Coordinating cardiomyocyte interactions to direct ventricular chamber morphogenesis

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Many organs are composed of complex tissue walls that are structurally organized to optimize organ function. In particular, the ventricular myocardial wall of the heart comprises an outer compact layer that concentrically encircles the ridge-like inner trabecular layer. Although disruption in the morphogenesis of this myocardial wall can lead to various forms of congenital heart disease and non-compaction cardiomyopathies, it remains unclear how embryonic cardiomyocytes assemble to form ventricular wall layers of appropriate spatial dimensions and myocardial mass. Here we use advanced genetic and imaging tools in zebrafish to reveal an interplay between myocardial Notch and Erbb2 signalling that directs the spatial allocation of myocardial cells to their proper morphological positions in the ventricular wall. Although previous studies have shown that endocardial Notch signalling non-cell-autonomously promotes myocardial trabeculation through Erbb2 and bone morphogenetic protein (BMP) signalling, we discover that distinct ventricular cardiomyocyte clusters exhibit myocardial Notch activity that cell-autonomously inhibits Erbb2 signalling and prevents cardiomyocyte sprouting and trabeculation. Myocardial-specific Notch inactivation leads to ventricles of reduced size and increased wall thickness because of excessive trabeculae, whereas widespread myocardial Notch activity results in ventricles of increased size with a single-cell-thick wall but no trabeculae. Notably, this myocardial Notch signalling is activated non-cell-autonomously by neighbouring Erbb2-activated cardiomyocytes that sprout and form nascent trabeculae. Thus, these findings support an interactive cellular feedback process that guides the assembly of cardiomyocytes to morphologically create the ventricular myocardial wall and more broadly provide insight into the cellular dynamics of how diverse cell lineages organize to create form.

The embryonic zebrafish heart comprises 200–300 cardiomyocytes when cardiac chambers form, and thus provides an opportunity to interrogate in detail how individual cardiomyocytes organize to create the nascent structures of the vertebrate embryonic ventricular wall. As a result, previous zebrafish studies have shown that distinct cardiomyocytes extend from the embryonic ventricular wall into the lumen to develop cardiac trabeculae, whereas others remain within this outer wall to create the primordial layer. Yet, how these cardiomyocytes are selected to form the distinct myocardial layers of the ventricular wall remains to be fully elucidated.

Because of the role of Notch signalling in regulating cell–cell interactions, we examined its dynamic activation during zebrafish embryonic ventricular morphogenesis using the Tg(Tp1: d2GFP) Notch reporter line, which expresses a destabilized green fluorescent protein upon Notch activation (Fig. 1 and Extended Data Fig. 1). As previously reported, we observed Notch signalling first in the ventricular endocardium at 24 hours post-fertilization (hpf), which then becomes restricted to the atrioventricular (AV) and outflow tract (OFT) endocardium at 48 hpf (Extended Data Fig. 1a–l). From 72 to 96 hpf when cardiac trabeculation initiates and 14 dpf, a subset of ventricular cardiomyocytes begins to express Notch-activated Tp1: d2GFP and remains

**Figure 1 | Notch signalling is dynamically activated in distinct myocardial clusters during cardiac morphogenesis.** Cardiac ventricles at 72 hpf, 96 hpf, and 14 dpf expressing (a–k, m–o) Tp1: d2GFP; myl7: mCherry or (q–s) Tp1: d2GFP; myl7: H2A–mCherry. a–k, Confocal slices; m–o, q–s, three-dimensional reconstructions. b, c, d, f, h, j–k, Magnifications of boxed areas in a, b, e, and i, respectively. Images c and d, g and h, b, and j and k are single channels from b, f, and i merged images, respectively. I, Schematic of myocardial Notch signalling. p, t, Quantification of (p) myocardial Tp1: d2GFP+ clusters and (t) cardiomyocytes per Tp1: d2GFP+ cluster, n. Number of embryos analysed per stage. White arrows, Tp1: d2GFP+ cardiomyocytes; white arrowheads, trabeculating cardiomyocytes; yellow arrows, cardiomyocytes in Tp1: d2GFP+ clusters. White and yellow asterisks, AV and OFT. Mean ± s.e.m. Scale bar, 25 μm.

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clones in trabeculae (red) and fewer in the outer ventricular wall (green), compared with control. Crosses, mean and s.e.m. *P < 0.05, by Student’s t-test. NS, not significant. u, Notch-altering mosaic cardiomyocyte studies. w, Constitutively activated Notch cardiomyocytes expressing NICD–P2A–Emerald are primarily located on the ventricular outer wall (n = 13/14 clones, Fisher’s exact test, P < 0.05); whereas (y) Notch-inhibited cardiomyocytes expressing dnSuH–P2A–Emerald are mainly found in trabeculae (n = 15/18 clones, Fisher’s exact test, P < 0.05). v, x. In controls lacking Tg(myl7:cre), mCherry+ cardiomyocytes are distributed equally between both layers (n = 11/21 and 14/26 clones in the outer wall). z, Quantitative analysis of v–y. Insets are magnifications of boxed areas. Arrowheads and arrows, trabeculae and outer wall cardiomyocytes. HS, heat shock. Scale bar, 25 μm.

We next investigated the role of Notch signalling in the endocardium and myocardium during ventricular morphogenesis through selectively perturbing Notch signalling at specific cardiac developmental stages. Treating zebrafish embryos with DAPT, which effectively decreases Trabeculae 3, is also increased in similarly DAPT-treated zebrafish (Extended Data Fig. 2), from 20 to 48 hpf when Notch signalling is activated in the endocardium, reduces cardiac trabeculation (Extended Data Fig. 2q–s) as previously described; however, treating from 60 to 72 hpf, when Notch signalling is present in the ventricular myocardium, results in increased trabecular formation (Fig. 2a, b, i, j). Consistent with these results, BMP signalling, which is activated in ventricular trabeculae, is also increased in similarly DAPT-treated zebrafish embryos from 60 to 72 hpf (Extended Data Fig. 4a–f). Furthermore, heat-shocking Tg(hsp70l:dnMAML–GFP) (abbreviated as hsp70l:dnM) embryos from 60 to 72 hpf, which induces dominant negative Mastermind-like (dnMAML) expression to block downstream Notch signalling, results in similar excessive trabeculation (Fig. 2c, d, k, l).

