Binding of LncRNA-DACH1 to dystrophin impairs the membrane trafficking of Nav1.5 protein and increases ventricular arrhythmia susceptibility

Zhenwei Pan (✉ panzw@ems.hrbmu.edu.cn)

Harbin Medical University <https://orcid.org/0000-0002-1011-0954>

Gen-Long Xue

Harbin Medical University

Yang Zhang

Harbin Medical University <https://orcid.org/0000-0002-6680-3098>

Jiming Yang

Harbin Medical University

Ying Yang

Harbin Medical University

Ruixin Zhang

Harbin Medical University

Desheng Li

Harbin Medical University

Tao Tian

Harbin Medical University

Xiaofang Zhang

Harbin Medical University <https://orcid.org/0000-0001-9866-2018>

Changzhu Li

Harbin Medical University

Xingda Li

Harbin Medical University

Jiqin Yang

Harbin Medical University

Kewei Shen

Harbin Medical University

Yang Guo

Harbin Medical University

Xuening Liu

Harbin Medical University

Guohui Yang

Harbin Medical University

Yanjie Lu

Harbin Medical University

Baofeng Yang

Harbin Medical University <https://orcid.org/0000-0002-0125-1608>

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Abstract

Dystrophin is a critical interacting protein of Nav1.5 that determines its membrane anchoring in cardiomyocytes. The study aims to explore whether lncRNA-DACH1(lncDACH1) can regulate the distribution of Nav1.5 by binding to dystrophin and participate in ventricular arrhythmogenesis. lncDACH1 was confirmed to bind to dystrophin. Cardiomyocyte-specific transgenic overexpression of lncDACH1(lncDACH1-TG) reduced the membrane distribution of dystrophin and Nav1.5 in cardiomyocytes. The opposite data were collected from lncDACH1 cardiomyocyte conditional knockout (lncDACH1-CKO) mice. Moreover, increased ventricular arrhythmia susceptibility was observed in lncDACH1-TG mice *in vivo* and *ex vivo*. The conservative fragment of lncDACH1 inhibited membrane distribution of dystrophin and Nav1.5 and promoted the inducibility of ventricular arrhythmia. Upregulation of dystrophin in lncDACH1-TG mice rescued the impaired membrane distribution of dystrophin and Nav1.5. The human homologue of lncDACH1 inhibited the membrane distribution of Nav1.5 in human iPS-differentiated cardiomyocytes. Collectively, lncDACH1 regulates Nav1.5 membrane distribution by binding to dystrophin and participates in ventricular arrhythmogenesis.

Introduction

The voltage gated sodium channel mediates the 0 phase depolarizing inward sodium currents of cardiomyocytes¹. The expression and function of sodium channel is regulated at multiple levels encompassing gene mutation, post-transcriptional modification, post-translational modification and protein trafficking etc^{1,2}. The disruption of either process is arrhythmogenic and occasionally causes sudden death^{2,3}.

The membrane targeting and localization of pore-forming subunit of sodium channel Nav1.5 was regulated by several interacting proteins such as ankyrin-G, MOG1, syntrophin and dystrophin etc^{2,4}. Dystrophin is a cytoplasmic protein that is encoded by duchenne muscular dystrophy (DMD) gene⁵. It distributes mainly on the cellular membrane of skeletal muscle cells and cardiomyocytes, and acts as a scaffold for Nav1.5⁶. In cardiomyocytes, dystrophin controls the expression and membrane anchoring of Nav1.5. Gavillet et al. showed that knockout of dystrophin in cardiomyocytes reduced peak sodium current, Nav1.5 protein expression and conduction velocity in mice⁶. Subsequently, they confirmed that knockout of dystrophin inhibits membrane distribution of Nav1.5 due to the disruption of dystrophin-syntrophin complex⁷.

Long noncoding RNAs (lncRNAs) are a new class of RNAs that are more than 200 nts long and possess little protein coding property.⁸ lncRNAs have been shown to regulate multiple biological processes and participate in the pathogenesis of various diseases including cardiac diseases⁹. lncRNAs were shown to regulate cardiac electrophysiological property by altering the function of different ion channels. For example, the increased expression of lncRNA-Kcna2as in heart failure reduced I_{ks} and prolonged action potential duration (APD)¹⁰. lncRNA-MALAT1 enhanced arrhythmia susceptibility by suppressing I_{to} and

prolonging APD¹¹. In a previous study, we found that lncRNA-CCRR (cardiac conduction regulatory) interacts with connexin-43 interacting protein 85(CIP85) to promote connexin-43 membrane distribution and improve the impaired cardiac conduction of failing hearts¹². However, to date, it remains unknown whether and how lncRNA regulates sodium channel.

lncDACH1 is an intronic lncRNA located on the first intron of *DACH1* gene¹³. We previously showed that lncDACH1 impairs cardiac function by promoting the degradation of sarco-endoplasmic reticulum ATPase 2a (SERCA2a), and exacerbates cardiac ischemia injury by inhibiting Yes-associated protein 1(YAP1) mediated proliferation of neonatal cardiomyocytes^{13, 14}. During analyzing the interacting proteins of lncDACH1 identified by mass spectrometry, we found that dystrophin is one of them, which drove us to hypothesize that lncDACH1 may be a critical regulator of sodium channel Nav1.5.

Therefore, in this study we explored whether lncDACH1 regulates Nav1.5 by interacting with dystrophin. We found that lncDACH1 inhibited the membrane trafficking of Nav1.5 by binding to dystrophin, which led to reduced sodium current and increased ventricular arrhythmia susceptibility. The study highlights a novel mechanism for the regulation of sodium channel trafficking, and reveals a potential therapeutic target for sodium channel dysfunction related cardiac arrhythmias.

Results

lncDACH1 binds to dystrophin and reduces Nav1.5 membrane distribution in cardiomyocytes of lncDACH1 transgenic mice

We firstly validated the binding between lncDACH1 and dystrophin. The RNA pulldown plus immunoblot assay confirmed that lncDACH1 can successfully pulldown dystrophin (Figure 1A). Conversely, the immunoprecipitation study showed that the antibody for dystrophin precipitated lncDACH1, while the negative control IgG did not (Figure 1B).

