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Cell cycle and apoptosis regulator 1, CCAR1, regulates enhancer-dependent nuclear receptor CAR transactivation

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Running title: CCAR as a gene-selective co-activator of CAR

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ABBREVIATIONS:

CAR, constitutive androstane receptor; CYP, cytochrome P450; DBD, DNA-binding domain; LBD, ligand binding domain; mRNA, messenger ribonucleic acid; NR, nuclear receptor; RXRα, retinoid X receptor α; TRIM24, tripartite motif containing 24; UGT, uridine diphosphate glucuronosyltransferase,

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Abstract

The nuclear receptor constitutive active/androstane receptor (CAR) controls genes involved in xenochemical metabolism. Although numerous cofactors have been reported to be involved in CAR-mediated transactivation, there remain unknown and poorly defined proteins recruited by CAR that have yet to be characterized. In this study, a novel CAR-interacting protein, cell cycle and apoptosis regulator 1 (CCAR1), has been identified by performing co-immunoprecipitation analysis using human hepatocarcinoma HepG2 cells expressing FLAG-epitope tagged CAR (FLAG-CAR). Here we demonstrated that CCAR1 can act as an enhancer-dependent coactivator of CAR. First, we showed that overexpression of CCAR1 enhanced CAR-induced reporter gene activity with triplicate consensus direct repeat 4 motif (DR4-Luc), xenobiotic responsive enhancer module (XREM)-enhancer of CYP3A4 (XREM-Luc), and phenobarbital-responsive enhancer module of UDP-glucuronosyltransferases 1A1 (UGT1A1) (gtPBREM)-enhancer of UGT1A1 (gtPBREM-Luc)-driven reporter plasmids, but not PBREM-enhancer of CYP2B6 (PBREM-Luc)-driven reporter activity. Furthermore, we showed that knockdown of CCAR1 suppressed CAR-induced *UGT1A1* mRNA expression, but did not affect CAR-induced *CYP2B6* mRNA expression in HepTR/CAR and HepaRG cells. Moreover, CCAR1 could be recruited to the gtPBREM of the *UGT1A1* enhancer by CAR, but not to the PBREM of the *CYP2B6* enhancer. Moreover, we showed that CCAR1 can act as a secondary coactivator by cooperating with the p160 family of steroid receptor coactivators (SRCs). These findings demonstrated CCAR1 to be a novel transcriptional

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cofactor for CAR, providing insight regarding the mechanism of CAR-mediated gene-selective transactivation.

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Introduction

The constitutive active/androstane receptor (CAR; NR1I3), a member of the nuclear receptor (NR) superfamily, was characterized as a xenosensor that plays a key role in cellular responses to xenochemicals. CAR protects cells from xenochemical toxicity by increasing xenobiotic/drug-metabolizing enzyme and transporter expression. CAR has important roles in drug metabolism and interactions by inducing target genes. However, it has been reported that CAR also regulates energy homeostasis including thyroid hormone metabolism (Maglich et al., 2004; Qatanani et al., 2005), glucogenesis (Ueda et al., 2002; Kodama et al., 2004; Miao et al., 2006), and lipogenesis (Roth et al., 2008). Generally, NRs, which are activated by ligands or activators, bind to a specific DNA motif of the regulatory region for target genes. The DNA-bound NRs recruit various cofactors, which regulates the active transcription complex on the transcription start site via chromatin remodeling. Such transcriptional activation by NRs is tightly controlled by a complex process involving DNA sequence, epigenetic modifications, chromatin structures, and coactivators.

Unlike general NRs, CAR exhibits constitutive transcriptional activity and is able to recruit coactivators without binding to ligands. CAR is normally sequestered in the cytoplasmic compartment of liver cells and translocates to the nucleus after exposure to an activator or ligand. Following nuclear translocation, CAR binds to response elements in the promoter regions of its target genes, forming a heterodimer complex with retinoid X receptor α (NR2B1). Thus, nuclear translocation and coactivator recruitment to

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the CAR-binding enhancer region of target genes constitute important steps for CAR-mediated activation.

Several coactivators that interact with CAR have been identified, such as steroid receptor coactivator 1 (SRC1) (Muangmoonchai et al., 2001), SRC2 (also known as glucocorticoid receptor-interacting protein 1 (GRIP1)) (Min et al., 2002), activating signal cointegrator 2 (Choi et al., 2005), growth arrest and DNA damage-inducible 45 beta (GADD45 beta) (Tian et al., 2011), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (Shiraki et al., 2003), tripartite motif containing 24 (TRIM24) (Kanno et al., 2018), DEAD box DNA/RNA helicase DP97 (Kanno et al., 2012), and protein arginine methyltransferase 5 (PRMT5) (Kanno et al., 2015). Previously, we reported that three coactivators of CAR, TRIM24, DP97, and PRMT5, contributed to CAR-mediated transcriptional activation and showed gene-selective transactivation. These results suggested that CAR-mediated transcriptional activation is regulated by divergent gene-selective cofactors. However, the gene-selective regulation mechanism of nuclear receptors is not yet well understood.

The aim of this study was to discover novel cofactors involved in the CAR-mediated gene regulation mechanism. To identify novel cofactors for CAR, we performed co-immunoprecipitation analysis using human hepatocarcinoma HepG2 cells expressing FLAG-epitope-tagged CAR (FLAG-CAR). Among the co-precipitated proteins, we focused on cell cycle and apoptosis regulator 1 (CCAR1) as a novel CAR-interacting protein. CCAR1, also known as CARP-1, was originally identified as a regulator of apoptosis

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signaling and cell proliferation (Rishi et al., 2003). CCAR1 is known to be associated with members of the steroid hormone receptor subfamily (NR3) of NR, such as estrogen receptor α, glucocorticoid receptor (GR), and androgen receptor (AR) (Kim et al., 2008). CCAR1 interacts with NRs and cooperates synergistically with the SRC coactivator complex. However, it has not been investigated whether CCAR1 acts as a coactivator of other NR subfamily members including CAR, which belongs to the NR1 subfamily. Here, we show that CCAR1 enhances transcriptional activity of CAR and acts as an enhancer-dependent selective cofactor of CAR for UGT1A1 expression.

