Title:
The tripartite motif containing 24 acts as a novel coactivator of the constitutive active/androstane receptor

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Running title: TRIM24 as a novel 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
DMD # 77693

Abstract

The constitutive active/androstane receptor (CAR) is a nuclear receptor that acts as a transcription factor for a variety of genes, including genes encoding xenobiotic, steroid and drug-metabolizing enzymes and transporters. Transactivation of a target gene by a transcription factor is generally mediated through the concerted and stepwise recruitment of various proteins termed co-regulators, including coactivators and corepressors. In this study, tripartite motif containing 24 (TRIM24; also known as transcriptional intermediary factor 1 alpha) was found to interact with the CAR. TRIM24 enhanced the CAR-dependent transactivation in reporter assays using the direct repeat-4 (DR4) motif, a binding site of the CAR. This enhancement was synergistically augmented in the presence of SRC1 or SRC2, both of which are coactivators of the CAR. In addition, TRIM24 was recruited to the CAR binding element of the CYP2B6 promoter together with the CAR. We also noted that knockdown of TRIM24 suppressed the CAR-induced CYP2B6 mRNA expression in HepTR/CAR and HepaRG cells and suppressed CAR-induced CYP3A4 mRNA expression in HepaRG cells but not HepTR/CAR cells. From these results, we suggest that TRIM24 is a novel coactivator of the CAR that is involved in cell- and/or promoter- selective transactivation.
Introduction

The nuclear receptor (NR) superfamily comprises a large superfamily of transcription factors, many of which regulate gene expression in a ligand-dependent manner (Mangelsdorf et al., 1995; Gronemeyer et al., 2004). Members of the NR superfamily are composed of several domains, including an N-terminal trans-activation domain containing activation function 1 (AF-1), a DNA-binding domain (DBD) containing zinc fingers that binds to specific DNA sequences, a hinge region, and a ligand-binding domain (LBD) containing ligand-dependent activation function 2 (AF-2). The transactivation ability of a NR is generally mediated through the concerted and stepwise recruitment of various proteins termed co-regulators, including coactivators and corepressors.

The constitutive active/androstane receptor (CAR; NR1I3), a member of the NR superfamily, plays a key role in cellular responses to xenochemical stimuli by inducing the gene expression of various xenobiotic-metabolizing enzymes and transporters. Its typical targets are the cytochrome P450 (CYP) 2B, 2C, and 3A subfamilies and uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) (Honkakoski et al., 1998; Sueyoshi et al., 1999; Sugatani et al., 2001). The CAR is predominantly localized in the cytoplasm of hepatocytes in vivo and in primary hepatocytes, and is translocated to the nucleus in response to the binding of ligands or indirect activators, such as 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and phenobarbital, respectively. Following nuclear translocation, the CAR forms a heterodimer with the retinoid X receptor α (RXRα) and binds to specific sequences (responsive elements) in the promoter regions of target genes (Baes et al., 1994). Unlike other NRs, the CAR has constitutive transcriptional activity. One reason for this is that the CAR is able to recruit coactivators without binding to ligands. Some coactivators that interact with the CAR have been identified, such as steroid receptor coactivator 1 (SRC1) (Muangmoonchai et al., 2001), SRC2 (also known as transcriptional intermediary factor 2) (Min et al., 2002), SRC3 (Chen et al., 2012), NCoA6 (Surapureddi et al., 2008), activating signal cointegrator 2 (ASC2) (Choi et al., 2005), GADD45 beta (Tian et al., 2011) and peroxisome proliferator-activated receptor-gamma coactivator 1 Alpha (PGC1α) (Shiraki et al., 2003). We have also previously identified two coactivators of the CAR, DEAD box DNA/RNA helicase DP97 (Kanno et al., 2012) and protein arginine methyltransferase 5 (PRMT5) (Kanno et al., 2015). However, the
mechanisms by which the CAR regulates gene expression are still being elucidated.

The aim of this study was to discover novel cofactor(s) involved in the mechanisms underlying the CAR-mediated gene regulation. To identify novel cofactors, we performed a co-immunoprecipitation analysis using a human hepatocellular carcinoma hepG2 cells expressing FLAG-epitope tagged CAR (FLAG-CAR). Among the co-precipitated proteins, we focused on tripartite motif containing 24 (TRIM24) as a novel CAR-interacting protein.

