Supplement Fig. 1: UCP2 as a mitochondrial protein in mice. A) Western blot indicating stable expression of UCP2 in subsarcolemmal (SSM) and interfibrillar (IFM) mitochondria isolated from heart ventricles. recPep indicates a control peptide. Spleen tissue from wild type mice (WT) and UCP2-/- mice was used to show specificity of the antibody. B) Effect of depletion of UCP2 in mice heart mitochondria on respiration. Complex I was tested with glutamate/malate as substrate, complex II with succinate as substrate. Data are shown individually (n=6) and as means ± SEM. Experiments were performed in the absence and presence of ADP. Two way ANOVA revealed no significant differences between genotypes but significant differences between absence and presence of ADP.
**Supplement Fig. 2:** Effect of mitochondrial UCP2 knockout on ROS formation. Data are shown individually (n=6) and as means ± SEM. Experiments were performed in the absence (left) and presence (right) of ADP. UCP was further inhibited by genipin. Two way ANOVA revealed no significant differences between genotypes and inhibition but significant differences between absence and presence of ADP.
Suppl. Fig. 3:

Top: UCP2 Blot with spleen samples from UCP2-/- mice and wildtype mice. Lane 1: MW marker; lane 2: UCP2 protein; lane 3/4: UCP2-/-; Lane 5/6: Wild Type Mice. The arrow indicates the corresponding band.

Middle: Original blot of RV samples after Sham (lane 3-6), PAB (lane 7-9), including recombinant UCP2 protein (lane 2). Lane 1: Molecular weight marker. Lane 10: RV tissue sample from UCP2-/- (see Fig. 2 E).

Bottom: GAPDH control blot for loading control of the RV samples (see Fig. 2E).
Protection against pressure overload-induced right heart failure by uncoupling protein 2 silencing

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Running Title: The role of UCP2 in right ventricular hypertrophy

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Material and Methods

Animals and ethic concerns

Adult male homozygous UCP2^+/− mice (B6.129S4-Ucp2^tmIlow^/J) were purchased from Jackson Laboratory (Bar Harbor, USA) and bred up to the 6th generation in our facility. Male C57BL/6J mice bought from Charles River Laboratories (Sulzfeld, Germany) were used as controls. All animal experiments were performed according to the regional authorities and ethics committees for animal research. The experiments were registered under the numbers GI20/2010 Nr. 86/2011, GI 20/10 Nr. 40/2011, and GI 20/1 Nr. 91/2017.

Induction of an acute increase in RV afterload by pulmonary artery banding (PAB)

Anesthesia was initiated by isoflurane 3-4% and maintained by isoflurane 1.5-2.5% supplemented with 100% oxygen. Afterwards, mice were intubated and mechanically ventilated using a mouse ventilator MiniVent type 845 (Hugo Sachs Elekronik, March-Hugstetten, Germany) while placed on a heating surface. Analgesic buprenorphine hydrochloride (Temgesic®, 0.1 mg/kg, Sigma-Aldrich, Germany) was given subcutaneously. After left anterolateral thoracotomy via the second left intercostal space, a small titanium clip (Hemoclip®, Edward Weck& Co., Inc., Research Triangle Park, NC, USA) with a width of 0.35 mm was placed around the pulmonary trunk with a special hemoclip applier in order to produce 65-70% constriction of the pulmonary artery. The chest and afterwards the skin were closed with 6.0 prolene sutures. Sham operated mice underwent the same procedure without applying the hemoclip. Mortality was

Echocardiography

Vevo770 high-resolution imaging system equipped by 30-MHz transducer (VisualSonics, Toronto) was used to perform transthoracic echocardiography 3 weeks after operation. For in vivo heart function evaluation, the right ventricular wall thickness (RVWT), right ventricular internal diameter (RVID), tricuspid annular plane systolic excursion (TAPSE), and cardiac output (CO) were measured as described before.¹

Invasive hemodynamic measurement

A series of pressure-volume loops was recorded with a micropressure conductance catheter (Millar instruments, Houston, USA). Right ventricular systolic pressure (RVSP) was measured through catheterisation under anaesthesia 3 weeks after PAB or sham operation.

The animals were anesthetized with 3-4% isoflurane supplemented with oxygen and ventilated with a rodent ventilator (Harvard Apparatus, Holiston, Massachusetts, USA). Maintenance of anaesthesia was done with 2-3% isoflurane supplemented with oxygen. The mice were laid supine on a heating platform with 3 legs taped to electrocardiogram electrodes for monitoring of heart rate (HR). A rectal thermometer (Indus Instruments, Houston, TX, USA) was used to control the body temperature. The heating pad helped to keep the body temperature at 36.5-37.5°C. The right jugular vein was cannulated for measurement of RVSP.

