SUPPLEMENTAL MATERIAL

The Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

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SUPPLEMENTAL METHODS

Nkx2.5-Cre and αMHC-Cre Genetic Fate Mapping

Nkx2.5$^{Cre/+}$;ROSA26^{LacZ} or *αMHC$^{Cre/+}$;ROSA26^{LacZ}* whole mouse E9.5 embryos were assessed for β-Galactosidase (lacZ) activity according to previously published protocol[1].

FACS Analysis of ESC Chimerism

For embryonic tissues, the heart, limbs, tail, head, or body were minced with a scalpel blade into 1-4 mm^3 pieces and individually digested in Hank's Balanced Salt Solution (HBSS) with Ca^{++}/Mg^{++} (Life Technologies, 14025-134) containing 10 mg/mL collagenase A&B (Roche; 11088785103, 11088823103) at 37º C for 30-60 minutes with periodic pipetting. Following centrifugation at 300xg for 30s, the supernatant was discarded and the digested cells were resuspended in 1X Red Blood Cell Lysis Solution (Miltenyi Biotec; 130-094-183) and incubated for 10 minutes at room temperature. Cells were again centrifuged and resuspended in HBSS with Ca^{++}/Mg^{++} containing 4% fetal bovine serum (FBS, Atlanta Biologicals, S11550) for flow cytometric analysis. For live-born mice, the same protocol was applied to tissue from the tail or ears at ~postnatal day 7. Following addition of propidium iodide to gate out dead cells, flow cytometric cell counting was performed on a FACSCalibur or FACSAria II flow cytometer (BD Biosciences) using CellQuest v3.3 software (BD Biosciences, San Jose, CA). Doublet discrimination and exclusion was performed by gating cells according to their FSC-H versus FSC-W and SSC-H versus SSC-W distributions. To determine the proportion of $eGFP^+/eGFP^-$ cells in each tissue sample, the data was analyzed with FlowJo v7.6 software (Tree Star, Ashland, OR). Degree of CPC or CM ablation was computed using the average percentage of $eGFP^+$ cell chimerism to tail and limb samples for embryonic mice or tail and ear samples for live-born mice.

Media Composition for Establishment of ESC Lines

ESC Derivation Media: DMEM with High Glucose/4.0 mM L-Glutamine (Thermo Scientific HyClone, SH30022), 20% KnockOut Serum Replacement (KOSR, Life Technologies, 10828), 4 mM Glutamax (Life Technologies, 35050), 100 µM MEM Non-Essential Amino Acids (Life Technologies, 11140), 50 U-µg/mL Penicillin-Streptomycin (Life Technologies, 15070), 100 µM 2-Mercaptoethanol (Sigma, M6250), and 500 U/mL leukemia inhibitory factor (LIF, Millipore, ESG1107).

ESC Maintenance Media: DMEM with High Glucose/4.0 mM L-Glutamine, 10% KOSR, 7.5% FBS, 4 mM Glutamax, 100 µM MEM Non-Essential Amino Acids, 50 U-µg/mL Penicillin Streptomycin, 100 µM 2-Mercaptoethanol, 500 U/mL LIF, and the "2i" inhibitors: 3 µM glycogen synthase kinase-3 inhibitor CHIR99021 (StemGent, 04-0004), and 1 µM mitogen-activated protein kinase inhibitor PD0325901 (Stem-Gent, 04-0006).

Media Composition for Ex Vivo Cardiac Cell Culture

Cardiomyocyte Maintenance Media: IMDM (Life Technologies, 12440), 15% FBS (Hyclone, SH30071.HI), 4 mM Glutamax (Life Technologies, 35050), 25 U-µg/mL Penicillin-Streptomycin (Life Technologies, 15070), 50 µg/mL Ascorbic Acid (Sigma, A4544), 100 µM Monothioglycerol (Sigma, M6145).

Ex vivo single-cell clonal analysis

Heart tubes were dissected on ice at E9.5 from CD-1 control embryos, or CD-1 embryos which had been chimerized by *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ or *αMHC*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ ESCs and individually transferred to microcentrifuge tubes containing 50 µL Hank's Balanced Salt Solution (HBSS) with Ca^{++}/Mg^{++} (Life Technologies, 14025-134). To each tube, 200 µL of 0.25% trypsin-EDTA solution (Life Technologies, 25200) was added and incubated for 5-10 minutes at 37°C with gentle dissociation by pipetting. Following inactivation of trypsin with FBS (Hyclone,

SH30071.HI), the cells were centrifuged and re-suspended in HBSS with Ca^{++}/Mg^{++} containing 4% FBS and placed on ice. Flow cytometric sorting was performed on a FACSAria II flow cytometer (BD Biosciences) and analyzed using FACSDiva v6.1.3 software (BD Biosciences, San Jose, CA). Doublet discrimination was performed by gating cells according to their FSC-H versus FSC-W and SSC-H versus SSC-W distributions. The proportion of $eGFP^+/eGFP^-$ cells in each tissue sample was determined as described in *FACS Analysis of ESC Chimerism* in **Supplementary Methods**. $eGFP^-$ single cardiac cells were FACS-purified and individually sorted into gelatinized 96-well tissue culture plates containing Cardiomyocyte Maintenance Media and cultured at 37°C in 5% CO_2 in humidified air for 7 or 14 days without media changes.

TUNEL Staining

Following deparaffinization, TdT-mediated dUTP nick end labeling (TUNEL) was performed on heart sections using an ApopTag Red *In Situ* Apoptosis Detection Kit (Chemicon, S7165, anti-digoxigenin rhodamine antibody) according to manufacturer's recommendations. The heating method was used for the DNA exposure step by incubating the slides in a pressure cooker for 3 minutes using citrate-based unmasking solution, pH 6.0 (Vector Labs, H-3300). Slides were subsequently washed in PBS and incubated overnight at 4º C in PBS with 10% goat serum, 1% BSA, 0.1% saponin, and primary antibodies to eGFP (Abcam, ab13970, 1:400 dilution) and *troponin T* (Thermo Scientific, MS-295, 1:500 dilution). Sections were washed with PBS and incubated at room temperature for 1 hour with corresponding secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, 1:200 dilution). Slides were then mounted in ProLong Gold Antifade Reagent containing DAPI (Life Technologies, P-36931). All staining was performed on 3-5 hearts/group, with 4 distinct sections/heart.

Wheat Germ Agglutinin (WGA) Staining

Following removal of OCT (E8.5 sections) or paraffin (E9.5 & adult heart sections), slides were washed in PBS and then incubated for 1 hour at room temperature with a primary antibody against WGA conjugated to FITC (Sigma, L4895, 5 µg/ml stock, 1:200 dilution). The slides were subsequently blocked and permeabilized with 10% goat serum, 1% BSA, and 0.1% saponin in PBS for 1 hour and incubated overnight at 4º C with a primary antibody to *troponin T* (Thermo Scientific, MS-295, 1:500 dilution). Sections were washed with PBS and incubated at room temperature for 1 hour with a secondary antibody conjugated to Alexa Fluor 546 (Invitrogen, 1:400 dilution). Slides were mounted in ProLong Gold Antifade Reagent containing DAPI (Life Technologies, P-36931). To quantify cardiomyocyte size, images at 32X magnification were captured and ImageJ was used to determine the average cell area by an investigator blinded to mouse genotype. Quantitative analyses involved counting a minimum of 3 fields from 3-4 independent hearts per group, and 2-4 distinct sections/heart (~30-50 cells per field assessed, total ~100-150 cells per sample).

