Coronary arterial development is regulated by a Dll4-Jag1-EphrinB2 signaling cascade

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Abstract Coronaries are essential for myocardial growth and heart function. Notch is crucial for mouse embryonic angiogenesis, but its role in coronary development remains uncertain. We show Jag1, Dll4 and activated Notch1 receptor expression in sinus venosus (SV) endocardium. Endocardial Jag1 removal blocks SV capillary sprouting, while Dll4 inactivation stimulates excessive capillary growth, suggesting that ligand antagonism regulates coronary primary plexus formation. Later endothelial ligand removal, or forced expression of Dll4 or the glycosyltransferase Mfng, blocks coronary plexus remodeling, arterial differentiation, and perivascular cell maturation. Endocardial deletion of Efnb2 phenocopies the coronary arterial defects of Notch mutants. Angiogenic rescue experiments in ventricular explants, or in primary human endothelial cells, indicate that EphrinB2 is a critical effector of antagonistic Dll4 and Jag1 functions in arterial morphogenesis. Thus, coronary arterial precursors are specified in the SV prior to primary coronary plexus formation and subsequent arterial differentiation depends on a Dll4-Jag1-EphrinB2 signaling cascade.

Introduction Coronary artery disease leading to cardiac muscle ischemia is the major cause of morbidity and death worldwide (Sanchis-Gomar et al., 2016). Deciphering the molecular pathways driving progenitor cell deployment during coronary angiogenesis could inspire cell-based solutions for revascularization following ischemic heart disease. The coronary endothelium in mouse derives from at least two complementary progenitor sources (Tian et al., 2015) that may share a common developmental origin (Zhang et al., 2016a; Zhang et al., 2016b). The sinus venosus (SV) commits progenitors to arteries and veins of the outer myocardial wall (Red-Horse et al., 2010; Tian et al., 2013), and the endocardium contributes to arteries of the inner myocardial wall and septum (Red-Horse et al., 2010; Wu et al., 2012; Tian et al., 2013). Regardless of origin, the endothelial precursors invest the myocardial wall along stereotyped routes and eventually interlink in a highly coordinated fashion. Subsequently, discrete components of the primitive plexus are remodeled into arteries and veins, and stabilized through mural cell investment and smooth muscle cell differentiation (Udan et al., 2013). Arterial-venous specification of endothelial progenitors is genetically pre-determined (Swift and Weinstein, 2009), whereas arterial differentiation and patterning depend on environmental cues, such as blood flow and hypoxia-dependent proangiogenic signals (le Noble et al., 2005; Jones et al., 2006; Fish and Wythe, 2015). Vascular endothelial growth factor (VEGF) binds
endothelial receptors and drives the expansion of the blood vessel network as a response to hypoxia (Dor et al., 2001; Liao and Johnson, 2007; Krock et al., 2011).

The Notch signaling pathway is involved in angiogenesis in the mouse embryo and in the postnatal retina. In this processes, the ligand Dll4 is upregulated by VEGF, leading to Notch activation in adjacent endothelial cells (ECs), vessel growth attenuation, and maintenance of vascular integrity (Blanco and Gerhardt, 2013). In contrast, the ligand Jag1 has a proangiogenic Dll4-Notch–inhibitory function, suggesting that the overall response of ECs to VEGF is mediated by the opposing roles of Dll4 and Jag1 (Benedito et al., 2009). Dll4-Notch1 signaling is strengthened in the presence of the glycosyltransferase Mfng (Benedito et al., 2009; D’Amato et al., 2016).

Our understanding of how coronary vessels originate, are patterned, and integrate with the systemic circulation to become functional is still limited. Coronary arteries are distinct from peripheral arteries in that they originate from SV and ventricular endocardium, which is a specialized endothelium that lines the myocardium. Moreover, SV endothelium has a venous identity, as opposed to the retina vascular bed, which has no pre-determined venous identity. Given these differences of developmental context, it is essential to evaluate the role of Notch in coronary arterial development, and importantly, the implications for heart development and repair.

Several components of the Notch pathway have been examined in the context of coronary artery formation. Inactivation of the Notch modifier Pofut1 results in excessive coronary angiogenic cell proliferation and plexus formation (Wang et al., 2017), while endothelial inactivation of Adam10, required for Notch signaling activation, leads to defective coronary arterial differentiation (Farber et al., 2019). Transcriptomics has shown that pre-artery cells appear in the immature coronary vessel plexus before coronary blood flow onset, and express Notch genes, including Dll4 (Su et al., 2018). Here, we examine the early and late requirements of Notch ligands Jag1 and Dll4, and their downstream effector EphrinB2, for coronary arterial development.

Results

Jag1 and Dll4 are expressed in SV endocardium and coronary vessels endothelium

We examined SV and coronary vessels for the expression of Jag1 and Dll4. At embryonic day 11.5 (E11.5), Jag1 was detected in SV ECs and in ECs extending into the right atrium (Figure 1A). Dll4 was also expressed in ECs emanating from the SV and in the endocardium lining the right atrium (Figure 1A). These results suggest that either ligand could potentially activate Notch1 in the SV endocardium (Figure 1A). At E12.5, N1ICD was detected in endomucin (Emcn)-positive ECs in subepicardial capillaries emerging from the SV (Figure 1A). At E13.5, Jag1 and Dll4 were expressed in ECs of the developing coronary arteries (intramyocardial vessels; Figure 1—figure supplement 1A). Dll4 and Mfng were also expressed in prospective veins (subepicardial vessels; Figure 1—figure supplement 1A). At E15.5, Jag1, Dll4, and Mfng were all expressed in arterial ECs, whereas Mfng was still found in subepicardial vessels (Figure 1—figure supplement 1B). Thus, Jag1 and Dll4 expression is found in discrete ECs in SV endothelium, abates in sub-epicardial veins and becomes restricted to intramyocardial coronary arteries at later developmental stages.

Nfatc1-positive progenitors give rise to the majority of subepicardial vessels

To inactivate Notch ligands in SV progenitors we used the Nfatc1-Cre driver line (Wu et al., 2012). To confirm the SV and endocardial specificity of this line, we crossed it with the Rosa26-LacZ reporter line (Soriano, 1999). X-gal-staining of heart sections of E11.5 embryos identified patchy LacZ expression in SV endothelium (Figure 1—figure supplement 2A). β-gal staining was consistent with Nfatc1 protein nuclear localization in a subset of ECs lining the SV (Figure 1—figure supplement 2A). Uniform β-gal staining was detected in ventricular endocardium and cushion mesenchyme derived from endocardial cells (Figure 1—figure supplement 2A). At E12.5, co-labelling with an anti-Pecam1 antibody revealed β-gal-positive staining in 60% of Pecam1-positive subepicardial vessels in the right ventricle and about 50% in the left ventricle (Figure 1—figure supplement 2B—Source data 1, sheet 1). Tracking the expression of Nfatc1-Cre-driven red fluorescent protein (RFP) and the endothelial-specific nuclear protein Erg at E12.5 indicated that about 80% of nuclei in the...
Figure 1. Endocardial Jag1 or Dll4 inactivation disrupts coronary plexus formation. (A) Jag1, Dll4, and N1ICD immunostaining (red) in E11.5 control hearts, sagittal views. Magnified views of boxed areas show details of sinus venosus (sv, arrowheads) and right atrium (ra, arrow). Whole-mount dorsal view of immunostainings for N1ICD (red) and Emcn (green) in E12.5 control heart. Magnified views show detail of sub-epicardial endothelium. Nuclei are counterstained with Dapi (blue). (B) Whole-mount dorsal view of immunostaining for Emcn (green) in E12.5 control, Jag1<sup>fl</sup>;Nfatc1-Cre, and Dll4<sup>fl</sup>;Nfatc1-Cre. Figure 1 continued on next page

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endothelial network were Nfatc1-positive and Erg-positive (Figure 1—figure supplement 2B—Source data 1, sheet 1). Thus, SV-derived Nfatc1-positive progenitors give rise to 50–80% of subepicardial vessels in the ventricular wall, consistent with previous reports (Chen et al., 2014; Cavallero et al., 2015; Zhang et al., 2016a).

To trace the fate of Nfatc1-positive cells relative to Notch activity, we crossed Nfatc1-Cre; Rosa26-RFP mice with the Notch reporter line CBF:H2B-Venus. At E11.5, a subset of nuclear-stained RFP ECs extending from the SV into the right ventricle were co-labelled with CBF:H2B-Venus (Figure 1—figure supplement 2D). At E12.5, some RFP-labelled capillaries on the dorsal side of the heart were co-labelled with CBF:H2B-Venus while others were labelled with CBF:H2B-Venus alone (Figure 1—figure supplement 2E), indicating that Notch signaling activity is present in both Nfatc1-positive and Nfatc1-negative populations of SV-derived ECs.

Opposing roles of Jag1 and Dll4 in coronary plexus formation from SV

Jag1 inactivation with the Nfatc1-Cre driver line, specific of SV and endocardium, resulted in death at E13.5 (Supplementary file 1). Whole-mount Endomucin (Emcn) staining at E12.5 revealed a well-formed vascular network covering the dorsal aspect of control hearts (Figure 1B, E—figure supplement 3A—Source data 1, sheet 2), whereas the vascular network in Jag1\textsuperscript{fluox};Nfatc1-Cre mutants was poorly developed, and exhibited numerous capillary malformations (Figure 1B, E—figure supplement 3B—Source data 1, sheet 2) with decreased endothelial branching (Figure 1B,E—Source data 1, sheet 2). N1ICD expression was also more prominent (Figure 1C) and EC proliferation reduced 60% relative to control (Figure 1D,E—Source data 1, sheet 2). Ventricular wall thickness in endocardial Jag1 mutants was reduced by 50–60% relative to controls (Figure 1—figure supplement 4A—Source data 1, sheet 3). Thus, endocardial Jag1 deletion causes the growth arrest of the primitive coronary plexus.

Deletion of Dll4 with the Nfatc1-Cre driver resulted in the death of high proportion of embryos at E10.5 (Supplementary file 1). Nonetheless, about a third of mutant embryos survived until E12.5 (Supplementary file 1). Whole-mount Ecmn immunostaining of E12.5 Dll4\textsuperscript{fluox};Nfatc1-Cre hearts revealed a comparatively denser capillary network (Figure 1B,E—Source data 1, sheet 2), characterized by numerous capillary malformations (Figure 1B and Figure 1—figure supplement 3A,C—Source data 1, sheet 2), and increased endothelial branching (Figure 1B,E—Source data 1, sheet 2). N1ICD expression was reduced (Figure 1C), as expected, and EC proliferation increased 30% relative to control (Figure 1D,E—Source data 1, sheet 2). These defects were associated with 50–60% reduction in ventricular wall thickness (Figure 1—figure supplement 4A—Source data 1, sheet 3). Thus, endocardial inactivation of Dll4 causes excessive growth of the primitive coronary plexus.
Endocardial Jag1 or Dll4 deletion results in a hypoxic and metabolic stress response

To determine the effect of early endocardial Jag1 or Dll4 inactivation on cardiac development we performed RNA-seq. This analysis yielded 211 differentially expressed genes (DEG) in the Jag1\textsuperscript{flox}; Nfatc1-Cre transcriptome (130 upregulated, 81 downregulated; Figure 2A—Supplementary file 2) and 274 DEGs in the Dll4\textsuperscript{flox};Nfatc1-Cre transcriptome (180 upregulated, 94 downregulated; Figure 2A—Supplementary file 2).

Ingenuity Pathway Analysis (IPA) identified enrichment of EC functions (Figure 2A, left plot, Supplementary file 3). The main terms overrepresented in both genotypes were angiogenesis and EC development, and capillary vessel density, possibly reflecting the lack of a normal-sized capillary network (Figure 2A—Supplementary file 3). EC proliferation and heart contraction were predicted to be upregulated in Jag1\textsuperscript{flox};Nfatc1-Cre embryos, while EC migration was upregulated in Dll4\textsuperscript{flox}; Nfatc1-Cre mice (Figure 2A—Supplementary file 3). Analysis of upstream regulators revealed activation of hypoxia (Hif1\textalpha{}), acute inflammatory response (Nf-\kappa{}b1\textalpha{}), intracellular stress pathways (Atf4), and response to metabolic stress pathways (Foxo), while cell cycle and DNA repair pathways (Myc, Tp53) were negatively regulated (Figure 2A, right plot).

