Notch and Bmp signaling pathways act coordinately during the formation of the proepicardium

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Abstract

Background: The epicardium is the outer mesothelial layer of the heart. It encloses the myocardium and plays key roles in heart development and regeneration. It derives from the proepicardium (PE), cell clusters that appear in the dorsal pericardium (DP) close to the atrioventricular canal and the venous pole of the heart, and are released into the pericardial cavity. PE cells are advected around the beating heart until they attach to the myocardium. Bmp and Notch signaling influence PE formation, but it is unclear how both signaling pathways interact during this process in the zebrafish.

Results: Here, we show that the developing PE is influenced by Notch signaling derived from the endothelium. Overexpression of the intracellular receptor of notch in the endothelium enhances bmp expression, increases the number of pSmad1/5 positive cells in the DP and PE, and enhances PE formation. On the contrary, pharmacological inhibition of Notch1 impairs PE formation. bmp2b overexpression can rescue loss of PE formation in the presence of a Notch1
1  |  INTRODUCTION

The epicardium is the mesothelial layer of the heart that covers the myocardium. During developmental stages, the epicardium protects and nurtures the underlying myocardium through paracrine signals that promote its growth.\(^1\),\(^2\) The embryonic epicardium also acts as a source of epicardial-derived cells (EPDCs), that can differentiate into other cell types\(^3\) such as adipocytes,\(^4\)\(^-\)\(^6\) vascular smooth muscle cells and cardiac fibroblasts.\(^7\)\(^-\)\(^9\) EPDCs are also involved in several aspects of tissue repair and regeneration after an injury. For example, they contribute to cardiac fibrosis, control the inflammatory response and promote neoangiogenesis and proliferation of cardiomyocytes.\(^10\)

The epicardium arises from an extracardiac structure called the proepicardium (PE). The PE is formed by clusters of cells that derive from the dorsal pericardial mesothelium (DP). It develops close to the venous pole of the heart tube, around the time of heart looping, and after the onset of heart beating.\(^11\),\(^12\) In zebrafish, the PE forms by a mechanism that involves the constriction of the DP layer and the extrusion of PE cells to the pericardial cavity.\(^13\) Once the PE clusters form, the pericardial fluid flow generated by the heartbeat allows PE cells to detach and reach the myocardium. PE cells spreads on top myocardium to form a new tissue layer: the epicardium.\(^14\),\(^15\)

Little is known about the molecular mechanisms involved in PE formation. In zebrafish, Bmp signaling is essential for PE specification. Animals lacking the Bmp receptor Acvr1l fail to form a PE, whereas \(bmp2b\) overexpression extends PE marker gene expression.\(^16\) The Bmp pathway affects actomyosin cytoskeleton rearrangements and promotes the constriction of the DP during the generation of the PE.\(^13\) \(Bmp2\) is also important for the generation of PE extrusion and the adhesion to the myocardial surface in the chicken.\(^17\) In human induced pluripotent stem cell cultures, temporally controlled activity of Bmp is necessary for the differentiation into an epicardial cell fate.\(^18\),\(^19\)

Another crucial regulator of cardiovascular development is the NOTCH pathway. NOTCH signaling controls numerous processes including endocardial cushion formation, proliferation of the endothelium, maturation of the myocardium, arterial-venous fate decisions and angiogenesis.\(^20\),\(^21\) Expression of \(Notch1\) in mesothelial EPDCs in the forming chick heart suggested a role of the NOTCH pathway in the developing epicardium.\(^22\) Moreover, loss of NOTCH function alters epicardium formation, and NOTCH signaling regulates smooth muscle differentiation of epicardium-derived cells.\(^23\),\(^24\) A relationship between NOTCH and Bmp2 pathways during cardiovascular development was reported in the mouse. On the one hand, ectopic \(Notch1\) activation in the myocardium expands the expression of its effector \(Hey1\) to non-chamber myocardium, which represses Bmp2 and disrupts valve tissue specification and epithelial to mesenchymal transition (EMT). On the other hand, conditional \(Bmp2\) inactivation in the myocardium impairs NOTCH1 activity, suggesting a functional link between these two signaling pathways.\(^25\) In endocardial cells NOTCH1 signaling induces the expression of \(Wnt4\), which upregulates \(Bmp2\) expression in the adjacent atrioventricular canal myocardium.\(^26\) Also in mice, NOTCH signaling within the PE inhibits Bmp2 signaling while myocardial Bmp2 activity induces endocardial \(Jag1\)-NOTCH signaling.\(^23\) It is unknown if the molecular mechanisms that lead to PE formation are conserved in the zebrafish.

