CLUH Couples Mitochondrial Distribution to the Energetic and Metabolic Status

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SUMMARY STATEMENT.
Mitochondrial distribution within the cell is critical in supplying ATP at specific sites. We show here that CLUH couples mitochondrial distribution to the energetic and metabolic status in human cells.
ABSTRACT
Mitochondrial dynamics and distribution are critical in supplying ATP in response to energy demand. CLUH is a protein involved in mitochondrial distribution, whose dysfunction leads to mitochondrial clustering, the metabolic consequences of which remain unknown. To gain insight into the role of CLUH on mitochondrial energy production and cellular metabolism, we have generated CLUH knockout cells using CRISPR/Cas9. Mitochondrial clustering was associated with a smaller cell size, and with decreased abundance of respiratory complexes, resulting in OXPHOS defects. This energetic impairment was found to be due to the alteration of mitochondrial translation and to a metabolic shift towards glucose dependency. Metabolomic profiling by mass spectroscopy disclosed increase in some amino-acids concentration witnessing a dysfunctional Krebs cycle, increased palmitoylcarnitine concentration indicating an alteration of fatty acid oxidation, and a dramatic decrease of phosphatidylcholine and sphingomyeline concentrations, consistent with the decreased cell size. Taken together, our study establishes a clear function for CLUH in coupling mitochondrial distribution to the control of cell energetic and metabolic status.
INTRODUCTION

Mitochondrial morphology and ultrastructural organization are known to vary depending on the tissue and the physiological states considered (Benard et al., 2006), and many studies indicate that physical rearrangements, which involve remodeling of the inner membrane to modulate the form of the cristae, and changes of the balance between mitochondrial fission and fusion, affect mitochondrial energy production (Benard and Rossignol, 2008; Zick et al., 2009). In addition, mitochondrial motility and location within the cell are crucial for the maintenance of cell homeostasis and specific functions. For instance, mitochondria in spermatozooids are specifically located at the most proximal part of the flagellum to supply ATP for sperm movement; while during apoptosis, active mitochondria aggregate near the nucleus, as an upstream event to cytochrome c release (Haga et al., 2003). Similar movements towards the nucleus were observed during the process of mitophagy, where ubiquitinated mitochondria recruit p62 and HDAC6 to promote their subsequent clearance (Lee et al., 2010). Further defects in mitochondrial transport and distribution were evidenced in neurons from a Miro1 deficient mouse, although mitochondrial respiration was normal (Nguyen et al., 2014). These examples demonstrate that both active and damaged mitochondria are subject to intense movements, which eventually could parallel metabolic modifications.

The CLU protein was first identified in the slime mould Dictyostelium discoideum as a factor required for the proper distribution of mitochondria within the cytoplasm (Zhu et al., 1997). So far, CLU deficiency induces mitochondrial clustering in all organisms tested, as the protist Dictyostelium discoideum (Fields et al., 2002; Zhu et al., 1997), the yeast Saccharomyces cerevisiae (Fields et al., 1998), the plant Arabidopsis thaliana (El Zawily et al., 2014; Logan et al., 2003), and the animal Drosophila melanogaster (Cox and Spradling, 2009), and in cell lines as mouse embryonic fibroblasts (Gao et al., 2014) and human cancer cells (Gao et al., 2014). While CLU function is clearly required to prevent mitochondrial clustering, the mechanism responsible remains elusive. Fields et al reported that in cluA-Dictyostelium cells, mitochondria were connected to neighboring mitochondria through narrow constrictions, suggesting impaired fission (Fields et al., 2002). However, in the Arabidopsis friendly CLU mutant, constrictions were not observed, but increased association time during the mitochondrial fusion process was reported.
to favor clustering (El Zawily et al., 2014). In Drosophila, the CLU orthologue clueless interacts genetically (Cox and Spradling, 2009) and physically with the PINK1-Parkin pathway (Sen et al., 2015), to promote Valosin-Containing Protein (VCP)-mediated Marf degradation, during the Parkin-dependent mitophagy (Wang et al., 2016). More recently, Gao et al reported that human CLUH interacts with a subset of mRNAs encoding mitochondrial proteins, suggesting that CLUH is also involved in mitochondrial biogenesis (Gao et al., 2014). Support for this role has also been provided by studies in Drosophila which demonstrated that CLU is a ribo-nucleo-protein binding ribosomal components at the mitochondrial outer membrane (Sen and Cox, 2016). Clearly, further studies are needed to understand whether alteration in mitochondrial distribution in CLUH depleted cells is primary effect or secondary to mitochondrial biogenesis defects.

Together, these data revealed a broad involvement of CLU in mitochondrial physiology, which were reinforced by bioinformatics analyses showing a coexpression of CLUH with a set of genes involved in mitochondrial metabolic pathways and oxidative phosphorylation (Gao et al., 2014). However, despite having clear effects on mitochondrial distribution, it is unclear to what degree CLU function is involved in energy production and cellular metabolism. To gain insight into this question, we have generated CLUH knockout HeLa cells using CRISPR/Cas9. Characterization of this CLUH-KO cell line revealed a decreased cell size with clustered mitochondria. In addition CLUH-KO cells showed abnormal cristae structures, OXPHOS defects resulting from altered mitochondrial translation, and drastic changes in metabolomic profiles.
RESULTS

CLUH knockout in HeLa cells leads to clustered mitochondria and reduced cell size.

To characterize the impact of CLUH on cellular metabolism, we generated CLUH knockout (KO) HeLa cells using CRISPR/Cas9. Plasmids encoding for Cas9 and for the guide-RNA targeting CLUH exon4 were cotransfected with a Puromycin-resistant gene flanked by homology arms upstream and downstream of the target site. Twenty-three colonies were screened by Western blot, among which three selected clones showed the absence of the CLUH protein (Fig. 1A and Fig. S1A, B). We then selected by qRT-PCR one cell line which further had a reduction of at least 80% of CLUH transcript compared to wild-type (WT) cells (Fig. 1B). In addition, we checked on genomic DNA that no PCR product was detectable using primers hybridizing to CLUH exon4 (Fig. 1C). Because CLU deficiency induces mitochondrial clustering in all models so far reported, we investigated mitochondrial distribution in CLUH-KO cells. Live confocal imaging of MitoTracker stained cells showed that mitochondria were clustered around the nucleus in CLUH-KO cells (Fig. 1D and Fig. S1C). In addition, we observed that these cells had drastically reduced size (Fig. 1E). To address an eventual correlation between the reduced cell size and the abnormal mitochondrial distribution, we standardized cell size using the CYTOO micro-patterns (Figs. 1F, S1D). After 12 hours, phase contrast microscopy showed that both WT and mutant cells occupied the whole surface of square-shaped CYTOO micropatterns (Figs. 1F, left panel, S1D). While WT cells showed the expected even mitochondrial distribution throughout the cytoplasm (Fig. 1F, right panel), the KO cells showed mitochondria clustered around the nucleus, despite the cell growth over the whole available surface (Figs. 1F, right panel, S1D). Quantification of mitochondrial clustering, calculated as the ratio of the mitochondrial surface divided by the whole cell area, revealed that 68% of CLUH KO cells show clustered mitochondria, while only 28% of WT cells show this phenotype (Fig. S1E,F). Moreover, analysis of mitochondrial network morphology using Imaris Filament Tracer demonstrated that mitochondria were more connected in the KO cells (Fig. 1F, right panel), although, the resolution of our fluorescent microscopy observations cannot distinguish whether mitochondria are truly fused or simply apposed. Because clustering of mitochondria could be related to cytoskeleton alterations, we investigated whether cell mobility, using a scratch test, and paclitaxel, a microtubule-stabilizing drug, would be affected
in KO cells. Interestingly, the absence of CLUH decreased the ability to migrate, and increased sensitivity to paclitaxel treatment (Fig. S2).

