The novel transmembrane protein Tmem2 is essential for coordination of myocardial and endocardial morphogenesis

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SUMMARY
Coordination between adjacent tissues plays a crucial role during the morphogenesis of developing organs. In the embryonic heart, two tissues – the myocardium and the endocardium – are closely juxtaposed throughout their development. Myocardial and endocardial cells originate in neighboring regions of the lateral mesoderm, migrate medially in a synchronized fashion, collaborate to create concentric layers of the heart tube, and communicate during formation of the atrioventricular canal. Here, we identify a novel transmembrane protein, Tmem2, that has important functions during both myocardial and endocardial morphogenesis. We find that the zebrafish mutation frozen ventricle (frv) causes ectopic atrioventricular canal characteristics in the ventricular myocardium and endocardium, indicating a role of frv in the regional restriction of atrioventricular canal differentiation. Furthermore, in maternal-zygotic frv mutants, both myocardial and endocardial cells fail to move to the midline normally, indicating that frv facilitates cardiac fusion. Positional cloning reveals that the frv locus encodes Tmem2, a predicted type II single-pass transmembrane protein. Homologs of Tmem2 are present in all examined vertebrate genomes, but nothing is known about its molecular or cellular function in any context. By employing transgenes to drive tissue-specific expression of tmem2, we find that Tmem2 can function in the endocardium to repress atrioventricular differentiation within the ventricle. Additionally, Tmem2 can function in the myocardium to promote the medial movement of both myocardial and endocardial cells. Together, our data reveal that Tmem2 is an essential mediator of myocardium-endocardium coordination during cardiac morphogenesis.

KEY WORDS: Zebrafish, Heart development, Atrioventricular canal, Cardiac fusion

INTRODUCTION
The embryonic heart tube is initially a two-layered structure: the outer layer of muscular myocardium contracts to propel circulation and the inner layer of endothelial endocardium provides continuity with the rest of the vasculature. Myocardial and endocardial cells originate in neighboring regions of the lateral mesoderm (Schoenebeck et al., 2007). During the process of cardiac fusion, both cell types migrate medially in a synchronized fashion and merge at the midline to assemble the heart tube (Bussmann et al., 2007; Holtzman et al., 2007; Moreno-Rodriguez et al., 2006). At later stages, cardiac maturation involves remodeling of both juxtaposed layers during valve formation and trabeculation (Armstrong and Bischoff, 2004; Hinton and Yutzey, 2010; Sedmera et al., 2000). Despite the continual proximity of the myocardium and endocardium, little is known about the mechanisms that coordinate their development.

The coordination of myocardial and endocardial development has been particularly well established in the context of atrioventricular canal (AVC) formation. Both the myocardium and endocardium undergo specialized differentiation in order to create the characteristic morphology of the AVC and to establish the endocardial cushions that will remodel into the atrioventricular valve (Armstrong and Bischoff, 2004; Beis et al., 2005; Chi et al., 2008; Hinton and Yutzey, 2010). Several studies indicate that myocardium-endocardium communication regulates these spatially coincident events (Armstrong and Bischoff, 2004; Hinton and Yutzey, 2010). For example, chick explant experiments suggest that signal transduction between atrioventricular myocardium and atrioventricular endocardium induces endocardial cushion formation (Mjaatvedt et al., 1987). Although several signaling pathways have been implicated in promoting endocardial cushion development (Armstrong and Bischoff, 2004; Beis et al., 2005; Hinton and Yutzey, 2010), it is less clear which genes are responsible for attenuating these signals so as to spatially restrict atrioventricular differentiation.

Myocardium-endocardium coordination is also crucial during cardiac fusion. The synchronization of myocardial and endocardial migration suggests that both tissues respond to the same cues in the extracellular environment. Some cues may emanate from the endoderm: both myocardial and endocardial fusion are inhibited when endodermal specification or morphogenesis is disrupted (e.g. Holtzman et al., 2007; Kikuchi et al., 2001; Kupperman et al., 2000). Additionally, either diminished or excessive deposition of extracellular matrix (ECM) can hinder myocardial and endocardial movement (e.g. Arrington and Yost, 2009; Garavito-Aguilar et al., 2010; Trinh and Stainier, 2004). Furthermore, the myocardium and endocardium may impact the behavior of one another. Cardiomyocytes display aberrant migration patterns in the absence of the endocardium.
of endocardial cells, implying that myocardium-endocardium communication influences the direction of cell movement (Holtzman et al., 2007). However, the molecular basis for myocardium-endocardium interactions during cardiac fusion is not yet clear.

