Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA

Biologia e Fisiologia Cellulare

Ciclo XX

Settore scientifico disciplinare di afferenza: BIO 18/ GENETICA

TITOLO TESI

Regulation of SRC-3 Localization and Dynamics By Phosphorylation During ERα-Dependent Transcriptional Activation

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Esame finale anno 2008

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ABSTRACT

Transcription is controlled by promoter-selective transcriptional factors (TFs), which bind to *cis*-regulatory enhancers elements, termed hormone response elements (HREs), in a specific subset of genes. Regulation by these factors involves either the recruitment of coactivators or corepressors and direct interaction with the basal transcriptional machinery (1). Hormone-activated nuclear receptors (NRs) are well characterized transcriptional factors (2) that bind to the promoters of their target genes and recruit primary and secondary coactivator proteins which possess many enzymatic activities required for gene expression (1,3,4).

In the present study, using single-cell high-resolution fluorescent microscopy and high throughput microscopy (HTM) coupled to computational imaging analysis, we investigated transcriptional regulation controlled by the estrogen receptor *alpha* (ERα), in terms of large scale chromatin remodeling and interaction with the associated coactivator SRC-3 (Steroid Receptor Coactivator-3), a member of p160 family (28) primary coactivators. ERα is a steroid-dependent transcriptional factor (16) that belongs to the NRs superfamily (2,3) and, in response to the hormone 17-ß estradiol (E2), regulates transcription of distinct target genes involved in development, puberty, and homeostasis (8,16). ERα spends most of its lifetime in the nucleus and undergoes a rapid (within minutes) intranuclear redistribution following the addition of either agonist or antagonist (17,18,19).

We designed a HeLa cell line (PRL-HeLa), engineered with a chromosome-integrated reporter gene array (PRL-array) containing multicopy hormone response-binding elements for ERα that are derived from the physiological enhancer/promoter region of the prolactin gene. Following GFP-ER transfection of PRL-HeLa cells, we were able to observe *in situ* ligand dependent (i) recruitment to the array of the receptor and associated coregulators, (ii) chromatin remodeling, and (iii) direct transcriptional readout of the reporter gene. Addition of E2 causes a visible opening (decondensation) of the PRL-array, colocalization of RNA Polymerase II, and transcriptional readout of the reporter gene, detected by mRNA FISH. On the contrary, when cells were treated with an ERα antagonist (Tamoxifen or ICI),

a dramatic condensation of the PRL-array was observed, displacement of RNA Polymerase II, and complete decreasing in the transcriptional FISH signal.

All p160 family coactivators (28) colocalize with ERα at the PRL-array. Steroid Receptor Coactivator-3 (SRC-3/AIB1/ACTR/pCIP/RAC3/TRAM1) is a p160 family member and a known oncogenic protein (4,34). SRC-3 is regulated by a variety of posttranslational modifications, including methylation, phosphorylation, acetylation, ubiquitination and sumoylation (4,35). These events have been shown to be important for its interaction with other coactivator proteins and NRs and for its oncogenic potential (37,39). A number of extracellular signaling molecules, like steroid hormones, growth factors and cytokines, induce SRC-3 phosphorylation (40). These actions are mediated by a wide range of kinases, including extracellular-regulated kinase 1 and 2 (ERK1-2), c-Jun N-terminal kinase, p38 MAPK, and IκB kinases (IKKs) (41,42,43). Here, we report SRC-3 to be a nucleocytoplasmic shuttling protein, whose cellular localization is regulated by phosphorylation and interaction with ERα. Using a combination of high throughput and fluorescence microscopy, we show that both chemical inhibition (with U0126) and siRNA downregulation of the MAP/ERK1/2 kinase (MEK1/2) pathway induce a cytoplasmic shift in SRC-3 localization, whereas stimulation by EGF signaling enhances its nuclear localization by inducing phosphorylation at T^{24}, S^{857}, and S^{860}, known partecipants in the regulation of SRC-3 activity (39). Accordingly, the cytoplasmic localization of a non-phosphorylatable SRC-3 mutant further supports these results. In the presence of ERα, U0126 also dramatically reduces: hormone-dependent colocalization of ERα and SRC-3 in the nucleus; formation of ER-SRC-3 coimmunoprecipitation complex in cell lysates; localization of SRC-3 at the ER-targeted prolactin promoter array (PRL-array) and transcriptional activity. Finally, we show that SRC-3 can also function as a cotransporter, facilitating the nuclear-cytoplasmic shuttling of estrogen receptor.

While a wealth of studies have revealed the molecular functions of NRs and coregulators, there is a paucity of data on how these functions are spatiotemporally organized in the cellular context. Technically and conceptually, our findings have a new impact upon evaluating gene transcriptional control and mechanisms of action of gene regulators.

Key words:

Transcriptional Coregulators; p160 Family Coactivators; SRC-3/AIB1; Estrogen Receptor *Alpha*; Nuclear Receptors; Chromatin Remodeling; Gene Transcription; High Throughput Microscopy; Human Cancer.

Mostly used Abbreviation:

AIB1 = Amplified in Breast Cancer 1; *CBP* = CREB (cyclic AMP response element binding protein) binding protein; *ER* = Estrogen Receptor; *EGF* = Epidermal Growth Factor; *GFP* = Green Fluorescent Protein; *GTF* = General Transcriptional Factor; *HER2* = Human Epidermal Growth Factor Receptor 2; *HRE* = Hormone Response Element ; *HTM* = High Throughput Microscopy; *NR* = Nuclear Receptor; *PTM* = Posttranscriptional Modification; *SRC* = Steroid Receptor Coactivator; *SD* = Standard Deviation; *SE* = Standard Error; *TF* = Transcriptional Factor.

INTRODUCTION

PART 1:
Nuclear Receptors and Transcriptional Control of Gene Expression

Nuclear receptors (NRs) comprise a variety of ligand-regulated transcriptions factors and play a central role in the body's ability to transduce the endocrine hormones signaling. Gene-specific NR-mediated transcriotion results from the ability of NRs to recognize specific sequences within the promoter/enhancer region of their target genes. The complex relationship of NRs with the RNA Polymerase II holoenzyme and the chromatin environment surrounding the gene is fundamental for the specificity of gene expression. Coregulators (coactivators or corepressors) are the nub of this critical aspect of transcriptional control.

Contents:

- Nuclear Receptor Superfamily
- Coactivators of Nuclear Receptors
- Corepressors of Nuclear Receptors
- Chromatin Dynamics and Gene Transcriptional Activation
- General Transcription Factors

Nuclear Receptor Superfamily

The mammalian nuclear receptors (NRs) are a family of approximately 50 members of transcriptional activator proteins (2). NRs regulate specific gene expression in response to the binding with small lipophilic molecules including steroid hormones, thyroid hormone, retinoic acid, vitamin D, fatty acids and eicosanoids. Genes regulated by NRs are involved in a broad variety of cellular processes (3,34) like metabolism, development, reproduction, growth and differentiation. Their primary function is to mediate transcriptional response in target cells to hormones and a variety of other metabolic ligands.

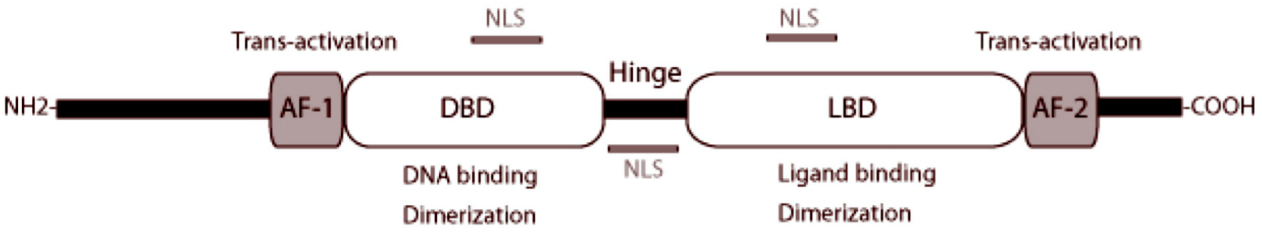

Figure 1. Canonical representation of functional domains of nuclear hormone receptors. Nuclear receptors share a modular structure constituted of a non-conserved amino-terminal domain that contains the transcriptional activation function 1 (AF-1), a conserved DNA-binding domain (DBD), a hinge region, and a carboxyl-terminal ligand-binding domain (LBD) that contains the transcriptional activation function 2 (AF-2). Nuclear localization sequences (NLS) and dimerization domains are also shown.

Three-dimensional structure analysis of different NRs reveals a common basic structure (9,16) (***Fig.1***), presenting three main functional domains: an high conserved DNA binding domain (DBD) that recognize a specific enhancer element called hormone response element (HRE); a carboxyl terminal ligand-binding domain (LBD), which binds the specific hormone, inducing the active conformation of the receptor; a less well defined amino terminal domain that is not conserved. The DNA binding domain (3,11) contains two zinc-

finger modules (10) coordinated with four conserved cysteine residues: a DNA recognition α-helix extends between the two zinc-fingers, making specific contacts in the major groove, while a second α-helix is required for the overall folding of the domain (***Fig.2.A***). This structural motif is a distinctive feature of all NRs. The LBD domain (12) usually consists of 12 α-helices folding with a conserved hydrophobic cavity in which the ligand is buried. Helix 12 extends away from the LBD core in the absence of ligand and undergoes a consistent repositioning in response to the hormone binding, sealing the entry site of the binding cavity and creating a hydrophobic surface that is recognized by primary co-activators (***Fig.2.B***). In addition to providing a binding pocket for the ligand, the LBD domain contains recognition sites for the interaction with chaperones (13) and possesses a dimerization function (14) (independent on the interaction with DNA) and a ligand-dependent transcriptional activation function (15), termed activation function AF-2. The DBD domain contains a second dimerization region that is dependent on DNA binding (21), with the role of stabilizing the receptor-DNA complex. The amino terminal domain is the most divergent with respect to length and amino acid sequence and little is known about its function. Some NRs, like steroid receptors, have a long N-terminus that retains a second trans-activation domain (22,23), called AF-1 (***Fig.1***). Through the activation domains the hormone-bound NR recruits coactivators to the target gene promoter.

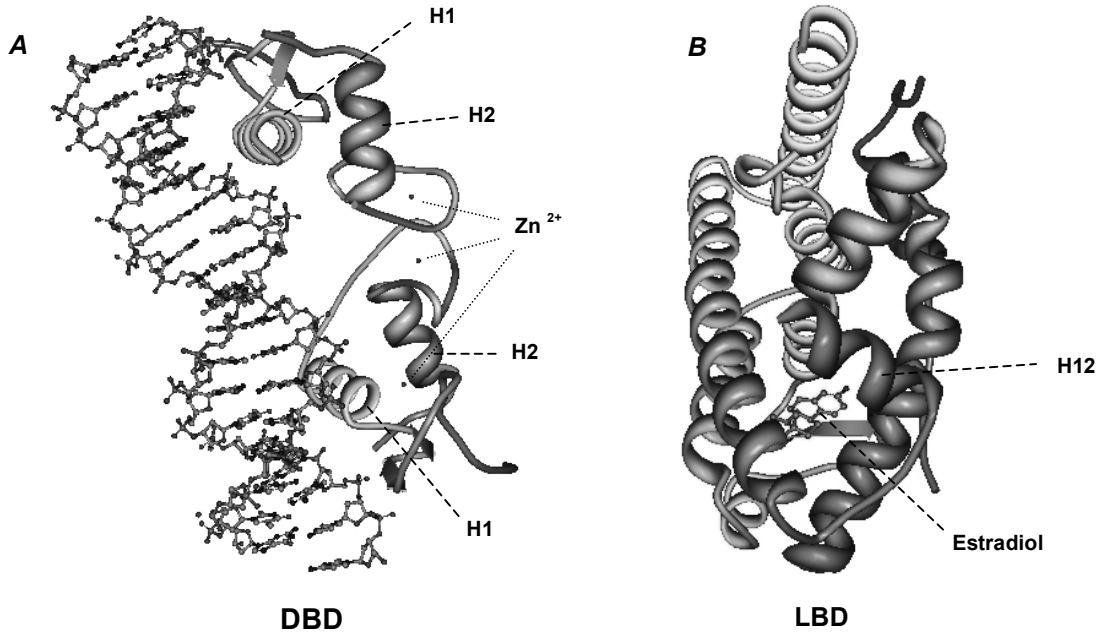

Figure 2. 3D molecular structure of DNA-binding and ligand-binding domain of NRs. (A) Conserved structure of the DNA-binding domain (DBD) of nuclear receptors, showing a dimer complexed to DNA. The recognition α-helix 1 (H1) fits into the major groove, while helix 2 (H2) is important to hold the overall structure and the zinc-conjugated dimerization complex. Zinc atoms are shown as balls while DNA is represented as a ball-and-stick diagram. (B) Conserved structure of the ligand-binding domain (LBD) of nuclear receptors. The crystal structure of estrogen receptor alpha LBD complexed with estradiol is depicted. Helix 12 contains the AF-2 transactivation function and folds over the ligand-binding pocket.

Member of the NRs family are classified both physiogenetically and by differences in their mechanism of action (2). NRs have a DNA-binding domain (DBD) that recognizes the specific enhancer element in at least three differing modes: according to this DNA-specificity, NRs are classified in three classes. Class I of NRs consists of steroid hormones receptors (SRs), like receptors for glucocorticoids (GR), progesterone (PR), androgen (AR), mineralocorticoids (MR) and estrogen (ER): they recognize a consensus sequence of 6 nucleotides arranged as an inverted repeat (24) and dimerize only after the binding to DNA, while in the absence of hormone SRs associate with a chaperone complex that help to keep the receptor in a folded state off of DNA until activated by the hormone (13,14).

Class II and class III of NRs (nonsteroid receptors) do not seam to form a stable association with chaperones and bind to DNA constitutively: activation occurs subsequently to DNA binding. Class II consists of vitamin D receptor (VDR), thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator activated receptor (PPAR) and bind to DNA generally as heterodimers with another element of the NR family, retinoid X receptor (RXR). Although RXR can function as a transcription factor itself in response to the binding to its ligand, 9-*cis*-retinoic acid, its main role is to act as a heterodimer partner (21) required for class II of NRs to bind with high affinity to the specific HRE. The class II RXR-heterodimer receptors recognize a hexanucleotide HRE arranged as direct repeat: variable nucleotide spacing in the half-site of the direct repeat provides the specificity of binding. Class III is the third and largest class of NRs and comprises orphan nuclear receptors (26), for which physiological ligand remain to be identify. Orphan receptors are capable of binding to DNA either as homodimers or as heterodimers with RXR (21). They can also stably bind to extend half-site of consensus sequence thanks to the presence of a so-called Grip Box in the C-terminal prolonged region of the DBD (27). The Grip Box forms a loop structure capable to lie in the minor groove of DNA, immediately after the 5' of the consensus hexanucleotide, providing a protein-DNA interaction in addiction to the core DBD recognition helix that lays in the major groove.

Class I receptors, in the absence of ligand, are sequestered by heat shock proteins and, in this state, are not thought to influence the rate of transcription at target genes. Conversely, class II receptors are able to bind DNA in the absence of ligand and often may exert a repressive effect upon the activation of their promoters, a phenomenon referred to as silencing (3).

Concerning to the actual model of mechanism of action in gene expression (1), DNA-bound NRs enable the recruitment of several different complexes of proteins (4,6), including either coactivators, which mediate transcriptional potential of activated NRs, or corepressors, which transduce the nonactivating function of NRs and trigger transcriptional repression. Accordingly, there is a combinatorial NR-mediated control of gene transcription (***Fig.3***): initial binding of activating ligand results in dissociation of corepressors from DNA-

bound NRs and recruitment of primary and secondary coactivator proteins (8) which locally remodel nucleosomal structure (5) and promote a readily accessible chromatin context for the assembly of preinitiation complex, resulting in the activation of RNA polymerase II and the basal transcriptional machinery (9), and initiation of transcription.

Combinatorial NR-mediated control of gene transcription

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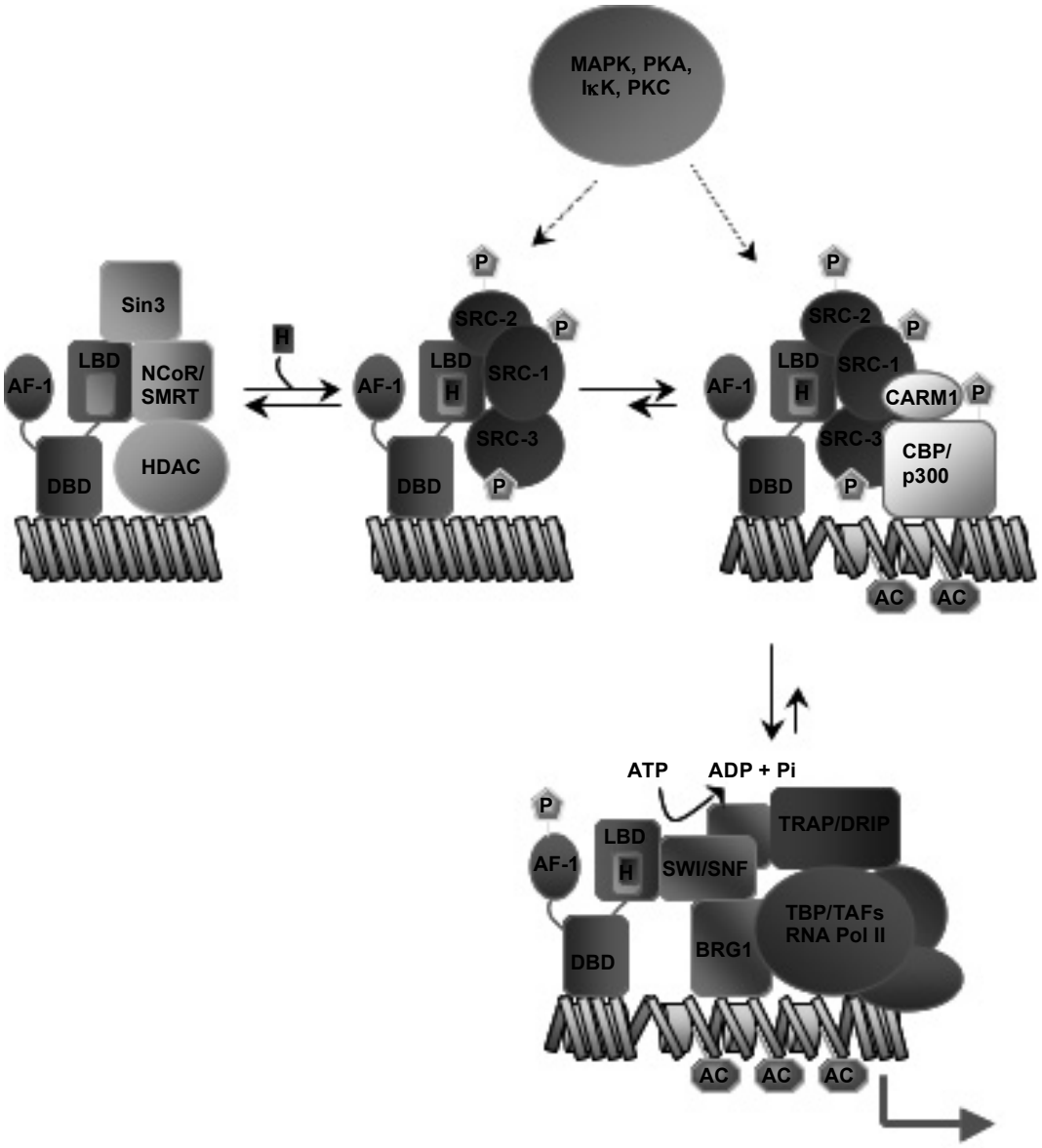

Figure 3. Combinatorial NR-mediated control of gene transcription. Representative model of gene transcription activation and assembling of transcriptional complex to the promoter. According to this model, coactivators and corepressors compete for the interaction with transcriptional factors. Chromatin is normally condensed and unexpressed. Following the specific cellular signal, transcriptional corepressors are displaced while transcriptional coactivators and activators are combinatorially recruited to the promoter. Binding of SRCs and CBP results in local acetyltransferase activity and chromatin opening. Recruitment of SWI/SNF remodeling complex causes the modification of the overall nucleosomal structure. TRAP/DRIP directly contacts components of the basal transcription machinery to effect transcriptional initiation. Us a result, the promoter region becomes accessible to the transcriptional machine and mRNA is transcribed from the initiation site. Local coactivator recruitment may vary: a promoter in a readily accessible chromatin context may not require significant chromatin remodeling or histone acetyltransferase activity for assembling of a preinitiation complex.

The p160 family members are primary coactivators (28) that directly bind to activated NRs and recruit secondary coactivators with local acetyl-transferase activity (CBP/p300) or methyl-transferase activity (CARM1). The SWI/SNF coactivator complex and other similar complexes have an ATP-dependent nucleosome remodeling activity, which contributes to a significant overall alteration of the chromatin structure. The TRAP/DRIP complex directly interacts with RNA polymerase II and apparently participates in the recruitment and activation of the polymerase. Transcription activation arises from a complex relationship between NRs, tens of distinct coregulators and the genes (***Fig.3***). Most importantly, coregulators are versatile factors that influence not only transcriptional initiation but also elongation, splicing, and translation. Additionally, they can also regulate a variety of biological processes outside of the nucleus, acting as propagators of post-translational modifications. This important concept underlines the sophisticated and dynamic epigenetic control that has been developed in the mammalian phenotype.

Coactivators of Nuclear Receptors

The NRs coactivators, as well as NRs corepressors, belong to that group of proteins termed coregulators (7). We conventionally define coactivators as molecules that interact with NRs and enhance their transactivation. Analogously, corepressors are molecules that lower the transcription rate at NRs target genes. Most coregulators are, by definition, rate limiting for nuclear receptors activation or repression, but do not significantly alter basal transcription.

It is now over a decade since the first NRs coactivator, SRC-1 was cloned and discovered (29,30) to be a new type of transcriptional factor that do not bind to DNA, but directly or indirectly bind to NRs, mediating their transcriptional activity. Initially, we believed that coactivators were simply adaptor proteins that stabilized the general transcription machinery at the TATA box. Over the past decade, we realized that they perform virtually all of the reactions needed for controlling the enhancer-dependent gene expression (35) and coordinate many aspects of hormone-receptor physiology (31,34), including the concepts of tissue specificity, hormone kinetics (32), integration of membrane and nuclear signaling (39), nuclear coordination of mitochondria (34), even mRNA translation (45) and cell motility processes (44). In fact, different coregulators appear to coordinately control many diverse DNA-binding transcriptional factors in order to implement major physiological processes within the cell such as inflammation (42,43), fat and carbohydrate metabolism (46), and cell growth (34,35).

Unlike general transcriptional factors (GTFs), coactivators interact directly or in close association with the enhancer DNA-binding transcriptional factors and are not constitutive of the RNA polymerase II holocomplex. Coactivator recruitment is usually, but not always, ligand dependent. Coactivators can be subdivided into two groups: primary coactivators and secondary coactivators, which actually could be referred to as co-coactivators (161). Co-coactivators are constituents of multisubunit coactivator complexes, which contribute to the enhancement of NR-mediated transcription but do not directly contact NRs, like primary coactivators (162). Coactivators always function in multiprotein complexes: they

are recruited to the target gene following NRs binding to the promoter and accomplish many enzymatic activities (28), like modification of chromatin structure (acetylation, methylation, phosphorylation), required for initiation of transcription, elongation of RNA chain, RNA splicing and finally termination of transcription. Even closely related coactivators (i.e. SRC-1 and SRC-3) have specific and different gene regulatory functions, associated with the control of metabolism, reproduction, cardiovascular system, neurology and they have great importance in many diseases, including cancer. In addiction, these factors may function as molecular gates to enable integration of diverse signal transduction pathways at NRs-regulated promoters. Over 270 coregulators have been identified till now and almost 160 of them are associated with some pathological state. The most frequent of the pathologies are a variety of cancers in which coregulators have been shown to serve as either oncogenes or tumor suppressors, depending upon signaling and cell context.

Corepressors of Nuclear Receptors

Originally corepressors were predicted to exist based upon the ability of the unliganded thyroid hormone receptor to function as a transcriptional repressor (164). Transcriptional repression (47) might in some way reflect transcriptional activation (48), representing its negative counterpart. In fact, an increasing number of studies have been providing new insights into how transcriptional corepressors, in addition to blocking activators entrance to the promoter site, may basically assemble repression complexes that include chromatin modification factors. Accordingly, NR corepressors (4), like NCoR or SMRT (49), are recruited by NRs either in the absence of ligand (50) or following the binding of NR to the antagonist (such as Tamoxifen). Normally, corepressors are supposed to repress gene expression primarily through their interaction with unliganded NR (163). Interaction with NR is mediated through amphipathic helical peptides, called CoRNR boxes (51), which are actually similar to the previously characterized NR-coactivator box. As a result, transcriptional corepressors are not only structurally similar but also functionally

comparable to coactivators. Histone deacetylation, for example, antagonizes coactivator acetyltransferase activity, although corepressors, lacking intrinsic deacetylase domains, require recruited factors such as Sin3 and histone deacetylases (HDACs) (52,53). Similarly to coactivators, corepressors are also targeted by posttranslational modifications. For example, intracellular localization of coregulators SMRT and NCoR is controlled by phosphorylation (169,170). Phosphorylation of SMRT results in its redistribution to the cytoplasm, neutralizing it corepressing function. Also, as observed in neural stem cells, NCoR is exported to the cytoplasm when phosphorylated by the Akt kinase, affecting the ability to differentiate into astrocytes (171). NCoR is subjected to SUMOylation too, which enhances its corepressing function (172).

NCoR (nuclear receptor corepressor), also know as RIP-3, is probably the most well established corepressor (50). It is a 270-KD protein that specifically binds unliganded TRα and RARα, while only partial interaction has been observed with RXR, ER, or GR (175). Mutational analysis of the TR LBD has identified a domain, termed the NCoR box, which is indispensable for the interaction of TR with NCoR (50). To substantiate the function of NCoR as a mediator of ligand-independent repression, fusion of NCoR to the Gal4-DBD effects potent repression at the reporter promoter bearing Gal4-DBD-binding sites (50). Moreover, deletion mutants have identified two receptor-interacting domains (RIDs) in the C-terminal portion of NCoR (175).

SMRT (silencing mediator for retinoic and thyroid hormone receptor) is the other important well-known corepressor of NRs. It was isolated on the basis of its interaction with RAR, RXR, and TR (176). Two C-terminal RID domains are present in the sequence of SMRT and are capable of differentially interact with individual receptors: for example, RARα binds RID-1 exclusively, whereas TR binds both domains with equal affinity (177).

NCoR and SMRT are structurally similar (50, 178) but also differ functionally in several respects. For example, the functional diversity among RAR isoforms has been ascribed to the differential interaction with SMRT or NCoR: whereas RARα and RARγ interact with both corepressors, RARβ exhibits no affinity for either SMRT or NCoR (177). These corepressors differ most notably, however, in the mediation of transcriptional repression by

certain orphan receptors. Orphan receptor DAX-1, which interacts with NCoR (179), does not interact with SMRT. Similarly, RevErb exclusively recruits NCoR to the DNA, but not SMRT to effect transcriptional repression (180).

TRUP (thyroid receptor-uncoupled protein) is another transcriptional repressor, which attenuates hormone-dependent transactivation by TR and RAR, but has no effects on ER and RXR transactivation (181).

Notably, steroid hormone receptors, like ER, PR or GR, have little DNA-binding activity in the absence of hormone; indeed, steroid receptors are sequestered in the cytoplasm by ternary interactions with hsp90 and hsp70 (3). Anyway, a number of studies have since demonstrated the interaction of nuclear receptor corepressors with antagonist-bound steroid receptors. SMRT has been shown to interact with ER in the presence of tamoxifen (182). NCoR is also likewise able to interact with antagonist-bound PR-LBD and overexpression of NCoR and SMRT markedly suppress the partial agonist activity of RU486 bound to PR (183).

Chromatin Dynamics and Gene Transcriptional Activation

Activation of gene transcription definitely occurs as a result of combinatorial control of gene expression accomplished by NR-mediated recruitment of functionally distinct coregulator complexes: multiple coactivators contribute in a complementary way to transcriptional activation. Hence, it has become clear that transcriptional coregulators act as master directors of chromatin remodeling and nucleosomal organization, communicating with each other and with the transcriptional apparatus through a complicate network of post-translational modifications. The entire process of NR-induced coactivators recruitment, assembly of transcription machinery, and initiation of transcription is dynamic and may happen in a cyclic fashion. For example, the ERα transcription complex appears to repeatedly cycle on and off of the target promoters in the presence of estrogen (85). The cycling is probably regulated by phosphorylation of the C-terminal region of RNA-Polymerase II, exchange of coactivators (especially CBP and p300), and

covalent modifications, phosphorylation and acetylation, of coregulators (especially SRCs) (78,86,87). Specifically, post-translational modification of coregulators has been emerging as an elegant mechanism for controlling tissue- and promoter-specificity of gene expression. Among several identified coregulators, p160 family members and the histone acetyltransferase CBP appear to be particularly prone to modifications by kinase-mediated signaling pathways (78). Kinase-mediated signaling may also communicate directly with NRs: AF-1 domain phosphorylation might serve to further consolidate ligand-dependent NR-SRC interaction or to recruit SRCs to the promoter in absence of ligand (***Fig.3***).

Currently, two secondary coactivators have been shown to be part of the p160 primary coactivator complex and collaborate with p160 coactivators to mediate transcriptional activation: the histone acetyltransferase CBP (or the related protein p300), and the histone methyltransferase CARM1. Accumulating data support a sequential mechanism for p160 function: CBP/p300, CARM1, pCAF, PRMT1, and other specific secondary components are recruited to the promoter following the direct interaction with ligand-bound NRs and specifically acetylate and methylate histones around the promoter region. In fact, posttranscriptional modifications of histones represent the major target for coregulator-mediate signaling and the final conduit to the regulation of gene expression (168). In the eukaryotic chromosome the histone represents the basic protein unit of the nucleosome, which constitutes the regularly repeating structure in which DNA is organized. The nucleosome core particle comprises a histone octamer, made up of two copies each of histones H2A, H2B, H3, and H4, around which is wrapped 1.7 turns of left-handed DNA superhelix (184). Much importantly, the net effect of this rearrangement is to create a thermodynamic barrier against the access of transcriptional factors to the DNA. Specifically, the amino-terminal tails of histones play an essential role in making internucleosomal contacts (185) and, for that reason, represent the major target of histone-modifying enzymes, such as acetylases and deacetylases, which ultimately regulate the access of transcriptional factors to the cognate DNA elements. Increased histone tails acetylation correlates with gene activation, whereas hypoacetylation or deacetylation has been associated with repression (187). The major effect of hyperacetylation of amino-

terminal histone tails, especially at selective lysine residues, is to reduce the positive charge and hence uncouple their interaction with negatively charged DNA, and thereby creating an environment more accessible to TFs (188). Specifically, p300 and CBP can acetylate all four core-histone types and, whereas CBP exhibits no substrate specificity, p300 HAT activity is directed primarily towards histone H3 (189,190). The intrinsic HAT activity of pCAF (p300/CBP associated factor) primarily targets histones H3 and H4 (191). pCAF interacts *in vitro* with p300/CBP (191), SRC-1 (120), SRC-3 (57), and nuclear receptors (192,193), interactions that serve to stabilize the functional complex of receptor ate the promoter. Additionally, HAT activity is also conserved in members of p160 family, including SRC-1 (120) and SRC-3 (57), although no such activity has been identified in SRC-2. However, SRCs HAT activity is only marginal and does not seams to be crucial for the modification of nucleosomal structure. Instead SRCs function in this context is to recruit at the promoter diverse combination of stronger HAT activities possessed by secondary coactivators.

As previously mentioned (***Fig.3***), a multistep model has been envisaged for transcriptional activation by nuclear receptors, in which binding of receptor to the enhancer region directs modification of the local chromatin structure into a transcriptionally permissive state, followed by recruitment of GTFs (see later) to for the preinitiation complex at the promoter. Basically the entire process id orchestrated by coactivators, which are thought to be fundamental for recruiting chromatin-modifying enzymes, acetylases and ATPases complexes. In fact, following the initial assembly of primary coactivators to the promoter, the SWI/SNF chromatin-remodeling complex is recruited through direct interaction with CBP/p300 and the CBP/p300-acetylated histone tails stabilizes the recruitment. The SWI/SNF complex possesses ATPase activity, causing active disruption of the chromatin state, in an ATP-dependent manner, and hence resulting in stable changes of DNA topology (81,82). The TRAP complex is in turn recruited to the chromatin through interactions with SRC/CBP/p300 preformed complex. The TRAP complex directly communicates with the basal transcriptional machinery loading initiation of transcriptional activation (83,84).

Broadly speaking, histone deacetylation represents a state of gene repression. The cloning of mammalian homologues of yeast deacetylases proteins (194,195,196), finally established a functional link between histone deacetylation and transcriptional regulation. Mammalian HDAC-1 (194), HDAC-2 (195), and Sin3 (196) proteins have been well documented to be involved in repression of transcription by nuclear receptors (197). Association between Sin3 and HDAC-1 and HDAC-2 has also been demonstrated *in vivo* (198). In addition, biochemical evidences suggest that Sin3 and HDACs exist in a stable preformed complex in mammalian cells (199,200). Moreover, anti-NCoR antibodies have been shown to specifically coimmunoprecipitate with histone deacetylases (53,201,54).
Collectively, these data support the idea that nucleosomal condensation through recruitment of corepressors and associated histone deacetylases is the mechanism by which unliganded NRs inhibit the assembly of the preinitiation complex, maintaining a transcriptionally inactive steady state. Ligand binding is thought to induce release of corepressors enable the receptor to recruit PCAF, p300/CBP, and SRCs to effect histone acetylation and promote the transcriptionally permissive environment at the promoter.

General Transcription Factors

Nuclear receptors regulate transcription through enhancer and promoter elements that may be located thousands or hundreds of base pairs from the transcription initiation site, at which transcription is mediated by RNA polymerase II (Pol II) and its several associated genera transcriptional factors (GTFs), which are constituents of the so-called Pol II holoenzyme. The initial step in mRNA transcription is the binding of TFIID to the promoter, at short distance from the transcriptional start site. TFIID functions as a multiprotein complex composed of TATA-binding protein (TBP) and the highly conserved TBP-associated factors (TAFs). Human TFIID complex has been shown to be composed of a core group of subunits, containing hTAFII250, hTAFII135, hTAFII30, hTAFII20, and hTAF18 (202,203). Following TFIID binding, TFIIB, another important TF is recruited to the TBP-DNA complex. TFIIB possesses affinity for single-stranded DNA and places in

proximity to TATA box in response to a critical change in DNA topology induced by TBP (204). Binding of TFIIB is necessary for recruitment of TFII-α, which is followed by RNA polymerase II entrance (205). TBP and TAFIIs interact functionally with specific nuclear receptors. Several interactions between TBP and NRs have been reported. Protein-protein interaction assay, such as yeast two-hybrid system and *in vitro* binding assay with recombinant proteins, have detected an association between TBP and AF-2 domain of RXR (205) and AF-1 and AF-2 domains of ER (207). A similar association has been found for PR with TFIID (208). Moreover, interactions of NRs with other general transcription factors have been described: between TFIIF and AR (209), TFIIH and RAR (210), and between TFIIB with VDR (211) and other nuclear receptors (212). Interaction of general transcriptional factors and nuclear receptors may be influential in modulating a DNA-bound ternary complex between receptor, TFIIs, and TBP-TAFs, contributing to the assembly of final transcriptional complexes at NRs target promoters. Consistent with the designation that TFs recruitment is a rate-limiting step, direct contacts between NRs and RNA Polymerase II-associated TFs may represent the endpoint of a stepwise aggregation of hundreds of factors. Essentially, it is by influencing the rate of assembly of such factors that nuclear receptors, in association with their coregulators, achieve activation of hormone-regulated genes.

PART 2:
Structure and Function of SRC Coactivators

The steroid receptor coactivator (SRC) gene family (also called p160 family) consists of three homologous members (SRC -1, -2, and -3), which acts as generic primary transcriptional coactivators for NRs and also other type of transcription factors. The major role of these coactivators is to specifically interact with hormone-activated NR, enanching their transcriptional activity and promoting the recruitment of chromatin modification enzymes to the promoter region. The molecular structure, as well as the fine spatio-temporal regulation, has been revealed to be essential for molecular mechanism of action and functional specificity of SRC family members. Most of the information concerning the biological function of p160 coactivators derives from the characterization of animal models, which revealed their fundamental role in development, organ maturation, endocrine regulation, and NR function. Accordingly, their disregulation and overexpression is critical for the progression of many type of cancer.

