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TRIM24 mediates ligand-dependent activation of androgen receptor and is repressed by a bromodomain-containing protein, BRD7, in prostate cancer cells

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Abstract

The androgen receptor (AR) is a ligand-dependent transcription factor that belongs to the family of nuclear receptors, and its activity is regulated by numerous AR coregulators. AR plays an important role in prostate development and cancer. In this study, we found that TRIM24/transcriptional intermediary factor 1 α (TIF1 α), which is known as a ligand-dependent nuclear receptor co-regulator, interacts with AR and enhances transcriptional activity of AR by dihydrotestosterone in prostate cancer cells. We showed that TRIM24 functionally interacts with TIP60, which acts as a coactivator of AR and synergizes with TIP60 in the transactivation of AR. We also showed that TRIM24 binds to bromodomain containing 7 (BRD7), which can negatively regulate cell proliferation and growth. A luciferase assay indicated that BRD7 represses the AR transactivation activity upregulated by TRIM24. These findings indicate that TRIM24 regulates AR-mediated transcription in collaboration with TIP60 and BRD7.

1. Introduction

Hormonal regulation of gene activity is mediated by nuclear receptors acting as ligand-activated transcription factors. Ligand-dependent activation of nuclear receptors requires intermediary factors that interact with the C-terminal activating domain AF-2, which is highly conserved in the nuclear receptor family [1]. The androgen receptor (AR) is a member of the nuclear receptor family and cooperates with various coregulators to modulate their target genes for proper function. AR upregulates or downregulates target gene expressions, depending on coactivators or corepressors [2]. Activities of AR and coregulators are regulated by posttranslational modifications, such as methylation, phosphorylation, acetylation, and ubiquitination [3-6]. Some of these coregulators contain E3 ligase activity, and AR transcriptional activity requires proteasome activity via regulation of AR nuclear translocation and interaction with coregulators in prostate cancer cells [7].

Tripartite motif (TRIM) proteins are characterized by the presence of a RING finger, one or two zinc-binding motifs named B-boxes, and an associated coiled-coil region. Many TRIM proteins have been reported to have a role in the ubiquitination process, and several TRIM family members are involved in various cellular processes, such as transcriptional regulation, cell growth, apoptosis, development, and oncogenesis. Alterations of these might disrupt growth regulations that lead to cancer formation [8].

TRIM24, one of the TRIM family proteins, has an N-terminal TRIM domain with potential self-assembly properties [9, 10], a nuclear receptor interaction box (NR box or LxxLL motif), and a C-terminal region containing a PHD finger and a bromodomain [11, 12], two well conserved signature motifs widely distributed among nuclear proteins

known to function at the chromatin level [13-15]. The latter two domains are also found in other transcriptional cofactors and chromatin-remodeling proteins [16] and are likely to mediate protein-protein interactions. TRIM24 associates with chromatin [17] and the association is mediated by bromodomain-DNA and bromodomain-nucleosome interactions [18]. TRIM24 belongs to the transcriptional intermediary factor 1 family that also includes TIF1 β , TIF1 γ , and TIF1 δ , all of which are believed to regulate chromatin structure [12, 16, 19-21]. TRIM24 is one of the coregulators that has been identified as a molecule interacting with retinoic acid receptor (RAR) in a ligand-dependent fashion [11] and has also been found to interact via a single LXXLL motif with the AF-2 transcriptional activation domain of several nuclear receptors, including thyroid (TR), vitamin D₃ (VDR), and estrogen (ER) receptors other than RAR [12, 22]. TRIM24 likely modulates their transcriptional activity either positively or negatively in a ligand-dependent fashion. TIF1 β interacts with heterochromatin-associated factors HP1 α , HP1 β and HP1 γ to promote silencing on euchromatic genes [23, 24], and colocalization of both TIF1 β and HP1 α/β with centromeres is required for induction of the parietal and visceral endoderm differentiation pathways [25, 26]. The interaction is thought to be involved in heterochromatin-mediated gene silencing [27]. TRIM24 has also been demonstrated to interact with HP1 α , HP1 β and HP1 γ [12]. Furthermore, it has been reported that TRIM24 is involved in modulating gene expression during the first wave of transcriptional activation in the mouse embryo [28] and that TRIM24 acts in mice as a potent liver-specific tumor suppressor by attenuating retinoic acid receptor α -mediated transcription [29].

Bromodomain containing 7 (BRD7) is a bromodomain-containing gene product

that was identified from nasopharyngeal carcinoma (NPC) cells by cDNA representational difference analysis. Expression of BRD7 was decreased or lost in NPC biopsies and cell lines, but the expression level was high in the normal nasopharyngeal epithelium [30]. Moreover, overexpression of BRD7 inhibits NPC cell growth and arrests the cells in G0/G1 phase by regulating transcription of some important molecules involved in ras/MEK/ERK and Rb/E2F pathways [31]. It has also been found that BRD7 can inhibit accumulation of β -catenin in the nucleus and plays a negative role in the MEK/ERK pathway [32]. It has been reported that BRD7 interacts with nuclear transcription factor IRF2 [33] and forms a triple complex with adenovirus nuclear protein E1B-AP5, thereby affecting the transcriptional activity of E1B-AP5 [34].

In this study, we showed that TRIM24 acts as a coactivator of AR in prostate cancer cells. Furthermore, we identified BRD7 as a novel TRIM24-interacting protein by yeast two-hybrid screening and found that BRD7 inhibits the transcriptional activity of AR upregulated by TRIM24.

2. Materials and methods

2.1. Cell culture

Prostate cancer cell lines CWR22Rv1, LNCaP-FGC and PC3 were provided by ATCC. CWR22Rv1 and LNCaP were cultured under an atmosphere of 5% CO₂ at 37°C in RPMI 1640 (Sigma) supplemented with 10% fetal bovine serum (Invitrogen). PC3, HEK293T and COS7 cell lines were cultured under the same conditions in DMEM (Sigma) supplemented with 10% fetal bovine serum.

2.2. Cloning of cDNAs and plasmid construction

Mouse TRIM24 cDNA was amplified from a mouse T cell cDNA library by PCR with BlendTaq (Takara). The amplified fragments did not contain the DNA sequence corresponding to amino acids 10-111, which encodes about 75% of a RING finger domain, and were utilized as TRIM24(Δ RING). Full-length TRIM24 was constructed by using the fragments amplified from a mouse testis cDNA library by PCR with KOD-plus (Toyobo). They were subcloned into pSC-A (Stratagene) and sequenced. Mouse BRD7 cDNA was amplified by PCR using pACT2-BRD7 isolated from yeast two-hybrid screening as a template and the amplified fragments were subcloned into pBluescript II SK⁺ (Stratagene). TRIM24 and BRD7 cDNAs were subcloned into p3xFLAG (Sigma) and pCGN-HA [35] for expression in eukaryotic cells and into pBTM116 and pACT2 (Clontech) for a yeast two-hybrid system. A deletion mutant of BRD7 cDNA containing amino acids 237-651 was subcloned from pCGN-HA-BRD7

by digesting with EcoR I. AR and TIP60 cDNAs were kindly provided by Dr. Sobue (Nagoya University) and Dr. Ikura (Tohoku University), respectively.

