PKC/Nedd4-2 Signaling Pathway Regulates the Cell Surface Expression of Drug Transporter hOAT1

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Running Title: PKC/Nedd4-2 Signaling Pathway Regulates hOAT1 Activity

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Non-standard Abbreviations:

Nedd4-2 – Neural precursor cell Expressed, developmentally down-regulated 4-2

PMA – phorbol 12-myristate 13-acetate

BUO – bilateral ureteral obstruction
ABSTRACT

Human organic anion transporter-1 (hOAT1) regulates the absorption, distribution, and excretion of a wide range of clinically important drugs. Our previous work demonstrated that hOAT1 is a dynamic membrane transporter, constitutively internalizing from and recycling back to cell plasma membrane. Short-term activation (<30 min) of protein kinase C (PKC) promotes the attachment of a lysine 48-linked polyubiquitin chain to hOAT1, a process catalyzed by ubiquitin ligase Nedd4-2. The Ubiquitination of hOAT1 then triggers an accelerated endocytosis of the transporter from plasma membrane, which results in a reduced hOAT1 expression at the cell surface and a decreased hOAT1 transport activity. In the present study, we investigated the long-term effect of PKC on hOAT1. We showed that long-term activation (>2 hrs.) of PKC significantly enhanced hOAT1 degradation, and such action was partially blocked by a ubiquitin mutant Ub-K48R, which has its lysine (K) 48 mutated to arginine (R) and is incapable of forming K48-linked polyubiquitin chain. The ubiquitin ligase Nedd4-2 was also found to augment hOAT1 degradation. These results suggest that PKC-regulated and Nedd4-2-catalyzed attachment of a lysine 48-linked polyubiquitin chain to hOAT1 is important for hOAT1 stability. We further showed through co-immunoprecipitation experiments that there was a direct association between hOAT1 and Nedd4-2, and such interaction was weakened when the WW3 and WW4 domains of the ligase were mutated. Mutating WW3 and WW4 domains of the ligase also impaired its ability to ubiquitinate hOAT1. Therefore, WW3 and WW4 domains of Nedd4-2 are critical for its association with and modulation of the transporter.
INTRODUCTION

Human organic anion transporter-1 (hOAT1) is localized at the basolateral membrane of the renal proximal tubule cells. It regulates the excretion of a wide range of environmental toxins, and clinical drugs, including anti-cancer drugs, anti-viral agents, diuretics, antibiotics, anti-hypertension drugs, and anti-inflammatories (You, 2002; Terada and Inui, 2007; Ahn and Nigam, 2009; Pelis and Wright, 2011; Wang and Sweet, 2013). As a cell membrane transporter, the transport activity of hOAT1 is critically dependent on its expression level at the plasma membrane. Our previous work demonstrated (Zhang et al., 2008) that hOAT1 is a dynamic membrane transporter, constitutively internalizing from and recycling back to cell surface. Short-term activation (<30 min) of PKC promotes the attachment of a lysine 48-linked polyubiquitin chain to hOAT1, a process catalyzed by ubiquitin ligase Nedd4-2 (Zhang et al., 2012; Xu et al., 2016b). The Ubiquitination of hOAT1 then triggers an accelerated endocytosis of the transporter from plasma membrane to intracellular endosomes, which results in a decreased hOAT1 expression at the plasma membrane and a decreased hOAT1 transport activity.

Recently, the post-translational modification by ubiquitin conjugation has become the major mechanism for regulating several membrane proteins in their internalization, intracellular sorting, and turnover rate (Staub and Rotin, 2006; Miranda and Sorkin, 2007). Ubiquitin, a highly conserved 8-kDa protein, forms a peptide bond between its glycine and lysine residue of the target protein. The ubiquitin conjugation can be either a single ubiquitin or a chain of ubiquitin proteins. The formation of a polyubiquitin chain occurs through the seven lysine residues of ubiquitin itself including K6, K11, K27, K29, K33, K48 and K63. Therefore, the ubiquitin conjugation can be classified as three types: monoubiquitination (attachment of one ubiquitin to one lysine on the target protein), multiubiquitination...
(attachment of several ubiquitin to several lysine on the target protein), or polyubiquitination (attachment of polyubiquitin chain(s) to one or more lysine on the target protein). Through mass spectrometry analysis, our lab recently demonstrated that PKC-promoted conjugation of ubiquitin to hOAT1 is the attachment of a lysine-48-linked polyubiquitin chain to the transporter (Zhang et al., 2012).

