-1347.
Zhao, Z., Tavoosidana, G., Sjolinder, M., Gondor, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Singh Sandhu, K., Singh, U.*, et al.* (2006b). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nat Genet *38*, 1341-1347.
Zink, D., Amaral, M.D., Englmann, A., Lang, S., Clarke, L.A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N.*, et al.* (2004). Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. J Cell Biol *166*, 815-825.
Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M.F., Ansorge, W., and Stelzer, E.H. (1998). Structure and dynamics of human interphase chromosome territories in vivo. Hum Genet *102*, 241-251.
Zou, A., Marschke, K.B., Arnold, K.E., Berger, E.M., Fitzgerald, P., Mais, D.E., and Allegretto, E.A. (1999). Estrogen receptor beta activates the human retinoic acid receptor alpha-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen. Molecular endocrinology (Baltimore, Md *13*, 418-430.

Figure Legends

Figure 1. Identification of long-range, estrogen induced chromosomal interactions by DSL (3D) and FISH analysis. (**A**) Diagram of the 3D technology. The initial steps are identical to the established 3C technology. A key extension is DNA capturing by using a specific biotinylated oligonucleotide followed by DNA selection and ligation to detect co-captured DNA fragments in a high-throughput and unbiased fashion. Specific signals were identified based on relative enrichment of DNA fragments linked by ligase in comparison to those from the parallel minus ligase control under an extensive dilution condition. **B**) Plot of 3D signals. The 1.5Mbp tiled region in chromosome 21 showing representative known genes with E_2-induced or repressed genes boxed in red or green, respectively. The ERα-binding profile in the tiled interval is illustrated on the top; the *TFF1* enhancer-captured signals are shown below the ERα-binding profile. Four independent captured experiments were performed with E_2-stimulated MCF-7 cells and signals above p-value of 0.05 are shown in the titled interval (see Experimental Procedures). Individual primers used for validation by 3C and the restriction fragments targeted by each primer (red bar) are illustrated at the bottom. Uppercase letters denote different loci within the 1.5 Mbp region, while lowercase letters indicate probes surrounding and including the *TFF1* gene proper. (**C**) Validation of the 3D signals by individual 3C assays using primers against individual loci in combination with the primer specific for the *TFF1* enhancer. All primers are designed to point to the same direction as that the *TFF1* primer does except two cases where the opposite orientation was used to avoid potential interference of PCR by repeat sequences. The right most 3D signal was not validated because the signal is surrounded by multiple repeats. 3C assays were performed on both mock-treated (-E_2) and E_2-stimulated (+E_2) MCF-7 cells. E_2-inducible 3C signals are boxed.

Randomly ligated DNA from a pool of four BACs corresponding to the tiled region was used to normalize PCR efficiency of individual primer pairs. Note that the BAC control for probe A and B were missing because the region is not present in the four BACs. **D.** Confirmation by FISH of data obtained by 3D, the first panel shows a positive E_2-induced interaction between *TMPRSS3* (J) and the *TFF1* enhancer; while the second panel shows a negative control (*K* vs. *TFF1*) performed with an intergenic region (*PDE9A* intron) and the *TFF1* promoter. Chr. 21 paint was used in left panel. Both graphs under the FISH data portray the percent of nuclei in which at least one allele colocalized in response to $E_{2..}$ ** denotes statistical significance of <0.001 (see Methods).

Figure 2. Survey of interchromosomal interactions predicted by 3D. **A.** Chromosomal location of predicted interactions depicting AcH3K9, an activation mark (red), as well as signal enrichment in 3D assay at the *GREB1* locus (chr. 2), including its predicted enhancers. **B & C.** Two negative controls from the 3D assay showing insignificant levels of AcH3K9 and no signal enrichment. The two controls are *CASP7* and *DIO1,* located in chromosomes 10 and 1, respectively. **D.** Confirmation of the interchromosomal interaction between *TFF1* promoter (chr. 21) and *GREB1* enhancers 1 and 2 (chr. 2). Upper left panel shows chromosomal locations of the loci; while lower left panel shows FISH data of the E_2-dependent interaction including kissing of the *TFF1* and *GREB1* chromosome territories in both alleles. The left panel shows an example of three-dimensional analysis of FISH, with deconvolution of the Z-stack, while the right panel shows examples of two dimensional FISH data in which chr. 21 and chr. 2 paints were used, with similar results. **E.** Percent of nuclei that colocalized upon treatment, among control (-E_2) and E_2-treated cells (45') in which one

allele (mono) or both alleles (bi) interacted. Together, 63% of nuclei exhibited E_2-induced *TFF1/GREB1* interchromosomal interactions. ** denotes statistical significance of <0.001(see Methods) as provided by the comparison of Pearson's coefficients. **F.** An example of DHT/androgen receptor (AR)-induced interchromosomal interactions in LNCaP prostate cancer cells between *KLK2* (chr. 19) and *TMPRSS2* (chr. 21) supporting the proposal that regulated interchromosomal interactions is a general strategy of the cell in response to ligands. **G.** ANOVA analysis of loci distances measured in both treated and untreated samples, again independently analyzing cells in which one (mono) or both (bi) alleles interacted. Together, 60% of the cells exhibited DHT-induced (*KLK2/TMPRSS2).* The asterisks indicate a population statistically different from the control samples (**P<0.001).

Figure 3. Kinetics of nuclear receptor-dependent movement of chromosome territories (CTs) **A.** ERα-mediated interchromosomal interaction between *TFF1* and *GREB1* in normal human mammary epithelial cells (HMEC). This panel includes a separate ANOVA analysis of loci distances measured in both treated and untreated samples, showing mono-allelic (~54%) and biallelic (~46%) E_2-dependent interactions. **B.** Bar graphs of the percent of nuclei that colocalized (both mono- and bi-allelic interactions) upon treatment per sampled time interval (0, 2, 5, 60 min) following addition of E_2. **C.** Convergence of *TFF1/GREB1* loci in HMECs at 60' paired promoter/enhancers in response to E_2. **D.** Convergence of ERα target genes using sites from 6 distinct loci from different chromosomes upon E_2 treatment, and including many chr.21 E_2-bound, 3D-positive regions; as shown in Fig. 1. The two separate green signals that did not colocalize upon E_2 treatment correspond to the intronic sequence of *PDE9A*, which served as negative control. **E.** ANOVA analysis of loci distances

measured in both treated and untreated samples. The absence of an asterisk indicates a population not statistically different from the control and treated samples. **F.** FISH analysis demonstrating dependency of the interaction on ERα and *FoxA1*. The data is accompanied by the corresponding ANOVA analysis. In all panels, ** denotes statistical significance of P<0.001.

Figure 4. Nuclear receptor coactivators are required for the interaction even prior to gene activation. **A.** Effect of nuclear microinjection of siRNA and antibody depletion of CBP/p300 in treated and untreated HMEC samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances all in which both alleles interacted. **B.** Effect of nuclear microinjection of *SRC1/pCIP* siRNA and single cell nuclear antibody microinjection of αSRC1/αpCIP IgG in treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances in control and E_2-treated (45') HMECs. **C.** Effect of nuclear microinjection of control or *PBP/P220* siRNA or single cell nuclear microinjection of control or αPBP/αP220 IgG in treated and untreated samples in control and E_2-treated (45') HMECs as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **D.** Effect of nuclear microinjection of control or *LSD1* siRNA and single cell nuclear antibody microinjection of control or αLSD1 IgG in treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. All panels show analysis of cells +/- E_2 in which both alleles exhibit chr. 21:chr. 2 interactions. ** denotes statistical significance of P<0.001.

Figure 5. An energy-dependent nucleoskeletal machinery is required for long-distance chromosomal interactions. **A.** ATP depletion (rotenone, Rot) abrogates the ability to form interchromosomal interactions; confirmed by ANOVA analysis of measured distances **B.** Sample of cells stained with the nuclear actin antibody 2G2. Each paired panel show higher and lower magnifications to appreciate the prominent nuclear staining in the absence of presence of E_2. **C.** Chemical disruption and stabilization of actin of E_2-induced (60') interactions by treatment with latrunculin (LA) and jaspaklinolide (JP), respectively with both drug treatments preventing E_2-induced interchromosomal interactions. **D.** Effect of single cells nuclear microinjection of control or *ARP2/3* siRNA and single cell nuclear antibody microinjection of control or αARP2/3 IgG in E_2-treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. All panels show analysis of cells in which both alleles exhibited interactions. **E.** Effect of nuclear microinjection of control or *Myosin1* siRNA or control or α nuclear Myosin 1 IgG in E_2-treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. ** denotes statistical significance of $P<0.001$.

Figure 6. Involvement of motor and actin binding proteins. **A.** Effect of single nuclear microinjection of control vs. dynein light chain I (DLC1) siRNA and control vs. αDLC1 IgG in E_2-treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **B.** Effect of single cell nuclear microinjection of control or *BAF53* siRNA, or control or αBAF53 IgG in E_2-treated and untreated HMECs (45') as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **C.** Effect of single cell nuclear

microinjection of control or *BAF57* siRNA and control or *αBAF57*/SMARCE1 IgG in E_2-treated and untreated samples as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **D.** Effect of single cell nuclear microinjection of control or *BAF155* siRNA and control or *αBAF155* IgG in E_2 treated and untreated HMECs as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **E.** Effect of single cell nuclear microinjection of control or *BAF170* siRNA and control or *αBAF170* IgG in E_2-treated and untreated HMECs as examined by FISH, accompanied by its corresponding ANOVA analysis of measured distances. **F.** Effects of treating with siRNAs against *DLCI, ARP2/3* and *BAF53* on control vs. E_2-induced activation of *TFF1*. Data is expressed as fold-induction +/-E_2 (+/- SEM).

Figure 7. Nuclear speckles colocalize with hubs for interchromosomal interactions. **A.** ImmunoFISH of *TFF1/GREB1* interaction (measured by FISH, red/green with arrows) with a marker for interchromatin granules, αSC35 IgG. Upon hormone treatment, interacting chromosomal loci converge with specific speckles. **B.** A similarly designed immunoFISH experiment as in A, but now using FISH probes to six FITC-tagged probes against ER regulated promoters on six different chromosomes (all stained green) each an ERα target gene present on a different chromosome and including one non-regulated gene intronic region (on chr. 21) as a control. All but these control regions exhibited colocalization with interchromatin granules. **C.** Chemical disruption and stabilization of actin by treatment with hormone and LA, + hormone + JP, respectively, inhibiting dynamic actin polymerization, prevented the interactions between the genomic loci and with the interchromatin granules/nuclear speckles. **D.** Percent of nuclei exhibiting the genomic interactions between *TFF1/GREB1* and

nuclear speckles in E_2-treatment, following nuclear microinjection of control, *ARP2/3*, *DLC1* or *BAF53* siRNAs. **P<0.001. **E.** Inhibition of transcription by 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) and α-amanitin (αAma) (60') disrupts the integrity of nuclear speckles as well as any long-range interactions. **F.** Microinjection of control vs. LSD1 siRNA revealed that LSD1 siRNA blocked E_2-dependent activation of the *TFF1* and *GREB1* transcription units (Garcia-Bassetts et al, 2006), but did not prevent their interchromosomal interactions. However the colocalized loci no longer associated with SC35-stained interchromatin granules. **G.** Percent of nuclei in which E_2-dependent genomic interactions of *TFF1/GREB1* was preserved; interactions with nuclear speckles were abolished by siRNA nuclear microinjection of LSD1. (+/- SEM) **H.** Proposed model of actin/myosin1/DLC1-dependent colocalization, and interactions with nuclear speckles in response to ligand in HMECs.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Signal enrichment as capture by the *TFF1e* probe upon 3D analysis. Probe "b" lies within the *TFF1* promoter and is largely enriched by increasing its molar ratio in comparison to the capture probe. Probes to regions "d" and "f" are located outside of the promoter region and the *TFF1e*, respectively; which are used as negative control and do not show significant levels of enrichment upon hormone treatment.

