Published in final edited form as:
Circ Res. 2010 July 9; 107(1): 84–95. doi:10.1161/CIRCRESAHA.110.216713.

EH domain proteins regulate cardiac membrane protein targeting

Hjalti Gudmundsson, M.D.[1,*], **Thomas J. Hund, Ph.D.**[1,*], **Patrick J. Wright, B.S.**[1], **Crystal F. Kline, Ph.D.**[1], **Jedidiah S. Snyder**[1], **Lan Qian**[1], **Olha M. Koval, Ph.D.**[1], **Shane R. Cunha, Ph.D.**[1], **Manju George, D.V.M., Ph.D.**[3], **Mark A. Rainey, Ph.D.**[3], **Farshid E. Kashef, M.D.**[1], **Wen Dun, M.D., Ph.D.**[4], **Penelope A. Boyden, Ph.D.**[4], **Mark E. Anderson, M.D., Ph.D.**[1,2], **Hamid Band, M.D., Ph.D.**[3], and **Peter J. Mohler, Ph.D.**[1,2]

[1]Department of Internal Medicine, Division of Cardiovascular Medicine; University of Iowa Carver College of Medicine, Iowa City, IA 52242

[2]Department of Molecular Physiology & Biophysics; University of Iowa Carver College of Medicine, Iowa City, IA 52242

[3]Eppley Institute for Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5950

[4]Department of Pharmacology, Center for Molecular Therapeutics, Columbia University, New York, NY 10032

Abstract

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 employed a tissue-wide screen for novel ankyrin-associated trafficking proteins and identified four 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 co-expressed with ankyrin-B in the myocyte perinuclear region. Notably, the expression of multiple EHD proteins is

Corresponding Author: Peter J Mohler, Ph.D., University of Iowa Carver College of Medicine, 285 Newton Road, CBRB 2283, Iowa City, IA 52242, peter-mohler@uiowa.edu, 319.335.9691, 319.353.5552 (fax).
*authors contributed equally

Disclosures
None.

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.

Keywords

trafficking; ion channel; ankyrin; EHD proteins; cytoskeleton; arrhythmia

Introduction

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. While 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.4 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, Na/K ATPase, and IP3 receptor.16 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.13, 18–20 While ankyrins 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 employed a tissue-wide screen for novel ankyrin-associated trafficking proteins and identified four 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.2, 5, 29 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 Na/Ca exchanger 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 mean ± SEM. P 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 <. 05. To analyze immunoblot densitometric measurements from multiple experiments on EHD expression across species and heart chambers, immunoblot bands were normalized to actin and then to a control (human or left ventricle), resulting in a mean value of one 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.5

In vitro binding

Protein products were *in vitro* translated using the TNT Coupled Reticulocyte Lysate System and labeled with [^{35}S]- methionine. Products were incubated with GST fusion proteins of ankyrin-B and ankyrin-G domains (MBD, SBD, CTD) in 500 μl binding buffer (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100 and Protease inhibitor overnight at 4 °C. GST-protein complexes were washed five times (50 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 500 mM NaCl, 0.1% Triton X-100), and analyzed by SDS-PAGE. To visualize equal loading of GST proteins, gels were stained with Coomassie Blue. [^{35}S]-labelled 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 due to the loss of membrane expression of a select group of critical cardiac ion channels and transporters.[18–20] As a first step in defining the key 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 two-hybrid library with the central region of ankyrin-B as bait for candidate interacting proteins with specific roles in protein trafficking, vesicle fusion, or organelle biogenesis. A screen of >two million independent clones identified one such candidate (Figure 1A). Sequencing of a ~500 base pair clone revealed a nucleotide match with NM_014600.1 that encodes residues 192–349 of the human Eps15 homology domain-containing 3 (EHD3) protein (Figure 1A). Previously unidentified in human cardiac tissue, this protein is a member of the four-member human EHD protein family (EHD1-4) with recently defined roles in membrane protein trafficking in other cell types.[22, 26] 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 products 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 [^{35}S]-methionine. Consistent with the two-hybrid screen, we observed interaction of radiolabeled EHD3 with GST-ankyrin-B (Figure 1B–C). Ankyrin-B is comprised of three structural domains including a membrane-binding domain that primarily associates with ion channels and transporters, a spectrin-binding domain that associates with cytoskeletal and signaling proteins, and a C-terminal domain that modulates ankyrin function *in vivo* (Figure 1B) We observed specific interaction of EHD3 with the ankyrin-B membrane-binding domain (Figure 1C). In contrast, GST-ankyrin-B spectrin-binding domain, GST-ankyrin-B C-terminal domain, and GST alone lacked EHD3 binding (Figure 1C). Moreover, despite ~75% amino acid identity with the ankyrin-B membrane-binding domain, the ankyrin-G (product of *ANK3* gene) membrane-binding domain lacked EHD3 binding activity (Figure 1C). We also observed no interaction of EHD3 with the ankyrin-G spectrin-binding domain or C-terminal domain (Figure 1C). Moreover, EHD3 did not associate with a third ankyrin-family gene product, ankyrin-R (not shown). Therefore, EHD3 directly associates with ankyrin-B *in vitro*. This interaction is specific to ankyrin-B versus other ankyrin-gene products and requires the ankyrin-B membrane-binding domain.

EH domain-containing proteins associate with ankyrin-B

While yeast and *in vitro* binding studies (Figure 1A–C) demonstrated that ankyrin- B can associate with EHD3, human EHD3 protein paralogs display high amino acid identity across all domains (Figure 1D–E). While EHD3 is most similar to EHD1 (87% identity), EHD2 and EHD4 sequences also closely resemble EHD3 (71% and 75% amino acid identity, respectively). We therefore tested whether ankyrin-B associates with multiple EHD proteins. 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–I). Specifically, purified GST-ankyrin-B membrane-binding domain interacts with radiolabeled full-length EHD1, EHD2, EHD3, and EHD4 (Figure 1F–I). 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 NTD 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–C). The EH domain consists of two EF hands of which the second is capable of binding to Ca^{2+}; this is thought to be important for the folding of the EH domain. In addition to associating with Ca^{2+}, lipid, and membrane trafficking proteins, EHD proteins form homo- and hetero-oligomers via their coiled-coil region. Nucleotide binding at the P-loop also regulates membrane-binding and oligomerization (Figure 2A–C). 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–E). In contrast, the EHD3 N-terminal region, dynamin-like domain, 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 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 non-cardiac sources (Figure 3A). Consistent with mRNA findings, we observed EHD1-4 proteins by immunoblot using isoform-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–F). Finally, immunoblots of normal human heart chambers revealed EHD1-4 expression in all cardiac chambers (Figure 3G–K). 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, co-immunoprecipitation studies using affinity-purified ankyrin-B Ig 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, as no EHD interaction was observed with control Ig (Online Figure I), or with ankyrin-G or ankyrin-R (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 peri-nuclear 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.8 While not fully differentiated, neonatal myocytes were chosen for these studies, as these cells are thin (~2 microns in X–Z dimension compared with ~20 microns 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–D, left panel). In addition, ankyrin-B is localized to the perinuclear region of cardiomyocytes (Figure 4A–D). 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.26, 33 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 polypeptides 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–E, n=7/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 EHD1-4 at the level of the single cell by examining the distribution of all EHD proteins in isolated primary cardiomyocytes lacking ankyrin-B. In agreement with immunoblot data, expression of all EHD–containing proteins (EHD1-4) was increased in the complete absence of ankyrin-B (Figure 5F–M). EHD1 expression was increased in the perinuclear region of ankyrin-B$^{-/-}$ neonatal cardiomyocytes (Figure 5F–G). Increased peripheral cytoplasmic staining was observed for both EHD2 and EHD3 in ankyrin-B$^{-/-}$ cardiomyocytes (Figure 5H–K). Finally, EHD4 showed a strong increase preferentially in the perinuclear area of ankyrin-B$^{-/-}$ cells (Figure 5L–M).

