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Repression of the Estrogen Receptor-α Transcriptional Activity by the Rho/Megakaryoblastic Leukemia 1 Signaling Pathway*

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Although involved in processes leading to the emergence and development of hormone-dependent breast cancers, the estrogen receptor α (ERα) also prevents transformed cells from progressing toward a more aggressive phenotype. The transcriptional activity of ERα is mediated through two transactivation functions, called activation function 1 and 2, whose respective involvement varies in a cell-specific manner. Here, we identify the Rho/megakaryoblastic leukemia 1 (MKL1) signaling pathway as a main actor in controlling the cell-specific activity of both transactivation functions of ERα. Notably, we show that, when the coregulator MKL1 is sequestered in an inactive form by unpolymerized actin, the transcriptional activity of ERα mainly relies on the activation function 1. The activation of MKL1, which results from its dissociation from unpolymerized actin, promoted by the ability of Rho to support polymeric actin accumulation, silences the activation function 1 of ERα and allows the receptor to mainly act through its activation function 2. Importantly, this switch in the respective contribution exerted by both transactivation functions is correlated with an impaired ability of ERα to efficiently transactivate estrogen-regulated reporter genes. MKL1 is further shown to be present on estrogen-responsive genes *in vivo*. Interestingly, the Rho/MKL1 signaling pathway is activated during the epithelial-mesenchymal transition. A reduced transactivation efficiency of ERα, resulting from the activation of this pathway, may therefore suppress the protective role exerted by ERα toward tumor progression and invasiveness.

The estrogen receptor α (ERα)[2] belongs to a class of structurally conserved transcription factors, the nuclear receptor superfamily. ERα regulates the transcription of specific genes upon binding to regulatory sequences, either directly through the association of its DNA-binding domain to specific sequences called estrogen-responsive elements (EREs), or indirectly through protein-protein interactions with activator protein 1 or Sp1 factors (1). ERα comprises an N-terminal transcriptional activation function, the activation function 1 (AF1), and a ligand-dependent transactivation function, the AF2, which is part of the C-terminal ligand-binding domain. Hormone binding to ERα triggers conformational changes that make these AFs available for interaction with transcriptional coregulators that, in turn, allow the regulation of target genes transcriptional activity. Several subclasses of NR coactivators have been identified, including members of the p160 family, the integrators CBP and p300, arginine methyltransferases, or components of the Mediator complex (2, 3).

Mediating estrogenic signaling, ERα plays critical roles in many physiological processes such as the development and function of the reproductive system, bone metabolism, and cardiovascular activity (4). It is also associated with major human pathologies, and notably estrogen-dependent cancers developed from reproductive organs. For instance, ERα mediates the mitogenic effects that estradiol (E_2) exerts on ~70% of primary breast tumors. However, contrasting with this harmful promotion of tumor growth during initial stages of carcinogenesis, ERα also has protective effects. ERα-positive breast tumors are indeed generally more differentiated and less invasive than ERα-negative cancers and, therefore, display a better prognosis (5). Unfortunately, ERα-positive tumors frequently acquire a resistance to steroid hormones-positive influence, which is accompanied with an amplification of growth factor receptor signaling. Notably, the overexpression of the human epidermal growth factor receptor HER2 in ERα-positive breast cancer cells results in a down-regulation of ERα transcriptional activity and expression, both *in vitro* and *in vivo* (6). The molecular basis of the loss of sensitivity of ERα-positive breast cancer cells to estrogens still remains elusive.

The relative contribution exerted by AF1 and AF2 on the transcriptional activity of ERα varies depending upon the promoter, but also upon the cell considered (7, 8). For instance, AF2 is the only active AF in cells that have achieved their epithelial-mesenchymal transition (9). Importantly, the loss of E-cadherin-mediated cell-cell junctions, a key process that occurs during the epithelial-mesenchymal transition, dramati-

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[2] The abbreviations used are: ERα, estrogen receptor α; CBP, CREB-binding protein; ERE, estrogen-responsive element; AF1, -2, activation functions 1 and 2; E_2, estradiol; MKL1, megakaryoblastic leukemia 1; SRF, serum response factor; CMV, cytomegalovirus; GFP, green fluorescent protein; EGFP, enhanced GFP; 4-OHT, 4-hydroxytamoxifen; ChIP, chromatin immunoprecipitation; ROCK, Rho kinase; ANOVA, analysis of variance; HA, hemagglutinin.

cally affects ERα functions by attenuating its transactivation efficiency. This occurs through the silencing of the activity of an AF1 subregion termed box 1, which forces the receptor to mainly act only via its AF2 (10). Disruption of cadherin-mediated intercellular adhesion, migration, and metastasis has been correlated with high activities of RhoA or of its target Rho kinase (or ROCK) (11). According to recent work, RhoA signaling influences the activity of the myocardin-related transcription factor MKL1 (megakaryoblastic leukemia 1, also termed MAL, BSAC, or MRTF-A), which is a coactivator of the serum response factor (SRF) (12, 13). The activity of MKL1 depends upon the integrity of epithelial cell junctions (14, 15) and is regulated by changes in actin dynamics. In serum-starved cells, MKL1 binds to unpolymerized actin ("globular" actin or G-actin) through its RPEL motifs and is thus sequestered in an inactive form. Upon Rho activation, accumulation of fibrillar actin ("fibrillar" actin or F-actin) leads to a G-actin depletion and release of MKL1, which then coactivate SRF transcriptional activities (12, 16).

We demonstrate in this report that the activation of the Rho/Actin/MKL1 signaling pathway impairs ERα-dependent transcriptional activation, mainly through the inhibition of the activity of the AF1 box 1. Our data thus provide important and novel insights into how actin-dependent processes such as cell shape changes or adhesion may deeply influence estrogenic signaling.

EXPERIMENTAL PROCEDURES

Plasmids and Constructs—The ERE-tk-LUC, *C3*-LUC, and c-*fos*-LUC reporter genes as well as the internal control CMV-βgal, the pCR-ERα, pCR-ERα Δ79, and pCR-ERα CFs (Δ173) have been previously described (9). Expression plasmids encoding the negative and positive dominant Myc-tagged forms of RhoA (Q63L and T19N), Rac1 and Cdc42 (Q61L and T17N) are a gift from Prof. William Harris (University of California). The p3×flag-MKL1, p3×flag-MKL1 ΔN200 and p3×flag-MKL1 ΔC301, p3×flag-MKL1 ΔB, p3×flag-MKL1 ΔQ, and p3×flag-MKL1 ΔSAP expression vectors were kindly provided by Prof. R. Prywes (Colombia University) (17). The pTAL-LUC, pEGFP, and the pEGFP-β-actin plasmids were purchased from Clontech. Open reading frames from the MKL1-, MKL1 ΔN200-, and MKL1 ΔC301-expressing vectors as well as a modified open reading frame of the actin-expressing vector were generated by PCR and subcloned into pCR3.1 or pCDNA3.1 plasmid (Invitrogen) to obtain pCR MKL1, pCR MKL1 ΔN200, pCR MKL1 ΔC301, and pCDNA-actin G13R. The pR-CMV-CBP-HA was a gift from Prof. F. Gannon (Heidelberg, Germany). The pGEX2T, pGEX2T-ERα AB, and pGEX2T-ERα DF plasmids have been previously described (18). The pGEX3X-MKL N330 plasmid was constructed by subcloning the sequence coding the 330 N-terminal amino acids of MKL1, generated by PCR, into the pGEX3X plasmid (Amersham Biosciences). All generated constructs were sequenced.

