# Cardiac Voltage-Gated Sodium Channel $Na_v1.5$ Is Regulated by Nedd4-2 Mediated Ubiquitination

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***Abstract***—$Na_v1.5$, the cardiac isoform of the voltage-gated $Na^+$ channel, is critical to heart excitability and conduction. However, the mechanisms regulating its expression at the cell membrane are poorly understood. The $Na_v1.5$ C-terminus contains a PY-motif (xPPxY) that is known to act as binding site for Nedd4/Nedd4-like ubiquitin-protein ligases. Because Nedd4-2 is well expressed in the heart, we investigated its role in the ubiquitination and regulation of $Na_v1.5$. Yeast two-hybrid and GST-pulldown experiments revealed an interaction between $Na_v1.5$ C-terminus and Nedd4-2, which was abrogated by mutating the essential tyrosine of the PY-motif. Ubiquitination of $Na_v1.5$ was detected in both transfected HEK cells and heart extracts. Furthermore, Nedd4-2–dependent ubiquitination of $Na_v1.5$ was observed. To test for a functional role of Nedd4-2, patch-clamp experiments were performed on HEK cells expressing wild-type and mutant forms of both $Na_v1.5$ and Nedd4-2. $Na_v1.5$ current density was decreased by 65% upon Nedd4-2 cotransfection, whereas the PY-motif mutant channels were not affected. In contrast, a catalytically inactive Nedd4-2 had no effect, indicating that ubiquitination mediates this downregulation. However, Nedd4-2 did not alter the whole-cell or the single channel biophysical properties of $Na_v1.5$. Consistent with the functional findings, localization at the cell periphery of $Na_v1.5$-YFP fusion proteins was reduced upon Nedd4-2 coexpression. The Nedd4-1 isoform did not regulate $Na_v1.5$, suggesting that Nedd4-2 is a specific regulator of $Na_v1.5$. These results demonstrate that $Na_v1.5$ can be ubiquitinated in heart tissues and that the ubiquitin-protein ligase Nedd4-2 acts on $Na_v1.5$ by decreasing the channel density at the cell surface. **(*Circ Res*. 2004;95:284-291.)**

**Key Words:** sodium channels ■ ubiquitin ■ Nedd4 ■ electrophysiology



Cardiac voltage-gated $Na^+$ channels ($Na_v$) initiate the action potential (AP), are essential for conduction of the electrical impulses, and contribute to the AP duration.[1] $Na_v1.5$ is the pore-forming $\alpha$-subunit of the predominant $Na^+$ channel found in the heart. The pivotal role of $Na_v1.5$ has been exemplified by the finding of more than 30 naturally occurring genetic variants[2] linked to cardiac phenotypes such as congenital and drug-acquired long QT syndromes, Brugada syndrome (BrS), conduction disorders, and sudden infant death syndrome.

Several mutations found in BrS patients alter the trafficking properties of $Na_v1.5$.[3,4] The molecular determinants of the targeting and trafficking of $Na_v1.5$, and other $Na_v$ channels, are however still poorly understood.

Ubiquitin is a 76 amino acid–long protein that can be covalently linked to target proteins, a process referred to as ubiquitination. The role of this posttranslational modification is to mark target proteins either for degradation[5] or transport toward other membrane compartments.[6] Recently, several membrane proteins have been found to be either mono- or polyubiquitinated.[6] Protein ubiquitination is achieved by specific ubiquitin-protein ligase enzymes (E3s) after ubiquitin has been carried by E1 and E2 enzymes in cascade. The E3 enzyme Nedd4-2 (neuronal precursor cell expressed developmentally downregulated[7]), belongs to the family of Nedd4/Nedd4-like proteins, which are characterized by the presence of a C-terminal HECT (homologous to E6-AP protein C-terminal) catalytic domain. Thus far, two kidney ion channels have been shown to be regulated by Nedd4/Nedd4-like proteins: the epithelial $Na^+$ channel (ENaC)[8] and the $Cl^-$ channel CLC-5.[9] For ENaC, it has been demonstrated that Nedd4-2, via its protein-protein interaction modules termed WW-domains, binds to specific regions of the ENaC subunits called PY-motifs (xPPxY).[8] This interaction leads to the internalization of ENaC subunits from the cell surface.[10] Mutations to the PY-motifs of ENaC subunits are linked to an inherited type of hypertension called Liddle syndrome.[10] Nedd4-2 is unable to bind to such mutated ENaC subunits,

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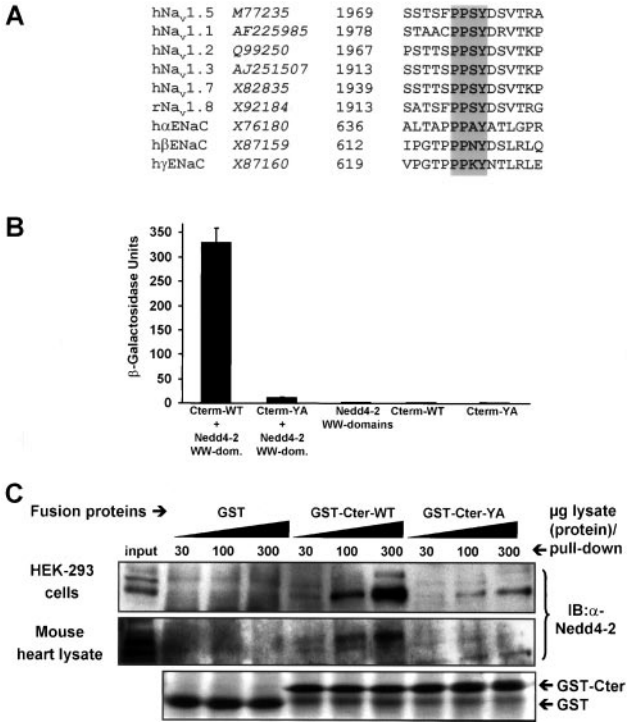


