J. Biochem. 2009;145(3)331–343
doi:10.1093/jb/mvn177

Repression of Estrogen Receptor alpha by CDK11^{p58} Through Promoting its Ubiquitin–Proteasome Degradation

Yanlin Wang, Hongliang Zong, Yayun Chi, Yi Hong, Yanzhong Yang, Weiying Zou, Xiaojing Yun and Jianxin Gu*

Gene Research Center, Shanghai Medical College and Institutes of Biomedical, Shanghai 200032, People's Republic of China

Received June 21, 2008; accepted December 2, 2008; published online January 2, 2009

Estrogen receptor α (ERα) is a ligand-dependent transcription factor that mediates physiological responses to 17β-estradiol (E_2). These responses of cells to estrogen are regulated in part by degradation of ERα. In this report, we found that CDK11^{p58} repressed ERα transcriptional activity. And we further demonstrated that ERα protein level was down-regulated by CDK11^{p58} in mammalian cells in a ligand independent manner. This effect could be abrogated by treatment with proteasome inhibitor MG132. Our results indicated that the ubiquitin/proteasome-mediated degradation of ERα was promoted by CDK11^{p58}. Furthermore, the interaction between ERα and CDK11^{p58} was detected. This interaction was necessary for the polyubiquitination and degradation of ERα. On the contrary, the other isoform of CDK11, CDK11^{p110} and the kinase dead mutant of CDK11^{p58}, D224N, did not associate with ERα and failed to reduce the ERα protein level. These data identified a new negative regulatory protein of ERα and provided a new pathway by which CDK11^{p58} negatively regulated cells.

Key words: CDK11p58, degradation, estrogen receptor α, steroid, ubiquitin–proteasome.

Abbreviations: ERa, estrogen receptor a; E2, 17b-estradiol; CDK, Cyclin-dependent kinase; AR, androgen receptor; CCND3, cyclin D3.

The estrogen receptor α (ERα) belongs to nuclear receptors (NR), a superfamily of structurally conserved transcription factors, which enhance the transcription of specific genes upon hormone binding (*1*). Upon 17β-estradiol (E_2) binding, the receptor interacts specifically with the estrogen response element (ERE), which is usually located upstream of the promoter and stimulates transcription of a variety of E_2-responsive genes (*2*). One important target of E_2 in breast cancer cells is the proto-oncogene c-myc (*3*). Protein–protein interaction screens have revealed a large group of proteins classified as co-activators, negative co-regulators and co-repressors, which function through binding to ERα (*4*). These proteins have an important role in ERα action and provide functional and physical links between ERα and the transcription apparatus. Some co-activators, such as SRC-1 (steroid receptor co-activator-1), TIF2 (transcriptional intermediary factor-2) and RAC3 (receptor-associated co-activator 3), have been validated to positively regulate ERα transcriptional activity. However, the transcriptional activity of ERα could also be negatively regulated by some co-repressors, such as SHARP (SMRT/HDAC-associated repressor protein), BRCA1 (Breast Cancer 1), HDACs (histone deacetylases) (*5*) But the roles of some other interacting proteins remain to be determined.

The ubiquitin–proteasome pathway is the major system in the eukaryotic cells for the selective degradation of short-lived regulatory proteins (*6*, *7*). Several members of the nuclear receptor superfamily are substrates for the ubiquitin–proteasome pathway (*8–13*), and ERα was first being identified (*14–16*). A common feature of proteasome-mediated protein degradation is the covalent attachment of ubiquitin, a highly conserved 8.6 kDa protein, to lysine residues of proteins targeted for degradation followed by the formation of polyubiquitin chains attached covalently to the targeted protein. Ubiquitinated ERα is recognized and degraded by the multisubunit protease complex, the 26S proteasome (*17*, *18*). The ubiquitin–proteasome pathway plays an important role in various cellular processes such as cell cycle regulation, signal transduction, differentiation and degradation of tumour suppressors (*17–20*). ER is degraded in a hormone-dependent manner and the proteasome inhibitor, MG132, promotes the *in vivo* accumulation of ER and blocks hormone-induced receptor degradation (*16*). The ubiquitination of ERα is involved in these processes and regulated by many different proteins (*21*).

Cyclin-dependent kinases (CDKs) are established to regulate the cell cycle in most eukaryotic cells (*22*). It was reported that estrogen-induced activation of CDK4 and CDK2 during G1-S phase progression (*23*). And inhibition of CDK2 and CDK4 modulated ERα-mediated transcriptional activation (*24*, *25*). CDK11, which contains a conserved PITSLRE motif within the protein kinase domain, has two major isoforms: CDK11^{p110} and

*To whom correspondence should be addressed. Tel: +86-21-54237704, Fax: +86-21-64164489, E-mail: jxgu@shmu.edu.cn

$CDK11^{p58}$. $CDK11^{p58}$ is produced by cell cycle-dependent translation initiation from internal ribosome entry sites (IRES) of the same transcript as $CDK11^{p110}$ (*26*, *27*). The $CDK11^{p110}$ isoform can be detected in all phases of the cell cycle, whereas the $CDK11^{p58}$ is mainly expressed in G2/M phase (*26*). It has been well documented that $CDK11^{p58}$ was closely related to cell apoptosis in a kinase-dependent manner (*28*), and it was reported by our group that cyclin D3/$CDK11^{p58}$ complex was involved in the negative regulation of androgen receptor (AR)-mediated transactivation (*29*). Thereinto, cyclin D3 (CCND3) was vital for the kinase activity of $CDK11^{p58}$ (*30*). Recent studies suggest that $CDK11^{p58}$ promotes centrosome maturation and bipolar spindle formation (*31*). In this article, we reported that $CDK11^{p58}$ also repressed ERα-mediated transcriptional activity and we further demonstrated that $CDK11^{p58}$ negatively regulates ERα protein level through ubiquitin/proteasome-mediated degradation. Moreover, the interaction between ERα and $CDK11^{p58}$ was required for the polyubiquitination and degradation of ERα. However, $CDK11^{p110}$ and the kinase dead mutant of $CDK11^{p58}$, D224N, did not associate with ERα and failed to reduce the ERα protein level. Our results provide a new mode by which $CDK11^{p58}$ represses ERα function and negatively regulates cell growth.

MATERIALS AND METHODS

Materials—Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma. LipofectAMINE™ 2000 Reagent, mouse monoclonal anti-myc antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-HA monoclonal antibody, PVDF membrane, protein G agarose, leupeptin, aprotinin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Roche. Rabbit polyclonal anti-PITSLRE, mouse monoclonal anti-ERα(D12), anti-ubiquitin (P4D1), anti-c-myc (A-14), anti-GAPDH, anti-GFP antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse IgG secondary antibodies were from Santa Cruz Biotechnology. The enhanced chemiluminescence (ECL) assay kit, [^{35}S]-Methionine and glutathione Sepharose 4B beads were purchased from Amersham Biosciences. Dual-luciferase reporter assay system, control pRL plasmid, TNT coupled reticulocyte lysate system was bought from Promega. The β-glycerolphosphate disodium and E_2 was purchased from Fluka.

Cell Culture, Transfections and Synchronization—The human MCF-7, T47D breast cancer cells and COS-1 cells were obtained from the Institute of Cell Biology Academic Sinica. COS-1 and T47D cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 50 μg/ml of streptomycin at 37°C under 5% CO_2 in humidified air. MCF-7 cells were cultured in DMEM containing 10% FCS, 10 μg/ml of insulin, 100 U/ml of penicillin and 50 μg/ml of streptomycin at 37°C under 5% CO_2 in humidified air. Cell transfection was performed with LipofectAMINE™ 2000 reagent according to the manufacture's instructions. To block cells in G2/M phase, cells were treated with 50 nM nocodazole for 20 h then washed twice and harvested.

Plasmids Construction and RNA Interference—ERα expression plasmid, pRL plasmid, 3×ERE reporter and recombinant pM containing 5×GAL4 reporter plasmids were kindly provided by Dr Hongliang Zong. $CDK11^{p58}$, $CDK11^{p110}$ and cyclin D3 expression plasmids were constructed as described previously (*29*). $CDK11^{p58}$D224N was generated by site-directed mutagenesis using MutanBEST kit (Takara) with the primers described previously (*28*). The siRNA designed for CDK11 is 5′-CCGGCAUCCUCAAGGUGGGUGACUU-3′, and 5′-AA GTCACCCACCTTGAGGATGCCGG-3′ (Invitrogen).

