

LKB1 Catalytic Activity Contributes to Estrogen Receptor α Signaling

Suchita Nath-Sain and Paola A. Marignani

Faculty of Medicine, Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H1X5

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The tumor suppressor serine-threonine kinase *LKB1* is mutated in Peutz-Jeghers syndrome (PJS) and in epithelial cancers, including hormone-sensitive organs such as breast, ovaries, testes, and prostate. Clinical studies in breast cancer patients show low *LKB1* expression is related to poor prognosis, whereas in PJS, the risk of breast cancer is similar to the risk from germline mutations in breast cancer (BRCA) 1/BRCA2. In this study, we investigate the role of *LKB1* in estrogen receptor α (ER α) signaling. We demonstrate for the first time that *LKB1* binds to ER α in the cell nucleus in which it is recruited to the promoter of ER α -responsive genes. Furthermore, *LKB1* catalytic activity enhances ER α transactivation compared with *LKB1* catalytically deficient mutants. The significance of our discovery is that we demonstrate for the first time a novel functional link between *LKB1* and ER α . Our discovery places *LKB1* in a coactivator role for ER α signaling, broadening the scientific scope of this tumor suppressor kinase and laying the groundwork for the use of *LKB1* as a target for the development of new therapies against breast cancer.

INTRODUCTION

Clinical evidence suggests a role for the multitasking tumor suppressor kinase *LKB1* (Marignani, 2005) in the development of breast carcinoma (Esteller *et al.*, 2000; Shen *et al.*, 2002; Fenton *et al.*, 2006). Early evidence in the literature suggests that a loss of *LKB1* expression leads to papillary breast carcinoma (Esteller *et al.*, 2000). More recently, a study that evaluated the expression of *LKB1* in 85 cases of breast cancers, the authors found that in a subset of high-grade in situ and invasive mammary carcinomas, the expression of *LKB1* was completely lost as determined by immunohistochemistry (Fenton *et al.*, 2006). In a separate study, 116 patients with confirmed breast carcinoma were evaluated for *LKB1* protein expression, of which expression was low in approximately one third of the patients compared with control population of women. The authors of this study concluded that low expression of *LKB1* correlated with higher histological grade, tumor size, and presence of lymph node status (Shen *et al.*, 2002). Both studies conclude that *LKB1* expression profile may serve as a prognostic marker for breast carcinoma; however, further investigation is warranted (Shen *et al.*, 2002; Fenton *et al.*, 2006). It should be noted that neither study determined whether *LKB1* was mutated in the patients. These recent findings suggest a role for *LKB1* in breast cancer however the molecular mechanisms by which this occurs is not fully understood.

Mutations in *LKB1* are responsible for Peutz-Jeghers syndrome (PJS) (Hemminki *et al.*, 1997; Ylikorkala *et al.*, 1999;

Marignani, 2005), an autosomal-dominant disease characterized by mucocutaneous pigmentation and benign gastrointestinal hamartomatous polyps (Hemminki, 1999). PJS patients are at a high risk of developing various cancers such as those of the gastrointestinal tract, breast, ovary, cervix, testis, lung, and pancreas (Avizienyte *et al.*, 1999; Hruban *et al.*, 1999; Wang *et al.*, 1999; Esteller *et al.*, 2000; Sanchez-Cespedes *et al.*, 2002; Lim *et al.*, 2003; Carretero *et al.*, 2004; Nakanishi *et al.*, 2004). PJS patients have a 54% greater risk of developing breast cancer than the general population (Giardiello *et al.*, 2000), as well as a reported loss of heterozygosity of *LKB1* in breast cancer (Bignell *et al.*, 1998; Chen *et al.*, 2000; Nakanishi *et al.*, 2004; Yang *et al.*, 2004). *LKB1* contains a nuclear localization sequence that allows for *LKB1* localization within the nuclear compartment of the cell (Nezu *et al.*, 1999; Tiainen *et al.*, 2002; Dorfman and Macara, 2008). Because a predominant number of mutations occur within the *LKB1* kinase domain, the tumor suppressor function of *LKB1* has been linked to its catalytic activity (Tiainen *et al.*, 1999; Ylikorkala *et al.*, 1999; Boudeau *et al.*, 2003). More recently, we showed that *LKB1* catalytic-deficient mutants exhibit oncogenic property, such that they are able to enhance the expression of the oncogene cyclin D1 in cells that lack expression cyclin D1-negative regulators p53 or p21^{WAF/CIP1} (Scott *et al.*, 2007).

Aberrant ER α signaling is a major contributor to the occurrence of ER α -positive breast cancer (Sommer and Fuqua, 2001). ER α is a member of the nuclear receptor (NR) family of transcription factors characterized by similar structural domains. There are two isoforms of ERs; ER α (Walter *et al.*, 1985) and ER β (Kuiper *et al.*, 1996). ER α regulates genes such as cathepsin D, trefoil factor (pS2), c-myc, and cyclin D1 (reviewed in O'Lone *et al.*, 2004). Whereas ER α interacts with the estrogen response elements (EREs) in the promoters of the first two genes (classical pathway), it indirectly regulates the latter genes by interaction with DNA-bound transcription factors (nonclassical pathway). In general, upon stimulation with 17- β -estradiol (E2), ER α dimerizes and translocate to the

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Address correspondence to: Paola A. Marignani (pmarigna@dal.ca).

Abbreviations used: 4-OHT, 4-hydroxy tamoxifen; E2, 17 β -estradiol; ER α , estrogen receptor α ; ERE, estrogen response element; PJS, Peutz-Jeghers syndrome; TnT, transcribed and translated.

nucleus in which the dimer interacts with DNA and coregulator proteins to regulate gene transcription. Because alterations in LKB1 expression have been linked to breast cancer, we investigated the molecular mechanism by which LKB1 regulates ER α -mediated signaling.

MATERIALS AND METHODS

Cell Culture and Transfection

MCF7 human breast cancer cells (American Type Culture Collection, Manassas, VA), G361 melanoma cells (gift from Dr. Alessi, Medical Research Council, Protein Phosphorylation Unit, University of Dundee), and human embryonic kidney (HEK) 293 were maintained in DMEM supplemented with 8% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were transfected with expression plasmids, as indicated, using Lipofectamine Plus reagent (Invitrogen).

Glutathione Transferase (GST) Pull-Down Assay

ER α expression plasmid was transcribed and translated (TnT) *in vitro* by using T7-coupled reticulocyte lysate and wheat germ extract (Promega, Madison, WI), in the presence of [³⁵S]methionine. Labeled proteins were incubated with purified recombinant fusion proteins, GST-LKB1 and GST, on glutathione-Sepharose beads for 4 h at 4°C as described previously (Marignani *et al.*, 2001). The samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

Western Blotting and Antibodies

Cells were harvested in lysis buffer as described previously (Scott *et al.*, 2007). Lysates were prepared for SDS-PAGE, and proteins were separated and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). ER α (HC-20), LKB1 (Ley-37D), Lamin B1 (H-90), p300 (H-272), BRCA1 (D-9), and actin (I-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ER α (AER314) was from NeoMarkers (Fremont, CA). Brg1, MP-activated protein kinase (AMPK), and pAMPK(T172) antibodies were from Cell Signaling Technology (Danvers, MA).

Immunoprecipitation Assay

MCF7 cells were maintained in tissue culture dishes (10 cm) in phenol-free (PF)-DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 5% charcoal-stripped fetal bovine serum (csFBS) and transfected with FLAG-LKB1, ER α expression plasmids. Cells were left untreated or treated with E2 (100 nM) for 45 min followed by lysis. LKB1 was immunoprecipitated (IP) from 500 μ g of precleared total cell lysate (TCL) by using anti-LKB1.

