CUE domain containing 2 regulates degradation of progesterone receptor by ubiquitin–proteasome

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Accumulated evidence indicates that progesterone receptors (PR) are involved in proliferation of breast cancer cells and are implicated in the development of breast cancer. In this paper, a yeast two-hybrid screen for PR led to the identification of CUE domain containing 2 (CUEDC2), whose function is unknown. Our results demonstrate that CUEDC2 interacts with PR and promotes progesterone-induced PR degradation by the ubiquitin–proteasome pathway. The inhibition of endogenous CUEDC2 by siRNA nearly abrogated the progesterone-induced degradation of PR, suggesting that CUEDC2 is involved in progesterone-induced PR ubiquitination and degradation. Moreover, we identify the sumoylation site Lys-388 of PR as the target of CUEDC2-promoted ubiquitination. CUEDC2 decreases the sumoylation while promoting ubiquitination on Lys-388 of PRB. We also show that CUEDC2 represses PR transactivation, inhibits the ability of PR to stimulate rapid MAPK activity, and impairs the effect of progesterone on breast cancer cell growth. Therefore, our results identify a key post-translational mechanism that controls PR protein levels and for the first time provide an important insight into the function of CUEDC2 in breast cancer proliferation.

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Introduction

The progesterone receptor (PR) is an important member of the nuclear receptor family of ligand-activated transcription factors (Evans, 1988). Accumulated evidence indicates that PR is involved in proliferation of breast cancer cells and is implicated in the development and progression of breast cancer (Hissom and Moore, 1987; Groshong *et al*, 1997;

Skildum *et al*, 2005). Moreover, PR is an important indicator of steroid hormone dependence and disease prognosis in breast cancer. The loss of PR signals development of an aggressive tumor phenotype, which is usually relative to acquiring enhanced sensitivity to growth factors (Horwitz and McGuire, 1975; Elledge *et al*, 1992). PR levels are influenced by ligand binding. After progesterone treatment, PR is extensively downregulated by mechanisms that remain unclear.

PR exists as two isoforms, PRA and PRB, which are identical except for an additional 164 N-terminal amino acids in the B upstream segment (Horwitz and Alexander, 1983). PRA and PRB are multidomain structures, which have a DNA-binding domain (DBD), a hormone-binding domain, and two transcriptional activation motifs: the C-terminal, ligand-dependent activation function (AF-2) and the N-terminal, more constitutively active AF-1. PRB contains an additional N-terminal AF-3 (Sartorius *et al*, 1994b). PRB is mostly reported as a stronger transactivator than PRA (Tung *et al*, 1993; Richer *et al*, 2002). The synergism between AF3 and the downstream AFs contributes to increased activity of PRB (Arnett-Mansfield *et al*, 2001). Recruitment of coactivators is important for transcription of PR, including SRC-1/NCoA-1, SRC-2/TIF2/GRIP1, and SRC-3/TRAM-1. CREB-binding protein and p300 interact with PR and its coactivators and are also found to upregulate PR transcription (Jenster, 1998; McKenna *et al*, 1999; Glass and Rosenfeld, 2000; Leo and Chen, 2000).

Aside from its regulation by specific ligands, PR can undergo covalent modifications, including phosphorylation, ubiquitinylation, and sumoylation, which have been shown to regulate PR function and stability (Weigel, 1996; Zhang *et al*, 1997; Lange *et al*, 2000; Abdel-Hafiz *et al*, 2002; Chauchereau *et al*, 2003). PR undergoes ligand-dependent downregulation via the ubiquitin–proteasome pathway leading to rapid and extensive loss of PR protein. Ligand-dependent downregulation of PR occurs by a mechanism involving phosphorylation of PR at Ser-294, targeting PR for ubiquitination and degradation by the 26S proteasome (Lange *et al*, 2000; Shen *et al*, 2001).

Until now, little is known about the function of CUEDC2 protein (GenBank: NM_024040). Bioinformatics analysis suggests CUEDC2 as a CUE domain-contained gene. Identified as ubiquitin-binding motifs, the CUE domains are small, moderately conserved domains of about 40-amino-acid residues that are found in a variety of eukaryotic proteins (Ponting, 2000; Donalson *et al*, 2003). CUE domains interact with both mono- and polyubiquitin, and have a dual role in mono- and polyubiquitin recognition as well as in facilitating intramolecular monoubiquitination (Shih *et al*, 2003).

In this paper, we demonstrate that CUEDC2 promotes PR degradation through ubiquitin–proteasome. Ligand-triggered downregulation of PR is nearly abrogated when endogenous CUEDC2 is silenced by its specific siRNA. In addition, CUEDC2 can interact with PR and repress PR transactivation.

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The transactivation of endogenous PR is preferentially enhanced by CUEDC2-siRNA. We also found that CUEDC2 impairs the effect of progesterone on breast cancer cell growth. Therefore, we propose that CUEDC2 is a novel negative regulator of PR and functions to promote the ubiquitin-dependent degradation of PR.

Results

CUEDC2 interacts with PRB in a ligand-independent manner

In the attempt to identify PRB-binding proteins that could regulate the role of PRB, we performed a yeast two-hybrid screen using the IF domain of PR as bait to screen a human mammary cDNA library. We identified two interacting clones containing a complete open reading frame (681 bp) for a 26 000 Da molecular weight gene product that has 100% amino acid identity to human CUEDC2. CUEDC2 is a novel member of the CUE family, whose function is unknown. This interaction was confirmed by inverse transformation of yeast cells with GAL4AD-PRB and GAL4DBD-CUEDC2 (Supplementary Figure S1). To test whether CUEDC2 interacts with PRB *in vitro*, we used a glutathione S-transferase (GST) pull-down assay. GST-CUEDC2 was expressed in bacteria and purified by glutathione-coupled beads. GST-CUEDC2 was then incubated with Myc-PRB protein expressed in 293T cells and subjected to SDS-PAGE and Western blot. Figure 1A shows that CUEDC2 interacts with PRB with or without progesterone *in vitro*.