To elucidate the molecular mechanisms underlying myocardium-endocardium communication, we have investigated the zebrafish mutation frozen ventricle (frv). Zygotic frv mutants display ectopic AVC characteristics, and maternal-zygotic frv mutants exhibit defects in cardiac fusion. Positional cloning indicates that the frv locus encodes Tmem2, a previously uncharacterized transmembrane protein. Tissue-specific rescue experiments suggest that Tmem2 acts in the endocardium to spatially restrict atrioventricular differentiation, whereas during cardiac fusion Tmem2 acts in the myocardium to promote the medial movement of both myocardial and endocardial cells. Together, our studies illuminate crucial roles of a novel molecule, Tmem2, in regulating the coordination of myocardial and endocardial morphogenesis.

MATERIALS AND METHODS

Zebrafish
We discovered the recessive lethal mutation frozen ventricle (frv<sup>sk38</sup>) through routine intercrosses in the Skirball zebrafish facility. To obtain maternal-zygotic frv embryos, we generated germline replacement chimeras through transplantation, as previously described (Ciruna et al., 2002). Donor embryos were generated by intercrossing frv heterozygotes, and wild-type host embryos were screened for gerrn cells derived from frv<sup>+</sup> donors. We raised 273 chimeric embryos and recovered eight fertile adult females. These females were bred to male frv heterozygotes to generate maternal-zygotic frv mutants.

In situ hybridization
We conducted in situ hybridization using previously reported probes for myl7 (ZDB-GENE-991019-3), notch1b (ZDB-GENE-990415-183), bmp4 (ZDB-GENE-980528-2059), versican (ZDB-GENE-011023-1), tbx2b (ZDB-GENE-990726-27) and cdh3 (ZDB-GENE-040816-1). Mutant embryos were identified by PCR genotyping.

Immunofluorescence
We performed MF20/S46 whole-mount immunofluorescence as previously described (Alexander et al., 1998). To detect Dm-grasp, we used the monoclonal antibody zn-5 (ZIRC; 1:10), rabbit polyclonal anti-GFP (Invitrogen; 1:100), goat anti-mouse IgG Alexa 594 (Invitrogen; 1:100) and goat anti-rabbit IgG Alexa 488 (Invitrogen; 1:100). Embryos were fixed in 4% PFA at 4°C overnight and were cut coronally posterior to the common cardinal vein to facilitate antibody penetration. Staining was performed in PBS with 0.5% Triton X-100 and 1% DMSO. Confocal images were obtained using a Zeiss LSM510 microscope, and z-stacks were rendered in three dimensions and analyzed with Velocity software (Perkin Elmer).

Positional cloning of frv
Meiotic mapping demonstrated that the frv mutation is located on chromosome 5, between SSLP markers z61852 and z22523. Analysis of SNPs narrowed the interval to a region containing five candidate genes. Sequencing of the coding region of tmem2 (GenBank HG97922) revealed a nonsense mutation in frv.

mRNA and morpholino injection
Embryos were injected at the one-cell stage with 200-500 pg mRNA or 8-13.5 ng anti-tmem2 morpholino (MO) (5′-AGACAGTCCAAAGCATACC-ATTACTCC-3′; Gene Tools). Capped mRNA was synthesized from a pcDNA vector containing the tmem2 or tmem2<sup>-effo</sup> coding sequence. The MO blocks splicing between exon 4 and intron 4-5, which is predicted to result in a truncated protein that contains the first 404 amino acids of Tmem2 and 44 amino acids encoded by intronic sequence.

Transgenes
We used the Gateway system (Kwan et al., 2007; Villefranc et al., 2007) to create the transgenes Tg(myl7:tmem2-gfp), Tg(kdrl:gal4vp16) and Tg(uas:tmem2-gfp). We employed Tol2 transposase-mediated transgenesis for both transient transgene expression and generation of transgenic founders (Fisher et al., 2006). Additional transgenic lines used were Tg(kdrl:GRCFP) (Cross et al., 2003) and Tg(fli:gal4ff<sub>PHAB</sub>), which drives endothelium-specific expression of the GAL4-VP16 derivative Gal4FF and is described elsewhere (Zygmont et al., 2011).

