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Extracellular Signal-regulated Kinase 8 (ERK8) Controls Estrogen-related Receptor α (ERRα) Cellular Localization and Inhibits Its Transcriptional Activity*[S]

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ERK8 (MAPK15) is a large MAP kinase already implicated in the regulation of the functions of different nuclear receptors and in cellular proliferation and transformation. Here, we identify ERRα as a novel ERK8-interacting protein. As a consequence of such interaction, ERK8 induces CRM1-dependent translocation of ERRα to the cytoplasm and inhibits its transcriptional activity. Also, we identify in ERK8 two L*XX*LL motifs, typical of agonist-bound nuclear receptor corepressors, as necessary features for this MAP kinase to interact with ERRα and to regulate its cellular localization and transcriptional activity. Ultimately, we demonstrate that ERK8 is able to counteract, in immortalized human mammary cells, ERRα activation induced by the EGF receptor pathway, often deregulated in breast cancer. Altogether, these results reveal a novel function for ERK8 as a *bona fide* ERRα corepressor, involved in control of its cellular localization by nuclear exclusion, and suggest a key role for this MAP kinase in the regulation of the biological activities of this nuclear receptor.

Mitogen-activated protein (MAP) kinases are a family of proline-directed serine/threonine kinases expressed in all eukaryotic cells, from yeast to human, and involved in key signaling pathways regulating cell proliferation, differentiation, apoptosis, and stress response (1, 2). ERK8 is the last identified member of the MAP kinase family. Along with a typical MAP kinase domain, it possesses a peculiarly long, unique C-terminal domain (3). Its activity can be modulated by serum, DNA damage, and activated human oncogenes such as BCR/ABL and RET/PTC3 (3–5). Still, although ERK8 has the classical signature Thr-Glu-Tyr activation motif of signal-dependent kinases such as ERK1, ERK2, and ERK5, it is not significantly activated by many extracellular stimuli that typically impinge on MAP kinases, and no MAP kinase kinase (MAPKK) has been identified that regulates its activity (6).[2] Interestingly, ERK8 has been recently involved in the transformation of human colon cancer cells (7) and in the maintenance of genomic integrity, by inhibiting proliferating cell nuclear antigen (PCNA) degradation (8). Recent data have also shown the ability of ERK8 to reduce the activity of nuclear receptors such as androgen and glucocorticoid receptors (9, 10). Moreover, ERK8 strongly enhances ubiquitin-dependent degradation of estrogen receptor α (ERα),[3] and loss of its expression has been correlated to breast cancer progression and increased ERα levels in mammary tumors (9). However, its targets and downstream effectors still remain almost completely unknown. Likewise, its biological functions have yet to be unraveled.

ERRα (NR3B1) was the first "orphan" nuclear receptor to be identified, on the basis of its similarity to ERα (11). It is a key regulator of mitochondrial biogenesis (12), energy metabolism (13), and bone formation and maintenance (14). ERRα binds to DNA and regulates transcription through specific ERR response elements (ERREs), also named SF-1 response elements (SFREs), as well as through classical estrogen receptor response elements (EREs) (15, 16). The natural ligand of ERs, 17β-estradiol (E_2), does not bind to ERRα (17). However, based on the ability of this steroid receptor to compete with ER in the binding to EREs and to heterodimerize with it, ERRα may play a role in the response of some genes to estrogen, thus leading to a possible overlap between ERRα and ERs transcriptional targets (15).

No endogenous ligand of ERRα has been reported to date. This observation, along with structural data showing that the putative ligand-binding pocket of ERRα is already locked in an agonist-bound conformation, led to considering ERRα as a "true orphan," constitutively active, nuclear receptor (18). However, despite the lack of natural ligands, the transcriptional activity of ERRα can be finely modulated by coregulatory "proteic ligands" such as coactivators and corepressors (19). As a matter of fact, such coregulators assume a huge importance in the modulation of orphan nuclear receptors, due to the lack of regulation by natural ligands. Indeed, the regulation of ERRα activity heavily relies on peroxisome proliferator-activated

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[2] M. Chiariello, unpublished observations.

[3] The abbreviations used are: ERα, estrogen receptor α; ERE, estrogen response element; LMB, leptomycin B; ERRE, ERR response element; SFRE, SF-1 response element; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; EGFR, epidermal growth factor receptor; TRITC, tetramethylrhodamine isothiocyanate.

receptor-γ coactivator-1α (PGC1α), a powerful coactivator that controls its role in cellular functions such as oxidative phosphorylation, mitochondrial biogenesis, and respiration (20, 21).

Evidence of ERRα roles in human malignancies, especially in breast cancer, is rapidly accumulating (22–26). Moreover, the EGF receptor and ErbB2 signaling pathways regulate ERRα transcriptional activity, leading to its hyperphosphorylation and enhancing its DNA binding (27, 28). Therefore the possibility to modulate ERRα activity is currently regarded as a valuable approach to target breast cancer as well as other aggressive human malignancies (23–25).

EXPERIMENTAL PROCEDURES

Antibodies and Western Blot Analysis—The following primary antibodies were used for Western blot experiments: HA (Covance), Lamin A/C, IκBα, ERK2, PGC1α (Santa Cruz Biotechnology), β-actin (Sigma), and ERK8 (Kinasource). HA (Santa Cruz Biotechnology) was used as the primary antibody for confocal microscopy experiments. The following primary antibodies were used for Western blot and confocal microscopy experiments: ERRα (Epitomics) and ERK8 (custom preparation). AU1 (Covance) was used as the primary antibody for Western blot, immunoprecipitation, and confocal microscopy experiments. Rabbit preimmune serum (Santa Cruz Biotechnology) was used as a negative control in immunoprecipitation experiments. The following secondary antibodies were used for Western blot experiments: HRP-conjugated anti-mouse IgG, HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology), and HRP-conjugated anti-sheep IgG (Calbiochem). For Western blot analysis, proteins derived from total lysates, immunoprecipitations, or affinity precipitations were loaded on SDS-PAGE, transferred to Immobilon-P PVDF membrane (Millipore), probed with appropriate antibodies, and revealed by enhanced chemiluminescence detection (ECL Plus; GE Healthcare). Bacterially expressed proteins were loaded on SDS-PAGE gels, stained with SimplyBlue SafeStain (Invitrogen), and revealed using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Expression Vectors—For two-hybrid screening, a DNA fragment encoding for the C-terminal domain of ERK8 from amino acids 267 to 544 was cloned in the pGBKT7 vector (Clontech). For bacterial expression of the C-terminal domain of the ERRα protein, the pGEX-4T3-ERRα_C-terminal plasmid was prepared by cloning a DNA fragment encoding for the murine ERRα C-terminal domain, specifically contained in the two-hybrid clone, in the pGEX-4T3 vector. The pCEFL HA ERK8 expression vector has been previously described (4). The pCEFL EGFP ERK8 expression vector was generated by subcloning the *ERK8* cDNA, obtained by restriction enzyme digestion from pCEFL HA ERK8, into the pCEFL EGFP vector. The pCEFL HA ERK8 3LA1, pCEFL HA ERK8 3LA2, and pCEFL HA ERK8 3LA1–2 expression vectors were generated with the QuikChange site-directed mutagenesis kit (Stratagene), using pCEFL HA ERK8 as a template. The pCEFL AU1 ERRα expression vector was generated by subcloning the *ERRα* cDNA, obtained by PCR from the pBlueScript ERRα vector (Clone ID 30336569) from the IMAGE Consortium, into the pCEFL AU1 vector. The pCEFL GST ERRα expression vector was generated by subcloning the *ERRα* cDNA, obtained by restriction enzyme digestion from pCEFL AU1 ERRα, into the pCEFL GST vector. The pCDNA3 HA JNK expression vector has been previously described (29). The pCEFL AU1 ERRβ and pCEFL AU1 ERRγ expression vectors were generated by subcloning the *ERRβ* and *ERRγ* cDNAs, obtained by PCR from pBlueScriptR ERRβ (Clone ID 30344716) and pENTR223.1 ERRγ (Clone ID 100015441) from Open Biosystems, into the pCEFL AU1 vector. The ERRE_Luc firefly luciferase reporter vector is a kind gift from J. M. Vanacker (16). The pcDNA4 PGC1α (Addgene plasmid 10974), pBABE5′ EGFR L858R (Addgene plasmid 11012), and pBABE5′ EGFR D770_N771 insNPG (Addgene plasmid 11016) expression vectors were obtained from Addgene (addgene.org). The identity and integrity of all vectors was confirmed by DNA sequencing.

Cell Culture and Transfection—HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 units/ml of penicillin-streptomycin at 37 °C in an atmosphere of 5% CO_2/air. MCF10A cells were maintained in DMEM/Ham's F-12 medium supplemented with 5% horse serum, 2 mM L-glutamine, 100 units/ml of penicillin-streptomycin, 10 ng/ml of epidermal growth factor (EGF), 0.5 μg/ml of hydrocortisone, 100 ng/ml of cholera toxin, and 10 μg/ml of insulin at 37 °C in an atmosphere of 5% CO_2/air. To generate the MCF10A cell lines stably expressing EGFP and HA ERK8, MCF10A cells were transfected with the pCEFL EGFP and pCEFL HA ERK8 expression vectors, respectively, and subjected to selection with G-418 sulfate for 2 weeks.

For Western blot and immunoprecipitation experiments, 1×10^6 cells were seeded in 6-cm plates and transfected with 1 μg of the different expression vectors using Lipofectamine LTX (Invitrogen). For luciferase assays, 1×10^5 cells were seeded in 12-well plates and transfected with 50 to 100 ng of the ERRE_Luc firefly luciferase reporter vector and 500 ng of the different expression vectors using Polyfect (Qiagen) for HeLa cells and Lipofectamine LTX (Invitrogen) for MCF10A cells. All experiments were performed 24 h after transfection. For confocal microscopy experiments, 2.5×10^4 cells were seeded on coverslips placed onto 12-well plates. For 293T cells, coverslips were pre-coated with polylysine (Sigma). Each sample was transfected with 200 to 500 ng of each plasmid using Lipofectamine LTX (Invitrogen).

