Cdon deficiency causes cardiac remodeling through hyperactivation of WNT/β-catenin signaling

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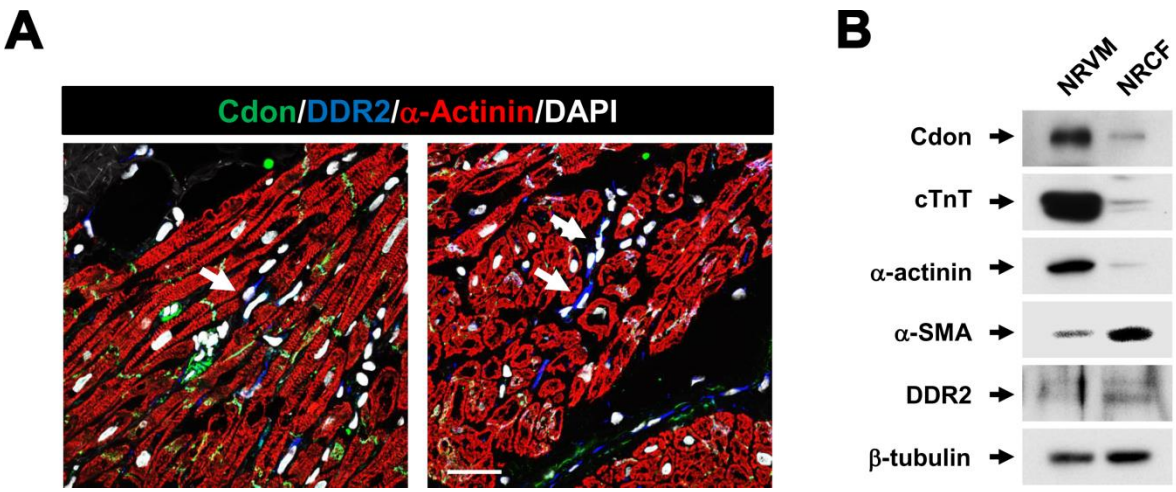
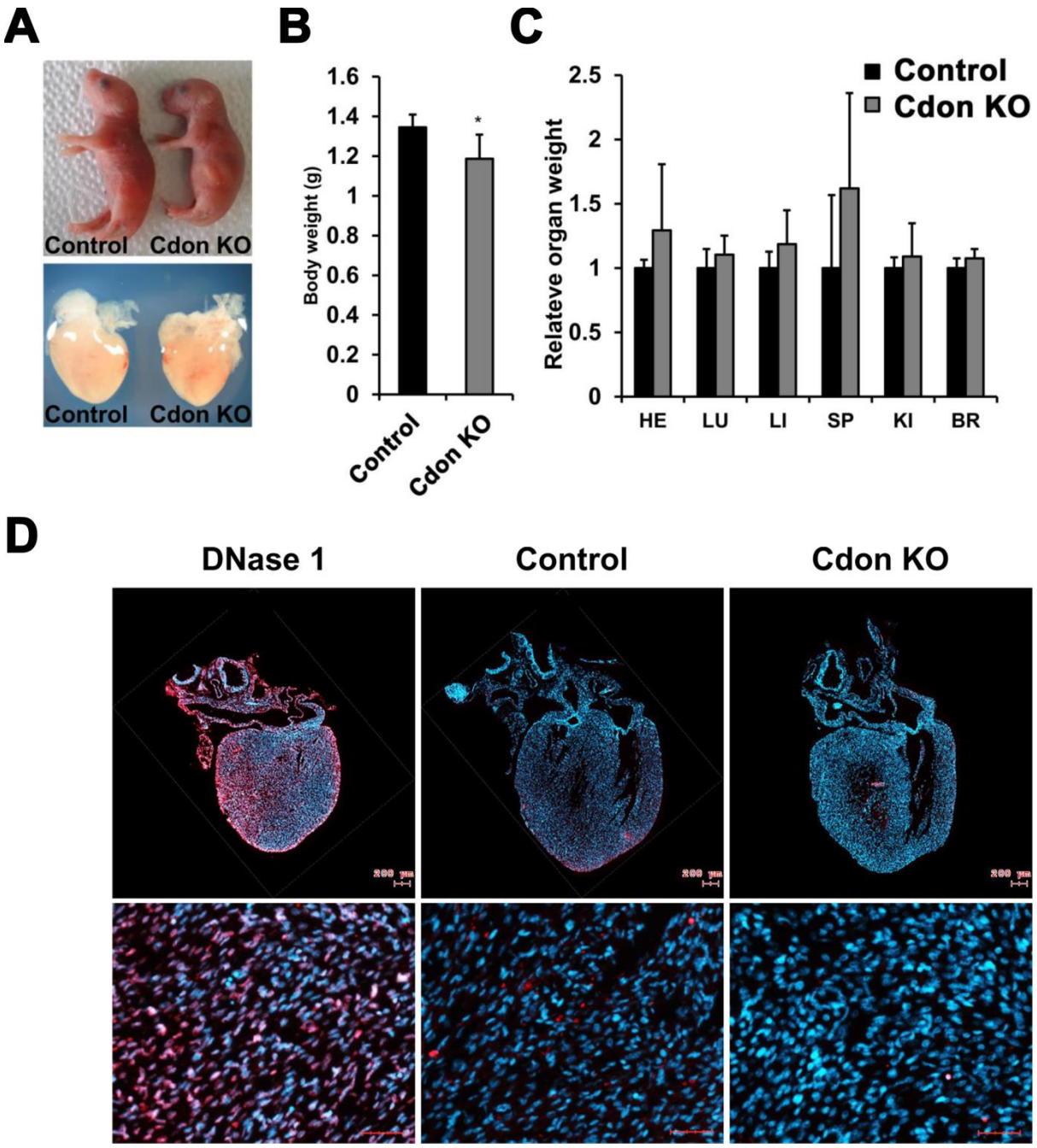
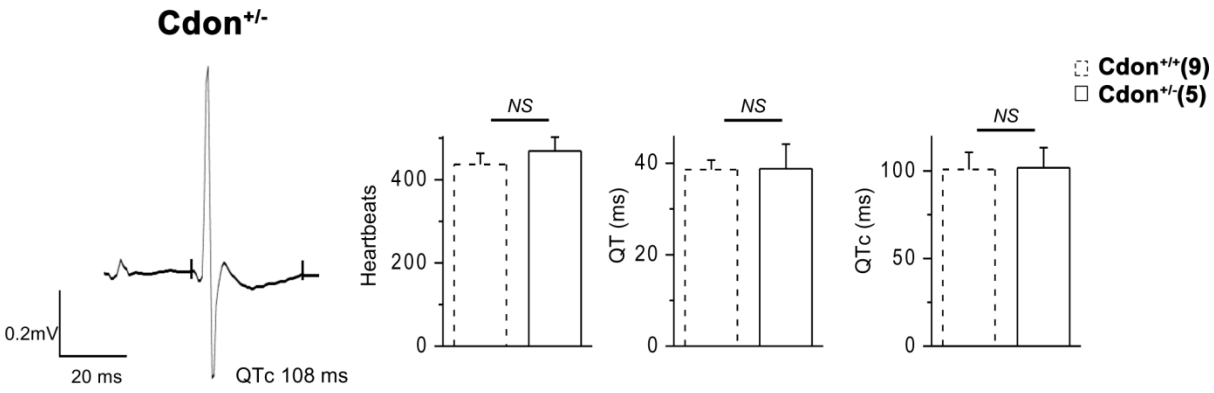
SI Appendix Figures



