EHD3-Dependent Endosome Pathway Regulates Cardiac Membrane Excitability and Physiology

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Rationale: Cardiac function is dependent on the coordinate activities of membrane ion channels, transporters, pumps, and hormone receptors to tune the membrane electrochemical gradient dynamically in response to acute and chronic stress. Although our knowledge of membrane proteins has rapidly advanced during the past decade, our understanding of the subcellular pathways governing the trafficking and localization of integral membrane proteins is limited and essentially unstudied in vivo. In the heart, to our knowledge, there are no in vivo mechanistic studies that directly link endosome-based machinery with cardiac physiology.

Objective: To define the in vivo roles of endosome-based cellular machinery for cardiac membrane protein trafficking, myocyte excitability, and cardiac physiology.

Methods and Results: We identify the endosome-based Eps15 homology domain 3 (EHD3) pathway as essential for cardiac physiology. EHD3-deficient hearts display structural and functional defects including bradycardia and rate variability, conduction block, and blunted response to adrenergic stimulation. Mechanistically, EHD3 is critical for membrane protein trafficking, because EHD3-deficient myocytes display reduced expression/localization of Na/Ca exchanger and L-type Ca channel type 1.2 with a parallel reduction in Na/Ca exchanger–mediated membrane current and Ca_{1.2}-mediated membrane current. Functionally, EHD3-deficient myocytes show increased sarcoplasmic reticulum [Ca], increased spark frequency, and reduced expression/localization of ankyrin-B, a binding partner for EHD3 and Na/Ca exchanger. Finally, we show that in vivo EHD3-deficient defects are attributable to cardiac-specific roles of EHD3 because mice with cardiac-selective EHD3 deficiency demonstrate both structural and electric phenotypes.

Conclusions: These data provide new insight into the critical role of endosome-based pathways in membrane protein targeting and cardiac physiology. EHD3 is a critical component of protein trafficking in heart and is essential for the proper membrane targeting of select cellular proteins that maintain excitability. (Circ Res. 2014;115:68-78.)

Key Words: ankyrins ■ cell biology ■ Ehd3 protein ■ electrophysiology ■ ion channels ■ mice ■ protein transport

Highly evolved and differentiated for excitation–contraction coupling, cardiac myocytes express a specific profile of ion channels, pumps, and transporters that maintain cardiomyocyte electric excitability. Collectively, these membrane proteins mediate action potential (AP) formation and response, Ca-induced Ca release, and the secretion of natriuretic peptides.1,2 Equally important is the set of hormone receptors localized to the sarcoplasmic membrane that regulate the activity and response of ion channels and pumps through specific second messenger pathways. These highly evolved systems are tightly synchronized to tune cardiac output to meet the changing demands placed on the heart by variable stresses. Like other complex cells, cardiac
membrane protein residency is in constant flux and primarily regulated by 3 general processes including synthesis and trafficking of new proteins through the endoplasmic reticulum and Golgi to specific membrane domains, membrane protein internalization and recycling, and ultimate membrane protein degradation. In metazoan cells, endothase-based protein machinery is indispensable for each of these functions. However, we know little to nothing about the in vivo components of the cardiac endosome system. The Eps15 homology domain (EHD)—containing protein family (EHD1–4) mediates endosome-based trafficking in nonexcitable and heterologous cells.3–5 Recently, we identified EHD3 in human heart.6 Furthermore, we identified that EHD3 levels are elevated in multiple forms of cardiovascular disease.7 Based on our initial findings, we hypothesized that the endothase-based EHD3 protein plays critical roles in cardiac membrane protein trafficking and physiology at baseline and in disease and that EHD3 deficiency would result in defects in both cardiac electric and functional phenotypes.

Here, we define the in vivo physiological and mechanistic roles of EHD3 in the heart. EHD3-deficient (EHD3−/−) mice display enlarged hearts and abnormal cardiac function. Furthermore, EHD3−/− mice exhibit bradycardia, atrioventricular conduction block, and heart rate (HR) variability and have a blunted response to β-adrenergic receptor (β-AR) stimulation. Adult ventricular myocytes isolated from EHD3−/− mouse hearts display a significant attenuation of AP duration (APD), increased total sarcoplasmic reticulum Ca concentration ([Ca]SRT) and Ca sparks, a blunted β-AR response, and reduced expression and function of the L-type Ca channel type 1.2 (Ca1.2) and Na/Ca exchanger (NCX). Confocal studies revealed improper localization of both the Ca1.2 and NCX, consistent with the role of EHD3 for targeting select membrane proteins. Moreover, ankyrin-B, an intermediate binding partner between EHD3 and the NCX, is decreased in EHD3−/− mice, providing the underlying mechanism between EHD3 and membrane proteins. Finally, EHD3−/− defects are attributable to cardiac-intrinsic roles of EHD3 because mice with cardiac-specific EHD3 deficiency demonstrate structural and electric phenotypes. These new data define a critical role for EHD3 in select protein trafficking in the heart as well as indicate the importance for subcellular protein targeting for cardiac excitability.

### Methods

For complete, expanded methods please refer to the Online Data Supplement. All animal studies were performed in accordance with the American Physiological Society Guiding Principles for Research Involving Animals and Human Beings and approved by The Ohio State University Institutional Animal Care and Use Committee. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Echocardiography

Transthoracic echocardiogram was performed on anesthetized wild-type (WT) and EHD3−/− mice as previously described to measure the in vivo function of the heart.

### Electrophysiology

Whole-cell recordings were obtained at room temperature with the use of standard patch clamp techniques, and cells from the WT and EHD3−/− groups had a similar membrane capacitance (Online Figure I).8

### ECG Experiments

ECG recordings of ambulatory mice were obtained using subcutaneously implanted radiotelemeters (DSI, St. Paul, MN).9 Recordings were obtained from mice that were conscious after exercise, after isoprenaline injection, and after exercise plus isoprenaline injection. For baseline HR analysis, continuous ECG data were collected from WT and EHD3−/− mice for 1 hour. Recordings were obtained every 48 hours at the same time of day. For stress tests, mice were run on a treadmill for a maximum of 45 minutes or until exhaustion and then immediately injected with isoprenaline (0.5 mg/kg). Nonsustained and sustained arrhythmias were identified using standard ECG analysis guidelines.10 In a separate group of mice than those implanted with radiotelemeters, surface ECG recordings were obtained under anesthesia with 1% to 2% isoflurane. Three needle electrodes were placed subcutaneously in the standard limb configuration. For each mouse, 15 minutes of continuous data were sampled at 4 kHz with a PowerLab 4/30 interface (AD Instruments, Colorado Springs, CO). Analysis was performed offline using LabChart 7 Pro (AD Instruments).

### SR Ca Load

SR Ca load and Ca handling were assessed from isolated ventricular myocytes as previously described.11 [Ca]SRT was calculated through the pseudoratio as previously described with [Ca] assumed to be 120 nmol/L for all mice.

