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UBC9 regulates cardiac sodium channel $Na_v1.5$ ubiquitination, degradation and sodium current density

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Abstract

Voltage-gated sodium channel $Na_v1.5$ is critical for generation and conduction of cardiac action potentials. Mutations and expression level changes of $Na_v1.5$ are associated with cardiac arrhythmias and sudden death. The ubiquitin (Ub) conjugation machinery utilizes three enzyme activities, E1, E2, and E3, to regulate protein degradation. Previous studies from us and others showed that Nedd4–2 acts as an E3 ubiquitin-protein ligase involved in ubiquitination and degradation of $Na_v1.5$, however, more key regulators remain to be identified. In this study, we show that UBC9, a SUMO-conjugating enzyme, regulates ubiquitination and degradation of $Na_v1.5$. Overexpression of UBC9 significantly decreased $Na_v1.5$ expression and reduced sodium current densities, whereas knockdown of UBC9 expression significantly enhanced $Na_v1.5$

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Author contributions

B.T., Q.C. and Q.K.W. designed the study; B.T., Y.H., Z.W., C.C., P.W., C.X., L.L., H.X., and C.L. performed experiments; Q.C., Q.K.W., C.X., B.T., Y.H., Z.W., C.C., P.W., L.L., H.X., and C.L. analyzed and/or interpreted the data; B.T., Q.C. and Q.K.W. drafted the manuscript; Q.C. and Q.K.W. critically revised the manuscript; all authors approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

expression and increased sodium current densities, in both HEK293 cells and primary neonatal cardiomyocytes. Overexpression of UBC9 increased ubiquitination of $Na_v1.5$, and proteasome inhibitor MG132 blocked the effect of UBC9 overexpression on $Na_v1.5$ degradation. Co-immunoprecipitation showed that UBC9 interacts with Nedd4–2. UBC9 with mutation C93S, which suppresses SUMO-conjugating activity of UBC9, was as active as wild type UBC9 in regulating $Na_v1.5$ levels, suggesting that UBC9 regulates $Na_v1.5$ expression levels in a SUMOylation-independent manner. Our findings thus identify a key structural element of the ubiquitin-conjugation machinery for $Na_v1.5$ and provide important insights into the regulatory mechanism for ubiquitination and turnover of $Na_v1.5$.

Keywords

UBC9; SCN5A; $Na_v1.5$ sodium channel; ubiquitination; Nedd4-2; arrhythmia

1. Introduction

$Na_v1.5$ is the α-subunit of the voltage-gated cardiac sodium channel and encoded by the *SCN5A* gene. $Na_v1.5$ is essential for the initiation of the cardiac action potential (AP) and conduction of electrical impulses [1–4]. The important role of $Na_v1.5$ has been exemplified by the discovery of more than 300 naturally occurring genetic mutations linked to various cardiac arrhythmias and sudden death, including Brugada syndrome (BrS) [5], long QT syndrome (LQTS) [3, 4, 6], and sick sinus syndrome [7–9]. As a plasma membrane protein, the expression level of $Na_v1.5$ on the cell surface is critical for its function because cell electrical excitability depends not only on its own activation but also on its expression levels [10].

$Na_v1.5$ degradation has been reported to be associated with Nedd4–2, a key component of the ubiquitin-proteasome system (UPS) [11, 12]. The UPS is an important degradation mechanism of cellular proteins including voltage-gated channels [13, 14]. Ubiquitin (Ub) is a small protein that can be covalently linked to a substrate protein [15]. The UPS contains ubiquitin, Ub-activating enzyme (E1), Ub-conjugating enzyme E2, and Ub-protein ligase E3, which together make membrane proteins mono- or poly-ubiquitinated and degraded [14, 15]. It was previously reported that $Na_v1.5$ contains the PY-motif (xPPxY), which can interact with the WW-domain of Nedd4–2, a ubiquitin-protein ligase (E3) characterized by the presence of a C-terminal HECT catalytic domain [16]. Ubiquitination is a prerequisite for endocytosis and degradation of plasma membrane proteins [15]. The ubiquitination of $Na_v1.5$ can be regulated by Nedd4–2, which leads to internalization and degradation of $Na_v1.5$ [16, 17].

UBC9 is a small ubiquitin-like modifier-conjugating enzyme E2 that helps ligation of SUMO to the substrate during SUMOylation [18, 19]. Like ubiquitination, SUMOylation is a post-translational modification process involved in protein quality control [18, 19]. UBC9 has been reported to participate in protein quality control and interact with some ubiquitin E3 ligases, such as muscle-specific RING finger 1 [20, 21]. In addition to SUMOylation, UBC9 also regulates gene expression through SUMOylation-independent pathways [22].

In this study, we assessed the effects of UBC9 on the regulation of the level of $Na_v1.5$. Surprisingly, we found that UBC9 regulates $Na_v1.5$ degradation in a SUMOylation-independent manner. We found that UBC9 regulated the ubiquitination of $Na_v1.5$ and cardiac sodium current densities in both a heterologous HEK293 cell expression system and neonatal cardiomyocytes. Moreover, we found that UBC9 interacted with Nedd4–2, which mediates $Na_v1.5$ degradation through the UPS. Therefore, we identified UBC9 as a key regulator of the Ub-conjugation machinery regulating $Na_v1.5$ ubiquitination and degradation.

2. Materials and methods

2.1. Plasmids, mutagenesis, siRNAs and t-CSM peptide

The expression construct for human cardiac sodium channel gene *SCN5A* in vector pcDNA3 (pcDNA3-SCN5A) was previously described [5, 6, 23–27]. The coding region of $Na_v1.5$ was excised from pcDNA3-SCN5A by restriction enzyme digestion and subcloned into the pIRES-EGFP vector, generating pEGFP-$Na_v1.5$. The *UBC9* gene was amplified by RT-PCR analysis from RNA samples from HEK293 cells and subcloned into the pCMV-HA vector to generate the pCMV-UBC9 plasmid. The *UBB* gene encoding ubiquitin was amplified by RT-PCR analysis from RNA samples from HeLa cells and subcloned into the pCMV-MYC vector, generating pCMV-MYC-UBB. All expression plasmids were verified by direct DNA sequencing analysis.

The C93S mutation was created in pCMV-UBC9 by an overlapping extension PCR mutagenesis method [28, 29], generating pCMV-UBC9-C93S. The sequences of *UBC9* siRNAs are UBC9-siRNA1 (Sense-GGAAUACAGGAACUUCUAA; Antisense-UUAGAAGUUCCUGUAUUCC), UBC9-siRNA2 (Sense-GCAGAGGCCUACACGAUUU; Antisense-AAAUCGUGUAGGCCUCUGC), and UBC9-siRNA3 (Sense-GGGAAGGAGGCUUGUUUAA; Antisense-UUAAACAAGCCUCCUUCCC). The t-CSM peptide (YGRKKRRQRRRGKMDENQ) was manufactured and purified by Genscript (CHINA) and was dissolved in double-distilled water.

2.2. Cell culture and transfection

A HEK293 cell line with stable expression of $Na_v1.5$ (HEK293-$Na_v1.5$) was described previously [23]. HEK293 and HEK293-$Na_v1.5$ cells were cultured in a DMEM medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, USA) and filtered by 0.22 μm membranes. Cells were incubated at 37°C in a humidified chamber with 5% CO_2.

Transfection of plasmid DNA (varying amount) and siRNA (10 nM) was carried out using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. Cells were seeded in 6-well plates overnight, and transfection was carried out when cells approached 80% confluence.

2.3. Real-time quantitative RT-PCR analysis

Total RNA was extracted from cultured cells using Trizol regent (TakaRa, USA), and real-time RT-PCR analysis was carried out with the FastStart Universal SYBR Green Master

(Roche, USA) as previously described [30–32]. The sequences of RT-PCR primers are Foward-ACCATTATTTCACCCGAATGTGT and Reverse-CTCGGACCCTTTTCTCGTACT for *UBC9*, and Forward-CACTGTGCCCATCTACGA and Reverse-GTAGTCAGTCGAGTCCCG for *ACTB* encoding β-actin.

2.4. Isolation of neonatal rat cardiomyocytes

Isolation of neonatal cardiomyocytes was carried out as previously described [33–35]. In brief, hearts were excised from eleven 0–3 day old Sprague-Dawley rats and washed in PBS to remove blood cells. Then, the heats were minced into small pieces and incubated in isolation buffer (0.05% collagenase B from Roche and 0.05% trypsin from Amresco in 1 × PBS). The isolated cardiomyocytes were cultured in the DMEM supplemented with 20% FBS for 24 h, and co-transfected with the pCMV-UBC9 expression plasmid or the pCMV-HA empty vector together with a GFP plasmid using Lipofectamine™ 3000 (Invitrogen, USA) according to the manufacture's instruction. Transfection of siRNAs was also carried out using Lipofectamine™ 3000. The study with animals was approved by the ethics committee of Huazhong University of Science and Technology.