Here, we investigated the role of Notch1 signaling during zebrafish PE formation. We found that Notch signaling inhibition impairs PE formation. Overactivation of Notch signaling in EPDCs did not lead to any alteration, but when overexpressed in the endothelium, PE formation was enhanced. We found that in the zebrafish, the effect of Notch activity on the PE was at least partially dependent on its role in promoting \(bmp2/4\) expression, which differs from mice. Thus, we describe a signaling relay mechanism through different tissues in which endothelial/endocardial Notch signaling augments \(bmp\) expression in the heart tube, ultimately promoting Bmp signaling in mesothelial cells forming PE clusters.

2  |  RESULTS

2.1  |  Endothelial Notch signaling promotes proepicardium formation

To determine whether Notch signaling regulates PE formation in the zebrafish, we treated embryos with the
Notch inhibitor RO4929097 (RO). To label the DP and PE we used the enhancer trap line Et(−26.5Hsa. WT1-gata2:EGFP)\textsuperscript{cn1} (hereafter epi:GFP)\textsuperscript{14} in which GFP expression is controlled by the wilms tumor 1a (wt1a) regulatory elements, and recapitulates wt1a expression pattern. Previously, we have illustrated the importance of actin dynamics for PE formation.\textsuperscript{13} Here, we observed that upon abrogation of Notch activity, F-actin was significantly decreased at 60 hr post fertilization (hpf) (Figure 1A,B). We also found that RO administration from 48 hpf onwards impaired PE formation by reducing the PE cluster size at 60 hpf (2 ± 2 cells vs. 8 ± 3 cells in controls) (Figure 1C). These results suggest that in the absence of Notch activity, actin cytoskeleton rearrangement in PE precursor cells is impaired, and as a result, PE size is reduced.

We next sought to test whether overactivation of the Notch pathway would induce the reciprocal phenotype. To this end, we used the transgenic line UAS:NICD\textsuperscript{-myc\textsuperscript{KCA3}}\textsuperscript{28}, which drives the expression of the intracellular domain of the Notch receptor (NICD) under the UAS promoter. When combined with a Gal4 transgene, this line allows overexpressing NICD in specific tissues or cell populations. To determine the cell type in which the

**FIGURE 1** Notch signaling in the endothelium is necessary for proepicardium formation. A, 3D projections, optical sections and zoomed images of a 60 hpf control zebrafish heart compared with a RO-treated animal. epi:GFP animals immunostained for GFP (green), F-actin is detected with fluorescently-labeled phalloidin (red) and nuclei counterstained with DAPI (blue). Arrowhead, PE cluster. Arrows, accumulation of F-actin in the PE. B, Quantification of actin intensity (arbitrary units) in PE cells from conditions shown in A. C, Quantification of PE cell number in A. D, Top panels, 3D projection of a 60 hpf zebrafish heart, middle panels optical section and zoomed images below. The DP was digitally isolated in 3D projections. Control compared to those overexpressing NICD in pericardial and proepicardial cells (wt1b:Gal4) or in endothelial cells (fli1a:Gal4). epi:GFP embryos immunostained for GFP (green), myosin heavy chain (MHC, red) and nuclei counterstained with DAPI (blue). Arrowheads, PE cluster. E, Quantification of PE cell number in D. at, atrium; DP, dorsal pericardium; hpf, hours post fertilization; PE, proepicardium; v, ventricle. Scale bar: 50 μm. Data are means ± SD. Unpaired two-tailed Student’s t-test in B and C. One-way ANOVA followed by Kruskal-Wallis significant difference test in E. *P < .05, **P < .001
activity of NICD might be needed to influence PE formation, we crossed UAS:NICD-myc<sup>RNAi</sup> transgenic fish with wt1b(BAC):Gal4FF animals to drive NICD expression in the DP and PE, or with flia:gal4<sup>ub33Tg</sup> to overexpress NICD only in endothelial and endocardial cells (Figure 1D). To determine the effect of Notch gain of function on PE formation, we performed these experiments in animals carrying the epi:GFP transgene. Whereas activation of the Notch pathway in wt1b<sup>+</sup> cells did not affect PE formation, we found that overexpression of NICD in endothelial cells resulted in a significant increase in PE cell numbers (13 ± 4 cells in flia<sup>+</sup> vs. 8 ± 3 cells in nontransgenic zebrafish) (Figure 1E).

Overall, our findings demonstrate that activation of the Notch signaling pathway in endothelial cells increased PE size, which suggests that paracrine signals from the underlying endothelium and endocardium may guide PE formation.

### 2.2 Notch signaling acts on endothelial cells

Our results predict that manipulating Notch signaling in the endothelium influences PE formation. Accordingly, we observed that the PE forms right over kdrl:mCherry<sup>+</sup> endocardial precursor cells located at the cardiac inflow tract (Figure 2A). To determine whether Notch signaling is active in this cell population, we examined kdrl: mCherry; ET33-mi60a animals in which the endothelium is marked by mCherry expression and expression of GFP.

