Nedd-4-2 is an archetypal HECT ubiquitin E3 ligase that disposes target proteins for degradation. Because of the proven roles of Nedd-4-2 in degradation of membrane proteins, such as epithelial Na\(^{+}\) channel, we examined the effect of Nedd-4-2 on the apical Ca\(^{2+}\) channel TRPV6, which is involved in transcellular Ca\(^{2+}\) transport in the intestine using the Xenopus laevis oocyte system. We demonstrated that a significant amount of Nedd-4-2 protein was distributed to the absorptive epithelial cells in ileum, cecum, and colon along with TRPV6. When co-expressed in oocytes, Nedd-4-2 and, to a lesser extent, Nedd4 down-regulated the protein abundance and Ca\(^{2+}\) influx of TRPV6 and TRPV5, respectively. TRPV6 ubiquitination was increased, and its stability was decreased by Nedd-4-2. The Nedd-4-2 inhibitory effects on TRPV6 were partially blocked by proteasome inhibitor MG132 but not by the lysosome inhibitor chloroquine. The rate of TRPV6 internalization was not significantly altered by Nedd-4-2. The HECT domain was essential to the inhibitory effect of Nedd-4-2 on TRPV6 and to their association. The WW1 and WW2 domains interacted with TRPV6 terminal regions, and a disruption of the interactions by D204H and D376H mutations in the WW1 and WW2 domains increased TRPV6 ubiquitination and degradation. Thus, WW1 and WW2 may serve as a molecular switch to limit the ubiquitination of TRPV6 by the HECT domain. In conclusion, Nedd-4-2 may regulate TRPV6 protein abundance in intestinal epithelia by controlling TRPV6 ubiquitination.

TRPV6 (transient receptor potential cation channels, vanilloid subfamily, subtype 6; also known as CaT1) is an epithelial Ca\(^{2+}\) channel expressed in the apical membrane of intestinal epithelial cells (1, 2). It mediates the first step of transcellular Ca\(^{2+}\) transport and is considered to be a gatekeeper for active Ca\(^{2+}\) absorption. The biosynthesis of TRPV6 is highly regulated by 1,25(OH)\(_2\) vitamin D\(_3\) at the transcriptional level (3). This is supported by the fact that multiple vitamin D-responsive elements have been identified in the TRPV6 gene promoter (4). Furthermore, a 90% reduction of duodenal TRPV6 mRNA was observed in vitamin D receptor knock-out (KO) mice (5). 1,25(OH)\(_2\) vitamin D\(_3\) levels were elevated in Trpv6 KO mice together with a 60% reduction in intestinal Ca\(^{2+}\) absorption, indicating a key role of vitamin D in regulating the synthesis of TRPV6 (6). TRPV6 is also induced by estrogens and the reproductive cycle at transcriptional levels (7). The trafficking and stability of TRPV6 are regulated by proteins such as S100A10-annexin II complex (8) and \(\beta\)-glucuronidase Klotho, respectively (9, 10). The serum- and glucocorticoid-induced kinases SGK1 and SGK3 have been reported to regulate TRPV6 (11). Our previous work showed that WNK3, a member of the WNK (with no lysine (K)) protein kinase family, increases complex glycosylated mature TRPV6 protein at the cell surface (12), whereas WNK4 has little effect (13). In contrast to biosynthesis, trafficking, and stability of TRPV6, little is known about the pathway via which TRPV6 protein is degraded. Identification of mechanisms responsible for TRPV6 degradation will provide new insights into the regulation of intestinal Ca\(^{2+}\) absorption.

Nedd-4-2 (neuronal precursor cell–expressed developmentally down-regulated 4-2) is an archetypal member of the E3 ligase family. It is well known for regulating cell surface stability of membrane proteins by the addition of ubiquitin chains to them, followed by endocytosis and lysosomal or proteasomal degradation (14–16). The best characterized Nedd-4-2 substrate is the epithelial Na\(^{+}\) channel (ENaC)\(^{4}\) (17, 18). Nedd-4-2 inhibits ENaC by binding to its PY motif (19). This down-regulation of ENaC is physiologically important because ENaC mutants lacking the PY motif disrupt Nedd-4-2 binding and cause Liddle syndrome, a hereditary hypertension caused by elevated ENaC stability/activity (17, 19, 20).

Nedd-4-2 is broadly expressed in many tissues, such as brain (21), lung (22), liver (21), prostate (23), kidney (22), and intestine (22, 24). In line with its distribution, it has a variety of membrane proteins as substrates. The ubiquitination mediated by Nedd-4-2 is required for the down-regulation of many membrane proteins, such as voltage-gated Na\(^{+}\) channel (25), neurotrophin receptor TrkA (26), potassium channel KCNQ (27, 28), Cl\(^{-}\) channel CIC-2 (29), Na\(^{+}\)-glucose co-transporter SGLT1 (30), and Na\(^{+}\)-phosphate co-transporter NaPi IIb (24). The list of ion channels/transporters regulated by Nedd-4-2 has grown longer since the identification of ENaC as a substrate of...
Nedd4-2. Nevertheless, physiologically relevant substrates of Nedd4-2 are yet to be identified.

