The CXCL12/CXCR4 Axis Plays a Critical Role in Coronary Artery Development

Graphical Abstract

Highlights

- Cxcl12 and Cxcr4 mutants lack intra-ventricular coronary arteries
- Coronary artery stem formation is impaired in Cxcl12 mutants
- Peritruncal blood vessels fail to penetrate the aortic endothelium
- CXCL12 signaling is required for anastomosis of peritruncal endothelial cells

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In Brief
Coronary artery stem formation depends on the establishment of connections between the peritruncal capillary network and the lumen of the developing aorta. Ivins et al. have shown that in mice deficient for the chemokine CXCL12, the formation of such connections is disrupted, leading to severe coronary artery defects.

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The CXCL12/CXCR4 Axis Plays a Critical Role in Coronary Artery Development

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SUMMARY

The chemokine CXCL12 and its receptor CXCR4 have many functions during embryonic and postnatal life. We used murine models to investigate the role of CXCL12/CXCR4 signaling in cardiac development and found that embryonic Cxcl12-null hearts lacked intra-ventricular coronary arteries (CAs) and exhibited absent or misplaced CA stems. We traced the origin of this phenotype to defects in the early stages of CA stem formation. CA stems derive from the peritruncal plexus, an encircling capillary network that invades the wall of the developing aorta. We showed that CXCL12 is present at high levels in the outflow tract, while peritruncal endothelial cells (ECs) express CXCR4. In the absence of CXCL12, ECs were abnormally localized and impaired in their ability to anastomose with the aortic lumen. We propose that CXCL12 is required for connection of peritruncal plexus ECs to the aortic endothelium and thus plays a vital role in CA formation.

INTRODUCTION

Formation of the coronary vascular system coincides with a period of rapid expansion in cardiac size between embryonic day (E) 11.5 and E13.5. Myocardial growth requires enhanced oxygen delivery and triggers an influx of endothelial cells (ECs) that undergo vasculogenesis to form a capillary plexus (Mikawa and Fischman, 1992). Coronary ECs invade the myocardium from a variety of sources, including the pro-epicardium (Katz et al., 2012), endocardium (Wu et al., 2012), and sinus venosus (Red-Horse et al., 2010; Tian et al., 2013b). Coronary lineage tracing has been utilized in a recent study to show both complementary (sinus venosus and endocardium) and cardiac-wide (pro-epicardium) distribution of coronary ECs from these three sources (Chen et al., 2014b). Extensive angiogenic remodeling of the vascular plexus takes place from E14.5, giving rise to the mature coronary vascular system (Kattan et al., 2004; Morabito et al., 2002).

Normally, two major coronary arteries (CAs) arise from the aorta, with their stems positioned at the left and right aortic valve sinuses. The origins of the CA stems were first elucidated in avian embryos, where it was found that they form from an ingrowth of ECs into the aorta (Bogers et al., 1989; Waldo et al., 1990). Further investigation showed that endothelial strands grow into the aorta from a ring of capillaries encircling the outflow tract (peritruncal plexus) and penetrate the aortic sinuses at multiple sites. By mechanisms that are unknown so far, the peritruncal vessels persist and remodel at only two of these sites, ultimately forming the CA stems (Alkawa and Kawano, 1982; Ando et al., 2004; Poelmann et al., 1993; Ratajska and Fiejka, 1999; Waldo et al., 1990). This ingrowth model of CA stem formation has recently been confirmed in mice (Tian et al., 2013a). The molecular signals underlying CA stem and ostia formation have yet to be fully elucidated, although factors such as vascular epidermal growth factor (VEGF), fibroblast growth factor 2 (FGF-2), and platelet-derived growth factor β (PDGFβ) have been found to be required (Tomanek et al., 2006, 2008; Van den Akker et al., 2008), indicating that multiple growth factors and signaling molecules are involved in this tightly orchestrated process. In particular, a role for chemokine activity in directing EC migration to the aortic sinuses has been suggested (Waldo et al., 1990).

CXCL12 and the G-protein-coupled cell surface receptor CXCR4 are a chemokine ligand and receptor pair that have been shown to regulate cell behavior and migration in numerous contexts (Kiefer and Siekmann, 2011; Teicher and Fricker, 2010). During development, CXCL12 drives migration of cells expressing CXCR4, including ECs (Li et al., 2013) and neural crest (Belmadani et al., 2005; Escot et al., 2013; Theveneau et al., 2010), while knockout studies have shown that mice lacking either CXCL12 or CXCR4 die in utero or perinatally, with identical defects in hematopoiesis and cerebellar development as well as a cardiac ventricular septal defect (VSD) (Nagasawa et al., 1996, Ma et al., 1998, Tachibana et al., 1998, Zou et al., 1998). CXCL12-CXCR4 signaling has been shown to play a significant role in angiogenesis in the gut (Ara et al., 2005; Tachibana et al., 1998), kidney (Takabatake et al., 2009), and brain, indicative of an organ-specific role in vascularization (Kiefer and Siekmann, 2011).

We have now identified a critical role for CXCL12-CXCR4 signaling in the development of CAs in mice. By examining the hearts of Cxcl12 and Cxcr4 mutant embryos late in development, we have uncovered a severe phenotype, with complete absence...
Figure 1. Mutation of Cxcl12 Causes Severe CA Defects

(A–B') Control (A) and Cxcl12−/− (B and B') E18.5 embryos; note the small size of the null embryo in (B) and the hemorrhagic appearance of the embryo in (B').

(C and D) H&E-stained E18.5 heart sections; boxed areas in control (C) and Cxcl12 null (D) are shown enlarged to the right (boxes i and ii). Arrows in (i) indicate intra-ventricular CAs; arrowheads in (ii) indicate dilated veins in the null.

(E and F) Frontal view of E18.5 Cxcl12+/+ (E) and Cxcl12−/− (F) hearts.

(G–I) Whole-mount SM22α antibody staining of CAs at E18.5. Arrow indicates left CA in a wild-type control (G); Cxcl12−/− (H) and Cxcr4−/− (I) hearts lack CAs.

(J–M') Analysis of CAs in E18.5 heart sections: SM22α marks CAs, EMCN labels endocardium, and nuclei are counterstained with DAPI. CAs are indicated by arrows in wild-type myocardium (J) but are absent in Cxcl12 null myocardium (J'). Control coronary ostia are positioned in the aortic valve sinuses (arrows in K). Arrowhead in (K') shows a single CA proximal to the aorta (Ao) in the null, asterisks indicate thickened aortic valve leaflets. Arrowheads in (L) indicate arteries in

(legend continued on next page)
of intra-ventricular CAs and abnormalities in the number and position of coronary ostia. Detailed expression analysis and confocal microscopy in Cxcl12 mutants have enabled us to trace the origins of the defects to the development of the peritruncal plexus from which the CA stems originate. Our data suggest a model whereby CXL12 secreted by cells of the aortic wall acts as a chemoattractant for CXCR4-positive peritruncal ECs and is required for anastomosis of peritruncal ECs with the lumen of the aorta. Impairment of this process in Cxcl12 or Cxcr4 mutants results in failure of arterialization of the main coronary plexus.

RESULTS

Severe Defects in CA Development in Cxcl12- and Cxcr4-Mutant Hearts

We carried out interbreeding of Cxcl12 heterozygous animals in order to investigate the cardiovascular phenotype of Cxcl12 null embryos. Despite the reported 50% lethality of Cxcl12 mutation at E18.5 (Nagasawa et al., 1996), we were able to recover viable mutant embryos at this stage at approximately Mendelian ratios. Cxcl12 mutants were stunted in growth to a variable degree, and a number of them showed evidence of hemorrhaging (compare Figure 1A with Figures 1B and 1B'). Furthermore, the hearts of a number of them showed evidence of hemorrhaging (compare mutant embryos at this stage at approximately Mendelian ratios.

The aortic valve leaflets also appeared profoundly abnormal in Cxcl12 mutants (Figure 1K'). In order to visualize the artery and valve defects more clearly, high-resolution episcopic microscopy (HREM) (Mohun and Weninger, 2012) was carried out on Cxcl12 null (n = 3) and wild-type (n = 3) hearts (Figures 1N–1O'; Movie S1). In the null hearts, the aortic valve leaflets appeared thickened and were positioned at differing levels within the aorta (Figure 1O'). Similar defects were observed in the pulmonary valves (Movie S1). Each HREM-examined null heart had a single, distally positioned ostium, with a short section of proximal CA that failed to branch into the ventricles and terminated in the IVS (Figures 1N and S1D–S1H'; Movie S1). In one mutant heart, the ostium was also displaced ventrally (Figure 1N). Thus, all Cxcl12 null hearts examined combined a lack of distal CAs with severe semi-lunar valve (SLV) defects and a failure of proximal artery/CA stem formation on at least one side.

Expression of Cxcl12 and Cxcr4 during Cardiac Vascularization

Given the CA phenotype described earlier, we wanted to examine cardiac expression of both Cxcl12 and Cxcr4 in detail from E11.5 onward. This developmental stage was selected as hearts are avascular before E10.5, while E11.5 marks the beginning of the expansion of the coronary plexus that reportedly invades the ventricular myocardium from E12.5 to E14.5 (Red-Horse et al., 2010; Tian et al., 2013b).

