

ORIGINAL ARTICLE

mTORC1 directly phosphorylates and activates ER α upon estrogen stimulation

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Breast cancer is the leading cause of cancer-related deaths among women. Approximately 75% of breast cancers are estrogen receptor- α (ER α) positive, underscoring the dependence of cancer cells on estrogen for growth and survival. Patients treated with endocrine therapy often develop resistance, either *de novo* or acquired, which in some cases is caused by aberrations within the growth factor signaling pathways. The mechanistic target of rapamycin complex 1 (mTORC1) has emerged as a critical node in estrogenic signaling. We have previously shown that mTORC1 can phosphorylate and activate ER α on S167 via its effector—the 40S ribosomal S6 kinase 1 (S6K1). Presently, we have uncovered a direct link between mTORC1 and ER α . We found that ER α binds to regulatory-associated protein of mTOR (Raptor) and causes it to translocate to the nucleus upon estrogen stimulation. In addition, we identified mTOR as the kinase that phosphorylates ER α on S104/106 and activates transcription of ER target genes. Our findings show a direct link between mTORC1 and ER α , which further implicates mTORC1 signaling in the pathogenesis of ER-positive breast cancer and provides rationale for FDA-approved use of mTORC1 inhibitors in combination with endocrine agents for treatment of this disease.

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INTRODUCTION

Estradiol is a steroid hormone that has an important role in the development of several tissues, including the mammary gland.¹ Approximately two-thirds of all breast cancer cases are estrogen receptor (ER) positive, which underscores the dependence of cancer cells on estrogen for growth and survival.² ER α is a nuclear transcription factor that upon binding of its ligand estrogen can promote expression of growth and survival genes. For this reason, endocrine therapy has been a standard of care for many women with ER-positive breast cancer. Endocrine therapy agents can antagonize the ligand binding to ER α (tamoxifen and other selective ER modulators), downregulate ER expression (fulvestrant) or block estrogen biosynthesis (aromatase inhibitors). However, response rates vary between 35 and 70%, and patients often develop resistance, either *de novo* or acquired, to endocrine treatments.^{3,4} The mechanism of resistance is not well understood, however, studies have shown that growth factor receptor signaling pathways have a significant role.²

Importantly, the mechanistic target of rapamycin complex 1 (mTORC1) emerged as a critical node in estrogenic signaling in breast cancer cells. Estrogen rapidly and potently activates mTORC1 signaling, and conversely, mTORC1 is a crucial activator of ER α transcriptional activity.^{5–8} mTOR is a conserved serine/threonine kinase that is a key regulator of cell growth and proliferation in response to nutrient availability and growth factor signaling.⁹ In addition to mTOR itself, mTORC1 is composed of the regulatory-associated protein of mTOR (Raptor) which recruits mTOR substrates to the complex,^{10,11} the positive regulator mammalian lethal with SEC13 protein 8 (mLST8) also known as G β L,¹² the negative regulators 40 kDa proline-rich Akt substrate (PRAS40)^{13,14} and DEP domain containing mTOR-interacting protein (DEPTOR).¹⁵

The growth factor input to mTORC1 is mainly relayed via the phosphoinositide 3-kinase (PI3K) signaling pathway resulting in

inhibition of the tuberous sclerosis complex protein TSC2.^{16–18} TSC2 heterodimerizes with TSC1, and negatively regulates mTORC1 activity by acting as a GTPase-activating protein for the small GTPase Rheb.^{19,20} Rheb directly binds to mTOR and activates it in a GTP-dependent manner.²¹ Therefore, phosphorylation and inhibition of TSC2 leads to activation of mTORC1. Akt, acting downstream of active PI3K, phosphorylates TSC2 at S939 and T1462.²² In addition, Ras-activated ERK1/2 phosphorylates TSC2 at S664, also leading to the inactivation of TSC2.²³ Finally, RSK, acting downstream of ERK, has also been shown to directly phosphorylate TSC2 on S1798.²⁴ Therefore, several growth factor-stimulated signaling pathways converge on TSC2.

ER α activation and transcriptional activity is mainly mediated by the binding of its ligand-17 β -estradiol. Growth factors can also activate ER α , leading to multi-site phosphorylation of the receptor and ligand-independent activation.²⁵ We have previously demonstrated that mTORC1 promotes growth factor-mediated ER α activation by direct phosphorylation on S167.^{6,7} This phosphorylation, which is mediated by the mTORC1 effector 40S ribosomal S6 kinase 1 (S6K1), is important for ER α dimerization, DNA binding and transcriptional activity, is associated with endocrine resistance and correlates with therapy response.^{26,27} Moreover, in response to estrogen, ER α promotes expression of S6K1, generating a feed-forward positive activation loop.⁵ This biochemical relationship between the mTORC1 and ER α signaling pathways provides rationale for FDA-approved use of mTORC1 inhibitors in combination with endocrine agents for treatment of advanced ER-positive breast cancer.²⁸

There are two aspects of the relationship between ER α and growth factor signaling pathways that should be considered. First, signaling via the MAPK/ERK and PI3K pathways leads to activation of mTORC1 and can mediate estrogen-induced, tamoxifen-induced and ligand-independent ER α transcriptional activity.² Second,

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estrogen activates many intracellular signaling pathways, including MAPK, PI3K and mTORC1, ultimately potentiating ER activation and contributing to development of endocrine therapy resistance.²

Because phosphorylation of ER α by S6K1 only partially contributes to growth factor-stimulated ER α activation, we set out to investigate additional mTORC1-mediated inputs into ER α regulation. In the present study, we describe estrogen-regulated interaction between mTORC1 and ER α , which allows for raptor translocation into the nucleus, and phosphorylation of ER α on S104/106. Identification of this additional point of cross-talk between mTORC1 and ER α will pave way for better understanding of mechanisms of breast cancer pathogenesis and therapy response.

