Loss of Glyoxalase 2 alters the glucose metabolism in zebrafish

Christoph Tobias Tabler[1], Elisabeth Lodd[1], Katrin Bennewitz[1], Chiara Simone Middel[1], Vanessa Erben[1], Hannes Ott[1], Tanja Poth[2], Thomas Fleming[3], Jakob Morgenstern[3], Ingrid Hausser[4], Carsten Sticht[5], Gernot Poschet[6], Julia Szendroedi[3], Peter Paul Nawroth[3], Jens Kroll[1*]

[1] Department of Vascular Biology and Tumor Angiogenesis, European Center for Angioscience (ECAS), Medical Faculty Mannheim, Heidelberg University, 68167 Mannheim, Germany

[2] CMCP - Center for Model System and Comparative Pathology, Institute of Pathology, Heidelberg University Hospital, 69120 Heidelberg, Germany

[3] Department of Internal Medicine I and Clinical Chemistry, Heidelberg University Hospital, 69120 Heidelberg, Germany

[4] Institute of Pathology IPH, EM Lab, Heidelberg University Hospital, 69120 Heidelberg, Germany

[5] NGS Core Facility, Medical Faculty Mannheim, Heidelberg University, 68167 Mannheim, Germany

[6] Metabolomics Core Technology Platform, Centre for Organismal Studies, Heidelberg University, 69120 Heidelberg, Germany

***Corresponding Author:**

Prof. Dr. Jens Kroll

European Center for Angioscience (ECAS)

Dept. of Vascular Biology & Tumor Angiogenesis

Medical Faculty Mannheim, Heidelberg University

Ludolf-Krehl-Str. 13-17, 68167 Mannheim, Germany

Phone: +49 (0) 621 383 71455

Fax: + 49 (0) 621 383 9961

Email: jens.kroll@medma.uni-heidelberg.de

Supplemental Material and Methods

Electron microscopy of the kidney

The kidneys were dissected in ice-cold PBS, as described under ”preparation of tissues” and directly transferred to a 3 % GA solution buffered by 0.1 M cacodylate (CAS 6131-99-3) at a pH of 7.4 for fixation. Subsequently, they were cut into pieces of ca. 1 mm^3, washed in buffer and postfixed for 1 h at 4 °C in 1 % aqueous osmium tetroxide (Sigma, CAS 20816-12-0). Afterwards rinsed with water and dehydrated using graded ethanol solutions. Then transferred to propylene oxide and embed in epoxy resin (glycidether 100). Semithin and ultrathin sections were cut using an ultramicrotome (Reichert Ultracut E). The semithin sections of 1 µm were stained with methylene blue and analyzed under a light microscope (Olympus) at 200 x magnification, to identify glomeruli. 60 - 80 nm ultrathin sections were treated with uranyl acetate (CAS 6159-44-0) and lead citrate and examined with a transmission electron microscope (JEM 1400), equipped with a 2k TVIPS CCD camera (TemCam F216). Pictures were taken at 3000 - 10.000 x magnification. Tilings of glomeruli were created from these pictures and analyzed using EMMeasure.

