αB-Crystallin Interacts with Na\textsubscript{v}1,5 and Regulates Ubiquitination and Internalization of Cell Surface Na\textsubscript{v}1,5*  

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Na\textsubscript{v}1,5, the pore-forming α subunit of the cardiac voltage-gated Na\textsuperscript{+} channel complex, is required for the initiation and propagation of the cardiac action potential. Mutations in Na\textsubscript{v}1,5 cause cardiac arrhythmias and sudden death. The cardiac Na\textsuperscript{+} channel functions as a protein complex; however, its complete components remain to be fully elucidated. A yeast two-hybrid screen identified a new candidate Na\textsubscript{v}1,5-interacting protein, αB-crystallin. GST pull-down, co-immunoprecipitation, and immunostaining analyses validated the interaction between Na\textsubscript{v}1,5 and αB-crystallin. Whole-cell patch clamping showed that overexpression of αB-crystallin significantly increased peak sodium current (I_{Na}) density, and the underlying molecular mechanism is the increased cell surface expression level of Na\textsubscript{v}1,5 via reduced internalization of cell surface Na\textsubscript{v}1,5 and ubiquitination of Na\textsubscript{v}1,5. Knock-out of αB-crystallin expression significantly decreased the cell surface expression level of Na\textsubscript{v}1,5. Co-immunoprecipitation analysis showed that αB-crystallin interacted with Nedd4-2; however, a catalytically inactive Nedd4-2-C801S mutant impaired the interaction and abolished the up-regulation of I_{Na} by αB-crystallin. Na\textsubscript{v}1,5 mutation V1980A at the interaction site for Nedd4-2 eliminated the effect of αB-crystallin on reduction of Na\textsubscript{v}1,5 ubiquitination and increases of I_{Na} density. Two disease-causing mutations in αB-crystallin, R109H and R151X (nonsense mutation), eliminated the effect of αB-crystallin on I_{Na}. This study identifies αB-crystallin as a new binding partner for Na\textsubscript{v}1,5. αB-Crystallin interacts with Na\textsubscript{v}1,5 and increases I_{Na} by modulating the expression level and internalization of cell surface Na\textsubscript{v}1,5 and ubiquitination of Na\textsubscript{v}1,5, which requires the protein-protein interactions between αB-crystallin and Na\textsubscript{v}1,5 and between αB-crystallin and functionally active Nedd4-2.

Na\textsubscript{v}1,5 is the pore-forming α subunit of the major cardiac voltage-gated Na\textsuperscript{+} channel complex. It generates the sodium current (I_{Na})\textsuperscript{4} plays an essential role in the initiation and propagation of the cardiac action potential (1–3). Mutations in the SCN5A gene (encoding Na\textsubscript{v}1,5) cause several inherited arrhythmias, including atrial fibrillation, Brugada syndrome, long QT syndrome, progressive cardiac conduction defect disease, sick sinus syndrome, and dilated cardiomyopathy (4).

Na\textsubscript{v}1,5 exists in vivo in a multiprotein complex, which interacts with the actin cytoskeleton and the extracellular matrix to provide an important functional link between channel complexes, cardiac structure, and electrical functioning (5, 6). Several proteins have been reported to bind to Na\textsubscript{v}1,5 (5–7). We have previously reported a small protein, MOG1, with a function in nucleocyttoplasmic protein transport that interacts directly with Na\textsubscript{v}1,5, promotes trafficking of Na\textsubscript{v}1,5 to the cell surface, and increases peak I_{Na} density (4, 6). Specifically, MOG1 facilitates export of Na\textsubscript{v}1,5 from the endoplasmic reticulum as well as targeting of Na\textsubscript{v}1,5 to caveolae on plasma membranes (4). Other Na\textsubscript{v}1,5-interacting proteins include four β-subunits, fibroblast growth factor homologous factor 1B, calmodulin, Nedd4-like ubiquitin-protein ligases (Nedd4-2), ankyrin-G, the Src family tyrosine kinase Fyn, syntrophin (associated with dystrophin), the protein tyrosine phosphatase PTPH1, and 14–3–3 β (5, 7). The majority of these proteins bind to Na\textsubscript{v}1,5 and affect its biophysical properties. MOG1, 14–3–3 β and ankyrin-G regulate Na\textsubscript{v}1,5 localization to the cell surface in cardiomyocytes (4, 7). Nedd4-2 interacts with Na\textsubscript{v}1,5 and regulates Na\textsubscript{v}1,5 degradation (8). However, the Na\textsubscript{v}1,5 protein

4 The abbreviations used are: I_{Na} sodium current; IP, immunoprecipitation; EGFP, enhanced GFP; MESNA, 2-mercaptoethanesulfonic acid sodium salt.
complex is highly sophisticated. Here, we aim to identify other important components of the Na\textsubscript{v}1.5 protein complex.

The \textit{CRYAB} gene encodes αB-crystallin, a small molecular weight heat shock protein widely expressed in many tissues, including the heart, lens, and skeletal muscle (9). Mutations in \textit{CRYAB} cause a number of inherited human disorders, including cataracts, skeletal muscle myopathy, and cardiomyopathy (9). In this study, using yeast two-hybrid screening and follow-up biochemical technologies, we have identified αB-crystallin as a new binding partner of Na\textsubscript{v}1.5. We further show that αB-crystallin interacts with Na\textsubscript{v}1.5 and enhances Na\textsubscript{v}1.5 cell surface expression by reducing ubiquitination of Na\textsubscript{v}1.5.

**Experimental Procedures**

\textbf{Plasmids, Mutagenesis, Antibodies, and Animals—}The cDNA for the human \textit{CRYAB} gene encoding αB-crystallin (NM_001885) was amplified by PCR using plasmid pcDNA3.1-αB-crystallin (10) as a template and subcloned into the pIREs2-EGFP vector between the XhoI and EcoRI restriction sites, resulting in expression plasmid pIREs2-CRYAB for \textit{CRYAB} or αB-crystallin. The disease-causing mutations in \textit{CRYAB}, including R11H, P20S, R56W, D109H, R120G, D140N, G154S, R151X, R157H, and A171T, were created into pIREs2-CRYAB using a DNA-based mutagenesis method (11, 12).

