Trabeculation is crucial for cardiac muscle growth in vertebrates. This process requires the Erbb2/4 ligand Neuregulin (Nrg), secreted by the endocardium, as well as blood flow/cardiac contractility. Here, we address two fundamental, yet unresolved, questions about cardiac trabeculation: why does it initially occur in the ventricle and not the atrium, and how is it modulated by blood flow/contractility. Using loss-of-function approaches, we first show that zebrafish Nrg2a is required for trabeculation, and using a protein-trap line, find that it is expressed in both cardiac chambers albeit with different spatiotemporal patterns. Through gain-of-function experiments, we show that atrial cardiomyocytes can also respond to Nrg2a signalling, suggesting that the cardiac jelly, which remains prominent in the atrium, represents a barrier to Erbb2/4 activation. Furthermore, we find that blood flow/contractility is required for Nrg2a expression, and that while non-contractile hearts fail to trabeculate, non-contractile cardiomyocytes are also competent to respond to Nrg2a/Erbb2 signalling.
Cardiac trabeculation, which allows the increase in muscle mass prior to the formation of coronaries, is one of the most important processes the vertebrate heart must undergo to form and function properly\(^1\). During this process, trabeculae, which are sponge-like muscular structures in the cardiac cavity, form as a result of cardiomyocyte delamination and proliferation\(^2-9\). Defects in this complex morphogenetic lead to embryonic lethality or adult onset dilated cardiomyopathies\(^5,7-12\) illustrating the importance of trabeculation in cardiac development and function.

The vertebrate heart initially consists of two cell layers, the endocardium and myocardium, separated by an acellular, gelatinous matrix called the cardiac jelly\(^13-15\). Ablating components of the cardiac jelly by injecting hyaluronidase in chick embryos has been reported to cause looping defects\(^16\), and it has been shown that physical forces are necessary in the developing heart, particularly during trabecular formation. Endocardial–myocardial interactions are known to orchestrate cardiac trabeculation; for example, in chick embryos, a myocardial-derived growth factor, Angiopoietin 1, has been reported to bind to its receptor Tie2 which is expressed by endocardial cells and modify their angiogenic behaviour which in turn affects the complexity of cardiac trabeculation\(^17\). Similarly, signalling from endocardial to myocardial cells is also necessary for trabeculation. For instance, mice lacking the endocardial-derived ligand Nrg1 or its receptors Erbb2 and Erbb4, which are expressed by myocardial cells, exhibit severe trabeculation defects\(^8,12,18\). Similarly, zebrafish \(erbb2\) mutants fail to form trabeculae\(^19\). Additional studies have shown that Nrg signalling can positively regulate cardiomyocyte proliferation and heart regeneration\(^19-22\).

Cardiac formation can also be influenced by its function\(^23-25\). Recent studies have shown the dependence of trabeculation on physical forces generated by cardiac contractility and blood flow\(^6,26,27\). For instance, several groups have reported that reduction of blood flow in the ventricular chamber markedly diminishes trabeculation in chick embryos and zebrafish larvae\(^6,26,28,29\). By manipulating cardiac contractility, another study presented data indicating that cardiac contraction promotes trabeculation through the regulation of Notch signalling in the ventricular endocardium\(^27\). These and other findings support the hypothesis that physical forces are necessary in the developing heart, particularly during trabecular formation.

In this study, we use the zebrafish model to address two fundamental questions pertinent to cardiac trabeculation; first, why trabeculae initially form in the ventricle and not the atrium, as is observed in zebrafish as well as in other vertebrates including ammioites\(^3,30\) and second, how trabeculation is modulated by physical forces. Using a genetic approach and high-resolution live imaging, we first show that Nrg2a is an essential ligand for cardiac trabeculation in zebrafish. By ectopically expressing \(\text{nrg2a}\) in cardiomyocytes, we further show that atrial cardiomyocytes, like ventricular cardiomyocytes, can respond to Nrg2a/Erbb2 signalling. Using loss- and gain-of-function approaches, we show that blood flow/contractility modulates endocardial \(\text{nrg2a}\) expression while it is not required for the expression or function of Nrg2a/Erbb2 signalling effectors in cardiomyocytes.

Results

**Nrg2a is required for cardiac trabeculation in zebrafish.** Given the role of Nrg1 in cardiac trabeculation in mouse\(^7,8,12,18\), we mutated zebrafish \(\text{nrg1}\) using TALEN technology\(^31\). Since \(\text{nrg1}\) has multiple isoforms\(^32\), we targeted exon 2 which encodes part of the highly conserved IGc2-domain, and identified a \(\Delta 14\) allele (Supplementary Fig. 1a) encoding a predicted truncated protein (Supplementary Fig. 1b) due to harbouring a premature stop codon within the same exon. Using brightfield microscopy, no gross morphological defects were observed in \(\text{nrg1} \Delta 14\) (\(\text{nrg1}^{\Delta 14}\)) homozygous mutant larvae (Supplementary Fig. 1c,d). Increasing \(\text{nrg1}^{\Delta 14}\) fish in the \(\text{Tg}(\text{myl7:LifeACT-GFP})\) background, a myocardial specific line in which F-actin (filamentous actin) is labelled with GFP\(^33\) we observed that trabeculation appears unaffected in \(\text{nrg1}^{\Delta 14}\) mutant larvae (Supplementary Fig. 1e–h), which can survive to become fertile adults.