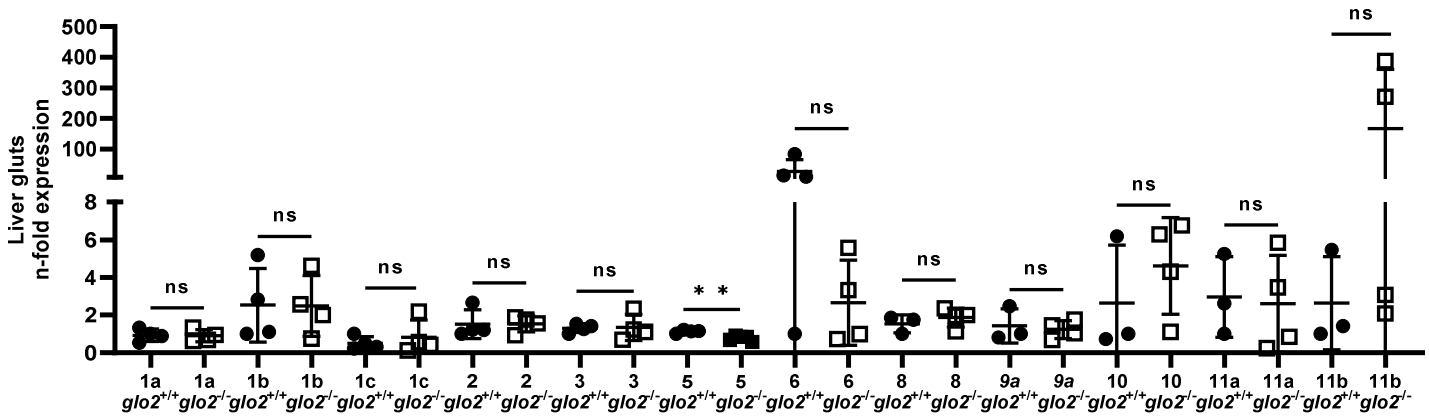
Confocal microscopy of the retina

For confocal imaging of the retina vasculature in *Tg(fli1:EGFP)* zebrafish, the animals were prepared as described under “preparation of tissues”. The heads of the zebrafish were fixed in 4 % PFA solution overnight. Afterwards, they were transferred to a Petri dish containing an agarose platform and covered with 1x PBS. The eyes were removed from the head using micro tweezers. Afterwards, the retina was detached from the eyes under a stereomicroscope using micro tweezers and a microlancet and washed twice. The washed retina was then transfer to a microscope slide, covered with mounting media and carefully covered with a cover slip. Following this, the retina was imaged using a confocal microscope (Leica DM6000B) with a Leica TCS SP5 DS scanner. The images were taken at 600 Hz, 1024 x 1024 pixels and a z-stack height of 1.5 µm, and analyzed using Leica Appliction Suite X, ImageJ and Gimp2.

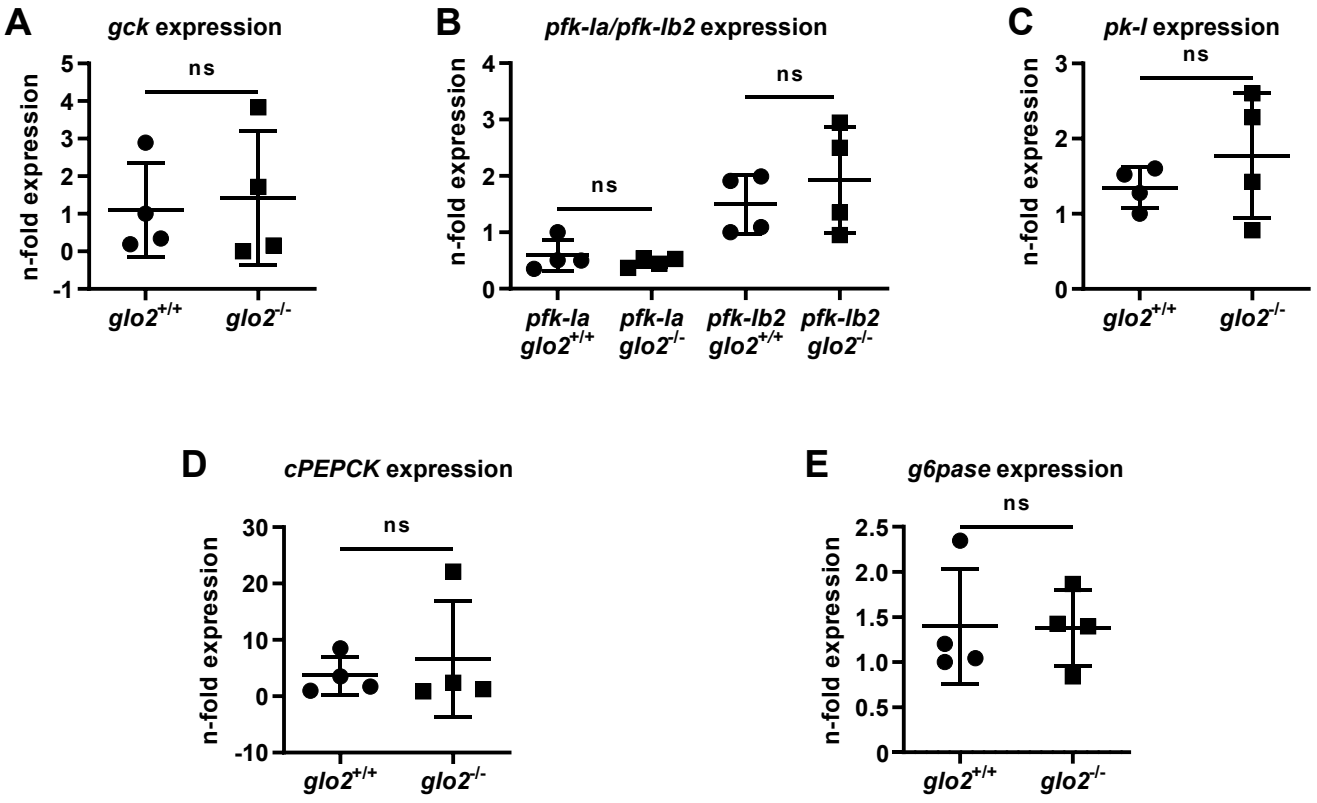
RNA-sequencing of the liver

For the RNA-sequencing of the liver, total RNA was isolated from adult livers, which had been prepared and flash frozen as described under “preparation of tissues”. The RNA was prepared using the RNeasy Mini Kit (QIAGEN). The quality control of the RNA and analysis of the sequencing data were conducted by the Bioinformatics Core-Facility. The library construction and strand-specific RNA sequencing were performed by the Beijing Genomic Institution

