TRIM68 Regulates Ligand-Dependent Transcription of Androgen Receptor in Prostate Cancer Cells

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Abstract

The androgen receptor (AR) is a transcription factor belonging to the family of nuclear receptors that mediate the action of androgen. AR plays an important role in normal development of the prostate, as well as in the progression of prostate cancer. AR is regulated by several posttranslational modifications, including phosphorylation, acetylation, and ubiquitination. In this study, we found that the putative E3 ubiquitin ligase TRIM68, which is preferentially expressed in prostate cancer cells, interacts with AR and enhances transcriptional activity of the AR in the presence of dihydrotestosterone. We also found that TRIM68 functionally interacts with TIP60 and p300, which act as coactivators of AR, and synergizes in the transactivation of AR. Overexpression of TRIM68 in prostate cancer cells caused an increase in secretion of prostate-specific antigen (PSA), one of the most reliable diagnostic markers for prostate cancer, whereas knockdown of TRIM68 attenuated the secretion of PSA and inhibited cell growth and colony-forming ability. Moreover, we showed that TRIM68 expression is significantly up-regulated in human prostate cancers compared with the expression in adjacent normal tissues. These results indicate that TRIM68 functions as a cofactor for AR-mediated transcription and is likely to be a novel diagnostic tool and a potentially therapeutic target for prostate cancer.

Introduction

Prostate cancer is the most frequently diagnosed malignancy and is the second leading cause of cancer deaths among men in the United States (1). Prostate cancer is a hormonally regulated malignancy, and androgen receptor (AR) plays an important role in disease progression (2). One of the most troubling aspects of prostate cancer progression is the conversion from an androgen-dependent state to an androgen ablation-resistant state, which, at present, defies any effective treatment (3). In majority of end-stage hormone-refractory tumors, AR continues to be expressed and seems to be activated under androgen ablation conditions. Elucidation of the mechanism of AR activation is essential for understanding process of the prostate cancer progression and for identifying possible targets for intervention (4). AR mediates androgen action as a transcriptional factor in collaboration with a number of coregulators. AR up-regulates or down-regulates target gene expressions, depending on coactivators or corepressors (5, 6). Furthermore, activities of AR and coregulators are regulated by posttranslational modifications, such as methylation, phosphorylation, acetylation, and ubiquitination (7–10). However, little is known about the contribution of such processes to AR function.

Ubiquitination is a versatile posttranslational modification mechanism used by eukaryotic cells. The ubiquitin-proteasome pathway involves ubiquitin modification of substrates and sequential degradation by the proteasome (11). Ubiquitin conjugation is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3; ref. 12). E3 is a scaffold protein that mediates between the ubiquitin-linked E2 and the substrate. The resulting covalent ubiquitin ligations form polyubiquitinated conjugates that are rapidly detected and degraded by 26S proteasome (13). E3 is thought to be most directly responsible for substrate recognition. E3 ubiquitin ligases thus far identified include members of the homologous to E6-AP carboxyl terminus (HECT), RING finger, and U-box protein families (14–16).

TRIM68 is a member of the tripartite motif-containing protein (TRIM) family defined by the presence of a common domain structure composed of a RING finger, a B-box, and a coiled-coil motif (17). In addition to these motifs, TRIM68 possesses a carboxy-terminal PRY/SPRY domain. TRIM family proteins are involved in a broad range of biological processes, and consistently, their alterations result in diverse pathologic conditions, such as genetic diseases, viral infection, and cancer development (18). It has been reported that TRIM68 is one of the autoantigens associated with Sjögren’s syndrome and is a new diagnostic marker for Sjögren’s syndrome (19). However, the function of TRIM68 has not been elucidated. Recently, TRIM68 has also been shown to be highly expressed in the prostate compared with its expression in other normal tissues. TRIM21, which is structurally similar to TRIM68 and is also one of the autoantigens associated with Sjögren’s syndrome, has been found to bind DNA and has been suggested to act as a transcription factor regulating gene expression (20, 21). TRIM21 has also been shown to be involved in cellular proliferation and cell death (22). Given the highly prostate-specific expression pattern of TRIM68 and the transcriptional function of its related protein, we hypothesized that TRIM68 plays a role in AR-dependent transcription.