Nuclear Cytoplasmic Fractionation

MCF7 cells were trypsinized followed by centrifugation (700 \times g), cells were resuspended in hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.1% NP-40, 1 mM NaF, and 4 μ g/ml protease inhibitors), incubated on ice, and centrifuged (700 \times g) to separate the cytoplasmic fraction. To obtain the nuclear fraction, cells were homogenized in nuclear lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.2% NP-40, 0.04% SDS, 0.1% sodium deoxycholate, 50 mM NaF, 1 mM Na₃VO₄, and 4 μ g/ml protease inhibitors), and ultracentrifuged (125,000 \times g) to remove cellular debris.

Reporter Assay

Reporter assays were performed using Dual Luciferase kit (Promega), and promoter activity was analyzed using an Auto Lumat Plus luminometer (Berthold Technologies, Bad Wildbad, Germany) as described previously (Scott *et al.*, 2007). Briefly, MCF7 or G361 cells were transfected with the following plasmids: LKB1 (50 ng), D194A/R304W (50 ng) LKB1 NR mutants (50 ng), pRL-tk (0.5 ng), ERE-luc (gift from Dr. Myles Brown, Harvard University, Boston, MA)/cyclin D1-luc (50 ng), ER α /ER β (Addgene plasmid 11356) (10 ng)/androgen receptor (AR; 10 ng), glucocorticoid receptor (GR; 50 ng), mouse mammary tumor virus luciferase (MMTV-luc; 50 ng) (gifts from Dr. Steven Balk, Harvard University), p300 (50 ng) (Addgene plasmid 10718; Addgene, Cambridge, MA), BRCA1, and p53 (indicated concentrations). Six hours after transfection, phenol-free DMEM 5% serum-stripped FBS (ssFBS) was used to replace the media. Twenty-four hours after transfection, cells were treated with E2 (10 nM), 4-hydroxy tamoxifen (4-OHT; 1 μ M), dexamethasone (100 nM), or testosterone (100 nM) (Sigma-Aldrich). Control cells were left untreated. Cells were washed in phosphate-buffered saline (PBS) and harvested after 24 h in passive lysis buffer according to manufacturer's protocol (Promega).

Small Interfering RNA (siRNA) Transfection

MCF7 cells were transfected with LKB1 siRNA (100 nM; Target sequences 1, GCUCUACGGCAAGGUGAA; 2, UGAAAGGGAUGCUUGAGUA; and 3

GAAGAAGGAAAUUCAACUA; Dharmacon, RNA Technologies, Lafayette, CO) by using DharmaFECT transfection reagent.

Flow Cytometry. MCF7 maintained in PF-DMEM 5% csFBS for 72 h before transfection with LKB1 siRNA and myr-green fluorescent protein (GFP)-cytomegalovirus (CMV) as described previously (Scott *et al.*, 2007). Twenty-four hours after transfection, cells were incubated in PF-DMEM, 3% ssFBS for 24 h before treatment with vehicle or E2 (100 nM) for 24 h and then harvested and prepared for flow cytometry. Propidium iodide was purchased from Sigma-Aldrich. Fluorescence-activated cell sorting (FACS) analysis was conducted on an FACSCalibur flow cytometer (Beckman Coulter, Fullerton, CA) with data acquired using CellQuest software. Cell cycle profiles were determined using ModFit LT.

Immunofluorescence

Cells were prepared for fluorescent microscopy as described previously (Marignani and Carpenter, 2001). Cells were incubated in primary antibody for 1 h, followed by incubation with appropriate secondary antibodies: Oregon green and rhodopsin (Invitrogen). Nuclear staining was accomplished using 4,6-diamidino-2-phenylindole (DAPI) for 2 min. Images were obtained using an Eclipse TE 2000-E inverted research microscope (Nikon, Tokyo, Japan), mounted with a Q-Imaging charge-coupled device camera and acquired using the Simple PCI software.

In Vitro Kinase Assay

LKB1 assays were conducted as described previously (Marignani *et al.*, 2007). Briefly, HEK293 cells were transfected with the following expression plasmids: LKB1pEBG-2T, R304WpEBG-2T, and pEBG-2T vector alone together with MO25 α -MYC-CMV and STRAD α -FLAG-CMV. Phosphotransferase activity of LKB1 toward the LKB1tide peptide (150 μ M) (Millipore, Billerica, MA) was determined using protein complex (0.5 μ g) in the presence of 0.1 mM [γ -³²P]ATP (PerkinElmer Life and Analytical Sciences, Boston, MA) at 30°C for 15 min. Kinase assays *in vitro* were performed as described previously (Marignani *et al.*, 2001). ER α , AMPK, p300, BRCA1, and Brg1 were immunoprecipitated with appropriate antibodies from MCF7 cells for 3 h followed by incubation with purified LKB1/Strad α /Mo25 complexes in the presence of [γ -³²P]ATP. Purified recombinant human AMPK was from Cell Signaling Technology. Phosphorylation was visualized by autoradiography.

RNA Isolation and Polymerase Chain Reaction (PCR)

RNA was isolated using TRIzol reagent (Invitrogen) and reverse transcribed as per manufacturer's protocol (Ambion, Austin, TX). PCR was conducted at 95°C for 15 min, 24 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1.5 min, and a final step of 72°C for 5 min (iCycler; Bio-Rad Laboratories, Hercules, CA) on cDNA by using primers listed on Supplemental Table 1.

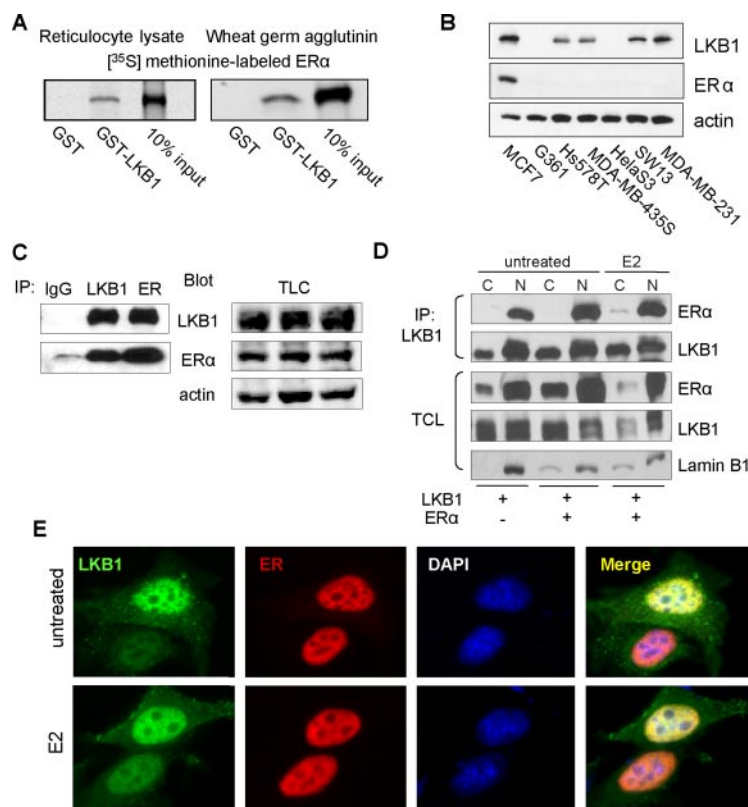
Chromatin Immunoprecipitation

G361 and MCF7 cells were grown in DMEM supplemented with 8% FBS or PF-DMEM with 5% charcoal-stripped FBS, respectively. MCF7 cells were left untreated or treated with 100 nM E2 for 45 min. G361 cells were transfected with ER α , LKB1, and R304W expression plasmids. Chromatin immunoprecipitation (ChIP) assays were conducted as described previously (Scott *et al.*, 2007). Immunoprecipitation was performed at 4°C overnight using anti-LKB1 or anti-mouse surface immunoglobulin G (mlgG) (control) antibodies. PCR was performed using primers for the promoters of pS2, c-myc, and cathepsin D as listed in Supplemental Table 1.

