Supporting Information (SI Appendix)

HIF-1α is required for development of the sympathetic nervous system

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SI Material and Methods

Animals. Animal experiments were conducted according to protocols approved by the Animal Care and Use Committee of the Institute of Molecular Genetics, Czech Academy of Sciences. All experiments were performed with littermates (males and females) cross-bred from two transgenic mouse lines: floxed *Hif1a*^{tm3Rsjo} with exon 2 of *Hif1a* gene flanked by loxP sites (*Hif1a*$^{loxP/loxP}$ maintained on B6/129 background; stock #007561 Jackson Laboratory) (1), and *Islet1-Cre* (Isl1$^{tm1(cre)Sev}$/$^{+}$; obtained from Dr. S. Evans; FVB background) (2). *Isl1-Cre* mice do not have any detectable phenotype (2-7). Breeding scheme: Female mice *Hif1a*$^{loxP/loxP}$ were crossed with *Hif1a*$^{loxP/+}$; *Isl1-Cre* males, in which, *Isl1-Cre* knock-in allele was inherited paternally to minimize the potential influence of maternal genotype and avoid maternal HIF-1α insufficiency effects on the developing embryos. *Hif1a*$^{loxP/+}$ or *Hif1a*$^{loxP/loxP}$ mice were used as the controls. The mouse line *Isl1-Cre* was bred with Cre-reporter line (allele: *Gt(ROSA)26Sor*^{tm1Sor}*;* background C57BL/6J, stock #3474, The Jackson Laboratory) to analyze Cre-recombination pattern. Genotyping was performed by PCR on tail DNA. The specific primers used were the following: *Isl1Cre* forward 5'-GCC TGC ATT ACC GGT CGA TGC AAC GA-3' and *Isl1Cre* reverse 5'-GTG GCA GAT GGC GCG GCA ACA CCA TT-3' with a 600-bp product; *Hif1a* forward 5'-TGC ATG TGT ATG GGT GTT TTG-3' and *Hif1a* reverse 5'-GAA AAC TGT CTG TAA CTT CAT TTC C-3' with a 99-bp product for the wild type allele and 120-bp product for the floxed allele. Mice were kept under standard experimental conditions with a constant temperature (23–24 °C) and fed on soy-free feed (LASvendi). The females were housed individually during the gestation period and the litter size was recorded.

Reverse Transcription-quantitative Real-time PCR. RT-qPCR was performed as described previously (8) using primers from PrimerBank (http://pga.mgh.harvard.edu/primerbank/) (9). The nucleotide sequences of the primers that were used are as follows: *Th* forward, 5'-GTC TCA GAG CAG GAT ACC AAG C-3' and *Th* reverse, 5'-CTC TCC TCG AAT ACC ACA GCC-3' with a 179-bp product; *Ngf* forward, 5'-CCA GTG AAA TTA GGC TCC CTG-3' and *Ngf* reverse 5'-CCT TGG CAA AAC CTT TAT TGG G-3' with a 142-bp product.

Coronary Artery Visualization by Micro-CT Imaging. The mice were injected intraperitoneally with 100 i.u. of heparin and 15 min later were killed by cervical dislocation. The hearts were quickly isolated and cannulated via the ascending aorta. Blood was flushed out using warm Tyrode's buffer followed by ice-cold cardioplegia solution with 5 mM adenosine to induce dilation of coronary arteries followed by injection of 2 ml of yellow Microfil (Flow Tech) as described (10). The hearts were kept overnight at -20^{o}C to allow polymerization and then fixed with 4% paraformaldehyde in PBS for 48 h, rinsed with PBS, and processed by micro-CT scanning (SkyScan 1272, Bruker) with following parameters: pixel size, 7 μm; Al 0.25 mm filter, frame averaging 3; acquisition time, approx. 1.5 h. Projection images were reconstructed using NRecon SW (Bruker). 3D visualizations were achieved via CTVox SW (Bruker). In some specimens CTAnalyser SW (Bruker) was applied for removal of excesses of extravascular contrast which limited image clarity. Branching of right and left coronary arteries and septal artery (SA) was evaluated using 3D visualizations. Afterwards, distances between branches of left coronary artery were measured in 3D.