EHD3 over-expression 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 Na/Ca exchanger 1 (NCX1), while the localization and function of the L-type Ca^{2+} channel, $Ca_v1.2$, is unaffected.[3, 16, 17, 19] 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 Na/Ca exchanger current (I_{NCX}) in ankyrin-B$^{-/-}$ cardiomyocytes, consistent with the notable perinuclear localization in ankyrin-B$^{-/-}$ myocytes (Figure 6A–C). Specifically, we observed differences in whole cell I_{NCX} at nearly all voltages in ankyrin-B$^{-/-}$ cardiomyocytes compared with wild-type myocytes (n=12 cells/genotype, $p<0.05$). Overexpression of human GFP-EHD3 increased I_{NCX} in wild-type cardiomyocytes at select voltages compared with untransfected myocytes (Figure 6D–I, n=12 cells/condition, $p<0.05$). Overexpression of GFP-EHD3 had no significant effect on I_{NCX} in ankyrin-B$^{-/-}$ myocytes (n=12 cells/condition, N.S.). We observed no difference in Na/Ca exchanger (or ankyrin-B) localization in wild-type or ankyrin-B$^{-/-}$ cardiomyocytes in the presence or absence of EHD3 overexpression. Finally, EHD3 overexpression had no affect on an ankyrin-B-independent myocyte current, $I_{Ca,L}$ (Figure 6J–K, n=12 cells/genotype/treatment, N.S.). Together, these data support the requirement of ankyrin-B for anterograde delivery of select myocyte membrane proteins to the cell surface. Moreover, these data demonstrate that EHD3-overexpression enhances this cellular pathway in wild-type cardiomyocytes.

EHD3 is required for Na/Ca exchanger membrane expression and function

We further probed the role of EHD3 for myocyte function by reducing EHD3 expression using EHD3-specific siRNA. Notably, myocytes lacking EHD3 expression (see Online Figure II) displayed striking reduction in targeting and function of I_{NCX} (Figure 7A,C–D,F–G, n=11 cells/treatment for I_{NCX} experiments, $p<0.05$). Moreover, EHD3-deficient cardiomyocytes displayed defects in the localization (Figure 7F–G, 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 as we observed no difference in membrane expression and function of the voltage-gated calcium channel, $Ca_v1.2$ (Figure 7B, E, n=9 cells/treatment, N.S.). Moreover, we observed no differences in expression or function of ankyrin-B, Na/Ca exchanger 1, or $Ca_v1.2$ in the presence of a control siRNA (Figure 7A–E, n=9 cells/treatment for $I_{Ca,L}$, 11 cells/treatment for I_{NCX}, N.S.). 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 (Na/Ca exchanger versus $Ca_v1.2$), as well as the direct affect of EHD3 knock-down on ankyrin-B expression strongly support 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. 18 Moreover, common variants in the ankyrin-B gene *(ANK2)* modulate cardiac function in the general human population.30 Finally, ankyrin-B expression is significantly reduced in a large animal model of arrhythmias following myocardial infarction (MI).10 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.10, 15 At 48 hours post-coronary artery occlusion, we observed an increase in EHD3 expression in a specific heart region where arrhythmias originate (infarct border zone) compared to control tissue (remote from infarct; remote EHD3 levels are no different from remote regions of non-infarcted hearts at 48 hrs or 5 days after occlusion: 1.19±0.05 and 1.11±0.10, respectively, p=N.S. compared to control, n=3) (Figure 8A–B). By five days post-occlusion (when action potential and ion channel changes are most prominent28), we observed significant increases in both EHD3 and EHD4 levels in the infarct border zone (Figure 8A–B). EHD1 and EHD2 levels were not significantly changed at 48 hours or five days post-occlusion (Figure 8A–B). Notably, ankyrin-B levels are reduced in this post-MI arrhythmia model (representative immunoblots shown in Figure 8). 10

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 co-expressed 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-$B^{-/-}$ myocytes, displaying both decreased expression and function of Na/Ca exchanger, but maintaining the expression and activity of $Ca_v1.2$. Finally, we demonstrate dramatic alteration of the EHD proteins following MI, suggesting that these proteins may play an important role in action potential and ion channel changes in heart disease. Our findings provide the initial functional characterization of a new class of membrane trafficking proteins in heart, and provide the first reported link between EHD proteins and a human disease model. We believe these findings open a new avenue to understand the molecular determinants of cardiac membrane excitability.

EHD proteins appear to regulate endosomal anterograde and retrograde trafficking, as well as membrane protein recycling and lipid homeostasis.2, 5, 12 Structural analysis has revealed a similarity of EHD2 with the dynamin superfamily. This similarity may translate into parallel functional roles of EHD-containing proteins with dynamin, including the ability to tubulate liposomes, dimerize, oligomerize around lipid tubules in ring-like structures, and to stimulate nucleotide hydrolysis in response to lipid-binding. 5 Among EHD proteins, EHD1 is the best characterized with roles in membrane recycling of the transferrin receptor, major histocompatibility class 1 receptor, and beta-1 integrin.12 Additionally, EHD1 associates directly with phosphatidylinositol compounds11, 24 and modulates cholesterol homeostasis.25 While molecular mechanisms underlying EHD1 function are still being identified, EHD1-binding partners including MICAL-L133, ARF6, and Rab effector proteins argue that EHD1 plays a central role in endosome/Golgi vesicular trafficking.2, 5, 21, 26 Similarly, EHD2 regulates glucose-stimulated GLUT4 membrane transport and endocytosis in adipocytes.9 Potentially relevant for cardiac muscle, EHD2 interactions with myoferlin are important for myoblast fusion.6 Additionally, EHD1 has been demonstrated to

interact with amphiphysin-127, a member of a protein family implicated in biogenesis of transverse-tubules in skeletal muscle.14 Finally, both EHD3 and EHD4 have roles in endosome vesicular targeting and recycling.23, 34 Specifically, EHD3 directly associates with critical trafficking proteins including Rab11-FIP2.26 Finally, two reports describe testicular phenotypes in mouse models of EHD1 and EHD4 deficiency. Deletion of EHD1 and EHD47, 31 result in reduction in testes size; however, deletion of EHD1 leads to male infertility while deletion of EHD4 does not. In summary, while much is unknown about the roles of these recently identified proteins, the clear linkage of EHD proteins with protein trafficking, endocytosis, and endocytosis, suggest 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 up-regulating the EHD/ankyrin pathway. EHD protein expression levels may also be regulated by the local signaling environment.1, 32 Thus, abnormal ion homeostasis created by the loss of ankyrin-B13, 19 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 myocardial infarction that also shows parallel loss of ankyrin-B (Figure 8).10 Clearly, additional studies in adult 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 tissues8, 29, we predict that loss of EHD-containing proteins in heart will affect on 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 3). 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 electrical remodeling in the damaged myocyte, due to 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, while 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.7, 31 Interestingly, a proportion of EHD1-deficient mice are neonatal lethal (Rainey et al, accepted) due to unknown causes. Future work will help unravel if this is due to defects in cardiac excitability. Thus, while 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 knock-out of multiple EHD protein isoforms.

Novelty and Significance

What is known?

- Cardiac excitability requires cellular pathways that tightly regulate integral membrane protein trafficking, endocytosis, and endocytic recycling.

- There is a fundamental lack of understanding of the cellular pathways and molecular mechanisms that control membrane protein trafficking and recycling in heart.