Dual-Luciferase Reporter Gene Assays—COS-1 or MCF-7 cells (3×10^4 cells per well in 24-well plates) was incubated in 5% FCS supplemented DMEM for 24 h prior to transfection. Cells were co-transfected with ERα (20 ng), an estrogen-responsive 3×ERE-luciferase reporter construct (200 ng), a control Renilla luciferase plasmid (pRL, 2 ng) and $CDK11^{p58}$ with indicated amounts using LipofectAMINE™ 2000 according to the manufacturer's instructions. The total plasmids were balanced up to 500 ng with pcDNA3 empty vector. At 24 h after transfection, the culture medium was replaced with DMEM containing 5% FCS and supplemented with EtOH or 100 nM E_2. After another 24 h, cells were lysed using Passive Lysis Buffer (Promega) according to the manufacturer's specifications and assayed immediately for reporter and control gene activities with the Dual-Luciferase Reporter Gene Assay (Promega) using a Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany).

GST Pull-down Experiments—GST-fused $CDK11^{p58}$ and ERα *in vitro* translated and ^{35}S labelled methionine with the TNT coupled reticulocyte lysate system. Labelled ERα and GST-fused $CDK11^{p58}$ were incubated together with glutathione-Sepharose beads in the binding buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 10 mM NaF, 1% Nonidet P-40, 1 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF) at 4°C for 4 h with gentle rotation. The beads were washed three times with the same buffer. Bound proteins were subjected to 10% SDS–PAGE. The gel was then dried and visualized with phosphoimaging (Fujifilm).

GST-fused $CDK11^{p58}$ was expressed in *Escherichia coli* and COS-1 cells were transfected with ERα or empty vector. Approximately 24 h after transfection, cells were cultured in the medium containing 5% FCS with 100 nM E_2 or EtOH. After another 24 h, cells were washed with ice-cold PBS and solubilized with 0.5 ml of lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF]. Lysate and GST-fused $CDK11^{p58}$ were incubated together with glutathione–Sepharose beads. GST protein was used as a negative control in the assays. The beads were then washed and boiled. The supernatants were subjected to 10% SDS–PAGE and blotted with anti-ERα and anti-PITSLRE antibodies.

Immunoprecipitation—Approximately 24 h after transfection, cells were cultured in the medium containing 10% FBS. After another 24 h, cells were washed with ice-cold PBS and solubilized with 0.5 ml of CoIP buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM EGTA, 15 mM $MgCl_2$, 60 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1 mM NaF, 0.1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF]. Each IP was carried out using 2 μg of antibody. The precipitated proteins were collected using proteinG agarose (Santa Cruz). After low-speed centrifugation to remove the supernatants, the agarose was washed with CoIP buffer, boiled in SDS sample buffer and subjected to SDS–PAGE and western blotting.

Reverse Transcriptase–Polymerase Chain Reaction—RNA was harvested using the TRIzol reagent (Invitrogen). Total RNA (1 μg) extracted was used as a template for cDNA synthesis, with a TaKaRa RNA PCR Kit and specific primers (ERα forward 5′-AACT CGAGATGGCTGGAGACATGAGAG-3′, reverse 5′- AAGA ATTCTCAGACCGTGGCAGGGAAA-3′. β-Actin forward: 5′-ATGGGTCAGAAGGATTCCTAT-3′, reverse: 5′-GCGCT CGGTGAGGATCTTCAT-3′). The primers of C3 and PS2 were constructed as described in reference (*32*). Amplification was carried out for 30 cycles under saturation, each at 94°C, 45 s; 60°C, 45 s; 72°C, 1 min in a 50 μl reaction mixture containing 2 μl each cDNA, 0.2 μM each primer, 0.2 mM dNTP and 2.5 U of Taq DNA polymerase. After amplification, 10 μl of each reaction mixture was analysed by 1% agarose gel electrophoresis, and the bands were then visualized by ethidium bromide staining.

Ubiquitination Assay—COS-1 cells transfected myc-ERα and other plasmids were pre-treated with 10 μM MG132 (Calbiochem, La Jolla, CA, USA) for 6 h to block proteasome activity. Following treatment, cells were lysed in buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 50 mM NaF, 1% NP-40 and 1% SDS. Lysates were boiled immediately for 10 min followed by centrifugation. Immunoprecipitations were carried out using with anti-myc antibody or control IgG and proteinG agarose overnight. Then agarose were washed three times in lysis buffer and boiled in SDS sample buffer, and subjected to SDS–PAGE and western blotting with anti-ubiquitin antibody or anti-HA antibody.

RESULTS

CDK11p^{58} Represses ERα-dependent Transcription—Our previous investigation showed that CDK11^{p58} was involved in the negative regulation of AR-mediated transactivation (*29*). To investigate whether CDK11^{p58} had the ability of directly regulating ERα-mediated transcription activity, we took advantage of a dual-luciferase assay system using the 3×ERE-LUC reporter plasmid containing multiple estrogen-responsive elements and the internal control plasmid pRL encoding a CMV promoter-regulated *Renilla* luciferase together. ERα-positive MCF-7 human breast cancer cells were transfected with 3×ERE-LUC, pRL, pcDNA3.0 or CDK11^{p58} expression plasmids. Figure 1A demonstrated that CDK11^{p58} repressed ERα-mediated transcription in a dose-dependent manner in MCF-7 cells. Estradiol-induced ERα transactivation was inhibited by 39.4% when 50 ng of CDK11^{p58} was transfected. In the presence of 100 ng of CDK11^{p58} expression plasmids, ERα activity was down-regulated by 48.3%.

Considering that CDK11^{p58} represses AR-mediated transactivation as well (*29*), we analysed an unrelated transcription factor Gal4 by using the similar artificial response element 5×GAl4-LUC to investigate whether CDK11^{p58} generally represses transcription. COS-1 cells were transfected with ERα, 3×ERE-LUC, pRL, vector or CDK11^{p58} expression plasmids. The dual-luciferase reporter assay showed that exogenous ERα-mediated transactivation was repressed by CDK11^{p58} as well. In order to investigate whether CDK11^{p58} has the same effect on different targets, two ERα transactive function domain AF1 and AF2 were fused in-frame downstream of the GAL4 DBD. The 5×GAL4-LUC plasmid contains a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the *Photinus pyralis* luciferase gene was employed as the reporter plasmid for GAL4 DBD-fused transactivators. Correspondingly, GAL4-AR-LBD was employed to compare with ERα domains. In the three GAL4 DBD-fused transactivators examined, ERα AF1 and AF2 mediated transactivation were repressed in response to CDK11^{p58} over-expression. And the transcription mediated by AR LBD was not affected by CDK11^{p58}. Thus, CDK11^{p58} is not a general repressor (Fig. 1B). Furthermore, CDK11 contains two major isoforms: CDK11^{p110} and CDK11^{p58}. To investigate whether CDK11^{p110} had the same effect with CDK11^{p58} on ERα-mediated transcription, the dual-luciferase reporter assay was also used. Surprisingly, different from CDK11^{p58}, CDK11^{p110} facilitated ERα-dependent transcription significantly (Fig. 1C).

CDK11p^{58} Negatively Regulates ERα Protein Level—To investigate the mechanism by which CDK11^{p58} represses ERα transactivation, we detected the ERα protein level. ERα, GFP and CDK11^{p58} were transfected in COS-1 cells and detected by western blot (Fig. 2A). As a result, ERα protein level was negatively regulated by CDK11^{p58} in a dose-dependent manner. Similar tendencies were observed in the presence of estrogen and the ERα protein had a shorter half-life in the presence of estrogen. This suggests that ERα was down-regulated in the presence of CDK11^{p58}.

Steroid receptor family proteins, including ER, AR, progestogen receptor (PR) and so on, have some similar characters. For example, many of them are substrates for the ubiquitin–proteasome pathway (*8–13*). Thus, we wondered whether CDK11^{p58} has the same effect on AR and other steroid receptor family proteins. COS-1 cells were transfected with AR and different amounts of CDK11^{p58}. Cell lysates were assessed by western blot with an anti-AR antibody. Compared with ERα, the protein level of AR was stable regardless of CDK11^{p58} no matter in the presence or absence of dihydrotestosterone (DHT). And AR was more stable in the presence of DHT as it is reported (*33*). ERα and AR seem to have opposite behaviour. This result gave the evidence that CDK11^{p58} specifically down-regulated ERα protein level.