In this study, we obtained evidence that TRIM68 is a novel AR-interacting protein and acts as a coactivator of AR, depending on its ubiquitin ligase activity. Furthermore, knockdown of endogenous TRIM68 expression by RNA interference (RNAi) results in suppression of the oncogenic properties of prostate cancer cells. In addition, we found that TRIM68 is significantly up-regulated in human prostate cancers, suggesting that TRIM68 is likely to be a novel diagnostic tool for prostate cancer.
Materials and Methods

Cell culture. Prostate cancer cell lines LNCaP-FGC, CWR22Rv1, and PC3 were obtained from the American Type Culture Collection. LNCaP and CWR22Rv1 were maintained under an atmosphere of 5% CO₂ at 37°C in RPMI 1640 (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS; Life Technologies Bethesda Research Laboratories). PC3 and HEK293T cell lines were cultured under the same conditions in DMEM (Sigma) with 10% FBS.

Cloning of cDNAs and plasmid construction. Human TRIM68 cDNA was amplified by PCR from HeLa cDNA (Clontech Laboratories, Inc.). The resulting fragment containing the human TRIM68 cDNA was ligated into the pcR3 vector (Invitrogen) with a FLAG tag and into the pFastBacHT vector (Invitrogen). Human AR cDNA was kindly provided by Dr. Sobue (Nagoya University). Deletion mutants of AR cDNA were generated by PCR. Human TIP60 cDNA was kindly provided by Dr. Ikura (Tohoku University).

Recombinant proteins, antibodies, and reagents. His-tagged TRIM68 was expressed in the SF9 insect cell line using a baculovirus protein expression system (Invitrogen). The recombinant TRIM68 protein was used as immunogen in rabbits. A rabbit polyclonal anti-TRIM68 antibody was generated and then affinity-purified using a recombinant TRIM68-conjugated Sepharose 4B column. Other antibodies used were as follows: mouse monoclonal anti-HA (HA11/16B12, Covance Research Products), mouse monoclonal anti-FLAG (M5, Sigma), mouse monoclonal anti-ubiquitin (P4D1, Santa Cruz Biotechnology), goat polyclonal anti-ubiquitin–specific antigen (PSA; Santa Cruz), mouse monoclonal anti-AR (Santa Cruz), and mouse monoclonal anti-α-tubulin (Zymed Laboratories). Dihydrotestosterone, dexamethasone, and 17β-estradiol were purchased from Sigma.

Ubiquitination assay. In vitro ubiquitination assays were performed as previously described (16). In brief, reaction mixtures containing 4 μg of the recombinant TRIM68 with 0.1 μg recombinant E1 (Boston Biomedica), 1 μg recombinant E2s, 0.5 unit phosphocreatine kinase, 1 μg ubiquitin (Sigma), 25 mmol/L Tris-HCl (pH 7.5), 120 mmol/L NaCl, 2 mmol/L ATP, 1 mmol/L MgCl₂, 0.3 mmol/L DTT, and 1 mmol/L creatine phosphate were incubated for 3 h at 30°C. The reaction was terminated by the addition of SDS sample buffer containing 4% β-mercaptoethanol and heating at 95°C for 5 min. Samples were subjected to immunoblotting with anti-ubiquitin and anti-TRIM68 antibodies.

Transfection, immunoprecipitation, and immunoblot analysis. HEK293T cells were transfected by the calcium phosphate method. After 48 h, the cells were 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 Na₂VO₃, 400 μmol/L EDTA, 10 mmol/L NaF, and 10 mmol/L sodium PPI. The cell lysates were centrifuged at 16,000 × g for 10 min at 4°C, and the resulting supernatant was incubated with antibodies for 2 h at 4°C. Protein A-Sepharose (Amersham Pharmacia) that had been equilibrated with the same solution was added to the mixture, and then the mixture was 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).

Establishment of stable transfectants by using a retrovirus expression system. Complementary DNAs were subcloned into pMX-puro (kindly provided by T. Kitamura, Tokyo University), the resulting vectors were used to transfect Plat A cells, and then recombinant retroviruses were generated (23). LNCaP cells were infected with the recombinant retroviruses and selected in medium containing puromycin (2 μg/mL; Sigma).

RNA interference. The pMX-puro II vector in which the U3 portion of the 3′ long terminal repeat was deleted was kindly provided by Dr. T. Kamura (Nagoya University; ref. 24). The hairpin sequences specific for human TRIM68 mRNAs corresponded to nucleotides 329 to 349 (siTRIM68-1) and 796 to 816 (siTRIM68-2) of the respective coding regions. The hairpin sequences specific for enhanced green fluorescent protein (GFP; Clontech) mRNA were used as a control. Recombinant retroviruses were generated and used to infect LNCaP cells as described above. After selection in medium containing puromycin (2 μg/mL), the resulting cell lines were checked by immunoblot analysis with anti-TRIM68 antibody.