RESULTS AND DISCUSSION

frv restricts atrioventricular differentiation to the atrioventricular canal
The zebrafish mutation frozen ventricle (frv) has a distinctive impact on cardiac morphology and function. At 48 hours post-fertilization (hpf), embryos homozygous for frv exhibited contractility and dysmorphic cardiac chambers, with poor demarcation of the AVC and an abnormal separation between the ventricular myocardium and endocardium (Fig. 1A-H). By 72 hpf, the frv mutant ventricle ceased to contract, although atrial function seemed unaffected, inspiring the use of frozen ventricle as the locus name.

Expression patterns of cardiac genes appeared normal during the initial steps of heart tube assembly in frv mutants (see Fig. S1 in the supplementary material). By contrast, defects in expression of the AVC markers notch1b, bmp4, versican, and tbx2b were evident in frv mutants by 48 hpf. In wild-type embryos, expression of each of these markers becomes restricted to the AVC (Hurlstone et al., 2003; Walsh and Stainier, 2001) (Fig. 1I-K, see Fig. S2A in the supplementary material). In frv mutants, none of these markers became properly restricted (Fig. 1L-N, see Fig. S2B in the supplementary material): notch1b expression was evident in the ventricular endocardium, bmp4 expression was expanded into the ventricular myocardium, and versican and tbx2b expression expanded into both the ventricular and atrial myocardium. Additionally, frv mutants exhibited expanded localization of the adhesion molecule Dm-grasp. Dm-grasp is normally detectable in differentiated endocardial cells within the AVC, butnot in the remainder of the endocardium (Beis et al., 2005) (Fig. 1O-Q). In frv mutants, Dm-grasp was found throughout the entire ventricular endocardium, as well as in the AVC endocardium (Fig. 1R-T). Thus, the presence of ectopic atrioventricular characteristics in the frv mutant ventricle indicates an important role for frv in the regional restriction of atrioventricular differentiation.

Positional cloning of frv reveals a novel transmembrane protein
To gain insight into the molecular mechanisms of frv function, we positionally cloned the gene encoded by the frv locus. Meiotic mapping of frv identified a 0.28 cM genetic interval, and the annotated zebrafish genome assembly indicated five candidate genes in this region (Fig. 2A). Examination of the coding sequence of one of these genes, transmembrane protein 2 (tmem2), revealed a nonsense mutation in frv mutant genomic DNA that is predicted to truncate the Tmem2 protein (Fig. 2B). To test the hypothesis that loss of tmem2 function is responsible for the frv mutant phenotype, we injected tmem2 mRNA into frv mutant embryos and found that this can rescue the frv mutant defects in chamber morphology, ventricular contractility and Dm-grasp distribution (see Table S1 and Fig. S3G-I in the supplementary material). Injection of tmem2 mRNA did not cause any detectable phenotype in wild-type embryos. Additionally, we found that injection of anti-tmem2 morpholinos (MOs) into wild-type embryos could cause...
dysmorphic chambers, reduced ventricular contractility and ectopic Dm-grasp (see Fig. S3J-L in the supplementary material). Together, the linkage, nonsense mutation, rescue and MO phenocopy data indicate that disruption of the \textit{tmem2} gene is the cause of the \textit{frv} phenotype.

\textit{Tmem2} is predicted to be a type II transmembrane protein with a single transmembrane helix, a short cytoplasmic tail and a long extracellular portion (Fig. 2B). A BLAST local alignment search detected two identifiable domains – a G8 domain and a GG domain – within the extracellular sequence (Fig. 2B), consistent with previous bioinformatic analysis of the human TMEM2 homolog (see Fig. S4 in the supplementary material) (Guo et al., 2006; He et al., 2006). The structure of \textit{Tmem2} is highly conserved: \textit{Tmem2} homologs are detectable in a wide variety of vertebrate genomes, including \textit{Homo sapiens}, \textit{Mus musculus}, \textit{Gallus gallus}, \textit{Xenopus tropicalis} and \textit{Takifugu rubripes}, all of which share at least 63\% amino acid similarity with zebrafish \textit{Tmem2} (see Fig. S4 in the supplementary material). However, we were unable to identify \textit{Tmem2} homologs in \textit{Caenorhabditis elegans} or \textit{Drosophila melanogaster}. Despite its conservation among vertebrates, no prior studies have investigated \textit{Tmem2} function. Thus, our data provide novel evidence of an essential role for \textit{Tmem2}.

