

Neuron Navigator 3 (NAV3) is Required for Heart Development in Zebrafish

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Research Article

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Abstract

As a tightly controlled biological process, cardiogenesis requires the specification and migration of a suite of cell types to form a particular three-dimensional configuration of the heart. Many genetic factors are involved in the formation and maturation of the heart, and any genetic mutations may result in severe cardiac failures. The neuron navigator (NAV) family consists of three vertebrate homologs (NAV1, NAV2, and NAV3) of the neural guidance molecule Uncoordinated-53 (UNC-53) in *Caenorhabditis elegans*. Although they are recognized as neural regulators, their expressions are also detected in many organs, including the heart, kidney, and liver. However, the functions of NAVs, regardless of neural guidance, remain largely unexplored. In our study, we found that *nav3* gene was expressed in the cardiac region of zebrafish embryos from 24 to 48 hours post-fertilization (hpf) by means of in situ hybridization (ISH) assay. A CRISPR/Cas9-based genome editing method was utilized to delete the *nav3* gene in zebrafish and loss-of-function of Nav3 resulted in a severe deficiency in its cardiac morphology and structure. The similar phenotypic defects of the knockout mutants could recur by *nav3* morpholino injection and be rescued by *nav3* mRNA injection. Dual-color fluorescence imaging of ventricle and atrium markers further confirmed the disruption of the heart development in *nav3*-deleted mutants. Although the heart rate was not affected by the deletion of *nav3*, the heartbeat intensity was decreased in the mutants. All these findings indicate that Nav3 was required for cardiogenesis in developing zebrafish embryos.

Background

Cardiogenesis is an indispensable process in vertebrates during embryogenesis, and the heart plays a vital role in the distribution of nutrients and oxygen in the embryos. Any failures during cardiogenesis will lead to cardiac malformations, further resulting in the death of the embryos. It is challenging to study heart development during embryogenesis. Compared with other mammalian models, such as mice, zebrafish offers several unique advantages for studying cardiac development (Gore et al., 2012; Liu and Stainier, 2012). First, the zebrafish embryos develop externally after fertilization, allowing direct non-invasive observation of heart development during cardiogenesis. Second, the optical transparency of zebrafish embryos allows high-resolution optical imaging. Third, zebrafish embryos can survive up to 7 days without a functional cardiovascular system to supply the nutrients and oxygen due to their tiny size, offering the possibility to analyze the phenotype of the cardiac defects. Besides, essential genes responsible for cardiac development are also highly conserved throughout vertebrates. Therefore, zebrafish is a valuable animal model for exploring the mechanisms underlying cardiac development.

In zebrafish, the formation of a mature heart includes a series of cellular processes, including cell migration, proliferation, and differentiation (Buckingham et al., 2005; Keegan et al., 2004). Numerous genes are involved in embryonic cardiogenesis, such as *bmp4* (Jiao et al., 2003), *nppa* (Grassini et al., 2018), *vcana* (Mittal et al., 2019), *amhc*, and *vmhc* (Berdougo et al., 2003; Yelon et al., 1999), as well as transcription factors *nkx2.5* (Targoff et al., 2013), *hand2* (Schindler et al., 2014), and GATA family members (Haworth et al., 2008; Holtzinger and Evans, 2007; Reiter et al., 1999). *Nav3*, the vertebrate homology of *C. elegans* gene uncoordinated-53 (*unc-53*), is one of the neural guidance genes, which plays a role in shaping the developing nervous system by modulating/guiding neural cell migration (Maes et al., 2002; Stringham et al., 2002). Moreover, NAV3 is identified as a microtubule-binding protein that regulates actin assembly in extensions of lamellipodia and filopodia (Klein et al., 2011). Although *nav* genes are mainly expressed in the brain, their slight expressions are also detected in the heart, kidney, and liver, suggesting potential roles of NAVs in organogenesis (Maes et al., 2002). In the present study, we focused on the effects of Nav3 on heart development in zebrafish. We found that *nav3* was temporally expressed in the cardiac region from 24 h post-fertilization (hpf) to 48 hpf through in situ hybridization (ISH) assay, indicating its function in ventricle-atrium differentiation (Bakkers, 2011).

Deletion of *nav3* in zebrafish via CRISPR/Cas9 genome editing technique resulted in severe defects in heart development. Fluorescent imaging of ventricle marker (*vmhc*) and atrium marker (*amhc*) further confirmed the malformed morphogenesis of the cardiac structure in *nav3*-null mutants. In addition to structural alterations, a significant decrease in heartbeat intensity (HI) was also detected in *nav3*^{-/-} mutants. Moreover, the phenotypic defects caused by loss-of-function of Nav3 could be rescued by *nav3* mRNA injection. Collectively, our current work revealed a novel role of Nav3 in heart development, which might provide potential strategies for heart disease therapy and cardiac regeneration.

