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Original Paper

YOD1 Deubiquitinates NEDD4 Involved in the Hippo Signaling Pathway

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Key Words
Deubiquitinating enzyme • Deubiquitination • Kidney • Post-translational modification • Ubiquitination

Abstract
Background/Aims: Deubiquitinating enzymes (DUBs) are crucially involved in controlling signal transductions, and reverse ubiquitination by removing the ubiquitin from protein substrates. The Hippo signaling has an important role in tissue growth, cell proliferation, differentiation, and apoptosis. Since disruption of the Hippo signaling is associated with a number of diseases, it is imperative to investigate the molecular mechanism of the Hippo signaling. ***Methods:*** DUB screening was performed using the kidney of the mouse unilateral ureteric obstruction (UUO) model to identify the cellular mechanism of the DUB-regulated Hippo signaling. In addition, kidney cells were used to confirm cell proliferation and protein levels in the Hippo signaling pathway. Densitometric analysis was conducted to compare the expression level of proteins using Image J. ***Results:*** We found that *YOD1,* also known as *OTU1*, is downregulated in the mouse UUO model. We also demonstrated that YOD1 binds to and deubiquitinates neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4). Furthermore, we observed that YOD1 suppresses NEDD4-induced cell proliferation. ***Conclusion:*** YOD1 regulates the Hippo signaling pathway through NEDD4, and the K63-linked polyubiquitin chain of NEDD4 plays an important role. Also, our results indicate that YOD1 plays an important role in kidney diseases.

Introduction

Ubiquitination is one of the post-translation modifications, where the C-terminus of ubiquitin binds to a lysine residue of the substrate through a cascade reaction of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) [1-3]. A ubiquitin protein consists of 76 amino acids containing 7 lysine residues (K6, K11, K27, K29, K33, K48, and K63) [4]. In addition, subsequent binding of the C-terminal ubiquitin

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to a lysine residue of another ubiquitin results in the formation of polyubiquitin chains [5, 6]. The role or destiny of substrate is determined on the usage of a lysine residue of ubiquitin: a 48-linked polyubiquitin chain induces proteasomal degradation of the substrate, whereas a K63-linked polyubiquitin chain regulates signal transduction, kinase activation, and endocytosis [7-9]. Deubiquitination is defined as the process where ubiquitin detaches from the target protein by deubiquitinating enzymes (DUBs) [10, 11]. DUBs play an important role in regulating stability and activity of proteins, various signal transductions, cell cycle, apoptosis, proliferation and so on [12-14].

The Hippo signaling pathway regulates the number of cells and organ sizes by controlling proliferation, cell survival, apoptosis, and differentiation [15-17]. The pathway is initiated by cell density, G protein-coupled receptors (GPCRs) or mechanical and stress signals [17]. This is followed by phosphorylation of Sav, LATS1/2, and Mob by MST1/2, and phosphorylation of YAP/TAZ by the phosphorylated LATS1/2 [15]. Phosphorylated YAP/TAZ then interacts with 14-3-3 and suppresses their activity, preventing transportation to the nucleus, resulting in subsequent cytoplasmic retention and ubiquitin-mediated degradation of YAP/TAZ [18, 19]. Dephosphorylated YAP/TAZ enters the nucleus and induces gene transcription, promoting cell survival and proliferation [19]. Thus, deregulation of the Hippo signaling pathway may result in unregulated cell proliferation and survival, with subsequent development of diseases [20-23]. YAP/TAZ is known to play a role in proliferation, junction assembly, and metabolism in sprouting angiogenesis and barrier maturation [24].

Neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) is an E3 ligase that participates in the Hippo signaling pathway [25, 26]. NEDD4 induces proteasomal degradation of LATS1 to negatively regulate the Hippo signaling pathway, and controls stem cell homeostasis by ubiquitinating LATS1/2 and WW45 in the Hippo signaling pathway [27, 28]. It is also known that NEDD4 can regulate stability through self-ubiquitination [29]. In the current study, we found that YOD1 (also known as OTU1) involved in the ovarian tumor (OTU) family of DUB is weakly expressed in the mouse unilateral ureteric obstruction (UUO) model. We also observed that YOD1 interacts with NEDD4; moreover, we demonstrate that YOD1 targets NEDD4 for deubiquitination and inhibits NEDD4-mediated proliferation. In summary, our studies indicate that YOD1 is a DUB that modulates the Hippo signaling pathway through NEDD4 regulation.

Materials and Methods

Construction of the expression vector

Commercial clone Flag-pcDNA3.1-*YOD1* was purchased (Invitrogen, Carlsbad, CA, USA). YOD1 was subcloned into the pcDNA3-6Myc vector, using the forward primer 5'-GAA TTC GGA TGT TTG GCC-3' and reverse primer 5'-CTC GAG TCA CAC TTC TCC-3'. The Flag-*NEDD4* cDNA was provided by Professor Jae Hong Seol (Seoul National University, Seoul, Republic of Korea). A catalytically inactive form of *YOD1* (C160S) was generated by site-directed mutagenesis. The forward primer 5'-GAC AAC TCT AGC CTC TTT-3' and the reverse primer 5'-AGT AAA GAG GCT AGA GTT-3' were used for replacing cysteine to serine at position 160 of YOD1 (Ubiprotein Corp., Seongnam, Republic of Korea). After conduction of PCR, Dpn I enzyme (Enzynomics, Daejeon, Republic of Korea) was added to the PCR product. The mutant of YOD1 (C160S) was confirmed by direct sequencing (Cosmogenetech, Seoul, Republic of Korea). The HA-tagged ubiquitins (WT, K6, K11, K27, K29, K33, K48, and K63) were provided by Professor Byung Joon Hwang (Kangwon National University, Chuncheon, Republic of Korea).