**CLUH deficiency decreases the abundance of OXPHOS complexes following the inhibition of mitochondrial translation.**

To gain further insights in the consequences of CLUH deficiency, we analyzed the expression of OXPHOS complex subunits with specific antibodies. Western blot analysis showed that ATP5A (C-V), UQRC2 (C-III), Cox II (C-IV) and NDUFB8 (C-I) subunits were all decreased in both whole cell lysate and mitochondrial lysate of KO cells, while VDAC expression level remained constant in both conditions (Fig. 2A). We then explored respiratory complex assembly using BN-PAGE with specific antibodies for each complex (Fig. 2B). Interestingly, although the abundance of complex II, which is composed only of nuclear encoded subunits, did not differ between WT and KO cells, the abundances of complexes I, III, IV and V, that are composed of subunits encoded by both nuclear and mitochondrial genes, were drastically decreased by 50%, 80%, 40% and 20%, respectively in KO cells (Fig. 2C). As these changes are frequently associated to a defect in the maintenance of the amount of the mitochondrial genome, we evaluated the integrity of the mtDNA. Indeed, by using qPCR with two sets of primers targeting nuclear and mitochondrial genome, we show that mtDNA amount was not affected in KO compared to the WT cells neither at steady state nor during recovering after ethidium bromide depletion (Fig. S3A, B). Moreover, using long fragment PCR, we did not evidenced any mtDNA deletions in KO cells (Fig. S3C). These results indicate that neither mtDNA quantity (Fig. S3B) nor quality (Fig. S3C) was compromised in KO cells. Thus, we further investigated the status of mitochondrial translation. To do this, we first performed an *in silico* co-expression analysis of CLUH in the NCI-60 database (Fig. 2D) using CellMiner (http://discover.nci.nih.gov/cellminer/) (Langer et al., 2010; Liu et al., 2010; Reinhold et al., 2012). The 258 genes that positively correlate with CLUH mRNA expression level, (correlation factors between 1 and 0.5; Table. S1), were classified using the functional annotation database PANTHER (http://pantherdb.org). Based on PANTHER sorting classifications by molecular function and biological process (Mi et al., 2013), the 258 genes are mainly involved on mitochondrial function (Fig. 2D). However, beside genes involved in
mitochondrial metabolic pathways and cellular respiration previously reported (Gao et al., 2014), the ontology based analysis revealed that genes involved in mitochondrial translation are highly correlated to CLUH expression, being enriched by 14 times (Fig. 2D). To corroborate this analysis, we analyzed the products of mitochondrial translation, by pulsing cells with radioactive $^{35}$S-methionine in the presence of emetine, an inhibitor of cytoplasmic translation. We found that mitochondrial translation was significantly decreased in KO cells (Fig. 2E). Indeed, despite the equal total protein loading shown by Coomasie staining (Fig. 2E, right panel), the autoradiography shows a clear decrease in the abundance of mitochondrial radiolabeled proteins in KO versus WT cells (Fig. 2E, left and middle panel). This defect could be due to the decrease in mitochondrial protein-synthesis machinery as Western blots on mitochondrial fractions revealed that the small MRPS27 and MRPS17 and large MRPL44 and MRPL13 ribosomal subunits, , the ERAL1 translation assembly factor and EFG1 and TUFM elongation factors were decreased in KO versus WT cells (Fig. 2F). Taken together, these results indicate that in addition to mitochondrial mis-localization, mitochondrial translation and OXPHOS complex assembly are significantly decreased in CLUH-KO cells.

**CLUH deficiency leads to altered mitochondrial ultrastructure and impaired respiratory activities.**

OXPHOS complexes contribute in shaping the inner membrane, particularly the ATPase which is involved in cristae formation (Davies et al., 2012). To determine whether decreased abundance of OXPHOS complexes in CLUH-KO cells parallels mitochondrial ultrastructure defects, we performed transmission electron microscopy (TEM) on WT and CLUH-KO cells (Fig. 3A). KO cells contained two populations of mitochondria distinguishable on the basis of their ultrastructure: one population contained few, disorganized cristae and an electron-light matrix (Fig. 3A, yellow arrowheads) and–second population enclosed small mitochondria and displayed an electron-dense matrix and many cristae (Fig. 3A, red arrowheads). These populations represent 40% and 60 % of total mitochondria respectively (Fig. S4). Moreover, we observed that some mitochondria, 2 % of quantified mitochondria, were connected in KO cells by thin tubules (Fig. 3A, black arrow) suggesting a failure of membrane to fully divide or to fuse. We next chose to investigate OXPHOS complex activities in isolated mitochondria from WT and KO cells (Fig. 3B). All activities were
normalized to the Krebs cycle enzyme citrate synthase, which is used as a reference for mitochondrial mass. The activities of complexes I, IV and V were each decreased in KO cells by 40 % (Fig. 3B), while complex II activity in KO cells was increased compared to control cells (Fig. 3B), in agreement with our measurements of complex abundance (Fig. 2). In general, alterations of mitochondrial respiration leads to the generation of reactive oxygen species (ROS), thus we investigated mitochondrial ROS generation by means of live cell imaging following incubation with the superoxide indicator MitoSOX Red, as MitoSOX fluorescence increases with mitochondrial ROS production (Mukhopadhyay et al., 2007). Only weak fluorescence was detected in WT cells, while the CLUH-KO cells clearly displayed increased fluorescence localized to mitochondria, indicating increased ROS production (Fig. 3C). FACS analysis (Fig. 3D) confirmed that one hour after MitoSOX staining, KO cells showed higher fluorescence intensity compared to WT cells (Fig. 3D). Taken together, these results demonstrate that the mitochondria from CLUH-KO cells display pronounced ultrastructural defects affecting cristae formation, reduced OXPHOS abundance and activity, and concomitant increased ROS production.

