

Supplemental Information

Peroxisome protein import deficiency causes heart failure in mouse and human

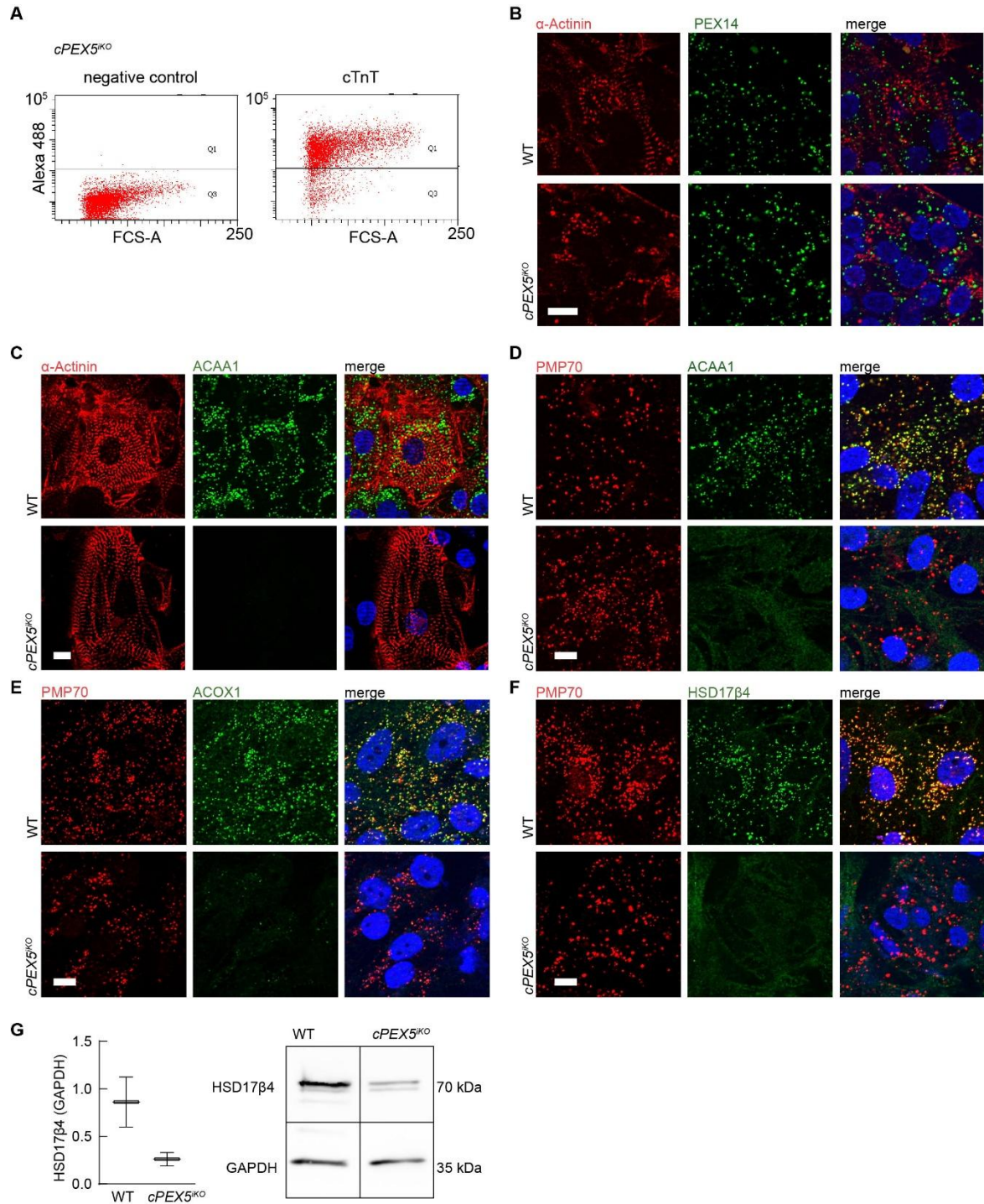
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The Supplemental Information contains