To determine whether PRB interacts with CUEDC2 *in vivo*, 293T cells were transfected with Myc-PRB and HA-CUEDC2 and cultured in the absence or presence of 100 nM progesterone (Figure 1B). HA-CUEDC2 was immunoprecipitated from cell lysates by anti-HA and analyzed for Myc-PRB binding by immunoblotting. As indicated in Figure 1B, Myc-PRB could be co-immunoprecipitated in a ligand-independent manner in the presence of HA-CUEDC2. With regard to the important relation between PR and ER, the interaction of CUEDC2 and ER was also examined. As shown in Supplementary Figure S2A, we found that CUEDC2 also interacted with ER α .

To further characterize the interaction of CUEDC2 with PR, CUEDC2 was immunoprecipitated with an anti-PR antibody from human T47D breast cancer cells, which express endogenous PR and CUEDC2, and the immunocomplexes were subjected to SDS-PAGE, followed by Western blot analysis. Figure 1C reveals that CUEDC2 is detected in the PR immunocomplexes in the presence or absence of 100 nM progesterone. We next asked whether PRB and CUEDC2 colocalize in living cells as visualized by confocal fluorescence microscopy, reflecting the possibility that PRB could physically associate with CUEDC2. As shown in Figure 1D (upper panel), when 293T cells were cultured in the absence of progesterone, PRB localizes in the cytoplasm, where unliganded PRB is normally located. In contrast, when cells were treated with progesterone for 2 h, liganded PRB translocated from the cytoplasm to the nucleus. Figure 1D (lower panel) shows that CUEDC2 is found throughout the whole cell, and is not affected by the presence of progesterone. In 293T cells cotransfected with PRB and CUEDC2, we found that PRB and CUEDC2 colocalize in the cytoplasm in the absence of progesterone, but colocalized in the nucleus in the presence of progesterone (Figure 1E, lower panel). These data suggest

that PRB and CUEDC2 could engage in a specific physical interaction both *in vitro* and *in vivo*.

Mapping of the PRB and CUEDC2 interacting domains

To define which domains of PRB are required for interaction with CUEDC2, different PRB mutants were created. As indicated in Figure 1F, the IF domain was found to be essential for the interaction of PRB with CUEDC2 in yeast. GST pull-down experiments were performed, and deletion of the IF domain abolished the ability of the PRB protein to bind CUEDC2 (Figure 1G). These results indicate that the interaction of PRB with CUEDC2 is mediated through the IF domain. Unlike PR, ER lacking the IF domain can interact with CUEDC2. We performed a GST pull-down assay and found that the DNA domain of ER (180–282 aa) was essential for the interaction between CUEDC2 and ER. Notably, the interaction of CUEDC2 with ER was ligand-independent and was similar to the interaction of PR (Supplementary Figure S2B).

To further delineate the regions of CUEDC2 that mediate the protein–protein interaction with PRB, a series of mutant GST-CUEDC2 proteins were used in GST pull-down experiments. As shown in Figure 1H, PRB binds full-length CUEDC2 and the two CUEDC2 fragments (1–180 and 100–226 aa) containing the CUE domain. In contrast, the CUEDC2 fragments (1–133 and Δ 133–180 aa) lacking the CUE domain failed to interact with PRB. These data indicate that the CUE domain might be indispensable for interaction with PRB.

CUEDC2 promotes PR degradation through proteasomes

CUE domains were identified as the ubiquitin-binding motifs (Ponting, 2000; Donalson *et al*, 2003) involved in the gp78-mediated ubiquitination and protein degradation (Chen *et al*, 2006). To explore the effects of CUEDC2 on PR function, we first examined whether CUEDC2 affects the levels of PR protein. As indicated in Figure 2A, we observed that increasing expression of CUEDC2 resulted in decreased levels of both endogenous PRA and PRB protein in a progesterone-dependent manner. To further verify the effect of CUEDC2 on PR protein level, 293T cells were transfected with a constant amount of Myc-PRB and increasing amounts of HA-CUEDC2 in the absence or presence of 10 nM progesterone. As expected, transfection of CUEDC2 resulted in reduced levels of exogenous PRB protein (Figure 2B) and PRA (data not shown), which was abrogated when progesterone was not added. The effect of CUEDC2 on other proteasome-regulated transcription factors was also examined. As shown in Supplementary Figure S3A, we found that CUEDC2 had an inhibitory effect on ER protein level, but had no effect on STAT5 and p53 proteins (Supplementary Figure S3B and C). Notably, the effect of CUEDC2 on ER was ligand-independent and was different from the effect on PR.