**CLUH deficiency increases the glycolytic rate.**

To assess the consequences of the respiratory dysfunctions in CLUH-KO cells on the energetic metabolism, we next evaluated parameters associated to the glycolysis. KO cells consumed glucose at a faster rate than WT (Fig. 4A), such that after two and four days of culture only 25 % and 4 %, respectively, of the original glucose remained in the medium of KO cells, while 75% and 40 % respectively of the original glucose remained in that of WT cells. In addition, measurement of extracellular lactate revealed that KO cells produced two times more lactate than WT cells (Fig. 4B), which correlated with the acidification of the medium, as indicated by the change from red to yellow of the phenol red indicator. In parallel, the extracellular acidification rates (ECAR), predominately the result of glycolysis, was increased in KO cells compared the WT cells (Fig. 4C). Finally, we investigated cell growth and death after glucose starvation using the IncuCyte ZOOM system, in which cell density and shape were monitored by photonic microscopy while cells are maintained under standard culture conditions (Fig. 4D). Using an adapted confluence mask, cell viability was assessed and the percentage of cell growth was calculated based on the
evolution of cell confluency in time course. Our analysis showed that 24 hours after glucose starvation, KO cell growth drastically slower compared to WT cells (Fig. 4E). In addition, they started dying after 36 hours of starvation and were all dead by 72 h, while WT cells grew normally for two days and were still alive after 3 days of starvation. Taken together these results demonstrate a metabolic shift in CLUH-KO cells, increasing glycolysis and glucose dependency.

**CLUH deficiency modulates cellular metabolic steady state.**

Mitochondria are crucial contributors to many metabolic pathways, as those leading to the anabolism of amino-acids, hormones and phospholipids. Whether these pathways are compromised by alterations of mitochondrial structure and distribution is an open question. Thus, we performed a targeted metabolic analysis using the AbsoluteIDQ p180 Kit (Biocrates, Innsbruck, Austria) to obtain a global view of the effects of CLUH function on cell metabolism. Metabolites, including acylcarnitines (n = 40), amino acids (n = 21), biogenic amines (n = 21), monosaccharides (1), sphingolipids (n = 15) and glycerophospholipids (n = 90), were measured by mass spectrometry (AB Sciex QTRAP 5500, Life Sciences SCIEX, Villebon sur Yvette, France). Flow-injection analysis (FIA-MS/MS) was used to quantify acylcarnitines, glycerophospholipids, sphingolipids and sugar, whereas liquid chromatography (LC-MS/MS) allowed the separation of amino acids and biogenic amines prior to detection with mass spectrometry. Cellular extractions from ten independent flasks for both WT and KO cells were used for this analysis and the metabolite concentrations were normalized with respect to protein cell extract concentrations. Multivariate data analysis showed a significant ($Q_{2cum} = 0.92$) separation between WT and CLUH-KO cells, as represented in an orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised multiple regression analysis, score plot (Fig. 5A). The volcano plot, presenting the most discriminant quantified metabolites, revealed that the steady states of two major groups of metabolites were changed in KO compared to WT cells: increased polar metabolites and decreased phospholipids (Fig. 5B). Indeed, non-essential amino acids (Fig. 5C,D), carnosine (Fig. 5E) and palmitoylcarnitine (Fig. 5F) were increased by two to three times in KO compared to the WT cells (Fig. 5C,D), as the polyamines, including spermine, spermidine and putrescine (Fig. 5G) that were increased by 76%, 37% and 66% respectively. Conversely, all lipids were less abundant in KO cells, including phosphatidylcholine, lyso-phosphatidylcholine
and sphingomyelin, which were decreased by 70%, 32% and 64%, respectively (Fig. 5H). These different metabolic signatures highlight the profound changes in cell metabolism that result from CLUH deficiency, and indicate the central role of CLUH in orchestrating cellular metabolism. Because increased concentrations in amino acid in KO cells could reflect a defective Krebs cycle, we evaluated the respiration rates in permeabilized cells after supplying different substrates of the Krebs cycle (Fig. 5I). We observed that in the presence of pyruvate and malate, respiration rates were significantly decreased in KO cells (Fig. 5I). Moreover, mass spectrometry analysis revealed that KO cells consume less pyruvate and produce less citrate compared to WT cells (Fig. 5J). Next, we observed that the addition of isocitrate failed to increase the respiration rate in KO cells, while it did in WT cells (Fig. 5I). Similar results were obtained after the sequential addition of glutamine, α-ketoglutarate and succinate (Fig. 5I).

Finally, to evaluate the β-oxidation, we measured the respiration rates in permeabilized cells after supplying carnitine, malate and palmytoyl-CoA. Again, coupling and uncoupling respirations were slower in KO cells compared to WT cells (Fig. 5K). Taken together, these results indicate that in CLUH KO cells, both the Krebs cycle and the β-oxidation are defective, leading to a profound cellular metabolism remodeling.
DISCUSSION

In most eukaryotic cells, mitochondria appear as a reticulated network spread out all over the cytoplasmic volume, in order to locally fulfill the energetic requirements. This strongly suggests that in the eukaryote ancestor that preceded the branching of the present eukaryote phyla, one of the main challenges in the generation of an efficient mitochondrial network consisted in the elaboration of a mechanism allowing proper mitochondrial distribution. In this respect, CLUH, a highly evolutionary conserved protein throughout the eukaryotic kingdom, is a key element, as its deletion leads to mitochondrial clustering in all models so far considered. To gain insights into CLUH functions, we generated a homozygous deletion of CLUH in HeLa cells using the CRISPR/Cas9. The data presented here confirmed that the absence of CLUH leads to mitochondrial clustering in HeLa cells. But importantly, our results connect CLUH to altered mitochondrial ultrastructure, translation, respiration and major changes in cellular metabolism.

We first identified a clear reduction of cell size and volume, although no modification of the cytoskeleton was evidenced. This observation parallels the fact that in the A. thaliana plant model, deletion of friendly, the CLUH orthologue, leads to a global reduction of leaf, root and branch sizes (El Zawily et al., 2014). Similarly, knockout of clueless in Drosophila leads to sterile adults with reduced size, and uncoordinated movements, abnormal wing position, and short life expectancy (Cox and Spradling, 2009).

As expected, observation of mitochondria in CLUH KO cells revealed clustered networks, but with heterogeneous mitochondria displaying two phenotypes one with a normal structure and dense matrix, and one with altered cristae structures and a light matrix. The peculiar mitochondrial phenotype was already observed in adult muscles from the drosophila cluless strain (Sen et al., 2015) but was absent in COS7 cells in which CLUH expression was silenced using siRNA (Gao et al., 2014), suggesting that stable CLUH-KO clones trigger adaptation processes that are not set in an acute model triggered by siRNA. The existence of two mitochondrial populations in KO cells could be related to the physical and functional interactions between CLUH and the PINK-Parkin pathway that promote the turn-over of mitochondria to eliminate the unfit ones (Cox and Spradling, 2009; Sen et al., 2015; Wang et al., 2016). Indeed, a cross-talk between CLUH and mitophagy in Drosophila was found associated to the degradation of the valosin-containing protein Mitofusine, in order to prevent the
fusion between healthy and altered mitochondria (Wang et al., 2016). This concept could support why some thin connections between mitochondria are observed in CLUH-KO cells, as already reported in the cluA- mutant of Dictyostelium (Fields et al., 2002), which might result from aborted fusion or defective fission. However, electron microscopy observations did not disclose an increase in mitochondrial size, excluding a major modification of the equilibrium between mitochondrial fusion and fission.

