

Hormone-dependent nuclear export of estradiol receptor and DNA synthesis in breast cancer cells

Maria Lombardi,¹ Gabriella Castoria,¹ Antimo Migliaccio,¹ Maria Vittoria Barone,² Rosina Di Stasio,¹ Alessandra Ciociola,¹ Daniela Bottero,¹ Hiroshi Yamaguchi,³ Ettore Appella,³ and Ferdinando Auricchio¹

¹Dipartimento di Patologia Generale, Il Università di Napoli, 80138 Naples, Italy

²European Laboratory for Food Induced Disease, Dipartimento di Pediatria, Università "Federico II," 80131 Naples, Italy

³Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

In breast cancer cells, cytoplasmic localization of the estradiol receptor α (ER α) regulates estradiol-dependent S phase entry. We identified a nuclear export sequence (NES) in ER α and show that its export is dependent on both estradiol-mediated phosphatidylinositol-3-kinase (PI3K)/AKT activation and chromosome region maintenance 1 (CRM1). A Tat peptide containing the ER α NES disrupts ER α -CRM1 interaction and prevents nuclear export of ER α - and estradiol-induced DNA synthesis. NES-ER α mutants do not exit the nucleus and inhibit estradiol-induced S phase entry; ER α -dependent transcription is normal. ER α is associated with Forkhead proteins

in the nucleus, and estradiol stimulates nuclear exit of both proteins. ER α knockdown or ER α NES mutations prevent ER α and Forkhead nuclear export. A mutant of forkhead in rhabdomyosarcoma (FKHR), which cannot be phosphorylated by estradiol-activated AKT, does not associate with ER α and is trapped in the nucleus, blocking S phase entry. In conclusion, estradiol-induced AKT-dependent phosphorylation of FKHR drives its association with ER α , thereby triggering complex export from the nucleus necessary for initiation of DNA synthesis and S phase entry.

Introduction

Steroid receptors, in addition to regulating the transcription of specific genes (Mangelsdorf et al., 1995), trigger rapid effects in the extra nuclear compartment (for review see Migliaccio et al., 2007a). It is expected that hormone action depends on the integration of these different receptor activities (Vicent et al., 2006).

Localization and action of estradiol receptor α (ER α) are regulated by multiple factors, including interaction with signaling effectors or other proteins, such as the metastatic tumor antigen or the modulator of nongenomic action of steroid receptors (MNAR; Kumar et al., 2002; Vadlamudi et al., 2005; Gururaj et al., 2006). Deregulation of these processes causes ER α mislocalization and may trigger tumor progression. Overexpression of the EGF receptor is a hallmark of aggressive human breast cancers (Slamon et al., 1989), and cross talk between ER α and

growth factor signaling has emerged as a critical factor in endocrine resistance through the control of the subcellular localization of ER α signaling components (Saporita et al., 2003; Gururaj et al., 2006). ER association with MNAR may sequester ER in the cytoplasm/membrane (Vadlamudi et al., 2005), thus enhancing the nongenomic effects of ER α and resulting in tumorigenesis, as well as the anti-hormone resistance of breast cancer cells (Vadlamudi et al., 2005; Gururaj et al., 2006). Expression of a shortened form of the metastatic tumor antigens sequesters ER α in the cytoplasm and leads to malignant phenotypes by enhancing ER α cytoplasmic functions in hormone-dependent breast cancer cells (Kumar et al., 2002). These data imply that cytoplasmic localization of ER α provides a mechanism to control signal transduction-dependent functions, such as DNA synthesis and anchorage-dependent growth of target cells. They suggest that the ER α localization has functional implications in breast cancer progression.

Steroid receptors undergo nucleocytoplasmic shuttling (for review see DeFranco, 2001). In the presence of a ligand, glucocorticoid receptors (GR), androgen receptors (AR), thyroid hormone receptors (TR), and progesterone receptors (PgR) rapidly shuttle between the nuclei and cytoplasm (DeFranco,

M. Lombardi and G. Castoria contributed equally to this paper.

Correspondence to A. Migliaccio: antimo.migliaccio@unina2.it

Abbreviations used in this paper: AR, androgen receptor; CRM1, chromosome region maintenance 1; ER, estradiol receptor; FKHR, forkhead in rhabdomyosarcoma; LMB, leptomycin B; NES, nuclear export signal; PgR, progesterone receptor; PI3K, phosphatidylinositol-3-kinase; wt, wild type.

The online version of this paper contains supplemental material.

2001). Hormone withdrawal from cells induces accumulation of GR and AR in the cytoplasm (Tyagi et al., 2000). ER α also shuttles from the nuclei to the cytoplasm, and the antiestrogen, ICI 182, 780, disrupts this process (Dauvois et al., 1993). Ligand binding and protein–protein interactions can significantly influence the nucleocytoplasmic shuttling of the chimeric GFP-ER α (Maruvada et al., 2003). However, although the mechanism of nuclear import of steroid receptors is well documented, the mechanism of export is still unclear (DeFranco, 2001).

The best-characterized nuclear export pathway uses chromosome region maintenance 1 (CRM1) as a receptor for proteins with leucine-rich nuclear export signals (NESs; Fornerod et al., 1997; Fukuda et al., 1997). Leptomycin B (LMB), through covalent binding to CRM1, inhibits CRM1-dependent nuclear export (Kudo et al., 1998). Conflicting data have been reported on inhibition of steroid receptor export by LMB treatment. Although it is generally accepted that steroid receptors lack classical leucine-rich NESs, they do have sequences with limited homology to NESs (Liu and De Franco, 2000). Because the exact spacing of the leucine/hydrophobic residues in the NES of each protein is subject to variation, it can be difficult to define a NES on this basis alone (Nigg, 1997). Under some circumstances, LMB treatment does indeed inhibit the nuclear export of steroid receptors (Savory et al., 1999; Pruffer and Barsony, 2002; Maruvada et al., 2003; Saporita et al., 2003).

Here, we find that in MCF-7 cells, ER α is a nucleocytoplasmic shuttling protein whose traffic out of nuclei is regulated by estradiol activation of the phosphatidylinositol-3-kinase (PI3K)–AKT pathway and depends on CRM1. By combining different approaches, we identified a hormone-dependent and CRM1-directed NES in the ER α hormone-binding domain. The potential interest of these findings is highlighted by the observation that the ER α NES shows significant homology with sequences of other steroid receptors. A peptide mimicking the NES-ER α specifically sequesters the receptor in the nuclei and interferes in hormone-triggered S phase entry in these cells. Site-directed mutagenesis of the ER α NES inhibits forkhead in rhabdomyosarcoma (FKHR) nuclear export, the estradiol-induced cytoplasmic relocalization of receptor, and DNA synthesis. Targeting ER α by siRNA retains FKHR in the nuclear compartment of estradiol-treated MCF-7 cells.

This study provides a new link between the rapid extra-nuclear action of estradiol and ER α nuclear export, as well as a proliferative role of receptor nuclear export, which is closely associated to FKHR export.

Results

Estradiol regulates ER α shuttling in MCF-7 cells

Localization of endogenous ER α in quiescent MCF-7 cells was verified by immunofluorescence using two different antibodies directed against either a C-terminal (H222 mAb; Katzenellenbogen et al., 1987) or N-terminal epitope of ER α (A314 mAb; Abbondanza et al., 1998). Fig. 1 a shows that, regardless of the antibody used, estradiol treatment of MCF-7 cells induces nuclear translocation of ER α after 30 min, followed by a decrease of the

nuclear receptor to the basal level by 1 h. Representative images of one of these experiments are presented in the supplemental materials, together with the parallel change in cytoplasmic redistribution of the receptor (Fig. S1 a and Fig. S1 b, left, available at <http://www.jcb.org/cgi/content/full/jcb.200712125/DC1>). Immunoblot analysis of cell lysates does not reveal any significant change in ER α level (Fig. S1 c), which suggests that the observed decrease in ER α nuclear localization is caused by nuclear export of the receptor.

Quiescent MCF-7 cells were next transiently transfected with a full-length ER α cDNA subcloned into the GFP plasmid (pEGFP-HEG0; HEG0 represents the wtER α). In Fig. 1 b, the quantitative count of cells with nuclear fluorescence shows that hormone treatment triggers nuclear accumulation of GFP-ER α after 30 min in the absence of any significant change in GFP-wtER α expression (Figs. 1 b and S1 c). By 1 h, the nuclear GFP-wtER α decreases to the basal level, whereas no trafficking of GFP-wtER α was detectable in untreated cells (Fig. 1 b). Representative images of one of these experiments are presented in Fig. S1 b (right). In addition, GFP alone was insensitive to estradiol treatment when overexpressed in MCF-7 cells (unpublished data). This pattern of trafficking is similar to that observed for endogenous ER α . Furthermore, the partial antagonist, 4-OH-tamoxifen, prevents the observed nucleocytoplasmic shuttling of GFP-wtER α in response to hormonal treatment (Fig. 1 b).

To exclude the potential contribution of de novo GFP-ER α synthesis to its reemergence in the cytoplasm 1 h after estradiol treatment, we included actinomycin D in the medium during hormone stimulation. No significant change in the estradiol-induced redistribution of GFP-wtER α (Fig. 1 c) or in protein expression was observed (Fig. S1 c). Therefore, the chimeric protein in cytoplasm is from nuclear export rather than from de novo synthesis. Because actinomycin D inhibits the nuclear import of the NES-containing REV protein (Henderson, 2000), and the GFP-wtER α dynamic redistribution is not modified by actinomycin D (Fig. 1 c), it is likely that cytoplasmic relocalization of the chimera depends on nuclear export rather than inhibition of nuclear import.

