**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 Material and Methods**

**Generating Plekhm2 floxed mice for Plekhm2 KO cell cultures**

Plekhm2tm1a mice were mated with a mouse (JAX 009086) that expresses the enzyme flippase (FLP**+/+**). The flippase deletes the sequence between the FRT sites, thus removing the LacZ+ Neo cassette, 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 get Plekhm2floxed/floxed /FLP**-/-** mice. Neonatal cells were isolated from Plekhm2floxed/floxed /FLP**-/-** micehearts and incubated with adenovirus expressing the Cre-recombinase enzyme (as we previously described {Segal, 2022 #124}) resulting in a deletion of exon 8 and Plekhm2 KO. Cells transfected with the control adenovirus express Plekhm2 protein normally.

**Echocardiography**

2D 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. 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 (F.S. - %) were done according to (LVDed-LVDes)/LVDed × 100. LV ejection fraction (LVEF) was calculated using planimetry as follows: EF = 100 × (LVD3ed - LVD3es / LVD3ed). The maximum duration of the echocardiography procedure was 15 minutes.

**Gene expression analysis by Real-time fluorescent quantitative PCR (qPCR)**

Total RNA was extracted from cells or hearts homogenized in TRI-reagent using Direct-zolTM RNA mini-prep kit (cat # R2050-1-50, Zymo research) according to the manufacturer's instructions. Concentration and purity of the RNA were measured by NanoDrop1000 spectrophotometer (Thermo Fisher scientific, MA, USA). Synthesis of cDNA was performed using random hexamers and Taqman reverse transcription reagents according to the manufacture's protocol (Quanta BioSciences MA, USA). Gene expression was examined with reverse transcriptase-quantitative polymerase chain reaction (qPCR) and PerfeCTa SYBR Green FastMix (Quanta BioSciences MA, USA) using QuantStudio5 Real Time PCR Systems (Thermofisher Scientific, MA, USA). Reactions were run in program of pre-incubation; 50°C for 3 min and 95°C for 3 min followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds and a final stage of dissociation. 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 GAPDH housekeeping gene. Each sample was tested in triplicate.

**Additional file 2. Supplementary Figures**

**Fig. 1S** Deterioration in PLK2-KO mice physiological parameters with aging. Echocardiography was measured at 3, 6 and 12-month-oldmice and several parameters were examined. **A.** Stroke Volume (mL); **B.** Cardiac Output (L/min); **C.** LV mass (mg) and **D.** Total body weight (gr). Statistics was calculated with GraphPad Prism, \* p<0.05 \*\* p<0.01 PLK2-KO vs WT at the same age.

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**Fig. 2S** Decrease in the expression of autophagy related genes in WT but not in Plekhm2 KO mice.mRNA levels of *becn1*, *atg5* and *ctsl* was evaluated in CQ-treated NMCFs and untreated cells. Calculating the delta expression before and after adding CQ demonstrates that CQ significantly reduces *atg5*, *becn1* and *ctsl* in control but not in KO cells. Statistic was examined as Wilcoxon signed-rank test, compare column median to a hypothetical value, using the GraphPad Prism software. # p<0.05 ## p<0.01 related to the hypothetical value of 0 and \* p<0.05 \*\* p<0.01 KO-NMCFs vs 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.** Schemes representing the Plekhm2tm1a(EUCOMM/Wtsi) mouse genotype, and **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 therefore representing KO mouse. When the lacZ-neo cassette is not present the PCR product will be of 478bp 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 300bp product **(C)**. Crossing these mice with Flp mice remove the sequence flanked by the FRT. These Plekhm2floxed/floxed /FLP**-/-** mice, will further be using for neonatal cardiac cells isolation. **D.** For deletion of Plekhm2 in neonatal cells we transfected cells with Cre-recombinase enzyme. Since primer Plekhm2 44411 R 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 in NMCMs and NMCFs (CRE) as compared to control cells (GFP). The molecular size marker on the right side of the photographs is O' GeneRuller 1Kb plus DNA ladder of ThermoScientific.

