Supplementary Materials and Methods

X. laevis is an allotetraploid species, which resulted from whole genome duplication events after interspecific hybridization of an ancestral diploid species (Uno et al., 2013). Many genomic loci have been duplicated, including *lhx9*. This resulted in a short (*lhx9.S*, Version 8.0 chr4S:74365055-74397926) and long form of *lhx9* (*lhx9.L*, Version 8.0 chr4L:102410656-102438303). Whilst the coding region sequence of *lhx9.S* and *lhx9.L* is highly conserved (DNA 95%, protein 96%), the genomic scaffolds are only 86% identical (NCBI Blast, ClustalW, Xenbase *X. laevis* genome browser v8.0).

To enable sufficient and reproducible gene function depletion, we designed and utilized two strategies. One was to microinject a translation-blocking Morpholino (MOT) for both *lhx9* loci (Fig. S1, Fig. S7, Supplemental Table 2) together with a 5 base mismatch control. The other strategy was to design two splice blocking MO that target exon 1/intron 1 from each loci (MO1) (Fig. S1, Supplemental Table 2). Both strategies resulted in significant reduction in Lhx9 translation and *lhx9* transcription (Fig. S7, Fig. S8), therefore confirming reproducible depletion of Lhx9 function using two independent methods. Note that both strategies deplete Lhx9α and Lhx9HD isoforms. Injection of the 5-base mismatch control MO lead to no obvious embryological defects whilst the phenotype observed by depleting Lhx9 by either MO strategy was highly comparable, supporting the specificity of each method. Interestingly, microinjecting each MO1 individually did not result in any epicardial defect (therefore serving as a control), further supporting the specificity of MO1 as well as suggesting both *lhx9.S* and *lhx9.L* genomic loci are developmentally regulated and actively required.

Supplemental Figures

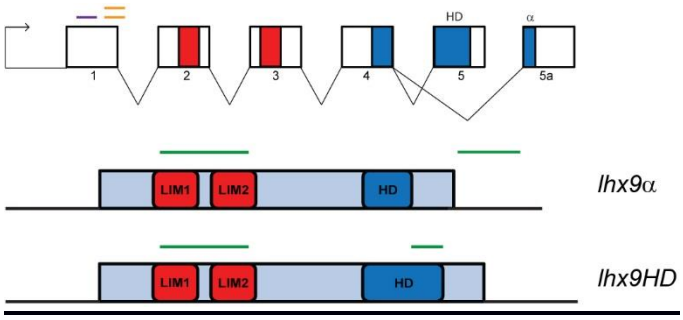

Figure S1. Lhx9 genomic loci and isoform organization

Schematics of *X. laevis* Lhx9 genomic locus (top) and mRNA (bottom), showing localization of LIM protein binding domains (red) in exon 2 and 3, and the DNA-binding homeodomain in exons 4 and 5 (blue). Not to scale. Note that *lhx9α* isoform harbors a truncated HD due to alternative splicing to exon 5a. Translation-blocking (purple) and splice-blocking (orange) Morpholinos are depicted on exon 1 of genomic locus. Green bars on mRNA schematics depict localization of in situ hybridization probes.

