**ABSTRACT**

Over the course of development, the vertebrate heart undergoes a series of complex morphogenetic processes that transforms it from a simple myocardial epithelium to the complex 3D structure required for its function. One of these processes leads to the formation of trabeculae to optimize the internal structure of the ventricle for efficient conduction and contraction. Despite the important role of trabeculae in the development and physiology of the heart, little is known about their mechanism of formation. Using 3D time-lapse imaging of beating zebrafish hearts, we observed that the initiation of cardiac trabeculation can be divided into two processes. Before any myocardial cell bodies have entered the trabecular layer, cardiomyocytes extend protrusions that invade luminaly along neighboring cell-cell junctions. These protrusions can interact within the trabecular layer to form new cell-cell contacts. Subsequently, cardiomyocytes constrict their abluminal surface, moving their cell bodies into the trabecular layer while elaborating more protrusions. We also examined the formation of these protrusions in trabeculation-deficient animals, including erbb2 mutants, tntt2a morphants, which lack cardiac contractions and flow, and myh6 morphants, which lack atrial contraction and exhibit reduced flow. We found that, compared with cardiomyocytes in wild-type hearts, those in erbb2 mutants were less likely to form protrusions, those in tntt2a morphants formed less stable protrusions, and those in myh6 morphants extended fewer protrusions per cell. Thus, through detailed 4D imaging of beating hearts, we have identified novel cellular behaviors underlying cardiac trabeculation.

**KEY WORDS:** Cardiac cell biology, Morphogenesis, Trabeculation, erbb2, Live imaging, Zebrafish

**INTRODUCTION**

The adult heart is composed of a number of substructures optimized for pumping blood. In order to create this efficient pump, the heart undergoes complex morphogenetic changes during development, all while continuing to beat and maintain blood flow to the developing animal. One early morphogenetic process is the development of the ventricular trabeculae. Ventricular trabeculae are ridge-like myocardial structures within the walls of the ventricles that form a complex network early in development (Sedmera et al., 2003; Stankunas et al., 2008; Liu et al., 2010; Peshkovsky et al., 2011; Staudt and Stainier, 2012). Trabeculae are crucial for heart function, as mutations that abrogate trabecular formation are embryonic lethal (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Suri et al., 1996; Morris et al., 1999; Tidcombe et al., 2003; Liu et al., 2010), and human patients with hypertrabeculation exhibit a form of heart failure (Jenni et al., 1999). The trabeculae serve to increase myocardial mass before the formation of coronary vessels and, as such, have been shown to contribute to ventricular contractility; they are also necessary for the formation of the conduction system (Sedmera et al., 2003; Liu et al., 2010). Although studies in mouse have started to reveal the genetic basis of cardiac trabeculation, how cardiomyocytes form these important structures while continuously contracting remains relatively unknown. Most challenging for these in vivo studies is the fact that the heart is constantly moving, and so obtaining high-resolution images over time required novel image-processing techniques. Zebrafish are amenable to long-term imaging of the developing heart (Beis and Stainier, 2006), thereby providing an ideal model system in which to study cardiac trabeculation with cellular resolution in real time.

A number of factors are known to play a role in trabeculation. Several signaling pathways, including the Angiopoietin/Tie2 (Suri et al., 1996), Semaphorin/Plexin (Toyofuku et al., 2004a; Toyofuku et al., 2004b) and the Neuregulin/ErbB2/ErbB4 (Gassmann et al., 1995; Lee et al., 1995; Morris et al., 1999; Tidcombe et al., 2003; Liu et al., 2010) pathways have been implicated in the process. Among these regulators of trabeculation, the ErbB2 pathway has been implicated to be important cell-autonomously in cardiomyocytes (Morris et al., 1999; Tidcombe et al., 2003; Liu et al., 2010). Additionally, mechanical factors such as blood flow and/or cardiac contractility contribute to trabeculation. Zebrafish larvae with severe defects in cardiac contractility due to disrupted sarcomeres fail to form trabeculae (Chi et al., 2008). Disrupting blood flow through the ventricle by disrupting atrial contractility leads to impaired trabeculation (Peshkovsky et al., 2011). Similarly, disrupting flow into the left ventricle of embryonic chick hearts by left atrial ligation decreases left ventricular trabecular volume (Sedmera et al., 1999). It is currently unknown how ErbB2 signaling or mechanical forces regulate trabeculation.