Recently, a Cluh knock-out mouse was generated, and its characterization evidenced the importance of Cluh in the regulation of nuclear mRNAs encoding mitochondrial proteins, which are required for metabolism reprogramming during nutriment deprivation. Indeed, although the fetal development was normal in Cluh-KO mouse, pups died at 0-1 day after birth, and liver analysis at E18.5 or liver-specific Cluh KO mouse at 8 weeks revealed severe metabolic alterations under starvation condition (Schatton et al., 2017). Thus, both, HeLa cells and mice deleted for Cluh revealed clustered mitochondria and hyper-sensitivity to glucose starvation. However, the worst mitochondrial ultrastructure defects were observed only after nutriment deprivation in the mouse model suggesting the importance of CLUH for specific metabolic status. Our data extend these results by showing that CLUH exerts a broader role in maintaining the whole cellular metabolism, as we found mitochondrial defects and changes in many metabolites, even in normal growth condition. Thus, both in vitro and in vivo models revealed that CLUH has an essential role in the coordination of the cellular metabolism.

In parallel, we have evidenced in CLUH-KO cells, a significant proportion of mitochondria with altered inner structures. This cristae disorganization may be linked to the reduced abundance of the respiratory complex subunits and their assembly into the respirasome, and explain why we disclosed reduced respiratory complexes enzymatic activities and increased ROS production. Frequently, the decrease in respiratory subunits abundance and complex assembly are associated to mtDNA depletion, which was found in the liver and heart of Cluh- KO mice, but not in their kidney and brain tissues (Schatton et al., 2017). In our cellular model, the mtDNA abundance remained unaffected, but the mitochondrial protein synthesis was significantly reduced in the absence of CLUH, suggesting a major alteration of the mitochondrial translation process. Beside the importance of CLUH on mRNA
translation of nuclear-encoded mitochondrial proteins (Gao et al., 2014; Sen and Cox, 2016), the evidence that PINK1 and Parkin also control the mRNA translation of the respiratory chain subunits at the outer mitochondrial membrane (Gehrke et al., 2015), suggests that a concerted regulation of all the respiratory components, encoded by the nuclear and mitochondrial genomes can be achieved by a crosstalk between CLUH and the PINK-Parkin pathway. Thus, the decrease in the mitochondrial translation machinery in CLUH-KO cells without mtDNA defects, questions both the stability of mitochondrial mRNA and the regulation of specific downstream consequences on the mRNAs that are targeted by CLUH (Schatton et al., 2017).

To further address the consequences related to the absence of CLUH on mitochondrial physiology, we characterized the energetic and metabolic status of these KO cells. We first observed a metabolic reprogramming, switching the energetic production from OXPHOS to a predominant glycolysis process, and disclosing a high dependency to glucose fueling. In parallel, using targeted mass spectrometry, we found a drastic change in the metabolomics signature. Multivariate analysis of our results evidenced a significant increase in polar metabolites, including the non-essential amino acids, carnosine, palmitoylcarnitine, and polyamines, and a dramatic decrease in some lipids’ amounts. The increase of carnosine (β−alanyl-L-histidine), which has an antioxidant effect (Kohen et al., 1988), might protect from the increased ROS production induced by defective mitochondria in KO cells. We also found an increase of many amino acids, including alanine, glycine, serine, arginine, proline, glutamine, glutamate and aspartate, as reported also by Schatton et al in the liver of E-18.5 Cluh-KO embryo and in the serum of 8 weeks starved Cluh-KO mice (Schatton et al., 2017). This can be related to the dysfunction of the Krebs cycle, as we observed decreased respiration rates when supplying different substrates of the Krebs cycle. This could also be related to a reduced anabolic status and protein synthesis, paralleling the reduction of the cell size. Altogether, these results indicate that CLUH-KO cells can not support normal respiration, and are unable to supply the Krebs cycle with its intermediates.

In addition, we observed an increase in palmitoyl-carnitine level in CLUH-KO cells that might be due to a β-oxidation defect, as the mitochondrial carnitine system is essential for long-chain fatty acids oxidation, by catalyzing their transport into the mitochondrial matrix. This result is in contradiction with that of Schatton et al, who
reported a decrease of palmitoyl-carnitine levels in liver lysates of starved *Cluh-KO* mice at E18.5, although CPT2, the first enzyme in the long fatty β-oxidation, that generates acyl-carnitines (Lee et al., 2015), and HADHA, the enzyme required for the three last steps of mitochondrial β-oxidation were decreased in the liver tissues. To conciliate these opposite results, we suppose that the differences in the biological origins of the samples analyzed and their energetic environment may explain this discrepancy in the palmitoyl-carnitine metabolism.

In addition, we disclosed an increase in polyamines such as putrescine, spermidine and spermine, which may reflect a compromised ability to survive of the *CLUH-KO* cells, as increased organic cations may restore conditions supporting cellular growth and viability (Nishimura et al., 2002; Pendeville et al., 2001). Finally, our metabolomics analysis disclosed dramatic decreased amounts of phospholipids in *CLUH-KO* cells, which could either be related to the smaller cell size and a lower protein to lipid ratio, or to altered mitochondrial metabolism of lipid precursors. These results could further explain the decrease by 10-15% of the body weight of the *Cluh-KO* mice.

Taken together, our study shows that through its crucial involvement in mitochondrial distribution, CLUH is central to a network of many functions related to mitochondrial quality control, mitochondrial ultrastructure, respirasome synthesis, energy production and metabolic pathways, to maintain a sustainable cellular homeostasis. Finally, how the different evolutionary conserved domains of CLUH contribute to these multiple functions remains a challenge to address in the future.
MATERIALS AND METHODS

Generation of CLUH-KO HeLa cells using CRISPR/Cas9.
HeLa were obtained from American Type Culture Collection and cultured in growth medium (DMEM; Gibco) supplemented with 10% heat-inactivated FBS (ThermoFisher) and 1 mM of sodium pyruvate and glutamine (ThermoFisher). Cells were verified by PCR and shown to be free of contamination with mycoplasma. Plasmids pX330 encoding for Cas9 and the guide-RNA targeting CLUH exon 4 (target site sequence: GTCTTCACCGACGGCGACCTGGG), were co-transfected with a Puromycin-resistant gene flanked by homology arms upstream and downstream of the target site. Transfected cells were selected with 1 μg/mL puromycin, 72 h after initial transfection. Secondary screen by Western blotting was carried out in 23 colonies to identify clones with different levels of CLUH expression. Three independent selected clones showed undetectable level of CLUH (Fig. S1, A and B). The PCR with primers upstream (Forward primer ATGTCCGAGACCTGCTCAAG) and downstream (Reverse primer GTCCATCTCAGCCGTCCTCT) of the target site confirmed the absence of the band in KO cells. For the exon 10, forward primer: CGTCCGAGACCCTACA. Reverse primer: AGCAGAGCTCAGCCTCT. Clone 2 was used as knockout clone in subsequent experiments.