2.3. Yeast two-hybrid screening

Complementary DNA encoding the full-length of mouse TRIM24 was fused in-frame to the nucleotide sequence for the LexA domain (BD) in the yeast two-hybrid vector pBTM116. To screen for proteins that interact with TRIM24, we transfected yeast strain L40 (Invitrogen) stably expressing the corresponding pBTM116 vector with a mouse brain cDNA library (Clontech).

2.4. Antibodies and reagents

The antibodies used were as follows: mouse monoclonal anti-HA (HA.11/16B12, Covance), rabbit polyclonal anti-HA (Y11, Santa Cruz), mouse monoclonal anti-FLAG (M2 or M5, Sigma), mouse monoclonal anti-TRIM24 (Abnova), mouse monoclonal anti-AR (Santa Cruz), rabbit polyclonal anti-BRD7 (Aviva) and mouse monoclonal anti- β -actin (AC15, Sigma). Dihydrotestosterone (DHT), bicalutamide and flutamide were purchased from Sigma.

2.5. Transfection, immunoprecipitation, and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method and lysed in a solution containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Nonidet P-40,

leupeptin (10 µg/mL), 1 mmol/L phenylmethylsulfonyl fluoride, 400 µmol/L Na₃VO₄, 400 µmol/L EDTA, 10 mmol/L NaF, and 10 mmol/L sodium pyrophosphate. The cell lysates were centrifuged at 16,000 x g for 15 min at 4°C, and the resulting supernatant was incubated with antibodies for 2 h at 4°C. Protein A-sepharose (Amersham Pharmacia Biotech) that had been equilibrated with the same solution was added to the mixture, which was then rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with primary antibodies, horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:10,000 dilution; Promega), and an enhanced chemiluminescence system (Amersham Pharmacia).

2.6. *Dual-luciferase assay*

Cells were seeded in 24-well plates at 1 x 10⁵ cells per well (CWR22Rv1 and LNCaP) or 5 x 10⁴ cells per well (PC3) and incubated at 37°C with 5% CO₂ for 24 h. The mouse mammary tumor virus-luciferase (MMTV-Luc) reporter plasmid and the pRL-TK Renilla luciferase plasmid (Promega) were transfected with TRIM24 and/or BRD7 expression vectors into CWR22Rv1, LNCaP or PC3 cells using Fugene HD reagent (Roche). Transfected cells were incubated in phenol-red free RPMI 1640 or DMEM (Invitrogen) supplemented with 10% charcoal-treated fetal bovine serum (Equitech-Bio) for 24 h and then incubated with DHT (10 nmol/L), bicalutamide (1 µmol/L) or flutamide (1 µmol/L) for 24 h, harvested, and assayed for luciferase activity with a Dual-Luciferase Reporter Assay System (Promega). The luminescence was quantified with a luminometer (Promega).

2.7. Pulse-chase analysis with cycloheximide

Transiently transfected HEK293T cells were cultured with cycloheximide at the concentration of 50 µg/mL and then incubated for various times. Cell lysates were then subjected to SDS-PAGE and immunoblot analysis with antibodies to HA, β-actin and FLAG.

2.8. Immunofluorescence staining

COS7 cells expressing FLAG-tagged TRIM24 (wild-type or ΔRING) or HA-tagged BRD7 grown on a glass cover were fixed for 10 min at room temperature with 2% formaldehyde in PBS and then incubated for 1 h at room temperature with primary antibody to FLAG or HA in PBS containing 0.1% bovine serum albumin and 0.1% saponin. They were then incubated with Alexa488-labeled goat polyclonal antibody to mouse immunoglobulin or Alexa546-labeled goat polyclonal antibody to rabbit immunoglobulin (Molecular Probes) at a dilution of 1:1,000. The cells were covered with a drop of VECTASHIELD (VECTOR) and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51 microscope.

2.9. Statistical analysis

The unpaired Student's *t* test was used to determine the statistical significance of experimental data.

3. Results

3.1. TRIM24 interacts with AR in vivo

Since it has been reported that TRIM24 is a transcriptional regulator of several nuclear receptors, we hypothesized that TRIM24 is involved also in the AR signaling pathway in prostate cancer cells. We checked the expression levels of TRIM24 and AR in prostate cancer cell lines including CWR22Rv1, LNCaP and PC3. Immunoblot analysis showed that these prostate cancer cell lines express TRIM24 and that CWR22Rv1 and LNCaP abundantly express AR, whereas PC3 expresses AR at barely detectable levels [36] (Fig. 1A). It has already been reported that CWR22Rv1 expresses a full-length AR isoform of 114 kDa containing an in-frame tandem duplication of exon 3 with H874Y mutation in the ligand-binding domain and a carboxy-terminally truncated form of 75-80 kDa that lacks a ligand-binding domain, and it has been shown that LNCaP expresses a full-length AR but that AR in LNCaP contains one point mutation (T877A) [37-39].

To determine whether TRIM24 physically interacts with AR in vivo, expression vectors encoding FLAG-tagged TRIM24 and HA-tagged AR were transfected into HEK293T cells. The cell lysates were subjected to immunoprecipitation with anti-FLAG antibody, and the resulting precipitates were subjected to immunoblot analysis with antibodies to HA and FLAG. We found that TRIM24 specifically interacts with AR (Fig. 1B). We also verified interaction between endogenous TRIM24 and AR in CWR22Rv1 cells with dihydrotestosterone (DHT) treatment by immunoprecipitation with anti-AR antibody (Fig. 1C).

3.2. TRIM24 enhances AR-mediated transcriptional activity

To examine whether TRIM24 affects AR-mediated transcriptional activity, we performed a luciferase reporter assay using an MMTV promoter-driven luciferase construct (MMTV-Luc) in CWR22Rv1 cells. FLAG-tagged TRIM24 expression vector and MMTV-Luc were transfected into CWR22Rv1 cells, and luciferase activity was measured with or without DHT. The luciferase assays showed that TRIM24 enhances androgen-dependent AR-mediated transcriptional activity in a dose-dependent fashion (Fig. 2A). We performed immunoblot analysis using anti-FLAG and anti-TRIM24 antibodies to compare the expression levels of endogenous TRIM24 and transfected FLAG-TRIM24, and the results suggested that overexpressed TRIM24 causes an increase in transcriptional activity (Fig. 2B).