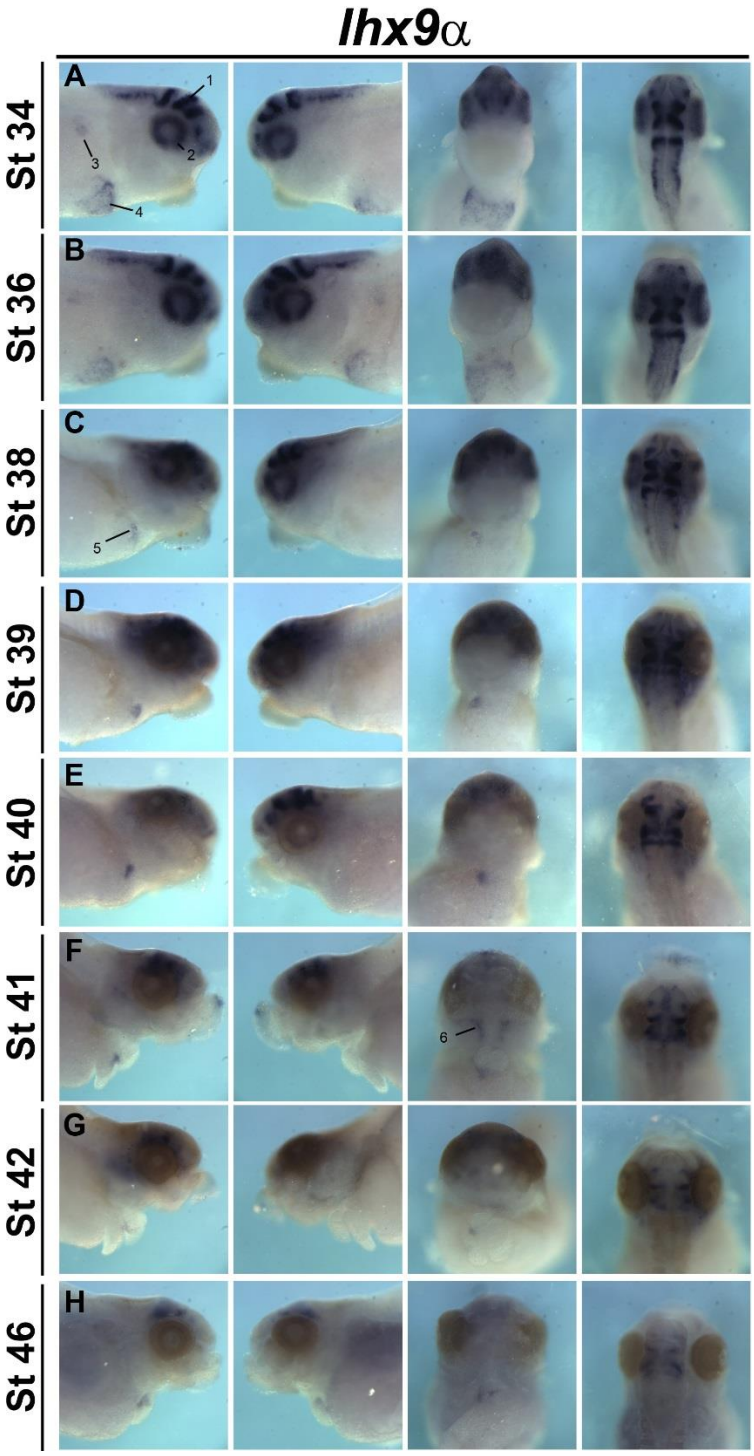

Figure S2. Spatio-temporal analysis of lhx9α during Xenopus embryogenesis.

In situ hybridization right, left, ventral and dorsal views of wild-type embryos showing *lhx9a* expression of the anterior region, from stage 34 to stage 46. 1; neural tube, 2; retina, 3; kidney, 4; septum transversum, 5; proepicardial cluster, 6; jaw cartilage.