Q-PCR analysis.

6 \times 10^6 cells were harvested, suspended in 100 μL of phosphate buffered saline (PBS) and total RNA was purified using RNeasy Mini Kit (Cat No./ID: 74104) according to the manufacturer’s protocol. 2 μg of RNA were reverse transcribed and quantitative PCRs were performed in triplicates in 96-wells reaction plates (Applied Biosystems). Each reaction (final volume 10 μl) contained 100 ng of cDNA, 5 μl of Power SYBR-Green PCR Master Mix (Applied Biosystems) and 0.5 μM of each forward and reverse primer. Two sets of primers were designed to measure mRNA expression level of CLUH. Primers sequences, forward 1: GTGGAGCAGAGGTACCTCCT and reverse 1: AGGGTCTGCTGTGCCGTC. Forward 2: CACACGAGGCTTTTACCTGAATC and reverse 2: CAATCCCATGCGATGCTCCG. GAPDH was used as control.

Live cell confocal imaging.
HeLa cells were seeded at the density of 20,000 cells/well in 4 well glass bottom µ-slide (80427, Ibidi) for 24 hours then mitochondria and nuclei were stained using
MitoTracker Green (ThermoFisher, M7514) and Hoechst solutions at 100 nM, 30 min before confocal cell live imaging. To measure mitochondrial reactive oxygen species (ROS), cells were stained with MitoSOX (ThermoFisher, M36008) at 5 μM, 30 min prior imaging. All the images were captured using a Leica TCS SP8 confocal microscope with a 40X/1.20 water objective lens (Leica Microsystems) equipped with a GaAsP Hybrid detector (HyD), at 37 °C. For FACS analysis, MitoSOX at 5 μM was added for 1 hour.

In order to standardize cell size, HeLa cells were seeded at the density of 60.000 cells on CYTOOchips with square or T micropatterns. Images were acquired with the inverted widefield NIKON Ti-E microscope equipped with an Andor NEO sCOMS camera controlled by NIS Element software. Each image was obtained with a 100x oil objective (Nikon Plan Apo100x, N.A. 1.45). On average, 21 image-planes were taken along the Z-axis at 0.2μm increments. To limit mitochondrial movements, the images were taken at a controlled-temperature (25°C). Huygens software (Scientific Volume Imaging) was used for deconvolution. The computational model of the mitochondrial network was done using Imaris 8 Bitplane AG, Zurich, Switzerland. The background of the raw fluorescence images was first corrected using the same algorithm in Huygens Essential® software (Scientific Volume Imaging, Hilversum, The Netherlands), which resulted in intensity-coded images of the mitochondrial network. Imaris 8.0® software was used for 3D processing and morphometric analysis. The colours define mitochondrial connectivity with purple referring to fragmented mitochondria and red to extremely connected mitochondria.

**Glucose starvation analysis using IncuCyte ZOOM**

To assess cell viability in response to glucose starvation we used IncuCyte ZOOM system in which the cell density and shape were observed by photonic microscopy while cells are maintained under standard culture conditions. HeLa cells were seeded at the density of 50.000 cells/well in a 24-well plate in regular medium (DMEM, 4.5g/L of glucose, 10% FBS, 1 mM sodium pyruvate and 1 mM glutamine). After 24 hours, the medium was removed and cells were incubated in DMEM without glucose supplemented with 10% FBS, 1 mM sodium pyruvate and 1 mM glutamine. Real time live cell images were taken every two hours using IncuCyte ZOOM. For analysis of cell viability, well confluence (%) was automatically calculated during 84 hours by using Basic Analyzer segmentation mask of the IncyCyte ZOOM software 2015A.
Despite the two filters used, cell area and eccentricity, the mask still recognized the dead cells, which explains the density for KO cells remains close to 100% at the end of the experiment.

**Electron Microscopy.**

HeLa cells were seeded at the density of 500,000 cells/well in a 6-well plate for 24 hours and fixed in 4% formaldehyde, 2% glutaraldehyde, 0.1M Cacodylate (pH 7.4) for 2 hour at room temperature then postfixed in 1% osmium tetroxide for 1 hour and stained in 0.5% uranyl acetate for another hour. The samples were then dehydrated in a graded series of 35%, 50%, 70%, 100% ethanol and exchanged to propylene oxide. After infiltration at 1:1 propylene oxide and epoxy resin (Poly/Bed 812, Polysciences, Warrington, PA) overnight, samples were embedded in 100% epoxy resin. Polymerization of resin was performed for 3 days at 55°C. Thin sections of 70 – 90 nm were cut with an ultramicrotome (Leica EM UC6, Leica Microsystems, Buffalo Grove, IL), stained with uranyl acetate and lead citrate, lightly carbon coated, and imaged in a Hitachi 7650 or 7600 transmission electron microscope (Hitachi High Technologies America, Gaithersburg, MD). Images were taken with 2k × 2k AMT digital camera (Advanced Microscopy Techniques, Woburn, MA).

**Western blotting.**

HeLa cells were cultured for 24h in 25 cm² flasks. Protein lysates were prepared using RIPA buffer supplemented with 1X protease inhibitor cocktail (Roche) and subjected to SDS-PAGE and transferred onto PVDF membranes (Amersham Hybond 0.45µm PVDF). The membranes were blocked for 1 h with 5% milk in TBST (Tris-buffered saline, Tween20 0.1%), then were incubated overnight with the following primary antibodies at 1:5000 dilutions: rabbit polyclonal eIF3X (CLUH) antibody (Novus Biologicals, NB100-93306), total OXPHOS Human WB antibody cocktail (Abcam, ab110411), mouse monoclonal α-Tubulin antibody (Sigma Aldrich, T9026), anti-VDAC1 antibody (Abcam, ab14734). The antibodies used for mitochondrial translation machinery are ERAL1 (Proteintech 11478-1-AP), MRPL44 (genetex GTX121263), MRPL13 (Proteintech 16241-1-AP), TUFM (Abnova H00007284-B01P), EFG1 (abcam ab204338), MRPS27 (Proteintech 17280-1-AP) and MRPS17 (Proteintech 18881-1-AP).

After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:10000 dilution) (GE Healthcare) for 1
hour and then washed with TBST. Immunoblots were detected using SuperSignal West Femto Maximum Sensitivity Substrate (11859290, Pierce).

