Estrogen receptor- α , RBCK1, and protein kinase C β 1 cooperate to regulate estrogen receptor- α gene expression

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Abstract

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

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Introduction

Estrogen receptor α (ER α) 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). ER α 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 ER α (Ali & Coombes 2002). Breast cancers overexpressing ER α can only partly be accounted for by ER α gene amplification, with associated increased ER α expression, as observed in ~50% of ER α -positive breast cancers (Holst *et al.* 2007). Thus, it has been suggested that other processes, such as control of ER α transcription and mRNA and protein stability, are deregulated in the remaining 50% of ER α overexpressing breast cancers (Fowler & Alarid 2007). Therapy for ER α -positive breast cancer includes receptor antagonists, such as tamoxifen, that inhibits ER α -mediated proliferation in breast cancer (Early Breast Cancer Trialists' Collaborative Group 2005). Approximately 80% of ER α -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 ER α signaling, and identifying regulators of ER α expression in breast cancer cells is one strategy to identify novel targets to inhibit proliferative ER α signaling in breast cancer. However, the knowledge of the regulation of the ER α gene in both normal cells and in breast cancer cells is relatively limited.

Multiple promoters have been described for the ER α 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 ER α expression via promoter B has been suggested, as ER α mRNA levels transcribed from this promoter correlates with increased ER α levels in breast cancer patients as well as in breast cancer cells in culture (Hayashi *et al.* 1997, Tanimoto *et al.* 1999). ER α binding sites have been identified in the ER α gene promoter B, as well as in upstream enhancer regions (Carroll *et al.* 2006). Consistent with this, it has been shown that ER α can regulate its own expression (Castles *et al.* 1997, Ellison-Zelski *et al.* 2009). Under normal physiological

conditions, expression of ER α and estrogen levels are inversely related and repression of the ER α promoter by recruitment of ER α upon estrogen stimulation has been reported (Ellison-Zelski *et al.* 2009). However, in breast cancer, this inverse relationship between ER α and its agonist is lost, indicating deregulation of ER α 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 C β 1 (PKC β 1)-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 PKC β 1 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 ER α target gene promoter (Nair *et al.* 2010).

We previously reported that RBCK1 is recruited to the ER α promoter B and that reduced RBCK1 levels lead to reduced ER α levels, reduced expression of ER α target genes, and cell cycle arrest in breast cancer cells (Gustafsson *et al.* 2010). Thus, RBCK1 is a modulator of ER α signaling, potentially by directly regulating ER α expression. The fact that RBCK1 is overexpressed in breast cancer samples and that its expression correlates with ER α 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 ER α 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 ER α expression has to be defined. In this study, we provide novel insight into the molecular mechanism of regulation of ER α 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 Shiu, 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 17- β 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 GAG-3' and the following antisense primers: RBCK1 1–447 (antisense 5'-AAA CTC GAG CTA GCG CAT GGC CTC GCC CTG CTG CA-3'), RBCK1 1–352 (antisense 5'-AAA CTC GAG CTA CTG GTA ATC CTC AGG GGT CAG GA-3'), RBCK1 1–282 (antisense 5'-AAA CTC GAG CTA CTC GGC GGC CTC CGT GTT CAG CA-3'), (antisense 5'-AAA CTC GAG CTA CCG TCC GGG TTC CTG GGG GAC-3'), RBCK1 1–184 (antisense 5'-AAA CTC GAG CTA GTC CTG CGT TGG GGA GAC CTC-3'). pcDNA3-ER α was provided by Nina Heldring (Karolinska Institute, Sweden). The full-length FLAG-ER α and FLAG-ER α amino acid 180–595 (ER α Δ AF1) constructs were cloned in a pcDNA3/pFRT vector and have been described previously (Matthews *et al.* 2007). FLAG-ER α 1–535 (ER α Δ H12) was constructed by introducing a stop codon in the full-length FLAG-ER α construct using site-directed mutagenesis. Correct sequences were verified using DNA sequencing. The pGL2-derived luciferase reporter plasmid ER α -promoter-luc was kindly provided by R J 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 (Poly-Plus-Transfection SAS, Illkirch, France) according to the manufacturer's instructions. Stealth Select siRNA Catalog# HSS145705 and HSS145706, referred to in this study as siRBCK1; ER α - Stealth Select siRNA Catalog# HSS103375 and HSS103376, referred to as siER α ; and a control siRNA – Individual Stealth Select siRNA, referred to as siControl (Invitrogen).