Reagents and Antibodies—Cytochalasin D, the ROCK inhibitor Y-27632, and the Rho inhibitor C3 exoenzyme were purchased from Calbiochem. $ICI_{182,780}$ (ICI) was provided by TOCRIS Bioscience. The anti-GFP antibody (JL-8) and the anti-phospho-RNA polymerase II antibody (CTD4H8) were purchased from BD Biosciences and Upstate Biotechnologies, respectively. 17 β-estradiol (E_2), 4-hydroxytamoxifen (4-OHT), the anti-β-actin (AC-15) and the anti-FLAG M2 antibodies were purchased from Sigma-Aldrich. The anti-Myc tag antibody (9B11) was purchased from Cell Signaling. Antibodies raised against the HA epitope (HA probe), ERα (HC20), and MKL1 (MRTF-A and C-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture and Transfection—HeLa, HepG2, MCF-7, or MDA-MB231 cells or MDA-MB231 stably expressing ERα (19) were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 5% or 10% (MCF-7) fetal calf serum (Biowest) and antibiotics (Sigma-Aldrich) at 37 °C in 5% CO_2. Transfections were carried out using FuGENE 6™ reagent according to manufacturer's instructions (Roche Applied Science). One day before transfection, cells were plated in 24-well plates at 50% confluence. 1 h prior transfection, the medium was replaced with phenol red-free Dulbecco's modified Eagle's medium-F12 (Sigma-Aldrich) containing 2.5% charcoal-stripped fetal calf serum (Biowest). Transfection was carried out with 100 ng of reporter gene, 100 ng of CMV-βGal internal control, and appropriate combinations of expression vectors. Plasmid mix was made up to 500 ng of total DNA per well with empty vector. Following an incubation overnight, cells were treated for 24 h with ligands or ethanol (vehicle control), and required drugs. The C3 exoenzyme (125 ng/ml) was added to the FuGENE/medium mix during the transfection step. Cells were then harvested, and luciferase and β-galactosidase assays were performed as previously described (18). Luciferase reporter gene activity was normalized to the β-galactosidase.

Protein Extracts and Western Blotting—To obtain whole cell extracts, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a mixture of protease inhibitors (Roche Applied Science). Subcellular fractionation was performed as described in Current Protocols (20). Western blots were performed as previously depicted (9).

Pulldown Assays—To determine Rho activity in cell lines, the Rho Activation Assay Kit (Upstate) was used in accordance with manufacturer's instructions.

Reverse Transcription-PCR, ChIP, and Sequential ChIP Assays—Reverse transcription-PCR, ChIP, and sequential ChIP assays were performed as previously described (21). The primers against promoter regions are available upon request.

RESULTS

Rho GTPases and Actin Dynamics Control ERα Transactivation Potency—We previously demonstrated that the transcriptional activity of ERα depends upon the differentiation stage of the cell, with the highest activity detected in differentiated cells (9). De-differentiation of epithelial cells is correlated with significant changes in the activity of members of the Rho GTPases family such as RhoA, Rac1, and Cdc42 (11). The impact of the Rho/actin signaling pathway on ERα transactivation efficiency was analyzed by transient transfection experiments in HepG2 and HeLa cells, which are two epithelial-like cell lines exhibiting divergent phenotypes. HeLa cells, originating from a cervix

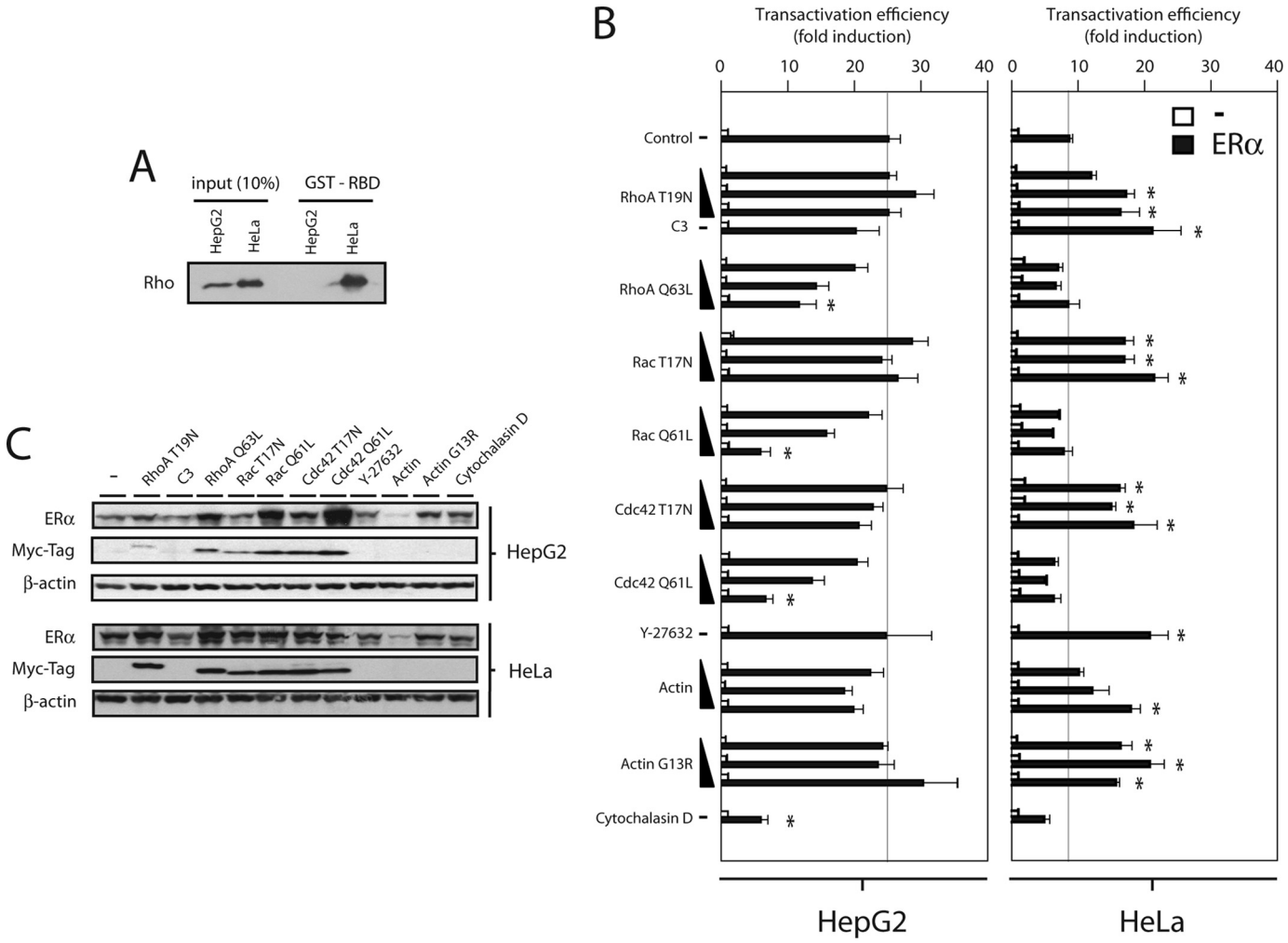


FIGURE 1. **Rho GTPases and actin dynamics influence ERα transcriptional efficiency.** *A*, glutathione *S*-transferase (*GST*) pulldown experiments using the glutathione *S*-transferase-Rho-binding domain (Rho-binding domain (*RBD*) of the human rhotekin protein) fusion protein were performed to measure the Rho activity contained within protein extracts prepared from confluent HeLa and HepG2 cells, as described under "Experimental Procedures." *B*, HeLa and HepG2 cells were transfected with the ERE-tk-LUC and CMV-βGal reporter genes together with 50 ng of pCR3.1 (−) or pCR ERα (*ERα*) vectors, in combination with increasing concentrations (50, 100, and 200 ng) of plasmids expressing dominant positive and negative Myc-tagged forms of RhoA (RhoA Q63L and RhoA T19N), Rac1 (Rac Q61L and Rac T17N), and Cdc42 (Cdc42 Q61L and Cdc42 T17N), pEGFP-β-actin or actin G13R. C3 exoenzyme (125 ng/ml) was added to the DNA mixes when required. Cells were treated for 24 h with 10 nM E_2 and, when mentioned, co-treated with the ROCK inhibitor Y-27632 (15 μM) or the actin-capping drug cytochalasin D (0.5 μM). Normalized luciferase activities were expressed as -fold increase above values measured with empty pCR3.1. Data correspond to the mean values ± S.E. of at least six separate transfection experiments. *, significantly different from the transcriptional activity of ERα measured in the absence of Rho GTPase, actin expression, or treatment ($p < 0.01$, as determined by ANOVA). *C*, Western blots controlling the expression of ERα, Myc-tagged Rho GTPases, and β-actin in HeLa and HepG2 cells transiently transfected as described in *B*. Only the protein extracts from cells transfected with the highest concentration of Myc-tagged Rho GTPases and actins were analyzed.

carcinoma, present a poorly differentiated phenotype, do not express the calcium-dependent cell-cell adhesion molecule E-cadherin, and strongly express vimentin, an intermediate filament protein that expression is associated with increased invasive and metastatic potency. In contrast, the hepatocarcinoma HepG2 cell line appears more differentiated, producing a high level of E-cadherin but no vimentin (9). Moreover, Rho activity was much higher in HeLa than in HepG2 cell extracts, as indicated by glutathione *S*-transferase pulldown experiments using as a bait the Rho-binding domain of the human rhotekin protein, which has the ability to retain only active Rho (Fig. 1*A*). Although no signal was observed within the HepG2 extracts, we cannot exclude that some active Rho is present in these cells but at really low levels that could not be detected by the procedure. The transactivation efficiency of ERα on an ERE-tk-LUC reporter gene is also much higher in HepG2 than in HeLa cells following treatment with 10^{-8} M E_2 (Fig. 1*B*), corroborating our previous data (9, 10).

