PDGF signaling directs cardiomyocyte movement toward the midline
during heart tube assembly

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ABSTRACT

Communication between neighboring tissues plays a central role in guiding organ morphogenesis. During heart tube assembly, interactions with the adjacent endoderm control the medial movement of cardiomyocytes, a process referred to as cardiac fusion. However, the molecular underpinnings of this endodermal-myocardial relationship remain unclear. Here, we show an essential role for platelet-derived growth factor receptor alpha (Pdgfra) in directing cardiac fusion. In both zebrafish and mouse, mutation of \textit{pdgfra} inhibits cardiac fusion and can lead to cardia bifida. Timelapse analysis of individual cardiomyocyte trajectories reveals misdirected cells in zebrafish \textit{pdgfra} mutants, suggesting that PDGF signaling steers cardiomyocytes toward the midline. Intriguingly, the ligand \textit{pdgfaa} is expressed in the endoderm medial to the \textit{pdgfra}-expressing myocardial precursors. Ectopic expression of \textit{pdgfaa} interferes with cardiac fusion, consistent with an instructive role for PDGF signaling. Together, these data uncover a novel mechanism through which endodermal-myocardial communication guides the cell movements that initiate cardiac morphogenesis.
IMPACT STATEMENT

Studies in zebrafish and mouse implicate the PDGF signaling pathway in the communication between the endoderm and the myocardium that drives medial myocardial movement and thereby initiates cardiac morphogenesis.
INTRODUCTION

Organogenesis relies upon the coordinated regulation of precisely defined patterns of cell movement. Multiple precursor cell populations must convene at the appropriate location and organize into the correct configuration in order to insure proper organ function. Differential adhesion and paracrine signaling between neighboring tissues often influence the specific routes traveled by precursor cells during morphogenesis (Scarpa and Mayor, 2016). However, the molecular mechanisms through which tissue interactions guide organ assembly remain poorly understood.

Heart formation requires the coordinated movement of myocardial precursor cells from their bilateral origins toward the embryonic midline, where they meet and merge through a process called cardiac fusion (Evans et al., 2010). Cardiac fusion is essential for the construction of the heart tube, which provides a fundamental foundation for subsequent steps in cardiac morphogenesis. During cardiac fusion, the medial movement of the myocardium is considered to be a collective cell behavior: the cardiomyocytes travel along relatively parallel paths with very little neighbor exchange (Holtzman et al., 2007) and simultaneously form intercellular junctions and create a primitive epithelial sheet (Linask, 1992; Manasek, 1968; Stainier et al., 1993; Trinh and Stainier, 2004; Ye et al., 2015). Whether these coherent patterns of myocardial movement reflect active migration or passive morphogenesis is not yet resolved (Aleksandrova et al., 2015; Dehaan, 1963; Varner and Taber, 2012; Xie et al.,...
2016; Ye et al., 2015). In either case, it is important to elucidate the specific
signals that dictate the medial direction of myocardial trajectories during cardiac
fusion.

Several lines of evidence indicate that cardiac fusion is mediated by
interactions between the myocardium and the adjacent anterior endoderm. In
both mouse and zebrafish, mutations that block endoderm formation or disrupt
endoderm integrity also inhibit cardiac fusion (Alexander et al., 1999; Holtzman et
al., 2007; Kawahara et al., 2009; Kikuchi et al., 2001; Kupperman et al., 2000; Li
et al., 2004; Mendelson et al., 2015; Molkentin et al., 1997; Osborne et al., 2008;
Ragkousi et al., 2011; Roebroek et al., 1998; Ye and Lin, 2013; Yelon et al.,
1999). Studies tracking both endodermal and myocardial movement in chick
have suggested that endodermal contraction provides a physical force that pulls
the myocardium toward the midline (Aleksandrova et al., 2015; Cui et al., 2009;
Varner and Taber, 2012). However, while endodermal forces may influence
initial phases of cardiac fusion, the observed patterns of endoderm behavior
seem insufficient to account for the entire path traversed by the moving
cardiomyocytes (Aleksandrova et al., 2015; Cui et al., 2009; Varner and Taber,
2012; Xie et al., 2016; Ye et al., 2015). Moreover, observations of myocardial
cell protrusions have suggested that these cells may actively migrate in response
to endodermal cues (Dehaan, 1963; Haack et al., 2014; Ye et al., 2015). While it
is clear that the endoderm plays an important role in facilitating cardiac fusion,
the molecular underpinnings of the endodermal-myocardial relationship are still
unknown.
Here, we reveal a novel connection between the endoderm and myocardium by discovering a new role for platelet-derived growth factor (PDGF) signaling. PDGFs signal through receptor tyrosine kinases and are well known for their mitogenic activity (Andrae et al., 2008), as well as for their role in guiding the migration of mesenchymal cells (Ataliotis et al., 1995; Yang et al., 2008). However, PDGF signaling has not been previously implicated in heart tube assembly, even though it is known to be important for later aspects of heart development, such as the contribution of cardiac neural crest cells to the outflow tract (Morrison-Graham et al., 1992; Schatteman et al., 1995; Tallquist and Soriano, 2003), the formation of the inflow tract (Bleyl et al., 2010), and the formation of epicardial derivatives (Smith et al., 2011).

Our analysis of early morphogenetic defects caused by mutation of the gene encoding PDGF receptor alpha (Pdgfra) uncovers an essential function for Pdgfra during cardiac fusion in both zebrafish and mouse. Notably, through live imaging of individual cell movements in zebrafish mutants, we find that pdgfra is crucial for guiding cardiomyocyte movement toward the midline. Furthermore, our studies suggest that expression of PDGF ligands by the anterior endoderm could facilitate interaction of this tissue with the pdgfra-expressing myocardial precursors. Thus, our work supports a model in which PDGF signaling underlies communication between the endoderm and myocardium and thereby directs the cell movements that initiate heart tube assembly. These insights into the regulation of cardiomyocyte behavior provide new ideas regarding the etiology of diseases associated with aberrant cell movement (Friedl and Gilmour, 2009),
including congenital heart diseases (CHDs) caused by defective myocardial morphogenesis (Bleyl et al., 2010; Briggs et al., 2012; Neeb et al., 2013; Samsa et al., 2013).
RESULTS

refuse-to-fuse (ref) mutants display cardiac fusion defects

In a screen for ethylnitrosourea-induced mutations that disrupt cardiac morphogenesis in zebrafish (Auman et al., 2007), we identified a recessive lethal mutation, refuse-to-fuse (ref), that causes abnormal cardiac chamber morphology. Instead of the normal curvatures of the wild-type ventricle (Fig. 1A), ref mutants often displayed a bifurcated ventricle at 48 hours post-fertilization (hpf) (Fig. 1B). This phenotype was the most common among a range of cardiac defects in ref mutants (seen in ~50% of morphologically evident mutants; Fig. 1-Supplement 1); on rare occasions, we found ref mutants with cardia bifida, a condition in which two separate hearts form in lateral positions (seen in ~1% of morphologically evident mutants; Fig. 1-Supplement 1). The observed bifurcated ventricle and cardia bifida phenotypes led us to hypothesize that the ref mutation might interfere with cardiac fusion. In wild-type embryos, cardiac fusion results in the formation of a ring of cardiomyocytes at the midline by the 22 somite stage (Fig. 1C). In contrast, ref mutant cardiomyocytes failed to fuse into a ring and instead remained in separate bilateral domains (Fig. 1D) or fused only in posterior positions, creating a horseshoe shape (Fig. 1E). Similar fusion defects were also observed when examining a broader portion of the anterior lateral plate mesoderm (ALPM) encompassing the heart fields (Fig. 1F,G and Fig. 5A-F).

