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Estrogenic regulation of S6K1 expression creates a positive regulatory loop in control of breast cancer cell proliferation

D. Myriam Maruani, BA¹, Tirtza N. Spiegel, BA¹, Emily N. Harris, BA¹, Avielle S. Shachter, BA¹, Helen A. Unger¹, Sandra Herrero-González, PhD², and Marina K. Holz, PhD^{1,3}

¹Department of Biology, Stern College for Women of Yeshiva University, New York, New York 10016

²Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

³Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Abstract

The 40S ribosomal S6 kinase 1 (S6K1) is an important regulator of cell growth. Expression of S6K1 is often elevated in breast cancer cells. However, the transcriptional mechanism of S6K1 overexpression is not understood. In this report, we demonstrate that estrogen activates expression of S6K1 via Estrogen Receptor (ER) α in ER-positive breast cancer cells. We also show that estrogen acts on the proximal promoter of the S6K1 gene in a mechanism involving the transcriptional factor GATA-3. Finally, we provide data that support the importance of estrogenic regulation of S6K1 expression in breast cancer cell proliferation. S6K1 directly phosphorylates and regulates ligand-independent activity of ER α , while ER α upregulates S6K1 expression. This S6K1-ER α relationship creates a positive feed-forward loop in control of breast cancer cell proliferation. Furthermore, the co-dependent association between S6K1 and ER α may be exploited in the development of targeted breast cancer therapies.

Keywords

S6K1; mTOR; estrogen; rapamycin; breast cancer

Introduction

The 40S Ribosomal S6 Kinase 1 (S6K1) is a key regulator of cell size control and cell proliferation downstream of the mammalian target of rapamycin (mTOR) [1, 2]. The activation of S6K1 by mTOR occurs in response to cellular energy status, nutrient levels, and mitogens. Full and sustained S6K activation requires multiple phosphorylation events

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Corresponding Author: Marina K. Holz, Department of Biology, Stern College for Women of Yeshiva University, 245 Lexington Ave., New York, NY 10016. Tel.: 212-340-7838; Fax: 212-340-7883; mholz@yu.edu.

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by phosphatidylinositol 3-kinase (PI3K)-dependent and -independent mechanisms [3]. Treatment with rapamycin, a potent inhibitor of mTOR complex 1 (mTORC1), results in rapid dephosphorylation and inactivation of S6K1 [4]. Results from *S6k1* mouse knockouts uncovered a specific role for S6K1 in regulation of cell growth [5]. S6K1 has been shown to drive the G1/S cell cycle progression, and overexpression of S6K1 provides a significant proliferative advantage in low serum conditions [1, 6, 7]. The study of cellular targets and interacting partners of S6K1 revealed a repertoire of proteins involved in transcription (CREM τ , UBF-1, and ER α), RNA processing (SKAR), protein translation (rpS6, eIF3, eIF4B, eEF2K, and PDCD4), survival (Bad and MDM2), and signaling feedback (IRS-1, mTOR, and rictor), [reviewed in 2].

The *RPS6KB1* gene is located in the 17q23 genomic region. 17q23 amplifications in breast cancer cause increased copy number of the *RPS6KB1* gene, resulting in elevated S6K1 expression [7–13]. While amplification of this region has been observed in several tumor types, high copy number amplification occurs in breast cancer specifically [12]. Furthermore, *RPS6KB1* amplification and S6K1 overexpression are associated with poor prognosis in breast cancer patients, supporting the role of S6K1 in disease development and/or progression [9, 14].

Clinically, up to 60% of breast cancers are Estrogen Receptor (ER)-positive. ER-positive breast cancers can be targeted therapeutically by antiestrogens (such as tamoxifen) or aromatase inhibitors. However, only about half of ER-positive breast cancers respond to endocrine treatments [15], and resistance frequently develops [16]. Thus, an important clinical strategy is to use a combination therapy approach with inhibitors of other signaling pathways, and to understand the underlying molecular mechanisms.

Targeting S6K1 may be a feasible strategy for the following reasons. First, 17q23 amplification strongly correlates with ER-positive status [13], and is one of the most frequent aberrations in ER-positive invasive ductal carcinoma [17]. Second, *RPS6KB1* was determined to be a gene whose gain in ER-positive tumors is prognostic of the metastatic capacity of human breast cancer [18]. Third, *RPS6KB1* amplification and S6K1 overexpression in ER-driven breast cancers may stem from its involvement in ER α regulation. Indeed, we and others have shown that S6K1 regulates ER α transactivational activity in control of breast cancer cell proliferation by directly phosphorylating ER α on Ser167 [7, 19, 20]. Therefore, maintaining high co-overexpression of both S6K1 and ER α may provide a selective advantage for breast cancer cells.

While many studies have addressed the regulation of S6K1 kinase activity, very little is known regarding the transcriptional regulation of S6K1 expression. Since ER α is a transcription factor that regulates expression of many growth- and proliferation-promoting genes, we asked whether ER α is involved in regulation of S6K1 expression. We found that in ER-positive breast cancer cells, estrogen regulates S6K1 expression via ER α . Our data support a model of a functional co-regulatory relationship between ER α and S6K1 that controls breast cancer cell proliferation. Most significantly, the identification of *RPS6KB1* as an estrogen-inducible gene may help to elucidate the mechanisms of breast cancer pathogenesis, and may lead to the development of new targeted therapies.

