Supplementary Information

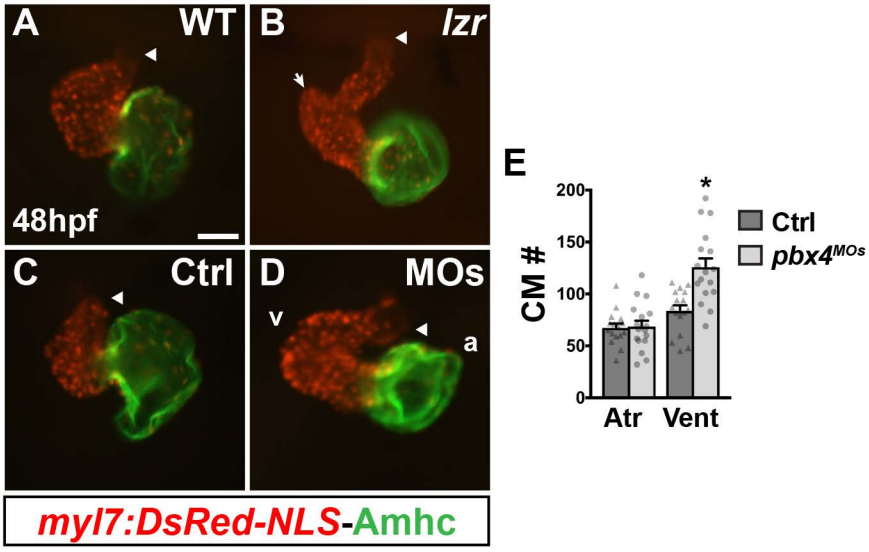

Fig. S1. Pbx4 depletion results in a specific increase in ventricular CMs equivalent to *lzr* mutants. (A,B) Hearts of WT and *lzr* mutant *myl7:DsRed2-NLS* embryos at 48 hpf. (C,D) Hearts of Control (Ctrl) and Pbx4-depleted *myl7:DsRed2-NLS* embryos at 48 hpf. IHC for DsRed2-NLS (CMs - red) and Amhc (atria – green). Images are frontal views. White arrow in B denotes ventricular protrusion. White arrowheads indicate the arterial pole of the hearts. Scale bar - 50 μm. (E) Number of CMs in hearts of WT and *lzr* mutant *myl7:DsRed2-NLS* embryos at 48 hpf. WT n=16, Pbx4-depleted n=17.

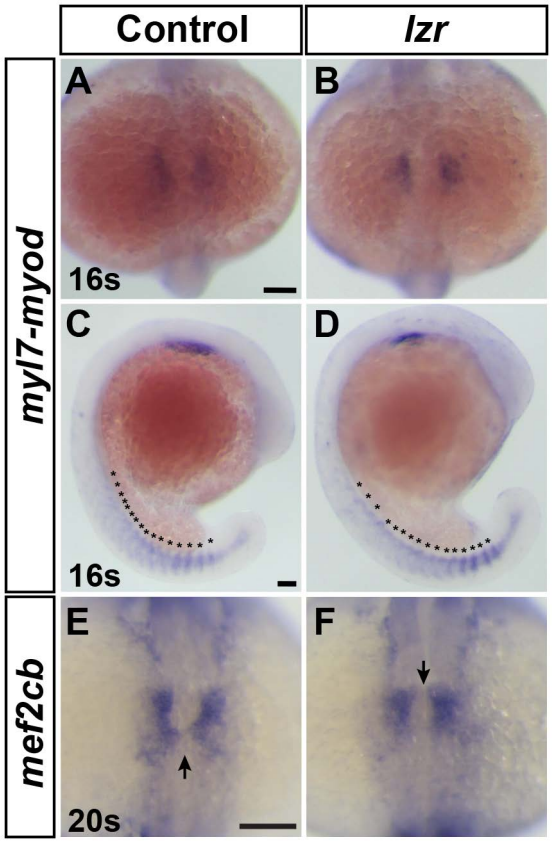

Fig. S2. Early CM differentiation is not affected in *lzr* mutants. (A-D) ISH for *myl7* and *myod* at 16s. Views in A and B are dorsal with anterior up and show *myl7*. Views in C and D are lateral with dorsal right of same embryos in A and B. Asterisks indicate somites. WT n=52, *lzr* n=4. (E,F) ISH for *mef2cb* at the 20s stage. Views in E and F are dorsal with anterior up. Arrows indicated sites of fusion of the cardiac cells forming the cone. WT n=2, *lzr* n=2. Scale bars - 100 μm.

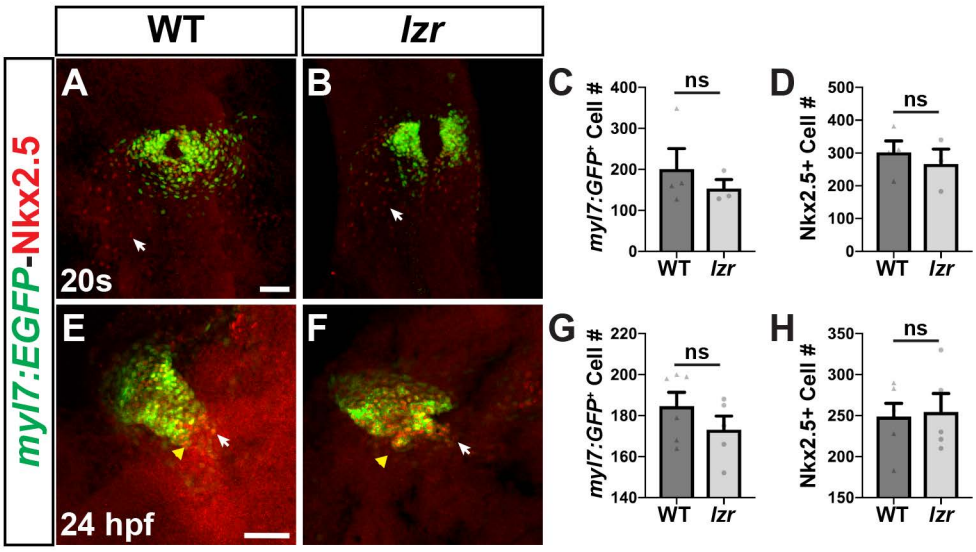

Fig. S3. Differentiating CMs and CM progenitors are not affected in *lzr* mutants. (A,B,E,F) IHC for *myl7:EGFP* (green) and Nkx2.5 (red) at the 20s stage and 24 hpf. Views are dorsal with anterior up. White arrows indicate Nkx2.5+ nuclei. Yellow arrows in E and F indicate arterial pole of the heart. Scale bars - 50 μm. (C,G) Number of *myl7:EGFP*+ CMs at 20s (WT n= 4, *lzr* n=3) and 24 hpf (WT n=5, *lzr* n=4). (D,H) Number of Nkx2.5+ cells at 20s (WT n=4, *lzr* n=3) and 24 hpf (WT n=5, *lzr* n=4).