The expression construct for the human cardiac sodium channel gene SCN5A (hH1a) in vector pcDNA3 (pcDNA3-SCN5A-Na\textsubscript{v}1.5) was previously described (13–15). The cDNAs for HA-tagged Na\textsubscript{v}1.5-Loop II (cytoplasmic Loop II between DII and DIII; amino acids 940–1200), HA-tagged Na\textsubscript{v}1.5-Loop III (cytoplasmic Loop III between DIII and DIV; amino acids 1471–1523), and HA-tagged Na\textsubscript{v}1.5-C terminus (C-terminal domain; amino acids 1773–2016) were amplified by PCR using plasmid pcDNA3-Na\textsubscript{v}1.5 as a template and subcloned into the pCMV-HA vector between the EcoRI and XhoI restriction sites. The expression construct for mutant SCN5A with mutation V1980A was generated using a PCR-based mutagenesis method (11, 12). The expression plasmids for wild type human Nedd4-2 (KIAA0439) and a catalytically inactive form of Nedd4-2 with mutation C801S in pcDNA3.1(−) were amplified using PCR and fused to GST in the pGEX-4T-1 vector (pGEX-4T-1-Na\textsubscript{v}1.5 LI) as described previously (6). The construct was verified by direct DNA sequencing analysis. Expression of GST and the GST-Na\textsubscript{v}1.5-Loop II fusion protein in \textit{Escherichia coli} BL21 cells was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 8 h at 26 °C. Following expression, the cells were harvested, resuspended in lysis buffer (0.5% Nonidet P-40, 50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA supplemented with 1× protease inhibitor Complete Mini EDTA-free mixture from Roche Applied Science), and sonicated on ice. The lysate was centrifuged at 13,000 × g for 30 min at 4 °C, and the supernatants were incubated with glutathione-Sepharose 4B beads to precipitate GST and GST-Na\textsubscript{v}1.5-Loop II with gentle agitation on a rotator overnight at 4 °C according to the manufacturer's instructions (GE Healthcare). After centrifugation, the beads were washed three times for 5 min in PBS containing 1% Triton X-100. An equal amount of soluble fractions from HEK293 cells transiently transfected with pIREs2-EGFP-αB-crystallin was incubated with the pre-washed beads coupled with GST or GST-Na\textsubscript{v}1.5-Loop II with gentle agitation on a rotator for 1 h at room temperature. The beads were centrifuged and washed three times for 5 min in NETN buffer (0.5% Nonidet P-40, 50 mM Tris/HCl, 900 mM NaCl, 1 mM EDTA). The bound proteins were eluted with 1× SDS-PAGE sample buffer and separated on a 15% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were subsequently probed with an anti-αB-crystallin antibody, and the rest of the procedures for Western blotting analysis were as described previously (6).

\textbf{Co-immunoprecipitation (Co-IP) Analysis—}Co-IP studies were performed as described previously (4, 6, 16, 17). A stable HEK293 cell line with constitutive expression of Na\textsubscript{v}1.5 (HEK/Na\textsubscript{v}1.5) was described previously (4, 6, 15). The HEK/Na\textsubscript{v}1.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1-glutamine (2 mM), penicillin G (100 units/ml), streptomycin (10 mg/ml), and G418 (200 μg/ml) (Gibco) in a humidified incubator with 5% CO\textsubscript{2} at 37 °C. HEK/Na\textsubscript{v}1.5 cells with 70–80% confluence in a 10-cm plate were transiently transfected with 10 μg of plasmid DNA (pIREs2-EGFP-αB-crystallin) using 20 μl of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and cultured for 48 h, harvested, and lysed in ice cold lysis buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1× protease inhibitor Complete Mini EDTA-free mixture from Roche Applied Science). The cell lysate was centrifuged at 13,000 × g for 30 min at 4 °C. Cell extracts in the supernatants (500 μg) were preabsorbed with 30 μl of Protein A/G PLUS-agarose (sc-2003, Santa Cruz Biotechnology) for 1 h at 4 °C and microcentrifuged at 4 °C. An equal volume of the supernatants was incubated with 1.5 μg of an anti-αB-crystallin antibody or the same amount of anti-mouse IgG on a rotator overnight at 4 °C and

\textbf{Yeast Two-hybrid Screening for Na\textsubscript{v}1.5-interacting Proteins—}Yeast two-hybrid screening for Na\textsubscript{v}1.5-interacting proteins was reported by us previously (6).

\textbf{GST Pull-down Analysis—}GST pull-down assays were carried out as described by us previously (6). A cDNA fragment encoding Na\textsubscript{v}1.5-Loop II (amino acids 940 and 1200) was amplified using PCR and fused to GST in the pGEX-4T-1 vector (pGEX-4T-1-Na\textsubscript{v}1.5 LI) as described previously (6). The construct was verified by direct DNA sequencing analysis. Expression of GST and the GST-Na\textsubscript{v}1.5-Loop II fusion protein in \textit{Escherichia coli} BL21 cells was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 8 h at 26 °C. Following expression, the cells were harvested, resuspended in lysis buffer (0.5% Nonidet P-40, 50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA supplemented with 1× protease inhibitor Complete Mini EDTA-free mixture from Roche Applied Science), and sonicated on ice. The lysate was centrifuged at 13,000 × g for 30 min at 4 °C, and the supernatants were incubated with glutathione-Sepharose 4B beads to precipitate GST and GST-Na\textsubscript{v}1.5-Loop II with gentle agitation on a rotator overnight at 4 °C according to the manufacturer's instructions (GE Healthcare). After centrifugation, the beads were washed three times for 5 min in PBS containing 1% Triton X-100. An equal amount of soluble fractions from HEK293 cells transiently transfected with pIREs2-EGFP-αB-crystallin was incubated with the pre-washed beads coupled with GST or GST-Na\textsubscript{v}1.5-Loop II with gentle agitation on a rotator for 1 h at room temperature. The beads were centrifuged and washed three times for 5 min in NETN buffer (0.5% Nonidet P-40, 50 mM Tris/HCl, 900 mM NaCl, 1 mM EDTA). The bound proteins were eluted with 1× SDS-PAGE sample buffer and separated on a 15% SDS-polyacrylamide gel and transferred to PVDF membranes. The membranes were subsequently probed with an anti-αB-crystallin antibody, and the rest of the procedures for Western blotting analysis were as described previously (6).
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then mixed with 30 µl of Protein A/G PLUS-agarose. The antibody-protein A/G PLUS-agarose complex was incubated on a rocker for 2 h at 4 °C, centrifuged at 1000 \( \times \) g for 5 min, and washed five times with washing buffer (lysis buffer with 0.1% Tween 20). The washed pellets were resuspended in 50 µl of 1× SDS loading buffer, incubated at 37 °C for 5 min, and electro-phoresed through 8–15% SDS-polyacrylamide gel using the Bio-Rad minigel system. The proteins were transferred onto a PVDF membrane (Millipore) overnight at 100 mA at 4 °C. The membrane was blocked with blocking buffer (3% BSA and 0.05% Tween in PBS) for 2 h at room temperature with gentle agitation and incubated with an anti-Na\textsubscript{v}1.5 antibody overnight at 4 °C with gentle agitation. After three washes with PBS (0.05% Tween in PBS) for 15 min at room temperature, the membrane was incubated with goat anti-rabbit HRP-conjugated secondary antibody for 2 h at room temperature. After three washes with PBS for 15 min at room temperature, the proteins were visualized using a SuperSignal West Pico Chemiluminescent Substrate (Pierce).