Yeast Two-hybrid Screening—To isolate potential ERK8 interacting proteins, we used a region comprising its C-terminal 277 amino acids (amino acids 267–544) as a bait to perform a yeast two-hybrid screening. The cDNA corresponding to such a region was cloned in the pGBKT7 plasmid (Clontech), in-frame with the yeast GAL4 DNA-binding domain, by PCR amplification followed by enzymatic digestion. Thanks to the presence of a MYC epitope in the resulting fusion protein (MYC-GAL4-ERK8 C-term), we confirmed the expression of such a protein in yeast. Before starting the screening we also confirmed that our bait was not able to activate, by itself, the transcription of reporter genes. In addition, we confirmed that our bait could not interact directly with the GAL4 transactivation domain contained in the plasmid employed to engineer the

cDNA library used for the screening. In this regard, we used a Clontech mouse brain cDNA library (Matchmaker GAL4 two-hybrid system 3).

For the screening, we co-transformed 500 μg of the pGBKT7 ERK8_C-term bait and 1 mg of library DNA in yeast strain PJ69-4A. Protein-protein interactions were assessed by streaking transformants on selective medium lacking Leu, Trp, and His with addition of 3–10 mM 3-aminotriazole. Potential interactors were sequenced from the pACT2 plasmid by using the Matchmaker 5′ and Matchmaker 3′ primers (Clontech).

Bacterial Expression of GST Fusion Proteins—The BL21 Lys strain of *Escherichia coli* was transformed with the pGEX-4T3 vector alone or encoding for the mouse ERRα C-terminal fusion protein. Bacterially expressed GST and GST fusion protein were purified as previously described (30).

Immunofluorescence, Confocal Microscopy, Intensitometric Analysis of Fluorescence, and Count of ERRα-positive Nuclei—Twenty-four hours after transfection, cells were washed with PBS, then fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized and blocked with a solution of 0.075% saponin (Sigma) and 0.2% gelatin (Sigma) in PBS for 20 min. Cells were incubated with appropriate primary antibodies for 1 h, washed three times with PBS, incubated with appropriate Cy2-conjugated and TRITC-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories), and then washed again three times with PBS. Nuclei were stained with a 15 μM solution of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS for 3 min. Coverslips were mounted in Fluorescence Mounting Medium (Dako). Samples were visualized on a TSC SP5 confocal microscope (Leica) adapted to an inverted LEICA DMI 6000CS microscope and equipped with an oil immersion PlanApo ×63 1.4 NA objective. Images were acquired using LAS AF acquisition software (Leica). Intensitometric analysis of fluorescence was performed using the Quantitation Module of Volocity software (PerkinElmer Life Science). For the count of ERRα-positive nuclei, the total number of ERRα-positive cells and the number of cells with nuclear ERRα staining were determined in 20 random fields; the results were expressed as percentages of the ratio between ERRα-positive nuclei and ERRα-positive cells. In samples co-transfected with ERRα and ERK8, only cells expressing both proteins were considered.

Luciferase Assays—HeLa cells were transfected with 50 ng of the ERRE_Luc firefly luciferase reporter vector and 500 ng of different expression vectors (unless otherwise indicated). MCF10A cells were transfected with 100 ng of the SFRE_Luc firefly luciferase reporter vector and 500 ng of different expression vectors. Twenty-four hours after transfection, cells were lysed in Passive Lysis Buffer (Promega) and luciferase activity in the cellular lysates was assessed on a Glomax 20/20 luminometer (Promega) using the Luciferase Assay System (Promega). Results were normalized for total protein content. All luciferase results represent the normalized average ± S.D. of at least two independent transfections. All samples were read in triplicate.

Knock-down of Endogenous ERK8—*ERK8*-specific siRNA (target sequence 5′-TTGCTTGGAGGCTACTCCCAA-3′) and control non-silencing siRNA (target sequence 5′-AATTCTCCGAACGTGTCACGT-5′) were obtained from Qiagen. HeLa cells were transfected with *ERK8*-specific or control non-silencing siRNA at a final concentration of 5 nM using HiPerFect (Qiagen), according to the manufacturer's instructions. Samples were collected 48 h after transfection.

Expression Analysis of ERRα Target Genes—Total RNA was purified using TRIzol Reagent (Invitrogen). Reverse transcrip-

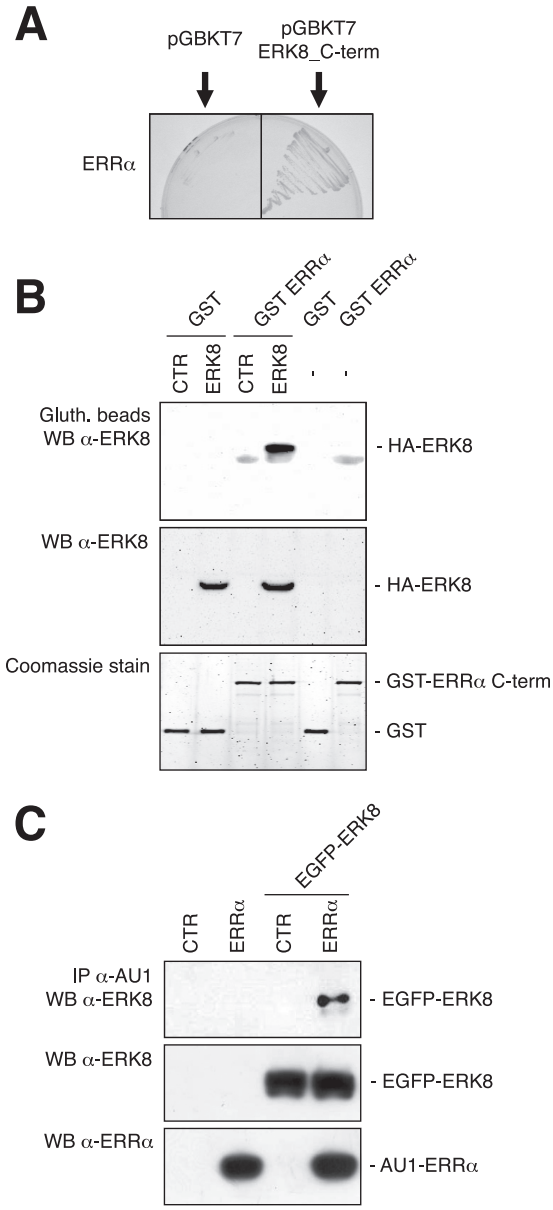
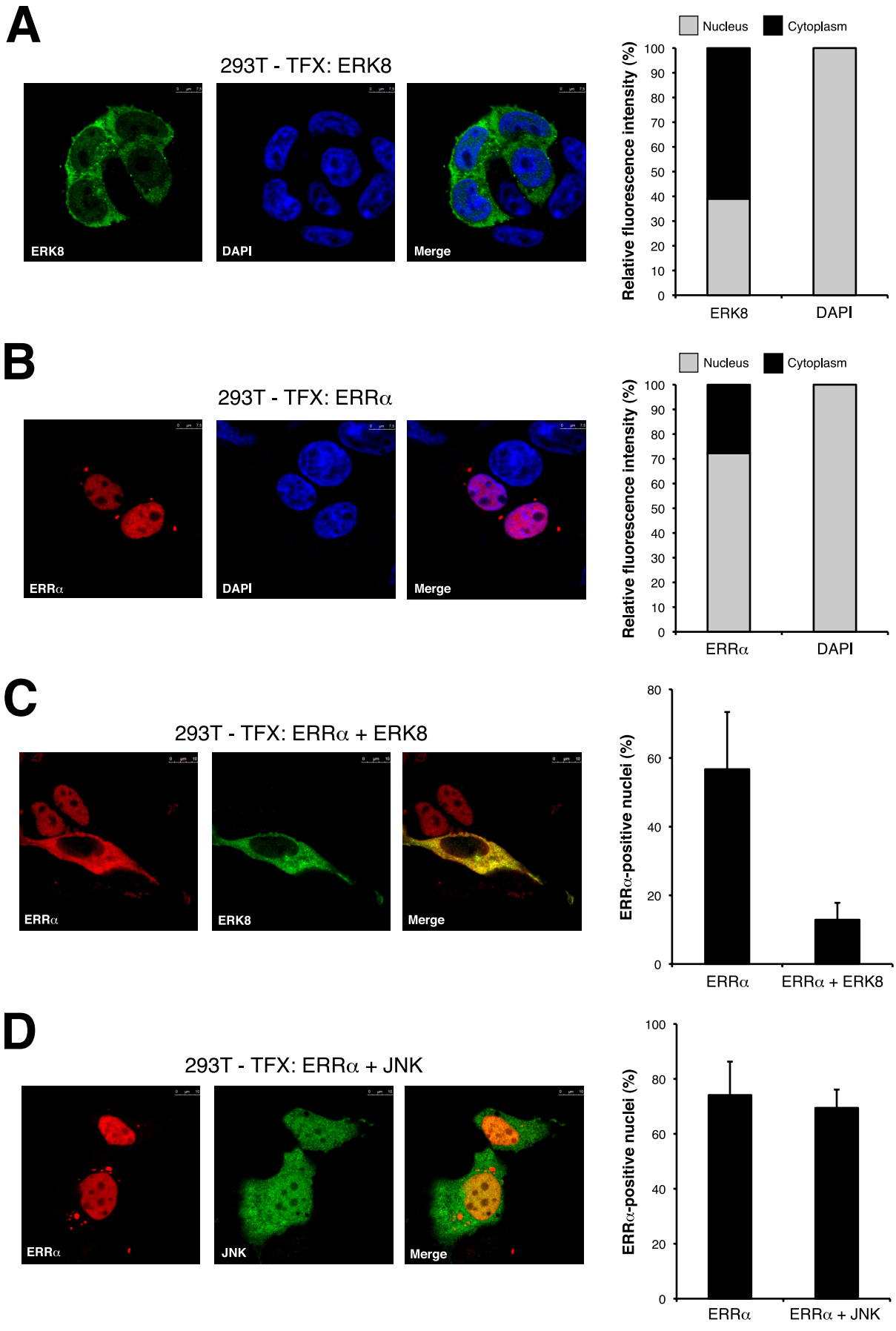