Cell culture and transfection

293T cells (transformed human embryonic kidney cells) and NIH3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) and 1% antibiotic-antimycotic reagent (Gibco, Grand Island, NY, USA). The cells were grown in a 5% CO2 incubator at 37°C. For transfection, 10 mM polyethyleneimine reagent (PEI, Polysciences, Inc., Warrington, PA, USA) and 150 mM NaCl were used.

Antibodies

Anti-β-actin (sc-47778, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HA (12CA5, 11 666 606 001, Roche, Basel, Switzerland), anti-Flag (M185-3L, Sigma-Aldrich, St. Louis, MO, USA), anti-Myc (sc40, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-YOD1 (25370-I-AP, Proteintech, Rosemont, IL, USA), anti-LATS1 (C66B5, Cell Signaling, Danvers, MA, USA), anti-p-YAP (S127, Cell Signaling, Danvers, MA, USA), anti-YAP (1A12, Cell Signaling, Danvers, MA, USA), and anti-NEDD4 (611480, BD Biosciences, San Jose, CA, USA) antibodies were used for Western blotting and immunoprecipitation.

Western blotting and immunoprecipitation

Cells were lysed using a lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100) and incubated for 20 min on ice. Samples were then centrifuged for 20 min at 16,200 g at 4°C, and the supernatant was boiled with 2X SDS sample buffer for Western blotting. The samples were resolved by SDS-PAGE and transferred onto microporous polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in TBS buffer containing 0.05% Tween 20 for 20 min at room temperature, and incubated overnight at 4°C with primary antibodies, in 3% skim milk in TBS buffer containing 0.05% Tween 20. This was followed by incubating the probed membranes with a secondary antibody for 1 hr at room temperature. Protein bands were visualized using the ECL reagent solution (Young In Frontier, Seoul, Republic of Korea).

For immunoprecipitation, cell lysates were incubated with antibodies at 4°C overnight. Protein A/G PLUS-agarose beads (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) were then added and incubated at 4°C for 2 hrs on a rotator. The samples were washed with TBS containing 0.05% Tween 20 and boiled in 2X SDS sample buffer.

Ubiquitination and deubiquitination assays

For the ubiquitination assay, Flag-*NEDD4*, Myc-*YOD1*, and HA-*Ub* were transfected into 293T cells. The cell lysates were used for immunoprecipitation with an anti-Flag antibody. The ubiquitination level of Flag-NEDD4 was detected through Western blotting. For the deubiquitination assay, Flag-*NEDD4*, Myc-*YOD1*, Myc-*YOD1* (C160S) and HA-*Ub* were transfected into 293T cells. The cell lysates were immunoprecipitated with an anti-Flag antibody and the ubiquitination level of Flag-NEDD4 was analyzed by performing Western blotting.

GST pull-down assay

GST-*YOD1* was transformed into BL21 (DE3) cells, once the bacterial culture achieved a density of $0.5_{O.D.600.}$ GST and GST-YOD1 proteins were then induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega, Madison, WI, USA) at 28°C overnight. The protein bound to GST-YOD1 was washed and boiled with 2X SDS buffer. The bound protein was detected through Western blotting, and GST and GST-YOD1 were visualized by staining with Coomassie Brilliant Blue R (SLBL7178V, Sigma-Aldrich, St. Louis, MO, USA) and G (SLBN7053V, Sigma-Aldrich, St. Louis, MO, USA) solution.

Cell proliferation assays

For the colony forming unit (CFU) assay, 100 cells were seeded in 100-mm dishes. After 14 days, the cells were stained with crystal violet. To evaluate cell viability, cells were seeded at a density of 5,000 cells per well in 96-well plates; after 12 hrs, the cells were incubated with medium containing CCK-8 (Dojindo, Kumamoto, Japan) for 4 hrs, followed by measurement of the absorbance at 450 nm using a microplate reader (Tecan Group Ltd. Seestrasse, Manndorf, Switzerland).

FACS analysis

293T cells were treated with 1 μg/ml of mitomycin C for 24 hrs, and subsequently transfected with Flag-*NEDD4* and HA-*Ub* (WT, K33, K48, and K63). The cells were fixed with 70% ethanol for 2 hrs, followed by permeabilization with 0.1% PBS-Triton X-100 for 15 min, incubation with an anti-Ki67 antibody (1:1,000) (Ab16667, Abcam, Cambridge, UK) at room temperature for 30 min, and subsequent incubation with a goat anti-rabbit IgG-FITC (1:1,000) (sc-2012, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min at room temperature. This was followed by the histogram analysis performed by FACS program (FlowJo, BD Biosciences, San Jose, CA, USA).

Statistical analysis

Densitometric analysis was conducted to compare the expression level of proteins using Image J (Version 1.4.3.67). Statistical results were analyzed by GraphPad Prism Version 5 (GraphPad Software, Inc., La Jolla, CA, USA). Independent experiments were performed at least three times. *T*-test and one-way analysis of variance followed by Tukey's multiple comparisons post hoc test were performed for analyzing statistical significance. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate statistically significant differences.

Results

YOD1 is downregulated in the mouse UUO model

In a previous study, we developed a screening platform to simultaneously compare the differential expression levels of various DUBs [28]. Moreover, it was reported that nuclear YAP and TAZ, both well-known factors for the Hippo signaling, are expressed in the mouse UUO model, but p-YAP levels were reported to be increased in sham-operated controls [30, 31]. Thus, to identify DUBs associated with the Hippo signaling, we decided to perform DUB screening with the mouse UUO model (Fig. 1A). RNA extracted from the kidneys of the control and the UUO model group were used to synthesize cDNA. cDNA was then used in DUB screening, which was performed to compare the expression level of DUBs of the two groups. Densitometric analysis was performed, and the mRNA level of each gene was normalized to *GAPDH* level (Fig. 1B). As a result, we observed that the expression level of *YOD1* was downregulated (Fig. 1C and 1D). Interestingly, a bioinformatics tool, BioGRID, revealed that NEDD4 binds to YOD1 (Table 1) [5]. NEDD4 is an E3 ubiquitin ligase that targets proteins for ubiquitination. It has previously been reported that NEDD4 targets LATS1 for ubiquitination and degradation, and is known to inhibit the Hippo signaling [25, 26, 32]. We therefore decided to investigate the cellular role of YOD1 in the Hippo signaling pathway.

