SUPPLEMENTARY INFORMATION

Loss of protein association causes cardiolipin degradation in Barth syndrome

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SUPPLEMENTARY RESULTS

Supplementary Table 1. BTHS increases the acyl turnover of CL in human lymphoblasts[a]

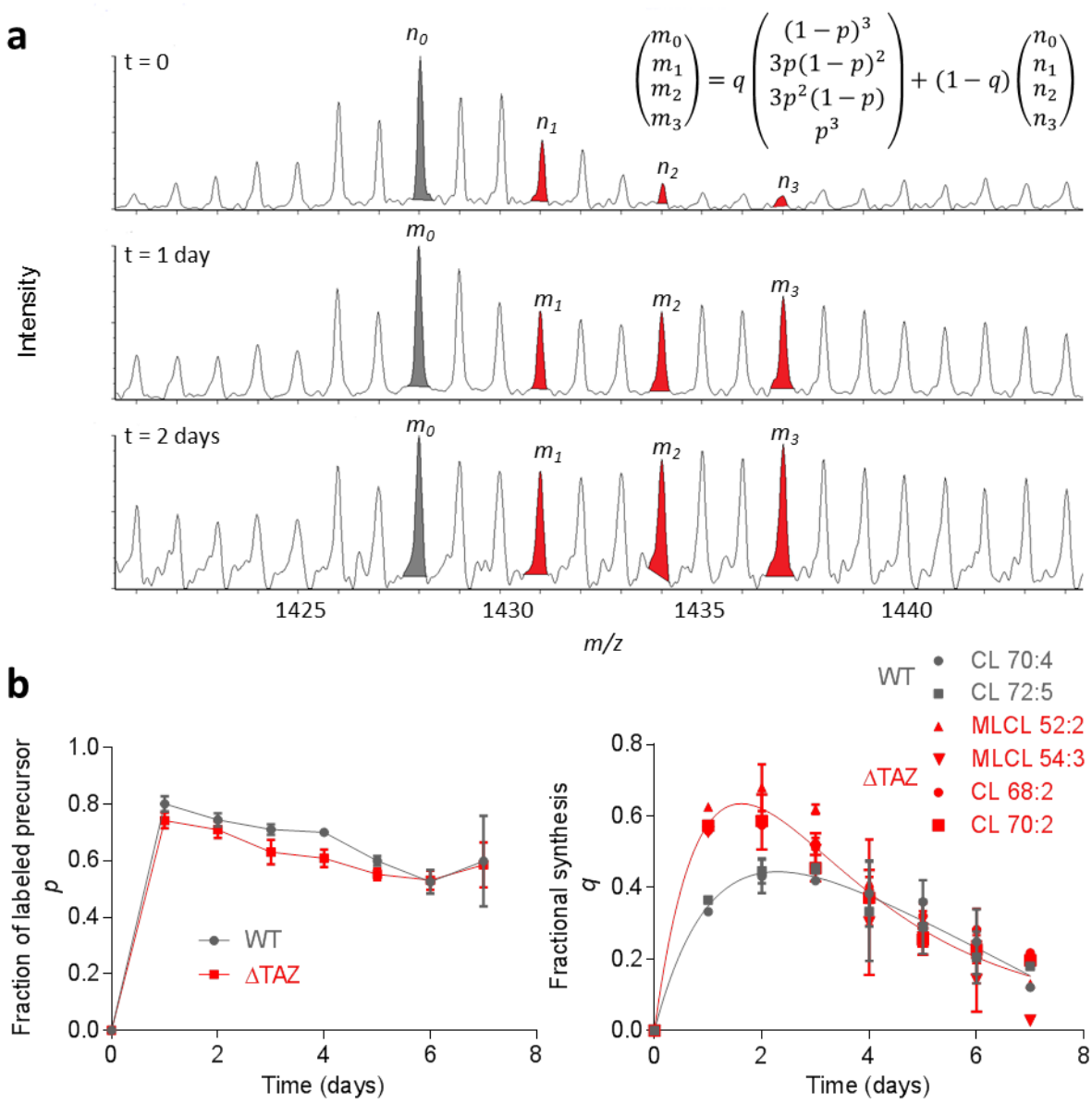
Phospholipid species	Control k (10^{-3} h^{-1})	Control t_h (h)	BTHS k (10^{-3} h^{-1})	BTHS t_h (h)
PC 36:2	200±4	3.5	249±11	2.8
PE 36:2	80±8	8.7	125±7	5.5
PE 38:2	100±12	6.9	104±13	6.7
PI 36:1	172±9	4.0	285±8	2.4
PI 36:2	191±18	3.6	307±29	2.3
CL 70:3			120±16	5.8
CL 70:4	37±3	19.0		
CL 72:6			151±18	4.6
CL 72:7	27±5	25.7		
CL 74:7	40±7	17.4	149±16	4.7

[a] Rate constants with standard errors (k) and half-life times (t_h) were estimated from q values in Fig. 1c by non-linear regression.