Nedd4-2, a member of Nedd4 family of HECT ubiquitin ligases, catalyzes the ubiquitination of various mammalian transporters and channels (Snyder et al., 2004; Sorkina et al., 2006; Vina-Vilaseca and Sorkin, 2010; Garcia-Tardon et al., 2012). Structurally, Nedd4 family proteins contain a catalytic HECT domain, C2 (Ca$^{2+}$/lipid binding) domain, and 2-4 WW domains. HECT domain at the C-terminus contains ubiquitin ligase activity. WW domains are involved in recognition and interaction with target proteins, while C2 domain carries the membrane targeting function.

Despite the significant progress made from our lab in understanding the short-term regulation of hOAT1 by PKC. The long-term effect of PKC on hOAT1 has not been explored. In the current study, we present evidence showing that Nedd4-2-catalyzed conjugation of a lysine 48-linked polyubiquitin chain to hOAT1 plays a significant role in the long-term PKC regulation of hOAT1 stability, and that WW3 and WW4 domains of Nedd4-2 are critical for its association with and modulation of the transporter.
MATERIALS AND METHODS

Materials — COS-7 cells were purchased from ATCC (Manassas, VA). [3H]-labeled p-aminohippuric acid (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). cDNA for human Nedd4-2 was generously provided by Dr. Peter M. Snyder from the College of Medicine, University of Iowa (Iowa City, IA). HA-tagged ubiquitin mutant HA-Ub-K48R was generously provided by Dr. Cam Patterson, Carolina Cardiovascular Biology Center, University of North Carolina, Chapel Hill, North Carolina, USA. Mouse anti-myc antibody and anti-β-actin were purchased from Roche (Indianapolis, IN). Mouse anti-ubiquitin antibody P4D1 was purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-FLAG (M2) antibody was purchased from Sigma-Aldrich (St. Louis, MO). Mouse anti-E-Cadherin (M168) antibody was purchased from Abcam (Cambridge, MA). PKC activator phorbol 12-myristate 13-acetate (PMA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and Transfection — hOAT1-expressing COS-7 cells were maintained in Dulbecco's modified Eagle's medium at 37 °C in 5% CO₂. The medium contained 10% fetal bovine serum. hOAT1 was tagged with an epitope Myc to its carboxyl terminus for the immuno-detection of the transporter (6). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for the transfection of the cDNA plasmids following the manufacturer's instructions. 48 hours after transfection, Cells were harvested for further experiments.

Site-Directed Mutagenesis — Ubiquitin lysine 48 mutant (K48R) and Nedd4-2 WW domain mutants
were generated in our lab using a QuickChange® site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA), followed by the confirmation of the mutant sequences using dideoxy chain termination method.

Measurement of hOAT1-mediated Transport — The uptake solution consisted of phosphate-buffered saline (PBS)/Ca^{2+}/Mg^{2+} (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_{2}HPO_{4}, 1.4 mM KH_{2}PO_{4}, 0.1 mM CaCl_{2}, and 1 mM MgCl_{2}, pH 7.3, and 20 µM [^{3}H]-PAH). The uptake solution was added to cells for indicated periods of time, and the uptake was terminated by removing the uptake solution and washing the cells with ice-cold PBS solution. The cells were then solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and subjected to liquid scintillation counting.

Cell Surface Biotinylation — The amount of hOAT1 at the plasma membrane was examined using the membrane-impermeable biotinylation reagent, sulfo-NHS-SS-biotin. Cells were incubated with NHS-SS-biotin for 20 min twice on ice. Afterwards, cells were washed with PBS/CM containing 100 mM glycine to quench the unreacted sulfo-NHS-SS-biotin. The cells were then lysed 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 mixture and 20 mM N-ethylmaleimide (NEM)). The cell lysates were subjected to centrifugation at 16,000g at 4 °C, followed by addition of streptavidin-agarose beads to the supernatant to isolate plasma membrane proteins. The surface proteins were then separated on SDS-PAGE, followed by immunoblotting using an anti-myc antibody to detect myc-tagged hOAT1.

Degradation of plasma membrane hOAT1— hOAT1-expressing cells were labeled with NHS-SS-biotin
as described above. The labeled cells were then incubated with or without PMA at 37°C. At designated
time, cells were lysed and subjected to centrifugation at 16,000g at 4 °C. The supernatant was mixed
with streptavidin-agarose beads to isolate cell membrane proteins. The surface proteins were then
separated on SDS-PAGE, followed by immunoblotting using an anti-myc antibody to detect myc-tagged
hOAT1.

**Immunoprecipitation** — Cells were lysed with lysis buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, 0.5%
Triton X-100, 2 mM EDTA, 10% glycerol, 1% of proteinase inhibitor cocktail, 20 mM NEM). Lysed cells
were precleared at 4 °C with protein G-agarose beads to minimize nonspecific binding. The pre-cleared
lysate was then mixed overnight with antibody-bound protein G-agarose beads. Proteins bound to the
protein G-agarose beads were eluted with urea buffer, followed by immunoblotting with appropriate
antibodies.