YOD1 binds to NEDD4

Since results from DUB screening and bioinformatics search indicated that YOD1 plays a role in the Hippo signaling pathway, and that NEDD4 might be a target of YOD1, we next assessed the interaction between YOD1 and NEDD4. Myc-YOD1 and Flag-NEDD4 were co-expressed in 293T cells, which were followed by immunoprecipitation. Our results show that YOD1 interacts with NEDD4 (Fig. 1E). Furthermore, we conducted GST pull-down assay with purified GST-YOD1 proteins. The cell lysates expressed with Flag-NEDD4 were incubated with GST-YOD1 proteins. The results revealed that YOD1 binds to NEDD4 (Fig. 1F), thus confirming the direct interaction between YOD1 and NEDD4.

NEDD4 is ubiquitinated

Since our previous experiment confirmed the interaction between YOD1 and NEDD4, we hypothesized that YOD1 as a DUB targets NEDD4 to decrease the level of ubiquitination of NEDD4. To demonstrate this, we first sought to confirm the ubiquitination of NEDD4. Since the function or destiny of a substrate is determined by the types of its ubiquitin chains, we further investigated which types of its ubiquitin chains are formed on NEDD4 by performing ubiquitination assay using specific ubiquitin mutant constructs (K6, K11, K27, K29, K33, K48, and K63) (Fig. 2A). Transfection of Flag-*NEDD4* and HA-*Ub* into 293T cells, and subsequent ubiquitination assay confirmed that Flag-NEDD4 is ubiquitinated (Fig. 2B). We observed that all mutant ubiquitin constructs were polyubiquitinated in NEDD4, indicating that NEDD4 E3 ligase can be ubiquitinated (Fig. 2B).

YOD1 targets NEDD4 for deubiquitination

After confirming the YOD1-NEDD4 interaction and ubiquitination of NEDD4, we performed the deubiquitination assay to check whether YOD1's interaction with NEDD4 suppresses ubiquitination of NEDD4. The results obtained indicate that overexpression of YOD1 decreases the ubiquitination level of NEDD4, while YOD1 (C160S), whose DUB activity