For reciprocal co-IP analysis, 1.5 µg of anti-Na\textsubscript{v}1.5 antibody was used for immunoprecipitation, and the anti-αB-crystallin antibody was used for Western blotting analysis. For the negative control, 1.5 µg of anti-rabbit IgG antibody was substituted for the anti-Na\textsubscript{v}1.5 antibody.

For co-IP analysis of αB-crystallin and Na\textsubscript{v}1.5 in cardiac cells, Sprague-Dawley rats were euthanized with nembutal (intraperitoneal injection of 162.5 units/kg body weight), and hearts were excised and rinsed with ice-cold Hanks’s buffer. The cardiac tissue samples were homogenized in lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1× protease inhibitor Complete Mini EDTA-free mixture from Roche Applied Science) on ice. The cardiac protein extracts were then used for co-IP analysis as described above.

Electrophysiological Studies—For patch clamping experiments, HEK/Na\textsubscript{v}1.5 cells were transfected with 0.8 µg of pIRE2-GFP-αB-crystallin DNA or pIRE2-GFP control DNA using 2 µl of GenJet\textsuperscript{TM} in vitro DNA transfection reagent. After 48 h of transfection, the cells expressing an approximately equal amount of GFPs were selected for electrophysiological studies for recordings of I\textsubscript{Na} (6, 18–21). 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 CaCl\textsubscript{2}, 10 mM HEPES, 10 mM glucose, and 1 mM MgCl\textsubscript{2} (pH 7.4-adjusted with CsOH). All reagents were obtained from Sigma-Aldrich. I\textsubscript{Na} was recorded using a whole-cell voltage clamp recording method with an Axon multiclamp700B patch clamp amplifier using the Digidata1440A digitizer (Axon Instruments, Sunnyvale, CA) on a desktop computer at room temperature (22 °C). I\textsubscript{Na} currents were filtered at 5 kHz with a 4-pole Bessel filter and sampled at 50 kHz. Pipette resistance ranged from 2 to 3 megaohms. The series resistance recorded in the whole cell configuration was compensated (80%) to minimize voltage errors. The holding potential for all pulse protocols was −120 mV, and the voltage dependence of the relative Na\textsuperscript{+} conductance activation, voltage-dependent inactivation, and recovery from inactivation were determined by means of custom voltage clamp protocols modified from those published previously. Details of each pulse protocol are given schematically in the related figures. I\textsubscript{Na} density was normalized using the cell capacitance. The data were analyzed using a combination of Clampfit version 10.2 (Molecular Devices), Microsoft Excel, and Origin version 8.5 (Microcal Software, Northampton, MA).

The steady-state activation and voltage dependence of inactivation curves were fitted with the Boltzmann equation, \( I/I_{\text{max}} = (1 + \exp((V - V_{1/2})/k))^{-1} \) to determine the membrane potential for half-maximal (in)activation (\( V_{1/2} \)) and the slope factor \( k \).

Recovery from inactivation was analyzed by fitting the data with a two-exponential equation, \( I/I_{\text{max}} = A_f \times \exp(-t/\tau_f) + A_s \times \exp(-t/\tau_s) \), to determine the fractions of the fast (\( A_f \)) and the slow (\( A_s \)) components of recovery from inactivation and the time constants for recovery from fast (\( \tau_f \)) and slow (\( \tau_s \)) inactivation, respectively.

Immunohistochemistry—The immunostaining analysis of neonatal rat cardiomyocytes and paraffin-embedded adult rat heart sections (8 µm) was performed with an anti-Na\textsubscript{v}1.5 antibody (ASC-013, Alomone) and an anti-αB-crystallin antibody (ADI-SPA-222, Enzo Life Science) according to standard protocols as described previously (6).

RNA Interference—The αB-crystallin siRNA targeting rat αB-crystallin (5′-GUGGAAUCCUCUCACUAAATTT’-3′) and scrambled control siRNA were designed and synthesized by GenePharma (Suzhou, Jiangsu, China). The scramble siRNA does not recognize any mRNA from H9C2 cells. H9C2 cells were cultured in DMEM supplemented with 15% fetal bovine serum and transfected with siRNA using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen). After 48 h of transfection, cells were harvested for assaying siRNA knockdown efficiency using quantitative real-time RT-PCR and Western blotting analysis as described (4, 22–25).

Sequences of primers used for quantitative real-time PCR analysis are listed in Table 1.

Table 1

| Primer/Sequence | Primer/Sequence |
|-----------------|-----------------|
| αB-Crystallin (rat) Forward | 5′-TGGGGCCACCTAGCTCGATTGA-3′ |
| αB-Crystallin (rat) Reverse | 5′-GGCTCTGTGTCGATTGTG-3′ |
| β-Actin (rat) Forward | 5′-CCGTAAGAGCTCTAAGCCCA-3′ |
| β-Actin (rat) Reverse | 5′-CCGACTCATGCTACTCTGCT-3′ |

Isolation of Cell Surface Proteins and Analysis of Expression Levels of Cell Surface Na\textsubscript{v}1.5—Transfected cells were used for preparation of plasma membrane protein extracts using EZ-Link Sulfo-NHS-SS-Biotin (Pierce) as described by us previously (4). HEK/Na\textsubscript{v}1.5 cells were transiently transfected with 10 µg of pIRE2-EGFP-αB-crystallin or pIRE2-EGFP using 20 µl of Lipofectamine 2000 (Invitrogen). H9C2 cells were transfected with αB-crystallin siRNA or control scramble siRNA, also using Lipofectamine 2000 (Invitrogen). After 48 h of transfection, cells were washed three times with ice-cold PBS and incubated for 30 min at 4 °C with 2 ml of biotin per 10-cm dish (1 mg/ml; EZ-Link Sulfo-NHS-SS-Biotin, Pierce) to label the cell surface proteins. After three washes with 100 mM glycine in PBS, the cells were incubated with 100 mM glycine for 15 min at
4 °C to quench the biotinylation reaction. After quenching, the cells were scraped and lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1× protease inhibitor Complete Mini EDTA-free mixture from Roche Applied Science). Cell lysates were centrifuged at 13,000 × g for 30 min at 4 °C, and the supernatants were incubated with NeutrAvidin-agarose resins (Pierce) overnight at 4 °C with gentle agitation. The protein-agarose complexes were washed three times with PBS and then resuspended in 1× SDS-PAGE sample buffer containing 50 mM DTT and analyzed by Western blotting with an anti-Na<sub>1.5</sub> antibody as described above. The plasma membrane protein Na<sup>+</sup>K<sup>-</sup>-ATPase was used as loading control to calibrate the cell surface proteins.