What New Information Does This Article Contribute?

- In the present study it was identified that the family of Eps15 homology (EH) domaincontaining proteins (EHD1-4) is critical for membrane protein trafficking in primary cardiomyocytes.
- EHD proteins interact with the previously identified ankyrin-dependent pathway for membrane protein targeting.
- EHD protein levels are altered in small animal models of ankyrin-B deficiency and in a large animal model of human cardiovascular disease.

Cardiac membrane excitability requires tightly regulated intracellular pathways that control anterograde and retrograde trafficking of membrane proteins, their stability at the cell surface, and their degradation. This study identifies four members of the Eps15 homology domain-containing protein family in cardiomyocytes that serve critical roles in endosome-based membrane protein targeting in other cell types. Cardiac EHD proteins were found to associate with the ankyrin-based pathway for membrane protein targeting in myocytes, and decreased EHD3 expression altered the membrane targeting and electrophysiological properties of select cardiac ion channels and transporters. Finally, altered EHD expression was identified in a large animal model of arrhythmias following myocardial infarction, suggesting that EHD proteins may serve as key nodal points for regulating membrane excitability in normal and diseased hearts. Collectively, these findings characterize a new class of cardiac membrane targeting proteins, identify key components of the ankyrin-based cellular pathway for membrane protein trafficking, and define potential new targets to regulate membrane excitability in disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

NIH (HL084583, HL083422 PJM; HL079031, HL62494, HL70250 MEA; HL66140 PAB; HL096805 TJH; HL092232 SRC; CA105489, CA87986, CA99163,CA116552 HB; HL007121 [Cardiovascular Interdisciplinary Research Fellowship] HG), Pew Scholars Trust (PJM), and Fondation Leducq Award (Alliance for Calmodulin Kinase Signaling in Heart Disease (PJM, MEA)).

References

1. Amit I, Citri A, Shay T, Lu Y, Katz M, Zhang F, Tarcic G, Siwak D, Lahad J, Jacob-Hirsch J, Amariglio N, Vaisman N, Segal E, Rechavi G, Alon U, Mills GB, Domany E, Yarden Y. A module of negative feedback regulators defines growth factor signaling. Nat Genet. 2007; 39:503–512. [PubMed: 17322878]
2. Caplan S, Naslavsky N, Hartnell LM, Lodge R, Polishchuk RS, Donaldson JG, Bonifacino JS. A tubular EHD1-containing compartment involved in the recycling of major histocompatibility complex class I molecules to the plasma membrane. Embo J. 2002; 21:2557–2567. [PubMed: 12032069]
3. Cunha SR, Bhasin N, Mohler PJ. Targeting and stability of Na/Ca exchanger 1 in cardiomyocytes requires direct interaction with the membrane adaptor ankyrin-B. J Biol Chem. 2007; 282:4875–4883. [PubMed: 17178715]

4. Cunha SR, Mohler PJ. Cardiac ankyrins: Essential components for development and maintenance of excitable membrane domains in heart. Cardiovasc Res. 2006; 71:22–29. [PubMed: 16650839]
5. Daumke O, Lundmark R, Vallis Y, Martens S, Butler PJ, McMahon HT. Architectural and mechanistic insights into an EHD ATPase involved in membrane remodelling. Nature. 2007; 449:923–927. [PubMed: 17914359]
6. Doherty KR, Demonbreun AR, Wallace GQ, Cave A, Posey AD, Heretis K, Pytel P, McNally EM. The endocytic recycling protein EHD2 interacts with myoferlin to regulate myoblast fusion. J Biol Chem. 2008; 283:20252–20260. [PubMed: 18502764]
7. George M, Rainey MA, Naramura M, Ying G, Harms DW, Vitaterna MH, Doglio L, Crawford SE, Hess RA, Band V, Band H. Ehd4 is required to attain normal pre-pubertal testis size but dispensable for fertility in male mice. Genesis.
8. George M, Ying G, Rainey MA, Solomon A, Parikh PT, Gao Q, Band V, Band H. Shared as well as distinct roles of EHD proteins revealed by biochemical and functional comparisons in mammalian cells and C. elegans. BMC cell biology. 2007; 8:3. [PubMed: 17233914]
9. Guilherme A, Soriano NA, Bose S, Holik J, Bose A, Pomerleau DP, Furcinitti P, Leszyk J, Corvera S, Czech MP. EHD2 and the novel EH domain binding protein EHBP1 couple endocytosis to the actin cytoskeleton. J Biol Chem. 2004; 279:10593–10605. [PubMed: 14676205]
10. Hund TJ, Wright PJ, Dun W, Snyder JS, Boyden PA, Mohler PJ. Regulation of the ankyrin-B-based targeting pathway following myocardial infarction. Cardiovasc Res. 2009; 81:742–749. [PubMed: 19074823]
11. Jovic M, Kieken F, Naslavsky N, Sorgen PL, Caplan S. Eps15 homology domain 1-associated tubules contain phosphatidylinositol-4-phosphate and phosphatidylinositol-(4,5)-bisphosphate and are required for efficient recycling. Mol Biol Cell. 2009; 20:2731–2743. [PubMed: 19369419]
12. Jovic M, Naslavsky N, Rapaport D, Horowitz M, Caplan S. EHD1 regulates beta1 integrin endosomal transport: effects on focal adhesions, cell spreading and migration. J Cell Sci. 2007; 120:802–814. [PubMed: 17284518]
13. Le Scouarnec S, Bhasin N, Vieyres C, Hund TJ, Cunha SR, Koval O, Marionneau C, Chen B, Wu Y, Demolombe S, Song LS, Le Marec H, Probst V, Schott JJ, Anderson ME, Mohler PJ. Dysfunction in ankyrin-B-dependent ion channel and transporter targeting causes human sinus node disease. Proc Natl Acad Sci U S A. 2008; 105:15617–15622. [PubMed: 18832177]
14. Lee E, Marcucci M, Daniell L, Pypaert M, Weisz OA, Ochoa GC, Farsad K, Wenk MR, De Camilli P. Amphiphysin 2 (Bin1) and T-tubule biogenesis in muscle. Science. 2002; 297:1193–1196. [PubMed: 12183633]
15. Lue WM, Boyden PA. Abnormal electrical properties of myocytes from chronically infarcted canine heart. Alterations in Vmax and the transient outward current. Circulation. 1992; 85:1175–1188. [PubMed: 1371431]
16. Mohler PJ, Davis JQ, Bennett V. Ankyrin-B Coordinates the Na/K ATPase, Na/Ca Exchanger, and InsP(3) Receptor in a Cardiac T-Tubule/SR Microdomain. PLoS Biol. 2005; 3:e423. [PubMed: 16292983]
17. Mohler PJ, Gramolini AO, Bennett V. The Ankyrin-B C-terminal Domain Determines Activity of Ankyrin-B/G Chimeras in Rescue of Abnormal Inositol 1,4,5-Trisphosphate and Ryanodine Receptor Distribution in Ankyrin-B (−/−) Neonatal Cardiomyocytes. J Biol Chem. 2002; 277:10599–10607. [PubMed: 11781319]
18. Mohler PJ, Le Scouarnec S, Denjoy I, Lowe JS, Guicheney P, Caron L, Driskell IM, Schott JJ, Norris K, Leenhardt A, Kim RB, Escande D, Roden DM. Defining the cellular phenotype of "ankyrin-B syndrome" variants: human ANK2 variants associated with clinical phenotypes display a spectrum of activities in cardiomyocytes. Circulation. 2007; 115:432–441. [PubMed: 17242276]
19. Mohler PJ, Schott JJ, Gramolini AO, Dilly KW, Guatimosim S, duBell WH, Song LS, Haurogne K, Kyndt F, Ali ME, Rogers TB, Lederer WJ, Escande D, Le Marec H, Bennett V. Ankyrin-B mutation causes type 4 long-QT cardiac arrhythmia and sudden cardiac death. Nature. 2003; 421:634–639. [PubMed: 12571597]
20. Mohler PJ, Splawski I, Napolitano C, Bottelli G, Sharpe L, Timothy K, Priori SG, Keating MT, Bennett V. A cardiac arrhythmia syndrome caused by loss of ankyrin-B function. Proc Natl Acad Sci U S A. 2004; 101:9137–9142. [PubMed: 15178757]

