mTORC1 directly phosphorylates and activates ERα upon estrogen stimulation

A Alayev[1], **R S Salamon**[1], **S M Berger**[1], **N S Schwartz**[1], **R Cuesta**[1], **R B Snyder**[1], and **M K Holz**[1,2]

[1]Department of Biology; Yeshiva University; New York, NY USA

[2]Department of Biology; Yeshiva University; New York, NY USA; Department of Molecular Pharmacology and the Albert Einstein Cancer Center; Albert Einstein College of Medicine; Bronx, NY USA

Abstract

Breast cancer is the leading cause of cancer-related deaths among women. Approximately 75% of breast cancers are estrogen receptor α (ERα) 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 ERα on S167 via its effector the 40S ribosomal S6 kinase 1 (S6K1). Presently, we have uncovered a direct link between mTORC1 and ERα. We found that ERα binds to regulatory-associated protein of mTOR (Raptor) and causes it to translocate to the nucleus upon estrogen stimulation. Additionally, we identified mTOR as the kinase that phosphorylates ERα on S104/106 and activates transcription of ER target genes. Our findings show a direct link between mTORC1 and ERα, 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.

Keywords

mTORC1; Raptor; ERα; Estrogen; Breast Cancer

Introduction

Estradiol is a steroid hormone that plays 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

Corresponding Author: Dr. Marina K. Holz, 245 Lexington Ave. Bio 341, New York, NY 10016, Phone: 212-340-7838, Fax: 212-340-7883, mholz@yu.edu.

Conflict of Interest: Authors declare no conflict of interest

on estrogen for growth and survival (2). ERα 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 ligand binding to ERα (tamoxifen and other selective ER modulators [SERMs]), downregulate ER expression (fulvestrant), or block estrogen biosynthesis (aromatase inhibitors [AIs]). 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 play 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 ERα 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 GβL (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 (GAP) 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 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.

ERα activation and transcriptional activity is mainly mediated by the binding of its ligand 17β-estradiol. Growth factors can also activate ERα, leading to multi-site phosphorylation of the receptor and ligand-independent activation (25). We have previously demonstrated that mTORC1 promotes growth factor-mediated ERα 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 ERα dimerization, DNA binding and transcriptional activity, is associated with endocrine resistance and correlates with therapy response (26, 27). Moreover, in response to estrogen, ERα promotes expression of S6K1, generating a feed-forward positive activation loop (5). This biochemical relationship between the mTORC1 and ERα 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 ERα 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 ERα transcriptional activity (2). Second, 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 ERα by S6K1 only partially contributes to growth factor-stimulated ERα activation, we set out to investigate additional mTORC1-mediated inputs into ERα regulation. In the present study, we describe estrogen-regulated interaction between mTORC1 and ERα, which allows for raptor translocation into the nucleus, and phosphorylation of ERα on S104/106. Identification of this additional point of cross-talk between mTORC1 and ERα will pave way for better understanding of mechanisms of breast cancer pathogenesis and therapy response.

Results

Estrogen stimulates nuclear raptor localization and interaction with ERα

We investigated whether a direct biochemical interaction between ERα and mTORC1 may explain the significant cross-talk between these signaling pathways. Raptor is the adapter protein that recruits mTOR kinase to its substrates (29, 30). Therefore, we first examined whether ERα 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 ERα (Figure 1a). Additionally, 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 ERα occurs rapidly within 10 minutes of estrogen stimulation (Figure 1c). We confirmed that ERα 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 ERα 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 ERα 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 ERα (Figure 1f). In contrast to estrogen, tamoxifen, an antagonist of ERα in the breast, was not able to promote translocation of raptor to the nucleus (Figure 2a). Furthermore, tamoxifen treatment disrupted the interaction of ERα and raptor (Figure 2b).

mTOR binds and phosphorylates ERα through TOS motif

Since raptor is an adaptor protein for mTOR kinase that we found to interact with ERα, to verify the biochemical relevance of this interaction, we confirmed that mTOR co-immunoprecipitates with ERα and raptor (Figure 3a). We also found that mTOR co-localizes with ERα in the nucleus upon estrogen stimulation (Figure 3b). We next asked whether ERα could be a substrate for the mTOR kinase. We examined ERα protein sequence for a putative TOR signaling (TOS) motif, which mediates the interaction with raptor and

subsequent phosphorylation by mTOR (29–33). Indeed, ERα 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 ERα 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 ERα and raptor, which is necessary for mTOR binding. Therefore, we mutated the phenylalanine in position 591 of ERα 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). Additionally, we found that raptor was unable to co-immunoprecipitate with TOS motif-deficient ERα (Figure 4d). Moreover, the interaction is specifically dependent on the TOS motif and not on transcriptionally-active ERα, since mutation of two other sites (S118 and S167) important for ERα activation did not abrogate the interaction with raptor (Figure 4d).

To determine whether mTOR recruitment to ERα results in phosphorylation, we analyzed phosphorylation of WT and F/A alleles of ERα. 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 34, 35). Our results indicate that mutation of F591 reduced S104/106 phosphorylation compared to wild-type ERα, however, F/A mutation did not affect phosphorylation at S118 (Figure 4e). Immune complex kinase assay showed that F/A mutation dramatically reduced ERα phosphorylation by mTOR *in vitro* (Figure 4f).