Reporter 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 ER α , polyclonal rabbit PKC β 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or FLAG (Sigma).

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 μ g 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 μ l

Protein G slurry and 1 μ g IgG for 1 h at 4 °C. The supernatant was collected and immunoprecipitated with ER α , RBCK1, IgG, or PKC β 1 antibodies (Santa Cruz Biotechnology) overnight at 4 °C. The following day, 40 μ l Protein G slurry was added for 2 h at 4 °C, then protein complexes were washed four times in 500 μ l lysis buffer, and lysed in 60 μ l Laemmli sample-loading buffer. Protein complexes were subjected to 10% SDS–PAGE followed by western blot analysis with ER α , RBCK1, or PKC β 1 antibodies (Santa Cruz Biotechnology).

Chromatin immunoprecipitation assays

MCF-7 cells were plated in 150 mm dishes and chromatin immunoprecipitation (ChIP) assays performed at indicated time points (as previously described). Input samples for RT-PCR and western blot analysis were removed before pre-clearing with 20 μ l 50% Protein G slurry for 1 h. Chromatin was collected and immunoprecipitated overnight with 2 μ g ER α , RBCK1, PKC β 1 (Santa Cruz Biotechnology), 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 ER α gene known not to bind ER α , RBCK1, or PKC β 1 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.

Results

RBCK1 enhances ER α promoter activity

Our previous data has shown that RBCK1 interacts with the ER α promoter B in MCF-7 breast cancer cells and that reducing RBCK1 expression leads to reduced ER α expression (Gustafsson *et al.* 2010). Figure 1A shows that transcripts derived from ER α 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 β-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. ($n = 3$).

we find it likely that recruitment of RBCK1 to promoter B also regulates the activity of promoter A. Therefore, the *ERα* luciferase reporter used in this paper contains both promoters A and B. Further evidence for the involvement of RBCK1 in regulation of *ERα* expression was demonstrated in an assay where overexpressed RBCK1 upregulated the *ERα* promoter reporter activity in a dose-dependent manner (Fig. 1B). To examine whether RBCK1 also upregulates the endogenous *ERα* promoter, we assayed endogenous *ERα* levels in response to RBCK1 overexpression. As shown in Fig. 1C, we observed an increase in *ERα* mRNA already 15 h after RBCK1 transfection. This was followed by an increase in *ERα* protein (Fig. 1D). 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.

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).

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).

Figure 2 RBCK1 and ER α interact and cooperate in regulation of the ER α promoter in MCF-7 cells. (A) Co-recruitment of RBCK1 and ER α was examined by sequential ChIP assays. Immunoprecipitated DNA with the RBCK1 antibody was subjected to reChIP with ER α antibody or IgG control. DNA was quantified by RT-PCR. $**P < 0.01$ for RBCK1-ER α vs RBCK1-IgG. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=3$). (B) Protein complexes were immunoprecipitated with RBCK1 antibody (IP1), ER α antibody (IP2), or IgG control. ER α and RBCK1 protein levels were determined by immunoblot. Shown is a representative of three independent experiments. (C) Schematic representation of the ER α B promoter, surrounding sequences, analyzed. The ER α promoter reporter construct includes the region +63 to -1977. Arrows indicate the positions of primers used to assay binding of investigated proteins to a region in the ER α promoter B. (D) Cells were transfected with 1 μ g ER α promoter luciferase reporter with or without 1 μ g RBCK1 and ER α plasmids. Luciferase activity was measured 48 h post-transfection. $**P < 0.01$; $***P < 0.001$ for RBCK1 and ER α vs RBCK1 or ER α alone. (E) Effect of agonist on RBCK1-induced ER α promoter activity. Cells were seeded in hormone-free media for 24 h, transfected with 1 μ g ER α promoter luciferase reporter with or without 1 μ g FLAG-RBCK1 deletion plasmids. 10 nM of E₂, 10 nM 4-OHT, or vehicle were added and luciferase activity was measured 16 h post-treatment. $***P < 0.001$ for RBCK1 vs FLAG. Columns represent the arithmetic mean of fold change compared with FLAG-transfected cells; bars, s.d. ($n=3$).

The functional interaction between RBCK1 and ER α was further explored in reporter assays. Co-transfection of RBCK1 and ER α led to a further upregulation of the ER α promoter reporter activity compared with what was observed upon transfection with RBCK1 or ER α alone (Fig. 2D).