It has been reported that PC3 cells transfected with AR can be used for AR-mediated transactivation assay [40-43]. To determine whether the effect of TRIM24 depends on the expression of AR, we performed a luciferase reporter assay with PC3 cells which do not express AR (Fig. 1A). Androgen-dependent AR-mediated transcriptional activity was not increased in PC3 cells, regardless of transfection of TRIM24. However, PC3 cells that were transiently transfected with an expression vector encoding AR showed androgen-dependent AR-mediated transcriptional activity and TRIM24 further enhanced the activity. These findings showed that the expression of AR is required for androgen-dependent transcriptional activation via TRIM24 (Fig. 2C).

3.3. TRIM24 lacking a RING finger domain also enhances AR-mediated transcriptional

activity

To investigate the role of a RING finger domain of TRIM24 in AR-mediated transcriptional activity, we used a deletion mutant lacking a RING finger domain of TRIM24 (TRIM24(Δ RING)) for a luciferase reporter assay (Fig. 3A). The TRIM24(Δ RING) mutant also enhanced androgen-dependent AR-mediated transcriptional activity in a dose-dependent fashion (Fig. 3B), but its effect was slightly weaker than that of wild-type TRIM24 (Fig. 3C), suggesting that the RING domain is partially important for AR-mediated transcription by TRIM24.

To confirm the physiologic role of TRIM24 in AR-mediated transcription, we used RNAi to down-regulate endogenous TRIM24 in CWR22Rv1 cells. Short interference RNAs (siRNA) targeting TRIM24 were introduced into CWR22Rv1 cells using a retroviral infection system. RNAi treatment resulted in significant silencing of TRIM24 at the protein level in CWR22Rv1 cells (Fig. S1A). To examine the effect of the depletion of TRIM24 on AR-mediated transcriptional activity, we performed a luciferase reporter assay in CWR22Rv1 cells transfected with TRIM24 siRNA. Down-regulation of TRIM24 caused a slight decrease in androgen-dependent AR-mediated transcriptional activity but did not show a statistically significant effect (Fig. S1B).

It has been reported that antiandrogen reagents inhibit AR-mediated transcriptional activity induced by androgen [44-46] and that the AR-coactivator CBP enhances AR-mediated transcriptional activity in the presence of the antiandrogens hydroxyflutamide and bicalutamide [47]. To examine whether TRIM24 acts as an agonist for androgen dependent-transcriptional activation in collaboration with

antiandrogens, we performed a luciferase reporter assay using the antiandrogens bicalutamide and flutamide. Luciferase reporter assays showed that both drugs affected androgen-dependent transcriptional activity but partially suppressed the transcriptional activation by TRIM24. However, neither bicalutamide nor flutamide acted as an agonist in the presence of TRIM24 (Fig. 3D and E).

3.4. TRIM24 cooperates with TIP60 to enhance AR-mediated transcriptional activity

Previous studies by us and others have shown that AR and coregulators are regulated by histone acetyltransferases, such as TIP60, to enhance AR-mediated transcriptional activity [5, 48]. Therefore, we hypothesized that TRIM24 physically or functionally interacts with TIP60. To determine whether TRIM24 physically interacts with TIP60, expression vectors encoding FLAG-tagged TRIM24 and HA-tagged TIP60 were transfected into HEK293T cells. The cell lysates were subjected to immunoprecipitation with anti-HA antibody, and then immunoblot analysis was performed with an antibody to FLAG or HA. An *in vivo* binding assay showed that TRIM24 specifically interacts with TIP60 (Fig. 4A). To determine whether TRIM24 functionally interacts with TIP60, we performed an AR transactivation assay. CWR22Rv1 cells were cotransfected with expression vectors encoding TIP60 and/or TRIM24 with MMTV-Luc as a reporter and treated with or without DHT, and then luciferase reporter assays were performed. TIP60 and TRIM24 individually enhanced AR-mediated transactivation by DHT. Most importantly, the combination of TIP60 and TRIM24 further enhanced AR-mediated transcriptional activity (Fig. 4B). These findings suggest that TRIM24 cooperates with the coactivator TIP60 for AR-mediated

transactivation.

3.5. TRIM24 interacts with BRD7

To identify novel proteins that interact with TRIM24, we performed yeast two-hybrid screening from a mouse brain cDNA library by using full-length TRIM24 as bait. Twenty-nine positive clones were isolated on Leu-Trp-His-deficient medium from 6.1×10^5 transformants. One of the positive clones was identified as bromodomain containing 7 (BRD7). We tested whether TRIM24 physically interacts with BRD7 *in vivo*. We expressed FLAG-tagged TRIM24 and HA-tagged BRD7 in HEK293T cells. Cell lysates were subjected to immunoprecipitation with anti-HA antibody, and the resulting precipitates were subjected to immunoblot analysis with an antibody to FLAG or HA. An *in vivo* binding assay showed that FLAG-tagged TRIM24 specifically interacts with HA-tagged BRD7 (Fig. 5A). Next, to examine whether a RING finger domain of TRIM24 and a bromodomain of BRD7 are required for the interaction between TRIM24 and BRD7, we constructed a BRD7 mutant lacking a bromodomain (BRD7(Δ BRD)) (Fig. 5B). An *in vivo* binding assay clarified that TRIM24(Δ RING) interacts with BRD7 (Fig. 5C) and that BRD7(Δ BRD) interacts with TRIM24 (Fig. 5D), indicating that a RING finger domain of TRIM24 and a bromodomain of BRD7 are dispensable for the interaction between TRIM24 and BRD7.

3.6. TRIM24 colocalizes with BRD7 but does not accelerate degradation of BRD7

Next, we performed immunofluorescence staining to determine the subcellular

localization of TRIM24 (WT or Δ RING) and BRD7. COS7 cells were transfected with expression vectors encoding FLAG-tagged TRIM24 (WT or Δ RING) or HA-tagged BRD7 and then fixed and stained with antibodies to FLAG or HA with Hoechst33258 to reveal the subcellular distribution of FLAG-tagged TRIM24 (WT or Δ RING) (green), HA-tagged BRD7 (red), and nuclei (blue). Immunofluorescence staining revealed that both FLAG-tagged TRIM24(WT) and HA-tagged BRD7 showed a predominantly nuclear localization pattern, whereas TRIM24(Δ RING) showed a predominantly cytosolic localization pattern (Fig. 6A).

Although it is likely that TRIM24 has an E3 ubiquitin ligase activity via its RING-finger domain [49], we could not find enhanced ubiquitination of BRD7 by TRIM24 (data not shown). We also performed pulse-chase analysis to determine whether TRIM24 affects the stability of BRD7. Pulse-chase analysis revealed that overexpression of TRIM24 does not accelerate the degradation of BRD7 (Fig. 6B and C).