Contents:

The p160 Primary Coactivators Family

According to the assumption that gene activation arises from a coactivators-driven signal transduction system, in which each component need to possess specific domains for interacting with upstream and downstream components, primary coactivators serve as the earliest and major nexus between DNA-bound transcriptional factors and cellular signaling pathways. These coactivators interact with ligand-bound nuclear receptors (1,5) recruiting histone acetyltransferases (HATs) and methyltransferases (DMTs) to specific enhancer regions (47), which facilitates chromatin remodeling and consequently assembly of general transcription factors.

The p160 steroid receptor coactivator (SRC) gene family was firstly identified in the 90's (29,30) and consists of three homologous members (28), which act as primary coactivators for NRs and certain other transcription factors. SRC-1 (full length SRC-1 or steroid receptor coactivator-1) was cloned through its ability to interact with the ligand bound PR, ER, or TR (29,30); SRC-2 (glucocorticoid receptor-interacting protein 1, transcriptional intermediary factor-2, or steroid receptor coactivator-2) was identified through its interactions with ligand binding domains of GR and ER (54,55); SRC-3 (p300/CBP - interacting protein, receptor-associated coactivator 3, acetyl transferase ACTR, amplified in breast cancer 1, thyroid hormone receptor activator molecule-1, or steroid receptor coactivator-3) was initially identified in an amplified chromosomal region of a human breast cancer cell line and subsequently characterized as a nuclear receptor coactivator homologous to SRC-1 and SRC-2 (56,57,58,59). All three members of the SRC family are able to interact with many NRs in a ligand-dependent manner and significantly enhance NR-activated gene transcriptional. In addition to NRs, SRC family members, when experimentally expressed in cultured cells, also interact and coactivate certain other transcription factors such as AP-1 (60), serum response factor (61), nuclear factor-kB (NF-kB) (62), and interferon-γ and cAMP regulatory element-binding protein (CREB) (63). Therefore, each member of SRC family seem to serve as a general coactivator for multiple NRs, providing an explanation for the functional redundancy of SRC members observed *in*

vivo.

The amino acid sequences and biological functions of all known SRC-related proteins are evolutionarily conserved among different species. Avian SRC-1 is 75% identical to mammalian SRC-1 protein. Except human and rodent, SRC-2 was also identified in zebrafish (zTIF2) and frog (64). SRC-3-related proteins where also identified in fruit fly and frog: in *Drosophila* a coactivator for ectysone receptor, called Taiman, was identified by a genetic approach and found sharing 48% of sequence identity with the N-terminal helix-loop-helix domain of human SRC-3 (65); the *Xenopus* SRC-3 (xSRC-3) is 72% identical to mammalian SRC-3 (66) and was demonstrated that it can coactivate mammalian NRs such as retinoid X receptor, RAR, and TR.

Structural and Functional Domains of SRC Family Proteins

Human and rodent SRC proteins are about 160kDa and they are encoded from genes with an overall sequence similarity of 50-55% and sequence identity of 43-48% between the three members (***Fig.4***). Their most conserved N-terminal bHLH/PAS domain (bHLH/Per/Ah receptor nuclear translocator (ARNT)/Sim domain) was originally discovered in *Drosophila*, where it is involved in DNA binding and heterodimerization between other proteins containing this kind of motif (67). In human, it has been shown that this domain is important for interaction of SRC-2 with myogenic factors (68). The central region of SRC family members is relatively conserved and contains three LXXLL (L, leucine; X, any amino acid) motifs that are responsible for the interaction with ligand-bound NRs (L1-L3 in ***Fig.4***) (57,63,69,70). The LXXLL motif forms an amphipathic α-helix that binds a hydrophobic cleft present in the ligand-binding domain of NRs and accessible only after binding ligands (3,8,71). Single mutation of any one of these three LXXLL motifs does not completely abolish the interaction with NRs, suggesting the idea that they collaborate for the high-affinity binding of SRCs to NRs (72). In the carboxyl-terminal region are present two transactivation domains (AD1 and AD2), defined by the two-hybrid system

assay, which cooperatively enhance the activation function domains of NRs (55,73). The AD1 region is responsible for interaction with general transcription coregulators, CBP and p300, but does not interact with NRs (***Fig.4***). Interestingly, AD1 also contains three LXXLL-like motifs (L4-L6 in ***Fig.4***) and mutations of one or more of these three motifs impair the interaction of SRCs with CBP and p300, indicating that the major role of AD1 is to recruit acetyltransferases for chromatin remodeling (fig.4) (9,70,74). The second transactivation domain, AD2, is responsible for interaction with histone methyltransferases, coactivator-associated arginine methyltransferase 1 and PRMT1 (75,76), recruitment of which is also a critical event for NR-directed local chromatin remodeling and assembly of the transcriptional machinery around the promoter region.

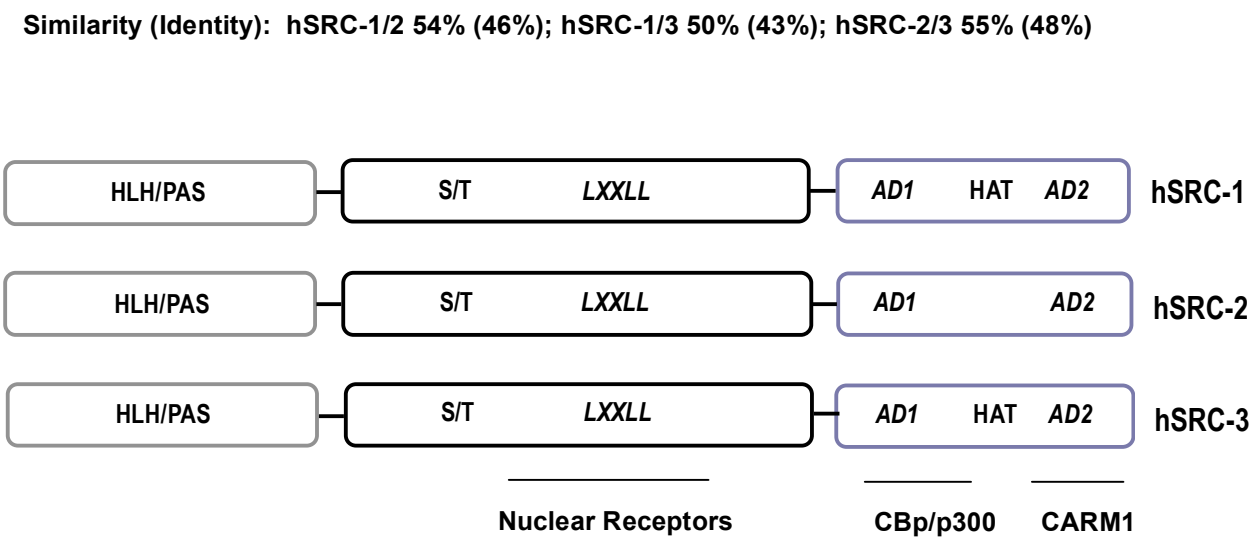

Figure 4. Structural and functional domains of SRC family members and sequence homology. The similarity and identity of amino acid sequences for full-length human SRC proteins are indicated above the bars. The letters within the bars indicate structural domains, and the lines under the bars indicate domains that interact with different factors. bHLH/PAS: helix loop helix Per/ARNT/Sim homologous domain; S/T: serine/threonine-rich regions; LXXLL: typical LXXLL α-helix motif; HAT: histone acetyltransferase domains identified in SRC-1 and SRC-3. AD1 and AD2: transcriptional activation domains 1 and 2.

Role of SRC Proteins in Chromatin Remodeling and Transcriptional Activation

The C-terminal domains of SRC-1 and SRC-3 contain histone acetyltransferase (HAT) activities, raising the possibility that SRC coactivators may play a direct role in chromatin remodeling during the process of NR directed initiation of transcription (57,77). However, the SRC HAT activity is much weaker than those in CBP, p300, and p/CAF and inactivation of the HAT activity in SRC-1 by site-directed mutagenesis does not significantly affect its coactivating function in an *in vitro* transcriptional assay (77). In fact, the intrinsic HAT activity of SRCs is not essential for initiation of transcription. Instead, SRCs may play a major role in assembling general transcription factors through direct and indirect recruitment of other coactivators (***Fig.3***), which results in acetylation and methylation of specific histones (79,80) and initiation of transcription. First, ligand binding-triggered interaction between SRCs and NRs results in the recruitment of secondary coactivators, such as CBP/p300 (78). Second, the SWI/SNF chromatin-remodeling complex is specifically recruited through the histone CBP/p300-acetylated regions and causes resistant remodeling of DNA topology (81,82). Third, the TRAP complex is recruited through the preformed SRC/CBP/p300 complex and more directly communicates with the transcriptional apparatus (83,84). Fourth, initiation of transcription, not only depends on progressively direct interaction of functional complexes, but is also regulated via the intercommunication between all these members, accomplished by many different secondary modifications (85,86). Importantly, coregulators always work as multiprotein complexes. For instance, the SRC family coactivators exist in a steady-state complexes consisting of six to ten stably associated components, which anyway are not static but rather are subjected to dynamic rearrangement related to specific post-translational modifications. Phosphorylation (87), acetylation (86), methylation (165,166), and ubiquitination (167) of coregulator complex members by their associated co-coregulators such as p300/CBP, CARM1, and the Fbw7α (part of SCF E3 ligase) can define the affinity of SRCs for NRs or other members of the coregulator complex. As an important

consequence of their existence as multiprotein complexes, coregulators are able to transmit signals from the environment by supplying a variety of enzymatic activities at the promoter of the genes.

Biological Function and Characterization of SRC Family Members

Our knowledge of SRC family members is based in part on expression patterns of mRNA and proteins and more specifically on phenotype of mouse models. Discussing each SRC member, both these aspects will be taken into account.

SRC-1 (NCoA-1)

The SRC-1 gene is located in chromosome 2 (p23) in humans and chromosome 12 (A2-3) in mice (88,89). SRC-1 was identified by yeast two-hybrid screen of a human B-lymphocyte cDNA library with bait encoding the PR LBD. SRC-1 gene is widely expressed in many tissues and cell types (90). During mouse embryonic development, SRC-1 is highly expressed starting from day 8.5 and it is important for a correct formation of different tissue (91,92). In adult mice, SRC-1 remains highly expressed in certain brain regions including olfactory bulb, hippocampus, piriform cortex, amygdala, hypothalamus, cerebellum and brainstem (92). Despite these tissue specific patterns in neuronal cells, no known human disease has been specifically linked to genetic defects of SRC-1 gene at this time. However, genetic models provided important clues for partial resistance to steroid hormones due to loss of SRC-1 function. For example, the estrogen-induced growth and estrogen- and progesterone- dependent uterine decidual response were decreased in ovariectomixed female SRC-1 -/- mice. Mammary gland ductal side branching and alveolar formation were reduced in ovariectomized female SRC-1 -/- mice treated with estrogen and progesterone. A similar partial response was observed from testosterone-1-stimulated prostate growth in castrated males SRC-1 mice (93). These observations in part indicate that SRC-1 serves as one of the *in vivo* coactivators to mediate transcriptional activity of

steroid receptors. SRC-1 possesses a broad range of interaction with nuclear receptors, including ER, PR, GR, TR, RXR (213), HNF-4 (214), and PPAR-γ (215). In addiction to nuclear receptors, SRC-1 modestly coactivates other transcription factors, including AP-1 (216), serum response factor (217), and NF-κB (218).

SRC-1 plays an important role in brain development (94). At birth, the gonads of male rats produce testosterone that is converted into dihydrotestosterone and estradiol in the brain. The dihydrotestosterone activates AR and results in behavioral masculinization. Estradiol activates ER and results in development of the sexually dimorphic nucleus (SDN) and behavioral defeminization in male rats. Interestingly, down-regulation of SRC-1 by infusion of antisense oligodeoxynucleotides (ODNs) into the neonatal stage significantly reduces the SDN volume, blocking behavioral defeminization. In contrast, SRC-1 down-regulation does not affect the masculinization on male sexual behavior, suggesting that SRC-1 plays an important role in ER-mediated SDN development and defeminization but not in AR-mediated masculinization.

In addition, SRC-1 is expressed at higher levels than other SRCs in the cerebella PC (95), and mice lacking SRC-1 exhibit a delay in PC development. The adult SRC-1 knockout mice also exhibit moderate motor learning dysfunction. Since TR and retinoid-related orphan nuclear receptor play important role in PC development, SRC-1 might be required for normal function of these nuclear receptors in PC development.

Intriguingly, multiple observations have suggested that the usage of SRC-1 by different TR isoforms is tissue specific. First, SRC-1 is important for both TRα- and TRβ-mediated body growth since both TRα/SRC-1 and TRβ/SRC-1 double-knockout mice exhibit more severe growth retardation than either TRα or TRβ single-knockout mice. Second, SRC-1 partially mediates the TH effects on heart rates by TRα and TRβ. Third, hypersensitivity to TH seen in TRα null mice, as demonstrated by overexpression of a TH regulated gene, 5' deoiodinase, in the liver, is abolished in SRC-1-/- mice, suggesting that the hypersensitivity in TRα null mice is due to TRβ function enhanced by SRC-1 (96). Fourth, SRC-1 is required for normal down-regulation of TSH by both TRα and TRβ in the pituitary. In the absence of TRα, SRC-1 expression is elevated in the pituitary, suggesting that the excess

amount of SRC-1 in TRα null mice may superactivate TRβ and cause TH hypersensitivity for downregulation of TSH (97).

The role of SRC-1 in peroxisomal proliferator-activated receptor-γ (PPARγ) function also exhibit certain levels of tissue specificity, which is probably due to relative amount PPARγ and SRC family members. In liver SRC-1 seems to be not required for expression of PPARγ-regulated genes. In contrast, in the brown fat, activation of PPARγ triggers the recruitment of coactivator complex containing PGC-1, SRC-1 and CBP/p300. Inactivation of SRC-1 impairs the thermogenic activity of PGC-1 in the brown fat, decreases the energy expenditure, and results in obesity following a high-fat diet (98).

SRC-2 (TIF-2/GRIP-1/NCoA-2)

The SRC-2 gene is located in chromosome 8 (q21 in humans and chromosome 1 (A3-5) in mice (99). SRC-2 mRNA and protein have been detected in many tissues, including placenta, testis, brain, heart, liver, pancreas, and uterus. SRC-2 protein is undetected in hepatocytes, thyroid gland, and striated muscle by immunohistochemistry. These studies indicate hat SRC-2 is widely expressed in many organs and its expression amount varies between cell types and organs. SRC-2 associates in a ligand-dependent manner *in vitro* with several receptor LBDs (55) and, *in vivo*, with RARα, ER, and PR in the presence of hormone (54,55,219).

Similar to SRC-1 -/- mice, SRC-2 -/- mice exhibit nearly normal somatic growth. However, the fertility is significantly reduced in both male and female SRC-2 null mice. Male hypofertility appears to be of Sertoli cell origin since SRC-2 is specifically expressed in this cell type in the testis and the absence of SRC-2 results in lipid accumulation and germ cells apoptosis. In agreement with the role of SRC-2 in mouse, some men with oligospermic infertility possess an AR mutation from methionine to valine that disrupts the interaction between AR and SRC-2 (100). The hypofertility of female SRC-2 mutant mice is due to a placental hypoplasia caused by the absence of SRC-2 in decidual stromal cells. These findings indicate that SRC-2 plays a critical role in reproductive behavior and functions.

Recent studies have shown that SRC-2 plays an important role also in lipid metabolism and energy balance (98). In the white adipose tissue (WAT), SRC-2 serves as a coactivator for PPARγ. In SRC-2 -/- mice, WAT expresses high levels of leptin and lower levels of genes responsible for antilipolysis and fatty acid uptake and trapping, such as the perilipins, fatty acid binding protein P2, lipoprotein lipase, and PPARγ, causing higher levels of lipolysis and a lower potential for fatty acid storage. In the brown adipose tissue (BAT), SRC-1 is a better coactivator than SRC-2 in the stabilization of PPARγ and PGC-1. The absence of SRC-2 in BAT facilitates the formation PPARγ/PGC-1/SRC-1 complex for PPARγ-dependent transactivation. Thus, BAT lacking SRC-2 expresses higher levels of uncoupling protein 1, PGC-1, and acetyl coenzyme A oxidase, causing higher energy expenditure due to enhanced fatty acid oxidation and uncoupling of respiration. As a result, SRC-2 null mice exhibit higher body temperature under cold conditions, less fat accumulation, lower levels of fasting glycemia and triglyceride, and higher insulin sensitivity. Collectively, these mice are better able to protect themselves against obesity induced by high-fat diet or hyperphagia. Interestingly, a high-fat diet induces SRC-2 expression in both WAT and BAT, which may reflect part of the molecular mechanism for the enhancement of fat accumulation (98).

SRC-2 is also expressed in proliferating myoblasts and postmitotic differentiated myotubes and potentiates skeletal muscle differentiation by acting as a critical coactivator for MEF-2 (68). However, no defect in skeletal muscle development was observed in SRC-2 deficient mice, suggesting that other SRCs may compensate for the loss of SRC-2 function in skeletal muscle development *in vivo*.

SRC-3 (NCoA3/pCIP/AIB1/ACTR/RAC-3/TRAM-1)

The SRC-3 gene is located in chromosome 20 (q12) in humans and chromosome 2 (H2-4) in mice (56,89). Similar to the other members of the family, experiments based on Northern blot analysis reveal that SRC-3 mRNA is also widely expressed. More notably, a knock-in mouse model harboring a β-galactosidase reporter downstream of the endogenous SRC-3 promoter reveled that the mouse SRC-3 gene is mainly expressed in mammary gland

epithelial cells, oocytes, vaginal epithelial layer (34), indicating that, although widely expressed, SRC-3 is selectively higher in some specific cell types.

SRC-3 -/- mice display growth retardation and reduced adult body size (101) with decreased white adipose tissue mass (46). Although the reproductive function of male SRC-3 -/- mice is only slightly reduced, the development and function of the female reproductive system is abnormal (34). First, estrogen levels are significantly lower in female SRC-3 -/-, which in turn causes a delay in pubertal development and mammary gland growth. Second, mammary development in response to combined stimulation of estrogen and progesterone is decreased in SRC-3 -/- female mice. Third, the ovulation capacity, after fertilization when mating with fertile males, is also substantially reduced, probably as a consequence of the poor follicular development due to the estrogen inefficiency or, partially, because of the absence of SRC-3 in the oocytes. Collectively, these results indicate that the major physiological functions of SRC-3 are distinct from those of SRC-1 and SRC-2.

Another aspect is that SRC-3 is coexpressed with ERα and ERβ also in the endothelial cells and vascular smooth muscle cells and may facilitate the estrogen-mediated vasoprotective effects, through inhibition of neointimal formation after vessel injury (102). For example, the neointimal growth in ovariectomized wild-type mice is almost completely inhibited by estrogen treatment, but only partially inhibited in ovariectomized SRC-3 -/- mice due to an insufficient suppression of vascular cell proliferation by estrogen (102).

Recently, SRC-3 was found to be associated with the IκB kinase (103). The IκB kinase can phosphorylate SRC-3 and promote nuclear localization of SRC-3. In addition, SRC-3 is able to promote NF-κB-mediated gene expression while the expression of known NF-κB target genes is reduced in SRC-3 -/- mice (103). These observations support a former discovery that SRC-3 is a NF-κB coactivator too (104), suggesting that SRC-3 can play an important role in inflammatory and immune response as well as cell survival mediated by NF-κB.

Functional Specificity of SRC Family Members

The SRC family is defined by an overall sequence similarity of 40% between the three members, reflecting their functional correlation (***Fig.4***). The most extending sequence conservation is in the N-terminal region, in which the bHLH/PAS domain is located. Importantly, the homology in this region for the SRC members is unique among PAS-containing proteins (110), identifying the SRC proteins as a distinct family of PAS factors. The bHLH-PAS domain serves as a protein-protein interaction surface (118) other than assist in the DNA-binding. Accordingly, like other PAS proteins, evidence suggests that SRC members are capable of forming heteromultimeric and homomultimeric complexes *in vivo* (220). Anyway, while the considerable sequence similarity between SRC family members indicates some functional redundancy, there is nevertheless sufficient sequence divergence to indicate also autonomy of function. Moreover, the putative use of alternative splicing junctions in the C-terminus give rise to more considerable sequence complexity. For example, SRC-1 and SRC-2 are distinguished by a 65-aminoacids deletion in SRC-2 with respect to SRC-1 (***Fig.4***).

Protein-protein interaction assays and *in vitro* or *ex vivo* experiments have made it clear that SRC family members may possess many common features, but also important functional and tissue specificity. On one hand, the viable and fertile phenotype of knockout mice lacking functional SRC-1, SRC-2, or SRC-3 further support the notion that SRC family members may be able to partially compensate each other's function *in vivo* (34,93), probably due to the similar structure of functional domains as well as their partially overexpression patters in certain tissues. Additionally, it could be equally possible that some biological functions are dependent on the total amount of SRC family members. However, several studies have demonstrated the existence of a precise specificity between NRs, in terms of select distinct downstream coactivators and, therefore, selective activation of target genes for different NRs may depend on differential recruitment of specific SRCs. For example, microinjection of expression plasmids for SRC-1 or SRC-2, but not SRC-3, was shown to rescue RAR-dependent transcription in SRC-1-immunodepleated cells, suggesting that SRC-3 cannot compensate for the function of

SRC-1 and SRC-2 in these assays (63). More interestingly, a recent study demonstrated that PR interacts preferentially with SRC-1 in breast cancer cells, which recruits CBP and enhances acetylation of histone H4. In contrast, GR interacts preferentially with SRC-2, which recruits p/CAF and results in histone H3 acetylation. This study suggests that selective recruitment of SRCs by different NRs may determine the specificity of coactivator complexes that in turns reflects the specificity of transcriptional signals (79). In addiction, variable tissue-specific expression patterns of SRC family members may be also responsible for their functional specificity. For example, SRC-3 has been found to be specifically overexpressed in primary breast tumors (56) against the relatively low expression of SRC-1 and SRC-2. Although members of SRC family are widely expressed, their expression levels are often tissues and cell type related. The differences in temporal and spatial expression of SRCs may explain, at least in part, the discrepancy between *in vitro* experiments where all SRCs enhance most NR-dependent transcription and *in vivo* experiments, where SRC-1, SRC-2 and SRC-3 knockout mice exhibit differing phenotypes.

PART 3:
SRC-3 Steroid Receptors Coactivator: Structure and Function

Steroid receptor coactivator-3 (SRC-3/pCIP/AIB1/ACTR/RAC3/TRAM-1) is a member of the p160 family. SRC-3 is importantly required for normal animal growth and development of sexual characters and it is often overexpressed or amplified in many type of cancers, especially human breast cancer. Specifically, SRC-3 is required for the complete functionality of ER. Therefore, it is likely that SRC-3 also plays an important role in estrogen-stimulated proliferation and hormone-related tumorigenesis. Nevertheless, it has been shown that SRC-3 can also modulate divers hormone-independent pathways. Phosphorylation, along with other posttranscriptional modifications, plays a pivotal role in regulating SRC-3 cellular functions and allows SRC-3 to act as an important integrator of many signaling pathway. For this reason, SRC-3 exemplifies the paradigm of the mammalian transcriptional program complexity.

Contents:

Physiological and Cellular Functions of SRC-3

Previous studies indicate that SRC-3 is required for normal animal growth and is often amplified or overexpressed in many cancers, including breast cancer (56,105), prostate cancer (106,107), ovarian cancer (101), endometrial carcinoma (108), and gastric cancer (109). SRC-3 was firstly localized in a frequently amplified chromosomal region, 20q12 (56) and named Amplified in Breast cancer 1 (AIB1) but is also known as NCoA3, pCIP (63), RAC-3 (110), ACTR (57), and TRAM-1 (58). Overexpression of SRC-3 is demonstrated to promote initiation and progression of cancer by affecting many important signaling pathways; anyway, the mechanism of SRC-3-mediated growth regulation remains still unclear. However, many evidences revealed that SRC-3 possesses different physiological roles in respect to the other two members of p160 family. SRC-1 knockout mice exhibit a partial resistance to steroid hormones and a reduction in growth of steroid target organs in response to hormonal treatment (93). Elimination of SRC-2 revealed that it plays a critical role in mouse reproductive function (100). Unlike SRC-1 and SRC-2, SRC-3-null mice are small in size and show delayed puberty, reduced female reproductive function, and reduced mammary gland development (101,103).

SRC-3 acts as a promiscuous coactivator in enhancing transcriptional activity of a great number of different NRs (63) in response to hormonal activation, regulating genes specifically involved in cellular proliferation and differentiation. SRC-3 interacts not only with estrogen receptor (8,40), progesterone receptor (111), and thyroid hormone receptor (112), but also with other transcriptional factors, like NF-κB (104), the signal transducer STAT (113) and the transcriptional activator E2F1 (114). Thus, it is conceivable that SRC-3 activity and regulation may influence a variety of cellular process, affecting the expression levels of many genes. Moreover, as described later, regulation of SRC-3 by phosphorylation has been revealed to be a sophisticated mechanism for specifically communicating external signals to the nucleus and represents a novel function for transcriptional regulators that is not directly restricted to the DNA level.

Role of SRC-3 in Mice Normal Growth and Sexual Functions

In mouse, SRC-3 is tissue-specifically expressed and distributed mainly in the oocytes, mammary glands, hippocampus, olfactory bulb, smooth muscle, hepatocytes, and vaginal epithelium (34). SRC-3-knockout mice exhibit a pleiotropic phenotype showing dwarfism, delayed puberty, reduced female reproductive function, and blunted mammary gland development. This phenotype could be explained by the fact that SRC-3 plays a role in both growth regulatory pathways and the production of estrogen, as proved by hormonal analysis. Moreover, these distinct pathologies observed in SRC-3-null mice, but not knockout mice for SRC-1 or SRC-2, suggest that the physiological role in development of SRC-3 is distinct from that of the other members of SRC family. Additionally, when compared with SRC-1, obvious differences in expression patters exist between the two genes (34). For example, in neocortex, SRC-1 is highly expressed, whereas SRC-3 is lowly expressed; in cardiac muscle, SRC-1 is highly expressed but SRC-3 is undetectable. These differential expression patterns reflect the distinct phenotypes observed in mice where the two genes have been individually knocked out.

Disruption of SRC-3 in mice basically results in alteration of the regulatory pathway for growth hormone (GH) function and reduced production of estrogen (E2). Knockout mice for SRC-3 also possess deficiency in IGF-1 production, although the mechanism that leads to this decreasing is unclear. These low levels of IGF-1 and GH most likely cause the observed growth retardation in SRC-3 -/- mice, resulting in dwarfism (34). In contrast, the low levels of systemic E2 are responsible for the pubertal delay in female SRC-3 -/- mice (34), because the timing for vaginal opening appears identical in both wild type and knockout mice after treating animals with E2. The mechanism by which SRC-3 absence causes estrogen deficiency may depend on a retardation of hormonal production in the ovary, where the majority of estrogen is synthesized. More specifically, the granulose cells in the ovary may have a reduced sensitivity to the follicle-stimulating hormone (FSH), due to a secondary effect of SRC-3, maybe through an unknown interaction between the granulsa and the oocytes, where SRC-3 is highly expressed. Consistent with the delayed puberty and the E2 insufficiency, reproductive function in SRC-3 -/- female mice is also

affected (34), in terms of ovulatory capacity and length of estrous cycle, as a direct consequence of the depletion of SRC-3 in the oocytes, where it is normally highly expressed. Moreover, SRC-3 is also highly expressed in the epithelial cells of mammary gland ducts and it may play an important role in mammary gland development (34). Indeed, mammary gland ductal growth is strongly reduced in SRC-3 -/- mice, also if the low level of E2 seems to be the major factor in growth retardation of mammary gland in null mice, because estrogen therapy successfully rescues the growth deficiency. On the other hand, mammary gland alveolar development in SRC-3 null mice is dramatically decreased also in response to E2 treatment.

Role of SRC-3 in Vasoprotective Effect of Estrogen

Vasoprotective effects of estrogen are well recognized and attributed to both systemic effects, such as decreasing in total serum cholesterol and lipoproteins, and direct effects on cardiovascular system, mediated by ER-α and ER-β (115). SRC-3 is expressed in vascular smooth muscle cells (VSMCs) and is required for the estrogen-dependent vasoprotective effect mediated by ERs (116) through the inhibition of neointima formation. Experiments in SRC-3 null mice show that the neointima formation is significantly higher in knockout female than in wild type female mice (117) following vessel injury. This difference diminishes after ovariectomy, suggesting that the difference in neointimal growth between wild type and knockout mice is dependent on ovarian hormones. Neointimal growth in ovariectomized wild type mice is almost completely inhibited following estrogen treatment but only partially in ovariectomized SRC-3 null mice (117), suggesting that loss of SRC-3 function decreases the estrogen-dependent inhibition of neointima formation. Therefore, SRC-3 may facilitate vasoprotection by ER after vascular trauma, and, since that a reduction of ER levels is observed human coronary diseases, loss of ER coactivator function may also result in increased susceptibility to cardiac diseases.

Molecular Structure of SRC-3

The SRC-3 gene encodes a 160-KDa protein that is 40% sequence similar to the other SRC family coactivators. Furthermore, SRC family members possess similar functional domains (***Fig.4***). The N-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain is the most conserved region (60% identity among the three SRCs) (56). The bHLH-PAS domain serves as a protein-protein interaction surface for various bHLH-PAS-containing factors (118) other than assist in the DNA-binding. It is also possible that this region can participate in intramolecular and intermolecular interactions to regulate the transcriptional activity (44). The conserved central region contains multiple LXXLL motifs (where L is leucine and X is any amino acid) that interact with a hydrophobic cleft in the nuclear receptors LBD, which is formed as a result of ligand-induced conformational change (71,119). In the C-terminus are located two transcriptional activation domains (AD1 and AD2) and constitute the receptor interaction domain of SRCs. The AD1 contains multiple LXXLL motifs that are responsible for the interaction with histone acetyltransferase (HAT) CBP and p300. Although SRC-3, like SRC-1, C-terminal domains possess HAT activity, it is weaker than that of CBP, p300 and p/CAF (120). AD2 can interact with protein arginine methyltransferases (PRMT), such as CARM1 and PRMT1 (75,121). Based on this molecular structure, it seems clear that SRCs serve as adaptor protein to recruit additional coactivators and basal transcription machinery to the promoter, which is critical for promoting local chromatin remodeling and NR-directed transcriptional activation.

Molecular Mechanism of Action of SRC-3

Hormone-dependent Signal Transduction

According to the fact that SRC-3 is a member of the steroid receptors coactivator family, it is actually necessary for the complete functioning of ER (122,123). Therefore, it is likely that SRC-3 also plays an important role in estrogen-stimulated proliferation and hormone-

related tumorigenesis. In fact, estrogen-signaling modulation is closely related to transcriptional expression of genes involved in the generation of breast, ovarian and prostate cancer. In line with this notion, suppression of SRC-3 leads to reduction of the recruitment of ER and polymerase II to the target promoters. Moreover, it seems that inhibition of SRC-3 protein expression has a more determinant effect on lowering ER-dependent transcription than does the inhibition of other p160 protein SRC-1 (124), underling the fact that SRC-3 may function as a specific coactivator for ER activity. Cyclin D1, which is frequently overexpressed in tumors, is an ERα target gene, and its expression is enhanced by SRC-3-driven functional interaction of ER and cyclin D1 promoter (125). The spicing variant SRC-3-Δ3 can increase the estrogen-dependent induction of neoplasia in a variety of tissues and contributing to development of hormone resistant breast cancers (126). All these findings suggest that SRC-3 is required for the complete functionality of steroid hormone receptors, especially for ER. Overexpression and/or amplification of SRC-3 are likely to facilitate transformation induced by ER signaling.

Hormone-independent Signal Transduction Pathway

SRC-3-driven transformation has also been detected in tumors that are not targeted by steroid hormones, such as gastric cancer and HCC (109,127,128). Overexpression of SRC-3 also exists in ER- and PR-negative breast cancer (129). This clinical evidence supports the hypothesis that SRC-3 can enhance also hormone-independent proliferation during tumorigenesis. Actually, extensive investigations reveal that SRC-3 can interact with a broad spectrum of transcriptional factors in addition to steroid receptors (60,113,114,130), indicating that different signal pathways, other than hormone signaling, can be affected by SRC-3 overexpression or deregulation (***Fig.6***). As we will present later, in the results section, phosphorylation of SRC-3 has been showing to be an important step for promoting its nuclear translocation and activating the transcriptional coativating function. SRC-3 can be phosphorylated in specific target residues upon interaction with steroid hormones (39,150). However, SRC-3 is predominantly phosphorylated in a steroid-

independent manner by many signal receptors in response to a variety of growth factors stimulation, integrating different signaling pathways.

E2F1 signal pathway. E2F1 transcriptional factor has been shown to regulate cell cycle progression by modulating the expression of proteins required for the G1/S transition and DNA synthesis. In the G0 and early G1 phases, RB binds to E2F1 suppressing its transcriptional activity. Phosphorylation of RB by CDK results in the release and activation of E2F1 (131). After heterodimerization with its binding partner DP-1 or DP-2, E2F1 promotes the transcription of target genes, including cyclin A, cyclin E, Cdk2, cdc25A, and E2F1 itself. E2F4, another E2F family member, appears to operate as a repressor by recruiting RB to E2F-regulated promoters and consequently it is required for cell cycle exit and differentiation (132). Recently, SRC-3 has been found to directly interact with E2F1, but not with E2F4 (114). Consequently, overexpression of SRC-3 may enhance the transcription of E2F1 target genes promoting cell proliferation in a hormone-independent mechanism.

Insulin-like growth factor-1/AKT pathway. Insulin-like growth factor-1 (IGF-1) has diverse roles in cell processes, promoting growth, proliferation, cell survival, and migration (133). The binding of IGF-1 to its cognate receptor, IGF1R, triggers phosphorylation of the receptor and activation of phosphatidylinositol 3-kinase (PI3K), which is followed by activation of AKT. Both *in vitro* and *in vivo* data reveal that SRC-3 expression level is closely associated with the IGF-1 expression level (34,101,134). Moreover, IGF1R expression level is reduced in SRC-3 knockdown mice breast cancer cells and is highly phosphorylated in tumors derived from MMTV-SRC-3 transgenic mice (134,135). Consistently, the activity of AKT is increased in the MMTV-SRC-3 mouse model (134) and as a result, downstream pathway of AKT are affected, mostly at phosphorylation level. In the MMTV-SRC-3 transgenic mice model, GSK-3β, the substrate of ATK, is strongly phosphorylated at Ser 9, resulting in reduced β-catenin phosphorylation level and translocation of β-catenin into the nucleus, leading to activation of target genes, such as cyclin D1 (134).

NF-κB signal pathway. Rel/Nuclear factor-κB (NF-κB) is a dimeric transcriptional factor

that plays important role in growth control, differentiation, and apoptosis (136). The inactive form of NF-κB is localized in the cytoplasm and consists of 3 subunits: the DNA-binding p50 and p65 subunits, and an inhibitory subunit, called IκB, which is bound to p65. Once IκB is released upon phosphorylation by IκB kinase (IKK), NF-κB will translocate into the nucleus and bind to target genes (136). Aberrant NF-κB activation is implicated in inducing cancer of the breast, prostate gastrointestinal tract, liver, pancreas and skin. SRC-3 can interact and coactivate p65/NF-κB (104). Recently SRC-3, but not SRC-1, has been reported to associate with IKK, suggesting the essential role of SRC-3 in the NF-κB pathway (103). In response to tumor necrosis factor (TNF)-α, SRC-3 is phosphorylated by IKK and complexes with NF-κB, translocating from cytosol to nucleus. As a result, some NF-κB target genes are activated, such as interleukin (IL)-6, which acts as an important autocrine cytokine for tumor progression and metastasis (137), especially prostate cancer (138). Introduction of wild type SRC-3 in SRC-3 null MEF cells can restore IL-6 induction by TNF-α, but not SRC-3 phosphorylation mutants (39), indicating that phosphorylated SRC-3 is tightly linked to NF-κB transcriptional activity.

