Estrogen receptor-α, RBCK1, and protein kinase C β1 cooperate to regulate estrogen receptor-α gene expression

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Abstract

Estrogen receptor α (ERα) is initially overexpressed in two-thirds of all breast cancers and is involved in its development and proliferation. We previously reported that the RanBP-type and C3HC4-type zinc finger containing 1 (RBCK1) interacts with the ERα promoter and that RBCK1 expression positively correlates with ERα levels, expression of ERα downstream target genes, and proliferation of breast cancer cells. Based on this, and that RBCK1 positively correlates with ERα expression in breast cancer samples, we propose RBCK1 as a potential therapeutic target in breast cancer acting as a modulator of ERα expression. To further explore this, the molecular mechanism by which RBCK1 regulates ERα expression has to be defined. Here, we show that ERα, RBCK1, and the RBCK1-interacting protein kinase C β1 (PKCβI) co-occupy a previously identified ERα binding region in the proximal ERα promoter. We describe a number of mechanistic details of this complex including that RBCK1 recruitment to the ERα promoter B is facilitated by ERα, which in turn facilitates PKCβI recruitment and PKCβI-dependent histone modifications. Furthermore, ERα regulation of its own mRNA expression is facilitated by RBCK1 recruitment, suggesting an ERα coactivator function of RBCK1. The interaction between RBCK1 and ERα was dependent on the E3 ubiquitin ligase domain of RBCK1 and the activating function-1 domain of ERα. The ligand-binding function of ERα does not influence the interaction with RBCK1. In summary, our data provide insight into the molecular mechanism by which ERα expression is modulated in breast cancer cells.

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Introduction

Estrogen receptor α (ERα) belongs to the nuclear hormone receptor superfamily of transcription factors and regulates genes including those involved in cell proliferation, differentiation, and motility in the breast (Dahlman-Wright et al. 2006). ERα expression in mammary tissue correlates with the risk of developing breast cancer (Fabris et al. 1987, Khan et al. 1994, Shaaban et al. 2002), and two-thirds of all breast cancers initially overexpress ERα (Ali & Coombes 2002). Breast cancers overexpressing ERα can only partly be accounted for by ERα gene amplification, with associated increased ERα expression, as observed in ~50% of ERα-positive breast cancers (Holst et al. 2007). Thus, it has been suggested that other processes, such as control of ERα transcription and mRNA and protein stability, are deregulated in the remaining 50% of ERα overexpressing breast cancers (Fowler & Alarid 2007). Therapy for ERα-positive breast cancer includes receptor antagonists, such as tamoxifen, that inhibits ERα-mediated proliferation in breast cancer (Early Breast Cancer Trialists’ Collaborative Group 2005). Approximately 80% of ERα-positive breast cancers are initially responsive to tamoxifen treatment; however, one-third of women treated with tamoxifen for 5 years will have recurrent disease within 15 years (McDonnell & Wardell 2010). Thus, there is a clear need to increase the knowledge of the molecular details regulating ERα signaling, and identifying regulators of ERα expression in breast cancer cells is one strategy to identify novel targets to inhibit proliferative ERα signaling in breast cancer. However, the knowledge of the regulation of the ERα gene in both normal cells and in breast cancer cells is relatively limited.

Multiple promoters have been described for the ERα gene, each initiating transcripts with a unique 5′-UTR. The promoters are subject to tissue- and cell-specific regulation (Flouriot et al. 1998, Kos et al. 2001). Breast cancer-selective regulation of ERα expression via promoter B has been suggested, as ERα mRNA levels transcribed from this promoter correlates with increased ERα levels in breast cancer patients as well as in breast cancer cells in culture (Hayashi et al. 1997, Tanimoto et al. 1999). ERα binding sites have been identified in the ERα gene promoter B, as well as in upstream enhancer regions (Carroll et al. 2006). Consistent with this, it has been shown that ERα can regulate its own expression (Castles et al. 1997, Ellison-Zelski et al. 2009). Under normal physiological
conditions, expression of ERz and estrogen levels are inversely related and repression of the ERz promoter by recruitment of ERz upon estrogen stimulation has been reported (Ellison-Zelski et al. 2009). However, in breast cancer, this inverse relationship between ERz and its agonist is lost, indicating deregulation of ERz expression (Khan et al. 1999).

