Supplemental Material

Methods

Animals. All animal experiments were approved by the Institutional Animal Investigation Care and Use Committee or governmental committee. *Cox4i2*$^{-/-}$ mice were generated as described[1]. Only littermates (WT or *Cox4i2*$^{-/-}$) were used for the experiments. Mice of either sex were studied at 2–3 months of age.

Hemodynamic measurements in isolated, perfused, and ventilated mouse lungs

Lungs of either WT or *Cox4i2*$^{-/-}$ mice were isolated, perfused and ventilated as described previously[2]. Briefly, mice were deeply anesthetized intraperitoneally with xylazine (20 mg/kg body weight, Bayer Healthcare, Leverkusen, Germany) and ketamine (100 mg/kg body weight, Pfizer, Karlsruhe, Germany), and anticoagulated with heparin (50,000 I.E./kg body weight). After reaching deep anesthesia mice were intubated via a tracheostoma and ventilated with room air with a tidal volume of 300 µl, at a frequency of 90 breaths/min and a positive end-expiratory pressure of 3 cm H_2O using a piston pump (Minivent Type 845, Hugo Sachs Elektronik, March-Hugstetten, Germany). Subsequently, midsternal thoracotomy was performed and catheters were inserted into the pulmonary artery and left atrium. Perfusion with sterile Krebs–Henseleit buffer (Serag-Wiessner, Naila, Germany) was initiated through the catheter in the pulmonary artery using a peristaltic pump (ISM834A V2.10, Ismatec, Glattbrugg, Switzerland), with a buffer temperature of 4°C and a flow of 0.2 ml/min. In parallel with the onset of artificial perfusion, ventilation was changed from room air to a pre-mixed gas (21% O_2 5.3% CO_2, balanced with N_2). The lungs were removed from the thorax while continuously ventilated and perfused and were freely suspended from a force transducer for the monitoring of organ weight in a temperature-equilibrated, humidified chamber. After rinsing the lungs, the perfusion circuit was closed for recirculation, left atrial pressure was set at 2.0 mm Hg, the flow was slowly increased to 2 ml/min and the entire system heated to 37°C. Pressures in the pulmonary artery and the left atrium were registered via small diameter catheters with pressure transducers.

For determination of acute HPV and the vasoconstrictive response to KCl the following protocol was applied. After an initial steady-state period, lungs were ventilated three times for 10 min with a hypoxic gas mixture (1% O_2, 5.3% CO_2, balanced with N_2), each time followed by a 15-min period of normoxic ventilation (21% O_2, 5.3% CO_2, balanced with N_2). Afterwards, a bolus of KCl was infused into the pulmonary artery three times each for 25 min during normoxic ventilation. The strength of the vasoconstrictor response to hypoxia or KCl was quantified from the second hypoxic or KCl challenge, respectively. For determination of the prolonged vasoconstrictor response to alveolar hypoxia, lungs were ventilated according to the following protocol. After the initial steady-state period, a short-term hypoxic ventilation was performed (10 min, 1% O_2), followed by a normoxic ventilation period of 15 min and subsequent hypoxic ventilation for 180 min (1% O_2).
In order to determine the effect of MitoTempo, S3QEL2 and SkQ1 on HPV, or the effect of MitoTempo and S3QEL2 on hypoxia-independent vasoresponsiveness of the pulmonary vasculature, six repetitive hypoxic or KClchallenges in absence or presence of increasing doses of MitoTempo, S3QEL2 or SkQ1, respectively, were performed. The effect on U46619 induced vasoconstriction was tested either with repetitive challenges in presence or absence of increasing doses of MitoTempo or by application of U46619 after the last repetitive hypoxic challenge in presence of the highest dose of S3QEL2 or SkQ1.

Arterial pO_2 measurement and airway fluid challenge

Anesthetized (1.5-2.5% isoflurane) and heparinized mice were artificially ventilated (MiniVent Type 845, Hugo Sachs Elektronik, March-Hugstetten/Germany) with a isofluran/oxygen mixture (tidal volume 250 ml, 60 breath per min, 21% oxygen). Blood gases drawn from the carotid artery were measured by an ABL 500 (Radiometer, Copenhagen, Denmark) directly before and 2 and 10 min after tracheal instillation of 25 µl of saline (0.9% NaCl), as described previously[2].

Mouse PASMC isolation

Mouse PASMCs were isolated from precapillary pulmonary arterial vessels as described previously[2]. Mice were anesthetized with xylazine (20 mg/kg body weight, Bayer Healthcare, Leverkusen, Germany) and ketamine (100 mg/kg body weight, Pfizer, Karlsruhe, Germany), anticoagulated with heparin

(50,000 I.E./kg body weight) and killed by exsanguination. A catheter was placed in the pulmonary artery and the pulmonary vasculature was rinsed with cold PBS following infusion of growth medium 199 (M199 containing Earl´s salts and 100 mg/l L-glutamine; Gibco, Invitrogen, De Schelp, Netherlands) containing 1% penicillin/streptomycin (10000 U/ml penicillin, 10 mg/ml streptomycin, PAN Biotech GmbH Aidenbach, Germany), 0.5% low-melting-point agarose (type VII, Sigma-Aldrich, Munich, Germany) and 0.5% Fe_3O_4 particles (Sigma-Aldrich, Munich, Germany). The lung was removed and placed in cold PBS until the agarose solidified. Afterwards the lung was mechanically chopped with scissors, lung fragments were suspended and washed twice with PBS by using a magnet to retain the iron-containing fragments. The lung pieces were incubating in M199 medium containing collagenase (80 U/ml) at 37°C for 1 hour. Finally, the digested lung pieces were disrupted by drawing them through 15 and 18 gauge needles 5-6 times each and collagenase digestion was stopped by washing 3 times with M199 medium containing 1% penicillin/streptomycin and 10% fetal calf serum (FCS, PromoCell, Heidelberg, Germany) by use of a magnetic holder. The tissue was resuspended in smooth muscle cell growth medium 2 containing supplement-mix (PromoCell, Heidelberg, Germany), normocin (1%, Invitrogen, De Schelp, Netherlands) and 20% FCS and transferred to cell culture dishes. PASMCs were incubated at 37°C in an atmosphere containing 21% O_2, 5% CO_2, balanced with N_2 for 5 to 10 days without passaging (passage 0). During this time the precapillary PASMCs migrate and adhere to the dish. Microvascular PASMCs in passage 0 (4 days) or 2 were used for experiments. We obtained 91.7 ± 1.7% of cells that were positive for α-smooth-muscle actin in each cell isolation.

Calcium measurement

The fluorescent dye Fura 2-AM (Sigma-Aldrich, Munich, Germany) was used for detection of changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in PASMCs (passage 0) isolated from WT and *Cox4i2*$^{-/-}$ mice. Cells were cultured on 60 µm glass bottom dishes (Ibidi, Martinsried, Germany) for 4 days under normoxic conditions (21% O_2, 5% CO_2). Cells were loaded with 5 µM Fura 2-AM in HEPES buffer solution (5.6 mM KCl, 136.4 mM NaCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 11 mM glucose, 10 mM HEPES; pH 7.4) with 0.1% BSA at 37°C for 30 min. After washing twice with HEPES buffer, dishes were mounted on the stage of an Olympus IX71 inverted fluorescence microscope (Hamburg, Germany) coupled to a Polychrome V monochromator and a sensicam camera (PCO, Kelkheim, Germany). Fura 2-AM was excited with 340 and 380 nm wavelengths at 37°C. In all experiments, $[Ca^{2+}]_i$ of multiple cells in a single field was measured every second. The recording protocol consisted of 3 min measurement with normal HEPES buffer (to record the baseline Ca^{2+} concentration), application of 2 ml normoxic (room air) or hypoxic (1% O_2, balanced with N_2) HEPES buffer at minute 2. The experiment was stopped after 10 minutes. Results are expressed as F_{340}/F_{380} ratios, (including background fluorescence correction), and normalized to initial F_{340}/F_{380} ratios measured in the initial minute of the experiment. The acquired images were processed with TillVision software (Till Photonics, Gräfelfing, Germany).

Site-directed mutagenesis

Cox4i2 mutant plasmids were generated using overlap extension polymerase chain reaction (PCR). Briefly, wild-type *Cox4i2* cDNA present in the pCINeo mammalian expression vector was amplified using RedTaq polymerase (Sigma) and primers containing select point mutations to alter codon sequences for cysteine to alanine or serine. Forward primers were C41A, 5'-TACGTTGACGCCTACGCCCAGCGCTCC-3'; C41S, 5'-TACGTTGACAGCTACGCCCAGCGCTCC-3'; C55A, 5'-ATGCCGGATGAGCCCTTCGCCACAGAGCTCA-3'; C55S, 5'-ATGCCGGATGAGCCCTTCTCCACAGAGCTCA-3'; C109A, 5'-GAATGGAAGACAGTGATGGGCGCCGTCTTCTTCTTCATT-3'; C109S, 5'-GAATGGAAGACAGTGATGGGCTCCGTCTTCTTCTTCATT-3' (mutated bases are underlined) to amplify the 3'-part of the cDNA using the counter-primer 5'-CCGGGTCGACTCTAGATCACTTTTTCCA-3', containing a SalI restriction site for cloning (underlined). Another set of reverser primers was generated containing the reverse-complement sequences of the above site-directed mutagenesis primers to amplify the 5'-part of the cDNA using the counter primer 5'-ACTGAATACGCTAGCCTCGAGATGTTTTCCAGAG-3' containing a XhoI restriction site. The resulting 100-300bp fragments were agarose gel purified using the Wizard SV Gel

Purificaton kit (Promega) and subjected to overlap extension PCR using the restriction site-containing primers. Fragments were digested with the corresponding restriction enzymes (New England Biolabs) and cloned into the pCINeo vector using T4 ligase (Roche) and transformed into competent DH5α *E. coli* cells following plasmid isolation with the QIAPrep spin kit (Qiagen). Isolated plasmids were sequence confirmed.

Lentivirus production and transduction of primary PASMCs
Full length Cox4i1, or wild type or mutated Cox4i2 were subcloned into the pWPXL plasmid (Addgene, Boston, USA) and packed with a second-generation lentivirus transduction system with pMD2.G as the envelope and psPAX2 as a packing vector (Addgene, Boston, USA). L*entiviral transduction* was performed with a titer of at least 1×10^7 particles according to established protocols (see http://tronolab.epfl.ch/ for more details). After three days of transduction, PASMCs were used for ROS measurements.