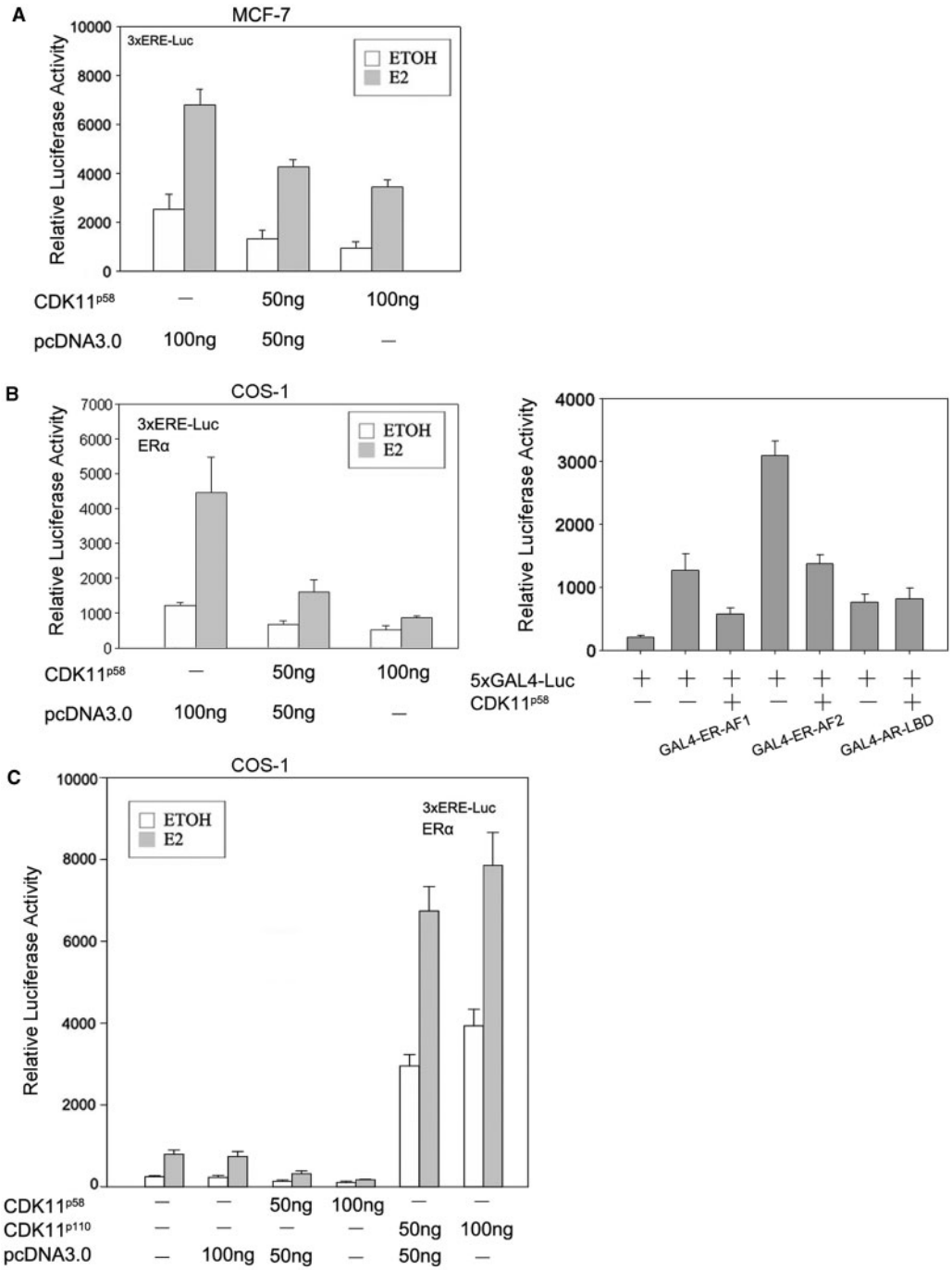

Fig. 1. **CDK11^{p58} represses ERα-dependent transcription in multiple human cell lines.** (A) Two nanograms of PRL and 200 ng of 3×ERE-LUC were transfected with indicated amount of CDK11^{p58} or mock vector pcDNA3.0 into MCF-7 breast cancer cells. The cells were treated with 100 nM ethanol (EtOH) or 100 nM E_2 at 24 h after transfection, and harvested after another 24 h. Luciferase activity was measured and normalized to Renilla luciferase activity. (B) A total of 20 ng of ERα, 2 ng of PRL and 200 ng of 3×ERE-LUC were transfected with indicated amount of CDK11^{p58} or vector pcDNA3.0 into COS-1 cells. The cells were treated with 100 nM of EtOH or 100 nM of E_2 after 24 h, and were harvested after another 24 h (left panel). A 200 ng 5×GAL4-LUC and 20 ng GAL4-ER-AF1, GAL4-ER-AF2 or GAL4-AR-LBD were co-transfected with 50 ng CDK11^{p58} or vector pcDNA3.0 into COS-1 cells. At 24 h post-transfection, cells were treated with 100 nM E_2 or 10 nM DHT for another 24 h. Luciferase activity was then detected (right panel). Luciferase activity was assayed as described above. (C) Indicated amount of CDK11^{p58}, CDK11^{p110} or vector were transfected into COS-1 cells. ERα, PRL, 3×ERE-LUC were transfected and luciferase activities were assayed as described. Data shown are the mean and standard error of four independent experiments.