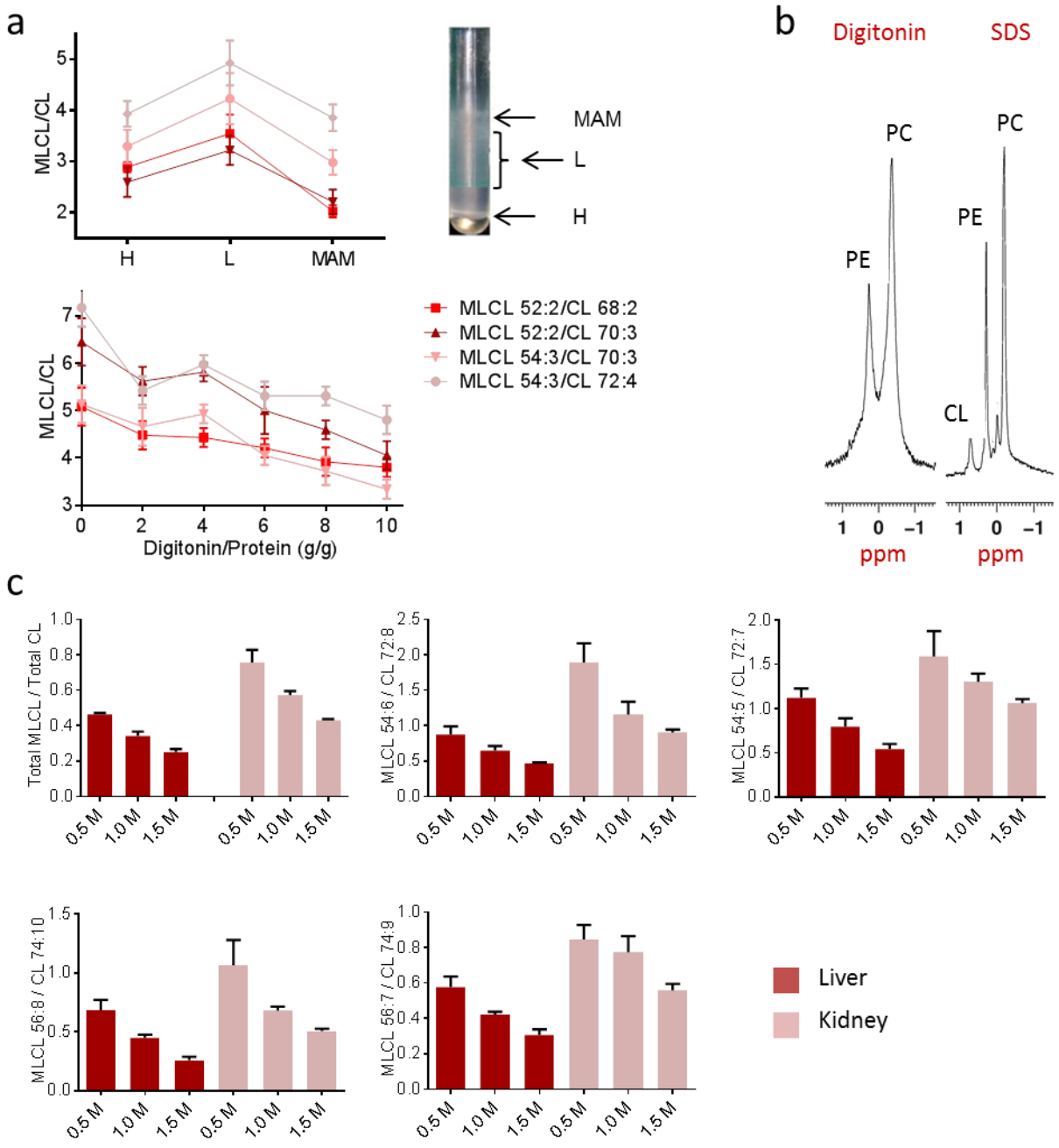
Supplementary Table 2. ^{31}P-NMR spectroscopy reveals motional restriction of CL but not of PC, PE, and MLCL[a]

Source of mitochondria	Genotype	Detergent	N	Signal intensity (Percent of total ^{31}P signal)			
				PC	PE	CL	MLCL
Mouse liver	Wild-type	Digitonin	5	28±3	12±1	0±0	0±0
		SDS	4	37±5	14±3	5±1	0±0
Mouse kidney	Wild-type	Digitonin	2	22±2	15±2	0±0	0±0
		SDS	2	24±2	21±2	4±1	0±0
	TAZKD	Digitonin	2	20±3	19±1	0±0	1.2±0.1
		SDS	2	25±4	28±1	5±1	1.8±0.2
Human lymphoblast	Control	Digitonin	1	26.5	15.5	0	0
		SDS	1	48.4	29.8	7.7	0
	BTHS	Digitonin	1	25.4	17.9	0	1.5
		SDS	1	46.8	33.1	4.5	2.7

[a] Membranes were prepared from mitochondria and solubilized with digitonin or SDS. ^{31}P-NMR spectra were acquired in a 700 MHz instrument equipped with a cryoprobe; the signals of individual phospholipids were quantified. In the presence of SDS, all phospholipids (PC, PE, CL, MLCL) produced resolvable signals. In the presence of digitonin, only PC, PE, and MLCL were detectable. Data are mean values with SEM of the indicated number of replicates (N).