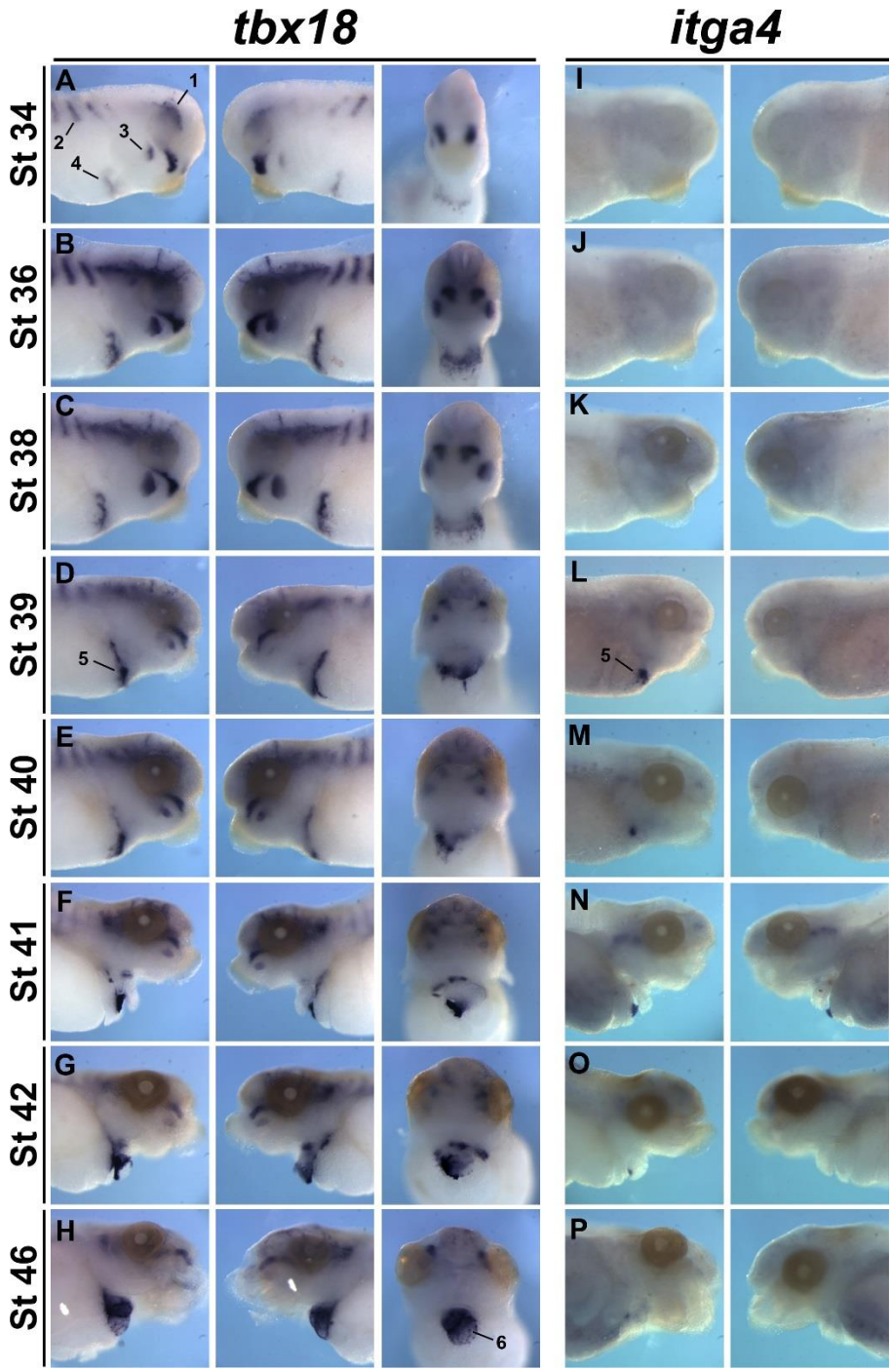

Figure S3. Spatio-temporal analysis of tbx18 and itga4 during Xenopus embryogenesis.
(A-H) In situ hybridization of *tbx18* showing right, left and ventral views of wild-type embryos from stage 34 to 46, anterior region of embryo. (I-P) In situ hybridization of *itga4* showing right and left views of anterior region from stage 34-46. 1; cranial mesoderm, 2; somites, 3; branchial arches, 4; septum transversum, 5; proepicardial cluster, 6; epicardium.

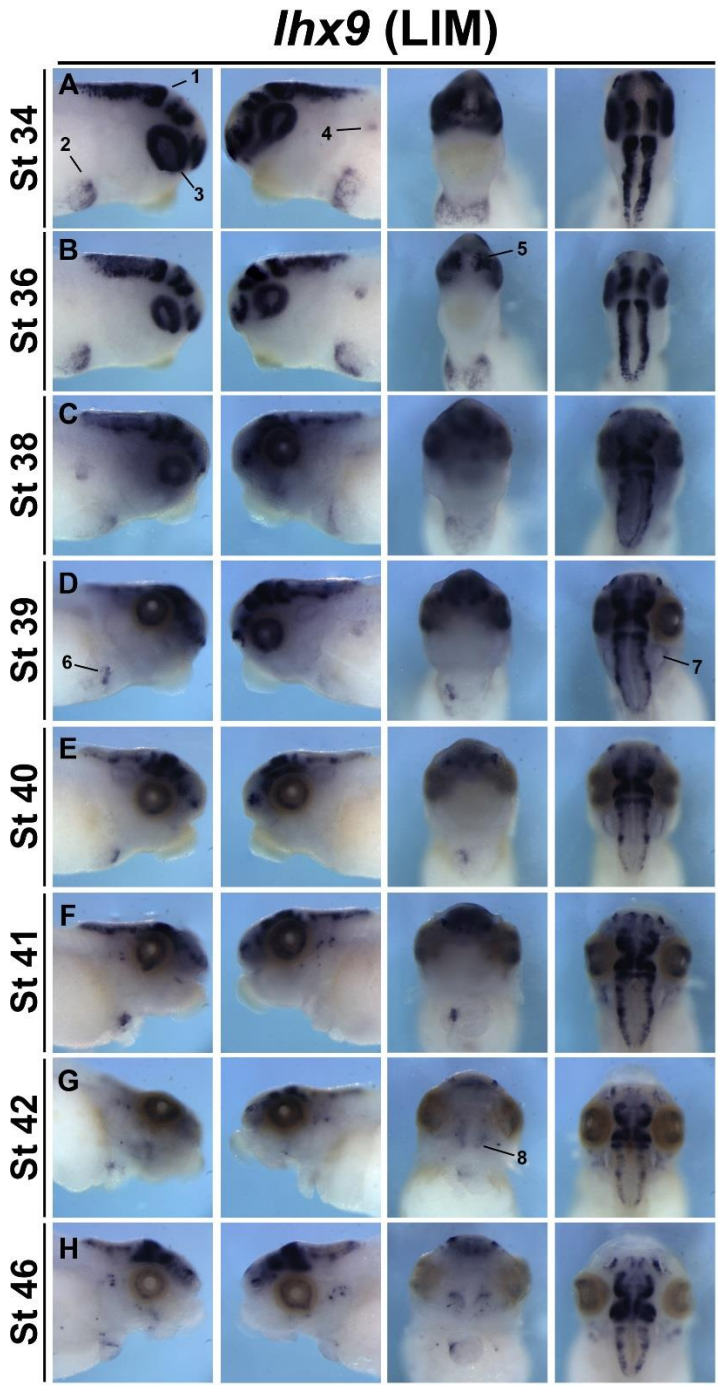

Figure S4. Spatio-temporal analysis of lhx9 during Xenopus embryogenesis.

