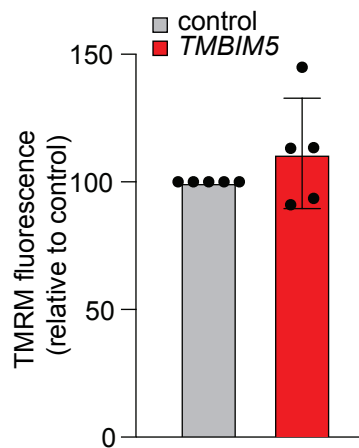
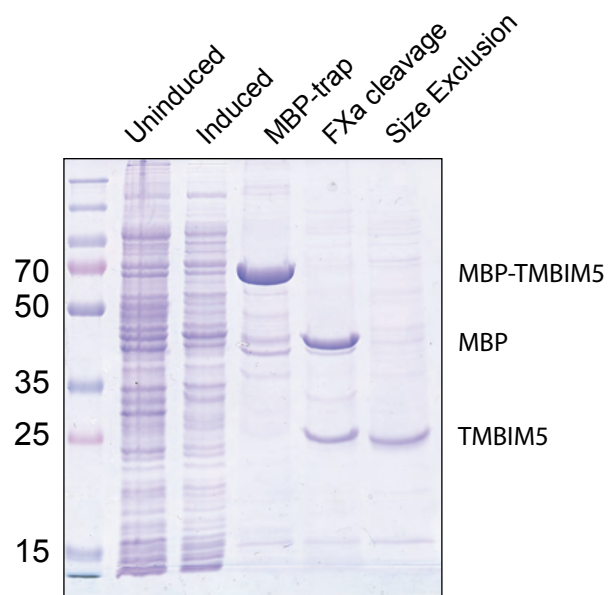
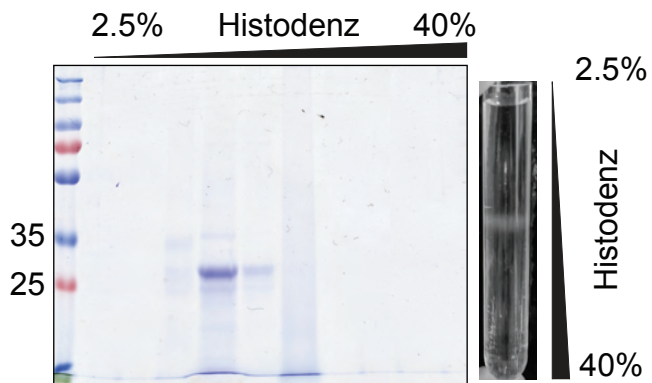