RBCK1 is an E3 ubiquitin ligase belonging to the RING-between rings-RING (RBR) family of ubiquitin ligases (Marin & Ferrus 2002, Tatematsu et al. 2008) and was originally identified as a protein kinase Cβ1 (PKCβ1)-interacting protein (Cong et al. 1997, Tokunaga et al. 1998). RBCK1 contains a transcriptional activation domain at its C-terminal domain. The C-terminal domain also includes a nuclear localization signal which, together with a nuclear export signal at the N-terminal domain, enables RBCK1 to shuttle between the nucleus and the cytoplasm (Tatematsu et al. 1998, 2005).

The RBCK1-interacting protein PKCβ1 was recently linked to transcriptional regulation during nuclear receptor target gene activation through maintenance of histone H3 dimethyl lysine 4 (H3K4me2) via phosphorylation of histone H3 threonine 6 (H3T6ph) (Metzger et al. 2010). H3K4me2 is a marker for gene activation and has been implicated in activation of the ERz target gene promoter (Nair et al. 2010).

We previously reported that RBCK1 is recruited to the ERz promoter B and that reduced RBCK1 levels lead to reduced ERz levels, reduced expression of ERz target genes, and cell cycle arrest in breast cancer cells (Gustafsson et al. 2010). Thus, RBCK1 is a modulator of ERz signaling, potentially by directly regulating ERz expression. The fact that RBCK1 is overexpressed in breast cancer samples and that its expression correlates with ERz mRNA expression in clinical samples (Richardson et al. 2006, Desmedt et al. 2007) provides further support for an important role of RBCK1 in breast cancer. We hypothesize that RBCK1 could constitute a molecular target to modulate ERz expression and estrogen signaling, ultimately leading to decreased breast cancer proliferation. However, to further explore RBCK1 as a potential therapeutic target, the molecular mechanism by which RBCK1 regulates ERz expression has to be defined. In this study, we provide novel insight into the molecular mechanism of regulation of ERz expression by RBCK1 in breast cancer cells.

Materials and methods

Cell culture and reagents

T-47D human breast cancer cell line and COS-7 monkey kidney fibroblast-like cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). MCF-7 human epithelial breast cancer cells developed at the Michigan Cancer Foundation (kindly provided by Dr Robert P C Shi, University of Manitoba, Canada, USA). T-47D cells were cultured in RPMI 1640 (Invitrogen) and MCF-7 and COS-7 cells were cultured in DMEM (Invitrogen). All cell culture media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen). Cells were grown at 37°C in 5% CO₂. For experiments involving treatment with 10 nM 17β estradiol (E2) or 4-hydroxytamoxifen (4-OHT; Sigma), cells were seeded 24 h before ligand treatment in DMEM without phenol-red supplemented with dextran-coated charcoal (DCC-FBS). The PKC inhibitor Go6976 was purchased from Tocris Biosciences (Bristol, UK), and dimethyl sulfoxide (Sigma) was used as control.

Plasmid construction

The cDNA encoding human RBCK1 (Invitrogen) was subcloned into pcDNA3-Flag (modified from Invitrogen) using standard PCR-based cloning. RBCK1 constructs were made using the sense primer 5'TTT GAA TTC ATG GAC GAG AAG ACC AAG AAA GCA 3 and the following antisense primers: RBCK1 1–447 (antisense 5’-AAA CTC GAG CTA GCG CAT GGC 3), RBCK1 1–352 (antisense 5’-AAA CTC GAG CTA GCG CAT GGC 3), RBCK1 1–282 (antisense 5’-AAA CTC GAG CTA GCG CAT GGC 3). RBCK1 1–352 (antisense 5’-AAA CTC GAG CTA GCG CAT GGC 3), RBCK1 1–282 (antisense 5’-AAA CTC GAG CTA GCG CAT GGC 3). pcDNA3-ERz was provided by Nina Heldring (Karolinska Institute, Sweden). The full-length FLAG-ERz and FLAG-ERz amino acid 180–595 (ERzΔAF1) constructs were cloned in a pcDNA3/pRFT vector and have been described previously (Matthews et al. 2007). FLAG-ERz 1–535 (ERzΔH12) was constructed by introducing a stop codon in the full-length FLAG-ERz construct using site-directed mutagenesis. Correct sequences were verified using DNA sequencing. The pGL2-derived luciferase reporter plasmid ERz-promoter-luc was kindly provided by RJ Wiegel (22).