Since prior studies in zebrafish have shown that defects in endoderm specification or morphogenesis can inhibit cardiac fusion (Alexander et al., 1999;
Holtzman et al., 2007; Kawahara et al., 2009; Kikuchi et al., 2001; Kupperman et al., 2000; Mendelson et al., 2015; Osborne et al., 2008; Ye and Lin, 2013; Yelon et al., 1999), we examined the status of the endoderm in ref mutants. During gastrulation stages, the specification and movement of endodermal cells appeared normal in ref mutants (Fig. 1-Supplement 2A-D). In addition, the differentiation and morphology of the anterior endoderm in ref mutants appeared intact during the stages when cardiac fusion takes place (Fig. 1-Supplement 2E-H). The normal appearance of the ref mutant endoderm was consistent with the unaltered progress of the endocardial precursor cells in ref mutants: endocardial cells require interactions with the anterior endoderm for their medial movement during cardiac fusion (Holtzman et al., 2007; Wong et al., 2012; Xie et al., 2016), and the ref mutant endocardium seemed to reach the midline normally (Fig. 1-Supplement 3). Taken together, our data suggest that defects in myocardial movement, as opposed to defects in the endoderm, cause the bifurcated cardiac morphology in ref mutants.

ref is a loss-of-function mutation in pdgfra

In order to identify the genomic lesion responsible for the ref mutant phenotype, we mapped the ref locus to a <0.1 cM region on linkage group 2 containing 6 annotated genes (Fig. 2A). Our examination of ref mutant cDNA revealed missplicing in one of these genes, platelet-derived growth factor receptor alpha (pdgfra) (Fig. 2B). Specifically, we noted that exon 14 was often omitted or truncated in the pdgfra messages detected in ref mutant cDNA.
Furthermore, ref mutant genomic DNA contained a G-to-A mutation in the first nucleotide of intron 15 of pdgfra (Fig. 2C,D). Since a G at the exon/intron boundary is an essential conserved feature of splice sites, we infer that this mutation would disrupt pdgfra splicing. The misspliced pdgfra messages found in ref mutants cause a frameshift in the coding sequence, resulting in a premature truncation prior to the transmembrane domain of Pdgfra (Fig. 2D). In concordance with the concept that premature stop codons often lead to nonsense-mediated decay, we detected a global reduction of pdgfra mRNA in ref mutants (Fig. 2E,F).

We next compared the ref mutant phenotype to the effects of another mutation in pdgfra, b1059. The b1059 allele is a missense mutation that disrupts a conserved residue within the tyrosine kinase domain of Pdgfra (Eberhart et al., 2008) (Fig. 2D). Previous studies of b1059 mutant embryos focused on their dorsal jaw defects (Eberhart et al., 2008); our analysis also uncovered dorsal jaw defects in ref mutants (Fig. 2-Supplement 2B,E), as well as cardiac fusion defects in b1059 mutants (Fig. 2G,I). Through complementation testing, we found that ref and b1059 fail to complement each other; transheterozygotes displayed defects in both cardiac fusion (Fig. 2J) and dorsal jaw formation (Fig. 1-Supplement 2C,F). Finally, we found that injection of a morpholino targeting pdgfra also interfered with cardiac fusion (Fig. 2H). Together, our mapping, sequencing, complementation testing, and morpholino data support the conclusion that the ref mutation causes inappropriate splicing of pdgfra, resulting in diminished pdgfra function and cardiac fusion defects.
1 **Mutation of Pdgfra disrupts heart tube assembly in mice**

2 Although prior work in mouse has revealed functions for PDGF signaling during later stages of heart development (e.g. Grüneberg and Truslove, 1960; Richarte et al., 2007; Schatteman et al., 1995), these studies did not report an earlier role for PDGFRα during cardiac fusion or heart tube assembly. In contrast, analysis of the Patch (Ph) mutant, carrying a chromosome deletion including Pdgfra, did reveal early cardiac phenotypes, as well as yolk sac defects (Orr-Urtreger et al., 1992). Exploration of the early functions of PDGFRα has been complicated by the variability of Pdgfra mutant phenotypes, due in part to genetic background (Grüneberg and Truslove, 1960; Orr-Urtreger and Lonai, 1992; Schatteman et al., 1995; Soriano, 1997; Tallquist and Soriano, 2003). Notably, the C57BL/6 background was reported to generate more severe phenotypes in the Ph mutant (Orr-Urtreger et al., 1992). We therefore chose to analyze mouse embryos carrying Pdgfra null alleles on the C57BL/6 background at E9.5, using expression of Nkx2-5 to highlight heart morphology. Our analysis revealed a range of early defects in cardiac morphogenesis in homozygous Pdgfra mutants (Fig. 3A-E’). By E9.5, wild-type hearts had undergone looping and exhibited distinct left and right atrial and ventricular chambers (Fig. 3A-A’). Some Pdgfra homozygous mutant hearts displayed relatively mild defects in heart looping as well as in the size and shape of the cardiac chambers and their inflow and outflow tracts (Fig. 3B,B’). Other Pdgfra mutant hearts displayed more severe disruptions that could be the consequence of abnormal cardiac fusion (Fig. 3C-E’): examples included embryos with a split inflow/common atrial region
connected to a single ventricle (Fig. 3C,D) or with near total cardia bifida (Fig. 3E,E’). The majority of Pdgfra mutants died by E10.5, slightly earlier than reported for the majority of Ph mutants (Orr-Urtreger et al., 1992). These data uncover a previously unappreciated influence of Pdgfra on the early stages of cardiac morphogenesis in mice; in combination with the phenotype of ref mutants, these studies suggest that Pdgfra plays a conserved role in regulating heart tube assembly.

**pdgfra is expressed within the ALPM while cardiac fusion is underway**

To further elucidate how Pdgfra influences heart tube assembly, we next examined the expression pattern of pdgfra during cardiac fusion in zebrafish. We found robust expression of pdgfra within the ALPM and in migrating neural crest cells (Fig. 4A-D). The domains of pdgfra expression in the ALPM matched those of hand2 (Fig. 4E-J), which is expressed in the territories that contain myocardial precursor cells and is excluded from the territories containing endocardial precursors (Schoenebeck et al., 2007). As cardiac fusion proceeds, hand2 continues to be expressed in the cardiomyocytes that reach the midline (Fig. 5A-C), while pdgfra expression appears to be absent from these cells (Fig. 4D).