Because data in Fig. 1 (a–c) show that ER α export shortly occurs upon estradiol treatment of MCF-7 cells, we hypothesized a role for a rapid action of estradiol in the ER α intracellular traffic. Activation of the PI3K–AKT pathway, for instance, modulates the trafficking of various proteins, including FKHR, nuclear factor κ B, glycogen synthase kinase-3 β , Mdm2, p27 (for review see Kau and Silver, 2003), and PTEN (phosphatase and tensin homologue deleted on chromosome; Liu et al., 2007). Because of the rapid activation of this pathway by estradiol treatment of breast cancer cells (Fig. S1 d; Castoria et al., 2001, 2004), we verified its contribution to the ER α nuclear export. Quiescent MCF-7 cells were then transiently transfected with the dominant-negative form of the regulatory subunit of PI3K, p85 α (Δ p85 α ; Dhand et al., 1994), or the catalytically inactive version of AKT (K179M; dominant-negative AKT). In Fig. 1 d, the count of transfected cells showing ER α nuclear fluorescence indicates that overexpression of either Δ p85 α or dominant-negative AKT induces nuclear retention of ER α in 60-min estradiol-treated MCF-7 cells. Transfection of cells with the Myc-tagged pSG5 control

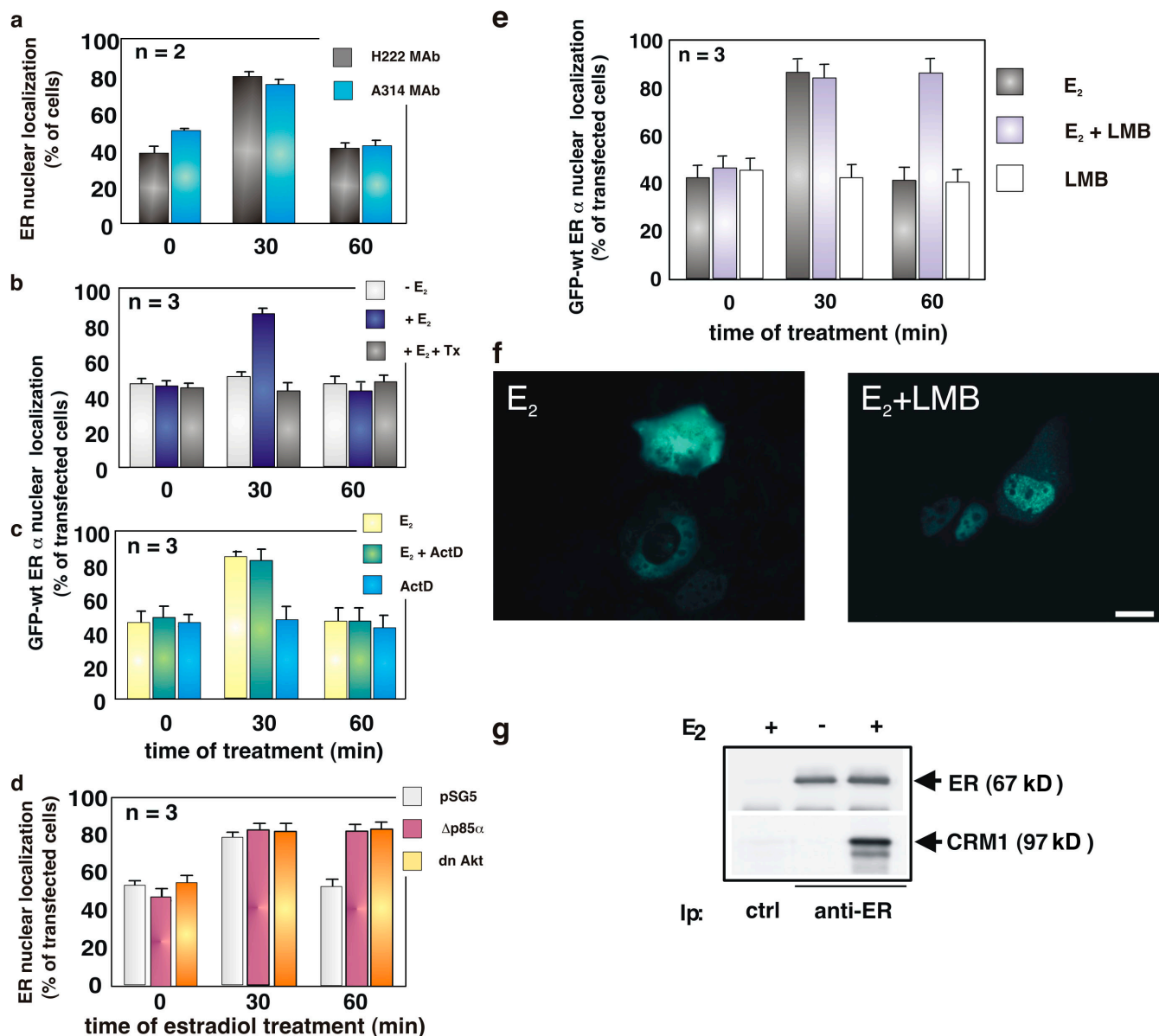


Figure 1. Estradiol induces nuclear export of ER α , which is regulated by the PI3K-AKT pathway and depends on CRM1. Quiescent MCF-7 cells were used. (a) Cells were untreated or treated with 10 nM estradiol (E₂) for the indicated times (min). ER α localization was analyzed by immunofluorescence using the indicated antibodies. (b and c) Cells were transfected with GFP-wtER α then left untreated or treated with the indicated compounds. OH-tamoxifen (Tx; AstraZeneca) was used at 0.1 μ M; actinomycin D (Act D) was added at 5 μ g/ml, 1 h before estradiol stimulation. GFP-wtER α localization was determined by fluorescence. (d) Cells were transfected with the indicated plasmids and left untreated or treated with 10 nM estradiol for the indicated times (min). The Myc-tagged pSG5, Δ p85 α , or Myc-His-tagged dominant-negative AKT ectopically expressed in MCF-7 cells were visualized by immunofluorescence, as described in Materials and Methods. ER α localization was analyzed by immunofluorescence using the rat H222 anti-ER α mAb. (e) Cells were transfected with GFP-wtER α and then left untreated or treated with 10 nM estradiol in the absence or presence of LMB (at 5 ng/ml). LMB was added 30 min before the hormone. Cells were also treated with LMB in the absence of hormone. GFP-wtER α localization was determined by fluorescence. (a, b, c, d, and e) Cells that fell into the category of exclusively nuclear fluorescence were scored, and data was expressed as a percentage of total cells (in a) or transfected cells (in b, c, d, and e). Data were derived from at least 1,000 scored cells. The results of several independent experiments were averaged; means and SEM are shown. *n* represents the number of experiments. (f) Images from one experiment in panel e were captured. Panels show GFP-wtER α localization in MCF-7 cells stimulated for 60 min with estradiol (E₂) in the absence or presence of LMB. Bar, 5 μ m. (g) ³⁵S-labeled HA-CRM1 was incubated with recombinant ER α in the absence or presence of 10 nM estradiol. The purified recombinant RanQ69L (at 1 μ M) was included in the incubation mixture of each sample. Proteins were immunoprecipitated with rabbit polyclonal anti-ER α antibody. Eluted proteins were immunoblotted with anti-ER α antibody (top) or revealed by fluorography (bottom).

plasmid does not interfere in the trafficking of ER α . Images of one experiment in Fig. 1 d are presented in Fig. S1 e.

To determine if CRM1 plays a role in ER α nuclear exit, MCF-7 cells were treated with LMB in addition to estradiol. This antifungal compound blocks the nuclear export of NES-

containing proteins by preventing their association with the CRM1 export receptor (Kudo et al., 1998). A quantitative analysis of transfected cells shows that LMB treatment results in maintenance of nuclear GFP-wtER α 1 h after estradiol stimulation (Fig. 1, e and f). LMB treatment alone does not have an effect

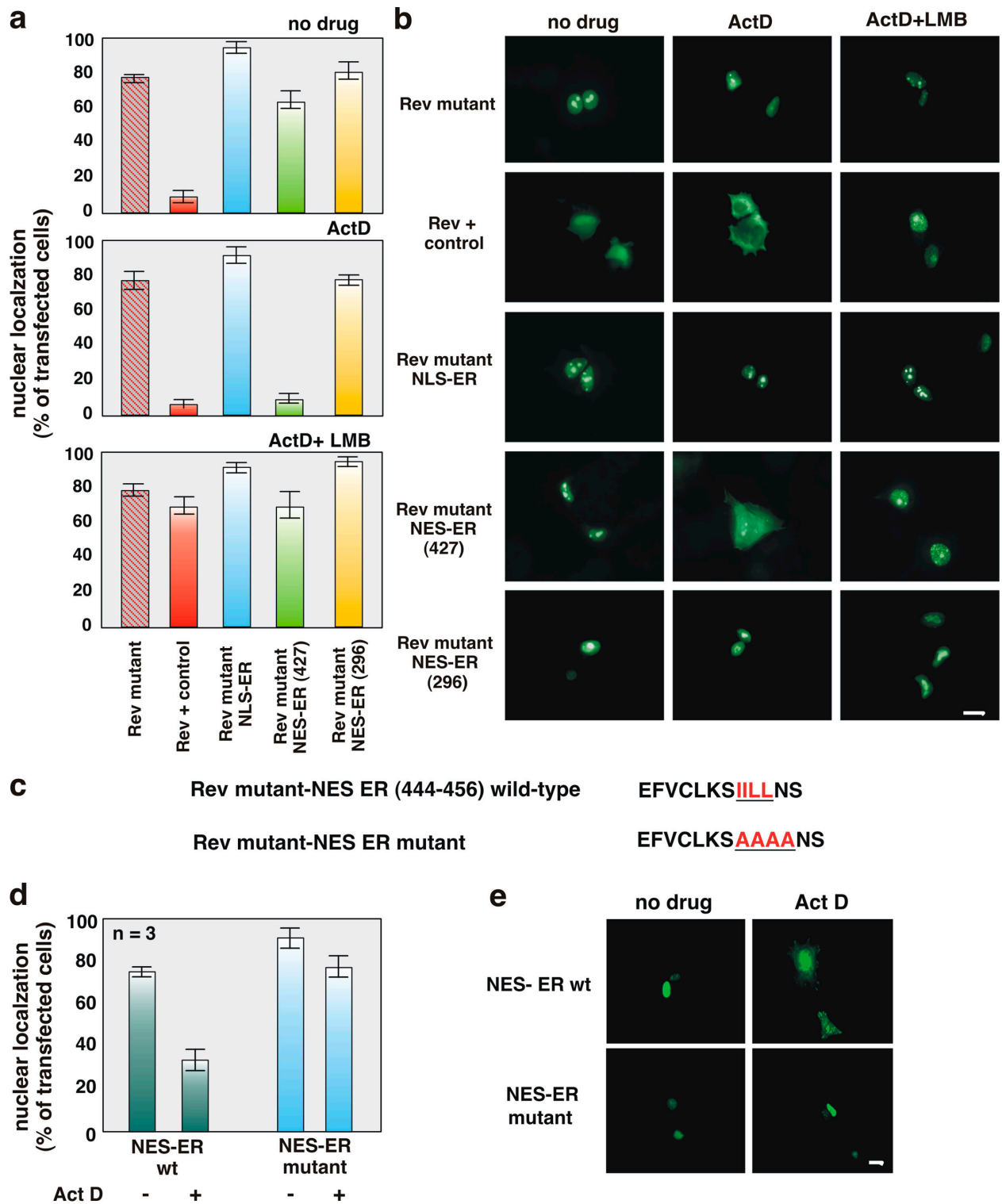


Figure 2. The ER α 444–456 sequence restores export activity of the NES-deficient REV1.4-GFP. Growing MCF-7 cells were used. (a and b) Cells were transfected with the indicated constructs. After transfection, the cells were left untreated (no drug) or treated with actinomycin D (ActD) at 5 μ g/ml, alone or together with 5 ng/ml LMB. The subcellular distribution of GFP proteins was determined by fluorescence microscopy, and cells that fell into the category of exclusively nuclear fluorescence were scored. Data are expressed as a percentage of transfected cells, with mean values taken from at least three experiments. For each experiment, at least 600 cells were scored. (b) Images of one experiment in panel a. (c) The wt ER α 444–456 sequence (NES ER α wt) as well as its mutated version (NES ER α mutant). The putative NES-ER α sequence is indicated by the underlined amino acids, which were substituted with alanine residues in the mutant sequence. The NES-ER α wt as well as the NES-ER α mutant subcloned into the Rev mutant were transfected (d and e) in growing MCF-7 cells. After transfection, the cells were left untreated (–) or treated with 5 μ g/ml Act D (+). The percentage of cells with nuclear GFP protein was determined by fluorescence microscopy and graphically shown in panel d. Data were derived from at least 600 scored cells. The results of several independent experiments were averaged. (a and d) Means and SEM are shown. *n* represents the number of experiments. (e) Images of one experiment in panel d. Bars, 5 μ m

