Apelin signaling dependent endocardial protrusions promote cardiac trabeculation in zebrafish

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Abstract

During cardiac development, endocardial cells (EdCs) produce growth factors to promote myocardial morphogenesis and growth. In particular, EdCs produce Neuregulin which is required for ventricular cardiomyocytes (CMs) to seed the multicellular ridges known as trabeculae. Defects in Neuregulin signaling, or in endocardial sprouting towards CMs, cause hypotrabeulation. However, the mechanisms underlying endocardial sprouting remain largely unknown. Here, we first show by live imaging in zebrafish embryos that EdCs interact with CMs via dynamic membrane protrusions. After touching CMs, these protrusions remain in close contact with their target despite the vigorous cardiac contractions. Loss of the CM-derived peptide Apelin, or of the Apelin receptor, which is expressed in EdCs, leads to reduced endocardial sprouting and hypotrabeulation. Mechanistically, Neuregulin signaling requires endocardial protrusions to induce extracellular signal-regulated kinase (Erk) activity in CMs and trigger their delamination. Altogether, these data show that Apelin signaling dependent endocardial protrusions modulate CM behavior during trabeculation.

Introduction

To meet the needs of the growing embryo, the vertebrate heart has to undergo a series of complex morphogenetic events to transform from a linear tube into a mature organ. During cardiac trabeculation, CMs in the outer curvature of the ventricles delaminate towards the lumen to form

multicellular sponge-like projections, called trabeculae (Sedmera and Thomas, 1996; Sedmera et al., 2000; Stankunas et al., 2008; Liu et al., 2010; Peshkovsky et al., 2011; Staudt et al., 2014). Cardiac trabeculae are crucial to achieve increased contractility as well as for the formation of the conduction system. Trabeculation defects are often associated with left ventricular noncompaction (Oechslin et al., 2000; Claudia and Josef, 2004), embryonic heart failure, and lethality (Gassmann et al., 1995; Lee et al., 1995; Lai et al., 2010; Liu et al., 2010; Rasouli and Stainier, 2017).

In zebrafish, as in other vertebrates, the early embryonic heart consists of two cell layers, the myocardium and the endocardium, separated by a specialized extracellular matrix (ECM) called the cardiac jelly (CJ) (Stainier and Fishman, 1992; Brutsaert et al., 1996). Recently, it has been shown that EdCs, similar to blood endothelial cells (ECs), form sprouts, and that these sprouts are mostly oriented towards the myocardium (Del Monte-Nieto et al., 2018). During sprouting angiogenesis, ECs first extend filopodia to sense the microenvironment for growth factors, then they migrate into avascular areas and form new blood vessels (Gerhardt et al., 2003). Due to its similarity to sprouting angiogenesis, the sprouting of EdCs has been termed endocardial sprouting. However, whether endocardial sprouting is regulated by the same signaling pathways as sprouting angiogenesis is not known.

Multiple signaling pathways have been implicated in cardiac trabeculation, including neuregulin (Nrg)/ErbB signaling. Mouse and zebrafish embryos lacking the endocardium derived ligand Nrg or the ErbB receptor, which is expressed by the myocardium, fail to form trabeculae (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Lai et al., 2010; Liu et al., 2010; Rasouli and Stainier, 2017). Furthermore, endocardial Notch signaling (Grego-Bessa et al., 2007; D'Amato et al., 2016; Del Monte-Nieto et al., 2018), angiopoietin 1/Tie2 signaling (Suri et al., 1996; Tachibana et al., 2005; Qu et al., 2019), and semaphorin 3E (Sema3E)/plexinD1 signaling (Sandireddy et al., 2019) are required for cardiac trabeculation in mouse. Of note, genetic deletion of the relevant receptors in the endocardium results in attenuated endocardial sprouting (Qu et al., 2019) and trabeculation defects (Grego-Bessa et al., 2007; D'Amato et al., 2016; Del Monte-Nieto et al., 2018; Qu et al., 2019; Sandireddy et al., 2019).

Cells communicate by a variety of mechanisms including paracrine and contact dependent signaling. More recently, a novel mechanism of cell communication by active transport of signaling molecules through filopodia-like actin rich membrane protrusions, also known as cytonemes, has been shown in different models including *Drosophila* (Ramirez-Weber and Kornberg, 1999; Roy et al., 2011; Huang et al., 2019), chick (Sanders et al., 2013), zebrafish (Stanganello et al., 2015), and mouse (Fierro-Gonzalez et al., 2013). Like filopodia, cytonemes depend on actin polymerization by various

effector proteins including formins, profilin and IRSp53, a substrate for the insulin receptor (Rottner et al., 2017).

In this study, we take advantage of the zebrafish model, as its transparency allows single-cell resolution and high-speed imaging of the beating heart, to analyze endocardial-myocardial communication during embryogenesis. By investigating *apelin* (*apln*) mutants, we found that endocardial protrusion formation is controlled by Aplin signaling. We also observed by *in vivo* imaging that endocardial protrusions promote cardiac trabeculation by modulating Nrg/ErbB/Erk signaling. Altogether, our results provide new insights into the role of endocardial protrusions during cardiac trabeculation.

Results

Endocardial-myocardial interactions in zebrafish

The early embryonic heart in vertebrates is composed of two cell types: endocardial cells and myocardial cells (Figure 1A-D). In order to analyze possible interactions between the endocardial and myocardial cells in zebrafish, we genetically labeled the actin cytoskeleton of the endocardial cells using the *TgBAC(cdh5:Gal4ff)* and *Tg(UAS:LIFEACT-GFP)* lines, and the membrane of myocardial cells with mCherry using the *Tg(myf7:mCherry-CAAX)* line. We observed endocardial protrusions extending towards the myocardium at 24 (Figure 1A-A'') and 48 (Figure 1B-B'', Figure 1-figure supplement 1A) hpf. Of note, we observed more endocardial protrusions in the ventricle than in the atrium at 48, 60 and 72 hpf (Figure 1-figure supplement 1B). Subsequently, these ventricular endocardial protrusions formed anchor points with the myocardium, and to be consistent with similar observations in mouse (Del Monte-Nieto et al., 2018) we will refer to them as touchdowns (Figure 1B-B''). Notably, these touchdowns are stable even during cardiac contractions (Figure 1E-H, Figure 1-video 1). Starting at around 60 hpf, CMs delaminate from the compact layer towards the lumen to seed the trabecular layer (Figure 1C, C', and C'''), as reported before (Liu et al., 2010; Peshkovsky et al., 2011; Staudt et al., 2014; Priya et al., 2020). At this stage, we observed that endocardial protrusions appear to extend along, and sometimes around, the delaminating CMs (Figure 1C'' and C''', Figure 1-video 2). Next, trabecular CMs start to assemble into trabeculae, "finger-like" multicellular projections, starting at 72 hpf (Figure 1D, D', and D'''). At this stage, we observed that endocardial protrusions can also be detected in close proximity to trabecular CMs (Figure 1D'' and D''', Figure 1-video 3). Cardiac trabeculae are mostly present in the outer curvature of the ventricle at early developmental stages in zebrafish (Liu et al., 2010). Interestingly, we observed a spatial correlation between endocardial protrusions and trabeculation. At 48 hpf, endocardial protrusions are mostly located in the outer curvature of the ventricle (Figure 1-figure