Results

Estrogen regulates S6K1 protein expression

We used the ER-positive MCF-7 breast cancer cell line, which serves as a model for studying the basic responses of breast cancer cells to estrogens. We observed that the addition of estrogen to MCF7 cells resulted in a dose-dependent increase in S6K1 protein levels. As shown in Figure 1A, elevated S6K1 expression correlated with increased activity of S6K1 as detected by phosphorylation of S6K1 target proteins, rpS6 at Ser240/244 and ER α at Ser167. In addition, we quantified the upregulation of S6K1 expression by estrogen in MCF7 cells using in-cell Western analysis. As depicted in Figure 1B, S6K1 expression increased with gradual dose increase of estrogen. Since the kinase activity of S6K1 is important for driving cellular proliferation, we also directly measured the activity of endogenous S6K1 in estrogen-treated MCF7 cells using an *in vitro* kinase assay (Figure 1C). We observed that the increase in S6K1 protein levels results in a corresponding increase in the enzymatic activity of S6K1. In parallel, we determined whether estrogen-induced S6K1 kinase activity correlates with ER α transcriptional activity. As shown in Figure 1D, we transfected MCF7 cells with a reporter construct that contains firefly luciferase gene under the control of three estrogen response elements (EREs) and a control construct expressing renilla luciferase under the control of SV40 promoter for luciferase activity normalization, and then starved and stimulated the cells as in Figure 1B. We found that estrogen-regulated transcriptional activity mirrored the S6K1 kinase activity we observed earlier. We next investigated whether estrogen-mediated regulation of S6K1 levels depends on the cell line's ER status. As illustrated in Figure 1E, estrogen addition resulted in increased S6K1 levels in other ER-positive cell lines, BT-474 and ZR-75-1, but not in the ER-negative cell lines MDA-MB-231 and MDA-MB-468. We also confirmed our observations in primary cells by analyzing S6K1 expression in mammary glands from ovariectomized mice treated with 17 β -estradiol for 12 and 18 hours. As depicted in Figure 1F, expression of total S6K1 protein, as well as its activated form (phosphorylated on Thr389), and phosphorylated rpS6 increased in murine mammary cells following estradiol administration *in vivo*. We also observed that estrogen regulates S6K1 mRNA expression, as determined by real-time RT-qPCR analysis (Figure 1G). Using Actinomycin D treatment, we determined that estrogen regulates transcription, rather than stability of the S6K1 transcript. Actinomycin D prevented estrogen-induced upregulation of the S6K1 mRNA in serum-starved cells, but we observed no significant decrease in mRNA levels in untreated growing cells. Collectively, these data indicate that estrogen regulates S6K1 expression.

Estrogen regulates *RPS6KB1* promoter activity

To determine whether *RPS6KB1* regulation by estrogen may occur through its interaction with ER α , we extracted the proximal promoter region of *RPS6KB1* from the UCSC Genome Bioinformatics database and performed a search for EREs and other transcription factor binding sites using publically available transcription factor binding sites predictor programs, such as TESS (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>) and TRANSFAC (gene-regulation.com). While the region spanning 2 kb upstream and downstream of the transcription start site did not contain consensus EREs, we identified an imperfect

palindrome, a half-ERE, as well as three GATA-3 sites, two SP-1 sites, an AP-1 site, and a TATA-box (Figure S1).

To test the hypothesis that estrogen may regulate *RPS6KB1* expression via the proximal promoter region, we obtained the 1kb promoter region upstream of the *RPS6KB1* transcription start site controlling firefly luciferase expression and measured the activity of the *RPS6KB1* promoter in ER+ and ER- cells. As depicted in Figure 2A, estrogen addition to ER+ MCF7 cells resulted in a significant dose-dependent increase in luciferase activity controlled by the *RPS6KB1* promoter. A similar increase in the *RPS6KB1* promoter activity was measured in another ER-positive cell line, BT-474 (Figure 2B). We then tested whether ER α directly binds the 1kb proximal promoter region of *RPS6KB1* using chromatin immunoprecipitation (ChIP) promoter tiling assay with primers spaced around every 100bp. However, ChIP assay did not reveal significant enrichment in ER α at the *RPS6KB1* 1kb promoter region in estrogen-stimulated MCF7 cells compared to binding to the control PS2 promoter (data not shown). However, others have detected ER α binding near the *RPS6KB1* gene [21], leading us to theorize that ER α may synergize with other transcription factors to regulate this promoter region. Since the 1kb promoter region contains several consensus GATA-3 binding sites, we investigated whether estrogen regulates the promoter via GATA-3. Indeed, following estrogen treatment we observed significant enrichment in GATA-3 throughout the *RPS6KB1* promoter, but not at the transcription start site (TSS) (Figure 2C). Moreover, we tested for enrichment in RNA polymerase II C-terminal tail phosphorylated at serine 5 (RNPII S5P) and histone H3 trimethylated at lysine 4 (H3K4Me3), which are markers of transcriptional activation. We detected estrogen-stimulated binding of RNPII S5P to the promoter of *RPS6KB1*, peaking strongly at TSS, and decreasing gradually away from the TSS in the 1kb region tested. Similarly, we detected estrogen-stimulated H3K4Me3 presence at TSS and up to several hundred bp away. In contrast, we did not detect RNPII S5P and H3K4Me3 presence within an exonic region, underscoring their role as markers of active promoter regions. As a control, total histone H3 binding was present in all sequences tested, and the levels were unchanged by estrogen treatment. Thus, estrogen may regulate the transcriptional activity of the *RPS6KB1* promoter in a mechanism involving recruitment of GATA-3.

Modulation of ER α overexpression affects S6K1 levels

To further determine whether estrogen regulates S6K1 expression via ER α , we overexpressed ER α in MCF7 cells. We observed constitutively upregulated S6K1 protein expression, which was no longer inducible by estrogen (Figure 3A). We next expressed different amounts of ER α in MCF7 cells and ER-negative HEK293E cells in the absence of estrogen (Figure 3B). We observed a dose-dependent increase in S6K1 expression, which correlated with increased activity of S6K1, as manifested by increased phospho-S6 levels. Expression of ER α with a mutation in serine 167, a residue phosphorylated by S6K1, an allele with defective transcriptional activity, was not able to induce S6K1 expression in ER-negative MDA-MB-231 cells (Figure 3C). We next interrogated the effect of estrogen on the *RPS6KB1* promoter in ER α -overexpressing cells using luciferase reporter assay. As depicted in Figure 3D, ER α overexpression in ER-negative HEK293E cells resulted in high basal promoter activity, which was further augmented by estrogen addition. In ER-negative MDA-

MB-231 cells, ER α overexpression increased basal *RPS6KB1* promoter activity, which was further augmented in the presence of estrogen (Figure 3E). Next, we lowered ER α expression by means of two different siRNA duplexes or a combination of the two siRNAs in MCF7 cells (Figure 4A). Reduced ER α expression resulted in decreased S6K1 protein levels. Moreover, both basal and estrogen-induced activity of the *RPS6KB1* promoter was severely reduced following ER α knockdown (Figure 4B).

