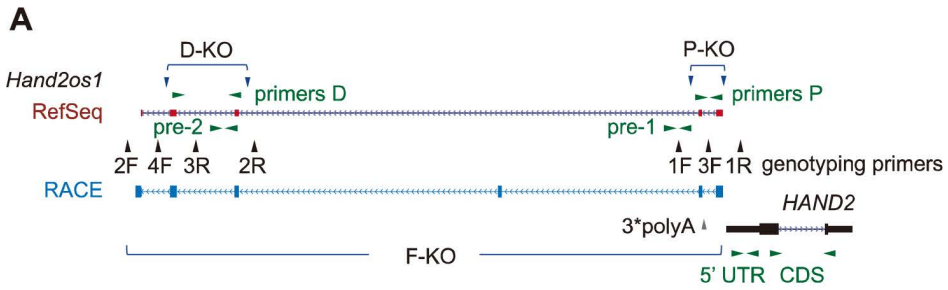
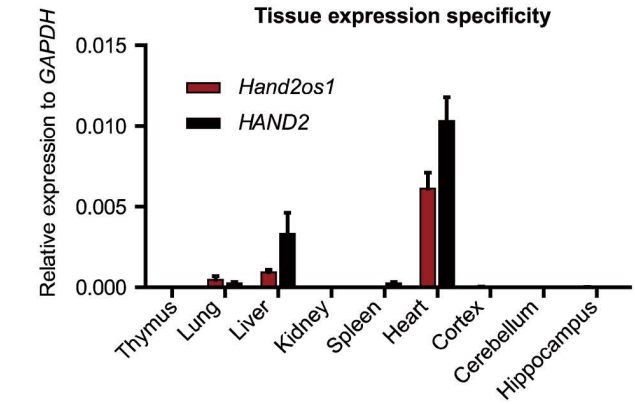
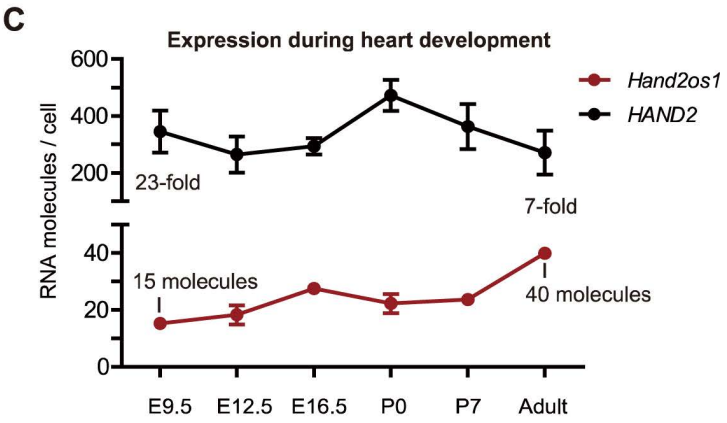
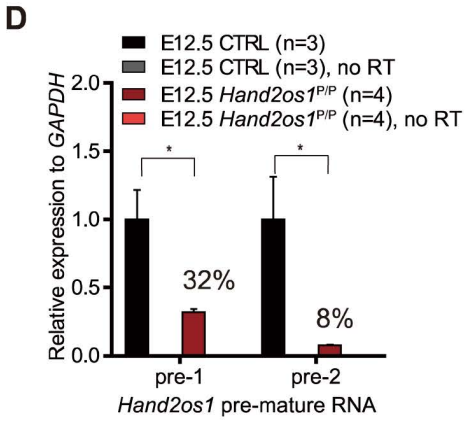