### Generation of EHD3−/− Mouse

EHD3−/− mice were generated as previously described.12 DNA was isolated from tail clips of 10-day-old mice, and mice were genotyped by polymerase chain reaction. Three primers in a single duplex polymerase chain reaction reaction amplified the WT allele (377 bp) and the deleted allele (488 bp, Figure 1A). To test the in vivo cardiac-intrinsic roles of EHD3, we used a conditional null mutant allele in which the 5′ untranslated region and exon 1 of the mouse EHD3 gene (Ehd3) were flanked by LoxP sites (Ehd3fl/+). In addition, the mouse EHD3 gene (Ehd3) were transplanted into the IP (in place) position. Therefore, the presence of Cre recombinase. We selectively eliminated EHD3 in cardiomyocytes by using alpha subtype myosin heavy chain (αMHC)-Cre knock-in mice13; homozygous conditional knockout (cKO) Ehd3f/f mice were born at expected Mendelian ratios and were healthy and fertile with body weights comparable with their WT littermates.

### Antibodies

The following antibodies were used to conduct this study: affinity-purified rabbit polyclonal antibody directed at human EHD4 (SHRKSLPKAD), rabbit polyclonal anti-EHD1 (abcam, Cambridge, MA), mouse monoclonal anti-NCX1 (Swant, Bellinzona, Switzerland), rabbit polyclonal anti–ankyrin-B,14 mouse monoclonal anti-Ca1.2,15 rabbit polyclonal anti-β1- and anti-β1-AR (Santa Cruz

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Biotechnology, Dallas, TX), rabbit polyclonal anti-Na\textsubscript{v}1.5,\textsuperscript{16} actin (Santa Cruz Biotechnology), rabbit polyclonal anti-SERCA (Santa Cruz), mouse monoclonal antiphospholamban (abcam, Cambridge, MA), and rabbit monoclonal anti–calsequestrin 2 (abcam).

**Immunoblots and Immunostaining**

Immunoblots of whole heart lysates were performed as described.\textsuperscript{17} Briefly, whole hearts were harvested from WT and EHD3\textsuperscript{−/−} adult, age-matched littermates and immediately placed into ice cold homogenization buffer (in mmol/L: 50 Tris-HCl, 10 NaCl, 320 sucrose, 5 EDTA, 2.5 EGTA; supplemented with 1:1000 protease inhibitor cocktail and 1:1000 phenylmethanesulfonyl fluoride [Sigma]). After quantification, tissue lysates were analyzed on Mini-PROTEAN tetra cell (BioRad) on a 4% to 15% precast TGX gel (BioRad). Gels were transferred to a nitrocellulose membrane using the Mini-PROTEAN tetra cell (BioRad). Membranes were blocked for 1 hour at room temperature using a 3% BSA solution or 5% milk solution and incubated with primary antibody overnight at 4°C. Densitometry analysis was done using ImageLab software (BioRad). For all experiments, protein values were normalized against an internal loading control (actin or GAPDH).

**Statistics**

All values are presented as mean±SEM. When appropriate, data were analyzed using a 2-tailed Student\textsubscript{t} test. NCX–mediated membrane current (\textit{INCX}) values were analyzed using a 1-tail Student\textsubscript{t} test with the WT myocytes predicted to have the larger mean value based on previous work.\textsuperscript{6} \textit{P} values <0.05 were considered significant.

**Results**

**EHD3\textsuperscript{−/−} Mice Display Chamber Dilation and Reduced Ejection Fraction**

To evaluate the role of EHD3 in cardiac physiology, we first examined the structure and function of the heart in EHD3\textsuperscript{−/−} and WT mice (Figure 1A). By gross examination, whole heart morphology was significantly altered in EHD3\textsuperscript{−/−} mice. Both atria and ventricles were larger in excised EHD3\textsuperscript{−/−} hearts compared with those of WT littermates (Figure 1B), and we observed increased heart weight to body weight ratio in EHD3\textsuperscript{−/−} mice compared with WT littermates (EHD3\textsuperscript{−/−}: 7.98±0.26 mg/g; WT: 6.79±0.21 mg/g; \textit{P}=0.004; Online Table I). We performed echocardiograms on age-matched WT and EHD3\textsuperscript{−/−} mice to assess whether EHD3 deficiency directly affected the development, structure, or contractility of the adult heart. Both fractional shortening and ejection fraction were decreased in EHD3\textsuperscript{−/−} mice (Figure 1F and 1G), although stroke volume was preserved (Figure 1H; likely attributable to a larger left ventricular diameter in EHD3\textsuperscript{−/−} hearts [Figure 1C]). Both anterior and posterior systolic wall thicknesses were decreased in EHD3\textsuperscript{−/−} mice (Figure 1D and 1E); however, we observed no difference in diastolic wall thickness between genotypes. Chamber dilation and stroke volume phenotypes were present...
in EHD3−/− mice as early as 4 weeks, although at this age we observed no difference in fractional shortening or ejection fraction between genotypes (Online Tables I and II).

**EHD3−/− Mice Display Bradycardia, HR Variability, and Cardiac Conduction Defects**

We next assessed the in vivo role of EHD3 in cardiac electric signaling. ECG data were acquired from conscious, ambulatory WT and EHD3−/− mice with subcutaneously implanted radiotelemeters. Notably, we observed a significant reduction in baseline HR in EHD3−/− mice and WT littermates (Figure 2A). We examined HR variability by conducting a fast Fourier transform analysis of HR. In addition to bradycardia, EHD3−/− mice displayed increased HR variability compared with controls, with the frequency of lower HRs more prominent in EHD3−/− mice (Figure 2B and 2C). Analysis of individual ECG traces revealed numerous electric phenotypes in EHD3−/− mice. Unlike WT mice (Figure 2D and 2E), EHD3−/− mice were prone to bursts of irregular HR reflected as increased variability in the R-R interval and sinus pause in selected segments of the ECG recordings (Figure 2C). EHD3−/−, but not WT, mice commonly displayed significant R-R interval variability for periods lasting >1 minute and as long as 5 minutes under baseline conditions. This phenotype became even more apparent when EHD3−/− mice were stimulated with isoprenaline injection (Figure 2E). With the exception of episodes of sinus pause or atrioventricular block, we did not observe significant differences in either P-R interval or QRS duration between WT and EHD3−/− mice (Online Figure II).

In addition to increased R-R variability, EHD3−/− mice consistently displayed type II atrioventricular conduction block, with exacerbation of the phenotype after isoprenaline stimulation (Figure 2F). The type II block was characterized by 2 P waves without subsequent QRS complexes followed by a single extended R-R interval and resumption of normal rhythm (Figure 2F). This pattern of atrioventricular block was maintained for as long as 15 minutes in EHD3−/− mice and was never observed in WT littermates. Collectively, we conclude that normal cardiac automaticity and conduction require EHD3.

