Phosphoproteome Analysis Reveals Estrogen-ER Pathway as a Modulator of mTOR Activity Via DEPTOR

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In Brief
The connection between estrogenic and PI3K/AKT/mTOR signaling pathways is frequently associated with poor outcome in ER-positive breast tumors. To characterize this connection, we performed a phosphoproteome analysis of ER-positive MCF7 cells. DEPTOR was identified as an estrogen-regulated mTORC1 target. DEPTOR is an mTORC1/2 inhibitory protein that is degraded in response to mitogen-induced and mTOR-mediated phosphorylation. We demonstrated that estrogen-ERα transcriptionally upregulate DEPTOR expression. Thus, despite decreased stability, increased DEPTOR levels act to modulate estrogen-induced activation of mTORC1 and mTORC2.

Highlights
- Phosphoproteome analysis identified estrogen-regulated mTORC1 targets in MCF7 cells.
- Estrogen-bound ERα transcriptionally upregulates DEPTOR expression.
- Elevated levels of DEPTOR modulate estrogen-induced activation of mTORC1 and mTORC2.
Phosphoproteome Analysis Reveals Estrogen-ER Pathway as a Modulator of mTOR Activity Via DEPTOR*

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ER-positive breast tumors represent ~70% of all breast cancer cases. Although their treatment with endocrine therapies is effective in the adjuvant or recurrent settings, the development of resistance compromises their effectiveness. The binding of estrogen to ERα, a transcription factor, triggers the regulation of the target genes (genomic pathway). Additionally, a cytoplasmic fraction of estrogen-bound ERα activates oncogenic signaling pathways such as PI3K/AKT/mTOR (non-genomic pathway). The upregulation of the estrogenic and the PI3K/AKT/mTOR signaling pathways are frequently associated with a poor outcome. To better characterize the connection between these two pathways, we performed a phosphoproteome analysis of ER-positive MCF7 breast cancer cells treated with estrogen or estrogen and the mTORC1 inhibitor rapamycin. Many proteins were identified as estrogen-regulated mTORC1 targets and among them, DEPTOR was selected for further characterization. DEPTOR binds to mTOR and inhibits the kinase activity of both mTOR complexes mTORC1 and mTORC2, but mitogen-activated mTOR promotes phosphorylation-mediated DEPTOR degradation. Although estrogen enhances the phosphorylation of DEPTOR by mTORC1, DEPTOR levels increase in estrogen-stimulated cells. We demonstrated that DEPTOR accumulation is the result of estrogen-ERα-mediated transcriptional upregulation of DEPTOR expression. Consequently, the elevated levels of DEPTOR partially counterbalance the estrogen-induced activation of mTORC1 and mTORC2. These results underscore the critical role of estrogen-ERα as a modulator of the PI3K/AKT/mTOR signaling pathway in ER-positive breast cancer cells. Additionally, these studies provide evidence supporting the use of dual PI3K/mTOR or dual mTORC1/2 inhibitors in combination with endocrine therapies as a first-line treatment option for the patients with ER-positive advanced breast cancer.

Estrogen Receptor α (ERα)\(^1\) is a transcription factor that promotes expression of growth and survival genes (1, 2). ERα activation and transcriptional activity is mainly mediated by the binding of its ligand 17β-estradiol. In addition to genomic ERα effects, estrogen also activates non-nuclear ERα that, in turn, activates membrane-associated and cytoplasmic kinases, such as IGF-1R/InsR, EGFR, Src, PI3K, and MEK. Growth factor-stimulated kinases can activate ERα as well, leading to multi-site phosphorylation of the receptor and ligand-independent activation (3, 4). The activation of ERα by growth factor signaling is also promoted in a feed-forward fashion, whereby ERα promotes the transcription of genes encoding ligands, receptor tyrosine kinases, and signaling adaptors (5).

There is strong evidence that ERα can localize kinases to the nucleus, stimulating estrogen-dependent transcription. Previous studies have described estrogen-activated ERα co-localization with ERK2 in the nucleus, which leads to receptor-mediated transcription of estrogen-dependent genes involved in cell proliferation (6, 7). We have recently shown a similar interaction between ERα and the mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase activity (8). Although much is known about the genomic function of estrogen-activated ERα, there exists a gap in knowledge regarding the global effects of estrogen on cytoplasmic and nuclear kinase-mediated signaling.

