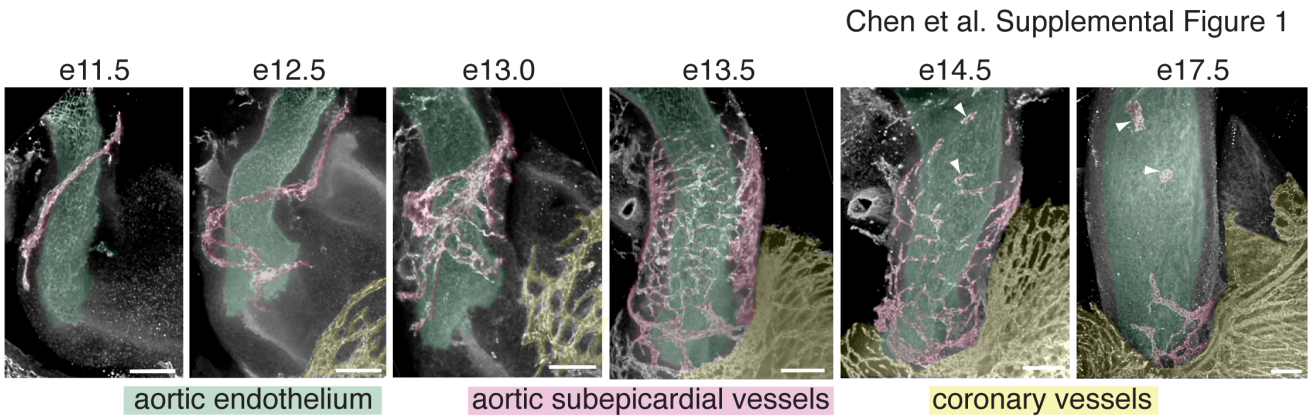
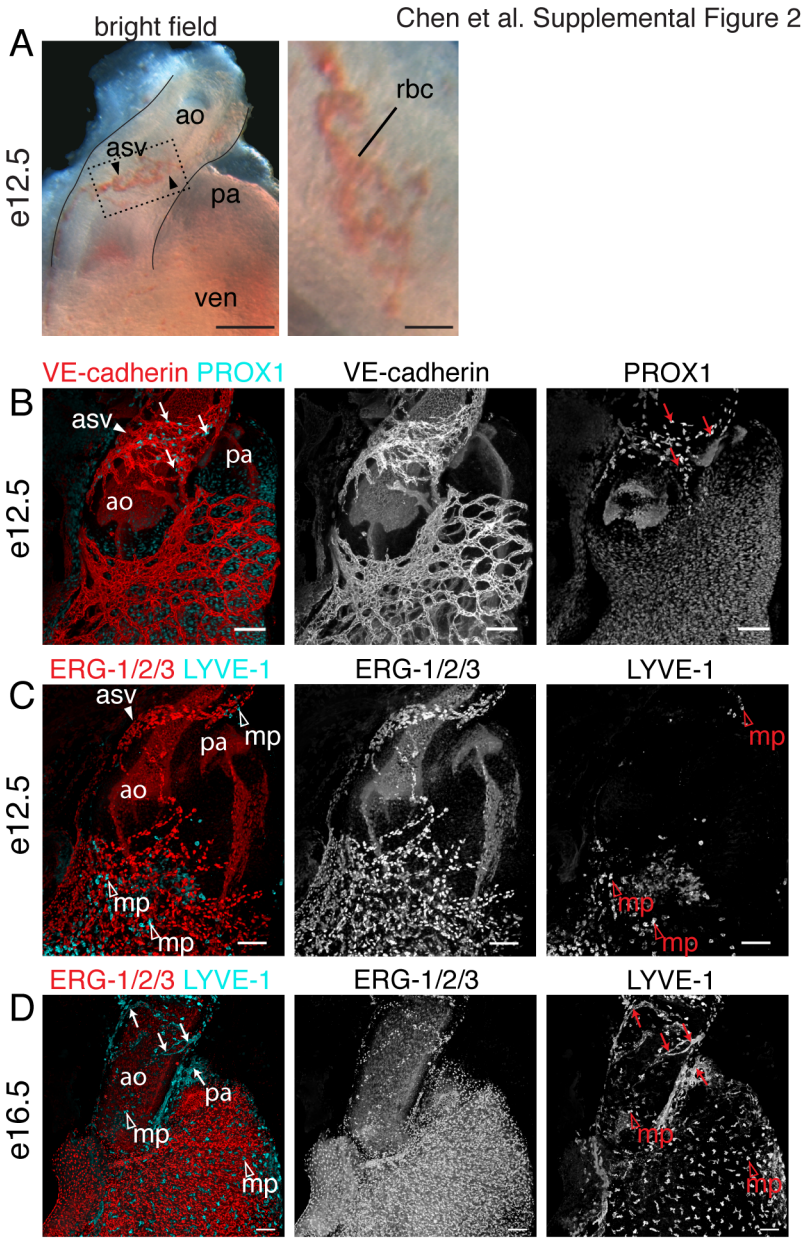
