

Phosphorylation by p38 Mitogen-Activated Protein Kinase Promotes Estrogen Receptor α Turnover and Functional Activity via the SCF^{Skp2} Proteasomal Complex

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The nuclear hormone receptor estrogen receptor α (ER α) mediates the actions of estrogens in target cells and is a master regulator of the gene expression and proliferative programs of breast cancer cells. The presence of ER α in breast cancer cells is crucial for the effectiveness of endocrine therapies, and its loss is a hallmark of endocrine-insensitive breast tumors. However, the molecular mechanisms underlying the regulation of the cellular levels of ER α are not fully understood. Our findings reveal a unique cellular pathway involving the p38 mitogen-activated protein kinase (p38MAPK)-mediated phosphorylation of ER α at Ser-294 that specifies its turnover by the SCF^{Skp2} proteasome complex. Consistently, we observed an inverse relationship between ER α and Skp2 or active p38MAPK in breast cancer cell lines and human tumors. ER α regulation by Skp2 was cell cycle stage dependent and critical for promoting the mitogenic effects of estradiol via ER α . Interestingly, by the knockdown of Skp2 or the inhibition of p38MAPK, we restored functional ER α protein levels and the control of gene expression and proliferation by estrogen and antiestrogen in ER α -negative breast cancer cells. Our findings highlight a novel pathway with therapeutic potential for restoring ER α and the responsiveness to endocrine therapy in some endocrine-insensitive ER α -negative breast cancers.

The nuclear hormone receptor estrogen receptor α (ER α) is a master regulator of gene expression and the proliferative program of breast cancer cells (18, 29, 36, 38, 50, 54) and, hence, is the main target of endocrine therapies. Approximately 70% of human breast tumors express ER α and depend on estrogens for growth, rendering these tumors amenable to treatment with drugs such as selective estrogen receptor modulators/antiestrogens (such as tamoxifen) and aromatase inhibitors, which are quite effective and have relatively few side effects. These ER α -targeted therapies (7, 27, 28, 40, 41) have resulted in a steady decline in the rate of mortality due to breast cancer but show effectiveness only against ER-positive breast tumors, while ER-negative tumors fail to respond. The regulation of the cellular level of ER α is therefore key to the effectiveness of endocrine therapies in breast cancer, and an understanding of its underlying mechanism is critical for the identification of novel drug targets for the design of combinatorial therapies.

ER α is unusual among nuclear hormone receptors in being a rapidly turning-over protein with a half-life of ca. 4 h in breast cancer cells and in normal target tissues such as the uterus (2, 16, 39), indicating dynamic regulation by modulating factors. The degradation of ER α , and several other nuclear receptors, has been shown to be under the control of the ubiquitin (Ub) proteasome system (2, 31, 32, 48, 51), yet many important aspects of this regulation remain unclear. In view of the importance of ER α in many target tissues and in breast cancer biology, prognosis, and responses to endocrine treatments, we have investigated the underlying mechanism for the cellular turnover of ER α and identify Skp2 (S-phase kinase-associated protein 2), an F-box protein (FBP), and a substrate recognition component of the SCF ubiquitin ligase complex (10) overexpressed in many cancers, including breast cancer (21, 23, 42–44, 46, 47), as a novel E3-ubiquitin ligase that regulates ubiquitination and the turnover of ER α upon specification by the p38 mitogen-activated protein kinase

(p38MAPK)-mediated phosphorylation of the receptor while positively regulating the functional activity of this receptor.

We were intrigued to examine the interrelationships between ER α and Skp2, because in our studies of the estrogen receptor and its coregulators, we observed that ER α and the E3 ubiquitin ligase Skp2 appeared to be inversely correlated. The SCF^{Skp2} complex is under tight bimodal regulation by the concerted actions of various kinases that modulate its activity by phosphorylating either its components (19, 22, 33) or its target proteins (26). Since there is compelling evidence for the requirement of substrate phosphorylation as a signal for SCF^{Skp2}-mediated protein turnover (57, 58), we have investigated the role of such posttranslational modifications in Skp2-mediated ER α turnover and identify the stress-activated kinase p38MAPK as a critical regulator.

Our work highlights the molecular mechanisms governing ER α turnover and the control of receptor proliferative and gene regulatory activities by the coordinated actions of Skp2 and p38MAPK. The findings further reveal a dynamic inverse relationship between ER α and Skp2 and/or active p38MAPK in human breast tumors and breast cancer cell lines and suggest potential new therapeutic strategies for restoring functional ER α protein in some endocrine-insensitive ER α -negative breast cancer cells.

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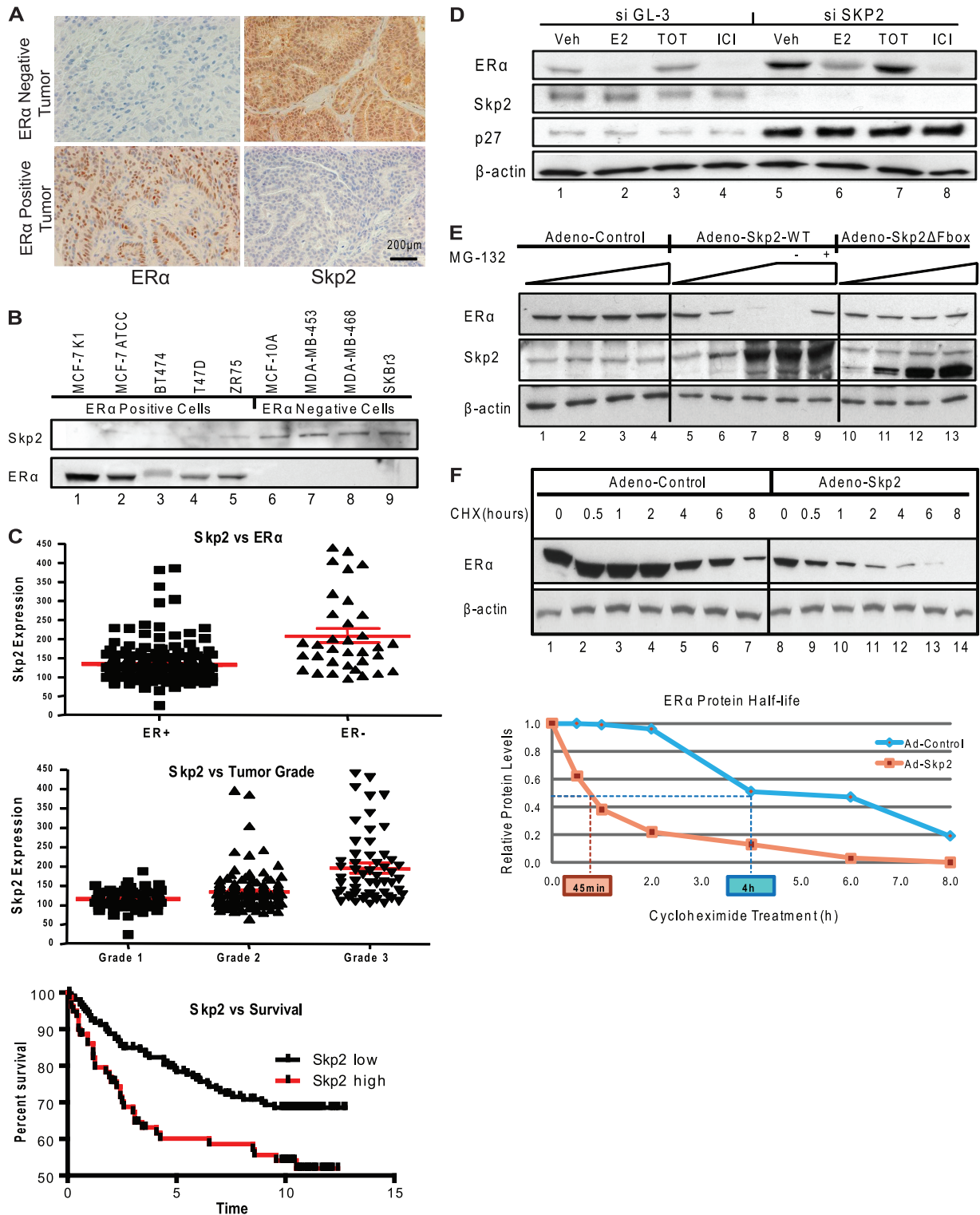
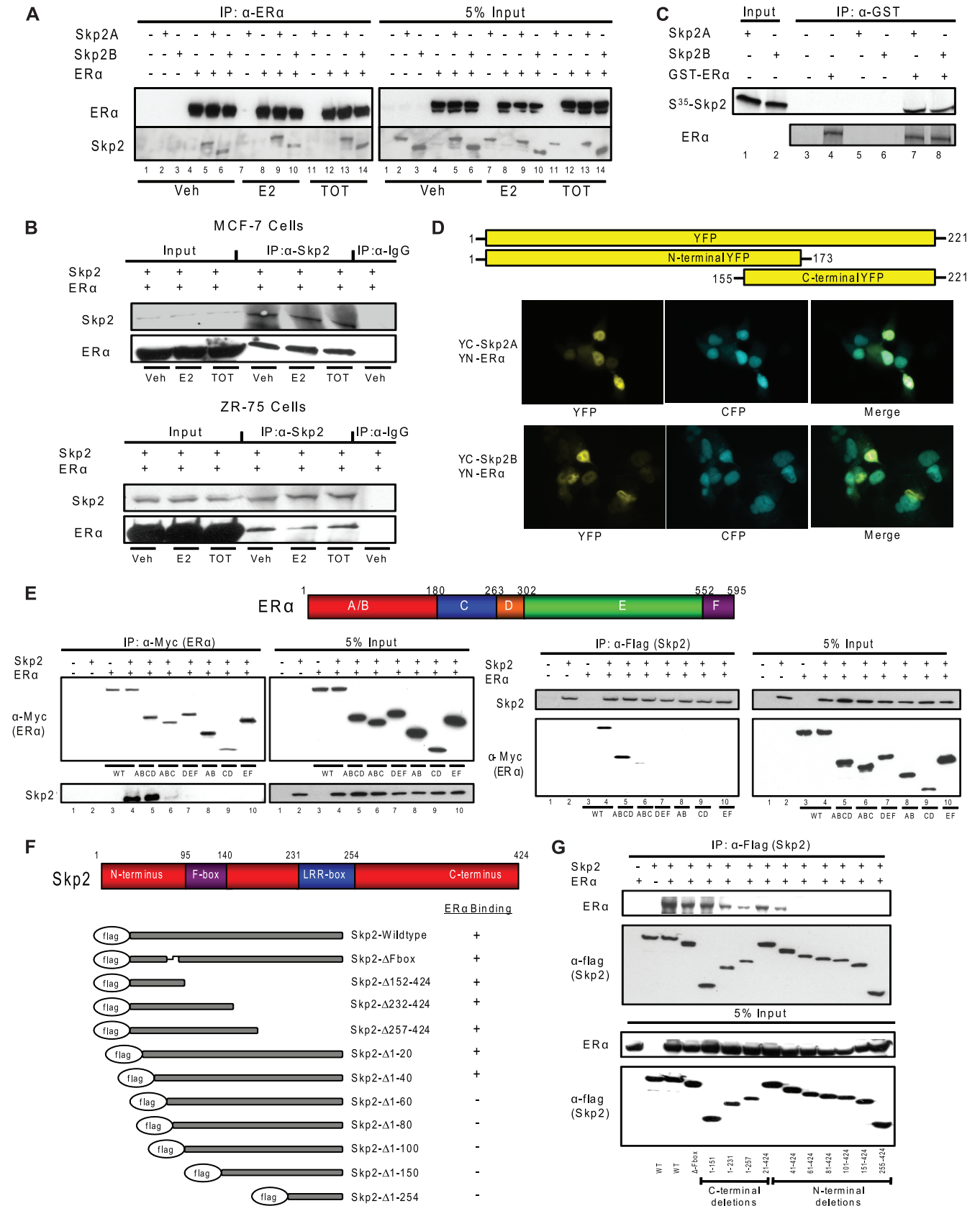