SI Appendix figure 1. The predominant expression of Cdon in cardiomyocytes, compared to cardiac fibroblasts. (**A**) Immunostaining of Cdon, DDR2 and α-Actinin for human heart. Cdon is mainly expressed and localized in intercalate disc area of cardiomyocytes (α-Actinin positive cells). Note little or no staining for Cdon in DDR2 positive cardiac fibroblasts. Scale bar = 100μm. (**B**) Western blot analysis for Cdon expression in NRVM and NRCF (new born rat cardiac fibroblast) cells. To isolation NRCF cells, isolated cardiac cells were enriched by prepalting for 1 hour, followed by subsequent removal of supernatant containing cardiomyocyte.

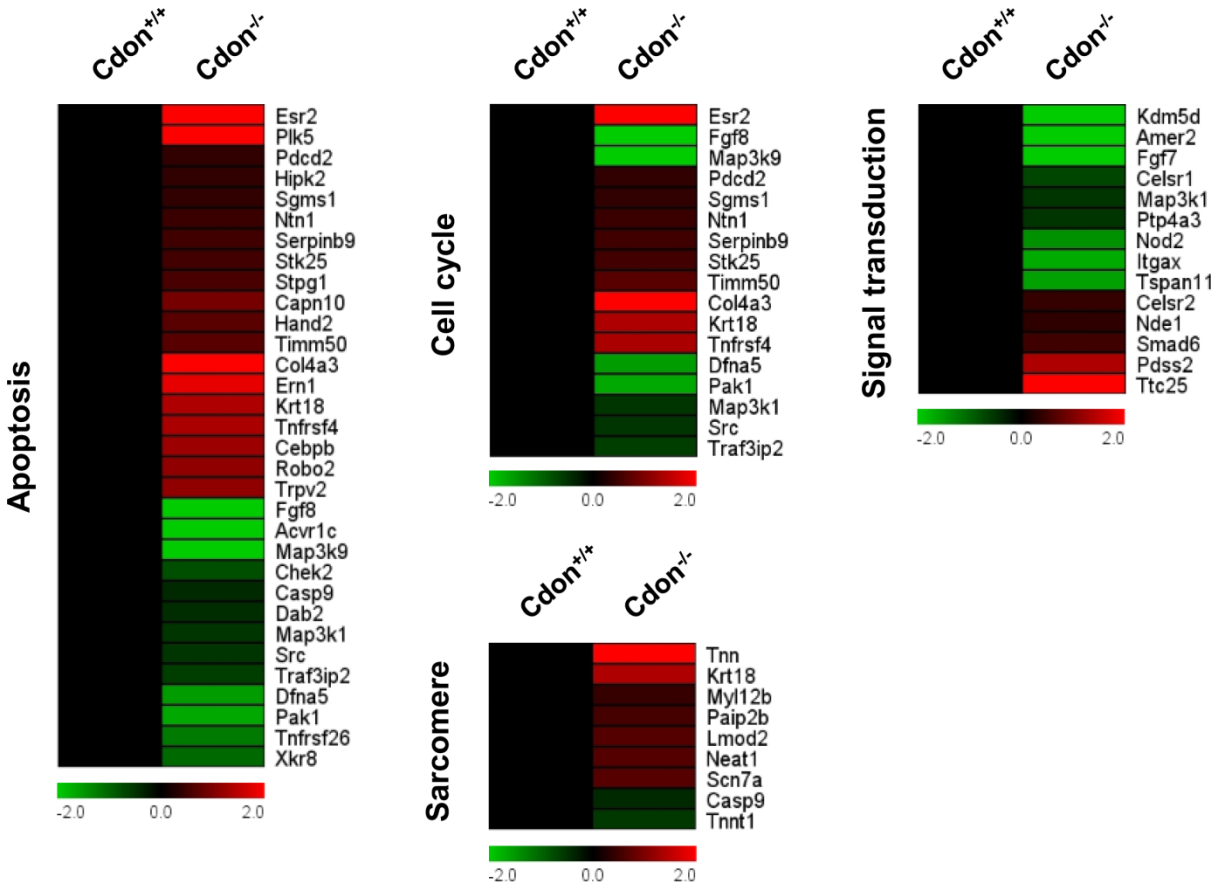


SI Appendix figure 2. The relative organ weights to body weights in control and *Cdon*$^{-/-}$ littermates. (**A**) Images of neonatal control *Cdon*$^{+/-}$ and *Cdon*$^{-/-}$ littermate mice and their hearts. (**B**) Body weights of control *Cdon*$^{+/+}$or *Cdon*$^{+/-}$ and *Cdon*$^{-/-}$ littermates. n=6, **P*<*0.05*. (**C**) The relative weight of organ weights to body weights from control *Cdon*$^{+/+}$or *Cdon*$^{+/-}$ and *Cdon*$^{-/-}$ littermates at the postnatal day 1 (P1). He: Heart, Lu: lung, Li: Liver, Sp: Spleen, Ki: Kidney and Br: Brain. **(D)** Images from TUNEL assay with P1 control and *Cdon*$^{-/-}$ hearts. DNase I was used as positive control for TUNEL assay. Scale bar = 200μm.

