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TITLE PAGE

Three Ubiquitination Sites of Organic Anion Transporter-1 Synergistically Mediate Protein Kinase C-dependent Endocytosis of the Transporter

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Running Title: Regulation of OAT1 by ubiquitination

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Nonstandard abbreviations:

OAT – Organic anion transporter

Ub – Ubiquitin

PMA – Phorbol 12-myristate 13-acetate

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ABSTRACT

Organic anion transporter-1 (OAT1) mediates the body disposition of a diverse array of clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories. Therefore, understanding the regulation of OAT1 has profound clinical significance. We previously established that OAT1 constitutively internalizes from and recycles back to cell surface, and that activation of protein kinase C (PKC) inhibits OAT1 activity by promoting ubiquitination of the transporter, which then leads to an accelerated internalization of the transporter from cell surface to intracellular compartments. In the current study, we demonstrated that PKC isoform PKC-alpha was responsible for OAT1 ubiquitination. To directly address the role of OAT1 ubiquitination, we then generated two OAT1 mutants, each having multiple lysines (K) simultaneously mutated to arginine (R). One mutant K163/297/303/315/321R lost sensitivities to PKC-induced inhibition of transport activity, to PKC-induced ubiquitination, and to PKC-induced acceleration of transporter internalization. Further dissecting each lysine within this mutant, we identified Lys297, Lys303 and Lys315 being the ubiquitin-conjugation sites. Interestingly, mutating any one of the three lysines prevented the ubiquitin-conjugation to the other two lysines, suggesting that Lys297, Lys303 and Lys315 may form an optimal structure to interact with ubiquitination machineries. This is the first demonstration that Lys297, Lys303 and Lys315 play synergistic role in PKC-regulated OAT1 ubiquitination, trafficking and transport activity.

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INTRODUCTION

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs, including anti-HIV therapeutics, anti-tumor drugs, antibiotics, anti-hypertensives, and anti-inflammatories (You, 2002; Dantzler and Wright, 2003; Srimaroeng et al., 2008; Ahn and Nigam, 2009; VanWert et al., 2010). Therefore, understanding the regulation of these transporters has profound clinical significance.

Ten OATs (OAT1-10) have been cloned, and their expressions identified in distinct tissues and cell membranes. In the kidney, OAT1 and OAT3 utilize a tertiary transport mechanism to move organic anions across the basolateral membrane into the proximal tubule cells for subsequent exit across the apical membrane into the urine for elimination. Through this tertiary transport mechanism, Na^+/K^+-ATPase maintains an inwardly directed (blood-to-cell) Na^+ gradient. The Na^+ gradient then drives a sodium dicarboxylate cotransporter, sustaining an outwardly directed dicarboxylate gradient that is utilized by a dicarboxylate/organic anion exchanger, namely OAT, to move the organic anion substrate into the cell. This cascade of events indirectly links organic anion transport to metabolic energy and the Na^+ gradient, allowing the entry of a negatively charged substrate against both its chemical concentration gradient and the electrical potential of the cell (You, 2002; Dantzler and Wright, 2003; Srimaroeng et al., 2008; Ahn and Nigam, 2009; VanWert et al., 2010).

All of the cloned OATs share several common structural features including 12 transmembrane domains flanked by intracellular amino- and carboxyl termini; multiple glycosylation sites localized in the first extracellular loop and multiple potential phosphorylation sites. Investigation from our laboratory on the structure-function relationship of OATs revealed that glycosylation is necessary for the targeting of these transporters to the plasma membrane (Tanaka et al., 2004).

The amount of OATs at the cell surface is critical for their drug transport activity. We previously established that OAT1 constitutively internalizes from and recycles back to cell surface, and that activation of PKC inhibits OAT1 activity by promoting ubiquitination of the transporter,

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which then leads to an accelerated internalization of the transporter from cell surface to intracellular compartments (Zhang et al., 2008; Zhang et al., 2013).

Ubiquitin is a highly conserved 76-amino-acid protein that forms an isopeptide bond between its C-terminal glycine and a lysine (K) residue on the target protein. In the current study, we employed an integrated strategy of site-directed mutagenesis and biochemical/functional analyses to identify ubiquitin-accepting lysine residues on OAT1 and their roles in PKC-regulated OAT1 trafficking and transport activity.

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MATERIALS AND METHODS

Membrane impermeable biotinylation reagent NHS-SS-biotin and streptavidin agarose beads were purchased from Pierce (Rockford, IL). cDNA for HA-tagged wild type ubiquitin was generously provided by Dr. Cam Patterson, Carolina Cardiovascular Biology Center, University of North Carolina, Chapel Hill, North Carolina, USA. Mouse anti-myc antibody (9E10) and mouse anti-HA antibody 12CA5 were purchased from Roche Diagnostics Corporation (Indianapolis, IN). Mouse anti-ubiquitin antibody P4D1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PKC-alpha-specific inhibitor Gö6976 was from LC Laboratories (Woburn, MA). PKC activator Phorbol 12-myristate 13-acetate (PMA) and all other reagents were purchased from Sigma (St. Louis, MO).

Cell Culture and transfection – COS-7 cells were grown in DMEM supplemented with 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and glucose (100 mg/ml) in a 5% CO_2 atmosphere at 37°C. Confluent cells were transfected with DNA plasmids using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA).

Site-directed Mutagenesis – Mutant transporters were generated by site-directed mutagenesis using human OAT1-*myc* as a template. hOAT1-*myc* contains a 10-amino acid c-*myc* tag at the C terminus of hOAT1. Previous studies from our laboratory (Zhang et al., 2008) showed that the *myc*-tagged protein retained the functional properties of the native (unmodified) structure. The mutant sequence was confirmed by the dideoxy chain termination method.

Ubiquitination Assay – Cells transfected with wild type hOAT1 or its lysine mutants were treated with or without 1 μM PMA at 37 ˚C for 30 min. Treated cells were lysed with lysis buffer I (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 1 mM NaF)

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freshly added with 1% of proteinase inhibitor cocktail and 20 mM *N*-ethylmaleimide (NEM, deubiquitination inhibitor). OAT1 was then immunoprecipitated with anti-myc antibody, followed by immunoblotting with anti-ubiquitin antibody P4D1 or anti-HA antibody in the case when HA-tagged ubiquitin were transfected into the cells.