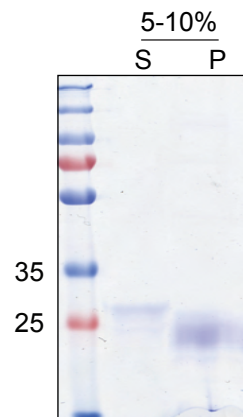
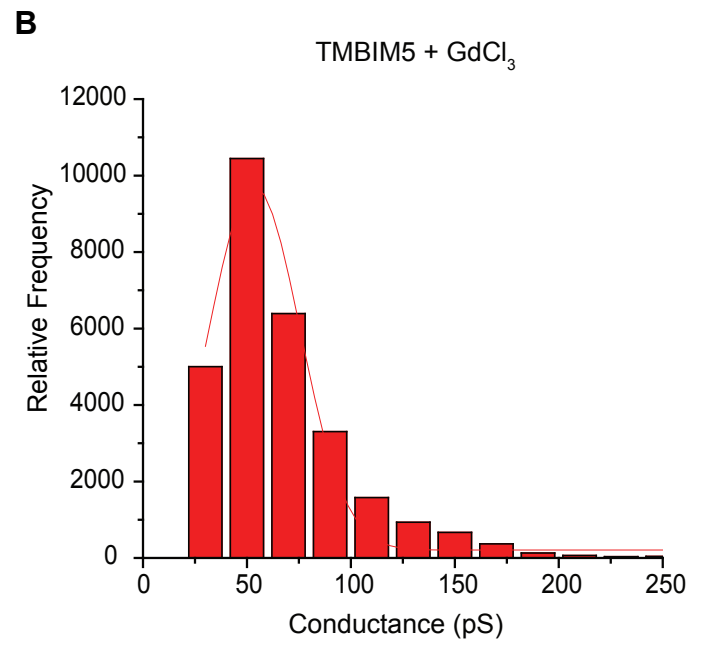
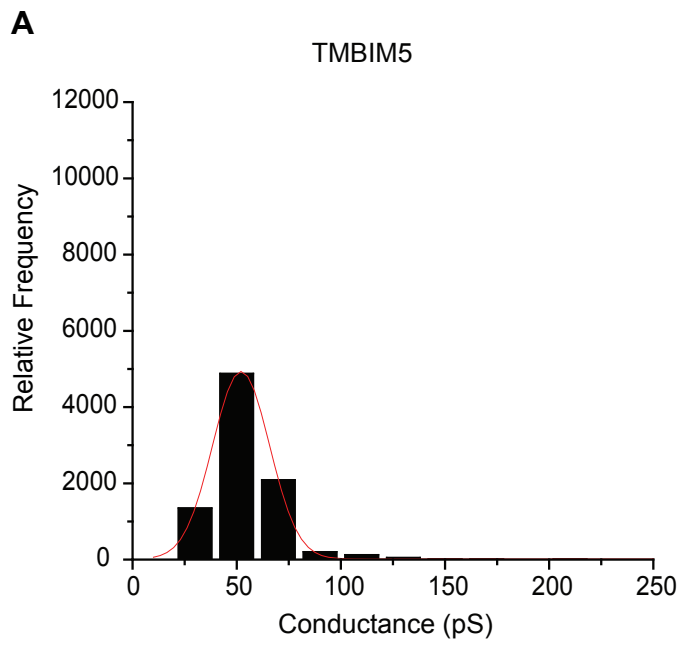