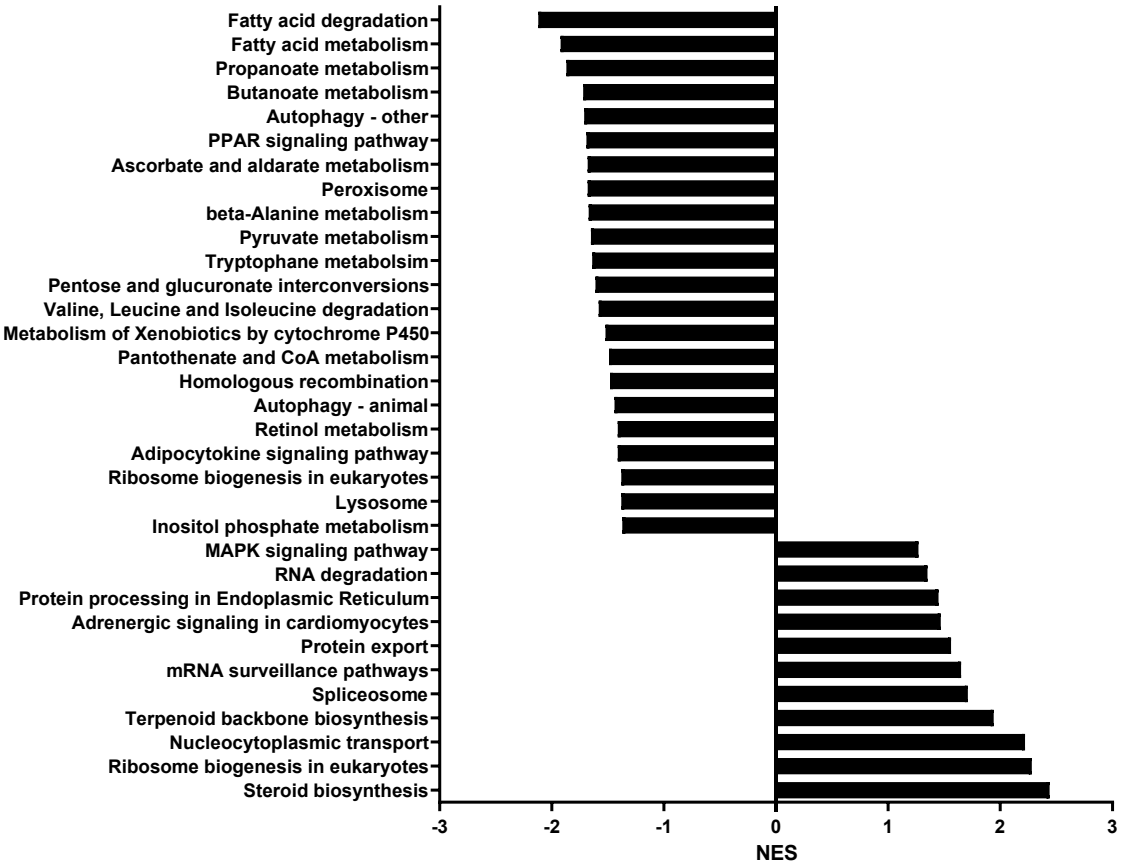
(www.bgi.com, BGI) using a BGISEQ-500. The sequencing analysis was performed by the Next-Generation Sequencing (NGS) Core facility of the Medical Faculty Mannheim of the University of Heidelberg.



