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Ubiquitin-specific Peptidase 8 Regulates the Trafficking and Stability of the Human Organic Anion Transporter 1

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Abstract

Background: Organic anion transporter 1 (OAT1) plays a vital role in avoiding the potential toxicity of various anionic drugs through the involvement of kidney elimination. We previously demonstrated that ubiquitin conjugation to OAT1 led to OAT1 internalization from cell surface, followed by degradation. Ubiquitination is a dynamic process, where deubiquitination is catalyzed by a class of ubiquitin-specific peptidases.

Methods: The role of ubiquitin-specific peptidase 8 (USP8) in hOAT1 function, expression and ubiquitination was assessed by conducting transporter uptake assay, biotinylation assay and ubiquitination assay.

Results: We demonstrated that USP8 overexpression in hOAT1-expressing cells led to an increased hOAT1 transporter activity and expression, which correlated well with a reduced hOAT1 ubiquitination. Such phenomenon was not observed in inactive USP8 mutant-transfected cells. In addition, the knockdown of endogenous USP8 by USP8-specific siRNA resulted in an increased hOAT1 ubiquitination, which correlated well with a decrease in hOAT1 expression and transport activity. Biotinylation experiments demonstrated that USP8-induced increase in hOAT1 expression and transport activity occurred through a deceleration of the rates of hOAT1 internalization and degradation.

Conclusions: These results indicated the regulatory role of USP8 in OAT1 function, expression, trafficking, and stability.

General significance: USP8 could be a new target for modulating OAT1-mediated drug transport.

Keywords

Drug Transport; Organic Anion Transporter; Ubiquitin-specific Peptidase; Deubiquitination; Regulation

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Conflicts of Interest:
The authors declare no conflicts of interest.

1. Introduction

Organic anion transporter 1 (OAT1) belongs to organic anion transporter family of solute carrier 22 family, which play vital roles in the elimination of various anionic drugs from the kidney, including anti-viral therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. OAT1 plays critical roles in the handling of numerous drugs through the kidney which affects pharmacokinetics and pharmacodynamics of drug profiles and thereby affecting drug efficacy and potential drug toxicity in the body [1–7].

OAT1 protein expression level on the cell surface critically contributes to their transport activity. It has been demonstrated by our lab that the members of OAT family endocytose/internalize from and recycle back to the cell membrane [8]. Ubiquitin conjugation to the lysine residues on the transporter is a vital event which accelerates OAT internalization. The ubiquitin attachment to the OAT at the cell membrane triggers OAT endocytosis from the cell membrane to the early endosomes [9]. Once in the endosomes, OAT either becomes deubiquitinated which leads to OAT recycling back to the cell surface or becomes degraded in the proteolytic system [8, 9]. Furthermore, we demonstrated that the activation of protein kinase C (PKC) enhanced the ubiquitination level of OAT, thereby accelerating the OAT endocytosis from the cell surface to the early endosomes and subsequent degradation without impacting the recycling rate. As a result, OAT protein expression level at the cell membrane is significantly decreased which leads to a significantly reduced OAT transport activity [8–11].

Similar to the opposing but coordinated functions of protein kinase and phosphatase activity, ubiquitination is a highly dynamic and reversible process, and the ubiquitination state of target proteins is also controlled by the deubiquitinating enzymes, which remove ubiquitin from them [12–14]. Deubiquitinating enzymes, also known as ubiquitin proteases, are a superfamily of nearly 100 enzymes which are classified into two main classes including cysteine proteases and metalloproteases. The two main classes can be further subdivided into five subfamilies with different structures and specificities for the isopeptide bond that links ubiquitin chains [15–17].

USP8, a cysteine protease, is a member of the ubiquitin-specific protease family of deubiquitinating enzymes which catalyze the removal of ubiquitin from several membrane proteins such as receptors and channels [18–22]. In this study, we assessed the role of USP8 in OAT1 deubiquitination, expression and function.

2. Materials and Methods

2.1. Materials

COS-7 cells were purchased from American Type Culture Collection (Manassas, VA). [^{3}H]-labeled para-aminohippurate ([^{3}H]-labeled PAH) was purchased from PerkinElmer (Waltham, MA). Membrane-impermeable biotinylation reagent sulfo-NHS-SS-biotin, streptavidin-agarose beads and protein G-agarose beads were purchased from Pierce (Rockford, IL). cDNAs for pCIneo-Flag-Vector (empty vector), pCIneo-Flag-WT-USP8 and pCIneo-Flag-USP8-C786A were generously provided by Dr. Chunying Du from University

of Cincinnati [23]. Mouse monoclonal anti-myc antibody was purchased from Roche (Indianapolis, IN). Rabbit polyclonal anti-USP8 antibody was purchased from Cell Signaling (Danvers, MA). Mouse monoclonal anti-E-cadherin antibody was purchased from Abcam (Cambridge, MA). Normal mouse lgG, mouse monoclonal anti-ubiquitin and mouse monoclonal anti-β-actin antibodies were purchased from Santa Cruz (Santa Cruz, CA). Human USP8 siRNA (Catalog NO.: J-005203–09-0005) and scrambled siRNA (control siRNA) were purchased from GE Healthcare Dharmacon (Lafayette, CO). All other reagents were from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and Transfection

Parental COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. Transfection with plasmids such as N-terminal myc-tagged human organic anion transporter 1 (N-myc-hOAT1) was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. COS-7 cells were transfected with non-targeting or USP8 siRNA (48-well plate: 12.5 pmol per well; 35 × 10 mm dish: 50 pmol per dish; 60 × 15 mm dish: 75 pmol per dish) for 48 h using Lipofectamine 2000.

2.3. Transport Measurements

Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consists of phosphate-buffered saline (PBS) with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4) and [^{3}H]-labeled PAH (20 μM). At the time points indicated, uptake was terminated by removing the uptake solution, followed by washing with ice-cold PBS twice. The cells were then lysed in 0.2 N NaOH, neutralized with 0.2 N HCl and transferred into scintillation vials for liquid scintillation counting. The relative expression levels of the hOAT1 between in 48-well plates and in 6-well plates at various transfection conditions are similar.

2.4. Cell Surface Biotinylation

Cell surface expression level of hOAT1 was examined using the membrane-impermeable biotinylation reagent, sulfo-NHS-SS-biotin. Cells were seeded in 6-well plates. Each well of cells was incubated with 1 ml of freshly made sulfo-NHS-SS-biotin (0.5 mg/ml in PBS/CM) in two successive 20 min incubations on ice with very gentle shaking. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine then incubated with the same solution for 20 min on ice, to ensure complete quenching of the unreacted sulfo-NHS-SS-biotin. The cells were then lysed on ice for 30 min in lysis buffer (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 with 1/100 protease inhibitor cocktail). Protease inhibitor cocktail consists of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), aprotinin, bestatin, trans-Epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), leupeptin and pepstatin A. The cell lysates were centrifuged at 16,000g at 4 °C. 40 μl of streptavidin-agarose beads was then added to the supernatant to isolate cell membrane proteins. hOAT1 was detected in the pool of surface proteins by SDS-PAGE and immunoblotting using anti-myc antibody.

2.5. Internalization Assay

We followed the procedure previously established in our lab [11]. hOAT1-expressing cells transfected with empty vector or USP8 wild type underwent biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin at 4 °C. Following biotinylation, hOAT1 internalization was initiated by incubating the cells (37 °C) in medium for designated periods of time. Residual cell surface biotin was stripped by incubating cells three times for 20 min with freshly prepared 50 mM MeSNa in NT buffer (150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin, 20 mM Tris, pH 8.6). Cells were lysed in lysis buffer with protease inhibitor cocktail. Biotinylated (internalized) proteins were separated from nonbiotinylated proteins by streptavidin pull-down from equivalent amounts of cellular proteins, followed by immunoblotting with anti-myc antibody.