on the localization of GFP-wtER α (Fig. 1, e and f). Thus, the effect of LMB treatment on ER α localization suggests that this receptor, when bound to the hormone, is exported from nuclei through the CRM1-exportin pathway. Estradiol induces a strong interaction between recombinant ER α and in vitro translated CRM1 (Fig. 1 g) when active Ran (Ran-GTP; Askjaer et al., 1999) was included as a GTPase-deficient RanQ69L mutant (Bischoff et al., 1994).

This set of experiments shows that estradiol activation of the PI3K-AKT pathway modulates nuclear export of ER α and that CRM1 contributes to estradiol-mediated ER α nuclear export in MCF-7 cells.

Identification of an ER α nuclear export sequence

Two ER α mutants, HEG15 and HEG14, were subcloned into pEGFP. HEG15 (Δ 282–595 HEG0) contains highly conserved NLSs (2 and 3; Guiochon-Mantel et al., 1991). It binds DNA but is unable to bind estradiol (Ylikomi et al., 1992). In turn, HEG14 (Δ 1–281) contains NLS1. It binds the hormone but does not bind DNA (Ylikomi et al., 1992). The resulting chimeras, GFP-HEG15 and GFP-HEG14, were transiently transfected into quiescent MCF-7 cells. Consistent with previous findings (Ylikomi et al., 1992), results in Fig. S2 (available at <http://www.jcb.org/cgi/content/full/jcb.200712125/DC1>) show that neither GFP-HEG15 nor GFP-HEG14 shuttle between the nucleus and the cytoplasm. Irrespective of the hormonal treatment, the first mutant mostly resides in the nuclear compartment. Although GFP-HEG14 contains the NLS1 sequence and binds the hormone, it does not enter nuclei even after estradiol addition. From these data, we speculated that the hormone-binding domain localized in the HEG14 mutant as well as NLSs (2 and 3) of ER α are both required for the estradiol-induced nuclear import of the receptor. We next subcloned HE241G into pEGFP. This mutant contains the hormone-binding domain and lacks the three NLSs. It is, in fact, prevalently localized in the cytoplasm regardless of estradiol treatment. The behavior of this mutant confirms that in the absence of NLS, the hormone-binding domain is not sufficient to induce nuclear translocation of the receptor. Because HEG15 is localized in the nuclear compartment and its localization is unaffected by hormonal treatment of cells, we reasoned that the addition of NLSs to HEG14 might restore the estradiol-induced import of ER α . Analysis of transfected cells with the resulting chimera, GFP-NLS/HEG14, showed not only nuclear import but also export similar to GFP-wtER α in response to estradiol treatment. These findings collected in Fig. S2 point to the presence of nuclear export sequences in the NLS-HEG14 construct, which is made up of the NLSs and the hormone-binding domain of ER α .

It has been found that NLSs of the PgR are responsible for its nuclear export (Guiochon-Mantel et al., 1994). Therefore, we studied whether NLSs are involved in ER α export using in vivo export assay (Henderson and Eleftheriou, 2000). In this assay, nuclear export sequences are identified by their ability to restore export activity of the NES-deficient REV1.4-GFP (Rev mutant) to levels similar to those observed with the wild-type (wt) pREV-GFP or the REV1.4-GFP NES (Rev positive control),

in which the NES is the canonical export sequence of the REV protein. The NLS sequences of ER α were subcloned into the Rev mutant and expressed in MCF-7 cells. After transfection, cells were incubated in the absence or presence of actinomycin D because it causes cytoplasmic accumulation of the putative NES-containing REV protein by preventing nuclear import of REV (Henderson, 2000). Irrespective of experimental conditions, the Rev mutant NLS localizes in nuclei of MCF-7 cells (Fig. 2, a and b). In the same experiment, the Rev-positive control completely shifted to the cytoplasm in the presence of actinomycin D, whereas the Rev mutant NLS sequences from the ER α showed nuclear, sometimes nucleolar, staining. These data indicate that the NLS sequences of ER α are inactive in this export assay.

We then subcloned different sequences of ER α containing leucine residues into a Rev mutant. These constructs were transfected into MCF-7 cells and then analyzed for their ability to restore the nuclear export of the Rev mutant. Using this assay, we verified that ER α residues 427–456 induced a nucleocytoplasm redistribution of Rev mutant in cells treated with actinomycin D (Fig. 2, a and b). LMB treatment blocked the export activity of the 427–456 ER α sequence (Fig. 2, a and b). Regardless of the experimental conditions, the leucine-rich 296–335 ER α sequence was unable to drive the Rev mutant to the cytoplasm (Fig. 2, a and b). These data show that the 427–456 region is involved in the export of ER α through CRM1/exportin binding.

A thorough sequence analysis showed homology between the 444–456 amino acids of ER α and the conserved leucine-rich and REV-like NES of p53 (residues 340–351; Stommel et al., 1999). A comparison of the homologous amino acids across the family of steroid receptors revealed a high level of sequence homology with ER β , PgR, AR, and GR. The alignment between the sequences is shown in Fig. S3 (a and b, available at <http://www.jcb.org/cgi/content/full/jcb.200712125/DC1>).

The possibility that residues 444–456 of the ER α sequence contain the NES-ER α was further investigated. The 444–456 ER α sequence were subcloned into the Rev mutant, as they were with a mutated version in which residues 444–456 were mutated from ILL to alanines (Fig. 2 c). The ER α wt sequence (NES-ER α wt) was able to shift the Rev mutant into the cytoplasm of MCF-7 cells, whereas the mutated form of ER α (NES-ER α mutant) failed to do so (Fig. 2, d and e). This difference is even more evident in the presence of actinomycin D.

These results show that a small sequence of the ER α hormone-binding domain contains a CRM1-mediated ER α NES.

A peptide mimicking the 444–456 ER α sequence sequesters the receptor in the nuclear compartment and inhibits estradiol-induced S phase entry in breast cancer cells

Small cationic peptides (also called protein transduction domains) derived from nucleic acid-binding proteins, such as HIV-Tat protein, deliver a myriad of peptides into animal models and represent a biological and potentially therapeutic tool for targeting proteins into the cells (Joliet and Prochiantz, 2004).

To trap ER α in the nuclear compartment of cells, we synthesized a Tat-conjugated peptide construct corresponding to the residues 444–456 of ER α (Fig. 3 a). Confocal microscopy analysis revealed in preliminary experiments that the carboxy-fluorescein-conjugated peptide translocated across the plasma membrane and, within 30 min, accumulated in nuclei of MCF-7 cells (unpublished data). This peptide displaced the estradiol-induced interaction between recombinant CRM1 and ER α (Fig. 3 b) and blocked the 60-min estradiol-induced nuclear export of GFP-wtER α , thus sequestering the receptor in nuclei (Fig. 3, c and d). The Tat alone did not affect both the ER α –CRM1 interaction (Fig. 3 b) as well as the trafficking of GFP-wtER α (unpublished data). Furthermore, the Tat-conjugated peptide did not affect p27 nuclear export or p53 subcellular localization (Fig. S3, c and d), which indicates that it specifically interferes in ER α localization.

The extranuclear activity of ER α triggers hormone-dependent DNA synthesis (Castoria et al., 1999), and ER α sequestration in the cytoplasm increases its nongenomic actions and drives neoplastic transformation (Kumar et al., 2002). Fig. 3 e shows that the Tat-conjugated peptide reduced estradiol-induced DNA synthesis in MCF-7 cells by 60%. A negligible effect was observed in cells treated with Tat alone. Furthermore, the Tat peptide did not interfere in serum-induced DNA synthesis in MCF-7 cells (Fig. 3 e), which indicates that the peptide specifically interferes in estradiol action. Addition of the Tat-conjugated peptide at different times after estradiol stimulation shows that after 60 min, when ER α is almost completely exported from nuclei, the peptide does not affect S phase entry in MCF-7 cells (Fig. 3 f), thus reinforcing the view that the estradiol-induced nuclear export of ER α plays a role in DNA synthesis.

ER α NES mutants do not exit nuclei and fail to mediate estradiol-induced DNA synthesis

Based on our findings (Fig. 2), two mutants of GFP-wtER α were prepared by site-directed mutagenesis to identify the NES-ER α sequence and analyze the biological effects induced by these mutations. Fig. 4 a shows a schematic representation of the GFP-wtER α NES and its mutated versions, which were transiently transfected into MCF-7 cells. Estradiol treatment induced nuclear export of GFP-wtER α , which contains the wild-type human ER α . In contrast, both mutants, GFP-ER α 4A and GFP-ER α IL, were unable to be exported from nuclei upon hormonal stimulation (Fig. 4 b). Images in Fig. 4 c show the diffuse, sometimes extranuclear localization of GFP-wtER α in MCF-7 cells stimulated for 60 min with estradiol. Under these conditions, a nuclear/nucleolar localization of both GFP-ER α 4A and GFP-ER α IL was detected.

We then verified the ability of the GFP-ER α IL mutant to activate estradiol-induced gene transcription. To this end, we used ER α -negative NIH3T3 fibroblasts (Castoria et al., 1999, 2003). The Western blot in Fig. 4 d (inset) confirms that NIH3T3 fibroblasts are indeed ER α negative. The cells were then transiently transfected with GFP-wtER α , GFP-ER α IL, or GFP alone. The ERE-Luc reporter gene was cotransfected and its activ-

ity assayed. Fig. 4 d shows that the constructs GFP-wtER α and GFP-ER α IL are equally efficient in activating gene transcription upon estradiol stimulation of the cells, with a sixfold induction of ERE-Luc activity. Transcriptional activation was almost undetectable in unstimulated cells or in cells expressing GFP alone.

