Artificial ALCAT1 overexpression affects supercomplex formation and increases ROS in respiring mitochondria

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Supplementary information

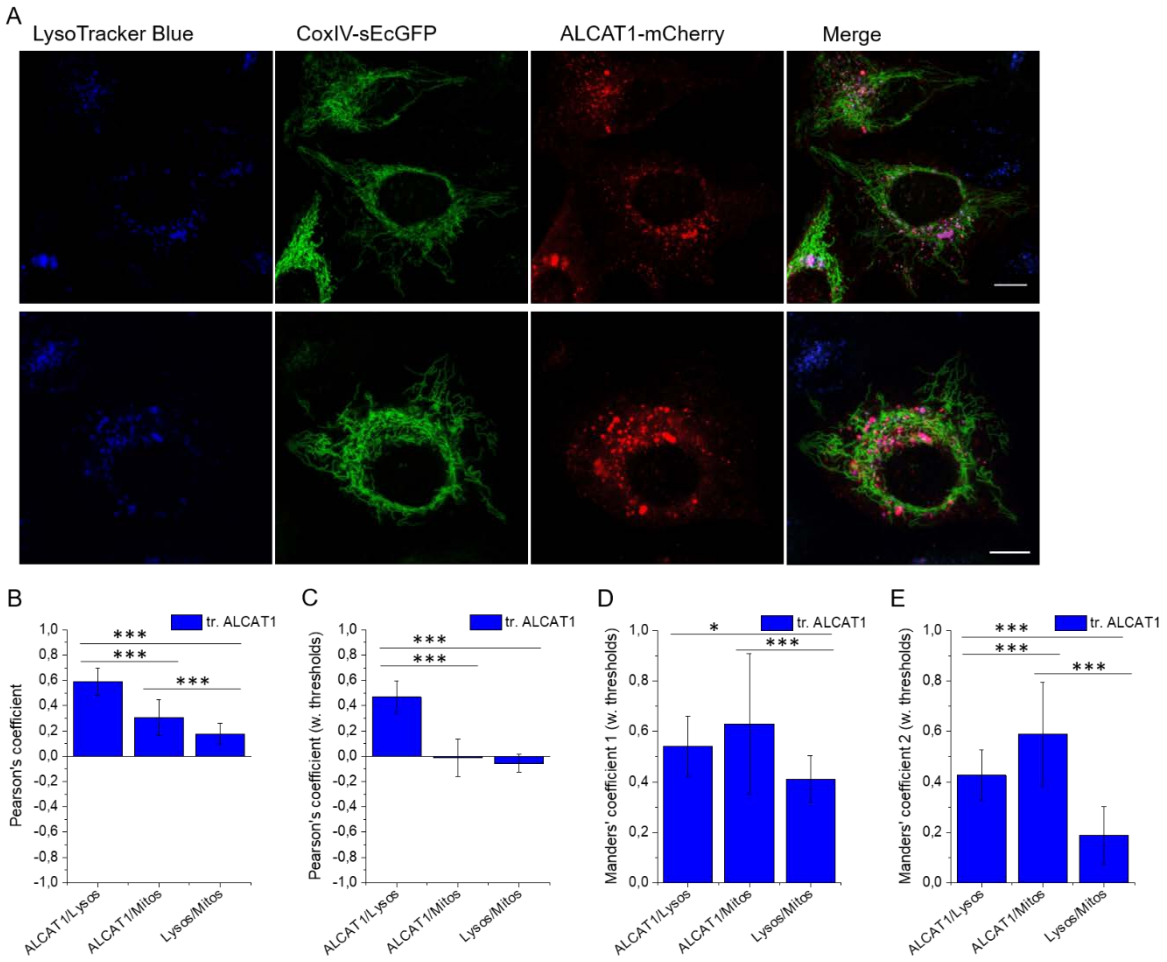
Co-localization analysis of ALCAT1. The quantification of the co-localization was performed using the Pearson coefficient, with a value of +1 for perfect correlation and -1 for perfect anticorrelation. According to these values, the proportion of ALCAT1 co-localization with lysosomes was significantly higher than the co-localization of ALCAT1 and mitochondria or lysosomes and mitochondria (Fig. S1B-C). Thresholding only made a significant difference for the last two groups (ALCAT1 and mitochondria compared to lysosomes and mitochondria), which were equal. In addition to the Pearson coefficient, the estimated split Manders colocalization coefficients with a value of +1 for perfect correlation and 0 for perfect anticorrelation were also analyzed (Fig. S1D-E). There is a coefficient for each of the two channels indicating the proportion of the signal in one channel colocalized with the other channel. In this case, there is not much difference between the two channels. While the Pearson coefficient uses the deviation from the mean, the Manders coefficients use the absolute intensities. With these coefficients, the overlap between ALCAT1 and lysosomes is still quite high. However, the overlap between ALCAT1 and mitochondria is higher in this case, while according to this analysis the overlap between lysosomes and mitochondria is lower (Fig. S1D-E).