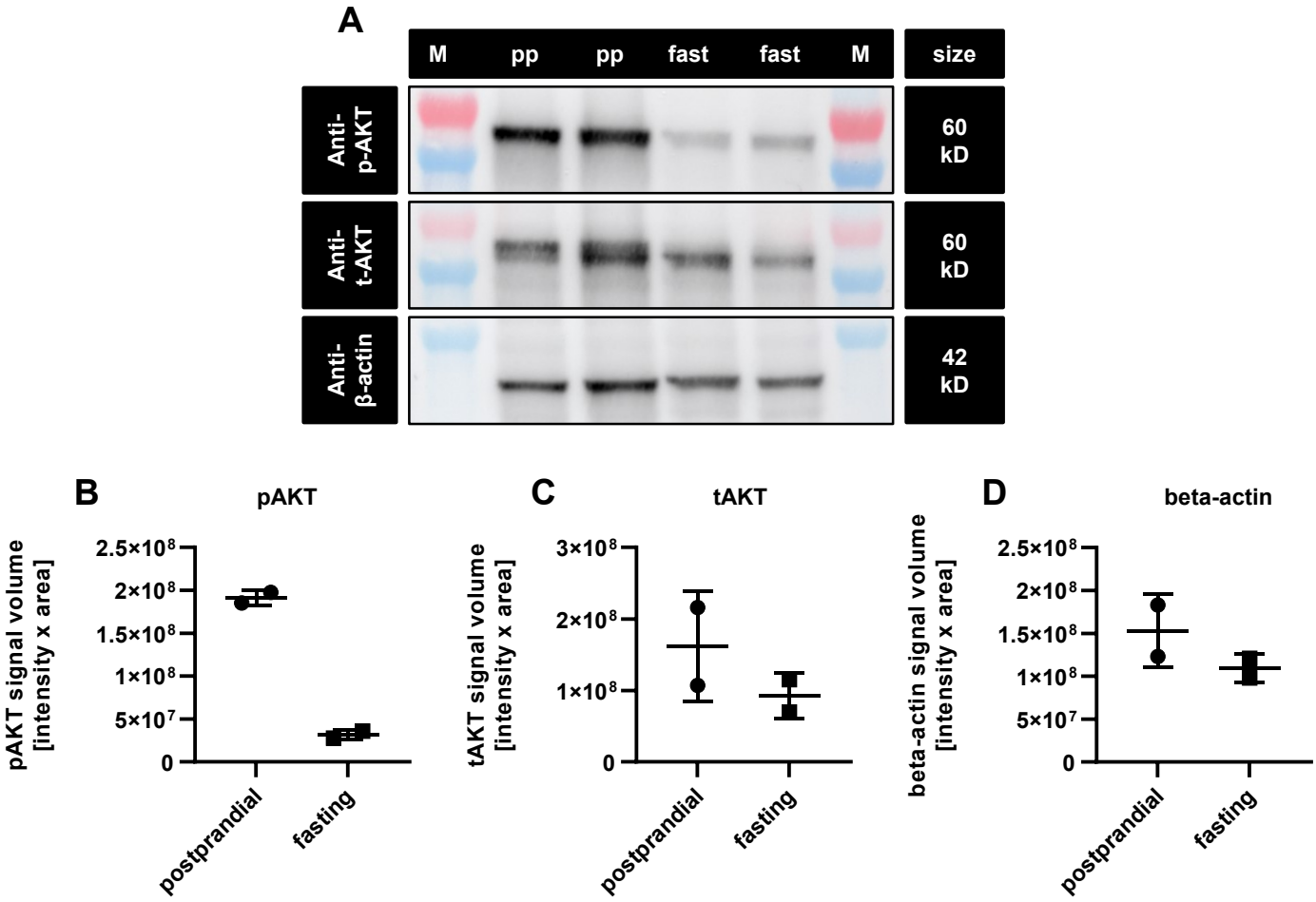
Supplemental Figure 1: The expression patterns of glucose transporters in adult *glo2*$^{-/-}$ liver appeared unaltered, while the expression of the fructose transporter *glut5* appears apears diminished. While the expression of the glucose transporters 1a, 1b, 1c, 2, 3, 6, 8, 9a, 10, 11a, 11b mRNA appeared to be unchanged in adult *glo2*$^{-/-}$ liver tissue, the fructose transporter *glut5* was significantly downregulated in the liver of *glo2*$^{-/-}$ zebrafish. N = 3 / 4. Measured via RT-qPCR. One datapoint represents the liver of one adult zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test, or Mann-Whitney-Test. Data presented as mean ± SD. ns = p > 0.05, glut: facilitated glucose transporter member.



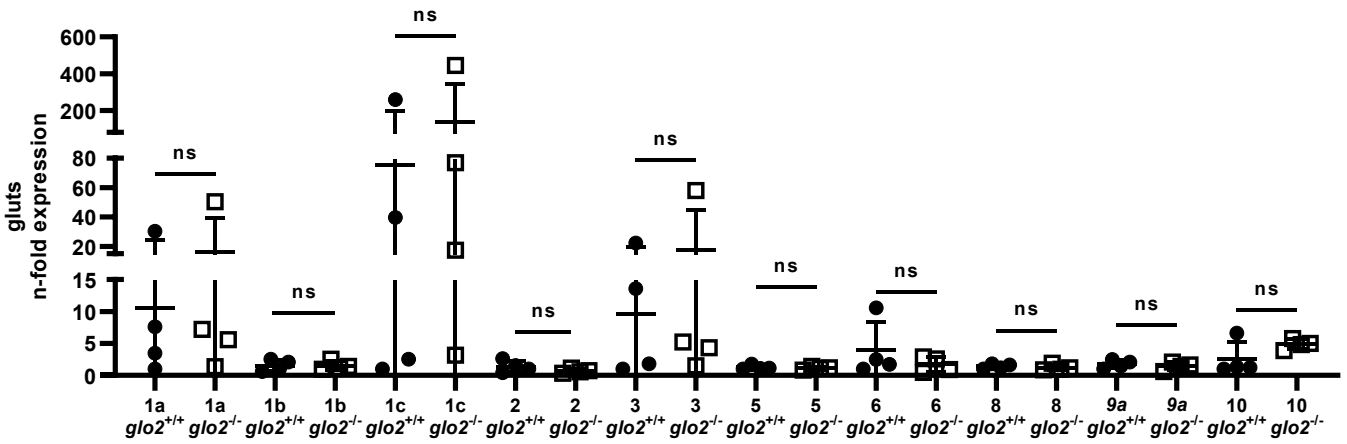
Supplemental Figure 2: **The gene expression of the rate-limiting enzymes of glycolysis and gluconeogenesis was unchanged in adult *glo2*$^{-/-}$ liver.** The gene expression of glucokinase (**A**), phosphofructokinase (**B**), pyruvate kinase (**C**), phosphoenolpyruvate carboxykinase (**D**) and glucose-6-phosphatase (**E**) was unchanged in the adult liver. N = 4. Measured via RT-qPCR. One datapoint represents RNA from the liver of one zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test (**A**, **B**, **C**), or Mann-Whitney-Test (**D**, **E**). Data presented as mean ± SD. ns = p>0.05. gck: glucokinase, pfk-la/lb2: 6-phosphofructokinase liver type a/b2, pk-l: pyruvatekinase liver / red blood cells, cPEPCK: phosphoenolpyruvate carboxykinase 1 (cytosolic), g6pase: glucose-6-phosphatase