In situ hybridization of whole embryo anterior region using probe specific for the LIM domains of *lhx9*. Right, left, ventral and dorsal views of wild-type embryos from stage 34 to stage 46. 1; neural tube, 2; septum transversum, 3; retina, 4; kidney, 5; nasal placode, 6; proepicardial cluster, 7; otic placode, 8; jaw cartilage.

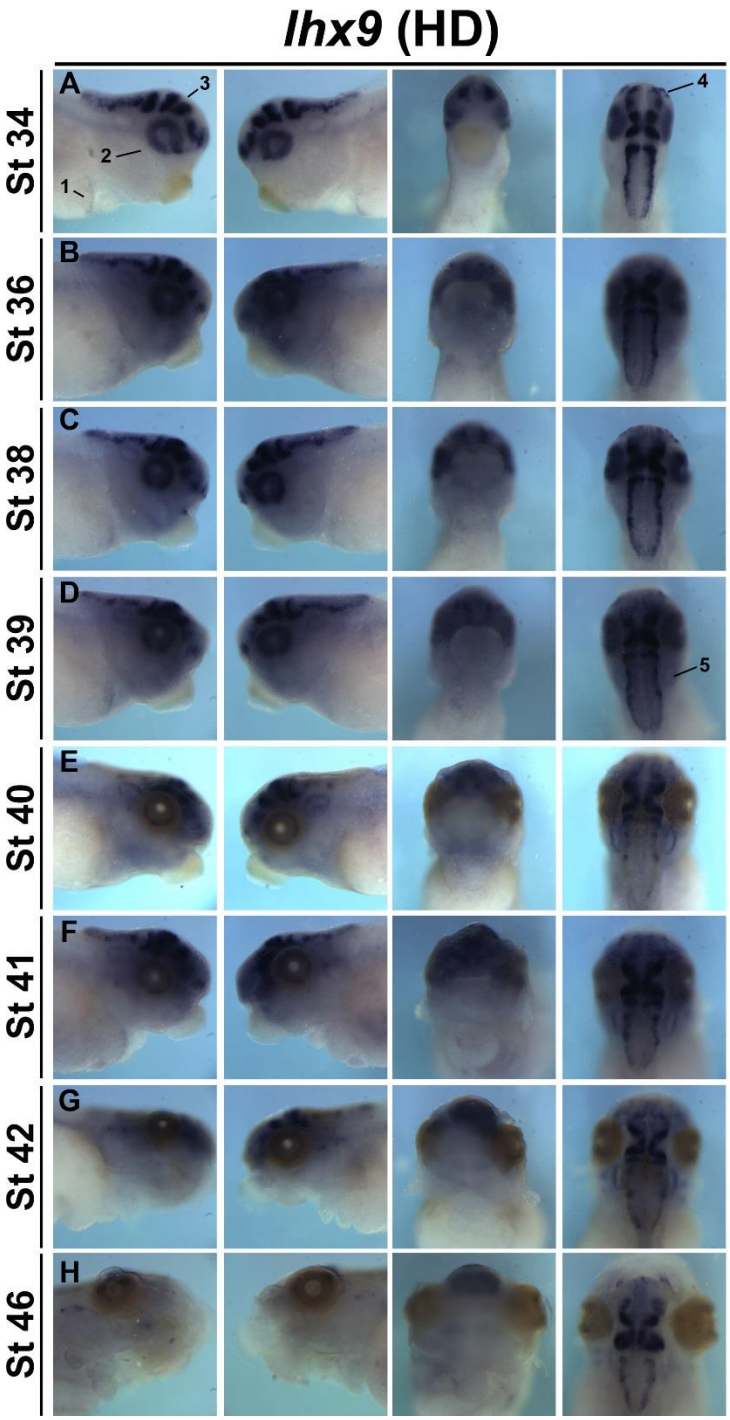

Figure S5. Spatio-temporal analysis of lhx9HD during Xenopus embryogenesis.

