Supplementary information

**PTEN Regulates Mitophagy and Maintains Mitochondrial Quality Control**

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**Figure S1.** Evaluation of mitochondria structure by transmission electron microscopy (TEM). (**A**) Representative TEM micrographs of 2-months-old and 4-months-old *Pten+/+* and *ptenm/m* mouse cardiac mitochondria in cross sections. Cardiac muscle (1 mm x 3 mm) was dissected from the left ventricle and fixed with 2.5% glutaraldehyde in 0.1 M Sorenson’s buffer (pH 7.2). Magnification: top, 10,000x; bottom, 20,000x. (**B**)Representative TEM micrographs of cardiac mitochondria from 4-months-old *Pten+/+* and *ptenm/m* mice. Representative vacuolated mitochondrion is outlined in white; arrowheads, remnant cristae; red arrowhead, lysosome around the degenerated mitochondrion; LD, lipid droplet. Magnification: upper left, 5000x; upper right, 10,000x; lower left, 10,000x; lower right, 20,000x. (**C**) mRNA levels of *Tfam*, *Nrf1* and *Ppargc1a*assessed with real-time PCR in heart tissue from 2-months-old mice (n=9). Total RNA was extracted from left ventricles. cDNA obtained by inverse transcription was used to amplify the indicated genes. Data are presented as mean ± SEM; n.s., not significant, *p*> 0.05 (two-tailed unpaired Student’s t-test).

**Figure S2.** Assessment of mitophagy and autophagy. (**A**) Representative confocal images of isolated cardiomyocytes from 4-months-old mice, labeled with MitoTracker Red and LysoTracker Green following treatment with DMSO or oligomycin (1 µM) and antimycin A (1 µM) for 3 h. Magnification: 100x; scale bars: 20 μm. (**B**)Numbers of colocalized lysosomes and mitochondria per cell. Cells (20) from 3 mice from each group were analyzed. Data are presented as mean ± SEM; \*\*\**p*< 0.001 comparing *Pten+/+*, *ptenm/m* groups, analyzed by two-way ANOVA followed by Bonferroni’s multiple comparisons test. (**C-D**) Evaluation of general autophagy by immunoblotting SQSTM1 and LC3B in heart tissues from 4-months-old mice. For I-R stress, the hearts were subjected to 30 min of ischemia followed by 30 min of reperfusion. After I-R, left ventricles were dissected and the homogenates were immunoblotted with SQSTM1 and LC3B antibodies. Relative expression of LC3B-I, LC3B-II, and SQSTM1 was quantified with ImageJ (n=3). n.s., not significant, *p*> 0.05; \*\*\**p*< 0.001 comparing *Pten+/+*, *ptenm/m* groups, analyzed by two-way ANOVA followed by Bonferroni’s multiple comparisons test.

**Figure S3.** Assessment of PRKN mitochondrial translocation**.** (**A**) Representative confocal images of isolated cardiomyocytes from 4-months-old mice, labeled with MitoTracker Red and a ubiquitin antibody following treatment with DMSO or oligomycin (1 µM) and antimycin A (1 µM) for 2 h. Magnification: 100x; scale bars: 20 μm. (**B**)Representative TEM images of autophagosome formation in *Pten+/+* and *ptenm/m* hearts. Isolated hearts from 4-months-old mice were perfused with DMSO or 1 M CCCP for 30 min, and cardiac muscle (1 mm x 3 mm) was dissected from the left ventricle and fixed with 2.5% glutaraldehyde in 0.1 M Sorenson’s buffer (pH 7.2). Mitochondria targeted for degradation were surrounded by membrane organelles. Magnification: 5000x; insets: 20,000x.