**Fig. S1**

**A****C****B**

TMBIM5 (Homo sapiens)	265	LPPTTVAGATLYSVAMYGGLVLFMSFLLYDTQKVIKRAEVSPMYGVQKYDPINSMLSIYMDTLNIFMRV
		P A YSV G ++FS+++LYD ++ R + V LS+Y+D +N+F+ +
YetJ (Bacillus subtilis)	145	FSPLNSAAMMAYSVI---GTIVFSLYIILYDLNQIKHRHITEDLIPVMA-----LSLYLDFINLFINL

**D****E**



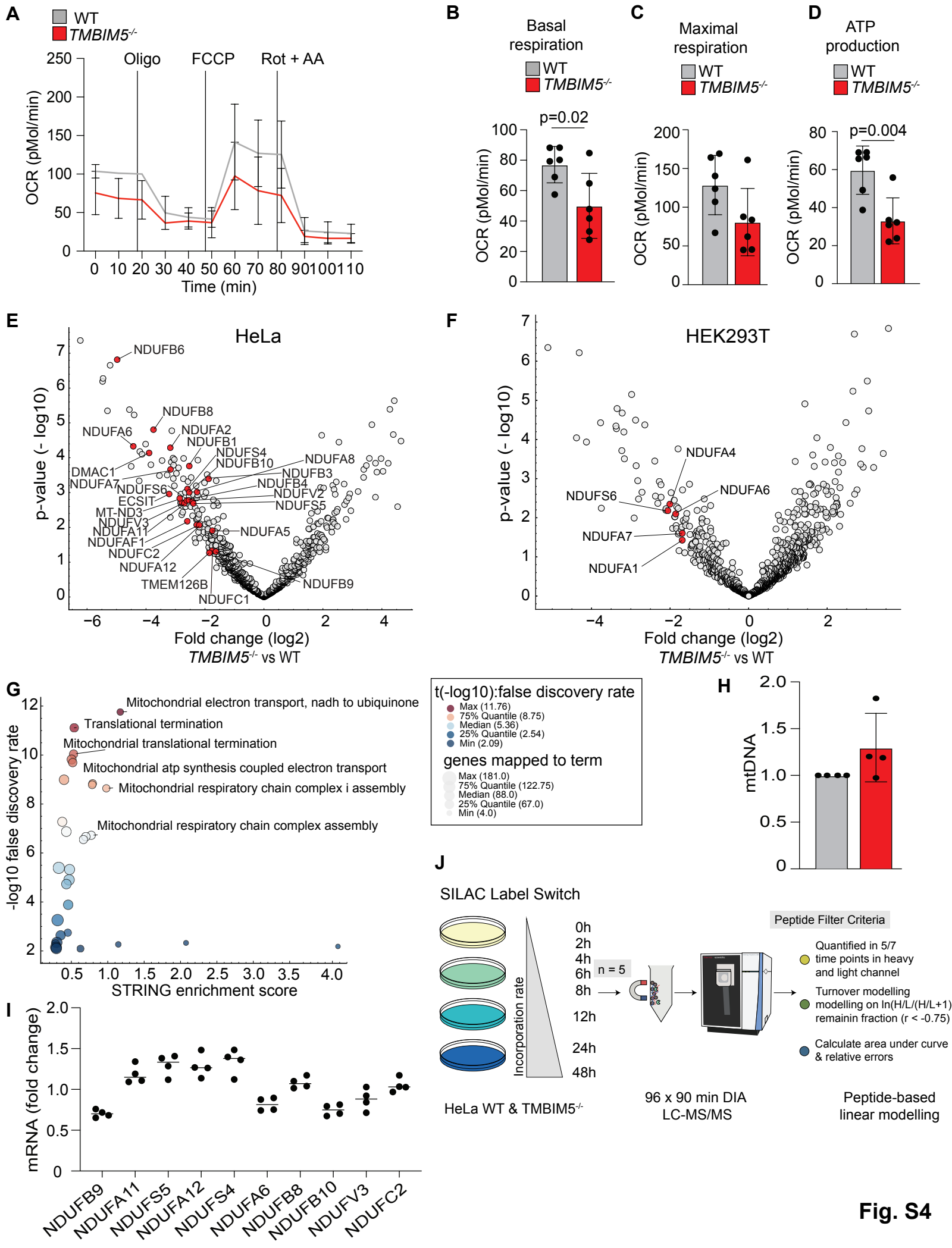


Fig. S4

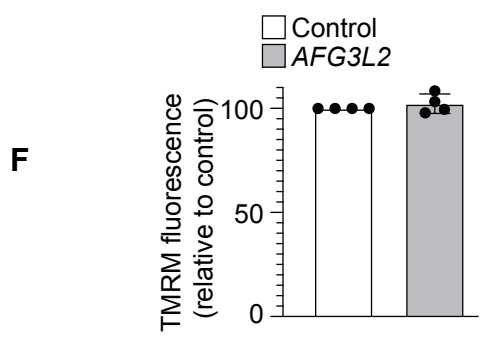
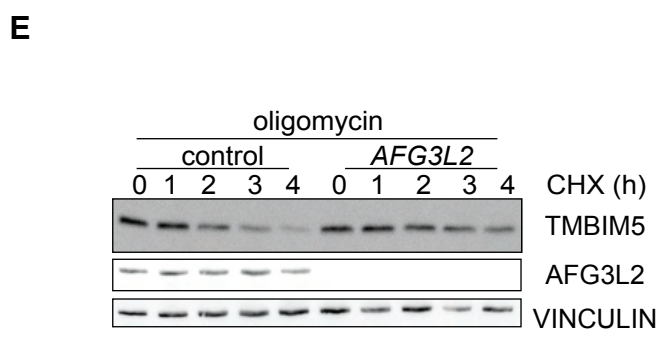
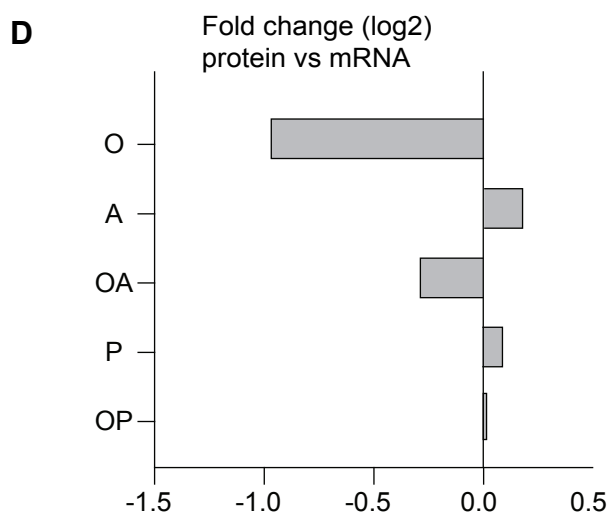
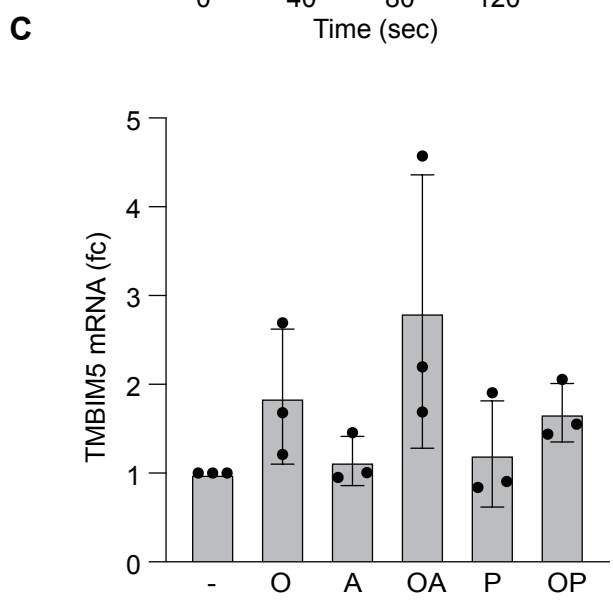
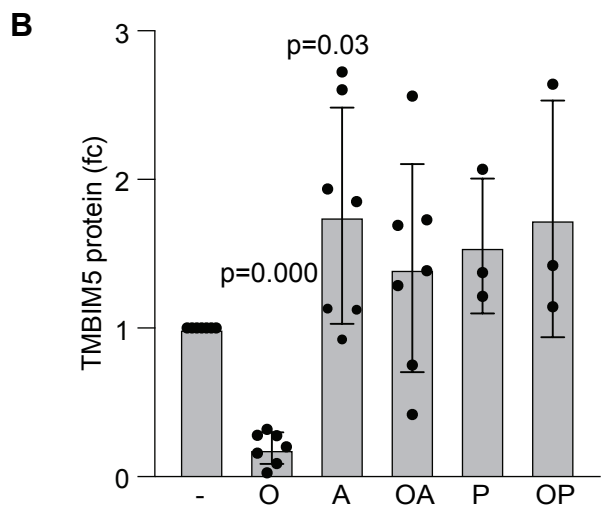
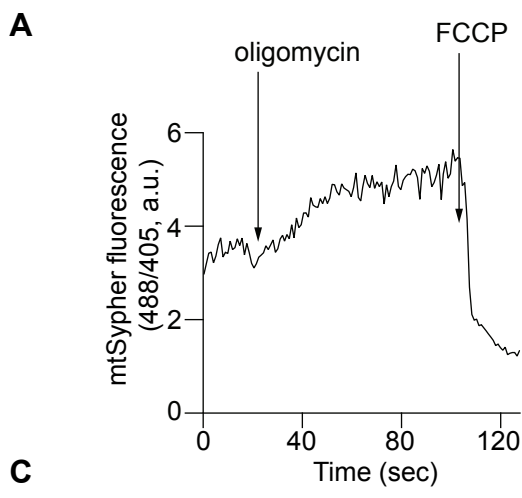