C/EBPβ signal pathway. There are two C/EBPβ isoforms: liver-enriched activating protein (LAP) and liver-enriched inhibitory protein (LIP). It is thought that LIP functions as a dominant negative. In the mammary gland, increased expression of LIP is associated with rapid mammary epithelial cell proliferation during pregnancy (139). In CMV-SRC-3-Δ3 transgenic mice, there is a reduced LAP/LIP ration, due to the induction of LIP expression level (135), which in turns can trigger hyperplasia in ER-independent manner. However, so far little is known about how overexpression of SRC-3 can directly induce LIP expression and whether SRC-3 can bind to C/EBPβ promoter.

HER2/neu/MAPK signaling pathway. Although deregulated SRC-3 does not affect MAPK pathway activity (122), it serves as a conduit from MAPK signaling to ER (fig.8. MAPKs regulate SRC-3 activity by phosphorylation and enhance ER transcriptional activity by increasing the recruitment of p300 and associated HATs to the promoters (40). In breast cancer patients, HER2 and SRC-3 expression levels are closely associated with development of tamoxifen resistance, suggesting a crosstalk between SRC-3 and HER2

(140).

As described above, SRC-3 is a well-established oncogene. First of all, use of MMTV-SRC-3 is sufficient to initiate tumorigenesis. Furthermore, SRC-3 overexpression is detected in a variety of cancer and affect many signaling pathway other that NRs, such as HER2/MAPK, IGF/AKT, NF-κB, which are frequently deregulated in many cancers. Reduction of SRC-3 in cancer cells by RNAi knockdown can markedly reduce tumor formation in nude mice (107) and deficiency of SRC-3 in mouse model can suppress tumorigenesis, when challenged with carcinogens (122). However, it is still to be clarified how SRC-3 activities are modulated during carcinogenesis. It has been suggested that phosphorylation of SRC-3 is strongly associated with SRC-3 oncogenic potential. Recently SRC-3 was also found to physically interact with ER81, a PEA3 family member, and drive MMP-1 expression, which might be involved in metastasis (141). Thus, it is not unlikely that SRC-3 interacts with other transcriptional factors or even other proteins unrelated to the transcriptional complex.

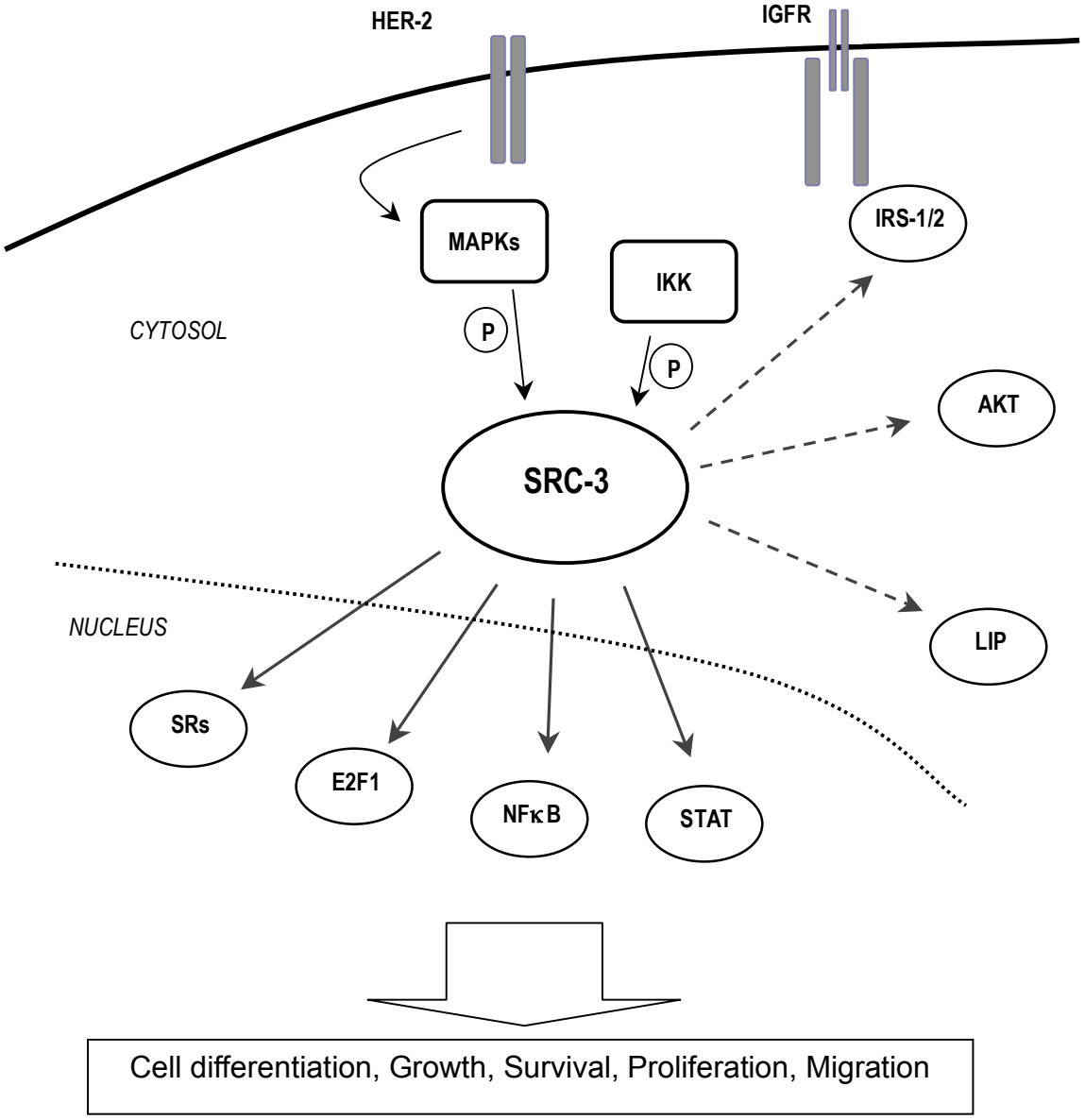

Figure 6. The broad range of SRC-3 interactants. SRC-3 interacts with multiple signaling pathways other than with steroids receptors (SRs). Tyrosine kinase receptors, like HER-2, phosphorylate SRC-3 through the MAPKs, whereas IKK complex can directly phosphorylate SRC-3. Phosphorylation acts as an activation switch for SRC-3 functional activity into the nucleus, where it can bind a broad range of nuclear receptors and transcriptional factors (TFs), depending on the cellular context. Some TFs have been characterized ad direct binding partners of SRC-3 for activating target genes (solid arrows). Some other proteins are not known as direct or indirect targets of SRC-3 (dashed arrows), including LIP (C/EBPb isoform) and several components of IGF-1/AKT pathway.

Selective Phosphorylation of SRC-3

The fact that SRC-3, in addiction to nuclear receptor, can coactivate a number of other distinct transcriptional factors, such as NF-κB, E2F, C/EBP, AP-1, STAT, p53 (60,113,114,130,140,142), suggests that it is involved a much broader role in cellular functions and diverse physiological processes. It is now evident that many phosphorylation pathways, including MAPKs, IKK and HER2/neu (41,103,140), specifically influence SRC-3 activity, but it is still unclear how different signaling pathways can exert different physiological functions. Consequently, it could be postulated that distinct cellular signals may differentially phosphorylate SRC-3 at distinct amino acids, preferentially directing its activity to different pathways. Recently, six phosphorylation sites have been characterized (39), which are specifically targeted by different kinases and all important for the optimal interaction of SRC-3 with transcriptional activators and other coactivators, as shown by mutational substitution of phosphoamino acids with non-phosphorytable alanine. Using siRNA, p38 has been identified as a kinase that phosphorylates S505, S543, S860, and S867 whereas IKK phosphorylates S857 in living cells (39). Interestingly, different combinations of phosphorylation in these sites are required for different physiological function of SRC-3, which could be not redundant with those of SRC-1 and SRC-2. In fact, amino acid sequence alignment reveled that not all of these six sites are conserved between the three p160 members: only phosphoamino acids T24, S505, and S543 are conserved in SRC-1 and only S505 and S543 are conserved in SRC-2. The remaining three amino acids, S857, S860, and S867, are unique for SRC-3 (39). In this regard, not all SRC-3 phosphorylation sites are equally required for maximal activity of SRC-3 in different signaling pathways. While phosphorylation at all six sites is important for interaction with ER and AR, it is not induced by TNF-α, which induces phosphorylation of all but the S860 site. Consequently, mutation to alanine at any of these sites impairs the ability of SRC-3 to coactivate E2-induced ER, while mutation at all six sites but S860 affects TNF-α-dependent interaction with NF-κB. Likewise, alanine-mutation of T24, S543, S857, and S867, but not S505 and S860 sites, negatively influences expression of IL-6 gene.

According to all these findings, it appears that SRC-3 phosphorylation is extremely important for achieving its complete function as a coactivator and probably specific phospho-conformations of SRC-3 differentially interacts with transcriptional activators, which in turn selectively regulate different target gene promoters. Concerning to this hypothesis, it seems also that SRC-3 possesses the capacity to act as a downstream effector for a wide variety of cellular signaling, functioning as a common phosphorylation target for different stimuli and integrating multiple signals to gene expression and growth responses.

Extranuclear Phosphorylation of SRC-3

As mentioned earlier, different stimuli induce distinct patterns of SRC-3 phosphorylation, and mutations at different phospho-sites have different downstream effects. How E2 induces phosphorylation of SRC-3 is poorly understand. E2 can induce cellular response through direct interaction with ER, but it can also induce a rapid and transient activation of various kinase cascades independently to prior gene transcription (143). Usually, this "nongenomic" action of E2 is mediated by the interaction with the cytoplasmic-localized ER (144), as shown for the activation of Src/ERK pathway through the interaction with MNAR (modulator of non-genomic activity of ER). Alternatively, in an ER-independent way, E2 has been shown to activate MAPK pathway through the membrane-bound G protein-coupled receptor (145). It is well established that ER shuttles between nucleus and cytoplasm, although it is mainly detected into the nucleus (146,147). In the absence of ligand, 5-10% of GFP-ER is localized in the cytoplasm (146,148), whereas E2 increases ER nuclear localization. It also has been shown that E2 induces a translocation of SRC-3 from cytoplasm to the nucleus (101,103,149). E2-induced phosphorylation of SRC-3 requires a direct interaction between SRC-3 and ER that occurs in the cytoplasm and it does not require DNA binding and nuclear localization as classically believed for the interaction of coactivators with transcriptional factors (150). Both the AF-1 and AF-2 domains of ER seem to be necessary for maximal E2-induced phosphorylation of SRC-3 and a direct interaction between ER-LBD domain and the coactivator is required. The

inability of 4-hydroxil tamoxifen (4HT) to induce SRC-3 phosphorylation further supports this point. However, the mechanism by which kinases are recruited to ER-SRC-3 complex is not clear. It is possible that E2-bound ER can first recruit kinases via its AF-1 and AF-2 domains, allowing subsequent phosphorylation of SRC-3, or, vice versa, SRC-3 or either ER recruit kinases to the ER-SRC-3 preexisting complex. Anyway, phosphorylation of the major phosphorylation sites of ER is not a prerequisite for SRC-3 phosphorylation (150). The important idea provided by these findings is that E2 can induce assembling of an extranuclear SRC-3-ER complex prior to promoter binding. This complex could serve as convergence point for cross-talking cell signaling pathways and ER transcriptional activity resulting in a much fine-tuning activity of transcriptional coactivators.

SRC-3 Cellular Degradation

Recently, it has been revealed that SRC-3 degradation is regulated by REGγ-directed proteasome 20S activity, in an ATP- and ubiquitin-independent manner (151). Usually, destruction of intact cellular proteins is largely orchestrated by ATP-dependent ubiquitination and subsequent degradation by 26S proteasome. The REG-20S proteasome only degrades short peptides. Li and coworkers (151) provided evidences that the REGγ-20S complex can selectively target an entire protein for destruction, in this case SRC-3. REGγ interacts directly with SRC-3 and its overexpression accelerates the turnover of SRC-3, whereas REGγ silencing through small interfering RNAs (siRNAs) results in accumulation of SRC-3. The most striking feature in Li's study is the biochemical evidence for the unconventional degradation of SRC-3 by REGγ-20S proteasome, which was thought only to degrade small peptides (152). SRC-3 was degrades in a cell-free proteasome proteolysis assay, in the presence of the 20S proteasome and heptameric REGγ, without exogenous ATP and ubiquitin pathway components. On the other hand, the REGα/β complex did not promote the degradation of SRC-3. Moreover, SRC-1 is incapable to bind REGγ and to be degraded by REGγ. Ultimately, given to the fact that

ATP and chaperons were absent in the cell-free proteolytic assay, the hypothesis is that internal unstructured sequences of SRC-3 may serve as endoproteolytic sites for the entry into the REGγ-20S complex. These internal SRC-3 sequences are predicted to be disordered with long loops and polyglutamine loops. Additionally, the N-terminal 38 residues and the C-terminal147 residues are also unfolded. It is thinkable that these disordered sequences may serve as cleavage sites for the 20S proteasome, given the ATP-independent nature of SRC-3 degradation. However, it is currently unclear if the 20S proteasome possesses any substrate specificity *in vivo* and it is known that SRC-3 can be also ubiquitinated and degraded by the 26S proteasome (153). Therefore, it is possible that SRC-3 stability is regulated by interplay of ubiquitin-dependent and -independent pathways, which could provide a much precise mechanism for controlling SRC-3 abundance. These observations give rise to the proposal that REGγ is required for a fine tumor-suppressive mechanism for restricting the uncontrolled accumulation of SRC-3, given to the fact that SRC-3 is a broad-range protein involved in many aspects of cell proliferation and survival, probably more that the other p160 members.

SRC-3 in Oncogenesis

SRC-3 gene is localized on a frequently amplified region, 20q12, and it is often found in many cancers, including breast, gastric, prostate, endometrial, and esophageal carcinoma. Consequently, many evidences indicate SRC-3 as an important oncogene and directly implicated as a causal factor in the genesis of human cancer. Accordingly, aberrant expression of SRC-3 has been associated with hormone-sensitive and -insensitive cancers.

Clinical Studies

In breast cancer biopsies, SRC-3 has been shown being amplified or overexpressed in 5%-10% and 30%-60% of cases, respectively (56,154). In tamoxifen-treated patients, SRC-

3 overexpression is associated with high levels of HER-2, tamoxifen resistance, and low survival expectative, underlining the important cross talk between SRC-3, HER-2 and ER (140,155). In prostate cancer, study of the expression of SRC-3 revealed that the expression level of SRC-3 correlate directly with tumor grade and stage of disease (106). Moreover, it was reported that the splicing variant SRC-3-Δ3, encoding a 130 kDa protein that lacks the N-Terminal HLH and a portion of the PAS domain, possesses a strong steroid receptor coactivator activity *in vitro*, even when present at low level, if compared with the full-length SRC-3 (126). This splicing variant of SRC-3 has been shown to be highly expressed in breast cancer biopsies (156). Additionally, a recent study has revealed that in breast cancer SRC-3 overexpression correlates with the absence of ER and PR (129), suggesting that SRC-3 may also act via other transcriptional factors that ER or PR during tumorigenesis. Consistent with this finding, SRC-3 is also found to be involved in many types of non-steroids-related tumors, such as pancreatic cancer, gastric cancer, colorectal carcinoma and hepatocellular carcinoma (109,127,128,157,158). SRC-3 is associated with cancer progression, metastatic potential, and tumor reassurance.

Transgenic Mouse Models

The most helpful model for SRC-3 studying in mice was the transgenically overexpressed SRC-3 gene under the control of mouse mammary tumor virus (MMTV) LTR (134). The overexpression of MMTV-SRC-3 was specifically associated with extremely high tumor incidence in aging animals, with an average latency of 16 months. High tumor incidence was found in mammary glands, pituitary, uterus and lung, consistently with the high ectopic expression of SRC-3 in these organs (134). Moreover, most of the mammary tumors were invasive and several adenocarcinomas were metastatic.

A second very powerful transgenic model for investigating SRC-3 function in cancer was the cytomegalovirus (CMV) promotering SRC-3-Δ3 (135). Mammary cell proliferation and ductal ectasia were found in CMV-SRC-3-Δ3 mice, and other phenotypes similar to those founded in MMTV-SRC-3 mice.

Expectantly, as mentioned above, knockout mouse model for SRC-3 show reduction in

mammary gland alveolar development during pregnancy, and resistance to growth hormones and estrogens (34,101). In addiction, SRC-3 deficiency significantly suppresses the incidence of MMTV-H-Ras induced mammary ductal hyperplasia, tumorigenesis and metastasis to the lung (159). Most of the SRC-3 +/+ and SRC-3 +/- H-Ras overexpressing mice develop many mammary neoplasia lesions by the age of 17 weeks and fifty percent of theme present breast tumors by the age of 30-40 weeks. All these mice develop palpable breast tumors by the age of 70 weeks. In comparison, 50% of SRC-3 -/- mice still have normal mammary gland morphology by the age of 80 weeks. Moreover, depletion of SRC-3 also significantly reduces the mammary tumorigenesis induced by chemical carcinogens (160). Parallel to defects in sexual organs, SRC-3 knockout mice display also metabolic defects, with reduction in adipose tissue deposition and growth retardation. In fact, SRC-3 exerts an important role in lipid metabolism by enhancing transcription of PPARγ2, essential for progression of adipocyte differentiation (46).

Biological Function of SRC-3 *in vivo*: Concluding Remarks. The Exemplification of Transcriptional Program Complexity

Similar to SRC-1 and SRC-2, *in vitro* and *ex vivo* experiments have clearly demonstrated that SRC-3 is a transcriptional coactivator for nuclear receptors and other transcription factors. SRC-3 functions by recruiting at the promoter both HATs (CBP, p300, pCAF) and histone methyltransferases (CARM1 and PRMT1), which are necessary for chromatin remodeling and gene transcriptional initiation. Firstly, SRC-3 has been shown to regulate somatic growth through affecting IGF-1 expression. Second, SRC-3 participates in the regulation of puberty, vasoprotection, and female reproductive functions, coactivating steroids receptors. Third, SRC-3 plays a role in regulation of ovulation too, through still undefined mechanism. Fourth, SRC-3 is involved in the regulation of glucose and lipid metabolism. Finally, SRC-3 has a general coativating function in hormonal regulation of cell proliferation and its altered expression and activity are strongly related to

tumorigenesis. However, SRC-3 has recently been found to regulate cellular processes other than transcription, functioning at the translational level of cytokine mRNAs and repressing their translation (173). All the functional roles that SRC-3 exerts *in vivo* are summarized in **Fig.7**. The most important question that still needs to be addressed concerns the *in vivo* relationship between the three p160-members and other classes of coactivators. It is now well accepted that coactivators always exert their function as multiprotein complexes. This concept underlines the complexity necessary to accommodate a broad range of developmental, physiological, and environmental programs. The equilibrium existing between the many different states of transcriptional control is fundamentally regulated at the level of coregulators and especially through posttranscriptional modifications of coregulators. SRC-3 serves as a good example to demonstrate how posttranscriptional modifications contribute to the biological complexity, signal integration, and the propagation of biological programs. SRC-3 in fact exists in a complex that includes kinases, ubiquitin ligases, ATPases, methyltransferases, acetylases. Initially, SRC-3 is likely to be SUMOylated (at aa 723 and 786) (167) and hypophosphorylated at serine/threonine residues (39), existing as an inactive protein in its basal state. After phosphorylation at serine 505 and 509, especially through a GSK3-dependent manner, SRC-3 becomes monoubiquitinated at residues 723 and 786 (167), becoming able to function as a transcriptional coactivator. The ubiquitination sites are progressively polyubiquitinated during subsequent rounds of transcription, ultimately leading to SRC-3 degradation through the 26S proteasome. Independent on this, other phosphorylation sites are targeted by other kinases, including p38, IKK, and MAPKs, and are necessary for SRC-3 to form divergent multiprotein complexes and coactivate its full range of transcription factors (39). Additionally, methylation and acetylation of SRC-3 (165,166), along with proteasome-mediated degradation, leads to the coregulator complex disassembly and, beside phosphorylation, contribute to the coregulator dynamics.

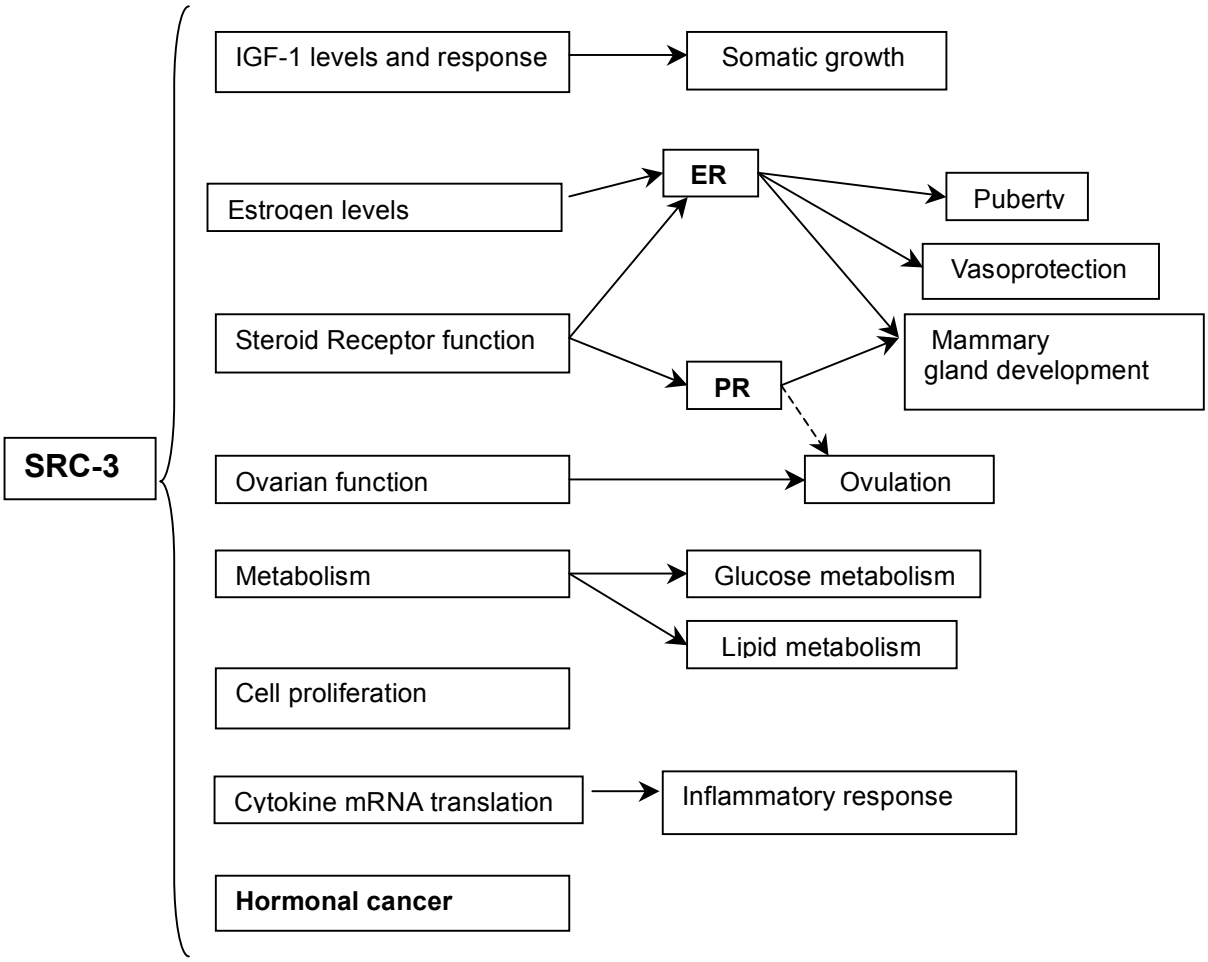

Figure 7. ***Biological effects of SRC-3 in vivo.*** Transgenic mouse models and cultured cell experiment have revealed that SRC-3 plays important and wide roles in different cellular processes, regulating as many functions as somatic growth, puberty, vasoprotection, mammary gland development, and metabolism. SRC-3 exerts its effect by interacting with transcriptional factors at the gene promoter level or interplaying with cytoplasmic signaling pathways.

PART 4:
TRANSCRIPTION BY ESTROGEN RECEPTOR AND MECHANISM OF ESTROGEN ACTION

Estrogen receptor (ER) primarily acts as a nuclear transcription factor upon binding with the specific ligand 17-β estradiol (E_2). However, emerging data have identified a separate pool of estrogen receptors in the plasma membrane compartment, where it (as well as other steroid hormone receptors) is thought to trigger rapid and non-genomic signaling from various kinase pathways. Moreover, there is increasing evidence that cell-signaling pathway and the phosphorylation status of transcription factors and associated coregulators determines the overall response to the hormone. ER is synthesized in many cell types as two forms, ERα and ERβ, which are products of separate genes. The two receptors do not completely overlap in the various tissues and recent evidence indicates that there are specific actions of E_2 that can be attributed to one receptor but not to the other.

Contents:

Signaling by Estrogen and Non-genomic Action of ER

Estrogens are a group of steroid compounds, named for their importance in woman estrous cycle, and function primarily as a female sex hormone, but they are also expressed in males, where regulate certain functions of the reproductive system and maturation of sperm. The principal estrogen hormone is 17-β estradiol, which is also converted into estrone and estriol that anyway are much weaker agonist of ER (221) and hence are thought to be inactive or suggested to possess some tissue specificity (222). Like other steroid hormones, E_2 readily diffuse across the cell membrane and inside the cell specifically interact with its cognate receptor, ER. Primarily, ligand binding to ER in the nucleus leads to conformational changes that regulate the ability of the receptor to recognize the specific DNA binding sequences (hormone response elements) and interact with related coactivators, promoting transcription (the so called genomic action) of target genes involved in cell proliferation, differentiation and homeostasis. However, although the majority of ER is localized and activated in the nucleus, there is clear evidence that a small fraction of the receptor is localized at the cell membrane, either in the presence or absence of E2 (223,224,225,226,227), where it would mediate rapid response to the estrogen through the activation of different signaling pathways (the so called non-genomic action), occurring in a cell-type specific manner and including activation of MAPKs (230,302,303,304,305,306,307,308) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway (309,310,311,312,313), induction of ion channel fluxes (229,314,315), G-protein-coupled receptor-mediated second messenger generation (cAMP and calcium) (316,317,318,319,320,321,322,323), as well as stimulation of growth factor receptors (324,325). The most accepted view is that these receptors are membrane associated by anchoring with scaffold proteins. Candidate interaction proteins are caveolin-1/-2 and the caveolin-binding protein streatin, a calmodulin-binding member. Caveolae are specialized regions of the plasma membrane that assemble and organize signaling protein complexes (228). Overexpression of caveolin-1 in MCF-7 cells, as well as overexpression of striatin, has been shown to increase the E2-dependent ERα localization to the plasma membrane

(229,326). Striatin has also been reported to serve as a scaffold for the formation of an ERα-Gαi complex (326). Other candidate molecules involved in formation of these signaling complexes include heat shock protein 90 (Hsp90), matrix metalloproteinases (MMPs), G-proteins (Gαi), modulator of non-genomic action of ER (MNAR), c-Src, and Shc. The Src-homology and collagen homology adaptor (Shc) binds to docking sites of many growth factor receptors and directly associates with ER through an interaction between its SH2 domain and the N-terminal part of ER (230). ERα, Shc and insulin-like growth factor (IGF-IR) have been shown to interact on the cell membrane in MCF7 cells and treatment with siRNA for Shc, or IGF-IR, attenuate the E_2-induced ERα translocation to cell membrane and E_2 stimulation of MAPKs (231). MNAR (327) acts as a scaffold protein and is anchored to c-Src via its SH3 domain interaction with a PXXP motif on MNAR. This complex is further stabilized by binding of c-Src's SH2 domain to a phosphorylated tyrosine of ERα and binding of ERα to a LXXL motif present in MNAR. Consequently, activation of c-Src kinase activity leads to downstream signaling by Ras and Raf to MAPKs (328). In addition to scaffold proteins, ER lipid modifications are also involved in ER membrane targeting. Specifically, cysteine 447 appears to be a critical palmitoylation site (232,233).

Rapid response mediated by estrogen is specific for different tissues. Within the cardiovascular system, rapid signaling pathway initiated by ER has been well defined. In fact, estrogen is able to exert rapid modulation of the vascular endothelium via the enhanced production of nitric oxide (NO), a vasoprotective molecule that promotes vasodilatation, inhibition of platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation, protecting the arteries against injury (48). ER uses scaffold molecules and localization to caveolae (329,330), as described above, to induce activation of eNOS via PI3K/Akt pathway (309,310). In MCF-7 breast cancer cells, E_2-deperndent activation of ERK via ERα association with Shc, c-Src, and Ras, results in cell cycle progression (230,302). Estrogen can also induce stimulation of IGF-IR by interaction with ER in breast cancer cells and consequent signaling through PI3K/Akt pathway, resulting in enhanced cell proliferation (331,332,333). G protein-coupled receptor (GPR30), an orphan receptor,

can also bind to ER in breast cancer cells and induce activation of PI3K in a way dependent to EGF-R (324,325). In neuronal cells, E_2, through membrane-associate ER action, rapidly triggers ERK-1/-2 activation (334), leading to the expression of cFos gene (335), and genes with neuroprotective effects like neurotensin. Acting through MAPK pathway, ER can also confer protection against β-amyloid peptide (309). In pituitary, through calcium flux ER induces a rapid depolarization at the plasma membrane, which promote release of prolactin (336,337). In osteocytes, E_2-triggered signaling confers protection against bone loss, inducing antiapoptotic mechanisms by activation of Src/Shc/ERK pathway (303,304). ERα may also interact with PKC to affect osteoblast differentiation (338).

Although ERs at the plasma membrane and in the nucleus appear to act by very different mechanisms, their cell biological roles may overlap and be complementary. In fact, kinase signaling can rapidly activate transcription by NRs and play an important role in posttranslational modification of both NRs and associated cofactors. ERα is phosphorylated in multiple serine and threonine sites (see later) by MAPKs, resulting in increased receptor stability and transcriptional activity (247). Members of p160 family are also specific targets of MAPKs. More importantly, by affecting many different intracellular pathways, in addition to its classical transcriptional activity, ER can mediate the extremely broad spectrum of its physiological function in different cell types.

Mechanism of Action of ER in the Nucleus

Primarily, estrogen receptor is a nuclear transcription factor (genomic action) and, like other steroid hormone receptors, is activated upon direct binding with specific ligand and also modulated by posttranslational modification including phosphorylation. In the absence of hormone, a fraction of ER monomers is transiently sequestered in the cytoplasm associated with heat shock protein (3,13). Following binding to the hormone, the receptor dimerizes and translocate to the nucleus, where is able to bind sequence-specific hormone

response elements (HRE). Typically, hormone binding and localization to the specific DNA sequence is accompanied by increasing in receptor phosphorylation (see ahead) and receptor recruits coactivator complexes and activates transcription of specific target genes by DNA Pol II.

Ex vivo expression of wild type and mutated mammalian ER (234) allowed functional analysis of ER structure and characterization of distinct functional domains (16,186,235,236,237). Similar to other NRs, the N-terminal region (A/B domain) of ER contains a constitutively active transactivation function (AF-1 region) (23,74). The most conserved region is the DNA binding domain (DBS, C domain) (11), which contains the P-box, a short motif responsible for DNA-binding specificity to a sequence typically containing a palindromic hexanucleotide 5'-AGGTCA-3', and is involved in ER dimerization (15,24). The less conserved D domain behaves as a flexible hinge region and contains the nuclear localization sequence (NLS), which extends into the C domain. Ligand-binding domain (12) (LBD, E domain) is the largest and possesses the conserved secondary structure of a three-layered 12-helices sandwich, typical of all NRs (186), in which is embedded the ligand-binding cavity. Helix 12 (H12) is the most important, orientating differentially in the presence or the absence of ligand (238). Following the binding of agonist, H12 adopts a position in which a conserved glutamate in the AF-2 and a conserved lysine in helix 3 grip the ends of the helical motifs that contain the LXXLL consensus sequence present in most coregulators (239). The leucine residues of the LXXLL helix pack into a specific hydrophobic pocket formed upon H12 reorientation, stabilizing the interaction with the receptor (186,240,241). Similar to coregulators, corepressors that include NCoR (50) and SMRT (176) interact with unliganded ER through an elongated helix of sequence LXXI/HIXXXI/L, called CoRNR-box (51,242,243), which in the absence of agonist occupies the same hydrophobic pocket contacted by LXXLL motif, because it can displace the H12. On the contrary, this extended helix of NCoR and SMRT is too long to accommodate in the hydrophobic pocket when H12 assumes the activated configuration in response to agonist binding. Thus, agonist binding reduces the affinity of ER, and NR in general, for the CoRNR-box of corepressors and increase the affinity for

the LXXLL motif of coregulators. The E domain is responsible of also other functions, possessing a dimerization interface, a second NLS, and a second transactivation function (AF-2) (22). The very C-terminal part of ER contains the F domain, which exerts a complex mudulatory function (244,245).

ER can associate with distinct subsets of cofactors (178,339). The majority of these cofactors bind to the LBD and so far only very few coactivators are likely to function in ER-specific manner, considering that the various NRs appears to utilize similar cofactors (340). With regard to ER, it has been found that, in the case of SRC-1 and -2, the NR-box 2 has the highest affinity for agonist-bound ERα, while in SRC-3 the NR-box 1 serves as the primary docking site (341). Additionally, acetylation of lysine residues adjacent to NR-box 1 in SRC-3 abolishes the interaction with AF-2 domain of ERα and may represent one important mechanism for attenuation and feedback regulation in ER-regulated gene expression (87). The interaction of coregulators with ER can be controlled at several levels. In the case of SRC-3, six phosphorylation sites are required for coactivation of ER (39) and different combination of phosphorylated residues modulate its interaction with co-coactivators partners (e.g. CBP and p-300), allowing specific regulation of endogenous genes by ER. On the other side, conventional corepressors, like NCoR and SMRT, can associate to antagonist-bound ER (342)and it has been proposed that they can play a role in regulating ER activity in tumors treated with antiestrogens (182). There is evidence that histone deacetylase activity required by corepressors complexes like NCoR/SIN3/HDAC2 is required for the transcriptional repression of tamoxifen-bound ER (49,182,343). However, direct binding of antagonist-bound ER to corepressors has not been demonstrated yet, indirect recruiting mechanisms are also possible and it also possible that ER antagonists, via realignment of H12 (344), may expose yet unidentified corepressor binding epitopes. Importantly, a putative ER-specific corepressor has been identified by two-hybrid screen (342), which is called REA (repressor of estrogen receptor activity) and competes with SRC-1 coativating activity.

The traditional model for transcriptional activation of specific genes by E_2-activated ER, envisage the direct binding of the receptor to the EREs. Today we know that ER can also

modulate gene expression without this direct binding to DNA, but rather via the participation in the formation of preinitiation complexes by other transcriptional factors. The most characterizes example is the physical interaction of ER, after hormone binding, with AP1 complex and stimulation of transcription through Jun/Fos transcription factors (246). Another example of indirect action on DNA is the interaction between ER and NFκB complex, which prevents expression of interleukin-6 promoter (345). Additionally, unliganded-ER can interact with Sp1 transcription factor, enhancing its affinity for DNA and promoting transcription of retinoic acid receptor (RAR-1) gene (346,347).

Result from these studies suggest that in the nucleus ER can mediate signaling from different pathways by a complex crosstalk between EREs-dependent or independent genomic action, and interacts with a repertoire of coregulators and other associated proteins, which in turn determine the expression level and the specificity of regulated genes.

Modulation of ER Function by Phosphorylation

Basically, steroid hormone receptors are hormone-activated transcriptional factors whose activities are also modulated by posttranscriptional modifications, including phosphorylation (247). In some cases, enhanced cell signaling is also sufficient to cause activation of receptors in culture medium depleted of steroids (248). All of the steroid receptors contain multiple phosphorylation sites. Normally, the receptors are partially phosphorylated in the absence of hormone and are more highly phosphorylaed after hormone treatment. Some of the sites exhibit enhanced phosphorylation in response to hormone, while other sites are phosphorylated exclusively in presence of hormone. Most of the sites are serines or threonins located in the N-terminal part of the receptor, although there is also a conserved region of Ser-Pro or Thr-Pro in the hinge region of the receptor. In addition to Ser/Thr sites, there is a limited evidence to support tyrosine phosphorylation too. In ERα, phosphorylation of Tyr^{537} has been described (249,250). Some of the

phosphorylation sites of steroid receptors are also conserved across species. In the case of ERα, eight phosphorylation sites (Ser 104, 106, 118, 167, 236, and 30, Thr 311 and Tyr 537) have specifically identified (250). Specific phosphorylation of these residues contribute to a variety of ER functions including receptor stability, nuclear localization, transcriptional activity, interaction with coregulators, splicing, and, in some cases, phosphorylation may determine whether a subsequent posttranslational modification will occur.