Stable expression of Cox4i2 and Cox4i1 in CMT 167 cells
Mouse cDNA of Cox4i1, wild-type, or of mutated Cox4i2 were subcloned in the pCI-neo plasmid. CMT 167 (mouse lung carcinoma cell line) cells were transfected with the corresponding plasmids using TurboFectin 8.0 transfection reagent. Transfected cells were passaged in flasks containing 0.5 mg/ml G418 (Geneticin, Thermo Fisher Scientific, Darmstadt, Germany) in 10% FCS, 1% penicillin/streptomycin Waymouth's medium (Thermo Fisher Scientific, Darmstadt, Germany). After 1-2 weeks, transfected cells were passaged in 6-well plates and these cells were further passaged two times in medium containing 0.5 mg/ml G418. At this time, colonies of stably transfected cells were obtained. These populations of cells were cultured under “light selective pressure” of medium containing 0.2 mg/ml G418 and used for experiments.

SOD activity assay
Briefly, the PASMCs from WT and Cox4i2$^{-/-}$ mice (70 000 cells each sample) were homogenized in ice-cold 0.1 M Tris/HCL (pH 7.4) containing 0.5% Triton X-100, 5 mM β-mercaptoethanol, and 0.1 mg/ml phenylmethanesulfonyl fluoride (PMSF) and centrifuged at 14000g for 5 minutes at 4°C to remove cell debris. The assay was performed as described in the manufacturer´s protocol (Abcam, Cambridge, MA, USA). After incubation at 37°C for 20 minutes absorbance was measured at 450 nm using a microplate reader.

siRNA transfection
Selective targeting of Cox4i2 in mouse and human PASMCs was performed using ON-TARGETplus siRNA against Cox4i2 (GE Healthcare, Little Chalfont, United Kingdom). As a control, a scrambled siRNA was employed that does not target any gene in the genome (GE Healthcare, Little Chalfont, United Kingdom). Mouse PASMCs in passage 2 or human PASMCs in passage 3-9 were transfected using lipofectamine (Thermo Fisher Scientific, Darmstadt, Germany). Experiments were conducted three days after transfection. To verify the efficiency of siRNA transfection real-time PCR was carried out.

RNA isolation, cDNA synthesis and real-time polymerase chain reaction (PCR)
Total RNA (1 µg) was extracted from mouse PASMCs by RNeasy Micro Kit (Qiagen N.V., Hilden, Germany), or human PASMC or human lung material (lung homogenate, bronchus, pulmonary artery, media, adventitia) by RNeasy Mini Kit (Qiagen). RNA was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, USA). Real-time PCR was performed using the iTaq SYBR Green supermix (Bio-Rad, Hercules, USA) and the Mx3000P QPCR System (Agilent Technologies, Santa Clara, USA). The Ct values for each target gene were calculated by Ct = Ct reference gene – Ct target gene. Primer sequences are given from (5’-3’): mouse β_2M (β2-microglobulin, NM_009735.3, reference gene) (FP: GGT GAT TTG GTG GCA GCG AG; RP: GCC CTG TAT GGG GTT GCT CT), mouse Cox4i2 (NM_053091.2) (FP: AGC CCA AGA CCG TCT ACT GG; RP: TTC TTT CTG CGT GCA TAA ATT G), mouse Kv1.2 (NM_008417) (FP: TGG AAA CCT TGC CCA TCT TC; RP: GGA GGT GGA CTG CTG GTA CC) mouse Kv1.5 (NM_145983) (FP: GGC TCC GCT CCT GCC TAG; RP: GGC AAG CAA AGA AAC GCA C), mouse Kv2.1 (NM_008420) (FP: CTC CAC CAT TGC CCT GTC AC; RP: CAC ACA GCC TCC ACG TGT G), mouse Kv3.1 (NM_008421) (FP: GAG ACT CAC

GAG CGC TTC AAC; RP: AGG CCT CCG TCT CTG CTT C), mouse Kv9.3 (NM_173417) (FP: ATA TGG GCA AGG TGG TCC AG; RP: TCA GTG TGG CCC CAA GAG AC), mouse Task1 (NM_010608.2) (FP: CCT TCT ACT TCG CCA TCA CCG; RP: ACA TGA CTA GTG TGA GCG GGA), mouse Cu/ZnSOD1 (NM_011434.1) (FP: GCA GGG AAC CAT CCA CTT CG; RP: CCT GCA CTG GTA CAG CCT TG), mouse MnSOD2 (NM_013671.3) (FP: AGG GTG GTG GAG AAC CCA AA; RP: TAT TGA AGC CAA GCC AGC CC), human PBGD (AH002926) (FP: CCC ACG CGA ATC ACT CTC AT; RP: TGT CTG GTA ACG GCA ATG CG), human Cox4i1 (NM_001318794.1) (FP: GGC CTT GCT CTC TTC CGG T; RP: ATT GCT CGC TTG CCA ACT AGG) and human Cox4i2 (NM_032609.2) (FP: GTT CTC AGT TGC TCG CTG GG; RP: GAG CTG TGC ATC CCT CGT CT). The primers were intron spanning.

Western blot analysis

For expression experiments of HIF-1α, PASMCs in passage 2 were exposed to hypoxia (1% O_2, 5% CO_2, balanced with N_2 at 37°C) in a water saturated hypoxic cell incubator (Thermo Fisher Scientific, Waltham, USA) for 36 h prior to protein extraction. Proteins from PASMCs were extracted using a cell scraper. Proteins were extracted from tissue samples by Precelly®24 Homogeniser (Peqlab, Erlangen, Germany) or by grinding in liquid nitrogen. PMSF containing RIPA buffer was added to PASMC, mitochondria or ground tissue. After incubation on ice, samples were centrifuged (20,000 x g, 15 minutes, 4°C). The protein concentration in the supernatant was determined by a spectrophotometric assay (BCA assay, Pierce). A 20µg/µL quantity of protein containing supernatant was used for Western blotting. Protein extracts were separated on a 12 or 15% sodium dodecyl sulfate (SDS) polyacrylamide gel, followed by electrotransfer to a 0.45 µm polyvinylidene fluoride membrane (PVDF, Pall Corporation, Dreieich, Germany). After blocking with 5-6% non-fat dry milk in TBS-T buffer (Tris Buffer Saline + 0.1% Tween 20) for 1 h, the membrane was incubated overnight at 4°C with anti-Cox4i2 (dilution 1:1000, Novus Biologicals, Littleton, USA), HIF-1α (1:1000 dilution; 10006421; Cayman Chemical Company, MI, USA),SOD1 (1:1000 dilution, ab16831; Abcam), SOD2 (1:1000 dilution, NB100-1992; novus Biologicals) or anti-β-actin (dilution 1:50000, Sigma-Aldrich, St. Louis, USA) antibodies. After washing the membranes in TBS-T buffer three times for 10 min, specific immunoreactive signals were detected by enhanced chemiluminescence (GE Healthcare, Little Chalfont, United Kingdom) using a proprietary secondary antibody coupled to horseradish-peroxidase (W4011; Promega, WI, USA) diluted 1:5000 (incubation was 1 h at room temperature).

Mitochondria isolation

Mitochondria from CMT cells were isolated according the manual of the mitochondria isolation kit for cultured cells (Thermo Fisher Scientific, Waltham, USA).

Measurement of superoxide by electron spin resonance spectroscopy

Intracellular and extracellular ROS concentration was measured using an EMXmicro Electron Spin Resonance (ESR) spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) using 0.5 mM of the spin probe CMH (1-hydroxy-3-methoxycarbonyl-2,2,5, 5-tetramethylpyrrolidine; Noxygen, Elzach, Germany). The superoxide portion of ROS was determined by subtracting the ESR signal of the sample with polyethylen-glycol conjugated superoxide dismutase (pSOD) from the sample incubated for 90 min without 45 U/ml pSOD in ESR-Krebs HEPES buffer (99.0 mM NaCl, 4.69 mM KCl**,** 2.5 mM $CaCl_2$ x $2H_2O$, 1.2 mM $MgSO_4$ x $7H_2O$, 25 mM $NaHCO_3$, 1.03 mM KH_2PO_4, 5.6 mM D(+) Glucose, 20 mM Na-HEPES, 25 µM deferoxamine, 5 µM diethyldithiocarbamate)[3, 4]. 100,000 precapillary mouse PASMCs in passage 2 from wild-type and *Cox4i2*$^{-/-}$ animals or CMT cells were used for each sample. After the treatment with or without pSOD, the samples were incubated with CMH for 30 min under normoxic conditions at 21% oxygen and 37°C. In parallel, acute hypoxia was applied by incubation of the cells in half of the samples in a hypoxic chamber (Coy Laboratory Products, Michigan, USA) for the last 5 min of the total CMH incubation time at 1% O_2 and 37°C. Following this procedure all cells were collected into 1 ml syringes and flash frozen in liquid nitrogen. The X-Band (9.65 GHz) ESR measurements were performed at room temperature (20 – 22°C). The experimental parameters were as follows: G-factor 2.0063, center field ~ 3360 G, microwave power 2,000 mW, receiver gain 50 dB, time constant 10,24 ms, modulation amplitude 2,999 G, modulation frequency 100 GHz.

For the measurements in presence of oligomycin or carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), the substances were dissolved in dimethylsulfoxid (DMSO) and applied 20 min prior to freezing. The respective amount of DMSO was applied as control.