**Analysis of Stability and Internalization of Cell Surface Na<sub>1.5</sub>—**The assay for stability of cell surface Na<sub>1.5</sub> was performed as described previously (4). Cells were transfected and cultured in 10-cm culture dishes for 48 h. Cell surface proteins were tagged with biotin at 4 °C and subsequently quenched with 100 mM glycine as described above. The culture media were then added, and biotin-tagged cells were cultured in a 37 °C incubator to allow biotinylated cell surface proteins to traffic inside cells (internalization) for 9 h. The biotin-tagged proteins remained on cell surface were stripped off with 50 mM MESNA (1392807, Sigma) in 100 mM Tris/HCl (pH 8.6) containing 100 mM NaCl and 2.5 mM CaCl<sub>2</sub> at 4 °C for 30 min and quenched with 5 mg/ml iodoacetamide in PBS at 4 °C for 15 min. The cells were then lysed in ice with cold lysis buffer, and the supernatants were incubated with 200 μl of precleared NeutrAvidin-agarose resins (Pierce) to precipitate the internalized biotinylated cell surface proteins for overnight at 4 °C with gentle agitation. The protein-agarose complexes were washed five times with lysis buffer and resuspended in 100 μl of 1× SDS-PAGE loading buffer (39001, Pierce) containing 50 mM DTT. The expression levels of internalized biotinylated cell surface proteins for 9 h were normalized to the total biotinylated proteins remaining on the cell surface.

**Statistical Analysis—**All data were from at least three independent experiments and expressed as means ± S.E. Statistical analysis was carried out using two-tailed paired or unpaired Student’s t tests between two groups. A p value of ≤0.05 was considered to be statistically significant. The differences between groups over a time period were analyzed by two-way analysis of variance.

**Results**

**Identification of αB-Crystallin as a New Protein That Interacts with Na<sub>1.5</sub>—**We have previously reported a yeast two-hybrid screen to identify candidate proteins that interact with Na<sub>1.5</sub> (6). MOG1 was identified as a Na<sub>1.5</sub>-interacting protein during the study and previously reported (6). In the same screen, we also identified a cDNA clone that encodes human αB-crystallin (NM_001885). In this study, we validated the interaction between αB-crystallin and Na<sub>1.5</sub> by an in vitro GST pull-down assay first and then by a co-immunoprecipitation assay. We constructed an expression plasmid that will express a GST-Na<sub>1.5</sub>-Loop II protein with GST fused to the cytoplasmic Loop II of Na<sub>1.5</sub> (between transmembrane domain II and III, amino acids 940–1200) in E. coli BL21 cells. The GST-Na<sub>1.5</sub>-Loop II protein was purified using glutathione beads. The affinity-purified GST or GST-Na<sub>1.5</sub>-Loop II was incubated with cell lysates from HEK293 cells transfected with a pIREs2-EGFP-αB-crystallin expression plasmid. Unbound proteins were washed off, and proteins pulled down were analyzed by Western blotting analysis using an anti-αB-crystallin antibody. As illustrated in Fig. 1A, GST-Na<sub>1.5</sub>-Loop II successfully pulled αB-crystallin down, whereas no binding for αB-crystallin was observed with GST alone (negative control). These results suggest that αB-crystallin interacts with Na<sub>1.5</sub>-Loop II in vitro.

To determine whether αB-crystallin interacts with Na<sub>1.5</sub> in vivo, a co-IP assay was performed using protein extracts from HEK293 cells and rat hearts. A HEK293 stable cell line that overexpresses Na<sub>1.5</sub> (HEK/Na<sub>1.5</sub>) was transfected with pIREs2-EGFP-αB-crystallin for 48 h. The HEK/Na<sub>1.5</sub> cell lysates were immunoprecipitated using an anti-Na<sub>1.5</sub> antibody (Fig. 1B, lane 2) or anti-rabbit IgG as a negative control (Fig. 1B, lane 3). The precipitates were analyzed by Western blotting analysis with an anti-αB-crystallin antibody. The anti-Na<sub>1.5</sub> antibody successfully precipitated αB-crystallin (Fig. 1B, lane 2), but the anti-rabbit IgG failed to precipitate αB-crystallin (Fig. 1B, lane 3). Consistently, reciprocal co-IP showed that the anti-αB-crystallin antibody, but not the control anti-mouse IgG, precipitated Na<sub>1.5</sub> (Fig. 1C). These results demonstrated that αB-crystallin interacted with Na<sub>1.5</sub> in HEK293 cells.

Similar to studies with HEK/Na<sub>1.5</sub> cells, a co-IP assay was also performed using protein extracts from adult rat hearts. Fig. 1D shows that the anti-Na<sub>1.5</sub> antibody, but not the control IgG or an anti-Na<sup>+K</sup>—ATPase antibody, pulled αB-crystallin down. Reciprocally, the anti-αB-crystallin antibody, but not the control IgG, precipitated Na<sub>1.5</sub> protein (Fig. 1E). These results demonstrated that αB-crystallin interacted with Na<sub>1.5</sub> in the rat heart.

We also assessed whether αB-crystallin interacts with Loop III (between transmembrane domain III and IV) and the C terminus of Na<sub>1.5</sub>. Interestingly, co-IP assays demonstrated that αB-crystallin interacted strongly with the C terminus of Na<sub>1.5</sub> but not with Loop III (Fig. 1F and G).

Because αB-crystallin interacted with Na<sub>1.5</sub>, we hypothesized that αB-crystallin co-localized with Na<sub>1.5</sub> in the cardiac tissue. To test the hypothesis, immunostaining was performed in adult rat hearts fixed in paraformaldehyde and embedded in paraffin. The paraffin sections were used for immunostaining using both anti-αB-crystallin and anti-Na<sub>1.5</sub> antibodies (Fig. 2A). Immunostaining without primary antibodies was used as negative controls (Fig. 2B). The anti-αB-crystallin produced red signals, whereas the anti-Na<sub>1.5</sub> produced green signals. When the two signals merged, a yellow signal was generated. The αB-crystallin protein showed strong expression in atrial and ventricular cardiomyocytes and co-localized with Na<sub>1.5</sub> (Fig. 2A). These results suggested the co-localization of αB-crystallin and Na<sub>1.5</sub> expression in cardiac tissue.
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αB-Crystallin Modulates Sodium Current in HEK293 Cells—To investigate the functional role of αB-crystallin in Na\textsubscript{v}1.5 physiology, electrophysiological studies were performed using the whole-cell patch clamp method. Plasmid pIRE\textsubscript{S2}-EGFP-α-crystallin or control vector pIRE\textsubscript{S2}-EGFP was transfected into HEK/Na\textsubscript{v}1.5 cells. Whole-cell sodium currents were then measured (Fig. 3A). Compared with the control, overexpression of α-crystallin significantly increased sodium current densities (Fig. 3B and C). Cells with α-crystallin overexpression significantly increased the peak sodium current density (−297.22 ± 45.27 pA/picofarads) at −25 mV compared with the cells with control EGFP (−202.05 ± 23.82 pA/picofarads, p < 0.01) (Fig. 3C). The voltage dependence of the activation and inactivation of sodium currents was also investigated. However, no differences were observed in the voltage dependence kinetics of activation and inactivation of sodium currents between cells with α-crystallin overexpression and those with expression of control EGFP (Fig. 3D). An investigation of time dependence of recovery from inactivation showed moderate deceleration of t\textsubscript{r} by 3.19 ms (from 12.88 to 9.69 ms for fast time constant of recovery) by αB-crystallin (Fig. 3E). The fitted data are shown in Table 2.