PR is a target of the estrogen receptor, and the addition of estrogen (E2) markedly promotes the expression of PR (Nancy and Tornesi, 1997). Further experiments showed that the E2-stimulated increase of PR protein expression was also inhibited by CUEDC2 expression (Figure 2C). To investigate the effect of endogenous CUEDC2 on PR protein stability, CUEDC2 siRNA was transfected into T47D cells. As shown in Figure 2D, PR was extensively downregulated by progesterone treatment (lane 2). However, inhibition of

Figure 1 CUEDC2 interacts with PRB *in vitro* and *in vivo*. (A) GST-CUEDC2 or GST was incubated with total cell lysates expressing Myc-PRB with or without 100 nM progesterone for 2 h (hours), and then detected using an anti-Myc. (B) 293T cells were transfected as indicated, and treated with progesterone as in (A). Cell lysates were immunoprecipitated (IP) using anti-HA, and the immunoprecipitates were probed with anti-Myc. (C) T47D cells were treated with progesterone as in (A). Lysates were subjected to IP with anti-PR or a control mouse IgG. The immunoprecipitates were analyzed by immunoblotting (IB) with anti-CUEDC2 and anti-PR. (D) 293T cells were transfected as indicated, and treated as in (B). The localization of either PRB or CUEDC2 was analyzed by confocal microscopy. Scale bar: 20 μ m. (E) 293T cells were transfected as in (D), except that cells were cotransfected with RFP-PRB and GFP-CUEDC2. Scale bar: 20 μ m. (F) The yeast strain AH109 was cotransformed with Gal4-AD-PRB or its mutants plus the Gal4-DBD-CUEDC2. A cotransformant from each plate was streaked on SD/-Trp/-Leu/-His/-Ade medium with β -galactosidase (+, positive interaction; -, no interaction). At the top of the graph is a schematic diagram of the PRA/B protein, illustrating the locations of various domains. (G) 293T cell lysates expressing Myc-PRB or its mutants were incubated with GST or GST-CUEDC2, and interacting proteins were detected with an anti-Myc. (H) 293T cell lysates expressing Myc-PRB were incubated with GST, GST-CUEDC2 or its mutants as indicated, and interacting proteins were detected with anti-Myc. At the top of the graph is a schematic diagram of the CUEDC2 protein, illustrating the regions of various mutants.

endogenous CUEDC2 by siRNA nearly abrogated the ligand-induced degradation of PR (lane 4). To further assess whether CUEDC2 also affected PR transcription, real-time RT-PCR was performed (Figure 2E). Transfection with HA-CUEDC2 resulted in no significant change in PR mRNA levels. In the chase experiment with cycloheximide analysis, transfection of CUEDC2 in 293T cells led to a clear reduction in the half-life of PRB compared to the empty vector, indicating that CUEDC2 actively increased the turnover of PRB (Figure 2F). As the half-life of PRB was considerably shortened by

CUEDC2 (Figure 2F), which was independent of mRNA effects (Figure 2E), the effect of CUEDC2 on PR stability could be occurring at the post-translational level. To test this hypothesis, 293T cells were cotransfected with Myc-PRB and HA-CUEDC2 and then treated with the proteasome inhibitor ALLN (*N*-acetyl-Leu-Leu-Nle-CHO; Figure 3A). The addition of ALLN effectively abrogated the effect of HA-CUEDC2 on PRB protein degradation (compare lane 6 to lane 4). Addition of the proteasome inhibitor lactacystin also blocked PRB degradation (Figure 3B), indicating that

Figure 2 CUEDC2 negatively regulates PRB expression. (A) HA-CUEDC2 (1.0 or 2.0 μ g) was transfected into T47D cells and treated with 10 nM progesterone for 24 h. Protein levels were assessed by Western blot as indicated. (B) 293T cells were cotransfected with 0.5 μ g of Myc-PRB and HA-CUEDC2 (1.0 or 2.0 μ g) and treated as in (A), and protein levels were assessed as indicated. (C) T47D cells were transiently transfected with 2.0 μ g of HA-CUEDC2 and treated with or without 10 nM estradiol for 24 h and then treated with 10 nM progesterone for another 24 h. The cell lysates were analyzed by immunoblot using anti-PR and anti-HA. (D) T47D cells were transfected with CUEDC2 siRNA or control siRNA. At 48 h after transfection, cells were treated as in (A), and protein levels were measured by Western blot as indicated. (E) T47D cells were transfected with HA-CUEDC2 (2.0 μ g) and treated with 10 nM progesterone for 24 h. Cells were harvested and PR mRNA levels were assessed by real-time RT-PCR. (F) The PRB protein levels in 293T cells cotransfected with Myc-PRB (0.5 μ g) and HA-CUEDC2 (2.0 μ g) were detected by immunoblotting at different times after exposure to the protein synthesis inhibitor cycloheximide (CHX, 20 μ g/ml) as indicated. The graph shows the quantification of the immunoblotting data (lower panel) ($\blacklozenge$ -P, $\blacksquare$ -+P, $\blacktriangle$ -C+P mean without progesterone, with progesterone, or with HA-CUEDC2 and progesterone, respectively).

CUEDC2 promotes PRB degradation via the proteasome pathway. To determine that the CUEDC2-promoted degradation of PR was a consequence of PR ubiquitination, 293T cells were transfected with Myc-PRB, HA-CUEDC2, and His-ubiquitin (Figure 3C). Immunoprecipitation with anti-Myc and immunoblotting with anti-Myc revealed that ubiquitinated species of PRB were obviously increased upon cotransfection of HA-CUEDC2 (Figure 3C). In order to investigate the role of endogenous CUEDC2 in the PRB-mediated ubiquitination, 293T cells were transfected with siRNA-CUEDC2 or a control siRNA. The results in Figure 3D demonstrate that knocking down CUEDC2 could reduce the ubiquitination of PRB induced by progesterone, suggesting that CUEDC2 targets PRB for ubiquitination and then degradation by proteasome.