Most of what we know about trabeculation comes from end point analyses of mutants, which can reveal whether or not embryos develop trabeculae. However, no studies have addressed how cardiomyocytes form trabeculae at the cellular level. Previous studies have reported the location of cardiomyocytes in the trabecular layer starting at 55 hours postfertilization (hpf) in zebrafish (Chi et al., 2008; Liu et al., 2010; Peshkovsky et al., 2011).
Additionally, although studies in mouse and chick indicate that trabecular cardiomyocytes are clonally related to cardiomyocytes in the outer, compact layer (Mikawa et al., 1992; Meilhac et al., 2003; Meilhac et al., 2004a; Meilhac et al., 2004b), our recent cell lineage-tracing data (Liu et al., 2010), as well as data from Gupta and Poss (Gupta and Poss, 2012), indicate that trabeculation occurs via a process of delamination, rather than asymmetric cell division. How cardiomyocytes delaminate from the compact layer remains unclear. Here, using a combination of clonal analysis and live cell imaging of beating hearts, we show that trabeculae are formed by a two-step delamination process. Cardiomyocytes first extend luminal protrusions over their neighbors, and then gradually constrict their abluminal surface to move their cell body into the trabecular layer. Further, we find that mutations that disrupt trabeculation cause distinct defects in the formation and behavior of these myocardial protrusions. In hearts that do not contract, cardiomyocytes extend short-lived protrusions, whereas in hearts with reduced blood flow, cardiomyocytes extend fewer protrusions per cell. Finally, in erbb2 mutants, fewer cardiomyocytes extend protrusions. Thus, we identify a differentially regulated cellular process that contributes to the first steps of trabeculation.

RESULTS
Cardiomyocytes extend long protrusions before the first trabeculae appear
Although many mutant studies have reported trabeculation defects, the dynamics of the process remain unclear. In order to study the cellular behaviors underlying the emergence of trabeculae, we employed a combination of high-speed spinning disk confocal microscopy and post-acquisition temporal alignment to acquire 3D time-lapse movies of beating hearts (Liebling et al., 2005; Ohn et al., 2009) (Fig. 1A-D; supplementary material Movie 1).
we used a spinning disk microscope run by a custom script to obtain confocal movies of beating zebrafish hearts at sequential z-levels. The script allowed for repeated imaging of multiple positions corrected for z-drift. z-stacks of movies were obtained at multiple developmental time points between 55 and 76 hpf. These movies were triggered without regard to the stage of the cardiac contraction cycle, and so were unsynchronized (Fig. 1B). Using previously published image-alignment algorithms (Liebling et al., 2005; Liebling et al., 2006; Ohn et al., 2009), these stacks of movies were synchronized post-acquisition, yielding 3D movies of hearts at regularly spaced intervals within the cardiac cycle, allowing us to focus on the heart at ventricular diastole, when the ventricle is most expanded (Fig. 1C,D). We could then examine cellular behaviors over the course of development without the complicating motion of normal contraction. Strikingly, live imaging of Tg(myl7:ras-GFP) embryos revealed that a large proportion of cardiomyocytes extend long protrusions along the surface of the heart (supplementary material Movie 2). Live imaging of mosaically marked ventricular cardiomyocytes revealed that ~48% extended protrusions (Fig. 5D; n=103 cardiomyocytes from 16 embryos). These protrusions extended slowly, taking 8-10 hours to reach their full length of 10-20 μm (Fig. 1E; supplementary material Movie 3). Occasionally, protrusions from different cardiomyocytes extended towards one another, made contact, and stayed connected for many hours (Fig. 1F; supplementary material Movie 4). Using mosaic analysis, we observed such cell-cell contacts in five pairs of cardiomyocytes out of 103 imaged.