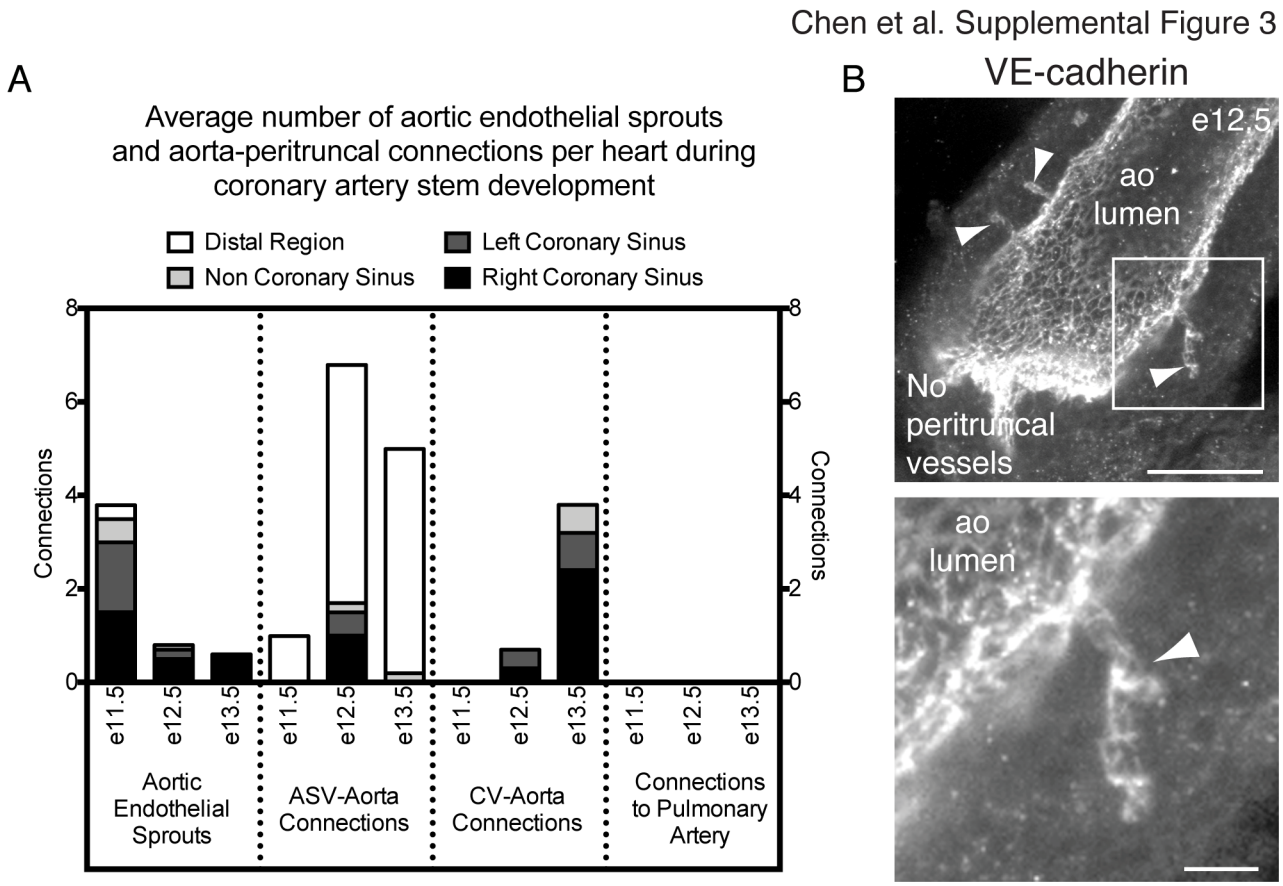


