EH Domain Proteins Regulate Cardiac Membrane Protein Targeting

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Rationale: Cardiac membrane excitability is tightly regulated by an integrated network of membrane-associated ion channels, transporters, receptors, and signaling molecules. Membrane protein dynamics in health and disease are maintained by a complex ensemble of intracellular targeting, scaffolding, recycling, and degradation pathways. Surprisingly, despite decades of research linking dysfunction in membrane protein trafficking with human cardiovascular disease, essentially nothing is known regarding the molecular identity or function of these intracellular targeting pathways in excitable cardiomyocytes.

Objective: We sought to discover novel pathways for membrane protein targeting in primary cardiomyocytes.

Methods and Results: We report the initial characterization of a large family of membrane trafficking proteins in human heart. We used a tissue-wide screen for novel ankyrin-associated trafficking proteins and identified 4 members of a unique Eps15 homology (EH) domain-containing protein family (EHD1, EHD2, EHD3, EHD4) that serve critical roles in endosome-based membrane protein targeting in other cell types. We show that EHD1-4 directly associate with ankyrin, provide the first information on the expression and localization of these molecules in primary cardiomyocytes, and demonstrate that EHD1-4 are coexpressed with ankyrin-B in the myocyte perinuclear region. Notably, the expression of multiple EHD proteins is increased in animal models lacking ankyrin-B, and EHD3-deficient cardiomyocytes display aberrant ankyrin-B localization and selective loss of Na/Ca exchanger expression and function. Finally, we report significant modulation of EHD expression following myocardial infarction, suggesting that these proteins may play a key role in regulating membrane excitability in normal and diseased heart.

Conclusions: Our findings identify and characterize a new class of cardiac trafficking proteins, define the first group of proteins associated with the ankyrin-based targeting network, and identify potential new targets to modulate membrane excitability in disease. Notably, these data provide the first link between EHD proteins and a human disease model. (Circ Res. 2010;107:84-95.)

Key Words: trafficking • ion channel • ankyrin • EHD proteins • cytoskeleton • arrhythmia

Membrane trafficking, endocytosis, and endocytic recycling of integral membrane proteins are critical cellular functions for normal membrane excitability and cardiomyocyte physiology. Mechanistically, these cellular pathways are controlled by highly organized protein networks that tightly regulate anterograde and retrograde trafficking of membrane proteins, their stability at the cell surface, and their degradation following internalization. Whereas the last few years have witnessed the identification and preliminary characterization of these protein networks in cultured cells, essentially nothing is known regarding these pathways in primary excitable cells, including cardiomyocytes. In fact, the field of molecular cardiology lacks fundamental understanding even of the identity of these critical trafficking proteins in primary cardiac tissue.

Ankyrins are a family of proteins required for membrane targeting and stability of membrane ion channels, transporters, cell adhesion molecules, and signaling proteins. The importance of ankyrins for normal physiology is illustrated by human diseases linked with ankyrin dysfunction and mice lacking ankyrin gene products. In heart, ankyrins have key roles in the membrane trafficking and regulation...
of sodium and calcium channels, Na/Ca exchanger (NCX), Na/K ATPase, and inositol 1,4,5-trisphosphate (IP3) receptor. Ankyrin-B dysfunction is associated with a human arrhythmia syndrome affecting multiple excitable cell types resulting in sinus node disease, atrial fibrillation, conduction defects, catecholaminergic polymorphic ventricular arrhythmia, and sudden death. Although ankysins are recognized as critical components for anterograde targeting of ion channels and transporters, little is known regarding the underlying molecular machinery and associated proteins required for ankyrin-based trafficking.

Here, we report the initial characterization of a family of membrane trafficking proteins in human heart. We used a tissue-wide screen for novel ankyrin-associated trafficking proteins and identified 4 members of a family of EH (Eps15 homology) domain-containing (EHD) gene products (EHD1-4) that provide critical roles for endosome-based membrane protein targeting and recycling in other cell types. Specifically, EHD proteins regulate endosomal anterograde and retrograde trafficking, as well as membrane protein recycling and lipid homeostasis. We report that EHD1-4 directly associate with ankyrin and provide the first information on the expression and localization of these molecules in cardiomyocytes. EHD1-4 are expressed with ankyrin-B in the myocyte perinuclear region and EHD expression is increased in ankyrin-B-deficient hearts. Myocytes deficient in EHD3 display striking loss of NCX membrane trafficking and function, thus establishing the first functional role for cardiac EHD proteins. Finally, we report modulation of EHD expression in a large animal model of arrhythmias, suggesting that these proteins may play a key role in regulating membrane excitability in normal and diseased heart. Together, our findings identify a new class of cardiac membrane trafficking proteins, define the first group of proteins associated with the cardiac ankyrin-based targeting network, and identify potential new targets to modulate membrane excitability in human disease.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Statistics**

Values are expressed as means±SEM. Probability values were assessed with a Student’s t test or ANOVA, as appropriate. The Bonferroni test (electrophysiological measurements) or least squares difference was used for post hoc testing. The null hypothesis was rejected for P<0.05. To analyze immunoblot densitometric measurements from multiple experiments on EHD expression across species, immunoblot bands were normalized to actin and heart chambers, immunoblot bands were normalized to actin and then to a control (human or left ventricle), resulting in a mean value of with no standard error for the control group.