Dual-luciferase assay. Cells were seeded in 24-well plates at 1 × 10⁵ per well (LNCaP) or 5 × 10⁴ per well (PC3 and CWR22Rv1) 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 the TRIM68 expression vector into LNCaP and CWR22Rv1 cells or with the AR expression vector into PC3 cells using Fugene HD reagent (Roche). Estrogen-response element reporter plasmid (ERE-Luc) was used for the estrogen receptor (ER)–luciferase assay. Transfected cells were incubated in 10% charcoal–treated FBS (EquitechBio) medium for 48 h and then washed and treated with or without 10 mmol/L dihydrotestosterone 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).

PSA expression and secretion assays. LNCaP cell lines were seeded in six-well plates and incubated in 10% charcoal–treated FBS medium for 48 h and then washed and treated with or without 10 mmol/L dihydrotestosterone for 24 h. The cells were lysed and subjected to immunoblotting with anti-PSA antibody. Cell culture media were collected and assayed for PSA concentration by ELISA analysis using a TOSOH II PA monoclonal immunoenzymeenzyme assay kit.

Cell proliferation assay. LNCaP cell lines were incubated in 10% charcoal–treated FBS medium for 48 h and then plated into 96-well plates at 5,000 per well in 100 μL media. Cells were treated with 10 mmol/L dihydrotestosterone and refed with fresh medium containing dihydrotestosterone every 2 d. MTS cell proliferation assay was performed using CellTiter 96 one solution (Promega) according to the manufacturer’s instructions.

Colonies formation assay in soft agar. LNCaP cells were plated at a density of 1 × 10³ cells in 60-mm dishes containing 0.4% top low-melting agarose and 0.5% bottom low-melting agarose medium. Colonies with a diameter of >0.1 mm were counted after 3 wk.

Reverse transcription and real-time quantitative PCR. Total RNA (3 μg) isolated from various cell lines, human prostate cancers, and adjacent normal tissues with the use of TRI Reagent (Sigma) was subjected to reverse transcription with MMLV Reverse Transcriptase (Invitrogen). The resulting cDNA was subjected to real-time quantitative PCR by TaqMan gene expression assays (Applied Biosystems) following the manufacturer’s instructions. The assays were performed with a TRIM68-specific TaqMan probe and primers (commercialized by Applied Biosystems) in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). TATA box-binding protein (TBP) was selected as an internal control to normalize the expression levels. Each sample was tested in triplicate.

Human tissue samples. Tissues from 35 cases of primary prostate cancer were surgically resected by radical prostatectomy. Written informed consent was obtained from each patient before surgery. The excised samples from tumor and adjacent normal tissues were obtained within 1 h after the operation. All excised tissues were immediately placed in liquid nitrogen and stored at −80°C until further analysis. Samples were then manually microdissected from frozen sections on microscope and histologically confirmed to be highly homogeneous cancer tissues by H&E staining of step sections.

Immunohistochemical analysis. Tissues were fixed in 4% formaldehyde for 3 d and then embedded in paraffin. Paraffin-embedded sections (3-μm thick) were mounted on silane-treated slides. After drying overnight at 37°C, paraffin was removed from the sections with xylene, and they were then rehydrated with a graded series of ethanol solutions. The tissues were then subjected to immunohistochemical staining with an antibody to TRIM68 (5 μg/mL) by a streptavidin-biotin immunoperoxidase method using an immunohistochemical detection kit (Vectastain Elite; Vector) and diaminobenzidine as a chromogen (Wako) according to the manufacturer’s instructions. Immunoreactivity was semiquantitatively classified. Two independent investigators reviewed and scored slides observed under a microscope by categorizing staining intensity of characteristic staining cells as negative, weak, medium, or strong (scored as 0–3). The final score was
obtained by multiplying the percentage of positive cells by the intensity score with an estimated score range of 0 to 300.

**Statistical analysis.** We used the unpaired Student’s *t* test and the Mann-Whitney *U* test to determine statistical significance of experimental data.

**Results**

TRIM68 has a ubiquitin ligase activity and is predominantly expressed in the prostate cancer cell line LNCaP. TRIM68 has a RING finger domain at its NH2 terminus and belongs to the TRIM family of proteins, some of which have been reported to be E3 ubiquitin ligases (18). To determine whether TRIM68 actually mediates an E3 ligase activity, we generated recombinant TRIM68 protein by using a baculovirus expression system and performed an *in vitro* ubiquitination assay with various combinations. Immunoblot analysis using an anti-ubiquitin antibody revealed that TRIM68 exhibits ubiquitination activity only in the presence of E1, E2 (Ubc4), ubiquitin, ATP, and TRIM68 (Fig. 1A, top). The lack of any of these components prevented autoubiquitination of TRIM68. In addition, an *in vitro* ubiquitination assay using anti-TRIM68 antibody showed that TRIM68 has an autoubiquitination activity (Fig. 1A, bottom). These findings indicate that TRIM68 is a bona fide E3 ligase. To further investigate the region of TRIM68 responsible for ubiquitination, we generated a deletion mutant lacking a RING finger domain (∆RING) of TRIM68 and then performed an *in vitro* ubiquitination assay (Fig. 1B). The *in vitro* ubiquitination assay showed that the deletion mutant has no E3 ligase activity, indicating that the RING finger domain is indispensable for E3 ubiquitin ligase activity of TRIM68.