EHD proteins have been implicated in membrane receptor expression, internalization, and recycling in other cell systems. We therefore assessed the impact of EHD3 deficiency on β-AR signaling in vivo. In conscious mice, the maximum HR in response to isoprenaline injection (either low or high dose) was equivalent in WT and EHD3−/− mice; however, the duration of this response was significantly shorter in the EHD3−/− mice (Online Figure IIIA and IIIB). To limit any variation in the data resulting from the physical handling of the mice, we performed surface ECG recordings on sedated mice with continuous monitoring of HR (Online Figure IIIC). After injection of isoprenaline (0.5 mg/kg), WT mice had a significantly larger increase in HR compared with EHD3−/− mice (79±10 versus 46±5 bpm; P<0.05; Online Figure IIID). Outside the sinus pause and atrioventricular block, we observed no other evidence of arrhythmias (ie, premature ventricular complexes, or atrial flutter or fibrillation) in EHD3−/− mice at baseline, after isoprenaline injection, or after isoprenaline injection and exercise.

Based on these data, we hypothesized that EHD3−/− myocytes would display altered β1-AR trafficking and membrane expression. However, by immunoblot, we observed increased expression of β1- and β2-ARs in EHD3−/− hearts (Online Figure IIIE–IIIH). Because immunobLOTS are unable to discriminate...
between proteins embedded in the surface membrane and those confined to internal compartments (including endosomes), we used radiolabeled β1-AR–specific antagonist, 3H-CGP-12177, to assay potential changes in cell surface β1-AR density in intact, isolated ventricular cardiomyocytes. Using this assay, we observed no significant difference in β1-AR surface density (Online Figure III A). Notably, however, by immunostaining and confocal analysis of WT and EHD3−/− adult ventricular cardiomyocytes, we observed a significant population of β1-AR expression in the perinuclear region of EHD3−/− myocytes (Online Figure IIIJ). Based on these data, we hypothesize that although anterograde membrane trafficking of the β1-AR is EHD3 independent, β1-AR endosomal recycling may be compromised in EHD3−/− myocytes.

**EHD3−/− Myocytes Display Abnormal Myocyte Excitability**

We directly evaluated the effect of EHD3 deficiency on cardiomyocyte membrane excitability. APD was significantly shorter in ventricular myocytes isolated from EHD3−/− hearts (Figure 3A, 3B, and 3D; n=7, both WT and EHD3−/−). At all intervals, APD was significantly shorter in EHD3−/− myocytes, although the shortening was most significant in the early phases of the AP (APD at 50% and 75% repolarization [APD50, APD75], Figure 3A, 3B, and 3D). Furthermore, consistent with in vivo data, EHD3−/− myocytes displayed a blunted β-adrenergic response to isoprenaline treatment (100 nmol/L) compared with WT littermates (Figure 3C–3E).

**EHD3−/− Myocytes Display Increased SR Ca Load and Ca Spark Frequency**

Given the abbreviated AP, but no reduction of the stroke volume in EHD3−/− mice, we hypothesized that EHD3−/− myocytes may have an increased [Ca]SRT. This higher Ca load would increase the Ca sensitivity of the SR Ca release channel, ryanodine receptor, making it more prone to release even with a smaller Ca concentration transients were observed between WT and EHD3−/− myocytes (Figure 4A–4D), although there was a significant increase in [Ca]SRT in the latter (186±25 versus 262±20 μmol/L; Figure 4E). Increased Ca sensitivity of the ryanodine receptor is further reflected by an increased spark frequency found in intact ventricular EHD3−/− myocytes (Figure 4F–4H). These data support the hypothesis that [Ca]SRT is upregulated to maintain contractility in EHD3−/− myocytes, indicating a role for EHD3 in Ca homeostasis.

**EHD3 Is Required for NCX and Ca1.2 Membrane Targeting in Heart**

As a first step toward determining the ionic basis for reduced APD, increased SR Ca2+ load, and increased spark frequency in EHD3−/− myocytes, we screened for likely candidates by performing parameter sensitivity analysis on a well-validated mathematical model of the mouse ventricular AP (Online Figure IV). This analysis revealed that, among sarcolemmal ion channels/transporters, the L-type Ca2+ current and NCX had the greatest influence on APD and SR Ca2+ load, respectively, in a manner consistent with experimentally measured changes. More specifically, the model predicts that loss of L-type Ca2+ channel membrane targeting would produce the greatest decrease in APD, whereas loss of NCX would result in the greatest increase in SR Ca2+ load.

Based on WT and EHD3−/− APD and [Ca]SRT data and subsequent mathematical predictions, we first evaluated the expression of NCX and Ca1.2 by immunoblot. Ca1.2 and NCX expression were reduced by 24% and 17%, respectively (Figure 5A and 5B; P<0.05). Conversely, no differences in sarco/endoplasmic reticulum Ca2+ ATPase 2, phospholamban, or calsequestrin expression were identified between genotypes (Online Figure V). Together with computational analysis, these results provide a possible mechanism for abnormal AP and SR Ca2+ properties in EHD3−/− myocytes.

Decreased expression of NCX and Ca1.2 was paralleled by decreased membrane expression of both NCX and Ca1.2 in EHD3−/− mice by immunostaining and confocal analysis of isolated ventricular cardiomyocytes. Representative images are shown in Figure 5C to 5H. In WT myocytes, the NCX

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**Figure 3. Eps15 homology domain 3-deficient (EHD3−/−) myocytes display shortened action potential duration (APD).** A, Representative baseline AP waveform in wild-type (WT; black) and EHD3−/− (gray) mice. B, APD at baseline. C, Representative AP waveform after application of 100 nmol/L isoprenaline (Iso) in WT (black) and EHD3−/− (gray) myocytes. D, APD at 50%, 75%, and 90% repolarization (APD50, APD75, and APD90; also (Iso)) in WT and EHD3−/− myocytes. E, Change in APD after Iso application. For data in figure, *P<0.05 vs WT; #P<0.05 vs WT+Iso; n=7 mice/genotype; **P<0.05 vs WT+Iso.
clearly localizes to both the sarcolemmal membrane and along the transverse-tubule network (Figure 5C and 5E). However, in EHD3−/− myocytes, the localization to these 2 domains is decreased with a striking perinuclear distribution that is not observed in WT cells (Figure 5D and 5F).

Cav1.2 localization was also disrupted by EHD3 deficiency. In WT myocytes, Cav1.2 staining shows a typical striated pattern (Figure 5G). This pattern was disrupted in EHD3−/− myocytes (Figure 5H). Although some striations were evident, Cav1.2-dependent staining was largely diffused and not localized to any specific subcellular domain. Using an antibody specific for N-cadherin and the lipophilic membrane marker, di-8-ANEPPS, we observed no structural changes to intercalated disc and the t-tubule system in EHD3−/− myocytes, indicating that myocyte ultrastructure is EHD3 independent (Figure 5I–5L).