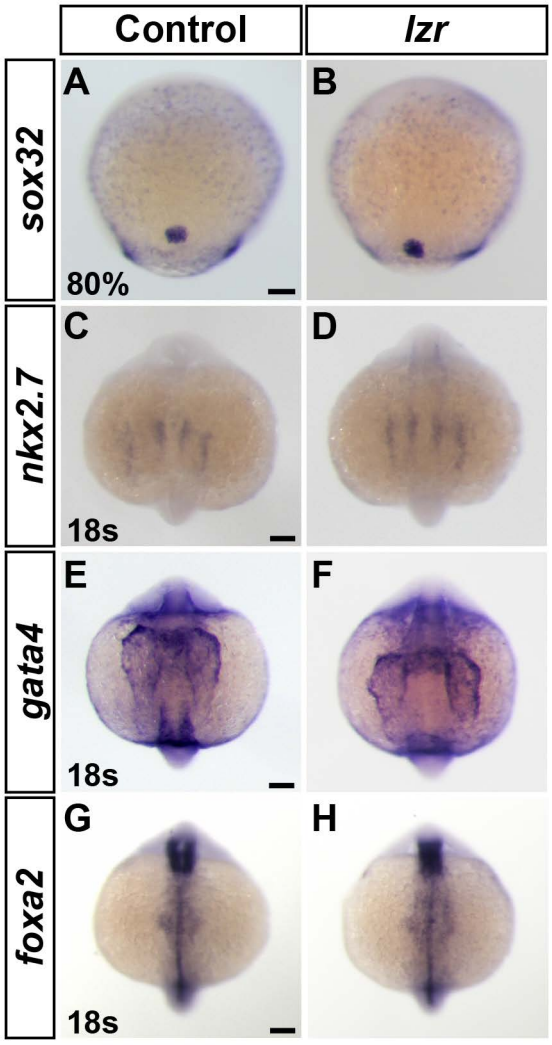

Fig. S4. Endodermal development is overtly normal in *lzr* mutants. (A,B) ISH for the endodermal marker *sox32* at 80% epiboly. Views are dorsal with the animal pole up. WT n=14, *lzr* n=2. (C,D) ISH for the cardiac (medial) and pharyngeal endoderm marker (lateral) *nkx2.7* at 18 hpf. WT n=32, *lzr* n=8. (E,F) ISH for the cardiac (medial) and endoderm marker *gata4* at 18 hpf. WT n=14, *lzr* n=4. (G,H) ISH for the endodermal marker *foxa2* at 18 hpf. WT n=17, *lzr* n=9. For C-H, views are dorsal with anterior up. Scale bars - 100 μm.

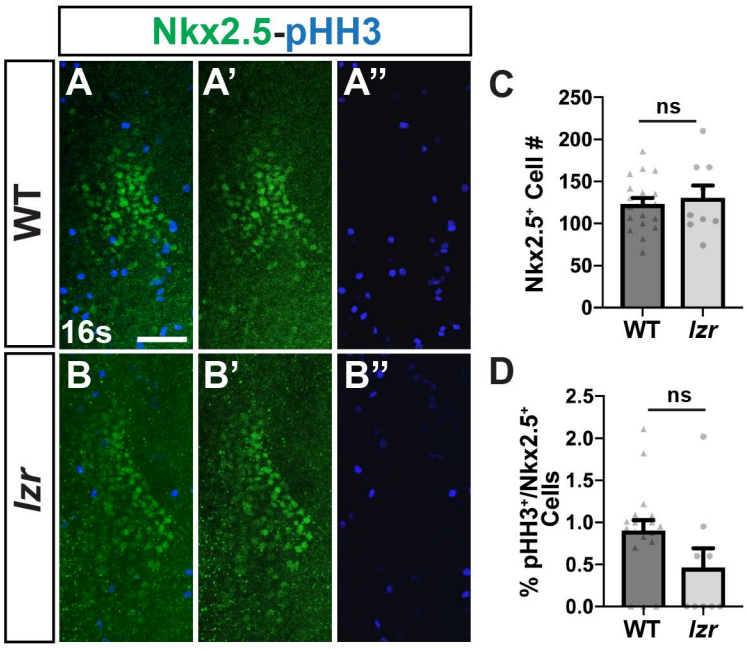

Fig. S5. The number of Nkx2.5+ cells during somitogenesis is unaffected in *lzr* mutants. (A-B") IHC for Nkx2.5+ and pHH3+ cells in WT and *lzr* mutant embryos at the 16s stage. View are dorsal with anterior up of one side of the embryo. Single sides of the embryos were counted to account for uneven flat-mounting that occasionally may have abrogated cells on a single side. Scale bars - 50 μm. (C) Number of Nkx2.5+ cells in WT and *lzr* mutants at the 16s stage. (D) Percentage of pHH3+/Nkx2.5+ cells in WT and *lzr* mutants at the 16s stage. WT n=19, *lzr* n=9.

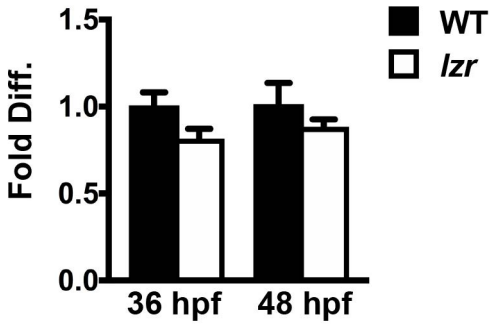

Fig. S6. *Amhc* expression is not affected in *lzr* mutants. RT-qPCR for *amhc* expression levels in WT and *lzr* mutants at 36 hpf and 48 hpf.