We next explored the influence of lncDACH1 on cellular distribution of dystrophin. The western blot data showed that the total protein of dystrophin did not change, while the membrane fraction was reduced, and the cytoplasmic fraction increased in the hearts of lncDACH1-TG mice than wild-type (WT) controls (Figure 1C). Consistently, the membrane and total protein levels of Nav1.5 were reduced, while cytoplasmic Nav1.5 increased in the hearts of lncDACH1-TG mice than WT controls (Figure 1D). The reduced membrane distribution of dystrophin and Nav1.5 in the cardiomyocytes of lncDACH1-Tg mice was further confirmed by immunofluorescent staining (Figure 1E). The mRNA levels of dystrophin and *SCN5A* did not change (Figure 1F). We then evaluated the functional change of sodium channel. Consistent with reduction of membrane Nav1.5, the peak I_{Na} was significantly decreased in the ventricular myocytes of lncDACH1-TG mice than WT controls, while the kinetics of I_{Na} (activation, inactivation, and recovery) did not change (Figure 1G). Meanwhile, the conduction velocity was slower in the hearts of lncDACH1-TG than WT mice (Figure 1H).

We then applied IncDACH1 adenovirus to cultured neonatal cardiomyocytes to confirm the regulation of IncDACH1 on sodium channel *in vitro*. Infection of adenovirus carrying IncDACH1 significantly upregulated the level of IncDACH1 (Figure 2A) and remarkably inhibited peak I_{Na} with no change in kinetics (Figure 2B, C). The membrane distribution of dystrophin and Nav1.5 was remarkably reduced by overexpression of IncDACH1 as indicated by immunofluorescent staining (Figure 2D). The mRNA levels of dystrophin and SCN5A were not altered by IncDACH1 overexpression (Figure 2E).

Inhibition of IncDACH1 in cardiomyocytes increased membrane Nav1.5 distribution

We then employed IncDACH1 cardiomyocyte conditional knockout(IncDACH1-CKO) mice to examine the regulation of IncDACH1 on dystrophin and Nav1.5. The western blot data showed that membrane distribution of dystrophin was increased in the hearts of IncDACH1-CKO mice than WT controls, while the total dystrophin protein and dystrophin mRNA did not change (Figure 3A). Consistently, the membrane and total level of Nav1.5 was increased in the hearts of IncDACH1-CKO mice than WT controls, with no change on SCN5A mRNA (Figure 3B). The change on dystrophin and Nav1.5 membrane distribution was further validated by immunofluorescent staining (Figure 3C). Meanwhile, the peak I_{Na} was larger in cardiomyocytes of IncDACH1-CKO mice than WT controls, while the kinetics of I_{Na} (activation, inactivation, and recovery) did not change (Figure 3D). Consistent with the increase of peak I_{Na} , the conduction velocity in the hearts of IncDACH1-CKO mice was faster than WT controls (Figure 3E).

We further confirmed effects of IncDACH1 knockdown with its siRNAs on sodium channel in cultured neonatal cardiomyocytes *in vitro*. Infection of adenovirus carrying siRNAs for IncDACH1 significantly reduced the level of IncDACH1 (Figure 4A). The patch-clamp recordings showed that knockdown of IncDACH1 significantly increased the current density of peak I_{Na} with no change on channel kinetics (Figure 4B, C). Membrane distribution of dystrophin and Nav1.5 were both increased after knockdown of IncDACH1 as indicated by immunofluorescent staining (Figure 4D). The mRNA levels of dystrophin and SCN5A were not altered by IncDACH1 knockdown (Figure 4E).

Transgenic overexpression of IncDACH1 is arrhythmogenic in mice

We next evaluated whether the inhibition of Nav1.5 by IncDACH1 is arrhythmogenic in IncDACH1-TG mice. Electrical pacing technique was employed to evaluate the arrhythmia susceptibility of intact hearts *in vivo* and isolated hearts *ex vivo*. The *in vivo* study showed that programmed pacing induced more ventricular arrhythmia in IncDACH1-TG mice than WT controls. Both induction rate and episodes of ventricular arrhythmia were higher in IncDACH1-TG mice (Figure 5A). In consistent, the *ex vivo* electrical pacing study demonstrated that ventricular arrhythmia was more frequently occurred in IncDACH1-Tg mice (Figure 5B). The optimal mapping study revealed that there are more breaking points in the perfused heart of IncDACH1-TG mice than WT controls (Figure 5C-E). Conversely, no ventricular arrhythmia was induced in the hearts of IncDACH1-CKO mice (Figure 5F).

The conservative fragment of IncDACH1 reduced peak sodium current and promoted ventricular arrhythmia

The sequence blasting data showed that the fragment of IncDACH1 from 835 to 2085 nts is conservative between human and mouse. We then cut IncDACH1 into different fragments (Figure 6A) to evaluate the sequence that is responsible for the binding with dystrophin. The data showed that only fragments containing the conserved sequence, fragments a and b, can pulldown dystrophin, indicating that the conserved sequence may be the functional region of IncDACH1 (Figure 6A).

We then examined the influence of the conserved sequence from 835 to 2085 nts (conserved fragment of IncDACH1, cF-IncDACH1) on cardiac sodium channel. The adenovirus carrying cF-IncDACH1 was constructed and administered to mice. The successful overexpression of cF-IncDACH1 was validated by qRT-PCR (Figure 6B). Administration of cF-IncDACH1 reduced the membrane distribution, and increased cytoplasmic expression of both dystrophin and Nav1.5 as indicated by western blot and immunofluorescent data (Figure 6C, D). The mRNA levels of dystrophin and Nav1.5 were not affected by cF-IncDACH1 (Supplemental Figure 1A). Overexpression of cF-IncDACH1 reduced peak I_{Na} currents (Figure 6E), and produced no influence on channel kinetics (Supplemental Figure 1B). The optical mapping data showed that administration of cF-IncDACH1 reduced conduction velocity and increased break points of ventricular arrhythmias (Figure 6F, G). The induction rate and episodes of ventricular tachycardia (VT) were higher in cF-IncDACH1 group than controls (Figure 6H).