Similarly, we found that mouse Pdgfra is expressed in the ALPM at E7.5 (Fig. 3F) and later becomes confined to the caudal aspect of the forming heart tube by E8.0 (Fig. 3G) and to the inflow tract of the looping heart (Fig. 3H) as well as the dorsal mesocardium (Prall et al., 2007) by E8.5. In more mature hearts, Pdgfra is expressed in the atrioventricular valves and epicardium (Chong et al.,...
2011; Orr-Urtreger et al., 1992). Even though Pdgfra mRNA levels had declined in the anterior cardiac mesoderm by the beginning of heart tube formation (Fig. 3G), we found persistent PDGFRα protein expression in the forming heart at this stage (Fig. 3I). PDGFRα was also found in the more caudal domains defined by Pdgfra mRNA expression, including the caudal aspect of the forming heart corresponding to its future inflow tract and coelomic mesothelium (Fig. 3I) (Bax et al., 2010).

We did not observe pdgfra expression within the anterior endoderm during cardiac fusion in either zebrafish or mouse (Figs. 3G, 4K-P; (Prall et al., 2007)). In zebrafish, comparison of axial and pdgfra expression demonstrated mutually exclusive expression domains (Fig. 4K-P). Lack of pdgfra expression in the anterior endoderm is also consistent with previous expression analysis in mouse (Orr-Urtreger and Lonai, 1992), as well as with the lack of anterior endoderm defects in ref mutant embryos (Fig. 1-Supplement 2). Altogether, the pdgfra expression patterns in both zebrafish and mouse indicate that pdgfra could act within the ALPM to regulate the progression of cardiac fusion.

Pdgfra controls the medial direction of cardiomyocyte movement during cardiac fusion

Although our analysis pointed toward a role for pdgfra within the ALPM during cardiac fusion, we also considered the possibility that pdgfra expression in the early embryo (Ataliotis et al., 1995; Liu et al., 2002; Mercola et al., 1990; Yang et al., 2008) could indirectly affect cardiac fusion by influencing processes
such as mesoderm involution during gastrulation (Yang et al., 2008). However, we did not observe any defects in the size, shape, or bilateral spacing of the ALPM in ref mutants at the 8-12 somite stages (Fig. 5A,D,G), indicating that early ALPM morphogenesis is intact in these embryos. Moreover, we found that pharmacological inhibition of Pdgfr activity at the tailbud stage can disrupt cardiac fusion (Fig. 5-Supplement 1), further supporting the conclusion that pdgfra activity influences cardiac fusion after gastrulation is complete.

To determine when cardiac fusion first goes awry in ref mutants, we began by comparing the distance between the left and right sides of the ALPM in wild-type and ref mutant embryos. Until the 15 somite stage, the spacing between the bilateral domains of the ALPM was normal in ref mutants (Fig. 5G). After the 15 somite stage, the ref mutants began to display an evident phenotype: whereas the two sides of the wild-type ALPM continued to move toward each other, the sides of the ref mutant ALPM stayed apart (Fig. 5B,C,E,F,G). Thus, although the initial stages of ALPM convergence are unaffected in ref mutants, the ref mutant ALPM is unable to approach the midline normally during cardiac fusion.

We next sought to elucidate the cellular defects responsible for the inhibition of cardiac fusion in ref mutants. Do ref mutant cardiomyocytes move at a sluggish rate or are they misdirected? Previous studies have shown that VEGF signaling can regulate the speed of endocardial precursor movement during cardiac fusion (Fish et al., 2011), suggesting the possibility that PDGF signaling might set the pace of myocardial precursor movement. Alternatively, PDGF signaling has been shown to control the direction of mesodermal movement
during gastrulation (Damm and Winklbauer, 2011; Nagel et al., 2004), suggesting that it could also guide the route taken by myocardial cells during cardiac fusion. To test these hypotheses, we tracked individual cell movements over time, using the myocardial reporter transgene $Tg(\text{myl7:egfp})$ (Holtzman et al., 2007; Huang et al., 2003) to follow the patterns of cardiomyocyte behavior in live embryos (Fig. 6).

We initiated our timelapse analysis at the 16 somite stage, the earliest timepoint when we could robustly detect $Tg(\text{myl7:egfp})$ expression. Consistent with our analysis of ALPM position (Fig. 5), the bilateral populations of cardiomyocytes in ref mutants were already slightly farther apart than their wild-type counterparts were at the 16 somite stage (Fig. 6A,C,E). Following the movements of these cells during cardiac fusion, we found that wild-type cardiomyocytes display a coherent pattern of collective movement without significant neighbor exchange (Fig. 6A,B), consistent with our prior work (Holtzman et al., 2007). Cardiomyocytes in ref mutants exhibited similar patterns of coherent movement (Fig. 6C-F). However, while wild-type cardiomyocytes moved progressively toward the midline (Fig. 6A,B; Video S1), the medial movement of ref mutant cardiomyocytes seemed severely diminished, even though these cells still appeared to be in motion (Fig. 6C-F; Videos S2-S3). In ref mutants with a relatively mild phenotype, a posterior subset of cardiomyocytes still exhibited sufficient medial movement to fuse at the midline (Fig. 6C,D; Video S2). In more severely affected ref mutants, medial movement...
appeared lost along the entire anterior-posterior extent of the myocardium (Fig. 6E,F; Video S3).

Lack of medial movement could be the result of defects in several aspects of cell behavior including speed, efficiency, and directionality. To distinguish between these possibilities, we performed quantitative analysis of individual cardiomyocyte trajectories. Compared to wild-type cardiomyocytes, ref mutant cardiomyocytes moved at a slightly slower average speed (distance/time) (Fig. 6G) and with a slightly reduced efficiency (displacement/distance) (Fig. 6H).

When examining velocity (displacement/time) along particular axes, we found no difference between the velocities of wild-type and ref mutant cardiomyocyte movement along the anterior-posterior axis (Fig. 6I). However, there was a substantial difference between the velocities of wild-type and ref mutant cardiomyocyte movement along the medial-lateral axis: the average velocity along the medial-lateral axis was 0.19 micron/min for wild-type cardiomyocytes, but was only 0.016 micron/min for ref mutant cardiomyocytes (Fig. 6J). This difference in cell behavior becomes even more striking when considering the variability in the ref mutant phenotype. Two of the six ref mutant embryos examined had a relatively mild phenotype, and the cardiomyocytes in these embryos exhibited an average medial-lateral velocity similar to that seen in wild-type embryos (Fig. 6K). In contrast, the other four ref mutant embryos displayed a more severe phenotype, and the cardiomyocytes in these embryos had an average medial-lateral velocity near or below zero (Fig. 6K). Further examination of the vectors of cell movement revealed that these deficiencies in medial-lateral
velocity reflect the misdirection of ref mutant cardiomyocytes. In our wild-type
timelapse data, almost all cardiomyocytes move in the medial direction, whereas
over half of the cardiomyocytes in our ref mutant timelapse data show no medial
movement, with many of these cells moving away from the midline (Fig. 6L).
Together, these data reveal that pdgfra plays an important role in steering
cardiomyocyte movement toward the midline during cardiac fusion.