**Protein Modeling**

Modeling of EHD2 was performed using Cn3D4.1 software based on the EHD2 structure of Daumke et al.

**In Vitro Binding**

Protein products were in vitro translated using the TNT Coupled Reticulocyte Lysate System and labeled with [35S]methionine. Products were incubated with glutathione S-transferase (GST) fusion proteins of ankyrin-B and ankyrin-G domains (MBD, SBD, CTD) in 500 μL of binding buffer (50 mmol/L Tris [pH 7.4], 1 mmol/L EDTA, 1 mmol/L EGTA, 150 mmol/L NaCl, 0.1% Triton X-100 and protease inhibitor) overnight at 4°C. GST–protein complexes were washed 5 times (50 mmol/L Tris [pH 7.4], 1 mmol/L EDTA, 1 mmol/L EGTA, 500 mmol/L NaCl, 0.1% Triton X-100), and analyzed by SDS-PAGE. To visualize equal loading of GST proteins, gels were stained with Coomassie Blue. [35S]-labeled products that bound GST–ankyrin fusion proteins were detected by phosphorimaging. All binding experiments were replicated >3 times.

**Results**

**Ankyrin-B Associates With Key Membrane Trafficking Protein**

Loss of ankyrin-B results in the development of a proarrhythmic substrate caused by the loss of membrane expression of a select group of critical cardiac ion channels and transporters. As a first step in defining the molecular components of the cardiac ankyrin-B–based membrane trafficking pathway, we performed a screen for novel ankyrin-binding proteins. We screened a human heart yeast 2-hybrid library with the central region of ankyrin-B as bait for candidate interacting proteins with specific roles in membrane trafficking, vesicle fusion, or organelle biogenesis. A screen of >2 million independent clones identified 1 such candidate (Figure 1A). Sequencing of a ∼500-bp clone revealed a nucleotide match with NM_014600.1 that encodes residues 192 to 349 of the human EHD3 protein (Figure 1A). Previously unidentified in human cardiac tissue, this protein is a member of the 4-member human EHD protein family (EHD1-4) with recently defined roles in membrane protein trafficking in other cell types. Notably, the ankyrin-binding partial sequence bears strong similarity to the corresponding sequences in EHD1 (86% identical), EHD2 (77% identical), and EHD4 (78% identical) (Figure 1A). Like EHD3, these other EHD proteins have not been previously studied in heart.

**EHD3 Directly Interacts With Ankyrin-B**

We confirmed the ankyrin-B/EHD3 interaction in vitro using radiolabeled full-length EHD3 and bacterially expressed and affinity-purified ankyrin-B fused to GST. Full-length EHD3 cDNA was cloned from a human heart cDNA library and in vitro translated in the presence of [35S]methionine. Consistent with the 2-hybrid screen, we observed interaction of radiolabeled EHD3 with GST–ankyrin-B (Figure 1B and 1C). Ankyrin-B is comprised of 3 structural domains, including a
EHD1-4 directly associate with GST–ankyrin-B but not GST. Binding data were replicated in 4 independent experiments.

EHD3 directly associates with the ankyrin-B MBD, but not ankyrin-G. Equal protein quantities were evaluated for each domain. All proteins were evaluated on the same gel by phosphorimaging, but ankyrin-G lanes were moved to the right of the image.

Consistent with the high homology of EHD proteins, particularly within the EHD central region, we observed association of ankyrin-B with all EHD proteins (Figure 1F through 1I). Specifically, purified GST–ankyrin-B membrane-binding domain interacts with radiolabeled full-length EHD1, EHD2, EHD3, and EHD4 (Figure 1F through 1I). In contrast, radiolabeled EHD proteins (EHD1-4) displayed no binding activity for GST alone. Similar to findings with EHD3 (Figure 1C), we observed no interaction of ankyrin-G with EHD1, -2, or -4.

EHD3 Interaction With Ankyrin Requires Coiled-Coil Domain

EHD proteins display a conserved primary structure consisting of a short N-terminal region (NT), a dynamin-like domain (DLD) that includes the nucleotide-binding “P-loop” known to bind ATP (together, N-terminal domain and DLD often referred to as “G-domain”), a “coiled-coil” region, and a C-terminal EH domain that binds to NPF motifs in target proteins (Figure 2A through 2C). The EH domain consists of 2 EF hands of which the second is capable of binding to Ca2+; this is thought to be important for the folding of the EH domain. In addition to associating with Ca2+, lipid, and membrane trafficking proteins, EHD proteins form homo- and heterooligomers via their coiled-coil region. Nucleotide binding at the P-loop also regulates membrane-binding and oligomerization (Figure 2A through 2C). Therefore, we investigated the structural requirements on EHD3 for ankyrin-binding activity. We generated a library of mutants corresponding to each EHD3 domain/region (Figure 2D). Additionally, we created mutants corresponding to all combinations of EHD3 domains (Figure 2D). Each EHD3 mutant was radiolabeled and incubated with purified GST–ankyrin-B or GST. We observed consistent interaction with ankyrin-B for all EHD3 mutants that contained the coiled-coil region (Figure 2D and 2E). In contrast, the EHD3 N-terminal region, DLD, and EH domain lacked ankyrin-binding activity. We observed no binding of any EHD3 mutant with GST (Figure 2E). Thus, we have defined the structural requirements on both ankyrin-B and EHD3 for interaction. Specifically, the ankyrin-B membrane-binding domain is critical for EHD3-binding activity, whereas the coiled-coil region mediates the EHD3 interaction with ankyrin-B. It is noteworthy that the association of the EHD coiled-coil region with ankyrin-B is