To explore whether Notch signalling functions in a cardiomyocyte-specific manner to directly guide myocardial cell fate position within the ventricle, we employed a myocardial-specific Cre (Extended Data Fig. 5a–d) strategy in combination with Tg(ubi:loxP-STOP-loxP-dnMAML–GFP) or Tg(hsp70l:loxP-STOP-loxp-NICD–P2A–Emerald) switch lines (abbreviated as ubi:RSdnM and hsp70l:RSN) to inhibit or activate Notch signalling in cardiomyocytes, respectively. As observed in DAPT-treated and heat-shocked Tg(hsp70l:dnM) zebrafish from 60 to 72 hpf, Tg(myl7:Cre; ubi:RSdnM) zebrafish display excessive cardiac trabeculation due to inhibition of myocardial Notch signalling (Fig. 2e, f, m, n). Conversely,
Heat-shocking Tg(myl7:Cerulean; hsp70:RSN) zebrafish, which induces myocardial Notch-intracellular domain (NICD) expression, between 60 and 120 hpf leads to cardiac ventricles without significant trabeculae because of constitutively activated Notch signalling throughout the myocardium (Fig. 2g, h, o, p). Moreover, constitutive myocardial Notch activation at later time points (80, 96, and 120 hpf to 7 dpf) prevents trabeculae from further sprouting and/or extending (Extended Data Fig. 6a–g); however, trabeculae continue to develop after the cessation of this myocardial Notch activity, but fail to recover to wild-type levels (Extended Data Fig. 6h, i).

In line with these findings, we discovered that Notch inhibition results in smaller ventricular areas (Fig. 2a–f and Extended Data Fig. 5e) and thicker ventricular myocardial walls (Fig. 2a–f and Extended Data Fig. 5f) due to increased cardiomyocytes within the trabecular layer (approximately two or three cells thick) (Fig. 2i–n, Extended Data Fig. 5g), whereas Notch activation gives rise to larger ventricular areas (Fig. 2g, h, Extended Data Fig. 5e) and thinner ventricular walls (Fig. 2g, h and Extended Data Fig. 5f) that are about one cell thick with no apparent trabecular cardiomyocytes (Fig. 2o, p and Extended Data Fig. 5g). Although these hearts do not exhibit a significant difference in overall cardiomyocyte numbers compared with control hearts (Extended Data Fig. 5h–p), we did discover that Notch inhibition promotes the redistribution of N-cadherin away from myocyte cell–cell contacts whereas Notch activation prevents this reorganization (Extended Data Fig. 7), suggesting that myocardial Notch signalling may control ventricular size and wall thickness through regulating the allocation of cardiomyocytes between the ventricular wall layers via cell–cell contacts. To further investigate this possibility, we monitored the fate of individual genetically labelled cardiomyocytes using a myocardial specific Brainbow system Tg(myl7:CreeER; priZm)4 (Fig. 2q–t). After confirming that adjacent cardiomyocytes were consistently labelled with different colours at 60 hpf before trabeculation (Extended Data Fig. 8), we treated zebrafish embryos with DAPT or dimethylsulfoxide (DMSO) from 60 to 72 hpf. DAPT treatment leads to increased numbers of trabeculating clones and conversely decreased numbers of non-trabeculating clones compared with DMSO-treated hearts; however, the total number of ventricular cardiomyocyte clones is not significantly different (Fig. 2r–t), further supporting the idea that Notch signalling segregates individual cardiomyocyte clones between the ventricular outer wall and inner trabecular layers.

To examine whether Notch signalling acts cell-autonomously to control cardiomyocyte sprouting, we perturbed Notch signalling in individual cardiomyocytes during trabeculation by injecting hsp70:loxP–mCherry–STOP–loxP–NICD–P2A–Emerald (hsp70:RNA, Notch activating) or hsp70:loxP–mCherry–STOP–loxP–dnSuH/P2A–Emerald15 (hsp70:RSdnS, dominant negative Suppressor of Hairless/Notch repressing) switch plasmids into Tg(myl7:Cerulean)16. Tg(myl7:Cerebral) zebrifish embryos (Fig. 2u). Heat-shocking these injected fish from 60 to 72 hpf resulted in most Notch-activated NICD-P2A–Emerald+ cardiomyocytes remaining in the ventricular outer myocardial wall (Fig. 2w, z), whereas Notch-inhibited dnSuH/P2A–Emerald+ cardiomyocytes reside primarily in trabeculae (Fig. 2y, z). Heat-shocking injected control fish lacking Tg(myl7:Cerebral) generated mCherry+ cardiomyocytes that were distributed equally between both myocardial layers (Fig. 2x, z), altogether revealing a myocardial cell-autonomous role for Notch signalling.

Because Neuregulin/ErbB2 and BMP10 signalling can promote cardiac trabeculation3,10,17, we investigated whether myocardial Notch may cross-talk with these signalling pathways to regulate cardiomyocyte selection between the ventricular wall layers. Inhibiting ErbB2 signalling with AG1478 treatment10 from 60 to 72 hpf prevents cardiac trabeculation and expression of Tp1:d2GFP in cardiomyocytes, although Tp1:d2GFP remains expressed in AV and OFT endocardial cells (Extended Data Fig. 9a, b). Consistent with these findings, both erbb2 morpholino (MO) knockdown and erbb2−/−mutant (erbb2−/−) Tg(Tp1:d2GFP) embryos, which exhibit similar trabecular defects10,18,
requirement for BMP signalling in the maintenance but not the initiation of cardiac trabeculation.

To explore whether Notch activation negatively regulates Erbb2 signalling to prevent trabeculation formation, we examined erbb2 expression in 72 hpf Tp1:d2GFP hearts and discovered that erbb2 is expressed in many ventricular cardiomyocytes but diminished in Tp1:d2GFP+ cardiomyocytes (Extended Data Fig. 9f–i). In support of these findings, constitutive myocardial Notch activation by heat-shocking Tg(myl7:Cerulean; hsp70l:RSN) fish between 60 and 120 hpf results in the dramatic reduction of erbb2 myocardial expression (Extended Data Fig. 9k, l, o, p). In contrast, Notch-inhibited hearts treated with DAPT from 60 to 72 hpf exhibit increased erbb2 myocardial expression (Extended Data Fig. 9m, n, q, r). Thus, myocardial Notch signalling may block Neuregulin/Erbb2 signalling by downregulating erbb2 expression to inhibit cardiomyocyte sprouting.

