SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Animals

All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at The University of Arizona (UA) and the University of California, San Diego (UCSD) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Systemic Cx40 knockout (KO) mice were kindly provided by Dr. Janis Burt from the UA[31], and C57BL/6J were used as a wild-type (Wt) control. Tie2-driven Cx40 Wt overexpressing (Cx40TG) mice and Tie2-driven Cx40 negative mutant overexpressing mice (Cx40-NM) were provided by Dr. Anthony Ashton from the University of Sydney[32]. Heterozygous parents were used as a breeder of Cx40TG mice, and homozygous parents were used for $Cx40^{NM}$ breeders. Homo- and heterozygosity was determined by Cx40 copy number. Mice without Tie2-Cx40TG gene or Tie2-Cx40-NM gene were used as Wt control. These mice were bred in the animal facility of the UA and UCSD. Male mice were used for experiments in this study. The primer sequence information for genotyping and copy number assessment is listed in **Table S1**. Mice were randomly allocated to experimental groups, and hypoxia-induced PH was induced by placing mice (8 weeks old) in the normobaric hypoxic chamber (10% O_2) for 4 weeks. Sugen-hypoxia PH mouse model (SuHx) was generated by injections of Sugen 5416 (20mg/kg, once a week) during the 4 weeks of hypoxic exposure. Cx40 overexpression was achieved by a single intravenous injection (i.v.) of CMV-Cx40 adenovirus[33] (Cx40-Adv) or control Adv (Cont-Adv, Adv vector without gene insertion) at 3×10^9 pfu/mouse. Three weeks after hypoxic exposure, mice received either Cx40-Adv or Cont-Adv and kept in the hypoxic chamber for an additional one week before experimentation. Lung dissection was performed under anesthesia with sodium pentobarbital (130 mg/kg, i.p.), and all efforts were made to minimize pain.

Isometric tension measurement in pulmonary arterial ring

Isometric tension in the PAs was measured and recorded as described previously[34]. Briefly, a large proximal PA (2nd order) and small distal PA (4th-5th order) were dissected from a left superior lobe and cut into 1-mm segments. The PA rings were mounted on a myograph (DMT-USA, Inc. Ann Arbor, MI, USA) using thin stainless wires (20 μm in diameter), and the resting tension was set at 0.1 g. PAs were allowed to equilibrate for 45 min with intermittent washes every 15 min. After equilibration, each PA ring was contracted by treatment with $PGF_{2\alpha}$ to generate a similar contraction level in all groups. Acetylcholine (ACh) or sodium nitroprusside (SNP, an NO donor) was administrated in a dose-dependent manner (1 nmol/l to 100 μmol/l), and the degree of vasodilatation was described as a percentage (%) decrease of $PGF_{2\alpha}$-induced contraction.

Right ventricular systolic pressure (RVSP) measurement

Mice were anesthetized with isoflurane (1%), and the catheter was inserted into the right external jugular vein and proceeded into the right ventricle (RV). Right ventricular pressure (RVP) was measured and recorded using an MPVS Ultra system (Millar Inc. Houston, TX, USA), as described in our previous manuscript[34]. RVSP, a surrogate measure of pulmonary artery systolic pressure, was measured and compared between

the groups. At the end of the experiment, the heart was dissected. The free wall of the RV was then isolated from the left ventricle and septum (LV+S) and weighed separately. The Fulton index was calculated as the weight ratio of RV/(LV+S).

Isolation of mouse pulmonary ECs

Mouse pulmonary ECs (MPECs) were isolated using a method previously described[34]. The purity of MPECs was evaluated by DiI-acLDL uptake and Bandeiraea Simplicifolia lectin-FITC (BS-I) staining. Efficient isolation yields approximately $4x10^4$ MPECs per mouse with over 80% purity.

RNA sequencing

mRNA from MPECs was isolated using a miRNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA). Frozen RNA samples (n_{mice} = 6 per group) were sent to QIAGEN for RNA sequencing. At the company, RNA samples were quantified, and sequencing libraries were generated using Illumina TruSeq stranded total RNA library preparation kit with rRNA depletion (Illumina, San Diego, CA, USA). The libraries' size distribution was validated, and the quality was inspected on a Bioanalyzer 2100 or BioAnalyzer 4200 TapeStation (Agilent Technologies, Santa Clara, CA, USA). Paired end-75bp read sequencing was conducted using an Illumina NextSeq500 (Illumina Inc.). The data analysis was performed using the Tuxedo software package by QIAGEN. The components of QIAGEN NGS data analysis pipeline for RNA-seq include Bowtie2 v.2.2.2, Tophat v2.0.11, and Cufflinks v2.2.1.