Next, we decided to investigate the role of \(\text{nrg2a}\), another member of the Nrg family, by using a conditional protein-trap line, \(\text{Tg}2\alpha^{\text{mdm2330}}\) (refs 34,35). Using brightfield microscopy, \(\text{nrg2a}\) mutants can be recognized by their aberrant jaws (Fig. 1a,b) as well as other defects in median fin fold (MFF) morphogenesis and swim bladder inflation\(^35\). As previously reported, the gene-breaking transgene (GBT) \(\text{nrg2a}\) allele generates a truncated \(\text{nrg2a}\) protein tagged with mRFP (\(\text{nrg2a}\)-mRFP) due to the in-frame integration of the mutagenic pGBT-RP2.1 (RP2) cassette in the intron between alternative exon 1C and exon 2 (ref. 35). Prior studies have also shown that the expression of \(\text{nrg2a}\)-mRFP recapitulates that of endogenous \(\text{nrg2a}\) (refs 34,35). Confocal imaging of larvae from \(\text{nrg2a}\)-mRFP incrosses in the \(\text{Tg}(\text{myl7:LifeACT-GFP})\) background (Fig. 1c–k) revealed that \(\text{nrg2a}\)-mRFP is highly expressed in the endocardium at early larval stages (Fig. 1d,e,g,h,j,k), and that \(\text{nrg2a}^{+/−}\) hearts fail to form trabecule by 75 h post fertilization (hpf) (Fig. 1c–e). Observation of \(\text{nrg2a}\) mutants at later stages, including 120 and 168 hpf (Fig. 1f–k), confirmed that their trabeculation defects appear as severe as those in \(\text{erbb2}\) mutants\(^5\). Overall, these data suggest that \(\text{nrg2a}\), and not \(\text{nrg1}\), is the crucial regulator of cardiac trabeculation in zebrafish.

Differential \(\text{nrg2a}\) expression in the ventricle and atrium. Trabeculae start to form in zebrafish around 60 hpf and become clearly apparent by 72 hpf\(^5,26\). To investigate why trabeculation occurs primarily in the ventricle and not the atrium\(^3,30\), we examined \(\text{nrg2a}\)-mRFP expression at several stages of cardiac development. We used high-resolution confocal microscopy to image live \(\text{nrg2a}^{+/−}\) animals in the \(\text{Tg}(\text{kdr:l:NLs-EGFP})\) background, in which the endothelial cell nuclei are labelled with GFP\(^36\), at 52, 78 and 120 hpf (Fig. 2a–i). \(\text{nrg2a}\)-mRFP expression appears in the endocardium at 52 hpf, particularly in the outer curvature of the ventricle (Fig. 2a–c, Supplementary Fig. 2a–c and 3a–c). Subsequently, \(\text{nrg2a}\)-mRFP expression becomes stronger in ventricular and atrial endocardial cells (Fig. 2d–i, Supplementary Fig. 2d–i, Fig. 3d–i), although it is weaker in the atrioventricular (AV) canal (Fig. 2d–i and Supplementary Fig. 4a–c). Accordingly, \(\text{nrg2a}\) mutants develop functional AV valve leaflets (Supplementary Fig. 4d–i) as was observed in \(\text{erbb2}\) mutants\(^5\), suggesting that \(\text{nrg2a}\)/Erbb2 signalling is not required for AV valve development.

Utilizing the Zeiss Efficient Navigation (ZEN) software, we measured the levels of \(\text{nrg2a}\)-mRFP expression as well as the number of \(\text{nrg2a}\)-mRFP positive endocardial cells at 78 hpf (Fig. 2j,k). These analyses show that ventricular endocardial cells express higher levels of \(\text{nrg2a}\)-mRFP than atrial or AV canal endocardial cells (Fig. 2j), and that there are more \(\text{nrg2a}\)-mRFP positive endocardial cells in the ventricle as compared to the other cardiac regions (Fig. 2k). We also found that on average (\(N=5\) hearts), 97% and 90% of endocardial cells in the ventricle and atrium were positive for \(\text{nrg2a}\)-mRFP expression, respectively (Supplementary Fig. 2j). Thus, ventricular cardiomyocytes might be exposed to a higher level of \(\text{nrg2a}\) ligands than atrial ones, although atrial endocardial cells are clearly positive for \(\text{nrg2a}\)-mRFP expression.
To examine whether the Nrg2a protein-trap expression in the endocardium accurately recapitulates endogenous nrg2a expression\textsuperscript{34,35}, we carried out \textit{in situ} hybridizations on 78 hpf hearts. We observed that nrg2a is more highly expressed in the ventricle, compared to the atrium, and that it is mainly restricted to the outer curvature (Supplementary Fig. 2k). This expression pattern is consistent with that of Nrg2a-mRFP.