FIG 1 Skp2 regulates ERα protein levels and stability in breast cancer cells. (A) Immunohistochemical staining for Skp2 and ERα proteins in ER-negative and -positive human breast tumors. (B) Western analysis of breast cancer cell lines. (C) Microarray data analysis of 209 ERα-positive and 77 ERα-negative human breast tumors (56) showing the relationship of Skp2 with ERα, tumor grade, and survival. (D) Western analysis of MCF-7 cells transfected with the control or Skp2 siRNA, followed by ligand treatment. Veh, vehicle. (E) Western analysis of MCF-7 cells infected with the control, Skp2-WT, or Skp2-ΔFbox adenovirus with or without MG-132. (F) Western analysis of MCF-7 cells infected with the control or Skp2 adenovirus and treated with cycloheximide (50 µg/ml) for the indicated times.



MATERIALS AND METHODS

Cell cultures, antibodies, and other reagents. Anti-Skp2 (N-19, H-435, and A-2), anti-ER α (HC-20 and F-10), anti-Ub (P4D1), antihemagglutinin (anti-HA) tag (F-7 and Y-11), and anti-glutathione S-transferase (GST) (Z-5) antibodies and horseradish peroxidase (HRP)-conjugated donkey anti-goat, donkey anti-mouse, and donkey anti-rabbit IgG secondary antibodies were obtained from Santa Cruz Biotechnology. Anti-Myc tag (catalog numbers 2272 and 2276), anti-p38MAPK (catalog numbers 9212 and 9228), anti-phospho-Thr-180/Tyr-182 p38MAPK (catalog numbers 9211 and 9216), anti-ATF2, and anti-phospho-ATF2 antibodies were obtained from Cell Signaling; anti-Flag rabbit and mouse antibodies were obtained from Sigma; and antiphosphoserine antibody (ab17465) was obtained from Abcam. Immunoprecipitations (IPs) were performed with antibodies obtained from Santa Cruz (ER α [HC-20], Skp2 [N-19], and RNA polymerase II [N-20]), Abcam (RNA polymerase II phospho-Ser-5 [ab5131]), and Bethyl Laboratories (p38MAPK [A310-212A]). The p38MAPK inhibitor SB203580 was obtained from Calbiochem.

All cell lines were obtained from the American Type Culture Collection (ATCC) and were maintained in culture as suggested by the ATCC, except for MCF-7 cells, which were grown in minimum essential medium (MEM) with phenol red supplemented with 5% heat-inactivated calf serum and were transferred into MEM without phenol red supplemented with 5% charcoal dextran-stripped calf serum prior to treatment, as described previously (18). Plasmid transfections were performed by use of LipofectAMINE 2000 (Invitrogen).

Plasmids and adenoviral constructs. Skp2 cDNA (Open Biosystems) was cloned into the pcDNA3-Flag expression vector. Various Skp2 deletion mutants were generated from this full-length construct by PCR-based subcloning. ER α was cloned into the pcDNA3-Flag expression vector and the pcDNA3.1-Myc/His expression vector from Invitrogen. Various ER α deletion mutants were generated from the full-length Myc-tagged construct by PCR-based subcloning. A site-directed mutagenesis kit (Stratagene) was used to introduce various Ser-to-Ala or -Glu point mutations in pcDNA3-Flag-ER α or Skp2 (full length). pCMV-ER α /ER β chimeras were generated previously in our laboratory (37). pCMV-HA-Ubiquitin was generated by subcloning from a GST-ubiquitin expression vector (Addgene plasmid 10861), originally generated by Peter Howley. pMEV-2HA-p38MAPK-WT (wild type [WT]) and the DN (dominant negative) mutant were obtained from Biomyx Technology. Myc-tagged cullins and HA-tagged Rbx1 and Rbx2 were generated by subcloning from cDNA vectors from Addgene. Adenoviral vectors encoding Skp2-WT or the Skp2- Δ Fbox mutant were constructed by cloning relevant sequences into the pAdTrack vector from Stratagene. Details on the generation of these expression vectors are available upon request.

Silencing by small interfering RNA. Cells at a confluence of 40 to 60% were transfected with the small interfering RNA (siRNA) duplexes siSkp2 (Qiagen); siER α ; sip38MAPK; siCull1, -2, -3, -4A, -4B, -5, or -7; siRbx1; siRbx2 (Dharmacon); or nontargeting control siGL-3 (Dharmacon or Qiagen), followed by ligand treatment, as indicated. siSkp2 transfections were performed by use of lipitoid reagent, kindly provided by Ronald Zuckermann at the Molecular Foundry, Lawrence Berkeley National Laboratory (25, 55). All other siRNA transfections were performed by use of Dharmafect transfection reagent (Dharmacon).

Expression analysis by quantitative real-time PCR and Western immunoblotting. RNA was isolated from cells by using TRIzol reagent (Invitrogen) and the mRNA for genes of interest was quantitated by SYBR green-based reverse transcriptase PCR (RT-PCR), using the ABI7600HT sequence detection system (Applied Biosystems). All mRNA quantities

were normalized to 36B4. Primer sequences are available upon request. In parallel experiments, total cellular proteins were harvested by using radioimmunoprecipitation assay (RIPA) buffer, followed by Western immunoblotting for proteins of interest.

In vitro kinase assays. For *in vitro* kinase assays, 1 μ g of recombinant active phospho-GST-p38MAPK or inactive GST-p38MAPK protein (Invitrogen) was incubated with 1 μ l of the *in vitro*-translated ER α -WT or ER α -S294A protein as the substrate in the presence of 1 \times kinase buffer (25 mM Tris-HCl [pH 7.5], 5 mM beta-glycerophosphate, 2 mM dithiothreitol [DTT], 0.1 mM Na₃VO₄, 10 mM MgCl₂ [Cell Signaling]) supplemented with 200 μ M ATP in a total reaction mixture volume of 50 μ l. The reaction mixtures were incubated at 30°C for 30 min for enzymatic catalysis to occur; thereafter, reactions were terminated by the addition of 3 \times SDS sample buffer to the mixtures, and the mixtures were subjected to Western blotting.

Immunohistochemical analysis of primary human breast tumor tissues. All protocols involving the use of human breast tumor tissues were approved by the University of Illinois and Carle Foundation Institutional Review Boards. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections were cut at a 0.2- μ m thickness, dried at 37°C overnight, and stained according to standard methods for microwave antigen retrieval. Sections were incubated for 12 h at 4°C with 1:50-diluted anti-ER α , anti-Skp2, or anti-phospho-p38MAPK antibody or a negative control (0.1% bovine serum albumin [BSA] in 1 \times phosphate-buffered saline [PBS]). After washing in PBS, sections were incubated with a biotinylated secondary antibody for 1 h at room temperature and then incubated with an avidin-biotin complex (Vector Laboratories) for 30 min. Reactivity was visualized by using diaminobenzidine (DAB) chromogen, and sections were counterstained with Mayer's hematoxylin.

Bimolecular fluorescence complementation assays. Bimolecular fluorescence complementation (BiFC) was performed as described previously (24). Cos-1 cells were transfected with the YN and YC fusion constructs alone or in combination and were then incubated for 24 h at 37°C in 5% CO₂ and humidified air. Cell nuclei were visualized with CFP-NLS (cyan fluorescent protein [CFP] fused to a nuclear localization signal), which was cotransfected along with the YN and YC fusion constructs. Yellow fluorescent protein (YFP) fluorescence was measured by excitation at 513 nm and emission at 527 nm. Fluorescence emissions were observed in live cells by using an inverted phase-contrast microscope.

GST fusion protein purification, in vitro translation, and GST pull-down assays. The purification of the GST-fused ER α protein from cultures of bacteria grown overnight and GST pulldown assays thereafter were performed as described previously (20). The *in vitro* translation of Skp2A and Skp2B and the incorporation of ³⁵S-labeled methionine (Perkin-Elmer) were performed by use of the TNT T7-coupled reticulocyte lysate system kit (Promega). Labeled Skp2A or Skp2B and GST-ER α (or GST alone) were incubated with glutathione-Sepharose beads (Amersham) in binding buffer (20 mM Tris [pH 7.5], 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM NaVO₄, 1 \times protease inhibitor cocktail [Roche], and 1 mM phenylmethylsulfonyl fluoride [PMSF]) at 4°C for 4 h with gentle rotation. The beads were washed three times with the same buffer. Bound proteins were eluted with Laemmli buffer containing SDS supplemented with β -mercaptoethanol and subjected to 10% SDS-PAGE. The gels were then dried and visualized by autoradiography.

Coimmunoprecipitation assays. Cos-1 cells seeded into 6-well plates at a confluence of 80 to 90% were transfected with plasmids of interest, followed by treatment with a vehicle (0.1% ethyl alcohol [EtOH]) or ligands, as indicated. At 24 h posttransfection, cells were harvested, washed

of GST-tagged ER α in the presence or absence of *in vitro*-translated ³⁵S-tagged Skp2. Coimmunoprecipitated Skp2 was analyzed by autoradiography. (D) Bimolecular fluorescence complementation (BiFC) analysis. Cos-1 cells were transfected with YN-ER α along with YC-Skp2A or YC-Skp2B and CFP fused with the nuclear localization signal (to visualize cell nuclei), and YFP fluorescence was monitored by confocal microscopy. (E) CoIP of the ER α deletion mutant with Flag-Skp2 in Cos-1 cells. (F) Schematic of Skp2 deletion mutants showing their relative abilities to interact with ER α . (G) CoIP of the Skp2 deletion mutant with ER α in Cos-1 cells. All data are representative of 3 experiments.