Supplemental Figures 1 to 9

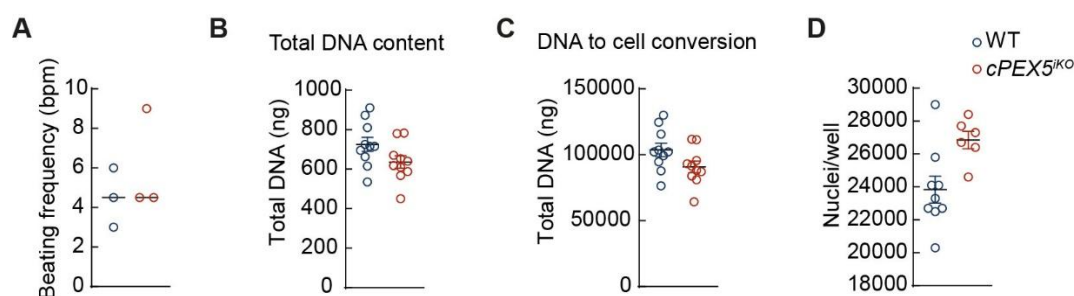
Supplemental Tables 1 and 2

Supplemental Videos 1 to 4



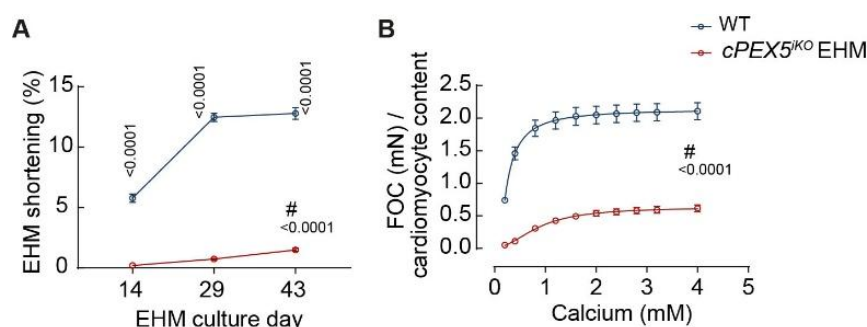
Supplemental Figure 1: hiPSC and hiPSC-CM characterisation, rel. to Figure 1.

(A) Flow cytometric analysis of cTnT stained hiPSC-CM was performed at day 60 post-cardiac differentiation. Original measurement from *cPEX5^{KO}*. (B) Staining of peroxisomal matrix protein PEX14 shows increased size and decreased number of peroxisomes in *cPEX5^{KO}*. Co-staining with α -Actinin. (C-G) Peroxisomal import defect was shown by co-staining of PMP70, a peroxisomal membrane protein, or sarcomeric α -Actinin with the peroxisomal matrix proteins ACAA1 (C,D), ACOX1 (E) and HSD17B4 (F) and Western blotting of the matrix protein HSD17B4 (G).



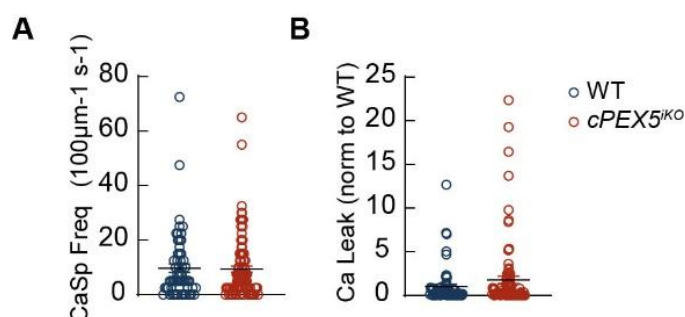
Supplemental Figure 2: Defects mitochondrial structure in *PEX5^{iKO}* hiPSC-CM , rel. to Figure 2

No differences in beating frequency (A), total DNA content (B), DNA to cell conversion (C) or cells per well (D) between WT and *PEX5^{iKO}* hiPSC-CM used for measurement of mitochondrial respiratory function.



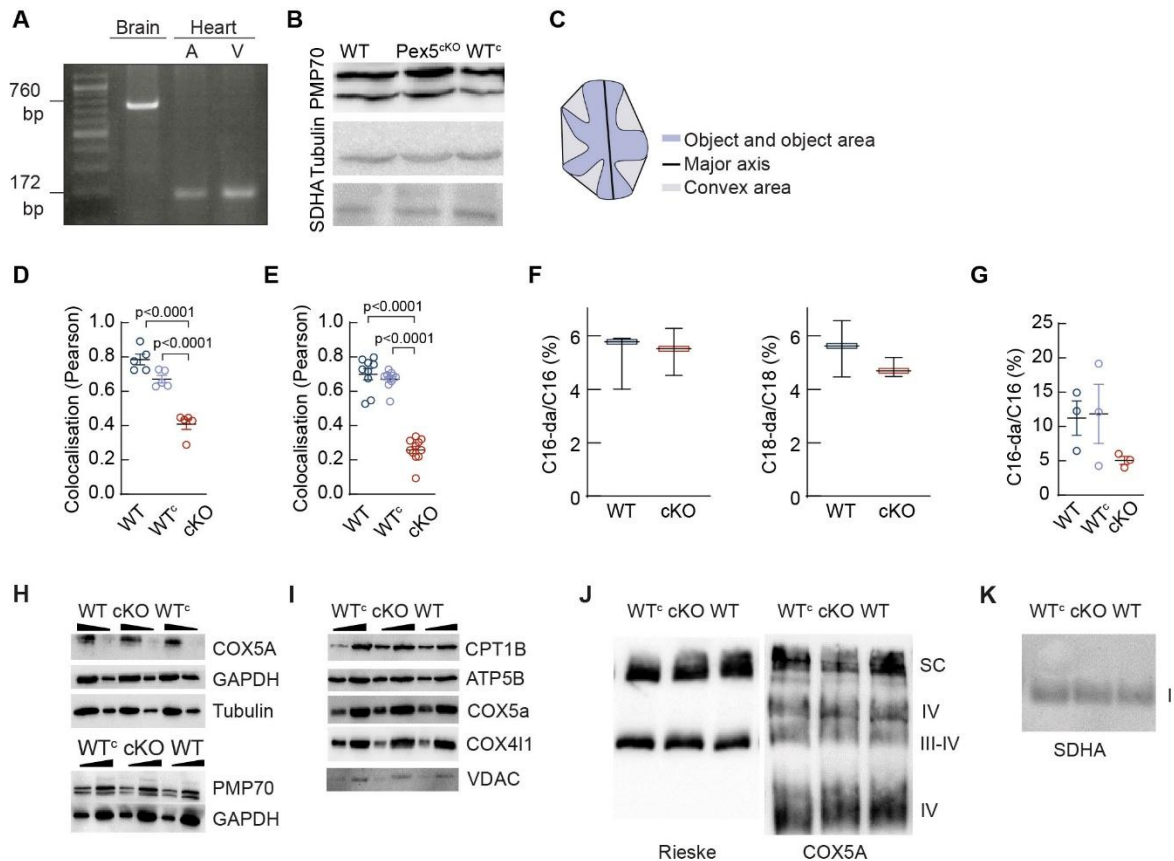
Supplemental Figure 3: EHM characterization, rel. to Figure 3.