The cardiac jelly and the onset of trabeculation. The cardiac jelly is known to be pivotal in endo-myocardial interactions required for cardiac morphogenesis\textsuperscript{4,37}, and its reduction during development has previously been reported in chicken and mice\textsuperscript{4,38}. Using high-resolution confocal microscopy, we decided to investigate more closely the dynamics of cardiac jelly reduction around the onset of trabeculation in \textit{Tg(kdrl:Hsa.HRAS-mCherry)}\textsuperscript{39};\textit{Tg(myl7:EGFP-Hsa.HRAS)}\textsuperscript{40} animals in which endocardial and myocardial membranes are labelled in red and green, respectively. We found that at 48 hpf the cardiac jelly fully separates the endocardial and myocardial layers (Fig. 3a), and that its thickness gradually decreases in both chambers but more clearly in the ventricle (Fig. 3b–h). The timing of this decrease correlates with the appearance of the first trabeculae in the outer curvature of the ventricle by 60 hpf (Fig. 3b,c, arrowhead). By 96 hpf, the cardiac jelly appears to be fully gone in the ventricle while it remains in the atrium for at least the first 6 days of development (Fig. 3d–h).

To investigate whether Nrg2a signalling modulates the thickness of the cardiac jelly, we incrossed \textit{nrg2a}\textsuperscript{+/+} animals in the \textit{Tg(kdrl:Hsa.HRAS-mCherry)};\textit{Tg(myl7:LIFEACT-GFP)} background with \textit{nrg2a}\textsuperscript{+/-} and \textit{nrg2a}\textsuperscript{-/-} to study the effects on cardiac jelly thickness.

Figure 1 | Nrg2a is required for cardiac trabeculation in zebrafish. (a,b) Zebrafish larvae from \textit{nrg2a}\textsuperscript{+/-} incrosses were imaged at 120 hpf; lateral view, anterior to the left; scale bars, 0.5 mm. \textit{nrg2a}\textsuperscript{-/-} larvae can be recognized by their defective jaws (asterisk in b). (c–k) \textit{nrg2a} mutants lack cardiac trabeculae. Confocal images (mid-sagittal sections) of larval hearts from \textit{Tg(myl7:LIFEACT-GFP);nrg2a}\textsuperscript{+/-} incrosses at 75 (c–e), 120 (f–h) and 168 hpf (i–k); ventricular outer curvature (dashed boxes) magnified in upper right corners; arrows and asterisks indicate trabeculated and non-trabeculated walls, respectively; scale bars, 50 μm.
background, and found no obvious differences between wild types and mutants (Supplementary Fig. 5a–h), similar to what we observed in \( nrg^{10a101} \) mutants and Erbb2 inhibitor-treated animals (Supplementary Fig. 5i–k), suggesting that Nrg/Erbb2 signalling is not involved in this process in zebrafish.

Altogether, these data show that the reduction of the cardiac jelly takes place in the ventricle as trabeculation starts, suggesting that it may determine the onset of this process, possibly by constituting a diffusion barrier for Nrg ligands.

\[ \text{nrg2a overexpression in cardiomyocytes.} \]

The current model is that endocardial-derived Nrg ligands traverse the cardiac jelly to activate cardiomyocyte behaviour and proliferation\(^{4,12,41,42}\). To

| \( \text{nrg2a}^{+/-} \) | \( \text{Nrg2a-mRFP} \) | \( \text{Tg(kdrl:NLS-EGFP)} \) | \( \text{Merge} \) |
|---|---|---|---|
| a | V | AV | At |
| 52 hpf |
| d | V | AV | At |
| 78 hpf |
| g | V | AV | At |
| 120 hpf |

\( \text{j} \) Cell-based mRFP intensity, measured with the ZEN Imaging Software and plotted as a graph, showing that Nrg2a-mRFP is more highly expressed in the ventricle compared to the AV canal and atrium; dots in this graph represent individual Nrg2a-mRFP expressing endocardial cells. \( \text{k} \) Nrg2a-mRFP positive endocardial cells counted in each chamber at 78 hpf, showing that there are more Nrg2a-mRFP positive endocardial cells in the ventricle than in the atrium at 78 hpf; dots in this graph represent individual hearts; \( N = 5 \) hearts; values represent means \( \pm \) s.e.m.; **\( P \leq 0.01, ***P \leq 0.001 \) by Student’s t-test.