2.6. Degradation Assay

hOAT1-expressing cells transfected with empty vector or USP8 wild type underwent cell-surface biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin at 4 °C. After biotinylation, each well was rinsed with 3 ml PBS pH8.0/CM containing 100 mM glycine and then incubated with the same solution for 20 min on ice, to ensure complete quenching of the unreacted sulfo-NHS-SS-biotin. The biotin-labeled cells were incubated in DMEM at 37 °C. Treated cells were collected at 0, 2, 4 and 6 h and lysed in lysis buffer with protease inhibitor cocktail. The cell lysates were centrifuged at 16,000 × g at 4 °C. 40 μl of streptavidin-agarose beads were then added to the supernatant to isolate cell membrane proteins. Samples were loaded on 7.5% SDS-PAGE minigels and analyzed by immunoblotting with anti-myc antibody.

2.7. Ubiquitination

Cells are pre-treated with MG132 (10 μM, 1h), a proteasome inhibitor, to enhance the basal hOAT1 ubiquitination signal. Then cells were lysed with lysis buffer (20 mM Tris/HCl, pH 7.5, 1% Triton X-100, 2 mM EDTA, and 25 mM NaF), freshly added with 1% of proteinase inhibitor cocktail and 2% of N-ethylmaleimide to obtain the cell total lysates. Cell membrane proteins were extracted using ProteoExtract Native Membrane Protein Extraction Kit. Cell total lysates and cell membrane lysates were precleared with protein G-agarose beads to reduce non-specific binding at 4 °C for 1.5 hours. Anti-myc antibody or normal mouse lgG was incubated with appropriate volume of protein G-agarose beads at 4 °C for 1.5 hours. The precleared protein sample was then mixed with antibody-bound protein G-agarose beads and underwent end-over-end rotating at 4 °C overnight. Proteins bound to the protein G-agarose beads were eluted with urea buffer containing β-mecaptoethanol and analyzed by immunoblotting with indicated antibodies.

2.8. Electrophoresis and Immunoblotting

Protein samples were resolved on 7.5% SDS-PAGE minigels and electroblotted on to polyvinylidene difluoride membranes. The blots were blocked for 1 hour with 5% non-fat dry milk in PBS-0.05% Tween 20, washed, and incubated overnight at 4 °C with appropriate primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura Extended Duration Substrate kit

(Pierce, Rockford, IL). Non-saturating, immuno-reactive protein bands were quantified by scanning densitometry with the FluorChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA).

2.9. Data Analysis

Each experiment was repeated a minimum of three times. The statistical analysis was from multiple experiments. Between two groups, statistical analysis was performed using Student's paired t-tests. Among multiple groups, one-way ANOVA or two-way ANONA, Tukey's test was applied by using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). A P-value of <0.05 was considered significant.

3. Results

3.1. Expression of USP8 and its inactive mutant in hOAT1-expressing cells

To investigate the role of USP8 in hOAT1 expression and function, we transfected cDNAs encoding USP8 wild type and its inactive mutant USP8/C786A. Our results showed that both USP8 wild type and USP8/C786A were effectively and equally transfected into the cells (Fig. 1), providing a useful tool for our following studies.

3.2. Effect of USP8 and its inactive mutant on hOAT1 transport activity

To examine the role of USP8 in hOAT1 transporter activity, we transfected hOAT1-expressing cells with USP8 wild type or its inactive mutant USP8/C786A, and then measured hOAT1-mediated uptake of [^{3}H]-labeled PAH, a prototypical substrate for OAT1. In Fig. 2, USP8 wild type significantly enhanced the uptake as compared to the uptake in control cells (empty vector-transfected cells), whereas the inactive mutant USP8/C786A did not impact the uptake.

3.3. Effect of USP8 and its inactive mutant on hOAT1 expression

We examined the transporter expression both at the cell surface and in the total cell lysates. We showed that transfection of USP8 wild type into hOAT1-expressing cells led to an increase in hOAT1 expression at the cell surface and its total expression (Fig. 3a, top panel, and Fig. 3c, top panel), whereas transfection of the inactive mutant USP8/C786A into hOAT1-expressing cells was without significant effect. The protein levels of cell membrane protein marker E-cadherin (Fig. 3a, bottom panel) and cell total protein marker β-actin (Fig. 3c, bottom panel) were not affected under these conditions, thereby indicating that the change in hOAT1 expression induced by USP8 wild type transfection was not due to the general perturbation of membrane and cellular proteins.

3.4. Effect of USP8 on hOAT1 ubiquitination

To examine whether USP8, a deubiquitination enzyme, specifically catalyzes the removal of ubiquitin from hOAT1, we transfected hOAT1-expressing cells with USP8 wild type. The transfected cells were lysed and anti-myc antibody (hOAT1 was tagged with epitope myc to facilitate the immunodetection) or normal mouse lgG was used for hOAT1 immunoprecipitation. And then the immunoblotting (IB) was performed with anti-ubiquitin

antibody. As shown in Fig. 4a, the level of ubiquitinated hOAT1 was much lower in USP8 overexpressing cells as compared to that in control cells (empty vector-transfected cells). To determine whether USP8 overexpression affects the ubiquitination of membrane hOAT1, the membrane proteins were extracted. Anti-myc antibody (hOAT1 was tagged with epitope myc to facilitate the immunodetection) was used for hOAT1 immunoprecipitation. And then the immunoblotting (IB) was performed with anti-ubiquitin antibody. As demonstrated in Fig. 4c, the level of ubiquitinated membrane hOAT1 was much lower in USP8 overexpressing cells as compared to that in control cells (empty vector-transfected cells).

3.5. Efficiency of knocking-down endogenous USP8 by USP8-specific siRNA

The previous results (Figs. 1–4) evaluated the impact of exogenous USP8 on hOAT1 transporter activity, protein expression and ubiquitination. Furthermore, we investigated the impact of endogenous USP8 on hOAT1 ubiquitination, transport activity and expression in the following experiments. First, we examined the knockdown efficiency of the endogenous USP8 by USP8-specific siRNA. In Fig. 5, the expression of endogenous USP8 was greatly decreased in cells transfected with USP8-specific siRNA as compared to that in control siRNA-transfected cells, indicating the specificity and effectiveness of the siRNA used for our studies.

3.6. Effect of USP8-specific siRNA on hOAT1 ubiquitination

We transfected hOAT1-expressing cells with USP8-specific siRNA or control siRNA. The transfected cells were lysed and anti-myc antibody (hOAT1 was tagged with epitope myc) or normal mouse lgG was used for hOAT1 immunoprecipitation. And then the immunoblotting (IB) was performed with anti-ubiquitin antibody. Our results (Fig. 6) illustrated that the level of ubiquitinated hOAT1 was much higher in cells transfected with USP8-specific siRNA as compared to that in control siRNA-transfected cells.

3.7. Effect of USP8-specific siRNA on hOAT1 transport activity

We transfected hOAT1-expressing cells with USP8-specific siRNA or control siRNA. The hOAT1-mediated uptake of [^{3}H]-labeled PAH was conducted in the transfected cells. As demonstrated in Fig. 7, the transport activity of hOAT1 was significantly decreased in USP8-specific siRNA-transfected cells as compared to that in control siRNA-transfected cells.

3.8. Effect of USP8-specific siRNA on hOAT1 expression

The hOAT1-expressing cells were transfected with USP8-specific siRNA or control siRNA. The cell surface expression and total protein expression of hOAT1 were then examined. The results demonstrated that the knockdown of endogenous USP8 by USP8-specific siRNA resulted in the reduced expression of hOAT1 at the cell surface (Fig. 8a) and in total cell lysates (Fig. 8c).

3.9. Effect of USP8 on the rate of hOAT1 internalization

Previously we demonstrated that ubiquitination of hOAT1 triggers an internalization of hOAT1 from cell surface to intracellular endosomes. In this experiment, we detected the effect of USP8 on the rate of hOAT1 internalization. hOAT1-expressing cells was transfected

with empty vector or USP8 wild type. The rates of hOAT1 internalization were measured using a biotinylation strategy as described in the section of methods. As demonstrated in Fig. 9, the rate of hOAT1 internalization was much slower in USP8 wild type-transfected cells as compared to the rate in control cells (empty vector-transfected cells).

