Na,K-ATPase is essential for embryonic heart development in the zebrafish

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Summary

Na,K-ATPase is an essential gene maintaining electrochemical gradients across the plasma membrane. Although previous studies have intensively focused on the role of Na,K-ATPase in regulating cardiac function in the adults, little is known about the requirement for Na,K-ATPase during embryonic heart development. Here, we report the identification of a zebrafish mutant, *heart and mind*, which exhibits multiple cardiac defects, including the primitive heart tube extension abnormality, aberrant cardiomyocyte differentiation, and reduced heart rate and contractility. Molecular cloning reveals that the *heart and mind* lesion resides in the α1B1 isoform of Na,K-ATPase. Blocking Na,K-ATPase α1B1 activity by pharmacological means or by morpholino antisense oligonucleotides phenocopies the patterning and functional defects of *heart and mind* mutant hearts, suggesting crucial roles for Na,K-ATPase α1B1 in embryonic zebrafish hearts. In addition to α1B1, the Na,K-ATPase α2 isoform is required for embryonic cardiac patterning. Although the α1B1 and α2 isoforms share high degrees of similarities in their coding sequences, they have distinct roles in patterning zebrafish hearts. The phenotypes of *heart and mind* mutants can be rescued by supplementing *α1B1*, but not *α2*, mRNA to the mutant embryos, demonstrating that α1B1 and α2 are not functionally equivalent. Furthermore, instead of interfering with primitive heart tube formation or cardiac chamber differentiation, blocking the translation of Na,K-ATPase α2 isoform leads to cardiac laterality defects.

Supplemental figure available online

Key words: Heart development, Zebrafish, Na,K-ATPase

Introduction

Vertebrate hearts develop from the fusion of bilaterally positioned cardiac precursors, followed by the growth of the primitive heart tube. In the zebrafish, complex morphogenic events transforming the embryonic heart from sheets of cardiac precursors into a three-dimensional tubular structure have been previously described (Stainier and Fishman, 1992; Yelon, 2001; Yelon et al., 1999). The proper growth of the primitive heart tube is an important factor for subsequent patterning of cardiac chambers, as demonstrated by studies on the zebrafish *heart and soul* mutation (Peterson et al., 2001; Yelon et al., 1999). However, detailed molecular and cellular mechanisms guiding primitive heart tube extension are still largely unknown.

Na,K-ATPase is an integral membrane protein that transports Na^+ and K^+ across the plasma membrane to establish proper chemical and electrical gradients (for reviews, see Blanco and Mercer, 1998; Therien and Blostein, 2000). Its activity is essential for maintenance of the physiological function of many cell types. In the heart, it is believed that Na,K-ATPase regulates cardiac function through interaction with the Na^+/Ca^{2+} exchanger. Blocking Na,K-ATPase activity increases intracellular Na^+ concentration, which inhibits the activity of Na^+/Ca^{2+} exchanger, increases intracellular Ca^{2+} concentration and, thereby, enhances cardiac contractility (for a review, see Schwinger et al., 2003). In fact, Na,K-ATPase inhibitors, such as cardiac glycosides, are often used to enhance cardiac contraction in heart failure patients, and abnormal expression levels of Na,K-ATPase in the heart have been detected in heart failure and arrhythmia patients (Mohler et al., 2003; Schwinger et al., 1999).

Both the cation- and ATP-binding sites essential for catalytic and transport activity of Na,K-ATPase are both located in the α subunit but the enzyme activity requires dimerization of the α- and β-subunits (Therien and Blostein, 2000). Four isoforms of the Na,K-ATPase α subunit have been identified in mammalian cells (Blanco and Mercer, 1998). These isoforms exhibit overlapping but distinct expression patterns, and have dramatically different affinities to cardiac glycosides, such as ouabain, suggesting specific functional roles for these isofoms in regulating cardiac contraction, despite the high degree of similarity in their sequences and enzymatic properties. In fact, mice heterozygous for the α1 and α2 isoforms have opposite physiological responses in the heart and the skeletal muscle (He et al., 2001; James et al., 1999). The hearts of α2 heterozygous mice are hypercontractile, whereas the hearts of α1 heterozygotes are hypocontractile. The enhanced contraction activity noted in the cardiomyocytes of α2 heterozygotes correlates with the increased intracellular Ca^{2+} level. No such fluctuation in cardiac cells was noted in α1 heterozygotes. These findings lead to the hypothesis that Na,K-ATPase α1 and α2 isoforms conduct unique functions, and that the α2, but not α1, isoform modulates Ca^{2+} signaling during cardiac contraction.

Although the requirement for Na,K-ATPase in adult hearts has been intensively studied, very little is known about its role in embryonic hearts. Genetic studies in the mouse suggest that Na,K-ATPase activity is important during embryogenesis, as α1 homozygous mice are embryonic lethal and α2 homozygotes die during the first day after birth (James et al., 1999). However, whether the lethality is caused by defects in heart development requires further investigation. Gene expression analyses in the chick and zebrafish suggest that Na,K-ATPase may have an important role in embryonic heart formation. In the zebrafish, three Na,K-ATPase isoforms, α1B1 (also known as α1a.1), α2 and β1a, are expressed in the developing heart (Rajarao et al., 2001; Serluca et al., 2001). In the chick, Na,K-ATPase is also expressed in cardiac precursors. More interestingly, the localization of Na,K-ATPase protein switches from an initial even distribution, to a polarized lateral position on the plasma membrane of cardiac precursors at the time of heart tube formation (Linask, 1992), which suggests that Na,K-ATPase may be involved in guiding the growth of the primitive heart tube.

We report the identification of a zebrafish mutation, *heart and mind* (*had*), which is defective in the Na,K-ATPase α1B1 isoform. The *had* mutation causes severe abnormalities in primitive heart tube extension, cardiomyocyte differentiation and embryonic cardiac function, indicating crucial roles for the Na,K-ATPase α1B1 isoform in zebrafish heart development. In addition, we found that the α1B1 and α2 isoforms conduct different functions in developing zebrafish hearts. Despite the high degree of homology in α1B1 and α2 coding regions, *had* phenotypes can only be rescued by wild-type *α1B1* mRNA. Blocking translation of the α2 isoform does not cause significant defects in early cardiac patterning or embryonic heart function, but disturbs the establishment of cardiac laterality, further support that the α1 and α2 isoforms of Na,K-ATPase are not functionally equivalent.