Expression of Cxcl12 and Cxcr4 was analyzed by carrying out in situ hybridization on serial sections. At E11.5, Cxcl12 was highly expressed in the wall of the outflow tract (OT) and at a lower level in the ventricular walls and trabeculated myocardium, while Cxcr4 expression was localized to the endocardial lining of the OT and atrioventricular cushions (AVCs) as previously reported (Siervo et al., 2007), (Figures 2A and 2A'). At E12.5, a number of vessels expressing Cxcr4 were observed, restricted to the myoendothelial layer of the ventricle walls and the peritruncal region (Figures 2B' and 2C'; data not shown). The sites of Cxcr4 expression correlated with the emerging intra-myocardial coronary plexus (Tian et al., 2013b). Cxcr4 was also expressed in the endothelium and underlying mesenchyme of the aortic and pulmonary valves, as well as in the AV valves from E12.5 onward (data not shown). High levels of Cxcl12 expression were seen in the walls of the aorta and pulmonary trunk at this developmental stage (Figure 2B), as well as in the left ventricle, with lower levels in right ventricular myocardium (Figures 2B' and 2C).

Expression of both genes was maintained at E13.5 (Figures 2D–2E'), with increasing numbers of Cxcr4-expressing vessels observed in the ventricular myocardial walls by E14.5 (Figure 2G'). At E13.5, Cxcr4 expression was also detected in peritruncal ECs, coinciding with a region of strong Cxcl12 expression in the aortic wall (Figures 2E and 2E'), while from E14.5 onward, Cxcr4 expression was observed in the maturing CAs and ostia (Figure 2F'; data not shown). Cxcl12 expression remained at

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Figure 2. Endothelial Expression of Cxcr4 Is Required for Coronary Arteriogenesis

(A–G) Cxcl12 and Cxcr4 expression in serial sections of E11.5–E14.5 wild-type hearts (in situ hybridization). (A and A') Sagittal sections at E11.5. Arrow in (A) indicates Cxcl12 in the inferior wall of the OT; arrowheads in (A') show Cxcr4 in the lining of OT and AVCs (bracketed). (B–C') Transverse sections at E12.5. (B and C) Cxcl12 is highly expressed in OT vessels and left ventricle (LV) and at a lower level in the right ventricle (RV). Arrows in (C') indicate Cxcr4-positive vessels in the ventricular wall; dotted line in (C') delineates ventricle wall. (D–E') Sagittal sections at E13.5. Inset in (D') shows enlargement of Cxcr4-positive vessels in the ventricular wall (boxed); arrowheads in (E') indicate Cxcr4 expression in the peritruncal plexus (PtP); note strong aortic Cxcl12 expression in the consecutive section in (E). (F–G') Transverse sections at E14.5. Arrows in (F') indicate Cxcr4 in the maturing LCA and ostium. Boxed area shown enlarged in (G and G') shows Cxcr4 and Cxcl12 expression in a large coronary vessel. Ao, aorta; PT, pulmonary trunk; tb, trabeculated myocardium.
high levels in both aorta and ventricular myocardium at E14.5 (Figures 2F and 2G), decreasing at later stages (data not shown).

In addition, from E14.5, larger intra-myocardial vessels were observed to express both Cxcr4 and Cxcl12 (Figures 2G and 2H; data not shown). Cxcr4 was not expressed in sub-epicardial ECs or vessels at this or any of the stages examined.

Antibody labeling of OT sections confirmed the expression of Cxcr4 in peritruncal plexus ECs (Figures 2H–2K). While anti-PECAM antibodies labeled ECs of the peritruncal plexus and aortic lumen (Figure 2I), Cxcr4 staining was restricted to peritruncal ECs and aortic valve endothelium (Figure 2J). Co-staining of E14.5 heart sections with Cxcr4 and PECAM antibodies corroborated the aforementioned in situ data, further showing that ventricular expression of Cxcr4 was confined to ECs localized in the myocardium (Figures 2L–2N). By E16.5, Cxcr4 expression was reduced or extinguished in most of the intra-myocardial capillary plexus, being retained mainly in larger vessels (Figures 2O–2Q; Figure 3E). Thus, Cxcr4 expression appeared to be linked to the re-modeling/maturation of the coronary network, declining as larger vessels developed.

To test whether deletion of Cxcr4 in the endothelial lineage alone was sufficient to recapitulate the CA phenotype found in Cxcl12/Cxcr4 mutants, we used the Tie2-Cre transgene and a floxed Cxcr4 allele (Nie et al., 2004) (Figures 2R–2V). Of seven E16.5 Tie2-Cre/Cxcr4fl/fl embryos examined by whole-mount immunolabeling, four were observed to display the same severe CA phenotype as Cxcl12 and Cxcr4 nulls (Figure 2S). Two had a milder phenotype, with CAs that followed a normal course over the left and right ventricles but that appeared shorter and less branched than in control hearts (particularly the left CA [LCA]; one example shown in Figure 2T), and one appeared normal (data not shown). Sections from another severely affected conditional null showed that, as well as lacking intra-ventricular CAs (Figure 2V), SLV defects were also present, along with a single, distally positioned ostium (Figures S2A' and S2B'). Thus, EC-specific deletion of Cxcr4 recapitulated the CA phenotype observed in Cxcl12 nulls.

As a proportion of the conditional mutants appeared to be less severely affected than full nulls, we investigated the efficiency of the Cre recombinase in Tie2-Cre/Cxcr4fl/fl hearts at E15.5. In one

Figure 3. ECs in Cxcl12-Mutant Hearts Are Normally Localized, but Fail to Downregulate Cxcr4 at E16.5, while Loss of Cxcr4 in the Smooth Muscle Lineage Does Not Affect CA Development

(A–B') In situ hybridization analysis shows that Cxcr4 expression is unaltered in E14.5 Cxcl12−/− myocardial vessels (A') compared to control (A). Arrowheads in (B) and (B') indicate coronary ostia; arrows indicate proximal CAs, LV, left ventricle; Ao, aorta.

(C and C') PECAM antibody staining shows that ECs are localized normally in Cxcl12−/− ventricular myocardium at E13.5, RV, right ventricle.

(D and D') NOTCH1 expression in control and Cxcl12−/− hearts.

(E–G') Cxcr4/PECAM antibody labeling of E16.5 wild-type and Cxcl12−/− heart sections; Cxcr4 is co-expressed with PECAM in all intra-myocardial (myo) vessels in the null (E'–G') in contrast to its more restricted expression in the control (E–G). Dotted lines delineate sub-epicardial (epi) and intra-myocardial regions of ventricle wall.

(H and H') Conditional deletion of Cxcr4 in the smooth muscle lineage using SM22α-Cre does not affect CA development. SM22α/EMCN/DAPI labeling shows normal CAs (arrows) present in E18.5 SM22α/Cxcr4fl/fl heart sections in (H).

Scale bars represent 100 μm. See also Figure S3.
of four conditional null hearts examined by in situ hybridization, strong Cxcr4 expression was still observed (Figure S2D), indicating a failure of Cre-mediated recombination in the endothelium. Therefore, inefficient recombination of the Cxcr4 floxed allele by the Tie2-driven Cre recombinase likely explains the reduced penetrance of the CA phenotype in the conditional nulls.

**CA Maturation Failure in Cxcl12 Mutants Is Not Caused by Defective EC Localization, Arterial Fate Specification, or vSMC Recruitment**

As described earlier, CXCR4 is expressed in ECs located within the compact myocardium of the ventricles. ECs localized in the ventricular myocardium are fated to form capillaries and arteries rather than veins (Red-Horse et al., 2010; Tian et al., 2013b; Wu et al., 2012). To investigate the possible mechanism(s) underlying the failure of CA formation in the Cxcl12 nulls, we first examined the localization of Cxcr4-expressing ECs in Cxcl12 mutant hearts. However, this was found to be normal, as determined by in situ hybridization at E14.5 (compare Figures 3A and 3A’). The main difference observed at this stage was the lack of any larger bore vessels on the left side of the aorta (a single ostium was observed on the right side) (compare Figures 3B and 3B’), consistent with the defects we observed at later stages. Similarly, immunolabeling with PECAM antibody at E13.5 (Figures 3C and 3C’) and E14.5 (data not shown) did not reveal abnormalities in the distribution of coronary ECs throughout the myocardial and sub-epicardial layers of Cxcl12 mutant hearts. Thus, CXCL12-CXCR4 signaling is not required for myocardial invasion by coronary ECs.

To test the possibility that the myocardially localized ECs were not correctly specified for arterial differentiation, we used NOTCH1 as a marker of arterial fate (del Monte et al., 2011). However, we found that NOTCH1 was expressed normally in Cxcl12 mutant hearts at E15.5 (Figures 3D and 3D’), consistent with correct arterial specification of coronary ECs. These data indicated that coronary ECs in Cxcl12 mutants are both correctly localized and specified for arterial differentiation; therefore, the lack of CA formation is likely caused by the failure of the coronary plexus to mature/re-model. Our observation that CXCR4 expression becomes restricted mainly to larger coronary vessels between E14.5 and E16.5 (Figures 2O–2Q) suggested that CXCR4 levels may serve as an indicator of the state of maturation of the coronary plexus. Therefore, we examined CXCR4 expression in Cxcl12 mutant hearts at E16.5 and found that, in contrast to control hearts (Figures 3E–3G), small CXCR4/PECAM co-stained vessels were visible throughout the myocardium of mutant ventricles (Figures 3E’–3G’) and IVS (data not shown). This was similar to the pattern observed in E14.5 wild-type hearts (Figure 2N), supporting the hypothesis that the vascular plexus is retained in an immature state in mutant hearts. Quantification of the number of ECs in mutant hearts confirmed the increase in the proportion of CXCR4-expressing ECs but showed that there was no difference in the overall number of ECs compared to controls (data not shown).