**Electrophoresis and Immunoblotting** — Protein samples were separated on 7.5% SDS-PAGE minigel,
and electroblotted on to PVDF membranes, followed by incubation overnight with appropriate primary
antibodies, and subsequently by incubation with horseradish peroxidase-conjugated secondary
antibodies. The SuperSignal West Dura Extended Duration Substrate kit (Pierce) was used to detect
the signals, followed by quantification using scanning densitometry with the FluorChem 8000 imaging
system (Alpha Innotech Corp., San Leandro, CA).

**Data Analysis** — We performed each experimental test for at least three times, and multiple
experiments were used to carry out the statistical analysis using Student’s paired t tests. A p-value<0.05
DMD # 75861

was indicated as "**".
RESULTS

Effect of long-term PKC activation on hOAT1 expression — We previously demonstrated (6) that short-term (<30 min) treatment of cells with PKC activator PMA leads to an inhibition of hOAT1-mediated transport by accelerating hOAT1 internalization from plasma membrane to intracellular endosomes. Consequently, the amount of hOAT1 at the plasma membrane is reduced, and hOAT1 transport activity is decreased. In the current investigation, we examined the effect of long-term PKC activation on hOAT1 expression. hOAT1-expressing cells were treated with PKC activator PMA for 30 min and 4 hrs. respectively, and hOAT1 expression at the plasma membrane and in total cell lysates were then compared. Our results showed that (Fig. 1a, Top panel and Fig. 1b) both short-term (30 min) and long-term (4 hrs.) treatment with PMA led to a decrease in hOAT1 expression at the plasma membrane. Furthermore, such a change in hOAT1 cell surface expression was not a result from the overall perturbation of plasma membrane proteins because there was no significant change in the expression of the cell surface protein marker E-cadherin (Fig. 1a, bottom panel). In contrast, short-term PMA treatment had no effect on the total expression of hOAT1 (Fig. 1c, top panel, and Fig. 1d), whereas long-term (4 hrs.) treatment with PMA resulted in a significant reduction of hOAT1 expression in total cell lysate (Fig. 1c, top panel, and Fig. 1d). Again, such a change in hOAT1 total expression was not a result from the general perturbation of cellular proteins because there was no change in the expression of cellular protein marker β-actin (Fig. 1c, bottom panel). The decrease in the total expression of hOAT1 after long-term PMA treatment suggests that hOAT1 stability was affected. Comparable results were obtained in HeLa cells (Fig. 2), suggesting that PKC regulation of hOAT1 is not cell type-specific.

Effect of long-term PKC activation on hOAT1 stability — The stability of cell surface hOAT1 was
subsequently assessed using a biotinylation approach. hOAT1-expressing cells were labeled with membrane-impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then incubated with or without PKC activator PMA at 37°C for 2, 4, and 6 hrs. respectively. After the treatment, cells were lysed and cell surface proteins were purified using streptavidin-agarose beads. Immunoblotting was then followed using anti-myc antibody (epitope myc was tagged to hOAT1 for immuno-detection).

Our results (Fig. 3a and Fig. 3b) showed that the rate of hOAT1 degradation increased significantly after 2-hr PMA treatment as compared to that of control. After 6-hr treatment with PMA, there was ~70% of hOAT1 degraded as compared to only ~30% of hOAT1 degradation in control samples. These results indicate that long-term PKC activation significantly decreases hOAT1 stability. Under the same condition, the expression of cell surface protein marker E-cadherin was not affected by PMA treatment (Fig. 3c). Our previously published work demonstrated that Nedd4-2, a ubiquitin ligase, mediates the PKC regulation of hOAT1 expression and activity (Xu et al., 2016a). We showed here that the expression of Nedd4-2 was not affected by PMA treatment (Fig. 3d).

**The role of K48-linked polyubiquitin chains in PKC-regulated hOAT1 degradation** — Our published work (7) revealed that short-term PKC activation accelerates hOAT1 internalization from the cell surface through promoting the conjugation of **K48-linked polyubiquitin chain to the transporter**. To explore the role of K48-linked polyubiquitin chains in long-term PKC regulation of hOAT1 stability, we transfected cells with wild type ubiquitin or ubiquitin mutant Ub-K48R. Ub-K48R has the lysine (K) at position 48 mutated to arginine (R) and therefore prevents the formation of K48-linked polyubiquitin chains. Transfected cells were then labeled with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin, followed by the treatment with or without PKC activator PMA for 4 and 8 hrs. respectively. The
degradation rates of cell surface hOAT1 in these cells were then determined as described above. As shown in Fig. 4a and Fig. 4b, the degradation of hOAT1 was much slower in mutant Ub-K48R-transfected cells than that in wild type ubiquitin (WT-Ub)-transfected cells. These results indicate that conjugation of K48-linked polyubiquitin chain to hOAT1 plays a significant role in PKC regulation of hOAT1 stability. Under the same condition, the expression of cell surface protein marker E-cadherin was not affected by the expression of WT-Ub and Ub-K48R (Fig. 4c).