In situ hybridization (ISH) showed reduced expression of HeyL and the Notch target Efnb2 in subepicardial vessels (Figure 2B). Fabp4, a member of the fatty-acid-binding protein family, was upregulated (Figure 2A). Fabp4 is a DLL4-NOTCH target downstream of VEGF and FOXO1 in human EC (Harjes et al., 2014), required for EC growth and branching (Elmasri et al., 2009). Fabp4 expression was found exclusively in the atrio-ventricular groove in Jag1\textsuperscript{flox};Nfatc1-Cre hearts (Figure 2B), but extended sub-epicardially into the base of the heart in Dll4\textsuperscript{flox};Nfatc1-Cre hearts (Figure 2B). Vegfa expression was not globally affected in mutant hearts (Figure 2B), despite being upregulated in the RNA-seq (Figure 2A). Cell cycle-associated genes such as Cdkn1b/p27, a negative regulator of cell proliferation, were also upregulated in both genotypes (Figure 2A), and p27 nuclear staining was increased twofold in compact myocardium (Figure 2C,D—Source data 1, sheet 4), indicating decreased cellular proliferation. Connexin 40 (Cx40) and Hey2, which label trabecular and compact myocardium respectively, showed no alteration in their expression domains by ISH (Figure 2B; Figure 1—figure supplement 4B), suggesting that chamber patterning was normal in these mutants. Likewise, the glycolytic marker genes Ldha and Pdk1 (Menendez-Montes et al., 2016) were confined, as normal, to the compact myocardium (Figure 2B; Figure 1—figure supplement 4B), indicating maintenance of ventricular chamber metabolic identity.

We examined E12.5 Jag1\textsuperscript{flox};Nfatc1-Cre and Dll4\textsuperscript{flox};Nfatc1-Cre mutants for evidence of hypoxia given that the gene signatures in the RNA-seq analysis suggested an ongoing hypoxic/metabolic stress response. The hypoxic response might also explain the defect of ventricular wall growth. We performed immunohistochemical detection of pimonidazole on E12.5 Jag1\textsuperscript{flox};Nfatc1-Cre and Dll4\textsuperscript{flox};Nfatc1-Cre heart sections (Figure 2—figure supplement 1). Pimonidazole hydrochloride (Hypoxyprobe) immunostaining for hypoxic tissues was detected most intensely in cells lying within the ventricular septum and atrio-ventricular (AV) groove in the subepicardial area where the primitive coronary endothelium emerges to cover the myocardium (Figure 2—figure supplement 1Ai, arrowheads). Immunostaining for Glut1 (Slc2a1), a direct HIF1 target, showed myocardial expression partially overlapping with hypoxyprobe distribution (Figure 2—figure supplement 1Aii). There was no Glut1 immunostaining in the subepicardial mesenchyme zone (Figure 2—figure supplement 1Aii). We found no difference in the intensities of hydroxyprobe or Glut1 staining in either the ventricular free wall, or in the ventricular septum in Jag1\textsuperscript{flox};Nfatc1-Cre and Dll4\textsuperscript{flox};Nfatc1-Cre hearts (Figure 2—figure supplement 1). There was no obvious change in hydroxyprobe immunostaining of endothelial cells emerging at the AV groove either (Figure 2—figure supplement 1Ai–Ci). These results indicate that Jag1\textsuperscript{flox};Nfatc1-Cre and Dll4\textsuperscript{flox};Nfatc1-Cre mutant hearts are not overtly hypoxic at E12.5, suggesting that the hypoxic/metabolic stress gene signatures may be due to a cell autonomous defect of endocardial/endothelial cells.

Defective coronary remodeling and maturation in endothelial Jag1 or Dll4 mutants

We next examined the requirements of endothelial Jag1 and Dll4 for coronary vessel remodeling and maturation. To circumvent the early lethality of Jag1\textsuperscript{flox}– or Dll4\textsuperscript{flox};Nfatc1-Cre mutants, we...
Figure 2. Transcriptome profiling of endocardial Jag1 and Dil4 mutant hearts. (A) Left, Total number of differentially expressed genes identified by RNA-seq (Benjamini-Hochberg adjusted p<0.05) in the indicated genotypes. Numbers indicate upregulated genes (red) and downregulated genes (blue). Center, circular plot of representative differentially expressed genes, presenting a detailed view of the relationships between expression changes (left semicircle perimeter) and IPA functions belonging to the Cardiovascular System Development and Function category (right semicircle). Figure 2 continued on next page.
crossed Jag1\(^{\text{flox}}\) and Dll4\(^{\text{flox}}\) mice with the vascular endothelium-specific Pdgfb-iCre\(^{\text{ERT2}}\) transgenic mice (Wang et al., 2010) to obtain the corresponding tamoxifen-inducible lines. Tamoxifen-induced Jag1 deletion at E12.5 resulted in 68% reduction in Jag1 expression (Figure 3—figure supplement 1A,C—Source data 1, sheet 5) and a complete absence of arteries at E15.5, whereas the veins appeared unaffected (Figure 3A,C—Source data 1, sheet 6). We measured NOTCH pathway activity by carrying out a N11CD staining that showed a 50% increase in endothelial N11CD (Figure 3B,C—Source data 1, sheet 6), consistent with Jag1 acting as an inhibitory Notch ligand. Next, we examined perivascular coverage of the Jag1\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) endothelial coronary tree. We used \(\alpha\)-smooth muscle actin (\(\alpha\)SMA) and Notch3 to measure the extent of coronary vessel smooth muscle cell differentiation and pericyte coverage, respectively (Volz et al., 2015). We found that the proportion of \(\alpha\)SMA- and Notch3-positive cells was significantly reduced in E15.5 Jag1\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) coronary arteries (Figure 3B,C—Source data 1, sheet 6) reflecting the lack of differentiation of perivascular cells. Although \(\alpha\)SMA is a commonly used marker of vascular smooth muscle cells, it is expressed more broadly in mesenchymal cells and cardiomyocytes throughout the embryonic heart prior to E16.5. To evaluate smooth muscle differentiation more specifically in E15.5 hearts, we used SM22a (Figure 3—figure supplement 2A,B). After co-staining with Notch3, we found that the proportion of SM22a and Notch3-positive cells in the Jag1\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) mutants was significantly reduced (Figure 3—figure supplement 2A,B,E—Source data 1, sheet 7), confirming that pericytes fail to properly differentiate into smooth muscle.

To examine Dll4 function in coronary artery formation, we crossed Dll4\(^{\text{flox}}\) mice with Pdgfb-iCre\(^{\text{ERT2}}\) to obtain Dll4\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) mice. Tamoxifen-induced Dll4 deletion from E12.5 or E13.5, resulted in embryonic lethality at E15.5, confirming that embryo survival is highly sensitive to reduction in endothelial Dll4 expression. However, induction at E14.5 resulted in a 82% reduction in Dll4 expression (Figure 3—figure supplement 1B,C—Source data 1, sheet 5) and a complete absence of arteries at E15.5, whereas veins were unaffected (Figure 3D,F—Source data 1, sheet 6). Endothelial N11CD staining was decreased by 60% compared with controls (Figure 3E,F—Source data 1, sheet 6), consistent with Dll4 activating Notch1 during angiogenesis. Furthermore, Dll4\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) mutants had deficient perivascular cell coverage as indicated by decreased \(\alpha\)SMA- and Notch3-positive cells (Figure 3E,F—Source data 1, sheet 6). This was confirmed by co-immunostaining with SM22a and Notch3 demonstrating near complete absence of smooth muscle differentiation in Dll4\(^{\text{flox}},\text{Pdgfb-iCre}^{\text{ERT2}}\) mutants (Figure 3—figure supplement 2C,D,E—Source data 1, sheet 7).

**Endocardium/endothelial Notch ligand inactivation impairs coronary artery formation and ventricular growth**

To confirm endothelial Jag1 requirement for coronary arterial formation, we crossed Jag1\(^{\text{flox}}\) mice with Cdh5-Cre\(^{\text{ERT2}}\) driver line (Wang et al., 2010) to obtain homozygous Jag1\(^{\text{flox}},\text{Cdh5}^{\text{CreERT2}}\) mice. Tamoxifen-induced Jag1 inactivation from E9.5 onwards (Figure 3—figure supplement 3A) resulted in reduced coronary artery coverage and marginally increased vein coverage (Figure 3—figure supplement 3A,C—Source data 1, sheet 8). N11CD staining in the endothelium was increased (Figure 3—figure supplement 3B,C—Source data 1, sheet 8), reflecting Jag1 inhibitory Notch function during angiogenesis. Furthermore, Jag1\(^{\text{flox}},\text{Cdh5}^{\text{CreERT2}}\) mutant heart coronaries had decreased

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Figure 3. Late endothelial Jag1 or Dll4 inactivation disrupts coronary plexus remodeling. (A) Dorsal view of whole-mount immunochemistry for IsoB4 (red), labelling arteries and Emcn (green), labeling veins and capillaries, in E15.5 control and Jag1^{lox};Pdgfb-iCre^{ERT2} mutant hearts, tamoxifen (Tx)-induced at E12.5. Scale bars, 100 μm. (B) E15.5 control and Jag1^{lox};Pdgfb-iCre^{ERT2} mutant heart sections. Left, Immunostaining for N1ICD (red) and IsoB4 (green).
perivascular cell coverage, as indicated by the reduced proportion of αSMA- and Notch3-positive cells (Figure 3—figure supplement 3B,C—Source data 1, sheet 8).

To confirm Dll4 requirement for coronary arterial formation and circumvent the lethality resulting from E12.5 tamoxifen administration in Dll4*Pdgfb-iCreERT2 hearts, we crossed Dll4* mice with Cdh5-CreERT2 driver line (Wang et al., 2010) to obtain homozygous Dll4*;Cdh5-CreERT2 mice (Figure 3—figure supplement 3D). Tamoxifen induction at E12.5 resulted in the reduction of coronary arterial coverage, unchanged vein coverage but reduced vein caliber (Figure 3—figure supplement 3D,F—Source data 1, sheet 8). N1ICD staining in the endothelium was decreased by 60% compared with controls (Figure 3—figure supplement 3E,F—Source data 1, sheet 8), likely due to reduced EC contribution to the arteries. Furthermore, Dll4*;Cdh5-CreERT2 mutant heart coronaries had decreased perivascular cell coverage as indicated by the reduced proportion of αSMA- and Notch3-positive cells (Figure 3—figure supplement 3E,F—Source data 1, sheet 8). The impact of impaired coronary vessel formation in endothelial Jag1- or Dll4- mutants was also indicated by their diminished ventricular wall thickness (Figure 3—figure supplement 4A—Source data 1, sheet 9).

To examine the effect of endothelial Dll4 deletion on cardiac gene expression, we performed RNA-seq on E15.5 Dll4*Cdh5-CreERT2 mutant ventricles. Therefore of 163 DEGs, 138 were upregulated, while 25 were downregulated (Figure 3—figure supplement 4B and Supplementary file 2, sheet 3). As expected, Dll4 was downregulated, while Vegfa was upregulated, suggesting cardiac hypoxia (Figure 3—figure supplement 4B—Supplementary file 2, sheet 3). Cdkn1a/p21 was upregulated, indicating impaired cell proliferation (Figure 3—figure supplement 4B—Supplementary file 2, sheet 3). IPA identified strong upregulation of endothelial cell functions (Figure 3—figure supplement 4B and Supplementary file 3), whereas analysis of upstream regulators identified an enrichment for transcriptional activators associated with innate inflammatory responses (Irf5 Stats), suggesting defective vascular integrity. Other upstream regulators were associated with cardiovascular disease, including hypertrophy (Nfatc2) and oxidative stress responses (Nfe212; Figure 3—figure supplement 4B right and Supplementary file 3). Moreover, ISh showed reduced endothelial expression of Efnb2 and Dll4 in smaller caliber vessels (Figure 3—figure supplement 4C), suggesting a loss of arterial identity, whereas Fapb4 was upregulated (Figure 3—figure supplement 4C) consistent with increased coronary vessel density of the un-remodeled coronary plexus..

Figure 3 continued

Right, α-smooth-muscle actin (SMA, red) and Notch3 (green). Dapi counterstain (blue). Arrowheads point to N1ICD-positive nuclei. Arrows point to αSMA-Notch3 co-immunostaining. Scale bars, 100 μm. (C) Quantified data from E15.5 control and Jag1*Pdgfb-iCreERT2 hearts: area of coverage by coronary arteries (IsoB4-positive vessels) and veins (Emcn-positive vessels); Data are mean ± s.d. (n = 3 control embryos and 4 mutant embryos. N1ICD-positive nuclei as a percentage of total endothelial nuclei; and SMA-Notch3 co-immunostaining in coronary arteries. Data are mean ± s.d. (n = 3 sections from 4 control embryos and from 4 mutant embryos. (D) Whole-mount dorsal view of immunohistochemistry for Emcn (green) and IsoB4 (red) in E15.5 control and Dll4*Pdgfb-iCreERT2 mutant hearts. Tx-induced at E14.5. (E) Left, immunohistochemistry for N1ICD (red) and IsoB4 (green); right, immunohistochemistry for SMA (red) and Notch3 (green) on control E15.5 WT and Dll4*Pdgfb-iCreERT2 mutant heart sections. Dapi counterstain (blue). Arrowheads indicate N1ICD-positive nuclei. Arrows point to SMA-Notch3 co-immunostaining. Scale bars, 100 μm. Microscopes: Nikon A1-R, Leica SP5. Softwares: NIS Elements AR 4.30.02. Build 1053 LO, 64 bits (Nikon); LAS-AF 2.7.3. build 9723 (Leica). Objectives: Plan Apo VC 20x/0.75 DIC N2 dry; Plan Fluor 40x/1.3 Oil DIC H N2 Oil (Nikon); HCX PL APO CS 10 × 0.4 dry. HCX PL APO lambda blue 20 × 0.7 multi-immersion (Leica). (F) Quantified data from E15.5 control and Dll4*Pdgfb-iCreERT2 hearts: area covered by coronary arteries (IsoB4-positive vessels); area covered by veins (Emcn-positive vessels); percentage of N1ICD-positive nuclei in endothelium as a percentage (% of total nuclei); and SMA-Notch3 co-immunostaining in coronary arteries. Data are mean ± s.d. (n = 3 sections from 3 control embryos and from three mutant embryos). *p<0.05, **p<0.01, by Student’s t-test; n.s. , not significant. Scale bars, 100 μm.