Figure 1. Ankyrin-B associates with EHD3. A, Yeast 2-hybrid screen of human heart library with ankyrin-B identified clone-HHL278 corresponding to the central region of human EHD3 (NM_014600.1). This clone was similar to the corresponding nucleotide sequence of EHD1 (86% identical), EHD2 (77% identical), and EHD4 (78% identical). B and C, EHD3 directly associates with membrane-binding domain of ankyrin-B, but not ankyrin-G. B, Ankyrin-B and ankyrin-G are comprised of 3 domains including a membrane-binding domain (MBD), spectrin-binding domain (SBD), and C-terminal domain (CTD; death domain and regulatory domain). C, Radiolabeled EHD3 directly associates with the ankyrin-B MBD, but not ankyrin-G. Equal protein quantities were evaluated for each domain. All proteins were evaluated on the same gel by phosphorimaging, but ankyrin-G lanes were moved to the right of the image. D, Domain organization of EHD proteins. E, Amino acid identity of EHD-containing protein domains compared with EHD3. F through I, Radiolabeled EHD1-4 directly associate with GST–ankyrin-B but not GST. Binding data were replicated in 4 independent experiments.
Figure 2. EHD3 coiled-coil domain is required for ankyrin-binding. A, EHD proteins are comprised of an N-terminal region (NT), DLD, a coiled-coil region (CC), and an EH domain (EHD). B and C, Depiction of ribbon and space filling model of an EHD3 homolog (EHD2) to illustrate potentially exposed sites on the polypeptide. Yellow denotes AMP-PNP molecule in structure. D, Library of protein mutants generated for mapping the ankyrin-binding region on EHD3. E, Purified GST-ankyrin-B membrane-binding domain (MB) associates with radiolabeled EHD3 mutants containing the coiled-coil domain. We observed background binding for all other mutants. Input (In) and control binding to GST alone (Cn) are also shown. All binding data were successfully replicated in 4 independent experiments. Note that the translated DLD/CC/EHD product displays a second, slightly smaller (migrates ~3 kDa lower) degradation product that also associates with ankyrin-B.
the first assigned non-EHD protein binding partner for this domain.

EHD Family Proteins Are Expressed in Vertebrate Heart

Four genes encode EHD proteins in vertebrates. However, to date, there is no detailed information on the cellular expression, localization, or function of any of these gene products in human heart. We first performed RT-PCR from normal human left ventricle to establish the presence of EHD1-4 message in human heart. All EHD mRNA products were observed in left ventricle, and sequencing revealed matches with full-length EHD1-4 cDNAs previously identified from noncardiac sources (Figure 3A). Consistent with mRNA findings, we observed EHD1-4 proteins by immunoblot using isofrom-specific antibodies on human left ventricle (Figure 3B). Parallel lysates prepared from mouse, rat, and canine left ventricle demonstrated expression of EHD1-4 (Figure 3B through 3F). Finally, immunoblots of normal human heart chambers revealed EHD1-4 expression in all cardiac chambers (Figure 3G through 3K). Together, these findings demonstrate that the all members of the EHD protein
family (EHD1-4) are expressed in heart across a range of vertebrates.

Finally, we tested the ability of each EHD-containing protein to interact with ankyrin-B in cardiac tissue lysates. In agreement with in vitro binding assays, ankyrin-B associates with all EHD-containing polypeptides in heart. Specifically, coimmunoprecipitation studies using affinity-purified ankyrin-B immunoglobulin and detergent-soluble lysates from adult mouse heart demonstrate interaction of ankyrin-B with EHD1-4 (Online Figure I). This interaction was specific to ankyrin-B, because no EHD interaction was observed with control immunoglobulin (Online Figure I), or with ankyrin-G or ankyrin-R (data not shown). Collectively, our data provide the first evidence for the cellular expression of the EHD protein family across vertebrates.

**EHD Proteins Are Localized to Cardiomyocyte Perinuclear Region**

To date, the cellular distribution of any EHD-containing protein in any cardiac cell type is unknown. We defined the subcellular distribution of EHD1-4 in heart using primary cardiomyocytes and EHD isoform–specific antibodies. Although not fully differentiated, neonatal myocytes were chosen for these studies, because these cells are thin (~2 μm in x-z dimension compared with ~20 μm in adult myocytes), which allows optimal imaging of the organelles involved in the membrane targeting pathway. In neonatal cardiomyocytes, ankyrin-B is localized to cytoskeleton-enriched domains underlying the myocyte membrane (Figure 4A through 4D, left). In addition, ankyrin-B is localized to the perinuclear region of cardiomyocytes (Figure 4A through 4D). EHD1-4–positive staining was observed in clusters near the perinuclear region (Figure 4). This is similar to the localization of EHD proteins in HeLa cells, where they reside in endosomes and regulate trafficking through this endocytic compartment. Together, these findings establish the subcellular distribution of EHD proteins in isolated primary ventricular cardiomyocytes.

