Material and methods

Cell culture, transfection and lentivirus

Pooled Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Verviers, Belgium) and cultured in EBM (Lonza) supplemented with 10% FBS (Invitrogen) and EGM-SingleQuots (Lonza). Cells were grown at 37°C and 5% CO2 and cell number was determined by NucleoCounter (ChemoMetec A/S). Lipofectamine RNAiMax (Life Technologies) was used to transfect 60-70% confluent HUVECs with 70nM siRNA (Sigma) against PFKFB3, AMPKα1, HK2 and PFK1 according to manufacturer’s instructions. Universal negative control siRNA (Sigma) was used as control. Cells were treated Nω-nitro-L-arginine-methyl ester hydrochloride (L-NAME, 1mM, Sigma). Long term overexpression of KLF2 and shRNA mediated silencing of KLF2 was done as previously described. PFKFB3 cDNA was obtained in pENTR221 (Source Bioscience) and shuttled into plenti4 vector (Invitrogen). Lentiviral particles were generated as previously described.

Shear stress

1x10⁵ HUVECs were plated overnight on µ -Slides I and exposed to laminar flow at a shear stress of 20 dynes/cm² for 72 hours controlled by an Ibidi perfusion system. HUVECs seeded on µ-Slides I (Ibidi) were used as static controls.

Cone plate viscometer setup

500,000 HUVECs were seeded overnight on 6cm dishes and were subjected to a fluid shear stress of 12 dyn/cm² for 48 hours in a cone plate viscometer as previously described. HUVECs maintained under static conditions served as controls.

In-vitro Glucose up-take

HUVECs were incubated with 100μM of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Life Technologies) for 1hour at 37°C and 2-NBDG fluorescence was measured in the FITC channel (FL-1) using a FACS Canto II device (BD Biosciences).

Glucose uptake imaging

1x10⁵ HUVECs were plated overnight on µ-slide y shaped (Ibidi) and exposed to laminar flow at a shear stress of 20 dynes/cm² for 72 hours controlled by an Ibidi perfusion system. Slides were incubated with 1mM of 2-NBDG (Life Technologies) for 30 min at 37°C and confocal micrographs were imaged on LSM 780, Axio Observer (Carl Zeiss) with a LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC M27 objective. 10 images each were analyzed from straight channel high shear region and branched low shear regions for 2-NBDG mean fluorescence and cell alignment using NIH ImageJ digital image analysis software.

Glucose transwell assay

Mock and KLF2 transduced cells were seeded on fibronectin coated 24-well ThinCert transwell PET inserts (Greiner bio-one). Cells were washed twice in Hank’s BSS (GE healthcare) and incubated with 100μM 2-NBDG (Life Technologies) and 10μM 20kDa -Dextran (TMR-Dextran, Sigma), which served as an internal control, in Hank’s BSS for 1 hour at 37°C. TMR-dextran fluorescence (Ex 525nm and Em 580-640nm) and 2-NBDG fluorescence (Ex 490nm and Em 510-570nm) were measured in duplicates from Hank’s BSS in the compartment underneath the cells using a GloMax®-Multi+ Microplate Multimode Reader with Instinct®
**RNA isolation and Real Time Quantitative Polymerase Chain Reaction**

Total RNA was isolated from cultured cells using miRNeasy kits (Qiagen) according to the manufacturer’s instruction. cDNA was synthesized from 0.5-1µg RNA using MuLV reverse transcriptase (Life Technologies) and random hexamers (Thermo Scientific). Real time quantitative PCR was carried out using Fast SYBR Green (Applied Biosystems) in a StepOnePlus machine (Applied Biosystems). Gene expression was normalized to RPLP0 or GAPDH. Primer sequences are listed in table 1.

**Mitochondrial DNA content measurement**

Total DNA was isolated from cells using DNeasy Blood and Tissue kits (Qiagen) as per manufacturer’s instruction. 30ng DNA was analyzed by real time qPCR for mitochondrial encoded gene ND1 and nuclear encoded gene RPLP0. The ratio of the relative levels of mitochondrial ND1 DNA and nuclear RPLP0 DNA was used to express mitochondrial DNA content.