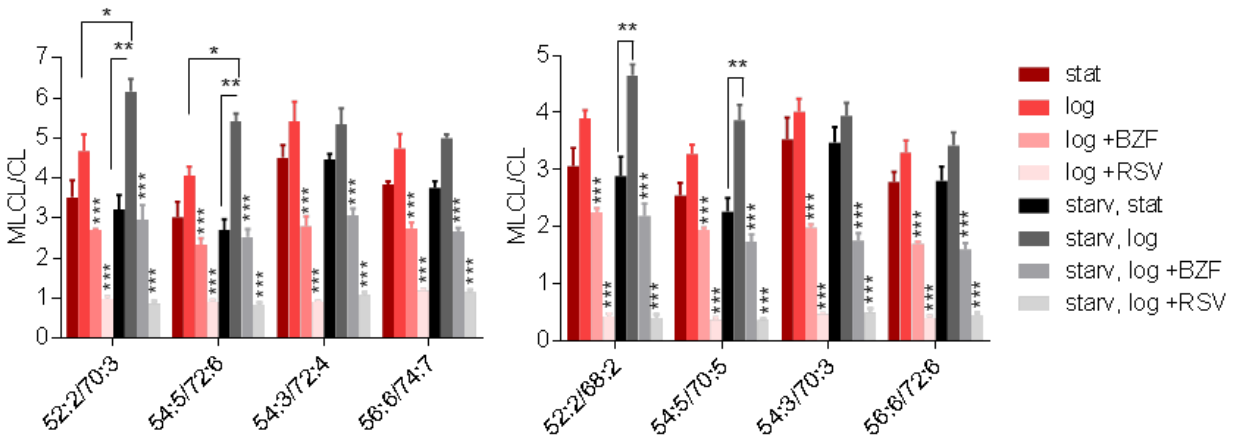


Supplementary Figure 1. Tafazzin deficiency increases the glycerol turnover of CL. Mouse embryonic fibroblasts were grown in the presence of D-[U-$^{13}C_6$]-glucose for 2 days, after which the medium was switched to unlabeled glucose. Lipids were extracted at different time points and analyzed by MS. (**a**) Spectra acquired during the labeling period show an increase of signals corresponding to CL 70:4 isotopomers with 1, 2, and 3 labeled glycerols. The equation shows the relation between spectral intensities (n_i, m_i), the fraction of labeled precursor (p) and the fraction of newly synthesized CL 70:4 (q) in the cells. (**b**) The fractional synthesis (q) of CL and MLCL species rose and vanished faster in tafazzin knock-out cells (ΔTAZ) than in wild-type cells (WT). The intracellular proportion of labeled precursor (p) was similar in both cell lines. Data are mean values with SEM (N=3).