siRNA transfection

siRNA transfections were carried out using a final concentration of 50 nM oligo (at 40–60% cell confluence) using INTERFERin transfection reagent (PolyPlus-Transfection SAS, Illkirch, France) according to the manufacturer’s instructions. Stealth Select siRNA Catalog# HSS145705 and HSS145706, referred to in this study as siRBCK1; ERz– Stealth Select siRNA Catalog# HSS103375 and HSS103376, referred to as siERz; and a control siRNA – Individual Stealth Select siRNA, referred to as siControl (Invitrogen).
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Reporters gene assay

Cells were seeded into six-well plates 24 h before transfection. Plasmid transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer’s instructions, all transfections included 20 ng of a β-galactosidase construct. In case of ligand treatment, ligands were added in cell media at the same time point as transfections and luciferase activity was assayed after 16 h treatment. Luciferase and β-galactosidase activities were measured using a TECAN luminometer instrument. Luciferase activity was normalized against β-galactosidase activity.

RNA isolation and quantitative real-time PCR (RT-PCR)

MCF-7 cells were seeded into six-well plates 24 h before transfection or treatment with Go6976. Total RNA was extracted using the RNeasy kit (Qiagen) and reversely transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen) with random hexamers. RT-PCR measurements were performed in triplicate using SYBR Green reagent (Applied Biosystems) in a 7500 ABI Real-Time PCR thermocycler (Applied Biosystems). Twofold serial dilutions of cDNA were used to produce standard curves, Ct values were converted to nanograms, and 36B4 was used as reference gene. Primer sequences have been published before (Gustafsson et al. 2010).

Western blot analysis

Cells were seeded into six-well plates, 100 mm plates, or 150 mm plates 24 h before transfection or treatment and lysed in Laemmli sample-loading buffer at indicated time points. The following primary antibodies were used for immunoblotting: monoclonal mouse β-actin antibody (Sigma–Aldrich), polyclonal goat RBCK1, polyclonal rabbit ERz, polyclonal rabbit PKCβ1 (Santa Cruz Biotechnology), Histone H3K4me3, Histone H3K4me2, Histone H3K4me3 (Active Motif, Inc., Carlsbad, CA, USA), or Histone H3T6ph (Abcam, Cambridge, UK) antibodies. Species-specific IgG was used as control in all ChIP assays. A region of the ERz gene known not to bind ERz, RBCK1, or PKCβ1 was used as a control. reChIP assays were performed as described previously (Kouskouti & Talianidis 2005). Precipitated chromatin regions were quantified by RT-PCR. Input samples were used to produce standard curves to calculate recruitment to DNA, and then fold recruitment over IgG was calculated. Recruitment to DNA was considered to be significant when fold enrichment was > 2 with a P value of < 0.05.

Statistical analysis

Unless otherwise stated, all experiments were carried out in triplicates. Student’s t-test was used for group comparisons. P value of < 0.05 was considered significant.

Immunoprecipitations

MCF-7 cells were plated in 100 mm dishes 48 h before immunoprecipitations for investigating endogenous protein interactions. COS-7 cells were seeded into 100 mm dishes 24 h before transfection with 4 μg plasmid, and cells were assayed for protein interactions 48 h post-transfection. Cells were pelleted for 3 min at 6000 g, then lysed in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 1% NP40, complete protease inhibitors (Roche),) and then incubated for 30 min on ice followed by centrifugation for 30 min at 18000 g. Ten percent of the supernatant was removed as input. Pre-clearing was done with 20 μl Protein G slurry and 1 μg IgG for 1 h at 4°C. The supernatant was collected and immunoprecipitated with ERz, RBCK1, IgG, or PKCβ1 antibodies (Santa Cruz Biotechnology) overnight at 4°C. The following day, 40 μl Protein G slurry was added for 2 h at 4°C, then protein complexes were washed four times in 500 μl lysis buffer, and lysed in 60 μl Laemmli sample-loading buffer. Protein complexes were subjected to 10% SDS–PAGE followed by western blot analysis with ERz, RBCK1, or PKCβ1 antibodies (Santa Cruz Biotechnology).