21. Naslavsky N, Boehm M, Backlund PS Jr, Caplan S. Rabenosyn-5 and EHD1 interact and sequentially regulate protein recycling to the plasma membrane. Mol Biol Cell. 2004; 15:2410–2422. [PubMed: 15020713]
22. Naslavsky N, Caplan S. C-terminal EH-domain-containing proteins: consensus for a role in endocytic trafficking, EH? J Cell Sci. 2005; 118:4093–4101. [PubMed: 16155252]
23. Naslavsky N, McKenzie J, Altan-Bonnet N, Sheff D, Caplan S. EHD3 regulates early-endosome-to-Golgi transport and preserves Golgi morphology. J Cell Sci. 2009; 122:389–400. [PubMed: 19139087]
24. Naslavsky N, Rahajeng J, Chenavas S, Sorgen PL, Caplan S. EHD1 and Eps15 interact with phosphatidylinositols via their Eps15 homology domains. J Biol Chem. 2007; 282:16612–16622. [PubMed: 17412695]
25. Naslavsky N, Rahajeng J, Rapaport D, Horowitz M, Caplan S. EHD1 regulates cholesterol homeostasis and lipid droplet storage. Biochem Biophys Res Commun. 2007; 357:792–799. [PubMed: 17451652]
26. Naslavsky N, Rahajeng J, Sharma M, Jovic M, Caplan S. Interactions between EHD proteins and Rab11-FIP2: a role for EHD3 in early endosomal transport. Mol Biol Cell. 2006; 17:163–177. [PubMed: 16251358]
27. Pant S, Sharma M, Patel K, Caplan S, Carr CM, Grant BD. AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling. Nat Cell Biol. 2009; 11:1399–1410. [PubMed: 19915558]
28. Pinto JM, Boyden PA. Electrical remodeling in ischemia and infarction. Cardiovasc Res. 1999; 42:284–297. [PubMed: 10533567]
29. Rapaport D, Auerbach W, Naslavsky N, Pasmanik-Chor M, Galperin E, Fein A, Caplan S, Joyner AL, Horowitz M. Recycling to the plasma membrane is delayed in EHD1 knockout mice. Traffic. 2006; 7:52–60. [PubMed: 16445686]
30. Sedlacek K, Stark K, Cunha SR, Pfeufer A, Weber S, Berger I, Perz S, Kaab S, Wichmann HE, Mohler PJ, Hengstenberg C, Jeron A. Common Genetic Variants in ANK2 Modulate QT Interval: Results From the KORA Study. Circ Cardiovasc Genet. 2008; 1:93–99. [PubMed: 20031550]
31. Sengupta S, George M, Miller KK, Naik K, Chou J, Cheatham MA, Dallos P, Naramura M, Band H, Zheng J. EHD4 and CDH23 are interacting partners in cochlear hair cells. J Biol Chem. 2009; 284:20121–20129. [PubMed: 19487694]
32. Shao Y, Akmentin W, Toledo-Aral JJ, Rosenbaum J, Valdez G, Cabot JB, Hilbush BS, Halegoua S. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. J Cell Biol. 2002; 157:679–691. [PubMed: 12011113]
33. Sharma M, Giridharan SS, Rahajeng J, Naslavsky N, Caplan S. MICAL-L1 links EHD1 to tubular recycling endosomes and regulates receptor recycling. Mol Biol Cell. 2009; 20:5181–5194. [PubMed: 19864458]
34. Sharma M, Naslavsky N, Caplan S. A role for EHD4 in the regulation of early endosomal transport. Traffic. 2008; 9:995–1018. [PubMed: 18331452]

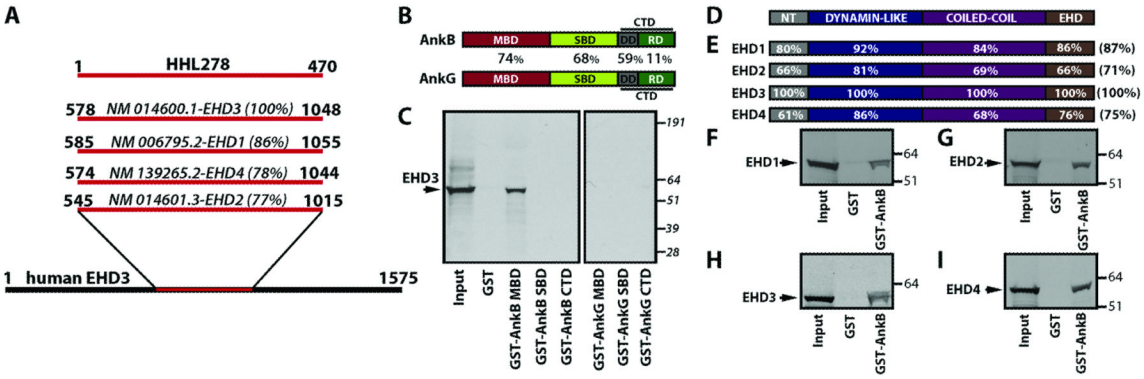

Figure 1. Ankyrin-B associates with EHD3

A) Yeast two-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 (78% identical), and EHD4 (77% identical). **B-C)** *EHD3 directly associates with membrane-binding domain of ankyrin-B, but not ankyrin-G*. **B**) Ankyrin-B and ankyrin-G are comprised of three 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 panel for clarity. **D**) Domain organization of EHD proteins. **E**) Amino acid identity of EHD-containing protein domains compared with EHD3. **F–I**) Radiolabeled EHD1-4 directly associate with GST-ankyrin-B but not GST. Binding data were replicated in four independent experiments.