ER α and S6K1 collaborate in control of cell proliferation

We have previously shown that S6K1 provides a proliferation advantage to cells growing in low-serum medium. These cells become dependent on the continuous activity of S6K1 in such conditions [7]. Therefore, we sought to determine whether cells are also dependent on ER α under restricted-serum conditions. As depicted in Figure 5A, we examined the effects of full and low serum, rapamycin treatment, and the role of ER α and S6K1 levels in MCF7 cells. First we focused on the effect of rapamycin on cell growth in full and low serum. As expected, MCF7 cells were sensitive to rapamycin treatment, however, the effect was slightly more pronounced in low serum, underscoring the dependence of these cells on S6K1 activity for continuous proliferation. Next, we examined the effect of ER α knockdown on MCF7 cell proliferation in full and low serum. As expected, reduction of ER α expression led a significant decrease of about 25% in cell proliferation in full serum, and this effect was even more pronounced in low serum conditions (about 50% reduction) compared to the control siRNA. Significantly, ER α knockdown in low serum together with rapamycin reduced cell proliferation more effectively than ER α knockdown in full serum in the presence of rapamycin, compared to the siRNA control. Finally, we investigated whether the effect of ER α knockdown on proliferation could be rescued by overexpression of S6K1. We found that S6K1 overexpression could partially rescue proliferation reduction brought by ER α knockdown. However, as expected, in the presence of rapamycin S6K1 was not longer able to rescue the knockdown.

Conversely, we examined the effect of ER α overexpression on S6K1 expression and proliferation in full and low serum. ER α induced basal S6K1 expression in MDA-MD-231 cells in serum-free conditions, and this effect was increased in low and full sera (Figure 5B). While in full serum ER α overexpression had no discernable effect on cell proliferation, a small, but significant effect on proliferation was observed in low serum, compared to vector-expressing control (Figure 5C). Importantly, ER α Ser167Ala mutant was not able to increase proliferation in low serum. We also made an interesting observation that ER α overexpression rendered the cells more sensitive to rapamycin treatment in both full and low serum conditions, similar to the phenotype observed with S6K1 overexpression [7].

Discussion

A better understanding of the molecular mechanisms by which estrogen stimulates cell growth can provide new insight into the diagnosis, treatment, and prevention of ER-positive breast cancer. For this reason, the identification of estrogen-responsive genes with oncogenic properties could provide new avenues for experimental investigation. In this report, we demonstrated that estrogen activates expression of S6K1 via ER α . The

identification of S6K1 as an estrogen-inducible gene is of great interest since it sheds light on the mode of transcriptional regulation of S6K1 expression and may help understand the role of S6K1 overexpression in the development and progression of breast cancer.

We found that estrogenic activation of S6K1 expression is mediated through ER α , as summarized in Figure 6. In general, ER binds DNA either directly via EREs or tethered through other transcription factors. Maximum ER transcriptional activity is thought to be displayed by the interaction of ER with the ERE consensus sequence (CAGGTCAnnnTGACCTG). While the ERE consensus sequence is rarely found in the promoter regions of human genes [22], the promoter regions of estrogen-responsive genes usually contain more than one imperfect palindromic sequence and widespread ERE half-sites, which have been shown to act synergistically [23]. While the promoter region of *RPS6KB1* contains what appear to be an imperfect palindrome and a half-site, we were not able to detect ER α binding. It is known that ER α may exert effects at non-ERE-containing chromatin targets through protein–protein interactions with DNA-bound transcription factors [21]. The presence of several canonical GATA boxes in the promoter region of *RPS6KB1* led us to hypothesize that GATA-3 may be involved in mediating estrogenic effects at this promoter. Using ChIP analysis we observed a significant enrichment in estrogen-induced GATA-3 presence at the *RPS6KB1* promoter, indicating a possible mechanism through which ER α may mediate its transcriptional effect. This is a revealing finding because estrogen stimulation of ER α -positive breast cancer cell proliferation has been previously reported to occur via the transcription factor GATA-3 [24, 25]. Moreover, analogous to S6K1, GATA-3 has also been shown to correlate in expression and activity with ER [24, 26, 27].

In addition to direct binding by GATA-3, published data suggest that estrogenic regulation of S6K1 expression may also occur via long-distance and secondary ER α effects that may regulate the *RPS6KB1* proximal promoter. About 95% of ER α binding sites are not located within the promoter proximal region, but rather are found in the intronic or distal locations of the transcription start site of a gene [28, 29]. *RPS6KB1* was found to be associated with half or full EREs in the 20kb region 5' to the transcription start site [30], and ER α was found to interact directly within an exonic region of *RPS6KB1* [31]. The molecular mechanism by which ER α synergizes with various transcription factors to contribute to estrogen-dependent *RPS6KB1* expression remains to be addressed.

We made the intriguing observation that S6K1 expression is maintained in two modes: a basal level of expression, typical of normal cells, and an estrogen-ER α specific upregulation. Correspondingly, we did not observe an increase in S6K1 expression in ER-negative cell lines. The significance of the co-stimulatory relationship between ER and S6K1 can be seen when examining the rate of cell proliferation. Estrogen-induced proliferation is largely dependent on mTOR signaling, and we observed that treatment of MCF7 cells with rapamycin reduced cell proliferation. The novel finding presented here was the effect of S6K1 and ER inhibition in low serum conditions. The ability to proliferate in such conditions is one of the hallmarks of neoplastic transformation. We have previously shown that S6K1 provides a proliferative advantage to cells in low serum conditions [7]. Here we found that rapamycin was more effective in reducing cell proliferation of cells