supplement 1C and F). At 60 and 72 hpf, respectively, 79 and 83% of all endocardial protrusions in the ventricle are located in the outer curvature (Figure 1-figure supplement 1D-F). 69% of all endocardial protrusions in the outer curvature are close to delaminating CMs at 60 hpf, and 91% of all endocardial protrusions in the outer curvature are close to trabecular CMs at 72 hpf (Figure 1-figure supplement 1D, E, and G). Moreover, 98% of delaminating CMs and 93% of trabecular CMs are in close proximity to endocardial protrusions at 60 and 72 hpf, respectively (Figure 1-figure supplement 1D, E, and H). Together these data lead us to speculate that endocardial protrusions play a role during endocardium-myocardium interactions.

Genetically blocking endocardial protrusion formation reduces myocardial trabeculation

Since we observed a correlation between the presence of endocardial protrusions and myocardial trabeculation, we next aimed to examine the function of endocardial protrusions during cardiac morphogenesis. To this aim, we generated a transgenic line, *Tg(UAS: irsp53^{dn}-p2a-RFP)*, to specifically block protrusion formation in the endothelium. IRSp53 regulates the actin cytoskeleton to enable cells to form different types of membrane extensions (Nakagawa et al., 2003; Millard et al., 2005; Scita et al., 2008). By crossing the *Tg(UAS: irsp53^{dn}-p2a-RFP)* line to the *TgBAC(cdh5:Gal4ff)* line to overexpress *Irsp53^{dn}* specifically in endothelial cells, we observed a 70% reduction in the number of endocardial protrusions at 48 hpf (Figure 2A, B, and E), while their distribution appeared mostly unaffected (Figure 2A, B, and F). To test the hypothesis that endocardial protrusions modulate myocardial trabeculation, we analyzed embryos overexpressing *irsp53^{dn}* in their endothelial cells in a CM membrane line (*Tg(myI7:BFP-CAAX)*). Upon *irsp53^{dn}* overexpression in ECs, we detected fewer endocardial touchdowns (Figure 2A and B), as well as a reduction in cardiac trabeculation (Figure 2C, D, G, and G'). In order to identify a possible effect of endocardial protrusions on CM proliferation, we overexpressed *irsp53^{dn}* in the endothelium in the context of the *Tg(myI7:mVenus-gmnn)* reporter to visualize cycling CMs. Compared with controls, endothelial overexpression of *irsp53^{dn}* led to significantly fewer mVenus-Gmnn⁺ CMs in the ventricle (Figure 2H and I).

Apelin signaling positively regulates endocardial protrusion formation and myocardial trabeculation

We have recently found that Apelin signaling regulates endothelial filopodia formation during angiogenesis in the zebrafish trunk (Helker et al., 2020). Therefore, we hypothesized that Apelin signaling also regulates endocardial protrusion formation. In order to visualize the expression of *apln* and *aplnrb* at single cell resolution in the heart, we examined the *TgBAC(apln:EGFP)* reporter line (Helker et al., 2020) and generated a novel *Tg(aplnrb:VenusPEST)* reporter line. We detected

apln:EGFP expression in the myocardium at 48 and 72 hpf (Figure 3A and B). Furthermore, we detected *aplnrb*:VenusPEST expression in the endocardium at 48 and 72 hpf (Figure 3C and D). These results suggest that *apln* is expressed in the myocardium while *aplnrb* is expressed in EdCs. Based on these results, we hypothesized that Apelin signaling plays a role during endocardium-myocardium interactions.

To test this hypothesis, we used mutants for *aplnra* (Helker et al., 2015), *aplnrb* (Helker et al., 2015), *apln* (Helker et al., 2015), and *apela* (Chng et al., 2013). Since *apela* mutants fail to form a heart (Chng et al., 2013), we did not analyze them. While most *aplnrb* mutants fail to form a heart (Scott et al., 2007; Zeng et al., 2007), a low number of them do. By analyzing *aplnrb* mutants that do form a heart, we observed that they exhibit a reduced number of endocardial protrusions at 48 hpf (Figure 4-figure supplement 1A and B) and a reduced number of trabeculae at 72 hpf (Figure 4-figure supplement 1C and D). In wild-type embryos, the CJ between the endocardium and myocardium in the outer curvature of the ventricle appears to be mostly degraded at 72 hpf (Figure 4-figure supplement 1C); however, the CJ in *aplnrb* mutants appears to be thicker at this stage (Figure 4-figure supplement 1D). In addition, *aplnra* mutants also exhibit a reduced number of trabeculae at 72 hpf (Figure 4-figure supplement 1E and F). We further observed that *apln* mutants exhibit a significantly lower number of endocardial protrusions at 24 and 48 hpf (Figure 4A-D, G-I). In line with fewer endocardial protrusions, *apln* mutants also exhibit a reduced number of endocardial touchdowns at 48 hpf (Figure 4C and D), and a reduced number of trabeculae at 72 hpf (Figure 4E, F, and J). Altogether, these results indicate that Apelin signaling regulates endocardial protrusion formation and myocardial trabeculation.

To further examine the function of Apelin dependent endocardial protrusions during cardiac trabeculation, we next analyzed CM proliferation in *apln* mutants using EdU labeling. Homozygous *apln* mutants exhibit a significantly decreased number of EdU⁺ CMs in their ventricle compared with *apln*^{+/+} siblings (Figure 4-figure supplement 2). In addition, *apln* mutants also display a significantly thicker CJ compared with *apln*^{+/+} siblings at 72 hpf (Figure 4C-F, K, and L). However, we did not observe any obvious defects in sarcomere formation (Figure 4-figure supplement 3A and B), heart rate, ejection fraction, or blood circulation in *apln* mutants (Figure 4-figure supplement 3C and D; Figure 4-video 1-2), indicating that the myocardial trabeculation phenotype is caused by the endocardial protrusion defect and is not secondary to cardiac dysfunction.

Notch signaling negatively regulates endothelial sprouting and protrusion formation in several vascular beds (Hellstrom et al., 2007; Leslie et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007). In order to determine whether Notch signaling also regulates endocardial protrusion formation, we treated embryos with the γ -secretase inhibitor RO4929097 and observed a decrease

in Notch reporter expression in the endocardium (Figure 4-figure supplement 4A and B) as well as an increased number of endocardial protrusions in the ventricle (Figure 4-figure supplement 4E, F, and H). However, and in line with the touchdown reduction phenotype in Notch deficient mice, we observed a reduction in endocardial touchdowns in Notch inhibitor treated zebrafish larvae at 48 hpf (Figure 4-figure supplement 4C, D, and G).