EHM were generated from human WT hiPSC-CM and *cPEX5^{iKO}*. (A) Optical analysis of EHM function in 48 well plate shows reduced shortening (% change of baseline pole distance) during EHM development. (B) After normalization to cardiomyocyte number, force of contraction remains lower in *cPEX5^{iKO}*. N = 8 EHM per group. #two-way ANOVA; Bonferroni's multiple comparisons.



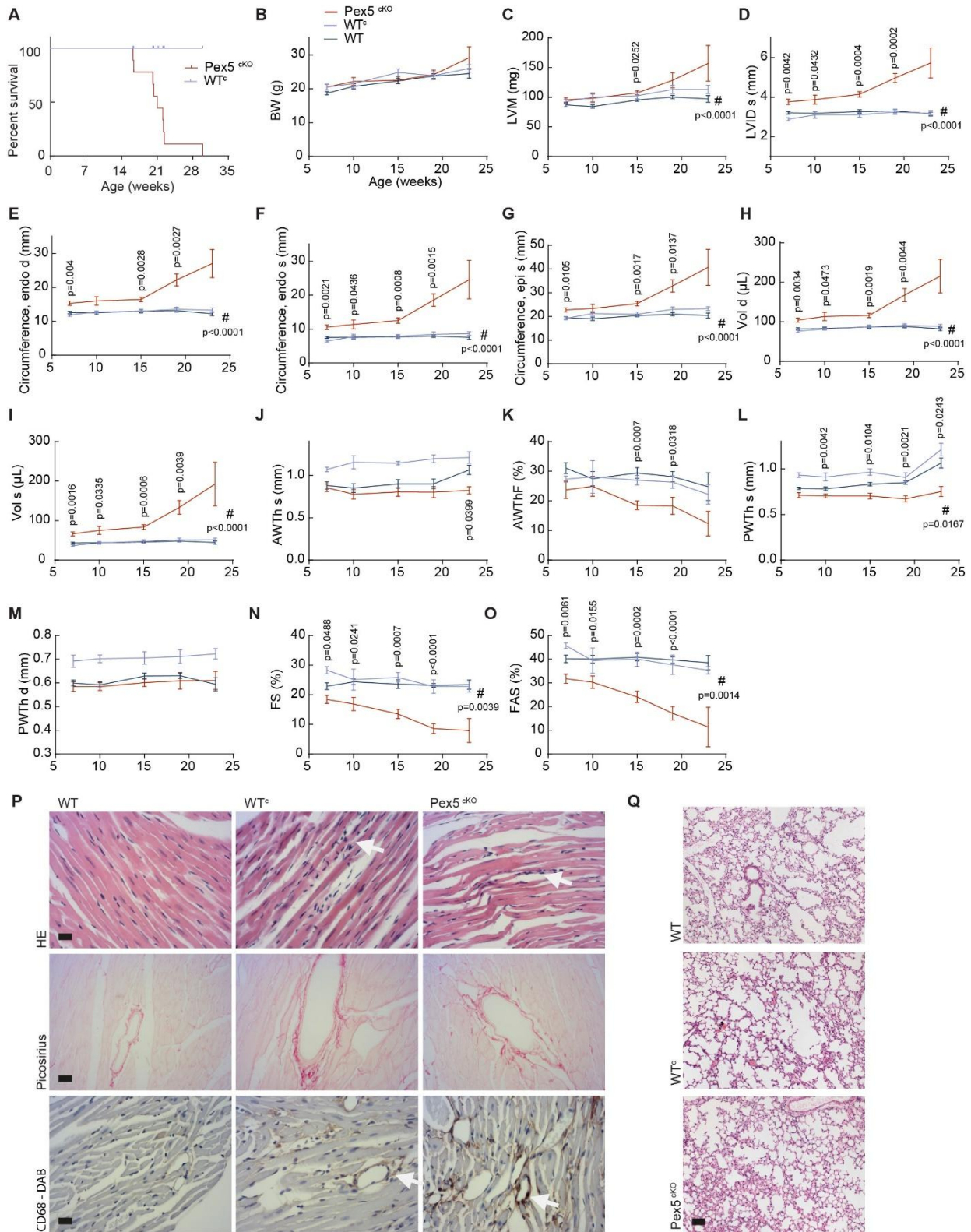
Supplemental Figure 4: Defects in *cPEX5^{iKO}* calcium handling, rel. to Figure 4.