Electron Microscopy

E10.5 embryos were dissected in 0.1M sodium cacodylate buffer, pH 7.4 and placed into electron microscopy fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde. 0.025% calcium chloride in a 0.1M sodium cacodylate buffer, pH 7.4) and allowed to fix overnight at 4ºC. The fixative was replaced with cacodylate buffer and the embryos were stored at 4ºC until further processing in a Leica Lynx automatic tissue processor. The embryos were post fixed with osmium tetroxide, en bloc stained with 2.0% uranyl acetate dehydrated in a graded ethanol series, embedded in pure epoxy resin and polymerized overnight at 60ºC. 1 µm thick sections were cut using glass knives and a Sorvall MT-1 (Dupont) ultramicrotome and floated on water droplets on glass slides. The slides were dried in a humidity chamber on a warm hot plate. Toluidine blue stain (0.5% toluidine blue in aqueous 0.5% sodium borate) was pipetted over the

sections and placed onto the hot plate until a slight gold rim could be seen around the stain droplet. The sections were rinsed in a stream of distilled water, dried, cover slipped and examined by light microscopy. Tissues representing the common ventricle of the heart were chosen, and the blocks trimmed accordingly. Thin sections were cut using a diamond knife and an LKB 2088 ultramicrotome and placed on copper grids. Sections were stained with lead citrate and examined in a FEI Morgagni transmission electron microscope. Images were captured with an Advanced Microscopy Techniques 2K digital CCD camera. Quantitative analysis involved measuring the average sarcomeric length and width per field on images acquired at a direct magnification of 10,000-20,000X by an investigator blinded to mouse genotype. Multiple fields were examined from 3 independent hearts per group, and 2 distinct sections/heart.

H&E Staining

Hematoxylin-eosin staining on adult heart sections was performed according to standard protocol at the MGH Endocrine Core Histology Facility.

SUPPLEMENTAL TABLES

Supplemental Table 1: Primers Utilized for PCR-based Genotyping

Primer	Direction	Sequence (5' to 3')
Cre	Forward	CGTATAGCCGAAATTGCCAG
	Reverse	CAAAACAGGTAGTTATTCGG
Y chromosome	Forward	TTGTCTAGAGAGCATGGAGGGCCATGTCAA
	Reverse	CCACTCCTCTGTGACACTTTAGCCCTCCGA

Supplemental Table 2: Antibodies Utilized for Immunohistochemistry

Gene	Species	Manufacturer	Catalog# / Clone	Dilution
β-catenin, nuclear	mouse monoclonal	DSHB	PY489	1:50
β-catenin, total	rabbit monoclonal	Cell Signaling	#8480	1:100
PECAM-1 (CD31)	rat monoclonal	Dianova	DIA-310 / SZ31	1:20
cleaved caspase-3 (Asp175)	rabbit polyclonal	Cell Signaling	#9661	1:100
Gata4	rabbit polyclonal	Santa Cruz	sc-9053 / H-112	1:100
GFP	chicken polyclonal	Abcam	ab13970	1:400
Islet-1	mouse monoclonal	DSHB	39.4D5	1:100
Ki67	rabbit monoclonal	Abcam	ab16667 / SP6	1:200
Mef2c	rabbit monoclonal	Cell Signaling	#5030	1:100
myosin light chain 2v	rabbit monoclonal	Abcam	ab92721 / EPR3741	1:200
phosphorylated histone H3 on Ser10 (pH3)	rabbit polyclonal	Cell Signaling	#9701	1:200
sarcomeric *α-actinin*	mouse monoclonal	Sigma	A7811 / EA-53	1:200-1:400
smooth muscle myosin heavy chain	rabbit polyclonal	Biomedical Technologies Inc. (BTI)	BT-562	1:300
troponin T	mouse monoclonal	Thermo Scientific	MS-295 / Ab-1, 13-11	1:500
Yap	rabbit polyclonal	Cell Signaling	#4912	1:200

SUPPLEMENTAL REFERENCES

1. Nagy A, Gertsenstein M, Vintersten K, Behringer R. Staining whole mouse embryos for β-galactosidase (lacz) activity. *CSH protocols*. 2007;2007:pdb prot4725

Figure S1

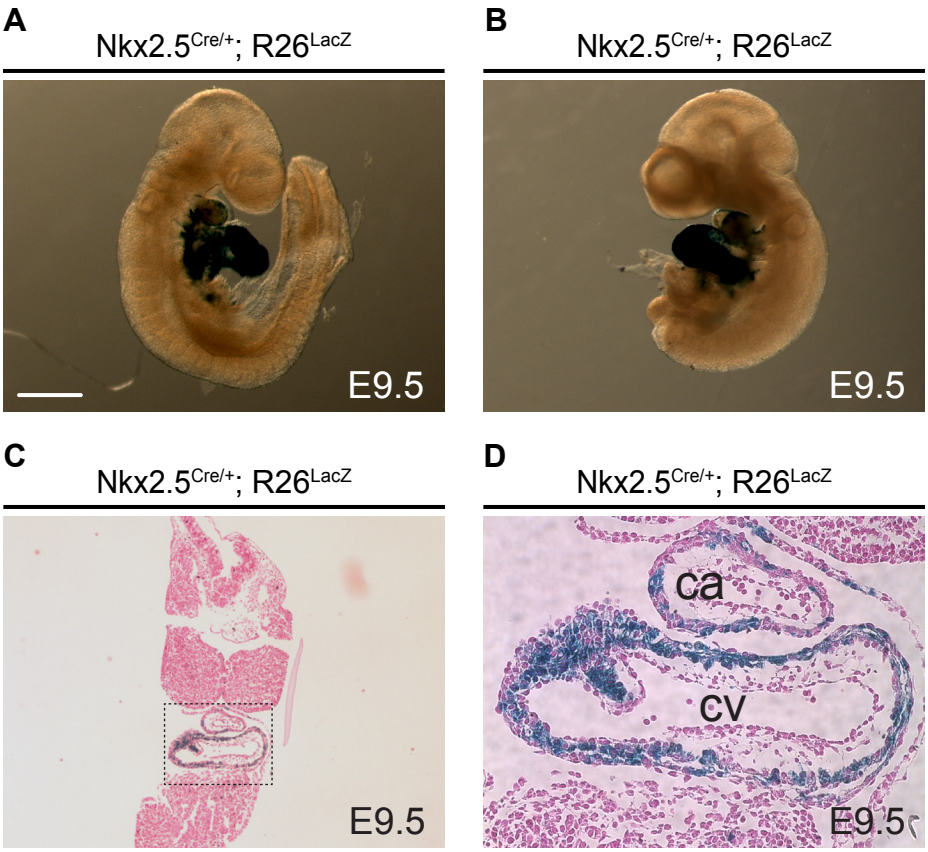

A
Nkx2.5Cre/+; R26LacZ
E9.5
B
Nkx2.5Cre/+; R26LacZ
E9.5
C
Nkx2.5Cre/+; R26LacZ
E9.5
D
Nkx2.5Cre/+; R26LacZ
ca
cv
E9.5

Figure S1 - Genetic fate mapping of *Nkx2.5* lineage cells, related to Figure 1. (A-B) Sagittal views of an E9.5 embryo. Robust β-galactosidase activity is evident throughout the common atrial and ventricular chambers, as well as pharyngeal arch ectoderm. (C-D) Transverse section of an E9.5 embryo. β-galactosidase is detected in the myocardium, with minimal activity in the endocardium. ca, common atrium; cv, common ventricle. Scale bar: 500 µm.