Recruitment of mural cells (pericytes and vascular smooth muscle cells [vSMCs]) to newly formed vessels is required to stabilize, remodel, and mature the primary vasculature. As expression of CXCR4 has been reported in both pericytes and vSMC progenitors (Song et al., 2009; Zernecke et al., 2005; Petit et al., 2007), we wanted to examine the potential role of the CXCR4/CXCL12 axis in the recruitment of mural cells to the coronary vasculature. Therefore, SM22α-driven Cre recombinase was used to inactivate Cxcr4 in vSMCs and pericytes (Anastasia et al., 2014). However, CAs were found to have developed normally in all conditional nulls examined at E18.5 (Figure 3H’; n = 8), although a high percentage of SLV defects was observed (87.5% of conditional nulls) (Figures S3A’ and S3B’). Coverage of CAs with vSMCs (as analyzed using SM22α/PECAM expression) was unaffected in SM22α-Cre;Cxcr4fl/fl hearts (Figure S3C’). Therefore, a lack of mural cell recruitment does not appear to be the underlying cause of the failure of coronary plexus maturation in Cxcl12 null hearts.

**Confocal Analysis of Peritruncal Plexus Development**

Another requirement for maturation of the intra-ventricular coronary plexus is blood flow into the coronary ostia; development of the tunica media of the arteries has been shown to proceed in a proximo-distal direction at the onset of coronary flow (Ando et al., 2004; Ratajska and Fiejka, 1999; Vrancken Peeters et al., 1997). Therefore, defective development of the CA stems/ostia, preventing blood flow into the coronary vessels, would abrogate the maturation process. Absent coronary ostia in Cxcl12 null hearts coupled with expression of Cxcr4 and Cxcl12 in peritruncal ECs and aortic wall, respectively (discussed earlier) suggest a potential role for CXCL12-CXCR4 signaling in CA stem formation.

In order to better understand a potential role for CXCL12-CXCR4 signaling in this process, we first made a detailed study of peritruncal plexus formation from E11.5 onward, using confocal microscopy of optically cleared whole-mount PECAM-stained wild-type hearts. Previous studies have not investigated peritruncal plexus formation at murine developmental stages prior to E12.5. At E11.5, we observed isolated clusters of ECs in the dorsal part of the OT wall anterior to the cushions, with multiple processes contacting the OT lumen (boxed region in Figure 4A; Figure 5A). The only other coronary ECs observed at this stage were in the sub-epicardial region of the ventricles (data not shown), consistent with previously reported observations (Tian et al., 2013b). Therefore, peritruncal plexus formation precedes development of the main intra-myocardial coronary plexus. By E12.5, the peritruncal plexus had expanded around the proximal part of the aorta, anterior to the cushions; clusters of interconnected EC strands were now observed on either side of the aorta, located close to the lumen as well as in the sub-epicardial compartment, again with multiple processes forming anastomoses with the aortic lumen (Figures 4B and 5D). At this stage, the peritruncal plexus was still not continuous with the developing intra-ventricular plexus, but inter-connecting vessels were observed at the base of the aorta at a slightly later stage (approximately E12.5–E13.0; arrows in Figure 4C). By E13.5, the peritruncal plexus had taken on a more organized, mature appearance, with some of the EC strands appearing wider and lumenized (Figure S4A). Multiple points of contact were still retained between the EC strands and both sides of the aortic lumen at various points level with, and distal to, the aortic valve, as previously described (Ando et al., 2004; Poelmann et al., 1993; Ratajska and Fiejka, 1999; Tian et al.,
2013a; Waldo et al., 1990), and the peritruncal plexus was now fully connected to the main coronary capillary network. A day later, at E14.5, multiple aorta-contacting vessels were still observed in some hearts (e.g., on the right side of the aorta, shown in Figure S4D, in contrast with the major vessel on the left side), but by E15.5, these vessels had resolved into a single major lumenized vessel on either side of the aorta, generally positioned at the level of the aortic valve (data not shown).

Peritruncal ECs in Cxcl12-Null Hearts Are Impaired in Their Ability to Anastomose with the Aortic Lumen

Peritruncal plexus development was then examined in Cxcl12 mutants. At E11.5, striking peritruncal plexus abnormalities were observed in five of seven mutants examined (71%). In the examples shown in Figures 5B and 5C, clusters of ECs were observed in the sub-epicardial layer of the OT (bracketed regions); however, the ECs lacked processes contacting the aortic lumen, in contrast to controls (n = 6; Figure 5A). In addition, in each mutant, a short vessel protruded from the lumen of the OT (asterisks in Figures 5B and 5C). Dramatic differences were also observed at E12.5. Where EC strands in control aortas were observed to form multiple Anastomoses on the left and right sides of the lumen (n = 12; Figure 5D), all Cxcl12 mutants (n = 11) were characterized by reduced or absent peritruncal EC connections. In many cases, a single contacting vessel/EC strand was present (7 of 11), and peritruncal EC strands/clusters were frequently localized solely in the sub-epicardial layer of the aorta (Figures 5E and 5F). Similar results were observed at E13.5 and E14.5 (Figure S4).

Sections through the OT at E12.5 confirmed that peritruncal ECs were not absent in Cxcl12 nulls but rather were mislocalized in sub-epicardial clusters and lacked anastomotic connections with the aortic endothelium (Figures 5H and 5J). This was in contrast to controls, where anastomosing EC strands were localized in close proximity to the aortic lumen (Figures 5G and 5I). A recent study has shown that endocardial sprouts can extend from the aortic lumen and make a small contribution to the future CA stems (Chen et al., 2014a). We also observed sprouts from the aortic lumen in 33% of wild-type E12.5 hearts examined (Figure 5K); however, sprouts were wider in diameter and more frequently observed in Cxcl12 nulls (66% of nulls; Figures 5J and 5L) and could be both proximally and much more distally positioned relative to the aortic valve (Figure 5L). Quantification of the number of peritruncal aortic connec-

Tions showed a significant reduction in Cxcl12 nulls, even when the more proximal sprouts were included (Figure 5M).

Targeting Cxcl12 in Ventricular Myocardium Is Insufficient to Induce CA Defects

The defects we observed in Cxcl12 nulls suggest that CXCL12 is required for anastomosis of peritruncal ECs in the aorta but not for the initial establishment of the peritruncal plexus. This role for CXCL12 signaling correlates with its high level of expression in the developing aorta. However, Cxcl12 is also expressed in the ventricular myocardium, and we wanted to determine whether this domain of Cxcl12 expression is also important in CA development. For example, ventricular CXCL12 could play a direct role in the remodeling of the vessel network, perhaps by modulating EC proliferation in response to coronary flow. We used two different Cre drivers in conjunction with a floxed Cxcl12 allele (Greenbaum et al., 2013) in an attempt to differentially target aortic and ventricular myocardial expression of Cxcl12. Nkx2.5-Cre is an established driver in cardiac chamber cardiomyocytes (CMs) and is now known to be active in both first and second heart fields (FHF and SHF, respectively), although the extent and degree of expression in the SHF is likely to be reporter and genetic background dependent (Ma et al., 2008; Moses et al., 2001). For example, a recent study using the same Nkx2.5-Cre allele as that used here showed inefficient recombination in the OT (Ramsbottom et al., 2014). The Met2c enhancer Cre drives recombination in anterior SHF lineages (Verzi et al., 2005) but would not be expected to effect recombination in left ventricular wall myocardium (Figure 6A). Analysis of Cxcl12 expression in the conditional mutants showed that, as expected, Nkx2.5-Cre efficiently knocked down Cxcl12 in the ventricular muscle (Figure 6B') but had much less effect in the OT (Figure 6C'). Conversely, Met2c-Cre was able to substantially reduce (but not completely abrogate) Cxcl12 expression in the OT, particularly the proximal portion (Figure 6E'), and knock it out in the right ventricle and IVS while leaving left ventricular wall expression largely intact (Figure 6D'). Targeting in the right ventricle was not completely effective, as residual expression was observed at the base of the pulmonary trunk (arrow in Figure 6D').

The two conditional mutants showed remarkably different phenotypes. While normal CAs and ostia were present in Nkx2.5-Cre;Cxcl12<sup>C0/C0</sup> mutant hearts (n = 8) (Figures 6F–6G'), with no apparent difference in CA length (Figure 6H), a range of much
Figure 5. Defective Anastomosis of the Peritruncal Plexus in Cxcl12-Null Hearts

(A–F) Confocal microscopic images of E11.5–E12.5 whole-mount PECAM-labeled control and Cxcl12−/− hearts showing the OT/aorta (Ao) and peritruncal plexus (bracketed region). (A–C) Images at E11.5. Arrowheads indicate multiple points of contact between ECs and OT in the control, shown in (A). In contrast, EC clusters localized in the sub-epicardial region of Cxcl12 nulls lack connections with the OT lumen, shown in (B and C); asterisks indicate vessels that appear to sprout from the OT lumen of nulls. (D–F) At E12.5, Cxcl12 nulls (E and F) show reduced/absent peritruncal EC anastomoses (arrowheads) on both sides of the aorta, compared to controls (D); disconnected EC strands/clusters (blue arrowheads in E and F) and sub-epicardially localized vessels (arrow in F) are also observed in nulls. Dotted lines delineate the edges of the aortic cushions (AoC).