To study the expression profile of TRIM68, we measured TRIM68 mRNA levels in various human cell lines of different origins by using real-time quantitative reverse transcription–PCR (RT-PCR). The gene expression of TRIM68 was found predominantly in the prostate cancer cell line LNCaP compared with its expression in other cell lines, including the prostate cancer cell lines CWR22Rv1 and PC3. B-cell lymphoma cell line Namalwa, embryonic kidney cell line 293, breast cancer cell lines T47D and MCF7, uterine epithelial carcinoma cell line HeLa, neuroblastoma cell line SH-SYSY, and hepatocellular carcinoma cell line HepG2 (Fig. 1C). Next, we compared the protein levels of TRIM68 by immunoblotting in hormone-related cancer cell lines, including LNCaP, 22Rv1, PC3, T47D, and MCF7. Consistent with the observed mRNA expression pattern, TRIM68 protein was found to be abundantly expressed in the prostate cancer cell line LNCaP compared with its expression in other cell lines (Fig. 1D).

**Interaction between TRIM68 and AR.** Given that TRIM68 is highly expressed in the androgen-responsive prostate cancer cell line LNCaP, we hypothesized that TRIM68 is associated with prostate cancer and particularly the AR signaling pathway. To test the possibility, we verified interaction between endogenous TRIM68 and AR in LNCaP cells by immunoprecipitation using antibodies to TRIM68 and AR (Fig. 2A). Furthermore, to determine the domain of AR that interacts with TRIM68, deletion mutants of AR were constructed for *in vitro* binding assays (Fig. 2B). HA-tagged AR mutants and FLAG-tagged TRIM68 were expressed in HEK293T cells, and the cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. The results showed that AR-L, including the ligand-binding domain, was coprecipitated with TRIM68 but AR-ND, including the amino-terminal domain and DNA-binding domain, was not, suggesting that the ligand-binding domain is responsible for interaction with TRIM68 (Fig. 2C).
Next, to confirm subcellular colocalization of TRIM68 and AR in the nucleus, we performed an immunoprecipitation assay using nuclear fractions of LNCaP cells treated or not treated with dihydrotestosterone. The immunoprecipitation assay showed that the nuclear interaction of TRIM68 with AR was enhanced by dihydrotestosterone treatment, suggesting that TRIM68 serves as a coregulator for androgen-dependent transcription (Fig. 2D).

**TRIM68 enhances AR-mediated transcriptional activity.** Having shown coprecipitation and colocalization of TRIM68 and AR, we next examined whether TRIM68 functionally affects AR-mediated transcription. To examine the effect of TRIM68 on AR-mediated transcriptional activity, we performed a luciferase reporter assay using an MMTV promoter-driven luciferase construct (MMTV-Luc). A TRIM68 expression vector and MMTV-Luc were transfected into LNCaP and CWR22Rv1 cells, and luciferase assays were then performed with and without dihydrotestosterone. The luciferase assays showed that TRIM68 enhances androgen-dependent AR-mediated transcriptional activity in a dose-dependent manner, whereas TRIM68ΔRING mutant, which lacks ubiquitin ligase activity, showed a dominant negative effect, suggesting that TRIM68 acts as a positive regulator for AR signaling and that ubiquitin ligase activity of TRIM68 is indispensable for AR transactivation (Fig. 3A).

To confirm the physiologic role of TRIM68 in AR-mediated transcription, we used RNAi to knockdown endogenous TRIM68 in LNCaP cells. Two different short interference RNAs (siRNA) targeting TRIM68 were introduced into LNCaP cells by using a retroviral infection system. RNAi treatment resulted in significant silencing of TRIM68 at protein level in LNCaP cells (Fig. 3B, left). To examine the effect of the depletion of TRIM68 on AR-mediated transcription, we performed a luciferase assay for AR signal in LNCaP cells transfected with TRIM68 siRNA. The relative luciferase activities of cells transfected with TRIM68 siRNA were decreased compared with those of cells transfected with the control siRNA (Fig. 3B, right).