The online version of this article includes the following figure supplement(s) for figure 3:

Figure supplement 1. Efficient tamoxifen-induced endothelial deletion of Jag1 and Dll4 using Pdgfb-iCreERT2 driver line.

Figure supplement 2. Induced endothelial Jag1 or Dll4 deletion disrupts coronary smooth muscle cell differentiation.

Figure supplement 3. Late endothelial Jag1 or Dll4 inactivation disrupts coronary plexus remodeling.

Figure supplement 4. Induced endothelial Jag1 or Dll4 deletion disrupts myocardial growth.
Thus, endothelial Jag1 or Dll4 signaling promotes remodeling and maturation of the coronary vascular tree, and in the absence of adequate vascular remodeling, cardiac growth is impaired.

**Forced endothelial Dll4 expression disrupts coronary vascular remodeling**

To further characterize the role of Notch in coronary development, we generated a transgenic line (Dll4^GOF) bearing a Rosa26-CAG-floxNeoSTOPflox-Dll4-6xMycTag expression cassette (see Materials and methods). Tie2-Cre-mediated removal of the floxed NeoSTOP sequences resulted in a mild 1.2-fold endothelial Dll4 overexpression that permitted survival of transgenic embryos bearing a single copy of the Dll4^GOF allele (not shown). At E14.5, forced Dll4 expression lead to marginally increased, sub-epicardial vessel (Emcn-positive) coverage (Figure 4A,F—Source data 1, sheet 10) and vascular malformations similar to those found in Dll4^floxflox;Pdgfrb-icreERT2 and Dll4^floxflox;Cdh5-CreERT2 mutants (Figure 1—figure supplement 3E). However, by E16.5 IsoB4-positive (arteries) intramyocardial vessels were substantially decreased (Figure 4B,F—Source data 1, sheet 10). Accordingly, Dll4 and Efnb2 expression was restricted to smaller caliber vessels in Dll4^GOF transgenics (Figure 4C). In contrast, superficial Emcn-positive vessels (veins) were more numerous and dense (Figure 4B,F—Source data 1, sheet 10). N1ICD expression was increased and extended to the prospective veins (Figure 4D,G—Source data 1, sheet 10), consistent with Notch1 gain-of-function in endothelium. Arterial smooth muscle cell coverage was also substantially reduced (Figure 4E,G—Source data 1, sheet 10), suggesting defective pericyte and/or smooth muscle differentiation. These coronary vascular defects were associated with below-normal cardiomyocyte proliferation and reduced myocardial thickness (not shown). Therefore, endothelial Dll4 overexpression blocks coronary artery formation, vessel remodeling and maturation, and impairs cardiac growth. These results were surprising, as our loss-of-function data support a pro-arteriogenic role for Dll4.

To support our Dll4^GOF results, we used a transgenic line conditionally overexpressing Mfng (Mfng^GOF) (D’Amato et al., 2016) to test whether increased Mfng activity favored Dll4-mediated signaling, and thus impaired arteriogenesis. At E11.5, Mfng^GOF;Tie2-Cre embryos displayed reduced SV sprouting, increased Notch1 signaling and myocardial wall thinning (Figure 4—figure supplement 1A,B). However, by E14.5 Mfng^GOF;Tie2-Cre embryos displayed an extensive vascular network (Figure 4—figure supplement 1C), although the arteries appeared atrophied (Figure 4—figure supplement 1C) and prospective veins appeared more numerous and intricately branched (Figure 4—figure supplement 1C). Mfng^GOF;Tie2-Cre embryos displayed numerous capillary malformations at E14.5 (Figure 1—figure supplement 3F), suggesting defective arterial-venous differentiation. The decrease in arterial coverage and increase in venous coverage in Mfng^GOF;Tie2-Cre embryos became more obvious by E16.5 (Figure 4—figure supplement 1C,G—Source data 1, sheet 11). N1ICD expression was markedly increased (Figure 4—figure supplement 1D,G—Source data 1, sheet 11), as expected, while the expression of the Notch targets HeyL and Efnb2 was restricted to smaller caliber vessels (Figure 4—figure supplement 1E). Vegfa appeared upregulated throughout (Figure 4—figure supplement 1E), suggesting that Mfng^GOF;Tie2-Cre hearts are hypoxic. Pericyte coverage and smooth muscle differentiation, as shown by αSMA and Notch3 co-immunostaining, were significantly decreased at E16.5 (Figure 4—figure supplement 1F,G—Source data 1, sheet 11), suggesting defective pericyte recruitment and/or differentiation. Ink injection confirmed that Mfng^GOF;Tie2-Cre coronaries were ‘leaky’ (Figure 4—figure supplement 1H), and therefore functionally deficient.

**EphrinB2 is required for coronary arteriogenesis and vessel branching**

Efnb2 is necessary for arterial-venous differentiation and vascular maturation (Kania and Klein, 2016) and is a Notch target during ventricular chamber development (Grego-Bessa et al., 2007). We find Efnb2 expression in ventricular endocardium at E10.5 (Figure 5—figure supplement 1A) but not in SV endothelium (not shown). At E13.5, Efnb2 was expressed in emerging arteries and prospective veins (Figure 5—figure supplement 1A), and at E16.5 was confined to arteries (Figure 5—figure supplement 1A). Therefore, Efnb2 is expressed dynamically in a pattern similar to Dll4 and Mfng.

To examine EphrinB2 function in coronary angiogenesis, we crossed mice bearing a conditional Efnb2^floxfloxfloxfloxfloxfloxfloxfloxfloxfloxfloxfloxfloxflox allele with the Nfatc1-Cre line. Whole-mount IsoB4 immunostaining revealed reduced...
ForcedDll4 expression in endothelium disrupts coronary arteriovenous differentiation and remodeling. (A) Dorsal view of whole-mount immunoochemistry for IsoB4 (red), labeling arteries, and Emcn (green), labeling veins and capillaries, in E14.5 control and Dll4GOF;Tie2-Cre heart. Scale bar, 100 μm. (B) Dorsal view of whole-mount immunoochemistry for IsoB4 (red) and Emcn (green) in E16.5 control and Dll4GOF;Tie2-Cre mutant heart. Scale bar, 100 μm. (C) ISH of Dll4 and Efnb2 on E16.5 control and Dll4GOF;Tie2-Cre heart sections. Arrowheads indicate coronary arteries. Scale bar, 50 μm. (D) Immunohistochemistry for N1ICD (red) and IsoB4 (green) on E16.5 control and Dll4GOF;Tie2-Cre heart sections. Dapi counterstain (blue). Arrowheads indicate N1ICD-stained nuclei. Scale bar, 50 μm. (E) Immunohistochemistry for SMA (red) and Notch3 (green) on E16.5 control and Dll4GOF;Tie2-Cre heart sections. Dapi counterstain (blue). The arrow points to a coronary vessel stained by SMA and Notch3. Scale bar, 50 μm. Microscope: Nikon A1-R. Software: NIS Elements AR 4.30.02. Build 1053 LO, 64 bits. Objectives: Plan Apo VC 20x/0.75 DIC N2 dry; Plan Fluor 40x/1.3 Oil DIC H N2 Oil. (F) Quantified data for control and Dll4GOF;Tie2-Cre hearts: E14.5, area covered by veins (Emcn-positive vessels). Data are mean ± s.d, (n = 2 control embryos and n = 3 mutant embryos); E16.5, area covered by coronary arteries (IsoB4-positive vessels), and area covered by veins (Emcn-positive vessels). Data are mean ± s.d, (n = 3 control embryos and n = 2 mutant embryos). (G) Quantified data of the percentage of N1ICD-positive nuclei in E16.5 endothelium as a percentage (%) of total nuclei; and SMA-Notch3 co-immunostaining in coronary arteries. Data are mean ± s.d, (n = 3 sections from 3 control embryos and n = 4 sections from 3 mutant embryos) *p<0.05, **p<0.01, by Student’s t-test; ns. not significant.
artery coverage in E15.5 Efnb2\textsuperscript{flox}\texthyp{;}Nfatc1-Cre hearts (Figure 5A,F—Source data 1, sheet 12), while vein coverage (Emcn-positive) was unchanged (Figure 5A,F—Source data 1, sheet 12). Histological examination showed that the compact myocardium in the ventricles was 30% thinner at E16.5 (Figure 5—figure supplement 1B,C—Source data 1, sheet 13), which could be attributed to the reduced proliferation observed at E14.5 (Figure 5—figure supplement 1C,D—Source data 1, sheet 13). The presence of smaller caliber arteries coincided with higher Notch1 activity (Figure 5B,F—Source data 1, sheet 12) and increased Hey1 expression (Figure 5C), suggesting negative feedback between EphrinB2 and Notch signaling. Although the patterning of prospective veins was unchanged, we found occasional malformations (Figure 1—figure supplement 3G,H) and transmural communications or shunts between the endocardium and epicardium, which we interpret to be arteriovenous fistulae (Figure 5—figure supplement 1E,F).

Accordingly, the venous marker EphB4 was expressed ectopically in a subset of intramyocardial vessels at E16.5 (Figure 5—figure supplement 1E), indicative of abnormal venous identity. Vegfa was upregulated throughout the heart (Figure 5—figure supplement 1F), suggesting that Efnb2\textsuperscript{flox}\texthyp{;}Nfatc1-Cre hearts are hypoxic. Arterial smooth muscle coverage was reduced substantially (Figure 5E,F—Source data 1, sheet 12), suggesting defective vessel maturation. Dye perfusion in Efnb2\textsuperscript{flox}\texthyp{;}Nfatc1-Cre hearts revealed absence of left anterior descending coronary artery at E16.5 (Figure 5—figure supplement 1G) and ink perfusion revealed vascular hemorrhaging in the most distal section of the coronary arterial tree at E18.5 (Figure 5—figure supplement 1H), consistent with vessel leakiness, while aortic connections were normal (Figure 5—figure supplement 1H). Thus, endocardial EphrinB2 is required for coronary arterial remodeling, vessel structural integrity, and cardiac growth.

**EphrinB2 mediates Jag1 and Dll4 signaling in arterial branching morphogenesis**

To model coronary angiogenesis ex vivo, we developed a ventricular explant assay (Figure 6A; (Zhang and Zhou, 2013). Compared with controls, Jag1\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants had a lower angiogenic potential, manifested as smaller caliber vessels, longer distances between branching points and a lower number of transmural endothelial branches (Figure 6B,C,K—Source data 1, sheet 14). Conversely, Dll4\textsuperscript{flox}\texthyp{;}Nfatc1-Cre or Notch1\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants yielded more densely interconnected vascular networks of larger vessel caliber, longer distances between vessel branching points, and more transmural endothelial branches (Figure 6B,D,E,K—Source data 1, sheet 14). Notch1\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants displayed a phenotype similar to Dll4\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants, although the differences in vessel caliber and endothelial branch number did not reach significance (Figure 6B,E,K—Source data 1, sheet 14). Mfng\textsuperscript{GOF};Tie2-Cre and Dll4\textsuperscript{GOF};Tie2-Cre explants showed a broadly similar phenotype, with substantial lengthening of branching point distance and a below-normal number of endothelial branches (Figure 6B,I,J,K—Source data 1, sheet 14). In contrast, Mfng\textsuperscript{GOF};Tie2-Cre explants had smaller caliber vessels than Dll4\textsuperscript{GOF};Tie2-Cre explants (Figure 6I,J,K—Source data 1, sheet 14). Efnb2\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants formed sparsely interconnected networks with decreased vessel caliber, reduced number of transmural endothelial branches, and increased distances between branching points, similar to those seen in Jag1\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants (Figure 6B,F,K—Source data 1, sheet 14).

