Ubiquitination-activating enzymes UBE1 and UBA6 regulate ubiquitination and expression of cardiac sodium channel Na\textsubscript{v}1.5

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Cardiac sodium channel Na\textsubscript{v}1.5 is associated with cardiac arrhythmias and heart failure. Protein ubiquitination is catalyzed by an E1–E2–E3 cascade of enzymes. However, the E1 enzyme catalyzing Na\textsubscript{v}1.5 ubiquitination is unknown. Here, we show that UBE1 and UBA6 are two E1 enzymes regulating Na\textsubscript{v}1.5 ubiquitination and expression. Western blot analysis and patch-clamping recordings showed that overexpression of \textit{UBE1} or \textit{UBA6} increased the ubiquitination of Na\textsubscript{v}1.5 and significantly reduced Na\textsubscript{v}1.5 expression and sodium current density, and knockdown of \textit{UBE1} or \textit{UBA6} expression significantly increased Na\textsubscript{v}1.5 expression and sodium current density in HEK293/Na\textsubscript{v}1.5 cells. Similar results were obtained in neonatal cardiomyocytes. Bioinformatic analysis predicted two ubiquitination sites at K590 and K591. Mutations of K590 and K591 to K590A and K591A abolished the effects of overexpression or knockdown of \textit{UBE1} or \textit{UBA6} on Na\textsubscript{v}1.5 expression and sodium current density. Western blot analysis showed that the effects of \textit{UBE1} or \textit{UBA6} overexpression on the ubiquitination and expression of Na\textsubscript{v}1.5 were abolished by knockdown of \textit{UBC9}, a putative E2 enzyme reported for Na\textsubscript{v}1.5 ubiquitination by us. Interestingly, real-time RT-PCR analysis showed that the expression level of \textit{UBE1}, but not \textit{UBA6}, was significantly up-regulated in ventricular tissues from heart failure patients. These data establish \textit{UBE1} and \textit{UBA6} as the E1 enzymes involved in Na\textsubscript{v}1.5 ubiquitination, and suggest that \textit{UBE1} and \textit{UBA6} regulate ubiquitination of Na\textsubscript{v}1.5 through \textit{UBC9}. Our study is the first to reveal the regulatory role of the \textit{UBE1} or \textit{UBA6} E1 enzyme in the ubiquitination of an ion channel and links \textit{UBE1} up-regulation to heart failure.

Introduction

The cardiac voltage-gated sodium channel Na\textsubscript{v}1.5 is encoded by the SCN5A gene, and generates the cardiac sodium current $I_{Na}$ important for the generation and conduction of the cardiac action potential (CAP) [1, 2]. Na\textsubscript{v}1.5 has four homologous transmembrane domains connected by intracellular loops [3, 4]. We and others reported that SCN5A mutations cause a variety of arrhythmic disorders, including ventricular tachycardia (VT), ventricular fibrillation (VF), Brugada syndrome (BrS), long QT syndrome (LQTS), atrial fibrillation (AF), progressive cardiac conduction, sick sinus syndrome, and dilated cardiomyopathy (DCM) [5–7].

Common VT and VF are also associated with myocardial ischemia, such as coronary artery disease (CAD) and myocardial infarction (MI), and heart failure. VT/VF causes 50–75% of sudden deaths associated with CAD, recurrent MI and heart failure [8]. Some reports suggest that common VT and VF
linked with ischemia and heart failure were associated with the down-regulation of Na\textsubscript{v}1.5 expression and reduced $I_{\text{Na}}$ [9–11]. Down-regulation of Na\textsubscript{v}1.5 expression can be caused by many factors, however, one key regulatory mechanism is ubiquitination, a post-translational modification involved in Na\textsubscript{v}1.5 degradation [12–14].

Ubiquitination involves the covalent attachment of ubiquitin (Ub) to a target protein (substrate) via an enzymatic cascade of Ub transfer to the substrate. The initial activation step is catalyzed by enzyme 1 (E1) (ubiquitin-activating enzyme). Then, Ub is covalently linked to the second enzyme (E2), a ubiquitin-conjugating enzyme. The final step is the transfer of Ub to the substrate amino group, which is usually facilitated by the third enzyme (E3), a ubiquitin ligase. The target protein is mono- or poly-ubiquitinated and destined for degradation [15–17]. For Na\textsubscript{v}1.5, Nedd4-2 was identified as the E3 ubiquitin ligase (E3), and found to interact with Na\textsubscript{v}1.5 and regulate its degradation [18]. Recently, studies from our laboratory suggest that UBC9, an E2 enzyme for SUMOylation [19], acts as the E2 conjugating enzyme for ubiquitination to significantly decrease Na\textsubscript{v}1.5 expression and $I_{\text{Na}}$ densities by interaction with Nedd4-2 [13].

The E1 enzyme for Na\textsubscript{v}1.5 ubiquitination is unknown. There are only two E1s, UBE1 and UBA6, in contrast with more than 40 E2s and more than 600 E3s in the human genome [20–22]. UBE1 was previously known as the sole E1 enzyme in animals and fungi, which activates ubiquitin in an ATP-dependent manner. However, in 2007, UBA6 was reported as another E1 enzyme and found to possess all properties of UBE1 [21, 23, 24]. UBE1 and UBA6 have distinct preferences for E2 enzymes, and share ~40% identity [21]. Both of them contain three functional domains: an adenylation domain to bind ATP and appropriate ubiquitin-like proteins, the catalytic cysteine domain as the acyl carrier for Ub, and the C-terminal ubiquitin-fold domain to recruit specific E2s [25–27]. In this study, we used biochemical and electrophysiological tools to characterize UBE1 and UBA6 to identify the E1 enzyme(s) for Na\textsubscript{v}1.5 ubiquitination. Together with previous findings [13, 18], this study completes the identification of key components of the E1–E2–E3 cascade of Ub enzymes for Na\textsubscript{v}1.5 ubiquitination.