Patch clamp recordings of cellular membrane potential and K_V-channel currents Using a perfusion insert for cell culture dishes (Warner Instruments, Hamden, Conneticut/USA), mouse (passage 0) or human PASMCs (PromoCell, Heidelberg, Germany), grown on glass bottom dishes (35/10 mm, Greiner Bio-One, Frickenhausen, Germany) were continuously perfused with extracellular analogous bath solution (composition in mM: 126.7 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 0.42 NaH_2PO_4, 22 $NaHCO_3$, 10 Glucose, pH 7.4, 0.5 ml/min). Bath solution was bubbled with either normoxic (21 % O_2, 5.3 % CO_2, rest N_2) or hypoxic (1 % O_2, 5.3 % CO_2, rest N_2) gas mixture and preheated to 37 °C *via* an in-line solution heater (SHM-8, Warner Instruments, Hamden, USA). Acute hypoxia was applied by switching from normoxic to hypoxic bath solution and the pO_2 near the cell was recorded by an optical needle-type oxygen sensor (Firesting, Pyro Science, Aachen, Germany).
Due to its instability, H_2O_2 was freshly dissolved for each individual experiment directly before application. For determining the effective H_2O_2-concentration inside the measurement chamber, H_2O_2 (to reach a calculated final concentration of 1µM) was added to the perfusate and delivered to the measurement chamber *via* the perfusion system. Samples were taken out of the measurement chamber immediately after infusion to determine effective H_2O_2-concentration by Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, De Schelp, Netherlands) according to the suppliers instructions.
Fire-polished patch pipettes with a tip resistance of 3 – 5 MΩ were pulled from borosilicate glass capillary tubes (Sutter instruments, Novato, California, USA) using a DMZ universal electrode puller (Zeitz, Martinsried, Germany) and filled with intracellular analogous solution containing (in mM): 105 K^+-asparate, 25 KCl, 4 NaCl, 1 $MgCl_2$, 4 Mg-ATP, 10 EGTA and 10 HEPES, pH adjusted to 7.2 with 1 M KOH. Experimentally determined liquid junction potential (+12.4 mV) was corrected electronically. Cells used for experiments were either relaxed or partially contracted and exhibited spindle-shaped morphology - round and fully contracted cells were discarded.
All patch clamp experiments were performed using an EPC10 USB single amplifier (HEKA, Lambrecht, Germany) controlled by Patchmaster software (HEKA, Lambrecht, Germany). Data were filtered at 2.9 kHz and sampled at 50 or 200 Hz (current clamp) and 10 kHz (voltage clamp), respectively. For analysis, Fitmaster (HEKA, Lambrecht, Germany) and IGOR Pro 6.37 (Wavemetrics, Lake Oswego, OR) were utilized.
Cellular membrane potential was recorded using the patch clamp technique in current clamp mode (I = 0, whole cell configuration). Stability of membrane potential was determined for at least 1 min before the recording was initiated. For determination of whole cell voltage-dependent K^+-currents (I_{Kv}), PASMCs were voltage-clamped at a holding potential of -70 mV. I_{Kv}-amplitudes were elicited by applying 1 sec voltage steps between -80 mV and +80 mV in 20-mV increments. Mean I_{Kv}-amplitudes were subjected to offline leak subtraction, normalized to cell size (whole-cell membrane capacitance), plotted against the respective test potential and fitted using the Boltzmann-equation. For isolating I_{Kv} from other K^+-conductances, paxilline (1 µM) and glibenclamide (10 µM) were present in the bath solution in order to inhibit Ca^{2+}-activated and ATP-sensitive K^+-channels, respectively.

Measurement of mitochondrial membrane potential by JC-1
Mitochondrial membrane potential was investigated by fluorescent microscopy using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) as described previously[5]. JC-1 is a lipophilic, cationic dye that accumulates within mitochondria in relation to mitochondrial membrane potential, where it forms JC-1 aggregates. Aggregation of JC-1 changes the emission spectrum from green (530 nm) to red (590 nm) upon excitation at 490 nm. Precapillary PASMCs of passage 2 seeded on coverslips were incubated in 50 nM JC-1 diluted in mouse growth medium for 20 min and were then transferred to a closed perfusion chamber (PeCon, Erbach, Germany). Acute hypoxia was prompted by switching from normoxic perfusion buffer (Hepes-Ringer buffer containing 136.4 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$, 2.2 mM $CaCl_2$, 10 mM Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 5 mM glucose, pH 7.4) to hypoxic buffer, which had been pregassed with N_2 resulting in a concentration of 1% O_2 in the measurement chamber. Flow rate was held constant at 1 ml/min. For control experiments

the normoxic perfusion buffer was switched to a second normoxic perfusion buffer. Fluorescent signal was analyzed by the Polychrome II monochromator coupled to IMAGO CCD camera (Till Photonics, Gräfelfing, Germany) and to an inverted microscope (IX70; Olympus, Hamburg, Germany). Changes in the ratio of red and green fluorescent signals were analyzed in comparison to baseline data.

Measurement of mitochondrial membrane potential by TMRM

To measure mitochondrial membrane potential tetramethylrhodamine methyl ester (TMRM), which is a lipophilic, cationic fluorescent dye that accumulates within mitochondria according to their $\Delta\psi_m$ in a Nernstian fashion, was used in non-quenching mode. PASMCs were loaded with 30nM TMRM in mouse growth medium for 1h at 37°C in cellular incubator. Afterwards, cells were washed three times with buffer (composition in mM: 126.7 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1.05 $MgCl_2$, 0.42 NaH_2PO_4, 22 $NaHCO_3$, 10 Glucose, pH 7.4). Acute hypoxia was induced by superflow of 0.5% O_2, 5% CO_2, rest N_2 hypoxic gas in a chamber (PeCon GmbH, Erbach, Germany) on the microscope stage. $\Delta\psi_m$ hyperpolarization caused an increase of mitochondrial TMRM concentration, which resulted in increased emission of light at 580nm, when excited with 550 nm measured by confocal microscopy (TCS SP5X, Leica Microsystems, Manheim, Germany). Images were acquired before and 2 minutes after hypoxic exposure. In experiments with S3QEL2, PASMC were incubated 5 min before the experiment with 20µM S3QEL2.

Determination of cytosolic hydrogen peroxide (H_2O_2) concentration by HyPer

For intracellular H_2O_2 detection, HyPer sensor subcloned into the pWPXL plasmid (distributed by Addgene, Boston, USA) was used from Evrogen Company (Moscow, Russian Federation). L*entiviral transduction* was carried out with a titer of at least 1×10^7 particles according to established protocols (see http://tronolab.epfl.ch/ for more details). Experiments were carried out as outlined for the JC1 imaging in precapillary PASMC seeded on coverslip 3 days after l*entiviral transduction.* HyPer has two excitation peaks with maxima at 420 nm and 500 nm, and one emission peak with a maximum at 516 nm. H_2O_2 increases the excitation at 500nm and decreases the excitations at 420nm of the HyPer construct.

Determination of mitochondrial matrix pH by SypHer

Mitochondria-targeted SypHer (plasmid #48251 from Addgene, Boston, USA) was used to measure matrix pH ([6]). SypHer is a genetically encoded fluorescent pH-indicator with a ratiometric readout and is targeted to mitochondrial matrix. 100 000 PASMCs in passage 1 were transfected with 0.5µg SypHer plasmid using electroporation by Nucleofector™ (Lonza Group, Basel, Switzerland), afterwards PASMCS were seeded on coverslip. 5 days after transfection matrix pH measurement were performed as described for JC measurement. SypHer was excited by 420 and 490 nm through a 515 dichroic filter. The increase of 490/420 fluorescence ration is reflected the increase of matrix pH, as the fluorescence intensity of SypHer increased with pH at excitation wavelength 490 nm.

High resolution respirometry

Oxygen consumption rate was determined at 37°C using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). PASMCs from passages 2 were trypsinized, washed two times, and resuspended in M199 medium containing 1% penicillin/streptomycin and 10% HEPES for measurement using intact cells, ESR-Krebs HEPES buffer for hypoxic measurements, or Mir05 buffer (0.5 mM EGTA, 3 mM $MgCl_2$ x $6H_2O$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l BSA, pH 7.1; Oroboros Instruments, Innsbruck, Austria) for measurement using permeabilized cells. Intact CMT cells were measured in Waymouth's medium (Sigma-Aldrich, Saint Louis, USA) containing 10% HEPES. In intact cells the endogenous unstimulated respiration (basal), oligomycin (2 µg/ml) inhibited respiration, FCCP (increasing concentrations in 0.5 µM steps up to a total concentration of 4 µM) stimulated respiration, and antimycin A (5 µM) dependent non-mitochondrial respiration were measured. In hypoxic experiments, oxygen concentration in the O-2k chamber was decreased by application of N_2 into the opened chamber, subsequent equilibration of the buffer at the air-liquid phase, and immediate closure of the chamber. After stabilization of the signal, pO_2 and oxygen consumption rate were determined during the course of the experiment until oxygen was completely consumed by the cells and the pO_2 value reached zero. For substrate-specific respiration of permeabilized cells a standard substrate-inhibitor protocol using pyruvate (5 mM), glutamate (10

mM) and malate (2 mM), ADP (1 mM), succinate (10 mM), FCCP (increasing concentrations in 0.05 µM steps up to a total concentration of 0.4 µM), and antimycin A (5 µM) was applied. Complex IV dependent oxygen consumption was determined by subtraction of the oxygen consumption rate in the presence of azide from the oxygen consumption rate after application of ascorbate (2 mM) and TMPD (0.5 mM). Oxygen consumption was calculated from the recorded data as the time derivative of the oxygen content in the chamber standardized to cell number using DatLab software (Oroboros Instruments, Innsbruck, Austria).