Based on the congruence of endocardial/endothelial Jag1, Dll4 and Efnb2 loss-of-function phenotypes, we tested whether EphrinB2 mediates Notch function in coronary angiogenesis. Thus, delivery of a lentivirus expressing Efnb2 to Jag1\textsuperscript{flox}\texthyp{;}Nfatc1-Cre ventricular explants normalized the reduced endothelial branch number to the number seen in control explants (Figure 6B,G,K—Source data 1, sheet 14). Likewise, Efnb2 expression restored the elevated number of endothelial branches in Dll4\textsuperscript{flox}\texthyp{;}Nfatc1-Cre explants to control levels (Figure 6B,H,K—Source data 1, sheet 14), showing that the branching defect could be normalized in both mutants.
Figure 5. Endocardial Efnb2 inactivation disrupts coronary artery differentiation and remodeling. (A) Dorsal view of whole-mount immunochemistry for IsoB4 (red), labelling arteries and Emcn (green), labeling veins and capillaries, in E15.5 control and Efnb2^{lox};Nfatc1-Cre hearts. Scale bars, 100 μm. (B) Immunohistochemistry of N1ICD (red) and Emcn (green) on E16.5 control and Efnb2^{lox};Nfatc1-Cre heart sections. Dapi-counterstain (blue). Arrowheads indicate N1ICD-stained nuclei. Scale bars, 100 μm. (C) ISH of Hey1 on E16.5 control and Efnb2^{lox};Nfatc1-Cre heart sections. Arrowheads indicate Hey1.
EphrinB2 functions downstream of Jag1 andDll4 in capillary tube formation

The ventricular explant assay assesses the collective behaviors of endocardium and coronary endothelial outgrowth. In order to determine endothelial behavior exclusively, we performed capillary tube formation assays with primary human endothelial cells (HUVEC). shRNA directed against Jag1, Dll4 or Efnb2 were lentivirally-transduced into HUVEC. This resulted in reduction of mRNA levels of 30% and 60% for Jag1 and Dll4, respectively (Figure 7A—Source data 1, sheet 15), and of 50% for Efnb2 (Figure 7—figure supplement 1A—Source data 1, sheet 16). The NOTCH target Hey1 was unchanged in shJag1-infected cells and 50% decreased in shDll4-infected cells (Figure 7A—Source data 1, sheet 15). The activity of Efnb2 lentivirus was verified by rescuing shRNA-mediated Efnb2 knockdown (Figure 7—figure supplement 1B,C—Source data 1, sheet 16). Efnb2 knockdown resulted in decreased capillary network complexity (Figure 7—figure supplement 1B,C—Source data 1, sheet 16). These parameters were restored to control levels by lentiviral-mediated Efnb2 overexpression (Figure 7—figure supplement 1B,C—Source data 1, sheet 16).

We next tested whether EPHRINB2 acts downstream of Jag1 and Dll4 in capillary tube formation. Jag1 knockdown inhibited capillary tube formation and decreased network complexity as determined by measuring EC junctions, nodes, segments and pieces that were below control, while the ‘total isolated branches length’ was above control (Figure 7B,C—Source data 1, sheet 15). However, these parameters were restored to control levels in presence of the Efnb2 transgene (Figure 7B,C—Source data 1, sheet 15), suggesting that EPHRINB2 compensates for Jag1 knockdown in this assay. In contrast, knockdown of Dll4 increased capillary tube formation and network complexity (junctions, nodes, segments and pieces; Figure 7B,C—Source data 1, sheet 15), while measurements were restored and even went beyond control by overexpressing Efnb2 (Figure 7B,C—Source data 1, sheet 15). Thus, Efnb2 overexpression not only compensates for the absence of Dll4 in this assay, but has an added effect as well.

Taken together, our observations with ventricular explants and HUVEC indicate that Jag1 and EPHRINB2 promote coronary vessel sprouting and branching, whereas Dll4, Mfng and Notch1 inhibit these processes. Moreover, EPHRINB2 mediates signaling from both Jag1 and Dll4 to regulate endothelial branching during coronary angiogenesis.

Discussion

The origin of the coronary endothelium from a venous source has prompted the suggestion that arteries need to be ‘reprogrammed’ from veins (Red-Horse et al., 2010). We found Jag1, Dll4, Mfng, and N1ICD expression in SV endothelium and sub-epicardial capillaries, with Dll4 and Mfng expression maintained subsequently in prospective veins. These Notch pathway elements are co-expressed with the venous marker endomucin, which is also expressed in the endocardium and SV (Cavallero et al., 2015). This implies that nascent subepicardial vessels have a mixed arterial/venous identity. During embryonic angiogenesis, Notch is necessary for artery-vein specification—the reversible commitment of ECs to arterial or venous fate—before the onset of blood flow (Swift and Weinstein, 2009). Our finding of SV endothelial cell heterogeneity in relation to arterial-venous identity is consistent with the notion that early vascular beds are phenotypically plastic during embryonic
Figure 6. EPFRINB2 rescues disrupted arterial branching in ventricular explants from Jag1 and Dll4 mutant hearts. (A) Ventricular explant assay procedure. (B–J) Representative images of E11.5 cultured ventricular explants from the following embryos: (B) control (n = 8–10), (C) Jag1<sup>flox</sup>;Nfatc1-Cre (n = 4), (D) Dll4<sup>flox</sup>;Nfatc1-Cre (n = 5), (E) Notch1<sup>flox</sup>;Nfatc1-Cre (n = 4), (F) Mfng<sup>GOF</sup>;Tie2-Cre (n = 3), (G) Dll4<sup>GOF</sup>;Tie2-Cre (n = 3), (H) Efnb2<sup>flox</sup>;Nfatc1-Cre (n = 5–7), (I) Dll4<sup>flox</sup>;Nfatc1-Cre infected with Efnb2-overexpressing lentivirus (n = 5–7). Microscope: Nikon A1-R. Software: NIS Elements AR 4.30.02. Figure 6 continued on next page.
development (Moyon et al., 2001; Chong et al., 2011; Fish and Wythe, 2015). Our results are consistent with recently published data showing that pre-arterial cells expressing Notch pathway elements are present in coronary endothelium (Su et al., 2018), prior to blood flow onset.

We show that Notch ligands Jag1 and Dll4 are required for SV sprouting angiogenesis, further supporting the notion that coronary arteries are specified by Notch prior to blood flow (Su et al., 2018) (Figure 8A). Dll4 is the key Notch ligand activator regulating embryonic (Duarte, 2004; Gale et al., 2004; Krebs et al., 2004; Benedito et al., 2008) and postnatal retinal angiogenesis (Hellström et al., 2007; Lobov et al., 2007), whereas Jag1 antagonizes Dll4-Notch1 signaling (Benedito et al., 2009) and acts downstream of Dll4-Notch1 to promote smooth muscle differentiation (Pedrosa et al., 2015). Jag1 or Dll4 inactivation in the SV results in arrested and excessive angiogenesis, respectively, resembling the situation in the retina (Benedito et al., 2009) (Figure 8B). Moreover capillary ‘entanglements’ suggestive of vascular malformations were present in the early plexus of Jag1<sup>flox</sup>;Nfatc1-Cre and Dll4<sup>flox</sup>;Nfatc1-Cre mutants. Vascular malformations in Notch mutants have been attributed to unresolved intermingling of arteries and veins during differentiation, or to failed maintenance of arterial and venous identities in the vascular bed (Gale et al., 2004; Krebs et al., 2004). The presence of these malformations in Jag1<sup>flox</sup>;Nfatc1-Cre and Dll4<sup>flox</sup>;Nfatc1-Cre mutants could therefore indicate that endothelial progenitors begin to differentiate into arteries and veins very soon after exiting the SV.

Consistent with the known roles of the Notch ligands in angiogenesis, the hierarchical organization of the coronary vascular tree was profoundly altered in the late-induced Jag1 and Dll4 mutants, implying that both Notch ligands are required for high-order complexity of the coronary vessels (Figure 8C). Jag1 or Dll4 inactivation during coronary arterial remodeling results in smaller diameter arteries, consistent with Notch promoting vascular remodeling and arterial fate commitment, as described in other developmental settings (Figure 8D; (Duarte, 2004; Gale et al., 2004; Krebs et al., 2004). However, gain of Notch function also led to smaller caliber arteries (Figure 8D), when the opposite outcome was anticipated (Uyttendaele et al., 2001; Trindade et al., 2008; Krebs et al., 2010). This result implies that non-physiological variations of Notch activity lead to the arrest of coronary artery differentiation. Dll4 mutants also showed coronary vessel hemorrhaging reminiscent of that found in vascular disorders (Park-Windhol and D’Amore, 2016) or tumors (Goel et al., 2011) and indicative of disrupted endothelial integrity.

We reasoned that the coronary maturation defects observed in Jag1 or Dll4 mutants are due, at least in part, to the loss of EphrinB2 function given that Efnb2 is a direct Dll4-Notch signaling target in ECs (Iso et al., 2006) and endocardium during ventricular development (Grego-Bessa et al., 2007). Moreover, EphrinB2 and VEGF are functionally linked during angiogenesis and lymphangiogenesis; EphrinB2 is a direct activator of VEGFR2 and VEGFR3, and therefore cooperates in the mechanism leading to tip cell extension and vessel sprouting (Sawamiphak et al., 2010; Wang et al., 2010). Thus, Efnb2 inactivation leads to coronary artery remodeling defects, similar to those resulting from Jag1 or Dll4 inactivation, suggesting that EphrinB2 functions downstream of Notch to promote coronary arterial remodeling (Figure 8D). This notion is supported by the angiogenic ventricular explant and EC capillary tube assays, in which opposite effects of Jag1 or Dll4 deficiency on vessel branching are rescued by transduction of an EFNB2-expressing lentivirus, identifying EphrinB2 as a Notch effector during coronary artery development.

A common feature among the endocardial or endothelial Notch loss- and gain-of-function models analyzed in our study is the thin ventricular wall (Figure 8B,D). Of interest is that myocardial inactivation of Jag1, or combined inactivation of Jag1 and Jag2, or Mib1, leads to thinner ventricular walls, accompanied by reduced cardiomyocyte proliferation, disrupted ventricular chamber patterning, and cardiomyopathy (Luxán et al., 2013; D’Amato et al., 2016). In contrast, chamber patterning is maintained in endocardial Jag1, Dll4, and Efnb2 mutants and endothelial Dll4 and Jag1 mutants. Our results suggest that disturbed ventricular wall growth in the earlier E11.5-E12.5 Jag1<sup>flox</sup>;Nfatc1-Cre is caused by altered endocardial-cardiomyocyte signaling, as suggested for Dll4<sup>flox</sup>;Nfatc1-Cre
Figure 7. EPFRN2 rescues defective capillary network formation resulting from shRNA-mediated silencing of JAG1 and DLL4 in HUVEC. (A) qRT-PCR of JAG1, DLL4 and HEY1 after transduction of shRNA. (B) Representative phase contrast images (1 of 2 experiments) of the HUVEC network, after transduction of indicated shRNA and rescue by EFNB2 analyzed by the Angiogenesis Analyzer from ImageJ. (C) Significantly changed measurements in the analyzed area: nodes surrounded by junctions (red dot surrounded by dark blue circle). Isolated elements (dark blue line). Segments (yellow).

Figure 7 continued on next page
mutants (D’Amato et al., 2016). In the later E14.5-E16.5 Jag1\textsuperscript{flox};Pdgfb-iCre\textsuperscript{ERT2}, Dll4\textsuperscript{flox};Pdgfb-iCre\textsuperscript{ERT2}, Jag1\textsuperscript{flox};Cdh5-Cre\textsuperscript{ERT2} and Dll4\textsuperscript{flox};Cdh5-Cre\textsuperscript{ERT2} mutant embryos, a thinner ventricular wall would be due to the lack of a well-formed coronary plexus (Figure 8D). At E12.5-E13-5, myocardial growth may also depend on 'angiocrine signals' from the un-perfused primitive coronary plexus (Rafii et al., 2016), as the diffusion limit of oxygen and nutrients from the endocardium is reached during the transition from endocardial to coronary myocardial perfusion. Therefore, ventricular compaction relies on two interconnected Notch-dependent processes: patterning and maturation of the chamber myocardium, and timely development of a functional coronary vessel network, as previously suggested (D’Amato et al., 2016). This may be clinically relevant to the study and treatment of cardiomyopathies.