We also assessed whether α-crystallin affects the late sodium currents generated by Na\textsubscript{v}1.5. Mutant ΔKPQ-Na\textsubscript{v}1.5 associated with long QT syndrome and known to generate large sodium currents was used as a control for easy detection of late sodium currents (26). Overexpression of α-crystallin did not show an effect on late I\textsubscript{Na} generated by wild type Na\textsubscript{v}1.5 compared with control cells transfected with pIRE\textsubscript{S2}-EGFP (Fig. 3, Fand G). The rate of decay of I\textsubscript{Na} was slowed by overexpression of α-crystallin (Fig. 3F), although it did not reach a significant level (Fig. 3G).

Overexpression of αB-Crystallin Increased the Amount of Na\textsubscript{v}1.5 on the Cell Surface—Given the significant role of the level of cell surface expression of Na\textsubscript{v}1.5 in sodium current densities, αB-crystallin overexpression may increase sodium current densities by increasing cell surface expression of Na\textsubscript{v}1.5. Forty-eight hours after transfection of HEK/Na\textsubscript{v}1.5 cells with pIRE\textsubscript{S2}-EGFP-α-crystallin or pIRE\textsubscript{S2}-EGFP, cell surface biotinylation assays were performed. Because Na\textsuperscript{+}K\textsuperscript{+}-ATPase is expressed on the plasma membrane of HEK293, it was used as a loading control for cell surface proteins. Western blotting analysis showed that compared with the control EGFP vector, overexpression of α-crystallin significantly increased the amount of Na\textsubscript{v}1.5 in plasma membranes (Fig. 4A). However, αB-crystallin overexpression did not affect the expression level of total Na\textsubscript{v}1.5 protein in cells (Fig. 4B). These data suggest that αB-crystallin regulates cell surface expression of Na\textsubscript{v}1.5 but not expression of total Na\textsubscript{v}1.5.

Knockdown of αB-Crystallin Decreased the Cell Surface Expression Level of Na\textsubscript{v}1.5 in H9C2 Cells—We next evaluated the role of endogenous αB-crystallin in regulation of Na\textsubscript{v}1.5 function. Because Western blotting analysis failed to detect αB-crystallin protein expression in HEK293 cells (data not shown), we studied H9C2 rat cardiac myoblasts. Small interfering RNA (siRNA) against αB-crystallin (si-Cryab) was transfected into H9C2 rat cardiac myoblasts to knock down the endogenous expression level of αB-crystallin. Quantitative real-time RT-PCR analysis of mRNA expression showed a significant reduction (about 57%) of αB-crystallin mRNA in cells transfected with si-Cryab as compared with control scrambled siRNA (Fig. 4C). Western blotting analysis showed that si-Cryab was able to significantly decrease the expression of αB-crystallin (Fig. 4D); therefore, it was used to determine the effects of knockdown of αB-crystallin on Na\textsubscript{v}1.5 cell surface expression.

Biotinylated cell surface protein extracts from H9C2 cells were analyzed by Western blotting analysis (Fig. 4E). Compared with control scrambled siRNA, si-Cryab significantly decreased the amount of Na\textsubscript{v}1.5 in the plasma membranes (Fig. 4E).
αB-Crystallin Overexpression Decreased Ubiquitination of Na\textsubscript{v}1.5 and Increased the Stability of Na\textsubscript{v}1.5 on the Cell Surface—The steady state level of Na\textsubscript{v}1.5 on the cell surface is determined by the rate of trafficking to the plasma membrane and the internalizing rate to intracellular organelles. Ubiquitination is one of the major processes that regulate the degradation of membrane proteins and their trafficking to other membrane compartments. Heat shock proteins, including Hsp70, Hsc70, and α-crystallin have been shown to be involved in regulating the cell surface expression level of epithelial sodium channels through regulating ubiquitination (27). Thus, we hypothesized that αB-crystallin also regulated the cell surface expression level of Na\textsubscript{v}1.5 by modulating ubiquitination of Na\textsubscript{v}1.5. Western blotting analysis was performed with cell extracts from HEK/Na\textsubscript{v}1.5 cells with overexpression of αB-crystallin or transfected with the EGFP vector and treated with a 10 μM concentration of the proteasome inhibitor MG132 to detect the ubiquitination level of Na\textsubscript{v}1.5 by the FK2 mouse monoclonal anti-ubiquitination antibody. First, we used the anti-Nav1.5 antibody to precipitate Nav1.5 from HEK/Nav1.5 cell extracts. Then the precipitates were analyzed by Western blotting analysis. The anti-Nav1.5 antibody was able to precipitate an approximately equal amount of Nav1.5 from cells with EGFP and αB-crystallin (Fig. 5A, top). Western blotting analysis showed that overexpression of αB-crystallin decreased the ubiquitination level of wild type Na\textsubscript{v}1.5 as compared with EGFP but had no effect on the ubiquitination level of mutant V1980A-Na\textsubscript{v}1.5 (Fig. 5A (bottom) and B). This experiment was repeated three times, and similar results were obtained (data not shown).

To determine whether αB-crystallin is involved in regulating Na\textsubscript{v}1.5 internalization from the plasma membranes, we tested whether αB-crystallin affects the stability of Na\textsubscript{v}1.5 on the cell surface. HEK/Na\textsubscript{v}1.5 cells transfected with pRES2-EGFP-αB-crystallin or pRES2-EGFP were labeled with biotin at 4 °C and returned to the culture incubator for 9 h at 37 °C. At the end of the incubation, the remaining cell surface biotin was stripped with MESNA, and the internalized biotin-labeled Na\textsubscript{v}1.5 was extracted and analyzed by Western blotting analysis with anti-Na\textsubscript{v}1.5 antibody. As shown in Fig. 5, C and D, compared with EGFP (vector), overexpression of αB-crystallin significantly decreased the amount of internalized Na\textsubscript{v}1.5.