RESULTS

LKB1 Interacts with ER α

We previously identified the first binding partner for LKB1, the SWI/SNF chromatin remodeling protein Brg1 (Marignani *et al.*, 2001). LKB1 interacts with the helicase domain of Brg1, facilitating the ATPase activity of this chromatin remodeler (Marignani *et al.*, 2001). Given that Brg1 is necessary for ER α -mediated gene transactivation (DiRenzo *et al.*, 2000) and LKB1 enhances Brg1-ATPase activity, we investigated whether LKB1 interacts with the nuclear hormone receptor ER α . Here, we TnT ER α expression plasmid *in vitro* in the presence of [³⁵S]methionine by using rabbit reticulocyte lysate and wheat germ agglutinin followed by GST pull-down assays (Figure 1A). We observed that recombinant GST-LKB1 protein bound to ER α , whereas control recombinant GST protein did not (Figure 1A). The interaction was confirmed in MCF7 cells that express both endogenous LKB1 and ER α (Figure 1B). Here, endogenous LKB1 and ER



IP using anti-LKB1 and anti-ER α antibodies followed by Western blot analysis to confirm coimmunoprecipitation (Figure 1C).

Nuclear Localization of LKB1-ER α Interaction

To determine the cellular localization of LKB1 and ER α interaction, MCF7 cells were transfected with LKB1 and ER α expression plasmids. Cells were treated with E2 (100 nM) for 45 min before harvesting. Nuclear and cytoplasmic fractions were prepared, followed by immunoprecipitation of equal amounts of protein from each fraction with anti-LKB1 antibody. We observed that the interaction between LKB1 and ER α was restricted to the nuclear fraction (Figure 1D). To further verify these observations, immunofluorescence microscopy was conducted in MCF7 cells left untreated or treated with E2. We observe the nuclear colocalization of endogenous LKB1 and ER α (Figure 1E). These results confirm our Western blot analysis that shows coimmunoprecipitation of LKB1 and ER α occurs in the nuclear fraction (Figure 1D).

LKB1 Functions as a Coactivator of ER α

Because LKB1 interacts with the ER α in the nuclear compartment of the cell, we investigated the possibility that LKB1 played a role in ER α -mediated gene transactivation by performing reporter assays. Because we were interested in whether the catalytic activity of LKB1 was required for this function, we used LKB1 null cells to facilitate in the interpretations of results in the absence of endogenous LKB1. In addition, previous work by others have shown that ectopic expression of ER α in cells that express endogenous ER α , such as MCF7, may lead to aberrant ER α signaling because the endogenous protein may function as a dominant-negative (Bocquel *et al.*, 1989; Webb *et al.*, 1992). Hence, we surveyed known ER α -negative breast cancer cell lines for

LKB1 expression. In contrast to previous reports (Shen *et al.*, 2002), we observed LKB1 expression in MDA-MB-435S melanoma-derived cell line and MDA-MB-231 breast cancer cells (Figure 1B). Therefore, we conducted ERE-luciferase reporter gene assays in G361 melanoma cell line that do not express either LKB1 or ER α . G361 cells were transfected with LKB1 and ER α expression plasmids pERE-luc and pRL-tk, (Figure 2A). In the presence of LKB1, ER α -mediated transactivation was enhanced in response to E2 treatment, compared with ER α alone and LKB1 alone. To determine whether increasing concentrations of LKB1 altered ER α -mediated transactivation of a reporter gene, G361 and MCF7 cells were transfected with increasing concentrations of LKB1 (50, 100, and 500 ng), followed by ERE-luc reporter assays. In G361 cells, optimal transactivation was observed at 50 ng LKB1, as squelching of the reporter gene, as described previously by Lee *et al.* (2005), occurred at higher concentrations (Figure 2B). In contrast, overexpression of LKB1 in MCF7 cells did not significantly alter ER α -mediated gene transactivation in response to E2 treatment (Figure 2D). In the presence of 4-OHT, a selective estrogen receptor modulator of ER α , as expected, transactivation of the reporter was abrogated both in the presence of ER α alone and ER α plus LKB1 (Figure 2, C and E). Knockdown of LKB1 expression in MCF7 cells using three separate siRNA duplexes suppressed ER α -mediated transcriptional activity (Figure 2F).

To investigate whether enhanced ER α -mediated transactivation was attributed to the introduction of LKB1 and not to changes in ER α expression levels due to the introduction of LKB1, G361 and MCF7 cells were transfected with increasing concentrations of LKB1 expression plasmid (0, 0.5, 1.5, and 2 μg), while simultaneously maintaining the concentration of ER α expression plasmid (2 μg) constant in G361 cells. Increased expression of LKB1 did not alter the