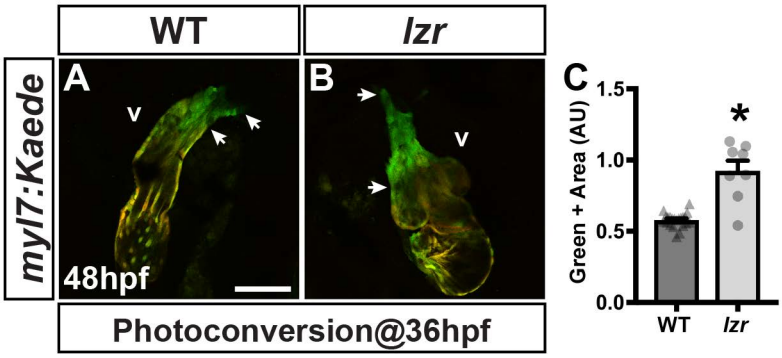

Fig. S7. *Lzr* mutants have an increase in later-differentiating ventricular CMs. (A,B) Confocal images of hearts from WT and *lzr* mutant *myl7:Kaede* embryos. Kaede was photoconverted at 36 hpf. Arrows indicate distance between green-only ventricular CMs and arterial pole of the ventricle. Scale bar - 100 μm. (C) Area (A.U.) of green-only cells in images. WT n=22, *lzr* n=8 embryos.

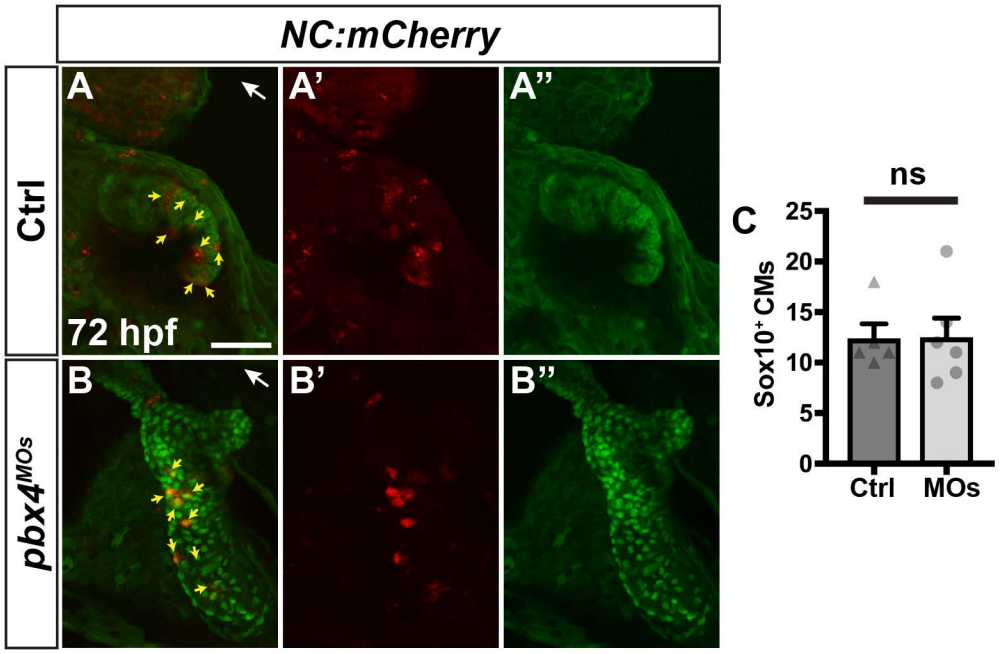

Fig. S8. NC-derived CMs are unaffected in Pbx4-depleted embryos. (A-B'') IHC of Control and Pbx4-depleted *NC:mCherry* embryos at 72 hpf. Yellow arrows indicated NC-derived CMs (red). Scale bar - 50 μm. (C) Number of *sox10*+-derived CMs. WT n=6, *lzr* n=5.

