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| Title | Ubiquitylation of epsilon-COP by PIRH2 and regulation of the secretion of PSA |
| Author(s) | Maruyama, Satoru; Miyajima, Naoto; Bohgaki, Miyuki et al. |
| Citation | Molecular and Cellular Biochemistry, 307(1-2), 73-82<br>https://doi.org/10.1007/s11010-007-9586-3 |
| Issue Date | 2008-01 |
| Doc URL | https://hdl.handle.net/2115/32324 |
| Rights | The original publication is available at www.springerlink.com |
| Type | journal article |
| File Information | PIRH2_Hatakeyama.pdf |

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# Ubiquitylation of ε-COP by PIRH2 and regulation of the secretion of PSA

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Running title: Ubiquitylation of ε-COP by PIRH2

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**Abstract**

Ubiquitylation appears to be involved in the membrane trafficking system including endocytosis, exocytosis and ER-to-Golgi transport. We found that PIRH2, which was identified as an interacting protein for androgen receptor or p53, interacts with and ubiquitylates the ε-subunit of coatmer complex, ε-COP. PIRH2 promotes the ubiquitylation of ε-COP in vitro and in vivo and consequently promotes the degradation of ε-COP. The interaction between PIRH2 and ε-COP is affected by the presence of androgen, and PIRH2 in the presence of androgen promotes ubiquitylation of ε-COP in vivo. Furthermore, overexpression of the wild type of PIRH2 in prostate cancer cells causes downregulation of the secretion of prostate-specific antigen (PSA), a secretory protein in prostate epithelial cells and one of diagnostic markers for prostate cancer. Our results indicate that PIRH2 functions as a regulator for COP I complex.

**Keywords** PIRH2 · ε-COP · ubiquitin · androgen receptor · PSA

## Introduction

The androgen receptor (AR), a member of the nuclear receptor superfamily, mediates androgen action by interacting with a number of coregulatory proteins, both coactivators and corepressors, to activate or repress transcription of androgen-responsive genes [1-4]. Tissue-specific expression of coregulators, promoter-specific protein interactions, and exposure to different androgens also determine the specificity of AR action [5, 6].

A novel human AR N-terminal-interacting protein (ARNIP) has recently been reported and is also known as p53-induced protein with a RING-H2 domain (PIRH2) [7]. It has also been shown that PIRH2 physically interacted with p53 and promoted ubiquitylation of p53 independently of Mdm2. PIRH2 repressed p53 functions, including p53-dependent transactivation and growth inhibition [8]. Other studies have shown that PIRH2 interacts with measles virus phosphoprotein and N-terminal kinase-like protein (NTKL) [9]. Recently, PIRH2 has been reported to interact with histone deacetylase 1 (HDAC1) and to promote its degradation, suggesting that PIRH2 is involved in the direct control of androgen-responsive genes [10]. These studies suggest that PIRH2 is a multifunctional protein for several cellular events.

ε-COP is a subunit of the COP I coatomer complex, which coats the Golgi-derived vesicles involved in protein transport from the Golgi apparatus to the ER and within the Golgi apparatus [11, 12]. When a COP I-coated vesicle reaches the acceptor membrane, GTP hydrolysis by the small GTPase ARF1 triggers coat disassembly, allowing vesicle

fusion [13, 14]. The COP I complex can be reversibly dissociated into two subcomplexes: F-COP I, composed of β-, γ-, δ- and ζ-COPs, and B-COP I, composed of α-, β´- and ε-COPs [15].

Ubiquitylation is a versatile post-translational modification mechanism used by eukaryotic cells [16]. Ubiquitin conjugation is catalyzed by enzymes designated as ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). The resulting covalent ubiquitin ligations form polyubiquitylated conjugates that are rapidly detected and degraded by 26S proteasome. E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition [17-23]. There is now compelling evidence that several cellular proteins with diverse functions use ubiquitin modification as their targeting signal. The addition of a ubiquitin molecule at one (monoubiquitylation) or multiple (multiubiquitylation) lysine residues of ion channels, receptors or junctional complexes determines their regulated internalization and sorting to the endocytic compartment [24].