Finally, to quantify potential functional differences, NCX (I_{NCX}) and Cav1.2 (I_{Cav, L}) currents were assessed in isolated ventricular myocytes using whole-cell patch clamp. Data in Figure 5M to 5Q show that both I_{Cav, L} and I_{NCX} were significantly reduced in EHD3−/− myocytes. I_{NCX} was significantly decreased in EHD3−/− myocytes compared with that in WT myocytes with a 47% reduction in peak current (Figure 5M and 5N). At baseline, I_{Cav, L} was decreased 60% in EHD3−/− myocytes (Figure 5O and 5Q). Treatment with isoproterenol (100 nmol/L) increased I_{Cav, L} similarly in both WT and EHD3−/− myocytes (Figure 5P and 5Q).

In summary, our data obtained using 3 different approaches support a mechanistic in vivo role of EHD3 for the membrane targeting of both NCX and the Cav1.2 in primary adult cardiomyocytes. Importantly, loss of membrane protein expression phenotypes was EHD3 dependent (versus a compensatory response to a failing myocardium) as we observed defects in channel or transporter expression in EHD3−/− primary cardiomyocytes (Online Figure VI) isolated at postnatal day 1 (weeks before observed functional phenotypes).

**Dysregulated Ankyrin-B Trafficking in EHD3−/− Myocytes**

Our findings implicate EHD3 for targeting select ion channels/transporters to the myocyte membrane. However, to further understand the mechanistic link between EHD3 and membrane protein targeting, we investigated the regulation of ankyrin-B, a membrane adapter protein previously linked with both NCX and EHD3.6,22–24 Notably, ankyrin-B expression is significantly decreased (≈40%) in EHD3−/− hearts (Figure 6A and 6B). Furthermore, EHD3−/− myocytes show altered ankyrin-B staining. We observed a decrease in overall intensity with increased perinuclear ankyrin-B staining in
Figure 5. Eps15 homology domain 3 (EHD3) regulates Na/Ca exchanger (NCX) and L-type Ca channel type 1.2 (Cav1.2) membrane targeting and function in heart. A, NCX expression is decreased by 17% in EHD3-deficient (EHD3−/−) hearts (left); representative immunoblot (right). Space between lanes denotes that data were collected from noncontiguous lanes of the same gel. B, Cav1.2 expression is decreased by 24% in EHD3−/− hearts (left); representative immunoblot (right); n=8 hearts/wild type (WT), n=9 hearts/EHD3−/− for immunoblots (*P<0.05 vs WT). C to L, Representative confocal images of WT and EHD3−/− isolated ventricular cardiomyocytes displaying localization of NCX, Cav1.2, N-cadherin, and di-8-ANEPPs (T-tubule marker). Bar=10 μm for all images. M and N, Whole-cell NCX-mediated membrane current (I_{NCX}) was decreased in EHD3−/− myocytes (n=10 myocytes/genotype). Specifically, peak I_{NCX} was decreased by 47% in EHD3−/− myocytes. O and P, EHD3−/− myocytes display reduced Cav1.2-mediated membrane current (I_{Ca,L}) compared with WT at baseline and after application of 100 nmol/L isoprenaline (Iso; n=6 myocytes/genotype, *P<0.05 vs WT). Q, Peak I_{Ca,L} is significantly decreased in EHD3−/− myocytes+Iso. #P<0.05 vs WT+Iso.
EHD3−/− myocytes compared with WT myocytes (Figure 6C–6J). Defects in ankyrin-B expression and localization are present from birth and are directly EHD3 dependent, because viral reintroduction of green fluorescent protein-EHD3 is sufficient to rescue the expression of ankyrin-B in EHD3−/− myocytes (Online Figure VII). Based on these findings, we predict that ankyrin-B plays a key nodal role for EHD3-dependent membrane targeting in heart.

Cardiac-Specific EHD3−/− Mice Display Structural and Electric Phenotypes

Global EHD3−/− mice display bradycardia, HR variability, conduction defects, and structural phenotypes (Figures 1 and 2). However, these parameters may be influenced by cardiac-extrinsic factors (ie, nervous system). To test the in vivo cardiac-intrinsic roles of EHD3 directly, we used a conditional null mutant allele in which exon 1 of the mouse EHD3 gene (Ehd3) was flanked by LoxP sites (Ehd3fl/fl) and therefore are deleted in the presence of Cre recombinase. We selectively eliminated EHD3 in cardiomyocytes by using αMHC-Cre knock-in mice13; homozygous conditional knockout mice are referred to as αMHC-Cre; Ehd3fl/fl or cKO.

Data presented in Figure 7 demonstrate a striking similarity between the EHD3−/− and the EHD3−/− cKO mice. Resting HR was equally depressed in the EHD3−/− cKO and EHD3−/− mice compared with WT (Figure 7A; P<0.05) with a shifted distribution of HR similar to the EHD3−/− model (Figure 7B). Moreover, we observed similar conduction defects in the EHD3−/− cKO mice: a high HR variability at baseline (Figure 7C), a high incidence of sinoatrial node (SAN) pause (Figure 7D), and atrioventricular conduction block (Figure 7E) after isoprenaline treatment. SAN pause and atrioventricular block were also present at a lower rate in the absence of isoprenaline stimulation (not shown). These conduction disorders were never observed in the WT mice. Importantly, at the level of the single myocyte, cKO mouse myocytes display significant loss of I\text{NCX} and I\text{Ca,L} (Figure 7F–7I). Specifically, EHD3−/− cKO peak I\text{NCX} was reduced ≈43%, and I\text{Ca,L} was reduced ≈53% compared with WT (both P<0.001). Beyond electric phenotypes, the structural and function phenotype of EHD3−/− global and cKO mice assessed by echocardiography were similar in nearly all respects (Online Table III). Together, these new data indicate that cardiac EHD3 is critical for normal cardiac structural and electric phenotypes. Furthermore, these data support cardiac-intrinsic roles for EHD3 in regulating normal cardiac structure and function.

Discussion

Anterograde and retrograde protein trafficking and endocytic protein recycling are often overlooked cellular systems. As an often membrane-centric discipline, we take for granted that these pathways are present and critical for cell function. Our lack of understanding may stem from the particular difficulties in studying these systems because endosomes undergo an incredibly complex maturation process that makes investigating particular processes or steps within these pathways problematic, particularly in vivo. Relatively little is known about the particular proteins and enzymes that may be involved in cell membrane trafficking processes. Research during the past 15 years has only just started to isolate single proteins and enzymes that are involved in trafficking. Proteins such as Arf6 (ADP-ribosylation factor 6), SNARE (soluble NSF attachment protein) complexes, BIN1 (amphiphysin 2), EHD1–4 among others are now the focus of efforts to further understand protein trafficking in all cell types.4,25–27 Recently, a mutation in EHD3 was linked to major depressive disorder in humans, indicating that trafficking systems simply do not maintain cellular health but may be the primary cause for medical disorders.28 We recently identified EHD3 in the heart and demonstrated an association of this protein with cardiac disease.7