**Methods**

**Plasmids, antibodies, and cell lines**

The full-length cDNA clones for the human UBE1 gene (NM-003334) and UBA6 (NM-018227) were kindly provided by Dr. Jiahuai Han at Xiamen University. The cDNA clones were used as templates for PCR to amplify UBE1 cDNA or UBA6 cDNA, which were inserted into the pCMV10-3xFLAG vector, generating the expression plasmid for FLAG-tagged UBE1 (UBE1-Flag) and FLAG-tagged UBA6 (UBA6-Flag).

The expression constructs for human cardiac sodium channel gene SCN5A (hH1a) in vector pcDNA3 (pHL3) was previously described [1, 2]. The mutant plasmids, Na\textsubscript{v}1.5-K590A and Na\textsubscript{v}1.5-K591A, were generated on pHL3 as a template using PCR-based mutagenesis.

A mouse anti-β-tubulin antibody (10068-1-AP, Proteintech, China) and a mouse anti-FLAG antibody (M185-3L, MBL, Japan) were used at a dilution factor of 1:2000. A rabbit anti-Na\textsubscript{v}1.5 antibody (ASC-005, Alomone, Israel) was used at a dilution factor of 1:1000. A rabbit anti-UBC9 antibody (sc-271057, Santa Cruz, U.S.A.) was used at a dilution factor of 1:1000. A rabbit anti-rabbit HRP-conjugated secondary antibody and a goat anti-mouse HRP-conjugated secondary antibody were from Millipore (U.S.A.) used at a dilution factor of 1:2000.

The stable cell line with stable expression of Na\textsubscript{v}1.5, HEK293/Na\textsubscript{v}1.5, was previously reported [13]. The tsA201 cell line was kindly provided by Dr. Charles Antzelevitch. Cell culture and transfection were performed as previously reported [28].

**Isolation of neonatal cardiomyocytes**

The procedure for the isolation of neonatal cardiomyocytes was described before [13, 28], and performed at Huazhong University of Science and Technology. In brief, 10–15 <3 days old Sprague-Dawley neonatal rats were sacrificed by cervical dislocation and decapitation. The skin was sterilized using 75% ethanol, and the hearts were dissected out, washed using ice-cold PBS, and cut into small pieces. The tissue was digested in enzyme buffer with 0.05% collagenase B from Roche (U.S.A.) and 0.05% trypsin from Amresco (U.S.A.) dissolved in 1x PBS. Myocardial cells were isolated using a method of differential adhesion by removing cardiac fibroblasts that adhere to wells faster. The isolated cardiomyocytes were cultured in DMEM medium supplied...
with 10% FBS (Gibco, U.S.A.). The study with animals was approved by the Ethics Committee of Huazhong University of Science and Technology.

RNA interference
The siRNAs specific for human UBE1 and UBA6 were synthesized by GenePharma (China). The siRNA sequences for human UBE1 and UBA6 were as previously reported [29]. The siRNA sequence specific for human UBC9 was as previously reported by us [13]. Transfection of siRNAs into cells was as described. The efficiency of each siRNA for knockdown of the respective gene was examined using the real-time RT-PCR analysis with an ABI 7900 System (Applied Biosystems, U.S.A.) as described [30].

Electrophysiology studies
Whole-cell voltage-clamp recording of sodium current was described previously [28]. The pipette was filled with a solution containing 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). All reagents were from Sigma–Aldrich (U.S.A.).

Proteasome inhibitor study
HEK293/Na1.5 cells were transfected with 2 μg of pCMV10-3xFLAG, UBE1-Flag, or UBA6-Flag when grown to a density of 80–90%. Twenty-four hours after transfection, cells were treated with 20 nM of MG132 (Abclone, U.S.A.) for 24 h, collected, and lysed in lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 2.0 mM EDTA, 1% Nonidet P-40, pH 7.5) supplemented with a cocktail of proteasome inhibitors (Roche, U.S.A.). The cell lysates were centrifuged at 13 000 g for 10 min at 4°C, and the supernatant was mixed with 5× SDS loading buffer. Protein extracts were resolved by polyacrylamide gels and analyzed by Western blot analysis as described [12, 13].

Ubiquitination of Na1.5
HEK293/Na1.5 cells were transfected as described above. After 24 h, cells were treated with 20 nM of MG132 for 12 h and lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, and 1% Nonidet P-40) with 1× protease inhibitor (Roche Applied Science). Cell lysates were centrifuged at 13 000 g for 30 min at 4°C. An equal volume of supernatant was incubated with 1.5 μg of an anti-Na1.5 antibody or the same amount of anti-rabbit IgG overnight on a rotator at 4°C. Thirty microliters of Protein A/G PLUS-Agarose (Santa Cruz, U.S.A.) was added and incubated for 2 h. The Protein A/G PLUS-Agarose was washed with lysis buffer for five times at 4°C, mixed with 50 μl 1× SDS loading buffer, and separated using 8% SDS–polyacrylamide gels. The proteins were transferred to a PVDF membrane, which was incubated with an anti-FK2 antibody (BML-PW8810, Enzo Life Science, U.S.A.) overnight at 4°C with gentle agitation. The subsequent steps were the same as previously reported [12, 13].