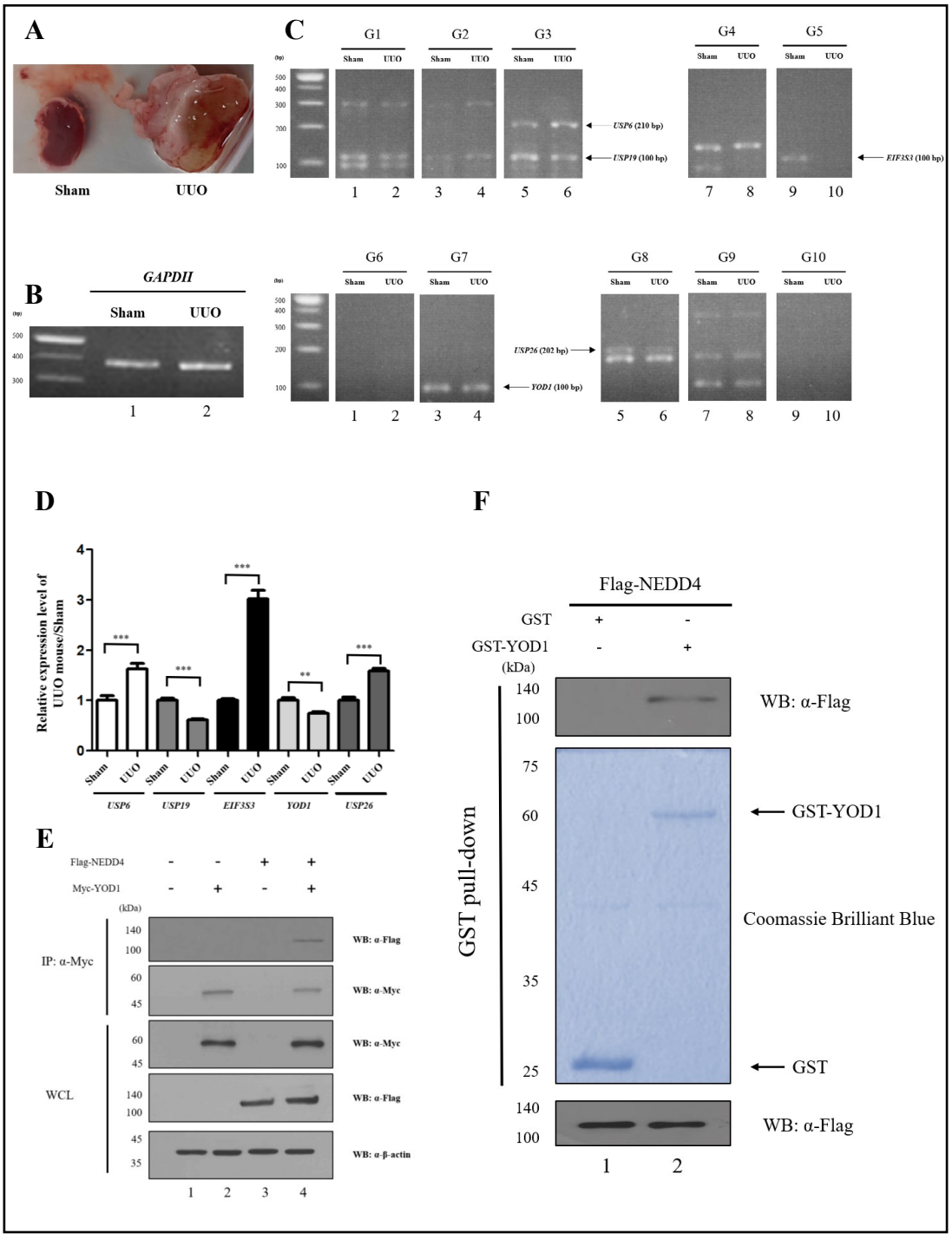

Fig. 1. (A) The kidneys of the control and UUO mice were extracted and confirmed. (B) The mRNA level of GAPDH was determined by RT-PCR and used as an internal control. (C) DUB screening was performed through multiplex PCR, and PCR product was loaded on 2% agarose gel. (D) Densitometric analysis was conducted using Image J to analyze the expression level of YOD1. The expression level of GAPDH was used to normalize YOD1 expression level. (E) Flag-NEDD4 and Myc-YOD1 were co-transfected into 293T cells. An anti-Myc antibody was used for immunoprecipitation. (F) Purified GST or GST-YOD1 was incubated with 293T cell lysates overexpressed with Flag-NEDD4. Protein bound to GST-YOD1 was detected through Western blotting. GST control and GST-YOD1 were visualized using Coomassie Brilliant Blue R/G staining solution.

Table 1. Putative protein candidates that may bind to YOD1

No.	Name	Full name	No.	Name	Full name
1	AMFR	autocrine motility factor receptor, E3 ubiquitin protein ligase	15	SYVN1	synovial apoptosis inhibitor 1, synoviolin
2	BLOC1S5	biogenesis of lysosomal organelles complex-1, subunit 5, muted	16	THOC3	THO complex 3
3	CCDC51	coiled-coil domain containing 51	17	TRA	T cell receptor alpha locus
4	DERL1	derlin 1	18	TRIM54	tripartite motif containing 54
5	ELAVL1	ELAV like RNA binding protein 1	19	TRIM55	tripartite motif containing 55
6	FAF2	Fas associated factor family member 2	20	TRIM63	tripartite motif containing 63, E3 ubiquitin protein ligase
7	FAM98B	family with sequence similarity 98, member B	21	UBC	ubiquitin C
8	FCF1	FCF1 rRNA-processing protein	22	UBE2J1	ubiquitin-conjugating enzyme E2, J1
9	GPSM1	G-protein signaling modulator 1 (AGS3-like, C. elegans)	23	UBE3A	ubiquitin protein ligase E3A
10	NEDD4	neural precursor cell expressed, developmentally down-regulated 4, E3 ubiquitin protein ligase	24	UFD1L	ubiquitin fusion degradation 1 like (yeast)
11	NPLOC4	nuclear protein localization 4 homolog (S. cerevisiae)	25	USP15	ubiquitin specific peptidase 15
12	PFKM	phosphofructokinase, muscle	26	USP21	ubiquitin specific peptidase 21
13	POLD1	polymerase (DNA directed), delta 1, catalytic subunit	27	VCP	valosin containing protein
14	RIPK1	receptor (TNFRSF)-interacting serine-threonine kinase 1			

was eliminated, is unable to deubiquitinate NEDD4 (Fig. 2C). Moreover, we observed the formation of various ubiquitin chains on NEDD4 (Fig. 2B). To determine which type of ubiquitin chain of NEDD4 is regulated by YOD1, we conducted a deubiquitination assay using specific ubiquitin constructs (K6, K11, K27, K29, K33, K48, and K63). As presented in Fig. 2D and 2E, we observed that YOD1 exerts its DUB activity on K33- and K63-linked polyubiquitin chains of NEDD4.

YOD1 inhibits NEDD4-promoted cell proliferation

It is reported that K48-linked polyubiquitin chains attract 26S proteasome for target protein degradation [33]. To confirm the half-life of NEDD4 mediated by YOD1, a dose-dependent transfection of *YOD1* cDNA was performed, followed by evaluating the expression level of NEDD4. We observed that there was no increase in the stability of NEDD4 by YOD1 (Fig. 3A), consisting with the result (Fig. 2D and 2E) that YOD1 deubiquitinates NEDD4 through K33- and K63-linkage. Hence, we hypothesized that YOD1 regulates the function of NEDD4, which in turn regulates the Hippo signaling. YAP is downstream of the Hippo signaling pathway, and the degree of cell proliferation changes with phosphorylation of YAP. As expected, the expression levels of p-YAP were downregulated in the UUO model (Fig. 3B), indicating that YOD1 inhibits the function of NEDD4, which in turn, decreases LATS1. The expression of NEDD4 was upregulated in the UUO model (Fig. 3C).

Furthermore, we investigated the biological effects of YOD1 and NEDD4 on cell proliferation. To do this, Flag-NEDD and Myc-YOD1 were co-expressed in 293T and NIH3T3 cells, and the colony forming unit (CFU) assay was performed. Overexpression of NEDD4 resulted in increased cell proliferation; however, YOD1 overexpression inhibited cell proliferation (Fig. 3D). In addition, YOD1 was observed to suppress NEDD4-induced cell proliferation. CCK-8 assay also showed a similar outcome (Fig. 3D and 3E). Taken together, the above results indicate that YOD1 binds to NEDD4 for deubiquitination and regulates the function of NEDD4, but does not control the proteasomal degradation of NEDD4.

