Mechanosensitive Notch-Dll4 and Klf2-Wnt9 signaling pathways intersect in guiding valvulogenesis in zebrafish

Graphical abstract

Highlights
- Atrioventricular valvulogenesis in zebrafish is regulated by Notch and Klf2 pathways
- Notch-mediated lateral inhibition singles out Delta-like-4-positive endocardial cells
- These cells ingress into the cardiac jelly in response to Erk5-Klf2-Wnt9a signaling
- Frizzled9b makes presumptive ingressing endocardial cells competent to respond to Wnt9a

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In brief
Paolini et al. show that lateral inhibition between endocardial cells, mediated by Notch, singles out Delta-like-4-positive endocardial cells. These Delta-like-4-positive cells ingress into the cardiac jelly during zebrafish cardiac valvulogenesis in response to Wnt9a, which is produced in parallel through an Erk5-Klf2-Wnt9a signaling cascade activated by blood flow.

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Mechanosensitive Notch-Dll4 and Klf2-Wnt9 signaling pathways intersect in guiding valvulogenesis in zebrafish

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SUMMARY

In the zebrafish embryo, the onset of blood flow generates fluid shear stress on endocardial cells, which are specialized endothelial cells that line the interior of the heart. High levels of fluid shear stress activate both Notch and Klf2 signaling, which play crucial roles in atrioventricular valvulogenesis. However, it remains unclear why only individual endocardial cells ingress into the cardiac jelly and initiate valvulogenesis. Here, we show that lateral inhibition between endocardial cells, mediated by Notch, singles out Delta-like-4-positive endocardial cells. These cells ingress into the cardiac jelly, where they form an abluminal cell population. Delta-like-4-positive cells ingress in response to Wnt9a, which is produced in parallel through an Erk5-Klf2-Wnt9a signaling cascade also activated by blood flow. Hence, mechanical stimulation activates parallel mechanosensitive signaling pathways that produce binary effects by driving endocardial cells toward either luminal or abluminal fates. Ultimately, these cell fate decisions sculpt cardiac valve leaflets.

RESULTS AND DISCUSSION

The Notch-Delta signaling pathway causes lateral inhibition between cells organized in tissue layers and biases equivalent cells toward different fates. This occurs when transmembrane ligands of the Delta family interact with the extracellular domain of Notch on opposing cells. Recent studies have shown that mechanical tension can sensitize this signaling by opening a site on Notch that makes it accessible for further processing and cleavage (Gordon et al., 2015; Hunter et al., 2019). This subsequently releases the Notch intracellular domain (NICD), which translocates into the nucleus and functions as a transcriptional regulator. Its target genes include the Notch receptor itself. Hence, the Notch-active cell expresses even more Notch, while the Delta-expressing cell produces less Notch and eventually takes on a different fate.

In the embryonic zebrafish heart, Notch activity is highest in prevalvular endocardial cells at the atrioventricular canal, which are exposed to strong fluid shear stress (Samsa et al., 2015; Vermot et al., 2009; Walsh and Stainier, 2001). This shows that mechanical forces are involved in activating Notch signaling. Yet, whether Notch-Delta-mediated lateral inhibition plays a role in this process has been unclear. Also, we do not have a good understanding how this pathway is integrated with the mechanosensitive Klf2 signaling pathway, which is also regulated by blood flow during cardiac valve development (Donat et al., 2018; Goddard et al., 2017; Steed et al., 2016; Vermot et al., 2009). Recent functional studies have demonstrated that Notch activity and Klf2 signaling within the atrioventricular endocardium are not contingent on each other (Fontana et al., 2020). It remains unclear how Notch signaling and the mechanosensitive Klf2 signaling pathway are integrated. In mice, hemodynamic forces activate endocardial Klf2 expression, which causes the secretion of Wnt9b and stimulation of canonical Wnt signaling in neighboring mesenchymal cells that go on to sculpt deeper portions of developing valve leaflets (Goddard et al., 2017). There is reason to believe this mechanism may also be found in zebrafish, because endocardial cells within deeper internal portions of valve leaflets exhibit Wnt activity (Pestel et al., 2016).

Valvulogenesis at the atrioventricular canal (AVC) (Figures 1A and 1A’) begins when individual endocardial cells exhibit filopodial protrusions and then ingress into the cardiac jelly at ~54 h post-fertilization (hpf) (Gunawan et al., 2019, 2020) (Figure 1A’). This initiates a series of morphogenetic events, which transform a monolayer of AVC endocardial cells into a functional multilayered valve leaflet (Figure 1B) (Gunawan et al., 2019, 2020). The process by which ingressing cells become selected within the prevalvular endocardium has been unclear. Zebrafish atrioventricular valvulogenesis requires Notch (Timmerman et al., 2004; Torregrosa-Carrion et al., 2019), which hinted that Notch-Delta-mediated lateral inhibition might contribute to this process. This system plays a crucial role in other developmental events. During zebrafish angiogenesis, for example, endothelial cells expressing the Delta ligand Delta-like 4 (Dll4) are singled out as tip cells and initiate sprouting morphogenesis, while those...
Figure 1. Dll4 and TCF reporters are co-expressed in ingressing endocardial cells

(A–B) Model depicting the zebrafish embryonic heart.

(A and A') At 48 hpf, the superior AVC endocardium (black square in A) is monolayered and blood flow is bi-directional (black arrow) (A').

(A') At 54 hpf, single cells ingress into the cardiac jelly.

(B) At 96 hpf, valve leaflets have formed, and blood flow is uni-directional (black arrow).

(C–E) Single confocal z section plane images of the superior AVC endocardium. (C and D) Single cells express Dll4 and TCF reporter at 48 and 55 hpf (asterisks).

(E and E) Similarly, only single cells express the TCF reporter at 54 hpf (white arrowhead). Those cells are always negative for Notch activity reporter expression.
cells with Notch activity acquire a stalk cell fate (Hellström et al., 2007; Leslie et al., 2007).

To assessDll4 expression in the prevalvular endocardium, we first compared the expression of a Dll4 reporter transgene TgDll4:Gal4tp120b22; Tg5xUAS:EGFPKANmuasg^tp12a (hereafter referred to as Dll4 reporter) to that of the Notch activity reporter Tg(TP1:VenusPEST)144D (Ninov et al., 2012) and canonical Wnt reporter Tg7x(TCF-βgal); Siam:nismCherry170 (hereafter referred to as TCF reporter) (Moro et al., 2012). The expression of the Dll4 reporter reflects the endogenous dll4 expression in the embryonic heart as previously described (Wang et al., 2013) (Figures S1A and S1A′). Imaging of the atrioventricular endocardium at single-cell resolution revealed a dispersed pattern of Dll4 activity in prevalvular endocardial cells at 48 hpf. Individual prevalvular cells expressing the TCF reporter always co-expressed the Dll4 reporter (100%, n = 10 embryos analyzed) (Figures 1C, 1B, and S1B; Video S1) but never the Notch activity reporter (100%, n = 36 embryos analyzed). By 54 hpf, those cells that ingressed into the cardiac jelly were Dll4 and TCF positive (100%, n = 36 embryos analyzed). By 54 hpf, those cells that ingressed

(F) Light-sheet microscopy time-lapse images of a TCF-positive ingressing endocardial cell (white arrows) at the superior AVC. (G–J) Single confocal z section plane images of the superior AVC endocardium at 55 hpf. (G and H) Upon Notch inhibition, the superior AVC endocardium remains monolayered and has a decreased expression of the Dll4 reporter. (I and J) Dll4 expression and singling out of AVC endocardial cells is prevented in absence of blood flow (tnnt2a MO).