**Loss of Ankyrin-B Affects EHD1-4 Expression and Localization in Heart**

To examine the functional relationship between ankyrin-B and EHD proteins, we evaluated the expression of EHD polypep-
tides in hearts of adult wild-type and ankyrin-B−/− mice (ankyrin-B−/− mice die immediately after birth). Interestingly, EHD1-4 levels were not reduced in ankyrin-B−/− hearts. Instead, we observed significant increases in all EHD protein levels (Figure 5A through 5E; n=7 experiments/genotype; *P<0.05). These findings strongly support a relationship between ankyrin-B and EHD proteins in heart. We further examined the relationship of ankyrin-B and EHD proteins in the perinuclear region of ankyrin-B−/− neonatal cardiomyocytes (Figure 5F and 5G). Increased peripheral cytoplasmic staining was observed for both EHD2 and EHD3 in ankyrin-B−/− cardiomyocytes (Figure 5H through 5K). Finally, EHD4 showed a strong increase preferentially in the perinuclear area of ankyrin-B−/− cells (Figure 5L and 5M).

**EHD3 Overexpression Selectively Increases Myocyte I_{NCX}**

Ankyrin-B is critical for targeting select cardiac ion channels and transporters. Myocytes lacking ankyrin-B expression display decreased membrane expression and function of NCX1, whereas the localization and function of the L-type

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Figure 5. EHD protein expression is increased by loss of ankyrin-B. A through E, Representative immunoblots (A) and densitometric measurements (B through E) of EHD1-4 from lysates prepared from adult wild-type (WT) and ankyrin-B−/− mouse left ventricle. Equal protein quantities were loaded for each genotype. Band densities were normalized to actin and expressed relative to wild type (n=7 experiments/genotype, *P<0.05). F through M, Ankyrin-B−/− cardiomyocytes display increased expression of EHD proteins. Localization of ankyrin-B and EHD proteins in wild-type (F, H, J, and L) and ankyrin-B−/− (G, I, K, and M) primary cardiomyocytes. All cells were stained and imaged using similar protocols. Scale bar indicates 10 µm. Cells are colabeled with topro-3AM to stain nuclei. Experiments were repeated from 4 mice/genotype and >100 myocytes/experiment were analyzed.
Ca\(^{2+}\) channel, Ca\(_{1.2}\), is unaffected\(^{2,5,14,15}\). We therefore assessed the ability of EHD overexpression to enhance ankyrin-B–associated protein membrane expression and function. As expected, whole-cell patch clamp recordings revealed a decrease in membrane NCX current (\(I_{\text{NCX}}\)) in ankyrin-B\(^{-/-}\) cardiomyocytes, consistent with the notable perinuclear localization in ankyrin-B\(^{-/-}\) myocytes (Figure 6A through 6C). Specifically, we observed differences in whole-cell \(I_{\text{NCX}}\) at nearly all voltages in ankyrin-B\(^{-/-}\) cardiomyocytes compared with wild-type myocytes (\(n=12\) cells/genotype, \(P<0.05\)). Overexpression of human green fluorescent protein (GFP)-EHD3 increased \(I_{\text{NCX}}\) in wild-type (\(n=12\) cells/treatment, \(P<0.05\) compared to wild-type [WT] untransfected) but not ankyrin-B\(^{-/-}\) myocytes (\(n=12\) cells/treatment, \(P=\text{NS}\) compared to ankyrin-B\(^{-/-}\) untransfected). We observed no difference in NCX (or ankyrin-B) localization in wild-type or ankyrin-B\(^{-/-}\) cardiomyocytes in the presence or absence of EHD3 overexpression. Finally, EHD3 overexpression had no effect on ankyrin-B–independent myocyte current, \(I_{\text{Ca,L}}\) (Figure 6J and 6K; \(n=9\) myocytes/experiment, \(P=\text{NS}\) compared to untransfected). Scale bars indicate 10 \(\mu\text{m}\).

**Figure 6.** EHD3 overexpression alters \(I_{\text{NCX}}\) in cardiomyocytes. A and B, Ankyrin-B (green) and NCX (red) expression in wild-type and ankyrin-B\(^{-/-}\) neonatal myocytes. Nuclei are stained in blue. C, \(I_{\text{NCX}}\) is significantly reduced in cardiomyocytes derived from ankyrin-B\(^{-/-}\) mice (\(P<0.05\), \(n=12\) myocytes/genotype). D through F, Control myocytes (D) and myocytes from wild-type (E) and ankyrin-B\(^{-/-}\) (F) mice expressing GFP-EHD3. Note untransfected cells lack signal on the green channel. G through I, EHD3-GFP overexpression increases \(I_{\text{NCX}}\) in wild-type (\(n=12\) cells/treatment, \(P<0.05\) compared to wild-type [WT] untransfected) but not ankyrin-B\(^{-/-}\) myocytes (\(n=12\) cells/treatment, \(P=\text{NS}\) compared to ankyrin-B\(^{-/-}\) untransfected). J and K, GFP-EHD3 overexpression does not affect myocyte \(I_{\text{Ca,L}}\) (\(n=9\) myocytes/experiment, \(P=\text{NS}\) compared to untransfected). Scale bars indicate 10 \(\mu\text{m}\).