Figure S2

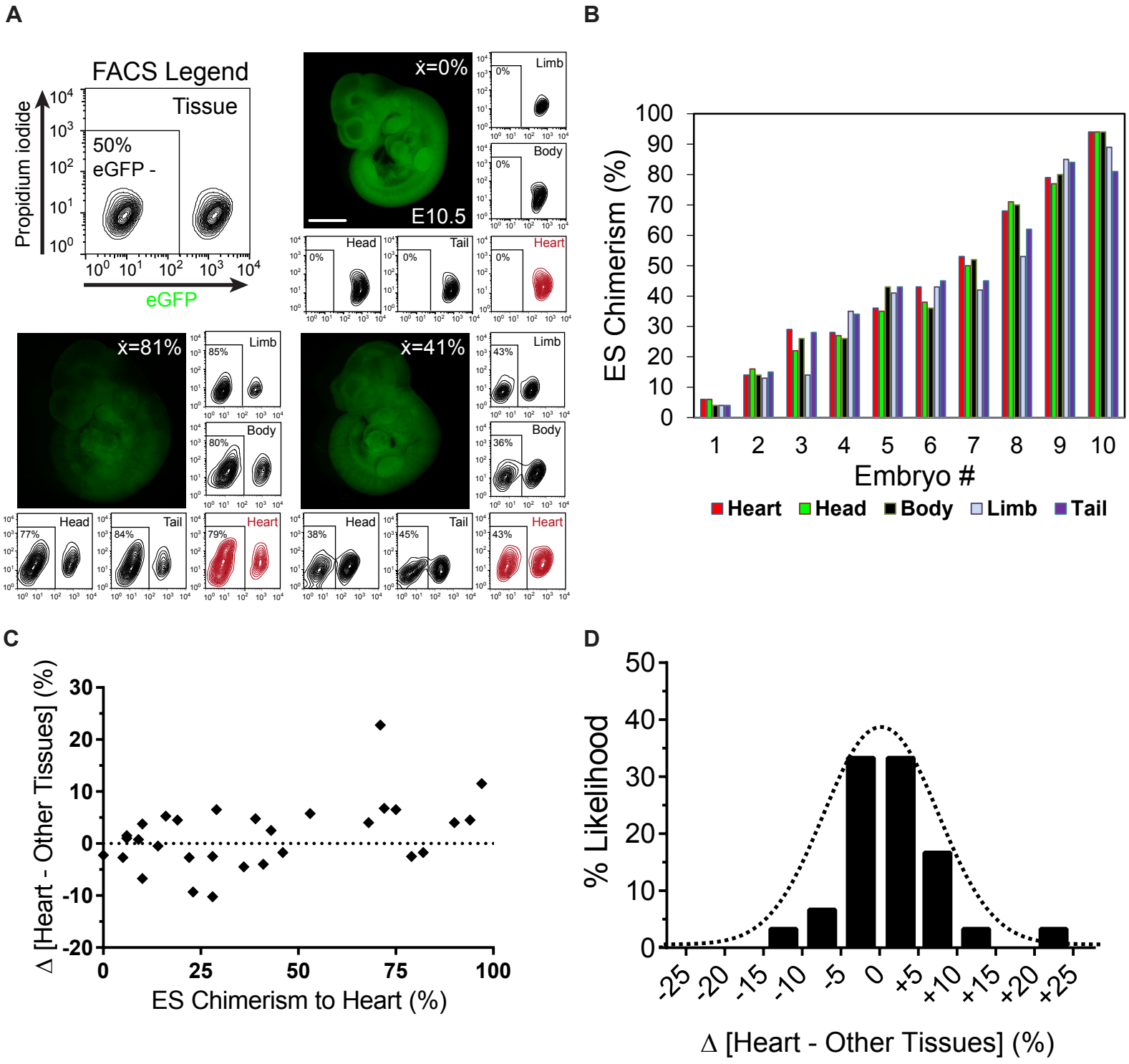
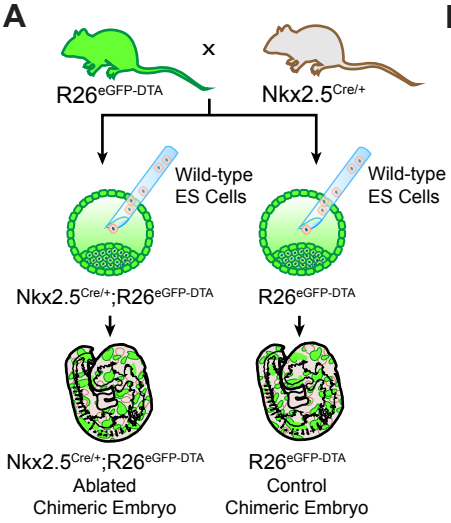

A
FACS Legend
Tissue
50%
eGFP -
Propidium iodide
eGFP
ẋ=0%
E10.5
Limb
Body
Head
Tail
Heart
ẋ=81%
ẋ=41%
B
ES Chimerism (%)
Embryo #
Heart
Head
Body
Limb
Tail
C
Δ [Heart - Other Tissues] (%)
ES Chimerism to Heart (%)
D
% Likelihood
Δ [Heart - Other Tissues] (%)

Figure S2 - Determination of the variance of ESC chimerism in the heart versus other tissues, related to Methods. (A) FACS contour plots showing distribution of $eGFP^+$ versus $eGFP^-$ cells from the tissues of three representative E10.5 $eGFP^+$ embryos that have been complemented with unlabeled wild-type ESCs. Mean ESC chimerism percentage is shown within the embryo image. Scale bar: 1 mm. (B) Graphical representation of flow cytometry-based cell counting in selected embryos demonstrating ESC chimerism in the heart correlates well with chimerism in other tissues. (C) Difference between percent chimerism in the heart and the mean extra-cardiac tissue chimerism (i.e. head, body, limb, tail). Each data point represents one embryo. (D) Likelihood of variation between extra-cardiac tissue chimerism and heart chimerism. 67% of embryos exhibited a variation ≤5%; 90% of embryos exhibited a variation ≤10%; and 97% of embryos exhibited a variation of ≤12%. For (C and D): n=30 embryos.