Cell Surface Biotinylation – Cell surface expression levels of hOAT1 and its mutants was examined using the membrane-impermeable biotinylation reagent, NHS-SS-biotin. Cells were plated in six-well plates. Each well of cells was incubated with 1 ml of NHS-SS-biotin (0.5 mg/ml in PBS) in two successive 20 min incubations on ice with very gentle shaking. The reagent was freshly prepared each time. After biotinylation, each well was briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine then incubated with the same solution for 30 min on ice, to ensure complete quenching of the unreacted NHS-SS-biotin. The cells were then lysed on ice for 1 h in 400 μl of RIPA lysis buffer (25 mM Tris-HCl, pH = 7.5, 150mM NaCl, 1 M EDTA, 0.1% SDS, 1% Triton-X 100, 1% sodium deoxycholate with 1/100 protease inhibitor cocktail). The cell lysates were cleared by centrifugation at 16,000 x g at 4^{o}C. 40 μl of streptavidin-agarose beads were then added to the supernatant to isolate cell membrane proteins. OAT1 was detected in the pool of surface proteins by immunoblotting using an anti-myc antibody 9E10.

Internalization Assay – We followed the procedure previously established in our lab (Zhang et al., 2008). Cells transfected with wild type hOAT1 and its lysine mutants underwent biotinylation with 0.5 mg/ml sulfo-NHS-SS-biotin at 4 °C. Following biotinylation, OAT1 internalization was initiated by incubating the cells (37 °C) in PBS containing either 1 μM PMA or vehicle 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

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(internalized) proteins were separated from nonbiotinylated proteins by streptavidin pull-down from equivalent amounts of cellular proteins, followed by immunoblotting with anti-myc antibody.

Transport Measurement – Cells were plated in 48-well plates. For each well, uptake solution was added. The uptake solution consisted of phosphate-buffered saline (PBS)/Ca^{2+}/Mg^{2+} (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] PAH. At the times indicated, uptake was stopped by aspirating off the uptake solution and rapidly washing the well with ice-cold PBS. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquotted for liquid scintillation counting. The uptake count was standardized by the amount of protein in each well. Values are means ± SE (n = 3).

Electrophoresis and Immunoblotting – Protein samples were resolved on 7.5% SDS-PAGE minigels and electroblotted on to PVDF membranes. The blots were blocked for 1 hour with 5% nonfat dry milk in PBS-0.05% Tween-20, washed, and incubated for 1 hour at room temperature with appropriate primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The signals were detected by SuperSignal West Dura Extended Duration Substrate kit (Pierce, Inc., Rockford, IL). Nonsaturating, immunoreactive protein bands were quantified by scanning densitometry with FluorChem 8000 imaging system (Alpha Innotech Corporation, San Leandro, CA).

Data Analysis – Each experiment was repeated a minimum of three times. The statistical analysis was from multiple experiments. Statistical analysis was performed using Student's paired t tests. A p value of <0.05 was considered significant.

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RESULTS

PKC-alpha is responsible for hOAT1 ubiquitination – We recently demonstrated that activation of PKC induces ubiquitination of hOAT1 (Zhang et al., 2013). Most cells express more than one PKC isoforms. Individual PKC isoform mediates distinct cellular processes in a cell type-dependent manner. PKC-alpha has been shown to be the PKC isoform for regulation of hOAT1 in response to angiotensin II (Li et al, 2009). To determine whether PKC-alpha is responsible for hOAT1 ubiquitination, we treated hOAT1-expressing cells with a general PKC activator PMA in the presence of either a general PKC inhibitor staurosporin or a PKC-alpha-specific inhibitor Go6976. hOAT1 was then immunoprecipitated followed by immunoblotting with anti-ubiquitin antibody. As shown in Fig. 1, both staurosporin (St) and Go6976 completely prevented PMA-stimulated hOAT1 ubiquitination, suggesting that PKC-alpha is mainly responsible for hOAT1 ubiquitination and other PKC isoforms may not play significant roles in this process.

Site-directed mutagenesis of intracellular lysine residues of hOAT1 – Ubiquitination occurs through the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine residue on the target protein. hOAT1 has 10 intracellular lysine residues (Fig. 2). To identify which lysine(s) serves as acceptor(s) for hOAT1 ubiquitination, we initially generated two lysine mutants by simultaneously replacing multiple lysine residues (K) with arginine (R) using site-directed mutagenesis approach. The mutant K523/525/535/547R had all the lysine residues at the carboxyl terminus mutated, whereas the mutant K163/297/303/315/321R had the rest of the lysine residues mutated. Lys382, a residue conserved across species, was not included in the mutants because it was previously shown that mutation of this residue to arginine resulted in complete loss of transport function of rat OAT3 and flounder OAT1 (Burckhardt et al., 2002; Feng et al, 2001). Mutant transporters were analyzed for their ability to transport PAH, a prototypical substrate of hOAT1. As shown in Fig. 3, both mutants retained transport activities and the levels of expression comparable to that of wild type transporter.

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Sensitivity of mutant transporters to PKC-induced inhibition of transport activity – We recently showed that activation of PKC inhibited hOAT1 activity by enhancing hOAT1 ubiquitination, which led to an acceleration of hOAT1 internalization from cell surface to intracellular compartments (Zhang et al., 2008; Zhang et al., 2013). To examine the sensitivity of mutant transporters to PKC-induced inhibition of transport activity, cDNAs for wild type hOAT1 and its mutants were individually transfected into the cells. Transfected cells were treated with or without PKC activator PMA (30 min), followed by measurement of PAH transport. As shown in Fig. 4, mutant K523/525/535/547R was equally sensitive to PKC-induced inhibition of transport activity as compared to that of the wild type transporter, whereas mutant K163/297/303/315/321R was insensitive to PKC-induced inhibition.