Figure 6. Dysregulation of ankyrin-B expression and localization in Eps15 homology domain 3-deficient (EHD3−/−) heart. A, Ankyrin-B expression is reduced ≈40% in EHD3−/− hearts (n=9 wild type [WT], 8 EHD3−/−; *P<0.05 vs WT). B, Representative immunoblot of ankyrin-B expression in WT and EHD3−/− hearts. Space between lanes denotes that data were collected from noncontiguous lanes of the same gel. C to J, Representative confocal images detailing ankyrin-B trafficking dysregulation in EHD3−/− myocytes (*P<0.05 vs WT).
This report is the first to detail the functional in vivo roles of EHD3 in the heart. The key findings of this investigation are as follows: (1) EHD3−/− hearts are significantly larger with preserved cardiac output; (2) EHD3−/− mice display bradycardia, HR variability, type II conduction block, and a blunted response to β-AR stimulation; (3) isolated EHD3−/− myocytes display a significantly abbreviated APD and a blunted response to β-AR stimulation; (4) EHD3−/− myocytes have a significant reduction in expression, targeting, and function of Cav1.2 and NCX; (5) EHD3−/− myocytes display a significantly larger [Ca]SRT and a higher frequency of sparks compared with WT; (6) ankyrin-B function is directly altered by EHD3 loss; and (7) EHD3−/− phenotypes can be directly linked with EHD3 expressed in myocytes. Together, these data highlight the importance of endosome-based pathways for normal cardiac function.

EHD proteins (EHD1–4) are key regulators of membrane protein targeting in other tissue and cell types.29–31 This family of proteins has high homology among the members (ranging from 71% to 86%), with EHD1 and EHD3 sharing the highest homology.32 This degree of homology infers similar and potentially redundant cellular function. For example, in a Caenorhabditis elegans model lacking the EHD ortholog, RME-1, expression of human EHD1–4 is sufficient to rescue RME-1–dependent protein trafficking.4 These findings seem relevant to our study. For example,
although select protein trafficking pathways are disrupted and EHD3⁺⁻ hearts display multiple in vivo phenotypes, the viability of EHD3⁺⁻ mice strongly support that additional pathways are present for ion channel and transporter membrane expression. Interestingly, EHD1 expression was significantly upregulated in the EHD3⁺⁻ mice (Online Figure VIII). We speculate that this upregulation may represent a compensatory response of the heart to maintain protein trafficking. We also observed reduced expression of EHD4 in the EHD3⁺⁻ hearts (Online Figure VIII). Although nothing is currently known regarding the role of either EHD1 or EHD4 proteins in heart, these data suggest that the EHD family may collaborate to regulate endosome-based trafficking pathways. EHD3⁺⁻ and EHD3⁻⁻ cKO mice display bradycardia, rate variability, and conduction defects. The SAN is the pacemaker of the heart, and the atrioventricular node is critical for the proper conduction of the AP from the atria to the ventricles. Both the SAN and atrioventricular node depend on voltage-gated ion channels and exchangers to maintain proper electric activity. In particular, the SAN is known to rely on both the I_{Ca,L} and I_{Na,CX} for automaticity, whereas I_{Ca,L} activity in the atrioventricular node is thought to be dispensable for conduction through this junction. Notably, EHD3 is expressed in the SAN of both mice and canine hearts (Online Figure IX). Although additional studies will be necessary to identify specific roles of EHD3 in these critical cardiac cell types, our initial findings link EHD3 function with cardiac automaticity.

EHD3-dependent trafficking in the heart may represent a nodal control pathway for regulating protein trafficking in response to acute or chronic stress. Here, we demonstrate that EHD3-dependent mechanisms are broadly implicated in the subcellular trafficking and localization of many of the proteins involved in excitation–contraction coupling. NCX expression is increased in nearly all reports examining protein expression in heart failure, including in humans. Although initially compensatory, as the severity of heart failure progresses, the increased NCX expression becomes maladaptive and arrhythmogenic leading to delayed afterdepolarizations. We previously reported that EHD3 expression is increased in numerous causes of heart failure. The evidence in this report strongly indicates that EHD3 mediates NCX trafficking in the heart. Together, I_{Na,CX} and I_{Ca,L} make up a substantial fraction of the whole-cell current during a typical AP. Based on modeling predictions, we expect that loss of I_{Ca,L} plays a critical role in abbreviation of APD in EHD3-null myocytes, especially in the early phases of AP development, whereas loss of I_{Na,CX} determines changes in SR Ca²⁺ load. Although EHD3 is critical for NCX, Ca,L, and ankyrin-B targeting, it is important to note that EHD3 also likely targets additional membrane proteins and that observed phenotypes in the EHD3⁺⁻ heart may not be related to calcium-based signaling pathways. Future experiments will be critical to define the full spectrum of EHD3 targets in heart.

Finally, additional work will be necessary to define the structural phenotypes observed in EHD3⁺⁻ hearts during development, as well as the relationship between structural and electric phenotypes. Despite chamber dilation, reduced ejection fraction, and reduced stroke volume, the myopathy phenotype in EHD3⁺⁻ mice is complex and may not simply represent a pure maladaptive or physiological hypertrophy phenotype. In fact, despite observing increased expression of slow skeletal muscle troponin (consistent with maladaptive hypertrophy, P<0.05), we did not observe elevated expression of atrial natriuretic peptide, brain natriuretic peptide, or β-myosin heavy chain expression mRNA in EHD3⁺⁻ hearts (P=NS). Future experiments will be critical to characterize the role of EHD3 fully in cardiac development as well as the specific roles of EHD3 in heart failure. Importantly, EHD3⁺⁻ NCX targeting phenotypes were directly attributable to EHD3 deficiency, and not a compensatory response to functional decompensation, because dysregulated NCX targeting was present from birth, and rhythm defects were observed as early as 4 weeks of age in EHD3⁺⁻ mice (before depressed cardiac function; Online Figures VI and X). Thus, our data support that EHD3 plays roles in targeting proteins involved in both cardiac structural and electric functions.

**Sources of Funding**

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Ion channels and transporters require complex trafficking and retention pathways to modulate cardiac cell excitability.
- In noncardiac cell types, endosome pathways play critical roles in membrane protein trafficking, internalization, and recycling.
- Eps15 homology domain (EHD) family gene products were recently identified in heart, associated with membrane protein trafficking in myocytes, and shown to be altered in multiple forms of heart disease.

**What New Information Does This Article Contribute?**

- Global deficiency of EHD3 in mice results causes in vivo defects in cardiac structure and electric function including chamber dilation, reduced ejection fraction, bradycardia, and conduction defects.
- EHD3-deficient myocytes display defects in ion channel and transporter expression, membrane localization, and function. These defects are associated with loss of the membrane adapter protein ankyrin-B.
- Mice that selectively lack EHD3 in cardiomyocytes show similar cardiac phenotypes of global EHD3-deficient mice, demonstrating a key role of the EHD3-based endosome pathway in cardiovascular physiology.