Materials and methods

Zebrafish

Zebrafish and embryos were maintained and staged as previously described (Westerfield, 1995). The *heart and mind* mutation was identified in the UCLA zebrafish colony during routine intercrosses.

Linkage and sequence analyses

We established the *had* map cross by mating a male *had* heterozygote to a female fish from the EK strain. We analyzed linkages between *had* and simple sequence-length polymorphism (SSLP) markers (Shimoda et al., 1999). Genomic DNA samples were extracted from pools of 50 homozygous mutant embryos and their wild-type siblings. mRNA was extracted from pools of 50 homozygous mutant embryos and their wild-type siblings (RNAwiz, Ambion) for cDNA synthesis (ACCESS RT-PCR system, Promega). Five sets of primers, forward (F) and reverse (R), were used to amplify the coding region of Na,K-ATPase α1B1:

1, 5′-CCAGCGGTGACCAAGGAGAG-3′ (F), 5′-CTCAATAG-AGATGGGGGTGC-3′ (R);

2, 5′-CTCTTTCAAGAATTTGGTTCCC-3′ (F), 5′-CTGTTGGG-GTTCTGGTGGATG-3′ (R);

3, 5′-GTTGGTCGCACCCCCATCTC-3′ (F), 5′-TAATTGCT-CAGGGCTCAGATCC-3′ (R);

4, 5′-CAAGGCCATTGCCAAGGGGG-3′ (F), 5′-GGCACGCA-ATGTCTTGGTTCA-3′ (R); and

5, 5′-GGATCTGAGCCCTGAGCAAT-3′ (F), 5′-GCAGTGAT-GATGGTAGGAAG-3′ (R).

PCR and RT-PCR products were subcloned using TOPO TA Cloning Kit (Invitrogen) for subsequent sequencing analysis.

In situ hybridization and antibody staining

Embryos for in situ hybridization and immunohistochemistry were raised in embryo medium supplemented with 0.2 mM 1-phenyl-2-thiourea to maintain optical transparency (Westerfield, 1995). Whole-mount immunohistochemistry using monoclonal antibody S46 (from F. Stockdale, Stanford University) was carried out as described (Chen and Fishman, 1996). Whole-mount in situ hybridization was performed as described (Chen and Fishman, 1996). The antisense RNA probes used in this study were *Na,K-ATPase α1B1*, *wt1*, *pax2* (from F. Serluca), *cmlc2*, *vmhc*, *versican* (from D. Y. Stainier), *nkx2.5* and *irx1*.

Histology

Fixed embryos were dehydrated, embedded in plastic (JB-4, polysciences), sectioned at 8 μm and stained with Hematoxylin.

Ouabain treatment

Wild-type zebrafish embryos were raised in embryo media for the first 5 hours of development. At 5 hpf, ouabain (Sigma) was added to the embryo media to a final concentration of 1 mM. These embryos were grown in the presence of ouabain until 24 hpf or 50 hpf, and then were fixed in 4% paraformaldehyde for whole-mount in situ hybridization and immunohistochemistry.

Morpholino injections

Morpholino antisense oligonucleotides (Gene-Tools), complementary to the translation start site and its flanking sequence of the Na,K-ATPase α1B1 (α1B1MO, 5′-CTGCCAGCTCATATTGTTCTC-GGCC-3′) and α2 (α2MO, 5′-TTTCATGTCCGTACCCTTT-CCCCAT-3′) isoforms, were synthesized to block the translation of the α1B1 and α2 isoforms. Morpholino oligonucleotides with a 5-base pair mismatch to α1B1MO (5′-CTGgCAcCTCATAaTGTT-gTCGcCC-3′) and to α2MO (5′-TTTgATcTCCGTAgCCTTT-gCCgAT-3′) were synthesized as controls (lowercase letters indicate mismatched bases). Wild-type embryos were each injected with 2 ng of the morpholino oligoculeotide at one- to two-cell stage. Cardiac phenotypes were examined by whole-mount in situ hybridization at 24 and 50 hpf.

Phenotypic rescue

Capped mRNA for Na,K-ATPase α1B1 and α2 was synthesized by in vitro transcription with the mMESSAGE mMACHINE™ Kit (Ambion). Embryos from *had* heterozygote crosses were injected with 100 pg of mRNA at the one- to two-cell stage. Cardiac phenotypes of the injected embryos were examined by whole-mount in situ hybridization at 24 hpf. All injected embryos were genotyped using primers flanking the deletion site of the *had* allele (6F, 5′-

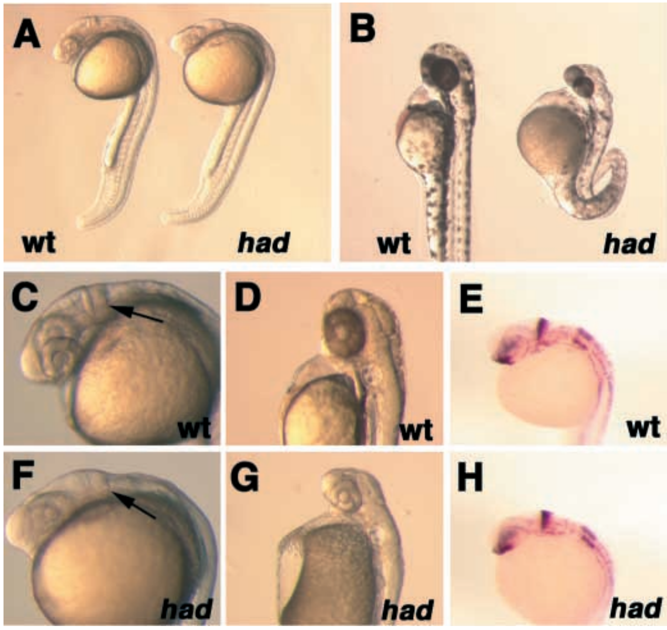

Fig. 1. Brain and body axis defects in *had* mutants. (A,B) Live wild-type (left) and *had* mutant (right) embryo. The body axis is normal in *had* mutants at 24 hpf (A) but becomes curly by 48 hpf (B). (C,F) By 24 hpf, the boundary of midbrain and hindbrain is distinctive in wild-type embryos (C, arrow), but not *had* mutants (F, arrow). (D,G) Two-day-old wild-type (D) and *had* mutant embryos (G). Note that two-day-old *had* mutant has developed edema. (E,H) *pax2* expression pattern in wild-type (E) and *had* mutant (H) embryos at 24 hpf.