Supplemental Figure 1. Characterization of peritruncal vessels in murine hearts. Time course of peritruncal vessel development showing pseudo-colored aortic luminal endothelium (green), coronary vessels (yellow), and aortic subepicardial vessels (ASVs)(pink). While coronary vessels progressively expand through embryogenesis, the ASVs grow to their maximum extent by e13.5 and begin to degrade thereafter. Scale bars, 100μm.

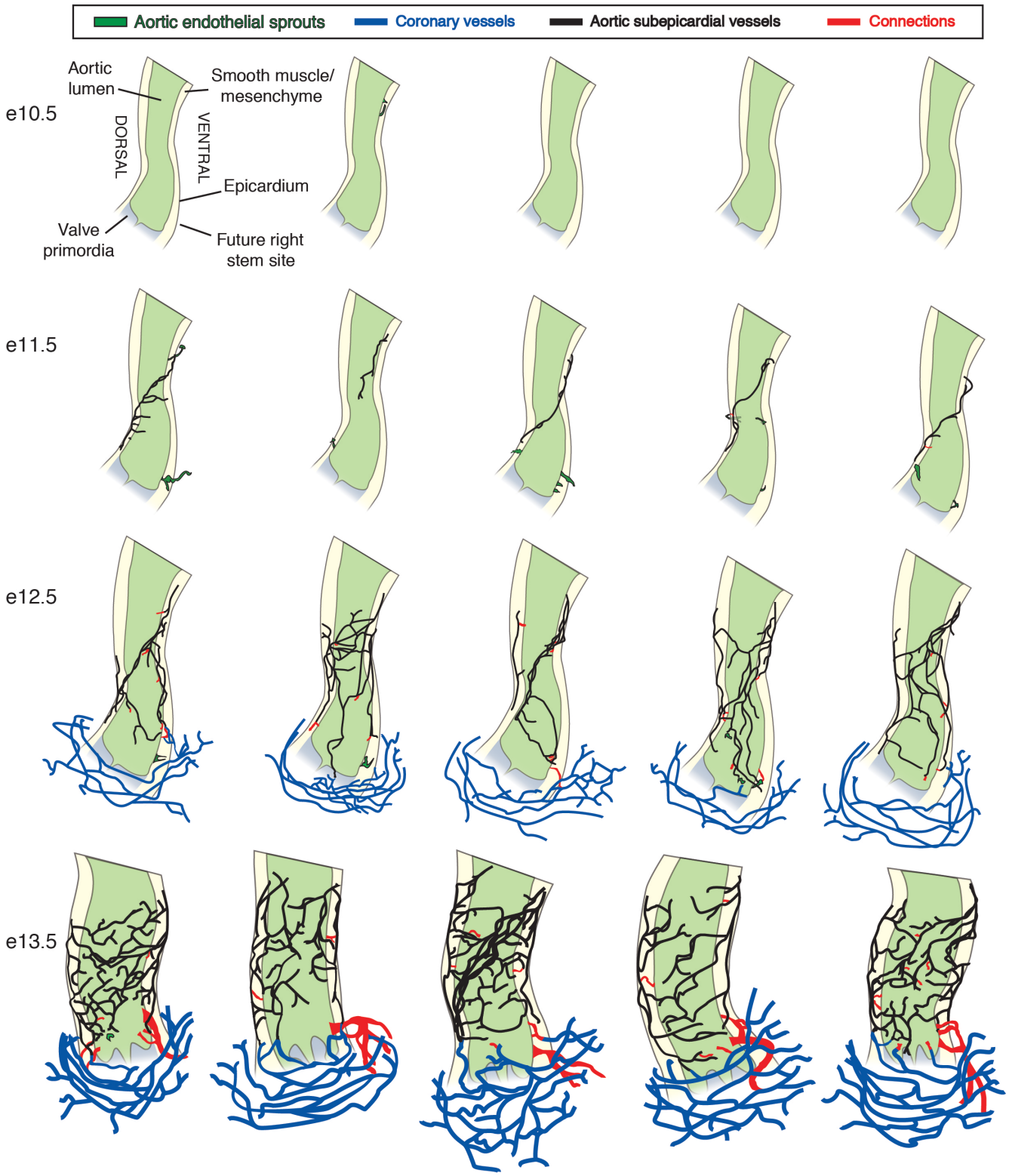


Supplemental Figure 2. Red blood cells and lymphatic markers in aortic subepicardial vessels of whole mount embryonic hearts imaged from the right lateral side. (**A**) Bright field stereomicrograph showing aortic subepicardial vessels (arrowheads) filled with red blood cells (rbc). Right panel is boxed region. (**B-D**) Confocal images showing lymphatic markers (blue, PROX1 or LYVE-1) in aortic subepicardial vessels (asv) at the indicated ages with VE-cadherin or ERG-1/2/3 (red) marking endothelial cells. At e12.5, a subset of aortic subepciardial vessels are