FIGURE 1. **ERK8 and ERRα interact both *in vitro* and *in vivo*.** *A*, one positive clone encoding for ERRα was tested to be devoid of autoactivation. Yeast cells were co-transformed with the pACT2 vector containing the *ERRα* cDNA, with pGBKT7 alone (*left*) or with pGBKT7 ERK8_C-term (*right*), and streaked on selective medium. *B*, *in vitro* GST pulldown. Bacterially expressed GST-tagged ERRα (*lanes 3 and 4*) or GST alone (*lanes 1 and 2*), immobilized on glutathione-Sepharose Beads 4B, were incubated for 2 h at 4 °C with lysates of 293T cells transiently transfected with a control vector or with HA-ERK8, then analyzed by Western blot (*WB*) with anti-ERK8 antibody (*top*). As additional negative controls, glutathione beads conjugated with GST alone or with GST ERRα were also loaded (*lanes 5 and 6*). 293T cellular lysates were analyzed by Western blot with anti-ERK8 antibody (*middle*) and bacterially expressed GST and GST ERRα were analyzed by Coomassie staining (*bottom*) for normalization purposes. *C*, *in vivo* GST pulldown. 293T cells were co-transfected with a control vector or with AU1-ERRα, in the presence or absence of EGFP-ERK8. Lysates (1 mg) were then immunoprecipitated with anti-AU1 antibody (4 h at 4 °C) and blotted with anti-ERK8 antibody (*top*). For normalization purposes of input samples, total cellular lysates (20 μg) were blotted with anti-ERK8 (*middle*) and anti-ERRα (*bottom*) antibodies.



tion was performed with the QuantiTect Reverse Transcription Kit (Qiagen). Real time PCR (RT-PCR) was performed with the FastStart SYBR Green Master Mix (Roche Applied Science) on a Rotor-Gene 6000 RT-PCR system (Corbett Life Science). The following primer pairs were used: *ERK8*, 5′-GGAGTTTGGGGACCATCC-3′ and 5′-GCGTTCAGGTCAGTGTCC-3′; *ERK2*, 5′-GCCCATCTTTCCAGGGAAGCATTA-3′ and 5′-AGAGCTTTGGAGTCAGCATTTGGG-3′; *CYP11A1*, 5′-CGCTTTGCCTTTGAGTCCATCACTA-3′ and 5′-TCTTGGTCCTGAACAGACGGAACA-3′; *CYP27A1*, 5′-TGGAGTGGACACGACATCCAA-3′ and 5′-ATGTGGGCAAAGTCCTTGTGCT-3′.

Subcellular Fractionation—Cytoplasmic and nuclear fractions were obtained by the Subcellular Protein Fractionation Kit (Thermo Scientific), according to the manufacturer's instructions.

RESULTS

ERK8 Interacts with ERRα in Vitro and in Vivo—We carried out a yeast-based two-hybrid screening to identify novel ERK8 interacting proteins. As ERK8 is characterized by a quite peculiar, long C-terminal domain, we decided to use this domain as bait for this screening. We chose to screen a mouse brain library, based on the evidence that ERK8 is highly expressed in the nervous system (3). Among the positive clones, we found multiple clones encoding for ERRα. One of these was confirmed to be devoid of autoactivation (Fig. 1*A*). Next, we performed pulldown experiments using full-length, HA-tagged ERK8 from a 293T cellular lysate and bacterially expressed, GST-tagged C-terminal domain of ERRα. As shown in Fig. 1*B*, the ERRα fragment corresponding to the clone identified by the two-hybrid screening readily interacted, *in vitro*, with the ERK8 protein. To determine whether ERK8 is able to interact also *in vivo* with ERRα, we performed co-immunoprecipitation experiments in 293T cells, co-transfecting EGFP-ERK8 with full-length, AU1-tagged, human ERRα. As shown in Fig. 1*C*, ERK8 evidently co-immunoprecipitated *in vivo* with ERRα. Altogether, these results indicate a physical interaction between ERK8 and ERRα.

ERK8 Promotes Re-localization of Nuclear ERRα to the Cytoplasm—To confirm the *in vivo* interaction of ERRα and ERK8, we next decided to investigate the cellular localization of these two proteins. Information about ERK8 subcellular localization is still limited. Therefore, we first sought to determine its subcellular localization in 293T cells, our experimental model. In these settings, ERK8 was mostly localized to the cytoplasm, whereas a much lower signal appeared in the nucleus (Fig. 2*A*, *left panels*). In turn, in the same model, we confirmed the already described (31, 32) predominantly nuclear localization of ERRα (Fig. 2*B*, *left panels*). To further support the aforementioned evidence and obtain quantitative information about the subcellular localization of ERK8 and ERRα, we measured fluorescence intensity in the nucleus and cytoplasm, as shown in the representative images in the *left panels* of Fig. 2, *A* and *B*, respectively. As expected, ERK8 was mainly cytoplasmic (Fig. 2*A*, *right panel*), whereas ERRα was mostly nuclear (Fig. 2*B*, *right panel*). Still, co-transfecting ERK8 and ERRα, we observed that they co-localized to the cytoplasm only in cells expressing both proteins, whereas ERRα maintained its nuclear localization in cells not expressing ERK8 (Fig. 2*C*, *left panels*). As a control, the overexpression of another MAP kinase, JNK, together with ERRα did not affect subcellular localization of this nuclear receptor (Fig. 2*D*, *left panels*). As an additional confirmation to these observations and to obtain quantitative data, we counted the number of ERRα-positive nuclei from cells expressing only ERRα or both ERRα and ERK8. ERK8 co-expression led to an ~80% reduction in the number of cells with ERRα-positive nuclei (Fig. 2*C*, *right panel*). As a control, no decrease in the number of cells with ERRα-positive nuclei occurred following JNK co-expression (Fig. 2*D*, *right panel*).

ERRα, ERRβ, and ERRγ belong to the same subfamily of nuclear receptors and share several features, such as the lack of an endogenous ligand and the ability to bind the same DNA consensus sequence (33). Nevertheless, co-expression with ERK8 did not interfere with nuclear localization of ERRβ and ERRγ, showing a clear difference in their behavior compared with ERRα (supplemental Fig. S1*A*). These observations therefore confirm our data showing a direct interaction between ERRα and ERK8 and strongly suggest a specific role for ERK8 in selectively determining the subcellular localization of ERRα.

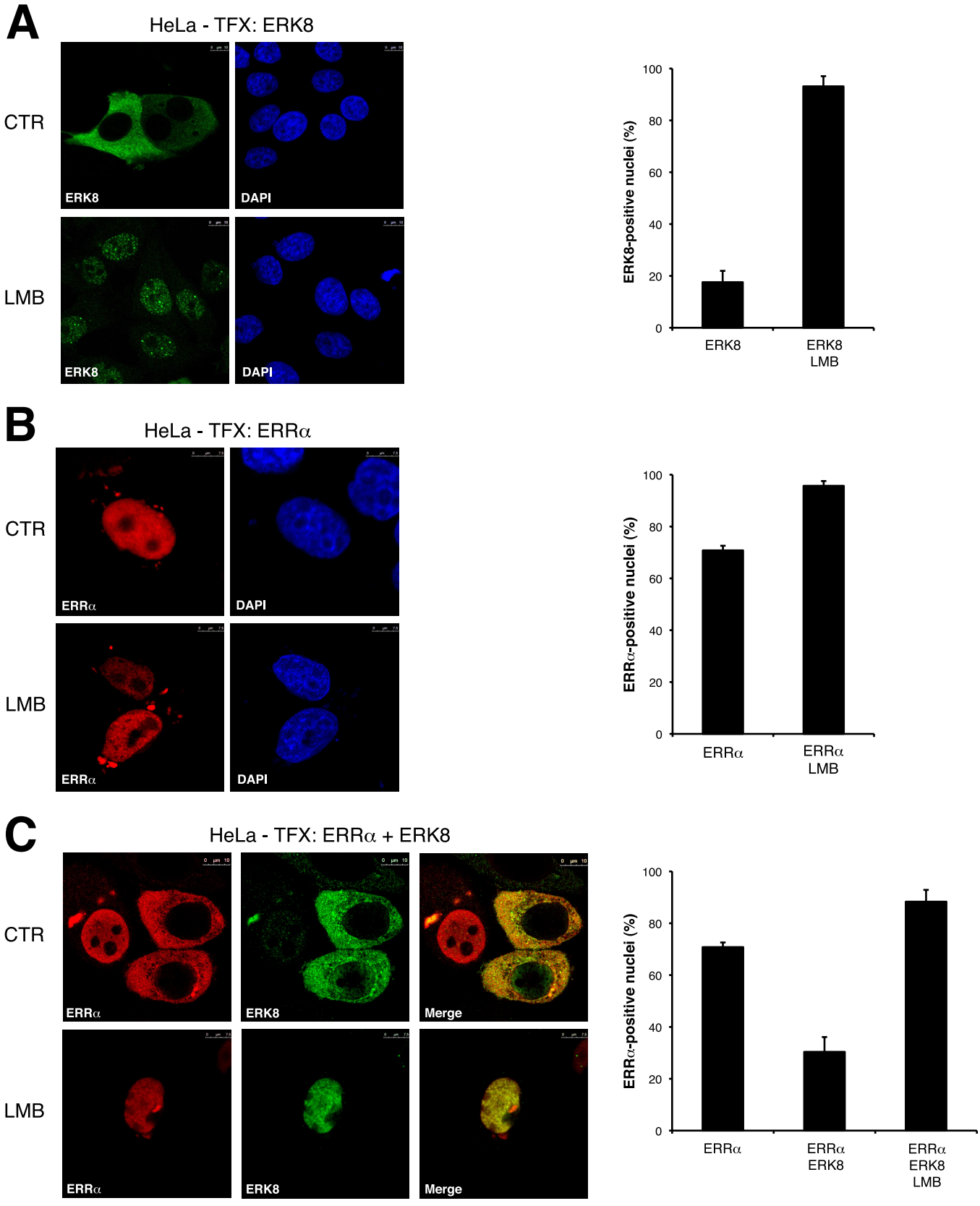
ERK8 Re-localizes Nuclear ERRα to the Cytoplasm with a CRM1-dependent Mechanism—The antifungal compound leptomycin B (LMB) specifically blocks the nuclear export of proteins by preventing their association with the CRM1 export receptor (34). Indeed, the use of LMB already implicated this karyopherin in the export of different nuclear receptors. One such example is ERα, whose nuclear export is inhibited by LMB in breast cancer cell lines (35, 36).