Quantification of hypoxia-induced pulmonary hypertension by in vivo hemodynamics, right ventricular morphometry, vascular remodeling, and echocardiography

Mice were exposed to normobaric hypoxia (10% O_2) for 4 weeks. Invasive quantification of pulmonary hypertension was performed as described previously[2]. Anesthesia was induced with 3% isoflurane in oxygen and maintained via nose cone with 1.5% isoflurane (balanced with oxygen). Mice were laid supine on a heating pad and connected to a small animal ventilator MiniVent type 845 (Hugo Sachs Elektronik, March-Hugstetten, Germany). The right jugular vein was cannulated by a fluid-filled Hyman mouse pressure catheter (Numed. Inc, Hopkinton, USA) for measurement of right ventricular systolic pressure (RVSP). The digital signals were recorded using LabTech Pro software. After RVSP was recorded, the animals were exsanguinated and lungs were flushed with saline. Lungs were fixed by perfusion with 3.5-3.7% formaldehyde (Otto Fischar GmbH&Co KG, Saarbruecken, Germany) with a constant pressure of 22 cm H_2O in the pulmonary artery and 11 cm H_2O in the trachea. The lung and the heart were removed *en bloc*. Lung lobes were embedded in paraffin, and sections of 3 µm were cut from all lobes. The degree of muscularization of small peripheral pulmonary vessels was assessed by double staining with an anti-α-smooth muscle actin antibody (1:900 dilution, clone 1A4, Sigma-Aldrich, Saint Louis, USA) and anti-human von Willebrand factor antibody (1:900 dilution, Dako, Hamburg, Germany). Sections were counterstained with methyl green and examined by light microscopy with the use of a computerized morphometric system (Qwin, Leica, Wetzlar, Germany). At 40x magnification, 100 intra-acinar vessels (10-50 µm in diameter) accompanying either alveolar ducts or alveoli were analyzed. Each vessel was categorized as non muscularized (no apparent smooth muscle), partially muscularized (with a partial smooth muscle layer), or fully muscularized (with a complete smooth muscle layer). The percentage of pulmonary vessels in each muscularization category was determined by dividing the number of vessels in that category by the total number counted. The right ventricle (RV) was dissected from the left ventricle and septum (LV+S), and these dissected samples were dried for 3 days at 50°C and weighed to obtain the right to left ventricle plus septum ratio (RV/LV+S).

Measurement of transthoracic echocardiography was performed in the mice prior to measurement of invasive hemodynamics with the Vevo2100 high-resolution imaging system equipped with a 40-MHz transducer (VisualSonics, Toronto, Canada). Anesthesia was induced with 3% isoflurane in oxygen and maintained via nose cone with 1.5% isoflurane (balanced with oxygen). The mouse was laid in a supine position on a heating platform while all four limbs were connected to ECG electrodes in order to monitor heart rate. The body temperature was monitored using a rectal thermometer (Indus Instruments, Houston, TX). After shaving the chest area and spreading pre-warmed ultrasound gel over the chest, echocardiographic studies were performed. For in vivo heart function evaluation, the cardiac index (CI) was measured as described previously[7].

BrdU proliferation assay

For assessment of proliferation after stimulation with recombinant mouse PDGF, mouse PASMCs (5000 cells per well) from passage 1 were seeded in 24-well plates in smooth muscle cell basal medium (starvation) containing normocin (1%, Invitrogen, De Schelp, Netherlands). After 24 hours of starvation, medium was replaced by either smooth muscle cell basal medium containing normocin (starvation) and Bromdesoxyuridin (BrdU, 1:1000 dilution, Roche, Basel, Switzerland) to measure newly synthesized DNA in proliferating PASMC or smooth muscle cell basal medium containing normocin, BrdU and recombinant mouse PDGF (10 ng/mL, R&D systems, Minneapolis, USA). Cells were either exposed to normoxia (21% O_2/ 5% CO_2) or hypoxia (1% O_2/ 5% CO_2) at 37°C in water saturated incubators (Thermo Fisher Scientific, Waltham, USA) for 24 hours. The proliferation assay was performed according the protocol of the Cell Proliferation ELISA (Roche, Basel, Switzerland). The absorbance of the substrate reaction was measured after 20 minutes incubation at 370nm (reference wavelength 492nm).

Statistics

Sample size for chronic hypoxic experiments were determined by SPSS statistics software (SPSS Inc., Chicago, USA). One experiment was excluded from the isolated lung experiments with MitoTempo (Fig. 2d) which showed an increase of PAP in response to the control application of KCl below the pre-established treshold of 0.4 mmHg. Two outlier detected by ROUT method using Prism 6 (GraphPad Software Inc., San Diego, USA) were removed in the experiments of Fig. 2 h, i and Fig. S 4 a, respectively, and one in each data set of Fig. 2 j and 4 b, d. Animals were randomly assigned for hypoxic or normoxic treatment. All analyses except analysis of vessel muscularization after chronic hypoxic exposure were performed unblinded. Normality distribution of the sample sets was determined by Shapiro Wilk normality test. For sample sets with Gaussian distribution, student's two-tailed *t*-test or 2-way-ANOVA were used. For the sample sets with a non-Gaussian distribution, Mann-Whitney-test and Kruskal-Wallis-test was used. Multiple comparisons, using the same group in more than one analysis or hypothesis, were adjusted using Bonferroni correction. All analyses were considered statistically significant at $p<0.05$. Statistical analysis was performed using Prism 6 (GraphPad Software Inc., San Diego, USA).

Supplemental References

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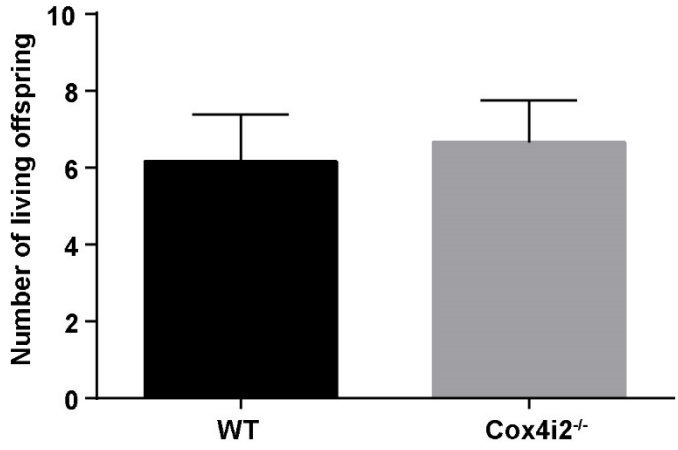
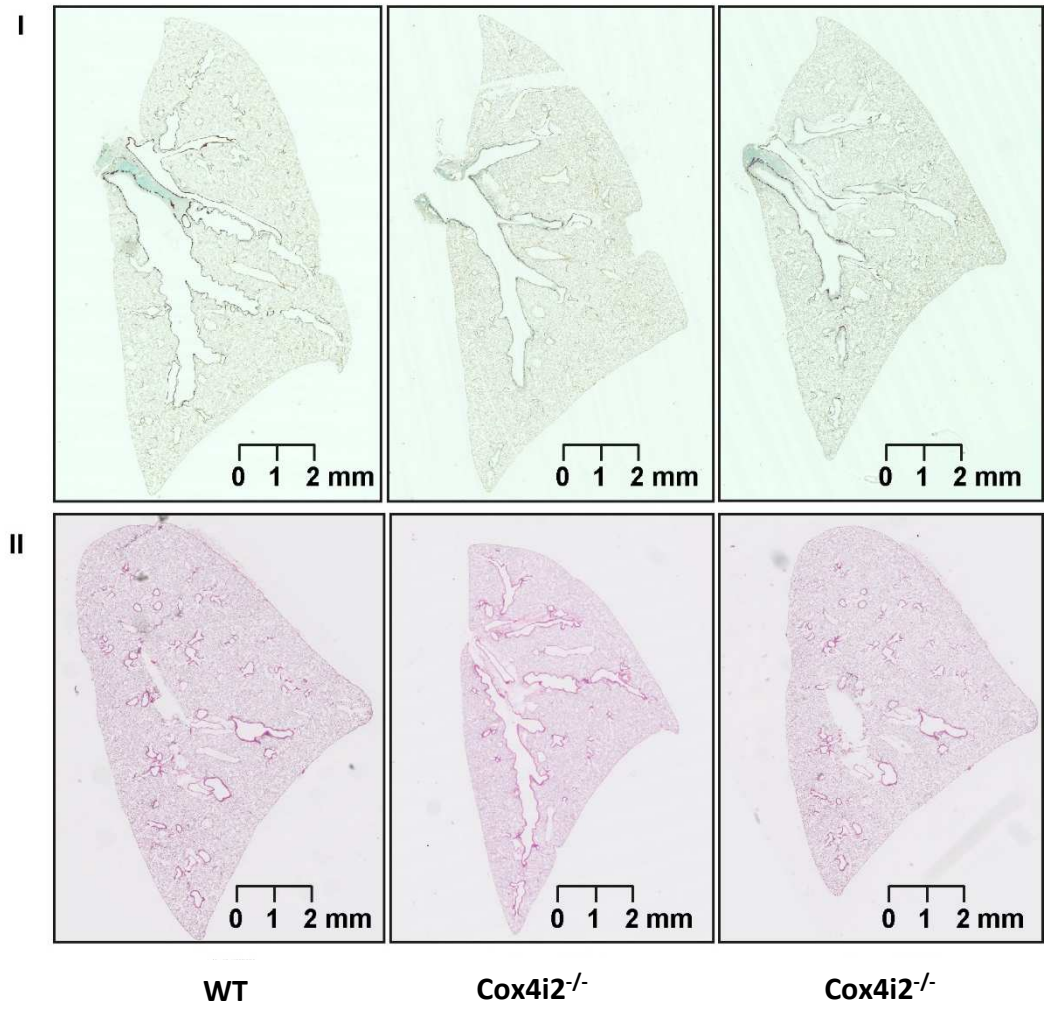
Supplemental Figures