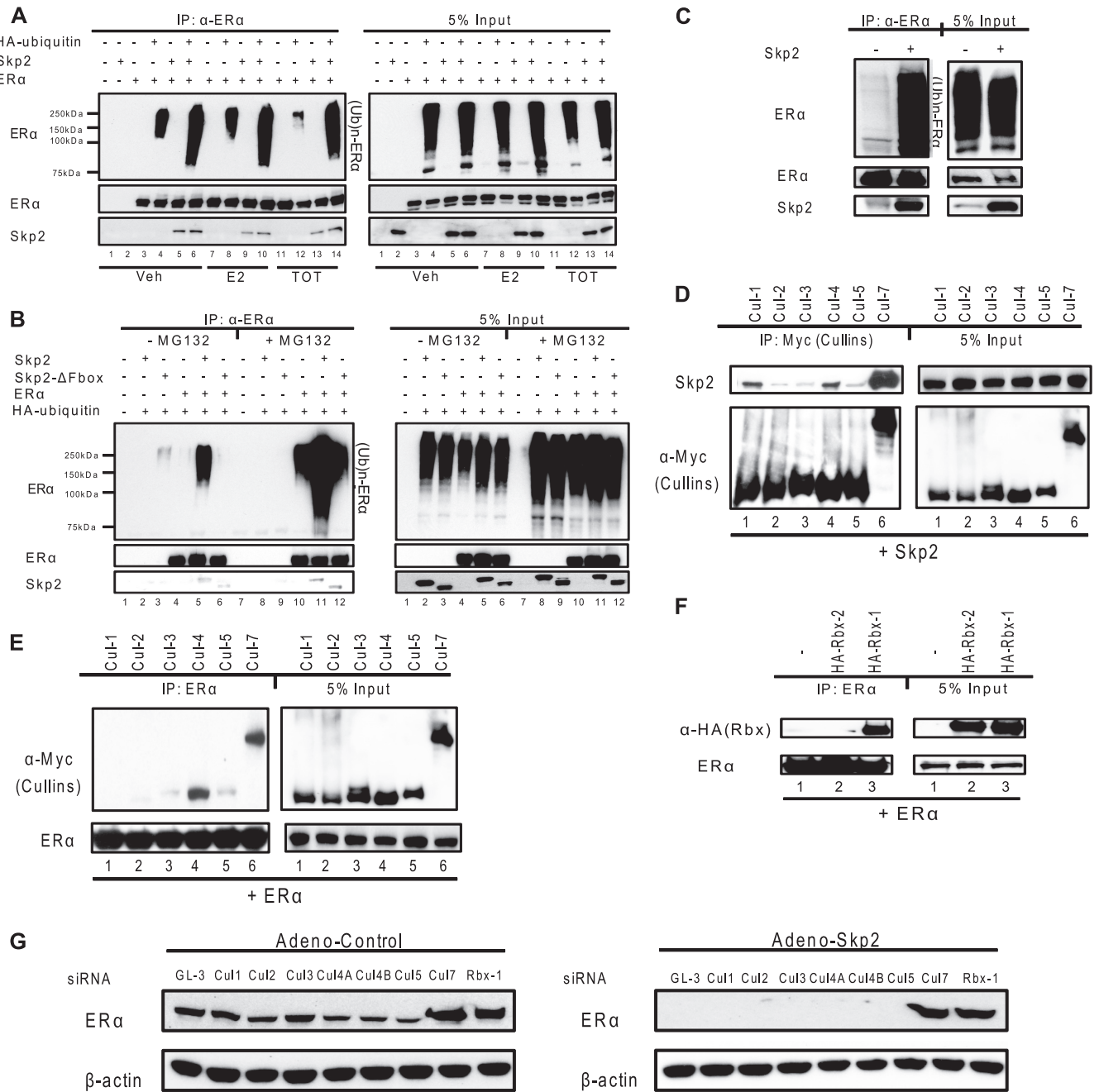


FIG 3 Skp2 mediates ligand-independent proteasomal degradation of ER α with Cul7 and Rbx1 as part of the SCF^{Skp2} complex. (A) Ubiquitination assay of ER α in Cos-1 cells in the presence of vehicle or ligand (0.1% ethanol vehicle or 10 nM *trans*-hydroxy-tamoxifen [TOT]) and/or Skp2. (B) Ubiquitination assay of ER α in Cos-1 cells with pCMV-Skp2 or Δ Fbox-Skp2. (C) Ubiquitination assay for endogenous ER α in MCF-7 cells infected with the control or Skp2 adenovirus. (D) CoIP of Flag-Skp2 and various cullin isoforms in Cos-1 cells. (E) CoIP of Flag-ER α and various cullin isoforms in Cos-1 cells. (F) CoIP of Flag-ER α and HA-tagged pCMV-Rbx1 or -Rbx2 in Cos-1 cells. (G) Western analysis of MCF-7 cells transfected with siGL-3 or siRNA against various cullin isoforms or Rbx1, followed by ligand treatment. (H) Western analysis of MCF-7 cells transfected with siGL-3 or siRNA against various cullin isoforms or Rbx1, followed by infection with the control or Skp2 adenovirus. All data are representative of 3 experiments.

with ice-cold PBS, and incubated with 0.5 ml coimmunoprecipitation (CoIP) buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 0.1 mM NaVO₃, 1 mM DTT, 1 mM PMSF, and 1 \times protease inhibitor cocktail from Roche). Immunoprecipitation was carried out by using 2 to 5 μ g specific antibody by incubation at 4 $^{\circ}$ C overnight with gentle rotation. The precipitated proteins were collected by incubation with protein G-Sepharose (GE Healthcare) for 2 h at 4 $^{\circ}$ C. After low-speed

centrifugation to remove the supernatant, the Sepharose beads were washed with CoIP buffer, boiled in SDS sample buffer, and subjected to SDS-PAGE and Western blotting. In the case of MCF-7 cells, endogenous proteins were immunoprecipitated from lysates obtained from 15-cm² plates as described above but without the exogenous expression of factors.

Ubiquitination assays. Cos-1 cells transfected with ER α and other plasmids were pretreated with 10 μ M MG-132 (Calbiochem) for 5 h to

block proteasome activity, before they were harvested at 24 h posttransfection. Cells were lysed in buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Nonidet P-40, 0.1 mM NaVO₃, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor cocktail from Roche. Immunoprecipitation was carried out by using ER α antibody by incubation at 4°C overnight with gentle rotation. The precipitated proteins were collected by incubation with protein G-Sepharose for 2 h at 4°C with gentle rotation. After low-speed centrifugation to remove the supernatant, the Sepharose beads were washed with CoIP buffer, boiled in SDS sample buffer, and subjected to SDS-PAGE and Western blotting with an anti-HA or anti-ER α antibody (Santa Cruz). For MCF-7 cells, endogenous ER α was immunoprecipitated from cell lysates after 48 h of expression of either β -galactosidase (β -gal) (control) or the Skp2 or p38MAPK adenovirus and 5 h of treatment with 10 μ M MG-132, and ubiquitinated ER α was detected with an antiubiquitin or anti-ER α antibody.

ChIP and sequential ChIP assays. Chromatin immunoprecipitation (ChIP) and ChIP-sequential ChIP (reChIP) assays were performed as described previously (54). The DNA isolated was subjected to quantitative real-time PCR with 36B4 used as an internal control, and a recruitment index was calculated (ratio of the specific antibody signal over the IgG signal). In ChIP-reChIP experiments, after the first pulldown, immunoprecipitated material was recovered with 10 mM dithiothreitol in IP buffer at 37°C for 30 min, diluted, and subjected to a second round of immunoprecipitation.

Synchronization of cells and fluorescence-activated cell sorter (FACS) analysis. MCF-7 cells were synchronized at various stages of the cell cycle (G₁ phase, G₁/S-phase boundary, S phase, and G₂/M-phase boundary), and the DNA content was analyzed by flow cytometry using a BD FACS Canto flow cytometer (BD Biosciences). To synchronize cells in the G₁ phase, the cells were serum starved for 48 h, fed with 10% serum-containing medium thereafter, and treated for 24 h with 100 μ M indole 3-carbinol, which induces G₁-phase cell cycle arrest by inhibiting CDK6 expression. To synchronize cells in the S phase, the cells were serum starved for 48 h to first synchronize them in the G₀/G₁ phase and then synchronize them at the G₁/S boundary by incubation with 1.5 μ g of 4-hydroxyurea/ml medium for 24 h in 10% serum-containing medium. The cells were thereafter washed with Hanks' buffered saline (HBS) and released into the S phase with serum-free medium. Synchronization in the G₂/M phase was carried out by synchronizing the cells at the G₁/S boundary with 1.5 μ g of 4-hydroxyurea/ml medium for 48 h in 10% serum-containing medium, washing the cells with HBS, and synchronizing the cells in the G₂/M phase by treatment with 1 μ g/ml nocodazole in 10% serum-containing medium for 24 h.

FACS analysis. MCF-7 cells were washed once in cold PBS, scraped from plates, and pelleted by centrifugation at 340 × g at 4°C. The cells were resuspended in 2 ml of 0.9% sodium chloride and fixed for 30 min in 5 ml of 90% ethanol. Ethanol was added dropwise with constant vortexing. After 30 min, the cells were centrifuged out of the fixing solution and resuspended in 1 ml propidium iodide (50 μ g/ml) diluted in PBS and supplemented with 100 μ g of RNase A (Roche), as described previously (5). The cells were incubated at 37°C for 15 min, and the cell cycle distribution was analyzed by flow cytometry using a BD FACS Canto flow cytometer (BD Biosciences).