GGGATTGTCCTGTAATCGTCA-3′; 6R, 5′-TTCTTCGGTGTT-CAACAGCAG-3′). The wild-type and mutant alleles can be distinguished by the size of PCR products. A 258 bp fragment is amplified from the wild-type allele, whereas a 201 bp fragment is amplified from the *had* allele.

Cell count

Embryonic cells are dissociated at 24 hpf using the mechanical dissociation method previously described (Westerfield, 1995). EGFP-positive cells were counted using a Zeiss Axioplan2 microscope.

Ventricular contractility analysis

Mutant embryos of *had* and their wild-type siblings were anesthetized for 5 minutes with Tricaine (0.16 mg/ml). These embryos were then transferred to a recording chamber perfused with modified Tyrode's solution (136 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.3) at 48 hpf. Cardiac contractions were recorded with a high-resolution video camera (Panasonic WV BL202) for 5 minutes. The lengths of ventricles in diastolic and systolic conditions were measured to calculate the ventricular shortening fraction (VSF). Values are presented as mean±s.e.m.

$$\text{VSF} = \frac{\text{Ventricular length at diastole} - \text{Ventricular length at systole}}{\text{Ventricular length at diastole}}$$

Results

The transparent nature of zebrafish embryos and the prominent ventral location of embryonic zebrafish hearts provide unique opportunities for the identification and characterization of

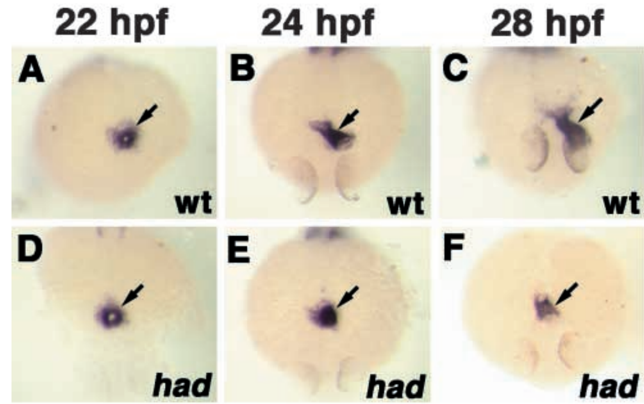

Fig. 2. Primitive heart tube extension is perturbed in *had* mutant embryos. (A-F) All panels show dorsal views, anterior to the bottom. Cardiac cells (arrows) are visualized by in situ hybridization analysis using a *vmhc* probe. (A) Bilateral cardiac primordia have fused at the midline in wild type zebrafish embryos at 22 hpf. (B) By 24 hpf, the fused heart has grown into a tubular structure, known as the primitive heart tube. (C) By 28 hpf, a long tubular heart is clearly visible in the wild types. In *had* mutant embryos, the fusion of bilateral cardiac primordia appears normal (D), but primitive heart tube extension is severely defective (E,F). (E) The primitive heart of the *had* mutant is a cone-shaped structure at the midline at 24 hpf, and the primitive heart tube is significantly shorter at 28 hpf (F), compared with that observed in wild-type siblings (C).

cardiovascular mutants (Alexander et al., 1998; Chen et al., 1996; Stainier et al., 1996). Here, we report the identification of a zebrafish mutation, named *heart and mind* (*had*). The body axis of one-day-old *had* mutants is normal, but these embryos manifest a curved body after two days of development (Fig. 1A,B). Defects of the developing brain is noted in *had* mutants by 24 hours post fertilization (hpf). Although the boundary between midbrain and hindbrain (MHB) is prominent in wild-type embryos, it is not as distinctive in *had* mutants (Fig. 1C,F). We used *Pax2* to analyze the development of MHB and found identical *Pax2* expression patterns in *had* mutants and their wild-type siblings (Fig. 1E,H), suggesting that the identity of MHB is specified in *had* mutants. In addition to the brain and body axis defects, cardiac development is severely affected by *had* mutation. The heart is small and the circulation is never established. It is likely that the severe cardiac patterning and functional abnormalities are primary causes of the embryonic lethality of *had* mutants after five days of development. Therefore, we focused on characterizing *had* cardiac defects.

had gene activity is required for primitive heart tube extension in the zebrafish

In the zebrafish, a morphogenic event known as primitive heart tube extension takes place immediately after the bilateral primordia fuse at the midline guiding cardiac precursors to develop from sheets of cells into a three-dimensional tubular structure (Stainier et al., 1993; Yelon et al., 1999). By 21 hpf, the bilateral cardiac primordia has fused at the midline, and a shallow cone consisting of myocardial precursors is placed at the midline with the apex of the cone raised dorsally (Fig. 2A). The cone soon shifts its axis from a dorsoventral plane to an anterioposterior plane. This tilt cone will then coalesce and extends into a tubular beating

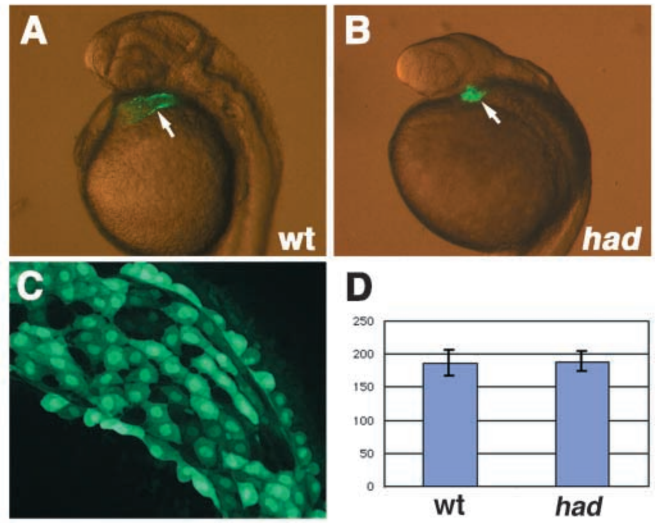

Fig. 3. The cmlc2-driven EGFP is expressed in cardiomyocytes. (A) The wild-type primitive heart is a long tubular structure. (B) The primitive heart tube is short in the *had* mutant embryo, but has a stronger EGFP signal. Arrows point to the heart. (C) A typical image of dissociated cardiomyocytes from a cmlc2:EGFP embryo at 24 hpf. (D) Comparison of the numbers of cardiomyocytes in wild-type and *had* mutant embryos at 24 hpf. The y-axis shows the number of EGFP-positive cells.