Sensitivity of mutant transporters to PKC-induced ubiquitination – We next examined whether mutants K523/525/535/547R and K163/297/303/315/321R were subjected to ubiquitination in response to PKC activation as that of wild type transporter. cDNAs for wild type hOAT1 and the mutants were transfected into the cells individually. Transfected cells were treated with or without PKC activator PMA. Treated cells were lyzed and hOAT1 was immunoprecipitated, followed by immunoblotting with anti-ubiquitin antibody. A shown in Fig. 5, PMA treatment resulted in significant increase in the ubiquitination of wild type hOAT1 and mutant K523/525/535/547R, whereas mutant K163/297/303/315/321R was insensitive to PMA-induced ubiquitination, suggesting that mutant K163/297/303/315/321R serves as an acceptor for PKC-induced ubiquitination of the transporter.

Sensitivity of mutant K163/297/303/315/321R to PKC-induced decrease in surface expression – cDNAs for wild type hOAT1 and mutant *K163/297/303/315/321R* were transfected into the cells individually. Transfected cells were treated with or without PMA (30 min), followed by cell surface biotinylation to isolate cell surface hOAT1. As shown in Fig. 6, Activation of PKC by PMA resulted in a reduction in cell surface expression of the wild type hOAT1 without affecting its total expression. In contrast, PMA had no effect on the surface expression of the mutant transporter.

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Sensitivity of mutant K163/297/303/315/321R to PKC-induced acceleration of transporter internalization – PKC-induced acceleration of transporter internalization was subsequently examined in both wild type transporter- and mutant-transfected cells. As shown in Fig. 7, PMA treatment (30 min) of cells resulted in an acceleration of wild type hOAT1 internalization, whereas no significance difference in the rate of mutant internalization was observed between PMA-treated and control cells.

Identification of single lysine residue(s) responsible for PKC-induced ubiquitination – The results above indicate that one or more lysine residue(s) within K163/297/303/315/321R is responsible for PKC-induced ubiquitination. To identify such residues, the five lysine residues were individually mutated into arginine: K163R, K297R, K303R, K315R, and K321R. PKC-induced ubiquitination of wild type hOAT1 and its mutants was subsequently examined. As shown in Fig. 8, PMA treatment resulted in significant increase in the ubiquitination of wild type hOAT1, and mutants K163R and K321R, whereas mutants K297R, K303R and K315R were insensitive to PMA-induced ubiquitination.

To further confirm our observation, we next examined the incorporation of ectopically expressed ubiquitin into wild type hOAT1 and its mutants. Cells were transfected with wild type hOAT1, K163R, K297R, K303R, K315R, and K321R individually together with HA-tagged ubiquitin. Transfected cells were treated with PMA. As shown in Fig. 9, HA-tagged ubiquitin significantly incorporated into wild type hOAT1, K163R, and K321R in response to PMA treatment, whereas no such incorporation was observed into mutants K297R, K303R and K315R.

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DISCUSSION

The organic anion transporter (OAT) family mediates the body disposition of a diverse array of environmental toxins, and clinically important drugs. Therefore, understanding the regulation of these transporters has profound clinical significance. Short-term (acute) regulation of OAT activity is particularly important when body has to deal with rapidly changing amounts of substances as a consequence of variable intake of drug, fluid, and meal as well as metabolic activity.

The amount of OATs at the cell surface is critical for their drug transport activity. We previously established that OAT1 constitutively internalizes from and recycles back to cell surface. The transporter in a dynamic rather than a static state is more primed for the input to initiate trafficking, therefore is capable of providing quick and efficient fine-tuning in body response to environmental changes. We further established that activation of PKC inhibits OAT1 activity by promoting ubiquitination of the transporter, which then leads to an accelerated internalization of the transporter from cell surface to intracellular compartments (Zhang et al., 2008; Zhang et al., 2013).

Modification of receptors and channels by ubiquitin conjugation has recently emerged as the major regulatory mechanism of internalization, intracellular sorting, and turnover of these membrane proteins (Varghese et al., 2008; Kumar et al., 2007; Bomberger et al., 2009; Zhou et al., 2007; Miranda et al., 2005). Ubiquitin moiety can be recognized by the components of plasma membrane internalization and endosomal sorting machinery. In the current study, we provide the first demonstration that three ubiquitination sites of OAT1 synergistically mediate PKC-dependent endocytosis of the transporter.

We choose COS-7 cells for our study because these cells offer many useful advantages. First, these cells are kidney origin and have been very useful in understanding other renal transport processes (Zhang et al., 2002; Nagai et al., 2006). Secondly, these cells possess endogenous PKC and PKA signaling pathways and provide a good experimental model system for studying the regulatory mechanisms underlying many transport processes (Kazanietz et al., 2001; Cobb et al., 2002) Lastly, the transport characteristics of OAT1 in these cells were in a good agreement with

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that observed in other systems (Zhang et al., 2008, Miller et al., 1998; Shuprisha et al., 2000).

Demonstration of the individual ubiquitination site through site-directed mutagenesis can be technically difficult due to the redundancy of ubiquitination sites, which requires multiple lysines be mutated. Yet, multiple mutations often lead to nonfunctional proteins. However, in our case, mutation of multiple lysines resulted in fully functional mutants, which delivered fully to the cell surface (Fig. 3).

Generation of OAT1 mutant K163/297/303/315/321R with minimal ubiquitination allows us to establish the strong correlation between PKC-dependent endocytosis and OAT1 ubiquitination. We previously demonstrated that activation of PKC inhibits OAT1-mediated drug transport by reducing OAT1 cell surface expression through accelerating its internalization from cell surface to intracellular compartments without affecting their recycling (Zhang et al., 2008). The insensitivities of K163/297/303/315/321R to PKC-induced inhibition in transport activity (Fig. 4), to PKC-induced reduction in cell surface expression (Fig. 6) and to PKC-induced acceleration of internalization (Fig. 7) all suggest that OAT1 ubiquitination is required for the PKC-dependent OAT1 trafficking and function.

The strong evidence on the involvement of Lys297, Lys303 and Lys315 in the PKC-dependent OAT1 ubiquitination came from our studies measuring the incorporation of both endogenously expressed and ectopically expressed ubiquitin into wild type hOAT1 and its mutants. Interestingly, mutating any one of the three lysine residues prevented the ubiquitin-conjugation to the other two lysine residues. These three lysine residues are clustered in the large intracellular loop between transmembrane domains 6 and 7, suggesting that Lys297, Lys303 and Lys315 may form an optimal structure for the interaction with ubiquitination machineries.