Based on the role of Nedd4-2 in degradation of membrane proteins, we tested the effect of Nedd4-2 on TRPV6 in this study. We show that TRPV6 and Nedd4-2 are co-expressed in the intestinal tract and that TRPV6 is degraded by Nedd4-2 via the ubiquitination pathway. We also found that an interaction between TRPV6 and Nedd4-2 WW1 and WW2 domains plays a critical role in controlling the degradation of TRPV6.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The human TRPV6 cDNA was described previously (31). The human Nedd4 and Nedd4-2 cDNAs (hNedd4, BC152562; hNedd4-2, BC032597 and BC019345) were purchased from Open Biosystems, Inc. (Huntsville, AL). The cDNAs were subcloned into the Xenopus laevis oocyte expression vector pLN (13, 32). To obtain the full-length hNedd4-2, the Xhol/EcoRV fragment of BC019345 was used to replace the corresponding segment in BC032597, which lacks 1067–1198 bp in the Nedd4-2 open reading frame. The resultant cDNA was cloned into our pLN vector as the full-length human Nedd4-2. To detect protein expression of the Nedd4-2 construct lacking the homology to the E6-associated protein C terminus (HECT) domain and for the purpose of co-immunoprecipitation, a hemagglutinin epitope (HA tag) was added to the N terminus of Nedd4-2 using a PCR-based approach. The Nedd4-2 C922S mutant was generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer’s instructions and confirmed by sequencing.

Ca\(^{2+}\) Uptake in X. laevis Oocytes—The animal protocol used in this study was approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. In vitro transcription, injection of the resultant capped synthetic cRNAs into oocytes, and a \(^{45}\)Ca\(^{2+}\) uptake assay in oocytes were conducted as described previously (12). cRNAs were injected at 12.5 ng/oocyte unless stated otherwise. When a combination of two or three cRNAs was required, we mixed the cRNAs in such a way that the concentration of individual cRNAs was maintained. Defolliculated X. laevis oocytes were kept at 18 °C in 0.5× L-15 medium (Invitrogen) supplemented with 10 mM HEPES (pH 7.6), 5% heat-inactivated horse serum, penicillin at 10,000 units/liter, streptomycin at 10 mg/liter, and amphotericin B at 25 μg/liter. Two days after injection, uptake experiments were carried out at room temperature (24 °C) for a period of 30 min. Standard uptake solution contained 100 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\) (including \(^{45}\)CaCl\(_2\) at 10 μCi/ml), and 10 mM HEPES, pH 7.5. Oocytes were washed six times with ice-cold standard uptake solution without \(^{45}\)CaCl\(_2\) after a 30-min uptake. They were then dissolved in 10% SDS solution. The incorporated \(^{45}\)Ca\(^{2+}\) was determined using a scintillation counter. Ca\(^{2+}\) uptake data are presented as mean values from at least three experiments with 7–9 oocytes/group, using the S.E. as the index of dispersion.

Two-microelectrode Voltage Clamp—Voltage clamp experiments were performed as described previously (33). The resistance of microelectrodes filled with 3 M KCl was 0.5–5 megohms. In experiments involving voltage jumping or holding, currents and voltages were digitized at 0.3 and 200 ms/sample, respectively. After −3 min of stabilization of membrane potential following perfuion with the microelectrodes, the oocyte was clamped at a holding potential of −50 mV. Then 100-ms voltage pulses between −160 and +60 mV, in 20-mV increments, were applied, and steady state currents were obtained as the average values 80–95 ms after initiation of the voltage pulses. The standard perfusion solution contained 100 mM choline chloride, 2 mM KCl, 1 mM MgCl\(_2\), and 10 mM HEPES, pH adjusted to 7.5 with Tris base and HCl. Choline chloride was substituted with NaCl when Na\(^+\)-evoked currents were tested.

Western Blot Analysis—Two days after injection with different cRNAs, oocytes were washed with modified Barth’s solution (MBS). Oocytes were lysed with lysis buffer (100 mM NaCl, 20 mM Tris-Cl, 1% Triton X-100 plus protease inhibitor mixture, pH 7.6) at 20 μl/oocyte and centrifuged at 2,500 × g for 10 min at 4 °C to remove the cellular debris and yolk proteins. Cell lysates were subjected to SDS-PAGE (7.5% acrylamide). In general, extract supernatant corresponding to one oocyte was loaded per lane. After SDS-PAGE, the proteins were transferred to PVDF membranes. Membranes were blocked with PBS containing 0.5% Tween 20 and 5% nonfat milk for 1 h at room temperature. Primary antibodies were incubated overnight at 4 °C, followed by multiple washes in PBS-Tween 20. Monoclonal anti-HA antibody (product H9658, 1:5,000) was purchased from Sigma-Aldrich. The rabbit anti-TRPV6 antisem (1:3000) was customer-raised with a synthetic peptide antigen corresponding to amino acids 359–377 of the first extracellular loop of TRPV6 by Open Biosystems, Inc. This antisem specifically recognizes TRPV6 without cross-reaction with TRPV5. The anti-Nedd4-2 antibody (ab46521, 1:2,000) was purchased from Abcam, Inc. (Cambridge, MA). The anti-β-actin antibody (sc-47778, 1:5,000) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, Santa Cruz Biotechnology, Inc.) were incubated in the blocking solution for 1 h at room temperature, followed by multiple washes with PBS-Tween 20. Chemiluminescence was detected using a SuperSignal West Femto maximum sensitivity substrate kit (Pierce) in accordance with the manufacturer’s protocol.