Most of the information has been obtained using transfected receptors but nevertheless it has clearly been showed that the actions of some kinases are receptor-specific and may also be cell-type specific and it is likely that many of the responses may be target gene-specifically. Estrogen receptor activity can be generally modulated by cyclin-dependent kinases (251), like Cdk2, which enhances the activity of various steroid receptors. On the other hand, p38 MAPK enhances specifically ERα activity by phosphorylation of Thr^{311}, increasing also its nuclear localization on endometrial cells (252). Phosphorylation of Ser^{104}, Ser^{106}, Ser^{118} appears to be particularly important for functional association with coregulators (253,254). Ser^{118} is also implied in interaction with splicing factors (255). Ser^{167} phosphorylation plays a role in optimal DNA binding *in vitro* and binding to endogenous promoter *in vivo* (254,264,257). This serine is target of casein kinase II (258), Rsk (259) as well as Akt (257). Ser^{305} phosphorylation increases transcriptional activity and has been implied in preventing acetylation of Lys^{303} (260). Interestingly, Lys^{303} is often mutated in breast cancer to Arg, and this mutation increases sensitivity to estrogen (261).

In addition, there are multiple pathways for hormone-independent estrogen receptor activation. One of the best characterized is the EGF (epidermal growth factor)-dependent activation of ERα in HeLa cells in the absence of hormone, by specific phosphorylation of Ser^{118} and Ser^{167} (262,263), typically resulting in the activation of MAPKs (259,264). Phosphorylation in the amino terminus of ERβ by p42/p44 MAPK also induces ligand-independent activation through enhancing the association with SRC-1 (265). Oppositely, Ser^{236} phosphorylation of ERα by PKA has been implied in inhibition of hormone-independent dimerization and DNA binding, but this inhibition is overcome by the addition

of estradiol (266). However, signaling pathways that induce hormone-independent action also potentiate the corresponding hormone-dependent activity.

Phosphatases also play a role in regulating activity of ER. It has been shown that overexpression of protein phosphatase 5 (PP5) reduces Ser^{118} phosphorylation and transcriptional activation of ERα, while reducing expression of PP5 enhances transcriptional activity (267).

In addition to directly modifying the receptor, there is increasing evidence that ER functions are regulated by changes in the phosphorylation state of associated coregulators. For example, the p160 coactivators are extensively phosphorylated by different kinases in multiple signaling pathways. Phosphorylation of SRC-1 is important for the interaction with receptors (268). Phosphorylation of SRC-2 by p42/p44 MAPK may potentiate the activation of receptors (269,270). SRC-3 is highly phosphorylated and plays a broader role in modulating the activity of many transcription factors. Steroid receptors require specific phosphorylation of SRC-3 sites to achieve complete potentiation of transcriptional activity (39,43). ERα itself can also induce phosphorylation of SRC-3 in the presence of estradiol (271). Phosphorylation of corepressors also influences their ability to repress transcription. Phosphorlation of SMRT, by MEKK1, causes nuclear export, relieving repression (170). MEKK1 also provoke dissociation of NCoR from androgen and estrogen receptor, enhancing transcriptional activity (272).

The simplest mechanism for influence phosphorylatyon of component of steroid receptor pathway is the direct interaction between kinases or phosphatases and receptor or coagulators. In some cases, anyway, these modifying enzymes are brought to the target by proteins that interact with the receptor. For example, IκK associates with SRC-3, which is also a substrate for phosphorylation, is important for the hormone-dependent phosphorylation of Ser^{118} in ERα (43,273). In the case of GR, Hsp90 is a mediator for P5 phopshatase interation with the receptor (274).

Cell signaling pathways that regulate phopshprylation of steroid receptors and their ceregulators are critical in determining the activity of the receptor under different physiological condition. All these data also point to the fact that posttranslational

modifications are as fundamental as hormonal induction for the optimal activity of steroid receptors and much importantly they regulate the specificity of target-gene transcription.

Tissue Distribution and Physiological Role of ERα and ERβ

Jensen and Jacobsen were the first to describe that the biological effects of estrogen are mediated by receptor proteins (276). The cloning of ERα was reported in 1986 (276,277), but in 1995 a second ER isoform, named ERβ, was cloned from a rat prostate cDNA library (278), revealing new significance of ER function, especially with respect to our understanding of estrogen action in male. Both the isoforms are widely expressed in the organism often at significantly different levels, accomplishing also non-overlapping role.

Estrogen exposure is fundamental for the correct development of mice urogenital tract, and lack of E2 action results in structural and functional alteration of male reproductive system (348,349,350,351). In these organs, ERβ is the highest expressed isoform in several species (352,353,354,355). In the testis, both ERα and ERβ are expressed, but differently localized (356,357,358). E2 appears to play a crucial role for normal epididymal and testicular function, where the hormone is synthesized by aromatase (350). ERβ is highly expressed also in the inner epithelial cell layer of rat urethra (279,280). In the prostate, ERβ is the predominant ER, as revealed by immunohistochemistry, while ERα has been detected only in the stromal compartment (280,281). Estrogen has been clearly linked with prostate pathologies, synergizing with androgen. In fact, animal experiments show that E2 plays a role in prostatic neoplasia, benign prostatic hyperplasia and prostate cancer (359,360,361), but the specific role is still unknown. However, knockout mice have revealed that the absence of ER leads to structural and functional alteration in the prostate (301).

The importance of E2 for the development of female breast tissue is well documented. The female mammary gland undergoes a surge of cell divisions during puberty, and throughout the adult like there is cyclical proliferation and involution during estrous cycle. Women aromatase deficient patients, unable to convert testosterone to estrogen, show no breast

development at the onset of puberty (282,283,284). ERα knockout female mice have lost the capacity to develop mammary gland tissue (301,362). E2 is as well fundamentally linked to the induction and progression of mammary carcinoma. More than 70% of primary breast cancer in women are ERα positive and show estrogen-dependent growth (285). The possible role of ERβ, which is also expressed in mammary gland, in normal breast tissue development or breast cancer development is however as yet unknown (286).

Estrogen and androgen play important role also in bone metabolism and homeostasis. During adolescence, they are involved in modeling of bone. In the adult, E2 is also involved in remodeling of bone and, particularly, it is crucial for post-menopausal women to protect from bone loss and development of osteoporosis (363,364,365). In osteoblast, E2 stimulates synthesis and secretion of IGF and inhibits that of cytokines, IL-1 and IL-6, which are involved in resorption (366,367). E2 also stimulates synthesis and secretion of osteoprotegrin, a protein with a critical role in inhibition of the function of osteoclasts (368,369). Suppression of osteoclastic bone resorption and stimulation of osteoblastic bone formation form the basis of the bone-preserving effect of E2. Loss of ERα in mice is associated with decreased longitudinal and radial limb growth in both sexes (370,371).

In the cardiovascular system, both ER isoforms are expressed in endothelial cells (287,288), smooth muscle cells (289,290), and myocardial cells (279,280). The risk of women development of cardiovascular diseases increases dramatically after menopause, suspected to be a consequence of the cessation of estrogen production by ovaries (116). E2 exerts both nongenomic effects on vasodilatation, by acting on ion channels function (292) and nitric oxide synthesis (293,294), and long-term effects, by modulation of prostaglandin synthase, nitric oxide synthase, and endothelin gene expression (295,296,297).

Estrogen is reported to influence a variety of function also in the central nervous system (298). Expression of ERα and ERβ differs in some areas but in others is overlapping (299,300). ERα is more expressed in the hypothalamus. Estrogen, through the hypothalamus-pituitary axis, regulates the expression and secretion of hormones such as luteinizing hormone, follicle stimulating hormone, growth hormone, and prolactin, from the

anterior pituitary gland (301).

Evidences accumulated suggest that ERα and ERβ regulate, at least in part, separate and distinct gene networks, particularly regarding tissue and cell specific effects of estrogen. Varying ratio between the two isoforms may be fundamental for obtaining the final hormone effect.

RESULTS

CHAPTER 1:
High Resolution Quantitative HTM Analysis of ERα Transcriptional Activity and Chromatin Remodeling

ERα is a representative member of nuclear receptor transcription factors. Approximately 70% of breast cancer patients are positive for elevated ERα expression at diagnosis. Several anti-estrogen have been developed and used in anti-cancer therapy and include 4-hydroxitamoxifen (4HT) and ICI 182,780 (ICI). These molecules act as transcriptional antagonist by promoting the repressed state of promoter-localized ER, which, in turns, generates a transcriptionally prohibitive environment and chromatin condensation. Differently, binding of agonist to ER increases the recruitment of multiple coactivators and chromatin remodeling proteins, leading to chromatin decondensation.

In this study, we have developed a model system (PRL-HeLa cell line) that is valuable to High throughput microscopy (HTM) analysis of chromatin remodeling and ERα functional response to ligand.

Contents:

Introduction to High Throughput Microscopy (HTM)

Traditional biochemical and reporter assay approaches are representative of an averaged evaluation of cellular responses. Recent microscopic single-cell analyses in some cases provide markedly different results relative to classical approaches, due to the fact that individual cells could exhibit quiet varied responses. High throughput microscopy combines automated image acquisition to qualitative analysis at the individual cell level. HTM couples rapid acquisition of multiple cell pictures by a robotic fluorescence-microscope (IC100, Beckman Coulter) to computational-imaging analysis software (Cytoshop, Beckman Coulter), under similar acquisition parameters (i.e. type of optics, number of fluorescent channels, exposure time, and number of optical fields) (see reference 372). Then, cells are easily identified basing on their DAPI staining (***Fig.8***). In this way, HTM allows quantitative study of variation in plenty of qualitative cellular features. HTM-coupled computational-imaging analyzer includes hundreds of cell morphological detection/measurement algorithms, which primarily allows to filter (or gate) the initial imaged cell population to obtain a more homogenous subpopulation of cell pictures, then susceptible for analysis. Specialized algorithms are supplied to measure specific phenomena, including protein subcellular localization. Finally, data are exported as excel files. These algorithms have already been applied for successfully quantify the cytoplasmic to nuclear translocation of NFκB-p65 subunit (375). Here, we have adopted HTM as an analytical instrument to study ERα-regulated transcriptional activation in transfected-cell system. Ahead, in PART-2 section, HTM will be greatly applied for measuring subcellular localization of SRC-3 upon phosphorylation and interaction with ERα.

HTM Analysis of Protein Expression Level in EGFP-ERα-transfected Cell Population

In this paragraph, we will introduce the basic tools needed for HTM analysis system, in terms of images acquisition, data extraction, filtering, and data analysis of a population of HeLa cells exogenously expressing EGFP-ERα (***Fig.8-10***). We will describe how to use HTM to properly select cells for analysis based on cell viable morphology and expression of exogenous EGFP-ERα.

Cells are imaged by the IC100 microscope using the 40X/0.90 resolution objective, which gives a good balance between field size and resolution, using an exposure time of approximately 0.10 ms for DAPI and 500 ms for GFP fluorescence. Afterwards, acquired pictures are transferred to the imaging-analysis software Cytoshop for computational imaging analysis, which requires application of appropriate algorithms throughout three sequential steps.

Step 1. Initially, cells are identified and extracted by generating a *nuclear mask* around the DAPI-stained nucleus, through a predefined radius range and an optimized image filter, which is used to create marked object-background contrast, followed by automatic "thresholding". In this way, effects due to background fluorescence are subtracted and the mean image intensity is corrected. The corresponding cell channel mask or *extraction correlation radius*, which is used for the approximate identification of cytoplasm, is calculated and defined as an intersection between the "thresholded" channel, the *Voroni tessellation polygon*, and the *nuclear mask* (***Fig.8***). The resulting total cell population is biologically heterogeneous, containing apoptotic or dividing cells, and clusters of cells, which are not defined as a single cell. This total cell population contains also cells with high level of expression of GFP-ERα. Morphologically regular cells and properly expressing GFP-ERα will be filtered and selected in step 2 and 3, respectively.

Step 2. Threshold filters standard to Cytoshop software are used for removing aberrant cell types and properly select cells for analysis, based on viable morphology (***Fig.9***). Three filters are simultaneously applied to the total imaged cell population: 1) "DNA Content

Outliner", which removes apoptotic and mitotic cells based on DAPI intensity; 2) "DNA Cluster", which removes cell clusters that could not be resolved as individual cells; 3) The "Keep Cells Acquired with Two Channels" filter is combined with the two previous morphological filters, which are derived for the first fluorescent channel (DAPI) and applied to the second channel (i.e. GFP), in order to produce a more homogenous cell sub-population differentially expressing GFP-ERα.

Step 3. Finally, the morphological-selected cell sub-population is filtered for proper levels of expression (near endogenous) of GFP-ERα, by examination of the mean intensity value of cellular fluorescence (***Fig.10***). Cells that don't express green fluorescence at all (presumed to be non-transfected with GFP-ERα) are removed as well. On account of the fact that HeLa cells do not endogenously express ER gene, the lower and upper values of expression levels in GFP-ERα cells are arbitrarily set within a range of cellular fluorescence intensity comparable to presumed endogenous expression. As a result, GFP-ERα over-expression artifacts are automatically eliminated and a final population gallery of selected cells is obtained. It is anyway important to note that the expression range values are not absolute, but differs between experiments, due to biological and optical variability. Moreover, note also that from the original total population of ~ 6000 identified and morphologically filtered cells, only ~ 300 are ultimately selected for possessing the adequate level of GFP-ER expression.

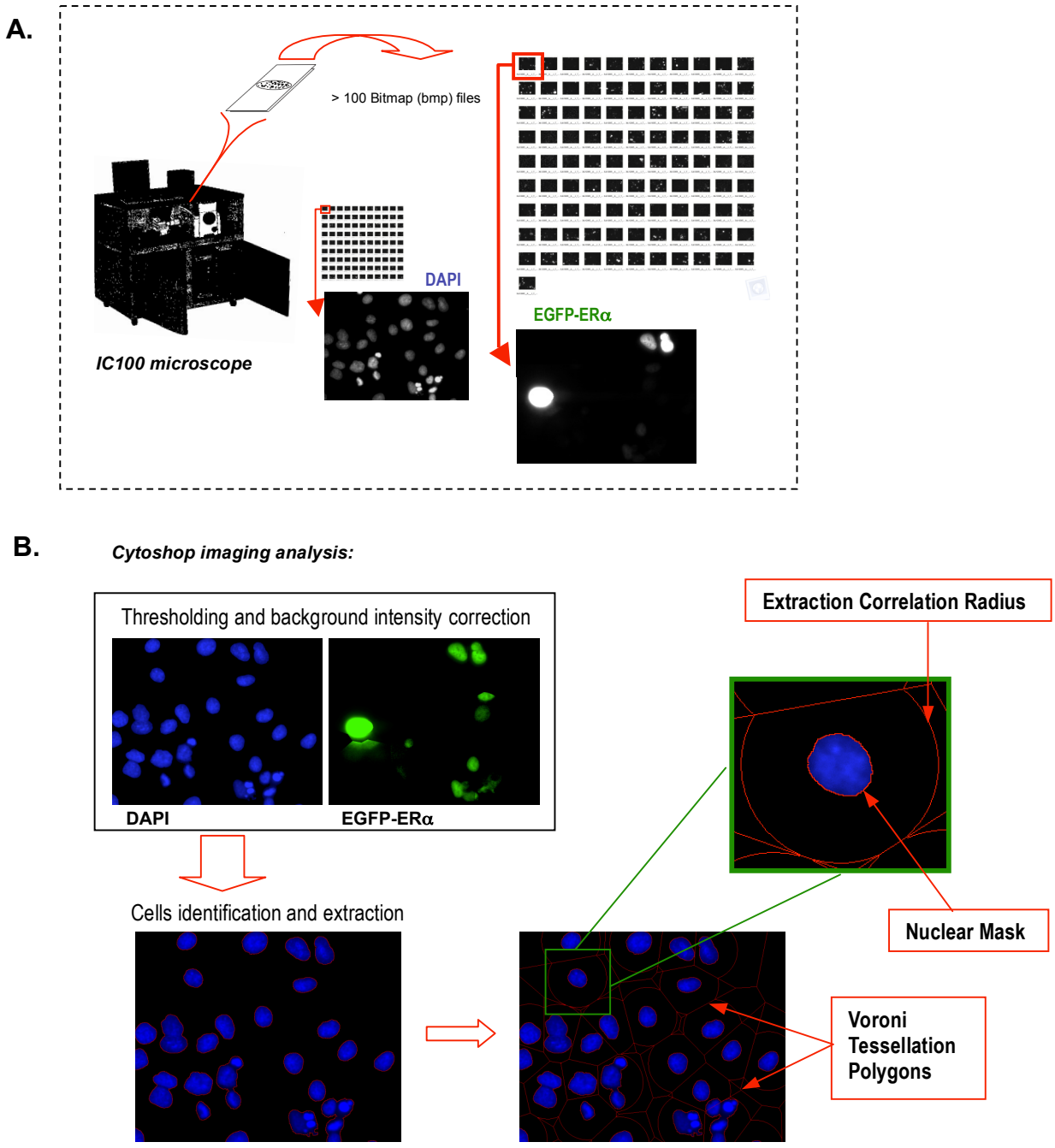

Figure 8. Step 1: HTM images acquisition, cell identification and extraction. (A) The two fluorescence channels, DAPI and GFP-488nm, are imaged in a single plane, using a 40X/0.90 NA objective. More than one hundred pictures are acquired per coverslip and transferred to the Cytoshop imaging software for data analysis. (B) An automatic threshold algorithm subtracts background fluorescence, allowing image intensity correction. Contrast measurements identify DAPI-stained nuclei (nuclear mask) and the Voroni tessellation polygons define cell boundaries, required for delineating the approximate cytoplasm by the extraction correlation radius.

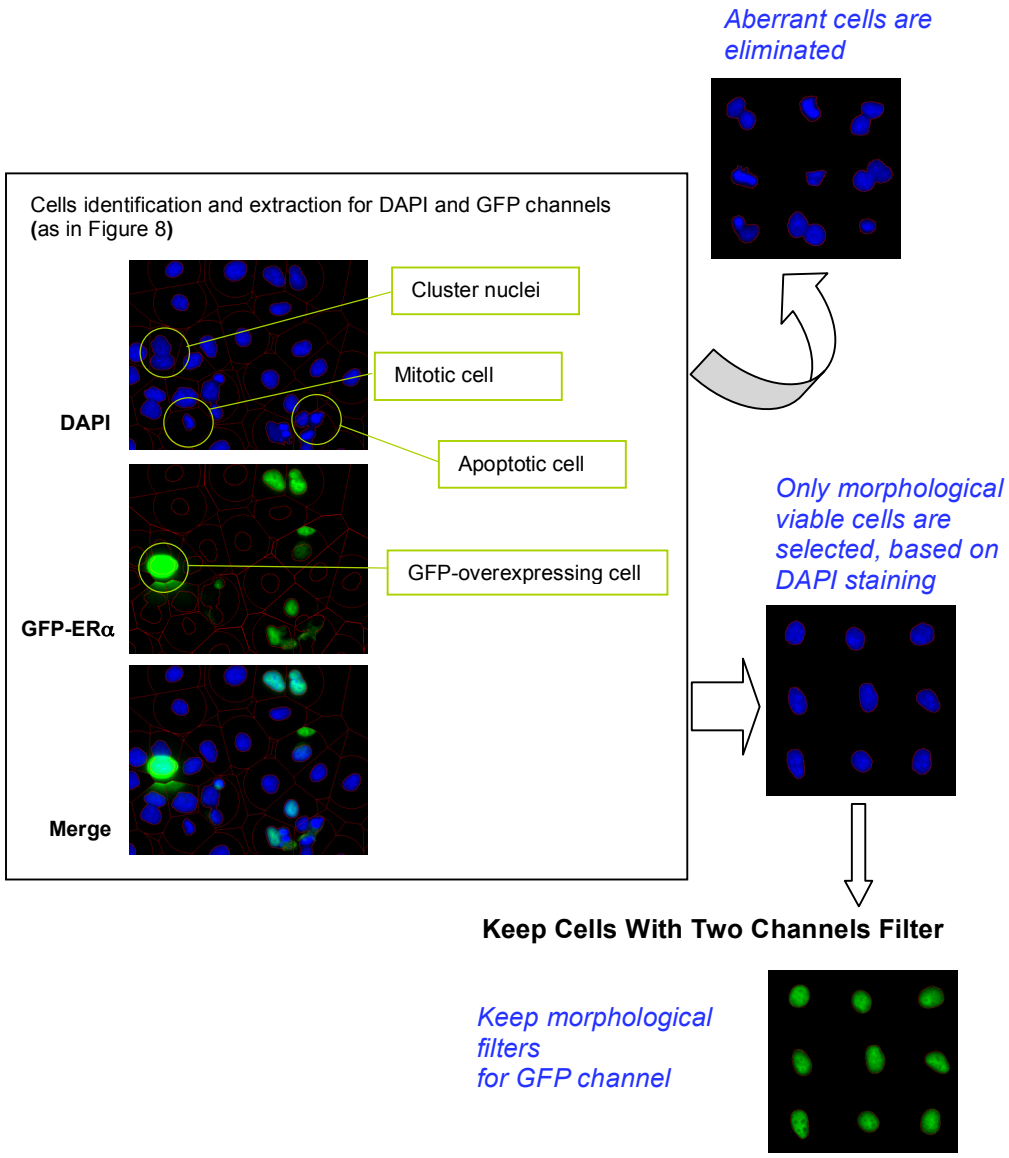

Figure 9. Step 2: Morphological Filtering of Total imaged Cell Population. After cell identification and extraction, the original cell population, containing apoptotic or mitotic cells, unresolved nuclei clusters, is filtered to obtain a morphological selected sub-population of viable interphase cells. "DNA Content" algorithm is applied to identify the apoptotic and mitotic cells. "DNA Cluster" algorithm is applied to identify the cluster of non-resolved nuclei. The "Keep Cells With Two Channels" filter is then combined with the two previous filters and applied to the total population of GFP-ERα-expressing cells, to generate a morphologically homogenous population of interphase cells in the GFP channel. Cell presenting over-expression of GFP-ERα will be then eliminated (step 3). Representative galleries of aberrant and selected cells for the DAPI channel, and the corresponding cells for GFP channel, are depicted.

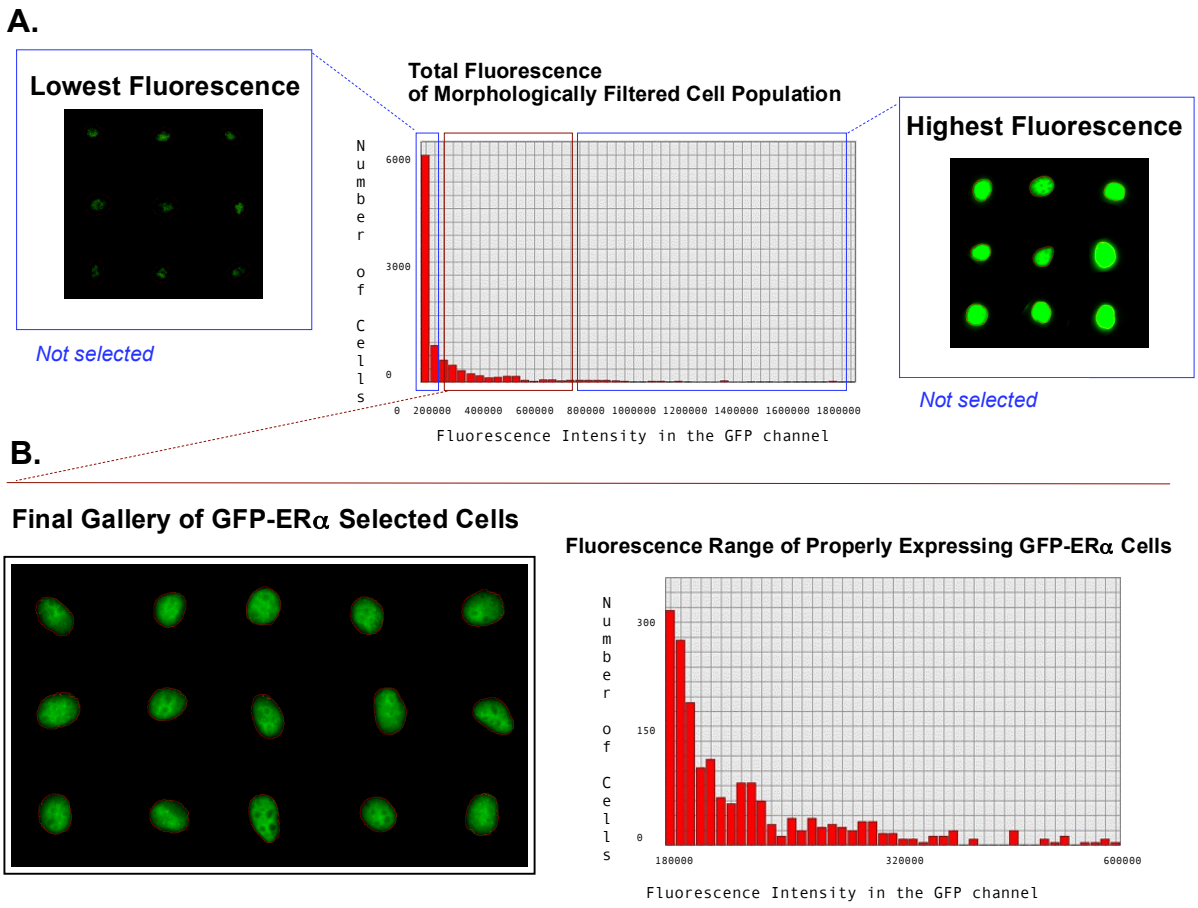

Figure 10. Step 3: Selecting Cell Properly Expressing GFP-ERα. HeLa cells transiently transfected with GFP-ERα are examined for expression levels. Channel 0 (DAPI) and channel 1 (GFP) are imaged in the same plane and morphologically filtered as in Figure 9. (A) Two galleries featuring low and high expressing cells are depicted. A histogram is presented that demonstrate the broad range of GFP-ERα fluorescence expressions with a maximum intensity of ~ $1.8x10^6$. The range of fluorescence intensity for properly expressing GFP-ERα cells is conventionally identified between ~ 180000 and ~ 600000. Cells with lower or higher values are automatically discarded. Note that the vast majority of cells are not transfected or don't express detectable level of GFP fluorescence intensity. (B) A GFP-ERα expression final histogram after morphological and expression filtering and a representative image gallery of selected HeLa cells transiently expressing GFP-ERα are presented. Note the absence of lower/higher expressing cells.

Engineered PRL-HeLa Cell Line for Studying Ligand-dependent Chromatin Remodeling by ERα

We developed a HeLa cell line (PRL-HeLa) specifically engineered for the study of ERα transcriptional function (20). PRL-HeLa cells contain multiple and stable genomic integrations of a replicated prolactin (PRL) enhancer-promoter (***Fig.11 A***). The multiple integrations (PRL array) are spatially confined and are visualized by the accumulation of GFP-tagged ERα and fluorescently immunolabeled coregulators or RNA Polymerase II. The enhancer-promoter within the PRL-array contains repetitions (52X) of Pit-1/ERE binding site for ERα, following the physiological PRL distal enhancer (-1807 to -1498), and the proximal promoter, fused to a reporter gene, which enables to assay ERα-dependent mRNA expression through RNA fluorescence *in situ* hybridization (FISH) detection.

Different cellular clones were developed and characterized for responsiveness to ligand treatment and binding of ER and coregulators. Sequential cloning steps generated by recombination events 2X, 4X, 8X, 13X, 26X, and 52X Pit-1/ERE repeated sequences (20). Finally two different clones have been selected for containing stably integrated the 52X PRL-array into the genome: in clone referred as 23, the construct is integrated into two distinct chromosomes, and visualized as two separate spots when GFP-ERα targeted; clone 19 presents a single chromosome-integration (***Fig.11 B***). Concerning to both clone 23 and clone 19, quantitative Southern blotting indicated that approximately 150-300 total copies of 52X PRL-array are integrated in the cell.

PRL-HeLa cells were transiently transfected with GFP-ERα, resulting in localization of GFP-ERα on the 52X PRL-array. Addition of ERα agonist (17-β estradiol, E2) for 30 minutes causes a visible opening (decondensation) of the PRL-array relative to no ligand treated cells. Cells were then fixed and immunoassayed for endogenous SRC-1, -2, -3 recruitment to the PRL-enhancer/promoter and co-localization with GFP-ERα. All of the three coactivators tested occupy the GFP-ER-targeted array (***Fig.12***). Treatment with E2 leads also to co-localization of RNA Polymerase II to the PRL-array (***Fig.13***), and to transcriptional readout of the reporter gene, with an increased FISH signal of about 2 fold

over the level of vehicle (ethanol) treated cells and about 8 fold over the basal level of non-transfected cells (***Fig.14***). On the contrary, treatment for 30 minutes with the ERα antagonist 4HT or ICI induces a marked lowering in size (condensation) and displacement of p160 coregulators and RNA Polymerase II from the GFP-ERα targeted PRL-array (***Fig.12*** and ***Fig.13***). As expected, condensed PRL-array has a FISH signal reduction of more than 10 fold relative to the vehicle and about 25 times lower relative to E2 stimulation (***Fig.14***). Importantly, PRL-HeLa cells that are not transfected with GFP-ERα also present a basal level of PRL-array chromatin decondensation and RNA Polymerase II localization (about 12% of the total cell population), which corresponds to a minor FISH signal (***Fig.12*** and ***Fig.14***). Additionally, PRL-HeLa cells transfected with GFP-ERα but vehicle treated also present a subpopulation (about 15%) of cells with a detectable GFP-ERα transcriptional activity to the PRL-array (***Fig.12*** and ***Fig.14***), which is assumed as the ligand-independent ability of ER to bind specific responsive elements and partially activate transcription.

It follows that array size in this cell line expressing ERα is an indicator of receptor transcriptional functionality in response to ligand treatment. Much importantly, these data indicate that chromatin decondensation and transcriptional activation are direct reflections of ERα dependent coregulators recruitment to the PRL-array. For this reason, PRL-HeLa cell system allows the ability to measure the multiple aspect of ERα transcriptional activity by HTM, as it is shown in the next paragraphs, which is not possible in conventional cell lines.

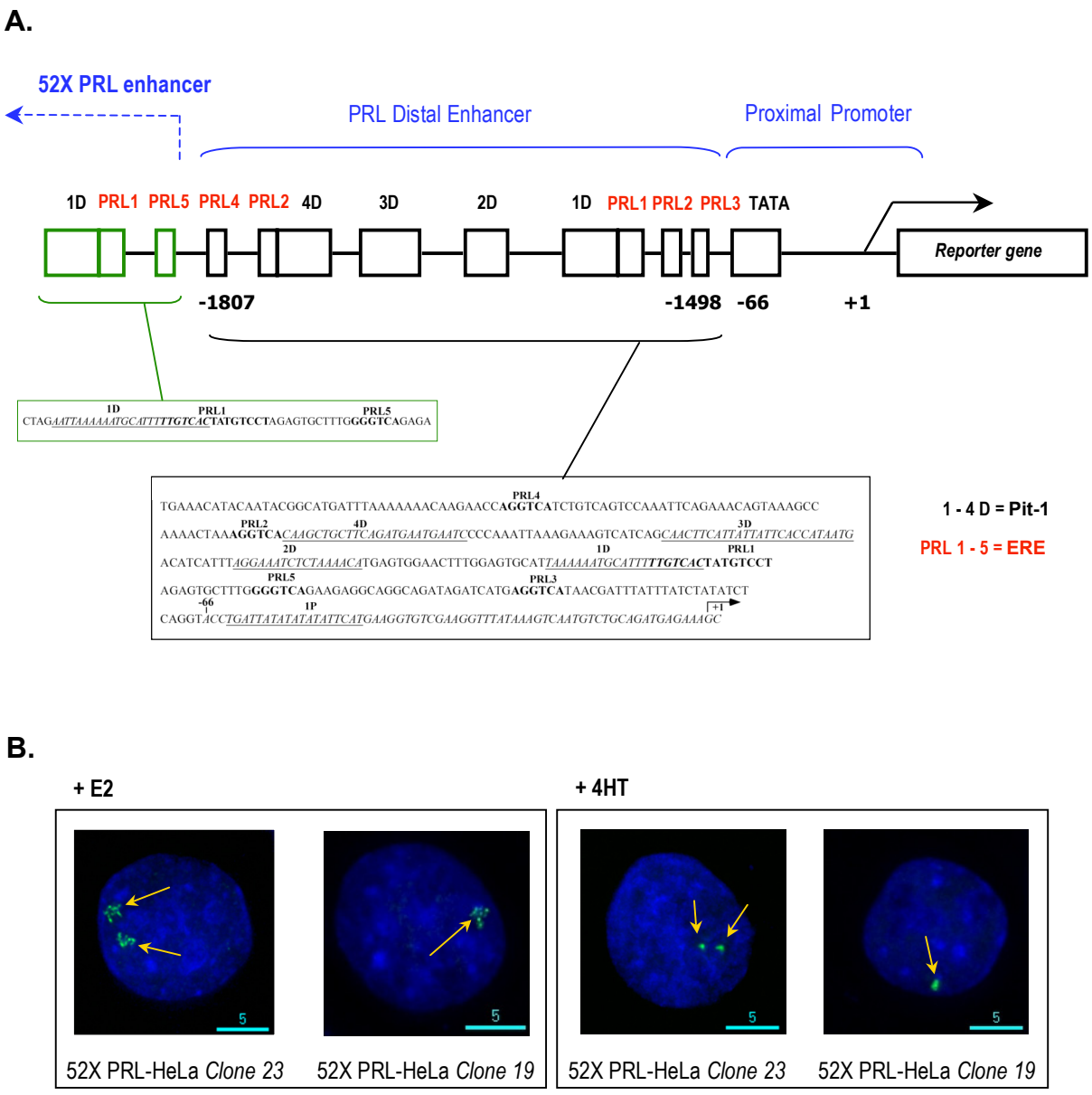

Figure 11. 52X Prolactin integrated enhancer-promoter in PRL-HeLa cells. PRL-HeLa engineered cell line contains a chromosomally integrated prolactin (PRL) enhancer-promoter array (PRL-array) that regulates the expression of a reporter gene. (A) Schematic representation of PRL-array. Multicopy repetition (52X PRL enhancer) of Pit-1/ERE binding site (1D-PRL1-PRL5) follows the physiological PRL distal enhancer (-1807 to -1498) and the proximal promoter, which is fused to the reporter gene. (B) Transfection of PRL-HeLa cells with GFP-ERα results in the localization of GFP-ERα on the integrated prolactin promoter (yellow arrows). Treatment with E2 leads to a visible opening of the GFP-ERα-targeted array, while antagonist 4HT causes a drastic condensation. Both clone 23 and clone 19 are shown.