Tissue sampling, RV hypertrophy assessment

Separation of right ventricular (RV) wall from left ventricular wall and ventricular septum (S) was done. After heart hypertrophy measurements the RV wall, LV wall and S were fixed in formalin
(3.5%-3.7%), dehydrated and finally paraffin embedded for histological analysis. Collagen staining was performed by Picro Sirius Red staining. A total amount of 0.2 g Sirius Red was dissolved in 200 ml of picric acid, the solution was filtered and finally the pH was adjusted to pH 2.0. After staining the slices were measured using Leica QWin standard imaging software. 40-90 pictures were taken per ventricle and red stained collagen was normalized to the total tissue amount.

Isolation and culture of adult mouse ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated from wildtype and UCP2<sup>−/−</sup> mice as described in detail elsewhere.<sup>2</sup> After deep ether anaesthesia, hearts were excised from the chest cavity, transferred rapidly to ice-cold saline and immediately mounted on the cannula of Langendorff perfusion system. Heart perfusion and next steps were all done at 37°C. Perfusion of the heart was performed with the non-circulating mode for 5 min at 10 ml/min with perfusate (perfusate in mmol/l: NaCl 110, KH<sub>2</sub>PO<sub>4</sub> 1.2, KCl 2.5, MgSO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 25, glucose 10). Afterwards, perfusion was continued with recirculation of 50 ml of the perfusate supplemented with 20 mg collagenase and 25 µM CaCl<sub>2</sub> at 5 ml/min. Ventricular tissue was minced after 25 min and incubated in 5 ml of the recirculating medium for 5 min. The prepared suspension was filtered through a 200-µm nylon mesh, washed four times by centrifugation (1 min, 25 x-g) and resuspended in the perfusate, in which the concentration of CaCl<sub>2</sub> was increased gradually (125 µM, 250 µM, 500 µM, and 1000 µM). Afterwards, the cell pellet was re-suspended in serum-free culture medium (medium 199 with Earle’s salts, 5 mM creatine, 2 mM L-carnitine, 5 mM taurine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were plated on 35 mm cell culture dishes (Falcon, type 3001) which were pre-coated with laminin (5 µg/ml). After 2 hours, cell cultures were washed with the serum free medium to remove non-attached and round cells. Cells were used either within the next two hours or cultured overnight where indicated in the result.

Load-free cell shortening

Cell contraction determination was carried out at room temperature and analysed using a cell-edge-detection system as previously described.<sup>3</sup> Stimulation of cells was done via two AgCl electrodes with biphasic electrical stimuli made of two equal but opposite rectangular 50-V stimuli of 0.5 ms duration 2 Hz for duration of 1 min was chosen for stimulation of each cell. Contractions were recorded after every 15 seconds. The mean of four measurements at a given frequency was used to calculate the cell shortening of each cell. A line camera (data recording at 500 Hz) was used to measure cell lengths. Cells were used in M199 with an extracellular calcium concentration of 1.25 mM. Data are shown as cell shortening normalized to diastolic cell length (ΔL/L (%)).

Calcium transient quantification

The fluorescent dye fura-2 AM was used to measure cytosolic calcium transients as described.<sup>4</sup> In brief, isolated cardiomyocytes were located on FCS-coated glass cover-slips and were loaded for 30 min at 37°C with the acetoxyethyl ester of fura-2 (2.5 µmol/l). Afterwards, cells were washed twice with medium 199 for 30 min. Cover-slips with loaded cells were introduced into a gastight, temperature-controlled (37°C), transparent perfusion chamber while positioned in the light path of an inverted microscope. Excitation of the fluorescence dye at wavelengths of 340/380 nm was changed with an AR-Cation system connected to the microscope. Light emitted (500-520 nm)
from an area of 10 x 10 µm within a single fluorescent cell was collected by an ION Optix Corp. imaging system. The data are analysed as the ratio of light emitted at 340-to-380 nm wavelength. Calcium transient of beating cells were determined while cells were submitted to field stimulation at 1 Hz.

Cell spreading

Quantification of cell spreading (building of pseudopodia-like structures from cultured mice cardiomyocytes) was analyzed by light microscopy as described before in detail. Mouse myocytes were cultured in a modified medium containing the following additions: 20% fetal calf serum, mM: NaCl 118, KH₂PO₄ 1.2, KCl 4.7, Glucose 5, MgSO₄ 0.8, HEPES 10, CaCl₂ 2.5, and pyruvate 1.9, gassed with carbogen. The pH was adjusted to 7.4.