![Figure 2](https://example.com/figure2.png)

**Figure 2** Notch signaling is active in endothelial cells. A, 3D projection and optical section of a 60 hpf epi:GFP; kdrl:mCherry double transgenic embryos. GFP<sup>+</sup> pericardium and PE are shown in green, the mCherry<sup>+</sup> endothelium in red. Arrowhead, PE cluster. B, Maximum projection and optical sections of a 60 hpf zebrafish heart. GFP<sup>+</sup> cells (green) colocalize with the endogenous mCherry<sup>+</sup> endothelium (red) of kdrl:mCherry. Arrows, lnfg:GFP<sup>+</sup> kdrl:mCherry<sup>+</sup> cells. C, Optical sections through the avc and vp heart regions of 60 hpf wt1b:Gal4;UAS:mCherry; lnfg:GFP embryos immunostained for mCherry (red), GFP (green), myosin heavy chain (MHC, white) and nuclei counterstained with DAPI (blue). Zoomed views including single channels are shown below. Arrowheads, PE cluster. D, Fluorescent in situ mRNA hybridization on a 60 hpf flia:GFP ventricular heart section with notch1b riboprobe (green) followed by immunofluorescence for GFP (red) and myosin heavy chain (MHC, gray). Nuclei are counterstained with DAPI (blue). at, atrium; avc, atrioventricular canal; DP, dorsal pericardium; hpf, hours post fertilization; PE, proepicardium; v, ventricle; vp, venous pole. Scale bar: 50 μm (25 μm in C middle images and in D; 10 μm in C zoomed images).
Overexpression in NICD increases the number of PE clusters at 80 hpf. In contrast, fli1a in enhaced PE cluster formation at 80 hpf (14 ± 8 PE cells). One possible explanation for the increase in PE clusters resulted in PE clusters maintenance at 80 hpf (Figure 3A). We further analyzed the impact of NICD or fli1a overexpression, it also resulted in the formation of PE clusters at later stages of development. In control animals, PE clusters appear around 60 hpf but are undetectable at 80 hpf when epicardial cells start to colonize the myocardial surface (Figure 3A). In agreement with our previous results, overexpression of NICD using the EPDCs driver wt1b:Gal4 failed to increase the number of PE clusters at 80 hpf. In contrast, NICD overexpression in fli1a+ endothelial cells still enhanced PE cluster formation at 80 hpf (14 ± 8 PE cells in fli1a+ vs. 1 ± 3 cells in wt1b+ animals; P < .0001) (Figure 3A,B). We previously described that NICD overexpression was sufficient to rescue PE formation under BDM treatment. Contrary to fli1a overexpression, PE formation was not rescued by NICD overexpression in fli1a+ endothelial cells at 60 hpf (Figure 3G,H). However, at 80 hpf, PE clusters were observed in BDM-treated animals that overexpressed Notch in fli1a+ cells (10 ± 2 cells vs. 0 ± 1 cells in BDM; P < .0001; Figure 3I,J). bmp2b overexpression also allowed PE maintenance at 80 hpf under BDM treatment. Thus, while ectopic bmp overexpression can rescue PE formation at 60 hpf, Notch overexpression rescues PE formation with a certain delay, at 80 hpf.

### 2.3 Notch activation rescues proepicardium formation upon Myosin-II inhibition

We next aimed to test whether experimental activation of the Notch signaling pathway was sufficient to promote the formation of PE clusters at later stages of development. In control animals, PE clusters appear around 60 hpf but are undetectable at 80 hpf when epicardial cells start to colonize the myocardial surface (Figure 3A). In agreement with our previous results, overexpression of NICD using the EPDCs driver wt1b:Gal4 failed to increase the number of PE clusters at 80 hpf. In contrast, NICD overexpression in fli1a+ endothelial cells still enhanced PE cluster formation at 80 hpf (14 ± 8 PE cells in fli1a+ vs. 1 ± 3 cells in wt1b+ animals; P < .0001) (Figure 3A,B). We previously described that NICD overexpression was sufficient to rescue PE formation under BDM treatment. Contrary to fli1a overexpression, PE formation was not rescued by NICD overexpression in fli1a+ endothelial cells at 60 hpf (Figure 3G,H). However, at 80 hpf, PE clusters were observed in BDM-treated animals that overexpressed Notch in fli1a+ cells (10 ± 2 cells vs. 0 ± 1 cells in BDM only; P < .0001; Figure 3I,J). bmp2b overexpression also allowed PE maintenance at 80 hpf under BDM treatment. Thus, while ectopic bmp overexpression can rescue PE formation at 60 hpf, Notch overexpression rescues PE formation with a certain delay, at 80 hpf.