Materials and methods

Key resources table

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Nowotschin et al., 2013) | CBF1:H2B-Venus |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Kisanuki et al., 2001) | Tie2-Cre |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Wu et al., 2012) | Nfatc1-Cre |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Wang et al., 2010) | Pdgfb-iCre\textsuperscript{ERT2} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Wang et al., 2010) | Cdh5-Cre\textsuperscript{ERT2} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Koch et al., 2008) | Dll4\textsuperscript{flox} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Mancini et al., 2005) | Jag1\textsuperscript{flox} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Grunwald et al., 2004) | Efnb2\textsuperscript{flox} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (Radtke et al., 1999) | Notch1\textsuperscript{flox} |                       |
| Genetic reagent                  | Mus Musculus (Mouse strain) | (D’Amato et al., 2016) | Mfng\textsuperscript{GOF} |                       |

Sequenced-based reagent

| FH1\_DLL4                        | 5’-GTTACACAGTGAAGAAGCGCC-3’ | KiCqStart_\_SIGMA qPCR primer |
|-----------------------------------|-----------------------------|-----------------------------|
| RH1\_DLL4                        | 5’-CTCTCCTCTGATATCAACAC-3’  | KiCqStart_\_SIGMA qPCR primer |
| FH1\_JAG1                        | 5’-ACTACTCTATGGCTTTGCG-3’   | KiCqStart_\_SIGMA qPCR primer |
| RH1\_JAG1                        | 5’-ATAGCTCTGGTACATTCCGG-3’  | KiCqStart_\_SIGMA qPCR primer |
| FH1\_HEY1                        | 5’-CCGGATCAATACAGTGTTCGC-3’ | KiCqStart_\_SIGMA qPCR primer |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Sequenced-based reagent           | RH1_HEY1    | 5'-CTTTTTCTAGCTTAGCAGATCC-3' | KiCqStart_ | Sigma qPCR primer |
| Sequenced-based reagent           | FH1_EFNB2   | 5'-AAAGTTGGACAAAGATGCAAG-3' | KiCqStart_ | Sigma qPCR primer |
| Sequenced-based reagent           | RH1_EFNB2   | 5'-TGACCAGCTTCTAGTTCTG-3' | KiCqStart_ | Sigma qPCR primer |
| Transfected construct (human)     | VSV-G       | Viral Vectors Unit, CNIC, Spain |             | Lentiviral construct to transfect shRNA |
| Cell line (include species here)  | HUVEC       | Lonza               |             |                        |
| Transfected construct (mouse)     | pRLL-IRESeGFP | Addgene          |             | Lentiviral construct to transfect eGFP or Full-length murine EphrinB2 |
| Transfected construct (human)     | shRNA to JAG1 | Sigma             | SHCLNG-NM_000214; Clone ID:NMI_000214.2-3357s21c1; Clone ID:NMI_000214.2-1686s21c1; | transfected construct (human) |
| Transfected construct (human)     | shRNA to DLL4 | Sigma             | SHCLNV-NM_019074; Clone ID:NMI_019074.2-2149s21c1; Clone ID:NMI_019074.2-2276s21c1; | transfected construct (human) |
| Antibody                          | Anti-CD31/Pecam1 (Monoclonal Rat) | BD Biosciences Phramingen | 550274 MEC13.3 | IF=1:100 |
| Antibody                          | Dll4 (Polyclonal Rabbit) | Santa Cruz Biotechnology | Sc-28915 | IF=1:100 |
| Antibody                          | Endomucin (Polyclonal Rat) | Santa Cruz Biotechnology | sc-65495 V.7C7 | IF=1:200 |
| Antibody                          | Jag1 (Monoclonal Rabbit) | Cell Signaling Technology | 2620 28H8 | IF=1:100 |
| Antibody                          | NFATc1 (Monoclonal Mouse) | Enzo Life Sciences | ALX-804-022-R100 7A6 | IF=1:100 |
| Antibody                          | Cleaved Notch1 (Val1744) (Monoclonal Rabbit) | Cell Signaling Technology | 4147 D3B8 | IF=1:100 |
| Antibody                          | p27 (Polyclonal Mouse) | Medical and Biological Laboratories | K0082-3 p27 Kip1 | IF=1:100 |
| Antibody                          | Notch 3 (Polyclonal Rabbit) | Abcam | ab23426 G00041 | IF=1:100 |
| Antibody                          | ERG (Monoclonal Rabbit) | Abcam | ab110693 EPR3863 | IF=1:100 |
| Antibody                          | Glut1 (Polyclonal Rabbit) | MERCK Millipore | 07-1401 C00222 | IF=1:100 |
| Antibody                          | BrdU (Monoclonal Mouse) | BD Biosciences | 347580 B-44 | IF=1:50 |
| Antibody                          | Isolectin IB4- Alexa Fluor 647 | Molecular Probes | i32450 | IF=1:300 |

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Mouse strains and genotyping

Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Madrid regional government (Ref. PROEX 118/15). All animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005. Mouse strains were CBFI:H2B-Venus (Nowotschin et al., 2013), Tie2-Cre (Kisanuki et al., 2001), Nfatc1-Cre (Wu et al., 2012), Pdgfb-iCreERT2 (Wang et al., 2010), Cdh5-CreERT2 (Wang et al., 2010), Dll4 flox (Koch et al., 2008), Jag1 flox (Mancini et al., 2005), Ehnb2 flox (Grunwald et al., 2004), Notch1 flox (Radtke et al., 1999), Mfn2 GOF (D’Amato et al., 2016). To generate the R26CAGDll4 GOF transgenic line, a full-length mouse Dll4 cDNA was obtained from clone IMAGE 6825525. The sequence was PCR amplified with Phusion High-Fidelity DNA Polymerase (NEB) and primers containing BamHI and ClaI sites and was cloned in-frame with 6 Myc Tag epitopes into the BamHI and ClaI sites of a pCS2-MT plasmid. The Dll4-MT fragment was subcloned into pCDNA3.1 with BamHI and EcoRI and excised with BamHI and NotI. We then modified pCCALL2 (Lobe et al., 1999) by cloning new XbaI sites before and after a CAG cassette, which includes a CMV enhancer/β-actin promoter and a rabbit β-globin polyA signal. The KpnI and NotI PCR Dll4-MT was cloned into the BglII-NotI sites of the modified pCCALL2. The XbaI-cassette containing CAG-loxP-β-Geo-loxP-Dll4-IREScGFP was obtained by digestion and cloned into the XbaI site of the pROSA26-1 plasmid (Soriano, 1999). The final construct was linearized with XhoI and electroporated into R1 mESCs derived from a cross of 129/Sv x 129/Sv-CP mice (Nagy et al., 1993). After G418 (200 μg/ml) selection for 7 days, 231 clones were picked. Homologous recombination was identified by Southern blot of EcoRV-digested DNA and hybridized with 5' and 3' probes. About 25% of the clones were positive, and we selected three clones to confirm karyotype. One positive clone was injected into C57/BL6C blastocysts to generate chimaeras that transmitted the transgene to their offspring. The resulting founders were genotyped by PCR of tail genomic DNA using primers targeting the R26 locus before and after the cloning site and the transgene polyA signal.
Figure 8. Coronary arterial development is regulated by a Dll4-Jag1-EphrinB2 signaling cascade. (A,B) Embryonic stages E11.5-E12.5. Development of the primitive coronary plexus. (A) Left: sinus venous (SV)-derived vessels covering the dorsal side of a wild-type heart. Zoomed view showing a subepicardial vessel with a subset of ECs expressing N1ICD (red). These N1ICD-expressing cells are equivalent to pre-artery cells defined by Su et al. (2018). Center: cross-section through the ventricular wall. SV-derived ECs invade the sub-epicardial space over the myocardium to cover the heart dorsally. Some pre-artery ECs invade the myocardium and begin to differentiate into arteries. Right: during EC sprouting and proliferation, a regulatory balance between Dll4/Mfng and Jag1 modulates Notch signaling output downstream of Vegf. (B) Left: E12.5 Jag1flox;Nfatc1-Cre (Jag1LOF; top) exhibit arrested coronary angiogenesis, Dll4flox;Nfatc1-Cre mutants (Dll4LOF; bottom) display increased angiogenesis, and both mutants display vascular malformations. The zoomed views show details of a sub-epicardial vessels with increased N1ICD expression in the Jag1LOF mutants (red), and decreased expression in the Dll4LOF mutants (blue). Center: the sub-epicardial capillary plexus is either poorly developed in Jag1LOF mutants or over-developed in Dll4LOF mutants, and with vascular malformations. Mutant hearts have a thin myocardial wall, but maintain myocardial patterning (grey-brown boundary). Right: Endocardial Jag1 deletion disrupts the regulatory balance with Dll4/Mfng, leading to increased N1ICD and arrested migration and proliferation. In contrast, endocardial Dll4 deletion results in decreased N1ICD signaling, and increased network complexity and proliferation. (C,D) Embryonic stages E14.5-E16.5. Arterial differentiation and plexus remodeling. (C) Left: coronary vasculature at the dorsal aspect of the wild-type heart. Zoomed view showing a coronary artery with an inner layer of ECs expressing N1ICD (red) and an outer layer of smooth muscle cells (yellow). Center: capillary differentiation and patterning give rise to large veins sub-epicardially and large arteries intra-myocardially. Right: systemic blood flow activates Dll4/Jag1/Mfng/N1ICD/EphrinB2 signaling to drive terminal arterial differentiation and remodeling. (D) Left: in coronary endothelial-Dll4flox;Pdgfb-iCreERT2 or -Jag1flox;Pdgfb-iCreERT2 mutants, the coronary vasculature is mis-patterned. Zoomed view detailing near absence of arteries in Dll4 mutants, and comparatively smaller caliber coronary artery in Jag1 mutants. The arteries in both mutants have an inner layer of a ‘leaky’ endothelium and an outer layer of poorly differentiated perivascular cells (white). Center: Dll4 mutants are characterized by the near-absence of coronary arteries while arteries in Jag1 mutants are decreased. In either case, veins are unaffected. Mutant hearts have a thin myocardial wall but maintain myocardial patterning (grey-brown boundary). Right: Coronary endothelial-Jag1 or Dll4LOF leads to increased or decreased N1ICD respectively, causing arrested arterial differentiation and remodeling. Inactivation of Efnb2 leads to a similar phenotype. Endothelial Notch gain-of-function (GOF), resulting from Dll4 or Mfng overexpression, also leads to increased N1ICD and disrupted arterial differentiation and remodeling.
Throughout the MS, Jag1$^{flox/flox}$,Nfatc1-Cre mice are called Jag1$^{flox/flox}$,Nfatc1-Cre for simplicity; this also applies to the Dll4$^{flox/flox}$,Nfatc1-Cre mice, Jag1$^{flox/Pdgfb-iCre^{ERT2}}$, mouse, Dll4$^{flox/Pdgfb-iCre^{ERT2}}$, Dll4$^{flox/Cdh5-Cre^{ERT2}}$, Jag1$^{flox/Cdh5-Cre^{ERT2}}$, Mfn2$^{GOF}$,Tie2-Cre mice (which carry two extra copies of Mfn2), and Efnb2$^{flox}$,Nfatc1-Cre mice, but not to Dll4$^{GOF}$,Tie2-Cre mice, which carry only one extra copy of Dll4.

**Tamoxifen induction**

Double heterozygous Cdh5-Cre$^{ERT2/+}$,Jag1$^{flox/+}$ females were crossed with homozygous Jag1$^{flox/flox}$ males, and pregnant females were administered by oral gavage 200 µl of tamoxifen solution (Sigma, T5648; 5 mg/ml; generated by diluting 50 mg of tamoxifen in ml of 95% ethanol plus 9 ml of Corn Oil). To obtain Jag1$^{flox/flox}$,Cdh5-Cre$^{ERT2/+}$ embryos, pregnant females were induced at E9.5 and opened at E16.5. To obtain Dll4$^{flox/flox}$,Cdhl5-Cre$^{ERT2/+}$ embryos, Dll4$^{flox/flox}$ males were crossed with double heterozygous Cdh5-Cre$^{ERT2/+}$,Dll4$^{flox/+}$ females, and pregnant females were induced at E12.5 and opened at E15.5. With Pdgfb-iCre$^{ERT2}$, double heterozygous Pdgfb-iCre$^{ERT2/+}$,Jag1$^{flox/+}$ (or Dll4$^{flox/+}$) males were crossed with homozygous Jag1$^{flox/flox}$ or Dll4$^{flox/flox}$ females. Pregnant females were induced at E12.5 and dissected at E15.5 (for Jag1$^{flox}$), or induced at E14.5 and dissected at E15.5 for Dll4$^{flox}$.

**Histology and in situ hybridization**

Hematoxylin and eosin (H & E) staining and in situ hybridization (ISH) on sections were performed as described (Kanzler et al., 1998). Details of probes will be provided on request.

**Whole-mount immunofluorescence**

Embryos were dissected and fixed for 2 hr in 4% paraformaldehyde (PFA) in PBS at 4°C, then permeabilized for 1 hr with 0.5% Triton X-100/PBS and subsequently blocked for 1 hr in Histoblock solution (5% goat serum, 3% BSA, 0.3% Tween-20 in PBS). After several washes in PBS-T (PBS containing 0.1% Tween-20), embryos were mounted in 1% agar in a 60 mm petri dish. For whole-mount immunofluorescence on E11.5-E12.5 CBF:H2B-Venus embryos endogenous GFP, was imaged with a NIKON A1R confocal microscope. Z-stacks were captured every 5 µm.