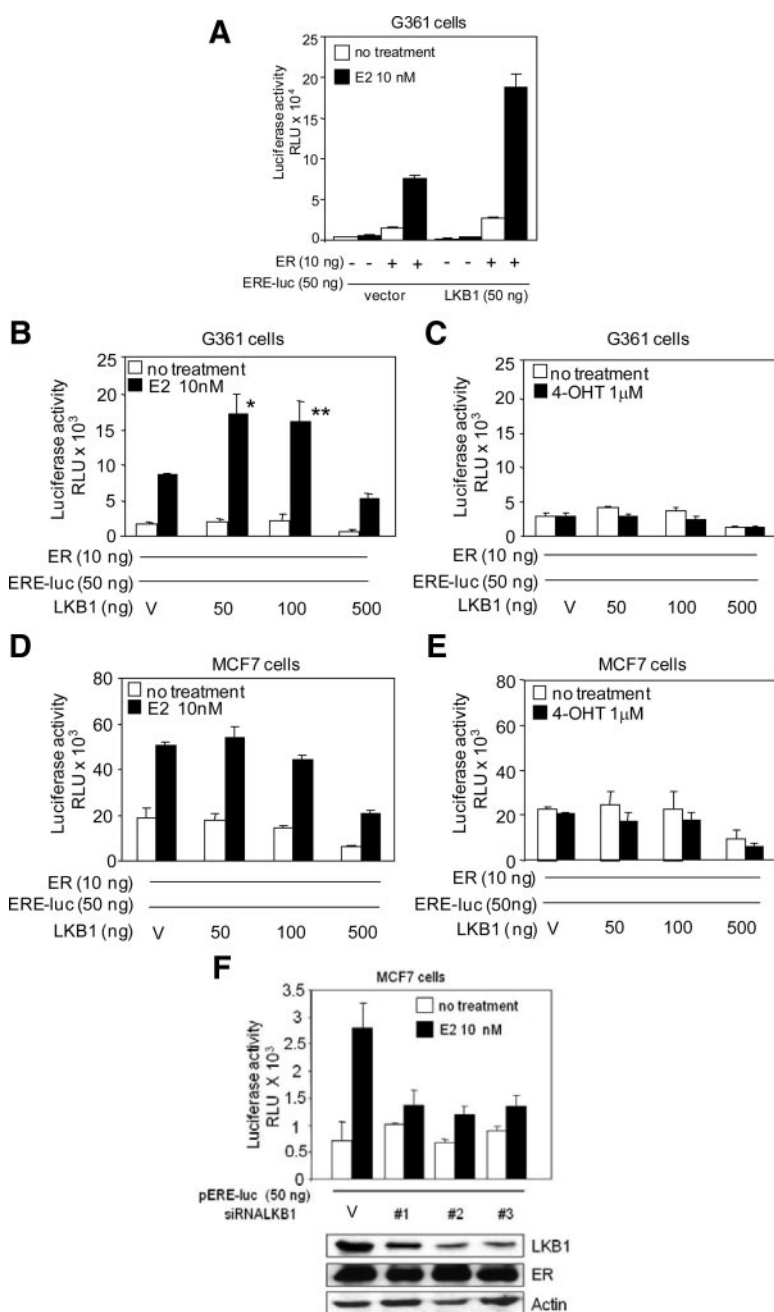


Figure 2. LKB1 enhances ER α activity in the presence of 17- β estradiol. (A) G361 cells were transfected with LKB1, ER α , ERE-luc, and pRL-tk expression plasmids or vector (V), left untreated or treated with E2 as described in *Materials and Methods*, followed by reporter assays. Data representative of two experiments in triplicate mean \pm SD. (B) G361 cells were transfected with ER α , ERE-luc, and pRL-tk expression plasmids, and increasing concentrations of LKB1 expression plasmid, left untreated, or treated with E2 or (C) 4-OHT as described in *Materials and Methods* followed by reporter assays. Results are three experiments in triplicate. Data are mean \pm SEM. * $p < 0.02$ compared with 0 ng; ** $p < 0.05$ compared with 0 ng. (D and E) MCF7 cells were transfected and treated as described in B and C. Results are three experiments in triplicate. Data are mean \pm SEM. (F) MCF7 cells were transfected with three different siRNA-LKB1 duplexes, ERE-luc, pRL-tk, and treated with E2 as described in *Materials and Methods* followed by reporter assays. Western blot analysis confirms abrogation of LKB1 expression by using anti-LKB1, -ER α , and -actin antibodies. Results are representative of two experiments in triplicate mean \pm SD.

expression of ectopic (G361 cells) or endogenous ER α (MCF7) as determined by reverse transcription (RT)-PCR and Western blot analysis (Figure 3A). Furthermore, knockdown of LKB1 expression in MCF7 cells by siRNA did not affect ER α expression (Figure 3A, bottom right). In addition, the introduction of increasing concentrations of ER α expression plasmid (0, 0.5, 1.5, and 2 μ g), while simultaneously maintaining the concentration of LKB1 expression plasmid (2 μ g) constant in G361 cells did not alter LKB1 expression of ectopic LKB1 in G361 or endogenous LKB1 in MCF7 cells (Figure 3B).

LKB1 Synergizes with p300

To study the effect of LKB1 on ER α activity in the presence of a known ER α coactivator protein p300 (Hanstein *et al.*, 1996), we conducted ERE-luc reporter assays as described

above. We first tested whether expression of p300 in G361 cells maintained coactivator function of ER α -ERE-luc (Figure 4A, left). When p300 and LKB1 expression plasmids were introduced simultaneously, we observe a synergistic effect, suggesting cooperation between LKB1 and p300 in enhancing ER α activity (Figure 4A, right). However, coactivator function of LKB1 in the presence of known corepressor proteins p53 and BRCA1 (Yu *et al.*, 1997; Fan *et al.*, 1999) was suppressed in a concentration-dependent manner (Figure 4, B and C).

Next, we examined whether the coactivator function of LKB1 applies to other members of the nuclear receptor family such as ER β , AR, and GR by performing reporter assays. G361 cells were transfected with LKB1, ER β , AR, or GR expression plasmids, pRL-tk and pERE-luc or MMTV-luc as indicated, left untreated, or treated with E2, testosterone, or dexamethasone

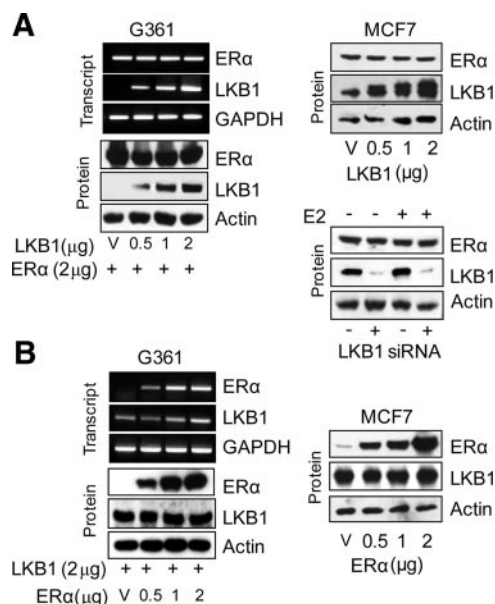


Figure 3. ER α expression is not altered by LKB1. (A) Left, G361 cells were transfected with expression plasmids or vector (V) as indicated. LKB1 and ER α expression were determined by PCR and Western blot analysis. Top, right, Western blot analysis of MCF7 cells transfected with increasing concentrations of LKB1. Bottom, right, Western blot analysis of MCF7 cells transfected with LKB1 siRNA-#2 duplex (100 nM). (B) Cells were transfected with expression plasmids as indicated. LKB1 and ER α expression were determined by PCR and Western blot analysis. Results are representative of three experiments.

(Figure 5). Transactivation of respective reporters by ER β , GR or AR did not significantly increase in the presence of LKB1.

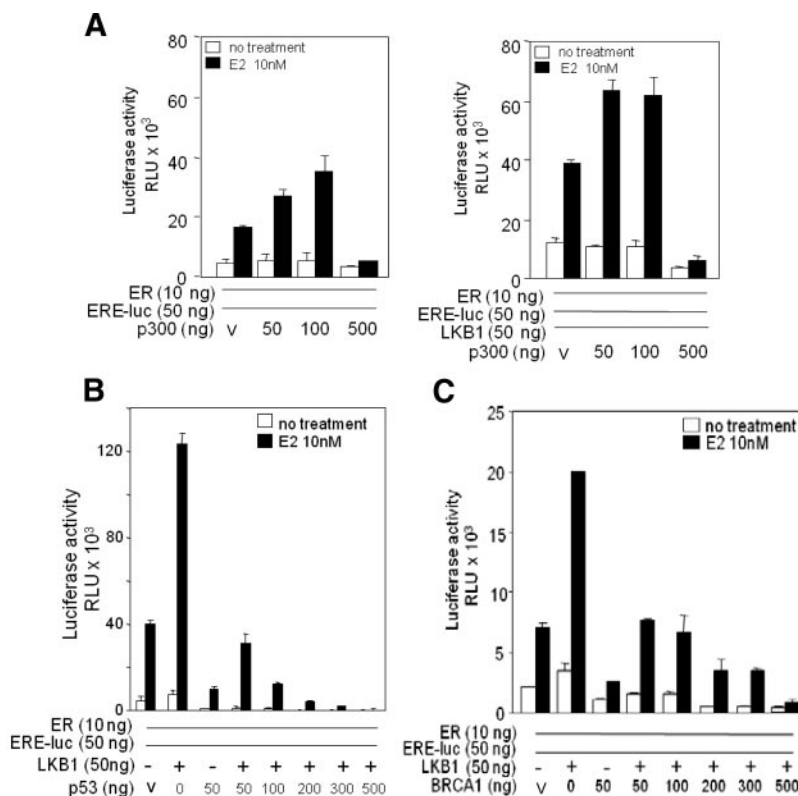
LKB1 Catalytic Activity Is Required

To determine whether LKB1 catalytic activity was necessary for ER α -mediated transactivation of the reporter, reporter assays were conducted using LKB1 catalytic-deficient mutants (LKB1 mutants) D194A and R304W described previously (Marignani *et al.*, 2007; Scott *et al.*, 2007). Both mutants did not alter ER α -mediated transactivation of ERE-luc compared with ER α alone, thereby implying that LKB1 catalytic activity contributes to ER α -mediated transactivation of ERE-luc (Figure 6A). In the presence of 4-OHT transactivation of the reporter was abrogated both in the presence of ER α alone and ER α plus LKB1 or mutants (Figure 6B).