3.10. Effect of USP8 on the rate of hOAT1 degradation

We previously demonstrated that after ubiquitination-dependent internalization of hOAT1 from cell surface to intracellular endosomes, excess of ubiquitination may lead the internalized hOAT1 to target to proteolytic system for degradation. In this experiment, we evaluated the effect of USP8 on the rate of hOAT1 degradation. hOAT1-expressing cells was transfected with empty vector or USP8 wild type. The rates of hOAT1 degradation were measured using a biotinylation strategy as described in the section of methods. In Fig. 10, USP8 slowed down the rate of hOAT1 degradation as compared to that in control cells (empty vector-transfected cells), suggesting that USP8 played a role in increasing hOAT1 stability.

4. Discussion

OAT-mediated transport of therapeutics is a major determinant of their efficacy and toxicity. Thus, investigation of the molecular and cellular mechanisms underlying OAT regulation is of clinical and pharmacological importance. Our current study investigated the role of ubiquitin-specific protease USP8 in the deubiquitination, transport activity and protein expression of OAT1 and revealed a critical role of USP8 in the regulation of this transporter.

Certain membrane proteins are post-translationally modified by ubiquitination process which leads to the alternation of protein stability and function. The ubiquitination includes three major enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. Ubiquitin has 76 amino acids and seven lysine residues itself (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63). A polyubiquitination chain forms with different linkages including Lys48 linkage and Lys63 linkage [24, 25]. Our previous studies demonstrated that E3 ubiquitin ligase, Nedd4–2, catalyzed the ubiquitination of OAT and therefore decreased OAT transport activity and protein expression [26–28]. The Nedd4–2-catalyzed ubiquitination of OAT accelerates the internalization and degradation of OAT. Furthermore, Nedd4–2-catalyzed and PKC-dependent OAT ubiquitination bears Lys48-linked polyubiquitin chains [8].

Interestingly, ubiquitination is a dynamic and reversible process. Deubiquitination is catalyzed by deubiquitinases (DUBs) which include Jab1/Mpn/Mov34 (JAMM) enzymes, ubiquitin C-terminal hydrolase (UCH), Machado-Joseph domain protease (MJD), ubiquitin-specific protease (USP) and ovarian tumor protease (OTU) [17]. USP8 belongs to ubiquitin-specific proteases (USPs) family and has been shown to play important roles in multiple physiological processes, such as Na^+ homeostasis, development and homeostasis of T cells and K^+ balance [18, 22, 29]. Additionally, USP8 is a key factor in multiple pathological development, thus USP8 is a target for some disease treatments. USP8 is a potentially therapeutic target for Cushing's disease, Alzheimer's disease, hypertension and lung cancer [21, 22, 30–32]. Ubiquitination and deubiquitination of receptors and channels by ubiquitin

conjugation and removal have emerged as important regulatory mechanisms of internalization, recycling and degradation of those membrane proteins. USP8 has been reported to regulate various receptors' and channels' trafficking process including epidermal growth factor receptor, chemokine receptor 4, vascular endothelial growth factor receptor 2, Na+ channel and KCa3.1 channel [18–20, 22, 33]. And USP8 catalyzes ubiquitin removal from both Lys48 linkage and Lys63 linkage polyubiquitination chains. Although ubiquitination of OAT has been well-studied, the knowledge on OAT deubiquitination is limited and USP8 regulation on OAT remains unclear. In addition, USP8 is expressed *in vivo* in proximal tubular cells [34], a site where OATs are expressed. Thus, it is important to examine the role of USP8 in the regulation of OAT function and trafficking and this work could provide insights into the in vivo relationship between USP8 and OAT1.

African green monkey kidney COS-7 cells were utilized in this investigation. Although COS-7 cells are not a polarized cell line, this cell line is a widely-used model for studying the mechanisms of various renal transport processes and is an excellent choice for its high transfection efficiency [35–38]. Furthermore, this cell line demonstrated the similar characteristics of OATs as compared to those *in vivo* and *ex vivo* [39–41]. Thus, the studies in these cells could pave the way for the further investigations of the roles of USP8 in OAT regulation *in vivo*.

Our current study is the first investigation of this protease on any of the drug transporters and it demonstrated that USP8 plays an important role in OAT1 function. First, we discovered that overexpression of USP8 in hOAT1-expressing cells led to an increase of hOAT1 expression at the cell surface and an increase in hOAT1-mediated drug transport, which closely correlated with the significant deubiquitination of the transporter. This phenomenon was further verified because a catalytically inactive mutant USP8/C786A was without any effect (Figs. 1–4). Secondly, the knockdown of endogenous USP8 in hOAT1-expressing cells by USP8-specific siRNA resulted in an opposite effect, which is a distinct increase in hOAT1 ubiquitination that correlated well with a reduction of hOAT1 expression at the cell surface and a reduction in hOAT1-mediated drug transport (Figs. 5–8). Our results provided strong evidence on the involvement of USP8 in regulating OAT1 ubiquitination, although we can't rule out the possibility that USP8 may affect the ubiquitination of other proteins which may, in turn, affect the level of ubiquitinated OAT1.

As mentioned above, ubiquitination-dependent down regulation of OAT1 expression and function occurs in two distinct steps. First step involves ubiquitination-dependent internalization of the transporter from cell surface into intracellular endosomes. Second step involves ubiquitination-dependent targeting of the transporter to proteolytic system for degradation. USP8 has been previously shown to be responsible for the slowing down of the degradation step of epithelial sodium channel (ENaC) whereas has no effect on the internalization of ENaC [22]. Interestingly, in our situation, USP8 seems to be responsible for regulating both the internalization and the degradation of hOAT1 (Figs. 9 and 10).

USP8 plays critical roles in various pathological developments and USP8 inhibitors are the potential treatments for those diseases including lung cancer, Cushing's disease and Alzheimer disease. Some USP8-specific inhibitors are developed in pre-clinical stage and

will be progressed into clinical therapies in the near future [42–46]. Downexpression of USP8 decreased OAT1 transport activity and OAT1 expression. Thus, patients treated with USP8 inhibitors could have impaired OAT1-mediated transport activity, and therefore the potential drug-drug interactions and other side effects including drug toxicity should be taken into consideration. Of course, we cannot exclude the possibility that the expression or activity of other membrane or intracellular proteins may also be regulated by USP8 activity, therefore suggesting that the overall effect of using these inhibitors may be complicated.

We have recently reported the crosstalk between different post-translational modifications (PTMs) on OAT, such as the interplay between ubiquitination and SUMOylation [41] . Ubiquitination and SUMOylation could occur on the same lysine residue of the target protein through a competitive manner. Ubiquitin and SUMO could also modify different lysine residues of the target protein and the conjugation of SUMO may interfere with the ubiquitin conjugation on another lysine residue of the target protein [47]. The wide network of crosstalk among various PTMs including ubiquitination, SUMOylation and potential other types of PTMs may be the underlying reason for the observation in our study that the marked overexpression or knockdown of USP8 is only associated with relatively moderate changes in hOAT1 activity. This is an interesting topic for further exploration. In addition, COS-7 cells have fair amount of endogenous USP8, which perhaps have already increased hOAT1 expression and activity to certain extent. Thus, transfection of additional USP8 might not induce a further increase at the magnitude comparable to the amount of USP8 transfected.

In conclusion, our results identify a functional role of USP8 in OAT1 expression, trafficking, stability, and function (Fig. 11) and suggest that USP8 could be a new target for modulating OAT1-mediated drug transport *in vivo*. *In vivo* investigation on the role of USP8 in OAT1 activity and regulation will be our future direction.