PROX1^{+} (arrows) (**B**) while none of them are LYVE-1^{+} (**C**). At all ages, macrophages (mp, open arrowheads) express LYVE-1 (**C-D**). At e16.5, LYVE-1^{+} lymphatic vessels (arrows) are visible on the aorta (ao) and at the ventral base of the pulmonary artery (pa) (**D**). ven, ventricle. Scale bars: **A**-**D**, 100μm; boxed region of **A**, 25μm.

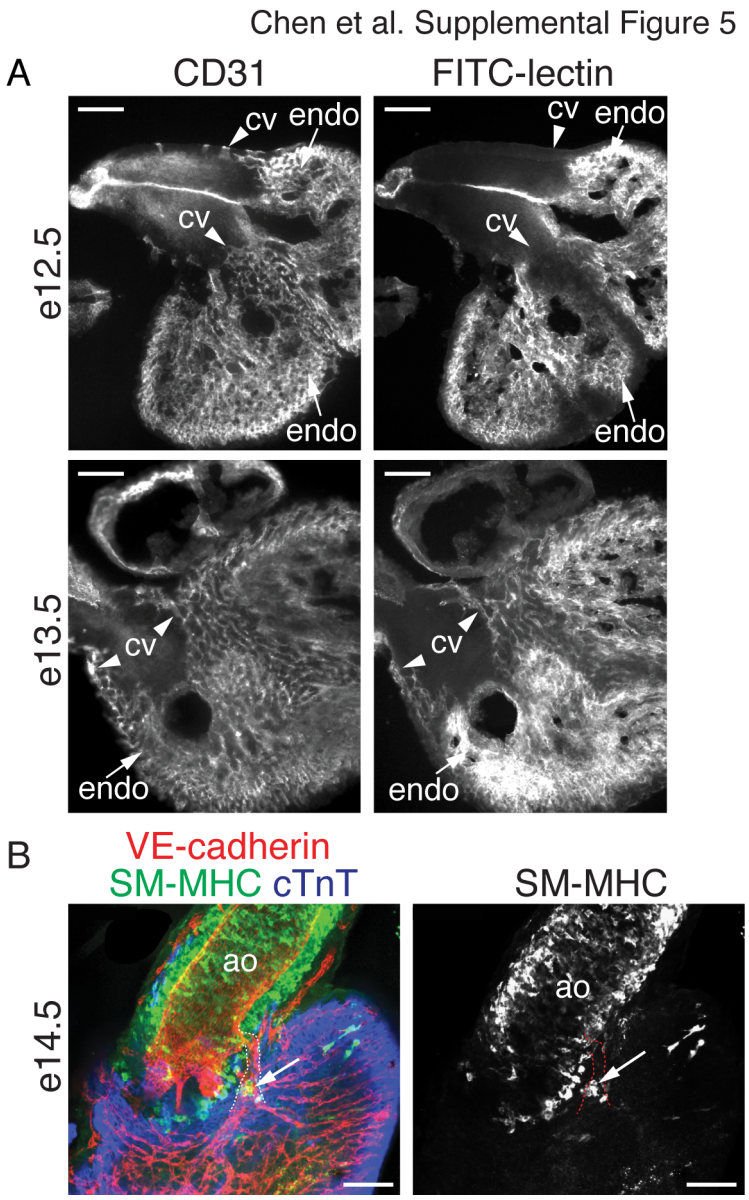


Supplemental Figure 3. Aortic sprouts and anastomoses during CA stem development. (**A**) Distribution of aortic endothelial sprouts and connections with peritruncal vessels at e11.5-13.5, categorized by their location at the left, right, and non-coronary valve sinuses or at regions distal to the valve (e11.5, n=6; e12.5, n=23; e13.5, n=5). (**B**) Confocal image of the aorta (ao) from an embryo injected with Axitinib to inhibit peritruncal vessels. Under these conditions, small aortic endothelial sprouts (arrowheads) form. Endothelial cells are labeled with VE-cadherin. Lower panel is boxed region. Scale bars: upper panel, 100 μm; lower panel, 25 μm.