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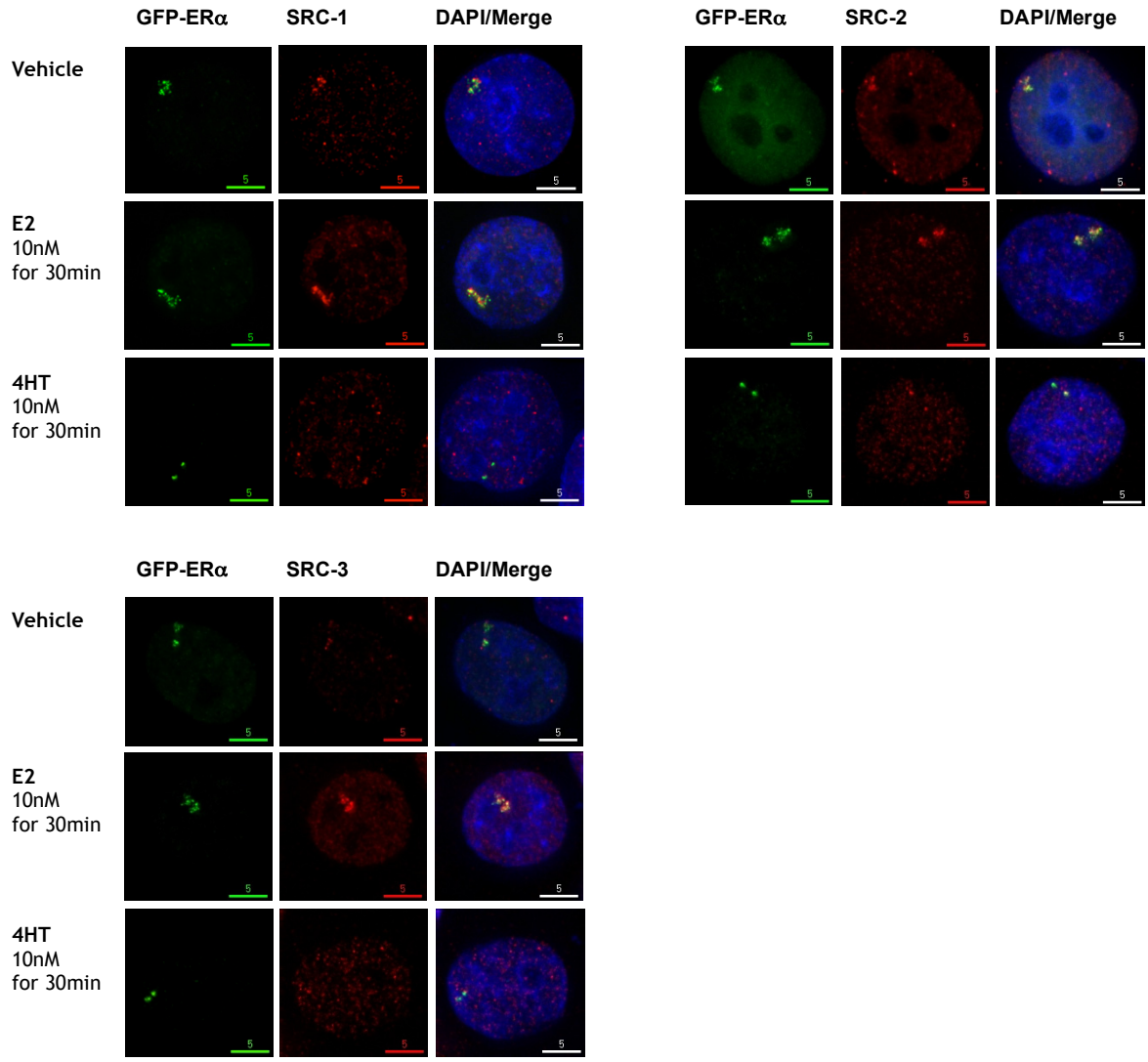


Figure 12. Localization of p160 family members on the 52X PRL-array. After 48 hours in hormone free media, Clone 23 PRL-HeLa cell line was transiently transfected with pEGFP-ERα expression vector and then treated with agonist (E2), antagonist (4HT or ICI), or vehicle, as indicated. Cells were then fixed and processed for immunostaining with antibodies specific for endogenous SRC-1, -2, or -3. Decondensed array are seen in vehicle and E2-treated cell, although E2 treatment results in a major decondensation. On the contrary, exposure to the antagonist leads to a visible condensation. All of the three p160-coactivators co-localize with GFP-ERα to the open PRL-array in presence of E2 but not to the condensed array. The DAPI channel is shown as merged with the GFP and the red channel. ICI-treated cells (not shown) display similar array condensation and coregulator displacement like 4HT-treated cells

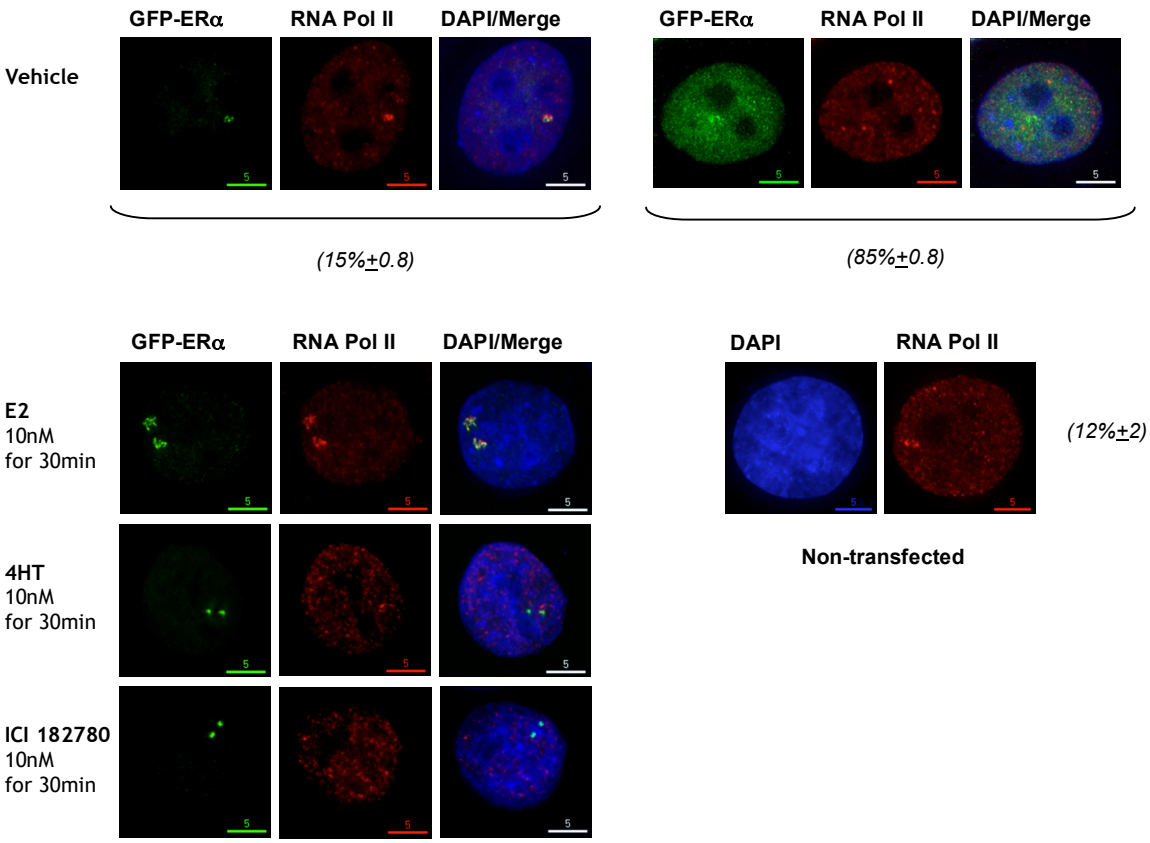


Figure 13. Localization of RNA Polymerase II on the 52X PRL-array. After 48 hours in hormone free media, Clone 23 PRL-HeLa cell line was transiently transfected with pEGFP-ERα expression vector and then treated with agonist (E2), antagonist (4HT or ICI), or vehicle, as indicated. Cells were then fixed and processed for immunostaining with antibodies specific for the endogenous RNA Polymerase II. Clearly decondensed and Polymerase-targeted array appears following E2 exposure in GFP-ERα transfected cells. Nevertheless, a small subpopulation of cells showing slight decondensation of the array is also present in vehicle-treated cells, while the vast majority of cells present cytoplasmic diffusion of ERα. Also, constitutive chromatin decondensation of the array is revealed in non-transfected cells by Polymerase localization. On the contrary, exposure to the antagonist leads to a visible condensation and Polymerase displacement. Percentages of subpopulations are indicated. The DAPI channel is shown as merged with the GFP and the red channel.

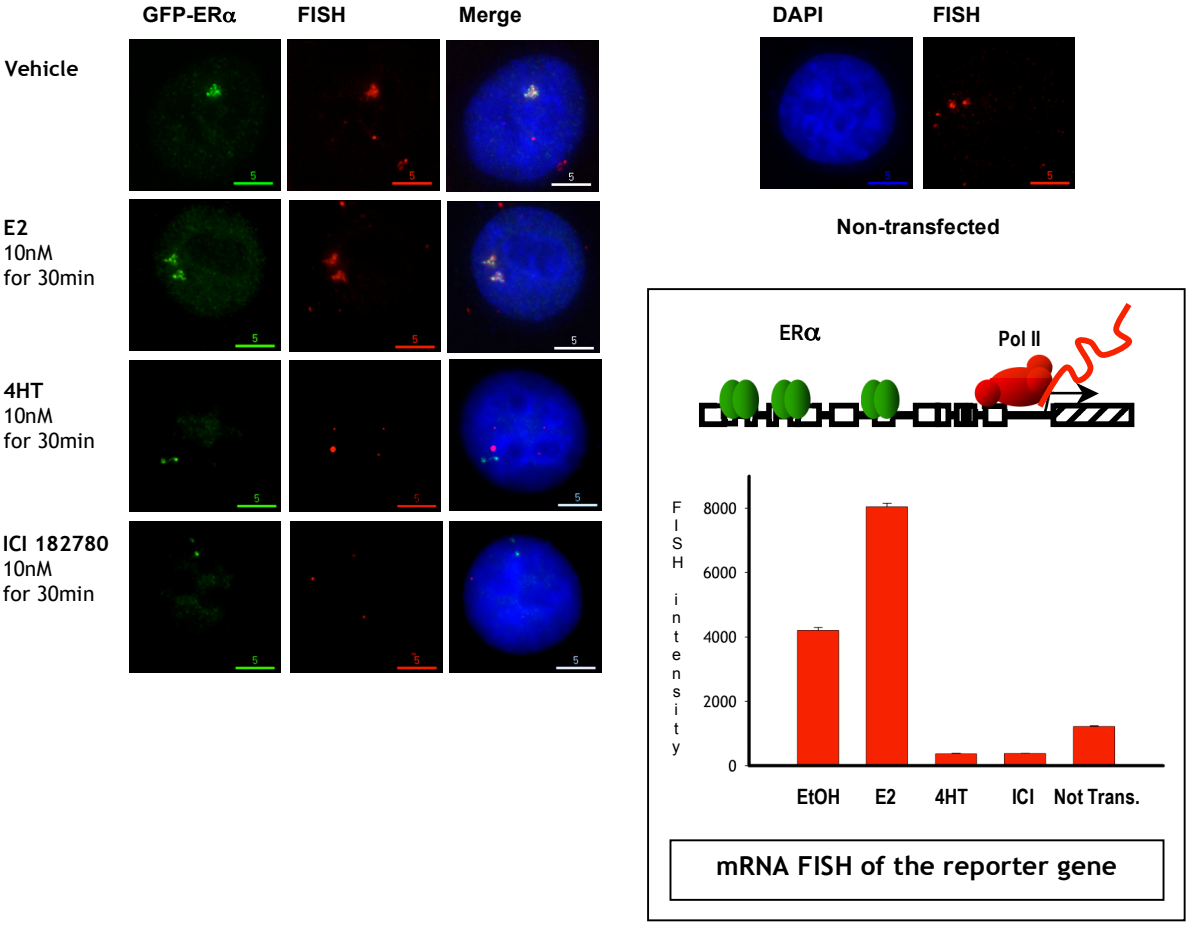


Figure 14. Analysis of the transcriptional response of the 52X PRL-array. After 48 hours in hormone free media, Clone 23 PRL-HeLa cell line was transiently transfected with pEGFP-ERα expression vector and then treated with agonist (E2), antagonist (4HT or ICI), or vehicle, as indicated. Cells were then fixed and processed for FISH and imaged as described in Methods. Fluorescence is shown in green and mRNA FISH signal is shown in red. The merge includes DAPI-staining in blue. Intensity of FISH signals at the PRL-array was quantified by the freeware-imaging program GimpShop (see Material and Methods) and graphed. The treatments are also indicated below the graphs. While there is a constitutive FISH signal in non-transfected cells, it increases significantly in cells with arrays targeted by GFP-ERα ($p<0.01$) and treated with E2. A small, but significant FISH signal is detectable also in vehicle-treated cells ($p<0.05$). Conversely, there is a significant decrease in FISH signal at the array in cells treated with antagonist ($p<0.001$).

Quantitative Determination of PRL-array Size via HTM

In our early experiments, we show that PRL-array shape and size may vary considerably and correlate with agonist or antagonist exposure of PRL-HeLa cells transiently expressing GFP-ERα. In this section, we will illustrate how high throughput microcopy can be a useful tool for quantifying the transcriptional response of 52X PRL-array to ERα ligands at the single cell level.

Transiently GFP-ERα-transfected PRL-HeLa cells were treated for 30 minutes with vehicle, 10nM of E2, or 10nM of antagonist (4HT or ICI), fixed and immunolabeled for anti-RNA Pol II, DAPI stained, and imaged on IC100 using the high numerical aperture 40X, 0.9 NA, objective. The secondary antibody is conjugated to Alexa 647 (Molecular Probes), which gives a signal in the far-red wavelength. Cells were imaged for DAPI, red immunofluorescence, and GFP fluorescence. Exposure time was approximately 0.1 ms for DAPI, 500 ms for immunolabeled RNA Polymerase II, and 2 seconds for GFP-ERα. Pictures from the total cell population, for the three different channels, were collected and analyzed with Cytoshop software, as described in previous sections and in Methods. Channel 0 (DAPI) was used to find the focus on nuclei; channel 1 visualizes GFP-ERα transfected cells and identified the PRL array; channel 2 for RNA Pol II. To maximize the number of PRL-array imaged in focus, the Z-plane section for GFP-ERα was offset 1 μm from the DAPI focal plane. Up to 100 fields per coverslip were imaged to obtain an unfiltered total cell population of at least 5000 cells. An aggregate algorithm was automatically applied to identify the GFP-ERα array (aggregate), before morphological filtering. The aggregate identification parameters are 350 (maxim area in pixel), 30 (object scale), 5 (minimum peak height). These parameters were determined to be optimal for identifying arrays in cells treated with either agonist (large array) or antagonist (small array) and for excluding non-array identified aggregates. Thereafter, mitotic and apoptotic cells, nuclei clusters, out of focus, and over-expressing transfected cells were removed from the images collection, using classical morphological and expression filters, and resulted in a gallery of selected cells (***Fig.15 A***). An additional filter (*Number of Aggregates*

Identified per Cell <*3*) was then applied to the morphology/expression-selected subpopulation, in order to include only cells with maximum of two aggregate per cell. Finally, a homogenous cell population was generated and visually and manually inspected for colocalization between GFP-ERα-targeted array and RNA Polymerase II immunostaining. Cellular parameters, like PRL-array size, were automatically measured at single cell level (for each ligand treatment) and directly exported as an excel data file.

HTM analysis shows a drastic difference in the PRL-array size, depending on the exposure to specific ERα ligands (agonist or antagonist), which we would call ERα-dependent chromatin remodeling. These results indicate a basal array size (vehicle control) in pixels of 78.8 ± 4.2 (***Fig.15 B***). Treatment with E2 causes a spatial expansion of the array (131.9 ± 4.5). Treatment with antagonist 4HT or ICI causes array contraction (42.7 ± 3.0 and 45.3 ± 2.6 respectively). Array size for each treatment is significantly different than vehicle control ($p<0.01$).

In conclusion, we could assume that PRL-array size is a reliable indicator of ERα ligand-dependent regulation of transcription, as shown in figure 14. Here, PRL-HeLa cell line and HTM has successfully demonstrated the capability to quantitatively measure the variation of the array size upon ligand treatment. In the next chapter, we will show HTM as a convenient tool for demonstrating distinct aspect of ERα transcriptional function in relationship with the coregulator SRC-3.

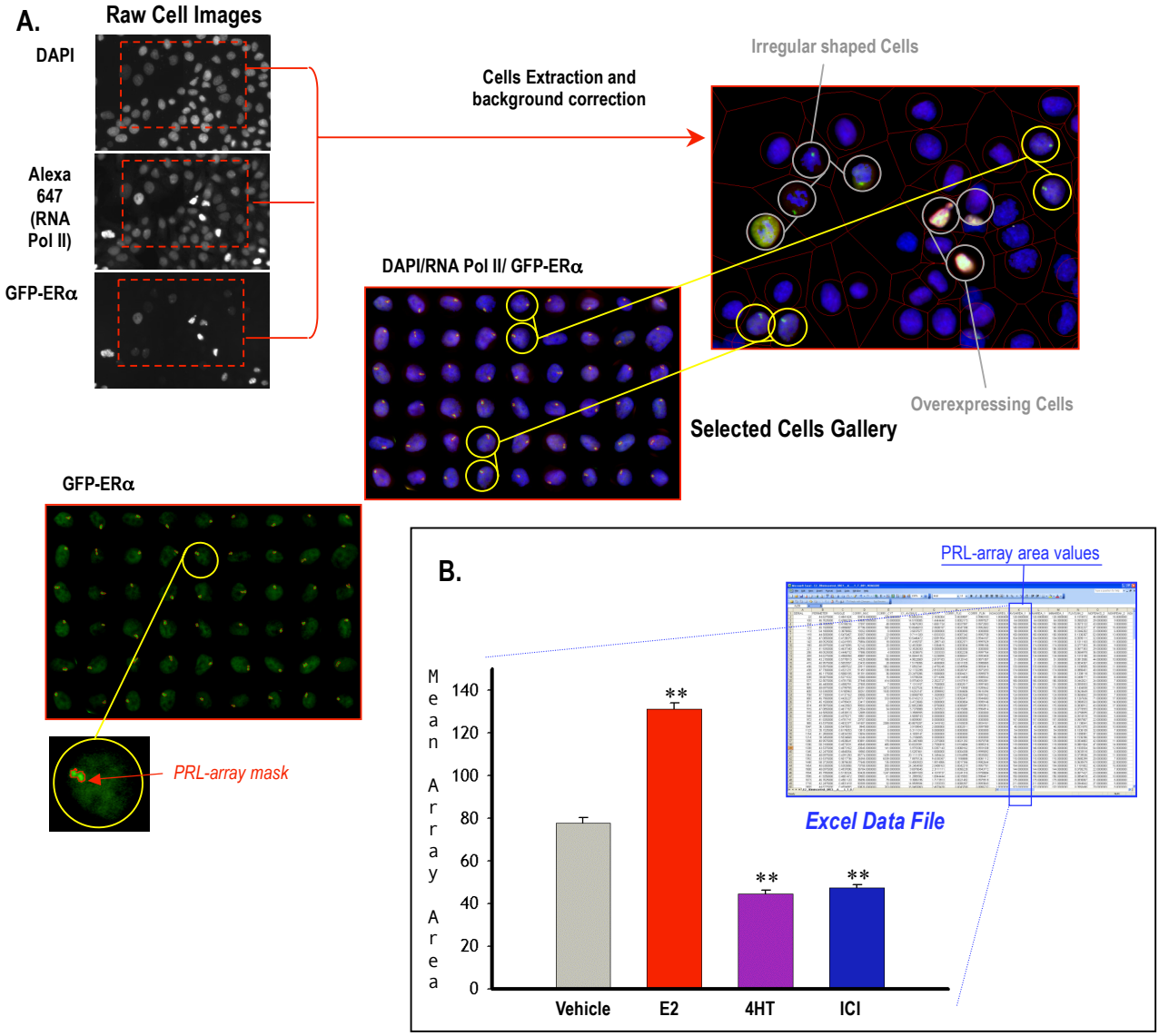


Figure 15. HTM analysis of PRL-array size. PRL-HeLa cells (Clone 23) were transiently transfected with GFP-ERα, fixed and DAPI/RNA Pol II stained upon treatment with ligands. Raw pictures from the total cell population were collected and analyzed with Cytoshop software. (A) A representative field of E2 treated cell images is presented. An aggregate algorithm was used to identify, mask (red) and quantify the PRL-array. Out of focus, over-expressed and irregular-shaped transfected cells (gray circles) are removed from the images collection, as previously shown in figures 8-10, resulting in a gallery of selected cells (yellow circles). An additional filter (*Number of Aggregates Identified per Cell <3*) was applied in order to remove cells with non-array aggregates identified by the aggregate algorithm. The selected cell population contains exclusively in-focus and correctly identified arrays. (B) From this gallery, cellular parameters, like PRL-array size were measured and automatically exported as an excel file. Size values obtained are graphed and presented as mean array area in pixels (n cells > 100 for each treatment). Error bars represent SE (**, P<0.01).

CHAPTER 2:
Regulation of SRC-3 Cellular Localization and Dynamics by Phosphorylation and ERα Interaction

SRC-3 belongs, together with SRC-1 and SRC-2, to p160/SRC (steroid receptor coactivators) family of general transcription coactivators of nuclear receptors (NRs). SRC-3 is strongly suggested to play an oncogenic role in cancer: it is amplified in ~10 % of human breast cancer and in the majority of tumors. SRCs are essentially localized in the nucleus. However, weak, but significant, expression is also observed in the cytoplasm. Here, with an extensive use of cellular microscopy, including quantitative high throughput microscopy (HTM), we will demonstrate that SRC-3 is in fact a primarily nuclear protein and that a fraction of the cellular pool shuttles between nucleus and cytoplasm. Moreover, phosphorylation state and interaction with ERα regulate the compartmental dynamics of SRC-3. We also demonstrate that inhibition of SRC-3 phosphorylation results in reduced ERα-dependent transcription in PRL-HeLa cell line and intranuclear ERα-SRC-3 complex formation. Finally, we suggest that the nuclear-cytoplasmic shuttling of SRC-3 may be important for facilitating nuclear translocation of estrogen receptor.

Contents:

SRC-3 Is Primarily a Nuclear Protein

To examine the subcellular distribution of endogenous SRC-3, we used HeLa cells immunolabeled with a monoclonal anti-SRC-3 antibody (***Fig.16A***). The vast majority of cell revealed an intense nuclear signal, although in a few cells (>8%) weak cytoplasmic staining was also observed.

We repeated the experiment using HeLa cells transiently transfected with GFP-SRC-3, confirming that SRC-3 is localized primarily in the nucleus (***Fig.16B***). However, in this case it was noted that numerous cellular globules (or speckles) accumulate in cells expressing high levels of GFP-SRC-3, probably due to non-physiological polymerization of the transfected protein when present at much higher-than-endogenous levels, as reported previously for several GFP-overexpression-based cytological studies (374,375). To avoid any possible alteration in our quantitative analyses (see later), we excluded all cells having more than three times the level of endogenous SRC-3 expression by HTM-automated filtering (as described in ref. 372).

Additionally, to eliminate concerns associated with the exogenous transfection of GFP-fused protein, we also generated a HeLa cell line stably expressing HA-SRC-3 under the control the tetracycline (Tet) promoter, which is reversibly turned on and off in the presence or absence of the antibiotic tetracycline, or its derivative doxycycline (DOX). HA-SRC-3 expression was repressed in the presence of 200 ng/ml DOX in hormone-free medium. To express HA-SRC-3, cells were grown in DOX-free medium for 48 hours. Immunolabeling with anti-HA antibody confirmed that SRC-3 is localized in the nucleus (***Fig.16C***).

Taken together, these results show a clear prominent nuclear localization of endogenous SRC-3 and transiently transfected SRC-3, when this is expressed at approximately physiological levels and without accumulation of cellular speckles.

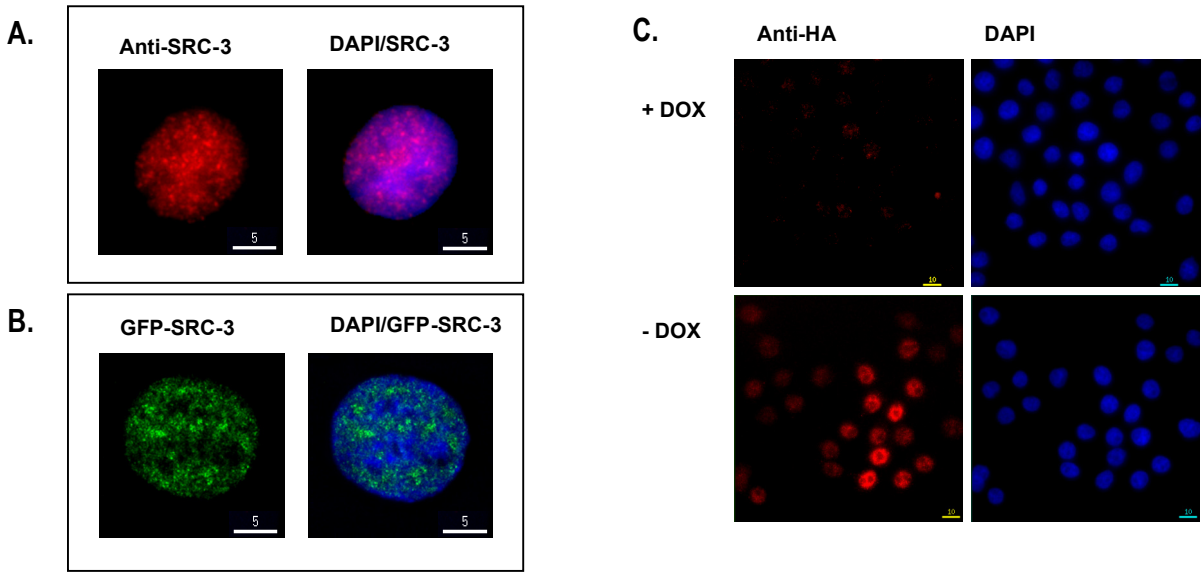
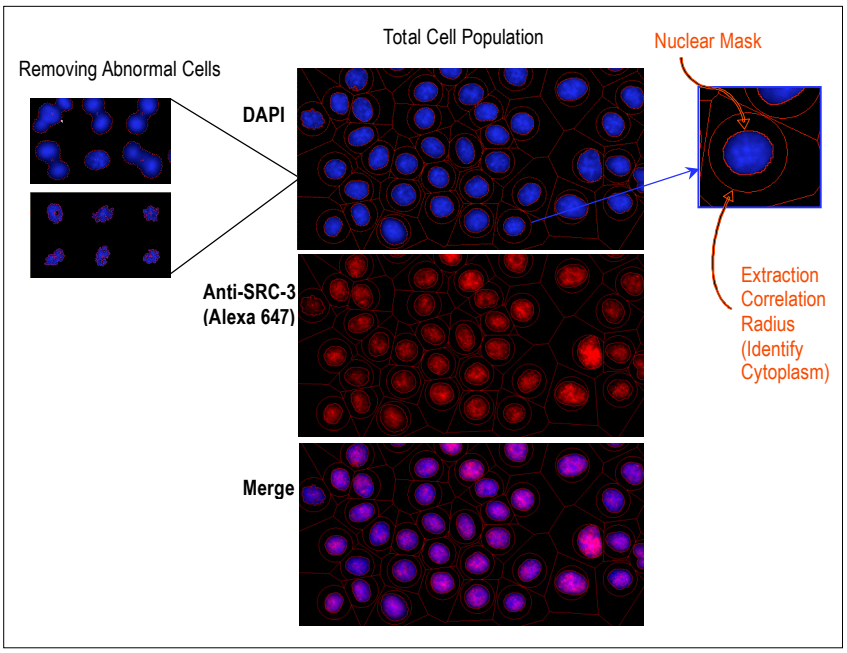


Figure 16. SRC-3 is a predominantly nuclear protein. (A) HeLa cells immunolabeled for endogenous SRC-3. (B) Transient expression of GFP-SRC-3 in HeLa cells. (C) HeLa cells stably expressing HA-SRC-3 under the control of Tet promoter. The expression of HA-SRC-3 is repressed in the presence of doxycycline (+DOX) and activated when cells grow in DOX-free medium (-DOX). HA-SRC-3 is expressed in the nucleus as shown by immunodetection using anti-HA antibody. DAPI staining delineates the nuclei. Deconvolution microscopy was performed either with 63X or 20X objective (see methods).

Quantification of SRC-3 Cellular Localization Using High-resolution HTM

To quantitatively characterize SRC-3 localization, accurate imaging analysis was performed by HTM. HeLa cells were immunolabeled for endogenous SRC-3, fixed and DAPI stained. The secondary antibody was conjugated to Alexa 647. Afterwards, cells were imaged for DAPI and red immunofluorescence by IC100 microscope, using the 40X/0.90 resolution objective, and computationally analyzed by Cytoshop. As previously described in chapter 1, morphological filters were applied to generate a final gallery of selected cells. Examination of the mean nuclear fluorescence of endogenous SRC-3 indicates a broad range of expression (***Fig.17***).



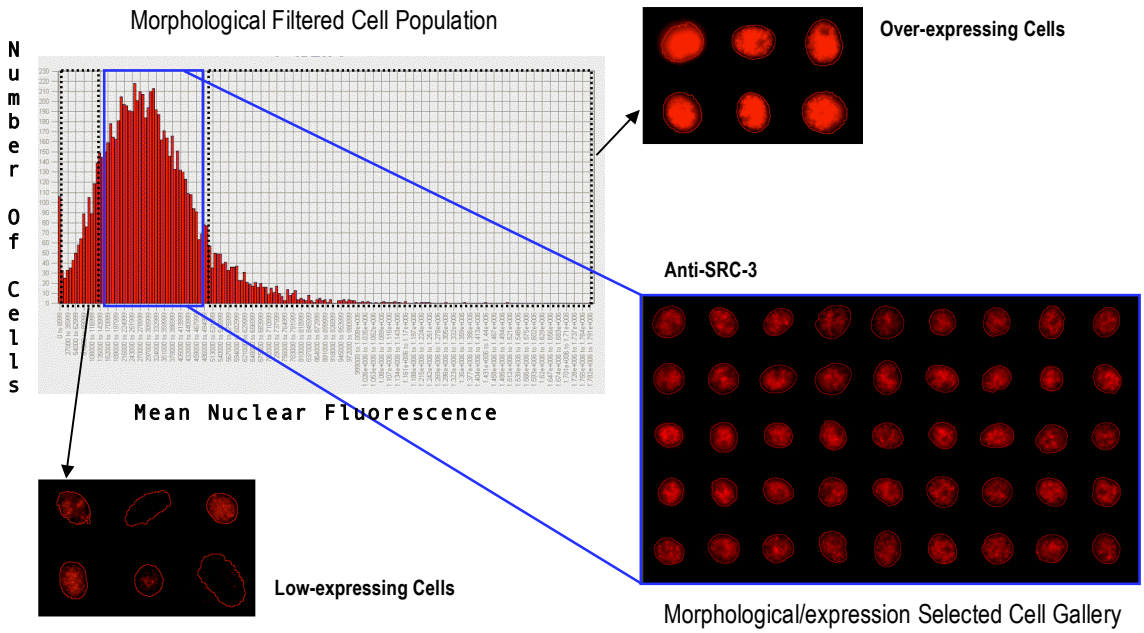


Figure 17. Analysis of endogenous SRC-3 nuclear localization by HTM. The expression level of endogenous SRC-3 in HeLa cells is examined by immunofluorescence and HTM. Two channels are imaged in a single plane, channel 0 (DAPI) and channel 1 (SRC-3), and the resulting cell population is morphologically filtered as in figures 8-10. A histogram is presented that demonstrates the broad range of SRC-3 nuclear immunofluorescence. A selected gallery containing cells with correct expression levels is shown. Two galleries featuring high and low expressing cells are also depicted.

In a second pivotal experiment, we examined the mean nuclear fluorescence of transiently expressed GFP-SRC-3 in HeLa cells. The pEGFP-SRC-3 transfected cells were imaged for DAPI, red immunolabeling for endogenous SRC-3, and GFP for exogenously expressed SRC-3, maintaining the same exposure time. Only cells expressing near-endogenous levels of coactivator were selected for analyses (as previously described in figure 10, concerning with GFP-ERα transient transfection), after morphological filtering. Notably, overexpression of GFP-SRC-3 causes the formation of non-physiological speckles. We excluded all cells in our analyses having more than 3 times the level of the average SRC-3 endogenous fluorescence. All cells included in the range of nuclear fluorescence corresponding to endogenous level of SRC-3 expression, selected through HTM filtering, do not exhibit speckles formation (see ref.6).

We used HTM analysis to show that both endogenous SRC-3 and transiently expressed GFP-SRC-3 are predominantly localized in the nucleus. The quantitative FLIN value, defined as "*Fractional Localized Intensity In the Nucleus*", is an accurate HTM parameter that measures the ratio between nuclear and total fluorescence of SRC-3 in the cell. This measurement is independent of cell-to-cell variations in signal intensities and it's defined as a percentage value from 0% (absence of nuclear fluorescence) to 100% (absence of fluorescence in the cytoplasm, which indicates that the protein is completely nuclear localized) (***Fig.18A***).

Measurements of thousands of cells expressing endogenous SRC-3 gave an average FLIN value of 96% ± 0.08, thus indicating that in an average cell, 96% of SRC-3 fluorescence is nuclear (***Fig.18B***). Importantly, when gated for a near-endogenous expression level in HeLa cells, exogenous GFP-SRC-3 also showed high FLIN values, similar to those of the endogenous protein (Average FLIN = 95% ± 0.11, ***Fig.18B***).

These results quantitatively confirm that SRC-3 in almost completely localized in the nucleus. Nevertheless, a small amount of protein is also detected in the cytoplasm (on average, about the 4% of total cellular amount). Moreover, they indicate that GFP-SRC-3 may be used as a reliable representation of endogenous SRC-3 function, when expressed at near-physiological levels in HeLa cells.

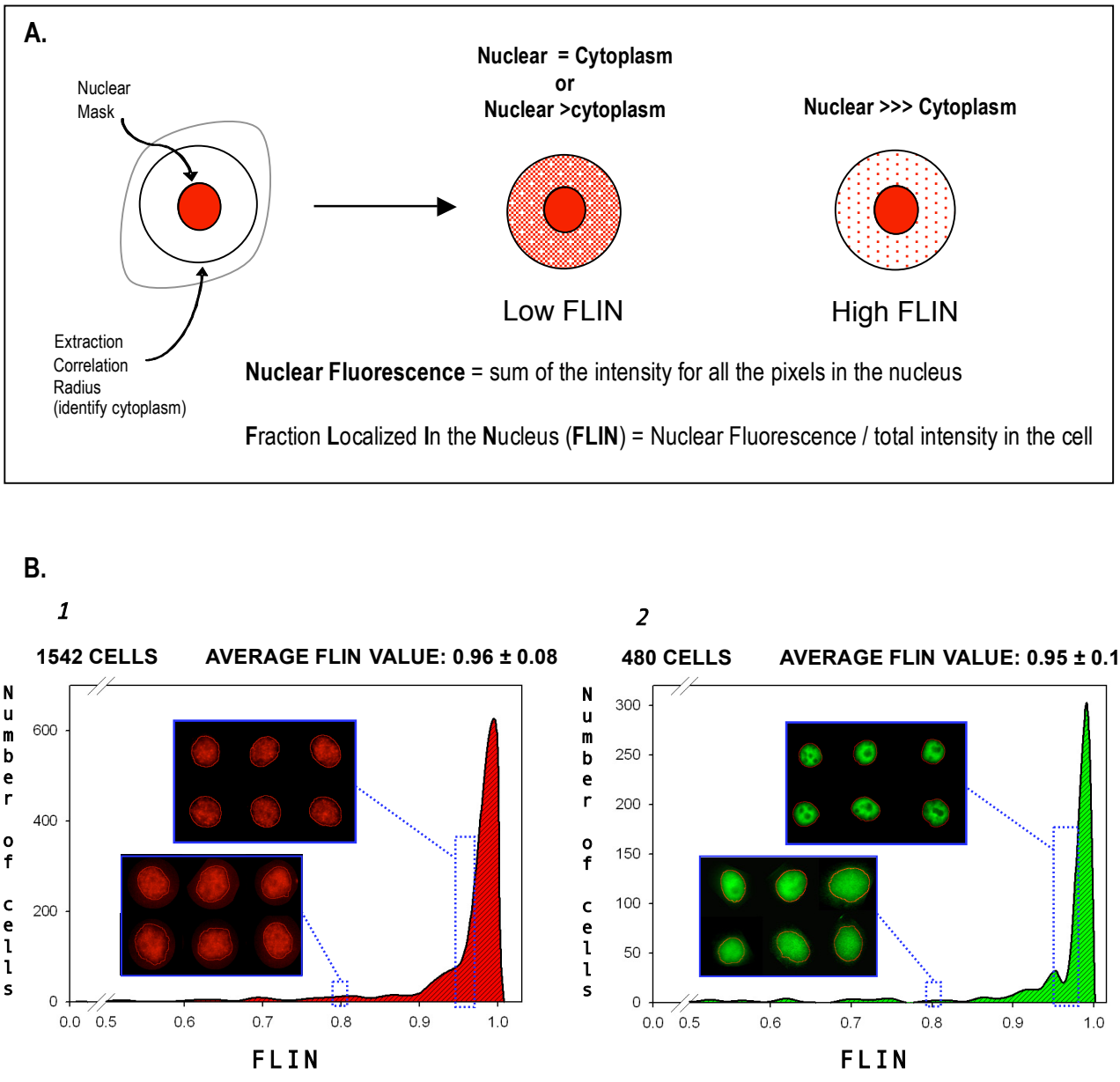


Figure 18. Automated quantification of SRC-3 subcellular localization using HTM. (A) Schematic depiction of FLIN value determination by HTM, defined as the ratio between nuclear and total fluorescence of a specific protein. Filters standard to Cytoshop were used to gate and extract the imaged cell population; nucleus and cytoplasm are identified. The degree of nuclear localization is represented by the fraction of total fluorescence measured in the nucleus. (B) HeLa cells were grown for 48 hours in hormone-free medium. Shown are filled-area graphs of the Fractional Localized Intensity In the Nucleus (FLIN) obtained for the cell population imaged. FLIN values were measured for endogenous SRC-3 (panel 1) and transiently expressed GFP-SRC-3 (panel 1). The numbers of cells analyzed and the FLIN averages are indicated. (Inset) Representative cell galleries which belong to the extracted cell subpopulation are shown as example.

