The small heart Mutation Reveals Novel Roles of Na\(^+\)/K\(^+\)-ATPase in Maintaining Ventricular Cardiomyocyte Morphology and Viability in Zebrafish

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Abstract—Forward genetic screens in zebrafish have been used to identify mutations in genes with important roles in organogenesis. One of these mutants, small heart, develops a diminutive and severely malformed heart and multiple developmental defects of the brain, ears, eyes, and kidneys. Using a positional cloning approach, we identify that the mutant gene encodes the zebrafish Na\(^+\)/K\(^+\)-ATPase α1B1 protein. Disruption of Na\(^+\)/K\(^+\)-ATPase α1B1 function via morpholino “knockdown” or pharmacological inhibition with ouabain phenocopies the mutant phenotype, in a dose-dependent manner. Heterozygosity for the mutation sensitizes embryos to ouabain treatment. Our findings present novel genetic and morphological details on the function of the Na\(^+\)/K\(^+\)-ATPase α1B1 in early cardiac morphogenesis and the pathogenesis of the small heart malformation. We demonstrate that the reduced size of the mutant heart is caused by dysmorphic ventricular cardiomyocytes and an increase in ventricular cardiomyocyte apoptosis. This study provides a new insight that Na\(^+\)/K\(^+\)-ATPase α1B1 is required for maintaining ventricular cardiomyocyte morphology and viability. (Circ Res. 2004;95:595-603.)

Key Words: apoptosis ▪ cardiac ▪ development ▪ heart failure ▪ organogenesis ▪ Na\(^+\)/K\(^+\)-ATPase ▪ Danio rerio

Formation of the vertebrate heart is a complex and dynamic biological process controlled by genetic and epigenetic factors.\(^1\) Numerous genes contribute to the processes of cell determination, migration, differentiation, and coordinated functions that are necessary for cardiogenesis. Perturbations of any of these processes may lead to cardiac dysmorphogenesis.

Human genetic studies have identified a number of genes that are associated with congenital heart defects, including several T-box genes and the nkx2.5 gene.\(^2,3\) Recently, the identification of additional genes involved in cardiac development has been aided by using model organisms, such as the zebrafish.\(^4\) The genes governing cardiac development are well-conserved between human and zebrafish, and the characteristics of the zebrafish embryo facilitate the identification and characterization of cardiac defects. The identities of some genes involved in cardiogenesis have been revealed by positional cloning of zebrafish genes mutated in large-scale genetic screens; these include orthologs of human genes implicated in cardiogenesis as well as genes not previously thought to be important in cardiac development.\(^5\)

The small heart (slh\(^{pm156}\)or slh\(^{−/−}\)) mutant was identified in a screen for genes involved in cardiovascular development; this same mutant was also recently reported as a spontaneous mutation by another laboratory and named heart and mind.\(^6\) Homozygous small heart mutants develop a diminutive heart, suggesting that the mutant gene plays an essential role in establishing appropriate organ size during cardiac development. The small heart gene encodes the zebrafish Na\(^+\)/K\(^+\)-ATPase α1B1 protein. In our studies, we have systematically investigated the mechanism of the small heart defect in the mutant. First, our findings begin with the identification of the mutation by positional cloning with a high-resolution genetic and physical map and the full genomic structure of the Na\(^+\)/K\(^+\)-ATPase α1B1 gene. Second, our experiments demonstrate that the efficiencies of the inhibition of the Na\(^+\)/K\(^+\)-ATPase α1B1 function and the overexpression rescue are dosage dependent. Importantly, heterozygous embryos are more sensitive to the inhibition treatments than wild-type embryos. Third, cellular analysis reveals that the reduced size of the mutant heart is caused by dysmorphic ventricular cardiomyocytes and an increase in ventricular cardiomyocyte apoptosis. These results provide direct in vivo evidence that genetic loss of function or inhibition of Na\(^+\)/K\(^+\)-ATPase α1B1 promotes ventricular cardiomyocyte apoptosis and deterioration of the heart. Finally, our data provides insight into a critical role for Na\(^+\)/K\(^+\)-ATPase α1B1 in organogenesis, beyond its role in formation of a functional heart of normal size.