CUEDC2-mediated PRB degradation reduces PRB transcriptional activity

To determine whether CUEDC2-mediated PRB degradation affects PRB transcriptional activity, HeLa cells lacking PRA/PRB were cotransfected with the mouse mammary tumor

virus containing reporter (MMTV-Luc), Myc-PRA/B, and increasing amounts of HA-CUEDC2. As shown in Figure 4A, PRA and PRB transcriptional activation was inhibited by CUEDC2 in a dose-dependent manner in the presence of progesterone. The results show that 1 μ g of HA-CUEDC2 inhibited the transcriptional activity of PRB by 93% and inhibited PRA transcriptional activity by 56%. Comparable results were obtained in 293T cells (Supplementary Figure S4A). We then examined whether inhibition of PRB transactivation by CUEDC2 was also a property of endogenous PRA/PRB in PR-positive T47D breast cancer cells. As shown in Figure 4B, CUEDC2 markedly represses progesterone-driven reporter gene transcription. Additional experiments were performed in MCF-7 breast cancer cells (Supplementary Figure S4B), showing that CUEDC2 functions as an inhibitor of PRB-mediated transactivation. Meanwhile, when the effect of CUEDC2 on other transcription factors was examined, we found that CUEDC2 could not inhibit the transactivation of STAT5 (Goh *et al*, 2002) and p53 (Dornan *et al*, 2004) (Supplementary Figure S4C and D), but could inhibit trans-

Figure 3 CUEDC2 promotes PRB degradation by ubiquitin-proteasome. (A, B) 293T cells were cotransfected with Myc-PRB (0.5 μ g) and HA-CUEDC2 (2.0 μ g). At 12 h after transfection, the cells were treated with the proteasome inhibitor ALLN (30 μ M) or lactacystin (10 μ M) for 30 min before treatment with progesterone (10 nM). Cells were cultured for additional 12 h and Western blot was performed with anti-Myc and anti-HA. (C) 293T cells were transfected as indicated and treated as in (A). Lysates were immunoprecipitated and detected with anti-Myc. (D) 293T cells were transfected with either CUEDC2 siRNA or control siRNA. At 48 h post-transfection, cells were cotransfected with Myc-PRB and His-ubiquitin plasmid and then treated as in (A).

Figure 4 CUEDC2 strongly inhibits the transactivation of PR. (A) Inhibition of PRB transcriptional activity by CUEDC2 in HeLa cells. All treatments were as follows, except when noted: cells were transiently transfected with pMMTV luciferase reporter (0.2 μ g) together with Myc-PRA or PRB (0.05 μ g), or various amounts of HA-CUEDC2 vectors, alone or in combination as indicated. After transfection, cultures were treated with or without 10 nM progesterone for 24 h, and luciferase activity was measured. (B) Inhibition of PR transactivation by CUEDC2 in T47D cells. (C) T47D cells were transfected with either CUEDC2 siRNA or control siRNA, and were cotransfected with pMMTV-luc reporter plasmid 48 h later and then treated with 10 nM progesterone or ethanol for 24 h, and luciferase reporter assays were performed. Western blots were analyzed with antibodies to CUEDC2 and actin. (D) Inhibition of PRB transactivation by CUEDC2 or its mutants in 293T cells. The results are means $\pm$ s.d. of three independent determinations.

activation of the estrogen receptor in a ligand-independent manner (Supplementary Figure S4E).

To investigate the role of endogenous CUEDC2 in PRB-mediated transcriptional activation, T47D cells were transfected with either CUEDC2 siRNA or control siRNA. As shown in Figure 4C (upper panel), PRB transactivation significantly increased in CUEDC2 siRNA-transfected cells. Western blot analysis demonstrated that CUEDC2 siRNA, but not control siRNA, specifically reduced the expression of endogenous CUEDC2 (lower panel). These results further suggest that CUEDC2 inhibits the transcriptional activity of PR.

CUE domain is required for the inhibitory effect of CUEDC2 on PRB

To further examine whether the inhibitory effect of CUEDC2 on PRB transactivation depends on interaction with PRB, the four Flag-CUEDC2 mutants (1–133, 1–180, 100–226, and Δ 133–180 aa) were cotransfected with the MMTV-Luc reporter and Myc-PRB into 293T cells. As shown in Figure 4D, the CUEDC2 mutants (1–133 and Δ 133–180 aa) lacking the CUE domain did not inhibit the transactivation of PRB, suggesting that the effect of CUEDC2 on PRB depends on interaction with PRB. To our surprise, the CUEDC2 mutant (1–180 aa) did not inhibit the transactivation of PRB even with the CUE domain, but the CUEDC2 mutant (100–226 aa) did inhibit the transactivation of PRB. These results indicate that the C-terminus of CUEDC2 (180–226 aa) contains a functional region essential for the inhibitory effect of CUEDC2 on PRB.

CUEDC2 decreases the expression of PR target genes

To corroborate the results of the luciferase reporter assay, the effects of CUEDC2 on the expression of PR target genes were examined. T47D cells were transiently transfected with HA-CUEDC2 in the absence or presence of 10 nM progesterone for the indicated times, and cyclin D1 and p21 mRNA levels were assessed by quantitative real-time RT-PCR. As shown in Figure 5A and B, the transcriptional levels of cyclin D1 and p21 were significantly lowered by CUEDC2. Consistent with the real-time RT-PCR results, the inhibitory effect of CUEDC2 on cyclin D1 and p21 was also observed at the protein level by Western blot (Figure 5C and D). As a negative control, the levels of Bax protein did not change (Figure 5D). Moreover, the decrease of cyclin D1 protein level by CUEDC2 was markedly inhibited when the proteasome inhibitor ALLN was added (Figure 5E). Because of the observed inhibitory effects of CUEDC2 on ER transactivation, we examined whether CUEDC2 could affect estrogen-induced cyclin D1 expression. As shown in Supplementary Figure S4F, CUEDC2 also inhibited the estrogen-induced upregulation of cyclin D1. Using luciferase reporter assays, we further observed that p21 mRNA decreased in response to CUEDC2 transfection in PR-positive T47D cells, but not in PR-negative HeLa cells (Figure 5F and G). These data indicate that CUEDC2 lowers the expression of PR-responsive genes while inhibiting PR transactivation.