Since Notch signalling has been shown to mediate cell fate position through lateral inhibition mechanisms, we investigated whether Erbb2 signalling non-cell-autonomously activates myocardial Notch signalling in neighbouring cardiomyocytes. Thus, we created mosaic embryos by transplanting Tg(myl7:Cerulean) wild-type donor blastomeres into erbb2−/− or control MO injected Tg(Tp1:d2GFP); Tg(myl7:H2A–mCherry) host embryos and assessed the ability of wild-type donor cells to contribute to the ventricular wall layers and activate myocardial Notch signalling. As previously reported, a greater percentage of donor-derived wild-type cardiomyocytes is present in the trabeculae of erbb2 knockdown embryos compared with control embryos (compare Fig. 3i with Fig. 3g; Extended Data Fig. 10a). Although non-transplanted erbb2 knockdown hearts fail to exhibit myocardial Notch activity (Fig. 3f, h), in vitro transplantation of erbb2 knockdown host hearts containing wild-type donor myocardial cells (myl7:Cerulean+) can activate myocardial Tp1:d2GFP expression (Fig. 3i and Extended Data Fig. 10b). Upon closer inspection, these host erbb2 knockdown Tp1:d2GFP+ cardiomyocytes (Fig. 3i, arrows) appear adjacent to donor wild-type myl7:Cerulean+ cardiomyocytes (Fig. 3i, arrowheads, and Extended Data Fig. 10c), supporting a role for Erbb2-responsive cardiomyocytes in activating Notch signalling in neighbouring cardiomyocytes.

On the basis of these results, we searched for potential Notch ligands mediating the activation of Notch signalling in neighbouring cardiomyocytes and discovered that jag2b is expressed in select ventricular cardiomyocytes at 72 hpf when myocardial Notch signalling is activated (Fig. 4a, b). This ventricular myocardial jag2b expression is reduced in erbb2−/− mutant hearts (Fig. 4c, d), suggesting that Erbb2 signalling may activate Notch signalling in neighbouring cardiomyocytes through Jag2b. In support of this possibility, we discovered that jag2b−/− mutant hearts exhibit not only increased trabeculation as observed in Notch-inhibited hearts but also reduced Tp1:d2GFP Notch reporter activity in the ventricular myocardium but not in the AV or OFT endocardium (Fig. 4e–h). Together these data support a model in which myocardial Erbb2 signalling non-cell-autonomously activates Notch signalling in neighbouring ventricular outer-wall cardiomyocytes through Jag2b, which in turn leads to the reduction of erbb2 expression, subsequent inhibition of Erbb2 signalling, and suppression of cardiomyocyte sprouting (Fig. 4i–k).

Overall, these findings reveal a molecular mechanism whereby Notch and Erbb2 signalling coordinates social cardiac interactions between cardiomyocytes that determine their morphological fate within the ventricular wall. Although previous studies have suggested that Notch signalling may be activated in the myocardium, our zebrafish studies illuminate the precise role of myocardial Notch activity in forming the ventricular wall. Similar to the receptor tyrosine kinase (RTK)-Notch lateral inhibition signalling mechanisms that regulate epithelial tip and stalk cell formation during branching morphogenesis, myocardial Notch acts in concert with the RTK Erbb2 to segregate embryonic cardiomyocytes into two functionally distinct classes of cells: (1) sprouting cardiomyocytes that respond to Neuregulin via...
Erbb2 and (2) non-sprouting Notch-activated cardiomyocytes, in which Notch signalling inhibits erbb2 expression. These roles appear not to be pre-specified, but rather are determined by social interactions between cardiomyocytes. Furthermore, recent studies have reported human Notch genetic variants linked to a wide spectrum of congenital heart diseases including non-compaction cardiomyopathy, which exhibit similar severe ventricular wall defects to those observed in our Notch studies. More broadly, our studies support a conserved role for intercellular cross-talk between RTKs and Notch signalling for allocating cells within organ substructures and might be particularly relevant in developing strategies for human pluripotent stem-cell tissue-specific developmental and disease modelling or regenerative therapies.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS
Zebrafish husbandry and strains. Zebrafish (Danio rerio) were raised under standard laboratory conditions at 28 °C. All animal work was approved by the University of California at San Diego Institutional Animal Care and Use Committee. The following established transgenic and mutant lines were used: Tg[EPV:Tp1-Mmu.Hbb:dn2GFP]mv70ex1(ref. 8) abbreviated as Tg(Tp1:dn2GFP); Tg(EPV:Tp1-Mmu.Hbb:dn2GFP)mv56ex14(ref. 12) abbreviated as Tg(Tp1:dn2GFP); Tg(BRE-AAN:rap2a-d2GFP)mv30ex12(ref. 19) abbreviated as Tg(BRE:Rap2a-d2GFP); Tg(sph70.dN-MAML–GFP)sc2205ex1(ref. 33) abbreviated as Tg(sph70.dN-MAML); Tg(myl7:Cre)sd38 (ref. 30); Tg(my17:R26–loxP–eGFP)tm12;23; Tg(my17:CreER)at1;37 (ref. 31); Tg(my17:Cerulean)195 (ref. 16); Tg(my17:eGFP–HRAS)gg853 (ref. 32) abbreviated as Tg(my17:ras–eGFP); Tg(my17:CreER)at33; Tg(β-act2:Brainbow1.0)Pj490 (ref. 4) abbreviated as Tg(β-zf2m); Tg(hsp70:loxP–mCherry–STOP-loxp–N CID-P2A–Emerald)ym059 (ref. 14) abbreviated as Tg(hsp70:loxp-RSN); Tg(β-act2:loxP–DsRed–STOP-loxp–eGFP)gg836 (ref. 33) abbreviated as Tg (ref. β-act2:RSO); erbB2gg50 (ref. 18), and jg195(gg385) (ref. 34).