In order to examine these protrusions more closely, we generated embryos mosaically expressing a random combination of fluorescent proteins in the cytoplasm of Tg(myl7:ras-GFP) cardiomyocytes by Tol2-mediated transgenesis (Kawakami, 2007). Plasmids were generated in which the myocardial myl7 promoter drives expression of either the red TagRFP-T (Shaner et al., 2008), blue TagBFP (Subach et al., 2008) or near-infrared IRFP (Filonov et al., 2011). Co-injection of these three plasmids generated numerous, multi-colored cardiomyocytes that could be uniquely tracked. The cytoplasmic fluorescent proteins allowed us to unambiguously identify cardiomyocytes throughout the 3D structure of the heart, while the membrane GFP allowed us to relate marked cardiomyocytes to their unmarked neighbors, as well as to the overall structure of the myocardium. This approach allowed us to identify these invading membrane-bound structures as protrusions from cardiomyocytes that remained in contact with the abluminal surface of the myocardial epithelium (Fig. 2A-B′). Interestingly, these protrusions tended to extend above cell-cell junctions of neighboring cardiomyocytes (Fig. 2B). To determine whether these protrusions persisted within the trabeculae, we used the same technique and examined hearts at 78 hpf, which is ~24 hours after trabeculation has begun. Cardiomyocytes with long protrusions were readily visible within the trabecular layer (Fig. 2C,C′). Thus, cardiomyocytes begin to extend protrusions before a trabecular layer is present, and trabecular cardiomyocytes can possess similarly long protrusions.

**Cardiomyocytes constrict their abluminal surface while extending protrusions to enter the trabecular layer**

Our observations suggest that cardiomyocytes start to extend protrusions before they begin entering the trabecular layer, but it is not clear how they actually translocate from the compact layer into the forming trabecular layer. To address this, we generated high-resolution time-lapse movies using a different technique to image the developing hearts. In brief, Tg(myl7:ras-GFP) fish mosaically labeled as above were anesthetized to the point where their heart stopped beating. After acquiring z-stacks of the stopped ventricles, we washed out the anesthetic, at which time the hearts began to beat again. Hearts were then allowed to develop 3-4 hours before being stopped again. The high-resolution images thereby obtained allowed us to unambiguously identify individual cardiomyocytes as they entered the trabecular layer. We found that cardiomyocytes progressively constricted their abluminal surface, eventually exiting the compact layer (Fig. 3A-E′). Interestingly, we observed cardiomyocytes extending new protrusions as they constricted their abluminal surface (Fig. 3C-H′). The total volume of each of these cardiomyocytes remained relatively constant, suggesting that they redistribute their abluminal volume into the new expanding protrusions (Fig. 3F-H′).

To examine this process further, we returned to imaging beating hearts. We first imaged cardiomyocytes in transverse section as they entered the trabecular layer (red cardiomyocyte in Fig. 4A; supplementary material Movie 5). We found that cardiomyocytes gradually constricted their abluminal surface and exited the compact layer over several hours. As mentioned above, cardiomyocytes entering the trabecular layer concurrently extended protrusions into the trabecular layer while constricting their abluminal surface (green

![Fig. 2. Cardiomyocytes extend protrusions luminally while still in the compact layer.](image-url)