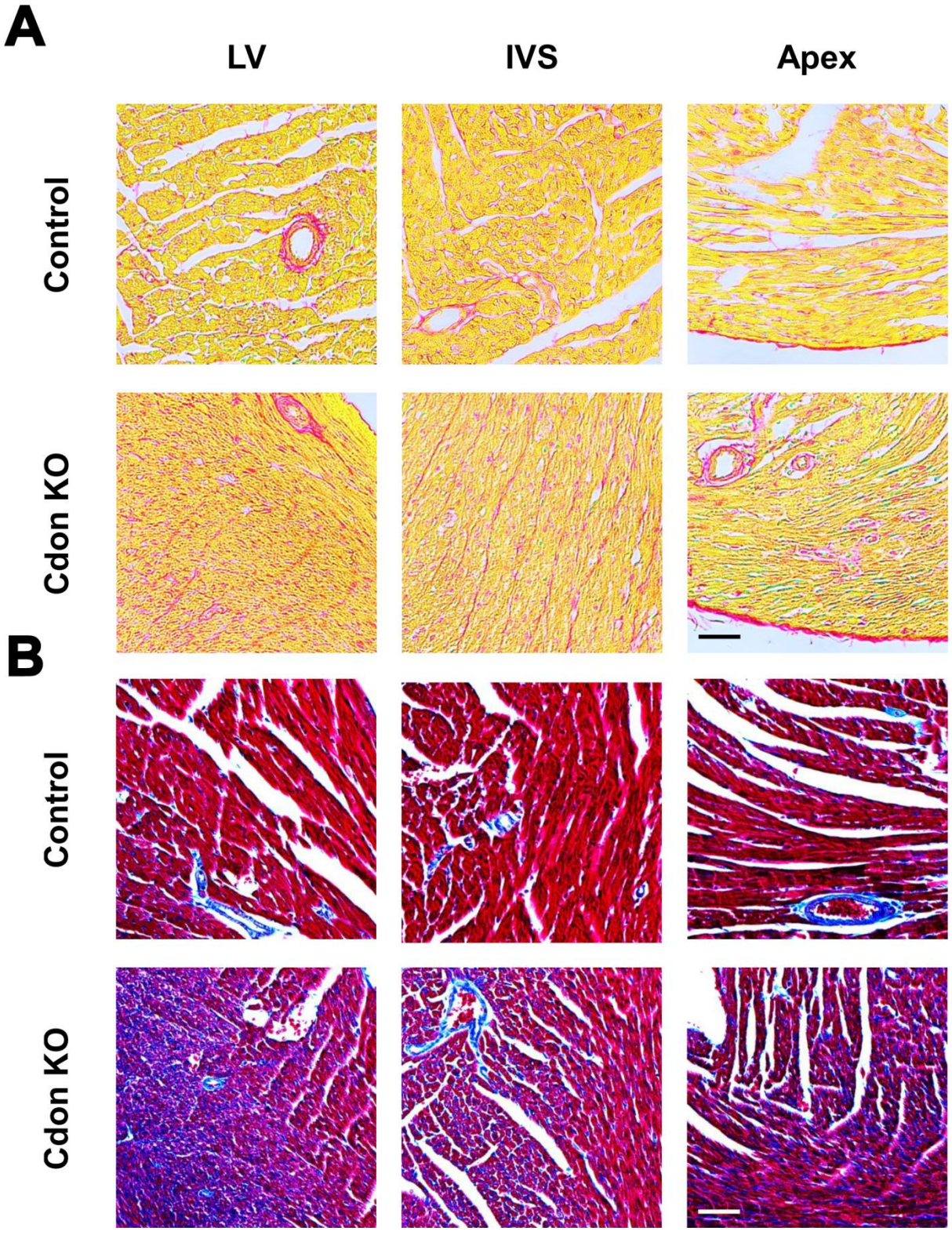


SI Appendix figure 3. No alteration of ECGs in *Cdon*$^{+/-}$ mice.