(G–J) PECAM/DAPI-stained sections (20 μm) show anastomoses (arrowheads, G and I) formed by peritruncal ECs in control hearts. Null sections lack anastomoses, and ECs are clustered away from the aortic lumen (white ovals in H and J). Arrow in (J) indicates a possible sprout from the aortic lumen.

(M) Graph showing the number of connections with the aortic lumen in control (Con) and null (Null) hearts at E12.5.

Legend continued on next page.
more severe phenotypes was observed in *Mef2c-Cre;Cxcl12fl/fl* mutant hearts (n = 9) (Table 1). CA abnormalities were seen in six mutants, including absent LCA and left ostium in four cases (Figures 6I–6K). In addition, one mutant exhibited CAs originating from the pulmonary trunk and the portion of right ventricle immediately proximal to the pulmonary valve (Figures 6L–6N), while in another mutant, both CAs branched from the right ostium (Figures S5G–S5L). These were frequently, but not always, accompanied by SLV defects (Figures S5A–S5C), which themselves could be variable in their severity (Figures S5D–S5F). SLV defects were observed in only two *Nkx2.5-Cre;Cxcl12fl/fl* mutants (an example is shown in Figure 6G”). The defects of CA formation and patterning observed in the *Mef2c-Cre;Cxcl12fl/fl* mutant, but not the *Nkx2.5-Cre;Cxcl12fl/fl* mutant, indicate that it is CXCL12 secreted by the aorta rather than the ventricular myocardium that plays the most significant role in CA development.

**DISCUSSION**

We have identified a crucial role for the CXCL12/CXCR4 axis in the development of the CAs. Confocal analysis of the OT at the earliest stages of CA stem development enabled us to identify abnormalities in the structure of the peritruncal plexus, the aortic capillary network from which the CA stems first develop. Peritruncal ECs were mislocalized and failed to connect to the aortic endothelium in *Cxcl12* mutants, resulting in defective CA stem development. Furthermore, although we showed *Cxcl12* and *Cxcr4* expression throughout the heart (in myocardium and ECs, respectively), conditional mutagenesis experiments identified the aorta as the source of CXCL12 critical for CA development. We propose that CXCL12 acts early in CA formation, guiding peritruncal plexus ECs toward the aortic lumen to form anastomoses, consistent with the known role of CXCL12/CXCR4 signaling in chemotraction and guidance of collective cell movements. Failure of this process results in absent or abnormally positioned coronary stems and ostia, as well as a subsequent lack of intra-ventricular CAs in late-gestation embryos.

Our confocal analysis of the OT of developing hearts revealed the characteristic structure of the peritruncal capillary network, which connects specifically to the aorta. This appears to develop as a separate plexus from E11.5, arising from vessels in the sub-epicardial region of the aorta and initially unconnected to the main intra-ventricular coronary plexus. This contrasts somewhat with previous studies that describe a ring of coronary vessels that surround the entire outflow region and grow toward the aorta (Ando et al., 2004; Bernanke and Velkey, 2002; Waldo et al., 1990). However, this difference could be accounted for by the earlier developmental stages at which we carried out our analyses. The ability of CXCL12 to direct migration of various cell types is well documented (Belmadani et al., 2005; Escot et al., 2013; Kasemeier-Kulesa et al., 2010; Li et al., 2013; Song et al., 2009; Theveneau et al., 2010), while the reduced anastomoses and mislocalization of peritruncal ECs observed in *Cxcl12* mutants are consistent with a lack of angiogenic guidance cues in the OT resulting from deficient CXCL12 signaling. The ectopic distal positioning of those vessels that managed to form and contact the lumen in some *Cxcl12* mutants may simply indicate the failure of anastomosis in the more proximal region of the aorta and/or may point to abnormalities of proximo-distal EC migration. However, it is not yet understood how the broad expression of CXCL12 in the aorta could correlate with this fine-tuning of CA stem positioning; this likely involves additional molecular signals, adding another layer of complexity to the process (discussed later).

In some *Cxcl12* nulls, single, high coronary ostia were associated with short proximal CAs that failed to branch. It is possible that, in contrast to normal vessels, these result from sprouting from the aortic endothelium, as large-diameter sprouting vessels were frequently observed in *Cxcl12* mutants both proximal and distal to the aortic valve. Endothelial sprouts were also observed in normal hearts, in agreement with the recent demonstration of a limited role for aortic sprouting in normal CA stem formation (Chen et al., 2014a) and in contrast to the accepted ingrowth model. It is feasible that this mechanism is over-activated in *Cxcl12* nulls in an attempt to compensate for the lack of anastomosis from peritruncal vessels. Although this is speculative, it could be tested by lineage tracing using a Cre driver specific for the endocardial lining of the aorta, such as *Nfatc1* (Wu et al., 2012). It is also unclear why the compensatory proximal stems would be unable to connect the main coronary plexus, unless this process also requires CXCL12-dependent migration of the latter toward the aorta.

Another explanation for the failure of the proximal CA stems to grow into the ventricles in the *Cxcl12* mutants could be that disrupted blood flow resulting from the abnormally distal ostia, combined with the defective aortic valve, reduces the hemodynamic forces associated with blood entering the CAs. Shear and strain forces induced by diastolic blood flow are generally accepted to act as a necessary stimulus to CA remodeling, causing an increase in vessel lumen size and inducing smooth muscle recruitment (Jones et al., 2006; Ratajska and Fiejka, 1999; Tomanek, 2005). However, the positioning of the coronary ostia has been shown to vary considerably in wild-type mouse hearts (Fernández et al., 2008) without causing disruption in intra-ventricular CA development, making this possibility less likely.

Finally, these CA stems could, in theory, form by normal means—i.e., peritruncal EC anastomosis—with the failure of the intra-ventricular CAs to mature resulting from an additional, ventricle-specific requirement for CXCL12-CXCR4 signaling. However, when ventricular expression of *Cxcl12* was targeted while expression in the aorta was left mostly intact (*Nkx2.5-Cre* conditional mutants), we did not observe major defects in CA.
Figure 6. Targeting Cxcl12 Expression in the OT Has a Severe Impact on CA Development

(A) Schematic of hearts comparing regions of Cxcl12 (blue) and Cre recombinase (as indicated by published lineage tracing) expression driven by either Nkx2.5 (red/pink) or Mef2c (green). Relative expression levels are indicated by shading. Ao, aorta; PT, pulmonary trunk; LV, left ventricle; RV, right ventricle.

(B–E') Cardiac expression of Cxcl12 was targeted using Nkx2.5 and Mef2c Cre drivers. In situ hybridizations (E14.5, heart sections) show effective targeting of Cxcl12 expression by Nkx2.5-Cre in the ventricles (B') but not the aorta (C'). Control sections are shown in (B) and (C). Mef2c-Cre largely abrogated Cxcl12 expression in aortic regions (D and D').

(legend continued on next page)
CA defects seen in the of CA stem formation. The reduced penetrance and severity of absent and mispatterned coronary ostia indicating a disruption considerably more profound impact on CA development, with (F–G'') Labeling with anti-SM22 (H) Scatter graph shows the relative length of LCAs and RCAs in control and infrequently (G''); asterisks indicate thickened aortic valve leaflets. Controls are shown in (F) and (G).

(I–N) CA defects in Arrowhead in (K') indicates an IVS vessel that branches from the RCA. (I) Control with CAs visible in left ventricle free wall. (J) Control with CAs in right ventricular free wall. (J') Example of one mutant with CAs formed within right ventricle free wall. (L–N) In one aCAs were observed in the ventral portion of the LV, branching from a CA located in the IVS.

Together with the observation of CA branches from the IVS growing into the ventral portion of the left ventricle (in the absence of a left ostium; Table 1), our results suggest that compensatory mechanisms of CA development are induced when normal CA stem development fails, perhaps facilitated by residual Cxcl12 expression in the conditional hearts.

SLV defects were always observed in Cxcl12 knockouts and frequently associated with the conditional null phenotype (SM22α-cre, MeF2c-cre, and, to a much lesser extent, Nkx2.5-Cre). Although we did not investigate the basis of the valve defects in this study, a role in endothelial-to-mesenchymal transformation (Endo-MT) seems likely, as Cxcr4 is expressed in the endothelial lining of the cardiac cushions as well as in coronary ECs. The second CXCL12 receptor, CXCR7 (Balabanian et al., 2005; Burns et al., 2006), is also expressed in the endocardial lining of the OT, while SLV defects have been identified in Cxcr7 mutants (Siervo et al., 2007; Yu et al., 2011). Therefore, a combined role for CXCR4 and CXCR7 in valve formation might be expected.