(A) Illustration of the orientation of the hearts shown in B and C. (B) Optical slice of a 60 hpf Tg(myl7:ras-GFP) heart with cardiomyocytes marked with multiple fluorescent proteins. Arrow points to a marked cellular protrusion (red), extending over another marked cardiomyocyte (purple). (B′) View of the same dataset as B rotated 90°, showing that this protrusion extends from a cardiomyocyte that is still mostly contained within the compact layer. (C) Optical slice of a 78 hpf Tg(myl7:EGFP) heart similarly marked with multiple fluorescent proteins. Arrow points to a protrusion extending from a trabecular cardiomyocyte (green). (C′) View of the same dataset as C rotated 90°, showing that the same cardiomyocyte extends multiple protrusions. AVC, atrioventricular canal; IC, inner curvature; OC, outer curvature; OFT, outflow tract. Scale bars: 10 μm.
cardiomyocyte in Fig. 4B,C, arrow in 4C; supplementary material Movie 6). Notably, although we could identify very occasional cardiomyocyte divisions within our time-lapse movies (1/103 clonally labeled cardiomyocytes observed; supplementary material Fig. S1, Movie 7), all cardiomyocytes observed entering the trabecular layer did so without undergoing cell division. Interestingly, in several cases, cardiomyocytes neighboring trabeculating cardiomyocytes exhibited dramatic cell shape changes, allowing them to fill the space vacated by the exiting cardiomyocyte (e.g. purple cardiomyocyte in Fig. 4B,C; supplementary material Movie 6).

**Trabeculation-deficient animals exhibit different modes of trabecular failure**

A number of mutants have been identified to exhibit defects in trabeculation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996; Suri et al., 1996; Jones et al., 2003; Liu et al., 2010; Peshkovsky et al., 2011). However, the affected cellular behaviors underlying the trabecular defects remain undefined. To determine how changes in cardiomyocyte behaviors lead to defects in trabeculation, we examined three models of trabecular failure in zebrafish: *tmnt2a* morphants, *myh6* morphants...
and erbb2 mutants. Consistent with previous observations, we never observed myocardial cell bodies enter the trabecular layer in these embryos during our live imaging analysis. Thus, we looked earlier, at the initial formation of trabecular protrusions. To examine protrusions in a quantitative manner, we performed clonal analysis in wild-type and trabeculation-deficient embryos via the transient transgenesis approaches outlined above, and imaged the marked cardiomyocytes over a 10-hour period. Cardiomyocytes were scored for extension of protrusions (Fig. 5A), number of protrusions extended (Fig. 5B), and for whether protrusions persisted over the entire 10-hour imaging period (Fig. 5C).

We and others have previously shown that the ErbB2 receptor tyrosine kinase is necessary for trabecular development in both mouse and zebrafish (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Kramer et al., 1996; Jones et al., 2003; Lai et al., 2010; Liu et al., 2010; Peshkovsky et al., 2011). We have also shown that erbb2 mutant cardiomyocytes exhibit a cell-autonomous defect in trabeculation (Liu et al., 2010). However, the cellular mechanisms that cause this defect remain unknown. Strikingly, cardiomyocytes in erbb2 mutants were less likely to extend protrusions than wild-type cardiomyocytes, with 29% of erbb2 mutant cardiomyocytes extending protrusions compared with 48% of wild-type cardiomyocytes (Fig. 5D; \( P=0.04 \)). erbb2 mutant cardiomyocytes also extended an average of 1.06 protrusions/cardiomyocyte, compared with 1.28 in wild type (Fig. 5E; \( P=0.03 \)). Additionally, erbb2 mutant protrusions tended to be less stable, with 58% of protrusions collapsing within the 10-hour imaging period compared with only 21% in wild type (Fig. 5F; \( P=0.07 \)). Thus, lack of ErbB2 function seems to inhibit the initial formation of protrusions and may decrease their stability as well.