Surface Expression and Internalization Measurement—Surface expression of TRPV6 was detected by an anti-TRPV6 antiserum, which recognizes the extracellular loop of TRPV6 using an approach described previously (12). Water-injected oocytes and oocytes injected with cRNA for TRPV6 alone or with Nedd4-2 were incubated at 18 °C for 2 days. The oocytes were then blocked in MBS with 1% BSA at 4 °C for 60 min, labeled with our anti-TRPV6 antiserum (1:10,000) in 1% BSA at 4 °C for 30 min, and washed at 4 °C (two times for 15 min each in MBS with 1% BSA; four times for 5 min each in MBS). For measuring the endocytosis rate of TRPV6, each group of oocytes was then incubated in MBS at room temperature to allow internalization of antibody-labeled surface TRPV6 proteins. At each time point (0, 15, and 30 min), oocytes were transferred to ice-cold MBS. After that, all of the oocytes were fixed in PLP fixative solution (2% paraformaldehyde, 0.075 m lysine, 0.037 m sodium phosphate buffer, and 0.01 m NaIO\(_4\), pH 7.4) at 4 °C for 1 h. The
fixed oocytes were washed with ice-cold MBS for 5 min and then with ice-cold MBS with 1% BSA for 15 min three times. The oocytes were incubated with HRP-coupled secondary antibody (sc-2004, goat anti-rabbit, 1:20,000, Santa Cruz Biotechnology) in 1% BSA for 30 min, followed by extensive wash with MBS at 4 °C (seven times for 5 min each). Individual oocytes were placed in 25 μl of SuperSignal West Pico chemiluminescent substrate (Pierce) and incubated at room temperature for exactly 1 min. Chemiluminescence was quantified using an FB12 single tube luminometer (Berthold Detection Systems, Pforzheim, Germany) and recorded by FB12/Sirius software. Background chemiluminescence signals from control oocytes were subtracted, and the levels of signal at time 0 were defined as 100%. Chemiluminescence signals were plotted as a function of time, and the curves were fitted using linear regression with SigmaPlot 10 (Systat Software, Chicago, IL). The slopes of the curves represent the rate of TRPV6 internalization.

Co-immunoprecipitation—HA-Nedd4-2 and TRPV6 were co-expressed in X. laevis oocytes. Two days after injection, oocytes were treated with 25 μM MG132 in 0.5× L-15 medium for 5 h. Oocytes were then washed with modified Barth’s solution and lysed with lysis buffer at 20 μl/oocyte and centrifuged at 2,500 × g for 10 min at 4 °C to remove the cellular debris and yolk proteins. Samples were precleared by adding Protein A/G-agarose (sc-2003, Santa Cruz Biotechnology, Inc.) to remove nonspecific agarose binders from the protein mixture. Our customer-made TRPV6 antiserum (1:20) was then added to the lysates and incubated at 4 °C for 2 h. Next, Protein A/G-agarose was added, and samples were incubated overnight at 4 °C. Immunoprecipitated proteins bound to the agarose beads were washed three times using PBS, and proteins were eluted from the beads by boiling the samples in loading buffer. The eluted samples were analyzed for the presence of HA-Nedd4-2 by Western blot analysis with anti-HA antibody.

Purification of GST Fusion Protein—Nedd4-2 C2, WW1, WW2, WW3, WW4, and HECT domains were amplified by PCR and then subcloned into pGEX-6P-1 vector (Amersham Biosciences) and transferred into competent Escherichia coli BL21 by the heat shock method. After incubation at 37 °C overnight, a one-fiftieth volume culture was transferred to 25 ml of fresh LB medium with 10 mg/ml ampicillin. Fusion protein expression was induced by adding IPTG to a final concentration of 0.1 mM after 0.5 h of induction. 35S-Labeled protein probes were synthesized using TNT® SP6 High-Yield Master Mix following the manufacturer’s instructions (Promega, Madison, WI).

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The translation products were analyzed by SDS-PAGE and radioautography.

To prepare membranes with Nedd4-2 domains or the TRPV6 C-terminal region, each GST fusion protein was separated by SDS-PAGE (12% acrylamide) and was transferred to PVDF membranes by standard procedures. The membranes were blocked at room temperature for 2 h in Blotto solution (20 mM HEPES, pH 7.5, 25 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.05% Nonidet P-40, 5% nonfat dry milk). Then the membranes were incubated in Hyb 75 (20 mM HEPES, pH 7.5, 75 mM KCl, 2.5 mM MgCl2, 1 mM CaCl2, 1 mM dithiothreitol, 0.05% Nonidet P-40, 1% nonfat dry milk) for 15 min at room temperature. 35S-Labeled probes were diluted in 3 ml of Hyb 75. Far Western experiments were carried out at 4 °C with gentle rocking for 4–5 h or overnight. The membranes were washed for 15 min three times with Hyb 75 and then air dried and exposed to x-ray films.