As no information is yet available about ERK8 nucleocytoplasmic transport, we first determined the behavior of this protein upon LMB treatment in HeLa cells, often used to investigate CRM1 activity. As described in Fig. 3*A*, *left*

FIGURE 2. **ERK8 promotes re-localization of ERRα to the cytoplasm.** *A, left panels*, confocal microscopy images showing the localization of ERK8, in 293T cells. Cells were transfected with HA-ERK8 (300 ng), incubated with anti-ERK8 antibody, and labeled with Cy2-conjugated secondary antibody. *A, right panel*, intensitometric analysis of ERK8 and DAPI fluorescence in the nuclear and cytoplasmic compartments of 293T cells; analysis was performed on the representative images in *A, left panels*. *B, left panels*, confocal microscopy images showing the localization of ERRα in 293T cells. Cells were transfected with AU1-ERRα (300 ng), incubated with anti-AU1 antibody, and labeled with TRITC-conjugated secondary antibody. *B, right panel*, intensitometric analysis of ERRα and DAPI fluorescence in the nuclear and cytoplasmic compartments of 293T cells; analysis was performed on the representative images in *B, left panels*. *C, left panels*, confocal microscopy images of 293T cells co-transfected with AU1-ERRα (300 ng) and HA-ERK8 (300 ng), then incubated with anti-AU1 and anti-ERK8 antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies. *C, right panel*, decrease in the number of 293T cells with AU1-ERRα-positive nuclei in the presence of HA-ERK8. 293T cells were transfected and labeled as in *C, left panels*, then cells with AU1-ERRα-positive nuclei were counted in 20 random fields. *D, left panels*, confocal microscopy images of 293T cells co-transfected with AU1-ERRα (300 ng) and HA-JNK (300 ng), then incubated with anti-ERRα and anti-HA antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies. *D, right panel*, the number of 293T cells with AU1-ERRα-positive nuclei is not affected by the presence of HA-JNK. 293T cells were transfected and labeled as in *D, left panels*, then cells with AU1-ERRα-positive nuclei were counted in 20 random fields. In all experiments involving the count of HA-ERK8- and AU1-ERRα-positive nuclei, each *bar* represents the average ± S.D. of three independent experiments counted by different operators. *TFX*, transfection.

panels, LMB induced nuclear compartmentalization of the otherwise predominantly cytoplasmic ERK8 protein, suggesting the existence of an active, CRM1-dependent mechanism of ERK8 nuclear export. As expected, LMB did not perturb the already nuclear localization of ERRα (Fig. 3*B*, *left panels*).

We next wished to determine whether CRM1 mediates ERK8-dependent nuclear export of ERRα. As depicted in Fig.



3*C*, *left panels*, *upper row*, ERRα was predominantly localized in the nucleus in HeLa cells not expressing ERK8, whereas cells co-expressing this MAP kinase showed ERRα localization to the cytoplasm, in line with our previous observations in 293T cells. However, treating HeLa cells with LMB, ERRα localization was completely restrained to the nucleus, despite ERK8 co-expression (Fig. 3*C*, *left panels*, *lower row*), definitively suggesting CRM1 involvement in ERRα nuclear exclusion induced by ERK8. As an additional confirmation to these observations and to obtain quantitative data, we counted the number of ERK8-positive nuclei from cells expressing only ERK8 (Fig. 3*A*, *right panel*), of ERRα-positive nuclei from cells expressing only ERRα (Fig. 3*B*, *right panel*) and of ERRα-positive nuclei from cells expressing both ERRα and ERK8 (Fig. 3*C*, *right panel*), in the presence or absence of LMB. As expected, in all cases LMB treatment led to an almost complete re-localization of both ERK8 and ERRα to the nucleus. Altogether, these data therefore allow us to conclude that ERK8 re-localizes ERRα to the cytoplasm through a CRM1-dependent mechanism.

ERK8 Inhibits ERRα Transcriptional Activity—Based on the previously described data and on ERK8 ability to control the activity of different nuclear receptors (9, 10), we next asked whether this MAP kinase was able to modulate ERRα transcriptional activity. It is known that ERRα and ERα can interact *in vitro* and induce transcription through both the classical ERE and the ERRα response element (ERRE/SFRE) (15, 33). To avoid potential biases due to cross-talk between ERRα and ERα, we therefore decided to use the ERα-negative HeLa cells, a typical model system used for functional studies on ERRα transcriptional activity (37, 38).

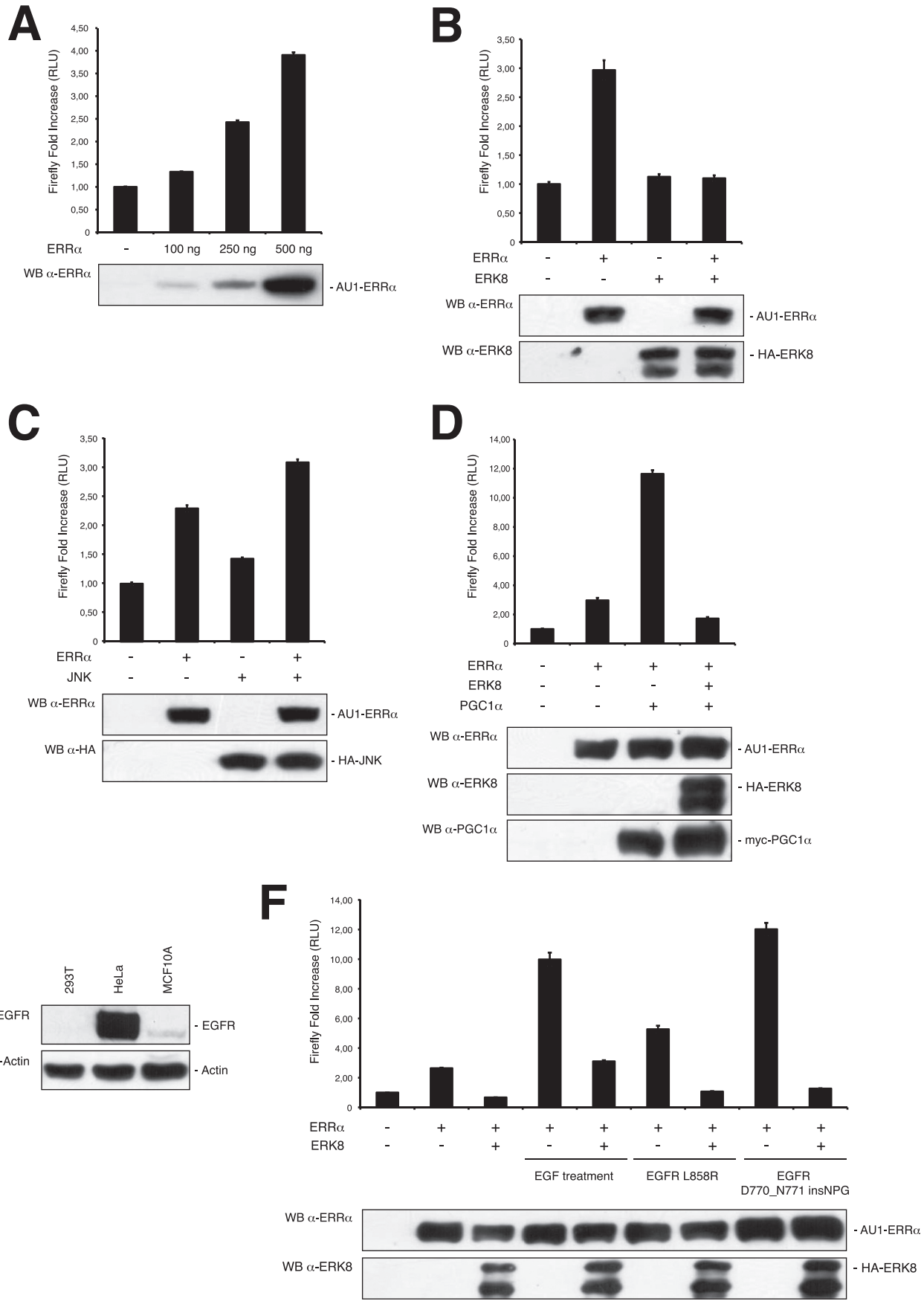
To investigate the transcriptional activity of this nuclear receptor, we used a firefly luciferase reporter vector, ERRE_Luc, in which the luciferase gene is under control of a minimal promoter harboring three ERRE/SFRE repeats (16). As expected (16), the activity of the reporter was dependent on ERRα expression (Fig. 4*A*). Next, we studied the effect of ERK8 on ERRα transcriptional activity by co-transfecting, together with the ERRE_Luc reporter, an ERRα expression vector alone or in combination with the ERK8 expression vector. Luciferase activity increased ~3-fold in the presence of ERRα but, remarkably, it returned to basal levels when ERRα and ERK8 were co-expressed (Fig. 4*B*), therefore indicating a corepressive role for ERK8 on ERRα transcriptional activity. As a control, the expression of ERK8 alone had no effect on the reporter (Fig. 4*B*). Furthermore, overexpression of other MAP kinases such as JNK could not counteract ERRα-dependent induction of the ERRE_Luc reporter (Fig. 4*C*). Although ERRβ was a very poor inducer of the reporter (39), ERRβ and ERRγ activities were not affected by ERK8 in HeLa cells (supplemental Fig. S1*B*), confirming the specificity of the ERK8 effect on ERRα.