grown in low serum than in full serum. This finding is in agreement with the role of S6K1 in driving proliferation in low serum conditions, whereby the cells are more dependent on the activity of this kinase, and thus are more acutely sensitive to its inhibition. Similarly, ER α knockdown was more effective in reducing cell proliferation in low serum than in full serum conditions, underscoring the importance of the S6K1-ER α relationship in these cells. In addition, S6K1 was able to partially rescue ER α knockdown. We observed that wildtype ER α , but not ER α lacking the S6K1-dependent phosphorylation site (Ser167Ala mutant), was able to induce S6K1 expression and promote proliferation in low serum. This observation is supported by work from others which found that wildtype ER α , but not the Ser167Ala mutant, was able to promote focus formation of cells grown in 1% serum [20]. We observed that increased estrogenic expression of S6K1 leads to increased kinase activity and phosphorylation of its downstream effectors, including ER α . Together, these data support our model of a positive co-regulatory loop between S6K1 and ER α . Importantly, S6K1 can promote ER α activation in the absence of estrogen, therefore, high expression of S6K1 might be one reason for acquiring ligand-independent proliferative ability [7, 19]. Moreover, estrogen and ER α -mediated stimulation of cell proliferation leads to accumulation of genetic damage, resulting in neoplastic transformation [32]. Thus, 17q23 chromosomal region gain will augment expression of *RPS6KB1* and activation of ER α , and will contribute to dysregulated proliferation of cells during the progression to carcinogenesis and breast cancer.

The future investigation of the relationship between S6K1 and ER α would lead to utilization of S6K1 as a prognostic marker and a therapeutic target, and would allow us to develop novel cancer treatments to inhibit downstream pathways of estrogen action. While combination of endocrine therapy and mTOR/S6K1 pathway inhibitors has been previously used [33], the molecular mechanism explaining the patient response has not been determined [34]. Based on the data presented above, we propose that the positive co-regulatory loop between S6K1 and ER α promotes cell proliferation in ER-positive breast cancer, and provides a rational approach for treatment of ER-positive and S6K1-overexpressing tumors.

Materials and Methods

Mammary cell extract preparation

Mouse mammary glands were kindly provided by Dr. Antonio Di Cristofano (Albert Einstein College of Medicine of Yeshiva University, Bronx, NY). Briefly, ovariectomy was performed on 5 to 9-week-old 129/Sv mice. Estradiol (Sigma) was injected s.c. at 1 μ g/mouse in corn oil for 12 or 18 h treatment. Abdominal mammary glands were removed and immediately frozen in liquid nitrogen. Tissue was homogenized in RIPA buffer, aliquoted, and frozen for further analysis by immunoblot.

Cell culture

MCF7 cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS). BT-474, HEK293E, ZR-75-1, and MDA-MB-231 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS. For experiments testing the effects of

estrogen, cells were grown for three days in phenol-red free media with charcoal-stripped (low-estrogen) FBS.

Cell transfection

pCMV5-FLAG-ER α (W. Lee Kraus, UT Southwestern Medical Center, Dallas, TX) and pRK7-S6K1 was transfected into cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's instructions. ER α Ser167Ala was generated by site-directed mutagenesis (Genewiz, Plainfield, NJ).

RNAi against ER α

For siRNA studies, double-stranded RNAs targeting two different sequences for ESR1 and a scrambled control were purchased from Qiagen. MCF7 cells were transfected with a total of 50 nM siRNA per 35-mm dish using Lipofectamine2000 (Invitrogen) according to the manufacturer's recommendations. 24 h post-transfection, cells were deprived of serum overnight, and treated with agents as indicated in the figure legend.

Reporter gene assays

1kb *RPS6KB1* promoter controlling firefly luciferase expression was purchased from SwitchDB/Promega. pGL2-3xERE-TATA-luc were kindly provided by Donald P. McDonnell via Addgene. (Duke University, Durham, NC). pIS2 *Renilla* luciferase reporter was kindly provided by David Bartel (MIT, Cambridge, MA) via Addgene. For luciferase reporter assays, cells were transfected using Lipofectamine2000 (Invitrogen) following the manufacturer's protocol with the *RPS6KB1* promoter or the pGL2-3xERE-TATA-luc reporter and control *Renilla* luciferase. At 24 h post-transfection, agents were added as indicated in figure legends. At 48 h post-transfection, cells were harvested using 1X Passive Lysis Buffer (Promega), and relative luciferase activity from three independent experiments performed in triplicate was measured using the Dual Luciferase Reporter Assay System and Glomax-multi luminometer (Promega).

Immunoblots

Cells were lysed using 1X Passive Lysis Buffer (Promega). Whole-cell lysates (10% of total cell extract) were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (4–15%). Proteins were transferred to a nitrocellulose membrane (Schleicher and Schuell), and blotted with the indicated antibodies. Anti-S6K1 antibody has been previously described [19]. Anti-phospho-S6K1 Thr389, anti-phospho-S6 Ser240/244, and anti-phospho-ER Ser167 antibodies were purchased from Cell Signaling Technology. Anti-ER and anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-FLAG antibodies were from Sigma. For immunoblotting, anti-rabbit, anti-mouse, and anti-goat horseradish peroxidase (HRP)-conjugated antibodies were purchased from Amersham, Chemicon, and Santa Cruz Biotechnology, respectively. Immunoblots were developed using enhanced chemiluminescence reagents (Pierce) and Chemidoc XRS imager with Quantity One software (Bio-Rad).

In-Cell Westerns

MCF7 cells were seeded in a 96-well plate, and starved in serum- and phenol-red free media for 24 hours. Cells were stimulated with the indicated amounts of estrogen or fed with full-serum media (untreated control). After 24 h, cells were fixed with 3.7% formaldehyde in 1X PBS, permeabilized by with 1X PBS containing 0.1% Triton® X-100, blocked and stained with the anti-S6K1 antibody (rabbit), followed by anti-rabbit IRDye® 800CW antibody and DRAQ5 cell-permeable DNA-interactive agent that was used for cell number normalization. The plate was scanned with detection in both 700 and 800 nm channels using an Odyssey® Imaging System (LiCOR), and the signal in the 700 nm channel (S6K1) was normalized to the signal in the 800 nm channel and quantified using the accompanying software.