In cultured neonatal cardiomyocytes, overexpression of cF-IncDACH1 reduced peak I_{Na} with no change in kinetics, inhibited membrane distribution of dystrophin and Nav1.5, and produced no influence on the mRNA levels of dystrophin and SCN5A (Supplemental Figure 2).

Activation of dystrophin transcription by dCas9-SAM system prevented the reduction of sodium current in IncDACH1 transgenic mice

As IncDACH1 reduced Nav1.5 membrane targeting by interacting with dystrophin, we reasoned that overexpression of dystrophin may rescue the inhibition of Nav1.5 by IncDACH1. To test this notion, we constructed the AAV9 virus carrying dCas9-SAM system that can activate dystrophin transcription (AAV9-Dys-Act) to perform rescuing experiments on IncDACH1-TG mice (Supplemental Figure 3). Tail vein injection of AAV9-Dys-Act virus significantly increased the mRNA level of dystrophin in the hearts of both WT and IncDACH1-TG mice (Figure 7A). The western blot data showed that overexpression of dystrophin with AAV9-Dys-Act virus increased both total and membrane protein expression of dystrophin, and rescued the reduction of dystrophin expression in IncDACH1-Tg mice (Figure 7A). The mRNA level of SCN5A was not influenced by AAV9-Dys-Act virus (Figure 7B). Strikingly, AAV9-Dys-Act virus administration restored total and membrane expression of Nav1.5 in IncDACH1-TG mice (Figure 7B). In addition, AAV9-Dys-Act virus injection rescued the reduction of peak I_{Na} current in IncDACH1-TG mice (Figure 7C). The kinetics of I_{Na} current did not change among groups (Figure 7C). Activation of dystrophin transcription with AAV9-Dys-Act virus restored the conduction velocity in IncDACH1-TG mice (Figure 7D).

Both *in vivo* and *ex vivo* data indicated that activation of dystrophin transcription reduced the susceptibility to ventricular arrhythmia of lncDACH1-TG mice (Figure 7E, F).

Hadhb binds to lncDACH1 and promotes its decay

Reduced Nav1.5 expression and reduction of peak I_{Na} in heart failure have been reported by a bunch of studies^{15, 16, 17, 18}. We therefore evaluated the contribution of lncDACH1 on sodium channel remodeling in transaortic constriction (TAC) induced heart failure model in mice. We found that lncDACH1 was increased in failing hearts than sham controls (Figure 8A). Although lncDACH1 was upregulated in failing hearts, the mRNA of its host gene DACH1 did not change (Figure 8B). This finding excluded the transcription related mechanism on lncDACH1 upregulation during heart failure. By analyzing the RNA Pulldown plus Mass Spectrometry data, we identified three potential interacting proteins of lncDACH1 that have been shown to regulate RNA stability. They are ANP32a (acidic leucine-rich nuclear phosphoprotein 32A), eIF4A1 (eukaryotic initiation factor 4A1) and hydroxyacyl-CoA dehydrogenase subunit beta (hadhb). We therefore speculated that it may be the change of RNA stability that renders to the expression change of lncDACH1. We then tested whether these proteins can affect lncDACH1 level by knocking down their expression with siRNA. The data showed that knockdown of hadhb increased the expression of lncDACH1, while knockdown of ANP32a and eIF4A1 produced no influence (Figure 8C). The influence of hadhb on lncDACH1 stability was further validated by the fact that knockdown of hadhb increased the decaying half-life of lncDACH1 (Figure 8D). Furthermore, the sense sequence of lncDACH1 successfully pulled down hadhb, and the antibody of hadhb precipitated lncDACH1 (Figure 8E). Additionally, the protein level of hadhb was reduced in mouse failing hearts (Figure 8F), which is inversely correlated to the upregulation of lncDACH1. The siRNA for hadhb reduced the expression of Nav1.5 (Figure 8G). These data indicated that hadhb is an upstream regulator of lncDACH1 which determines the stability of lncDACH1.

We lastly evaluated the human conserved sequence of lncDACH1 (hcF-lncDACH1) on Nav1.5 distribution of human iPS induced cardiomyocytes. We found that overexpression of hcF-lncDACH1 reduced the membrane distribution of dystrophin and Nav1.5 (Figure 8H).

Discussion

In this study, we discovered that lncDACH1 is critical regulator of sodium channel in the heart. lncDACH1 binds to dystrophin and thus inhibits membrane trafficking of Nav1.5, which leads to the reduction of peak sodium current and impairment of cardiac conduction. Therefore, upregulation of lncDACH1 increased the susceptibility to ventricular arrhythmia (Fig 8I).

lncRNAs have been established to be critical regulators of various biological processes¹⁹. The action modes of lncRNAs are complex. One major mechanism for them to exert their biological function in the cytoplasm is to interact with the molecules such as proteins, miRNAs, mRNAs to alter protein translation, enzyme activity, protein degradation, etc²⁰. For instance, lncRNA-CCRR was shown to inhibit the

endocytic trafficking of connexin-43 by binding to CIP85¹². LncDACH1 mainly distributes in the cytoplasm, and can bind to SERCA2a to promote its ubiquitination and degradation¹³. LncDACH1 can also bind to protein phosphatase 1 catalytic subunit alpha (PP1A) to inhibit its dephosphorylation activity on yes-associated protein 1 (YAP1), leading to the cytoplasmic sequestration of YAP1¹⁴. The unraveling of the molecular mechanism of Nav1.5 expression and function is critical for the insightful understanding of sodium channel function under physiological and pathological conditions. Several interacting proteins have been demonstrated to determine membrane distribution of Nav1.5 and sodium channel function⁴. Dystrophin is a well-characterized Nav1.5 partner protein. It indirectly interacts with Nav1.5 via syntrophin, which binds with the C-terminus of dystrophin and with the SIV motif on the C-terminus of Nav1.5^{6, 21}. In this study, we found that lncDACH1 binds to dystrophin and leads to the impairment of Nav1.5 trafficking and reduced membrane distribution. This is so far the first evidence verifying the regulation of lncRNA on cardiac sodium channel.