Due to the lack of a natural agonist, the induction of ERRα transcriptional activity heavily relies on "proteic coactivators," the best characterized of which is PGC1α (40). Therefore, we sought to determine whether ERK8 could counteract PGC1α-dependent ERRα activation as well. Fig. 4*D* clearly shows that, despite the remarkable induction of ERRα transcriptional activity by PGC1α, ERK8 was still able to inhibit such activation.

ERRα is phosphorylated *in vivo* on multiple sites (27, 28). It has been recently reported that some of these phosphorylation events are responsive to epidermal growth factor receptor (EGFR) stimulation and are able to enhance ERRα binding to DNA and, therefore, its transcriptional activity (28). As HeLa cells express high EGFR levels (Fig. 4*E*), we tested the ability of ERK8 to interfere with the activity of ERRα induced by the EGF signaling pathway in this experimental model. As shown in Fig. 4*F*, ERK8 retained its corepressive function on ERRα even when this nuclear receptor was activated by EGF. Similarly, ERRα stimulation induced by two human-activated EGFR oncogenic mutants (EGFR L858R and EGFR D770_N771 insNPG) (41) was also completely abolished by ERK8 expression (Fig. 4*F*). Altogether, our data therefore demonstrate that ERK8 is a *bona fide* ERRα corepressor, exerting such function in a highly specific manner on ERRα, both on its basal activity and in situations in which this nuclear receptor is induced by proteic coactivators, such as PGC1α, or by well characterized stimuli, such as activation of the EGF signaling pathway.

ERK8 Modulates the Expression of ERRα Target Genes—Based on evidence that ERK8 inhibits ERRα transcriptional activity in a luciferase reporter system, we sought to study the ability of ERK8 to control the endogenous expression of ERRα transcriptional targets as well. To this purpose, ERK8 expression was silenced in HeLa cells using a validated (5) siRNA to score, by real time PCR, the effects of ERK8 depletion on the expression levels of two well established ERRα target genes, *CYP11A1* and *CYP27A1* (42). In these conditions, whereas *ERK8* mRNA levels underwent a 50% reduction (Fig. 5*A*) we observed a ~2- and ~2.5-fold increase in *CYP11A1* (Fig. 5*B*) and *CYP27A1* mRNA expression (Fig. 5*C*), respectively. As a control, *ERK8* mRNA depletion had no effect on the expression of a gene whose expression does not depend on ERRα, namely *ERK2* (Fig. 5*D*). These data therefore support our previous

FIGURE 3. **CRM1-dependent cytoplasmic localization of ERRα induced by ERK8, in HeLa cells.** *A*, *left panels*, confocal microscopy images of cells transfected with HA-ERK8 (300 ng), left untreated (*top row*), or treated with 5 nM LMB for 24 h (*bottom row*). Cells were incubated with anti-ERK8 antibody and labeled with Cy2-conjugated secondary antibody and DAPI for nuclear staining. *A, right panel*, increase in the number of 293T cells with HA-ERK8-positive nuclei after treatment with LMB. 293T cells were transfected, treated, and labeled as in *A, left panels*, then cells with HA-ERK8-positive nuclei were counted in 20 random fields. *B, left panels*, confocal microscopy images of cells transfected with AU1-ERRα (300 ng), left untreated (*top row*), or treated with 5 nM LMB for 24 h (*bottom row*). Cells were incubated with anti-AU1 antibody and labeled with TRITC-conjugated secondary antibody and DAPI for nuclear staining. *B, right panel*, increase in the number of 293T cells with AU1-ERRα-positive nuclei after treatment with LMB. 293T cells were transfected, treated, and labeled as in *B, left panels*, then cells with AU1-ERRα-positive nuclei were counted in 20 random fields. *C, left panels*, confocal microscopy images of cells co-transfected with AU1-ERRα (300 ng) and HA-ERK8 (300 ng), left untreated (*top row*), or treated with 5 nM LMB for 24 h (*bottom row*). Cells were incubated with anti-AU1 and anti-ERK8 antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies. *C, right panel*, increase in the number of ERK8-transfected 293T cells with AU1-ERRα-positive nuclei after treatment with LMB. 293T cells were transfected, treated, and labeled as in *C, left panels*, then cells with AU1-ERRα-positive nuclei were counted in 20 random fields. In all experiments involving the count of HA-ERK8- and AU1-ERRα-positive nuclei, each *bar* represents the average ± S.D. of three independent experiments counted by different operators. *TFX*, transfection.



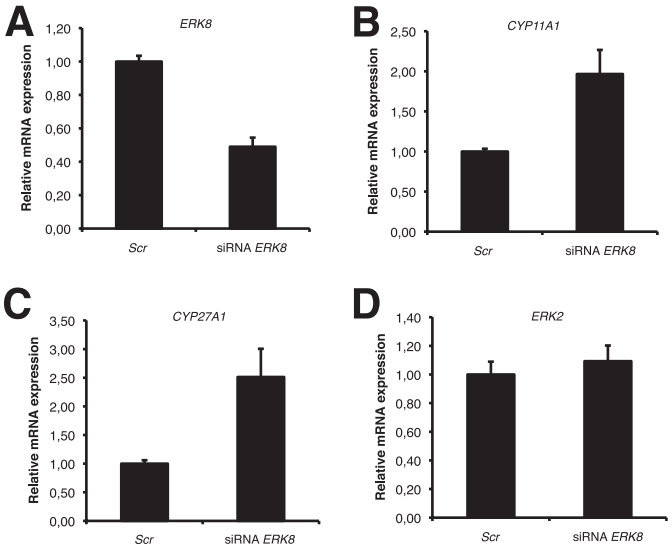


FIGURE 5. **Effect of ERK8 knockdown on the expression of ERRα target genes.** Expression levels of *ERK8* (*A*), *CYP11A1* (*B*), *CYP27A1* (*C*), and *ERK2* (*D*) mRNAs 48 h after scramble (*scr*) or *ERK8*-specific siRNA transfection.

observations and confirm the role of ERK8 in modulating ERRα transcriptional activity, *in vivo*.

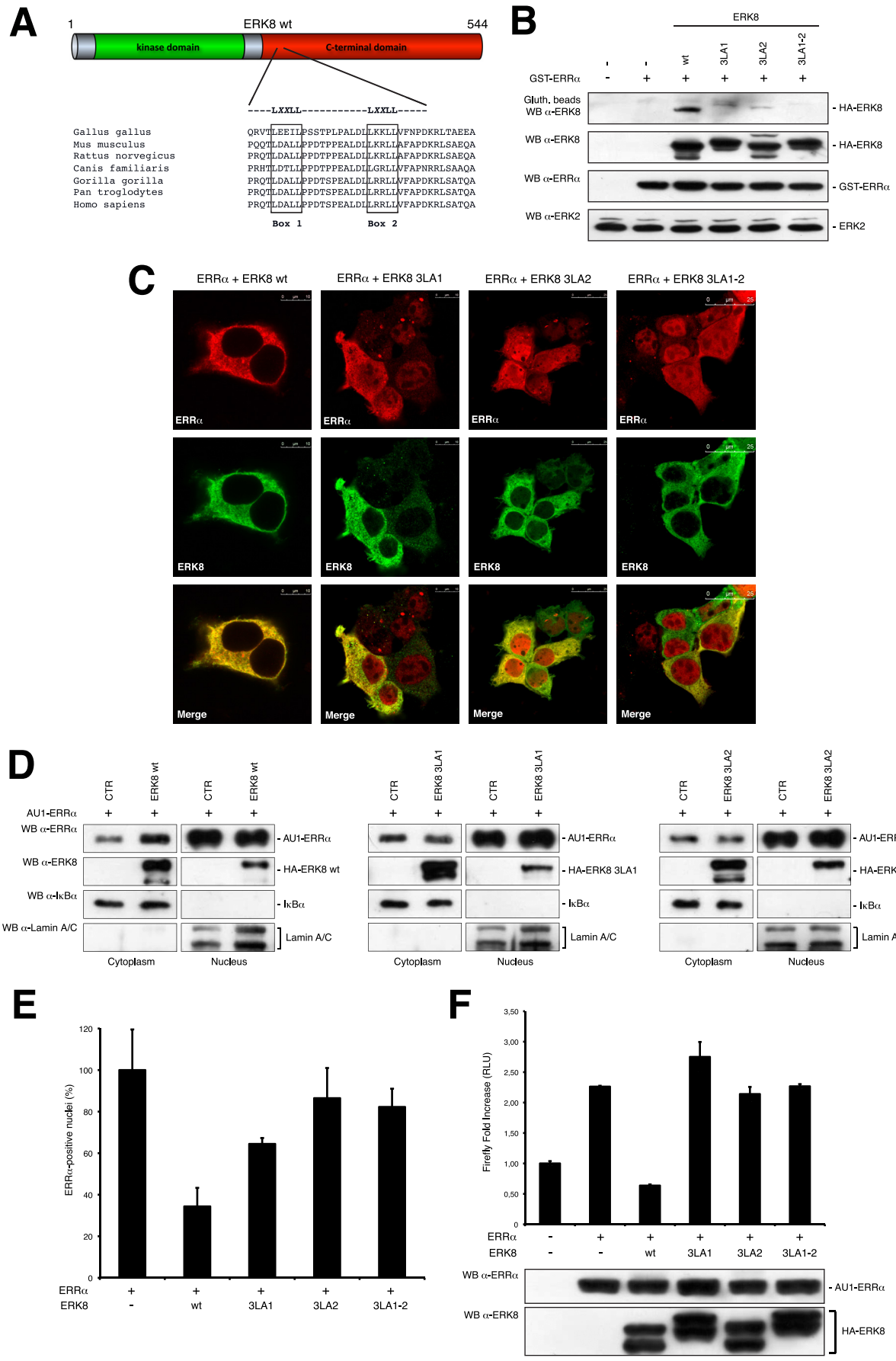
ERK8 Binds to ERRα through LXXLL Motifs Typical of Agonist-bound Nuclear Receptor Corepressors—Interactions of nuclear receptors with coregulatory proteins are mediated by conserved motifs, well defined for both coactivators and corepressors. Classical corepressors, which prevent unliganded nuclear receptors from being activated by ligands, are characterized by the "corepressor nuclear receptor" (CoRNR) box, typically having a sequence of (L/V)*XX*(I/V)I or alternatively L*XXX*(I/L)*XXX*(I/L) (where L is leucine, V is valine, I is isoleucine, and *X* is any amino acid) (43). Ligand binding favors the displacement of this kind of corepressors and the recruitment of coactivators, characterized instead by the L*XX*LL box (44). Still, a different family of corepressors that are able to inhibit active, ligand-bound, nuclear receptors through an L*XX*LL box, hence named "agonist-bound nuclear receptor corepressors," has been recently described (45).