Immune complex kinase assays

S6K1 was immunoprecipitated with anti-S6K1 antibody and protein A/G sepharose beads (Sigma). Immunoprecipitates were stringently washed once in 1 ml each of buffers A (10 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 100 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 2 mM dithiothreitol, 40 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 5 µg/ml pepstatin, pH 7.2), B (same as buffer A, except for 0.1% NP-40 and 1 M NaCl), and ST (50 mM Tris-HCl, 5 mM Tris base, 150 mM NaCl), and resuspended in 20 mM HEPES pH 7.2, 10 mM MgCl₂, 0.1 mg/ml BSA, 3 mM 2-mercaptoethanol.

Kinase assays were carried out by using a GST fusion of the last 32 amino acids of 40S ribosomal protein S6 as a substrate, by addition of S6K1 in a reaction containing 20 mM HEPES, 10 mM MgCl₂, 50 µM ATP, 5 µCi of [γ -³²P]ATP, 3 ng/µl protein kinase A inhibitor, pH 7.2, in a linear range of substrate phosphorylation for 10 min at 30°C. The reaction products were resolved using SDS-PAGE, the gels dried and exposed to X-Ray film, and the kinase activity was quantified by autorad using QuantityOne software (BioRad).

RT-PCR

MCF7 cells were starved in phenol-red-free media for 24 h, and then treated with estrogen for 4, 8, 16 or 24 h, or pre-treated with actinomycin D (10 µM, Sigma). Cells were lysed and total RNA was isolated using RNAeasy kit (Qiagen). Conventional RT-PCR was performed using the Easy-A® One-Tube RT-PCR System (Stratagene) using the following primers: S6K1 forward: 5'-AGAAATGCTGCTTCTCGTCTGGGA-3', reverse: 5'-GGTGTTCTGTTGGGCTGCCAATAAAT-3'; GAPDH forward: 5'-CCACCCATGGCAAATTCCATGGCA-3', reverse: 5'-TCTAGACGGGCAGGTCAGGTCCACC-3'. For Real-Time PCR (qPCR), cDNA synthesis was carried out using the iScript kit (BioRad) according to the manufacturer's instructions. mRNA levels of S6K1 were measured by qPCR with GAPDH as the internal standard; qPCR was performed using the SYBR Green PCR (BioRad) and processed in the CFX96 real-time thermal cycler (BioRad). The oligonucleotides used were: S6K1 forward: 5'-CTCTGAGGATGAGCTGGAGG-3', S6K1: 5'-TTCTCACAATGTTCCATGCC-3'; GAPDH forward: 5'-CATGTTCCAATATGATTCCACC-3', reverse: 5'-GATGGGATTTCCATTGATGAC-3' The relative gene expression was calculated by

$2^{-\Delta\Delta CT}$ using the CFX Data Manager Software, where CT = fluorescence threshold value; $\Delta CT = CT$ of the target gene – CT of the reference gene (GADPH); and $\Delta\Delta CT = CT$ of the target sample – CT of the reference sample (cells growing in full media). The results were expressed in n-fold differences in mRNA expression relative to the expression of GAPDH and the reference sample.

Chromatin immunoprecipitation and qPCR

MCF-7 cells were grown for at least 3 days phenol-red free media with charcoal-stripped (low-estrogen) FBS. Cells at ~90% confluency were starved in serum- and phenol-red free media for 24 h and 10nM estrogen was added for 24 h, were indicated. The cells were cross-linked with 1% formaldehyde in phosphate-buffered saline at 37 °C for 12 min immediately prior to harvesting. CHIP assays were performed as described previously [35] using polyclonal antibodies against ER α and GATA-3 (Santa Cruz Biotechnologies), histone H3, trimethylated histone H3, and RNA pol II phosphorylated Ser5 (Abcam), as well as “no antibody” controls. The resulting ChIP DNA material was used in qPCR analyses (the primer sequences are listed in the supplemental data). The no antibody signals from the ChIP assays for each of the primers sets were subtracted from individual antibody signals. Each experiment was run at least twice to ensure reproducibility.

Cell Proliferation Measurements

Cell proliferation was assayed using the WST-1 reagent (Millipore). Briefly, cells were seeded in quadruplicate at a density of 5000 cells/well in 96-well plates and grown overnight. Media were changed to assay media with or without the agents, as indicated in the figure legends. Cell proliferation was assayed after 96 h and absorbance was recorded with a microtiter plate spectrophotometer. Cell density was averaged and plotted using Excel.

Statistical analysis

Comparisons between experimental conditions and controls were made using Excel by two-tailed paired Student's *t* test. The data are presented as mean \pm S.D. Statistical difference was denoted as follows: *, $p < 0.05$; **, $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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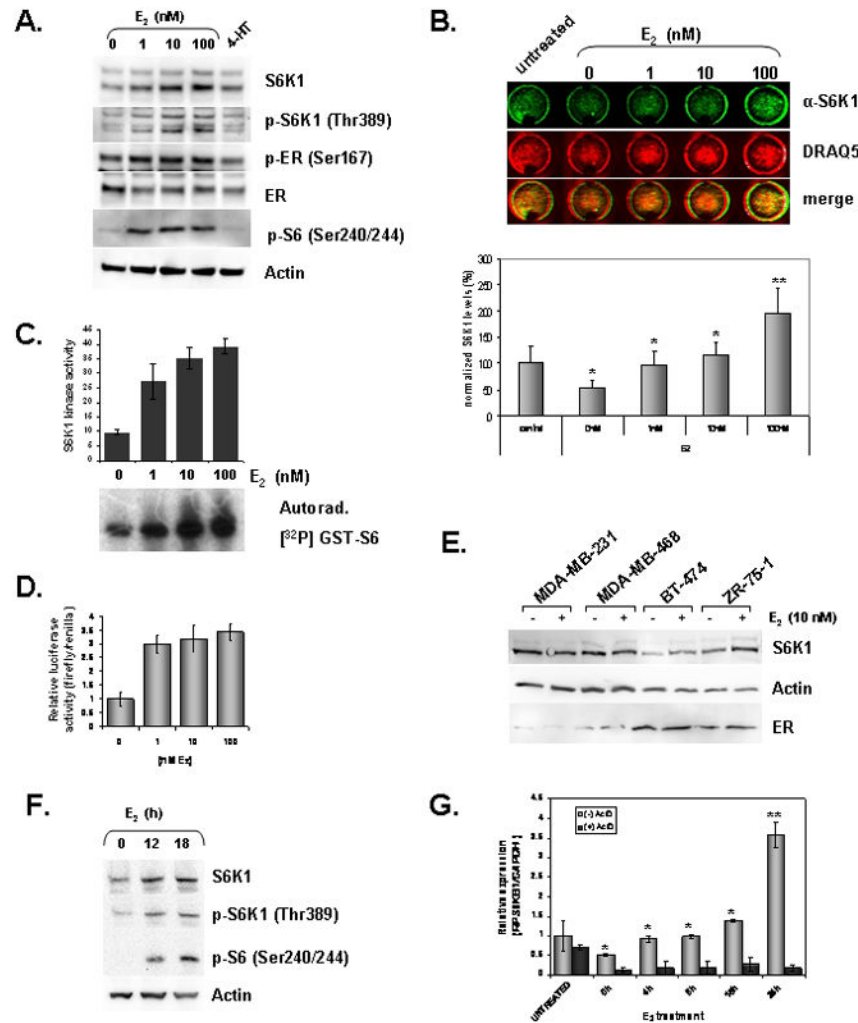