**Figure 1.** A, Alignment of the PY-motif of $Na_v$ channels and ENaC subunits. PY-motifs (shaded box) are found in the cardiac ($Na_v1.5$) and most neuronal $Na_v$ isoforms. It is absent in $Na_v1.4$, $Na_v1.9$, and Nax (GeneBank numbers in italics). Similar PY-motifs are also found in the 3 ENaC subunits. B, Interaction between the WW-domains of Nedd4-2 and the C-terminus of $Na_v1.5$ in yeast. After transformation with the corresponding plasmids, yeast cells were plated on growth media. β-Galactosidase activity, indicative of protein-protein interaction, was measured in lysates from cells grown from 3 independent colonies. $Na_v1.5$/Nedd4-2 interaction was disrupted by the YA mutation of the PY-motif. Bars are mean+SEM (n=3). C, Pulldown experiments showing the interaction between the PY-motif of $Na_v1.5$ and Nedd4-2. HEK cells transiently transfected with Nedd4-2 and mouse heart tissue were lysed as described in Materials and Methods. Samples of soluble fractions containing increasing amounts of protein were mixed with GSH-Sepharose beads containing either GST or each of the two GST-Cter fusion proteins (WT or YA mutant); see bottom panel. Bound Nedd4-2 was detected by Western blot analysis.

resulting in an accumulation of the channel at the apical membrane of kidney epithelial cells and concomitant increased tubular $Na^+$ reabsorption.

Inspection of the amino-acid sequence of $Na_v$ channels reveals the presence of a conserved PY-motif similar to those found in ENaC (Figure 1A). Because the ubiquitin-protein ligase Nedd4-2 is expressed in the heart,[11,12] we hypothesized that Nedd4-2 may be involved in the regulation of the density of $Na_v1.5$ channels at the plasma membrane. To investigate this hypothesis, we have (1) examined the biochemical interaction between $Na_v1.5$ and Nedd4-2, (2) tested for Nedd4-2–dependent ubiquitination of $Na_v1.5$, and (3) studied the functional consequences of Nedd4-2 activity. The present study provides evidence that $Na_v1.5$ can be ubiquitinated and that its surface density is likely regulated by the ubiquitin-protein ligase Nedd4-2.

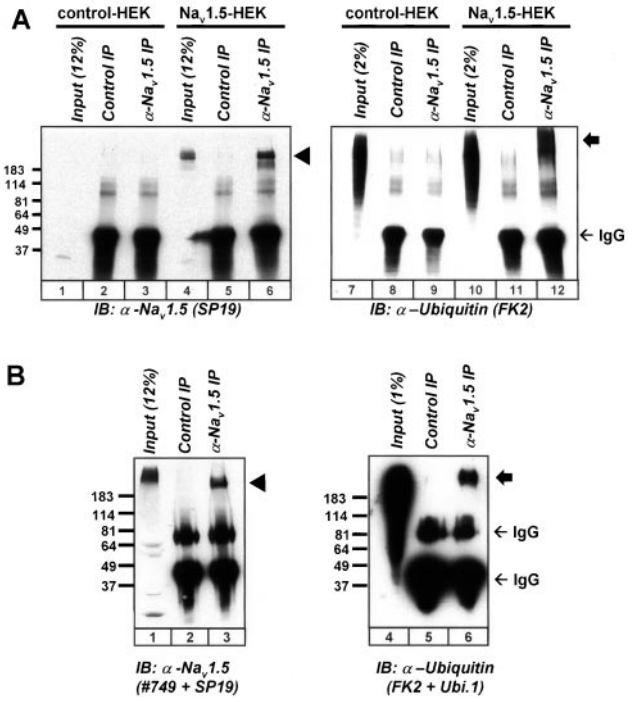


**Figure 2.** Detection of ubiquitinated forms of $Na_v1.5$. Membrane fractions from either control or $Na_v1.5$ stably-transfected HEK cells (A) or from mouse heart (B) were solubilized in Triton-X100 containing buffer. Samples containing 0.5 mg (HEK cells) or 1 mg (heart tissue) of total protein were incubated with either control or anti-$Na_v1.5$ sera plus protein-A Sepharose beads (see Materials and Methods). Bound proteins were released from the beads by boiling and split into 2 gels. Total $Na_v1.5$ (arrowheads) and ubiquitinated $Na_v1.5$ (arrows) were detected by western blot using, respectively, anti-$Na_v$ and anti-ubiquitin antibodies. For mouse heart, detection was enhanced by probing the corresponding immunoblots with a mix of either two anti-$Na_v$ antibodies (in B, 1 through 3) or two anti-ubiquitin antibodies (in B, 4 through 6).

## Materials and Methods

### Antibodies

Two anti-ubiquitin monoclonal antibodies were used: FK2 (Affiniti Research) and Ubi.1 (Zymed). Rabbit serum against human $Na_v1.5$ C-terminus (no. 749, raised against a GST-fusion protein comprising the residues 1978 to 2016) was a gift from Alomone (Jerusalem, Israel). The specificity of this serum was confirmed by the experiments presented in Figure 2. SP19 anti–pan-$Na_v$ rabbit polyclonal antibody was from Upstate; anti–Nedd4–1 and anti–Nedd4-2 antibodies have been described.[12]

### DNA Constructs and Cell Lines

Human $Na_v1.5$ cDNA was a gift of Dr M. Keating (University of Utah, Salt Lake City, Utah), and human Nedd4–1 (KIAA0093) and Nedd4-2 (KIAA0439) cDNAs were gifts of Dr T. Nagase (Kazusa Institute, Japan). Mutant constructs were generated using the QuickChange Mutagenesis Kit (Stratagene) and verified by sequencing. Stably transfected HEK cell lines expressing either wild-type (WT) or Y1977A (YA) mutated $Na_v1.5$ were generated using Zeocin (Invitrogen) as previously reported.[13]

### Yeast Two-Hybrid Assays

cDNA fragments encoding the 66 last amino acids of either WT or YA $Na_v1.5$ were amplified by PCR and cloned into the yeast expression vector pBTM116. A fragment encompassing the four WW-domains of Nedd4-2 but not its HECT domain was cloned into pACT2 (Clontech). Plasmids were transfected into yeast, selected on

appropriate media and assayed for protein-protein interaction using a liquid β-galactosidase assay (Clontech).