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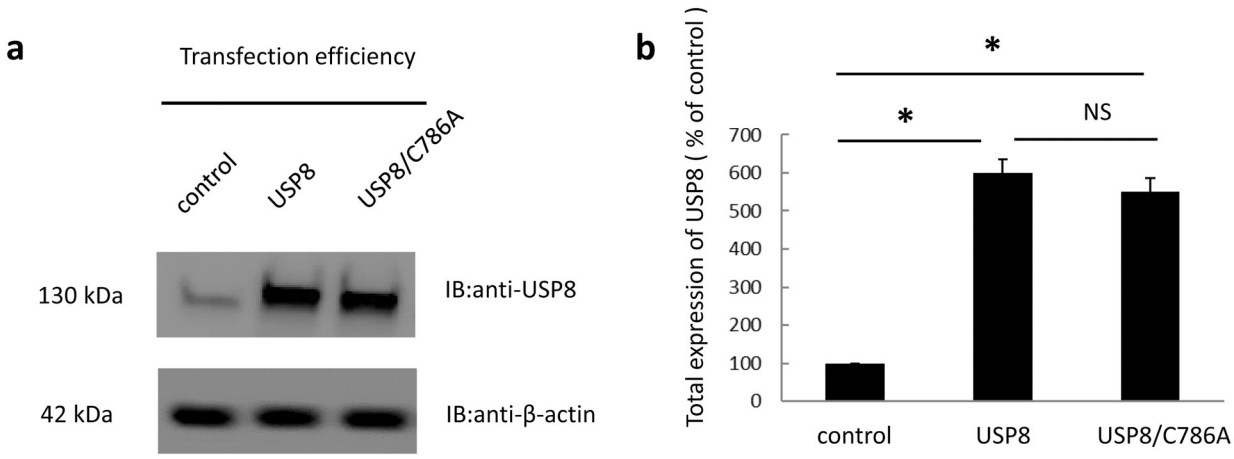

Fig. 1. Expression of USP8 and its inactive mutant in hOAT1-expressing cells.

(a). *Top panel:* USP8 wild type and its inactive mutant USP8/C786A were transfected in hOAT1-expressing cells. Empty vector-transfected cells were used as control. Transfected cells were lysed and, followed by immunoblotting (IB) with anti-USP8 antibody. *Bottom panel:* The same blot as the top panel was re-probed with anti-β-actin antibody. (b). Densitometry plot of results from Fig. 1, top panel, as well as from other repeat experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using one-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). Values are means ± SE; n = 3. *P < 0.05.

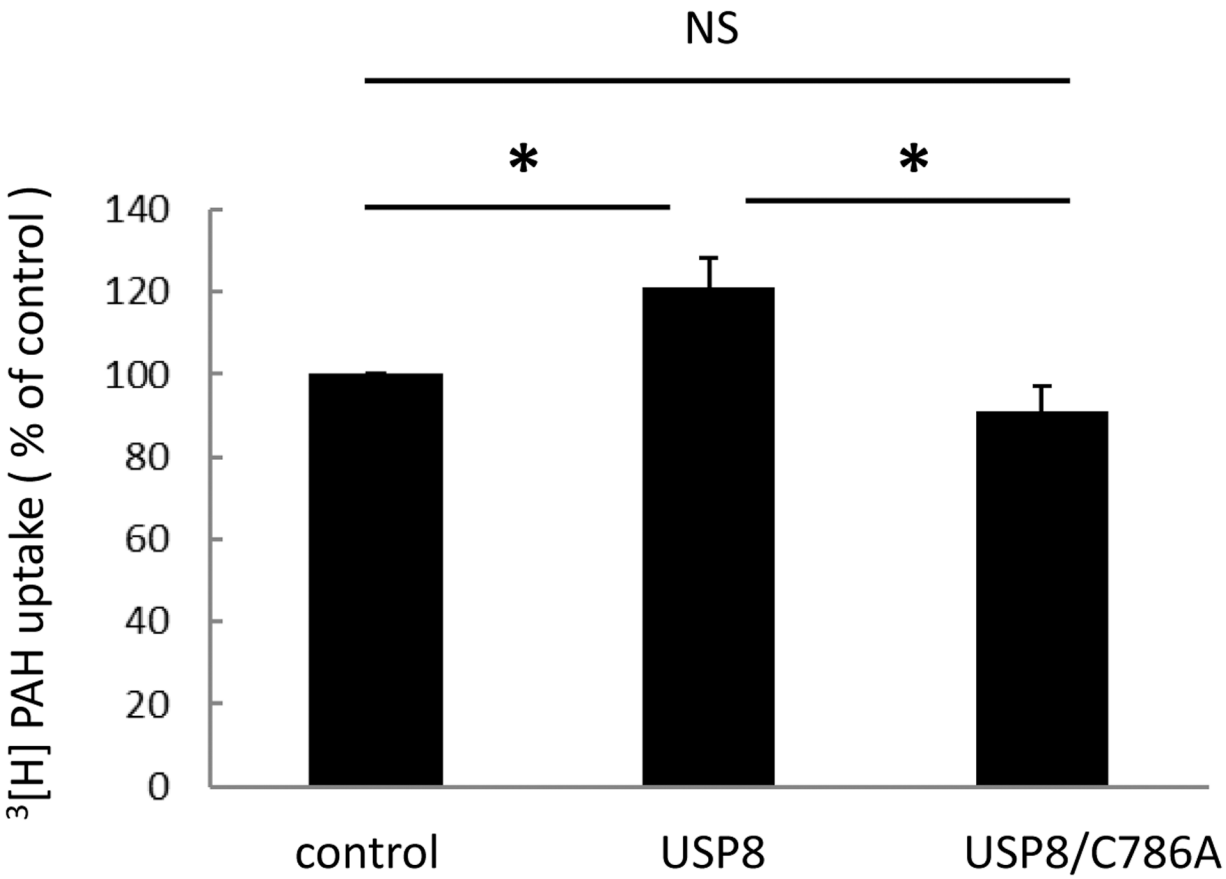

Fig. 2. Effect of USP8 and its inactive mutant on hOAT1 transport activity.

Cells were plated in 48-well plates. hOAT1-expressing cells were transfected with USP8 wild type or its inactive mutant USP8/C786A, followed by the measurement of the uptake of [^{3}H]-labeled PAH (4min, 20 μM). Empty vector-transfected cells were used as control. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT1-expressing cells minus uptake into mock cells (parental COS-7 cells). Statistical analysis was performed using one-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). Values are mean ± S.E. (n = 3). *P<0.05.