Methods

Zebrafish husbandry and strains

The zebrafish (TU line) in this study was obtained from China Zebrafish Resource Centre and maintained in Jiangsu key laboratory of neuroregeneration of Nantong university. The transgenic zebrafish lines *Tg(vmhc:mCherry::amhc:EGFP)* and *Tg(myf7:mCherry)* were kindly provided by Dr. R. Zhang and Dr. T. Zhong, respectively. *Tg(mef2a:EGFP)* transgenic line was constructed by our lab previously (Lv et al., 2017). *Tg(vmhc:mCherry::amhc:EGFP)* and *Tg(myf7:mCherry)* were outcrossed with *nav3*-null mutants to generate the *Tg(nav3^{-/-}-vmhc:mCherry::amhc:EGFP)* and *Tg(nav3^{-/-}-mef2a-EGFP)* homozygous lines. All zebrafish lines were maintained at 28.5°C.

Whole-mount in situ hybridization (WISH)

WISH with antisense RNA probes was performed as previously described (Krueger et al., 2011). The probe for the detection of NAV3 (NM_001045143.2) was cloned from cDNA fragments. The *nav3* cDNA fragment was amplified with a forward primer: 5'-TCACCTTCTGACTCCACCAG-3' and a reverse primer: 5'-GTTTCGATGTTACGCGCTCAC-3'. After hybridization, images of the embryos were acquired with an Olympus stereomicroscope MVX10 equipped with an Olympus DP71 camera.

Generation of *nav3*-null mutants

A CRISPR/Cas9-mediated approach was used to generate *nav3*-deleted mutants. The target site of CRISPR/Cas9 designed to identify the sequence in the second exon of *nav3* was 5'-CATACCGATGGATTCTGCTGG-3' (underlined was the PAM sequence). The template sequence of sgRNA for *in vitro*

transcription was 5'-

TAATACGACTCACTATAGCATACCCGATGGAGTTCTGCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGG 3'. For the sgRNA synthesis, a forward primer (5'-TAATACGACTCACTATAGCATACCCGATGGAGTTCTGCGTTTTAGAGCTAGAAATAGC-3') containing a T7 promoter region and a *nav3* gene-targeting region, and a universal reverse primer (5'-AAAAAAGCACCGACTCGGTGCCAC-3') were used in the PCR amplification with pT7 plasmid as the template to obtain the sgDNA, which was then transcribed into sgRNA *in vitro* using the T7 mMessage mMachine kit (Ambion). Micro-injection was performed with 1-cell stage zebrafish embryos, and each embryo was co-injected with 100 pg sgRNA and 200 pg Cas9 mRNA. G0 generations were examined by PCR, followed by Sanger sequencing. A mutant with a 55-bp deletion at the second exon was identified, leading to the occurrence of a stop codon downstream of the Cas9 cutting site. The heterozygous *nav3*^{+/-} mutants were incrossed to obtain the homozygous F2 progenies.

Morpholino-mediated gene deletion of *nav3* in zebrafish

The *nav3* gene-specific morpholino (Gene Tools, LLC) was used to block the splicing of *nav3* pre-mRNA. The sequence of the morpholino antisense oligomer was 5'-CAGCCCATGTGCCATGCTGCTCTTG-3'. In the present study, 2 nL of 0.3 mM morpholino oligo was micro-injected into the embryos at the 1-cell stage.

mRNA rescue experiment

In the rescue experiment, 200 pg *nav3* mRNA was co-injected into the embryos with the *nav3* antisense morpholino. The cDNA fragment of *nav3* was first subcloned into pCS2+ vector. To make the mRNA, the DNA plasmid was linearized with an appropriate restriction enzyme and transcribed *in vitro* using the SP6 mMessage mMachine kit (Ambion).

Survival rate quantification

Zebrafish embryos of WT and *nav3*-null mutants were respectively collected after fertilization and divided into 10 tanks (100 embryos in each tank). The counts of surviving fishes per tank were determined at 1, 2, 3, 20, and 60 days post-fertilization (dpf). Afterward, the proportions of fish surviving at each time point were assessed.

Confocal imaging and quantification

Zebrafish embryos of *Tg(mef2a:EGFP)*, *Tg(nav3^{-/-}-mef2a:EGFP)*, *Tg(vmhc:mCherry::amhc:EGFP)* and *Tg(nav3^{-/-}-vmhc:mCherry::amhc:EGFP)* were anaesthetized with egg water/0.16 mg/mL tricaine/1% PTU (Sigma) and embedded in low-melting agarose. Living imaging was performed with Nikon A1R confocal microscopy.