**EHHD3 Is Required for NCX Membrane Expression and Function**

We further probed the role of EHD3 for myocyte function by reducing EHD3 expression using EHD3-specific small inter-
ferring (si)RNA. Notably, myocytes lacking EHD3 expression (see Online Figure II) displayed striking reduction in target-
ing and function of $I_{\text{NCX}}$ (Figure 7A, 7C, 7D, 7F, and 7G; n=11 cells/treatment for $I_{\text{NCX}}$ experiments, $P<0.05$). Moreover, EHD3-deficient cardiomyocytes displayed defects in the localization (Figure 7F and 7G; note decreased expression and prominent large cytosolic puncta) and expression (see Online Figure II) of ankyrin-B. This loss was specific to the ankyrin-B–associated cellular trafficking pathway because we observed no difference in membrane expression or function of the voltage-gated calcium channel Ca,1.2 (Figure 7B and 7E; n=9 cells/treatment, $P=NS$). Moreover, we observed no differences in expression or function of ankyrin-B, NCX1, or Ca,1.2 in the presence of a control siRNA (Figure 7A through 7E; n=9 cells/treatment for $I_{\text{Ca,L}}$, 11 cells/treatment for $I_{\text{NCX}}$, $P=NS$). Together, these data provide the first functional evidence of a role for EHD3 in the membrane trafficking of a critical cardiac transporter. Moreover, the notable selectivity of the targeting defect (NCX versus Ca,1.2), as well as the direct affect of EHD3 knockdown on ankyrin-B expression, strongly supports a role for EHD3 in the cardiac ankyrin-B membrane protein trafficking pathway.

EHD Proteins Are Differentially Regulated in Cardiovascular Disease

Aberrant ankyrin-B regulation is observed in rare forms of congenital arrhythmia. Moreover, common variants in the ankyrin-B gene (ANK2) modulate cardiac function in the general human population. Finally, ankyrin-B expression is significantly reduced in a large animal model of arrhythmias following myocardial infarction (MI). In contrast, there are no data on the role of EHD1-4 in human arrhythmia or in animal models of human disease. As a first step in identifying a potential role of EHD proteins in disease, we examined the expression of EHD1-4 in a well-validated large-animal (canine) model of arrhythmias following MI. Forty-eight hours after coronary artery occlusion, we observed an increase in EHD3 expression in a specific heart region where
expression phenocopy ankyrin-B in increased EHD1-4 expression. Myocytes lacking EHD3 are affected by ankyrin-B loss. In fact, loss of ankyrin-B results of ankyrin-B–associated proteins that are not negatively region of cardiomyocytes. Our findings define the first group heart and are coexpressed with ankyrin-B in the perinuclear coiled-coil domain. EHD proteins are expressed in human directly associates with EHD1-4 via a conserved sequence in based targeting pathway in heart. Specifically, ankyrin-B plays a central role in endosome/Golgi vesicular trafficking. Similarly, EHD2 regulates glucose-stimulated GLUT4 membrane transport and endocytosis in adipocytes. Potentially relevant for cardiac muscle, EHD2 interactions with myoferrin are important for myoblast fusion. Additionally, EHD1 has been demonstrated to interact with amphiphysin-1,27 a member of a protein family implicated in biogenesis of transverse tubules in skeletal muscle. Finally, both EHD3 and EHD4 have roles in endosome vesicular targeting and recycling. Specifically, EHD3 directly associates with critical trafficking proteins including Rab11-FIP2. Finally, 2 reports describe testicular phenotypes in mouse models of EHD1 and EHD4 deficiency. Deletion of EHD1 and EHD4 result in reduction in testes size; however, deletion of EHD1 leads to male infertility, whereas deletion of EHD4 does not. In summary, although much is unknown about the roles of these recently identified proteins, the clear linkage of EHD proteins with protein trafficking, endocytosis, and endocytosis suggests that these proteins may have critical roles in controlling trafficking of membrane proteins that maintain cardiac excitability.

Loss of ankyrin-B in this study resulted in increased EHD family protein expression. Based on these findings, we hypothesize that that the EHD/ankyrin in vivo interaction occurs either upstream or concurrently with the interaction of ankyrin with membrane-associated proteins. Thus, in response to loss of ankyrin-B–associated proteins at the membrane, the myocyte may attempt to augment anterograde membrane trafficking by upregulating the EHD/ankyrin pathway. EHD protein expression levels may also be regulated by the local signaling environment.33,34 Thus, abnormal ion homeostasis created by the loss of ankyrin-B3,5 may trigger a parallel ankyrin-independent EHD-containing protein pathway to accelerate anterograde membrane protein trafficking. Interestingly, EHD3 and EHD4 expression was increased in the canine model of MI that also shows parallel loss of ankyrin-B (Figure 8). Clearly, additional studies in adult arrhythmias originate (infect border zone) compared to control tissue (remote from infarct; remote EHD3 levels are no different from remote regions of noninfarcted hearts at 48 hours or 5 days after occlusion: 1.19±0.05 and 1.11±0.10, respectively; *P=NS compared to control; n=3) (Figure 8A and 8B). By 5 days after occlusion (when action potential and ion channel changes are most prominent19), we observed significant increases in both EHD3 and EHD4 levels in the infarct border zone (Figure 8A and 8B). EHD1 and EHD2 levels were not significantly changed at 48 hours or 5 days after occlusion (Figure 8A and 8B). Notably, ankyrin-B levels are reduced in this post-MI arrhythmia model (representative immunoblots shown in Figure 8).17