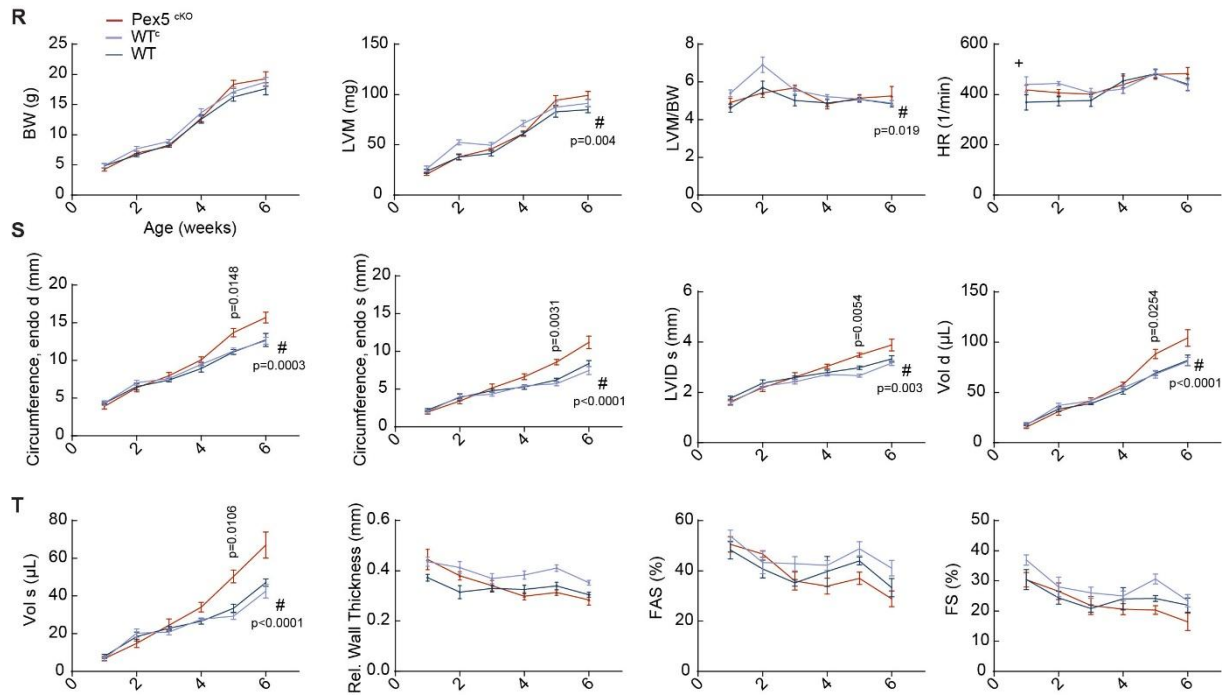
(A,B) Ca^{2+} spark frequency (A) and Ca^{2+} leak (B) were not changed in *cPEX5^{iKO}* compared to WT.



Supplemental Figure 5: Characterization of Pex5^{cKO}, rel. to Fig. 5.

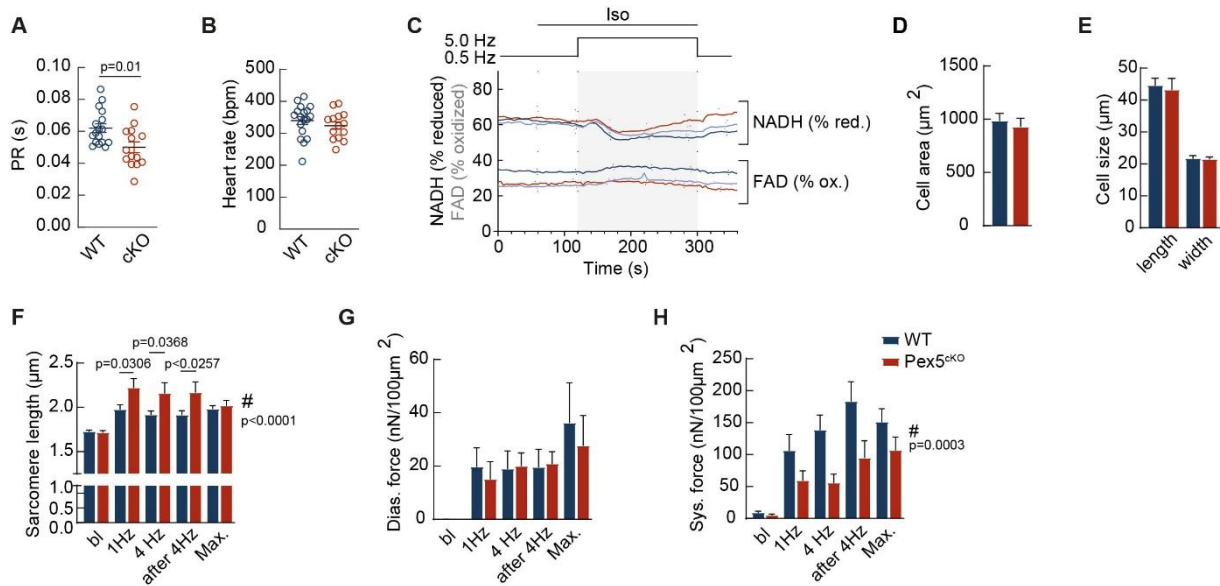
(A) Tissue-specific knockout of *Pex5* in atria (A) and ventricles (V), but not in brain of Pex5^{cKO} mice confirmed by PCR. (B) Equal PMP70 expression in Pex5^{cKO} and WT ventricles. (C) Schematic of an object analysis from confocal and STED images in Figure 5F,G. Object area, major axis and convex area are used in the calculation of shape parameters of peroxisomes. (D,E) Loss of DAP-AT (D) and PHYH (E) in peroxisomes of isolated cardiomyocytes of fasted Pex5^{cKO} mice. Quantification of Figure 5 K and M, Pearson coefficient. One-way ANOVA, Tukey's multiple comparisons. N = 5-9 cells per group. (F) No difference in C16 and C18 plasmalogen levels analyzed in EDTA blood samples from WT and Pex5^{cKO} mice. (G) Non-significant reduction of C16 plasmalogens in Pex5^{cKO} hearts. (H) Western blot analyses of cardiac tissue lysates using antibodies against mitochondrial protein COX5A and peroxisomal PMP70. Cytosolic proteins Tubulin and GAPDH were used as a control. (I) Isolated mitochondria were analyzed by Western blotting using indicated mitochondrial proteins CPT1B, ATP5a, COX5a and COX4I1. Vdac was used as a loading control. (J) Isolated mitochondria were subjected to blue native analysis to separate native respiratory chain complexes. Indicated respiratory chain complexes were analyzed by Western blot analysis against the complex III component Rieske and complex IV component COX5A. (K) Blue Native analysis of succinate dehydrogenase (complex II).