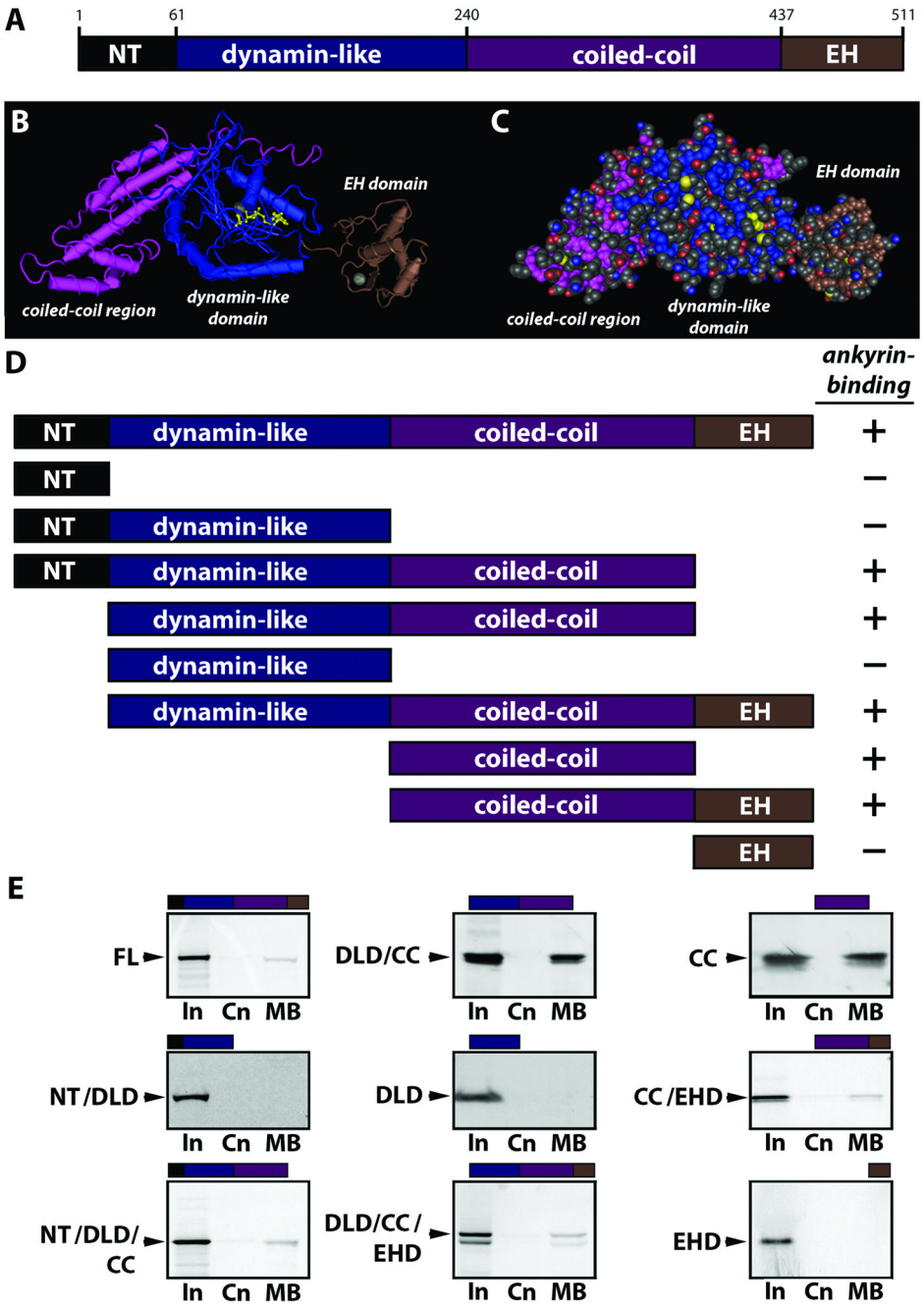

Figure 2. EHD3 coiled-coil domain is required for ankyrin-binding

A) EHD proteins are comprised of an N-terminal region (NT), dynamin-like domain (DLD), a coiled-coil region (CC), and an EH domain (EHD). **B–C)** Depiction of ribbon and space filling model of an EHD3 homolog5 (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 central helical 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 four independent experiments. Note that the translated DLD/CC/EHD product

displays a second, slightly smaller (migrates ~3 kD lower) degradation product that also associates with ankyrin-B.

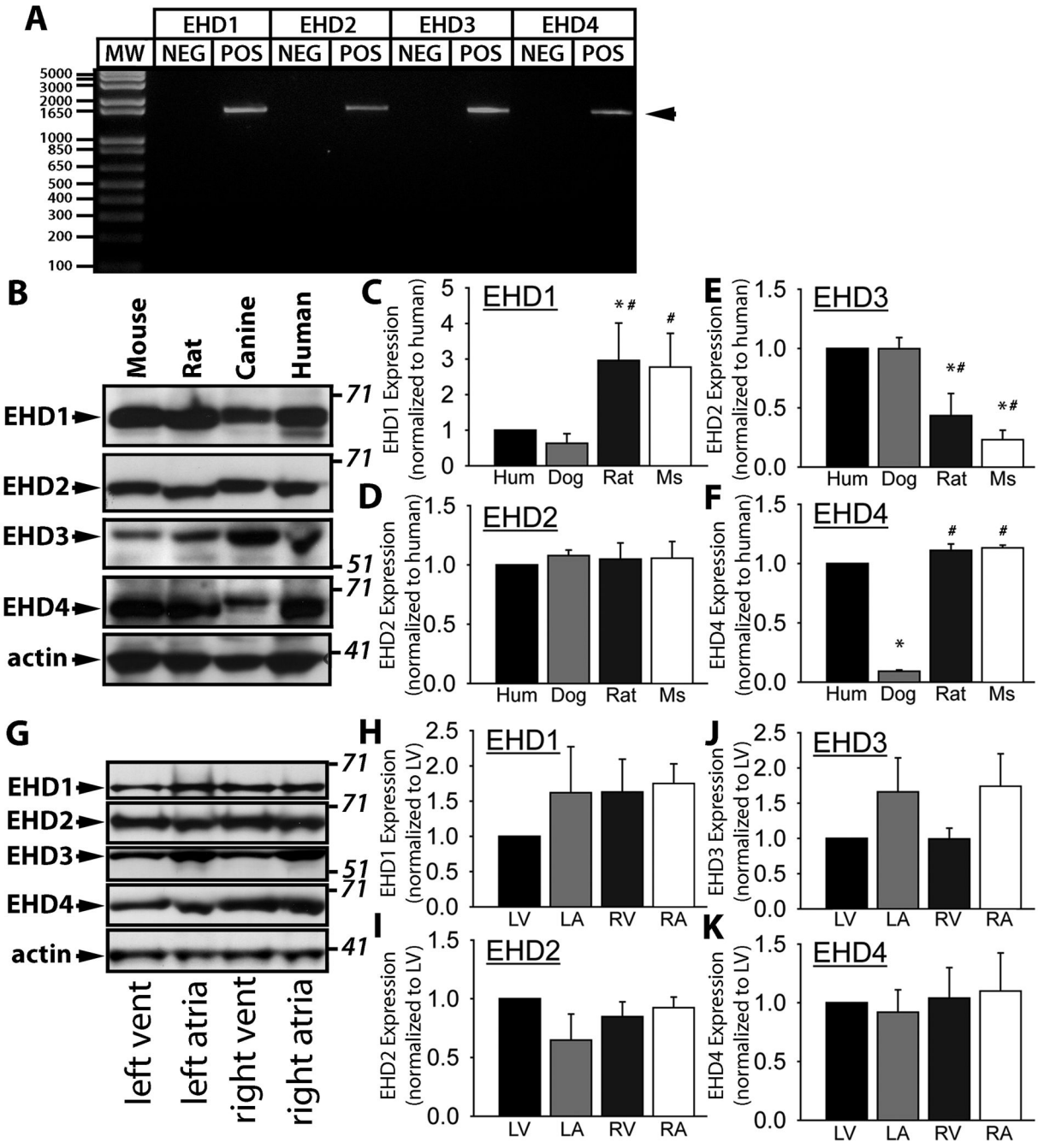

Figure 3. EHD proteins are expressed in human heart

A) RT-PCR of mRNA prepared from human left ventricle demonstrates expression of full-length EHD1-4. Identical results were obtained from three independent experiments. **B–F)** Immunoblots and densitometric measurements of EHD1, EHD2, EHD3, and EHD4 from total protein lysates of mouse, rat, canine, and human left ventricle. Band densities were normalized to actin and expressed relative to human heart for comparison across experiments. Data in C–F represent n=4 normal independent samples. Asterisk denotes $p<0.05$ compared to human heart, whereas the pound symbol represents $p<0.05$ compared with dog. **G–K**) EHD proteins are expressed throughout the heart. Immunoblots and summary data from analyses of total protein lysates of normal human heart chambers for

EHD1-4. Actin was utilized as a loading control and expression is normalized to left ventricle. Data in H–K represent n=3 normal independent samples (p=N.S. for all groups).