Constitutively active Myc-tagged forms of RhoA (Q63L), Rac1 (Q61L), or Cdc42 (Q61L), as well as their respective inactive forms (RhoA T19N, Rac1 T17N, and Cdc42 T17N), were then used to study the contribution of these Rho GTPases in transcriptional regulations mediated by ERα. In HepG2 cells, the transfection of increasing concentrations of plasmids encoding inactive RhoA, Rac1, and Cdc42 forms, which inhibit endogenous Rho GTPase activity, has little effect on ERα-mediated induction of the ERE-tk-LUC reporter. In contrast, the expression of these mutated Rho GTPases in HeLa cells increases 2- to 3-fold the transcriptional response of the reporter gene to ERα (Fig. 1*B*). Importantly, in HepG2 cells, although the transfection of low amounts of plasmids encoding the three constitutive Rho GTPase forms has little impact on

ERα transactivation potency, the latter is severely reduced at higher amounts of plasmids. In contrast, in HeLa cells, the constitutively active forms have no obvious effect on the ERα-mediated stimulation of ERE-tk-LUC reporter, as expected (Fig. 1*B*). The use of the C3 exoenzyme, which is an inhibitor of the three Rho proteins (A, B, and C), restores the situation observed with the inactive form of RhoA (T19N) (Fig. 1*B*). Finally, we analyzed the expression level of ectopically expressed proteins in both cell lines through Western blots (Fig. 1*C*). These experiments showed no correlation between results from gene reporter assays and any variation in the amounts of ERα or Rho GTPase forms. Moreover, the increased amount of ERα detected within HepG2 cells transfected with the three constitutive Rho GTPase forms is inversely correlated with its activity. Altogether, these results indicate that ERα transcriptional efficiency is negatively regulated by active Rho GTPases.

Rho GTPases are mainly studied for their roles in regulating the actin cytoskeleton (22). Activation of RhoA induces the polymerization of actin, partly through its effector ROCK (23). We consequently evaluated the transcriptional activity of ERα in both cell lines following an inhibition of ROCK by the Y-27632 compound. In agreement with data presented before, the Y-27632 treatment enhances ERα transcriptional activity in HeLa cells only (Fig. 1*B*). Contribution of actin dynamics in ERα transcriptional regulation was then further evaluated by disrupting the intracellular levels of actin monomers (G-actin). For this purpose, cells were transfected with increasing concentrations of expression vectors encoding wild-type or unpolymerizable mutant (G13R) of β-actin, the overexpression of which enhances the G-actin level (24). When expressed in HeLa cells, both actin forms potentiate the induction of the ERα-dependent reporter gene. In contrast, ERα activity in HepG2 cells was not really modified in their presence (Fig. 1*B*). Western blots indicate that β-actin levels are not obviously affected by the transfections of both actin forms in both cell lines (Fig. 1*C*). In contrast, ERα expression level is slightly reduced by the overexpression of the wild-type β-actin form, while it remains active. Together, these results indicate that actin dynamics influence ERα activity. To substantiate this observation, we next used cytochalasin D, an actin-binding drug that mimics the coactivation of SRF by Rho (25). As expected, cytochalasin D strongly inhibits ERα transcriptional activity in HepG2 cells, thereby mimicking the situation observed upon the overexpression of constitutively active forms of Rho GTPases (Fig. 1*B*). Altogether, these results strongly suggest that Rho GTPases control ERα transcriptional activity by modulating actin dynamics.

Rho/Actin Regulation of ERα Transactivation Potency Involves ERα AF1 Box 1 Activity—In epithelial cells, the attenuation of ERα activity observed following the loss of E-cadherin-mediated cell contacts partly results from a silencing of the ERα AF1 box 1 activity (10). We therefore evaluated the respective contribution exerted by ERα AFs toward the transcriptional activity of the receptor, upon changes of the Rho/actin pathway activity. Cell permissiveness to either ERα AFs was determined by comparing the transcriptional activity of ERα with those of ERα Δ79 (deletion of AF1 box 1) and ERα CF (additional deletion of AF1 box 2/3). Importantly, the ERE-tk promoter mainly used in our transient transfection experiments exhibits no intrinsic preference for a specific AF (9). In HepG2 cells, the main region involved in ERα transcriptional activity is the AF1 box 1 (ERα Δ79 *versus* ERα, Fig. 2*B*), the remaining activity depending upon the AF1 box 2/3, as expected (10). In contrast, the AF2 (ERα CF) represents ~60% of the whole ERα transcriptional potency in HeLa cells. The AF1 box 2/3 (ERα Δ79 *versus* ERα CF) is responsible for the remaining 40% of the activity (Fig. 2*B*).

The overexpression of the constitutively active form of RhoA (Q63L) specifically abolishes the activity of ERα AF1 box 1 in HepG2 cells (ERα *versus* ERα Δ79) without significantly affecting the activities of the other AFs. In contrast, in HeLa cells, RhoA Q63L does not change the relative contribution exerted by the different AFs, as indicated by the similar activity promoted by the ERα, ERα Δ79, and ERα CF proteins (Fig. 2*B*). Thus, in the presence of a constitutively active form of RhoA, the transcriptional activity of ERα is similar in both cell types. As expected, the overexpression of the T19N-inactive form of RhoA had no effect on the transcriptional properties of the different ERα proteins in an AF1 box 1 permissive context such as HepG2 cells (Fig. 2*B*). As a consequence, the overexpression of this mutant in HeLa cells would have been expected to allow an activity of the AF1 box 1, but this is not the case (Fig. 2*B*). Similar results were observed following the overexpression of dominant positive and negative forms of Rac1 and Cdc42 (data not shown). To confirm the involvement of ERα AF1 in the regulation of ERα transactivation potency by the Rho/Actin pathway, we then evaluated the relative contribution of the different AFs toward ERα activity following the inhibition of the RhoA effector ROCK by the Y-27632. Importantly, while this treatment had no effect in HepG2 cells, it allowed HeLa cells to become permissive to an AF1 box 1 activity, as revealed by the higher transcriptional activity of full-length ERα *versus* ERα Δ79 (Fig. 2*B*). Importantly, this treatment did not affect the contribution of AF1 box 2/3 in HeLa cells, indicating that pathways initiated by RhoA were targeting the AF1 box 1 activity. Corroborating this conclusion, the overexpression of unpolymerizable β-actin (G13R) activated the AF1 box 1 activity in HeLa cells, as revealed by the different transcriptional potency of full-length ERα and ERα Δ79 proteins. In contrast, cytochalasin D totally abolished the activity of the AF1 box 1 in HepG2 cells and dictated a strict AF2 permissiveness in HeLa cells. Finally, as assessed by Western blots (Fig. 2*C*), although some ERα variants exhibited different expression levels, they were not affected upon the specific treatments that modify ERα activity in both cell lines.

These data demonstrate that Rho/actin axis controls ERα transcriptional activity, at least through the modulation of the respective contribution of each AFs toward the activity of the receptor. Notably, the activation of RhoA and subsequent actin polymerization inhibited the activity of the AF1 box 1 of ERα.