In this study, we found that PIRH2 promotes the ubiquitylation and proteasome-mediated degradation of ε-COP and that its interaction is regulated by AR. We also found that overexpression of PIRH2 diminishes the secretion of PSA in the prostate cancer cell line LNCaP.FGC, suggesting that PIRH2 functions as a regulator of retrograde transport from the Golgi apparatus to endoplasmic reticulum (ER) in androgen-dependent cells such as prostate cancer cells.

## Materials and methods

### Cell culture

HEK293T and LNCaP.FGC cells were cultured under an atmosphere of 5% $CO_2$ at 37°C in Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) or RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), respectively.

### Cloning of cDNAs and plasmid construction

Human PIRH2 and ε-COP cDNAs were amplified from human renal cell cancer cDNA and human liver cDNA, respectively, by the polymerase chain reaction (PCR) with ExTaq (Takara, Tokyo, Japan) using primers 5'-GAGATGGCGGCGACGGCCCGGGAA-3' (PIRH2 sense), 5'-TCGTCATTGCTGATCCAGTGAAAT-3' (PIRH2 antisense), 5'-GAAATGGCGCCTCCGGCCCCCGGC-3' (ε-COP sense), and 5'-GCCTCAGGCGCTGGGAGCGTACTG-3' (ε-COP antisense). The amplified fragments were subcloned into pBluescript II $SK^+$ (Stratagene, La Jolla, CA), and their sequences were verified. The FLAG-, HA-, or Myc-tagged PIRH2 and ε-COP cDNAs were then subcloned into pCR, pCGN, pcDNA3 (Invitrogen), pET30a (Merck KGaA, Darmstadt, Germany), pGEX-6P1 (GE Healthcare Bioscience Corp., Piscataway, NJ),

pBTM116 and pACT2 (Clontech, Mountain View, CA). To generate a mutant (C145A) of PIRH2, we performed a site-directed mutagenesis with a QuikChange kit (Stratagene). Human AR cDNA was obtained from Dr. G Sobue (Nagoya University).

Transfection, immunoprecipitation, and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method, cultured for 16 hours, and then treated with MG132 (10 μM; Merck). After 8 hours, the cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM $Na_3VO_4$, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were immunoprecipitated with antibodies and Protein G-Sepharose (GE Healthcare). The resin was washed five times with ice-cold lysis buffer and then boiled in SDS sample buffer. Immunoblot analysis was performed with the following primary antibodies: anti-c-Myc (1 μg/ml; 9E10, Covance Research Products, Berkeley, CA), anti-FLAG (1 μg/ml; M2 or M5, Sigma), anti-HA (1 μg/ml; HA.11/16B12, Covance), anti-hexa-histidine (1 μg/ml; H-15, Santa Cruz Biotechnology, Santa Cruz, CA) anti-HSP90 (1 μg/ml; 68, BD Transduction Laboratories, San Jose, CA), anti-ubiquitin (1 μg/ml; P4D1, Santa Cruz), anti-PIRH2 (1 μg/ml; Bethyl Laboratories, Montgomery, TX), anti-ε-COP (1 μg/ml; E-20, Santa Cruz), anti-α-tubulin (1 μg/ml; TU-01, Zymed), and anti-AR (1 μg/ml; 441, Santa Cruz). Immune complexes were detected with horseradish peroxidase-conjugated antibodies to mouse, rabbit or goat immunoglobulin

G (1:10,000 dilution, Promega) and an enhanced chemiluminescence system (ECL, GE Healthcare). To fractionate cytosolic or nuclear extracts, cell lysates were treated with a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany).

Yeast two-hybrid screening

Complementary DNA encoding amino acids 1 to 129 of human PIRH2 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 PIRH2, we transfected yeast strain L40 (Invitrogen) stably expressing the corresponding pBTM116 vector with a human HeLa Matchmaker cDNA library (Clontech) by the lithium acetate method.