Although the membrane distribution of both dystrophin and Nav1.5 was inhibited by lncDACH1, the total protein level of dystrophin was not affected, while Nav1.5 was reduced. The mechanism for the differential influence of lncDACH1 on total protein levels of dystrophin and Nav1.5 is unclear. One explanation may be that Nav1.5 is a membrane channel protein. If they failed to target on the plasma membrane, they may be regarded as unnecessary protein and undergo the process of protein degradation. The E1-E3 enzymes in the ubiquitination systems have been shown to regulate the degradation of Nav1.5, which includes E1 enzyme UBE1 (Ubiquitin-activating Enzyme1), UBA6 (Ubiquitin-like modifier-activating enzyme 6), E2 enzyme, UBC9 (Ubiquitin-Conjugating Enzyme 9), and E3 ligase Nedd4-2 (neuronal precursor cell expressed developmentally downregulated 4-2)^{22, 23, 24}. LITAF (lipopolysaccharide-induced tumor necrosis factor-alpha factor), a protein encoding a regulator of endosomal trafficking, was shown to reduce surface Nav1.5 by promoting degradation of NEDD4-2²⁵. Therefore, cytoplasmic Nav1.5 that failed to target on plasma membrane may be quickly distinguished and then degraded by these ubiquitination enzymes.

The dysfunction of sodium channel is associated with various arrhythmias. Reduced peak I_{Na} due to SCN5A loss-of-function mutation can cause a series of arrhythmias such as atrial fibrillation, Brugada syndrome, long QT syndrome, sudden cardiac death, and ventricular tachycardia etc^{26, 27}. Consistently, we found that accompanied with the reduction of peak I_{Na} , overexpression of lncDACH1 reduced ventricular conduction velocity and increased the susceptibility to ventricular arrhythmia in mice. The increased peak I_{Na} due to SCN5A gain-of-function mutation is associated arrhythmias such as atrial fibrillation, long QT syndrome; polymorphic ventricular complexes and ventricular arrhythmia^{26, 27}. In this study, although the peak I_{Na} increased in lncDACH1-CKO mice, the susceptibility to arrhythmia did not increase. One difference of our data with SCN5A gain-of-function mutation is that the kinetics of peak I_{Na} often change during mutation, while it is not the case of lncDACH1 knockout. One possible explanation may be that the increase of peak I_{Na} has not reach the limit to break the electrophysiological balance of the heart.

Sodium channel remodeling is commonly occurred in cardiac diseases especially heart failure. Despite of some discrepancy, the main observations are that the peak I_{Na} , SCN5A mRNA and Nav1.5 protein are reduced during heart failure of both human patients and animal models^{15,16,17,18}. In this study, we found that lncDACH1 was increased during heart failure, indicating that it may contribute to sodium channel remodeling and arrhythmogenesis during heart failure by interfering the action of dystrophin. Interestingly, we found that activation of dystrophin with dCas9-SAM system restored the membrane distribution of Nav1.5 in cardiomyocytes of lncDACH1-TG mice, which implies its potential in counteracting sodium channel remodeling of patients with heart failure.

The low sequence conservation of lncRNAs among species is a critical issue that limits the extrapolation of data from animal studies to human beings^{28,29}. In this study, we found that the conserved fragment of lncDACH1 exhibits the same effect as lncDACH1 on Nav1.5 trafficking and arrhythmogenesis. Moreover, the human conservative homologous fragment of lncDACH1 can inhibit the membrane distribution of Nav1.5 in cardiomyocytes derived from induced pluripotent stem cells (iPS-CMs). These findings hint the clinical relevance of lncDACH1, which holds the potential to become a therapeutic target for treating sodium channel remodeling in clinic.

In conclusion, lncDACH1 is a novel regulator of sodium channel, which suppresses the membrane trafficking of Nav1.5 by disturbing the function of dystrophin. The current work enriched our understanding of the biology of sodium channel trafficking and function, and indicated that lncRNAs possess the potential to become therapeutic targets for ventricular arrhythmias.

Methods

Animals

Neonatal (within 3-day post born) and adult C57BL/6 mice (8 to 10 weeks old) were provided by the animal center at the Second Affiliated Hospital of Harbin Medical University. Use of animals was approved by the Ethic Committees of Harbin Medical University and conformed 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).

Neonatal cardiomyocytes preparation

Neonatal cardiomyocytes were isolated from 3-day-old mice in accordance with the following procedures. Briefly, after dissection, hearts were washed and minced in 0.25% trypsin. Pooled cell suspensions were centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM Hyclone, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The suspension was incubated in culture flasks for 90 min, which makes fibroblasts preferentially adhere to the bottom of the culture flasks. Neonatal cardiomyocytes were removed from the culture flasks and the medium was changed. Cell cultures were incubated for 48 h at 37 °C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide before any experimentation.

Generation of cardiac myocyte-specific IncDACH1 overexpressing mice

Cardiomyocyte-specific IncDACH1 overexpressing mice driven by murine α MHC promoter on a C57BL/6 background was generated by Biocytogen Co., Ltd (Beijing, China) as demonstrated in previous study¹³.

Generation of cardiomyocyte-specific IncDACH1 knockout mice

IncDACH1 conditional KO mice (IncDACH1 Flox/Flox) was generated by using CRISPR/Cas9 technique on C57BL/6 background mice by Biocytogen Co., Ltd (Beijing, China) as demonstrated in previous study¹³.