Figure S2. ANOVA analysis of loci distances measured in both treated and untreated samples. Note that uppercase letters denote different loci within the 1.5 Mbp region, while lowercase letters indicate probes surrounding and including the *TFF1* gene proper. Probes to regions "G, J" show a positive induction upon E_2-treatment;

while "K" is not affected by the hormone. Two positive controls are included for inter- and intra-chromosomal interactions. The asterisks indicate a population statistically different (**P<0.001) from the control samples.

Figure S3. Time course of inter-chromosomal interaction. A. Kinetic Interaction of *TFF1* and *GREB1* as examined by FISH analysis following addition of E_2 at subsequent sampled time intervals. **B.** Kinetic Interaction of Chr21 and Chr2 as examined by FISH analysis following addition of E_2 at subsequent sampled time intervals. **C.** Interactions of *GREB1* and *PDZK1* as examined by FISH analysis following addition of E_2 at subsequent sampled time intervals. **D.** Interactions of Chr2 and Chr1 as examined by FISH analysis following addition of E_2 at subsequent sampled time intervals. **E.** Statistical analysis of the kinetics of E_2-induced interactions**.** The null hypothesis (NH) of significance is calculated by the software and its dependence on the standard deviation and the average absolute deviation from the median. Values below NH indicate insignificant colocalization events, whereas values above NH indicate statistically significant colocalization events. Top two panels indicate statistics obtained from H +/- E_2 with chromosome paints (chr. 2 & 21) and an individual locus probes (*GREB1 & TFF1*), respectively. The third panel indicates statistics obtained from HMEC +/- E_2 with *GREB1/PDZK1* locus probes.

Figure S4. Abrogation of observed of *TFF1* and *GREB1* interaction by early inhibition of transcription with 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) and α-Amanitin (αAma) (60'), respectively.

Figure S5. ATP definition of studies. A. Plot of standard curve of ATP values as determined by a luciferin-luciferase assay of untreated (blue) and treated (yellow) samples. Note that more ATP is consumed by the cells upon hormone treatment, and therefore, there is less free ATP measured by the luciferase assay and the curve is lower than that of the vehicle (ethanol). **B.** Measurement of ATP before and after chemical change of energy levels by depletion of glucose and addition of the protonophore rotenone (reduction of intracellular ATP levels) by luciferase assay; such reduction decreases or completely abolishes interchromosomal interactions.

Figure S6. Chemical disruption and stabilization of actin by treatment with hormone + LA, or hormone + JP, respectively. Both chemicals disrupt the interchromosomal interactions even in E_2-treated cells. The plot shows ANOVA analysis of measured distances of a control population in comparison to hormone and chemical treated samples, counting cells in which both alleles interacted ($P<0.001$).

Figure S7. Verification of nuclear delivery of antibody by microinjection. The dextran marker was coinjected with the antibody. The first panel shows representation of nuclear staining of a Texas Red conjugated dextran, while the second panel shows nuclear stain of the injected antibodies (α-nuclear myosin1 and α-ARP2/3) which were FITC conjugated. The last panel shows DAPI staining in blue indicating cells within the field..

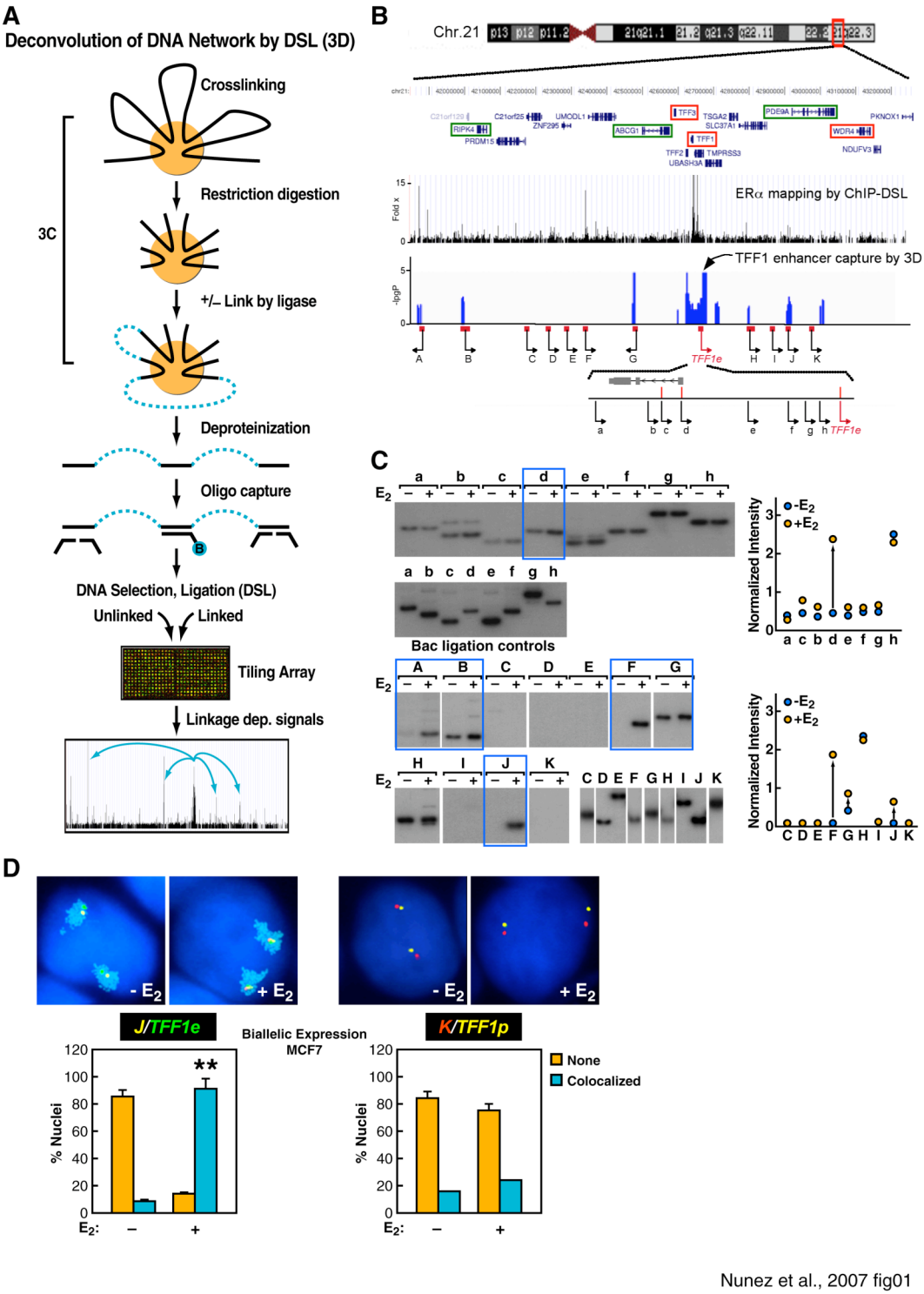
Figure S8. A. Effects of specific factors on nuclear speckles____colocalization. ImmunoFISH of *TFF1/GREB1* interaction with a marker for interchromosomal

granules, αSC35 IgG. An untreated nucleus revealing neither E_2-dependent interchromosomal interactions nor colocalization with SC35 staining. Following pictures show E_2-treated nuclei with control siRNA and siRNAs against *ARP2/3*, *DLC1* and *BAF53*, respectively. Upon hormone treatment, the interacting chromosomal loci converged with specific speckles, and this convergence was prevented by specific siRNAs. B. Validation of *siRNAs* by qPCR, in MCF7 cells (48 hr siRNA treatment).

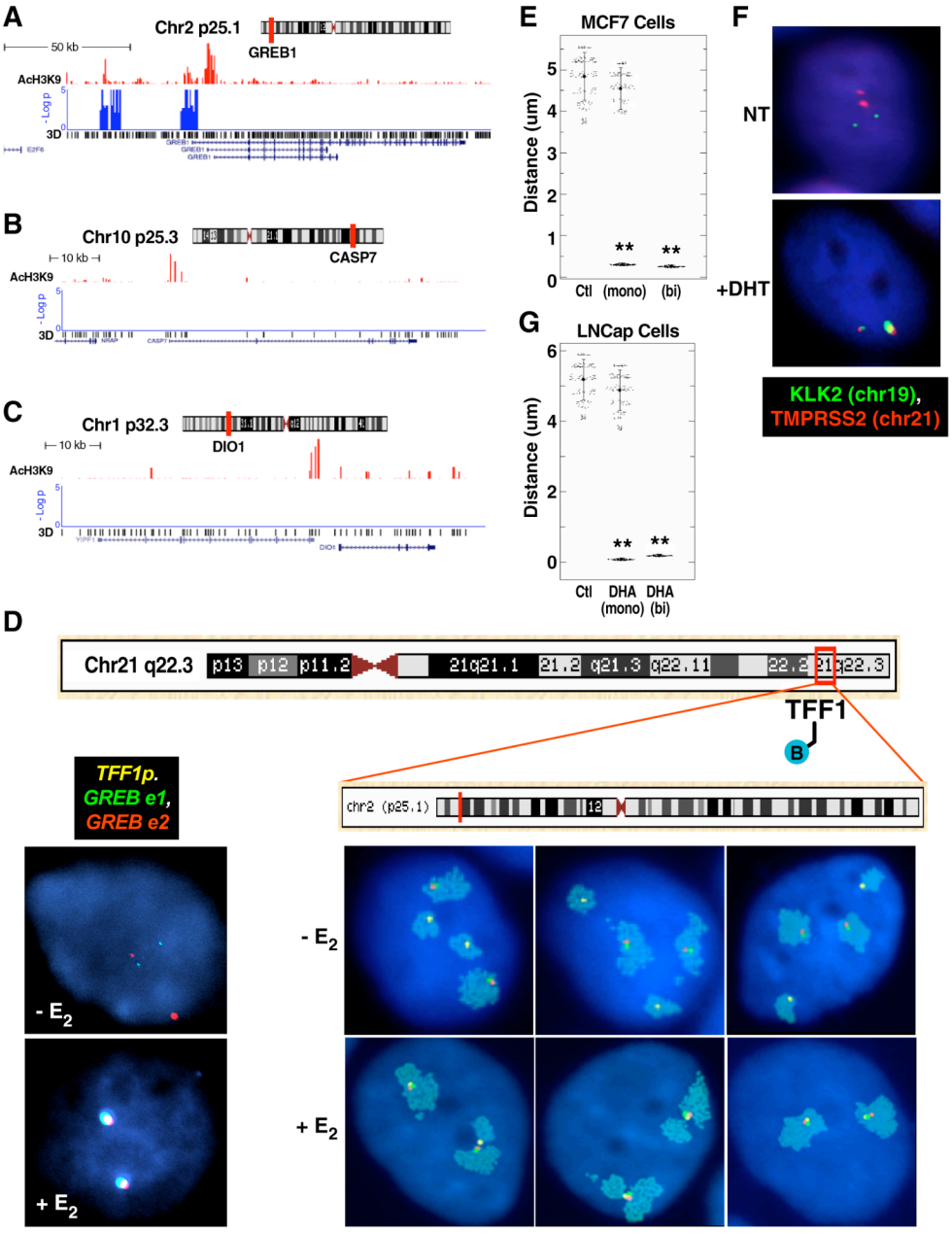
Figure S9. A. Deconvolved Z-stack of control samples showing that the interchromosomal interaction and the convergence with SC35 staining does not occur in E_2-untreated nuclei. **B.** Deconvolved Z-stack of E_2-treated cell samples displaying interchromosomal interaction as well as convergence with SC35 staining.

Figure S10. List of oligonucleotide probes used.

Figure S11. Partial list of antibodies employed.



