**Additional Files.**

**Plekhm2 acts as an autophagy modulator in murine heart and cardiofibroblasts but is not vital for myocardial function under stress**

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**Abbreviated title:** Roles of Plekhm2 in the heart

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**Additional file 1. Supplementary Materials and Methods**

**Generation of Plekhm2 floxed mice for Plekhm2 KO cell cultures**

Plekhm2tm1a mice were mated with a mouse (JAX 009086) expressing the enzyme flippase (FLP**+/+**). The flippase deletes the sequence between the FRT sites, thereby removing the LacZ+ Neo cassette and leaving only the LoxP sites flanking exon 8 (named Plekhm2tm1c = floxed Plekhm2 gene). Mice born from this pairing were interbred with WT mice (FLP**−/−**) to obtain Plekhm2floxed/floxed /FLP**−/−** mice. Neonatal cells were isolated from Plekhm2floxed/floxed/FLP**−/−** mouse hearts and incubated with adenovirus expressing the Cre-recombinase enzyme (as we previously described {Segal, 2022 #124}), resulting in the deletion of exon 8 and Plekhm2 KO. Cells transfected with the control adenovirus express Plekhm2 protein normally.

**Echocardiographic analysis**

Two-dimensional images of the left ventricle were obtained in parasternal long- and short-axis views. Long- and short-axis M-mode images were taken at the mid-papillary muscle level with cursor penetration at the papillary muscle tip. The LV end-systolic diameter (LVDes) and LV end-diastolic diameter (LVDed) were evaluated from the long-axis M-mode trace. Calculations of LV fractional shortening (FS, %) were conducted using (LVDed − LVDes)/LVDed × 100. LV ejection fraction (LVEF) was calculated using planimetry as follows: EF = 100 × (LVD3ed − LVD3es/LVD3ed). The maximum duration of the echocardiographic procedure was 15 minutes.

**Gene expression analysis by real-time fluorescent quantitative PCR**

Total RNA was extracted from cells or hearts homogenized in TRI reagent using a Direct-zolTM RNA Mini-Prep kit (#R2050-1-50, Zymo Research) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop1000 spectrophotometer (Thermo Fisher Scientific, MA, USA). cDNA synthesis was performed using random hexamers and TaqMan reverse transcription reagents according to the manufacturer's protocol (Quanta BioSciences, MA, USA). Gene expression was examined with reverse transcription-quantitative polymerase chain reaction (qPCR) and PerfeCTa SYBR Green FastMix (Quanta BioSciences) using a QuantStudio5 Real Time PCR System (Thermo Fisher Scientific). Reaction programs comprised pre-incubation of 50°C for 3 minutes and 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds and a final dissociation stage. Relative gene expression was calculated by the efficiency 2−ΔΔCT method or as relative measurements (2−ΔCT) with the expression of the genes of interest (Table 3S) normalized to that of the housekeeping gene GAPDH. Each sample was tested in triplicate.

**Additional file 2. Supplementary Figures**

**Fig. 1S** Deterioration in the physiological parameters of PLK2-KO mice with aging. Echocardiography was conducted in 3-, 6-, and 12-month-old mice and several parameters were examined. **A.** Stroke volume (mL). **B.** Cardiac output (µL/min). **C.** LV mass (mg). **D.** Total body weight (g). Statistical analyses were conducted with GraphPad Prism. \*p<0.05 and \*\*p<0.01 for same-age PLK2-KO versus WT.

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**Fig. 2S** Decrease in the expression of autophagy-related genes in WT but not Plekhm2 KO mice. mRNA levels of *becn1*, *atg5,* and *ctsl* were evaluated in CQ-treated NMCFs and untreated cells. Calculation of the delta expression before and after CQ addition demonstrated that CQ significantly reduced *atg5*, *becn1*, and *ctsl* in control but not KO cells. Statistical analysis was conducted with a Wilcoxon signed-rank test and the column median was compared to a hypothetical value using GraphPad Prism software. #p<0.05 and ##p<0.01 versus the hypothetical value of 0, and \*p<0.05 and \*\*p<0.01 for KO-NMCFs versus control following CQ (Mann–Whitney nonparametric test).