NEDD4 is activated by the K63-linked ubiquitin chain

Our deubiquitination assay demonstrated that K33 and K63-linked chains were reduced by YOD1 (Fig. 2D). We therefore performed FACS analysis to find polyubiquitin chains of NEDD4 that increase the function of NEDD4 (Fig. 4A). FACS analysis showed that K63-linked polyubiquitin chains of NEDD4 increase cell proliferation. Next, we evaluated the protein

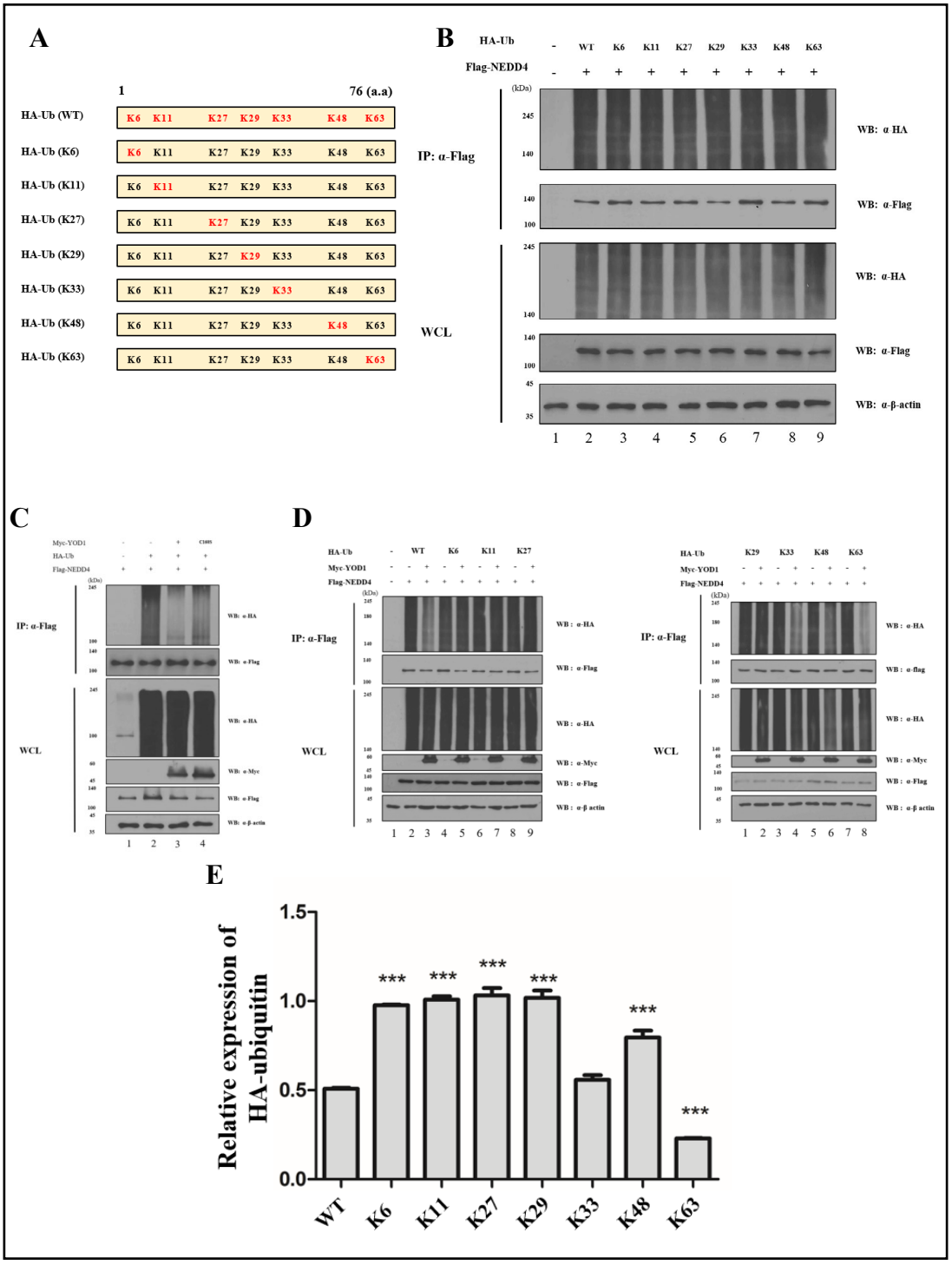

Fig. 2. (A) A diagram of specific ubiquitin constructs. (B) HA-Ub and specific mutant ubiquitin constructs were transfected into 293T cells. The cell lysates were immunoprecipitated with an anti-Flag antibody. (C) HA-Ub, Flag-NEDD4, Myc-YOD1, and Myc-YOD1 (C160S) were transfected into 293T cells. An anti-Flag antibody was used to immunoprecipitate Flag-NEDD4. (D) 293T cells were transfected with HA-Ub, specific mutant ubiquitin constructs, and Myc-YOD1. The cell lysates were used with an anti-Flag antibody. (E) To analyze the differential expression of HA-tagged ubiquitin with and without YOD1 expression, densitometric analysis was performed using Image J.