Western blot analysis

Protein levels were analyzed using SDS-PAGE separation and electrophoretic transfer to nitrocellulose membranes. Primary antibodies used in this study are listed in **Table S1**.

GJ activity measurement

Human pulmonary ECs were plated in a 12-well plate. Next day, the plate was placed in a hypoxic incubator (3% O_2) or regular normoxic incubator (21% O_2) and cultured for 48 hours. Dye transfer assay was performed as described in our previous manuscript[35]. Briefly, Lucifer yellow (0.5 mg/ml) was added to each well at the end of 48 hours incubation, and the confluent cells were scratched by a scalpel in the middle of the well to allow dye to get into cells at the edge of the scrape. The dye solution was left for 20 min in the well, and then cells were washed and fixed. Images of dye transfer were captured using an EVOS FL Auto Imaging System (Thermo-Fisher Scientific, Waltham, MA, USA). The distance of dye transfer from the scraping site to the farthest site of cells showing visual uptake of dye was measured using Image Pro-Plus 7.0 software (Media Cybernetics, Inc. Rockville, MD, USA). The data were normalized to the averaged distance of dye transfer in normoxia-exposed ECs.

Systemic arterial pressure and heart rate measurement

Mice were anesthetized with isoflurane (1%), and the catheter was inserted into the right carotid artery. Systemic arterial pressure and heart rate were measured using an MPVS Ultra system and data were collected using PowerLab data acquisition system

(Sydney, Australia).

Quantification of muscularization in pulmonary vessels

Lungs were inflated, perfused with OTC compound, and frozen. Lung sections (6 μm in thickness) were stained with antibodies against von Willebrand factor (vWF, an EC marker, conjugated with Alexa488) and α-smooth muscle cell actin antigen (αSMA, a SMC marker, conjugated with Cy3). The lung image was taken with a Nikon Eclipse Ti-E 3D Deconvolution microscope (Nikon Corp. Tokyo, Japan). Vessels sized between 20 μm and 70 μm in the entire lung were scored based on αSMA-staining: highly-muscularized = >50% of αSMA-staining, partially-muscularized = 1% < αSMA-staining < 50%, non-muscularized = 0% of αSMA-staining.

Lung angiograph

Pulmonary angiography was conducted as previously described[34]. The lungs were perfused with 0.5 mL/min for 2 min through the pulmonary artery (PA) and fixed at 4°C overnight. Then the lungs filled with Microfil were bathed in serial concentrations of ethanol, placed in methyl salicylate, and photographed with a digital camera (MD600E, Amscope, Irvine, CA, USA). We made binary images using NIH ImageJ 1.47v software on the peripheral pulmonary vasculature (the areas between the edge of the lung and 1 mm inside from the edge of the lung) using Photoshop CS software (Adobe Inc. San Jose, CA, USA); the images were then skeletonized for analyzing the number of vessel branches, the number of junctions and the total length. These data were normalized by the area selected in each lung image.

Statistics

We conducted data analysis in a blinded fashion wherever possible and set proper controls for every experimental plan. The mouse numbers and independent experiment numbers are described in the figure legends. Statistical analysis was performed using GraphPad Prism 7.04 (La Jolla, CA, USA). Data are presented as mean ± SEM. After the data passed a normality test, the two-tailed Student's *t*-test was used for comparisons of two groups, and one-way ANOVA was used for multiple comparisons. If the data did not pass the normality test, a non-parametric test (Mann-Whitney for two groups, Kruskal-Wallis for multiple comparisons) was used. Statistical comparison between dose-response curves was made by two-way ANOVA with Bonferroni post hoc test. Differences were considered to be statistically significant when $P<0.05$. In RNAseq data, *q*-values are obtained from adjusted *p*-values using the Benjamini-Hochberg False Discovery Rate approach to correct for multiple testing. The fold changes with *q*-values below 0.05 are considered significant.