To determine whether the effect of TRIM68 is specific to AR-mediated transcription, we transfected expression vectors encoding TRIM68 and wild-type AR, glucocorticoid receptor (GR) or ER into the androgen-independent prostate cancer cell line PC3 and performed luciferase reporter assays using each reporter system for AR, GR, and ER. Luciferase assays showed that TRIM68 markedly enhances AR-mediated transcription in PC3 cells transfected with AR, whereas TRIM68 enhances GR-mediated transcription to a lesser degree and has no effect on ER-mediated transcription (Fig. 3C). These findings indicate that TRIM68 is a comparatively specific coactivator for AR.

It has been reported that ubiquitin modification of substrates and the sequential degradation by the proteasome is involved in transcription activity of AR (25). To determine whether proteasome activity is required for TRIM68-mediated transcriptional activity of AR, we performed a luciferase assay for AR transactivation in the presence or absence of a proteasome inhibitor, MG132. TRIM68 enhanced AR transactivation in the absence of MG132, whereas the effect of TRIM68 was dramatically suppressed in the presence of MG132, indicating that the proteasome activity is required for the effect of TRIM68 on AR transcriptional activity (Fig. 3D).
vectors encoding FLAG-tagged TRIM68 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 using anti-FLAG antibody. An \textit{in vivo} binding assay showed that TRIM68 specifically interacts with TIP60 (Fig. 4A). To further determine whether TRIM68 functionally interacts with TIP60, we performed an AR transactivation assay. LNCaP cells were cotransfected with expression vectors encoding

![Image: Figure 3. TRIM68 enhances AR-mediated transcriptional activity. A, TRIM68 enhances AR-mediated transcriptional activity in a dose-dependent manner. MMTV luciferase reporter vector (MMTV-Luc) and various amounts of TRIM68 expression vector (wild type or ΔR) were transfected into LNCaP (left and right) and CWR22Rv1 (middle) cells. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone were defined as 1. Columns, mean of values from three independent experiments; bars, SD. *, statistically significant based on attaining \( P \)'s of <0.05 (unpaired Student's \( t \) test). B, knockdown of TRIM68 causes attenuation of AR-mediated transcriptional activity. Two different siRNAs targeting TRIM68 (siTRIM68-1 or siTRIM68-2) or targeting GFP (siGFP, used as a control) were introduced into LNCaP cells by a retrovirus expression system. Knocked-down LNCaP cell lines with siTRIM68 were analyzed by immunoblotting using anti-TRIM68 and anti-\( \alpha \)-tubulin (an internal control) antibodies (left). Knocked-down LNCaP cell lines with siTRIM68 were transfected with MMTV-Luc reporter vector. Cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with siGFP expression vector and then treated with dihydrotestosterone were defined as 1. Columns, mean of values from three independent experiments; bars, SD. C, effects of TRIM68 on the transcriptional activities of AR, GR, and ER. PC3 cells were transiently transfected with steroid receptor expression vectors (AR, GR, or ER), reporter vectors (MMTV-Luc for AR and GR, ERE-Luc for ER) and TRIM68 expression vector. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without a cognate ligand (10 nmol/L dihydrotestosterone, 10 nmol/L dexamethasone, or 10 nmol/L 17\( \alpha \)-estradiol) for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone were defined as 1. Columns, mean of values from three independent experiments; bars, SD. D, proteasome activity is required for the effect of TRIM68 on AR-mediated transactivation. MMTV-Luc and TRIM68 expression vectors were transfected into LNCaP cells. Transfected cells were incubated in 10% charcoal–treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 8 h. The cells were then harvested and assayed to detect luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with dihydrotestosterone were defined as 1. Columns, mean of values from three independent experiments; bars, SD.

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transcriptional level is regulated by AR, we hypothesized that PSA gene contains an androgen-responsive element (ARE) and its usually increased in patients with prostate cancer (28). Because the PSA is a secretory glycoprotein that acts as a serine protease and for AR-mediated transactivation.

**TRIM68 cooperates with TIP60 and p300 to enhance AR-mediated transactivation.** A. Physical interaction between TRIM68 and TIP60. Expression vectors encoding FLAG-tagged TRIM68 and HA-tagged TIP60 were transfected into HEK293T cells as indicated. After 48 h, cells were lysed and subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting with anti-FLAG and anti-HA antibodies. Whole-cell lysates were also subjected to immunoblotting to confirm the expression of TRIM68 and TIP60 (right). B. TRIM68 cooperates with TIP60 or p300 to enhance AR-mediated transactivation. LNCaP cells were cotransfected with expression vectors encoding TIP60, p300, and TRIM68 with MMTV-Luc as a reporter. Transfected cells were incubated in 10% charcoal-treated FBS medium for 48 h and then treated with or without 10 nmol/L dihydrotestosterone for 24 h. The cells were then harvested and assayed for luciferase activity. Relative luciferase activities of cells that had been transfected with an empty vector and then treated with or without dihydrotestosterone were defined as 1. Columns, mean of values from three independent experiments; bars, SD. *p < 0.05 (unpaired Student’s t test).