Additionally, 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 ERα on S104/106 was greatly reduced in the mutant but not the wild-type ERα (Figure 4h), further indicating the importance of raptor recruitment to the nucleus for ERα phosphorylation and activation.

Raptor regulates ERα target genes

To demonstrate that raptor is necessary for ERα 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 (Figure 5a and b). Raptor knockdown also reduced ERα 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 ERα activation. Next, to investigate the effect of raptor on ERα 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 ERα activation, we examined effects of raptor knockdown on ERα target gene expression. We observed that knockdown of raptor reduced estrogen-stimulated expression of ERα target genes such as CyclinD1 and TFF1, both on the levels of mRNA (Figure 5e) and protein (Figure 5f). Additionally, we noted that the TOS motif is necessary for TFF1 activation because the F591A mutant of ERα had reduced ability to induce estrogen stimulated TFF1 mRNA (Figure 5g) and protein expression (Figure 5h). Thus our results indicate that the interaction between ERα and raptor is necessary to recruit raptor to the nucleus, where mTOR phosphorylates and activates ERα. Additionally, 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 5j). 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.

Discussion

Hereby we describe a novel mechanism of estrogenic activation of ERα and directly implicate mTOR in ERα phosphorylation. Previous work from our lab has shown that there is a close interaction between mTORC1 and ERα signaling pathways, particularly through S6 kinase 1 phosphorylation and activation of ERα (6, 7). Additionally, it was found that Akt can phosphorylate ERα on S167 (36) and that PI3K mediates AP-1/ERα cooperative transcription (37). An additional link between PI3K/Akt/mTOR and ER signaling pathways hinged on the fact that Akt can activate ERα pathway in the absence of estrogen, and the combination of endocrine therapy with mTOR inhibitors was able to overcome 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 TAMRAD (39) and BOLERO-2 (28) showed that combination of everolimus with either tamoxifen or aromatase inhibitors improved progression-free survival of ER-positive breast cancer patients. However, significantly improvement in overall survival was not observed, underscoring the need for additional translational research into the mechanisms of mTOR inhibition in this setting (40).

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 antiestrogen resistance was reversed with the use of PI3K inhibitors (36, 42). Additionally, 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 ERα, further underscoring the close relationship between these signaling pathways. Specifically, we showed that in response to estrogen, ERα co-localizes with raptor in the nucleus. It is important to note that using biochemical and microscopy methods we observed ERα to be present both in the nucleus and the cytoplasm, while raptor was only observed in the cytoplasm of serum-starved MCF7 and BT474 cells. However, ERα translocated to the nucleus and rapidly co-localized with raptor upon estrogen stimulation. In contrast, in HEK293E cells we noted that overexpression of ERα results in constitutive nuclear localization of ERα and co-localization with mTORC1.

Interestingly, we found that interaction between raptor and ERα is mediated by a TOS motif within the extreme C-terminus of ERα, expanding the repertoire of known TOS motif-containing mTORC1 substrates (47). We determined that TOS motif is necessary for phosphorylation of ERα on S104/106. These residues have been previously shown to be estrogen-sensitive and phosphorylated by MAPK (25, 48). Because mTOR phosphorylates similar sequence motifs within 4E-BP1, it is reasonable that mTOR may also capable of phosphorylating S104/106 in ERα. Therefore, we propose a model whereby in the presence of estrogen, ERα binds to raptor via the TOS motif and recruits mTORC1 to the nucleus where mTOR phosphorylates ERα, 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 (49), HIF1α (33), TFEB (50), ERRα (51), PGC1α and YY-1 (52). Moreover, mTOR can associate with chromatin to regulate expression of rRNA genes (53). Previous studies focused on understanding the cross-talk between ERα and MAPK signaling pathways have described a similar mechanism, whereby estrogen-activated ERα co-localizes with ERK2 in the nucleus and leads to receptor-mediated transcription of estrogen-dependent genes involved in cell proliferation (54, 55). Together with these studies, our work supports the model of ERα nuclear localization in association with its activating kinase. Though it is unclear whether ERα-raptor interaction is mediated through estradiol-mediated activation of membrane-bound ERα or directly through unbound ERα (2), Chambliss et. al. showed that MCF7-cell breast cancer xenoraft growth was specifically stimulated by estrogen and not by the estrogen-dendrimer conjugate (EDC), which is excluded from the nucleus, further indicating that it is the nuclear ERα functions that are activated in breast cancer (56).

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 crosstalk 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.

Materials and Methods

Cell Culture and Treatment

HEK293E cells were kindly provided by John Blenis (Weill Cornell Medical College, New York, NY). MCF7 and BT474 cells were obtained from American Type Culture Collection (ATCC). HEK293E, MCF7 and BT474 cells were cultured in a humidified incubator with 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). For experiments, cells were grown in phenol red-free media with 10% charcoal-stripped FBS for 3 days. Where indicated, cells were starved in serum-free media for 24h and stimulated with 10nM estrogen or 100nM 4-hydroxy-tamoxifen (in ethanol).

Transfection

HEK293E cells were transfected using calcium phosphate method as previously described (57). MCF7 cells were transfected with plasmid DNA using FuGENE® HD Transfection Reagent (Promega). For siRNA transfection, 50pmol of siRNA was transfected using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to manufacturer's protocol. Cells were lysed 48h 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). 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).