Materials and Methods

Plasmid construction and chemicals

The construction of expression plasmids encoding N-terminal myc or FLAG epitope-tagged human CAR proteins (myc-CAR or FLAG-CAR) has been reported previously (Kanno et al., 2012; Kanno et al., 2005; Kanno et al., 2007). The expression plasmid encoding a GAL4-DBD/CAR-LBD fusion protein was constructed as reported previously (Kanno et al., 2010). The pEBMultipuro/FLAG-CAR plasmid was constructed by inserting a FLAG-epitope tagged human CAR fragment into the multiple cloning site of pEBMultipuro (Wako, Osaka, Japan), which carries Epstein-Barr virus nuclear antigen 1 to allow episomal replication of the plasmid without integration into the host genome. It also included the puromycin N-acetyltransferase gene to enable selection of transfectants by puromycin. Full-length human TRIM24 cDNA was amplified by RT-PCR using mRNA prepared from HepG2 cells. The expression plasmid encoding FLAG epitope-tagged TRIM24 (FLAG-TRIM24) was constructed based on the pCMV-3Tag-6 vector (Stratagene, Santa Clara, CA, USA) using an In-Fusion HD Cloning Kit (Clontech, CA, USA). The direct repeat-4 (DR4)-driven luciferase reporter plasmid (pDR4-Luc) was constructed by inserting three tandem repeats of the DR4 motif sequence (AGTTCA|TGGC|AGTTCA), into pGL4.24 (Promega, Madison, WI, USA), which has a minimal promoter (Kanno et al., 2012). The GAL-driven luciferase reporter (pG5-luc) was purchased from Promega. The expression plasmids encoding siRNA to knock down TRIM24 expression were generated by inserting the following shTRIM24-#1 or shTRIM24-#2 annealed double-strand fragments into the BamHI-HindIII sites of pBasi-hU6 Pur, which contains an RNA polymerase III promoter for the expression of siRNA (Takara, Japan): shTRIM24-#1; 5′-GAT CCG CAT GAA ATG AGC CTG GCT TTC TCG AGA AAG CCA GGC TCA TTT CAT GCT TTT
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TA-3’ and 5’-AGC TTA AAA AG C ATG AAA TGA GCC TGG CTT TCT CGA GAA AGC CAG GCT CAT TTC ATG CG-3’, shTRIM24-#2; 5’-GAT CCG CCA TGA AAT GAG CCT GGC TTT CTC GAG AAA GCC AGG CTC ATT TCA TGG CTT TTT A-3’ and 5’-AGC TTA AAA AGC CAT GAA ATG AGC CTG GCT TTC TCG AGA AAG CCA GGC TCA TTT CAT GGC G-3’.

Tetracycline (Tet), PK11195, CITCO and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and cell culture

The HepTR/hCAR cell line, derived from HepG2 cells, was previously established using the T-REx system (Invitrogen), in which the expression of hCAR can be induced by tetracycline (Tet)-treatment (Kanno et al., 2012). HEK293, MCF-7, HepG2, HepTR/hCAR cells were cultured in Dulbecco’s modified Eagle’s medium (Wako, Osaka, Japan) containing 10% fetal bovine serum and penicillin–streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. 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% CO₂ and 95% air according to the manufacturer’s instructions.

