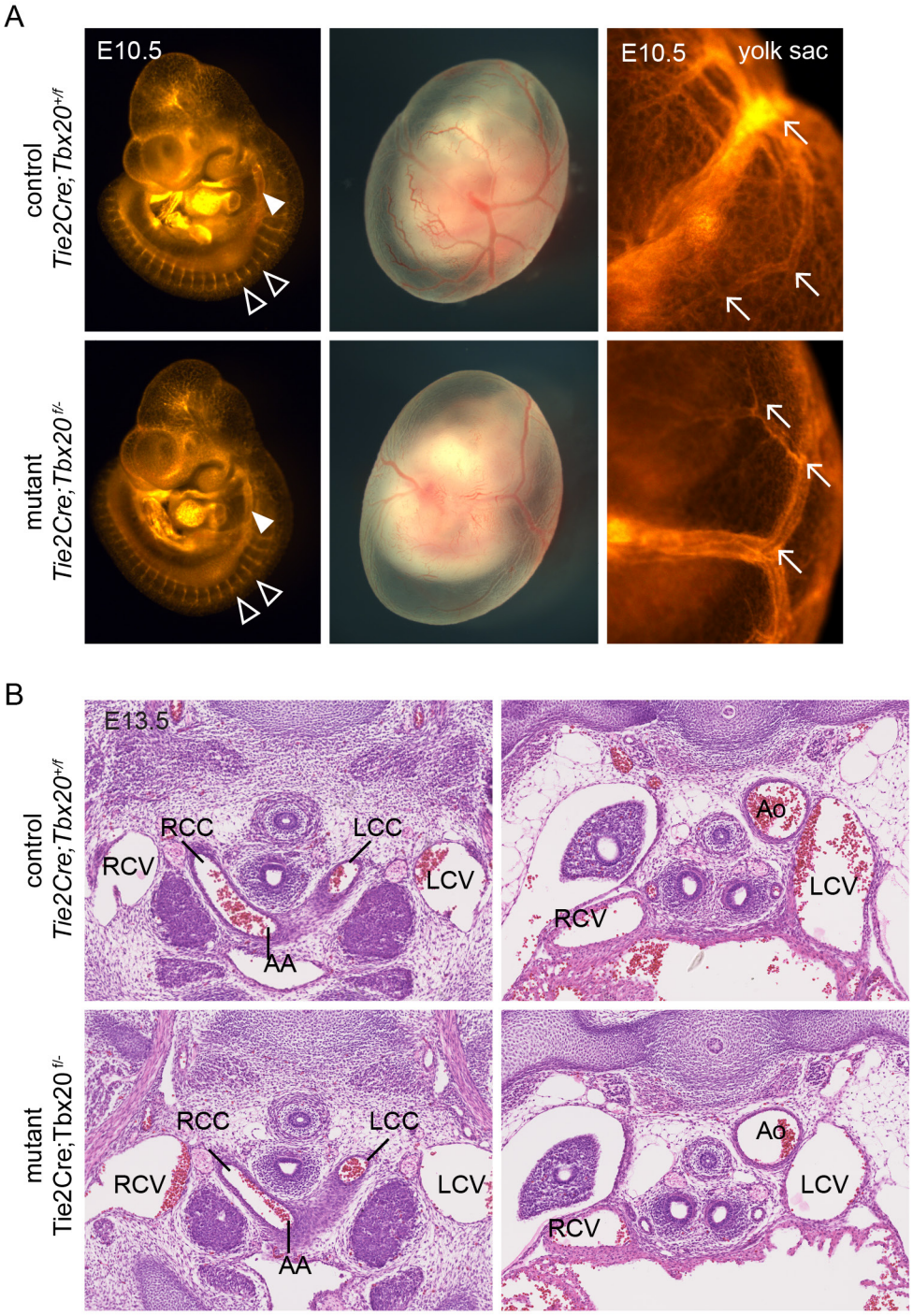
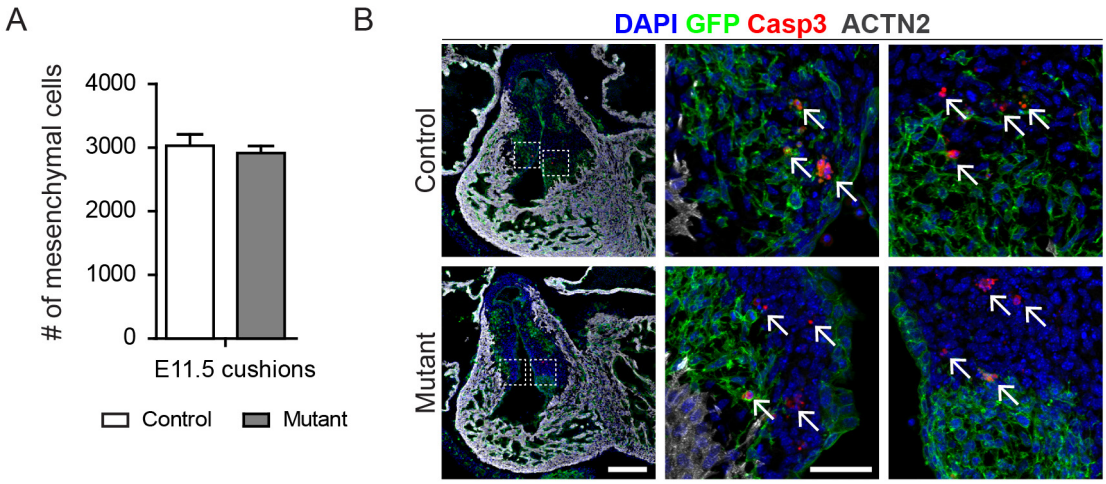
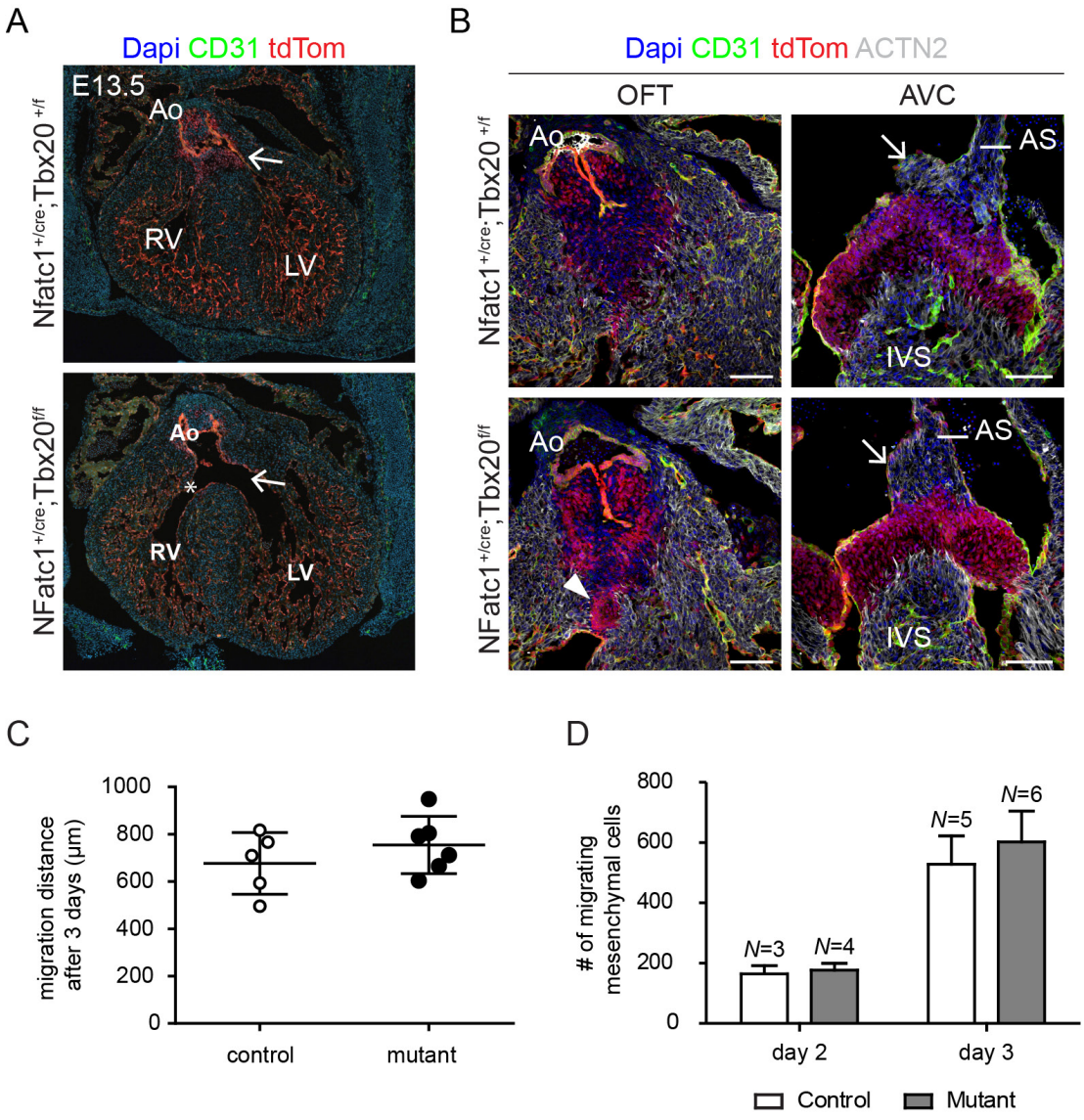


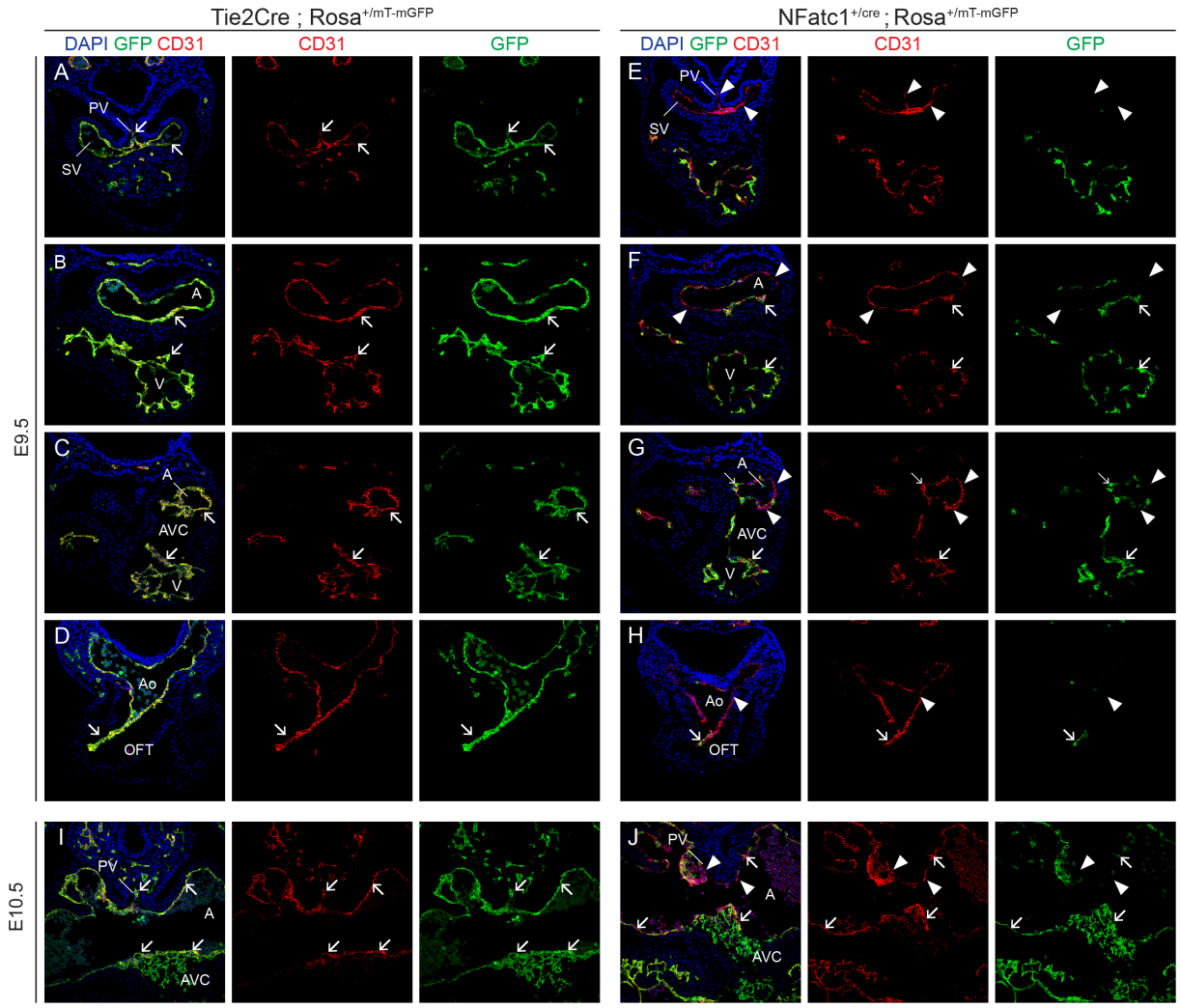
Supplemental Figure 1. Vasculature development in Tie2Cre;Tbx20 mutants and littermate controls. (A) At E10.5, Tie2Cre lineage tracing indicates that vascular patterning is grossly normal in Tie2Cre;Tbx20 mutant embryos and control littermates. Open arrowheads mark intersomitic vessels, filled arrowheads mark dorsal Aorta. Mutant yolk sacs are highly vascularized, with an interconnected network of large, medium and small sized branching vessels (arrows) and capillary beds. (B) H&E staining of two representative sections showing position and size of major vessels in E13.5 Tie2Cre;Tbx20 mutant and littermate control embryos. Right (R) and left (L) common carotid arteries (CC), caval veins (CV), rostral extremity of the aortic arch (AA), and Aorta (Ao).