**Mice experiments**

All mice experiments were carried out in accordance with the principles of laboratory animal care as well as according to the German national laws. The studies have been approved by the local ethical committee (Regierungspräsidium Darmstadt, Hessen). Cdh5-CreERT2 mice 5 and KLF2 flox/flox 6 were described previously and kindly provided by Dr. Adams (Münster, Germany) and Dr. Sebzda (Vanderbilt University, TN), resp. Animals were administered with seven injections of 2 mg tamoxifen base each (Sigma) intraperitoneally over a period of two weeks and sacrificed in the third week.

**Ex-vivo glucose up-take in mice hearts**

Mice hearts were subjected to Langendorff mediated perfusion and digestion as previously described 7 along with 50µM 2-NBDG. The digest was incubated with 200µM 2-NBDG and 5ul CD31-APC antibody (BD Pharmingen) for 30 min at 37°C and analyzed for CD31+ endothelial cell population and 2-NBDG fluorescence using a FACS Canto II device.

**Measurement of cellular metabolism**

HUVECs were seeded overnight at 6x10^4 cells per well on fibronectin (Sigma) coated Seahorse XF96 polystyrene tissue culture plates (Seahorse Bioscience). The plate was incubated in unbuffered DMEM assay medium (Sigma) for 1 hour in a non-CO2 incubator at 37°C before measuring in an XF96 extracellular flux analyzer (Seahorse Bioscience). Both OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were measured over 4 min periods with a mixing of 2 min in each cycle, with five cycles in total. Inhibitors and activators were used at the following concentrations: Glucose (10mM), Oligomycin (3µM), 2-DG (100mM), FCCP (1µM), Antimycin A (1.5µM) and Rotenone (3µM). Cellular DNA content using DAPI (Roche) was measured on a microplate reader (TECAN) and the data is represented as OCR or ECAR normalized to DNA content (RFU). Each measurement was averaged from triplicate wells.

**Mitochondrial activity measurement**
Cells were incubated with 0.5mg/ml of MTT (Dimethylthiazol-zyl-diphenyltetrazoliumbromide) for 4 hours at 37°C. After a wash with PBS, cells were lysed 30 min at room temperature and absorbance of the cleared lysate was photo metrically measured at 550nm.

**ATP assay**
Cell titer Glo luminescent cell viability assay kit (Promega) was used to quantify ATP levels in cells. 3x10⁴ cells in 100µl culture media were incubated with 100µl Cell-Titer-Glo Reagent (Promega) for 10 min at room temperature. Luminescence signal was measured with FlourChem M system (Proteinsimple) and signal normalized to background media control. All conditions were assayed in triplicates.

**Mitochondrial membrane potential measurement**
Mitochondrial membrane potential in HUVECs was assessed using MitoPT® TMRM Assay Kit (Immunochemistry Technologies LLC). Cells were incubated with 100nM TMRM for 20 min at 37°C and the orange red fluorescence (FL2) in cells was analyzed using FACS Canto II device (BD Biosciences).

**Proliferation assay**
Cells were incubated with 10mM BrdU for 45 min and subsequent staining was performed using BrdU Flow Kit (BD Pharmingen) as per manufacturer’s instruction. Cells were stained with 2.5µl of anti-BrdU-V450 for 20min and 10µl of 7-AAD for 10min, both at room temperature and analyzed using FACS Canto II device (BD Biosciences)

**Caspase 3/7 activity**
Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega) was used as per manufacturer’s instruction and Caspase 3/7 activity was analyzed by measuring fluorescence at with an excitation of 490nm and emission of 510-570nm using a GloMax®-Multi+ Microplate Multimode Reader with Instinct® (Promega). Mock transduced cells treated with 200nM of staurosporine for 4 hours were used as a positive control.

**Apoptosis measurement**
Mock- and KLF2 transduced cells were stained with Annexin V V450 (BD Biosciences) and 7-AAD (BD Biosciences) in binding buffer (BD Biosciences) for 15 min at room temperature as per manufacturer’s instructions. Apoptosis was quantified by flow cytometry analysis on a FACS Canto II device (BD Biosciences)

**Senescence staining**
Acidic β-gal staining mixture was added onto cells for 24 hours as per manufacturer’s instruction (Cell signaling). Cells were imaged on a bright field microscope (Axiovert 100, Zeiss) and 5 image fields were analyzed per condition for positive β-gal staining using digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss).