Materials and Methods

Plasmid construction and chemicals

The construction of expression plasmids encoding N-terminal FLAG epitope-tagged human CAR proteins (FLAG-CAR) has been reported previously (Kanno et al., 2012; Kanno et al., 2005; Kanno et al., 2007). Full-length human *CCAR1* cDNA was amplified by reverse transcription-polymerase chain reaction (RT-PCR) using cDNA prepared from human hepatoma HepG2 cells. The expression plasmids encoding FLAG epitope-tagged CCAR1 (FLAG-CCAR1), SRC-1, SRC-2, and GR were constructed based on the pCMV-3Tag-6 vector (Stratagene, Santa Clara, CA, USA) using an In-Fusion HD Cloning Kit (Clontech, Mountain View, CA, USA). The expression plasmid encoding VP16-CCAR1 was constructed based on the pcDNA-VP16 vector using an In-Fusion HD Cloning Kit. The direct repeat-4

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(DR4)-driven luciferase reporter plasmid (DR4-Luc) was constructed by inserting three tandem repeats of the DR4 motif sequence (AGTTCATGGCAGTTCA), to which CAR binds to mediate transactivation, into pGL4.24 (Promega, Madison, WI, USA), which has a minimal promoter (Kanno et al., 2012). The phenobarbital-responsive enhancer module (PBREM) of the CYP2B6-driven luciferase reporter plasmid (PBREM-Luc) has also been described previously (Kanno et al., 2005). The CYP3A4 XREM-reporter plasmid (CYP3A4.XREM-Luc.) containing the proximal 362 bp of the *CYP3A4* gene promoter and a distal enhancer (−7836 to −7208 and −362 to +53) was constructed based on pGL4.14 (Promega). The phenobarbital-responsive enhancer module of *UGT1A1* (gtPBREM)-driven luciferase reporter plasmid (gtPBREM-Luc.) containing a 290-bp distal enhancer module (−3570/−3180) was constructed based on pGL3-Promoter (Promega). Tetracycline (Tet) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The CAR agonist CITCO was purchased from Calbiochem (San Diego, CA, USA).

Cell lines and cell culture

fCAR cells, which comprise stably N-terminal FLAG-tagged CAR-expressing HepG2 cells, were previously established (Kanno et al., 2018). The HepTR/CAR cell line, derived from human hepatoma HepG2 cells, was previously established using the T-REx system (Invitrogen), in which the expression of hCAR can be induced by Tet-treatment (Kanno et al., 2012; Kanno et al., 2016). HepG2, fCAR and

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HepTR/hCAR cells were cultured in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Differentiated HepaRG cells (KAC, Kyoto, Japan) were maintained in Williams' medium E (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, 5 μg/ml insulin, and 50 μM hydrocortisone (WAKO) at 37°C under 5 % CO_2 and 95% air according to the manufacturer's instructions.

CoImmunoprecipitation and immunoblot analysis

Nuclear extracts of fCAR cells were collected using Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 0.5% Nonidet P-40) and Buffer C (20 mM HEPES, pH 7.9, 0.2M NaCl, 1 mM EDTA, and 1 mM EGTA). Nuclear extract was incubated with Anti-DDDDK (FLAG)-tag mouse monoclonal Ab (MBL, Nagoya, Japan) or normal mouse IgG (Cell Signaling Technology, Danvers, MA, USA) for 2 h. After incubation, the immune complex was precipitated with 20 □l Protein G Magnet beads (BioRad, Hercules, CA, USA) for 2 h. Coprecipitated proteins were eluted by FLAG-M2 peptide (Sigma Aldrich).

The proteins were separated by SDS-PAGE and detected by immunoblotting using anti-CCAR1 (A300-435; Bethyl Laboratories, Montgomery, TX, USA), anti-CAR (Perseus Proteomics Inc,, Tokyo, Japan), horseradish peroxidase(HRP) conjugated anti-mouse IgG (Cell Signaling Technology), and anti-DDDDK

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(FLAG)-tag-HRP-DirecT(MBL) antibodies. The signals were captured using a LuminoGraph (ATTO, Tokyo, Japan).

Luciferase reporter assay

The appropriate expression plasmids, reporter plasmids, and pGL4.74 (hRluc/TK; Promega) carrying the *Renilla* luciferase gene as an internal standard were transfected into HepG2 cells using PEI Max reagent (Polysciences., Warrington, PA, USA). After 48 h, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The activities of firefly luciferase were normalized against those of *Renilla* luciferase.

CCAR1 knockdown and quantitative RT-PCR

HepTR/hCAR and HepaRG cells were transfected with CCAR1-targeting or control small interfering RNA (siRNA; Sigma Aldrich) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. The next day, cells were treated with Tet or CITCO. After 48 h, total RNA was isolated using ISOGEN II (Nippon Gene, Tokyo, Japan) and cDNA was synthesized using a ReverTraAce qPCR RT Kit (Toyobo, Osaka, Japan). Quantitative PCR (qPCR) was performed using the KOD SYBR qPCR Mix (Toyobo) according to the manufacturer's protocol on a 7500 Fast system (Applied Biosystems, Foster City, CA, USA). The specific PCR primers used were as follows: *CYP2B6*

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(5′-AAG CGG ATT TGT CTT GGT GAA-3′ and 5′-TGG AGG ATG GTG GTG AAG AAG-3′), *CYP3A4* (5′-CCA AGC TAT GCT CTT CAC CG-3′ and 5′-TCA GGC TCC ACT TAC GGT GC-3′), *UGT1A1* (5′-AGTGGATGGCAGCCACTGGCT-3′ and 5′-CAGTAAGTGGGAACAGCCAGA-3′), *CCAR1* (5′-AGTGCTCCGTGAATCTTGCT-3′ and 5′-TGAGGCCAACATTTTCTTCC-3′), *CAR* (5′-CCAGCTCATCTGTTCATCCA-3′ and 5′-GGTAACTCCAGGTCGGTCAG-3′), and β-actin (5′-TCC TCCTGA GCG CAA GTA CTC-3′ and 5′-CTG CTT GCT GAT CCA CAT CTG-3′).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using a Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Immunoprecipitation was performed using antibodies directed against CAR (Perceus Proteomics Inc., Tokyo, Japan), CCAR1 (ab70244; Abcam, Cambridge, MA, USA) (Mizuta et al., 2014) or normal IgG (Cell Signaling Technology) as a negative control. After immunoprecipitation, DNA fragments were subjected to qPCR using primers containing PBREM of the *CYP2B6* promoter (forward 5′- CTG CAA TGA GCA CCC AAT CTT -3′ and reverse 5′- ACA CAT CCT CTG ACA GGG TCA -3′), gtPBREM of the *UGT1A1* promoter (forward 5′- TACACTAGTA AAGGTCACTC AATT -3′ and reverse 5′-CTAGCCATTCTGGATCCCTTGCTG -3′), and a CAR-free region of the *CYP2B6* gene (forward 5′-CAG CTG GAG GGG TCA TCA AA -3′ and reverse 5′-GCT AGC CAG AGA CCC TTC AC -3′).