Online Figure I

A



B

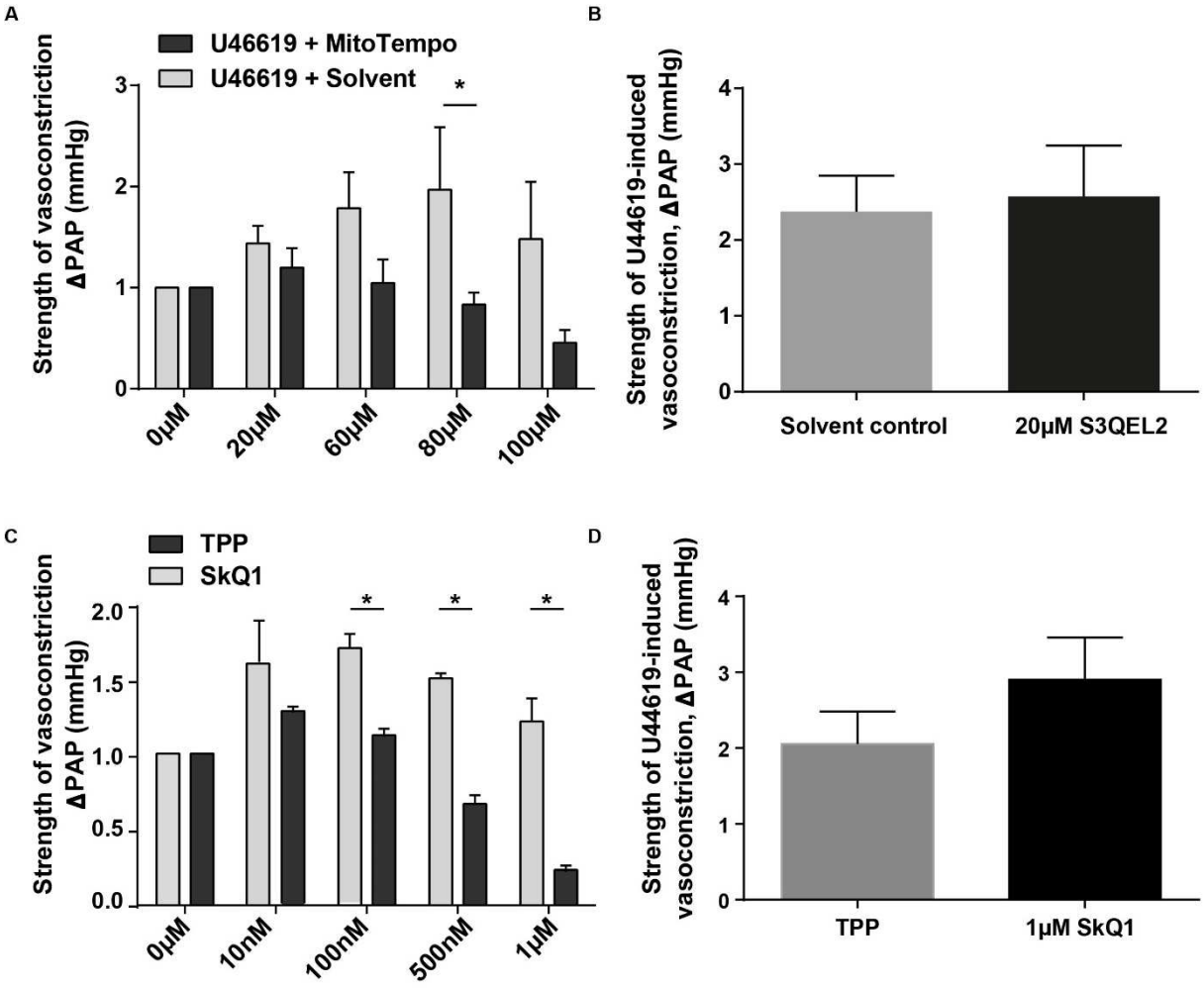


Online Fig. I. Phenotypical characteristics of Cox4i2$^{-/-}$ mice

(A) Average number of living offspring per litter (WT: n=6 litters from 3 matings, Cox4i2$^{-/-}$: n=9 litters from 4 matings).

(B) Representative histological lung sections of WT and Cox4i2$^{-/-}$ mice of 2-3 months of age. I: von Willebrand (brown: endothel), α-smooth muscle actin (violet) and methyl-green stain, II: hematoxylin and eosin stain.

Online Figure II



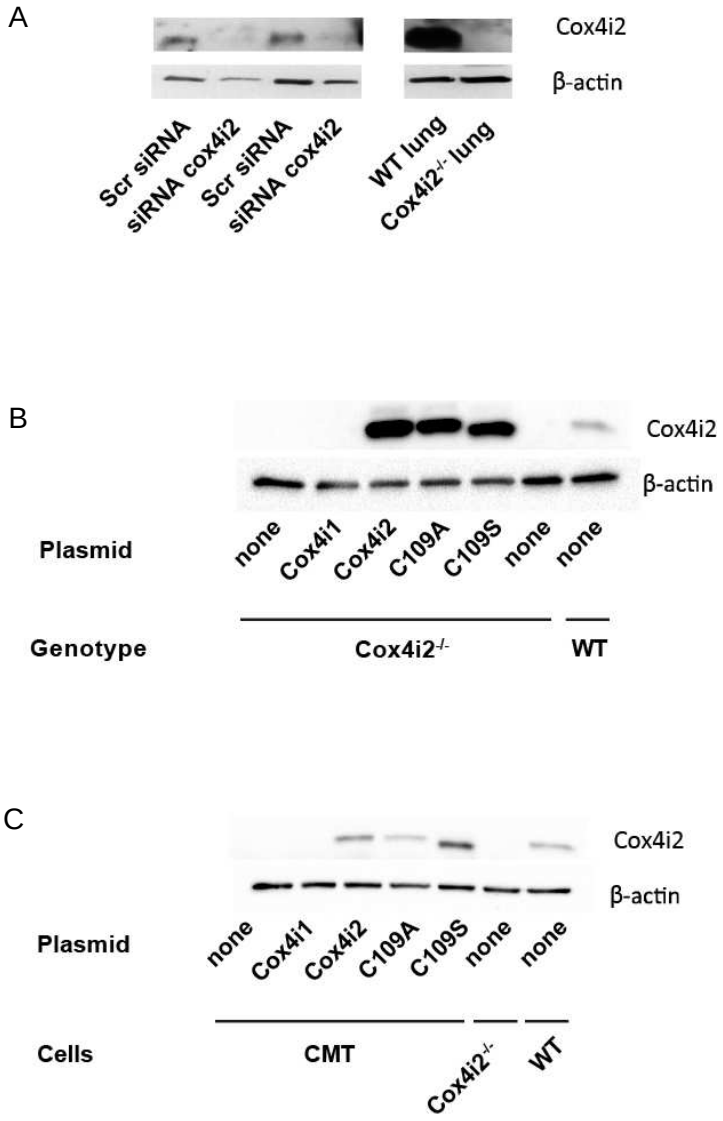
Online Fig. II: Effect of mitochondrial superoxide inhibitors on pulmonary vasoconstriction induced by the thromboxane mimetic U46619 and the effect of the mitochondrial antioxidant SkQ1 on pulmonary vasoconstriction

(A) Strength of pulmonary vasoconstriction induced by the thromboxan mimetic U46619 (n=4 isolated lungs for solvent, n=6 isolated lungs for MitoTempo) determined as an increase of pulmonary arterial pressure (ΔPAP) in presence (MitoTempo) and absence (solvent group) of MitoTempo in WT mice. MitoTempo was applied in increasing concentrations in the same lung 5 min before each repetitive maneuver of U46619 application, respectively, and compared to the respective increase of PAP in presence of the solvent only. *p<0.05, analyzed by two-tailed Mann-Whitney test.

(B) Strength of pulmonary vasoconstriction induced by the thromboxan mimetic U46619 (n=4 per group) determined as an increase of pulmonary arterial pressure (ΔPAP) in presence and absence of 20 µM S3QEL2 in WT mice. U46619 was applied after the repetitive hypoxic maneuvers.

(C, D) Strength of hypoxic pulmonary vasoconstriction (HPV) (C) and potassium chloride (KCl)- or U46619-induced pulmonary vasoconstriction (D) determined as an increase of pulmonary arterial pressure (ΔPAP) in presence of SkQ1 or the control substance, TPP^+, in WT lungs (n=4 isolated lungs per group). SkQ1 or TPP^+ was applied in increasing concentrations in the same lung 5 min before each repetitive maneuver of hypoxic ventilation. U46619 was applied after the last hypoxic maneuver. *p<0.05, analyzed by two-tailed Mann-Whitney test.

Online Figure III



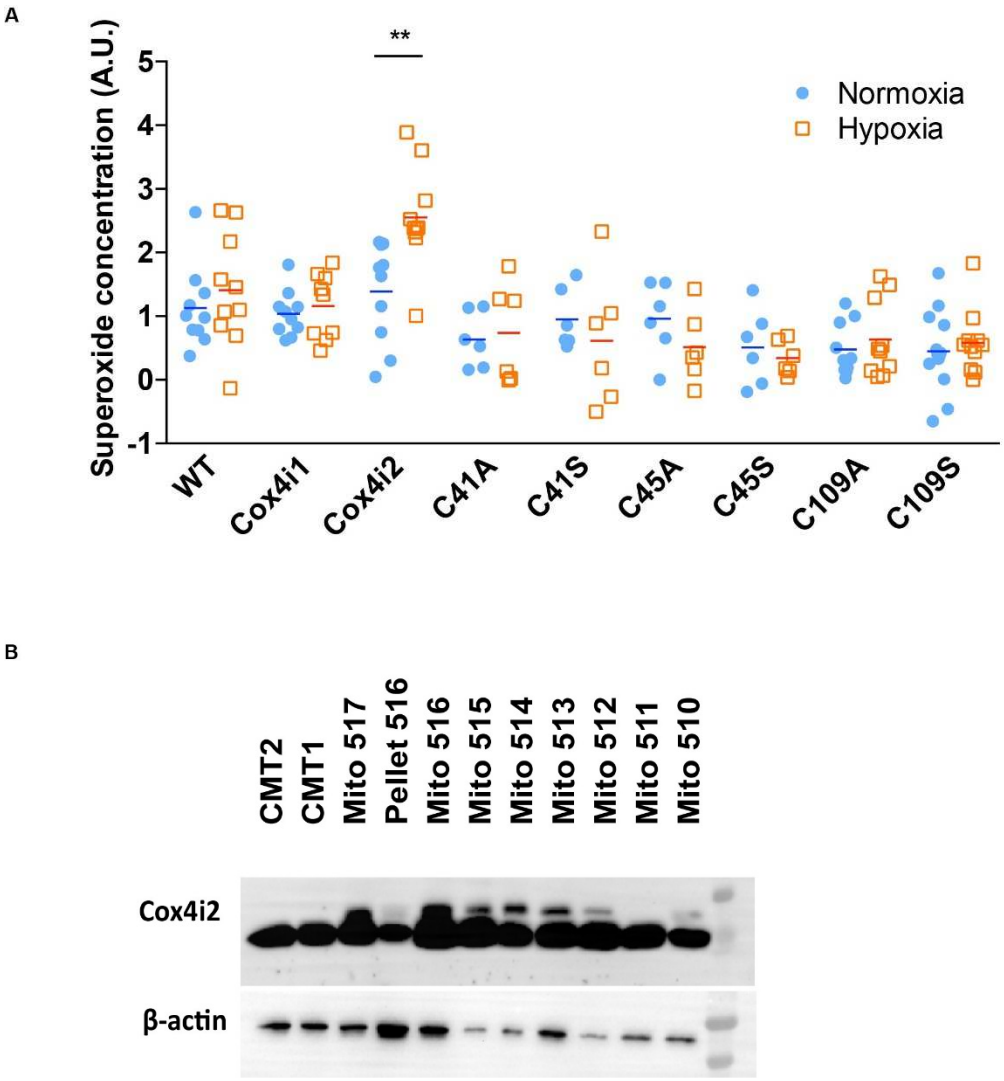
Online Fig. III. Expression of Cox4i2 after knockdown and overexpression.