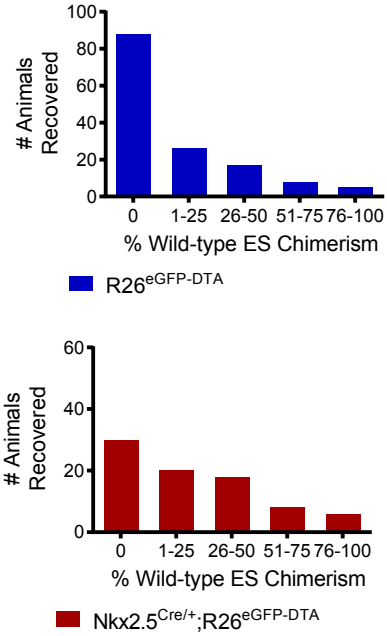
Figure S3

A



B

ES Line	Blastocysts Transferred	Animals Recovered	eGFP+ Animals Recovered	eGFP+ Genotype	Animals Recovered with Genotype
V6.5	512	323	192	Nkx-Cre -	120
				Nkx-Cre +	72
R1	127	71	34	Nkx-Cre -	24
				Nkx-Cre +	10
Total	**639**	**394**	**226**	**Nkx-Cre -**	**144**
				Nkx-Cre +	**82**



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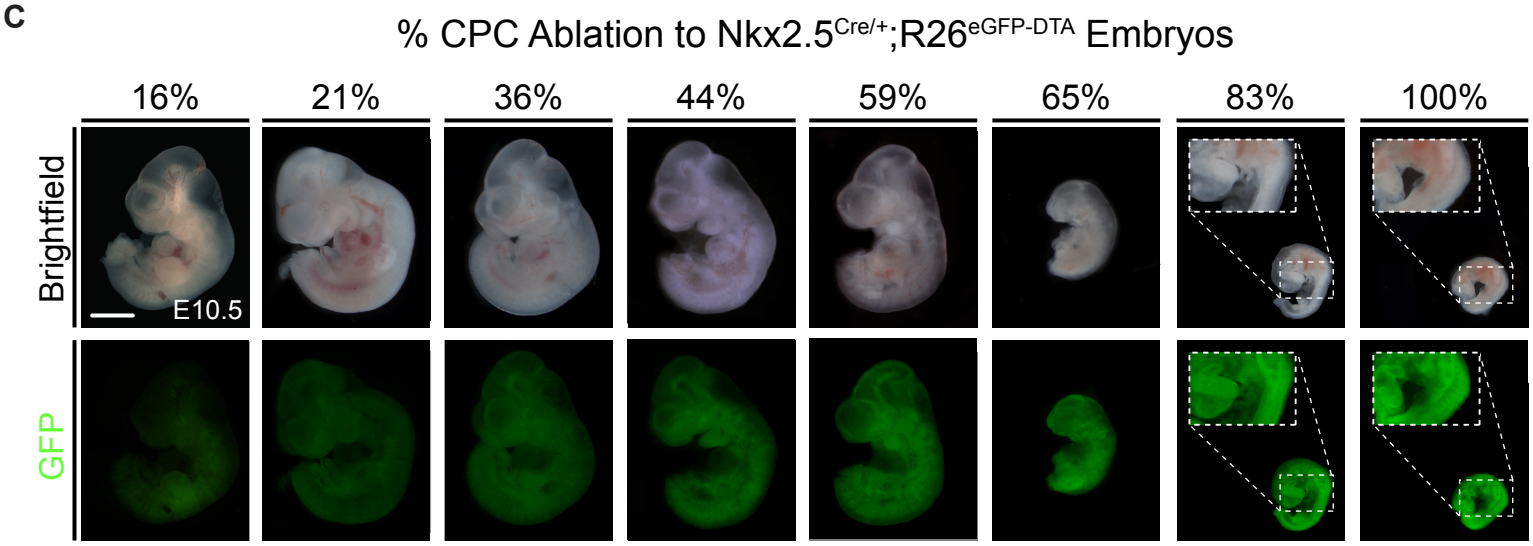
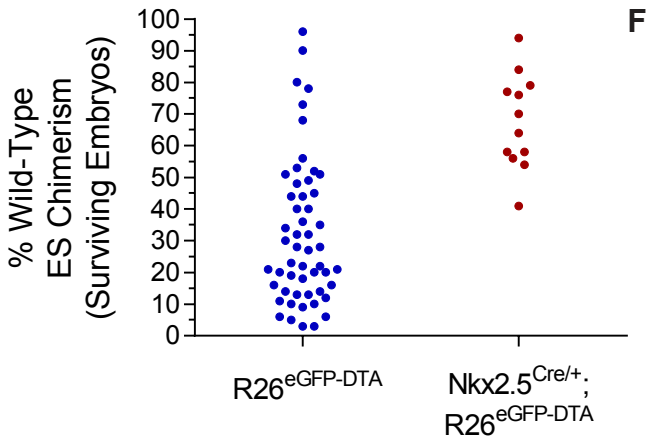
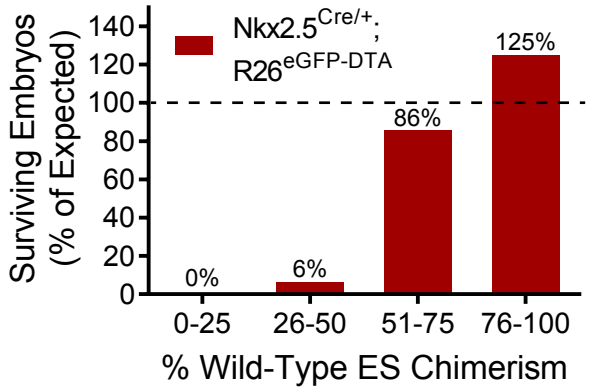