Clinically, up to 75% of breast cancers are ER-positive, indicative of estrogen dependence for cancer cell growth (5), and thus can be targeted by endocrine therapies. However, >20% of ER-positive breast cancers do not respond to endocrine treatments, and resistance often occurs (9, 10). To define the drivers of endocrine therapy response and resistance, we must fully understand the basic mechanisms of estrogenic signaling. We now know that estrogenic signaling is tightly connected to the action of oncogenic kinases, such as mTORC1, the focus of this work.
mTOR is a conserved protein kinase that is a key regulator of cell growth and proliferation in response to extracellular cues, including nutrient availability and growth stimuli. mTOR exists in two complexes in eukaryotic cells, mTORC1 and mTORC2, which consist of distinct sets of proteins and perform non-redundant functions (11). mTORC1 is a central integrator of multiple signaling pathways in the cell. mTORC1 is activated by a variety of upstream signals and in turn, phosphorylates many target proteins either directly or via its effector kinase S6K1. mTORC1 regulates anabolic processes such as cell growth, proliferation, protein and nucleotide synthesis, autophagy, and others (11, 12). mTORC1 has also emerged as a critical node of estrogenic signaling in the cell. We and others have shown that estrogen rapidly and potently activates mTORC1 (13–15), and determined that mTORC1 is a critical direct activator of ERα transcriptional activity (8, 12, 16–19). Thus, the biochemical relationship between the mTORC1 and estrogen signaling pathways provides the rationale for the FDA-approved use of mTORC1 inhibitors in combination with endocrine agents for treatment of ER-positive advanced breast cancer (20) to enhance the efficacy of endocrine therapy and suppress the development of resistance (20). Studies such as TAMRAD (21) and BOLERO-2 (20) showed that combination of mTORC1 inhibitor everolimus with either tamoxifen or exemestane improved progression-free survival of ER-positive breast cancer patients. However, significant improvement in overall survival was not observed (22), underscoring the need for additional research into the mechanisms of estrogen-mTORC1 relationship.

To better understand the connection between estrogen and mTORC1 pathways, we performed a phosphoproteome analysis of ER-positive MCF7 cells treated with estrogen or estrogen and the mTORC1 inhibitor rapamycin. We identified DEP domain-containing mechanistic target of rapamycin (mTOR)-interacting protein (DEPTOR) as an mTORC1 target regulated by estrogen. Peterson et al. (23) described DEPTOR as an mTOR-interacting protein that inhibits mTOR kinase activity, and showed that on mitogen-stimulation, active mTORC1 and mTORC2 phosphorylate and promote DEPTOR degradation. Although mTORC1 phosphorylated DEPTOR in estrogen-stimulated cells, we did not detect a reduction of DEPTOR levels. In contrast, we observed that estrogen-stimulated cells, we did not detect a reduction of degradation. Although mTORC1 and mTORC2, which consist of distinct sets of proteins and perform non-redundant functions (11). mTORC1 is a central integrator of multiple signaling pathways in the cell. mTORC1 is activated by a variety of upstream signals and in turn, phosphorylates many target proteins either directly or via its effector kinase S6K1. mTORC1 regulates anabolic processes such as cell growth, proliferation, protein and nucleotide synthesis, autophagy, and others (11, 12). mTORC1 has also emerged as a critical node of estrogenic signaling in the cell. We and others have shown that estrogen rapidly and potently activates mTORC1 (13–15), and determined that mTORC1 is a critical direct activator of ERα transcriptional activity (8, 12, 16–19). Thus, the biochemical relationship between the mTORC1 and estrogen signaling pathways provides the rationale for the FDA-approved use of mTORC1 inhibitors in combination with endocrine agents for treatment of ER-positive advanced breast cancer (20) to enhance the efficacy of endocrine therapy and suppress the development of resistance (20). Studies such as TAMRAD (21) and BOLERO-2 (20) showed that combination of mTORC1 inhibitor everolimus with either tamoxifen or exemestane improved progression-free survival of ER-positive breast cancer patients. However, significant improvement in overall survival was not observed (22), underscoring the need for additional research into the mechanisms of estrogen-mTORC1 relationship.

To better understand the connection between estrogen and mTORC1 pathways, we performed a phosphoproteome analysis of ER-positive MCF7 cells treated with estrogen or estrogen and the mTORC1 inhibitor rapamycin. We identified DEP domain-containing mechanistic target of rapamycin (mTOR)-interacting protein (DEPTOR) as an mTORC1 target regulated by estrogen. Peterson et al. (23) described DEPTOR as an mTOR-interacting protein that inhibits mTOR kinase activity, and showed that on mitogen-stimulation, active mTORC1 and mTORC2 phosphorylate and promote DEPTOR degradation. Although mTORC1 phosphorylated DEPTOR in estrogen-stimulated cells, we did not detect a reduction of DEPTOR levels. In contrast, we observed that estrogen-bound ERα promoted DEPDC6 gene transcription, which resulted in DEPTOR protein accumulation. Consequently, we demonstrated that the activation of mTORC1 and mTORC2 induced by the cytoplasmic nongenomic estrogen-ERα pathway is partially counterbalanced by the transcriptional upregulation of DEPTOR expression mediated by the nuclear genomic estrogen-ERα pathway. These results support the combined use of endocrine therapy and dual mTORC1/mTORC2 inhibitors for the treatment of ER-positive advanced breast cancer.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—MCF7 cell line was originally obtained from the ATCC (Flowery Branch, GA). MCF7 cells were maintained in DMEM (Corning, NY), supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals), and penicillin/streptomycin (Corning). For estrogen stimulation experiments, cells were grown in phenol red-free DMEM (Corning) with 5% or 10% charcoal-stripped FBS (Atlanta Biologicals) for 3 days. 17 β-estradiol (Sigma-Aldrich), St Louis, MO), rapamycin (Sigma-Aldrich), everolimus (Selleckchem, Houston, TX), pp242 (EMD Millipore, Burlington, MA), (Z)-4-hydroxytamoxifen (Sigma-Aldrich), fulvestrant (Sigma-Aldrich), and cycloheximide (Sigma-Aldrich) were used as indicated in figure legends.