PRB mutants abrogate the inhibitory effect of CUEDC2 on PR

Our and others previous studies have reported that SUMO-1 modification of PRB can occur at Lys-388 (Abdel-Hafiz *et al*, 2002; Chauchereau *et al*, 2003; Man *et al*, 2006). Moreover,

PRB mutation at Lys-388 is resistant to progesterone-induced degradation. In this study, we showed that CUEDC2 promotes PRB degradation by the ubiquitin-proteasome pathway. We next addressed whether CUEDC2 could promote PRB K388R degradation. We transfected Myc-PRB or lysine mutant into 293T cells in the absence or presence of CUEDC2 expression. The results demonstrated that the protein levels of K388R mutant were nearly not affected by CUEDC2 (Figure 6A). Consistent with the results, the transactivity of PRB K388R was inhibited only slightly by CUEDC2. These results suggest that Lys-388 is a key site for regulating CUEDC2 inhibition of PRB.

To exclude the effects inherent to transient transfection systems, we screened cell populations that stably express relatively equal levels of wild-type and mutant PRB K388R. Consistent with the results shown in Figure 6A, when Lys-388 was mutated, the protein level of PRB was not reduced by CUEDC2 (Figure 6B). We performed time courses of PRB loss in progesterone-untreated and treated cell populations stably expressing similar levels of wild-type and mutant PRB. The results suggest that CUEDC2 has no effect on the mutant PRB K388R (Supplementary Figure S5). As shown in Figure 6C, the PRB mutant could still interact efficiently with CUEDC2. Figure 6D also showed that progesterone could not induce the ubiquitination of the Lys-388 mutant, and CUEDC2 cannot promote the ubiquitination of the Lys-388 mutant albeit CUEDC2 can promote the ubiquitination of the wide-type PRB.

As both ubiquitination and sumoylation do not occur when Lys-388 of PRB is mutated, perhaps SUMO-1 and ubiquitin compete for conjugation to the same lysines. A similar mechanism has been observed for I κ B α (Desterro *et al*, 1998), Mdm2 (Buschmann *et al*, 2000), and Huntingtin (Steffan *et al*, 2004). We performed an inverse correlation assay. The results show that ubiquitination attenuates sumoylation at Lys-388 of PR (Figure 6E). To further verify the effect of CUEDC2 on PRB sumoylation, we detected PRB sumoylation in the presence of CUEDC2, and the results showed that CUEDC2 decreases the sumoylation of wide-type PRB while promoting the ubiquitination of wide-type PRB (Figure 6F). These data suggest that CUEDC2 inhibits PRB transactivation by promoting ubiquitin-dependent degradation of PRB and that Lys-388 might be the target of CUEDC2-promoted ubiquitination.

CUEDC2 inhibits progesterone-induced cell proliferation

Upon progesterone treatment, T47D cells have an increase of S phase in the cell cycle (Clarke and Sutherland, 1990; Lange *et al*, 1999). In order to investigate the effects of CUEDC2 on the cell cycle, T47D cells were transfected with HA-CUEDC2 or HA vector and cultured with or without 10 nM progesterone. For cell cycle analysis, cells were harvested, stained with propidium iodide (PI) and DNA content was examined by flow cytometry. After progesterone treatment for 24 h, the cells had a larger S population of 34.12%, compared with 12.24% of cells treated with ethanol (Figure 7A, columns 1 and 2). However, the increase of the cells in S phase was inhibited by CUEDC2 expression (Figure 7A, column 4). To further test the role of endogenous CUEDC2 on cell cycle, T47D cells were transfected with either CUEDC2 siRNA or control siRNA. As shown in Figure 7B, the cells in S phase were increased when CUEDC2 was silenced. These results

Figure 5 CUEDC2 downregulates expression of PR target genes. (A, B) T47D cells were transiently transfected as indicated and treated with 10 nM progesterone for 6 h (cyclin D1) or 24 h (p21). The cells were harvested and total RNA was extracted. Real-time RT-PCR was performed as described. Cyclin D1 and p21 mRNA levels were assessed and normalized to GAPDH mRNA. (C, D) T47D cells were transfected and treated as described in (A) and (B); cyclin D1, p21, and Bax expression was detected using specific antibodies. (E) T47D cells were transiently transfected as indicated. At 18 h post-transfection, cells were treated with 10 nM progesterone for 6 h, and the proteasome inhibitor ALLN (30 μ M) was added 30 min before progesterone treatment. Cells were cultured and Western blot was performed with anti-cyclin D1 and anti-HA. (F, G) p21-Luc (0.2 μ g) and HA-CUEDC2 (2.0 μ g) were cotransfected into T47D and HeLa cells as indicated. The cells were treated as in (A) and (B). Cell lysates were prepared and measured for luciferase activity as described above.

suggested that CUEDC2 inhibited progesterone-induced cell proliferation. It is reported that progestin-induced S-phase entry and cyclin D1 upregulation are MAPK-dependent events (Migliaccio *et al*, 1998; Skildum *et al*, 2005). To determine if CUEDC2 inhibits MAPK activity, we transfected CUEDC2 into T47D cells and detected the phosphorylation levels of p42 and p44. As shown in Figure 7C, CUEDC2 decreases the phosphorylation level of MAPK p42 and p44, suggesting that CUEDC2 inhibits progesterone-induced cell proliferation by decreasing MAPK activity.