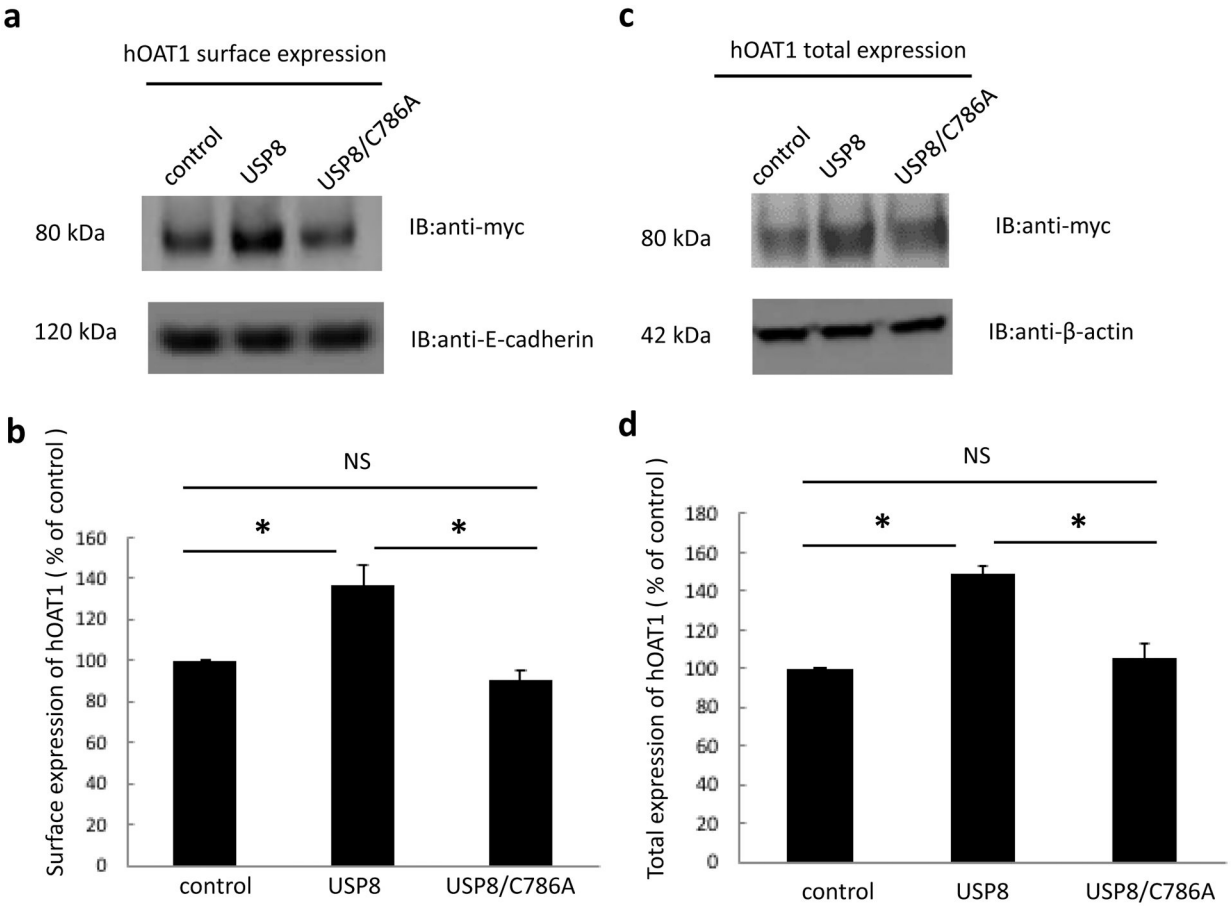

Fig. 3. Effect of USP8 and its inactive mutant on hOAT1 expression.

(a). Cell surface expression of hOAT1. Cells were plated in 6-well plates. *Top panel:* hOAT1-expressing cells were transfected with USP8 wild type or its inactive mutant USP8/C786A. Empty vector-transfected cells were used as control. Transfected cells were labeled with biotin. Biotinylated/cell surface proteins were separated with streptavidin beads, followed by immunoblotting (IB) with anti-myc antibody (epitope myc was tagged to hOAT1). *Bottom panel:* The same blot as the top panel was re-probed with anti-E-cadherin antibody. E-cadherin is a cell membrane marker protein. (b). Densitometry plot of results from Fig. 3a, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using one-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). The values are mean ± S.E. (n = 3). *P<0.05. (c). Total expression of hOAT1. Cells were plated in 6-well plates. *Top panel:* hOAT1-expressing cells were transfected with USP8 wild type or its inactive mutant USP8/C786A. Empty vector-transfected cells were used as control. Cells were lysed, followed by immunoblotting (IB) with anti-myc antibody. *Bottom panel:* The same blot as the top panel was re-probed with anti-β-actin antibody. β-actin is a cellular protein marker. (d). Densitometry plot of results from Fig. 3c, top panel, as well as from other experiments. Statistical analysis was performed using one-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). The expression level was expressed as percentage of that of control. The values are mean ± S.E. (n = 3). *P<0.05.

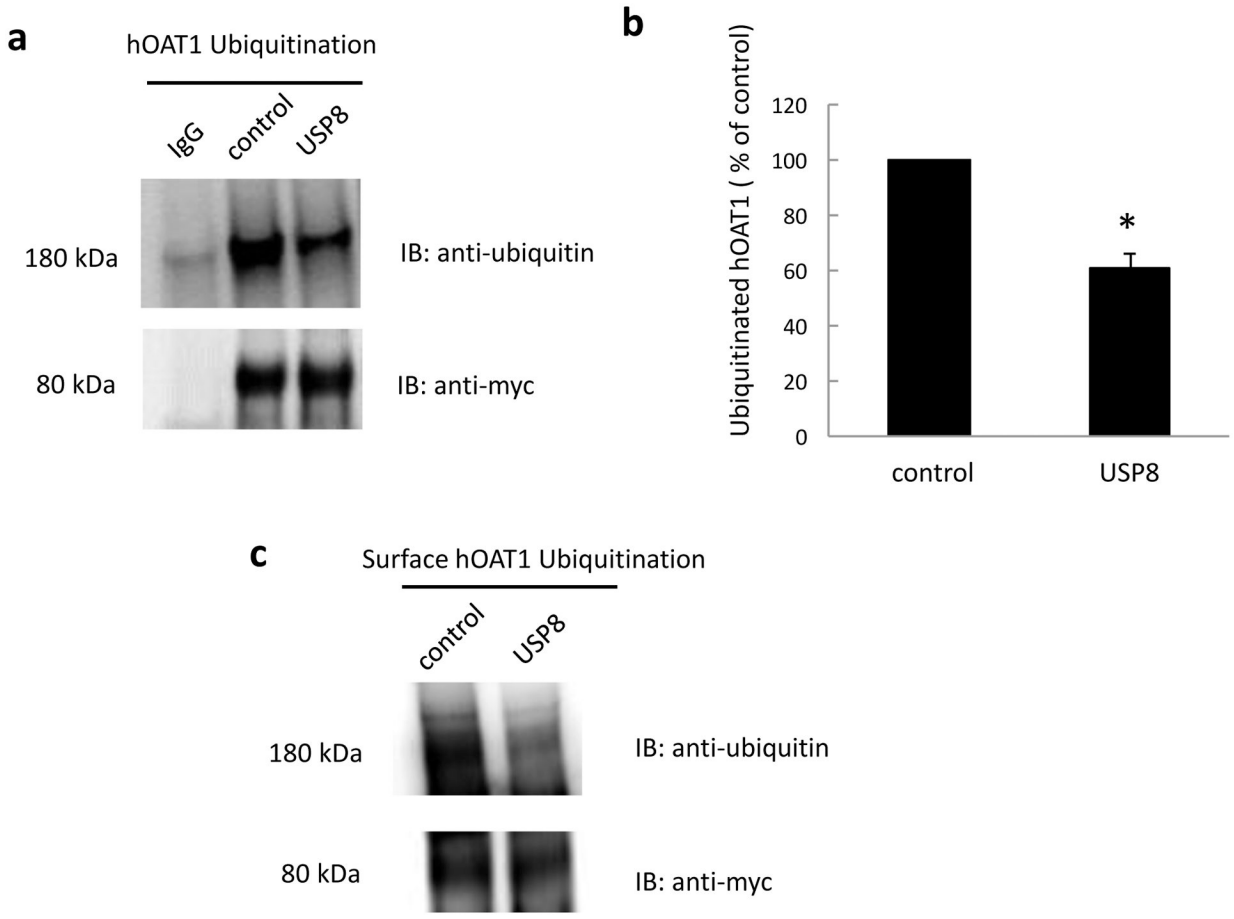

Fig. 4. Effect of USP8 on hOAT1 ubiquitination.

(a). *Top panel:* hOAT1-expressing cells were transfected with USP8 wild type. Empty vector-transfected cells were used as control. Cells were pre-treated with MG132 (10 μM, 1h), a proteasome inhibitor, to enhance the basal hOAT1 ubiquitination signal and then lysed. hOAT1 was immunoprecipitated by anti-myc antibody (epitope myc was tagged to hOAT1) or normal mouse IgG (as negative control), followed by immunoblotting (IB) with anti-ubiquitin antibody to detect ubiquitinated hOAT1. *Bottom panel*: The same blot as the top panel was re-probed by anti-myc antibody. (b). Densitometry plot of results from Fig. 4a, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using Student's paired t-tests. The values are mean ± S.E. (n = 3). *P<0.05. (c). *Top panel:* hOAT1-expressing cells were transfected with USP8 wild type. Empty vector-transfected cells were used as control. Cells were pre-treated with MG132 (10 μM, 1h), a proteasome inhibitor, to enhance the basal hOAT1 ubiquitination signal and then lysed. The membrane proteins were extracted and hOAT1 was immunoprecipitated by anti-myc antibody (epitope myc was tagged to hOAT1), followed by immunoblotting (IB) with anti-ubiquitin antibody to detect ubiquitinated hOAT1. *Bottom panel*: The same blot as the top panel was re-probed by anti-myc antibody.