3.7. Expression patterns of TRIM24 and BRD7 in various cell lines

To study the expression profiles of TRIM24 and BRD7, we compared the protein levels of TRIM24 and BRD7 in various human cell lines from different origins: prostate cancer cell lines CWR22Rv1, LNCaP and PC3, acute myeloid leukemia cell lines KG-1, HL-60, NKM-1, J111, HEL, KMOE-2 and CMK, chronic myeloid leukemia cell line K562, T cell leukemia cell line Jurkat, embryonic kidney cell line HEK293T, uterine epithelial carcinoma cell line HeLa, and hepatocellular carcinoma cell line HepG2 (Fig. 7). Immunoblot analysis using several cell lines showed various expression patterns of

TRIM24 and BRD7.

3.8. BRD7 represses AR-mediated transcriptional activity upregulated by TRIM24

To examine whether BRD7 functionally affects AR-mediated transcription in CWR22Rv1 cells, we performed a luciferase reporter assay. BRD7 expression vector and MMTV-Luc were transfected into CWR22Rv1 cells, and luciferase activity was measured with or without DHT (Fig. 8A). The luciferase assays showed that BRD7 represses androgen-dependent transcriptional activity in a dose-dependent fashion.

We also examined whether BRD7 modulates the effect of TRIM24 in the AR signaling pathway. CWR22Rv1 cells were cotransfected with expression vectors encoding BRD7 and/or TRIM24 with MMTV-Luc as a reporter and treated with or without DHT, and then luciferase assays were performed. TRIM24 enhanced AR-mediated transactivation by DHT. However, AR-mediated transcriptional activity by the combination of TRIM24 and BRD7 was much less than that by TRIM24 (Fig. 8B). These findings suggest that BRD7 represses AR-mediated transcriptional activity by TRIM24.

4. Discussion

AR signaling has key roles in the development and progression of prostate cancer. Like other steroid receptors, the activity of AR is regulated by a series of cofactors that either enhance or suppress AR-mediated gene transcription [50]. It has been shown that TRIM24 has various functions, including activation of AF-2 of nuclear receptors such as RAR α [11]. Since AR is also a ligand-dependent nuclear receptor, TRIM24 may promote modification of chromatin and formation of a bridge between the DNA-bound nuclear receptor and the basal transcriptional machinery recruiting or stabilizing the chromatin remodeling proteins to the nuclear receptor target gene. Moreover, it has been reported that TRIM24 is a phosphoprotein that undergoes ligand-dependent hyperphosphorylation upon nuclear receptor binding *in vivo* and that it also has a kinase activity that can phosphorylate basal transcription factors (TFIIE α , TAFII28, and TAFII55) *in vitro* [51]. However, the specific molecular mechanism of TRIM24 as a coactivator and the relation to other coactivators involved in transcriptional activation of endogenous genes remain to be determined.

In this study, we showed that TRIM24 acts as a coactivator of AR in prostate cancer cells. We found that TRIM24 physically interacts with AR and enhances androgen-dependent AR-mediated transcriptional activity in a dose-dependent fashion. TRIM24 lacking a RING finger domain also has an activity for AR-mediated transcription, but its activity is weaker than that of wild-type TRIM24. The difference in subcellular localization likely causes the attenuation of transcriptional activity by deletion of a RING finger domain: wild-type TRIM24 predominantly localizes in the nucleus, whereas TRIM24(Δ RING) localizes also in the cytosol. A previous study

showed that TRIM24 does not function as a coactivator by itself but only functions in cooperation with GRIP1 and CARM1 in AR transcriptional activity induced by DHT in the monkey kidney epithelial cell line CV-1 [16]. However, we showed that TRIM24 enhances transcriptional activity of AR by DHT alone in the prostate cancer cell line CWR22Rv1. This discrepancy may be due to the differences between the AR signaling pathways in a kidney epithelial cell line and a prostate cancer cell line. Otherwise, CWR22Rv1 cells may endogenously express GRIP1 and CARM1.

It has been reported that TIP60 is crucial for AR-mediated transactivation [52, 53]. We also showed that TRIM24 physically interacts with TIP60 and that TRIM24 cooperates with TIP60 for AR-mediated transactivation. We previously have reported that TRIM68, another TRIM family protein, enhances AR-mediated transactivation in collaboration with TIP60 [54], suggesting that nuclear TRIM family proteins other than TRIM68 and TRIM24 function as regulators for transcription. In other nuclear receptor signaling, TRIM25/EFP has an activity to enhance estrogen-dependent ER-mediated transcription [55]. Therefore, it is likely that TRIM family proteins have important roles in regulations of the transcriptional activity by nuclear receptors.

BRD7 plays a critical role in cellular growth, cell cycle progression, and signal-dependent gene expression. A previous study has shown that BRD7 plays a role in transcriptional regulation by binding to acetylated histone H3, and thereby chromatin remodeling for transcription is regulated by BRD7 [56]. We showed that BRD7 interacts with TRIM24 in this study. We compared the protein levels of TRIM24 and BRD7 in various human cell lines from different origins and found their expression patterns varied. Since it is important to clarify the relation between TRIM24 and BRD7, we should further analyze the expression profiles of TRIM24 and BRD7 using various

cancer tissues or developing tissues. More interestingly, TRIM24 did not enhance ubiquitination of BRD7 (data not shown) or accelerate the degradation of BRD7. However, BRD7 repressed AR-mediated transcriptional activity upregulated by TRIM24. The molecular mechanism of inhibition of TRIM24-mediated transcription by BRD7 remains to be determined. Future studies aimed at elucidation of the relation between TRIM24 and BRD7 should be helpful for establishing novel therapeutic tools for advanced hormone refractory and metastatic prostate cancer.

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Figure legends

Fig. 1. TRIM24 physically interacts with AR in vivo. (A) Expression of endogenous TRIM24 and AR in several prostate cancer cell lines. Whole cell lysates from prostate cancer cell lines were immunoblotted (IB) with an antibody to TRIM24, AR or β -actin. (B) TRIM24 interacts with AR in vivo. An in vivo binding assay between TRIM24 and AR. FLAG-tagged TRIM24 and HA-tagged AR expression vectors were transfected into HEK293T cells. Whole cell lysates (WCL) were immunoprecipitated (IP) with anti-FLAG antibody and immunoblotted with an antibody to HA or FLAG. (C) Interaction between endogenous TRIM24 and AR in prostate cancer cells. CWR22Rv1 cells were treated with dihydrotestosterone (DHT) and lysed. Then WCLs were immunoprecipitated with anti-AR antibody and immunoblotted with an antibody to TRIM24 or AR.

Fig. 2. TRIM24 enhances AR-mediated transcriptional activity in prostate cancer cells. (A) TRIM24 enhances AR-mediated transcriptional activity in CWR22Rv1 cells. MMTV luciferase reporter vector (MMTV-Luc) and various amounts of FLAG-tagged TRIM24 expression vector were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (B) A comparison of the protein levels of endogenous and transfected TRIM24. Whole cell lysates used in the experiment for which results are

shown in Fig. 1A were immunoblotted with an antibody to TRIM24, FLAG or β -actin. (C) AR-dependent transcriptional activation of TRIM24. MMTV-Luc and TRIM24 expression vectors were transfected into PC3 cells with or without AR expression vector. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test.