SRC-3 Localization Is Regulated by Phosphorylation

To analyze the upstream effectors that may regulate the subcellular localization of SRC-3, we studied the impact of the phosphorylation status of SRC-3 at the cellular level. Previous studies have shown that the phosphorylation of SRC-3 is important for its coactivation function and that this phosphorylation can be mediated by ERK1/2 (p44/p42) *in vitro* (39,40). Thus, we treated HeLa cells with the MEK inhibitor U0126, which is a noncompetitive inhibitor of MEK-dependent ERK phosphorylation (376). At the same time, treatment by cycloheximide prevented new protein synthesis and SRC-3 distribution was analyzed by immunolabeling. HTM was use to quantify the redistribution of the coactivator in response to U0126 through determination of the FLIN value. Exposure to U0126 resulted in a partial redistribution of SRC-3 to the cytoplasm (FLIN value of ~ 0.87), while in the same experiment, FLIN value for SRC-1 did not significantly change (FLIN value of ~ 0.98), meaning that the effect of the inhibitor is specific to SRC-3 (***Fig.19***).

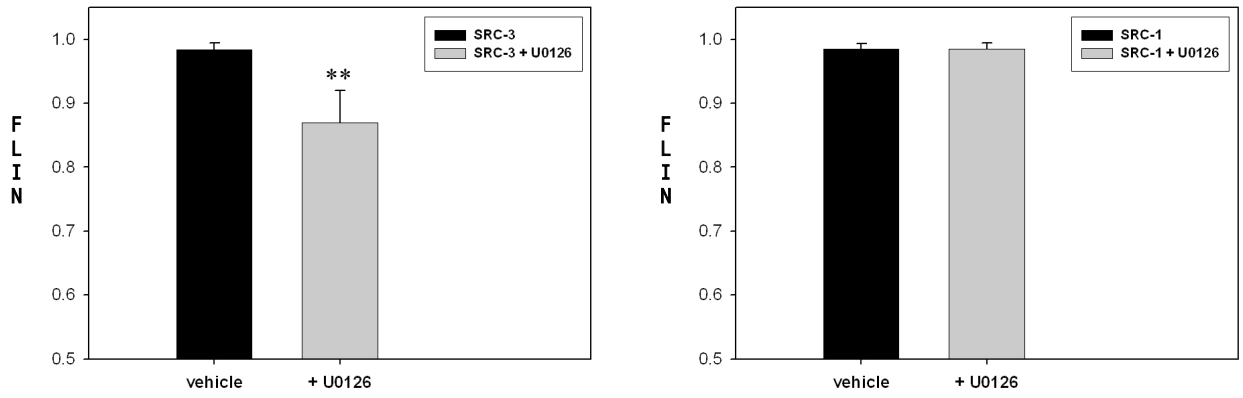


Figure 19. Inhibition of SRC-3 phosphorylation impedes its nuclear localization. HeLa cells were cultured 48 hours in hormone-free medium and then incubated for 4 hours with MEK kinase inhibitor U0126 (45μM) or vehicle, as a control, both in the presence of cycloheximide (50μg/ml). HTM quantification of nuclear localization of endogenous either SRC-3 or SRC-1 is reported as histograms, showing the average FLIN values collected for each treatment. Error bars represent SD (**, P<0.01).

To confirm that the effect of U0126 on SRC-3 localization was due to the inactivation of ERK1/2, we used RNAi to knock down the expression of ERK1/2 in HeLa cells (***Fig.20A***). The reduction of both ERK1 (p44) and ERK2 (p42) together resulted in a significant shift of SRC-3 to the cytoplasm, corresponding to a FLIN value of ~ 0.78 (***Fig.20B***), without affecting the expression level of SRC-3 (***Fig.20A***). Also in this case, the effect on SRC-3 relocation was specific since the subcellular distribution of the other p160 coactivator, SRC-1, was not changed upon the same RNAi treatment (FLIN of ~ 0.98) (***Fig.20B***).

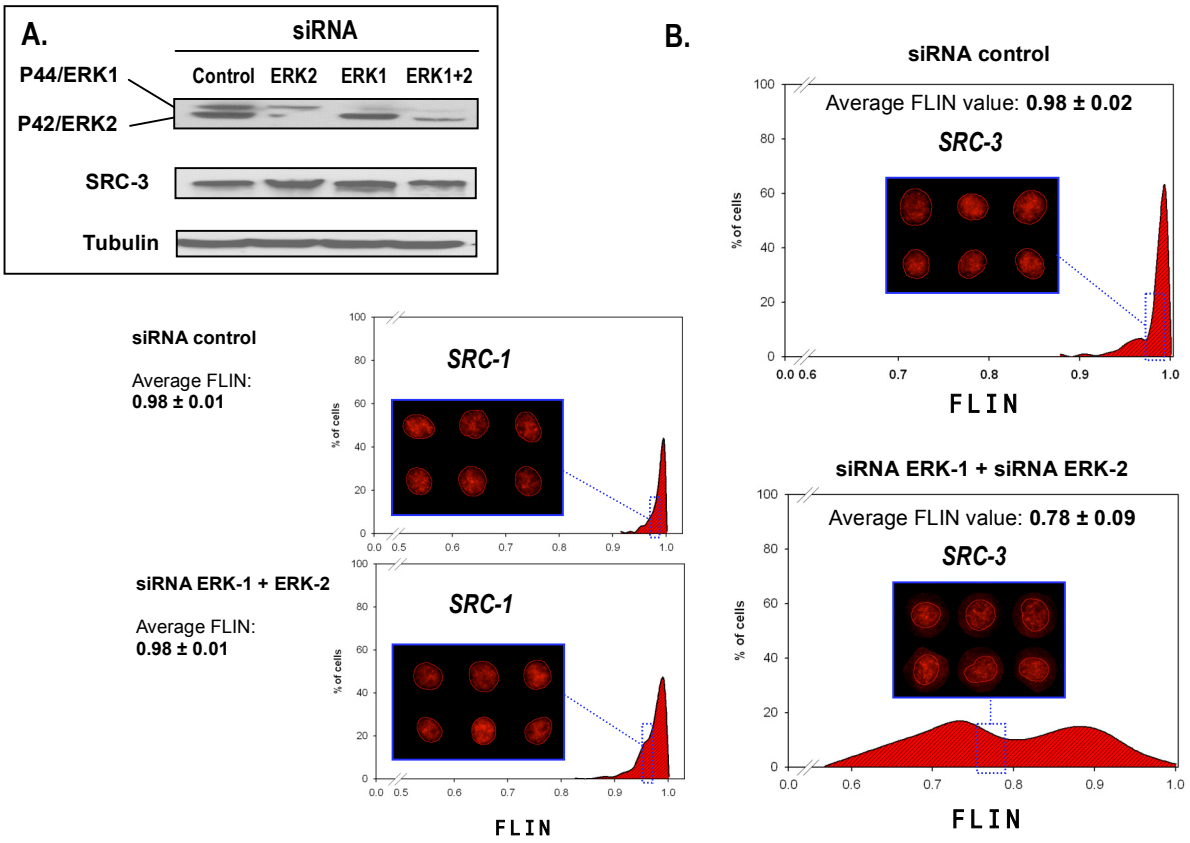


Figure 20. Knock down of both ERK1 and ERK2 induces cytoplasm relocation of SRC-3. (A) HeLa cells were transfected with siRNA for ERK1, ERK2, both together (ERK1+2), or a control siRNA. 72 hours after transfection, reduction of protein levels for both kinases was demonstrated by immunoblotting with anti-ERK1/2 antibodies. Tubulin levels were determined as a loading control. (B) Coverslips corresponding to siRNA control and siRNA ERK1+2 were immunolabeled for anti-SRC-3 or anti-SRC-1 and then scanned with HTM. Corresponding FLIN value were determined and reported a fill-area histograms, demonstrating that RNAi of ERK1 and 2 result in a much decreased nuclear localization of endogenous SRC-3, compared to control RNAi or to the same treatment for endogenous SRC-1. Representative cell galleries are also shown.

Finally, to further investigate the phosphorylation-dependent localization of SRC-3, we used a GFP-tagged SRC-3 mutant in which six previously identified phosphorylation sites (T^{24}, S^{505}, S^{543}, S^{857}, S^{860}, S^{867}) were replaced by alanines (GFP-SRC-3 A_{1-6}) (39,151). This mutant is partially and visibly localized in the cytoplasm (***Fig.21A***). Likewise, wild-type GFP-SRC-3 shows similar cytoplasmic localization following treatment with U0126. Cycloheximide was added to the medium at the same time as the inhibitor, in order to prevent synthesis of new coactivator, demonstrating the partial accumulation of SRC-3 within the cytoplasm. HTM analysis was performed and indicated that the average FLIN value for GFP-SRC-3 A_{1-6} was ~ 0.82, compared to the value of ~ 0.98 obtained for the wild-type GFP-SRC-3 (***Fig.21B***), corresponding to a average redistribution of ~15% of the GFP-SRC-3 A_{1-6} pool from the nucleus to the cytoplasm. When cells were treated with U0126, wild-type GFP-SRC-3 also presented a similar relocation in the cytoplasm (FLIN value of ~ 0.83). In addition, the FLIN value was compared to that obtained in the case of wild-type GFP-SRC-1 upon treatment with U0126, showing that cellular localization of GFP-SRC-1 is not affected (FLIN value of ~ 0.98) by the MEK-inhibitor (***Fig.21B***), whereas the effect is specific to SRC-3.

Both the U0126 and RNAi studies clearly suggest a role for ERK1/2-directed phosphorylation in regulating the residency of SRC-3 in the nucleus. Moreover, the non-phosphorylatable GFP-SRC-3 mutant is more localized in the cytoplasm, compared to the wild type. Together, these data suggest not only that phosphorylation regulates SRC-3 localization but also that the main phosphorylation sites involved are situated within these six mutated sites, previously identified.

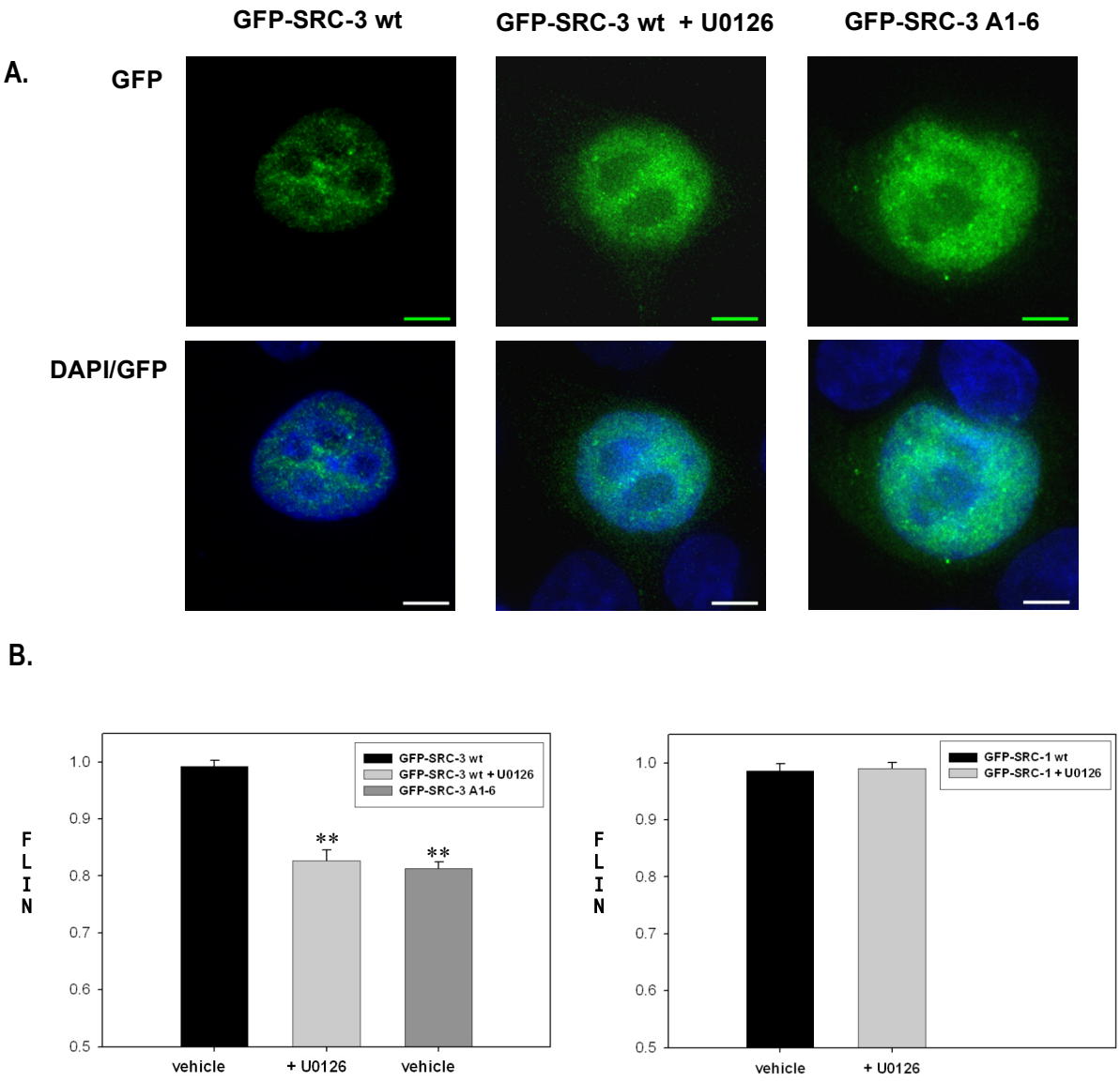


Figure 21. The non-phosphorylatable SRC-3 mutant display altered subcellular localization. (A) HeLa cells were transiently transfected with wild type GFP-SRC-3 or GFP-SRC-3 A_{1-6} mutant and pictures were taken with deconvolution microscope using a 63X objective lens. DAPI staining delineates the nucleus. GFP-SRC-3 A_{1-6} shows slight but significant cytoplasmic localization in most of the cells observed. Similarly, GFP-SRC-3 was partially shifted to the cytoplasm in the presence of the kinase inhibitor U0126. Bar, 5 μm. (B) HeLa cells transiently expressing GFP-SRC-3 (wild type), GFP-SRC-3 A_{1-6}, or GFP-SRC-1, were cultured 48 hours in hormone-free medium and then incubated for 4 hours with U0126 (45μM) or vehicle, both in the presence of cycloheximide (50μg/ml). HTM quantification of FLIN is reported as histograms. Error bars represent SD (**, P<0.01).

EGF Induces Phosphorylation of SRC-3 and Promotes SRC-3 Nuclear Localization

To further investigate how nuclear localization of SRC-3 is regulated by phosphorylation, we took advantage of the fact that the epidermal growth factor receptor (EGFR) is an essential link in the receptor-mediated ERK1/2 activation pathway (39,377). Indeed, if our previous conclusion is correct, EGF treatment should induce an enhancement in SRC-3 nuclear localization. To test our hypothesis, we used HMT analysis of HeLa cells transfected with GFP-SRC-3, GFP-SRC-3 A_{1-6}, or GFP-SRC-1. Compared to control experiments, where cells were exposed to vehicle only, the addition of EGF (100 ng/ml) for 1.5 hours stimulated a significant increase of GFP-SRC-3 accumulation within the nucleus, corresponding to an increase of the measured average nuclear intensity from 116383 to 188613 (indicating an increase of about 38%) (***Fig.22***). Conversely, pretreatment of cells with U0126 (for 1 hour) prior to EGF abrogated the nuclear accumulation of GFP-SRC-3. Differently, treatment with EGF alone or preincubation with U0126 had no significant effects on GFP-SRC-3 A_{1-6} or GFP-SRC-1 localization (***Fig.22***).

Note that the increased observed in nuclear fluorescence after EGF treatment is, at least in part, due to an increase in the average FLIN value, referred to translocation of SRC-3 from cytoplasm to nucleus. Indeed, although already high in untreated cells (~ 0.95), the SRC-3 FLIN value further increased to ~ 0.97 after EGF treatment (data not shown). However, we believe that a part of the increased nuclear intensity is also due to the nuclear accumulation of a diffuse pool of SRC-3 in the cytoplasm, which is not detectable with the FLIN algorithm, whereas increase in the average nuclear intensity is easier measurable.

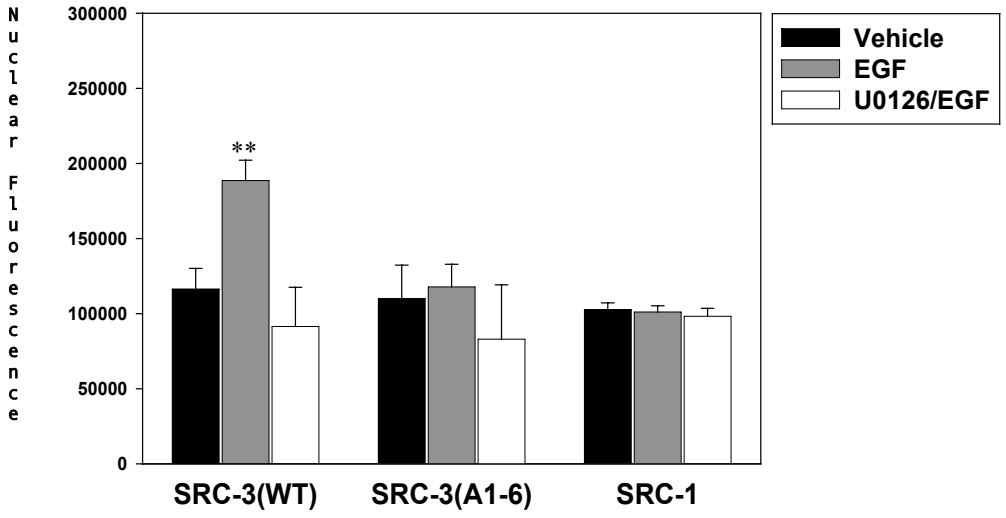


Figure 22. High-resolution HTM quantification of SRC-3 nuclear localization upon EGF treatment. HeLa cells were transfected with GFP-SRC-3, GFP-SRC-3 A_{1-6}, or GFP-SRC-1 and treated for 1.5 hours with vehicle, EGF (100 ng/ml) or U0126 (45 μg/ml for 1 hour) + EGF (100 ng/ml for 1.5 hours). Cells were fixed, DAPI stained and analyzes by HTM. The histograms show the nuclear fluorescence intensity values. Error bars represent SD (**, P<0.01).

These results indicate the hypothesis that EGF-induced phosphorylation of SRC-3 leads to an increased residency time of SRC-3 in the nuclear compartment. To definitively demonstrate the link between EGF-induced nuclear localization of SRC-3, we investigated the effect of EGF on SRC-3 phosphorylation using site-specific antibodies in HEK293 cells transfected with Flag-tagged SRC-3. The phosphorylation of SRC-3 at threonine 24, and at serines 857 and 860, was enhanced in the presence of EGF (***Fig.23A***). However, pretreatment with U0126 reduced the EGF-dependent phosphorylation of serine 857 and serine 860 (***Fig.23B***), but not of threonine 24, suggesting that these two serine sites are major targets of EGF-induced phosphorylation through ERK1 and ERK2. Interestingly, when these two sites were in combination, but not independently, mutate to alanine in a GFP-tagged SRC-3 (GFP-SRC-3 A_{4-5}), the coactivator partially localized in the cytoplasm (FLIN value of ~ 0.87) (***Fig.24***). This result indicates the importance of serines 857 and 860 in regulating nuclear localization of SRC-3 only when simultaneously phosphorylated, while individual phosphorylation is not sufficient to induce nuclear retaining of SRC-3.

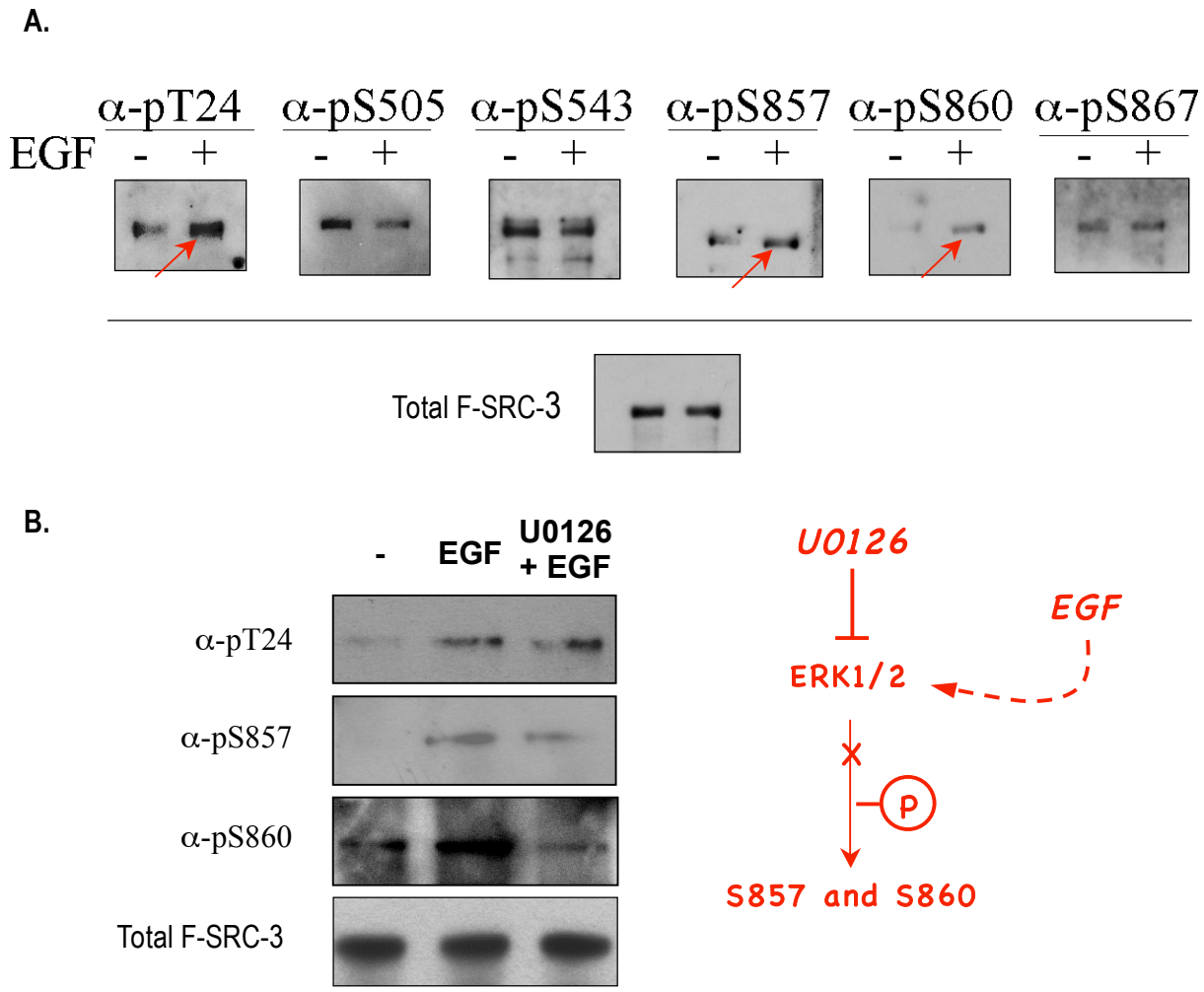


Figure 23. EGF induces phosphorylation of SRC-3 at specific sites. (A) HEK293 cells grown in phenol red-free DMEM (supplemented with 5% charcoal-dextran stripped FBS) were cotransfected with an expression plasmid for wild-type Flag-SRC-3. After transfection, cells were grown in medium containing 0.5% FBS for 36 hours and stimulated with EGF (100 ng/ml) for 1 hour before harvest. Flag-SRC-3 was immunoprecipitated by anti-Flag antibodies and separated on an 8% SDS-PAGE gel. Immunoblotting was performed using the indicated phosphorylation state-specific antibodies. Anti-Flag antibody was used as control for protein loading (total F-SRC-3). Arrows indicate phosphorylation status at threonine 24, and serines 857 and 860. (B) Cells were treated as described above, except for the right lane, where the cells were preincubated with U126 (45 μg/ml for 1hour). Immunoblotting was performed using the indicated phosphorylation site-specific antibodies, demonstrating that U126 causes a specific reduction of the EGF-induced phosphorylation at serine 857 and serine 860 sites, but not at threonine 24. The hypothesis is also schematically depicted.

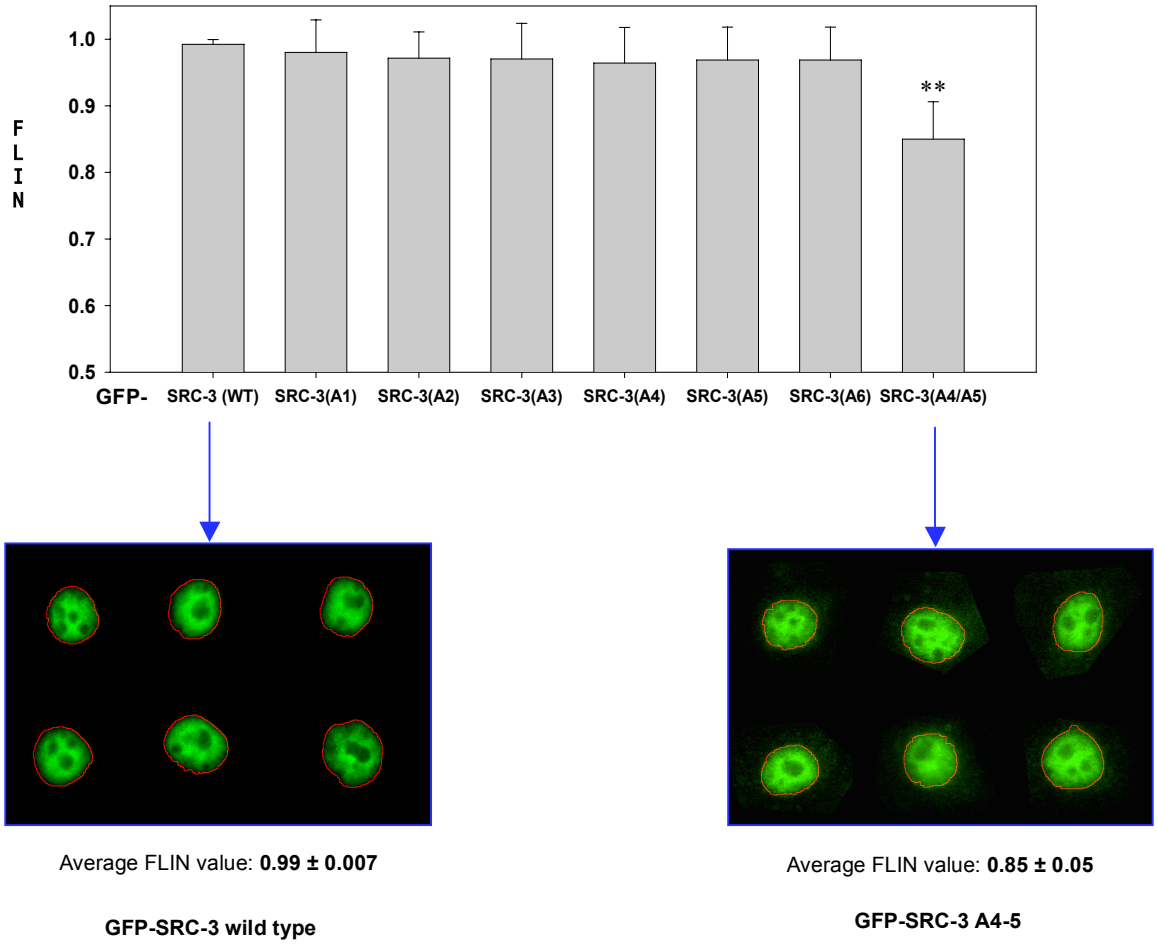


Figure 24. HTM analysis of cellular localization of GFP-SRC-3 A4-5 double mutant. HeLa cells were transiently transfected with GFP-SRC-3 (wild type), GFP-SRC-3 single mutant for each phosphorylation site (T^{24}, S^{505}, S^{543}, S^{857}, S^{860}, S^{867}), or GFP-SRC-3 $A_{4\text{-}5}$ (double mutation in S^{857} and S^{860}). Cells were then cultured 48 hours in hormone-free medium, with any treatment, and HTM quantification of FLIN was performed and here reported as histograms. Error bars represent SD (**, P<0.01). Representative cells galleries are also reported for evidencing the partial cytoplasmic localization of GFP-SRC-3 $A_{4\text{-}5}$ with respect to the wild type GFP-SRC-3.

Phosphorylation of SRC-3 Regulates Interaction and Colocalization with ERα

To determine the role of SRC-3 phosphorylation in the context of ER interactions at the single cell level, we next examined the effect of U0126 inhibition in cells co-expressing ERα and SRC-3. HeLa cells were cotransfected with GFP-SRC-3 and HcRed-ERα and incubated with 10 nM E2 for 2 hours with or without a 1-hour pre-incubation with U0126. A Z-series of focal planes were digitally imaged and deconvolved with the DeltaVision constrained iterative algorithm to generate high-resolution images.

In E2-treated cells, we observed co-reorganization of GFP-SRC-3 and HcRed-ERα into a hyper-speckled colocalization pattern (***Fig.25A***). Strikingly, when the cells were pre-treated with U0126, nuclear GFP-SRC-3 and HcRed-ERα showed much less colocalization, suggesting that in the presence of ERα, U0126 does not alter SRC-3 nuclear detainment in general, but it may specifically inhibit the colocalization with nuclear partners.

To accurately quantify this phenomenon, we used HTM and measured the variance of the fluorescent signal in the nucleus (NVAR) of GFP-SRC-3 and HcRed-ERα upon hormone or inhibitor treatment. The NVAR value is another HTM algorithm that specifically detects the *Nuclear Variation* of speckled pattern, measuring the statistical variation in pixel brightness of channels of interest (GFP and HcRed in this case) in the nucleus. Hence, NVAR values are directly linked to the presence or absence of nuclear hyper-speckling. We observed that, in the absence of hormone, the average NVAR value of both proteins was very low (0.24 and 0.14 for GFP-SRC-3 and HcRed-ERα, respectively, ***Fig.25B***). On the contrary, in presence of estradiol, the average fluorescence variation of both GFP-SRC-3 and HcRed-ERα increased to 0.9 and 0.13, which correspond to an increase of about four and nine times, respectively. However, when cells were pretreated with U0126, this increase was abolished. We also found that E2 was not able to induce the co-reorganization of the GFP-SRC-3 $A_{1\text{-}6}$ mutant and HcRed-ERα into a hyper-speckled pattern (***Fig.26A***). Accordingly, the corresponding NVAR values were no significantly modified (***Fig.26B***).

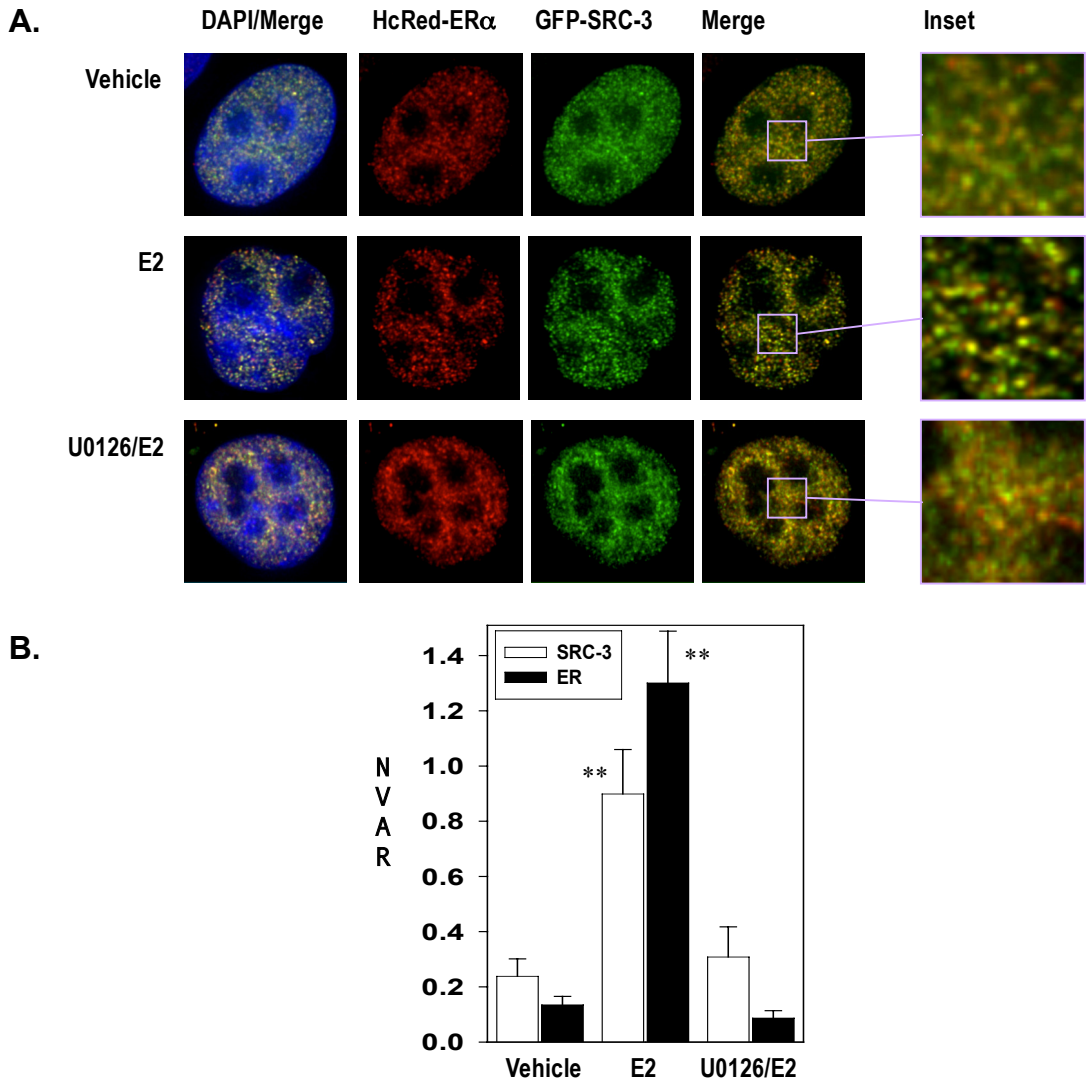


Figure 25. SRC-3 phosphorylation by ERK1/2 is necessary for colocalization with ERα. HeLa cells expressing GFP-SRC-3 and HcRed-ERα were incubated in hormone-free medium for 48 hours. Cells were then treated for 2 hours with vehicle or 10nM E2. Where indicated (U0126/E2) cells were pre-treated for 1 hour with U0126 (45 μM) and then treated with E2 for 2 hours. The inset shows a magnification of the selected subnuclear region (white square). Note that addition of E2 resulted in a redistribution of both proteins into the same foci only in the absence of U0126. (B) Automated quantification of the variation in the subnuclear pattern of SRC-3 and ER. HeLa cells transiently expressing GFP-SRC-3 and HcRed-ERα were treated as described in (A) and analyzed by HTM with the 63X high-resolution objective (>100 cells). The histograms show the subnuclear variation measurement (NVAR X 10^{-2}), which is the statistical variation in pixel brightness for each channel (GFP-SRC-3 and HcRed-ERα) in the nuclear compartment. Note that the increase in NVAR for both SRC-3 and ER in the presence of hormone is inhibited by pretreatment with U0126. Error bars represent SD (**, P<0.01).