The Pdgfra ligand pdgfaa is expressed in the anterior endoderm, adjacent
to the ALPM

We next evaluated whether the expression patterns of genes encoding
Pdgfra ligands could provide insight into how PDGF signaling confers
directionality to cardiomyocyte movement. Interestingly, we found that pdgfaa is
expressed in bilateral domains within the anterior endoderm between the 10-16
somite stages, positioned near the lateral edges of this tissue (Fig. 7A-J). The
expression of pdgfaa within the anterior endoderm in zebrafish is grossly
consistent with prior studies demonstrating expression of Pdgfa in the mouse
foregut (Palmieri et al., 1992). We readdressed this issue in mouse and found
Pdgfa expression in endoderm at the rim of the foregut pocket (Fig. 3J) as well
as in the pharyngeal floor and pharyngeal pouches (Fig. 3J'), regions closely
associated with the forming heart tube at E8.0 and earlier stages. Moreover, the
pdgfaa expression domains in zebrafish are medially adjacent to the positions of
the myocardial precursors within the ALPM (Fig. 7K-P), suggesting the possibility
of a paracrine relationship between Pdgfaa in the endoderm and Pdgfra in the ALPM.

To investigate whether the spatially restricted expression of pdgfaa is important for the regulation of cardiac fusion, we induced pdgfaa expression throughout the embryo using the heat-inducible transgene Tg(hsp70l:pdgfaa-2A-mCherry). Following heat shock at the tailbud stage, transgenic embryos displayed cardiac fusion defects similar to those observed in ref mutants (Fig. 7Q-S). The ability of ectopic pdgfaa expression to disrupt cardiac fusion indicates that PDGF signaling has the potential to serve as an instructive influence in directing cardiomyocytes towards the midline.
DISCUSSION

Taken together, our studies point to a model in which the PDGF signaling pathway facilitates communication between the endoderm and the myocardium and thereby directs cardiomyocytes toward the midline during cardiac fusion. We propose that PDGF ligands, produced by the anterior endoderm, signal through the Pdgfra receptor in the ALPM in order to control the directionality of cardiomyocyte behavior. This connection parallels other examples in which PDGF ligand-receptor pairs in adjacent tissues influence cell movements (e.g. in the kidney, neural crest, and gastrulating mesoderm (Eberhart et al., 2008; Lindahl et al., 1998; Yang et al., 2008)), highlighting a paradigm for how tissue-tissue interactions establish the landscape of organogenesis (Andrae et al., 2008; Hoch and Soriano, 2003). Importantly, this new function for PDGF signaling represents the first insight into a pathway that guides the medial direction of cardiomyocyte movement. Moreover, these findings uncover a previously unappreciated molecular basis for the interactions between the endoderm and the myocardium that govern cardiac fusion.

How might PDGF signaling confer directionality on the collective behavior of the cardiomyocytes? In the absence of pdgfra function, myocardial movement is no longer directed toward the midline, implicating PDGF signaling in the arrangement of the forces that steer this epithelial tissue. One intriguing possibility is that PDGF signaling could direct medial movement via polarized Pdgftra activation that controls oriented formation of active protrusions, akin to the
activity of the PDGF/VEGF receptor Pvr in *Drosophila*, which directs the
collective movement of the epithelial border cells during oogenesis and the
epidermal cells during dorsal closure (Duchek et al., 2001; Garlena et al., 2015).
Alternatively, PDGF signaling could promote other types of epithelial
reorganization that could facilitate directional movement, such as the
rearrangement of adherens junctions or extracellular matrix at the medial edge of
the myocardium, causing epithelial deformations that drive movement forward
(Xu et al., 2005; Yang et al., 2008). Future examination of the relationship of
PDGF signaling to the subcellular characteristics of the myocardium during
cardiac fusion will help to elucidate the precise morphogenetic consequences of
Pdgfra activity.

It is likely that the PDGF signaling pathway works in parallel with
additional pathways to influence cardiac cell movement during cardiac fusion.
Although *ref* mutants fail to undergo proper cardiac fusion, they do not fully
phenocopy mutants with primary endoderm defects (e.g. *casanova* (*cas; sox32*)
or *miles apart* (*mil; s1pr2*) (Alexander et al., 1999; Kikuchi et al., 2001;
Kupperman et al., 2000; Ye and Lin, 2013; Yelon et al., 1999)). In *cas* and *mil*
mutants, both the endocardial and myocardial precursors fail to move to the
midline (Holtzman et al., 2007; Wong et al., 2012; Xie et al., 2016); moreover,
their myocardial movement defects can be detected prior to the 8 somite stage
(Ye et al., 2015). In contrast, the endocardial precursors seem to reach the
midline normally in *ref* mutants, and the *ref* myocardial movement defects
emerge only after the 15 somite stage. Most likely, other factors, such as VEGF
signaling to the endocardium (Fish et al., 2011) or mechanical forces from the endoderm (Aleksandrova et al., 2015; Varner and Taber, 2012), collaborate with PDGF signaling to control distinct aspects of endocardial and myocardial cell behavior during earlier and later phases of cardiac fusion.

The cardiomyocyte movements that occur during cardiac fusion, guided by interactions with the endoderm, establish a foundation of proper tissue orientation and morphology upon which to assemble the initial heart tube. Our studies in zebrafish and mouse reveal a conserved influence of PDGF signaling on heart tube assembly. This influence is likely to be relevant to CHD in humans, since defects in cardiac morphology can originate in the cardiac precursor populations involved in cardiac fusion and heart tube assembly (Prall et al., 2007; Vincent and Buckingham, 2010). More generally, the molecular mechanisms that control the direction of cardiomyocyte movement are likely to be relevant to the etiology of disorders that are caused by anomalous cell movement, potentially including ventricular septal defects, atrial septal defects, outflow tract defects, and trabeculation abnormalities (Bax et al., 2010; Bruneau, 2008; Ding et al., 2004; Neeb et al., 2013; Samsa et al., 2013), as well as inflow tract defects known to be associated with mutations in PDGFRA (Bleyl et al., 2010). PDGFRA is deleted in a number of human families showing total anomalous pulmonary venous return (TAPVR), in which the pulmonary arteries connect with the systemic venous system instead of the left atrium, a defect replicated in mouse and chick loss-of-function models (Bleyl et al., 2010). TAPVR occurs in 1 in 15,000 live births and, while life-threatening, is at the mild end of the spectrum of
morphogenetic defects that we have observed in Pdgfra knockout mice. Thus, our studies suggest the possibility of a broader involvement of PDGFRA mutations in CHD, specifically through their effects on heart tube assembly, and more globally as part of the spectrum of diseases associated with aberrant cardiac cell movements.
MATERIALS AND METHODS

Zebrafish

We used the following transgenic and mutant strains of zebrafish:

- Tg(myl7:egfp)\textsuperscript{twu34} (Huang et al., 2003), Tg(fli1a:negfp)\textsuperscript{y7} (Roman et al., 2002),
- Tg(sox17:egfp)\textsuperscript{ha01} (Mizoguchi et al., 2008), pdgfra\textsuperscript{b1059} (Eberhart et al., 2008),
- and ref (pdgfra\textsuperscript{sk16}; this paper). The Tg(hsp70l:pdgfaa-2A-mCherry)\textsuperscript{sd44} transgene was assembled using the D-Topo vector (Invitrogen) with a pdgfaa cDNA lacking the stop codon (Eberhart et al., 2008), in combination with established Gateway cloning vectors (Kwan et al., 2007). The final destination vector was created by inserting a Cryaa:CFP cassette (Hesselson et al., 2009) into the pDestTol2pA4 vector (gift from K. Kwan). Transgenic founders were established using standard techniques for Tol2-mediated transgenesis (Fisher et al., 2006). We analyzed F2 embryos from four separate transgenic lines to evaluate the effect of pdgfaa overexpression on cardiac fusion. Embryos were heat shocked at 38°C for 45 min beginning at the tailbud stage and were then returned to 28°C. Transgenic and nontransgenic sibling embryos were distinguished based on their expression of mCherry following heat shock. All zebrafish work followed protocols approved by the UCSD IACUC.

Mice

Pdgfra null embryos were generated by intercrossing heterozygous Pdgfra\textsuperscript{tm11(EGFP)Sor} (Hamilton et al., 2003) mutant mice on the C57BL/6 background. In situ hybridization and immunohistochemistry were performed.
using standard protocols (Prall et al., 2007), and genotyping was performed as
described for Jax stock #007669 (https://www.jax.org/strain/007669). Images
were captured using a Leica M125 microscope outfitted with a Leica DFC295
camera and processed using Adobe Photoshop. All mouse experiments were
overseen by the Garvan Institute of Medical Research/St. Vincent's Hospital
Animal Ethics Committee.

8 **Positional cloning and genotyping**

9 Meiotic recombinants were mapped using polymorphic SSLP and SNP
markers to identify a small critical interval on linkage group 20; PCR primers
used for mapping are provided in Figure 2-Supplement 1. Sequence analysis of
candidate genes was performed on cDNA from homozygous wild-type and ref
mutant embryos.

14 PCR genotyping was used to identify ref mutant embryos following
phenotypic analysis. The primer pair 5'-GTAGGTAAAAAGTAAAGCTGGTA-3' and
5'-CAAGGGTGTGTGGAACCTGA-3' amplifies a 136 bp PCR product flanking the
e14i15 boundary in the pdgfra locus and creates a KpnI restriction site within the
wild-type allele, but not within the ref mutant allele. Digestion of the wild-type
PCR product with KpnI creates fragments of 113 and 23 bp.

**Morpholinos and inhibitors**

A pdgfra morpholino (5'-CAGTCGAATAATCAGACCTCCTGAT-3') was
designed to disrupt the splicing of exon 11 and thereby lead to premature
truncation of Pdgfra prior to its kinase domain. We injected 12 ng of morpholino at the 1-cell stage; this dose did not induce visible toxicity. Furthermore, injection of this morpholino into ref mutants did not increase the frequency or severity of their cardiac fusion defects.

For pharmacological inhibition of PDGF signaling (Kim et al., 2010), we incubated embryos in Pdgfr inhibitor V (Calbiochem 521234) from the tailbud stage until the 22 somite stage. Three separate experiments were performed, using doses of 0.25-0.4 μM.

**In situ hybridization, immunofluorescence, and Alcian blue staining**

The following probes and antibodies were used: myl7 (ZDB-GENE-991019-3), axial/foxa2 (ZDB-GENE-980526-404), sox17 (ZDB-GENE-991213-1), hand2 (ZDB-GENE-000511-1), pdgfra (ZDB-GENE-990415-208), pdgfaa (ZDB-GENE-030918-2), anti-GFP (Abcam ab13970; 1:1000), anti-ZO-1 (Zymed 33-149100; 1:200), and donkey anti-mouse Alexa 488 (Invitrogen; 1:300). Standard in situ hybridization, fluorescent in situ hybridization, and immunofluorescence were performed using established protocols (Alexander et al., 1998; Brend and Holley, 2009; Yelon et al., 1999). Fluorescent in situ hybridization was combined with immunofluorescence as previously described (Zeng and Yelon, 2014). Standard in situ hybridization was combined with visualization of transgene expression by creating transverse sections following in situ hybridization, using standard cryoprotection, embedding, and sectioning techniques (Garavito-Aguilar et al., 2010) and then performing standard immunofluorescence for GFP on sections.
Alcian blue staining was performed as previously described (Kimmel et al., 1998). Trunks were removed for genotyping prior to Alcian staining. Images were captured using Zeiss M2Bio, AxioZoom and AxioImager microscopes outfitted with Axiocam cameras and processed with Adobe Photoshop. Confocal stacks were collected using a Leica SP5 confocal laser-scanning microscope and processed using Imaris (Bitplane).

Timelapse imaging and cell tracking

*Tg(myl7:egfp)* embryos at the 14 somite stage were mounted head down in 0.8% low-melt agarose and placed on a coverslip bottom dish in wells made from a layer of 3% agarose. Timelapse images were collected using a Leica SP5 confocal microscope with a 20X objective, in a chamber heated to 28°C. Confocal stacks of GFP and brightfield images were collected every 4 min for 2-3 hrs, starting around the 16 somite stage. In each stack, 30 confocal slices spanning the expression of *Tg(myl7:egfp)* were collected at ~3 µm intervals. Embryos were retained after completion of imaging, and we only analyzed data from embryos that appeared healthy for 24 h following the timelapse. Image processing and cell tracking was performed on three-dimensional reconstructions generated with Imaris, using the semi-automated cell tracking module. In each embryo, we tracked 20-30 cells from the 2 most medial columns of cardiomyocytes on each side. Only tracks in which a cell position could be determined for each timepoint were used for further analysis. We also tracked the tip of the notochord in brightfield images at each timepoint. Although we
observed a slight posterior retraction of the notochord over the course of our
timelapse analysis, we found that this was the most consistent landmark to use
as a reference point to correct for drift that occurred during imaging. Thus, the
movement of the tracked notochord tip was subtracted from the movement of
each tracked cardiomyocyte. Our wild-type tracking data were largely consistent
with our prior studies (Holtzman et al., 2007), including the velocity of movement,
coherence of movement, lack of cell movement in the Z-axis, and direction of
wild-type cardiomyocyte trajectories. Subtle differences between these two data
sets are likely due to our current use of the notochord as a reference point and
the slightly later stage at which we initiated these timelapse experiments.