Results

RBCK1 enhances ERα promoter activity

Our previous data has shown that RBCK1 interacts with the ERz promoter B in MCF-7 breast cancer cells and that reducing RBCK1 expression leads to reduced ERα expression (Gustafsson et al. 2010). Figure 1A shows that transcripts derived from ERz promoter B, and also to a lower extent A and E2, are expressed in MCF-7 breast cancer cells. Additionally, depletion of RBCK1 leads to decreased expression of transcripts derived from promoters A and B (Gustafsson et al. 2010). As the distance is small between promoters B and A (Fig. 1A),
Figure 1 RBCK1 activates ERα expression in breast cancer. (A) Upper panel, schematic representation of the ERα promoters and upstream exons. Lower panel, relative expression of promoter-associated ERα exon expressions in MCF-7 cells, as determined by RT-PCR. Reverse primer was set to the common acceptor splice site at position +163 unless otherwise stated, primer sequences have been described earlier (Nilsson et al. 2007). (B) MCF-7 cells were co-transfected with 1 µg ERα promoter luciferase reporter and indicated amounts of FLAG-RBCK1 or control FLAG. Luciferase activity was measured after 48 h. (C and D) MCF-7 cells were transfected with 1 µg FLAG-RBCK1 or FLAG. (C) ERα mRNA levels were determined by quantitative RT-PCR for indicated time points. (D) ERα and FLAG-RBCK1 protein levels were determined after 48 h by immunoblot with α-actin used as loading control. Image J was used to perform quantitative densitometry of ERα expression. A representative of three independent experiments is shown. (E) T-47D cells were transfected with 1 µg ERα promoter luciferase reporter and 1 µg FLAG-RBCK1 or FLAG. Luciferase activity was measured after 48 h. *P<0.05, **P<0.01, ***P<0.001 for RBCK1 vs FLAG. Columns represent the arithmetic mean of fold change compared to FLAG-transfected cells; bars, s.d. (n=3).

As RBCK1 and ERα co-occupy the same chromatin region in the ERα promoter B, we assayed protein–protein interactions between endogenous RBCK1 and ERα. MCF-7 cells were subjected to immunoprecipitation with an RBCK1 antibody followed by detection of ERα by western blot analysis (Fig. 2B, IP1) or immunoprecipitation with an ERα antibody followed by detection of RBCK1 by western blot analysis (Fig. 2B, IP2). RBCK1 and ERα co-precipitated in both IP1 and IP2, whereas control IgG did not precipitate RBCK1 or ERα (Fig. 2B).

RBCK1 and ERα functionally interact at the ERα promoter B

RBCK1 is recruited to the ERα promoter B, which is the same promoter region that has previously been shown to recruit ERα (Carroll et al. 2006, Gustafsson et al. 2010). We, therefore, investigated the potential cross talk between RBCK1 and ERα with regard to interaction with the ERα promoter B and regulation of ERα promoter activity. Sequential ChIP was employed to demonstrate co-occupancy of RBCK1 and ERα at the ERα promoter B. Using an RBCK1 antibody for ChIP, followed by reChIP with an ERα antibody, we observed that RBCK1 and ERα co-localize to the same region in the ERα promoter B in MCF-7 cells (Fig. 2A). Co-localization of RBCK1 and ERα at the ERα promoter B was also observed under reciprocal conditions initially performing ChIP with an ERα antibody followed by reChIP with an RBCK1 antibody and also in T-47D cells (data not shown). ERα binding sites have also been identified in upstream enhancer regions of the ERα gene (Carroll et al. 2006). However, we did not observe any recruitment of RBCK1 to these regions, indicating that RBCK1 and ERα specifically co-occupy a region of the ERα promoter B (data not shown).

In order to determine whether the activation of the ERα promoter by RBCK1 is restricted to MCF-7 cells, we cotransfected RBCK1 and the ERα promoter reporter into ERα-positive T-47D breast cancer cells. Figure 1E shows that RBCK1 also upregulates the ERα promoter activity in this cell line. Thus, RBCK1 activation of the ERα promoter is not restricted to MCF-7 cells.
The functional interaction between RBCK1 and ERα was further explored in reporter assays. Co-transfection of RBCK1 and ERα led to a further upregulation of the ERα promoter reporter activity compared with what was observed upon transfection with RBCK1 or ERα alone (Fig. 2D).