ER α Is Not a Substrate of LKB1

Because we show that LKB1 catalytic activity enhances ER α -mediated transactivation (Figure 2), and LKB1 mutants attenuated ER α -mediated transactivation (Figure 6), we tested whether ER α was a substrate for LKB1. LKB1/Strad α /MO25, R304W/Strad α /MO25 complexes, and GST were prepared from HEK293 cells as described previously (Marignani *et al.*, 2007; Scott *et al.*, 2007) (Figure 7A) and tested for catalytic activity toward the LKBtide peptide (Figure 7A) and purified recombinant human AMPK protein followed by Western blot analysis using anti-phospho-AMPK antibody (pT172) (Figure 7B). Endogenous ER α was immunoprecipitated from MCF7 cells (Figure 7C, top) followed by incubation with purified complexes in the presence of [γ - 32 P]ATP (Figure 7C, middle). To test whether known coregulator proteins of ER α may be substrates for LKB1, BRCA1, p300, Brg1 (negative control) (Marignani *et al.*, 2001), and AMPK (positive control) (Hawley *et al.*, 2003) were immunoprecipitated from MCF-7 cells by using the appropriate antibodies (Figure 7D, top) followed by incubation with purified LKB1 complex (Figure 7A) followed by kinase assays *in vitro* (Figure 7D, bottom).

Figure 4. LKB1 and p300 synergize to enhance ER α activity. (A) Left, G361 cells were transfected with increasing concentrations of p300 expression plasmids as indicated; ER α , ERE-luc, and pRL-tk, or vector (V); left untreated or treated with E2 for 24 h followed by reporter gene assays. (A) Right, G361 cells were transfected with increasing concentrations of p300 expression plasmids, a constant concentration of LKB1 as indicated; ER α , ERE-luc, and pRL-tk or V; left untreated or treated with E2 for 24 h followed by reporter gene assays. Results are two experiments in triplicate. Data are mean \pm SD. (B) G361 cells were transfected with pRL-tk, ER α , ERE-luc, and increasing concentrations of p53, or LKB1 expression plasmids, left untreated or treated with E2 for 24 h followed by reporter gene assays. Data representative of three experiments in triplicate. Data are mean \pm SD. (C) Transfection conditions similar to B; however, in the presence of increasing concentrations of BRCA1. Data are representative of three experiments in triplicate mean \pm SD.



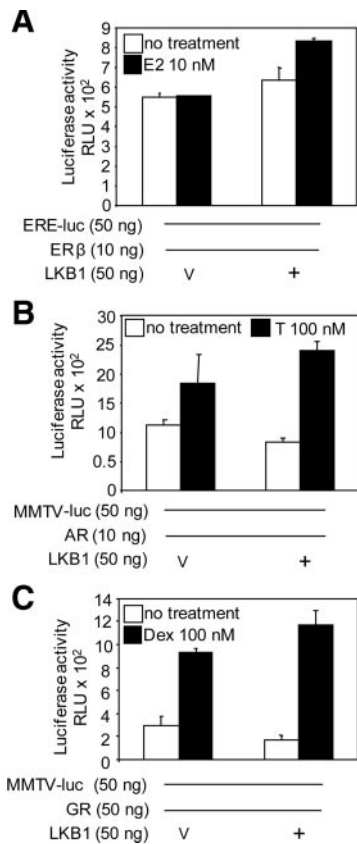


Figure 5. LKB1 does not alter ERβ, GR, or AR activity. (A) G361 cells were transfected with LKB1, ERβ, ERE-luc, and pRL-tk expression plasmids or vector (V). Cells were left untreated or treated with E2 (10 nM) for 24 h before harvesting for reporter assay. (B) Cells were transfected with LKB1, AR, MMTV-luc, and pRL-tk expression plasmids or V, followed by testosterone (T; 100 nM) treatment. (C) Cells were transfected with LKB1, GR, MMTV-luc, and pRL-tk expression plasmids or V followed by treatment with dexamethasone (Dex; 100 nM). Results are three experiments in triplicate mean ± SEM.

LKB1 Enhances Nonclassical ERα Signaling

To examine whether LKB1 alters ERα-mediated transcription of genes through the nonclassical pathway, whereby ERα tethers to transcription factors that bind to the promoters of genes, reporter assays were conducted using cyclin D1 luciferase (CD1-luc) (Scott *et al.*, 2007). We observed that ERα enhances CD1-luc in the presence of E2 consistent with a previous report (Sabbah *et al.*, 1999), whereas coexpression of LKB1 and ERα followed by E2 treatment enhanced transactivation of cyclin D1-luc compared with LKB1 alone or ERα alone (Figure 8A). Transactivation of cyclin D1-luc by LKB1 mutants did not differ from ERα alone. Given that G361 cells are wild type for expression of cyclin D1 regulators p53 and p21^{WAF1/CIP1}, we did not expect LKB1 mutants to function as described previously in DLD1^{p21-/-p53-/-} colorectal cancer (Scott *et al.*, 2007).

Because we observe that LKB1 enhances ERα-mediated transcription in G361 cells (Figure 2A), we tested whether LKB1 could alter ERα-ligand dependent cell cycle progression (Ikeda and Inoue, 2004). For these studies, LKB1 expression in MCF7 cells was ablated using siLKB1#2 (Figure 2F), followed by treatment with E2 (100 nM) for 24 h, and then flow cytometry to evaluate the cell cycle profile of asynchronous cells. As expected, abrogation of LKB1 expression re-

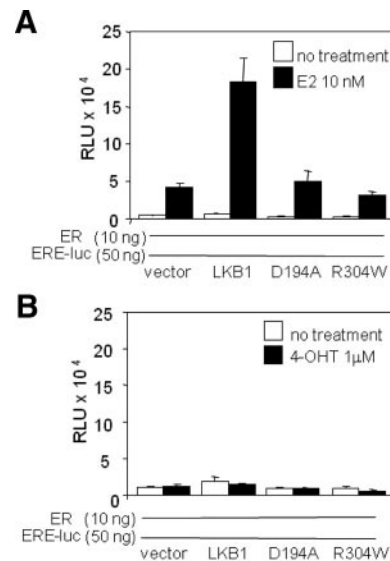


Figure 6. LKB1 catalytic activity is necessary for ERα transactivation. (A and B) G361 cells were transfected with LKB1, D194A, R304W, ERα, ERE-luc, and pRL-tk expression plasmids or vector (V), left untreated or treated with E2 or 4-OHT for 24 h before harvesting for reporter assay. Results are representative of two experiments in triplicate. Data are mean ± SD.

sulted in an overall increase in the percentage of cells found in S phase for untreated cells with a corresponding reduction in