structure, known as the primitive heart tube, by 24 hpf (Fig. 2B). By using *ventricular myosin heavy chain* (*vmhc*) as a marker, we found that the *had* mutant heart remains as a shallow cone-shaped structure at 24 hpf (Fig. 2E). A small heart with no contraction was eventually detected in *had* mutants after 28 hpf (Fig. 2F). To investigate whether cardiac defects of *had* mutants manifest prior to the formation of primitive heart tube, we used early cardiac markers to analyze the heart primordia. We did not detect any difference in *vmhc* expression pattern between wild-type and *had* mutant embryos up to the stage when the bilateral cardiac primordia fuse at the midline (Fig. 2A,D). Similar results were obtained with *nkx2.5* and *cmlc2* probes (data not shown), suggesting that *had* gene activity is essential for primitive heart tube extension, but is not required for early cardiac cell fate determination or the fusion of cardiac primordia.

It is possible that the small heart phenotype observed in one-day-old *had* mutants is caused by general developmental delay, lack of sufficient cardiac cells or a blockage in tube extension. To distinguish these possibilities, we used *pax2*, *wt1* and *pdx1* to analyze the development of eye, pronephric, glomeruli and gut primordia. Expression patterns of *pax2* (Fig. 1E,H), as well as of *wt1* and *pdx1* (not shown), are identical between *had* mutants and wild-type embryos at 24 hpf, suggesting that the *had* mutation does not cause general developmental delay. We further analyzed cardiac cell numbers by crossing the *had* mutation into TG(cmlc2:EGFP) (Huang and Tsai, 2003). In this transgenic line, a *cmlc2* (*mylc2a* – Zebrafish Information Network) minimum promoter drives EGFP expression specifically in cardiomyocytes, which allows us to quantify cardiomyocytes by counting GFP-positive cells. As shown in Fig. 3, the primitive heart of *had* mutants is significantly shorter, but the GFP signal is much more intense compared with that of the wild-type heart, suggesting that *had* mutants may have similar numbers of cardiac cells as their wild-type siblings. To test this hypothesis, we counted GFP-positive cells, and detected similar numbers in one-day-old *had* mutants (188±15, *n*=12) and their wild-type siblings (186±19, *n*=16). These data clearly demonstrate that sufficient cardiomyocytes are produced in *had* mutants, but these cells maintain close contact to each other and fail to grow into a long tubular structure, which suggests that the primitive heart defect observed in *had* mutants is caused by the blockage of tube extension.

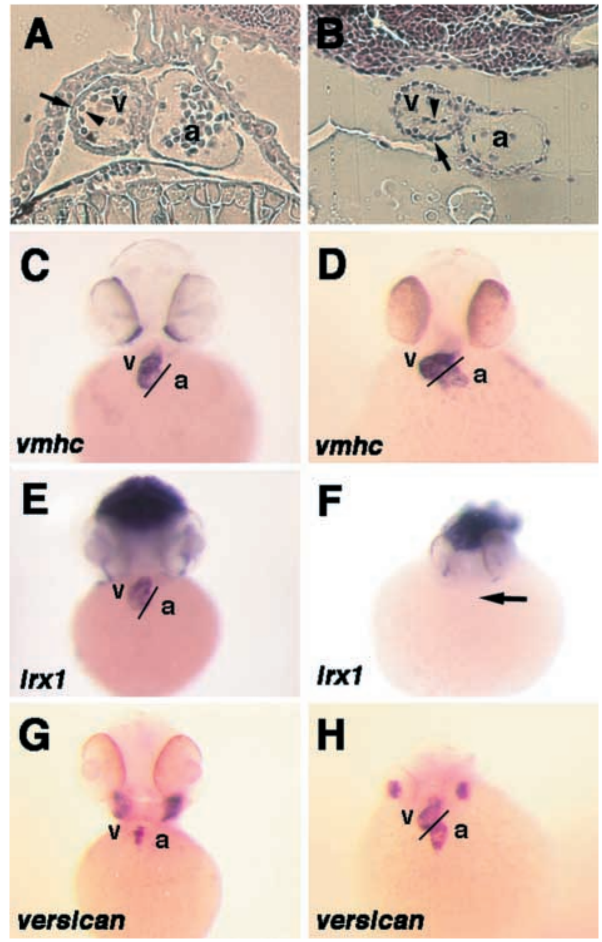

Fig. 4. Cardiomyocyte differentiation defects in *had* mutants. (A,B) Transverse sections of two-day-old wild-type (A) and *had* mutant (B) heart. Arrow indicates myocardium; arrowhead, endocardium. (C-H) Ventral views of 50 hpf embryos, head to the top, solid line marks the site of the constriction between the ventricle (v) and the atrium (a). Cardiac expression of *vmhc* (C,D), *irx1* (E,F) and *versican* (G,H) was detected by in situ hybridization. The hearts of *had* mutants are smaller and dysmorphic (B,D,F,H) compared with those in wild types (A,C,E,G). By 50 hpf, expression of *vmhc* is restricted to ventricles in wild types (C), but expression of this gene extends to the atrium in *had* mutants (D). Cardiac expression of *irx1* is restricted to ventricles in wild-type embryos (E), but is severely reduced in the heart of *had* mutants (F, arrow). *versican* is expressed at the boundary of the atrium and the ventricle in wild-type embryos (G), but is expressed throughout *had* mutant hearts (H).