(K–N) Quantification of klf2a:YFP levels in luminal versus TCF-positive cells at 48 hpf (K–L) and 55 hpf (M–N). The corrected total cell fluorescence (CTCF) of TCF-positive ingressing cells (white arrowheads) is compared with the CTCF of neighboring TCF-negative cells (yellow arrowheads) (48 hpf, n = 16 embryos; 55 hpf, n = 12 embryos). Single values are shown in a boxplot. Lines inside the box represent mean values. The lower and upper whiskers indicate minimum and maximum values, respectively (⁎p < 0.01 by paired Student’s t test).

(O) Model depicting the superior AVC endocardium at stages when singled-out cells ingress into the cardiac jelly. Scale bars, 5 μm.

Our finding that ingressing endocardial cells express both Dll4 and TCF reporters pointed at some interaction between the mechanosensitive Notch-Dll4 and Klf2a-Wnt9 signaling pathways. This posed the question of how these signaling pathways are spatially distributed and how they might interact in the development of AVC endocardial cells. Levels of klf2a expression are a sensitive measure of fluid shear stress due to blood flow on endocardial cells. As they ingress into the cardiac jelly, endocardial cells express only low levels of klf2a. In contrast, luminal endocardial cells express klf2a at graded levels that correlate with their exposure to blood flow (Steed et al., 2016). We used the Tg(klf2a:YFPmut07) reporter transgene (Sugden et al., 2017) to quantify klf2a levels in endocardial cells at single-cell resolution. This revealed TCF-reporter-positive cells consistently had lower klf2a expression levels than their direct neighbors at 48 hpf (Figures 1K, 1K′, and 1L) and 54 hpf (Figures 1M, 1M′, and 1N). In summary, these results suggested that Dll4-/TCF-positive cells are exposed to lower blood-flow-dependent shear stress prior to their ingress into the cardiac jelly. Apparently, Notch-Dll4 lateral inhibition and paracrine signaling by the Klf2a-Wnt9 pathway intersect in a few Dll4-/TCF-positive cells within the prevalvular endocardium (Figure 1O).

Next, we performed functional studies to clarify how the Notch-Dll4 and the Klf2a-Wnt9 pathways might interact. First, we tested whether canonical Wnt signaling was required for the ingress of atrioventricular endocardial cells. For this, we overexpressed a dominant-negative TCF (dnTCF) transgene to block Wnt target gene expression (Kübler et al., 2018). We triggered a heat-shock-induced overexpression of EGFP-dnTCF specifically in endothelial tissues by using an endothelial-specific Cre recombinase (Bertrand et al., 2010) to remove a stop cassette from the Tg(hsp70:loxP-S-STOP-loxP-EGFP-dntcftmu107) reporter transgene. When the heat shock to overexpress dnTCF was induced at 26 hpf, this caused a depletion of TCF reporter expression in the atrioventricular endocardium at 54 hpf in 40% of treated embryos (n = 20 embryos analyzed). Under this condition, endocardial cells failed to ingress into the cardiac jelly and the atrioventricular endocardium remained monolayered (Figures 2A, 2B, S2A, S2B, and S2B′).

This experiment demonstrated that endocardial Wnt signaling plays an important role in atrioventricular valvulogenesis, but the mode by which Klf2a-Wnt9 expression is activated in response to blood flow was unknown, as was which Wnt9 ligands are involved in paracrine signaling within the AVC endocardium of
zebrafish. We wondered whether components of the Klf2-Wnt9 signaling pathway might be involved in activating canonical Wnt signaling in this tissue. First, we tested whether the process of Wnt pathway activation was dependent on Klf2. At 54 hpf, in double mutants of klf2ash317 (Novodvorsky et al., 2015) and klf2bpbb42 (Fontana et al., 2020) (hereafter referred to as klf2 mutants), there was a complete loss of expression from the TCF reporter and ingression of atrioventricular endocardial cells into the cardiac jelly was abolished (Figures 2C, 2D, S2C, S2C, S2D, and S2D) (100%, n = 13 embryos analyzed). Instead, the endocardium remained a luminal monolayered tissue. This demonstrated that canonical Wnt activity within the atrioventricular endocardium requires Klf2 signaling.

Within endothelial cells, levels of klf2 are controlled by the Mekk3 downstream effector Erk5 (Zhou et al., 2015). Since Erk5 is activated by laminar flow within endothelial cells (Kim et al., 2012), we wondered whether it may also be involved in the activation of Klf2 during zebrafish valvulogenesis. We applied the Erk5 inhibitor XMD8-92 (Otten et al., 2018) from 30 hpf and found that it significantly lowered the expression of the Tg(klf2a:YFP)mu107 reporter transgene within the superior side of the atrioventricular endocardium at 48 hpf and 54 hpf (Figures 2E–2J). This had the expected result from a loss of Klf2 activity: the expression of the TCF reporter was almost entirely abolished in the atrioventricular endocardium (Figures 2E, 2F, 2H, 2I, and S2E–S2L) (73%, n = 44 embryos analyzed). Notably, no sprouting cells were detected in the atrioventricular endocardium at 54 hpf and the tissue remained monolayered (Figures 2I, S2H, and S2L) (83%, n = 23 embryos analyzed). Erk5 inhibition did not, however, affect Notch activity (100%, n = 16 embryos analyzed) (Figures S2E–S2H) nor the expression of the Dll4 reporter (100%, n = 9 embryos analyzed) (Figures S2I–S2L), confirming that these two pathways are regulated separately. This demonstrated that zebrafish valvulogenesis involves Erk5-Klf2 signaling.