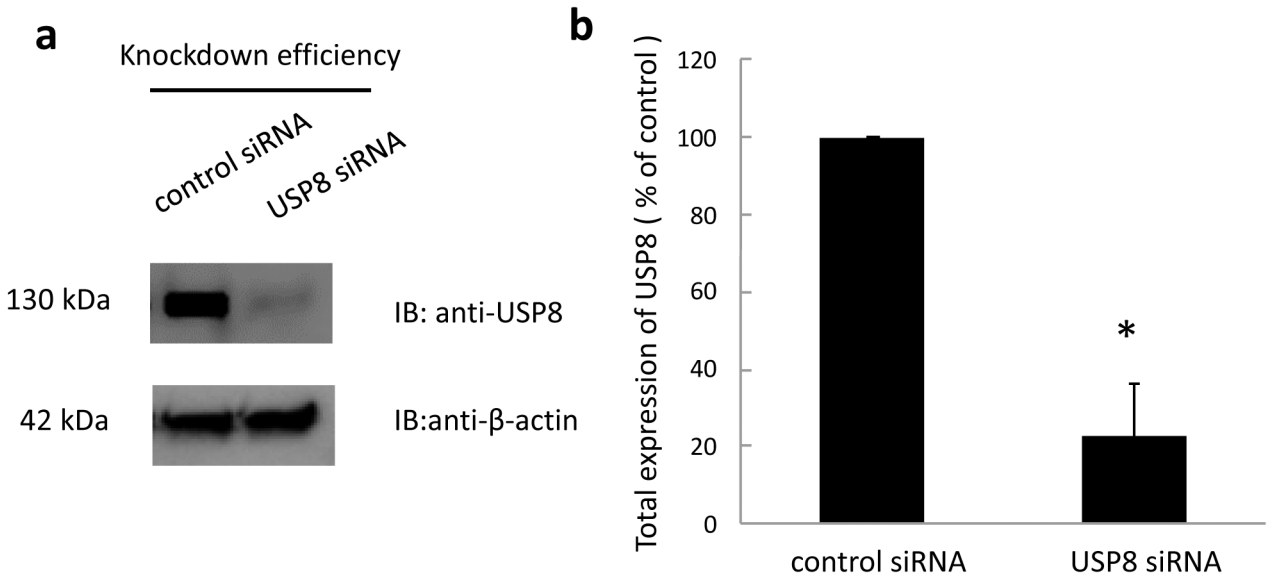

Fig. 5. Effect of USP8-specific siRNA on the expression of endogenous USP8.

(a). *Top panel:* hOAT1-expressing cells were co-transfected with scrambled siRNA (control siRNA) or with USP8-specific siRNA. The effectiveness of USP8-specific siRNA was tested by immunoblotting (IB) of the lysis sample with anti-USP8 antibody. *Bottom panel:* The same blot as the top panel was re-probed with anti-β-actin antibody. (b). Densitometry plot of results from Fig. 5a, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using Student's paired t-tests. The values are mean ± S.E. (n = 3). *P<0.05.

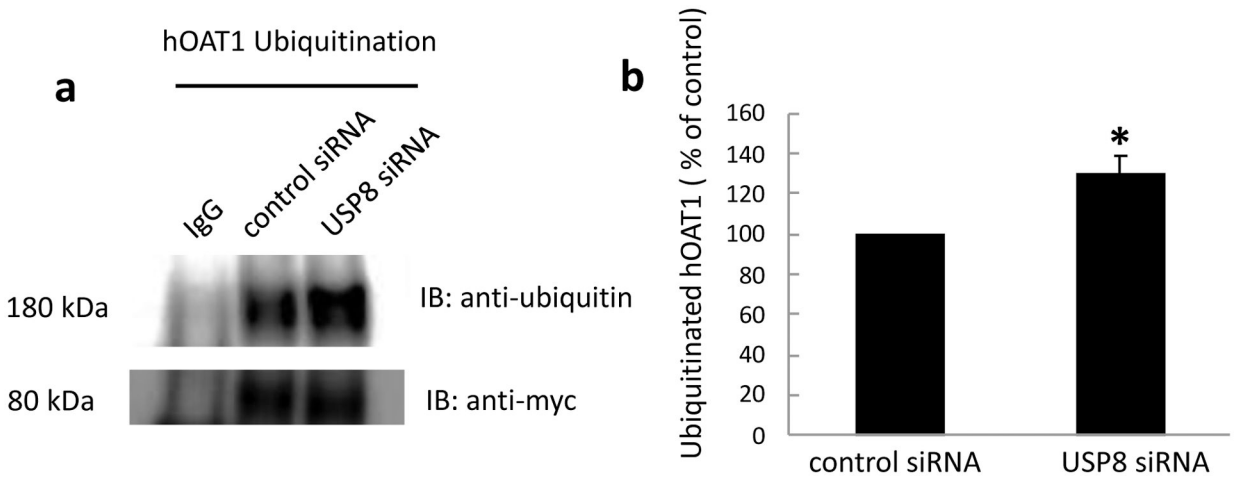

Fig. 6. Effect of USP8-specific siRNA on hOAT1 ubiquitination.

(a). *Top panel:* hOAT1-expressing cells were transfected with scrambled siRNA (control siRNA) or with USP8-specific siRNA. Cells were pre-treated with MG132 (10 μM, 1h), a proteasome inhibitor, to enhance the basal hOAT1 ubiquitination signal and then lysed. hOAT1 was immunoprecipitated by anti-myc antibody (epitope myc was tagged to hOAT1) or normal mouse IgG (as negative control), followed by immunoblotting (IB) with anti-ubiquitin antibody. *Bottom panel:* The same blot as the top panel was re-probed by anti-myc antibody. (b). Densitometry plot of results from Fig. 6a, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using Student's paired t-tests. The values are mean ± S.E. (n = 3). *P<0.05.