Construction of adeno-associated virus 9 (AAV9) carrying deactivated clustered regularly interspaced short palindromic repeats associated protein 9 nuclease- synergistic activation mediator(dCas9-SAM) system to activate the transcription of dystrophin

Adeno-associated virus 9(AAV9) carrying dCas9-SAM system to activate the transcription of dystrophin was constructed as reported previously with brief modifications³⁰. The sgRNA targeting on the promoter region of dystrophin was designed and cloned into the multiple cloning site of plasmid GV639 (EFS-NLS-dSaCas9-NLS-VP64-bGHpA-U6). The constructed plasmid was packaged into AAV9 virus. The sequence of sgRNA is: 5'- CGCTTCCGCGGCCCGTTCAA -3'; The mock-sgRNA target sequence (5'- CGCTTCCGCGGCCCGTTCAA -3') was used as negative control. The obtained AAV9 virus volume was administered into C57BL/6 mice via tail vein injection at 1×10^{11} genome containing particles (GC)/animal in 100 μ l.

Construction of adenovirus carrying cF-IncDACH1 and in vivo gene delivery

Adenovirus vectors carrying cF-IncDACH1(OE- cF-IncDACH1) and a negative control (NC) and a CAG promoter conjugated with green fluorescent protein (GFP) were constructed by Genechem Co., Ltd. (Shanghai, China). OE- cF-IncDACH1, control constructs at 1×10^9 genome containing particles (GC)/animal in 100 μ l volume was administered into C57BL/6 mice with body weights ranging from 18~22g via tail vein injection. Seventy-two hours after injection, the mice were subjected to further study.

Construction of adenovirus carrying IncDACH1, IncDACH1 siRNA, conserved fragment of human IncDACH1 and infection

Adenovirus vectors carrying IncDACH1(OE-IncDACH1), a short RNA fragment for silencing IncDACH1 (Si-IncDACH1) or conserved fragment of human IncDACH1(hcF-IncDACH1) and a CAG promoter conjugated with green fluorescent protein (GFP) were constructed by Genechem Co., Ltd. (Shanghai, China). Neonatal cardiomyocytes were infected with adenovirus for 48 hours, and then subjected to subsequent study.

Transfection of Hadhb, Eif4a1, and Anp32 siRNA.

siRNA for Hadhb (siHadhb), Eif4a1 (siEif4a1), Anp32 (siAnp32) and a scrambled negative control RNA (siNC) were synthesized by Generalbiol (Chuzhou, Anhui, China). These siRNAs were transfected at a final concentration of 100 nM into NMVMs using the X-treme GENE Transfection Reagent (Roche, Indianapolis, USA) according to the manufacturer's protocols. The cardiomyocytes were collected for total RNA isolation or protein purification. The sequences are: siHadhb, sense 5'- GCUCUCAGAUCUUCUAUAATT-3' and antisense 5'- UUAUAGAAGAUCUGAGAGCTT-3'; siEif4a1, sense 5'- GCUCACCGAGAAGAUGCAUTT-3' and antisense 5'- UUAUAGAAGAUCUGAGAGCTT-3'; siAnp32, sense 5'- GCCUCACCUCCAUUUCCAATT-3' and antisense 5'- UUGGAAAUGGAGGUGAGGCTT-3'.

Induction of ventricular arrhythmia

C57BL/6 mice were anesthetized with 2,2,2-tribromoethanol (200 mg/kg, i.p.). An octapolar electrophysiological catheter (1.1F, SciSense Inc., Canada) was inserted into the right ventricle via the jugular vein. Intracardiac pacing was performed using an automated stimulator interfaced with the data acquisition system (GY6000; HeNan HuaNan Medical Science & Technology Ltd., Zhengzhou, China). The surface recording electrode was fixed on LV epicardium to record pseudo-ECG. Inducibility of ventricular tachycardia (VT) was determined by applying a train of ten consecutive electrical pulses with a coupling interval of 80 ms (S1), followed by two extra stimuli (S2 and S3) at coupling intervals of 2 ms, respectively. Successful induction of VT was defined as the appearance of rapid nonsinus rhythm ventricular activations lasting for three beats or more.

Optical mapping recording

Mice were heparinized and euthanized by 2,2,2-tribromoethanol (200 mg/kg, intraperitoneal injection; Sigma, St Louis, MO, USA). The heart was isolated and Langendorff perfused with Tyrode's solution (NaCl 128.2 mM, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 1.3 mM, KCl 4.7 mM, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1.85 mM, $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ 1.19 mM, Na_2CO_3 20 mM, and glucose 11.1 mM; pH 7.35) at 37 °C. After 10 min of stabilization, the hearts were stained with RH237 (10 μM) for membrane voltage (V_m) mapping. The dye was excited at 710 nm using monochromatic light-emitting device. The fluorescence was filtered and recorded simultaneously with a MiCAM05 CMOS camera (SciMedia, USA) at 1ms/frame and 100x100 pixels with a spatial resolution of 0.35x0.35 mm² per pixel. Blebbistatin (10 μM , Selleckchem, Houston, TX, USA) was used to inhibit motion artifact during optical mapping.

Experimental protocol of optical mapping

A pair of hook bipolar electrodes was inserted into apex of heart for pacing. A pseudo-ECG was obtained with widely spaced bipolar electrodes to determine ventricular rhythm. The ventricles were initially paced at a constant pacing cycle length (PCL) of 200ms. The PCLs were progressively shortened (200, 100, 60, 40, 30, 20ms) with a duration of 1-2 s until ventricular tachycardia (VT) was induced or the loss of 1:1 capture of the ventricles. Optical recording was performed after 20 beats of stable pacing at each PCL. Optical recordings were then performed during VT.

Patch-clamp recording

Whole-cell configuration of the patch-clamp technique was used to record peak I_{Na} . Experiments were performed at room temperature (22~23°C) by using a MultiClamp 700B (Alembic Instruments) amplifier. Pipettes (tip resistance 1 to 2 MΩ) were filled with a solution containing (in mM): NaCl 5, CaCl₂ 2, MgCl₂ 2, CsCl 130, HEPES 10, EGTA 15 and MgATP 4 (pH 7.2 with CsOH). Myocytes were bathed with a solution containing (in mM): NaCl 25, CaCl₂ 2, MgCl₂ 2.5, CsCl 108.5, HEPES 10, CoCl₂ 2.5 and glucose 10 (pH 7.4 with CsOH).