Cranial Morphometry. Since the body weight of the mutant was affected by hindlimb hypoplasia, the weight of the hearts was normalized to the length of the cranial base (LCB) measured by micro-CT for each individual mouse used for analysis. Whole mouse cadavers were scanned on a MikroPET/CT scanner Albira (Bruker) with the following settings: voltage 45 kV, current 400 μA, 600 projections/frame, 125 micrometers/voxel. Micro-CT images were used for the measurement of LCB skull dimension (distance between the anterior point of premaxilla and occipital condyles). Skulls were reoriented in 3D using DataViewer SW (Bruker) to reach the same position for all the specimens. Distances were measured in the sagittal plane (LCB) of 3D visualization.

Immunohistochemistry and Morphological Evaluation. For vibratome sections, dissected tissues were fixed in 4% paraformaldehyde, embedded in 4% agarose gel and sectioned at 80 μm on a VT1000S vibratome (Leica). The primary antibodies used were: anti-CX43 at 1:2000 dilution (C6219, Sigma-Aldrich), anti-tyrosine hydroxylase at 1:750 dilution (AB152, Merck Millipore), anti-TUJ1 at 1:400 dilution (T6793, Sigma-Aldrich), anti-HCN4 at 1:500 dilution (APC-052, Alomone Labs), anti-HIF1α at 1:100 dilution (NB100-105, NB100-479, Novus Biologicals), anti-NeuN at 1:500 dilution (ab177487, Abcam), anti-Ki67 at 1:400 dilution (9129, Cell Signaling), anti-Caspase3 at 1:100 dilution (9661, Cell Signaling), anti-ISL1 at 1:200 dilution (39,4D5, NICHD Developmental Hybridoma Bank, University of Iowa), and anti-chromograninA at 1:100 dilution (ab15160, Abcam). The nuclei were counterstained with Hoechst 33342. Image acquisition was completed using the LSM 880 NLO scanning confocal and Axio ZoomV16 microscopes, with ZEN lite program (Zeiss). Whole-mount TH immunostaining of cardiac innervation was visualized using plugin 3D viewer ImageJ (NIH) and quantified using the thresholding tool in ImageJ. The quantification of TH^+ labeled fibers was done only in the right and left ventricles of the heart. $Cx43^+$, TH^+, $ISL1^+$, and $Caspase3^+$ areas were assessed using the thresholding tool in ImageJ. $Cx43^+$ areas were determined in the compact myocardium of the LV in transverse medial sections of control and *Hif1aCKO* hearts, and expressed as a percentage of total cross-sectional area of the compact myocardium of the LV (4-6 fields per section for 3 embryos per genotype). TH^+ areas were expressed as a percentage of the $ISL1^+$ area in the motor neuron columns of 3 sections from each of 3 embryos per genotype. The average percentage of $Caspase3^+$ area per $ISL1^+$ area was determined in the ventral neural tube of five embryos per genotype. Ki-67^+ cells per $ISL1^+$ cells were calculated from sections of two ganglia from each of five different embryos per genotype using the Cell Counter plug-in of ImageJ. The area of the secondary sympathetic ganglia was determined using ImageJ. The average neuronal density was assessed by counting $NeuN^+$ nuclei using the Cell Counter plug-in of ImageJ per volume of the stellate ganglion (two stellate ganglia from each of five embryos per genotype). For the evaluation of immunolabeling of adrenal chromaffin cells, TH^+ and chromogranin A^+ areas were quantified using ImageJ and expressed as a percentage of the total adrenal gland area.