## Cardiac Tissue Preparation

Heart ventricles of 4-to-5–month mice (129Sv strain, in-house bred) were excised and rinsed with chilled PBS containing 1 mmol/L phenyl-methylsulfonyl fluoride (PMSF), and 10 mmol/L *N*-ethlylmaleimide (NEM) before being transferred into heart lysis buffer (HLB): 20 mmol/L Tris/HCl, pH 7.5, 0.32 mol/L sucrose, 1 mmol/L EGTA, 1 mmol/L PMSF, 10 mmol/L NEM, and Complete protease inhibitor cocktail (Roche). Tissue was homogenized using a Polytron for 1 minute. The insoluble fraction from a 10-minute centrifugation (1000*g*) was resuspended using a Teflon/glass homogenizer and recentrifuged. Supernatants from both low-speed centrifugation steps were pooled and centrifuged for 30 minutes at 50 000*g*. Soluble fractions were used as a source of Nedd4-2 in pulldown assays. $Na_v1.5$ was solubilized from membrane pellets in buffer containing 1% Triton-X100, and recovered in the supernatant after 15 minutes centrifugation at 13 000*g* (4°C).

This study was performed in accordance with Swiss law.

## Transfection and Homogenization of HEK Cells

HEK cells either nontransfected or stably expressing $Na_v1.5$ were transiently transfected with Nedd4-2 and/or $Na_v1.5$ constructs using calcium phosphate. Two days after transfection, cells were solubilized as described for cardiac membranes.

## Pulldown Assays

WT or mutant $Na_v1.5$ cDNAs encoding the last 66 residues of the channel were cloned into pGEX-4T1 (Amersham). Expression of GST-fusion proteins in *E. coli* cells was induced with 0.2 mmol/L IPTG for 4 hours at 22°C. Cells were harvested by centrifugation and resuspended in lysis buffer. Soluble fractions from a 15-minute centrifugation at 13 000*g* (4°C) were rotated for 1 hour in the presence of GSH-Sepharose at 4°C. Beads containing bound fusion proteins were recovered after washing and used in pulldown experiments. GST-pulldown assays of soluble fractions from either Nedd4-2 (WT or C801S inactive mutant) transfectants or heart lysates was performed using GSH-Sepharose beads containing either GST or one of the two GST-$Na_v1.5$-Cter fusion proteins. After incubation for 1 hour by rotation (4°C) and washing, bound Nedd4-2 was detected by Western blot. GST-fusion protein of the ubiquitin-binding proteasomal subunit S5a (GST-S5a) was obtained as described above from a pGEX construct kindly provided by Dr R. Layfield (University of Nottingham, UK).[14] Triton-soluble lysates from HEK cells transiently transfected with either $Na_v1.5$ alone or together with Nedd4-2 were incubated for 2 hours with either GST or GST-S5a bound to GSH-Sepharose beads. After extensive washing, bound $Na_v1.5$ was analyzed by Western blotting using anti-$Na_v1.5$ serum (no. 749).

## Immunoprecipitation Studies

Triton-X100 soluble fractions from either HEK cells or mouse heart membranes were incubated for 2 hours by rotation at 4°C with either anti-$Na_v1.5$ (no. 749) or an unrelated control rabbit serum. After addition of protein-A-Sepharose beads (Amersham), incubation followed for 1 hour. After washing of the beads, IP-fractions were analyzed by Western blot.

## Electrophysiology

For electrophysiological studies, HEK cells stably expressing either WT or YA mutant $Na_v1.5$ were transiently transfected in T25 flasks with either WT, CS-mutated Nedd4-2 cDNAs (1.6 μg), or empty vector. Alternatively, HEK cells were transiently transfected with 0.3 μg $Na_v1.5$ and 1.4 μg Nedd4-2 or Nedd4-1 constructs. $Na_v$ β-subunits were not cotransfected. All transfections included 0.8 μg cDNA encoding CD-8 antigen as a reporter gene. Cells were incubated with the transfection mix (Lipofectamine or calcium phosphate for 6 or 18 hours, respectively). After 24 hours, cells were split at low density. Anti-CD8 beads (Dynal) were used to identify transfected cells, and only decorated cells were analyzed. A detailed description of the whole-cell and single channel experiments and analysis is presented in the online data supplement.

## Confocal Imaging

HEK cells were transiently transfected with 0.025 μg of $Na_v1.5$-YFP construct[15] (kind gift from Dr T. Zimmer, University of Jena, Germany) and with GFP-Nedd4-2 (1.4 μg), which was obtained by subcloning Nedd4-2 into peGFP-C1 (Clontech). In this set of experiments, we had to reduce by 10-fold the amount of transfected DNA, compared with standard transfections, because under the latter conditions, the localization of the protein was mainly restricted to intracellular compartments.[15] Two days after transfection, fluorescent proteins were visualized by confocal microscopy (Zeiss LSM 510) on living cells. Optical sections were obtained at 512×512 pixels resolution, and analyzed using LSM software (Zeiss). Under these cotransfection conditions, the vast majority of cells expressed both fusion proteins.

Data are represented as mean±SEM. Two-tailed Student *t* test was used to compare means.

An expanded Materials and Methods section is available in the online data supplement available at http://circres.ahajournals.org.