We next investigated the potential influence of ERα, the agonist E2, or the antagonist 4-OHT on the RBCK1-mediated activation of the ERα promoter. After transfections, cells were maintained in steroid-free media for 24 h before ligand treatment. Treatment with E2 or 4-OHT did not influence the activation of the ERα promoter reporter by RBCK1 compared with vehicle (Fig. 2E), suggesting that functional interactions between RBCK1 and ERα involved in regulating ERα promoter activity are not influenced by ligand modulation of ERα. In line with this, protein–protein interactions between RBCK1 and ERα were not influenced by E2 or 4-OHT (data not shown). In summary, these data identify RBCK1 as a novel ERα-interacting protein and suggest that interactions between RBCK1 and ERα enhance activation of the ERα promoter. Furthermore, our results do not support a role of ERα ligands in interactions between RBCK1 and ERα.

Characterization of ERα and RBCK1 interaction domains

To begin to dissect regions of RBCK1 that are required for interaction with ERα, RBCK1 deletion variants were assayed for interaction with ERα. A schematic representation of the RBCK1 domain structure is shown in Fig. 3A. Full-length RBCK1 and RBCK1 (1–447) lacking the C-terminal putative RING finger interacted with ERα whereas deletion of the IBR-domain, RBCK1 (1–352), abolished the interaction with ERα (Fig. 3B).

To determine whether regions of RBCK1 required for interaction with ERα were overlapping with regions involved in activation of the ERα promoter, RBCK1
deletion variants were assayed for their capacity to activate the ERα promoter. The RBCK1-dependent upregulation of the ERα promoter was drastically reduced by deletion of the C-terminal putative RING domain, RBCK1 (1–447) (Fig. 3C). Thus, sequences of RBCK1 required for activation of the ERα promoter extends beyond those required for interaction with ERα.

To determine regions of ERα that are critical for interaction with RBCK1, we employed ERα deletion constructs in co-immunoprecipitation assays. We focused on the two activation functions described for ERα, with either deletion of helix 12 (H12) that is critical for ligand-dependent transcriptional activation or deletion of the activating function-1 (AF-1) domain, which corresponds to the non-ligand-dependent transcriptional activation function. We observed that the interaction between RBCK1 and ERα was intact upon deletion of H12 but completely abolished upon deletion of the AF-1 domain (Fig. 3D). Thus, the ligand-binding function of ERα does not influence the interaction with RBCK1, which is consistent with our data that ligands do not influence the RBCK1–ERα interaction.

ERα recruits RBCK1 to the ERα promoter B with RBCK1 enhancing ERα expression

To further elucidate the molecular mechanism of RBCK1 regulation of the ERα promoter B, we investigated the importance of ERα for RBCK1 recruitment to the ERα promoter B. Figure 4A (left panel) shows that reducing ERα levels reduced the recruitment of RBCK1 to the ERα promoter B. Importantly, RBCK1 protein levels were not changed (Fig. 4A, right panel), demonstrating that the decreased promoter occupancy of RBCK1 is not due to reduced RBCK1 protein levels.