Nunez et al., 2007 fig01



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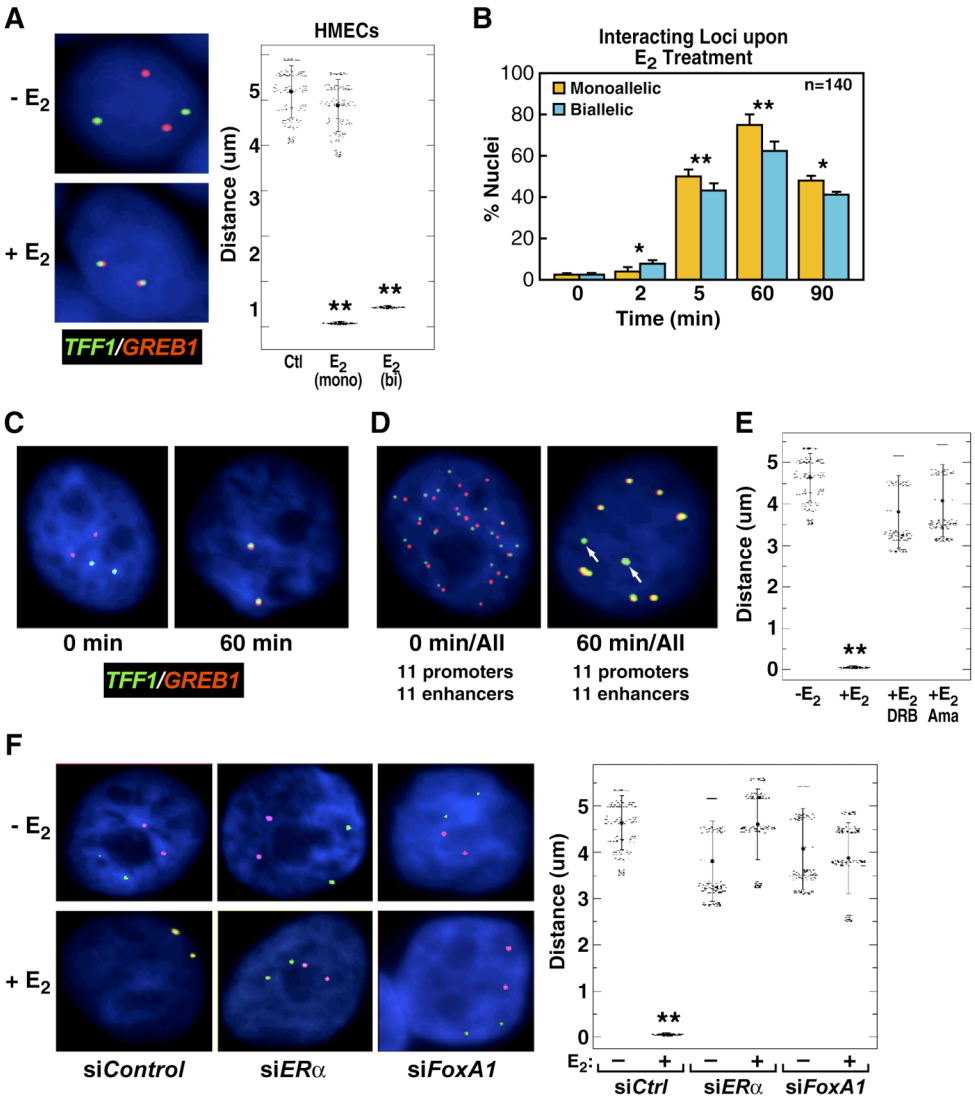

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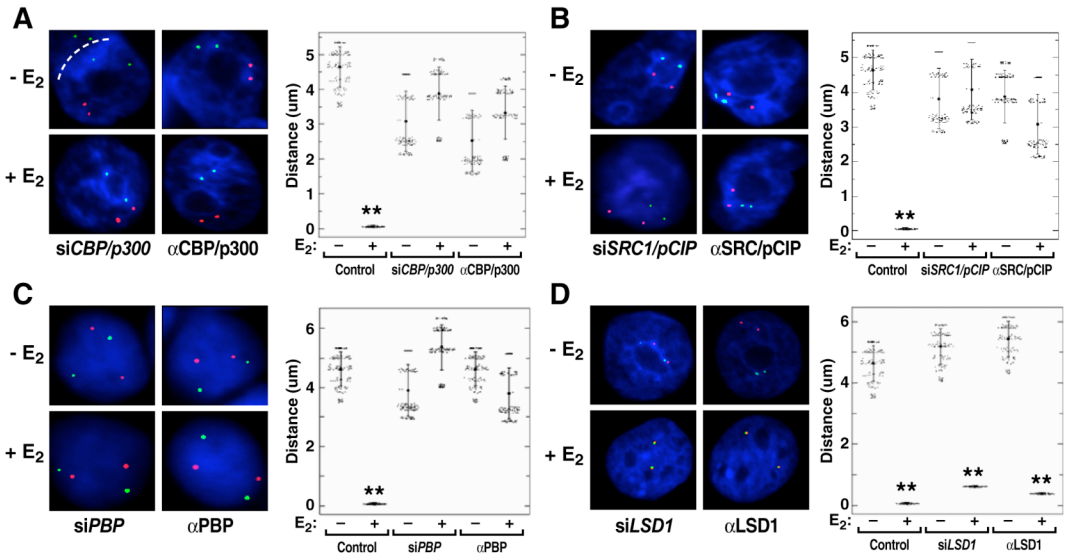
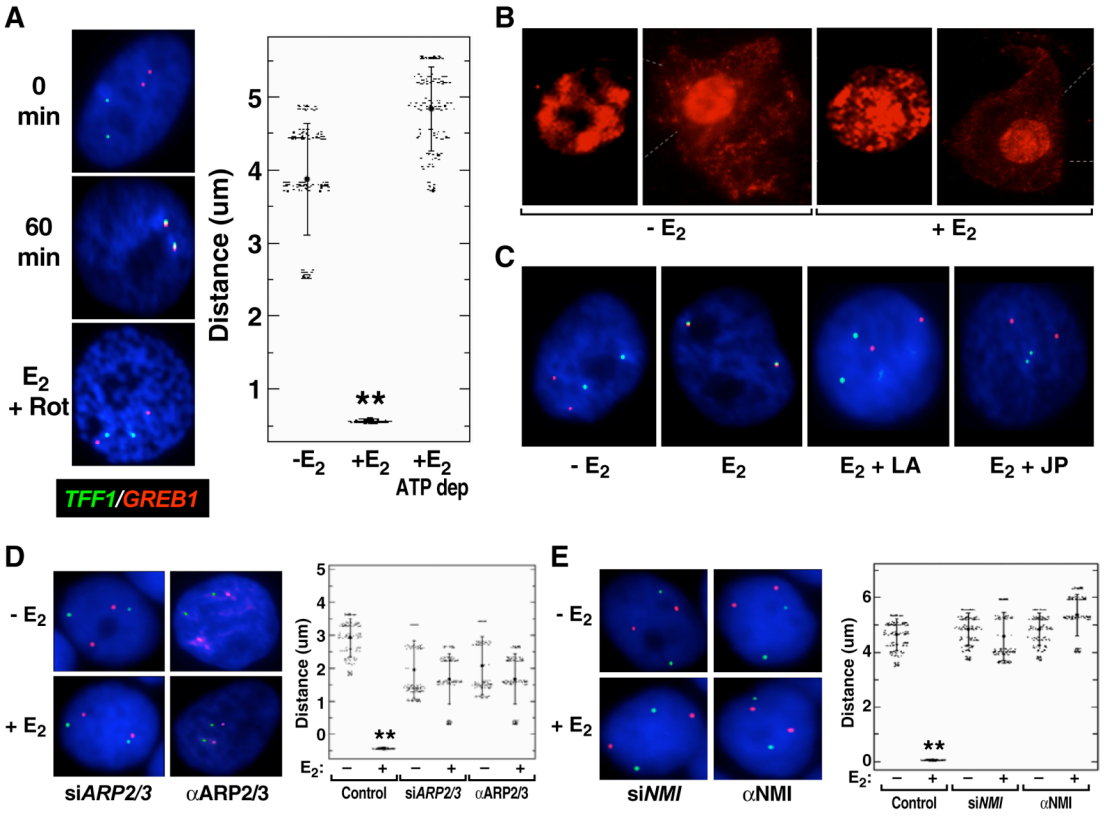
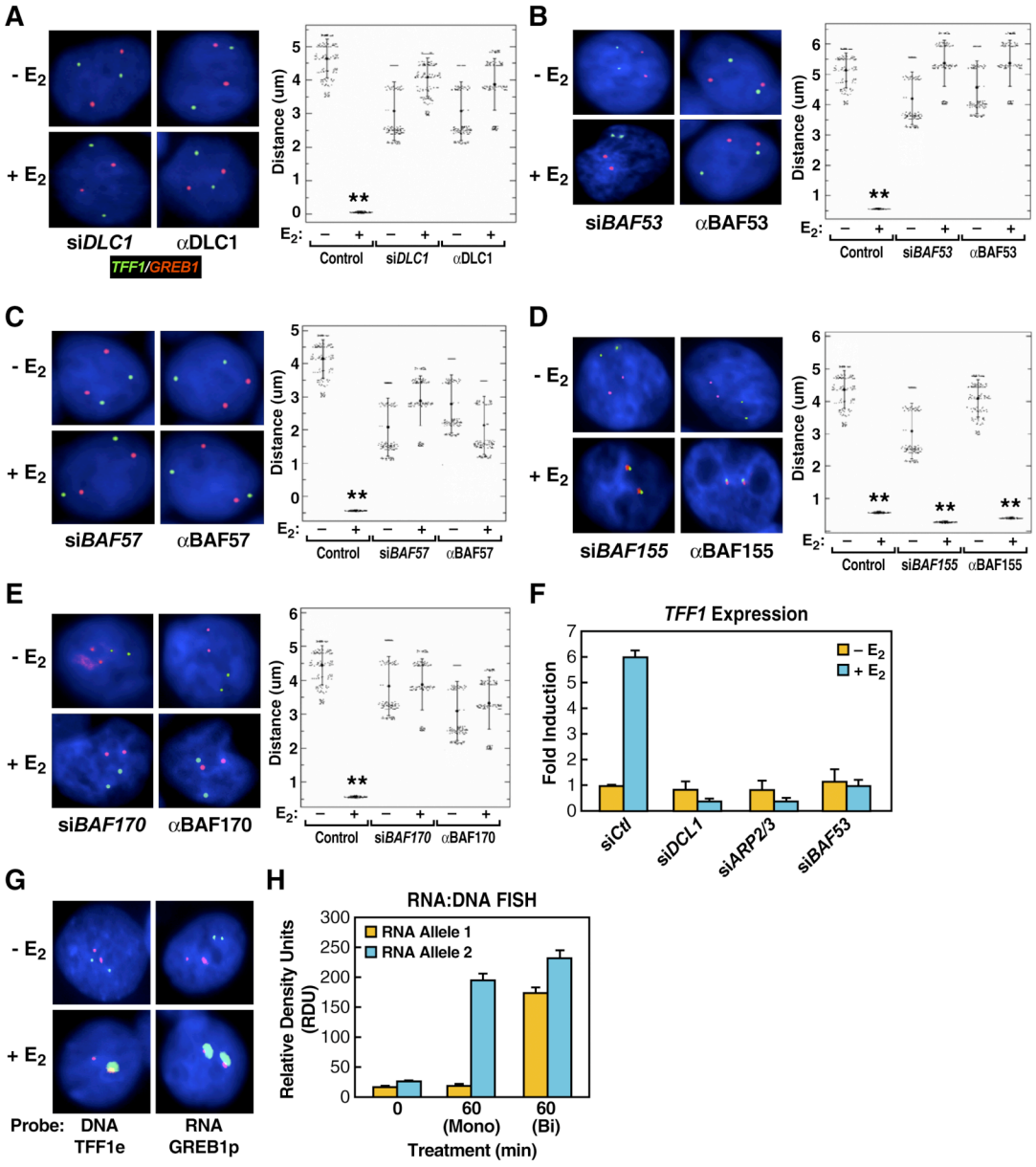