Figure 2. Erk5-Klf2a-Wnt signaling within AVC endocardium is essential for valvulogenesis
(A–D) Single confocal z section plane images of the superior AVC endocardium at 54 hpf. (A and B) The endothelial-specific knockdown of canonical Wnt signaling prevents AVC endocardial cells from ingressing into the cardiac jelly. (A) Presence of a TCF-positive ingressed cell when TCF is not blocked (asterisk). Note the presence of TCF-positive cells in the myocardium of treated hearts (B), which confirms the tissue-specificity of this treatment. (C and D) TCF-positive endocardial cells (asterisks in C) are absent in in klf2ash317;klf2bpbb42 double mutants. (E–J) Single confocal z section plane images of the superior AVC endocardium at 54 hpf. (E and F) Quantification of Klf2a:YFP net fluorescent intensity in the superior AVC endocardium of DMSO- and XMD8-92-treated embryos at 48 hpf (E, F, and G) (DMSO-treated, n = 8 embryos; XMD8-92-treated, n = 9 embryos) and 54 hpf (H–J) (DMSO-treated, n = 10 embryos; XMD8-92-treated, n = 8 embryos). Single values are shown in a boxplot. Lines inside the box represent mean values. The lower and the upper whiskers indicate minimum and maximum values, respectively (*p < 0.05 and ****p < 0.0001 by unpaired Student’s t test). (E, F, H, I, and J) The presence of single TCF-positive cells (asterisks in E and H) is prevented by treating embryos with XMD8-92 (F’ and I’). Scale bars, 5 μm.
In search of further potential Wnt ligands, we performed whole-mount in situ hybridizations in zebrafish, and found that the wnt9a paralog is expressed in the AVC at 48 hpf (Figure 3A) in a pattern similar to wnt9b (Goddard et al., 2017). To test whether Klif2a controls the expression of wnt9a, we overexpressed Klif2a in zebrafish endothelium by crossing Tg(fli1a:GAL4FF)absc (Herwig et al., 2011) with Tg(UAS:klif2a)97 (Renz et al., 2015) and analyzed wnt9a expression by whole-mount in situ hybridization. This revealed that the endothelial-specific overexpression of Klif2a causes wnt9a expression throughout both cardiac chambers (100%, n = 20 embryos analyzed) (Figure 3B).

Klif2 might activate expression of Wnt9a and Wnt9b that, upon secretion, could trigger canonical Wnt signaling within neighboring Dll4-positive endocardial cells. However, the combination of the two loss of function mutants wnt9a<sup>sd49</sup> (Grainger et al., 2016) and wnt9b<sup>sa20083</sup> resulted in an early cardiac phenotype, which prevented the analysis of valve leaflet morphogenesis (data not shown). In comparison, the loss of function allele wnt9a<sup>sd49</sup> alone did not result in abnormal atrioventricular valvulogenesis by 72 hpf and did not impact Wnt pathway signaling (Figures 3C and 3D). This might be due to genetic compensation (Rossi et al., 2015). Instead, the antisense oligonucleotide morpholino (MO)-mediated knockdown of wnt9a (Figure S3) (100%, n = 9 embryos analyzed). The effects of MO-mediated knockdown of wnt9a were suppressed upon injection into wnt9a<sup>sd49</sup> mutants (Figure 3F) (90%, n = 10 embryos analyzed). This provides further strong evidence for the specificity of this MO during early phases of atrioventricular valvulogenesis and the activation of genetic compensation in the wnt9a<sup>sd49</sup> mutant background. The MO-mediated knockdown of wnt9a also prevented the ingression of atrioventricular endocardial cells at 54 hpf (55%, n = 22 embryos analyzed) and resulted in the permanent arrest of valve leaflet formation (Figures 3G and 3H). Contrarily, the MO-mediated knockdown of wnt9b was not as efficient (data not shown). This all demonstrated an essential role for Wnt9a during endocardial ingression into the cardiac jelly (Figures 3I and 3J).

We knew that when inhibition of the Erk5-Klif2 pathway impaired Wnt canonical signaling, it would not affect Notch activity or the expression of the Dll4 reporter (Figure S2). Nevertheless, could canonical Wnt signaling be dependent on Notch activity? To explore this possibility, we used the γ-secretase inhibitor RO4929097 to inhibit Notch-Dll4 signaling and monitored the expression of the TCF reporter. Treatment with the inhibitor from 30 hpf led to a loss of TCF reporter expression at 54 hpf in superior atrioventricular endocardial cells and abolished endocardial cell ingression into the cardiac jelly (Figures 4A, 4B, and 4C).
Figure 4. Notch-Dll4 signaling singles out cells that are competent to respond to Wnt9a

(A–D) Single confocal z section plane images of the superior atrioventricular endocardium at 54 hpf (A and B) and 55 hpf (C and D). (B) Treating embryos with the Notch inhibitor RO4929097 prevents TCF expression and ingression of single endocardial cells (asterisk in A). (C and D) Unlike in wild type (C), overexpression of the Notch intracellular domain within endocardium (nfatc > NICD) prevents TCF expression and ingression of endocardial cells (D) (see asterisks in C).

(E–G) Quantification of chloride(YFP) net fluorescence intensity in the superior AVC endocardium of DMSO- versus RO4929097-treated embryos at 54 hpf (DMSO-treated, n = 10 embryos; RO4929097-treated, n = 10 embryos). Single values are shown in a boxplot. Lines inside the box represent mean values. Lower and upper whiskers indicate minimum and maximum values, respectively (ns = not significant by unpaired Student’s t test).

(H) Whole-mount in situ hybridization of fz9b cardiac expression at 54 hpf. fz9b expression in the superior AVC endocardium is highlighted by a black arrow.

(I) Quantitative real-time PCR quantifications of kdrl and fz9b mRNAs in npas4l morphants when normalized to wild-type hearts. Minimum to maximum values are shown (n = 3 experiments; *p < 0.05 and **p < 0.001 by ratio paired Student’s t test).

(J and K) Single confocal z section plane images of the superior AVC endocardium at 55 hpf. (J) The expression of endocardial cells is marked with asterisks. (K) Endocardial TCF reporter expression is lost upon MO-mediated knockdown of fz9b, and ingression of endocardial cells is prevented.

(L–Q) Single confocal z section plane images of the superior AVC endocardium at 72 hpf. Shown are representative cell clones in Tg(fli1a:GAL4FF)ubs3 embryos injected with either UAS:H2B-GFP (L) and treated with RO4929097 (M) or injected with UAS:Dll4-H2B-GFP DNA constructs (N) and treated with RO4929097 at 72 hpf (O). Cell clones overexpressing DNA constructs are marked with red asterisks.

(P) Quantifications of ratio of clones that ingressed into the abluminal side with respect to the total number of clones integrated in the superior AVC endocardium (UAS:H2B-GFP-injected, n = 27 embryos; UAS:Dll4-H2B-GFP-injected, n = 22 embryos; UAS:Dll4-H2B-GFP injected and RO4929097-treated, n = 16 embryos). Single values are shown in a boxplot. Lines inside the box represent mean values. Lower and upper whiskers indicate minimum and maximum values, respectively. (**p < 0.01 by unpaired Student’s t test).