In conclusion, ubiquitination has been shown to regulate internalization, post-internalization sorting and degradation of other membrane proteins. Our current investigation focuses on the first step: the internalization step. The major finding from our studies is that Lys297, Lys303 and Lys315 play synergistic role in PKC-regulated OAT1 ubiquitination, trafficking and transport activity.

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To the best of our knowledge, this report is the first mechanistic study to demonstrate a site-specific role of ubiquitination in the regulation of any drug transporters. The physiological significance of our studies can be implied with bilateral ureteral obstruction (BUO). BUO is a serious and common clinical condition, and an important cause of acute renal failure (Seldin and Giebisch, 2000; Villar et al., 2005). It is shown (Villar et al., 2005) that in BUO rats, elimination of drugs was impaired partly due to a redistribution of OAT1 from cell surface to intracellular compartment. In BUO, angiotensin II has elevated level of expression (Seldin and Giebisch, 2000; Klahr et al., 1998; Klahr and Morrissey, 2002). We recently reported that angiotensin II inhibits OAT1 activity through activation of PKC in cultured cells (Li et al., 2009). Therefore, Ang II may, via activation of PKC, enhance OAT1 ubiquitination, leading to an internalization of OAT1 from cell surface. Our current studies may provide important insight into the molecular, cellular, and clinical bases underlying BUO.

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Authorship Contributions

Participated in research design: Zhang, Li and You

Conducted experiments: Li, and Zhang

Performed data analysis: Li, Zhang, and You

Wrote or contributed to the writing of the manuscript: You

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1. PKC-alpha-regulated hOAT1 ubiquitination. hOAT1-expressing COS-7 cells were pre-treated with a general PKC inhibitor staurosporin (St, 2μM, 5 min) or PKC-alpha-specific inhibitor Go6976 (1μM, 20 min), and then treated with PKC activator PMA (1 μM) in the presence of staurosporin (St, 2μM) or Go6976 (1μM) for 30 min. Treated cells were then lysed and OAT1 was immunoprecipitated with anti-myc antibody, followed by immunoblotting (Ib) with anti-ubiquitin antibody. This is a representative blot from two experiments.

Fig. 2. Predicted transmembrane topology of hOAT1. Twelve transmembrane domains are numbered from *1 to 12.* Potential glycosylation sites are denoted by tree-like structures. Intracellular lysine residues are indicated by • and numbered.

Fig. 3. Characterization of hOAT1 wild type (Wt) and its lysine mutants. A. Transport activity. cDNAs for hOAT1 Wt and its lysine mutants were transfected into COS-7 cells. Transport of ^{3}H-PAH (20 μM, 3 min) in transfected cells was then measured. Uptake activity was expressed as % of the uptake measured in Wt. The results represent data from three experiments, with triplicate measurements. The uptake values in mock cells (parental COS-7 cells) were subtracted. B. Cell surface and total expression. *Top panel*: *Cell surface expression.* Cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). *Bottom panel: Total cell expression.* Cells were lysed, and their proteins were separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). C. Densitometry plot of results from Fig. 3B, *Top panel*, as well as from other experiments. The expression level was expressed as % of that of Wt. Values are mean ± S.E. (*n* = 3).

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Fig. 4. Effect of PMA on transport activities of hOAT1 wild type (Wt) and its lysine mutants. cDNAs for hOAT1 Wt and its lysine mutants were transfected into COS-7 cells. Transfected cells were treated with PKC activator PMA (1 μM, 30 min), followed by measurement of ^{3}H-PAH transport (20 μM, 3 min). Uptake activity was expressed as % of the uptake measured in non-treated cells. The uptake values in mock cells (parental COS-7 cells) were subtracted. The results represent data from three experiments, with triplicate measurements. *P < 0.05.

Fig. 5. Effect of PMA on ubiquitination of hOAT1 wild type (Wt) and its lysine mutants. cDNAs for hOAT1 Wt and its lysine mutants were transfected into COS-7 cells. Transfected cells were treated with PKC activator PMA (1 μM, 30 min). OAT1 was then immunoprecipitated by anti-myc antibody, followed by immunoblotting (Ib) with anti-ubiquitin antibody (anti-Ub). This is a representative blot from two experiments.

Fig. 6. Effect of PMA on cell surface and total expression of hOAT1 wild type (Wt) and its lysine mutant K163/297/303/315/321R. A. *Top panel*: *Cell surface expression.* cDNAs for hOAT1 Wt and its lysine mutant were transfected into COS-7 cells. Transfected cells were treated with PKC activator PMA (1 μM, 30 min). Treated cells were biotinylated, and the labeled cell surface proteins were precipitated with streptavidin beads, separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). *Bottom panel: Total cell expression.* Cells were lysed, and their proteins were separated by SDS-PAGE, followed by immunoblotting with anti-myc antibody (1:100). B. Densitometry plot of results from Fig. 6A as well as from other experiments. Surface OAT1 in PMA-treated cells was expressed as % of surface OAT1 in control cells. Values are mean ± S.E. (n = 3). *P < 0.05.

Fig. 7. Effect of PMA on the internalization of hOAT1 wild type (Wt) and its lysine mutant. A. Internalization of hOAT1 Wt. cDNA for hOAT1 Wt was transfected into COS-7 cells. Internalization