Figure 1. Estrogen upregulates S6K1 expression

(A) MCF7 cells were starved and treated with the indicated amounts of estrogen or 100nM 4-hydroxytamoxifen (4-HT). Cell lysates were immunoblotted with the indicated antibodies. (B) MCF7 cells were grown in full-serum media (untreated) or starved and treated with the indicated amounts of estrogen. In-cell western was performed by staining the cells against S6K1 (green), while DRAQ5 (red) was used as a cell normalization control. Normalized levels of S6K1 (yellow) in the merged signal image from three independent experiments are quantified in the graph, where the untreated condition is set as the 100% control. Statistical differences are shown between control and estrogen-deprived cells, as well as between estrogen-treated and estrogen-deprived cells. (C) S6K1 was immunoprecipitated from MCF7 cells treated as in (B). An *in vitro* kinase assay towards recombinant GST-rpS6 was performed as described in *Materials and Methods*, and a representative gel is shown. Levels of [γ -³²P]ATP incorporation from three independent experiments were determined by autorad, quantified using the QuantityOne software and plotted using Excel. (D) MCF7 cells were transfected with plasmids encoding ERE-firefly luciferase construct, and control *Renilla* luciferase. 24 h after transfection, cells were serum-starved, and treated with the

indicated amounts of estrogen for an additional 24 h. Cells were lysed, and firefly luciferase expression was measured and normalized to control *Renilla* luciferase. The data are presented as mean \pm S.D. of three independent experiments performed in triplicate. (E) Cells were grown in serum-free media for 24h, and 10nM estrogen or the ethanol vehicle was added for an additional 24h. Cell extracts were prepared, and lysates were immunoblotted with the indicated antibodies. (F) Immunoblot analysis of S6K1 levels in the mammary glands of mice subjected to estrogen treatments. (G) MCF7 cells were grown in full-serum media (untreated) or starved and stimulated with 10 nM estrogen for the indicated periods of time with or without pre-treatment with Actinomycin D. Total mRNA was isolated, and RT-qPCR analysis of S6K1 gene expression normalized to GAPDH was performed in triplicate for each sample. Statistical differences are shown between untreated and estrogen-deprived cells, as well as between estrogen-treated and estrogen-deprived cells.

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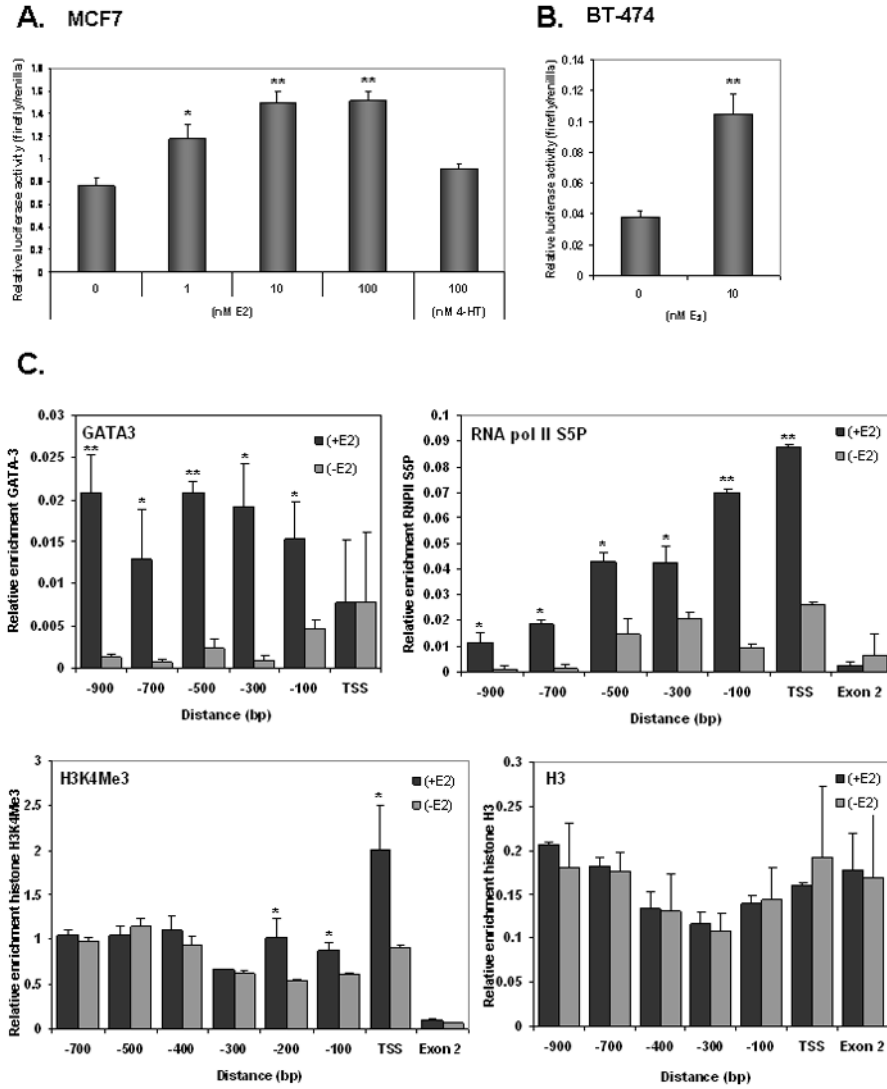