**Figure 3** Domain analysis of RBCK1 and ERα interacting domains and RBCK1 domain required for ERα promoter activation. (A) Schematic representation of the full-length RBCK1 domain structure. (B) COS-7 cells were transfected with ERα and RBCK1 deletion constructs and interactions of ERα and FLAG-RBCK1 constructs were examined by immunoprecipitations with ERα antibody. FLAG antibody was used for immunoblot to confirm interactions. Shown is a representative of three independent experiments. (C) Requirement for the putative RING finger in RBCK1 for transcriptional activation of the ERα promoter. 1 μg ERα promoter reporter was cotransfected with 1 μg FLAG or FLAG-RBCK1 constructs as indicated. Luciferase activity was measured 48 h post-transfection. *P < 0.05; **P < 0.01; for RBCK1 constructs vs FLAG. Columns represent the arithmetic mean of fold change compared with FLAG-transfected cells; bars, s.d. (n=3). (D) MCF-7 cells were transfected with FLAG-ERα deletion constructs and interactions of deleted variants of FLAG-ERα with endogenous RBCK1 were assayed by immunoprecipitations with RBCK1 antibody followed by immunoblot analysis using a FLAG antibody to detect immunoprecipitated FLAG-ERα constructs. Shown is a representative of two independent experiments.
We next examined the effect of RBCK1 depletion on ERα occupancy at the ERα promoter B. To avoid decreased ERα protein levels as a consequence of RBCK1 silencing (Gustafsson et al. 2010), which could result in decreased ERα recruitment to its own promoter, we performed the assay at a time point when RBCK1 depletion had not yet affected ERα protein levels. RBCK1 protein levels began to decrease at 24 h after siRBCK1 transfection (Fig. 4B, left panel); however, ERα protein levels remained unchanged up to 30 h (Fig. 4B, right panel). Thus, the assay was conducted 24 h after RBCK1 siRNA transfection. At this time point, occupancy of RBCK1 on the ERα promoter B was significantly decreased in RBCK1-depleted cells but recruitment of ERα remained unchanged (Fig. 4C), suggesting that the occupancy of ERα at the ERα promoter B is not dependent on RBCK1. In a parallel analysis of ERα mRNA expression, we observed that ERα mRNA started to decrease after 25 h of siRBCK1 treatment (Fig. 4D), even though ERα recruitment to the promoter remained unchanged up to 30 h post-siRBCK1 (data not shown). Together, these data suggest a model where ERα is necessary for association of RBCK1 to the ERα promoter B with formation of an ERα–RBCK1 complex important for maintaining ERα expression.

RBCK1-dependent recruitment of PKCβ1 and histone modifications at the ERα promoter B

Previous studies have demonstrated that the RBCK1-interacting protein PKCβ1 activates transcription through maintenance of histone H3 dimethyl lysine 4 (H3K4me2) via phosphorylation of histone H3 threonine 6 (H3T6ph), suggesting a potential role for PKCβ1 in mediating RBCK1-dependent histone modifications.

As interactions between RBCK1 and PKCβ1 (Tokunaga et al. 1998, Tatematsu et al. 2008) have not been investigated in breast cancer cells, we first assayed potential interactions between RBCK1 and PKCβ1 in MCF-7 cells. Immunoprecipitation with an RBCK1 antibody followed by western blot analysis for PKCβ1 revealed protein–protein interactions between endogenous RBCK1 and PKCβ1 (Fig. 5A).

Furthermore, ChIP assay with RBCK1 antibody followed by reChIP with PKCβ1 antibody revealed co-occupancy of RBCK1 and PKCβ1 at the ERα promoter B (Fig. 5B). To determine whether PKCβ1 recruitment to the ERα promoter B is dependent on the presence of RBCK1 at the promoter, RBCK1 levels were reduced by siRNA followed by a ChIP assay with PKCβ1 antibody. Importantly, we observed reduced occupancy of PKCβ1 on the ERα promoter B in RBCK1-depleted

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**Figure 4** ERα recruits RBCK1 to the ERα promoter B. (A) MCF-7 cells were transfected with siControl or siERα for 24 h. Recruitment of ERα and RBCK1 was examined by ChIP and DNA was quantified by RT-PCR. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. (n=3). *P<0.05 for siERα vs siControl. 10% of input was used for determination of ERα and RBCK1 protein levels by immunoblot, β-actin was used as loading control. Shown is a representative of two independent experiments. (B, C and D) MCF-7 cells were transfected with RBCK1 siRNA for indicated time points. (B) ERα and RBCK1 was examined by ChIP and DNA was quantified by RT-PCR. Columns represent the arithmetic mean of fold change over siControl; bars, s.d. (n=2). *P<0.05; **P<0.01 for siRBCK1 vs siControl.