**Effects of TRIM68 on the expression and secretion of PSA.** PSA is a secretory glycoprotein that acts as a serine protease and exists exclusively in prostate epithelial cells (27). Serum PSA level is usually increased in patients with prostate cancer (28). Because the PSA gene contains an androgen-responsive element (ARE) and its transcriptional level is regulated by AR, we hypothesized that TRIM68 also affects the expression of PSA. LNCaP cells, which express AR and secrete PSA, were used for the PSA expression and secretion assays. LNCaP cells stably expressing TRIM68 or TRIM68ΔRING by a retroviral expression system were treated with or without dihydrotestosterone, and then cell lysates were subjected to immunoblot analysis with anti-PSA antibody (Fig. 5A). Furthermore, cell culture supernatants were collected and assayed for PSA concentration by ELISA analysis (Fig. 5B). TRIM68 increased both the expression and secretion of PSA by treatment with dihydrotestosterone, whereas TRIM68ΔRING did not. To confirm the relationship between TRIM68 and PSA production, we used LNCaP cells transfected with TRIM68 siRNA and analyzed the expression and secretion levels of PSA. Knockdown of TRIM68 decreased both the expression and secretion of PSA in LNCaP cells (Fig. 5C and D). These findings suggest that TRIM68 contributes to the expression and secretion of PSA in prostate cancer cells.

**TRIM68 is overexpressed in human prostate cancer.** Given that TRIM68 modulates AR-mediated transcription, we hypothesized that TRIM68 affects androgen-dependent cell growth. An MTS cell proliferation assay was performed to examine the effects of TRIM68 on cell growth. Knockdown of TRIM68 significantly inhibited the growth of LNCaP cells, whereas overexpression of TRIM68 slightly increased the growth of LNCaP cells (Fig. 6A). These results suggest that TRIM68 has a significant effect on the androgen-dependent growth of LNCaP cells.

To examine the effect of TRIM68 on oncogenic phenotype in prostate cancer cells, we performed an anchorage-independent colony formation assay in soft agar. Overexpression of TRIM68 marginally increased colony-forming rate, whereas knockdown of TRIM68 significantly inhibited colony formation of LNCaP cells, indicating that TRIM68 has a significant effect on the oncogenic properties of prostate cancer cells (Fig. 6B). These findings may indicate that TRIM68 is required for oncogenic properties of prostate cancer cells but is not sufficient to enhance oncogenic phenotypes (Fig. 6A and B).

Considering the involvement of TRIM68 in prostate cancer cell proliferation and PSA production, we hypothesized that TRIM68 is aberrantly expressed in human prostate cancers. TRIM68 mRNA levels of 35 cases of human prostate cancer and adjacent normal tissue, which were surgically resected by radical prostatectomy in patients with primary prostate cancer, were quantified by real-time quantitative RT-PCR. TBP was selected as an internal control to normalize the expression levels, because there are no known retro-pseudogenes for it and TBP is not differentially expressed in tumor and normal prostate tissues (29, 30). Relative mRNA levels of TRIM68 were significantly increased in the majority of human prostate cancers compared with the levels in normal prostate tissues (Mann-Whitney U test, P < 0.05; Fig. 6C). These results indicate that TRIM68 gene expression is up-regulated in human prostate cancer.

Next, to examine the protein expression levels of TRIM68 in human prostate cancers, prostate cancer tissues and adjacent normal tissue, which were surgically resected by radical prostatectomy in patients with primary prostate cancer were analyzed by immunohistochemistry using anti-TRIM68 antibody. TRIM68 staining in tissues was detected mainly in the nucleus of epithelial cells, as observed in LNCaP cells treated with dihydrotestosterone. TRIM68-expressing cells were more abundant in cancer tissues than in normal tissues (Fig. 6D, a–f). TRIM68 expression in the benign sample group was low or absent (mean staining score, 91). In cancer samples, on the other hand, TRIM68 exhibited mainly a moderate or high level of expression (mean staining score, 220), indicating that TRIM68 immunoreactivity was significantly higher.
in cancer tissues than in benign tissues (Fig. 6D, g). These findings indicate that TRIM68 is overexpressed in human prostate cancer and may serve as a significant marker protein for prostate cancer.