Altogether, these results indicate that myocardial derived Apelin promotes endocardial protrusion formation while Notch signaling inhibits it. Furthermore, Apelin signaling is required for cardiac trabeculation, possibly via the formation of endocardial protrusions.

The function of endocardial *nrg2a* in trabeculation requires endocardial protrusions

Nrg-ErbB signaling is indispensable for cardiac trabeculation in mouse and zebrafish (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Lai et al., 2010; Liu et al., 2010; Peshkovsky et al., 2011; Rasouli and Stainier, 2017). To determine whether endocardial protrusions are required for Nrg-ErbB signaling, we first overexpressed *nrg2a* in the endothelium using the *Tg(fli1a:nrg2a-p2a-tdTomato)* line (Rasouli and Stainier, 2017). Overexpression of *nrg2a* in the endothelium results in hypertrabeculation as well as a multilayered myocardium (Figure 5A, B, and E-G; Figure 5-figure supplement 1A and B). Strikingly, overexpressing *nrg2a* in the endothelium while blocking endocardial protrusion formation by endothelial overexpression of *irsp53^{dn}* is not sufficient to restore cardiac trabeculation or induce CM multilayering (Figure 5C-C''' and E-G). In line with these results, overexpressing *nrg2a* in the endothelium of homozygous *apln* mutants is not sufficient to restore cardiac trabeculation or induce CM multilayering (Figure 5D-D''' and E-G). Importantly, we did not detect a change in the expression levels of *nrg2a* in *apln* mutant hearts at 48 hpf (Figure 5-figure supplement 1C). Taken together, these data indicate that endocardial protrusions are required for Nrg-ErbB signaling during cardiac trabeculation.

Genetically blocking endocardial protrusion formation attenuates Erk signaling in cardiomyocytes

An important molecule in the Nrg/ErbB signaling pathway is the extracellular signal-regulated kinase Erk (Lai et al., 2010). In order to visualize Erk activity in CMs in living zebrafish, as a readout of ErbB signaling, we generated novel reporter lines (*Tg(myf7:ERK-KTR-Clover-p2a-H2B-tagBFP)* and *Tg(myf7:ERK-KTR-Clover-p2a-H2B-mScarlet)*) that use the kinase translocation reporter (KTR) technology (Regot et al., 2014; de la Cova et al., 2017). When Erk is inactive, the KTR is unphosphorylated and Clover can be detected in the nucleus; in contrast, when Erk is active, the KTR is phosphorylated and Clover can be detected in the cytoplasm (de la Cova et al., 2017). We observed that most ventricular CMs in wild-type larvae display active Erk signaling with cytoplasmic Clover expression (Figure 6A). Treating embryos expressing the reporter with a MEK inhibitor led to

an increased number of ventricular CMs with nuclear Clover expression (i.e., inactive Erk signaling) indicating that the reporter is functional (Figure 6-figure supplement 1). Next, we treated embryos expressing the reporter with an ErbB2 inhibitor and found an increased number of ventricular CMs with nuclear Clover expression (Figure 6B). To determine whether endocardial protrusions modulate myocardial Erk signaling activity, we genetically blocked endocardial protrusion formation via endothelial overexpression of *irsp53^{dn}* and observed more ventricular CMs with nuclear Clover expression (Figure 6C and E) compared with control (Figure 6A and E), indicating more ventricular CMs with inactive Erk signaling. In line with these results, we observed more ventricular CMs with inactive Erk signaling in homozygous *apln* mutants (Figure 6D and E) compared with *apln^{+/+}* siblings (Figure 6A and E). Altogether, these observations indicate that Apelin signaling dependent endocardial protrusions promote Nrg/ErbB/Erk signaling in CMs.

Discussion

Endocardial protrusions are required for trabeculation

Cardiac trabeculation is initiated, at least in zebrafish, by individual CMs delaminating from the myocardial monolayer and protruding into the lumen (Liu et al., 2010; Peshkovsky et al., 2011; Staudt et al., 2014; Jimenez-Amilburu et al., 2016; Priya et al., 2020). In contrast, the myocardium in mouse is multilayered at the onset of trabeculation. Several studies have reported that the endocardium plays an important role during cardiac trabeculation (Grego-Bessa et al., 2007; Lai et al., 2010; D'Amato et al., 2016; Rasouli and Stainier, 2017; Del Monte-Nieto et al., 2018; Qu et al., 2019). Furthermore, it has recently been shown that EdCs, similar to ECs, undergo sprouting (Del Monte-Nieto et al., 2018). However, in comparison with endothelial sprouting, little is known about the morphogenetic events underlying endocardial sprouting and their effect on cardiac morphogenesis including trabeculation.

In mouse, endocardial sprouting and touchdown formation occur early during cardiac trabeculation (Del Monte-Nieto et al., 2018). These observations are in line with our data in zebrafish, suggesting that the morphogenetic events of cardiac trabeculation are evolutionarily conserved. However, endocardial sprouts in mouse appear to be cellular (Del Monte-Nieto et al., 2018), whereas endocardial protrusions in zebrafish appear more similar to filopodia, although these differences may be due to the fact that fixed tissue was used for the mouse work compared with live tissue for the zebrafish work. CM delamination and trabeculation occur in the outer curvature of the ventricle (Liu et al., 2010; Jimenez-Amilburu et al., 2016; Rasouli and Stainier, 2017). Consistent with these observations, we find that endocardial protrusions are mostly located in the outer curvature of the ventricle and extend along delaminating CMs as well as trabecular CMs. The temporal and spatial

correlation between the emergence and location of endocardial protrusions and CM delamination therefore suggests a role for endocardial protrusions in cardiac trabeculation.

Molecular regulators of endocardial sprouting

During sprouting angiogenesis, so-called tip cells lead the new sprouts (Gerhardt, 2008). Tip cells dynamically extend filopodia to identify growth factors in their environment (Gerhardt, 2008). Apelin and Notch signaling have been previously identified as regulators of endothelial filopodia formation (Hellstrom et al., 2007; Suchting et al., 2007; Helker et al., 2020). In contrast, the pathways regulating endocardial sprouting are largely unknown. Only Tie2 signaling has been identified to date as a regulator of endocardial sprouting, and *Tie2* deficient mice exhibit fewer endocardial touchdowns (Qu et al., 2019). We have recently shown that Apelin signaling regulates filopodia formation during sprouting angiogenesis in the trunk (Helker et al., 2020). In line with these published observations, we now show that Apelin regulates endocardial filopodia formation and endocardial sprouting (Figure 6-figure supplement 2), revealing a conserved role for Apelin signaling during endothelial and endocardial sprouting.

Consistent with the negative regulation of sprouting angiogenesis by Notch signaling in ECs (Hellstrom et al., 2007; Leslie et al., 2007; Siekmann and Lawson, 2007; Suchting et al., 2007), we found that Notch signaling also negatively regulates endocardial protrusion formation. Interestingly, inhibition of Notch signaling also leads to an increased number of delaminated CMs and trabeculae, although Notch signaling may also affect CM behavior cell-autonomously (Han et al., 2016; Priya et al., 2020).