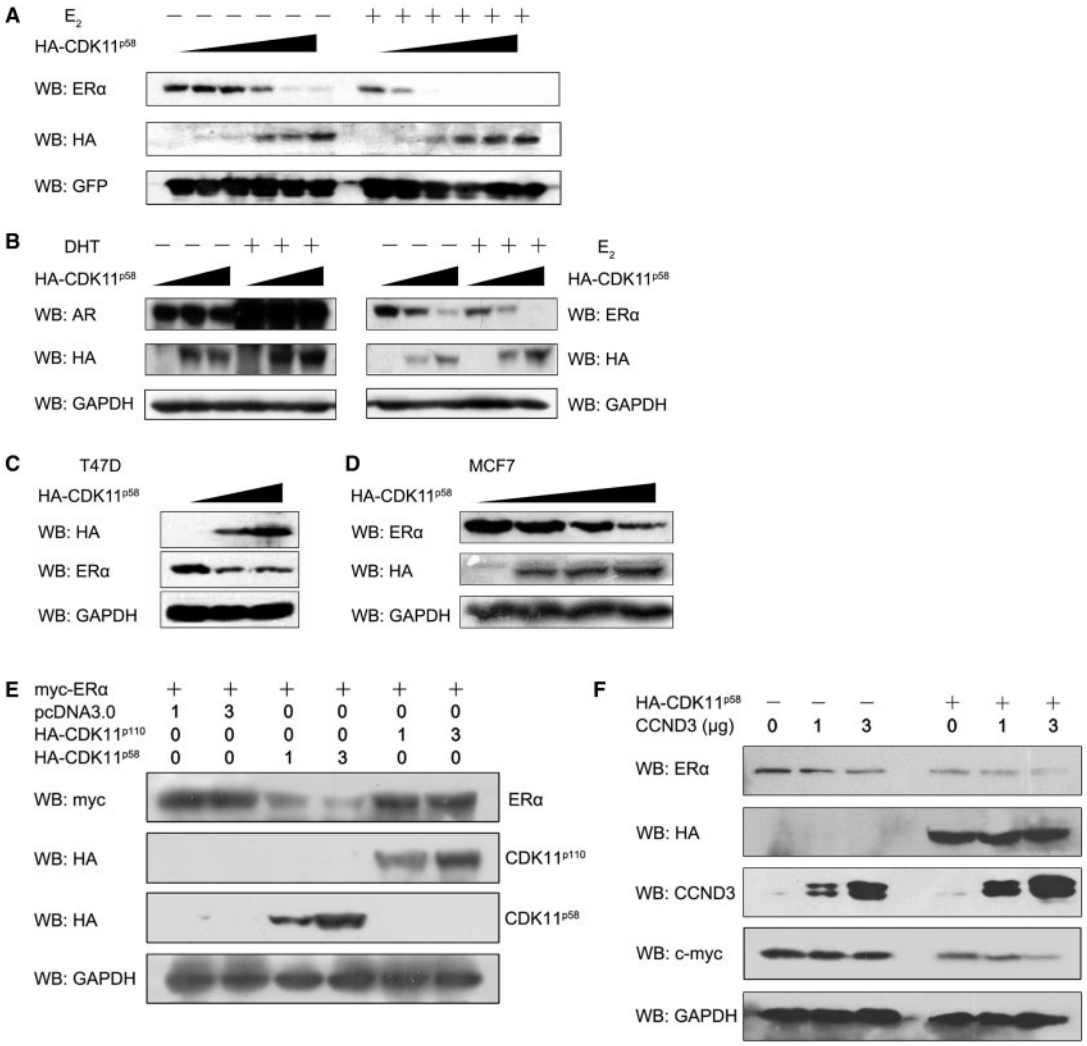

Fig. 2. **CDK11^{p58} negatively regulates ERα protein level.** (A) COS-1 cells were transfected with ERα and GFP, in combination with different dose of HA-CDK11^{p58} (0, 0.25, 0.5, 1, 2, 4 μg) and vector to fill dose up. The cells were treated with 100 nM EtOH or 100 nM E_2 at 24 h after transfection and harvested after another 24 h. The lysates were quantified and blotted with anti-ERα, anti-HA and anti-GFP antibodies. (B) COS-1 cells were transfected with AR or ERα in combination with different dose of HA-CDK11^{p58} (0, 1, 3 μg) and vector to fill dose up. The cells were treated with ethanol (EtOH) or 10nM DHT or 100 nM E_2 at 24 h after transfection, and harvested at 24 h after hormone treatment. The lysates were quantified and blotted with anti-AR, anti-ERα, anti-HA and anti-GAPDH antibodies. (C) T47D breast cancer cells were transfected with different dose of HA-CDK11^{p58} (0, 1, 3 μg) or vector to fill dose up. The cells were harvested at 48 h after transfection. Lysates were quantified and blotted with anti-HA, anti-ERα, and anti-GAPDH antibodies. (D) MCF-7 breast cancer cells were transfected with different dose of HA-CDK11^{p58} (0, 0.5, 1, 2 μg) or vector to fill dose up. The cells were assayed as described in (C). (E) COS-1 cells were transfected with myc-ERα and different dose of vector pcDNA3.0, HA-CDK11^{p58}, HA-CDK11^{p110} (1, 3 μg) and harvested at 48 h after transfection. The lysates were quantified and blotted with anti-myc, anti-HA and anti-GAPDH antibodies. (F) MCF-7 breast cancer cells were transfected with different dose of cyclin D3 (0, 1, 3 μg) or vector to fill dose up with 1 μg of HA-CDK11^{p58} or without. The cells were harvested at 48 h after transfection. Lysates were quantified and blotted with anti-ERα, anti-HA, anti-CCND3, anti-c-myc and anti-GAPDH antibodies. (G) MCF-7 breast cancer cells were transfected with vector, scrambled siRNA and siCDK11. 28 h after transfection, cells were treated with 50 nM nocodazole for 20 h and washed twice before harvest. Lysates were quantified and blotted with anti-ERα, anti-PITSLRE and anti-GAPDH antibodies (left panel). Relative ERα protein levels are shown in corresponding histogram (lower panel). Two nanograms of PRL and 200 ng of 3×ERE-LUC were cotransfected with scrambled siRNA or siCDK11 into MCF-7 breast cancer cells. The cells were treated with 100 nM EtOH or 100 nM E_2 at 24 h after transfection, and harvested after another 24 h. Luciferase activity was measured and normalized to Renilla luciferase activity. Data shown are the mean and standard error of four independent experiments (right panel).

As reported earlier (*34*, *35*), estrogen reduces ERα protein level by promoting its degradation through the ubiquitin–proteasome pathway. As shown in Fig. 2A and B, CDK11^{p58} promoted the down-regulation of ERα with or without estrogen, and ERα protein level were further reduced at the present of both E_2 and CDK11^{p58}. Thus, CDK11^{p58} negatively regulated ERα protein levels in a hormone-independent manner. To analyse this effect, we transfected CDK11^{p58} into breast cancer cell lines T47D and MCF7 cells, in which ERα protein is

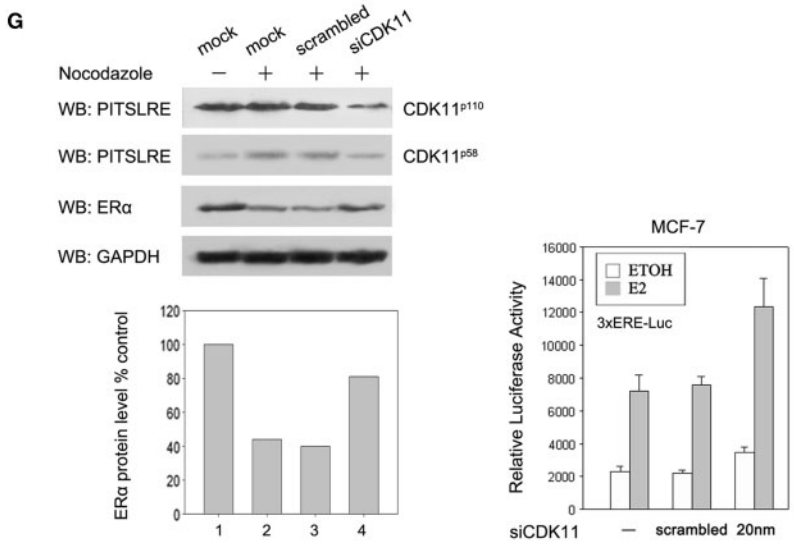

Fig. 2. Continued.

endogenously expressed. The same tendency was observed that ERα could be negatively regulated by CDK11^{p58} in a dose-dependent manner (Fig. 2C and D). These results suggested that CDK11^{p58} induced both exogenous and endogenous ERα degradation.

Considering that CDK11^{p110} significantly facilitated ERα-dependent transcription (Fig. 1C), we assessed the effect of CDK11^{p110} on ERα protein stability. Myc-ERα was transfected into COS-1 cells with 1μg or 3μg of HA-CDK11^{p58} or HA-CDK11^{p110}. Cell lysates were assessed by western blot with anti-myc and anti-HA antibody. Compared with CDK11^{p58}, CDK11^{p110} did not affect the ERα protein level obviously (Fig. 2E). These observations suggested that CDK11^{p110} facilitated ERα-dependent transcription by another unknown mechanism.

It was reported by our group that the elevated expression of cyclin D3 affected CDK11^{p58} cellular distribution and greatly enhanced the kinase activity of CDK11^{p58} (*30*). To assess the effect of cyclin D3 on endogenous ERα, different amounts of cyclin D3 were transfected into the MCF-7 cells and cell lysates were assessed by western blot with anti-ERα antibody. As a result, ERα protein level was also negatively regulated by cyclin D3 in a dose-dependent manner. Moreover, when 1μg of CDK11^{p58} was co-transfected with different amounts of cyclin D3, ERα protein level was reduced significantly (Fig. 2F). Since c-myc is an important target of E_2 in breast cancer cells (*3*), we assessed its protein level by western blot with an anti-c-myc antibody. It is suggested that the repression of c-myc by cyclin D3 and CDK11^{p58} has the same trend as the repression of ERα (Fig. 2F).

Considering the endogenous CDK11^{p58} was in a low level and mainly expressed in G2/M phase (*26*), we incubated MCF-7 cells with nocodazole to synchronize the cell cycle. Then CDK11^{p58} was detected by western blot with an anti-PITSLRE antibody. As a result of the accumulation of CDK11^{p58} in G2/M phase, the protein level of ERα was also down-regulated. Reversely, ERα protein level was elevated when endogenous CDK11^{p58} was knockdown by CDK11-siRNA (Fig. 2G). Because CDK11^{p58} and CDK11^{p110} are translated from the same transcript, the endogenous CDK11^{p110} was knockdown at the same time. But it did not affect ERα level as the previous experiment (Fig. 2E). Relative ERα protein levels are shown in corresponding histogram (Fig. 2G, lower panel). To investigate the effects of siCDK11 on ERα-mediated transcription, the dual-luciferase reporter assay was assessed in the MCF-7 cells. Compared with the scrambled control, siCDK11 facilitated ERα-dependent transcription (Fig. 2G, right panel).