In silico analysis of the ERK8 protein sequence led us to identify two putative L*XX*LL boxes starting at amino acids 265 and 281, respectively (referred to as L*XX*LL Box 1 and L*XX*LL Box 2 herein), in the C-terminal domain of ERK8. Of note, the two putative L*XX*LL boxes appeared to be very well conserved among different species (Fig. 6*A*), the conservation of this particular structural motif being suggestive of its importance in the function of ERK8, otherwise exhibiting very low homology with its orthologs, especially in its C-terminal domain (3). Hence, to investigate whether the two L*XX*LL boxes are involved in the interaction with ERRα, we generated ERK8 mutants in which we substituted leucine residues with alanines in L*XX*LL Box 1 (ERK8 3LA1 mutant), in L*XX*LL Box 2 (ERK8 3LA2), and in both (ERK8 3LA1–2), respectively. As shown in Fig. 6*B*, interaction of these mutants with ERRα was barely detectable. Next, based on the ability of ERK8 to prevent nuclear localization of ERRα (see above), we wanted to determine whether the L*XX*LL mutants, whose interaction with ERRα is hindered, could still affect the subcellular localization of this nuclear receptor. As shown in Fig. 6*C*, the ERK8 L*XX*LL mutants, whereas showing the same localization of wild-type ERK8 (*middle row*), lost the ability to re-localize ERRα to the cytoplasm. Biochemical fractionation in Fig. 6*D* also confirmed the increase of ERRα in the cytoplasm in the presence of wild-type ERK8 as well as the inability of the ERK8 L*XX*LL mutants to efficiently promote the nuclear export of ERRα. Accordingly, 293T cells transfected with the nuclear receptor showed a more modest decrease in the number of ERRα-positive nuclei when co-expressing the ERK8 L*XX*LL mutants, compared with cells co-expressing the wild-type ERK8 (Fig. 6*E*). Next, to ascertain whether the interaction between these two proteins is necessary for ERK8 corepressive function, we tested the ERK8 L*XX*LL mutants for their ability to inhibit ERRα transcriptional activity. We therefore co-transfected the ERRE_Luc reporter vector along with ERRα and either wild-type ERK8 or the different mutants. Fig. 6*F* clearly shows that all ERK8 mutants lost the ability to repress ERRα transcriptional activity, thus supporting the conclusion that the L*XX*LL boxes are essential for ERK8 corepressive function.

Altogether, our data identify two L*XX*LL motifs contained in the ERK8 C-terminal domain as necessary features for ERK8 to interact with ERRα, to regulate its subcellular localization and to inhibit its transcriptional activity. Based on previous data indicating ERRα as a constitutively active nuclear receptor (18), such features also suggest ERK8 as a new member of the family of agonist-bound nuclear receptor corepressors, unique in its ability to control the functions of orphan members of this family of proteins, whose structure is already locked in a transcriptionally active conformation, even in the absence of a natural ligand.

ERK8 Negatively Regulates Endogenous ERRα Activity and Controls Its Cellular Localization, in Human Breast Epithelial Cells—Next, we decided to evaluate the effect of ERK8 on endogenous ERRα, in a biologically relevant cellular system, the human immortalized mammary epithelial MCF10A cell line. These cells are in fact often used to recapitulate differentiation and transformation of the human breast epithelium (46, 47), a tissue whose malignant proliferation is indeed affected by ERRα

FIGURE 4. **Effect of ERK8 on ERRα transcriptional activity in HeLa cells.** *A*, activation of ERRE_Luc luciferase reporter vector after transfection with increasing concentrations of AU1-ERRα expression vector, as indicated; 20 μl of the same cellular lysate was subjected to Western blot (*WB*) with anti-ERRα antibody. *B*, AU1-ERRα-induced ERRE_Luc luciferase reporter activity is inhibited by HA-ERK8; 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies. *C*, AU1-ERRα-induced ERRE_Luc luciferase reporter activity is not inhibited by HA-JNK; 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα and anti-HA antibodies. *D*, HA-ERK8 inhibits AU1-ERRα-induced ERRE_Luc luciferase reporter activity following stimulation of AU1-ERRα with myc-tagged PGC1α; 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα, anti-ERK8, and anti-PGC1α antibodies. *E*, expression levels of endogenous EGFR. Cellular lysates (30 μg) from 293T, HeLa, and MCF10A cells were subjected to SDS-PAGE followed by Western blot with anti-EGFR and anti-Actin antibodies. *F*, HA-ERK8 inhibits AU1-ERRα-induced ERRE_Luc luciferase reporter activity following stimulation of AU1-ERRα with EGF (100 μg/ml, 4 h) or transfection with two constitutively active mutants of EGFR (EGFR L858R and EGFR D770_N771 insNPG); 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies. *RLU*, relative light units.



(22, 23, 26). Importantly, MCF10A cells express high levels of ERRα but no ERα (Fig. 7*A* and Ref. 9), allowing us to avoid possible biases due to cross-talk between ERRα and ERα (see above). Therefore, we first confirmed in these mammary epithelial cells our findings obtained in 293T and HeLa, by showing that ERK8 induced the cytoplasmic re-localization (Fig. 7*B*) and inhibited the transcriptional activity (Fig. 7*C*) of ectopically expressed ERRα. Next, to determine whether our reporter assay was able to detect the signal generated by the endogenous ERRα, we treated ERRE_Luc-transfected MCF10A cells with the ERRα-specific inverse agonist XCT790. As a result of drug action on both ERRα transactivation potential and protein levels (25), the activity of the luciferase reporter underwent a marked dose-dependent decrease, thus indicating that the basal ERRE_Luc luciferase signal observed in MCF10A is mostly due to endogenous ERRα (Fig. 7*D*). Based on this information, we observed a highly significant decrease in the ERRE_Luc reporter activity in MCF10A cells transfected with ERK8, indicating that ERK8 can indeed inhibit the transcriptional activity of endogenous ERRα (Fig. 7*E*). Next, based on the ability of ERK8 to prevent nuclear localization of exogenously expressed ERRα, we wanted to determine whether this MAP kinase could also affect the subcellular localization of the endogenous nuclear receptor. Indeed, as shown in Fig. 7*F*, only MCF10A cells expressing ERK8 showed cytoplasmic localization of the ERRα protein. In line with this observation, cell fractionation experiments showed a clear increase of ERRα levels in the cytoplasmic fraction of MCF10A cells stably expressing the ERK8 protein (Fig. 7*G*, *left panels*). As MCF10A cells also express endogenous ERK8 (8, 9), we next confirmed the interaction between endogenous ERK8 and ERRα proteins. Indeed, in line with our results on ectopically expressed proteins (Figs. 1*C* and 6*B*), endogenous ERK8 evidently co-immunoprecipitated *in vivo* with endogenous ERRα (Fig. 7*H*). Ultimately, we set up to evaluate the ability of ERK8 to counteract activation of endogenous ERRα by stimuli relevant for breast cancer, *e.g.* activation of the EGFR pathway (48). To this aim, as MCF10A cells express very low levels of EGFR (see Fig. 4*E*), we decided to overexpress in these cells the constitutively active EGFR mutants, EGFR L858R and EGFR D770_N771 insNPG, which, although typically found in non-small cells lung carcinomas (41), well recapitulate activation of the EGFR pathway in human cancer. In agreement with our findings in HeLa cells (see Fig. 4*F*), activation of the EGFR signaling pathway in MCF10A cells increased the activity of endogenous ERRα, whereas ERK8 retained its repressory effect even on the endogenous nuclear receptor stimulated by EGFR-derived activated oncogenes (Fig. 7*I*). These results therefore propose ERK8 as a corepressor of endogenous ERRα, with the ability to promote its cytoplasmic localization and counteract its activation by positive cellular stimuli often deregulated in human cancers.