Discussion
Our findings identify the EHD family of endosome-associated proteins as novel components of the ankyrin-B–based targeting pathway in heart. Specifically, ankyrin-B directly associates with EHD1-4 via a conserved sequence in the coiled-coil domain. EHD proteins are expressed in human heart and are coexpressed with ankyrin-B in the perinuclear region of cardiomyocytes. Our findings define the first group of ankyrin-B–associated proteins that are not negatively affected by ankyrin-B loss. In fact, loss of ankyrin-B results in increased EHD1-4 expression. Myocytes lacking EHD3 expression phenocopy ankyrin-B7–9 myocytes, displaying both decreased expression and function of NCX, but maintaining the expression and activity of Ca,2.11,29,30 EHD proteins follow-
cardiomyocytes and in vivo will be necessary to carefully evaluate the functional relationship between EHD proteins and ankyrin-B.

Finally, based on the demonstrated roles of EHD proteins in other tissues, we predict that loss of EHD-containing proteins in heart will affect cardiac membrane protein regulation and excitability. In support of this hypothesis and in agreement with our findings in neonatal cardiomyocytes, we observed striking perinuclear staining for all EHD proteins (EHD1–4) in adult cardiomyocytes (Online Figure III). Our findings that EHD proteins are upregulated in animal models of cardiovascular disease suggest that this pathway may be a nodal point for the regulation of electric remodeling in the myocyte damaged by aberrant Na\(^+\) or Ca\(^{2+}\) homeostasis or myocyte membrane damage/ischemia. However, based on previous findings in other tissues and high sequence identity, together with our results that ankyrin-B interacts with all EHD proteins, we predict that cardiac EHD-containing proteins may display functional redundancy in the constitutive absence of a single family member in the context of an animal. For example, whereas EHD4-deficient mice display defects in germ cell development and testis size, changes in the levels of EHD1–3 likely prevents EHD4\(^{-/-}\)/mice from displaying more overt phenotypes. Interestingly, a proportion of EHD1-deficient mice are neonatal lethal because of unknown causes. Future work will help unravel whether this is attributable to defects in cardiac excitability. Thus, although siRNA experiments may be effective in assessing phenotypes in isolated cells acutely (Figure 7), elucidating the physiological relevance of EHD proteins in vivo may in fact require knockout of multiple EHD protein isoforms.

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Disclosures
None.

References


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Expanded Methods

Yeast two-hybrid. The central region of human 220 kD ankyrin-B (C-terminus of membrane-binding domain and spectrin-binding domain\(^7\)) was cloned into pAS2-1 (Clontech) to create the GAL4-ankyrin-B bait. The bait plasmid was transformed into AH109 yeast (ADE2, HIS3, lacZ selection) using a standard lithium acetate protocol.\(^8\) Autoactivation was not observed on adenine-, leucine-, tryptophan-deficient (-ALT) or adenine-, leucine-, tryptophan-, histidine-deficient (-AHLT) medium plates for the bait plasmid. The resulting yeast (in the AH109 strain) were mated with pre-transformed yeast containing the Clontech human heart Matchmaker cDNA library (pACT2 vector). The mated yeast were supplemented with nutrient rich medium, followed by subsequent incubation in -AHLT medium. Clones displaying colony growth on –AHLT medium in less than three days were re-plated on –AHLT plates to confirm selection. Interaction bait/prey controls for the screen included TD1-1/pLAM5 (negative, Clontech) and pTD1-1/pVA3 (positive). Approximately 2.21 x10^6 independent clones were screened.

Co-immunoprecipitations and immunoblots. Adult heart immunoprecipitations were performed as described.\(^6\),\(^8\) Briefly, adult mouse heart was dissected and rinsed in PBS plus 0.32 M sucrose and 2 mM EDTA. Tissue was flash frozen in liquid nitrogen and ground into a fine powder. The powder was resuspended in 4 volumes of 50 mM Tris HCl (pH 7.35), 10 mM NaCl, 0.32 M sucrose, 5 mM EDTA, 2.5 mM EGTA, 1 mM PMSF, 1 mM 4-(2-aminomethyl) benzenesulfonylfluoride hydrochloride (AEBSF), 10 µg/ml leupeptin, and 10 µg/ml pepstatin using a Dounce homogenizer. The homogenate was centrifuged at 1,000g to remove nuclei. Triton X-100 and deoxycholate were added to the post-nuclear supernatant for final concentrations of 1.5% Triton X-100 and 0.75% deoxycholate. The lysate was pelleted at 100,000g for 1 hour at 4°C and the supernatant was re-cleared at 100,000g for 1 hr to remove residual large membranes or vesicles. The resulting supernatant was used for immunoprecipitation, immunoblots, or for binding experiments. Co-immunoprecipitation and immunoblot data represent at least three experiments. Band densities from non-saturated immunoblots were measured with Adobe Photoshop 8.0, normalized to actin, and expressed relative to a control for comparison between experiments. Protein was isolated from human heart muscle tissue from three healthy donor hearts not suitable for transplantation (subclinical atherosclerosis, age, no matching recipients) through the Iowa Donors Network and the National Disease Research Interchange. The investigation conforms with principles outlined in the Declaration of Helsinki. Age and sex were the only identifying information acquired from tissue providers, and the Iowa Human Subjects Committee deemed that informed consent from each patient was not required. None of the patients died from cardiac-related causes.