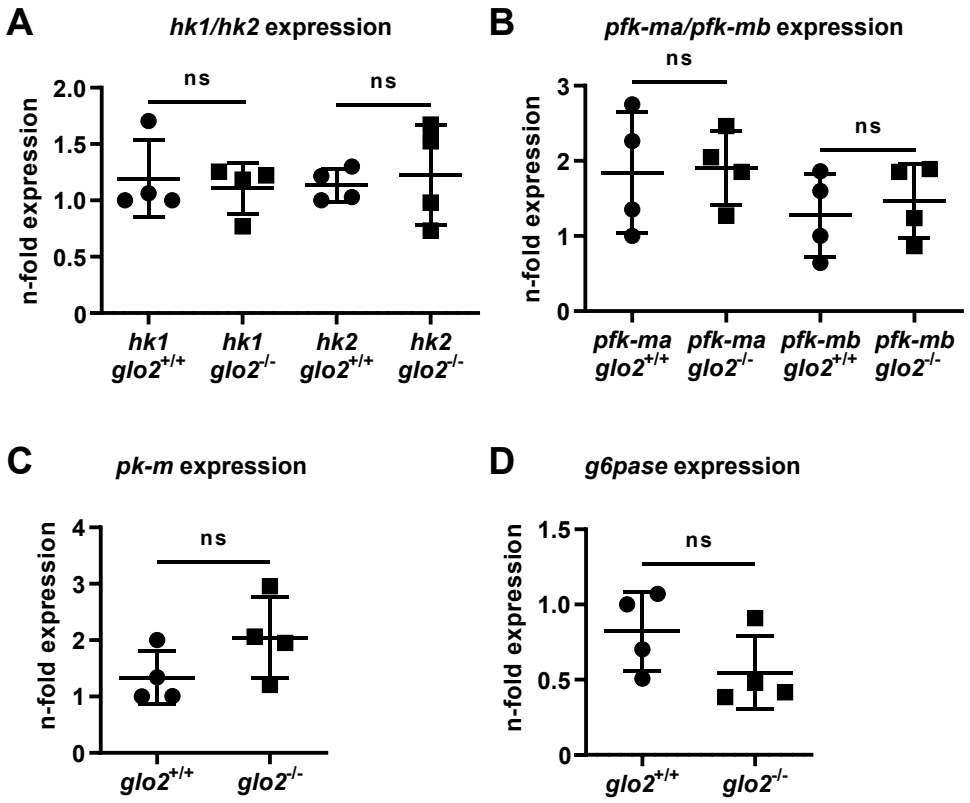
Supplemental Figure 3: *glo2*$^{-/-}$ zebrafish showed a strong downregulation of fatty acid metabolism in the liver as well as an increase of steroid biosynthesis and MAPK signaling. Graph depicting all significantly up- and downregulated KEGG pathways in adult liver in *glo2*$^{-/-}$ compared to *glo2*$^{+/+}$ samples. N = 6. KEGG: Kyoto encyclopedia of genes and genomes, NES: normalized enrichment score, PPAR: peroxisome-proliferator-activating receptor, CoA: coenzyme A, MAPK: mitogen activated protein kinases.