RNA Isolation and RT-PCR—Intestinal tissues were harvested from healthy adult rats immediately after anesthesia. Approximately 1.5–2 cm tissues were excised from duodenum, jejunum, ileum, cecum, and colon. Tissues were washed by ice-cold PBS to remove food residues. Using TRIzol reagent, total RNA was isolated according to the instructions of the manufacturer (Invitrogen). One microgram of total RNA was reverse-transcribed with oligo(dT) primer and the SuperScript III first strand kit (Invitrogen). A housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), served as a control gene to verify the consistency of reverse transcription. The rat TRPV6 fragment was amplified by PCR using the following primers: 5′-CATCTTCCAGACAGAGGCC-3′ (1527–1547 of open reading frame, sense) and 5′-TTAGTC-TGTACTCCAGCCT-3′ (2184–2162, antisense). For rat Nedd4-2, the primers were 5′-CTGAAAGCCTATGGTT-CAG-3′ (2332–2350, sense) and 5′-TTATACACCTCTC-GAAGCC-3′ (2892–2872, antisense). For rat Nedd4-4, the primers used were 5′-GACATGAGGGCTGATAGTG-3′ (1984–2005, sense) and 5′-CTAACAAACCATCAG-3′ (2670–2650, antisense). Amplification reactions were performed with Premix fTaq (Takara) for 25 cycles using Mastercycler (Eppendorf, Westbury, NY).

Immunofluorescent Staining—Fresh rat intestinal tissues were cut into 0.2–0.3 cm pieces. X. laevis oocytes were washed with Barth’s solution 48 h after injection. Tissues and oocytes were fixed in PLP fixative at room temperature for 2 h. After the removal of PLP fixative, the tissues were incubated in PBS solution with 15% sucrose at 4 °C overnight. Tissues and oocytes were embedded in optimal cutting temperature compound and frozen in –42 °C cold 2-methylbutane. The tissues were sectioned at 6-μm thickness using a Leica Reichert Jung Frigocut 2800 cryostat (Leica Instruments GmbH, Heidelberg, Germany). After being fixed in cold acetone at –20 °C for 20 min and washed with PBS (pH 7.5) at room temperature for 15 min, sections were incubated with blocking solution (PBS with 0.5% Triton X-100 and 5% BSA) at room temperature for 1 h. Sections were then incubated with anti-Nedd4-2 or TRPV6 antiserum (1:100 dilution in blocking solution) in a humidified chamber overnight at 4 °C. After being washed with PBS (pH 7.5) three times for 5 min each, sections were incubated with...
goat anti-rabbit IgG-FITC antibody (sc-2012, 1:500, Santa Cruz Biotechnology, Inc.) or Alexa Fluor 594 goat anti-rabbit IgG (H+L) (A11012, 1:1,000, Invitrogen) in a humidified chamber at room temperature for 2 h in darkness. Photographs were captured by using a Leica DC500 12-megapixel camera and Leica DMIRB fluorescence microscope (Leica Microsystems, Heerbrugg, Switzerland) with an FITC filter (blue, 450–490 nm/LP515) for Nedd4-2 and then with a rhodamine filter (green, 515–560 nm/LP590) for TRPV6.

RESULTS

Nedd4-2 Co-localizes with TRPV6 in Rat Intestine—Compared with the well known distribution of Nedd4-2 in the kidney, less is known about its distribution in the intestinal tract. We thus investigated the distribution pattern of Nedd4-2 in the rat intestinal tract and compared it with that of TRPV6. First, we used RT-PCR approach to assess the mRNA level of Nedd4-2, Nedd4, and TRPV6 in rat intestinal tract tissue. As shown in Fig. 1A, Nedd4-2 mRNA was strongly expressed in ileum, cecum, and colon and weakly expressed in duodenum and jejunum. In contrast, the mRNA level of Nedd4, another E3 HECT family member, did not vary significantly along the intestinal tract. TRPV6 was strongly expressed in duodenum, cecum, and colon, weakly expressed in jejunum, and undetectable in ileum (Fig. 1A). This is consistent with the TRPV6 mRNA distribution pattern described previously with Northern blot and in situ hybridization approaches (1). Next, we validated the Nedd4-2 and TRPV6 antisera. As shown in Fig. 1B, the antiserum against Nedd4-2 recognized only Nedd4-2 but not Nedd4 exogenously expressed in X. laevis oocytes by Western blot analysis. A band with the same size as Nedd4-2 and two bands of lower molecular weight were recognized by this antiserum in rat colon lysate. Immunofluorescent staining of oocytes injected with cRNAs for TRPV6, Nedd4-2, Nedd4, or water (as controls) with antiserum against TRPV6 and Nedd4-2, respectively. TRPV6 and Nedd4-2 antiserum recognized TRPV6 and Nedd4-2 expressed in the plasma membrane and cytoplasm of oocytes without specific staining in water or Nedd4 cRNA-injected oocytes. A low level of endogenous Nedd4-2 in X. laevis oocytes was detected by this antiserum as well (Fig. 1B). The Nedd4-2 antiserum also recognized a band of identical molecular weight to Nedd4-2 and its splice variants. The TRPV6 antiserum, but not the preimmune serum, recognized signals along the plasma membrane and inside oocytes expressing TRPV6 exogenously, but no specific signal was detectable in the control oocytes (Fig. 1C). Nedd4-2 antiserum recognized Nedd4-2 along the plasma membrane and intracellularly, and no specific signal was detectable in Nedd4-expressing and control oocytes (Fig. 1C). Thus, these two antisera were well suited for detecting TRPV6 and Nedd4-2, respectively, by immunofluorescent staining. As previously reported in mouse intestine (2), TRPV6 protein was detected in all of the segments of the rat intestine tested, with the strongest expression in the cecum and colon (Fig. 1D). Although TRPV6 mRNA was not detected by RT-PCR (25 cycles) in the ileum (Fig. 1A), TRPV6 protein was detectable by immunostaining in the same segment (Fig. 1D, top). A similar phenomenon was reported in mice using a dif-
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Different antibody (2). Because we did detect TRPV6 mRNA expression in ileum by RT-PCR at higher cycle numbers (35–40 cycles; data not shown), it is likely that TRPV6 protein was more stable, or the translation efficiency of TRPV6 was high in the ileum. Parallel immunofluorescent staining experiments were performed with Nedd4-2 antiserum using adjacent sections of intestinal tissues (Fig. 1D). Consistent with the level of mRNA (Fig. 1A), Nedd4-2 proteins were strongly expressed in ileum, cecum, and colon, moderately in jejunum, and weakly in duodenum (Fig. 1D). Whereas TRPV6 exhibited staining at the plasma membrane, Nedd4-2 showed both membrane and intracellular distribution. Nedd4-2 and TRPV6 were highly colocalized in the cecum, colon, and ileum (Fig. 1D).