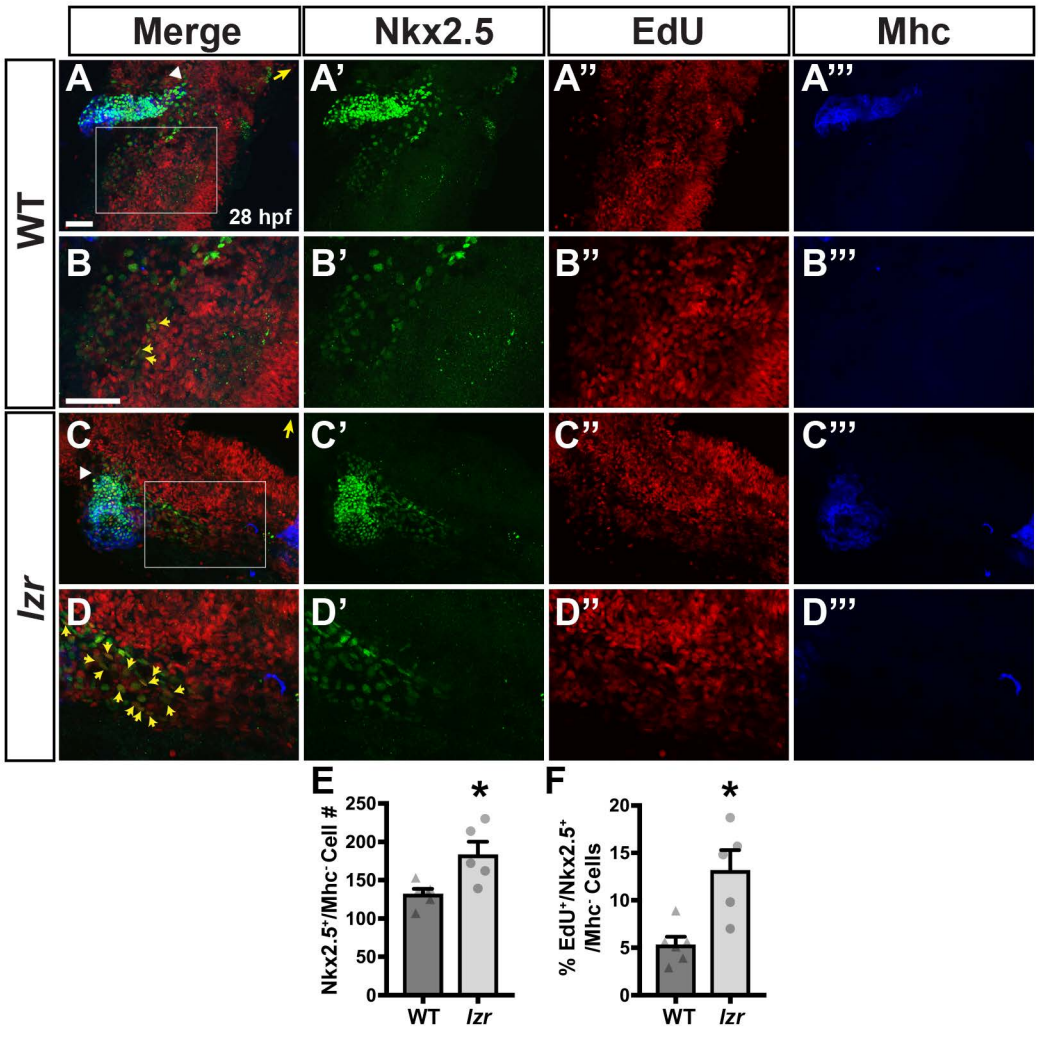

Fig. S9. *Lzr* mutants have an increase in proliferating SHFPs. (A-D''') IHC for EdU +, Nkx2.5+, and Mhc+ cells at 28 hpf. B and D are higher magnification images of boxes in A and C. White arrowheads in A and C indicate border of Nkx2.5+/Mhc+ and Nkx2.5+/Mhc- cells at the arterial pole of hearts. Yellow arrows in A and C indicate the direction of the arterial pole of the hearts. Yellow arrows in B and D denote Nkx2.5+/ Mhc-cells co-labeled with EdU as determined using Imaris. Scale bars - 50 μm. (E) Number of Nkx2.5+/Mhc- cells. (F) Percentage of EdU+/Nkx2.5+/Mhc- cells. For E and F, WT n=6, *lzr* n=5.

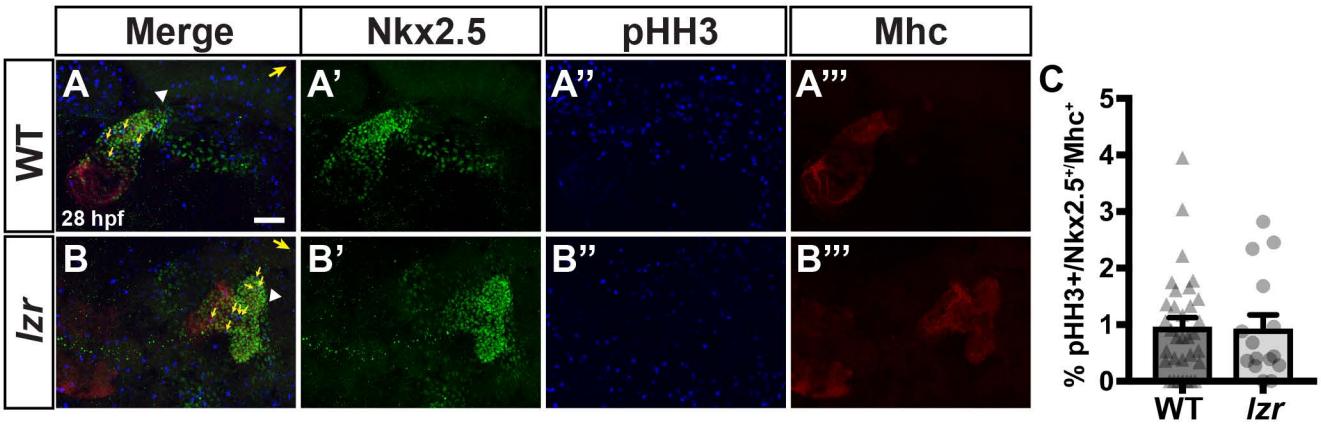

Fig. S10. Proliferation of differentiated CMs is unaffected in *lzr* mutants. (A-B''') IHC for Nkx2.5+, pHH3+, and Mhc+ cells in WT and *lzr* mutant embryos at 28 hpf. White arrowheads in A and B indicate border of Nkx2.5+/Mhc+ and Nkx2.5+/Mhc- cells at the arterial pole of hearts. Yellow arrows in A and B indicate the direction of the arterial pole of the hearts. Scale bars - 50 μm. (C) Percentage of Nkx2.5+/Mhc+ cells co-stained for pHH3. Yellow arrows denotes differentiated CMs expressing pHH3. WT n=33, *lzr* n=15.

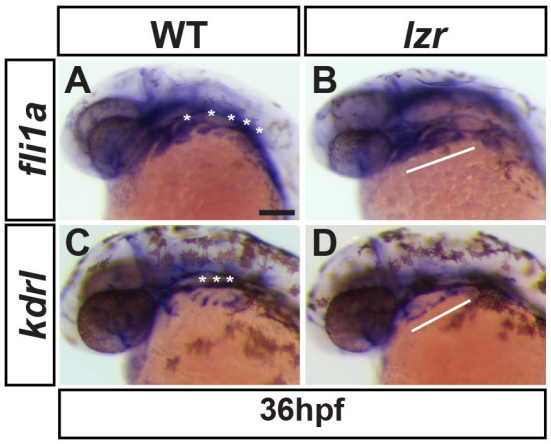

Fig. S11. Aggregates of EC progenitors in the pPAAs are lost in *lzr* mutants. (A-D) ISH for the EC markers *fli1a* and *kdrl* at 36 hpf in WT and *lzr* mutant embryos. Views are lateral with anterior left. Asterisks denote aggregates of EC progenitors within developing posterior arches. White line depicts *fli1a*+ and *kdrl*+ cells that extend to the dorsal aorta in *lzr* mutants. Scale bars - 100 μm. *fli1a* - WT n=3, *lzr* n=5; *kdrl* - WT n=10, *lzr* n=6.