# Results

## $Na_v1.5$ Interacts With Nedd4-2

Most $Na_v$ channels display in their C-termini a conserved PY-motif (Figure 1A), a potential binding site for proteins bearing WW-domains,[16] such as the Nedd4/Nedd4-like ubiquitin-protein ligases.[7] The PY-motifs of $Na_v$ channels are similar to those found in the three subunits of ENaC (Figure 1A), which are regulated by Nedd4-2.[11,12] In a preliminary study using *Xenopus laevis* oocytes, we reported that *Xenopus* Nedd4-2 modulates rat $Na_v1.5$ mediated $I_{Na}$.[17] However, the molecular mechanisms underlying this finding, such as a potential ubiquitination of $Na_v1.5$, were not investigated. Therefore, we first tested for an interaction between the human isoforms of Nedd4-2 and $Na_v1.5$ by yeast-two hybrid analysis. Expression of a protein bearing all 4 WW-domains of Nedd4-2 together with the last 66 residues of $Na_v1.5$ revealed a strong interaction between these two proteins, which was robustly reduced with the $Na_v1.5$-YA protein, harboring a mutation in the PY-motif (Figure 1B). This interaction was also confirmed by in vitro GST-pulldown assays. GST and GST-fusion proteins, containing the last 66 residues of $Na_v1.5$ (GST-Cter-WT and GST-Cter-YA), were incubated with lysates of either Nedd4-2 transfected HEK cells or mouse cardiac tissue. As shown in Figure 1C, GST did not bind Nedd4-2 from either lysate, in comparison to GST-Cter-WT, which bound efficiently to Nedd4-2 from both HEK cells and mouse heart lysates in a dose-dependent fashion. The YA-mutation of $Na_v1.5$ strongly decreased this interaction, illustrating that the association of Nedd4-2 to $Na_v1.5$ is mediated by way of the PY-motif.

## $Na_v1.5$ Is a Substrate for Ubiquitination

Ubiquitination of membrane proteins is a modification that has been proposed to play a role in their degradation and/or internalization.[6] We therefore wished to determine whether $Na_v1.5$ was also a target for ubiquitination. For this purpose, $Na_v1.5$ was immunoprecipitated (IP) from HEK cells stably expressing $Na_v1.5$ using an anti-$Na_v1.5$ isoform–specific serum (no. 749). Western blot analysis was then performed

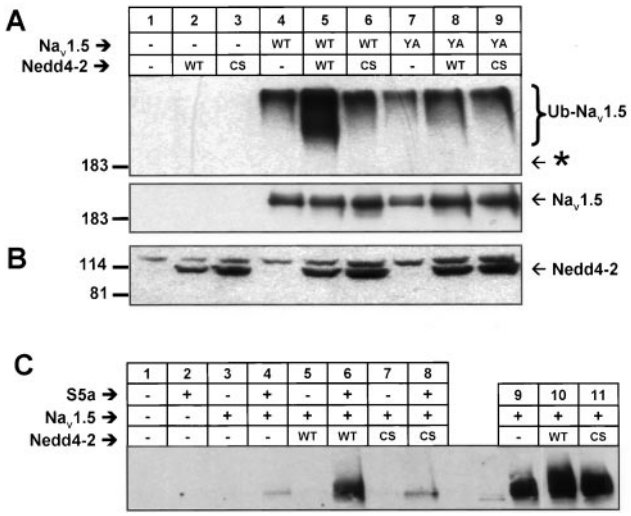


**Figure 3.** Nedd4-2 ubiquitinates $Na_v1.5$. HEK cells were transiently transfected with WT or YA-$Na_v1.5$ and/or WT or CS-Nedd4-2 as indicated. A, $Na_v1.5$ was immunoprecipitated and immunoblotted against ubiquitin as in Figure 2A. *Position of unmodified $Na_v1.5$. After stripping, the membrane was probed with anti–pan-$Na_v$ antibody (bottom). B, Anti-Nedd4-2 Western blot of soluble lysates from the various transfectants as a control for exogenous expression of Nedd4-2. C, Solubilized proteins of HEK cells expressing $Na_v1.5$ and/or WT or CS-Nedd4-2 as indicated were precipitated using GST-S5a fusion proteins in order to recover ubiquitinated proteins. Input of the S5a-precipitation experiments is shown in lanes C9–11 as a control of $Na_v1.5$ expression. Precipitated and soluble fractions were immunoblotted using anti-$Na_v1.5$. Fraction of bound $Na_v1.5$ was significantly higher in cells cotransfected with WT Nedd4-2 (C6), suggesting enhanced ubiquitination of the channel.

using either an anti–pan-$Na_v$ antibody (SP19) or an anti-ubiquitin antibody (FK2). Both total $Na_v1.5$ and ubiquitinated $Na_v1.5$ were detected in IP-fractions (Figure 2A). Ubiquitinated $Na_v1.5$ displayed an upward-shift relative to the total $Na_v1.5$ band (compare lanes 6 and 12), likely reflecting an increase in its molecular weight resulting from ubiquitination. The diffuse nature of the band is probably the result of multiple forms of $Na_v1.5$ carrying various amounts of ubiquitin moieties. However, such ubiquitinated forms most likely represent a small fraction of the total $Na_v1.5$ pool and are therefore not detected under our blotting conditions (lane 6).

Similar results were also obtained with mouse heart extracts. $Na_v1.5$ could be immunoprecipitated (Figure 2B, lane 3) and an ubiquitinated band detected in this fraction (Figure 2B, lane 6) demonstrating that $Na_v1.5$ is a physiological substrate for ubiquitination.

To investigate the role of Nedd4-2 in $Na_v1.5$ ubiquitination, we transiently transfected HEK cells with $Na_v1.5$-WT alone or together with Nedd4-2. Basal ubiquitination of the channel was detected (Figure 3A, lane 4), similar to that seen in HEK cells stably expressing $Na_v1.5$ (Figure 2A). Importantly, a robust increase in incorporated ubiquitin was observed when Nedd4-2-WT was cotransfected (Figure 3A, lane 5). This effect was not seen with an inactive Nedd4-2 (lane 6) in which cysteine 801 of the catalytic site was replaced by a serine (Nedd4-2-CS).[18] Although $Na_v1.5$-YA mutant channels were also found to be endogenously ubiquitinated (lane 7), $Na_v1.5$-YA was, in contrast to the WT channel, not further ubiquitinated by cotransfecting Nedd4-2-WT (lane 8). Thus, Nedd4-2–dependent enhancement of $Na_v1.5$ ubiquitination requires both the catalytic activity of Nedd4-2 and an intact PY-motif on the channel.

As an alternative approach to assess changes in the ubiquitination of $Na_v1.5$ upon Nedd4-2 cotransfection, lysates from cells obtained as described were incubated with GST-S5a, a fusion protein of the proteasomal subunit responsible for the binding of ubiquitinated proteins.[14] Western blots of the pulldown fractions showed an enhancement of S5a-bound $Na_v1.5$ in cells cotransfected with Nedd4-2-WT (Figure 3C, lane 6) but not with Nedd4-2 CS.