**Mitochondrial extraction and Blue Native PolyAcrylamide Gel Electrophoresis (BN-PAGE).**

HeLa cells were cultured in 75 cm² flasks. 7\(^{10}\) cells were harvested and suspended in 100 µL of phosphate buffered saline (PBS). 100 µL of 4 mg/mL digitonine diluted in PBS were added and cells were incubated for 10 min at 4°C under agitation. The mixture was washed twice with 1 mL PBS and mitochondria were harvested by centrifugation at the speed of 10,000 g for 10 min at 4°C. The mitochondrial pellet was suspended in AC/BT buffer (amino-caproic acid 1.5M, bis tris 75 mM, pH=7) for an approximate final protein concentration of 10 mg/mL. 25 µg of mitochondrial proteins were suspended in AC/BT for a final volume of 25 µL and 75 µg of lauryl maltoside were added to samples which were then incubated under agitation for 10 min at 4°C. Samples were centrifuged at 20,000 g for 20 min at 4°C and supernatants were submitted to BN-PAGE in 3-12% gels. The gel was transferred on PVDF membrane. The membrane was blocked for 1 hour with 5% milk TBS-Tween20 0.1%. Then, the membrane was blotted with the following antibodies at 1:1000 dilutions: Anti-NDUFS2 antibody (Abcam, ab110249), Anti-UQCRC2 antibody (Abcam, ab14745), Anti-MTCO1 antibody (Abcam, ab14705), Anti-SDHA antibody (Abcam, ab14715), Anti-OXPHOS complex V subunit (Invitrogen, 12005645). The quantification of the different respiratory complexes was done using Image J.

**Mitochondrial Enzymatic Activities.**

The activities of the mitochondrial OXPHOS complexes and citrate synthase (CS) were measured in WT and CLUH KO mitochondrial-enriched fractions at 37°C using UVmc2 spectrophotometer (SAFAS, Monaco). Activities of the succinate ubiquinone reductase (complex II), ubiquinol cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and citrate synthase (CS) were measured according to standard methods (Medja et al., 2009). Complex I activity was measured as described by Desquiret-Dumas et al. (Desquiret-Dumas et al., 2013) and F0F1-ATPase activity (Complex V) was determined according to Rustin et al. (Rustin et al., 1994).

**Quantification of mtDNA copy number**

For mtDNA quantification, total DNA was isolated from WT and CLUH KO HeLa cells using DNeasy Blood and tissues Kit (QIAGEN). Quantitative PCRs were
performed in triplicates in 96-Well reaction plates (Applied Biosystems). Each reaction (final volume 10 μl) contained 25 ng DNA, 5 μl of Power SYBR-Green PCR Master Mix (Applied Biosystems) and 0.5 μM of each forward and reverse primer. ND1, mitochondrial encoded gene, was amplified and β2 microglobulin (β2m), nuclear encoded gene, was used as normalizing control. ND1-F: AAGTCACCCTAGCCATCATTCTAC, ND1-R: GCAGGAGTAATCAGAGGTGTTCTT. β 2m-F: TGCTGTCTCCATGTTTGATGTATCT, β 2m-R: TCTCTGCTCCCCACCTCTAAGT.

MtDNA recovery after ethidium bromide depletion.

WT and CLUH KO HeLa cells were seeded in six-well plates at 20% confluence. After 24 h, cells were incubated with or without 0.1 µg/mL ethidium bromide. After 3 days, cells were washed three times with 2 mL of medium without ethidium bromide. After washing, Cells were incubated in medium without ethidium bromide and mtDNA was quantified as described below after 2, 4, 6 and 8 days of recovery.

MtDNA damage

MtDNA damage was quantified by long range PCR to assess mtDNA deletions. One very long fragment PCR, covering the whole mtDNA genome and two long fragments PCR, designed to cover the whole mtDNA genome, were amplified. PCR products were analysed by agarosis gel at 0.8%. Primers1 (Forward ND5 to reverse COX1) and primers3 (Forward COX1 and reverse ND5). Primers2 cover the whole mtDNA on one very long PCR product.

Extracellular acidification rate (ECAR) measurement using a Seahorse.

WT and CLUH KO cells were plated on XF96 plate (Seahorse Biosciences) at 5000 and 20000 cells/well and incubated for 24 hours. ECAR was measured using XF96 Extracellular Flux Analyser according to the suggested protocols by Seahorse Bioscience. Krebs cycle analysis

Oxygraphic analyses were performed at 37 °C on a high-resolution oxygraph (Oroboros, Innsbruck, Austria) as described previously by Desquiret-Dumas et al (Desquiret-Dumas et al., 2013). Briefly, cells were permeabilized using digitonin at 20 µg/106 cells, then after washing in respiratory buffer, 3x10⁶ permeabilized cells were placed in 2 ml respiratory buffer in an oxygraphic chamber. Respiration rates were measured on cells after adding substrates or inhibitors.
-Measurement of pyruvate consumption and citrate production:
Pyruvate (2.5 mM) and malate (5 mM) were added in the oxygraphic chamber and 1 ml aliquot was immediately removed for acid organic analysis. Five minutes after substrate additions, sub-saturating ADP concentration (0.5 mM) was added, that allowed a stimulation of phosphorylating respiration at nearly 50% of the the maximal mitochondrial respiratory rate (Vmax) in control cells. Precisely 30 minutes after ADP addition, a new 1 ml aliquot was taken from the oxygraphic chambers for the acid organic analysis. Oxygen consumption was controlled during all the experiment. For both aliquots, 200 µl of 7% perchloric acid was added to quench the reactions. After 10 seconds of stirring, the aliquots were immediately frozen in liquid nitrogen and thawed at 37°C to lyse mitochondria. Then, tubes were centrifuged for 5 minutes, 20 000 g at 4°C. The pellet was used for protein concentration and the supernatant was conserved at -80°C until analysis. The metabolites were extracted by ethyl acetate, evaporated, silylated and injected in a gas chromatography/mass spectrophotometer (GCMS-2010C, Shimadzu). Peaks were identified and quantified using GC solution software (Shimadzu) with an existing library.

-Measurement of respiration after adding Krebs Cycle substrates:
Respirations rates were measured on permeabilized cells after adding sequentially: 1: 2.5 mM pyruvate and 5 mM malate (M+P). 2: 5 mM isocitrate (M+P+I). 3: 5 mM glutamate (M+P+I+G). 4: 5 mM Alpha-Ketoglutarate (M+P+I+G+KG), 5: 10 mM succinate (M+P+I+G+KG+S) and 6: 5 µM rotenone (M+P+I+G+KG+S+R) to inhibit CI activity and thus checked the maximal CII-linked respiration. All the respirations rates were conducted at saturating ADP concentration (1 mM ADP) to induce the maximal phosphorylating respiration.

β-oxidation was investigated by following the respirations rates in 6x10⁶ permeablized cells after supplying 1mM carnitine, 5 mM malate and 100 µM palmytoyl-CoA.