The effect of Nedd4-2 on hOAT1 stability — We previously established that PKC-stimulated ubiquitination of hOAT1 is catalyzed by ubiquitin ligase Nedd4-2 (Xu et al., 2016b). In this experiment, we examined the effect of Nedd4-2 on hOAT1 stability. hOAT1-expressing cells were transfected with or without Nedd4-2. The degradation rates of plasma membrane hOAT1 in these cells were then determined using a biotinylation approach as described above. As shown in Fig. 5a and Fig. 5b, the degradation of hOAT1 was much faster in Nedd4-2-transfected cells than that in control cells, suggesting that Nedd4-2-mediated ubiquitination plays an important role in hOAT1 stability. Under the same condition, the expression of cell surface protein marker E-cadherin was not affected by the transfection of Nedd4-2 (Fig. 5c).

Interaction of Nedd4-2 with hOAT1 — Nedd4-2 interacts with its target proteins, either directly or indirectly, through its four WW domains (WW1-4). We mutated two amino acids in each domain separately (Mut-WW1: V91W/H93G; Mut-WW2: V283W/H285G; Mut-WW3: I395W/H397G; Mut-WW4: I446W/H448G). It was previously proven that the mutation of these amino acids inactivates the WW domains and thus, interferes with the binding of Nedd4-2 to its substrates. The cDNAs of Nedd4-2
mutants were transfected into the cells. hOAT1 was then pulled down by anti-myc antibody (myc was tagged to hOAT1), and subjected to immunoblotting with anti-FLAG antibody. Epitope FLAG was tagged to wild type Nedd4-2 and its mutants so that the exogenously expressed Nedd4-2 and its mutants could be immuno-detected. As shown in Fig. 6, although wild type Nedd4-2 and its mutants were all efficiently and equally expressed in hOAT1-expressing cells (not shown), the amounts of Mut-WW3 and Mut-WW4 detected in hOAT1 immunoprecipitates were much less in comparison to that of wild type Nedd4-2, Mut-WW1, and Mut-WW2 (Fig. 6a, top panel, and Fig. 6b), indicating that less amounts of Mut-WW3 and Mut-WW4 were associated with hOAT1. The association of Nedd4-2 with hOAT1 was further reduced when its WW3 and WW4 domains were mutated simultaneously (Mut-WW3/WW4) (Fig. 6c, top panel, and Fig. 6d). The different amounts of Nedd4-2 mutants detected in hOAT1 immunoprecipitates were not results from different amounts of hOAT1 immunoprecipitated because there were similar amounts of hOAT1 immunoprecipitated in all samples under these conditions (Fig. 6a, bottom panel, and Fig. 6c, bottom panel). These results suggest that WW3 and WW4 domains are critical for the binding of Nedd4-2 to hOAT1.

Effect of mutations of Nedd4-2 WW domains on hOAT1 ubiquitination — The data shown above (Fig. 6) demonstrated that mutations at WW3 and WW4 domains of Nedd4-2 weakened the binding of Nedd4-2 to hOAT1. To test whether such mutations affect hOAT1 ubiquitination, we transfected cells with cDNAs for wild type Nedd4-2 or for Nedd4-2 WW domain mutant Mut-WW3/WW4. hOAT1 was then pulled down by anti-myc antibody, followed by immunoblotting with anti-ubiquitin (anti-Ub) antibody. Our results showed that (Fig. 7a, top panel and Fig. 7b) hOAT1 ubiquitination was significantly decreased in Mut-WW3/WW4-transfected cells in comparison to that in wild type Nedd4-2-transfected
cells. Furthermore, the different amounts of ubiquitinated hOAT1 were not due to the different amounts of hOAT1 immunoprecipitated because there were similar amounts of hOAT1 immunoprecipitated in both samples under the same conditions (Fig. 7a, bottom panel). These data suggest that a decreased binding affinity of Mut-WW3/WW4 for hOAT1 results in a decreased hOAT1 ubiquitination.