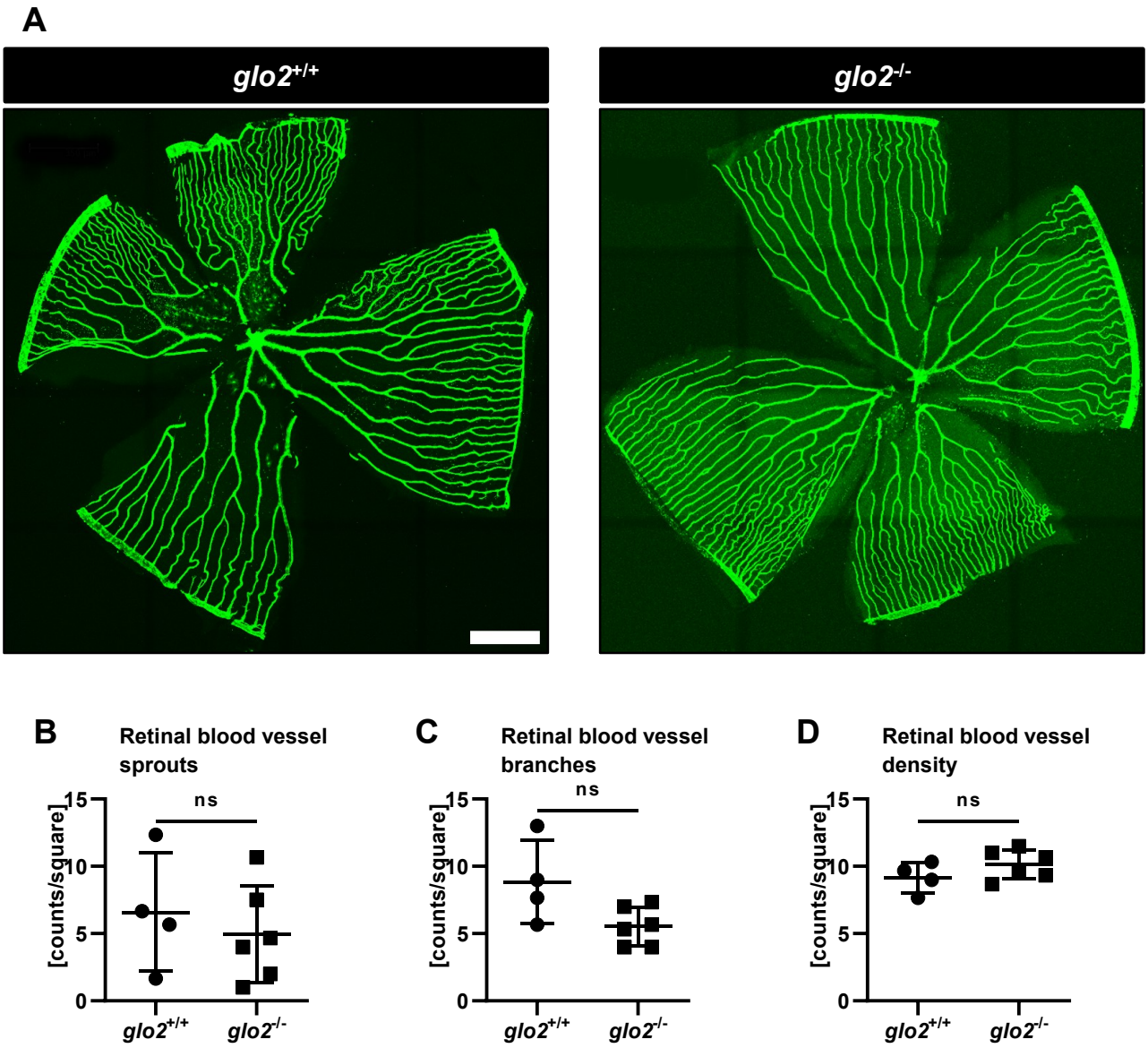
Supplemental Figure 4: Postprandial AKT activation in the liver is evident in wildtype zebrafish. A) The liver of wildtype zebrafish showed a strong increase in phosphorylation of AKT, measured via Western blot two hours postprandially, indicating increased insulin signaling acitvity. Original image has been cropped. **B)** The quantification of the pAKT Western blot showed a strong increase in phosphorylation in the postprandial liver compared to the liver of fasting zebrafish. **C)** The total amount of AKT was unchanged in postprandial and fasting states. **D)** The quantification of the housekeeping gene β-actin showed no significant difference in signal volume, indicating equal loading in all samples. One datapoint represents one zebrafish liver. N = 2. For statistical analysis unpaired t-test were used (**B-D**). Data presented as mean ± SD. M: marker, pp: postprandial, fast: fasting, pAKT: phosphorylated AKT, tAKT: total AKT, kD: kilodalton.