### 2.4 Notch signaling acts upstream of Bmp pathway to control proepicardium formation

Given that Bmp2b is able to rescue the formation of the PE upon BDM treatment at 60 hpf, and that PE formation requires more time to recover by NICD overexpression (observed at 80 hpf but not 60 hpf), we hypothesize that Notch signaling may function upstream of Bmp pathway activation to control PE formation.

To determine whether these two pathways act coordinately during the formation of the PE, we first analyzed bmp2b and bmp4 expression levels in the heart tube upon altering Notch signaling. Treatment with the Notch inhibitor RO from 48 hpf reduced heart tube bmp4 expression levels at 60 hpf (n = 22/26 in control and 14/20 presented this phenotype in RO-treated animals) (Figure 4A). In addition, the overexpression of NICD in fli1a+ endothelial cells at 60 hpf increased bmp4 levels in the heart tube (n = 9/13) (Figure 4A). When we overexpressed NICD in fli1a+ cells at 80 hpf, bmp4 as well as bmp2b expression levels increased in the heart (n = 21/21 and n = 20/20, respectively) (Figure 4B). On the contrary, RO-treated animals revealed reduced bmp4 and bmp2b expression levels at 80 hpf (Figure 4B).

To study if Notch-induced augmented bmp4/2b in the heart results in increased Bmp signaling activation in PE cells, we first examined the presence of pSmad1/5+ DP and PE cells in NICD overexpressing animals. pSmad1/5 is a downstream effector of the Bmp pathway and its expression reflects the activation of the signaling
FIGURE 3 Legend on next page.
At 60 hpf, we observed that animals with ectopically activated Notch signaling in \textit{fli1a} \textsuperscript{+} cells, but not in \textit{wt1b} \textsuperscript{+} cells, exhibited more pSmad1/5/\textsuperscript{+} DP and PE cells than controls at 60 hpf (Figure 4C–E) as well as at 80 hpf (Figure 4F–H).

To further address the relationship between Notch and Bmp signaling pathways, we combined various Notch and Bmp gain- and loss-of-function scenarios. First, we assessed how Bmp inhibition influences Notch- and Bmp gain- and loss-of-function scenarios. For this, we treated embryos overexpressing \textit{NICD} in \textit{fli1a} \textsuperscript{+} cells with the Bmp inhibitor LDN-193189 \textsuperscript{34,35} from 48 hpf onwards. LDN impairs PE formation, and \textit{bmp2b} overexpression rescues the number of PE cells to a wild-type situation. \textsuperscript{13} Here, we found that PE cluster formation was not rescued by \textit{NICD} overexpression after LDN treatment. We counted 2 ± 3 cells in LDN treated vs. 3 ± 2 cells in LDN treated-overexpressing \textit{NICD} animals (Figure 5A,B); while the PE comprises 8 ± 3 cells in nontransgenic zebrafish (Figure 1C). We then tested the effect of overexpressing \textit{bmp2b} upon Notch signaling inhibition with RO. We found that while 1 ± 2 cells comprised a PE in RO treated animals, in RO treated \textit{bmp2b}-overexpressing animals this number raised to 9 ± 4 cells (Figure 5A,B). Thus, \textit{bmp2b} overexpression can override the negative impact of the lack of Notch signaling on the formation of the PE. Next, we evaluated how the different treatments affected pSmad1/5/\textsuperscript{+} cell number in the DP. LDN treatment reduced the number of pSmad1/5/\textsuperscript{+} cells in the DP, and \textit{NICD} overexpression in \textit{fli1a} \textsuperscript{+} cells could not rescue the number of pSmad1/5/\textsuperscript{+} cells upon LDN treatment (Figure 5C). Even at 80 hpf, \textit{NICD} overexpression in \textit{fli1a} \textsuperscript{+} cells could neither rescue PE cluster formation nor the number of pSmad1/5/\textsuperscript{+} cells upon LDN treatment (Figure 5D–F). On the contrary, the reduction in the number of PE cells and pSmad1/5/\textsuperscript{+} cells upon RO administration could be recovered by \textit{bmp2b} overexpression (Figure 5D–F).

Overall, we conclude that the Notch signaling pathway acts in the endothelium/endocardium to regulate Bmp expression in the heart tube, which is necessary for the formation of the PE.

### DISCUSSION

We propose a model in which Notch activity in endothelial cells leads to the expression of \textit{bmp2b} and \textit{bmp4} in the heart tube, which subsequently signals to PE precursor cells promoting PE cluster formation.