To generate the Tg(my17:Cre)gg18 transgenic line, a 900-base-pair fragment of the myl7 promoter was cloned upstream of the Cre recombinase gene into a multi-cloning site flanked by L-SceI sites in the pBluescript-SK vector. Standard 1-SceI meganuclease transgenesis was used to create transgenic founders which were screened for myocardial Cre recombinase activity by crossing to the Tg(β-act2:RSO)gg18 line. Three independent founders were identified, all with similar levels of Cre recombinase activity and matching the expression of Tg(my17:Cerulean)gg18 (Extended Data Fig. 5a–d). A single representative founder was propagated further.

To generate the Tg(ubl:loxP–mKate2–STOP-loxp–dnMAML–GFP)gg16 strain, abbreviated as Tg(ubl:RsdsnM), gateway cloning technology (Life Technologies) was used to conduct an LR recombination reaction with the pENTR5′_ubi, pME-loxP–mKate2–STOP-loxp, p3E-dnMAML–GFP entry vectors and the pDESTol2pA2 destination vector. The pME-loxP–mKate2–STOP-loxp entry vector was created by replacing the AmCyan complementary DNA in the pME-loxP-AmCyan–STOP-loxp vector with a complementary DNA encoding mKate2 (Evrogen) using In-Fusion HD cloning (Clontech Laboratories). The p3E-dnMAML–GFP entry vector was generated by conducting a PCR recombination reaction between a PCR product encoding a fusion protein between dnMAML and GFP amplified from pME-dnMAML–GFP and the Gateway Donor Vector pDONR2P-3. Altogether the ubl:loxP–mKate2–STOP-loxp–dnMAML–GFP construct was co-injected with Tol2 transposase mRNA into one-cell stage embryos to generate independent founders which were screened for mKate2 and then GFP upon Cre-mediated recombination. Founders with both mKate2 and GFP were propagated further.

Embyronic immunofluorescence and live imaging studies. Wholemount embryonic studies were performed as previously described44, with the following modifications. After initial fixation, any pre-existing fluorescence was quenched by incubating embryos in 2 M HCl at 37 °C for 30 min and washing with double-distilled H2O and phosphate buffer saline with 0.1% Tween-20 (PBST). The antibodies used were anti-ME2/C-21 (rabbit, Santa Cruz Biotechnology, 1:100), anti-ME/HRAS20 (mouse, Developmental Studies Hybridoma Bank, 1:100) and anti-N-cadherin (rabbit, GeneTex, 1:100) followed by anti-rabbit IgG-Alexa 488 (goat, Life Technologies 1:200). The following secondary antibodies were used: anti-mouse IgG-Alexa 405 (goat, Life Technologies, 1:200) and anti-chicken IgG-Alexa 488 (goat, Life Technologies, 1:200) and anti-rabbit IgG-Alexa 568 (goat, Life Technologies, 1:200) and anti-chicken IgG-Alexa 648 (goat, Life Technologies, 1:200). Alexa Fluor 594-conjugated wheat germ agglutinin (Life Technologies, 50 μg ml−1) was used to stain the extracellular matrix. DAPI (1 μg ml−1) staining was used to identify nuclei. Notably, we discovered that eGFP from the Tg(Tp1:leGFPP)gg11 transgene persisted for a longer period in the ventricular outer myocardial wall (Extended Data Fig. 3) than d2GFP from the Tg(Tp1:dn2GFP)gg11 transgene (Extended Data Fig. 1).

Notch signalling studies. Notch inhibition studies were performed using DAPT (a chemical inhibitor of ~secretase) or dnMAML mis-expression (dominant negative mastermind-like 1). DAPT: zebrafish embryos were incubated in 100 μM DAPT (Sigma) or 0.1% DMSO alone (control) at specified developmental stages and time intervals and then quickly washed (two or three times) with egg water (60 μg ml−1 Instant Ocean salts) for further analysis. The ability of 100 μM DAPT treatment to inhibit Notch signalling was validated by examining Tp1:dn2GFP expression after DAPT treatment (Extended Data Fig. 2i–p). dnMAML: The Tg(hsp70:dnM) was used to globally express dnMAML at specified time points. Heat-shock induction was conducted by placing Tg(hsp70:loxnM) or wild-type siblings into a 37 °C incubator for 30 min, followed by 3 min in a 42 °C water bath. Embryos were heat-shocked twice every 24 h to maintain the induction of dnMAML–GFP throughout the embryo. This protocol was highly efficient at inducing dnMAML–GFP expression and produced minimal lethality. To inhibit Notch signalling in cardiomyocytes only, the Tg(ubl:loxP–mKate2–STOP-loxp–dnMAML–GFP) line was crossed with the Tg(my17:Cre) line to produce embryos which express dnMAML–GFP only in the myocardium. Induction of dnMAML–GFP was verified by examining GFP fluorescence 5–6 h after heat shock or Cre-mediated recombination45.

Notch activation was performed by expressing NICD. Heat shocking embryos containing both Tg(hsp70:loxP–mCherry–STOP-loxp–N CID-P2A–Emerald)gg14 and Tg(my17:Cro) transgenes produced NICD–P2A–Emerald only in the myocardium. Heat shock was performed as described above.

Ventricular wall thickness was measured to quantify the effect of perturbing Notch signalling and was determined by drawing five representative lines perpendicular to the ventricular wall in a representative confocal slice. Thickness was measured as the distance along the line between the lateral and medial edge of the myocardial wall. All hearts were imaged in the same orientation and comparable confocal slices were chosen for analysis. Six hearts were measured for each condition.

To determine the effect of altering Notch signalling on cardiomyocyte cell numbers within the ventricular outer wall and trabecular layers, ventricular cardiomyocyte nuclei were counted from hearts exposed to specified experimental conditions using three-dimensional reconstructions of confocal slices from embryos with myl7:H2A–mCherry or from embryos stained with the Me2 antibody. Me2 immunostaining was used in embryos containing transgenes with fluorophores that overlapped with H2A–mCherry, such as ubl:RsdsnM or hsp70:loxp-RSN. For these analyses, the cells within the trabeculae could be separated from the ventricular outer wall using the post-image processing procedure described in the ‘Image processing and statistical analysis’ section below. To calculate the number of cardiomyocyte nuclei in the total ventricle and the number of cardiomyocyte nuclei within the trabeculae, the number of cardiomyocyte nuclei in the ventricular outer wall was calculated by subtracting the number of cardiomyocytes in the trabeculae from the total.