Cardiomyocyte differentiation is affected in *had* mutants

Both cardiac chambers (atrium and ventricle) and both cardiac cell types (myocardium and endocardium) are developed in two-day-old *had* mutants. However, the heart is small, the ventricular wall is thin, and the space between the myocardial and endocardial cells is enlarged in *had* mutants (Fig. 4A,B). To investigate whether cardiomyocytes are properly differentiated, we used multiple cardiac chamber specific markers to analyze two-day-old *had* mutant hearts. Transcripts of *vmhc*, *irx1* and *versican* (*cspg2* – Zebrafish Information Network) are initially present throughout the primitive heart tube. However, after two days of development, cardiac expression of *vmhc* and *irx1* become ventricle-specific, whereas *versican* is localized at the boundary of the atrium and ventricle (Fig. 4C,E,G) (Chen and Fishman, 1996; Cheng et al., 2001; Walsh and Stainier, 2001; Yelon et al., 1999). These chamber-specific expression patterns are disrupted in *had* mutant hearts. Significant *vmhc* transcripts are detected in the atrium as well as in the ventricle of *had* mutant hearts (Fig. 4D). The expression of *irx1* in cardiac ventricle is drastically reduced in *had* mutant hearts (Fig. 4F), and transcripts of *versican* can be found throughout the heart (Fig. 4H). These results support the notion that chamber-specific differentiation of cardiomyocytes is incomplete in *had* mutant embryos.

had mutant hearts are bradycardiac and hypocontractile

In addition to cardiac patterning defects, we observed functional abnormalities in *had* mutant hearts. No beating heart was observed in *had* mutant embryos at 24 hpf, and a slow heart with weak contraction is evident in *had* embryos after two days of development. The heartbeat of two-day-old wild-type zebrafish embryos is strong and fast, ensuring circulation throughout the body. In *had* mutants, the heart rate drops, from the average of 120.1±1.1 beats per minute (bpm) (n=42) observed in the wild-type siblings, to 96.7±2.5 bpm (n=27) (P<0.001). Additionally, cardiac contraction is apparently reduced and no circulation is ever established in *had* mutant hearts. We quantified cardiac contractility by measuring the ventricular shortening fraction (VSF). The average VSF of the wild-type siblings is 19.9±1.1% (n=28), whereas the VSF value is 8.8±0.7% in *had* mutants (n=18) (P<0.001), demonstrating that, in addition to cardiac patterning, *had* possesses an important role in regulating cardiac contractility.

had encodes Na,K-ATPase α1B1

We mapped *had* to zebrafish Linkage Group 1 (LG1) (Shimoda et al., 1999). No recombination between *had* and the genetic marker z6384 was detected in 848 meiosis (Fig. 5A). A zebrafish EST (fa03c03), which shows significant homology to the Na,K-ATPase α subunit, is also mapped to the same region of LG1 (http://zfrhmaps.tch.harvard.edu/ZonRHmapper/Maps.htm). Nine isoforms of the Na,K-ATPase α subunit have previously been cloned in the zebrafish (Blasiole et al., 2002; Rajarao et al., 2001; Serluca et al., 2001). Interestingly, expression of the Na,K-ATPase α1B1 isoform (also known as α1a.1) was detected in the developing zebrafish heart (Canfield et al., 2002; Serluca et al., 2001), and was previously mapped to LG1 (Rajarao et al., 2001; Serluca et al., 2001). We therefore considered Na,K-ATPase α1B1 to be a good candidate gene for *had*. To further analyze whether the *had* lesion resides in α1B1, we amplified α1B1 from wild-type and *had* mutant embryos by the RT-PCR approach. Whereas a single fragment was amplified from wild-type RNA using primers 5F and 5R, two truncated fragments were obtained from *had* mutant RNA (Fig. 5D). Sequencing of genomic DNA revealed 4 exons between primers 5F and 5R (depicted as exon A-D in Fig. 5B) in the wild-type, and a 2 bp insertion followed by a 59 bp deletion in *had* mutants. This deletion encompasses the 3′ exon-intron boundary of exon C and presumably results in aberrant splicing.

We sequenced 20 random clones generated from the *had* mutant RT-PCR product. Nineteen clones have a 330 bp internal deletion, corresponding to the lower band detected by RT-PCR. This splice variant lacks exons B and C, and results in a 93 amino acid in-frame internal deletion. Three of the ten transmembrane domains of α1B1 (TM5-TM7) are missing in this mutated protein (Fig. 5E) (Lingrel and Kuntzweiler, 1994). One clone from the 20 sequenced contains a 120 bp deletion, missing exon C alone. The predicted protein of this minor splice variant has a stop codon at amino acid 822, which results in a truncated protein missing C-terminal sequences, including TM7-TM10 (Fig. 5E). Furthermore, *α1B1* mRNA level is significantly reduced in *had* mutants. The low level of *α1B1* transcripts was detected in *had* mutant embryos up to the 20-somite stage (data not shown), but no signal was detected in the heart of *had* mutants by in situ hybridization after two days of development (Fig. 5F). Similar results were obtained by RT-PCR analysis (data not shown). These findings suggest that the *had* mutation might be a null, or severely hypomorphic, allele.

Blocking Na,K-ATPase activity phenocopies *had*

To confirm that the mutation in α1B1 causes *had* phenotypes, we blocked Na,K-ATPase activity by applying ouabain, a Na,K-ATPase inhibitor, to wild-type zebrafish embryos, and by injecting a morpholino antisense oligonucleotide targeting the α1B1 translation initiation site (α1B1MO) to wild-type zebrafish embryos at the one-cell stage (Nasevicius and Ekker, 2000). Both ouabain treatment and α1B1MO injection yielded embryos essentially identical to *had* mutants (Fig. 6B,C,D). We further analyzed the effects of these treatments on heart tube extension and cardiac chamber differentiation by in situ hybridization using the *vmhc* probe. After one day of development, 63% of ouabain-treated embryos (n=104) and 94% of α1B1MO-injected embryos (n=106) show severe defects in heart tube extension similar to those observed in *had* mutants (Fig. 6G,H,I). After two days of development, cardiac expression of *vmhc* is no longer restricted to the ventricle in 74% of ouabain-treated embryos (n=82) and 82% of α1B1MO injected embryos (n=99) (Fig. 6M,N), as was observed in *had* mutants (Fig. 6L). No such phenotypes were observed in untreated control embryos (n=105), nor in embryos injected with the 5 bp-mismatch α1B1 control morpholino (n=72). Therefore, inhibition of Na,K-ATPase α1B1 activity produces *had* mutant phenotypes, strongly supporting the hypothesis that mutation in α1B1 is responsible for the *had* phenotype. Furthermore, we noted that in order to completely phenocopy *had* phenotypes, ouabain treatment needed to be conducted prior to the onset of gastrulation, suggesting that Na,K-ATPase α1B1 activity is required during early embryogenesis.