CUEDC2 is expressed in human breast cancer cells and other tumor cells

To determine whether CUEDC2 was expressed in tumor cells, we prepared mRNA from the tumor cells as indicated and performed RT-PCR using primers for the CUEDC2 region. As

shown in Figure 7D, we could detect CUEDC2 mRNA in all cells. In addition to CUEDC2 mRNA, CUEDC2 protein expression was also detected by Western blot (Figure 7E). The results show that CUEDC2 proteins are expressed in nearly all tumor cells tested except SAOS-2 and MDA-MB-231 cells.

Discussion

Our study demonstrated that CUEDC2 acts as a novel regulator of PR and promotes PR degradation through the ubiquitin-proteasome pathway. CUEDC2 interacts with PR, repressing the transcriptional activity of PR and blocking progesterone signaling. The transcriptional activity of endogenous PR in T47D breast cancer cells was preferentially enhanced by CUEDC2 siRNA. Moreover, CUEDC2 expression affected the expression of the progesterone-response genes

Figure 6 Mutated PRB resists the inhibitory effect of CUEDC2. (A) 293T cells were transfected with 0.2 μ g of pMMTV-Luc reporter, 1.0 μ g of HA-CUEDC2, and 0.05 μ g of Myc-PRB or its mutant, and treated with or without 10 nM progesterone for 24 h. Cells were harvested and luciferase activity was measured, and cell lysates were probed with anti-HA and anti-Myc (lower panel). (B) HEK-293 cells stably expressing wild-type and mutant PRB K388R were treated as in (A). (C) Total cell lysates expressing Myc-PRB or its mutant were incubated with GST-CUEDC2 or GST. Bound proteins were detected by anti-Myc. (D) 293T cells were transfected as indicated and treated with 10 nM progesterone for 12 h, and the proteasome inhibitor ALLN (30 μ M) was added 30 min before progesterone treatment. Cells were cultured for an additional 12 h and Western blot was performed with antibodies as indicated. (E) 293T cells were transfected with 1.0 μ g Myc-PRB, 1.0 μ g HA-SUMO, and 1.0 μ g His-ubiquitin as indicated. Cells were treated as in (D) and cultured for an additional 12 h. Western blot was performed with antibodies as indicated. (F) 293T cells were transfected with 1.0 μ g Myc-PRB, 1.0 μ g HA-SUMO, 1.0 μ g His-ubiquitin, and 0.5 μ g HA-CUEDC2 as indicated. Cells were treated as in (D) and cultured for an additional 12 h. Western blot was performed with antibodies as indicated and ubiquitination of PRB was detected as in (D). P + C means HA-CUEDC2 and progesterone.

cyclin D1 (Sicinski and Weinberg, 1997) and *p21* (Lange *et al.*, 1999; Skildum *et al.*, 2005). Notably, the ligand-triggered downregulation of PR could almost be abrogated when endogenous CUEDC2 was silenced by specific siRNA.

The effect of CUEDC2 on PR was dependent on its interaction with PR. We characterized the relevant domain for the PR-CUEDC2 interaction by GST pull-down assays. We found that the CUE domain (133–180 aa) is essential for the interaction of PRB and CUEDC2, and the CUEDC2 mutant lacking the CUE domain could not interact with PRB and inhibit transactivation. The CUEDC2 mutant (1–180 aa) encompassing the CUE domain could interact with PRB, but did not inhibit the transactivation of PRB. However, the CUEDC2

mutant (100–226 aa) encompassing the CUE domain could interact with PRB and inhibit its transactivation. Thus, we propose that the 180–226 aa region of CUEDC2 is required for the efficient repression of PR transcriptional activity, and its 133–180 aa CUE domain is sufficient for association with PR. Furthermore, the interaction between CUEDC2 and PR is independent of progesterone. In fact, for both GST pull-down and co-immunoprecipitation assays, progesterone did not markedly affect the interaction of PR with CUEDC2. Progesterone is one of the factors that influence PR levels, and the occupation of PR by its ligand triggers a rapid and extensive downregulation of PR. However, our findings show that the ligand-induced downregulation of PR could almost

Figure 7 CUEDC2 inhibits progesterone-induced cell proliferation. (A) T47D cells were transfected. After 6 h, the cultures were treated with 10 nM progesterone for 24 h and percentage of cells in S phase was determined by PI staining and FACS. (B) T47D cells were transfected as indicated. At 48 h after transfection, cells were treated and analyzed as in (A). (C) T47D cells were transfected with HA-CUEDC2 and treated with progesterone as indicated. Cells were harvested and detected by antibodies as indicated. (D, E) Expression of CUEDC2 in human breast cancer and other tumor cells. RT-PCR from the selected cell lines was performed as described. The tumor cells were also prepared and analyzed by anti-CUEDC2.

be abrogated when endogenous CUEDC2 was silenced by its specific siRNA. These findings clearly indicate the important role of CUEDC2 in the ligand-induced downregulation of PR.

It is interesting to speculate on the mechanisms by which CUEDC2 downregulates PR protein levels. Several studies have revealed the potential mechanisms by which a regulator might regulate the protein level of steroid receptors: decreasing the mRNA levels (Kastner *et al*, 1990) and post-translational modifications, including phosphorylation, ubiquitylation, and sumoylation, which have been shown to regulate PR function and stability (Weigel, 1996; Zhang *et al*, 1997; Lange *et al*, 2000; Abdel-Hafiz *et al*, 2002; Chauchereau *et al*, 2003). In our study, transfection of CUEDC2 resulted in downregulation of PR protein. Consistently, knockdown of CUEDC2 expression by CUEDC2 siRNA caused an accumulation of PRB protein. Furthermore, we found that CUEDC2 degraded PRB expression in a ligand-dependent manner, and this degradation was effectively blocked by inhibitors of ubiquitin-proteasome pathway. PR has been reported to be modified by ubiquitination. In our study, we found that CUEDC2 could markedly promote the ubiquitination of PR and target PR for degradation. Phosphorylation of PR at S294 has been reported to target PR for degradation, and the mutant PRS294A almost abrogated the degradation of PR protein (Lange *et al*, 2000). We examined the interaction of CUEDC2 and PRBS294A by GST pull-down assay, and found that phosphorylation at Ser-294 did not affect the interaction between PRB and CUEDC2 (Supplementary Figure S6A). Further experiments revealed that CUEDC2 could not promote degradation of PRBS294A (Supplementary Figure S6B). These results show that phosphorylation of PRB at Ser-294 is a critical step in the initiation

of PR degradation by CUEDC2. At the same time, we did not observe significant changes in PR mRNA levels. Therefore, protein degradation rather than transcriptional regulation should account for this effect of CUEDC2 on PR.