D



E



F

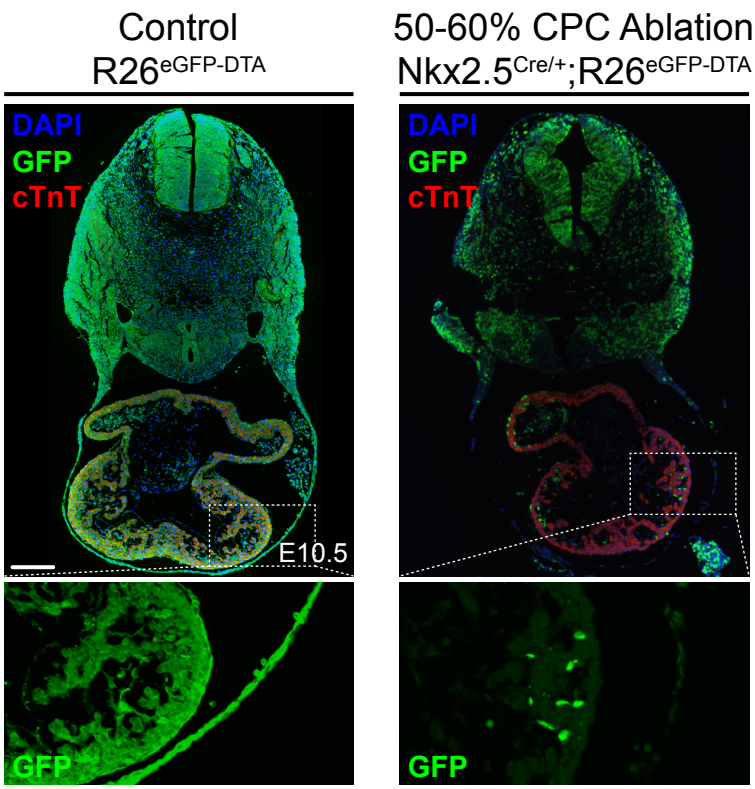
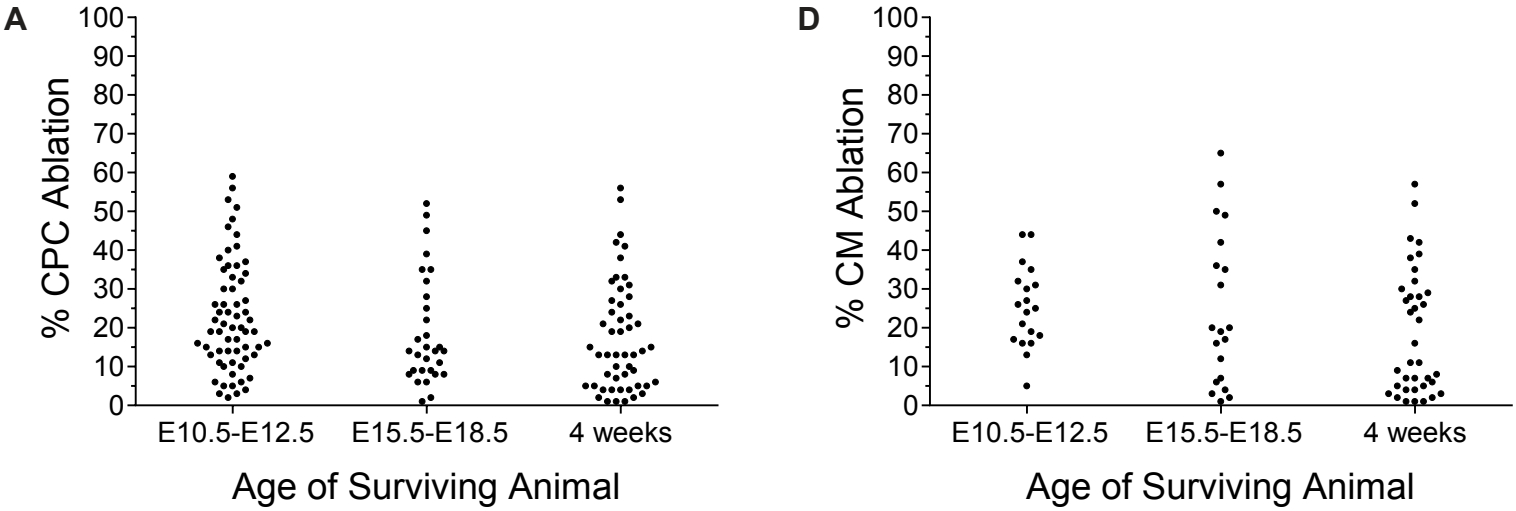
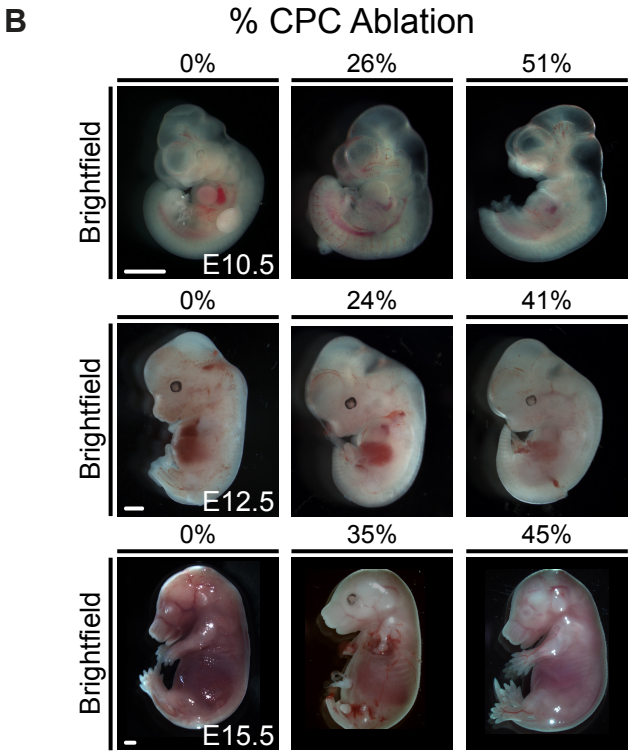


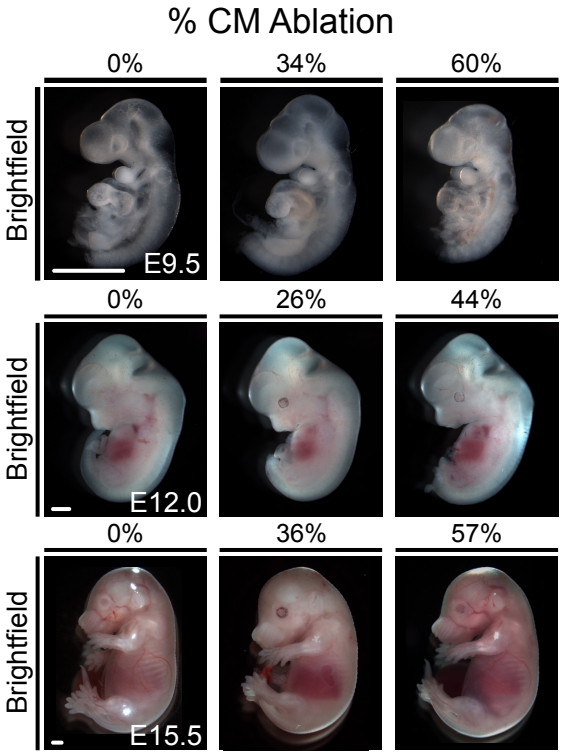
Figure S3 - Recapitulation of the CPC ablation phenotype using a reverse complementation approach, related to Figure 1. (A) Strategy for rescue of CPC ablation phenotype by wild-type ESC chimerism. *Nkx2.5*$^{Cre/+}$ mice are mated to *ROSA26*$^{eGFP\text{-}DTA}$ mice to generate eGFP^{+} embryos carrying either *ROSA26*$^{eGFP\text{-}DTA}$ only (control) or *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ (ablated) alleles. Unlabeled wild-type ESCs are injected into eGFP^{+} blastocyst-stage embryos from each group to generate chimeras. (B) Summary table demonstrating the number of embryos and live-born mice recovered from wild-type ESC injection into control and CPC-ablating blastocysts. The number of animals derived from injection into the control genotype is expected to be higher due to early lethality of CPC-ablated embryos with low ESC chimerism. (C) Brightfield and GFP images of E10.5 chimeric *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ embryos. Embryos with >60% ablation show developmental arrest. (D) Degree of wild-type ES chimerism found in recovered surviving embryos or mice with *ROSA26*$^{eGFP\text{-}DTA}$ or *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ alleles. (E) Rate of recovery of surviving *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ chimeric animals relative to the number of un-ablated control (i.e. *ROSA26*$^{eGFP\text{-}DTA}$) chimeric embryos stratified by degree of wild-type ES chimerism. Note the drastic reduction in the percentage of recoverable *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ live animals when ES chimerism is <50%. (E) eGFP, troponin T (cTnT), and DAPI staining of a control *ROSA26*$^{eGFP\text{-}DTA}$ and a chimeric *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ embryo. Only rare eGFP^{+} endocardial cells are apparent in the ablated heart. Scale bars: 1 mm for (C); 200 μm for (F).

Figure S4



Ablation Tolerance at Various Developmental Stages





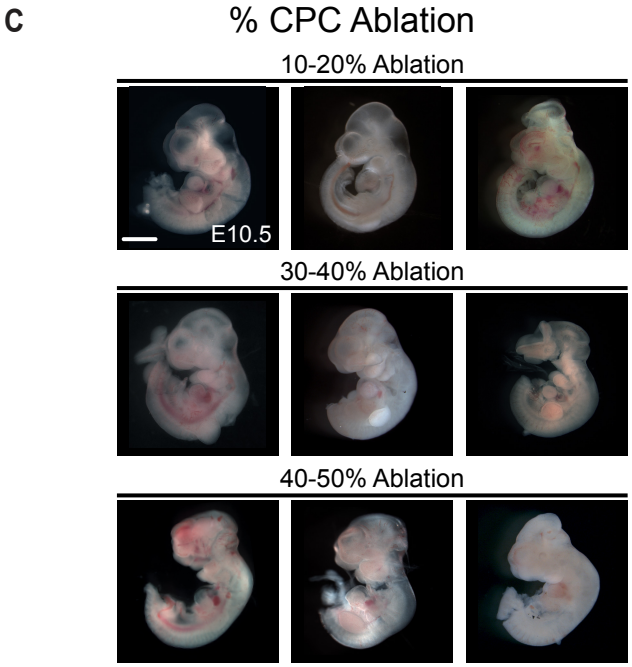


Figure S4 - Embryonic survival and additional embryo images, related to Figure 1 and 3.