In vitro ubiquitylation assay

Reaction mixtures (20 μl) each containing 1 μg of recombinant PIRH2 and ε-COP, 0.1 μg of E1 (Boston Biochem, Cambridge, MA), 1 μg of E2, 0.5 U of phosphocreatine kinase, 1 μg of bovine ubiquitin (MBL, Nagoya, Japan), 25 mM Tris-HCl (pH 7.5), 120 mM NaCl, 2 mM ATP, 1 mM $MgCl_2$, 0.3 mM dithiothreitol, and 1 mM creatine phosphate were incubated for 2 hours at 30°C and subjected to immunoblot analysis with mouse monoclonal antibodies to ubiquitin or GST.

Pulse-chase analysis with cycloheximide

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 an antibody to Myc. The percentages of expression levels are shown as means ± S.D. of values from three independent experiments.

Immunofluorescence staining

LNCaP.FGC cells grown on a glass cover were fixed for 20 minutes at room temperature with 4% formaldehyde in PBS and then incubated for 1 hour at room temperature with primary antibodies to AR, PIRH2 or Golgi-58K (58K-9, Sigma) in PBS containing 0.1% bovine serum albumin and 0.1% saponin. They were then incubated with Alexa546-labeled goat polyclonal antibodies to mouse immunoglobulin, Alexa488-labeled goat polyclonal antibodies to rabbit immunoglobulin or Alexa546-labeled goat polyclonal antibodies to mouse immunoglobulin (Molecular Probes, Eugene, OR) at a dilution of 1:2,000 and then stained with Hoechst33258 (Wako, Osaka, Japan). The cells were covered with a drop of GEL/MOUNT (Biomeda, Foster City, CA) and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51 microscope.

Retroviral infection

Retroviral expression vectors for FLAG-tagged PIRH2 were constructed with pMX-puro, which was obtained from Dr. T Kitamura (University of Tokyo). For retrovirus-mediated gene expression, LNCaP.FGC cells were infected with retroviruses produced by Plat-A packaging cells and then cultured in the presence of puromycin (0.2 μg /ml; Sigma).

Real-time PCR

Total RNA (3 μg) isolated from LNCaP.FGC cells with the use of TRI Reagent (Sigma) was subjected to RT with an M-MLV Reverse Transcriptase (Invitrogen). The resulting cDNA was subjected to PCR with TaqMan Universal PCR Master Mix in an ABI-PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

PSA secretion assay

Cells were left untreated or stimulated with $10^{-8}$ M DHT for 24 hours. For each condition, the medium was collected and the concentration of PSA was measured using a TOSOH II PA monoclonal immunoenzyme assay kit (Tosoh, Tokyo, Japan). The concentrations are shown as means ± S.D. of values from three independent experiments.

## Results

### Identification of a protein that interacts with PIRH2 by using a yeast two-hybrid system

We screened a HeLa cDNA library for the PIRH2-interacting proteins by using pBTM116-hPIRH2(1-129) plasmid as a bait, since the full-length hPIRH2 or the C-terminal half of hPIRH2 without the RING-H2 domain has auto-transcriptional activities. We obtained 25 cDNA positive clones from 1.0 x $10^6$ transformants. Three clones (clone #12, 16, 23) out of the 25 positive clones have sequence identities with human ε-COP cDNA (accession number: BC017285), which is a subunit of the heptameric coatmer protein complex I (COP I), mediates the transport between the Golgi complex and ER (Fig. 1A). We tested whether the full-length PIRH2 and a RING finger domain mutant (PIRH2(CA)) in which the first cysteine 145 was substituted to alanine physically interact with ε-COP. We expressed the FLAG-tagged PIRH2 together with Myc-tagged ε-COP in HEK293T cells. Cell lysates were subjected to immunoprecipitation with antibodies to Myc, and the resulting precipitates were subjected to immunoblot analysis with antibodies to FLAG. Wild type or mutant (CA) type of FLAG-tagged PIRH2 was co-precipitated by the antibody to Myc, indicating that PIRH2 specifically interacts with ε-COP in human cell lines (Fig. 1B).