Materials and Methods

Zebrafish Strains

The small heart mutant was discovered in ENU-mutagenesis screens conducted at the MGH/Cardiovascular Research Center. Genetic mapping lines were generated by outcrossing heterozygote fish (TL...
strain) to a wild-type WIK strain. Embryos were raised and staged as previously described.7

Genetic and Physical Mapping of the Mutant Gene
Bulk segregant analysis was conducted on pools of genomic DNA from mutant and wild-type embryos to establish initial genetic linkage,8 and the genetic interval was defined by analysis of individual embryos using additional markers from the locus. Single strand conformation polymorphic markers were designed against ESTs (expressed sequence tags) mapped to this region. Genetic and physical maps were coordinated with the Goodfellow radiation hybrid panel.9

Detection of the Small Heart Mutation
Reverse transcription of total RNA extracted from wild-type or mutant embryos at 48 hours post-fertilization (hpf) was performed using an RT-PCR kit (Promega). We amplified and cloned seven overlapping fragments of the Na+/K+-ATPase α 1B1 coding region from both wild-type and mutant cDNA pools. Two abnormal RT-PCR fragments in the mutants were found using the primers F (5’-GTGCCATTTGACCCCAAC-3’) and R (5’-TCCCTGCT-GAAGACGACT-3’). Genomic DNA fragments were amplified using these same primers and sequenced. Based on the Sanger Center Genomic Sequence Databases, we identified the full genomic structure of zebrafish Na+/K+-ATPase α 1B1 gene.

Morpholino Injection and Inhibitor Treatment
To confirm the identity of the mutant gene, morpholino antisense oligonucleotides were designed against the translational start site (M1: 5’-TCTCCCTGCTCCCATTITGCTGCTTT-3’) and internal splice donor site (M2: 5’-GATGCTTACCGATCTGACCGTAGGC-3’) of the gene (GeneTools, LLC). The morpholinos were diluted to 50 to 500 pmol/L (0.42 to 4.2 ng) with Danieau’s solution, and injected into one-cell stage wild-type embryos.

To test pharmacological inhibition of the Na+/K+-ATPase α 1B1, wild-type embryos were treated at 6 or 48 hpf with different concentrations (1 to 5 mmol/L) of ouabain (Sigma), a specific inhibitor of Na+/K+-ATPase. The effects of the inhibitors were determined by genotyping.

mRNA Injection
Full-length Na+/K+-ATPase α 1B1 cDNAs were generated by RT-PCR with primers including Cld/α 1B1, α 1B1-Primer: 5’-CCATCGATGAGCCGTC CCTCACGCCTGACCAA-3’; XbaI-Primer: 5’-GCT/CTAGA GGCCACAAGGCAGCGCAATGT-3’. The PCR fragments were subcloned into pCS2’. The pCS2’ constructs were linearized by NotI and capped mRNA was transcribed in vitro using the SP6 message machine kit (Ambion). Embryos were injected at the one-cell stage with 50 to 100 pg mRNA. The embryos phenotypically rescued by mRNA injection were confirmed as being mutants by genotyping.

BrdU Assay
Embryos were collected at 6 or 48 hpf and incubated with 10 mmol/L BrdU (Sigma)+15% DMSO in culture medium on ice for 20 minutes. Embryos recovered at room temperature for two hours and were then incubated at 28.5°C until the desired stage. BrdU was detected by an anti-BrdU antibody (Roche).

Morphological Assays
Embryos were fixed in 4% paraformaldehyde/PBS, except embryos for staining with the α5 antibody, an anti-Na+/K+-ATPase subunit-specific antibody (Developmental Studies Hybridoma Bank), which were fixed in 100% methanol at 4°C overnight. The anti-atrial myosin heavy chain S46 antibody was obtained as a generous gift from Dr Frank Stockdale (Stanford University, Calif). Whole-mount in situ hybridization was performed as described previously.11 The fragmented DNA of apoptotic cells was detected by terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) (Intergen). In some cases, embryos were labeled immuno-