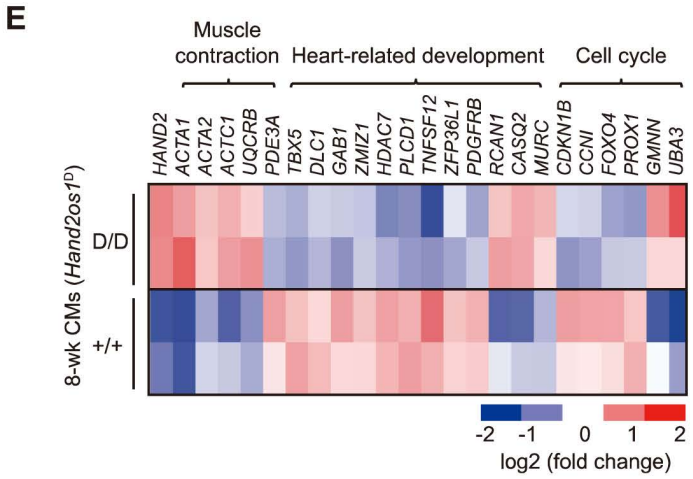
Genotyping strategies

	MUT allele	WT allele
P-KO	1F / 1R (290 bp)	3F / 1R (519 bp)
D-KO	4F / 2R (546 bp)	4F / 3R (841 bp)
F-KO	2F / 1R (759 bp)	3F / 1R (519 bp)









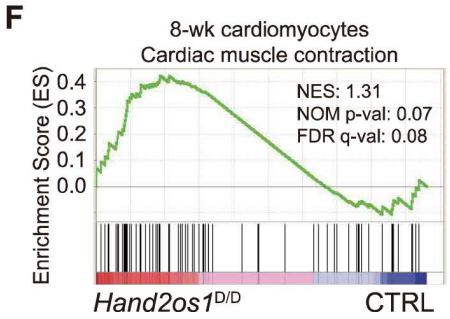


Fig. S1. Genetic and survival characterization of *Hand2os1* KO mice

(A) *Hand2os1* and *HAND2* primers for RT-qPCR are shown with horizontal green arrowheads. In *Hand2os1*, primers P and D are located within the proximal and distal deletions, respectively. In *HAND2*, primers 5' UTR are located in the first 500 bp of *HAND2* mRNA, and primers CDS flank the CDS region in exon 1 and exon 2. Genotyping primers for the three *Hand2os1* KO lines are shown with vertical black arrowheads. The three deletion alleles were characterized by PCR with different combinations of genotyping primers, as shown in the right panel. (B) RT-qPCR analysis of *HAND2* and *Hand2os1* expression (primers D) across the indicated tissues in adult mouse (8-weeks old). The y axis shows expression relative to *GAPDH*. Data are shown as mean ± s.e.m.. (C) Analysis of transcript abundance of *HAND2* and *Hand2os1* by RT-qPCR in the bulk heart from the embryonic stage E9.5 to the adult (8-wk). *Hand2os1* RT-qPCR used primers D. The y axis shows relative numbers of RNA molecules per cell. Data are shown as mean ± s.e.m.. The abundance of transcripts per cell was estimated by normalizing *HAND2* and *Hand2os1* PCR signals to primer-specific PCR efficiency and the numbers of cells used for RT-qPCR and to *18S* rRNA. In embryos, cardiac expression of *Hand2os1* continues to rise, while that of *HAND2* decreases from E9.5 to E16.5. Similarly, after birth, *Hand2os1* shows significantly increased expression from postnatal day P0 to adult (8-wk), while *HAND2* expression appears to be slightly decreased. The abundance of *Hand2os1* transcripts is about 7- to 23-fold lower than that of *HAND2* in the time course analyzed. (D) RT-qPCR analysis of *Hand2os1* pre-mature RNA in E12.5 hearts from the control (CTRL, wild-type and heterozygote littermates) and *Hand2os1*$^{P/P}$ mice. No signal was detected in samples without reverse transcriptase (no RT) in RT-qPCR. The pair of primers 'pre-1' locate at 5' end of *Hand2os1* intron 2. The pair of primers 'pre-2' locate within the exon 4 and intron 4 of *Hand2os1* (panel A). 3' end of *Hand2os1* pre-mature RNA showed a more dramatic downregulation compared to 5' end. These results indicate residual transcription of *Hand2os1* in *Hand2os1*$^{P/P}$ mutant hearts. The y axis shows expression relative to *GAPDH* (normalized to CTRL). Data are shown as mean ± s.e.m.. *, $p<0.05$. n, number of analyzed mice. (E) Heatmap shows representative genes that are dysregulated in 8-wk cardiomyocytes of *Hand2os1*$^{D/D}$ (rep1 and rep2) compared to wild-type (rep1 and rep2) littermates (n=2). Genes shown are involved in muscle contraction, heart-related development and cell cycle. The colored bar indicates log2 (fold change over average FPKM of each gene across 4 samples). Other samples that are not in the same litter are not considered. (F) GSEA of 8-wk cardiomyocytes from CTRL (wild-type and heterozygote littermates) (n=7) and *Hand2os1*$^{D/D}$ (n=6) mice. Enrichment plot shows that genes related to cardiac muscle contraction (KEGG PATHWAY: mmu04260) are upregulated in *Hand2os1*$^{D/D}$ mice compared to controls. NES, normalized enrichment Score.