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Statistical analysis

Statistical comparisons were performed with one-way analysis of variance followed by Dunnett's multiple comparison test as the post hoc test, and differences were considered statistically significant at *p < 0.05.

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Results

CCAR1 is a CAR-interacting protein and enhances the transactivation activity of CAR

Previously, we identified candidate CAR-interacting proteins in fCAR cells, which comprise N-terminal FLAG-tagged CAR stably expressing cells, using immunoprecipitation and LC-MS/MS analysis (Kanno et al., 2018). CCAR1 represented one of several candidate CAR-interacting proteins from the obtained complexes. To confirm whether CCAR1 constitutes a novel CAR-interacting protein, nuclear extracts from fCAR cells were immunoprecipitated with an anti-FLAG antibody or normal IgG. CCAR1 was coimmunoprecipitated with the anti-FLAG antibody but not normal IgG (Fig. 1). This result demonstrated that CCAR1 specifically interacts with CAR. To investigate whether the CAR/CCAR1 interaction is direct or indirect, we performed a pulldown assay using *in vitro* translated proteins. However, CAR co-precipitation with CCAR1 was not observed (Supplemental Fig.1), suggesting that the CAR/CCAR1 interaction is not direct.

To determine whether CCAR1 might act as a coactivator for CAR, we performed a reporter gene assay in which HepG2 cells were transfected with Flag-tagged CCAR1 (Fig. 2A). We used four CAR-driven reporter plasmids, which contained the consensus element or enhancer regions of CAR target genes (Fig. 2B). Overexpression of CCAR1 enhanced CAR-induced reporter gene activity on triplicate consensus DR4 motif (DR4-Luc), XREM-enhancer of CYP3A4 (XREM-Luc), and gtPBREM-enhancer of UGT1A1 (gtPBREM-Luc)-driven reporter plasmids. Conversely, PBREM-enhancer of CYP2B6 (PBREM-Luc)-driven reporter activity was not affected (Fig. 2B).

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CCAR1 selectively activates CAR-mediated transactivation via the gtPBREM enhancer

To investigate whether the transactivation effect was directly or indirectly performed (e.g., by increasing other coactivators or transcriptional factors), we performed a mammalian 2-hybrid assay using GALDBD-CCAR1/VP16-CAR or GAL4DBD-CAR/VP16-CCAR1. However, no transactivation response was detected (data not shown). Figure 3 shows the result of a modified mammalian one-hybrid assay, which utilized a CAR-responsive-enhancer reporter plasmid with VP16-fused CCAR1. After 24 h, VP-fused CCAR1 enhanced only CAR-mediated transactivation of the gtPBREM-Luc reporter (Fig. 3). These observations suggested that a specific enhancer region, such as neighboring transcriptional factors, is required for CAR/CCAR1 interaction.

CCAR1 depletion selectively affects CAR target gene expression in HepTR/hCAR cells

To assess the functional involvement of CCAR1 in CAR-mediated target gene regulation, we next examined whether CCAR1 is required for CAR-induced target gene expression by using CCAR1-targeting siRNA in HepTR/CAR cells (Fig. 4A). HepTR/CAR cells were established previously as HepG2 cells expressing a Tet-inducible human CAR. Depletion of *CCAR1* mRNA by siRNA and induction of *CAR* mRNA by Tet-treatment were observed in HepTR/CAR cells. Induction of *CYP3A4* and *UGT1A1* by Tet was attenuated upon CCAR1 depletion whereas *CYP2B6* induction was not affected (Fig. 4B).

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CCAR1 depletion selectively affects CAR target gene expression in HepaRG cells

Furthermore, we investigated the effect of knockdown of CCAR1 on CAR-mediated induction in HepaRG cells (Fig. 5A). The HepaRG cell line constitutes highly differentiated cells, which serves as a good model for studies of CAR activation. Treatment with CITCO, an agonist of CAR, increased *CYP2B6*, *CYP3A4*, and *UGT1A1* mRNA expression (Fig. 5B). Induction of *UGT1A1* mRNA was not observed following CCAR1 depletion, whereas *CYP2B6* and *CYP3A4* induction was not affected. These observations suggest that CCAR1 regulates CAR-mediated transactivation in a gene-selective manner.

CCAR1 is selectively recruited to gtPBREM of the *UGT1A1* promoter together with CAR but not to PBREM of the *CYP2B6* promoter

CCAR1 regulates CAR-mediated mRNA expression (Fig. 4, 5) and reporter activity (Fig. 2, 3) of UGT1A1, but not CYP2B6. We speculated that CCAR1 is differentially recruited to CAR-responsive enhancer regions. To investigate this hypothesis, we carried out ChIP experiments in HepTR/CAR cells. ChIP assay revealed that CAR was recruited to both the PBREM (*CYP2B6*) and gtPBREM (*UGT1A1*) enhancer region by Tet treatment in HepTR/CAR cells, whereas Tet treatment only recruited CCAR1 to gtPBREM but not PBREM (Fig. 6 and Supplemental Fig. 2). These observations demonstrated that recruitment of CCAR1 by CAR was performed in an enhancer-dependent manner.

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CCAR1 synergistically enhances CAR-mediated transactivation with other coactivators

Previous studies have shown that CCAR1 enhances NR-activity by coordinating with SRCs, secondary coactivators (p300, coiled-coil coactivator (CoCoA), and CARM1), and mediator complexes (Kim et al., 2008; Seo et al., 2013). We tested whether CCAR1 cooperated with known CAR coactivators (SRC1 and SRC2) using a DR4-Luc reporter plasmid. CCAR1 enhanced CAR-activity synergistically with known CAR coactivators (Fig. 7A). This observation suggested that CCAR1 can enhance CAR-activity under overexpression of primary coactivators, such as SRC1 and SRC2. To investigate this speculation, the effect of CCAR1-overexpression was compared using reporters containing enhancer regions of different genes, gtPBREM of *UGT1A1* and PBREM of *CYP2B6*, in the presence of SRC1 and SRC2. For the gtPBREM-reporter, CCAR1 could enhance CAR-mediated transactivity by cooperating with overexpressed SRC1 or SRC2 (Fig. 7B). In contrast, CCAR1 overexpression did not affect CAR-mediated transactivity in the PBREM-reporter (Fig. 7C). These observations suggested that the selectivity of cofactor recruitment was determined by its enhancer region.