Nedd4-2 Decreases TRPV6 Activity and Protein Level—We tested the effects of Nedd4-2 on TRPV6-mediated Ca2+ uptake by performing co-expression studies in X. laevis oocytes. When TRPV6 was expressed in oocytes alone, it mediated a robust increase in Ca2+ uptake over the control oocytes (injected with water) at 2 days after injection. When co-expressed with Nedd4-2, TRPV6-mediated Ca2+ uptake was decreased by 47.9 ± 1.4% compared with oocytes expressing TRPV6 alone (Fig. 2A, top). Nedd4, another ubiquitin E3 ligase, also reduced TRPV6-mediated Ca2+ uptake by 28.3 ± 1.8% (Fig. 2A). Measuring TRPV6 Ca2+ current in X. laevis oocytes is hindered by the endogenous Ca2+-activated Cl− channel; however, in the absence of extracellular Ca2+, TRPV6 is permeable to Na+, and therefore TRPV6-mediated Na+ current can be used as a measure of TRPV6 activity (12). The Na+ current mediated by TRPV6 was also decreased by Nedd4-2 and, to a lesser extent, by Nedd4, as assessed by voltage clamp (Fig. 2B). Consistent with the reduction in TRPV6 activity, TRPV6 protein level was also decreased by Nedd4-2 and, to a lesser extent, by Nedd4 (Fig. 2A, lower panel). Different N-glycosylated forms of TRPV6 could be detected by Western blot analysis (12, 13); the two bands below 75 kDa represent unglycosylated and core-glycosylated forms, and the group of bands higher than 75 kDa represent complex-glycosylated forms. Nonspecific bands, especially the one that corresponds to the unglycosylated TRPV6, may be detected in control oocytes, although their abundance varied from batch to batch. Nedd4-2 and Nedd4 decreased both bands of TRPV6 bands but had little effect on nonspecific bands (Fig. 1B).

We also determined the effects of Nedd4-2 and Nedd4 on TRPV5 (Fig. 2, C and D). TRPV5 is another epithelial channel that shares 75% amino acid identity with TRPV6 (35, 36). Unlike TRPV6, TRPV5 is specifically expressed in the distal convoluted tubule and connecting tubule, where it is responsible for the fine tuning of Ca2+ reabsorption (37, 38). In the presence of Nedd4-2 or Nedd4, TRPV5-mediated Ca2+ uptake decreased by 51.9 ± 1.7 or 26.2 ± 4.8% (Fig. 2C, top). TRPV5-mediated Na+ currents were also similarly decreased by Nedd4-2 and Nedd4, respectively (Fig. 2D). Consistent with the decreased activity of TRPV5, the protein level of TRPV5 was decreased by Nedd4-2 and Nedd4 correspondingly (Fig. 2C, bottom). In all cases, Nedd4-2 exhibited stronger inhibitory effects than Nedd4 on both TRPV6 and TRPV5.

Nedd4-2 inhibited TRPV6 dose-dependently. TRPV6 protein level decreased gradually as the level of Nedd4-2 protein increased (Fig. 3A). Similarly, TRPV6-mediated Ca2+ uptake was further decreased as the level of Nedd4-2 increased (Fig. 3B). We used 12.5 ng/oocyte of Nedd4-2 cRNA in the following experiments because this dosage of Nedd4-2 exhibited a maximal inhibitory effect on TRPV6, although Nedd4-2 exerted a significant effect on TRPV6-mediated Ca2+ influx at a cRNA level as low as 3.2 ng/oocyte (Fig. 3B).

Nedd4-2 Decreases TRPV6 Stability—Because Nedd4-2 can target proteins for degradation, we determined the effects of Nedd4-2 on TRPV6 stability. We examined the stability of TRPV6 protein by treating the oocytes with 100 μg/ml cycloheximide, an inhibitor of protein synthesis, for 0, 3, 6, or 9 h at 2 days after injection. Lysates were analyzed by Western blot with the TRPV6 antiserum, and the band densities were quantified by Gel-Pro Analyzer 4.0 software (Media Cybernetics, Singapore).
Inc., Bethesda, MD). The amount of protein was loaded within a linear range of detection, as determined with different loading levels of lysates from oocytes expressing TRPV6 by Western blot analysis (Fig. 4B). Ned4-2 significantly increased the TRPV6 degradation rate (Fig. 4A). After cycloheximide treatment for 9 h, 69.6 ± 5.3% of the total TRPV6 was degraded by Ned4-2, whereas only 33.3 ± 9.9% of TRPV6 protein was degraded in the absence of Ned4-2 (Fig. 4C).