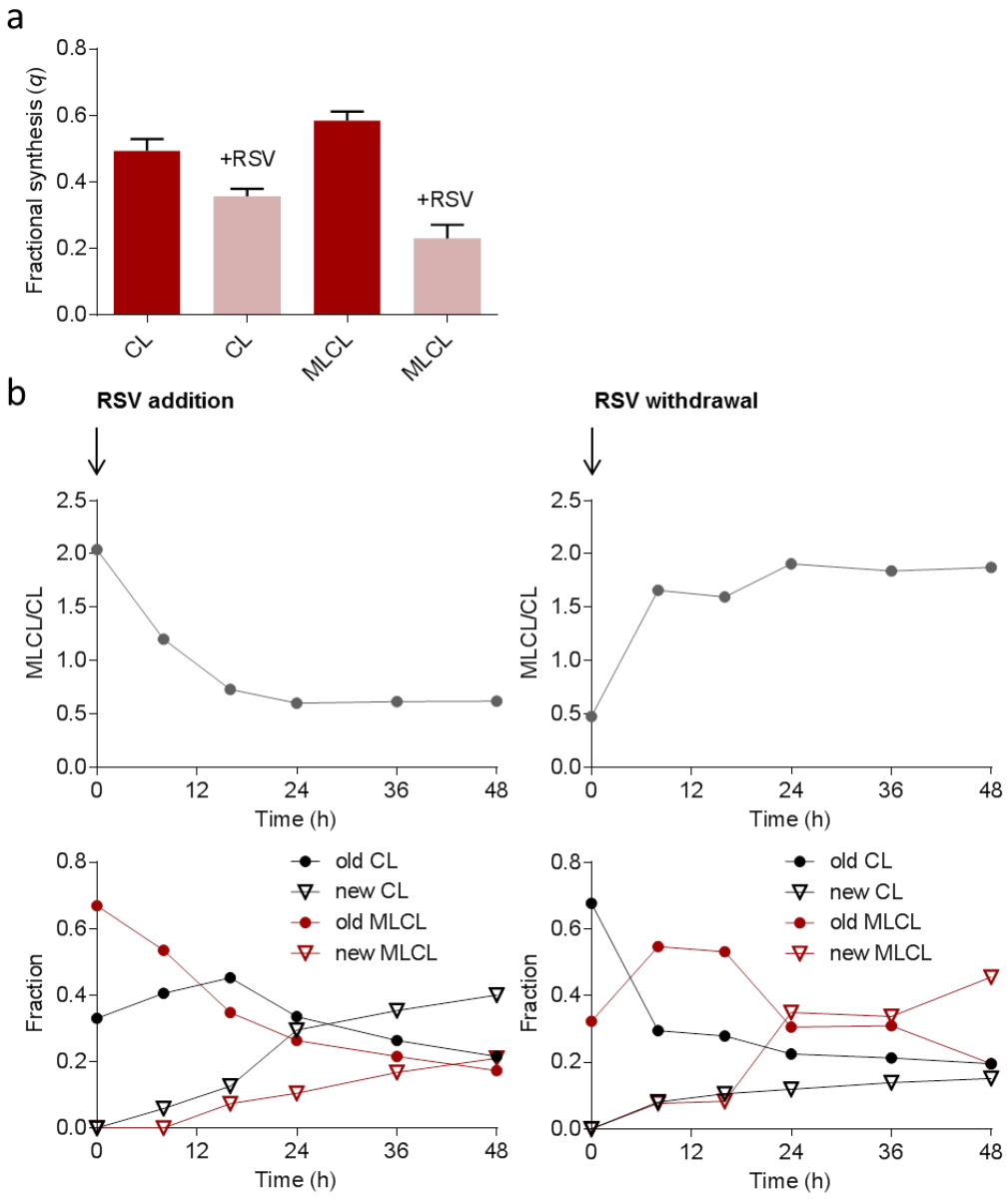


Supplementary Figure 2. CL but not MLCL is associated with proteins. (**a**) Mitochondria were isolated from lymphoblasts of BTHS patients and sub-fractionated by Percoll density gradient centrifugation into high-density mitochondria (H), low-density mitochondria (L), and mitochondria-associated membranes (MAM). The MLCL/CL ratios were higher in low-density mitochondria than in high-density mitochondria ($P<0.004$, paired *t*-test) and in MAM ($P<0.002$, paired *t*-test). Data are means with SEM (N=3). Mitochondria were also treated with different amounts of digitonin followed by centrifugation at 100,000 g. The MLCL/CL ratios were determined in the non-solubilized pellets. Digitonin solubilized MLCL more readily than CL,

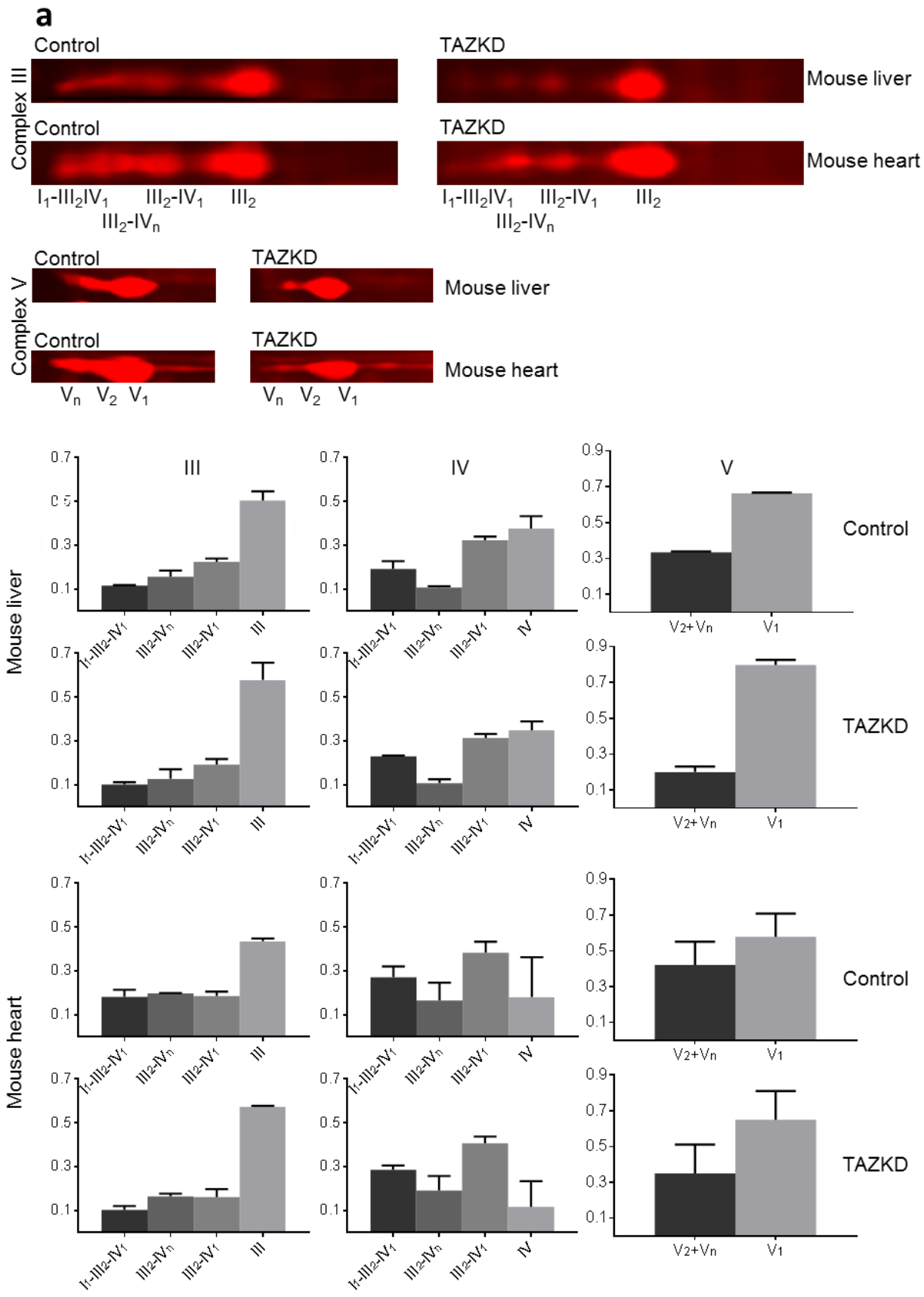
resulting in a concentration-dependent decrease of the MLCL/CL ratio in the pellets (P<0.001, ANOVA). Data are mean values with ranges of duplicate determinations. (**b**) Mouse liver mitochondria (20 mg protein per sample) were incubated in hypotonic medium (15 mM Tris, pH 7.4) for 30 min. Membranes were re-collected by centrifugation (39,000 g, 15 min) and solubilized with either digitonin (3 g/g protein) or SDS (1.5 g/g protein). Static ^{31}P-NMR spectra of the samples (volume 0.6 mL) were acquired on a 700 MHz spectrometer equipped with a cryo-probe. In the presence of SDS, ^{31}P signals of the major mitochondrial phospholipids PC, PE, and CL were detectable. In the presence of digitonin, the CL signal was not detectable because of line broadening, which indicates tight protein association. (**c**) Mitochondria were isolated from liver and kidney of TAZKD mice, solubilized with n-dodecyl β-D-maltoside (DDM, 2.5 g/g protein) and loaded on a sucrose density gradient. The gradient was spun for 20 hours at 158,000 g at 4°C and fractions were analyzed by MS. The MLCL/CL ratio decreased from low to high density (P<0.005 for liver; P<0.02 for kidney, ANOVA). Data are means with SEM (N=3).