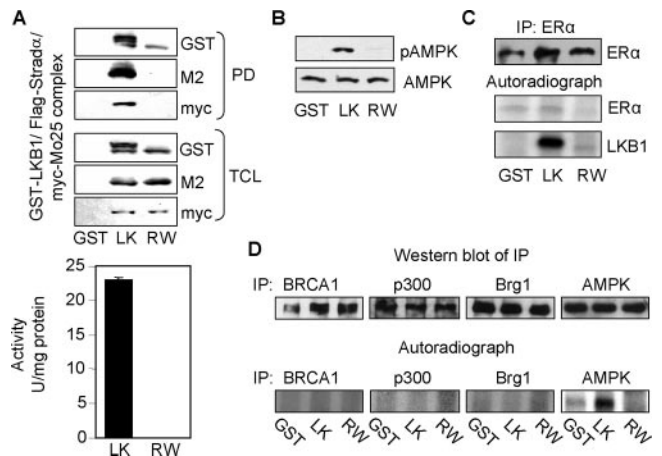


Figure 7. LKB1 does not phosphorylate ERα. (A) Expression of purified GST-LKB1/FLAG-STRADα/Myc-MO25α complex from HEK293 cells was confirmed by Western blot analysis (top). Confirmation of catalytic activity of complex toward LKBtide peptide (bottom) and (B) purified recombinant human AMPK incubated with complex followed by Western blot analysis using anti-pAMPK(T172) and anti-AMPK for total expression. (C) ERα was IP using anti-ERα antibody from MCF7 cells followed by incubation with purified complex in the presence of [γ-³²P]ATP. Top panel confirms IP of ERα by Western blot analysis; bottom panels are corresponding autoradiographs. (D) BRCA1, p300, Brg1 (negative control), and AMPK (positive control) were immunoprecipitated from MCF7 cells by using the appropriate antibodies followed by incubation with complex described in A, in the presence of [γ-³²P]ATP. Top panel confirms IP of BRCA1, p300, Brg1, and AMPK by Western blot analysis. Bottom panel represent autoradiographs. Results are representative of three separate experiments. Purified complexes: LK, GST-LKB1/STRADα/Myc-MO25α; RW, GST-R304W/STRADα/Myc-MO25α.

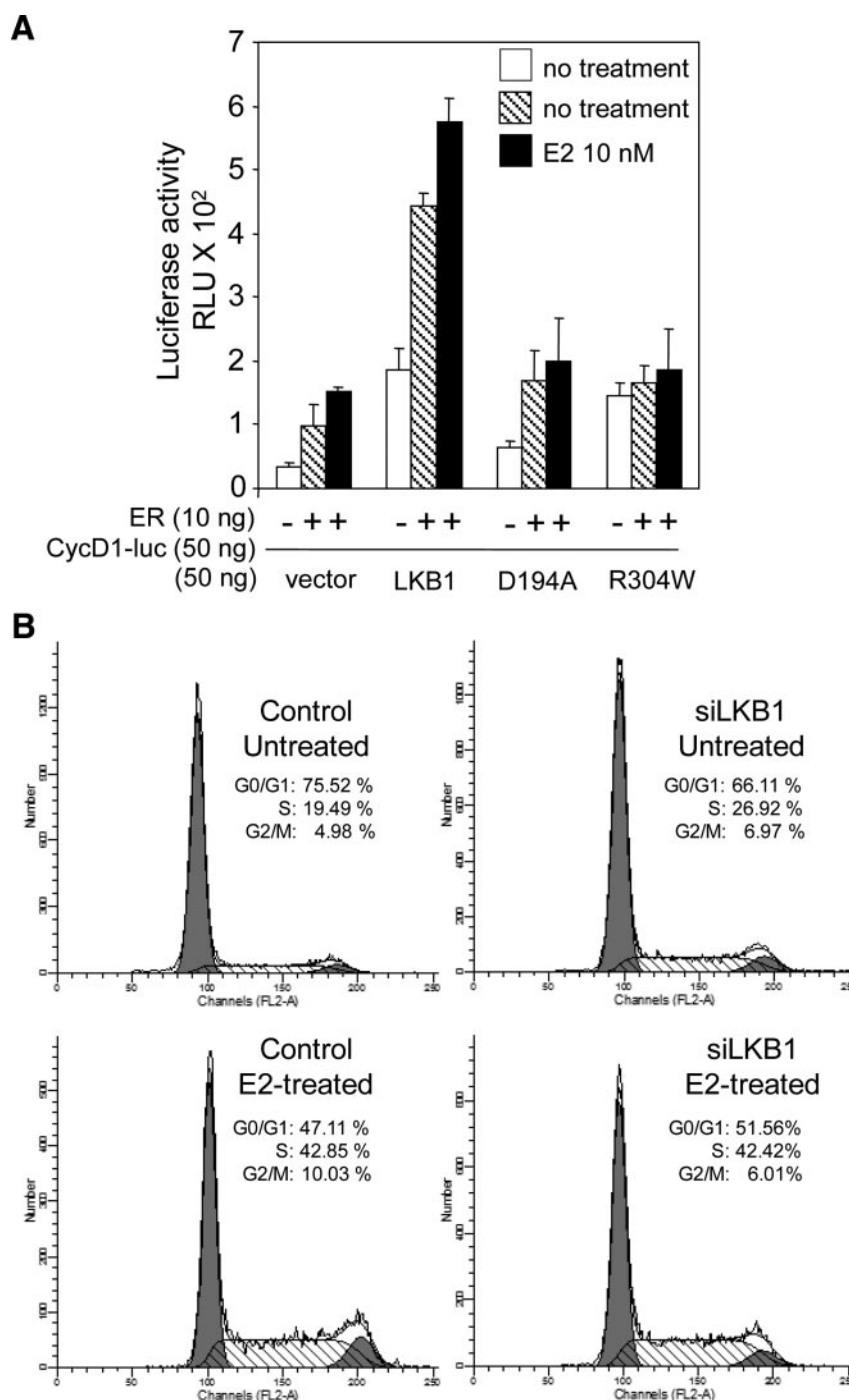


Figure 8. LKB1 enhances nonclassical ER α signaling. (A) G361 cells were transfected with LKB1, D194A and R304W, ER α , cyclin D1-luc and pRL-tk, expression plasmids, or vector. Cells were left untreated or treated with E2 (10 nM) 24 h before harvesting for reporter assay. Results are representative of three experiments in triplicate. Data are mean \pm SD. (B) Cells were transfected with siLKB1#2 duplex and GFP-empty vector, left untreated or treated with E2 (100 nM) for 24 h, followed by FACS analysis. DNA profiles for PI/GFP-positive cells were recorded and analyzed. Data as shown are representative of three experiments.

the percentage of cells in G0/G1 phase (Figure 8B), agreeing with previous work that shows the introduction of LKB1 into LKB1 null G361 cells arrests cells in G1 phase of cell cycle (Tiainen *et al.*, 1999). Compared with control E2-treated cells, we did not observe a difference in the percentage of cells in S phase from siLKB1 cells. Similar cell cycle profiles were observed for siLKB1#1 and #3 (data not shown).