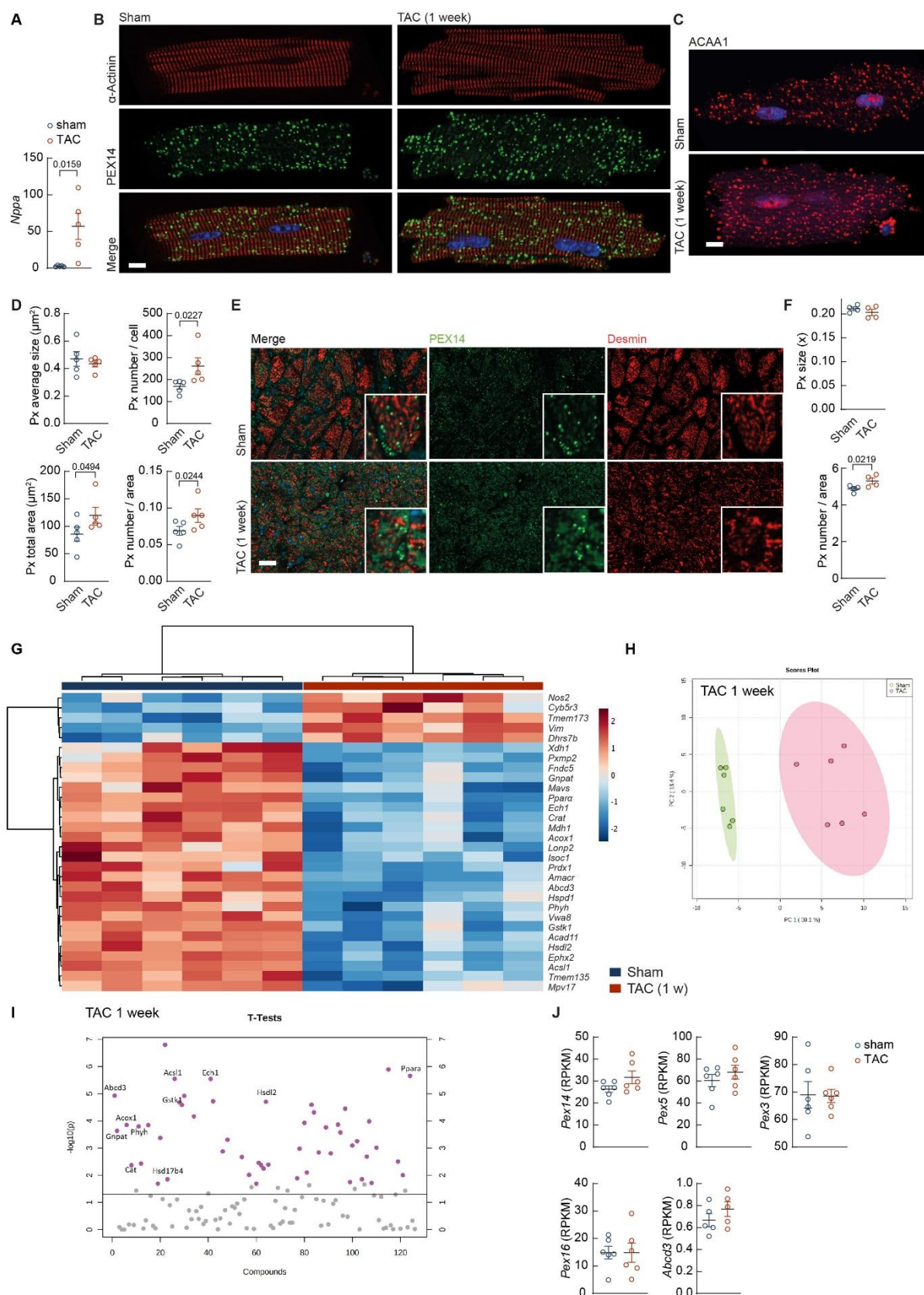
Supplemental Figure 6: Characterization of Pex5^{cKO} heart function, rel. to Fig. 6.