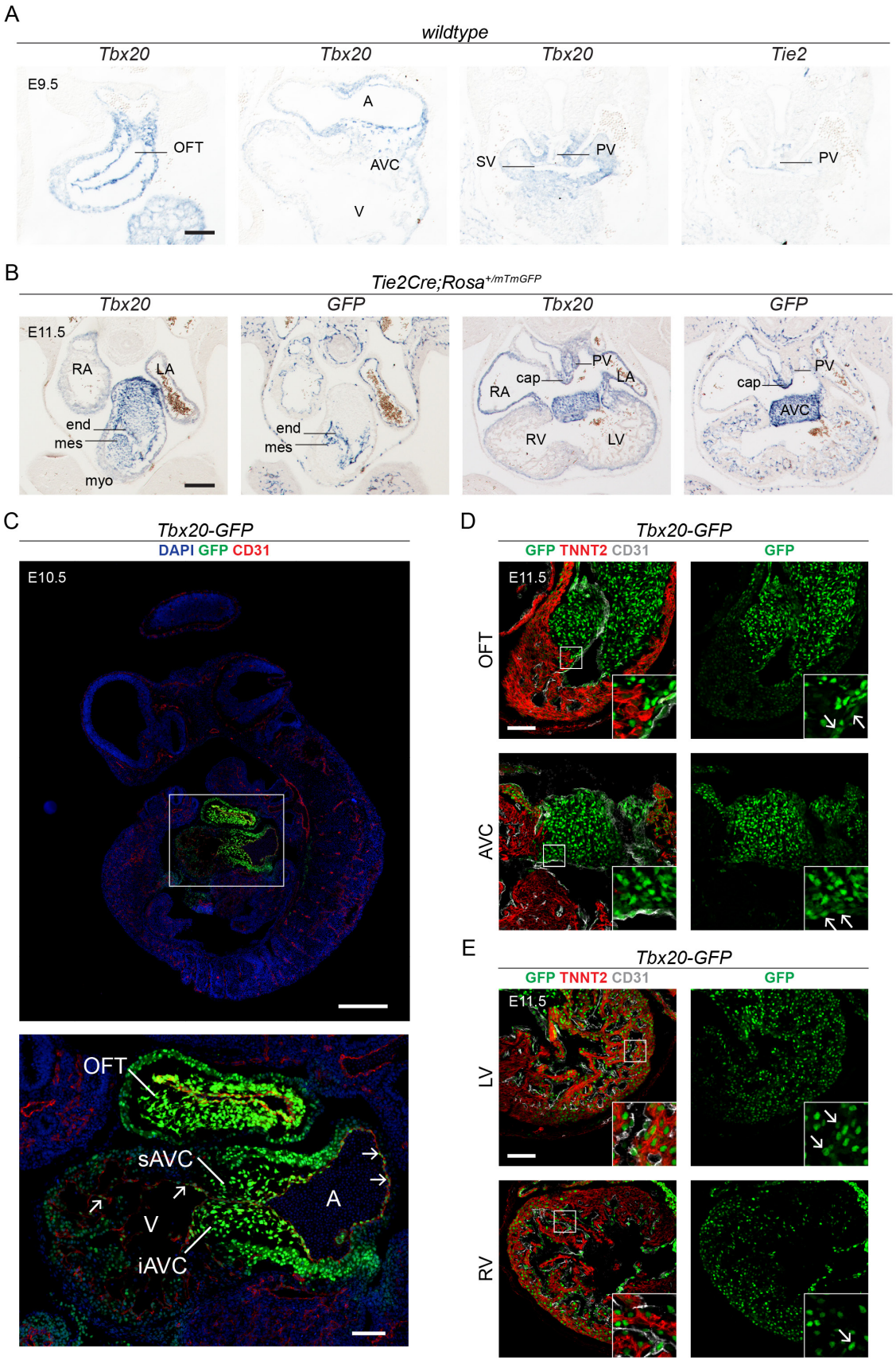
Supplemental Figure 2. (A) Quantification of mesenchymal cells in vivo in E11.5 *Tie2Cre*;*Tbx20* mutant hearts (N=3) and littermate controls (N=3), obtained by counting every other section of entire E11.5 hearts. (*B*) Apoptosis marked by cleaved Caspase 3 (Casp3) (arrows) is similar between control and mutant hearts. ACTN2: α-actinin.



Supplemental figure 3. Outflow tract alignment defects and defective cell migration in E13.5 *Nfatc1Cre;Tbx20* mutants. (*A)* Aorta is connected to left ventricle (arrow) in controls, whereas a connection between RV and Ao persists in *Nfatc1Cre;Tbx20* mutants at this stage. (*B*) Clustered endocardial derived *Nfatc1Cre;Tbx20* mutant cells display migration defects in proximal outflow tract cushions (arrowhead) in *Rosa*^{tdTom} lineage tracing analysis, coincident