Immunofluorescence

Cells were plated on 18mm Poly-L-Lysine coated cover slips (Fisher Scientific). Following treatment, cells were fixed in 4% parafalmaldehyde 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). Images were collected using EVOS FL Auto microscope (Invitrogen, Grand Island, NY) under 60X magnification.

Cell Lysis

Cells were lysed in ice-cold buffer containing 10 mM KPO_4, 1 mM EDTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 5 mM EGTA, 0.5% Nonidet P-40 [NP-40], 0.1% Brij 35, 1 mM sodium orthovanadate, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin. For co-immunoprecipition experiments to detect mTOR, cells were lysed in buffer containing 40mM HEPES, pH 7.5, 120mM NaCl, 50mM NaF, 1mM EDTA, 50mM β-glycerophosphate, 0.3% CHAPS, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 10 μg/ml aprotinin). Lysates were cleared of

insoluble material by centrifugation at 15,000*g* for 10 min at 4°C. NE-PER kit (Pierce) was used for nuclear-cytoplasmic fractionation.

Protein concentration in cell extracts was measured by Bradford reagent (BioRAD) according to the manufacturer's protocol using Eppendorf BioPhotometer (Eppendorf, Hauppauge, NY). 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). Membranes were probed with the following primary antibodies: myc (9E11 Covance), AU-1 (MMS130R Covance), FLAG (F3165 Sigma), PARP (ab32071 Abcam), raptor (sc81537 Santa Cruz Biotechnology), ER (sc8005 Santa Cruz Biotechnology), cyclin D1 (sc718 Santa Cruz Biotechnology), actin (sc1615 Santa Cruz Biotechnology), p-ER S104/106 (2517S Cell Signaling Technologies), p-ER S118 (2511S Cell Signaling Technologies), TFF1 (12419S 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). 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, 5mM EGTA, 1 mM sodium orthovanadate, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 10 µg/ml aprotinin), buffer B (10 mM HEPES, 50 mM β-glycerophosphate, 50 mM NaCl, 1 mM sodium orthovanadate, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 5 µg/ml pepstatin, 10 µg/ml aprotinin) and resuspended in 10mM HEPES. Samples were incubated in kinase buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 50mM β-glycerophosphate and 10mM $MnCl_2$), and 1mM ATP at 30°C for 1 h while shaking. 5 µCi of [γ-^{32}P]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 ^{32}P incorporated was assessed by autoradiography using phosphoimaging analysis (Bio-Rad) or by immunoblotting with phospho-specific antibodies.

Luciferase Reporter Assay

MCF7 cells were transiently transfected with a reporter plasmid containing 3 estrogen response elements (ERE) controlling 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, Madison, WI). Assays were performed in triplicates and results were analyzed and plotted using Excel.

Quantitative RT-PCR

RNA was isolated using RNeasy® Mini Kit (Qiagen) and 1μg 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, Hercules, CA) 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′
>
> 18s F: 5′ TTC GAA CGT CTG CCC TAT CAA 3′
>
> 18s R: 5′ ATG GTA GGC ACG GCG ACT A 3′

Wound Healing Assay

Cells were seeded in 12-well plates in complete DMEM media, transfected with either scrambled siRNA or siRNA against raptor and grown to confluency in monolayer overnight. Diagonal wound/scratch was created using a 200μl 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 100nM (in ethanol) for 20h. 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 10X magnification. Scale bars represent 400μm.

Statistical analysis

Data are presented as mean ± S.D. and n = 3. Statistical significance was determined by paired Student's t-test using Microsoft Excel.

Acknowledgments

References

1. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine reviews. 1999; 20(3):358–417. [PubMed: 10368776]

2. Miller TW, Balko JM, Arteaga CL. Phosphatidylinositol 3-kinase and antiestrogen resistance in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011; 29(33):4452–61. [PubMed: 22010023]

3. Ellis M. Overcoming endocrine therapy resistance by signal transduction inhibition. The oncologist. 2004; 9 (Suppl 3):20–6. [PubMed: 15163844]

4. Miller WR, Larionov AA. Understanding the mechanisms of aromatase inhibitor resistance. Breast cancer research : BCR. 2012; 14(1):201. [PubMed: 22277572]

5. Maruani DM, Spiegel TN, Harris EN, Shachter AS, Unger HA, Herrero-Gonzalez S, et al. Estrogenic regulation of S6K1 expression creates a positive regulatory loop in control of breast cancer cell proliferation. Oncogene. 2012; 31(49):5073–80. [PubMed: 22286763]

6. Yamnik RL, Digilova A, Davis DC, Brodt ZN, Murphy CJ, Holz MK. S6 kinase 1 regulates estrogen receptor alpha in control of breast cancer cell proliferation. The Journal of biological chemistry. 2009; 284(10):6361–9. [PubMed: 19112174]

7. Yamnik RL, Holz MK. mTOR/S6K1 and MAPK/RSK signaling pathways coordinately regulate estrogen receptor alpha serine 167 phosphorylation. FEBS letters. 2010; 584(1):124–8. [PubMed: 19925796]