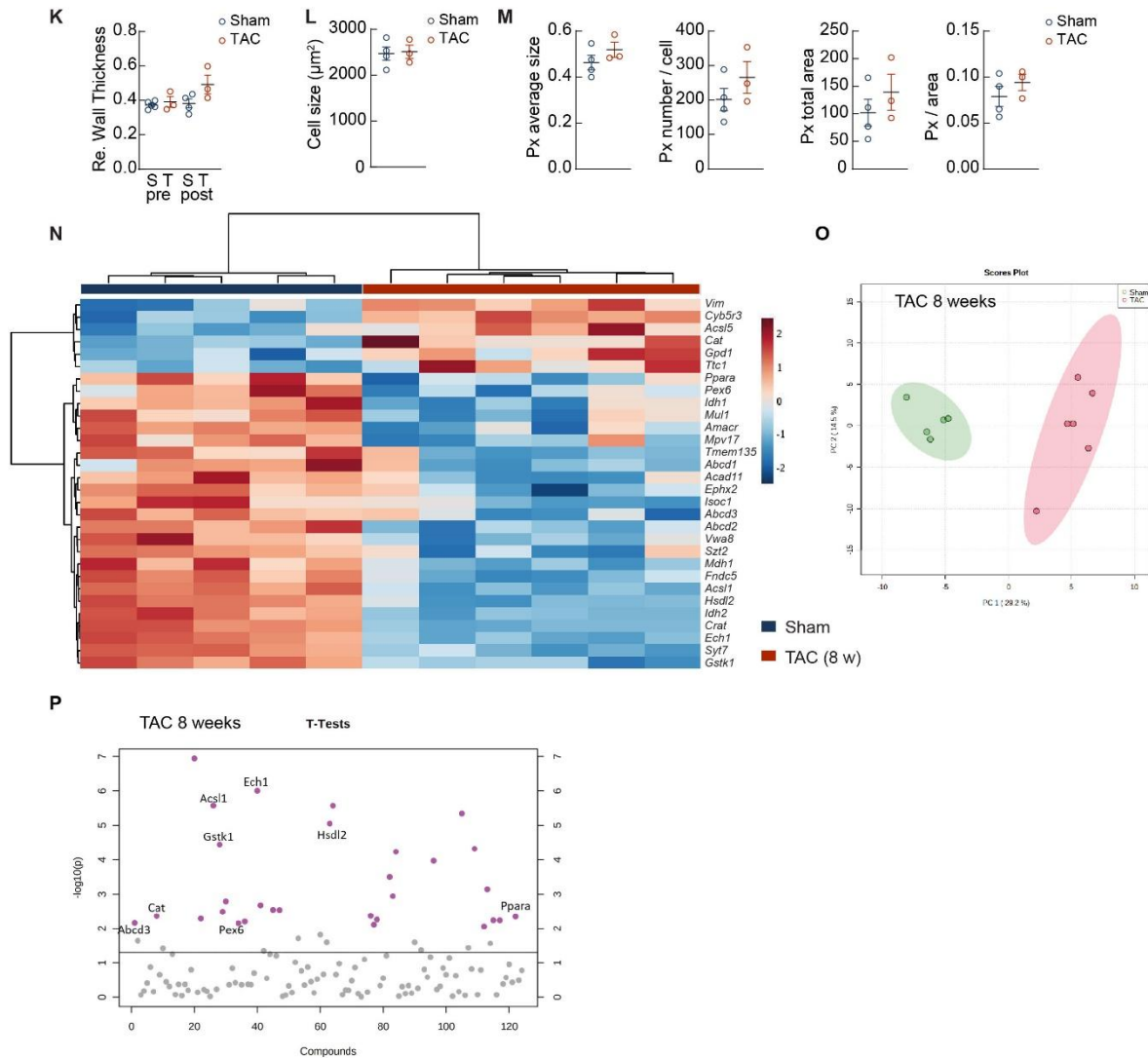
(A) Kaplan Maier survival curve shows early death of Pex5^{cKO} mice, but Cre-expressing WT^c mice show normal life span. $p < 0.01$. $N = 5-7$ mice per group. (B-O) Echocardiography of 7- to 23-week old mice shows dilated hearts with decreased contraction indicating severe DCM in Pex5^{cKO} mice. $N = 3-16$ mice per group and time point, one-way ANOVA, Tukey's multiple comparisons. (P) Picrosirius staining shows no signs of fibrosis in Pex5^{cKO} hearts. HE- and CD68-DAB staining (highly expressed by macrophages) of heart tissue sections show inflammation and macrophage infiltration (white arrows) in Pex5^{cKO} and WT^c mouse hearts. (Q) No signs of pathology in HE-staining of Pex5^{cKO} and WT^c lung tissue. Scales: 20 μ m. (R-T) Echocardiography of 1- to 6-week old mice shows no functional differences in the first 6 weeks of age, but first signs of dilation in 4 week old Pex5^{cKO} mice. $N = 5-7$ mice per group and time point, one-way ANOVA, Tukey's multiple comparisons. BW = body weight, LVW = left ventricular weight, HR = heart rate, AWT_{hd} = anterior wall thickness diastole, AWT_{hs} = anterior wall thickness systole, PWT_{hd} = posterior wall thickness diastole, PWT_{hs} = posterior wall thickness systole, LVID_d = left ventricular internal diameter end diastole, LVID_s = left ventricular internal diameter end systole, FS = fractional shortening, FAS = fractional area shortening, AWT_{hF} = anterior wall thickening fraction, PWT_{hF} = posterior wall thickening fraction, Vol s = volume systole, Vol d = volume diastole, Epi = epicardium.