To determine whether PIRH2 ubiquitylates ε-COP, we generated and purified recombinant GST-ε-COP and hexahistidine ($His_6$)-tagged-PIRH2 as a substrate and an E3 ligase, respectively, for in vitro ubiquitylation assay. We assayed the E3 ligase

activity of $His_6$-tagged PIRH2 against recombinant GST-ε-COP substrate in the presence of E1, E2, ubiquitin (Ub) and ATP. Polyubiquitylation of GST-ε-COP was observed only in the additional presence of the $His_6$-tagged PIRH2 (Fig. 1C). However, we could not determine whether this ubiquitylation of ε-COP by PIRH2 is polyubiquitylation or multiple monoubiquitylation. To further investigate the ubiquitylation of ε-COP in cells by in vivo ubiquitylation assay, FLAG-tagged PIRH2, Myc-tagged ε-COP and HA-tagged ubiquitin were transfected in HEK293T cells. Though Myc-tagged ε-COP was slightly ubiquitylated even without the overexpression of PIRH2, introduction of PIRH2 significantly enhanced the polyubiquitylation of Myc-tagged ε-COP (Fig. 1D). These findings demonstrated that PIRH2 indeed promotes ubiquitylation of ε-COP.

Promotion of ε-COP degradation by PIRH2 in vivo

To examine the possible effect of PIRH2 on the degradation of ε-COP in vivo, HEK293T cells were transfected with vectors for Myc-tagged ε-COP and for either the wild type or CA mutant of FLAG-tagged PIRH2, and the cells were cultured with cycloheximide and then cell lysates at indicated times were analyzed by immunoblotting with anti-Myc. Pulse-chase analysis revealed that the expression of wild-type PIRH2 promoted the degradation of ε-COP and that mutant PIRH2(CA) delayed the degradation of ε-COP in vivo (Fig. 2A and B), suggesting that PIRH2(CA) has a dominant negative activity for degradation of ε-COP. These results thus reveal

that PIRH2 contributes to the turnover of ε-COP in cells.

Subcellular localization of PIRH2 in the presence of DHT

It is known that AR possesses a feature of androgen-dependent nuclear localization and that PIRH2 physically interacts with AR. Dihydrotestosterone (DHT), an androgen, significantly promotes the nuclear translocation of AR. Next, we examined the intracellular localization of AR and PIRH2 in the presence or absence of DHT by immunostaining using the prostate cancer cell line LNCaP, which expresses endogenous AR. DHT caused accumulation of AR in the nucleus but did not affect the localization of PIRH2 (Fig. 3A). To further confirm the change in localization of PIRH2 in the absence or presence of DHT, we performed subcellular fractionations by a biochemical procedure. The ratio of expression level of PIRH2 in the nucleus was not changed in the presence of DHT, whereas the nuclear fraction of AR was increased by treatment with DHT (Fig. 3B). These findings suggest that PIRH2 dissociated with AR in the presence of DHT remains in the cytosol.

ε-COP is efficiently ubiquitylated and degraded in the presence of androgen and PIRH2

Immunoprecipitation was performed to quantify the affinity between PIRH2 and AR in the presence or absence of DHT. Expression vectors for FLAG-tagged PIRH2 and HA-tagged AR were transfected into HEK293T cells, and the cells were cultured in the