Seahorse XF Cell Mito Stress Test Kit. The Seahorse XF Cell Mito Stress Test Kit enables metabolic profiling of mitochondria measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). It uses sequential injection of inhibitors (oligomycin, FCCP, rotenone/antimycin A) of the ETC complexes to measure basal respiration, proton leak, ATP production and non-mitochondrial respiration (Fig. S2). After measuring the basal respiration, oligomycin, an inhibitor of the ATP synthase, is injected and causes a decreased OCR that characterizes the ATP-linked mitochondrial respiration. FCCP is an uncoupling compound that intercepts the membrane potential so that the electron flow through the ETC complexes is faster and the maximal amount of oxygen is reduced by CIV. The measured OCR shows the maximal respiration. The injection of rotenone and antimycin A inhibits the activity of CI and CIII. As a result, no electrons are transported by the ETC and the mitochondrial respiration shuts down. This enables the measurement of non-mitochondrial respiration. Moreover, the ratio of ATP production to basal respiration represents the coupling efficiency of the mitochondrial respiration (XF Cell Mito Stress Test Kit User Guide).

Determination of mitochondrial membrane potential. Tetramethylrhodamine ethyl ester (TMRE) is a fluorescent and lipophilic cation that accumulates within mitochondria dependent on the negative mitochondrial membrane potential (MMP). Consequently, a higher mitochondrial fluorescence indicates a more negative MMP (O'Reilly et al., 2003). The cells were incubated in medium containing 7 nM TMRE under normal growth conditions (37 °C, 5 % CO_2). After 30 min of incubation with the dye, the samples were used for fluorescence microscopic analysis (ex. 559 nm, em. 650-710 nm). The cells were imaged using the following settings: $\lambda_{ex.}$ 579 nm, $\lambda_{em.}$ 590-610 nm.