While this article was in its revision stages, the K. Red-Horse laboratory published a study describing the role of VEGF-C in peritruncal vessel growth and described a capillary plexus they termed the “aortic sub-epicardial vessels,” or ASVs, which circle the lateral side of the aorta, expanding as development proceeds, and connect to the aortic lumen (Chen et al., 2014a). We believe that these ASVs are continuous with the peritruncal vessels we describe, as we have also observed superficial vessels on the ventral side of the aorta connecting to the deeper peritruncal vessels (data not shown). However, we focused our attention more closely on the vessels deep in the aortic wall that, we consider, form the critical connections with the lumen. Chen et al. showed that VEGF-C is required for the initial growth and expansion of the peritruncal vessels and went on to describe a population of CMs specific to the aorta. The CMs are predicted to provide the angiogenic signal required for anastomosis of the peritruncal vessels with the aortic endothelium, as well as playing a role in patterning the CA stems (Chen et al., 2014a). Our results show that while CXCL12 provides an angiogenic signal for peritruncal ECs, its expression is not restricted to this minor population of CMs described; therefore, it is unlikely to be wholly

Table 1. CA and SLV Defects in MeF2c-Cre;Cxcl12fl/− Mutant Hearts

| Mutant | LCA | RCA | Ostia | SLV Defects |
|--------|-----|-----|-------|-------------|
| 1      | X   | ✓   | right ostium only | ✓           |
| 2      | X   | ✓   | right ostium only | ✓           |
| 3      | X   | ✓   | right ostium only | X           |
| 4      | X   | ✓   | right ostium only | ✓           |
| 5      | ✓   | ✓   | LCA and RCA branch from right ostium | partial   |
| 6      | ✓   | ✓   | LCA and RCA connect to RV and PT, respectively | ✓           |
| 7–9    | ✓   | ✓   | left and right ostia | ✓           |

X indicates that no defects were detected. PT, pulmonary trunk; RV, right ventricle.

aCAs were observed in the ventral portion of the LV, branching from a CA located in the IVS.

Left aortic valve leaflet was normal.

A third ostium was formed by a CA arising from the IVS and connecting to the aorta.

development. This seems to preclude a significant role for ventricular CXCL12 in CA development, although analysis of greater numbers may be required to uncover a more subtle role, e.g., in CA growth.

Targeting of Cxcl12 expression in the aorta (MeF2c-Cre) had a considerably more profound impact on CA development, with absent and mispatterned coronary ostia indicating a disruption of CA stem formation. The reduced penetrance and severity of CA defects seen in the MeF2c conditional, compared to full, Cxcl12 nulls was not unexpected, as patches of low level Cxcl12 expression remained in and around the OT in addition to the untargeted left ventricular expression. However, it is worth noting that left ventricular Cxcl12 was not sufficient to rescue LCA formation in a number of conditional mutants, underlining the critical role of OT-specific Cxcl12 expression in CA stem formation. Intriguingly, one mutant exhibited CAs connected to the pulmonary trunk. This is an unusual phenotype, although a similar scenario has been observed in a rare but serious human congenital cardiac anomaly known as anomalous origin of the pulmonary trunk. This is an unusual phenotype, although a similar scenario has been observed in a rare but serious human congenital cardiac anomaly known as anomalous origin of the pulmonary trunk (ALPACA) (Hauser, 2005; Wesselhoeft et al., 1968). Peritruncal vessels do not normally penetrate the pulmonary trunk for reasons that are not well understood, but may involve differential gene expression (Bernanke and Velkey, 2002; González-Iriarte et al., 2003). Clearly, expression of Cxcl12 is insufficient to regulate this differential invasion of the aorta, as it is expressed at equally high levels in the aorta and the pulmonary trunk; therefore, repulsive factors preventing EC migration toward the pulmonary trunk may also contribute. It is not known how such repulsion is overcome in the MeF2c-cre Cxcl12 conditional mutant, but it is possible that uneven recombination of the floxed Cxcl12 allele in the OT, leading to an altered balance of CXCL12 in the outflow vessels, may play a role.

expression in the right ventricle and IVS (D’); arrow in (D’) indicates residual Cxcl12 expression at the base of the pulmonary trunk. Cxcl12 was considerably reduced in both proximal (bracketed) and distal regions of the aorta (E’). Control sections are shown in (D) and (E). (F–G’’) Labeling with anti-SM22α antibody shows normal CAs and ostia in Nkx2.5-Cre;Cxcl12fl/− hearts (arrows in F’, G’, and G’’). SLV defects were observed infrequently (G’’); asterisks indicate thickened aortic valve leaflets. Controls are shown in (F) and (G).

(H) Scatter graph shows the relative length of LCAs and RCAs in control and Nkx2.5-Cre;Cxcl12fl/− hearts. Relative CA length was variable in both controls and conditional nulls, with no overall difference between the two groups.

(I–N) CA defects in MeF2c-Cre;Cxcl12fl/− hearts; arrows indicate CAs. Absence of the LCA and left ostium (I’ and K) was observed in several conditional nulls. Arrowhead in (K’) indicates an IVS vessel that branches from the RCA. (I) Control with CAs visible in left ventricle free wall. (J) Control with CAs in right ventricular free wall. (J’) Example of one mutant with CAs formed within right ventricle free wall. (L–N) In one MeF2c-Cre;Cxcl12fl/− heart, CAs originated from the pulmonary trunk (arrow in N) and the right ventricle, just proximal to the pulmonary trunk valve (arrows in M and M’). Boxed region in (M) is shown enlarged in (M’). A third ostium was formed in the aorta of the same mutant by a CA that branched into the IVS (arrowhead in L). Scale bars represent 100 μm. See also Figure S3.
responsible for the fine positioning of the stems. Thus, we reason that the aortic CMs may secrete an additional factor that co-operates with CXCL12 or perhaps serves to antagonize anti-angiogenic signals acting in the walls of the OT, as suggested in Chen et al.’s report. So far, preliminary analyses of Cxcl12 null OTs have not revealed major differences in the distribution of CMs (data not shown), although further investigation is required. The recent work on VEGF-C adds to previous reports by Tomanek and colleagues of a requirement for VEGF signaling in CA stem formation (Tomanek et al., 2002, 2006). Taken together, the combined data suggest a model of CA stem formation whereby VEGF-C and CXCL12 act sequentially to direct growth and anastomosis of the peritruncal vessels, respectively.

Our results have shown that the CXCL12/CXCR4 signaling pathway plays an essential role in CA development; failure of aterigenesis in Cxcl12 and Cxcr4 mutant hearts most likely explains the lethality caused by loss of either gene. Our data showing the role of CXCL12 in the anastomosis of peritruncal ECs provide insight into the molecular processes that control the earliest stages of CA development, which could potentially inform future therapeutic interventions to regenerate coronary vessels and effect cardiac repair. For instance, sequential, timed activation/repression of multiple signaling pathways, including CXCL12, e.g., using modified RNAs (Zangi et al., 2013), may well be more effective at inducing neovascularization of ischemic tissue than treatment with a single effector.

EXPERIMENTAL PROCEDURES

Mutant Mouse Breeding

Cxcl12 mutants (Cxcl12-GFP; Ara et al., 2003) and Cxcl12+/− (Greenbaum et al., 2013), Cxcr4+/− (Nie et al., 2004), Tie2-Cre (Kisanuki et al., 2001), SM22a-Cre (Holtwick et al., 2002), Nkx2.5-Cre (Moses et al., 2001), and Mef2c-Cre (Verzi et al., 2005) mice have been described previously. Generation of Cxcr4 nulls and Cxcl12 conditional mutants is described in Supplemental Experimental Procedures. Animal work was carried out according to UK Home Office regulations.

Immunolabeling

For whole-mount immunolabeling, hearts were permeabilized in PBS with 0.1% Tween 20 (PBST), blocked for 1 hr in PBST/10% goat serum, and incubated overnight at 4°C with primary antibody in blocking buffer. Primary antibodies anti-PECAM-1 (Mec13.3, Pharmingen) and anti-SM22α (Abcam) were diluted in blocking buffer at 1:50 and 1:250, respectively. Hearts were washed several times in PBST (1-hr washes) before incubation overnight at 4°C with secondary antibody (Alexa Fluor 594 conjugate, Life Technologies, Abingdon, UK). Images were captured on a Zeiss Axio Lumar.V12 stereomicroscope.

Immunolabeling of 10-μm paraffin sections and 12-μm frozen sections was carried out according to standard protocols. Briefly, sections were incubated in blocking buffer for 1 hr at room temperature (PBS/10% BSA/1% goat serum for wax sections and PBS/10% BSA/10% goat serum/0.1% Triton X-100 for cryosections) followed by primary antibody in blocking buffer at 4°C overnight. Secondary antibodies Alexa Fluor conjugated antibodies (Life Technologies, Abingdon, UK), secondary antibodies were Alexa Fluor conjugated antibodies (Life Technologies, Abingdon, UK), and DNA was counterstained using DAPI (Sigma). Images were captured on a Zeiss Axiosmager Z1 microscope. Further details, including details of antigen retrieval and antibodies used, can be found in Supplemental Experimental Procedures.

HREM

Embryos were bled out for 5 min, and then hearts were dissected out and fixed for 30 min in 4% paraformaldehyde. The hearts were then soaked in a large volume of water for 60 min (with three or four changes of water) to lyse the blood cells and transferred back to fix. HREM was carried out as described else-

where (Mohun and Weninger, 2012). See also Supplemental Experimental Procedures.

In Situ Hybridization

In situ hybridization was carried out on 10-μm paraffin sections using standard methods. Hybridization temperature was 70°C. Digoxigenin-labeled probes for Cxcl12 and Cxcr4 were synthesized from full-length IMAGE clones (IMAGE:3483088 and IMAGE:3592479, respectively). See Supplemental Experimental Procedures for further details.

Confocal Analysis

Immunolabeled hearts from control (wild-type or Cxcl12+/−) and Cxcl12−/− (E12.5–E15.5) embryos were dehydrated through a methanol series before clearing with one part benzyl alcohol/two parts benzyl benzoate solution (BABB; Sigma). Embryos were examined by epifluorescence on an inverted LSM710 confocal system mounted on an AxioObserver Z1 microscope (Carl Zeiss, UK). See Supplemental Experimental Procedures for further details.