Supplemental Figure 7: Characterization of Pex5^{cKO} cardiac mitochondrial energetics and force production, rel. to Fig. 7.

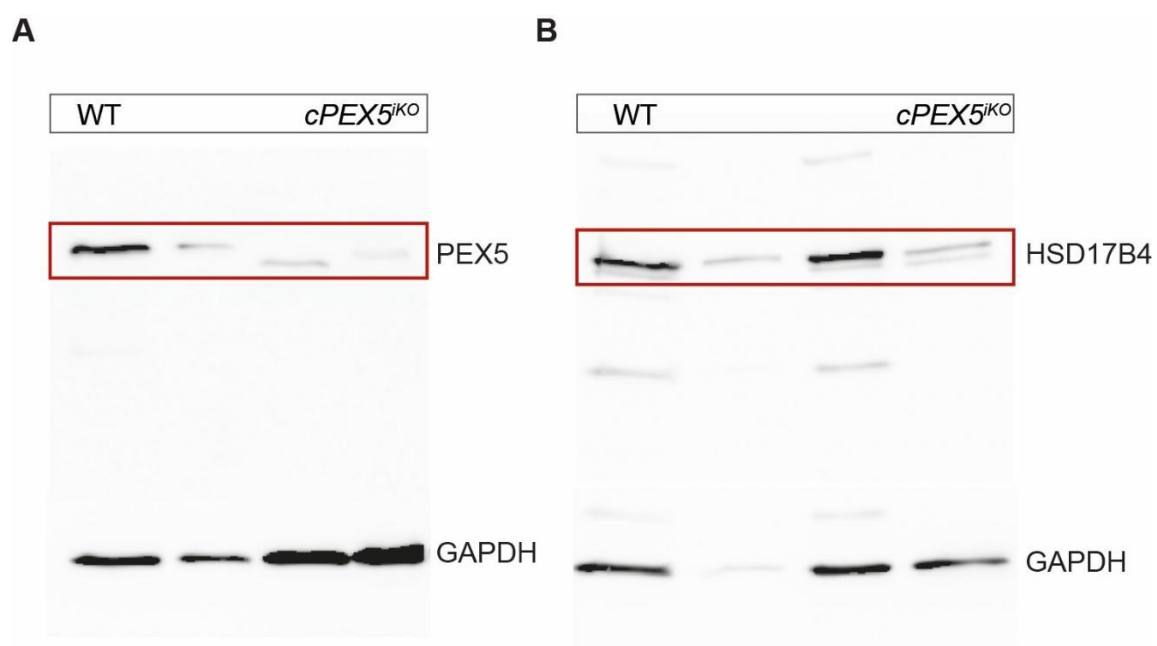
(A,B) Statistical analysis of the PR interval, 50.1 ± 0.3 ms vs. 63.7 ± 0.3 ms (A) and the spontaneous beating rate (B) in cardiac electrograms of explanted hearts from WT and Pex5^{cKO} . (C) Autofluorescences of NAD(P)H and FAD reveal a trend towards a more reduced redox status of Pex5^{cKO} compared to WT cells. (D-H) Force measurement in unloaded cardiomyocytes. Similar cell area (D) and size (E) during stretch were acquired to ensure that the cells were stretched equally and force is therefore theoretically acquired from the same amounts of sarcomeres. Cell length corresponds to the distance between the two holders that stretch the cell and measure the force. Pex5^{cKO} cells show increased sarcomere length (F). Diastolic force (G) is not changed, whereas systolic force (H) is significantly decreased in Pex5^{cKO} myocytes. Two-way ANOVA, Bonferroni's multiple comparisons.





Supplemental Figure 8: Peroxisome abundance and composition in the failing heart, rel. to Fig. 8.

(A) *Nppa* mRNA expression significantly increased one week after TAC. qPCR. (B,C) Peroxisome distribution in isolated CM from sham-operated mouse hearts and mouse hearts isolated 1 week after transverse aortic constriction (TAC). Staining of PEX14 and α -Actinin (B) or ACAA1 (C) in isolated CM 1 week after TAC. (D) Analysis of ACAA1-stained peroxisomes (Px) from (C). N = 4-5 mice per group. (E) Paraffin-embedded sections stained with anti-PEX14 and anti-Desmin. Scale: 10 μm . (F) Peroxisome number per area increased significantly 1 week after TAC, peroxisome size did not change. Quantification of (E). N = 4 mice. (G-I) RNA sequencing one week after TAC. Heatmap (G), PCA plot (H) and (I) T-tests. (J) *Pex14*, *Pex5*, *Pex3*, *Pex16* and *Abcd3* mRNA expression unchanged one week after TAC. Data from RNA sequencing. (K) Echocardiographic analysis of WT mice before (pre) and 8 weeks after TAC (post) shows no significant change in relative wall thickness; N=4 WT and 3 *Pex5*^{CKO} mice. S = sham, T = TAC. (L,M) Analysis of peroxisome staining of isolated CM from sham and 8 week-TAC mouse hearts. Peroxisomes were stained by anti-PEX14 (L) or anti-ACAA1 (M). No change in cell size, peroxisome size or peroxisome number was observed. N = 4 WT, 3 TAC mice. (N-P) RNA sequencing 8 weeks after TAC. (N) Heatmap, (O) PCA plots and (P) T-tests.