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**Table 1**

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<th>Time-point</th>
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**Fold enrichment**
ER effects on histone modifications, thereby activating a model where PKC\(\text{H3K4me2}\) and \(\text{H3T6ph}\) modifications. This suggests (Fig. 5E). It should be noted that the level of active promoters, was decreased by siRBCK1 consistent with a highly active promoter region. H3K4me2 and H3K4me3 recruitment is very high, and PKC\(\text{promoter B}\) is necessary for H3K4me3 modification of PKC\(\text{depleted cells. Thus, RBCK1 is needed for recruitment}

Figure 5D shows that H3K4me2 (left panel, white bars) and H3T6ph (right panel, white bars) are significantly decreased in the ER\(\text{promoter B}\) region of RBCK1-depleted cells. Thus, RBCK1 is needed for recruitment of PKC\(\text{to the ER}\alpha\) promoter and for active histone modifications that have been shown to be maintained by PKC\(\text{Additionally, H3K4me3, a recognized marker of active promoters, was decreased by siRBCK1 (Fig. 5E). It should be noted that the level of H3K4me2 and H3K4me3 recruitment is very high, consistent with a highly active promoter region.}

In summary, RBCK1 occupancy on the ER\(\alpha\) promoter B is necessary for H3K4me3 modification and PKC\(\text{association, as well as PKC\(\text{dependent H3K4me2 and H3T6ph modifications. This suggests a model where PKC\(\text{mediates the RBCK1-induced effects on histone modifications, thereby activating ER\(\alpha\) transcription (Fig. 6).}

PKC\(\beta\) kinase activity modulates ER\(\alpha\) signaling

We investigated the importance of a functional PKC\(\beta\) kinase activity for H3T6 phosphorylation and subsequently ER\(\alpha\) expression by treating MCF-7 cells with the PKC\(\beta\) kinase inhibitor Go6976. Figure 7A shows that H3T6ph is significantly decreased in the ER\(\alpha\) promoter B region in Go6976-treated cells. Furthermore, we observed decreased ER\(\alpha\) mRNA levels in Go6976-treated cells (Fig. 7B), supporting a critical role for the PKC\(\beta\) kinase activity for the regulation of ER\(\alpha\) promoter B activity. Furthermore, cells treated with Go6976 displayed significantly decreased ER\(\alpha\), but not RBCK1, protein levels (Fig. 7C). Consistent with Go6976 downregulating ER\(\alpha\) expression, the compound downregulated the expression levels of the ER\(\alpha\) target genes cyclin D1 (Fig. 7D, upper panel) and c-MYC (MYC) (Fig. 7D, lower panel).

Discussion

We have previously reported that RBCK1 recruitment to the ER\(\alpha\) promoter B positively correlates with ER\(\alpha\)
expression, estrogen signaling, and cellular proliferation in breast cancer cells. Based on this, we hypothesize that inhibitors of RBCK1 could constitute a novel strategy for inhibiting proliferative ERα signaling in ERα-positive breast cancer via regulation of the expression of the receptor. To develop this hypothesis further, it is necessary to increase the knowledge about the molecular mechanism by which RBCK1 affects ERα expression and this has been the focus of this study. Our analysis of the molecular mechanism by which RBCK1 affects ERα expression via promoter B in breast cancer cells suggests that ERα and RBCK1 interact via the AF-1 and RBR domains respectively. Furthermore, the presence of RBCK1 is necessary for PKCβ1 to associate to the promoter, stimulating active histone modifications and activating ERα gene expression (Fig. 6).

Although the molecular mechanisms of ERα target gene activation have been extensively studied, regulation of the ERα gene itself in both normal cells and in breast cancer cells remains less understood. This may in part be due to the complexity of the ERα gene regulatory regions, which include multiple upstream exons with associated promoters (Kos et al. 2001). Furthermore, potential autoregulation of ERα expression, mediated via ERα binding sites in the promoter and upstream enhancers (Carroll et al. 2006, Ellison-Zelski et al. 2009), provides an additional level of complexity when studying regulation of ERα expression.

Both pure antagonists such as Fulvestrant and partial antagonists such as tamoxifen have been shown to downregulate ERα mRNA levels (Pink & Jordan 1996, Wittmann et al. 2007). Although our studies do not support that anti-estrogens modulate the ability of RBCK1 to regulate ERα, mRNA expression, future studies should take a broader approach to, in detail, address the potential influence of RBCK1 and/or PKCβ1 on the modulation of ERα, transcription by anti-estrogens.