Figure 1. Phenotypes of the mutant and wild-type embryos. In comparison with wild-type embryos (A), the mutant embryos (B) show curved tails (arrow). A wild-type embryo (C) at 24 hpf shows a clear midbrain-hindbrain boundary and otic vesicles containing otoliths, but the mutant embryo (D) at 24 hpf shows a flat brain, midbrain-hindbrain boundary developmental delay, small eyes, and an otolith defect (arrows). At 72 hpf, a wild-type embryo (E). A mutant embryo (F) at 72 hpf with a curved tail and pericardial edema. The size of wild-type heart (G) is much larger than the mutant heart (H) (arrows). Also, the mutant heart has a malformed atrium and ventricle, indicates atrium; mhb, midbrain-hindbrain boundary; op, otic placode; v, ventricle.

nohistochemically after in situ hybridization or TUNEL staining. Transmission electron microscopy was performed as described previously.12

Results
Isolation of the Small Heart Mutant
A recessive embryonic lethal mutant was isolated from an ENU-mutagenesis screen. The mutant embryos did not exhibit an overt phenotype until ~18.5 hpf when a delay in formation of otic vesicle with no otoliths was observed. At 24 hpf, the mutants differ from wild-type siblings in that there is no discrete morphological midbrain-hindbrain junction, the embryos have a curved body axis and fail to hatch (Figure 1A through 1F). There is no evidence of circulation in the mutants, and the heart is markedly reduced in size by 24 hpf. The size of the brain and eyes are also reduced. And otoliths do not develop in the mutant otic vesicles. The cardiac phenotype is striking in that by 24 hpf, the mutant failed to form a properly elongated and functional heart tube. By 72 hpf, the mutant heart consists of a thin-walled chamber and an aggregate of clustered cells with an atrioventricular junction defect (Figure 1G and 1H).
Figure 2. Positional cloning of the mutation. A, Integrated genetic/physical map on linkage group 1 showing that the number of recombinant events detected by Z-markers, ESTs (fk24c12.y; fa03c03.s1; fc69d02.x1; fc38a10.x1), and BAC (B165D01, Incyte Genomics, and HUKGB735PP31, RZPD) end markers from 3192 meioses toward the telomeric side and 1080 meioses toward the centromeric side. Critical genetic interval containing the mutant locus is spanned by two overlapping BACs. One recombinant on the telomeric side represents intragenic recombination events. B, Full genomic structure of the Na^+/K^+-ATPase alpha 1B1. The red arrow points to the mutant region. C, Deletion leads to two aberrantly spliced mRNA variants (V1 and V2). Box with red dashed lines indicates the deletion. Black arrows indicate where the RT-PCR primers were designed. D, Two abnormal splice variants were identified by RT-PCR analysis, normal splicing in wild-type embryos (W, arrow), abnormal splice variant 1 (V1, arrow) with missing exon 17, which causes a frame-shift and premature translational termination (Red, TGA), and abnormal splice variant 2 (V2, arrow), which lacks exon 16 and exon 17. Comparison of genomic sequences from the mutant and wild-type embryos (E). A 57-bp deletion (black dashed lines) was identified (E, Left). GT with red underlined bar indicates the region of the normal splicing donor site. Genomic DNA fragments were amplified by PCR (E, right). F, Schematic diagram of the Na^+/K^+-ATPase alpha B1 protein. Predicted protein domains affected by the mutation. Wild-type Na^+/K^+-ATPase alpha B1 protein transverses the cell membrane 10 times (M1-M10). Purple indicates unaffected regions; blue indicates the region affected by V2 splicing. V1 splicing causes premature termination of translation, which leads to protein truncation. Truncation starts within the M6 domain (red arrow, blue) and continues through the M7-M10 domains (blue and gray).
Small Heart Mutation Is in the \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) Gene

We identified the small heart gene using a positional cloning approach (Figure 2A). Bulk segregant analysis identified linkage with the marker Z9217 on zebrafish LG 1, and the locus was refined to an interval between the markers, Z6384 and Z2915. This interval was further reduced using polymorphic markers designed from local sequences mapped by radiation hybrid analysis (see http://zfishmaps.tch.harvard.edu/ZonRHmapper/Maps.htm). One local EST (AA495435) was a partial \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) sequence. \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) emerged as a candidate for the mutant gene as it is expressed in the heart and other organs affected in the small heart embryos.\(^{1,14}\) We compared cDNA and genomic DNA sequences produced from mutant and wild-type embryo templates. We found a 57 bp deletion extending from the splice donor site of exon 17 (\(-5\text{bp}\)) into intron 17 (\(-52\text{bp}\)) (Figure 2E), which results in the production of two aberrantly spliced mRNAs. One splice variant (V1) introduces a premature stop codon at base pair 2769 in exon 18 and the other splice variant (V2) results in the loss of exons 16 and 17 (Figure 2C and 2D).