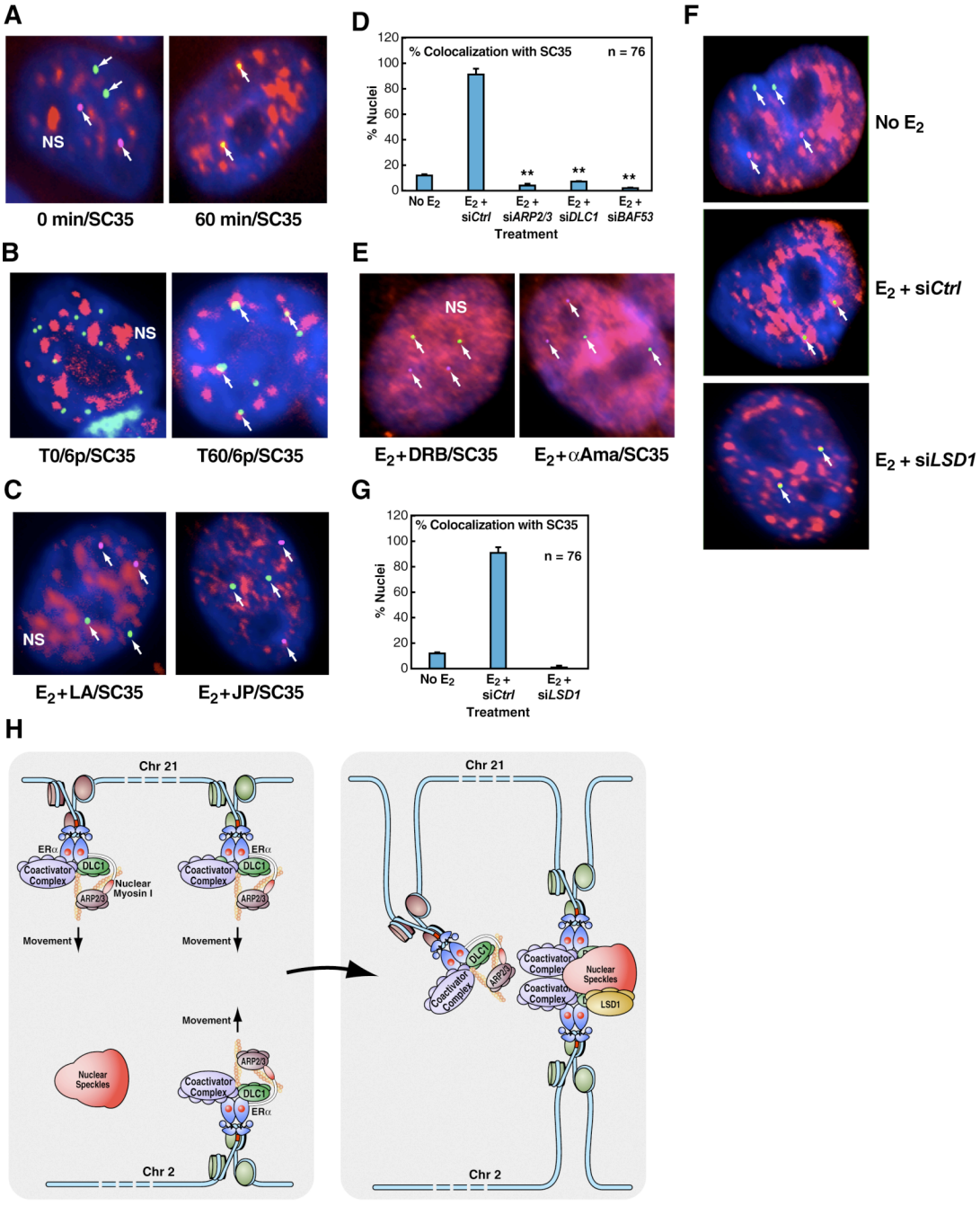
Nunez et al., 2007 fig04



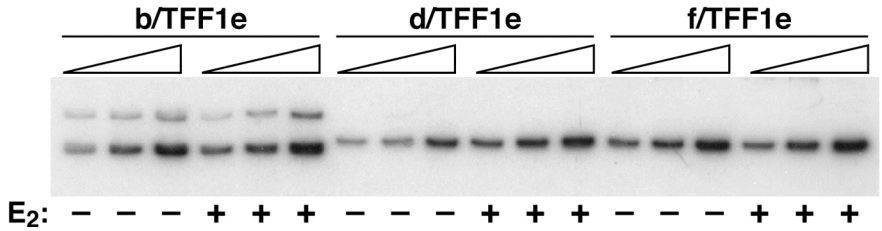
Nunez et al., 2007 fig05



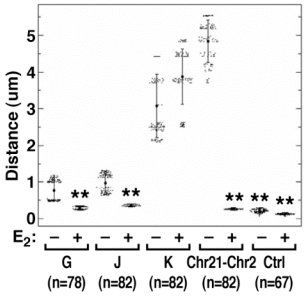
Nunez et al., 2007 fig06



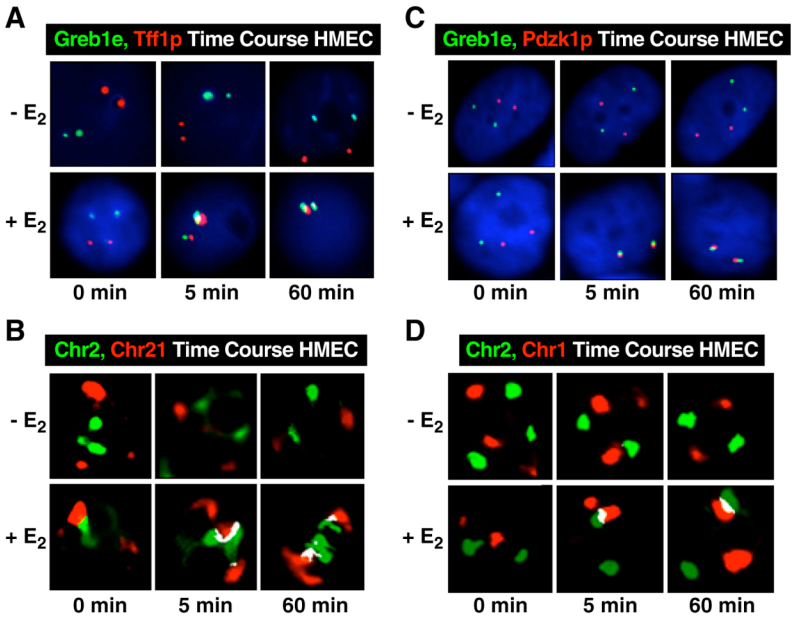
Nunez et al., 2007 fig07



Nunez et al., 2007 sup01



Nunez et al., 2007 sup02



E

Stats – HMEC ± E_2 (GREB1e, TFF1p)

	T0 (n=80)	T2 (n=80)	T5 (n=81)	T10 (n=80)	T30 (n=80)	T60 (n=80)
Null Hypothesis	1	1	1	1	1	1
Standard Deviation	3.27	2.41	2.03	3.27	2.41	2.03
Average Absolute deviation from Median	0.67	1.67	1.83	2.00	1.77	1.82
Significant Colocalization at T45'	No	Yes	Yes	Yes	Yes	Yes

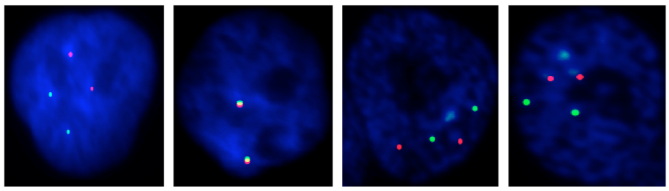
Stats – HMEC ± E_2 (Chr.2, Chr.21)

	T0 (n=70)	T2 (n=70)	T5 (n=70)	T10 (n=70)	T30 (n=70)	T60 (n=70)
Null Hypothesis	1	1	1	1	1	1
Standard Deviation	3.27	2.37	2.03	2.99	3.03	2.56
Average Absolute deviation from Median	0.53	1.67	1.90	2.01	1.78	1.30
Significant Colocalization at T45'	No	Yes	Yes	Yes	Yes	Yes

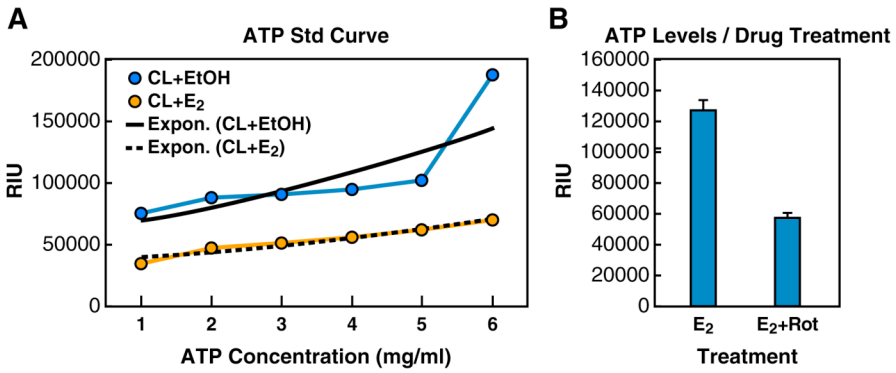
Stats – HMEC ± E_2 (GREB1e, PDZK1p)

	T0 (n=76)	T2 (n=76)	T5 (n=76)	T10 (n=76)	T30 (n=76)	T60 (n=76)
Null Hypothesis	0.53	1	1	1	1	1
Standard Deviation	0.724	4.37	5.3	3.4	1.77	3.4
Average Absolute deviation from Median	0.6	3.2	2.1	2.1	2.56	2.3
Significant Colocalization at T45'	No	Yes	Yes	Yes	Yes	Yes

Nunez et al., 2007 sup03



Nunez et al., 2007 sup04



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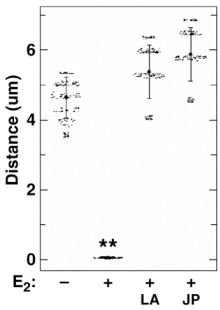
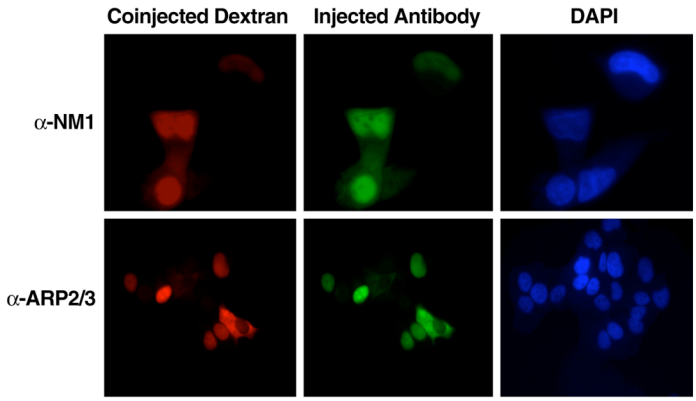
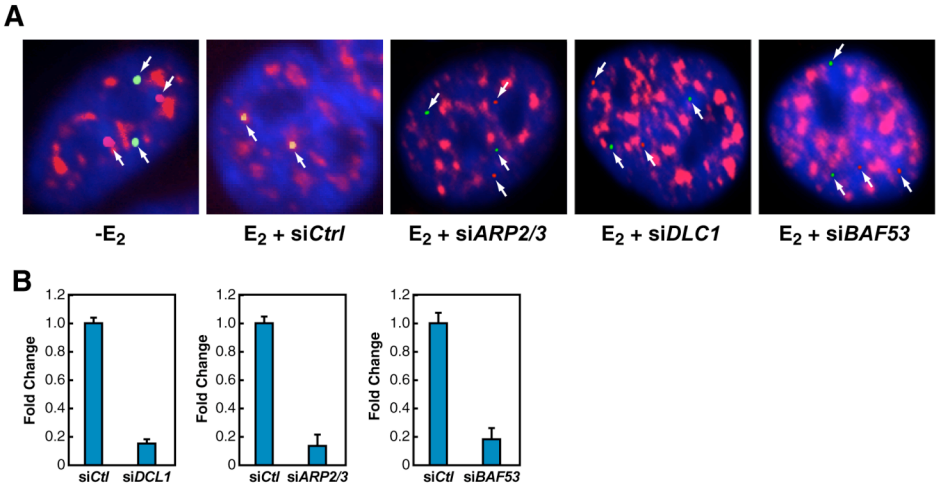