RESULTS

Estrogen stimulates nuclear raptor localization and interaction with ER α

We investigated whether a direct biochemical interaction between ER α and mTORC1 may explain the significant cross-talk between these signaling pathways. Raptor is the adaptor protein that recruits mTOR kinase to its substrates.^{29,30} Therefore, we first examined whether ER α and raptor interact in MCF7 cells, an ER-positive breast cancer cell line. Indeed, we found that estrogen potently stimulates raptor to translocate from the cytoplasm to the nucleus, where it co-localizes with ER α (Figure 1a). In addition, we found that raptor translocates to the nucleus upon estrogen stimulation in BT474 cells, another ER-positive breast cancer cell line (Figure 1b). Furthermore, we observed that the nuclear interaction between raptor and ER α occurs rapidly within 10 min of estrogen stimulation (Figure 1c). We confirmed that ER α and raptor interact by immunoprecipitation in transfected HEK293E whole-cell lysates (Figure 1d). Importantly, this interaction was stimulated by estrogen in MCF7 cells (Figure 1e). Because ER α is a transcription factor that translocates to the nucleus upon estrogen stimulation, we prepared nuclear and cytoplasmic cell fractions. As expected, estrogen-induced translocation of ER α into the nuclear fraction (Figure 1f). Using co-immunoprecipitation, we also found that estrogen is necessary for raptor recruitment to the nucleus and interaction with ER α (Figure 1f). In contrast to estrogen, tamoxifen, an antagonist of ER α in the breast, was not able to promote translocation of raptor to the nucleus (Figure 2a). Furthermore, tamoxifen treatment disrupted the interaction of ER α and raptor (Figure 2b).

mTOR binds and phosphorylates ER α through TOS motif

Since raptor is an adaptor protein for mTOR kinase that we found to interact with ER α , to verify the biochemical relevance of this interaction, we confirmed that mTOR co-immunoprecipitates with ER α and raptor (Figure 3a). We also found that mTOR co-localizes with ER α in the nucleus upon estrogen stimulation (Figure 3b). We next asked whether ER α could be a substrate for the mTOR kinase. We examined ER α protein sequence for a putative TOR signaling (TOS) motif, which mediates the interaction with raptor and subsequent phosphorylation by mTOR.^{29–33} Indeed, ER α protein contains a putative TOS motif in the extreme C-terminal region with a high degree of similarity to TOS motifs of other known mTOR substrates (Figure 4a). The putative TOS motif of ER α is also conserved among mammalian species (Figure 4b), further indicating potential functional significance for this domain.

Next, we sought to determine whether the TOS motif may mediate the interaction between ER α and raptor, which is necessary for mTOR binding. Therefore, we mutated the phenylalanine in position 591 of ER α to alanine (F/A ER), since it was previously shown that this residue is critical for the functionality of the TOS motif.^{29,30} Indeed, we found that unlike the wild-type (WT) ER, the F/A ER was severely handicapped in its ability to co-localize with raptor and recruit it to the nucleus

(Figure 4c). In addition, we found that raptor was unable to co-immunoprecipitate with TOS motif-deficient ER α (Figure 4d). Moreover, the interaction is specifically dependent on the TOS motif and not on transcriptionally-active ER α , since mutation of two other sites (S118 and S167) important for ER α activation did not abrogate the interaction with raptor (Figure 4d).

To determine whether mTOR recruitment to ER α results in phosphorylation, we analyzed phosphorylation of WT and F/A alleles of ER α . In order to determine the putative phosphorylation site, we employed a candidate approach. S104/106 are proline-directed sites, while S118 resides within a sequence that resembles a hydrophobic motif, which are phosphorylated by mTOR in its substrates 4E-BP1 and S6K1, respectively (reviewed in Fingar and Blenis,³⁴ and Hay and Sonenberg³⁵). Our results indicate that mutation of F591 reduced S104/106 phosphorylation compared to wild-type ER α , however, F/A mutation did not affect phosphorylation at S118 (Figure 4e). Immune complex kinase assay showed that F/A mutation dramatically reduced ER α phosphorylation by mTOR *in vitro* (Figure 4f).

In addition, we observed that mTOR is specifically responsible for S104/106 phosphorylation using immune kinase complex assay coupled with immunoblotting, as phosphorylation of these sites was greatly impaired in the F/A mutant (Figure 4g). We also determined that nuclear phosphorylation of ER α on S104/106 was greatly reduced in the mutant but not the wild-type ER α (Figure 4h), further indicating the importance of raptor recruitment to the nucleus for ER α phosphorylation and activation.

Raptor regulates ER α target genes

To demonstrate that raptor is necessary for ER α activation, we reduced expression of raptor using siRNA. We tested the efficacy of two siRNAs against raptor and observed approximately 60% reduction of raptor protein levels (Figures 5a and b). Raptor knockdown also reduced ER α localization to the nucleus in response to estrogenic stimulation as well as its phosphorylation on S104/106 (Figure 5c), further indicating that raptor is necessary for ER α activation. Next, to investigate the effect of raptor on ER α activity, we performed a luciferase reporter assay using reporter construct containing 3 estrogen response elements (ERE). We observed that raptor co-transfection significantly activated ERE transcription even in the absence of estrogen, and that this effect was further enhanced by estrogen stimulation. Importantly, co-transfection with siRNA against raptor significantly reduced ERE transcription to levels below those of the control and estrogen was unable to rescue it (Figure 5d).