**Phosphoproteome Analysis**—Phosphoproteome analysis was performed as described in supplemental Data.

**Plasmids and Transfections**—pPRK5 Flag human DEPTOR plasmid was kindly provided by David Sabatini (24) (Addgene, Watertown, MA; plasmid #21334).

Scrambled siRNA (DS Scrambled Neg) and siRNAs to target DEPTOR (hs.Ri.DEPTOR.13.1, hs.Ri.DEPTOR.13.2, and hs.Ri.DEPTOR.13.3) were obtained from Integrated DNA Technologies (IDT, Coralville, IA).

MCF7 cells were transfected using Fugene HD (Promega, Madison, WI) according to the manufacturer’s protocol. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was used for transfection of siRNAs following the manufacturer’s protocol.

**Immunoblot Analysis and Immunoprecipitation**—Cell lysates were prepared by incubating the cells in ice-cold lysis buffer (10 mM K2PO4, 1 mM EDTA, 10 mM MgCl2, 5 mM EGTA, 50 mM β-glycerophosphate, 0.5% Nonirot P-40, 0.1% Brij, 40 μg/ml PMSF, 10 μg/ml leupeptine, 5 μg/ml pepstatin, and 10 μg/ml aprotinin) for 20 min on ice, followed by centrifugation at 10,000 rpm at 4 °C for 10 min. Equal amounts of whole-cell extracts were resolved by SDS-PAGE (4–12% gradient), and transferred to nitrocellulose membrane. Indicated proteins were detected by immunoblot analysis using specific antibodies: anti-phospho-mTOR (Ser-2448), anti-phospho-S6K1 (Thr-389), anti-S6K, anti-phospho-AKT, anti-DEPTOR, anti-phospho-S6 (Ser-235/6), anti-S6, anti-4EBP1, anti-phospho-4EBP1 (Ser-65), and anti-phospho-4EBP1 (Thr-37/46) (Cell Signaling Technology, Danvers, MA); anti-actin (C-11), anti-phospho-Serine (16B4), anti-ERα (HC-20), and anti-mTOR (N-19) (Santa Cruz Biotechnology, Dallas, TX); anti-Flag M2 (Sigma-Aldrich). Anti-goat IRDye, anti-mouse IRDye, and anti-rabbit IRDye (LI-COR Biosciences, Lincoln, NE) were used as secondary antibodies.

For immunoprecipitation assays, equal amounts of protein were precleared with 20 μl of 50% protein G-Sepharose 4 bead slurries for 1 h at 4 °C followed by incubation with anti-Flag (2 μl) overnight at 4 °C. Protein G-Sepharose beads, previously blocked with BSA, were added to the samples and incubation continued for 1 h at 4 °C. Beads were washed five times with lysis buffer for 5 min at 4 °C and collected by centrifugation at 3,000 rpm for 3 min. Immunocomplexes were resolved and detected as described above. For mTOR immunoprecipitation, cell extracts were prepared and processed as described by Peterson et al. (24).

**Chromatin Immunoprecipitation (ChIP) Assays**—ChIP analyses were performed as previously described by Saint-Andre et al. (25). Antibodies were: rabbit IgG and anti-ERα (HC-20) (Santa Cruz Biotechnology). Immunoprecipitated DNA and 10% of chromatin input...
were analyzed by qPCR using the following primers: DEP ERE F: 5’-GGCTCACAGTCTGAGCAGATG-3’, DEP ERE R: 5’-GAATGGGACTGGTTTTGCTG-3’, DEP DIS F: 5’-CGGGCGCTTCTTTGATTACGAC-3’, DEP DIS R: 5’-CTGACAAAGGCACGTAGGAG-3’, DEP PRO F: 5’-CCCGCAGAGTTTATATTGATG-3’, DEP PRO R: 5’-CTGACGAGCATTGCTGAGG-3’, TFF F: 5’-CCCGCCATCTCTCAGACTA-3’, and TFF R: 5’-CCTGGGAGCGAGGCAAATAC-3’. Percentage of input chromatin enriched by each antibody was calculated.

Quantitative RT-PCR—RNA was purified using PureLink™ RNA Mini kit (Invitrogen) and 0.5 µg of RNA were reverse transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). For qPCR, cDNA was amplified with IQ™SYBR® Green Supermix (Bio-Rad) in a CFX96™ Real-Time System (Bio-Rad). Primers used: TFF1 f: 5’-ATCGAGTCCTCTCCAGAGAAG-3’, TFF1 r: 5’-CTCTGGGAAGCTCATAT-3’, DEPTOR 1F: 5’-GTCTACCAGTCATTTGATG-3’, DEPTOR 2R: 5’-GCCAGTCAGGAATTCAGATG-3’, GAPDH f: 5’-ATCCACCTCAGGAGGGA-3’, and GAPDH r: 5’-CCTGGGAGCGAGGCAAATAC-3’. Experimental Design and Statistical Rationale—Samples were prepared from three biological replicates.