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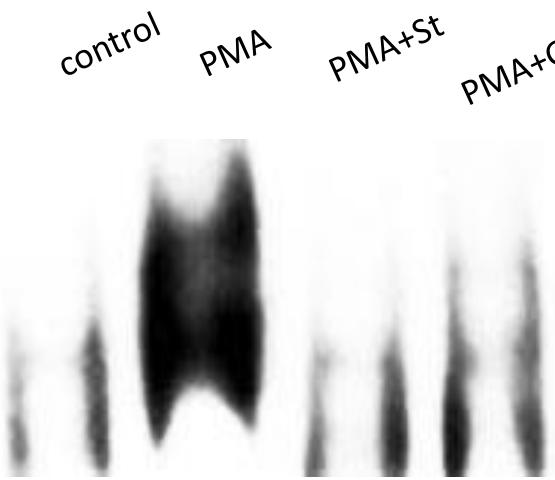
(5 min and 10 min) was analyzed as described in “Materials and Methods” section in the presence and the absence of PMA followed by immunoblotting using anti-myc antibody. This is a representative blot from three experiments. B. Densitometry plot of results from Fig. 7A as well as from other experiments. Internalized OAT1 was expressed as % of total cell surface OAT1 pool before initiation of internalization. Values are mean ± S.E. (n = 3). The significant difference between PMA-treated value and non-treated value at the same time point was indicated by * (*P < 0.05). C. Internalization of hOAT1 lysine mutant. cDNA for hOAT1 lysine mutant was transfected into COS-7 cells. Internalization (5 min and 10 min) was analyzed in the presence and the absence of PMA followed by immunoblotting using anti-myc antibody. This is a representative blot from three experiments. D. Densitometry plot of results from Fig. 7C, as well as from other experiments. Internalized OAT1 mutant was expressed as % of total cell surface OAT1 mutant pool before initiation of internalization. Values are mean ± S.E. (n = 3).

Fig. 8. Effect of PMA on the incorporation of endogenous ubiquitin into hOAT1 wild type (Wt) and its lysine mutants. cDNAs for hOAT1 Wt and its lysine mutants were transfected into COS-7 cells respectively, followed by treatment with or without PMA (1 μM, 30 min). Treated cells were lysed. OAT1 was immunoprecipitated by anti-myc antibody, followed by immunoblotting with anti-ubiquitin antibody. This is a representative blot from three experiments.

Fig. 9. Effect of PMA on the incorporation of ectopically expressed ubiquitin into hOAT1 wild type (Wt) and its lysine mutants. cDNAs for hOAT1 Wt and its lysine mutants were co-transfected with HA-ubiquitin into COS-7 cells respectively, followed by treatment with or without PMA (1 μM, 30 min). Treated cells were lysed. OAT1 was immunoprecipitated by anti-myc antibody, followed by immunoblotting with anti-HA antibody. This is a representative blot from three experiments.

Fig. 1



Ib: anti-Dvl2b

Fig. 2

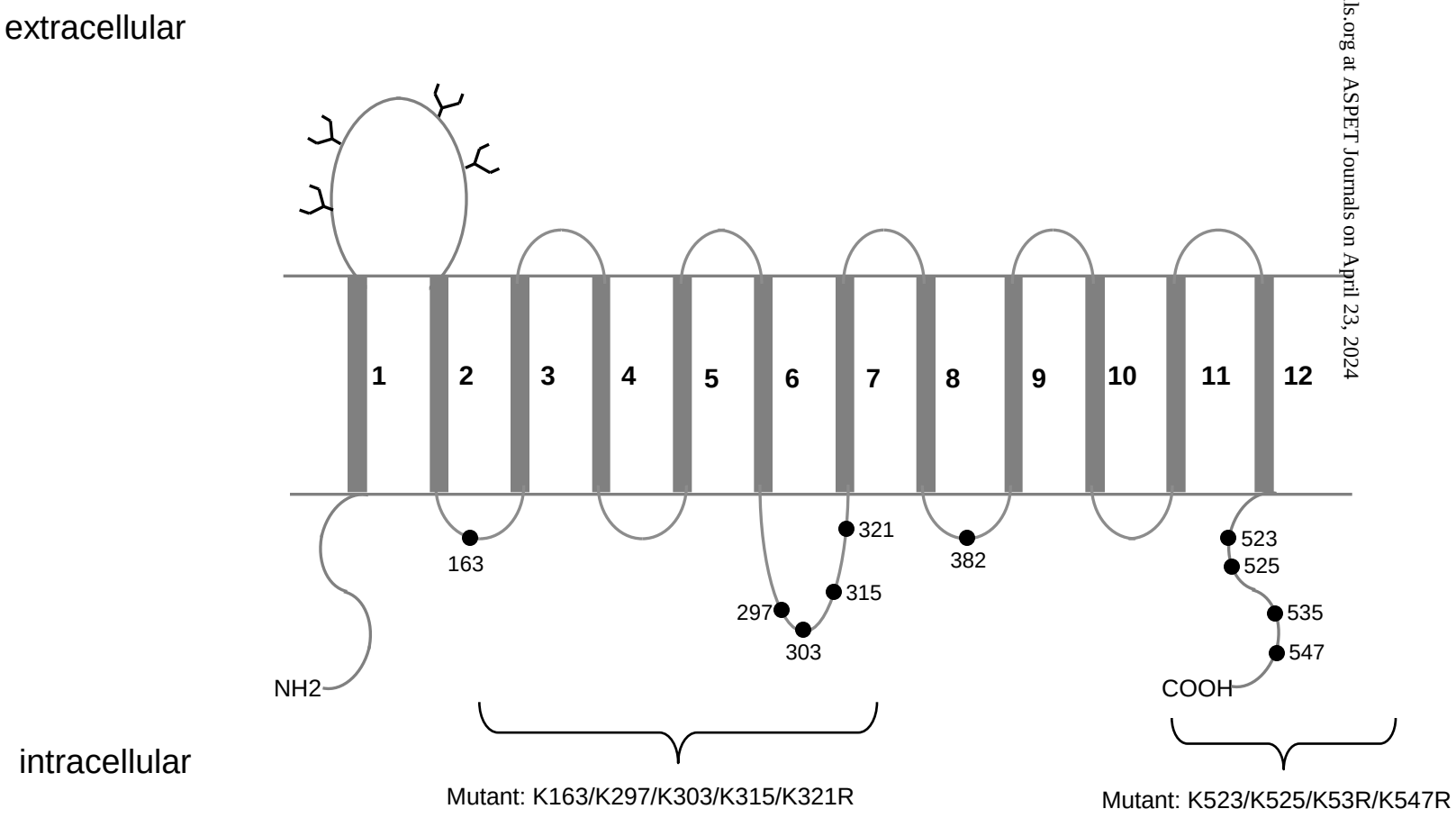
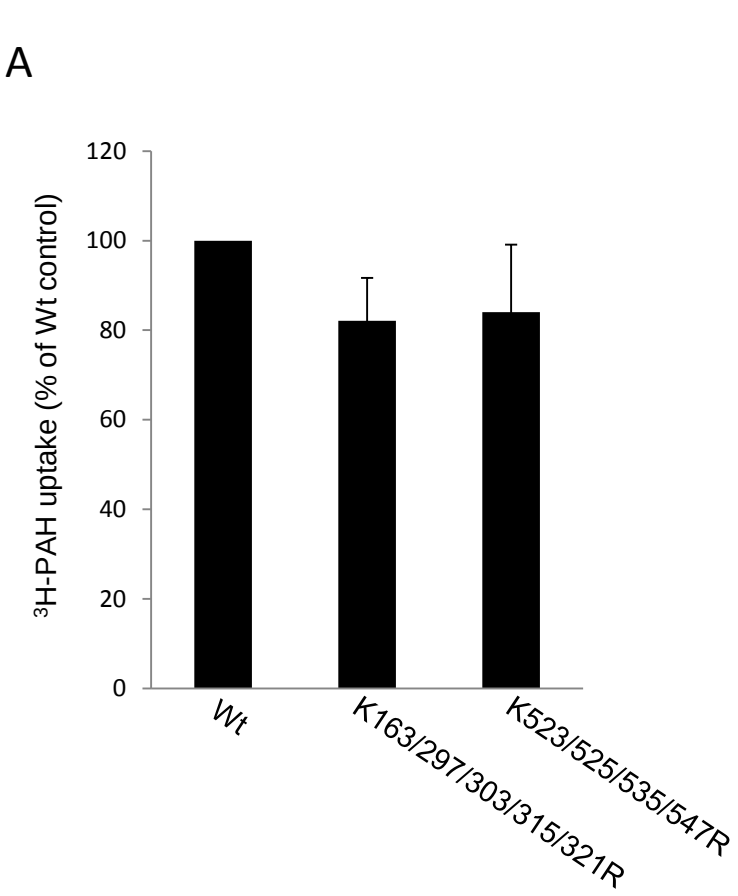