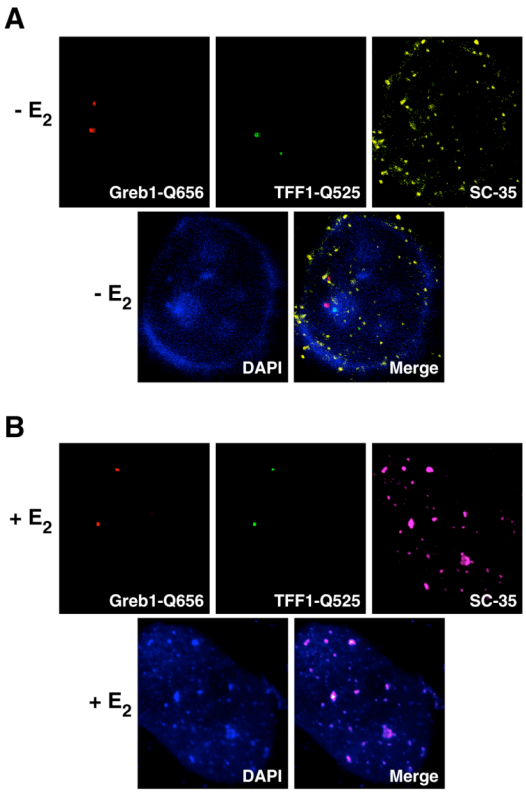
Nunez et al., 2007 sup06



Nunez et al., 2007 sup07



Nunez et al., 2007 sup08



Nunez et al., 2007 sup09

	Locus	50mer Probe	Chr.
1	TFF1_p	GCAGACCGTTGATCCATTCTGCAGGTAAAGGAGTTGAGATGCAAACACTT	21
2	TFF1_e	CTCTCCTGACTGCTGCCTCTCATCCCTGGCTCCCAGCGAGGTTCCAGCCC	21
3	GREB1_e1	GAGCTGACCTTGTGGTAGGCACGGGCATGACTGGGCTGGGTGCCCGTTTT	2
4	GREB1_e2	TTGGGGTTTTCCTCAGTTCATATATTTGCTCCTTTAGTAGTTTATCTTTA	2
5	GREB1_e3.1	TAGCTCCTGGGGAGGAATTTGTGTCTTGGGAATCATCATTTAATTTAGTA	2
6	GREB1_e3.2	CCAGGTATTATGGCCAGTGCAGAGGGAACAGAGATTGAATTGGATAGAGA	2
7	GREB1_p1.1	GGGGACTTCAAAGACGATGATGAAGTGCACCTTCACCAGAACCTGCATGT	2
8	GREB1_p1.2	CCCTTCTCCTGTGTCAGCTCCACATTCCCTCTCTAGTCTGGCTTCTGGAA	2
9	GREB1_p2.1	AGTTTCCCACCGTCACACACGGTGCCCTGAGTGGCAGAAGGCACGGGGCA	2
10	GREB1_p2.2	TTTCTGCTTCCCTGGCTTTATTCTGAAGGTCCTTCTCTTCCATTCAGTTC	2
11	NRIP1_p	ACCTTCCATCGCAATCAGAGAGAGACGTACTGTTACATTCTGTCCAAGAT	21
12	NRIP1_e1	TTGACTGTGTGCCTCCTCACTAACTAAATATTTGTGAATGAGCGTATTTT	21
13	NRIP1_e2	AGGCAGGGTAGCACTGTTGTCTGTAAACAGCATGCTATCTCTGAGACTCC	21
14	NRIP1_e3	TCAGAGGGATTAGGCAATGGGGCATGGACTGCAATGCTCCAGCCCCACCC	21
15	TMPRSS3_p	AGAACCAGGCAAATCTGGTGGAAACAATATGTAAGGAGTGTCCAGATGCC	21
16	E2F6_p	CTGGAATTCTCGACGGTGATAACTGAAAGTTCTGAGGTGATAACTGAAAG	2
17	RIPK4_p	ATTACCCAACAGCAAATCTAAAGTCCATTTCAAATGCAAGAGTTCCCTCA	21
18	PDE9A_p	GCGGCGCGGCCACGCTCAGCCCAGCGCTGGCTTAGGAGGGACGGGCTGCG	21
19	PDE9A1 intron 2	GCTTGACCCAGATCAACACTGGGTGAAAGGCTGACTTCAGAAATGGGTTG	21
20	NDUFV3_p	GCCCAGCGCAGACGCCTGAGAGGGGTGCCCAGCAGCCCCCGCAGGTCTCC	21
21	PDZK1_p	GAAGTCTGCAGCAAATTACTCAGGGTCTTTGAATTTCTGTGTTATGATAG	1
22	ESR1_p	GTGAAACTCAGCCTCTATCCAGCAGCGACGACAAGTAAAGTAAAGTTCAG	6
23	FOXA1_p	GTTTCATGCCCTTCCATCTTCACAGTTCCTAACATCCTGGAGCCACCCTG	14
24	WISP2_p	GTGACCTCACAGCTGCCGGAACATAAAGACTCACAGGTCCGCCTCCCAGG	20

Nunez et al., 2007 sup10

	Antibody	Source
1	Qdot 525 Streptavidin Conjugate	Invitrogen
2	Qdot® 605 anti-Digoxigenin Conjugate	Discontinued
3	Qdot® 655 anti-Fluorescein Conjugate	Invitrogen
4	Texas Red®-X	Invitrogen
5	SC35	Fu and Maniatis, 1990
6	Nuclear actin 2G2	Progen
7	Alexa Fluor® 594	Invitrogen
8	Anti-Myosin I beta (Nuclear)	Sigma
9	Human DLC1	Abcam
10	ARP2	Santa Cruz
11	ARP3	Santa Cruz
12	ARP2/3 Complex (p34-ARC)	Santa Cruz
13	BAF53	Santa Cruz
14	BAF155	Santa Cruz
15	BAF170	Santa Cruz
16	BAF250	Santa Cruz
17	FoxA1	Santa Cruz
18	ESR1 (F-10)	Santa Cruz
19	ESR1 (H-184)	Santa Cruz
20	LSD1	Abcam

Nunez et al., 2007 sup11

DISCUSSION

The identification of the set of cis-acting target of a trans-acting factor such as the estrogen receptor across the whole genome provides an important new resource for the study of gene regulation. The estrogen receptor is the master transcriptional regulator of breast cancer phenotype, then the complete set of estrogen receptor binding sites across the genome is a new resource to understanding estrogen action in breast cancer. For this reason our first step was to identify putative functional estrogen receptor binding sites on the genome scale by computational approaches. Initially we tried to identify this set of gene using the matrix M00191 present in TRANSFAC but our approach was not efficient because we identified as ERE containing genes almost 57% of the genes in the genome. This happened because the matrix M00191 is unbalanced (Fig. 24), fact that impairs the identification of a complete site.

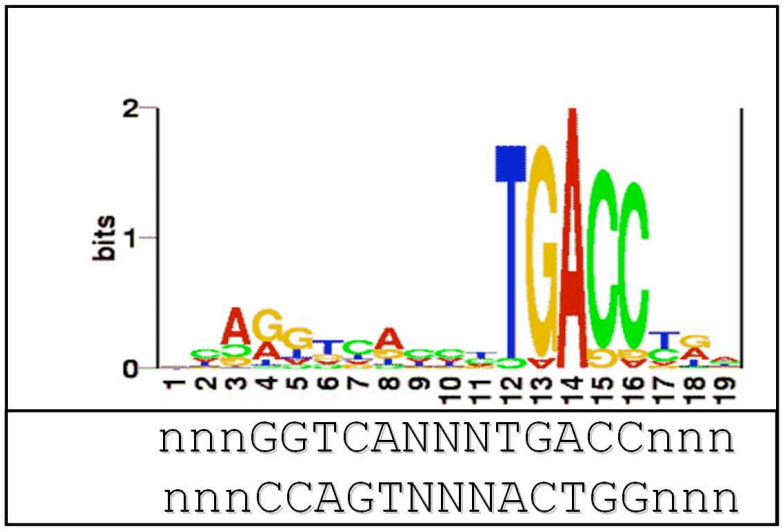


Figure 17 SLOGOS of the matrix M00191 present in TRANSFAC.

Therfore, we decided to improve the alignment matrix in order to obtain a more efficient genome-wide analysis.

To build a new ERE weight matrix we implemented the data generated from chromatin immunoprecipitation (ChIP) on chip experiment (Kwon et al., 2007) (Lin et al., 2007) using the algorithm MakeMatrix. The using of these experimental data allowed us to build a new matrix (ERE-m) more balanced (Fig. 25) than the matrix M00191 and with a higher IC (Tab.1).

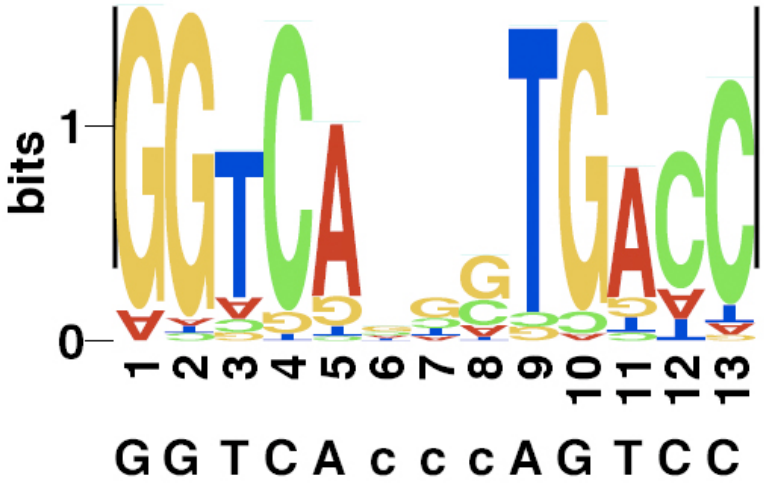


Figure 18 SLOGOS of ERE-m.

Although the new matrix improved our analysis, the number of ERE containing genes was still too high to be significant and, for this reason we scanned also the murine and rat's genome. Indeed, it has been shown that the selection of regulatory elements can be improved if they are conserved over the course of evolution (Loots et al., 2000). Using this approach we did improve gene selection, obtaining 6984 genes that have a ERE conserved in both human and mouse or human and rat. Although this number is biologically significant it is still hard to know if these genes are target of ER alpha. To avoid this problem we decided to cross this set with genes that were identified as modulated by estrogen.

Since, genes identified as differential expressed in ER+ and ER- breast cancers are mostly involved in regulation of cell proliferation an apoptosis and estrogen antagonists are used as therapy for reduction of the tumor mass in ER+ tumors, we decided to use the genes identified by Scafoglio and coworker because they also analyzed the effect of some estrogen antagonist on gene expression. Moreover, since most of the work that has been done in the last 5 years on the investigation of estrogen-regulated transcription has been focused on up-regulated genes we focused our attention on down-regulated genes. Using this approach we identified 134 genes that have a putative ERE and that are down-regulated by estrogen. These 134 gene are mostly involved in the regulation of cellular growth and proliferation and this is concordant with the fact that estrogen

stimulate proliferation. Moreover some of these genes are involved in cell death and, interestingly, in cell migration and invasion. Since when the tumor becomes aggressive the cells start to proliferate and migrate to perform the functional validation assay of our computational screening, we chose genes involved in these pathways, as for example BCL2L1 which is an pro-apoptotic genes and CLDN4 or INTB4, required for cell-cell contact. We included also genes that are linked to estrogen action (GRP30) and also the interesting transcription factor GATA2.

When we looked at gene expression after estrogen treatment, all of the genes analyzed were indeed repressed by estrogen and this was concordant with their biological function and with the hormonal effect. The fact that we observe repression does not authomatically validate the role of the identified ERE in regulation. To prove this, we have investigated the in vivo recruitment of ER alpha on these promoters by ChIP. The results obtained testify in 6 out of the 8 cases examined (including E-Cadherin) that ER alpha is actually bound to the promoter, most likely through interaction with the identified ERE. The additional gene studied, E-Cadherin, provides a proof that the ERE can be the responsible of this recruitment: in fact, the use of mutant promoters with altered ERE proved that the regulation is lost. These results provide further evidence that our computational approach can be used to improve the discovery of both known and new regulatory element across the genome.