Endocardium-myocardium communication is essential for trabeculation

Cell communication between the endocardial and myocardial cells is required for cardiac trabeculation (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995; Lai et al., 2010; Liu et al., 2010; Rasouli and Stainier, 2017; Gunawan et al., 2021). Several studies have shown that endocardial derived Nrg is required to activate ErbB receptor complexes on CMs (Gassmann et al., 1995; Meyer and Birchmeier, 1995; Grego-Bessa et al., 2007; Rasouli and Stainier, 2017). In zebrafish, *nrg2a*, which is expressed in the developing endocardium, is required for trabeculation (Rasouli and Stainier, 2017), and *nrg2a* overexpression in the endothelium results in an increased number of trabeculae and trabecular CMs (Figure 5), indicating that endocardial *nrg2a* is necessary and sufficient for trabeculation. Notably, blocking endocardial protrusion formation compromises the function of endocardial *nrg2a* during trabeculation, suggesting that endocardial protrusions are required for *nrg2a* function during trabeculation. The membrane-bound form of Nrg2a has a molecular weight of 55 kilodaltons, and after cleavage, the presumed secreted form has a molecular

weight of 22 kilodaltons. In contrast, the most active form of Apelin is 13 amino acids long, with a molecular weight of 1.52 kilodaltons. Therefore, it is possible that due to its small size, Apelin is able to diffuse through the cardiac jelly, while Nrg2a needs to be transported by endocardial protrusions towards the myocardium.

Like other receptor tyrosine kinases, ErbB receptors activate multiple signaling cascades, including the MAPK cascade, upon ligand stimulation, leading to the phosphorylation of ERK1/2 (Sweeney et al., 2001; Wee and Wang, 2017). Accordingly, attenuated ERK phosphorylation is observed in CMs in Nrg1/ErbB signaling deficient mice (Lai et al., 2010). By analyzing a novel reporter of Erk activity in CMs, we observed that the inhibition of endocardial protrusion formation, as well as the genetic inactivation of Apelin signaling, leads to attenuated Erk phosphorylation in CMs. Altogether, these data suggest that Apelin signaling dependent endocardial protrusions modulate ErbB signaling in CMs (Figure 6-figure supplement 2).

It has recently been shown that EC filopodia modulate neurogenesis by affecting progenitor cell proliferation in the developing brain of mice and zebrafish (Di Marco et al., 2020; Taberner et al., 2020). Of interest, ErbB signaling is also known for its function within the nervous system (Buonanno and Fischbach, 2001). Thus, one might speculate that Nrg/ErbB signaling also plays a role during the modulation of neurogenesis by endothelial filopodia. Several studies have reported cell to cell communication by cytonemes in different animal models (Ramirez-Weber and Kornberg, 1999; Holzer et al., 2012; Luz et al., 2014; Sagar et al., 2015). Whether endocardial protrusions qualify as cytonemes needs further analysis. However, our data indicate that Apelin dependent endocardial protrusions are required for the communication between endocardial and myocardial cells via Nrg/ErbB signaling (Figure 6-figure supplement 2).

In summary, our work describes how endocardial sprouting is required for Nrg/ErbB signaling during cardiac trabeculation. Furthermore, we identify Apelin signaling as a positive regulator of endocardial sprouting.

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Competing interests

298 Didier YR Stainier: Senior editor, *eLife*. The other authors declare that no competing interests exist.

299 **Materials and methods**

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Key resources table				
Reagent type (species) or resource	Designation	Source or reference	Identifiers	Additional information
genetic reagent (<i>D. rerio</i>)	<i>TgBAC(apln:EGFP)^{bns157}</i>	(Helker et al., 2020)	ZFIN: <i>bns157</i>	
genetic reagent (<i>D. rerio</i>)	<i>TgBAC(cdh5:Gal4ff)^{mu101}</i>	(Bussmann et al., 2011)	ZFIN: <i>mu101</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(UAS:LIFEACT-GFP)^{mu271}</i>	(Helker et al., 2013)	ZFIN: <i>mu271</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(fli1a:nrg2a-p2a- tdTomato)^{bns199}</i>	(Rasouli and Stainier, 2017)	ZFIN: <i>bns199</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(myf7:mCherry-CAAX)^{bns7}</i>	(Uribe et al., 2018)	ZFIN: <i>bns7</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(myf7:BFP-CAAX)^{bns193}</i>	(Guerra et al., 2018)	ZFIN: <i>bns193</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(myf7:MKATE-CAAX)^{sd11}</i>	(Lin et al., 2012)	ZFIN: <i>sd11</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(kdr1:HsHRAS-mCherry)^{s896}</i>	(Chi et al., 2008)	ZFIN: <i>s896</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(myf7:HRAS-EGFP)^{s883}</i>	(D'Amico et al., 2007)	ZFIN: <i>s883</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(tp1-MmHbb:EGFP)^{um14}</i>	(Parsons et al., 2009)	ZFIN: <i>um14</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(myf7:mVenus-gmnn)^{ncv43Tg}</i>	(Jimenez-Amilburu et al., 2016)	ZFIN: <i>ncv43Tg</i>	
genetic reagent (<i>D. rerio</i>)	<i>Tg(UAS: irsp53^{dn}-p2a- tagRFP)^{bns440}</i>	This paper	<i>bns440</i>	See Materials and methods section
genetic reagent (<i>D. rerio</i>)	<i>TgBAC(aplnrb:VenusPEST)^{mr13}</i>	This paper	<i>mr13</i>	See Materials and methods section

genetic reagent (<i>D. rerio</i>)	<i>Tg(-0.8myl7:ERK-KTR-Clover-p2a-Hsa.H2B-tagBFP)</i>	This paper		See Materials and methods section
genetic reagent (<i>D. rerio</i>)	<i>Tg(-0.8myl7:ERK-KTR-Clover-p2a-Hsa.H2B-mScarlet)^{bns565}</i>	This paper	<i>bns565</i>	See Materials and methods section
genetic reagent (<i>D. rerio</i>)	<i>apln^{mu267}</i> mutant	(Helker et al., 2015)	ZFIN: <i>mu267</i>	
genetic reagent (<i>D. rerio</i>)	<i>aplnrb^{mu281}</i> mutant	(Helker et al., 2015)	ZFIN: <i>mu281</i>	
genetic reagent (<i>D. rerio</i>)	<i>aplnra^{mu296}</i> mutant	(Helker et al., 2015)	ZFIN: <i>mu296</i>	
antibody	Alexa Fluor TM 488 anti-Chicken IgG (H+L) (goat polyclonal)	Thermo Fisher Scientific	Cat# A-11039	(1:500)
antibody	Alexa Fluor TM 568 anti-Mouse IgG (H+L) (goat polyclonal)	Thermo Fisher Scientific	Cat# A-11004	(1:500)
antibody	Alexa Fluor TM 647 anti-Rabbit IgG (H+L) (goat polyclonal)	Thermo Fisher Scientific	Cat# A-21244	(1:500)
antibody	Anti-GFP (chicken polyclonal)	AvesLab	Cat#: GFP-1020	(1:500)
antibody	Anti-mCherry (mouse monoclonal)	Takara Bio Clontech	Cat# 632543	(1:500)
chemical compound, drug	Agarose, low gelling temperature	Sigma	A9414-25g	
chemical compound, drug	EdU	Thermo Fisher Scientific	Cat# A10044	(1 mM)
chemical compound, drug	ErbB2 inhibitor PD168393	Sigma	Cat# PZ0285	(10 µM)
chemical compound, drug	MEK inhibitor PD0325901	Sigma	Cat# PZ0162	(1 µM)
chemical	RO 4929097	MedChemExpress	Cat# HY-11102	(1 µM)