Figure 2 | Nrg2a-mRFP expression during embryonic and larval cardiac development. (a–i) Two-dimensional (2D) confocal images (mid-sagittal sections) of zebrafish hearts from \( \text{Tg(kdrl:NLS-EGFP);nrg2a}^{+/-} \) outcrosses at 52 (a–c), 78 (d–f) and 120 hpf (g–i) showing that Nrg2a-mRFP expression is clearly visible in the ventricular endocardium by 52 hpf, mainly in the outer curvature (a–c), and that it becomes stronger in both ventricular and atrial chambers at 78 and 120 hpf (d–i); however, it is weak in the atrioventricular canal; arrowheads point to the superior valve leaflet (a–i); AV, atrioventricular canal; At, atrium; V, ventricle; scale bars, 50 μm. (j) Cell-based mRFP intensity, measured with the ZEN Imaging Software and plotted as a graph, showing that Nrg2a-mRFP is more highly expressed in the ventricle compared to the AV canal and atrium; dots in this graph represent individual Nrg2a-mRFP expressing endocardial cells. (k) Nrg2a-mRFP positive endocardial cells counted in each chamber at 78 hpf, showing that there are more Nrg2a-mRFP positive endocardial cells in the ventricle than in the atrium at 78 hpf; dots in this graph represent individual hearts; \( N = 5 \) hearts; values represent means \( \pm \) s.e.m.; **\( P \leq 0.01, ***P \leq 0.001 \) by Student’s t-test.
determine whether ectopic overexpression of nrg2a in cardiomyocytes enables them to form trabeculae in nrg2a mutants, we collected embryos from Tg(myl7:LIFEACT-GFP);nrg2a+/+ incrosses and injected them with a myl7:nrg2a-p2a-tdTomato plasmid at the one-cell stage (Fig. 4a,b). This approach allows one to identify the nrg2a overexpressing cardiomyocytes by virtue of their expression of tdTomato. Confocal microscopy analysis showed that ectopic overexpression of nrg2a in nrg2a mutant ventricular cardiomyocytes could drive them to form a multilayered wall in most tdTomato positive hearts (13 < 15) (Fig. 4c–e). To determine whether Nrg2a signals via Erbb2 in cardiomyocytes, we ectopically overexpressed nrg2a in erbb2st61 mutant cardiomyocytes. Our results show that mosaic myocardial nrg2a overexpression did not rescue the trabeculation defects in erbb2 mutants (Fig. 4f–i), indicating that Nrg2a must signal through Erbb2 in cardiomyocytes.

We also generated a stable myocardial-specific Nrg2a transgenic line by injecting wild-type embryos with the myl7:nrg2a-p2a-tdTomato plasmid. Although a majority of the injected animals survived to adulthood, some exhibited pericardial edema due to the presence of a much enlarged heart as compared to those of non-injected animals, indicating that mosaic expression of nrg2a in cardiomyocytes leads to cardiomegaly (Supplementary Fig. 6a–d), as expected from data in mouse using a transgene encoding constitutively active Erbb2 (ref. 21) and in zebrafish using nrg1 overexpression22. Overall, these data indicate that ventricular cardiomyocytes are capable to respond to the myocardial overexpression of nrg2a.

**Atrial cardiomyocytes can respond to Nrg2a signalling.** To further investigate the correlation between cardiac jelly thickness and the onset of trabeculation, we used our newly generated Tg(myl7:nrg2a-p2a-tdTomato) line to evaluate the behaviour of cardiomyocytes in the presence or absence of Nrg2a signalling (Fig. 5a). The cardiac jelly is present for at least the first 6 days of development in the zebrafish atrium, physically separating the endocardial and myocardial walls (Fig. 3), and thus possibly preventing myocardial cells from receiving endocardial-derived Nrg2a molecules. A myocardial specific nrg2a overexpression approach allows one to circumvent the hypothetical barrier function of the cardiac jelly and test whether atrial cardiomyocytes are in fact competent to respond to Nrg signalling. To better visualize cardiomyocyte boundaries, Tg(myl7:nrg2a-p2a-tdTomato) fish were outcrossed to the myocardial specific membrane line Tg(myl7:EGFP-Hsa.HRAS). Compared to Tg(myl7:nrg2a-p2a-tdTomato) negative animals, we observed that nrg2a overexpression in cardiomyocytes pushed
them to form a bilayered wall in the ventricle as well as the atrium starting at 46 and 78 hpf, respectively (Fig. 5b–e, Supplementary Fig. 7a,b). Subsequently, constitutively active Nrg2a signalling resulted in cardiomyocyte multilayering in both ventricular and atrial walls (Fig. 5f–i). However, this cardiomyocyte response was blocked by Erbb2 inhibitor treatment starting at 36 hpf (Supplementary Fig. 8a–d). By crossing the Tg(myl7:nrg2a-p2a-tdTomato) line with a Fucci line (Tg(myl7:mVenus-gmnn)) which labels proliferating cardiomyocytes in green43, we observed that ectopic myocardial expression of nrg2a increased the number of proliferating cardiomyocytes (Supplementary Fig. 9a–f). Overall, these data indicate that both ventricular and atrial cardiomyocytes can respond to Nrg/Erbb2 signalling.

**Contractility regulates endocardial nrg2a expression.** Physical forces generated by contractility have been previously shown to modulate cardiac trabeculation6,26,27. Taking advantage of the