We next investigated the potential influence of ER α , the agonist E₂, or the antagonist 4-OHT on the RBCK1-mediated activation of the ER α 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 ER α promoter reporter by RBCK1 compared with vehicle (Fig. 2E), suggesting that functional interactions between RBCK1 and ER α involved in regulating ER α promoter activity are not influenced by ligand modulation of ER α . In line with this, protein-protein interactions between RBCK1 and ER α were not influenced by E₂ or 4-OHT (data not shown). In summary, these data identify RBCK1 as a novel

ER α -interacting protein and suggest that interactions between RBCK1 and ER α enhance activation of the ER α promoter B. Furthermore, our results do not support a role of ER α ligands in interactions between RBCK1 and ER α .

Characterization of ER α and RBCK1 interaction domains

To begin to dissect regions of RBCK1 that are required for interaction with ER α , RBCK1 deletion variants were assayed for interaction with ER α . 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 ER α whereas deletion of the IBR-domain, RBCK1 (1–352), abolished the interaction with ER α (Fig. 3B).

To determine whether regions of RBCK1 required for interaction with ER α were overlapping with regions involved in activation of the ER α promoter, RBCK1

Figure 3 Domain analysis of RBCK1 and ER α interacting domains and RBCK1 domain required for ER α promoter activation. (A) Schematic representation of the full-length RBCK1 domain structure. (B) COS-7 cells were transfected with ER α and RBCK1 deletion constructs and interactions of ER α and FLAG-RBCK1 constructs were examined by immunoprecipitations with ER α 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 ER α promoter. 1 μ g ER α promoter reporter was cotransfected with 1 μ g 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-ER α deletion constructs and interactions of deleted variants of FLAG-ER α with endogenous RBCK1 were assayed by immunoprecipitations with RBCK1 antibody followed by immunoblot analysis using a FLAG antibody to detect immunoprecipitated FLAG-ER α constructs. Shown is a representative of two independent experiments.

deletion variants were assayed for their capacity to activate the ER α promoter. The RBCK1-dependent upregulation of the ER α 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 ER α promoter extends beyond those required for interaction with ER α .

To determine regions of ER α that are critical for interaction with RBCK1, we employed ER α deletion constructs in co-immunoprecipitation assays. We focused on the two activation functions described for ER α , 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 ER α was intact upon deletion of H12 but completely abolished upon

deletion of the AF-1 domain (Fig. 3D). Thus, the ligand-binding function of ER α does not influence the interaction with RBCK1, which is consistent with our data that ligands do not influence the RBCK1–ER α interaction.

ER α recruits RBCK1 to the ER α promoter B with RBCK1 enhancing ER α expression

To further elucidate the molecular mechanism of RBCK1 regulation of the ER α promoter B, we investigated the importance of ER α for RBCK1 recruitment to the ER α promoter B. Figure 4A (left panel) shows that reducing ER α levels reduced the recruitment of RBCK1 to the ER α 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 4 ER α recruits RBCK1 to the ER α promoter B. (A) MCF-7 cells were transfected with siControl or siER α for 24 h. Recruitment of ER α 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 siER α vs siControl. 10% of input was used for determination of ER α 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) ER α and RBCK1 protein levels were determined by immunoblot, β -actin was used as loading control. Shown is a representative of three independent experiments. (C) Recruitment of RBCK1 and ER α was analyzed by ChIP after 24 h. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=3$). * $P<0.05$ for siRBCK1 vs siControl. (D) RBCK1 and ER α mRNA levels were determined by quantitative 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.

We next examined the effect of RBCK1 depletion on ER α occupancy at the ER α promoter B. To avoid decreased ER α protein levels as a consequence of RBCK1 silencing (Gustafsson *et al.* 2010), which could result in decreased ER α recruitment to its own promoter, we performed the assay at a time point when RBCK1 depletion had not yet affected ER α protein levels. RBCK1 protein levels began to decrease at 24 h after siRBCK1 transfection (Fig. 4B, left panel); however, ER α 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 ER α promoter B was significantly decreased in RBCK1-depleted cells but recruitment of ER α remained unchanged (Fig. 4C), suggesting that the occupancy of ER α at the ER α promoter B is not dependent on RBCK1. In a parallel analysis of ER α mRNA expression, we observed that ER α mRNA started to decrease after 25 h of siRBCK1 treatment (Fig. 4D), even though ER α recruitment to the promoter remained unchanged up to 30 h post-siRBCK1 (data not shown). Together, these data suggest a model where ER α is necessary for association of RBCK1 to the ER α promoter B with formation of an ER α -RBCK1 complex important for maintaining ER α expression.