Real-time RT-PCR and Western blot analyses
For quantitative real-time RT-PCR analysis, total RNA was isolated using the Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two micrograms of total RNA samples were reverse-transcribed using a reverse transcription kit (Promega, U.S.A.), and quantitative PCR was performed using SYBR Green PCR master mix (Roche, U.S.A.) and specific primers were either described previously [13] or as follows: UBE1, forward primer 5′-GACAACCAACACACGG-3′, and reverse primer 5′-AGAACCCGGATCAG-3′; UBA6, forward primer 5′-CCAACAATGTTGTCAGGGA-3′, and reverse primer 5′-CCAAATCAATGTTGTCAGGGA-3′. The primers for heart failure samples are as follows: hUBE1 F 5′-CCAGTCCGTTGTCAGGGAAG-3′, hUBE1 R 5′-CCACACAGAGTATGTTCC-3′, hUBA6 F 5′-GGGGACTGGCAGCACAATA-3′, hUBA6 R 5′-CCAAACCAAGACCCCCAATTAA-3′, hUBC9 F 5′-CTGTGCACTGTTTCCACAAA-3′, hUBC9 R 5′-ATTITGGTGCAGGACAGATGGA-3′, hCRYAB F 5′-CTTTGACCAGTTCATCGGAG-3′, hCRYAB R 5′-CCTCAATCACATCTCCACAC-3′, hNedd4-2 F 5′-AGTTGAGCTGTCCTTACTGAT-3′, hNedd4-2 R 5′-TAGGGTCGTCCATGTTG-3′, hNEDD4-1 F 5′-CCAGTTGGAGAAGAAAAAC-3′, hNEDD4-1 R 5′-ATTTTACGCTGGTGTCAC-3′.

Western blot analysis was carried out as described previously [13].
Human tissue samples
Human ventricular tissues without any identifiable health information (PHI) were obtained from explanted hearts and unmatched or rejected donor hearts through the Cleveland Clinic Heart Transplant Program. This study was approved by the Cleveland Clinic Institutional Review Board (IRB) on Human Subject Research, and written consent was obtained from the subjects. This study abides by the Declaration of Helsinki principles.

Statistical analysis
All data were obtained from at least three independent experiments and presented as mean ± SD (standard deviation). Statistical analysis was performed using a two-tailed Student’s t-test to compare means between two groups using SPSS or GraphPad PRISM 8. A P value of ≤0.05 was considered significant.

Results
Ubiquitin-activating enzymes UBE1 and UBA6 modulate the protein expression level of Na1.5 in HEK293/Na1.5 cells
The E1 enzyme(s) of the ubiquitin-proteasome system (UPS) regulating the degradation of cardiac sodium channel Na1.5 is not known. There are two E1 enzymes that activate the extensive downstream ubiquitination cascades, including UBE1 and UBA6. To determine whether one of the two E1 enzymes or both of them initiate the ubiquitination process of Na1.5, we transfected a stable HEK293 cell line with stable expression of Na1.5, HEK293/Na1.5 cells, with an expression plasmid for UBE1, an expression plasmid for UBA6 or the empty vector as control, and analyzed their effect on the expression of Na1.5 using Western blot analysis (Figure 1). We found that the level of Na1.5 expression was significantly decreased by overexpression of either UBE1 or UBA6 compared with the vector control (Figure 1A,B).

We also examined the effect of knockdown of UBE1 or UBA6 on the expression of Na1.5 in HEK293/Na1.5 cells transfected with two different UBE1-siRNAs or two UBA6-siRNAs (scrambled siRNA NC as negative control). Western blot analysis showed that knockdown of either UBE1 or UBA6 significantly increased Na1.5 expression levels compared with NC (Figure 1C,D). The effectiveness of UBE1-siRNAs and UBA6-siRNAs was demonstrated using real-time RT-PCR analysis (Figure 1E, left 3 panels for UBE1; right 3 panel for UBA6). UBE1-siRNA2 and UBA6-siRNA1 were chosen for the rest of experiments. Together, these data indicate that both UBE1 and UBA6 regulate the cellular expression level of Na1.5.

Then, we investigated the relationship of UBE1 and UBA6 on Na1.5. As shown in Figure 1F, the effect of the knockdown of both UBE1 and UBA6 on the expression of Na1.5 was more than the combined additive effects of knockdown of UBE1 or UBA6 alone, suggesting that UBE1 and UBA6 had a synergistic effect. However, the synergistic effect was not obvious in the case of overexpression of both UBE1 and UBA6 (Figure 1G). Interestingly, in the case of overexpression of both UBE1 and UBA6, the effect of UBE1 or UBA6 on Na1.5 is dose-dependent. As the amount of either UBE1 or UBA6 expression plasmid DNA in transfection was increased, Western blot analysis showed that the expression level of Na1.5 was decreased in a dose-dependent manner (Figure 1H-J).

Both UBE1 and UBA6 regulate the density of sodium current in HEK293/Na1.5 cells
Given the significant effects of either overexpression or knockdown of UBE1 and UBA6 on the protein expression level of Na1.5, we investigated the function role of UBE1 or UBA6 in Na1.5 physiology by whole-cell patch-clamping in HEK293/Na1.5 cells transfected with expression plasmid UBE1-Flag or UBA6-Flag (empty vector as negative control). Compared with the vector, overexpression of either UBE1 or UBA6 significantly decreased the cardiac sodium current density (−416.25 ± 153.31 for vector vs. −208.89 ± 71.88 for UBE1-Flag and −209.053 ± 88.32 for UBA6-Flag) (Figure 2A–C). There was no difference observed for the voltage dependence kinetics of activation and inactivation (Figure 2D) or time dependence of recovery from inactivation (Figure 2E).