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Discussion

Nuclear receptor CAR plays key roles in drug/xenobiotic sensing and metabolizing cascades as a xenosensor. Through ligand binding, CAR binds to the enhancer regions of target genes and elicits transcriptional activation following the recruitment of coregulators. In particular, it is reported that CAR-mediated transcriptional activation is regulated in a cell-, cellular context-, and gene-dependent manner. In this study, we provide insights into a novel mechanism of CAR-mediated gene regulation by the coregulator CCAR1. We defined that CCAR1 differently regulates individual enhancer modules. Using HepTR/CAR cell lines, we showed that knockdown of CCAR1 decreased CAR-induced *CYP3A4* and *UGT1A1* mRNA expression. Luciferase reporter assay demonstrated that CCAR1 directly transactivates the gtPBREM enhancer of *UGT1A1*, but not the PBREM enhancer of *CYP2B6*. Although the XREM enhancer of *CYP3A4* was also enhanced by overexpression and decreased by knockdown of CCAR1, VP-fused CCAR1 could not enhance XREM-reporter activity. These observations suggested that overexpression or knockdown of CCAR1 indirectly regulates XREM-reporter gene activity. Notably, knockdown of CCAR1 upregulated *CYP2B6* mRNA expression in the absence of CAR. Hence, it is difficult to understand the effect of CCAR1 on CAR-induced *CYP2B6* mRNA expression. However, the PBREM-Luc reporter assay showed that CCAR1 overexpression and knockdown did not affect reporter activity. Furthermore, a ChIP assay demonstrated that CCAR1 is recruited to the gtPBREM enhancer

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region along with CAR, but not to the PBREM enhancer region. These observations suggested that CCAR1 is an enhancer-selective coactivator of CAR.

CCAR1 was firstly identified as a CoCoA C-terminal binding protein, interacting indirectly with NRs through the p160 coactivators (Kim et al., 2008). It was shown that CCAR1 contributes to NR-induced recruitment of mediator components and RNA pol II to target gene promoters. CCAR1 is also known as a regulator of other transcriptional factors, such as p53, GATA1, and b-catenin-regulated factors (Mizuta et al., 2014; Ou et al., 2009). GATA2, a pioneer factor for AR, comprises a large portion of the AR binding complex. GATA2 facilitates AR binding to AREs and enhances AR-mediated transcription (Wang et al., 2007). Specifically, it is reported that CCAR1 stabilizes this association between AR and GATA2 (Seo et al., 2013).

In the present study, we investigated whether CCAR1 was involved in SRC (SRC1 and SRC2)-mediated CAR transactivation. CCAR1 was able to enhance CAR-induced DR4-reporter activity by overexpression of SRC1 or SRC2 (Fig. 7), suggesting that CCAR1 serves as a secondary (indirect) coactivator of CAR. In the gtPBREM enhancer, we demonstrated that CAR-mediated transcriptional activation was coordinately activated by CCAR1/SRCs. In contrast, the PBREM enhancer of *CYP2B* was only slightly affected by CCAR1/SRCs overexpression. Recently, increasing evidence suggests that gene-, chromatin-, and cell- dependent transcriptional regulation by NRs is related to coregulators. For example, Hic-5 constitutes a well-characterized gene-selective coregulator of GR (Chodankar et al.,

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2014; Chodankar et al., 2015). Hic-5 facilitates GR regulation of some genes but blocks others. Hic-5 facilitates MED1 and RNA pol II recruitment with GR by Dex in Hic-5 modulated genes. Conversely, in blocked genes, GR binding is prevented by Hic-5 whereas Hic-5 depletion allows transcriptional activation of these genes by GR and coregulator binding. Differential Hic-5 expression may therefore contribute to tissue- and cell-specific gene regulation by GR. In contrast, our result suggested that gene-selectivity of CCAR1 for *CAR* was determined by whether it was recruited to the enhancer region. This is the first report of selective recruitment of a coactivator to CAR-responsive enhancers.

Taken together our results suggested that CAR can selectively recruit CCAR1 to the gtPBREM enhancer of *UGT1A1*, through cooperating with SRC1/2. In contrast, *CYP2B6* is CCAR1-independently regulated by CAR, because CCAR1 is not recruited to the PBREM enhancer of the *CYP2B6* promoter. The selectivity of CCAR1 recruitment to CAR-responsible enhancer regions might thereby contribute to gene-selective induction by CAR.

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Authorship Contributions

Participated in research design: Yuichiro Kanno

Conducted experiments: Yuichiro Kanno, Shuai Zhao, Naoya Yamashita, Aoi Ujiie, Rie Iijima, and Nami Kikawa

Contributed new reagents or analytic tools: Yuichiro Kanno

Performed data analysis: Yuichiro Kanno and Yoshio Inouye

Wrote or contributed to the writing of the manuscript: Yuichiro Kanno, Kiyomitsu Nemoto, and Yoshio Inouye

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Chodankar R, Wu D-Y, Gerke DS, and Stallcup MR (2015) Selective coregulator function and restriction of steroid receptor chromatin occupancy by Hic-5. Mol Endocrinol **29**:716–29.

Chodankar R, Wu D-Y, Schiller BJ, Yamamoto KR, and Stallcup MR (2014) Hic-5 is a transcription coregulator that acts before and/or after glucocorticoid receptor genome occupancy in a gene-selective manner. Proc Natl Acad Sci U S A **111**:4007–12.

Choi E, Lee S, Yeom SY, Kim GH, Lee JW, and Kim SW (2005) Characterization of activating signal cointegrator-2 as a novel transcriptional coactivator of the xenobiotic nuclear receptor constitutive androstane receptor. Mol Endocrinol **19**:1711–9.

Kanno Y, Aoki S, Mochizuki M, Mori E, Nakahama T, and Inouye Y (2005) Expression of constitutive androstane receptor splice variants in rat liver and lung and their functional properties. Biol Pharm Bull **28**:2058–62.

Kanno Y, Inajima J, Kato S, Matsumoto M, Tokumoto C, Kure Y, and Inouye Y (2015) Protein arginine methyltransferase 5 (PRMT5) is a novel coactivator of constitutive androstane receptor (CAR). Biochem Biophys Res Commun **459**:143–7.

Kanno Y, Kure Y, Kobayashi S, Mizuno M, Tsuchiya Y, Yamashita N, Nemoto K, and Inouye Y (2018) Tripartite motif containing 24 acts as a novel coactivator of the constitutive active/androstane receptor. Drug Metab Dispos **46**:46–52.

Kanno Y, Serikawa T, Inajima J, and Inouye Y (2012) DP97, a DEAD box DNA/RNA helicase, is a target

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gene-selective co-regulator of the constitutive androstane receptor. Biochem Biophys Res Commun **426**:38–42.

Kanno Y, Suzuki M, Miyazaki Y, Matsuzaki M, Nakahama T, Kurose K, Sawada J, and Inouye Y (2007) Difference in nucleocytoplasmic shuttling sequences of rat and human constitutive active/androstane receptor. Biochim Biophys Acta - Mol Cell Res **1773**:934–44.