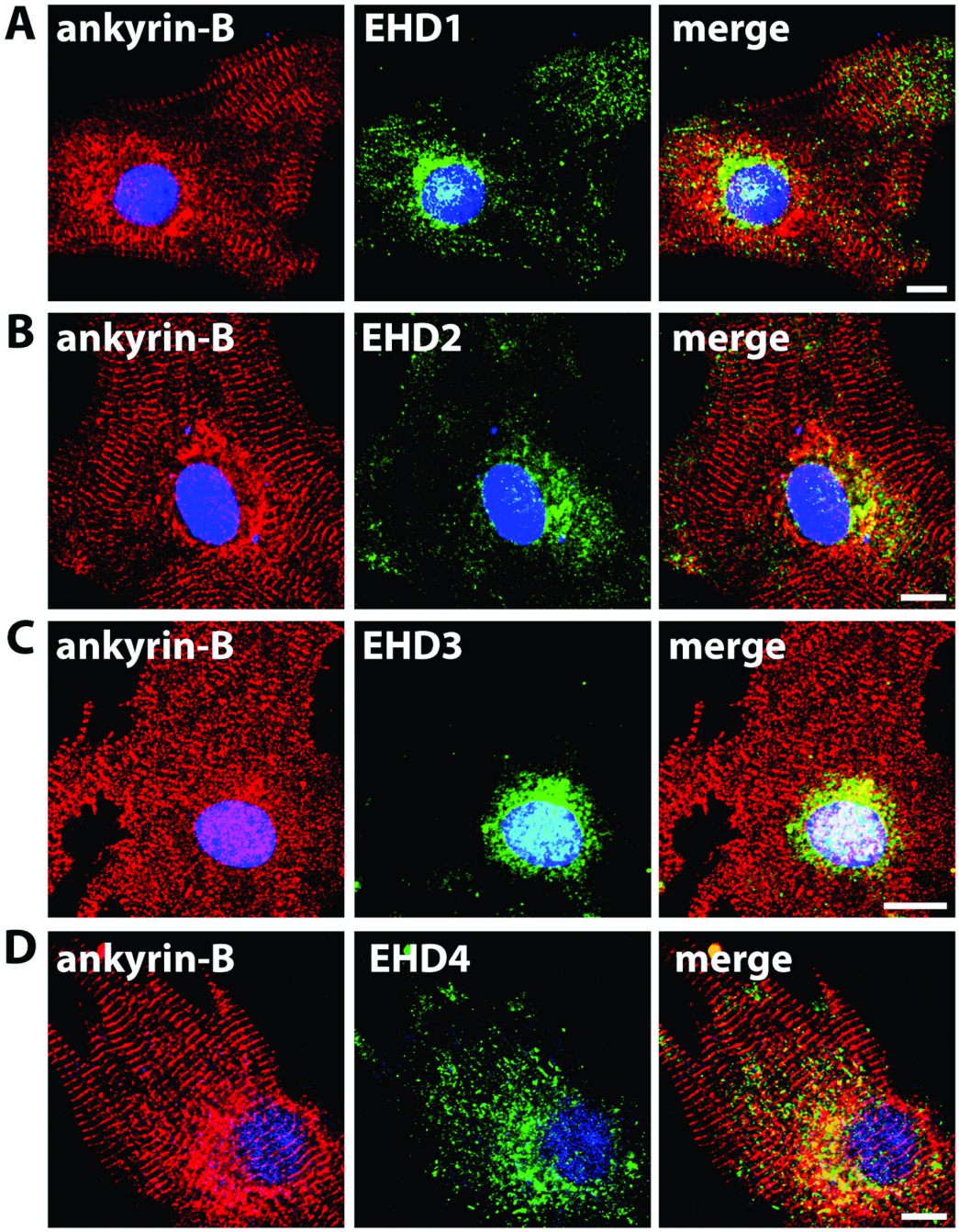

Figure 4. EHD proteins are expressed in the myocyte perinuclear region

Mouse neonatal cardiomyocytes were immunostained for ankyrin-B (red) and EHD1-4 (green). Cells were co-labeled with topro-3AM to stain nuclei (blue). Scale bar=ten microns. Experiments were repeated four times for each EHD protein from four different wild-type mice (>100 individual myocytes/experiment were analyzed for each EHD).