\(\text{Na}^+/\text{K}^+\)-ATPases function as heterodimeric transmembrane proteins assembled from one \(\alpha\) and one \(\beta\) subunit.\(^{15}\) The catalytic \(\alpha\) subunit traverses the cell membrane ten times (Figure 2F). If translated, the mutant V1 protein would lack a portion of the carboxyl-terminal region including M7-M10, a region with 90% identity to human \(\text{ATP}1\text{A}1\), and the mutant V2 protein would lack 93 amino acids (from M5-M7). These mutant proteins are expected to be nonfunctional.

Phenocopy by Morpholino Injection

To confirm the identity of the small heart gene, we used antisense morpholino oligonucleotide inhibition of mRNA translation\(^{16}\) or pre-mRNA splicing.\(^{17}\) Morpholinos (MOs) were injected into wild-type embryos at the one-cell stage. A low dose of 0.42 ng MO phenocopied the otolith defect of small heart mutants in 94% of injected embryos (116/124). Injections with a dose range from 2.1 to 4.2 ng completely phenocopied both the heart and otolith deficiencies (Figure 3C) in 96% of injected embryos (139/144). RT-PCR was used to confirm that injection of MO2 results in the same splice variants as the small heart genetic mutant (data not shown).

Phenocopy by Ouabain Treatment

To further confirm that the mutant deficiencies are caused by a loss of \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) function, we treated wild-type embryos with ouabain at 6 hpf. Treated embryos showed the mutant otolith defect (18/80), but not the heart defect, at a 1 mmol/L ouabain dose. A 1.5 mmol/L ouabain dose induced the otolith defect (98/98) and heart malformation (39/98). Embryos treated with 2.5 mmol/L ouabain reproduced the small heart phenotype in 100% of the cases (81/81) (Figure 3D). Heterozygosity for the mutation sensitizes embryos to ouabain treatment. When heterozygous mutant and homozygous wild-type embryos were treated at 48 hpf with 2.5 mmol/L ouabain, 68.7% (44/64) of heterozygous embryos and 19% (6/31) of homozygous wild-type embryos showed a heart defect. When a 4.5 mmol/L ouabain dose was used to treat embryos, 90% (54/60) of homozygous wild-type embryos had ventricular deterioration (Figure 3F).

mRNA Rescue

To confirm that the \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) gene is mutated in the small heart genetic mutant fish, we rescued the mutant phenotype by microinjecting wild-type zebrafish \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) mRNA. The eye size in mutants is incompletely rescued by mRNA injection (Figure 4A through 4D). Injection of 100 pg mRNA fully restores the otoliths and completely rescued by mRNA injection (Figure 4A through 4D). Injection of 100 pg mRNA fully restores the otoliths and completely rescued by mRNA injection (Figure 4A through 4D). Injection of 100 pg mRNA fully restores the otoliths and completely rescued by mRNA injection (Figure 4A through 4D). Injection of 100 pg mRNA fully restores the otoliths and completely rescued by mRNA injection (Figure 4A through 4D). Injection of 100 pg mRNA fully restores the otoliths and completely rescued by RNA injection (Figure 4A through 4D).

Na\(^+\)/K\(^+\)-ATPase \(\alpha\text{B}1\) Expression Is Downregulated in the Mutants