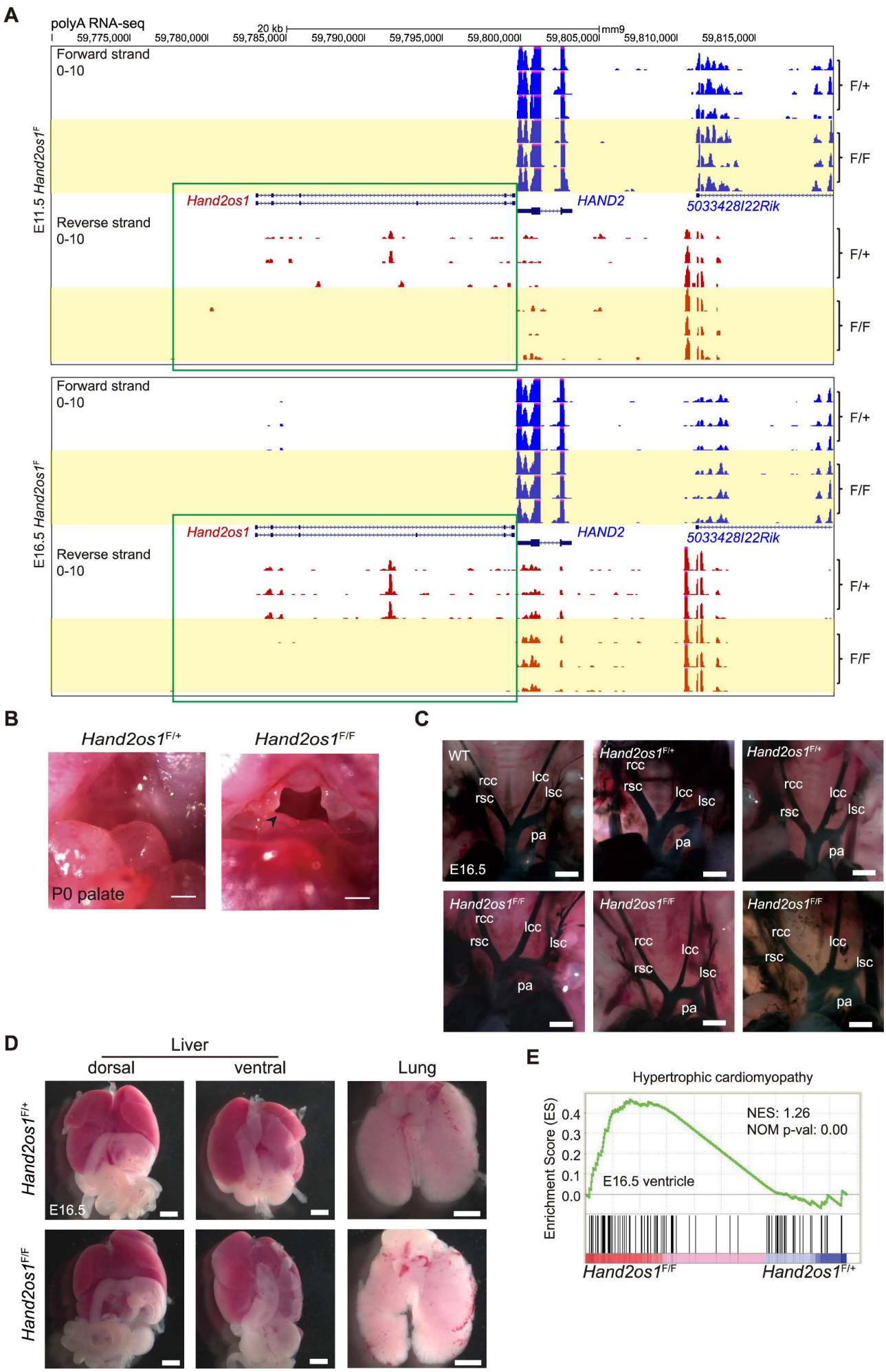


Fig. S2. Morphological characterization of *Hand2os1*$^{F/F}$ embryos

(A) The genome browser view shows expression (strand-specific polyA RNA-seq signal) of *Hand2os1* and *HAND2* in E11.5 hearts (top) and E16.5 ventricles (bottom) from *Hand2os1*$^{F/F}$ mutants compared to *Hand2os1*$^{F/+}$ littermates (n=3 for each genotype). The yellow shadings indicate *Hand2os1*$^{F/F}$ mutants. The green boxes indicate *Hand2os1* expression signal. (B) Gross morphological analysis of P0 palate of *Hand2os1*$^{F/+}$ and *Hand2os1*$^{F/F}$ mice reveals craniofacial defects (arrowhead) in *Hand2os1*$^{F/F}$ newborns. A suckling defect is not the cause of death in *Hand2os1*$^{F/F}$ pups, as these animals die within hours after birth. Mice lacking the branchial arch enhancer have craniofacial defects and cannot suckle, and die with an empty stomach 24 hours after birth. Scale bar, 500 µm. (C) Vasculature visualization by Chinese ink injection into the left ventricle of *Hand2os1*$^{F/F}$ and CTRL (*Hand2os1*$^{+/+}$ and *Hand2os1*$^{F/+}$) embryos at E16.5. lcc, left common carotid artery; lsc, left subclavian artery; pa, pulmonary artery; rcc, right common carotid artery; rsc, right subclavian artery. Scale bar, 500 µm. (D) Gross morphological examination of liver and lung of *Hand2os1*$^{F/+}$ and *Hand2os1*$^{F/F}$ embryos at E16.5. Scale bar, 1000 µm. (E) GSEA of E16.5 ventricles of *Hand2os1*$^{F/+}$ and *Hand2os1*$^{F/F}$ mice (n=3 for each genotype). Enrichment plot shows that genes related to hypertrophic cardiomyopathy (KEGG PATHWAY: mmu05410) are upregulated in *Hand2os1*$^{F/F}$ mice compared to *Hand2os1*$^{F/+}$ controls.