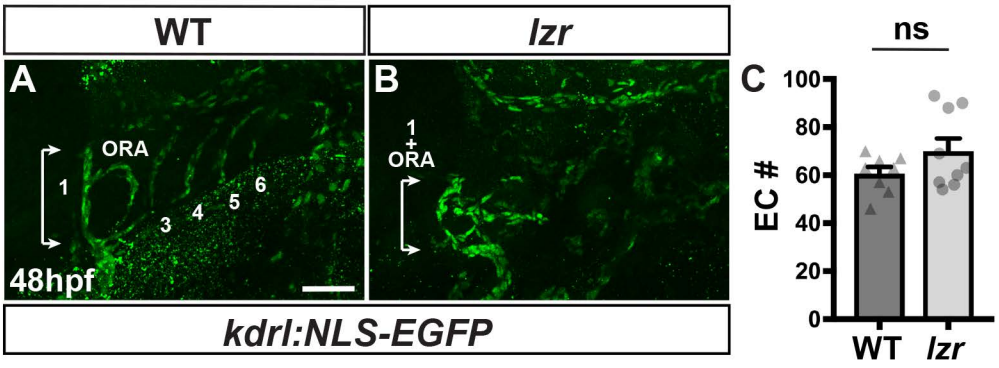

Fig. S12. EC number within anterior PAAs of *lzr* mutants is unchanged. (A, B) IHC of ECs in the PAAs of WT and *lzr* mutant *kdrl:NLS-EGFP* embryos. Views are lateral with anterior left. Numbers designate the PAAs. ORA – opercular artery. Brackets indicate anterior arches. Scale bars - 50 μm. (C) EC number in the 1st and 2nd arches of WT embryos and remaining anterior PAAs. WT n=8, *lzr* n=9.

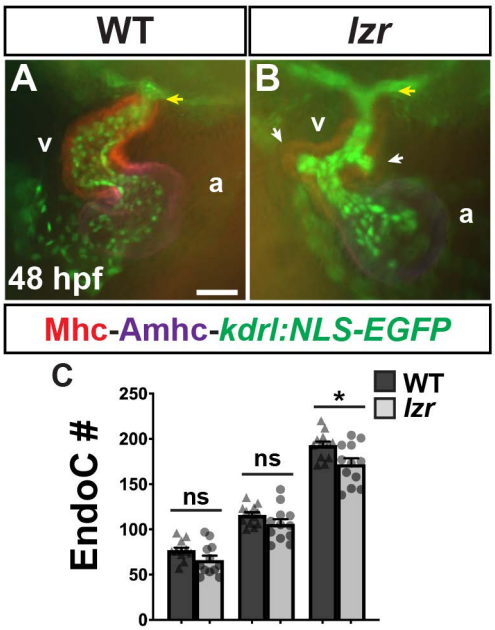

Fig. S13. Endocardial cells are not increased in *lzr* mutants. (A,B) IHC of hearts from WT and *lzr kdrl:NLS-EGFP* embryos at 48 hpf. Views are frontal. Mhc – red. Amhc – Purple. Yellow arrows indicate arterial pole of the heart. White arrows indicate ventricular protrusions. v – ventricle. a – atrium. Scale bar - 50 μm. (C) Endocardial cell (EndoC) number in WT and *lzr* mutant embryos. WT n=13, *lzr* n=12.

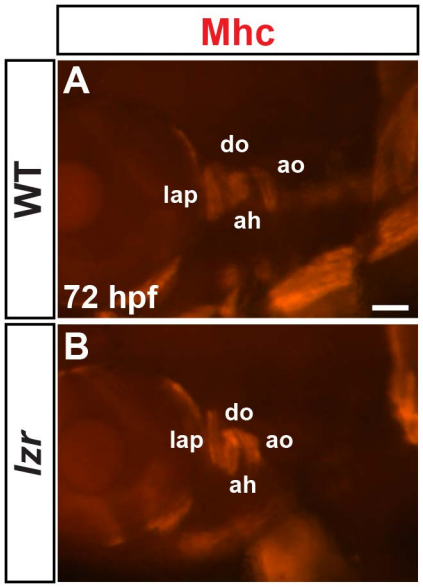

Fig. S14. Anterior craniofacial muscles are overtly unaffected in *lzr* mutants. (A,B) IHC for Mhc in WT and *lzr* mutants. Views are lateral with anterior left. lap - levator arcus palitini, do - dilator opercula, ah - adductor hyoideus, ao - adductor operculi. WT n=35 and *lzr* n=12 embryos that were examined. Scale bar - 50 μm.