RBCK1-dependent recruitment of PKC β 1 and histone modifications at the ER α promoter B

Previous studies have demonstrated that the RBCK1-interacting protein PKC β 1 activates transcription through maintenance of histone H3 dimethyl lysine 4 (H3K4me2) via phosphorylation of histone H3 threonine 6 (H3T6ph), suggesting a potential role for PKC β 1 in mediating RBCK1-dependent histone modifications.

As interactions between RBCK1 and PKC β 1 (Tokunaga *et al.* 1998, Tatematsu *et al.* 2008) have not been investigated in breast cancer cells, we first assayed potential interactions between RBCK1 and PKC β 1 in MCF-7 cells. Immunoprecipitation with an RBCK1 antibody followed by western blot analysis for PKC β 1 revealed protein-protein interactions between endogenous RBCK1 and PKC β 1 (Fig. 5A).

Furthermore, ChIP assay with RBCK1 antibody followed by reChIP with PKC β 1 antibody revealed co-occupancy of RBCK1 and PKC β 1 at the ER α promoter B (Fig. 5B). To determine whether PKC β 1 recruitment to the ER α promoter B is dependent on the presence of RBCK1 at the promoter, RBCK1 levels were reduced by siRNA followed by a ChIP assay with PKC β 1 antibody. Importantly, we observed reduced occupancy of PKC β 1 on the ER α promoter B in RBCK1-depleted

Figure 5 RBCK1 affects histone modifications and PKC β_1 recruitment. (A) Interaction between RBCK1 and PKC β_1 was analyzed by immunoprecipitation. Protein complexes were co-precipitated with RBCK1 antibody or IgG control. PKC β_1 and RBCK1 protein levels were determined by immunoblot. Shown is a representative of two independent experiments. (B) Co-recruitment of RBCK1 and PKC β_1 was examined by sequential ChIP assays. Immunoprecipitated DNA with the RBCK1 antibody was subjected to reChIP with PKC β_1 antibody or IgG control. DNA was quantified by RT-PCR, data are expressed as fold enrichment over IgG. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=3$). (C, D and E) RBCK1 was depleted in MCF-7 cells for 24 h by siRNA. (C) ChIP was then performed with PKC β_1 antibody or IgG. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=3$). * $P<0.05$ for siRBCK1 vs siControl. (D) ChIP performed using histone H3K4 dimethylation antibody (left graph) or histone H3T6 phosphorylation antibody (right graph). Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=3$). * $P<0.05$; ** $P<0.01$ for siRBCK1 vs siControl. (E) ChIP performed using histone H3K4 trimethylation antibody. Columns represent the arithmetic mean of fold enrichment over IgG; bars, s.d. ($n=2$).

cells (Fig. 5C), suggesting that RBCK1 is necessary for PKC β_1 association to the ER α promoter B.

As PKC β_1 maintains H3K4me2 via H3T6ph (Metzger *et al.* 2010), which is related to gene activation, we investigated the status of these histone modifications for the ER α promoter B region upon RBCK1 silencing. Figure 5D shows that H3K4me2 (left panel, white bars) and H3T6ph (right panel, white bars) are significantly decreased in the ER α promoter B region of RBCK1-depleted cells. Thus, RBCK1 is needed for recruitment of PKC β_1 to the ER α promoter and for active histone modifications that have been shown to be maintained by PKC β_1 . 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 α promoter B is necessary for H3K4me3 modification and PKC β_1 association, as well as PKC β_1 -dependent H3K4me2 and H3T6ph modifications. This suggests a model where PKC β_1 mediates the RBCK1-induced effects on histone modifications, thereby activating ER α transcription (Fig. 6).