Table S1. Major Resources.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mouse	Jackson Laboratory, (ME, USA)	C57BL/6J, Stock #: 000664	M	jax.org/jax-mice-and-services/find-and-order-jax-mice/most-popular-jax-mice-strains/aged-b6

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Cx40$^{-/-}$ (Parent)	Mouse	Jackson Laboratory, (ME, USA)	C57BL/6	Cx40 knockout mice Stock #: 025697	https://www.jax.org/strain/025697
Cx40$^{Tg/+}$ (Parent)	Mouse	University of Sydney, (NSW, Australia)	C57BL/6	Tie2-Cx40 (wild type) overexpressing mice (hetero)	https://www.ncbi.nlm.nih.gov/pubmed/23471232
Cx40-NM	Mouse	University of Sydney, (NSW, Australia)	C57BL/6	Tie2-Cx40 negative mutant overexpressing mice (homo)	https://www.ncbi.nlm.nih.gov/pubmed/25547341

Antibodies

Target antigen	Vendor or Source	Catalog #	Dilution Rate	Persistent ID / URL
Cx37	Thermo-Fisher Scientific (MA, USA)	424400	1:500	https://www.thermofisher.com/antibody/product/Connexin-37-Antibody-Polyclonal/42-4400
Cx40	Santa Cruz Biotechnology Inc. (TX, USA)	sc-20466	1:2000	https://www.scbt.com/p/connexin-40-antibody-c-20?requestFrom=search
Cx43	Thermo-Fisher Scientific (MA, USA)	710700	1:1000	https://www.thermofisher.com/antibody/product/Connexin-43-Antibody-Polyclonal/71-0700
IK	Santa Cruz Biotechnology Inc. (TX, USA)	sc-365265	1:1000	https://datasheets.scbt.com/sc-365265.pdf
Actin	Santa Cruz Biotechnology Inc. (TX, USA)	sc-1616	1:4000	https://www.scbt.com/p/actin-antibody-i-19?requestFrom=search
CD31	BD Biosciences (CA, USA)	553370	1:500	https://www.bdbiosciences.com/us/solrSearch?text=553370
vWF	Santa Cruz Biotechnology Inc. (TX, USA)	sc365721-488	1:500	https://www.scbt.com/p/vwf-antibody-c-12?requestFrom=search
αSMA	Sigma Aldrich, (MO, USA)	C6198	1:1000	https://www.sigmaaldrich.com/catalog/product/sigma/c6198?lang=en&region=US

Cultured Cells

Name	Vendor or Source	Catalog #	Lot#	Sex (F, M, or unknown)
HPAEC	Cell Applications Inc. (CA, USA)	302-05a	2439	F

Chemicals

Description	Source	Catalog #
Medium 199	Thermo-Fisher Scientific, (MA, USA)	MT10060-CV
Streptomycin/penicillin	Thermo-Fisher Scientific, (MA, USA)	MT-30-002-CI
Trypsin/EDTA	Thermo-Fisher Scientific, (MA, USA)	MT25052-CI
Endothelial Cell Growth Supplement	Thermo-Fisher Scientific, (MA, USA)	356006
Fetal Bovine Serum	Thermo-Fisher Scientific, (MA, USA)	MT35010CV
Iron-Supplemented Calf Serum	Thermo-Fisher Scientific, (MA, USA)	SH30072.04
Matrigel	Thermo-Fisher Scientific, (MA, USA)	356237
Dynabeads ® Sheep Anti-Rat IgG	Thermo-Fisher Scientific, (MA, USA)	11035
Collagenase I	Worthington Biochemical Corp. (NJ, USA)	LS004196
Dispase II	Worthington Biochemical Corp. (NJ, USA)	LS02109
Sodium pentobarbital	Henry Schein(NY, USA)	VINV-CIII-0001
Dil-acLDL	Thermo-Fisher Scientific, (MA, USA)	L3484
Lectin-FITC	Sigma Aldrich (MO, USA)	L9381
miRNeasy Mini Kit	Qiagen (CA, USA)	217004
RT^2 First Strand Kit	Qiagen (CA, USA)	330404
$PGF_{2\alpha}$	Sigma Aldrich (MO, USA)	P0424-5 mg
Acetylcholine	Sigma Aldrich, (MO, USA)	A9101
Sodium Nitroprusside	Sigma Aldrich, (MO, USA)	71778
L-NAME	Cayman Chemical (MI, USA)	80210
indomethacin	Sigma Aldrich, (MO, USA)	I7378
Lucifer Yellow	Thermo-Fisher Scientific, (MA, USA)	L453
Microfil	Flow Tech Inc. (MA, USA)	MV-122
Isoflurane	Henry Schein(NY, USA)	1169567762
Other general chemicals	Sigma Aldrich, (MO, USA)	

Table S2. Primers used for genotyping and copy number assessment.