We further analyzed the PR lysine residues that influenced the CUEDC2-promoted degradation of PR protein, and found that the sumoylation site Lys-388 was targeted by CUEDC2-promoted ubiquitination. The mutation of lysine at 388 made PR resistant to CUEDC2-promoted degradation through the ubiquitin-proteasome pathway. In contrast, CUEDC2 could promote wild-type PRB degradation, indicating that Lys-388 plays an important role in PR ubiquitin-proteasome degradation promoted by CUEDC2. Consistently, CUEDC2 expression significantly inhibited the transactivation of wild-type PRB, but not the PRB mutant at Lys-388 as indicated in Figure 6. Notably, the mutation at Lys-388 in the IF domain of PRB did not affect the interaction with CUEDC2, but made PR insensitive to CUEDC2-promoted ubiquitination. These results support that CUEDC2 promotes ubiquitin-dependent degradation of PRB and inhibits transactivation. Interestingly, SUMO-1 modification could also occur at Lys-388 (Abdel-Hafiz *et al*, 2002; Chauchereau *et al*, 2003), and our results show that CUEDC2 attenuates sumoylation of Lys-388 while promoting ubiquitination. The inverse correlation between ubiquitination and sumoylation suggests that the sumoylation site Lys-388 was also the site targeted by CUEDC2-promoted ubiquitination.

An intricate array of coactivators, corepressors, and coin-tegrators are recruited to receptor-bound promoters such as PR and other nuclear receptors, and exert regulatory effects (Wagner *et al*, 1998; Maldonado *et al*, 1999; Wardell *et al*, 2002). To date, a number of these coactivators have been

identified and characterized. For instance, SRC-1 and JDP-2 are well-known steroid receptor coactivators (Wardell *et al*, 2002) that interact with the AF2 domain of PR and increase PR transcriptional activity in a ligand-dependent manner. Although coregulator studies have focused mainly on transcriptional activation, transcriptional repression is also critical to understanding gene regulation (Abdel-Hafiz *et al*, 2002). In our study, the repression of PR transcriptional activity by CUEDC2 appeared not to involve these mechanisms that transcriptional repression involves blockade of coactivator binding, and collaboration with corepressor (Maldonado *et al*, 1999). Our results demonstrated that CUEDC2 expression did not affect the association of JDP-2 and SMRT with PR (Supplementary Figure S7A). Furthermore, our results showed that CUEDC2 expression did not abolish the effects of SRC-1 and JDP-2 on PR transactivation (Supplementary Figure S7B and C), and that CUEDC2 did not inhibit PRB transactivation through corepressors NcoR and SMRT (Supplementary Figure S7D). These results indicate that the mechanisms by which CUEDC2 represses PR transactivation are not associated with the currently known activities of coregulators.

Hissom and Moore first reported the proliferative effect of progesterone on T47D cells. Indeed, the immediate response of asynchronous cultured T47D human breast cancer cells to a single pulse of progesterone is proliferation (Clarke and Sutherland, 1990; Musgrove *et al*, 1991; Sartorius *et al*, 1994a). There is transient induction of gene associated with cell cycle progression, including increased expression of cyclin D1 (Groshong *et al*, 1997) in the first 12 h. Our results show that CUEDC2 greatly reduces expression of the progesterone-regulated endogenous gene *cyclin D1*. In addition, Skildum *et al* reported that PR induced proliferation through activation of cytoplasmic kinases, which was blocked by inhibition of MAPK. We found that CUEDC2 attenuates the ability of PR to stimulate rapid MAPK activity, which significantly inhibits the transient growth stimulatory effects of progesterone and accelerating cells to the G1 checkpoint in the cell cycle. Consistent with its role in regulating PR stability and transactivation, CUEDC2 expression impairs the effect of progesterone on breast cancer cell growth, reflecting its functional significance.

In conclusion, our results demonstrate that CUEDC2 interacts with PR and promotes progesterone-induced PR degradation by the ubiquitin-proteasome pathway. CUEDC2 also inhibited the ability of PR to stimulate rapid MAPK activity and impaired the effect of progesterone on breast cancer cell growth. Therefore, our results identify a key post-translational mechanism that controls PR protein levels and for the first time provide important insight into the function of CUEDC2 in breast cancer proliferation.