PE and epicardium formation has been widely studied mainly in three animal species: the mouse, the chicken and the zebrafish. In the mouse and the chick, the PE is formed by a cauliflower-like structure with a core of precardiac mesoderm and a mesothelial lining. \textsuperscript{11,12} In the zebrafish, the PE is formed by small groups of mesothelial cells. \textsuperscript{13,14,36} In mice, PE cysts are thought to be released into the pericardial cavity, \textsuperscript{37} similar to what has been observed in the zebrafish. \textsuperscript{14} In the chick, the PE forms a bridge to the myocardium and PE cells are transferred in this way to the myocardial surface. \textsuperscript{38}

Here, we studied the role of Notch on PE formation in the zebrafish and found some similarities but also species-specific differences. While cell-autonomous effects on PE formation and epicardium were previously found in mice, \textsuperscript{22-24} we did not observe enhanced PE formation when overexpressing \textit{NICD} in PE cells using the \textit{wt1b:Gal4} line. Notch receptors are expressed in the adult epicardium \textsuperscript{39} and in the endocardium, \textsuperscript{40,41} suggesting that these two cardiac layers should be Notch responsive. It is possible that the \textit{wt1b:Gal4} line is insufficient to robustly overexpress \textit{NICD} in all PE cells and that this is the reason why we did not observe the same result as observed in the mouse. However, we clearly showed that overexpressing \textit{NICD} in the endothelium is sufficient to

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**FIGURE 3** Notch signaling rescues proepicardium formation upon Myosin-II inhibition at 80 hpf. A, E, and G, \textit{epic}GFP embryos immunostained for GFP (green), myosin heavy chain (MHC, red) and nuclei counterstained with DAPI (blue). Top panels, 3D projections and lower panels optical sections. The DP was digitally isolated in 3D projections. A, 80 hpf control zebrafish heart compared with those overexpressing \textit{NICD} in pericardial and PE cells (\textit{wt1b:Gal4}), endothelial cells (\textit{fli1a:Gal4} or \textit{bmp2b} overexpressing embryos. Arrowheads, PE cluster. Arrows, epicardial cells. B, Quantification of PE cell number in A. C, Number of PE release events per larva observed from 58 to 65 hpf. D, Number of PE cells released per event of cell release per larva from 58 to 65 hpf. E, Quantification of epicardial cell number in A. F, Top panels, maximal projection of three optical sections and zoomed views are shown below. 5 days post fertilization (5 dpf) control zebrafish heart compared with those overexpressing \textit{NICD} in endothelial cells (\textit{fli1a:Gal4} or \textit{bmp2b} overexpressing embryos. Asterisk mark the region of PE formation at 60 hpf. Arrows, epicardial cells. G, 60 hpf BDM-treated control zebrafish heart and heart from embryo overexpressing \textit{NICD} in \textit{fli1a} \textsuperscript{+} endothelial cells. H, Quantification of PE cell number of conditions as shown in G. I, 80 hpf BDM-treated control heart, heart from embryo overexpressing \textit{NICD} in \textit{fli1a} \textsuperscript{+} endothelial cells and heart from an embryo overexpressing \textit{bmp2b}. Arrowheads, PE cluster. J, Quantification of PE cell number of conditions shown in I. at, atrium; BDM, 2,3-butanedione monoxime; DP, dorsal pericardium; hpf, hours post fertilization; PE, proepicardium; v, ventricle. Scale bars: 50 μm. Data are means ± SD, one-way ANOVA followed by Kruskal-Wallis significant difference test, unpaired two-tailed Student’s t-test in H. ***P < .001, ns, nonsignificant
FIGURE 4  Legend on next page.
enhance PE formation, indicating a non-cell-autonomous role for Notch signaling occurring in the zebrafish. The different tissue composition of the PE—being purely mesothelial in nature in the zebrafish, while harboring a mesoderm core in the mouse or chicken—might be one reason for the observed differences in Notch activity in the PE between these species.

BMP2 signaling has also been previously studied in the context of PE and epicardium formation, mostly in the chicken. There, BMP signaling has a concentration-dependent effect on the specification of the precardiac mesoderm toward a PE or myocardial fate. In the mouse, BMP signaling has been extensively studied in the context of epicardial EMT. In support to previous findings, we confirmed that Bmp2b is required for PE cell extrusion during the time window of PE formation. BMP is also necessary to allow formation of PE extrusions and attachment of PE cells to the myocardial surface in chicken. In our previous article, we found that overexpression of bmp2b indeed led to an increase not only of PE formation but also of epicardialisation. At 60 hpf, hearts of fish overexpressing bmp2b revealed more epicardial cells than controls. However, here, at 80 hpf, we could not find differences in the amount of epicardial cell number in bmp2b overexpressing animals compared to controls. We suspect that 80 hpf is a time point too close to complete epicardialisation that would make it difficult to see differences. Also, since the myocardial surface, compensatory mechanism such as less or more proliferation of epicardial cells might come into play.

