Zebrafish early cardiac connexin, Cx36.7/Ecx, regulates myofibril orientation and heart morphogenesis by establishing *Nkx2.5* expression

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Heart development is a precisely coordinated process of cellular proliferation, migration, differentiation, and integrated morphogenetic interactions, and therefore it is highly susceptible to developmental anomalies such as the congenital heart disease (CHD). One of the major causes of CHD has been shown to be the mutations in key cardiac transcription factors, including *nkx2.5*. Here, we report the analysis of zebrafish mutant *ftk* that showed a progressive heart malformation in the later stages of heart morphogenesis. Our analyses revealed that the cardiac muscle maturation and heart morphogenesis in *ftk* mutants were impaired because of the disorganization of myofibrils. Notably, we found that the expression of *nkx2.5* was down-regulated in the *ftk* heart despite the normal expression of *gata4* and *tbx5*, suggesting a common mechanism for the occurrence of *ftk* phenotype and CHD. We identified *ftk* to be a loss-of-function mutation in a connexin gene, *cx36.7/early cardiac connexin* (*ecx*), expressed during early heart development. We further showed by a rescue experiment that Nkx2.5 is the downstream mediator of Ecx-mediated signaling. From these results, we propose that the cardiac connexin Ecx and its downstream signaling are crucial for establishing *nkx2.5* expression, which in turn promotes unidirectional, parallel alignment of myofibrils and the subsequent proper heart morphogenesis.

cardiomyocyte | congenital heart disease | trabeculation | *futka* | positional cloning

Normal development and function of the heart are indispensable for the survival of vertebrates. Cardiogenesis involves a precisely coordinated process of cellular proliferation, migration, and differentiation, and integrated morphogenetic interactions. Because of the complexity of these embryonic processes, heart development is highly susceptible to developmental anomalies collectively known as congenital heart diseases (CHD). The syndrome threatens the lives of as many as 1% of newborns (1, 2). Studies have uncovered that several transcription factors, including TBX5, NKX2.5, GATA4, and SALL4, cooperate to direct the cardiac cell lineage commitment and/or morphogenesis through the regulation of proteins characteristic of cardiomyocytes and that mutations in these transcription factors are responsible for many cases of CHD (3–7). Recent data have also suggested the significant roles of these transcription factors for maintenance of the functional heart in postnatal life as well (8–12). In the past decade, zebrafish (*Danio rerio*) has emerged as a valuable model system to study many developmental processes, including heart development, and a number of important insights have been gained from analyses of zebrafish mutants (13–17). In addition to the early determination and differentiation of heart primordium, zebrafish mutations are also expected to contribute to the clarification of a molecular mechanism that governs myofibrillogenesis and morphogenesis of the heart. The unidirectional alignment of myofibrils is essential for proper functioning of striated muscle such as skeletal and heart muscles (18). Despite a well characterized morphology, the molecular mechanisms of myofibril formation are poorly understood (19).

Here, we report the analysis of *futka* (*ftk*), a newly identified zebrafish mutant that displays abnormalities in myofibrillogenesis and morphogenesis of the heart. We show that the characteristics of *ftk* phenotypes, including progressive abnormalities in cardiac muscle maturation and heart morphogenesis, are in common with some types of CHD. In particular, the expression of key cardiac transcription factor, *nkx2.5*, which is one of the responsible genes in CHD, is severely down-regulated in the *ftk* heart that exhibits anisotropy of myofibrils. We identify that the *ftk* mutation is a loss-of-function allele of the connexin gene *cx36.7/early cardiac connexin* (*ecx*), expressed during early heart development. Furthermore, we show that the forced expression of *nkx2.5* can rescue all *ftk* phenotypes, thus demonstrating that Nkx2.5 is the downstream mediator of Ecx function required for proper heart morphogenesis and maturation. Our analysis of *ftk* mutant reveals that Ecx is a unique regulator for establishing *nkx2.5* expression at an early stage of heart development, which is essential for heart morphogenesis, including ordered alignment of myofibrils in cardiomyocytes and trabeculation of heart muscles.

Results and Discussion

Progressive Heart Phenotype in *ftk* Mutant. A number of zebrafish heart mutations have been generated and analyzed so far (13–17, 20, 21); however, only a relatively small number of mutants have shown specific phenotypes in the later processes of heart formation, such as cardiomyocyte maturation, trabeculation of the cardiac chamber walls, and coordination of heartbeats, which are processes that occur after the initial formation of basic body plan of the heart. In our small-scale haploid mutant screening, we identified the zebrafish mutant *ftk*, which had no recognizable abnormality during early development and morphogenesis but showed progressive development of abnormalities in heart morphogenesis and function (Fig. 1). Early development of *ftk* embryos seemed to be normal until ≈24 hours postfertilization (hpf), i.e., around the time when the heart has completed tube