Mass spectrometry analysis of ER α . Semiquantitative tandem mass spectrometry (MS/MS) analysis of posttranslational modifications was performed on the ER α protein in MCF-7 cells in the absence or presence of overexpressed Skp2 or p38MAPK. MCF-7 cells from five 150-mm dishes, 90% confluent, with or without the overexpression of Skp2 or p38MAPK were lysed by using CoIP buffer, sonicated, and then subjected to blocking by use of an excess of salmon sperm DNA and nonspecific IgG for 5 h at 4°C, followed by immunoprecipitation using a specific antibody against ER α or nonspecific IgG (negative control) at 4°C overnight. All ER α -bound protein complexes were then washed three times with CoIP buffer, and the specific ER α -bound protein complexes were extracted by using Laemmli sample buffer. The ER α protein band at 66 kDa in SDS-

PAGE gels was visualized by Coomassie blue staining. Gel slices were excised, destained, lyophilized, and digested with trypsin (Promega, Madison, WI) overnight at 37°C. The tryptic peptides were extracted from gel slices by using 70% acetonitrile containing 5% formic acid and were further purified through PepClean C₁₈ Spin columns (Pierce, Rockford, IL). The peptide solution was lyophilized and reconstituted in 0.1% formic acid prior to nanoflow reversed-phase liquid chromatography (nanoRPLC) MS/MS analysis using either an LTQ Orbitrap XL or an LTQ-FT mass spectrometer (ThermoElectron, San Jose, CA). The samples were injected onto a nanoRPLC column, and the peptides were eluted by use of a gradient of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) under the following conditions: 2% mobile phase B at 500 nl/min for 20 min, linear increases of 2 to 42% mobile phase B at 250 nl/min in 40 min and 42 to 98% mobile phase B at 250 nl/min in 10 min, and 98% mobile phase B at 500 nl/min for 18 min. The mass spectrometer was operated in a data-dependent mode, where the seven most abundant peptide molecular ions in every MS scan were sequentially selected for fragmentation and the acquisition of MS² spectra. The acquisition of neutral-loss MS³ spectra was triggered when a neutral loss of phosphoric acid (H₃PO₄) was detected with a loss of *m/z* 98, 49, or 32.7 Da among the peptide fragment ions in MS² scans. The normalized collision energy was set at 35% for both MS² and MS³ collision-induced dissociations (CIDs). Dynamic exclusion was enabled to minimize the repeated selection of peptides previously selected for CID. The ion source capillary voltage and temperature were set at 45 V and 160°C, respectively. The electrospray voltage was set at 1.7 kV.

Tandem mass spectra were searched against the human database by using SEQUEST software (ThermoFinnigan, San Jose, CA). Fully tryptic cleavage constraints and two missed cleavage sites were applied for the search. The phosphorylation of serine, threonine, and tyrosine (+79.9663 Da); the acetylation of lysine (+42.0106 Da); and the oxidation of methionine (+15.9949 Da) residues were included as dynamic modifications when searching MS² spectra, whereas a loss of water at serine and threonine (−18.0106 Da) residues was added as a dynamic modification when searching MS³ spectra. The SEQUEST filtering criteria for the initial identification of peptides were cross correlation (X_{corr}) scores of 1.9 for [M + H]¹⁺, 2.2 for [M + 2H]²⁺, and 3.1 for [M + 3H]³⁺ ions and a minimum delta correlation (ΔC_n) value of 0.08. The MS² and MS³ spectra of eligible peptides were manually inspected to confirm the identification of the correct peptide sequence and modifications.

Cell proliferation assays. Assays were performed with ER α -positive MCF-7 cells or ER α -negative MDA-MB-453 and -468 cells in the absence or presence of the overexpressed Skp2-WT, Skp2-S64A mutant, or Skp2-S64E mutant protein; siGL-3 or siSkp2; vehicle (0.1% dimethyl sulfoxide [DMSO]); or the p38MAPK inhibitor (1 μ M), as indicated, followed by treatment with the vehicle or ligands indicated and analysis of cell density by using WST-1 reagent (Roche Applied Science; catalog number 11644807001) according to the manufacturer's protocols.

Statistics. All data were analyzed by using an unpaired, 2-tailed Student *t* test or analysis of variance (ANOVA), as appropriate. A *P* value of less than 0.05 was considered significant.

RESULTS

Skp2 regulates ER α protein levels and stability in breast cancer cells: inverse correlation between ER α and Skp2. We observed ER α and Skp2 protein levels to be inversely correlated in multiple breast cancer cell lines and also in primary human breast tumors (Fig. 1A and B, and see Fig. S1A in the supplemental material). In addition, data from an analysis of publically available breast tumor database information were consistent with our findings of higher Skp2 levels in ER-negative tumors (Fig. 1C). The Skp2 level was also higher in higher-grade tumors, and elevated Skp2 expression levels were associated with reduced patient survival (Fig. 1C), implying an increased aggressiveness of tumors with higher Skp2

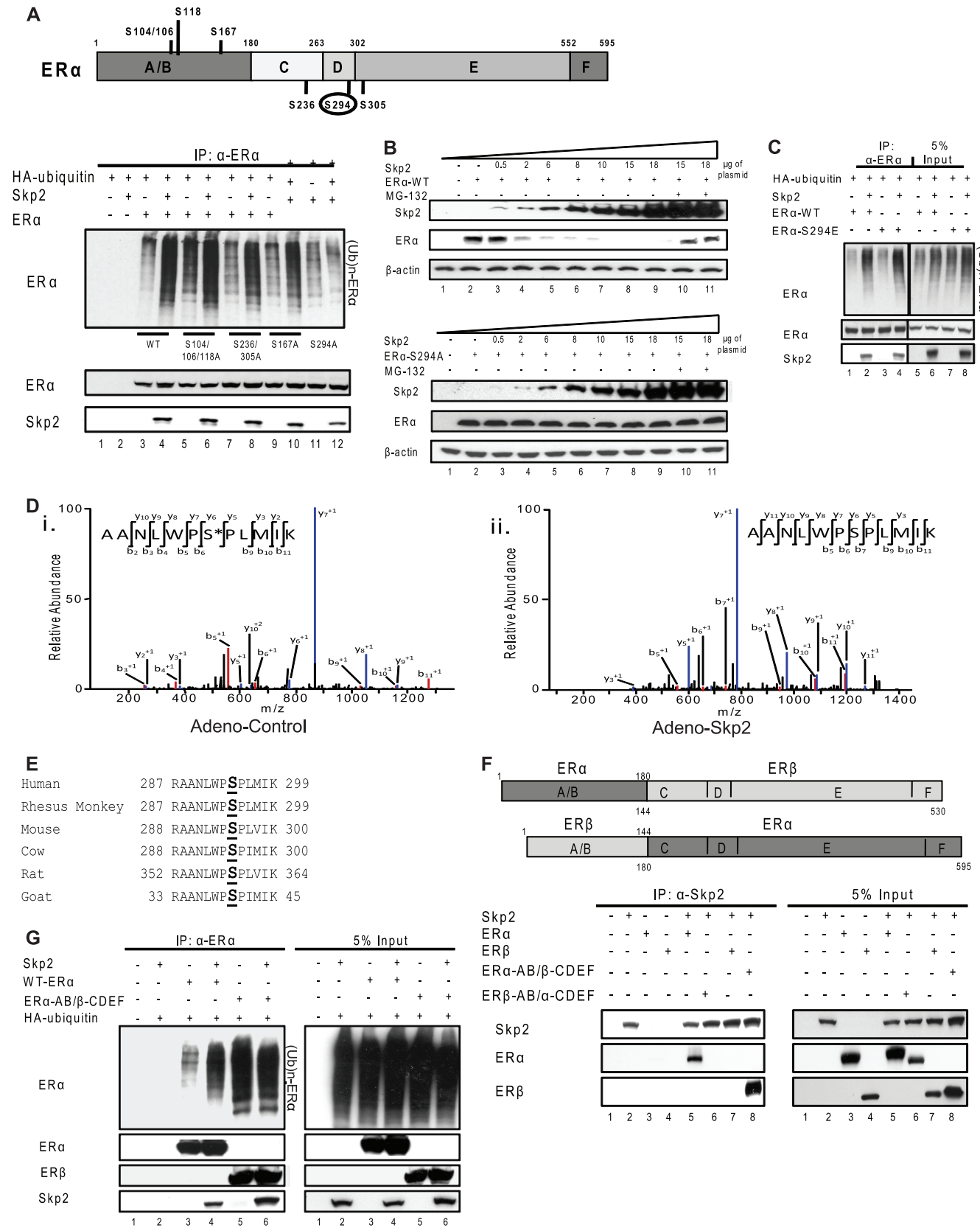


FIG 4 Phosphorylation of ERα at serine 294 is required for its ubiquitination by Skp2. (A) Ubiquitination assay for ERα-WT or various serine-to-alanine point mutants in Cos-1 cells in the absence or presence of added Skp2. (B) Western analysis of Cos-1 cells transfected with increasing doses of pCMV-Skp2 along with constant levels of pCMV-ERα-WT or pCMV-ERα-S294A. Samples in lanes 10 and 11 were also treated with MG-132. (C) Ubiquitination assay for the ERα-WT

levels, also a hallmark of ER-negative tumors. We thus hypothesized that Skp2, by virtue of its E3-ubiquitin ligase activity, might be regulating the level of ER α and contributing to the increased aggressiveness of ER-negative breast cancers. To investigate this, we performed an siRNA-mediated knockdown of Skp2 in ER α -positive MCF-7 (and ZR-75 [data not shown]) breast cancer cells (Fig. 1D). This siSkp2 exposure reduced Skp2 protein levels to ~10% of the levels in control siGL-3 (luciferase)-treated cells and produced a 4-fold increase in the cellular level of the ER α protein. This regulation of ER α by Skp2 appeared to be ligand independent (Fig. 1D, and see Fig. S1B in the supplemental material).

Notably, with the exogenous expression of the Skp2-WT (wild type) or Skp2- Δ Fbox (dominant negative Skp2 lacking the F-box domain) protein, we observed a progressive decrease in ER α protein levels with increasing Skp2-WT expression levels that did not occur with dominant negative Skp2 (Fig. 1E, and see Fig. S1C in the supplemental material) and was blocked by the proteasomal inhibitor MG-132 (Fig. 1E, lane 9). To explore the regulation of the ER α protein half-life by Skp2, we blocked *de novo* protein synthesis using cycloheximide (CHX) and monitored ER α protein levels in the presence or absence of exogenously expressed Skp2. The half-life of ER α , which was about 4 h in control virus-infected cells, was shortened to about 45 min with overexpressed Skp2 (Fig. 1F, and see Fig. S1D in the supplemental material), implying SCF^{Skp2} E3-ligase to be a regulator of ER α protein turnover.

Skp2 and ER α interact in the cell nucleus through their N-terminal regions. To elucidate the mechanism of ER α degradation by Skp2, we examined the interaction of the two proteins. Coimmunoprecipitation assays of ER α with Skp2A and Skp2B, isoforms that differ in their C-terminal protein sequences, showed a ligand-independent interaction of ER α with both isoforms (Fig. 2A). Data from coimmunoprecipitation studies with endogenous proteins in MCF-7 and ZR-75 cells were consistent with this finding (Fig. 2B), and *in vitro* GST pulldown assays confirmed a direct interaction between ER α and Skp2 (Fig. 2C).