We analyzed the \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) transcription pattern in mutants and wild-type sibling embryos from 6 to 72 hpf during zebrafish organogenesis. Initially, \(\text{Na}^+/\text{K}^+\)-ATPase \(\alpha\text{B}1\) expression was uniformly distributed throughout the embryo. With the onset of formation of the otic placode, the expression level is increased in the regions of the otic vesicle, pronephric duct, eyes, and brain; and is lowered in the somites. The gene expression in the cardiac domain becomes...
detectable at the stage when the bilateral cardiac progenitor fields fuse at the ventral midline (Figure 5A). Na\(^+/K^+\)-ATPase α1B1 expression is greater in the ventricular myocardium than in the atrial myocardium (Figure 5C). Although Na\(^+/K^+\)-ATPase α1B1 is expressed in mutant embryos, the transcription is weak (Figure 5B and 5D). These results are confirmed by Na\(^+/K^+\)-ATPase protein expression analysis. For example, Na\(^+/K^+\)-ATPase α subunit protein can be detected in the heart tube of wild-type embryo at 24 hpf by the α5 antibody (an anti-Na\(^+/K^+\)-ATPase α subunit antibody; Figure 5E); but the protein is not detected in the heart field of the mutant or morpholino injected embryos (Figure 5F and 5G).

**Figure 4.** Na\(^+/K^+\)-ATPase α1B1 mRNA overexpression rescues the mutant defects in a dose-dependent manner. All embryos were injected at the one-cell stage and examined at 50 hpf. A, Wild-type embryo. B, Mutant after injection with 50 pg mRNA shows rescue of the otolith defect (o, otoliths, arrow). C, Injection of 100 pg mRNA rescues defects in otoliths, brain, heart, and trunk (arrows). D, An uninjected mutant embryo. a indicates atrium; e, eye; t, trunk; v, ventricle.

**Figure 5.** Comparison of Na\(^+/K^+\)-ATPase α1B1 gene and protein expression patterns. A, Expression pattern of Na\(^+/K^+\)-ATPase α1B1 mRNA is clearly detected in the eyes, otic placode, pronephric duct, and heart tube of wild-type embryos at 24 hpf (arrows), but the expression in the mutants (B) at 24 hpf is weakly detectable (arrows). At 72 hpf, the level of the gene expression in the ventricle of wild-type embryos is higher than in the atrium (C, arrows), but no Na\(^+/K^+\)-ATPase α1B1 transcription is detected in the mutant hearts at 72 hpf (D, arrows). Na\(^+/K^+\)-ATPase α1B1 protein can be detected by the α5 antibody (an anti-Na\(^+/K^+\)-ATPase α subunit antibody), Na\(^+/K^+\)-ATPase α1B1 protein expression (E) is shown in the wild-type heart tube at 24 hpf (arrow), but the protein expression is not detected in mutant (F) or MO-injected embryos (G) at 24 hpf (arrow), a indicates atrium; e, eyes; ht, heart tube; op, otic placode; pd, pronephric duct; v, ventricle.

**Figure 6.** Electron microscopy revealed abnormal aggregated cardiomyocyte mass with weak contractility. Although the mutant precardiac cells can differentiate into cardiac myocytes with expression of S46 and vmhc (Figure 6F), the aggregated ventricular cardiomyocytes progressively deteriorate, lose contractility, and eventually result in a small nonfunctional heart. In addition, the atrial myocardium in some cases appears to cross the atrioventricular canal into a portion of the ventricular region (Figure 6F).

Cell number is dramatically decreased in the ventricular chamber of the mutant hearts at 72 hpf (50±11.4) compared with the wild-type hearts (250±58.3) (Figure 7A). There is no significant difference in the number of atrial cardiomyocytes between the mutant (132±24.7) and wild-type (169±31.9) hearts. On histological observation, the ventricular cardiomyocytes of the mutants appear shrunken and clustered together with a small or nonexistent ventricular cavity (Figure 7C).
ventricular myocytes with reduced cytoplasm (Figure 7F through 7I). In addition, the mutant cardiomyocytes exhibited evidence of typical pathological changes such as mitochondria defects and a reduction in sarcomeric assembly.

Cell Proliferation Not Prevented
Because the number of ventricular cardiomyocytes is fewer in mutants compared with wild-type embryos, we investigated whether loss of Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) function affects cell proliferation. A BrdU assay of embryos treated with BrdU at 6 hpf and analyzed at 22 hpf demonstrates that there is no significant difference in cell proliferation activity in the cardiac field and adjacent region in the mutant, morpholino-injected, or ouabain-treated embryos compared with wild-type embryos (online Figure IA and IB, available in the online data supplement at http://circres.ahajournals.org). Also, cell proliferation in the heart progressively diminishes in both wild-type embryos and mutants embryos (online Figure IC and ID).