Nedd4-2 Increases Ubiquitination of TRPV6—To confirm the effect of ubiquitin ligase activity of Ned4-2 on TRPV6, we co-injected HA-ubiquitin and TRPV6 with or without Ned4-2. Twenty-four hours after injection, oocytes were treated with 25 μM MG132, a proteasome inhibitor, and then harvested 5 h later. After the immunoprecipitation by anti-HA antibody, ubiquitinated TRPV6 was detected with antibody against HA. Ubiquitination of TRPV6 was dramatically increased by Ned4-2 (Fig. 5A).

Ligase activity of E3 ligase family members roots in the HECT domain (14), and mutation of a conserved cysteine (amino acid 922 in human Ned4-2) in this domain results in ubiquitin ligase deficiency (39). To determine the role of Ned4-2 ubiquitin ligase activity in the degradation of TRPV6, we mutated cysteine 922 to serine (C922S). The effects of wild-type and C922S Ned4-2 on TRPV6 were compared in the X. laevis oocyte system. The C922S mutant lost its ability to inhibit TRPV6 (Fig. 5B). Thus, the inhibitory effect of Ned4-2 on TRPV6 was dependent on its ubiquitin ligase activity.

Nedd4-2 Increases the Proteasomal Degradation of TRPV6—The regulation of TRPV6 by Ned4-2 could be via monoubiquitination and endocytosis or polyubiquitination and proteasomal degradation. We do not know the nature of the ubiquitination of TRPV6 by Ned4-2 because both polyubiquitination of a single lysine and monoubiquitination of multiple lysines on TRPV6 can result in the high molecular weight bands in Fig. 5A. We thus evaluated the endocytosis rate of TRPV6 and the pathway for TRPV6 degradation induced by Ned4-2.

Using an antiserum against part of the first extracellular loop of TRPV6, we were able to detect TRPV6 expressed at the surface of oocytes. In the presence of Ned4-2, surface-expressed TRPV6 was decreased by 73.5 ± 7.7% (Fig. 6A). The rates of TRPV6 endocytosis were 3.2 ± 0.6 and 2.5 ± 0.3%/min in the presence and absence of Ned4-2, respectively (Fig. 6B). They were not significantly different. The presence of lysosome inhibitor chloroquine (100 μM) had no effect on the action of
Nedd4-2 Interacts with TRPV6 Directly—To further demonstrate a direct interaction between Nedd4-2 and TRPV6, a Far

Nedd4-2, whereas proteasome inhibitor MG132 (25 μM) alleviated the inhibitory effects of TRPV6 Ca\(^{2+}\) uptake and TRPV6 protein abundance (Fig. 6C).

HECT Domain Is Necessary for the Association between Nedd4-2 and TRPV6—We next assessed which domain of Nedd4-2 is essential for its action on TRPV6. Nedd4-2 consists of an N-terminal C2 (calcium-dependent lipid binding) domain, followed by four WW domains, which interact with target proteins, and a C-terminal ubiquitin ligase HECT domain. We deleted C2, WW1-2, WW3-4, and HECT domains individually and evaluated the effect of each construct on TRPV6 using the X. laevis oocyte system (Fig. 7, A and B). Deletion of the C2 (amino acids 1–166) or WW1-2 domain (amino acids 194–398) resulted in an enhanced inhibitory effect on TRPV6. Deletion of the WW3-4 domain (amino acids 478–561) slightly alleviated the inhibitory effect, whereas removal of the HECT domain (amino acids 618–955) completely abolished the inhibitory effect of Nedd4-2 on TRPV6. In fact, TRPV6-mediated Ca\(^{2+}\) uptake was significantly increased by the HECT-deleted Nedd4-2 construct over the control group expressing TRPV6 alone, indicating that this construct may have a dominant negative effect on the endogenous Nedd4-2 because a Nedd4-2-like band was detected in control oocytes (Fig. 1B). It is worth noting that the WW1-2-deleted construct made the oocytes unhealthy at the regular dose of cRNA injection (12.5 ng of cRNA/oocyte). At 1.6 ng of cRNA/oocyte, the action of this construct was much more effective on TRPV6 than the wild-type Nedd4-2 (Fig. 7B).