On the other hand, knockdown of either UBE1 or UBA6 significantly increased the cardiac sodium current density in HEK293/Na1.5 cells compared with scrambled siRNA NC (−75.94 ± 34.05 for NC vs. −181.01 ± 130.24 for UBE1-siRNA and −204.75 ± 129.50 for UBA6-siRNA) (Figure 3A–C). Knockdown of UBE1 or UBA6 did not affect the voltage dependence kinetics of activation and inactivation (Figure 3D) or the time dependence of recovery from inactivation (Figure 3E).
Figure 1. UBE1 and UBA6 overexpression reduces Na\textsubscript{1.5} expression, whereas their knockdown increases Na\textsubscript{1.5} expression in HEK293/Na\textsubscript{1.5} cells. (A) Western blot analysis showing that overexpression of FLAG-tagged UBE1 or UBA6 significantly decreased the expression level of Na\textsubscript{1.5} in HEK293/Na\textsubscript{1.5} cells transfected with an expression plasmid for UBE1 or UBA6. An anti-Tubulin antibody was used as loading control. An anti-FLAG antibody was used to detect the expression level of FLAG-tagged UBE1 or UBA6 derived from transfected plasmids. (B) Quantified data from Western blot images as in (A). (C) Western blot analysis showing that the expression level of Na\textsubscript{1.5} was significantly increased by two siRNAs for UBE1 and UBA6.
Figure 1. Two siRNAs for UBA6 compared to negative control siRNA (NC) in HEK293/Na,1.5 cells. (D) Quantified data from Western blot images as in (C). (E) Real-time RT-PCR analysis showing that siRNAs targeted to UBE1 and UBA6 were effective in knocking expression of respective genes down in HEK293/Na,1.5 cells (UBE1: left 3 panels, UBA6: right 3 panels). (F) Western blot analysis showing that knockdown of the expression of both UBE1 and UBA6 in HEK293/Na,1.5 cells showed a much larger effect on Na,1.5 expression than the combined effects of knockdown of UBE1 and UBA6 alone (synergistic effect). (G) Western blot analysis showing that overexpression of both UBE1 and UBA6 in HEK293/Na,1.5 cells had a larger effect on Na,1.5 expression than overexpression of either UBE1 or UBA6 alone. (H–J) Western blot analysis showing that the effect of UBE1 or UBA6 overexpression on Na,1.5 expression was concentration-dependent. *P < 0.05, **P < 0.01, n = 3–6 samples or experiments/group.

Figure 2. Overexpression of UBE1 and UBA6 significantly decreases cardiac sodium current density. HEK293/Na,1.5 cells were transfected with an expression plasmid for FLAG-tagged UBE1 or UBA6 (empty vector as control), and characterized by patch-clamping electrophysiology studies. The voltage-clamp protocol is shown in the inset. (A) Representative whole-cell sodium current traces recorded from HEK293/Na,1.5 cells transfected with the empty vector or a UBE1 or UBA6 expression plasmid. (B) The relationship of average current densities and voltages (current normalized to cell capacitance). (C) The peak current density of each group at −20 mV. (D) The steady-activation and inactivation of curves. The holding potential was −120 mV. (E) Time course of recovery from inactivation was studied using a two-pulse protocol from −20 to −120 mV holding potentials. *P < 0.05, **P < 0.01, n = 10–12 cells/group.
UBE1 and UBA6 regulate sodium current density of Nav1.5 in neonatal rat cardiomyocytes

To further demonstrate the effect of UBE1 and UBA6 on the cardiac sodium current, we isolated and characterized neonatal rat cardiomyocytes transfected with UBE1 or UBA6 expression plasmids or siRNAs. Because the transfection efficiency for neonatal cardiomyocytes was low (5–10%), each plasmid was co-transfected with an EGFP plasmid. Only the GFP-positive cells with successful transfection were selected for patch-clamping as described previously [28]. As shown in Figure 4A,B, overexpression of either UBE1 or UBA6 significantly decreased endogenous cardiac sodium current density in cardiomyocytes (−372.12 ± 66.77 for control vector vs. −149.85 ± 66.55 for UBE1 and −145.09 ± 59.40 for UBA6). We also characterized the effect of knockdown of either UBE1 or UBA6 on cardiac sodium current in rat neonatal cardiomyocytes. The UBE1-siRNA and
UBE1 or UBA6-siRNA was co-transfected with an EGFP plasmid into rat neonatal cardiomyocytes, and only the GFP-positive cells with successful transfection were selected for patch-clamping. Knockdown of the expression of UBE1 or UBA6 significantly increased the cardiac sodium current density in cardiomyocytes (−382.03 ± 97.08 for scrambled NC vs. −593.26 ± 114.481 for UBE1-siRNA and −593.68 ± 122.16 for UBA6-siRNA) (Figure 4D,E).

Figure 4. UBE1 and UBA6 overexpression reduces Na_{1.5} expression, whereas their knockdown increases Na_{1.5} expression in primary neonatal rat cardiomyocytes.

(A) The relationship of average current densities and voltages (current normalized to cell capacitance) in neonatal cardiomyocytes transfected with a UBE1 or UBA6 expression plasmid (empty vector as control). (B) The peak current density at −25 mV. (C) Real-time RT-PCR analysis showing that the UBE1-siRNA and UBA6-siRNA successfully knocked down the expression of UBE1 and UBA6, respectively, as compared with negative control scrambled siRNAs (NC). (D) The relationship of average current densities and voltages (current normalized to cell capacitance) in neonatal cardiomyocytes with knockdown of UBE1 or UBA6 using specific siRNAs (scrambled siRNA NC as control). (E) The peak current density at −25 mV. *P < 0.05, n = 10 cells/group.
Overexpression of UBE1 and UBA6 increases ubiquitination of Na$_{v}$1.5

To determine how UBE1 and UBA6 affect the expression level of Na$_{v}$1.5, we overexpressed UBE1 or UBA6 in HEK293/Na$_{v}$1.5 cells, treated the cells with MG132, a potent and reversible 26S proteasome inhibitor, and then assessed their effects on expression of Na$_{v}$1.5. We found that the reduction in Na$_{v}$1.5 expression by overexpression of either UBE1 or UBA6 was blocked by MG132 treatment (Figure 5A,B). The data suggest that overexpression of either UBE1 or UBA6 affects the ubiquitin-dependent proteasome degradation pathway of Na$_{v}$1.5.

**Figure 5.** Overexpression of UBE1 or UBA6 promotes ubiquitination of Na$_{v}$1.5 and degradation by the ubiquitin-proteasome pathway.