Trabecular area was measured from a confocal slice of a ventricle containing a cytoplasmic fluorophore such as myl7:HmCherry or ubl:RsdsnM or hsp70:loxp-RSN. Confocal slices at the level of the AV canal were analysed. Non-trabecular tissue in these images was masked manually and then the total number of fluorescent pixels was measured using the IDL program (Research Systems). All images were taken at the same dimensions. Ventricle area was determined by measuring the total pixels outlined in the ventricle region using ImageJ software.

Clonal analysis. Cardiomyocyte clones were genetically labelled by combining the myl7:CreER and priZm(β-act2:Brainbow1.0) transgenes and then treating with 4-hydroxytamoxifen (4-HT, Sigma). Specifically, zebrafish embryos with these transgenes were treated at 48 hpf, when the zebrafish heart consists of a single cardiomyocyte thick wall and is looped but has not initiated cardiac trabeculation, are represented in a scatter plot (Extended Data Fig. 1r) and used to overlay a linear regression line.

Adult immunofluorescence and imaging studies. Immunofluorescence studies were conducted on cryosections of adult zebrafish hearts. These hearts were cryoprotected, mounted, sectioned, and stained as performed previously46. The following primary antibodies were used: anti-MHC/MF20 (mouse, Developmental Studies Hybridoma Bank, 1:100); anti-α-actinin (mouse, Diagnostic BioSystems, 1:100); anti-Raldh2 (rabbit, Abmart, 1:100); and anti–GFP (Chieken, Aves Labs, 1:100). The following secondary antibodies were used: anti-rabbit IgG–Alexa 594 (goat, Life Technologies, 1:200), anti-rabbit IgG–Alexa 568 (goat, Life Technologies, 1:200) and anti-chicken IgG–Alexa 488 (goat, Life Technologies, 1:200). Alexa Fluor 594-conjugated wheat germ agglutinin (Life Technologies, 50 μg ml−1) was used to stain the extracellular matrix. DAPI (1 μg ml−1) staining was used to identify nuclei. Notably, we discovered that eGFP from the Tg(Tp1:leGFPP)gg11 transgene persisted for a longer period in the ventricular outer myocardial wall (Extended Data Fig. 3) than d2GFP from the Tg(Tp1:dn2GFP)gg11 transgene (Extended Data Fig. 1).
with 10µM 4-HT or 0.1% ethanol (control) for 6h at 28°C and then washed with fresh egg water several times. The dose and length of incubation of 4-HT was titrated to create small distinct clones (one or two cells) before trabeculation (Extended Data Fig. 8). The total numbers of cardiomyocyte clones were counted from three-dimensional reconstructions of confocal slices from hearts containing myl7:CreER and pziZm transgenes. Visualization and counting of clones solely within the trabeculae were analysed using the post-imaging processing procedure described in the 'Image processing and statistical analysis' section below.

**Mosaic analysis by DNA injection.** To create the hsp70:lox::m-Cherry::STOP-loxp-dsUaH-P2A-Emerald plasmid (abbreviated as hsp70:RdsH5), the dominant negative Suppressor of Hairless (dnSuH) DNA construct was PCR amplified with flanking Ascl and SacII restriction sites. After sequence verification, this dsSuH product was subcloned into the hsp70:lox::m-Cherry::STOP-loxp-NICD-P2A::Emerald construct (hsp70:RdsH5), replacing the NICD sequence and generating the hsp70:lox::m-Cherry::STOP-loxp-dsUaH-P2A::Emerald (hsp70:RdsH5) construct for subsequent injection studies (see below). To generate cardiomyocyte clones with constitutively activated or inhibited Notch signalling, the I-SceI enzyme was co-injected with either the hsp70:Rsn plasmid (25 pg) or the hsp70:RdsH5 (25 pg) plasmid into one-cell stage embryos containing Tg(myl7::Cre; myl7::Cerulean) or only Tg(myl7::Cerulean). Embryos were then heat-shocked (as described above) at 60 hpf and imaged at 72 hpf. Cardiomyocytes containing either plasmid were detected by the co-expression of m-Cherry or Emerald and Cerulean. The location of Notch-altered Emerald+ cardiomyocyte clones in either the ventricular outer wall or the trabeculae was determined using the method described within the 'Image processing and statistical analysis' section below.

**Erbb2 and BMP loss of function studies.** The activity of Erbb2, a tyrosine kinase receptor, was inhibited using (1) homozygous erbb2+/− mutants10, (2) a splice morpholino targeting erbb2 (erbb2 MO)39, or (3) the tyrosine kinase inhibitor AG1478 (Calbiochem). (1) The erbb2+/− homozygous mutant embryos were identified by the previously characterized aberrant cardiac morphology18. (2) The erbb2+/− embryos were previously characterized and shown to be specific10. We injected 570 pg of the erbb2 morpholino (erbb2 MO) or a mismatched control morpholino (control MO) into one-cell stage embryos as previously described10. (3) AG1478 (5µM; Calbiochem) or 0.1% DMSO (control) was added to embryos as previously described40. Scale bars for all images represent 25 µm. Measurements comparing the ventricular outer wall with the trabeculae were performed with post-image processing of confocal slices. Visualization of all cardiomyocytes and clones within the ventricle (comprising both the ventricular outer wall and the trabeculae) were made using three-dimensional reconstructions (Nikon NIS Elements) of confocal slices. However, to visualize only the trabeculae, cardiomyocytes within the ventricular outer wall in individual confocal slices were identified by their outer location and orientation and then masked manually. Three-dimensional reconstructions with these masked confocal slices then allowed the visualization and measurement of trabeculae alone. Measurements for the ventricular outer wall alone were calculated by subtracting the measurements of the trabeculae from the total ventricular cardiomyocytes. No statistical methods were used to predetermine sample size. Animals were assigned to experimental groups using simple randomization, without investigator blinding. Unpaired two-tailed Student’s t-tests or Fisher’s exact tests were used to determine statistical significance. P < 0.05 was considered to be statistically significant, as indicated by an asterisk. Error bars, s.e.m.