X-gal Staining. The mouse line *Isl1-Cre* was bred with reporter R26R-*lacZ* (*Gt(ROSA)26Sor^{tm1Sor}*, The Jackson Laboratory) and animals carrying both loci were subjected to X-gal staining as described (3). Whole mounts were observed under a MZFLIII stereomicroscope (Leica).

SI References

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SI FIGURES

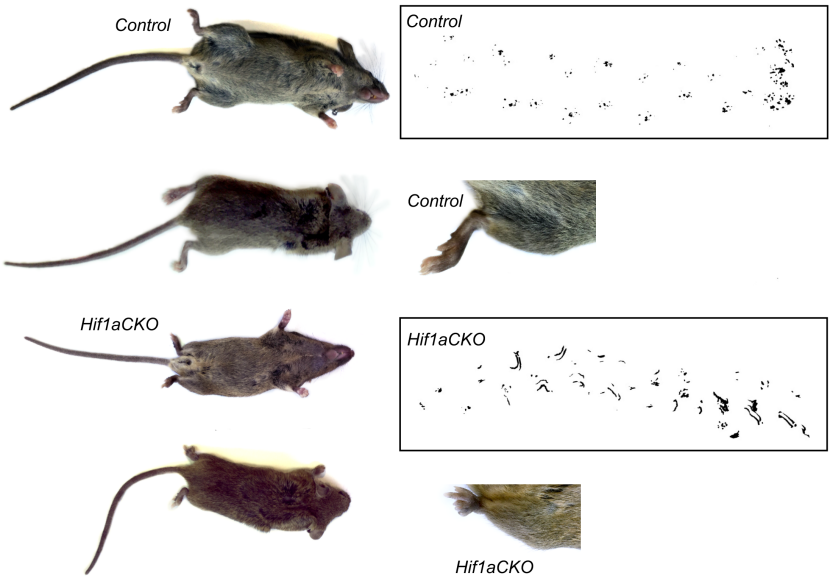

Fig. S1. Abnormal development of hind-limbs in *Hif1CKO* mice. *Hif1aCKO* have shorten hind-limbs with abnormal morphology of toes. Representative footprints show gait abnormalities in *Hif1aCKO* compared to control mice.

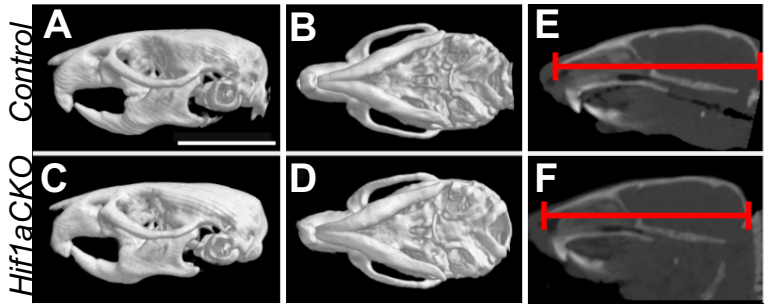

Fig. S2. Micro-CT visualization of skulls (*A*-D) and length of the cranial base (LCB) from virtual 2D sagittal sections (red line in *E* and *F*). Scale bar, 10 mm.

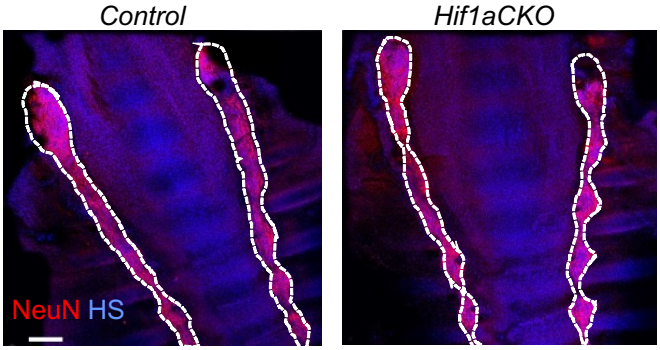

Fig. S3. Whole-mount NeuN immunostaining of the spinal cord to visualize the sympathetic ganglia at E14.5. HS, Hoechst stain. Scale bar, 200 μm.