Identification of TRIM24 by co-immunoprecipitation

pEBMultipuro/FLAG-hCAR was transfected into HepG2 cells, then a clone stably expressing FLAG-hCAR was isolated by puromycin selection, named fCAR cells. The clone cells were harvested and suspended in lysis buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 1% Triton X-100) containing a protease inhibitor cocktail (Roche Diagnostics). The recovered supernatant was incubated with Anti-DDDDK-tag mAb-magnetic beads (MBL, Aichi, Japan), which are equivalent to anti-FLAG-tag monoclonal antibody-magnetic beads, at 4°C for 4 h to concentrate the proteins co-immunoprecipitated with FLAG-hCAR. Co-immunoprecipitated proteins were identified using a mass spectrometry service provided by Oncomics (Nagoya, Japan). Among the candidate proteins interacting with the CAR, we decided to further analyze the properties of
Co-immunoprecipitation using HEK293 cells was also performed to confirm that TRIM24 can interact with the CAR. HEK293 cells were seeded in 60-mm plates and transfected with expression plasmids for myc-hCAR and/or FLAG-TRIM24 using the PEI Max reagent (Polysciences, Warrington, PA, USA). At 48 h post-transfection, cells were harvested after being washed twice with ice-cold phosphate-buffered saline (PBS) and were suspended in lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics). The supernatants were incubated with Anti-DDDDK-tag mAb-magnetic beads at 4°C for 4 h. The beads were washed three times with wash buffer (50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 650 mM NaCl, and 0.1% Triton X-100) and then suspended in sodium dodecyl sulfate (SDS) sample buffer (10% glycerol, 2% SDS, 62.5 mM Tris-HCl (pH 6.8), and 1 mM DTT). The co-immunoprecipitated proteins were then separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and detected by western blotting using anti-myc-tag- and anti-DDDDK (FLAG)-tag-HRP-Direct antibodies. The signals were captured by a LuminoGraph II (ATTO).

**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 the PEI Max reagent (Polysciences). After 48 h, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The activities of firefly luciferase were normalized to those of *Renilla* luciferase.

**TRIM24 knockdown and quantitative RT-PCR**

HepTR/hCAR cells and HepaRG cells were transfected with TRIM24-targeting or control small interfering RNA (siRNA; Sigma Aldrich) using the Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. 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). The specific PCR primers used were as follows: CYP2B6 (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′-AGT GGA TGG CAG CCA CTG GCT-3′ and 5′-CAG TAA GTG GGA ACA GCC AGA-3′), CAR (5′-CCAGCTCATCTGTTCATCCA-3′ and 5′-GGTACTCCAGGTCGGTCAG-3′) and β-actin (5′-TCC TCCTGA GCC TAA 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 Signalling Technology, Danvers, MA). Immunoprecipitation was performed using antibodies directed against the CAR (N4111, Perceus Proteomics Inc., Tokyo, Japan) or TRIM24 (14208, Proteintech, Chicago, IL), or samples were incubated with normal IgG (Cell Signalling Technology) as a negative control. After immunoprecipitation, DNA fragments were subjected to quantitative PCR (qPCR) using primers containing the phenobarbital responsive enhancer module (PBREM) of the CYP2B6 promoter (forward 5′- CTG CAA TGA GCA CCC AAT CTT and reverse 5′- ACA CAT CCT CTG ACA GGG TCA-3′).

Statistical analysis
Statistical comparisons were performed with a 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.

Results
TRIM24 was identified as a CAR-interacting protein
To identify CAR-interacting proteins, we established stably N-terminal FLAG-tagged CAR expressing HepG2 cells (fCAR cells). CAR-protein complexes were immunoprecipitated with an anti-FLAG antibody from the lysates of fCAR cells. Immunoprecipitated proteins were analyzed
by LC-MS/MS systems (Supplemental material S1). Identified proteins contained previously reported CAR-interacting proteins, such as CAR, Hsp90, and Hsp70 (Yoshinari et al., 2003; Timsit YE and Negishi M, 2014). An analysis of the complexes obtained from these experiments identified TRIM24 as a novel candidate CAR-interacting protein. TRIM24, also known as transcriptional intermediary factor 1α (TIF1α), belongs to the tripartite motif (TRIM) family and contains a RING finger, two B-box zinc finger domains, and a coiled-coil region. TRIM24 has been reported to serve as a co-regulator of several nuclear receptors, including the glucocorticoid receptor (GR), estrogen receptor (ER), androgen receptor (AR), retinoic acid receptor (RAR), thyroid hormone receptor (TR), and vitamin D receptor (VDR) (Le Douarin et al., 1995; Le Douarin et al., 1996; Vom et al., 1996; Kikuchi et al., 2009; Teyssier et al., 2006).