CDK11p^{58} Promotes Ubiquitin/Proteasome-mediated Degradation of Erα—To analyse the mechanism by which CDK11^{p58} negatively regulates ERα protein level, we explored whether CDK11^{p58} affects the mRNA level of ERα. MCF7 cells were transfected with mock vector or CDK11^{p58}. Then mRNA was extracted and detected by quantitative reverse transcription–PCR assay. The mRNA level of ERα did not change in the presence or absence of CDK11^{p58} among MCF-7 cells transfected with mock vector (Fig. 3A, left panel, lane 1) or with different doses of CDK11^{p58} (Fig. 3A, left panel, lanes 2 and 3). Relative ERα mRNA levels from two independent experiments were shown in corresponding histogram with actin as an internal control (Fig. 3A, right panel). This suggested the degradation in the ERα protein by CDK11^{p58} was not due to a reduction in the ERα mRNA level.

Then, we put our focus on the regulation of ERα protein levels by CDK11^{p58}. It has been reported that ERα is rapidly degraded through the ubiquitin–proteasome pathway in mammalian cells in an estrogen-dependent manner (*34*). And treatment of mammalian cells with the proteasome inhibitor MG132 inhibited the activity of the proteasome and thus blocked ERα degradation (*16*). COS-1 cells were transfected with vector or CDK11^{p58} plasmid and subsequently incubated with either DMSO (vehicle) or MG132 both in the absence

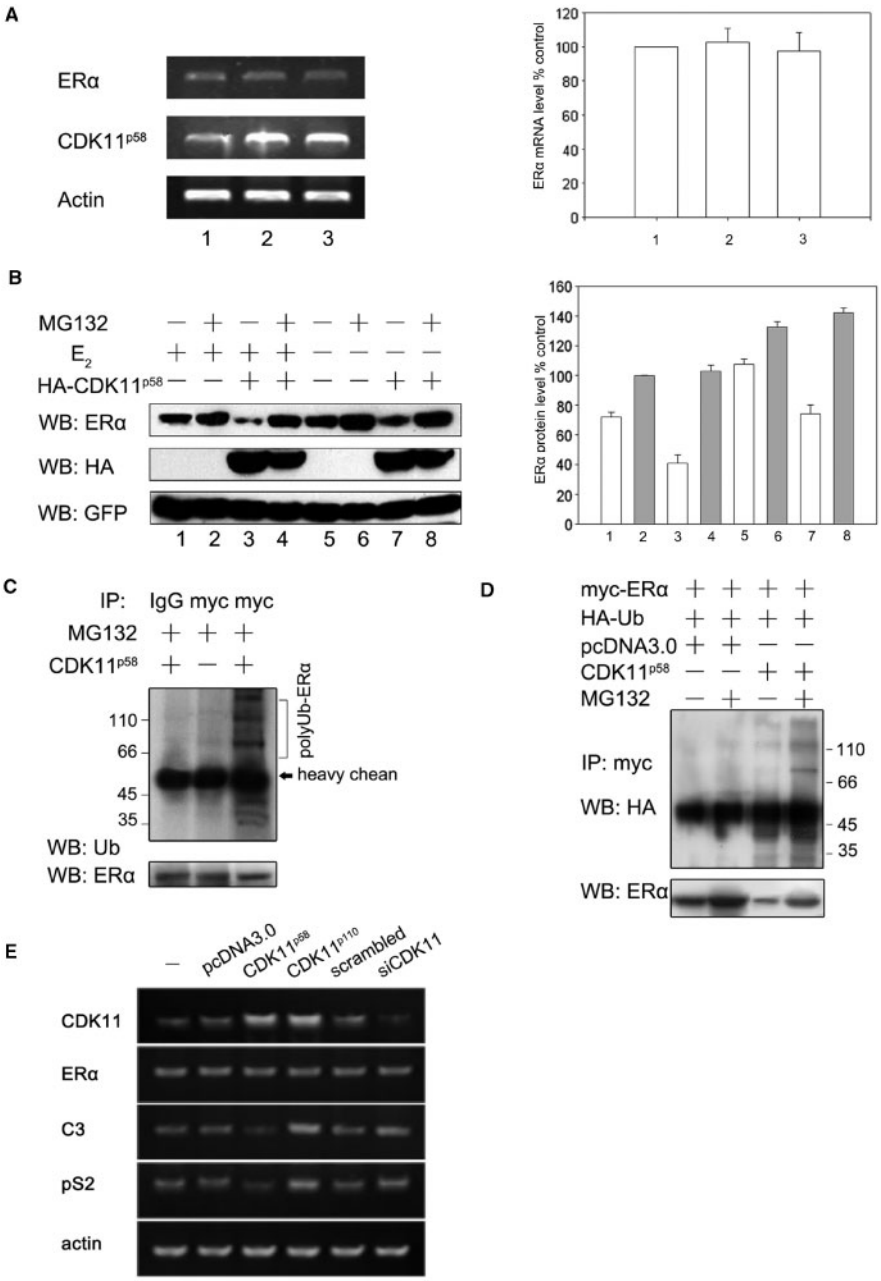

Fig. 3. **CDK11^{p58} promotes ERα ubiquitin–proteasome degradation.** (A) MCF-7 breast cancer cells were transfected with different dose of HA-CDK11^{p58} (0, 1, 2 μg) or vector to fill dose up. One microgram of total RNA extracted from the transfected cells was subjected to quantitative reverse transcription–PCR to assess the mRNA level of ERα, CDK11^{p58} and actin. The expressions of these mRNAs were analysed by gel electrophoresis (left panel). Relative ERα mRNA levels in cells co-transfected with vector or CDK11^{p58} from two independent experiments are shown in corresponding histogram. Actin was used as an internal control to correct it (right panel). (B) COS-1 cells were transfected with 1 μg of ERα and vector or CDK11^{p58} then treated with EtOH or 100 nM E_2 at 24 h after transfection. After another 18 h, cells were incubated with DMSO or MG132 for 6 h and subsequently harvested. The effects of CDK11^{p58}, E_2 and MG132 on ERα protein levels were analysed by western blot with anti-ERα, anti-HA and anti-GFP antibodies (left panel). Relative ERα protein levels in cells treated with DMSO (white) or MG132 (grey) from two independent experiments are shown in corresponding histogram. GFP were used as an internal control to correct it (right panel). (C) COS-1 cells were transfected with 2 μg of myc-ERα and CDK11^{p58} or vector and incubated with MG132 or DMSO at the time of transfection. After 24 h the cells were harvested. Cell lysates were boiled immediately at harvest in lysis solution containing 1% SDS. ERα was then immunoprecipitated with anti-myc antibody or control mouse IgG. Ubiquitinated myc-ERα samples in the precipitated immunocomplex were blotted with anti-ubiquitin antibody and cell lysates were blotted with anti-ERα antibody. (D) COS-1 cells were transfected with 2 μg of myc-ERα, HA-ubiquitin and CDK11^{p58} or vector. Cells were incubated with MG132 or DMSO at the time of transfection. After 24 h the cells were harvested. Cell lysates were treated with the same operation as above. Ubiquitinated myc-ERα samples were blotted with anti-HA antibody and cell lysates were blotted with anti-ERα antibody. (E) MCF-7 cells were transfected with vector, CDK11^{p58} CDK11^{p110} scrambled siRNA and siCDK11. One microgram of total RNA extracted from the transfected cells was subjected to quantitative reverse transcription–PCR to assess the mRNA level of CDK11, ERα, C3, pS2 and actin. The expressions of these mRNAs were analysed by gel electrophoresis.

and presence of estrogen. The effect of CDK11^{p58}, hormone and MG132 on ERα protein levels was analysed by western blot. As shown in Fig. 3B, the DMSO-treated control cells (lanes 1, 3, 5, 7) exhibited lower levels of ERα protein compared with that of MG132-treated cells (lanes 2, 4, 6, 8). Addition of CDK11^{p58} reduced the level of ERα protein compared with cells that were not transfected with CDK11^{p58} in both the presence (lane 3 versus lane 1) and the absence (lane 7 versus lane 5) of estrogen. However, MG132 blocks the CDK11^{p58}-induced degradation of the ERα protein (lane 4 versus lane 8). Relative ERα protein levels from two independent experiments are shown in corresponding histogram with GFP as an internal control (Fig. 3B, right panel). This suggests CDK11^{p58} promotes ERα degradation through ubiquitin–proteasome pathway in a hormone-independent manner.