We next used bimolecular fluorescence complementation (BiFC) to visualize the intracellular location of the ER α -Skp2 interaction in living cells. This technique exploits the reconstitution of yellow fluorescent protein (YFP) from nonfluorescent N-terminal (YN) and C-terminal (YC) YFP fragments when brought together by two interacting proteins fused to the YFP fragments. Upon expressing ER α -YN and Skp2-YC fusion constructs in Cos-1 cells, we found YFP fluorescence to be localized almost exclusively to the cell nucleus, as confirmed by the colocalization with CFP-NLS (cyan fluorescent protein fused to a nuclear localization signal) (Fig. 2D). No fluorescence was detected in control cells transfected with c-Jun-YN and c-Fos-mut-YC (c-Fos mutant lacking the c-Jun interaction domain), a control for background fluorescence.

To identify the regions of interaction between ER α and Skp2, we generated a series of Myc epitope-tagged ER α deletion constructs and Flag epitope-tagged Skp2 deletion derivatives. The de-

letion of the C-terminal region of ER α did not diminish its interaction with Skp2, whereas truncations in the N-terminal region resulted in a loss of interactions, with a dramatic reduction with the ABC region alone and a complete loss with the AB domain only, indicating that ABCD is the smallest portion of ER α that gives an efficient interaction (Fig. 2E). For Skp2, the deletion of either the C-terminal region with or without the leucine-rich-repeat (LRR)-box or the F-box domain had no effect on its interaction with ER α . However, the deletion of the N-terminal regions between amino acids 40 and 60 was very detrimental (Fig. 2F and G). Thus, we conclude that ER α and Skp2 directly interact, primarily in the cell nucleus, and that the N-terminal regions of both proteins are essential for their mutual interaction.

Skp2 mediates proteasomal degradation of ER α with Cul1 and Rbx1 as part of the SCF^{Skp2} complex. To further understand Skp2-mediated ER α protein turnover, we conducted ubiquitination assays for ER α and observed a significant increase in ubiquitinated ER α levels upon the overexpression of Skp2 (Fig. 3A). Furthermore, the F-box domain in Skp2 was critical for this regulation (Fig. 3B). We also observed an increased ubiquitination of endogenous ER α in MCF-7 cells in the presence of elevated Skp2 levels (Fig. 3C).

We next characterized the SCF^{Skp2} complex targeting ER α . Immunoprecipitation studies revealed an interaction of Skp2 with Cul1, Cul4, and Cul7 (Fig. 3D) and an interaction of ER α with Cul4 and Cul7 (Fig. 3E). The immunoprecipitation of ER α with HA-tagged derivatives of the two estradiol (E2) (ubiquitin-conjugating enzyme) proteins Rbx1 and Rbx2, known to be a part of the SCF^{Skp2} complex, revealed that ER α interacted specifically with Rbx1 but not with Rbx2 (Fig. 3F). The siRNA-mediated knockdown of various cullins (each decreased by >80% with specific siRNA) known to be expressed in MCF-7 cells, and of Rbx1, showed that the depletion of Cul7 and Rbx1 prevented the loss of ER α protein upon the overexpression of Skp2 (Fig. 3G, and see Fig. S2C in the supplemental material).

Phosphorylation of ER α at serine 294 is required for its ubiquitination and degradation by Skp2: mutagenesis and mass spectrometry analysis. Skp2 is a member of the FBL (L for leucine-rich repeat) subclass of F-box proteins that often require phosphorylation signals on their target proteins to elicit target ubiquitination. We investigated this specific phosphodegron code for the ubiquitination of ER α by Skp2 by generating multiple serine point mutants of ER α at sites previously characterized as being important for ER α functions in breast cancer and found serine 294 in ER α to be critical for its ubiquitination by Skp2 (Fig. 4A). To confirm this, we overexpressed Skp2 in Cos-1 cells in the presence of either the ER α -WT or ER α -S294A protein and observed a dose-dependent decrease in the level of the ER α -WT protein with the overexpression of Skp2 (reversed by the proteasome inhibitor MG-132), whereas the ER α -S294A mutant was resistant to Skp2-mediated degradation (Fig. 4B, and see Fig. S3A in the supplemental material). A similar resistance to the degradation of the ER α -S294A mutant versus the ER α -WT protein was

or ER α -S294E protein in Cos-1 cells in the absence or presence of added Skp2. (D) Mass spectrometry analysis of ER α in MCF-7 cells. Shown is an MS² spectrum of an ER α peptide showing modification by phosphorylation at serine 294 (*) in the control (i) and the same but unmodified peptide upon the overexpression of Skp2 (ii). (E) Sequence alignment for ER α across various species showing the highly conserved serine residue 294. (F) CoIP of ER α -WT or the ER α and ER β chimera with pCMV-Skp2 in Cos-1 cells. (G) Ubiquitination assay of ER α -WT or the ER α and ER β chimera with pCMV-Skp2 in Cos-1 cells. All data are representative of 3 experiments.

observed over time in experiments conducted with ER α -negative MDA-MB-468 cells (see Fig. S3B in the supplemental material). Of note, the S294E mutant, which acts as a phosphomimic, showed enhanced ubiquitination upon Skp2 overexpression, as did the wild-type receptor (Fig. 4C). Consistent with this, mass spectrometry analysis of ER α with or without the overexpression of Skp2 in MCF-7 cells revealed a loss of ER α peptides phosphorylated at Ser-294 (Fig. 4Di and ii, shown in red) with elevated Skp2 levels, presumably reflecting an enhanced degradation of the receptor by Skp2 (Fig. 4D, and see Fig. S3D in the supplemental material). It is noteworthy that the sequence alignment of the serine 294-containing peptide in ER α indicates that the entire 13-amino-acid sequence, including serine 294, is highly conserved across species (Fig. 4E).

Skp2 targets ER α but not ER β for degradation. Having identified Skp2 as a novel E3-ubiquitin ligase for ER α , we next investigated if this regulation was selective for ER α by checking its closely related receptor, estrogen receptor β (ER β) (Fig. 4F, lane 7), and a few other nuclear receptors and transcription factors, including thyroid receptor, retinoic acid receptor, glucocorticoid receptor, progesterone receptor (PgR), and cyclic AMP (cAMP) response element binding protein, none of which interacted with Skp2 (data not shown), suggesting a great specificity of the interaction of Skp2 with ER α .

ER α and ER β proteins share considerable sequence homology; however, the most variable domain is the AB domain, which we have shown to be essential for interactions with Skp2 (Fig. 2E). Therefore, we investigated whether the swapping of the AB domains between the two ER proteins would alter their abilities to interact with Skp2. For this, we used two ER α /ER β chimeras, one with the AB domain of ER α and the CDEF domain of ER β (ER α -AB/ER β -CDEF) and the other with the AB domain of ER β and the CDEF domain of ER α (ER β -AB/ER α -CDEF). We indeed found that just by interchanging the AB domains, we were able to alter the abilities of the two receptors to interact with Skp2 (Fig. 4F, lanes 6 and 8). Intrigued by this, we investigated whether the transfer of the interaction function to the ER α -AB/ER β -CDEF chimera would also render it as a target for Skp2-mediated ubiquitination. Whereas Skp2 did increase the ubiquitination of ER α , the ER α -AB/ER β -CDEF chimera showed high basal ubiquitination levels with no apparent increase with Skp2 (Fig. 4G). This finding suggests that the Skp2-mediated degradation of ER α is a two-step process, the first step being the interaction between the two proteins and the next step being the ubiquitination of ER α , which may require a very precise posttranslational modification(s) of the ER α protein on residues that probably are not conserved between ER α and ER β . In fact, an examination of the sequences of ER α and ER β reveals that the important phosphorylation residue, serine 294 in ER α , is not conserved in ER β , consistent with the lack of increased ubiquitination of the ER α -AB/ER β -CDEF chimera by Skp2.

p38MAPK-mediated phosphorylation of ER α at serine 294 is required for ER α ubiquitination by Skp2. To investigate the kinase regulating ER α phosphorylation at Ser-294, we performed a bioinformatic analysis of the ER α protein sequence, which revealed the Ser-294 residue to be a putative cyclin-dependent kinase 2 (CDK2) or MAPK family target site. We therefore examined ER α ubiquitination by Skp2, this time also with or without specific inhibitors of various CDK family and MAPK family members, including phosphatidylinositol 3-kinase (PI3K); protein ki-

nases A, B, and C; JNK/stress-activated protein kinase (SAPK); and p38MAPK. Data are shown only for the p38MAPK inhibitor (Fig. 5A), since only in the presence of this kinase inhibitor were we able to prevent the Skp2-mediated ubiquitination of ER α . Inhibitors of CDK2 (Fig. 5A) and all other kinases examined were without effect. As expected, Skp2 had no effect on the ER α -S294A mutant (Fig. 5A).

The dose-dependent increase in Skp2-mediated ER α ubiquitination with the overexpression of wild-type p38MAPK was blocked by the dominant negative p38MAPK mutant (Fig. 5B), consistent with our postulate that the phosphorylation of ER α by p38MAPK is essential for its ubiquitination by Skp2. Since ER α interacted with both wild-type and mutant kinases, the block of Skp2-mediated ER α ubiquitination in the presence of dominant negative p38MAPK appeared to be solely because of the inability of the mutant kinase to phosphorylate ER α . In MCF-7 breast cancer cells, we likewise observed a marked increase in ubiquitinated ER α levels in the presence of elevated p38MAPK levels (Fig. 5C). Furthermore, with the p38MAPK inhibitor, Skp2 was ineffective in eliciting ER α degradation (Fig. 5D, and see Fig. S3D in the supplemental material), and with *in vitro* kinase assays, we detected the phosphorylation of ER α but not the ER α -S294A mutant in the presence of active p38MAPK (Fig. 5E). Consistent with this, semiquantitative mass spectrometry analysis of MCF-7 cells revealed an increased level of phosphorylation of ER α at serine 294 upon the overexpression of p38MAPK (see Fig. S3B in the supplemental material). Furthermore, the phosphorylation of Skp2 at serine 64 by p38MAPK (S. Bhatt and B. S. Katzenellenbogen, unpublished data) appeared to be critical for the binding of Skp2 to ER α (see Fig. S5A in the supplemental material) and also for the regulation of its function (see Fig. S5B and S5C in the supplemental material). These findings support a critical role for the p38MAPK-dependent phosphorylation of ER α in regulating its turnover by Skp2. Interestingly, as observed for Skp2, levels of active p38MAPK inversely correlated with ER α protein levels in multiple breast cancer cell lines (Fig. 5F) and in primary human breast tumors (Fig. 5G). We also tested the possibility of the regulation of Skp2 and/or p38MAPK by ER α in breast cancer cells (MCF-7 and ZR-75) and also mammary tissue from wild-type versus ERKO (ER α knockout) mice. Interestingly, the loss of the ER α protein was associated with increased expression levels of the Skp2 protein in both breast cancer cells and mammary tissues, whereas no effect on p38MAPK protein levels was observed (see Fig. S5 in the supplemental material).