Strain	Gene	Forward	Reverse
Genotype			
$Cx40^{-/-}$	Wt	TGGAGCCACAGTTGCAATGGT	TCTCTGACTCCGAAAGGCAAG
	$Cx40^{-/-}$		GCACGAGACTAGTGAGACGTG
$Cx40^{TG}$	$Cx40^{TG}$-IRES	CCAGGGCACCCTACTCAACA	AGGGGCGGATCTCGAATCAA
$Cx40^{NM}$	$Cx40^{NM}$-IRES	CCAGGGCACCCTACTCAACG	AGGGGCGGATCTCGAATCAA
Copy number			
Cx40	Cx40	CCACAGTCATCGGCAAGGTC	CTGAATGGTATCGCACCGGAA
GAPDH	GAPDH	TGACCTCAACTACATGGTCTACA	CTTCCCATTCTCGGCCTTG

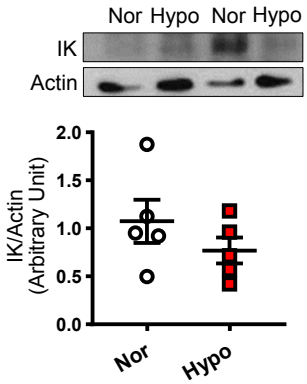

Figure S1. IK channel protein levels in mouse pulmonary endothelial cells (MPECs) isolated from normoxic control mice (Nor, n_{mice}=5) and hypoxia-exposed mice (Hypo, n_{mice}=5) determined by Western Blot. Data are mean ± SEM. Unpaired Student's *t*-test (2-tailed) was used for comparisons of two experimental groups and there is no significant difference between two groups.

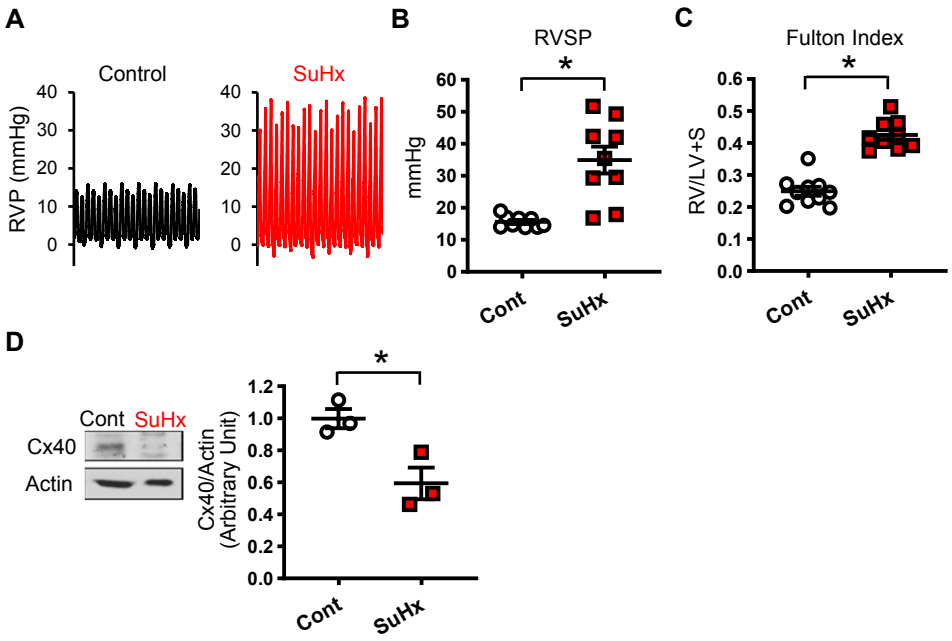

Figure S2. Characterization of mice with Sugen-hypoxia (SuHx)-induced pulmonary hypertension (PH). **A**: Typical record of right ventricular pressure (RVP). **B**: Dot plot shows right ventricular systolic pressure (RVSP) in mice exposed to 4wks-Hypoxia and Sugen5416 (SuHx, n_{mice}=9) or control mice for SuHx (Cont, n_{mice}=9). **C**: Fulton Index (RV/LV+S). Cont, n_{mice}=10; SuHx, n_{mice}=9. **D**. Cx40 protein levels in mouse pulmonary endothelial cells (MPECs) determined by Western Blot. N_{mice}=3 per group. Data are mean ± SEM. **P*<0.05 vs. Cont. Unpaired Student's *t*-test (2-tailed) was used for comparisons of two experimental groups.