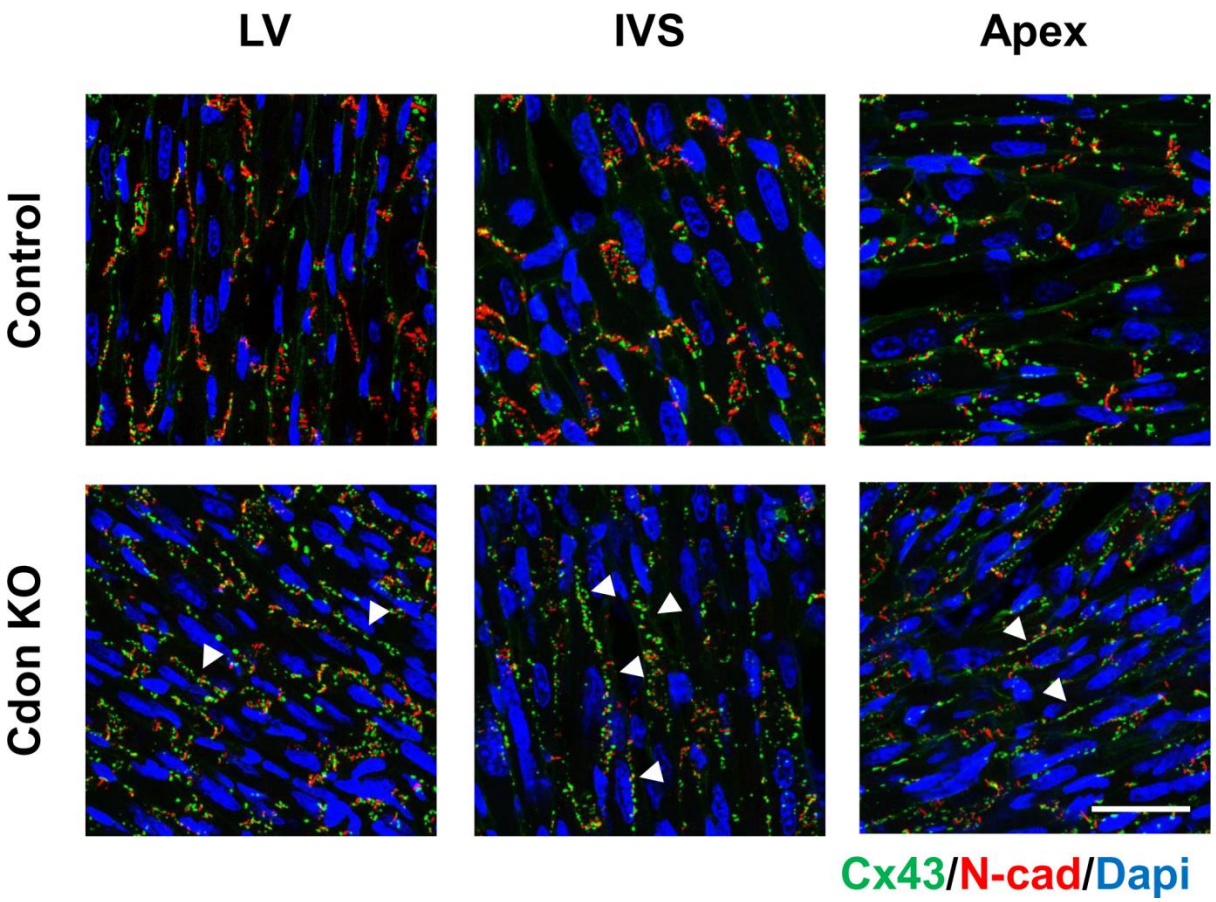
Left, Representative trace of ECGs of *Cdon*$^{+/-}$ mice at P14. Right, Bar graphs revealno significant change in HR, QRS duration, and QTc interval, compared with *Cdon*$^{+/+}$ mice.



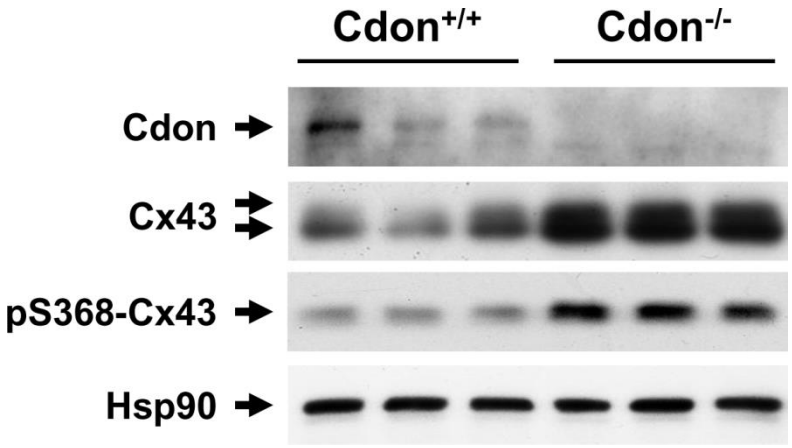
SI Appendix figure 4. GO analysis for RNA transcription level of *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ neonatal hearts. Heat maps for GO terms including apoptosis, cell cycle, signal transduction and sarcomere were shown. (>1.3 fold, average of normalized RC log2 >2, p-value <0.05)



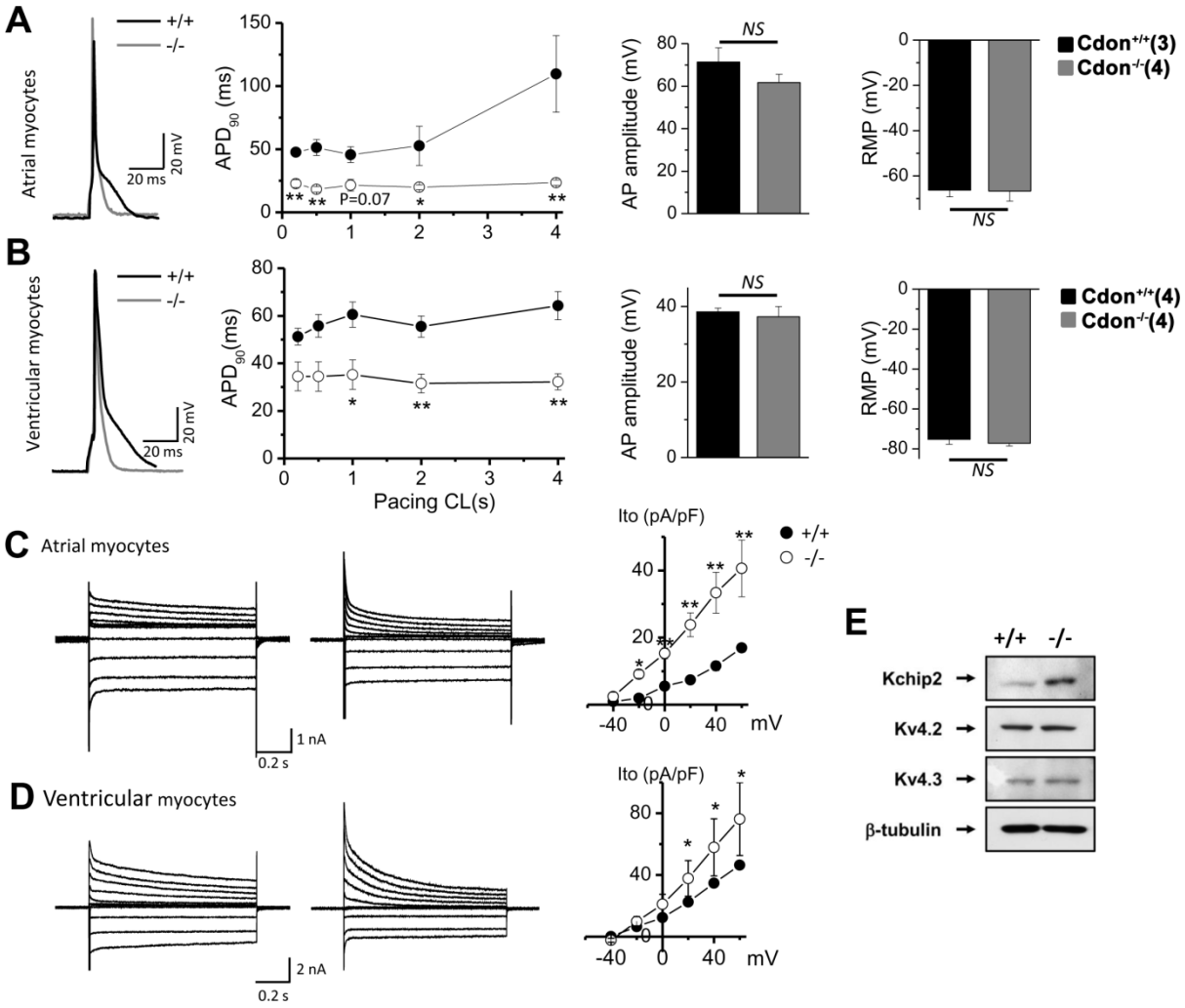
SI Appendix figure 5. Cdon depletion can induce cardiac fibrosis. Sirius red staining (**A**) and Masson's trichrome staining (**B**) were performed for 2 weeks old control and *Cdon*$^{-/-}$ hearts. LV; left ventricle, IVS; interventricular septum. Scale bar=100um.