Fig. 3



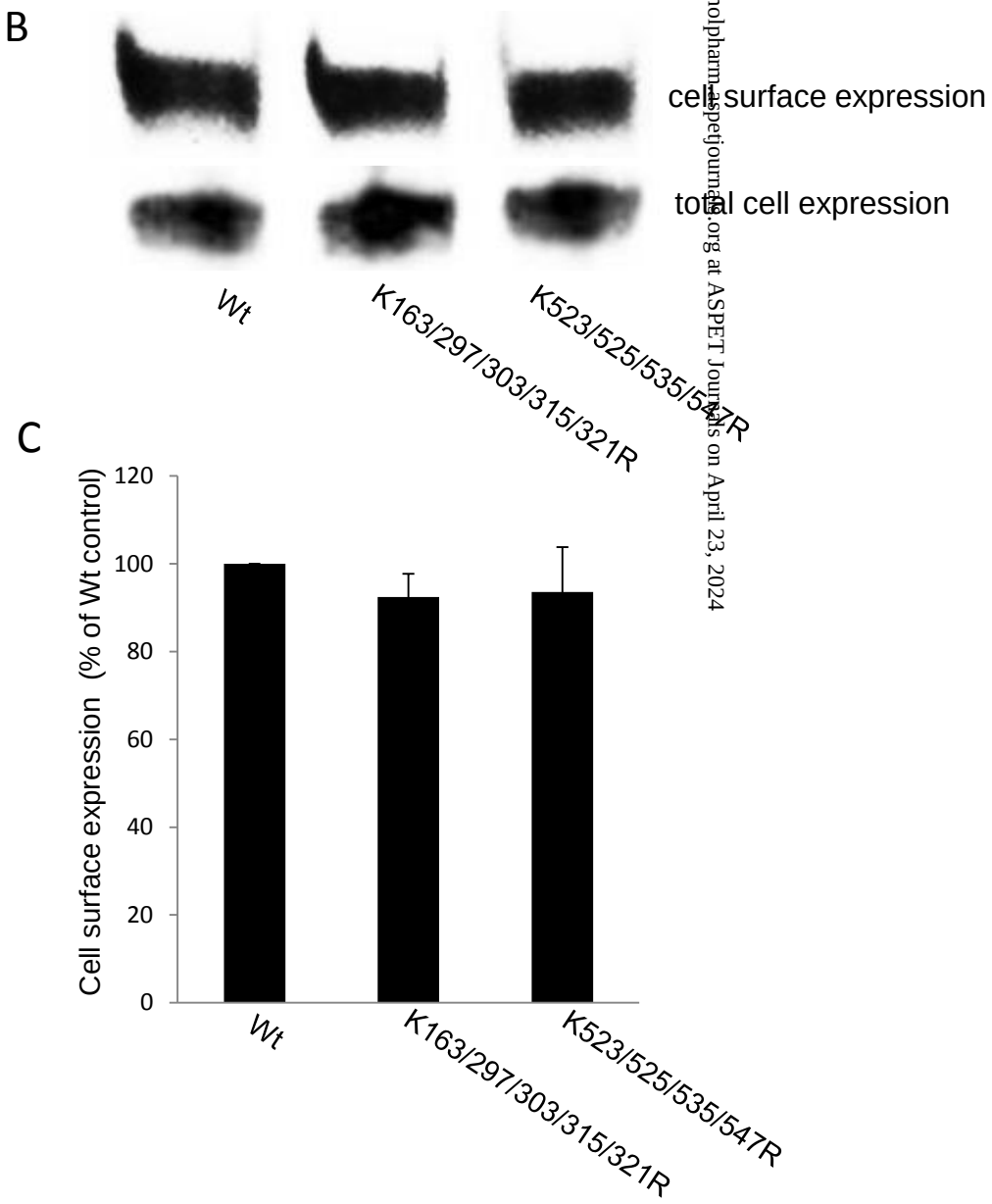


Fig. 4

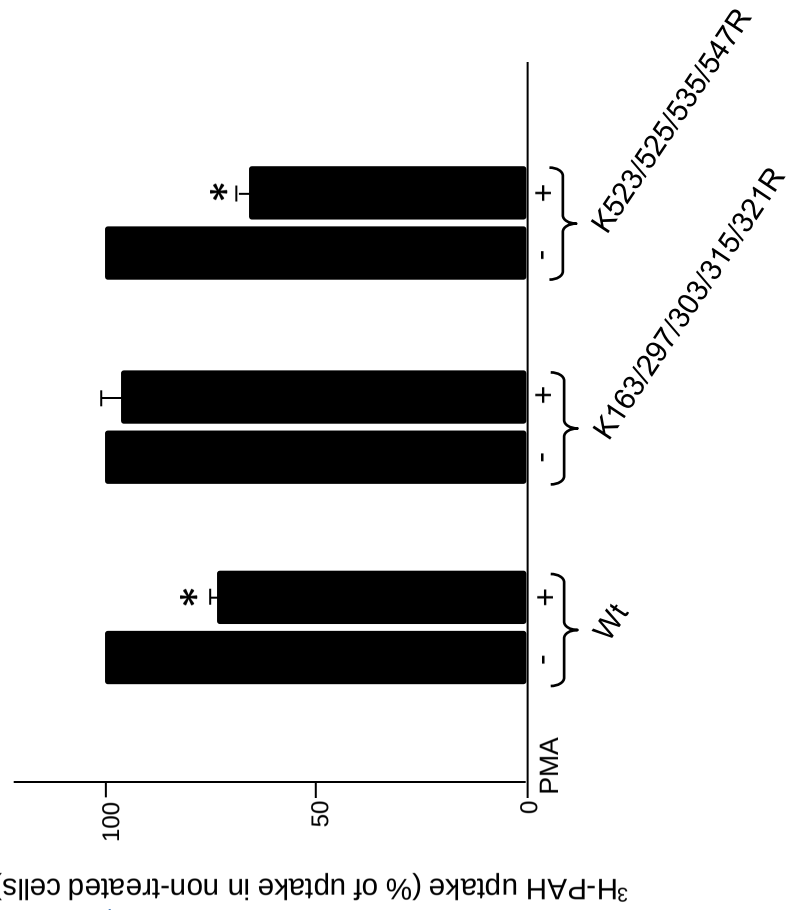