presence or absence of DHT. The cell lysates were then immunoprecipitated with anti-HA or anti-FLAG antibodies, and anti-FLAG or anti-HA immunoblotting was performed to detect interaction between PIRH2 and AR. PIRH2 strongly interacted with AR in the absence of androgen, whereas the interaction was imperceptible in the presence of androgen (Fig. 4A). Next we examined whether androgen affects the ubiquitylation of ε-COP by an in vivo ubiquitylation assay. Expression vectors for Myc-tagged ε-COP, HA-tagged ubiquitin and AR were transfected into HEK293T cells, and the cells were cultured in the absence or presence of DHT and then anti-HA immunoblotting was performed for immunoprecipitates with anti-Myc antibody. Myc-tagged ε-COP was ubiquitylated extensively in the presence of DHT (Fig. 4B), suggesting that DHT induces the nuclear translocation of AR and then PIRH2 remaining in the cytosol interacts with ε-COP and expedites its ubiquitylation. Next pulse-chase analysis for ε-COP was carried out in the absence or presence of DHT and/or flutamide, a selective androgen receptor modulator (SARM) that binds to and modulates androgen receptors. Flutamide directly acts on the proliferation of prostate cancer cells by competitively blocking the binding of endogenous and exogenous androgen to AR [25]. HEK293T cells were transfected with vectors encoding Myc-tagged ε-COP, AR and FLAG-tagged PIRH2 in the absence or presence of DHT and/or flutamide, and then the cells were cultured with cycloheximide and the cell lysates at indicated times were immunoblotted with anti-Myc antibody. Pulse-chase analysis revealed that the degradation of ε-COP was promoted in the presence of DHT but inhibited by flutamide. Overexpression of wild-type PIRH2 and administration of

DHT enhanced the degradation of ε-COP, but flutamide dampened the effect yet again (Fig. 4C). These results indicate that androgen and PIRH2 are likely to control the turnover of ε-COP in cells that express AR such as prostate cancer cells.

PIRH2 inhibits the secretion of PSA from prostate cancer cells

PSA is a secretory glycoprotein that acts as a serine protease and exists exclusively in prostate epithelial cells. PSA can be measured in serum and its measurement is used as a clinical assay for monitoring prostate cancer [26, 27]. Since PIRH2 mediates the degradation of ε-COP, we hypothesized that PIRH2 affects the intracellular membrane traffic system for secretory proteins such as PSA, and we carried out an ELISA assay for secreted PSA in culture supernatant. The human prostate carcinoma cell line LNCaP.FGC, which expresses AR and secretes PSA, was used for the establishment of cell lines stably overexpressing FLAG-tagged PIRH2 by a retroviral expression system (Fig. 5A). The expression levels of FLAG-tagged PIRH2 in this cell line were not changed in the absence or presence of DHT. Logan et al. showed that PIRH2 upregulates AR-mediated transcription for target genes such as the PSA gene, whereas Beitel et al. reported that PIRH2 does not significantly up- or downregulate AR transcriptional activation [7, 10]. First, we checked the mRNA level of PSA in LNCaP.FGC cells overexpressing PIRH2 by real-time PCR with/without DHT. The cells were starved in six-well plates with androgen-free culture medium for 24 hours, and then some of the cells were further treated with DHT ($10^{-8}$ M) for 24 hours. The

mRNA from these cell lines was evaluated by real-time PCR. Quantitative analysis of mRNA revealed that androgen-dependent transcription of PSA was slightly enhanced in cells overexpressing wild-type PIRH2 compared with that in the control (Fig. 5B). Next, the secretory levels of PSA in culture medium were quantified. The cells were starved in six-well plates with androgen-free culture medium for 24 hours, and then some of cells were further treated with DHT ($10^{-8}$ M) for 24 hours. The culture medium was then harvested, and the concentration of PSA was evaluated by ELISA. This PSA secretion assay revealed that androgen-dependent secretion of PSA was inhibited in cells overexpressing wild-type PIRH2 compared with that in the control, suggesting that PIRH2 negatively affects the membrane traffic system including COP I complex (Fig. 5C).