**Phospho kinase proteome profiling**
5x10⁶ mock- and KLF2- transduced cells were processed using Human Phospho-Kinase Array Kits (Proteome Profiler™ R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. The phosphorylation level of proteins was analyzed by quantification of membrane profile panels by NIH ImageJ digital image analysis software with a Dot Blot Analyzer macro
plugin. The signal values were subtracted from negative control values (PBS) and further normalized to whole membrane signal intensity.

**AMPKα1 phosphorylation measurement**

2x10^7 mock- and KLF2- transduced cells were analyzed using DuoSet IC Human Phospho AMPKα1 (T174) elisa kits (R&D Systems) according to manufacturer’s instruction. Absorbance was measured at 450nm using a GloMax®-Multi+ Microplate Multimode Reader with Instinct® (Promega) and wavelength correction was set to 560nm. Concentration was determined from a standard curve performed along with the samples.

**RNA sequencing**

For RNA sequencing, 0.5μg total RNA isolated from static HUVEC and shear stress exposed HUVEC were used. Sequencing libraries were prepared as described previously. The deep sequencing data was mapped against the reference genome GRCH37 by applying tophat2. Afterwards the transcript abundance was calculated by Cufflinks v2.2 based on the Ensemble annotation. The sequence data have been deposited in the NCBI GEO database under accession number GSE54384.

**Western blotting**

HUVECs were lysed in RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche) for 20min on ice. Western blot analysis was performed as described previously by using antibodies against PFKFB3, HK2, PFKP (1:1000, Cell signaling), KLF2 and Tubulin (1:2000, Thermo Scientific). All protein levels were normalized to tubulin signal.

**Luciferase assay**

Luciferase promoter reporter experiments were performed as described. Specifically, the region bearing the putative KLF2 binding site in PFKFB3 promoter or a mutated version of the KLF2 binding site (see Figure S3) were cloned into firefly luciferase reporter plasmid pGL4.10 according to manufacturer’s instructions (Promega). Cells were co-transfected with luciferase plasmid and pGL4 Renilla plasmid (Promega) as control for transfection efficiency by electroporation using Neon transfection system (Invitrogen). The activity of Luciferase and Renilla was assessed after 24 hours with the Dual Luciferase reporter assay system (Promega).

**Tube formation assay**

HUVECs were cultured at a density of 1x10^5 on Matrigel basement membrane matrix (BD) and tube formation was analyzed after 24 hours. Cumulative tube length was quantified from microscopic images taken from 5 random fields for every condition. Quantification was carried out by digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss).

**Spheroid based angiogenic assay**

Endothelial cell spheroids were generated as previously described. Cumulative length of sprouts was quantified from 10 spheroids for every condition. Quantification was carried out by digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss).

**Aortic Ring Outgrowth Assay**

Aortae were isolated from WT (KLF2^fl/fl^) and EC-KO (Cdh5-CreERT2;KLF2^fl/fl^) mice and cultured as previously described. Briefly, cleaned 1 mm long aortic tissue was embedded in rat...
tail collagen type I gel (1mg/ml, Millipore) in a 96 well plate and cultured in DMEM/F-12 medium (Life technologies) containing 2.5% FBS and treated with or without 3-PO (50µM, Merck Millipore). Endothelial sprouts were allowed to develop over 7 days, which were fixed with 4% PFA and stained with biotin Isolectin B4 (Vector laboratories) and streptavidin AlexaFluor 488 (Molecular Probes). Photomicrographs of sprouts from aortic rings were taken with Axio Observer.Z1 microscope (Zeiss) and cumulative outgrowth length from 3 rings per condition was quantified using NIH Image J digital image analysis software.

Statistical analysis
Data are expressed as mean ± S.E.M. GraphPad Prism 5 software was used to assess statistical significance by student t-test or Mann-Whitney U test when comparing two groups or analysis of variance (ANOVA) followed by Bonferroni’s correction or Kruskal-Wallis test with Dunns correction for multiple comparisons. Statistical significance was defined as follows: *p<0.05, **p<0.01, ***p<0.001

Table 1. Oligonucleotide primer sequence list

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<tr>
<th>Target gene</th>
<th>Species</th>
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<th>Reverse primer</th>
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References