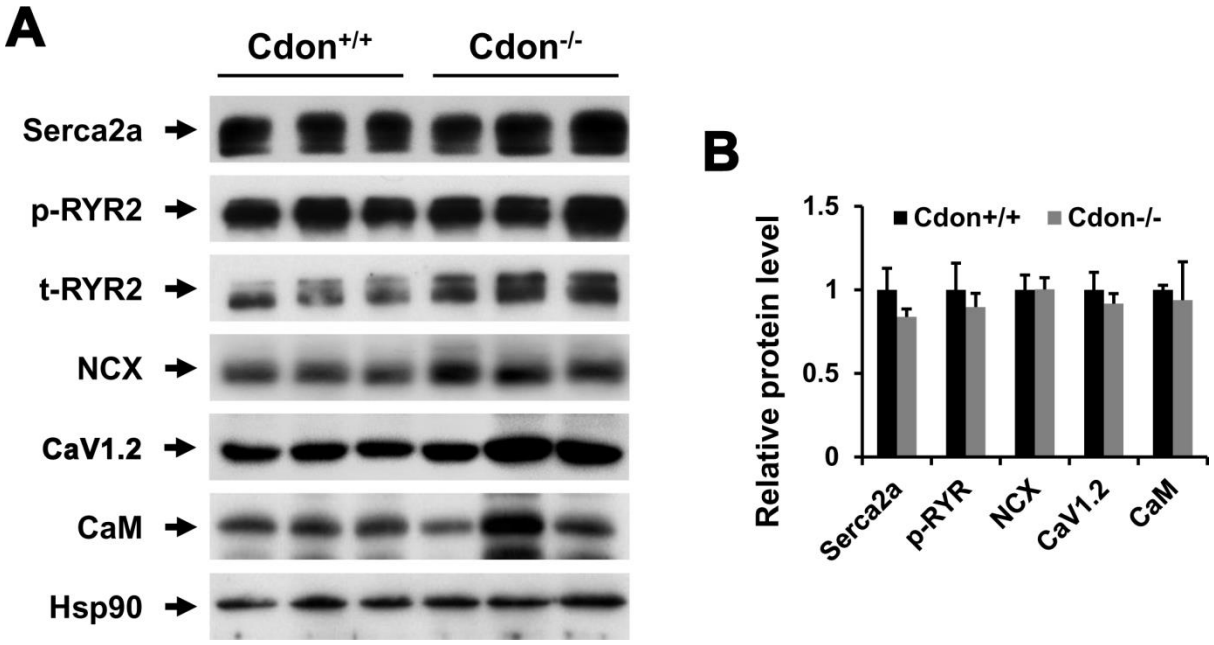
SI Appendix figure 6. **Abnormal localization of Cx43 in various regions of** *Cdon*$^{-/-}$ **hearts**. Representative images for the immunostaining of Cx43 (green) and N-cadherin (Red) for 2 weeks old control and Cdon KO hearts were shown, LV; left ventricle, IVS; interventricular septum. Lateralization of gap-junction was indicated with arrow heads in *Cdon*$^{-/-}$ heart images. Scale bar=50μm