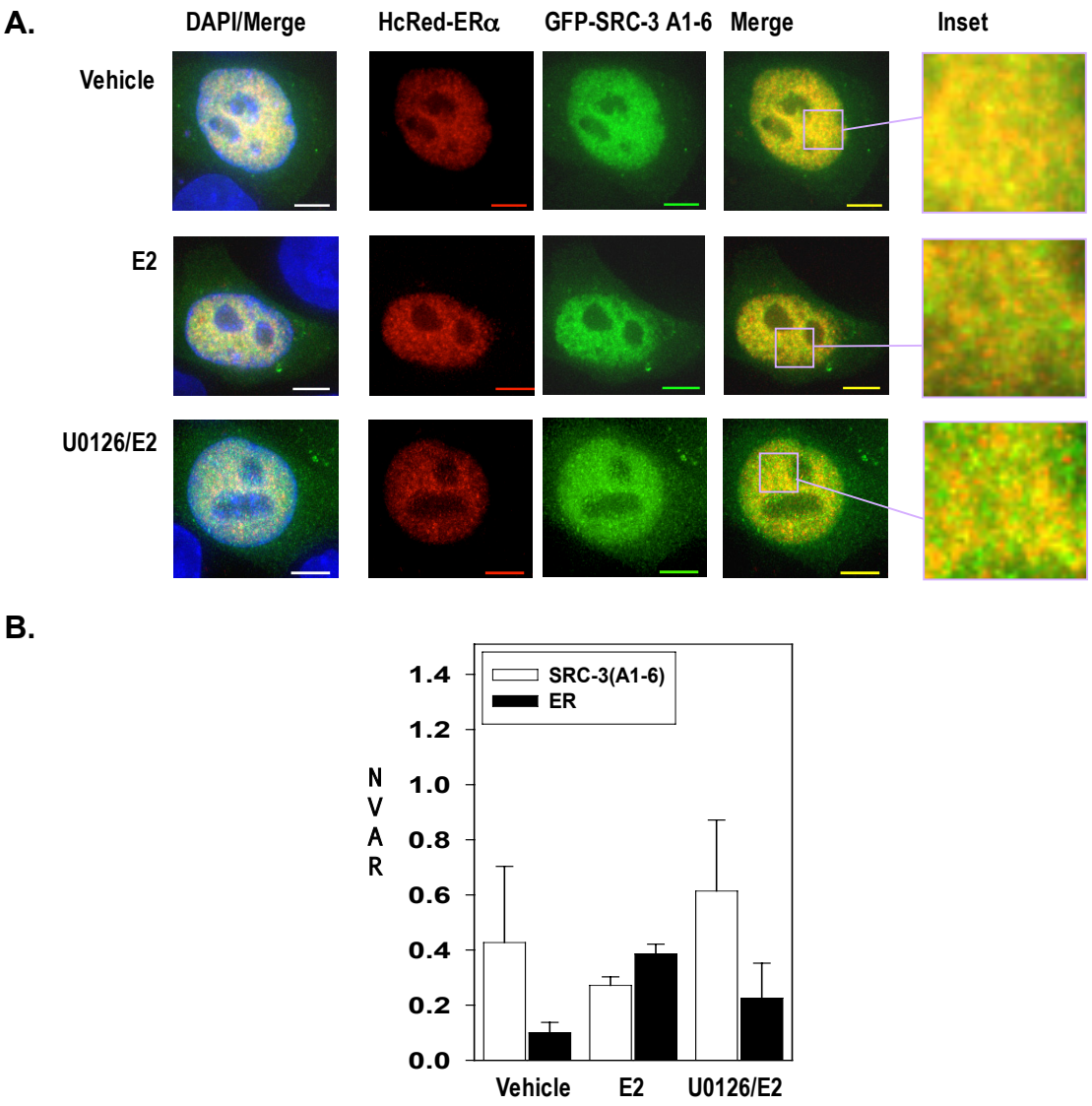


Figure 26. SRC-3 A1-6 phosphorylation mutant is unable to colocalize with ERα. (A) HeLa cells expressing GFP-SRC-3 A_{1-6} and HcRed-ERα were treated and analyzed as described above in figure 26A. (B) Automated HTM quantification of the variation in the subnuclear pattern (NVAR) of GFP-SRC-3 A_{1-6} and HcRed-ERα was performed as described above in figure 26B. In this case there are no significant divergence between the different treatments. Error bars represent SD.

The E2-induced phosphorylation of ERα by ERK1/2 at serine 118 is still controversial (42,378,379). However, to test whether the inhibitor effect of U0126 on SRC-3 and ERα colocalization is due to a decrease in ERK1/2 phosphorylation of SRC-3 alone not involving ER phosphorylation, we abrogated the direct regulation of ER by ERK1/2 phosphorylation through the use of the S118A mutation (235). The result shows that in the presence of E2, the GFP-ERα 118A can still colocalize with SRC-3 (***Fig.27A***) at a level comparable to that of wild type ER, quantified through HTM calculation of NVAR value (***Fig.27B***). This data indicate that the phosphorylation of ERα at serine 118 by ERK1/2 is not a prerequisite for promoting ERα/SRC-3 colocalization.

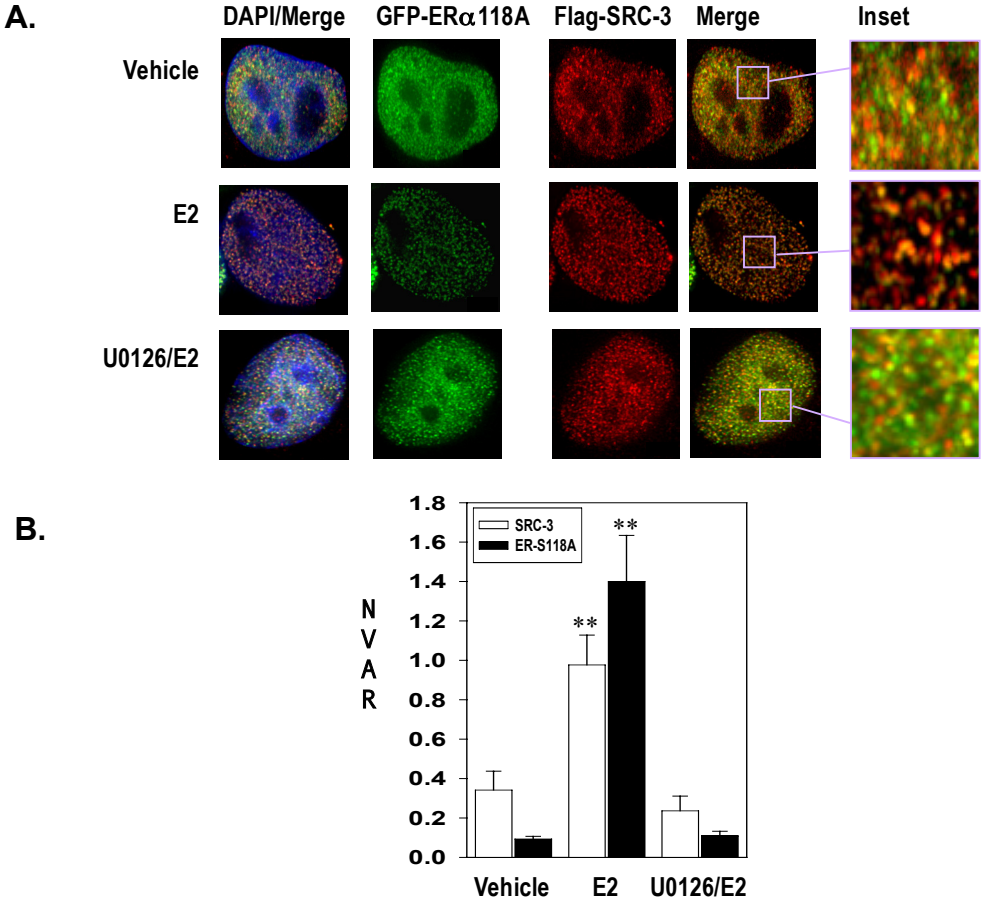


Figure 27. Inhibition of ERα by ERK1/2 doesn't prevent colocalization with SRC-3. (A) HeLa cells expressing Flag-SRC-3 (in red) and GFP-EαR118A (in green) were treated and analyzed as described above in figure 26A, except that after fixation and immunodetection of SRC-3 by anti-Flag antibody. (B) Automated HTM quantification of the variation in the subnuclear pattern (NVAR) of Flag-SRC-3 and GFP-ERα118A was performed as described above in figure 26B. Error bars represent SD (**, P<0.01).

Since the NVAR values are directly linked to the presence of subnuclear hyperspeckles, these results in part demonstrate that the ERK1/2 phosphorylation of SRC-3 is necessary for both its intranuclear organization and concomitant interaction with ER. To confirm the interaction between SRC-3 and ERα, hormone induction was carried out in MCF-7 cells, which express both endogenous SRC-3 and endogenous ERα. Following immunoprecipitation with an anti-ERα antibody, the co-precipitated SRC-3 was detected by Western blotting with anti-SRC-3 antibody. As expected, in the absence of hormone wild-type SRC-3 interacted minimally with ERα, and this interaction was greatly enhanced by E2 (***Fig.28***). However, when the cells were pre-incubated with the U0126 inhibitor, a marked reduction in the interaction between ERα and coactivator was observed (***Fig.28***). This reduction was not due to a decreased protein expression since the total amount of SRC-3 was constant.

Taken together, these results indicate that the lower levels of transcriptional activation previously observed for the SRC-3 phospho-mutant (39) are due, at least in part, to a reduced interaction between the SRC-3 mutant and ERα. Finally, this result shows that phosphorylation events are important for regulating the interaction between ERα and SRC-3 in the cell.

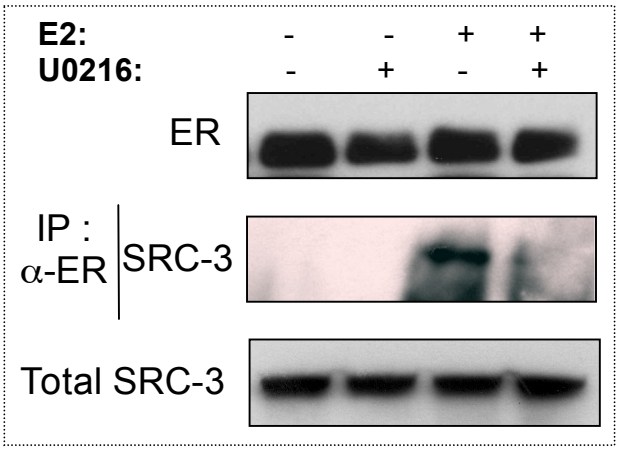


Figure 28. SRC-3 and ERα physically interact in MCF-7 cells. MCF-7 cells were incubated for three days in 5% stripped-dialyzed serum. E2 (10nM) and U0126 (45μM) were added for 1 hour (indicated by a +). Preincubation with U0126 was of 2 hours (lane 4). Coimmunoprecipitation assay was performed using anti-ERα antibody (top), and coprecipitated SRC-3 was detected by Western blotting using anti-SRC-3 antibody (middle). Note that the E2-induced ER/SRC-3 interaction is reduced in the presence of U0126.

SRC-3 Phosphorylation Is Required for Promoter Interaction with ERα and Transcriptional Readout at the PRL-array

The results described above indicate that a considerable fraction of the nuclear pools of SRC-3 and ERα can interact in an ERK1/2 phosphorylation-dependent manner. However, those studies do not allow us to discriminate the minute fraction (e.g., a small percent of the total nuclear volume) involved in transcriptional activation of genes from the whole pool of nuclear SRC-3 and ERα. Moreover, phosphorylation events might be important for increasing the rate of interaction of both proteins in the bulk nucleus, but it is not necessarily certain that this post-translational modification is also imperative for the co-recruitment of both NR and coactivator to a promoter during gene activation. To answer this question, we next visually evaluated the amount of receptor and coactivator involved in transcriptional activation using the PRL-HeLa cell line, previously described in chapter 1. This cell line (Clone 19) (see chapter 1 and Material and Methods) carries the chromosomally integrated ERα-regulated reporter gene array (PRL-array), which allows us to visualize the recruitment of ERα, SRC-3, or SRC-1, and RNA Polymerase II, and accordingly, the transcriptional readout of mRNA FISH signal upon transcription activation. When cells were treated with E2, the PRL-array became visible within a few minutes, as revealed by GFP-ERα and endogenous SRC-3 and SRC-1 colocalization (see ***Fig.12***). This accumulation was also accompanied by a clear recruitment of RNA Polymerase II (see ***Fig.13***) and mRNA transcription from the reporter gene (FISH signal > 10 fold over control) (see ***Fig.14***). However, when cells were pretreated with U0126 for 1 hour, whereas ERα still accumulated at the PRL-array, SRC-3 recruitment decreased (***Fig.29A***) and a marked reduction of the colocalization pattern with ERα was detected, using the Pearson's colocalization coefficient (Rr) (see Material and Methods) (***Fig.29B***). Interestingly, the PRL-array remained decondensed, indicating that in this system, although interaction between SRC-3 and ERα was abolished in the presence of U0126, ERα was still able to recruit other coregulators involved in chromatin remodeling, such as SRC-1 (***Fig.29A*** and ***Fig.29B***).

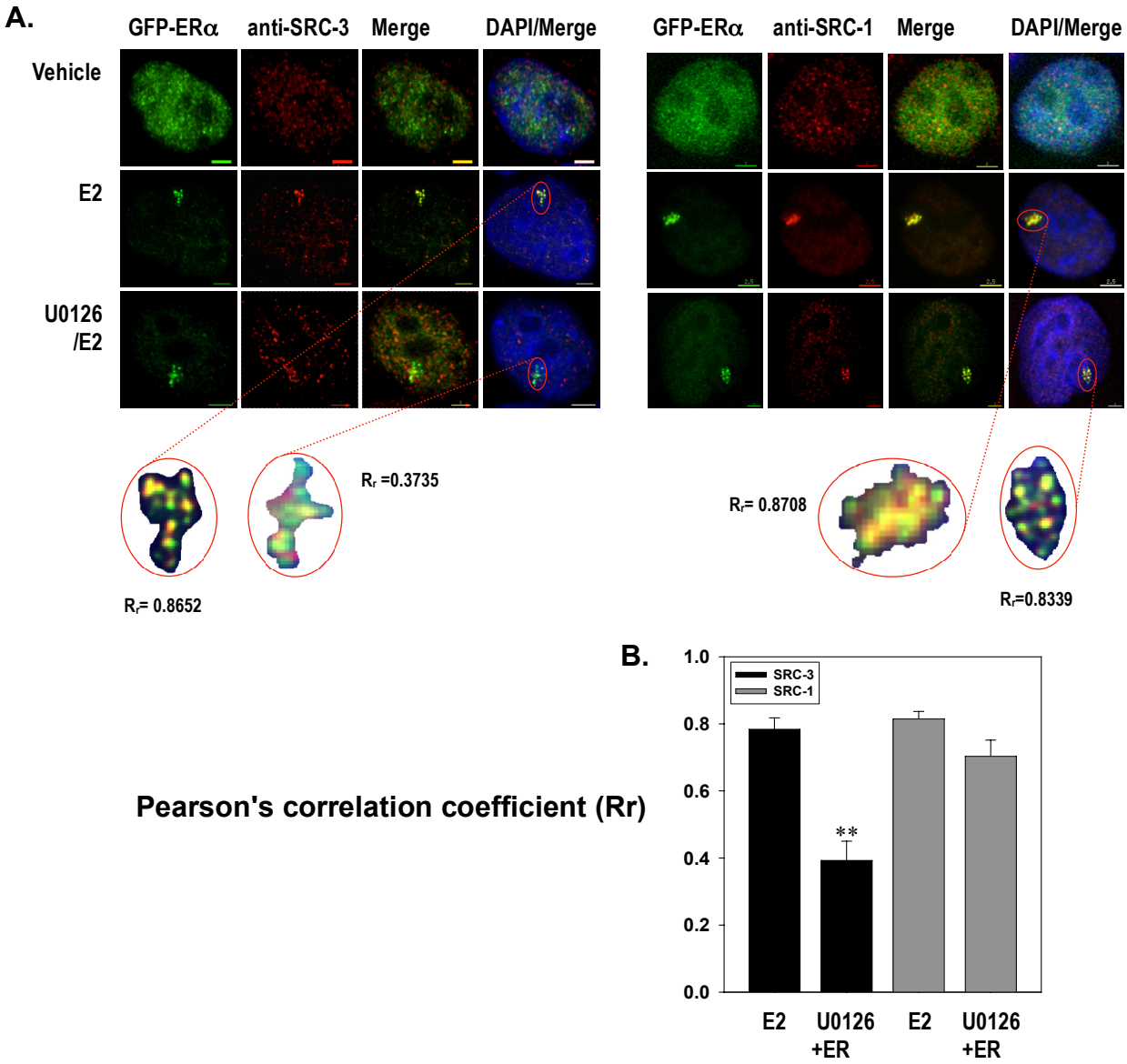


Figure 29. Recruitment of endogenous SRC-3 at the PRL-enhancer/promoter is abrogated by U0126. (A) The PRL-HeLa cells (Clone 19) were transiently transfected with GFP-ERα and treated with E2 (10nM) or vehicle, fixed and immunolabeled for endogenous SRC-3 or SRC-1. Where indicated, cells were pretreated with U0126 (45μM) and then further incubated with E2 (U0126/E2). A Z-series of focal planes was digitally imaged and deconvolved with the DeltaVision algorithm. Circles indicate the accumulation of ER or SRC-3/SRC-1 at the PRL-array. Note that, in contrast to SRC-1, the ligand-dependent recruitment of SRC-3 is inhibited by the presence of U0126. Bar, 2.5 μm. (B) Quantification of the colocalization pattern between endogenous SRC-3 (or SRC-1) and GFP-ERα at the PRL-array using the Pearson's colocalization parameter (Rr) (see Material and Methods). Error bars represent SD (**, P<0.01). The inset box shows example of areas of PRL-array that are selected for calculation and the corresponding Rr value.

To further confirm the importance of phosphorylation for SRC-3 recruitment at the promoter, GFP-SRC-3 wild type was compared with its mutant GFP-SRC-3 A_{1-6}. Both the constructs were cotransfected with HcRed-ERα in PRL-HeLa cells clone 19. Cells were then incubated for 30 minutes with E2 or vehicle.

As expected, the results show that GFP-SRC-3 was successfully corecruited at the PRL-array with HcRed-ERα (***Fig.30A***) in the 97.8% of the cell population expressing both the ER and SRC-3 constructs (***Fig.30B***). In contrast, when GFP-SRC-3 A_{1-6} was coexpressed with HcRed-ERα, we saw a marked reduction of cells with HcRed-ERα and GFP-SRC-3 A_{1-6} colocalizing at the PRL-array, upon E2 treatment (37.7% of the cell population) (***Fig.30A*** and ***Fig.30B***). Interestingly, we also found that transcriptional activation at the PRL-array was markedly reduced in cells coexpressing HcRed-ERα and GFP-SRC-3 A_{1-6}, compared to that of cells transfected with GFP-SRC-3, as reflected by the mRNA fluorescent *in situ* hybridization signal observed at the array (***Fig.30A***). In fact, a decrease of about two fold in FISH intensity was quantified in cells with arrays targeted by GFP-SRC-3 A_{1-6} and treated with E2 (***Fig.30C)***. Moreover, HTM automated quantification of PRL-array size revealed a corresponding reduction of the average measured area of GFP-SRC-3 A_{1-6} -targeted array (119.9 ± 7.8 pixels), compared to that of GFP-SRC-3 -targeted array (69.1 ± 11.8 pixels) (***Fig.31***), revealing a lowered ability of chromatin remodeling by activated ER as a result of impaired SRC-3 phosphorylation.

Taken together, these results could reflect the consequence that recruitment of other coactivators (e.g. CBP) (39) is affected by defects in SRC-3 localization at the PRL-promoter. Also, these results clearly underscore the importance of SRC-3 phosphorylation as a condition for an optimal localization at the promoter and are consistent with previous observation showing that phosphorylation sites governed by the MAPK pathway are required for the coactivation of ER (39).

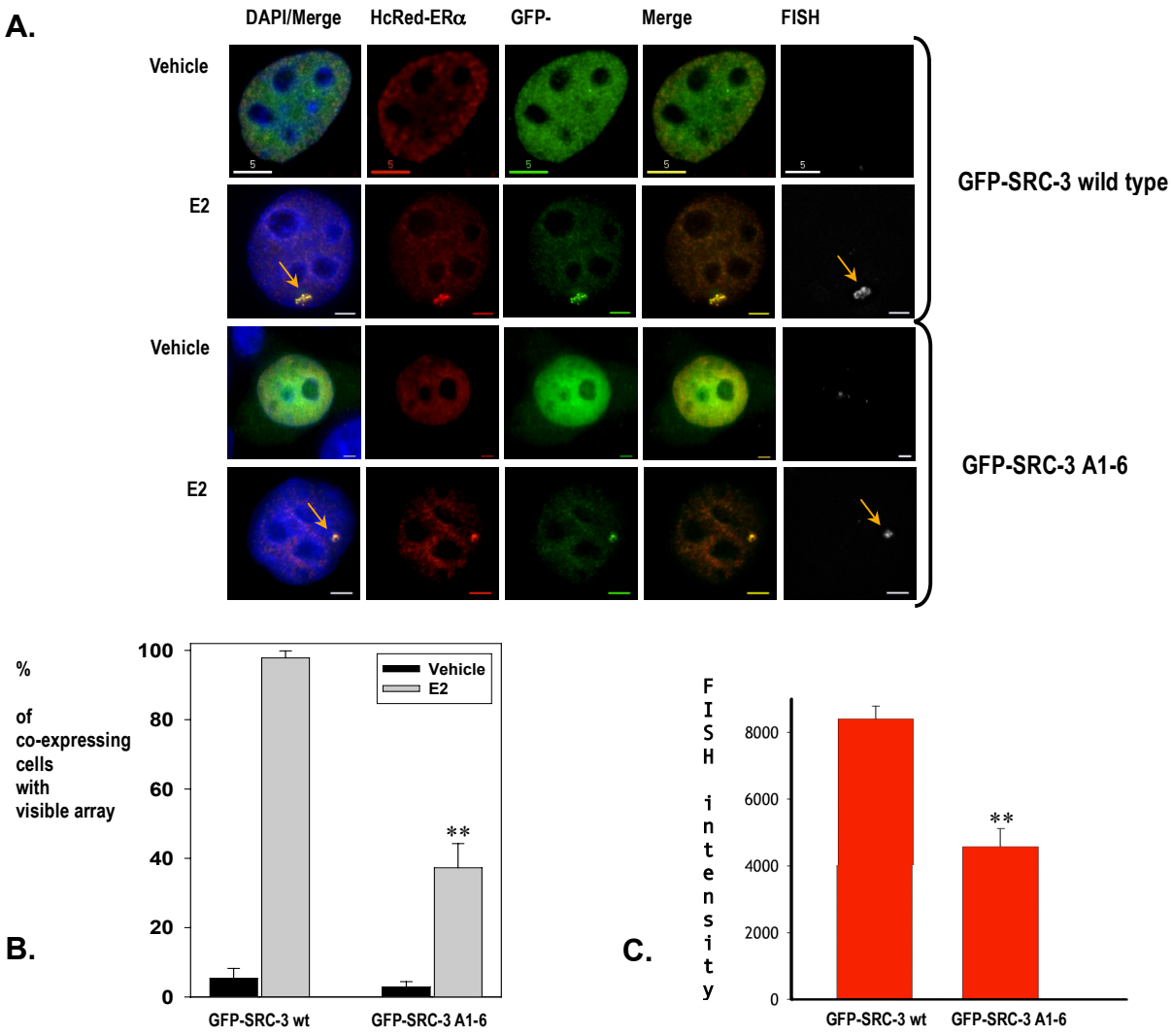


Figure 30. SRC-3 phosphorylation is essential for recruitment at the PRL-promoter and coactivation of ER-dependent transcription activation. (A) PRL-HeLa cells (Clone 19) transiently coexpressing HcRed-ER and GFP-SRC-3 or GFP-SRC-3 A1-6 were treated with E2 (10nM) or vehicle, and then fixed and DAPI stained. A Z-series of focal planes was digitally imaged and deconvolved with the DeltaVision constrained algorithm. Arrows indicate the accumulation of ER or SRC-3 at the PRL-array and the FISH signal. Note that in contrast to GFP-SRC-3 (wt), the non-phosphorylatable mutant GFP-SRC-3 A1-6 is not completely corecruited with ER. Bar, 5 μm. (B) Histogram represents the proportion of the cotransfected cell population that has visible accumulation of both HcRed-ER and GFP-SRC-3 (or GFP-SRC-3 A1-6) at the PRL-array in response to E2 stimulation (cells counted > 100). (C) FISH signal of the PRL-array was quantified by the freely available GimpShop software (see Material and Methods). Histogram represents the FISH intensity calculated for the cell population depicted in (A), following E2 stimulation and presenting a visible array. Error bars represent SD (**, P<0.01).

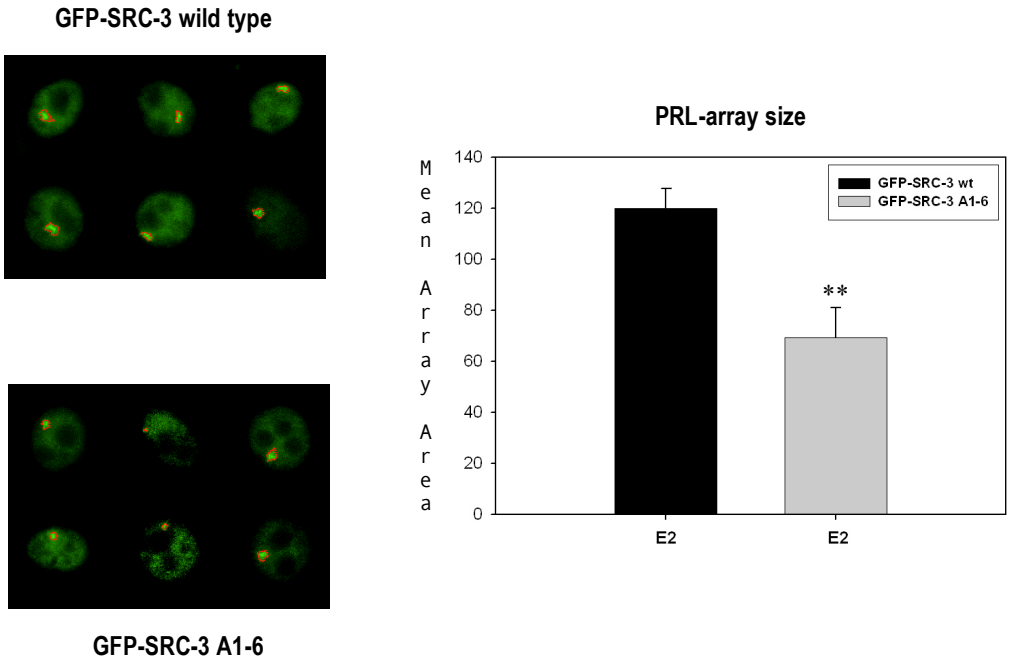


Figure 31. HTM quantification of PRL-array size upon GFP-SRC-3-targeting. PRL-HeLa cell population described in figure 30 was also scanned by HTM and the PRL-array size was determined by Cytoshop algorithms (as previously described in Chapter 1 and in Material and Methods). Size values, obtained for PRL-HeLa cells expressing either GFP-SRC-3 (wt) or GFP-SRC-3 A1-6 together with HcRed-ER, in the presence of E2, are graphed and show the mean array area in pixels (n cells > 50). Error bars represent SE (**, P<0.01). Representative cell galleries are also shown for comparison. PRL-array is identified through the localization of GFP-SRC-3 or GFP-SRC-3 A1-6, and masked in red. Note the visible reduction in the PRL-array area when the GFP-SRC-3 A1-6 mutant targets the array.

SRC-3 Shuttles Between Nucleus and Cytoplasm Affecting Estrogen Receptor Translocation into the Nucleus

The ability of coactivators to regulate gene expression is not only dependent upon their dynamics within the nucleus, but also upon their general cellular compartmentalization. This important concept points to the supposition that coactivators can move between different compartments to gain access to transcriptional sites, interacting with transcriptional factors, and deeply impacts the understanding of coactivators mechanism of action.

In order to first determine whether a fraction of SRC-3 shuttles between nucleus and cytoplasm, we used an indirect approach by incubating cells with a specific inhibitor of nuclear export, leptomycin B (380), and then analyzing the effect of the drug on nuclear compartmentalization of SRC-3. For these experiments we used T47D cells, which express endogenous SRC-3, showing a slightly lower FLIN value (87%) (***Fig.32*** left panel) than HeLa cells (see ***Fig.18B***). We exploited this difference and used HTM to assess the response to inhibition of nuclear export. When T47D cells were cultured in hormone-free medium for 48 hours and then incubated with leptomycin B for 4 hours, FLIN levels increased to 97% (***Fig.32*** right panel), suggesting that the small pool of cytoplasmic SRC-3 is in part the result of active nuclear export.

This observation suggests the idea that, if SRC-3 is able to shuttle from the nucleus to cytoplasm, a dynamic situation could exist during which the coactivator transiently interacts with cytoplasmic proteins. If this hypothesis is true, and the interaction between SRC-3 and its cytoplasmic partner is strong enough, then SRC-3 may be able to return to the nucleus with its associated protein. To verify this hypothesis, an GFP-ERα mutant, referred as ER(ΔNLS) and lacking amino acids 250 to 303 (151), which encompasses the ER nuclear localization signal (NLS), was used in cotransfection assay with Flag-SRC-3. Once transfected with SRC-3, ER(ΔNLS) appeared to be cytoplasmic in the absence of hormone E2 (***Fig.33A***), with a calculated average FLIN value of about 0.46 (***Fig.33B***). However, upon administration of E2, the receptor was shifted in the nucleus (FLIN value of

~ 0.95) (***Fig.33A*** and ***Fig.33B***). Therefore, in the presence of E2, ER(ΔNLS) may be transported in the nucleus by a "piggyback" mechanism involving the cytoplasmic fraction SRC-3. Consequently, a Flag-SRC-3 mutant, referred as SRC-3(ΔNES), that lacks amino acid 1031 to 1130, encompassing the nuclear exporting sequence (NES), failed to carry ERΔ(NLS) in the nucleus also in the presence of E2 (FLIN value of ~ 0.58) (***Fig.33A*** and ***Fig.33B***).

Taken together, these results indicate that SRC-3 location reflects a dynamic state in which the coactivator continuously crosses the nuclear membrane, interacts with cytoplasmic estrogen receptors, and potentially helps them to shuttle into the nucleus.

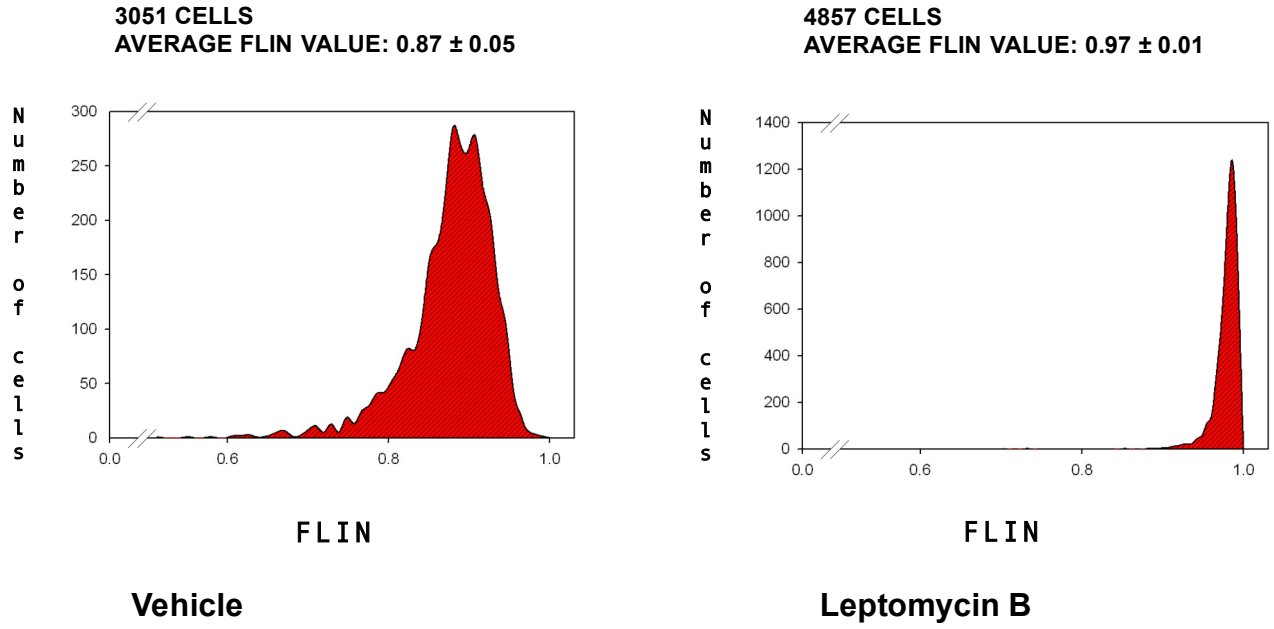


Figure 32. SRC-3 shuttles from nucleus to cytoplasm. T47D cells were cultured for 48 hours in hormone-free medium and the incubated with leptomycin B (40nM) or vehicle for 4 hours. Cell were fixed, immunolabeled for endogenous SRC-3, and then analyzed by HTM. FLIN values were quantified and the corresponding fill-area graphs are plotted. The average FLIN values are also indicated in addition to the number of cells analyzed. Note the shift of the graph in the presence of leptomycin B, which is indicative of an increased nuclear localization of SRC-3.