Ventricular Cardiomyocyte Death
We examined whether apoptosis might occur as a consequence of the small heart mutation. The mutant, morpholino-injected, ouabain-treated, and wild-type embryos were examined at different developmental stages using TUNEL analysis. The results demonstrate an increase in the number of apoptotic cells associated with mutation or inhibition of Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\). Interestingly, more apoptotic cells were observed in the region adjacent to the heart fields in mutant embryos at the time of heart tube formation (Figure 8B and 8F). Importantly, 97% of the mutant embryos (32/33) exhibited ventricular cardiomyocytes undergoing apoptosis compared with 2% of wild-type embryos. To confirm the localization of apoptotic cells, we performed double-labeling in the embryos with the TUNEL assay and a ventricular or atrial marker (Figure 8N, 8O, and 8P). These experiments provide direct in vivo evidence that genetic loss of function or inhibition of Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) promotes ventricular cardiomyocyte apoptosis and deterioration of the heart leading to the small heart phenotype.

Discussion
Functional Domains of Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\)
Na\(^{+}/K\(^{-}\)\)-ATPases are responsible for the energy-dependent maintenance of intracellular ion homeostasis, transporting Na\(^{+}\) and K\(^{-}\) ions across the cell membrane.\(^{15}\) These functions play an important role in many fundamental processes including the control of contractility, excitability, and cell volume regulation. Whereas the function of the intact “pump” has been extensively studied, little is known of the specific functions of individual protein domains. In this study, we describe a deletion in a zebrafish Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) gene that results in the production of aberrantly spliced mRNAs. One mutant splice variant causes a frameshift that produces a premature stop codon and protein lacking part of domain M7 and all of domains M8–10, a region including phosphorylation sites for protein kinase A.\(^{16}\) The second mutant splice variant results in protein lacking part of M5 and all of domains M6 and M7; cation binding residues are included in this protein region.\(^{19}\)

Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) Functions in Cardiac Morphogenesis
The role of Na\(^{+}/K\(^{-}\)\)-ATPase has been intensively studied in cardiomyocyte physiology, but its role in cardiac morphogenesis has only recently been reported, leaving several crucial open questions.\(^{6}\) Targeted disruption of the murine Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) isoform leads to an early embryonic lethality with heart defect,\(^{20}\) but the pathology of the cardiovascular defect of these mutant mice remains undetermined. In this study, we provide direct evidence that the Na\(^{+}/K\(^{-}\)\)-ATPase \(\alpha1B1\) is required for regulation of early heart tube formation and ventricular function. Cardiac progenitors in the mutant form a concentric cardiac cone, but the cone fails to elongate properly into a full-length heart tube. The problems at the concentric cone stage may reflect increased apoptosis in the developing ventricle, but may also reflect other abnormalities induced by the loss of Na\(^{+}/K\(^{-}\)\)-ATPase function. The apparent loss of S46 atrial restriction suggests that atrial or ventricular myocyte differentiation is dysregulated, or that migration of specified precursors is disrupted.
Prolonged ouabain inhibition of Na⁺/K⁺-ATPase causes cells to detach from each other and substrate; thus, it is conceivable that cell adhesion and migration are disrupted in the mutant zebrafish. A change in osmotic balance likely disrupts the extracellular matrix, which normally provides attachment sites used for guiding migrating cells into defined pathways. The atrioventricular defects in the mutant may reflect secondary effects, but it is possible that the specification or migration of a critical cell population is lost.

Interestingly, the heart defect in the small heart mutant is similar to that of an atypical protein kinase C (PKCα) mutant called heart and soul. This similarity opens up a new direction to pursue further details about how these molecules interrelate to regulate heart tube formation during cardiogenesis.