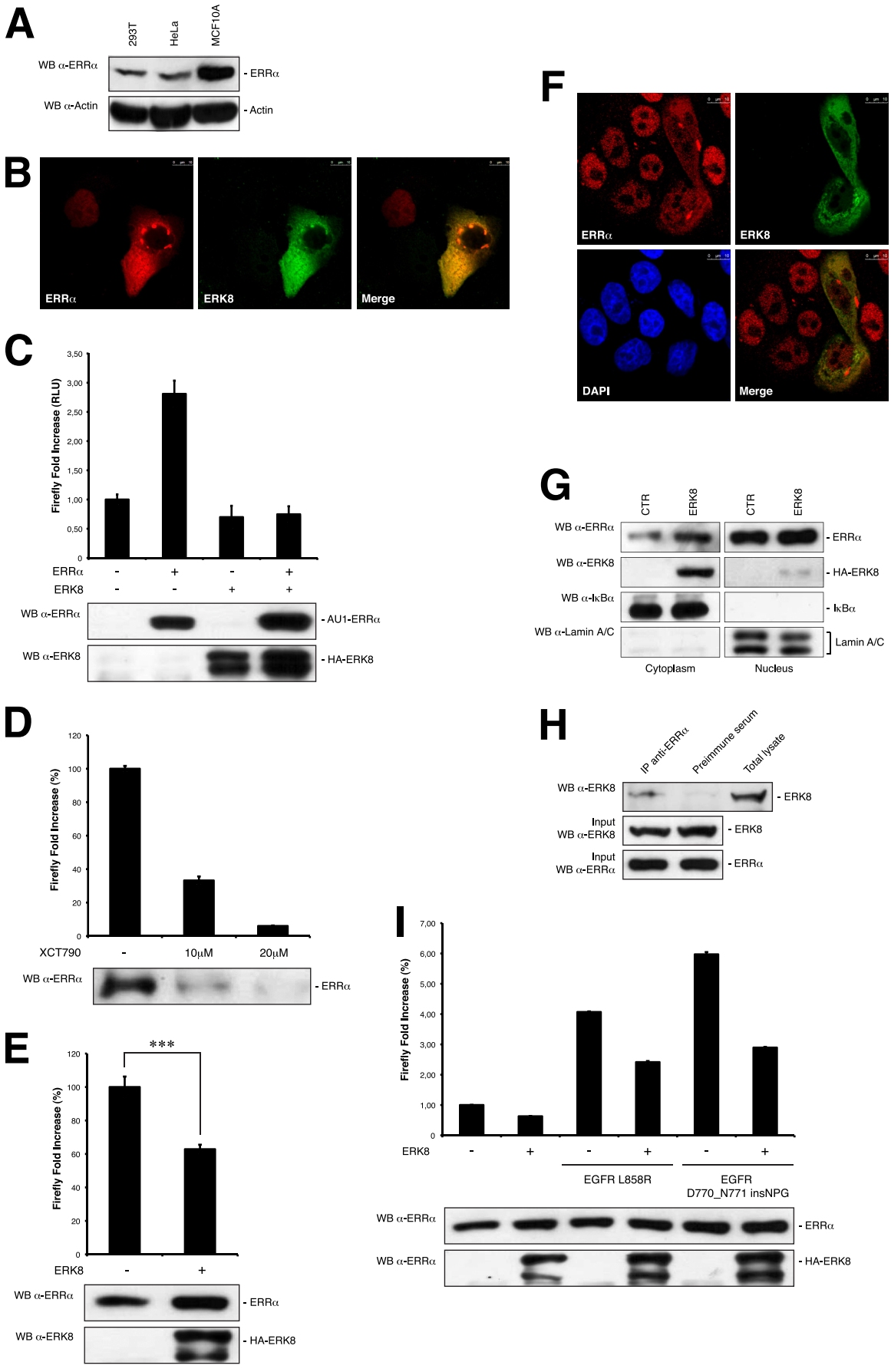
DISCUSSION

In this paper we demonstrate that ERK8, a member of the MAP kinase family of proteins, is a *bona fide* ERRα corepressor, able to modulate the subcellular localization and activity of this nuclear receptor. Indeed, our data support a model in which ERK8 binds to ERRα, inhibits both its basal and stimulated (by PGC1α and the EGFR pathway) activity, and induces its localization to the cytoplasm (Fig. 8).

Recent work has already revealed that ERK8 negatively regulates the activity of androgen and glucocorticoid receptors by interacting with the HIC-5 coactivator (10), and that the product of its murine ortholog gene enhances the degradation of ERα (9). However, differently from what was demonstrated for these nuclear receptors, we show a direct interaction between ERK8 and ERRα. Also, unlike ERα, we observed no decrease in the protein levels of ERRα in the presence of ERK8. Therefore, it appears that ERK8 is a multifunctional corepressor for different nuclear receptors. Still, its corepressive activity is certainly not aspecific, as we demonstrate that it is able to discriminate among very similar proteins such as different members of the ERR subfamily. In this context, it is worth noting that ERK8 is able to influence the expression of ERRα target genes such as *CYP11A1* and *CYP27A1* (42). Interestingly, *CYP11A1* encodes for the cholesterol side chain cleavage enzyme (P450scc), which mediates the initial and rate-limiting step in steroidogenesis, converting cholesterol to pregnenolone, the precursor of androgens, estrogens, and progesterone, and which has been heavily associated to prostate (49), breast (50), and endometrial (51) cancer risk. On the other hand, *CYP27A1* encodes for sterol 27-hydroxylase, a cytochrome P450 family member implicated in the metabolism of vitamin D. Overall, the presence of ERK8 may therefore coordinately modulate, by multiple and different mechanisms, the activity of several nuclear receptors, at multiple levels, possibly in response to specific cellular stimuli.

Coregulatory proteins are key modulators of nuclear receptor-dependent transcription of target genes (52). In nuclear receptors, in fact, helix 12 (also referred to as activation func-

FIGURE 6. **The L*XX*LL motifs of ERK8 are necessary for its ability to control ERRα cellular localization and its corepressor activity.** *A*, schematic representation of the ERK8 protein, showing the position of the two identified L*XX*LL motifs, and sequence alignment of the region of ERK8 flanking the two L*XX*LL motifs (*boxed*) in various species. *B*, *in vivo* GST pulldown to test the interaction of ERRα with the ERK8 L*XX*LL mutants. 293T cells were co-transfected with GST-ERRα (1 μg) and HA-ERK8 (1 μg) or ERK8 mutants (1 μg) on the first (*3LA1*), second (*3LA2*), or both L*XX*LL motifs (*3LA1–2*). Cellular lysates were pulled down with glutathione-Sepharose beads 4B (3 h at 4 °C) and analyzed by Western blot (*WB*) with anti-ERK8 antibody (*top*). 293T total cellular lysates were blotted with anti-ERK8 (*middle top*), anti-ERRα (*middle bottom*), and anti-ERK2 (*bottom*) antibodies for normalization purposes. *C*, confocal microscopy images of 293T cells co-transfected with AU1-ERRα and HA-ERK8 (*left*), HA-ERK8 3LA1 (*middle left*), HA-ERK8 3LA2 (*middle right*), or HA-ERK8 3LA1–2 (*right*). Cells were incubated with anti-AU1 and anti-ERK8 antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies. *D*, HA-ERK8 expression (*left*), but not HA-ERK8 3LA1 (*middle*) or HA-ERK8 3LA2 expression (*right*), increases the amount of AU1-ERRα in the cytoplasmic fraction of HeLa cells. HeLa transfected with AU1-ERRα along with EGFP, HA-ERK8, HA-ERK8 3LA1, or HA-ERK8 3LA2 (1×10^6 cells) were subjected to fractionation using the Subcellular Protein Fractionation Kit (Thermo Scientific); lysates were subjected to SDS-PAGE followed by Western blot with anti-ERRα (*top*), anti-ERK8 (*middle top*), anti-IκBα (*middle bottom*), and anti-Lamin A/C (*bottom*) antibodies. *E*, decrease in the number of 293T cells with AU1-ERRα-positive nuclei in the presence of HA-ERK8, but not in the presence of the ERK8 L*XX*LL mutants. Cells were transfected and labeled as in *C*, then cells with AU1-ERRα-positive nuclei were counted in 20 random fields; each *bar* represents the average ± S.D. of three independent experiments counted by different operators. *F*, ERK8 L*XX*LL mutants fail to inhibit AU1-ERRα-induced ERRE_Luc luciferase reporter activity; 20 μl of the same cellular lysate used for the luciferase assay was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies.



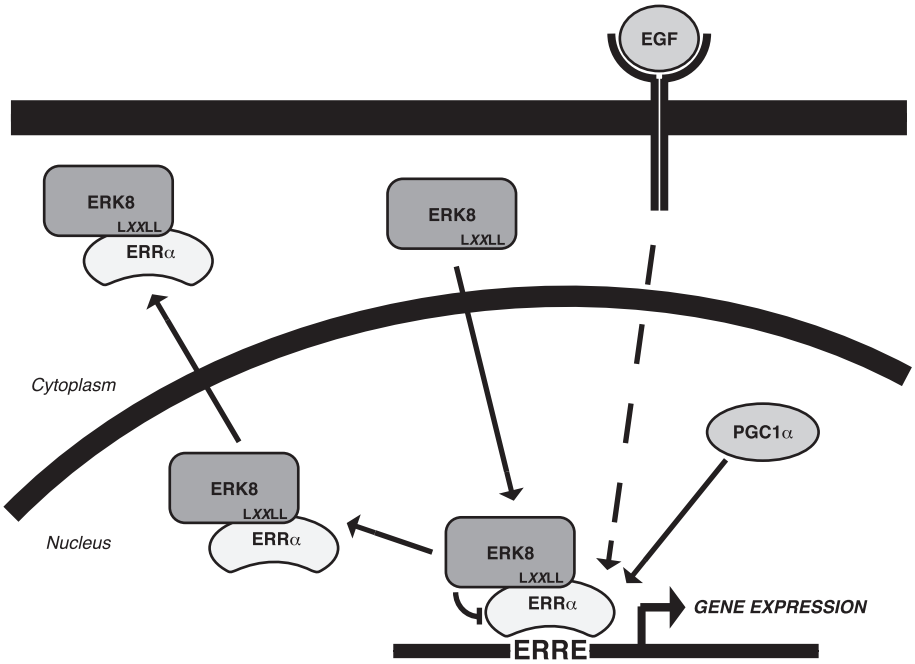


FIGURE 8. **Mechanism of ERK8 action on ERRα.** ERRα is a constitutively active orphan nuclear receptor whose activity can be further increased by proteic coactivators (*PGC1α*) (40) or EGFR-dependent signaling (27, 28). ERK8 acts as a *bona fide* ERRα-specific corepressor interacting with the nuclear receptor through its L*XX*LL motifs and promoting its re-localization from the nucleus to the cytoplasm.