For quantitative analysis of cardiomyocyte movement, we extracted the X
and Y position of each cell at each timepoint along its track, as previously
described (Holtzman et al., 2007). Cell movement properties, including overall
speed (distance/time), efficiency (displacement/distance), velocity
(displacement/time), and direction, were then calculated for each individual
cardiomyocyte. Velocity measurements were split into their X (medial-lateral)
and Y (anterior-posterior) components. Cells along the anterior-posterior axis
were further divided into top, middle, and bottom subsets, as in our prior work
(Holtzman et al., 2007). Direction was calculated as \text{arctan}[abs(y-
\text{displacement})/(x\text{-displacement})], after aligning movement between the left and
right sides. Graphs were made using Matlab (Mathworks) and Prism (Graphpad)
software.
1 Statistics and replication

2 All statistical analyses were performed using a two-tailed unpaired
3 Student’s T-test. No statistical methods were used to predetermine sample sizes.
4 Instead, sample sizes were determined based on prior experience with relevant
5 phenotypes and standards within the zebrafish and mouse communities. All
6 results were collected from at least two independent experiments (technical
7 replicates) in which multiple embryos, from multiple independent matings, were
8 analyzed (biological replicates).

9
10
ACKNOWLEDGEMENTS

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COMPETING INTERESTS

No competing interests declared.

AUTHOR CONTRIBUTIONS

JB: Conception and design, acquisition of data, analysis and interpretation of data, writing the manuscript. RS: Conception and design, acquisition of data, analysis and interpretation of data, writing the manuscript. OWJP: Conception and design, acquisition of data, analysis and interpretation of data. ACD: Acquisition of data. MV: Acquisition of data. CL: Acquisition of data. RPH: Conception and design, analysis and interpretation of data, writing the manuscript. DY: Conception and design, analysis and interpretation of data, writing the manuscript.

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1  Australian Research Council Strategic Initiative in Stem Cell Science [Stem Cells Australia SR110001002]. JB was supported by an American Heart Association Postdoctoral Fellowship [12POST11660038]. OWJP was supported by an Australian Heart Foundation Career Development Fellowship [CR 08S 3958].

2  RPH was supported by an NHMRC Australia Fellowship [573705].
FIGURE LEGENDS

Figure 1. Cardiac fusion defects in *refuse-to-fuse* (*ref*) mutants.

**(A,B)** Three-dimensional reconstructions depict wild-type (wt) and *ref* mutant hearts expressing the myocardial reporter transgene *Tg(myl7:egfp)* at 48 hpf. In contrast to the normal contours of the wt heart (A), the *ref* mutant heart (B) often displays a bifurcated, two-lobed ventricle and a misshapen atrium. See also Fig. 1-Supplement 1 for a description of the range of cardiac phenotypes in *ref* mutants at 48 hpf. A: atrium; V: ventricle.

**(C-G)** Dorsal (C-E) and ventral (F,G) views, anterior to the top, of wt (C,F) and *ref* (D,E,G) mutant embryos displaying the expression of *myl7* at the 22 somite stage (22 s; C-E) and the localization of ZO-1 at the 18 somite stage (18 s; F,G). (C-E) In *ref* mutants, cardiomyocytes fail to fuse at the midline (D; 8 out of 20 *ref* mutants) or only fuse posteriorly (E; 7 out of 20 *ref* mutants) by 22 s. The *ref* mutation is incompletely penetrant; 5 out of 20 homozygous mutant embryos exhibited successful cardiac fusion at 22 s. (F,G) ZO-1 localization highlights junctions forming within the maturing epithelium of the ALPM in both wt and *ref* mutant embryos. The ventral portion of the neural tube located at the midline is also visible. By 18 s, the wt ALPM (F) has initiated fusion at the midline, whereas the two sides of the *ref* mutant ALPM are still separate (G). Scale bars: 60 µm.
Figure 1 – Figure Supplement 1. *ref* mutant embryos display a range of cardiac defects.

**(A,B)** Lateral views of live wild-type (wt, A) and *ref* mutant (B) embryos at 48 hpf. *ref* mutants exhibit a mild pericardial edema.

**(C-F)** Lateral views from the right side of wt (C) and *ref* mutant (D-F) hearts reveal a variety of abnormal cardiac morphologies in *ref* mutants. In our analysis of 454 *ref* mutants with morphologically evident phenotypes, 229 had a bifurcated ventricle (D), 74 did not undergo normal looping (E), 147 had a severely shrunken heart (F), and 4 had cardia bifida (not shown). Furthermore, the *ref* mutation is incompletely penetrant: approximately 10% of the progeny from intercrosses of heterozygotes display abnormal cardiac morphology at 48 hpf (n=58/522).

**(C’-F’)** Cartoons outline the cardiac morphologies shown in C-F, with the ventricle in magenta and the atrium in green.
Figure 1 – Figure Supplement 2. The anterior endoderm forms normally in ref mutant embryos.

(A-D) Lateral views, dorsal to the right, depict the expression of axial at 8.5 hpf (A,B) or sox17 at 8 hpf (C,D). The number and distribution of endoderm precursor cells is similar in wt (A,C) and ref mutant (B, n=8/8; D, n=14/14) embryos during gastrulation stages.

(E-H) Dorsal views, anterior to the top, depict the anterior endoderm, visualized with the expression of axial at 24 hpf (E,F) or the endodermal reporter transgene Tg(sox17:egfp) at 18 s (G,H). The width and morphology of the anterior endoderm is similar in wt (E,G) and ref mutant (F, n=13/13; H, n=7/7) embryos during the stages when cardiac fusion takes place. Scale bars: 60 µm.
Figure 1 – Figure Supplement 3. Endocardial cells move to the midline in \textit{ref} mutant embryos.

\textbf{(A,B)} Dorsal views, anterior to the top, depict the expression of the endothelial reporter transgene \textit{Tg(fli1a:negfp)} in the endocardial precursor population at 18 s (n=8). In both wt (A) and \textit{ref} mutant (B) embryos, the endocardial precursors move to the midline during the process of cardiac fusion. Scale bars: 60 \textmu m.
Figure 2. *ref* is a loss-of-function mutation in *pdgfra*.

(A) Polymorphic markers (z21170, kdr_e28, 5’scfd2, z14614) were used to map meiotic recombination events, narrowing the region containing the *ref* mutation to <0.1 cM on linkage group (LG) 20. (See also Fig. 2-Supplement 1.) Fractions indicate frequencies of proximal (magenta) and distal (green) recombination between markers and *ref*. Six annotated genes are present in this region (GRCv10); sequence analysis of *kdr, kita, gsx2, lnx1, and fip1l1a* in *ref* mutants revealed only missense mutations that led to conserved amino acid changes.

(B) RT-PCR spanning exons 13-19 of *pdgfra* generates a single, properly spliced product from homozygous wt embryos and multiple, smaller products from *ref* mutant embryos. Sequencing revealed that exon 14 was either omitted or truncated in these smaller products; in all cases, the observed missplicing would result in a frameshift followed by a premature stop codon. RT-PCR of *gapdh* demonstrates use of comparable amounts of template.

(C,D) Sequencing the e14i15 exon-intron boundary of *pdgfra* revealed that *ref* mutant genomic DNA contains a G-to-A mutation in a conserved intronic nucleotide required for proper splicing. Chromatograms (C) and sequence alignment (D) show position of the mutation relative to reference sequences. Schematics (D) depict the proteins predicted to result from the wt, *ref*, and *b1059* alleles of *pdgfra*; immunoglobulin (magenta), transmembrane (green), and tyrosine kinase (blue) domains are shown.