(Q) Model of blood-flow-dependent molecular mechanisms that result in singling out of atrioventricular endocardial cells at the beginning of valve morphogenesis. Scale bars, 5 μm.
intracardially (Scheer et al., 2002) Notch activator transgene to over-express the NICD within endocardium (Figures 4C, 4D, and S4C–S4D) (82%, n = 17 embryos analyzed). These results showed that Notch-Dll4 signaling is essential for canonical Wnt signaling within endocardial cells at the AVC. This effect could be due to changes in Klf2a signaling. To test such a possibility, we measured the expression levels of Tg(klf2a:YFP)mut072 in the superior AVC endocardium of RO4929097-treated embryos at 54 hpf. However, a lack of Notch activity did not alter klf2a expression levels in the superior AVC endocardium (Figures 4E–4G), which suggested that both pathways are regulated independently.

These findings suggested that, while Klf2a regulates the expression of the ligand Wnt9a, Dll4 controls the competence to respond to this paracrine signal by biasing a different cell fate that is characterized by the expression of a Wnt receptor. For instance, this could be some Frizzled receptor such as Fzd9b, which has been suggested as a specific Wnt9a receptor in the zebrafish vascular endothelium (Graninger et al., 2019) and the sinoatrial region of the zebrafish heart (Burkhard and Bakers, 2018). Indeed, fzd9b was expressed in the 54-hpf AVC endocardium, as revealed by whole-mount in situ hybridization (Figure 4H). We also quantified fzd9b mRNA levels by quantitative real-time PCR (qPCR) analysis in hearts lacking the endocardium due to the MO-mediated knockdown of npas4l (Reischauer et al., 2016; Stainier et al., 1995). This revealed a strong decrease of fzd9b mRNA levels by qPCR (fold change, 0.43 ± 0.018 SEM; p = 0.0003; n = 4 replicates). This suggested the involvement of this Wnt receptor in valvulogenesis, we performed a MO-mediated knockdown of fzd9b. This prevented the activation of Wnt canonical signaling within the superior AVC, which remained monolayered (Figures 4J, 4K, S4E, and S4F) (54%, n = 28 embryos analyzed). Next, we assayed whether the MO-mediated knockdown of Dll4 would impact fzd9b expression. Indeed, upon this knockdown, we detected a significant decrease of fzd9b mRNA levels by qPCR (fold change, 0.43 ± 0.018 SEM; p = 0.0003; n = 4 replicates). This suggested the expression of fzd9b was affected by Dll4. These same embryos also lacked expression of TCF and Notch activity reporters within the AVC endocardium (Figures S4G and S4H) (57%, n = 14 embryos analyzed), which was organized as a monolayer (Figure S4H) (100%, n = 14 embryos analyzed).

These findings suggested that Dll4 provides atrioventricular prevalvular cells with the competence to respond to Wnt9a. To assess whether the activity of Dll4 was sufficient to trigger ingestion of atrioventricular endocardial cells into abluminal positions, we injected a UAS:Dll4–H2B–GFP DNA construct into Tg(fli1a:GAL4FF)ubss embryos. Indeed, endocardial cells overexpressing Dll4 were more likely to contribute to the abluminal side of the valve leaflets at 54 hpf (Figures S4I–S4K) and 72 hpf (Figures 4L–4N and 4P) compared to endocardial cells overexpressing an H2B–GFP control construct. Notably, most ingressing clones were also expressing the TCF reporter (Figures 4N and S4J). However, this experiment did not address whether Notch activity was involved in the ingestion of Dll4-overexpressing endocardial cells. We tested this possibility by pharmacological inhibition of Notch, which completely abolished endocardial sprouting induced by Dll4 overexpression (100%, n = 16 embryos analyzed) (Figures 4M and 4O). This suggested, some Notch activity is required in Dll4-overexpressing cells. However, an overactivation of the NICD within endocardium, while causing a broader expression domain of dll4 as assessed by whole-mount in situ hybridization (Figures S4L and S4M), did not cause increased TCF reporter expression or endocardial sprouting (Figure 4D). These results suggested that low levels of Notch activity are required within Dll4-positive endocardial cells. This enables these cells to express Fzd9b receptor and ingress into the cardiac jelly in response to paracrine Wnt9a signaling. Our findings suggest that Wnt canonical signaling within the AVC endocardium is controlled by Notch-Dll4 activity and, in parallel, by Erk5-Klf2-Wnt9a paracrine signaling. These signaling events cause Dll4-positive prevalvular cells to acquire a different fate.

A key process in early zebrafish atrioventricular valvulogenesis comes when cells of the endocardium ingress into the cardiac jelly. Only few cells exhibit this behavior, and it has been unclear how they are singled out. Our work suggests that this is the result of mechanical forces, which arise when blood flow triggers a lateral inhibition mediated by Notch-Dll4 signaling. Once they have been selected, Dll4-positive cells acquire a competence to respond to paracrine Klf2-Wnt9a signaling, which triggers their ingressation into the cardiac jelly. We found that this process requires some Notch activity, which is similar to valvulogenesis in mice where NICD activation induces an endothelial-to-mesenchymal transition (endoMT) via direct upregulation of endoMT genes (Luna-Zurita et al., 2010; Luxán et al., 2016). This is required for endocardial cell delamination and cushion formation (Person et al., 2005). It will be an important question for future studies to characterize the precise role of Notch in ingressing endocardial cells in zebrafish and to test whether this involves an endoMT program as well.

Work in mice by Goddard and colleagues (Goddard et al., 2017) showed that blood flow triggers Klf2 activity and Wnt9b secretion and that mesenchymal cells are positive for TCF signaling. Our work revealed that TCF signaling is active already prior to cell ingestion and that endocardial cell invasion into the extracellular matrix (ECM) requires Wnt signaling by the ligand-receptor pair Wnt9a–Fzd9b. Dissecting molecular pathway signaling at the single-cell level has helped to clarify how two biomechanically regulated pathways, Notch and Erk5-Klf2-Wnt9a, intersect to bias cells toward luminal versus abluminal binary cell fate decisions. It reveals how parallel and complementary mechanosensitive signaling pathways cooperate during the sculpting of valve leaflets. While Notch-Delta-mediated lateral inhibition has generally been considered a stochastic process, this provides evidence that it can be biased by mechanical tension in a way that can result in highly stereotypical and invariant patterns.

**STAR METHODS**

**Detailed methods are provided in the online version of this paper and include the following:**

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2021.109782.