Mitochondrial protein synthesis
The labelling of mitochondrial newly translated proteins was performed as described previously (Chomyn, 1996). Briefly, cells were seeded in 6 wells plates and treated with 100 µg/mL emetine for 20 min to inhibit cytosolic protein synthesis. 35S-methionine was then added to the cells and incubated for 45’. Cells were then collected, lysed and 25 µg proteins were separated by SDS-PAGE. Gels were stained.
with Coomassie blue, dried and exposed to phosphor plates for 72 hours. The 35S signal was read using Typhoon™ Phosphorimager (GE Healthcare).

**Metabolites extraction.**

WT and CLUH-KO HeLa cells were cultured in 10 different 75 cm² flasks. When 70% confluence was obtained, the culture medium was removed and cells were harvested by trypsination and washed twice in a cold, phosphate-buffered saline 1X (PBS-1X) solution and 10% fetal bovine serum (ThermoFisher). After centrifugation (300 g for 5 minutes at 4°C), the supernatant was eliminated and the cell pellet was dissolved in 100 µL of a cold PBS-1X solution. Fifty microliters were used for protein dosage, the remaining 50 µL (~ 2.10⁶ cells) being used for the extraction of metabolites. The metabolic quenching was completed by adding 100 µL of a cold ethanol/PBS-1X (85:15, v/v) solution to the cell pellet obtained after a second run of centrifugation (300 g for 5 minutes at 4°C) and elimination of the supernatant. After vortexing, the mixture was transferred to a 0.5 ml homogenizer tube prefilled with ceramic beads. Cell lysis and protein extraction was achieved in a Precellys®24 homogenizer (Bertin Corp., Rockville, MD, USA) by two cycles of grinding (40 seconds at 6500 rpm, followed by 30 seconds at 6000 rpm) at 4°C. The resulting homogenate was centrifuged at 20 000 g for 10 min at 4°C and the supernatant was stored at -80°C until analysis. We applied a targeted, quantitative metabolomic approach to the cell extracts using the Biocrates AbsoluteIDQ p180 kit (Biocrates Life sciences AG, Innsbruck, Austria). This kit, in combination with a AB Sciei QTRAP 5500 (Life Sciences SCIEX, Villebon sur Yvette, France) mass spectrometer, enables quantification of up to 188 different endogenous molecules, including acylcarnitines, amino acids, biogenic amines,, glycerophospholipids, sphingolipids, and sugar. Samples were prepared according to the Biocrates Kit User Manual. After validation of the three levels of quality controls (QCs) used in the kit, the metabolite concentrations were normalized with respect to protein cell extract concentrations.

**Metabolomics data analysis.**

Orthogonal partial least squares discriminant analysis (OPLS-DA), a supervised method of pattern recognition, was further used to maximize the variations between groups and to determine the contributing variables. The quality of models was validated by determining two parameters: $R^2$ (goodness of fit) and $Q^2$ cumulated (goodness of prediction). In the model with the best predictive capabilities, variables were selected on the basis of the variable importance in the projection (VIP) and...
loading values. VIP values summarize the importance of each variable for the OPLS-DA model, whereas loading values summarize the importance of each X variable in approaching the variance of X captured by the latent variable. Variables with a VIP value greater than 1 are considered important for group discrimination in predictive models. Plotting VIP versus loading values – a procedure called the “volcano” plot – allows the selection of important variables in PLS models. Multivariate data analysis was conducted using the SIMCA-P software 13.0 (Umetrics, Umeå, Sweden).
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COMPETING INTERESTS

No competing interests declared

AUTHOR CONTRIBUTIONS

JM designed, performed and analyzed the experiments. JM also contributed to writing the paper. DG performed the In-Silico analysis and contributed to writing the paper. RP performed and analyzed confocal imaging microscopy. NG analysed BN-PAGE experiments. VDD performed vector sequencing. JMCB and SC performed and analyzed the metabolomics study. IDR performed and analyzed the mitochondrial translation experiment. FM performed the electron microscopy images. MLM performed and analyzed the respiratory chain complexes activity. AC performed and analyzed standardized CYTOOchips live-cell microscopy images. VP, DB and PR contributed to analyzing and writing the paper. DCL, GL and SK designed, analyzed the results, wrote and approved the final version of the manuscript.