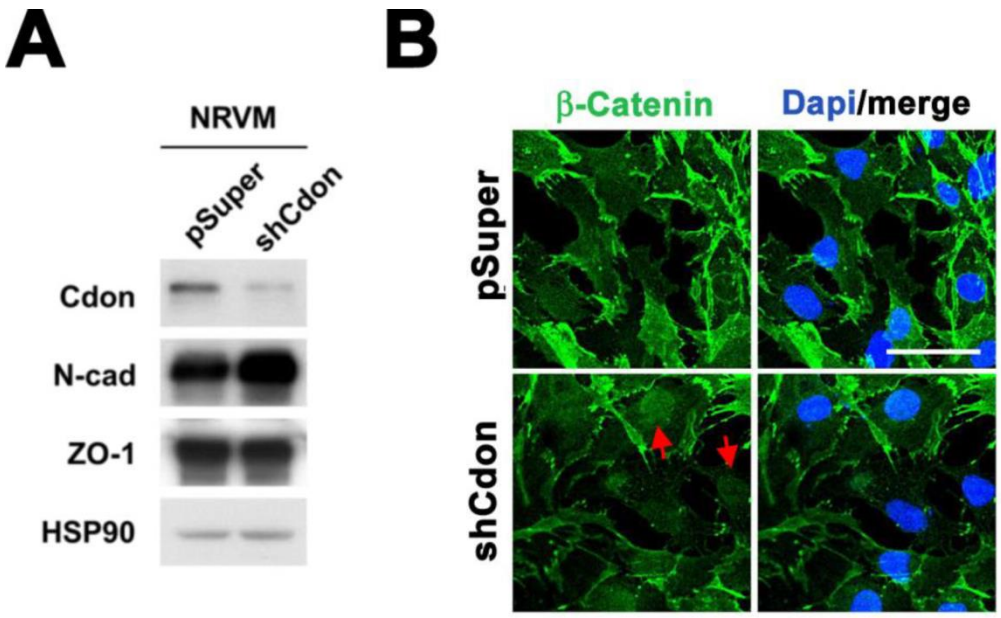
SI Appendix figure 7. Immunoblotting analysis for Cx43 in ***Cdon***$^{+/+}$ **and** ***Cdon***$^{-/-}$ **heart sample**. Note overall enhanced Cx43 levels in *Cdon*$^{-/-}$ hearts, including slow-migrating (phosphorylated) forms and a phosphorylated form of Cx43 at serine 368 (pS368-Cx43), relative to control WT hearts.



SI Appendix figure 8. Cdon deficiency causes reduction of action potential duration (APD). (**A, B**) Examples of APs of atrial myocytes (A) or ventricular myocytes (B) from *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ mice. Middle, plot of APD_{90} over a range of pacing cycle lengths in *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ myocytes. Right, AP amplitude and resting membrane potential (RMP) in atrial (A) and ventricular myocytes (**B**) from *Cdon*$^{-/-}$ mice, compared to WT mice. Mean ± S.E.M. *n*, the number of cells tested.(**C, D**) Left, Representative K^+ currents recorded in atrial myocytes (C) or ventricular myocytes (D) from *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ mice. All currents were recorded in the whole-cell configuration of the patch clamp. Right, The IV relationships for I_{to} currents reveal an increase in I_{to}. I_{to} current was measured by subtracting the amplitude of the current measured at the end of the depolarizing voltage pulse (sustained current) from peak current amplitude. Current amplitude of each cell was normalized to its capacitance (*current density*; pA/pF). Filled circles indicate current density in WT, and open circles indicate current density in *Cdon*$^{-/-}$ myocytes. (**E**) Immunoblot analysis of KChIP2, Kv4.2, and Kv4.3 in *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ hearts at postnatal day 7. *$p< 0.05$, **$p< 0.01$ vs control.