Contractility and/or blood flow play an important role in cardiac development (Berdougo et al., 2003; Bartman et al., 2004; Chi et al., 2008; Vermot et al., 2009; Chi et al., 2010; Peshkovsky et al., 2011), and have specifically been implicated in trabecular development (Chi et al., 2008; Peshkovsky et al., 2011). Notably, tnt2a mutant hearts, which fail to form functional sarcomeres and thus fail to contract (Sehnert et al., 2002), do not form trabeculae (Chi et al., 2008). However, since both contractility and blood flow are disrupted in tnt2a mutants, it is difficult to separate out the role that each might play in trabeculization. Interestingly, weak atrium (myh6) mutants, which exhibit disrupted atrial contraction and thus reduced blood flow through the ventricle, also have defects in trabeculization (Peshkovsky et al., 2011). To study the role that physical forces play in trabeculization, we performed clonal analysis in embryos injected with morpholinos targeting tnt2a or myh6, which phenocopy the respective mutants (Sehnert et al., 2002; Berdougo et al., 2003). The two morphants exhibited different phenotypes in terms of their myocardial protrusions. In tnt2a morphants, a similar number of cardiomyocytes extended protrusions as in wild type (52% in tnt2a morphants versus 48% in wild type, \( P=0.8 \)), but the protrusions were less stable and retracted more frequently (62% retracting in tnt2a morphants versus 21% in wild type, \( P=0.02 \)). By contrast, in myh6 morphants, the cardiomyocytes showed a trend towards extending fewer protrusions than in wild type (24% in myh6 morphant versus 48% in wild type, \( P=0.07 \)), but the protrusions they did extend were as stable as in wild type (16% retracting in myh6 morphants versus 21% in wild type, \( P=0.02 \)).
morphants versus 21% in wild type, \( P=0.81 \)). Notably, whereas wild-type cardiomyocytes occasionally extended more than one protrusion per cell (average of 1.28 protrusions/cardiomyocyte in wild type), the cardiomyocytes in myh6 morphants uniformly extended only one protrusion (\( P=0.004 \)).

**DISCUSSION**

In this study, we combined clonal analysis with powerful live imaging techniques to identify myocardial cell behaviors leading to trabeculation. We identified a previously unknown capability of actively contracting cardiomyocytes to extend long luminal protrusions, even before they translocate to form the trabeculae. Further, our data show that cardiomyocytes enter the trabecular layer through abluminal constriction, extending protrusions as they enter. While cardiomyocytes constrict their abluminal surface and enter the trabecular layer, neighboring cardiomyocytes move into the space left behind to maintain a cohesive compact layer. Finally, we found that cardiomyocytes in non-contractile hearts fail to stabilize their protrusions, which is likely to contribute to their failure to form trabeculae, and that cardiomyocytes in erbb2 mutants fail to form trabeculae despite forming stable, though fewer, protrusions.

Previous studies in zebrafish have shown that cardiomyocytes change shape depending on their location within the heart (Auman et al., 2007; Chi et al., 2010; Lin et al., 2012). However, given the necessity to maintain the integrity of the myocardial epithelium, it has been generally assumed that it was a very stable and static structure. Thus, it was surprising to find that cardiomyocytes exhibit such a dynamic behavior. The combination of live imaging, clonal analysis and image processing allowed us to reveal this process for the first time. How trabecular and compact layer cardiomyocytes are specified remains unknown. The identification of myocardial protrusions that invade luminally before cardiomyocytes translocate into the trabecular layer raises a number of hypotheses about their function. The early extension of protrusions could allow cardiomyocytes to sample their environment, seeking limiting supplies of fate-determining morphogens for example. In addition, these protrusions could provide cardiomyocytes with extra space to redistribute their volume as they constrict their abluminal surface. Interestingly, we noted that cardiomyocyte protrusions preferentially extend over cell-cell junctions of the compact layer. This arrangement could provide a mechanism for strengthening weak attachments or aligning cytoskeletons among multiple cardiomyocytes.