Considering the effect of MG132 in Fig. 3B, it was necessary to examine the effect of CDK11^{p58} on ERα ubiquitination. COS-1 cells were co-transfected with myc-ERα and CDK11^{p58} or vector. Then cells were treated with MG132 or DMSO at the time of transfection. Cell lysates were denatured immediately at harvest in lysis solution containing 1% SDS to maximize preservation of ubiquitylated ERα species. ERα was then immunoprecipitated with an anti-myc antibody or control IgG. And the levels of ubiquitinated ERα in the precipitated immunocomplex were assessed by western blot with an ubiquitin antibody. The polyubiquitinated ERα exhibited a ladder of higher molecular weight species (Fig. 3C, lane 3). As controls, cells transfected with vector and transfected with CDK11^{p58} but immunoprecipitated with control IgG did not exhibit any significant ladder (Fig. 3C, lanes 2 and 1). To eliminate the non-specific effect of endogenous ubiquitin, COS-1 cells were co-transfected with ERα and exogenous haemagglutinin (HA)-tagged ubiquitin, along with vector or CDK11^{p58}. Then cells were treated with MG132 or DMSO at the time of transfection. The similar empirical method was performed as above except cell harvest after 24 h to avoid invalidation of MG132. Then the levels of ubiquitinated ERα in the precipitated immunocomplex were assessed by western blot with an anti-HA antibody. The poly-ubiquitinated ERα ladder was only detected in the both presence of CDK11^{p58} and MG132 (Fig. 3D). These results suggest that CDK11^{p58} promotes ERα polyubiquitination and degradation through ubiquitin–proteasome pathway.

In breast cancer cells, estrogen-dependent activation enhances transcription of several ERα-responsive promoters, such as complementation 3 (C3) and pS2 (*36*, *37*). To analyse the effect of CDK11^{p58} on endogenous ER target genes, MCF7 cells were transfected with vector, CDK11^{p58}, CDK11^{p110}, scrambled siRNA and siCDK11 in the presence of estrogen. Then mRNA was extracted and detected by quantitative reverse transcription–PCR assay (Fig. 3E). The levels of ERα mRNA were stable. However, the mRNA levels of C3 and pS2 were repressed by CDK11^{p58} and facilitated by CDK11^{p110}. And compared with scrambled siRNA, siCDK11 promoted the mRNA levels of C3 and pS2. These results were consistent with the dual-luciferase reporter assays assessing ERα-mediated transcription activities.

CDK11p^{58} Interacts with ERα in Vitro and in Mammalian Cells—To investigate mechanism of ERα degradation promoted by CDK11^{p58}, we examined the association between these two proteins. GST pull-down and co-immunoprecipitation assays were performed. GST-CDK11^{p58} and ERα proteins were synthesized and isotopically labelled with [^{35}S]-Methionine in vitro. GST-CDK11^{p58} and ERα were incubated with or without estrogen. GST was used as a negative control. The protein mixtures were bound to glutathione Sepharose beads, washed and subjected to SDS–PAGE. The resulting gel was then exposed. ERα was detected after incubation with GST-CDK11^{p58} (Fig. 4A, lanes 5 and 6). As a negative control, no ERα was found after incubation with GST (Fig. 4A, lane 4).

Furthermore, GST-CDK11^{p58} was expressed and purified from *E. coli*, pre-immobilized onto glutathione

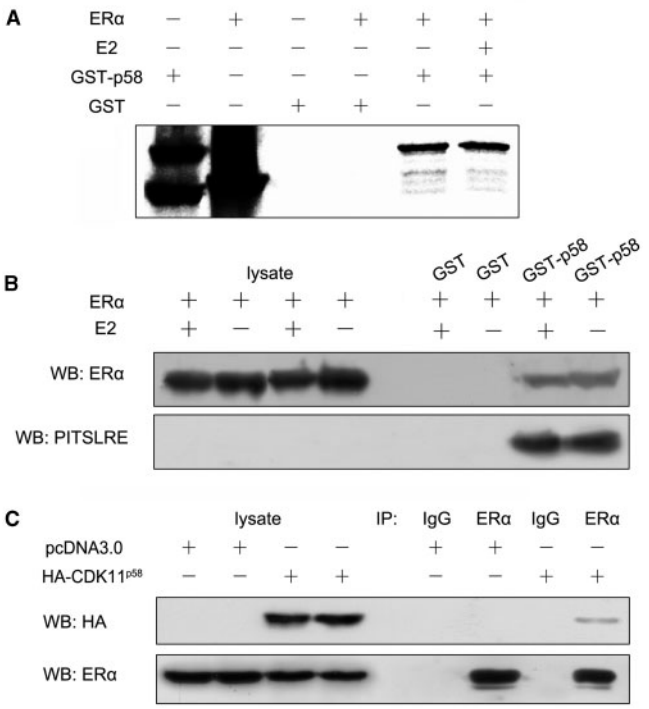

Fig. 4. **CDK11^{p58} interacts with ERα *in vitro* and in mammalian cells.** (A) GST, GST-CDK11^{p58} and ERα were translated with TNT-coupled reticulocyte lysate system *in vitro*. They were all labelled with [^{35}S] methionine. GST-pull down experiment was carried out as described in MATERIALS AND METHODS section. The translated proteins labelled with [^{35}S] methionine were viewed by autoradiography (left lanes). Bound GST-CDK11^{p58} and bound ERα was detected in the treatment with or without E_2 (right lanes upper bands and lower bands). (B) CDK11^{p58} was bacterially expressed and purified as a GST-fusion protein. Whole-cell lysates (WCL) of COS-1 cells transfected with ERα were quantified and incubated with 2 μg of pre-immobilized GST-CDK11^{p58} or GST control in the treatment with or without E_2. The beads were washed and eluted with SDS sample buffer. Cell lysates and the eluates were blotted with anti-ERα and anti-PITSLRE antibodies. (C) MCF-7 cells were transfected with pcDNA3.0 or HA-CDK11^{p58}. Immunoprecipitation was carried out using mouse monoclonal anti-ERα antibody or normal mouse IgG. Cell lysates and immunoprecipitate samples were quantified and blotted with anti-HA and anti-ERα antibodies.

Sepharose 4B beads, and incubated with lysates derived from COS-1 cells transfected with ERα in the presence or absence of estradiol. The protein complex on beads was detected by western blot with an anti-ERα antibody. These data demonstrated the direct binding of ERα and CDK11^{p58} in both the absence and the presence of estradiol (Fig. 4B). The presence of estradiol reduced the stability of ERα protein, but did not affect the interaction markedly.

To further characterize the *in vivo* interaction of CDK11^{p58} and ERα, immunoprecipitation assay was performed. Vector pcDNA3.0 and HA-CDK11^{p58} were transfected in MCF-7 cells and endogenous ERα was co-immunoprecipitated with the anti ERα antibody or normal mouse IgG as control. Samples were analysed by western blot with the anti-HA antibody. Consistently, HA-CDK11^{p58} was co-immunoprecipitated with endogenous ERα specifically (Fig. 4C).

Interaction Between ERα and CDK11p^{58} is Required for Ubiquitin/Proteasome-mediated Degradation of Erα—Our previous study has reported the interaction between CDK11^{p58} and AR. But its kinase dead mutant, CDK11^{p58D224N}, does not associate with AR (*29*). In searching for the relationship between ERα and CDK11^{p58D224N}, we performed co-immunoprecipitation assays in COS-1 cells. Vector pcDNA3.0, HA-CDK11^{p58}, HA-CDK11^{p58D224N} and ERα were transfected and the cell lysates were immunoprecipitated with the anti-HA antibody or normal mouse IgG as control. Compared with wild-type HA-CDK11^{p58}, we found that ERα could not be co-immunoprecipitated with HA-CDK11^{p58D224N} (Fig. 5A). This result showed that D224N can not bind ERα *in vivo*.