Kanno Y, Tanuma N, Yazawa S, Zhao S, Inaba M, Nakamura S, Nemoto K, and Inouye Y (2016) Differences in gene regulation by dual ligands of nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in HepG2 cells stably expressing CAR/PXR. Drug Metab Dispos **44**:1158–63.

Kim JH, Yang CK, Heo K, Roeder RG, An W, and Stallcup MR (2008) CCAR1, a key regulator of mediator complex recruitment to nuclear receptor transcription complexes. Mol Cell **31**:510–9.

Kodama S, Koike C, Negishi M, and Yamamoto Y (2004) Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. Mol Cell Biol **24**:7931–40.

Maglich JM, Watson J, McMillen PJ, Goodwin B, Willson TM, and Moore JT (2004) The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. J Biol Chem **279**:19832–8.

Miao J, Fang S, Bae Y, and Kemper JK (2006) Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolism is mediated by

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competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1alpha. J Biol Chem **281**:14537–46.

Min G, Kemper JK, and Kemper B (2002) Glucocorticoid receptor-interacting protein 1 mediates ligand-independent nuclear translocation and activation of constitutive androstane receptor in vivo. J Biol Chem **277**:26356–63.

Mizuta S, Minami T, Fujita H, Kaminaga C, Matsui K, Ishino R, Fujita A, Oda K, Kawai A, Hasegawa N, Urahama N, Roeder RG, and Ito M (2014) CCAR1/CoCoA pair-mediated recruitment of the Mediator defines a novel pathway for GATA1 function. Genes Cells **19**:28–51.

Muangmoonchai R, Smirlis D, Wong SC, Edwards M, Phillips IR, and Shephard EA (2001) Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1. Biochem J **355**:71–8.

Ou CY, Kim JH, Yang CK, and Stallcup MR (2009) Requirement of cell cycle and apoptosis regulator 1 for target gene activation by Wnt and beta-catenin and for anchorage-independent growth of human colon carcinoma cells. J Biol Chem **284**:20629–37.

Qatanani M, Zhang J, and Moore DD (2005) Role of the constitutive androstane receptor in xenobiotic-induced thyroid hormone metabolism. Endocrinology **146**:995–1002.

Rishi AK, Zhang L, Boyanapalli M, Wali A, Mohammad RM, Yu Y, Fontana JA, Hatfield JS, Dawson MI,

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This article has not been copyedited and formatted. The final version may differ from this version.

Majumdar APN, and Reichert U (2003) Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437. J Biol Chem **278**:33422–35.

Roth A, Looser R, Kaufmann M, Blättler SM, Rencurel F, Huang W, Moore DD, and Meyer UA (2008) Regulatory cross-talk between drug metabolism and lipid homeostasis: constitutive androstane receptor and pregnane X receptor increase Insig-1 expression. Mol Pharmacol **73**:1282–9.

Seo WY, Jeong BC, Yu EJ, Kim HJ, Kim SH, Lim JE, Kwon GY, Lee HM, and Kim JH (2013) CCAR1 promotes chromatin loading of androgen receptor (AR) transcription complex by stabilizing the association between AR and GATA2. Nucleic Acids Res **41**:8526–36.

Shiraki T, Sakai N, Kanaya E, and Jingami H (2003) Activation of orphan nuclear constitutive androstane receptor requires subnuclear targeting by peroxisome proliferator-activated receptor gamma coactivator-1 alpha. A possible link between xenobiotic response and nutritional state. J Biol Chem **278**:11344–50.

Tian J, Huang H, Hoffman B, Liebermann DA, Ledda-Columbano GM, Columbano A, and Locker J (2011) Gadd45β is an inducible coactivator of transcription that facilitates rapid liver growth in mice. J Clin Invest **121**:4491–502.

Ueda A, Hamadeh HK, Webb HK, Yamamoto Y, Sueyoshi T, Afshari CA, Lehmann JM, and Negishi M (2002) Diverse roles of the nuclear orphan receptor CAR in regulating hepatic genes in response to phenobarbital. Mol Pharmacol **61**:1–6.

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Wang Q, Li W, Liu XS, Carroll JS, Jänne OA, Keeton EK, Chinnaiyan AM, Pienta KJ, and Brown M (2007) A hierarchical network of transcription factors governs androgen receptor-dependent prostate cancer growth. Mol Cell **27**:380–92.

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Footnotes

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Figure legends

Fig. 1 CCAR1 interacts with CAR

Nuclear extracts from fCAR cells were immunoprecipitated (IP) with an anti-FLAG antibody and normal IgG. Coprecipitants were resolved by SDS-PAGE and detected by immunoblot analysis using anti-CCAR1, anti-CAR, and anti-DDDDK (α-FLAG) antibodies. The data show one of two independent experiments.

Fig. 2 CCAR1 enhances the transactivation activity of CAR.

HepG2 cells were transiently co-transfected with various CAR-driven luciferase reporter plasmids (0.1 μg) and pGL4.74 (0.01 μg) carrying the *Renilla* luciferase gene and expression plasmids for CAR (0.05 μg) or CCAR1 (0.1(+) or 0.2(++) μg) or empty plasmid, as indicated.

Cells were harvested at 48 h post-transfection.

(A) Whole cell lysates prepared from transfected cells were resolved by SDS-PAGE and detected by western blot analyses using anti-FLAG and anti-Tubulin antibodies.

(B) Luciferase activity was measured. Results are presented as the *Renilla*-normalized luciferase activity. The experiments were performed in three-independent wells (n = 3) and repeated at least two times. Representative results of one repeat are shown as the means ± SD. (**$p < 0.01$, *$p < 0.05$).

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Fig. 3 CCAR1 selectively activates CAR-mediated transactivation of the gtPBREM enhancer.

HepG2 cells were transiently co-transfected with various CAR-driven luciferase reporter plasmids (0.1 μg) and pGL4.74 (0.01 μg) carrying the *Renilla* luciferase gene and expression plasmids for CAR (0.05 μg) or VP-CCAR1 (0.1 μg) or empty plasmid, as indicated. Results are presented as the *Renilla*-normalized luciferase activity. The experiments were performed in three-independent wells (n = 3) and repeated at least two times. Representative results of one repeat are shown as the means ± SD.

Fig. 4 CCAR1 depletion selectively affects CAR target gene expression in HepTR/hCAR cells.