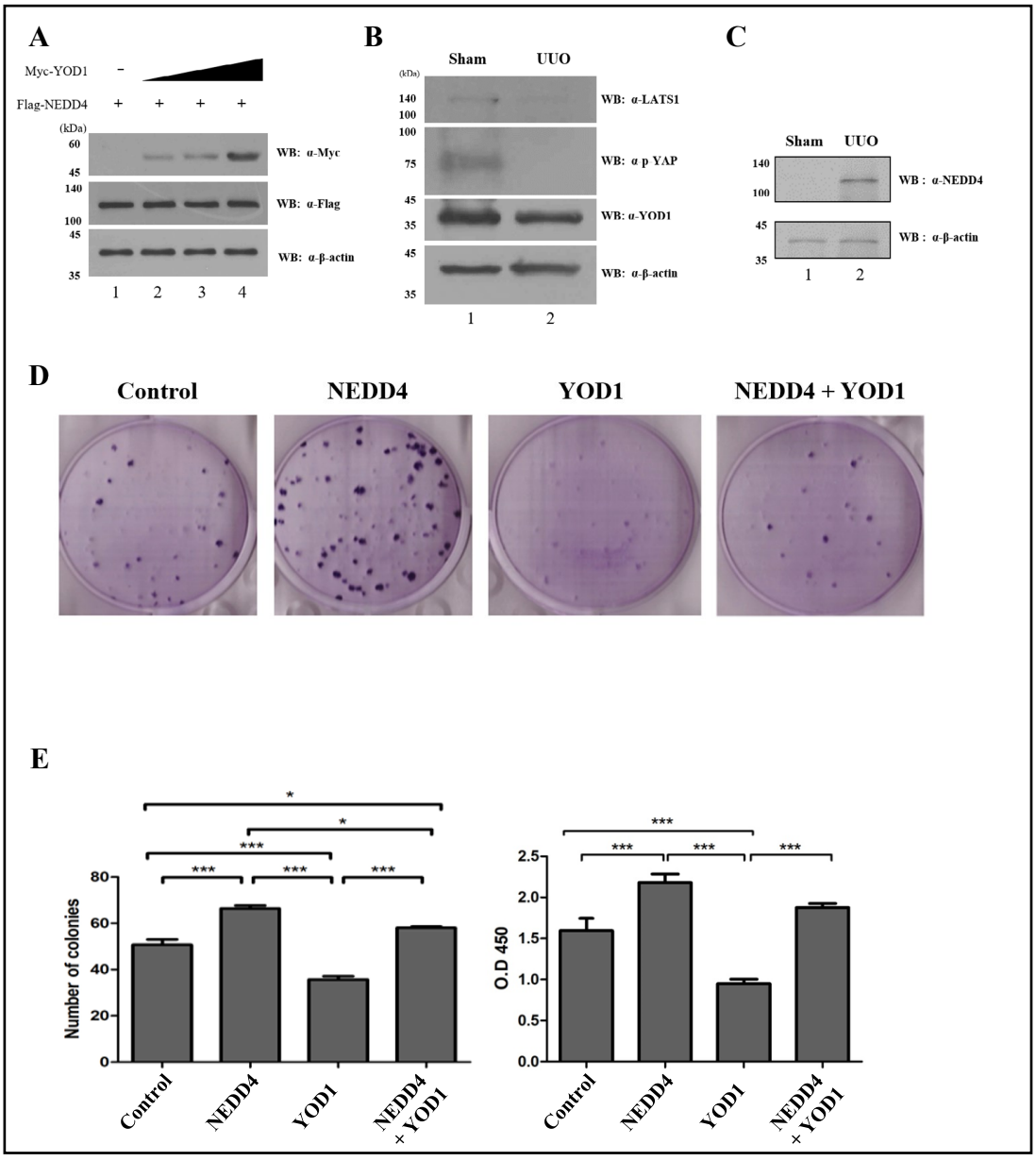

Fig. 3. (A) Flag-NEDD4 was expressed in 293T cells, and Myc-YOD1 was transfected in a dose-dependent manner. (B) Using mouse samples, Western blotting was performed to analyze the expression levels of YOD1, NEDD4, and p-YAP. (C) Using mouse samples, Western blotting was performed to analyze the expression levels of NEDD4. (D) The cell seeding density was 100 cells, grown in 100-mm dishes for 14 days, and stained with crystal violet. (E) 5,000 cells were transferred into 96-well plates. After 12 hrs, the cells were incubated with a medium containing CCK-8 for 4 hrs and OD was measured at 450 nm.

level of YAP and p-YAP using cells transfected with Ub (K63), NEDD4, and YOD1. Western blot analysis revealed that p-YAP protein levels are increased in the cells transfected with Ub (K63), NEDD4, and YOD1, compared to the cells transfected with just Ub (K63) and NEDD4 (Fig. 4B). This result supports the tendency that p-YAP increases as YOD1 increases and P-YAP decreases as YOD1 decreases. This suggests that when YOD1 increases, LATS increases phosphorylation of YAP. This demonstrates the need of K63-linked polyubiquitin chains to increase the activity of NEDD4.