8. Yu J, Henske EP. Estrogen-induced activation of mammalian target of rapamycin is mediated via tuberin and the small GTPase Ras homologue enriched in brain. Cancer research. 2006; 66(19): 9461–6. [PubMed: 17018601]

9. Alayev A, Holz MK. mTOR signaling for biological control and cancer. Journal of cellular physiology. 2013; 228(8):1658–64. [PubMed: 23460185]

10. Hara K, Maruki Y, Long X, Yoshino K, Oshiro N, Hidayat S, et al. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 2002; 110(2):177–89. [PubMed: 12150926]

11. Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, et al. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 2002; 110(2):163–75. [PubMed: 12150925]

12. Kim DH, Sarbassov DD, Ali SM, Latek RR, Guntur KV, Erdjument-Bromage H, et al. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Molecular cell. 2003; 11(4):895–904. [PubMed: 12718876]

13. Sancak Y, Thoreen CC, Peterson TR, Lindquist RA, Kang SA, Spooner E, et al. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Molecular cell. 2007; 25(6):903–15. [PubMed: 17386266]

14. Vander Haar E, Lee SI, Bandhakavi S, Griffin TJ, Kim DH. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nature cell biology. 2007; 9(3):316–23. [PubMed: 17277771]

15. Peterson TR, Laplante M, Thoreen CC, Sancak Y, Kang SA, Kuehl WM, et al. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 2009; 137(5):873–86. [PubMed: 19446321]

16. Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nature cell biology. 2002; 4(9):648–57. [PubMed: 12172553]

17. Potter CJ, Pedraza LG, Xu T. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol. 2002; 4(9):658–65. [PubMed: 12172554]

18. Tee AR, Anjum R, Blenis J. Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin. The Journal of biological chemistry. 2003; 278(39):37288–96. [PubMed: 12867426]

19. Garami A, Zwartkruis FJ, Nobukuni T, Joaquin M, Roccio M, Stocker H, et al. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Molecular cell. 2003; 11(6):1457–66. [PubMed: 12820960]

20. Zhang Y, Gao X, Saucedo LJ, Ru B, Edgar BA, Pan D. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nature cell biology. 2003; 5(6):578–81. [PubMed: 12771962]

21. Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. Rheb binds and regulates the mTOR kinase. Current biology : CB. 2005; 15(8):702–13. [PubMed: 15854902]

22. Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Molecular cell. 2002; 10(1):151–62. [PubMed: 12150915]

23. Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell. 2005; 121(2):179–93. [PubMed: 15851026]

24. Roux PP, Ballif BA, Anjum R, Gygi SP, Blenis J. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Proceedings of the National Academy of Sciences of the United States of America. 2004; 101(37): 13489–94. [PubMed: 15342917]

25. Le Romancer M, Poulard C, Cohen P, Sentis S, Renoir JM, Corbo L. Cracking the estrogen receptor's posttranslational code in breast tumors. Endocrine reviews. 2011; 32(5):597–622. [PubMed: 21680538]

26. Yamashita H, Nishio M, Toyama T, Sugiura H, Kondo N, Kobayashi S, et al. Low phosphorylation of estrogen receptor alpha (ERalpha) serine 118 and high phosphorylation of ERalpha serine 167 improve survival in ER-positive breast cancer. Endocrine-related cancer. 2008; 15(3):755–63. [PubMed: 18550720]

27. Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, et al. Phosphorylation of estrogen receptor alpha serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast cancer research : BCR. 2005; 7(5):R753–64. [PubMed: 16168121]

28. Baselga J, Campone M, Piccart M, Burris HA 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. The New England journal of medicine. 2012; 366(6):520–9. [PubMed: 22149876]

29. Schalm SS, Blenis J. Identification of a conserved motif required for mTOR signaling. Current biology : CB. 2002; 12(8):632–9. [PubMed: 11967149]

30. Schalm SS, Fingar DC, Sabatini DM, Blenis J. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Current biology : CB. 2003; 13(10):797–806. [PubMed: 12747827]

31. Oshiro N, Takahashi R, Yoshino K, Tanimura K, Nakashima A, Eguchi S, et al. The proline-rich Akt substrate of 40 kDa (PRAS40) is a physiological substrate of mammalian target of rapamycin complex 1. The Journal of biological chemistry. 2007; 282(28):20329–39. [PubMed: 17517883]

32. Fonseca BD, Zakaria C, Jia JJ, Graber TE, Svitkin Y, Tahmasebi S, et al. La-related Protein 1 (LARP1) Represses Terminal Oligopyrimidine (TOP) mRNA Translation Downstream of mTOR Complex 1 (mTORC1). The Journal of biological chemistry. 2015; 290(26):15996–6020. [PubMed: 25940091]

33. Land SC, Tee AR. Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. The Journal of biological chemistry. 2007; 282(28):20534–43. [PubMed: 17502379]

34. Fingar DC, Blenis J. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene. 2004; 23(18):3151–71. [PubMed: 15094765]

35. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004; 18(16):1926–45. [PubMed: 15314020]

36. Campbell RA, Bhat-Nakshatri P, Patel NM, Constantinidou D, Ali S, Nakshatri H. Phosphatidylinositol 3-kinase/AKT-mediated activation of estrogen receptor alpha: a new model for anti-estrogen resistance. The Journal of biological chemistry. 2001; 276(13):9817–24. [PubMed: 11139588]