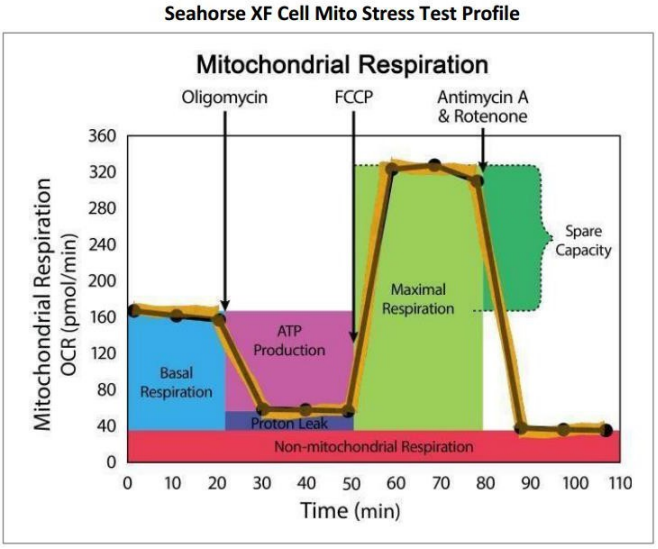
Supplementary Figures

Supplementary Figure S1



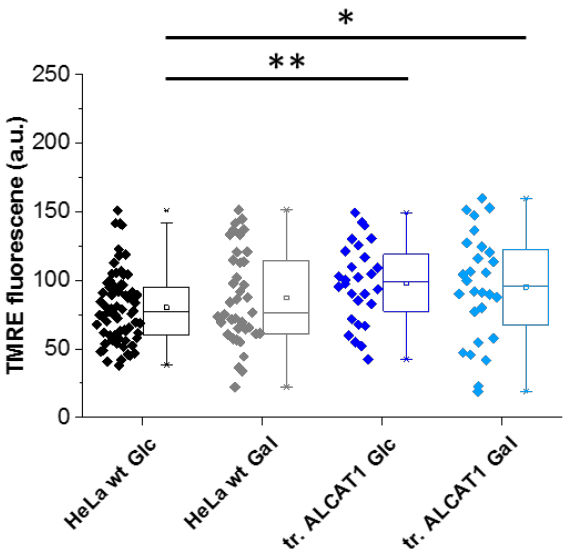
Suppl. Figure 1. Intracellular localization of overexpressed ALCAT1 in HeLa cells. (A) Triple color fluorescence microscope analysis of transiently transfected HeLa cells expressing the mitochondrial protein CoxIV-sEcGFP and ALCAT1-mCherry, stained with LysoTracker blue™. The images were processed with ImageJ to enhance the contrast. (B) Pearson's coefficients and (C) Pearson's coefficients using thresholds for both channels and (D-E) thresholded Manders' split colocalization coefficients for Co-localization analysis (N=2, 21 cells). The ImageJ plugin "Colocalization threshold" was used for calculation of the coefficients, automatically determining thresholds for each channel. One Way ANOVA with post hoc Tukey (***P < 0,001; **P < 0.01;*P < 0.05), SD are shown. Scale bars: 10 µM (A).

Supplementary Figure S2



Suppl. Figure 2. Seahorse XF Cell Mito Stress Test Profile. The serial injection of ETC complex inhibitors enables the measurement of basal respiration, proton leak, maximal respiration and non-mitochondrial respiration. (XF Cell Mito Stress Test Kit User Guide)

Supplementary Figure S3



Suppl. Figure S3. Mitochondrial membrane potential in ALCAT overexpressing cells. Mitochondria in cells were stained with TMRE: (λex. 559 nm, λem. 650-710 nm. (A and B) Quantitative analysis of fluorescence intensities displayed as box plots with the measured fluorescence intensities of 20-30 measured cells on the left. The experiments were performed at RT without temperature control. N=2 (***P < 0,001; **P < 0.01;*P < 0.05).

Supplementary Information

Table 1. Primary antibodies used for Western Blot analysis

Name	Species reactivity	Host	poly/mono	kDa	Dilution for WB	Company
Anti-ALCAT1	Human	Rabbit	poly	49	1:2000	abcam
Anti-ATP5b [Suβ]	Human, Rat, Mouse	Rabbit	poly	56	1:2000	proteintech
Anti-ATP5I [Sue]	Human, Rat, Mouse	Rabbit	poly	8	1:500	abcam
Anti-VDAC	Human, Rat, Mouse	Rabbit	poly	32	1:2000	Cell signaling

The peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch, dianova) was used as a secondary antibody at a final dilution of 1:2000.

Gene sequence of pSems-ALCAT1-2A-GFP-NL

Molecule Features

Molecule: pSems-ALCAT1-2A-GFP-NLS, 7301 bps DNA Circular
File Name: pSems-ALCAT1-2A-GFP-NLS.cm5, dated 23 Feb 2016
Description:

Start	End	Name	Feature Key	Draw
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2166	2231	2A		Gene
2232	2945	EGFP		Gene
2952	3020	NLS		Gene
3988	4788	Neomycin		Gene

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Plasmid map and gene sequence of pSems-ALCAT1-HA

Molecule Features

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File Name: pSems-ALCAT1-HAtag.cm5, dated 11 Feb 2016 Description: Ligation of product ALCAT1* into pSems-1-26-Halo7-AN-HAtag*

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Plasmid map and gene sequence of pSems-ALCAT1-mCherry

Molecule Features

Molecule:	pSems-ALCAT1-mCherry, 7154 bps DNA Circular

File Name: pSems-ALCAT1-mCherry.cm5, dated 07 Jun 2016

Description:

Start	End	Name	Feature Key	Draw
921	2162	ALCAT1		Gene
2169	2876	mCherry		Gene
3841	4641	Neomycin		Gene

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCT
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TTGTGTGTT

GGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTAC
AACAAGGCAAG
GCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAG
GCGTTTTGCG

CTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT
GATTATTGAC

TAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATA
GCCCATATA

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TCCCATAGT

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