Fig. 3. TRIM24 lacking a RING finger domain also enhances AR-mediated transcriptional activity. (A) Schematic representation of wild-type (WT) TRIM24 and TRIM24(Δ RING). TRIM24 has a RING finger, two B-boxes, a coiled-coil region, a PHD domain and a bromodomain. (B) TRIM24(Δ RING) enhances AR-mediated transcriptional activity. MMTV-Luc and various amounts of TRIM24(Δ RING) expression vector were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (C) TRIM24(Δ RING) retains partial activity compared with that of wild-type TRIM24. MMTV-Luc and TRIM24 (WT or Δ RING) expression vectors were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for

luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (D and E) Antiandrogens suppress AR-mediated transcriptional activity via TRIM24. MMTV-Luc and TRIM24(WT) expression vectors were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (1 or 10 nmol/L), bicalutamide (1 μ mol/L), and flutamide (1 μ mol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test.

Fig. 4. TRIM24 cooperates with TIP60 to enhance AR-mediated transactivation. (A) Physical interaction between TRIM24 and TIP60. Expression vectors encoding FLAG-tagged TRIM24 and HA-tagged TIP60 were transfected into HEK293T cells as indicated. After 24 h, cells were lysed and subjected to immunoprecipitation (IP) with anti-HA antibody followed by immunoblotting (IB) with an antibody to FLAG or HA. Whole cell lysates (WCL) were also subjected to immunoblotting to confirm the expressions of FLAG-tagged TRIM24 and HA-tagged TIP60. (B) TRIM24 cooperates with TIP60 to enhance AR-mediated transactivation. CWR22Rv1 cells were cotransfected with expression vectors encoding TIP60 and TRIM24 with MMTV-Luc as a reporter. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the

indicated comparisons were determined by Student's *t* test.

Fig. 5. TRIM24 interacts with BRD7. (A) An in vivo binding assay between TRIM24 and BRD7. FLAG-tagged TRIM24 and HA-tagged BRD7 expression vectors were transfected into HEK293T cells. Whole cell lysates (WCL) were immunoprecipitated (IP) with anti-HA antibody and immunoblotted (IB) with an antibody to FLAG or HA. (B) Schematic representation of wild-type (WT) BRD7 and BRD7(Δ BRD). BRD7 has a bromodomain. (C, D) A RING finger domain of TRIM24 and a bromodomain of BRD7 are not required for the interaction between TRIM24 and BRD7. HEK293T cells were transfected with expression vectors encoding either FLAG-tagged TRIM24(WT) or FLAG-tagged TRIM24(Δ RING) and HA-tagged BRD7(WT) or HA-tagged BRD7(Δ BRD), immunoprecipitated with anti-HA antibody, and immunoblotted with an antibody to FLAG or HA.

Fig. 6. Colocalization of TRIM24 and BRD7. (A) Localization of TRIM24 and BRD7 determined by immunofluorescence analysis. COS7 cells were transfected with an expression vector encoding FLAG-tagged TRIM24(WT), FLAG-tagged TRIM24(Δ RING) or HA-tagged BRD7. After 48 h, the cells were fixed and stained with antibodies to FLAG or HA as well as Hoechst33258 to reveal the subcellular distribution of FLAG-tagged TRIM24 (WT or Δ RING) (green), HA-tagged BRD7 (red), and nuclei (blue). Micrographs were obtained at 400 x magnification (Scale bars, 20 μ m). (B) Pulse-chase analysis of BRD7 with TRIM24. HEK293T cells were transfected with expression vectors encoding HA-tagged BRD7 and FLAG-tagged TRIM24 or an empty vector (Mock). After 48 h, the cells were cultured in the presence of

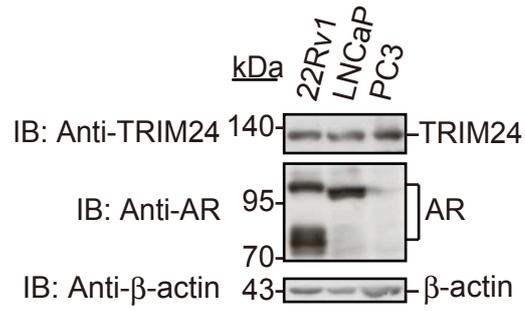
cycloheximide (50 $\mu\text{g}/\text{mL}$) for the indicated times. Cell lysates were then subjected to immunoblot (IB) analysis with anti-HA, anti- β -actin or anti-FLAG antibody. (C) Intensity of the HA-BRD7 bands in Fig. 6B was normalized by that of the corresponding β -actin bands and then expressed as a percentage of the normalized value at the beginning of the chase period. Data are means \pm SD of values from three independent experiments.

Fig. 7. Expression of TRIM24 and BRD7 in various cell lines. Cell lysates from prostate cancer cell lines CWR22Rv1, LNCaP and PC3, acute myeloid leukemia cell lines KG-1, HL-60, NKM-1, J111, HEL, KMOE-2 and CMK, chronic myeloid leukemia cell line K562, T cell leukemia cell line Jurkat, embryonic kidney cell line HEK293T, uterine epithelial carcinoma cell line HeLa, and hepatocellular carcinoma cell line HepG2 were subjected to immunoblot (IB) analysis with anti-TRIM24, anti-BRD7 or anti- β -actin antibody.

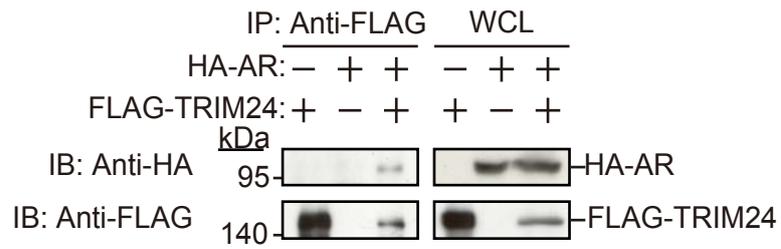
Fig. 8. BRD7 represses AR-mediated transcriptional activity. (A) MMTV-Luc and various amounts of BRD7 expression vector were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (B) BRD7 and/or TRIM24 expression vectors with MMTV-Luc were transfected into CWR22Rv1 cells. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum

for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test.