The heart rate of the embryo was defined as the beats per minute and counted under a dissecting microscope (Olympus). To examine the HI, the cardiac-labeled transgenic zebrafish lines *Tg(mef2a:EGFP)* and *Tg(nav3^{-/-}-mef2a:EGFP)* were used to visualize the hearts of WT and mutants, respectively. The cross-sectional areas of the chambers in GFP-labeled hearts in the dilated state (A_d) and contracted state (A_c) were respectively measured with ImageJ (Rueden et al., 2017). The HI was calculated by the equation as follows, $HI = A_d - A_c$. In our present analysis, five WT and mutant fishes were respectively selected, and each fish was measured for 20 successive heartbeats.

Data analysis

A two-tailed, unpaired Student's t-test was used to determine statistical significance when comparing independent groups. $P < 0.05$ was considered statistically significant. All results were presented as mean \pm SD.

Results

Zebrafish *nav3* is expressed in the heart during embryogenesis

Zebrafish Nav3 has been previously reported as an ortholog of UNC-53 in *C. elegans*. Compared with other vertebrate NAV3 in humans, mice, and rats, as well as UNC-53 in *C. elegans*, zebrafish Nav3 was highly conserved among all these species (Supplemental Fig. S1). NAV3 is a huge protein consisting of more than 2,000 amino acids. It contains several conserved domains, including a putative calponin-homology domain (CH-domain), an LKK actin-binding domain, two Src Homology 3 (SH3) domains, and an ATP/GTP-binding AAA domain (Schmidt et al., 2009) (Fig. 1A). The CH-domain is essential for the interaction with actin filaments, suggesting its role in the formation of lamellipodia and filopodia, and it would further drive cell movements. Phylogenic alignment also revealed the close relationship between zebrafish Nav3 and NAV3 of other species (Fig. 1B).

ISH was performed with zebrafish embryos from 18 hpf to 96 hpf to explore the spatiotemporal expression of zebrafish *nav3* during embryogenesis. The *nav3* mRNA transcripts could be detected at an early embryonic stage (18 hpf), while its expression was mainly restricted to the brain and somites (Fig. 1C). A slight expression was also presented in cardiac primordium at the same developmental stage (Fig. 1D, E). As *nkx2.5* is specifically expressed in cardiac primordium, the ISH experiment with *nkx2.5* probe could further confirm the expression of *nav3* in the region of cardiac primordium (Fig. 1F). At 24 hpf, *nav3* was more specifically expressed in the brain, heart, and somites (Fig. 1G-I). However, the expression of *nav3* in somites became weaker, and it was rarely detected from 48 hpf. At 48 hpf, its expression was highly regionalized to the brain and heart (Fig. 1J-L). At 96 hpf, there was almost no expression of *nav3* in the heart, and its expression was found to be accumulated in other tissues, such as the gill arch, swim bladder, and intestine (Fig. 1M-O), suggesting that its potential roles were not only restricted to nervous or cardiovascular system.

Nav3 loss-of-function in zebrafish exhibits severe phenotypic defects and low survival rates

To explore the effects of Nav3 deletion in heart development, a CRISPR/Cas9-based genome editing method was utilized to generate a *nav3*-deleted mutant (Fig. 2A). A 55-bp deletion at the second exon of *nav3* led to the occurrence of a truncated protein (a 79-amino acid sequence versus the full-length sequence with 2,270 amino acids). The homozygous *nav3*^{-/-} mutants were further obtained through genetic selection. An obvious heart malformation phenotype was observed in *nav3*-null zebrafish embryos. Compared with the wild-type (WT) counterparts, the *nav3*^{-/-} mutants displayed severe pericardial edemas not only at the early developmental stage but also in adult zebrafishes, although more than 70% of the *nav3*^{-/-} mutants died within 24 hpf (Fig. 2B-H). Afterwards, only 8% of the mutants could successfully develop into adults (Fig. 2H).

Heart morphogenesis and function are disrupted in *nav3* mutant zebrafish embryos

To further evaluate the changes in cardiac morphology, structure, and function in *nav3*-null mutants, the fluorescently labeled atrium-specific marker *amhc* and ventricle-specific marker *vmhc* were respectively adopted. Transgenic lines *Tg(vmhc:mCherry::amhc:EGFP)* and *Tg(nav3^{-/-}-vmhc:mCherry::amhc:EGFP)* were used to compare the heart morphological differences between WT and mutants. Both atrium and ventricle in *nav3*^{-/-} mutants at 72 hpf exhibited a long tubular-shaped morphology, and the boundary line between atrium and ventricle was not as clear as the WT (Fig. 3A-F). Next, we compared the heart rate as well as the HI between WT and *nav3*^{-/-} mutants to check whether the heart functions were affected upon Nav3 loss-of-function. We found that the heart rate was not affected by the deletion of *nav3*. The frequency was within a range of 150 to 200 times per minute in both WT and mutant strains (Fig. 3S). However, the HI in WT and mutant strains was different. Here, we introduced a parameter defined as HI to indicate cardiac capacity. The areas of the GFP-labeled heart were measured in the dilated and contracted states (Fig. 3G-R). The area difference between these two states was regarded as HI. The calculation formula was shown in **Methods**. The HI was significantly lower in the mutants compared with the WT siblings (Fig. 3T). However, there was no obvious difference in heart rate between WT and mutants (Fig. 3U). All these findings indicated that Nav3 deficiency resulted in heart development defects during zebrafish embryogenesis, including the cardiac structure and functional disruption.