Materials and methods

Plasmid constructions

PRA and PRB cDNAs were gifts from Professor O'Malley and were further cloned into pGADT7, pXJ40-Myc, and pcDNA3.0-Flag vectors. To construct pXJ40-HA-CUEDC2, full-length cDNA fragment was generated by PCR from human mammary cDNA library and transferred into pXJ40-HA vector. Myc- or Flag-tagged PRB mutants K388R and S294A were generated by PCR-mediated site-directed mutagenesis. Plasmids pEGFP-N1 and pDsRed-N1 were from Clontech. For subcellular localization assay, the CUEDC2 and

PRB cDNAs were transferred into the pEGFP-N1 and pDsRed-N1 vectors respectively. For mapping assay, PRA, PRB and its mutants $\Delta 1-165$ aa (Δ AF3), $\Delta 155-475$ aa (Δ IF), $\Delta 455-556$ aa (Δ AF1), $\Delta 536-652$ aa (Δ DBD), $\Delta 632-933$ aa (Δ AF2) were generated by PCR and cloned into pGADT7 and pXJ40-Myc vectors, and CUEDC2 cDNA was inserted into pGBKT7 vectors (Clontech Laboratories Inc.). To generate bacterial expression vector for GST-CUEDC2 and the mutants, the corresponding CUEDC2 cDNAs (1-226, 1-133, 1-180, 100-226, and $\Delta 133-180$ aa) were cloned in-frame into pGEX-KG vector (Amersham Pharmacia Biotech). The relative amplified CUEDC2 and its mutant fragments were also cloned into pcDNA3.0-Flag vectors. The *Renilla* vector (pRL-TK) was from Promega and the reporter pMMTV-Luc was kindly provided by Professor Palvimo Jorma.

Yeast two-hybrid assay

The bait plasmid was created by inserting the IF domain of PRB into pGBKT7, resulting in a fusion with the Gal4DNA-binding domain. The resultant plasmid and a mammary cDNA library were simultaneously transformed into AH109 yeast strains as previously described (Pan *et al*, 2006).

Fluorescence microscopy

293T cells were seeded in 6 cm plates on glass coverslips and cultured in medium containing phenol red-free DMEM (Hyclone) supplemented with 5% charcoal-filtered fetal bovine serum (Biochrom AG, German). At 24 h after transfection with GFP-CUEDC2 and RFP-PRB, cells were treated and analysis performed as described in our previous study (Man *et al*, 2006).

RNA interference

The small interfering RNA (siRNA) to target CUEDC2 was chemosynthesis and the target sequence was 5'-TAGGGGACATGATGCAGAA-3' and the scrambled sequence was 5'-GACGGTCCAGATTCTGTGA-3'. The 1.5 μ g siRNA was transfected into T47D cells. After 48 h, the cells were transfected with pMMTV-Luc reporter (0.2 μ g) and siRNA. After being treated with 10 nM progesterone for another 24 h, cells were lysed for PRB-luciferase reporter assay. The nonspecific RNA was used as a control. The relative expression of endogenous CUEDC2 was detected by anti-CUEDC2 (from our laboratory) Western blot from control or siRNA-treated cells.

Transfection and luciferase assay

MCF-7, NIH3T3, HeLa, T47D, and 293T cells were transfected using Lipofectamine2000 (Invitrogen) with 0.2 μ g of pMMTV-Luc reporter, ERE-TK-Luc, p53-LUC, Spi2.1-GLE1-CAT luciferase reporter or p21 promoter, 0.02 μ g of *Renilla* reporter pRL-TK, with or without 0.05 μ g of pXJ40-Myc-PRB (wild type or mutant) or pXJ40-Myc-PRA, and various amounts of the pXJ40-HA-CUEDC2 expression vectors. After treatment with 10 nM progesterone as indicated, the transfected cells were harvested. Luciferase activity was determined as described in a previous study (Man *et al*, 2006). All experiments were repeated at least three times.

Immunoprecipitation and immunoblotting

293T cells were transfected as indicated and then lysed in IP lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM DTT, 1 $\times$ cocktail). After brief sonication, the lysates were cleared by centrifugation at 4°C. Supernatants were incubated with anti-Myc or anti-HA for 4 h and protein A/G-Sepharose beads for 2 h at 4°C. The immunocomplexes were washed three times, boiled in sample buffer, and immunoblotted with anti-Myc and anti-HA (Santa Cruz Biotechnology). For CHX chase assays, cells were treated with 20 μ g/ml CHX and with or without 10 nM progesterone 18 h after transfections, collected at indicated time points and cell lysates were subjected to Western blot.

Real-time RT-PCR analysis

T47D cells were transfected with 2.0 μ g of pXJ40-HA-CUEDC2 and treated with 10 nM progesterone for 6 h (for cyclin D1) or 24 h (for p21). Cell pellets were collected and RNA was extracted by Trizol (Sigma). The diluted RNA was analyzed as described in our previous study (Man *et al*, 2006), and PCR was performed specifically with cyclinD1, p21, PR, CUEDC2, and GAPDH primers.

Ubiquitylation assay

293T cells were transfected with Myc-PRB or its mutant (2.0 µg) and His-ubiquitin (2.0 µg) in the presence or absence of HA-CUEDC2 (2.0 µg) for 12 h in DMEM with 5% charcoal-filtered serum. Then, the cells were treated with or without progesterone (10 nM) for additional 12 h in the presence of proteasome inhibitor ALLN (Sigma) and subsequently harvested using IP lysis buffer. PRB proteins were immunoprecipitated with an anti-Myc antibody and subjected to SDS-PAGE, followed by Western blot with anti-Myc antibody.

Flow cytometry

T47D cells were plated in six-well plates in phenol red-free DMEM supplemented with 5% charcoal-filtered serum for 2 days and transfected with 2.0 µg of HA-CUEDC2 or CUEDC2 siRNA as indicated. After 6 h, the cultures were treated with or without 10 nM progesterone for 24 h. The cells were harvested and resuspended in 0.3 ml PBS containing 10% FBS, and ice-cold 100% ethanol (0.7 ml). The cells were incubated with RNase A (10 µg/ml) and 50 µg/ml PI (Sigma). DNA fluorescence of nuclei

was measured with a FACScan flow cytometer (Becton Dickinson), and percentages of S phase of cell cycle were analyzed using FACScan software programs.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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