Since the loss of E-cadherin is an important event for the invasion of epithelial tumor cells we decided to study the molecular events associated with hormone-induced negative regulation, that are much less well understood than positive regulation, by focusing on estrogen repression of E-Cadherin in breast cancer cells.

Our results demonstrate that E-Cadherin is an estrogen down-regulated gene and that this effect on E-cadherin expression is mediated by E2 responsive region of E-cadherin promoter (-164/-152) by recruitment of ER alpha. Moreover, we demonstrated that mutation of the half-ERE greatly reduced ER binding and abrogated E2-mediated repression.

Although repression of E-cadherin gene transcription is mediated by many transcription factors including the most famous SLUG and SNAIL repressors, (Huber et al., 2005; Peinado et al., 2007) the open question is: “there is a co-operation or a functional hierarchy of the different repressors in order to down-regulate E-cadherin expression?”

Our finding of a sequential recruitment of N-CoR and CtBP complexes after E2

treatment suggests a time checkpoint regulation of E-Cadherin repression. Our proposed model is that E2-ER interaction induces first the recruitment of N-CoR at the E-Cadherin promoter. Formation of this first complex leads to hypo-acetylation of histones (Jepsen and Rosenfeld, 2002) and, after that, the recruitment of CtBP complexes induces hypermethylation of histones (Peinado et al., 2004), such as histone H3 on the lysine 9, which causes stabilization of the nucleosome structure through recruitment of HP1, limiting accessibility to the basal transcriptional machinery and thus repressing E-Cadherin gene expression.

Moreover, we found that the half-ERE at the E-Cadherin promoter is clustered in a GC-rich regions; it is known that GC-rich regions are involved in ER-mediated repression at the p21/WAF1 and VEGF gene promoters, where interplay with members of the Sp1 family of transcription factors seems to occur (Varshochi et al., 2005). Furthermore, direct ER and Sp1 binding is well documented in estrogen-stimulated genes (Porter et al., 1997). Indeed, through chromatin immunoprecipitation experiments, we were able to show that both Sp1 and ER alpha bind to the responsive region of E-Cadherin promoter. Of note, E2 treatment markedly enhanced ER association, whereas Sp1 was decreased in the presence of E2 but was still present at the E-cadherin promoter like the situation described at the p21/WAF1 promoter. These findings suggest the importance of both the half-ERE and Sp1 sites in the recruitment of ER alpha to the E-cadherin promoter. Since we demonstrated that unliganded ER alpha is required for the basal expression of E-cadherin and it has been shown that Sp1 is required to promote E-cadherin expression (Liu et al., 2005) our hypothesis is that in absence of estrogen ER alpha can bind on the half ERE as heterodimer with Sp1 and bring coactivator to activate the expression of E-cadherin. Moreover, E2-ER alpha binding can lead to a conformational change that allows the increase of ER alpha recruitment on the promoter and also the recruitment of corepressor such N-CoR to repress E-cadherin expression.

E-cadherin plays a critical role in establishing cell polarity and cellular differentiation and maintaining normal tissue morphology (Berx and Van Roy, 2001). Indeed, in many types of epithelial cancers, the ability to undergo metastasis has been associated with a loss of epithelial features and acquisition of mesenchymal properties leading to migration of individual cells, a process known as epithelial-to-mesenchymal transition (EMT).

This observation together with our finding that constitutive activation of mitogenic-activated protein kinase RON (Bardella et al., 2004) in estrogen receptor alpha-positive breast cancer cells induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors suggest a new possible role of ER alpha.

Since we demonstrated that the inhibition of kinase activity of sfRON can revert the molecular epithelial morphology paralleled by expression of both E-cadherin and ER alpha and recruitment of ER alpha on the E-cadherin promoter, our hypothesis is that ER alpha plays a role as ligand-independent activator essential for the determination of epithelial morphology.

We demonstrated that ER alpha bound to the same sequence (ERE) can determinate activation or repression at different gene contexts and that these responses can be very rapid. These results raised two hypotheses, first that the differential transcriptional response to the same stimulus of distinct subsets of genes can be regulated by transcriptional complexes with distinct composition exist in the nuclei and second that genes with a common mode of regulation in response to stimuli can share the same transcriptional machinery. In order to validate these hypotheses, I joined the laboratory of M.G. Rosenfeld, who was examining the functional relationships between nuclear structure and gene expression using as a model the well-characterized regulation of ER alpha target genes in response to hormone and studying the dynamic responses of the cell to different signals resulting in changes in chromatin compaction levels and movement of genomic loci.

The genome-wide identification of DNA binding sites for ER alpha described in both Carroll et al, 2005 and Bassets et al, 2007 clearly show that ER alpha bind to both gene promoters and many other remote intergenic sites such as enhancers. To investigate how those remote binding sites can communicate with appropriate target genes we developed an unbiased high-throughput chromosome conformation capture (3D) assay, which is used in conjunction with a quantum dot (qdot)-based FISH method, to delineate new paradigms of long-distance chromosomal interactions. Using this technique we find that multiple ER alpha binding sites are organized into specific chromosomal hubs, rather than pair-wise promoter/enhancer interactions, suggesting a three-dimensional architectural component of regulated gene expression in mammalian cells. The 3D technology has uncovered a previously unrecognized DNA network involving multiple ER alpha binding sites, which have been extensively validated by

the conventional 3C assay and by a qdot-FISH. Together, the results suggest nuclear architecture as an additional critical determinant for regulated gene expression in mammalian cells. Indeed, dynamic interactions between proximal and distal regions are induced upon estradiol treatment and different E2-regulated gene sets are engaging in distinct network interactions in the nucleus.

In order for the chromosome to change position there must also be a nucleoskeletal transport mechanism. Indeed, conventional actin is found in the nucleus and it has a role in diverse nuclear activities such as transcription, mRNA export and Chromatin remodeling. Moreover it has been found a a nuclear isoform of myosin I, Nuclear Myosin I (NMI) that interacts with WSTF and SNF2 components of the chromatin remodeling complex and, like actin, it also colocalizes with RNA Polymerase II. Since all myosins are actin-activated ATP hydrolases and acto-myosin complexes function as molecular motors in the cytoplasm, it is logical to predict that actin and NMI also act together in the nucleus. Indeed our results demonstrated that ligand induces rapid interchromosomal interactions among subsets of ER alpha bound transcription units. This interaction required nuclear actin/myosin-1 transport machinery, dynein light chain 1, and a specific subset of transcriptional coactivators and chromatin remodeling complexes.

Moreover, we demonstrated that these interactions are necessary for ER alpha trans-activation activity because the inhibition of nucleoskeletal transport machinery by both siRNA and drugs treatments blocking chromosomal interaction also block the transcriptional response to estrogen, while not impairing *per se* the recruitment of the transcriptional machinery at gene promoters. These data finally suggest a molecular mechanism by which the hormone-induced interchromosomal interactions are finalized to achieve enhanced, coordinated transcription and RNA splicing for nuclear receptor target genes.

BIBLIOGRAPHY

Bailey, T.L., and Gribskov, M. (1998). Methods and statistics for combining motif match scores. J Comput Biol *5*, 211-221.

Bardella, C., Costa, B., Maggiora, P., Patane, S., Olivero, M., Ranzani, G.N., De Bortoli, M., Comoglio, P.M., and Di Renzo, M.F. (2004). Truncated RON tyrosine kinase drives tumor cell progression and abrogates cell-cell adhesion through E-cadherin transcriptional repression. Cancer research *64*, 5154-5161.

Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., and Nilsson, S. (1998). Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. Molecular pharmacology *54*, 105-112.

Berg, O.G., and von Hippel, P.H. (1987). Selection of DNA binding sites by regulatory proteins. Statistical-mechanical theory and application to operators and promoters. Journal of molecular biology *193*, 723-750.

Berx, G., and Van Roy, F. (2001). The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res *3*, 289-293.

Bourdeau, V., Deschenes, J., Metivier, R., Nagai, Y., Nguyen, D., Bretschneider, N., Gannon, F., White, J.H., and Mader, S. (2004). Genome-wide identification of high-affinity estrogen response elements in human and mouse. Molecular endocrinology (Baltimore, Md *18*, 1411-1427.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engstrom, O., Ohman, L., Greene, G.L., Gustafsson, J.A., and Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Buck, M.J., and Lieb, J.D. (2004). ChIP-chip: considerations for the design, analysis, and application of genome-wide chromatin immunoprecipitation experiments. Genomics *83*, 349-360.

Bussemaker, H.J., Foat, B.C., and Ward, L.D. (2007). Predictive modeling of genome-wide mRNA expression: from modules to molecules. Annual review of biophysics and biomolecular structure *36*, 329-347.

Cai, S., and Kohwi-Shigematsu, T. (1999). Intranuclear relocalization of matrix binding sites during T cell activation detected by amplified fluorescence in situ hybridization. Methods (San Diego, Calif *19*, 394-402.

Carroll, J.S., Liu, X.S., Brodsky, A.S., Li, W., Meyer, C.A., Szary, A.J., Eeckhoute, J., Shao, W., Hestermann, E.V., Geistlinger, T.R*., et al.* (2005). Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell *122*, 33-43.

Carroll, J.S., Meyer, C.A., Song, J., Li, W., Geistlinger, T.R., Eeckhoute, J., Brodsky, A.S., Keeton, E.K., Fertuck, K.C., Hall, G.F*., et al.* (2006). Genome-wide analysis of estrogen receptor binding sites. Nature genetics *38*, 1289-1297.

Caselle, M., Di Cunto, F., and Provero, P. (2002). Correlating overrepresented upstream motifs to gene expression: a computational approach to regulatory element discovery in eukaryotes. BMC bioinformatics *3*, 7.

Cawley, S., Bekiranov, S., Ng, H.H., Kapranov, P., Sekinger, E.A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A.J.*, et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell *116*, 499-509.

Chambeyron, S., and Bickmore, W.A. (2004). Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. Genes & development *18*, 1119-1130.

Cicatiello, L., Scafoglio, C., Altucci, L., Cancemi, M., Natoli, G., Facchiano, A., Iazzetti, G., Calogero, R., Biglia, N., De Bortoli, M.*, et al.* (2004). A genomic view of estrogen actions in human breast cancer cells by expression profiling of the hormone-responsive transcriptome. Journal of molecular endocrinology *32*, 719-775.

Cook, P. (1998). Duplicating a tangled genome. Science (New York, NY *281*, 1466-1467.

Cora, D., Di Cunto, F., Caselle, M., and Provero, P. (2007). Identification of candidate regulatory sequences in mammalian 3' UTRs by statistical analysis of oligonucleotide distributions. BMC bioinformatics *8*, 174.

Cora, D., Herrmann, C., Dieterich, C., Di Cunto, F., Provero, P., and Caselle, M. (2005). Ab initio identification of putative human transcription factor binding sites by comparative genomics. BMC bioinformatics *6*, 110.

Couse, J.F., and Korach, K.S. (1999). Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine reviews *20*, 358-417.

Cowley, S.M., and Parker, M.G. (1999). A comparison of transcriptional activation by ER alpha and ER beta. The Journal of steroid biochemistry and molecular biology *69*, 165-175.

Creighton, C.J., Hilger, A.M., Murthy, S., Rae, J.M., Chinnaiyan, A.M., and El-Ashry, D. (2006). Activation of mitogen-activated protein kinase in estrogen receptor alpha-positive breast cancer cells in vitro induces an in vivo molecular phenotype of estrogen receptor alpha-negative human breast tumors. Cancer research *66*, 3903-3911.

Croft, J.A., Bridger, J.M., Boyle, S., Perry, P., Teague, P., and Bickmore, W.A. (1999). Differences in the localization and morphology of chromosomes in the human nucleus. The Journal of cell biology *145*, 1119-1131.