Fig. S5

## Supplementary Figures

### Figure S1. Mitochondrial Ca<sup>2+</sup> overload triggers apoptotic death of *TMBIM5*<sup>-/-</sup> cells

#### Related to Figure 1

- (A) Representative immunoblot of immunoprecipitates using anti-FLAG M2 beads of mitochondrial lysates which were isolated from Flp-In HEK293T-REx cells expressing AFG3L2<sup>E408Q</sup> FLAG when indicated.
- (B) Representative immunoblot of WT and *TMBIM5*<sup>-/-</sup> HeLa cells treated with the indicated drugs for 16 h. Control (0.1% DMSO), actinomycin D (1 μM), venetoclax (1 μM), A-1155463 (1 μM), staurosporine (1 μM), thapsigargin (2 μM). (n=3 independent experiments).
- (C) Representative transmission electron microscopy images of WT and *TMBIM5*<sup>-/-</sup> HeLa cells. Mitochondrial morphology is shown. Scale bar 1000 nm. (n=4 independent experiments).
- (D) Quantification of the electron microscopy images of HeLa WT and *TMBIM5*<sup>-/-</sup> cells. Mitochondrial parameters (area, length and cristae) were calculated with Fiji.
- (E) Quantification of the electron microscopy images of WT and *TMBIM5*<sup>-/-</sup> HeLa cells as in Figure S1F. The ratio between the long length and the short length of mitochondria is shown.
- (F) Quantification of electron microscopy images of WT and *TMBIM5*<sup>-/-</sup> HeLa cells as in Figure S1F. Number of cristae per mitochondrial length (μM) is shown.
- (G) Quantification of Figure 1C. The ratio between cleaved PARP (cPARP; 89 kDa) and PARP (116 kDa). WT and *TMBIM5*<sup>-/-</sup> HeLa cells transfected with scrambled siRNA (control) or siRNA targeting *MCU* for 72 h. When indicated, samples were treated with staurosporine (STS; 1 μM) for 16 h. (n=3 independent experiments).

**(H)** Quantification of WT and *TMBIM5*<sup>-/-</sup> HeLa cells with cytoplasmic cytochrome c. After transfection with scrambled siRNA (control) or siRNA targeting *MCU* for 72 h cells were incubated for 16 h with staurosporine (0.1 μM) in the presence of Z-VAD-FMK (50 μM) and epoxomicin (1 μM) to prevent apoptosis. Cells were analyzed by immunofluorescence microscopy using antibodies directed against cytochrome c (green) and TOMM20 (magenta). (n=3 independent experiments; number of cells > 150 in each experiment).

## **Figure S2. Reconstitution of TMBIM5 into liposomes**

### **Related to Figure 2**

- (A)** Mitochondrial membrane potential was monitored by TMRM staining in WT HeLa cells transfected with scrambled siRNA (control) or siRNA targeting *TMBIM5* for 72 h. Fluorescent intensity was calculated relative to the value upon CCCP addition (15 μM). Values are expressed as mean relative to control. Measurements of 5 different preparations were performed in triplicate.
- (B)** Sequence alignment of TMBIM5 (*Homo sapiens*) and YetJ (*Bacillus subtilis*). Conserved amino acids between the two species are written in the middle line. We used red color to highlight the amino acids forming the di-aspartyl dyad.
- (C)** Representative Coomassie-stained SDS-polyacrylamide gel (12.5%) demonstrating enrichment of recombinant TMBIM5 protein upon purification.
- (D)** Flotation assay demonstrating co-migration of recombinant TMBIM5 protein with the liposomes after ultracentrifugation in a discontinuous histodenz gradient. A representative Coomassie-stained SDS-polyacrylamide gel (12.5%) is shown. The ultracentrifugation tube containing TMBIM5-liposomes in discontinuous histodenz gradient after ultracentrifugation but before fractionation is shown on the right.

**(E)** Sodium bicarbonate extraction. Supernatant (S) and pellet (P) fractions of TMBIM5-proteoliposomes after treatment with 0.1 M sodium bicarbonate (pH 11.5) analyzed by SDS-polyacrylamide gel (12.5%) electrophoresis. Pellet (P), and supernatant (S) are indicated in the figure. The presence of TMBIM5 in the pellet fraction indicates its integration into the lipid bilayer.

**Figure S3. TMBIM5 acts as a  $\text{Ca}^{2+}/\text{H}^{+}$  exchanger.**

**Related to Figure 3**

- (A)** TMBIM5 conductance state histogram in  $\text{CaCl}_2$  buffer. (n=3 independent experiments).
- (B)** TMBIM5 conductance state histogram in  $\text{CaCl}_2$  buffer containing  $\text{GdCl}_2$ . (n=3 independent experiments).