For EdU immunofluorescence, pregnant females at E12.5 were injected intraperitoneally with 100 µl EdU nucleotides (2 mg/ml in PBS). The mice were euthanized 1 hr later and fixed in 4% PFA. EdU incorporation was detected with the Click-iT EdU Imaging Kit (Thermo Fisher Scientific, C10340) according to manufacturer’s instructions. For IsoB4 and Endomucin immunostaining on E11.4-E16.5 embryos, imaging by confocal z-projection of the deeper section of the myocardium captures the arteries (IsoB4-stained) with the outer section corresponding to the veins (IsoB4-Endomucin-stained). N1ICD embryos were fixed for 5 hr at 4°C in frozen Methanol, washed and incubated 20 min at 98°C in DAKO target retrieval solution pH6 (Agilent). After washing in H2O, hearts were fixed again in acetone at −20°C for 10 min. After washing in PBT, the hearts were blocked in 10% FBS, 5% BSA, 0.4% TritonX100 for 3 hr at RT under gentle rocking. Hearts were incubated with primary N1ICD-antibody at 1:500 for 2 days at 4°C and allowed to settle at RT for 1 hr. This was followed by washing 5 hr in PBS/0.4% TritonX100 at RT. Hearts were then incubated in presence of anti-rabbit HRP (1:500), DAPI (1:1500) and anti-VE cadherin (1:500) overnight at 4°C. After resting for 1 hr at RT, hearts were washed in PBT, and 30 min in presence TSA (tyramides) diluted 1:200 in PBS. Quantifications were carried out using ImageJ software. The main coronary trees were selected with the tool ‘Freehand selection’ manually and measured directly from the autoscaled images obtained by Z-projection. The selected area was quantified in µm². Confocal microscopy analysis was carried out on a Nikon A1R or Leica LAS-AF 2.7.3.

**Immunofluorescence on sections**

Paraffin sections (7 µm) were incubated overnight with primary antibodies, followed by 1 hr incubation with a fluorescent-dye–conjugated secondary antibody. N1ICD, Dll4, Jag1, and p27 were immunostained using tyramide signal amplification (TSA) (Del Monte et al., 2007); see Supplementary file 4 for antibodies. All immunostainings were performed in the same way except for co-immunostaining of SM22a (+ secondary chicken anti-goat-594) and Notch3 (+secondary donkey anti-rabbit-488), which required continued permeabilization in PBS 1X + 0.3% TritonX100.
Images were processed using ImageJ software. For p21 and N1ICD quantification, the number of positive nuclei was divided by the total number of nuclei counted on sections (≥3). For αSMA and Notch3 quantification, the number of mural cells surrounding intramyocardial vessels expressing both SMA+ and Notch3+ (double positives) was counted and divided by the total number of intramyocardial vessels defined by Iso B4 staining in the same section. For SM22a and Notch3 quantification, the number of double- SM22 and Notch3 positive cells was divided by the total number Notch3-positive cells counted on sections (≥3).

**Hypoxia analysis**

Pimonidazole hydrochloride (Hypoxyprobe) was injected intraperitoneally at 60 mg/kg to E12.5 pregnant females. After 2.5 hr, embryos were dissected in cold PBS and fixed O/N in 4% PFA. Embryos were paraffin embedded. Sections at 7 μm were deparaffinized and boiled in 10 mM, pH = 6 Na Citrate solution. For immunofluorescence, sections were permeabilized with 0.4% Triton in PBS, blocked with 5% FBS and incubated anti-Glut1 (1:100) and anti-Pymonidazole (1:200) antibodies O/N at 4˚C. Secondary antibodies (anti-Rabbit Alexa568 for Glut1 and anti-mouse biotinylated for Pymonidazole 1:400, respectively) were incubated for 1 hr at RT. Pymonidazole signal was amplified using Fluorescein Tyramides (1:100). Counterstaining: IB4 (endocardium/endothelium) 1:200; DAPI (nuclei) 1:3000.

**Mouse ventricular explant culture and immunofluorescence**

Heart ventricular explants were performed as previously described with minor modifications (Wu et al., 2012). Ventricles were dissected from E10.5 or E11.5 embryos (with removal of the outflow tract and atria), rinsed with PBS to remove circulating cells, and placed in Nunc four-well plates. Matrigel (Corning Matrigel Basement Membrane Matrix, *LDEV-Free, 10 mL growth factor reduced, BD Biosciences 354234) was diluted 1:1 with DMEM plus 10% FBS and 10 ng/ml Vegf (Human-Vegf 165 Peprotech). Each well had a total volume of 400 μl, and 3–4 hearts were cultured for 6 days and then fixed in 4% PFA4% 10 min and washed twice with PBS1X for 15 min. Explants were permeabilized with 0.5% Triton X-100 for 1 hr, blocked with Histoblock solution (FBS Histoblock) for 2–3 hr at RT, and incubated with anti-CD31 1:100 (Purified Rat Anti-Mouse CD31 BD Biosciences Pharmingen) overnight at 4˚C. Anti-rat biotinylated was used as secondary antibody and diluted in BSA 1:150. Staining was amplified with the ABC kit and 3 min of TSA (tyramides) diluted 1:100 in PBS.

**Quantification of compact myocardium thickness**

The method used was a modification of that described by Chen et al. (2009); Yang et al. (2012). Briefly, 7 μm paraffin sections from E12.5, E15.5 and E16.5 wild type (WT) and mutant hearts were stained with anti-CD31 or Endomucin (Emcn) and anti-MF20 antibodies to visualize ventricular structures. Confocal images were obtained with a NIKON A1R confocal microscope. Measurements were made using ImageJ software. In E15.5 and E16.5 heart sections, endocardial cells were stained with anti-Emcn and myocardium with anti-cTnT. Left and right ventricles were analyzed separately. For each measurement, settings were kept constant for all images, using the scale bar recorded in each image as the reference distance. The thickness of the compact myocardium was measured by dividing the ventricle into left and right parts. Several measurements were taken in each region and the mean was expressed in μm.

**Quantification of Jag1 and Dll4 expression in sections**

Quantification of ISH signal were carried out using ImageJ software. Compact myocardium area was selected manually with the tool ‘Polygon selection’. Coronary area was identified in this selection by color intensity using ‘Color Threshold plugin’ with the settings: Hue: Min = 145 Max = 199, Saturation: Min = 60 Max = 255, Brightness: Min = 0 Max = 255. Positive coronary area for Dll4 or Jag1 expression was calculated dividing the coronary area positive for each gene by the total compact myocardium area. Statistical analysis was assessed by Student’s t-test.

**India ink perfusion**

Embryos were collected from E15.5 to E18.5 in PBS containing 2 μg/ml heparin. The thoracic cavities were immediately cut and transferred to DMEM containing 10%FBS and heparin on ice. Hearts were
carefully dissected from the surrounding tissue and then placed on an inverted Petri dish and gently dried to avoid movement during perfusion. India ink or red tempera was diluted in PSB/heparin and injected from the ascending aorta with a borosilicate glass tube thinned to the appropriate diameter and attached to a mouth pipette. India ink was slowly injected by minute puffs of breath during diastolic intervals. Hearts were fixed in 4% PFA, dehydrated, cleared in BABB (benzyl alcohol: benzyl benzoate, 1:1) and imaged with a stereomicroscope.

**Lentiviral production**

Bacterial glycerol stocks for JAG1 and DLL4 MISSION shRNA were purchased from SIGMA. MISSION shRNAs: shRNA JAGGED1_2: clone ID: NM_000214.2–3357 s21c1; shRNA JAGGED1_3: clone ID: NM_000214.2–1686 s21c1; shRNA DLL4_1: clone ID_ NM_019074.2–2149 s21c1; shRNA DLL4_1: clone ID_ NM_019074.2–821 s21c1. Concentrated lentiviral particles were produced by triple transfection in HEK293T cells. Briefly, HEK293T cells were cultured in 150 mm plates for transfection of the lentiviral vectors. The psPAX2 packing plasmid, the pMD.G envelope plasmid coding for the VSV-G glycoprotein (Viral Vectors Unit, CNIC, Spain) and the LTR-bearing shuttle lentiviral plasmids were cotransfected using the calcium phosphate method. The transfection medium was replaced with fresh medium 16 hr post-transfection. Viral supernatants were harvested at 72 hr post-transfection, filtered through a 0.45 μm filter (Steriflip-HV, Millipore, MA) and concentrated by ultracentrifugation. Precipitated viruses were resuspended in pre-chilled 1X PBS, aliquoted and stored at –80°C for further use. Viral titre was measured on viral genomes by qPCR using the standard curve method with primers against the LTRs. Forward primer: 5’-AGCTTGCCTTGAGTGCTTCAA-3’; Reverse primer: 5’-AGGGTCTGAGGGATCTCTAGTTA-3’. Full-length murine EphrinB2 (Efnb2), kindly provided by Ralf Adams (MPI for Molecular Biomedicine, University of Münster, Germany), was subcloned into the lentiviral vector pRLL-IRES-eGFP (Addgene). Concentrated lentiviruses expressing pRLL-IRES-eGFP or pRLL-Mfng-IRES-eGFP were obtained as described (Esteban et al., 2011). Viruses were titrated in Jurkat cells, and infection efficiency (GFP-expressing cells) and cell death (propidium iodide staining) were monitored by flow cytometry.

**Culture and infection on HUVEC**

Human umbilical vein endothelial cells (HUVECs) were maintained in supplemented EGM2 medium (EGM2 Bulletkit, K3CC-3162 Lonza). Cells were transduced on suspension at a multiplicity of infection (m.o.i.) of 80 with combinations of 2 shRNAs against JAG1 or DLL4 together with GFP or EFNB2 overexpressing lentivirus and seeded onto 24-well plates or 12-well plates at a density of 3 × 104 or 6 × 104 cells/well respectively. Infection efficiency was near 100%. HUVECs were harvested after 48 hr. Gene expression analysis was performed using KCQStart SYBR Green predesigned primers (Sigma-Aldrich).

**Angiogenesis assays on matrigel**

Angiogenesis in vitro was investigated using endothelial cell tube formation assay on Matrigel (#354234 Corning) as previously described (Grant et al., 1991). Briefly 50 μl of ice cold Matrigel was coated on a 96 micro-well plate as a base for tube formation. After allowing the gel to settle in the incubator for 30 min at 37°C, 5% CO2, HUVECs in basic EGM medium (EBM2 H3CC-3156, Lonza) were seeded in triplicate at a density of 12.5 × 104 on Matrigel and incubated at 37°C, 5% CO2. After 5 hr, tube formation was monitored by phase-contrast microscope. The tube networks were quantified using NIH Image J with Angiogenesis plugin software (http://image.bio.methods.free.fr/ImageJ/?angiogenesis-analyzer-for-imageJ). Excel data were represented on Graphpad Prism seven as mean with SD. For statistical analysis a one-way ANOVA and Tukey post-hoc tests were performed.

**Statistical analysis**

Statistical analysis was carried out using Prism 7 (GraphPad). All statistical tests were performed using two-sided, unpaired Student’s t-tests, except for Figures 1, 2 and 7 and figures Supplements 4, 9 and 12 where we performed one-way ANOVA to assess statistical significance with a 95% confidence interval, where numerical data are presented as mean ± SD; results are marked with one asterisk (*) if p<0.05, two (**) if p<0.01, and three (***) if p<0.001. Sample size was chosen empirically.
according to previous experience in the calculation of experimental variability. No statistical method was used to predetermine sample size. All experiments were carried out with at least three biological replicates. The numbers of animals used are described in the corresponding figure legends. Animals were genotyped before the experiment and were caged together and treated in the same way. Variance was comparable between groups throughout the manuscript. We chose the appropriate tests according to the data distributions. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

### RNA-sequencing

RNA was isolated at E12.5, from whole hearts of $Jag^{1}\text{floox};Nfatc1-\text{Cre}$ and $Dll4^{\text{floox}};Nfatc1-\text{Cre}$ embryos, as well as their WT counterparts (four replicate samples for each condition, pooling four embryos per sample, in all cases). At E15.5, RNA was isolated from ventricles of $Dll4^{\text{floox}};Cdh5-\text{Cre}^{\text{ERT2}}$ (three replicate samples, pooling three embryos per sample), as well as their WT counterparts (four replicate samples, pooling three embryos per sample). RNA-Seq data for $Jag^{1}\text{floox};Nfatc1-\text{Cre}$, $Dll4^{\text{floox}};Nfatc1-\text{Cre}$, and $Dll4^{\text{floox}};Cdh5-\text{Cre}^{\text{ERT2}}$ experiments was generated by CNIC’s Genomics Unit. RNA-Seq sequencing reads were pre-processed by means of a pipeline that used FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to assess read quality and Cutadapt v1.6 (Martin, 2011) to trim sequencing reads, thus eliminating Illumina adaptor remains, and to discard reads shorter than 30 bp. Resulting reads were mapped against the mouse transcriptome (GRCm38 assembly, Ensembl release 76) and quantified using RSEM v1.2.20 (Li and Dewey, 2011).