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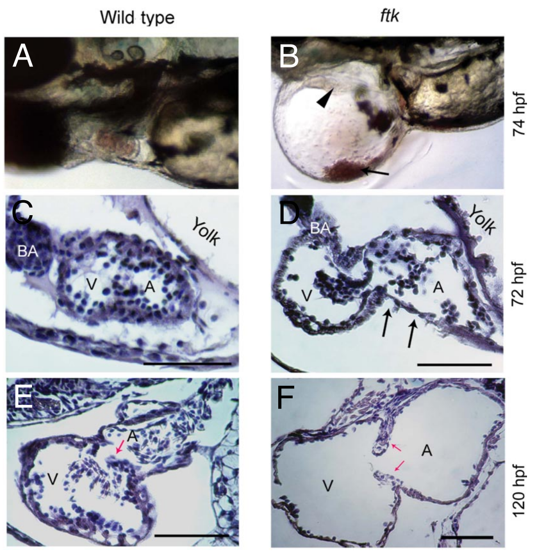

Fig. 1. Progressive heart malformation in zebrafish *ftk* mutant. (*A* and *B*) Gross heart morphology of *ftk* mutant. Wild-type (*A*) and *ftk* mutant embryos (*B*) at 74 hpf. Arrowhead indicates heart perforations in the *ftk* mutant; arrow points to blood cells accumulated in the pericardiac cavity. (*C*–*F*) Sagittal sections of the heart. At 72 hpf (*C* and *D*), a dilated atrium (A) and ventricle (V) are evident in the *ftk* mutant. The cardiac muscles appear to be thinner (arrows in *D*), and the dilation of heart chambers is under way. At 120 hpf (*E* and *F*), the heart chamber dilation becomes more severe in *ftk* mutants with thinner ventricular and atrial walls. Red arrows indicate the valve leaflets. (Scale bars: 150 μm.)

formation and starts beating. The *ftk* phenotype was first recognized as a growing, but subtle, pericardiac edema at ≈28 hpf, which became clearer by 36 hpf [see supporting information (SI) Fig. 6]. After 48 hpf, *ftk* mutants developed severe heart phenotypes such as dilated heart chambers (Fig. 1 *B* and *D*). In addition to their abnormal heart morphology, blood flow was abnormal such that it often flowed back to adverse direction. Moreover, heartbeats were also irregular and slower in *ftk* mutants; the number of heartbeats was less than half of wild-type (Table 1 and SI Movies 1 and 2). Despite these heart anomalies, the *ftk* mutant could survive until ≈7 dpf.

In *ftk* mutants, circulating blood cells often leaked into the pericardiac cavity (Fig. 1*B*), resulting in a lack of circulating blood cells in the mutants. By close examination, the blood leakage was caused by perforations in the heart wall (Fig. 1*B*), suggesting that the mutant hearts were fragile and were not resistant to the blood pressure. These defects could account for the pericardiac edema phenotype in *ftk* mutants and progressive increase of its severity.

To evaluate further the defects in the mutant hearts, we examined histological sections. It has been reported that the trabeculation in ventricle occurs between 72 and 120 hpf of zebrafish heart (22). Consistent with this report, the ventricle wall was found gradually thickened from 72 to 120 hpf for the wild-type larvae (Fig. 1 *C* and *E*). In contrast, the thickness of cardiac chamber wall in *ftk* mutants was unsynchronized and partly thinner (Fig. 1*D*). At 120 hpf, the mutant heart walls became thinner and severely dilated, and proper heart chamber morphogenesis was not accomplished (Fig. 1 *E* and *F*). Thus, the process of cardiac muscle maturation and heart morphogenesis appeared to be impaired in *ftk* mutants.

The backflow of bloodstream raised the possibility that the development of valve leaflets could be deformed in *ftk* mutants, which might have affected the blood flow and in turn resulted in the abnormal heart morphogenesis. However, we did not observe any significant defects in the valve leaflets of atrioventricular valves and bulbus arteriosus in larvae at 5 days postfertilization (dpf) (Fig. 1*F* and data not shown), in which the leaflets are clearly recognized in the atrioventricular region around this time (Fig. 1*E*) (17). This finding suggests that the dilated abnormal chamber morphology may be the cause of blood backflow. Taken together, from gross morphological inspection, the initial basic design of the heart such as the chamber specification and valve formation appeared to be normal in *ftk* mutants, but the mutant heart chambers were thinner and dilated, lacking the trabeculation of heart muscles. Thus, the *ftk* mutation appeared to affect primarily the maturation of cardiac muscles and subsequent proper heart morphogenesis.

Table 1. Abnormal heartbeat in *ftk* mutant

Larvae	*n*	48 hpf Atrium	48 hpf Ventricle	72 hpf Atrium	72 hpf Ventricle
Wild-type	15	114 ± 3	144 ± 3	206 ± 4	206 ± 5
*ftk**	12	108 ± 5	59 ± 5	80 ± 6	62 ± 4
ftk morphant	12	114 ± 5	54 ± 5	83 ± 5	57 ± 5

Quantitative analysis of heartbeat (per minute) at 48 hpf and 72 hpf at 28°C is shown. Measurement was performed by using the recorded movies.