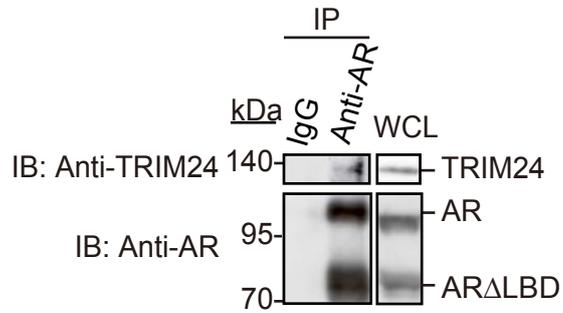
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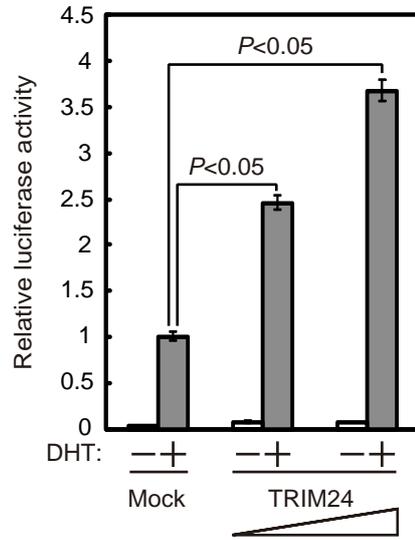
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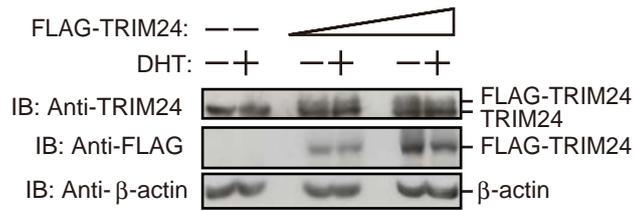
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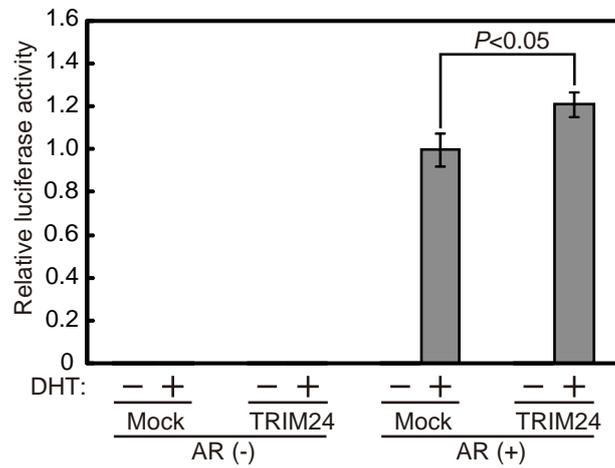
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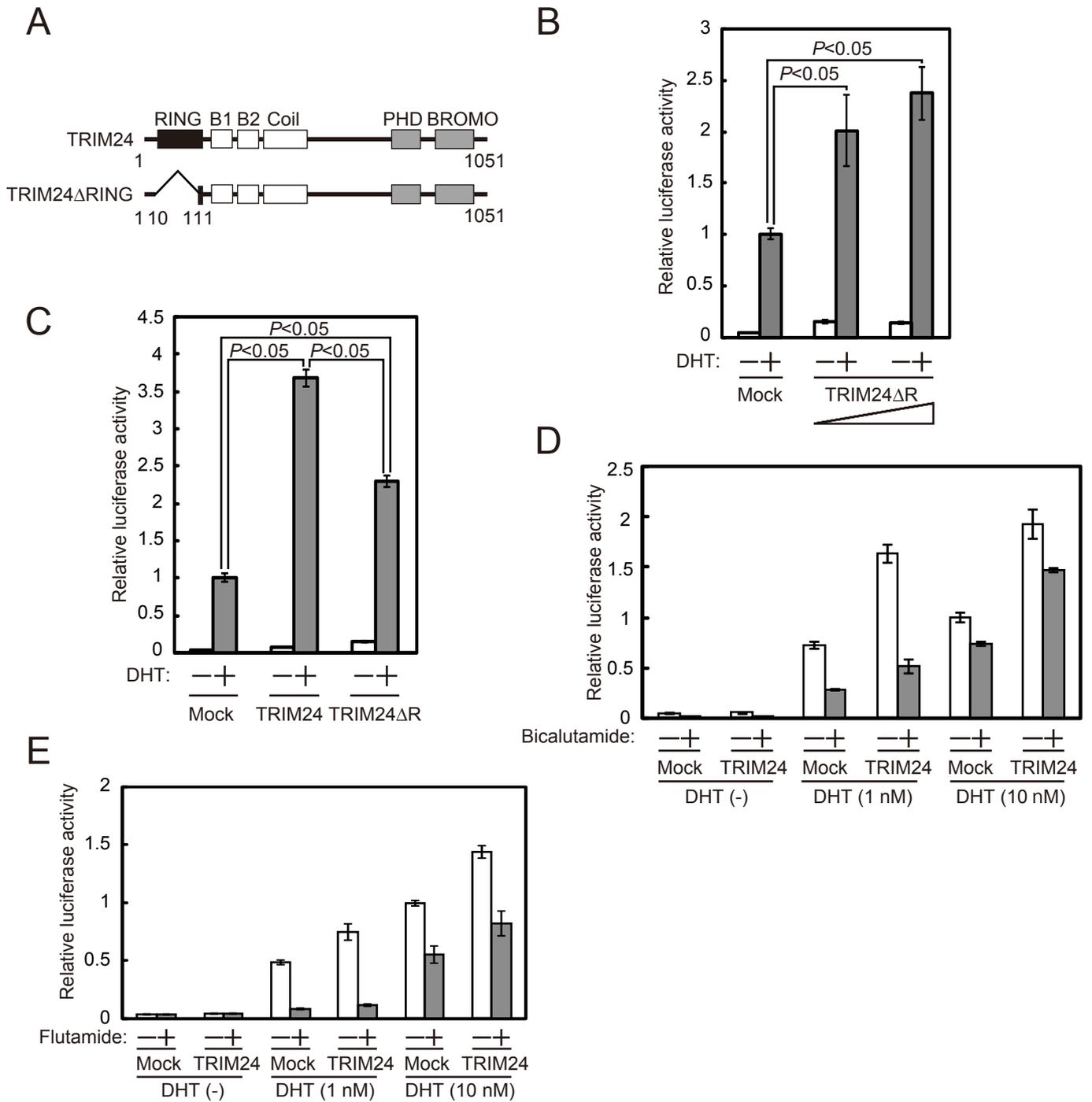