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Extended Data Figure 1 | Notch signalling is dynamically activated in the endocardium and myocardium during heart development.

a–f, Confocal slices of Tg(Tp1:d2GFP; myl7:mCherry) hearts reveal that Notch signalling is in the ventricular endocardium (yellow arrows) but not in the myocardium at 24 hpf (n = 11) and 36 hpf (n = 8), but (g–i) becomes restricted to the AV and OFT endocardium by 48 hpf (n = 12).

j–o, Tg(Tp1:d2GFP; kdrl:ras–mCherry) confocal imaging confirms that Tp1:d2GFP is expressed in the ventricular endocardium at (j–l) 24 hpf (n = 8) but becomes localized to the AV or OFT endocardium as well as non-endocardial cells in the outer ventricular myocardial wall (white arrows) by (m–o) 96 hpf (n = 10).

p, q, Three-dimensional confocal reconstructions of the (p) exterior and (q) interior regions of 72 hpf Tg(Tp1:d2GFP; myl7:mCherry) hearts reveal that Notch-activated Tp1:d2GFP+ cells are present in cardiomyocyte clusters (green, numbers in parentheses) and excluded from nascent cardiac trabeculae (pseudocolour magenta, numbers). r, Graph shows that the number of cardiac trabeculae (x axis) and Tp1:d2GFP+ cardiomyocyte clusters (y axis) are similar within the ventricle (n = 30) at 72 hpf. Size of dots indicates the number of embryos with a particular number of trabeculae and Tp1:d2GFP+ clusters. Line represents a linear regression fitted to the data.

s, t, Myocardial anti-MHC/MF20 immunostaining of Tg(Tp1:d2GFP) hearts reveals a loss of myocardial Tp1:d2GFP Notch reporter signal at 30 and 90 dpf hearts (n = 5 hearts per stage). White arrows, likely Tp1:d2GFP+ cardiomyocytes; yellow arrows, Tp1:d2GFP+ endocardial cells; white and yellow asterisks, AV and OFT. Dashed line in s outlines ventricle. V, ventricle; A, atrium. Scale bar, 25 μm.
Extended Data Figure 2 | DAPT treatment validates that the Notch reporter Tp1:d2GFP monitors dynamic Notch signalling more closely than Tp1:eGFP, and reveals opposing roles of Notch signalling on trabeculation at different developmental stages. a–d. At 48hpf, (a) Tp1:d2GFP expression is restricted to the AV and OFT endocardium (n = 8/8 embryos) whereas (c) Tp1:eGFP is expressed in the ventricular, AV and OFT endocardium (n = 6/6). However, gfp mRNA is primarily expressed in the AV and OFT regions in both (b) Tg(Tp1:d2GFP; myl7:mCherry) (n = 10/10) and (d) Tg(Tp1:eGFP; myl7:mCherry) embryos (n = 5/5), revealing that Tp1:d2GFP expression most closely matches Notch reporter activity. e–h. After 24 h DAPT treatments of (e, f) Tg(Tp1:d2GFP; myl7:H2A–mCherry) and (g, h) Tg(Tp1:eGFP; myl7:H2A–mCherry) embryos at 72 hpf, (f) Tp1:d2GFP is more diminished throughout the heart at 96 hpf (n = 8/10) compared with (h) Tp1:eGFP (n = 6/7), confirming Tp1:d2GFP signal more faithfully recapitulates Notch signalling dynamics. m–p, Tg(Tp1:d2GFP; myl7:mCherry) hearts DAPT-treated from 60 to 72 hpf exhibit increased trabeculation (white arrowheads) and diminished Tp1:d2GFP Notch reporter activity (n = 12/16) than (i–l) DMSO-treated hearts (n = 0/20). However, (r) Tg(myl7:mCherry) hearts DAPT-treated from 20 to 48 hpf exhibit reduced trabeculae at 120 hpf (n = 12/15) than (q) DMSO-treated hearts (n = 0/20). s, Graph represents trabeculae/total ventricular area in embryos treated with DMSO or DAPT in q and r. White and yellow arrows, myocardial and endocardial Notch reporter activity; white arrowheads, trabeculae; white and yellow asterisks, AV and OFT. Scale bar, 25 μm. Mean ± s.e.m. *P < 0.05 by Student’s t-test.
Extended Data Figure 3 | Tp1:eGFP labels the ventricular outer wall during early cardiac development, which becomes the distinctive ventricular primordial myocardium in adults. Using the Tp1:eGFP Notch reporter, which exhibits greater fluorescence perdurance than Tp1:d2GFP, we performed limited fate mapping of Notch activated cardiac cells during ventricular morphogenesis. a, b, Tp1:eGFP is expressed not only in ventricular cardiomyocytes (red nuclei, white arrows) at 72 hpf but also throughout the ventricular endocardium because of eGFP perdurance (yellow arrows) (n = 12). c, d, Although diminishing in the ventricular endocardium (yellow arrows) at 96 hpf (n = 14), Tp1:eGFP expands in the outer ventricular myocardial wall (white arrows), yet is notably absent from myocardial trabeculae (white arrowheads). e, f, By 30 and 45 dpf (n = 6, n = 5), Tp1:eGFP remains in the peripheral ventricular (primordial) myocardial layer, which is one cardiomyocyte thick (myl7:H2A–mCherry+/red and MF20+/blue), but is reduced in the ventricular but not the AV or OFT endocardium. g–i, At 60 dpf (n = 5), new cardiomyocytes (cortical layer, yellow arrowheads) form over the Tp1:eGFP+ primordial myocardium (white arrows) at the ventricular myocardial base (yellow box in g) and extend towards the apex where (j) Tp1:eGFP+ cardiomyocytes (white arrows) still remain the outer most layer of the ventricular myocardium (white box in g). j, However, by 90 dpf (n = 5), this new cortical myocardial layer (yellow arrowheads) spreads over the apical Tp1:eGFP+ ventricular primordial myocardium (white arrows). k–m, In adult hearts (90 dpf), Tp1:eGFP is primarily found in the (k, n = 5) myl7:H2A–mCherry+ primordial myocardium but not in the (l, n = 5) endocardium marked by kdrl:ras–mCherry, nor (m, n = 3) epicardium marked by Raldh2 localization. n–t, Adult hearts (6 months) were further examined to assess the cellular attributes of the primordial layer. n, Anti-MHC/MF20 immunostaining confirms that Tp1:eGFP+ cardiac cells are myocardial (n = 5). o, Anti-α-actinin immunostaining reveals that trabecular (white arrowheads) and cortical (yellow arrowheads) cardiomyocytes display organized sarcomeric structures but the Tp1:eGFP+ primordial cardiomyocytes (arrows) do not (n = 7). p–t, Wheat germ agglutinin (WGA) staining shows that (p, q) Tp1:eGFP+ primordial myocardial layer is surrounded by extensive extracellular matrix (n = 5) and that (r–t) Tg(myl7:ras–eGFP) primordial cardiomyocytes display a thin cellular morphology compared with other ventricular cardiomyocytes (n = 10). q, An X–Z reconstruction of confocal stacks from Tp1:eGFP and wheat germ agglutinin stainings at the dashed line shown in p, b, d, h–i, t, Magnifications of the boxed areas in a, c, g, s, respectively. White and yellow arrows, myocardial and endocardial Tp1:eGFP; white and yellow arrowheads, trabeculae and cortical layer; white and yellow asterisks, AV and OFT. Scale bar, 25 μm.
Extended Data Figure 4 | BMP signalling, which marks trabeculae, is required for expanding but not initiating trabeculae formation and has no effect on myocardial Notch activity. a–l, Tg(BRE:d2GFP; myl7:mCherry) hearts were treated with (a–c) DMSO, (d–f) DAPT, (g–i) AG1478, or (j–l) Dorsomorphin at 60 hpf and imaged at 72 hpf. a–c, DMSO-treated hearts express the BRE:d2GFP BMP reporter in trabeculae (arrowheads) and in the AV myocardium (yellow arrows, n = 11/11 embryos). d–f, DAPT-treated hearts exhibit increased trabeculation and BRE:d2GFP expression in these forming trabeculae (arrowheads, n = 9/12). g–i, AG1478-treated hearts fail to form trabeculae (n = 9/10) and only express the BRE:d2GFP BMP reporter in the AV myocardium (yellow arrow). j–l, Dorsomorphin-treated hearts form cardiac trabeculae (arrowheads) but fail to express the BRE:d2GFP BMP reporter in both cardiac trabeculae and the AV myocardium (n = 10/12). m–p, Treating Tg(Tp1:d2GFP; myl7:mCherry) embryos with Dorsomorphin from 60 to 72 hpf did not affect the initiation of trabeculae (arrowheads) nor the activation of myocardial Notch signalling (white arrows, n = 13/16) compared with treating with DMSO (see Extended Data Fig. 2i–l). q, r, Although Tg(myl7:mCherry) hearts treated with (q) DMSO or (r) Dorsomorphin from 60 hpf to 7 dpf form similar numbers of trabeculae (arrowheads), Dorsomorphin-treated hearts display trabeculae that are stunted/reduced in size (n = 12/15) compared with DMSO-treated control hearts (n = 0/15). s, Graph reveals a significant reduction in the trabecular/ventricular area ratio in Dorsomorphin-treated fish compared with DMSO-treated controls. Arrowheads, trabeculae; yellow arrows, AV myocardium; white arrows, Tp1:d2GFP + myocardium. White asterisks, AV. Mean ± s.e.m. *P < 0.05 by Student’s t-test. Scale bar, 25 μm.
Extended Data Figure 5 | Altering myocardial Notch signalling affects ventricular size and wall thickness but not total number of ventricular cardiomyocytes. **a–d,** The Tg(myl7:Cre) transgenic line used to specifically perturb Notch signalling in the myocardium was validated by confirming that Cre expression is restricted to the myocardium. Activity of myl7:Cre, as visualized by (c) GFP expression from the switch line, β-act2:RSG, exclusively overlaps with (b, d) myl7:Cerulean expression at 120 hpf (n = 10 embryos). Quantitative analyses of (e) ventricular size and (f) wall thickness performed on confocal images from Fig. 2a–h reveal that myocardial Notch signalling restricts ventricular size while promoting ventricular wall thickness. 