*Compared with wild-type larvae, *ftk* mutants show unsynchronized contractions of the atrium and ventricle in addition to the slower heart beating.

Abnormal Myofibril Organization in *ftk* Cardiac Muscles. To characterize the abnormalities in the *ftk* cardiomyocytes further, we examined the subcellular ultrastructure of cardiomyocytes. It had been reported that the ordered pattern of myofibers becomes evident in wild-type zebrafish cardiomyocytes at ≈48 hpf (23); therefore, we performed the transmission electron microscopy (TEM) analysis at ≈58 hpf. Normally, mature cardiomyocytes at this stage of development contain highly developed mitochondria and a well organized thick assembly of myofibrils (Fig. 2*A*). When sections were made along the longitudinal axis of heart, we observed that most of the myofibrils in a single cell were organized in the same orientation in this stage of wild-type larvae, and the myofibers in adjacent cardiac muscles were also in the same orientation (Fig. 2 *A* and *B*). However, in *ftk* mutants, we found that the myofibril organization was irregular such that the myofibril bundles were oriented randomly (Fig. 2 *C* and *D*; $n = 10$). In sections along the longitudinal axis of heart, many myofibril bundles, each of which seemed to be smaller than those of wild-type, run perpendicular to the plane of section (asterisks in Fig. 2*D*) in addition to many fiber bundles running in the plane of section (double-headed arrows in Fig. 2*D*).

Aside from the disorganized myofibril orientation, other subcellular structures characteristic of the cardiac muscles seemed to be normal. Intriguingly, in contrast to the disorganized heart myofibrils, the myofibrils themselves in the mutant skeletal muscles were assembled normally (SI Fig. 7), indicating that *ftk* mutation affected only myofibrils in the cardiac muscles. Overall, it is reasonable to assume that the myofibril organization may affect the heartbeat, cardiac muscle trabeculation, and heart morphogenesis; thus, our observations suggested that the *ftk* phenotypes were derived from a primary defect in myofibril organization.

Down-Regulation of *Nkx2.5* Transcription in *ftk* Mutants. We further investigated the *ftk* phenotype at the molecular level by looking at the expression of genes for cardiac structural proteins such as Cmlc2 and Vmhc, signaling molecules such as Bmp2b, Bmp4, and Notch1b, and transcription factors such as MyoD, Gata2, Gata4, Gata5, Tbx5, Mef2c, NFATc1, and Nkx2.5. Surprisingly, only *nkx2.5* expression was severely down-regulated in *ftk* mutants from the early stage of heart development (Fig. 2*E*),