To further understand the mechanism of raptor mediated ER α activation, we examined effects of raptor knockdown on ER α target gene expression. We observed that knockdown of raptor reduced estrogen-stimulated expression of ER α target genes such as CyclinD1 and TFF1, both on the levels of mRNA (Figure 5e) and protein (Figure 5f). In addition, we noted that the TOS motif is necessary for TFF1 activation because the F591A mutant of ER α had reduced ability to induce estrogen-stimulated TFF1 mRNA (Figure 5g) and protein expression (Figure 5h). Thus our results indicate that the interaction between ER α and raptor is necessary to recruit raptor to the nucleus, where mTOR phosphorylates and activates ER α . In addition, we showed that raptor is necessary for estrogen-induced cell migration, because knockdown of raptor in MCF7 cells significantly reduced cell migration as measured by the wound-healing assay (Figures 5i and j). Importantly, tamoxifen treatment of raptor knockdown cells did not further effect cell migration, indicating that in this setting, raptor and estrogen signaling function in the same pathway, further supporting our findings.

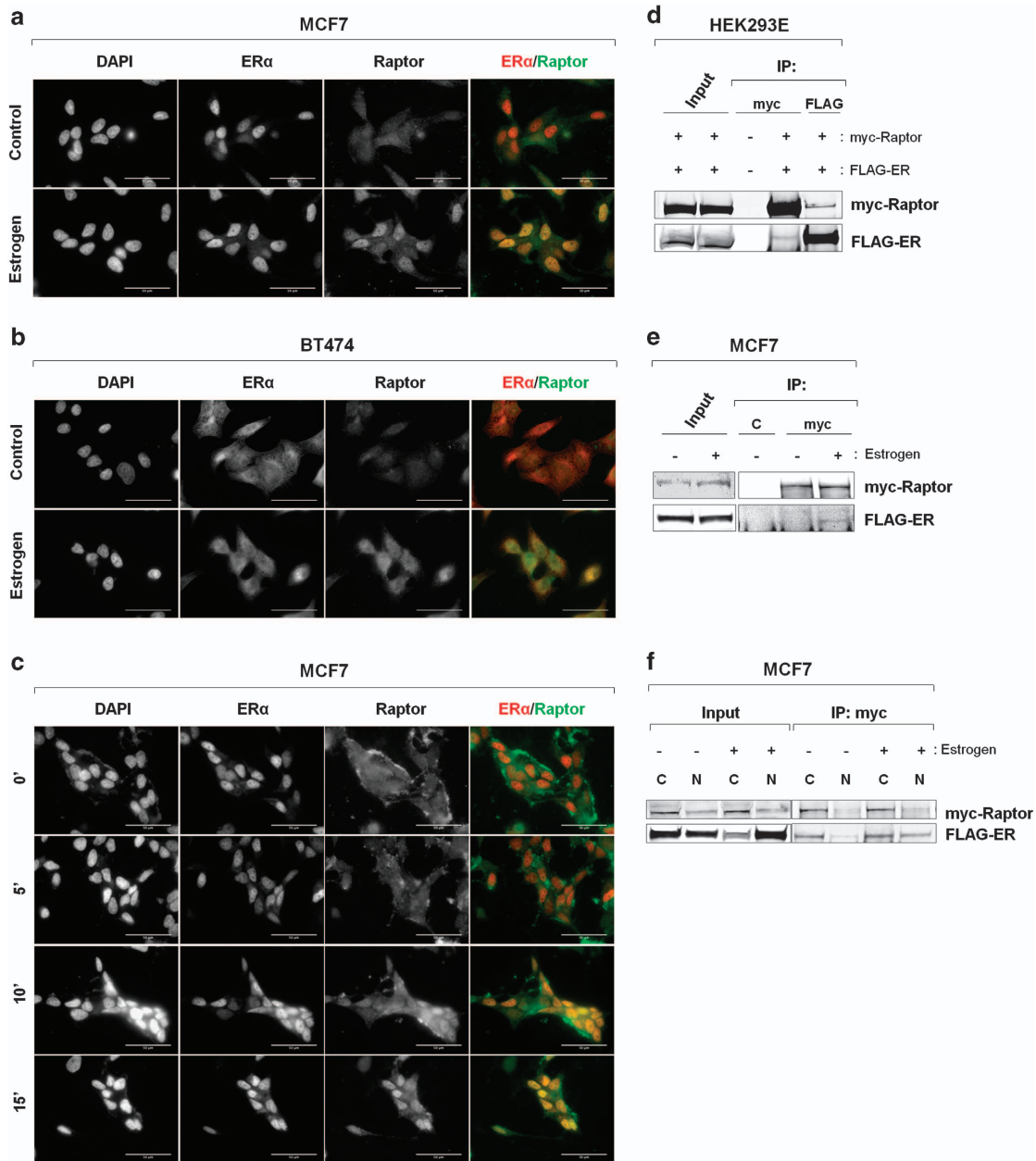


Figure 1. Raptor co-localizes with ER α in the nucleus upon estrogen stimulation. **(a)** MCF7 cells were serum-starved or stimulated with estrogen for 30 min. Immunofluorescence was performed as described in the Materials and methods section. Scale bar, 50 μ m. **(b)** BT474 cells were treated with estrogen as indicated and processed as described in **a**. **(c)** MCF7 cells were treated with estrogen as indicated, and processed as described in **a**. **(d)** HEK293E cells were co-transfected with myc-raptor and FLAG-ER constructs as indicated. FLAG or myc was immunoprecipitated as described in the Materials and methods section and proteins were detected by immunoblot. **(e)** MCF7 cells were co-transfected with myc-raptor and FLAG-ER and stimulated with estrogen for 30 min, as indicated. Raptor was immunoprecipitated and interaction with ER α is shown by immunoblot analysis. **(f)** MCF7 cells were co-transfected with myc-raptor and FLAG-ER, and nuclear (N) and cytoplasmic (C) fractionation was performed as described in the Materials and methods section. Raptor was immunoprecipitated and interaction with ER α was detected by immunoblot assay.