Skp2 regulates ER α target gene expression, and the two proteins cooperate in driving S-phase entry of MCF-7 cells. Since Skp2 is an S-phase-regulated E3-ligase, we tested the possibility of Skp2 mediating the ubiquitination-dependent regulation of ER α function driving gene expression and the S-phase entry and proliferation of breast cancer cells. We overexpressed Skp2 or p38MAPK in MCF-7 cells and examined the expressions of several ER α target genes known to have roles in regulating the estrogen response and the proliferation of these cells (TFF1/pS2, p21, and GREB1). We observed greatly upregulated (TFF1 [Fig. 6A] and GREB1 [not shown]) or downregulated (p21) basal expression levels, with a further enhancement of responses in the presence of estradiol, upon the overexpression of either Skp2 or p38MAPK (Fig. 6A and B). This effect of Skp2 on ER α target gene expression was reversed either by cotreatment with the pure antiestrogen ICI 182,780 (shown for p21 in Fig. 6C) or by the depletion of ER α by

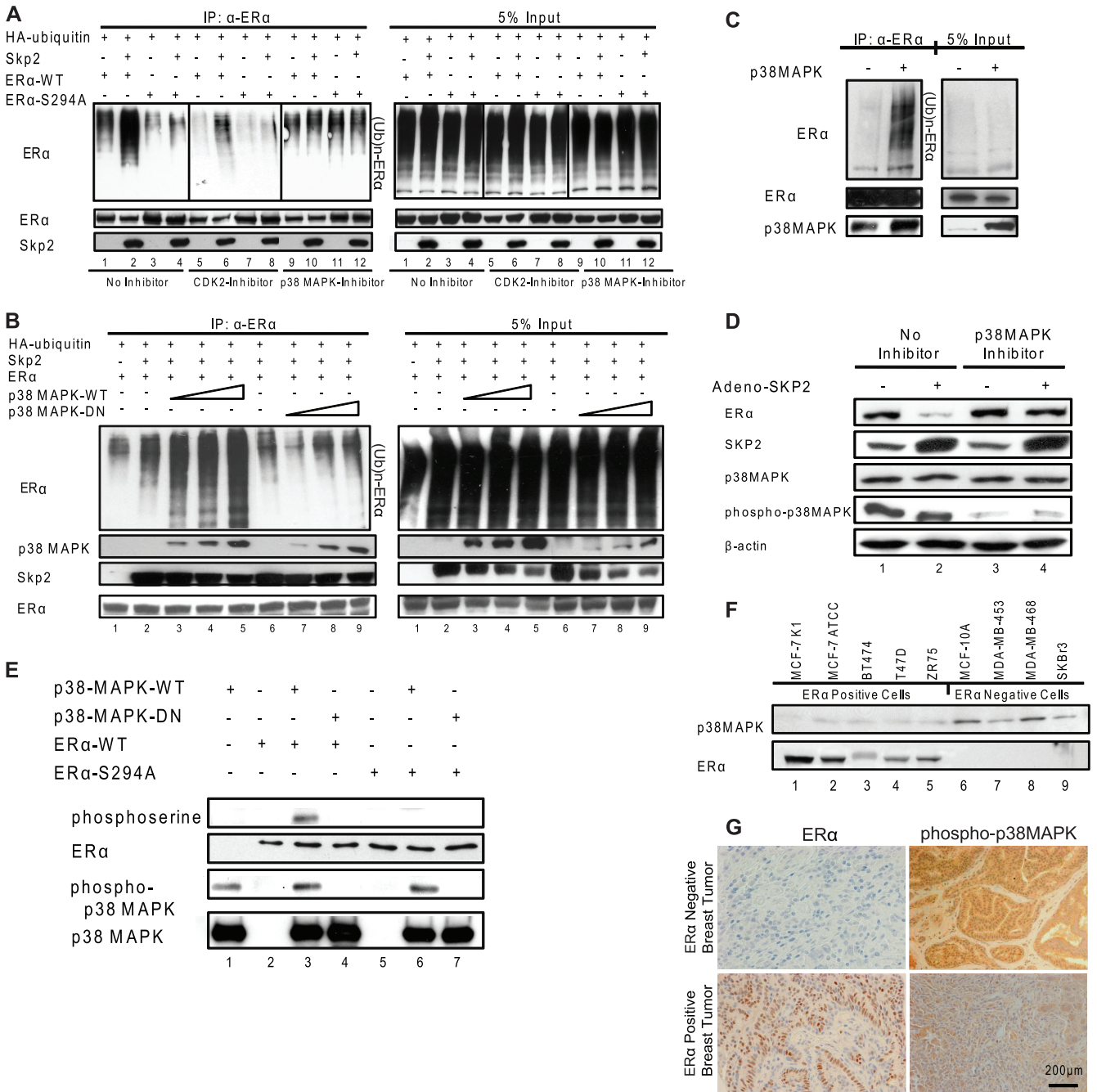
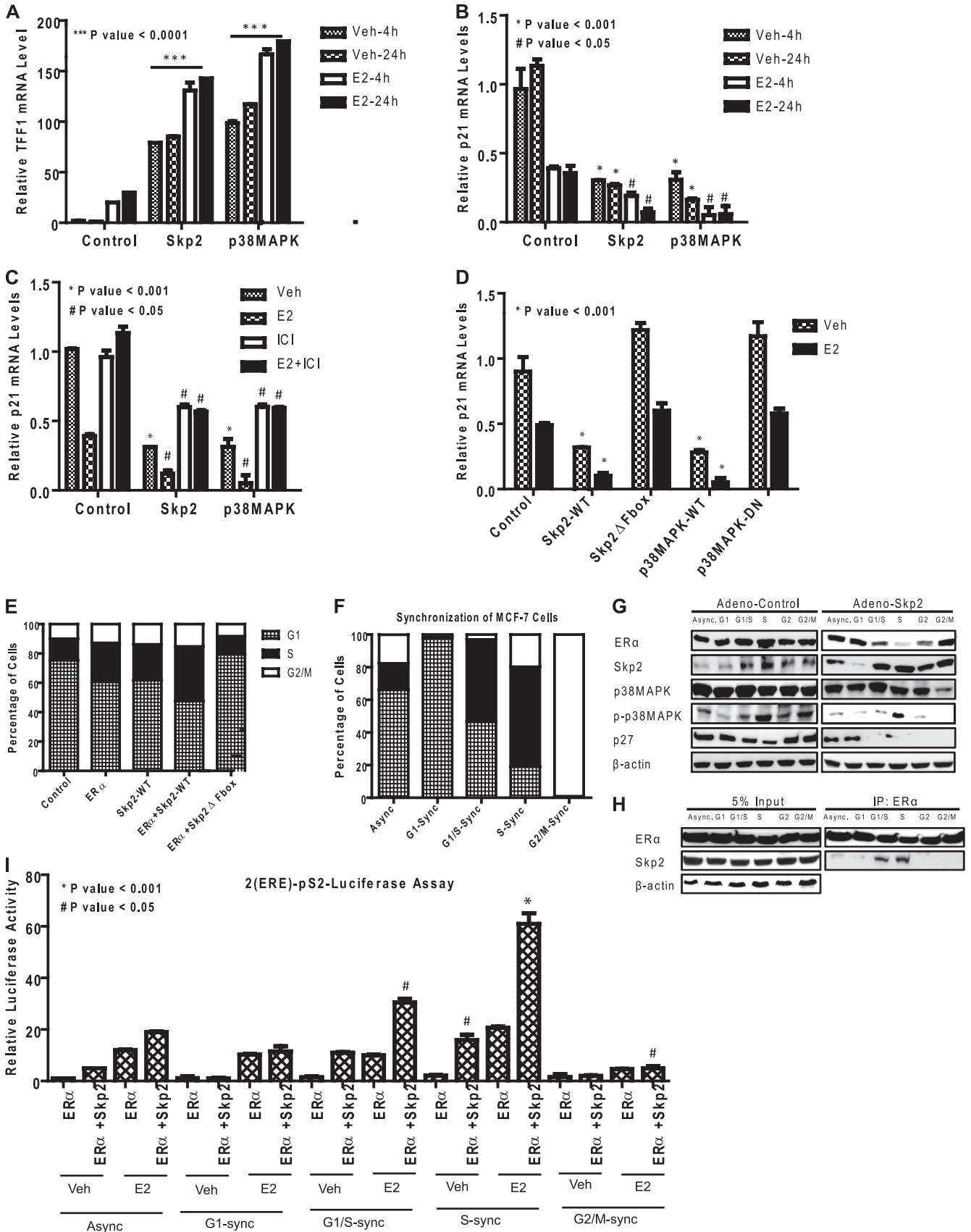


FIG 5 p38MAPK-mediated phosphorylation of ERα at serine 294 is required for its ubiquitination by Skp2. (A) Ubiquitination assay for the ERα-WT or ERα-S294A protein in Cos-1 cells exposed to the vehicle control, the cyclin-dependent kinase 2 (CDK2) inhibitor (1 μM), or the p38MAPK inhibitor (1 μM) in the absence or presence of Skp2. (B) Ubiquitination assay for ERα in Cos-1 cells in the presence of increasing amounts of either pCMV-p38MAPK-WT or p38MAPK-DN. (C) Ubiquitination assay for endogenous ERα in MCF-7 cells infected with the control or p38MAPK adenovirus. (D) Western analysis of MCF-7 cells infected with the control or Skp2 adenovirus and treated or not with the p38MAPK inhibitor. (E) *In vitro* kinase assay using an active or inactive recombinant GST-p38MAPK protein with *in vitro*-translated ERα-WT or ERα-S294A protein as the substrate. (F) Western analysis of p38MAPK and ERα proteins in multiple breast cancer cell lines. (G) Immunohistochemical staining for phospho-p38MAPK and ERα proteins in ER-negative and -positive human breast tumors. All data are representative of 4 experiments.

specific ERα siRNA (see Fig. S4A and S4B in the supplemental material), indicating these gene regulations to be ERα dependent. Also, the Skp2 mutant (Skp2ΔFbox), which was unable to degrade ERα, or the dominant negative p38MAPK mutant (p38MAPK-DN), which was unable to phosphorylate ERα, had no effect on

the transcriptional downregulation of the p21 gene by estradiol-bound ERα (Fig. 6D). As expected, in contrast to what was observed with the overexpression of Skp2 or p38MAPK in MCF-7 cells, the depletion of endogenous Skp2 or p38MAPK, which elevates ERα levels, was found to reduce the estrogen stimulation of



the pS2/TFF1 and GREB1 genes in these cells (see Fig. S8 in the supplemental material). Also of interest, ER α with the S294A mutation, which is resistant to Skp2-mediated turnover, showed compromised transcriptional activity compared to the activity of the wild-type receptor (see Fig. S3C in the supplemental material). Both of these findings suggest an important role for dynamic ER α turnover in supporting ER α transcriptional activity, as suggested previously by others (48).