HepTR/hCAR cells were transfected with CCAR1-targeting or control siRNA. Cells were treated with Tet or solvent (−) for 48 h. (A) Whole cell lysates prepared from cells were resolved by SDS-PAGE and detected by western blot analyses using anti-CCAR1, anti-CAR, and anti-Tubulin antibodies. (B) The levels of *CCAR1*, *CAR*, *CYP2B6*, *CYP3A4*, and *UGT1A1* mRNA were measured by qRT-PCR. Results were normalized against those for β-actin. The experiments were performed in three-independent wells (n = 3) and repeated at least two times. Representative results of one repeat are shown as the means ± SD.

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Fig. 5 CCAR1 depletion selectively affects CAR target gene expression in HepaRG cells.

HepaRG cells were transfected with CCAR1-targeting or control siRNA. The next day, cells were treated with CITCO or solvent (−) for 24 h. (A) Whole cell lysates prepared from cells were resolved by SDS-PAGE and detected by western blot analyses using anti-CCAR1 and anti-Tubulin antibodies. (B) The levels of *CCAR1*, *CYP2B6*, *CYP3A4*, and *UGT1A1* mRNA were measured by qRT-PCR. Results were normalized against those for β-actin. The experiments were performed in three-independent wells (n = 3) and repeated at least two times. Representative results of one repeat are shown as the means ± SD.

Fig. 6 CCAR1 is selectively recruited to gtPBREM of the *UGT1A1* promoter together with CAR but not to PBREM of the *CYP2B6* promoter.

HepTR/hCAR cells were treated with Tet or solvent for 24 h. Cells were used to perform ChIP with anti-CAR, anti-CCAR1, and normal IgG antibodies and analyzed by qPCR with primers flanking PBREM of the *CYP2B6* promoter, gtPBREM of the *UGT1A1* promoter, and the CAR-free region of the *CYP2B6* intron. Results are presented as the means of fold-enrichment by Tet treatment (n = 3) ± S.D. Statistically significant recruitment differences by Tet treatment compared to IgG are shown by an asterisk (*$p < 0.05$).

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Fig. 7 CCAR1 synergistically enhances CAR-mediated transactivation with other coactivators. HepG2 cells were co-transfected with the reporter plasmid (0.1 μg), pGL4.74 (0.01 μg), and expression plasmids for CAR (0.05 μg), CCAR1 (0.1 μg), SRC1 (0.05 μg), and SRC2 (0.05 μg), and empty plasmid, as indicated. The reporter plasmids used were indicated as DR4-(A), gtPBREM-(B) and PBREM-(C). Cells were harvested and luciferase activity was measured at 48 h post-transfection. Results are presented as the *Renilla*-normalized luciferase activity. The experiments were performed in three-independent wells (n = 3) and repeated at least two times. Representative results of one repeat are shown as the means ± SD.