## Nedd4-2 Decreases $Na_v1.5$ Currents

The functional role of Nedd4-2 on $Na_v1.5$ was analyzed in HEK cells using patch-clamp experiments. Transient overexpression of Nedd4-2 in cells stably expressing $Na_v1.5$ significantly decreased whole-cell $I_{Na}$ (Figure 4A), resulting in a $65\pm6\%$ reduction of peak $I_{Na}$ density (Figure 5A). No effect was observed when an inactive Nedd4-2-CS was coexpressed (Figures 4A and 5A), despite similar levels of expression to that of Nedd4-2-WT (Figure 5B). This indicates that $I_{Na}$ downregulation is dependent on the catalytic activity of Nedd4-2.

To assess whether the decrease in $I_{Na}$ was the result of an alteration of the biophysical properties of $Na_v1.5$, we analyzed the macroscopic $I_{Na}$ in the presence and absence of Nedd4-2. Nedd4-2 did not modify the voltage-dependence of steady-state activation and inactivation of the channels remaining at the cell membrane (Figure 4B). Similarly, recovery from fast inactivation (Figure 4C) and entry into the intermediate inactivated (Im) state were not altered by Nedd4-2 (Figure 4D). Moreover, single channel properties were not modified by Nedd4-2 (see online data supplement). These data therefore suggest that the $I_{Na}$ decrease is likely caused by a reduction of the cell membrane channel density.

## Role of the PY-Motif

To assess the importance of the PY-motif of $Na_v1.5$ for Nedd4-2 to exert its action, similar experiments were performed using HEK cells stably expressing the mutant $Na_v1.5$-YA. As anticipated, neither Nedd4-2-WT nor Nedd4-2-CS influenced the mutant $I_{Na}$ (Figure 5A). Because transfection of Nedd4-2 did not reduce the global expression of $Na_v1.5$ (Figures 3A and 5B), this suggests that, under these conditions, Nedd4-2 targets preferentially a small subpopulation of channels.

Recent structural studies[19,20] indicated that a hydrophobic residue in position +3 after the Tyr of the PY-motif is involved in the binding to the WW-domain pocket, hence forming an "extended" PY-motif. In $Na_v1.5$, this position is occupied by Val-1980. To test the importance of this residue in Nedd4-2 regulation, Val-1980 was mutated into Ala, Asp, or Arg. As illustrated in Figure 6A and 6B, pulldown experiments using mutant PY-motif GST-fusion proteins indicate that Nedd4-2 binding is strongly reduced when charged residues are substituted at position 1980. Functional experiments using $Na_v1.5$ forms mutated at Val-1980 corrob-

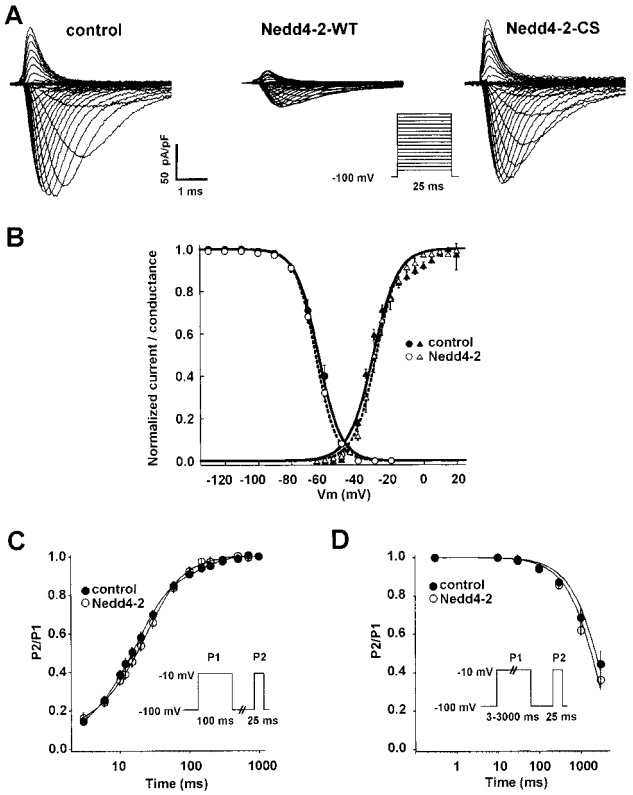


**Figure 4.** Nedd4-2-WT decreases $I_{Na}$ in HEK cells expressing $Na_v1.5$. A, Current traces obtained with a current/voltage protocol (inset) from control, Nedd4-2-WT or mutant Nedd4-2-CS transfected cells. Extracellular $Na^+$ concentration was reduced to 20 mmol/L for a better voltage control. B, Steady-state activation (triangles) and inactivation (circles) curves. Activation properties were determined from *I*/V relationships by normalizing peak $I_{Na}$ to driving force and maximal $I_{Na}$, and plotting normalized conductance vs Vm. Boltzmann curves were fitted to steady-state activation data: control $V_{1/2}=-32.7\pm0.7$ mV, $K=6.5\pm0.4$; Nedd4-2 $V_{1/2}=-31.1\pm2.0$ mV; $K=6.0\pm0.6$ (n=5). Voltage-dependence of steady-state inactivation (25-ms test pulse to −10 mV after a 500-ms conditioning pulse) was studied: control $V_{1/2}=-63.5\pm1.5$ mV, $K=6.4\pm0.3$, Nedd4-2 $V_{1/2}=-64.7\pm1.0$ mV; $K=5.8\pm0.2$ (n=5). C, Recovery from inactivation (protocol in inset) was fitted using a biexponential function; time constants and relative weights on averaged data are as follows: for control (●), $\tau_{fast}=16.8$ ms, $a_{fast}=0.78$, $\tau_{slow}=132$ ms, $a_{slow}=0.12$; for Nedd4-2 (○), $\tau_{fast}=23.7$ ms, $a_{fast}=0.78$, $\tau_{slow}=110$ ms, $a_{slow}=0.12$, n=10 to 11 cells. D, Time dependence of the onset of Im was measured using a two-pulse protocol (see inset), and was fitted using a monoexponential function; time constants on averaged data are as follows: for control (●), $\tau=3270$ ms and Nedd4-2 (○), $\tau=2551$ ms, n=6 cells. Fitted parameters obtained from individual cells (experiments described in C and D) were statistically not different.