Differentiation of human induced pluripotent stem cells(hiPSCs) to cardiomyocytes

Undifferentiated hiPS cells (AC-iPSC) were cultured on Matrigel-coated plates in an E8 medium (CA1001500, CELLAPY). Differentiation basal medium composed of RPMI1640 medium (C11875500BT, Thermo Fisher Scientific) and B27 minus insulin (A1895601, Thermo Fisher Scientific) was used to induce cardiomyocyte differentiation. Specifically, the 70~80% confluent hiPSCs were incubated in differentiation basal medium added with CHIR-99021 (HY-10182, MCE) for 1 day and Wnt-C59 (S7037, Selleck Chemicals) for 2 days. Then, the cells were cultured in RPMI1640 basal medium containing B27 (17504044, Thermo Fisher Scientific), which was replaced with fresh medium every 1~2 days. Beating cells were observed after 8 days of differentiation induction and used for further study.

Construction of truncated LncDACH1 fragment plasmids

The sequence of LncDACH1 was divided into five fragments. The cDNA of each fragment was inserted into the pCDNA3.1, respectively. The first 417 nts of the entire sequences was cut off and constructed as fragment-a (418-2085 nts). Another 417 nts was cut off to generate fragment-b (835-2085 nts). The third 417 nts was cut off to generate fragment-c (1251-2085 nts). Fragment-d is from 835-1668 nts, and fragment-e is from 835-1251 nts.

Isolation of cardiac myocytes

Hearts were rapidly excised, cannulated, and perfused with Ca²⁺-free Tyrode solution (in mM): NaCl 137, KCl 5.4, NaH₂PO₄ 0.16, glucose 10, CaCl₂ 1.8, MgCl₂ 0.5, HEPES 5.0, and NaHCO₃ 3.0 (pH 7.4 adjusted with NaOH) for 5 min. The heart was then perfused with a solution containing collagenases B and D (Roche) and protease XIV (Sigma) until digestion was complete. Tissue was dissociated using forceps, and dissociated left ventricular cardiomyocytes were gradually exposed to Ca²⁺ (from 50 to 500 μM over 40 min) and plated in culture chambers for further studies.

Immunocytochemistry of isolated mouse ventricular myocytes.

Cardiomyocytes were fixed for 10 min with 4% paraformaldehyde in PBS, and then washed in PBS for 10 min (2 times). The cells were permeabilized with 0.5% Tween 20 for 30 min. After washed out with PBS for 10 min (3 times), cardiomyocytes were incubated with primary antibodies against Nav1.5 (ASC005,

Alomone, 1;200) and dystrophin (MANDYS8, SIGMA, 1;300) overnight at 4°C. Following washout with PBS (10 min, 3 times), cells were incubated with secondary antibodies for 1h and washed with PBS (10 min, 3 times). The cover slips were mounted onto frosted slides in a solution composed of 90% FluorSave Reagent (Calbiochem, La Jolla, CA, USA) and 10% 10X PBS.

Fluorescent in situ hybridization (FISH)

In situ hybridization was performed with a Fluorescent in Situ Hybridization (FISH) Kit (RiboBio, Guangzhou, China). Briefly, isolated cardiomyocytes were fixed in 4% formaldehyde at 4°C for 10 min and dried out on the slides at room temperature (RT). The slides were rinsed and permeabilized with 0.5% Triton-100 in PBS at RT for 30 min, washed with PBS solution, and prehybridized at 37°C for 30 min before hybridization. The prehybridized slides were then incubated with lncRNA-probe in hybridization solution at 37°C for 16 h. After hybridization, the slides were washed six times with prewarmed wash buffer and PBS solution. Finally, the slides were counterstained with DAPI and visualized using a confocal laser-scanning microscope (Zeiss 800, Germany).

Quantitative Real-time RT-PCR

Total RNA was extracted by using Trizol reagent (Invitrogen, USA) according to manufacturer's protocol. Total RNA (0.5µg) was reverse transcribed by using the TransScript reverse transcriptase (GMO technology, Beijing) to obtain cDNA. The RNA levels were determined using SYBR Green I incorporation method on ABI 7500 fast Real Time PCR system (Applied Biosystems, USA), the expression levels of mRNA were calculated by relative quantitative $2^{-\Delta\Delta CT}$ method. The primers are: Mus lncDACH1 Forward: 5'-AAGATAGGATGTTGGGGCAG-3' Mus lncDACH1 Reverse: 5'-ACCATAGCACAAACACTTCC-3' Mus Dystrophin Forward: 5'-CGGGTTGGCTTTGAATGCTC-3' Mus Dystrophin Reverse: 5'-AGTCTTTGGGTGGCTGAGTG-3' Mus DACH1 Forward: 5'-AACCGCAAGAGACAGCATCG-3' Mus DACH1 Reverse: 5'-GGACAGGCCATCAGGAAACAG-3' Mus SCN5a Forward: 5'-GAAGGAACGCAGCACAGACAG-3' Mus SCN5a Reverse: 5'-CATCGCCCTTGACCCATACTA-3' Mus Hadhb Forward: 5'-CCAAGAAGGCACAGGATGAAG-3' Mus Hadhb Reverse: 5'-CCAGTGAGGAAGGACGGATG-3'

Western blot

The total, membrane and cytoplasmic protein samples were extracted from cardiac tissues of C57BL/6 mice for immunoblotting analysis. Total protein was collected with the treatment of RIPA lysis buffer (Beyotime, Beijing, China) and a protease inhibitor cocktail (Roche, Basel, Switzerland) at 4°C followed by centrifugation. Extraction of surface and cytoplasmic proteins was conducted using the Surface and Cytoplasmic Protein Reagent Kit (Cat#P0033; Beyotime, Shanghai, China) according to the manufacturer's instructions. Protein samples were fractionated by SDS-PAGE and then transferred to PVDF membrane. The membranes were blocked in Tris-buffered saline containing 5% milk and then incubated with primary antibodies at 4°C overnight. The primary antibodies include anti-Nav1.5 (ASC005, Alomone, 1:200), anti-dystrophin (MANDYS8, SIGMA, 1:500). The anti-β-actin (1:20000 dilution, 66009-1-Ig, Proteintech) and anti-N-cadherin antibody (Cat#ab76011, 1:5000; Abcam, Cambridge, UK) were used

as internal controls. Western blot bands were captured on the Odyssey Infrared Imaging System (LI-COR Biosciences, USA) and quantified with Odyssey v1.2 software by measuring the band intensity (area $\times$ OD) in each group. The band intensity was normalized to the internal control. All antibodies were diluted in PBS buffer.