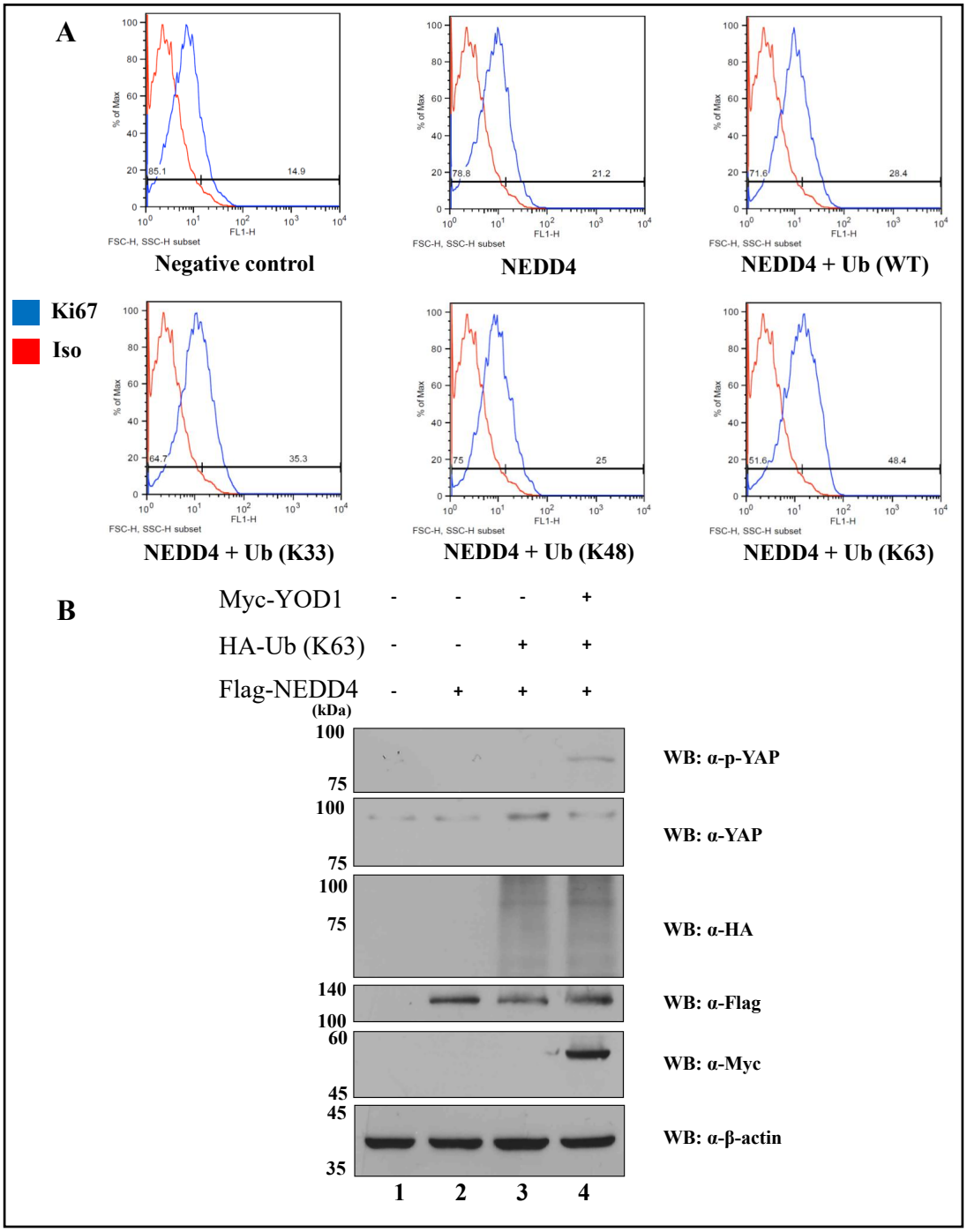

Fig. 4. (A) FACS analysis was performed using Ki67 Ab to confirm cell proliferation by polyubiquitin chain. (B) Western blot analysis for the K63-polyubiquitin chain of NEDD4 was performed to determine the effect of the Hippo signaling pathway.

Discussion

DUB acts as a ubiquitin releasing factor and is known to regulate the degradation of a number proteins. In addition, any abnormality in DUB is known to cause various diseases including cancer [34]. Over the years, various studies have investigated diseases triggered by aberrant functions of DUBs. To identify differential changes in DUB expression in the kidney during the onset of ureteral stones, we screened the changes in mRNA levels of DUBs in the UUO mouse model. We found that ureteral stones result in increased size of the kidney. It was therefore suggested that this phenomenon might be related to the Hippo signaling pathway, which is a key factor for organ size and tumor formation [35]. The pathway is also closely related to renal diseases such as polycystic kidney disease and renal cell carcinoma [36, 37]. NEDD4 is expressed in most tissues including the kidney [38]. It was found that YOD1 deubiquitinates and stabilizes E3 ligase ITCH, which mediates the Hippo signaling pathway through YAP/TAZ [39]. At low cell density, YOD1 is highly expressed and induces proteasomal degradation of ITCH and dephosphorylation of YAP/TAZ, leading to gene transcription for cell proliferation [39]. At high cell density, increased miR-21 expression level reduces the expression level of YOD1, which results in suppressing cell survival [39]. We have investigated the role of YOD1 in the kidney and have identified patterns that differ from previous studies. In the current study, we found downregulation of YOD1 in the kidney of mouse UUO model, and identified that YOD1 interacts with NEDD4, which regulates ubiquitination of LATS1 to inhibit the Hippo signaling pathway. NEDD4 is one of E3 ligases, such as ITCH, and is known to play a critical role in proliferation and metastasis of cancers [40]. In addition, NEDD4 is also involved in the Hippo signaling pathway [26]. Recent studies have reported the role of the Hippo signaling pathway in the kidney; hence, we decided to investigate the interaction between YOD1 and NEDD4 in the Hippo signaling pathway. We found that NEDD4 is ubiquitinated, whereas YOD1 decreases the ubiquitination level of NEDD4 but is unable to regulate the stability of NEDD4. It is of interest that NEDD4 is highly expressed in the kidney of UUO samples whereas the Sham model hardly revealed NEDD4 expression (Fig. 3C). Therefore, it is possible that a half-life of LATS can be regulated by both YOD1 and NEDD4. This synergistic effect may regulate the size of the kidney and the half-life of LATS in the UUO mouse (Fig. 1A and 3B).

In addition, an increase in the expression level of YOD1 leads to an increase in p-YAP (Fig. 4B), indicating that YOD1 regulates the function of NEDD4 to mediate the Hippo signaling pathway. YAP/TAZ is associated with proliferation, junction assembly, and metabolism in sprouting angiogenesis and barrier maturation [24]. Finally, overexpression of YOD1 suppresses cell proliferation and inhibits NEDD4-mediated cell proliferation, suggesting that YOD1 controls cell proliferation via inhibition of NEDD4. Our results achieved with YOD1 were consistent with the report that YOD1, which is the target of miR-373, suppresses cancer cell growth [41]. It is also known that miR-182 increases cell proliferation in renal cancer through YOD1 [42]. However, in contrast to the previous report, Kim et al. showed that YOD1 is required for transcriptional activity of YAP/TAZ and promotes cell survival [6]. Functions in other organs require more verification, but our data in kidneys show a similar pattern as reported by other studies. We think that it is possible for YOD1 to show opposing patterns in other organs compared to kidneys.