Figure 2. Estrogen stimulates S6K1 promoter activity

(A) MCF7 cells were transfected with plasmids encoding *RPS6KB1* promoter-regulated firefly luciferase, and control *Renilla* luciferase. 24 h after transfection, cells were serum-starved, and treated with the indicated amounts of estrogen for an additional 24 h. Cells were lysed, and firefly luciferase expression was measured and normalized to control *Renilla* luciferase. The data are presented as mean \pm S.D. of each experiment performed in quadruplicate; statistical differences are calculated between estrogen-treated and estrogen deprived cells. (B) Same as in (A) with BT-474 cells. (C) ChIP analysis of promoter occupancy by GATA-3, RNA pol II phosphorylated serine 5, trimethylated histone H3 at lysine 4 and total histone H3 in MCF7 cells with or without 10 nM estrogen treatment.

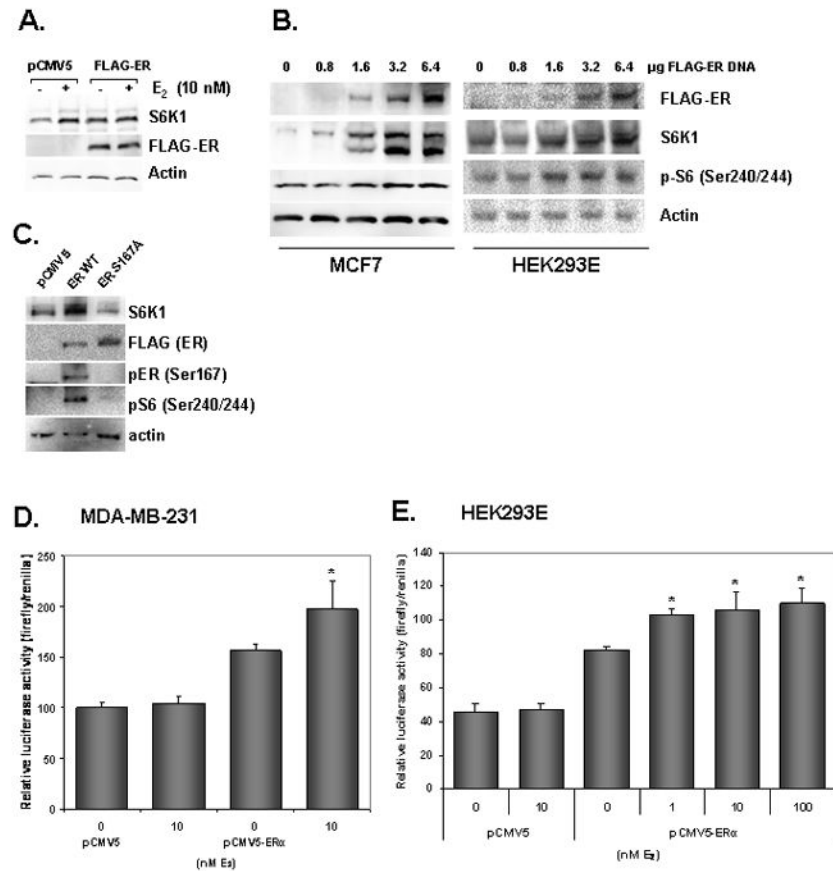


Figure 3. Overexpression of ER α stimulates *RPS6KB1* promoter activity

(A) MCF7 cells were transfected with plasmids encoding ER α or empty control. 24 h after transfection, cells were incubated with serum-free media for 24 h, and treated with 10nM estrogen. Cells were lysed, and protein expression was measured by immunoblot. (B) MCF7 or HEK293E cells were transfected with a vector control or the indicated amounts of ER α -encoding plasmid. 24 h after transfection, cells were incubated in serum-free media for an additional 24 h, lysed and assayed for the indicated protein expression using immunoblot. (C) MDA-MB-231 cells were transfected with a vector control or the indicated allele of ER α -encoding plasmid. 24 h after transfection, cells were incubated in serum-free media for an additional 24 h, lysed and assayed for the indicated protein expression using immunoblot. (D) MDA-MB-231 cells were transfected with plasmids encoding ER α , *RPS6KB1* promoter firefly luciferase construct, and control *Renilla* luciferase. 24 h after transfection, cells were serum-starved, and treated with the indicated amounts of estrogen for an additional 24 h. Cells were lysed, and firefly luciferase expression was measured and normalized to control *Renilla* luciferase. The data are presented as mean \pm S.D. of each experiment performed in quadruplicate; statistical differences are calculated between estrogen-treated and estrogen-deprived cells. (E) Same as in (C) with HEK293E cells.

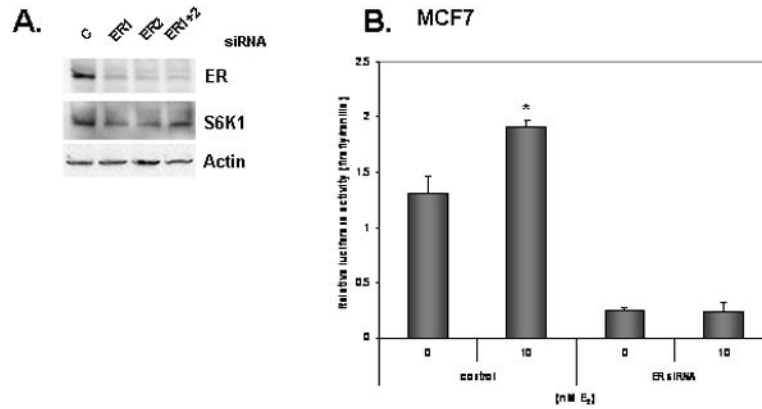


Figure 4. Down-regulation of ER α reduces S6K1 expression and abrogates estrogen-dependent *RPS6KB1* promoter expression

(A) MCF7 cells were transfected with two different siRNAs targeting ER α , alone or in combination, or a scrambled control. 48 h post-transfection, cells were lysed, and protein levels in cell extracts were determined by immunoblot. (B) MCF7 cells were co-transfected with siRNA targeting ER α , the firefly luciferase *RPS6KB1* reporter and *Renilla* luciferase control vector. 24 h after transfection, cells were serum-starved, and treated with the indicated amounts of estrogen for an additional 24 h. Cells were lysed, and firefly luciferase expression was measured and normalized to control *Renilla* luciferase. The data are presented as mean \pm S.D. of each experiment performed in quadruplicate; statistical differences are calculated between estrogen-treated and estrogen-deprived cells.