(A) Knockdown of Cox4i2 with siRNA in PASMCs cultivated under normoxia and hypoxia (See Fig. 2B).

(B) Overexpression of Cox4i1 and Cox4i2 mutants in *Cox4i2*$^{-/-}$ PASMCs (See Fig. 2F).

(C) Overexpression of Cox4i1 and Cox4i2 mutants in CMT cells (See Fig. 2G).

Online Figure IV

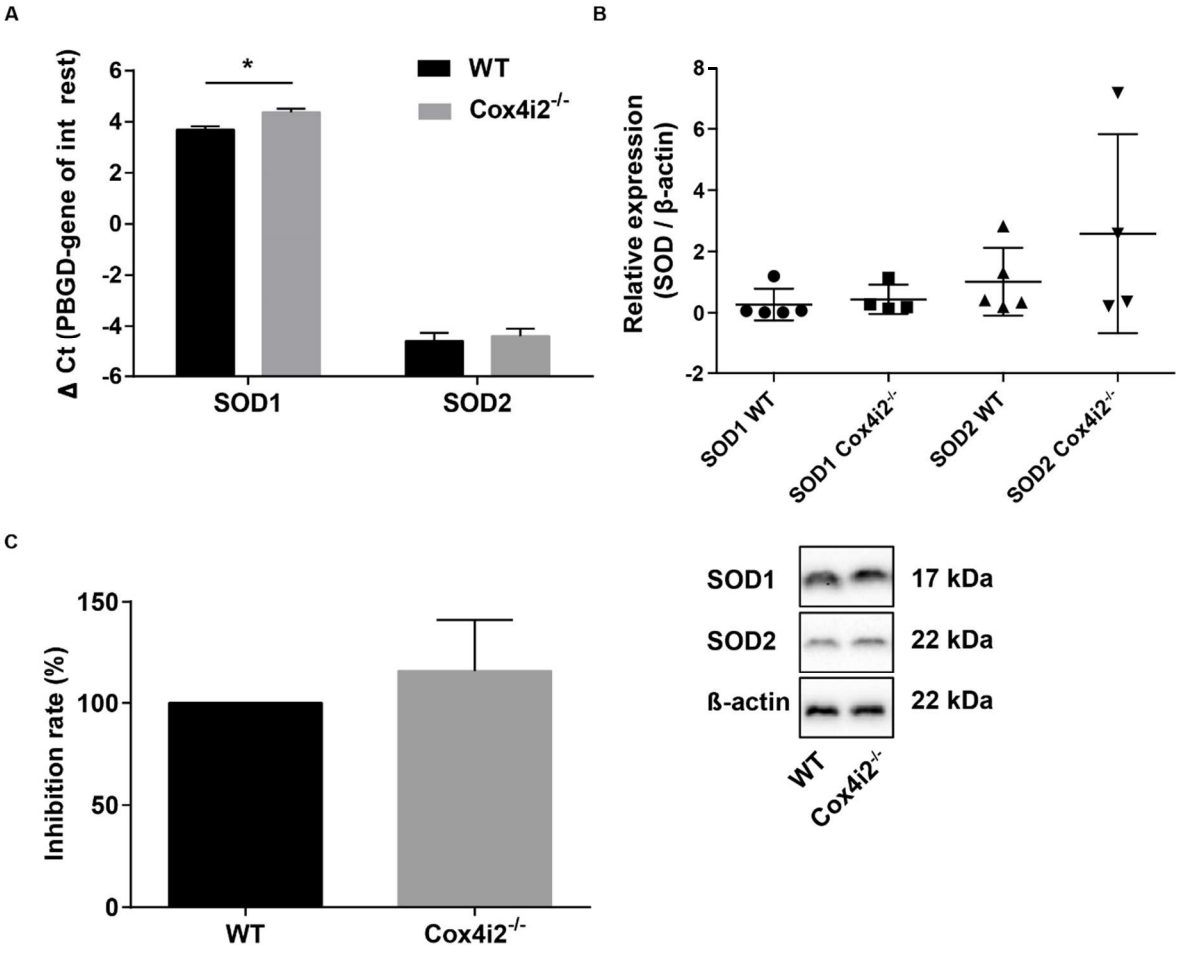


Online Fig. IV: Effect of mutation of Cox4i2 on hypoxia-induced superoxide release in CMT cells.

(A) Intracellular superoxide concentration during hypoxic exposure (1% O_2, 5 min) of CMT cells. n=6-13 per group. Data are from at least 3 independent experiments. The WT, Cox4i1, Cox4i2, C109A and C109S groups are the same as in Figure 2I. **p<0.05 comparing the normoxic and hypoxic group (analysis by t-test). Data are presented as mean and individual values.

(B) Lack of hypoxia-induced superoxide production was not due to failing expression or increased degradation of mutated proteins because Western blot analysis showed that the mutated Cox4i2 was expressed in the mitochondrial fraction of the cells.

Online Figure V



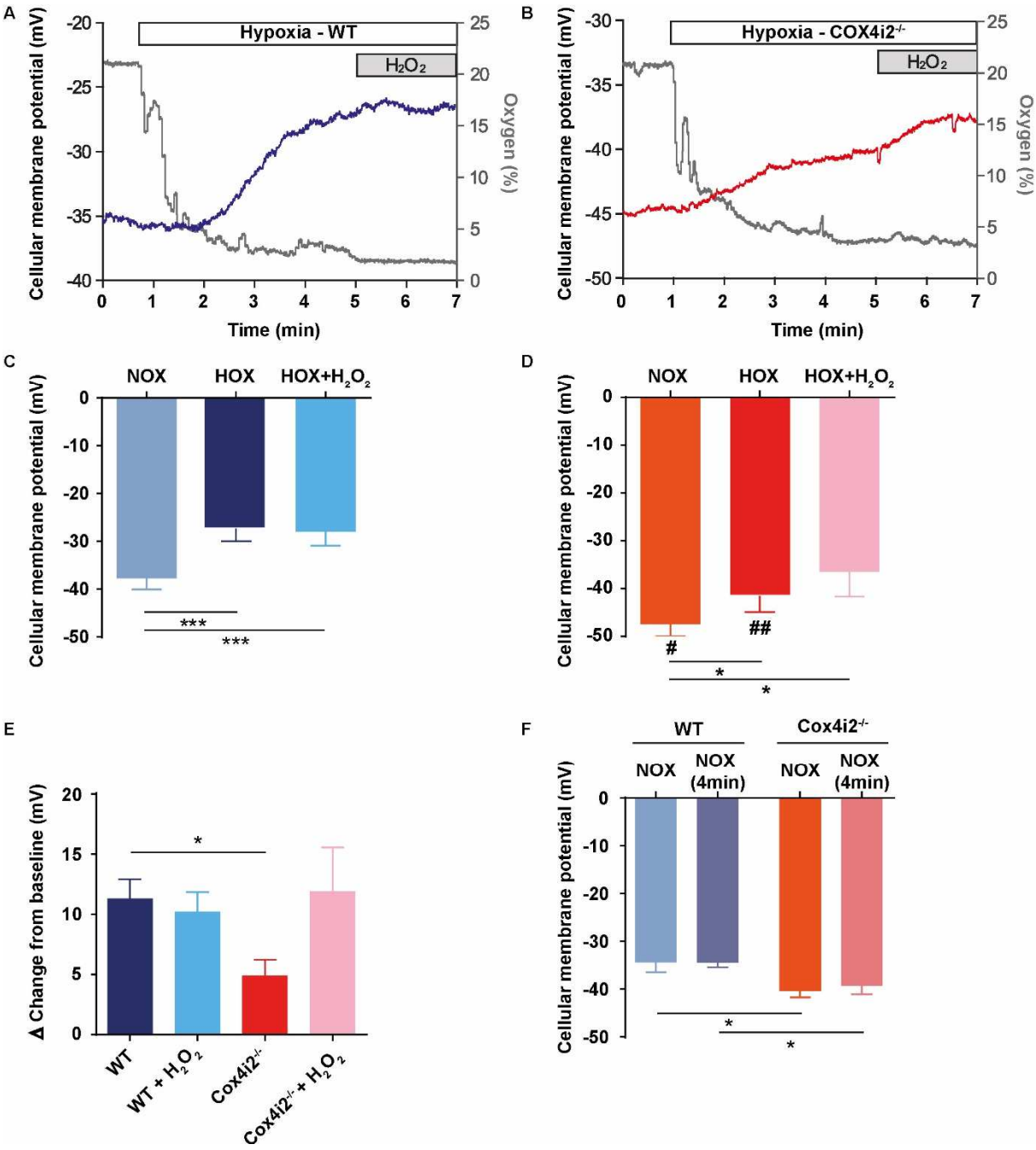
Online Fig. V. Expression superoxide dismutase in Cox4i2$^{-/-}$ PASMCs.

A) Expression of mRNA of the superoxide dismutase (SOD) 1 and 2 in mouse PASMCs displayed as difference of the Ct value of porphobilinogen deaminase (PBDG) and the respective gene (WT: n=5, Cox4i2$^{-/-}$: n=4). *p<0.05.

B) Protein expression of SOD1 and 2 in mouse PASMCs displayed as relative expression compared to β-actin (WT: n=5, Cox4i2$^{-/-}$: n=4).

C) Activity of the superoxide dismutase in mouse PASMCs (n=4 each group).

Online Figure VI



Online Fig. VI. Partial restoration of hypoxia-induced cellular membrane potential in Cox4i2$^{-/-}$ PASMCs with low dose application of H_2O_2.