DISCUSSION

Hereby we describe a novel mechanism of estrogenic activation of ER α and directly implicate mTOR in ER α phosphorylation. Previous work from our lab has shown that there is a close interaction between mTORC1 and ER α signaling pathways, particularly through S6K1 phosphorylation and activation of ER α .^{6,7} In addition, it was found that Akt can phosphorylate ER α on S167³⁶ and that PI3K mediates AP-1/ER α cooperative transcription.³⁷ An additional link between PI3K/Akt/mTOR and ER signaling pathways hinged on the fact that Akt can activate ER α pathway in the absence of

estrogen, and the combination of endocrine therapy with mTOR inhibitors was able to overcome the endocrine therapy resistance.³⁸ These studies provided a mechanistic rationale for clinical use of aromatase inhibitors in conjunction with mTORC1 inhibitors.²⁸ Studies such as TAMRAD³⁹ and BOLERO-2²⁸ showed that combination of everolimus with either tamoxifen or aromatase inhibitors improved progression-free survival of ER-positive breast cancer patients. However, significant improvement in overall survival was not observed, underscoring the need for additional translational research into the mechanisms of mTOR inhibition in this setting.⁴⁰

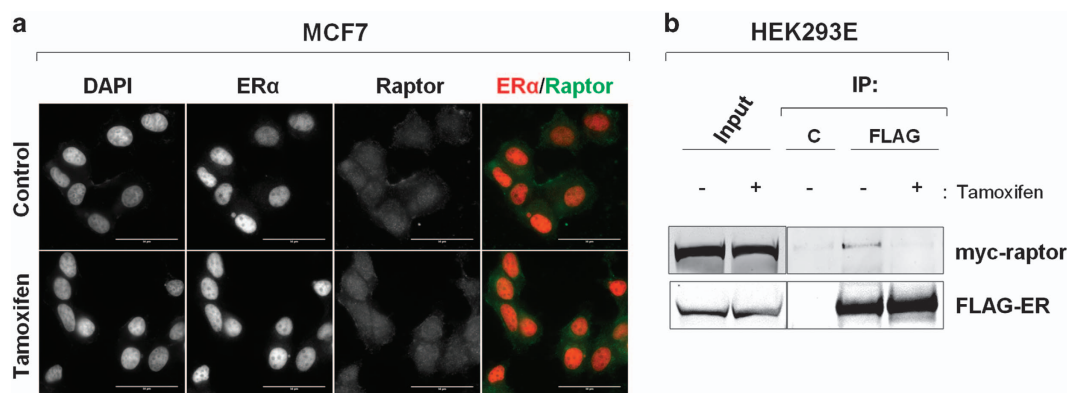


Figure 2. Tamoxifen disrupts ER α -Raptor interaction. (a) MCF7 cells were treated with tamoxifen for 1 h and immunofluorescence was performed as described in the Materials and methods section. Scale bar, 50 μ m. (b) HEK293E cells were co-transfected with myc-raptor and FLAG-ER. Cells were treated with tamoxifen for 1 h as indicated. ER was immunoprecipitated and interaction with raptor was detected by immunoblot assay.

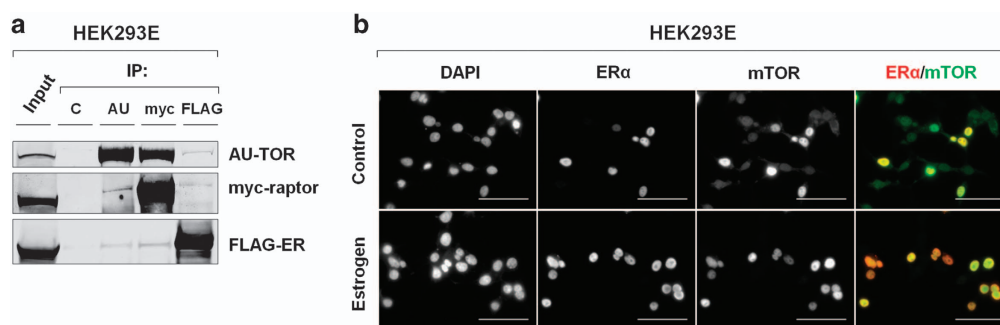


Figure 3. mTOR forms a complex with Raptor and ER α . (a) HEK293E cells were co-transfected with AU-TOR, myc-raptor and FLAG-ER, and immunoprecipitated. (C is no antibody control). The indicated proteins were detected by immunoblot assay. (b) HEK293E cells were transfected with FLAG-ER, and after serum starvation, cells were stimulated with estrogen for 30 min. Immunofluorescence was performed as described in the Materials and methods section. Scale bar, 50 μ m.

Studies of patients with hormone receptor-positive advanced breast cancer showed that acquired resistance to endocrine therapy is associated with aberrations within the PI3K/Akt/mTORC1 signaling pathways.⁴¹ Specifically, overexpression of oncogenes that activate this pathway was able to confer resistance to estrogen deprivation in ER-positive breast cancer cells and the anti-estrogen resistance was reversed with the use of PI3K inhibitors.^{36,42} In addition, long-term estrogen deprivation of ER-positive breast cancer cells resulted in hyperactivation of the PI3K/Akt/mTOR signaling and cell growth was inhibited with PI3K/mTOR inhibitors.⁴³ Clinical studies show that estrogen deprivation may be partially able to suppress PI3K/Akt/mTORC1 signaling in ER-positive breast cancer.^{44,45} For this reason, the PI3K/Akt/mTORC1 pathway is associated with endocrine therapy resistance and multiple inhibitors of this pathway are being tested in pre-clinical and clinical trials.⁴⁶

In our current work we identified a direct interaction between mTORC1 and ER α , further underscoring the close relationship between these signaling pathways. Specifically, we showed that in response to estrogen, ER α co-localizes with raptor in the nucleus. It is important to note that using biochemical and microscopy methods we observed ER α to be present both in the nucleus and the cytoplasm, whereas raptor was only observed in the cytoplasm of serum-starved MCF7 and BT474 cells. However, ER α translocated to the nucleus and rapidly co-localized with raptor upon estrogen stimulation. In contrast, in HEK293E cells we noted that overexpression of ER α results in constitutive nuclear localization of ER α and co-localization with mTORC1.