There are many isoforms of Na⁺/K⁺-ATPase functioning in the developing embryo. Fifteen isoforms of Na⁺/K⁺-ATPase have been isolated from zebrafish; of these, α1B1, α2, and β1a are expressed in the embryonic heart. The α1B1 and β1a isoforms are expressed in the heart at 24 hpf, and by 48 hpf the α2 isomorf is also expressed there. The specific phenotype arising in this mutant reflects the particular expression localization and timing of the α1B1 isoform. The α1B1 isoform is expressed in the otic placode, optic cup, brain, pronephric tubule and duct, mucous cells, and in the heart with enriched expression in the ventricle. The phenotypes seen in the mutant are recapitulated in the embryos treated with ouabain at 6 hpf and assayed at 72 hpf; this suggests that the Na⁺/K⁺-ATPase α1B1 isoform is active in the embryo during this critical period of organogenesis. A novel finding presented in this study is the defects in various organs of the developing zebrafish embryo that result from mutation of Na⁺/K⁺-ATPase α1B1. For example, the brain, ears, and eyes are dysmorphic in the mutant embryos, and the kidneys do not develop properly based on histological observation (data not shown) and an assay of Na⁺/K⁺-ATPase α1B1 protein expression (online Figure II).

**Na⁺/K⁺-ATPase α1B1 Regulates Formation of Heart Size**

Organ size determination is not well understood. In cardiac myocytes, hypertrophy plays a significant role in determining heart mass. The small heart mutation implicates Na⁺/K⁺-ATPase α1B1 activity in the regulation of heart size determination, affecting both heart cell size and heart cell number. The size of several organs (heart, brain, eyes, etc) is considerably smaller in the mutants than wild-type embryos. These data are concordant with previous in vitro studies on the effects of sodium pump inhibition on cell size, including cell shrinkage in cultured cardiomyocytes. Blocking Na⁺/K⁺-ATPase function has been shown to reduce intracellular K⁺, whereas increasing intracellular Na⁺ and Ca²⁺. It has been suggested that this intracellular K⁺ depletion may play a key role in cell shrinkage and apoptotic death.

In addition to cell size, cell number is also reduced in the mutant caused by an increase of cardiomyocyte apoptosis. In this study, we show that Na⁺/K⁺-ATPase is directly implicated in the regulation of apoptotic events in vivo. There are many potential pathways through which such regulation...
might be mediated. The tractability of the zebrafish allows for the possible dissection of these pathways, in the context of native developmental cues.

**Potential Insights Into Heart Failure**

A characteristic feature of heart failure is the progressive deterioration of left ventricular cardiomyocyte function. The mechanisms that mediate the pathogenesis of heart failure are poorly understood. Recently, cardiomyocyte apoptosis has been noted in failing human hearts. Also, increasing evidence have shown that human heart failure is associated with a reduction of Na⁺/K⁺-ATPase.

There is little doubt that Na⁺/K⁺-ATPase function is important in the adult vertebrate heart. Modulation of this protein complex using glycosides is a major part of the therapeutic armamentarium in human heart failure, and toxicity of these compounds remains a significant clinical problem. There is also evidence that there may be endogenous ouabain-like activities with functions that can only be speculated on at this time. Our discovery of this mutation in the zebrafish provides an in vivo model system in which a loss of function of Na⁺/K⁺-ATPase α1B1 may be studied in the context of the intact organism. Such studies might unravel the specific roles for Na⁺/K⁺-ATPase activity during organogenesis and also shed light on downstream pathways involved in the function of the adult vertebrate cardiomyocyte in health and disease.

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Supplementary figure legends

Supplementary figure 1. Cell proliferation assay indicates that a loss of Na⁺/K⁺-ATPase α1B function does not prevent cell proliferation. Wildtype (A) and mutant embryos (B) were treated with BrdU at 6 hpf, fixed at 22 hpf, and labeled with an anti-BrdU antibody (arrows, brown). No significant difference in cell proliferation activity in the cardiac field and adjacent regions was detected (arrows) (A compared to B). Since cell proliferation in the heart progressively diminishes during embryonic development, when wildtype (C) and mutant embryos (D) were treated with BrdU at 48 hpf, and labeled at 72 hpf, few BrdU-labeled cells were ever visible in examined hearts (C, D) (arrows). v, ventricle; a, atrium.

Supplementary figure 2. Kidney development is affected in mutant, MO-injected, and ouabain treated embryos. Embryos were labeled with α-5 antibody, which marks brain, ear, and kidney including tubules (t, red arrows), anterior duct, and pronephric ducts (pd, black arrows) in wildtype embryos at 72 hpf (A). The α-5 staining in the anterior ducts and tubules is absent in mutant (B), MO-injected (C) and ouabain treated (D) embryos at 72 hpf.
Supplementary Figure 1.
Supplementary Figure 2.