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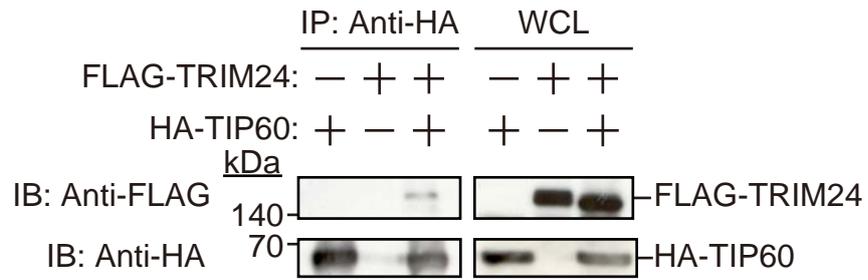
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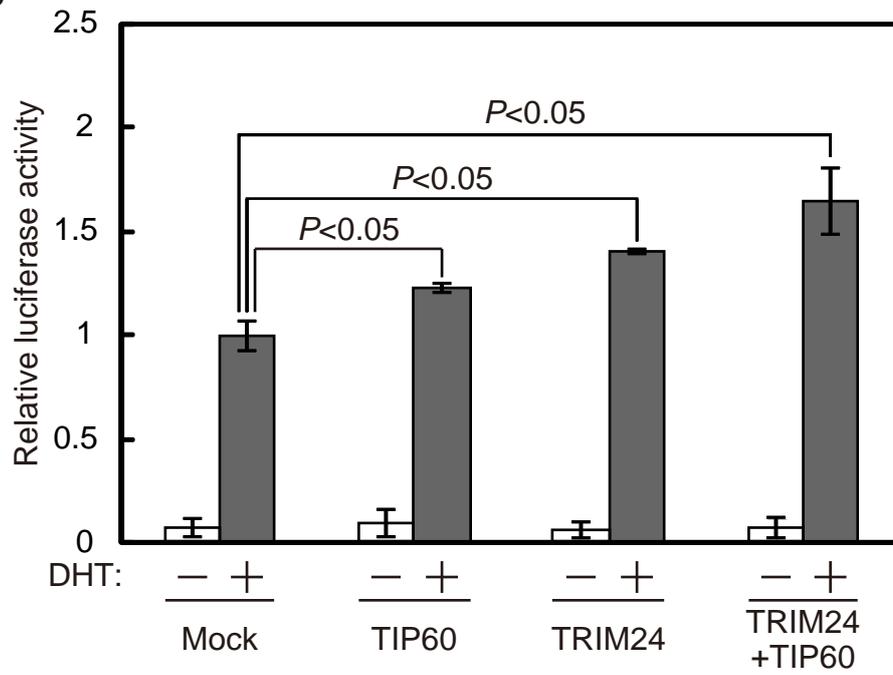


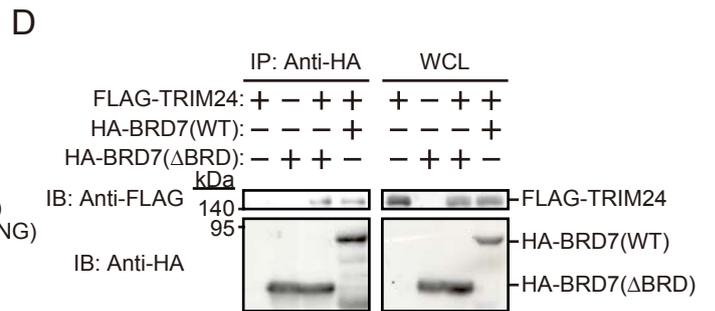
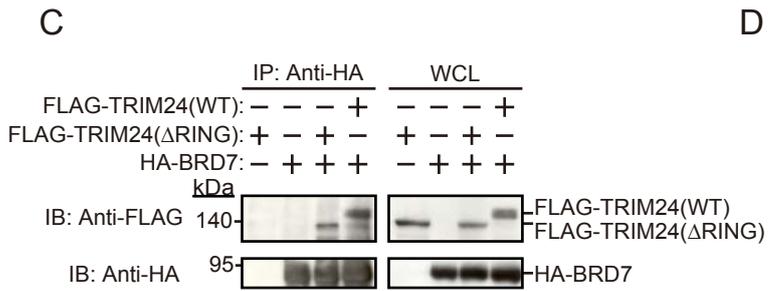
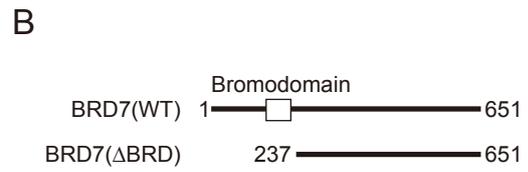
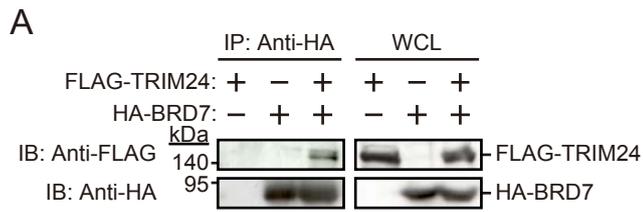
Kikuchi et al. Figure 3

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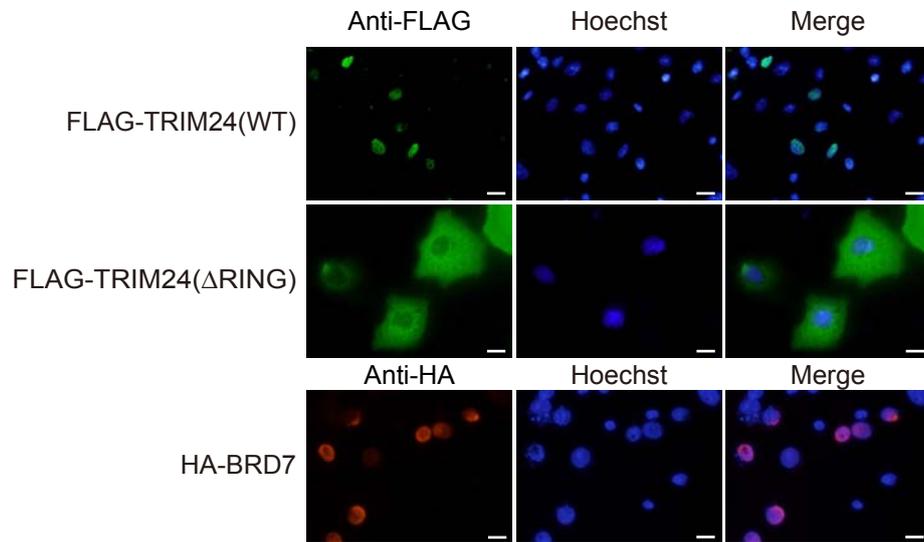


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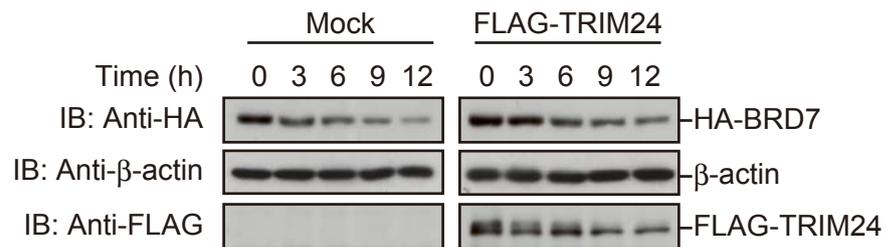