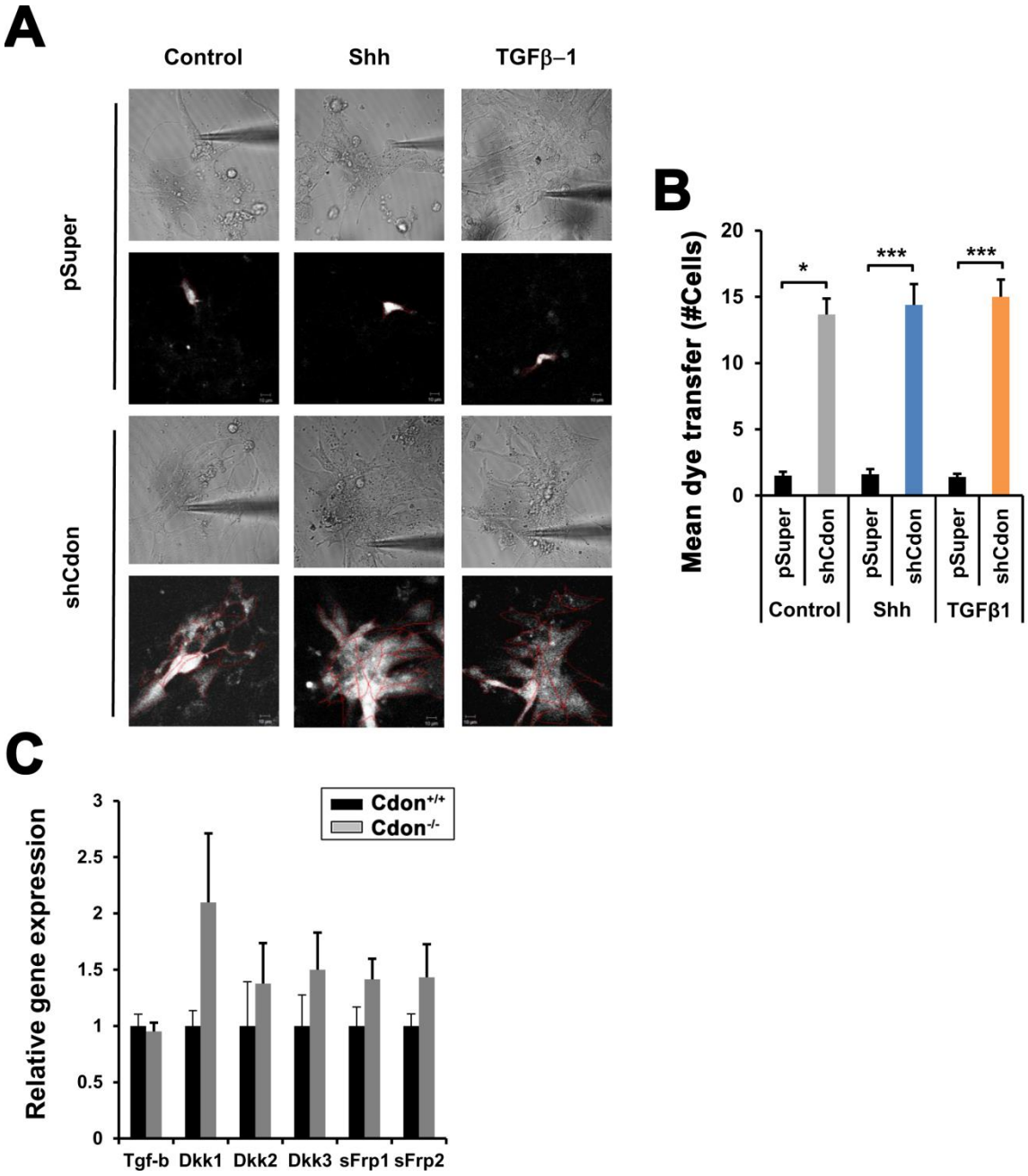


SI Appendix figure 9. Immunoblotting analysis for calcium cycling proteins of *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ heart lysates. (**A**) 2 weeks old *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ heart lysates were subjected to western blot analysis. Hsp90 used as loading control. (**B**) Quantification of relative protein levels. n=4 for each group.

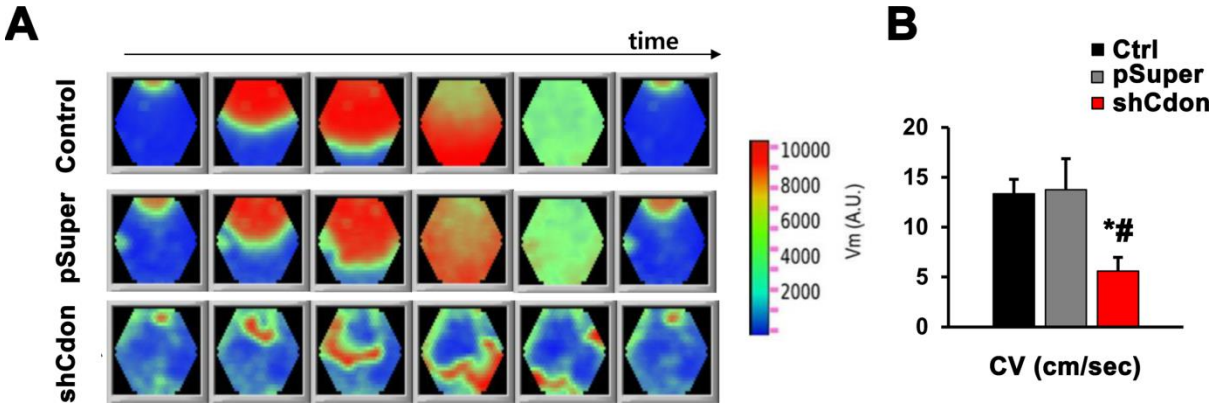


SI Appendix figure 10. Cdon deficiency causes elevated N-cadherin expression and nuclear β-catenin accumulation in NRVM cells.

(A) Immunoblot analysis of NRVM cells transfected with the control shRNA (pSuper) or Cdon shRNA (shCdon) vectors for N-cadherin, ZO-1 and Cdon. (**B**) Immunostaining of β–catenin in NRVM cells transfected with pSuper or shCdon plasmids. Arrows indicate nuclei with accumulation of β-catenin. Note that Cdon depleted cells have more nuclear β-catenin accumulation. Scale bar = 20 μm.



SI Appendix figure 11. Treatment of Shh or TGF-β has no effect on LY dye transfer in control or Cdon depleted NRVM cells. (**A**) Representative images for LY dye transfer in control, Shh (250ng/ml) or hTGF-β (2ng/ml) treated control or Cdon-depleted NRVM. Scale bar = 10μm. (**B**) Quantification of the mean dye transfer in panel A. *$p< 0.05$, ***$p< 0.001$ vs control. N=4 per each sample. (**C**) qRT-PCR analysis of RNAs isolate from *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ hearts for Wnt signaling modulator including TGF-β, Dkk1, Dkk2, Dkk3, sFrp1 and sFrp2. N=4 for each group.



SI Appendix figure 12. Optical mapping analysis for NRVM cells control or transfected with, pSuper or shCdon vectors. (**A**) Snapshots of the propagation of voltage wave through the monolayers of NRVM. From left to right, each row shows the propagation of one wavefront from initiation until it dies out. The last frame of each row is the initiation of a second voltage wave. Time intervals are arbitrary and not equal between different frames. (**B**) Quantification for conduction velocity of NRVM cells in panel A. *$*P<0.005$ vs control*, $^{\#}P<0.05$ vs pSuper.* N=6 (control); 3 (pSuper); 5 (shCdon).

SI Appendix Table 1. ECG parameters in *Cdon*$^{-/-}$ mice

	Cdon $^{+/+}$	*Cdon* $^{+/-}$	*Cdon* $^{-/-}$	*P value*
Number	9	5	12	
RR interval (ms)	140.3±8.1	139.4±1	138.5±9	*NS*
PR interval (ms)	15.4±2.2	15.1±3.8	14.4±3.2	*NS*
P wave (ms)	14.2±1.3	16.4±2.5	17.2±3.8	*NS*
QRS complex (ms)	15.4±1.8	15.3±1.6	11±0.6	*NS*
QT interval (ms)	38.6±2.1	38.8±1.3	29±2.1	<0.05
QTc (ms)	100.8±9.8	101.8±11.4	79.7±7.1	<0.05

SI Appendix Table 2. The gene lists of GO analysis with *Cdon*$^{+/+}$ and *Cdon*$^{-/-}$ P1 hearts.