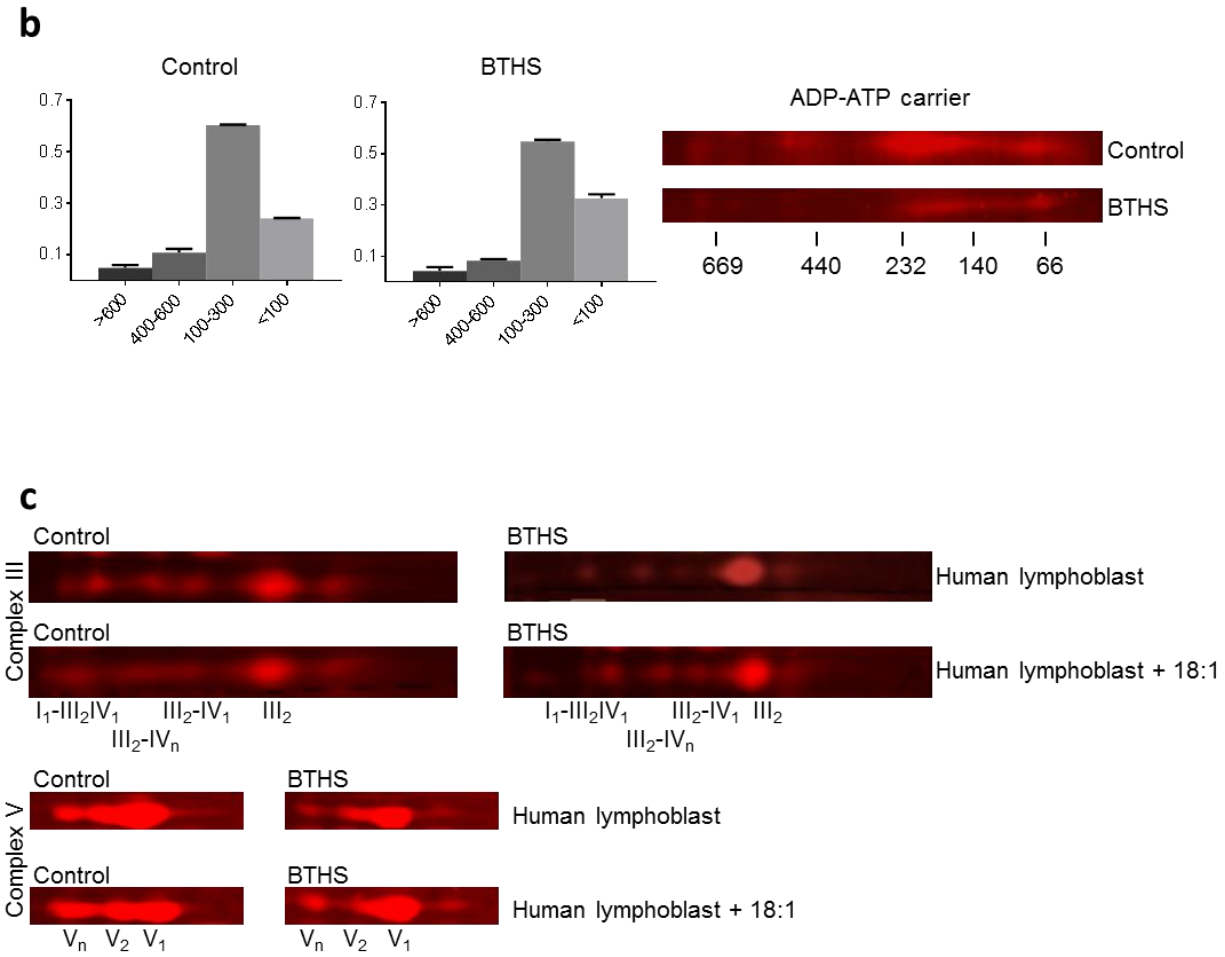


Supplementary Figure 3. The MLCL/CL ratio is reduced by stationary growth, bezafibrate, and resveratrol. Lymphoblasts from BTHS patients were cultured in normal medium or in starvation medium (starv, 3% fetal bovine serum) and harvested in the logarithmic (log) or stationary (stat) phase of growth. Lipids were analyzed by MS to determine the MLCL/CL ratios of corresponding molecular species (CL-MLCL pairs formed by loss of 18:1 or 16:0). Starvation increased the MLCL/CL ratio in the logarithmic growth phase (**P*<0.05). Logarithmic growth increased the MLCL/CL ratio under starvation condition (***P*<0.01). Treatment with 400 µM bezafibrate (BZF) or 40 µM resveratrol (RSV) decreased the MLCL/CL ratio (****P*<0.001). Data (means±s.e.m., N=3) were analyzed by *t*-test.