MKL1 Links Rho/Actin Pathway to ERα Transcriptional Activity—RhoA and changes in actin dynamic are integral components of a T cell factor-independent pathway leading to the activation of SRF. One mediator of this pathway is the coactivator MKL1 (12, 13). The organization of MKL1 is shown in Fig. 3*A*. MKL1 was expressed in both cell lines (Fig. 3*B*), therefore, we sought to evaluate the influence of this protein on ERα tran-

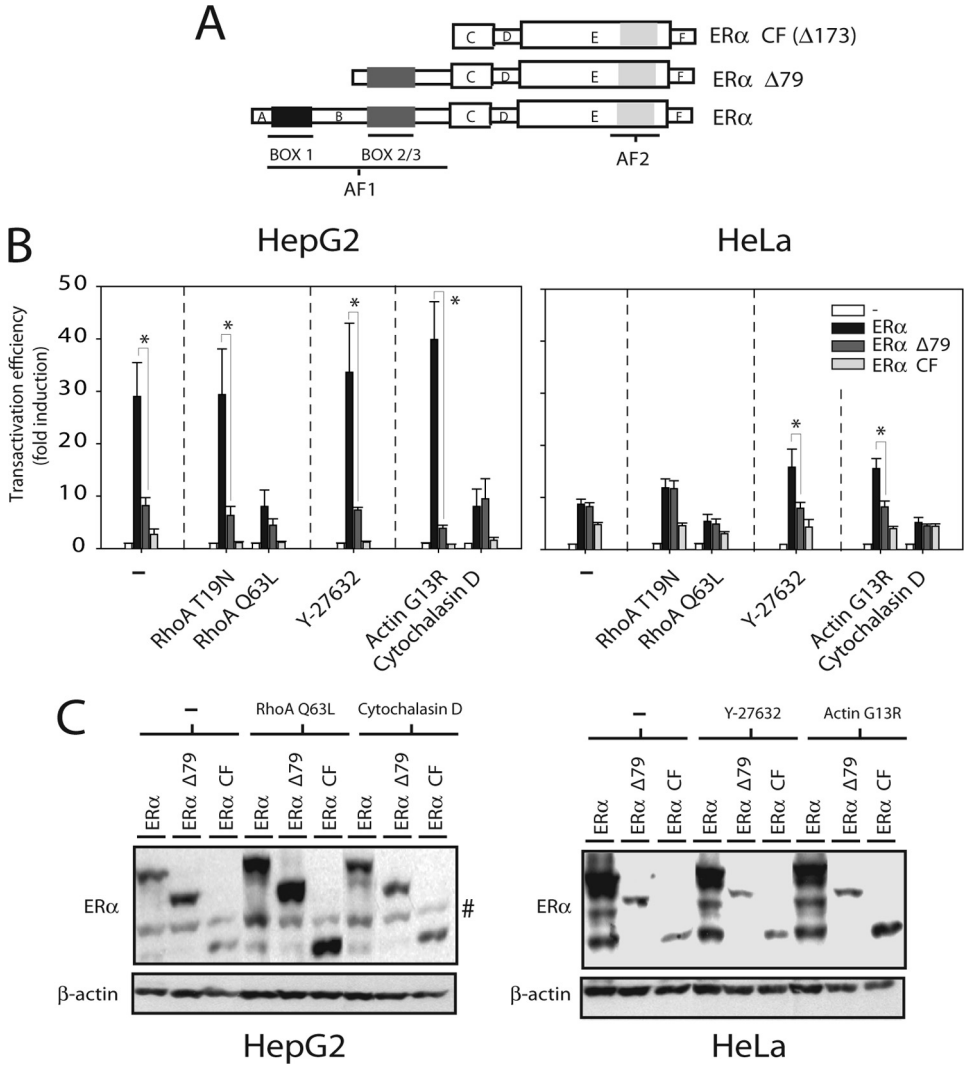


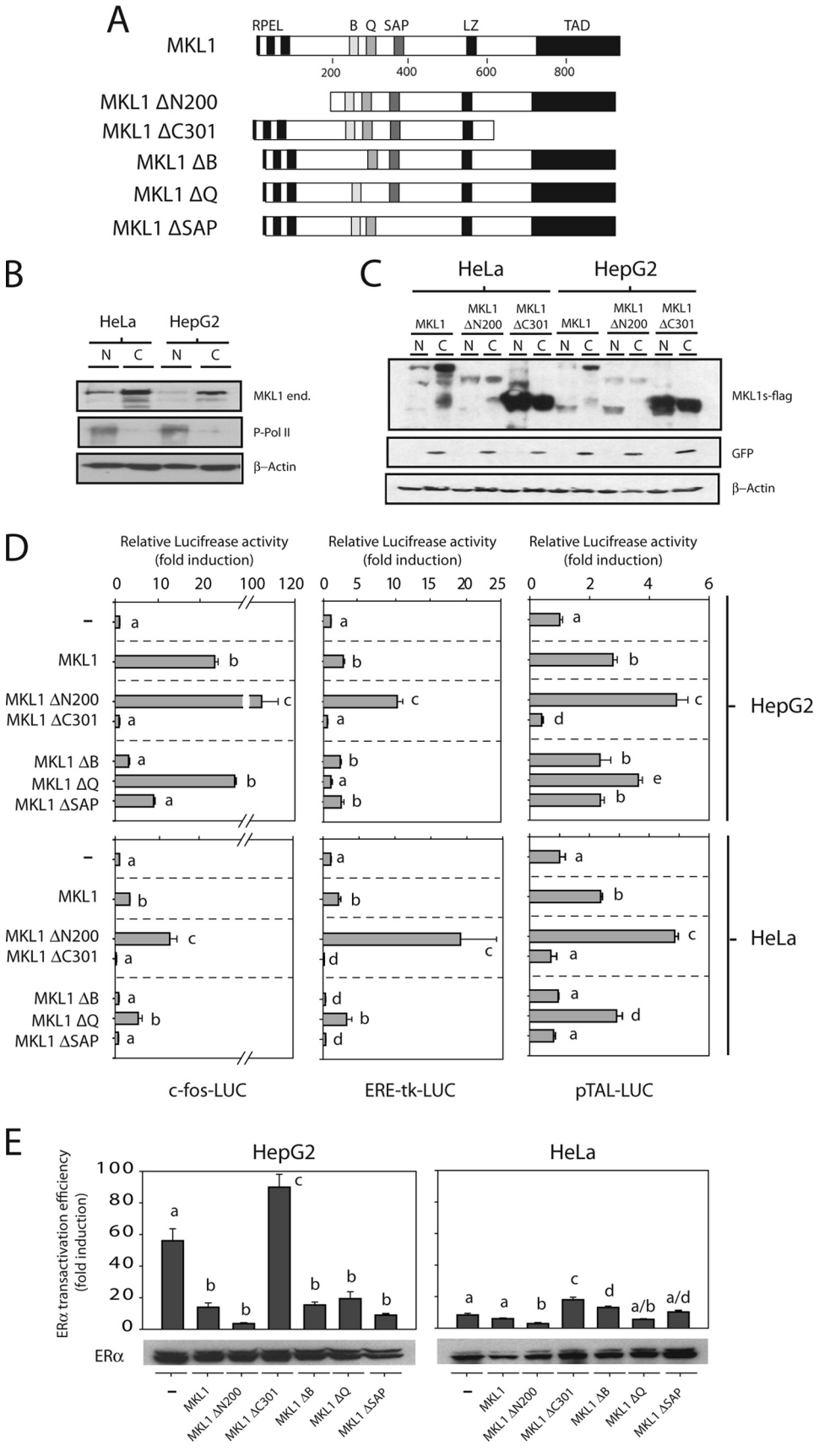
FIGURE 2. **Rho GTPases and actin dynamics influence the relative activity of ERα AFs.** *A*, schematic illustration of the sequence of ERα and of the two N-terminal truncated forms, ERα Δ79 and ERα CF, used in our experiments. *B*, HeLa and HepG2 cells were transfected with the ERE-tk-LUC and CMV-βGal reporter genes, together with 50 ng of pCR3.1 (−), pCR ERα (*ERα*), pCR ERα Δ79 (*ERα Δ79*), or pCR ERα CF (*ERα CF*), in combination with 200 ng of plasmids expressing dominant positive and negative forms of RhoA (RhoA Q63L and RhoA T19N) or actin G13R. Cells were treated for 24 h with 10 nM E_2 and, when mentioned, co-treated with the ROCK inhibitor Y-27632 (15 μM) or the actin-capping drug cytochalasin D (0.5 mM). For each treatment, transactivation efficiency corresponds to normalized luciferase activities expressed as -fold increase above values measured with the empty pCR3.1. Data correspond to averages ± S.E. of values obtained from at least six separate transfection experiments. *, ERα transactivation efficiency significantly differs from ERα Δ79 transactivation efficiency, which indicates a significant AF1 box 1 activity ($p < 0.005$, as determined by ANOVA followed by Fisher's post hoc test). *C*, Western blots controlling the expression level of β-actin, ERα, ERα Δ79, and ERα CF in HeLa and HepG2 cells following treatments affecting ERα activity, as determined in *B*. #, unspecific band.