To confirm the interaction of TRIM24 with the CAR, a co-immunoprecipitation assay was performed using lysates from a human embryonic kidney cell line, HEK293, expressing myc epitope-tagged CAR (myc-CAR) and/or FLAG-epitope tagged TRIM24 (FLAG-TRIM24). As shown in Fig. 1, the anti-FLAG antibody immunoprecipitate from the lysate of cells expressing both myc-CAR and FLAG-TRIM24 reacted with the anti-myc antibody, confirming that TRIM24 interacts with the CAR.

**TRIM24 enhances CAR transactivity**

We next examined the role(s) of TRIM24 in the CAR-mediated transcripntional regulation in HepG2 cells. It has previously been reported that transformed and immortalized human cells, such as HepG2 cells, lack the ability to retain the CAR in the cytoplasm (Kawamoto et al., 1999; Kanno et al., 2005). Thus, the CAR spontaneously accumulates in the nucleus without activator or ligand treatment. We found that the transfection of increasing amounts of either a TRIM24 expression plasmid or a CAR expression plasmid increased the luciferase reporter activity controlled by the direct repeat-4 (DR4) motif, a binding element of the CAR (Fig. 2A). Similar results were observed in other cell lines, including human breast cancer MCF-7 (Fig. 2B) and human embryonic kidney HEK293 cells (data not shown). We also evaluated the effects of TRIM24 knockdown on the transactivation of the CAR. A TRIM24-targeting shRNA (shTRIM24-#1 or -#2) expression plasmid, the DR4-driven luciferase reporter plasmid, and the CAR expression plasmid were co-transfected
into HepG2 cells and the luciferase activity was measured. The results showed that knocking down TRIM24 reduced the luciferase activity in cells expressing the CAR (Fig. 2C), further supporting that TRIM24 is an activator of the CAR. These results suggest that TRIM24 acts as co-activator of the CAR.

**TRIM24 enhances the transactivity of CAR through the LBD of the CAR**

The AF-2 domain in the LBD of the CAR plays an essential role in the high constitutive activity of the CAR in the absence of ligand by facilitating the active conformation of the CAR and recruiting coactivators, such as SRC1 and SRC2 (Windshugel et al., 2005; Jyrkkarinne et al., 2012). Moreover, the GAL4 DNA-binding domain-CAR LBD fusion protein (GAL4/CAR LBD) can activate transcription in response to the GAL responsive element (GALRE) without a CAR ligand in the mammalian two-hybrid system (Kanno et al., 2010). In the present study, the significance of the CAR AF-2 domain in the function of TRIM24 was evaluated using TRIM24 and GAL4 DBD/CAR LBD expression plasmids and a GALRE-luciferase reporter plasmid. Overexpression of TRIM24 increased the luciferase activity (Fig. 3), suggesting that TRIM24 enhances CAR transactivity through the CAR LBD.

**TRIM24-mediated enhancement of the CAR transactivity is synergistically augmented in the presence of SRC1 or SRC2**

Several studies have reported that TRIM24 is a secondary coactivator, which indirectly activates nuclear receptors in cooperation with several coactivators (Teyssier et al., 2006). To evaluate this possibility for the CAR, we investigated the cooperative effects of TRIM24 with SRC1 and SRC2, which are known coactivators of the CAR. The overexpression of each coactivator increased the luciferase activity from a DR4-driven luciferase reporter plasmid in HepG2 cells expressing the CAR. Simultaneous overexpression of TRIM24 significantly and more strongly augmented the increase in luciferase activities compared to the cells overexpressing SRC1 or SRC2 alone (Fig. 4).

**TRIM24 is co-recruited with the CAR to the PBREM region of the CYP2B6 promoter**
Next, we carried out a ChIP analysis to determine whether TRIM24 was co-recruited with the CAR to the PBREM region of the CYP2B6 promoter containing a CAR-response element. We used HepTR/hCAR cells, which are HepG2 cells carrying a tetracycline (Tet)-inducible CAR gene. A ChIP analysis revealed that the CAR and TRIM24 both recruited to the PBREM region in the presence of Tet treatment (Fig. 5). This result indicated that TRIM24 was recruited with the CAR to the PBREM region.