RNA pulldown and immunoblotting

The RNA pull-down was performed as described in the previous study¹³. Briefly, Biotin-labeled, full length IncDACH1 RNA and antisense RNA were prepared with the Biotin RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche). Biotinylated RNAs were treated with RNase-free DNase I (Invitrogen) and purified on G-50 Sephadex Quick Spin columns (Roche). Biotinylated RNA (17 μ g) was heated to 65°C for 10 min and slowly cooled to 4°C. Then the RNA was mixed with tissue extracts in pulldown buffer supplemented with tRNA (0.1 μ g/ μ l) and incubated at 4°C for 2 h. Washed Streptavidin agarose beads (60 μ l, Invitrogen) were added to each binding reaction and further incubated at 4°C for 1 h. Beads were washed briefly five times in pulldown buffer and boiled in SDS buffer, and the retrieved protein was visualized by immunoblotting.

RNA immunoprecipitation (RIP)

RNA immunoprecipitation (RIP) experiments were performed by using a Magna RIPTM RNA-Binding Protein Immunoprecipitation Kit (Millipore, USA) as previously reported¹³. Briefly, heart tissue was pieced and lysed in 220 μ l of lysis buffer containing protease inhibitors and RNase Inhibitor and centrifuged at 14,000 $\times$ g for 10 min. The supernatants were incubated with anti-dystrophin, anti-hadhb and anti-rabbit IgG antibody for overnight at 4°C with gentle rotation. Protein G magnetic beads (50 μ l) were added and incubated at RT with gentle rotation for 3 h. RNA was extracted with 400 μ l phenol:chloroform:isoamyl alcohol (125:24:1, pH = 4.3) according to the manufacturer's instructions before quantitation by RT-qPCR.

Mouse models of heart failure (HF) by transaortic constriction (TAC) and by coronary artery ligation

Mice were randomly divided into sham and TAC groups. In each group, mice were anesthetized with 2,2,2-tribromoethanol (200 mg/kg, i.p.) for TAC model. The animal was orally intubated with 20-gauge tube, and ventilated (mouse ventilator, UGO BASILE, Biological Research Apparatus, Italy) at the respiratory rate of 100 breaths/min with a tidal volume of 0.3 ml. The transverse aorta was constricted by a 6-0 silk suture ligature tied firmly against a 27-gauge needle between the carotid arteries. Then, the needle was promptly removed to yield a constriction of 0.4 mm in diameter. For sham group mice, the animals received the same procedures without aorta constriction.

Statistics

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using unpaired Student's t test or One-Way Analysis of Variance (ANOVA) followed by Tukey's post-hoc analysis. A $P < 0.05$ was considered statistically different.v

Declarations

Author contributions

Genlong Xue, YZ, JY, performed experiments, analyzed data, and prepared the manuscript. Ying Yang, RZ, DL, TT,XZ, CL, XL, JY, KS, YG, XL and GY helped perform experiments and collect data. BY and YJL oversaw the project and proofread the manuscript. ZP designed the project, oversaw the experiments and prepared the manuscript.

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Competing interests

The authors declare no competing interests.

Data availability

The data that support the findings of this study and unique materials are available from the corresponding authors upon reasonable request. Source data are provided with this paper. Additional data related to this paper may be requested from the authors.

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Figures

Figure 1

Binding of lncDACH1 to dystrophin and the effects of cardiomyocyte-specific transgenic overexpression of lncDACH1 (lncDACH1-TG) on the expression and function of sodium channel. (A) Blotting of dystrophin pulled-down by lncDACH1. (B) lncDACH1 precipitated by anti-dystrophin antibody. N=4. * $P < 0.05$ vs IgG by one-way ANOVA, followed by Tukey's post-hoc analysis. (C, D) The total, membrane and cytoplasm levels of dystrophin and Nav1.5 by Western blot. N-cadherin is the loading control for

membrane extracts. N=10-11 for total protein; N= 7-14 for membrane protein; N= 8-14 for cytoplasm protein. *P<0.05 versus WT group. *P<0.05 versus WT group. P-values were determined by unpaired t test. (E) Distribution of IncDACH1, dystrophin and Nav1.5 in isolated cardiomyocytes. (F) The mRNA levels of dystrophin and SCN5A. N=8-11. (G) Peak INa currents, I-V curve and kinetics of INa. N=9-15 cells from 3 or more mice. (H) Conduction velocity of perfused hearts by optical mapping recordings. N=7. * P<0.05 versus WT group. P-values were determined by unpaired t test.

Figure 2

Effects of lncDACH1 overexpression on sodium channel expression and function in cultured neonatal cardiomyocytes. (A) Verification of the expression of lncDACH1 after transfection of adenovirus carrying lncDACH1. N=7-8 from 3 independent cultures. *P<0.05 versus NC (negative control, empty plasmid). P-values were determined by unpaired t test. (B, C) Peak INa currents, I-V curves and kinetics of INa. N=12-16 cells from 3 independent cultures. (D) Distribution of Nav1.5 and dystrophin by immunofluorescent staining. (E) The mRNA levels of dystrophin and SCN5A. N=8-10 from 3 independent cultures.