SUPPLEMENTAL TABLES AND SUPPORTING INFORMATION

Table S1: Number of collected embryos and litters at different embryonic ages

Embryonic day	Embryos (N)/litters	*Control**	*Hif1aCKO heterozygous**	*Hif1aCKO**	absorbed
E7.5	24/3	14	6	4	0
E8.5	51/6	30	12	9	3
E9.5	81/10	44	18	19	6
E10.5	180/21	88	46	46	2
E11.5	96/11	45	22	29	7
E12.5	78/9	40	20	18	2
E13.5	41/5	24	4	13	6
E14.5	184/22	109	39	36	22
E15.5	91/11	44	23	24	1
E16.5	52/7	27	13	12	1
E17.5	24/3	15	3	6	0
E18.5	14/2	5	3	6	0

**Control* genotype: $Hif1a^{loxP/+}$ or $Hif1a^{loxP/loxP}$

**Hif1aCKO* heterozygous mice genotype: *Isl1-Cre;*$Hif1a^{loxP/+}$

**Hif1aCKO* genotype: *Isl1-Cre;*$Hif1a^{loxP/loxP}$

Table S2: Body and heart weight

Group	Body weight [g]	Heart weight [g]	LV weight [g]	RV weight [g]	LV/ heart [g/g]	LCB [mm]	Heart/LCB [g/mm]
Control	27.13	0.164	0.0609	0.0242	0.4035	23.12	0.0071
SEM (N)	0.75 (40)	0.0052 (32)	0.0029 (23)	0.0015 (23)	0.0110 (15)	0.16 (15)	0.0002 (15)
Hif1aCKO	20.16*	0.141*	0.055*	0.0199*	0.4096	22.84	0.0063*
SEM (N)	0.59 (37)	0.0059 (29)	0.0024 (21)	0.0009 (21)	0.0172 (13)	0.12 (13)	0.0003 (13)

*P < 0.05 *t*-test; LV, left ventricle; RV, right ventricle; LCB, cranial base length

Table S3: Basal left ventricular echocardiographic parameters

parameters	*Control*	*Hif1aCKO*
LVDd (mm)	3.92 ± 0.03	3.43 ± 0.06*
AVTd (mm)	0.83 ± 0.02	0.76 ± 0.02*
PVTd (mm)	0.83 ± 0.02	0.77 ± 0.02*
LVDs (mm)	2.47 ± 0.05	2.29 ± 0.04*
AVTs (mm)	1.21 ± 0.02	1.05 ± 0.02*
PVTs (mm)	1.21 ± 0.02	1.05 ± 0.02*
FS (%)	37.0 ± 0.94	33.3 ± 0.42*
HR (beats/min)	524 ± 7	487 ± 16

*P < 0.05, *t*-test; Values are mean ± SEM (*Control* n = 10, *Hif1CKO* n = 12)

Abbreviations: LVDd, left ventricle diastolic cavity diameter; AWTd, diastolic anterior wall thickness; PWTd, diastolic posterior wall thickness; LVDs, left ventricle systolic cavity diameter; AWTs systolic anterior wall thickness; PWTs, systolic posterior wall thickness; FS%, fractional shortening; HR, heart rate.

Table S4: Distance between branching points of left coronary artery

Control	*Hif1aCKO*
0.99	0.79
0.74	0.63
0.83	0.86
0.88	0.83
0.72	1.05
0.74	0.81
0.81	0.85
1.07	1.1
0.83	1.45
0.94	0.86
0.88	1.15
0.65	
0.76	

Mean ± SD *Control*: 0.83 ± 01, *Hif1aCKO*: 0.94 ± 0.2; *t*-test, p = 0.14