nav3 mRNA injection can rescue the cardiac defects in *nav3*-deleted embryos

To further confirm whether the abnormal phenotype of the mutant was caused by the deletion of *nav3*, we injected a morpholino against *nav3* mRNA into transgenic line *Tg(myf17:mCherry)* to delete *nav3*. *Tg(myf17:mCherry)* line was under a WT background, and the cardiac marker *myf17* (myosin light chain 7) was fluorescently labeled with mCherry fluorescent protein. Upon *nav3* expression was down-regulated through morpholino injection, WT zebrafish embryos displayed a similar phenotype to *nav3*^{-/-} mutants. Moreover, fluorescent imaging of the heart in morpholino-injected embryos mimicked the abnormal cardiac morphology of *nav3*-null mutants (Fig. 4).

To verify that the phenotype of *nav3*-morphants was attributed to the loss-of-function of Nav3 rather than non-specific effects, we performed a rescue experiment by injecting *nav3* mRNA together with *nav3* morpholino into 1-cell-stage zebrafish embryos of *Tg(myf17:mCherry)*. The *nav3* mRNA injection partially rescued the phenotypic defects caused by the down-regulation of *nav3*. Upon *nav3* mRNA injection, the tubular-shaped ventricle and atrium structure of the *nav3*-morphants were almost recovered to a normal state. However, compared with the WT counterparts, the rescued heart was slightly misshaped in the morphology, such as tubular-shaped ventricle. Additionally, the boundary between the ventricle and atrium became observable after *nav3* mRNA injection (Fig. 4).

Discussion

As a complicated but coordinated biological process, the assembly of an intact heart involves the proliferation, differentiation, and migration of cardiomyocytes to form a functional contractile organ (Buckingham et al., 2005; Keegan et al., 2004; Liu and Stainier, 2012). A cohort of genes participate in this process and play important roles. *Nav3*, the vertebrate homology of *unc-53* in *C.elegans*, is first discovered as a neural guidance gene that is functional in cell migration and outgrowth of axons. Because cell movement is highly relevant to embryonic organogenesis and NAV3 is also involved in liver development (Klein et al., 2011), we hypothesized that NAV3 played a novel role in cardiogenesis. In our present study, we first examined the expression of *nav3* in zebrafish during embryogenesis. The specific spatiotemporal expression of *nav3* in the zebrafish heart region suggested its potential function in embryonic heart development beyond the nervous system. To confirm our prediction, we generated the *nav3*-deleted mutants. Loss-of-function of Nav3 in zebrafish embryos displayed severe heart development defects, such as pericardial edemas and chamber malformations. Moreover, the function of the heart was affected by the deletion of Nav3. For instance, the HI was significantly decreased in mutants. However, the heart rates were similar in both WT and mutant embryos. The functional analysis revealed that loss of Nav3 resulted in an abnormal dilation and contraction in the heart.

To confirm whether the phenotypic defects of the *nav3*-null mutants were caused by the loss-of-function of Nav3, a morpholino-mediated gene knockdown experiment and a *nav3* mRNA rescue experiment were performed. The pericardial edemas and heart morphogenesis malformations were reproduced in *nav3*-deleted mutants and could also be partially rescued by co-injection of morpholino and *nav3* mRNA. Additionally, the mRNA rescue experiment also recapitulated that the severe cardiac edema was not caused by the off-target effects triggered by sgRNA or morpholino injection.

In our present work, we also noticed that the survival rates of *nav3*-deleted mutants were significantly lower compared with their WT counterparts, especially from the very early developmental stage (from 24 hpf). Since zebrafish could survive up to 7 days without a functional cardiovascular system, the high mortality upon Nav3 deletion might not result from the cardiac defects and more probably be attributed to its dysfunction in the nervous system or immunity (Carlsson et al., 2013; Karenko et al., 2005).