scriptional activity. For this purpose, flagged versions of wild-type or deletion mutants of MKL1 were overexpressed in the cells. The MKL1 ΔN200 protein was deleted from the N-terminal RPEL motifs (Fig. 3*A*), which are critical for actin binding, and acted as a constitutively active variant on SRF target genes such as c-*fos*. The MKL1 ΔC301 protein was deleted from the C-terminal transactivation domain and behaves as a dominant negative form on c-*fos* regulation (12, 17). The correct expression of these proteins, their cellular localization, and their appropriate behavior on a c-*fos*-LUC reporter gene were first established as shown in Fig. 3, *C* and *D*. The amount of the plasmids encoding MKL1 variants used was previously determined by a dose-response experiment (data not shown). Their impact on the ERE-tk-LUC reporter gene was then analyzed. Wild-type and mutated forms of MKL1 dramatically change the basal activity of the ERE-tk-LUC measured in the absence of ERα, paralleling variations observed on the c-*fos*-LUC reporter (Fig. 3*D*). The constitutively active mutant MKL1 ΔN200, and to a lesser extent MKL1, enhanced the basal activity of the ERE-tk-LUC, whereas the dominant negative form MKL1 ΔC301 had an opposite effect, notably in HeLa cells (Fig. 3*D*). Similar results were observed on other ERE-driven reporter genes as well as on ERE-less reporters (Fig. 3*D* and data not shown). Thus, besides exhibiting different amplitudes of their response to MKL1 variants, with the ERE-less pTAL reporter gene being the less sensitive, the responses of all reporters were similar, in the presence or absence of an ERE. Finally, the overexpression of MKL1 variants internally deleted of short basic (ΔB), glutamine-rich (ΔQ), or SAP (SAF-A/B, Acinus, and PIAS) (ΔSAP) regions, which are conserved within the myocardin-related transcription factor family (17), also modify the basal activity of the reporter genes in a cell- and promoter-dependent manner (Fig. 3*E* and data not shown).

To facilitate the analysis of the influence of MKL1 forms on ERα transcriptional activity and minimize the interpretation bias created by the variations in the basal transcriptional activity of the ERE-tk-LUC observed following MKL1 expression, ERα transactivation potency were expressed, for each MKL1 forms, as the -fold increase above reporter gene activity measured in the absence of receptor. The results of these assays clearly show that the overexpression of MKL1 and MKL1 ΔN200 dramatically reduced the transactivation efficiency of ERα in HepG2 cells (Fig. 3*E*), whereas the dominant negative MKL1 ΔC301 almost doubled the activity of ERα. The overexpression of MKL1 and MKL1 ΔN200 proteins has a similar impact on ERα transcriptional potency in HeLa cells, but to a lesser extent. Additionally, as in HepG2 cells, MKL1 ΔC301 also enhanced by 2-fold the transcriptional activity of ERα in HeLa cells (Fig. 3*E*). On the other hand, the internal deletions of MKL1 B, Q, or SAP regions influenced ERα activity in a cell-dependent manner (Fig. 3*E*).

The impact of MKL1 forms on cell permissiveness to either ERα AFs was then determined by comparing ERα, ERα Δ79, and ERα CF transcriptional activity. These experiments demonstrate that, in HepG2 cells, MKL1 and MKL1 ΔN200 dramatically reduced (MKL1) or even abolished (ΔN200) the activity of the AF1 box 1 activity. This is revealed by the decrease of full-length ERα activity to levels similar to those induced by ERα Δ79 (Fig. 4*A*). In HeLa cells, the reduction of ERα activity provoked by these two MKL1 proteins was apparently a consequence of an inhibition of the AF1 box 2/3, which generated a cell context permissive only to ERα AF2 (Fig. 4*A*). These results therefore parallel those observed following the overexpression of dominant positive RhoA (Q63L) and cytochalasin D treatments (Fig. 2*B*). Importantly, the stimulation of ERα activity observed following the overexpression of MKL1 ΔC301 in both cell lines seems to be a consequence of the de-repression of an AF1 box 1 activity in HeLa cells, and of the stimulation of its contribution toward ERα activity in HepG2 cells (compare ERα with ERα Δ79 in Fig. 4*A*). Lastly, the overexpression of MKL1 variants internally deleted of B, Q, or SAP regions abolished the AF1 box 1 activity of ERα in HepG2 cells (data not shown). In HeLa cells, these deleted forms of MKL1 had a similar impact on ERα AF1 box 2/3 activity as full-length MKL1 (data not shown).

To confirm the role exerted by MKL1 in controlling the transactivation potency of ERα AF1 box 1, we used another system that relies on the response of the human complement *C3* gene (*C3*) promoter to the agonist activity of 4-OHT. This response indeed depends exclusively upon ERα AF1 box 1 activity (9, 10, 26). Importantly, the overexpression of the dominant negative form of MKL1 (ΔC301) renders HeLa cells permissive to the AF-1 agonistic activity of 4-OHT (Fig. 4*B*). Correspondingly, the dominant positive MKL1 ΔN200 abolishes the



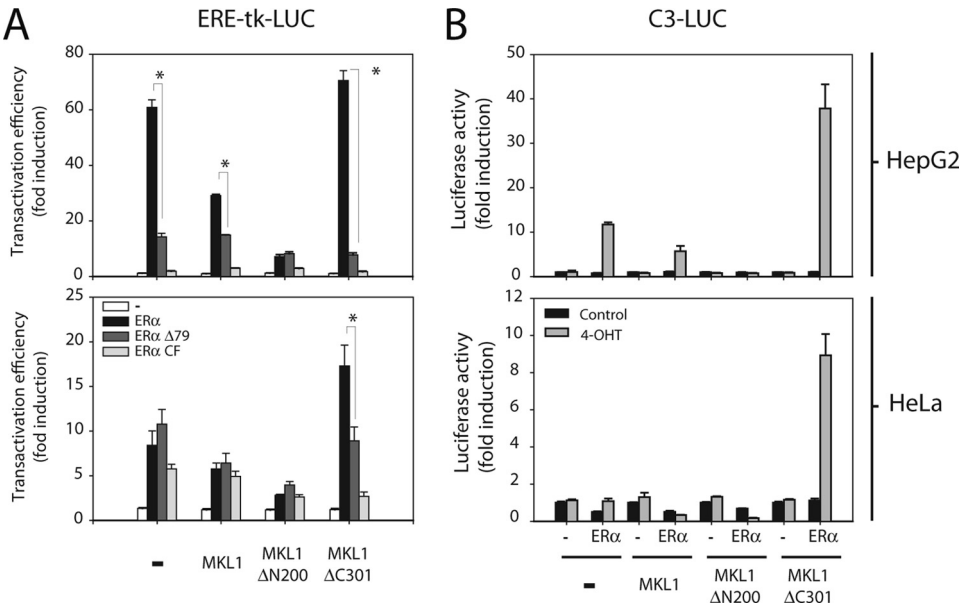


FIGURE 4. **MKL1 influences the relative contribution exerted by both AFs on ERα transcriptional activity.** *A*, HepG2 and HeLa cells were transfected using the ERE-tk-LUC together with CMV-βGal and 50 ng of pCR3.1 (−), pCR-ERα (*ERα*), pCR-ERα Δ79 (*ERα Δ79*), or pCR-ERα CF (*ERα CF*), in the absence or presence of 200 ng of p3×flag constructs expressing wild-type and variant MKL1 proteins. *B*, cells were transfected using the *C3*-LUC together with CMV-βGal and 50 ng of pCR3.1 or pCR-ERα (*ERα*), in the absence or presence of 200 ng of p3×flag constructs expressing MKL1 proteins. Twelve hours following transfection, cells were treated for 24 h with either 10 nM E_2 (*A*) or 2 μM 4-OHT or ethanol (control) (*B*). Luciferase activities were normalized to β-galactosidase and expressed for each MKL1 forms as the -fold increase above levels measured in the absence of ERα forms (−, *panel A*) or in ethanol-treated cells (control, *panel B*). Data correspond to the means ± S.E. of at least three separate transfection experiments. *, ERα transactivation efficiency significantly differs from ERα Δ79 transactivation efficiency ($p < 0.005$, as determined by ANOVA followed by a Fisher's post hoc test).