In situ hybridization right, left, ventral and dorsal views of wild-type embryos showing *lhx9HD* expression of the anterior region over time, from stage 34 to stage 46. 1; septum transversum, 2; retina, 3; neural tube, 4; nasal placode, 5; otic placode.

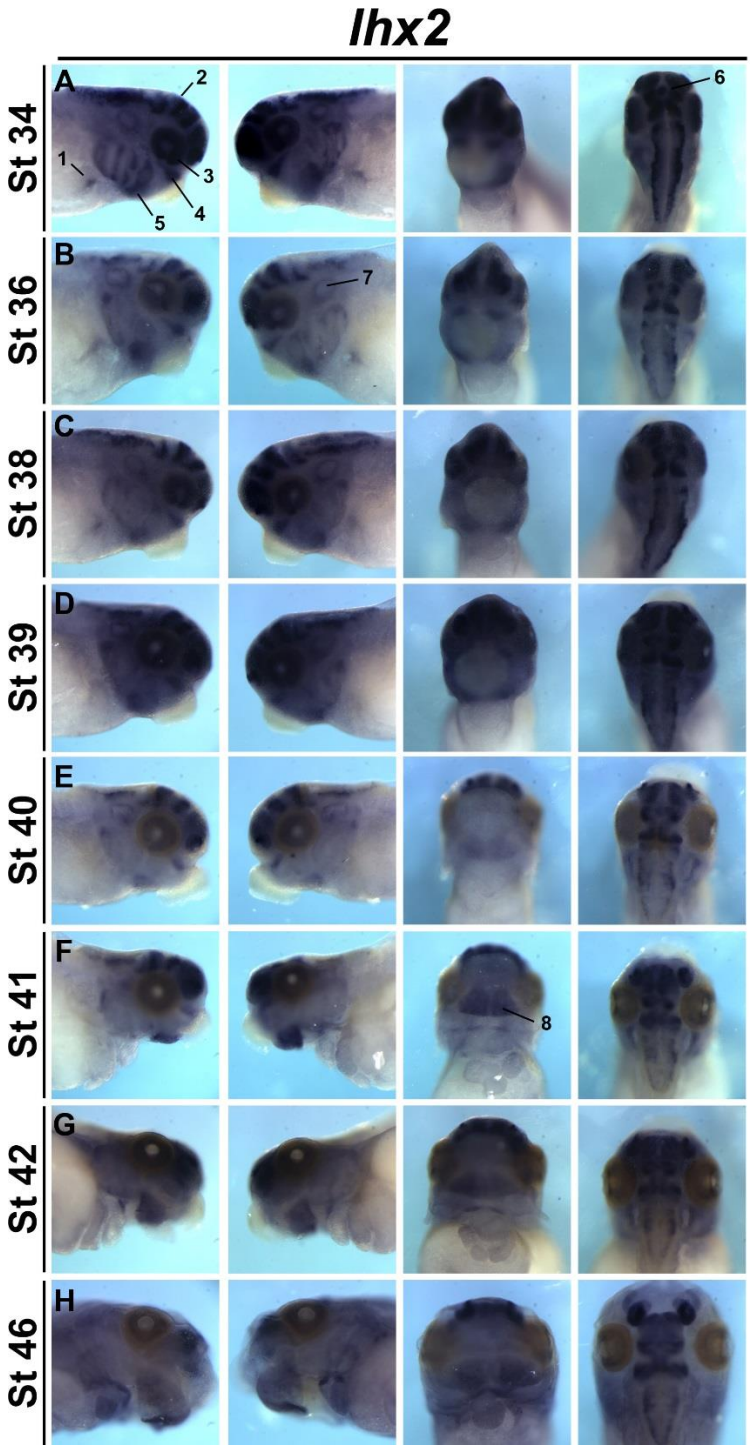

Figure S6. Spatio-temporal analysis of lhx2 during Xenopus embryogenesis.

In situ hybridization right, left, ventral and dorsal views of wild-type embryos showing *lhx2* expression of the anterior region over time, from stage 34 to stage 46. 1; lung bud, 2; neural tube, 3; retina, 4; mandibular arch, 5; branchial arches, 6; pineal gland, 7; otic placode, 8; lower jaw.