Discussion

Recent advances have indicated that AR-mediated transactivation is regulated by posttranslational modification, including phosphorylation, acetylation, and ubiquitination. Ubiquitination involves degradation of AR and coregulators, and ubiquitination also has a nonproteolytic role in transcription (10). E3 ubiquitin ligases should play an important role in the regulation of AR-mediated transcriptional activity. However, only a few E3 ligases for AR, coactivators, or corepressors have been identified.

Recently, AR NH2 terminal–interacting protein, also known as p53-induced protein with a RING-H2 domain (PIRH2), has been reported to interact with histone deacetylase 1 and promote its degradation (31). In addition, E6-AP, a HECT type ubiquitin ligase, has been shown to enhance the hormone-dependent transcriptional activity of AR (32). The coactivating function may result from the ability of E6-AP to target nuclear receptor corepressor (NcoR) for degradation (33). However, PIRH2 and E6-AP do not display tissue-specific expression in prostate, and the effects are not restricted to the regulation of AR activity.

The present study is the first study to provide evidence that TRIM68, which is preferentially expressed in prostate cancer cells, is a novel AR-interacting protein and acts as a coactivator of AR depending on its ubiquitin ligase activity. TRIM68 possesses E3 ubiquitin ligase activity in collaboration with E2, including Ubc4 and UbcH5. TRIM68 physically associates with AR and enhances the transcriptional potential of AR. Interaction of TRIM68 with AR in the nucleus was further enhanced by dihydrotestosterone treatment, indicating that TRIM68 behaves as a coregulator that assembles into an AR-associated transcription factor complex. Overexpression of TRIM68 enhanced AR-mediated transactivation in various prostate cancer cell lines, whereas knockdown of TRIM68 gene expression using RNAi caused suppression of AR-mediated transactivation. These findings indicate that TRIM68 is an intrinsic cofactor for AR activation in prostate cancer cells.

It has been reported that regulation of AR activity by ubiquitination is divided into two roles: a proteolytic role of AR and its coregulators linked to proteasome machinery and a nonproteolytic role without proteasome machinery (34, 35). We showed that the proteasome activity is also required for the effect of TRIM68 on AR-mediated transactivation. Therefore, coactivating function of TRIM68 may be involved in the proteolytic role for AR or AR-associated proteins. However, TRIM68 could not directly ubiquitinate AR (data not shown). Hence, TRIM68 may ubiquitinate one of the corepressors for AR-mediated transcription.

Cyclical recruitment of transcriptional coregulators is now an established phenomenon that is intrinsic to transcriptional activation by steroid receptors (36). However, few molecules that

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**Figure 5.** Effects of TRIM68 on PSA expression and secretion. 

A, TRIM68 increases PSA expression in prostate cancer cells. LNCaP cells stably expressing FLAG-tagged TRIM68 or TRIM68ΔR by a retroviral expression system were incubated in 10% charcoal–treated FBS medium for 48 h, washed, and treated with or without 10 nmol/L dihydrotestosterone for 24 h. Cells were lysed and subjected to immunoblotting with anti-PSA, anti-FLAG, and anti–α-tubulin antibodies. 

B, TRIM68 increases PSA secretion in prostate cancer cells. Cell culture media in A were collected and assayed for quantifying PSA concentration using ELISA analysis. Columns, mean of values from three independent experiments; bars, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student’s *t* test).

C, knockdown of TRIM68 attenuates PSA expression in prostate cancer cells. Stably knocked-down LNCaP cell lines with siTRIM68 or siGFP were incubated in 10% charcoal–treated FBS medium for 48 h, washed, and treated with or without 10 nmol/L dihydrotestosterone for 24 h. Cells were lysed and subjected to immunoblotting with anti-PSA, anti-TRIM68, and anti–α-tubulin antibodies. 

D, knockdown of TRIM68 decreases PSA secretion in prostate cancer cells. Cell culture media in C were collected and assayed for quantifying PSA concentration using ELISA analysis. Columns, mean of values from three independent experiments; bars, SD. *, statistically significant based on attaining *P*'s of <0.05 (unpaired Student’s *t* test).
regulate the recruitment and activation of coregulators have been identified. We showed that TRIM68 is associated with TIP60 and p300, which act as coactivators of AR, and cooperates in enhancing AR-mediated transcriptional activity. This raises the possibility that TRIM68 assembles into an AR complex with coactivators, such as TIP60 and p300, and ubiquitinate corepressors, leading to exchange of corepressors for coactivators after ligand binding.