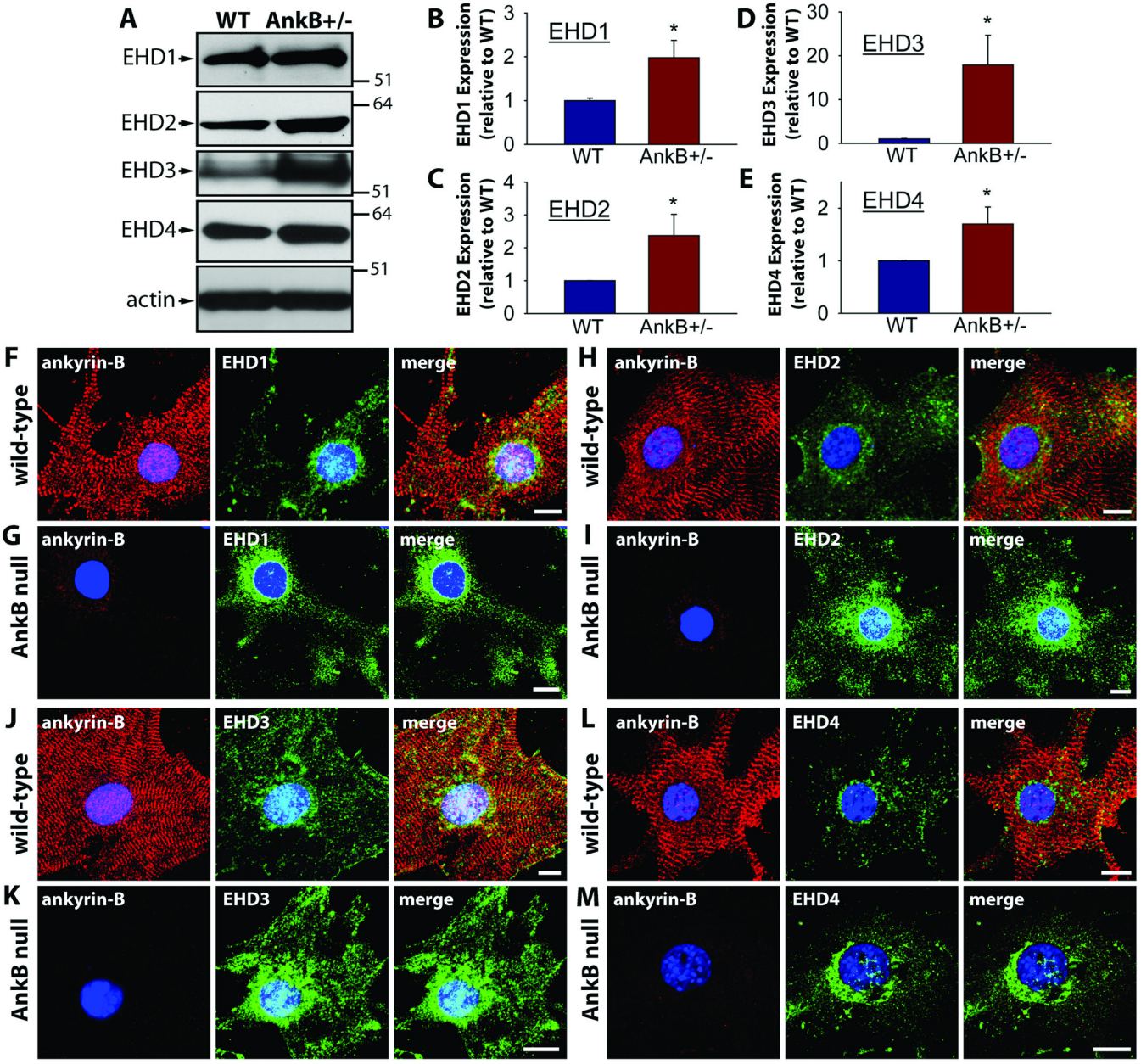

Figure 5. EHD protein expression is increased by loss of ankyrin-B

A) Representative immunoblots and **B–E)** densitometric measurements of EHD1-4 from lysates prepared from adult wild-type and ankyrin-B$^{+/-}$ mouse left ventricle. Equal protein quantities were loaded for each genotype. Band densities were normalized to actin and expressed relative to WT (n=7 experiments/genotype, *p<0.05). **F–M)** *Ankyrin-B$^{-/-}$ cardiomyocytes display increased expression of EHD proteins*. Localization of ankyrin-B and EHD proteins in wild-type (**F, H, J, L**) and ankyrin-B$^{-/-}$ (**G, I, K, M**) primary cardiomyocytes. All cells were stained and imaged using similar protocols. Scale bar=10 microns. Cells are co-labeled with topro-3AM to stain nuclei. Experiments were repeated from four mice/genotype and >100 myocytes/experiment were analyzed.

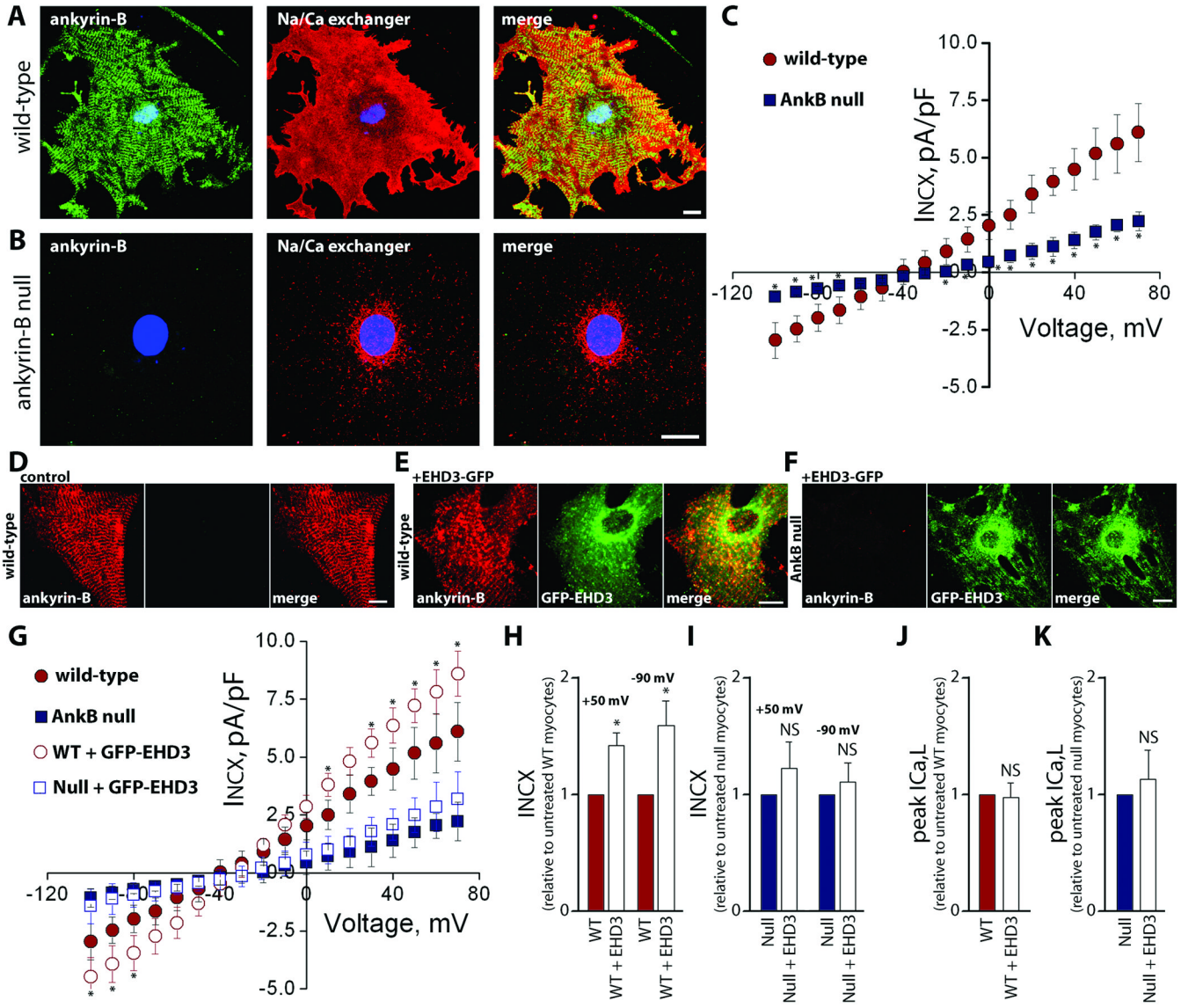

Figure 6. EHD3 overexpression alters I_{NCX} in cardiomyocytes

A–B) Ankyrin-B (green) and Na/Ca exchanger (red) expression in wild-type and ankyrin-$B^{-/-}$ neonatal myocytes. Nuclei are stained in blue. **C**, Na/Ca exchanger current (I_{NCX}) is significantly reduced in cardiomyocytes derived from ankyrin-$B^{-/-}$ mice ($p<0.05$, n=12 myocytes/genotype). **D–F)** Control myocytes (**D**), and myocytes from wild-type (**E**) and ankyrin-$B^{-/-}$ mice (**F**) expressing GFP-EHD3. Note untransfected cells lack signal on the green channel. **G–I)** EHD3-GFP overexpression increases INCX in wild-type (n=12 cells/treatment, *$p<0.05$ compared to WT untransfected), but not ankyrin-$B^{-/-}$ myocytes (n=12 cells/treatment, p = N.S. compared to ankyrin-$B^{-/-}$ untransfected). **J–K**) GFP-EHD3 overexpression does not affect myocyte $I_{Ca,L}$ (n=9 myocytes/experiment, p = N.S. compared to untransfected). Scale bars=10 microns.