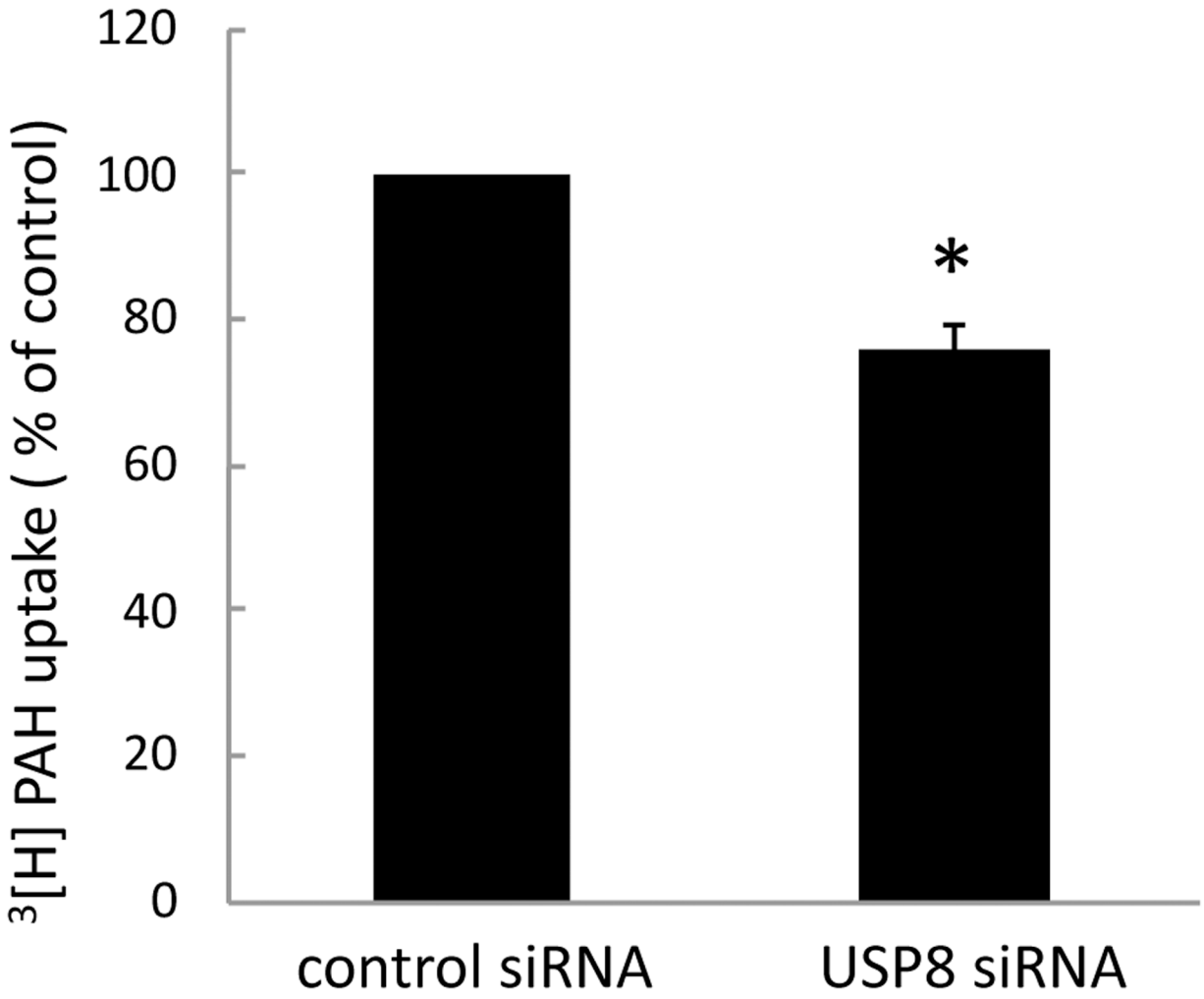

Fig. 7. Effect of USP8-specific siRNA on hOAT1 transport activity.

hOAT1-expressing cells were transfected with scrambled siRNA (control siRNA) or with USP8-specific siRNA. Transfected cells were then measured for the uptake of [^{3}H]-labeled PAH (4min, 20 μM). The data represent uptake into hOAT1-expressing cells minus uptake into mock cells (parental COS-7 cells). Uptake activity was expressed as a percentage of the uptake measured in control cells. Statistical analysis was performed using Student's paired t-tests. Values are mean ± S.E. (n = 3). *P < 0.05.

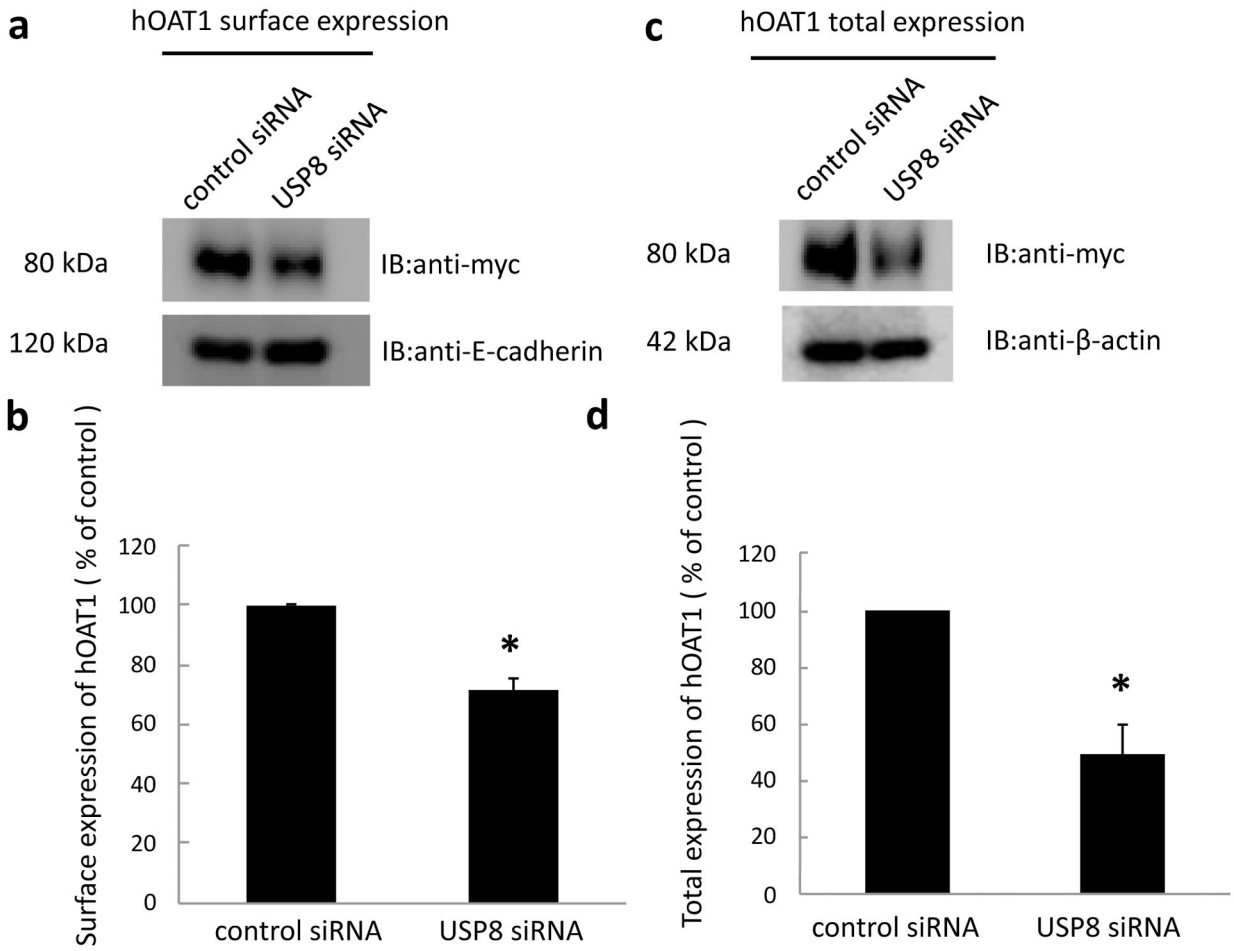

Fig. 8. Effect of USP8-specific siRNA on hOAT1 expression.

(a). *Top panel:* Cell surface expression of hOAT1. hOAT1-expressing cells were transfected with scrambled siRNA (control siRNA) or with USP8-specific siRNA. Then cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by immunoblotting (IB) with anti-myc antibody (epitope myc was tagged to hOAT1). *Bottom panel:* The same blot as the top panel was re-probed by anti-E-cadherin antibody. E-cadherin is a membrane protein marker. (b). Densitometry plot of results from Fig. 8a, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using Student's paired t-tests. The values are mean ± S.E. (n = 3). *P<0.05. (c). *Top panel:* Total cell expression of hOAT1. hOAT1-expressing cells were transfected with scrambled siRNA (control siRNA) or with USP8-specific siRNA. Transfected cells were lysed, and then proteins were separated by SDS-PAGE, followed by immunoblotting (IB) with anti-myc antibody. *Bottom panel:* The same blot as the top panel was re-probed by anti-β-actin antibody. β-actin is a cellular protein marker. (d). Densitometry plot of results from Fig. 8c, top panel, as well as from other experiments. The expression level was expressed as percentage of that of control. Statistical analysis was performed using Student's paired t-tests. Values are means ± SE (n = 3). *P<0.05.