Furthermore, we found D224N failed to reduce ERα in the assay above. To investigate its function, COS-1 cells were transfected with ERα, GFP and respectively transfected with vector, CDK11^{p58} and D224N. Cell lysates were assessed by western blot with the anti-ERα antibody and anti-PITSLRE antibody. Compared with CDK11^{p58}, D224N did not reduce the protein level of ERα (Fig. 5B).

To further confirm the different function between wild-type CDK11^{p58} and its mutant D224N, ERα ubiquitination was examined. Compared with CDK11^{p58} (Fig. 5C, lane 4), the polyubiquitinated ERα ladder was not obvious in the presence of D224N (Fig. 5C, lane 6). These results suggest that interaction between ERα and CDK11^{p58} is required for ubiquitin/proteasome-mediated degradation of ERα.

To investigate the effect of the kinase dead mutant on ERα-mediated transactivation, COS-1 cells were transfected with ERα, 3 × ERE-LUC, pRL, vector or CDK11^{p58}, CDK11^{p58D224N}, CDK11^{p110}, CDK11^{p110D224N} expression plasmids. The dual-luciferase reporter assay showed that CDK11^{p58D224N}, CDK11^{p110}, CDK11^{p110D224N} all facilitated ERα-dependent transcription (Fig. 5D).

DISCUSSION

Estrogens and ERα are indispensable for the development, regulation and maintenance of female phenotype and reproductive physiology (*1*). Although the ERα signalling pathway is required for normal mammary gland development, it has been hypothesized that aberrant signaling could lead to abnormal cellular proliferation and survival, potentially participating in the development and progression of breast cancer (*38*). For example, stimulation of c-myc oncogene expression associates with estrogen-induced proliferation of human breast cancer cells (*3*). It was reported that the breast cancer had increased by 51% in the Chinese women in the past 25 years (*39*). So the basic study about ERα is still exigent. Binding of ERα to estrogens results in a change in receptor conformation, dissociation from heat shock proteins, nuclear localization, dimerization, interaction between functional domains, DNA binding, recruitment of co-activators, initiation of transcription, and rapid degradation through ubiquitin–proteasome pathway (*40*). ERα degradation serves to control physiological responses in estrogen target tissues by limiting the expressions of estrogen responsive genes, such as complementation 3 (C3) and pS2 (*36*, *37*). In addition to the role in ERα degradation, ubiquitination may serve regulatory functions such as ERα-mediated transcription (*41*). Several proteins possessing ubiquitin ligase activity (e.g. E6AP, BRCA1, MDM2 and SUG1) have been shown to associate with ERα and modulate receptor signalling (*42–45*). These observations suggested that proteasome-mediated receptor degradation was important for ERα function.

ERα action is accommodated by many regulators and affected by several cellular signalling pathways (*46*). It was well established that cyclin-dependent kinases (CDKs) controlled the transitions between successive phases of the cell cycle in all eukaryotic cells (*22*). It was reported that estrogen-induced activation of CDK4 and CDK2 during G1-S phase progression is accompanied by increased cyclinD1 expression and decreased cyclin-dependent kinase inhibitor association with cyclin E-CDK2 (*23*). And transcriptional activation by the ERα is modulated through inhibition of CDK2 and CDK4 (*24*, *25*). These studies provided a view of the CDKs in the regulation of ERα. CDK11, which contains a conserved PITSLRE motif within the protein kinase domain, has two major isoforms: CDK11^{p58} and CDK11^{p110} (*26*, *27*). The data we show here demonstrated a distinct mechanism of CDK11^{p58} from that of other CDKs in the regulation of ERα-mediated transcription. CDK11^{p58}, not CDK11^{p110}, negatively regulated the ERα protein level specifically. Our previous researches showed that CDK11^{p58} was involved in the negative regulation of AR-mediated transactivation but not due to reducing AR protein level (*29*). The reduction of protein level resulted in the repression of ERα-mediated transcription activity. However, CDK11^{p110} did not affect ERα protein stability and facilitated ERα-dependent transcription significantly by some unknown mechanisms. Interestingly, D224N, the kinase dead mutant of CDK11^{p58}, lost the ability of repressing ERα protein stability and facilitated ERα-dependent transcription just like CDK11^{p110}. It suggests that the kinase activity of CDK11^{p58} is essential to the repression of ERα. So cyclin D3, which was vital for the kinase activity of CDK11^{p58} (*30*), negatively regulated ERα protein stability in MCF-7 cells as our results shown. And it was