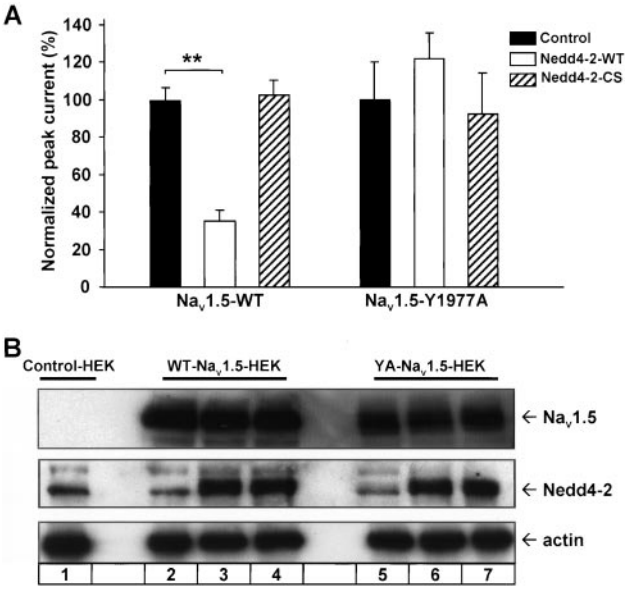


**Figure 5.** Nedd4-2-WT decreases $I_{Na}$ in cells expressing $Na_v1.5$-WT, but not PY-motif mutated channels. A, Peak $I_{Na}$ density from $Na_v1.5$-HEK cells transiently transfected with Nedd4-2-WT or CS (see Materials and Methods); n=15 cells from 3 experiments, $^{**}P<0.01$. B, Western blots of cell lysates transfected as in A showing the overexpression of either Nedd4-2-WT (lanes 3 and 6) or Nedd4-2-CS (lanes 4 and 7) in cells expressing either WT (lanes 2 to 4) or YA-mutated (lanes 5 to 7) channels. Western blots of control, nontransfected HEK cells (lane 1) is shown as reference. Protein loading was controlled by anti-actin immunoblotting.

orate the binding experiments (Figure 6C), thus supporting a role for residue Tyr +3 in the binding to Nedd4-2. Note that the membrane expression of Val-1980 mutant channels was comparable to WT $Na_v1.5$ (Figure 6D).

## Specificity of Nedd4-2 Effect

The ubiquitin-protein ligase Nedd4-1 belongs to the same family as Nedd4-2.[7] However, in *Xenopus* expression system Nedd4-1 did not regulate ENaC-mediated currents.[12] Because this Nedd4 isoform is also expressed at the RNA level in heart,[12] we tested whether coexpression of Nedd4-1 may modulate $Na_v1.5$-mediated currents. In contrast to Nedd4-2, Nedd4-1 was unable to downregulate $I_{Na}$ (Figure 6E) despite being expressed at similar levels to that of Nedd4-2 (Figure 6F).

## Modulation of $Na_v1.5$ Cellular Localization by Nedd4-2

In order to analyze whether Nedd4-2 reduces the density of $Na_v1.5$ at the plasma membrane, the channel was coexpressed in HEK cells as a fusion protein carrying a C-terminal YFP together with Nedd4-2 fused to the GFP protein. $Na_v1.5$-YFP yielded currents similar to that measured with WT $Na_v1.5$, and was down-regulated by GFP-Nedd4-2 to a similar extent as its native counterpart (data not shown). In control experiments, $Na_v1.5$-YFP was confined predominantly to the periphery of transfected HEK cells as observed by confocal microscopy (Figure 7A). In stark contrast however, $Na_v1.5$-YFP fluorescence in the presence of Nedd4-2 was clearly distributed homogenously over the cytosol, suggesting that Nedd4-2 can indeed reduce channel density at the plasma membrane (Figure 7B).

# Discussion

In this study, we investigated the molecular determinants and functional consequences of $Na_v1.5$ ubiquitination. The three main findings are as follows: (1) the ubiquitin-protein ligase Nedd4-2, expressed in cardiac cells, binds to the PY-motif of the cardiac sodium channels; (2) Nedd4-2 ubiquitinates and likely downregulates $Na_v1.5$ at the cell membrane; and (3) ubiquitinated fractions of $Na_v1.5$ are found in heart. To our