Figure 3

Conditional knockout of IncDACH1(IncDACH1-cKO) in cardiomyocytes increased peak sodium current, membrane Nav1.5 expression. (A) The total, membrane and cytoplasm levels of dystrophin by Western blot and dystrophin mRNA by qRT-PCR. N-cadherin is the loading control for membrane extracts. N=10 for total protein; N= 10 for membrane protein; N= 15 for cytoplasm protein. *P<0.05 versus WT group. P-values were determined by unpaired t test. (B) The total, membrane and cytoplasm levels of Nav1.5 by Western blot and SCN5A mRNA by qRT-PCR. N-cadherin is the loading control for membrane extracts. N=10 for total protein; N= 8 for membrane protein; N= 8 for cytoplasm protein. *P<0.05 versus WT group. P-values were determined by unpaired t test. (C) Distribution of IncDACH1, dystrophin and Nav1.5 in isolated cardiomyocytes. (D) Peak INa currents, I-V curve and kinetics of INa. N=10-15 cells from 3 or more mice. (E) Conduction velocity of perfused hearts by optical mapping recordings. N=7 and 5. * P<0.05 versus WT group. P-values were determined by unpaired t test.

Figure 4

Effects of lncDACH1 knockdown on sodium channel expression and function in cultured neonatal cardiomyocytes. (A) Verification of the expression of lncDACH1 after infection of adenovirus carrying lncDACH1 siRNAs. N=15 from 3 independent cultures. *P<0.05 versus NC (negative control, empty plasmids). P-values were determined by unpaired t test. (B, C) Peak INa currents, I-V curve and kinetics of INa. N=9-15 from 3 independent cultures. (D) Distribution of Nav1.5 and dystrophin by

immunofluorescent staining. (E) The mRNA levels of dystrophin and SCN5A. N=11-15 from 3 independent cultures.

Figure 5

Increased arrhythmia susceptibility in LncDACH1-TG mice. (A) Ventricular fibrillation (VF) induced by S1S2 pacing in intact mice. N=10-16.(B) Ventricular fibrillation (VF) induced by S1S1 pacing of perfused hearts.(C) Break points during VT of WT and LncDACH1-TG mice by optical mapping. Consecutive phase

maps sampled at 10-ms interval during VF from WT and TG mice. Phase singularities (wavebreaks) are indicated by phase maps. Upper panels showed corresponding optical recording of VF at asterisk site. n=8. (D) Representative of dominant frequency of VF from WT and TG mice. (E) The number of phase singularities and dominant frequency of WT and TG mice. (F) Ventricular fibrillation (VF) induced by S1S1 pacing in perfused hearts. N=5-7 mice.

Figure 6

The conserved fragment of IncDACH1(cF-IncDACH1) inhibited sodium channel function in mice. (A) Pulldown of dystrophin by fragments of IncDACH1 as indicated. (B) Verification of the expression of cF-IncDACH1 after injection of adeno-virus carrying cF-IncDACH1. N=5. *P<0.05 versus NC (negative control, Adeno-virus carrying empty plasmid). P-values were determined by unpaired t test. (C) The total, membrane and cytoplasm levels of dystrophin and Nav1.5 by Western blot. N-cadherin is the loading control for membrane extracts. N=11-12 for total protein; N= 10 for membrane protein; N= 5-11 for cytoplasm protein. *P<0.05 versus NC group. P-values were determined by unpaired t test. (D) Distribution of dystrophin and Nav1.5 in isolated cardiomyocytes. (E) Representative traces and I-V curve of peak INa currents. N=12-15 from 3 or more mice. (F) Conduction velocity of perfused hearts by optical mapping recordings. N=9-10. * P<0.05 versus NC group. P-values were determined by unpaired t test. (G) Break points during VT by optical mapping. H. Ventricular (VF) induced by S1S2 pacing in intact mice.

Figure 7

Activation of dystrophin transcription by AAV9 virus carrying dCas9-SAM system (AAV9-Dys-Act) rescued the remodeling of sodium channel in LncDACH1-TG mice. (A) The mRNA level of dystrophin by real-time PCR (N=12-17) and the total, membrane and cytoplasm protein levels of dystrophin by western blot. N-cadherin is the loading control for membrane extracts. N=7 for total protein; N= 8 for membrane protein; N= 8 for cytoplasm protein. *P<0.05 versus NC group. NC, AAV9 virus carrying dCas9-SAM system with

control sgRNA; Dys, AAV9 virus carrying dCas9-SAM system with sgRNA targeting dystrophin promoter. (B) The mRNA level of Nav1.5 by real-time PCR (N=10-12) and the total, membrane and cytoplasm protein levels of Nav1.5 by western blot. N-cadherin is the loading control for membrane extracts. N=10 for total protein; N= 5 for membrane protein; N= 6 for cytoplasm protein. *P<0.05 versus NC group. (C) Representative traces, I-V curves and kinetics of peak INa currents. N=11-20 cells from 3 or more mice. (D) Conduction velocity of perfused hearts by optical mapping recordings. N=6-9. * P<0.05 versus NC group. P-values were determined by unpaired t test. (E) Ventricular fibrillation (VF) induced by S1S1 pacing in perfused hearts. N= 7-10. (F) Ventricular fibrillation (VF) induced by S1S2 pacing in intact mice. N= 10-13. The data are analyzed by one-way ANOVA followed by Tukey's post-hoc analysis.

Figure 8

Hadhb binds to lncDACH1 and promotes its decay. (A) The expression level of lncDACH1 in the hearts of TAC mice. N=5-8. * $P < 0.05$ by unpaired t test. (B) The mRNA level of DACH1 in the hearts of TAC mice. N=12-14. (C) Effects of siRNAs for anp32a, eif4a1 and hadhb on the expression of lncDACH1. N=5-12 from 3 independent cultures. * $P < 0.05$ by unpaired t test. (D) The effects of hadhb siRNA on the decay of lncDACH1. N=6-15 from 3 independent cultures. (E) The effects of hadhb siRNA on the protein expression of Nav1.5 and dystrophin. (F) Blotting of hadhb pulled-down by lncDACH1, and precipitation of lncDACH1 by anti-hadhb antibody. N=4. * $P < 0.05$ vs IgG by one-way ANOVA followed by Tukey's post-hoc analysis. (G) lncDACH1 inhibits Nav1.5 in human iPS differentiated cardiomyocytes. I. Schematic summary of the signaling pathway of hadhb/lncDACH1/dystrophin/Nav1.5.

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