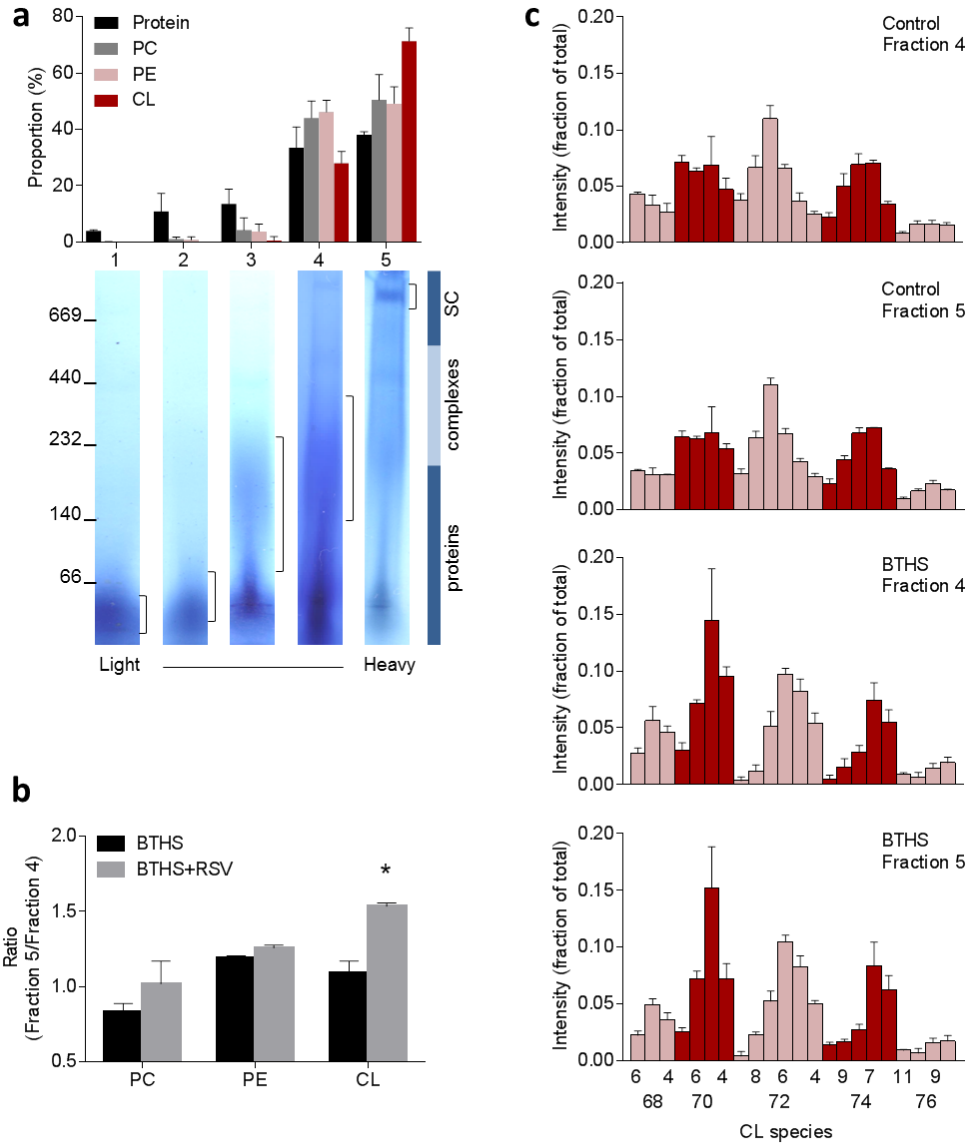


Supplementary Figure 4. Resveratrol stabilizes CL. (**a**) BTHS lymphoblasts were cultured ±40 µM resveratrol (RSV) for 36 hours. The fractional synthesis of CL and MLCL was estimated from the ^{13}C labeling pattern after incubation with D-[U-$^{13}C_6$]-glucose for 24 hours. RSV decreased the fractional synthesis of CL (P<0.01) and MLCL (P<0.003). Data (mean values with SEM, N=3) were analyzed by t-test. (**b**) BTHS lymphoblasts were incubated with D-[U-$^{13}C_6$]-glucose. The MLCL/CL ratio (upper graphs) and the proportion of old and newly synthesized CL and MLCL (lower graphs) were determined by MS. Upon addition of RSV (40 µM), the MLCL/CL ratio decreased over 24 hours. This transition was caused by the stabilization of preexisting CL. Upon withdrawal of RSV, the MLCL/CL ratio returned to the pre-RSV level within 24 hours. This transition was caused by rapid clearance of preexisting CL.