Moreover, we observed that YOD1 deubiquitinates K33- and K63-linked polyubiquitin chains of NEDD4. It was reported that different types of polyubiquitin chains regulate different signal transduction pathways. For example, K33-linked polyubiquitination of TGF-β receptor I promotes SAMD2/3 recruitment to the plasma membrane, which is inhibited by USP2a [43]. K63-linked polyubiquitin chains regulate cell proliferation via TRAF6 to modulate the TGF-β signaling pathway as well as cancer cell survival [44]. K48- and K63-branched polyubiquitination regulates NF-κB signaling [45]. Unlike the K48-linked polyubiquitin chain, the K63-linked polyubiquitin chain does not promote degradation of the protein [46]. For example, NEDD4 binds to the K63-linked polyubiquitin chain and is involved in molecular signaling, and K63-linked polyubiquitination is known for targeting the misfolded proteins

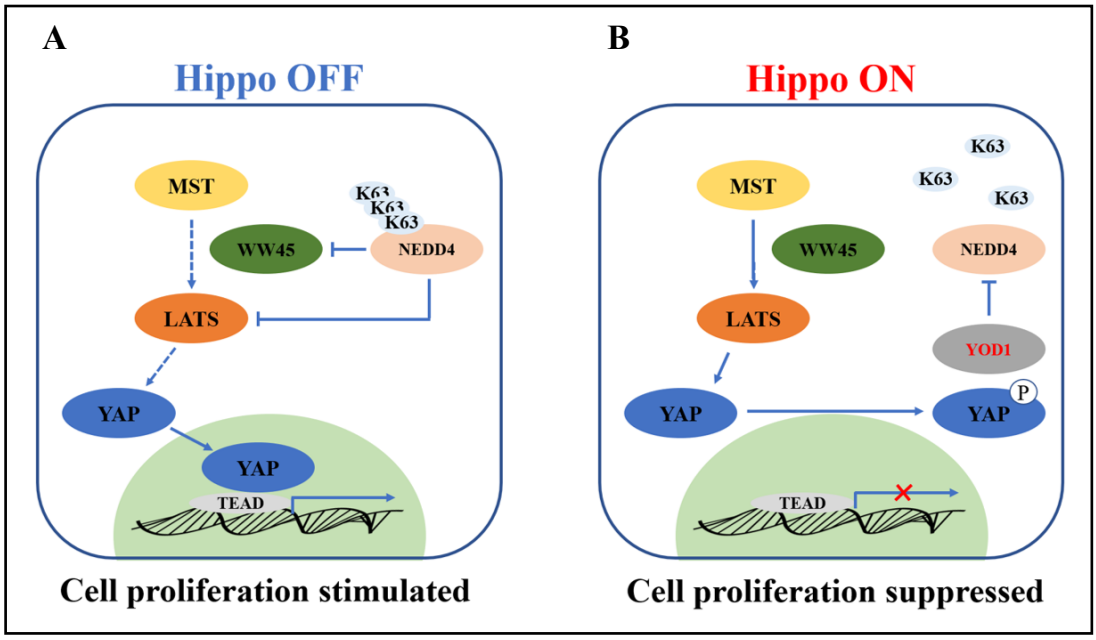

Fig. 5. The Hippo signaling pathway is mediated by a kinase cascade and regulates cell proliferation and apoptosis. WW45 and LATS protein levels are maintained at low levels by NEDD4-mediated degradation, which confirms the OFF state of Hippo signaling pathway. YOD1 activates the Hippo pathway through repression of NEDD4. When activity of NEDD4 is inhibited by YOD1, YAP is phosphorylated by LATS1. At this time, YAP cannot enter into the nucleus and cell proliferation is inhibited.

to remove them. Thus, although K48-linked polyubiquitination of NEDD4 was decreased by YOD1, the K48-linked polyubiquitin chains could be branched with K63-linked polyubiquitin chains of NEDD4, thus YOD1 might be capable of modulating ubiquitination of NEDD4 to regulate the Hippo signaling pathway and cell proliferation, but is unable to regulate NEDD4 stability (Fig. 5). In addition, YOD1 is also known to deubiquitinate K63 ubiquitin-linked proteins such as MAVS [47, 48]. Although there are many questions that have yet to be answered, identification of specific polyubiquitination at various lysine sites of ubiquitin is of great significance for the future investigation.

Conclusion

In conclusion, YOD1 binds to and deubiquitinates NEDD4. YOD1 is unable to regulate the protein degradation of NEDD4, but inhibits the E3 ligase activity of NEDD4 to increase the LATS1 protein level. As shown in Fig. 4, YOD1 may regulate the function of NEDD4 through K63-linked polyubiquitination. This result gives a new perspective of the YOD1 function in the Hippo signaling pathway. Currently, small molecule DUB inhibitors are being investigated as therapeutic agents for cancer [49]. Through the identification of these inhibitors and further studies with animal models, a strategy to treat kidney-related diseases, such as renal cell carcinoma, may be provided.

Abbreviations

DUB (deubiquitinating enzyme); USP (ubiquitin-specific protease); LATS1 (Large tumor suppressor kinase1); YAP (Yes associated protein-1); UUO (unilateral ureteric obstruction); NEDD4 (neural precursor cell expressed developmentally down-regulated protein 4).

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Statement of Ethics
The authors have no ethical conflicts to disclose.

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Author Contributions
JHP designed the research, performed the experiments and analyzed the data. SYK analyzed the data and wrote the manuscript. HJC and SYL designed the research and performed the experiments. KHB designed the research and, wrote and edited the manuscript. All authors have read and approved the final manuscript submitted for publication.

Disclosure Statement

The authors have no conflicts of interest to declare.

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