In another set of experiments, we assessed the ability of GFP-wtER α and GFP-ER α IL to mediate estradiol-induced AKT activation as well as DNA synthesis when ectopically expressed in ER α -negative NIH3T3 fibroblasts. We first verified that GFP-wtER α and its export mutant GFP-ER α IL both activate AKT upon estradiol stimulation of NIH3T3 cells (Fig. 4 e). Although able to activate the nongenomic pathway usually engaged by estradiol to transmit its mitogenic signal, the mutant GFP-ER α IL failed to induce S phase entry in fibroblasts treated with estradiol (Fig. 4 f). The mutant did not affect S phase entry induced by the receptor-independent serum stimulation (Fig. 4 f). In contrast, the GFP-wtER α induced a robust BrdU incorporation in hormone-treated cells (Fig. 4 f). As observed in MCF-7 cells (Fig. 3 e), addition of Tat-conjugated peptide inhibited the estradiol-induced S phase entry in fibroblasts transfected with GFP-wtER α . Here again, the peptide did not interfere in DNA synthesis induced by serum stimulation of the same cells (Fig. 4 f, asterisks).

These experiments demonstrate that the leucine-rich sequence 444–456 of ER α contains a functional NES. Mutations in the core (ILL) of this sequence impair the estradiol-induced nuclear export of ER α and DNA synthesis without affecting the signal transduction pathway or gene transcription regulation by estradiol.

Estradiol regulates FKHR/ER α export

Estradiol activation of PI3K is required to drive MCF-7 cells into S phase (Castoria et al., 2001). Activation of the PI3K–AKT pathway by estradiol regulates ER α nuclear export (Fig. 1), and FKHR nuclear export depends on its phosphorylation by AKT (Biggs et al., 1999). In addition, previous studies reported that an estradiol-dependent interaction between ER α and FKHR occurs *in vitro* (Schoor et al., 2001). Thus, we hypothesized a role for FKHR in both estradiol-regulated ER α nuclear export and cell cycle arrest mediated by the NES-ER α mutant. To analyze the role of FKHR in estradiol-induced DNA synthesis and ER α nuclear export, quiescent MCF-7 cells were transiently transfected with wt FKHR (GFP-FKHR wt) or a mutant containing a triple alanine substitution (GFP-FKHR AAA) that localizes in nuclei and blocks FKHR phosphorylation by AKT, thereby inducing G1 arrest of cells (Nakamura et al., 2000). Fig. 5 a shows that expression of this mutant reduced estradiol-triggered BrdU incorporation in MCF-7 cells, whereas expression of GFP-FKHR wt did not. We then used confocal microscopy to analyze the role of FKHR in the estradiol-regulated subcellular distribution of ER α . Although ectopically expressed GFP-FKHR wt or GFP alone did not modify the 60-min estradiol-induced nuclear export of ER α , the mutant GFP-FKHR AAA sequestered ER α in the nuclear compartment at that time (Fig. 5, b and d). Conversely, overexpression of the tagged NES-ER α mutant, Myc-ER α IL, resulted in retention of GFP-FKHR wt in the nuclear

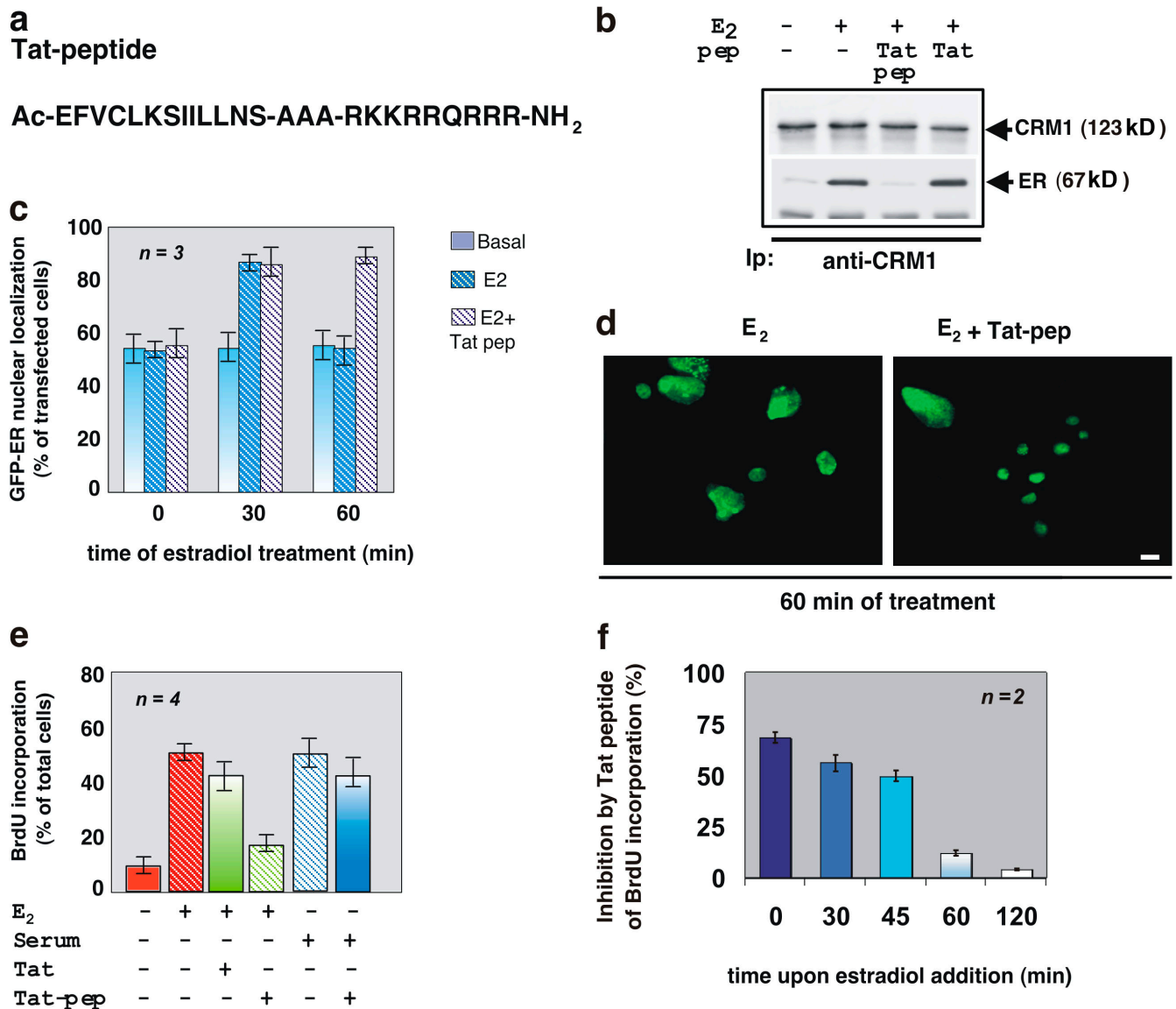


Figure 3. A peptide mimicking the ER α 444–456 sequence displaces the CRM1–ER α interaction, sequesters the receptor into nuclei, and inhibits S phase entry in estradiol-treated MCF-7 cells. (a) The amino acid sequence of the Tat-conjugated ER α (444–456) peptide. (b) ³⁵S-labeled HA-CRM1 was incubated with recombinant ER α from baculovirus in the absence or presence of 10 nM estradiol alone or together with a 200-fold excess of the Tat-conjugated peptide (Tat-pep). A 200-fold excess of a nonspecific peptide (Tat) was used as a control. CRM1 was immunoprecipitated with the anti-HA mAb, and proteins in immunocomplexes were revealed by fluorography (top) and immunoblotting with the anti-ER α antibody (bottom). (c) Quiescent MCF-7 cells transfected with GFP-HEGO were incubated for 1 h with the Tat-conjugated peptide (Tat pep). Thereafter, the cells were left untreated or treated for the indicated times with 10 nM estradiol. The proportion of cells with nuclear GFP-wtER α protein was determined by fluorescence microscopy and graphically shown. For each experiment, at least 200 cells were scored. The results of several independent experiments were averaged. *n* represents the number of experiments. (d) Images of one experiment in panel c. This shows the localization of GFP-wtER α in MCF-7 cells treated for 60 min with estradiol in the absence or presence of Tat-pep. Bar, 5 μ m. (e) MCF-7 cells on coverslips were made quiescent or serum starved (maintained for 24 h in 0.5% FCS). The cells were incubated for 24 h with 10 nM estradiol or 20% serum in the absence or presence of the Tat-conjugated ER α (444–456) peptide (Tat-pep) or Tat alone. After in vivo pulse with BrdU, DNA synthesis was analyzed and BrdU incorporation was expressed as a percentage of total nuclei. For each experiment, at least 200 cells were scored. The results of several independent experiments were averaged. *n* represents the number of experiments. (f) Quiescent MCF-7 cells were stimulated with 10 nM estradiol for 24 h, and the Tat peptide was added after the addition of the hormone at the time points indicated in the figure. After an in vivo pulse with BrdU, DNA synthesis was analyzed and expressed as a percentage of BrdU incorporation inhibition. For each experiment, at least 300 cells were scored. The results of two independent experiments were averaged. (c, e, and f) Means and SEM are shown.

compartment 60 min after estradiol treatment of MCF-7 cells (Fig. 5, c and d). Thus, estradiol simultaneously regulates ER α - and FKHR-associated nuclear export.

We then verified that silencing of ER α by siRNA retains GFP-FKHR wt in the nuclear compartment of 60-min hormonal-treated MCF-7 cells (Fig. 5 e). These findings confirm that, in

ER α -positive breast cancer cells, FKHR moves from nucleus to the cytoplasm by a mechanism depending on ER α .