The coordinated activity of NOTCH and BMP2 signaling during cardiovascular development has previously been studied in the mouse. In this species, ectopic expression experiments indicate that myocardial BMP2 activates JAG1-NOTCH1 signaling in the endocardium of the valve territory, while BMP2 signaling abrogation disrupts endocardial NOTCH signaling in this tissue. During the formation of the cardiac valve primordium, BMP2 and NOTCH1 act in concert to activate Snail expression and favors its nuclear localization that drive EMT. Also, ectopic Notch1 expression throughout the myocardium represses Bmp2 expression in this tissue. In cardiomyocytes, NOTCH activity has also been described in the myocardium, where it represses BMP signaling. Consistently, blocking NOTCH signaling leads to the increase of BMP2 signaling in the PE, and NOTCH and BMP2 actively repress the formation of the PE. In zebrafish embryos, our results suggest an opposing mechanism, Notch signaling acts upstream of the Bmp pathway promoting PE formation and promote PE formation. Interestingly, an interaction between Notch and Bmp signaling has been reported during zebrafish cardiac regeneration. Upon genetic myocardium ablation endocardial Notch upregulation occurs, and subsequently induces bmp10 expression in the myocardium. This is similar to what we observed in the context of embryonic development, suggesting that developmental mechanisms are reused during regeneration.

All these findings, and our own results on the interaction between Notch and Bmp signaling, show that the crosstalk between both pathways depends on the spatial-temporal context, as well as on the species analyzed. Our finding also shows an example of how signaling arising from the luminal endothelium can influence the formation of the outermost cardiac layer, the epicardium. Collectively, our results reveal an example for the coordinated action of signaling molecules in controlling tissue morphogenesis across multiple tissue layers.

### FIGURE 4

Endothelial Notch signaling enhances cardiac Bmp2/4 expression levels and induces pSmad 1/5 in the dorsal pericardium and proepicardium. A and B, Whole-mount in situ hybridization for bmp4 or bmp2b in control, RO-treated and animals overexpressing NICD in endothelial cells (fli1a:Gal4). A, 60 hpf. B, 80 hpf. White arrows, venous pole. Black arrows, atrioventricular canal of the heart tube. C, Quantification of pSmad 1/5+ DP cell numbers at 60 hpf. D, Quantification of pSmad1/5+ PE cell numbers at 60 hpf. E, epi:GFP embryos immunostained for GFP (green), myosin heavy chain (MHC, red), pSmad1/5 (white) and nuclei counterstained with DAPI (blue). Top panels, optical sections of a 60 hpf control zebrafish heart compared with hearts from zebrafish overexpressing embryos immunostained for GFP (green), myosin heavy chain (MHC, red), pSmad1/5 (white) and nuclei counterstained with DAPI (blue). Arrowheads, PE cluster. Arrows, epicardial cells. Yellow asterisks, pSmad1/5+ cells. F, Quantification of DP pSmad1/5+ cell number at 80 hpf. G, 3D projections, optical sections and zoomed images of 80 hpf control zebrafish heart compared to those overexpressing NICD in pericardial and proepicardial cells (fli1a:Gal4) or in endothelial cells (fli1a:Gal4). Zoomed views are shown below. Arrowheads, PE cluster. Yellow asterisks, PE pSmad1/5+ cells. H, Quantification of PE pSmad 1/5+ cell number at 80 hpf. at, atrium; DP, dorsal pericardium; hpf, hours post fertilization; PE, proepicardium; v, ventricle. Scale bar: 50 μm (25 μm in zoomed images E, G). Data in C, D, F, and H are means ± SD, one-way ANOVA followed by Kruskal-Wallis significant difference test. *P < .05, **P < .01, ***P < .001
Spain; and the “Amt für Landwirtschaft und Natur” from the Canton of Bern, Switzerland. Animals were housed and experiments performed in accordance with Spanish and Swiss bioethical regulations for the use of laboratory animals. Fish were maintained at a water temperature of 28°C. The following fish were used: wild-type AB strain; Et(−26.5Hsa.WT1-gata2:EGFP)\textsuperscript{en1} (epi:GFP)\textsuperscript{14}; Tg(hsp70: bmp2b)\textsuperscript{fr13} 49; Tg(uas::myc-Notch1a-intra)\textsuperscript{kca3} 28; Tg(fli1a: gal4)\textsuperscript{ubs3}Tg\textsuperscript{29}; Tg(kdr::mCherry)\textsuperscript{f15} (from Elke Ober’s laboratory), Et(krt4:EGFP)\textsuperscript{ysqet13}m60A \textsuperscript{50}; Tg(5xUAS:mRFP)\textsuperscript{51};
Tg(Tp1:CreERT2)s951,52; Tg(−3.5ubb:LOXP-EGFP-LOXP-mCherry)cz170253 and Tg(fli1a:eGFP-F).54

