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**FIG S1.** Them5 gene appeared later in evolution but shares similar structure with Them4. (A) Stylized representation of hThem4 and hThem5 genes. Genomic (upper panel) and exon-intron structure and mRNA cartoon representation (lower panel) of hThem4 and hThem5 genes (4HBT - 4-hydroxybenzoyl-CoA thioesterase domain, MTS - mitochondrial targeting sequence). (B) Philogenetic tree of Them4 and Them5 orthologs in different species. Them4 othologs are found in lower eukaryotes, such as yeasts and chordata (Them4 orthologs upper group); Them5 orthologs, however, are present only in mammals (lower group).

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SSGVEL	SSGVFI	SSGVEL	SSGVFL	SSGVFL	CSGIFL	CSGVEF	ASGTFI	VSGVFI	ATSLFI	ATALF I	ATALE 1	ATGLF 1	ATALE 1	ATALF 1	ATVLEV	ATAARS
DTVYAK	<b>DTVYAK</b>	KTVYAK	RTVYAK	DIVYAR	DTVYAK	<b>DTVYAK</b>	<b>DTVYAK</b>	<b>WLFAK</b>	KTLYSE	XTLYTE	KTLHTQ	KTLYSE	SILVAT	SMLHTE	SKVYTE	ALHTE
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UL IPUD	NL IFVG	NL IEVG	KXVQII	NLIPLG	NL IPVG	NL IEVG	NLIFLG	NL IEVG	RPIFLO	KP IPLL	KP IPLL	RPVPLC	VSLPLN	NP IFLG	SPVELG	SPIPLG
NIRFR	NIKFR	INTREE	MVRFK	SIRFK	NIRFR	MIRFR	FUIRER	NIKFR	NENYK	NITYK	NIDYE	MINER	SINYE	TVD YES	NTNYR	NINYR
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SVLEG	PYLEG	PYLEC	PYLEG	PYLEG	PYLEG	PYLEG	PYLEC	PYLEC	PYLEC	LHLOG	LHLOG	P VL QG	PYTEC	PYLEC	HLLEG	HLLEG
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DEKSV	SERKSV	D-KKSV	REKEN	SEKKSV	SEKKSI	KKKSI	SECKSI	<b>NKKKSV</b>	EERMV	TUS	VERRIV	ERTV	SEEKLH	VERRMV	EDICI	AQKCVI
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THEM4_Mus_musculus	29	SEKVIRKDYA	L <mark>P N P S W</mark> J	TKDLRLL	FDQFMK	CEDGSWE	K R M P S H	IRQNP	E	TRAIQEF
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## E Steady-state kinetic constants for hThem5 active site mutants

	Enzyme	Substrate	K <sub>m</sub> (µM)	k <sub>cat</sub> (s⁻¹)	k <sub>cat</sub> /K <sub>m</sub> (M <sup>-1</sup> s <sup>-1</sup> )
hThem5	T183A	C18:2	0.6	0.2	3.6x10 <sup>5</sup>
hThem5	G160A	C18:2	7.6	0.3	4.6x10 <sup>4</sup>
hThem5	G159A/T183A	C18:2	n.d. <sup>*</sup>	0.1	n.d.

\* - not possible to determine Km

FIG S2. Protein sequence and structural analysis of Them4/5 and their orthologs.

(A) ClustalW multiple sequence alignment of Them4/5 and their orthologs in other species used for ConSurf computational analysis (http://consurf.tau.ac.il/). The alignment shows conserved residues (highlighted in dark-grey and grey). Also note the lack of conservation in mitochondrial targeting sequences (N-terminal part of the sequences) between Them4 and Them5 orthologous groups. (B) ClustalW multiple sequence alignment of selected Them5 and Them4 sequences (without predicted MTS). Conserved residues are boxed in red, secondary structure elements in the  $\Delta$ 34Them5 and  $\Delta$ 36Them4 crystal structures are indicated at the top and bottom of the alignment, respectively. The first residues present in the crystallographic models are highlighted with open arrows. Disordered stretches with no electron density, not included in the models, are shown in yellow shading; mutations used in this study are highlighted with red triangles (dimerization) and a red asterisk (enzymatically inactive). (C) Cartoon representation of superimposed crystal structures of  $\Delta$ 34Them5 (blue and orange) and  $\Delta$ 36Them4 (gray, transparent). N- and C-terminal residues in the  $\Delta$ 34Them5 structure are labeled, and disordered stretches not included in the model are shown as dotted lines. (D) Cartoon representation of the putative  $\Delta$ 36Them4 active site. Homodimer subunits are shown in cyan and gray, residues expected to be involved in catalysis and substrate recognition displayed as sticks (atom colors). (E) Steady-state kinetic constants for hThem5 active site mutants in the hydrolysis reaction with linoleyl-CoA, obtained at 37°C, pH 7.5. T183A mutant displays reduced turnover and k<sub>cat</sub> (due to the interferes with catalysis), G160A mutant has reduced turnover and k<sub>cat</sub> (due to the sterical interference in the active site), and G159A/T183A mutant has no activity.