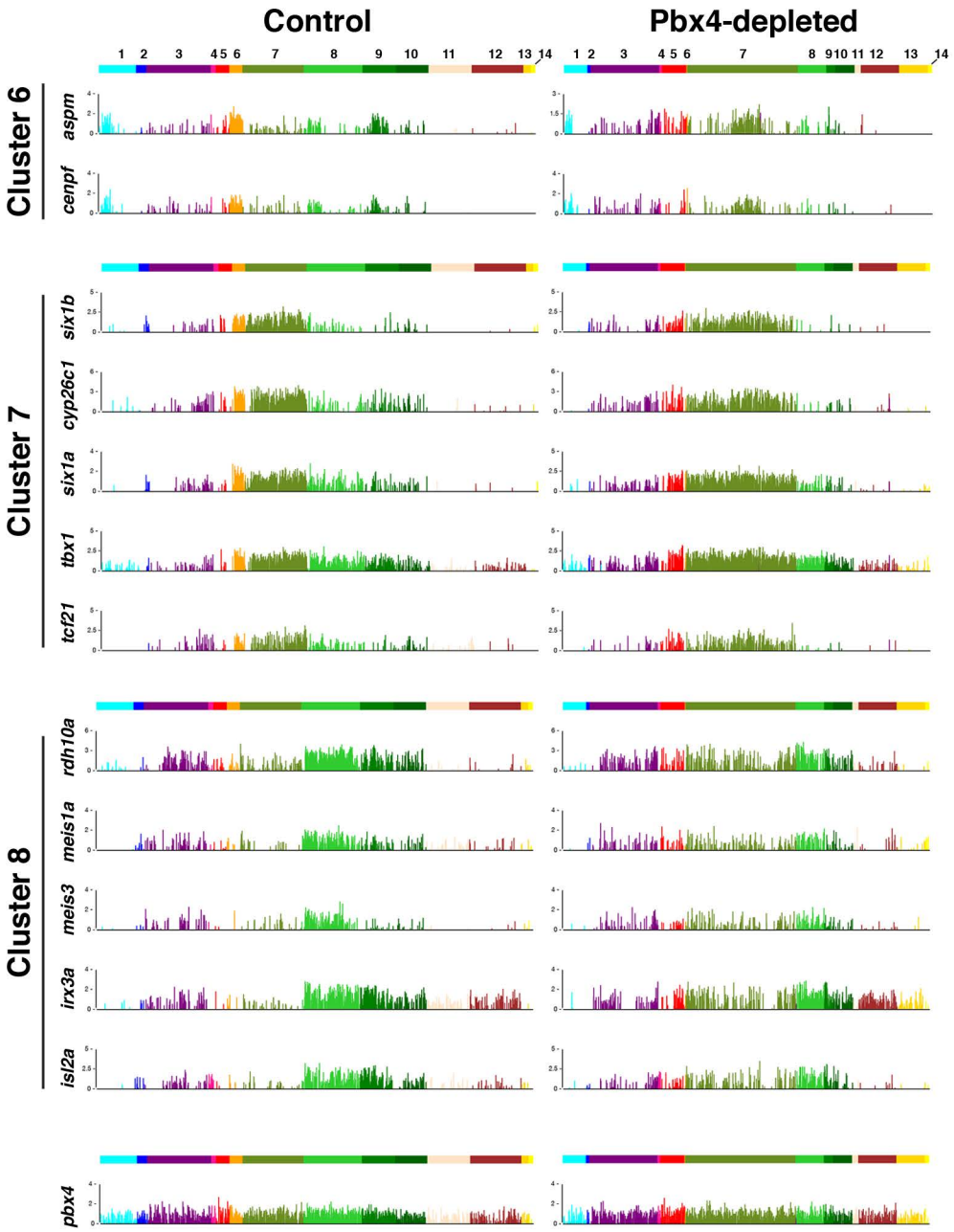

Fig. S15. Expression of additional genes enriched in clusters 6-8 from scRNA-seq of *nkx2.5:ZsYellow*+ cells. Graphs were generated using the online Pbx4-depleted expression viewer.

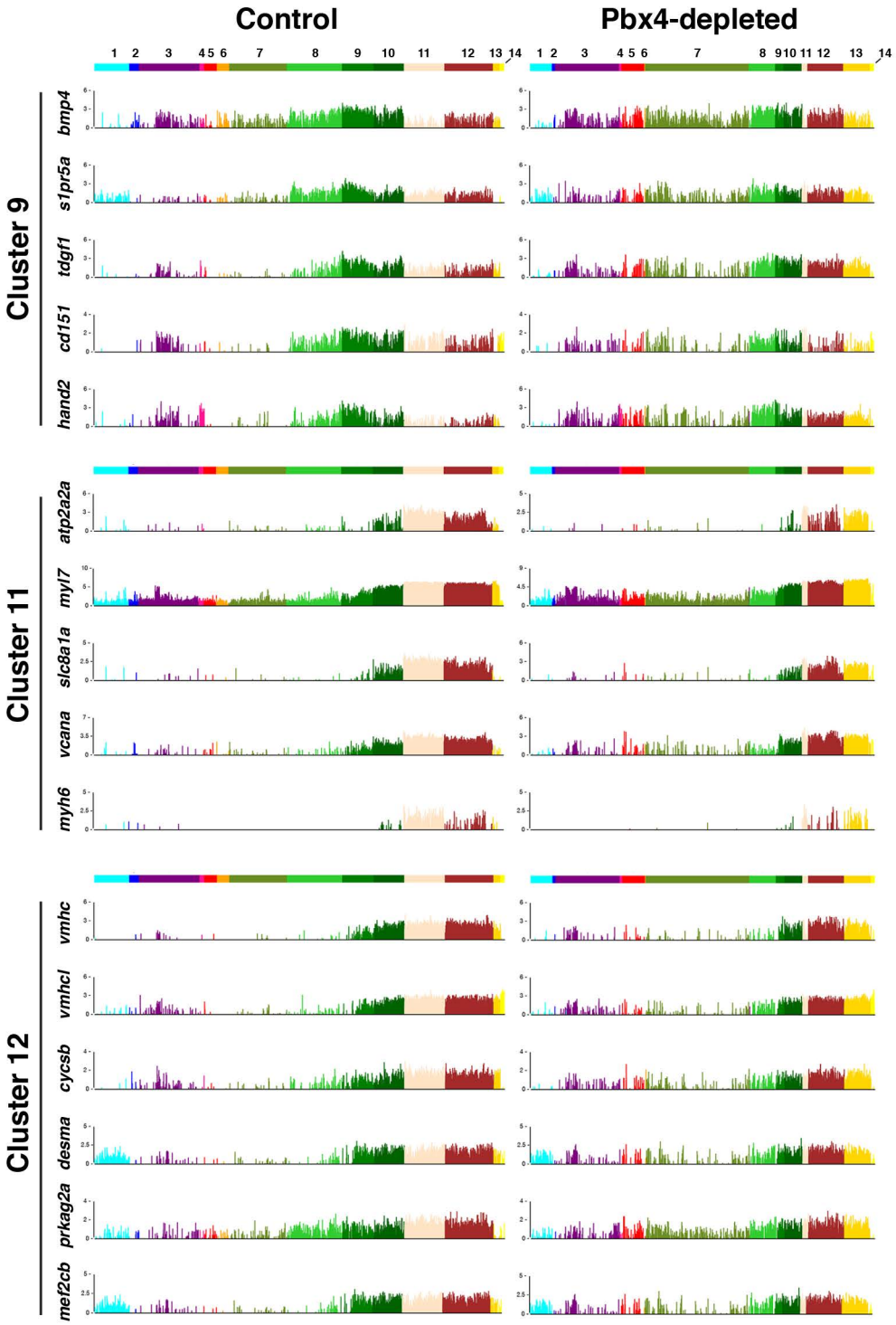

Fig. S16. Expression of additional genes enriched in clusters 9, 11, and 12 in *nkx2.5:ZsYellow*+ cells. Graphs using the online Pbx4-depleted expression viewer.