---

**Figure 4 | Myocardial nrg2a overexpression induces cardiomyocyte multilayering in nrg2a mutants.** (a) Cartoon of myocardial specific nrg2a construct. (b) Schematic representation of Nrg2a protein tagged by tdTomato. Due to the presence of the P2A peptide, cleavage occurs right after protein translation to separate the Nrg2a from the tdTomato fluorescent protein. (c–e) 2D confocal images (mid-sagittal sections) of Tg(myl7:LIFEACT-GFP);nrg2a+/− hearts injected with myocardial specific nrg2a construct (myl7:nrg2a-p2a-tdTomato) at the one-cell stage. Mosaic overexpression of nrg2a in nrg2a+/− cardiomyocytes led to the formation of a multilayered myocardial wall which is outlined by a white dashed box and magnified (c–e); arrows point to nrg2a overexpressing cardiomyocytes. (f–i) Confocal images (mid-sagittal sections) of 120 hpf Tg(myl7:EGFP-Hsa.HRAS) hearts from erbb2+/− incrosses injected with the myl7:nrg2a-p2a-tdTomato construct (h–i). Magnified images of dashed boxes are shown below c–i; arrows point to nrg2a overexpressing cardiomyocytes, arrowheads point to trabeculae and asterisks indicate lack of trabeculae. At, atrium; V, ventricle; scale bars, 50 μm.
nrg2a protein-trap line, we wanted to investigate how cardiac contractility/blood flow regulates nrg2a expression in the zebrafish heart. At 78 hpf, we observed that endocardial Nrg2a-mRFP is strongly expressed in the outer curvature of wild-type ventricles, where trabeculae are present, while it is clearly weaker in other endocardial cells (Fig. 6a–c,j). The levels of Nrg2a-mRFP expression in the heart were strongly reduced in the absence of cardiac contractility in tntt2a morphants44 (Fig. 6d–f,k) and 2,3-butanedione monoxime (BDM)-treated larvae (Fig. 6g–i,l), indicating that contraction and/or blood flow is required for cardiac expression of Nrg2a-mRFP. To investigate whether there is also a role for cardiac contractility in regulating Erbb2 downstream signalling pathways, we injected Tg(myl7:EGFP-Hsa.HRAS);Tg(myl7:nrg2a-202-p2a-tdTomato) embryos with tntt2a morpholinos and imaged them by confocal microscopy at 78 hpf. Notably, we observed that myocardial overexpression of nrg2a resulted in cardiomyocyte multilayering even in the absence of contractility (Fig. 6m,n). However, overexpressing nrg2a throughout the endothelium, including the endocardium, by using a stable Tg(fli1a:nrg2a-p2a-tdTomato) line did not cause a cardiomyocyte multilayering phenotype in tntt2a morphants (Supplementary Fig. 10). By injecting low amount of myl7:nrg2a-p2a-tdTomato plasmid DNA in tntt2a morphants, we also observed that mosaic expression of nrg2a recruits both nrg2a expressing and non-expressing cardiomyocytes to form a multilayered wall suggesting that nrg2a affects the behaviour of cardiomyocytes via both autocrine and paracrine signals (Supplementary Fig. 11). Furthermore, it has been recently reported that N-cadherin relocalizes to the basal side of cardiomyocytes during trabeculation15. We observed that nrg2a overexpression in non-contractile cardiomyocytes was sufficient to drive the relocalization of Cdhn2-EGFP molecules to
Figure 6 | Contractility/blood flow is required for endocardial expression of nrg2a but not the ability of cardiomyocytes to respond to nrg2a.

(a–i) Maximum intensity z-projections (25–30 z-stacks, mid-sagittal sections) of Tg(kdrl:NLS-EGFP);nrg2a+/− hearts from non-treated (a–c), tnnt2a MO injected (d–f) and BDM treated (g–i) 78 hpf larvae; scale bars, 50 μm. (j–l) Graphs showing cell-based Nrg2a-mRFP intensity in outer curvature (yellow box) and inner curvature (green box) of hearts from non-treated (j), tnnt2a MO (k) and BDM treated (l) 78 hpf larvae; dots represent individual Nrg2a-mRFP expressing endocardial cells. Values represent means ± s.e.m.; **P < 0.01, ***P < 0.001, NS (not significant), by Student’s t-test. (m,n) 2D confocal images (mid-sagittal sections) of 78 hpf Tg(myl7:EGFP-Hsa.HRAS) (m) or Tg(myl7:EGFP-Hsa.HRAS);Tg(myl7:nrg2a-p2a-tdTomato) hearts (n) showing that myocardial overexpression of nrg2a can induce cardiomyocyte multilayering in tnnt2a morphants; asterisks and arrows indicate single-layered and multilayered ventricular walls, respectively. (o–r) Maximum intensity z-projections of TgBAC(cdh2:cdh2-EGFP) hearts from non-injected (o), injected with tnnt2a MO alone (p) or injected with tnnt2a MO and myl7:nrg2a-p2a-tdTomato plasmid (q,r) 96 hpf larvae. (r) 2D confocal image (sagittal section) of heart shown in q. Magnified images of dashed boxes are shown below (o–r); arrowheads and lozenges indicate presence and absence of Cdh2-EGFP proteins on the basal side of cardiomyocytes, respectively; At: atrium; V, ventricle; scale bars, 50 μm. (s) Schematic diagram of modulation of Nrg2a/Erbb2 signaling by cardiac contractility/blood flow.
that in zebrafish cardiac contraction is required for cardiac nrg1 expression but not for cardiac erbb2 expression. Here, we showed that contractility/blood flow is essential for cardiac expression of nrg2a. Interestingly, although nrg1 upregulation by broad expression of a Notch intracellular domain was not sufficient to rescue trabeculation defects in tnnt2a morphants, we could induce cardiomyocyte multilayering in these animals by myocardial overexpression of nrg2a. Moreover, we also showed that ectopic overexpression of nrg2a in non-contractile cardiomyocytes led to the relocalization of Cdh2-EGFP to their basal side. Overall, these data indicate that contractility/blood flow is indispensable for cardiac expression of nrg2a but not the ability of cardiomyocytes to respond to nrg2a.