\textbf{Endocardial expression of \textit{tmem2} represses inappropriate atrioventricular differentiation}

The \textit{frv} phenotype indicates that \textit{Tmem2} prevents atrioventricular differentiation in the ventricular endocardium, but it is not clear in which tissue \textit{Tmem2} acts to execute this role. Human and mouse \textit{Tmem2} homologs are expressed in a wide array of tissues (Scott et al., 2000). Consistent with this, we found broad expression of \textit{tmem2} throughout the early zebrafish embryo, and we also detected a maternal supply of \textit{tmem2} (see Fig. S5 in the supplementary material).
Since the expression pattern of \textit{tmem2} does not clarify its site of action, we designed tissue-specific transgenes to test whether expression of \textit{tmem2} in the myocardium or endocardium could rescue the \textit{frv} mutant phenotype. These transgenes drive expression of a fusion protein in which the C-terminus of Tmem2 is tagged with GFP. Tmem2-GFP appears functionally equivalent to Tmem2: injection of \textit{tmem2-gfp} mRNA yielded results similar to injection of \textit{tmem2} mRNA (see Table S1 in the supplementary material).

Expression of \textit{tmem2} throughout the myocardium, driven by the stably integrated transgene \textit{Tg(myl7:tmem2-gfp)} (see Fig. S6A-C in the supplementary material), was not sufficient to rescue the ectopic atrioventricular characteristics in \textit{frv} mutants (see Fig. S7 in the supplementary material). Expression of \textit{tmem2} in the endocardium was more challenging to attain. Since no endocardium-specific driver is available, we employed endothelial drivers (Jin et al., 2005; Villefranc et al., 2007). First, we used the transgenes \textit{Tg(kdrl:gal4vp16)} and \textit{Tg(uas:tmem2-gfp)} to generate transient mosaic expression within the endocardium (Fig. 3A-C). Expression of \textit{tmem2} in atrial endocardium did not alter the \textit{frv} mutant phenotype (Fig. 3D-F; \( n = 4 \)). By contrast, we observed loss of ectopic Dm-grasp in ventricular endocardial cells exhibiting mosaic \textit{tmem2} expression (Fig. 3G-I; \( n = 5 \)). In an effort to generate a more thorough rescue of the \textit{frv} endocardium, we next employed the stably integrated driver \textit{Tg(fliep:gal4ff)} (Zygmunst et al., 2011). Unfortunately, stable integration of both \textit{Tg(fliep:gal4ff)} and \textit{Tg(uas:tmem2-gfp)} did not result in uniform expression of \textit{tmem2} throughout the endocardium (Fig. 3J-L, see Fig. S6D in the supplementary material).
supplementary material), potentially owing to epigenetic silencing of Tg(uas:tmem2-gfp) (Goll et al., 2009). Accordingly, the effects of this transgene combination in frv mutants were comparable to the effects of mosaic expression of Tg(kdrl:gal4vp16) and Tg(uas:tmem2-gfp) (n=3); however, in one frv mutant, broad expression of tmem2 in the ventricular endocardium eliminated the ectopic Dm-gpasp (Fig. 3M-O). These data indicate that the activity of Tmem2 in the ventricular endocardium is sufficient to prevent the ectopic assumption of atrioventricular characteristics. Moreover, our data imply the existence of repressive mechanisms that restrain atrioventricular differentiation in the ventricle.

Tmem2 facilitates medial migration of both myocardium and endocardium

Given the contrast between the broad expression pattern of tmem2 and the specific defects in frv mutants, we suspected that the maternal supply of tmem2 partially compensates for the loss of zygotic tmem2 function. To uncover additional roles of tmem2, we generated maternal-zygotic frv (MZfrv) mutant embryos via germline replacement (Ciruna et al., 2002). MZfrv mutants underwent gastrulation normally and began to display morphological defects during somitogenesis; by contrast, maternal frv (Mfrv) mutants appeared normal (Fig. 4A,B, see Fig. S8 in the supplementary material). Cardiac defects emerged in MZfrv mutants at an earlier stage than in zygotic frv mutants. Whereas cardiac fusion proceeded normally in Mfrv mutants, the medial migration of MZfrv cardiomyocytes was significantly hindered (Fig. 4C,D). However, the bilateral populations of cardiomyocytes were not permanently separated, and they eventually formed two linked sets of dysmorphic chambers (see Fig. S9 in the supplementary material). Additionally, MZfrv mutants displayed abnormal endocardial migration. Instead of meeting at the midline to form an endocardial tube, MZfrv endocardial cells were substantially delayed in their medial migration and remained bilaterally separate (Fig. 4E,F). Therefore, Tmem2 is crucial for facilitating cell movement during cardiac fusion.