compound, drug				
other	DAPI	Sigma	Cat# D9542	(1 µg/ml)
commercial assay or kit	Alexa Fluor™ 568 Phalloidin	Thermo Fisher Scientific	Cat# A12380	(1:100)
commercial assay or kit	Click-iT EdU Cell Proliferation Kit for Imaging, Alexa Fluor 647 dye	Thermo Fisher Scientific	Cat# C10340	
commercial assay or kit	DyNAmo ColorFlash SYBR Green qPCR Mix	Thermo Fisher Scientific	Cat# F416S	
commercial assay or kit	In-Fusion HD Cloning Plus	Takara Bio	Cat# 638910	
commercial assay or kit	Maxima First Strand cDNA kit	Thermo Fisher Scientific	Cat# K1641	
commercial assay or kit	RNA clean and concentrator™-5	Zymo Research	R1016	
software, algorithm	Fiji Image J	(Schindelin et al., 2012)	RRID:SCR_002285	
software, algorithm	GraphPad Prism 8	GraphPad Software	RRID:SCR_002798	
software, algorithm	Imaris-version 9.6.0	Bitplane	RRID:SCR_007370	
software, algorithm	ZEN Digital Imaging	Zeiss	RRID:SCR_013672	

301 Zebrafish lines

302 All zebrafish housing and husbandry were performed under standard conditions in accordance with
303 institutional (Max Planck Society) and national ethical and animal welfare guidelines approved by the
304 ethics committee for animal experiments at the Regierungspräsidium Darmstadt, Germany, as well
305 as the FELASA guidelines (Aleström et al., 2020). Embryos were staged by hours post-fertilization
306 (hpf) at 28.5°C (Kimmel et al., 1995).

307 Transgenic lines used in the study: *TgBAC(apln:EGFP)^{bns157}* (Helker et al., 2020),
308 *TgBAC(cdh5:Gal4ff)^{mu101}* (Bussmann et al., 2011), *Tg(UAS:LIFEACT-GFP)^{mu271}* (Helker et al., 2013),
309 *Tg(fli1a:nrg2a-p2a-tdTomato)^{bns199}* (Rasouli and Stainier, 2017), *Tg(myf7:mCherry-CAAX)^{bns7}* (Uribe et
310 al., 2018), *Tg(myf7:BFP-CAAX)^{bns193}* (Guerra et al., 2018), *Tg(myf7:MKATE-CAAX)^{sd11}* (Lin et al., 2012),

311 *Tg(kdrl:HsHRAS-mCherry)^{s896}* (Chi et al., 2008), *Tg(myf7:HRAS-EGFP)^{s883}* (D'Amico et al., 2007), *Tg(tp1-*
312 *MmHbb:EGFP)^{um14}* (Parsons et al., 2009), *Tg(myf7:mVenus-gmnn)^{ncv437g}* (Jimenez-Amilburu et al.,
313 2016), *Tg(UAS:irsp53^{dn}-p2a-tagRFP)^{bns440}* (this study), *TgBAC(aplnrb:VenusPEST)^{mr13}* (this study), *Tg(-*
314 *0.8myf7:ERK-KTR-Clover-p2a-Hsa.H2B-tagBFP)* (this study, abbreviated as *Tg(myf7:ERK-KTR-Clover-*
315 *p2a-H2B-tagBFP)*) and *Tg(-0.8myf7:ERK-KTR-Clover-p2a-Hsa.H2B-mScarlet)^{bns565}* (this study,
316 abbreviated as *Tg(myf7:ERK-KTR-Clover-p2a-H2B-mScarlet)*).

317 Mutant lines used in the study: *apln^{mu267}* (Helker et al., 2015), *aplnrb^{mu281}* (Helker et al., 2015),
318 *aplnra^{mu296}* (Helker et al., 2015).

319 **Generation of transgenic lines**

320 To generate the *Tg(UAS:irsp53^{dn}-p2a-tagRFP)* line, a dominant negative form of *irsp53* (Millard et al.,
321 2005; Meyen et al., 2015) was amplified by PCR using the primers: forward, 5'-
322 TTCGAATTAGATCTGTCGACCGCCACCATGTCTCGACCGACGAGGT-3'; reverse, 5'-
323 GTAGCTCCGCTTCCACGCGTCTGTGCAAAGCCTGCCATGC-3'. The amplification was cloned into a
324 5xUAS-p2a vector upstream of tagRFP.

325 To generate the *aplnrb* bacterial artificial chromosome (BAC) construct, we used the BAC clone
326 CH211-102K containing the *aplnrb* locus. All recombineering steps were performed as described in
327 (Bussmann and Schulte-Merker, 2011) with the modifications as described in (Helker et al., 2019).
328 The following homology arms were used to generate the targeting PCR product of the Venus-
329 pest_Kan cassette: *aplnrb_HA1_GFP_fw*:
330 GAGCACATGACAAACAATTCTCTGTGATCACTTCAAAGATTTCTTGAAACCATGGTGAGCAAGGGCGAGGA
331 G and *aplnrb_HA2_kanR_rev*:
332 TCGTCGAAGTAATCTGGGCTATAGTCAGCAGTCATGTTGTCCATGGCATTTCAGAAAGTAGTGAGGAG. The
333 Kanamycin cassette was removed with a flippase.

334 To generate the *Tg(-0.8myf7:ERK-KTR-Clover-p2a-Hsa.H2B-tagBFP)* and *Tg(-0.8myf7:ERK-KTR-Clover-*
335 *p2a-Hsa.H2B-mScarlet)* lines, ERK-KTR-Clover was amplified from addgene plasmid #59150 using
336 forward primer: GCAAAGCAGACAGTGAACAAGCTTGCTAGCCCACCATGAAGGGCCGAAAGCCTCGGG and
337 reverse primer: GTTAGTAGCTCCGCTTCCGTCGACGCGGCGGTACGAACTCCAGCAGG. The PCR
338 product was cloned into a Tol2 enabled vector containing p2a and H2B. Of note, no F1 adults were
339 recovered for the *Tg(-0.8myf7:ERK-KTR-Clover-p2a-Hsa.H2B-tagBFP)* line.