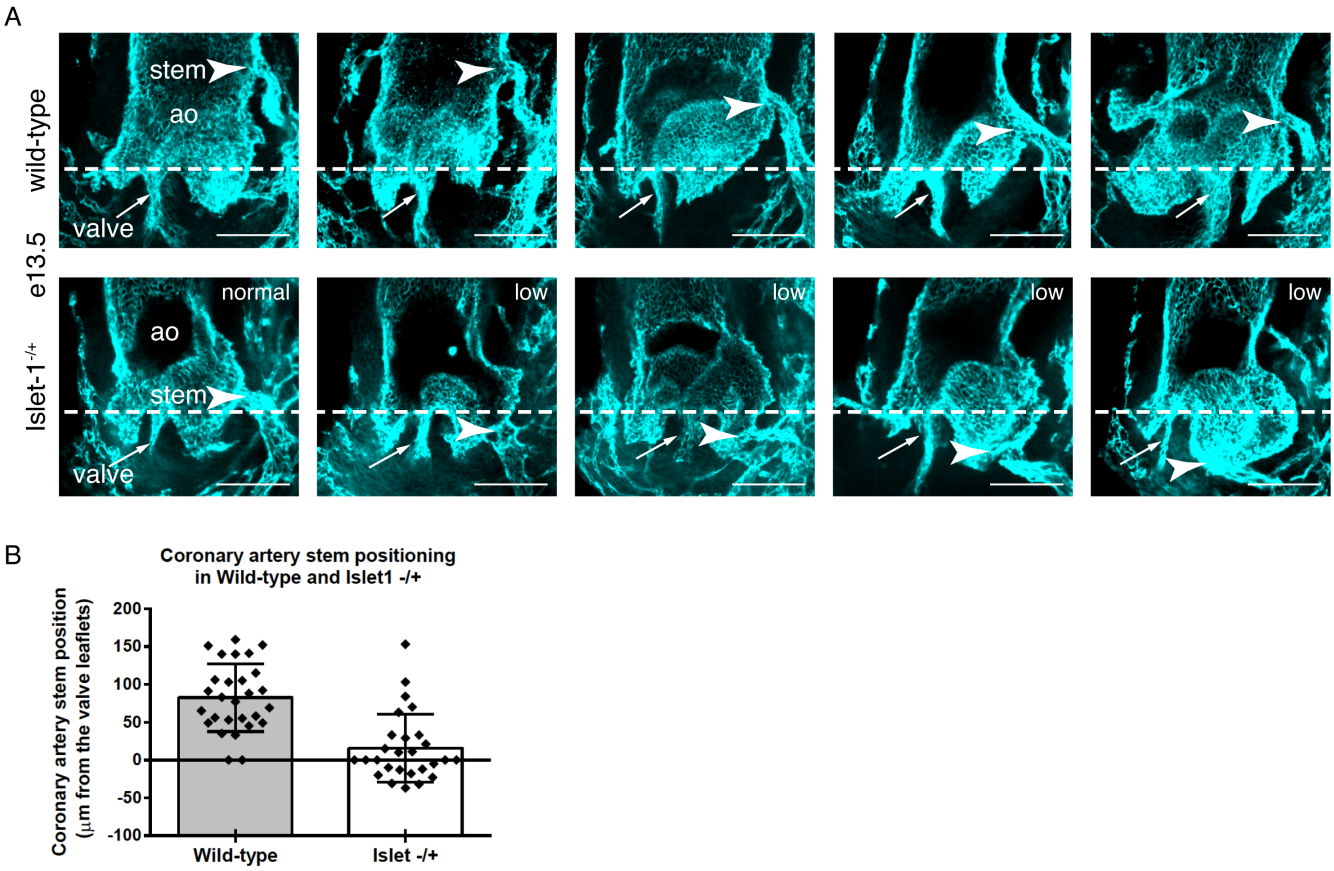
Chen et al. Supplementary Figure 4



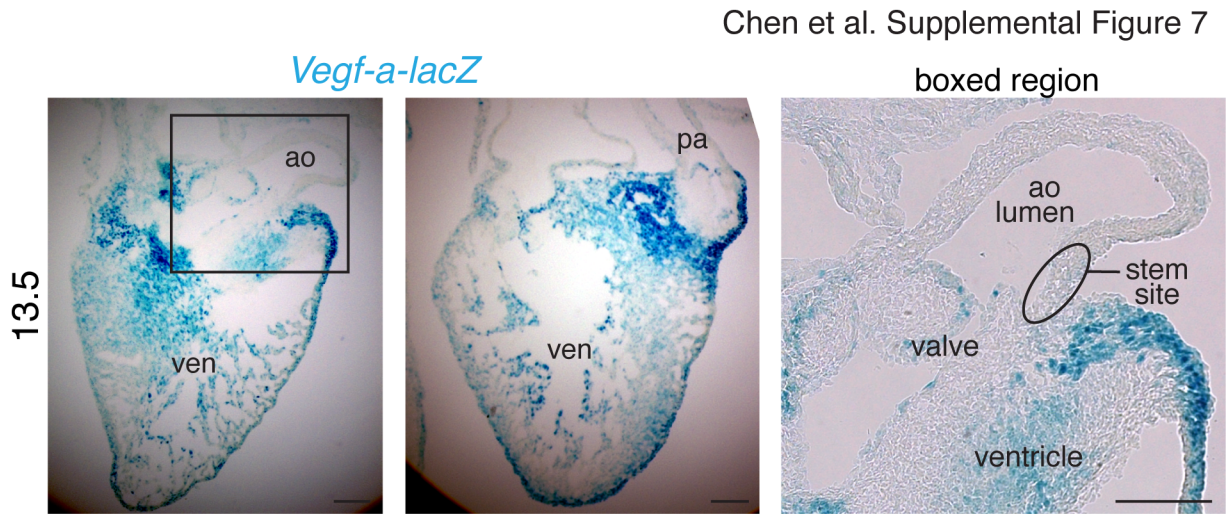
Supplemental Figure 4. Vessel patterns during CA stem development as visualized from the right lateral side of the heart. Schematics from five representative hearts per age, e10.5-13.5, showing location of aortic endothelial sprouts and connections with peritruncal vessels (coronary vessels and aortic subepicardial vessels).



Supplemental Figure 5. Onset of perfusion and smooth muscle acquisition during CA stem development. (**A**) Coronary vessel blood flow is established at e13.5. Embryos were perfused with FITC-lectin to reveal patent blood vessels followed by immunostaining for CD31 to label coronary endothelial cells (cv, arrowheads) and the endocardium (endo, arrows). Coronary vessels were only co-labeled with FITC-lectin and CD31 at e13.5. (**B**) Whole mount confocal image of e14.5 heart immunostained for SM-MHC-specific antibodies to highlight vascular smooth muscle (arrow, green) around developing CAs (dotted lines). Endothelial cells are in red (VE-cadherin); myocardium is in blue (cTnT). Right panel shows SM-MHC channel alone. Scale bars, 100 μm.