Gene annotation	Status	Official gene symbol	No. of genes
Cell adhesion	Up-regulated genes	Erf, Myl12b, Ntn1, Nhej1, Gp1ba, Pcdhb20, Pcdhb19, Tnfrsf4, Pcdh11x, Il7r, Vnn1, Col4a3, Cntn5, Crtam	14
	Down-regulated genes	Cass4, Cd86, Ctnnd2, Gp9, Otoa, Itgax, Micall2, Pcdh17, Angpt1, Frem2, Celsr1, Cdh11, Src, Tek	14
Regulation of transferase activity	Up-regulated genes	Ankrd54, Itprip, Agap2, Sdc4, Gp1ba, Irs1, Spry4, Tnfrsf4, Errfi1, Fam72a, Serinc2	11
	Down-regulated genes	Tlr1, Map3k9, Ghrl, Fgd2, Cdk5r2, Pak1, Angpt1, C1qtnf2, Pkia, Map3k1, Src, Csf1r, Ctc1, Tek	14
Regulation of cell motion	Up-regulated genes	Timp1, Ntn1, Ppargc1a, Sdc4, Dock5, Irs1, Tmeff2, Hoxa7, Bcl6	9
	Down-regulated genes	Mmp28, Fgf7, Pak1, Igfbp5, Myo1f, Angpt1, Scai, Arap1, Map3k1, Src, Csf1r, Tek	11
Response to hormone stimulus	Up-regulated genes	Timp1, Ppargc1a, Slc25a33, Irs1, Errfi1, Lipc, Tox2, Ptprn, Lct, Aldob, Fgf23, Esr2	12
	Down-regulated genes	Agrp, Ghrl, Pak1, Igfbp5, Sdc1, Src, Tek, Med12	8
Regulation of cell migration	Up-regulated genes	Timp1, Ntn1, Ppargc1a, Sdc4, Dock5, Irs1, Tmeff2, Hoxa7	8
	Down-regulated genes	Mmp28, Fgf7, Pak1, Igfbp5, Myo1f, Angpt1, Scai, Map3k1, Src, Csf1r, Tek	11
Enzyme linked receptor protein	Up-regulated genes	Chn1, Irs1, Col4a3, Dlx5, Fgf23	5
	Down-regulated genes	Fgf8, Fgf7, Angptl1, Angpt1, Arap1, Map3k1, Wfikkn2, Src, Csf1r, Tek	10
Peptide metabolic process	Up-regulated genes	Gsr, Pcsk5, Eef1e1, Slc25a33, Eif2s3x, Pcsk1	6
	Down-regulated genes	Ddx3y, Eif2s3y, Gm4832, Gstt1, Bace2, Slc25a14	6
Response to extracellular stimulus	Up-regulated genes	Ppargc1a, Atf3, Lipc, Oprm1, Lct, Aldob, Fgf23	7
	Down-regulated genes	Ghrl, Gstt1, Src	3
Response to nutrient	Up-regulated genes	Lct, Fgf23	2
	Down-regulated genes	Gstt1	1

SI Appendix Table 3. KEGG pathway analysis with RNA sequencing data.

Pathway	Gene Symbol
Metabolic pathways	Aldob, Asl, Bckdhb, Cda, Cers4, Lct, Lipc, Lipt2, Man1a2, Mvd, Mnmt, Pigv, Pla2g4c, Pld4, Prodh, Rbks, Sardh, Sat2, Sgms1
PI3K-Akt signaling pathway	Angpt1, Col4a3 ,Creb3l1 ,Csf1r, Fgf23, Fgf7, Fgf8, Gm5741, Il7r, Irs1, Osmr, Tek
Regulation of actin cytoskeleton	Fgf23, Fgf7, Fgf8, Gm5741, Itgax, Myl12b, Pak1, Pip4k2c, Src, Ssh3
RAP1 signaling pathway	Angpt1, Apbb1ip, Calm3, Csf1r, Efna4, Fgf23, Fgf7, Fgf8, Mapk12, Pard6g, Plcb4, Rac3, Src, Tek
MAPK signaling pathway	Fgf23, Fgf7, Fgf8, Gm5741, Illr2, Map3k1, Pak1, Pla2g4c, Ptpn7
RAS signaling pathway	Angpt1, Calm3, Csf1r, Efna4, Fgf23, Fgf7, Fgf8, Gm5741, Gng3, Pak1, Pla2g4c, Rac3, Tbk1, Tek

SI Appendix Table 4. Primer sequences for qRT-PCR analysis

Gene		5' to 3'	Tm (℃)
Nppb	Forward	AAATGGCCCAGAGACAGCTC	58
	Reverse	CAACTTCAGTGCGTTACAGCC	58
Col4a3	Forward	ATTCCAGAAGCTGGATGCCC	59
	Reverse	CTGCCTACGGATGGTTCTCC	58
Timp3	Forward	TTCTGCAACTCCGACATCGT	58
	Reverse	TTTCAGAGGCTTCCGTGTGA	58
Gja5	Forward	AAGCAGAAATTGCGGGATGC	58
	Reverse	CTACGATGAAGGCCACCTCC	58
Ace	Forward	GATGCCATGAAGCTGGGCTA	59
	Reverse	CTTCTGCGCGAGCGGT	59
Ctgf	Forward	TCCACCCGAGTTACCAA	53
	Reverse	TTAGGTGTCCGGATGC	51
Gja1	Forward	CCAGCCCTTAGCTATCGTGG	58
	Reverse	CCTCCACGGGAACGAAATGA	58
Timp1	Forward	CTACGATGAAGGCCACCTCC	58
	Reverse	ACTCTTCACTGCGGTTCTGG	58
TGF-b	Forward	TCGACATGGAGCTGGTGAAA	55
	Reverse	CTGGCGAGCCTTAGTTTGGA	57
Dkk1	Forward	CCAACGCGATCAAGAACCTGC	60
	Reverse	CACATAGCGTGCCTCATGCAG	60
Dkk2	Forward	CACTATTCCAACCATGACCTGGGA	60
	Reverse	CCTTTGCACAGTCACACCTCTGG	61
Dkk3	Forward	ATGCTATGCACCCGAGACAGT	58
	Reverse	CCTTCAGGCTCCAGTTCCCA	60
sFRP1	Forward	CAAGAAGATGGTGCTGCCCAAC	60
	Reverse	GAAGCCGAAGAACTGCATGACC	60
sFRP2	Forward	GAGACCATCCAGCCGTGTCA	60
	Reverse	GCAGGCTTCACACACCTTGG	60