(A) Degree of CPC ablation found in recovered surviving embryos or mice at mid (E10.5-E12.5) or late (E15.5-E18.5) gestation, and early life (4 weeks). (B) Brightfield images demonstrate the tolerance of ablated embryos to survive throughout gestation. Note: embryos derived from both chimeric rescue approaches are shown. (C) Brightfield images demonstrate consistency of normal embryo phenotype at various ranges of ablation. (D) Degree of CM ablation found in recovered surviving embryos or mice at mid (E10.5-E12.5) or late (E15.5-E18.5) gestation, and early life (4 weeks). (E) Brightfield images demonstrate stability of CM ablation tolerance throughout development. Scale bars: 1 mm.

Figure S5

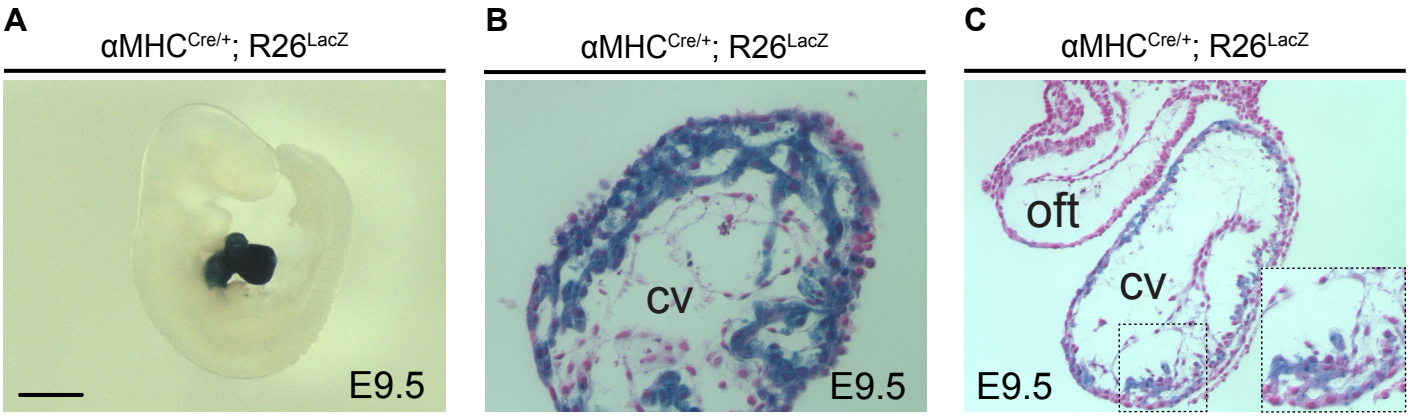

A
αMHC$^{Cre/+}$; R26^{LacZ}
E9.5
B
αMHC$^{Cre/+}$; R26^{LacZ}
cv
E9.5
C
αMHC$^{Cre/+}$; R26^{LacZ}
oft
cv
E9.5

Figure S5 - Genetic fate mapping of *αMHC* lineage cells, related to Figure 3. (A) Sagittal view of an E9.5 embryo. Robust β-galactosidase activity is evident throughout the atrial and ventricular chambers. (B) Transverse section through the mid-ventricle of an E9.5 embryo. β-galactosidase is detected throughout the myocardium. (C) Transverse section through the outflow tract and common ventricle of an E9.5 embryo. cv, common ventricle; oft, outflow tract. Scale bar: 500 μm.