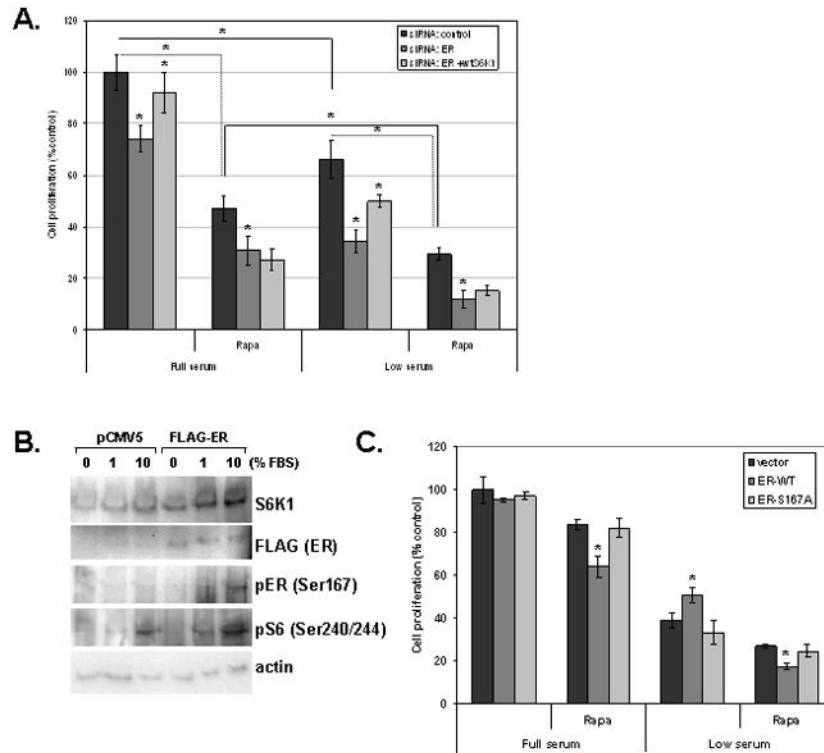


Figure 5. ER α and S6K1 modulation regulates cell proliferation in full and low-serum media (A) MCF7 cells were grown in full or low (1% FBS) serum media with or without rapamycin for 96 hr after siRNA transfection targeting ESR1, or siRNA knockdown together with HA-tagged S6K1 overexpression. Cell proliferation was determined using WST-1 assay. Statistical differences of three independent experiments performed in quadruplicate are calculated between the rapamycin treatment conditions and the control, as well as the siRNA knockdown versus the control or S6K1 overexpression conditions. (B) FLAG-tagged ER was overexpressed in MDA-MB-231 cells, which were subsequently grown in media with the indicated amount of serum, and assayed for protein expression by immunoblot. (C) MDA-MB-231 cells were grown in full or low (1% FBS) serum media with or without rapamycin for 96 hr after transfection of FLAG-tagged alleles of ER. Cell proliferation was determined using WST-1 assay. Statistical differences of three independent experiments performed in quadruplicate are calculated between the ER-overexpression conditions and the control.

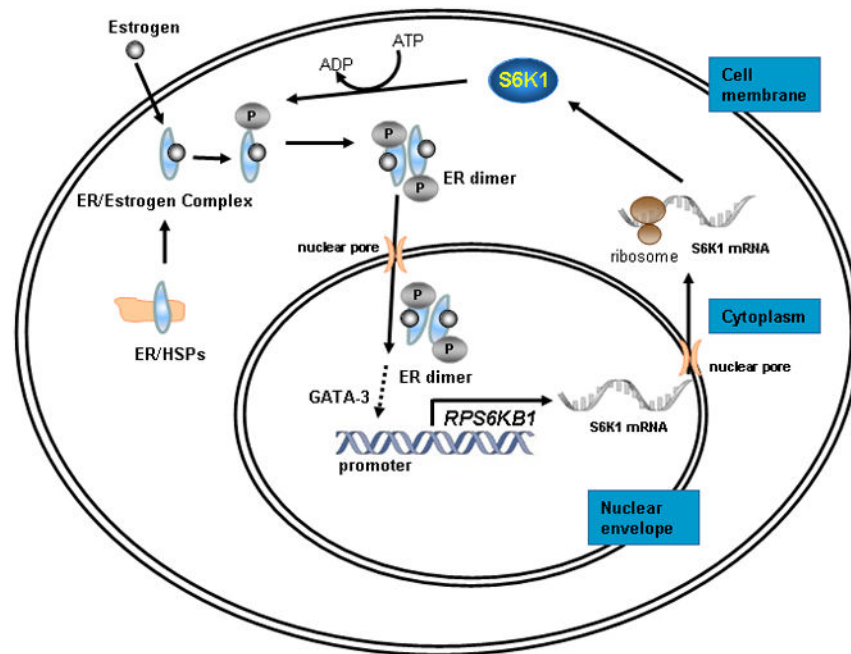


Figure 6. S6K1 and ER α participate in a positive co-regulatory relationship

ER α is activated by binding to its ligand estrogen, leading to dissociation of Heat shock proteins (HSPs), receptor dimerization, activation, and translocation to the nucleus. Phosphorylation by S6K1 promotes ligand-independent activation of ER α . In the nucleus, ER α activates the promoter region of *RPS6KB1* and upregulates transcription of the S6K1 mRNA. S6K1 protein produced as a result of ER α transactivation leads to increased activation of ER α , establishing a positive feed-forward loop.