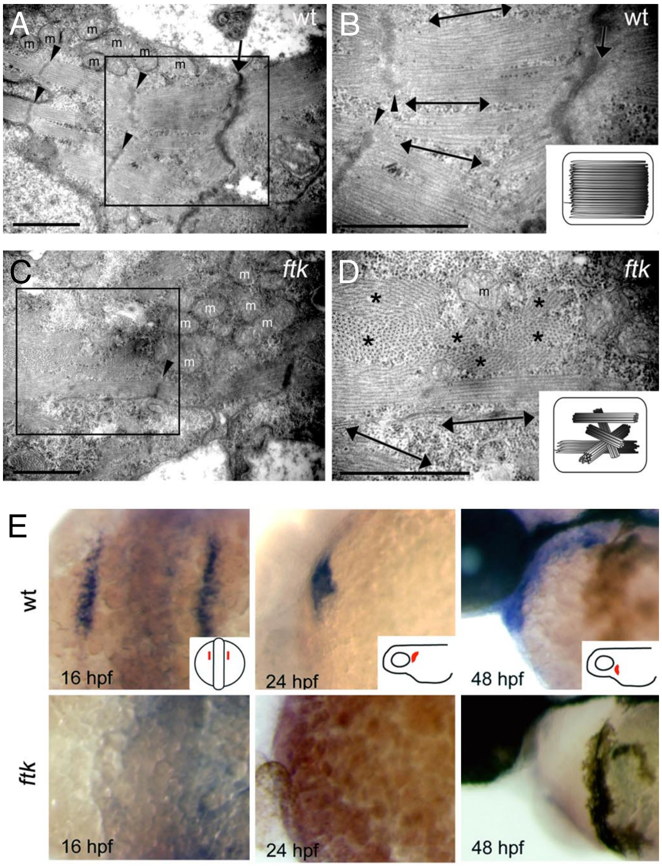

Fig. 2. *ftk* mutation primarily affected the myofibril organization and *nkx2.5* expression. (*A–D*) Myofibril organization examined by TEM analysis. In wild-type embryos at 58 hpf (*A* and *B*), myofibrils assemble as thick parallel bundles with orientation toward the longitudinal direction of the cardiomyocyte (double-headed arrows). In contrast, myofibrils in the *ftk* heart (*C* and *D*) are oriented in irregular directions. Double-headed arrows and asterisks indicate the myofibril bundles sectioned parallel and perpendicular to the long axis of the cells, respectively. Arrowheads, Z-lines; arrows, cell–cell contact sites; m, mitochondria. (Scale bars: 500 nm.) (*B* and *D Insets*) Schematic drawings of myofibril organization. (*E*) Whole-mount *in situ* hybridization analysis of *nkx2.5* expression. In wild-type zebrafish embryos (*Upper*) The expression is detected in the developing heart; however, it is not detectable in *ftk* mutants (*Lower*). Mutants at early stages were genotyped by RFLP analysis. (*Insets*) Orientations of embryos.

whereas the expression of other cardiac genes was not affected (data not shown).

A number of studies have established that Nkx2.5 is a key transcription factor that regulates the differentiation, maturation, and maintenance of cardiomyocytes throughout life (24) and that the gene is mutated in a class of human CHD (4–7, 12, 25). Although the *nkx2.5* gene knockout mice showed a severer phenotype than *ftk* in terms of cardiomyocyte commitment (26, 27), the overall phenotypes of *ftk*, including the loss of Nkx2.5, were reminiscent of those of *nkx2.5* knockout mice. To confirm that *ftk* phenotype is caused by the loss of *nkx2.5* expression, we examined the phenotype produced by the knockdown of *nkx2.5* (SI Fig. 8*A* and SI Table 2). The cardiac phenotypes, including the severe pericardiac edema, irregular heartbeat, reverse blood flow (SI Movie 3), and disorganized myofibril orientation (SI Fig. 8*B*; $n = 8$), were similar to those in *ftk* mutants, demonstrating that the entire cardiac phenotype of *ftk* can be explained by loss of *nkx2.5* expression. Taken together, our observations suggest a link between phenotypes of the *ftk* mutant and CHD; and hence we considered that the zebrafish *ftk* mutant could serve as a useful model for studying the mechanism of CHD and heart morphogenesis.

The *ftk* Locus Encodes a Unique Cardiac Connexin Gene. In exploring the molecular basis of the *ftk* mutant, we decided to determine the *ftk* gene by the positional cloning approach. By using single-strand conformation polymorphism analysis, we mapped the *ftk* locus to a region on zebrafish linkage group (LG) 7 (Fig. 3*A*) that houses six putative ORFs. The region neither contained *nkx2.5* gene, which is on LG 14, nor any similar *nkx* family genes. We amplified cDNAs of these ORFs from *ftk* mutants and wild-type embryos and determined their sequences. By sequencing analysis, we found that an adenine (A) to thymine (T) missense mutation, which results in a substitution of an aspartic acid (D) residue with a valine (V) at position 12 in the N-terminal domain, occurred in the connexin gene (Figs. 3*B* and 4 *A* and *B*). We designated this connexin as Cx36.7 according to its calculated molecular weight. To date, the connexin gene family comprises 20 members in humans, 19 in mice, and 37 in zebrafish (28). Judging from the phylogeny, the zebrafish Cx36.7 appeared to be the close homolog of human CX31.9 or CX40.1 (SI Fig. 9*A*), and it is more likely to be the ortholog of CX31.9 from the synteny conservation between corresponding genomic regions of human and zebrafish chromosomes (SI Fig. 9*B*). Although it has been shown that the CX31.9 gene is expressed in adult human heart (29), the expression during heart development has not been demonstrated.

To confirm that *cx36.7* and *ftk* were the same gene, we tested whether or not the *cx36.7* expression could rescue the *ftk* phenotypes. When the mRNA encoding the wild-type Cx36.7 was injected into *ftk* embryos, the *ftk* phenotype, including the heart dilation and severe pericardiac edema, was rescued at doses >30 pg of mRNA without affecting other parts of embryos (Fig. 3 *C* and *D* and SI Table 3), thus establishing that *cx36.7* is the responsible gene for the *ftk* mutant.

To verify the direct role of *ftk* in the developing heart further, we determined the expression of *cx36.7* during stages between 30% epiboly to 5 dpf. The expression was detectable in the ventral half of the marginal zone in 50% epiboly stage embryo (SI Fig. 10) and confined to the region of cardiac precursors in the following stages. By 16 hpf, the expression was localized in the myocardial precursor cells on both sides of the midline (Fig. 3*E*) and distributed to the whole heart in the later stages. Such expression of *cx36.7* is similar to that of *nkx2.5* expression (30); however, the expression started to decline by 36 hpf and was barely detectable at 48 hpf. Thus, the zebrafish Cx36.7 appeared to function in the developing heart, with a peak of expression corresponding to the time when the *ftk* phenotypes arise. We also observed that cx36.7 gene was normally expressed in *ftk* mutants. For its unique expression in developing heart, we called this connexin as "early cardiac connexin" (Ecx) and use this name in the following sections.