340 All constructs were injected into AB embryos at the one-cell stage (30 pg/embryo) together with Tol2
341 mRNA (25 pg/embryo) to establish the line.

342 **Live imaging of stopped hearts**

343 Zebrafish embryos and larvae were mounted in 1.4% low-melt agarose containing 1.6 mg/ml tricaine,
344 to stop the heartbeat, on glass-bottom dishes. The samples were imaged with a Zeiss LSM800
345 confocal microscope using a 40x/1.1 W objective or a Zeiss LSM880 confocal microscope using a
346 20x/1.1 W objective.

347 **Live imaging of beating hearts**

348 Zebrafish embryos were mounted in 0.8% low-melt agarose containing 0.2 mg/ml tricaine on glass-
349 bottom dishes and kept in water containing 0.2 mg/ml tricaine through the experiment. Videos were
350 acquired at 100 frames per second with a Hamamatsu ORCA flash 4.0 sCMOS camera and the image
351 was binned 4x4 to achieve a calculated pixel resolution of 0.7 μ m (40x objective).

352 **Inhibitor treatments**

353 Zebrafish embryos were treated with 1 μ M Notch inhibitor RO 4929097 for 24 h (from 24 to 48 hpf),
354 washed twice with egg water containing 0.1% (w/v) 1-phenyl-2-thiourea (PTU) and mounted in 1.4%
355 low-melt agarose with 1.6 mg/ml tricaine for imaging.

356 Zebrafish embryos were treated with 1 μ M MEK inhibitor PD 0325901 or 10 μ M ErbB2 inhibitor PD
357 168393 from 56 to 72 hpf, washed twice with egg water containing 0.1% (w/v) 1-phenyl-2-thiourea
358 (PTU) and mounted in 1.4% low-melt agarose with 1.6 mg/ml tricaine for imaging.

359 **EdU staining**

360 Zebrafish embryos were treated with 1mM EdU dissolved in 1% DMSO for 24 h (from 48 to 72 hpf) or
361 44 h (from 28 to 72 hpf) in egg water containing 0.1% (w/v) PTU. After treatment, embryos and
362 larvae were washed twice with egg water containing 0.1% (w/v) PTU, anaesthetized with 0.2% (w/v)
363 tricaine for 5 min and fixed in 4% PFA at room temperature for 2 h. The CLICK-IT reaction for EdU
364 labeling was performed as per manufacturer's protocol (Invitrogen). Samples were processed for
365 immunostaining with anti-GFP, anti-mCherry, and DAPI using the procedure described below.

366 **Immunostaining**

367 Zebrafish embryos and larvae were collected at different stages and fixed in 4% PFA at room
368 temperature for 2 h. Next, animals were washed with PBS/1% BSA/1%DMSO/0.5% Triton X-100
369 (PBDT) and blocked with PBDT/10% goat serum for 1 h before incubating in primary antibody at 4°C
370 overnight. Samples were washed in PBDT for 30 min x 4 times and incubated in secondary antibody
371 for 2 h at room temperature and then incubated with 1 μ g/ml DAPI for 10 min and washed with
372 PBS/0.1% Tween.

Primary antibodies used were GFP and mCherry. Phalloidin Alexa Fluor 568 was used to mark F-actin. Secondary antibodies were goat anti-chicken Alexa Fluor 488, goat anti-mouse Alexa Fluor 568, and goat anti-rabbit Alexa Fluor 647.

Image processing

Confocal data were processed on Imaris x64. Images were prepared using Adobe Photoshop.

Quantification and statistical analysis

The number of endocardial protrusions at 24 hpf was quantified from maximum intensity projections of the ventricle. The number of endocardial protrusions at 48 hpf was quantified from the mid-sagittal plane of the ventricle. The ratio of trabecular CMs was quantified as previously described (Jimenez-Amilburu et al., 2016). The number of trabeculae in the outer curvature of the ventricle was quantified from the mid-sagittal plane, and delaminating CMs were also counted as trabeculae. The number of trabecular CMs in the outer curvature of the ventricle was quantified from the mid-sagittal plane. The number of multilayered CMs in the outer curvature of the ventricle was also quantified from the mid-sagittal plane. EdU^+ and Gmnn^+ CMs were quantified in the entire ventricle. To quantify the volume of the CJ, the drawing tool paintbrush in Fiji was used to mark the space between the endocardium and myocardium in the outer curvature of the ventricle, followed by a 3D surface reconstruction by Imaris. Systolic and diastolic ventricular areas were measured to calculate ejection fractions. Sample sizes are indicated in each figure, one dot representing one sample. All quantifications were analyzed by the Student's *t*-test (two tailed) and considered significant at $p < 0.05$. *P* values are indicated in the figures.

Figure legends

Figure 1. Endocardial-myocardial interactions during zebrafish heart development. (A-D) Confocal projection images of the heart of *Tg(myl7:mCherry-CAAX); Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP)* zebrafish at 24 **(A)**, 48 **(B)**, 60 **(C)** and 72 **(D)** hpf. **(A-A'')** Endocardial protrusions (arrows) towards the myocardium at 24 hpf. **(B-B'')** Endocardial protrusions (arrows) and touchdowns (asterisks) with the myocardium at 48 hpf. **(C-C'')** Endocardial protrusions (arrows) during CM delamination (arrowheads) at 60 hpf. **(C''')** 3D surface rendering of the area in the yellow box in **C'**. **(D-D''')** Endocardial protrusions (arrows) during trabecular assembly and expansion (arrowheads) at 72 hpf. **(D''')** 3D surface rendering of the area in the yellow box in **D'**. **(A''''-D''''')** Schematics of endocardial protrusions, endocardial touchdowns, CM delamination, and trabecular expansion. Black asterisks indicate delaminating CMs; purple asterisks indicate trabeculae. **(E-H)** Still images from a spinning disc time-lapse movie of a 48 hpf *Tg(myl7:mCherry-CAAX); Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP)*

heart; white asterisks indicate endocardial touchdowns; numbers in the bottom right corner refer to seconds. All images are ventral views, anterior to the top. V, ventricle; A, atrium.