As mentioned above, Nav3 loss-of-function could lead to severe defects in zebrafish heart development. However, it remains largely unknown whether some essential cardiac-related genes are altered in *nav3*-null mutants. In our preliminary experiments, we examined the expressions of *nkx2.5*, *hand2*, *vmhc*, and *amhc* in *nav3*^{-/-} mutants and WT zebrafish embryos using ISH. We found that the expressions of *nkx2.5* and *hand2* were slightly decreased after the deletion

of *nav3* (Supplemental Fig. S2). Although they both play roles in embryonic heart development, each of them also owns a distinct and specific function in regulating this process. *Nkx2.5* determines the cardiomyocyte identity and can further maintain the vascular and atrial chamber morphology (Targoff et al., 2013). *Hand2* is implicated as a regulator of cardiomyocyte production, which promotes the generation of a proper number of cardiomyocytes during cardiogenesis (Schindler et al., 2014). As key regulators in heart development, the lower expressions of *nkx2.5* and *hand2* in *nav3*^{-/-} mutants supported its deficiency in cardiac development. This finding was consistent with the previous view that *nkx2.5* maintains cardiac chamber identity, and *hand2* regulates cardiac differentiation and morphogenesis (Schindler et al., 2014; Targoff et al., 2013). Although the structure and morphology of the atrium and ventricle were severely affected by the loss of *nav3*, the expressions of both their markers remained unchanged (Supplemental Fig. S2). We assumed that the cardiac-related myosin chain genes were not affected by the deletion of *Nav3*, and the heart morphogenesis defects might result from other factors, such as *nkx2.5*, or *hand2*.

Although *nav3* is initially discovered as a neural guidance gene in the nervous system, the NAV family members have also been widely accepted as regulators in cell migration among several species (Cohen-Dvashi et al., 2015; Klein et al., 2011; Maes et al., 2002; Schmidt et al., 2009; Stringham et al., 2002). Moreover, it has been reported to act as a positive modulator in regulating the actin assembly in the extension of filopodia and lamellipodia during zebrafish liver development (Klein et al., 2011). As a complicated but orchestrated morphogenetic process, heart formation involves specification, differentiation, and migration of cardiac progenitor cells. Therefore, NAV3 might also play a role in heart development by facilitating the migration of cardiac progenitor cells or cardiomyocytes. Besides, crosstalk between NAV3 and other essential factors, such as *nkx2.5*, might be involved in these crucial processes during cardiac development. Further efforts are needed to decipher the mechanism of NAV3-regulated cardiogenesis. Taken together, NAV3 was a potential regulator in cardiac development during embryogenesis.

Conclusions

The *nav3* gene can be expressed in a variety of tissues or organs although it is initially identified as a neural guiding factor. Gene knockout of *nav3* in zebrafish leads to severe development malformation of the heart. Moreover, the defective heart development can be rescued by *nav3* mRNA injection. All these findings indicate that *Nav3* was required for cardiogenesis in the development of zebrafish embryos.

Abbreviations

CH: calponin homology

dpf: days post-fertilization

HI: heartbeat intensity

hpf: hours post-fertilization

ISH: in situ hybridization

NAV: Neuron Navigator

SH3: Src Homology 3

UNC53: Uncoordinated-53

WISH: whole-mount in situ hybridization

WT: wild-type

Declarations

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Competing interests

The authors declare that they have no competing interests.

Ethics approval

All animal-related experiments were carried out following the NIH Guidelines for the care and use of laboratory animals (<http://oacu.od.nih.gov/regs/index.htm>), and animal protocols were ethically approved by the Administration Committee of Experimental Animals of Nantong

University, Jiangsu Province, China (Approval ID: 20180608-Z001). Transgenic zebrafish lines provided by others were approved by the owners with written informed consent.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Code availability

Not applicable.

Authors' contributions

CC and DL designed and conceptualised the project. FL, XG, PQ, and XL performed the experiment. FL and CC analyzed the data. CC wrote the manuscript. All authors read and approved the final manuscript.

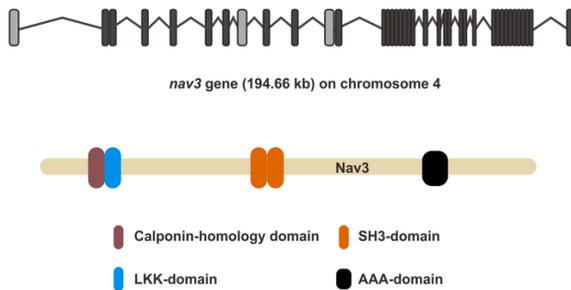
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Figures