RNA isolation and Real-Time PCR

Total RNA was extracted from RV and LV by RNeasy Micro Kit (Qiagen N.V., Hilden, Germany) and the extracted mRNA was reverse-transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Berkeley, California). The mRNA expression of UCP2 and UCP3 was quantified by master mix for RT PCR (iTaq SYBR Green supermix withROX, Bio-Rad, Berkeley, California) and Mx3000P QPCR Systems (AgilentTechnologies, Santa Clara, USA). Primer efficiency for UCP2 and UCP3 was 78% and 79%, respectively. Hypoxanthine phosphoribosyltransferase (HPRT) was as housekeeping gene. The used reaction condition was 1× (95°C, 10 min) followed by 45×(95°C, 5 s; 60°C, 5 s, 72°C, 10 s) and the extension phase was done at 72°C. Measurement of each gene was carried out as duplicate. Confirmation of the desired products formation was done by dissociation curves. The calculation of ΔCt values was done by subtracting the Ct values of the target gene from the endogenous control [ΔCt = Ct (endogenous control) –Ct (target)], and the fold change according 2ΔΔCt formula was calculated.

Western Blot

Western blots were performed with 40 µg total protein from lysates of isolated mitochondria, cells or total tissue extracts. After transfer to a nitrocellulose membrane samples were incubated with either an antibody directed against UCP2. Specificity was tested in tissue samples from splen of UCP2–/– mice and wild type mice, respectively (Suppl. Fig. 1). Control peptide for UCP2 was a recombinant hUCP2. Pan-Actin (Cell signalling technology, #4968), GAPDH (Calbiochem. #CD1001) or VDAC (Abcam, #ab34726) were used as loading controls. Signals were detected by horseradish peroxidise conjugated antibody (Abcam, ab6940).

Proliferation assay

The non-myocyte fraction of the isolated cells was used to separate cardiac fibroblasts. Non-myocytes were placed on a culture dish and the dishes were washed after 30 min to eliminate non attached cells (mainly endothelial cells). Proliferation was quantified by bromo-uridine incorporation. Cells were lysed after 12 and 48 h and stained with a fluorescence antibody. Quantification was subsequently performed via ELISA (5-Bromo-2’-deoxy-uridine Labeling and Detection Kit I; Merck, Darmstadt, Germany).

Amplex Ultra Red Measurements of mitochondria
The isolation of cardiac mitochondria from mice hearts has been described before. SSM and IFM were isolated as previously described. Mouse hearts were removed, washed in buffer A (100 mM KCl, 50 mM 3-[N-Morpholino]-propanesulfonic acid (MOPS), 5 mM MgSO4, 1 mM ATP, 1 mM EGTA, pH 7.4) and weighed. Ventricles were minced in 10 ml/g buffer B [buffer A + 0.04% bovine serum albumin (BSA)] with scissors and then with five strokes of a teflon pistill in a glass potter. The homogenate was centrifuged for 10 min at 800g. For isolation of SSM, the supernatant was centrifuged for 10 min at 8,000g, the sediment was resuspended in buffer A, centrifuged for 10 min at 8,000g, and the resulting sediment was resuspended in a small volume of buffer A. The sediment of the first centrifugation (used for isolation of IFM) was resuspended in buffer B (10 ml/g tissue) and after addition of 8 U/g of the protease nagarse incubated for 1 min on ice. The tissue was homogenized with five strokes of a teflon pistill in a glass potter and centrifuged for 10 min at 800g. The supernatant was centrifuged for 10 min at 8,000g, the resulting mitochondria were washed in buffer A, and finally resuspended in a small volume of buffer A. For subsequent Western blot analysis, SSM and IFM were further purified by percoll gradient ultracentrifugation. Mitochondria were lysed in cell lysis buffer [Cell Signaling, Beverly, MA, containing in mM: Tris pH 7.5 20, NaCl 150, EDTA 1, EGTA 1, Na pyrophosphate 2.5, β-glycerolphosphate 1, Na3VO4 1, Triton X-100 1%, Leupeptin 1 µg/ml, supplemented with 19 Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland)], and subsequently centrifuged at 13,000g for 10 min. The protein concentration of the supernatant was determined using the Dc protein assay (BioRad, Hercules, CA, USA). 25 µg of proteins were subjected to Western blot analysis. The quantification of ROS was performed by amplex UltraRed (Invitrogen, Eugene, USA) and 0.1 U/ml horseradish peroxidase as described before. Mitochondrial oxygen consumption was quantified with a Clarke-type oxygen electrode (Oxygen meter 782, Strathkelvin, Glasgow, UK) as described before.

Statistical analysis

All data were expressed as boxes representing the 25%, 50%, and 75% quartile with whiskers indicating the range of all data. Statistical analysis was performed using SPSS22.0. The different experimental groups were statistically analyzed by 1-way ANOVA and Newman-Keuls post hoc test for multiple comparisons. P values of < 0.05 were considered as statistically significant.

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