**e,** Ventricular size measurements were normalized to respective controls for each condition. 

**f,** Individual measurements (dots) of myocardial thickness were taken across the outer curvature of the ventricle (n = 30 measurements, 6 measurements were taken per embryo, 5 embryos per condition). Dashed line represents the ventricular wall thickness that distinguishes trabeculated myocardial thickness from ventricular outer wall myocardial thickness in control hearts. Crosses denote mean and s.e.m. 

**g–p,** Quantitative analysis of (g) trabecular cardiomyocytes and (p) total ventricular cardiomyocytes was calculated by counting myocardial nuclei labelled with myl7:H2A–mCherry or anti-Mef2 immunostaining using embryos from Fig. 2i–p for g, or from three-dimensional reconstructions in h–o for p. In g, the number of trabecular/total ventricular cardiomyocytes was used to calculate the percentage of trabecular cardiomyocytes for each condition. In p, total ventricular cardiomyocytes were normalized to respective controls for each condition. n, Number of embryos analysed per condition. Mean ± s.e.m. *P < 0.05 by Student’s t-test. NS, not significant. Scale bar, 25 μm.
Extended Data Figure 6 | Myocardial Notch activation can inhibit the formation and expansion of cardiac trabeculae at various cardiac developmental stages. a–g, Tg(myl7:Cre; hsp70l:RSN) and Tg(hsp70l:RSN) (control) embryos were heat-shocked (HS) during various developmental time windows as indicated and imaged at 7 dpf to assess the effects of constitutive myocardial Notch signalling on cardiac trabeculae formation. a, Red arrows in schematic indicate the time points at which embryos in the corresponding panels were heat-shocked. b, Control Tg(hsp70l:RSN) embryos heat-shocked from 60 hpf to 7 dpf ubiquitously express mCherry but do not overexpress myocardial NICD. They form cardiac trabeculae (arrowheads) similar to wild-type embryos (control, n = 14/15). c, However, Tg(myl7:Cre; hsp70l:RSN) embryos heat-shocked from 60 hpf to 7 dpf overexpress NICD-P2A–Emerald throughout the myocardium and fail to form cardiac trabeculae (n = 9/12). Although Hsp70l:RSN embryos heat-shocked from 60 to 120 hpf initially inhibit trabeculae formation, (i) the ventricular myocardium (detected by anti-MHC/ MF20 immunostaining, magenta) can still form trabeculae, albeit at reduced numbers (n = 4/5) by 30 dpf after stopping NICD overexpression compared with (h) heat-shocked Tg(hsp70l:RSN) hearts (control, n = 0/8). HS, heat-shock; white arrowheads, trabeculae. Scale bar, 25 μm. Mean ± s.e.m. *P < 0.05 by Student’s t-test.
Extended Data Figure 7 | Notch signalling regulates cardiomyocyte cell junctions during cardiac trabeculae formation. a–d, In DMSO-treated (control) 72 hpf wild-type hearts, N-cadherin is localized at cell junctions of cardiomyocytes within the ventricular outer wall (arrows) but redistributes away from these cell–cell contacts in cardiomyocytes that extend into the lumen to form trabeculae (arrowheads) (n = 12/12).