αB-Crystallin Interacts with Nedd4-2 and Decreases the Interaction between Nedd4-2 and Na\textsubscript{v}1.5—Nedd4-2 was shown to interact with Nav1.5 and regulate degradation of Nav1.5 by regulating ubiquitination (8). Considering that αB-crystallin decreases ubiquitination of Na\textsubscript{v}1.5, we investigated whether αB-crystallin interacts with Nedd4-2. Co-IP analysis showed that an anti-αB-crystallin antibody was able to precipitate Nedd4-2 in HEK293 cells overexpressing αB-crystallin (Fig. 6A). Reciprocal co-IP analysis showed that an anti-Nedd4-2 antibody also was able to precipitate αB-crystallin (Fig. 6B). These data suggest that αB-crystallin forms a complex with Nedd4-2.

Mutation C801S (Nedd4-2-CS) is a dominant negative mutant that affects the catalytic activity of Nedd4-2 (8). Co-IP analysis showed that the interaction between Nedd4-2 and αB-crystallin was reduced by the C801S mutation in Nedd4-2 (Fig. 6, C and D). In a separate study, we showed that overex-
expression of αB-crystallin did not affect the expression level of Nedd4-2 (Fig. 6, E and F). Together, these data suggest that functionally active Nedd4-2 is required for interaction between αB-crystallin and Nedd4-2.

We also assessed whether Nedd4-2 affects the interaction between Na$_{1.5}$ and αB-crystallin. Overexpression of wild type Nedd4-2 significantly reduced the interaction between Na$_{1.5}$ and αB-crystallin (Fig. 6, G and H), which is consistent with the reported finding that wild type Nedd4-2 reduced sodium current density (8). However, mutant Nedd4-2-CS did not affect the interaction between Na$_{1.5}$ and αB-crystallin or slightly increased the interaction (Fig. 6, G and H), which is also consistent with the reported finding that Nedd4-2-CS did not affect sodium current density (8).

Next, we investigated whether αB-crystallin affects the interaction between Nedd4-2 and Na$_{1.5}$. Co-immunoprecipitation analysis showed that, compared with EGFP (vector), overexpression of αB-crystallin reduced the interaction between Nedd4-2 and Na$_{1.5}$ (Fig. 6, I and J).

αB-Crystallin Modulates I$_{Na}$ by Affecting Its Interaction with Nedd4-2 and Nedd4-2-mediated Ubiquitination of Na$_{1.5}$—Electrophysiological studies were then used to further assess the effects of αB-crystallin on sodium currents by regulating Nedd4-2. In HEK/Na$_{1.5}$ cells with overexpression of wild type Nedd4-2, additional overexpression of αB-crystallin significantly increased the peak sodium current density (Fig. 7A). However, in HEK/Na$_{1.5}$ cells with overexpression of mutant Nedd4-2-CS, additional overexpression of αB-crystallin did not

FIGURE 3. Functional effects of αB-crystallin on I$_{Na}$ in HEK/Na$_{1.5}$ cells. A, representative whole-cell sodium currents recorded from HEK/Na$_{1.5}$ cells transfected with the control vector or a αB-crystallin overexpression plasmid. The voltage clamp protocol is shown in the inset. B, the relationship of average current densities (current normalized to cell capacitance) and voltage. C, peak sodium current densities at −110 mV. D, steady-state activation and inactivation curves. The holding potential was −120 mV. The protocol for recording the steady-state activation and inactivation curves is shown in the inset. E, time course of recovery from inactivation was studied using a two-pulse protocol of −20 mV at the −120 mV holding potential as in the inset. F and G, representative late I$_{Na}$ recorded at the −20 mV test potential from HEK293 cells with co-expression of wild type SCN5A with the control vector or the αB-crystallin expression plasmid. The ΔKPQ mutation associated with long QT syndrome, which is known to generate large late I$_{Na}$, was used as a positive control. Note that overexpression of αB-crystallin did not impact the generation of late I$_{Na}$; αB-crystallin slowed the rate of decay, although the effect did not reach a significant level. Data are shown as means ± S.E. (error bars). NS, not significant; *, p < 0.05. All studies were repeated at least three times. pF, picofarads.
TABLE 2
Summary data on effects of overexpression of αB-crystallin on cardiac sodium current $I_{Na}$

| Sodium current parametera | Control vector EGFP | αB-Crystallin |
|---------------------------|---------------------|--------------|
| Activation $V_{1/2}$ (mV) | $n = 12$             | $n = 12$     |
| $k$                       | $-34.73 \pm 0.79$    | $-34.72 \pm 0.61$ |
| Steady-state inactivation $V_{1/2}$ (mV) | $n = 10$            | $n = 11$ |
| $k$                       | $5.74 \pm 0.12$      | $5.29 \pm 0.10$ |
| Recovery from inactivation $A_f$ | $n = 15$   | $n = 14$ |
| $\tau_f$                 | $0.120 \pm 0.08$     | $0.156 \pm 0.01^b$ |
| $A_s$                     | $9.69 \pm 0.40$      | $12.88 \pm 0.45^b$ |
| $\tau_s$                 | $0.87 \pm 0.01$      | $0.831 \pm 0.00^b$ |
| $\tau_i$                 | $158.033 \pm 16.76$  | $165.454 \pm 14.95$ |

- $V_{1/2}$ voltage of half-maximal activation; $k$, slope factor of voltage dependence of (in)activation; $\tau_f$, time constant for development of slow inactivation; $\tau_s$, slow time constant of recovery from inactivation; $A_f$, fraction of the fast component of recovery from inactivation; $A_s$, fraction of the slow component of recovery from inactivation. Data were shown as mean ± S.E. 

- $^a$ p < 0.5 versus vector EGFP (Student’s t test). The experiment was repeated at least three times.

Affect the peak sodium current density (Fig. 7B). Together with the co-immunoprecipitation data above, these data suggest that the interaction between αB-crystallin and functionally active Nedd4-2 is important for the function of Na$_{1.5}$.

The valine residue at position 1980 in Na$_{1.5}$ (V1980A) has been shown to interact with Nedd4-2 and facilitate Nav1.5 ubiquitination (8). Mutating Val-1980 to Ala (V1980A) significantly increased the $I_{Na}$ density (8). Interestingly, electrophysiological analysis showed that overexpression of αB-crystallin could increase the sodium current density only from the wild type Na$_{1.5}$ (Fig. 7C). It failed to increase the sodium current density from the mutant V1980A-Na$_{1.5}$ channel (Fig. 7D).

Effects of Disease-associated Mutations of αB-Crystallin on Sodium Currents—Multiple mutations in αB-crystallin have been associated with a variety of diseases. These mutations include R11H, P20S, R56W, R120G, D140N, R151X, G154S, R157H, and A171T. To determine whether these naturally occurring mutations can alter the physiological roles of αB-crystallin in Na$_{1.5}$, we analyzed these αB-crystallin mutations for their effects on sodium current densities.