Differential expression was determined using a two-sided t test between conditions and considering phosphopeptides with a p value lower than 0.05 to be significant. Phosphopeptide abundance was normally distributed.

Pathway analysis was performed using a t test between the abundance of the members of each pathway in each condition. p values were adjusted using a Benjamini-Hochberg correction for multiple hypothesis testing and pathways with an adjusted p value lower than 0.05 were further considered. A combination of KEGG, Reactome, and Biocarta pathways were used derived from the MSIGdb (26).

Statistical analysis was performed using the Prism Graphpad 8.0 software. Significance was determined by paired two-tailed Student’s t test. p values lower than 0.05 were considered significant.

RESULTS

Characterization of Estrogen- and Rapamycin-regulated Phosphorylated Proteins—To quantitatively determine that estrogen signals to mTOR, we used a 10-plexed tandem mass tag (TMT) isobaric labeling approach, which allowed for significantly improved detection sensitivity and provides deep quantitative profiling of protein abundances and site-specific phosphorylation levels, including low-abundance species (27). ER-positive MCF7 cells were grown in hormone-depleted medium for 72 h, followed by 24 h of serum starvation. Cells were pretreated with vehicle (DMSO) or the mTORC1 inhibitor rapamycin (20 nM) for 30 min, and/or stimulated with estrogen (10 nM) for 30 min. Samples were prepared in 3 biological replicates, processed, and subjected to high pH reversed phase LC fractionation before final LC-MS/MS to achieve high throughput and deep coverage (28–30). Labeled mixed samples were split and 5% was used for global proteome analysis and the remainder for phosphoproteome analysis using immobilized metal affinity chromatography (IMAC) enrichment approach for global phosphorylation analysis. A common reference (cell lysate) was used in each TMT-10 run to facilitate comparison across multiple experiments. We conducted MS-GF+ searches (31) to below a 0.5% false discovery rate. The phosphoproteomics workflow is shown in Fig. 1.

We identified a total of 9733 unique phosphopeptides in this study and a breakdown of identifications for singly and multiply phosphorylated peptides and site specificity are shown in Fig. 1. Replicates agreed well with each other (Fig. 1) and differences were observed across treatments.

Analysis of mTORC1 Signaling in Response to Estrogen Treatment—Analysis of the resulting phosphoproteome measurements (Fig. 2) shows a small number of estrogen responsive sites that are all upregulated relative to control (blue dots) and a much larger number of rapamycin responsive sites that vary in direction (red dots). We focused on the estrogen-stimulated and rapamycin-sensitive phosphopeptides, which represented putative mTORC1 targets (upper left quadrant).

As expected, we identified well-known mTORC1 pathway members (yellow dots), which validate the experimental conditions used for the phosphoproteome analysis (Fig. 2 and Table I). Additionally, we detected novel sites and selected the most biological relevant ones, according to our criteria, for further characterization (Fig. 2 and Table I).

To assess the biological processes altered by estrogen and rapamycin treatment, proteins with one phosphorylation site and a quantification value greater than one standard deviation from mean were subjected to bioinformatic analysis (see Experimental Procedures). Because signal-regulated phosphorylation is dynamic in manifesting biological responses, we examined estrogen- and rapamycin-upregulated and -downregulated sites to identify Gene Ontology (GO) enrichment (Fig. 3). Transcription, a process canonically regulated by estrogen-ER complexes, was heavily enriched in our analysis. Globally, enriched processed could be grouped into a signaling and a metabolism category. Among signaling pathways, mTOR was featured prominently as an estrogen-upregulated and rapamycin-sensitive pathway. Other two pathways, Rac1 and TGF-β, which have an important role in breast cancer, were also enriched.

Regulation of DEPTOR by Estrogen—DEPTOR was identified as an mTORC1 target regulated by estrogen in our phosphoproteome analysis. As shown in Table I, we detected phosphorylation of a peptide containing serines 179, 181, and 185 in DEPTOR isoform 2, which correspond to serines 280, 282, and 286 in the isoform 1. DEPTOR was initially characterized as an mTOR-binding protein that inhibits mTORC1 and mTORC2 kinase activities (24). In response to mitogens, mTOR-mediated phosphorylation of DEPTOR on serines 293 and 299 promotes the phosphorylation on serines 286, 287, and 291 by CK1α, which facilitates the binding of the E3 ubiquitin ligase β-TrCP to DEPTOR and its subsequent degradation (23). Phosphorylation on serines 286, 287, and 291 by S6K1 and RSK1 has been reported, although its effect on DEPTOR degradation is controversial (23).