TRIM24 is an important factor for the promoter-selective transactivity of the CAR

The effects of TRIM24 on the CAR-induced expression of the CYP2B6 and CYP3A4 genes were also assessed following TRIM24 knockdown in HepTR/hCAR cells. In the HepTR/hCAR cells, the knockdown of TRIM24 by siRNA and overexpression of the CAR by Tet were confirmed by western blotting (Fig. 6A) and the mRNA expression of CYP2B6 and CYP3A4 mRNAs was also examined (Fig. 6B). Knocking down TRIM24 suppressed the Tet-inducible CYP2B6 mRNA expression, but did not affect CYP3A4 mRNA expression. We further investigated the effects of TRIM24 on the CAR-inducible mRNA expression using HepaRG cells as a highly-differentiated model for studies of the CAR activation. Treatment with CITCO, an agonist of the CAR increased the CYP2B6 and CYP3A4 mRNA expression (Fig. 7). The induction of CYP2B6 and CYP3A4 mRNA by CITCO was suppressed by TRIM24 knockdown, although CYP3A4 showed downregulated basal expression (in the absence of CITCO).

Discussion

The identification and characterization of coregulators that modulate individual NRs is important to understand the physiological functions of the NRs. The nuclear receptor CAR plays an important role in xenobiotic/drug metabolism. We previously identified DP97 and PRMT5 as gene (or promoter)-selective CAR coactivators (Kanno et al., 2012; Kanno et al., 2015). In the present study, we showed that knocking down TRIM24 reduced the CAR-mediated upregulation of CYP2B6 mRNA, whereas the CAR-mediated up-regulation of CYP3A4 mRNA was insensitive to TRIM24 knockdown in HepTR/CAR cells. Similarly, it was observed that knocking down TRIM24 reduced the CITCO-mediated CYP2B6 mRNA induction in HepaRG cells. Although CITCO-treated group
of CYP3A4 mRNA was decreased by TRIM24 knockdown in HepaRG cells, fold-response was not suppressed. These results suggest that TRIM24 is a novel coactivator of the CAR, which is involved in cell- and/or promoter- selective transactivation.

Previous reports showed that TRIM24 associated simultaneously with SRC2 and coactivator-associated arginine methyltransferase 1 (CARM1), and synergic transcriptional activation of the GR was observed with these coactivators (Teyssier et al., 2006). Because TRIM24 apparently lacks a transcriptional activation function, the role of TRIM24 was unknown. It was reported that the PHD-Bromo region of TRIM24 acts as a reader of dual histone marks (Tsai et al., 2010). TRIM24 recognizes unmodified histone H3 at lysine 4 (H3K4) and acetylated histone 3 at lysine 23 (H3K23ac) within the same histone tail. It was suggested that this function as a histone mark reader is important for co-activation. The CAR-binding enhancer element of CYP2B6 at −1800 bp, called the PBREM, contains two DR4 elements (NR1 and NR2) that act as high-affinity binding sites for the CAR/RXRα (Sueyoshi et al., 1999). CYP3A4 also has a CAR-binding enhancer element at −7800 bp, called the xenobiotic responsive enhancer module (XREM), which contains the distal direct repeat 3-type nuclear receptor binding element (dNR1) and the DR4-type essential distal nuclear receptor binding element for CYP3A4 induction (eNR3A4) (Goodwin et al., 1999; Toriyabe et al., 2009). However, whether there is histone modification in these enhancer elements upon CAR-mediated transactivation is unknown. The difference in TRIM24-mediated coactivation between CYP2B6 and CYP3A4 might be due to differences in the histone marks in these enhancer elements. Further investigation into histone modification by CAR is required to understand the TRIM24-selective coactivation.

TRIM24 was shown to suppress tumorigenesis during RAR activation to prevent liver cancer in a murine model. The TRIM transcription cofactors, TRIM24, TRIM28, and TRIM33, associate to form regulatory complexes that repress murine hepatocellular carcinoma (Herquel et al., 2011; Khetchoumian et al., 2007). Furthermore, TRIM24 knockout mice spontaneously develop hepatic lipid-filled lesions, steatosis, hepatic injury, fibrosis, and hepatic carcinoma since TRIM24 suppress hepatic lipid accumulation, inflammation, fibrosis, and damage (Jiang et al., 2015). Because there is increasing evidence that the CAR is involved in energy metabolism and tumorigenesis, the relationship between the CAR and TRIM24 with regard to energy metabolism
and tumorigenesis should be further analyzed.