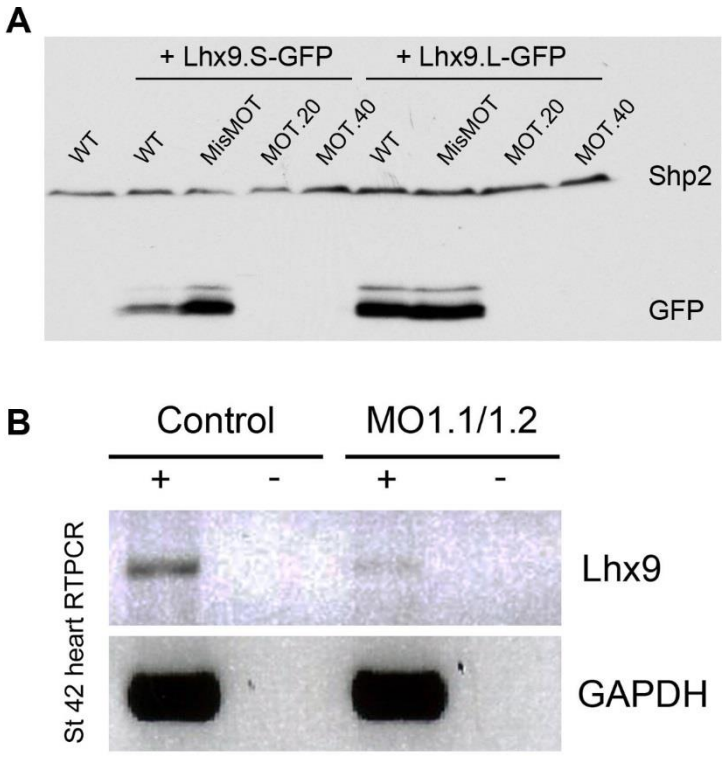

Figure S7. Validation of Lhx9 depletion assays.

(A) Validation of MO-specific inhibition of Lhx9 translation by GFP western blot on stage 11 embryos, injected with 1ng Lhx9-5'UTR-GFP RNA and MO at various concentrations (20-40 ng). Shp2 is used as a protein loading control. MOT targets the translational start site from both the short and long genomic versions of *X. laevis* Lhx9. (B) RT-PCR analysis of cardiac cDNA from stage 42 embryos injected with both MO1 (30ng each) targeting the short and long genomic versions of *X. laevis* Lhx9. Negative control lanes (-) are without superscript II enzyme, GAPDH PCR as loading control.

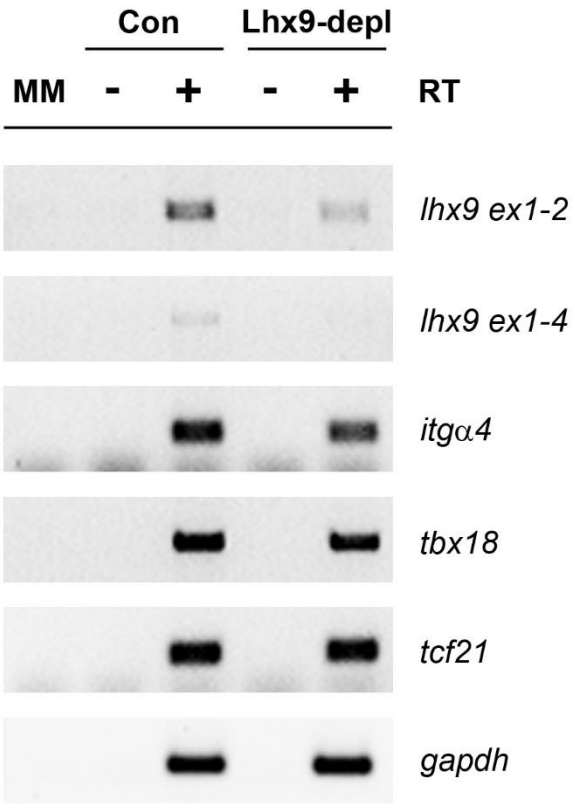

Figure S8. RT-PCR validation of epicardial marker expression in Lhx9-depleted hearts. RT-PCR was performed on equivalent amounts of RNA from control or Lhx9-MO1-depleted hearts from stage 41-42. *Lhx9* is significantly depleted by two PCR amplification methods, as well as decreased *itgα4*. *Tbx18* and *tcf21* appeared indistinguishable. *Gapdh* used as loading control.