A



B

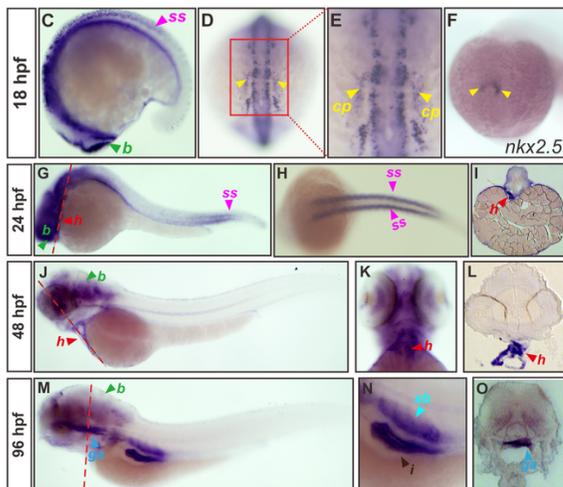
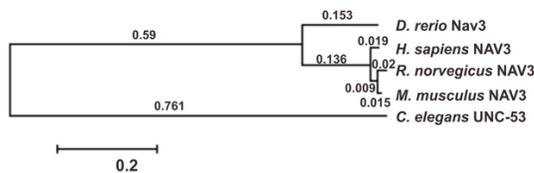


Figure 1

Phylogeny of NAV3 among species and the spatiotemporal expression of zebrafish *nav3* during embryogenesis. (A) Schematic representation of essential domains in NAV3 protein. It contains a calponin-homology domain, an LKK actin-binding domain, two putative SH3 domains, and ATPase domains of the AAA type. (B) Zebrafish Nav3 is closely related to other vertebrate homologs. A neighbor-joining tree was produced with MEAG5.0 software, displaying the relationships of NAV3 full-length amino acid sequences in *Danio rerio* (XP_021330780.1), *Homo sapiens* (NP_001019554.1), *Rattus norvegicus* (NP_001178711.1), *Mus musculus* (NP_001074504.1), and *Caenorhabditis elegans* (Q7YSI9). *Nav3* expression was detected with WISH at 18 (C-E), 24 (G-I), 48 (J-L), and 96 hpf (M-O). (C) *Nav3* expression was detected in the brain (*b*, green arrowhead) and somites (*ss*, magenta arrowhead). (D) Dorsal view of zebrafish embryo at 18 hpf. (E) A magnification view of the boxed area in D. *Nav3* was expressed in the cardiac primordium (*cp*, yellow arrowhead). (F) The expression of *nkx2.5* was in the cardiac primordium in zebrafish embryos at 18 hpf. At 24 hpf, *nav3* expression could be detected in the heart region (G, lateral view) and somites (H, dorsal view). Heart, *h*. (I) Images of the cross-section of zebrafish embryos. The red dashed lines indicate the cutting positions. (J-L) Expression of *nav3* was restricted to the heart at 48 hpf. Images were taken with a lateral view (J), dorsal view (K), and cross-sectional view (L). (M) Lateral view of *nav3* expression at 96 hpf. *Nav3* RNA transcripts were accumulated in the gill arch (*ga*, blue arrowhead). (N) Magnification view of the boxed area in M exhibits obvious *nav3* expression in the swim bladder (*sb*) and intestine (*i*). (O) Image of the cross-sectional area indicates the *nav3* expression in *ga*.