The effect of mutations of WW domains in Nedd4-2 on the cell surface expression of hOAT1—We next assessed the effect of Nedd4-2 wild type and its mutant Mut-WW3/WW4 on the cell surface expression of hOAT1. Our results (Fig. 8a, Top panel and Fig. 8b) showed that hOAT1 expression was ~30% lower in wild type Nedd4-2-transfected cells as compared to that in control cells. Nedd4-2 mutant Mut-WW3/WW4 significantly reversed the effect of wild type Nedd4-2 on hOAT1 expression. The expression of cell surface protein marker E-cadherin was not affected by the transfection of wild type Nedd4-2 or its mutant Mut-WW3/WW4 (Fig. 8a, bottom panel).

The effect of mutations of WW domains in Nedd4-2 on hOAT1 transport activity —To explore whether WW domain mutations of Nedd4-2 affect hOAT1 function, we measured hOAT1-mediated transport of $^3$H-labeled PAH in cells transfected with cDNAs for wild type Nedd4-2 or for its mutant Mut-WW3/WW4. We showed that (Fig. 9) overexpression of wild type Nedd4-2 inhibited hOAT1-mediated transport ~40%, whereas such level of inhibition was partially reversed in cells-transfected with Mut-WW3/WW4.
hOAT1 plays a pivotal role in drug efficacy and toxicity. The transport activity of hOAT1 is critically dependent on its expression level at the plasma membrane. We previously established that hOAT1 at the plasma membrane is dynamic, constitutively internalizing from and recycling back to plasma membrane. Short-term (30 min) PKC activation inhibits hOAT1 transport activity by promoting the attachment of a lysine 48-linked polyubiquitin chain to hOAT1, a step catalyzed by ubiquitin ligase Nedd4-2. The polyubiquitin chain attached to hOAT1 can be recognized by endocytosis machinery and triggers an accelerated endocytosis of the transporter from plasma membrane. Consequently, the amount of hOAT1 at the plasma membrane is reduced and hOAT1 transport activity is decreased. In the present study, we investigated the long-term effect of PKC on hOAT1.

To carry out our investigation, we chose monkey kidney COS-7 cells, a model cell system that has been widely used for mechanistic studies of many kidney transport processes. Our studies in these cells will pave the path for the future exploration in assessing whether similar mechanisms are working in vivo.

From our investigation, we obtained several pieces of important information. First, there was sharp contrast between the short-term and long-term effects of PKC on hOAT1: short-term PKC activation promoted hOAT1 to traffic from plasma membrane to intracellular endosomes without influencing the total expression of the transporter. However long-term activation of PKC led hOAT1 to degrade. Secondly, PKC-induced hOAT1 degradation was partially blocked by ubiquitin mutant Ub-K48R. Ub-K48R is defective in forming K48-linked polyubiquitin chain. Thirdly, the ubiquitin ligase Nedd4-2 also
promoted hOAT1 degradation. These results suggest that Nedd4-2-catalyzed conjugation of K48-linked polyubiquitin chain to hOAT1 not only plays an important role in short-term PKC regulation of hOAT1 trafficking but also is critical in long-term PKC effect on hOAT1 stability. These observations provide important insights into the molecular basis underlying the defective drug transport in bilateral ureteral obstruction (BUO). Clinically, BUO causes acute renal failure (Klahr, 1998; Klahr and Morrissey, 2002). In the rat model of this disease, elimination of drugs was impaired partly as a result of a decreased OAT1 expression (Villar et al., 2005). In BUO, the level of angiotensin II is elevated (Klahr, 1998; Klahr and Morrissey, 2002), and it was shown that angiotensin II is a physiological PKC activator (Duan et al., 2010). Therefore, angiotensin II may degrade OAT1 through Nedd4-2-mediated PKC regulation of OAT1 ubiquitination.