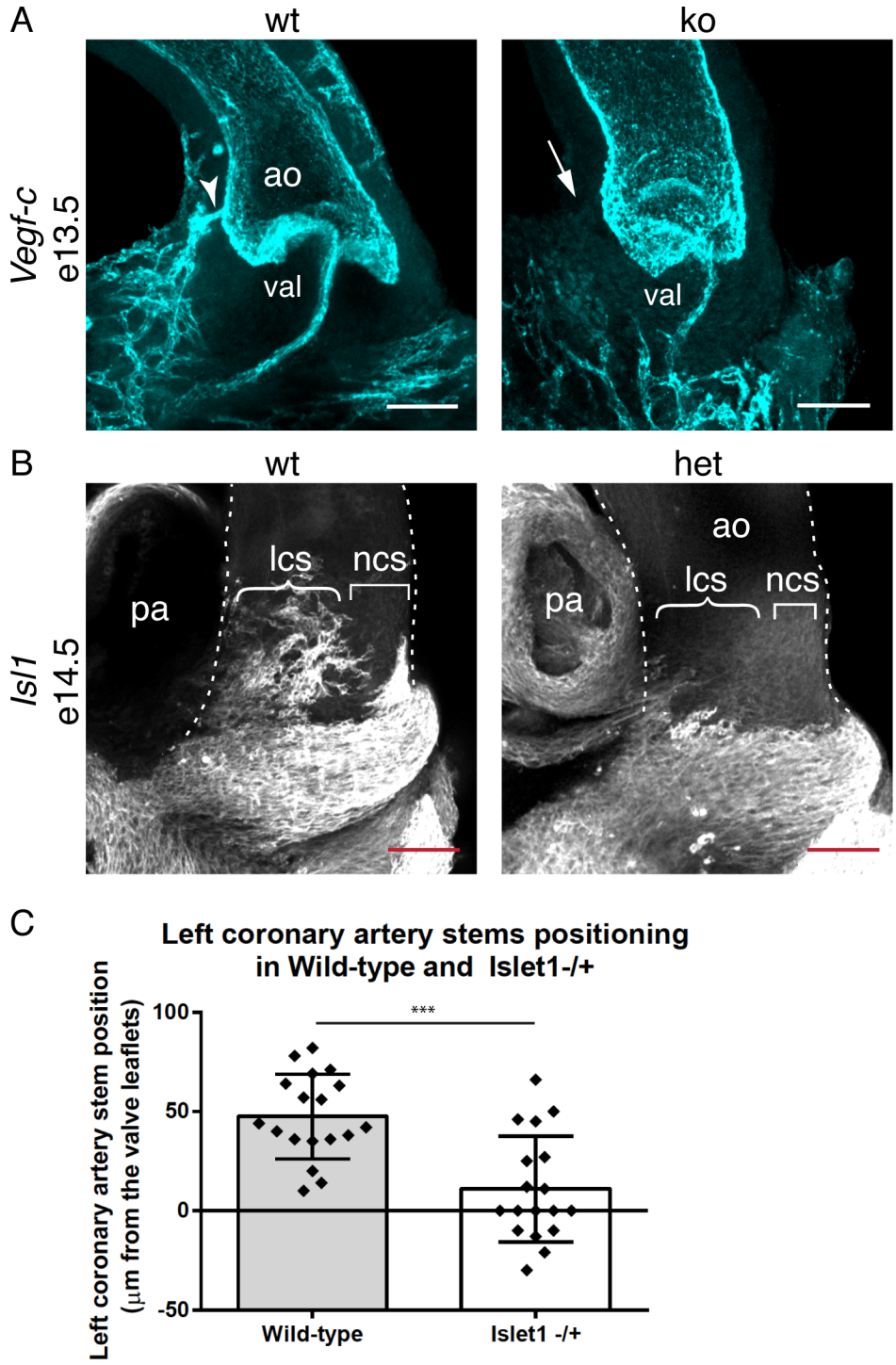


Supplemental Figure 6. Variability in CA stem attachment sites. (**A**) Confocal images of five e13.5 wild-type and five *Isl1* heterozygous aortas (ao) representing the variability in CA stem attachment sites (arrowheads). The position of the stem is measured as the length above or below the point where the valve leaflets come together (dotted lines). (**B**) Quantification indicates that at e13.5 normal stems are usually above the level of the valve leaflets (line) while mutants are frequently at or below. Data represent mean ± SD. Scale bars, 100 μm.