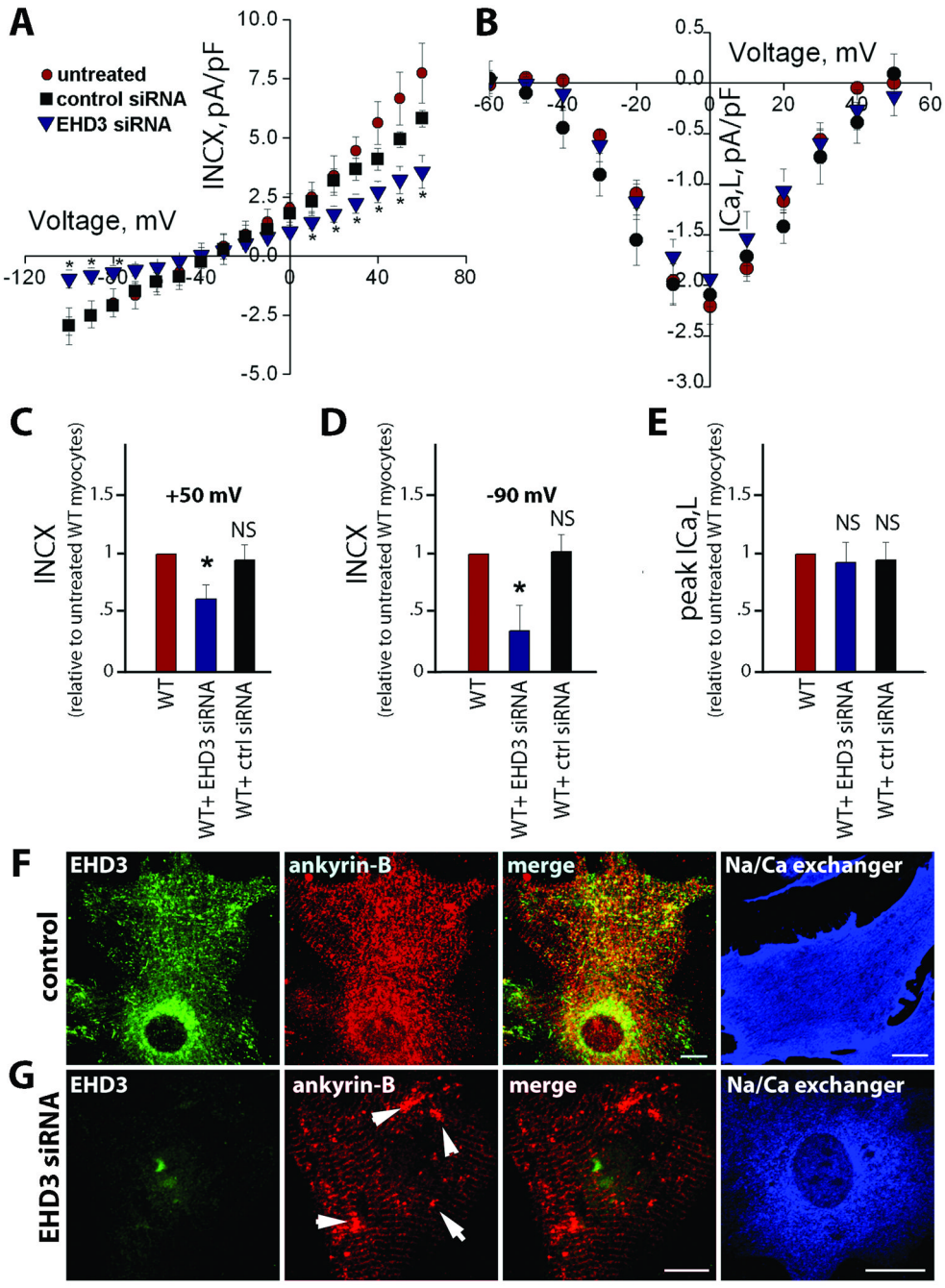

Figure 7. EHD3 knockdown alters ankyrin-B distribution and NCX1 targeting and function

A, C-D) I_{NCX} (pA/pF) in wild-type myocytes following EHD3 siRNA treatment (also untreated, control siRNA). Data represent 11 myocytes/treatment, *p<0.05 compared to control or untreated. **B,E)** EHD3 siRNA treatment does not affect $I_{Ca,L}$. Data represent 9 myocytes/treatment, p=N.S. for all groups **F–G)** Expression and distribution of EHD3, ankyrin-B, and Na/Ca exchanger in control and EHD3 siRNA-treated cardiomyocytes. Images are representative of >100 examined cells; no defects in myocyte proteins were observed in the presence of the control siRNA. Note abnormal distribution, decreased expression, and large ankyrin-B-positive puncta in EHD3 siRNA treated myocytes. Bars=10 microns.

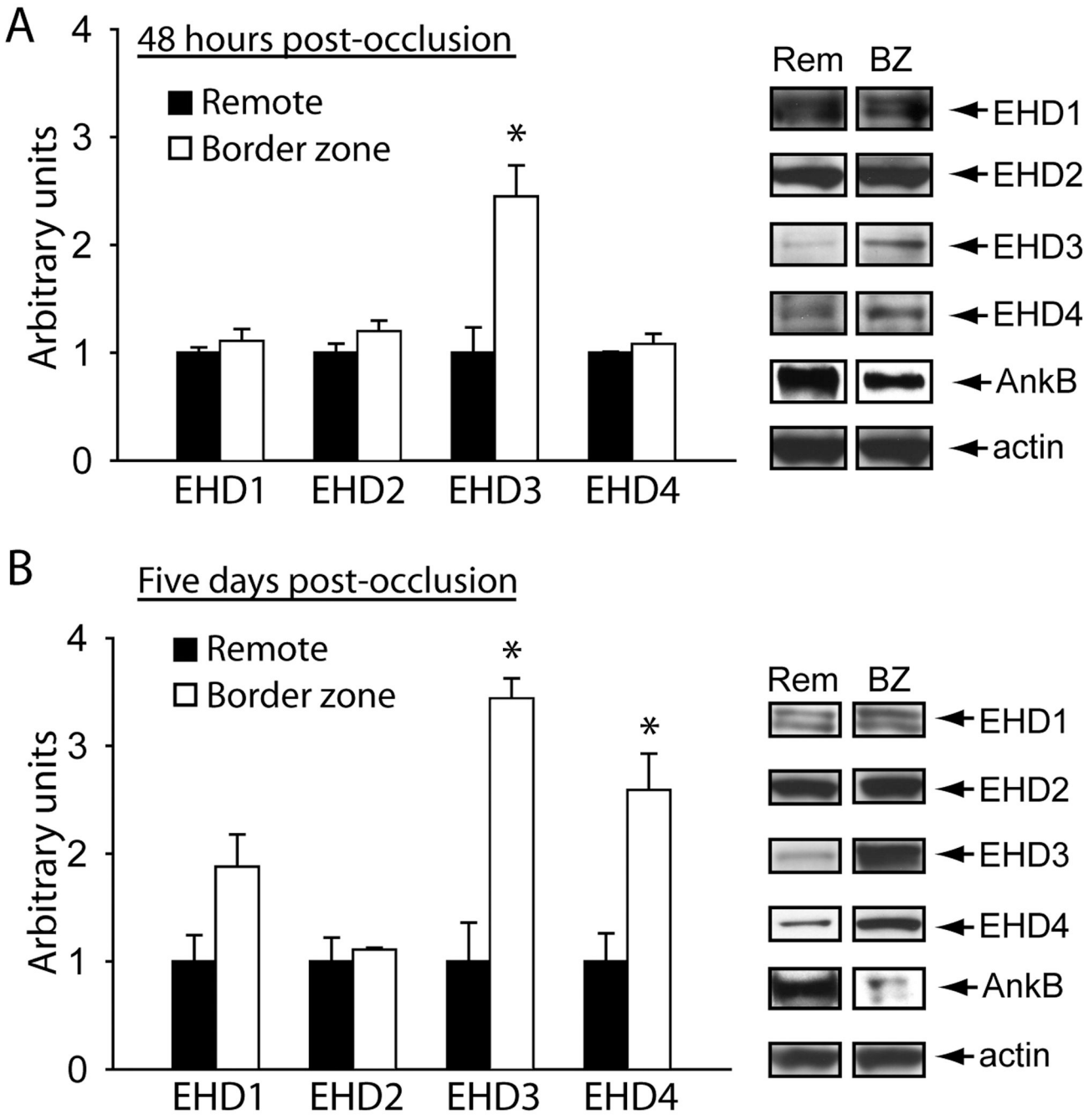

Figure 8. EHD3 and EHD4 expression levels are elevated following myocardial infarction

EHD1-4 densitometric measurements (left) and representative immunoblots (right) from canine border zone and remote tissues 48 hours (**A**) or five days (**B**) post coronary artery occlusion (n=4 for remote and n=3 for BZ, *p<0.05 compared to remote).