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REFERENCES


Figure 1. Generation of CLUH KO cells and characterization of mitochondrial distribution. (A) Generation of CLUH KO HeLa cells using CRISPR/Cas9 and Western blot of whole-cell lysate using CLUH-specific antibody. Tubulin was included as a loading control. (B) Relative mRNA expression levels of CLUH in WT and CLUH KO HeLa cells in four independent experiments. (C) Representative gel of the PCR product of CLUH exon four, targeted by Cas9, and exon 10 was used as control. (D) Live light (top panels) and fluorescence (lower panels) images of cells
stained with MitoTracker Green in WT (left) and CLUH-KO (right) cells (Scale bar: 1 μm). (E) Left panels, live cell light and fluorescence (DAPI) images. Right panels, quantification of cell size using ImageJ (https://imagej.net/Downloads), by measuring cell areas as highlighted with yellow line in the images. 200 cells were measured in each condition and in three independent experiments. ***: p≤0.001, paired t-test. Tow-tailed. (mean ±s.e.m.). (F) Live phase contrast images (left panels) and surface reconstitution of fluorescence images (right panels) of individual WT and KO cells in CYTOO square-shaped micro-patterns. Experiments were replicated four times in laboratory. Mitochondria were stained with MitoTracker green and color encoded using Imaris Imaris Software (Bitplane, Zurich, Switzerland) according to their length. Imaris Filament Tracer Colors codes indicating mitochondrial network shapes: red for long mitochondria and purple for fragmented mitochondria. All the experiments were replicated four times in our laboratory.
Figure 2. Mitochondrial OXPHOS complex abundance and mitochondrial translation are decreased in CLUH KO cells. (A) left panel: Western blots show, with specific antibodies, the expression level in whole cell lysates (WCL) and in mitochondrial lysate (ML) of ATP5A (Complex-V), UQCRC2 (Complex-III), Cox II (Complex-IV) and NDUFB8 (Complex-I) subunits. VDAC expression level was used as a loading control. Right panel: quantification of OXPHOS complex subunits amounts of four independent experiments. (B) BN-PAGE analysis of the five OXPHOS complexes with specific antibodies for each complex. (C) Quantification of OXPHOS complex amounts by BN-PAGE analysis. Quantification was done with four independent experiments. ***: P < 0.001, *: P < 0.05, paired t-test. Tow-tailed. (mean ±s.e.m.). (D) DAVID Gene ontology analysis of the 258 genes positively highly correlated with CLUH mRNA expression level in NCI-60. The 258 genes were sorted using the functional annotation database PANTHER (http://pantherdb.org), which are presented with fold enrichment according to biological processes and p-values. (E) Mitochondrial translation products were labeled with 35S-methionine in
the presence of emetine, a cytoplasmic translation inhibitor. Left panel shows the autoradiography of the radiolabeled neo-synthesized mitochondrial proteins, while the right panel shows the Coomassie staining, demonstrating equal protein loading of WT and KO cell lysates. All the experiments were replicated four times in our laboratory. (F) Western blots of cell lysates using specific antibodies disclose in CLUH-KO cells a decrease in mitochondrial small MRPS27 and MRPS17 and large MRPL44 and MRPL13 ribosomal subunits, in the translation assembly factor ERAL1 and in mitochondrial elongation factors EFG1 and TUFM. GAPDH was used as loading control.
Figure 3. Mitochondrial ultrastructure and activities are defective in CLUH KO cells leading to ROS overproduction. (A) Representative TEM images of WT and two independent CLUH-KO cells (left). 50 cells were analyzed in each condition. Enlarged views of the boxed areas (right) show the mitochondrial ultrastructure. Yellow arrowheads indicate disorganized cristae, while red arrowheads indicate homogeneous dense cristae. Right: black arrowheads indicate connected mitochondria. (B) OXPHOS complexes I, II, IV and V activities in WT and CLUH KO cells. Data were normalized to citrate synthase activity. Quantification was done with four independent experiments. *: P < 0.05, paired t-test. Tow-tailed. (mean
±s.e.m.). (C) Representative images of stained mitochondria with MitoTracker Green (left panels) and MitoSOX (right panels) of WT and CLUH KO cells. (D) FACS analysis of WT and KO cells after MitoSOX staining. All the experiments were replicated four times in our laboratory.
Figure 4. KO cells shift their metabolism to glycolysis, with increased glucose dependency. Measurements of glucose (A) and of lactate (B) concentrations in medium after one, two and four days of cell culture. (C) Left panel: color shift from red to yellow of the medium after 24 hours of culture of WT and KO cells, seeded at increasing confluences. Right panel: the extracellular acidification rates (ECAR) measurement using Seahorse. Quantification was done with four independent experiments. **: p < 0.005, paired t-test. Tow-tailed. (mean ± s.e.m.). (D) Representative real time images, using IncuCyte, after maintaining WT and CLUH KO cells for three days without glucose. (E) Quantification of cell growth using IncuCyte, during glucose starvation for 84 hours. All the experiments were replicated four times in our laboratory.
Figure 5. **CLUH deficiency modulates cellular metabolic steady state.** (A) The orthogonal partial least squares discriminant analysis (OPLS-DA) model comparing 10 cultures of WT and CLUH KO cells shows a clear distinction between the two groups. (B) Volcano plot showing the distribution of the quantified metabolites. (C-H) Bar plots represent the mean relative concentrations of the metabolites. (C) alanine (Ala) and proline (Pro). (D) serine (Ser), arginine (Arg), glutamine (Gln), lysine (Lys) and aspartate (Asp). (E) Carnosine. (F) Palmitoylcarnitine. (G) Putrescine, spermidine and spermine. (H) Phosphatidylcholine (PCae and PCaa), lyso-phosphatidylcholine (Lyso-PC) and sphingomyelins (SM). *: p < 0.05, paired t-test. Tow-tailed. (mean ±s.e.m.). (I) Respirations rates in permeablized cells after supplying different substrates of Krebs cycles sequentially indicated by numbers in the scheme (left panel). The added substrates were pyruvate and malate (1), isocitrate (2), glutamine (3), α-ketoglutarate (4) and succinate (5). Rotenone, complex I inhibitor, was added at the end of the experiment (6) (Right Panel). (J) Consumption of pyruvate and production of citrate in WT and KO cells were measured using mass spectrometry analysis. Metabolites were followed for 30min in media after stimulation of the phosphorylating respiration to 50% of the maximal mitochondrial respiratory rate (Vmax) in WT and KO cells. Pyruvate and citrate amounts metabolized per minute were normalized to protein concentrations (mg). *: P < 0.05, paired t-test. Tow-tailed. (mean ±s.e.m.). All the experiments were replicated four times in our laboratory. (K) β-oxidation was investigated by following the respirations rates in permeablized cells after supplying carnitine, malate and palmytoyl-CoA. All the experiments were replicated four times in our laboratory.
SUPPLEMENTARY INFORMATION

CLUH Couples Mitochondrial Distribution to the Energetic and Metabolic Status

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Figure S1: Clone selection of CLUH-KO cells after CRISPR/Cas9 transfection. (A) Western blot of 23 clones lysate using CLUH specific antibody. (B) Western blots showing the absence of CLUH in three selected clones used for further investigations. (C) Live cell fluorescence images of the three selected clones after Mitotracker red staining showing similar phenotype with altered distribution of mitochondria throughout the cytoplasm compared to the WT cells. (D) CYTOOchips with T micropattern and M size (CYTOOchips CW-M-A, #10-005-00-18) were placed on 6 wells plates and 60 000 cells were seeded in each well. 12 hours later, cells were stained with MitoTracker red and analyzed by confocal microscopy. (E) At least 50 cells were quantified in each condition using ImageJ. To do so, mitochondrial fluorescence area was measured and (F) divided by the whole cell area. Cells with a ration under 0.35 were considered as having clustered mitochondria. Using this approach, we show that 68% of CLUH-KO cells displayed clustered mitochondria. *: P < 0.05, paired t-test. Tow-tailed. (mean ±s.e.m.).
Figure S2: CLUH KO cells show decrease migration capacity and hypersensitivity to paclitaxel. (A) Scratch test to assess cell migration. (B) Quantification of cells migration using ImageJ in three independent experiments. 50 images were analyzed in each experiment. ***: P < 0.001, paired t-test. Tow-tailed. (mean ±s.e.m.). (C) Cell viability using PrestoBlue cell viability assay after paclitaxel treatment for three days. (D) Quantification of the half maximal inhibitory concentration (IC50) of paclitaxel following 3 days treatment in three independent experiments. *: P < 0.05, paired t-test. Tow-tailed. (mean ±s.e.m.).
Figure S3: Neither mtDNA quantity nor its quality were affected in CLUH-KO cell.

(A) mtDNA copy number in WT and KO cells. MtDNA copy number is expressed relative to WT cells (set as 1). Quantification was done in four independent experiments. (mean ±s.e.m.). (B) mtDNA copy number recovering at indicated days after ethidium bromide (EB) treatment. Quantification was done in four independent experiments. (C) Long fragment PCR to assess mtDNA deletions. Primers1 (forward ND5 and reverse COX1) and primers3 (forward COX1 and reverse ND5) were design to cover the whole mtDNA by two long PCR products. Primers2 cover the whole mtDNA on one very long PCR product. All the experiments were replicated four times in our laboratory.
**Figure S4:** CLUH KO cells show two populations of mitochondria. (A) TEM images showing the two distinct populations of mitochondria in CLUH-KO cells: swollen with light matrix content (red arrow heads) and small with dense matrix (yellow arrow heads). The abundance of each phenotype was quantified among 1000 mitochondria in KO cells (right panel). (C) Quantification of mitochondrial area (size) using ImageJ.

**Table S1:** 258 positively and highly correlated genes with CLUH mRNA expression level in NCI-60

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