Interestingly, we found that interaction between raptor and ER α is mediated by a TOS motif within the extreme C-terminus of

ER α , expanding the repertoire of known TOS motif-containing mTORC1 substrates.³² We determined that TOS motif is necessary for phosphorylation of ER α on S104/106. These residues have been previously shown to be estrogen-sensitive and phosphorylated by MAPK.^{25,47} Because mTOR phosphorylates similar-sequence motifs within 4E-BP1, it is reasonable that mTOR may also be capable of phosphorylating S104/106 in ER α . Therefore, we propose a model whereby in the presence of estrogen, ER α binds to raptor via the TOS motif and recruits mTORC1 to the nucleus where mTOR phosphorylates ER α , leading to its activation and resulting in upregulation of estrogenic gene transcription (Figure 6). Our findings are further supported by previous work that showed that mTOR can directly regulate activity of other transcription factors such as STAT3,⁴⁸ HIF1 α ,³³ TFEB,⁴⁹ ERR α ,⁵⁰ PGC1 α and YY-1.⁵¹ Moreover, mTOR can associate with chromatin to regulate expression of rRNA genes.⁵² Previous studies that focused on understanding the cross-talk between ER α and MAPK signaling pathways have described a similar mechanism, whereby estrogen-activated ER α co-localizes with ERK2 in the nucleus and leads to receptor-mediated transcription of estrogen-dependent genes involved in cell proliferation.^{53,54} Together with these studies, our work supports the model of ER α nuclear localization in association with its activating kinase. Though it is unclear whether ER α -raptor interaction is mediated through estradiol-mediated activation of membrane-bound ER α or directly through unbound ER α ,² Chambliss *et al.*⁵⁵ showed that MCF7-cell breast cancer xenograft growth was specifically stimulated by estrogen and not by the estrogen-dendrimer conjugate, which is excluded from the nucleus, further indicating that it is the nuclear ER α functions that are activated in breast cancer.