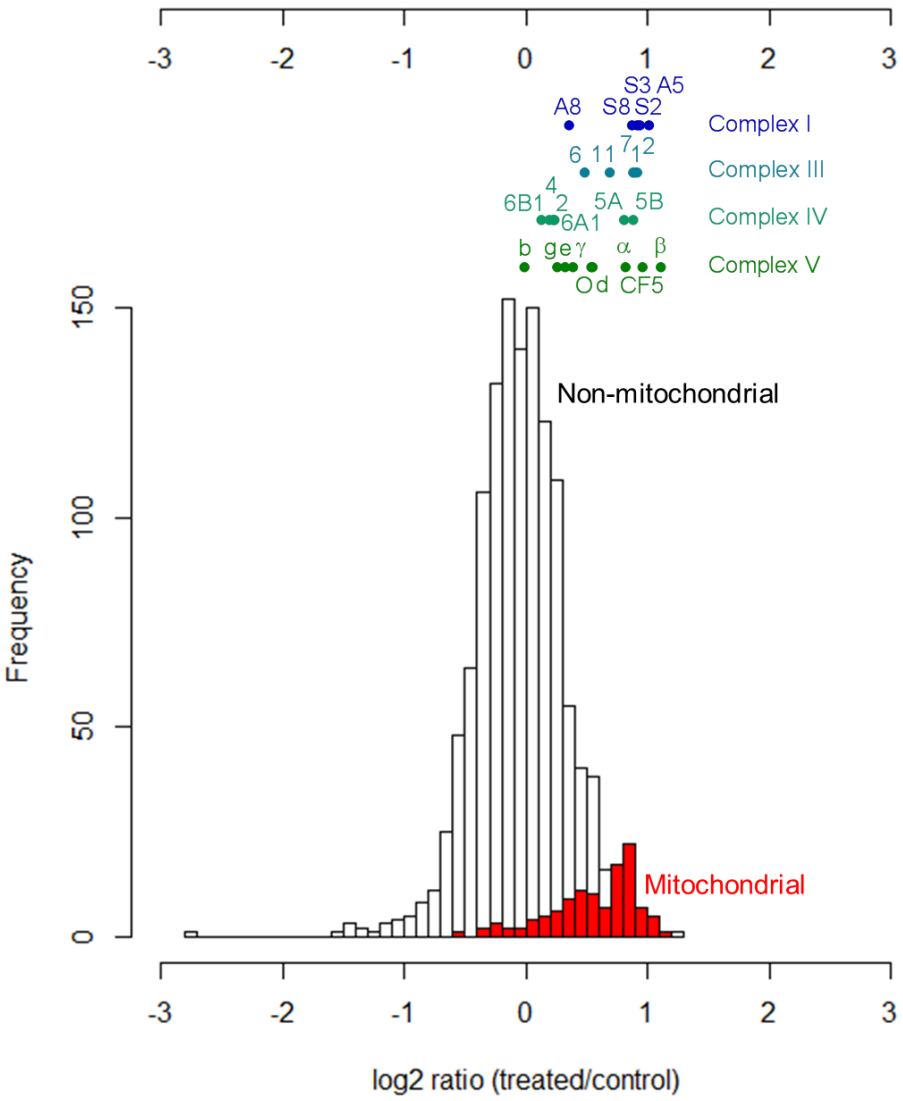

a
Control
TAZKD
Complex III
Mouse liver
Mouse heart
I1-III2IV1
III2-IV1
III2
III2-IVn
Complex V
Vn
V2
V1
III
IV
V
Mouse liver
Mouse heart
Control
TAZKD
V2+Vn



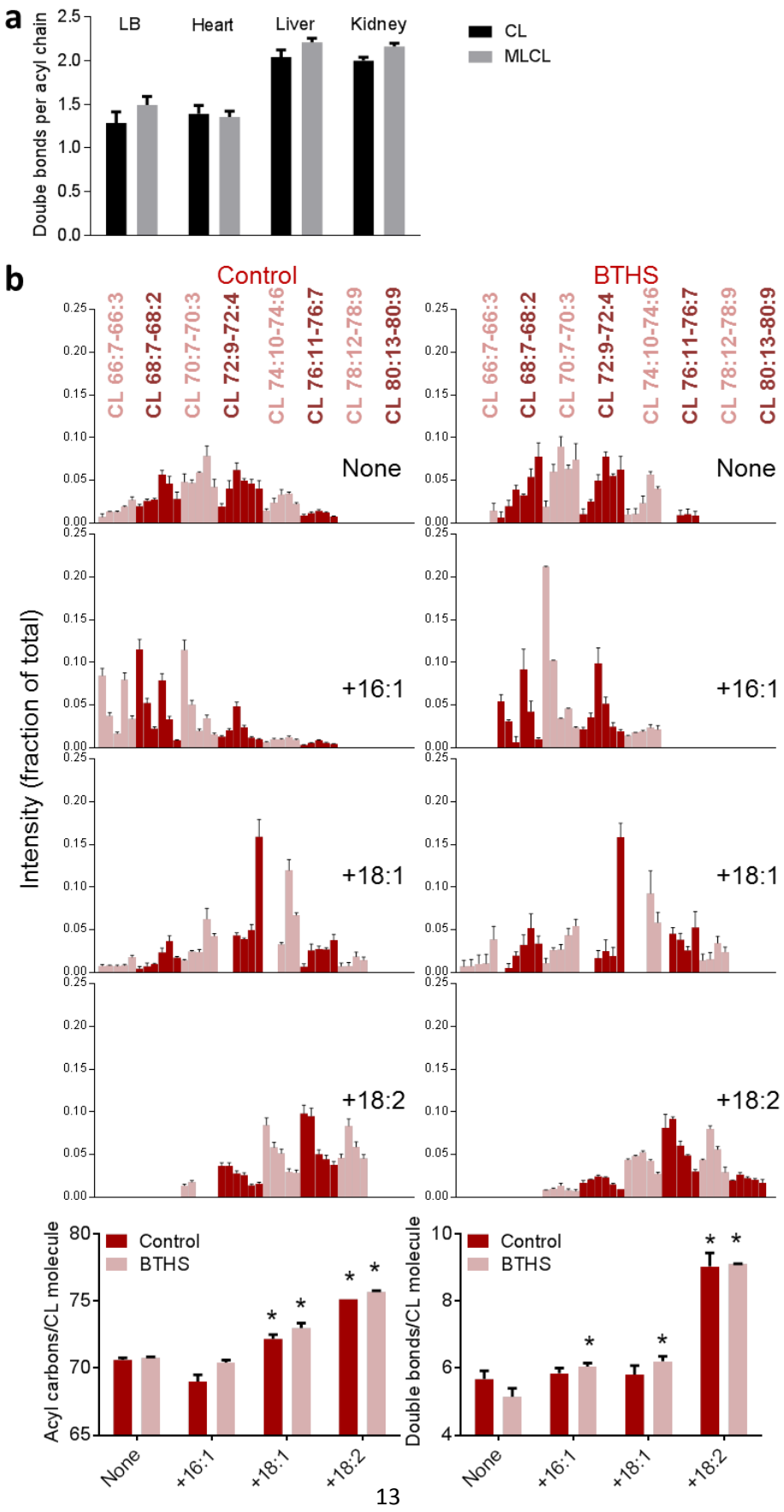
Supplementary Figure 5. Tafazzin deficiency affects supercomplexes. (**a**) Mitochondria were isolated from liver and heart of TAZKD mice and controls. Mitochondria were solubilized with digitonin and separated by 2D-Blue Native/SDS-PAGE followed by quantitative Western Blot analysis with the LiCor system, using fluorescent secondary antibodies. The primary antibodies recognized UQCRC2 (complex III), MtCO1 (complex IV), and F1α (complex V). Data are mean values with ranges from 2 experiments. Tafazzin knockdown reduced the supercomplex-associated portion of complex III and V. (**b**) Mitochondria were isolated from human lymphoblasts of BTHS patients and controls. The distribution of the ADP-ATP carrier (ANT1) was analyzed by 2D-Blue Native/SDS-PAGE as above. The positions of molecular weight markers are indicated (masses in kDa). The carrier was found in 4 regions of the gel. BTHS increased the proportion of the ADP-ATP carrier recovered below 100 kDa from 24±2% to 33±3%. Data are mean values with ranges from 2 experiments. (**c**) Human lymphoblasts were cultured ± exogenous oleic acid (18:1) for 4 days. Mitochondria were isolated and analyzed by 2D-Blue Native/SDS-PAGE followed by Western Blot analysis. BTHS reduced the supercomplex-associated portion of complex III and V, which was partially reversed by oleic acid.