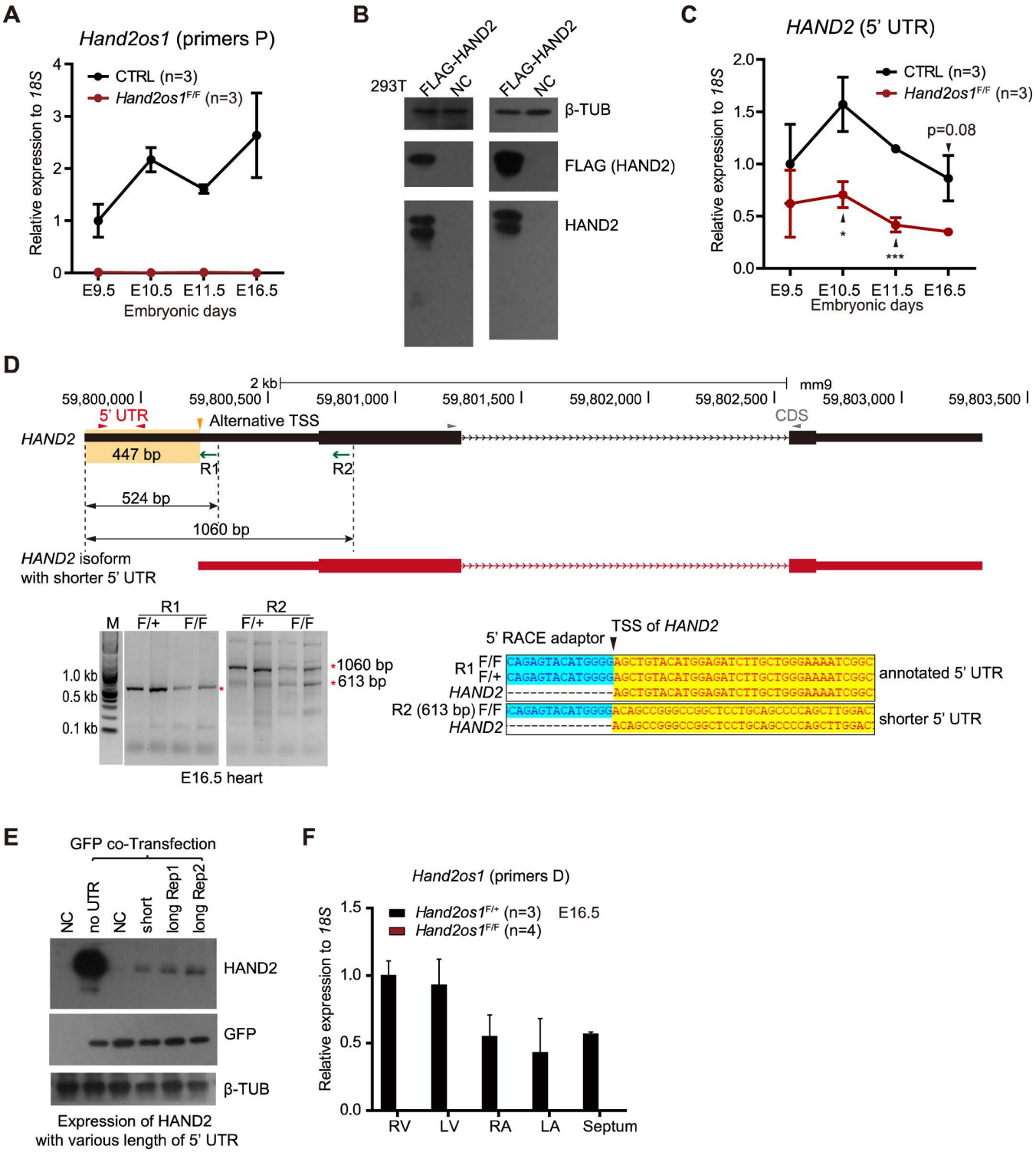


Fig. S3. Molecular analysis of *Hand2os1*$^{F/F}$ embryos

(A) RT-qPCR analysis of *Hand2os1* (primers P) in embryonic hearts at various stages from CTRL (*Hand2os1*$^{+/+,\ F/+}$) and *Hand2os1*$^{F/F}$. The y axis shows expression relative to *18S* (normalized to *Hand2os1* expression in CTRL embryos at E9.5). Data are shown as mean ± s.e.m..

(B) Western blot validation of the HAND2 antibody in 293T cells transfected with Flag-HAND2. β-TUBULIN is the loading control.

(C) RT-qPCR analysis of *HAND2* (5' UTR) in embryonic hearts at various stages from CTRL (*Hand2os1*$^{+/+,\ F/+}$) and *Hand2os1*$^{F/F}$. The y axis shows expression relative to *18S* (normalized to *HAND2* 5' UTR expression in CTRL embryos at E9.5). Data are shown as mean ± s.e.m.. *, $p<0.05$; ***, $p<0.001$.