By ChIP assays, we observed the recruitment of both Skp2 and p38MAPK to target gene promoters regulated by ER α , such as p21 (see Fig. S4C in the supplemental material), but not to promoters of genes closely related in function that were not regulated by ER α (p27) (see Fig. S4D in the supplemental material). By ChIP-re-ChIP, we showed the presence of Skp2 and p38MAPK together with ER α at target gene promoters (see Fig. S4E and S4F in the supplemental material). Flow cytometry analysis revealed a cooperativity between ER α and Skp2-WT in promoting the progression of cells through the G₁/S-phase checkpoint and their entry into the S phase, while the Skp2 mutant (Skp2 Δ Fbox) was unable to do so (Fig. 6E, and see Fig. S4G in the supplemental material).

Skp2 interacts with and ubiquitinates ER α specifically in the G₁/S and S phases of the cell cycle. Since Skp2 is known to regulate cell cycle progression through the coordinated control of factors regulating the G₁/S checkpoint, we investigated whether the Skp2 interaction with and regulation of ER α were cell cycle stage dependent. We therefore synchronized MCF-7 cells in the early G₁, G₁/S, S, and G₂/M phases of the cell cycle, as shown in Fig. 6F and Fig. S4H in the supplemental material, and monitored the levels of ER α , Skp2, and p38MAPK interactions between Skp2 and ER α at these stages. As shown in Fig. 6G, we found Skp2 levels to be low in the G₁ phase, in accordance with data from previous reports (4), and we were unable to detect an Skp2 interaction with ER α at this stage (Fig. 6H). An interaction of ER α with Skp2 could be detected in the G₁/S and S phases and was not detectable when cells moved into the G₂/M phase (Fig. 6H). Interestingly, we found ER α downregulation by Skp2 to occur primarily in the S phase of the cell cycle, a phase when the phospho-p38MAPK level was high (Fig. 6G).

In our analysis of synchronized breast cancer cell populations, we observed a significant increase in ER α -mediated (ERE)₂-pS2-Luciferase activity in the G₁/S and S phases of the cell cycle, which appeared to be Skp2 and estradiol dependent (Fig. 6I). These data imply that the regulation of ER α by Skp2, which occurs principally in the G₁/S and S phases, is important for the *trans*-activation functions of this nuclear hormone receptor and for the increased entry of cells into the S phase.

Restoration of functional ER α protein in ER-negative breast cancer cells by treatment with p38MAPK inhibitor or knock-

down of Skp2. In view of our findings for the involvement of p38MAPK and Skp2 in the reduction of ER α protein levels, we investigated whether we might be able to restore ER α levels in some ER-negative breast cancer cell lines by treatment with a p38MAPK inhibitor or the knockdown of Skp2. As shown in Fig. 7A and B, we observed a recovery of ER α protein levels in ER-negative MDA-MB-468 and MDA-MB-453 cells in the presence of either the p38MAPK inhibitor or the proteasomal inhibitor alone or in combination. ER α protein was seen by 4 h (Fig. 7A), and levels remained elevated over the 72 h examined (Fig. 7B). By comparing the level of reexpressed ER α protein in these cells to known amounts of ER α protein in MCF-7 cells, we observed a level of ER α in MDA-MB-453 cells that was ca. one-third of that of MCF-7 cells and nearly half of that in MDA-MB-468 cells after 72 h of p38MAPK inhibitor treatment (Fig. 7C). This receptor showed a normal response to ER ligands with a reduced level in the presence of E2 and ICI 182,780 and stabilization with tamoxifen (Fig. 7D), as we and others have reported previously for ER α -positive breast cancer cell lines (13, 14, 30). siSkp2 treatment also elicited increased ER α protein levels showing the same response to ER ligands (Fig. 7E). Of note, the depletion of Skp2, which increased ER α protein levels, did not change the ER α mRNA level in these cells (see Fig. S7 in the supplemental material). Thus, the regulation of ER by Skp2 is at the protein, and not the mRNA, level. Furthermore, it is of interest that the ER protein downregulates the production of the ER mRNA, and this occurs only when the ER protein is present, i.e., after the downregulation of Skp2 or the inhibition of p38MAPK, as shown in Fig. S7 in the supplemental material. Furthermore, the ER α protein was functional and enabled a marked estradiol stimulation of the well-known ER target genes progesterone receptor (PgR) and GREB1 (Fig. 7F), which was blocked by the antiestrogen *trans*-hydroxy-tamoxifen (TOT) or ICI 182,780. In addition, p38MAPK inhibition or Skp2 knockdown rendered the cells capable of responding to the E2 stimulation of cell proliferation, which could be suppressed by the antiestrogen tamoxifen or ICI 182,780 (Fig. 7G).

DISCUSSION

Our study reveals that two proteins important for the regulation of the cell cycle and other cellular activities, Skp2 and p38MAPK, play pivotal roles in controlling the levels and functional activities of ER α in breast cancer cells. Our findings are presented schematically in Fig. 7H and are discussed further below.

Selective regulation of ER α but not ER β by Skp2 and the ubiquitin-proteasome pathway. ER α is a rapidly turning-over protein with a half-life of about 4 h in breast cancer cells (16, 39), and in the present study, we have identified Skp2 as a novel E3-

FIG 6 Skp2 regulates ER α target gene expression, and the two proteins cooperate in driving S-phase entry of MCF-7 cells. (A to C) Real-time quantitative PCR analysis of TFF1 mRNA (A) or p21 mRNA (B) in MCF-7 cells infected with the control, Skp2, or p38MAPK adenovirus for 24 h followed by control, vehicle, or estradiol (E2) (10 nM) treatment for 4 h or 24 h and reversal of the E2 effect by the antiestrogen (1 μ M) ICI 182,780 (C). (D) Real-time RT-PCR analysis of p21 mRNA in MCF-7 cells infected with control, Skp2-WT, Skp2 Δ Fbox, p38MAPK-WT, or p38MAPK-DN adenovirus for 24 h, followed by vehicle or ligand treatment. (E) FACS analysis. MCF-7 cells were infected with the control, ER α alone, Skp2 alone, or ER α and Skp2 adenovirus followed by 48 h of E2 (10 nM) treatment and propidium iodide staining to monitor the DNA content. (F) FACS analysis. MCF-7 cells were synchronized at various stages in the cell cycle, as shown in Fig. S5H in the supplemental material, and the percentages of cells in the different cell cycle stages in asynchronous (Async) and synchronized (sync) populations are shown. (G) Western analysis of the indicated proteins in MCF-7 cells synchronized at various stages of the cell cycle and infected with the control or Skp2 adenovirus. (H) Skp2 interacts with ER α preferentially in the G₁/S and S phases of the cell cycle, as observed for synchronized MCF-7 cell populations. (I) Luciferase assay. Synchronized MCF-7 cells were transfected with the (ERE)₂-pS2-Luciferase expression plasmid along with ER α and β -galactosidase, treated for 24 h with either the vehicle or E2 (10 nM), and monitored for luciferase activity. Data are from 3 experiments and are represented as means \pm standard deviations (SD).

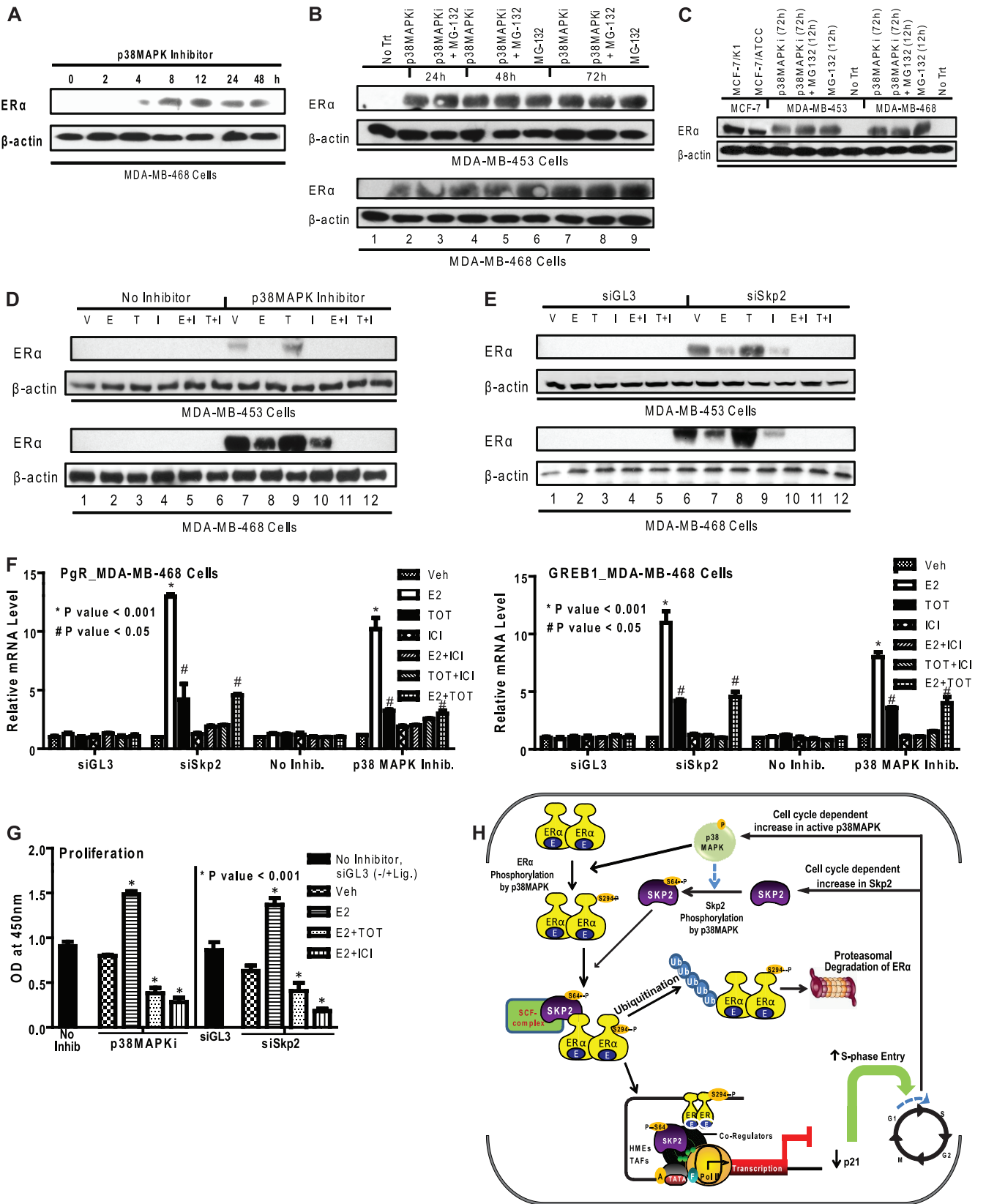


FIG 7 Restoration of ERα protein and functional activities in ERα-negative breast cancer cells by treatment with a p38MAPK inhibitor or knockdown of Skp2. (A) Western analysis for ERα in MDA-MB-468 cells treated with a p38MAPK inhibitor (SB203580) (1 μM) for the indicated times. (B) Western analysis for ERα in MDA-MB-453 and -468 cells receiving no inhibitor treatment (vehicle only) (No Trt), the p38MAPK inhibitor alone or in combination with MG-132 (10 μM), or MG-132 (10 μM) alone. (C) Western analysis for comparison of ERα in the ERα-positive cell lines MCF-7/K1 and MCF-7/ATCC and ERα-negative cell lines