Mutations R11H, P20S, R56W, R120G, G154S, R157H, and A171T in αB-crystallin did not affect the capability of αB-crystallin in enhancing the sodium current density (Fig. 8A). However, the R109H mutation, which is linked to multisystemic disease, including cataracts, myofibrillar myopathy, and cardiomyopathy, and the R151X mutation, which is linked to proximal and distal leg muscle weakness, abolished the function of αB-crystallin to increase the sodium current density (Fig. 8A).

We selected one αB-crystallin mutation that reduced the sodium current density (R151X) and one mutation that did not reduce the density (R120G) and examined their effects on αB-crystallin interaction with Na$_{1.5}$ and Nedd4-2. Mutation R151X significantly reduced the interaction between αB-crystallin and Na$_{1.5}$, whereas mutation R120G had little effect on the interaction (Fig. 8B). Together, these data suggest that the interaction between αB-crystallin and Na$_{1.5}$ plays an important role in increasing the sodium current density by αB-crystallin. Mutation R151X, but not R120G, also significantly reduced αB-crystallin interaction with Nedd4-2, but the effect was small (Fig. 8C).

Discussion

In this report, we describe the identification of a new protein factor, αB-crystallin, which interacts with Na$_{1.5}$ and increases peak $I_{Na}$ density. The cardiac sodium channel is in a multipro-
Regulation of $\alpha_{v}1.5$ by $\alpha$B-Crystallin

The interaction between $\alpha$B-crystallin and $\alpha_{v}1.5$ results in increased sodium current densities. Whole-cell patch clamping revealed that overexpression of $\alpha$B-crystallin significantly increased $I_{Na}$ densities (Fig. 3). We further showed that the interaction between $\alpha$B-crystallin and $\alpha_{v}1.5$ played an important role in increasing the sodium current density by $\alpha$B-crystallin. As shown in Fig. 8, $\alpha$B-crystallin mutation R115X reduced the interaction between $\alpha$B-crystallin and $\alpha_{v}1.5$, which was accompanied by significantly reduced sodium current density. On the other hand, R120G did not affect the interaction between $\alpha$B-crystallin and $\alpha_{v}1.5$ and did not have any effect on the sodium current density. Previously, we reported...
that MOG1 interacts with Na\(_{1.5}\) and increases \(I_{Na}\) densities by increasing expression of Na\(_{1.5}\) on the cell surface (4, 6). Therefore, similar to MOG1, one molecular mechanism by which \(\alpha B\)-crystallin increases \(I_{Na}\) densities is the increased expression of Na\(_{1.5}\) on the cell surface. Western blotting analysis with biotinylated cell surface proteins showed that overexpression of \(\alpha B\)-crystallin significantly increased cell surface expression of Na\(_{1.5}\) (Fig. 4A), and knockdown of \(\alpha B\)-crystallin expression significantly decreased the cell surface expression level of Na\(_{1.5}\) (Fig. 4E). In contrast to MOG1, which increases the cell surface expression level of Na\(_{1.5}\) by facilitating ER export of Na\(_{1.5}\) (4), \(\alpha B\)-crystallin reduces turnover of cell surface Na\(_{1.5}\) by slowing down internalization of Na\(_{1.5}\) for degradation (Fig. 5, C and D).

\(\alpha B\)-crystallin belongs to the family of \(\alpha\)-crystallins. The \(\alpha\)-crystallin family is composed of \(\alpha A\)-crystallin and \(\alpha B\)-crystallin and belongs to the heat shock protein family, which exhibits important molecular chaperone activities. Recent studies showed that overexpression of \(\alpha A\)-crystallin accelerated the degradation of misfolded membrane proteins, including a mutant cystic fibrosis transmembrane conductance regulator (CFTR\(\Delta 508\)) and epithelial Na\(^+\) channels via a process referred to as ER-associated degradation (27, 28). However, the effect of \(\alpha B\)-crystallin on ion channels is unknown. In this study, for the first time, we demonstrated that \(\alpha B\)-crystallin modulates the function of the cardiac sodium channel, Na\(_{1.5}\). Because \(\alpha B\)-crystallin decreased internalization of Na\(_{1.5}\) for degradation, we investigated the effect of \(\alpha B\)-crystallin on ubiquitination of Na\(_{1.5}\). Interestingly, overexpression of \(\alpha B\)-crystallin decreased ubiquitination of Na\(_{1.5}\) (Fig. 5, A and B). This finding is consistent with the report showing that knock-out of \(\alpha B\)-crystallin expression increased ubiquitination of VEGF-A in RPE cells (29).

Ubiquitination of Na\(_{1.5}\) was previously reported to be mediated by the ubiquitin-protein ligase Nedd4-2, which binds to the PY-motif at the C terminus of Na\(_{1.5}\) (8). Nedd4-2 can increase Na\(_{1.5}\) ubiquitination and reduce \(I_{Na}\) densities (8). When the PY motif of Na\(_{1.5}\) was mutated (V1980A-SCN5A), we found that the effect of overexpression of \(\alpha B\)-crystallin on \(I_{Na}\) densities was blocked (Fig. 7B). These data suggest that the effect of \(\alpha B\)-crystallin on Na\(_{1.5}\) function is dependent on the Nedd4-2 binding site. Furthermore, we found that \(\alpha B\)-crystallin interacted with Nedd4-2 (Fig. 6). Nedd4-2 was shown to decrease peak \(I_{Na}\) density by 65\%, but its effect on Na\(_{1.5}\) with the V1980A mutation in the PY motif was reduced (8). In contrast, the catalytically inactive mutation, Nedd4-2-C801S, failed to decrease peak \(I_{Na}\) density (8). We found that Nedd4-

**FIGURE 7. Analysis of peak \(I_{Na}\) in A and B, functional effects of overexpression of \(\alpha B\)-crystallin on \(I_{Na}\) densities from wild type Na\(_{1.5}\) in the presence of wild type Nedd4-2 or catalytically inactive mutant Nedd4-2 (Nedd4-2-C801S). Nedd4-2-C801S disrupts the activity of Nedd4-2 on ubiquitination of Na\(_{1.5}\). HEK/Na\(_{1.5}\) cells were co-transfected with wild type Nedd4-2 or mutant Nedd4-2-C801S as well as with the control vector and an expression plasmid for \(\alpha B\)-crystallin. C and D, functional effects of overexpression of \(\alpha B\)-crystallin on \(I_{Na}\) densities from wild type Na\(_{1.5}\) or mutant Na\(_{1.5}\) with mutation V1980A at the interaction site for Nedd4-2. HEK293 cells were co-transfected with an expression plasmid for either wild type SCN5A or mutant SCN5A with mutation V1980A together with an expression plasmid for \(\alpha B\)-crystallin or control empty vector. Data were shown as means ± S.E. (error bars). NS, not significant; *, significant with \(p < 0.05\). All studies were repeated at least three times.