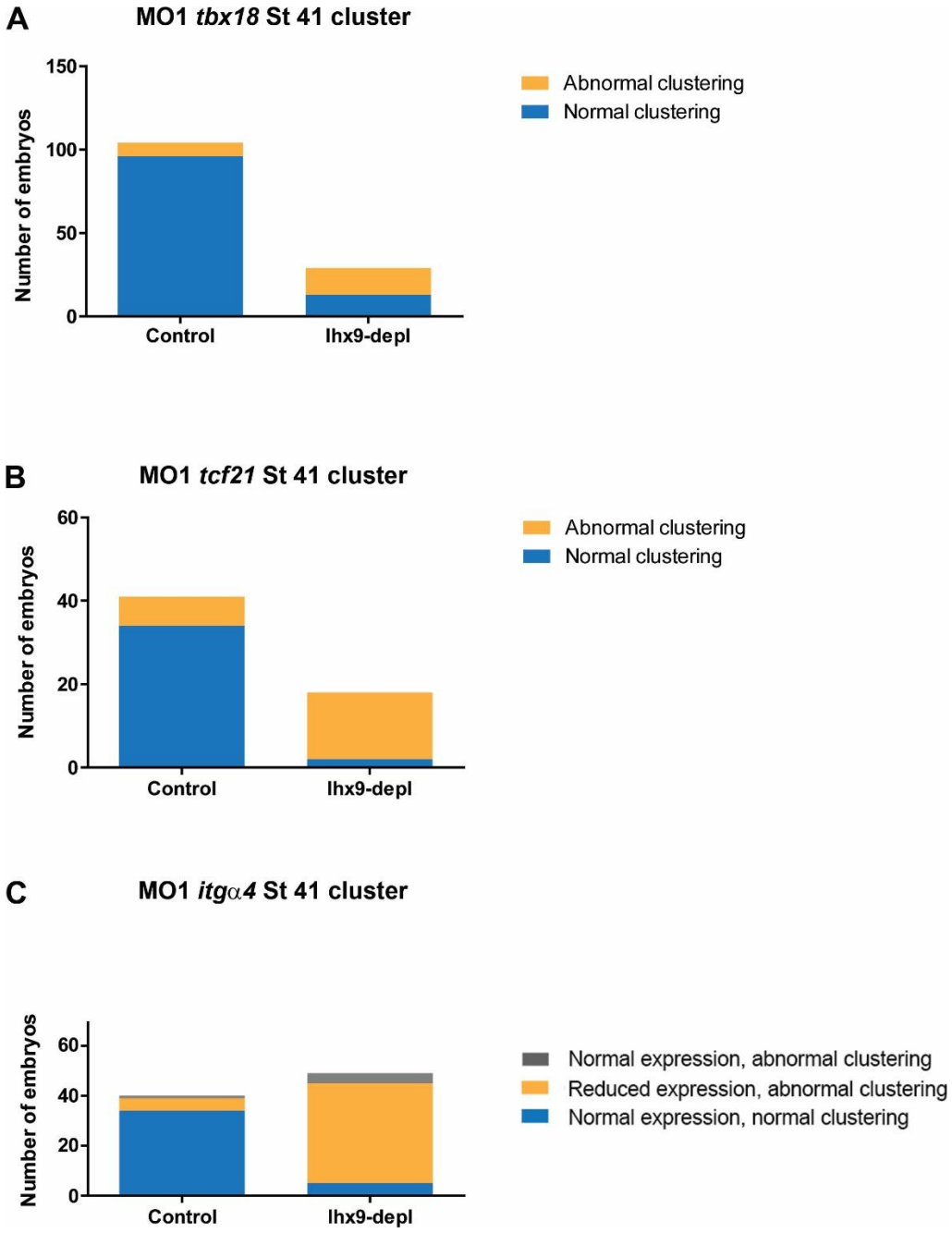

Figure S9. Lhx9 splice-blocking MO depletion strategy gives comparable PE clustering defects to translation-blocking MO.

Clustering defects at stage 41 as assessed by (A) *tbx18* and (B) *tcf21* whole embryo in situ hybridization expression were present in Lhx9-MO1-depleted embryos (Fishers exact test p = <0.0001). (C) Defects observed in *itga4* expression and localization was significant by Chi-square test (p = <0.0001) in Lhx9-MO1-depleted embryos. From three independent experiments.

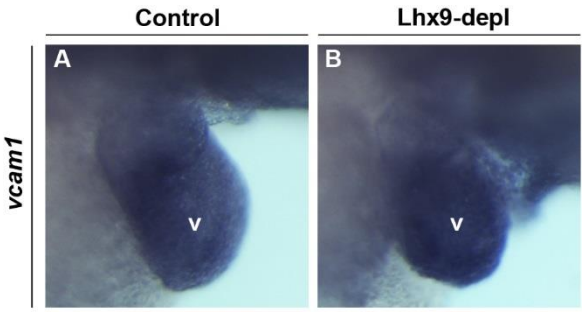

Figure S10. Lhx9 depletion has no obvious effects on vcam1 expression

Lhx9-depletion at stage 41(B) did not significantly alter the expression of *vcam1* in the heart compared to controls (A) by in situ hybridization, 6 embryos per condition. v; ventricle.