37. Petz LN, Ziegler YS, Loven MA, Nardulli AM. Estrogen receptor alpha and activating protein-1 mediate estrogen responsiveness of the progesterone receptor gene in MCF-7 breast cancer cells. Endocrinology. 2002; 143(12):4583–91. [PubMed: 12446585]

38. Paplomata E, Zelnak A, O'Regan R. Everolimus: side effect profile and management of toxicities in breast cancer. Breast cancer research and treatment. 2013; 140(3):453–62. [PubMed: 23907751]

39. Bachelot T, Bourgier C, Cropet C, Ray-Coquard I, Ferrero JM, Freyer G, et al. Randomized phase II trial of everolimus in combination with tamoxifen in patients with hormone receptor-positive, human epidermal growth factor receptor 2-negative metastatic breast cancer with prior exposure to aromatase inhibitors: a GINECO study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012; 30(22):2718–24. [PubMed: 22565002]

40. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2dagger. Annals of oncology :

official journal of the European Society for Medical Oncology / ESMO. 2014; 25(12):2357–62. [PubMed: 25231953]

41. Paplomata E, O'Regan R. New and emerging treatments for estrogen receptor-positive breast cancer: focus on everolimus. Therapeutics and clinical risk management. 2013; 9:27–36. [PubMed: 23345981]

42. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. Journal of the National Cancer Institute. 2004; 96(12):926–35. [PubMed: 15199112]

43. Miller TW, Hennessy BT, Gonzalez-Angulo AM, Fox EM, Mills GB, Chen H, et al. Hyperactivation of phosphatidylinositol-3 kinase promotes escape from hormone dependence in estrogen receptor-positive human breast cancer. The Journal of clinical investigation. 2010; 120(7): 2406–13. [PubMed: 20530877]

44. Generali D, Fox SB, Brizzi MP, Allevi G, Bonardi S, Aguggini S, et al. Down-regulation of phosphatidylinositol 3′-kinase/AKT/molecular target of rapamycin metabolic pathway by primary letrozole-based therapy in human breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2008; 14(9):2673–80. [PubMed: 18451231]

45. Baselga J, Semiglazov V, van Dam P, Manikhas A, Bellet M, Mayordomo J, et al. Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009; 27(16):2630–7. [PubMed: 19380449]

46. Paplomata E, O'Regan R. The PI3K/AKT/mTOR pathway in breast cancer: targets, trials and biomarkers. Therapeutic advances in medical oncology. 2014; 6(4):154–66. [PubMed: 25057302]

47. Fonseca BD, Zakaria C, Jia JJ, Graber TE, Svitkin Y, Tahmasebi S, et al. La-related protein 1 (LARP1) represses terminal oligopyrimidine (TOP) mRNA translation downstream of mTOR complex 1 (mTORC1). The Journal of biological chemistry. 2015

48. Thomas RS, Sarwar N, Phoenix F, Coombes RC, Ali S. Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-alpha activity. Journal of molecular endocrinology. 2008; 40(4):173–84. [PubMed: 18372406]

49. Yokogami K, Wakisaka S, Avruch J, Reeves SA. Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR. Current biology : CB. 2000; 10(1):47–50. [PubMed: 10660304]

50. Martina JA, Chen Y, Gucek M, Puertollano R. MTORC1 functions as a transcriptional regulator of autophagy by preventing nuclear transport of TFEB. Autophagy. 2012; 8(6):903–14. [PubMed: 22576015]

51. Chaveroux C, Eichner LJ, Dufour CR, Shatnawi A, Khoutorsky A, Bourque G, et al. Molecular and genetic crosstalks between mTOR and ERRalpha are key determinants of rapamycin-induced nonalcoholic fatty liver. Cell metabolism. 2013; 17(4):586–98. [PubMed: 23562079]

52. Cunningham JT, Rodgers JT, Arlow DH, Vazquez F, Mootha VK, Puigserver P. mTOR controls mitochondrial oxidative function through a YY1-PGC-1alpha transcriptional complex. Nature. 2007; 450(7170):736–40. [PubMed: 18046414]

53. James MJ, Zomerdijk JC. Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. The Journal of biological chemistry. 2004; 279(10):8911–8. [PubMed: 14688273]

54. Madak-Erdogan Z, Lupien M, Stossi F, Brown M, Katzenellenbogen BS. Genomic collaboration of estrogen receptor alpha and extracellular signal-regulated kinase 2 in regulating gene and proliferation programs. Molecular and cellular biology. 2011; 31(1):226–36. [PubMed: 20956553]

55. Madak-Erdogan Z, Ventrella R, Petry L, Katzenellenbogen BS. Novel roles for ERK5 and cofilin as critical mediators linking ERalpha-driven transcription, actin reorganization, and invasiveness in breast cancer. Molecular cancer research : MCR. 2014; 12(5):714–27. [PubMed: 24505128]

56. Chambliss KL, Wu Q, Oltmann S, Konaniah ES, Umetani M, Korach KS, et al. Non-nuclear estrogen receptor alpha signaling promotes cardiovascular protection but not uterine or breast cancer growth in mice. The Journal of clinical investigation. 2010; 120(7):2319–30. [PubMed: 20577047]