Around 80–90% of the reads participated in at least one reported alignment. Expected expression counts calculated with RSEM were then processed with an analysis pipeline that used Bioconductor package Limma (Ritchie et al., 2015) for normalization and differential expression testing in six pairwise contrasts involving mutant versus WT comparisons. We discarded two of the $Dll4^{\text{floox}};Nfatc1-\text{Cre}$ samples based on preliminary analyses of $Dll4$ expression levels and clustering patterns in diagnostic principal component analysis plots. Changes in gene expression were considered significant if associated with a Benjamini-Hochberg (BH) adjusted $p$-value <$0.05$. The number of differentially expressed genes detected in comparisons between $Jag^{1}\text{floox};Nfatc1-\text{Cre}$, $Dll4^{\text{floox}};Nfatc1-\text{Cre}$ and $Dll4^{\text{floox}};Cdh5-\text{Cre}^{\text{ERT2}}$ and their control counterparts was 205, 257 and 156, respectively. Enrichment analyzes were performed with IPA (Ingenuity Pathway Analysis, Qiagen, http://www.ingenuity.com).

IPA was used to identify collections of genes associated with canonical pathways, common upstream regulators, or functional terms significantly overrepresented in the sets of differentially expressed genes; statistical significance was defined by Benjamini-Hochberg adjusted $p$-value <$0.05$. Circular plots summarizing the association between genes and enriched pathways, upstream regulators, and functional terms were generated with GOplot (Walter et al., 2015).

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Ethics
Animal experimentation: Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Madrid regional government (Ref. PROEX 118/15). All animal procedures conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforced in Spanish law under Real Decreto 1201/2005.

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Additional files
Supplementary files
• Source data 1. Numerical data that are represented as a graph in a Figure or figure supplement, distributed in sheets corresponding to a given Figure or figure supplement by their order of appearance in the text, where they are also referred to.
• Supplementary file 1. Lethality phases of Jag1<sup>flox</sup>;Nfatc1-Cre, Dll4<sup>flox</sup>;Nfatc1-Cre, Jag1<sup>flox</sup>;Pdgfb-iCre<sup>ERT2</sup>, Dll4<sup>flox</sup>;Cdhh5-iCre<sup>ERT2</sup>, Dll4<sup>flox</sup>;Cdhh5-Cre<sup>ERT2</sup>, Mfn2<sup>flox</sup>;Tie2-Cre, Efnb2<sup>flox</sup>;Nfatc1-Cre embryos.
• Supplementary file 2. Lists of differentially expressed genes identified after analysis of RNA-seq data, for contrasts between E12.5 Jag1<sup>flox</sup>;Nfatc1-Cre (Jag1Nfatc1 211 Deg); E12.5 Dll4<sup>flox</sup>;Nfatc1-Cre (Dll4Nfatc1 274 Deg); E16.5 Dll4<sup>flox</sup>;Cdhh5-Cre<sup>ERT2</sup> (Dll4Cdhh5 163 Deg) and their control counterparts. Uprregulated genes appear in red background and downregulated genes in blue.
• Supplementary file 3. Results obtained with IPA for the sets of genes detected as differentially expressed (adjusted P value < 0.05) in contrasts between Jag1<sup>flox</sup>;Nfatc1-Cre (JAG1NF), Dll4<sup>flox</sup>;Nfatc1-Cre (Dll4NF), Dll4<sup>flox</sup>;Cdhh5-Cre<sup>ERT2</sup> (Dll4Cdhh5) and their control counterparts. Upstream Regulator analyses results, in sheets labelled as ‘UpRegTransc’, are restricted to molecules of type ‘transcription regulator’. For each predicted upstream regulator, tables describe expression log ratio (when available), predicted activation state, activation z-score, enrichment p_value and the collection
of differentially expressed genes that are targets of the regulator. Downstream Effect analyses results, in sheets labelled as ‘CardSystDevFunc’, are restricted to functions of category ‘Cardiovascular System Development and Function’. For each functional term, tables describe enrichment p value, predicted activation state, activation z-score and the collection of differentially expressed genes associated to that particular function. Positive and negative z-score values suggest activation or inhibition of the corresponding upstream regulator or function in the mutant or control condition, respectively; abs(z-score)>2 and p value < 0.05 are considered significant.

- Supplementary file 4. List of primary and secondary antibodies used in this study to immunodetect proteins in whole-mount or paraffin sections.
- Transparent reporting form

**Data availability**

Sequencing data have been deposited in GEO under accession code GSE110614.

The following dataset was generated:

| Author(s)            | Year | Dataset title                                                                 | Dataset URL                                                                 | Database and Identifier               |
|----------------------|------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------|---------------------------------------|
| Travisano SI, MacGrogan D, de la Pompa JL | 2018 | Coronary arterial development is regulated by a Dll4-Jag1-EphrinB2 signaling cascade | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE110614 | NCBI Gene Expression Omnibus, GSE110614 |

**References**

- Benefido R, Trindade A, Hirashima M, Henrique D, da Costa L, Rossant J, Gill PS, Duarte A. 2008. Loss of notch signalling induced by Dll4 causes arterial calibre reduction by increasing endothelial cell response to angiogenic stimuli. *BMC Developmental Biology* 8:117. DOI: https://doi.org/10.1186/1471-213X-8-117
- Benefido R, Roca C, Sörenslen I, Adams S, Gossler A, Fruttiger M, Adams RH. 2009. The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**:1124–1135. DOI: https://doi.org/10.1016/j.cell.2009.03.025
- Blance R, Gerhardt H. 2013. VEGF and notch in tip and stalk cell selection. *Cold Spring Harbor Perspectives in Medicine* **3**:a006569. DOI: https://doi.org/10.1101/cshperspect.a006569
- Cavalleri S, Shen H, Yi C, Lien C-L, Kumar SR, Suc HM. 2015. CXCL12 signaling is essential for maturation of the ventricular coronary endothelial plexus and establishment of functional coronary circulation. *Developmental Cell* **33**:469–477. DOI: https://doi.org/10.1016/j.devcel.2015.03.018
- Chen MJ, Yokomizo T, Zeiger BM, Dzierzak E, Speck NA. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* **457**:887–891. DOI: 10.1038/nature07619
- Chen H, Sharma B, Akerberg BN, Numi HJ, Kivela R, Saharinen P, Aghajanian H, McKay AS, Bogard PE, Chang AH, Jacobs AH, Epstein JA, Stankunas K, Altaloo K, Red-Horse K. 2014. The sinus venous contributes to coronary vasculature through VEGFC-stimulated angiogenesis. *Development* **141**:4500–4512. DOI: https://doi.org/10.1242/dev.113639
- Chong DC, Koo Y, Xu K, Fu S, Cleaver O. 2011. Stepwise arteriovenous fate acquisition during mammalian vasculogenesis. *Developmental Dynamics* **240**:2153–2165. DOI: https://doi.org/10.1002/dvdy.22706
- Del Monte G, Grego-Bessa J, González-Rajal A, Bolós V, De La Pompa JL. 2007. Monitoring Notch1 activity in development: evidence for a feedback regulatory loop. *Developmental Dynamics* **236**:2594–2614. DOI: https://doi.org/10.1002/dvdy.21246
- Dor Y, Camenisch TD, Itin A, Fishman GI, McDonald JA, Carmeliet P, Keshet E. 2001. A novel role for VEGF in endocardial cushion formation and its potential contribution to congenital heart defects. *Development* **128**:1531–1538. PMID: 11290292
- Duarte A. 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes & Development* **18**:2474–2478. DOI: https://doi.org/10.1101/gad.1239004
- D’Amato G, Luxán G, del Monte-Nieto G, Martínez-Poveda B, Torroja C, Walter W, Bochter MS, Benefido R, Cole S, Martínez F, Hadjantonakis A-K, Uemura A, Jiménez-Borreguero Lj, de la Pompa JL. 2016. Sequential notch activation regulates ventricular chamber development. *Nature Cell Biology* **18**:7–20. DOI: https://doi.org/10.1038/ncb3280
- Elmasri H, K provisional, Tepi Y, Ghelfi E, Weng M, Ince TA, Kozakewich H, Bischoff J, Cataltepe S. 2009. Fatty acid binding protein 4 is a target of VEGF and a regulator of cell proliferation in endothelial cells. *The FASEB Journal* **23**:3865–3873. DOI: https://doi.org/10.1096/fj.09-134882
- Esteban V, Méndez-Barbero N, Jesús Jiménez-Borreguero L, Roqué M, Novensá L, Belén García-Redondo A, Salaices M, Vila L, Arbonés ML, Campanero MR, Redondo JM. 2011. Regulator of calcineurin 1 mediates pathological vascular wall remodeling. *The Journal of Experimental Medicine* **208**:2125–2139. DOI: https://doi.org/10.1084/jem.20110503

- Pompa JL proteins in whole-mount or paraffin sections.
- Grogan D, de la Pompa JL respectively; abs(z-score)>2 and p value < 0.05 are considered significant.
- Esteban V, MacLennan D, MeCrediba H, Scura A, Meñigean M, Guzmán G, Ryan D, Koon RM, Guesdon J-L, Gribb F, Hooper M, Hogg N, Joffre V, Ondarza J, Fernández-Luna J. 2013. The Notch ligand Dll4 regulates fistula formation and arteriogenesis for hair follicle growth. *Nature Cell Biology* **15**:1527–1534. DOI: https://doi.org/10.1038/ncb3280
Farber G, Parks MM, Lustgarten Guahmich N, Zhang Y, Monette S, Blanchard SC, Di Lorenzo A, Blobel CP. 2019. ADAM10 controls the differentiation of the coronary arterial endothelium. Angiogenesis 22:237–250. DOI: https://doi.org/10.1007/s10456-018-9653-2

Fish JE, Wythe JD. 2015. The molecular regulation of arteriovenous specification and maintenance. Developmental Dynamics 244:391–409. DOI: https://doi.org/10.1002/dvdy.24525, PMID: 25641373

Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM, Murphy AJ, Adams NC, Lin HC, Holash J, Thurston G, Yancopoulos GD. 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. PNAS 101:15949–15954. DOI: https://doi.org/10.1073/pnas.0407290101

Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, Jain RK. 2011. Normalization of the vasculature for treatment of Cancer and other diseases. Physiological Reviews 91:1071–1121. DOI: https://doi.org/10.1152/physrev.00038.2010

Grant DS, Lelkes PI, Fukuda K, Kleinman HK. 1991. Intracellular mechanisms involved in basement membrane induced blood vessel differentiation in vitro. In Vitro Cellular & Developmental Biology - Animal 27:327–336. DOI: https://doi.org/10.1086/vcb.27.4.1152

Grego-Bessa J, Luna-Zurita L, del Monte G, Bolós V, Melgar A, Arandilla A, Garratt AN, Zang H, Mukouyama Ysuke, Chen H, Shou W, Ballester E, Esteller M, Rojas A, Pérez-Pomares JM, de la Pompa JL. 2007. Notch signaling is essential for ventricular chamber development. Developmental Cell 12:415–429. DOI: https://doi.org/10.1016/j.devcel.2006.12.011

Grunwald IC, Korte M, Adelmann G, Plueck A, Kullander K, Adams RH, Frotscher M, Bonhoeffer T, Klein R. 2004. Hippocampal plasticity requires postsynaptic ephrinB3. Nature Neuroscience 7:33–40. DOI: https://doi.org/10.1038/nn1164

Harjes U, Bridges E, McIntyre A, Fielding BA, Harris AL. 2014. Fatty Acid-biding protein 4, a point of convergence for angiogenic and metabolic signaling pathways in endothelial cells. Journal of Biological Chemistry 289:23168–23176. DOI: https://doi.org/10.1074/jbc.M114.576512

Hellström M, Phng L-K, Hofmann JJ, Wallgard E, Coutlas L, Lindblom P, Alva J, Nilsson A-K, Karlsson L, Gaiano N, Yoon K, Rossant J, Iruela-Arispe ML, Kalén M, Gerhardt H, Betsholtz C. 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature 445:776–780. DOI: https://doi.org/10.1038/nature05571

Iso T, Maeno T, Oike Y, Yamazaki M, Doi H, Arai M, Kurabayashi M. 2006. Dll4-selective notch signaling induces ephrinB2 gene expression in endothelial cells. Biochemical and Biophysical Research Communications 341:708–714. DOI: https://doi.org/10.1016/j.bbrc.2006.01.020

Jones EA, le Noble F, Eichmann A. 2006. What determines blood vessel structure? genetic prespecification vs. hemodynamics. Physiology 21:388–395. DOI: https://doi.org/10.1152/physiol.00020.2006, PMID: 17119151