LKB1 Is Recruited to the Promoters of ER-regulated Genes

Because LKB1 enhances ER α transcriptional activity, we examined whether endogenous LKB1 interacts with the pro-

motors of ER α activated genes pS2, cathepsin D (Augereau *et al.*, 1994), and c-myc (Dubik and Shiu, 1988) (Figure 9A). We conducted ChIP in MCF7 cells that were either left untreated or were treated with E2. When endogenous LKB1 was immunoprecipitated using anti-LKB1 antibody, we observed recruitment of LKB1 to the pS2, cathepsin D, and c-myc promoters in both untreated and E2-treated cells, compared with corresponding control regions (cr) (Figure 9B). To determine whether catalytic-deficient mutants were recruited to these promoters, G361 cells were transfected with ER α , LKB1, and R304W expression plasmids or vector control followed by ChIP. Both LKB1 and R304W were

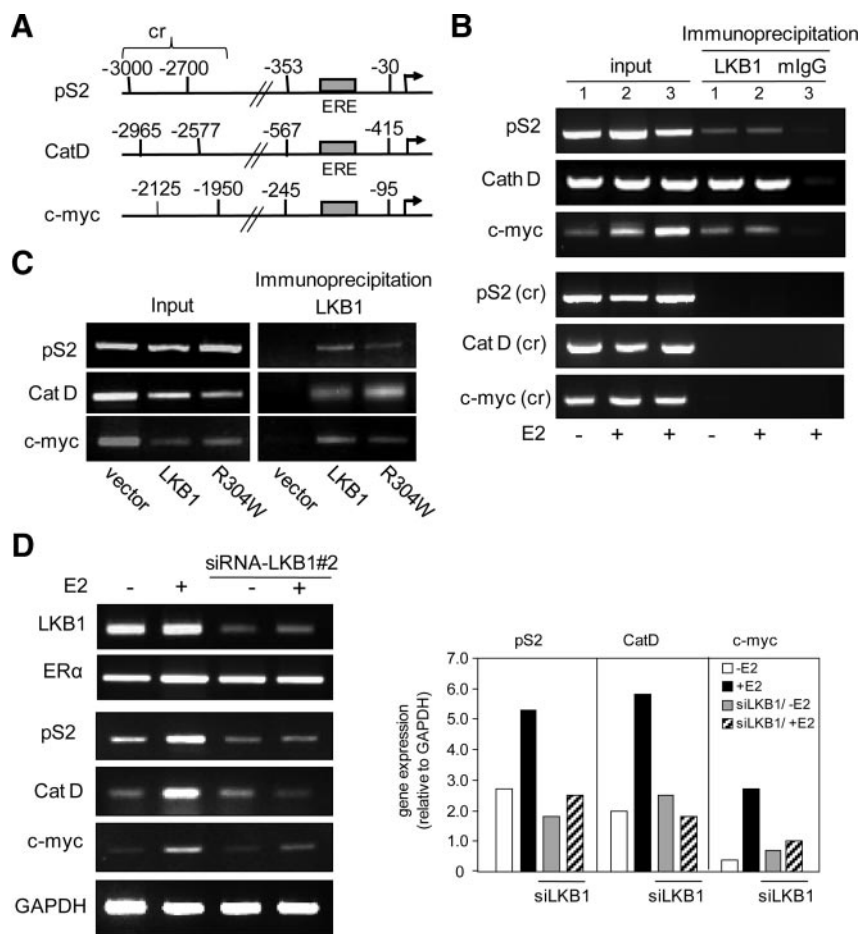


Figure 9. Recruitment of LKB1 to promoters. (A) Schematic representation of pS2, cathepsin D (Cat D), and c-myc promoters and control regions (cr). (B) ChIP analysis using anti-LKB1 antibody showing recruitment of LKB1 to the promoters of pS2, Cat D, and c-myc in the presence and absence of E2 treatment (100 nM) in MCF-7 cells. Control region (cr) of pS2 (–3000 to –2700), Cat D (–2965 to –2577), and c-Myc (–2125 to –1950) (C) G361 cells lacking endogenous LKB1 and ER α expression were transfected with ER α , plus LKB1 or R304W expression plasmids followed by ChIP using anti-LKB1 antibody. (D) MCF7 cells were transfected with siLKB1#2 duplex, left untreated or treated with E2 (100 nM). Expression of ER α -responsive genes pS2, Cat D, and c-myc was determined by RT-PCR (left) and expressed relative to GAPDH as determined by densitometry (right). Results are representative of three separate experiments.

recruited to all three gene promoters, compared with the vector control (Figure 9C).

Because we observed recruitment of LKB1 to the promoter of ER α -responsive genes, we asked whether LKB1 altered the expression of pS2, cathepsin D, and c-myc. To test this, we depleted the expression of LKB1 in MCF7 cells by siRNA-LKB1#2 (Figure 2F), followed by RT-PCR (Supplemental Table 1). In cells depleted of ~75% of LKB1 (Figure 9D, left), we observed a >50% reduction in pS2, cathepsin D, and c-myc expression in response to E2 treatment, compared with cells not depleted of LKB1 expression (Figure 9D, right; RT-PCR expressed relative to glyceraldehyde-3-phosphate dehydrogenase [GAPDH]).

DISCUSSION

We describe a novel function for LKB1, a regulator of ER α signaling. We demonstrate that LKB1 binds to ER α in a ligand-independent manner (Figure 1A) in the nuclear compartment of the cell (Figure 1, B and C). A function of this interaction is that LKB1 enhances ER α -mediated transactivation in a ligand-dependent manner (Figure 2, A–C), because abrogation of LKB1 expression by siRNA resulted in a suppression of ER α activity (Figure 2F). We confirmed by mRNA and protein analysis that the introduction of LKB1 did not alter the overall expression of ER α (Figure 3); thus, the observed enhancement in ER α activity was not due to increased ER α levels but due to the interaction of LKB1 with ER α . Equally important, the introduction of ER α into cells

did not alter the overall expression of LKB1 (Figure 3). Our discovery is consistent with a previous report (Dutertre and Smith, 2003) that demonstrates coactivator proteins such as p160 interacting with ER α independently of ligand binding; however, p160-enhanced ER α function was dependent of ligand (Dutertre and Smith, 2003). Furthermore, we show that LKB1 did not alter the transcriptional activity of ER β , AR, or GR (Figure 5), suggesting that LKB1 is selective for ER α .

Because coregulator proteins function to alter ER α activity, we investigated the effect of LKB1 in the presence of known ER α coregulatory proteins. In the presence of ER α coactivator p300 (Hanstein *et al.*, 1996), LKB1 synergizes ER α -mediated transactivation of ERE-luc (Figure 4A). Synergy between ER α coactivator proteins has been described, such as GRIP1, CARM1, and p300 (Chen *et al.*, 2000), and between SRC1 and CBP (Smith *et al.*, 1996). In the presence of ER α corepressor proteins p53 and BRCA1 (Yu *et al.*, 1997; Fan *et al.*, 1999), the enhanced transactivation we observe with LKB1 and ER α alone is antagonized when either of the ER α corepressor proteins is introduced, suggesting that these corepressor proteins compete with LKB1 for ER α (Figure 4, B and C). Previously, our laboratory discovered a functional interaction between LKB1 and Brg1, whereby the interaction enhanced Brg1 ATPase activity, necessary for chromatin remodeling and therefore gene transactivation (Marignani *et al.*, 2001). Furthermore, LKB1 induces p21^{WAF/CIP1} (Tiainen *et al.*, 2002), which in turn enhances ER α activity (Fritah *et al.*, 2005). Coregulator proteins function by participating in chromatin remodeling or assist in the

assembly of other coregulatory proteins. Because LKB1 exhibits both these functions, it is likely that LKB1 may be part of coactivator complexes with functionally diverse roles that are dependent on the availability of other coregulatory proteins (Szapary *et al.*, 1999).