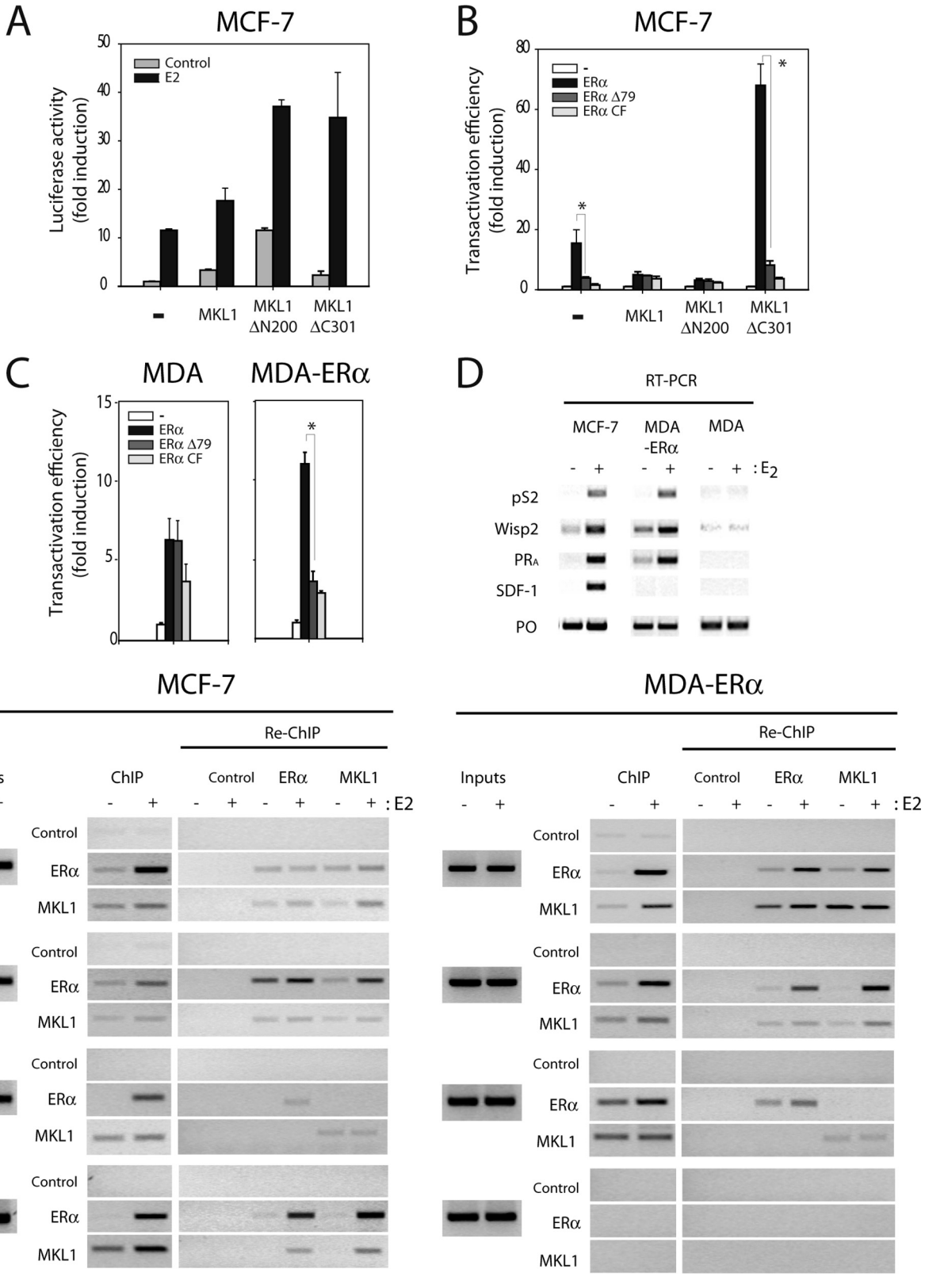
C3 promoter response to 4-OHT in HepG2 cells (Fig. 4*B*). Interestingly, upon the overexpression of MKL1 ΔN200 in HeLa cells, ERα bound to 4-OHT represses the reporter gene activity. These results ultimately highlight the role of MKL1 in regulating ERα AF1 box 1 activity. Altogether, these results complete the demonstration that the Rho/actin/MKL1 signaling pathway plays a major role in modulating ERα transactivation efficiency through the modification of the respective activities of ERα AF subregions.

MKL1 Is Present on Estrogen-responsive Promoters in Vivo and Is a Potential ERα-interacting Protein—We next explored the hypothesis that MKL1 could regulate ERα activity in cells endogenously expressing the receptor. The ERα-positive breast carcinoma cell line MCF-7 was selected for this purpose. Firstly, the impact of transiently transfected forms of MKL1 on the transactivation efficiency of endogenous ERα was measured on the ERE-tk-LUC reporter. As shown in Fig. 5*A*, the constitutively active mutant MKL1 ΔN200 and MKL1, to a lesser extent, enhance the basal activity of the reporter gene measured in the absence of E_2. Importantly, the transcriptional activity of endogenous ERα is up- or down-regulated by dominant negative or dominant positive forms of MKL1, respectively. These results are in total agreement with those previously observed for transfected ERα in HepG2 and HeLa cells. As expected from their differentiated phenotype (E-cadherin-positive and vimentin-negative) (9), MCF-7 cells exhibited a cell context that was mainly permissive to the ERα AF1 box 1 activity (compare ERα with ERα Δ79 and ERα CF in Fig. 5*B*). The overexpression of MKL1 and MKL1 ΔN200 totally abolished this permissiveness, whereas the dominant negative form of MKL1 (ΔC301) enhanced it (Fig. 5*B*). These results clearly indicated that MCF-7 cells provide a similar context to HepG2 cells, in terms of ERα transactivation efficiency on an ERE-driven reporter gene. In contrast, the dedifferentiated ERα-negative breast cancer MDA-MB 231 cell line (MDA) was insensitive to AF1 box 1 activity, as HeLa cells (Fig. 5*C*). Importantly, the stable re-expression of ERα into these cells (MDA- ERα), which induced a more differentiated phenotype, restored an AF-1 box 1-sensitive cell context (Fig. 5*C*).