The ventricular trabeculae form an intricate, interconnected network in zebrafish (Sedmera et al., 2003; Chi et al., 2008; Peshkovsky et al., 2011), as well as in chick, mouse and human (Sedmera et al., 2000). How cardiomyocytes interact to form this network is poorly understood. In theory, the trabecular network

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*Fig. 5. Trabecular-deficient animals exhibit different modes of trabecular failure. (A-C) Examples of distinct cardiomyocyte behavior: (A) a cardiomyocyte extending a single protrusion (green); (B) a cardiomyocyte extending two protrusions (purple and yellow); and (C) a cardiomyocyte extending a protrusion which then retracts over the next 2 hours (red). Scale bars: 10 \( \mu \)m. (D-F) Quantification of the properties and behavior of myocardial protrusions in trabecular-deficient backgrounds. Error bars indicate s.e.m. Data were collected from 97 WT cells (14 embryos, 59 processes), 40 tnt2a morphant cells (seven embryos, 32 processes), 37 erbb2 mutant cells (six embryos, 14 processes) and 20 amhc morphant cells (four embryos, eight processes). (D) The number of cardiomyocytes extending protrusions as a proportion of the total number of cardiomyocytes counted. (E) The number of protrusions per cardiomyocyte that extended at least one protrusion. (F) The number of protrusions that retracted in length during a 10-hour movie, as a proportion of the total number of protrusions observed in each set of embryos. *\( P<0.05 \), **\( P<0.005 \). WT, wild type.*
organization could form in two broad ways. Trabecular ridges could be specified in the compact layer, and multiple neighboring cardiomyocytes could translocate to the trabecular layer to form a pre-assembled ridge. Alternatively, trabecular cardiomyocytes could be specified intermittently throughout the compact layer and form new connections with other trabecular cardiomyocytes to create the trabecular network. Our live imaging identified instances in which protrusions from two non-neighboring cardiomyocytes came together to form a new contact between the cells. Although the role of these new contacts remains to be explored, this observation provides a clue that the trabecular network could form through the assembly of new cell-cell connections within the forming trabecular layer.

Our previous work had suggested that trabeculation involves cardiomyocyte migration, rather than oriented cell division (Liu et al., 2010), although the exact mechanisms of migration remained unknown. Our live imaging of cardiomyocyte delamination reveals that cardiomyocytes enter the trabecular layer via constriction of their abluminal surface, coupled to a redistribution of their cell volume into luminal protrusions. Importantly, cell divisions were never seen to accompany this migration, confirming that delamination is independent of cardiomyocyte proliferation. The abluminal constriction of cardiomyocytes resembles the process of apical constriction, which is involved in a number of important morphogenetic events (Sawyer et al., 2010). Indeed, if the apical domain of cardiomyocytes remains unchanged from the 20-somite stage, when it faces away from the endocardium (Trinh and Stainier, 2004; Trinh et al., 2005), this process might represent apical constriction.

Delamination following apical constriction has been observed in a number of different systems. Generally, there are two non-exclusive mechanisms that can drive cellular constriction and delamination. First, delaminating cells can constrict their own apical surface through actin rearrangements and myosin-mediated contraction, as observed in C. elegans gastrulation (Lee et al., 2006). Second, their neighbors could coordinate to form an actin ring to extrude the delaminating cardiomyocyte out of the compact layer, as is seen in crawling-induced delamination from the fly notum (Marinari et al., 2012). The combination of these two mechanisms has also been observed in the extrusion of apoptotic cells from epithelial layers (Rosenblatt et al., 2001; Slattum et al., 2009; Gu et al., 2011). Our study cannot distinguish between these three possibilities. Our previous study has shown that trabeculation can be inhibited cell-autonomously (Liu et al., 2010), although this finding cannot rule out a mechanism in which delaminating cardiomyocytes signal to their neighbors to form a contractile ring. Additionally, it is currently unclear whether compact cardiomyocytes rearrange to occupy the space left behind by their delaminating neighbors by changing their morphology, migrating, rearranging their cell junctions or through some other mechanism.

Further studies are needed to clarify the precise roles of future trabecular cardiomyocytes and their neighbors during the delamination process.