ubiquitin ligase that is important in regulating the level of ER α in these cells. Our work is the first evidence for the involvement of an SCF^{F-box} family E3-ubiquitin ligase in regulating the cellular level and functional activity of ER α . The therapeutic importance of this regulation stems from the strong inverse correlation between ER α and Skp2 levels in human breast tumors. Interestingly, Skp2 was found to selectively target ER α and not the closely related receptor ER β or several other nuclear hormone receptors and transcription factors that we examined, making it an attractive target for pharmacological intervention, because ER β in breast tumors shows antiproliferative activity, whereas ER α is proproliferative (11, 18).

Insights into the specificity of the interaction of Skp2 with ER α came through our analyses which implicated the N-terminal AB domain, the region least conserved between the two ERs, in the ER α interaction with Skp2. However, the inability of the ER α -AB/ER β -CDEF chimera to be ubiquitinated by Skp2, despite regained interactions, suggested an additional aspect controlling ER ubiquitination by Skp2 upon their mutual interaction. We showed that this involved the p38MAPK-dependent phosphorylation of ER α at serine 294, a residue not conserved between the two ERs. These data highlight the importance of posttranslational modifications in the unique regulation of these two homologous nuclear receptors. We also analyzed several other ubiquitin ligases of a similar F-box family (data not shown), e.g., β TrCP (beta-transducin repeat-containing protein), which failed to interact with ER α or regulate its level, or CHIP or Stub1, which regulated basal ER α levels, as previously reported (6), but did not regulate ER α through p38MAPK and was insensitive to the S294 phosphorylation of ER α , suggesting the specificity of this regulation toward Skp2.

Involvement of p38MAPK in phosphorylation of ER α and its targeting for degradation by Skp2. Our observations of ER α phosphorylation site mutants, kinase inhibitors, and dominant negative kinase and mass spectrometry analyses have highlighted a novel function of the phosphorylation of ER α at serine 294 by p38MAPK in controlling the ubiquitination of the receptor by Skp2. Although this serine residue was recently reported to be phosphorylated in MCF-7 cells (3), no functional aspects of this phosphorylation were explored. Hence, it is of interest that we found that the ER α -S294A mutant was not degraded by Skp2 and was compromised in its ability to upregulate the expression of estrogen-stimulated genes. Since preclinical studies have shown tamoxifen resistance to be associated with elevated levels of p38MAPK (1, 49), the dynamic inverse relationship between ER α , Skp2, and p38MAPK observed in our studies suggests that the degradation of ER α by Skp2, as signaled by its selective phosphor-

ylation by p38MAPK, may be a possible underlying mechanism of this endocrine resistance. Furthermore, our findings imply that the suppression of Skp2 or p38MAPK activity might enable the restoration of an ER α -positive status in some ER-negative breast cancers.

Although our studies have focused on ER α regulation by Skp2 and p38MAPK, there is considerable evidence that other ubiquitin ligases and protein kinases are also involved in ER protein regulation. For instance, the cullin regulation of the ER α protein was previously reported to be mediated through S118 in ER α (8). ER α has also been shown to be ubiquitinated at K302 by BRCA1/BARD1, a ubiquitin ligase (15, 35), although the effect of this on ER α stability was not reported. Nephew and associates also reported previously that lysines 302 and 303 in ER α impact receptor interactions with the E3-ubiquitin ligase CHIP and the proteasome-mediated degradation of the receptor (6, 17). ER α protein turnover is also regulated by p27, a well-established Skp2 substrate, and by Src kinases (12).

Since Skp2 (9, 47, 52) and ER α each have critical roles in regulating cell proliferation and cancer progression, the cooperativity between Skp2 and ER α in driving the progression of breast cancer cells into the S phase of the cell cycle was noteworthy, a phenomenon not elicited by the dominant negative Skp2 Δ Fbox mutant, which was incapable of degrading ER α . Furthermore, our work revealed that the ectopic expression of Skp2 or p38MAPK greatly increased basal and estradiol-stimulated ER α target gene expression levels. Consistent with this, several previous studies have demonstrated the requirement for the continuous turnover of nuclear receptors, including ER α , on target gene promoters for successful transcription (34, 48, 50, 53). This regulation involved the recruitment of Skp2 and p38MAPK to ER α target gene promoters while modulating the expressions of key cell cycle- and growth-regulating genes, like p21, presumably through the posttranslational modification (phosphorylation and ubiquitination) of the nuclear receptor.

Multilevel regulation of ER α and restoration of ER α protein and hormone responsiveness in ER-negative breast cancer cells by inhibition of p38MAPK or downregulation of Skp2. It is becoming increasingly understood that ER α , a critical factor in breast cancer prognosis and responsiveness to endocrine therapies, is regulated at multiple levels. These levels include the transcriptional regulation of ER mRNA production, the translational regulation of ER α mRNA involving, at least in part, microRNAs (miRNAs) (59), and the regulation of ER α protein turnover (6, 15, 45). We now report Skp2 to be an important E3-ubiquitin ligase regulating the ER α protein and functional activities in breast cancer cells.

MDA-MB-453 and -468 treated with the vehicle, with the p38MAPK inhibitor for 72 h with or without MG-132 for the last 12 h, or with MG-132 alone. Equal amounts of cell protein were loaded onto each lane. (D) Western analysis of MDA-MB-453 and -468 cells treated with the vehicle (V) or the p38MAPK inhibitor followed by the vehicle or ligand treatments indicated (10 nM E2 [E], 10 nM TOT [T], 1 μ M ICI 182,780 [I] alone or E2 and ICI 182,780, or TOT and ICI 182,780). (E) Western analysis of ER α in MDA-MB-453 and -468 cells transfected with siGL-3 or siSkp2 followed by the vehicle or ligand treatments indicated. (F) Real-time RT-PCR analysis to monitor PgR and GREB1 mRNAs in MDA-MB-468 cells after siSkp2 or control siGL-3 treatment for 48 h or treatment with no inhibitor or the p38MAPK inhibitor for 48 h, followed by treatment with the vehicle or the indicated ligands for 24 h. (G) Proliferation of MDA-MB-468 cells in the presence or absence of the p38MAPK inhibitor or after siGL-3 or siSkp2 exposure for 48 h, followed by a 6-day treatment with the indicated ligands. Cell numbers were analyzed by a WST reagent assay. Data are from 3 experiments and are represented as means \pm SD. OD, optical density. (H) Model showing the regulation of ER α turnover and functional activity by Skp2 and p38MAPK. The posttranslational modification of ER α by the p38MAPK-dependent phosphorylation of ER α at Ser-294 controls the turnover of ER α by the E3-ubiquitin ligase Skp2 and also the regulation of gene expression and enhanced cell cycle progression. Furthermore, in some ER α -negative breast cancer cells, the inhibition of p38MAPK or the depletion of Skp2 restores cellular ER α protein levels and the responsiveness to estrogen- and antiestrogen-regulated gene expression and cell proliferation.

Of note, we were able to restore substantial levels of functional ER α protein in ER-negative breast cancer cells (MDA-MB-453 and MDA-MB-468) with p38MAPK inhibitor treatment or with the knockdown of Skp2, and this ER α was effective in eliciting estrogen- and antiestrogen-regulated cell proliferation and target gene expression. These ER α -negative breast cancer cell lines are ER α protein negative (59) but do express ER α mRNA (our unpublished findings). In contrast, we found p38MAPK inhibitor treatment or Skp2 downregulation to be ineffective in restoring ER α protein levels in SKBr3 cells that are ER α mRNA and protein negative, consistent with our observations that p38MAPK and Skp2 are regulating ER α at the protein level. We also observed that the absence of the ER α protein in MDA-MB-231 breast cancer cells does not appear to be Skp2 dependent, as we found the Skp2 expression level in these cells to be low and comparable to that in the ER α -positive cell line MCF-7, and the further downregulation of Skp2 with siRNA did not restore ER α protein levels in these cells. However, in the other ER α -negative cell lines that we tested (MDA-MB-468, MDA-MB-453, and MCF-10A), our proposed mechanism seems to hold true. This suggests that although Skp2 appears to be an important regulator of the ER α protein in some breast cancer cells, there may be other mechanisms independent of Skp2 that might be responsible for the loss of ER α protein in some breast cancers, including those possibly due to the methylation of the ER α promoter. Our observations may have therapeutic significance and appear to be worthy of further consideration in the clinical setting, where the possible drug targeting of p38MAPK and Skp2 might restore ER α protein levels in a subset of ER-negative breast cancers that express the ER α mRNA but not the protein, in order to render them amenable to endocrine therapy. Collectively, our findings reveal a novel process regulating intracellular ER α protein levels that requires receptor phosphorylation by p38MAPK that is critical for targeting ER α for degradation by Skp2 and for regulating ER α functional activities in breast cancer cells.

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S.B. conceived of and designed the studies, carried out the experiments and data analysis, interpreted the data, and wrote drafts of the manuscript. Z.X. and Z.M. carried out some of the experimental studies and data analysis and contributed to the writing of the manuscript. B.S.K. conceived of and designed the studies, analyzed and interpreted the data, and wrote the manuscript. All authors read, made suggestions for, and approved the final manuscript.

We declare that we have no conflict of interest.

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