Cunha, G.R., Young, P., Hom, Y.K., Cooke, P.S., Taylor, J.A., and Lubahn, D.B. (1997). Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. Journal of mammary gland biology and neoplasia *2*, 393-402.

de Lanerolle, P., Johnson, T., and Hofmann, W.A. (2005). Actin and myosin I in the nucleus: what next? Nature structural & molecular biology *12*, 742-746.

Dekker, J., Rippe, K., Dekker, M., and Kleckner, N. (2002). Capturing chromosome conformation. Science (New York, NY *295*, 1306-1311.

Dennis, A.P., and O'Malley, B.W. (2005). Rush hour at the promoter: how the ubiquitin-proteasome pathway polices the traffic flow of nuclear receptor-dependent transcription. The Journal of steroid biochemistry and molecular biology *93*, 139-151.

Edelmann, P., Bornfleth, H., Zink, D., Cremer, T., and Cremer, C. (2001). Morphology and dynamics of chromosome territories in living cells. Biochimica et biophysica acta *1551*, M29-39.

El-Tanani, M.K., and Green, C.D. (1997). Two separate mechanisms for ligand-independent activation of the estrogen receptor. Molecular endocrinology (Baltimore, Md *11*, 928-937.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J.A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. The Journal of clinical endocrinology and metabolism *82*, 4258-4265.

Ensminger, A.W., and Chess, A. (2004). Coordinated replication timing of monoallelically expressed genes along human autosomes. Human molecular genetics *13*, 651-658.

Fakan, S., and Bernhard, W. (1971). Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. Experimental cell research *67*, 129-141.

Foster, H.A., and Bridger, J.M. (2005). The genome and the nucleus: a marriage made by evolution. Genome organisation and nuclear architecture. Chromosoma *114*, 212-229.

Frasor, J., Danes, J.M., Komm, B., Chang, K.C., Lyttle, C.R., and Katzenellenbogen, B.S. (2003). Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology *144*, 4562-4574.

Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nature reviews *3*, 362-374.

Fu, X.D., and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature *343*, 437-441.

Fujita, N., Jaye, D.L., Kajita, M., Geigerman, C., Moreno, C.S., and Wade, P.A. (2003). MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell *113*, 207-219.

Galien, R., and Garcia, T. (1997). Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. Nucleic acids research *25*, 2424-2429.

Garcia-Bassets, I., Kwon, Y.S., Telese, F., Prefontaine, G.G., Hutt, K.R., Cheng, C.S., Ju, B.G., Ohgi, K.A., Wang, J., Escoubet-Lozach, L.*, et al.* (2007). Histone methylation-dependent mechanisms impose ligand dependency for gene activation by nuclear receptors. Cell *128*, 505-518.

Ghosh, M.G., Thompson, D.A., and Weigel, R.J. (2000). PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer research *60*, 6367-6375.

Glass, C.K., and Rosenfeld, M.G. (2000). The coregulator exchange in transcriptional functions of nuclear receptors. Genes & development *14*, 121-141.

Gonsior, S.M., Platz, S., Buchmeier, S., Scheer, U., Jockusch, B.M., and Hinssen, H. (1999). Conformational difference between nuclear and cytoplasmic actin as detected by a monoclonal antibody. Journal of cell science *112 (Pt 6)*, 797-809.

Gruenbaum, Y., Margalit, A., Goldman, R.D., Shumaker, D.K., and Wilson, K.L. (2005). The nuclear lamina comes of age. Nat Rev Mol Cell Biol *6*, 21-31.

GuhaThakurta, D. (2006). Computational identification of transcriptional regulatory elements in DNA sequence. Nucleic acids research *34*, 3585-3598.

Gunawardena, S., and Rykowski, M.C. (2000). Direct evidence for interphase chromosome movement during the mid-blastula transition in Drosophila. Curr Biol *10*, 285-288.

Handwerger, K.E., and Gall, J.G. (2006). Subnuclear organelles: new insights into form and function. Trends in cell biology *16*, 19-26.

Harrington, W.R., Sheng, S., Barnett, D.H., Petz, L.N., Katzenellenbogen, J.A., and Katzenellenbogen, B.S. (2003). Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Molecular and cellular endocrinology *206*, 13-22.

Hernandez-Verdun, D. (2006). The nucleolus: a model for the organization of nuclear functions. Histochemistry and cell biology *126*, 135-148.

Hertz, G.Z., and Stormo, G.D. (1999). Identifying DNA and protein patterns with statistically significant alignments of multiple sequences. Bioinformatics (Oxford, England) *15*, 563-577.

Hewitt, S.L., High, F.A., Reiner, S.L., Fisher, A.G., and Merkenschlager, M. (2004). Nuclear repositioning marks the selective exclusion of lineage-inappropriate transcription factor loci during T helper cell differentiation. European journal of immunology *34*, 3604-3613.

Higgs, H.N., and Pollard, T.D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annual review of biochemistry *70*, 649-676.

Hoch, R.V., Thompson, D.A., Baker, R.J., and Weigel, R.J. (1999). GATA-3 is expressed in association with estrogen receptor in breast cancer. International journal of cancer *84*, 122-128.

Holzinger, A. (2001). Jasplakinolide. An actin-specific reagent that promotes actin polymerization. Methods in molecular biology (Clifton, NJ *161*, 109-120.

Hu, J., Li, B., and Kihara, D. (2005). Limitations and potentials of current motif discovery algorithms. Nucleic acids research *33*, 4899-4913.

Huber, B.R., and Bulyk, M.L. (2006). Meta-analysis discovery of tissue-specific DNA sequence motifs from mammalian gene expression data. BMC bioinformatics *7*, 229.

Huber, M.A., Kraut, N., and Beug, H. (2005). Molecular requirements for epithelial-mesenchymal transition during tumor progression. Current opinion in cell biology *17*, 548-558.

Inadera, H., Dong, H.Y., and Matsushima, K. (2002). WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochemical and biophysical research communications *294*, 602-608.

Jepsen, K., and Rosenfeld, M.G. (2002). Biological roles and mechanistic actions of co-repressor complexes. Journal of cell science *115*, 689-698.

Kato, S. (2001). Estrogen receptor-mediated cross-talk with growth factor signaling pathways. Breast cancer (Tokyo, Japan) *8*, 3-9.

Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E.S. (2003). Sequencing and comparison of yeast species to identify genes and regulatory elements. Nature *423*, 241-254.

Klein-Hitpass, L., Schorpp, M., Wagner, U., and Ryffel, G.U. (1986). An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. Cell *46*, 1053-1061.

Klinge, C.M., Bodenner, D.L., Desai, D., Niles, R.M., and Traish, A.M. (1997). Binding of type II nuclear receptors and estrogen receptor to full and half-site estrogen response elements in vitro. Nucleic acids research *25*, 1903-1912.

Kosak, S.T., Skok, J.A., Medina, K.L., Riblet, R., Le Beau, M.M., Fisher, A.G., and Singh, H. (2002). Subnuclear compartmentalization of immunoglobulin loci during lymphocyte development. Science (New York, NY *296*, 158-162.

Koszewski, N.J., and Notides, A.C. (1991). Phosphate-sensitive binding of the estrogen receptor to its response elements. Molecular endocrinology (Baltimore, Md *5*, 1129-1136.

Kreiman, G. (2004). Identification of sparsely distributed clusters of cis-regulatory elements in sets of co-expressed genes. Nucleic acids research *32*, 2889-2900.

Kuiper, G.G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences of the United States of America *93*, 5925-5930.

Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., and Gustafsson, J.A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology *139*, 4252-4263.

Kwon, Y.S., Garcia-Bassets, I., Hutt, K.R., Cheng, C.S., Jin, M., Liu, D., Benner, C., Wang, D., Ye, Z., Bibikova, M.*, et al.* (2007). Sensitive ChIP-DSL technology reveals an extensive estrogen receptor alpha-binding program on human gene promoters. Proceedings of the National Academy of Sciences of the United States of America *104*, 4852-4857.

Laganiere, J., Deblois, G., and Giguere, V. (2005). Functional genomics identifies a mechanism for estrogen activation of the retinoic acid receptor alpha1 gene in breast cancer cells. Molecular endocrinology (Baltimore, Md *19*, 1584-1592.

Lamond, A.I., and Spector, D.L. (2003). Nuclear speckles: a model for nuclear organelles. Nat Rev Mol Cell Biol *4*, 605-612.

Lin, C.Y., Vega, V.B., Thomsen, J.S., Zhang, T., Kong, S.L., Xie, M., Chiu, K.P., Lipovich, L., Barnett, D.H., Stossi, F.*, et al.* (2007). Whole-Genome Cartography of Estrogen Receptor alpha Binding Sites. PLoS Genet *3*, e87.

Ling, J.Q., Li, T., Hu, J.F., Vu, T.H., Chen, H.L., Qiu, X.W., Cherry, A.M., and Hoffman, A.R. (2006). CTCF mediates interchromosomal colocalization between Igf2/H19 and Wsb1/Nf1. Science (New York, NY *312*, 269-272.

Liu, Y.N., Lee, W.W., Wang, C.Y., Chao, T.H., Chen, Y., and Chen, J.H. (2005). Regulatory mechanisms controlling human E-cadherin gene expression. Oncogene *24*, 8277-8290.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif *25*, 402-408.

Lomvardas, S., Barnea, G., Pisapia, D.J., Mendelsohn, M., Kirkland, J., and Axel, R. (2006). Interchromosomal interactions and olfactory receptor choice. Cell *126*, 403-413.

Loots, G.G., Locksley, R.M., Blankespoor, C.M., Wang, Z.E., Miller, W., Rubin, E.M., and Frazer, K.A. (2000). Identification of a coordinate regulator of interleukins 4, 13, and 5 by cross-species sequence comparisons. Science (New York, NY *288*, 136-140.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P.*, et al.* (1995). The nuclear receptor superfamily: the second decade. Cell *83*, 835-839.

Martinez, E., and Wahli, W. (1989). Cooperative binding of estrogen receptor to imperfect estrogen-responsive DNA elements correlates with their synergistic hormone-dependent enhancer activity. The EMBO journal *8*, 3781-3791.

Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A.E., Kel-Margoulis, O.V.*, et al.* (2003). TRANSFAC: transcriptional regulation, from patterns to profiles. Nucleic acids research *31*, 374-378.
McDonnell, D.P., Clemm, D.L., Hermann, T., Goldman, M.E., and Pike, J.W. (1995). Analysis of estrogen receptor function in vitro reveals three distinct classes of antiestrogens. Molecular endocrinology (Baltimore, Md *9*, 659-669.

McGrath, J.L. (2005). Dynein motility: four heads are better than two. Curr Biol *15*, R970-972.

McInerney, E.M., Weis, K.E., Sun, J., Mosselman, S., and Katzenellenbogen, B.S. (1998). Transcription activation by the human estrogen receptor subtype beta (ER beta) studied with ER beta and ER alpha receptor chimeras. Endocrinology *139*, 4513-4522.
McKenna, N.J., and O'Malley, B.W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. Cell *108*, 465-474.

Murrell, A., Heeson, S., and Reik, W. (2004). Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nature genetics *36*, 889-893.

Nass, S.J., Herman, J.G., Gabrielson, E., Iversen, P.W., Parl, F.F., Davidson, N.E., and Graff, J.R. (2000). Aberrant methylation of the estrogen receptor and E-cadherin 5' CpG islands increases with malignant progression in human breast cancer. Cancer research *60*, 4346-4348.

Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Pettersson, K., Warner, M., and Gustafsson, J.A. (2001). Mechanisms of estrogen action. Physiological reviews *81*, 1535-1565.

Nishidate, T., Katagiri, T., Lin, M.L., Mano, Y., Miki, Y., Kasumi, F., Yoshimoto, M., Tsunoda, T., Hirata, K., and Nakamura, Y. (2004). Genome-wide gene-expression profiles of breast-cancer cells purified with laser microbeam microdissection: identification of genes associated with progression and metastasis. International journal of oncology *25*, 797-819.

Oesterreich, S., Deng, W., Jiang, S., Cui, X., Ivanova, M., Schiff, R., Kang, K., Hadsell, D.L., Behrens, J., and Lee, A.V. (2003). Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. Cancer research *63*, 5203-5208.