We also observed that unlike myocardial specific nrg2a overexpression, endocardial specific nrg2a overexpression did not induce cardiomyocyte multilayering in tnnt2a morphants, likely due to the presence of a substantial gap between the endocardial and myocardial layers. These data further suggest that the thickness of the cardiac jelly may play an important role in determining where trabeculation starts.

In conclusion, our data indicate that trabeculae may initially form only in the ventricle in part because the cardiac jelly in the atrium does not get degraded as fast as it does in the ventricle. Atrial cardiomyocytes exhibit another kind of behaviour as they form the inner pectinate myofibres, and it will be interesting to investigate the underlying signalling pathways. We also found that biomechanical forces work upstream of Nrg2a/Erbb2 signalling to modulate trabeculation in the zebrafish heart by modulating nrg2a expression in the endocardium. It will be important to identify the proteins involved in sensing these forces as well as the transcriptional effectors regulating nrg2a expression.

Methods
Zebrafish husbandry. Embryonic and adult zebrafish were raised and maintained under standard conditions and all animal experiments were done according to German Animal Protection Laws approved by the local governmental animal protection committee.

Zebrafish transgenic and mutant lines. In this study, we used the following transgenic and mutant lines: Tg(kdr:Ha.Hsa.HRAS-mCherry)209 (ref. 39), Tg(mytl7:EGFP-Hsa.HRAS)1683 (ref. 40), Tg(mytl7:LINE-ACT-GFP)1776 (ref. 33), Tg(kdr:LS-EGFP)1776 (ref. 36), Tg(acz:tdGFP)1699 (ref. 41), Tg(acz:tdGFP)1699 (ref. 63) abbreviated as TgBAC(dhc2:tdGFP), Tg(mytl7:nrg2a202-p2a-tdTomato)8880 (ref. 71), Tg(fli1a:nrg2a202-p2a-tdTomato)1699 abbreviated as Tg(fli1a:nrg2a-p2a-tdTomato) and Tg(mytl7:ves012:tm01472) (ref. 43) abbreviated as Tg(mytl7:ves-munsn). Embryonic and adult zebrafish were raised and maintained under standard conditions and all animal experiments were done according to German Animal Protection Laws approved by the local governmental animal protection committee.

Generation of nrg2aI3 mouse mutants. nrg2aI3 mutants were generated by TALEN mutagenesis targeting exon 2. The following TALEN arms were constructed and assembled using the Golden Gate method and TALEN arm1: NG NG NG NN NN HD NI NI NN NI NI NI HD HD DD HD NI NN TALEN arm2: HD NG NG NG HD HD DD HD NG NG HD NG NN NI HD DD. An amount of 100 pg of total TALEN RNA and 50 pg of GFP RNA (used to monitor injection efficiency) were coinjected into the cell at the one-cell stage. The nrg2aI3 allele was genotyped using high-resolution melt analysis (HRMA) with an Eco Real-Time PCR System (Illuma). The following HRMA primers were used for PCR reactions: nrg1-exon2–F1 5’TGMCATTTGGCGAAGAACCG-3’, nrg1-exon2–R1 5’TCTTATGCTTTGGTGTTG-3’. Mosaic and stable overexpression of nrg2a. To generate a mosaic specific nrg2aI3 overexpressing construct, the nrg2aI3-202 coding sequence followed by p2a-tdTomato was cloned in a mini toI2 plasmid harbouring a myr7 promoter using the Cold Fusion Cloning Kit (MCI01A-1-SB). For mosaic expression of nrg2aI3, 15 pg of myr7:nrg2aI3-202-p2a-tdTomato plasmid DNA was cotransfected with 10 ng of toI2 RNA into the cell at the one-cell stage. To establish the Tg(mytl7:nrg2aI3-202-p2a-tdTomato) line, injected larvae (F0) were screened for myocardial tdTomato fluorescence and raised to adulthood.

Discussion
During trabeculation, cardiomyocytes undergo several morphogenetic changes required for the luminal growth and maintenance of the myocardial wall. Taking advantage of high-resolution live imaging in the translucent zebrafish larvae, we investigated, using loss- and gain-of-function approaches, why trabeculation takes place primarily in the ventricle and how it is controlled by cardiac contractility/blood flow.

Given the importance of Nrg1 in cardiac trabeculation in mouse, we investigated the role of Nrg1 in zebrafish and found that it is not required for trabeculation, a finding consistent with a very recent publication. Instead we found that Nrg2a is required for trabeculation in zebrafish while loss of Nrg2 does not lead to any obvious cardiac defects in mouse. Nrg signalling plays many essential roles in vertebrate development and homeostasis, and it will be interesting to investigate ligand selection across evolutionary time. We also observed that Nrg1 and Nrg2a signalling are not required for AV valve formation in zebrafish, consistent with previous observations of erbb2 mutants.

In mouse, Erbb2–Erbb3 heterodimer activity is required for AV valve formation, whereas it is weaker in other endocardial cells suggesting that they are different stability than the endogenous Nrg2a protein. Prior studies have also revealed the critical role of the cardiac jelly in endo-myocardial interactions. Investigating the dynamics of cardiac jelly degradation during early cardiac development, we observed that by the time trabeculae appear, little cardiac jelly is left in the ventricle. By ectopic myocardial overexpression of nrg2a, we found that atrial cardiomyocytes could also respond to Nrg2a/Erbb2 signalling. We speculate that atrial cardiomyocytes receive less Nrg ligands, which are expressed by endocardial cells, due to the thickness of the cardiac jelly in the atrium and consequently do not form a multilayered wall.