Statistical Analysis

When comparing the number of anastomoses formed with the aortic lumen in Cxcl12 nulls versus controls, we calculated statistical significance using an unpaired Student’s t test. Significance was accepted when p < 0.05.

Quantification of Relative CA Length

E16.5 hearts in paraffin were sectioned at 12-μm and stained with anti-SM22α antibody to label smooth muscle cells. The length of the CAs relative to the total distance from the left or right ostium to the tip of each heart was calculated as follows: the number of sections from the tip of the heart to the left or right coronary ostium was multiplied by the section thickness, giving a “heart length” figure. The number of consecutive sections with visible SM22α-stained arteries in the left or right ventricles was similarly multiplied by the section thickness and subsequently divided by heart length to give a relative measure of CA length.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.03.026.

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REFERENCES

Akawa, E., and Kawano, J. (1982). Formation of coronary arteries sprouting from the primitive aortic sinus wall of the chick embryo. Experientia 38, 816–818.

Anastasia, A., Deinhardt, K., Wang, S., Martin, L., Nichol, D., Irnady, K., Trinh J., Parada, L., Rafi, S., Hempstead, B.L., and Kermani, P. (2014). TrkB signaling in pericytes is required for cardiac microvessel stabilization. PLoS ONE 9, e87406.

Ando, K., Nakajima, Y., Yamagishi, T., Yamamoto, S., and Nakamura, H. (2004). Development of proximal coronary arteries in quail embryonic
heart; multiple capillary beds penetrating the aortic sinus fuse to form main coro-
nary trunk. Circ. Res. 94, 346–352.
Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., and Nagasawa, T. (2003). Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). Proc. Natl. Acad. Sci. USA 100, 5319–5323.
Ara, T., Tokoyoda, K., Okamoto, R., Koni, P.A., and Nagasawa, T. (2005). The role of CXCL12 in the organ-specific process of artery formation. Blood 105, 3155–3161.
Balabanian, K., Lagane, B., Infantino, S., Chow, K.Y., Harriague, J., Moepps, B., Arenaza-Seixedos, F., Thelen, M., and Bacherie, F. (2005). The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. J. Biol. Chem. 280, 35760–35766.
Belmadani, A., Tran, P.B., Ren, D., Assimacopoulos, S., Grove, E.A., and Miller, R.J. (2005). The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. J. Neurosci. 25, 3995–4003.
Bermanke, D.H., and Velkey, J.M. (2002). Development of the coronary blood supply: changing concepts and current ideas. Anat. Rec. 269, 198–208.
Bogers, A.J., Gittenberger-de Groot, A.C., Poelmann, R.E., Pauwels, B.M., and Huysmans, H.A. (1989). Development of the origin of the coronary arteries; a matter of ingrowth or outgrowth? Anat. Embryol. (Berl.) 180, 437–441.
Burns, J.M., Summers, B.C., Wang, Y., Melkian, A., Berahovich, R., Miao, Z., Penfold, M.E., Sunshine, M.J., Littman, D.R., Kuo, C.J., et al. (2006). A novel chemokine receptor for SDF-1 and ITAC involved in cell survival, cell adhesion, and tumor development. J. Exp. Med. 203, 2201–2213.
Chen, H.I., Poduri, A., Numi, H., Kivelä, R., Saharinen, P., McKay, A.S., Raftrey, B., Churko, J., Tian, X., Zhou, B., et al. (2014a). VEGF-C and aortic cardiomyocytes guide coronary artery stem development. J. Clin. Invest. 124, 4899–4914.
Chen, H.I., Sharma, B., Akerberg, B.N., Nuni, H.J., Kivelä, R., Saharinen, P., Aghajanian, H., McKay, A.S., Bogard, P.E., Chang, A.H., et al. (2014b). The si
rnx venous contributes to coronary vasculature through VEGFC-stimulated angiogenesis. Development 141, 4500–4512.
del Monte, G., Casanova, J.C., Guadix, J.A., MacGrogan, D., Burch, J.B., Pérez-Pomares, J.M., and de la Pompa, J.L. (2011). Differential Notch signaling in the epicardium is required for cardiac inflow development and coronary vessel morphogenesis. Circ. Res. 108, 824–836.
Escot, S., Blavet, C., Härtle, S., Duband, J.L., and Fournier-Thibault, C. (2013). Misregulation of SDF1–CXCR4 signaling impairs early cardiac neural crest cell migration leading to conotruncal defects. Circ. Res. 113, 505–516.
Fernández, B., Durán, A.C., Fernández-McClelland, J., García, A., and Sans-Costa, V. (2000). The coronary arteries of the C57BL/6 mouse embryo. J. Anat. 207, 559–568.
Kattan, J., Dettman, R.W., and Bristow, J. (2004). Formation and remodeling of the coronary vascular bed in the embryonic avian heart. Dev. Dyn. 230, 34–43.
Katz, T.C., Singh, M.K., Degenhardt, K., Rivera-Feliciano, J., Johnson, R.L., Epstein, J.A., and Tabin, C.J. (2012). Distinct compartments of the proepicardial organ give rise to coronary vascular endothelial cells. Dev. Cell 22, 639–650.
Kiefer, F., and Siekmann, A.F. (2011). The role of chemokines and their recep-
tors in angiogenesis. Cell. Mol. Life Sci. 68, 2811–2830.
Kisian, Y.Y., Hamner, R.E., Miyazaki, J., Williams, S.C., Richardson, J.A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: a new model for endo-
thal cell-cell-lineage analysis in vivo. Dev. Biol. 230, 230–242.
Li, W., Kohara, H., Uchida, Y., James, J.M., Soneji, K., Cronshaw, D.G., Zhou, Y.R., Nagasawa, T., and Mukoyama, Y.S. (2013). Peripheral nerve-derived CXCL12 and VEGF-A regulate the patterning of arterial vessel branching in developing limb skin. Dev. Cell 24, 359–371.
Ma, Q., Jones, D., Borchersani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. (1998). Impaired B-lymphopoiesis, myelo-
poiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-defi-
tent mice. Proc. Natl. Acad. Sci. USA 95, 9448–9453.
Ma, Q., Zhou, B., and Pu, W.T. (2008). Reassessment of Isl1 and Nkx2-5 car-
diact fate maps using a Gata4-based reporter of Cre activity. Dev. Biol. 323, 98–104.
Mikawa, T., and Fischman, D.A. (1992). Retroviral analysis of cardiac morpho-
genesis: discontinuous formation of coronary vessels. Proc. Natl. Acad. Sci. USA 89, 9504–9508.
Mohun, T.J., and Weninger, W.J. (2012). Episcleral three-dimensional imaging of embryos. Cold Spring Harb. Protoc. 2012, 641–646.
Morabito, C.J., Kattan, J., and Bristow, J. (2002). Mechanisms of embryonic coronary artery development. Curr. Opin. Cardiol. 17, 235–241.
Moses, K.A., DeMayo, F., Braun, R.M., Reecy, J.L., and Schwartz, R.J. (2001). Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. Genesis 31, 176–180.
Nagasawa, T., Hirotl, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXCR4 chemokine PBSF/SDF-1. Nature 382, 635–638.
Nie, Y., Waite, J., Brewer, F., Sunshine, M.J., Littman, D.R., and Zou, Y.R. (2004). The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. J. Exp. Med. 200, 1145–1156.
Petit, I., Jin, D., and Rafii, S. (2007). The SDF-1–CXCR4 signaling pathway; a molecular hub modulating neo-angiogenesis. Trends Immunol. 28, 299–307.
Poelmann, R.E., Gittenberger-de Groot, A.C., Mentink, M.M., Bökenkamp, R., and Rogers, B. (1993). Development of the cardiac coronary vascular endo-
thelium, studied with antienoid antibodies, in chicken-quail chimeras. Circ. Res. 73, 559–568.
Ramsbottom, S.A., Sharma, V., Rhee, H.J., Eley, L., Phillips, H.M., Rigby, H.F., Dean, C., Chaudhry, B., and Henderson, D.J. (2014). Vangl2-regulated polarisation of second heart field-derived cells is required for outflow tract lengthening during cardiac development. PLoS Genet. 10, e1004871.
Rataj, A., and Fiejka, E. (1999). Prenatal development of coronary arteries in the rat: morphologic patterns. Anat. Embryol. (Berl.) 200, 533–540.
Red-Horse, K., Leno, H., Weissman, I.L., and Krasnow, M.A. (2010). Coronary arteries form by developmental reprogramming of venous cells. Nature 464, 549–553.
Sierro, F., Biben, C., Martinez-Muñoz, L., Mellado, M., Ransohoff, R.M., Li, M., Woehl, B., Leung, H., Groom, J., Batten, M., et al. (2007). Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. Proc. Natl. Acad. Sci. USA 104, 14759–14764.
Song, N., Huang, Y., Shi, H., Yuan, S., Ding, Y., Song, X., Fu, Y., and Luo, Y. (2009). Overexpression of platelet-derived growth factor-BB increases tumor pericyte content via stromal-derived factor-1alpha/CXCR4 axis. Cancer Res. 69, 6057–6064.
The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393, 591–594.