Catalytically deficient LKB1 mutants D194A and R304W failed to enhance ER α -mediated transactivation, indicating that LKB1 catalytic activity is necessary for this function (Figure 6). Thus, we tested whether LKB1 phosphorylated ER α because previous reports suggest phosphorylation of ER α enhances transcription activity by several mechanisms (Weigel and Moore, 2007). From kinase assays, we determined that LKB1 does not phosphorylate ER α (Figure 7B). Others have reported that alteration in the phosphorylation state of coregulatory proteins modulate nuclear receptor activity (Frigo *et al.*, 2006). It is conceivable that LKB1 phosphorylates an ER α coregulator protein, thereby enhancing ER α activity. A previous study suggested that a novel nuclear protein kinase that interacts with AR enhances AR activity by phosphorylating coregulatory proteins and not AR (Moilanen *et al.*, 1998). We demonstrate that LKB1 does not phosphorylate ER α coregulatory proteins p300 and BRCA1, compared with Brg1 (negative control) (Marignani *et al.*, 2001) and AMPK (positive control) (Lizcano *et al.*, 2004; Sakamoto *et al.*, 2004) (Figure 7C), indicating that a plausible substrate of LKB1 may be a coregulatory protein of hormone receptor signaling other than the coregulatory proteins we tested in our system.

In addition to the classical pathway of gene regulation, ER α regulates genes such as cyclin D1 by an alternative mechanism (Sabbah *et al.*, 1999; Castro-Rivera *et al.*, 2001), that is, the nonclassical pathway (Nilsson *et al.*, 2001; Sommer and Fuqua, 2001), whereby ER α associates with transcription factors that bind to DNA. Increases in cyclin D1 expression leads to enhanced cellular proliferation, promoting atypical lobular-alveolar development and contributing to the formation of mammary adenocarcinomas (Wang *et al.*, 1994). Clinical studies show >50% of breast tumors express elevated levels of cyclin D1 (Barnes and Gillett, 1998) and display elevated levels of ER α activity (Shoker *et al.*, 2001). Previously, we showed recruitment of LKB1 to the promoter of cyclin D1 in cells lacking the expression of cyclin D1 negative regulators p53 and p21^{WAF1/CIP1} (Scott *et al.*, 2007). Our current results demonstrate that LKB1 enhances ER α -mediated transactivation of the cyclin D1 promoter in G361 cells (Figure 8A). Hence, it is possible that recruitment of LKB1 to the promoter of cyclin D1 facilitates the protein-DNA or protein-protein interactions necessary for ER α activity. Previous work by others show that ER α associates with the p160-CBP coactivators, which are in turn recruited by jun/fos to the AP-1 site (Kushner *et al.*, 2000). Perhaps LKB1 may function in a similar manner by recruiting ER α to the cyclin D1 promoter, along with additional coactivator proteins. Interestingly, ablation of LKB1 expression in MCF7 cells by siRNA resulted in an increase in the percentage of cells transitions to S phase of cell cycle compared with control untreated cells, as measure by flow cytometry (Figure 8B). In response to E2 treatment, however, the percentage of cells in S phase of cell cycle did not differ between control and siLKB1 cells (Figure 8B). These data are supported by recent studies conducted in three-dimensional acini cultures of 4-OHT-inducible MCF10A-MycERtm cells. In this study, the reduction of LKB1 expression by short hairpin RNA led to an increase in c-myc-mediated cell proliferation as measured by Ki-67 labeling of acini (Partanen *et al.*, 2007). The authors of this study correlate reduced LKB1 expression with disruption of quiescent 4-OHT-induced MCF10A-MycERtm cell architec-

ture for up to 10 d, confirming cell polarity plays a protective role against apoptosis (Partanen *et al.*, 2007). Together, these data suggest that the tumor suppressor activity of LKB1 is likely distinct from its hormone receptor coactivator function and further highlight the multitasking and therefore multifunctional activities of LKB1 (Marignani, 2005).

We show that LKB1 is recruited to the promoters of ER α -regulated genes independently of E2 treatment, specifically cathepsin D, pS2/TFF1, and c-Myc (Shang and Brown, 2002; Métivier *et al.*, 2003) (Figure 9, B and C). Furthermore, we confirmed by siRNA that LKB1 contributes to the expression of cathepsin D, pS2/TFF1, and c-Myc because abrogation of LKB1 expression attenuated expression of pS2 in the absence of ligand and expression of all three ER α targets—pS2, cathepsin D, and c-myc—in the presence of E2 ligand (Figure 9D). In addition to wild-type LKB1, ChIP assays confirm the recruitment of catalytic deficient mutant R304W to the promoters of cathepsin D, pS2/TFF1, and c-Myc, supporting our position that a plausible substrate of LKB1 may be a coregulatory protein of hormone receptor signaling. Overall, these results suggest that LKB1 through its association with ER α is involved in the expression of downstream targets of ER α and that LKB1 tumor suppressor function, that is, preventing cells from transitioning from G1 phase to S phase of cell cycle (Tiainen *et al.*, 1999) is distinct from its ER α coactivator function.

Our discovery defines a new function for the tumor suppressor serine threonine kinase LKB1, as a coactivator of ER α . It is paradoxical that LKB1, a tumor suppressor, enhances ER α activity, which would likely contribute to breast cancer risk. This said, there are reports in the literature for tumor suppressor proteins functioning as nuclear receptor coactivators. Briefly, despite being the archetypical tumor suppressor, retinoblastoma (Rb) also has the distinction of being a coactivator of nuclear hormone receptors ER α , AR, progesterone receptor (PR), and GR (Singh *et al.*, 1995; Lu and Danielsen, 1998; Yeh *et al.*, 1998; Batsche *et al.*, 2005). Interestingly, Rb is the first example of a tumor suppressor to function in a role as an activator of nuclear hormone receptor signaling. The regulation of the GR by Rb is mediated through Rb association with SWI/SNF chromatin remodeling complex, specifically Brg1 (Singh *et al.*, 1995), whereas up-regulation of AR-mediated transcription by Rb is independent of Rb-Brg1 interaction (Lu and Danielsen, 1998). A second tumor suppressor, p21^{WAF1/CIP1}, was found to bind to ER α , enhancing ER α -dependent transcription of target genes by forming a ternary complex composed of p21^{WAF1/CIP1}, CREB-binding protein, and ER α (Fritah *et al.*, 2005). The tumor suppressor tuberous sclerosis 2 (TSC2) has also been shown to function as a coactivator of nuclear hormone receptors, specifically the retinoid X receptors, peroxisome proliferator receptor (PPAR) α , and vitamin D receptor (VDR) (Henry *et al.*, 1998). In these studies, TSC2 weakly associated with the receptors and up-regulated both PPAR α - and VDR-mediated transactivation. However, TSC2 functioned as a corepressor of GR-mediated transcription (Henry *et al.*, 1998). Finally, in addition to binding to LKB1 (Marignani *et al.*, 2001), Brg1 is reported to bind to nuclear hormone receptors including the ER α , AR, GR, and PR (Trotter and Archer, 2008). Interestingly, when bound to ER α , AR, and PR, Brg1 functions as a coactivator protein and when bound to GR, Brg1 functions as a corepressor protein for this receptor (Trotter and Archer, 2008).

In summary, we demonstrate for the first time a functional link between LKB1 and the hormone receptor ER α . Our discovery now places LKB1 in a coactivator role for ER α signaling, broadening the scientific scope of this tumor sup-

pressor kinase and laying the groundwork for further investigations into the role of LKB1 in breast disease.

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