An association with TRPV6 might be necessary for the action of Nedd4-2 on TRPV6. TRPV6 and Nedd4-2 are co-expressed in the intestine (Fig. 1D), and a physical association between TRPV6 and Nedd4-2 is possible although no PY motif was identified in TRPV6. We first evaluated the association between Nedd4-2 and TRPV6 using a co-immunoprecipitation approach. Oocytes were co-injected with TRPV6 and HA-Nedd4-2 and treated with 25 μM MG132 for 5 h 1 day after injection. The equal expression of HA-Nedd4-2 in the
Western approach was employed. \(^{35}\)S-Labeled N-terminal (amino acids 1–327) and C-terminal (amino acids 578–725) regions of TRPV6 were synthesized in vitro (Fig. 8C). GST fusion Nedd4-2 C2 region (amino acids 1–166, including C2 domain 21–124), WW1-2 region (amino acids 167–438), WW3-4 region (amino acids 439–590), and HECT region (amino acids 591–955, including the HECT domain 618–955) probed with \(^{35}\)S-labeled TRPV6 N-terminal (top) or C-terminal region (middle). Purified GST fusion proteins stained with Coomassie Blue are shown at the bottom. Note that the bands with lower molecular weights were present in lanes loaded with WW1-2 and HECT fusion proteins (top bands), respectively. The nature of these bands was unknown, but they may represent degraded products. B, autoradiography of individual GST fusion Nedd4-2 WW domains probed with \(^{35}\)S-labeled TRPV6 N-terminal (top) or C-terminal (middle) region. Purified GST fusion WW domains stained with Coomassie Blue are shown at the bottom. C, autoradiography of in vitro synthesized \(^{35}\)S-labeled TRPV6 N-terminal region (1–327 amino acids) and C-terminal region (578–725 amino acids) that were used as probes for A and B. D, autoradiography of GST fusion C-terminal region of TRPV6 (amino acids 597–725; the fusion protein was close to the 43 kDa top band) probed with \(^{35}\)S-labeled TRPV6 C-terminal (amino acids 591–955, including the HECT domain 618–955) probed with \(^{35}\)S-labeled TRPV6 N-terminal (top) or C-terminal region (middle). Purified GST fusion proteins stained with Coomassie Blue are shown at the bottom. B, D204H mutation in WW1 significantly attenuated the interaction between the TRPV6 N-terminal and WW1 domain. C, D204A mutation in WW1 also attenuated the interaction between the TRPV6 N-terminal region and the WW1 domain. The H539D mutation in the WW4 domain increased the interaction between the TRPV6 N-terminal region and WW4 domain. His\(^{184}\) in WW4 is the counterpart of Asp\(^{204}\) in WW1. D, D376H mutation in the WW2 domain also attenuated the interaction between the TRPV6 N-terminal region and WW2 domain. Asp\(^{376}\) in WW2 is the counterpart of Asp\(^{204}\) in WW1.

Because the C-terminal region of TRPV6 interacted more strongly with the HECT domain, which is critical for the regulation of TRPV6 by Nedd4-2, we evaluated the interactions between the C-terminal region and the Nedd4-2 constructs lacking C2, WW1-2, WW3-4, and the HECT region, respectively (Fig. 8D). Deletion of the C2 or the WW3-4 region attenuated the interaction; deletion of WW1-2 increased the interaction; deletion of the HECT region greatly reduced the interaction. This is consistent with the co-immunoprecipitation result showing the importance of the HECT domain in its association with TRPV6 (Fig. 7C).

Asp\(^{204}\) Is Essential to the Interaction between WW1 and TRPV6 N-terminal Region—We next set out to identify residues in the WW1 and WW2 domains critical for their interaction with TRPV6 cytoplasmic terminal regions. To this end, the amino acid sequences of four WW domains were aligned (Fig. 9A). Five amino acid residues in WW1 that are conserved in WW1-2 but not in WW3-4 domains were mutated to their corresponding residues in WW3-4. As shown by Far Western analysis, binding between the TRPV6 N-terminal region and the WW1 domain was largely unaffected when Tyr\(^{216}\), Asn\(^{213}\), His\(^{222}\), and Arg\(^{223}\) of WW1 were mutated to their correspond-
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Ca\(^{2+}\) increased the ability of Nedd4-2 to reduce TRPV6-mediated uptake result (Fig. 10), ubiquitination of TRPV6 was increased compared with the wild-type Nedd4-2. Details of the experiments were similar to those described in the legend to Fig. 5A. Error bars, S.E.

**DISCUSSION**

Degradation is as important as the biosynthesis of a protein in maintaining a functional protein pool in the cell. The degradation of TRPV6 probably plays a role in regulating TRPV6-mediated active Ca\(^{2+}\) absorption. However, little is known about the proteins involved in TRPV6 degradation. In this study, we provided some evidence that the ubiquitin E3 ligase Nedd4-2 plays a role in TRPV6 degradation. We showed that Nedd4-2 is co-expressed with TRPV6 in the rat intestinal epithelial cells. TRPV6 activity was reduced by Nedd4-2 due to the decreased TRPV6 protein abundance. Decreased TRPV6 stability and increased TRPV6 ubiquitination were also observed in the presence of Nedd4-2. The interaction between the HECT domain and the C-terminal region of TRPV6 is essential for the association between the two proteins and the action of Nedd4-2 on TRPV6. In addition, we identified Asp\(^{204}\) as a critical amino acid residue in the WW1-TRPV6 interaction, which is involved in controlling the efficiency of TRPV6 protein ubiquitination and degradation by Nedd4-2.

Nedd4-2 has been shown to be expressed in human small intestine (24). We further described the distribution of Nedd4-2 in various segments of the rat intestinal tract at both mRNA and protein levels. In the small intestine, both Nedd4-2 mRNA and protein levels increase gradually from the proximal to the distal small intestine, with the highest level in the ileum. This is opposite to TRPV6, which shows a decreasing abundance from proximal to distal small intestine. In contrast, the Nedd4 mRNA level is relatively constant along the small intestine. The opposite pattern of TRPV6 and Nedd4-2 expression gradients in the small intestine may further strengthen the high level expression of TRPV6 in the proximal intestine by exerting a minimal effect in this segment. In contrast to the small intestine, both TRPV6 and Nedd4-2 are very abundant in cecum and colon at both mRNA and protein levels. Therefore, TRPV6 in
the large intestine is more likely subjected to regulation by Nedd4-2. The complementary distribution pattern between TRPV6 and Nedd4-2 and the stronger effect of Nedd4-2 on TRPV6 suggest a more significant role of Nedd4-2 in regulating TRPV6, although this does not exclude Nedd4 as a potentially important regulator of TRPV6 and TRPV5.