with the region in which cardiomyocytes (ACTN2+) have not yet managed to fully colonize the outflow tract septum. Atrioventricular septation has occurred in *Nfatc1Cre;Tbx20* mutants, with evident contribution of DMP (arrow) to the atrioventricular septal complex. (C) Migration distance of *Tie2Cre* lineage traced mesenchymal cells over the surface of collagen gels after 3 days of culturing of *Tie2Cre;Tbx20* mutant (N=6) and littermate control (N=5) outflow tract cushions (2-sided t-test; mean + SEM; *n*=5 (control) *n*=6 (mutant); *P*=0.85) (D) Quantification of the number of migrating mesenchymal on the surface of collagen gels after 2 days of culturing and after 3 days of culturing, indicating no significant difference between *Tie2Cre;Tbx20* mutant explants and controls (2-sided t-test; mean + SEM; *P*=0.73 (2 days), *P*=0.61 (3 days)).



Supplemental figure 4. Comparison of *TieCre andNfatc1Cre* lineage tracing in developing heart using *Rosa* ^{mTmGFP}. (A-D) At at E9.5, *Tie2Cre* lineage tracing efficiently labels CD31+ endothelial cells and endothelial derived cells (arrows) throughout the heart, including sinus venosus (SV), progenitors of the midpharyngeal endothelial strand that will form the pulmonary vein (PV), atrium (A), atrioventricular cushion (AVC), outflow tract (OFT) and Aorta (Ao). (E-H) At E9.5, *Nfatc1Cre* labels CD31+ endocardium (arrows), but does not efficiently label all endothelial cells of SV and PV (arrowheads E), A (arrowheads in F,G), and AVC (arrowheads in G). (I) At E10.5, *Tie2Cre* efficiently labels CD31+ endothelial cells and CD31 derived lineages throughout the heart. (J) At E10.5, *Nfatc1Cre* labels the majority of endothelial cells in heart (arrows) but not all endothelial cells in atrial wall and PV.