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Figure 1

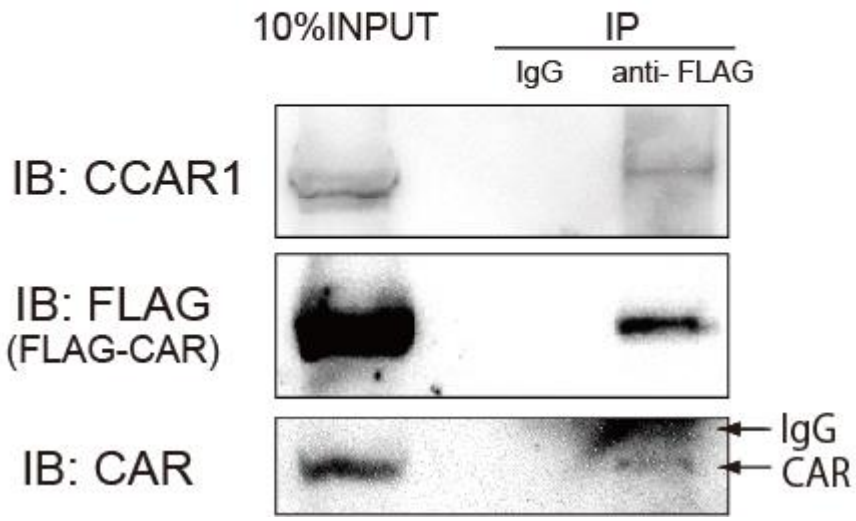

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Figure 2

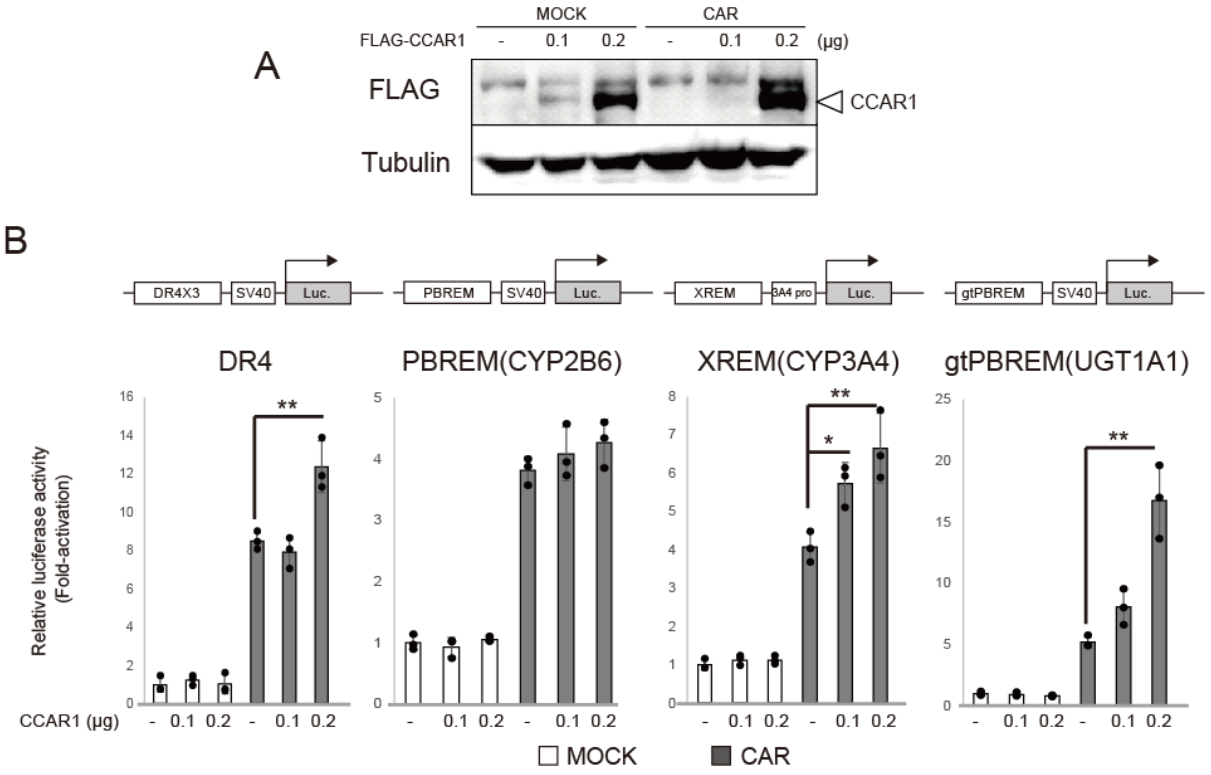

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Figure 3

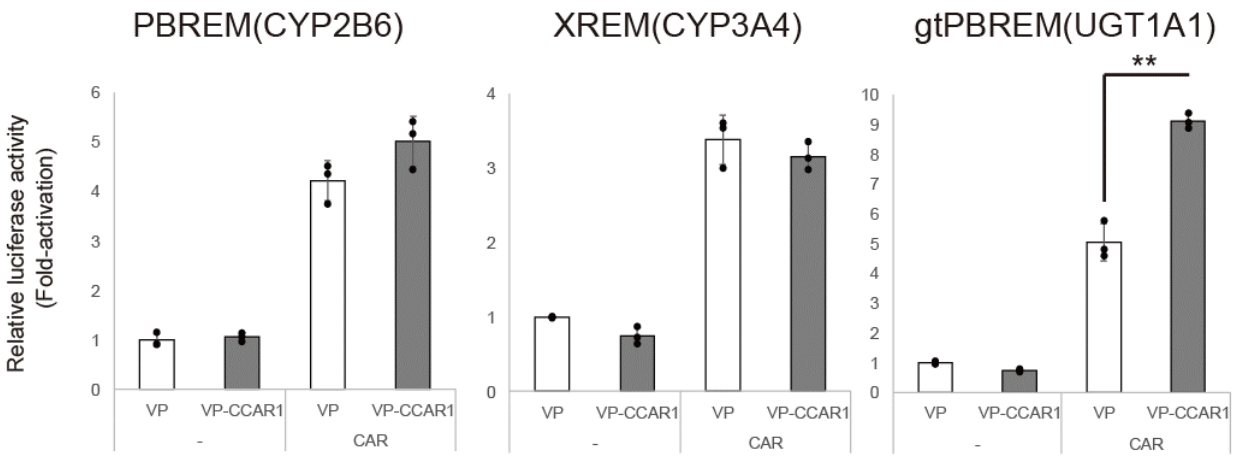

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Figure 4