Mouse neonatal cardiomyocytes. To generate primary cardiomyocyte cultures, hearts were dissected from P1 mice and placed in 2 ml of Ham's F-10.\(^1\) Atrial tissue was removed and ventricular chambers were rinsed to remove any remaining blood. Hearts were transferred into 1.5 ml of 0.05% Trypsin, 200 µM EDTA in Ham's F-10 medium. Hearts were minced into approximately forty small pieces using forceps and small scissors and incubated in trypsin/EDTA medium at 37 °C. Following 15 min, heart pieces were gently triturated and incubated for an additional 15 min. A mixture of 200 µl soybean trypsin inhibitor (2 mg/ml; Worthington) and 200 µl of collagenase (0.2 mg/ml; 1980 units/mg; Sigma) was incubated with cells for 50 min at 37 °C and the cell
suspension was pelleted, resuspended in Complete Medium (40% DMEM, 40% Ham’s F-10, 20% FBS), and plated on plastic dishes. Following five hours, non-adhered cells (cardiomyocytes) were aspirated, pelleted, resuspended in Complete Medium, and plated on fibronectin-coated (Roche) coverslips or glass tissue culture plates (Mattek). Following twenty-four hours, the cardiomyocytes were washed with Ham’s F-10 and Complete Medium was replaced with Defined Medium to prevent fibroblast overgrowth. 100X Defined Medium consists of 100 \( \mu \)g/ml insulin, 500 \( \mu \)g/ml transferrin, 100 nM LiCl, 100 nM NaSeO\(_4\), and 10 nM thyroxine. Medium was replaced every 24 hours.

**Electrophysiology.** Na/Ca exchanger current (\( I_{\text{NCX}} \)) recordings were obtained from neonatal wild-type and ankyrin-B\(^{-}\) neonatal cardiomyocytes as described.\(^5\), \(^11\) Whole-cell recordings were obtained at room temperature using standard patch-clamp techniques. Membrane current was assessed using an Axopatch-200B amplifier and a CV-203BU head stage (Axon Instruments). PClamp 8.0 with the Digidata 1320A acquisition system (Axon Instruments) was used for experimental control, data acquisition, and data analysis. Patch pipettes were pulled from thin-walled glass capillary tubes and heat polished. Electrode resistance ranged from 2 to 4 M\( \Omega \). The external solution contained (in mmol/L): NaCl 145, MgCl\(_2\) 1, HEPES 5, CaCl\(_2\) 2, CsCl 5, and glucose 10 (pH 7.4, adjusted with NaOH). The internal solution contained (in mmol/L): CsCl 65, NaCl 20, Na\(_2\)ATP 5, CaCl\(_2\) 6, MgCl\(_2\) 4, EGTA 21, HEPES 10, and tetraethyl ammonium chloride 20 (pH 7.2, adjusted with CsOH). Membrane currents were elicited by applying standard voltage ramp protocol from +50 mV to –100 mV with ramp duration 100 ms and holding potential –40 mV. The protocol was applied every 12 seconds. \( I_{\text{NCX}} \) was measured as the Ni-sensitive current. Ni\(^2+\) (5 mmol/L) was added to define the fraction of current that derives from NCX (the difference between total current and post-Ni\(^2+\) current). Membrane capacitance was read directly from the membrane test of PClamp 8.0 before compensating for series resistance and membrane capacitance.

**Experimental model of myocardial infarction.** Myocardial infarction (MI) was produced in healthy mongrel dogs (12 to 15 kg, 2 to 3 years old) by total coronary artery occlusion, as described.\(^2\), \(^4\), \(^10\) A cardiectomy was performed 48 hours or five days post-occlusion after surgery and thin tissue slices from visible epicardial BZ (border zone) and a remote area away from the infarct (left ventricular base) were flash frozen for immunoblot analysis. Ventricular lysates were prepared for immunoblot analysis as described.\(^4\) Equal quantities of protein were analyzed by SDS-PAGE (3-8% Tris acetate gels) and immunoblotting using affinity-purified polyclonal antibodies to ankyrin-B\(^7\) and EHD1-4.\(^3\) Slight differences in protein loading were corrected using an internal control standard (rabbit polyclonal antibody to actin (Santa Cruz)). This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (Pub. No. 85-23,1996).