Myocardial expression of tmem2 promotes both myocardial and endocardial fusion

The failure of both myocardial and endocardial fusion in MZfrv mutants suggested the possibility that both phenotypes result from a primary defect in endoderm formation. However, endodermal specification and morphogenesis appeared normal in MZfrv mutants (data not shown). We therefore hypothesized that Tmem2 functions in migrating cardiac cells. Using the stably integrated transgene Tg(my17:tmem2-gfp), we found that myocardial expression of tmem2 could rescue myocardial migration in MZfrv mutants; although morphogenesis was still slightly delayed in rescued embryos, cardiac fusion was complete and heart tube extension was under way by 26 hpf (Fig. 4G-J, see Fig. S10A-D in the supplementary material; n=11). Remarkably, the positive effects of Tg(my17:tmem2-gfp) expression extended to the endocardium. Myocardial expression of tmem2 rescued endocardial migration in MZfrv mutants, such that the endocardial cells reached the midline and extended into a tube (Fig. 4K-N, see Fig. S10E-H in the supplementary material; n=12). These data demonstrate that Tmem2 activity in the myocardium is sufficient to facilitate both myocardial and endocardial fusion. Moreover, our data provide the first evidence for a myocardial factor that influences the migration of endocardial cells.

Tmem2 is essential for the coordination of myocardial and endocardial morphogenesis

Our studies provide the first demonstration that the previously unappreciated transmembrane protein Tmem2 has multiple important functions during development. Notably, Tmem2 is
essential for two crucial aspects of cardiac morphogenesis: the spatial restriction of atrioventricular differentiation during AVC formation and the medial migration of cardiac cells during cardiac fusion. In each context, we have identified one location where Tmem2 can function—in the endocardium during AVC formation and in the myocardium during cardiac fusion—although our results do not rule out additional sites for Tmem2 activity during these processes. Our data suggest intriguing mechanisms for the coordination of myocardial and endocardial morphogenesis by Tmem2, and it is attractive to speculate that Tmem2 might play similar roles during AVC differentiation and cardiac fusion.

The transmembrane localization of Tmem2 evokes the hypothesis that it could facilitate myocardium-endocardium signaling. Perhaps certain signals repress atrioventricular differentiation in the ventricle, and Tmem2 plays a permissive role in facilitating signal reception by the endocardium. Similarly, Tmem2 could act to limit the activity of inductive signals that specify AVC endocardium; for example, the similarities between frv mutants and apc mutants suggest a possible role of Tmem2 in restricting Wnt signaling (Hurlstone et al., 2003; Verhoeven et al., 2011). During cardiac fusion, Tmem2 might facilitate the transduction of an unknown myocardial-to-endocardial motility cue; however, this scenario seems unlikely because the endocardium can move to the midline in hand2 mutants, which possess very few cardiomyocytes (Garavito-Aguilar et al., 2010). Alternatively, rather than influencing specific signaling pathways, Tmem2 might impact myocardial and endocardial morphogenesis by modulating the extracellular environment. Perhaps Tmem2 influences ECM organization between the endocardium and myocardium in a manner that normally insulates the ventricular endocardium from myocardial signals that induce atrioventricular differentiation. Likewise, since specific parameters of ECM composition are known to be fundamental for cardiac fusion (Arrington and Yost, 2009; Garavito-Aguilar et al., 2010; Trinh and Stainier, 2004), Tmem2 could play a permissive role in ensuring the appropriate ECM organization to facilitate both myocardial and endocardial movement. Whatever the molecular role of Tmem2, its activity, as described here, broadens our comprehension of the mechanisms that underlie the coordination of myocardial and endocardial development. Elucidating the biochemical basis for Tmem2 function will further advance our understanding of the roles of this novel developmental regulator.

Note added in proof
An accompanying paper (Smith et al., 2011) characterizes the phenotype caused by zebrafish mutations referred to as wickham (wkm) and reports that the wkm locus encodes tmem2. Complementation tests performed by breeding frv heterozygotes to wkm heterozygotes have demonstrated that the two mutant alleles fail to complement each other: summarizing the results of five independent clutches, 27.7% (49/177) of the progeny display a phenotype indistinguishable from that of zygotic frv mutants. These data further reinforce the conclusion that the frv and wkm mutations disrupt the same gene: tmem2.

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Competing interests statement
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