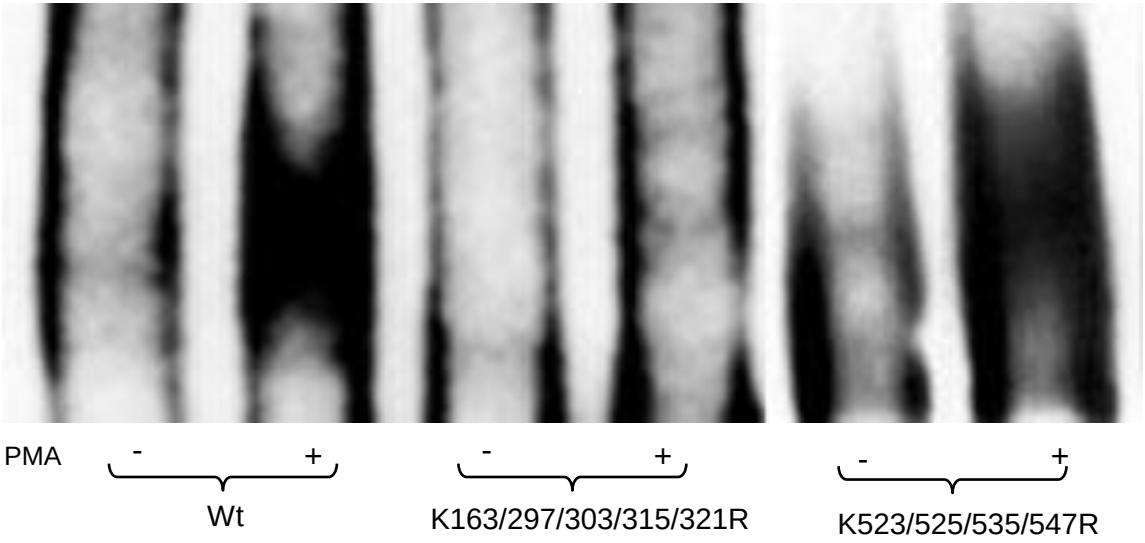
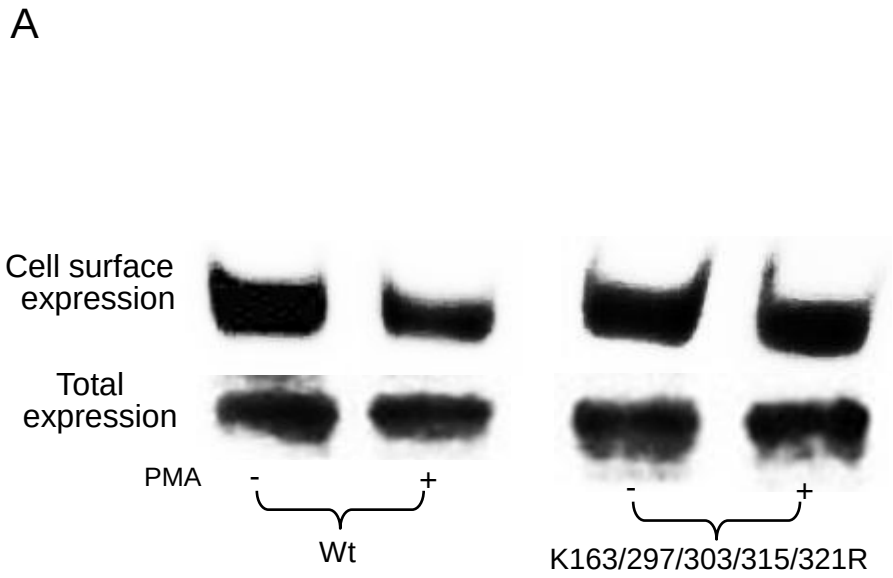