When we overexpressed nrg2a in cardiomyocytes using the myl7 promoter, we observed the formation of multiple continuous layers rather than the formation of trabeculae, possibly because of the high levels, even distribution and premature overexpression of nrg2a. We hypothesize that the expression of lower levels of nrg2a in single cardiomyocytes starting at the right stage would lead them to delaminate and seed trabeculae as observed in wild-type embryos, and new transgenic tools will be required to test this hypothesis. In addition, higher levels of Nrg2a signalling might drive cardiomyocyte proliferation.

Several groups have shown the importance of physical forces in cardiac morphogenesis. It has recently been reported that their basal side (Fig. 6o–r, Supplementary Movie 1) while they remained mostly localized on the lateral sides of non-nrg2a expressing cardiomyocytes, further indicating that cardiac contraction is not necessary for cardiomyocytes to respond to Nrg2a signalling. Altogether, these results support a model in which contractility/blood flow is required for endocardial expression of nrg2a but not the expression or function of Nrg2a/Erbb2 signalling effectors (Fig. 6s).
Afer identifying founders, F1 embryos positive for fluorescence in their cardiomyocyte were reared to adulthood.

**Morpholino injections.** To stop cardiac contraction, 0.5 ng of a tnt2a morpholino (5’-CATGGTTTTGCTCTAGCTGACGGCA-3’) was injected into one-cell stage embryos44.

**Chemical treatments.** To prevent contractility for 6 h (short term treatment), 72 hpf zebrafish larvae were exposed to 20 mM BDM65. To block ErbB2 signalling, dechorionated embryos were treated with 10 μM of the ErbB2 inhibitor PD 168393 (Calbiochem) from 36 to 96 hpf.

**In vivo confocal imaging and data processing.** Zebrafish embryos and larvae were mounted and anesthetized in 1.5% low-melt agarose (Sigma) containing 0.2 per cent tricaine on glass-bottom dishes. Before the gel solidified, the samples were manually oriented towards the microscope lens to enhance optical access to the heart. Images were captured with a Zeiss LSM780 or a spinning disk (CSU-X1) confocal microscope using ×40 (1.1 NA) and ×40 (1.15 NA) water immersion lenses, respectively. The confocal data were then processed with the ZEN 2012 software (black edition). Nrg2a-mRFP intensity per cell was measured with the ZEN 2012 software (blue edition).

**Whole mount in situ hybridization.** To perform whole mount in situ hybridization66, 78 hpf zebrafish larvae were fixed in 4% paraformaldehyde overnight at 4 °C and subsequently dehydrated in 100% methanol at –20 °C. Larvae were rehydrated with 1 × PBS and permeabilized by digestion with proteinase K (10 μg/ml) at room temperature for 30 min. After washing with 1 × PBST (1 × phosphate buffered saline (PBS), 0.1% Tween 20 (vol/vol)), larvae were hybridized with 200 ng of nrg2a antisense DIG-labelled RNA probe overnight at 70 °C. The hybridized probes were then detected with alkaline phosphatase-conjugated anti-Digoxigenin antibody (Roche, dilution 1:1,000) for three hours at room temperature and the signal was visualized with BM purple (Roche). The probe for nrg2a was amplified from 78 hpf heart cDNA using nrg2a forward 5′-TCTGTTGGCTCTTATGTTG-3′ and reverse 5′-GTGGCCG AGTCTGTTGCTGTG-3′ primers. The PCR fragment was subcloned into pGEM-T.

**Statistical analysis.** Data were processed with the Prism5 software. Values are presented as mean ± s.e. P values (**P**≤0.05, ***P***≤0.01, ****P****≤0.001) were calculated using Student’s t-test.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