2.5. Western blotting and co-immunoprecipitation (Co-IP) analyses

Cells were transfected for 48 hours, and lysed in lysis buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and protease inhibitor cocktail) (Roche, USA)) to prepare cell extracts for Western blot analysis and Co-IP analysis as described previously [17, 36, 37]. A rabbit anti-Na_V1.5 antibody (Alomone Labs, Jerusalem BioPark) was used at a dilution factor of 1:1000. A mouse anti-tubulin (Millipore, USA) antibody was used at a dilution factor of 1:5000. A goat anti-rabbit HRP-conjugated secondary antibody and a goat anti-mouse HRP-conjugated secondary antibody were all from Millipore (USA) and used at a dilution factor of 1:20,000. A rabbit anti-UBC9 antibody (Santa Cruz, USA) was used at a dilution factor of 1:1000. A mouse anti-Myc antibody (MBL, JAPAN) was used at a dilution factor of 1:1000. A rabbit anti-Na,K-ATPase antibody (Cell Signaling Technology, USA) was used at a dilution factor of 1:1000. A mouse anti-FLAG antibody, a rabbit anti-HA antibody and a rabbit anti-GFP antibody were all from MBL (JAPAN). The goat anti-rabbit IgG and goat anti-mouse IgG were from Santa Cruz Biotechnology. The anti-ubiquitin mouse monoclonal antibody FK2 (BMLPW8810, Enzo Life Science) was used at a dilution factor of 1:800.

2.6. Isolation of cell-surface proteins and analysis of expression level of cell surface Na_V1.5

Transfected cells were washed three times with ice-cold PBS, resuspended in 5 ml Buffer I (25 mM Tris-HCl, pH7.5, 5 mM EDTA, 5 mM EGTA), sonicated on ice using a 45W sonicator with a setting of 20 cycles of 3 seconds on and 5 seconds off (JY-92-IIN SCIENTZ, CHINA), and centrifuged at 13,000 x g at 4°C for 10 minutes. Supernatant was collected and centrifuged at 100,000 x g for 1 hour at 4°C. The pellets were resolved in Buffer II (20 mM Tris-HCl, pH7.4, 137 mM NaCl, 10% v/v glycerol, 1% v/v Nonidet P-40), incubated at 4°C for 30 minutes, and then centrifuged at 13,000 x g at 4°C for 5 minutes. The supernatant contains the cell membrane proteins, and used for Western blot analysis with an anti-$Na_v1.5$ antibody and an anti Na,K-ATPase antibody as control (Cell signaling technology, USA) as described previously [26, 27].

2.7. Proteasome inhibitor study

HEK293 cells were co-transfected with plasmids pcDNA3-SCN5A, PCMV-UBC9, or empty vector PCMV-HA for 36 hours. Cells were treated with MG132 (a potent, reversible, and cell-permeable proteasome inhibitor) for 12 hours, and then harvested for Western blot analysis as described above.

2.8. Determination of ubiquitination of $Na_v1.5$

HEK293 cells at 70%–80% confluence in a 10 cm plate were transiently co-transfected with pcDNA3-$Na_v1.5$ and pCMV-Myc-UBB plasmids with or without pCMV-HA-UBC9 for 48 hours, and lysed. The lysates were centrifuged at 13,000 x g at 4°C for 30 minutes. The supernatant was incubated with 2 μg of anti-$Na_v1.5$ antibody or anti-GFP antibody on a rotator at 4°C overnight and then mixed with 30 μl of Protein A/G PLUS-agarose (Santa Cruz Biotechnology, USA). The antibody-protein A/G PLUS agarose complex was incubated on a rocker at 4°C for 2 hours, centrifuged at 1000 x g for 2 minutes, and washed three times with lysis buffer. The washed pellets were resuspended in 50 μl of 1 x SDS loading buffer containing 5% (V/V) β-mercaptoethanol and then electrophoresed through SDS-polyacrylamide gels for Western blot analysis.

2.9. Electrophysiological studies

Patch-clamping experiments were carried out as previously described [5, 26, 27, 32, 38, 39]. The pipette solution contained 20 mM NaCl, 130 mM CsCl, 10 mM EGTA, and 10 mM HEPES (pH 7.2 adjusted with CsOH). The bath solution contained 70 mM NaCl, 80 mM CsCl, 5.4 mM KCl, 2 mM CaCl2, 10 mM HEPES, 10 mM glucose, and 1 mM MgCl2 (pH 7.4 adjusted with CsOH).

2.10. Statistical analysis

All data were from at least three independent experiments and expressed as means ± SEM. Statistical analysis was performed using Student's *t*-tests. A *P*-value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Overexpression of UBC9 downregulates the $Na_v1.5$ protein level

To analyze the potential role of *UBC9* in $Na_v1.5$ expression, we co-transfected pCMV-UBC9 and pcDNA3-SCN5A plasmids into HEK293 cell. Over-expression of *UBC9* led to a significant decrease in $Na_v1.5$ expression levels (Fig. 1A). Similar results were obtained from HEK293-$Na_v1.5$ cells (with stable expression of $Na_v1.5$) transfected with pCMV-UBC9 (Fig. 1B). To determine whether the effects of *UBC9* on $Na_v1.5$ expression was dose-dependent, we co-transfected HEK293 cells with pcDNA3-SCN5A and different amounts of pCMV-UBC9. Western blot analysis showed that the amount of $Na_v1.5$ decreased when the amount of pCMV-UBC9 increased in a dose-dependent manner (Fig. 1C). To determine whether *UBC9* affects the amount of cell surface expression of $Na_v1.5$, we performed Western blot analysis with plasma membrane extracts. Fig. 1D showed that overexpression of *UBC9* led to a significant decrease of plasma membrane $Na_v1.5$.

3.2. Overexpression of UBC9 reduces sodium current densities in HEK293-$Na_v1.5$ cells

To investigate the functional role of *UBC9* in $Na_v1.5$ physiology, whole-cell sodium currents were recorded in HEK293-$Na_v1.5$ cells transfected with pCMV-UBC9 or pCMV-HA empty vector. We found that overexpression of *UBC9* significantly decreased densities of sodium currents compared to the empty vector control (n>10, p=0.0087) (Figs. 2A and 2B). However, there were no significant changes in the time-dependence curves of recovery from inactivation (Fig. 2C) or voltage-dependent kinetics of activation and inactivation of sodium currents between cells with *UBC9* overexpression and those with the control plasmid (Fig. 2D).

3.3. Knockdown of UBC9 upregulates $Na_v1.5$ protein levels

To determine whether knockdown of *UBC9* affects $Na_v1.5$ expression levels, we tested the effectiveness of three different siRNAs against *UBC9* in HEK293-$Na_v1.5$ cells. After 48 hours of transfection, the gene expression level of *UBC9* was quantified by real time RT-PCR analysis. Fig. 3A showed that compared to the control siRNA (NC), UBC9 siRNA2 and siRNA3, but not siRNA1, significantly decreased *UBC9* mRNA levels. Western blot analysis further confirmed the knockdown of UBC9 by siRNA2 and siRNA3 (Fig. 3B). When *UBC9* was knocked down by siRNA2 or siRNA3, the expression level of $Na_v1.5$ was significantly elevated compared to control NC-siRNA (Fig. 3C). On the other hand, siRNA1 did not reduce *UBC9* expression, and thus did not affect the expression level of $Na_v1.5$ compared to control NC-siRNA (Fig. 3C).

3.4. Knockdown of UBC9 increases sodium current densities in HEK293-$Na_v1.5$ cells

To determine the effects of knockdown of *UBC9* on the function of $Na_v1.5$, *UBC9* siRNA3 was transfected into HEK293-$Na_v1.5$ cells, and whole-cell sodium currents (I_{Na}) were recorded. Compared to NC siRNA, *UBC9* siRNA led to a significant increase of I_{Na} densities (n>10, p=0.0386) (Figs. 4A and 4B). Knockdown of *UBC9* did not affect the time-dependence recovery from inactivation (Fig 4C) and voltage-dependent activation and inactivation kinetics of I_{Na} (Fig 4D).

3.5. Knockdown or overexpression of UBC9 regulates $Na_v1.5$ sodium current densities in neonatal rat cardiomyocytes

To determine whether UBC9 regulates $Na_v1.5$ and I_{Na} in cardiomyocytes, we isolated neonatal cardiomyocytes from rats, which were transfected with pCMV-UBC9 or control pCMV-HA empty vector. Whole-cell sodium currents were then recorded. Cardiomyocytes with overexpression of *UBC9* showed significantly decreased I_{Na} densities compared to cells with the empty vector control (p=0.0445, n>10) (Fig. 5A and 5B). To determine the effects of knockdown of *UBC9* on the function of $Na_v1.5$ and I_{Na} in cardiomyocytes, *UBC9*-siRNA was transfected into neonatal rat cardiomyocytes, and whole-cell sodium currents were recorded. Compared to NC siRNA, knockdown of UBC9 expression by *UBC9* siRNA led to a significant increase of I_{Na} densities (p=0.0326, n>10) (Fig. 5C and 5D).