(A) Western blot analysis showing that reduced Na$_{v}$1.5 expression by overexpression of UBE1 or UBA6 was abolished in HEK293/Na$_{v}$1.5 cells treated with 20 nM of MG132, a 26S proteasome inhibitor. (B) Quantified data from Western blot images as in (A). NS, not significant; n = 4 samples or experiments/group. (C) Overexpression of UBE1 or UBA6 promotes the ubiquitination of Na$_{v}$1.5. Protein extracts from HEK293/Na$_{v}$1.5 cells transfected with an expression plasmid for UBE1 or UBA6 (empty vector as control) and treated with MG132 were immunoprecipitated using an anti-Na$_{v}$1.5 antibody and the precipitates were used for Western blot analysis using a FK2 antibody to detect the ubiquitination level of Na$_{v}$1.5. An anti-Na$_{v}$1.5 antibody was used to calibrate the level of Na$_{v}$1.5, and an anti-FLAG antibody was used to assess whether the transfection was successful. (D) Quantified FK2/Na$_{v}$1.5 data from Western blot images as in (C). *P < 0.05, **P < 0.01, n = 3–6 samples or experiments/group.
To assess the direct effect of overexpression of either UBE1 or UBA6 on ubiquitination of Na\(_{v}1.5\), we used an anti-Na\(_{v}1.5\) antibody to immunoprecipitate Na\(_{v}1.5\) in HEK293/Na\(_{v}1.5\) cells with overexpression of UBE1 or UBA6, and then used FK2, a mouse anti-ubiquitin monoclonal antibody, to detect ubiquitin-bound Na\(_{v}1.5\) (Figure 5C). As shown in Figure 5C,D, the ubiquitination level of Na\(_{v}1.5\) was increased by overexpression of UBE1 or UBA6 as compared with the vector control. These data suggest that UBE1 and UBA6 regulate Na\(_{v}1.5\) expression levels through Na\(_{v}1.5\) ubiquitination and the ubiquitination-proteasome pathway.

**UBC9 is necessary for regulation of Na\(_{v}1.5\) ubiquitination by UBE1 and UBA6**

We previously reported that UBC9, the E2 enzyme for protein SUMOylation, actually acts as the E2 enzyme for ubiquitination of Na\(_{v}1.5\) [13]. Thus, we hypothesized that the E1 enzymes UBE1 and UBA6 modulate Na\(_{v}1.5\) expression levels through UBC9. To test the hypothesis, we knocked UBC9 expression down in HEK293/Na\(_{v}1.5\) cells transfected with a UBE1 expression plasmid or a UBA6 expression plasmid. Real-time RT-PCR analysis and Western blot analysis showed that the UBC9 siRNA successfully knocked the expression level of UBC9 down (Figure 6A,B) as reported before [13]. Western blot analysis showed that the level of Na\(_{v}1.5\) expression was not decreased by overexpression of either UBE1 or UBA6 (Figure 6B,C). The ubiquitination assay with immunoprecipitation using an anti-Na\(_{v}1.5\) antibody and Western blot analysis with FK2 showed that the ubiquitination level of Na\(_{v}1.5\) was not increased by overexpression of either UBE1 or UBA6 (Figure 6D,E). These data suggest that the regulation of Na\(_{v}1.5\) ubiquitination by UBE1 or UBA6 is dependent on UBC9.

**Identification of two potential ubiquitination sites on Na\(_{v}1.5\) at K590 and K591**

To identify the ubiquitination site(s) on Na\(_{v}1.5\), we performed bioinformatic analysis to predict potential sites using BDM-PUB (Prediction Ubiquitination Sites with Bayesian Discriminant Method), and found two putative sites at K590 and K591 with high BDM-PUB scores (Figure 7A). We mutated each site to an alanine residue, K590A and K591A, in an expression plasmid for Na\(_{v}1.5\), and determined their effects on the expression level of Na\(_{v}1.5\) in tsA201 cells with UBE1 or UBA6 knocked down or overexpressed. We co-transfected tsA201 cells with wild type Na\(_{v}1.5\) or mutant Na\(_{v}1.5\) (K590A or K591A) together with UBE1-siRNA or UBA6-siRNA in tsA201 cells. Western blot analysis showed that knockdown of UBE1 or UBA6 did not affect the expression level of mutant Na\(_{v}1.5\)-K590A or Na\(_{v}1.5\)-K591A (Figure 7B,C). Similarly, overexpression of UBE1 or UBA6 did not affect the expression level of mutant Na\(_{v}1.5\)-K590A or Na\(_{v}1.5\)-K591A (Figure 7D,E). These data suggest that the two predicted ubiquitination sites K590 and K591 are important for the regulatory effect of UBE1 and UBA6 on the expression level of Na\(_{v}1.5\).

To further confirm the above finding, we performed patch-clamping to record sodium currents in tsA201 cells co-transfected with mutant Na\(_{v}1.5\) (K590A or K591A) together with UBE1-siRNA or UBA6-siRNA, or a UBE1 or UBA6 expression plasmid. As shown in Figure 8A,B, knockdown of UBE1 or UBA6 did not affect the sodium current density of mutant Na\(_{v}1.5\)-K590A (−279.51 ± 148.30 for scrambled siRNA NC vs. −341.62 ± 148.20 for UBE1-siRNA and −299.66 ± 161.27 for UBA6-siRNA) or Na\(_{v}1.5\)-K591A (−346.87 ± 187.79 for NC vs. −308.40 ± 193.84 for UBE1-siRNA and −282.88 ± 137.95 for UBA6-siRNA). Similarly, overexpression of UBE1 or UBA6 did not affect the sodium current density of mutant Na\(_{v}1.5\)-K590A (−232.40 ± 115.55 for empty vector control vs. −243.17 ± 100.63 for UBE1 and −285.17 ± 95.66 for UBA6) or Na\(_{v}1.5\)-K591A (−282.87 ± 149.17 for vector control vs. −268.42 ± 143.67 for UBE1 and −220.58 ± 97.05 for UBA6) (Figure 8C,D). These results confirm that the two predicted ubiquitination sites K590 and K591 are important for the regulatory effect of UBE1 and UBA6 on the expression level and function of Na\(_{v}1.5\).