Our present studies also indicate that the effect of Nedd4-2 on hOAT1 partly arises from a direct association between the WW-3/WW-4 domains of Nedd4-2 and the transporter. The WW domains are well known to bind proline-rich sequences in the target proteins (Espanel and Sudol, 1999; Kamynina et al., 2001; Fotia et al., 2003; Vina-Vilaseca and Sorkin, 2010). There are four WW domains in Nedd4-2. These domains are known to have different selectivity and affinity to its target proteins. For instance, Nedd4-2 plays critical roles in the function of epithelial sodium channel ENaC, and its inhibition of ENaC activity depends on WW domains 3 and 4, and to a less extent WW domain 2 (Harvey et al., 1999; Farr et al., 2000). Yet, WW domains 1 and 2 of Nedd4-2 are important for ubiquitinating a calcium channel TRPV6 (Zhang et al., 2010). Our present study also uncovered that the binding affinity of the WW domains of Nedd4-2 to hOAT1 was substantially selective. WW domains 3 and 4 but not WW domains 1 and 2 were essential for the association of Nedd4-2 to hOAT1. When WW domains 3 and 4 in Nedd4-
2 was inactivated individually or in combination through site-directed mutagenesis, the binding affinity of these Need4-2 mutants (Mut-WW3, Mut-WW4, and Mut-WW3/WW4) for hOAT1 was significantly weakened (Fig. 6). We noticed that the effect of Mut-WW3/WW4 on hOAT1 ubiquitination, cell surface expression, and transport activity was not as dramatic as the effect on their binding to hOAT1 (Fig. 7, Fig. 8, and Fig. 9). The possible explanation is that in the study examining the binding of Mut-WW3/WW4 to hOAT1, only the FLAG-tagged exogenously expressed Nedd4-2 mutant was probed. In the study examining the effect of Mut-WW3/WW4 on hOAT1 ubiquitination and transport activity, we were looking at the combinational effect of Mut-WW3/WW4 and the endogenously expressed and fully functional Nedd4-2.

Finally, it should be noted that hOAT1 was shown in our study to interact with Nedd4-2 despite the lack of conventional WW domain-binding (L/P)PXY motifs. This indicates that the interaction occurs through non-canonical sequences in hOAT1. This is consistent with findings from other labs that Nedd4-2 is physically associated with the glutamate transporter GLT-1 (Garcia-Tardon et al., 2012) and mammalian dopamine transporter DAT (Sorkina et al., 2006), none of which possesses the conventional WW domain-binding motifs.

In conclusion, we provided the first demonstration that Nedd4-2-catalyzed conjugation of a lysine 48-linked polyubiquitin chain to hOAT1 plays a significant role in the long-term PKC regulation of hOAT1 stability, and that WW domains 3 and 4 of Nedd4-2 are critical in its association with and regulation of the transporter (Fig. 10). Our study offers important insights into understanding the molecular and cellular foundation of hOAT1 regulation in vivo.
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Authorship Contributions

Participated in research design: Xu, Zhang, J., You.

Conducted experiments: Xu, Zhang, J., Zhang, Q, Fan, Liu

Performed data analysis: Xu, Zhang, J., You.

Wrote or contributed to the writing of the manuscript: Xu, You.

Ψ: Both authors (Chenchang Liu and Yunzhou Fan) contributed equally
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Footnotes

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FIGURE LEGEND

Fig. 1. Comparison of the short-term and long-term effects of PKC on hOAT1 expression at the cell surface and in total cell lysates in COS-7 cells. (a) hOAT1 expression at the cell surface. Top panel: hOAT1-expressing cells were treated with or without PKC activator PMA for 30 min and 4 hrs. respectively. Cell surface biotinylation was then performed. Biotinylated/cell surface proteins were separated with streptavidin beads and analyzed by immunoblotting (IB) with an anti-myc antibody (myc was tagged to hOAT1 for immuno-detection). Bottom panel: The same immunoblot from top panel was re-probed by anti-E-cadherin antibody to determine the expression of the cell surface protein marker E-cadherin. (b) Densitometry plot of results from (a, top panel) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05. (c) hOAT1 expression in total cell lysate. Top panel: hOAT1-expressing cells were treated with or without PKC activator PMA for 30 min and 4 hrs. respectively. Treated cells were lysed, and hOAT1 total expression was analyzed by immunoblotting (IB) with an anti-myc antibody. Bottom panel: The same immunoblot from top panel was re-probed by cell protein marker anti-β-actin antibody. (d) Densitometry plot of results from (c, top panel) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05

Fig. 2. Comparison of the short-term and long-term effects of PKC on hOAT1 expression at the cell surface and in total cell lysates in HeLa cells. (a) hOAT1 expression at the cell surface. hOAT1-expressing cells were treated with or without PKC activator PMA for 30 min and 4 hrs. respectively. Cell surface biotinylation was then performed. Biotinylated/cell surface proteins were separated with streptavidin beads and analyzed by immunoblotting (IB) with an anti-myc antibody (myc was tagged to hOAT1 for immuno-detection). (b) Densitometry plot of results from (a) as well as from other
independent experiments. The values are mean ± S.E. (n = 3). *P<0.05. (c) hOAT1 expression in total cell lysate. hOAT1-expressing cells were treated with or without PKC activator PMA for 30 min and 4 hrs. respectively. hOAT1 total expression was analyzed by immunoblotting (IB) with an anti-myc antibody. (d) Densitometry plot of results from (c, top panel) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05

Fig. 3. Effect of PKC activation on the degradation rate of cell surface hOAT1. (a) hOAT1-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with or without PKC activator PMA at 37°C for 2, 4, and 6 hrs. respectively. Treated cells were lysed and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-myc antibody. (b) Densitometry plot of results from (a) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05 (between PMA-treated and control at the same time point). (c) hOAT1-expressing cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with or without PKC activator PMA at 37°C for 6 hrs. Treated cells were lysed and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-Ecadherin antibody. (d) hOAT1-expressing cells were treated with or without PKC activator PMA at 37°C for 30 min. Treated cells were lysed, followed by immunoblotting (IB) with anti-Nedd4-2 antibody.