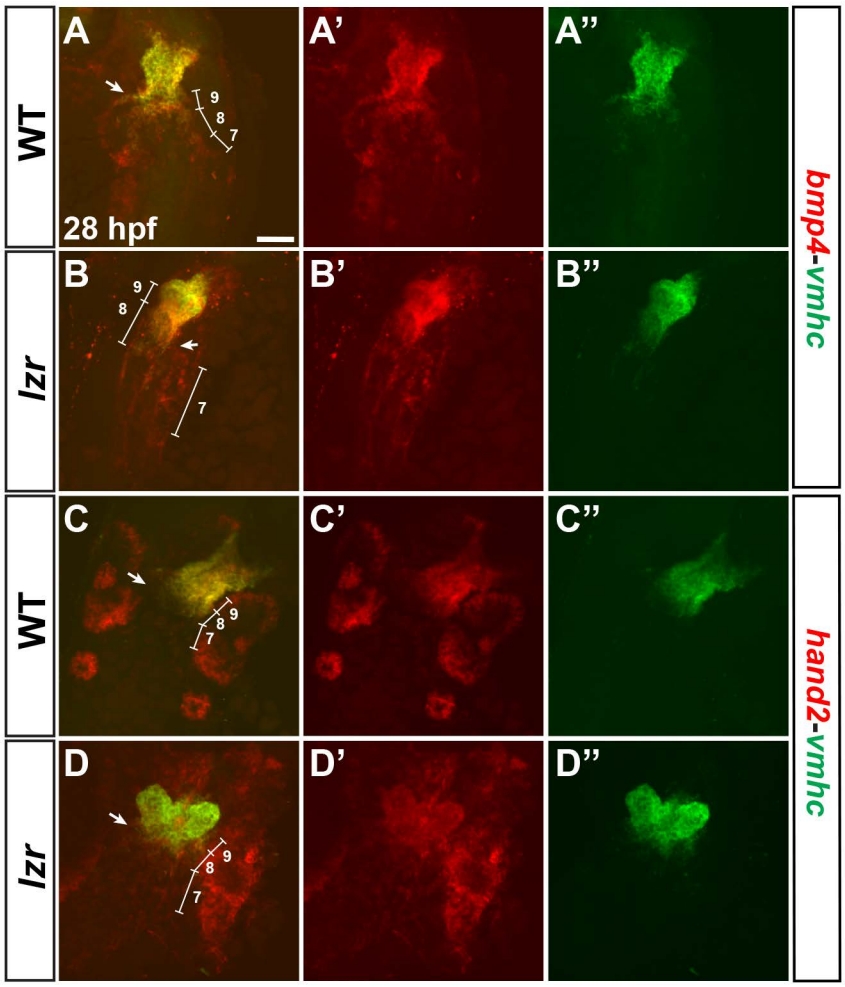

Fig. S17. *Bmp4* and *hand2* expression domains are expanded in lzr mutants. (A-D) FISH for *bmp4* and *hand2* with *vmhc* in WT and *lzr* mutant embryos at 28 hpf. Views are dorsal. Arrows indicate arterial pole of the hearts. Brackets and numbers indicate the predicted cluster populations from the scRNA-seq analysis. Scale bar - 50 μm.

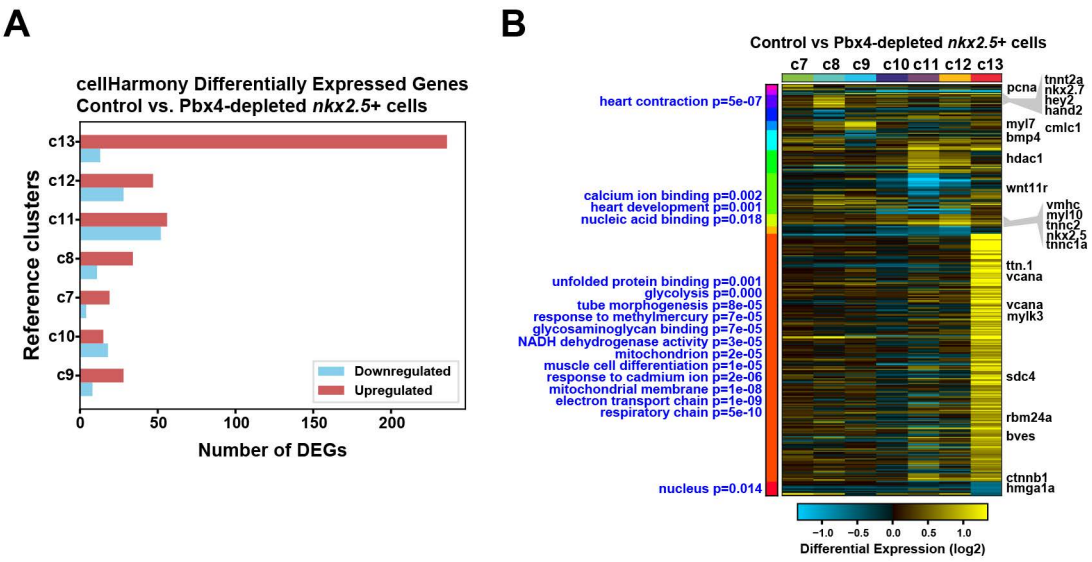

Fig. S18. Pairwise comparison of gene expression within cardiac clusters (C7-13) from control and Pbx4-depleted *nkx2.5:ZsYellow*+ cells. (A) Number of differentially expressed genes in each cluster. (B) Heat-map of pairwise comparison from cellHarmony showing enriched biological pathways and associated differentially expressed genes.