In confirmation of previous studies, we observed that cardiomyocytes in erbb2 mutants, or in tmt2a or myh6 morphants, fail to delaminate into the trabecular layer, at least during the stages that we imaged. However, we wished to determine whether the mechanisms leading to trabecular failure differed between these deficient states. Using a combination of clonal analysis and live imaging, we were able to extend our analysis to look at whether these deficiencies differentially affected the formation of myocardial protrusions. Interestingly, each of these three manipulations affected the protrusions differently. A mutation in erbb2 led to roughly half as many cardiomyocytes extending protrusions, and perhaps also to less stable protrusions. This suggests that erbb2 contributes to the formation of protrusions and perhaps to their stability. Combined with our previous finding that erbb2 is necessary for cardiomyocyte proliferation during zebrafish development (Liu et al., 2010), these data suggest that erbb2 mutants have deficiencies in the initiation of trabeculation as well as in the subsequent growth of these structures.

Injection of morpholinos that change the mechanical environment of the heart produces noticeably different effects on the myocardial protrusions. Comparing the tmt2a and myh6 morphants provides an opportunity to distinguish the effects of blood flow from those of contractility on trabecular development. The notable instability of the protrusions in tmt2a morphants, as compared with the relatively unaffected stability in myh6 morphants, suggests that contractility and/or the contractile structures themselves contribute to the stability of the protrusions. Paradoxically, decreasing blood flow through the ventricle via the myh6 morpholino actually decreases the number of protrusions that cardiomyocytes extend, whereas no difference from wild type is noted in the tmt2a morphants. Although there are many possible explanations for these seemingly conflicting observations, we currently favor a model in which sarcomeres stabilize protrusions at their current length, preventing either collapse or further extension. Dynamic rearrangements in sarcomeric structures can thus modulate the extension or collapse of protrusions. In erbb2 mutant cardiomyocytes, actin appears to be sequestered in, or around, sarcomeric pools compared with wild type (S. Reischauer and D.Y.R.S., unpublished observations). This sequestration might decrease the frequency of myocardial protrusions. Previous work has shown that myh6 mutant cardiomyocytes have an altered sarcomere distribution (Lin et al., 2012). This altered sarcomeric pattern might negatively impact the extension of myocardial protrusions. In tmt2a morphants, the cardiomyocytes cannot make sarcomeres and thus their protrusions extend without ‘inhibition’, but also cannot be stabilized. Additional reagents and experiments will be required to test these hypotheses.

In conclusion, our study demonstrates the power of combining clonal analysis and live imaging to probe cardiac development in zebrafish. Expanding this approach will provide a unique tool to address in vivo further cell biological questions during the development of the heart and other fast-moving tissues.

MATERIALS AND METHODS

Zebrafish and morpholino injections

Embryonic and adult zebrafish were maintained according to standard laboratory conditions (Westerfield, 2000) and IACUC license. We used the following transgenic and mutant lines: Tg(myl7:ras-eGFP)803 (D’Amico et al., 2007) and erbb2081 (Lyons et al., 2005; Liu et al., 2010). To generate tmt2amorphants, we injected 1 nl of a mixture of myl7:TagRFP-T plasmid at 5-10 ng/μl and To2 RNA at 25 ng/μl into Tg(myl7:ras-eGFP)803 embryos. For multi-color mosaic analysis, three different DNA constructs each at 5-10 ng/μl were injected into Tg(myl7:eras-eGFP)803 embryos. For multi-color mosaic analysis, three different DNA constructs each at 5-10 ng/μl were co-injected with 25 ng/μl To2 RNA. Embryos were screened for fluorescence and normal morphology at 55 hpf before being selected for imaging.
Embryos were then temporally aligned using previously published Matlab scripts. Developmental time points were collected every hour, and the rate of development, the imaging environment was heated to 31°C using a custom script. To increase the translation of the heart was corrected live using a custom script. Multiple embryos were imaged using a motorized stage (Prior). The laser exposure and motion blur, lasers were pulsed for 2-5 ms/frame using an Acousto-Optic Tunable Filter (Neos) gated on the camera trigger. Multiple embryos were imaged using a motorized stage. The microscope was controlled using mManager software (Edelstein et al., 2010), and z-translating the heart of the heart was corrected live using a custom script. To increase the rate of development, the imaging environment was heated to 31°C using a thermally controlled enclosure (In Vivo Scientific). Generally, movies of 90 frames each were collected at 50 z-positions 1 μm apart for each developmental time point. Developmental time points were collected every 15-30 minutes for 12-19 hours, depending on the experiment. The assembled datasets were then temporally aligned using previously published Matlab software (Liebling et al., 2005; Liebling et al., 2006; Ohn et al., 2009), and the aligned files were viewed and analyzed in Fiji (Schindelin et al., 2012).