(E,F) Lateral views depict expression of *pdgfra* at 11 s. Expression levels are lower in *ref* mutants (F; n=5/5) than in wt (E).
(G-J) Dorsal views, anterior to the top, of myl7 expression at 22 s. In contrast to wt (G), cardiac fusion defects are evident in embryos injected with a pdgfra morpholino (MO) (H; n = 5/12), b1059 homozygous mutant embryos (I; n = 12/13), and ref/b1059 transheterozygous mutant embryos (J; n = 12/12). Scale bars: 60 µm.
**Figure 2 – Figure Supplement 1. Primers used to map recombinants.**

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Figure 2 – Figure Supplement 2. *ref* mutants display craniofacial defects.

(A-F) Lateral (A-C) and ventral (D-F) views of embryos at 6 days post-fertilization (dpf), stained with Alcian blue to show the dorsal and ventral cartilage in the head. Embryos homozygous for *ref* (B,E; n=9/9) or transheterozgous for *ref* and *b1059* (C,F; n=5/6) display similar dorsal cartilage formation defects. Specifically, the dorsal ethmoid plate is diminished or absent in these mutants. Scale bars: 60 µm.

(D'-F') Cartoons illustrate the dorsal cartilage structures shown in D-F.
Figure 3. *Pdgfra* mouse mutants display defects in heart tube assembly.

(A-E') Ventral views (A,B,D,E), lateral views (A',B',C), and transverse sections (A'',E') depict Nkx2-5 expression in wt (A-A'') and *Pdgfra* homozygous mutant (B-E') mice on the C57BL/6 strain background at E9.5. Some *Pdgfra* mutants display relatively mild cardiac defects (B,B'; 4 out of 14 homozygous mutants), including defects in heart tube rotation. Other *Pdgfra* mutants display severe cardiac defects that could result from hindered cardiac fusion (C-E'; 9 out of 14 homozygous mutants). These defects include incomplete heart tube assembly (C,D) with two inflow/common atrial regions (red arrows) and a single ventricle (black arrowhead) or with cardia bifida (E,E'; arrows indicate unfused ventricles and atria). Note that we encountered *Pdgfra* null mutants on the C57BL/6 background less often than predicted by a Mendelian ratio (14 mutants recovered from a total of 108 embryos at E9.5), in keeping with other studies implicating *Pdgfra* in pre-implantation embryogenesis (Artus et al., 2013; Artus et al., 2010), as well as during gastrulation (Nagel et al., 2004; Yang et al., 2008). Also, *Pdgfra* mutant embryos on the C57BL/6 background were often smaller than wild-type littermates, consistent with previous observations (Orr-Urtreger et al., 1992); however, the observed severe cardiac defects (C-E') are not likely a result of general developmental delay, as these mutant phenotypes do not resemble wild-type cardiac morphology at younger stages. A: atrium; AVC: atrioventricular canal; LA: left atrium; LV: left ventricle; OFT: outflow tract; RA: right atrium; RV: right ventricle; V: ventricle. Scale bars: 100 µm.

(F-I) Ventral views depict localization of *Pdgfra* mRNA (F-H) and PDGFRα.
protein (I) in wt embryos from E7.5 to E8.5. *Pdgfra* expression is seen in anterior lateral plate mesoderm (ALPM) at E7.5 (F) and persists in the caudal region of the forming heart at E8.0 (G; arrows) and in the inflow tract at E8.5 (H; arrows). *Pdgfra* is also expressed in somites (S) and caudal lateral plate mesoderm (LPM) at E8.0-E8.5. Although mRNA levels are diminished, PDGFRα protein localization is maintained throughout the forming heart at E8.0 (I). FH: forming heart; H: heart.

(J,J') Ventral view (J) and transverse section (J') depict the localization of *Pdgfa* mRNA at E8.0. Black line in J indicates plane of transverse section shown in J'. At this stage, *Pdgfa* expression is seen in the foregut endoderm (Fg) and lateral ectoderm.
Figure 4. *pdgfra* is expressed within the ALPM while cardiac fusion is underway.

**(A-D)** Dorsal views, anterior to the top, depict *pdgfra* expression in wt embryos at 10 s (A), 12 s (B), 14 s (C), and 18 s (D). Arrows (A) indicate *pdgfra* expression in the ALPM and the neural crest (NC). Asterisk (D) denotes position of the myocardium by 18 s; although *pdgfra* is expressed in the myocardial precursors within the ALPM at earlier stages, its expression in these cells appears to be gone by this time point.

**(E-J)** Comparison of *hand2* (green) and *pdgfra* (red) expression patterns demonstrates their overlap in the wt ALPM at 10 s. (E-G) Three-dimensional confocal reconstructions of dorsal views, anterior to the top, focused on the left side of the ALPM (area outlined by a dashed box in A). Arrows (E) indicate *pdgfra* expression in the ALPM and the NC. (H-J) Single transverse (XZ) sections from (E-G), respectively. Yellow arrow (H) indicates overlap of *hand2* and *pdgfra* expression in the ALPM.

**(K-P)** Comparison of *axial* (green) and *pdgfra* (red) expression patterns demonstrates lack of *pdgfra* expression in the *axial*-expressing anterior endoderm in wt embryos at 12 s. (K-M) Three-dimensional confocal reconstructions of dorsal views, anterior to the top; arrows (K) indicate *pdgfra* expression in the ALPM and the neural crest. (N-P) Single transverse (XZ) sections from (K-M), respectively. Arrowheads (N) indicate *axial* expression in the anterior endoderm, adjacent to, but not overlapping with, *pdgfra* expression in the ALPM. Scale bars: 60 µm.
**Figure 5.** *pdgfra* influences the movement of the ALPM after the 15 somite stage.

**(A-F)** Dorsal views, anterior to the top, depict expression of *hand2* in the wt (A-C) and *ref* mutant (D-F) ALPM from 12-20 s. The morphology and position of the ALPM are indistinguishable in wt (A) and *ref* mutant (D) embryos at 12 s. After 15 s (B,C,E,F), disrupted movement of the ALPM is evident in *ref* mutants. Scale bars: 60 µm.

**(G)** Graph illustrates the average distance between the two sides of the ALPM in wt and *ref* mutant embryos from 8-20 s. In each embryo, the distance between the sides of the ALPM was calculated by measuring the distance between the medial edges of the *hand2*-expressing domains at three equidistant points (200 µm apart) along the anterior-posterior axis. The largest of those three measurements was selected as representative of the maximum distance between the bilateral ALPM domains for that embryo. Dots represent the selected measurements from individual embryos. The distance between the bilateral sheets in *ref* mutant embryos begins to diverge significantly from wt after 15 s. Error bars represent the standard error. Asterisks indicate p<0.05 (Student’s T-test): p=0.99 at 8 s; p=0.58 at 12 s; p=0.30 at 14 s; p=0.053 at 15 s; p=0.012 at 16 s; and p=0.00012 at 20 s.
Figure 5 – Figure Supplement 1. PDGF signaling is required after gastrulation for proper cardiac fusion.