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AUTHOR CONTRIBUTIONS

A.P. and S.A.-S. contributed to the conception and design of the experiments. F.F., V.-C.P., and C.J.R. contributed data. A.P., F.F., V.-C.P., and C.J.R. collected the data. A.P. and S.A.-S. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-Zn-8/Alcam | Developmental Studies Hybridoma Bank | Cat# zn-8; RRID: AB_531904 |
| Alexa Fluor 633 Goat anti-mouse IgG (H+L) | Thermo Fisher Scientific | Cat# A-21052; RRID: AB_2353719 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Agarose, Low Melting Point | Lonza | 50081 |
| 1-phenyl-2-thiourea | Sigma-Aldrich | 22283438 |
| Tricaine (3-amino benzoic acid methylester) | Sigma-Aldrich | A-5040 |
| Tween 20 | Serva | 39796 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D2650 |
| Paraformaldehyde (PFA) | Sigma-Aldrich | P6148 |
| RO4929097 | Selleckchem | S1575 |
| XMD8-92 | Selleckchem | S7525 |
| DAPI | Sigma-Aldrich | D954 |
| Alexa Fluor 647-Phalloidin | Thermo Fisher Scientific | A22287 |
| SlowFade Gold | Thermo Fisher Scientific | S36936 |
| Normal goat serum | Sigma-Aldrich | NS02L |
| TRIZol | Thermo Fisher Scientific | 15596026 |
| Triton X-100 | Serva | 37240 |
| **Critical commercial assays** | | |
| Quick RNA micro-prep | Zymo Research | R1050 |
| RevertAid H Minus First Strand cDNA Synthesis kit | Thermo Fisher Scientific | K1631 |
| KAPA Sybr Fast qPCR kit | Roche | 07959397001 |
| In-Fusion Cloning | TakaraBio | 638948 |
| DIG RNA Labeling kit | Roche | 11 175 025 910 |
| **Experimental models: Organisms/strains** | | |
| klf2as317 | Novodvorsky et al., 2015 | ZFIN: sh317 |
| klf2bpb42 | Fontana et al., 2020 | ZFIN: pbb42 |
| wnt5a2989 | Grainger et al., 2016 | ZFIN: sd49 |
| Tg(Dll4:G4al4)3a965 | This study | ZFIN: pbb65Tg |
| Tg(SxUAS:EGFP/nkaasgfp1a) | Asakawa et al., 2008 | ZFIN: nkauasgfp1a |
| Tg(7xTCF-Xla.Siam:nls-mCherry)2065 | Moro et al., 2012 | ZFIN: ia5 |
| Tg(EVP.Tp1-Mmu.Hbb:Venus-Mmu.Odc1)13840 | Ninov et al., 2012 | ZFIN: s940 |
| Tg(kdrl:EGFP)5643 | Jin et al., 2005 | ZFIN: s843 |
| Tg(kdrl:YFP)5u107 | Sugden et al., 2017 | ZFIN: mu107 |
| Tg(kdrl:Crel)6898 | Bertrand et al., 2010 | ZFIN: s898 |
| Tg(hsp70:loxP-STOP-loxP-EGFP-dntcf)-mu200 | Hübner et al., 2018 | ZFIN: mu200 |
| Tg(fli1a:Gal4FF)[ubs3] | Hervig et al., 2011 | ZFIN: ubs3 |
| Tg(UAS-klf2a)5g1 | Renz et al., 2015 | ZFIN: ig1 |
| Tg(BAC(nfatc1:GAL4)mu286 | Pestel et al., 2016 | ZFIN: mu286 |
| Tg(SxUAS-E1b:6xMYC-notch1a intra)kca3 | Scheer et al., 2002 | ZFIN: kca3 |
| **Oligonucleotides** | | |
| For primers sequences, see Table S1 | This study | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents may be directed to and will be fulfilled by the lead contact, Prof. Dr. Salim Seyfried (salim.seyfried@uni-potsdam.de).

Materials availability
Newly generated items are available from the lead contact without restrictions.

Data and code availability
All the data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish
Handling of zebrafish was done in compliance with German and Brandenburg state law, carefully monitored by the local authority for animal protection (LUGV, Brandenburg, Germany; Animal protocol #2347-18-2015). The following strains of zebrafish were maintained under standard conditions as previously described (Westerfield et al., 1997): klf2a<sup>sh317</sup> (Novodvorsky et al., 2015), klf2b<sup>pbb-42</sup> (Fontana et al., 2020), wnt9a<sup>sad49</sup> (Grainger et al., 2016), Tg(Dll4:Gal4)<sup>pbb-65</sup> (this study), Tg(5xUAS:EGFP)<sup>nkuasgfp1a</sup> (Asakawa et al., 2008), Tg(Dll4:Cre)<sup>s898</sup> (Bertrand et al., 2010), Tg(hsp70:loxP-STOP-loxP-EGFP-dntctf)<sup>mu206</sup> (Hübner et al., 2018), Tg(5xUAS-E1b:6xMYC-notch1a intra)<sup>o2a3</sup> (Scheer et al., 2002). The developmental stage of the embryos used is indicated for each experiment in the results and figure legends.

METHOD DETAILS

Antisense oligonucleotide Morpholino injections
The following morpholinos were injected into the yolk at the one-cell stage in 1 nL total volume: tntt2a (5'-CATTTTGTCTTGATCA-3') (1 ng/embryo) (Sehnert et al., 2002), wnt9a (5'-AAGGTGCTTGCTGTCTGGAAGTGA-3') (3 ng/embryo), cloche/npas4f (5'-GAGTCTCCGAGCTCTCACAAC-3') (1 ng/embryo) (Reischauer et al., 2016), fzd9b (5'-GATGAGTGGTCTGCTGCACTGGA-3') (2 ng/embryo) (Grainger et al., 2019), dll4 (5'-GAGTAAGTATTGGACTTC-3') (6 ng/embryo) (Hogan et al., 2009). Control embryos (WT) were always injected with 1 nL of water.

Generation and injection of UAS:Dll4-H2B-GFP DNA construct
Plasmid UAS:Dll4-p2A-H2B-EGFP was generated by In-Fusion cloning (TakaraBio), from a linearized plasmid (UAS:p2A-H2B-EGFP) by PCR with AP008 and AP004 primers (Table S1) and the Delta-like 4 coding sequence (ENSEDRG00000070425) with AP003 and AP002 primers (Table S1). To create UAS:H2B-EGFP control plasmid, UAS:Dll4-p2A-H2B-EGFP was linearized by PCR with AP008 and CP196 primers (Table S1) and re-circularized by In-Fusion cloning. 15pg of plasmid was injected into 1-cell stage embryos with 15pg of Tol2 transposase mRNA.
Characterization of Tg(Dll4:Gal4)pbb65

This transgene, of unknown origin, was a donation from the laboratory of Holger Gerhardt (MDC, Berlin). To characterize this transgene, fish were genotyped via PCR, using a forward primer blasting ~500 bp upstream of the dll4 (ENSDARG000000970425) start codon, and a reverse primer blasting the final part of the Gal4 DNA binding domain coding region. This PCR gave a fragment of ~1000 bp. Sequencing of the isolated fragment confirms the presence of the Gal4 DNA binding domain coding region downstream of the dll4 putative promoter region (Data S1) (Primers are listed in Table S1). This sequence was submitted to ZFIN: https://zfin.org/ZDB-ALT-201014-5

Heat shock and chemical treatments

To knock-down canonical Wnt signaling in vascular endothelium, transgenic fish carrying Tg(kdrf:Cre) were crossed to Tg(hsp70I:loxP-STOP-loxP-EGFP-dntcf); Tg(Tp1:VenuesPESCT); Tg(7xTCF-Xla.Siam:nlsc-mCherry) transgenic fish and the resulting embryos were heat-shocked at 30 hpf (45 min at 39 °C). These embryos were also genotyped to verify the combined presence of the two transgenes Tg(kdrf:Cre) and Tg(hsp70I:loxP-STOP-loxP-EGFP-dntcf); since the EGFP expression was too weak to be unambiguously detected (Primers are listed in Table S1). To inhibit Notch signaling, embryos were treated with 20 μM RO4929907 (Selleckchem) between 30-54 hpf, or 30-72 hpf, pending the developmental stage of interest. To inhibit Erk5 signaling, embryos were treated with 20 μM XMD8-92 (Selleckchem) between 30-48 hpf or 30-54 hpf. Control embryos were always treated with 0.4% DMSO.