The *ftk* Mutation Abolishes the Connexin Function. Connexins oligomerize to form gap junction, a cell surface channel involved in communication between neighboring cells and/or between the extracellular–intracellular milieus (31); however, they are atypical membrane proteins lacking an apparent signal peptide, and their translocation mechanism to plasma membrane is poorly understood. When the amino acid sequences of Ecx and several connexins were compared, the amino acid residue at position 12, corresponding to the mutated residue in the *ftk* mutant, was conserved or substituted with another acidic residue, glutamic acid (Fig. 4 *A* and *B*). It has been shown that this residue is crucial for the connexin function (32); therefore, it is reasonable to postulate that the D12V mutation may be a loss-of-function mutation of Ecx. To confirm this hypothesis, we introduced the C-terminally FLAG-tagged Ecx proteins in HeLa cells. Remarkably, the mutant Ecx did not reach the cell surface, in contrast to the dense cell surface distribution of

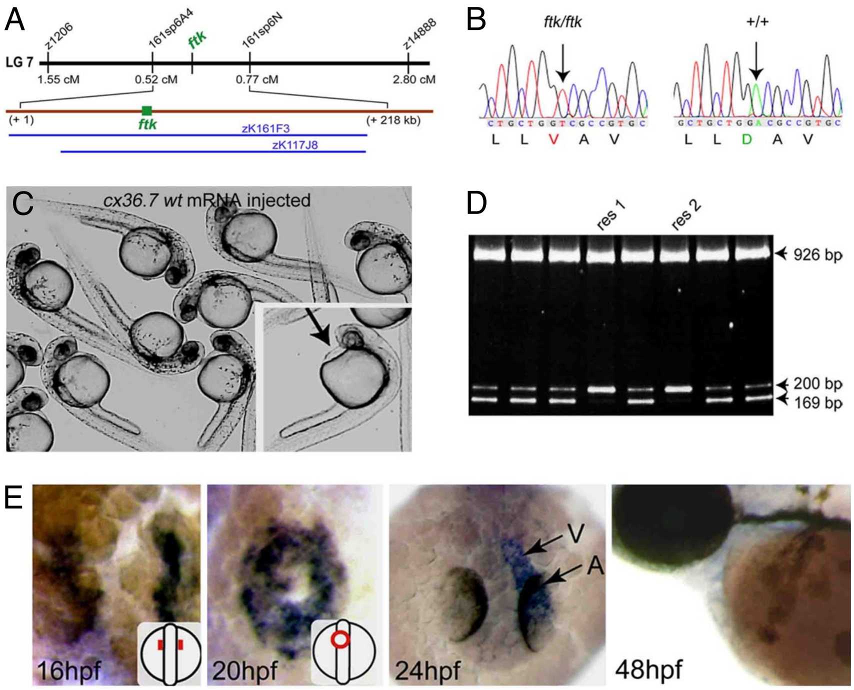

Fig. 3. Molecular characterization of *ftk* gene. (*A*) Chromosome map of the *ftk* locus on LG 7 between markers z1206 and z14888. An enlarged map (brown line) and overlapping BAC clones (blue lines) are shown below. (*B*) Missense mutation in the zebrafish *cx36.7* gene of the *ftk* mutant. (*C* and *D*) Rescue of the *ftk* phenotype by *cx36.7* mRNA injection. Embryos obtained from crosses of *ftk* heterozygotes and injected with *cx36.7* mRNA (30 pg) did not show the *ftk* phenotype at 36 hpf (*C*). (*Inset*) Sibling uninjected *ftk* mutant at 28 hpf, which displays the growing pericardiac edema (arrow). Genotyping of mRNA-injected embryos by RFLP analysis confirmed that two of eight embryos were genotypically *ftk* mutants (res1, res2) (*D*). (*E*) Whole-mount *in situ* hybridization analysis of *cx36.7* expression during heart development. (*Insets*) Orientation of embryos.

the wild-type Ecx protein (Fig. 4*C*), supporting our notion that the mutant *ecx* could be a loss-of-function allele caused by the inability of protein trafficking to the cell surface.

To confirm further that *ftk* was a loss-of-function mutation of *ecx*, we knocked down the *ecx* gene expression by injecting an antisense morpholinooligonucleotide (MO) for this gene into wild-type embryos. The resultant morphant displayed *ftk*-like phenotypes, such as the heart chamber dilation and irregular and slower heartbeat, which were indistinguishable from those of *ftk* (97%; n = 720; Fig. 4*D*, Table 1, and SI Movie 4). These data strongly supported that the *ftk* mutation was a loss-of-function phenotype. Importantly, we also confirmed by the TEM analysis that the *ecx*-morphants had disorganized myofibril bundles as in the *ftk* mutant (Fig. 4*D*, *Right*; n = 10) and that *nkx2.5* expression in the morphant heart was down-regulated as in *ftk* mutants (SI Fig. 11). Taken together, these facts indicated that Ecx function was required for the formation of ordered and parallel myofibrils in cardiomyocytes.