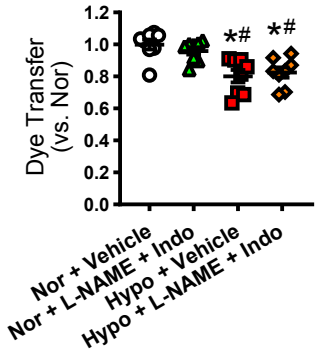

Figure S3. Gap junction (GJ) activity in human pulmonary ECs assessed by Lucifer Yellow dye transfer experiment. The dot plot shows summarized data of dye transfer (the distance compared to control) in normoxia-exposed vehicle-treated ECs (Nor + Vehicle), normoxia-exposed L-NAME (an eNOS inhibitor, 10μM) and indomethacin (a cyclooxygenase inhibitor, 100μM)-treated ECs (Nor + L-NAME + Indo), hypoxia-exposed vehicle-treated ECs (Hypo + Vehicle), hypoxia-exposed L-NAME and indomethacin-treated ECs (Hypo + L-NAME + Indo). Inhibitors were added to the cells for 20 min before dye transfer experiment. Experimental number is $n_{experiments}$=8 per group. Data are mean ± SEM. *P<0.05 vs. Nor + Vehicle. $^{\#}P$<0.05 vs. Nor + L-NAME + Indo. Statistical comparison between four groups was made by one-way ANOVA with Bonferroni *post hoc* test.

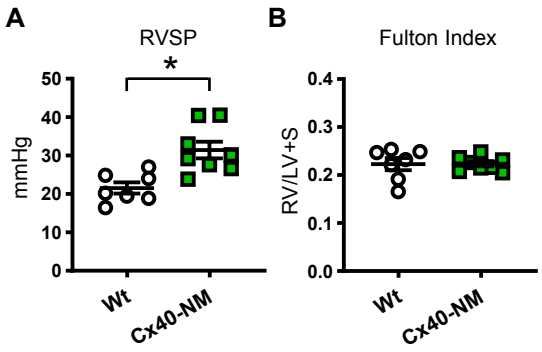

Figure S4. Cx40 negative mutant (NM) knock-in mice develops PH. **A**: RVSP. Wild type (Wt), n_{mice}=7; Cx40-NM, n_{mice}=8. **B**: Fulton Index (RV/LV+S). Wt, n_{mice}=7; Cx40-NM, n_{mice}=8. Data are mean ± SEM. **P*<0.05 vs. Wt. Unpaired Student's *t*-test (2-tailed) was used for comparisons of two experimental groups.