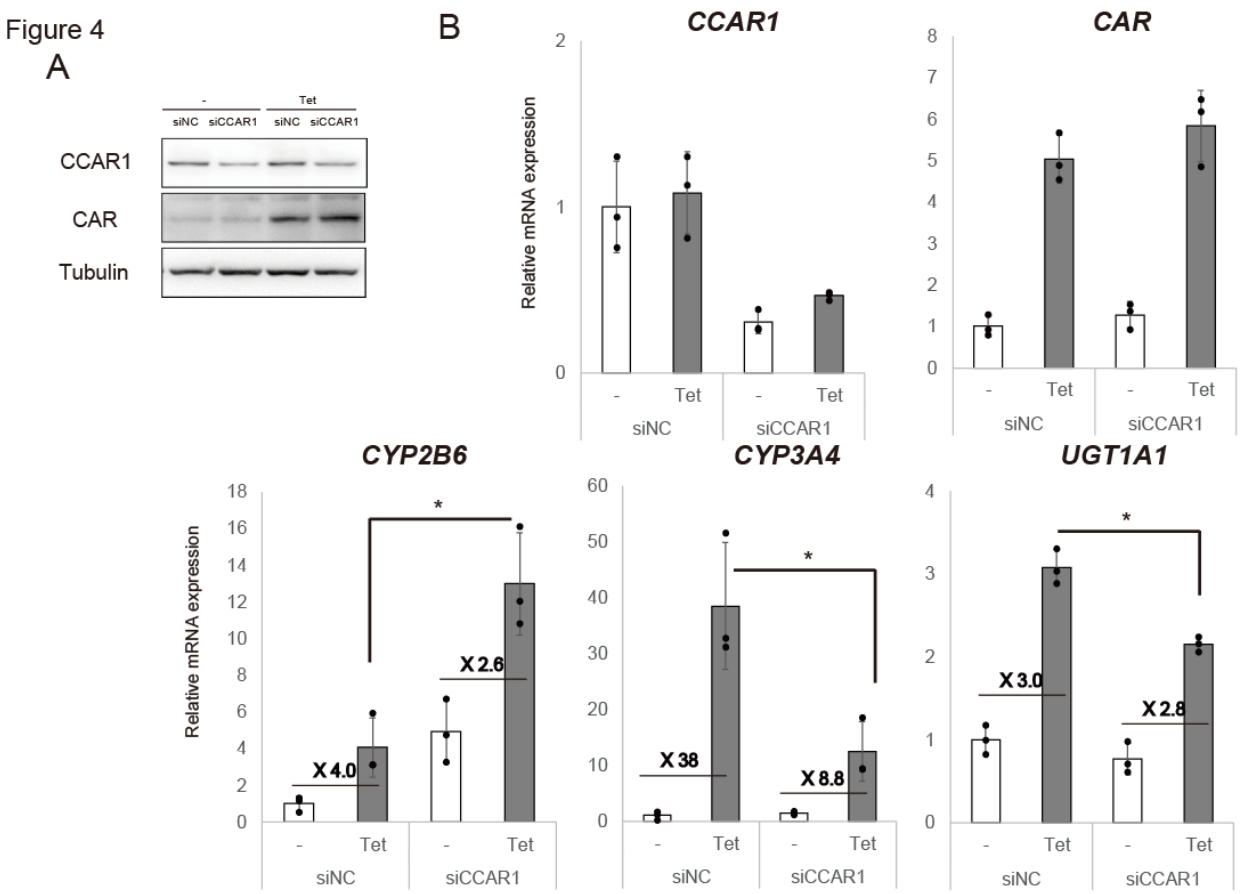

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Figure 5

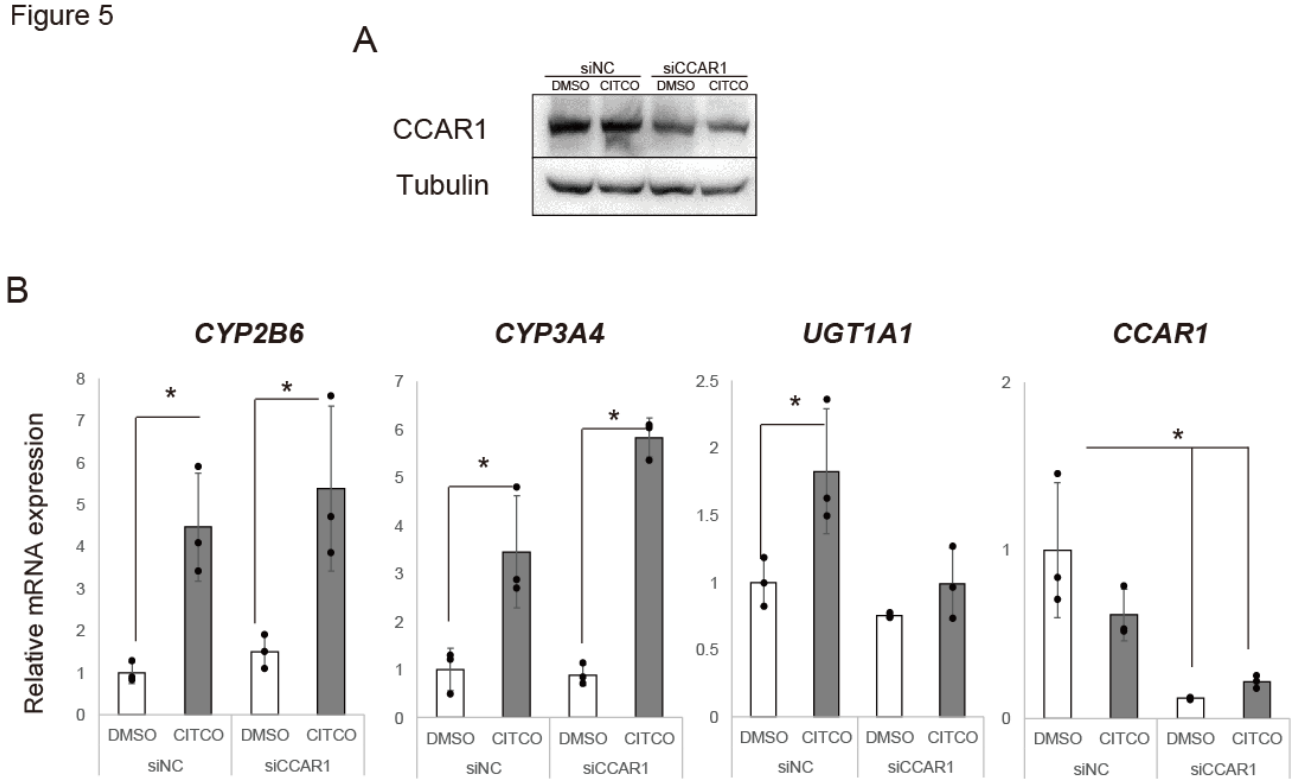

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Figure 6

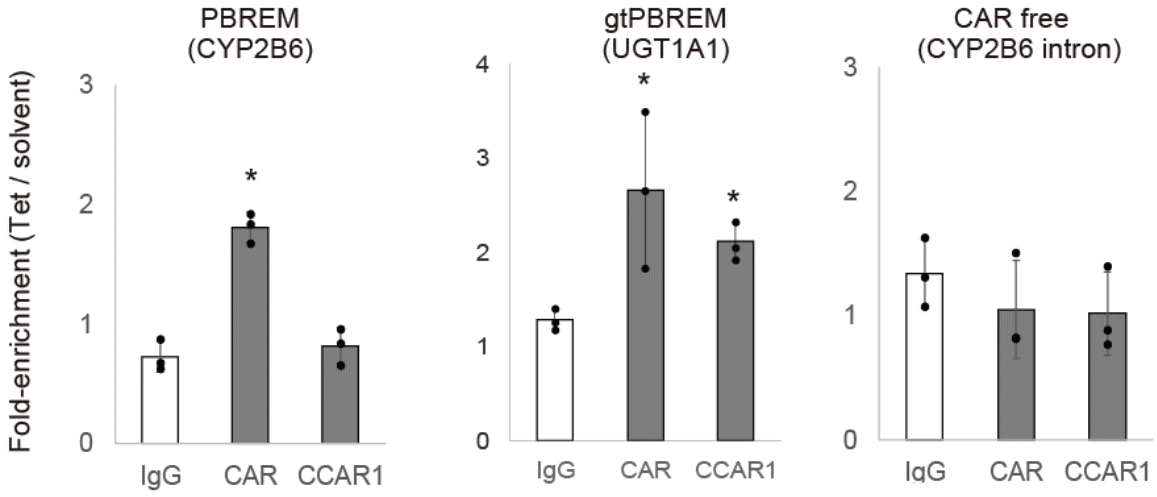

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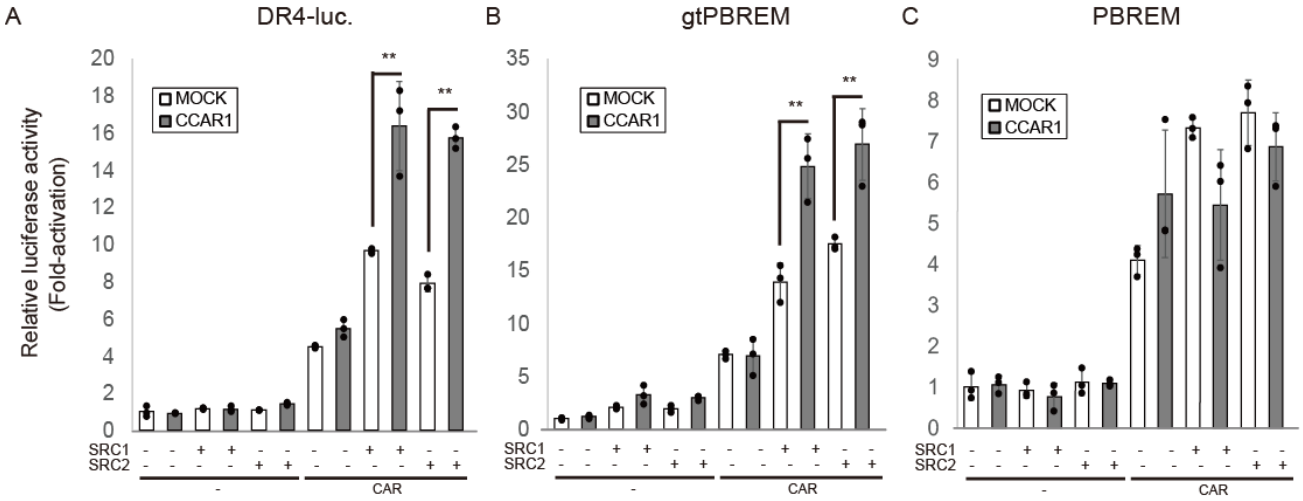
Figure 7



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