Connexin Function Is Directly Required for Heart Morphogenesis Through the Regulation of *Nkx2.5* Expression. Connexins have a major function in cell–cell coupling of cardiac muscles, which raises the possibility that loss-of-Ecx function could affect primarily the cardiac conduction system and lead to the phenotypes in heartbeat, myofibrils, and heart morphogenesis. To check whether *ftk* mutation affected primarily the conduction system, we visualized the calcium wave in the heart by using the calcium sensitive fluorescent dye. In *ftk* mutants, the Ca^{2+} wave that represents the electrical conduction between the cardiac muscles normally and smoothly sweeps across the heart from the atrium terminal to the ventricle terminal despite the uncoordinated and irregular contraction of chambers of *ftk* mutant (SI Movies 5 and 6). This result suggested that the irregular heartbeat in *ftk* mutants is not caused by the impaired cellular coupling between cardiomyocytes but rather by the inability of cardiomyocytes themselves to respond properly to the electrical conduction.

Thus, our results suggested that connexin function in cell–cell coupling may not be responsible for the *ftk* phenotypes but rather that other connexin functions, such as hemichannel activity or nonchannel functions relating to interactions with cytoplasmic proteins, are required for proper myofibril organization and heart morphogenesis. Indeed, in accordance with the above data, we also found through a dye uptake assay that Ecx could function as a hemichannel, but the mutant Ecx did not (SI Fig. 12). As we have suggested, there are significant similarities between the *ftk* mutant and CHD in their anomalies in heart morphogenesis and loss of Nkx2.5 function. Additionally, previous studies in mice have shown that the null mutant mice of *nkx2.5* exhibited an array of heart phenotypes such as impaired chamber morphogenesis and defective cardiomyocyte trabeculation (26, 27). These studies raise the possibility that Nkx2.5 is a downstream mediator of Ecx-derived signaling during heart morphogenesis.

To test further the possibility that *ftk* phenotypes are caused solely by the loss of Nkx2.5, we examined the ability of Nkx2.5 to compensate the loss of Ecx. We coinjected *nkx2.5* mRNA along with 2 ng of *ecx* MO, which was sufficient to cause the *ftk* phenotype in nearly all embryos (SI Table 4), and assayed the *ftk* phenotypes. Remarkably, as small as 10 pg of *nkx2.5* mRNA was enough to prevent all *ftk* phenotypes, including the heart morphogenesis (Fig. 5*A* and SI Table 4), myofibril organization (Fig. 5*B*; n = 8), and slower heartbeat (SI Movies 7 and 8). Similarly, *nkx2.5* mRNA injection could rescue *ftk* mutants obtained by crosses of *ftk* heterozygotes (SI Table 4). More intriguingly, *nkx2.5* mRNA injection rescued a significant percentage ($\approx$50%) of *ftk* mutants to viability up to 21 dpf (SI Table 5), suggesting that the early *nkx2.5* expression is sufficient for proper heart formation. Possibly, signaling mechanisms other than that of Ecx may maintain *nkx2.5* expression at later stages. From