tion 2) is critical in determining which kind of coregulator docks. Inactive nuclear receptors, unbound to ligands, have helix 12/activation function 2 in a conformation that provides access to corepressors interacting via the so-called corepressor nuclear receptor box (52), typically having a sequence of (L/V)*XX*(I/V)I or L*XXX*(I/L)*XXX*(I/L) (53). Upon agonist binding, helix 12/activation function 2 is repositioned in a conformation that no longer allows interaction of the corepressor nuclear receptor box with the corepressor-binding area, although favoring the interaction with coactivator boxes, typically having an L*XX*LL sequence (52). Therefore, typical corepressors lack the structural means to repress agonist-bound, active nuclear receptors. This model was initially challenged by the discovery of receptor interacting protein 140 (RIP140), which has the structural features of a coactivator, and included L*XX*LL sequences determining the interaction with the receptors, but acts as a corepressor (45). The discovery of a few more such coregulators gave rise to a small family of agonist-dependent corepressors whose interaction with activated nuclear receptors depends on L*XX*LL boxes (54). Moreover, ERRα, even if not bound to any ligand, is constitutively active and arranged in a structure typical of ligand-bound receptors (18). It is therefore not surprising for us to find two functional L*XX*LL motifs in ERK8 mediating its corepressive functions on ERRα. Together, these data led us to the conclusion that ERK8 is the first representative of a new class of corepressors that are able to control, through L*XX*LL boxes, the functions of orphan nuclear receptors whose structure is already locked in a transcriptionally active conformation, even in the absence of a natural ligand. In this context it is important to note that the two L*XX*LL boxes of ERK8 are perfectly conserved in mammals and birds, five of the six leucines are conserved in zebrafish, and the L*XX*LL box 2 is perfectly conserved down to *Drosophila melanogaster* (data not shown). Such remarkable conservation

FIGURE 7. **ERK8-dependent cytoplasmic re-localization of endogenous ERRα and inhibition of its transcriptional activity, in MCF10A cells.** *A*, expression levels of endogenous ERRα in our experimental cellular models. Total cellular lysates (30 μg) from 293T, HeLa, and MCF10A cells were subjected to SDS-PAGE followed by Western blot (*WB*) with anti-ERRα and anti-Actin antibodies. *B*, confocal microscopy images of MCF10A cells co-transfected with AU1-ERRα (500 ng) and HA-ERK8 (1 μg), then incubated with anti-AU1 and anti-ERK8 antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies. *C*, ERRα-induced ERRE_Luc luciferase reporter activity is inhibited by HA-ERK8 in MCF10A cells; 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies. *D*, ERRE_Luc basal activity in MCF10A cells is due to the transactivation potential of endogenous ERRα. MCF10A cells were transfected with the ERRE_Luc luciferase reporter vector and treated with 10 or 20 μM XCT790; after 24 h, cells were lysed and assayed for luciferase activity; 20 μl of the same cellular lysate was subjected to Western blot with anti-ERRα antibody. *E*, HA-ERK8 inhibits ERRE_Luc luciferase reporter activity induced by endogenous ERRα in MCF10A cells (***, $p < 0.001$ according to unpaired Student's *t* test); 20 μl of the same cellular lysates was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies; *F*, confocal microscopy images of MCF10A cells transfected with HA-ERK8 (1.5 μg), then incubated with anti-ERRα and anti-HA antibodies and labeled with TRITC- and Cy2-conjugated secondary antibodies and DAPI for nuclear staining. *G*, ERK8 expression increases the amount of ERRα in the cytoplasmic fraction of MCF10A cells; MCF10A stably expressing EGFP or HA-ERK8 (5×10^6 cells) were subjected to fractionation using the Subcellular Protein Fractionation Kit (Thermo Scientific); lysates were subjected to SDS-PAGE followed by Western blot with anti-ERRα (*top*), anti-ERK8 (*middle top*), anti-IkBα (*middle bottom*), and anti-Lamin A/C (*bottom*) antibodies; *H*, interaction between endogenous ERK8 and ERRα proteins; total cellular lysate (1 mg) from MCF10A cells was subjected to immunoprecipitation with anti-ERRα antibody or with rabbit preimmune serum (4 h at 4 °C), then subjected to SDS-PAGE and blotted with anti-ERK8 antibody (Kinasource) (*top*). Total cellular lysates (50 μg) were blotted with anti-ERK8 (*middle*) and anti-ERRα (*bottom*) antibodies for normalization purposes. *I*, HA-ERK8 inhibits ERRE_Luc luciferase reporter activity induced by endogenous ERRα following stimulation of the nuclear receptor with two constitutively active mutants of EGFR (EGFR L858R and EGFR D770_N771 insNPG); 20 μl of the same cellular lysates was subjected to Western blot with anti-ERRα and anti-ERK8 antibodies.

appears even more relevant when compared with the otherwise extremely low conservation score of the ERK8 protein throughout evolution.

Subcellular localization and dynamic movements between cellular compartments are important mechanisms used by cells to regulate the activity of different transcription factors (55). As concerns nuclear receptors, several groups have demonstrated that they continuously shuttle between the cytoplasm and the nucleus, as a consequence of a fine balance between operational strengths of nuclear localization signals and nuclear export signals (55, 56). The subcellular compartmentalization and the timely and efficient localization of these transcription factors are usually modulated through the binding of specific ligands, such as steroid or thyroid hormones (55). In principle, all nuclear receptors, when bound to their natural ligands, translocate to the nucleus and become transcriptionally active, being the nuclear localization necessary for their direct action on target genes. On the other hand, unliganded, inactive receptors appear to have a more diverse localization pattern, from predominantly cytoplasmic to uniformly distributed between the cytoplasm and nucleus (57–59) to predominantly nuclear (60–62). Moreover, steroid receptor localization has also been extended to mitochondria (63, 64) and plasma membrane (65–67). Interestingly, some nuclear receptors, such as the ERα, have even higher complexity, requiring a cytoplasmic localization, even when ligand-bound and active, to exert specific "non-genomic" functions alternative to direct promoter binding and necessary for cellular transformation (35). As mutations of ERK8 that prevent interaction also abolish its corepressive functions and ERRα cytoplasmic localization, we can state that direct interaction is a prerequisite for inhibition of ERRα activity as well as for its nuclear exclusion. However, the relationship between ERRα activity and cytoplasmic localization needs further investigation. Indeed, whereas ERRα nuclear exclusion prevents the interaction of this nuclear receptor with the promoters of its target genes and therefore may participate to the inhibition of its constitutive transcriptional activity, we cannot exclude that ERRα may also control, in the cytoplasm, alternative, non-genomic functions contributing to its biological activity, as described for ERα (35, 68, 69) and progesterone receptor (70). In this context, it is important to note that a naturally occurring short form of the metastatic tumor antigen 1 (MTA1s) is a characterized ERα corepressor that sequesters this steroid receptor to the cytoplasm through an L*XX*LL motif, enhancing non-genomic functions such as ERK2 activation (71). The analogies between the short form of the metastatic tumor antigen 1-ERα and the ERK8-ERRα situations may therefore suggest that ERK8, whereas negatively regulating ERRα transcriptional activity, may control as yet to be described non-genomic functions of this protein by regulating its nucleocytoplasmic shuttling. Further work will be required to establish the precise functions exerted by ERRα in the cytoplasm as well as the role of ERK8 in controlling such activities.

In addition to its physiological functions in cellular metabolism and in responses to stressors requiring shifts in energy production and utilization (12, 13, 72, 73), an increasingly important role for ERRα in human malignancies is emerging. Silencing of ERRα and the use of specific pharmacological inhibitors suggest a critical role for this nuclear receptor in the growth of highly aggressive forms of breast cancer (22–25). Moreover, it is known that ERRα and ERα can interact *in vitro* and regulate transcription through the classical ERE (15, 33), and that ERRα behaves as a repressor or an activator of ER-regulated transcription in a cell type-dependent manner (74). Hence, it has been proposed that, whereas ERRα tightly regulates estrogen responsiveness in normal breast cells, it may functionally replace ER in ER-negative breast tumors, thereby constitutively activating ERE-regulated transcription (74). Thus, the conversion of ERRα from a repressor to an activator may be a critical step in the progression of breast tumors to a hormone-independent phenotype, suggesting that ERK8 may provide opportunities for novel therapeutic approaches for the treatments of these tumors.

Evidences are accumulating about the involvement of ERK8 in human cancer (7, 9). In the context of breast cancer, in particular, high ERK8 expression has been demonstrated in normal human mammary cells, whereas loss of its expression has been correlated with breast cancer progression and increased ERα levels (9). Our findings therefore suggest that, in breast, ERK8 may participate in maintaining tissue homeostasis not only by regulating ERα protein degradation (9) but also by controlling the activity and cellular localization of ERRα, whose role in cellular metabolism and transformation has now been well established (28, 75, 76). Hence, this MAP kinase might regulate estrogen transcriptional targets both directly, by enhancing ERα degradation, and indirectly, by modulating the physical and functional interactions of this nuclear receptor with ERRα. Based on the well established role of estrogens in breast cancer progression, through the expression of cellular proto-oncogenes (*e.g.* c-MYC) as well as extracellular matrix molecules (*e.g.* syndecan-2 and metalloproteinase-9) (77, 78), the ability of ERK8 to control and integrate signals from ERα and ERRα receptors in normal and transformed mammary cells warrants further investigation.

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