Olave, I.A., Reck-Peterson, S.L., and Crabtree, G.R. (2002). Nuclear actin and actin-related proteins in chromatin remodeling. Annual review of biochemistry *71*, 755-781.

Osborne, C.K. (1998). Steroid hormone receptors in breast cancer management. Breast cancer research and treatment *51*, 227-238.

Paech, K., Webb, P., Kuiper, G.G., Nilsson, S., Gustafsson, J., Kushner, P.J., and Scanlan, T.S. (1997). Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. Science (New York, NY *277*, 1508-1510.

Paixao, T., Carvalho, T.P., Calado, D.P., and Carneiro, J. (2007). Quantitative insights into stochastic monoallelic expression of cytokine genes. Immunology and cell biology *85*, 315-322.

Pavesi, G., Mauri, G., and Pesole, G. (2001). An algorithm for finding signals of unknown length in DNA sequences. Bioinformatics (Oxford, England) *17 Suppl 1*, S207-214.

Peinado, H., Ballestar, E., Esteller, M., and Cano, A. (2004). Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. Molecular and cellular biology *24*, 306-319.

Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nature reviews *7*, 415-428.

Percipalle, P., and Farrants, A.K. (2006). Chromatin remodelling and transcription: be-WICHed by nuclear myosin 1. Current opinion in cell biology *18*, 267-274.

Percipalle, P., and Visa, N. (2006). Molecular functions of nuclear actin in transcription. The Journal of cell biology *172*, 967-971.

Perez-Moreno, M.A., Locascio, A., Rodrigo, I., Dhondt, G., Portillo, F., Nieto, M.A., and Cano, A. (2001). A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. The Journal of biological chemistry *276*, 27424-27431.

Perissi, V., Aggarwal, A., Glass, C.K., Rose, D.W., and Rosenfeld, M.G. (2004). A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. Cell *116*, 511-526.

Perissi, V., and Rosenfeld, M.G. (2005). Controlling nuclear receptors: the circular logic of cofactor cycles. Nat Rev Mol Cell Biol *6*, 542-554.

Planas-Silva, M.D., and Waltz, P.K. (2007). Estrogen promotes reversible epithelial-to-mesenchymal-like transition and collective motility in MCF-7 breast cancer cells. The Journal of steroid biochemistry and molecular biology *104*, 11-21.

Porter, W., Saville, B., Hoivik, D., and Safe, S. (1997). Functional synergy between the transcription factor Sp1 and the estrogen receptor. Molecular endocrinology (Baltimore, Md *11*, 1569-1580.

Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995). MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic acids research *23*, 4878-4884.

Rayala, S.K., den Hollander, P., Balasenthil, S., Yang, Z., Broaddus, R.R., and Kumar, R. (2005). Functional regulation of oestrogen receptor pathway by the dynein light chain 1. EMBO reports *6*, 538-544.

Razandi, M., Pedram, A., and Levin, E.R. (2000). Plasma membrane estrogen receptors signal to antiapoptosis in breast cancer. Molecular endocrinology (Baltimore, Md *14*, 1434-1447.

Revankar, C.M., Cimino, D.F., Sklar, L.A., Arterburn, J.B., and Prossnitz, E.R. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signaling. Science (New York, NY *307*, 1625-1630.

Rizk, R.S., and Walczak, C.E. (2005). Chromosome dynamics: actin's gone fishing. Curr Biol *15*, R841-842.

Rochefort, H., Platet, N., Hayashido, Y., Derocq, D., Lucas, A., Cunat, S., and Garcia, M. (1998). Estrogen receptor mediated inhibition of cancer cell invasion and motility: an overview. The Journal of steroid biochemistry and molecular biology *65*, 163-168.

Rosenfeld, M.G., and Glass, C.K. (2001). Coregulator codes of transcriptional regulation by nuclear receptors. The Journal of biological chemistry *276*, 36865-36868.

Rosenfeld, M.G., Lunyak, V.V., and Glass, C.K. (2006). Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes & development *20*, 1405-1428.

Russo, J., Ao, X., Grill, C., and Russo, I.H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast cancer research and treatment *53*, 217-227.

Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W.W., and Lenhard, B. (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic acids research *32*, D91-94.

Sandve, G.K., and Drablos, F. (2006). A survey of motif discovery methods in an integrated framework. Biology direct *1*, 11.

Scafoglio, C., Ambrosino, C., Cicatiello, L., Altucci, L., Ardovino, M., Bontempo, P., Medici, N., Molinari, A.M., Nebbioso, A., Facchiano, A.*, et al.* (2006). Comparative gene expression profiling reveals partially overlapping but distinct genomic actions of different antiestrogens in human breast cancer cells. Journal of cellular biochemistry *98*, 1163-1184.

Schneider, T.D., and Stephens, R.M. (1990). Sequence logos: a new way to display consensus sequences. Nucleic acids research *18*, 6097-6100.

Schneider, T.D., Stormo, G.D., Gold, L., and Ehrenfeucht, A. (1986). Information content of binding sites on nucleotide sequences. Journal of molecular biology *188*, 415-431.

Schwabe, J.W., Chapman, L., Finch, J.T., and Rhodes, D. (1993). The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements. Cell *75*, 567-578.

Shang, Y., Hu, X., DiRenzo, J., Lazar, M.A., and Brown, M. (2000). Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell *103*, 843-852.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., and Greene, G.L. (1998). The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell *95*, 927-937.

Siitonen, S.M., Kononen, J.T., Helin, H.J., Rantala, I.S., Holli, K.A., and Isola, J.J. (1996). Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. American journal of clinical pathology *105*, 394-402.

Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., and Liao, J.K. (2000). Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature *407*, 538-541.

Sinha, S., and Tompa, M. (2003). YMF: A program for discovery of novel transcription factor binding sites by statistical overrepresentation. Nucleic acids research *31*, 3586-3588.

Smith, C.L. (1998). Cross-talk between peptide growth factor and estrogen receptor signaling pathways. Biology of reproduction *58*, 627-632.

Soulez, M., and Parker, M.G. (2001). Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. Journal of molecular endocrinology *27*, 259-274.

Spector, D.L. (1993). Macromolecular domains within the cell nucleus. Annual review of cell biology *9*, 265-315.

Spector, D.L., Fu, X.D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. The EMBO journal *10*, 3467-3481.

Spiegelman, B.M., and Heinrich, R. (2004). Biological control through regulated transcriptional coactivators. Cell *119*, 157-167.

Spilianakis, C.G., Lalioti, M.D., Town, T., Lee, G.R., and Flavell, R.A. (2005). Interchromosomal associations between alternatively expressed loci. Nature *435*, 637-645.

Stormo, G.D. (2000). DNA binding sites: representation and discovery. Bioinformatics (Oxford, England) *16*, 16-23.

Stormo, G.D., and Hartzell, G.W., 3rd (1989). Identifying protein-binding sites from unaligned DNA fragments. Proceedings of the National Academy of Sciences of the United States of America *86*, 1183-1187.

Sun, G., Porter, W., and Safe, S. (1998). Estrogen-induced retinoic acid receptor alpha 1 gene expression: role of estrogen receptor-Sp1 complex. Molecular endocrinology (Baltimore, Md *12*, 882-890.

Thiery, J.P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nature reviews *2*, 442-454.

Tompa, M., Li, N., Bailey, T.L., Church, G.M., De Moor, B., Eskin, E., Favorov, A.V., Frith, M.C., Fu, Y., Kent, W.J.*, et al.* (2005). Assessing computational tools for the discovery of transcription factor binding sites. Nature biotechnology *23*, 137-144.

Varshochi, R., Halim, F., Sunters, A., Alao, J.P., Madureira, P.A., Hart, S.M., Ali, S., Vigushin, D.M., Coombes, R.C., and Lam, E.W. (2005). ICI182,780 induces p21Waf1 gene transcription through releasing histone deacetylase 1 and estrogen receptor alpha from Sp1 sites to induce cell cycle arrest in MCF-7 breast cancer cell line. The Journal of biological chemistry *280*, 3185-3196.

Vega, V.B., Lin, C.Y., Lai, K.S., Kong, S.L., Xie, M., Su, X., Teh, H.F., Thomsen, J.S., Yeo, A.L., Sung, W.K.*, et al.* (2006). Multiplatform genome-wide identification and modeling of functional human estrogen receptor binding sites. Genome biology *7*, R82.
Webb, P., Nguyen, P., Shinsako, J., Anderson, C., Feng, W., Nguyen, M.P., Chen, D., Huang, S.M., Subramanian, S., McKinerney, E.*, et al.* (1998). Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Molecular endocrinology (Baltimore, Md *12*, 1605-1618.

Wiesen, J.F., Young, P., Werb, Z., and Cunha, G.R. (1999). Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. Development (Cambridge, England) *126*, 335-344.

Williams, R.R., Azuara, V., Perry, P., Sauer, S., Dvorkina, M., Jorgensen, H., Roix, J., McQueen, P., Misteli, T., Merkenschlager, M.*, et al.* (2006). Neural induction promotes large-scale chromatin reorganisation of the Mash1 locus. Journal of cell science *119*, 132-140.

Zeps, N., Bentel, J.M., Papadimitriou, J.M., D'Antuono, M.F., and Dawkins, H.J. (1998). Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. Differentiation; research in biological diversity *62*, 221-226.

Zhang, G., Taneja, K.L., Singer, R.H., and Green, M.R. (1994). Localization of pre-mRNA splicing in mammalian nuclei. Nature *372*, 809-812.

Zhao, Z., Tavoosidana, G., Sjolinder, M., Gondor, A., Mariano, P., Wang, S., Kanduri, C., Lezcano, M., Sandhu, K.S., Singh, U.*, et al.* (2006). Circular chromosome conformation capture (4C) uncovers extensive networks of epigenetically regulated intra- and interchromosomal interactions. Nature genetics *38*, 1341-1347.

Zink, D., Amaral, M.D., Englmann, A., Lang, S., Clarke, L.A., Rudolph, C., Alt, F., Luther, K., Braz, C., Sadoni, N.*, et al.* (2004). Transcription-dependent spatial arrangements of CFTR and adjacent genes in human cell nuclei. The Journal of cell biology *166*, 815-825.

Zink, D., Cremer, T., Saffrich, R., Fischer, R., Trendelenburg, M.F., Ansorge, W., and Stelzer, E.H. (1998). Structure and dynamics of human interphase chromosome territories in vivo. Human genetics *102*, 241-251.

Zou, A., Marschke, K.B., Arnold, K.E., Berger, E.M., Fitzgerald, P., Mais, D.E., and Allegretto, E.A. (1999). Estrogen receptor beta activates the human retinoic acid receptor alpha-1 promoter in response to tamoxifen and other estrogen receptor antagonists, but not in response to estrogen. Molecular endocrinology (Baltimore, Md *13*, 418-430.

MEETING AND PUBBLICATION

Cardamone MD, Bardella C, Menini N, Di Renzo MF, De Bortoli M
Mechanisms of E-cadherin suppression in cells transformed by an active tyrosine kinase
13th Meeting FISV – Riva del Garda September 30- October 3, 2004

Cardamone MD, Bardella C, Gutierrez A, Di Croce L, Di Renzo M.F, De Bortoli M.
Mechanisms of E-Cadherin repression in epithelial-mesenchimal transition (EMT) like model
Abstract selected for oral presentation at the meeting "Epigenetic and Transcription" in Milan May 25-27, 2005

Cardamone MD, Cordero F, Lazzarato F, Cicatiello G, Scafoglio C, Cimino D, Basile W, Weisz A, Calogero RA, De Bortoli M
Bioinformatic and Functional Analysis of ERE-containing Genes.
EMBO Conference NUCLEAR RECEPTORS: FROM CHROMATIN TO DISEASE
Villa Alba, Gardone Riviera, Lake Garda (Italy) September 29- October 1, 2005

Nunez E, Kwon Y-S, Hutt KR, Hu Q, **Cardamone MD**, Garcia-Bassets I, Rose DW, Glass CG, Rosenfeld MG, and Fu K-D
Nuclear Receptor-Activated Transcription Requires Nuclear Actin/Myosin-mediated Interchromosomal Gene Networking in Interchromatin Granules
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