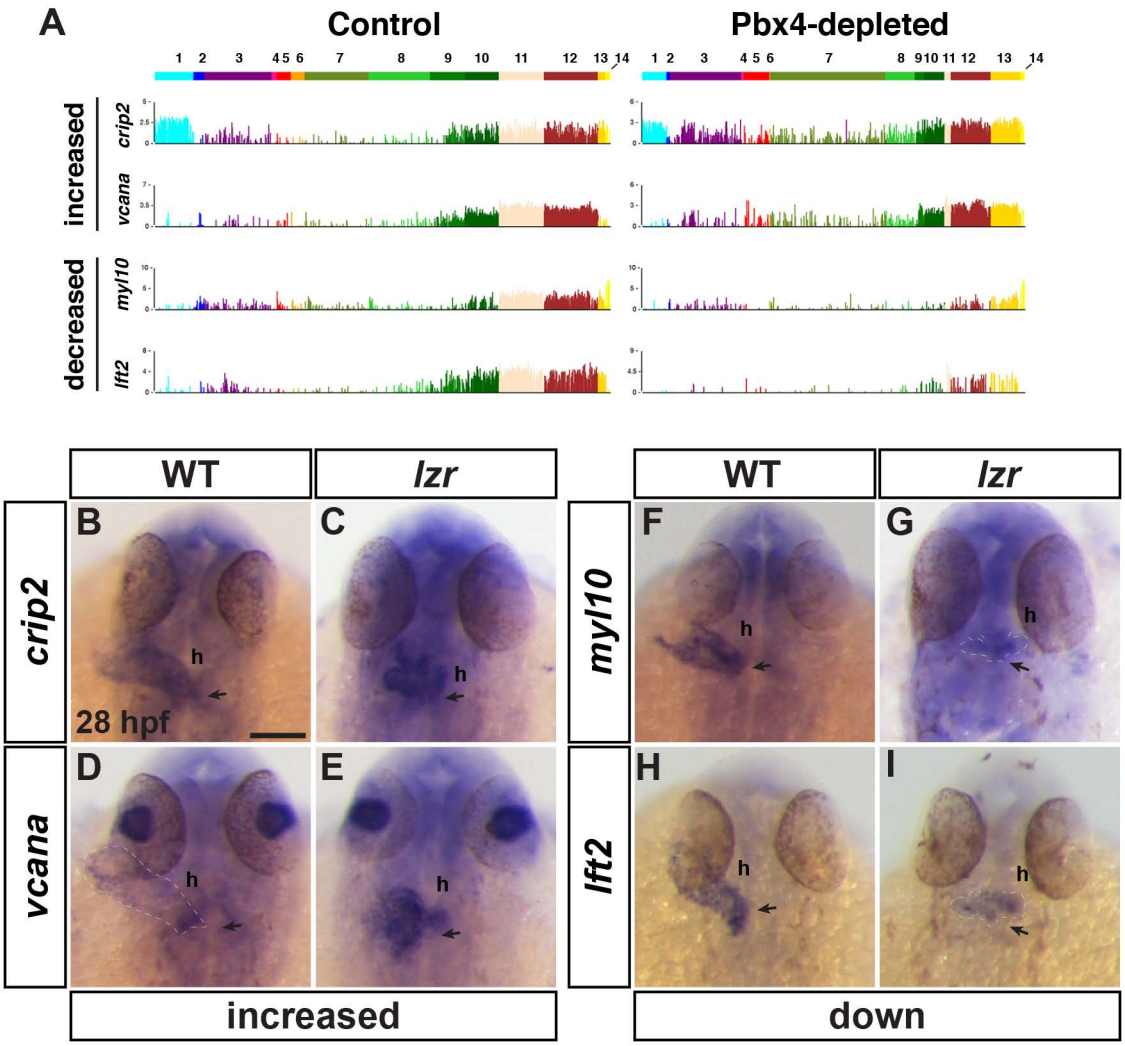

Fig. S19. Differentially expressed genes within the hearts of *lzr* mutants. (A) Expression of *crip2*, *vcana*, *myl10*, and *lft2* in single *nkx2.5:ZsYellow*+ cells from control and Pbx4-depleted embryos. (B-I) ISH for *crip2*, *vcana*, *myl10*, and *lft2* in WT and *lzr* mutant embryos. Dashed lines outline the heart tube (h). Images are dorsal views with anterior up. Arrows indicate arterial pole of the hearts. Scale bar - 100 μm.

Table S1. Markers genes found to be enriched in each of the cell clusters.

Click here to Download Table S1

Table S2. Genes found that were differentially expressed in cardiac clusters between control and Pbx4-depleted cells.

Click here to Download Table S2

Table S3. Primers used in experiments.

gene	genotyping primer	sequence 5'- -3'
lzr	forward	ACTCGGCGGACTCTCGCAAGC
lzr	reverse	GGCTCTCGTCGGTGATGGCCATGATCTTCT
gene	**qPCR primer**	**sequence 5'- -3'**
myh6	forward	GCAGGTAGCGATGAAAGGAG
myh6	reverse	CCTCGTCCGTCTGATAGGTC
β -actin	forward	TACAGCTTCACCACCACAGC
β -actin	reverse	AGGAAGGAAGGCTGGAAGAG
cdkn1a	forward	GGAGAAAACCCCAGAGAAGAGC
cdkn1a	reverse	AACGCTGCTACGAGAAGACGAATGC
cdkn2c	forward	TGCGATTGGGGATCTGATGG
cdkn2c	reverse	AGGTTGCCGTCGTTGTCTAG
ltbp3	forward	CGCCCAAACAGGCTTGTAGTAGT
ltbp3	reverse	CACTCTTCGGTGAAAACGG
mef2cb	forward	CTATGGAAACCACCGCAACT
mef2cb	reverse	TGCGCAGACTGAGAGTTGTT
nkx2.5	forward	GCATCAGAGCTTGGTGAACA
nkx2.5	reverse	ATGCGCACGCATAAACATTA
vmhc	forward	AGTCAACACCCTCACCAAGG
vmhc	reverse	TGCTGCTTGTCATTTTCCAG

Table S4. Antibodies used in experiments.

	Antibody	Supplier	Product	Dilution	Procedure
Primary	Rabbit anti-DsRed	Clontech	632496	1:1,000	IHC
	Mouse anti-Myh6	University of Iowa Hybridoma Bank	S46	1:10	IHC
	Chicken anti-GFP	Abcam	ab13970	1:250	IHC
	Rabbit anti-Nkx2.5	Gene Tex	128357	1:250	IHC
	Mouse anti-Sarcomeric myosin (MHC)	University of Iowa Hybridoma Bank	MF20	1:10	IHC
	Mouse anti-phospho Histone H3 (S10)	Abcam	ab14955	1:1000	IHC
	Sheep anti-Fluorescein-POD	Sigma-Aldrich	11426346910	1:50	FISH
	Sheep anti-DIG-POD	Sigma-Aldrich	11207733910	1:50	FISH
	Mouse anti-TRITC (rhodamine)-POD	Lifespan Biosciences	LS-C147273	1:500	FISH
	Rabbit anti-Elastin b	YenZym	N/A	1:100	IHC
	Chicken anti-GFP	Life Technologies	A10262	1:250	IHC
Secondary	Goat anti-rabbit IgG (H & L) TRITC	Southern Biotech	4050-03	1:100	IHC
	Goat anti-mouse IgG1 FITC	Southern Biotech	1070-02	1:100	IHC
	Goat anti-Chicken IgG FITC	Southern Biotech	6100-02	1:100	IHC
	Goat anti-Rabbit IgG(H+L) Alexa Fluor 488	Southern Biotech	4050-03	1:100	IHC
	Goat anti-mouse IgG2b TRITC	Southern Biotech	109003	1:100	IHC
	Goat anti chicken IgY(H+L) Alexa Fluor 488	Southern Biotech	6100-30	1:100	IHC
	Goat anti-mouse IgG (H & L) Alexa Fluor 405	Life Technologies	A31553	1:100	IHC
	Goat anti-mouse IgG1-DyLight 405	BioLegend	409109	1:100	IHC