**References**

1. Staudt, D. & Stainier, D. Uncovering the molecular and cellular mechanisms of cardiac development using the zebrafish. *Annu. Rev. Genet.* 46, 397–418 (2012).
2. Sedmera, D. & Thomas, P. S. Trabeculation in the embryonic heart. *Bioessays* 16, 607–607 (1996).
3. Sedmera, D., Pexieder, T., Vuillemin, M., Thompson, R. P. & Anderson, R. H. Developmental patterning of the myocardium. *Anat. Rec.* 258, 319–337 (2000).
4. Stankunas, K. et al. Endocardial Br1 represses ADAMTS1 to maintain the microenvironment for myocardial morphogenesis. *Dev. Cell* 14, 298–311 (2008).
5. Liu, J. et al. A dual role for ErbB2 signaling in cardiac trabeculation. *Development* 137, 3867–3875 (2010).
6. Staudt, D. W. et al. High-resolution imaging of cardiomyocyte behavior reveals two distinct steps in ventricular trabeculation. *Development* 141, 585–593 (2014).
7. Gassmann, M., Casagrande, F., Orioli, D. & Simon, H. Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390 (1995).
8. Lee, K.-F. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394–398 (1995).
9. Jenni, R., Rojas, I. & Oechslin, E. Isolated noncompaction of the myocardium. *N. Engl. J. Med.* **340**, 966–967 (1999).
10. Morris, J. K. et al. Rescue of the cardiac defect in ErbB2 mutant mouse reveals essential roles of ErbB2 in peripheral nervous system development. *Neuron* **23**, 273–283 (1999).
11. Crone, S. A. et al. ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat. Med.* **8**, 459–465 (2002).
12. Lai, D. et al. Neuregulin 1 sustains the gene regulatory network in both trabecular and nontrabecular myocardium. *Circ. Res.* **107**, 715–727 (2010).
13. Brutsaert, D. L. et al. The cardiac endothelium: functional morphology, development, and physiology. *Prog. Cardiovasc. Dis.* **39**, 239–262 (1996).
43. Jiménez-Amilburu, V. et al. In vivo visualization of cardiomyocyte apicobasal polarity reveals epithelial to mesenchymal-like transition during cardiac trabeculation. Cell Rep. 17, 2687–2699 (2016).
44. Sehnert, A. J. et al. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. Nat. Genet. 31, 106–110 (2002).
45. Cheriaux, A. V., Fukuda, R., Augustine, S. M., Maischein, H.-M. & Stainier, D. Y. N-cadherin relocalization during cardiac trabeculation. Proc. Natl Acad. Sci. USA 113, 7569–7574 (2016).
46. Kramer, R. et al. Neuregulins with an Ig-like domain are essential for mouse myocardial and neuronal development. Proc. Natl Acad. Sci. USA 93, 4833–4838 (1996).
47. Wadugu, B. & Kühn, B. The role of neuregulin/ErbB2/ErbB4 signaling in the heart with special focus on effects on cardiomyocyte proliferation. Am. J. Physiol. Heart Circ. Physiol. 302, H2139–H2147 (2012).
48. Samsa, L. A., Ito, C. E., Brown, D. R., Qian, L. & Liu, J. IgG-containing isoforms of Neuregulin-1 are dispensable for cardiac trabeculation in zebrafish. PLoS ONE 11, e0166734 (2016).
49. Britto, J. M. et al. Generation and characterization of neuregulin-2-deficient mice. Mol. Cell. Biol. 24, 8221–8226 (2004).
50. Horie, T. et al. Acute doxorubicin cardiotoxicity is associated with miR-146a-induced inhibition of the neuregulin-ErbB pathway. Cardiovasc. Res. 87, 656–664 (2010).
51. Cantó, C. et al. Neuregulins increase mitochondrial oxidative capacity and insulin sensitivity in skeletal muscle cells. Diabetes 56, 2185–2193 (2007).
52. Camenisch, T. D., Schroeder, J. A., Bradley, J., Klewer, S. E. & McDonald, J. A. Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. Nat. Med. 8, 850–855 (2002).
53. Armstrong, E. J. & Bischoff, J. Heart valve development endothelial cell signaling and differentiation. Circ. Res. 95, 439–470 (2004).
54. Gitter, A. D., Lu, M. M., Jiang, Y. Q., Epstein, J. A. & Gruber, P. J. Molecular markers of cardiac endocardial cushion development. Dev. Dyn. 228, 643–650 (2003).
55. Carraway, III K. L. et al. Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. Nature 387, 512–516 (1997).
56. Nakamura, A. & Manasek, F. J. An experimental study of the relation of cardiac jelly to the shape of the early chick embryonic heart. Development 65, 235–256 (1981).
57. Mellman, K., Huiskens, J., Dinsmore, C., Hoppie, C. & Stainier, D. Y. Fibrillin-2b regulates endocardial morphogenesis in zebrafish. Dev. Biol. 372, 111–119 (2012).
58. Hove, J. B. et al. Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. Nature 421, 172–177 (2003).
59. Dietrich, A.-C., Lombardo, V. A., Veerkamp, J., Priller, F. & Abdolah-Seyfried, S. Blood flow and Bmp signaling control endocardial chamber morphogenesis. Dev. Cell 30, 367–377 (2014).
60. Foglia, M. J., Cao, J., Tornini, V. A. & Poss, K. D. Multicolor mapping of the cardiomyocyte proliferation dynamics that construct the atrium. Development 143, 1688–1696 (2016).
61. Singleman, C. & Holtzman, N. G. Analysis of postembryonic heart development and maturation in the zebrafish, Danio rerio. Dev. Dyn. 241, 1993–2004 (2012).
62. Westerfield, M. in The Zebrafish Book (University of Oregon Press, 2000).
63. Revenu, C. et al. Quantitative cell polarity imaging defines leader-to-follower transitions during collective migration and the key role of microtubule-dependent adherens junction formation. Development 141, 1282–1291 (2014).
64. Lyons, D. A. et al. erbB3 and erbB2 are essential for schwann cell migration and myelination in zebrafish. Curr. Biol. 15, 513–524 (2005).
65. Higuchi, H. & Takeomi, S. Butanedione monooxide suppresses contraction and ATPase activity of rabbit skeletal muscle. J. Biochem. 105, 638–643 (1989).
66. Thiese, C. & Thiese, B. High-resolution in situ hybridization to whole-mount zebrafish embryos. Nat. Protoc. 3, 59–69 (2008).