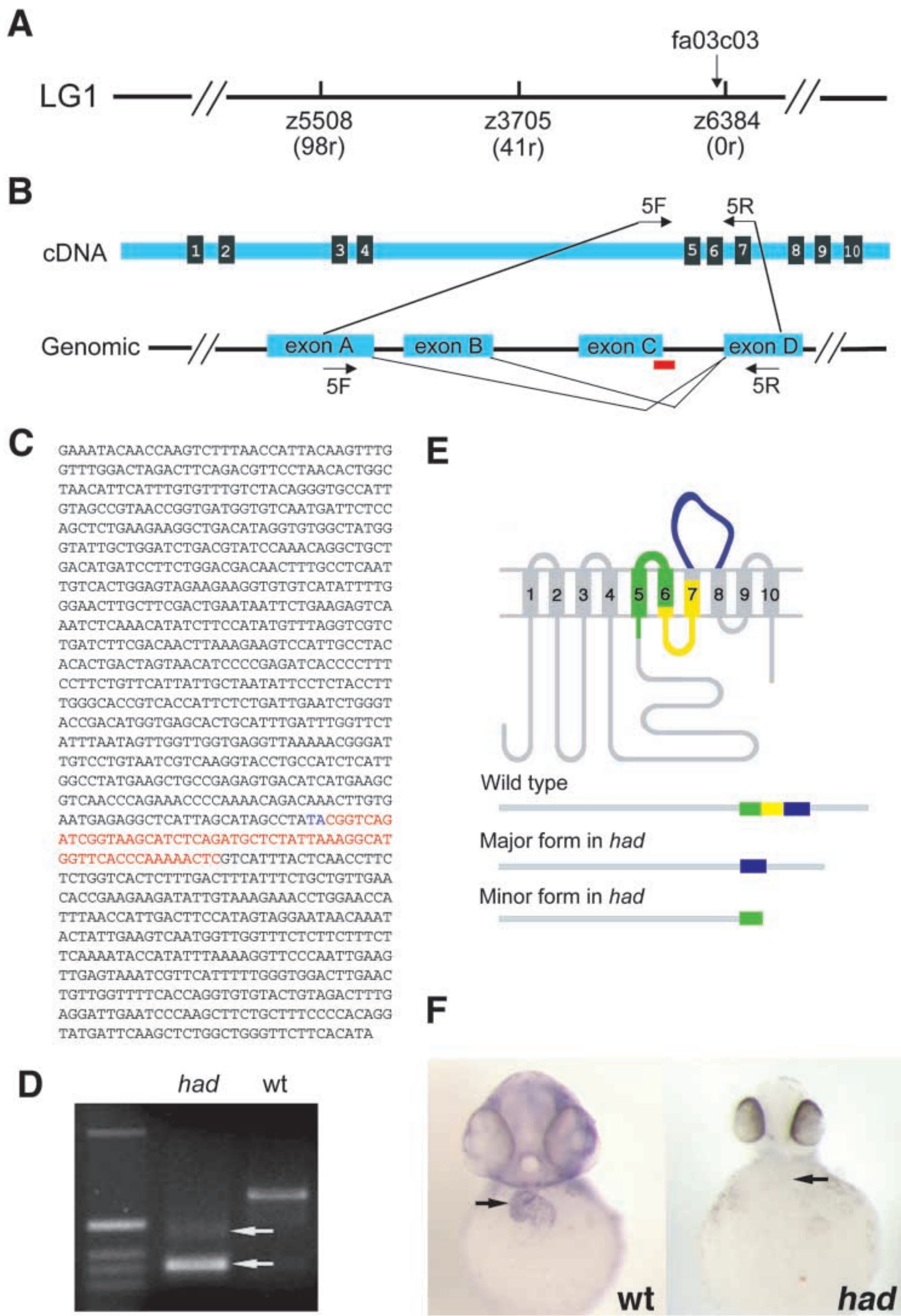

Fig. 5. *had* encodes the zebrafish Na,K-ATPase α1B1 isoform. (A) Linkage analysis shows 98 recombinants to z5508, 41 recombinants to z3705 and no recombinants to Z6384 of LG1 in 848 meiosis. (B) Schematic cDNA and genomic structures of zebrafish Na,K-ATPase α1B1. Dark boxes depict transmembrane domains (TM1-TM10). Arrows indicate positions of primers (5F and 5R) used for PCR analysis. Four exons (depicted by blue-boxes, labeled as exon A-D) were amplified from the genomic DNA using the 5F and 5R primers. A deletion at the 3′ boundary of exon C (red bar) was detected in *had* mutants. (C) Genomic sequence of α1B1 between the 5F and 5R primer sequences. The 2 bp-insertion is shown in blue and sequences deleted in *had* are shown in red. (D) RT-PCR analysis shows two alternative splicing variants (arrows) in *had* mutants. (E) Predicted structure of the Na,K-ATPase α1B1 protein. Amino acids encoded by exon B are shown in green and those encoded by exon C are in yellow. The purple region represents sequences between TM7 and TM8, which include crucial elements for the interaction of the α and β subunits. (F) The α1B1 isoform is highly expressed in the two-day old wild-type embryonic heart, but is not detectable in *had* mutant heart. Arrows point to the heart.

Overexpression of Na,K-ATPase α1B1 rescues *had* cardiac phenotypes

To provide additional confirmation that mutation in the α1B1 isoform is responsible for *had* phenotypes, we investigated whether injecting *α1B1* mRNA could rescue the *had* mutant phenotype. We microinjected 100 pg of *α1B1* mRNA to embryos collected from *had* heterozygous crosses at the one-cell stage. All *α1B1* mRNA-injected embryos were genotyped, and the primitive heart tube extension phenotypes were analyzed by in situ hybridization using *vmhc* as a probe. We found that microinjection of 100 pg of *α1B1* mRNA did not lead to any morphological changes in wild-type and *had* heterozygous embryos (data not shown), but was sufficient to rescue the brain and primitive heart tube extension phenotypes of one-day-old *had* homozygotes (80% fully rescued, *n*=39) (Fig. 7C). Interestingly, *had* cardiac phenotypes gradually become apparent in those 'rescued' embryos after two days of development. The heart failed to beat rigorously to generate proper circulation, and *vmhc* transcripts can be detected in the atrium (55%, *n*=20) at 50 hpf, by which time the injected mRNA is presumably degraded. These data demonstrate that mutation in the Na,K-ATPase α1B1 isoform is indeed responsible for the cardiac defects observed in *had* mutants, and that Na,K-ATPase α1B1 activity is required continuously during zebrafish embryonic heart development.