Supplemental Figure 9: Uncropped Western Blots.

(A) PEX5 expression, Fig.1 L (B) HSD17 β 4, Fig. S1G.

Supplemental Table 1: Antibodies.

First antibody	Firma	Order No.	Dilution
PEX14	Proteintech	10594-1-AP	1:500, 1:50 (IHC)
ACAA1	Proteintech	12319-2-AP	1:500
PMP70	Sigma	SAB4200181	1:250
Desmin	Progen	10519	1:100 (IHC)
CD68	Dako	110876	1:200
CAV3	BD	610420	1:250
RYR2	Thermo	MA3-916	1:200
PHYH	Santa Cruz	sc-376727	1:100
DAP-AT	Proteintech	14931-1-AP	1:100
HSD17B4	Proteintech	15116-1-AP	1:100, 1:100 (WB)
ACOX1	Proteintech	10957-1-AP	1:100
α -Actinin	Sigma	A7811	1:500
CAV3	BD	610420	1:250
RYR2	Thermo	MA3-916	1:200
OCT	BD Biosciences	560329	1:100
NANOG	Thermo	MA1-017	1:200
TRA1-60	BD Biosciences	560173	1:100
PEX5	Novus Biologicals	NBP2-38443	1:500
GAPDH	Millipore	MAB374	1:10000
Anti-cTnT	Invitrogen	MA5-12960	1:500
Anti-mouse IgG-Alexa Fluor® 633	Invitrogen	A-21053	1:1000
Anti-rabbit IgG-Alexa Fluor® 488	Life technologies	A-11008	1:1000
Anti-mouse IgG-Alexa Fluor® 488	Invitrogen	A-21202	1:1000
Anti-mouse IgG-CyTM3	Jackson ImmunoResearch	715-166-150	1:1000
Star 635P rabbit	Abberior	ST635P	1:1000
Star 580 mouse	Abberior	ST580	1:1000
ECL Anti-Rabbit IgG, HRP	Jackson ImmunoResearch	111-035-003	1:10000
ECL Anti-Mouse IgG, HRP	Jackson ImmunoResearch	715-035-150	1:10000

Supplemental Table 2: Primers used for qPCR (Murine genes).

Primers	Sequence for	Sequence rev	Product size (bp)
<i>Anp</i>	TCGTCTTGGCCTTTTGGCT	TCCAGGTGGTCTAGCAGGTTCT	106
<i>Ppara</i>	TCGGCGAACTATTTCGGCTG	GCACTTGTGAAAACGGCAGT	106
<i>Cat</i>	CCGACCAGGGCATCAAAA	GAGGCCATAATCCGGATCTTC	74
<i>Phyh</i>	TGCCAAGTGATAGGATGGTTTC	TCAGAATCTCGGGGAGAAGG	87
<i>Gnpat</i>	GGAGTCCACCACTGGATTGT	GCAGTGATACAGGCTCTCCG	92
<i>Pex11a</i>	ACTGGCCGTAAATGGTTCAGA	CGGTTGAGGTTGGCTAATGTC	119
<i>Hsd12</i>	CTGTTCCACTGCCACCAAGT	ATCCCGCTAGCTTCCCAGT	70
<i>Acs1</i>	TCCTACAAAGAGGTGGCAGAACT	GGCTTGAACCCCTTCTGGAT	68
<i>Pln</i>	TGCCCAGCTAAGCTCCCATA	TTTGTTGTGCAGACTGAAGCG	116
<i>Gapdh</i>	GAAGATGGTGATGGGATT	GAAGGTGAAGGTCGGAGT	89
<i>Gusb</i>	CACGGCGATGGACCCAAGAT	CCCATTACCCACACAACCTGC	86

Supplemental Video 1. Contraction of WT EHM. Representative videos of spontaneously beating EHM generated from WT hiPSC-CM. Related to Figure 3.

Supplemental Video 2. Contraction of *PEX5*^{KO} EHM. Representative videos of spontaneously beating EHM generated from *cPEX5*^{KO}. Related to Figure 3.

Supplemental Video 3. Optical Mapping of WT EHM.

Representative video of optical mapping of WT EHM. Colors representing the time point of maximal signal amplitude after electrical point stimulation (see Figure 4I). Stimulation electrode is situated in the inferior left corner of the tissue. Related to Figure 4.

Supplemental Video 4. Optical Mapping of *PEX5*^{KO} EHM.

Representative videos of optical mapping of *PEX5*^{KO} EHM. Colors representing the time point of maximal signal amplitude after electrical point stimulation (see Figure 4I). Stimulation electrode is situated in the inferior left corner of the tissue. Related to Figure 4.