Figure 2. Blocking endocardial protrusion formation reduces cardiac trabeculation. (A-D) Confocal projection images of the heart of *Tg(myf7:BFP-CAAX); Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP); +/- Tg(UAS:irsp53^{dn}-p2a-tagRFP)* zebrafish at 48 **(A-B)** and 72 **(C-D)** hpf. **(A-B)** Endocardial protrusions (white arrows) and touchdowns (white asterisks) are reduced in embryos with endothelial overexpression of *irsp53^{dn}*. **(C-D)** Cardiac trabeculation (arrowheads) is reduced in larvae with endothelial overexpression of *irsp53^{dn}*; **(C''-D'')** 3D rendering. **(E)** Quantification of the number of endocardial protrusions in wild-type and in embryos with endothelial overexpression of *irsp53^{dn}* at 48 hpf. **(F-F')** Illustration of the division of the 48 hpf ventricle into 4 regions **(F)**. Distribution and average number of endocardial protrusions in different regions of mid-sagittal sections of the ventricle from 48 hpf wild-type and *irsp53^{dn}* embryos **(F')**. **(G-G')** Illustration of the division of the 72 hpf ventricle into the outer and inner curvature **(G)**. Quantification of the percentage of trabecular CMs in the outer curvature of wild-type and *irsp53^{dn}* larvae at 72 hpf **(G')**. **(H-H')** 72 hpf larvae with endothelial overexpression of *irsp53^{dn}* display a reduced number of *myf7:mVenus-Gmnn⁺* CMs (yellow arrows) in their ventricle. **(I)** Quantification of the number of *mVenus-Gmnn⁺* CMs in the ventricle of wild-type and *irsp53^{dn}* larvae at 72 hpf. All images are ventral views, anterior to the top. V, ventricle; A, atrium. Data in graphs expressed as mean $\pm$ SEM.

Figure 3. Expression pattern of Apelin signaling pathway components. (A-D) Confocal projection images of the heart of *TgBAC(apln:EGFP); Tg(myf7:MKATE-CAAX)* **(A, B)** and *TgBAC(aplnrb:VenusPEST); Tg(kdr1:HsHRAS-mCherry)* **(C, D)** zebrafish at 48 **(A, C)** and 72 **(B, D)** hpf. **(A''-D'')** Maximum intensity projections. **(A-B)** *TgBAC(apln:EGFP)* expression is detectable in the myocardium at 48 **(A)** and 72 **(B)** hpf. **(C-D)** *TgBAC(aplnrb:VenusPEST)* expression is detectable in the endocardium with higher expression in the ventricular endocardium at 48 **(C)** and 72 **(D)** hpf. All images are ventral views, anterior to the top. V, ventricle; A, atrium.

Figure 4. Loss of Apelin signaling leads to reduced endocardial protrusion and reduced myocardial trabeculation. (A-F) Confocal projection images of the heart of *Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP)* zebrafish at 24 hpf **(A-B)** and of the heart of *Tg(myf7:mCherry-CAAX); Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP)* **(C-F)** zebrafish at 48 **(C-D)** and 72 **(E-F)** hpf. Maximum intensity projections **(A-B)** and mid-sagittal sections **(C-F)**. **(A)** Endocardial protrusions (arrows) in *apln^{+/+}* embryos at 24 hpf. **(B)** The number of endocardial protrusions (arrows) is reduced in *apln^{-/-}* siblings at 24 hpf. **(C-D)** The numbers of endocardial protrusions (arrows) and touchdowns (white asterisks) are reduced in *apln^{-/-}* embryos **(D)** at 48 hpf compared with *apln^{+/+}* siblings **(C)**. **(E-F)** *apln^{-/-}* larvae **(F)** exhibit reduced trabeculation (arrowheads) and thicker CJ (yellow asterisks) at 72 hpf compared with *apln^{+/+}* siblings

(E). (G-H) Quantification of the number of endocardial protrusions in the ventricle of *apln*^{+/+} and *apln*^{-/-} siblings at 24 (G) and 48 (H) hpf. (I) Distribution and average number of endocardial protrusions in different regions of mid-sagittal sections of the ventricle from 48 hpf *apln*^{+/+} and *apln*^{-/-} siblings. (J) Quantification of the percentage of trabecular CMs in the outer curvature of *apln*^{+/+} and *apln*^{-/-} siblings at 72 hpf. (K-K') Maximum intensity projections. *apln*^{-/-} larvae (K') exhibit a thicker CJ at 72 hpf compared with *apln*^{+/+} siblings (K). (L) Quantification of the CJ volume in the outer curvature of *apln*^{+/+} and *apln*^{-/-} siblings at 72 hpf. All images are ventral views, anterior to the top. V, ventricle; A, atrium; +/+, *apln*^{+/+}; -/-, *apln*^{-/-}. Data in graphs expressed as mean ± SEM.

Figure 5. Endocardial protrusions are necessary for *nrg2a* function. (A-D) Confocal projection images of the heart of *Tg(myl7:HsHRAS-EGFP)* larvae at 72 hpf. (A-B) Overexpression of *nrg2a* in the endothelium (B) leads to an increased number of trabeculae (arrowheads) and the multilayering of CMs (brackets) compared with wild-type (A). (C) Larvae with endothelial overexpression of *nrg2a* and *irsp53*^{dn} exhibit a reduced number of trabeculae (arrowheads) and of multilayered CMs (brackets) compared with larvae with endothelial overexpression of *nrg2a* alone (B). (D) *apln* mutant larvae with endothelial overexpression of *nrg2a* exhibit a reduced number of trabeculae (arrowheads) and of multilayered CMs (brackets) compared with wild-type larvae with endothelial overexpression of *nrg2a* (B). (E) Quantification of the number of trabeculae. (F) Quantification of the number of trabecular CMs. (G) Quantification of the number of multilayered CMs in the ventricle. Brackets indicate multilayered CMs. All images are ventral views, anterior to the top. V, ventricle. Data in graphs expressed as mean ± SEM.

Figure 6. Blocking endocardial protrusion formation reduces myocardial Erk signaling activity. (A-D) Maximum intensity projections of confocal images of the heart of *Tg(myl7:ERK-KTR-Clover-p2a-H2B-tagBFP/mScarlet)* larvae at 72 hpf. (A) Visualization of Erk activity by a CM specific ERK-KTR reporter. Nuclear Clover expression (arrows) indicates CMs with inactive Erk signaling. (B) Larvae treated with an ErbB2 inhibitor exhibit an increased number of CMs with inactive Erk signaling (arrows) compared with control larvae (A). (C) Larvae with endothelial overexpression of *irsp53*^{dn} exhibit an increased number of CMs with inactive Erk signaling (arrows) compared with control larvae (A). (D) *apln* mutant larvae exhibit an increased number of CMs with inactive Erk signaling (arrows) compared with *apln*^{+/+} siblings. (E) Quantification of the percentage of ventricular CMs with nuclear Clover expression. All images are ventral views, anterior to the top. V, ventricle. Data in graphs expressed as mean ± SEM.

Figure 1-figure supplement 1. Endocardial protrusions in the ventricle are mostly located in the outer curvature and are close to delaminating or trabecular CMs. (A) Quantification of the direction of endocardial protrusions; most endocardial protrusions extend towards the myocardium. (B)

473 Quantification of the average number of endocardial protrusions in the ventricle and atrium. **(C-D)**
 474 Confocal projection images of the heart of *Tg(myI7:mCherry-CAAX); Tg(cdh5:Gal4ff)*;
 475 *Tg(UAS:LIFEACT-GFP)* zebrafish at 60 **(C)** and 72 **(D)** hpf. Arrows point to endocardial protrusions;
 476 arrowheads point to delaminating CMs and trabecular CMs close to endocardial protrusions. **(E)**
 477 Quantification of the percentage of endocardial protrusions in the outer curvature of the ventricle.
 478 **(F)** Quantification of the percentage of endocardial protrusions in close proximity to delaminating
 479 CMs or trabecular CMs. **(G)** Quantification of the percentage of delaminating CMs or trabecular CMs
 480 in close proximity to endocardial protrusions. de CM, delaminating CM; TL CM, trabecular layer CM.