(A,B) Dorsal views, anterior to the top, display myl7 expression at 22 s in representative embryos treated with DMSO (A) or Pdgfr inhibitor V (B) from the tailbud stage until 22 s. DMSO-treated control embryos exhibited normal cardiac fusion (19/22 treated embryos), whereas treatment with Pdgfr inhibitor V disrupted cardiac fusion (22/29 treated embryos). Scale bar: 60 µm.
Figure 6. *pdgfra* regulates the directionality of cardiomyocyte movement.

(A-F) Representative timelapse experiments indicate patterns of cell movement in wt (A,B) and ref mutant (C-F) embryos carrying the *Tg(myl7:egfp)* transgene. (A,C,E) Three-dimensional confocal reconstructions of select timepoints within each timelapse depict the typical changes in myocardial morphology seen over time in wt (A), mildly affected ref mutant (C) and severely affected ref mutant (E) embryos. (B,D,F) Tracks show the movements of the innermost cardiomyocytes in these embryos over the course of a ~2 h timelapse. Cell tracks are colored from blue to red, indicating the location of each cell from the beginning to the end of its trajectory. See also Videos S1-S3. Scale bars: 60 µm.

(G-L) Quantitative analysis of cardiomyocyte movements. 168 and 137 cells were analyzed from 8 wt and 6 ref mutant embryos, respectively. Graphs depict the average speed of individual cells (G, distance/time), the average efficiency index of individual cells (H, displacement/distance), the average velocity (displacement/time) of individual cells along the anterior-posterior axis (I) and along the medial-lateral axis (J), the average medial-lateral velocity per embryo (K), and the direction of the overall trajectory of individual cells (L). Dots in (J) are colored to depict the embryo to which they belong, and the same color scheme is used in (K). In (L), individual cells are grouped into 10 bins based on their net direction of movement; length of each radial bar represents the number of cells in each bin. The velocity along the medial-lateral axis (J,K) and the direction of cell trajectories (L) were significantly altered in ref mutants, indicating the misdirection of ref mutant cardiomyocytes and implicating *pdgfra* in steering...
the medial direction of cardiomyocyte movement. Error bars represent the 
standard deviation; p values were determined using Student’s T-test.
Figure 7. pdgfaa is expressed in the anterior endoderm, medially adjacent to the ALPM.

(A-G) Fluorescent in situ hybridization and immunofluorescence compare the expression of pdgfaa (magenta) and Tg(sox17:egfp) (green) in wt embryos at 13 s. (A-C) Dorsal views, anterior to the top, of a three-dimensional reconstruction show that pdgfaa is expressed in bilateral domains of the anterior endoderm, near the lateral edges of the endodermal sheet. Expression of pdgfaa is also evident in cranial rhombomeres. (D-F) A coronal (XY) slice of the same embryo demonstrates the overlap between pdgfaa and Tg(sox17:egfp) expression. (G) A sagittal (ZY) slice of the same embryo provides a lateral view.

(H-J) Transverse cryosections compare the expression of pdgfaa (blue) and the expression of Tg(sox17:egfp) (green) in wt embryos at 13 s, showing that pdgfaa is expressed in lateral domains of the endodermal sheet.

(K-P) Comparison of hand2 (green) and pdgfaa (red) expression patterns demonstrates that pdgfaa is expressed medially adjacent to the domains of hand2 expression in the ALPM. (K-M) Three-dimensional confocal reconstructions of dorsal views, anterior to the top. (N-P) Single transverse (XZ) slices from (K-M), respectively.

(Q-S) Dorsal views, anterior to the top, display the expression of myl7 at 22 s in nontransgenic (Q) or Tg(hsp70l:pdgfaa-2A-mCherry) (R,S) embryos, following heat shock at the tailbud stage. Ectopic expression of pdgfaa causes cardiac fusion defects (defects seen in 23/34 transgenic embryos, compared to 3/36 nontransgenic siblings). Scale bars: 60 \( \mu \)m.
SUPPLEMENTARY VIDEO LEGENDS

Supplementary Video S1. Cardiomyocytes in a wild-type embryo undergo medially directed movement during cardiac fusion.

(A,B) Representative timelapse movie (A) and associated tracks (B) of cardiomyocyte movement occurring during cardiac fusion in a wild-type embryo carrying the Tg(myl7:egfp) transgene. (A) Movie of drift-corrected three-dimensional reconstructions of 30 confocal slices taken at ~4 min intervals for ~2 h, starting when eGFP could first be detected in the ALPM. (B) The movements of individual cardiomyocytes at the innermost region of the ALPM were tracked (dots, B) at each time point. Their positions over the previous 80 min are depicted as connected colored tracks (blue-to-red, beginning-to-end). Blank frames indicate brief pauses in acquisition for refocusing. Arrows indicate initial starting position of cardiomyocytes. Asterisks indicate GFP+ cells that are not cardiomyocytes.

Scale bar: 40 µm.
Supplementary Video S2. Not all cardiomyocytes in a mildly affected ref mutant embryo undergo medially directed movement during cardiac fusion. (A,B) Representative timelapse movie (A) and associated tracks (B) of cardiomyocyte movement in a mildly affected ref mutant embryo carrying the Tg(myl7:egfp) transgene. Images were acquired as described for Video S1; however, drift correction was not applied to this movie and thus its tracks were not included in further quantitative analysis. In mildly affected ref mutant embryos, posterior cardiomyocytes display sufficient medial movement to fuse at the midline, while anterior cardiomyocytes do not. Arrows indicate initial starting position of cardiomyocytes. Asterisks indicate GFP+ cells that are not cardiomyocytes. Scale bar: 40 µm.
Supplementary Video S3. Cardiomyocytes in a severely affected ref mutant embryo fail to display medially directed movement during cardiac fusion.

(A,B) Representative timelapse movie (A) and associated tracks (B) of cardiomyocyte movement in a severely affected ref mutant embryo carrying the Tg(myl7:egfp) transgene. Images were acquired as described for Video S1, with drift correction. In severely affected ref mutant embryos, none of the cardiomyocytes display measurable medial movement. Arrows indicate initial starting position of cardiomyocytes. Blank frames indicate brief pauses in acquisition for refocusing. Asterisks indicate GFP+ cells that are not cardiomyocytes.

Scale bar: 40 µm.
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FIGURE 1 - FIGURE SUPPLEMENT 3

A (wt) and B (ref) show images of fluorescent protein expression in 18 seconds. The expression is labeled as fli1a:negfp.
FIGURE 2 – FIGURE SUPPLEMENT 1: Primers used to map recombinants

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FIGURE 5

A 12 s  wt

B 16 s

C 20 s  hand2

D ref

E

F

G Distance between sides of the ALPM

wt

ref

µm

8 s  12 s  16 s  20 s
FIGURE 5 - FIGURE SUPPLEMENT 1

A. DMSO

B. Pdgfr Inhibitor V (tailbud)

22 s

myl7