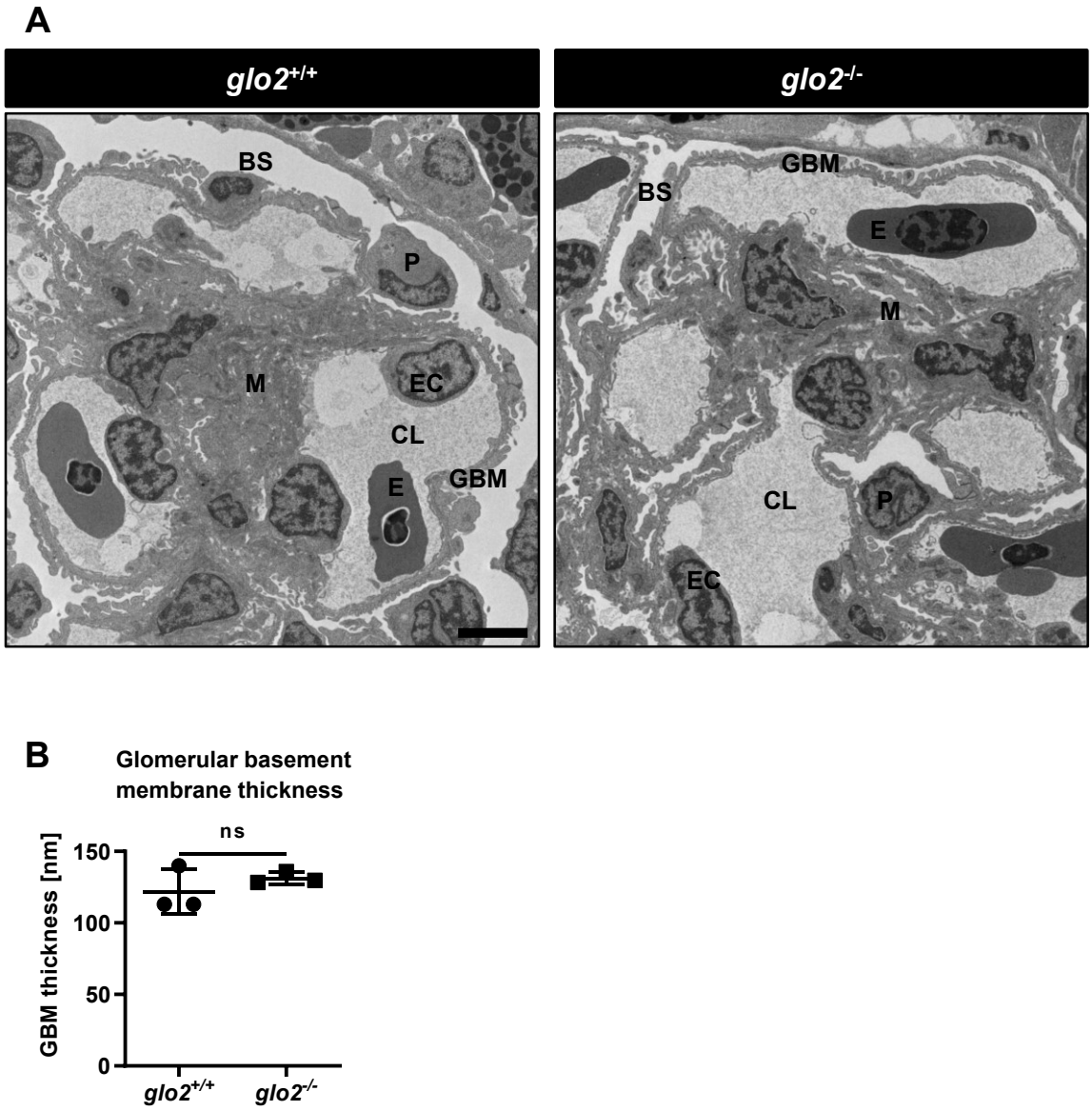
Supplemental Figure 5: The expression patterns of glucose transporters in adult *glo2*$^{-/-}$ skeletal muscle appeared unaltered. The expression of the glucose transporters 1a, 1b, 1c, 2, 3, 6, 8, 9a, 10, as well as the fructose transporter *glut5* mRNA appeared to be unchanged in adult *glo2*$^{-/-}$ skeletal muscle tissue. N = 3 / 4. Measured via RT-qPCR. One datapoint represents skeletal muscle tissue of one adult zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test, or Mann-Whitney-Test. Data presented as mean ± SD. ns = p>0.05, glut: facilitated glucose transporter member.



Supplemental Figure 6: **The expression of rate-limiting enzymes of glycolysis and gluconeogenesis was unchanged in adult *glo2*$^{-/-}$ skeletal muscle.** The gene expression of hexokinase (**A**), phosphofructokinase (**B**), pyruvate kinase (**C**), and glucose-6-phosphatase (**D**) remained unchanged. N = 4. Measured via RT-qPCR. One datapoint represents skeletal muscle tissue of one zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test (**A**, **B**, **C**, **D**), or Mann-Whitney-Test (**A**). Data presented as mean ± SD. ns = p>0.05. Hk1/2: hexokinase 1/2, pfk-ma/mb: 6-phosphofructokinase muscle type a/b, pk-m: pyruvate kinase muscle, g6pase: glucose-6-phosphatase



Supplemental Figure 7: Confocal imaging of adult retina revealed no alterations of the vascular structure. A) Confocal imaging of EGFP-marked retinal vasculature showed normal vascular architecture in *glo2*$^{-/-}$ zebrafish. Scale bar = 350 µm. **B)** Blood vessel sprouts appeared unaltered in the *glo2*$^{-/-}$ retina. **C)** Blood vessel branches appeared unaltered in the *glo2*$^{-/-}$ retina. **D)** Blood vessel density appeared equally unaltered in the *glo2*$^{-/-}$ retina. Counted in 350 µm squares, datapoints of several squares were averaged for every zebrafish. N = 4 / 6. One datapoint represents the retina of one zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test (**B**, **C**, **D**). Data presented as mean ± SD. ns = p>0.05.