3.6. t-CSM peptide does not affect I_{Na} densities

As previously reported, UBC9 can interact with CRMP2, and thus affect the $Na_v1.7$ sodium current [40]. CRMP2 (Collapsin Response Mediator Protein 2) is a microtubule-binding protein necessary for regulating neuronal polarity, axon/dendrite fate and axonal outgrowth [41]. To determine whether the effect of UBC9 on $Na_v1.5$ is through the UBC9-CRMP2 interaction, we used the t-CSM peptide to block the UBC9-CRMP2 interaction [40]. T-CSM is a tat-conjugated cell-penetrating peptide containing the SUMOylation consensus site of CRMP2 fused to the transduction domain of the HIV-1 tat protein [40]. HEK293-$Na_v1.5$ cells were treated with or without 20 μM of t-CSM for 12 h, and whole-cell sodium currents were recorded. Compared to the control, the t-CSM peptide did not affect the $Na_v1.5$ sodium current (Fig. 6A and 6B). Identical results were obtained in HEK293-$Na_v1.5$ cells with overexpression of UBC9 (Fig. 6C and 6D). These data suggest that UBC9 does not affect the $Na_v1.5$ function through the UBC9-CRMP2 interaction.

3.7. SUMO-conjugase activity is unnecessary for regulation of $Na_v1.5$ expression levels by UBC9

Since UBC9 is a well-characterized SUMO-conjugase, we examined if SUMOylation is involved in downregulation of $Na_v1.5$ by UBC9. We created the mutant UBC9-C93S, which was reported to suppress the SUMO-conjugating activity of endogenous UBC9 via a mutation of a cysteine residue critical for SUMO-conjugating activity [22, 42]. Wild type UBC9 or mutant UBC9 with mutation C93S was co-expressed with GFP-tagged p53 and HA-tagged SUMO1 in HEK293 cells. GFP-tagged p53 was immunoprecipitated using an anti-GFP-antibody, and the precipitates were analyzed using immunoblotting with an anit-HA antibody recognizing HA-SUMO1. As shown in Fig. 7A (left panel), overexpression of UBC9 increased the level of SUMOylated-p53, however, the effect was blocked by the C93S mutation. A similar observation was made with Western blot analysis using cell extracts and an anti-HA antibody (Fig. 7, right panel). However, no effect was observed for mutant UBC9-C93S on downregulation of $Na_v1.5$ levels by wild type UBC9 (Fig. 7B). These data suggest that UBC9 regulates $Na_v1.5$ expression levels in a SUMO-independent manner.

3.8. UBC9 regulates the expression level of $Na_v1.5$ through a ubiquitination-proteasome pathway

Because UBC9 does not reduce $Na_v1.5$ expression levels via SUMOylation, we determined whether the proteasome pathway was involved in UBC9 regulation of $Na_v1.5$ degradation using MG132, a potent and reversible proteasome inhibitor. Overexpression of *UBC9* in HEK293-$Na_v1.5$ cells reduced the level of $Na_v1.5$ protein, however, the reduction was blocked by MG132 treatment with a 10 μM concentration (Fig. 8A). As UBC9 regulation of $Na_v1.5$ levels was through proteasomal degradation, we analyzed the level of ubiquitination of $Na_v1.5$ by UBC9. We used the anti-$Na_v1.5$ and anti-GFP antibodies to immunoprecipitate $Na_v1.5$ and GFP-$Na_v1.5$, respectively, in HEK293 cells with co-overexpression of $Na_v1.5$ and Myc-ubiquitin (Myc-UB) with or without *UBC9* overexpression (Fig. 8B) or cells with co-overexpression of GFP-$Na_v1.5$ and Myc-UB and with or without *UBC9* overexpression

(Fig. 8C). We then used an anti-Myc antibody for immunoblotting to detect ubiquitin-Na_v1.5. Na_v1.5 is approximately 250 kDa, however, the ubiquitinated Na_v1.5 molecules were displayed in a large area of higher molecular weight than the unmodified Na_v1.5. Fig. 8B and 8C showed that the amount of ubiquitinated-Na_v1.5 was clearly increased in cells overexpressing *UBC9*. These results suggest that UBC9 can stimulate the ubiquitination of Na_v1.5.

To further demonstrate the effect of UBC9 on ubiquitination of Na_v1.5, we performed a uniquitin assay using FK2, an anti-ubiquitin mouse monoclonal antibody. HEK293-Na_v1.5 cells were transfected with an overexpression plasmid for UBC9 or an empty vector as control, and treated with MG132. The cell lysates were immunoprecipitated using an anti-Na_v1.5 antibody or a control rabbit IgG, and Western blot analysis was performed with the precipitates and FK2. As shown in Fig. 8D, overexpression of *UBC9* increased the level of ubiquitinated-Na_v1.5. The data with FK2 further show that UBC9 can stimulate the ubiquitination of Na_v1.5.

3.9. UBC9 interacts with Nedd4–2

Nedd4–2 was shown to be a candidate E3 ligase for the Ub-conjugation machinery involved in regulation of Na_v1.5 degradation [16]. We tested the hypothesis that UBC9 interacts with Nedd4–2 to regulate the ubiquitination of Na_v1.5. To test the hypothesis, we co-expressed HA-UBC9 and FLAG-Nedd4–2 in HEK293-Na_v1.5 cells, and performed co-immunoprecipitation (Co-IP) analysis. An anti-HA antibody recognizing HA-UBC9 successfully immunoprecipitated FLAG-Nedd4–2 recognized by an anti-FLAG antibody (Fig. 9A). Reciprocal Co-IP showed that an anti-FLAG antibody recognizing FLAG-Nedd4–2 precipitated UBC9 recognized by an anti-UBC9 antibody (Fig. 9B). Overexpression of UBC9 did not affect the expression level of Nedd4–2 (Fig. 9C). These data demonstrate that UBC9 interacts with Nedd4–2.

4. Discussion

Post-translational modifications by Ub regulate protein turnover and degradation involved in numerous cellular processes [15]. The Ub conjugation system is a complex machinery composed of three basic enzymatic activities, E1, E2, and E3, which mediate the transfer of Ub via an E1–E2–E3 cascade to various protein substrates to form polyUb chains [15]. The protein substrates carrying polyUb chains are targeted to the 26S proteasome for degradation [15]. In mammalian cells, there are two E1 activating enzymes, about 40 E2 Ub conjugating enzymes, and >600 E3 ubiquitin ligases [19, 43]. This generates high heterogeneity for the ubiquitin system so that the degradation of numerous protein substrates can be properly regulated. However, little is known about the specifics of ubiquitin enzymes involved in ubiquitination of Na_v1.5. van Bemmelen et al [16] showed that an E3 ubiquitin ligase Nedd4–2 was able to bind to the PY-motif at C-terminus of Na_v1.5, increased Na_v1.5 ubiquitination and reduced cardiac sodium current densities. These data suggest that Nedd4–2 is an E3 ubiquitin ligase of the Ub conjugation machinery involved in ubiquitination of Na_v1.5. Interestingly, we recently found that the activity of Nedd4–2 can be modulated by heat-shock chaperon αB-crystallin [16]. αB-crystallin showed interaction with Nedd4–2, decreased ubiquitination of $Na_v1.5$, and reduced internalization of plasma membrane $Na_v1.5$, resulting in an increased level of cell surface $Na_v1.5$ and increased cardiac sodium current densities although the detailed molecular mechanism remains to be further defined [16].

In the present study, we found that UBC9 is involved in the ubiquitination of $Na_v1.5$. UBC9 is the single E2 enzyme of the SUMOylation machinery, which is also a post-translational modification system composed of three E1, E2, and E3 enzymatic activities [18]. Different from the Ub conjugation system, SUMOylation is mediated by only one heterodimeric E1 enzyme (SAE1/SAE2), a single E2 enzyme (UBC9), and a limited number of E3 ligases [18].

To study the role of SUMOylation in regulation of $Na_v1.5$ levels, we overexpressed UBC9 in HEK293-$Na_v1.5$ cells and knocked its expression down, and then analyzed their impact on the level of $Na_v1.5$ and densities of cardiac sodium currents. Our results showed that overexpression of UBC9 significantly decreased the protein levels of $Na_v1.5$ channels (Fig. 1) and reduced cardiac sodium current densities (Fig. 2). Moreover, knockdown of UBC9 expression significantly increased the protein levels of $Na_v1.5$ channels (Fig. 3) and increased cardiac sodium current densities (Fig. 4). Most importantly, overexpression and knockdown of UBC9 affected cardiac sodium current densities in neonatal rat cardiomyocytes (Fig. 5). UBC9 was previously shown to interact with CRMP2 to regulate the function of $Na_v1.7$. However, our studies with the t-CSM peptide that disrupts the UBC9-CRMP2 interaction showed that the UBC9-CRMP2 interaction did not affect the $Na_v1.5$ function with or without overexpression of UBC9 (Fig. 6).