Cardiac excitability is governed by the synchronized activities of a host of membrane-bound ion channels, transporters, and receptors. Although the field has gained significant insight into the pathways governing membrane protein structure and biochemical function in health and disease, little is known about the cellular mechanisms that regulate membrane protein expression, trafficking, and internalization at baseline or in response to acute or chronic stress. Because these mechanisms are fundamental for cardiac structural and electric remodeling in heart failure, new in vivo studies focused on these pathways are essential for generating new therapeutic targets for disease. Our results using new in vivo models demonstrate key roles for the cardiac endosome-based system for membrane protein targeting. Furthermore, these data show that lack of the endosome EHD3 protein results in both cardiac structural and electrical phenotypes. Together, our findings provide new in vivo data that link endosome-based intracellular protein trafficking pathways with the expression, membrane targeting, and function of key cardiac membrane proteins. Because altered EHD3 levels have been previously linked with multiple forms of heart failure, our findings may help identify new markers and therapeutic avenues for the diagnosis and treatment of cardiovascular disease.
EHD3-Dependent Endosome Pathway Regulates Cardiac Membrane Excitability and Physiology
Jerry Curran, Michael A. Makara, Sean C. Little, Hassan Musa, Bin Liu, Xiangqiong Wu, Iuliia Polina, Joseph S. Alecsan, Patrick Wright, Jingdong Li, George E. Billman, Penelope A. Boyden, Sandor Gyorke, Hamid Band, Thomas J. Hund and Peter J. Mohler

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Supplemental Material

Methods

**Mathematical modeling and sensitivity analysis** Sensitivity analysis was performed, as described\(^1\) on a mathematical model of the mouse ventricular myocyte action potential and calcium transient to identify likely candidates for observed changes in AP and SR Ca\(^{2+}\) load\(^2\). Briefly, maximal conductances of sarcolemmal ion channels/transporters in the model were perturbed one parameter at a time +15% and -15%. Action potential duration at 90% repolarization (APD\(_{90}\)) and maximal diastolic Ca\(^{2+}\) concentration in the junctional sarcoplasmic reticulum ([Ca\(^{2+}\)]\(_{JSR}\)) were determined following steady-state (change in APD < 0.1%) pacing at a cycle length of 500 ms. For each property (X) and parameter (p), sensitivity was calculated according to equation [1] and expressed relative to maximal value for all parameters.

\[
S_{X,p} = \frac{X_{p,+15\%} - X_{p,-15\%}}{0.3X_{\text{con}}} \quad [1]
\]

**Generation of EHD3\(^{-/-}\) mouse.** LoxP sequences were inserted to flank exon 1. Cre/loxP-mediated recombination resulted in exon deletion. DNA was isolated from tail clips of 10 day old mice, and mice were genotyped by PCR. Three primers in a single duplex PCR reaction amplified the WT allele (377 bp) and the deleted allele (488 bp). Primers were as follows: 5’ CAA CAA GAG TGT CAG GAA ACC TGA ACT A-3’; 5’-CTG GGA AAC TGC AGA ACA TCA GGG AAC A-3’; 5’-ATG AGG GAC TCA AGG GGC AAG TCC TGG A-3’. PCR products were separated by agarose gel electrophoresis and imaged on a BioRad ChemiDoc XRS+ (BioRad, Hercules, CA). EHD3\(^{-/-}\) deficiency was confirmed by immunoblot using EHD3-specific antibodies.
**Echocardiography.** Transthoracic echocardiogram was performed on WT and EHD3<sup>−/−</sup> mice to measure the in vivo function of the heart using the Vevo 2100 (Visualsonics, Toronto, Ontario, Canada). The mice were anesthetized using 2.0 % isoflurane in 95% O<sub>2</sub> / 5% CO<sub>2</sub> at a rate of ~0.8 L/min. Anesthesia was maintained by administration of oxygen and ~1% isoflurane. Electrode gel was placed on the EKG sensors of the heated platform and the mouse was placed supine on the platform to monitor electrical activity of heart. A temperature probe was inserted into the rectum of the mouse to monitor core temperature of ~37°C. The MS-400 transducer was used to collect the contractile parameters of the heart in the short axis M-mode. Heart rates were continuously monitored, and echocardiography data acquired at heart rates below 450 bpm was excluded.

**Electrophysiology.** Membrane currents were assessed by use of an Axopatch-200B amplifier and a CV-203BU head stage (Axon Instruments). Experimental control, data acquisition, and data analysis were accomplished with the use of software package PClamp 10 with the Digidata 1440A acquisition system (Axon Instruments). Patch pipettes were pulled from thin-walled glass capillary tubes (Sutter Instruments). The electrode resistance ranged from 2 to 4 MΩ. The external solution contained the following (in mmol/L): NaCl, 145; CsCl, 4; MgCl<sub>2</sub>, 1; CaCl<sub>2</sub>, 2; HEPES, 5; glucose, 10 (pH 7.4, adjusting with NaOH). Ouabain (0.02 mmol/L), nifedipine (0.01 mmol/L) and niflumic acid (0.02 mmol/L) were added to the solution. The internal solution contained the following (in mmol/L): CsCl, 65; NaCl, 10; MgCl<sub>2</sub>, 4; CaCl<sub>2</sub>, 6; tetraethyl ammonium chloride, 20; HEPES, 10; Na<sub>2</sub>ATP, 5; EGTA, 11 (pH 7.2, adjusting with CsOH). Membrane currents were elicited with the use of standard voltage ramp protocol. From a holding potential of -40 mV, a 100-ms step depolarization to +50 mV was followed by a descending voltage ramp (from +50 mV to -110 mV at 100 mV/s). The protocol was applied every 12 seconds. I<sub>NCX</sub> was measured as the Ni-sensitive current. Ni<sup>2+</sup> (5 mmol/L) was added to define the fraction of current that derives from NCX (the difference between total current and post-Ni<sup>2+</sup> current). Membrane
capacitance was read directly from the membrane test of Pclamp10 before compensating for series resistance and membrane capacitance. For \( I_{\text{Ca,L}} \) measurements electrodes were fashioned from borosilicate glass capillaries (World Precision Instruments) and were filled with an internal solution containing: 150 cesium methane sulfonate (CsMeSO₃), 5 CsCl, 10 HEPES, 10 EGTA, 1 MgCl₂, and 4 MgATP (pH to 7.2 with NaOH). Pipettes typically had a resistance of 3-4 MΩ before series compensation. To form gigaohm seals and for initial break-in to the whole-cell configuration, cells were perfused with normal Tyrode solution: 138 NaCl, 4 KCl, 2 CaCl₂, 1 MgCl₂, 0.33 NaH₂PO₄, 10 HEPES, 1.8 CaCl₂, 0.5 MgCl₂, 25 CsCl (pH adjusted to 7.4 with NMG). Data traces were acquired at a repetition interval of 2 s from -70 to +50 mV with a holding potential of -80 mV.