Kania A, Klein R. 2016. Mechanisms of ephrin-Eph signalling in development, physiology and disease. Nature Reviews Molecular Cell Biology 17:240–256. DOI: https://doi.org/10.1038/nrm.2015.16, PMID: 26790531

Kanzler B, Kuschert SJ, Liu YH, Mallo M. 1998. Hoxa-2 restricts the chondrogenic domain and inhibits bone formation during development of the branchial area. Development 125:2587–2597, PMID: 9636074

Kisanoaki YY, Hammer RE, Miyazaki J-Ichi, Williams SC, Richardson JA, Yanagisawa M. 2001. Tie2-Cre transgenic mice: a new model for endothelial Cell-Lineage analysis in vivo. Developmental Biology 230:230–242. DOI: https://doi.org/10.1006/dbio.2000.0106

Koch U, Fiorini E, Benedito R, Besseyrias V, Schuster-Gossler K, Pierres M, Manley NR, Duarte A, MacDonald HR, Radtke F. 2008. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. The Journal of Experimental Medicine 205:2515–2523. DOI: https://doi.org/10.1084/jem.20080829

Krebs LT, Shutter JR, Tanigaki K, Honjo T, Stark KL, Gridley T. 2004. Haploinsufficient lethality and formation of arteriovenous malformations in notch pathway mutants. Genes & Development 18:2469–2473. DOI: https://doi.org/10.1101/gad.1239204, PMID: 15466160

Krebs LT, Starling C, Chervonsky AV, Gridley T. 2010. Notch1 activation in mice causes arteriovenous malformations phenocopied by ephrinB2 and EphB4 mutants. Genesis 48:146–150. DOI: https://doi.org/10.1002/dvg.20599, PMID: 20101399

Krock BL, Skuli N, Simon MC. 2011. Hypoxia-induced angiogenesis: good and evil. Genes & Cancer 2:1117–1133. DOI: https://doi.org/10.1177/1947601111423654, PMID: 22866203

Le Noble F, Fleury V, Pries A, Corvall P, Eichmann A, Reneman RS. 2005. Control of arterial branching morphogenesis in embryogenesis: go with the flow. Cardiovascular Research 65:619–628. DOI: https://doi.org/10.1016/j.cardiores.2004.09.018, PMID: 15664388

Li B, Dewey CN. 2011. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics 12:323. DOI: https://doi.org/10.1186/1471-2105-12-323

Liao D, Johnson RS. 2007. Hypoxia: a key regulator of angiogenesis in Cancer. Cancer and Metastasis Reviews 26:281–290. DOI: https://doi.org/10.1007/s10555-007-9066-y, PMID: 17603752

Lobe CG, Koop KE, Kreppner W, Lameli H, Gertsenstein M, Nagy A. 1999. Z/AP, a double reporter for Cre-Mediated recombination. Developmental Biology 208:281–292. DOI: https://doi.org/10.1006/dbio.1999.2909

Lobov IB, Renard RA, Papadopoulos N, Gale NW, Thurston G, Yancopoulos GD, Wiegand SJ. 2007. Delta-like ligand 4 (DLL4) is induced by VEGF as a negative regulator of angiogenic sprouting. PNAS 104:3219–3224. DOI: https://doi.org/10.1073/pnas.0611206104

Lúñan G, Casanova JC, Martínez-Poveda B, Prados B, D’Amato G, MacGrogan G, González-Rajal A, Dobarro D, Torroja C, Martínez F, Iziqierdo-García JL, Fernández-Friera L, Sabater-Molina M, Kong YY, Pizarro G, Ibañez
B, Medrano C, García-Pavía P, Gimeno JR, Monserrat L, et al. 2013. Mutations in the NOTCH pathway regulator MI1B cause left ventricular noncompaction cardiomyopathy. Nature Medicine 19:193–201. DOI: https://doi.org/10.1038/nm.3046, PMID: 23314057

Mancini Stephane J. C., Mantei N, Dumortier A, Suter U, MacDonald HR, Radtke F. 2005. Jagged1-dependent notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood 105:2340–2342. DOI: https://doi.org/10.1182/blood-2004-08-3207

Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBO Journal 17:10–12. DOI: https://doi.org/10.1463/17.1.200

Menéndez-Montes J, Escobar B, Palacios B, Gómez MJ, Izquierdo-Garcia JL, Flores L, Jiménez-Borreguero LJ, Aragones J, Ruiz-Cabello J, Torres M, Martin-Puig S. 2016. Myocardial VHL-HIF signaling controls an embryonic metabolic switch essential for cardiac maturation. Developmental Cell 39:724–739. DOI: https://doi.org/10.1016/j.devcel.2016.11.012, PMID: 27997827

Moyon D, Pardanaud L, Yuan L, Bréant C, Eichmann A. 2001. Plasticity of endothelial cells during arterial-venous differentiation in the avian embryo. Development 128:3359–3370. PMID: 11546752

Nagy A, Rossant J, Nagy R, Abramow-Newley W, Roder JC. 1993. Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. PNAS 90:8424–8428. DOI: https://doi.org/10.1073/pnas.90.18.8424, PMID: 8378314

Nowotchin S, Xenopoulos P, Schrode N, Hadjantonakis AK. 2013. A bright single-cell resolution live imaging reporter of notch signaling in the mouse. BMC Developmental Biology 13:15. DOI: https://doi.org/10.1186/1471-213X-13-15, PMID: 23617465

Park-Windhol C, D’Amore PA. 2016. Disorders of vascular permeability. Annual Review of Pathology: Mechanisms of Disease 11:251–281. DOI: https://doi.org/10.1146/annurev-pathol-012615-044506, PMID: 26907525

Pedroso AR, Trindade A, Fernandes AC, Carvalho C, Gigante J, Tavares AT, Diégoz-Hurtado R, Yagita H, Adams RH, Duarte A. 2015. Endothelial Jagged1 antagonizes Dll4 regulation of endothelial branching and promotes vascular maturation downstream of Dll4/Notch1. Arteriosclerosis, Thrombosis, and Vascular Biology 35:1134–1146. DOI: https://doi.org/10.1161/ATVBAHA.114.304741, PMID: 25762724

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR, Aguet M. 1999. Deficient T cell fate specification in mice with an induced inactivation of Notch1. Immunity 10:547–558. DOI: https://doi.org/10.1016/S1074-7613(00)80054-0, PMID: 10367900

Rafii S, Butler JM, Ding BS. 2016. Angiocrine functions of organ-specific endothelial cells. Nature 529:316–325. DOI: https://doi.org/10.1038/nature17040, PMID: 26791722

Red-Horse K, Ueno H, Weissman IL, Krasnow MA. 2010. Coronary artery formation by developmental reprogramming of venous cells. Nature 464:549–553. DOI: https://doi.org/10.1038/nature08873

Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. 2015. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Research 43:e47. DOI: https://doi.org/10.1093/nar/gkv007, PMID: 25605792

Sanchis-Gomar F, Perez-Quilis C, Leischik R, Lucia A. 2016. Epidemiology of coronary heart disease and acute coronary syndrome. Annals of Translational Medicine 4:256. DOI: https://doi.org/10.21037/atm.2016.06.33, PMID: 27500157

Sawamiphak S, Seidel S, Essmann CL, Wilkinson GA, Pitulescu ME, Acker T, Acker-Palmer A. 2010. Ephrin-B2 regulates VEGFR2 function in developmental and tumour angiogenesis. Nature 465:487–491. DOI: https://doi.org/10.1038/nature08995

Soriano P. 1999. Generalized lacZ expression with the ROSA26 cre reporter strain. Nature Genetics 21:70–71. DOI: https://doi.org/10.1038/3007

Su T, Stanley G, Sinha R, D’Amato G, Das S, Rhee S, Chang AH, Poduri A, Raftrey B, Dinh TT, Roper WA, Li G, Butler JM, Ding BS. 2016. Angiocrine functions of organ-specific endothelial cells. Developmental Biology 404:549–553. DOI: https://doi.org/10.1016/j.ydbio.2016.01.028, PMID: 29973725

Swift MR, Weinstein BM. 2009. Arterial-venous specification during development. Circulation Research 104:576–588. DOI: https://doi.org/10.1161/CIRCRESAHA.108.188805, PMID: 19286613

Tian X, Hu T, Zhang H, He L, Huang X, Liu Q, Yu W, He L, Yang Z, Zhang Z, Zhong TP, Yang X, Yang Z, Yan Y, Baldini A, Sun Y, Lu J, Schwartz RJ, Evans SM, Gittenberger-de Groot AC, et al. 2013. Subepicardial endothelial cells invade the embryonic ventricle wall to form coronary arteries. Cell Research 23:1075–1090. DOI: https://doi.org/10.1038/cr.2013.83, PMID: 23797856

Tian X, Pu WT, Zhou B. 2015. Cellular origin and developmental program of coronary angiogenesis. Circulation Research 116:515–530. DOI: https://doi.org/10.1161/CIRCRESAHA.116.305097, PMID: 25634974

Trindade A, Kumar SR, Schemet JS, Lopes-da-Costa L, Becker J, Jiang W, Liu R, Gill PS, Duarte A. 2008. Overexpression of delta-like-4 induces arterialization and attenuates vessel formation in developing mouse embryos. Blood 112:1720–1729. DOI: https://doi.org/10.1182/blood-2007-09-112748, PMID: 18559979

Udan RS, Culver JC, Dickinson ME. 2013. Understanding vascular development. Wiley Interdisciplinary Reviews: Developmental Biology 2:327–346. DOI: https://doi.org/10.1002/wdev.91, PMID: 23799579

Uyttendaele H, Ho J, Rossant J, Kitajewski J. 2001. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. PNAS 98:5643–5648. DOI: https://doi.org/10.1073/pnas.091584599
Volz KS, Jacobs AH, Chen Hl, Poduri A, McKay AS, Riordan DP, Kofler N, Kitajewski J, Weissman I, Red-Horse K. 2015. Pericytes are progenitors for coronary artery smooth muscle. eLife 4:e10036. DOI: https://doi.org/10.7554/eLife.10036, PMID: 26479710

Walter W, Sánchez-Cabo F, Ricote M. 2015. GOplot: an R package for visually combining expression data with functional analysis. Bioinformatics 31:2912–2914. DOI: https://doi.org/10.1093/bioinformatics/btv300, PMID: 25964631

Wang Y, Nakayama M, Pitulescu ME, Schmidt TS, Bochenek ML, Sakakibara A, Adams S, Davy A, Deutsch U, Lüthi U, Barberis A, Benjamin LE, Mäkinen T, Nobes CD, Adams RH. 2010. Ephrin-B2 controls VEGF-induced angiogenesis and lymphangiogenesis. Nature 465:483–486. DOI: https://doi.org/10.1038/nature09002

Wang Y, Wu B, Lu P, Zhang D, Wu B, Varshney S, Del Monte-Nieto G, Zhuang Z, Charafeddine R, Kramer AH, Sibinga NE, Frangogiannis NG, Kitsis RN, Adams RH, Alitalo K, Sharp DJ, Harvey RP, Stanley P, Zhou B. 2017. Uncontrolled angiogenic precursor expansion causes coronary artery anomalies in mice lacking Pofut1. Nature Communications 8:578. DOI: https://doi.org/10.1038/s41467-017-00654-w, PMID: 28924218

Wu B, Zhang Z, Lui W, Chen X, Wang Y, Chamberlain AA, Moreno-Rodriguez RA, Markwald RR, O’Rourke BP, Sharp DJ, Zheng D, Lenz J, Baldwin HS, Chang CP, Zhou B. 2012. Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. Cell 151:1083–1096. DOI: https://doi.org/10.1016/j.cell.2012.10.023, PMID: 23178125

Yang J, Bücker S, Jungblut B, Böttger T, Cinnamon Y, Tchorz J, Müller M, Bettler B, Harvey R, Sun QY, Schneider A, Braun T. 2012. Inhibition of Notch2 by Numb/Numblike controls myocardial compaction in the heart. Cardiovascular Research 96:276–285. DOI: https://doi.org/10.1093/cvr/cvs250, PMID: 22865640

Zhang H, Pu W, Li G, Huang X, He L, Tian X, Liu Q, Zhang L, Wu SM, Sucov HM, Zhou B. 2016a. Endocardium minimally contributes to Coronary endothelium in the embryonic ventricular free walls. Circulation Research 118:1880–1893. DOI: https://doi.org/10.1161/CIRCRESAHA.116.308749

Zhang H, Pu W, Tian X, Huang X, He L, Liu Q, Li Y, Zhang L, He L, Liu K, Gillich A, Zhou B. 2016b. Genetic lineage tracing identifies endocardial origin of liver vasculature. Nature Genetics 48:537–543. DOI: https://doi.org/10.1038/ng.3536, PMID: 27019112

Zhang Z, Zhou B. 2013. Accelerated coronary angiogenesis by vegfr1-knockout endocardial cells. PLOS ONE 8:e70570. DOI: https://doi.org/10.1371/journal.pone.0070570, PMID: 23894673