(D) Strategy for *HAND2* 5' RACE is shown. R1 and R2, gene specific primers for 5' RACE PCR amplification, are shown together with length of their PCR products. Annotated and shorter *HAND2* isoforms are indicated in black and red, respectively. Left, DNA gel of 5' RACE PCR products. *HAND2* specific PCR bands are indicated by red asterisks. Right, Sanger sequencing results of *HAND2* 5' end revealed by 5' RACE PCR. 5' RACE analysis revealed an alternative TSS of *HAND2*, which is 447 bp downstream to the annotated TSS and 477 bp prior to CDS of *HAND2*.

(E) Western blot shows HAND2 expression from constructs with various lengths of *HAND2* 5'UTR or without the 5'UTR. Transfections were performed in mouse ESCs. β-TUBULIN is the loading control and GFP is the transfection control.

(F) RT-qPCR analysis of *Hand2os1* (primers D) in dissected E16.5 embryonic hearts (RV, LV, RA, LA and septum) from *Hand2os1*$^{F/+}$ and *Hand2os1*$^{F/F}$. The y axis shows expression relative to *18S* (normalized to *Hand2os1* expression in RV of *Hand2os1*$^{F/+}$ embryos). Data are shown as mean ± s.e.m..

n, number of analyzed mice.

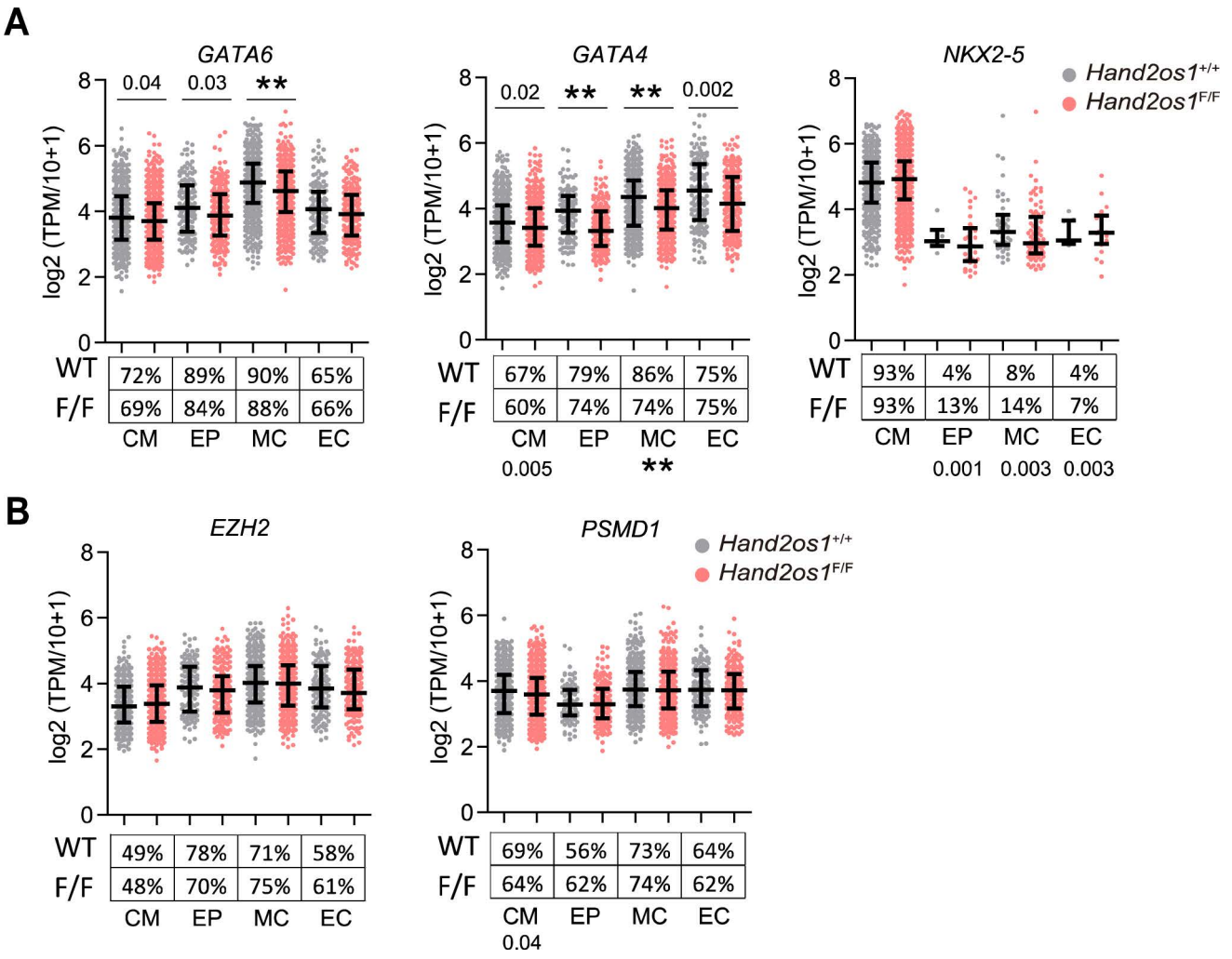


Fig. S4. Gene expression in *Hand2os1*$^{F/F}$ embryos

(A-B) Scatter plots show expression level and frequency of *GATA6*, *GATA4* and *NKX2-5* (A), and *EZH2* and *PSMD1* (B) in cardiac cells of *Hand2os1*$^{F/F}$ and wild-type embryonic hearts. Data are shown as median and interquartile range. The *p*-values are indicated: 0.0001<*p*<0.05; **, *p*<0.0001. The percentages of expressing cells (TPM>0) in each cell type are shown under the plots.

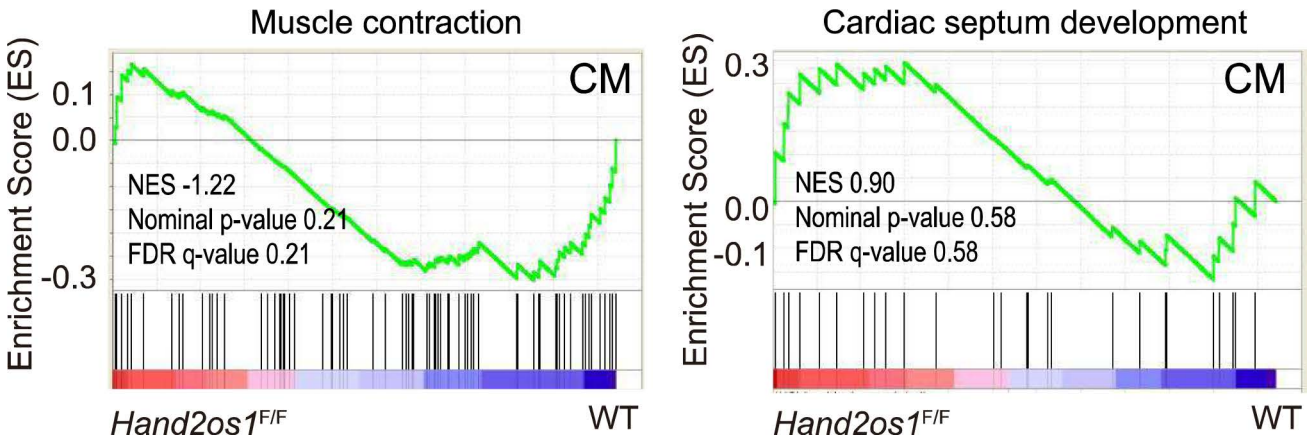


Fig. S5. Aberrant cardiac gene programs in *Hand2os1*$^{F/F}$ embryos

Non-significant changes of genes related to muscle contraction (GO: 0006936) and cardiac septum development (GO: 0003279) in CMs of *Hand2os1*$^{F/F}$ versus wild-type E11.5 hearts by GSEA.