First, we validated mTOR-induced phosphorylation of DEPTOR in estrogen-stimulated MCF7 cells. Cells expressing Flag-tagged DEPTOR were treated with vehicle, estrogen, or estrogen and the mTORC1 inhibitor everolimus for 30 min. After isolation of Flag-DEPTOR, we observed increased
phosphorylation of DEPTOR on serine residues in estrogen-treated cells, which was prevented by everolimus (Fig. 4A). As expected, the phosphorylation of known mTORC1 targets, such as S6K1 Thr-389, S6 Ser-235/6, and 4E-BP1 Thr-37/46 was enhanced by estrogen and reversed by everolimus (Fig. 4A).

Because the identified phosphopeptide contains Ser-286 (Ser-185 in isoform 2), we evaluated the effect of the estrogen-promoted phosphorylation on DEPTOR protein levels. Surprisingly, we observed the accumulation of DEPTOR in cells treated with estrogen for 24 h compared with vehicle-treated cells (Fig. 4B), which was independent of mTORC1

Fig. 1. Phosphoproteomic analysis of serum-starved, estrogen- and estrogen plus rapamycin-treated MCF7 cells. Workflow to discover estrogen-stimulated and rapamycin-sensitive phosphorylation events. MCF7 cells were incubated in phenol red-free DMEM supplemented with 10% charcoal-treated FBS for 3 days, serum-starved for 24 h, and treated with vehicle or 20 nM rapamycin (red) for 30 min before stimulation with vehicle (control) or 10 nM estradiol (blue) for 30 min. Cells were harvested and processed (see Experimental procedures), trypsinized, and isotopically labeled for TMT-10 analysis. Peptides were subjected to IMAC and enriched phosphopeptide-fractions were analyzed by LC-MS/MS. The MS-GF+ algorithm and Ascore were used for identification of and confident site localization of post-translational modifications, respectively. Numbers of single, doubly, or triply phosphorylated peptides are indicated, as are numbers of phosphorylation sites. The boxplot shows the distribution of relative abundances (x axis) of phosphopeptides in the three difference conditions (y axis).
activity. This result suggested estrogen-mediated regulation of DEPTOR expression by a mechanism other than phosphorylation.

To better characterize the effect of estrogen on DEPTOR expression, we determined the levels of DEPTOR at different time points on stimulation of MCF7 cells with 0.2% or 10% charcoal treated FBS and vehicle (ethanol) or estrogen. As shown in Fig. 5, serum-induced activation of mTORC1 resulted in decreasing levels of DEPTOR (Fig. 5A and 5C), which was prevented by the co-treatment with estrogen (Fig. 5B and 5D). These results suggested an important positive effect of estrogen on DEPTOR expression, because its protein levels increased even though mTORC1 activity was further upregulated.

Estrogen-ER Pathway Promotes DEPTOR Expression—Next, we investigated the function of the estrogen receptors (ERs) in the regulation of DEPTOR expression by estrogen. Using tamoxifen, a selective ER modulator, or fulvestrant, a selective ER degrader, we confirmed the role of estrogen-bound ER in this process because the levels of DEPTOR decreased in cells treated with fulvestrant or tamoxifen compared with estrogen-stimulated cells (Fig. 6). Although both drugs prevented estrogen-ER-mediated activation of mTORC1 and, therefore, DEPTOR degradation, we still observed an important reduction in the levels of DEPTOR. These results supported our previous observations (Fig. 5)

**TABLE I**

| Known mTORC1 pathway substrates and phosphorylation sites | Site         |
|---------------------------------------------------------|--------------|
| Protein                                                |              |
| AKT1S1 (PRAS40)                                        | S183         |
| eIF4EBP2                                               | T70          |
| eIF4B                                                  | S383 (S422 in isoform 1) |
| mTOR                                                   | S2454        |
| Raptor                                                 | S705 (S963 in isoform 1) |
| rpS6                                                   | S235/S236/S244 |
| S6K1                                                   | S371/T389    |

| Known mTORC1 pathway substrates with novel predicted phosphorylation sites | Site                                 |
|--------------------------------------------------------------------------|-------------------------------------|
| Protein                                                                 |                       |
| DEPTOR                                                                  | S179/S181/S185 (S280/S282/S286 in isoform 1) |
| eIF4B                                                                  | S385/S386 (S424/S425 in isoform 1) |
| eIF4EBP2                                                               | T36/T41                           |
| IRS2                                                                   | S365                               |
| LARP1                                                                  | T779/T781 (T856/T858 in isoform 1) |
| S6K1                                                                   | T367/S375/T376/S424/S429           |

**Fig. 2. Distribution of differentially regulated phosphopeptides by estrogen compared with serum starved versus the rapamycin- and estrogen-treated conditions.** MCF7 cells were grown in phenol red-free DMEM supplemented with 10% charcoal-treated FBS for 3 days, followed by 24 h of serum starvation. Cells were pretreated with vehicle (DMSO) or rapamycin (20 nM) for 30 min, and stimulated with vehicle (ethanol) or estradiol (10 nM) for 30 min. Samples were prepared in 3 biological replicates. The blue colored dots are significant phosphopeptides in the estrogen-starved comparison (y axis) and the red colored dots are significant in the rapamycin-estrogen comparison (x axis). Yellow dots are known mTOR pathway members, with those listed on Table I indicated by labels.
suggesting a more prominent role of the mTORC1-independent estrogen-ER pathway on DEPTOR expression in MCF7 cells.