Supplementary Figure 6. CL is enriched in the supercomplex fraction. (**a**) Mitochondrial membranes were isolated from normal lymphoblasts, solubilized with digitonin, and separated by sucrose density gradient centrifugation. Protein concentration was measured and 1D-Blue Native PAGE was performed in the 5 gradient fractions. Protein complexes were recovered in fraction 4, and supercomplexes (SC) in fraction 5. Lipids were analyzed by MS; CL was recovered in fractions 4 and 5; PC and PE were recovered in fractions 3-5. (**b**) The same experiment was done with BTHS lymphoblasts treated ±40 µM resveratrol (RSV) for 24 hrs. RSV treatment increased the proportion of CL in fraction 5 but did not alter the distribution of PC and PE. (**c**) The molecular species composition of CL was identical in fractions 4 and 5. All experiments were performed in triplicates. Data are mean values with SEM (* P<0.001, t-test).



Supplementary Figure 7. 3-Bromopyruvate increases the relative abundance of mitochondrial proteins. Normal lymphoblasts were cultured in SILAC medium containing either light or heavy-labeled amino acids. Each culture was divided in two aliquots one of which was treated with 80 µM 3-bromopyruvate for 5 hrs. Subsequently, two SILAC proteomics analyses were performed. For the forward analysis, heavy-labeled treated and light-labeled untreated cells were mixed in equal proportions and for the reverse analysis, heavy-labeled untreated and light-labeled treated cells were mixed in equal proportions. The graph shows a histogram of the lymphoblast proteome, in which mitochondrial proteins are shown in red bars and non-mitochondrial proteins in open bars. 3-Bromopyruvate treatment induced an about 1.7-fold increase in the relative abundance of mitochondrial proteins. The SILAC ratios of the identified subunits of complexes I, III, IV, and V are indicated above the histogram. The raw MS files are accessible through the link ftp://massive.ucsd.edu/MSV000079600/raw/Barth_syndrome/.


a
Doube bonds per acyl chain
LB
Heart
Liver
Kidney
CL
MLCL
b
Control
BTHS
CL 66:7-66:3
CL 68:7-68:2
CL 70:7-70:3
CL 72:9-72:4
CL 74:10-74:6
CL 76:11-76:7
CL 78:12-78:9
CL 80:13-80:9
None
+16:1
+18:1
+18:2
Intensity (fraction of total)
Acyl carbons/CL molecule
Double bonds/CL molecule
Control
BTHS
None
+16:1
+18:1
+18:2

Supplementary Figure 8. Exogenous fatty acids alter the molecular composition of CL. (**a**) CL and MLCL were analyzed in lymphoblasts of BTHS patients and in heart, liver and kidney of TAZKD mice. The average number of double bonds per acyl chain was similar in CL and MLCL. Data are mean values with SEM (N=4). (**b**) Human lymphoblasts were grown in the presence of 0.1 mM palmitoleic acid (16:1), oleic acid (18:1), or linoleic acid (18:2) for 4 days. The molecular composition of CL was determined by MS. Data (means with SEM, N=3) of treated samples were compared to controls by *t*-test (*P<0.05). All exogenous fatty acids increased the unsaturation of CL in BTHS but not in controls. 18:1 and 18:2 increased the chain length in BTHS and controls.