**Additional file 3. Supplementary Tables**

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

|  |  |  |
| --- | --- | --- |
| **PLK2-KO (n=20)** | **WT (n=17)** | **Echocardiogram**  **Measurements** |
| 25.8 ± 0.48 **\*** | 28.4 ± 0.66 | Weight |
| 457.3 ± 8.88 | 476.5 ± 12.9 | HR |
| 3.86 ± 0.08 | 3.91 ± 0.1 | lLVIDd |
| 2.68 ± 0.09 | 2.71 ± 0.11 | LVIDSs |
| 0.85 ± 0.03 | 0.93 ± 0.09 | LVPWd |
| 1.19 ± 0.04 | 1.28 ± 0.08 | LVPWs |
| 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 |
| 21.3± 2.06 | 23.9 ± 1.77 | CO |
| 0.90 ± 0.02 | 0.98 ± 0.05 | RWT |
| 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 (gr) |
| 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 physiology parameters and gravimetric measurements were examined in PLK2-KO and WT normal siblings' male mice at young age of 3-month old. HR; Heart rate, LVIDd; Left Ventricular internal diameter in diastole (mm), LVIDSs; Left ventricular internal diameter in systole (mm), LVPWd; Left ventricular posterior wall in diastole (mm), LVPWs; left ventricular posterior wall in systole (mm), EF; Ejection fraction (%), FS; Fractional shortening (%), LVVd; Left ventricular volume in diastole (L), LVVs; Left ventricular volume in systole (L), SV; Stroke volume (L), CO: Cardiac output (L/min), RWT; Relatively wall thickness (mm). BW; Body weight, HW; Heart weight, TL; Tibia length. **\*** represent KO *vs* WT p<0.05.

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

|  |  |  |
| --- | --- | --- |
| **PLK2 KO** **(n=6)** | **WT (n=6)** | **6-month old** |
| 21.4 ± 1.74 **\*** | 26.6 ± 0.65 # | Weight |
| 457.8 ± 13.6 | 485.2 ± 12.1 | HR |
| 3.52 ± 0.17 | 3.94 ± 0.12 | LVIDd |
| 2.53 ± 0.24 | 2.95 ± 0.19 | LVIDSs |
| 0.80 ± 0.03 | 0.80 ± 0.04 # | LVPWd |
| 1.02 ± 0.08 | 1.11 ± 0.09 | LVPWs |
| 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 |
| 36.5 ± 7.2 | 42.9 ± 4.6 | LVVs |
| 35.6 ± 2.5 | 41.3 ± 2.9 | SV |
| 16.6 ± 0.8 **\*** | 19.9 ± 1.1 | CO |
| 0.80 ± 0.02 | 0.83 ± 0.03 | RWT |
| 81.6 ± 5.07 \*# | 103.9 ± 6.4 # | LV mass |

Basic physiology parameters and gravimetric measurements were examined in PLK2-KO and WT normal siblings' female mice at 6-month old. HR; Heart rate, LVIDd; Left Ventricular internal diameter in diastole (mm), LVIDSs; Left ventricular internal diameter in systole (mm), LVPWd; Left ventricular posterior wall in diastole (mm), LVPWs; left ventricular posterior wall in systole (mm), EF; Ejection fraction (%), FS; Fractional shortening (%), LVVd; Left ventricular volume in diastole (L), LVVs; Left ventricular volume in systole (L), SV; Stroke volume (L), CO: Cardiac output (L/min), RWT; Relatively wall thickness (mm). BW; Body weight, HW; Heart weight, TL; Tibia length. **\*** represents KO *vs* WT (same age p<0.05), # represents WT or KO at 6-month old *vs* WT 3-month (p<0.05).

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

|  |  |
| --- | --- |
| 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 |