57. Holz MK, Blenis J. Identification of S6 kinase 1 as a novel mammalian target of rapamycin (mTOR)-phosphorylating kinase. The Journal of biological chemistry. 2005; 280(28):26089–93. [PubMed: 15905173]
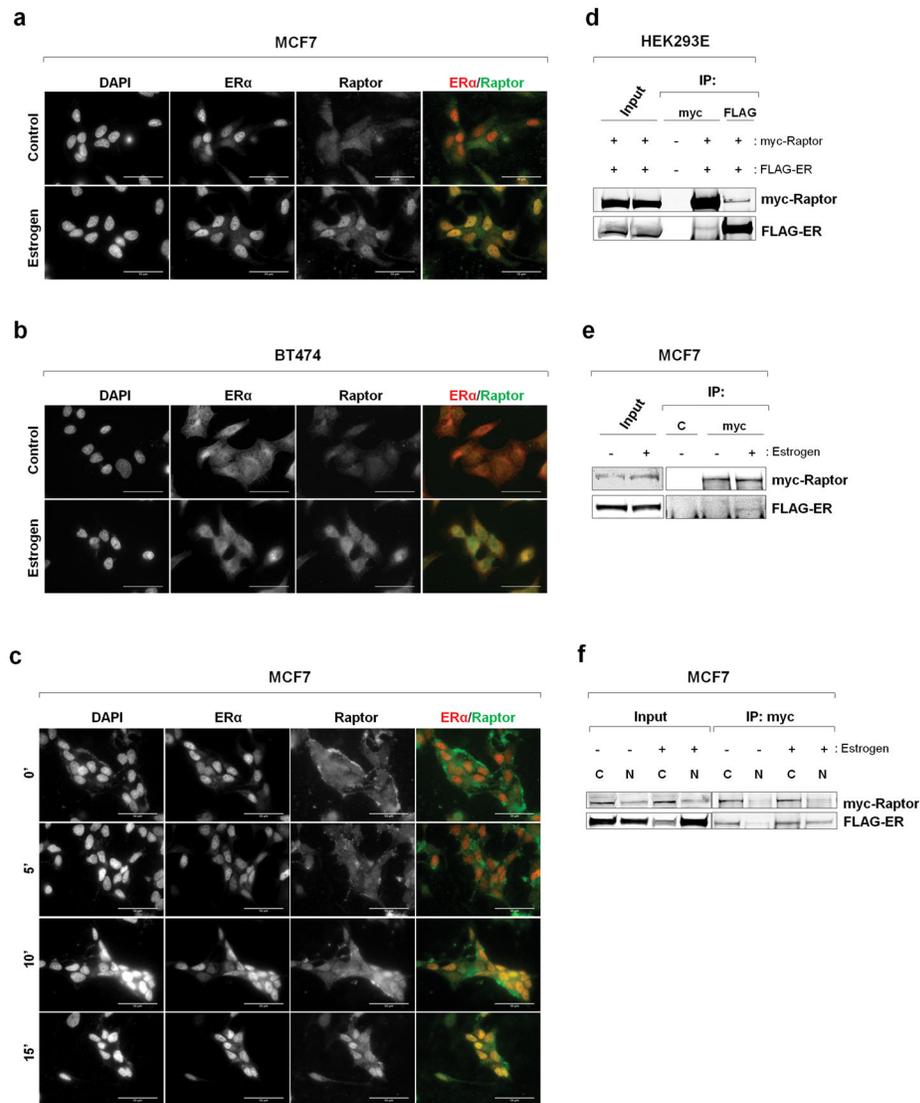
Figure 1. Raptor co-localizes with ERα in the nucleus upon estrogen stimulation

(a) MCF7 cells were serum-starved or stimulated with estrogen for 30 min. Immunofluorescence was performed as described in *"Materials and Methods"*. Scale bar represents 50μm. (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 *"Materials and Methods"* 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 ERα 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 *"Materials and Methods"*. Raptor was immunoprecipitated and interaction with ERα was detected by immunoblot.

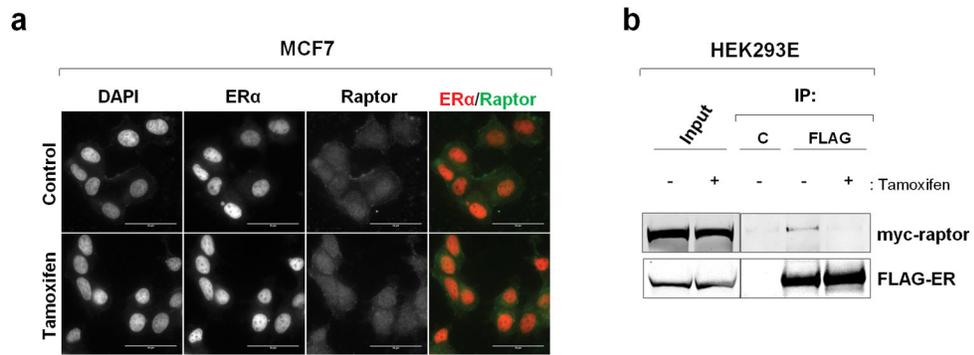**Figure 2. Tamoxifen disrupts ERα-Raptor interaction**
(a) MCF7 cells were treated with tamoxifen for 1h and immunofluorescence was performed as described in *"Materials and Methods"*. Scale bar represents 50μm. (b) HEK293E cells were co-transfected with myc-raptor and FLAG-ER. Cells were treated with tamoxifen for 1h as indicated. ER was immunoprecipitated and interaction with raptor was detected by immunoblot.