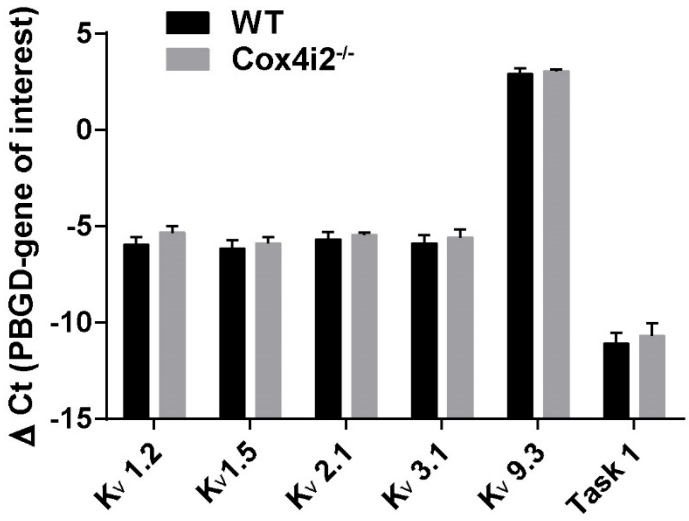
(A, B) Representative tracings of hypoxia-induced cellular membrane depolarization in WT (A) and *Cox4i2*$^{-/-}$ PASMCs (B) in presence and absence of hydrogen peroxide (H_2O_2) H_2O_2 was applied in a dosage that should result in a concentration of 150nM in the buffer fluid. However (see Figure xy), using the same protocol an amount that should result in a concentration of 1µM only reached a final concentration of 124 nM due to a loss of H_2O_2 related to technical reasons of the application The gray tracings show the oxygen concentration in %, the blue and red tracing the cellular membrane potential in WT (A) and *Cox4i2*$^{-/-}$ PASMCs (B), respectively.

(C, D) Cellular membrane potential during normoxia (NOX), acute hypoxia (HOX) and acute hypoxia after application of hydrogen peroxide (HOX+H_2O_2, same concentration as in A,B) in WT (C) and *Cox4i2*$^{-/-}$ PASMCs (D). n=14 for WT, and n=10 for *Cox4i2*$^{-/-}$ PASMCs from 3 and 2 individual isolations, respectively. *p<0.05, **p<0.01 compared to respective NOX group. #p<0.05, ##p<0.05 compared to WT.

(E) Change of cellular membrane potential compared to normoxia in WT and *Cox4i2*$^{-/-}$ PASMCs, and *Cox4i2*$^{-/-}$ PASMCs in presence of H_2O_2. Data are calculated as delta values from measurements of Fig. 3C and D.

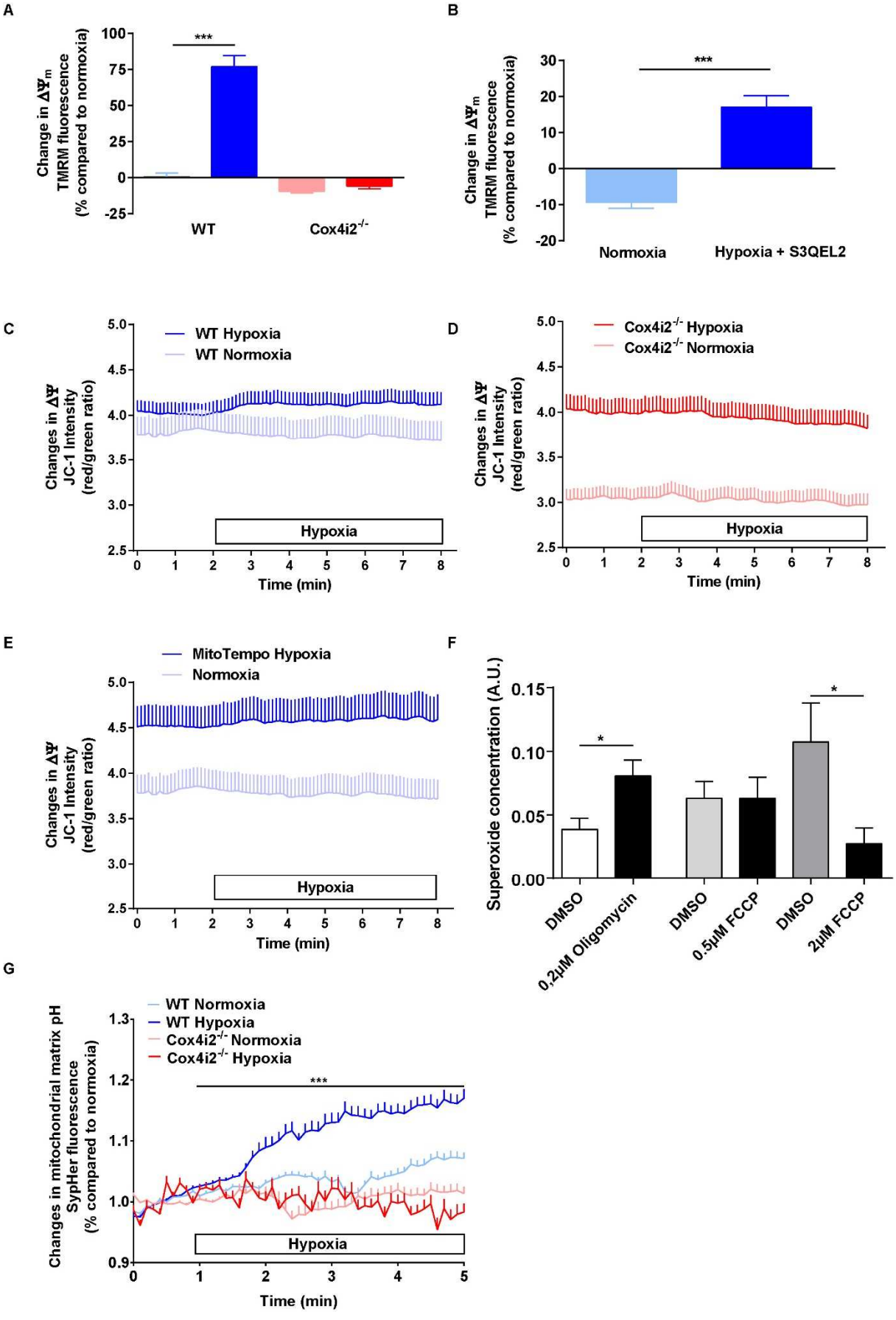
(F) Cellular membrane potential during the course of normoxia. Data are taken during baseline normoxia (NOX) and at minute 4 of the normoxic measurement (NOX 4min) to parallel the protocol of the hypoxic experiment. n=6 PASMC each from two respective cell isolations.

Online Figure VII



Online Fig. VII. Expression of cellular potassium channels and superoxide dismutase in Cox4i2$^{-/-}$ PASMCs. Expression of mRNA of different voltage dependent potassium channels (K_v) and the potassium channel subfamily K member 3 channel (TASK1) in mouse PASMCs displayed as difference of the Ct value of porphobilinogen deaminase (PBGD) and the respective gene (n=3-5).

Online Figure VIII



Online Fig. VIII. Hypoxia-induced hyperpolarization of the mitochondrial membrane potential

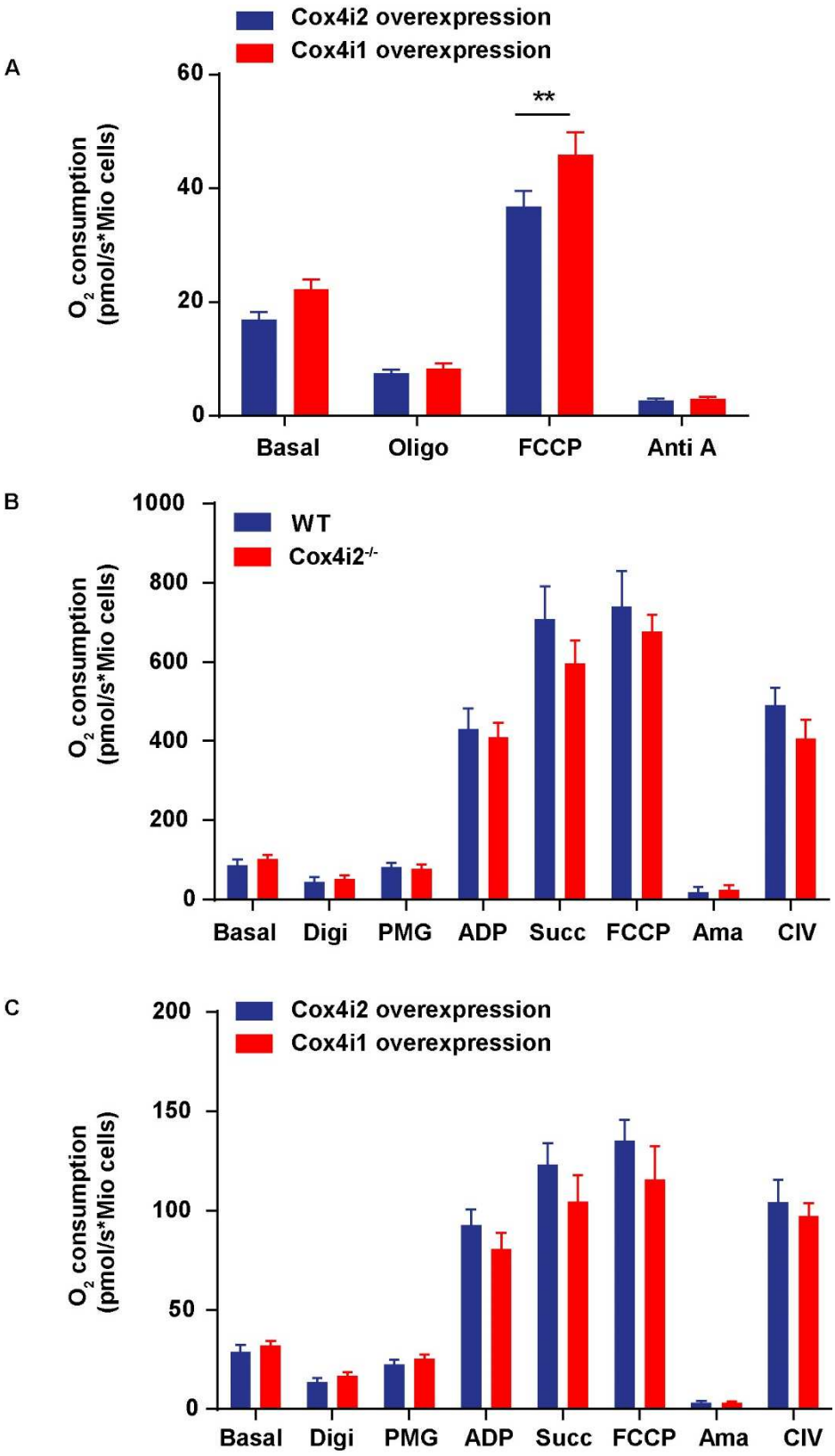
(A, B) Mitochondrial membrane potential determined as fluorescence intensity of TMRM staining, given as percent (%) of the normoxic value at minute 2 in PASMCs from WT mice in absence and presence of S3QEL2 (B). Data are from n=158/105 cells (hypoxia: WT/*Cox4i2*$^{-/-}$) or n=85/123 cells (normoxia: WT/*Cox4i2*$^{-/-}$) for Fig. A and n=31/59 cells (normoxia/hypoxia) for Fig. B from at least three individual PASMC isolations. *** significant difference ($p<0.001$) between normoxia and hypoxia analyzed by two-tailed Mann-Whitney-test comparing averaged values from minute 2 to 3.