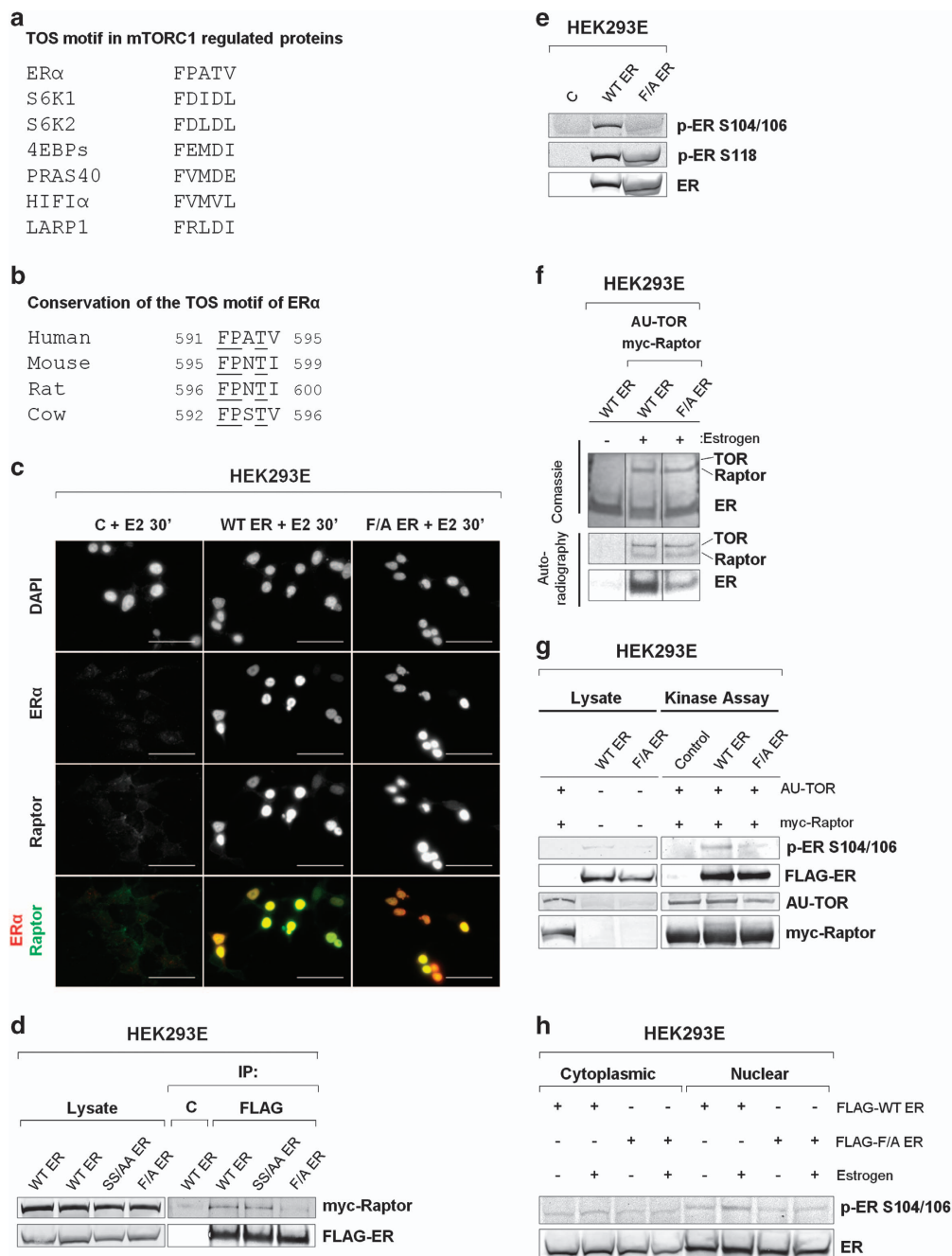


Figure 4. mTORC1 interacts with ERα via the TOS motif and phosphorylates ERα on S104/106. **(a)** Alignment of TOS motif of ERα with TOS motifs of known mTOR targets. **(b)** Conservation of ERα TOS motifs. **(c)** HEK293E cells were transfected with WT or F/A ER. Immunofluorescence was performed as described in the Materials and methods section with the indicated antibodies. **(d)** HEK293E cells were transfected with WT, F/A ER or S118A/S167A (SS/AA) alleles of ER and immunoprecipitated with FLAG. (C is no antibody control). The indicated proteins were detected by immunoblot assay. **(e)** HEK293E cells were transfected with WT or F/A ER. (C is empty vector). Lysates were generated as described in the Materials and methods section and the indicated proteins were detected by immunoblot. **(f)** Cells were treated with estrogen for 30 min as indicated. Immune complex kinase assay was performed as described in the Materials and methods section using WT or F/A ER as substrate. **(g)** Cells were treated with estrogen for 30 min, immune complex kinase assay using WT or F/A ER as substrate was performed as described in the Materials and methods section and the indicated proteins were detected by immunoblot. **(h)** HEK293E cells were transfected with WT or F/A ER and stimulated with estrogen for 30 min as indicated. Nuclear and cytoplasmic extraction was performed as described in the Materials and methods section and indicated proteins were detected by immunoblot.

Our results are exciting because we demonstrate that ERα is activated by estrogen via direct interaction with mTORC1, and by S6K1 in response to growth factors, illustrating the robustness of mTORC1 contribution to ERα activation. Thus, a growing body of evidence supports the importance of the cross-talk between ERα and

PI3K/Akt/mTORC1 signaling to ensure robust activation of oncogenic signaling in breast cancer cells. This knowledge will allow us to advance our understanding of the pathogenesis of hormone-positive breast cancers, as well as the mechanisms of response and resistance to endocrine therapy, paving way for future advances in the clinic.