The PSA gene is known to contain an ARE, and its transcriptional level is regulated by AR and coregulators, including TIP60 (37). We showed that TRIM68 increases both expression and

Figure 6. TRIM68 is overexpressed in human prostate cancer. A, TRIM68 affects prostate cancer cell growth. LNCaP cell lines stably expressing TRIM68, an empty vector (mock), siTRIM68, or siGFP were incubated in 10% charcoal–treated FBS medium for 48 h and then plated in 96-well plates (5,000 per well). Cells were treated with 10 nM dihydrotestosterone and refed with fresh medium containing dihydrotestosterone every 2 d. Relative cell number was assayed at various times using the MTS assay. Absorbances at 490 nm versus time for each treatment were plotted. Points, mean of six replicates; bars, SD. B, anchorage-independent colony formation assay. Equal numbers of LNCaP cells stably expressing TRIM68, TRIM68 siRNA, or their respective controls were plated in 0.4% soft agar and cultured for 3 wk, and then colonies were counted microscopically. Columns, mean of values from three independent experiments; bars, SD. *, statistically significant based on attaining P's of <0.05 (unpaired Student’s t test). C, TRIM68 gene expression is up-regulated in human prostate cancer. TRIM68 mRNA levels were compared in human prostate cancers and adjacent normal tissues of 35 cases by real-time quantitative RT-PCR. Samples were surgically resected by radical prostatectomy in patients with primary prostate cancer. The expression level of TRIM68 mRNA was normalized to that of TBP mRNA and shown as relative expression level. The boxes within the plots represent the 25th to 75th percentiles. The horizontal line in the boxes indicates median value. White circles indicate outlier values outside of the 10th and 90th percentiles. *, statistically significant based on attaining P's of <0.05 (Mann-Whitney U test). D, immunohistochemistry of human prostate tissues with anti-TRIM68 antibody. Samples were surgically resected by radical prostatectomy in patients with primary prostate cancer. Prostate cancer tissues (d–f) and adjacent normal tissues (a–c) were stained with H&E (a and d) or with anti-TRIM68 antibody (b, c, e, and f). c and f are higher magnification views of the rectangles in b and e, respectively. Magnifications, 200× (a, b, d, and e) and 400× (c and f). TRIM68 immunoreactivities were compared in human prostate cancers and adjacent normal tissues of 35 cases by immunohistochemistry (g). The immunointensity of samples was categorized as negative (score of 0), weak (score of 1), medium (score of 2), or strong (score of 3), and the final score was obtained by multiplying the percentage of positive cells by the intensity score. Columns, mean of values; bars, SD. *, statistically significant based on attaining P's of <0.05 (Mann-Whitney U test).
secretion of PSA in LNCaP cells, whereas knockdown of TRIM68 decreases both expression and secretion of PSA. Furthermore, we showed that knockdown of TRIM68 significantly attenuates prostate cancer cell growth. These findings imply that TRIM68 does play an important role in AR transcriptional complexes that regulate gene expression, including expression of the PSA gene, or in cell proliferation.

We showed that TRIM68 is predominantly expressed in LNCaP cells among various human cell lines, including sex hormone-related prostate and breast cancer cell lines. Thus, we speculate that effects of TRIM68 are restricted in prostate cancer cells and are particularly involved in AR-mediated transcription. Furthermore, we showed by immunohistochemistry and real-time quantitative RT-PCR that TRIM68 expression is significantly up-regulated in the majority of primary human prostate cancers compared with its expression in adjacent normal prostate tissues. As observed in LNCaP cells with dihydrotestosterone treatment, TRIM68 staining was dominantly detected by immunohistochemistry in the nuclear compartment in human prostate cancer tissues compared with the staining in adjacent normal prostate tissues. These results indicate the possibility that TRIM68 is a potential regulator for prostate carcinogenesis and cancer development.

It has been reported that autoantibodies to TRIM68 are frequently found in the sera of patients with Sjögren’s syndrome (19). However, the clinical significance of these autoantibodies in human cancers has not been investigated. Considering the overexpression of TRIM68 in prostate cancers, autoantibodies to TRIM68 may be found in the sera of patients with prostate cancer and could be used as an additional diagnostic tool for prostate cancer.

We showed that TRIM68 is a positive modulator of transcriptional activity of AR. We suggest that ubiquitination activity of TRIM68 regulates the functions of AR or corepressors critically involved in proliferation, differentiation, or oncogenesis of prostate epithelial cells. Thus, it is probably important to identify physiologic substrates of TRIM68 and pharmacologic inhibitors of AR-associated ubiquitin ligases, including TRIM68 for establishing novel therapeutic tools for advanced hormone refractory and metastatic prostate cancer.

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TRIM68 Regulates Ligand-Dependent Transcription of Androgen Receptor in Prostate Cancer Cells

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