The cysteine residue at codon 93 of UBC9 was shown to be required for the formation of a thiol ester bond with SUMO1, and a dominant negative mutation at the site, pCys93S, disrupts a thiol ester bond formation and SUMOylation of target proteins [42]. We showed that mutant UBC9 with the C93S mutation inhibited the SUMOylation of p53 (Fig. 7A), however, it did not affect the function of UBC9 regulation on the level of $Na_v1.5$ (Fig. 7B). The data suggest that the impact of UBC9 on the level of $Na_v1.5$ and densities of cardiac sodium currents is not associated with SUMOylation of $Na_v1.5$. We further showed that UBC9 interacts with Nedd4–2, which supports a role of UBC9 in $Na_v1.5$ ubiquitination (Fig. 9). Moreover, we found that UBC9 promoted $Na_v1.5$ ubiquitination and degradation through 26S proteasomes (Fig. 8). Altogether, our data implicate UBC9 as the E2 conjugating enzyme for the Ub-conjugating system.

In conclusion, the data in this study indicate that UBC9 promotes the ubiquitination and degradation of $Na_v1.5$ and regulates cardiac sodium current density. Because overexpression and knockdown of *UBC9* significantly affect cardiac sodium current densities, agents or tools that can modulate *UBC9* expression and function may help treatment or therapeutic interventions of human diseases associated with $Na_v1.5$ mutations and altered expression.

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Highlights

- UBC9 overexpression decreases $Na_V1.5$ expression and sodium current densities.
- Knockdown of UBC9 expression enhances $Na_V1.5$ expression and sodium current densities.
- UBC9 promotes $Na_V1.5$ ubiquitination and degradation, but not SUMOylation.
- UBC9 interacts with NEDD4–2, an E3 ligase for ubiquitination and degradation of $Na_V1.5$.
- UBC9 is a key structural element of the ubiquitin-conjugation machinery for $Na_V1.5$.
- *UBC9* may be a target for developing treatment or therapeutic interventions of human diseases associated with Nav1.5 mutations and altered expression.

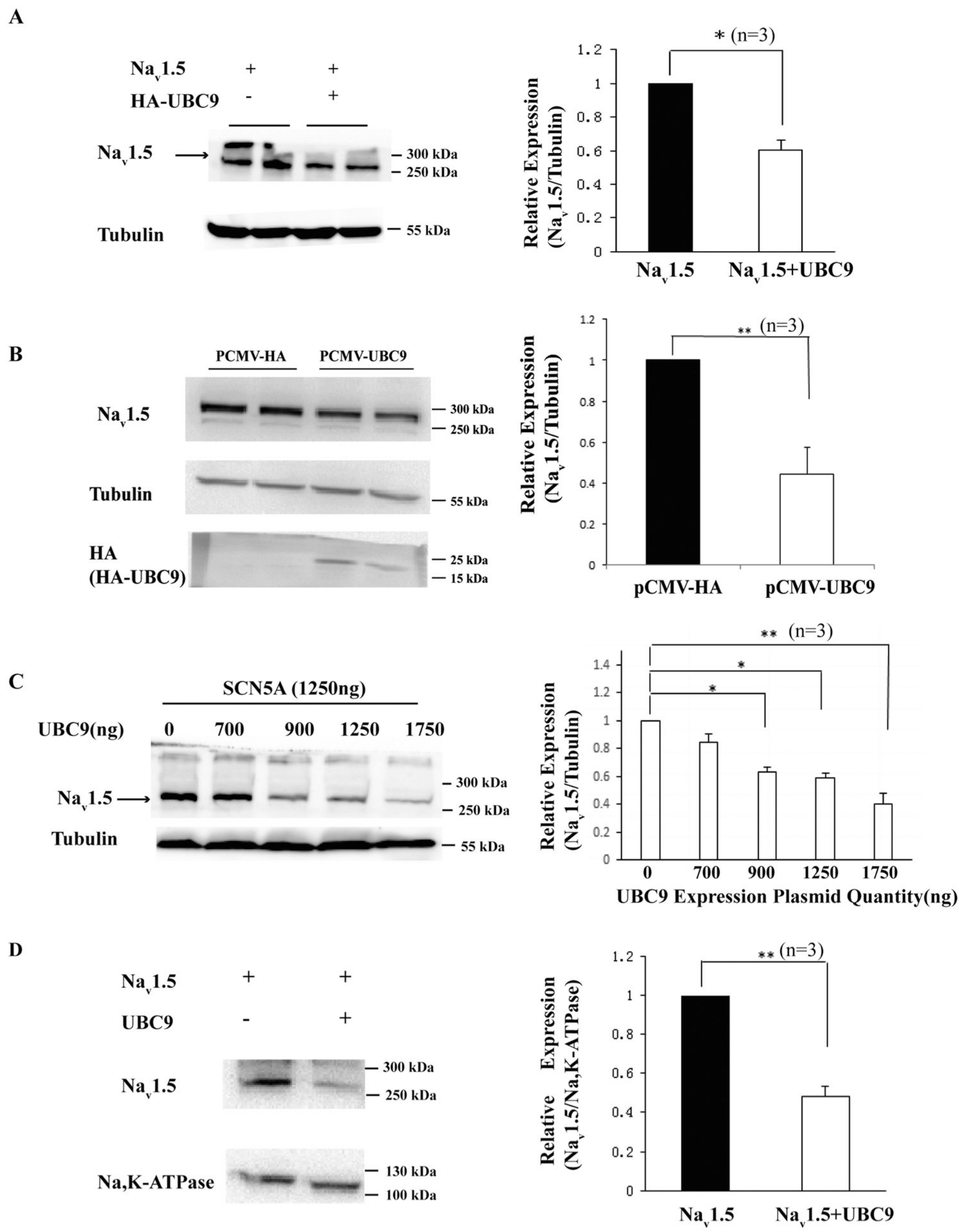

Fig. 1.

Effects of overexpression of *UBC9* on Na_V1.5 expression levels. Western blot analysis was carried out for Na_V1.5 using HEK293 cells co-transfected with pcDNA3-SCN5A and pCMV-UBC9. (A) Overexpression of *UBC9* significantly reduced the expression level of Na_V1.5. Tubulin served as a loading control. The nature of the band above 300 kDa is unknown, and may represent modified Na_V1.5 or Na_V1.5 aggregates. (B) Western blot analysis with protein extracts from HEK293-Na_V1.5 cells (with stable expression of Na_V1.5) transfected with pCMV-UBC9 or control plasmid pCMV-HA. (C) The effects of *UBC9* overexpression on Na_V1.5 expression levels were concentration-dependent. (D) Overexpression of *UBC9* significantly decreased the cell surface expression of Na_V1.5.

Na,K-ATPase was used as a loading control. Left panel: Western blot images; Right panel: quantification of Western blot images. *P<0.05, **P<0.01, n=3/group.

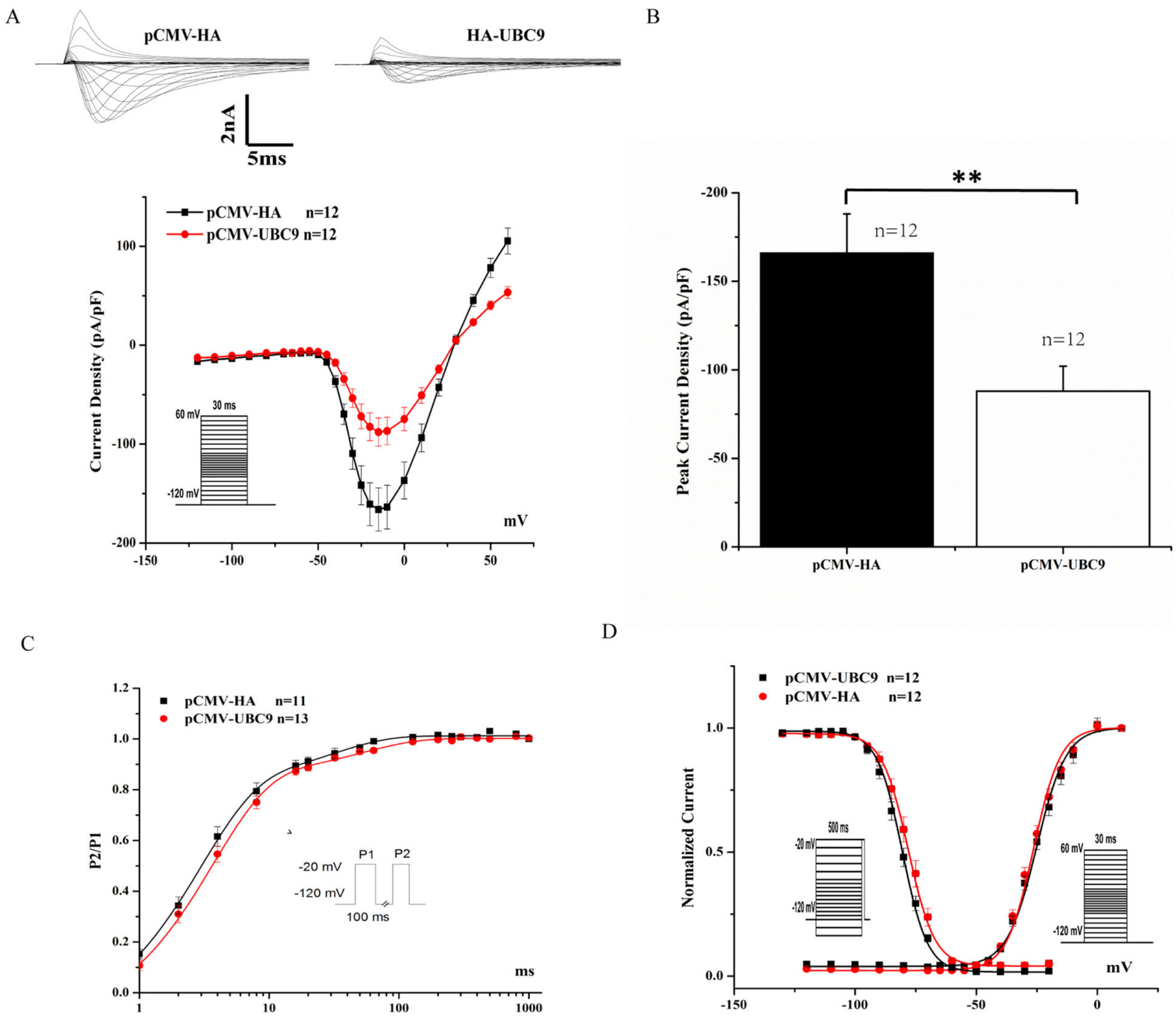

Fig. 2.