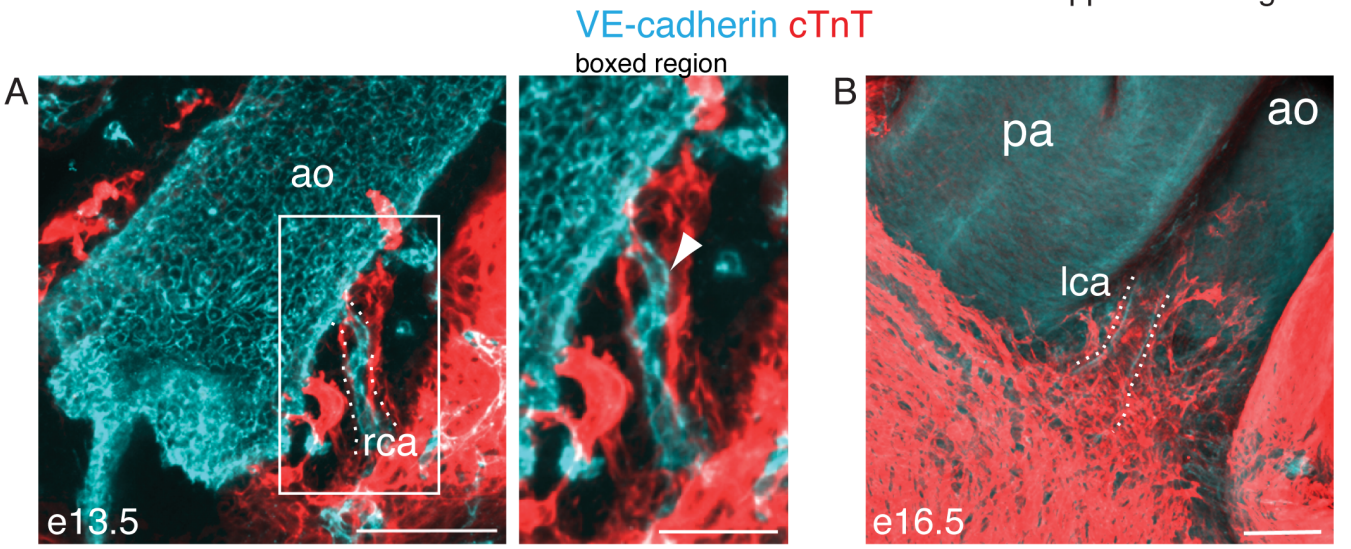


Supplemental Figure 7. Expression of VEGF-A during CA stem formation. Tissue sections through an e13.5 *Vegf-a-lacZ* heart treated with X-gal to show areas expressing VEGF-A (blue). Sections through ventricles (ven) at the level of the aorta (ao) and pulmonary artery (pa) are shown. Right panel is boxed region where *Vegf-a-lacZ* is at the base of the outflow vessels, but not at the CA stem site. Scale bars, 100 μm.

Chen et al. Supplemental Figure 8

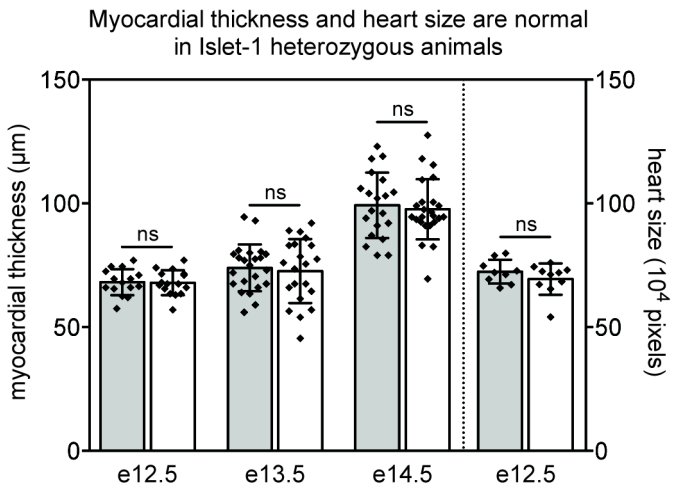


Supplemental Figure 8. Left coronary artery phenotypes in VEGF-C and *Isl1* hearts. (**A**) The left coronary artery (arrowhead) present in wild-type (wt) animals is missing in VEGF-C knockout (ko) hearts at e13.5. (**B**) Aortic cardiomyocytes (white) are lowered at the left coronary artery sinus (lcs) in *Isl1* heterozygous hearts. The dotted line indicates the aorta (ao). (**C**) Measurements show that left coronary artery stems are significantly lower on the *Isl1* heterozygous aorta. Data represent mean ± SD. ***, $P<0.001$. ncs, non-coronary sinus; pa, pulmonary artery; val, valve. Scale bars, 100μm.



Supplemental Figure 9. Cardiomyocytes are associated with CA stems throughout gestation in murine hearts. (**A** and **B**) Cardiomyocytes (red, cTnT^{+}) adjacent to CA stems (dotted line, arrowhead in high magnification) at e13.5 (**A**) and e16.5 (**B**). The right CA stem (rca) is shown in **A** while the left (lca) is shown in **B**. Endothelial cells are blue (VE-cadherin^{+}). Ao, aorta; pa, pulmonary artery. Scale bars: **A**-**B**, 100μm; boxed region of **A**, 50μm.

Chen et al. Supplemental Figure 10



Supplemental Figure 10. Myocardial thickness and heart size are normal in *Isl1* heterozygous hearts during stem formation. Chart showing myocardial thickness measurements (left y-axis) and total heart size (right y-axis) at the indicated ages. Each point represents an individual sample. Data represent mean ± SD. ns, not significant with *P value* ≥ 0.05.