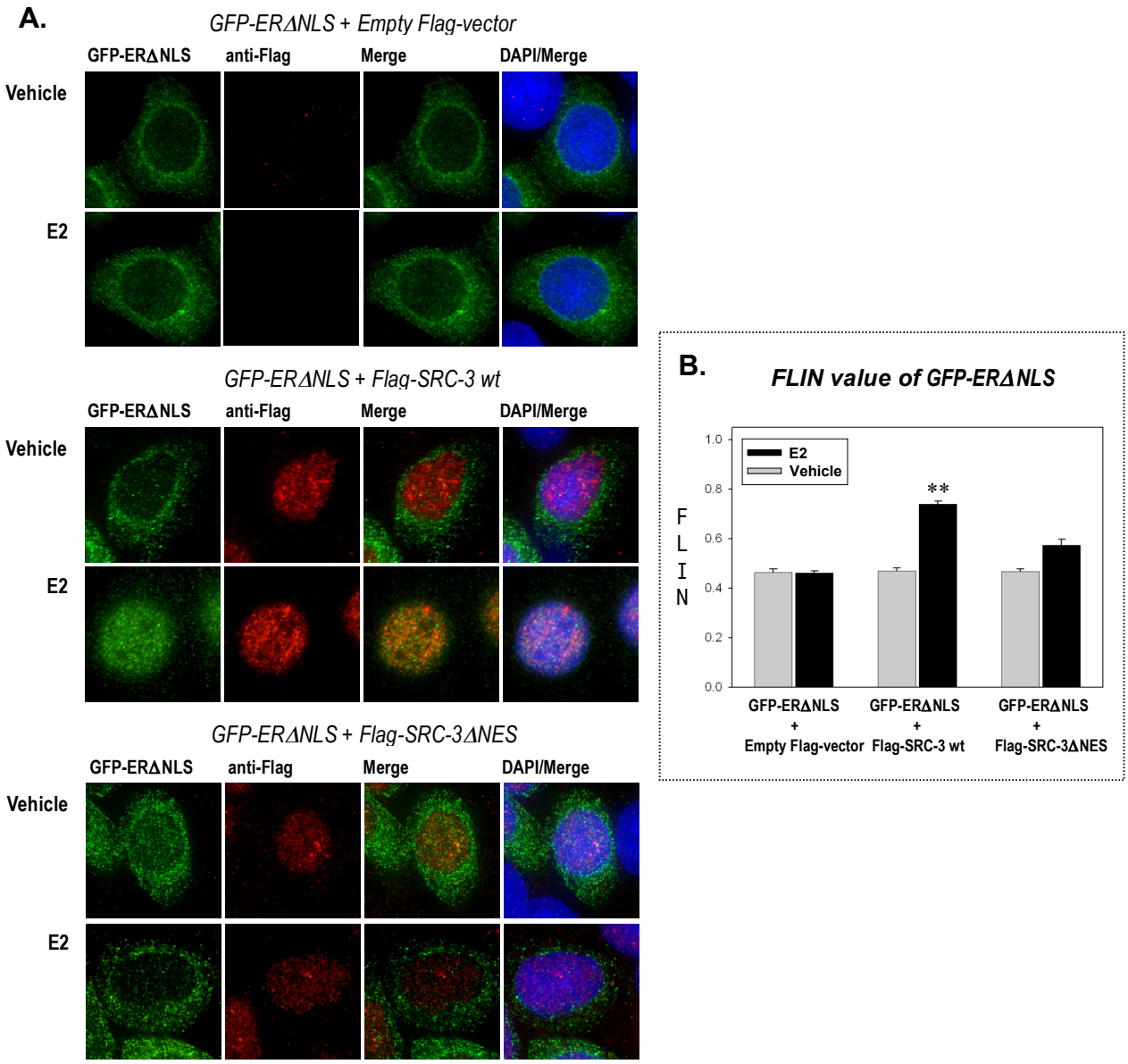


Figure 33. Hormone-dependent interaction between SRC-3 and ER(ΔNLS) results in the cotransportation of coactivator/receptor into the nucleus. (A) GFP-ER(ΔNLS) mutant was cotransfected with empty Flag-vector, Flag-SRC-3 wild type, or the Flag-SRC-3(ΔNES) mutant. 48 hours after transfection, cells were incubated with E2 (10nM), or vehicle, for 8 hours in presence of cycloheximide (50μg/ml). Then cells were fixed, and immunolabeled with anti-Flag antibodies. A Z-series of focal planes was digitally imaged and deconvolved with the DeltaVision algorithm. DAPI staining delineates the nuclei. Note that E2-induced the shift of ER(ΔNLS) in the nucleus do not occurs when coexpressed with SRC-3(ΔNES). (B) Automated quantification of ER(ΔNLS) subcellular localization using high resolution HTM. The histograms show the average FLIN values obtained for ER(ΔNLS) (n cells > 100). Error bars represent SE (**, P<0.01).

DISCUSSION

CHAPTER 1:
HTM Analysis of ERα Transcriptional Activity and Chromatin Remodeling

We have described the use of a chromosome-integrated reporter gene array (PRL-array) in HeLa cells (PRL-HeLa). These genomic integrations derive from the enhancer-promoter region of the prolactin gene and contain multicopy hormone response-binding elements for the estrogen receptor alpha (ERα). PRL-array can be visualized by the accumulation of GFP-tagged ERα and colocalization of fluorescently immunolabeled p160 family coregulators, and RNA Polymerase II. This colocalization appears typically ligand-dependent.

In our experiments, we investigated chromatin remodeling at the PRL-array and the transcriptional activation of the reporter gene in response to treatments of PRL-HeLa cells with agonist or antagonist or ERα. We were able to observe remarkable differences is the array size (closing and opening), when it was targeted by GFP-ER ERα, and we interpret these changes as a reflection of alteration in the large-scale chromatin state (chromatin remodeling). The chromatin remodeling that we observed at the PRL-array, in response to agonist (E2) treatment, correlates well with colocalization of coactivators and RNA Polymerase II, and with the transcriptional readout of the reporter gene, detected by mRNA FISH. The rapid loss of these factors from the array in response to antagonists (4HT or ICI) is consistent with striking reduction in the array size and undetectable levels of FISH signal. It follows that PRL-array size is an indicator of ER transcriptional activity in response to ligand. Moreover, we demonstrated PRL-array to be a physiologically relevant construct for visualizing gene transcription, chromatin remodeling, DNA targeting, and coregulator recruitment. For these reasons, we think that PRL-HeLa cell line described here would be an extremely useful model system for the single-cell analysis of multiple events associated with transcriptional activation, or repression, directed by the estrogen

receptor and associated coregulators.

Importantly, we also detailed the realization of high throughput microscopy (HTM) as a valid method to quantify ER functionality at the PRL-array. HTM combines automated image acquisition of thousands of cells to computational high-resolution analysis of diverse cellular parameters, achieved through specialized algorithms constrained to the software. Cells are identified basing on DAPI staining and examined for morphology, protein expression and localization.

In this study, HTM has been successfully demonstrated to be an extremely useful implement to quantitatively measure the variation of the PRL-array size upon ligand treatment. Moreover, HTM allows the simultaneous study of numerous aspects of transcriptional regulation, including protein subcellular localization, nuclear translocation, and proteins colocalization.

CHAPTER 2:
Regulation of SRC-3 Cellular Localization and Dynamics by Phosphorylation and ERα Interaction

During last decade, an extensive amount of work has been directed toward the understanding of how posttranscriptional modification (PTM) of coregulators could influence expression of genes. In fact, PTM represents the major conduit for coregulator-mediated regulation of transcription, through the ability to guide a variety of transcriptional factors with which the coregulator interacts, because the role of coregulators is to assemble the multiple complexes of the transcriptional apparatus. The important idea is that DNA, chromatin proteins, transcriptional factors, coregulators, RNA holoenzyme, and signaling enzymes all communicate with each other through the reversible network of epigenetic modifications. In this way, coregulators can be “differentially coded” by PTMs and, acting both as targets and as propagators of PTMs, allow the extremely broad degree of combinatorial control of gene transcription existing. Biological decision can be

implemented in very fine proportion due to the great combinatorial possibilities inherent in the “PTM coregulator code”.

It is estimated that about 25,000 human genes exist, differential splicing of which yields more than 125,000 different coding transcripts and potential proteins. When we consider that about 300 coregulators exist, and we suppose that, in any single coregulator, approximately eight PTMs of six different chemical types can occur, and we also consider that at least six different coregulators are contained within one transcriptional complex, then the potential combinations of combinatorial control employed could be terrifically high. In addition, an important concept, that is increasingly emerging, is that coregulators also control a variety of biological processes outside the nucleus. This underlines the extraordinary complexity of physiological, developmental, and evolutional program of mammalian phenotype.

According to laws of chemical equilibrium, coregulator control of transcriptional is dictated by molecular interaction and specific affinity with functional partners, as well as by the local concentration of these two interactants in the cell. SRC-3 provides a good example of how PTM of a coregulator contributes to the biological complexity, by signal integration and propagation of transcriptional program (6). SRC-3 specific phosphorylation at multiple residues by diverse kinases ultimately results in the recruitment of coactivator-associated proteins, including CBP, p300, CARM1, which leads to the selective engagement of distinct transcriptional factors, such as nuclear receptors, NF-κB, STAT, or E2F1.

In our study, we have shown that SRC-3 is capable of trafficking from the nucleus to the cytoplasm at a steady state and without any stimuli, and that this property confers SRC-3 the capability to interact with cytoplasmic partners (i.e. cytoplasmic pool of ERα). Phosphorylation plays a role in the cellular location and functionality of SRC-3. Indeed, we found that EGF modulates the SRC-3 “phosphocode” at specific threonine/serine residues and consequently enhances its nuclear localization and interaction with nuclear receptors (i.e. ERα). Finally, we have established that SRC-3 phosphorylation is not only important for colocalization with ER in the nucleus, but also for functional ER/SRC-3 complex formation and transcriptional activation of a model promoter (PRL-array).

SRC-3 phosphorylation can be integrated in a comprehensive cellular network (***Fig.34***) where it coordinates coactivation function of ER, influencing functional localization of the receptor, and promoter occupancy.

SRC-3 subcellular localization.

Making extensive use of high-resolution microscopy and HTM, we established that SRC-3 is primarily a nuclear protein. Only a small amount of protein was detected in the cytoplasm (on average, about the 4% of total cellular pool), which is in fact in accordance with the presence of a nuclear localization signal (150,381). Our findings are also consistent with various immunohistochemical observations (64,155,382) and with the its well established role as coactivator of NRs (111).

However, using a biochemical approach, other groups reported SRC-3 to be mainly localized in the cytoplasm (43,150). We could explained this discrepancy as a result of condition-dependent SRC-3 extractability, by the observation that >50% of nuclear proteins are lost from the nucleus within 10 min of cell disruption (383), a phenomenon that has also been observed for some steroid receptors (384,385). Nevertheless, some reports indicate the presence of SRC-3 in the cytoplasm directly in tissue sections (150,155,160). To reconcile these observations, we should consider several issues. First, SRC-3 can shuttle between the nucleus and the cytoplasm over the course of an hour. Furthermore, Kuang and colleagues (160) examined the subcellular localizations of SRC-3 in the mammary gland and suggested that cellular concentrations and the subcellular localization of the coactivator in this organ are both developmental-dependent and malignant state specific. In line with these findings, using breast epithelium of transgenic mouse model, Avivar et al. have shown that SRC-3 is cytoplasmic in non proliferative cells whereas nuclear in dividing cells (386). In addition, by comparing the expression of SRC-3 in normal and malignant breast tissue, List and colleagues found that there is an increased nuclear retention, with concomitant decrease of cytoplasmic SRC-3, as normal epithelium progresses to a more tumorigenic phenotype (155).

The differential localization of SRC-3 is of interest, since changes in subcellular

compartmentalization have also been described for other transcription or replication factors relevant to breast cancer, such as BRCA1 and p53 (387,388). SRC-3 contains both a NLS and a NES (150,381), and developmental or oncogenic functions of SRC-3 might be activated or repressed by cellular events favoring one of these two types of signal pathways: nuclear import or nuclear export. Our results, showing the effect of a MEK1/2 inhibitor (U0126) on SRC-3 subcellular localization, strongly suggests that activation of cytoplasmic kinases such as ERK1/2, perhaps through membrane-initiated pathways, could be a key to modulating the cellular location of SRC-3 and its function in the nucleus of normal versus malignant cells.

Physiological relevance of SRC-3 nuclear-cytoplasmic shuttling.

The nucleocytoplasmic shuttling properties of proteins have been ascribed to many nuclear receptors (389,390). These proteins have almost exclusively nuclear functions and, therefore, it has been difficult to explain the physiological relevance of the phenomenon. In contrast, our observations about SRC-3 subcellular localization appear to be critical for a better understanding of the biological function of this transcriptional coactivator.

An increasing number of observations reveal that SRC-3/AIB1 has a role in extranuclear events or is modified in the cytoplasmic compartments (39,43,392). For example, in a recent study, SRC-3 has been shown to play an unexpected role in repression of mRNA cytokine production at the translational level (174). This translational repression is dependent on the cytoplasmic association of SRC-3 with known translational repressors TIA-1 (T cell intracellular antigen 1) and TIA-1-related protein. It has also been shown that SRC-3 is involved in ovary cell migration and promotes metastasis in breast tumor cells, perhaps by assisting proper localization of FAK (focal adhesion kinase) to cellular extensions and membrane protrusions (392). These observations may imply that, at some point, a fraction of the coactivator has to be cytoplasmic to achieve these functions. Furthermore, nucleocytoplasmic shuttling properties may also provide a nexus for crosstalk between SRC-3 and cytoplasmic kinase pathways. Indeed, Wu et al. and Wang et al. have independently shown that SRC-3 interacts with three members of the IκB

kinase complex (IKK) (39,393). Two of these kinases (IKKα and NEMO) have been to shown be exclusively localized in the cytoplasm (394,395), implying that a fraction of the coactivator is necessarily cytoplasmic.

Finally, nucleocytoplasmic shuttling capabilities of SRC-3 might also serve as a regulatory process for the cell to modulate SRC-3 transcriptional function. For example, SRC-3 may be associated with different proteins at the steady-state level depending on its localization. Consequently, this phenomenon might lead to a compartment-specific activity of the coactivator as it has been reported that SRC-3 HAT activity was very robust when nuclear, but is dramatically decreased when it is cytoplasmic (141).

Our observations suggest that SRC-3 has a nuclear coactivation role as a main function, but may also have biological function in the cytoplasm. Concerning to this hypothesis, SRC-3 nuclear function can be regulated by membrane-initiated pathways and posttranslational modification occurring in the cytoplasm (e.g. phosphorylation). In the same way, SRC-3 cytoplasmic function can be regulated by posttranslational modification and/or interaction with specifically cytoplasmic localized protein (e.g. cytoplasmic ERs). We observed that the small pool of cytoplasmic SRC-3 is in part the result of active nuclear export, whereas phosphorylation increases its nuclear retention. Consequently, we suggest a dynamic state in which the coactivator continuously crosses the nuclear membrane, interacts with cytoplasmic localized ERα, and eventually, if the interaction between SRC-3 and its cytoplasmic partner is strong enough, SRC-3 may be able to return to the nucleus with its associated protein.

Cellular compartmentalization of SRC-3 is regulated by phosphorylation through the ERK1/2 signaling pathway.

Previous studies have shown that the phosphorylation of SRC-3 is important for its coactivation function and that this phosphorylation can be mediated by ERK1/2 (p44/p42) *in vitro* (39,40). We demonstrated that ERK1/2 kinases regulate the cellular localization of SRC-3 at several levels: nuclear retention, subnuclear speckling and promoter occupancy. Consistent with these findings, we found that a SRC-3 mutant, with alanine substitutions at

six previously identified phosphorylation site (T^{24}, S^{505}, S^{543}, S^{857}, S^{860}, S^{867}), showed a greatly enhanced tendency to localize in cytoplasm. Our data suggest not only that phosphorylation regulates SRC-3 localization but also that the main phosphorylation sites involved are situated within these six mutated sites.

ERK1/2 are the terminal effectors of the classical mitogen activated protein kinase (MAPK) cascade, which consists of signal transduction from the MAPKKKs (A-, B-, and C-Raf) to MAPKKs (MEK1/2), and finally to the MAPKs (ERK1/2). ERK1 and ERK2 have 83% of amino acid identity and are variably expressed in all tissues (reviewed in ref. 396). They are strongly activated by growth factors, like EGF (397). Indeed, in our studies, we shown that the EGF-stimulated phosphorylation of SRC-3, at threonine 24, serine 857 and serine 860, increased its nuclear localization. Interestingly, threonine 24 is located within the NLS of SRC-3 (residues 16 to 38) (381)and is also conserved in the NLS of SRC-1, which also can shuttle between the nucleus and cytoplasm (398)**.** Accordingly, through the use of MEK specific inhibitor U0126, which is a noncompetitive inhibitor of MEK-dependent ERK phosphorylation (376), we identified serine 857 and serine 860 as potentially distinct targets of ERK1/2. HTM was use to quantify the redistribution of the coactivator in response to U0126 through determination of the FLIN value. Exposure to U0126 resulted in a partial redistribution of SRC-3 to the cytoplasm (FLIN value of ~ 0.87), while in the same experiment, FLIN value for SRC-1 did not significantly change (FLIN value of ~ 0.98), meaning that the effect of the inhibitor is specific to SRC-3. Interestingly, when serine 857 and serine 860 were in combination, but not independently, mutate to alanine in a GFP-tagged SRC-3, the coactivator partially localized in the cytoplasm (FLIN value of ~ 0.87). Finally, we confirmed the specificity of ERK1/2 pathway to induce nuclear localization of SRC-3 (but not SRC-1) using ERKs-directed RNAi and quantifying the cellular distribution of SRC-3 through HTM analysis.

We cannot exclude the possibility that other kinases may also be involved in SRC-3

subcellular localization. The inhibitory effect of U0126 starts to be observed at a low concentration (~15 μM); higher concentrations (100 μM) increase cytoplasmic localization of SRC-3, but other kinases could be inhibited at this level of inhibitor (376,399), further reducing SRC-3 phosphorylation. However, we shown that the specific inhibition of ERK1/2 by RNAi visibly increased the cytoplasmic localization of SRC-3.

Importantly, U0126 leads also to a marked reduction in the colocalization complexes between ERα and SRC-3 under E2 stimulation, measured at the level of nuclear speckles formation. The same result is obtained when the SRC-3 non-phosphorylatable is used. As expected, coimmunoprecipitation assay confirmed that the two proteins could physically interact. These data suggest a role for phosphorylation in nuclear organization of SRC-3 and transient interaction with hormone-bound NR.

Finally, to complete our understanding of the phosphorylation state of SRC-3 in terms of functional complexes with NR, we used a transcriptional model (PRL-array), based on the prolactin promoter/enhancer region, to visualize and quantify the transcriptional activation based upon ERα stimulation. In this way, we demonstrated that phosphorylation is a key post-translational event, not only for regulating interactions between SRC-3 and other coactivators, nuclear targeting, and subnuclear colocalization with ER, but also for stabilize the interaction with ER at the promoter level.

These findings offer new information for the understanding of SRC-3 function in the cellular context. At one level, the distinct spatiotemporal phosphorylation of SRC-3 causes its nuclear localization, shifting the equilibrium of molecular probabilistic interactions towards transiently stable, and functional, complexes formation at the promoter level. Alternatively, dephosphorylation of SRC-3 leads to the dissociation of functional complexes and the return of coactivator and receptor to the cellular pool (***Fig.34***).

ERK signaling pathway and SRC-3 links to cancer.

It is interesting to consider our observations in the light of compelling evidence implicating SRC-3 as a causal factor in the genesis of human cancers. The role of SRC-3 phosphorylation in oncogenic stimulation was established by Wu et al. (39), who studied the oncogenic potential of SRC-3 and found that wild-type SRC-3 collaborated with Ras to transform SRC-$3^{-/-}$ MEFs, whereas non-phosphorylatable SRC-3 mutants caused only minimal transformation. Thus, it is plausible that, in normal tissues, a basal constant stimulation of SRC-3 leads to a neoplastic phenotype as a consequence of disregulation at any one of the multiple regulatory steps involved in its cellular compartmentalization or interaction with NRs. For example, both normal mammary development and breast cancer growth are under the influence of ER, which regulates cell proliferation (400). In addition to estrogens, many growth factors, like EGF, may also play an important role in regulating the growth of breast cancer cells by autocrine, paracrine, or endocrine mechanisms (401,402). As breast cancer cells have membrane receptors for the several growth factors they secrete, it has been proposed these growth factors activate the ERK signaling pathway (403). ERK1/2 activation, in turn, may induce SRC-3 phosphorylation, its retention in the nucleus and stimulation of ER activity (40,391). Interestingly, a clinical study of breast cancer patients showed that SRC-3 and HER2/neu (EGF receptor family member) expression levels are closely associated with worse prognosis, suggesting that a critical link between HER2/neu and SRC-3 might exist in breast cancer (141). Our current study further supports this notion by suggesting that constitutively active HER2 signaling (and hence ERK1/2 activation) would result in the increased phosphorylation of SRC-3 and its consequent prolonged nuclear residing time and longer coactivation of partner NRs.

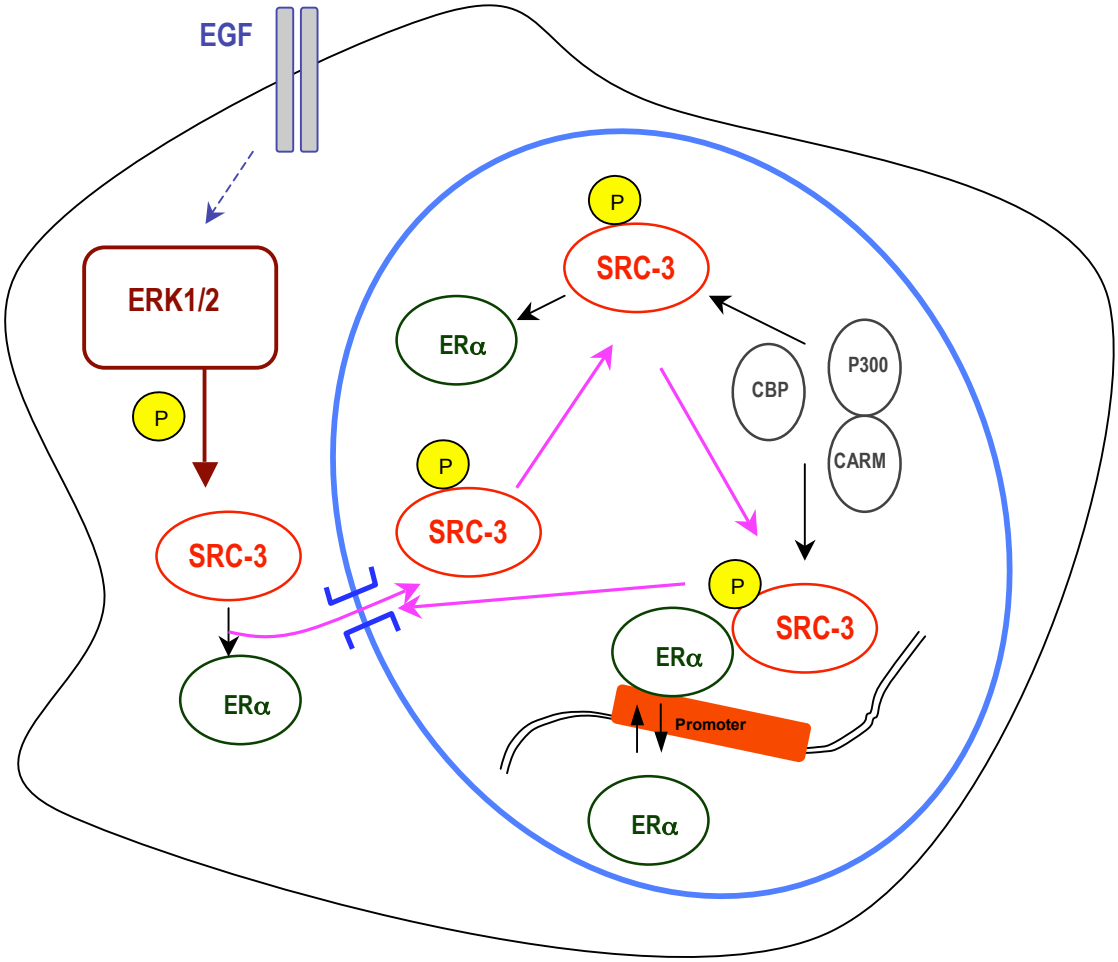


Figure 34. Phosphorylation regulates the equilibrium of SRC-3 subcellular location and functionality. The majority of SRC-3 is resident in the nucleus, but both cytoplasmic and nuclear pool are dynamic and in a finely regulated equilibrium. SRC-3 possesses an NLS and an NES that guide its nuclear import and export, respectively. External stimuli activate the cytoplasmic ERK1/2 signaling pathway, which in turn enhances SRC-3 phosphorylation and results in an increased nuclear localization. In the nucleus, phosphorylated SRC-3 has high-affinity interaction with ERα, and other coregulators (e.g. CBP, p300, CARM1). These interactions lead to the formation of transiently associated protein complexes, which are generally not associated with transcription sites. When functionally bound to target DNA, ERα can specifically interacts with phosphorylated SRC-3, and recruits other member of the chromatin remodeling machinery to initiate transcriptional activation. Phosphorylation drives these equilibria and increases the probability of forming functional complexes. In the context of SRC-3 life cycle phosphorylation is critical for nuclear interaction and ERα-regulated gene expression. Moreover, the minor pool of cellular SRC-3 that shuttle from the nucleus to the cytoplasm, beside other functions, can eventually operate as cotransporter to the nucleus for the cytoplasmic localized ERα.

MATERIAL AND METHODS

Plasmid

The expression plasmid pCMV-Flag-SRC-3 was generated as described previously (39,271). pCMV-Flag-SRC3(ΔNES) code for a mutant lacking 1031 to 1130 and was generated by PCR. The expression plasmids for GFP-SRC-3, and HA-SRC-3 were generated by inserting SRC-3 fragment from pCMV-Flag-SRC-3 (43) into pEGFP-C3 and pTRE2hyg2-HA vectors, respectively (CLONTECH Laboratories, Inc). The 6 constructs GFP-SRC-3(A1) to (A6) (Individual alanine mutation of the 6 identified SRC-3 Phosphorylation Sites (39), as well as the GFP-SRC3(A1-6) construct (expression plasmid in which all six identified phosphorylation sites have been mutated to alanine (271), and the were generated by subcloning SRC-3(A4-5) fragment from pCMV-Flag-SRC3(A1-6) into pEGFP-C3. The expression plasmids GFP-ER and GFP-SRC-1 were made as described previously (17). GFP-ER(ΔNLS) have been already described (271). GFP-ER-S118A was generated by inserting ER(S118A) fragment from pCMV5-ER(S118A) (404) into pEGFP-C3.

Hormone and Inhibitors

In all experiments, estradiol (E2) was added to a final concentration of 10^{-8} M. Stock solutions of U0126 MEK kinase inhibitor (Promega) were made in DMSO (10mM), stored at -20ºC, and used within 7 days. Where indicated, transfected cells were first pre-treated with 45 µM U0126 for 1-3 hours and then throughout the duration of the experiment. EGF (Invitrogen) was dissolved in ethanol and used at 100 ng/ml. Leptomycin B (Sigma) was used at 40 nM, a concentration efficient to inhibit nuclear export of a NES-containing protein (405).

Cell Cultures

Grow cells in the appropriate growth media and serum (usually 5% fetal bovine serum) to 90-95% confluency. Refresh the growth media one day prior to sub-culturing the cells onto cover slips or well plates following standard trypsin digestion protocols. For NR experiments, the sub-cultured cells are grown in media that contains stripped and dialyzed serum that lacks hormones that can obfuscate results. The appropriate seeding density is dependent on many factors and must be empirically determined for each experiment. The cells are grown for 24-48 hours on the cover glass until ligand treatment or transfection of expression vectors. For routine culture, cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS). For transfection experiment, cells were plated one day before in DMEM medium w/o red phenol (Invitrogen) containing 5% dextran charcoal-stripped-dialyzed (FBS).

Generation of Stable Cell Lines

HeLa tet-off cell line expressing HA-SRC-3 were generated by transfecting a Hela tet-off cell line (CLONTECH Laboratories, Inc) with the expression vector pTRE2hyg2-HA-SRC-3. Stable transformants were selected in Hygromycin (200 μg/ml) and Neomycin (100 μg/ml) and resistant clones were screened for expression of SRC-3 by Western Blotting.

The Production of the PRL array-HeLa cell line was performed as described for a similar cell line (20). Briefly, to establish a stable line bearing a chromosomally integrated array of p52X-PRL-DS-Red2-SKL plasmid, HeLa cells were plated in a 100 mm dish to a density of ~60% confluency in OptiMEM medium supplemented with 4% FBS. The cells were co-transfected with p52X-PRL-DS-Red2-SKL and pTK-Hygro (Clontech) at a 100:1 ratio, using a three-fold excess (by volume) of Fugene6 (Roche) reagent to DNA. After ~18

hours incubation with the DNA-lipid mixture, the cells were rinsed, detached, and split into ten 100-mm dishes. From this point, the cells were selected in OptiMEM supplemented with 4% FBS plus 400 µg/ml hygromycin. Approximately 100 colonies were picked and expanded directly into 24-well dishes, and then into a 35-mm dish containing a 12-mm diameter coverslip. To assess integration of p52X-PRL-dsRED2-SKL, coverslips were transiently co-transfected with GFP-ER and GFP-Pit-1 (both were tagged with GFP to maximize the fluorescent signal) and examined microscopically. A substantial percentage of dsRED2-SKL-positive cells with low diffuse levels of GFP signal in the nucleus had a single (sometimes two) bright intranuclear focus of fluorescence. These data suggested ER and/or Pit-1 interactions with an integrated 52XPRL array (presumably multicopy), and provided encouragement for the next step designed to separate cells based on reporter expression. To this end, we expanded several heterogeneous colonies of 52X-PRL HeLa cells. The clone [HeLa/52X-DM66-Red2-PTS #19] shown in this study was one of them and was named “PRL-HeLa clone 19”. Note that, in contrast to the previously desdcribed [HeLa/52X-DM66-Red2-PTS #23], the clone 19 used in this study was purified from a second generation clonal cell line and showed much less basal activation in absence of hormone.

Immunoprecipitation and Western Blotting Analysis

MCF-7 or HEK293 cells were lysed in lysis buffer (20 mM Tris-HCl [pH 8.0], 125 mM NaCl, 0.5% NP-40, 2 mM EDTA, 0.2 mM NaF, 0.2 mM Na3VO4, protease inhibitor cocktail) for 15 min and the debris was cleared by centrifugation at 13,400 x g for 15 min at 4°C. Lysates were incubated overnight (4°C) with the indicated antibodies [anti-ER clone 60C (Upstate Biotechnology), or anti-Flag M2 (Sigma)]. The antibody was allowed to bind to protein A/G beads (Santa Cruz Biotechnologies) for 30 min and then washed extensively with lysis buffer. For Western blot analysis, the samples were resolved by SDS PAGE and

transferred to nitrocellulose membranes (Bio-Rad). The indicated antibodies were diluted in TBST buffer (5% non-fat dry milk, 50 mM Tris-HCl, 150 mM NaCl [pH 7.5], 0.1% Tween 20) and added to the membranes for 1 hour at room temperature (RT) or overnight at 4°C followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies for 30 minutes at RT (Sigma). All proteins were detected with ECL Plus Detection Reagents (Amersham) and visualized by chemiluminescence.

DNA Transfection

Perform transfections by standard calcium phosphate precipitation protocols or by commercially available lipid based transfection reagents following the manufacturer's protocol. If cells are not to be transfected then perform the ligand treatments and proceed to the fixation and immunolabeling protocol. Remove the media containing the transfection reagent and expression vector and replace with media containing the appropriate ligand and control treatments. In our experience, empirically evaluating a variety of transfection reagents and protocols is required for optimal results, which can be significantly cell line specific.

RNA-mediated interference

MAPK1 (p42/ERK2)and MAPK3 (p44/ERK1) siRNAs were purchased from from the Validated Stealth RNAi Collection (Invitrogen). Cells were seeded in free-hormone media 1 day before transfection at 60% confluence in a 6 well-plate format (for Western Blot) containing glass coverslips (for HTM analysis). One hundred pmol of RNAi per well were transfected with Lipofectamine 2000 (Invitrogen) following the manufacturer's instruction. Seventy-two hours after transfection, coverslips were removed from the wells and proceed for immunochemistry, while remaining cells were collected in lysis buffer for Western Blot analysis.

Immunocytochemistry

Cells were grown on acid-etched and poly-D-lysine-coated glass coverslips (12 mm diameter). After the specified treatment, cells were removed from the incubator and immediately rinsed in ice-cold PBS, then fixed with a solution of 4% formaldehyde in PEM buffer (0.1 M PIPES, 2 mM EGTA, 3 mM MgCl2) and permeabilized in 0.5% Triton X-100 (in PEM, 30 min), then washed and quenched in sodium borohydride (0.5 mg/ml, 10 min in PEM). The cells were then washed and incubated for 1 hour (RT) in 5% non-fat dry milk in TBST (0.1M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% v/v Tween-20) before incubation overnight with primary antibody (at 4˚C) and subsequently with fluorophores conjugated to appropriate secondary antibodies (goat anti-mouse Alexa 555, Molecular Probes). The primary antibodies used were: anti-HA (Clone 3F10) (200 ng/ml), anti-RC-1 (1 µg/ml), and anti-AIB1/SRC-3 (0.5 µg/ml) obtained from Roche Molecular Biochemicals, Upstate Biotechnology and BD Biosciences, respectively. After first and secondary antibody labeling, cells were post-fixed and quenched (as above), counter-stained with DAPI (0.5 mg/ml, ~1min), rinsed quickly in water, and then mounted on slides (ProLong Gold, Molecular Probes).

High Throughput Microscopy (HTM)

Cells were imaged using the Cell Lab IC 100 Image Cytometer from Beckman Coulter, Inc with a Nikon 40X Plan S fluor 0.90 NA objective except for the experiment described in Figure 25,2 6, and 37, where a Nikon 63X Plan Apo 1.20 NA objective was used. Three channels were imaged: channel (DAPI) was used to find the focus and nuclei; channel 1, for imaging GFP-SRC-3, GFP-SRC-3(A1-6), GFP-SRC-3(A4-5), GFP-ER and GFP-ERS118A, channel 2 for imaging endogenous SRC-3, SRC-1, or HcRed-ER. A Correlated

Channel Segmentation Algorithm was used to identify and quantify the localization and level of fluorescence of SRC-3/ER in the nucleus and the cytoplasm. After image acquisition and application of the Correlated Channel Segmentation algorithm, the total cell populations for each treatment were progressively filtered (gated) using the following criteria: non-transfected cells, nuclei clusters, mitotic cells, and apoptotic cells were filtered out from the total cell population using an intersection of DNA content gate, DNA clusters gate, and “keep 2 channels” gate. Filtration of low level expression of GFP-SRC-3 gates was generated as described in reference and applied to produce the final cell population to be analyzed. The images and masks were visually inspected for accuracy.

Fluorescence Deconvolution Microscopy and Quantitative Colocalization

Deconvolution microscopy was performed with a Zeiss AxioVert S100 TV microscope and a DeltaVison Restoration Microscopy System (Applied Precision, Inc.). Cells were imaged using either the 63X lens (both 1.40 NA). A Z-series of focal planes were digitally imaged and deconvolved with the DeltaVision constrained iterative algorithm to generate high-resolution images.

Quantitative colocalization analysis (figure 29) were done with the Colocalizer Pro Software, using the Pearson's correlation coefficient (Rr). The estimation of Rr is one of the standard techniques applied for matching one image to another in order to describe the degree of overlap between the two patterns. The Rr coefficient accounts only for the similarity of shapes between the two images, and does not depend upon image pixel intensity values. Briefly, 10 images in each condition were acquired and analyzed by deconvolution microscopy. The Rr coefficient was calculated for each array transgene in each image and the average calculated values for each condition were ploted on the histogram.

Fluorescent In Situ Hybridization (FISH)

The methods used here, including procedures for non-isotopic probe preparation and fluorescent in situ hybridization, have been already published in numerous publication. Briefly, coverslips with adherent cells were rinsed twice in PBS, dipped in cytoskeleton (CSK) buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM PIPES, pH 6.8) (406) extracted on ice for 5 minutes in CSK buffer containing 0.5% Triton X-100 and 2 mM vanadyl-ribonucleoside complex (VRC; Gibco-BRL), rinsed in CSK/VRC, fixed in 4% paraformaldehyde/PBS for 10 minutes, rinsed again in PBS and stored in 0.4% paraformaldehyde at 4˚C until use. Hybridization to RNA was carried out at 37˚C in standard buffers containing 5 µg/ml probe and 50% formamide overnight. After incubation, samples were rinsed in a series of SSC buffers, detected for biotin using streptavidin, Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR) and rinsed in a series of PBS washes. Intensity of FISH signal at the PRL-promoter by the use of the free available software GimpShop.

ACKNOWLEDGMENTS

This study was conducted at the Baylor College of Medicine in Houston (TX) under the supervision of prof. Michael A. Mancini, which I gratefully acknowledge for the didactic guide and friendship. I especially want to thank Larbi Amazit, for the important instructive and educational support, for all the encouragement, and the sincere feeling of reciprocal affection. I greatly thank my Italian tutors, prof. Giuliano Della Valle and prof. Marialuisa Melli, which allowed me to achieve this study in the U.S.A. that has been essential to complete my PhD program. For this reason I also thank La Fondazione Marco Polo for the initial financial support.
I also thank J. Zhong, K. M. Antalis, D. Townley, and M.G.Mancini for excellent technical contribution, friendship, and personal help.

This work was funded by DK-55622 (M.Mielke), Cain Cancer 1080001204 (B.W.O'Malley), HD08818 (B.W.O'Malley), and DK61589 (M.G.Mancini). The Integrated Microscopy Core used throughout this study was supported in part by U54 HDFIG 96008 (B. W. O'Malley) and P01 CA-06425 (D. Medina) and the Dan L. Duncan Cancer Center of Baylor College of Medicine.

The majority of the work presented in this thesis has been published on M.C.B. Oct.2007, p.6913-69-32 (Amazit *et al*.).

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