A previous study has shown that temperature-sensitive ε-COP mutant cells (ldlF cells) exhibit phenotypes including endocytic missorting and disruption of ER-Golgi transport. Immunofluorescent staining with an antibody to the Golgi-associated protein mannosidase II indicated that Golgi-associated immunostaining of mannosidase II in ldlF cells gradually dispersed into a diffuse pattern after the cells had been transferred to a nonpermissive temperature. According to our results showing that PIRH2 degrades ε-COP via the ubiquitin-proteasome system, overexpression of PIRH2 may affect subcellular localization of Golgi markers. To examine the subcellular distribution of a Golgi marker, immunofluorescent staining was performed using anti-Golgi-58K antibody. LNCaP cells stably overexpressing FLAG-tagged PIRH2 by a retroviral expression system were treated with DHT ($10^{-8}$ M) for 24 hours and then stained with

anti-Golgi-58K antibody. Golgi-58K immunoreactivity in mock cell lines was distributed in a compact perinuclear pattern of a short rod-like structure, regardless of the presence of DHT, whereas that of PIRH2-overexpressing cells dispersed into a diffuse punctate pattern even without DHT treatment (Fig. 6). These findings indicate that overexpression of PIRH2 causes morphological change of TGN.

## Discussion

We showed that PIRH2 directly interacts with ε-COP, resulting in the ubiquitylation of ε-COP. We hypothesized that the interaction between PIRH2 and AR affects the interaction between PIRH2 and ε-COP, because PIRH2 was originally isolated as a novel androgen receptor-interacting protein (ARNIP). We demonstrated that PIRH2 inadequately interacts with AR in the presence of androgen but tightly binds in the absence of androgen in vivo. AR has been reported to be ligand-dependently translocated from the cytoplasm into the nucleus [28, 29]. These findings suggest that AR is retained in the cytoplasm by binding to PIRH2 or molecular chaperones, including HSP90, in a resting state. In the presence of DHT, AR coupled with DHT is activated and translocates into the nucleus and binds the androgen-responsive element (ARE) sequences in the promoter or enhancer of target genes. Conversely, PIRH2 left in the cytosol interacts with another partner, ε-COP, followed by the ubiquitylation and degradation of ε-COP (Fig. 7).

The proteolytic interaction of PIRH2 with ε-COP strongly suggests novel functions for PIRH2 in intracellular membrane trafficking. Recently, it has been reported that other proteolytic system, calpain interacts and proteolyzes another component of the COP I complex, β-COP [30]. These findings demonstrate that the protein degradation system, including the calpain system and ubiquitin-proteasome system, may be involved in membrane trafficking of differentiated secretory cells such as mucous cells in the stomach and prostate epithelial cells, via interactions with coat proteins.

The PSA gene is known to contain ARE, and its transcriptional levels is regulated by AR and Tip60 [31-33]. It has been shown that siRNA against PIRH2 caused an increase of HDAC1 protein in response to PIRH2 depletion, leading to down-regulation of AR-mediated transcription for target genes such as the PSA gene, in which the level of PSA mRNA was shown by quantitative PCR [10]. We found a slight increase in PSA mRNA by the overexpression of PIRH2, whereas the secretory level of PSA was attenuated. A recent study has shown that Rab27a, a small GTPase that is involved in vesicle-regulated secretion in several tissues, plays another important role in the secretory pathway for PSA in LNCaP.FGC cells [34]. Therefore, the level of PSA secretion seems to be regulated by several complicated mechanisms. The differences in transcriptional, translational and posttranslational levels of PSA in our results may be due to differences in regulatory systems, in which PIRH2 participates at distinct locations including the nucleus, cytoplasm and intracellular membranes (Fig. 5).

Previous studies demonstrated that PIRH2 interacts with several proteins that are involved in cell proliferation and gene activation [5, 8, 10, 35] and that PIRH2 is highly expressed in human non-small cell lung cancer and prostate cancer [10, 36]. It is difficult to reconcile the interaction between PIRH2 and ε-COP with the proliferation by PIRH2. The interaction between PIRH2 and epsilon-COP would have nothing to do with cell proliferation, but our data suggest that it affects protein secretion system. Interactions of PIRH2 with p53 and HDAC1 have been reported, and these interactions are probably critical for cell proliferation [8, 10]. However, it is significant for clinical diagnosis to reveal the control system of PSA secretion in prostate cancer cells. In cases

of advanced prostate cancer, ablative hormone therapy remains the mainstay of treatment, while chemotherapy is used for patients with hormone-refractory prostate cancer (HRPC) in which PSA may not be controlled by androgen, but the level of PSA in serum is as high as hormone-responsive prostate cancer [37, 38]. Taken together, post-translational and intracellular trafficking systems by PIRH2 may contribute to the secretory level of PSA in prostate cancer cells.