Figure S6

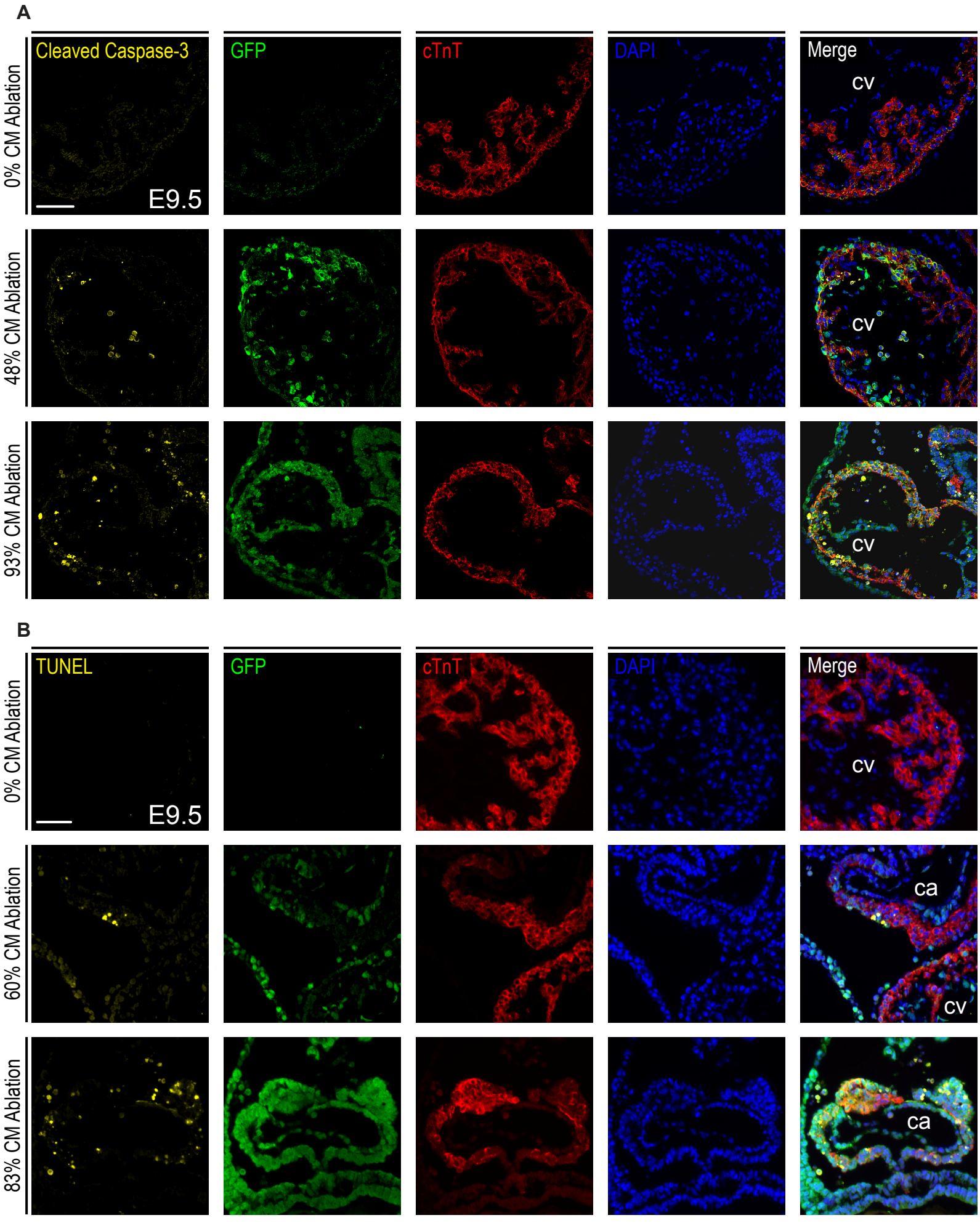
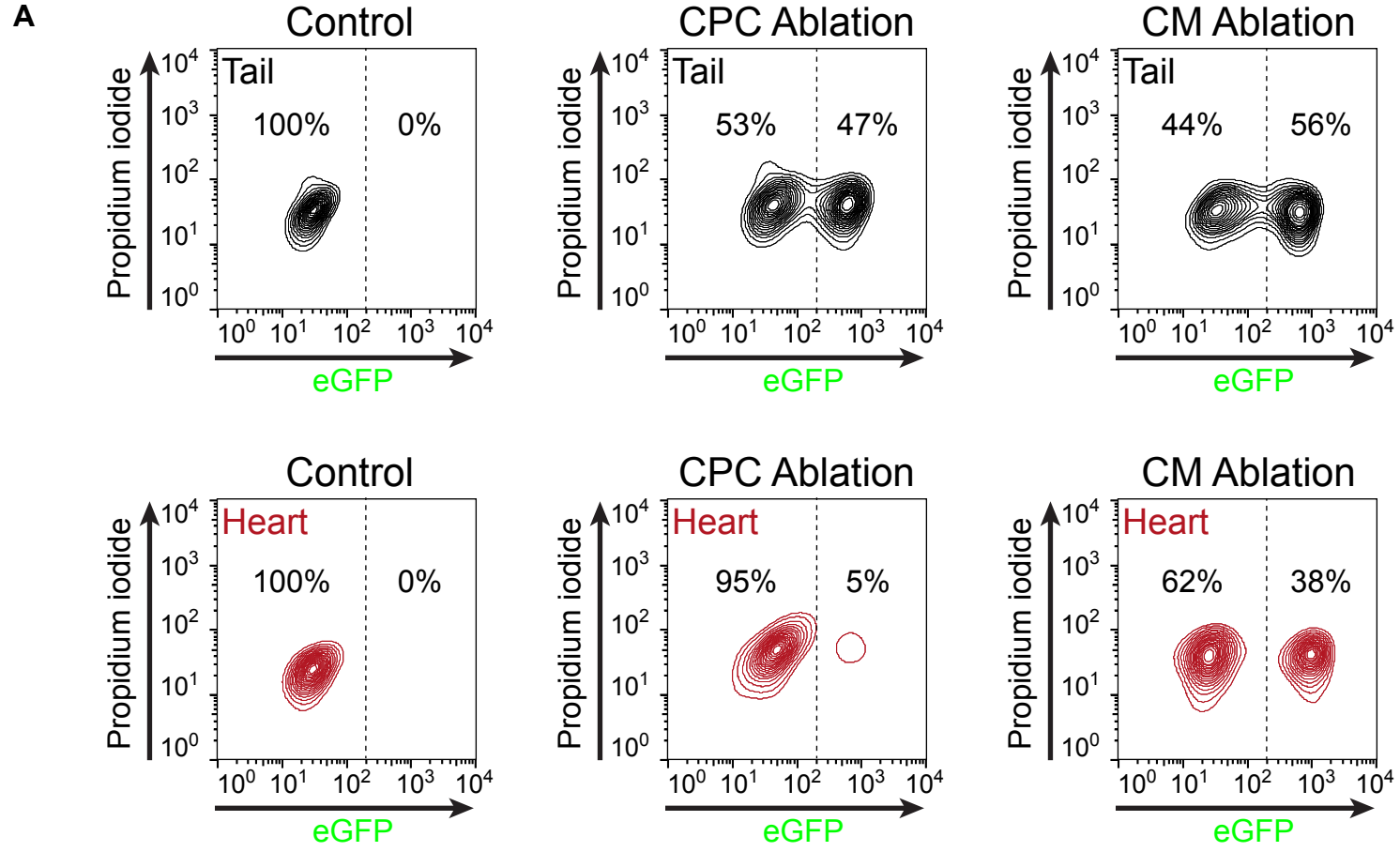
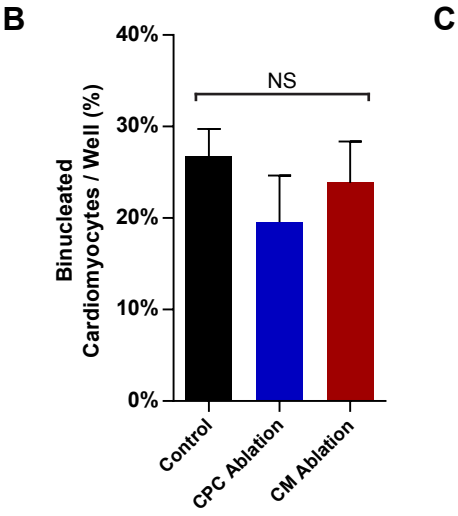

A
Cleaved Caspase-3
GFP
cTnT
DAPI
Merge
0% CM Ablation
E9.5
cv
48% CM Ablation
cv
93% CM Ablation
cv
B
TUNEL
GFP
cTnT
DAPI
Merge
0% CM Ablation
E9.5
cv
60% CM Ablation
ca
cv
83% CM Ablation
ca

Figure S6 - Cardiomyocyte apoptosis during CM ablation, related to Figure 3. (A-B) Cleaved caspase-3 (cCsp3, A) or TdT-mediated dUTP nick end labeling (TUNEL, B), eGFP, troponin T (cTnT), and DAPI staining of E9.5 embryos undergoing CM ablation. In the absence of CM ablation, no $cCsp3^{+}$ (A) or $TUNEL^{+}$ (B) cardiomyocytes are observed (upper row). However, a growing number of $cCsp3^{+}$ or $TUNEL^{+}$ cardiomyocytes can be identified as the severity of ablation increases (middle, lower rows). ca, common atrium; cv, common ventricle. Scale bar: 50 µm.

Figure S7


A
Control
CPC Ablation
CM Ablation
Tail
Heart
100%
0%
53%
47%
44%
56%
95%
5%
62%
38%
Propidium iodide
eGFP


B
NS
Binucleated Cardiomyocytes / Well (%)
Control
CPC Ablation
CM Ablation

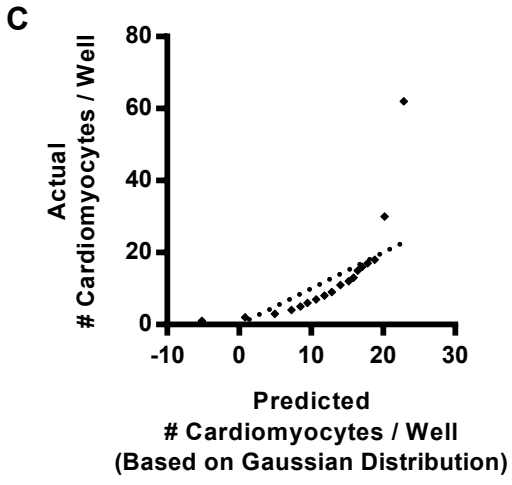

C
Actual # Cardiomyocytes / Well
Predicted # Cardiomyocytes / Well (Based on Gaussian Distribution)