481 **Figure 4-figure supplement 1. *aplnrb* mutants exhibit reduced endocardial protrusion formation**
 482 **and reduced trabeculation, and *aplnra* mutant exhibit a mild reduction in trabeculation. (A-F)**
 483 Confocal projection images of the heart of *Tg(kdrl:HsHRAS-mCherry)* **(A-B)** and *Tg(myI7:HsHRAS-*
 484 *EGFP); Tg(kdrl:HsHRAS-mCherry)* **(C-F)** zebrafish at 48 **(A-B)** and 72 **(C-F)** hpf. **(A-B)** *aplnrb*^{-/-} embryos
 485 exhibit fewer endocardial protrusions (arrows) compared with *aplnrb*^{+/+} siblings **(A)** at 48 hpf. **(C-D)**
 486 *aplnrb*^{-/-} larvae **(D)** exhibit reduced trabeculation (arrowheads) and thicker CJ (asterisk) compared
 487 with *aplnrb*^{+/+} siblings **(C)** at 72 hpf. **(E-F)** *aplnra*^{-/-} larvae **(F)** exhibit a mild reduction in trabeculation
 488 compared with *aplnra*^{+/+} siblings **(E)** at 72 hpf. V, ventricle.

489 **Figure 4-figure supplement 2. Apelin signaling regulates CM proliferation in the ventricle. (A-D)**
 490 Confocal projection images of the heart of *Tg(myI7:HsHRAS-EGFP)* larvae at 72 hpf. **(A-B)** Maximum
 491 intensity projections of confocal images. **(C-D)** Mid-sagittal sections of A and B, respectively. *apln*^{-/-}
 492 larvae **(D)** exhibit fewer EdU⁺ CMs (arrows) in their ventricle compared with *apln*^{+/+} siblings **(C)**. **(E)**
 493 Quantification of the percentage of EdU⁺ CMs in the ventricle of *apln*^{+/+} and *apln*^{-/-} siblings. V,
 494 ventricle. Data in graphs expressed as mean ± SEM.

495 **Figure 4-figure supplement 3. Wild-type like sarcomere structure and heart function in *apln*^{-/-}**
 496 **larvae. (A-B)** Confocal projection images. Maximum intensity projections of confocal images of the
 497 heart of 72 hpf larvae stained with Phalloidin. Sarcomere formation does not appear to be affected
 498 in *apln*^{-/-} larvae **(B)** compared with *apln*^{+/+} siblings **(A)** (*apln*^{+/+}, n=5; *apln*^{-/-}, n=4). **(C-D)** Quantification
 499 of heart rate **(C)** and ejection fraction **(D)** of *apln*^{+/+} and *apln*^{-/-} siblings. V, ventricle.

500 **Figure 4-figure supplement 4. Notch signaling represses endocardial protrusion formation. (A-D)**
 501 Confocal projection images of the heart of *Tg(myI7:mCherry-CAAX); Tg(tp1-MmHbb:EGFP)* **(A-B)** and
 502 *Tg(cdh5:Gal4ff); Tg(UAS:LIFEACT-GFP)* **(C-D)** embryos at 48 hpf. **(A-B)** Treatment with 1 μM of the
 503 Notch inhibitor RO4929097 from 24 to 48 hpf blocks the expression of the *Tg(tp1-MmHbb:EGFP)*
 504 Notch reporter in the endocardium. **(C-D)** Embryos treated with the Notch inhibitor exhibit fewer
 505 endocardial touchdowns (asterisks). **(E-F)** Embryos treated with the Notch inhibitor exhibit more

endocardial protrusions (arrows). **(G)** Quantification of the number of endocardial touchdowns in the ventricle of DMSO and Notch inhibitor treated embryos at 48 hpf. **(H)** Quantification of the number of endocardial protrusions in the ventricle of DMSO and RO4929097 treated embryos at 48 hpf. All images are ventral views, anterior to the top. V, ventricle; A, atrium. Data in graphs expressed as mean $\pm$ SEM.

Figure 5-figure supplement 1. *nrg2a* expression does not appear to be affected in *apln* mutants.

(A-B) Schematic illustration showing delaminating CMs and trabecular CMs vs multilayered CMs. **(C)** *nrg2a* mRNA levels in extracted hearts from wild types and *apln* mutants at 48 hpf (from RNA-seq data). Data in graphs expressed as mean $\pm$ SEM.

Figure 6-figure supplement 1. Validation of the Erk reporter line using MEK inhibitor treatment.

(A-B) Confocal projection images. Maximum intensity projections of the heart of *Tg(myl7:ERK-KTR-Clover-p2a-H2B-mScarlet)* larvae at 72 hpf. **(B)** Larvae treated with the MEK inhibitor PD 0325901 from 56 to 72 hpf exhibit an increased number of CMs with inactive Erk signaling (arrows) compared with larvae treated with DMSO **(A)**. **(C)** Quantification of the percentage of ventricular CMs with nuclear Clover. All images are ventral views, anterior to the top. V, ventricle. Data in graphs expressed as mean $\pm$ SEM.

Figure 6-figure supplement 2. Schematic model. Schematic model depicts that inhibiting the formation of endocardial protrusions results in cardiac trabeculation defects via affecting Nrg/ErbB signaling.

Supplemental videos

Figure 1-video 1. Endocardial touchdowns during cardiac contraction. Related to Figure 1E-H.

Beating 48 hpf zebrafish heart. Magenta, myocardium; white, endocardium.

Figure 1-video 2. Endocardial protrusions extend along delaminating CMs at 60 hpf. Related to Figure 1C'''.

3D surface rendering of a 60 hpf ventricle. Magenta, myocardium; white, endocardium; yellow, endocardial protrusions extending along delaminating CMs.

Figure 1-video 3. Endocardial protrusions are in close proximity to trabecular CMs at 72 hpf. Related to Figure 1D'''.

3D surface rendering of a 72 hpf ventricle. Magenta, myocardium; white, endocardium; yellow, endocardial protrusions in close proximity to trabecular CMs.

Figure 4-video 1. *apln*^{+/+} blood circulation at 48 hpf. Related to Figure 4-figure supplement 3.

Figure 4-video 2. *apln*^{-/-} blood circulation at 48 hpf. Related to Figure 4-figure supplement 3.

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Figure 1

Figure 2

Figure 3

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Figure 5

Figure 6

Figure 1-figure supplement 1

Figure 4-figure supplement 1

Figure 4-figure supplement 2

Figure 4-figure supplement 3

Figure 4-figure supplement 4

Figure 5-figure supplement 1

Figure 6-figure supplement 1

Figure 6-figure supplement 2