Fig. 5



Ib: anti-Ub

Fig. 6



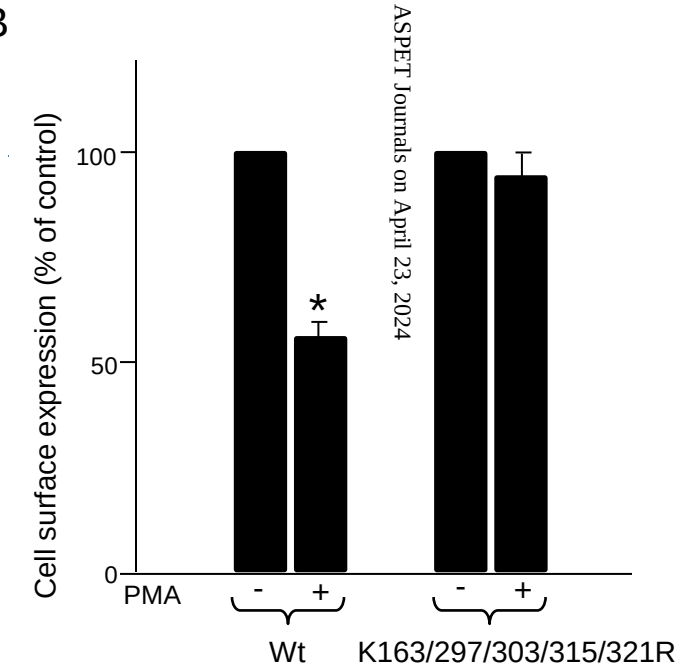


Fig. 7

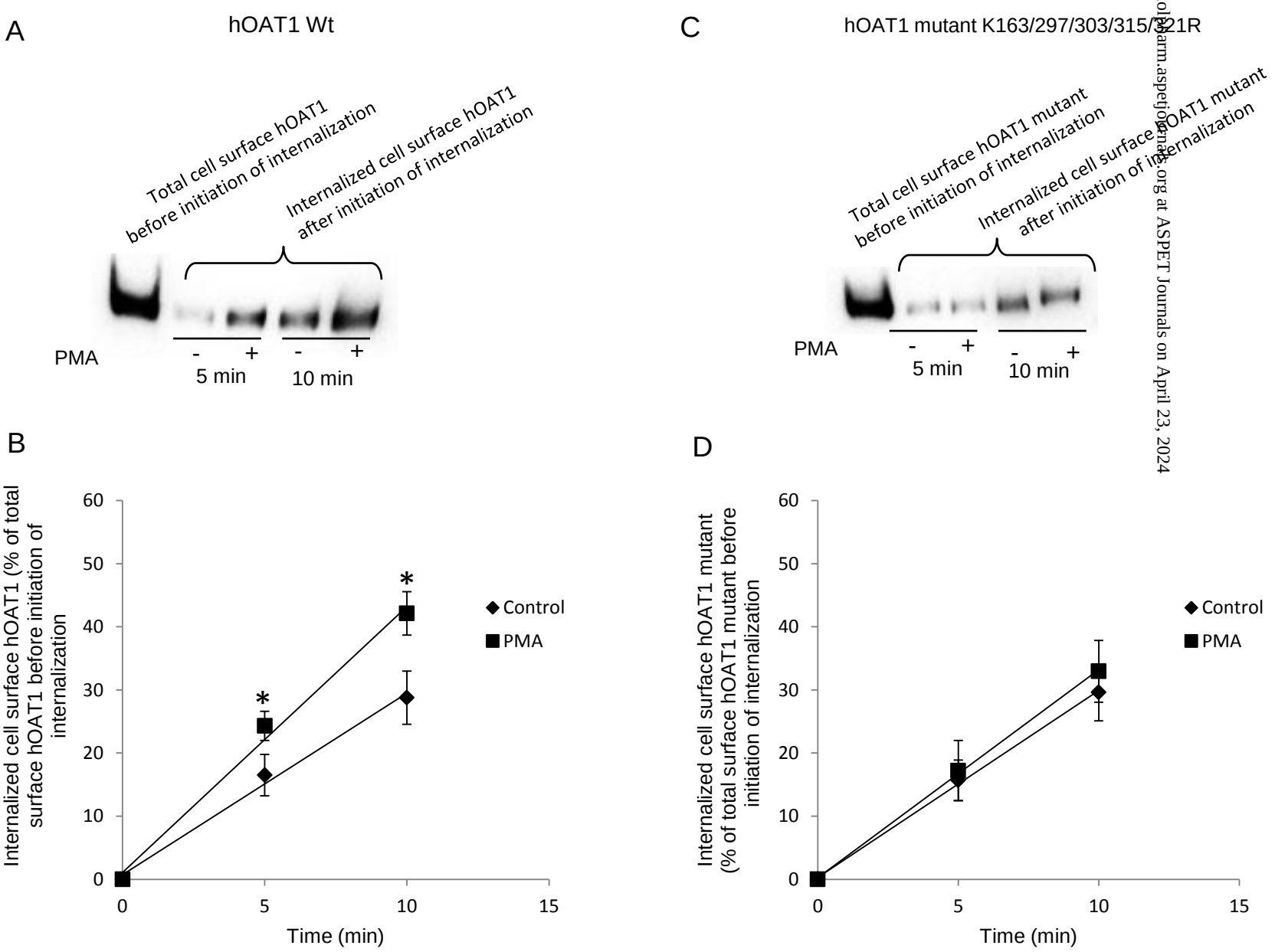


Fig. 8

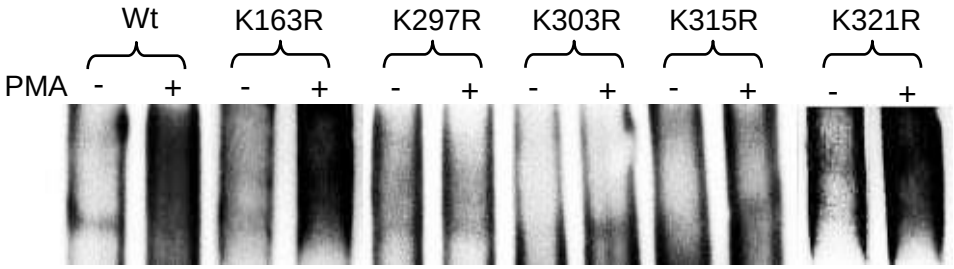


Fig. 9