A previous study has shown that serum PSA level is relatively low in patients with poorly differentiated or undifferentiated prostate cancer [39]. It has recently been revealed that PIRH2 enhanced AR signaling and was highly expressed in poorly differentiated prostate cancer [10]. These findings suggest that PIRH2 is involved in regulation of the level of PSA secretion from poorly differentiated or undifferentiated prostate cancer cells. Therefore, PIRH2 is probably noteworthy for a new prognostic factor or a target of therapy for prostate cancer. It may be important to investigate the expression levels of ε-COP and PIRH2 in clinical specimens of prostate cancers. Analysis by a genetic approach using transgenic or knock-out mice to determine whether PIRH2 promotes cell cycle progression and regulates PSA secretion is also needed.

**Acknowledgements** We thank G. Sobue, and T. Kitamura for the plasmids and cell lines and Y. Soida for helping with the preparation of the manuscript. The work was supported in part by a research grant from Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology (18013001), the Osaka Cancer Research Foundation and the Akiyama Foundation.

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**Figure legends**

**Fig. 1** PIRH2 mediates the ubiquitylation of ε-COP. **(A)** The clones (clones 12, 16 and 23) isolated from yeast two-hybrid screening using PIRH2 as a bait. **(B)** In vivo binding assay between PIRH2 and ε-COP. FLAG-tagged PIRH2 and Myc-tagged ε-COP expression vectors were transfected into HEK293T cells. Cell lysates (WCL) were subjected to immunoprecipitation (IP) with antibodies to Myc, immunoblotted (IB) and probed with antibodies to FLAG. A portion of the cell lysate corresponding to 3% of the input for immunoprecipitation was also subjected to immunoblot analysis. **(C)** Promotion of ε-COP polyubiquitylation in vitro by PIRH2. An in vitro ubiquitylation assay was performed with $His_6$-tagged PIRH2 and GST-ε-COP. Reaction mixtures were subjected to immunoblot analysis with anti-GST. **(D)** In vivo ubiquitylation assay for ε-COP with PIRH2. Expression vectors for Myc-tagged ε-COP, FLAG-tagged PIRH2 and HA-tagged Ub were transfected into HEK293T cells, and the cell lysates were immunoprecipitated with anti-Myc antibody and then anti-HA immunoblotting was performed to detect the ubiquitylation of ε-COP.

**Fig. 2** Pulse chase analysis of ε-COP with PIRH2. **(A)** HEK293T cells were transfected with expression plasmids encoding Myc-tagged ε-COP and either FLAG-tagged PIRH2(WT), FLAG-tagged PIRH2(CA) or the mock (empty vector). Twenty-four hours after transfection, the cells were cultured in the presence of cycloheximide (50 μg/ml) for the indicated times. Cell lysates were then subjected to immunoblot analysis with

anti-Myc and anti-α-tubulin. **(B)** The intensity of the Myc-tagged ε-COP bands in (A) was normalized by that of the corresponding α-tubulin bands and was then indicated as a percentage of the normalized value at 0 hour. Data are means ± SD of values from three independent experiments.

**Fig. 3** Subcelluar localization of PIRH2. **(A)** Immunofluorescence analysis of AR and PIRH2. LNCaP.FGC cell lines were incubated with anti-AR (mouse) or anti-PIRH2 (rabbit) antibodies. They were reacted with Alexa546-labeled goat polyclonal antibodies to mouse immunoglobulin and Alexa488-labeled goat polyclonal antibodies to rabbit immunoglobulin and then stained with Hoechst33258 to visualize the nuclei. **(B)** Intracellular localization of AR and PIRH2 either in the absence or presence of DHT. Subcellular fractions were separated biochemically, and the expression levels of AR and PIRH2 were analyzed by immunoblotting. Anti-α-tubulin and anti-histone H1 antibodies were used for controls of cytoplasmic and nuclear fractions, respectively. C: cytoplasmic fraction, N: nuclear fraction.