Having shown that MKL1 regulates ERα transcriptional activity, we next investigated whether MKL1 is present on endogenous estrogen-responsive promoters. We therefore performed ChIP experiments and sequential ChIP in MCF-7 cells to determine the (co)occupancy of several gene promoters with both ERα and MKL1. As shown in Fig. 5*E*, MKL1 was recruited

FIGURE 3. **The actin-sensitive coregulator MKL1 influences genes reporter activity and ERα transactivation efficiency.** *A*, schematic representation of wild-type and deleted forms of MKL1 with identified functional domains highlighted. The N-terminal region of MKL1 contains three RPEL motifs that mediate actin binding. The central region of the protein contains successively basic (*B*), glutamine-rich (*Q*), "SAF-A/B Acinus, and PIAS" (*SAP*), and leucine zipper (*LZ*) domains. The 300 C-terminal amino acids of the protein are depicted as a transactivation domain (*TAD*). *B*, Western blot analysis of endogenous MKL1 levels in nuclear and cytoplasmic fractions of HeLa and HepG2 cells. Fractionation and loading controls were performed using anti-phosphorylated Polymerase II (*P-Pol II*) and anti-β-actin antibody, respectively. *C*, Western blot analysis controlling the correct expression of FLAG-tagged MKL1, MKL1 ΔN200, and MKL1 ΔC301 transiently expressed in HeLa and HepG2 cells. Efficient transfection and fractionation controls were assessed by probing the expression of coexpressed pEGFP (cytoplasmic localization of the GFP). β-Actin was used as the loading control. *D*, impact of wild-type and mutated forms of MKL1 on c-*fos*-LUC, ERE-tk-LUC, or pTAL-LUC reporter genes. HeLa and HepG2 cells were transfected with the corresponding reporter genes together with CMV-βGal and 200 ng of pCR 3.1 or p3×flag constructs expressing wild-type and variant MKL1 proteins, as depicted. Thirty-six hours after transfection, luciferase activities were measured and normalized with β-galactosidase and are shown as the -fold increase above levels measured with empty pCR 3.1. *E*, impact of wild-type and mutated forms of MKL1 on ERα transactivation efficiency. Cells were transfected using the ERE-tk-LUC together with CMV-βGal and 50 ng of pCR 3.1 or pCR ERα in the absence or presence of 200 ng of p3×flag-expressing MKL1forms. Twelve hours following transfection, cells were treated for 24 h with 10 nM E_2. For each MKL1 form, ERα transactivation efficiency corresponds to the luciferase activity (normalized with β-galactosidase) obtained with pCR ERα and expressed as the -fold increase above values measured with empty pCR3.1. The impact of MKL1 proteins on the expression of ERα forms in both cell lines was probed by Western blot. In *D* and *E*, data correspond to the mean values ± S.E. from at least three separate transfection experiments. Columns with different superscripts differ significantly ($p < 0.01$, as determined by ANOVA followed by a Fisher's post hoc test).

to the promoter of all tested E_2-regulated genes (control reverse transcription-PCR within Fig. 5*D*), including *pS2/TFF1* (trefoil factor 1), *Wisp2* (WNT1-inducible signaling pathway protein 2), PR_A (progesterone receptor, A isoform), and *SDF-1* (stromal cell derived factor-1) in the absence or presence of E_2. Furthermore, with the exception of PR_A, a co-recruitment of MKL1 and ERα may occur on the three other analyzed promoters, as revealed through sequential ChIP (Fig. 5*E*). However, the association of ERα and MKL1 on these three promoters seems to exhibit some differences respective to the treatment of the cells



with E_2. For instance, MKL1 appears constitutively associated with *pS2/TFF1* and *Wisp2* promoters, in conjunction with ERα of which the recruitment is stimulated by E_2. In contrast, the recruitment of MKL1 on the *SDF-1* promoter seems enhanced by E_2 and appears associated with ERα only in the presence of E_2. This reflects the strict E_2 dependence of ERα recruitment on this promoter. These results were partly confirmed on the MDA-ERα cells, which offer a different system to analyze the association of MKL1 with the promoter region E_2-regulated genes. However, differing from the MCF-7 cells, the recruitment of MKL1 on the *pS2/TFF1* promoter appears to be enhanced by E_2. Among the tested genes, only *SDF-1* was not expressed in these cells (Fig. 5*D*). Importantly, the lack of expression of SDF-1 in MDA-ERα cells is reflected by its inability to recruit MKL1 or ERα, likely illustrating a closed chromatin conformation (Fig. 5*E*). This provides an interesting additional control of the specificity of the signals observed. In conclusion, these data indicate that ERα and MKL1 can be corecruited and are likely to cooperate on the promoter region of several E_2-regulated genes.

DISCUSSION

Transactivation efficiency of ERα results in part from the respective contribution exerted by its two activation functions, whose activity are tightly regulated in a cell-specific manner (7). For instance, we and others frequently use HeLa and HepG2 cell lines, which represent strict AF2- and AF1-permissive contexts, respectively. The relative sensitivity of a cell line to AF1 and AF2 can be defined by a comparison of the transcriptional activity of transfected wild-type ERα protein with that of an AF1-deleted form (ERα CF). A similar activity of both receptors defines a strict AF2-permissive cell context, whereas a lower transcriptional activity of ERα CF is inherent to a cell context permissive to AF1. However, differences observed in the expression level of some of the variants used in a given cell line (for instance in HeLa cell line (Ref. 10 and Fig. 2*C*)) might weaken the interpretation of the results. Nevertheless, the use of a second approach, based on the strict dependence of the partial agonistic activity of 4-OHT on the human complement C3 promoter toward a cell context sensitive to AF1 (precisely to AF1 box 1 (Refs. 9, 10 and Fig. 4*B*)), allowed us to confirm the respective contributions exerted by the two activation functions in a given cell line.

Through these approaches, we have shown that a high transactivation potency of ERα is correlated with an AF1-permissive cell context generally found in most differentiated epithelial-like cell lines (10). Accordingly, AF2 is the only active AF in cells that have achieved their epithelial-mesenchymal transition (9). A main step in epithelial-mesenchymal transition is the loss of E-cadherin-mediated cell contacts, which is also associated with tumor dedifferentiation and metastasis (27, 28). We recently demonstrated that the existence of intercellular junctions is required for a cell line to be permissive to AF1 (10). Rho proteins and the actin/MKL1 effector pathway, which are also reported to be involved in the epithelial-mesenchymal transition (15, 29, 30), were shown in this study to have a dramatic impact on ERα transcriptional activity.

High activities of Rho or of its target ROCK have been correlated with the disruption of cadherin-mediated intercellular adhesion, cell migration, and metastasis (11, 31). In accordance with previous work (32), the expression of constitutively active forms of RhoA, Rac1, and Cdc42 reduced ERα transactivation efficiency in a cell-specific manner, whereas dominant negative forms of the Rho GTPases enhanced ERα activity. Because the overexpression of such specific proteins might result in a multiplicity of effects that may indirectly influence ERα transcriptional activity, specific inhibitors of RhoA and ROCK were used to confirm the regulation. This effect involves a modulation of the activity of the AF1 box 1 activity, which was previously identified as the main domain involved in the cell context-dependent contribution of the AF1 (10). Rho GTPases control cellular processes that are connected with actin cytoskeleton, such as changes in cell shape, adhesion, and migration (22, 23). A direct impact of such processes on gene expression has been recently illustrated by the control exerted by RhoA on SRF-regulated genes, such as the immediate early gene c-*fos*. This process involves Rho GTPases promoting F-actin accumulation, thereby changing the cellular levels of actin monomers (G-actin) (25) and inducing the activity of the transcription factor MKL1 (12, 16). We demonstrate here that this actin/MKL1 pathway influences ERα transactivation efficiency by controlling the respective contributions of AF1 and AF2 toward ERα transcriptional activity (*integrative model* illustrated in Fig. 6). This was evidenced through (i) changing the amount of cellular G-actin through cell treatment with an actin-capping drug, or by overexpressing wild-type or unpolymerizable β-actin, and (ii) the use of dominant positive and negative forms of MKL1. In all conditions leading to MKL1 activation, the cellular context became refractory to ERα AF1. The physiological function of this AF is therefore likely to require low or no MKL1 activity.