In conclusion, we herein demonstrated that TRIM24 is a novel coactivator of the CAR, which acts synergistically with SRC1 and/or SRC2 to enhance CAR-mediated transcription. Further, TRIM24 regulates the promoter-selective coactivation of target genes. TRIM24 may therefore be a key regulator of selective gene induction by the CAR. However, further investigations are required to understanding for selective regulation mechanism.
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Authorship Contributions

Participated in research design: Yuichiro Kanno and Yoshio Inouye
Conducted experiments: Yuichiro Kanno, Yuki Kure, Saori Kobayashi, Mariko Mizuno, Naoya Yamashita, Yumi Tsuchiya
Contributed new reagents or analytical tools: Yuichiro Kanno
Performed data analysis: Yuichiro Kanno, Yuki Kure, Saori Kobayashi, Mariko Mizuno, Naoya Yamashita, Yumi Tsuchiya
Wrote or contributed to the writing of the manuscript: Yuichiro Kanno, Kiyomitsu Nemoto and Yoshio Inouye
References


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Footnotes

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Figure legends

Fig. 1. TRIM24 interacts with the CAR.

HEK293 cells were transfected with expression plasmids for myc-CAR and/or FLAG-TRIM24, or with an empty plasmid, as indicated. Whole cell lysates prepared from transfected cells were immunoprecipitated using anti-DDDDK-tag mAb-magnetic beads. Co-precipitants were resolved by SDS-PAGE and detected by western blot analyses using anti-myc (α-myc) and anti-DDDDK (α-FLAG) antibodies. The data shows one of two independent experiments.

Fig. 2. TRIM24 enhances the transactivational activity of the CAR.

(A) HepG2 cells and (B) MCF-7 cells were transiently co-transfected with the DR4-driven luciferase reporter plasmid (0.1 µg), pGL4.74 (0.01 µg) carrying the Renilla luciferase gene, and expression plasmids for the CAR (0.05 µg), TRIM24 (0.1, 0.15, or 0.2 µg) or an empty plasmid, as indicated. (C) HepG2 cells were co-transfected with the DR4-luciferase reporter plasmid (0.1 µg), pGL4.74 (0.01 µg), expression plasmids for the CAR (0.05 µg), shRNA expression plasmids for TRIM24-#1 (sh24-#1), TRIM24-#2 (sh24-#2) (0.1 µg), or GFP (shGFP) as a control, as indicated. Cells were harvested and the luciferase activity was measured at 48 h post-transfection. The results are presented as the average Renilla-normalized luciferase activity (n=4) and are expressed as the means ± S.D. (**p < 0.01, *p < 0.05). The graph presents one of three independent experiments.

Fig. 3. TRIM24 enhances the transactivation activity of the CAR through the LBD of the CAR.

HepG2 cells were co-transfected with the GALRE-luciferase reporter plasmid (0.1 µg) carrying the GAL-responsive element and pGL4.74 (0.01 µg) and expression plasmids for the GAL4-DBD/CAR-LBD fusion protein (0.05 µg), TRIM24 (0.1 or 0.2 µg) or an empty plasmid, as indicated. Cells were harvested and the luciferase activity was measured at 48 h post-transfection. The results are presented as the average of Renilla-normalized luciferase activity (n=4) and are expressed as the mean ± S.D. (*p < 0.05). The graph presents the results of one of three independent experiments.
Fig. 4. TRIM24 synergistically enhances the CAR-mediated transactivation with other coactivators.
HepG2 cells were co-transfected with the DR4-luciferase reporter plasmid (0.1 µg), pGL4.74 (0.01 µg), and expression plasmids for the CAR (0.05 µg), TRIM24 (0.1 µg each), SRC1 and/or SRC2 (0.1 or 0.2 µg), or an empty plasmid, as indicated. Cells were harvested and the luciferase activity was measured at 48 h post-transfection. The results are presented as the average of the Renilla-normalized luciferase activity (n=4) and are expressed as the means ± S.D (**p < 0.01 vs CAR-mediated transactivation, column 2). The graph presents the data for one of three independent experiments.