**Figure S4.** Protein interaction of PTENwith PRKN and domain analysis. (**A**)O-A treatment promotes interaction of PTEN and PRKN. Plasmids encoding FLAG-PTEN, and PRKN-GFP were co-transfected into HEK293T cells. Cells were treated with 10 μM CCCP for 1 h or 10 μM oligomycin and 4 μM antimycin A for 4 h before harvest. Cell homogenates were immunoprecipitated with FLAG antibody and immunoblotted with anti-FLAG and anti-GFP. (**B**)Endogenous interaction of PRKN and PTEN**Cardiac homogenates from 2-months-old wild-type mice were immunoprecipitated with PTEN N-terminus-specific antibody (-N) or IgG and immunoblotted with PRKN antibody. WHL, whole heart lysate. (**C**)Schematic diagram of domains of human PTEN. Asterisks indicate the poly-alanine and poly-arginine sequences. MBH, membrane binding helix region. (**D**) Schematic plot of PTEN truncations. PTEN[6A], PTEN lacking alanine (amino acids [aa] 12-17); PTEN[6R], PTEN lacking arginine (aa 47-52); PTEN[N], PTEN lacking amino acids 1-144; PTEN[MBH], PTEN lacking the membrane binding helix (aa 145-176); PTEN[PHD], PTEN lacking the phosphatase domain; and PTEN[C2], PTEN lacking the C2 domain. (**E**)Schematic plot of PRKN truncations. PRKN[FL], full-length PRKN; PRKN[C], PRKN lacking the RING2 domain; PRKN[NT], PRKN with UBL domain and linker; PRKN[N], PRKN lacking the UBL domain and linker region; PRKN[RBR], PRKN with RING1, IBR and RING2 domains; and PRKN[CT], PRKN with IBR and RING2 domains.(**F**)Interaction of FLAG-PTEN and its truncations with PRKN-GFP in HEK293T cells without CCCP treatment. Plasmids encoding FLAG-PTEN and its truncations were co-transfected with a plasmid encoding PRKN-GFP into HEK293T cells. Cells were harvested 24 h after transfection. Lysates immunoprecipitated with anti-FLAG were immunoblotted with anti-GFP or anti-FLAG. IgH, heavy chain of IgG. (**G**)Interaction of PRKN-FLAG and its truncations with PTEN-GFP in HEK293T cells without CCCP treatment. A dish of cells transfected with PRKN[FL] were treated with 10 μM CCCP, BS other cells were treated with DMSO for 1 h before harvest as indicated. Lysates immunoprecipitated with anti-FLAG were immunoblotted with anti-GFP or anti-FLAG.

**Figure S5.** PTENbinds PRKN in mitochondrial fraction. (**A**)Association of PTENand PRKN in subcellular location. PRKN-FLAG or control vector were co-transfected with PTEN-GFP in HEK293T cells. Cells were treated with 10 M CCCP for 1 h before harvest. Cytoplasmic (Cyto) and mitochondrial (Mito) fractions were separated according to a standard protocol (see Materials and Methods). Lysates immunoprecipitated with anti-FLAG were immunoblotted with anti-GFP or anti-FLAG.

**Figure S6.** Evaluation of PTEN mitochondrial localization. (**A**) Mitochondrial localization of PTEN and its truncations. Plasmids encoding PTEN-GFP or its truncations were transfected into HEK293T cells. Cytoplasmic (Cyto) and mitochondrial (Mito) fractions were separated 24 h after transfection according to a standard protocol. Lysates were immunoblotted with anti-GFP, TUBA1A or TOMM20. (**B**) Representative result of assessment of PTENlocalization through subcellular fractionation of ischemic heart tissue. Subcellular fractionation assays in cardiomyocytes were carried out to test PTEN localization changes after cardiac ischemia (30 min). Lysates were immunoblotted with PTEN, TUBA1A or CYCS antibodies. Lower panel is the quantified analysis of PTEN levels in mitochondrial and cytoplasmic fractions of 3 independent experiments. \**p*< 0.05 comparing indicated groups, analyzed by unpaired Student’s t test.

**Figure S7.** Endogenous interaction of PTEN, PINK1, and PRKN and abolition of PTENin HeLa cells.(**A**)Endogenous immunoprecipitation of PTEN with PINK1 and PRKN in control and ischemic hearts. Endogenous interaction of PTEN, PINK1, and PRKN were evaluated in ischemic heart lysates. After immunoprecipitation with anti-PTEN antibody, which was raised in our laboratory, PINK1 and PRKN were individually immunoblotted using commercial antibodies. (**B**)Sequences of PTEN 5’ UTR and CRISPR guide RNA. Colored bases represent the sequence of CRISPR guide RNA, and the black box shows the initiation codon for PTEN. (**C**)Immunoblot analysis of PTEN and PTEN in *PTEN+/+* and *PTEN-/-* HeLa cells.

**Figure S8.** Colocalization of LC3B with mitochondria in HeLa cells with or without PRKN. (**A**)CCCP induced PRKN translocation onto mitochondria in *PTEN+/+* (WT), *PTEN-/-* (KO) and PTEN re-expressing HeLa cells. A plasmid encoding PRKN-GFP was transfected into HeLa cells. Cells were treated with 10 μM CCCP for 2 h and PRKN was immunoblotted in mitochondrial (Mito) and cytosolic (Cyto) fractions. GAPDH is a cytosolic marker and COX4I1is a mitochondrial marker. Protein levels of mitochondrial and cytosolic PRKN were quantified with ImageJ (n=3). \*\**p*< 0.01comparing the indicated groups, analyzed by paired Student’s t test.(**B**) Time-dependent mitochondrial protein degradation following CCCP treatment. Plasmids encoding FLAG-PTEN and PRKN-GFP were co-transfected into *PTEN+/+* or *PTEN-/-* HeLa cells. Cells were treated with 10 μM CCCP and potential mitochondrial targets were evaluated by western blotting at corresponding time points. Protein levels of MFN2 were quantified with ImageJ (n=3) and the percentage of remaining MFN2 was drawn as a curve. (**C**)CCCP induced colocalization of GFP-LC3B with mitochondria. HeLa cells expressing PRKN-MYC or empty vector were transfected with a plasmid encoding GFP-LC3B for 48 h and labeled with MitoTracker Red. Magnification: 100x; scale bars: 10 μm.