**SR Ca load and Ca spark measurements.** Briefly, myocytes were loaded with Ca-dependent fluorescent dye, fluo-4 AM (10 µM), for 30 min at RT. Myocytes were electrically field stimulated at 0.5 Hz for at least 5 min before data acquisition to assure steady-state Ca handling. Stimulation occurred in Tyrode solution (in mM): 140 NaCl, 4 KCl, 1 MgCl₂, 10 glucose, 5 HEPES, 1 CaCl₂ (pH adjusted to 7.4 with NaOH). A rapid solution switch to 0 Na/0 Ca NT solution + 10 mM caffeine (140 LiCl substituted for NaCl) was applied for 2 s to empty the SR of Ca. The difference between basal and peak cytosolic \([\text{Ca}^{2+}]\) in the presence of caffeine is considered the total SR \([\text{Ca}^{2+}]\). \([\text{Ca}]_{\text{SRT}}\) was calculated through the pseudo-ratio as previously described³ with \([\text{Ca}]_{\text{d}}\) assumed to be 120 nM for all mice. through the pseudo-ratio as previously described³ with \([\text{Ca}]_{\text{d}}\) assumed to be 120 nM for all mice. Ca sparks measurement were acquired in intact ventricular myocytes loaded with 10 µM fluo-3 AM for 30 min. at RT. Imaging was performed using an Olympus Fluoview 1000 confocal microscope equipped with a 60X 1.4 N.A. objective in line-scan mode at a rate of 2 or 5 ms per line. Fluo-3 was excited by 488-nm line of an argon-ion laser, and fluorescence was acquired at wavelengths 500-530 nm. Ca sparks were analyzed using IDL software as previously described.⁴
**Surface Receptor Density.** Surface membrane density assay of the β-AR was adapted from Limas et al. and Yonemochi et al.\(^5,6\) Isolated ventricular myocytes from both EHD3\(^{−/−}\) and WT mice were isolated as described. The resulting myocyte suspension was divided into two equal aliquots were washed in ice cold assay solution (in mM: 137 NaCl; 5.4 KCl, 4 KCl; 0.16 NaH\(_2\)PO\(_4\); 3 NaHCO\(_3\) 20 HEPES, 10 glucose; supplemented with 650 mg taurine, pH 7.4). One aliquot was used to assess β-AR specific signal; the second was used to assess non-specific radioligand binding (background). To minimize the potential for surface receptor internalization, myocytes were kept on ice for the duration of the assay. Intact myocytes were counted by hemocytometer and a volume equal ~100,000 myocytes was added to a 5 mL BD Falcon tube. Sample was volume up to 500 µL with assay buffer. Radiolabeled β-AR specific antagonist \(^3\)H-CGP-12177 (Perkin Elmer) was then added to each tube at a final concentration of 15 nM. Samples were incubated for 16 h at 4° C. To terminate radioligand binding, 4.5 mL of ice cold assay buffer was added the samples. The samples were filtered over a Whatman GF/C filter and washed three times with ice cold assay buffer. Radioligand binding was then quantified on a scintillation counter. To account for non-specific binding of \(^3\)H-CGP-12177 to cell membranes, simultaneous experiments were conducted with the addition of 10 µM propranolol, a general β-AR agonist. The signal acquired in the presence of \(^3\)H-CGP-12177 and propranolol was considered the general non-β-AR-specific signal (or background) and was subtracted from all scintillation counts. All experiments for all treatments were conducted in triplicate and averaged.
Supplemental References


Online Figure I. Whole-cell recordings were obtained at RT with the use of standard patch-clamp techniques, and cells from the WT and EHD3<sup>−/−</sup> groups had a similar membrane capacitance.
Online Figure II

EHD3−/− mice display normal PR and QT interval. Radiotelemetry in conscious, ambulatory mice reveal no differences in A) average PR interval (WT = 35.4 ± 0.5, EHD3−/− = 35.8 ± 0.4,) or B) average QRS complex duration (WT = 12.0 ± 0.56, EHD3−/− = 10.9 ± 0.38 ms, n = 5 mice each).
**Online Figure III.** *EHD3*−/− mice display altered β-AR-dependent signaling. (A) Maximum heart rate achieved after low (left) or high dose (right) Iso injection in conscious, ambulatory WT and EHD3−/− mice. (B) Duration of Iso response (time from peak HR to return to baseline) after low dose (left) or high dose (right) Iso injection in conscious, ambulatory mice. (C) Baseline heart rate in anesthetized mice before Iso injection. (D) Change in heart rate after Iso injection in anesthetized mice is blunted in EHD3−/− mice. For A-D, n=7 WT and n=8 EHD3−/− mice, p<0.05. (E-F) Both β1-AR and β2-AR protein expression are increased EHD3−/− mice (n=5 mouse myocyte preps/genotype; p<0.05). Representative immunoblots for E and F are shown in (G-H). (I) β1-AR surface receptor density was no different between WT and EHD3−/− mice (n=5 mouse myocyte preps/genotype). (J) Immunostaining of the β1-AR receptor in WT (left) and EHD3−/− myocytes (right). EHD3−/− myocytes display no overt difference in membrane protein localization, but show significant increase in perinuclear staining compared with WT myocytes.
Online Figure IV. Sensitivity analysis to define likely candidates for decreased APD and increased SR Ca\(^{2+}\) load in EHD3\(^{-/-}\) myocytes. Relative sensitivities of steady-state (A) APD\(_{90}\) and (B) [Ca\(^{2+}\)]\(_{JSR}\) to maximal conductances of sarcolemmal ion channels, transporters and pumps in a mathematical model of the mouse ventricular AP. The L-type Ca\(^{2+}\) channel (\(I_{CaL}\)) and Na\(^{+}/Ca^{2+}\) exchanger (\(I_{NCX}\)) had the greatest influence on APD\(_{90}\) and [Ca\(^{2+}\)]\(_{JSR}\), respectively, in a manner consistent with observed defects (e.g. due to loss of membrane targeting would produce change in property consistent with observed defects in EHD\(^{-/-}\), designated by green bars). Abbreviations are as follows: \(I_{CaL}\) - L-type Ca\(^{2+}\) channel; \(I_{Cab}\) - Background Ca\(^{2+}\) current; \(I_{K1}\) - Inward rectifier K\(^{+}\) current; \(I_{KSS}\) - Non-inactivating steady-state K\(^{+}\) current; \(I_{Kur}\) - Ultrarapid delayed rectifier K\(^{+}\) current; \(I_{Na}\) - Fast Na\(^{+}\) current; \(I_{Nab}\) - Background Na\(^{+}\) current; \(I_{NaK}\) - Na\(^{+}/K^{+}\) ATPase; \(I_{NCX}\) - Na\(^{+}/Ca^{2+}\) exchanger; \(I_{ns}\) - Nonspecific leak current; \(I_{pCa}\) - Sarcolemmal Ca\(^{2+}\) pump; \(I_{io}\) - Transient outward K\(^{+}\) current.
Online Figure V. Summary data immunoblots assessing phospholamban, calsequestrin, and SERCA2a in whole heart lysates isolated from WT and EHD3⁻/⁻ mice (n=5 each). Representative immunoblots showing protein of interest (top) and loading control (actin, bottom) are shown for each data set. Space between lanes denotes that data were collected from noncontiguous lanes of the same gel.
Online Figure VI. Immunostaining of alpha-actinin and NCX in WT (A-C) and EHD3-/- (D-F) post-natal day 1 primary cardiomyocytes. Note peri-nuclear localization of NCX in EHD3-/- myocytes. Bar equals 10 microns.
Online Figure VII. Loss of ankyrin-B in EHD3−/− myocytes is rescued by viral transduction of GFP-EHD3. (A) Non-transduced EHD3−/− myocytes show lack of GFP signal as well as reduced ankyrin-B immunostaining. (B) GFP-EHD3 transduction results in peri-nuclear distribution of EHD3 as well as rescue of ankyrin-B expression. Bar equals 10 microns.
Online Figure VIII.