4.2 | Generation of the TgBAC(wt1b:GAL4)cn18 transgenic line

The translational start codon of wt1b in the BAC clone CH73-186G17 was replaced with the galff-polyA-KanR cassette by Red/ET recombineering technology (GeneBridges) as described.55 To generate the targeting PCR product, wt1b-specific primers were designed to contain 50 nucleotide homology arms around the ATG with 20 nucleotide ends to amplify the galff-polyA-KanR cassette. To facilitate transgenesis, the BAC-derived loxP site was replaced with the iTol2-AmpR cassette56 using the same technology. The final BAC was purified with the HiPure Midiprep kit (Invitrogen) and coinjected with Tol2 mRNA into Tg(UAS:GFP) embryos.51 The full name of this transgenic line is TgBAC(wt1b:GALFF). Primers used to generate the wt1b-GALFF targeting PCR product were the following:

wt1b_HA1_Gal4-For: gacattttgaactcagatattctagttttgcaac ccagaaaatccgtcaccATGAAGCTACTGTCTTCTATCGAAC.
wt1b_HA2-KanR-Rev: gcgctcaggtctctgacatccgatcccatcggg ccgcacggctctgtcagTCAGAAGAACTCGTCAAGAA.
(lower case indicates homology arms).

The line has been deposited at ZFIN with the name Tg(wt1b:Gal4)cn18.

4.3 | Heat shock

Heat shock (HS) was performed on the embryos at 39°C in preheated water for 1 hr. When we overexpressed bmp2b by HS using Tg(hsp70l:bmp2b)fr13 line HS was performed at 26, 35 and 48 hpf.

Animals treated with heat shock were genotyped after analysis. This allowed unbiased comparison and blinded quantification of experimental and control groups.

4.4 | Immunofluorescence

Embryos were fixed overnight in 4% paraformaldehyde in PBS, washed in 0.01% PBS-Tween-20 (Sigma) and permeabilized with 0.5% Triton-X100 (Sigma) in PBS for 20 min. Several washing steps were followed by 2 hr blocking with 5% goat serum, 5% BSA, 20 mM MgCl2 in PBS followed by overnight incubation with the primary antibody at 4°C. Secondary antibodies were diluted 1:500 in PBS and incubated for 3 hr. Nuclei were counterstained with DAPI (Invitrogen) for 30 min. Embryos were mounted in Vectashield (Vector).

Immunofluorescence staining on paraffin sections were performed as described in reference 57.

The antibodies and stains for immunofluorescence detection were as follows: anti-myosin heavy chain, MHC, (MF20, ab_2147781 DSHB) at a 1:20 dilution, anti-GFP (1010, Aveslab) at 1:1000, anti-mCherry (Abcam) at 1:500 and anti-pSmad1/5 (9516 Cell Signaling Technology) at 1:100, Phalloidin-488 (A12379, Thermo Scientific). Secondary antibodies were the following: anti-mouse IgG2b-Alexa 568 (A21144, Thermo Scientific), antichicken Alexa 488 (A11039, Thermo Scientific), antirabbit Alexa 647 (A11036, Thermo Scientific), all diluted at 1:500.

Embryos were imaged with a Zeiss 780 confocal microscope fitted with a ×20 objective 1.0 NA with a dipping lens. Z-stack images were acquired every 3–5 μm. Maximum intensity projections of images were 3D reconstructed in whole-mount views using IMARIS software (Bitplane Scientific Software). The pericardial ventral tissue was digitally removed to provide a clearer view of the heart. Optical sections of 1–3 z-slices were also reconstructed.

4.5 | Quantification of DP and PE cells

PE cells have been described to emerge from two main regions of the DP: the avcPE appears close to the atrioventricular canal (avc), and the vpPE around the venous pole (vp). We counted each cell in each z plane using DAPI nuclear counterstain and GFP expression using the line epi:GFP as described.13 We took care not to count any cell twice. Cells with a round morphology at the vp or avc region were counted as PE cells, cells with a flat morphology in the DP were counted as DP cells.

4.6 | Digital isolation of the dorsal pericardium

In epi:GFP embryos all pericardial cells express GFP. To visualize DP cells in 3D the VP was removed from all images of a Z-stack. For single time-point 3D reconstructions the surface function in Imaris was used and a mask was created to isolate the DP digitally.