Effects of *UBC9* overexpression on sodium current densities. (A) Representative original traces of sodium currents and the current-voltage (I-V) relationship between average current densities and voltages from HEK293-$Na_V1.5$ cells transfected with pCMV-UBC9 or a control plasmid. Overexpression of *UBC9* decreased sodium current densities. (B) Peak sodium current densities at −15 mV. (C) Time course of recovery from inactivation was studied using a two-pulse protocol of −20 mV at −120 mV holding potential. (D) Steady-state activation and inactivation curves. The holding potential was −120 mV. $^{**}P<0.01$, n=11–13 cells/group.

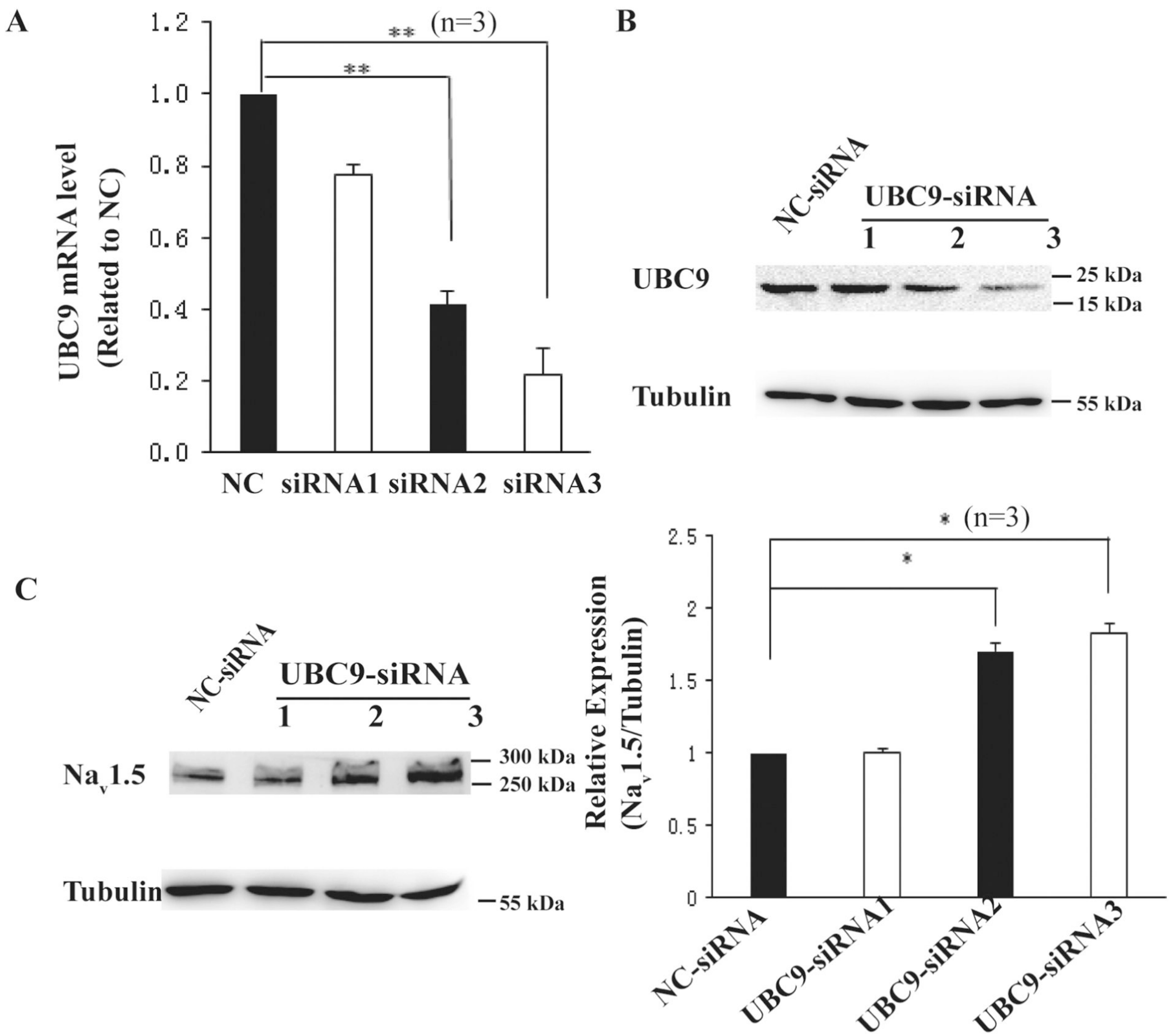

Fig. 3.

Effects of *UBC9* knockdown on $Na_V1.5$ expression levels. (A, B) Quantitative real-time RT-PCR analysis (A) and Western blot analysis (B) of *UBC9* in HEK293-$Na_V1.5$ cells transfected with siRNAs against *UBC9* (UBC9 siRNA1, siRNA2 and siRNA3) or nonspecific negative control siRNA (NC siRNA). (C) Western blot analysis of $Na_V1.5$ in HEK293-Nav1.5 cells transfected with siRNAs against *UBC9* or NC siRNA. Note that siRNA2 or siRNA3 knocked *UBC9* expression down and significantly increased $Na_V1.5$ expression, whereas siRNA1 did not knock *UBC9* expression down and did not affect $Na_V1.5$ expression. *P<0.05, n=3/group.