Fig. 4. Effect of ubiquitin mutant Ub-K48R on the degradation rate of cell surface hOAT1. (a) hOAT1-expressing cells were transfected with wild type ubiquitin (WT-Ub) or ubiquitin mutant (Ub-K48R). Transfected cells were biotinylated with membrane impermeable biotinylation reagent sulfo-
NHS-SS-biotin. Labeled cells were then treated with or without PKC activator PMA at 37°C for 4 or 8 hrs. respectively. Cells were lysed afterwards and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-myc antibody. (b) Densitometry plot of results from (a) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05 (between WT-Ub- and Ub-K48R-transfected cells at the same time point). (c) hOAT1-expressing cells were transfected with wild type ubiquitin (WT-Ub) or ubiquitin mutant (Ub-K48R). Transfected cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then treated with or without PKC activator PMA at 37°C for 6 hrs. Cells were lysed afterwards and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-Ecadherin antibody.

**Fig. 5. Effect of Nedd4-2 on the degradation rate of cell surface hOAT1.** (a) hOAT1-expressing cells were transfected with control vector or with Nedd4-2. Transfected cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then put in the cell incubator at 37°C for 2, 4, and 6 hrs. respectively. Cells were lysed afterwards and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-myc antibody. (b) Densitometry plot of results from (a) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05 (Between Nedd4-2-transfected and control at the same time point). (c) hOAT1-expressing cells were transfected with control vector or with Nedd4-2. Transfected cells were biotinylated with membrane impermeable biotinylation reagent sulfo-NHS-SS-biotin. Labeled cells were then put in the cell incubator at 37°C for 6 hrs. Cells were lysed afterwards and cell surface proteins were isolated using streptavidin-agarose beads, followed by immunoblotting (IB) with anti-Ecadherin antibody.
antibody.

**Fig. 6. Interaction between Nedd4-2 WW domain mutants and hOAT1.** (a) *Top Panel:* Two amino acid residues in each of the four WW domains of Nedd4-2 were mutated (Mut-WW1: V91W/H93G; Mut-WW2: V283W/H285G; Mut-WW3: I395W/H397G; Mut-WW4: I446W/H448G). Mutant-transfected cells were lysed, and hOAT1 was then immunoprecipitated (IP) with anti-myc antibody, followed by immunoblotting (IB) with anti-FLAG antibody. Epitope FLAG was tagged to wild type Nedd4-2 and its mutants for the immuno-detection of exogenously expressed Nedd4-2 and its mutants. *Bottom panel:* The same immunoblot from *top panel* was re-probed by anti-myc antibody to determine the amount of hOAT1 immunoprecipitated. (b) Densitometry plot of results from (a, *top panel*) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05. (c) *Top panel:* WW domain 3 and WW domain 4 were simultaneously mutated (Mut-WW3/WW4). Wild type Nedd4-2- and mutant Mut-WW3/WW4-transfected cells were lysed, and hOAT1 was then immunoprecipitated (IP) with anti-myc antibody, followed by immunoblotting (IB) with anti-FLAG antibody. *Bottom panel:* The same immunoblot from *top panel* was re-probed by anti-myc antibody to determine the amount of hOAT1 immunoprecipitated. (d) Densitometry plot of results from (c, *top panel*) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05.

**Fig. 7. Effect of Nedd4-2 WW domain mutant Mut-WW3/WW4 on hOAT1 ubiquitination.** (a) *Top panel:* hOAT1-expressing COS-7 cells were transfected with cDNAs for wild type Nedd4-2 or for Nedd4-2 WW domain mutant (Mut-WW3/WW4). Transfected cells were then lysed, and hOAT1 was
immunoprecipitated (IP) with anti-myc antibody, followed by immunoblotting (IB) with anti-ubiquitin antibody (anti-Ub). **Bottom panel:** The blot from top panel was re-probed with anti-myc antibody to determine the amount of hOAT1 immunoprecipitated. (b) Densitometry plot of results from (a, top panel) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05.