**Upregulation of mTORC1 Activity by Estrogen Does Not Result in Increased DEPTOR Degradation**—To determine the effect of estrogen-ER-induced activation of mTORC1 on DEPTOR degradation, we first analyzed the levels of DEPTOR in cells stimulated with 10% charcoal-treated FBS and vehicle or estrogen in the absence or the presence of the ATP-competitive inhibitor of mTOR pp242 (Fig. 7A). As previously shown, estrogen increased the amount of DEPTOR compared with vehicle-treated cells. mTOR inhibition resulted in DEPTOR accumulation in both vehicle- and estrogen-treated cells, which confirmed the role of mTOR complexes in DEPTOR degradation. However, we did not observe a significantly higher increase in DEPTOR levels in estrogen-treated cells.

Next, we determined the half-life of DEPTOR using the same treatment conditions in the presence of cycloheximide. As shown in Fig. 7B, estrogen-stimulation did not influence DEPTOR stability, although mTOR was upregulated. These results indicated that serum-induced activation of mTOR was responsible for DEPTOR degradation, but
ER observed a levels of DEPTOR mRNA were measured by RT-qPCR in MCF7 as a transcription factor, estrogen-bound ER

Gene regulation (2, 32). We identified an ERE-like sequence in the promoter proximal region of DEPTOR gene. Additionally, the binding of ERα to the promoter distal region (−2555 to −2252 from transcription start site) was determined by CHIP-seq as described in the ENCODE, although it does not contain ERE or ERE-like sequences. To evaluate the binding of estrogen-ERα complexes to these DEPTOR promoter regions, we performed chromatin immunoprecipitation (ChIP) assays using nuclear extracts from MCF7 cells treated with vehicle, estrogen, or tamoxifen for 45 min (Fig. 8B). We observed increased binding of ERα to both distal and proximal regions (−2.5 and −4-fold over background, respectively) in estrogen-stimulated cells, but not in vehicle- or tamoxifen-treated cells. However, we did not detect a significant interaction of ERα with the region containing the transcription start site. These results confirmed the binding of ERα to DEPTOR promoter and supported its role as a DEPTOR gene transcriptional activator. Using CHIP-on-chip, Mason et al. (32) identified three ERE-like sequences within 100 kbp of DEPTOR transcription start site that may play a role as distal enhancer elements facilitating the recruitment of ERα to the proximal promoter region by chromatin looping. We postulate that all these ERE-like sequences cooperate to ensure estrogen-ERα-mediated transcription of DEPTOR gene.

As a positive control, we determined the interaction of ERα with the TFF1 gene promoter (Fig. 8C). As expected, we detected strong binding of ERα in estrogen-stimulated cells compared with vehicle- or tamoxifen-treated cells. Unlike DEPTOR, TFF1 promoter contains an ERE-like sequence that diverges from canonical ERE in only one nucleotide, and therefore, binds to ERα with much higher affinity.

Estrogen-ERα-induced Upregulation of DEPTOR Modulates mTOR Activity—DEPTOR was characterized as an inhibitor of mTOR kinase activity, although its specific effect on mTORC1 and mTORC2 depends on the cellular context (23). To investigate how DEPTOR overexpression affects mTOR function in MCF7 cells, we first confirmed the interaction of DEPTOR with mTOR by immunoprecipitation assays (Fig. 9A). We observed that estrogen-induced accumulation of DEPTOR resulted in increased binding of DEPTOR to mTOR. However, mTORC1 and mTORC2 were still activated by estrogen as determined by the phosphorylation of S6K1 on Threonine 389 and AKT on Serine 473, respectively (Fig. 9B). The silencing of DEPTOR with specific siRNAs resulted in upregulation of mTORC1 and mTORC2 in both vehicle- and estrogen-treated cells, which confirmed the inhibitory effect of DEPTOR on these two complexes (Fig. 9B). These results demonstrated the critical role of estrogen-ERα pathway as a modulator of mTOR activity in ER-positive MCF7 breast cancer cells. Thus, the activation of mTOR by the cytoplasmic nongenomic estrogen-ERα pathway is partially counterbalanced by the upregulation of DEPTOR expression mediated by the genomic estrogen-ERα pathway.

Estrogen-ERα is an mTOR Modulator via DEPTOR
Estrogen-ERα is an mTOR Modulator via DEPTOR

Fig. 5. Estrogen promotes DEPTOR expression. A–D, MCF7 cells were grown in phenol red-free DMEM supplemented with 10% charcoal-treated FBS for 3 days, serum-starved for 24 h, and treated with 0.2% charcoal-stripped FBS (A), 0.2% charcoal-stripped FBS and estradiol (100 nM) (B), 10% charcoal-stripped FBS (C), or 10% charcoal-stripped FBS and estradiol (100 nM) (D) for the indicated times. Cell extracts were prepared and resolved by SDS-PAGE. Indicated proteins were analyzed by immunoblotting.