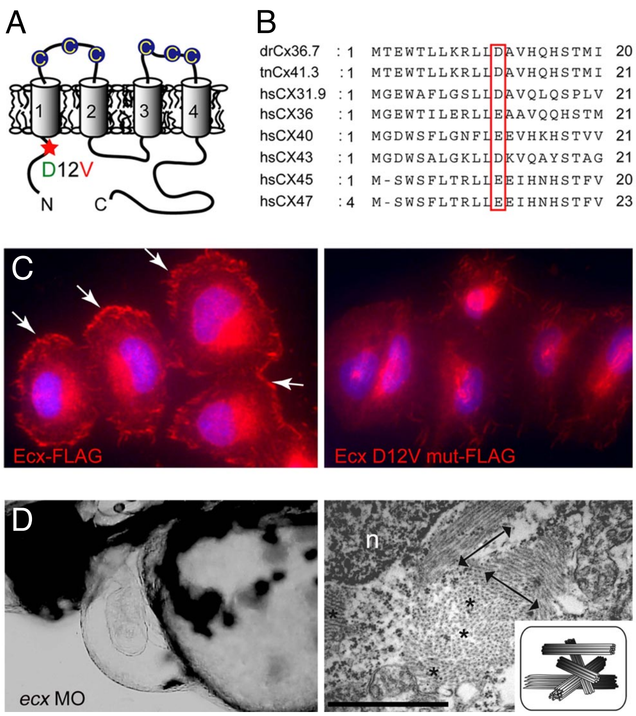

Fig. 4. *ftk* is a loss-of-function mutation of *ecx/cx36.7*. (*A*) Putative topology of Ecx protein. Extracellular cysteine residues are indicated by circles. The D12V mutation located in the N-terminal intracellular region of Ecx is indicated with a red asterisk. (*B*) Alignment of the N-terminal domain of zebrafish Ecx with *Tetraodon* Cx41.3 and six human connexins. (*C*) Subcellular localization of FLAG-tagged Ecx proteins in HeLa cells. Wild-type Ecx is localized at the cell surface (*Left*, arrows), whereas the mutant protein is not recruited to the membrane surface (*Right*). Nuclei were stained with Hoechst 33342 (blue). (*D*) Production of *ftk* phenotype with the *ecx* MO. (*Left*) Severe pericardiac edema and dilated heart chambers are observed in larvae injected with *ecx* MO. Lateral view at 56 hpf is shown. (*Right*) Disorganized myofibrils as seen in *ftk* mutants were also caused by the *ecx* MO. Double-headed arrows and asterisks indicate the myofibril bundles sectioned parallel and perpendicular to the long axis of the cells, respectively. (Scale bar: 500 nm.) *n*, nucleus. (*Inset*) Schematic of myofibril organization.

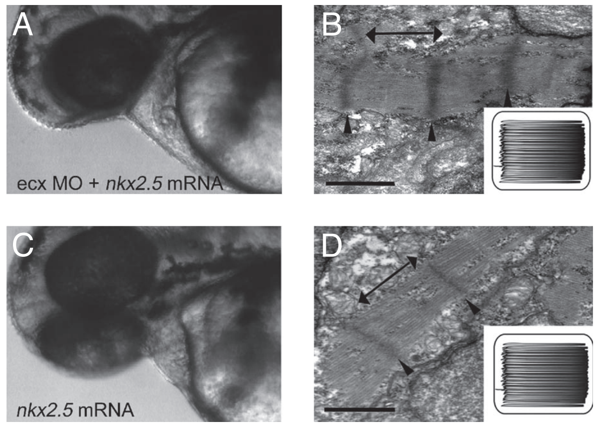

Fig. 5. Rescue of *ftk* phenotype by *nkx2.5* expression. (*A–D*) Embryos injected with *nkx2.5* mRNA plus *ecx* MO (*A* and *B*) or *nkx2.5* mRNA alone (*C* and *D*). Embryos were examined for their external phenotypes at 48 hpf (*A* and *C*; lateral views are shown in the lower right) and myofibril organization at 56 hpf by TEM analysis (*B* and *D*). The *ftk* phenotypes in the heart caused by *ecx* MO were apparently attenuated by coinjection with *nkx2.5* mRNA. Coinjection of *nkx2.5* mRNA also rescued the *ftk*-like abnormality in myofibril organization. Arrowheads, Z-lines; double-headed arrows, myofibril bundles. (*Insets*, *B* and *D*) Schematic of myofibril organization.

these data, we concluded that Nkx2.5 was the downstream mediator of Ecx-derived signaling and responsible for all *ftk* phenotypes that were seen during heart morphogenesis.

Concluding Remarks. Our work revealed a role of connexin in cardiomyocyte maturation through the establishment of *nkx2.5* expression. Without the Ecx function and the Ecx-mediated signal, the consequent down-regulation of Nkx2.5 leads to the abnormalities of myofibril organization, cardiomyocyte maturation, and heart morphogenesis. However, in contrast to sustained expression of *nkx2.5* in the heart, *ecx* is only expressed before 48 hpf (Fig. 3*E*), hence some other mechanism may be required for the sustained expression of *nkx2.5*. Studies have suggested that Bmp2 and Bmp4 (33, 34), Fgf (35), and phosphatidylinositol 3-kinase signaling (36) may also be the upstream regulators of *nkx2.5* transcription. In particular, Bmp signaling may induce cardiac *nkx2.5* expression through the activation of the composite enhancer by Smads in collaboration with Gata4 (37). These factors and/or signals may have a role for the later regulation of *nkx2.5* transcription in combination with Ecx-derived signal.

In addition, an epigenetic regulation for sustained *nkx2.5* transcription is also suggested (38). In mice deficient for *Polycomb* group gene *Rae 28*, *Nkx2.5* expression was initiated normally but not sufficiently sustainable. Furthermore, a possible mechanism for the maintenance of *nkx2.5* may be that other cardiac connexins expressed in the subsequent stages may take over the role of Ecx in later stages and maintain the *nkx2.5* expression (39, 40). Intriguingly, connexins such as *cx40* and *cx43* have been suggested to be the downstream target of Nkx2.5 (27, 41). A feedback mechanism between connexins and Nkx2.5 might exist for the sustained *nkx2.5* expression. Such mechanisms regulating the *nkx2.5* transcription may be included in the machinery that controls and maintains the functional heart.