Fig. 5. TRIM24 is recruited to the PBREM of the CYP2B6 promoter together with the CAR.
HepTR/hCAR cells were treated with Tet or solvent for 24 h. Cells were subjected to ChIP with anti-CAR and TRIM24 antibodies and analyzed by qPCR with primers flanking the PBREM of the CYP2B6 promoter. The results are presented as the average fold-enrichment induced by Tet treatment (n=4) and are expressed as the means ± S.D. Significant differences in recruitment by Tet treatment compared to IgG are indicated with an asterisk (*p < 0.05).

Fig. 6. TRIM24 knockdown suppress CAR-regulated gene expression in HepTR/hCAR cells.
HepTR/hCAR cells were treated with a TRIM24-targeting or control siRNA. Cells were then treated with Tet or solvent for 48 h. (A) The protein expression levels of TRIM24 and the CAR were detected by western blotting. The data shows one of two independent experiments. (B) The levels of CYP2B6 and CYP3A4 mRNA were measured by RT-qPCR. The results were normalized to those for β-actin (n=3). Individual values are shown along with the mean ± SD (# p < 0.05, ## p<0.01 vs (-) siNC, * p<0.05 vs (tet) siNC ). The graph presents one of three independent experiments.

Fig. 7. TRIM24 knockdown suppress CAR-regulated gene expression in HepaRG cells.
HepaRG cells were transfected with a TRIM24-targeting or control siRNA. On the following day, the cells were treated with CITCO or solvent (-) for 24 h. The levels of TRIM24, CYP2B6 and CYP3A4 mRNA were measured by RT-qPCR. The results were normalized to those for β-actin (n=3). Individual values are shown along with the mean ± SD (# p < 0.05 vs (-) siNC, * p<0.05 vs (CITCO) siNC ).
Fig. 1

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Fig. 2

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B

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Fig. 3
Fig. 5

Bar chart showing fold enrichment of PBREM (CYP2B6)

- IgG
- CAR
- TRIM24

Fold enrichment (Tet/solvent)

* indicates significant difference
Fig. 7

HepaRG

CYP2B6

CYP3A4

TRIM24

Relative mRNA expression

- CPTCO
  sINC
  siTRIM24

- CPTCO
  sINC
  siTRIM24

- CPTCO
  sINC
  siTRIM24
Supplemental Data

Journal Title

Drug Metabolism and Disposition

Article Title

The tripartite motif containing 24 acts as a novel coactivator of the constitutive active/androstane receptor

Authors

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Supplemental Material S1

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Buffer A; 2% Acetonitrile, 0.1%TFA
Buffer B; 80% Acetonitrile, 0.1%TFA

Figure legend of Supplemental material S1

LC-MS/MS systems at Oncomics Co., Lts as a custom service.

Ultrafiltration of co-immunoprecipitated proteins were used by ultrafiltration cartridge (AMICON UKTRA 0.5 3K, Millipore). Proteins were Cys. Alkylated and trypsin digested.

**Equipment and condition**

Nano-LC DiNa System (KYA TECH Corp.)
MS/MS TripleTOF® 5600(AB Sciex)
  Time; 100min

**Data analysis**

ProteinPilotTM Software 4.5 (AB Sciex)
  Sample type: Identification
  Cys. Alkylation: Iodoacetamide
  Digestion: Trypsin
Figure legend of Supplemental material S2

a) HEK293 cells were transfected with expression plasmids for 3tag6 (empty) or 3tag6-TRIM24.

b) HEK293 cells were transfected with expression plasmids for shRNA expression plasmids for TRIM24-#1 (sh24-#1), TRIM24-#2 (sh24-#2) (0.1 µg), or GFP (shGFP).

After 48hr, whole cell lysates were resolved by SDS-PAGE, and proteins were detected by immunoblotting using antibodies against TRIM24 and Tubulin.