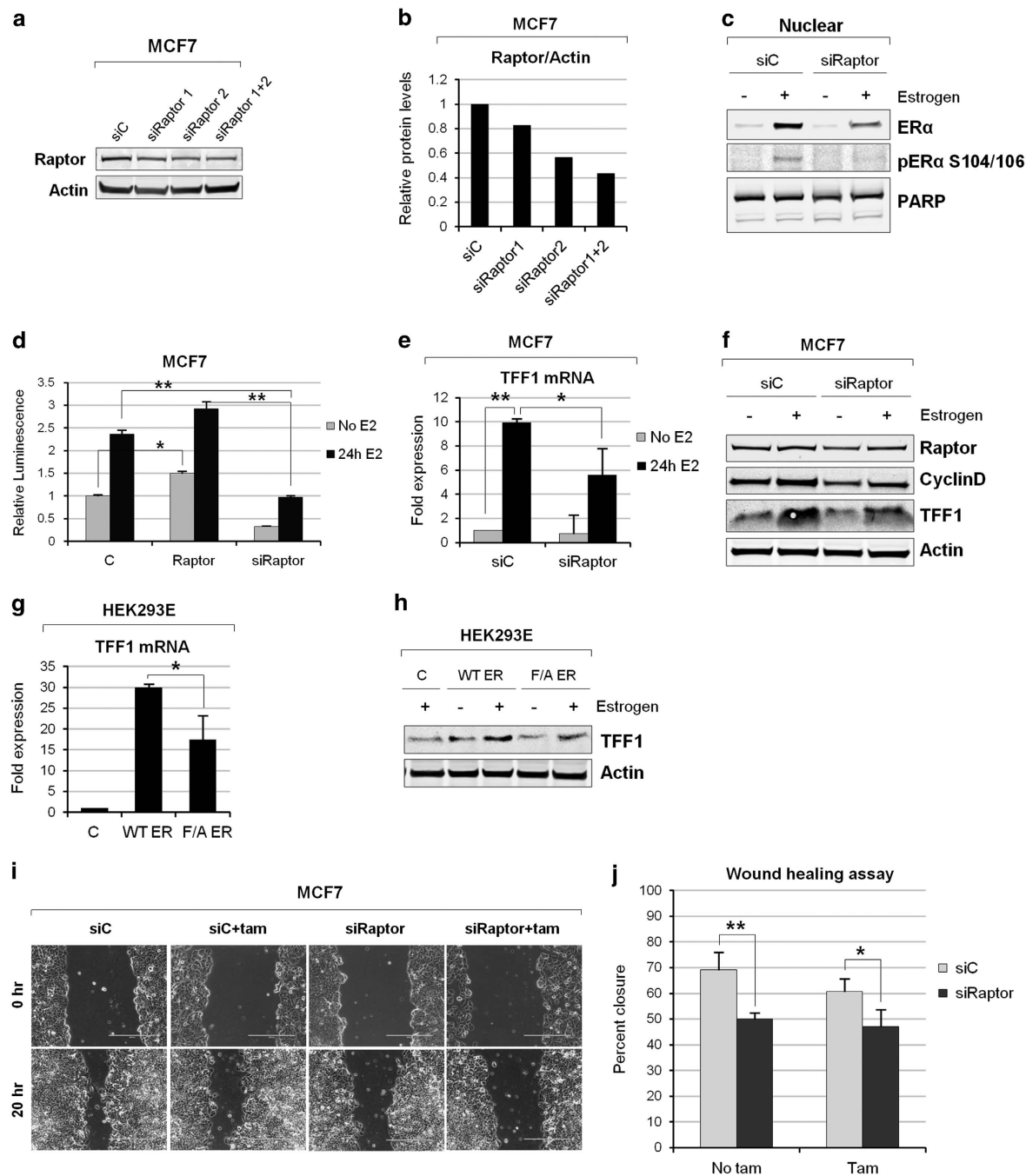


Figure 5. Raptor regulates ER α activity. **(a)** MCF7 cells were transfected with scrambled siRNA, or siRNAs against raptor alone or in combination and probed with the indicated antibodies. **(b)** Quantification of raptor normalized to actin from the western blot in **a** was performed using Odyssey Image Studio Version 4.0 and graphed using Excel. **(c)** MCF7 cells were transfected with either scrambled siRNA or siRNA against raptor. Cells were stimulated with estrogen for 30 min as indicated and probed with the indicated antibodies. **(d)** MCF7 cells were transfected, stimulated with estrogen for 30 min as indicated and Luciferase reporter assay was performed as described in the Materials and methods section. Data was plotted using Excel. $*P < 0.05$ and $**P < 0.01$. **(e)** MCF7 cells were transfected with non-specific si or si against Raptor. RT-qPCR was performed as described in the Materials and methods section and data were plotted using Excel. $*P < 0.05$. $**P < 0.001$ $n = 3$. **(f)** MCF7 cells were transfected as described in the Materials and methods section, stimulated with estrogen for 24 h as indicated and protein levels were detected using immunoblot assay. **(g)** HEK293E cells were transfected with WT or F/A ER. (C is empty vector). RT-qPCR was performed as described in the Materials and methods section and data were plotted using Excel. $*P < 0.05$. $n = 3$. **(h)** HEK293E cells were transfected as described in the Materials and methods section, stimulated with estrogen for 24 h as indicated and protein levels were detected by immunoblot assay. **(i)** MCF7 cells were transfected with either scrambled siRNA or siRNA against raptor and treated with 4-hydroxy-tamoxifen (tam, 100 nM) as indicated. Wound-healing assay was performed as described in the Materials and methods section. **(j)** Quantification of the wound-healing assay from **i** was plotted using Excel. $*P < 0.05$. $**P < 0.01$.

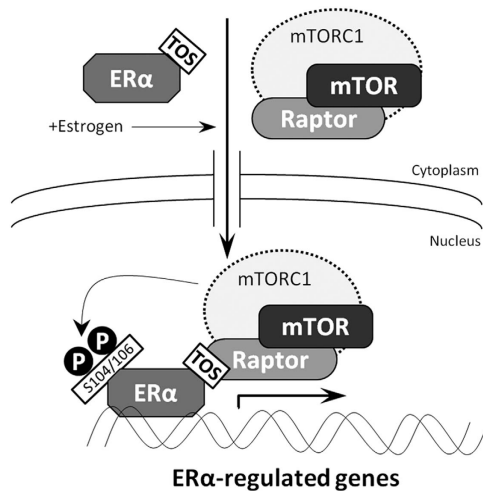


Figure 6. Mechanism of ER α -raptor interaction. In the presence of estrogen, ER α binds to raptor via the TOS motif and recruits mTORC1 to the nucleus where mTOR phosphorylates ER α , leading to its activation and resulting in upregulation of estrogenic gene transcription.

MATERIALS AND METHODS

Cell Culture and Treatment

HEK293E cells were kindly provided by John Blenis (Weill Cornell Medical College, New York, NY, USA). MCF7 and BT474 cells were obtained from American Type Culture Collection (Manassas, VA, USA). HEK293E, MCF7 and BT474 cells were cultured in a humidified incubator with 5% CO₂ at 37 °C in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For experiments, cells were grown in phenol red-free media with 10% charcoal-stripped fetal bovine serum for 3 days. Where indicated, cells were starved in serum-free media for 24 h and stimulated with 10 nM estrogen or 100 nM 4-hydroxy-tamoxifen (in ethanol).

Transfection

HEK293E cells were transfected using calcium phosphate method as previously described.⁵⁶ MCF7 cells were transfected with plasmid DNA using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA). For siRNA transfection, 50 pmol of siRNA was transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Grand Island, NY, USA) according to manufacturer's protocol. Cells were lysed 48 h post-transfection.

Constructs

siRNAs against Raptor (1 and 2) were ordered from Intergrated DNA technologies (HSC.RNAI.N001163034.12 and HSC.RNAI.N020761.12.1). Reporter plasmids used for the luciferase assay were previously described.⁶ The mTOR and raptor expression constructs were kindly provided by John Blenis (Weill Cornell Medical College, New York, NY, USA). The pCMV5-FLAG-WT ER plasmid was previously described.⁵ The S118A/S167A and F591A ER constructs were generated by site-directed mutagenesis performed by GeneWiz (South Plainfield, NJ, USA).

Immunofluorescence

Cells were plated on 18 mm Poly-L-Lysine-coated cover slips (Fisher Scientific, Waltham, MA, USA). Following treatment, cells were fixed in 4% paraformaldehyde and permeabilized in 0.2% Triton-X. After blocking and incubation with primary and secondary antibodies (as indicated), cover slips were mounted using DAPI-Fluoromount G mounting media (Southern Biotech, Birmingham, AL, USA). Images were collected using EVOS FL Auto microscope (Invitrogen) under $\times 60$ magnification.