Na,K-ATPase α1B1 and α2 isoforms have distinct functions in cardiac patterning

In addition to α1B1, the α2 isoform of Na,K-ATPase is expressed in the developing zebrafish heart (Canfield et al.,

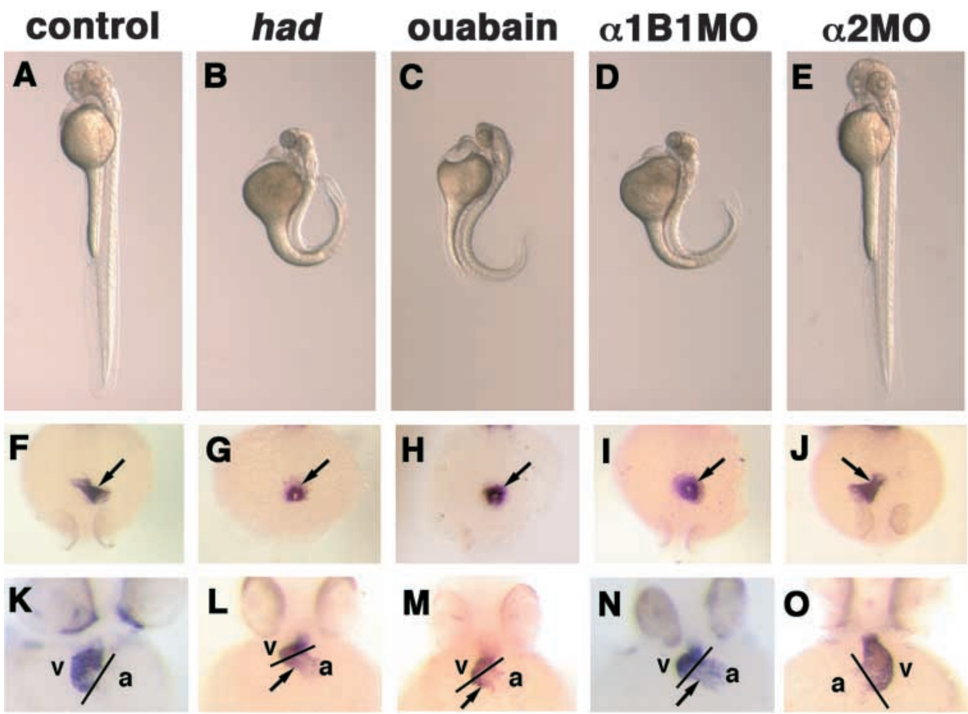

Fig. 6. Functional analyses of cardiac Na,K-ATPase isoforms. (A-E) Lateral views of 50 hpf embryos, head to the top. Ouabain treatment (C) and α1B1MO injection (D) create embryos morphologically indistinguishable from *had* mutants (B). The gross morphology of α2MO-injected embryos (E) is similar to that of wild-type controls (A). (F-O) Cardiac tissues are visualized by in situ hybridization using a *vmhc* probe. (F-J) Dorsal views of embryos at 24 hpf, head to the bottom; the arrows point to the heart. (K-O) Ventral views of 50 hpf embryos, head to the top; the solid line marks the constriction between the atrium (a) and the ventricle (v). (F) Wild-type control embryos at 24 hpf. Ouabain treatment (H) and α1B1MO (I) injection perturbed primitive heart tube extension in one-day-old embryos similar to the *had* mutation (G). (K) *vmhc* expression is restricted to the ventricle of wild-type control embryos at 50 hpf. The expression of *vmhc* extends into the atrium of *had* (L), ouabain-treated (M) and α1B1MO-injected (N) embryos. (J) Cardiac jogging is affected by α2MO. Some α2MO-injected embryos have the primitive heart tube placed on the right side of the embryos. After two days of development, abnormal cardiac looping is observed in α2MO-injected embryos. Some fail to loop and some have the ventricles on the left of the atrium (O).

2002; Serluca et al., 2001). These isoforms share a high degree of similarity in their coding sequences (83% identity). To analyze whether the α1 and α2 isoforms are functionally equivalent in the zebrafish heart, we injected 100 pg of *α2* mRNA to *had* homozygous embryos and their wild-type siblings. As with *α1B1* mRNA, overexpression of α2 did not cause notable morphological defects in wild-type and *had* heterozygous embryos. However, cardiac phenotypes of *had* mutant embryos injected with 100 pg of *α2* mRNA were indistinguishable from those observed in uninjected *had* mutants (*n*=55) (Fig. 7D), demonstrating that α2 could not compensate the loss of α1B1 activity, and indicating that α1B1 and α2 might conduct different functions during heart development.

To investigate the role of the Na,K-ATPase α2 isoform in zebrafish heart development we created a morpholino antisense oligonucleotide targeting the α2 translation initiation site (α2MO). We tested the inhibition ability of α2MO in vivo by injecting α2MO together with *α2-RFP* mRNA. *α2-RFP* is a chimera of Na,K-ATPase α2 with the coding region of Red Fluorescent Protein (RFP) fused in frame at the C terminus. We detected a strong RFP signal in embryos injected with 50 pg *α2-RFP* mRNA, but this signal is completely suppressed when co-injected with 2 ng of α2MO (see Fig. S1 at http://dev.biologists.org/supplemental/). This data demonstrates the effectiveness of α2MO in inhibiting translation of the Na,K-ATPase α2 isoform in vivo.

We injected one-cell stage wild-type zebrafish embryos with the α2MO to inhibit the translation of Na,K-ATPase α2 isoform, and observed neither primitive heart tube extension defects nor a reduction in the contractility of the embryonic hearts (*n*=120). The gross morphology of the α2MO-injected embryos appeared normal after two days of development (Fig. 6E). However, upon more careful analysis, we discovered a novel role for the α2 isoform in regulating cardiac laterality. In the zebrafish, the primitive heart tube is placed on the left side of the embryo by 24 hpf (cardiac jogging). This left-jogged heart gradually swings back to the midline before the ventricle bends to the right of the atrium (cardiac looping) (Chen et al., 1997). We found that 51% of α2MO-injected embryos analyzed failed to jog to the left (18% right, 33% midline, *n*=88) (Fig. 6J). 49% of α2MO-injected embryos analyzed (*n*=113) exhibited abnormal looping (18% had a reversed looping and 31% failed to loop and remained as a straight heart tube) (Fig. 6O). Such laterality defects were not observed in the control groups. Only 3% of uninjected control embryos (*n*=71) and 10% of embryos injected with the α2 5-bp-mismatch control morpholino oligonucleotide (*n*=96) exhibit cardiac looping abnormality. These results indicate that α1B1 and α2 isoforms are not functionally equivalent and that α2 activity is required for establishing cardiac laterality.