Fig. 6. Estrogen-ER pathway induces DEPTOR expression. MCF7 cells were cultured in phenol red-free DMEM supplemented with 10% charcoal-treated FBS for 3 days, serum-starved for 24 h, and treated with vehicle (ethanol), estradiol (100 nM), and estradiol and Fulvestrant (10 nM), or tamoxifen (100 nM) for 24 h in phenol red-free DMEM supplemented with 0.2% charcoal-treated FBS (left panels) or 10% charcoal-stripped FBS (right panels). Cell extracts were obtained and resolved by SDS-PAGE. Indicated proteins were analyzed by immunoblotting.

DISCUSSION

In recent years, an increasing number of studies have demonstrated that the combination of the mTORC1 inhibitor everolimus with either tamoxifen or the aromatase inhibitor exemestane, respectively, improved progression-free survival of advanced ER-positive HER-negative breast cancer patients. However, the overall survival was not improved (22). Therefore, it is critical to delineate the connection between the estrogenic and mTOR pathways to design more effective combined therapeutic strategies.

To better understand the relationship between estrogen-ERα and mTORC1 pathways, we performed analysis of the phosphoproteome in ER-positive MCF7 cells treated with estrogen or estrogen and the mTORC1 inhibitor rapamycin (Fig. 1). We identified multiple mTORC1 targets regulated by estrogen (Table I and Fig. 2).

As expected, most of these proteins are involved in transcriptional regulation or are components of the mTORC1 pathway, but factors implicated in other signaling and metabolic pathways are enriched as well (Fig. 3). Thus, we identified estrogen-stimulated and mTORC1-sensitive components of the Rac1 pathway. Rac1 is a member of the Rho GTPase family that plays a critical role in the proliferation of breast cancer cells bearing PI3K-activating mutations such as MCF7 cells (34). PI3K-activated Rac1 may bind to mTOR and modulate its kinase activity (35, 36). Because mTORC1-sensitive phosphorylation of Rac1 and PAK proteins is detected in our screening, we speculate that mTORC1 in turn regulates Rac1 activity. Interestingly, Rac1 also enhances ERα expression (37). Additionally, we observed phosphorylation of JAK2 and STAT1, components of the IL4 and IL2rb pathways, and propose a negative effect on their activity. JAK2 is a negative regulator of ERα that promotes ERα degradation (38). From our results, we speculate that estrogen-induced and mTORC1-mediated phosphorylation of JAK2 at Thr-530 may inhibit JAK2 activity and prevent ERα degradation. Supporting this idea,
phosphorylation of the same region of JAK2 (Ser-523) by ERK results in reduced kinase activity (39). Enriched phosphorylation of components of the NFAT pathway is also detected in our screening. Like JAK2, we propose a negative effect of the estrogen-induced and mTORC1-mediated phosphorylation on NFAT pathway. Accordingly, the binding of ERα/H9251 to NFAT3 or the phosphorylation of NFATc1 by mTORC1 repress their transcriptional activity (40, 41). For the metabolic pathways, as expected, we observed mTORC1-mediated upregulation of glycolytic pathways and downregulation of fatty acid oxidation (11, 42).

From the phosphoproteome analysis, we selected DEPTOR for further characterization. DEPTOR is an mTOR-interacting protein that inhibits mTORC1 and mTORC2 kinase activities (24). On mitogen-stimulation, active mTOR complexes phosphorylate DEPTOR, which facilitates the binding of the E3 ubiquitin ligase β-TrCP to DEPTOR and its subsequent degradation (43). Although DEPTOR is phosphorylated by mTORC1 in estrogen-stimulated MCF7 cells (Fig. 4A), we did not observe reduced levels of DEPTOR (Fig. 7). In contrast, we detected the accumulation of DEPTOR mRNA and protein in estrogen-treated cells compared with vehicle-treated cells (Fig. 4B, 5, 6, and 8). These results suggested estrogen-mediated transcriptional upregulation of DEPTOR expression. Accordingly, we demonstrated the specific interaction of estrogen-bound ERα with the proximal region of DEPDC6 promoter, the gene encoding DEPTOR (Fig. 8). ERα binds to two different regions within DEPDC6 promoter, although an ERE-like sequence is only identified in one of them. Surprisingly, this is a common characteristic of estrogen-regulated genes. In most of these genes, multiple half EREs, ERE-like sequences, or non-ERE sequences located up to 200 kbp from the transcription start site are required for estrogen-ERα-mediated transcriptional regulation (2, 32). Three other ERE-like sequences have been identified by CHIP-on-chip within 100 kbp from DEPDC6 transcription start site (32). We propose that these distal ERE-like sequences may play a role as distal enhancer elements and facilitate the recruitment of ERα to the proximal promoter region of DEPDC6 gene by chromatin looping. Thus, the lower affinity of ERα for the ERE-like sequences is compensated by the presence of several sequences and together with other cofactors stabilize the binding of ERα to the DNA and promote the transcription of target genes (2, 44). However, we observed much higher affinity of ERα for the ERE-like sequence within TFF1 promoter, which better matches with the consensus ERE sequence, and consequently, a faster response to estrogen (Fig. 8). Higher levels of DEPTOR mRNA have been detected in other ER-positive breast cancer cell lines and in ER-positive breast tumors compared with ER-negative disease (45). These results corroborate the relationship between ERα and DEPTOR, and suggest a critical role of DEPTOR in the biology of ER-positive breast tumors. Pavani et al. (45) have demonstrated a dual role of DEPTOR in the progression and metastasis of ER-negative breast tumors. Thus, low levels of DEPTOR in primary tumors are critical for the acquisition of a mesenchymal (EMT) phenotype and cell migration and invasion, but high levels in...
metastatic lesions are essential for cell survival and chemotherapy.