**UBE1, but not UBA6 or other genes involved in Na\(_{v}1.5\) ubiquitination, is up-regulated in heart failure**

Down-regulation of Na\(_{v}1.5\) expression was reported in patients with DCM and heart failure [9–11]. As ubiquitination is a key regulator of Na\(_{v}1.5\) stability and expression levels, we used quantitative real-time RT-PCR analysis to analyze the expression levels of UBE1, UBA6, and other genes encoding enzymes and regulators of Na\(_{v}1.5\) ubiquitination, such as UBC9, NEDD4.1, NEDD4.2, and CRYAB, in left ventricular tissues from nine patients with heart failure and 10 study subjects without heart failure matched by age and sex. As shown in Figure 9, the expression level of UBE1 was significantly higher in failing hearts than non-failing hearts. No significant difference was found for expression of other genes between the failing hearts and non-failing hearts (Figure 9). These data suggest that up-regulation of UBE1 is correlated with the down-regulation of Na\(_{v}1.5\) expression in heart failure patients.
Discussion
This study identifies the E1 ubiquitin-activating enzymes responsible for regulating ubiquitination of Na$_{\text{v}}$1.5. There are two E1 enzymes in the human genome, UBE1 and UBA6. To our surprise, both UBE1 and UBA6 are required for ubiquitination of Na$_{\text{v}}$1.5 and regulation of Na$_{\text{v}}$1.5 expression levels and the density of cardiac sodium current (Figures 1–4). The data suggest that UBE1 and UBA6 do not play a redundant role in the...
Figure 7. Mutations at two putative Na$_{\text{A1.5}}$ ubiquitination sites, K590A and K591A, abolished the effects of overexpression or knockdown of UBE1 or UBA6 on the expression level of Na$_{\text{A1.5}}$.

Bioinformatic analysis revealed two lysine residues at amino acid positions of 590 and 591 as ubiquitination sites on Na$_{\text{A1.5}}$ by UBE1 and UBA6. (A) Schematic diagram showing two predicted lysine residues (K) as the Na$_{\text{A1.5}}$ ubiquitination sites by bioinformatic analysis. Each K residue was mutated into an alanine (A) residue in an expression plasmid for Na$_{\text{A1.5}}$ (K590A and K591A). The tsA201 cells were co-transfected with wild type or mutant (either K590A or K591A) Na$_{\text{A1.5}}$ expression plasmid together with siRNA for either UBE1 or UBA6 (B, C) or an overexpression plasmid for UBE1 or UBA6 (D, E), and used for Western blot analysis. (B) Western blot analysis showing that the effect of knockdown of UBE1 or UBA6 by siRNAs on the expression level of Na$_{\text{A1.5}}$ was abolished by mutant K590A or K591A compared with scrambled siRNA NC. (C) Quantified data from Western blot images as in (B). (D) Western blot analysis showing that the effect of overexpression of UBE1 or UBA6 on the expression level of Na$_{\text{A1.5}}$ was abolished by mutant K590A or K591A. (E) Quantified data from Western blot images as in (D). NS, not significant; n = 4 samples or experiments/group.
ubiquitination of Na\textsubscript{\textit{v}}1.5. More interestingly, Western blot analysis showed that UBE1 and UBA6 acted synergistically in regulating Na\textsubscript{\textit{v}}1.5 expression in the knockdown studies of UBE1 and UBA6 with siRNAs (Figure 1F). Together with previous findings of Nedd4-2 as the E3 ligase [12, 14, 18] and UBC9 as the E2

**Figure 8.** Mutations at two Na\textsubscript{\textit{v}}1.5 ubiquitination sites, K590A and K591A, abolished the effects of overexpression or knockdown of UBE1 or UBA6 on sodium current density.

The tsA201 cells were co-transfected with either a K590A or K591A mutant Na\textsubscript{\textit{v}}1.5 expression plasmid together with siRNA for either UBE1 or UBA6 (A,B) or an overexpression plasmid for UBE1 or UBA6 (C,D), and used for patch-clamping of sodium current. (A,B) Current–voltage relationship of sodium current in tsA201 cells with knockdown of UBE1 or UBA6 using specific siRNAs (scrambled siRNA NC as control). The relative peak sodium density at \(-25\) mV is plotted below. (C,D) Current–voltage relationship of sodium current in tsA201 cells with overexpression of UBE1 or UBA6. The relative peak sodium density at \(-25\) mV is plotted below. NS, not significant; \(n=12–17\) cells/group.

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conjugating enzyme [13], the present study completes the identification of all three major components of the ubiquitination machinery responsible for ubiquitination and degradation of Nav1.5 (Figure 10). As shown in a working model, ubiquitination of Nav1.5 involves three major steps (Figure 10). First, ubiquitination of Nav1.5 is initiated by binding of UBE1 and UBA6 to ATP and ubiquitin, which catalyze ubiquitin adenylation with release of pyrophosphate. Then, a UBE1- and UBA6-S-ubiquitin thioester bond is formed with the C-terminal glycine of ubiquitin. Second, the activated ubiquitin is transferred to UBC9, a low-molecular mass E2 conjugation enzyme, and covalently attached to E2 by the formation of the second thioester bond with the cysteine residue of E2 (Figure 10). There are over 40 different E2 enzymes [31, 32]. Our earlier study identified UBC9 as an E2 enzyme for ubiquitination of Nav1.5 [13]. In this study, we showed that UBC9 is required for