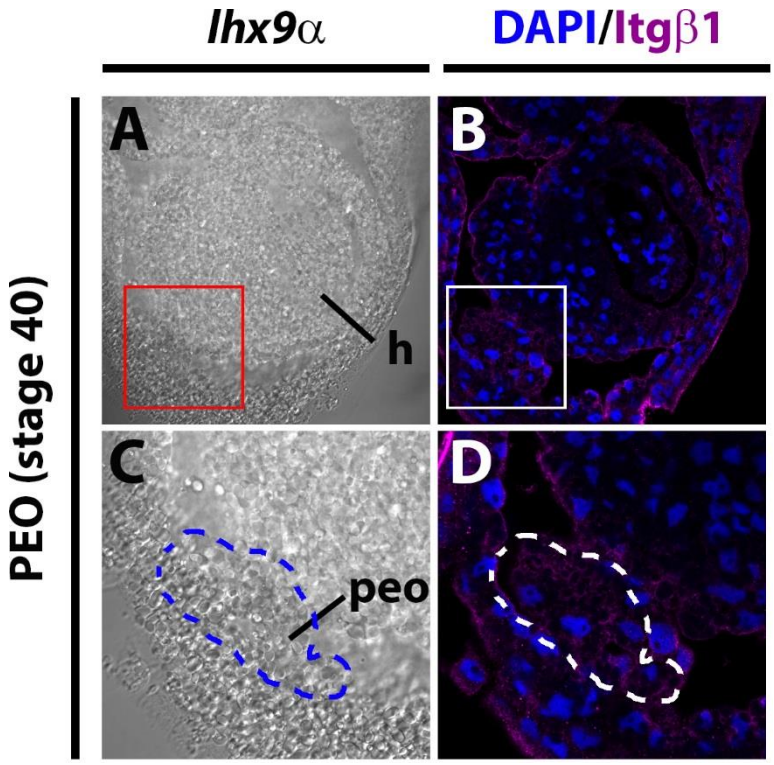

Figure S11. Lhx9α expression correlates with epicardial maker Integrin β1

Transverse agarose sections demonstrate the co-localization of *lhx9α* in situ hybridization (A, C) with the epicardial cell marker Itgβ1 (B, D, magenta) and DAPI (blue) at stage 40. Magnified images (C, D) shown in boxes (A, B). h, heart; peo, proepicardial organ.

Table 1. List of primers used for RT-PCR or plasmid construct

Primer	**Assay**	**Primer sequence 5'-3'**
gapdh_RTPCR_ F	RTPCR, 159 bp	ttgaagggaggtgccaag
gapdh _RTPCR_R		gatgacctttgcgagaggag
lhx9_RTPCR_ex1F	RTPCR, 275 bp	atatggaaattgtggggtgc
lhx9_RTPCR_ex2R		cttgtccacagccaagaggt
lhx9_RTPCR_ex1F	RTPCR, 836 bp	gtgcagagcagacgaaagtg
lhx9_RTPCR_ex4R		tcattgtacggagctggtga
lhx9_alpha3'UTR_ISH.F	ISH, 3'UTR of alpha isoform (short 98 %, long 98 %)	ctacaaccacgcttgcaaaa
lhx9_alpha3'UTR_ISH.R		gcagctggagtaattgcaca
lhx9_LIM_ISH.F	ISH, LIM domain 474 bp (short 100 %, long ~ 96%, Lhx2 78 %)	gcagacgaaagtgcctatcc
lhx9_LIM_ISH.R		tccggcagtagacaaggttt
lhx9_HD_ISH.F	ISH, homeodomain 254 bp (short 99 %, long 95 %)	ggtttcaaaacgcacgag
lhx9_HD_ISH.R		ttaaaaaaggttggttagtg
lhx2_ISH.F	ISH, 5'UTR 667 bp (short 80 %, long 100 %)	accctcctcccccattactc
lhx2_ISH.R		cctaagccatgcaccgaata
lhx9_5'UTRex1_long.F	MOT western blot assay, cloned 5'UTR and 1st exon (326 bp) from long alloallele, fused to GFP (pEGFP-N1, Clontech)	ctcaccgagcaagttccgcg
lhx9_5'UTRex1_long.R		gagtttctctgccattgacct
lhx9_5'UTRex1_short.F	MOT western blot assay, cloned 5'UTR and 1st exon (297 bp) from short alloallele, fused to GFP (pEGFP-N1, Clontech)	cattctcagccgggcaagtt
lhx9_5'UTRex1_short.R		gagtttctctgctattgactt

Table 2. Morpholino sequences.

Target	**Sequence (complimentary)**
Lhx9 translation-blocking (5'UTR and start site, short and long)	TCTGCACCCCACAATTTCCATATAC
Lhx9 translation-blocking 5 base mismatch control	TATGAACACCAAAATTTCAATATAC
Lhx9.S splice-blocking (donor exon 1)	TGCCCAGACGCCAGACTTACAGTTT
Lhx9.L splice-blocking (donor exon 1)	CGCGGCTACTTACAGTTTCTCTGCC