Quantitation of protrusions
Cardiomyocytes were mosaically labeled as described above in wild type, erbb2 morphants, tntn24a morphants or myb6 morphants and imaged live for 10 hours. To identify erbb2 mutants before imaging, embryos were sorted according to a tail phenotype consisting of a pointed caudal fin and a poorly formed “wavy” dorsal fin. Genotype was subsequently confirmed by PCR (Lyons et al., 2005). 3D movies from ventricular diastole were manually identified for each embryo. 2-projections were generated from each 3D movie, and the filenames were randomized to enable blinded scoring. Each 3D movie was scored for (1) the number of countable cardiomyocytes, (2) the number of cardiomyocytes extending protrusions, (3) the total number of protrusions and (4) the number of protrusions that retracted. Cardiomyocytes were deemed scorable if their edges were clearly distinct from the next closest cardiomyocyte. Protrusions were defined as subdomains of cardiomyocytes that had a clearly identifiable base and were more than 2 μm in length at some point in the movie and changed throughout the 10-hour movie. Protrusions were counted as retraction if their length began decreasing and reduced to less than one half of their maximal length during the course of the movie. The original z-stack was used to confirm the presence or absence of protrusions. The number of protrusions per cardiomyocyte, the percentage of cardiomyocytes extending protrusions, and percentage of protrusions that retracted were calculated for each embryo, and the various mutants and morphants were compared with wild type using a two-tailed t-test.

Periodic stopped heart imaging
Embryos were mounted in agarose as above, then anesthetized to the point of cardioptelgia with 0.2% tricaine. A z-stack was then acquired on the microscope described above using a 40×/1.15 water immersion objective (Nikon). In this case, the camera was not binned, yielding a calculated resolution of 227.5 nm/pixel. The anesthetic was then washed out, and the embryos were monitored until the heart resumed normal beating. To increase the rate of development, embryos were then incubated at 31°C for 3 hours, at which time anesthetic was reapplied to induce cardioptelgia, and the process repeated. Each dataset consisted of four separate time points. The cardiac health of the embryos was visually assessed after imaging to ensure normal function.

Imaging of stopped hearts
Embryos were mounted in 1% agarose containing 0.2% (w/v) tricaine. Embryos were then imaged using a Zeiss LSM5 confocal microscope.

Image processing
Most 3D datasets were rendered in Fiji using the Z projection tool or the 3D Viewer plugin. When noted, surface renderings were created in Imaris (Bitplane). When a noticeable drift in the x, y or z dimension was noticed, it was corrected using the Correct 3D Drift plugin in Fiji. In some instances, the StackReg plugin (Fiji) was used on the time-lapse of z-projections to allow better analysis of individual cardiomyocytes.

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Competing interests
The authors declare no competing financial interests.

Author contributions
D.W.S. designed and performed the experiments, analyzed data and wrote the paper. J.L. designed experiments and analyzed data. D.Y.R.S. designed experiments and wrote the paper. D.W.S., K.S.T. and N.S. set up the live microscopy platform. M.L. wrote the post-acquisition alignment program. All authors commented on the paper.

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Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.088632/-/DC1

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