A
wildtype
Tbx20
Tbx20
Tbx20
Tie2
E9.5
OFT
A
AVC
V
SV
PV
PV
B
Tie2Cre;Rosa+/mTmGFP
Tbx20
GFP
Tbx20
GFP
E11.5
RA
LA
end
mes
myo
end
mes
PV
cap
LA
RA
RV
LV
PV
cap
AVC
C
Tbx20-GFP
DAPI GFP CD31
E10.5
OFT
sAVC
A
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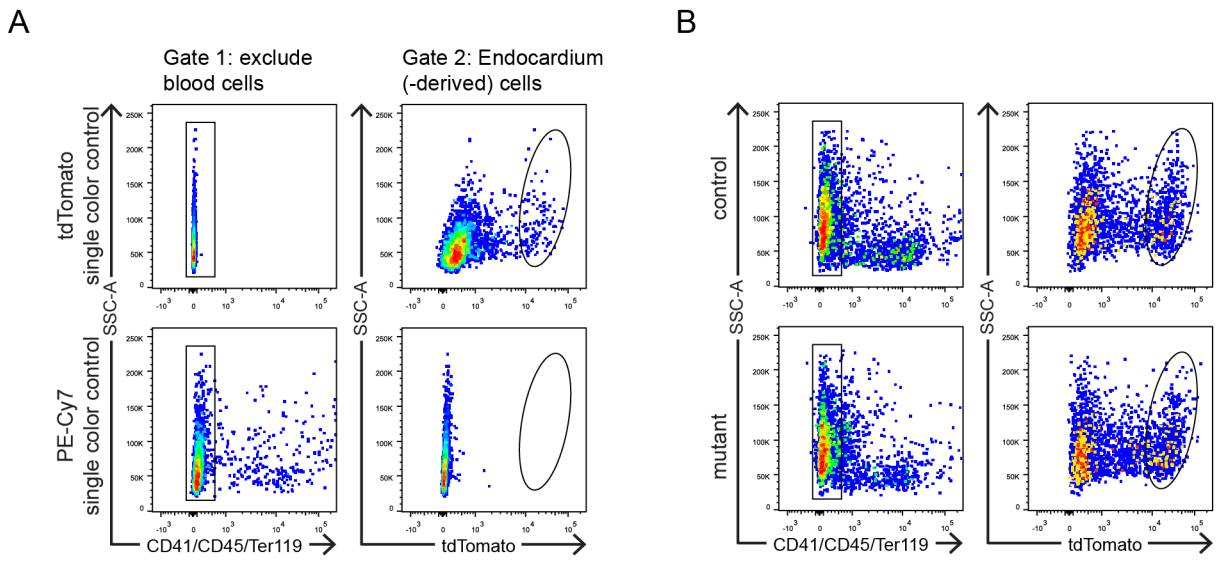
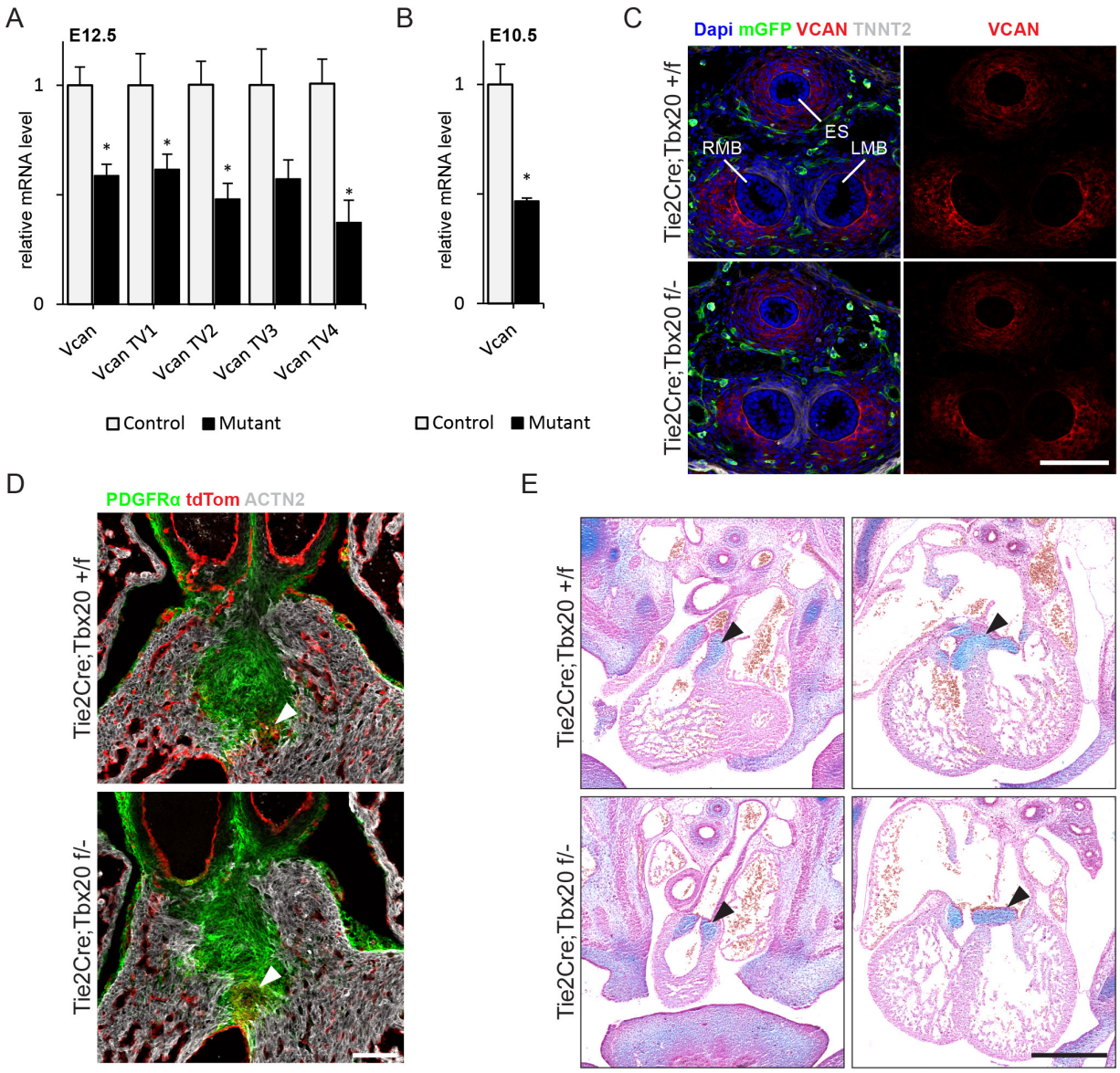
D
Tbx20-GFP
GFP TNNT2 CD31
GFP
E11.5
OFT
AVC
E
Tbx20-GFP
GFP TNNT2 CD31
GFP
E11.5
LV
RV

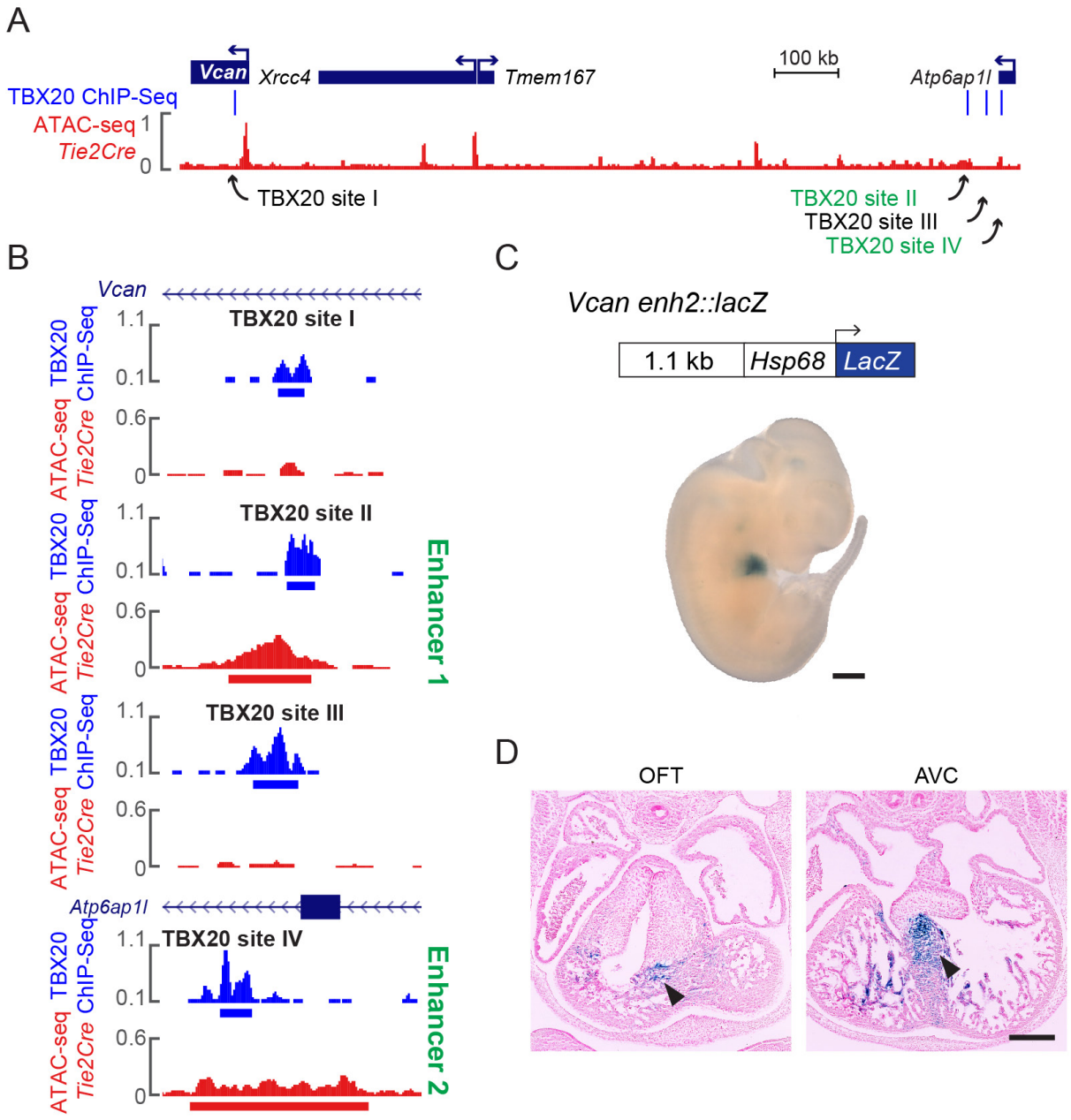
Supplemental figure 5. *Tbx20* and TBX20-GFP are expressed in endocardial lineages during heart development (A) *Tbx20* is expressed throughout E9.5 heart, including OFT and AV cushions and endothelial cells of sinus venosus (SV) and pulmonary vein progenitors of the midpharyngeal endothelial strand (PV), marked by expression of *Tie2*. (B) At E11.5, *Tbx20* is expressed in endothelial cells (end) and endothelial derived mesenchymal cells (mes) of the OFT, AVC and mesenchymal cap of the primary atrial septum (cap). Endothelial lineages are visualized in neighboring sections using *GFP* probe on *Tie2Cre;Rosa*^{mTmGFP} lineage traced tissue. (C) TBX20-GFP is expressed in E10.5 heart, with abundant levels detected in inferior and superior AV cushions (iAVC and sAVC respectively), outflow tract cushions (OFT) and CD31+ endothelial cells throughout the heart (arrows). (D) At E11.5, TBX20-GFP is expressed in endocardial and mesenchymal cells in OFT and AVC. (E) Tbx20-GFP is expressed in endocardium of the right and left ventricular chambers (LV, RV; arrows in insets).