**Fig. 4.** Androgen affects the interaction between PIRH2 and AR and affects the ubiquitylation and degradation of ε-COP. **(A)** Androgen inhibits the interaction between PIRH2 and AR. HEK293T cells were transfected with expression plasmids encoding FLAG-tagged PIRH2 and HA-tagged AR and then cultured with/without DHT ($10^{-8}$ M) for 24 hours. Cell lysates (WCL) were subjected to immunoprecipitation (IP) with antibodies to FLAG or HA and immunoblotted (IB) with antibodies to HA or FLAG.

**(B)** Androgen enhances the ubiquitylation of ε-COP. HEK293T cells were transfected with expression plasmids encoding Myc-tagged ε-COP, HA-tagged Ub and AR and then cultured with/without DHT ($10^{-8}$ M) for 24 hours. Cell lysates (WCL) were subjected to immunoprecipitation with antibodies to Myc and immunoblotted with antibodies to HA or Myc. **(C)** Pulse chase analysis of ε-COP with DHT and/or flutamide. HEK293T cells were transfected with expression plasmids encoding Myc-tagged ε-COP, AR and either FLAG-tagged PIRH2 or the mock. Twenty-four hours after transfection, the cells were cultured in the presence of cycloheximide (50 μg/ml), DHT ($10^{-8}$ M) and/or flutamide ($10^{-8}$ M) for the indicated times. Cell lysates were then subjected to immunoblot analysis with anti-Myc or anti-HSP90.

**Fig. 5.** PIRH2 inhibits the secretion of PSA from LNCaP.FGC cells. **(A)** LNCaP.FGC cells expressing FLAG–tagged PIRH2 or the mock were cultured with/without DHT ($10^{-8}$ M), and the cell lysates were subjected to immunoblot analysis with antibodies to PIRH2 or HSP90. **(B)** LNCaP.FGC cells expressing FLAG–tagged PIRH2 or the mock (empty vector) were cultured with/without DHT ($10^{-8}$ M), and the mRNA was quantified by real-time PCR. **(C)** Inhibition of PSA secretion from LNCaP.FGC cells by overexpression of PIRH2. LNCaP.FGC cells that stably overexpressed PIRH2 were cultured with androgen-free culture medium for 24 hours. Cells were left untreated or stimulated with $10^{-8}$ M DHT for 24 hours. The concentrations of PSA in each culture supernatant were evaluated using ELISA. Data are means ± SD of values from three independent experiments. The P values for the indicated comparisons were determined

by Student's t test.

**Fig. 6.** Subcelluar localization of Golgi-58K in LNCaP.FGC cells overexpressing PIRH2. Immunofluorescence analysis of AR and PIRH2. LNCaP.FGC cells expressing FLAG–tagged PIRH2 or mock cells were cultured with/without DHT ($10^{-8}$ M) for 24 hours, and the cells were stained with antibodies to Golgi-58K. They were reacted with Alexa546-labeled goat polyclonal antibodies to mouse immunoglobulin and then stained with Hoechst33258 to visualize the nuclei. Scale bars, 50 μm

**Fig. 7.** Schematic illustration of a model for ubiquitylation of ε-COP by PIRH2. In a resting state, AR is retained in the cytoplasm by binding to molecular chaperones such as HSP90 or PIRH2. In the presence of DHT, activated AR with DHT translocates into the nucleus and binds the ARE sequences in the promoter or enhancer of target genes (PSA gene). On the other hand, PIRH2 left in the cytosol interacts with ε-COP, resulting in the ubiquitylation and degradation of ε-COP.