An important question remained is what signaling molecule(s), regulated by Ecx, modulate(s) early transcription of *nkx2.5*. Because Ecx can act as a functional hemichannel and gap junction (data not shown), we suspect that Ecx may mediate extracellular and/or intercellular signaling through molecules such as Ca^{2+} or inositol 1,4,5-trisphosphate (42, 43) to activate the *nkx2.5* transcription. Further comprehensive studies will be needed to elucidate the mechanism for early induction and establishment of *nkx2.5* expression in the heart. The zebrafish *ftk* mutant may serve as a useful model for understanding the signaling mechanisms underlying proper morphogenesis and maintenance of a functional heart, the dysfunction of which leads to CHD.

Materials and Methods

Zebrafish Strains. The wild-type strain TL was used for mutagenesis with *N*-ethyl-*N*-nitrosourea. Tü and WIK strains were used for genetic mapping. The *ftk* mutant, *jk65*, was identified by a small-scale haploid mutant screen performed at the University of Tokyo (A.K., unpublished data). From the mutant morphology, which is similar to blown pufferfish, we termed the *ftk* mutant as *futka*, a local name of pufferfish in the Ganges basin from which the zebrafish originated.

Positional Cloning. Positional cloning was performed by a standard method by scoring 1,500 mutant embryos. Briefly, we mapped the *ftk* mutation to the vicinity of z8252 on LG 7. We further performed fine mapping and identified two closely linked markers, z1206 and z14462. By chromosomal walking with the bacterial artificial chromosome (BAC) library, we identified zK161F3 as a clone that covered the entire *ftk* locus. The whole genomic sequence of zK161F3 was obtained from the assembled zebrafish genome sequence. The putative candidate cDNAs encoding MARCH III, gap junction connexin, SH3 containing the Grb2 SH3 domain, ferritin, serum amyloid A, and 60S acidic ribosomal protein P2 were amplified by RT-PCR to identify the mutation. The sequence and mutation were verified in genomic sequences.

Transfection. Constructs with 3×FLAG tags at the C terminus were made by subcloning the wild-type and mutant *ecx* into pcDNA3.1 (Invitrogen). The constructs were used to transfect HeLa cells, and the cells were fixed and subjected to immunological staining with anti-FLAG antibody (Sigma). Nuclei were stained with Hoechst 33342.

Electron Microscopy. Embryos were fixed at ≈58 hpf with 2.5% glutaraldehyde and 4% paraformaldehyde in PBS (pH 7.0). After fixation was carried out in 0.5% OsO_4 and 0.8% $K_3Fe(CN)_6$, the specimens were processed by a standard procedure for TEM. Ultrathin sections (80 nm) were stained with uranyl acetate and lead citrate and analyzed with an electron microscope.

***In Situ* Hybridization.** Whole-mount *in situ* hybridization was carried out by using a standard protocol (44). Respective plasmids for probes were made by the PCR based on the reported sequences in the GenBank and cloned into pBluescript.

Knockdown. Antisense *ecx* MO (5′-GGAGAGCGAAGGTGCCATCACTGCT-3′) and *nkx2.5* MO (5′-CATTTGGCTAGAGAACATTGCCATG-3′) (Gene Tools) targeting the ATG initiation sites of respective transcripts were used. The MOs were dissolved in 1× Danieau buffer, and aliquots of 2 ng (*ecx* MO) or the concentrations indicated in SI Table 2 (*nkx2.5* MO) were injected into embryos at the one- to two-cell stage.

mRNA Injections. mRNAs were synthesized *in vitro* by using an mMESSAGE mMACHINE kit (Ambion) and injected into one- to two-cell stage embryos. Some embryos were genotyped by Hin1I restriction fragment-length polymorphism (RFLP) analysis.

Dye-Uptake Assay. The dye-uptake assay for Ecx-expressing cultured cells was performed according to the procedure as described in ref. 45 with some minor modification. Stocks of fluorescent dyes were prepared in PBS (pH 7.4) at 2 mg/ml for Lucifer yellow CH lithium salt (557 molecular weight; Invitrogen) and 25 mg/ml for the dextran–rhodamine (10,000 molecular weight, Invitrogen), which was used for labeling the dead cells. The HeLa cells (≈10^5) were transfected with the construct encoding Ecx (either wild-type or mutant). Transfection efficiency was evaluated by cotransfection with constructs encoding EGFP and was found to exhibit an expression efficiency of ≈80%. At 48 h after transfection, transfected cells were rinsed twice with PBS on ice and mechanically stimulated with a continuous drip of 800 μl of dye released from a height of ≈4.5 cm above the culture dish. The stimulation was repeated three times, and the dye-covered cells were incubated on ice for 5 min. Cultures were washed 10 times with ice-cold PBS and examined under a fluorescent microscope. Phase-contrast and fluorescent images were collected around the drip target site. Four independent transfections were assayed for dye uptake.

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