**Figure S4. TMBIM5 controls oxidative phosphorylation**

**Related to Figure 4**

- (A)** Oxygen consumption rate (OCR) of WT and *TMBIM5*<sup>-/-</sup> HEK293T cells in glucose media. Traces are mean  $\pm$  SD of 6 independent experiments, each one run at least in triplicate. Labeled lines denotes injections of oligomycin (Oligo, 2  $\mu\text{M}$ ), FCCP (0.5  $\mu\text{M}$ ), rotenone and antimycin A (Rot + AA; both 0.5  $\mu\text{M}$ ).
- (B)** Basal respiration calculated from OCR experiment of WT and *TMBIM5*<sup>-/-</sup> HEK293T cells in (A). Two-tailed *t*-test. A p-value of <0.05 was considered statistically significant.
- (C)** Maximal respiration calculated from OCR experiment of WT and *TMBIM5*<sup>-/-</sup> HEK293T cells in (A).



- (D)** ATP production calculated from OCR experiments of WT and *TMBIM5*<sup>-/-</sup> HEK293T cells in (A). Two-tailed *t*-test. A *p*-value of <0.05 was considered statistically significant.
- (E)** Volcano plots of mitochondrial protein changes in *TMBIM5*<sup>-/-</sup> HeLa cells when compared to WT HeLa cells. Significantly enriched mitochondrial complex I proteins at an FDR cutoff of 0.05 are colored in red. (n=6 independent experiments). *P*-values were calculated from a two-sided *t*-test followed by a permutation-based FDR controlling to 0.05. See also Table S2.
- (F)** Volcano plots of mitochondrial protein changes in *TMBIM5*<sup>-/-</sup> HEK293T cells when compared to WT HEK293T cells. Significantly enriched mitochondrial complex I proteins at an FDR cutoff of 0.05 are colored in red. (n=5 independent experiments). *P*-values were calculated from a two-sided *t*-test followed by a permutation-based FDR controlling to 0.05. See also Table S3.
- (G)** Scatter plot comparing the STRING enrichment score versus the -log<sub>10</sub> transformed false discovery rate (color encoded). The number of genes mapped to the enriched GOBP term is encoded by size.
- (H)** mtDNA levels from HeLa cells assessed by qPCR amplification of mitochondrial *CYTB* (n=4 independent experiments).
- (I)** Expression of complex I subunits monitored by RT-qPCR. (n=4 independent experiments).
- (J)** Workflow of SILAC chase experiments in WT and *TMBIM5*<sup>-/-</sup> HeLa cells.

## Figure S5.

Related to Figure 6

- (A) Representative Sypher3mito traces of HeLa cells treated with oligomycin (10  $\mu$ M) or FCCP (10  $\mu$ M) at the indicated timepoints. Ratios of the absorbance at 488 nm and 405 nm are shown in arbitrary units (a.u.).
- (B) Quantification of Figure 6C. WT HeLa cells treated with the indicated drugs for 16 h. antimycin A (A; 10  $\mu$ M), piericidin (P; 10  $\mu$ M) and oligomycin (O; 10  $\mu$ M). (n=7 independent experiments).
- (C) Quantification of *TMBIM5* mRNA monitored by RT-qPCR of HeLa cells treated as in Figure 6C. (n=3 independent experiments).
- (D) Bar graph displaying log<sub>2</sub> fold changes between transcript (mRNA) (Figure S6C) and protein (Figure S6B) changes for *TMBIM5* in cells treated as indicated.
- (E) Representative immunoblot of WT HeLa cells transfected with scrambled siRNA (control) or siRNA targeting *AFG3L2* for 48 h. Samples were treated with the protein synthesis inhibitor cycloheximide (CHX; 10  $\mu$ g/ml) and oligomycin (10  $\mu$ M) and analyzed at the indicated time points (n=3 independent experiments).
- (F) Mitochondrial membrane potential was monitored with TMRM in WT HeLa cells transiently transfected with scrambled siRNA (control) and siRNA targeting *AFG3L2* for 48 h. Fluorescent intensity was calculated relative to the value in the presence of CCCP (15  $\mu$ M). Values are expressed as mean relative to control. Each measurement was performed in triplicate from 4 different preparations.