Figure S7 - *Ex vivo* clonal analysis of cultured cardiomyocytes following cardiac ablation, related to Figure 4. (A) Representative FACS contour plots of dissociated tail and heart samples from E9.5 embryos used for single-cell clonal analysis. The proportion of $eGFP^+/eGFP^-$ cells in these tissues is shown from an embryo with no ESC chimerism (control), an embryo chimerized with *Nkx2.5*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ ESCs (CPC ablation), and an embryo chimerized with *αMHC*$^{Cre/+}$;*ROSA26*$^{eGFP\text{-}DTA}$ ESCs (CM ablation). Few $eGFP^+$ cardiac cells remain in the CPC-ablated embryo by this stage of development. In the CM-ablated embryo, the fraction of $eGFP^+$ cells in the heart relative to the tail is reduced due to the loss of $eGFP^+$ cardiomyocytes. (B) Mean percentage of binucleated cardiomyocytes per well among wells containing cardiomyocyte colonies. Error bars represent mean ± s.e.m. (C) Q-Q normality plot of actual versus predicted single-cell derived cardiomyocyte colony size demonstrates marked systematic deviation from linearity indicating lack of conformity to a Gaussian distribution.

Figure S8

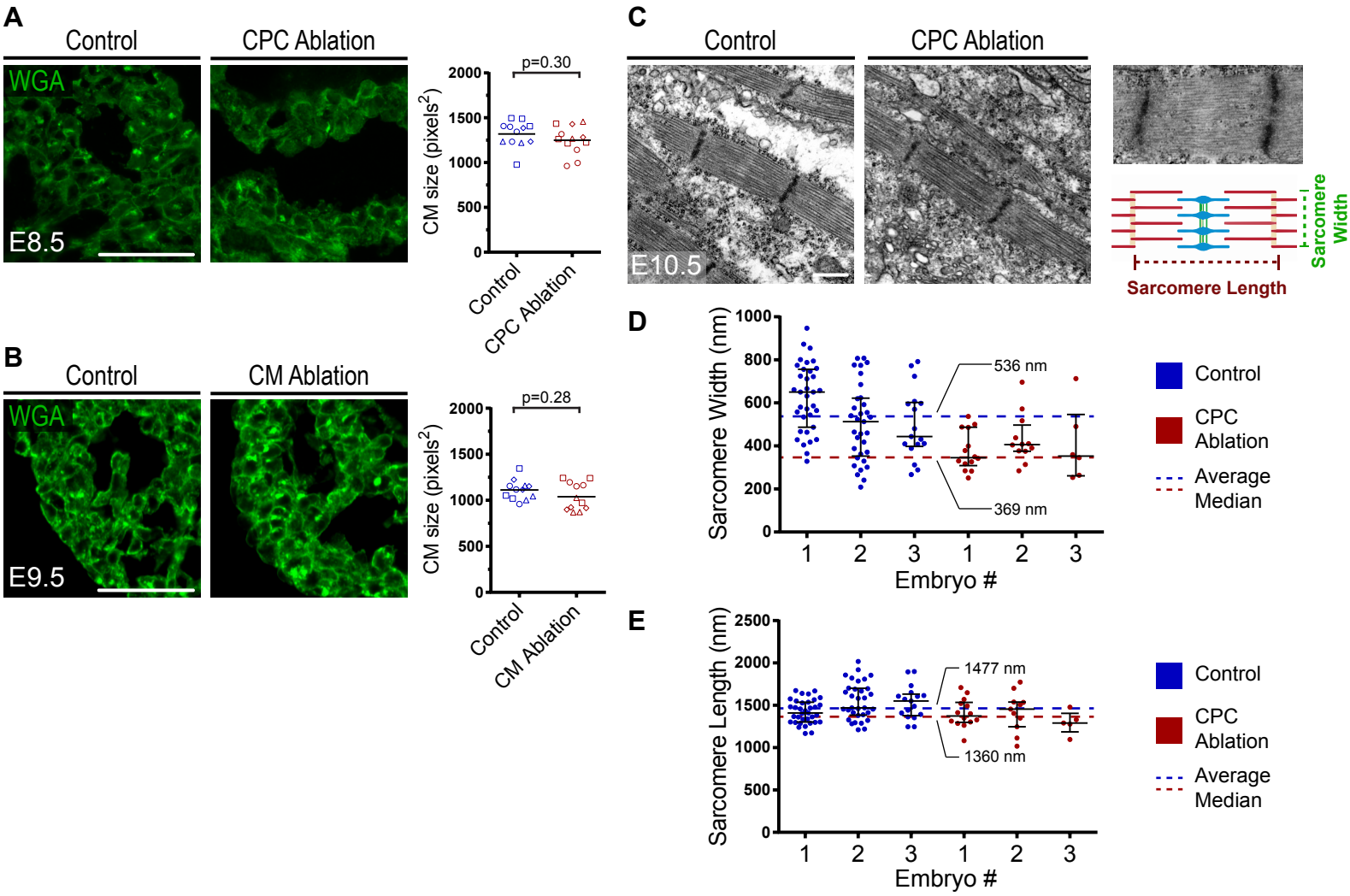

A
Control
CPC Ablation
WGA
E8.5
p=0.30
CM size (pixels²)
2000
1500
1000
500
0
Control
CPC Ablation
B
Control
CM Ablation
WGA
E9.5
p=0.28
CM size (pixels²)
2000
1500
1000
500
0
Control
CM Ablation
C
Control
CPC Ablation
E10.5
Sarcomere Width
Sarcomere Length
D
Sarcomere Width (nm)
1000
800
600
400
200
0
536 nm
369 nm
1
2
3
1
2
3
Embryo #
Control
CPC Ablation
Average
Median
E
Sarcomere Length (nm)
2500
2000
1500
1000
500
0
1477 nm
1360 nm
1
2
3
1
2
3
Embryo #
Control
CPC Ablation
Average
Median

Figure S8 - Embryonic cardiomyocyte size and ultrastructural features following ablation, related to Figure 4. For (A-E): experimental variables are represented as follows [group: # of mice examined, mean percent ablation ± SD]. (A-B) Wheat germ agglutinin (WGA) staining and dot plots displaying mean CM size for CPC-ablated hearts at E8.5 (A) and CM-ablated hearts at E9.5 (B). Each symbol in the dot plot represents the mean CM size in an individual heart section; a minimum of three 32X fields per section were counted; each symbol shape represents an independent mouse; a horizontal bar indicates the mean value for each group. Statistical comparisons were performed using a likelihood ratio test via ANOVA on a linear mixed-effects model. E8.5 embryos were embedded frozen in OCT; E9.5 embryos were embedded in paraffin. [control: n=4; CPC ablation: n=4, 41 ± 9%; CM ablation: n=4, 53 ± 12%]. (C) Transmission electron micrographs (EM) showing sarcomeric structure from a control and a CPC-ablated heart section at E10.5. (D-E) Dot plots of sarcomere width (D) and length (E) based upon EM analysis. [control: n=3; CPC ablation: n=3, 40 ± 7%]. Each point represents the average sarcomere width/length from a single field; bars represent median ± interquartile range; dashed lines represent the average of the three median values for each group. Scale bars: 50 μm for (A-B); 500 nm for (C).