**RNA isolation and reverse-transcription.** RNA was isolated from human heart tissue or primary cardiac cells using the RNeasy Kit (Qiagen) and the concentration was quantified by spectrophotometry. cDNA was amplified using SuperScript III Reverse Transcriptase (Invitrogen) and oligo-dT was used to prime for cDNA production. Polymerase chain reaction (PCR) was performed in 20 \( \mu \)L reaction volumes. Sense and antisense primers were used at a concentration of 10 \( \mu \)M. PCR was performed using Taq polymerase and 30 cycles of 95 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. PCR reactions were run on a 1% agarose gel with no-template controls.
In vitro transcription/translation. Eukaryotic cell-free protein expression was generated using the TNT Coupled Reticulocyte Lysate System (Promega). The transcription/translation reaction was assembled with 1.0 ug of EHD pcDNA3.1(+) plasmid templates, TNT reaction components (T7 RNA polymerase, rabbit reticulocyte lysate) and 20 μCi of L-[35S]-Methionine. Reactions were incubated at 30°C for 90 minutes, and radiolabeled proteins were analyzed by SDS-PAGE and phosphorimaging.

Immunofluorescence. Neonatal cardiomyocytes were washed with phosphate-buffered saline (PBS, pH 7.4) and fixed in warm 2% paraformaldehyde (37 °C). Cells were blocked/permeabilized in PBS containing 0.075% Triton X-100 and 3% fish oil gelatin (Sigma), and incubated in primary antibody overnight at 4 °C. Following washes (PBS plus 0.1% Triton X-100), cells were incubated in secondary antibody (Alexa 488, 568; and Topro-3AM Alexa 633) for 8 hours at 4 °C and mounted using Vectashield (Vector) and #1 coverslips. Images were collected on Zeiss 510 Meta confocal microscope (40 power oil 1.40 NA (Zeiss), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment. Imaging experiments were performed at least four times for each experimental protocol, and over 100 myocytes were examined from each experimental set. Myocytes displayed in figures represent typical staining patterns observed from all myocytes analyzed.

Molecular Biology. PCR primers were designed to TOPO-clone full-length coding regions of EHD1, EHD2, EHD3 and EHD4 from both human heart cDNA library (BD Biosciences) and reverse transcribed cDNA from human heart tissue, based on GenBank sequences (NM_006795, NM_014601, NM_014600, and NM_139265, respectively). To generate EHD and ankyrin-B constructs for in vitro transcription/translation, fusion protein expression and binding assay, cDNAs were engineered in-frame into pcDNA3.1(+) (Invitrogen), and pGEX6P1 (GE healthcare). Vectors were completely sequenced. EHD3 domains (N-terminus, dynamin-like domain, central helical domain, and EH domain) were cloned into pcDNA3.1(+) to facilitate EHD3-ankyrin-B site mapping. GFP-EHD3 was created by inserting human full-length EHD3 in frame into pEGFP-C1 (Clontech; GFP on EHD N-terminus).

Myocyte transfection and RNAi. Myocytes were transfected 2 days post-isolation for 9.5 hours at 37°C. For EHD3 silencing, a well-validated siRNA (Dharmacon) directed against EHD3 was transfected (10 pmol/coverlip) using Effectene according to manufacturer’s instructions. Validation experiments for the EHD3 and control siRNA to reduce EHD3 mRNA and protein levels are shown in Online Figure II. Due to the low transfection efficiency of primary ventricular cardiomyocytes, validation experiments were performed in primary cardiac fibroblasts from mice. Both mRNA and protein levels were reduced >75%. siRNA for EHD3 were confirmed in primary ventricular myocytes from mice by immunostaining using EHD3-specific antibody. The siRNA (GCTGGACATCTCCGATGAG; represents bp 950-968 of NM.020578.2) was engineered and purified in both unlabeled and labeled forms. The labeled form was constructed with a DY547 (Cy3 alternate) tag to allow detection in live cells for electrophysiology experiments. We noted consistent EHD3 knockdown at 40 hours post-transfection. Control siRNA experiments were performed identically using an unrelated control siRNA from Dharmacon (D-001210-01-20). For over-expression experiments, GFP- and GFP-EHD3 (human) were transfected as described above.
References for Extended Methods


Online Figure I. Ankyrin-B interacts with EHD1, EHD2, EHD3, and EHD4 in co-immunoprecipitation experiments from detergent-soluble lysates prepared from mouse left ventricle. We observed no interaction of EHD proteins with beads alone, or with ankyrin-G or ankyrin-R lgs. Data were replicated in three independent experiments.
Online Figure II. Previously validated siRNA reduces EHD3 protein and mRNA levels in primary mouse cardiac cells. Non-transfected and transfected cells (control siRNA and EHD3 siRNA) were analyzed for EHD3 mRNA (A) and protein (B). RNA levels in A represent EHD3 mRNA levels (n=3; p<0.05; arbitrary units) relative to untransfected cells. HPRT levels were also utilized to confirm equal loading. For immunoblot analysis, samples in B were also analyzed for ankyrin-B (C) and actin (control for equal immunoblot loading). Immunoblot data were replicated in three independent experiments.
Online Figure III. *EHD protein expression in adult cardiomyocytes.* A-D) Adult mouse cardiomyocytes were immunolabeled with EHD1-4 (red), topro-3AM (blue, nuclei), and alpha-actinin (green) to denote the myocyte Z-line. Note that EHD1-4 display perinuclear distribution (arrows) as well as peripheral cytoplasmic staining. Scale bars equal 10 microns.