e–h, Notch inhibition by DAPT treatment promotes N-cadherin redistribution and results in increased trabeculation (n = 8/11).
m–p, Conversely, myocardial Notch activation by heat shocking (HS) Tg(myl7:Cre; hsp70:RSN) leads to diminished N-cadherin redistribution and reduced trabeculation (n = 7/10) compared with (i–l) heat-shocked Tg(hsp70:RSN) control hearts (n = 0/10). Nascent cardiac trabeculae were pseudo-coloured magenta in c, g and k. b, d, f, h, j, l, n, p. Magnifications of boxed areas in a, c, e, g, i, k, m, o, respectively. Arrowheads, N-cadherin redistributed from cell–cell contacts; arrows, N-cadherin at cell–cell contacts within outer wall. Scale bar, 25 μm.
Extended Data Figure 8 | Tamoxifen treatment of Tg(myl7:CreER; priZm) embryos at 48 hpf labels adjacent individual cardiomyocytes with combinations of distinct fluorescent colours. Tg(myl7:CreER; priZm) embryos were treated with 4-HT at 48 hpf and confocal imaged at 60 hpf before the initiation of cardiomyocytes forming trabeculae. Individual cardiomyocytes (arrowheads) are labelled with distinct combinations of fluorescent proteins allowing for tracking of specific cardiomyocyte clones (n = 6). White arrowheads, cardiomyocytes; V, ventricle; A, atrium; white asterisk, AV. Scale bar, 25 μm.
Extended Data Figure 9 | Notch and Erbb2 signalling pathways form a feedback loop during cardiac trabeculation. a, b, Compared with (a) DMSO-treated Tg(Tp1:d2GFP; myl7:mCherry) (controls) embryos, (b) inhibiting Erbb2 function with AG1478 from 60 to 72 hpf blocks trabeculation and myocardial Notch signalling \((n = 14/17)\), confirming erbb2 MO and mutant phenotypes. c, However, Notch inhibition using DAPT cannot reverse the AG1478/Erbb2 inhibition effect on trabeculae formation \((n = 11/12)\). d, e, Consistent with these results, (d) control MO-injected Tg(hsp70l:dnM; myl7:mCherry) embryos expressing heat-shock induced dnMAML from 60 to 72 hpf display increased trabeculation \((n = 9/11)\); (e) however, erbb2 MO-injected embryos expressing heat-shock induced dnMAML fail to display trabeculae \((n = 9/12)\) as similarly observed in erbb2 MO-injected embryos alone (Fig. 3). f–j, The erbb2 fluorescent in situ hybridization and GFP co-immunostaining performed on 72 hpf Tg(Tp1:d2GFP) hearts reveal that erbb2 is expressed in an intermittent pattern across the ventricular wall and is specifically diminished in Tp1:d2GFP+ cells (arrows) \((n = 6/6)\). l, p, Heat-shocked (HS) Tg(myl7:Cre; hsp70l:RSN) hearts, which exhibit constitutively activated myocardial Notch signalling (NICD) from 60 to 120 hpf, minimally express erbb2 in the myocardium \((n = 8/11)\) compared with (k, o) heat-shocked Tg(hsp70l:RSN) control hearts \((n = 0/20)\) at 120 hpf. Compared with (m, q) DMSO-treated control hearts \((n = 0/10)\), (n, r) Notch-inhibited hearts by DAPT treatment from 60 to 72 hpf exhibit increased myocardial erbb2 expression as well as more trabeculae at 72 hpf \((n = 8/10)\), supporting the idea that Notch signalling inhibits erbb2 expression. h, i–j, Magnifications of boxed areas in g, h, respectively. Arrowheads, trabeculae; arrows, Tp1:d2GFP+ cardiomyocytes; white and yellow asterisks, AV and OFT. Scale bar, 25 μm.
Extended Data Figure 10 | Transplanted wild-type cardiomyocytes non-cell-autonomously activate Notch signalling in \( \text{erbb2} \) morphant host cardiomyocytes. 

**a**, On the basis of mosaic embryo studies from Fig. 3f–i, wild-type donor cardiomyocytes contribute equally to the outer ventricular wall (14/26 clones) or the trabeculae (12/26 clones) when transplanted into control MO host embryos (\( n = 12 \) embryos). However, when wild-type donor cells are transplanted into \( \text{erbb2} \) MO host embryos (\( n = 10 \) embryos), they contribute more to the trabecular layer (19/23 clones) than to the ventricular outer wall (4/23 clones, \( P < 0.05 \) by Fisher’s exact test).

**b**, On the basis of mosaic embryo studies from Fig. 3f–i, transplanting wild-type donor cells increases the number of \( \text{erbb2} \) MO host cardiomyocytes expressing \( Tp1:d2GFP \) (\( n = 10 \) embryos) compared with non-transplanted \( \text{erbb2} \) MO embryos (\( n = 16 \) embryos), but had no effect on the number of control MO host cells expressing \( Tp1:d2GFP \) (\( n = 12 \) embryos) compared with non-transplanted controls (\( n = 11 \) embryos).

**c**, Quantitative data for Fig. 3f–i reveal that transplanted wild-type donor cardiomyocytes are primarily adjacent to host \( Tp1:d2GFP^+ \) cardiomyocytes in \( \text{erbb2} \) MO hearts (\( n = 10 \) embryos). Mean ± s.e.m. *\( P < 0.05 \) by Student’s \( t \)-test. NS, not significant.