Takabatake, Y., Sugiyama, T., Kohara, H., Matsusaka, T., Kurihara, H., Koni, P.A., Nagasawa, Y., Hamano, T., Matsui, I., Kawada, N., et al. (2009). The CXCL12 (SDF-1)/CXCR4 axis is essential for the development of renal vasculature. J. Am. Soc. Nephrol. 20, 1714–1723.

Teicher, B.A., and Fricker, S.P. (2010). CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin. Cancer Res. 16, 5965–5972.

Theveneau, E., Marchant, L., Kuriyama, S., Gull, M., Moepps, B., Parsons, M., and Mayor, R. (2010). Collective chemotaxis requires contact-dependent cell polarity. Dev. Cell 19, 39–53.

Tian, X., Hu, T., He, L., Zhang, H., Huang, X., Poelmann, R.E., Liu, W., Yang, Z., Yan, Y., Pu, W.T., and Zhou, B. (2013a). Peritruncal coronary endothelial cells contribute to proximal coronary artery stems and their aortic orifices in the mouse heart. PLoS ONE 8, e80857.

Tian, X., Hu, T., Zhang, H., He, L., Huang, X., Liu, Q., Yu, W., He, L., Yang, Z., Zhang, Z., et al. (2013b). Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. Cell 151, 1083–1096.

Tomanek, R.J. (2005). Formation of the coronary vasculature during development. Angiogenesis 8, 273–284.

Tomanek, R.J., Holifield, J.S., Reiter, R.S., Sandra, A., and Lin, J.J. (2002). Role of VEGF family members and receptors in coronary vessel formation. Dev. Dyn. 225, 233–240.

Tomanek, R.J., Ishii, Y., Holifield, J.S., Sjogren, C.L., Hansen, H.K., and Mikawa, T. (2006). VEGF family members regulate myocardial tubulogenesis and coronary artery formation in the embryo. Circ. Res. 98, 947–953.

Tomanek, R.J., Hansen, H.K., and Christensen, L.P. (2008). Temporally expressed PDGF and FGF-2 regulate embryonic coronary artery formation and growth. Arterioscler. Thromb. Vasc. Biol. 28, 1237–1243.

Van den Akker, N.M., Winkel, L.C., Nisancioglu, M.H., Maas, S., Wisse, L.J., Armulik, A., Poelmann, R.E., Lie-Venema, H., Betsholtz, C., and Gittenberger-de Groot, A.C. (2008). PDGF-B signaling is important for murine cardiac development: its role in developing atrioventricular valves, coronaries, and cardiac innervation. Dev. Dyn. 237, 494–503.

Verzi, M.P., McCulley, D.J., De Val, S., Dodou, E., and Black, B.L. (2005). The right ventricle, outflow tract, and ventricular septum comprise a restricted expression domain within the secondary/anterior heart field. Dev. Biol. 287, 134–145.

Vrancken Peeters, M.P., Gittenberger-de Groot, A.C., Mentink, M.M., Hungerford, J.E., Little, C.D., and Poelmann, R.E. (1997). The development of the coronary vessels and their differentiation into arteries and veins in the embryonic quail heart. Dev. Dyn. 208, 338–348.

Waldo, K.L., Willner, W., and Kirby, M.L. (1990). Origin of the proximal coronary artery stems and a review of ventricular vascularization in the chick embryo. Am. J. Anat. 188, 109–120.

Wesselhoeft, H., Fawcett, J.S., and Johnson, A.L. (1988). Anomalous origin of the left coronary artery from the pulmonary trunk. Its clinical spectrum, pathology, and pathophysiology, based on a review of 140 cases with seven further cases. Circulation 88, 403–425.

Wu, B., Zhang, Z., Liu, W., Chen, X., Wang, Y., Chamberlain, A.A., Moreno-Rodriguez, R.A., Markwald, R.R., O’Rourke, B.P., Sharp, D.J., et al. (2012). Endocardial cells form the coronary arteries by angiogenesis through myocardial-endocardial VEGF signaling. Cell 151, 1083–1096.

Yu, S., Crawford, D., Tsuchihashi, T., Behrens, T.W., and Srivastava, D. (2011). The chemokine receptor CXCR7 functions to regulate cardiac valve remodeling. Dev. Dyn. 240, 384–393.

Zangi, L., Lui, K.O., von Gise, A., Ma, Q., Eberlin, W., Ptasek, L.M., Späther, D., Xu, H., Tabeordbar, M., Gorbatov, R., et al. (2013). Modified mRNA directs the fate of heart progenitor cells and induces vascular regeneration after myocardial infarction. Nat. Biotechnol. 31, 898–907.

Zernecke, A., Schober, A., Bot, I., von Hundelshausen, P., Liehn, E.A., Möpps, B., Mericskay, M., Gierschik, P., Biessen, E.A., and Weber, C. (2005). SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells. Circ. Res. 96, 784–791.

Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393, 595–599.
Developmental Cell
Supplemental Information

The CXCL12/CXCR4 Axis Plays a Critical Role in Coronary Artery Development

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Inventory of Supplemental Information

Supplemental Figures S1-S5

**Figure S1, related to Figure 1.** Coronary artery and ostia positioning defects in Cxcl12 mutants.

**Figure S2, related to Figure 2.** Deletion of Cxcr4 in the endothelial lineage results in coronary ostia and SLV defects.

**Figure S3, related to Figure 3.** Deletion of Cxcr4 in the vSMC lineage results in SLV defects but smooth muscle coverage of CAs is unaffected.

**Figure S4, related to Figure 5.** Confocal analysis of the peritruncal plexus in Cxcl12 mutants at E13.5-E14.5.

**Figure S5, related to Figure 6.** Variable SLV and CA defects in Mef2c-Cre;Cxcl12fl/fl hearts.

**Legend for Movie S1, related to Figure 1.** HREM analysis of E17.5 hearts.

Supplemental Experimental procedures

References
Figure S1

(A-C) Wholemount labelling of E18.5 hearts with anti-SM22α antibody. The right CA (arrows in A) is absent in both Cxcl12 and Cxcr4 null hearts. Asterisks indicate coronary veins on the dorsal surface of the heart. (D-H’) HREM analysis of ostia defects in Cxcl12−/− hearts (dynamic reslice of aortic region in ImageJ). Asterisks indicate coronary ostia in wild type control (right and left ostia in D and E respectively). Three nulls (F-H) each have a single, distally positioned ostium (asterisks). The red arrow in heart (G) shows the sub-epicardial location of its proximal CA. In (H) and (H’) the distal ostium gives rise to a short stretch of CA that passes along the exterior surface of the aorta (black arrow in H’).

Scale bars, 100μm. Ao, aorta; CA, coronary artery; coronary leaflets: L (left), R (right) and NC (non-coronary).

Figure S1, related to Figure 1. Coronary artery and ostia positioning defects in Cxcl12 mutants
Figure S2

Deletion of Cxcr4 in the endothelial lineage results in coronary ostia and SLV defects

(A-B') SM22α-labelling of OT regions of control and Cxcr4 conditional null E16.5 hearts. Arrows in (A) and (B) indicate coronary ostia in control heart; the ostium shown in (B) is slightly distal to the aortic valve. Defective SLVs (asterisks indicate valve leaflets) are clearly visible in the Tie2-Cre;Cxcr4fl/fl heart (A'); arrowhead in (A') indicates a single coronary artery (ostium shown in B'). (C-E) Cxcr4 expression in the ventricular septal region of E14.5 control and conditional null hearts (in situ hybridisation). Note Cxcr4 expression was variably down-regulated as high levels of Cxcr4 were present in the Tie2-Cre;Cxcr4fl/fl heart shown in (D), while in other cases there was virtually no detectable expression (E). This likely accounts for the variability of phenotype observed in this series of conditional mutants.

Scale bars, 100µm. Ao, aorta, OT, outflow tract; PT, pulmonary trunk; SLV, semi-lunar valve.
Figure S3

Figure S3, related to Figure 3. Deletion of Cxcr4 in the vSMC lineage results in SLV defects but smooth muscle coverage of CAs is unaffected.

(A-B') SM22α/EMCN-labelling of OT regions of control (Cxcr4<sup>fl/fl</sup>) and SM22α<sup>Cre</sup>;Cxcr4<sup>/fl</sup> E16.5 hearts (transverse sections). Asterisks in (A') and (B') indicate defective valve leaflets in the aorta (Ao) and pulmonary trunk (PT) of the SM22α<sup>Cre</sup>;Cxcr4<sup>/fl</sup> heart respectively. Arrows indicate CAs. (C, C') PECAM/SM22 staining shows CA coverage with smooth muscle cells is unimpaired in SM22α<sup>Cre</sup>;Cxcr4<sup>/fl</sup> hearts.

Scale bars, 100μm in (A) and (B), otherwise as indicated. CAs, coronary arteries; ECMN, endomucin; OT, outflow tract.
Figure S4, related to Figure 5. Confocal analysis of the peritruncal plexus in Cxcl12 mutants at E13.5-E14.5.