**A)** EHD1 is increased 30% in EHD3−/− hearts.

**B)** EHD4 expression is decreased by 31% in EHD3−/− hearts (n = 9 WT; 8 EHD3−/−, p < 0.05).

*Online Figure VIII. EHD1 and EHD4 are differentially expressed in EHD3−/− mice. A) EHD1 is increased 30% in EHD3−/− hearts. B) EHD4 expression is decreased by 31% in EHD3−/− hearts (n = 9 WT; 8 EHD3−/−, p < 0.05).*
Online Figure IX. *EHD3 is expressed in sinoatrial node.*  

**A)** EHD3 is expressed in mouse sinoatrial node cell lysates with SAN marker HCN4.  

**B)** EHD3 is expressed in canine sinoatrial node lysates with SAN marker HCN4.  

**C)** Control experiment to validate specificity of EHD3 antibody on EHD3-/- ventricular lysates.
Online Figure X. Sinus node dysfunction in 4 week old EHD3$^{-/-}$ mouse. SAN dysfunction was not observed in WT mice.
**Online Table I.** Echocardiographic parameters of WT and EHD3⁻/⁻ mice. In all, seven WT and 6 EHD3⁻/⁻ mice were assessed at 8 weeks of age. (*p<0.05 vs. WT).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT hearts 8 weeks (n=5)</th>
<th>EHD3⁻/⁻ hearts 8 weeks (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>3.72 ± 0.09</td>
<td>4.05 ± 0.09*</td>
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<tr>
<td>LV systolic diameter (mm)</td>
<td>2.4 ± 0.11</td>
<td>2.95 ± 0.08*</td>
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<tr>
<td>LV diastolic volume (µL)</td>
<td>58.17 ± 3.52</td>
<td>72.60 ± 3.83*</td>
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<tr>
<td>LV systolic volume (µL)</td>
<td>20.51 ± 2.46</td>
<td>33.91 ± 2.28*</td>
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<tr>
<td>Anterior diastolic wall thickness (mm)</td>
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<td>0.74 ± 0.02</td>
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<td>1.09 ± 0.02</td>
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<tr>
<td>Posterior diastolic wall thickness (mm)</td>
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<td>0.63 ± 0.02</td>
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<tr>
<td>Posterior systolic wall thickness (mm)</td>
<td>1.06 ± 0.04</td>
<td>0.89 ± 0.03*</td>
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<tr>
<td>Stroke Volume (µL)</td>
<td>38.47 ± 3.27</td>
<td>38.69 ± 1.89</td>
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<tr>
<td>Ejection Fraction</td>
<td>65.41 ± 2.14</td>
<td>53.59 ± 1.34*</td>
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<tr>
<td>Fractional Shortening</td>
<td>35.4 ± 1.23</td>
<td>27.34 ± 0.86*</td>
</tr>
<tr>
<td>Heart weigh/Body weight (mg/g)</td>
<td>6.79 ± 0.21</td>
<td>7.98 ± 0.26*</td>
</tr>
</tbody>
</table>
### Online Table II

Echocardiographic parameters of WT and EHD3<sup>−/−</sup> mice assessed at 4 weeks of age. (*p<0.05 vs. WT).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT hearts 4 weeks (n=5)</th>
<th>EHD3&lt;sup&gt;−/−&lt;/sup&gt; hearts 4 weeks (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV diastolic diameter (mm)</td>
<td>3.38 ± 0.03</td>
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<td>LV systolic diameter (mm)</td>
<td>2.20 ± 0.05</td>
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<tr>
<td>LV diastolic volume (µL)</td>
<td>46.7 ± 1.04</td>
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<tr>
<td>LV systolic volume (µL)</td>
<td>16.35 ± 0.89</td>
<td>25.15 ± 2.26*</td>
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<tr>
<td>Anterior diastolic wall thickness (mm)</td>
<td>0.7 ± 0.03</td>
<td>0.75 ± 0.02</td>
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<tr>
<td>Anterior systolic wall thickness (mm)</td>
<td>1.03 ± 0.03</td>
<td>1.16 ± 0.02*</td>
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<tr>
<td>Posterior diastolic wall thickness (mm)</td>
<td>0.66 ± 0.04</td>
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<td>Posterior systolic wall thickness (mm)</td>
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<tr>
<td>Stroke Volume (µL)</td>
<td>30.35 ± 0.68</td>
<td>35.55 ± 0.99*</td>
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<td>58.89 ± 2.73</td>
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<tr>
<td>Parameter</td>
<td>WT hearts 8 weeks (n=5)</td>
<td>EHD3⁻/⁻ MHC 8 weeks (n=5)</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>-------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
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<td>4.46 ± 0.13*</td>
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<tr>
<td>LV systolic diameter (mm)</td>
<td>2.4 ± 0.11</td>
<td>3.4 ± 0.09*</td>
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<tr>
<td>LV diastolic volume (μL)</td>
<td>58.17 ± 3.52</td>
<td>90.90 ± 6.33*</td>
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<td>LV systolic volume (μL)</td>
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<td>47.67 ± 2.98*</td>
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<tr>
<td>Anterior diastolic wall thickness (mm)</td>
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<td>0.9 ± 0.06</td>
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<tr>
<td>Anterior systolic wall thickness (mm)</td>
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<td>Posterior diastolic wall thickness (mm)</td>
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<td>0.66 ± 0.03</td>
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<tr>
<td>Posterior systolic wall thickness (mm)</td>
<td>1.06 ± 0.04</td>
<td>0.91 ± 0.07</td>
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<tr>
<td>Stroke Volume (μL)</td>
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<td>43.23±4.96</td>
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<tr>
<td>Ejection Fraction</td>
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<tr>
<td>Fractional Shortening</td>
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<td>23.7±1.71*</td>
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<td>Heart weigh/Body weight (mg/g)</td>
<td>6.79 ± 0.21</td>
<td>8.22±0.21*</td>
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**Online Table III.** Echocardiographic parameters of WT and EHD3⁻/⁻ cKO mice assessed at 8 weeks of age. (*p<0.05 vs. WT).