FIG S3. Generation of Them5 knockout mice. (A) Presence of mThem5 genomic DNA in BAC clone was verified by PCR of individual exons (cloned mThem5 cDNA is positive control). BAC clone was used as PCR template for generating left and right homology region of targeting vector. (B) Transcript structure of mTHEM5 gene in mice. White areas represent untranslated regions (Ensembl database). (C) Targeted mThem5 allele. A targeting vector was generated that contains a 3.7-kb 5' homology region, an IRES/lacZ/neo cassette, and a 5-kb 3' homology region. A genomic DNA fragment of about 1.3 kb, including the ATG start codon in exon 1 and the full sequence of exon2, is deleted in the targeting vector. The targeting vector was linearized with Notl and electroporated into 129/Ola ES cells. (D) Screening of ES cell clones was performed by Southern blotting. DNA was digested with EcoRV and probed with an external probe (sequence 16980-17663). An internal probe was then used on Ndel digested DNA (sequence 9652-10154) for further characterization of ES cell clones positive for homologous recombination. Correctly targeted ES cells (highlighted in red) were used to generate chimeras. Male chimeras were mated with wild-type C57BL/6 females to obtain Them5<sup>+/-</sup> mice, which were intercrossed to produce Them5 homozygous mutants. (E) Progeny were genotyped for the presence of a targeted allele by multiplex PCR. The following primers were used for genotyping: P1-as 5'-GCA GCA GGC TGA ACT GAC TGA GG-3'; P2/KO-s 5'- GCT GCC TCG TCC TGC AGT TCA TTC-3"; P3/WT-s 5'-CAG GCG GCT GGA TTA AAC TAC C-3'. One reaction amplifies a 500-bp fragment from the targeted allele and the second reaction amplifies a 330-bp fragment from the wild-type allele.







WT

m/z

m/z

m/z

KO

WT

m/z

HET

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1000

970.1

975

941.1

950

925

HET

1600

1500





D

**FIG S4**. Mass spectrometry analysis of phospholipid composition. Lipid extracts were prepared from Them5 WT (upper panel), HET (middle) and KO (lower) mouse liver mitochondria and analyzed by mass spectrometry in the positive mode [6]. (A) Detailed species composition in the cardiolipin region, analyzed by MS in the positive mode. The lipid profile shows limited, predominantly quantitative differences in the CL profile between Them5 WT (top panel) and KO (bottom panel) liver mitochondria. P – palmitoyl, S – stearoyl, O – oleoyl, L – lineoyl, A – arachidoyl. (B) Species composition in the MLCL/CL region, as analyzed by MS in positive mode. P – palmitoyl, S – stearoyl, A – arachidoyl. (C) Increase in major monolysocardiolipin (MLCL) over cardiolipin (CL) levels upon Them5 ablation. (D) Total spectra analysis of WT and KO samples without major changes between WT and KO. (E) Species composition in the phospholipid region does not show major differences between WT (top) and KO (bottom) samples. Representative MS profiles are shown. PE – phosphatidylethanolamine, PC – phosphatidylcholine, PI – phosphatidylinositol.

Α

Liver weight relative to body weight, %







scale bar 3  $\mu m$ 





**FIG S5.** Loss of Them5 leads to fatty liver development and changes in mitochondria morphology. (A-B) Effect of Them5 loss on fatty liver development, reflected in (A) increased liver weights in Them5 HET and KO mice, as compared to WT littermates (n=8-12), and (B) progressive development of liver steatosis in Them5 HET and KO mice. Hematoxylin-eosin staining (top) and Oil Red O staining (bottom) of liver sections from 7-month-old mice (right). (C) Representative electron micrographs of liver cells with 3D reconstructed mitochondria (male mice 3-4 months of age, fasted). (D) Increased mitochondria volume in *Them5<sup>-/-</sup>* and *Them5<sup>+/-</sup>* hepatocytes of fasted mice, compared to WT controls (min. 15 mitochondria per cell/mouse were reconstructed, 3 cells/per mouse, n=2).