Finally, we observed by Western blot analysis that estradiol stimulation of quiescent MCF-7 cells rapidly induces FKHR phosphorylation in Ser256 (Fig. 6 a). To complement colocalization experiments, we next analyzed the interaction

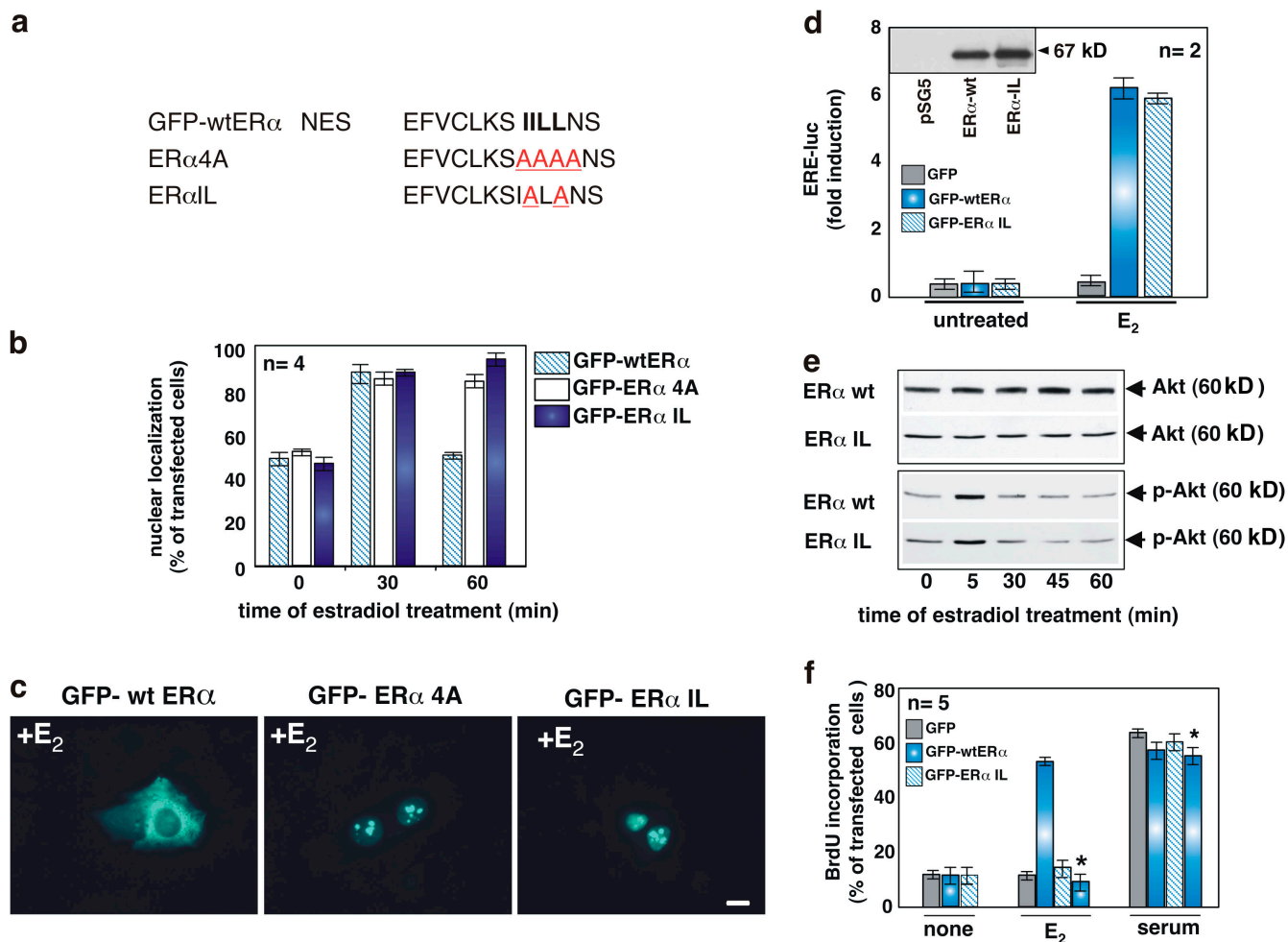


Figure 4. Mutations of NES-ER α impair nuclear export of full-length ER α and prevent S phase entry in MCF-7 cells stimulated by estradiol. (a) The wild-type NES-ER α endowed in the amino acid 444–456 sequence is shown in bold. Mutated amino acids are underlined. The NES mutants, GFP-HEG4A and GFP-HEG1L, were prepared as described in Materials and methods. (b) Quiescent MCF-7 cells were transfected with the indicated plasmids and then left unstimulated or stimulated with 10 nM estradiol for the indicated times. The percentage of cells with nuclear GFP protein was determined by fluorescence microscopy and shown graphically. For each experiment, at least 150 cells were scored. The results of different independent experiments were averaged; *n* represents the number of experiments. (c) Images of one experiment in panel b that shows the intracellular distribution of GFP-wtER α , GFP-ER α 4A, or GFP-ER α IL in MCF-7 cells treated for 1 h with estradiol. Bar, 5 μ m. (d–f) Growing NIH3T3 fibroblasts were used. The Western blot in panel d shows that NIH3T3 fibroblasts are ER α negative. In the graph in panel d, cells were transfected with an ERE-Luc construct along with the indicated plasmids. After transfection, the cells were made quiescent and then left unstimulated or stimulated with 10 nM estradiol. Luciferase activity was assayed, normalized using β -gal as an internal control, and expressed as fold induction. (e) Cells were transfected with the indicated plasmids and made quiescent. The cells were left unstimulated or stimulated for 5 min with 10 nM estradiol. Lysates were analyzed for AKT activation using the anti-P-Ser-473 antibody (bottom). The nitrocellulose filters were then probed with anti-AKT antibody (top). (f) Cells on coverslips were transfected with the indicated plasmids and made quiescent. The cells were left unstimulated or stimulated for 18 h with 10 nM estradiol or 20% serum. In cells transfected with GFP-wtER α , the inhibitory action of Tat peptide on estradiol-induced BrdU incorporation was analyzed by including this compound (at 1 μ M) to the cell medium (bars with asterisks). After in vivo pulse with BrdU, DNA synthesis was analyzed by immunofluorescence. In transfected cells, BrdU incorporation was calculated by the formula (percentage of BrdU-positive cells = [number of transfected-positive cells/number of transfected cells] \times 100) and compared with BrdU incorporation of untransfected cells from the same coverslips. For each plasmid, data were derived from at least 500 scored cells. The results of several independent experiments were averaged; *n* represents the number of experiments. (b, d, and f) Means and SEM are shown.

between ER α and FKHR in coimmunoprecipitation experiments. Data in Fig. 6 b show that 30-min hormonal stimulation increases the association of FKHR wt with ER α in MCF-7 cells (Fig. 6 b, left). Whatever the experimental condition, the mutant FKHR AAA does not coimmunoprecipitate with ER α (Fig. 6 b, right), therefore implicating that phosphorylation of FKHR induced by estradiol is required for its association with the receptor.

These findings led us to hypothesize the model of ER α export and its interplay with FKHR depicted in Fig. 6 c.

Discussion

Nucleocytoplasmic shuttling of proteins plays a critical role in cell function (for review see Kau and Silver, 2003). Most of the ER α is localized in nuclei of hormone target cells (Stenoien et al., 2001), and its best-known function as a ligand-activated transcription factor requires nuclear localization (Mangelsdorf et al., 1995). Much evidence, however, has demonstrated rapid, extranuclear action of ER α (for review see Migliaccio et al., 2007a). Ligand stimulation of different cell types recruits different

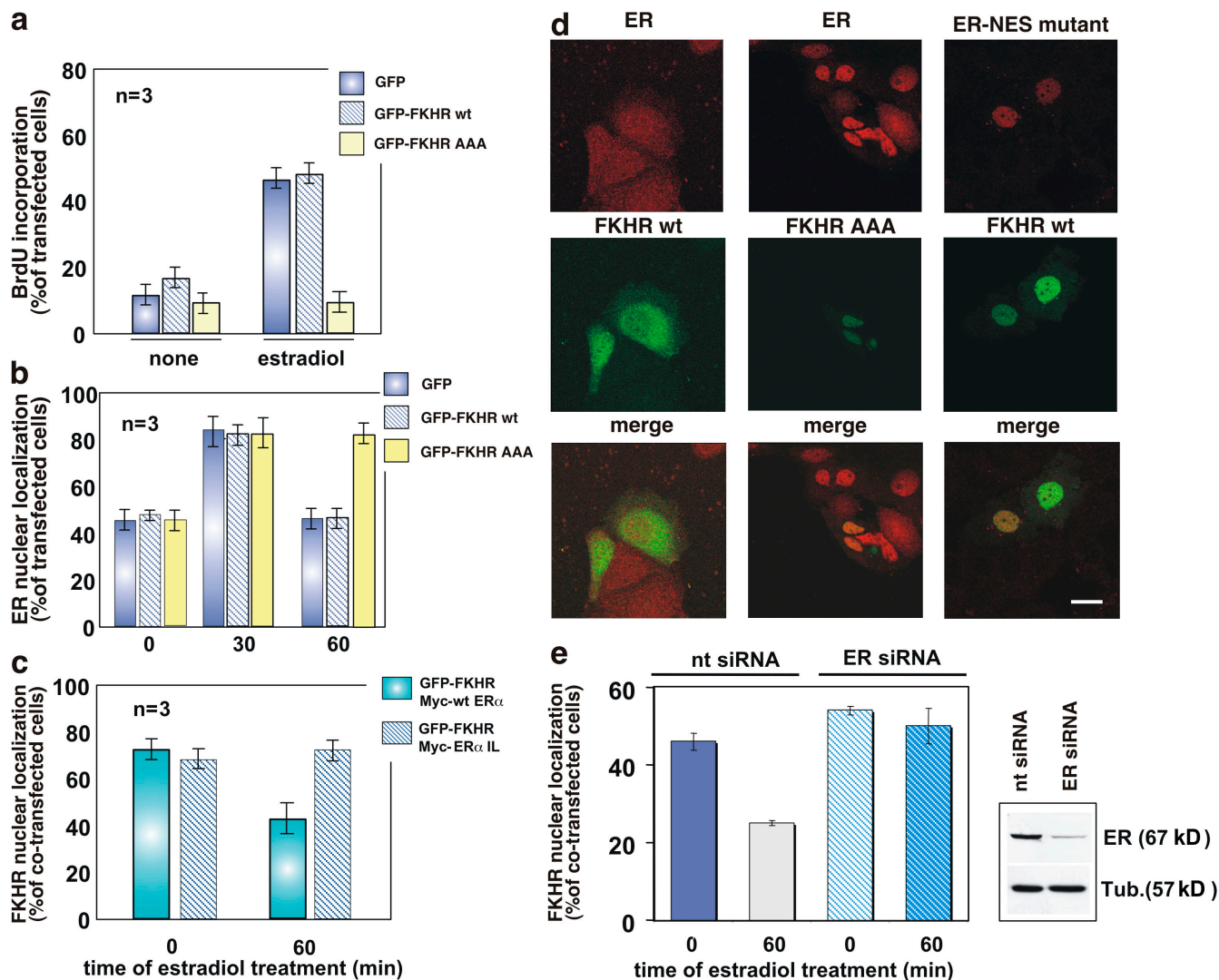
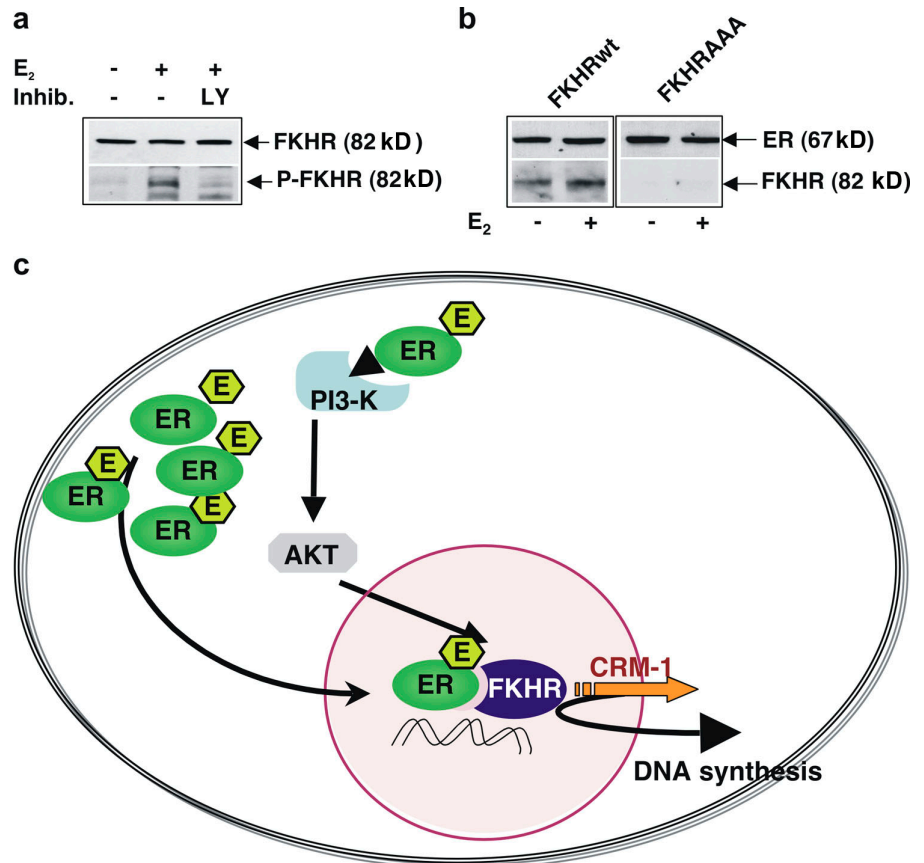