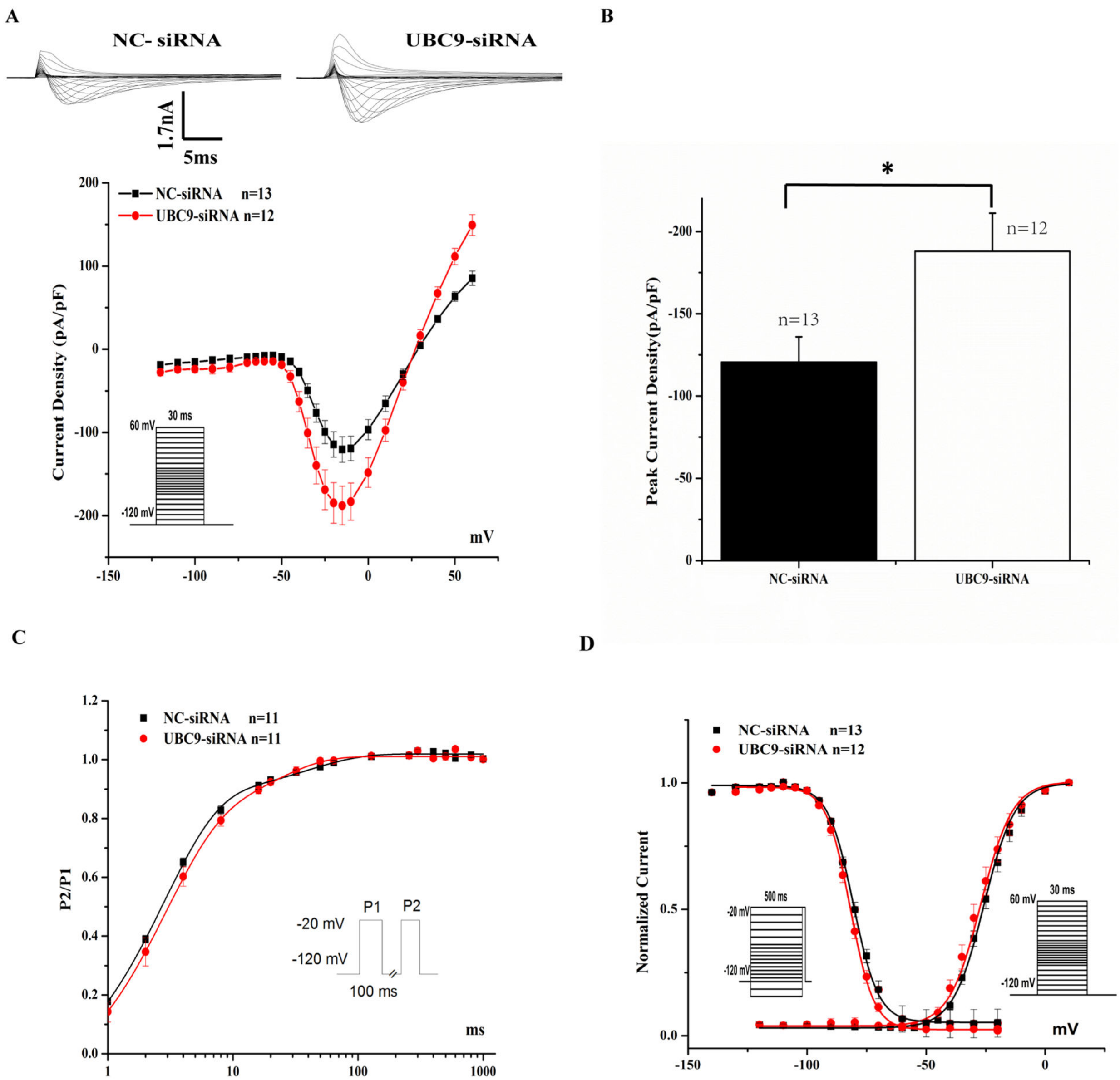

Fig. 4.

Effects of *UBC9* knockdown on sodium current densities in HEK293-Na_V1.5 stable cells. (A) Representative original traces of sodium currents and the current-voltage (I-V) relationship. (B) Peak sodium current densities at −15 mV. (C) Time course of recovery from inactivation. (D) Steady-state activation and inactivation curves. The holding potential was −120 mV. *P<0.05, n=11–13 cells/group.

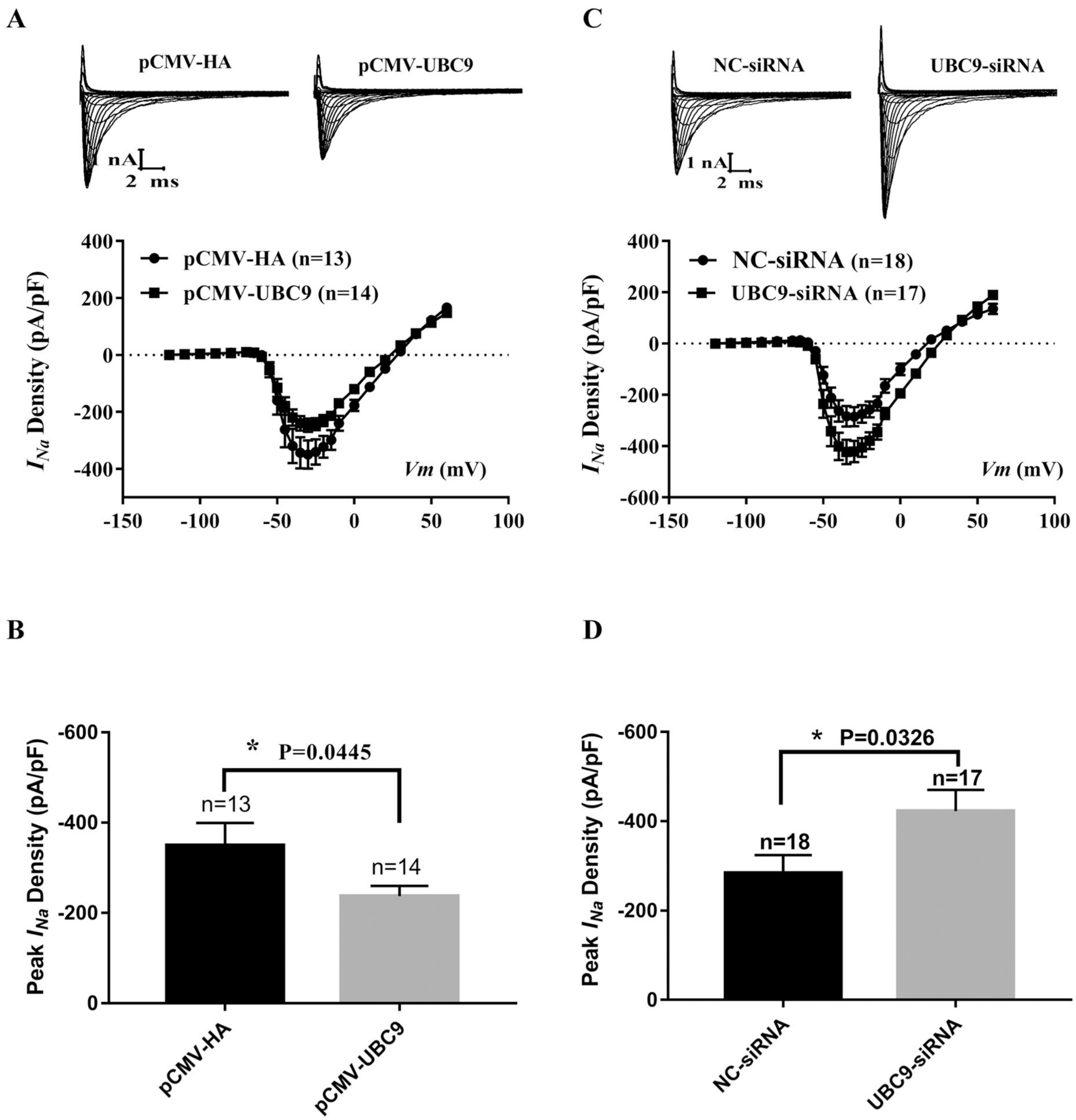

Fig. 5.

Effects of overexpression or knockdown of UBC9 on I_{Na} in neonatal rat cardiomyocytes. Whole-cell sodium currents recorded from neonatal rat cardiomyocytes transfected with an expression plasmid for UBC9 or its control empty vector or (A and B), and UBC9 siRNA or its negative control NC siRNA. (A) Representative original traces of sodium currents and the relationship between the average current density (current normalized to cell capacitance) and voltage from cardiomyocytes with or without overexpression of UBC9. (B) The relative peak sodium current density at −30 mV. (C) The relationship between the average current density (current normalized to cell capacitance) and voltage from cardiomyocytes with or without knockdown of UBC9 by siRNA. (D) The relative peak sodium current density at −30 mV. Data are shown as mean ± SEM. *$P < 0.05$, n=13–18 cells/group.

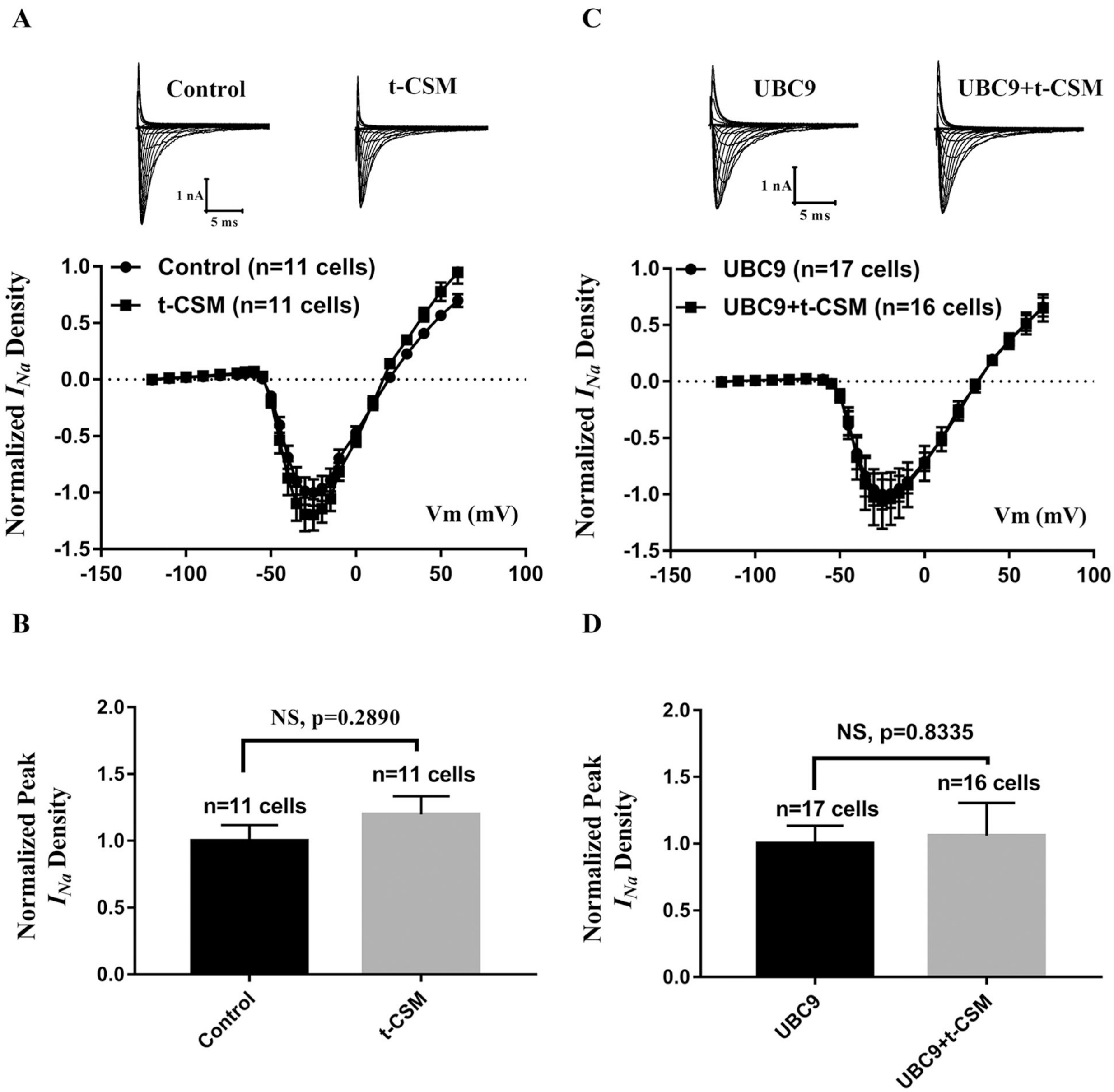

Fig. 6.