To inhibit pigmentation, embryos were treated with 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich) after 24 hpf.

Heart isolation, RNA extraction, and real time-qPCR

For cardiac extractions, ~50 hearts/replicate were extracted from 54 hpf WT, cloche/npas4l, or dll4 morphant embryos. Whole-embryo extractions were performed with ~20 WT or wnt9a morphant embryos at 48 hpf as described (Lombardo et al., 2015). Heats were pooled for each condition (at least three biological replicates) and transferred into 100ml of RNA lysis buffer from Quick RNA micro-prep (Zymo kit). RNA was isolated according to manufacturer’s instructions and the corresponding cDNA was synthesized from total RNA with the RevertAid H Minus First Strand cDNA Synthesis kit (ThermoFisher Scientific). Real Time PCR experiments were performed as described (Renz et al., 2015) using 2 ng cDNA/technical replicate and the KAPA Sybr Fast qPCR kit (Roche) on a PikoReal 96 Real-Time PCR System (ThermoFisher Scientific). Cycle threshold (Ct) values were determined by PikoReal software 2.2 (ThermoFisher Scientific). eif1b was used as housekeeping gene for normalization. Control sample values were normalized to 1, using the comparative threshold cycle method (2–DDCT) (Livak and Schmittgen, 2001). As each single biological replicate represents an independent experiment from an independent clutch of embryos, ratio paired t tests were performed, using Prism 9 (GraphPad). For the experiment in Figure 4I, the mean values of the fold changes are: kdrf, 0.11 ± 0.029 SEM (p = 0.0005); fzd9b, 0.50 ± 0.083 SEM (p = 0.012). All primers are listed in Table S1.

For RT-PCR reactions (Figure S3B), embryos were pooled for each condition and total RNA was extracted with Trizol (Sigma, following the manufacturer’s instructions). The corresponding cDNA was synthesized as described above. Primers used to test the efficiency of the wnt9a splicing morpholino are listed in Table S1. eif1a was used as housekeeping gene for normalization.

Whole-mount in situ hybridization, immunohistochemistry, and image acquisition

For whole-mount in situ hybridization, zebrafish embryos were collected at 48 hpf or 54 hpf and fixed with 4% paraformaldehyde overnight at 4 °C. The wnt9a and fzd9b antisense in situ probes were generated by PCR amplification from 48 hpf WT cDNA, with the reverse primers containing a T7 promoter overhang sequence (Primers listed in Table S1). Antisense RNA was synthesized using the DIG RNA Labeling kit (Roche) and T7 polymerase. For generating the dll4 antisense in situ probe, a pGEM-Teasy vector containing the dll4 full-length coding sequence was linearized with NotI (New England BioLabs) and the antisense RNA synthesized with SP6 polymerase. Whole-mount in situ hybridization was performed as previously described (Jowett and Lettice, 1994). Images were recorded with 20x objectives on an Axioskop (Zeiss) with an EOS 5 D Mark III (Canon) camera and processed using Adobe Camera Raw and Adobe Photoshop (Adobe Creative Cloud).

Whole-mount immunohistochemistry was performed on 48, 54, 72, and 96 hpf embryos, using mouse anti-Zn-8/Alcam (1:25, Developmental Studies Hybridoma Bank) as primary antibody. The secondary antibody used was Alexa Fluor 633-conjugated goat anti-mouse (1:200, Thermo Fisher Scientific). Embryos were fixed with 4% PFA overnight and permeabilized with Ice-cold Acetone for 10–15 min at –20 °C. After one hour of incubation in blocking solution (PBST with 1% DMSO and 5% NGS), embryos were incubated with primary antibody diluted in PBST with 1% DMSO and 1% NGS. In Figures 1G–1J and Figures 2C, and 2D, nuclei were visualized by DAPI (4,6-diamidino-2-phenylindole; Sigma) (1:1000) staining. For F-actin staining in Figures 1E, S1B, and S2A–S2D, after fixation, embryos were permeabilized with PBS + 2% Triton X-100 for 1.5 hours, then incubated at 4 °C ON with 1:20 Alexa Fluor 647-Phalloidin (Thermo Fisher Scientific) with PBS + 2% Triton X-100. All embryonic hearts were manually extracted and mounted in SlowFade Gold (Thermo Fisher Scientific). Images were recorded on LSM 710, LSM 780 or LSM 880 confocal microscopes (Zeiss) and processed with Fiji (Schindelin et al., 2012), in order to minimize the background and highlight the signal.

Live imaging

Live imaging was performed using a Lightsheet Z.1 Microscope (Zeiss). Embryos were manually dechorionated, anesthetized in 0.16 mg/ml Tricaine in egg water (60 μg/ml sea salt) (this solution was also used to fill the light sheet chamber during imaging), transferred
into 1% low-melting agarose (Lonza 50081) containing 0.16 mg/ml Tricaine in egg water and withdrawn into the black-labeled glass capillaries using a metal plunger. Embryos were positioned with the heart facing the outer wall of the agarose cylinder. Imaging was performed with a Lightsheet Z.1 system (Carl Zeiss, Germany) equipped with a water immersion 20x detection objective lens (W Plan Apochromat, NA 1.0), dual sided 10x illumination objective lenses (LSMF, NA 0.2), a pco.edge scientific CMOS camera (PCO) and ZEN software. For the 2D time-lapse datasets of beating hearts, z stacks encompassing the entire AVC, a z-interval of 0.6 μm, exposure of 7-15 msec, and a time interval of 5 min for 200 cycles were used. From z stack datasets, single planes were manually analyzed with Fiji.