**FIGURE 8. Functional effects of \(\alpha B\)-crystallin mutations associated with human diseases on peak \(I_{Na}\) densities and the interaction of \(\alpha B\)-crystallin with either Na\(_{1.5}\) or Nedd4-2. A, HEK/Na\(_{1.5}\) cells were transfected with an expression plasmid for wild type \(\alpha B\)-crystallin or mutant \(\alpha B\)-crystallin and then used for whole-cell patch clamp recordings of \(I_{Na}\). The peak sodium current density was normalized to the sodium current density recorded from HEK/Na\(_{1.5}\) cells transfected with wild type \(\alpha B\)-crystallin and plotted. B, co-IP analysis for interaction between wild type \(\alpha B\)-crystallin or two mutant \(\alpha B\)-crystallin proteins and Na\(_{1.5}\). C, co-IP analysis for interaction between wild type \(\alpha B\)-crystallin or two mutant \(\alpha B\)-crystallin proteins and Nedd4-2. NS, not significant; *, significant with \(p < 0.05\). Data are shown as means ± S.E. (error bars) All studies were repeated at least three times.
Regulation of Na\textsubscript{\textalpha}.1.5 by \alphaB-crystallin

2-C801S reduced the interaction between \alphaB-crystallin and Nedd4-2 (Fig. 6, C and D) and also blocked the increase of peak $I_{\text{Na}}$ density by \alphaB-crystallin (Fig. 7A). The data suggest that the interaction between \alphaB-crystallin and Nedd4-2 is critical to increased $I_{\text{Na}}$ density by \alphaB-crystallin. As discussed earlier, the interaction between \alphaB-crystallin and Na\textsubscript{1.5} is also critical to increased $I_{\text{Na}}$ density by \alphaB-crystallin (Fig. 8, A and B).

Genetic studies have linked mutations in \alphaB-crystallin to cataracts, skeletal muscle myopathy, and cardiomyopathy (9). In particular, mutations G154S and R157H in \alphaB-crystallin were identified in a Japanese patient and an Italian patient with dilated cardiomyopathy, respectively, but both patients did not present with cataracts or arrhythmias (30, 31). Later, mutation G154S was also reported in a 73-year-old German patient with late onset distal vacuolar myopathy but without cardiac involvement or cataracts (32). Due to the critical importance of Na\textsubscript{1.5} in cardiac physiology and human disease, including dilated cardiomyopathy, it would thus be interesting to determine whether these mutations affect function of Na\textsubscript{1.5}. We studied the effects of mutations G154S and R157H on the function of \alphaB-crystallin on peak $I_{\text{Na}}$ density, but no significant difference was found when compared with wild type \alphaB-crystallin (Fig. 8A). Similarly, mutations R11H, P20S, R56W, A171T, and R120G associated with cataracts or muscle myopathy did not have a significant effect on the function of Na\textsubscript{1.5} when compared with the wild type \alphaB-crystallin (Fig. 8A).

However, mutation R109H, linked to multisystemic disease, including cataract, myofibrillar myopathy, and cardiomyopathy, and mutation R151X, linked to proximal and distal leg muscle weakness, significantly inhibited the function of \alphaB-crystallin on Na\textsubscript{1.5} and peak $I_{\text{Na}}$ density (Fig. 8A). Because Na\textsubscript{1.5} mutations were found to be associated with cardiomyopathy (33), it should be interesting to further study how \alphaB-crystallin mutation R109H causes cardiomyopathy by affecting the function of Na\textsubscript{1.5}. Moreover, because reduced peak $I_{\text{Na}}$ density has been linked to Brugada syndrome, progressive cardiac conduction disease, sinus node disease, and atrial fibrillation, carriers with mutations R109H and R151X of \alphaB-crystallin are predicted to be at risk of these arrhythmic disorders. Although no arrhythmias were examined or detected in patients with mutations R109H and R151X, they should be monitored for a potential risk of arrhythmias. Moreover, mutational analysis may identify \alphaB-crystallin mutations in patients with Brugada syndrome, ventricular arrhythmias, cardiac conduction disease, sick sinus syndrome, and atrial fibrillation.

There are limitations to the present study. Although overexpression of \alphaB-crystallin significantly increased cell surface expression of Na\textsubscript{1.5} and $I_{\text{Na}}$ densities in HEK293 cells, future studies are needed to explore \alphaB-crystallin expression levels in pathological states and to assess the physiological role of \alphaB-crystallin interaction with Na\textsubscript{1.5} in cardiomyocytes. The low transfection efficiency of HL-1 cells and cardiomyocytes with plasmid DNA makes it difficult to achieve a sufficiently high overexpression of \alphaB-crystallin to observe the functional effects of \alphaB-crystallin with Western blotting analysis and patch clamping studies. Moreover, we found that wild type Nedd4-2 reduced the interaction between Na\textsubscript{1.5} and \alphaB-crystallin, whereas the mutant Nedd4-2-C801S did not have an effect on the interaction or slightly increased the interaction (Fig. 6, G and H). The data are consistent with the previous report that wild type Nedd4-2, but not mutant Nedd4-2-C801S, reduced $I_{\text{Na}}$ density (8). However, we could not distinguish the two possible mechanisms by which wild type Nedd4-2 reduces the interaction between \alphaB-crystallin and Na\textsubscript{1.5}: 1) the competition for the complex formation with Na\textsubscript{1.5} by \alphaB-crystallin and Nedd4-2 or 2) the possibility that increased Nedd4-2 leads to increased ubiquitination and degradation of Na\textsubscript{1.5}, resulting in less Na\textsubscript{1.5} and less \alphaB-crystallin:Na\textsubscript{1.5} complex formation.

Conclusions

This study identifies a new binding partner, a small heat shock protein \alphaB-crystallin, for Na\textsubscript{1.5}. We show that \alphaB-crystallin interacts with Na\textsubscript{1.5} and increases $I_{\text{Na}}$ densities by increasing cell surface expression levels of Na\textsubscript{1.5} via inhibition of its internalization. Moreover, we show that \alphaB-crystallin also interacts with functionally active Nedd4-2, which has been reported to interact with Na\textsubscript{1.5} to regulate ubiquitination of Na\textsubscript{1.5} and $I_{\text{Na}}$ densities. Considering the critical roles of Na\textsubscript{1.5} in cardiac physiology, cardiac arrhythmias, and sudden death, our finding of a critical role of \alphaB-crystallin in regulation of Na\textsubscript{1.5} predicts that \alphaB-crystallin may play a role in cardiac physiology, cardiac arrhythmias, and sudden death, too. This study may also provide an interesting target for developing new therapeutic strategies to treat lethal arrhythmias associated with reduced Na\textsubscript{1.5} function and $I_{\text{Na}}$.

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