The effect of the UBC9-CRMP2 interaction on cardiac I_{Na} from $Na_v1.5$. (A, B) Whole-cell sodium currents were recorded from HEK293-$Na_v1.5$ cells treated with t-CSM that disrupts the UBC9-CRMP2 interaction. (A) Representative original traces of sodium currents and the relationship between the average current density (current normalized to cell capacitance) and voltage. (B) The relative peak sodium current density at −25 mV. NS, not significant, n=11 cells/group. (C, D) Whole-cell sodium currents were recorded from HEK293-$Na_v1.5$ cells transfected with an expression plasmid for UBC9 and treated with t-CSM or without t-CSM. (C) The relationship between the average current density (current normalized to cell capacitance) and voltage. (D) The relative peak sodium current density at −25 mV. NS, not significant, n=16–17 cells/group.

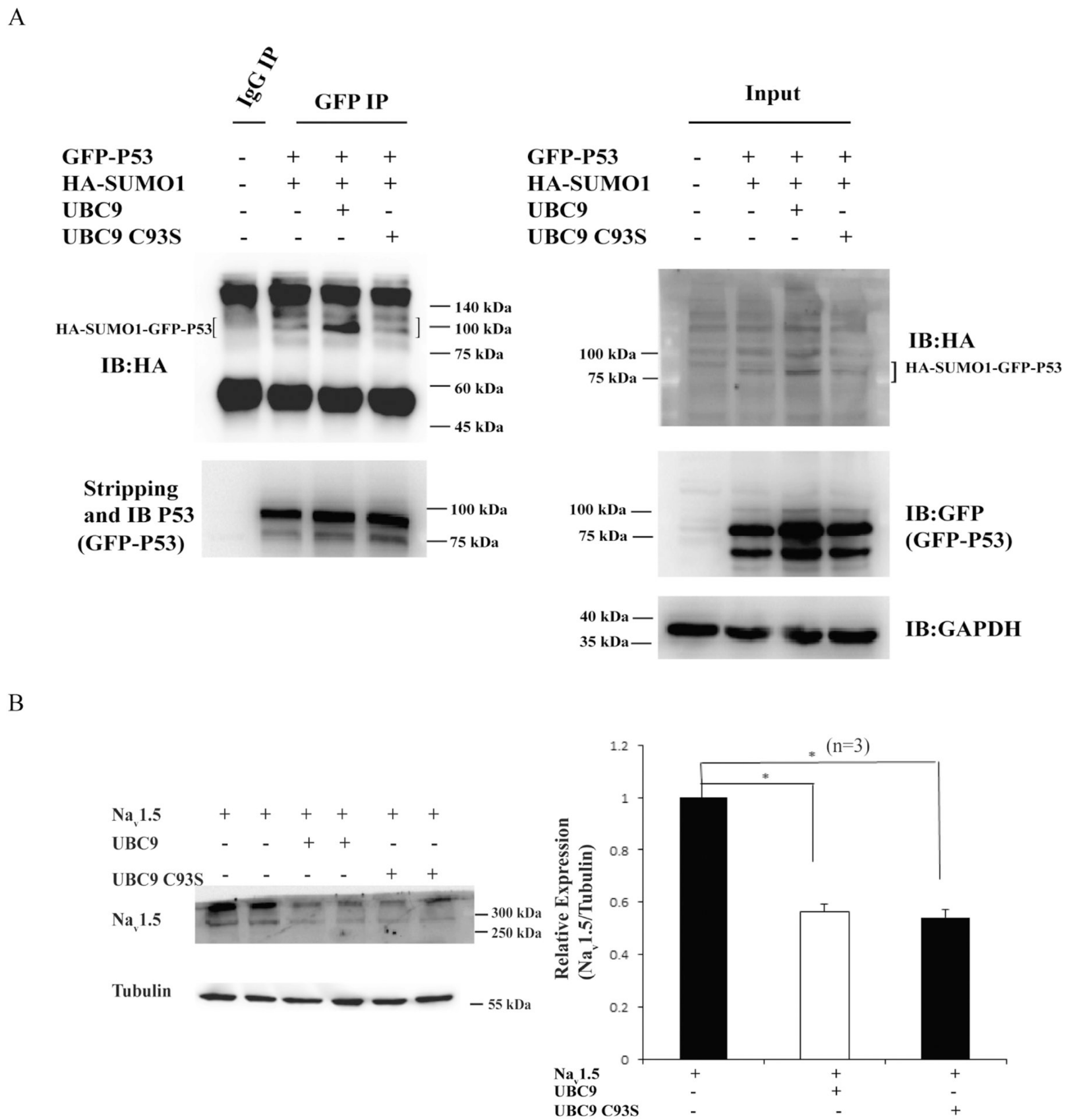

Fig. 7.

UBC9 SUMOylation is not involved in regulation of Na$_V$1.5 levels. (A) A positive control study showing that wild type UBC9 increases SUMO1 overexpression-induced SUMOylation of p53, but the effect was inhibited by mutant UBC9 with mutation C93S. HEK293 cells were co-transfected with multiple expression plasmids as indicated on the top, and lysed. Left panel: The cell lysates were immunoprecipitated with an anti-GFP antibody recognizing GFP-p53, and immunoblotting (IB) was carried out with an anti-HA antibody recognizing HA-SUMO1. After stripping of membranes, immunoblotting was carried out with an anti-p53 antibody. Right panel: the cell lysates were immunoblotted with an anti-HA antibody recognizing HA-SUMO1 antibody, and then with an anti-GFP antibody recognizing GFP-p53 or an anti-GAPDH antibody as loading controls after stripping of the membranes. SUMOylated p53 is indicated by a bracket around 100 kDa. (B) HEK293 cells were co-transfected with pcDNA3-SCN5A and pCMV-UBC9 or pCMV-UBC9-C93S, and used for Western blot analysis of $Na_v1.5$. Tubulin was used as a loading control. Overexpression of *UBC9* reduced the level of $Na_v1.5$, but the $Na_v1.5$ expression levels did not differ between wild type UBC9 and mutant UBC9-C93S lacking SUMO-conjugating activity. The nature of the band above 300 kDa is unknown, and may represent modified $Na_v1.5$ or $Na_v1.5$ aggregates. Data are shown as mean ± SEM. *$P < 0.05$, n=3/group.