4.7 | Pharmacological treatments

Embryos were manually dechorionated and incubated with compounds from 48 hpf onwards. The following
compounds were used: BDM (20 mM; Sigma), LDN-193189 (20 μM; Sigma), RO4929097 (10 μM; Selleckchem).

### 4.8 In situ hybridization

ISH on whole-mount embryos was performed as described\(^5^8\) using riboprobes against full coding sequence of *bmp4* or *bmp2b* cDNAs. Embryos at 60 hpf or 80 hpf were fixed in 4% PFA overnight, dehydrated in methanol series and stored at −20°C until its use. On day 1, embryos were bleached in 1.5% of H\(_2\)O\(_2\) in methanol, rehydrated, washed in TBS with 0.1% Tween20 (TBST), digested with proteinase K 10 μg mL\(^{-1}\) for 17 min, rinsed in TBST, blocked the endogenous alkaline phosphatase with triethanolamine 0.1 M pH 8 with 0.25% of acetic anhydride for 20 min, washed in TBST, refixed in 4% PFA for 20 min. After washing again in TBST, embryos were prehybridized at 68°C for at least 1 hr. The antisense riboprobe was added at 0.5 μg mL\(^{-1}\). After overnight hybridization, two washes with 50% Formamide/5xSSC plus 2% Tween20 and four washes with 2xSSC plus 0.2% Tween20, all at 68°C were performed. Then, embryos were transferred to RT, washed in TBST and incubated with 10% heat inactivated goat serum, 1.2% of blocking reagent (Roche, 11096176001) in maleic acid buffer (MABT). Then, embryos were incubated overnight with 1:4000 dilution of anti-digoxigenin-AP antibody (Roche, 11093274910) in blocking solution. After overnight incubation, embryos were washed in MABT and developed in BM-Purple until signal was detected.

Fluorescent in situ hybridization were performed using riboprobe against full coding sequence of *notch1b* cDNAs\(^5^9\) combined with immunostaining on paraffin sections. Sections were deparaffinized, postfixed 20 min with PFA 4%, washed with PBS, treated with proteinase K 10 μm L\(^{-1}\) for 10 min at 37°C, washed with PBS, postfixed with PFA 4% for 5 min, washed with PBS, treated with HCl 0.07 N for 15 min, washed with PBS, treated with 0.25% acetic anhydride in triethanolamine 0.1 M pH 8 for 10 min, washed with PBS, washed with RNase free water and then hybridized with the probe in prehybridization buffer overnight at 65°C. The following day sections where washed twice with posthybridization buffer 1 (50% Formamide, 5xSSC, 1% SDS) for 30 min at 65°C and twice with posthybridization buffer 2 (50% Formamide, 2xSSC, 1% SDS). Then, sections were washed with MABT buffer at room temperature, and incubated at least 2 hr in blocking solution at room temperature. They were next incubated overnight with anti-digoxigenin-POD antibody (1:500) in blocking solution. The third day, they were washed with MABT for several hours, after that sections were incubated with 1:200 tyramides (Perkin-Elmer, NEL701A001KT), washed with PBST. Afterwards, immunofluorescence on sections was performed using anti-GFP and anti-myosin heavy chain (MHC) antibodies (overnight incubation at 4°C) and followed by PBS 0.1% Tween20 washes and incubation with secondary antibodies (as described above). After a second round of PBS 0.1% Tween20 washes sections were counterstained with DAPI.

### 4.9 Statistical analysis

Student's unpaired *t*-test for comparisons between two groups or one-way ANOVA analysis of variance for comparisons between more than two groups was used when normal distribution could be assumed. When the normality assumption could not be verified with a reliable method, the Kruskal-Wallis test was used. Model assumptions of normality and homogeneity were checked with conventional residual plots. The specific test used in each comparison is indicated in the figure legend. Calculations were made with Microsoft Excel and GraphPad. *P*-values are indicated either in the figure legends or the main text or summarized.

Raw Data leading to the Figures of this article has been deposited in Mendeley under DOI: 10.17632/g9xgy9s7hn.1.

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AUTHOR CONTRIBUTIONS
Laura Andrés-Delgado: Conceptualization; formal analysis; funding acquisition; investigation; methodology; validation; visualization; writing-original draft; writing-review and editing. Maria Galardi-Castilla: Investigation; methodology; writing-review and editing. Juliane Münch: Investigation; methodology; writing-review and editing. Federico Tessadori: Investigation; methodology; writing-review and editing. Luis Santamaría: Supervision; writing-review and editing. Juan Manuel González-Rosa: Resources; writing-review and editing. Alexander Ernst: Investigation; validation; writing-review and editing. Julieán Vermot: Funding acquisition; supervision; writing-review and editing. José Luis de la Pompa: Funding acquisition; supervision; writing-review and editing.

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