(D-F) Mitochondrial membrane potential determined as fluorescence intensity of red/green ratio of JC-1 staining in PASMCs from WT (A) or *Cox4i2*$^{-/-}$ (B) mice, and in WT PASMCs in absence and presence of MitoTempo (C). The horizontal bar indicates the presence of hypoxic medium for the hypoxic group. Data are from n=180/207 cells (hypoxia: WT/*Cox4i2*$^{-/-}$) or n=107/95 cells (normoxia: WT/*Cox4i2*$^{-/-}$) from at least three individual PASMC isolations for Fig. A and B and n=31 cells from one PASMC isolation for the MitoTempo group in Figure 3C. The normoxic group in Figure 3C is identical to and taken from Figure 3A.

(G) Effect of mitochondrial hyperpolarization (Oligomycin) and depolarization (FCCP) on superoxide concentration in PASMCs (n=6-24). *$p<0.05$ compared to the respective solvent (DMSO) concentration.

(H) Mitochondrial matrix pH determined as fluorescence intensity of SNARF1 staining, given as percent (%) of the normoxic value during minute 1 in PASMCs from WT (blue lines) or *Cox4i2*$^{-/-}$ (red lines) mice. The horizontal bar indicates the presence of hypoxic medium for the hypoxic group. Data are from n=22/23 cells (hypoxia: WT/*Cox4i2*$^{-/-}$) or n=32/39 cells (normoxia: WT/*Cox4i2*$^{-/-}$) from at least three individual PASMC isolations. *** significant difference ($p<0.05$ or $p<0.001$) between normoxia and hypoxia analyzed by two-tailed Mann-Whitney-test comparing values averaged over one minute.

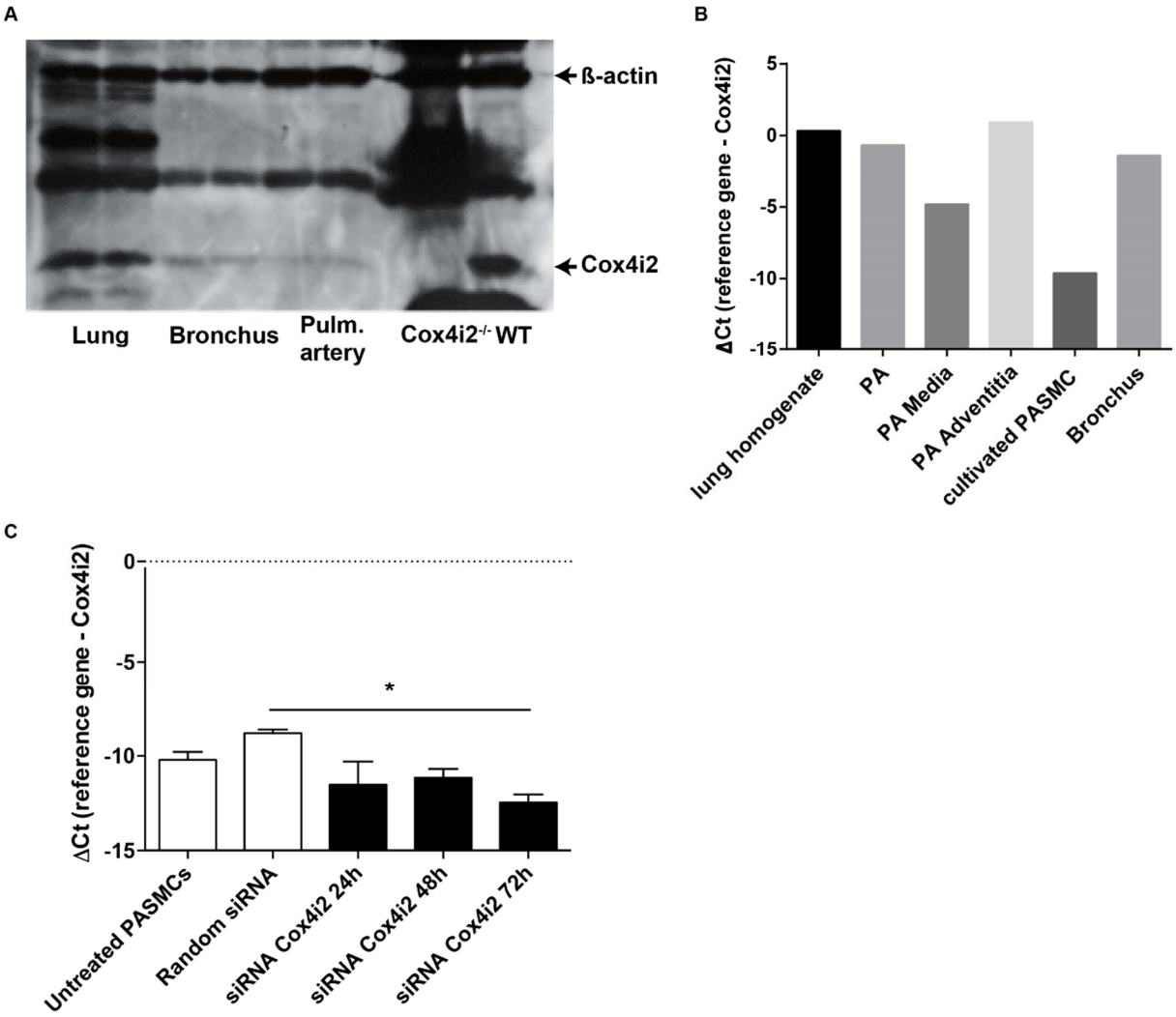
Online Figure IX



Online Fig. IX. Respiration of PASMCs isolated from WT and *Cox4i2*$^{-/-}$ mice (Supplemental Information for Fig. 3D-F).

(A) Respiration in intact CMT cells overexpressing either Cox4i2 or Cox4i1 given as oxygen consumption (O_2 consumption) in pmol/s per million (Mio) cells under unstimulated conditions (basal) and in presence of oligomycin (oligo), FCCP, and antimycin A (Anti A). Data are from n=11 experiments per group containing 100,000 to 300,000 cells isolated from one mouse per experiment. Data were analyzed by 2-way-ANOVA with Bonferroni posthoc test. **p<0.01.
(B) Respiration in permeabilized PASMCs cells isolated from WT and *Cox4i2*$^{-/-}$ mice defined as in (A) after permeabilization with digitonin (digi), and in presence of pyruvate/malate/glutamate (PGM), ADP, Succinate (Succ), FCCP, antimycin A (Anti A) or complex IV specific respiration (CIV). Data are from n=11 experiments per group containing 100,000 to 300,000 cells isolated from one mouse per experiment.
(C) Respiration in permeabilized CMT cells. Details as in (B).
All data are given as mean+SEM

Online Figure X



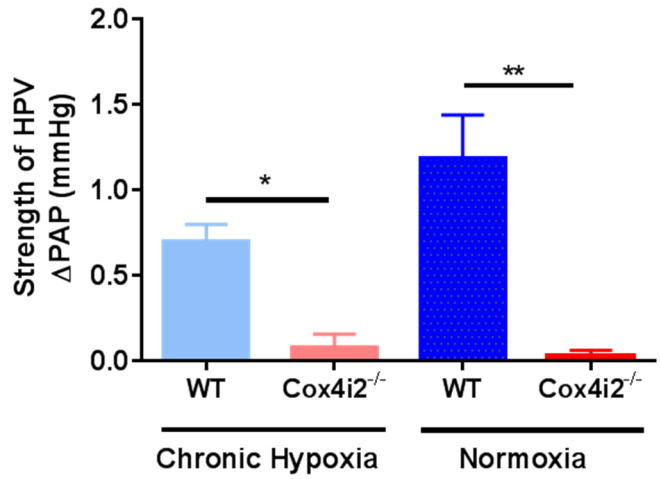
Online Fig. X. Expression of Cox4i2 in the human lung

(A) Protein expression of Cox4i2 in lung homogenate, the bronchus, and the pulmonary artery (pulm. artery) of a human donor lung (double samples), and in lung homogenate of Cox4i2$^{-/-}$ and WT mouse lungs as controls.

(B) Expression of mRNA of Cox4i2 in lung homogenate of human donor lung, the human pulmonary artery (PA), the media of the human PA, the adventitia of the human PA, primary cultivated human PASMCs and the bronchus of a human donor lung.

(C) Knockdown of Cox4i2 in human PASMCs incubated with siRNA for 24, 48 and 72 h.

Online Figure XI



Online Fig. XI. Hypoxia-induced pulmonary vasoconstriction (HPV) after exposure of WT (n=5 isolated lungs) and Cox4i2$^{-/-}$ (n=3 isolated lungs) mice to chronic hypoxia (4 weeks, 10 %O_2). Data of normoxic lungs are the same as presented in Figure 1 D. *p<0.5 (by Mann-Whitney-test), **p<0.01 (by two-tailed student's t-test).