Figure 5. FKHR nuclear export: regulation by estradiol and a role in hormone-induced DNA synthesis in MCF-7 cells. Quiescent MCF-7 cells on coverslips were used. (a) Cells were transfected with the indicated plasmids then left unstimulated or stimulated for 24 h with 10 nM estradiol. After *in vivo* pulse with BrdU, DNA synthesis was analyzed by immunofluorescence and BrdU incorporation was calculated as in Fig. 4. (b) Cells were transfected with the indicated plasmids then left unstimulated or stimulated with 10 nM estradiol for the indicated times. Endogenous ER α localization as well as expression of GFP, GFP-FKHR wt, or GFP-FKHR AAA mutant was monitored by confocal microscopy. Cells that fell into the category of exclusively ER α nuclear fluorescence were scored, and data were expressed as a percentage of transfected cells. (c) Cells were cotransfected with the indicated plasmids then left unstimulated or stimulated with 10 nM estradiol for 60 min. Localization of GFP-FKHR wt, Myc-HEGO, or Myc-HEGIL mutant was monitored by confocal microscopy. Cells that fell into the category of exclusively FKHR nuclear fluorescence were scored and the data were expressed as a percentage of cotransfected cells. For each experiment in panels a–c, data were derived from at least 500 scored cells. The results of several independent experiments were averaged; *n* represents the number of experiments. (d) Images from one experiment in b or c are shown. They represent the staining of endogenous ER α (red) in MCF-7 cells expressing GFP-FKHR wt (left, green) or the mutant, GFP-FKHR AAA (middle, green), and treated for 60 min with estradiol. (right) The staining of Myc-tagged NES-ER α mutant (red) in MCF-7 cells coexpressing GFP-FKHR wt (green) and treated for 60 min with estradiol. Merged images are also shown on the bottom. Bar, 5 μ m. (e) The cells were cotransfected with ER α siRNA (ER α siRNA) or nontargeting siRNA (nt siRNA) and GFP-FKHR wt. The cells were then left unstimulated or stimulated with 10 nM estradiol for the indicated times. GFP-FKHR wt localization was monitored by confocal microscopy. Cells that fell into the category of exclusively GFP-FKHR wt nuclear fluorescence were scored and data were expressed as a percentage of transfected cells. Data were derived from at least 200 scored cells. The results of two independent experiments were averaged. The blot in panel e confirms the silencing of ER α in MCF-7 cells transfected with ER α siRNA (top). The bottom shows the blot of loading proteins revealed using the anti-tubulin antibody. (a, b, c, and e) Means and SEM are shown.

signaling effectors to ER α or ER β , which in turn leads to signal transduction pathway activation (Migliaccio et al., 2000; Castoria et al., 2001). A transcriptionally inactive ER α mutant, permanently residing in the cytoplasm, mediates S phase entry triggered by estradiol activation of the Src–Ras–ERK and PI3K–AKT pathways (Castoria et al., 1999, 2004). These findings suggest a proliferative function for this extranuclear receptor that is independent of its transcriptional activity. This view is

supported by the observation that a classical mouse AR, which is expressed at a very low level in NIH3T3 fibroblasts, does not enter nuclei and does not activate gene transcription in androgen-stimulated cells. Nevertheless, it recruits several signaling effectors that control hormone-induced S phase entry and cytoskeleton changes in fibroblasts (Castoria et al., 2003). Rat uterine stromal cells, although they express low levels of transcriptionally incompetent PgR, respond to progestin with

Figure 6. Estradiol-induced FKHR activation and ER α -FKHR complex assembly in MCF-7 cells. Model of ER α nuclear export. Quiescent MCF-7 cells were used. (a) Cells were left unstimulated or stimulated for 5 min with 10 nM estradiol in the absence or presence of LY294002 (at 5 μ M; Qbiogene). Lysates were analyzed for FKHR phosphorylation using the anti-P-Ser-256-FKHR antibody (bottom). The nitrocellulose filter was then re probed with anti-FKHR antibody (top). (b) The cells were transfected with the indicated plasmids (FKHR wt or FKHR AAA) then left unstimulated or stimulated for 30 min with 10 nM estradiol. Lysates were immunoprecipitated with anti-ER α antibody. Immunocomplexes were immunoblotted using the antibodies against the indicated proteins. (c) The model of ER α nuclear export is depicted. Estradiol stimulates the PI3K-AKT pathway, leading to FKHR phosphorylation and triggering the functionally associated export of FKHR-ER α . This export results in stimulation of DNA synthesis.



active proliferation (Vallejo et al., 2005). In addition, the extranuclear cross talk between ER α -AR and the EGF receptor regulates EGF-elicited responses such as actin changes and DNA synthesis in breast and prostate cancer cells (Migliaccio et al., 2005). Direct evidence of the proliferative role of the ER α -AR complex association with Src in mammary and prostate cancer cell xenografts has been recently described (Migliaccio et al., 2007b; Varricchio et al., 2007). These and other similar findings point to the critical role of steroid receptor extranuclear localization in steroid hormone or growth factor action.

In this study, we analyzed the nucleocytoplasmic shuttling of ER α in MCF-7 cells, focusing on its export from the nucleus. We observed that estradiol induces CRM1-dependent nuclear export of ER α . Thus, identification of the ER α sequence responsible for its nuclear export was initially performed by a nuclear export assay (Henderson and Eleftheriou, 2000). Previous studies found that the outward movement of PgR from the nuclear compartment is mediated by its NLSs (Guiochon-Mantel et al., 1994). We then assayed the ability of ER α NLSs to shift the REV mutant into the cytoplasm. Our data show that NLSs are not involved in the nuclear export of ER α . It has also been hypothesized that the nuclear exit of ER α is regulated by its phosphorylation on Thr311 (Lee and Bai, 2002). This residue is present in a putative leucine-rich ER α NES (305–322 of ER α), which shares homology with the p53 N-terminal NES (Zhang and Xiong, 2001). We found that the leucine-rich 296–335 ER α sequence does not drive the REV mutant into the cytoplasm. Using the same approach, we observed that the leucine-rich

444–456 sequence of ER α contains a functional NES, which is sensitive to LMB treatment. A Tat-conjugated peptide mimicking this amino acid sequence displaces the *in vitro* interaction between ER α and CRM1. These findings support the conclusion that the 444–456 sequence contains a putative NES. Mutations in the core of this sequence impair estradiol-induced nuclear export of full-length ER α in MCF-7 cells. The 444–456 sequence shows homology with the conserved leucine-rich and REV-like NES of p53 (340–351; Fig. S3 a; Stommel et al., 1999). Remarkably, the 444–456 region of ER α is conserved in other steroid receptors, such as ER β , PgR, AR, GR, and MR (Fig. S3 b), which suggests that such a sequence is responsible for the CRM1-dependent nuclear export of most steroid receptors. This view is in agreement with the inhibition of nuclear export of different steroid receptors by LMB treatment described by several groups under different experimental conditions (Savory et al., 1999; Pruffer and Barsony, 2002; Maruvada et al., 2003).

ER α localization was also modulated by a Tat-conjugated peptide mimicking the ER α 444–456 sequence. It specifically traps ER α in the nuclear compartment of MCF-7 cells and reduces the estradiol-induced S phase entry in these cells, thus confirming that ER α nuclear export plays a key role in DNA synthesis of target cells. This view is reinforced by the finding that addition of the peptide to the cell medium 1 h after hormonal stimulation, when ER α nuclear export is almost complete, does not affect estradiol-induced S phase entry in MCF-7 cells. Experiments with NIH3T3 fibroblasts show that an NES-ER α mutant fails to induce S phase entry in these cells, whereas it

After transfection, the cells were incubated at 37°C for 24 h and then used for the indicated experiments.