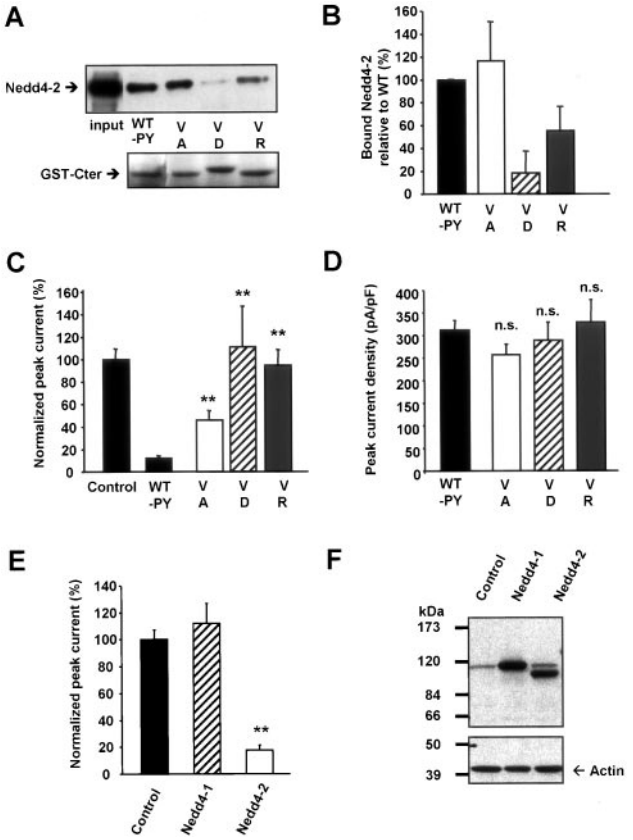


**Figure 6.** Val-1980 of $Na_v1.5$ participates to the binding of Nedd4-2. A, Pulldown experiments were performed as described in methods using lysates of Nedd4-2–transfected HEK cells (as in Figure 1B). Changing Val-1980 into charged residues (VD for aspartic acid and VR for lysine) clearly decreased the amount of bound Nedd4-2 as compared with the WT protein. Equal amounts of fusion proteins were used in pulldown assays (bottom). B, Quantification of three pulldown experiments as described in A. C, Charged residues (VD and VR) in position 1980 completely abolished the Nedd4-2–dependent downregulation seen with the $Na_v1.5$ (black column), and alanine in this position (white column) partially blunted this effect; n=10 to 15 cells from 2 to 4 experiments, $^{**}P<0.01$ vs WT-PY. D, Peak $I_{Na}$ densities were measured in HEK cells after transient transfection of WT and the three mutant $Na_v1.5$ constructs. Mutant constructs yielded $I_{Na}$ that were not significantly different from WT $Na_v1.5$ (WT-PY); n=10 to 15 cells from 2 to 4 experiments. E, Transient cotransfections of HEK cells stably expressing $Na_v1.5$ with Nedd4–1 or Nedd4-2 were performed as described in Materials and Methods. Only Nedd4-2 decreased $I_{Na}$, n=10 cells from 2 experiments, $^{**}P<0.001$. F, Control by immunoblotting of the expression of both Nedd4 isoforms in the conditions used for the experiments in E using a serum cross-reacting with Nedd4-1 and Nedd4-2. Protein loading was controlled by anti-actin immunoblotting.

knowledge, this study provides for the first time evidence that ion channels can be found ubiquitinated in native tissues.

## Nedd4-2 Associates With $Na_v1.5$

Ubiquitin protein-ligases of the Nedd4/Nedd4-like family are involved in many different cellular processes such as proteasome-mediated cytosolic protein degradation,[21] virus-mediated cell membrane budding,[22] and regulation of neuronal growth cone dynamics.[23] In addition, two ion channels are also regulated by Nedd4/Nedd4-like proteins,[8,9] although no direct evidence exists for a Nedd4-dependent ubiquitination of either of these two channels. The best studied example is ENaC that comprises a PY-motif in each of its three subunits.[24] Similar PY-motifs are found in the intracellular C-terminus of most $Na_v$ channels, suggesting that Nedd4-like proteins could also bind to and regulate sodium channels from excitable cells. In this study, we observed using both the yeast two-hybrid and GST-pulldown assays, that Nedd4-2, which is expressed in cardiac tissues,[11,12] can bind to $Na_v1.5$, and that this interaction is dependent on the integrity of the PY-motif.

Kanelis et al[20] investigated the interaction between the PY-motif of the $\beta$-subunit of ENaC and different Nedd4-WW-domains. The dissociation constants of these interactions were in the range of 20 to 160 $\mu$mol/L, providing a possible rationale for our failure to coimmunoprecipitate both proteins. Indeed, such low-affinity interactions are likely transient, and may be observed only in conditions where both proteins are found at concentrations higher than those attained in coimmunoprecipitation experiments. However, our ubiquitination and functional experiments indicate that both proteins associate in the cells.

Recent work[19] provided evidence that the PY-motif of ENaC can be extended to the amino acid residue found in position +3 after the Tyr, suggesting an extended PY-motif PPxYxx$\phi$ ($\phi$ being a hydrophobic residue). Pulldown experiments performed with mutant PY-motifs of $Na_v1.5$ are in close agreement with this model because mutation of either Tyr-1977 into Ala or substitution of Val-1980 (+3 after Tyr) with charged amino acids both resulted in a strong reduction in Nedd4-2 binding. However, our functional results suggest that the observed residual binding is not sufficient to result in Nedd4-2-dependent regulation of $Na_v1.5$ (Figures 5A and 6C). Because the Yxx$\phi$ motif is known as a potential binding site for proteins involved in endocytosis,[25] these mutations could be expected to have other effects on the expression of the channel as has been observed in the case of connexin43.[26] However, the observation that none of the mutants displayed an $I_{Na}$ significantly different to WT channels in the absence of Nedd4-2 argues against this possibility (Figure 6D).

## Ubiquitination of $Na_v1.5$

Our findings demonstrate that a fraction of the $Na_v1.5$ channels are ubiquitinated, both in HEK cells and in the heart. Furthermore, we observed that Nedd4-2 is able to enhance $Na_v1.5$ ubiquitination. These novel findings suggest that $Na_v1.5$ ubiquitination is playing a role in the trafficking and/or targeting of this channel in cardiac cells. It is interesting to note that the band of ubiquitinated $Na_v1.5$ found in cardiac cells (Figure 3A) is less diffuse than in HEK cells, which may suggest that mono- or oligoubiquitination is more important in native tissues. It should, however, be pointed out that multiple ubiquitination pathways mediated by different types of ubiquitin-ligases might be active in the cell at the same time as illustrated by a basal ubiquitination of $Na_v1.5$ in HEK cells. However, the finding showing that Nedd4-1 does not downregulate $Na_v1.5$ currents speaks for a specific role of Nedd4-2.