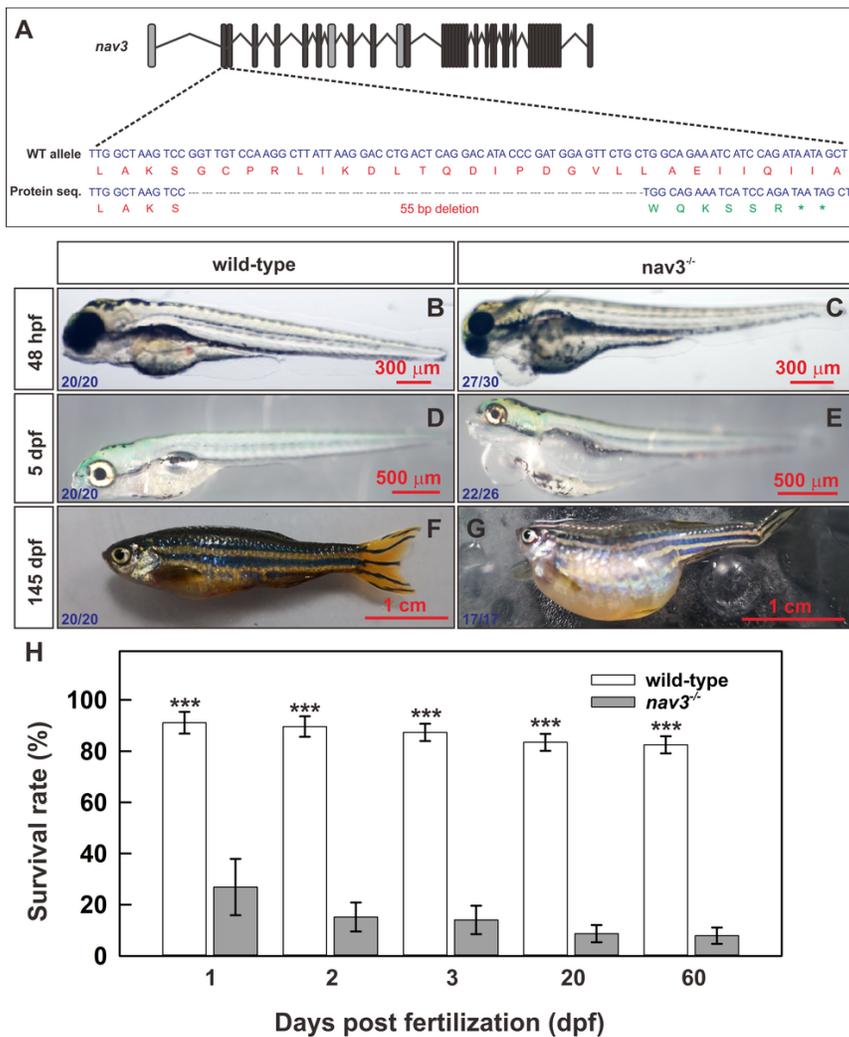


Figure 2

Deletion of zebrafish Nav3 leads to phenotypic defects in embryos and adults. (A) Schematic representation of *nav3* deletion in zebrafish through CRISPR/Cas9. The designed CRISPR sequence locates at the second exon of *nav3*. (B-G) Lateral views of WT (B, D, and F) and *nav3*-null mutants (C, E, and G) at 48 hpf (B and C), 5 dpf (D and E), and 145 dpf (F and G). The digits at the lower left position of each panel indicate the number of fishes with typical phenotype in total observed ones. (H) Survival rates of WT and *nav3*^{-/-} mutants raised at 28.5°C within 1, 2, 3, 20, and 60 dpf. Error bars indicate standard deviation. ***, p<0.001.