Nuclear export and transactivation assays

The nuclear export assay was performed into growing MCF-7 cells as described previously (Henderson and Eleftheriou, 2000). In brief, each putative NES-ER α was subcloned into the REV (1.4)-GFP (Rev mutant, provided by B. Henderson, Westmead Institute for Cancer Research, University of Sydney and Westmead Millennium Institute at Westmead Hospital, Westmead, Australia) and then transfected (at 2 μ g) by SuperFect into growing cells. After 18 h, the cells were incubated with cycloheximide (at 15 μ g/ml; EMD) and left untreated or treated for 8 h with actinomycin D (at 5 μ g/ml; EMD). When indicated, LMB (at 5 ng/ml; EMD) was added 30 min before the addition of actinomycin D. Growing NIH3T3 fibroblasts were plated on Petri dishes. They were then transfected by SuperFect using 2 μ g of either pEGFP, pEGFP-HEGO, or pEGFP-HEGIL. For transactivation assay, 2 μ g of pGL2 ERE-Luc (Bonapace et al., 1996) provided by L. Altucci (University of Naples Federico II, Naples, Italy) were cotransfected. 12 h after transfection, cells were made quiescent and left untreated or treated with 10 nM estradiol for 16 h. Lysates were prepared and the luciferase activity was measured using a luciferase assay system (Promega). The results were corrected using CH110-expressed β -galactosidase activity (GE Healthcare).

DNA synthesis analysis and peptides

Quiescent MCF-7 or NIH3T3 cells on coverslips were left unstimulated or stimulated for 18 h with the indicated compounds. Estradiol was used at 10 nM and serum was added at 20%. The Tat-peptide conjugated to the 444–456 amino acid sequence of ER α (Tat-pep) as well as the Tat control peptide were used at 1 μ M. The peptides were N-terminal acetylated and C-terminal amidated. After a 6-h pulse with 100 μ M BrdU (Sigma-Aldrich), BrdU incorporation was analyzed as described previously (Castoria et al., 1999) using the mouse monoclonal anti-BrdU antibody (clone BU-1 from GE Healthcare). Diluted (1:200 in PBS) Texas red-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) was used to detect the primary antibody.

Immunofluorescence and confocal microscopy

Cells on coverslips were fixed for 10 min with paraformaldehyde (3% wt/vol in PBS), washed with PBS, and then analyzed by fluorescence microscopy for the subcellular localization of GFP-wtER α , its mutants, as well as putative NES-ER α constructs. Similar analysis was performed to detect GFP-FKHR wt or its mutant GFP-FKHR AAA. In all other experiments, the cells on coverslips were fixed and permeabilized as described previously (Castoria et al., 1999). Endogenous ER α was visualized as described previously (Castoria et al., 1999) using the A314 mouse mAb or the H222 rat mAb. Mouse antibody was detected using diluted (1:200 in PBS) Texas red-conjugated goat anti-mouse antibodies (EMD). Diluted (1:200 in PBS containing 0.2% BSA) Texas red-conjugated AffiniPure anti-rat IgG (Jackson ImmunoResearch Laboratories) was used to detect the H222 rat monoclonal antibody. The dominant-negative Δ p85 α and Myc-His-tagged dominant-negative AKT were detected using diluted (1:100 in PBS) either anti-p85 α or anti-Myc tag (both from United Biotech Inc.) rabbit polyclonal antibodies. Diluted (1:200 in PBS containing 0.2% BSA) anti-rabbit fluorescein-conjugated antibodies (Jackson ImmunoResearch Laboratories) were added (Castoria et al., 2001). The Myc-tagged pSG5 or wt ER α or ER α IL were detected as described previously (Castoria et al., 2004) using the mouse anti-Myc tag monoclonal antibody (Clontech Laboratories, Inc.). Diluted (1:200 in PBS) Texas red-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories) was used as a secondary antibody. Endogenous p27 was visualized as described previously (Castoria et al., 2004) using the anti-p27 mAb (BD Biosciences). Endogenous p53 was visualized using the mouse monoclonal anti-p53 antibody (DO-1; Santa Cruz Biotechnology, Inc.). Diluted (1:200 in PBS) anti-mouse fluorescein isothiocyanate-conjugated antibodies (EMD) were added to detect the primary mouse antibodies. Coverslips were finally stained with Hoechst 33258, inverted, and mounted in Mowiol (EMD). Fields were analyzed with a fluorescent microscope (DMBL; Leica) using HCX PL Fluotar 40 \times , HCX PL Apo 63 \times oil, and HCX PL Fluotar 100 \times oil objectives (Leica). Images were captured using a DC200 or DC480 camera (Leica) and acquired using IM1000 or FW4000 software (Leica). For confocal microscopy, fields were analyzed and acquired using a confocal microscope (LSM510; Carl Zeiss, Inc.) using a Plan-Neofluar 40 \times immersion objective (Carl Zeiss, Inc.). Images were magnified two times.

Purified proteins and in vitro protein-protein interactions

Ran Q69L expressed in JM109 bacteria was purified using the Ni-NTA agarose (QIAGEN) and recombinant human ER α (2800 pmol/ml; Invitrogen). S³⁵-labeled HA-CRM1 was produced in rabbit reticulocyte lysate (Promega), and protein-protein interaction was performed as described previously (Migliaccio et al., 2000). Immunoprecipitation was performed using the rabbit polyclonal anti-ER α Ab (G-20 1 μ g/ml; Santa Cruz Biotechnology, Inc.) or the mouse monoclonal anti-HA antibody (at 2 μ g/ml; Covance). Eluted proteins were analyzed by immunoblotting or fluorography.

Lysates, immunoprecipitation, and immunoblotting

Lysates were prepared as described previously (Migliaccio et al., 2000), and ER α was immunoprecipitated according to the same protocol. Rabbit polyclonal anti-ER α antibody (G-20; Santa Cruz Biotechnology, Inc.) was used to detect ER α . P-Ser473-AKT and AKT from cell lysates were detected as described previously (Castoria et al., 2001). P-Ser256-FKHR and FKHR were revealed by Western blotting using the appropriate antibodies (Cell Signaling Technology). Expression and production of Ran Q69L was verified using the mouse monoclonal anti-His tag antibody (QIAGEN). When indicated, ER α and the chimera GFP-ER α were detected using either the rat monoclonal (H222; Abbott Labs) anti-ER α antibody or the mouse monoclonal anti-GFP antibody (Clontech Laboratories, Inc.). Immunoreactive proteins were revealed by the ECL detection system (GE Healthcare).

Online supplemental material

Fig. S1 shows the cytoplasmic localization of endogenous ER α in indirect IF staining of untreated or estradiol-treated MCF-7 cells. Corresponding cell lysates do not show significant change in ER α or GFP-ER α levels. The figure also presents images from experiments shown in the paper as well as estradiol activation of Akt in MCF-7 cells. Fig. S2 shows that the NLS-HEG14 construct, which is made up of the nuclear localization signals and the hormone binding domain of ER α contains the NES-ER α . Fig. S3 shows the homology between the 444–456 amino acids of ER α and the conserved leucine-rich and REV-like NES of p53. It also shows that the 444–456 sequence of ER α is highly conserved among the members of steroid receptor family. The specificity of Tat-conjugated peptide is also presented. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200712125/DC1>.

We thank L. Altucci, P. Chambon, A. Diehl, J. Downward, B. Henderson, and I. Mattaj for plasmids and reagents.

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (National and Regional grants) and Ministero dell'Università e della Ricerca Scientifica (PRIN 2006; grant Nos. 2006065483 and 2006062798_002). We declare that we do not have competing financial interests.

Submitted: 20 December 2007

Accepted: 27 June 2008

References

- Abbondanza, C., V. Rossi, A. Roscigno, L. Gallo, A. Belsito, G. Piluso, N. Medici, V. Nigro, A.M. Molinari, B. Moncharmont, and G.A. Puca. 1998. Interaction of vault particles with estrogen receptor in the MCF-7 breast cancer cell. *J. Cell Biol.* 141:1301–1310.
- Alt, J.R., J.L. Cleveland, M. Hannink, and J.A. Diehl. 2000. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. *Genes. Dev.* 14:3102–3114.
- Askjaer, P., A. Bachi, M. Wilm, F.R. Bischoff, D.L. Weeks, V. Ogniewski, M. Ohno, C. Niehrs, J. Kjems, I.W. Mattaj, and M. Fornerod. 1999. Ran-regulated interactions of CRM1 with nucleoporins and a shuttling DEAD-Box helicase. *Mol. Cell. Biol.* 19:6276–6285.
- Biggs, W.H. III, J. Meisenhelder, T. Hunter, W.K. Cavenee, and K.C. Arden. 1999. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. *Proc. Natl. Acad. Sci. USA.* 96:7421–7426.
- Birkenkamp, K.U., and P.J. Coffey. 2003. Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors. *Biochem. Soc. Trans.* 31:292–297.
- Bischoff, F.R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl. 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc. Natl. Acad. Sci. USA.* 91:2587–2591.
- Bonapace, I.M., R. Addeo, L. Altucci, L. Cicatiello, M. Bifulco, C. Laezza, S. Salzano, V. Sica, F. Bresciani, and A. Weisz. 1996. 17 beta-estradiol overcomes a G1 block induced by HMG-CoA reductase inhibitors and fosters