FIGURE 5. **MKL1 impacts endogenous ERα transcriptional activity and is recruited on E_2-sensitive genes.** *A*, MCF-7 cells were transfected with the ERE-tk-LUC together with CMV-βGal and 200 ng of p3×flag constructs expressing MKL1 proteins. Twelve hours following transfection, cells were treated or not for 24 h with 10 nM E_2. Luciferase activities were normalized to β-galactosidase and expressed as the -fold increase above levels measured in the absence of E_2 and MKL1 forms. *B*, MCF-7 cells were transfected using the *C3*-LUC together with CMV-βGal and 50 ng of pCR3.1 (−), pCR-ERα (*ERα*), pCR-ERα Δ79 (*ERα Δ79*), or pCR-ERα CF (*ERα CF*), in the absence or presence of 200 ng of p3×flag constructs expressing wild-type and variant MKL1 proteins. *C*, MDA-MB 231 (*MDA*) and MDA-MB 231 stably expressing ERα (*MDA-ERα*) were transfected with the *C3*-LUC together with CMV-βGal and 50 ng of pCR3.1 (−), pCR-ERα (*ERα*), pCR-ERα Δ79 (*ERα Δ79*), or pCR-ERα CF (*ERα CF*). In *B* and *C*, cells were treated for 24 h with 10 nM E_2. Transactivation efficiency corresponds to normalized luciferase activities expressed as -fold increase above values measured in the absence of ERα forms (empty pCR3.1). Values correspond to the mean ± S.E. from at least three different experiments. *, ERα transactivation efficiency significantly differs from ERα Δ79 transactivation efficiency ($p < 0.005$, as determined by ANOVA followed by a Fisher's post hoc test). *D*, reverse transcription-PCR assays were performed on MCF-7, MDA-MB-231, or MDA-ERα cells that were starved for 48 h in a steroid-free medium and then treated 8 h with 10 nM E_2. Targeted mRNAs are indicated on the *left side* of the *images*. The mRNA encoding the acidic ribosomal protein PO was used as a control. *E*, chromatin prepared from MCF-7 or MDA-ERα cells treated as in *D* was used in ChIP and sequential ChIP (*Re-ChIP*) experiments. These assays were performed using anti-ERα, anti-MKL1, or control (anti-HA) antibodies. PCR was performed on immunoprecipitated DNA, using primer pairs targeting the amplification of proximal promoter sequences of indicated genes. Experiments were repeated three times with identical results.

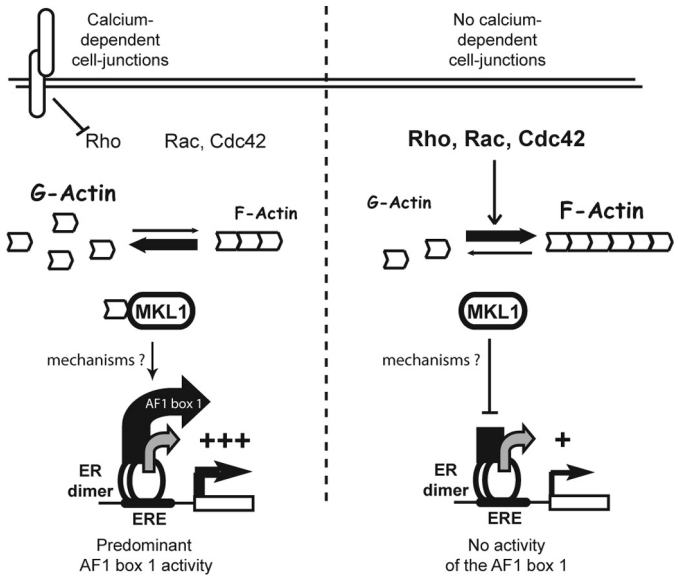


FIGURE 6. **Integrative model of the regulation of ERα transcriptional activity by MKL1.** Differentiated epithelial cells express proteins involved in calcium-dependent contacts such as E-cadherins. In these cells, the establishment of such contacts leads to a low activity of Rho GTPases, which displaces the equilibrium between G- and F-actin toward the generation of an important pool of G-actin. This form of actin interacts with MKL1 which, in this case, promotes the transcriptional activity of ERα through the potentiation of an AF1 box 1 activity. In contrast, in undifferentiated cells that have accomplished their epithelial to mesenchymal transition, the high activity of Rho GTPases triggers the polymerization of actin, which reduces the amounts of G-actin present in these cells. Actin-unbound MKL1 then inhibits the activity of the AF1 box 1 and weakens the transactivation potency of ERα.

According to our model, MKL1 adopts distinct functional states in HeLa and HepG2 cell lines, which exhibit a differential permissiveness for each of ERα AF. Therefore, how might MKL1 control the transcriptional activity of ERα and act to modify the relative contribution of AF1 and AF2? Differences in the expression of MKL1, or its subcellular localization, which is supposed to regulate its activity (12), were not observed between both cell lines. Post-translational modifications of MKL1 may thus provide additional cell-specific mechanisms controlling its activity, such as phosphorylation (12), or sumoylation (33). Results obtained through ChIP experiments show that MKL1 is associated with the promoter region of estrogen-responsive genes in the ERα-positive MCF-7 cells and the MDA-MB 231 cells manipulated to stably express ERα (MDA-ERα). Interestingly, both cell lines were demonstrated to be mainly permissive to AF1 transactivation. Following our model, we therefore hypothesize that MKL1 is recruited to the promoters of E_2-responsive genes in AF1 contexts which, by definition, would require low or no MKL1 activity. In these conditions, MKL1 is expected to be co-recruited with ERα, as demonstrated by our sequential ChIPs on given promoters. This may support a direct action of MKL1 on ERα activity by modulating the mobilization of coactivators and/or corepressors. For instance, one possible mechanism relies on the fact that the target of the Rho/actin/MKL1 pathway is the ERα AF1 box 1, located between amino acids 38 and 79, which is also involved in the control of ERα activity through E-cadherin-mediated cell contacts (10). This AF1 subregion constitutes an interaction surface for cofactors of the p160 and p300 families and synergistically bridges AF1 and AF2 activities (18, 34, 35). Importantly, the overexpression of Rho guanine nucleotide dissociation inhibitor potentiates the action of CBP/p300 on ERα transcriptional activity (36). Therefore, upon activation by Rho, MKL1 might inhibit the coactivation of ERα by CBP/p300. Rather than having an impact on cofactor recruitment, MKL1 action could also involve corepressors. Indeed, in HeLa cells, the dominant positive form of MKL1 allowed 4-OHT-bound ERα to repress the activity of the *C3*-LUC reporter gene. A high activity of MKL1 might therefore allow ligand-bound ERα to recruit corepressors such as the nuclear receptor corepressor or the silencing mediator of retinoic acid and the thyroid hormone receptor, which are known actors of the antagonistic actions of selective estrogen receptor mediators such as 4-OHT (37). Finally, MKL1 might act alternatively through a mechanism that is independent of its interaction with ERα. Such a hypothesis is strengthened by the fact that the association of MKL1 with the promoters of endogenous E_2-target genes appears to be mainly constitutive. In the absence of estradiol, MKL1 is always recruited to the tested promoters, whereas this is not systematically the case for ERα. Furthermore, the sequential ChIP experiments performed on the PR_A promoter indicate that MKL1 is likely to be recruited onto a subset of regulatory elements independently of ERα. Thus, MKL1 might regulate epigenetic marks such as histone acetylation or methylation, which indirectly could have an impact on ERα activity. Corroborating this hypothesis, we observed important variations in the basal activity of the luciferase reporter genes following overexpression of MKL1 and its deleted variants. Notably, ERE-driven (ERE-tk-LUC) and ERE-less reporter genes (c-*fos*-LUC and pTAL-LUC) behaved in a similar manner in response to MKL1 mutants while displaying totally different plasmid backbones. Only the amplitude of the responses differed. Accordingly, MKL1 might directly or indirectly affect the folding of the chromatin-like structure adopted by plasmids in the nucleus. Nevertheless, the correlation between a reduced transactivation potency of ERα and an increased basal activity of the reporter gene is not systematic. For instance, both ΔB- and ΔQ-deleted MKL1 forms reduced ERα activity but exerted opposite effects on the basal activity of some reporter genes. Consequently, the mechanisms underlying the MKL1 influence on ERα activity are likely different from those responsible for the variations of the basal activity of reporter genes. Studying more extensively the combinatorial recruitments of coactivators and corepressors on natural ERα target promoters as well as epigenetic regulations, in conditions where MKL1 exhibits distinct functional state, is now awaited to obtain further insights into the cell-specific mechanisms engaged by MKL1 to influence ERα transcriptional activity.

In conclusion, we provide evidence that the respective contribution exerted by AF1 and AF2 toward ERα transcriptional activity is controlled by the Rho/actin/MKL1 pathway. This implies that the transcriptional activity of ERα is greatly modified during the epithelial-mesenchymal transition, through a switch from a dominant AF1- to an AF2-permissive cell context. Changes in actin-dependent cell process, which occur during cell transformation and tumorigenesis, are thus likely to

have broad impacts on the transcriptional activity of estrogen receptors.

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