Figure 9. UBE1 expression is significantly up-regulated in human heart failure tissues.
Left ventricular (LV) tissue samples from nine heart failure patients (failing) vs. 10 age/sex-matched non-failing subjects (normal) were used for quantitative real-time RT-PCR analysis to evaluate the mRNA expression levels of six genes involved in Nav1,5 ubiquitination.
increased ubiquitination of Na\textsubscript{\textit{a},1.5} by overexpression of either \textit{UBE1} or \textit{UBA6} as the effect of either \textit{UBE1} or \textit{UBA6} was abolished by knockdown of \textit{UBC9} expression (Figure 6). Our data provide further evidence to support that \textit{UBC9} is the E2 enzyme for ubiquitination of Na\textsubscript{\textit{a},1.5}. However, it should be interesting to determine whether \textit{UBC9} is the sole E2 enzyme for ubiquitination of Na\textsubscript{\textit{a},1.5} or other E2 enzymes are also involved. Third, ubiquitin is covalently attached to Na\textsubscript{\textit{a},1.5} via its C-terminus to the lysine residues on Na\textsubscript{\textit{a},1.5} with the formation of an isopeptide bond by an E3 enzyme, and poly-ubiquitination may occur by the linkage of the ubiquitin C-terminus to the previous ubiquitin (Figure 10). Mono- or poly-ubiquitinated Na\textsubscript{\textit{a},1.5} is then targeted to the 26S proteasome for degradation (Figure 10). There are over 600 E3 ligases that co-ordinate the final step of ubiquitination [22, 32]. Nedd4-2, an ubiquitin ligase, was reported to regulate ubiquitination of Na\textsubscript{\textit{a},1.5} [12, 14, 18]. Two members of the ubiquitin protein ligase E3 component N-recogin (UBR) family, UBR3 and UBR6, were also reported to regulate ubiquitination of Na\textsubscript{\textit{a},1.5}, the expression level of Na\textsubscript{\textit{a},1.5}, and cardiac sodium current [33]. Future studies may identify other E3 ligases that are also involved in regulation of ubiquitination of Na\textsubscript{\textit{a},1.5}. Moreover, bioinformatic analysis and follow-up biochemical and electrophysiological studies identified two potential ubiquitination sites on Na\textsubscript{\textit{a},1.5}, including the lysine residue at an amino acid position of 590 and the other lysine residue at position 591 (Figures 7 and 8). On the other hands, there may be additional ubiquitination sites on Na\textsubscript{\textit{a},1.5} that can be identified in the future.

There are similarities and differences between \textit{UBE1} and \textit{UBA6} with regard to their effects on Na\textsubscript{\textit{a},1.5}. For similarities, overexpression of either \textit{UBE1} or \textit{UBA6} decreased the level of Na\textsubscript{\textit{a},1.5} and sodium current density by about two-fold compared with the control (Figures 1A,B, 2A–C, and 4A,B). Both \textit{UBE1} and \textit{UBA6} can increase the ubiquitination of Na\textsubscript{\textit{a},1.5} when overexpressed (Figure 5C,D). In dose experiments, \textit{UBE1} and \textit{UBA6} showed similar effects on regulation of Na\textsubscript{\textit{a},1.5} (Figure 1H,J). For differences, in the same transfection experiment in HEK/Na\textsubscript{\textit{a},1.5} cells, the expression level of \textit{UBE1} was 5–14 times higher than \textit{UBA6} (Figures 1A, 5A, and 6B), although the end effect on Na\textsubscript{\textit{a},1.5} expression was similar. The reason for the difference of \textit{UBE1} and \textit{UBA6} expression in a transient transfection system is unknown. Both genes were expressed using the same expression vector, and DNA isolation for all plasmids was carried out at the same time for each transfection. Therefore, a difference on the stability of \textit{UBE1} and \textit{UBA6} mRNAs or proteins may account for their different expression levels. Moreover, the effect of \textit{UBE1} on ubiquitination of Na\textsubscript{\textit{a},1.5} was less than \textit{UBA6} (Figure 5C,D). Overall, \textit{UBE1} and \textit{UBA6} may play a synergistic role in ubiquitination of Na\textsubscript{\textit{a},1.5}, however, the effect of \textit{UBA6} appeared to be stronger than \textit{UBE1}.

Considering the important role of \textit{UBE1} and \textit{UBA6} in ubiquitination and protein homeostasis, it is surprising that there has been no report on involvement of \textit{UBE1} or \textit{UBA6} in regulation of ubiquitination of ion...
channels to date. Therefore, to the best of our knowledge, our study is the first study to establish an important role of UBE1 or UBA6 in regulation of expression levels and ubiquitination of an ion channel. We showed that overexpression of either UBE1 or UBA6 significantly decreased the protein expression level of Na\(_{\text{v}}\)1.5, and reduced the density of cardiac sodium current in both HEK293/Na\(_{\text{v}}\)1.5 cells and neonatal rat cardiomyocytes by increasing ubiquitination of Na\(_{\text{v}}\)1.5, whereas knockdown of UBE1 or UBA6 expression by siRNAs caused the opposite effect (Figures 1–5). It remains to be determined whether UBE1 and UBA6 can regulate ubiquitination of other ion channels in cardiomyocytes and other cell types.