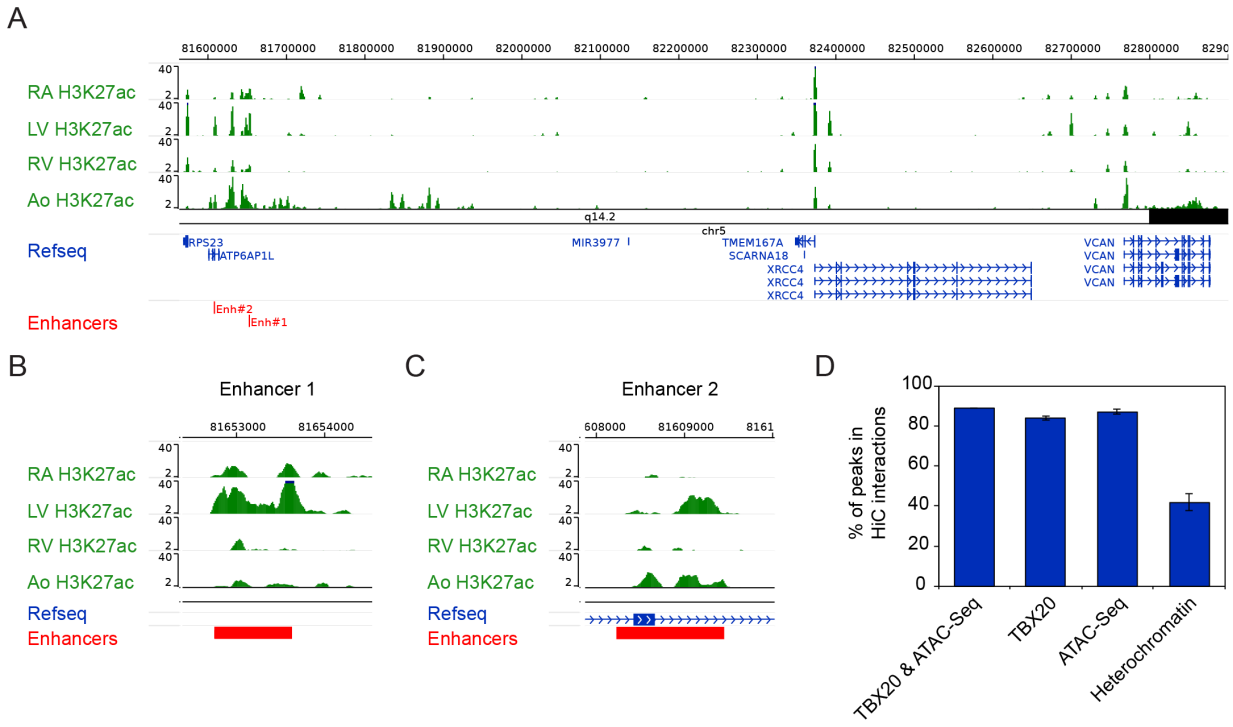
Supplemental Figure 6. Sorting strategy to obtain purified endocardial lineages from embryonic hearts. (A) Single color controls for tdTomato and PE-Cy7. First, CD41-PE-Cy7, CD45-PE-Cy7 and Ter199-PE-Cy7 were used in a cocktail to remove any potential *Tie2Cre* lineage trace blood cells, as previously described (Van Handel et al., 2012), after which tdTomato expression was used to select endocardial and endocardial derived cells. (B) Representative examples of FACs plots for E12.5 *Tie2Cre*;*Tbx20* mutant hearts and control littermates.



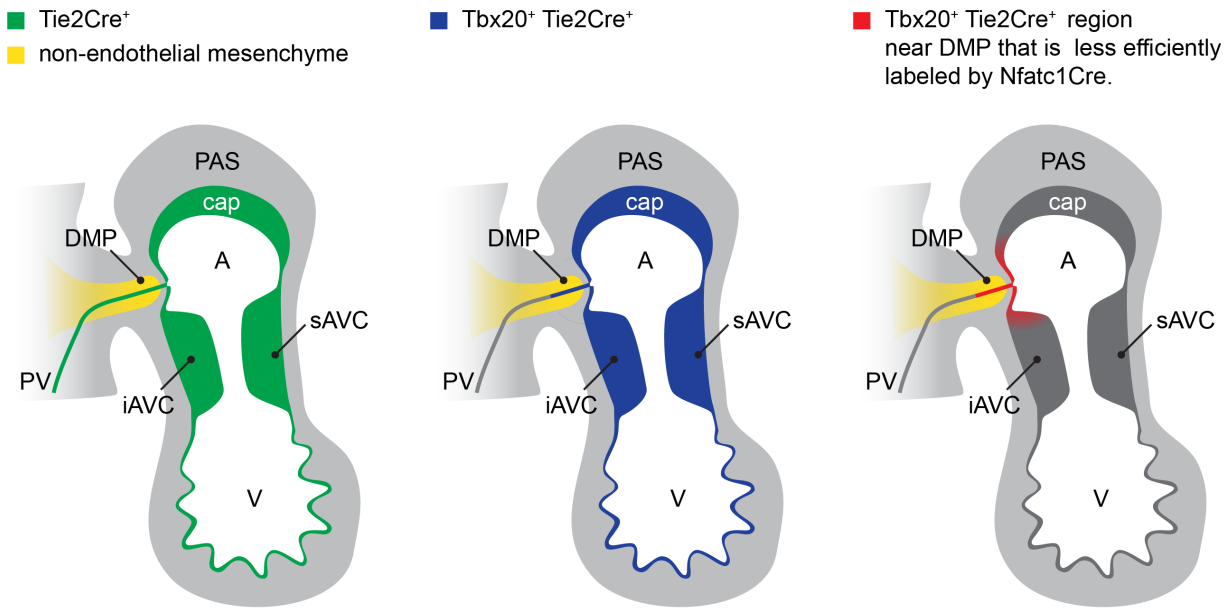
Supplemental Figure 7. Analysis of *Vcan* expression in *Tie2Cre;Tbx20* mutants. (*A*) qPCR analysis of *Vcan* transcript variants in E12.5 *Tie2Cre* lineage (* P<0.05; ** P<0.01; *n*=3; *t*-test) (*B*) qPCR analysis of *Vcan* in E10.5 FACS sorted endocardial cells (*C*) VCAN immunostaining in right and lift main bronchus (RMB, LMB) and esophagus (ES) regions indicates levels are highly similar between controls and mutants. (D) Mesenchymal marker PDGFRalpha is comparably expressed in *Tie2Cre;Tbx20* mutant cells and controls (arrowheads). (E) Alcian blue staining of OFT and AVC cushions (arrowheads). Scale bars: 100 µm (C,D), 500 µm (E).