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**Fig. 3S** Confirmation of Plekhm2 ablation in mice and primary murine cardiac cells. **A.** Schematics representing the Plekhm2tm1a(EUCOMM/Wtsi) mouse genotype. **B.** The positions of the primers used for genotyping. The primers Plekhm2 44411 F and Plekhm2 44411 R are on the normal genomic DNA flanking the cassette. The distance between them is too large to produce a PCR fragment when the cassette is inserted, representing KO mouse. When the lacZ-neo cassette is not present, the PCR product will comprise 478 bp, representing WT mouse. An additional primer, named CAS R1 Term, was used with Plekhm2 44411 F to verify the presence of the cassette. This will result in a 300-bp product **(C)**. When these mice are crossed with Flp mice, the sequence flanked by the FRT is removed. These Plekhm2floxed/floxed/FLP**−/−** mice will further be used for neonatal cardiac cell isolation. **D.** For deletion of Plekhm2 from neonatal cells, we transfected the cells with Cre-recombinase enzyme. Because Plekhm2 44411 R primer is located between the LoxP sites, it will be removed by the Cre enzyme. Thus, no PCR product is expected for the KO cells. Plekhm2 deletion from NMCMs and NMCFs (CRE) versus control cells (GFP). The molecular size marker on the right side of the photographs is GeneRuler 1 kb Plus DNA Ladder of Thermo Fisher Scientific.

**Additional file 3. Supplementary Tables**

**Table 1S** Basic physiological parameters of 3-month-old male WT and PLK2-KO mice

|  |  |  |
| --- | --- | --- |
| **PLK2-KO (n=20)** | **WT (n=17)** | **Echocardiographic****measurements** |
| 25.8 ± 0.48\* | 28.4 ± 0.66 | Weight (g) |
| 457.3 ± 8.88 | 476.5 ± 12.9 | HR (bpm) |
| 3.86 ± 0.08 | 3.91 ± 0.1 | LVIDd (mm) |
| 2.68 ± 0.09 | 2.71 ± 0.11 | LVIDs (mm) |
| 0.85 ± 0.03 | 0.93 ± 0.09 | LVPWd (mm) |
| 1.19 ± 0.04 | 1.28 ± 0.08 | LVPWs (mm) |
| 58.5 ± 1.96 | 58.7 ± 2.3 | EF (%) |
| 30.7 ± 1.32 | 30.9 ± 1.6 | FS (%) |
| 43.2 ± 1.9\* | 49.9 ± 1.44 | SV (µL) |
| 21.3± 2.06 | 23.9 ± 1.77 | CO (µL/min) |
| 0.90 ± 0.02 | 0.98 ± 0.05 | RWT (mm) |
| 4.46 ± 0.18 | 4.91 ± 0.23 | LV mass/BW |

|  |  |  |
| --- | --- | --- |
| **PLK2-KO** **(n=10)** | **WT (n=6)** | **Gravimetric analysis** |
| 27.3 ± 0.46\* | 28.9 ± 0.70 | BW (g) |
| 119.4 ± 4.71 | 128.0 ± 6.0 | HW (mg) |
| 18.3 ± 0.24 | 18.4 ± 0.21 | TL (mm) |
| 6.67 ± 0.35 | 6.73 ± 0.19 | HW/TL |

Basic physiological parameters and gravimetric measurements were examined in young (3-month-old) male PLK2-KO and their WT normal sibling. HR, heart rate; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; EF, ejection fraction; FS, fractional shortening; LVVd, left ventricular volume in diastole; LVVs, left ventricular volume in systole; SV, stroke volume; CO, cardiac output; RWT, relative wall thickness; BW, body weight; HW, heart weight; TL, tibia length. \*p<0.05 for KO versusWT.

**Table 2S** Deterioration in physiological parameters with aging

|  |  |  |
| --- | --- | --- |
| **PLK2 KO (n=6)** | **WT (n=6)** | **6 months old** |
| 21.4 ± 1.74\* | 26.6 ± 0.65# | Weight (g) |
| 457.8 ± 13.6 | 485.2 ± 12.1 | HR (bpm) |
| 3.52 ± 0.17 | 3.94 ± 0.12 | LVIDd (mm) |
| 2.53 ± 0.24 | 2.95 ± 0.19 | LVIDs (mm) |
| 0.80 ± 0.03 | 0.80 ± 0.04# | LVPWd (mm) |
| 1.02 ± 0.08 | 1.11 ± 0.09 | LVPWs (mm) |
| 55.3 ± 5.7 | 49.9 ± 5.3 | EF (%) |
| 28.8 ± 3.6 | 25.5 ± 3.8 | FS (%) |
| 70.3 ± 6.6 | 84.3 ± 42.9 | LVVd (µL) |
| 36.5 ± 7.2 | 42.9 ± 4.6 | LVVs (µL) |
| 35.6 ± 2.5 | 41.3 ± 2.9 | SV (µL) |
| 16.6 ± 0.8\* | 19.9 ± 1.1 | CO (µL/min) |
| 0.80 ± 0.02 | 0.83 ± 0.03 | RWT (mm) |
| 81.6 ± 5.07\*,# | 103.9 ± 6.4# | LV mass (mg) |