Discussion

heart and mind encodes the Na,K-ATPase α1B1 isoform

The *heart and mind* mutant exhibits strong defects in the patterning and function of developing zebrafish hearts, starting from the stage when cardiac precursors coalesce and extend into a tubular structure, and progressing to manifest in a small heart with aberrant cardiac chamber differentiation, and bradycardiac and hypocontractile phenotypes. Molecular

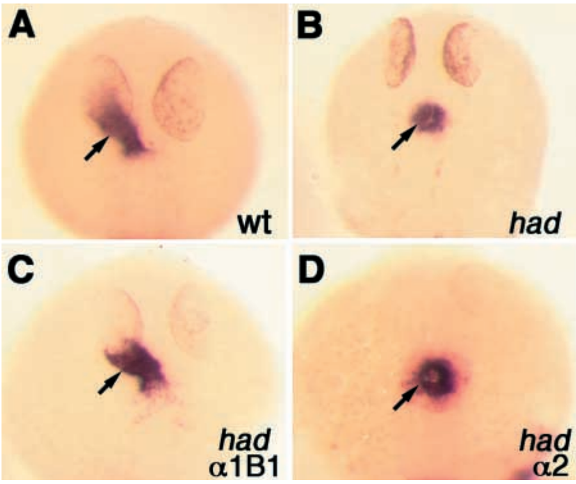

Fig. 7. Rescue of *had* cardiac phenotypes. At 24 hpf, the heart is a tubular structure in the wild-type embryos (A), but remains as a shallow cone in *had* mutant embryos (B). (C) Injecting *α1B1* mRNA into *had* mutant embryos results in the development of a normal tubular heart. (D) Hearts of *had* embryos injected with *α2* mRNA remain as a shallow cone.

cloning reveals a small deletion in the Na,K-ATPase α1B1 isoform, which induces aberrant splicing in *had* mutant embryos. Blocking α1B1 activity by ouabain treatment and a morpholino knockdown approach phenocopies the patterning and functional defects observed in *had* mutants. These data clearly indicate that *heart and mind* encodes Na,K-ATPase α1B1, and demonstrate for the first time that Na,K-ATPase α1B1 activity is required during embryonic heart formation.

Na,K-ATPase activity is required for embryonic cardiac function

A large number of physiological studies have firmly established Na,K-ATPase as a crucial component in regulating postnatal cardiac function (for a review, see Schwinger et al., 2003). Recent studies on cardiac and skeletal muscle contraction of Na,K-ATPase α1 and α2 heterozygous mice suggest that these isoforms have different roles in regulating Ca^{2+} signaling, which lead to the opposite physiological effects observed in α1 and α2 heterozygotes (He et al., 2001; James et al., 1999). The bradycardiac and hypocontractile phenotypes observed in *had* mutants are similar to the reduced contractility phenotype observed in adult α1 heterozygous mice (James et al., 1999), suggesting that the zebrafish Na,K-ATPase α1B1 isoform may regulate embryonic cardiac function in a Ca^{2+} independent manner, as mouse α1 does in adult hearts.

Na,K-ATPase α1B1 regulates embryonic cardiac patterning

Our studies on *had* mutants revealed previously undiscovered roles of Na,K-ATPase in cardiac morphogenesis. We found that the Na,K-ATPase α2 isoform is important for establishing cardiac laterality, and that the α1B1 isoform is required for primitive heart tube extension and cardiomyocyte differentiation. The discovery of the involvement of Na,K-ATPase α1B1 in primitive heart tube extension is an exciting one, because it provides a handle for future molecular and cellular studies on mechanisms governing heart tube extension. There are two equally plausible, and not mutually exclusive, cellular mechanisms by which Na,K-ATPase regulates primitive heart tube extension. One possibility is that Na,K-ATPase α1B1 regulates primitive heart tube extension by rearranging the cytoskeleton, as the Na,K-ATPase α subunit is known to be associated with multiple cytoskeletal proteins (for a review, see Therien and Blostein, 2000). The other possibility is that the polarity of cardiomyocytes is involved in primitive heart tube formation. Na,K-ATPase assumes a polarized position in epithelial cells, as well as in cardiac precursors during primitive heart tube formation in the chick (Linask, 1992). A recent study has shown that the basolateral distribution of Na,K-ATPase requires functional atypical PKC (Suzuki et al., 2001). Therefore, we are intrigued to note that mutation in the zebrafish atypical PKCλ results in phenotypes similar to those caused by the *heart and mind* mutation. Both mutations manifest in a primitive heart tube extension defect, as well as brain defects and an upwardly curved body (Horne-Badovinac et al., 2001; Peterson et al., 2001; Stainier et al., 1996; Yelon et al., 1999). Similarities in the spectrum of phenotypes shared between these two mutants suggest that Na,K-ATPase α1B1 and aPKCλ may direct primitive heart tube extension by regulating the polarity of cardiac cells.

Divergent functions of Na,K-ATPase isoforms in heart development

Multiple isoforms of Na,K-ATPase are expressed in mammalian hearts, and these isoforms conduct different functions in regulating cardiac function (James et al., 1999). We found a similar situation in the developing zebrafish hearts. Both the α1B1 and α2 isoforms are expressed in the developing zebrafish heart and they have distinct roles in heart development, as demonstrated by the morpholino knockdown and mRNA rescue experiments. The α1B1 isoform regulates the early patterning and contractility of developing zebrafish hearts, whereas the α2 isoform is required for proper cardiac laterality. It is not clear how molecules sharing such a high degree of similarity in their coding sequences and enzymatic activities assume such different functions. Identifying interacting proteins and signaling networks of each Na,K-ATPase isoform will provide further understanding of cardiomyocyte function and differentiation. Moreover, recent studies suggest that Na,K-ATPase isoforms have significant differences in their affinities for cardiac glycosides (Muller-Ehmsen et al., 2001), and that cellular physiological responsiveness to ouabain is dosage dependent (Aizman et al., 2001). As Na,K-ATPase inhibitors are often used to enhance cardiac contractility, studying the diverse functions of the Na,K-ATPase isoforms and their signaling network may lead to better treatment for heart diseases, and to the design of drugs that have more precisely targeted actions.

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