Cell Lysis

Cells were lysed in ice-cold buffer containing 10 mM KPO₄, 1 mM EDTA, 10 mM MgCl₂, 50 mM β -glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40 (NP-40), 0.1% Brij 35, 1 mM sodium orthovanadate, 40 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, 10 μ g/ml aprotinin. For co-immunoprecipitation experiments to detect mTOR, cells were lysed in

buffer containing 40 mM HEPES, pH 7.5, 120 mM NaCl, 50 mM NaF, 1 mM EDTA, 50 mM β -glycerophosphate, 0.3% CHAPS, 40 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, 10 μ g/ml aprotinin. Lysates were cleared of insoluble material by centrifugation at 15 000 g for 10 min at 4 °C. NE-PER kit (Pierce, Grand Island, NY, USA) was used for nuclear-cytoplasmic fractionation.

Protein concentration in cell extracts was measured by Bradford reagent (BioRAD, Hercules, CA, USA) according to the manufacturer's protocol using Eppendorf BioPhotometer (Eppendorf, Hauppauge, NY, USA). Samples were equalized for protein concentration and denatured using LDS Sample buffer and Reducing agent (Invitrogen) at 70 °C for 10 min. Samples were resolved using Bis-Tris Plus gels (Invitrogen) and transferred onto nitrocellulose membrane (GE Healthcare, Pittsburgh, PA, USA). Membranes were probed with the following primary antibodies: myc (9E11; Covance, Princeton, NJ, USA), AU-1 (MMS130R Covance), FLAG (F3165; Sigma, St Louis, MO, USA), PARP (ab32071; Abcam, Cambridge, UK), raptor (sc81537; Santa Cruz Biotechnology, Dallas, TX, USA), ER (sc8005; Santa Cruz Biotechnology), cyclinD1 (sc718; Santa Cruz Biotechnology), actin (sc1615; Santa Cruz Biotechnology), p-ER S104/106 (25175; Cell Signaling Technologies, Danvers, MA, USA), p-ER S118 (25115; Cell Signaling Technologies), TFF1 (124195; Cell Signaling Technologies).

Signal detection and quantification was accomplished using IRDye-conjugated anti-rabbit (LI-COR, 827-08365), anti-mouse (LI-COR, 926-68070) or anti-goat (LI-COR, 926-68074) secondary antibodies using Odyssey infrared detection instrument (LI-COR, Lincoln, NE, USA). All immunoblots were performed at least thrice to ensure reproducibility.

Immunoprecipitations and Immune Complex Kinase Assays

Lysates were immunoprecipitated with the indicated antibodies. Proteins were resolved and immunoblotted as described above. For kinase assays, immunoprecipitates were stringently washed once in 1 ml each of buffer A (20 mM Tris, 500 mM NaCl, 1 mM EDTA, 20 mM β -glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 40 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, 10 μ g/ml aprotinin), buffer B (10 mM HEPES, 50 mM β -glycerophosphate, 50 mM NaCl, 1 mM sodium orthovanadate, 40 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 5 μ g/ml pepstatin, 10 μ g/ml aprotinin) and resuspended in 10 mM HEPES. Samples were incubated in kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 50 mM β -glycerophosphate and 10 mM MnCl₂), and 1 mM ATP at 30 °C for 1 h while shaking. In all, 5 μ Ci of (γ -³²P)ATP was added to the mix, where indicated, and samples were incubated at 30 °C for 30 min. Reactions were subjected to electrophoresis on 4–12% SDS-polyacrylamide gels, and the amount of ³²P incorporated was assessed by autoradiography using phosphorimaging analysis (BioRad) or by immunoblotting with phospho-specific antibodies.

Luciferase Reporter Assay

MCF7 cells were transiently transfected with a reporter plasmid containing 3 EREs controlling the expression of firefly luciferase, and a control *Renilla* luciferase construct as previously described.⁶ Raptor expression plasmid or siRNA against raptor were co-transfected, as indicated in figure legends. Luciferase expression following cell treatment with estrogen was assayed using a dual luciferase kit and GloMax 20/20 Luminometer (Promega). Assays were performed in triplicates and results were analyzed and plotted using Microsoft Excel (Redmond, WA, USA).

Quantitative RT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) and 1 μ g of RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (BioRAD) and C1000 thermal cycler (BioRAD). For qPCR, cDNA was amplified with iQ SYBR Green Supermix (BioRAD) in CFX96 Real-Time PCR Detection System (BioRAD) with CFX Manager analysis on-board software.

TFF1 F: 5'-ATC GAC GTC CCT CCA GAA GAG-3'
TFF1 R: 5'-CTC TGG GAC TAA TCA CCG TGC TG-3'
18sF: 5'-TTC GAA CGT CTG CCC TAT CAA-3'
18sR: 5'-ATG GTA GGC ACG GCG ACT A-3'

Wound-Healing Assay

Cells were seeded in 12-well plates in complete Dulbecco's modified Eagle's media, transfected with either scrambled siRNA or siRNA against raptor and grown to confluency in monolayer overnight. Diagonal wound/scratch was created using a 200 μ l pipette tip. Cell debris were removed by

washing once with PBS, followed by addition of fresh media containing in the presence or absence of tamoxifen 100 nM (in ethanol) for 20 h. For each condition three different areas were imaged and three measurements were taken per each image. Images were collected and migration distance was measured using an EVOS FL Auto microscope and software at $\times 10$ magnification. Scale bars, 400 μ m.

Statistical analysis

Data are presented as mean \pm s.d. and $n=3$. Statistical significance was determined by paired Student's *t*-test using Microsoft Excel.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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