Low levels of DEPTOR mRNA are frequently detected in human cancers and support its role as a tumor suppressor, but some exceptions are found. For example, high expression of DEPTOR was observed in Multiple Myelomas (MM), T-cell acute lymphoblastic leukemia (T-ALL), thyroid carcinoma, hepatocellular carcinoma, esophageal squamous cell carcinoma, and osteosarcomas, and was linked to poorer survival (23). Initial studies in MM cells (23) showed that high DEPTOR levels were associated with reduced mTORC1 activity as determined by the decreased phosphorylation of S6K1 at Thr-389, but increased signaling of PI3K toward mTORC2 as indicated by the elevated phosphorylation of AKT at Ser-473. These results suggest that DEPTOR-mediated inhibition of mTORC1 relieves the feedback inhibitory mechanisms to PI3K, which triggers the activation of PI3K/AKT pathway. In this cell context, activation of PI3K/AKT dominates over DEPTOR for the control of mTORC2 activity. The role of DEPTOR in the inhibition of mTORC1 and the activation of PI3K/AKT pathway is essential for the proliferation and survival of MM cells (24). Interestingly, we observed that estrogen-induced upregulation of DEPTOR affects both mTORC1 and mTORC2 complexes, because the phosphorylation of S6K1 at Thr-389 and AKT at Ser-473 increases in estrogen-treated cells compared with vehicle-treated. As discussed above, our results also suggest that the partial inhibition of mTORC1 by DEPTOR may result in the activation of PI3K and consequently, mTORC2 complex, because we detected a
strong phosphorylation of AKT at Ser-473 in estrogen-stimulated control cells (Fig. 9). Unlike MM (46), PI3K-activating mutations are frequently detected in ER-positive breast tumors and cancer cells such as MCF7 cells (47, 48). Consequently, both mTOR complexes are activated in this cell line, which may explain the different effect of DEPTOR overexpression in these cells compared with MM cells. We observed that estrogen stimulation of MCF7 cells bearing PI3K-activating mutations hyperactivates mTOR complexes, which would result in an increased rate of high energy-demanding processes such as synthesis of proteins, lipids, and nucleotides. By inducing DEPTOR expression, the estrogen-ERα pathway modulates mTOR activity to preserve cellular homeostasis. Other genetic abnormalities that indirectly affect the mTORC1 pathway are frequently detected in ER-positive breast tumors (e.g. mutations in TP53 or components of the MAPK pathway) (47). In these tumors, estrogen-induced expression of DEPTOR may regulate mTOR activity by a mechanism like the one described in MM cells. These studies propose a critical role of estrogen-ERα pathway in the balance of mTORC1 and mTORC2 activities in ER-positive breast cancer cells. Therefore, therapeutic targeting of ER may contribute to aberrant mTOR activity in certain cell contexts, which has been associated with cancer cell proliferation and survival, and resistance to endocrine therapy. Accordingly, hyperactivation of the PI3K pathway is frequently associated with antiestrogen resistance (5). This pathway integrates the signaling from receptor tyrosine kinases such as HER2 or IGF1, which are frequently amplified or overexpressed in resistant tumors, to overcome the inhibitory effects of endocrine therapies (49). Additionally, hyperactivation of PI3K correlates with reduced expression of ERα, which is frequently observed in resistant cells (50). Consequently, our results support the combination of mTOR inhibitors and endocrine therapy for the treatment of advanced ER-positive breast cancers but predict that dual PI3K/mTOR or mTORC1/2 inhibitors together with endocrine therapy might be a more effective therapeutic strategy. Many ongoing clinical trials are evaluating different PI3K and/or mTOR inhibitors in combination with endocrine therapy (51).

Acknowledgments—The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (52) partner repository with the dataset identifier PXD013503 and 10.6019/PXD013503.

* This work was supported by American Cancer Society Grant RSG-13-287-01-TBE; NIGMS, National Institutes of Health Grant GM128675; NCI, National Institutes of Health Grant CA151112, and a research award from the Atol Charitable Trust to M.K.H.

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Author contributions: R.C., J.E.M., and M.K.H. designed research; R.C. performed research; R.C., V.A.P., A.K.S., C.-F.T., J.E.M., and M.K.H. analyzed data; R.C., J.E.M., and M.K.H. wrote the paper; M.A.G. and T.L. proteomics analysis.

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