PKC β_1 kinase activity modulates ER α signaling

We investigated the importance of a functional PKC β_1 kinase activity for H3T6 phosphorylation and subsequently ER α expression by treating MCF-7 cells with the PKC β_1 kinase inhibitor Go6976. Figure 7A shows that H3T6ph is significantly decreased in the ER α promoter B region in Go6976-treated cells. Furthermore, we observed decreased ER α mRNA levels in Go6976-treated cells (Fig. 7B), supporting a critical role for the PKC β_1 kinase activity for the regulation of ER α promoter B activity. Furthermore, cells treated with Go6976 displayed significantly decreased ER α , but not RBCK1, protein levels (Fig. 7C). Consistent with Go6976 downregulating ER α expression, the compound downregulated the expression levels of the ER α 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 α promoter B positively correlates with ER α

Figure 6 The model proposed for the complex of RBCK1, ER α , and PKC β_1 on the ER α promoter B including the property of this complex to stimulate active ER α transcription through histone H3 lysine 4 dimethylation and T6 phosphorylation. ER α then promotes cell cycle progression from the G1 to S phase, stimulating breast cancer proliferation.

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 ER α signaling in ER α -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 regulates ER α expression and this has been the focus of this study. Our analysis of the molecular mechanism by which RBCK1 affects ER α expression via promoter B in breast cancer cells suggests that ER α and RBCK1 interacts via the AF-1 and RBR domains respectively. Furthermore, the presence of RBCK1 is necessary for PKC β_1 to associate to the promoter, stimulating active histone modifications and activating ER α gene expression (Fig. 6).

Although the molecular mechanisms of ER α target gene activation have been extensively studied, regulation of the ER α 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 ER α gene regulatory regions, which include multiple upstream exons with associated promoters (Kos *et al.* 2001). Furthermore, potential autoregulation of ER α expression, mediated via ER α 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 ER α expression.

Both pure antagonists such as Fulvestrant and partial antagonists such as tamoxifen have been shown to downregulate ER α 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 ER α mRNA expression, future studies should take a broader approach to, in detail, address the potential influence of RBCK1 and/or PKC β_1 on the modulation of ER α transcription by anti-estrogens.

The model proposed in Fig. 6 of the complex, including ER α , RBCK1, and PKC β_1 , on the ER α promoter B, is consistent with the data presented in this manuscript: i) RBCK1 recruitment to the ER α promoter B is facilitated by ER α , ii) ER α regulation of its own mRNA expression is facilitated by RBCK1 recruitment, suggesting an ER α coactivator function of RBCK1, and iii) PKC β_1 recruitment to the ER α promoter B is facilitated by RBCK1. The model is also consistent with the known direct DNA-binding

Figure 7 Inhibition of PKC β_1 kinase activity affects histone H3T6 phosphorylation status and downregulates ER α signaling in MCF-7 cells (A, B, C and D). Cells were treated with DMSO (vehicle) or 4 μ M 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) ER α mRNA levels were determined by quantitative RT-PCR with each mRNA quantification performed in triplicate. (C) ER α and RBCK1 protein levels were determined by immunoblot, β -actin was used as loading control. Shown is a representative of three independent experiments. (D) ER α 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 ER α . However, many details of this complex remain to be investigated such as whether RBCK1 interacts directly with DNA as has been suggested and whether ER α interacts with PKC β_1 . Future studies should address whether this ternary complex is recruited in a sequential order or preformed before interaction with the ER α promoter B. Finally, RBCK1 occupancy is necessary for PKC β_1 association, as well as PKC β_1 -dependent H3K4me2 and H3T6ph modifications (Figs 4, 5 and 7).

Our results demonstrate that the function of RBCK1 as an activator of ER α 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 ER α interaction and activation of the ER α 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 ER α promoter B and the potential inclusion of other components of the LUBAC complex in the model shown in Fig. 6.

Given that ER α recruits RBCK1 to the ER α binding region in the ER α gene promoter, we investigated the possible recruitment of RBCK1 to other ER α binding regions in ER α target gene promoters. ChIP was performed for three known ER α target regions in the promoters of the *TFF1*, *IL20*, and *ADORA1* genes respectively (Carroll *et al.* 2006). Recruitment of ER α 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 ER α -mediated gene regulation. Based on these data, RBCK1 may not only affect ER α signaling by regulating ER α expression but also as a coregulator at ER α target gene promoters. However, more investigations are needed to clarify whether RBCK1 is a more general cofactor for ER α .

To identify novel therapeutic strategies for ER α -positive breast cancer, modulation of ER α 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 ER α , providing a valuable platform for future studies aimed at further exploring RBCK1 as a potential therapeutic target for inhibition of proliferative ER α signaling in breast cancer.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/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 to draft the manuscript. All authors read and approved the final manuscript.

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