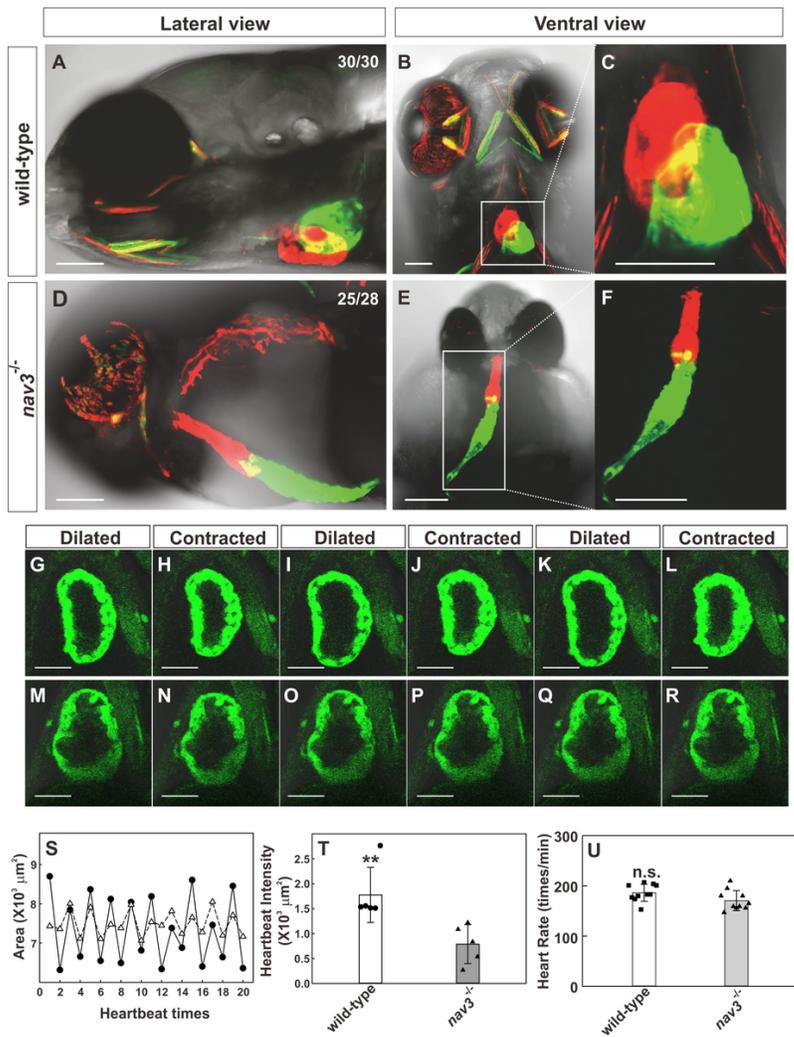


Figure 3

Nav3-deleted zebrafish embryos exhibit cardiac defects during cardiogenesis. (A-F) Lateral and ventral views of WT siblings and *nav3*-deleted mutants at 72 hpf. The atrium and ventricle were fluorescently labeled with GFP (green) and mCherry (red), respectively. Scale bars, 100 μm. The digits in the upper right corner of each panel indicate the number of zebrafish larvae with typical phenotype in total observed ones. (G-U) Functional analyses of heart in zebrafish embryos. (G-R) Fluorescently labeled heart chambers were imaged in the dilated and contracted states, respectively. (S) The difference in the heart chamber area between a contiguous dilated and contracted state was defined as the HI. Scale bars, 50 μm. (T and U) HI and heart rate of WT and *nav3*^{-/-} mutants at 72 hpf (n=10 in both WT and mutants). Error bars indicate standard deviation. **, p<0.01.

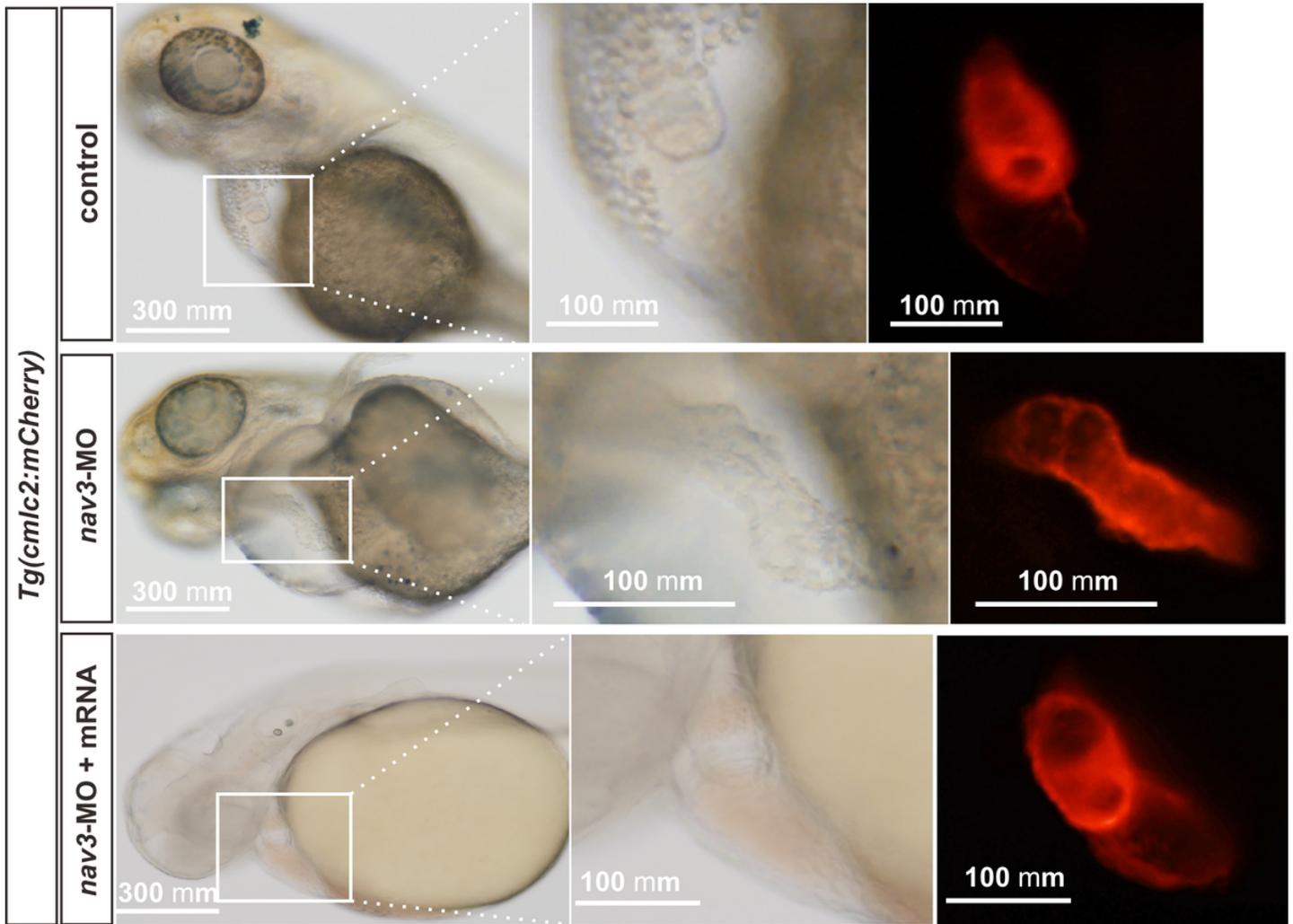


Figure 4

Supplementary Files

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- [supplementaryfigure.pdf](#)