The model proposed in Fig. 6 of the complex, including ERα, RBCK1, and PKCβ1, on the ERα promoter B, is consistent with the data presented in this manuscript: i) RBCK1 recruitment to the ERα promoter B is facilitated by ERα, ii) ERα regulation of its own mRNA expression is facilitated by RBCK1 recruitment, suggesting an ERα coactivator function of RBCK1, and iii) PKCβ1 recruitment to the ERα promoter B is facilitated by RBCK1. The model is also consistent with the known direct DNA-binding

**Figure 6** The model proposed for the complex of RBCK1, ERα, and PKCβ1 on the ERα promoter B including the property of this complex to stimulate active ERα transcription through histone H3 lysine 4 dimethylation and T6 phosphorylation. ERα then promotes cell cycle progression from the G1 to S phase, stimulating breast cancer proliferation.

**Figure 7** Inhibition of PKCβ1 kinase activity affects histone H3T6 phosphorylation status and downregulates ERα signaling in MCF-7 cells (A, B, C and D). Cells were treated with DMSO (vehicle) or 4 µM Go6976 overnight. (A) ChiP was performed using histone H3T6 phosphorylation antibody, columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. (n=2), *P<0.05; for Go6976 vs vehicle. (B) ERα mRNA levels were determined by quantitative RT-PCR with each mRNA quantification performed in triplicate. (C) ERα and RBCK1 protein levels were determined by immunoblot, β-actin was used as loading control. Shown is a representative of three independent experiments. (D) ERα target gene mRNA cyclin D1 and c-MYC levels were determined by quantitative RT-PCR. Columns represent the arithmetic mean of fold change over vehicle-treated cells; bars, s.d. (n=3), *P<0.05; **P<0.01 for Go6976 vs DMSO.
properties of ERα. However, many details of this complex remain to be investigated such as whether RBCK1 interacts directly with DNA as has been suggested and whether ERα interacts with PKCβ. Future studies should address whether this ternary complex is recruited in a sequential order or preformed before interaction with the ERα promoter B. Finally, RBCK1 occupancy is necessary for PKCβ association, as well as PKCβ-dependent H3K4me2 and H3T6ph modifications (Figs 4, 5 and 7).

Our results demonstrate that the function of RBCK1 as an activator of ERα expression (Fig. 2) was dependent on an intact RBR domain (Fig. 3). This is consistent with a previous study showing that the RBR domain has the potential to act as an activator of gene expression. The RBR domain is also associated with E3 ubiquitin ligase activity (Tatematsu et al. 2008). The current lack of information regarding specific amino acids involved in E3 ubiquitin ligase activity (Eisenhaber et al. 2007) restricts us from correlating RBCK1 functions in ERα interaction and activation of the ERα promoter with an E3 ubiquitin ligase function. Interestingly, RBCK1 is part of the recently described ubiquitin ligase complex called linear ubiquitin chain assembly complex (LUBAC; Ikeda et al. 2011, Tokunaga et al. 2011). Thus, future studies should define the possible role of other components of the LUBAC complex for activation of the ERα promoter B and the potential inclusion of other components of the LUBAC complex in the model shown in Fig. 6.

Given that ERα recruits RBCK1 to the ERα binding region in the ERα gene promoter, we investigated the possible recruitment of RBCK1 to other ERα binding regions in ERα target gene promoters. ChIP was performed for three known ERα target regions in the promoters of the TFF1, IL20, and ADORA1 genes respectively (Carroll et al. 2006). Recruitment of ERα and RBCK1 was observed for all three binding regions (Supplementary Figure 1, see section on supplementary data given at the end of this article), supporting a broader role of RBCK1 in ERα-mediated gene regulation. Based on these data, RBCK1 may not only affect ERα signaling by regulating ERα expression but also as a coregulator at ERα target gene promoters. However, more investigations are needed to clarify whether RBCK1 is a more general cofactor for ERα.

To identify novel therapeutic strategies for ERα-positive breast cancer, modulation of ERα expression constitutes one approach to inhibit estrogen signaling and associated cellular proliferation. This study gives novel insight into the molecular mechanism for regulating transcription of ERα, providing a valuable platform for future studies aimed at further exploring RBCK1 as a potential therapeutic target for inhibition of proliferative ERα signaling in breast cancer.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1550/JME-12-0073.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N G S participated in the design and coordination of the study, carried out the experiments, interpreted the data, and drafted the manuscript. N H produced reagents, read, and commented on the manuscript. K D-W conceived of the study, participated in the design of the experiments, in the interpretation of the data, and drafted the manuscript. All authors read and approved the final manuscript.

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