Table S1. Summary for cardiac *HAND2* knockout/overexpression mouse models

KO/OE strategy		Loss/gain of *HAND2* expression	Phenotype
Synthetic KO		complete loss	failed right ventricle formation and lethality at E10.5
Conditional KO (*cre*)	*ISLET1*	early SHF cells	phenocopying *HAND2* synthetic KO hearts
	CTNT	myocardium	lethality at E12.5, with a single clearly defined ventricle
	NKX2-5	RV and LV chambers after E8.5	heart formed, slightly smaller RV and LV at E9.5 and lethality at E12.5
	WNT1	neural crest-derived cells	die at E12.5 with severe cardiovascular and facial defects; embryos survived to later stages with drugs, misalignment of OFT and aortic arch arteries, DORV with a membranous VSD
	MEF2C	a subset of the SHF-derived cells (OFT and RV, IVS)	smaller RV at E9.5 and a hypoplastic RV with thinner myocardium, lacking the anlage of a tricuspid valve and displaying VSDs at E12.5, lethality at E13.5
	TIE2	endothelial cells from E8.5	no patent tricuspid valve, with IVS defects, and hypotrabeculated ventricles, lethality at E14.5
	NFATC1	endocardium from E9.0	defects in trabeculation, malformed IVS and atresia, large protrusions of myocardium and multiple IVSs
	TBX1	distal OFT and pulmonary artery	a shortened OFT with normal RV at E10.5, smaller RV and outflow tracts by E13.5, lethality at E15.5
Transgenic OE	*MYH7*	whole ventricles of embryo	complete absence of the IVS
	MYH6	postnatal myocardium	pathological hypertrophy
Chromosome duplication		*RIM4*	severe VSD (80%), perinatal lethality (80%)
miRNA-1-2 KO		4-fold increased at protein level	VSD(50%), perinatal death with 50% lethality by weaning.

SHF, second heart field; OFT, outflow tract; RV, right ventricle; IVS, interventricular septum; VSD, ventricular septal defect; DORV, double outlet right ventricle

Synthetic KO (Srivastava et al., 1997); Conditional KO with specific promoter-*cre* (*ISLET1* (Tsuchihashi et al., 2011), *CTNT* (Morikawa and Cserjesi, 2008), *NKX2-5* (Tsuchihashi et al., 2011), *WNT1* (Holler et al., 2010; Morikawa and Cserjesi, 2008; Morikawa et al., 2007), *MEF2C* (Tsuchihashi et al., 2011), *TIE2* (VanDusen et al., 2014), *NFATC1* (VanDusen et al., 2014), *TBX1* (Tsuchihashi et al., 2011)); Transgenic OE (*MYH7* (Dirkx et al., 2013), *MYH6* (Togi et al., 2006)); Chromosome duplication (Tamura et al., 2013); *miRNA-1-2* KO (Zhao et al., 2007)

Table S2. Echocardiography data of *Hand2os1*$^{D/D}$ and *Hand2os1*$^{P/P}$ mice

Click here to Download Table S2

Table S3. Gene sets for GSEA

Click here to Download Table S3

Table S4. Dysregulated genes in 8-wk cardiomyocytes of *Hand2os1*$^{D/D}$ mice

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Table S5. Dysregulated genes in E11.5 hearts of *Hand2os1*$^{F/F}$ embryos

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Table S6. Dysregulated genes in E16.5 ventricles of *Hand2os1*$^{F/F}$ embryos

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Table S7. Single-cell clustering

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Table S8. 1750 differentially expressed genes in four types of cardiac cells

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Table S9. Dysregulated genes in four cardiac cell types in E11.5 hearts of *Hand2os1*$^{F/F}$ embryos

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Table S10. Oligos (sgRNAs, genotyping primers, RT-qPCR primers, ISH probe, 5' RACE primers, 3C primers)

Click here to Download Table S10