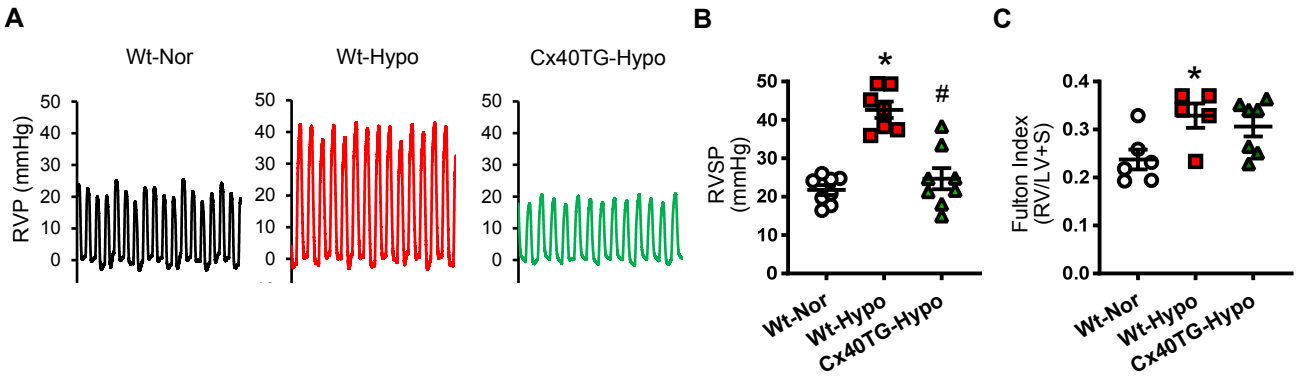

Figure S5. Typical record of RVP in normoxia-exposed Wt mice, hypoxia-exposed Wt mice, and hypoxia-exposed Cx40-overexpressing transgenic (Cx40TG) mice. **B**: RVSP. Wt-Nor, n_{mice}=8; Wt-Hypo, n_{mice}=7; Cx40TG-hypo, n_{mice}=8. **C**: Fulton Index. Wt-Nor, n_{mice}=6; Wt-Hypo, n_{mice}=5; Cx40TG-hypo, n_{mice}=7. Data are mean ± SEM. $^{*}P<0.05$ vs. Wt-Nor. $^{\#}P<0.05$ vs. Wt-Hypo. Statistical comparison between three groups was made by one-way ANOVA with Bonferroni *post hoc* test.

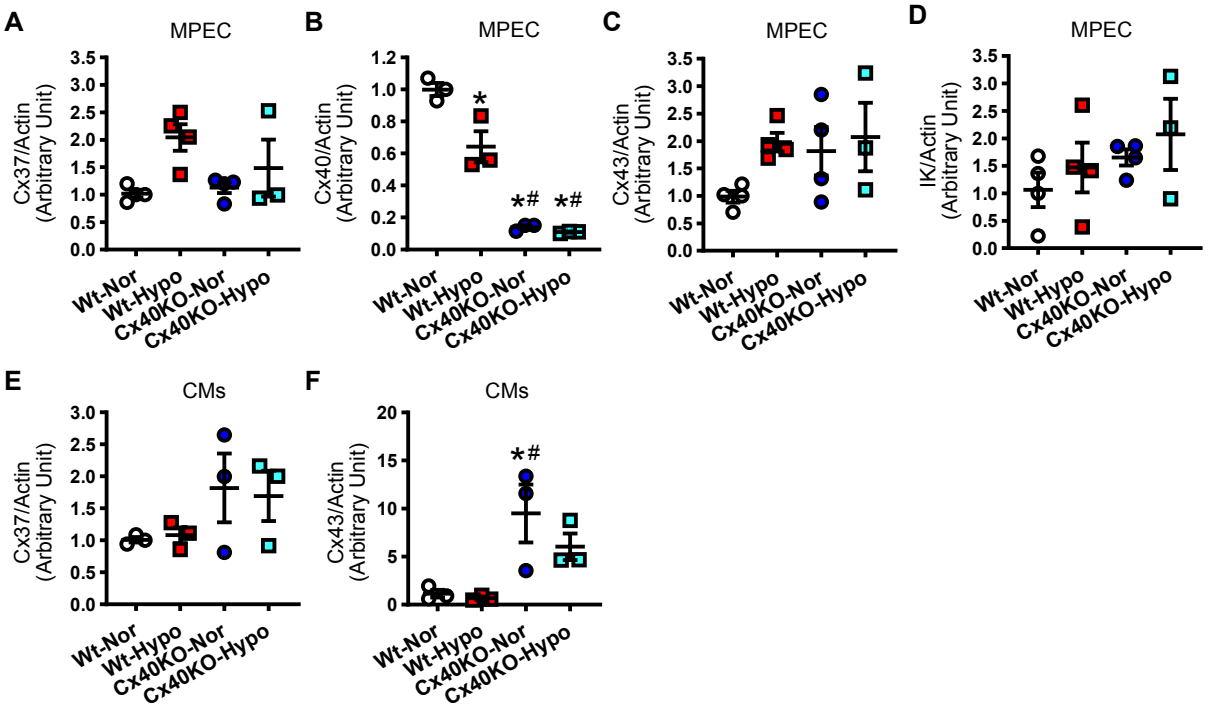

Figure S6. Protein levels of Cxs in MPECs (**A-B**) and cardiac myocytes (CMs, **E-F**) isolated from Wt and Cx40KO mice with or without 4wks-hypoxic exposure. **A:** Cx37 in MPECs. **B:** Cx40 in MPECs. **C:** Cx43 in MPECs. **D:** IK levels in MPECs. **E:** Cx37 in CMs. **F:** Cx43 in CMs. CMs were collected from digested heart materials after coronary endothelial cells were removed. N_{mice}=3-4 per group. Data are mean ± SEM. **P*<0.05 vs. Wt-Nor. #*P*<0.05 vs. Wt-Hypo. Statistical comparison between four groups was made by one-way ANOVA with Bonferroni *post hoc* test.