Supplemental Figure 8. Overview of Tbx20 binding sites in *Vcan* locus. (*A*) *Vcan* locus with TBX20 ChIP-Seq peaks (blue) and ATAC-Seq regions of open chromatin (Red). (*B*) Read counts (plots) and peaks (bars under plots) for TBX20 binding sites. TBX20 sites II and IV are within the enhancers tested in vivo, enhancer1 and enhancer 2 respectively. TBX20 sites I and III are in a region without significant ATAC-Seq read counts, indicating it is closed chromatin in endocardial lineage. (C) 7/7 *Vcan enh2::lacZ* transgenic embryos displayed strong cardiac specific X-gal staining pattern. (D) Section analysis revealed *Vcan enh2::lacZ* was specifically expressed in myocardium, and not cardiac cushions or endocardium (7/7). Scalebars: 1mm (C), 200µm (D).



Supplemental Figure 9. Human *VCAN* transcriptional landscape. (A) 1.2MB region upstream of *VCAN* with tracks for the enhancer and promoter associated chromatin mark acetylated Histone H3 Lysine27 (H2K27ac), from human right atrium (RA), left ventricle (LV), right ventricle (RV) and Aorta (Ao). Alignment of the homologous mouse Enhancer1 and Enhancer2 are shown in red. (B) H3K27Ac marks in human heart overlap with Enhancer 1 (C) H3K27ac marks in human hearts overlap with Enhancer 2. (D) Number of TBX20 and ATAC-seq peaks overlapping either end of a Hi-C interaction from human fibroblast Hi-C (Jin et al., 2013). Coordinates for Hi-C interactions from Supplemental data 3 and 4 from Jin et al (2013) were converted to hg19 using the UCSC Genome browser liftOver tool. Mouse Tbx20 ChIP-seq and ATAC-seq peaks generated in the present work were converted to hg19 coordinates also using the liftOver tool (-minMatch=0.1) and the mm9-to-hg19 chain. The central coordinates of Tbx20 and ATAC-seq peaks were intersected with Hi-C interaction coordinates using bedtools pairtobed. To directly compare the number of peaks common in our Tbx20/ATAC-seq experiments overlapping Hi-C coordinates, the same number (793) of central coordinates were randomly sampled 100 times and averaged for Tbx20 ChIP-seq, ATAC-seq peaks and fetal heart heterochromatin regions (Epigenome Roadmap 9_Heterochromatin chromHMM data downloaded from http://egg2.wustl.edu/roadmap/data/byFileType/chromhmmSegmentations/ChmmModels/coreMarks/jointModel/final/all.mnemonics.bedFiles.tgz). TBX20 & ATAC-seq means peaks overlapping each other by at least 1bp.



Supplemental Figure 10. Model displaying endothelial regions of *Tbx20* expression that could explain defective DMP development in *Tie2Cre;Tbx20* mutants. *Tie2Cre* lineage (green) includes pulmonary vein, atrial endocardium and mesenchymal cells of the inferior and superior atrioventricular cushions (iAVC and sAVC, respectively), mesenchymal cap of the atrial septum (cap) and sinus venosus endothelium (not shown). *Tbx20* is expressed (blue) in endothelial lineages including the proximal pulmonary vein, atrial endocardium and mesenchymal cap and AV cushions. Endocardial lineages near developing DMP that do express *Tbx20* but are not labeled efficiently by *Nfatc1Cre* include proximal pulmonary vein, atrial endocardium, cells of the iAVC and mesenchymal cap and sinus venosus endothelium (not shown).

Supplemental References

Jin, F., Li, Y., Dixon, J.R., Selvaraj, S., Ye, Z., Lee, A.Y., Yen, C.-A., Schmitt, A.D., Espinoza, C.A., Ren, B., 2013. A high-resolution map of the three-dimensional chromatin interactome in human cells. Nature 503, 290–4. doi:10.1038/nature12644

Van Handel, B., Montel-Hagen, A., Sasidharan, R., Nakano, H., Ferrari, R., Boogerd, C.J., Schredelseker, J., Wang, Y., Hunter, S., Org, T., Zhou, J., Li, X., Pellegrini, M., Chen, J.-N., Orkin, S.H., Kurdistani, S.K., Evans, S.M., Nakano, A., Mikkola, H.K.A., 2012. Scl represses cardiomyogenesis in prospective hemogenic endothelium and endocardium. Cell 150, 590–605. doi:10.1016/j.cell.2012.06.026