Basic physiological parameters and gravimetric measurements were examined in 6-month-old) female PLK2-KO and their WT normal siblings. HR, heart rate; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVPWd, left ventricular posterior wall in diastole; LVPWs, left ventricular posterior wall in systole; EF, ejection fraction; FS, fractional shortening; LVVd, left ventricular volume in diastole; LVVs, left ventricular volume in systole; SV, stroke volume; CO, cardiac output; RWT, relative wall thickness; BW, body weight; HW, heart weight; TL, tibia length. \*p<0.05 for same-aged KO versusWT; #p<0.05 for 6-month-old WT or KO versus 3-month-old WT.

**Table 3S** Primer sequences used in this study

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| Primer sequence (5'-3')-R | Primer sequence (5'-3')-F |
| Plekhm2r TCGTCCAGCTTGGTCTTTTT | Plekhm2f GCGTCATAACCCCTTCAATG |
| Plekhm1r AGCTTGGGTCGTACAAAGGA | Plekhm1f ACTTGGTGGGAGTCTGGATG |
| Plekhm3r GGCAGGGGACAGACTTTTCT | Plekhm3f CTGTAACAACGGCGAGATCC |
| mActa1r CCACCGATCCACACTGAGTA | Acta1f AAGTGCGACATCGACATCAG |
| mActa2r CACCAGGGCTGTGCTGTCTT | Acta2f AGCCAGTCGCTGTCAGGAA |
| Nppar AATGTGACCAAGCTGCGTGA | Nppaf GCTGCAACAGCTTCCGGTA |
| Nppbr TGGTCCTTCAAGAGCTGTCTC | Nppbf AGGTGCTGTCCCAGATGATT |
| Myh7r TCCACGATGGCGATGTTCT | Myh7f CCTCCAGAGTCTGCTGAAGGA |
| TGFb1r TGGTTGTAGAGGGCAAGGAC | TGFb1f TTGCTTCAGCTCCACAGAGA |
| LCBr CGCCGTCTGATTATCTTGATG | LCBf CCACCAAGATCCCAGTGATTATAG |
| P62r TGGGAGAGGGACTCAATCAG | P62f ACAGATGCCAGAATCGGAAG |
| BCIn1r ATCTTGCCTTTCTCCACGTC | BCIn1f TTTGACCATGCAATGGTAGC |
| Col1a1r GACGTGCTTCTTTTCCTTGG | Col1a1f TGACTGGAAGAGCGGAGAGT |
| Col3a1r GTCACCATTTCTCCCAGGAA | Col3a1f CAATATGCCCACAGCCTTCT |
| Col1a2r TGGGACCATCAACACCATC | Col1a2f TGCTCAGCTTTGTGGATACG |
| ATG5r CGGAACAGCTTCTGGATGA | ATG5f CAACCGGAAACTCATGGAAT |
| Mctsl1r CATAGCCATAGCCCACCAAC | Mctsl1f TCTGTTGCTATGGACGCAAG |
| KIF5Br TTCTACAATCCCAAGGAATAGAGG | KIF5Bf GGAGGCAAGCAGTCGTAAAC |
| KLC1r CCATGCTCTCAGGGTCATTT | KLC1f AGCTGCAGAGACATTGGAAGA |
| Arl8Br CTTCTCCGGGATTTTGAGTG | Arl8Bf GCCTTGGATGAGAAACAGCTA |
| Rab7ar CTGGCCTGGATGAGAAACTC | Rab7af CTGACCAAGGAGGTGATGGT |
| Gapdhr CCAATACGGCCAAATCCGT | Gapdhr TCTTGTGCAGTGCCAGCCT |