- cell cycle progression without inducing ERK-1 and -2 MAP kinases activation. *Oncogene*. 12:753–763.
- Castoria, G., M.V. Barone, M. Di Domenico, A. Bilancio, D. Ametrano, A. Migliaccio, and F. Auricchio. 1999. Non-transcriptional action of estrogen and progesterin triggers DNA synthesis. *EMBO J.* 18:2500–2510.
- Castoria, G., A. Migliaccio, A. Bilancio, M. Di Domenico, A. de Falco, M. Lombardi, R. Fiorentino, L. Varricchio, M.V. Barone, and F. Auricchio. 2001. PI3-kinase in concert with Src promotes the S-phase entry of estradiol-stimulated MCF-7 cells. *EMBO J.* 20:6050–6059.
- Castoria, G., M. Lombardi, M.V. Barone, A. Bilancio, M. Di Domenico, D. Bottero, F. Vitale, A. Migliaccio, and F. Auricchio. 2003. Androgen-stimulated DNA synthesis and cytoskeletal changes in fibroblasts by a nontranscriptional receptor action. *J. Cell Biol.* 161:547–556.
- Castoria, G., A. Migliaccio, M. Di Domenico, M. Lombardi, A. de Falco, L. Varricchio, A. Bilancio, M.V. Barone, and F. Auricchio. 2004. Role of atypical PKC in estradiol-triggered G1/S progression of MCF-7 cells. *Mol. Cell. Biol.* 24:7643–7653.
- Dauvois, S., R. White, and M.G. Parker. 1993. The antiestrogen ICI 182780 disrupts estrogen receptor nucleocytoplasmic shuttling. *J. Cell Sci.* 106:1377–1388.
- DeFranco, D.B. 2001. Nuclear export: DNA-binding domains find a surprising partner. *Curr. Biol.* 11:R1036–R1037.
- Dhand, R., K. Hara, I. Hiles, B. Bax, I. Gout, G. Panayotou, M.J. Fry, K. Yonezawa, M. Kasuga, and M.D. Waterfield. 1994. PI3-kinase: structural and functional analysis of intersubunit interactions. *EMBO J.* 13:511–521.
- Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*. 90:1051–1060.
- Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and E. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature*. 390:308–311.
- Guiochon-Mantel, A., P. Lescop, S. Christin-Maitre, H. Loosfelt, M. Perrot-Appianat, and E. Milgrom. 1991. Nucleocytoplasmic shuttling of the progesterone receptor. *EMBO J.* 10:3851–3859.
- Guiochon-Mantel, A., K. Delabre, P. Lescop, and E. Milgrom. 1994. Nuclear localization signals also mediate the outward movement of proteins from the nucleus. *Proc. Natl. Acad. Sci. USA*. 91:7179–7183.
- Gururaj, A.E., S.K. Rayala, R.K. Vadlamudi, and R. Kumar. 2006. Novel mechanisms of resistance to endocrine therapy: genomic and nongenomic considerations. *Clin. Cancer Res.* 12:1001s–1007s.
- Henderson, B.R. 2000. Nuclear-cytoplasmic shuttling of APC regulates β -catenin subcellular localization and turnover. *Nat. Cell Biol.* 2:653–660.
- Henderson, B.R., and A. Eleftheriou. 2000. A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals. *Exp. Cell Res.* 256:213–224.
- Izaurrealde, E., U. Kutay, C. von Kobbe, I.W. Mattaj, and D. Gorlich. 1997. The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.* 16:6535–6547.
- Joliet, A., and A. Prochiantz. 2004. Transduction peptides: from technology to physiology. *Nat. Cell Biol.* 6:189–196.
- Katzenellenbogen, B.S., J.F. Elliston, F.J. Jr Monsma, P.A. Springer, Y.S. Ziegler, and G.L. Greene. 1987. Structural analysis of covalently labeled estrogen receptors by limited proteolysis and monoclonal antibody reactivity. *Biochemistry*. 26:2364–2373.
- Kau, T.R., and P.A. Silver. 2003. Nuclear transport as a target for cell growth. *Drug Discov. Today*. 8:78–85.
- Kumar, R., R.A. Wang, A. Mazumdar, A.H. Talukder, M. Mandal, Z. Yang, R. Bagheri-Yarmand, A. Sahin, G. Hortobagyi, L. Adams, et al. 2002. A naturally occurring MTA1 variant sequesters oestrogen receptor- α in the cytoplasm. *Nature*. 418:654–657.
- Kudo, N., B. Wolff, T. Sekimoto, E.P. Schreiner, Y. Yoneda, M. Yanagida, S. Horinouchi, and M. Yoshida. 1998. Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.* 242:540–547.
- Lee, H., and W. Bai. 2002. Regulation of estrogen receptor nuclear export by ligand-induced and p38-mediated receptor phosphorylation. *Mol. Cell. Biol.* 22:5835–5845.
- Liu, J., and D.B. De Franco. 2000. Protracted nuclear export of glucocorticoid receptor limits its turnover and does not require the exportin1/CRM-directed nuclear export pathway. *Mol. Endocrinol.* 14:40–51.
- Liu, J.L., Z. Mao, T.A. LaFortune, M.M. Alonso, G.E. Gallick, J. Fueyo, and W.K. Yung. 2007. Cell cycle-dependent nuclear export of phosphatase and tensin homologue tumor suppressor is regulated by the phosphoinositide-3-kinase signaling cascade. *Cancer Res.* 67:11054–11063.
- Mangelsdorf, D.J., C. Thummel, M. Beato, P. Herrlich, G. Schütz, K. Umesono, B. Blumberg, P. Kastner, M. Mark, P. Chambon, and R.M. Evans. 1995. The nuclear receptor superfamily: the second decade. *Cell*. 83:835–839.
- Maruvada, P., C.T. Baumann, G.L. Hager, and P.M. Yen. 2003. Dynamic shuttling and intranuclear mobility of nuclear hormone receptors. *J. Biol. Chem.* 278:12425–12432.
- Mazumdar, A., and R. Kumar. 2003. Estrogen regulation of Pak1 and FKHR pathways in breast cancer cells. *FEBS Lett.* 535:6–10.
- Migliaccio, A., G. Castoria, M. Di Domenico, A. de Falco, A. Bilancio, M. Lombardi, M.V. Barone, D. Ametrano, M.S. Zannini, C. Abbondanza, and F. Auricchio. 2000. Steroid-induced androgen receptor-oestradiol receptor b2Src complex triggers prostate cancer cell proliferation. *EMBO J.* 19:5406–5417.
- Migliaccio, A., M. Di Domenico, G. Castoria, M. Nanayakkara, M. Lombardi, A. de Falco, A. Bilancio, L. Varricchio, A. Ciociola, and F. Auricchio. 2005. Steroid receptor regulation of EGF signaling through Src in breast and prostate cancer cells: steroid antagonist action. *Cancer Res.* 65:10585–10593.
- Migliaccio, A., G. Castoria, and F. Auricchio. 2007a. Src-dependent signalling pathway regulation by sex-steroid hormones: Therapeutic implications. *Int. J. Biochem. Cell Biol.* 39:1343–1348.
- Migliaccio, A., L. Varricchio, A. De Falco, G. Castoria, C. Arra, H. Yamaguchi, A. Ciociola, M. Lombardi, R. Di Stasio, A. Barbieri, et al. 2007b. Inhibition of the SH3 domain-mediated binding of Src to the androgen receptor and its effect on tumor growth. *Oncogene*. 26:6619–6629.
- Nakamura, N., S. Ramaswamy, F. Vazquez, S. Signoretti, M. Loda, and W.R. Sellers. 2000. Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN. *Mol. Cell. Biol.* 20:8969–8982.
- Nigg, E.A. 1997. Nucleocytoplasmic transport: signals, mechanism and regulation. *Nature*. 386:779–787.
- Prufer, K., and J. Barsony. 2002. Retinoid X receptor dominates the nuclear import and export of the unliganded vitamin D receptor. *Mol. Endocrinol.* 16:1738–1751.
- Saporita, A.J., Q. Zhang, N. Navai, Z. Dincer, J. Hahn, Z. Cai, and Z. Wang. 2003. Identification and characterization of a ligand-regulated nuclear export signal in androgen receptor. *J. Biol. Chem.* 278:41998–42005.
- Savory, J.G., B. Hsu, I.R. Laquian, W. Giffin, T. Reich, R.J. Hache, and Y.A. Lefebvre. 1999. Discrimination between NL1 and NL2 mediated nuclear localization of the glucocorticoid receptor. *Mol. Cell. Biol.* 19:1025–1037.
- Schuur, E.R., A.V. Loktev, M. Sharma, Z. Sun, R.A. Roth, and R.J. Weigel. 2001. Ligand-dependent interaction of estrogen receptor- α with members of the forkhead transcription factor family. *J. Biol. Chem.* 276:33554–33560.
- Slamon, D.J., W. Godolphin, L.A. Jones, J.A. Holt, S.G. Wong, D.E. Keith, W.J. Levin, S.G. Stuart, J. Udove, and A. Ullrich. 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 244:707–712.
- Stenoien, D.L., K. Patel, M.G. Mancini, M. Dutertre, C.L. Smith, B.W. O'Malley, and M.A. Mancini. 2001. FRAP reveals that mobility of oestrogen receptor- α is ligand- and proteasome-dependent. *Nat. Cell Biol.* 3:15–23.
- Stommel, J.M., N.D. Marchenko, G.S. Jimenez, U.M. Moll, T.J. Hope, and G.M. Wahl. 1999. A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. *EMBO J.* 18:1660–1672.
- Tora, L., A. Mullick, D. Metzger, M. Ponglikitmongkol, I. Park, and P. Chambon. 1989. The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.* 8:1981–1986.
- Tyagi, R.K., Y. Lavrovsky, S.C. Ahn, S.C. Song, B. Chatterjee, and A.K. Roy. 2000. Dynamics of intracellular movement and nucleocytoplasmic recycling of the ligand-activated androgen receptor in living cells. *Mol. Endocrinol.* 14:1162–1174.
- Vadlamudi, R.K., B. Manavathi, S. Balasenthil, S.S. Nair, Z. Yang, A.A. Sahin, and R. Kumar. 2005. Functional implications of altered subcellular localization of PELP1 in breast cancer cells. *Cancer Res.* 65:7724–7732.
- Vallejo, G., C. Ballaré, J.L. Barañao, M. Beato, and P. Saragüeta. 2005. Progesterin activation of nongenomic pathways via cross talk of progesterone receptor with estrogen receptor β induces proliferation of endometrial stromal cells. *Mol. Endocrinol.* 19:3023–3037.
- Van Der Heide, L.P., M.F. Hoekman, and M.P. Smidt. 2004. The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J.* 380:297–309.
- Varricchio, L., A. Migliaccio, G. Castoria, H. Yamaguchi, A. de Falco, M. Di Domenico, P. Giovannelli, W. Farrar, E. Appella, and F. Auricchio. 2007. Inhibition of estradiol receptor/Src association and cell growth by an estradiol receptor α tyrosine-phosphorylated peptide. *Mol. Cancer Res.* 5:1213–1221.
- Vicent, G.P., C. Ballaré, A.S. Nacht, J. Clausell, A. Subtil-Rodríguez, I. Quiles, A. Jordan, and M. Beato. 2006. Induction of progesterone target genes requires activation of Erk and Msk kinases and phosphorylation of histone H3. *Mol. Cell.* 24:367–381.

- Won, C.K., H.H. Ji, and P.O. Koh. 2006. Estradiol prevents the focal cerebral ischemic injury-induced decrease of forkhead transcription factors phosphorylation. *Neurosci. Lett.* 398:39–43.
- Ylikomi, T., M.T. Bocquel, M. Berry, H. Gronemeyer, and P. Chambon. 1992. Cooperation of proto-signals for nuclear accumulation of estrogen and progesterone receptors. *EMBO J.* 11:3681–3694.
- Zhang, Y., and Y. Xiong. 2001. A p-53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science.* 292:1910–1915.
- Zhao, H.H., R.E. Herrera, E. Coronado-Heinsohn, M.C. Yang, J.H. Ludes-Meyers, K.J. Seybold-Tilson, Z. Nawaz, D. Yee, F.G. Barr, S.G. Diab, et al. 2001. Forkhead homologue in rhabdomyosarcoma functions as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions. *J. Biol. Chem.* 276:27907–27912.