**Measurements of fluorescence intensity**

The corrected total cell fluorescence (CTCF) (McCloy et al., 2014) shown in Figure 1K-N was calculated to compare the klf2a levels [using Tg(klf2a:YFP)nu107] of cells expressing TCF with neighboring cells in the atrioventricular endocardium at 48 hpf, and the klf2a levels of the ingressing TCF positive cell with the most proximal and fully visible (in the selected z plane) luminal cell at 54 hpf. Images were acquired maintaining fixed recording settings, using the LSM 710 confocal microscope (Zeiss) and elaborated with Fiji. The CTCF, given by the integrated density – (area of selected tissue x mean fluorescence of background readings), was calculated using Excel 2016 (Microsoft Office). Next, all CTCF values collected for the TCF negative and the TCF positive cells were averaged and a paired Student’s t test was performed with GraphPad Prism (version 9). All the average and p values are indicated in Table S2.

The YFP net intensity of Tg(klf2a:YFP)nu107 shown in Figures 2E–2J and Figures 4E–4G was calculated to compare klf2a levels of the entire superior atrioventricular endocardium of DMSO-treated and Erk5 or Notch inhibitor-treated hearts at 48 and 54 hpf. Images were acquired maintaining fixed recording settings. From a 3D confocal image, the z-section corresponding to the entire superior AV endocardium was selected using ALCAM as marker. The mean gray value of YFP of the superior AV endocardium was measured on a single z-plane every 3 μm, until the full portion of the AV endocardium was fully covered. To each value, the mean gray value of the background (myocardium) was subtracted, and the average of the net mean gray values (net intensity) was obtained. The averages between different conditions were compared and the statistical significance of the resulting difference was determined by an unpaired Student’s t test, using Excel 2016. All average and p values are indicated in Table S3. The differences between mean values from the 2 different experiments is due to the use of two different microscopes.

**Quantification of clonal distribution at luminal versus abluminal positions of the forming valve leaflets**

The ratios of abluminal to the total number of clones integrated in the superior AVC shown in Figures 4L–4P and Figures S4I–S4K was calculated to assess the influence of dll4 on the ingression of ECs. Images were acquired with fixed recording settings. From 3D confocal image z stacks, the z section corresponding with the entire superior AV endocardium was selected based on the expression of ALCAM, an AVC endocardial marker (Beis et al., 2005), and the total number of GFP-positive clones was determined. Hearts in which only one clone integrated into the superior AV endocardium were excluded from the analysis. At 72 hpf, cell clones were counted as abluminal when present between myocardium and luminal endocardium. At 55 hpf, cell clones were considered abluminal when being TCF-positive and in positions that initiate endocardial sprouting behaviors (e.g., the most left clone highlighted with a red asterisk in Figures S4J–S4J). For each embryo, the ratio of abluminal to the total number of integrated clones was determined. A ratio value of 1 corresponds to a scenario with all clones integrated in the superior AV endocardium being in abluminal positions. All the ratios for each condition were averaged, compared, and the statistical significance of the resulting difference was determined by an unpaired Student’s t test (using Prism 9). All average and p values are indicated in Table S4.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical analyses were performed either with GraphPad Prism (version 6 or 9) or with Excel 2016 (Microsoft Office). Data representations and p values calculations are indicated in the figure legends and method details. All indicated p values are two-tailed and significance was defined as p value < 0.05.
Supplemental information

Mechanosensitive Notch-Dll4
and Klf2-Wnt9 signaling pathways intersect
in guiding valvulogenesis in zebrafish

Alessio Paolini, Federica Fontana, Van-Cuong Pham, Claudia Jasmin Rödel, and Salim Abdelilah-Seyfried
Figure S1
Figure S1. The Dll4 and TCF reporter expressing cell is surrounded by Notch activity-positive cells, related to Figure 1.

(A) Confocal maximum projection of a 54 hpf zebrafish heart expressing Tg(Dll4:Gal4)^pbb65;Tg(5xUAS:EGFP)^nuasgfp1a. Black dashed lines (A) highlight the outline of the heart as indicated by the ALCAM-positive myocardium (A').

(B,C) Single-channel images related to Fig. 1C (B,B') and Fig. 1D (C,C').

(D) Confocal z-section images of an ingressing TCF-positive cell (highlighted by white arrowheads in Figure 1E). Scale bars: 30 µm in A,A' and 5 µm in B-D
Figure S2
Figure S2. Erk5 inhibition does not affect Notch/Dll4 signaling in the superior AVC endocardium, related to Figure 2.

(A-D) Single-channel images related to Fig. 1A (A,A’), Fig. 1B (B,B’), Fig. 1C (C,C’), and Fig. 1D (D,D’).

(E-L) Single confocal z-section plane images of the superior AVC endocardium. Treatment of embryos with XMD8-92 does not affect Notch signaling in the superior AVC endocardium at 48 hpf (E,G) or 54 hpf (G,H). Similarly, Dll4 reporter expression remains unaltered upon XMD8-92 treatment at 48 hpf (I,J) and 54 hpf (K,L). (E’-L’). The presence and ingression of TCF-positive cells (asterisks in E’,G’,I’,K’) is prevented by treating embryos with XMD8-92 (F’,H’,J’,L’). Scale bars: 5 µm
Figure S3

Panel A: Diagram showing the expression of wnt9a MO. The diagram includes a gene structure with exons (exon1, intron1, exon2) and a mutation symbol indicating the effect of wnt9a MO.

Panel B: Gel electrophoresis showing the expression levels of wnt9a e1e2 and eif1a under different conditions (WT, 4ng wnt9a MO, WT, 0.8 ng wnt9a MO).
**Figure S3. Characterization of the wnt9a splicing morpholino, related to Figure 3.**

(A) Schematic model depicting the mode of action of the wnt9a splicing morpholino used for the experiments illustrated in Fig. 3E,F,H. The morpholino targets the exon 1/intron 1 splice site of wnt9a. This causes an intron retention and generates an in frame STOP codon. Black arrows indicate the positions of RT-PCR primers used to check the efficacy of this morpholino.

(B) RT-PCR analysis to assess the efficacy of the wnt9a splice morpholino in preventing correct splicing at the exon1/intron1 boundary at 48 hpf. Primers (black arrows in A) were designed to target exons 1 and 2. In wild-type, this primer pair produces a PCR amplification product of 311 bp (wnt9a e1e2). In morphants, intron 1 retention prevents an RT-PCR amplification.
Figure S4

**A, A’** DMSO control

**B, B’** RO4929097 Notch inhibitor

**C, C’** Wild type WT

**D, D’** nfatc>NICD

**E, F, G, H** ftol9b MO

**I, I’, I’’** ftol1a>H2B-GFP

**J, J’, J’’** ftol1a>dll4-H2B-GFP

**K** Bar graph showing the ratio of abluminal to total number of clones in the superior AVC

**L, M** nfatc>NICD

**AVC** Atrial Ventricular Canal
Figure S4. Notch-Dll4 signaling singles-out cells that are competent to respond to Wnt9a, related to Figure 4.

(A-F) Single-channel images related to Fig. 4A (A,A’), Fig. 4B (B,B’), Fig. 4C (C,C’), Fig. 4D (D,D’), Fig. 4J (E), and Fig. 4K (F).