**Fig. 8. Effect of Nedd4-2 WW domain mutant Mut-WW3/WW4 on hOAT1 cell surface expression.** (a) **Top panel:** hOAT1-expressing COS-7 cells were transfected with cDNAs for wild type Nedd4-2 or for Nedd4-2 WW domain mutant (Mut-WW3/WW4). Cell surface biotinylation was then performed. Biotinylated/cell surface proteins were separated with streptavidin beads and analyzed by immunoblotting (IB) with an anti-myc antibody. **Bottom panel:** The same immunoblot from top panel was re-probed by anti-E-cadherin antibody to determine the expression of the cell surface protein marker E-cadherin. (b) Densitometry plot of results from (a, top panel) as well as from other independent experiments. The values are mean ± S.E. (n = 3). *P<0.05.

**Fig. 9. Effect of Nedd4-2 WW domain mutant on hOAT1 transport activity.** hOAT1-expressing cells were transfected with cDNAs for wild type Nedd4-2 or for Nedd4-2 WW domain mutant Mut-WW3/WW4. Transfected cells were then measured for 3-min uptake of [3H] PAH (20 µM) at room temperature. Uptake activity was expressed as a percentage of the uptake measured in control cells. The data represent uptake into hOAT1-transfected cells minus uptake into mock cells (parental cells). Values are mean ± S.E. (n = 3). *P<0.05

**Fig. 10. PKC/Nedd4-2 signaling pathway regulates the activity of drug transporter hOAT1.**
Figure 1

(a) hOAT1 expression at the cell surface

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0.5</th>
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</thead>
<tbody>
<tr>
<td>PMA</td>
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</tbody>
</table>

IB: anti-Myc

IB: anti- E-Cadherin

(b) Cell surface hOAT1 (% of control)

<table>
<thead>
<tr>
<th>PMA</th>
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<tbody>
<tr>
<td>Time (h)</td>
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</table>

* indicates statistical significance.

(c) hOAT1 expression in total cell lysates

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<tr>
<th>Time (h)</th>
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<tbody>
<tr>
<td>PMA</td>
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IB: anti-Myc

IB: anti-β-Actin

(d) Cell total hOAT1 (% of control)

<table>
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<tbody>
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* indicates statistical significance.
Figure 2

(a) hOAT1 expression at the cell surface

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<th>Time (h)</th>
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<tbody>
<tr>
<td>PMA</td>
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IB: anti-Myc

(b) Cell surface hOAT1 (% of control)

<table>
<thead>
<tr>
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* indicates significant difference

(c) hOAT1 expression in total cell lysates

<table>
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<tr>
<th>Time (h)</th>
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<tbody>
<tr>
<td>PMA</td>
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IB: anti-Myc

(d) Cell total hOAT1 (% of control)

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* indicates significant difference
Figure 4

(a) Undegradated cell surface hOAT1

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IB: anti-Myc

(b) Undegraded cell surface hOAT1

Undegradated cell surface (% of control)

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<td>Ub-K48R</td>
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(c) Undegradated cell surface Ecadherin

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IB: anti-Ecadherin
**Figure 5**

**a**

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<td>Nedd4-2</td>
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**Undegradated cell surface hOAT1**

IB: anti-Myc

**b**

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Undegraded cell surface hOAT1 (% of control)

**c**

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</table>

**Undegradated cell surface Ecadherin**

IB: anti-Ecadherin
Figure 6

Nedd4-2 mutants associated with hOAT1

- control
- Nedd4-2
- Mut-WW1
- Mut-WW2
- Mut-WW3
- Mut-WW4

IB: anti-FLAG
IP: anti-Myc

Nedd4-2 mutant associated with hOAT1

IB: anti-FLAG
IP: anti-Myc

Graphs showing the percentage of Nedd4-2 mutants associated with hOAT1:

- a) Western blots for Nedd4-2 mutants
- b) Bar graph showing the percentage of Nedd4-2 mutants
- c) Western blots for Nedd4-2 mutant
- d) Bar graph showing the percentage of Nedd4-2 mutant

* indicates significant difference.
**Figure 7**

(a) Ubiquitinated hOAT1

- Nedd4-2
- Mut-ww3ww4

IB: anti-Ub
IP: anti-Myc

(b) Ubiquitinated hOAT1 (% of control)

- Nedd4-2
- Mut-ww3ww4

Ubiquitinated hOAT1 (% of control)

*
Figure 8

(a) Cell surface expression of hOAT1

IB: Anti-myc
IB: Anti-Ecadherin

(b) Cell surface hOAT1 (% of control)

Control  Nedd4-2  Mut-WW3WW4
Figure 10

Ubiquitination

Internalization

Degradation

OAT1

Nedd4-2

Plasma membrane

Endosome

Lysosome

PKC

C2

WW1

WW2

WW3

WW4

HECT

Ubiquitin

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