(A-F) Confocal microscopic images of E13.5-E14.5 wholemount PECAM-labelled control and Cxcl12−/− hearts showing the aorta (Ao) and peritruncal plexus (maximum intensity z-projections of confocal stacks). For clarity, dotted lines have been used to delineate the edges of the aortic valves (AoV). Arrowheads indicate points of contact between ECs/vessels and aortic lumen. (A-C) At E13.5 the control peritruncal plexus (bracketed region in A) still forms multiple contacts with the aortic lumen (arrowheads), and is connected to the intra-ventricular coronary plexus (small arrows). (B) shows a Cxcl12−/− aorta with sub-epicardially-localised vessels (arrows) forming single, distal connections on either side of the aortic lumen (arrowheads). In (C) an ectopic vessel region crosses over the aortic lumen (large arrowhead) and a wide, lumenised vessel buds from one side of the aorta (small arrowhead). By E14.5 the peritruncal plexus is disappearing (D). The large arrow in (D) shows a single major vessel connecting on left side of the control aorta; two smaller vessels are connected to the right side (small arrows). The Cxcl12−/− in (E) lacks any vessels in the aorta, whilst in (F), vessels connect either side of the aorta (arrows) but fail to branch into the ventricles. Scale bars, 100µm.
Figure S5, related to Figure 6. Variable SLV and CA defects in Mef2c-Cre;Cxcl12^{fl/fl} hearts.

(A-C) Anti-SM22α antibody labelling of E16.5 sections through the aorta; CA defects are not always accompanied by aortic valve defects in Mef2c-Cre;Cxcl12^{fl/fl} hearts. The conditional null in (B) shows thickened left (L) and right (R) aortic valve leaflets whereas in another null (C) the valve leaflets
appear normal. (D-F) A further null shows a combination of thickened (E) and normal (F) valve leaflets. In the same conditional null both left and right CAs are present (lca, rca, arrows in H, I, K, L) as well as a CA branching into the IVS (arrowhead in H). However only a single ostium (arrowhead) is formed on the right side (K). Corresponding control sections are shown in (G) and (J).

Scale bars, 100µm. Ao, aorta; coronary valve leaflets: left (L), right (R) and non-coronary (NC); IVS, inter-ventricular septum; PT, pulmonary trunk.
**Movie S1, Related to Figure1. HREM analysis of E17.5 hearts.**

Front erosion through E17.5 wild type (left) and Cxcl12^{-/-} (right) hearts. Wild type: note pulmonary valve (PV) visible at 11s, coronary arteries connecting to aorta at 15s (OS), and aortic valve (AoV) leaflets from 17-19s. Intra-ventricular arteries are visible from 14s onwards. In the mutant, defective pulmonary and aortic valve leaflets (PV and AoV) can be observed from 8-12s and 17-21s respectively. Note that the aorta over-rides a VSD (19s). A single, short coronary artery can be observed on the ventral side of the aorta (14-15s), the ostium it connects to (OS) is both laterally and longitudinally mis-positioned (15s). Intra-ventricular arteries are absent.
Supplemental Experimental Procedures

Mutant mouse breeding

Cxcr4 null mice were obtained by crossing Cxcr4*/* mice with βactin-Cre mice (Lewandoski et al., 1997). Offspring carrying both Cre and recombinated Cxcr4 alleles were bred again with wild types to obtain offspring carrying only the recombinated Cxcr4 allele. Mice carrying two copies of the recombinated Cxcr4 allele are referred to in the text as Cxcr4+/− or Cxcr4 null. Tie2-Cre;Cxcr4*/* mice were generated by crossing Tie2-Cre;Cxcr4*/* mice with Cxcr4*/* mice and SM22α-Cre; Cxcr4*/* mice by crossing SM22α-Cre; Cxcr4*/* mice with Cxcr4*/* mice. Similarly, Nkx2.5-Cre;Cxcl12*/* and Mef2c-Cre;Cxcl12*/* conditionals were generated by crossing Cxcl12*/* mice with Nkx2.5-Cre;Cxcl12*/* and Mef2c-Cre;Cxcl12*/* mice respectively. All mice were maintained on a C57Bl/6 background. Animal work was carried out according to UK Home Office regulations.

Fixation of embryonic hearts

Whole-mount hearts were fixed in 4% paraformaldehyde (PFA) for 15-30 minutes. For immunolabelling or in situ hybridisation on paraffin sections, hearts were fixed overnight in 10% neutral-buffered formalin or 4% PFA respectively at 4°C. For frozen sections, hearts were fixed for 15-60 minutes in 4% PFA, incubated overnight in 30% sucrose at 4°C, then mounted in optical cutting temperature compound (OCT, RA Lamb) and snap-frozen.

Immunolabelling

For paraffin sections, heat-induced antigen retrieval in Target Retrieval Solution Citrate pH 6 (Dako) was carried out after de-paraffinisation and rehydration, and
sections were blocked for one hour. The following antibodies were used: anti-PECAM (Abcam) at 1:50, anti-endomucin (EMCN) (V.737, Santa Cruz), anti-SM22α as above, and anti-NOTCH1 (Cell Signalling) at 1:400. Signal was detected using Alexa Fluor®-594 and 488 conjugated secondary antibodies (Life Technologies) at 1:500. For PECAM staining in combination with anti-SM22, rat anti-CD31 (RM0032-1D12, Abcam) was used at 1:100. In this case, antigen retrieval was carried out by proteinase K digestion for 30 minutes at room temperature (10µg/ml). The PECAM signal was amplified using anti-rat biotin-conjugated secondary (Thermo Scientific Pierce) at 1:500 followed by Streptavidin, Alexa Fluor® 594 conjugate (Life Technologies).

Frozen sections were permeabilised for 5 minutes in 0.5% Triton X-100 (Sigma) prior to blocking in 10% BSA/10% goat serum/0.1% Triton X-100 in PBS). Anti-CXCR4 (UMB2, Abcam) was used at 1:300 and anti-PECAM (MA3105, Thermo Scientific Pierce) at 1:400. The following anti-Armenian Hamster secondary antibodies were used to detect PECAM signal: Alexa Fluor 594 conjugate (Jackson ImmunoResearch) at 1:400 and Alexa Fluor 488 conjugate (Abcam) at 1:500. All incubation of sections with primary antibodies were carried out at 4°C overnight. Staining with secondaries was for one hour at room temperature; washes were carried out using PBS.

**In situ hybridisation**

Paraffin sections were de-waxed (Histoclear, National Diagnostics) and re-hydrated through an ethanol series. Sections were permeablisised using Proteinase K (Sigma) at 20µg/ml for 8 min. This was followed by glycine at 2mg/ml for 5 min, washing in PBS and post-fixing in 4% paraformaldehyde for 20 min. After rinsing in PBS,
sections were pre-hybridised in hybridisation buffer (50% de-ionised formamide (Promega), 5xSSC pH 5.0, 50µg/ml yeast tRNA (Sigma), 1% SDS and 50 µg/ml heparin (Sigma)) for one hour at 70°C. Probe incubation was carried out at 70°C overnight followed by washes at 65°C as follows: 3x 15 min in 50% formamide/5x SSC/1% SDS and 2 x 15 min in 50% formamide/2x SSC. The samples were allowed to cool to room temperature then washed twice for 10min each in MABT (100mM maleic acid, 150mM NaCl, 0.1% Tween-20, pH 7.5) with 2mM tetramisole hydrochloride (levamisol, Sigma). Blocking was carried out in 2% Blocking Reagent (Roche Life Science)/5% sheep serum/5% goat serum (in MABT) for one hour at room temperature before incubating with anti-Digoxygenin-AP antibody at 1:2000 (4°C overnight). After removal of the antibody the sections were washed in MABT/levamisol and equilibrated in alkaline phosphatase buffer (2 x 5 min washes) before staining with BM Purple solution (Roche Life Science)

High Resolution Episcopic Microscopy (HREM)

For further details of HREM see (Mohun and Weninger, 2012a) and (Mohun and Weninger, 2012b). Movies were created using Osirix software.

3D reconstruction

Image stacks containing the aorta were cropped in Fiji (Schindelin et al., 2012) and the 3D reconstructions were created using Imaris (Bitplane AG) surpass module with the projection mode set to blend. Snapshots of similar 3D orientation were selected for the figures.
Confocal Analysis

For whole-mount hearts, images were captured with a LD LCI Plan-Apochromat 25x/NA 0.8 water immersion DIC objective or a LD LCI Plan-Apochromat 10x/NA 0.3 air DIC objective (Carl Zeiss Ltd, United Kingdom). The Alexa Fluor 594 dye was excited with a 594 nm diode. The pinhole aperture was set to image a 3.4 µm thick optical section and z-stack images were acquired with 1.7-2.2 µm spacing between optical slices. The images files were exported into ImageJ (Rasband, 1997-2009, http://rsb.info.nih.gov/ij/) where they were processed for publication.

For analysis of sections, Alexa Fluor® 488 and Alexa Fluor® 594 dyes were sequentially excited with a 488 nm Argon laser and 561nm diode, and images were captured with the LD LCI Plan-Apochromat 10x/NA 0.3 air DIC objective. For DAPI and Alexa Fluor® 594-stained sections, 405 nm and 561nm laser diodes were used; images were captured with the Zeiss Plan-Apochromat 20x/0.8 M27 air objective.

Supplemental References

Lewandoski, M., Meyers, E.N., and Martin, G.R. (1997). Analysis of Fgf8 gene function in vertebrate development. Cold Spring Harb Symp Quant Biol 62, 159-168.

Mohun, T.J., and Weninger, W.J. (2012a). Episcopic three-dimensional imaging of embryos. Cold Spring Harb Protoc 2012, 641-646.

Mohun, T.J., and Weninger, W.J. (2012b). Generation of volume data by episcopic three-dimensional imaging of embryos. Cold Spring Harb Protoc 2012, 681-682.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods 9, 676-682.