a

HEK293E

b
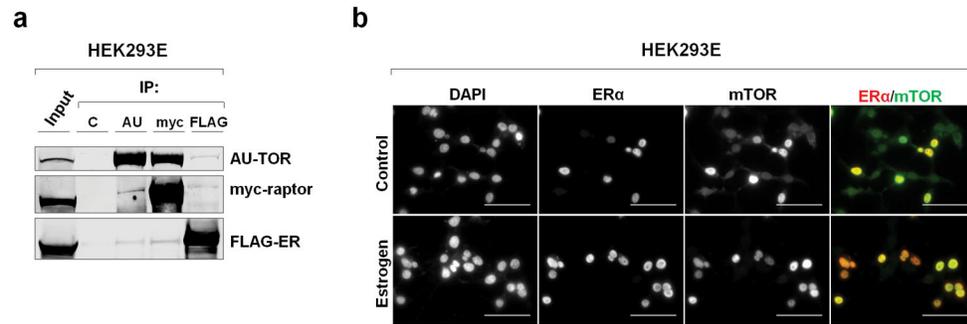
HEK293E

Figure 3. mTOR forms a complex with Raptor and ERα

(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. (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 *"Materials and Methods"*. Scale bar represents 50μm.

a

TOS motif in mTORC1 regulated proteins

ERα	FPATV
S6K1	FDIDL
S6K2	FDLDL
4EBPs	FEMDI
PRAS40	FVMDE
HIFIα	FVMVL
LARP1	FRLDI

b

Conservation of the TOS motif of ERα

Human	591	FPATV	595
Mouse	595	FPNTI	599
Rat	596	FPNTI	600
Cow	592	FPSTV	596

c

d

e

f

g

h

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 *"Materials and Methods"* 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. (e) HEK293E cells were transfected with WT or F/A ER. (C is empty vector). Lysates were generated as described in

"Materials and Methods" and the indicated proteins were detected by immunblot. (f) Cells were treated with estrogen for 30 min as indicated. Immune complex kinase assay was performed as described in *"Materials and Methods"* 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 *"Materials and Methods"* 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 *"Materials and Methods"* and indicated proteins were detected by immunoblot.
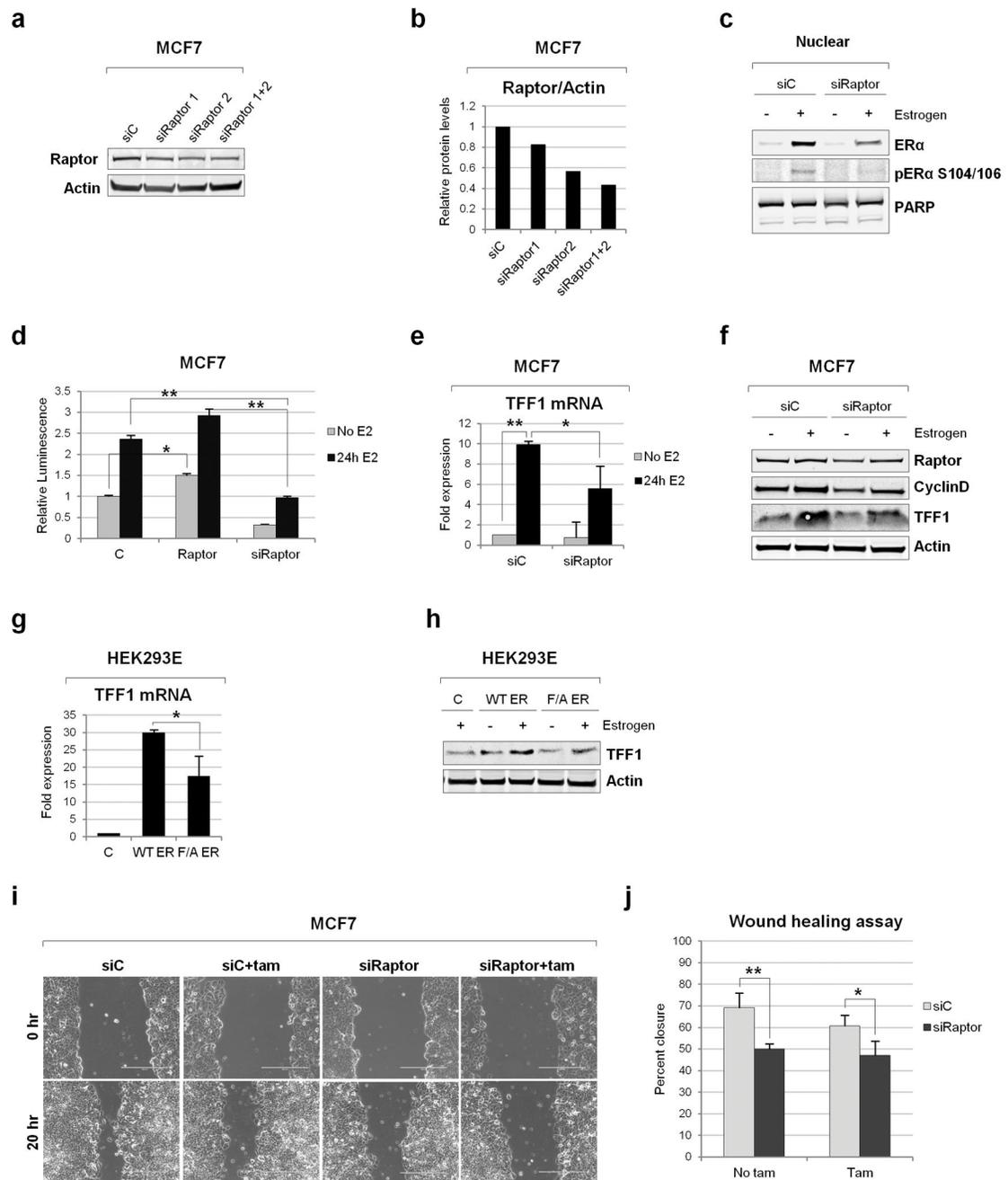
Figure 5. Raptor regulates ERα activity