The effect of Nedd4-2 on TRPV6 appears to be similar to those of Nedd4-2 on other membrane transporters, receptors, and ion channels (14). As an archetypal E3 ligase, Nedd4-2 is well known for regulating cell surface stability of the channels by attaching ubiquitin chains to them, followed by endocytosis and lysosomal or proteasomal degradation (14–16). Under the regulation of Nedd4-2, TRPV6 is ubiquitinated and, in turn, degraded via an MG132-sensitive proteasome pathway. We found no evidence that this process occurs only to TRPV6 proteins in the plasma membrane because the rate of TRPV6 endocytosis is not significantly affected by Nedd4-2. Although we cannot exclude the possibility that Nedd4-2 can mediate monoubiquitination on TRPV6, these data suggest that Nedd4-2 mediates polyubiquitination of TRPV6 and facilitates its degradation via a proteasome pathway.

WW domains mediate most of the binding of the Nedd4-like family members with their substrates. The well known binding motif of WW domains within their target proteins, such as ENaC (17), Notch (40), Snap3 (41), KCNQ1 (28), or TrkA neurotrophin receptor (26), is the proline-based recognition sequence PXXY (PY motif). The binding of PY motif to Nedd4-2 mostly occurs at the WW3 and WW4 domains (42, 43). However, not every protein directly binds with the WW domain of Nedd4-type ubiquitin E3 ligases through a PY motif. A chemokine receptor, CXCR4, which has no PY motif, binds with the WW1 or WW2 domains of E3 ubiquitin ligase AIP4 via a 324/325 double serine in its C-terminal region (44). PTEN even interacts with the C2 and HECT domains of Nedd4-4 (45). Nedd4-2 interacts with TRPV6 with different domains, including the C2, WW, and HECT domains.

Although all domains of Nedd4-2 tested exhibited interactions with either the N-terminal, the C-terminal, or both regions of TRPV6, the HECT domain appears to be the most critical domain for the association between Nedd4-2 and TRPV6, as demonstrated by co-immunoprecipitation and Far Western approaches. The C2 domain exhibited a significant interaction with the C-terminal region of TRPV6. The increased inhibition of TRPV6 by the ΔC2 construct (Fig. 7A) suggests that the effect of Nedd4-2 on TRPV6 may be Ca$^{2+}$-dependent. Most Nedd4-2-targeted proteins interact with its WW3-4 domains. Interestingly, our study revealed that the N-terminal region of TRPV6 interacts with WW1 and WW2 domains, a situation similar to the interaction between CXCR4 and AIP4 (44). However, the interaction between WW1-2 domains and TRPV6 is not indispensable for the association between Nedd4-2 and TRPV6, because other domains, such as the HECT and C2 domains, significantly interact with the TRPV6 C-terminal region.

Our study revealed an important role of the WW1-2 domains in Nedd4-2 function and regulation. WW1-2 exhibited the strongest interaction with the TRPV6 N-terminal region, although it also interacted moderately with the TRPV6 C-terminal region. The removal of WW1-2 or the disruption of the interaction between WW1-2 and TRPV6 terminal regions by the D204H/D376H mutation each resulted in an increase in inhibitory effects on Ca$^{2+}$ uptake and protein level of TRPV6 (Figs. 7 and 10). A decrease in association with TRPV6 was observed in the D204H/D376H mutant (Fig. 11); however, this does not necessarily imply a reduction in the interaction between HECT domain and TRPV6. On the contrary, the removal of the WW1-2 region increased the interaction between the TRPV6 C-terminal region and Nedd4-2 possibly through the HECT domain. The increased TRPV6 ubiquitination by the D204H/D376H mutant is consistent with an increase in efficiency of Nedd4-2 action on TRPV6. Thus, WW1 and WW2 probably act as a switch. When they bind to TRPV6 terminal regions, the interaction between HECT domain and TRPV6 C-terminal region is limited, and therefore, TRPV6 is ubiquitinated at a low speed; when WW1 and WW2 dissociate from TRPV6 terminal regions, the interaction between the HECT domain and the TRPV6 C-terminal region increases. This turns on the turbo mode of Nedd4-2 and, as a consequence, the efficiency of TRPV6 ubiquitination increases. In addition, the WW1-2 region may also regulate the E3 ligase activity of Nedd4-2 not specifically to TRPV6 because we have noticed that the expression of the ΔWW1-2 construct and, to a lesser extent, the D204H/D376H mutant alone rendered the oocytes unhealthy, although the situation worsened when TRPV6 was co-expressed.

Our data suggest that Nedd4-2 may regulate TRPV6 ubiquitination and degradation at different rates by regulating their interaction. The physiological cues that may strengthen or weaken these interactions are yet to be identified. Like Nedd4-2, Nedd4 appears to inhibit TRPV6 albeit to a lesser extent. Both Nedd4 and Nedd4-2 also inhibit TRPV5. The physiological significance of these regulations warrants further studies.

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