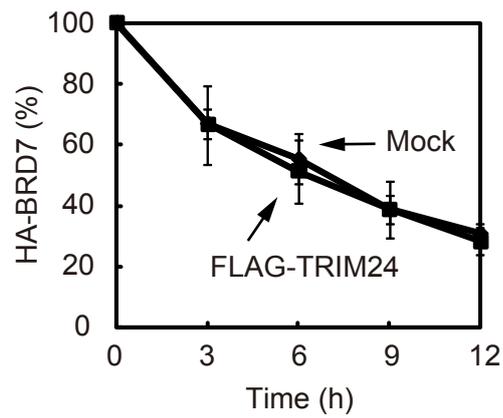
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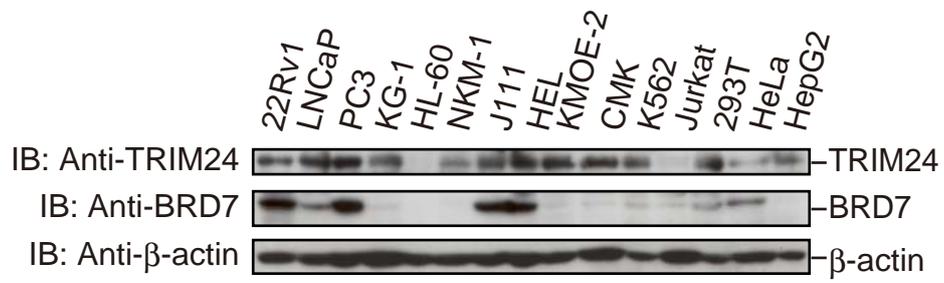


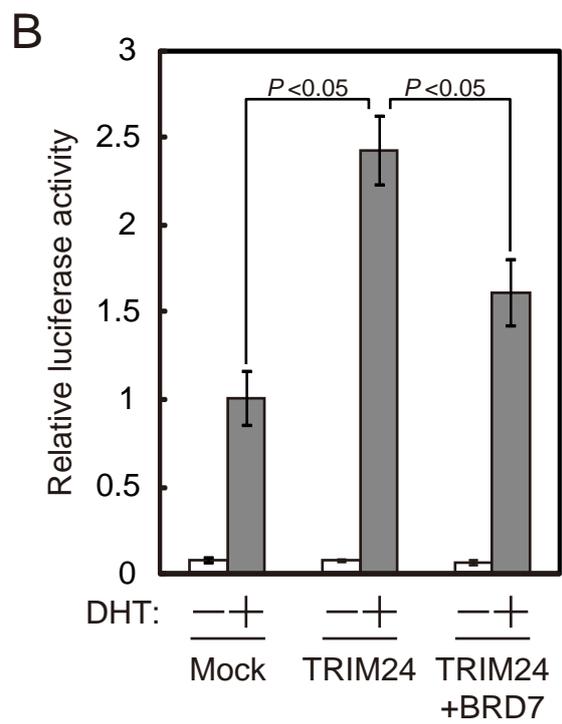
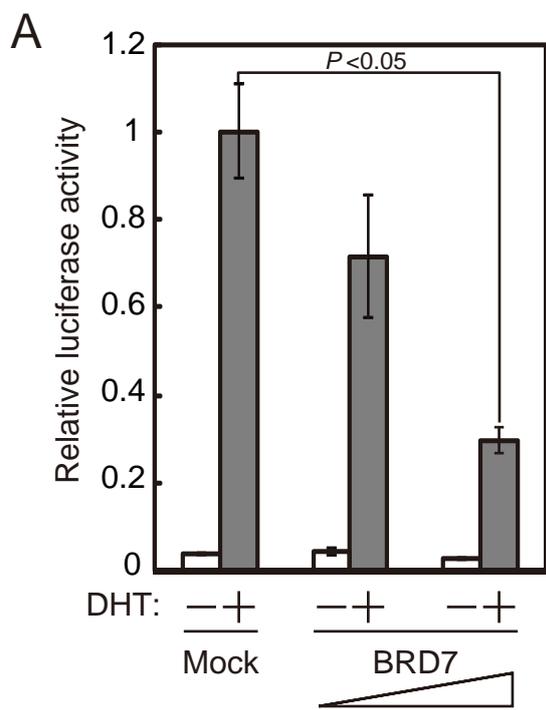
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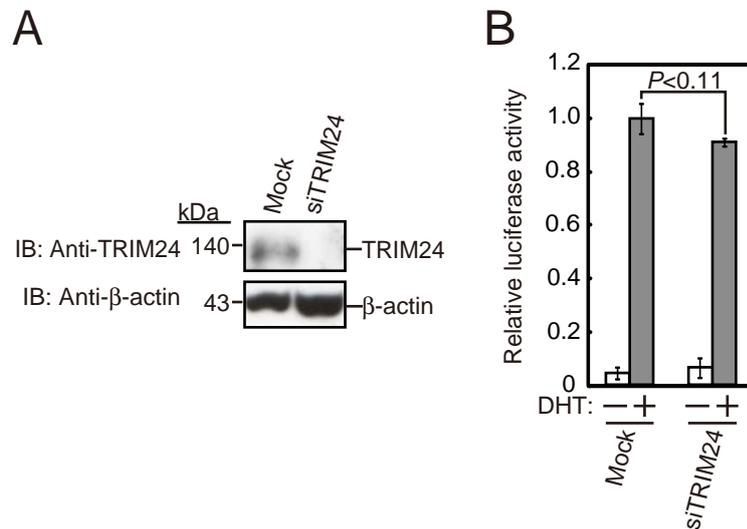


Fig. S1. Knock-down of TRIM24 in CWR22Rv1 cells. (A) Knocked-down CWR22Rv1 cell lines with siTRIM24 were analyzed by immunoblotting using anti-TRIM24. Short interference RNAs (siRNA) targeting TRIM24 were introduced into CWR22Rv1 cells by a retroviral infection system. Down-regulated CWR22Rv1 cell lines with siTRIM24 and non-targeting siRNA as a control (Mock) were analyzed by immunoblotting with an antibody to TRIM24 or β -actin. (B) Down-regulation of TRIM24 caused a slight decrease in androgen-dependent AR-mediated transcriptional activity. MMTV-Luc was transfected in down-regulated CWR22Rv1 cell lines with siTRIM24 and mock. Transfected cells were incubated in culture medium containing 10% charcoal-treated fetal bovine serum for 24 h and then incubated with or without DHT (10 nmol/L) for 24 h. The cells were then harvested and assayed for luciferase activity. Data are means \pm SD of values from three independent experiments. P values for the indicated comparisons were determined by Student's t test.