(G,H) Single confocal z-section plane images of the superior AVC endocardium. (G) Ingressing endocardial cells are marked with asterisks. (H) Endocardial Notch and TCF reporter expression is lost upon MO-mediated knock-down of dll4 and the ingestion of single endocardial cells is prevented.

(I,J) Single confocal z-section plane images of the superior AVC endocardium. Shown are representative clones (red asterisks) in Tg(fli1a:GAL4FF)ubs3 embryos injected with UAS:H2B-GFP or UAS:Dll4-H2B-GFP DNA construct at 55 hpf.

(K) Quantifications of ratio of clones that ingressed into the abluminal side with respect to the total number of clones integrated in the superior AVC endocardium (UAS:H2B-GFP injected, n = 12 embryos; UAS:Dll4-H2B-GFP injected, n = 13). Single values are shown in a box plot. Lines inside the box represent mean values. Lower and upper whiskers indicate min and max values, respectively (**p < 0.01 by unpaired Student’s t-test).

(L,M) Whole-mount in situ hybridization of dll4 cardiac expression at 54 hpf. (L) dll4 is expressed mainly within ventricle and at the atrioventricular canal (AVC) in wild-type (black arrowhead). (M) Upon endocardial-specific overexpression of the Notch-intracellular domain (nfatc>NICD), dll4 expression increases at the AVC and within the ventricle.
| Name                  | Sequence 5’-3’                                                                 |
|-----------------------|-------------------------------------------------------------------------------|
| kdrl qPCR Fw          | CAGATCACGGTGATGTAATT                                                         |
| kdrl qPCR Rv          | AAGAGGAGAGGAGAGAGAG                                                          |
| fzdkb qPCR Fw         | GGGGAGCGCTACCTGACATGTC                                                       |
| fzdkb qPCR Rv         | GGTCTCCCTGACCTGACATGTC                                                       |
| eif1b qPCR Fw         | CAGAACCTCCAGTTTGATGTC                                                        |
| eif1b qPCR Rv         | GCAGGCAAATTTCTTTTTGAAGGC                                                     |
| wnt9a ISH Fw          | 5’-GAGTAATACGACTCAGATGTCAGGCGATCAGG-3’                                     |
| wnt9a ISH Rv          | 5’-GGTGAGATTATATTGATAGTAGCATTTGTTGAGG-3’                                     |
| fzdkb ISH Fw          | GGTGAGATTATATTGATAGTAGCATTTGTTGAGG-3’                                       |
| fzdkb ISH Rv          | GAGTAATACGACTCAGATGTCAGGCGATCAGG-3’                                         |
| kdrl-cre geno Fw      | CATATCAACAGGAACATAGAG                                                      |
| kdrl-cre geno Rv      | ACCAGGCCAGGTATCTCTG                                                          |
| hs-dnTCF geno Fw      | TCTGGATCTGACATGGAAG                                                        |
| hs-dnTCF geno Rv      | TCGTCCCTGACAGAAGATGTG                                                       |
| Dll4:Gal4 geno FW     | GGTGGTATCTTATGCTAGTGGATATCTATTGCTGAGAAG                                     |
| Dll4:Gal4 geno RV     | TCAACTGCTTTTGAAGGTACTTACCTCTCTTTCC                                    |
| AP008-10xUAS-R        | GGGGCTGCAGGAAATTCG-3’                                                      |
| AP004-p2A-H22B-EGFP-F | GGGAGATCTGTTTTTTATTCTGTTGCTGCTACTAATTTTTTCT                                  |
| AP003-dll4-cDNA-F     | CACACGAATTTCATGCGCCATGCGACCTGACCTGACCTG-3’                                  |
| AP002-dll4-cDNA-3noSTOP-R | AGAAATAAAAACGACTCCTCTTTACCTCAGTTGC                                      |
| CP196-remove-dll4-F   | GATTCTGACGCCCATGCGAGGCGAGGCGAGG-3’                                         |
| wnt9a_exon1 exon2_FW  | CCCCGAGGCTACAAAT                                                         |
| wnt9a_exon1 exon2_RV  | CGAAGACTGCTGCGCCACTC                                                       |
| eif1a FW              | CATATCAACAGGAAGATGCTGAGAAG                                             |
| eif1a RV              | GGTAGTACCGCTAGCAATTTCTTCTCCTTTG |
Table S2. CTCF average values (*klf2a* levels) of TCF negative and positive cells at 48 and 54 hpf. Related to STAR Methods and Figure 1 K-N

| Stage          | n  | Mean CTCF | SEM  | Mean of differences | SEM of differences | p     |
|----------------|----|-----------|------|---------------------|--------------------|-------|
| TCF negative   | 8  | 312.1     | 54.75| -146.4              | 34.44              | 0.0038|
| TCF positive   | 8  | 165.7     | 35.57|                     |                    |       |
| TCF negative   | 6  | 253.8     | 42.97| -177.4              | 35.55              | 0.0041|
| TCF positive   | 6  | 76.47     | 25.39|                     |                    |       |
Table S3. *Tg(klf2a:YFP)*<sup>mu107</sup> net intensity values of the superior AV endocardium following different chemical treatments. Related to STAR Methods and Figure 2 E-J and 4 E-G.

| Treatment | Stage  | n | YFP net intensity | SEM  | p    |
|-----------|--------|---|-------------------|------|------|
| DMSO      | 48 hpf | 8 | 8.69              | 1.65 | 0.011|
| XMD8-92   | 48 hpf | 9 | 2.36              | 0.36 |      |
| DMSO      | 54 hpf | 10| 6.62              | 1.40 | 0.0002|
| XMD8-92   | 54 hpf | 8 | 1.27              | 0.53 |      |
| DMSO      | 54 hpf | 10| 149.70            | 6.15 | 0.20 |
| RO4929097 | 54 hpf | 10| 94.50             | 7.90 |      |
Table S4. Ratio of abluminal to the total number of clones integrated in the superior AV endocardium. Related to STAR Methods and Figure 4 P and S4 K.

| DNA construct injected | Stage | n   | Ratio abluminal / total | SEM  | p       |
|------------------------|-------|-----|-------------------------|------|---------|
| UAS:H2B-GFP            | 55 hpf| 12  | 0.38                    | 0.056| 0.002   |
| UAS:dll4-H2B-GFP       | 55 hpf| 13  | 0.68                    | 0.066|         |
| UAS:H2B-GFP            | 72 hpf| 27  | 0.43                    | 0.053| 0.0073  |
| UAS:dll4-H2B-GFP       | 72 hpf| 22  | 0.61                    | 0.032|         |
Data S1. Detailed partial sequence of the transgene *Dll4:Gal4*. Related to STAR Methods.

The chromosome region indicated (chromosome:GRCz11:20:28245045:28245586), upstream of the coding sequence for the Gal4 DNA binding domain, is the endogenous sequence upstream of the start codon of *dll4* (ENSDARG00000070425). Primers used are highlighted in purple.