Previous studies showed that loss of UBE1 function had a detrimental effect on cell cycle regulation and DNA repair [34–36]. UBE1 also plays a role in leukemia, myeloma, and colon cancer [32, 37, 38]. Disruption of UBE1 in neurons led to neurodegenerative diseases of spinal muscular atrophy and Parkinson’s disease [32]. Mutations in UBE1 were identified in patients with X-linked infantile spinal muscular atrophy (XL-SMA) [39]. Homozygous knockout mice of UBA6 was embryonically lethal, indicating its important role in embryogenesis [23]. Neuron-specific knockout mice of UBA6 displayed abnormal neuronal development, spine architecture, and behavioral phenotypes [40]. UBA6 was shown to plays a role in meiosis initiation and cancer development [41, 42]. However, to date, little is known about the role of UBE1 or UBA6 in the development of cardiovascular diseases. The data in this study suggest that UBE1 or UBA6 may be involved in regulation of heart rhythm and cardiac arrhythmias by regulating ubiquitination of Na\(_{\text{v}}\)1.5. Indeed, we found that the expression of UBE1, but not UBA6, NEDD4.1, NEDD4.2, UBC9, and CRYAB, was significantly up-regulated in left ventricular tissues from patients with heart failure (Figure 9), which may be a factor for down-regulation of Na\(_{\text{v}}\)1.5 in heart failure. Our findings may provide a new therapeutic approach for treatment of arrhythmias and heart failure caused by Na\(_{\text{v}}\)1.5 mutations and deregulation. Loss-of-function mutations of Na\(_{\text{v}}\)1.5 cause BrS, idiopathic VT and VF, sick sinus syndrome, and other arrhythmic disorders, which may be treated by increased Na\(_{\text{v}}\)1.5 expression and cardiac sodium current density [5, 43]. As knockdown of UBE1 or UBA6 expression significantly increased Na\(_{\text{v}}\)1.5 expression and cardiac sodium current density (Figures 1, 3, and 4), UBE1 or UBA6 inhibitors (e.g. PYZD-4409, TAK-243, and others) [38] may provide a potential therapy for patients with loss-of-function Na\(_{\text{v}}\)1.5 mutations or reduced Na\(_{\text{v}}\)1.5 expression.

Ubiquitinated proteins can be directed to either the 26S proteasome for partial or complete degradation or lysosomes for complete degradation. The ubiquitin-proteasome pathway is the major pathway for degradation of ubiquitinated proteins, in particular, poly-ubiquitinated proteins [44]. Some proteins, in particular, mono-ubiquitinated proteins, undergo degradation by the endosome–lysosome pathway [44]. For ubiquitinated Na\(_{\text{v}}\)1.5, the ubiquitin–proteasome pathway is involved as the effect of UBE1 or UBA6 on down-regulation of Na\(_{\text{v}}\)1.5 expression was inhibited by MG132 (Figure 5). However, the ubiquitin–lysosome pathway may also be involved in degradation of Na\(_{\text{v}}\)1.5. Previously, we showed that small chaperon protein αB-crystallin interacted with Nedd4-2 and regulated Na\(_{\text{v}}\)1.5 ubiquitination, whereas ubiquitination of Na\(_{\text{v}}\)1.5 led to increased internalization of cell surface Na\(_{\text{v}}\)1.5, thereby resulting in reduced cell surface expression of Na\(_{\text{v}}\)1.5 [12]. As internalization of ubiquitinated Na\(_{\text{v}}\)1.5 may involve the endosome–lysosome pathway, it is likely that the ubiquitin–lysosome pathway is also involved in the degradation of ubiquitinated Na\(_{\text{v}}\)1.5. More future studies are needed to distinguish the contribution of the ubiquitin–proteasome pathway and the ubiquitin–lysosome pathway in ubiquitination of Na\(_{\text{v}}\)1.5.

It is interesting to note that UBE1 showed significant up-regulation in left ventricular tissue samples from heart failure patients (Figure 9). Previous studies reported association between post-transcriptional and post-translational modifications of Na\(_{\text{v}}\)1.5 and heart failure, including alternative mRNA splicing and Na\(_{\text{v}}\)1.5 phosphorylation [45, 46]. Another study reported up-regulation of Nedd4-2 and increased co-localization of Na\(_{\text{v}}\)1.5 with ubiquitin or Nedd4-2 in volume-overload rat hearts, a model for heart failure [47]. More studies are needed to fully characterize the role of the Na\(_{\text{v}}\)1.5 ubiquitination system in heart failure.

In conclusion, this study identifies the E1 activating enzymes responsible for the ubiquitination of Na\(_{\text{v}}\)1.5. We show that both UBE1 and UBA6 E1 enzymes are required for regulating ubiquitination and expression of Na\(_{\text{v}}\)1.5 and the density of cardiac sodium current in both a HEK293/Na\(_{\text{v}}\)1.5 stable cell line and primary neonatal rat cardiomyocytes. Interestingly, the effects of overexpression of UBE1 or UBA6 on the ubiquitination and expression of Na\(_{\text{v}}\)1.5 were abolished by knockdown of UBC9, further validating the role of UBC9 as the E2 enzyme for ubiquitination of Na\(_{\text{v}}\)1.5. To the best of our knowledge, our study is the first to identify a regulatory role for UBE1 and UBA6 in homeostasis of ion channels. The findings in this study may lead to the development of a therapy based on E1 or ubiquitination inhibitors for cardiac arrhythmias and heart failure associated with reduced Na\(_{\text{v}}\)1.5 expression or Na\(_{\text{v}}\)1.5 mutations.
Competing Interests
The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution
Y.H., Q.C., C.X., and Q.K.W. designed the study; Y.H., X.B., C.Z., S.C., H.X., and Z.W. performed experiments; Y.H., B.T., G.Y., C.X., Q.K.W., and Q.C. analyzed the data; Y.H., Q.C., and Q.K.W. wrote this paper.

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Abbreviations
AF, atrial fibrillation; BrS, Brugada syndrome; CAD, coronary artery disease; CAP, cardiac action potential; DCM, dilated cardiomyopathy; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; HEK293/Na1.5, a stable HEK293 cell line with stable expression of Na1.5; INa, sodium current; LQTS, long QT syndrome; MG132, carbobenzoxy-Leu-Leu-leucinal, a proteasome inhibitor; MI, myocardial infarction; Na1.5, voltage-gated sodium channel alpha subunit 5; Nedd4-2, Neuronal precursor cell-expressed developmentally down-regulated 4 isoform 2; SUMO, small ubiquitin-related modifier; Ub, ubiquitin; UBA6, ubiquitin-like modifier activating enzyme 6; UBC9, ubiquitin-conjugating enzyme 9; UBE1, ubiquitin-like modifier activating enzyme 1; Ub-Na1.5, ubiquitinated Na1.5; VF, ventricular fibrillation; VT, ventricular tachycardia.

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