**Figure S9.** Rescue experiments in HeLa cells and cardiomyocytes. (**A**)PRKN translocation onto mitochondria in *PTEN-/-* HeLa cells transfected with plasmids encoding PTEN and its truncations. *PTEN-/ -* HeLa cells co-transfected with plasmids encoding PRKN-GFP and PTENor its truncations were treated with 10 μM CCCP for 30 min. Colocalization of PRKN-GFP and mitochondria (TOMM20) is indicated by yellow dots. Magnification: 100x; scale bars: 20 μm.(**B**) Evaluation of LC3B isoforms and SQSTM1 expression in Nam fed *ptenm/m* mice. After Nam feeding for 20 days, ventricles were dissected and the homogenates were immunoblotted with SQSTM1 and LC3B antibodies. Relative expression of LC3B-I, LC3B-II, and SQSTM1 was quantified with ImageJ (n=4). \*\* p< 0.01; \*\*\*p< 0.001 comparing H2O and Nam feeding groups, analyzed by unpaired Student’s t test. (**C**)TTC staining in I-R hearts of *Pten+/+*, or *ptenm/m* mice fed with water or 0.5% nicotinamide (Nam). The picture is representative of 4 independent experiments. The infarcted (white) area was analyzed with ImageJ software as a percentage of total area (n=4). \**p*< 0.05, \*\*\**p*< 0.001 comparing the indicated groups, analyzed with the unpaired Student’s t test.

**Table S1.** Echo data.

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| --- | --- | --- | --- |
|  | **+/+（n=8）** | **m/m（n=8）** | ***p* value** |
| EF, % | 64.±4.0 | 55±3.7 | 0.13 |
| FS, % | 32±2.7 | 28±2.5 | 0.21 |
| LVIDd, mm | 3.6±0.17 | 3.7±0.11 | 0.40 |
| LVPWd, mm | 0.59±0.021 | 0.60±0.027 | 0.78 |
| LVAWd, mm | 0.82±0.033 | 0.78±0.035 | 0.39 |
| LVEDV, l | 56±6.2 | 62±4.4 | 0.45 |
| LVM, mg | 89±5.1 | 74±8.8 | 0.24 |

EF, ejection fraction; FS, fractional shortening; LVIDd, left ventricular internal diameter at end-diastole; LVPWd, left ventricular posterior wall thickness at end- diastole; LVAWd, left ventricular anterior wall thickness at end-diastole; LVEDV, left ventricular end-diastolic volume; LVM, left ventricular mass.

**Table S2.** Primers of real-time PCR.

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| --- | --- |
| *Ppargc1a*\_forward | GGAGCTCCAAGACTCTAGACA |
| *Ppargc1a*\_reverse | CCAAAGTCTCTCTCAGGTAGC |
| *Nrf1*\_forward | TTACAGGGCGGTGAAATGAC |
| *Nrf1*\_reverse | GTTAAGGGCCATGGTGACAG |
| *Tfam*\_forward | CCCTGGAAGCTTTCAGATACG |
| *Tfam*\_reverse | AATTGCAGCCATGTGGAGG |
| *mt-Cytb*\_forward | ATTCCTTCATGTCGGACGAG |
| *mt-Cytb*\_reverse | ACTGAGAAGCCCCCTCAAAT |
| *H19*\_forward | GTCCACGAGACCAATGACTG |
| *H19*\_reverse | GTACCCACCTGTCGTCC |
| *Actb*\_forward | AAGATCAAGATCATTGCTCCTCC |
| *Actb*\_reverse | AAAGCCATGCCAATGTTGTC |
| *Nppa*\_forward | ATTTCAAGAACCTGCTAGACCA |
| *Nppa*\_reverse | CCAATCCTGTCAATCCTACCC |
| *Nppb*\_forward | AAGTCCTAGCCAGTCTCCAG |
| *Nppb*\_reverse | AACTTCAGTGCGTTACAGCC |