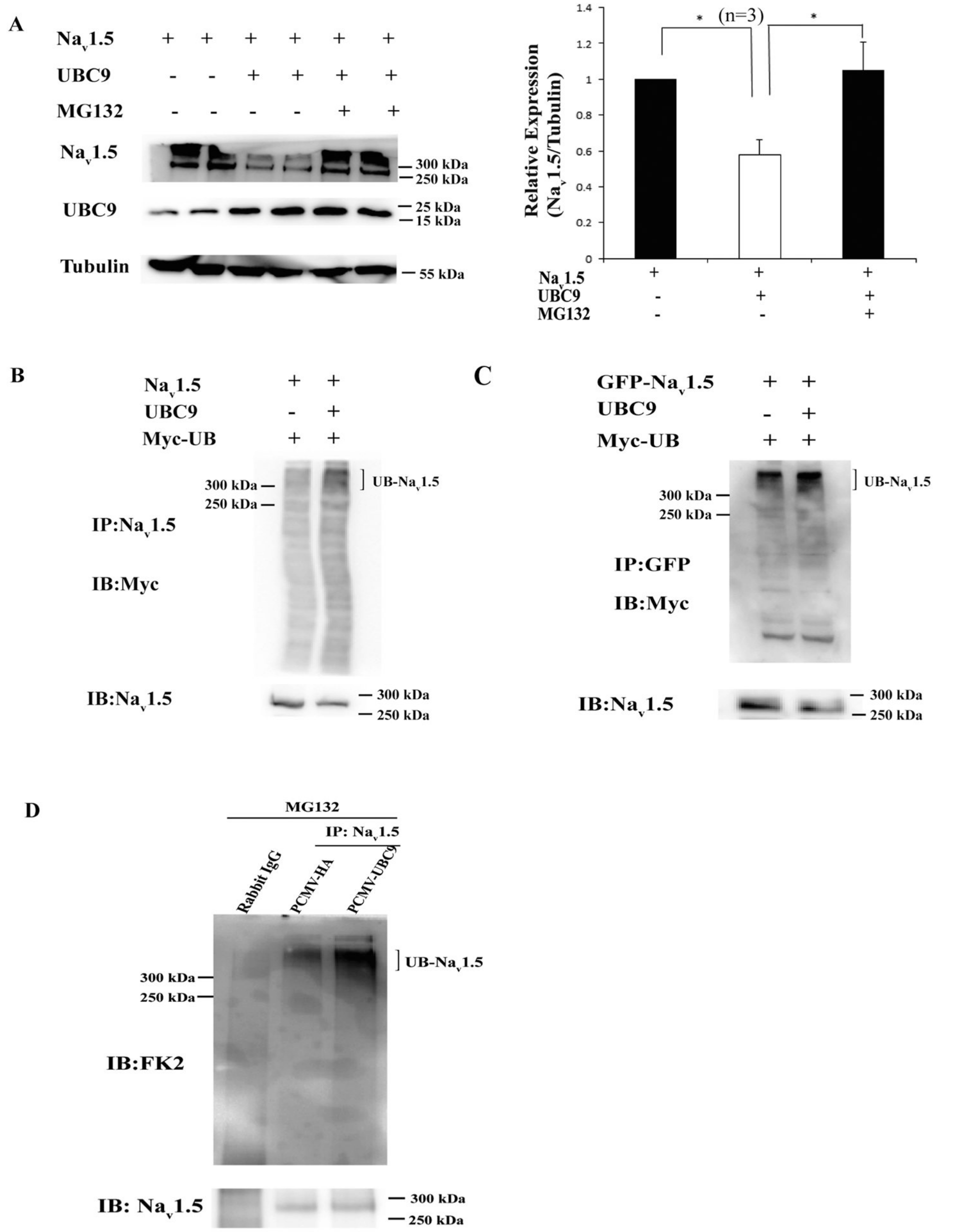

Fig. 8.

Effects of *UBC9* on Na$_V$1.5 expression involve the proteasome pathway and ubiquitination. (A) Western blot analysis of Na$_V$1.5 and UBC9 in HEK293 cells co-transfected with pcDNA3-SCN5A together with or without pCMV-UBC9. Proteasome inhibitor MG132 increased Na$_V$1.5 expression levels. Western blot images in the left were quantified and plotted on the right. The nature of the band above 300 kDa is unknown, and may represent modified Na$_V$1.5 or Na$_V$1.5 aggregates. Data are shown as mean ± SEM. *$P < 0.05$, n=3/group. (B) Immunoprecipitation analysis of Na$_V$1.5 protein ubiquitination in HEK293 cells co-transfected with pcDNA3-SCN5A (overexpression of Na$_V$1.5) and pMyc-UBB (overexpression of Myc-tagged Ub, Myc-UB) together with or without pCMV-UBC9. Cell lysates were immunoprecipitated with an anti-Na$_V$1.5 antibody (IP: Na$_V$1.5), and immunoblotting was carried out with anti-Myc (IB: Myc-UB) or anti-Na_v1.5 (IB: Na_v1.5) antibodies. Ubiquitinated-Na_v1.5 (UB-Na_v1.5) is indicated by a bracket above 250 kDa. (C) Immunoprecipitation analysis of Na_v1.5 protein ubiquitination in HEK293 cells co-transfected with pEGFP-Na_v1.5 and pMyc-UBB together with or without pCMV-UBC9. Cell lysates were immunoprecipitated with an anti-GFP antibody (IP: GFP-Na_v1.5), and immunoblotting was carried out with anti-MYC (IB: Myc-Ub) or anti-Na_v1.5 (IB: Nav1.5) antibodies. Overexpression of UBC9 enhanced Na_v1.5 ubiquitination. Ubiquitinated-Na_v1.5 (UB-Na_v1.5) is indicated by a bracket above 250 kDa. (D) Confirmation of the effect of UBC9 on ubiquitination of Na_v1.5 using the FK2 antibody. HEK293-Na_v1.5 stable cells were transfected with pCMV-UBC9 or control pCMV-HA empty vector, and lysed. Cell lysates were immunoprecipitated with an anti-Na_v1.5 antibody (IP: Na_v1.5) or rabbit IgG control, and immunoblotting was carried out with FK2 recognizing ubiquitin. After stripping, the membrane was re-probed with an anti-Na_v1.5 antibody (IB: Nav1.5). Ubiquitinated-Na_v1.5 (UB-Na_v1.5) is indicated by a bracket above 250 kDa.

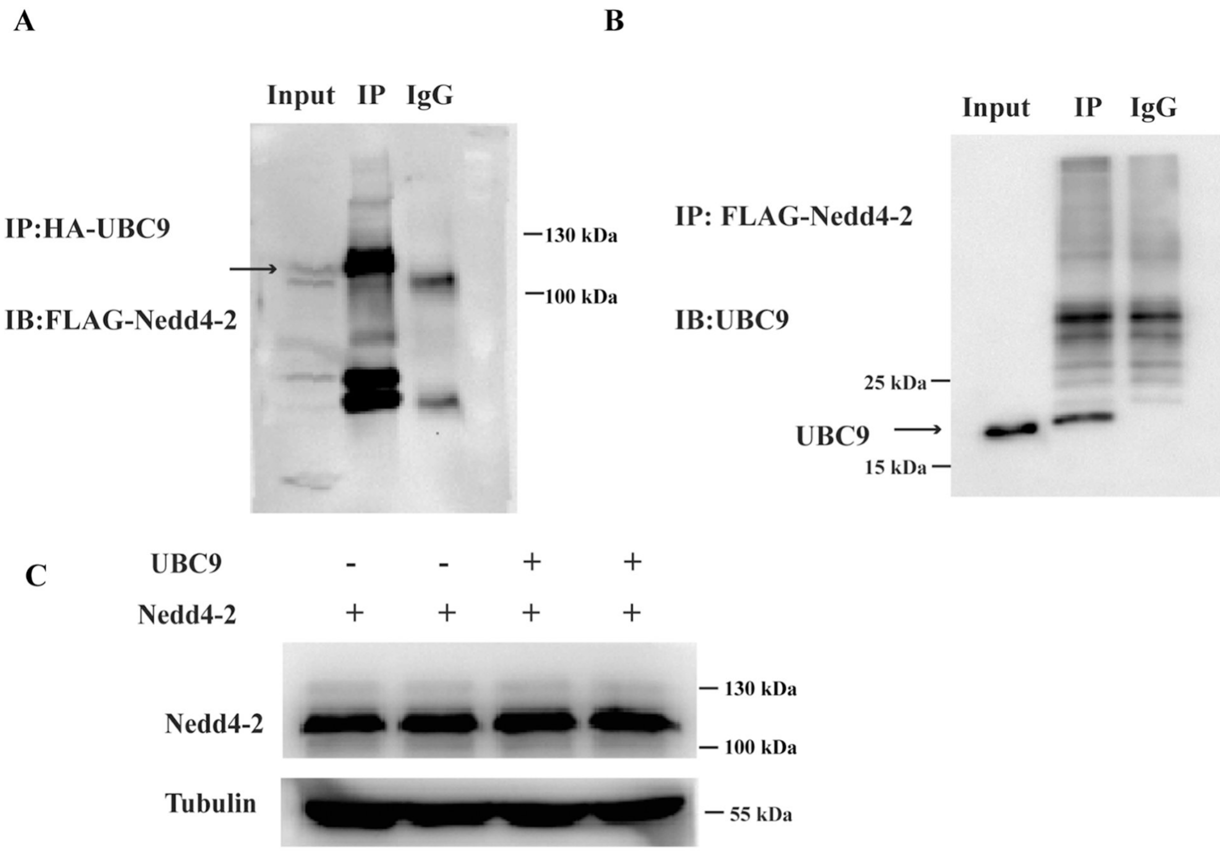

Fig. 9.

UBC9 interacts with NEDD4–2. (A) Co-IP analysis of interaction between HA-tagged UBC9 and FLAG-tagged Nedd4–2. Immunoprecipitation was carried out with an anti-HA antibody, and immunoblotting was with an anti-FLAG antibody. Anti-HA-UBC9 successfully precipitated FLAG-Nedd4–2. (B) Co-IP analysis of interaction between FLAG-tagged Nedd4–2 and UBC9. Immunoprecipitation was carried out with an anti-FLAG antibody, and immunoblotting was with an anti-UBC9 antibody. Anti-FLAG-Nedd4–2 successfully precipitated UBC9. (C) Western blot analysis of Nedd4–2 in HEK293 cells with or without overexpression of UBC9. Overexpression of *UBC9* did not affect the level of Nedd4–2 expression.