(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 *"Materials and Methods"*. Data was plotted using Excel. * represents p<0.05 and **

represents $p<0.01$. (e) MCF7 cells were transfected with non-specific si or si against Raptor. RT-qPCR was performed as described in *"Materials and Methods"* and data was plotted using Excel. * represents $p<0.05$. ** represents $p<0.001$. n=3. (f) MCF7 cells were transfected as described in *"Materials and methods"*, stimulated with estrogen for 24 h as indicated and proteins levels were detected using immunoblot. (g) HEK293E cells were transfected with WT or F/A ER. (C is empty vector). RT-qPCR was performed as described in *"Materials and Methods"* and data were plotted using Excel. * represents $p<0.05$. n=3. (h) HEK293E cells were transfected as described in *"Materials and Methods"*, stimulated with estrogen for 24 h as indicated and protein levels were detected by immunoblot. (i) MCF7 cells were transfected with either scrambled siRNA or siRNA against raptor and treated with 4-hydroxy-tamoxifen (tam, 100nM) as indicated. Wound Healing Assay was performed as described in *"Materials and Methods"*. (j) Quantification of the Wound Healing Assay from *(i)* was plotted using Excel. * represents $p<0.05$. ** represents $p<0.01$.
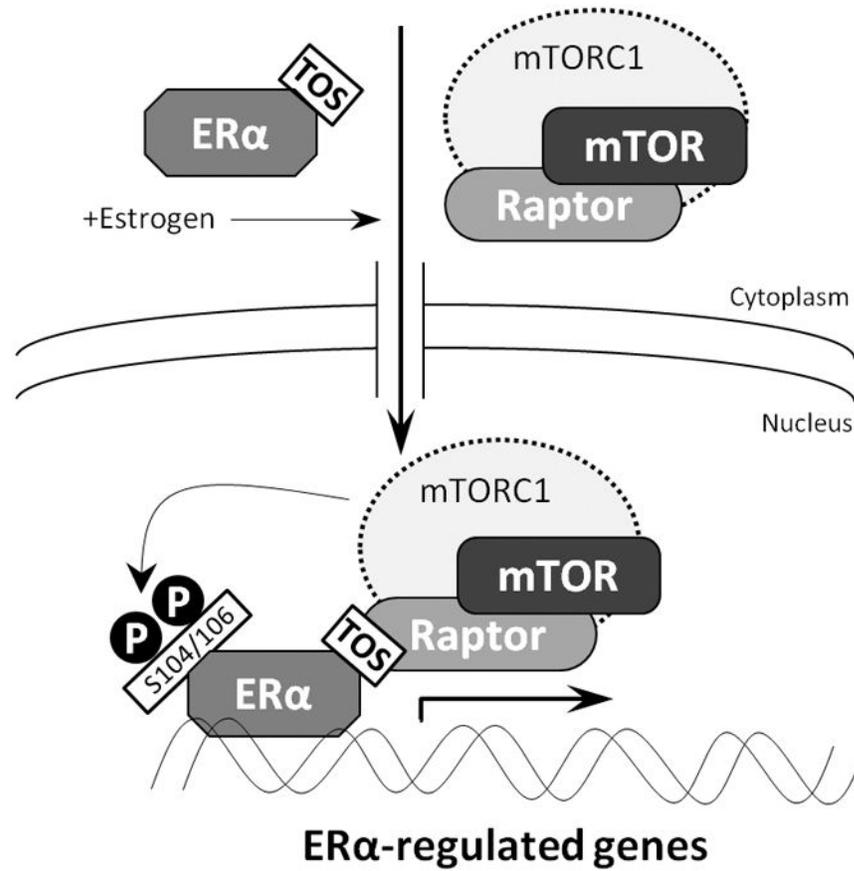
Figure 6. Mechanism of ERα-raptor interaction
In the presence of estrogen, ERα binds to raptor via the TOS motif and recruits mTORC1 to the nucleus where mTOR phosphorylates ERα, leading to its activation and resulting in upregulation of estrogenic gene transcription.