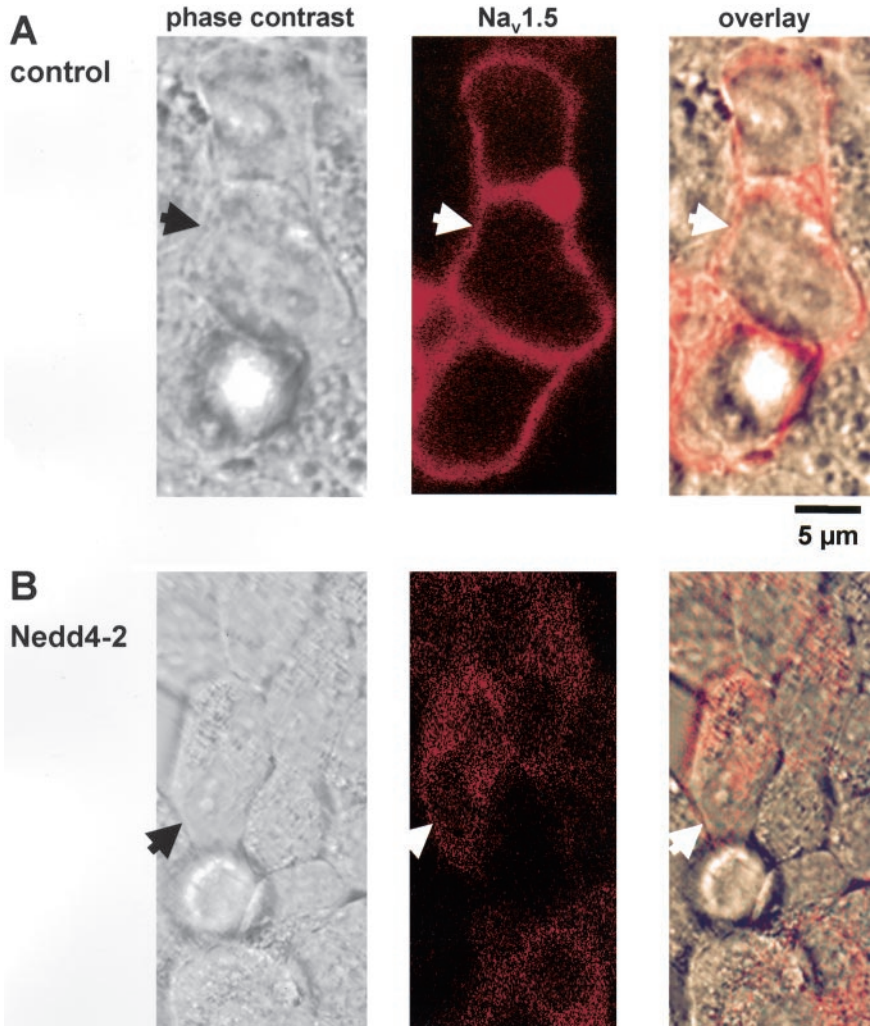


**Figure 7.** Peripheral localization of $Na_v1.5$ channels is reduced by Nedd4-2. Cellular localization of $Na_v1.5$ was studied in HEK cells transiently transfected with $Na_v1.5$-YFP with (A) our without (B) Nedd4-2-GFP constructs as described in Materials and Methods. Clear peripheral localization of the $Na_v1.5$-YFP proteins (shown in red for better visualization) was reduced on Nedd4-2 cotransfection. A similar pattern was observed in more than 100 cotransfected cells. Arrows show plasma membrane localization.



### $Na_v1.5$ Cell Surface Density Is Modulated by Nedd4-2

$Na_v1.5$ currents measured in HEK cells are decreased on coexpression of WT but not inactive Nedd4-2, implying that a ubiquitination step underlies this phenomenon. $Na_v1.5$ biophysical properties were not altered upon Nedd4-2 overexpression, suggesting that only the channel density was reduced. Imaging experiments performed using $Na_v1.5$-fluorescent fusion proteins (Figure 7) clearly support this model. These findings are in agreement with the proposed mode of Nedd4-2 action on ENaC,[8] and suggest that Nedd4-2 controls either the internalization or the externalization rate of $Na_v1.5$ channels, or both. An alternative mechanism could be that Nedd4-2 is regulating the intracellular pool of channels by, for instance, targeting them for lysosomal or proteasomal degradation. Our binding and functional data support a direct modulation of $Na_v1.5$ membrane density by Nedd4-2. However, indirect effects mediated through other cellular targets of Nedd4-2 cannot be excluded.

### Potential Roles of Nedd4-2 Regulation of $Na_v1.5$

Changes in $I_{Na}$ have been documented in in vivo models of cardiac disorders. A reduction of $I_{Na}$ in dog cardiac cells isolated from the epicardial border zone surrounding infarcted areas has been reported.[27] Similarly, a decrease in $Na_v1.5$ expression has been demonstrated in a dog model of atrial fibrillation.[28] Ahmmed et al[29] reported an increase of 30% to 80% $I_{Na}$ density in cardiomyocytes from guinea pigs with cardiac hypertrophy and failure. Even if, in some cases, such alterations in $Na_v1.5$ expression correlated with mRNA changes, it is clear that other regulatory pathways might be activated in parallel. Consequently, it would be interesting to analyze Nedd4-2 expression levels in pathological states. Interestingly, Nedd4-2 is negatively regulated through phosphorylation by the serum and glucocorticoid-dependent kinase 1 (SGK1),[30] which is well expressed in human heart.[31] SGK1 is regulated by endocrine factors[32] that may, via the activation of SGK1, modulate cardiac $I_{Na}$.

The penetrance and expressivity of *SCN5A* mutations, the gene encoding $Na_v1.5$, are known to be variable,[2] suggesting that other genetic or epigenetic factors modulate, for instance, the cell surface expression of $Na_v1.5$. This is exemplified by BrS, in which the channel membrane density is an important determinant of the clinical phenotype.[3,4] Our present findings suggest that ubiquitin-protein ligases represent potential modifier genes capable of modulating the phenotypic expression of genetic disorders, by modulating the number of mutant $Na_v1.5$ channels at the cell membrane.

In conclusion, our work provides strong evidence that Nedd4-2 ubiquitinates $Na_v1.5$, thereby regulating the channel density at the plasma membrane. Moreover, it shows that the effect of Nedd4-2 requires the PY-motif of $Na_v1.5$, a motif which is conserved in most $Na_v$ channels. These observations likely indicate that the type of regulation described in this work may apply to the physiological regulation of $Na_v1.5$ in the heart, as well as to neuronal $Na_v$ channels.

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