Supplemental Figure 8: Transmission electron microscopy of the adult *glo2*$^{-/-}$ kidney revealed a normal morphology of glomeruli. A) Tilings of glomeruli showing an overview of the glomerular structure. Original pictures taken at 3000 x magnification. Scale bar = 3 µm. BS: bowman space, P: podocyte, M: mesangium, EC: endothelial cell, CL: capillary lumen, E: erythrocyte, GBM: glomerular basement membrane. **B)** The glomerular basement membrane thickness was unchanged in all zebrafish. N = 3. One datapoint represents the averaged data of the glomeruli of one zebrafish. For statistical analysis normality tests were used for all samples, followed by unpaired t-test (**B**). Data presented as mean ± SD. ns = p>0.05.

Supplemental Table 1: Primer list

Primer name	Application	Forward primer sequence	Reverse primer sequence
Glo2	Genotyping	GCGGGCTCTATCTCTGGTTT	GCATGACCAGCTGACTTCAA
Glo2	Genotyping	TCTTAGATGGGATGGTGGGC	ACAATGCATGACCAGCTGAC
Glo2	qPCR	GCACCAGCTGTGTTTACGGG	ACACACGCGTTTCTGGTGGA
b2m	qPCR	ACTGCTGAAGAACGGACAGG	GCAACGCTCTTTGTGAGGTG
Insra	qPCR	AGAGGCCAGCGAGCTCTAC	CACTTGTGTGGGGGCTCT
Insrb	qPCR	GCCTCTGCGGATCACTACAT	CTCCTGCGTGGTCTTGAAC
mPEPCK	qPCR	TGCCTGGATGAAATTTGACA	GGCATGAGGGTTGGTTTTTA
glut1a	qPCR	TGACCGGCCCATACGTTTTC	ATCATCTCGGTTATATTTATCTGCC
glut1b	qPCR	CCATTTCTCCTGGGCTTTACCTTTA	CAGATTTGGCTTTGCTTTCCTCGTT
glut1c	qPCR	CATCCGTAATATTCAGGTGCTAGTG	ATTTTCAGCAGAGGTGGAAAGAG
glut2	qPCR	GCAGAAGAACCCTCACTC	TCTCCGCCACAATAAACC
glut3	qPCR	TCGTCAATGTCTTGGCTCTG	CAACATACATTGGCGTGAGG
glut5	qPCR	TCTCTGGTTGCTGGATTTGGT	CAAGAGGGTGAGGAGATTGTCC
glut6	qPCR	TTGCTATTGCAGCCAGTTTG	CAGGCCGTCTGTTAGGGTAA
glut8	qPCR	CATTTTGTCTGGTGTCGTCATGT	CCTGCAATGAAAAAGCCCAT
glut9a	qPCR	GAGGCCGGAGCAGAGAAAGCGTTC	AGCATTCAGTCCACACAGCTGATA
glut10	qPCR	GCACTTCAGTAGGACCGCAT	GTGCCCACATTAAAGCAGTTGA
glut11a	qPCR	CCCTGGGAACTATCCCTCAT	TCCACTGATTGCCAACACAT
glut11b	qPCR	AAGGATGAGTACTGGCCGATCCTC	AATGCCGAGAGCGCTGACCCTTTC
gck	qPCR	AATCACCGCTGACCTGCTAT	GCCACTTCACATACGCAATG
pfk-la	qPCR	ACTGCCACTCCAGCGTTAAA	CAGAGCTGGAGTTCACCCTC
pfk-lb2	qPCR	GCCGTTCAACATTCACGACC	TGCAGTCGAACACTCCTTGG
pk-l	qPCR	TCCTGGAGCATCTGTGTCTG	GTCTGGCGATGTTCATTCCT
cPEPCK	qPCR	ATCACGCATCGCTAAAGAGG	CCGCTGCGAAATACTTCTTC
g6Pase	qPCR	TCACAGCGTTGCTTTCAATC	AACCCAGAAACATCCACAGC
hk1	qPCR	ATGATAGCGGCACAGCTTCT	GTTGGTGTCTCGTGCCAATC
hk2	qPCR	TGAGGTCAGTCTCGTCCAGT	TCTTAATCGACAGGCCACCG
pfk-ma	qPCR	CTCTGTGTAATCGGCGGTGA	ATGGAGCCAACCATACCCAC
pfk-mb	qPCR	ACTGTCGGTTTGCTGTACTC	GCTCGAACAGCAGCATTCAT
pk-m	qPCR	TGGGCTTATTAAGGGCAGTG	TGCACCACCTTTGTGATGTT