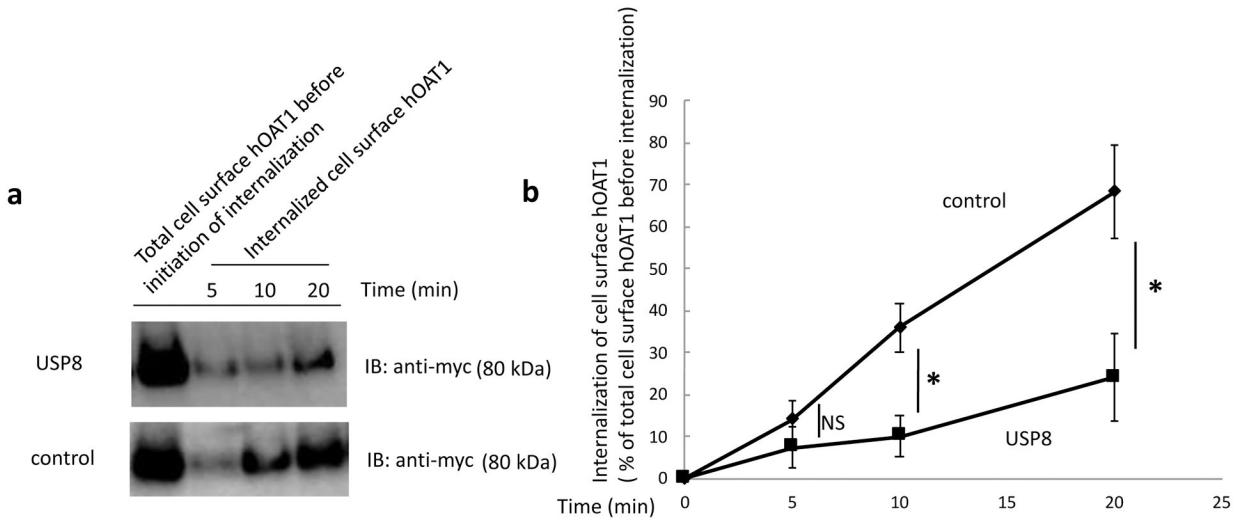

Fig. 9. Effect of USP8 on the rate of hOAT1 internalization.

(a). hOAT1 internalization (0, 5, 10 and 20 min) was analyzed as described under "Materials and Methods" with the transfection of USP8 wild type (top panel) or empty vector (bottom panel), followed by immunoblotting (IB) using anti-myc antibody (epitope myc was tagged to hOAT1). Empty vector-transfected cells were used as control. (b). Densitometry plot of results from Fig. 9a, as well as from other experiments. The expression level was expressed as percentage of total cell surface hOAT1 expression before internalization. Statistical analysis was performed using two-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). Values are mean ± S.E. ($n = 3$). *P<0.05.

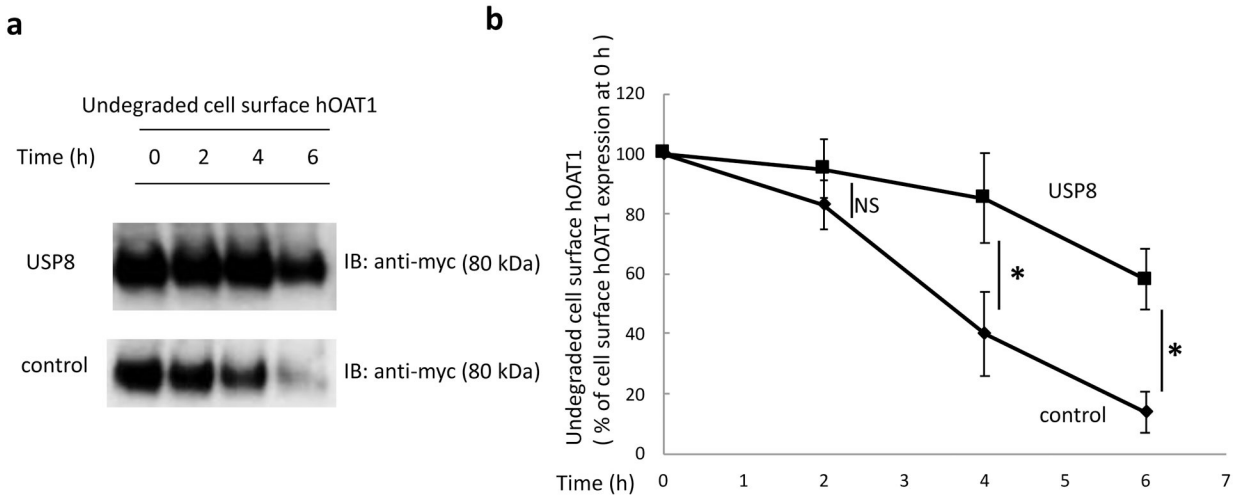

Fig. 10. Effect of USP8 on the rate of hOAT1 degradation.

(a). hOAT1 degradation (0, 2, 4 and 6 h) was analyzed as described under "Materials and Methods" with the transfection of USP8 wild type (top panel) or empty vector (bottom panel), followed by immunoblotting (IB) using anti-myc antibody (epitope myc was tagged to hOAT1). Empty vector-transfected cells were used as control. (b). Densitometry plot of results from Fig. 10a, as well as from other experiments. The expression level was expressed as percentage of cell surface hOAT1 expression at 0 h. Statistical analysis was performed using two-way ANOVA, post-hoc Tukey's test (GraphPad Software Inc., San Diego, CA). Values are mean ± S.E. (n = 3). *P<0.05.

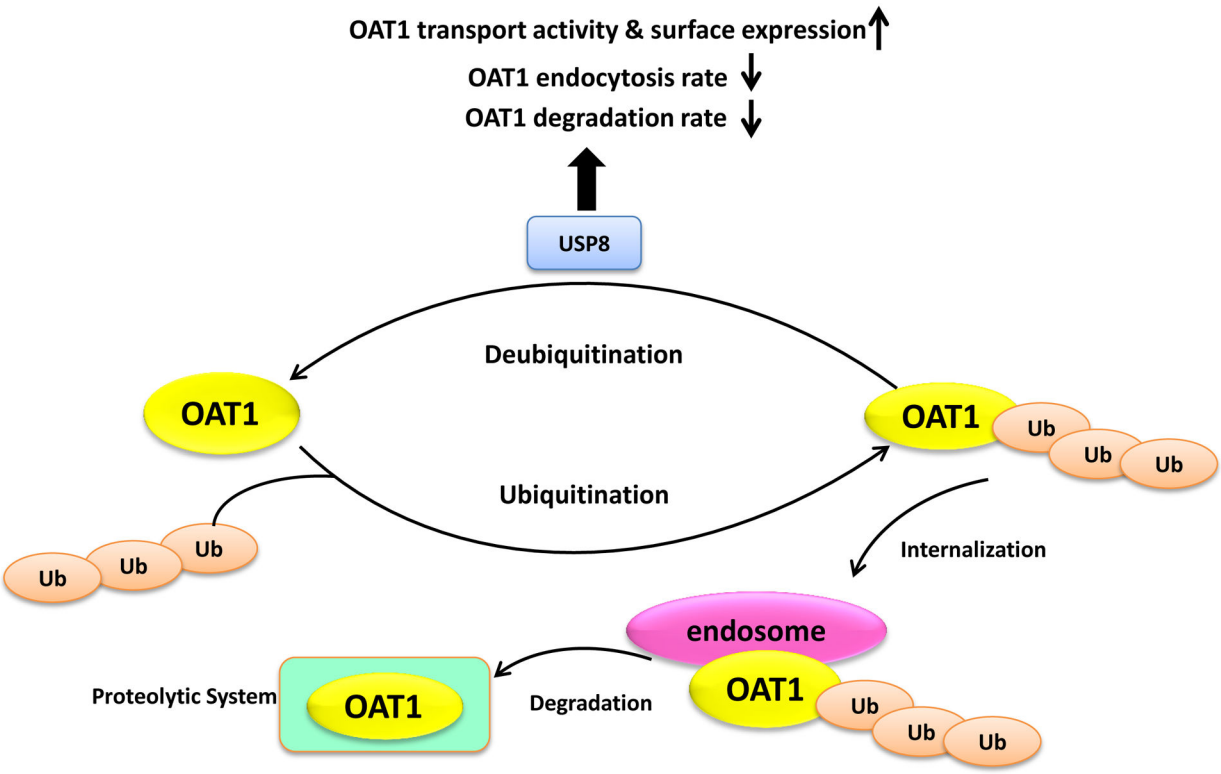

Fig. 11. The role of USP8 in the regulation of OAT1 ubiquitination, function and expression.
Ub: ubiquitin.