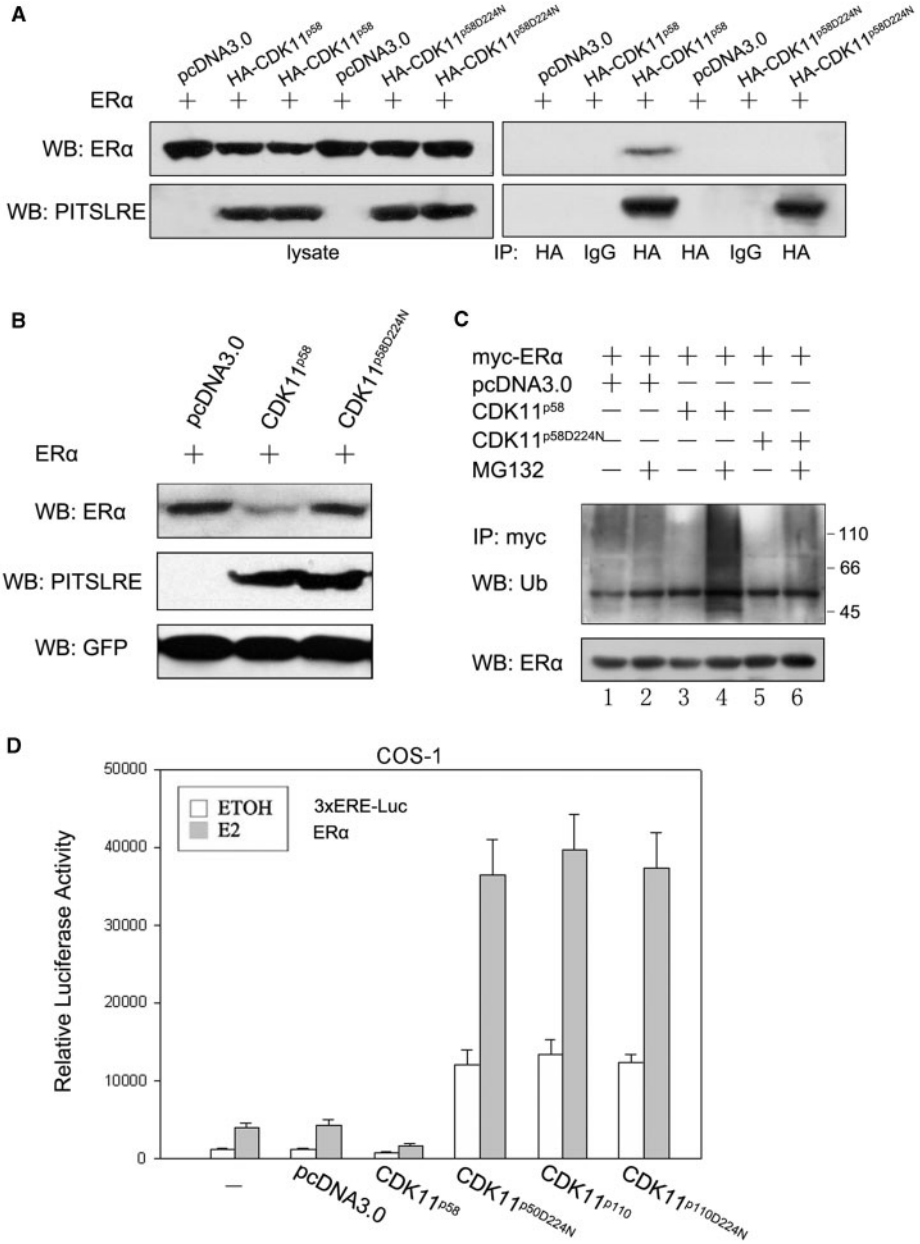

Fig. 5. **Interaction between ERα and CDK11^{p58} is required for ubiquitin/proteasome-mediated degradation of ERα.** (A) COS-1 cells were transfected with 6 μg of ERα in combination with 2 μg of pcDNA3.0, HA-CDK11^{p58} or HA-CDK11^{p58D224N}. At 48 h after transfection, cell lysates were immunoprecipitated with mouse monoclonal anti-HA antibody or normal mouse IgG. Cell lysates and immunoprecipitate samples were quantified and blotted with anti-ERα and anti-PITSLRE antibodies. (B) COS-1 cells were transfected with 2 μg of ERα and GFP, in combination with 2 μg of pcDNA3.0, HA-CDK11^{p58} or HA-CDK11^{p58D224N}. The cells were harvested at 48 h after transfection and the lysates were quantified and blotted with anti-ERα, anti-PITSLRE and anti-GFP antibodies. (C) COS-1 cells were transfected with 2 μg of myc-ERα, and 2 μg of vector or CDK11^{p58} or CDK11^{p58D224N}. At 42 h after transfection, cells were incubated with MG132 or DMSO for 6 h and subsequently harvested. Cell lysates were boiled immediately at harvest in lysis solution containing 1% SDS. Myc-ERα was immunoprecipitated with anti-myc antibody. Ubiquitinated myc-ERα samples in the precipitated immunocomplex were blotted with anti-ubiquitin antibody and cell lysates were blotted with anti-ERα antibody. (D) A 2 ng of PRL, 20 ng of ERα and 200 ng of 3×ERE-LUC were co-transfected with pcDNA3.0 or CDK11^{p58}, CDK11^{p58D224N}, CDK11^{p110}, CDK11^{p110D224N} expression plasmids into COS-1 cells. The cells were treated with 100 nM EtOH or 100 nM E_2 at 24 h after transfection, and harvested after another 24 h. Luciferase activity was measured and normalized to Renilla luciferase activity. Data shown are the mean and standard error of four independent experiments.

reported by our group that D224N retained the ability to interact with cyclin D3 and blocked the CDK11^{p58}–cyclin D3 interaction (*29*). Then over-expression of D224N competitively repressed the function of CDK11^{p58} and subsequently enhanced AR-mediated transactivation. So D224N and CDK11^{p110} facilitated ERα-dependent transcription by the similar mechanism probably. As a result of the accumulation of CDK11^{p58} through

synchronizing the cell cycle to G2/M phase, ERα protein stability was also down-regulated. Reversely, ERα protein level was elevated when endogenous CDK11^{p58} was knockdown by CDK11-siRNA. Actually, CDK11^{p110} and CDK11^{p58} can be both knockdown by CDK11-siRNA. Considering CDK11^{p110} had no effect on ERα protein stability, CDK11-siRNA affected ERα protein level just through the CDK11^{p58} pathway. But ERα-dependent transcription was affected by CDK11^{p110} and CDK11^{p58} in opposite manners and the dual-luciferase reporter assay showed that CDK11-siRNA facilitated ERα-dependent transcription as a whole. It suggested that CDK11^{p58} could have dominant effect on CDK11^{p110} substrates. Then those ERα-responsive promoters, such as c-myc, C3 and pS2, which have important functions in estrogen-induced proliferation of human breast cancer cells (*3*, *36*, *37*) may be repressed by CDK11^{p58}. These results provide a new pathway in which CDK11^{p58} negatively regulates cells.

There are two possible mechanisms of regulation on the ERα protein level. One is affecting its mRNA stabilization and the other is just affecting the protein stabilization (*42*). In elucidating the mechanism, we found that ERα mRNA stabilization was not influenced by CDK11^{p58} over-expression. So the protein stabilization of ERα was to be detected. It was confirmed that ERα was degraded in an ubiquitin/proteasome-dependent manner and the proteasome inhibitor, MG132, promotes the *in vivo* accumulation of ERα and blocks estrogen-induced receptor degradation (*42*). Thus, we found MG132 could block the CDK11^{p58}-mediated degradation of ERα protein. Furthermore, ERα ubiquitination assay was performed and a ladder of higher molecular weight species of ERα was visible only in the over-expression of CDK11^{p58}. These results suggested that CDK11^{p58} might act as a regulator which promoted ubiquitin/proteasome-mediated degradation of ERα. And our data showed that CDK11^{p58} affected ERα in an estrogen-independent manner. Considering that ERα is activated by estrogen (*1*), it illustrates that CDK11^{p58} function in whether activated or inactivated condition of ERα. Since CDK11^{p58} is a cyclin-dependent kinase, some proteins are phosphorlated by it as substrates, such as HBO1 which we reported previously (*47*). Then ERα is a potential substrate of CDK11^{p58}. So some phosphorylation experiments have been performed. But we can not confirm whether CDK11^{p58} phosphorlates ERα directly yet. Further research is needed. At present, their interaction has been confirmed and this binding maybe changes the molecular conformation of ERα. So that it would be dissociated from the molecular chaperones or facilitated to bind some kinds of E3 ubiquitin–protein ligases. Therefore, ERα protein stability will be affected. Importantly, their interaction is essential to this phenomenon because the kinase dead mutant D224N has no interaction with ERα and has no effect on ubiquitination or protein negative regulation. The same phenomenon was detected with CDK11^{p110}, which had no interaction with ERα (data not shown) and failed to reduce its protein stability. Furthermore, they seem to have opposite behaviour to CDK11^{p58} on ERα-mediated transcription activity. Accordingly, D224N does not associate with AR so that it does not repress AR-mediated transactivation (*29*).

So we consider that CDK11^{p58} represses ERα transcriptional activity as a result of the obvious promotion of ubiquitin/proteasome-mediated degradation. But the regulation of ERα transactivation is complicated and dynamic. It was reported that proteasome dependent degradation of ERα is required to the cyclical recruitment on its responsive promoters. There is interdependence between ERα-mediated transactivation and proteasome-mediated degradation of ERα. And inhibition of ERα degradation with MG132 rather impaired transcriptional activity of ERα (*48*). In fact, we also found the repression of transactivation by MG132 in our experiments. To explain this inconsistency, we think the ubiquitination of ERα keeps a dynamic balance in normal status. Suitable ubiquitination of ERα is needed for normal transcriptional activity. But when ubiquitin/proteasome-mediated degradation of ERα becomes more rapid and intense, the protein level will be too low to maintain the normal transactivation. So the dynamic balance is affected. In addition, CDK11^{p58} interacts with ERα and changes its molecular conformation likely. So it will be a regulation for transcription-dependent involvement of the proteasome in ERα degradation. In our experiment, inhibition of CDK11^{p58} by siRNA removed the impairment on ERα level, but did not completely keep ERα from ubiquitination. So the ERα transactivation was still increased.

CDK11 is encoded by chromosome band 1p36 (*49*, *50*). It has been reported that CDK11 is essential to cell viability and normal early embryonic development (*51*). And deletion of CDK11 has been found in several tumors including non-Hodgkin lymphoma, melanoma and childhood lymphoma (*49*, *50*, *52*). Recent study has detected the various genomic alterations of 1p36 in ERα-positive breast cancers. It was reported that patients in the recurrence group showed a significantly different pattern of chromosomal gain and loss than patients in the non-recurrence group. Copy number loss of 1p36 is significantly associated with distant recurrence of the disease within 5 years of diagnosis (*53*). Thus the deletion of CDK11 will be further identified in breast cancers. Additionally, ERα degradation mediated by CDK11^{p58} may serve to limit the expression of estrogen responsive genes such as proto-oncogene c-myc (*3*). Thus CDK11^{p58} controls physiological responses in estrogen target tissues. Then loss of repression by CDK11^{p58} might lead to abnormal cellular proliferation and survival, potentially participating in the development and progression of breast cancer.

In a word, we identified CDK11^{p58} as a new negative regulatory protein of ERα and provided a new signalling pathway of CDK11^{p58} in the cells negative regulation.

FUNDING

The State Key Program of Basic Research of China '973' (2002CB512803); Scientific Technology Development Foundation of Shanghai (074307038); Shanghai Leading Academic Discipline Project, Project Number: B110;

National Natural Scientific Foundation of China (30470442, 30330320 and 30770426).

CONFLICT OF INTEREST

None declared.

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