CLUH granules coordinate translation of mitochondrial proteins with mTORC1 signaling and mitophagy

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Abstract

Mitochondria house anabolic and catabolic processes that must be balanced and adjusted to meet cellular demands. The RNA-binding protein CLUH (clustered mitochondria homolog) binds mRNAs of nuclear-encoded mitochondrial proteins and is highly expressed in the liver, where it regulates metabolic plasticity. Here, we show that in primary hepatocytes, CLUH coalesces in specific ribonucleoprotein particles that define the translational fate of target mRNAs, such as Pcx, Hadha, and Hmgs2, to match nutrient availability. Moreover, CLUH granules play signaling roles, by recruiting mTOR kinase and the RNA-binding proteins G3BP1 and G3BP2. Upon starvation, CLUH regulates translation of Hmgs2, involved in ketogenesis, inhibits mTORC1 activation and mitochondrial anabolic pathways, and promotes mitochondrial turnover, thus allowing efficient reprogramming of metabolic function. In the absence of CLUH, a mitophagy block causes mitochondrial clustering that is rescued by rapamycin treatment or depletion of G3BP1 and G3BP2. Our data demonstrate that metabolic adaptation of liver mitochondria to nutrient availability depends on a compartmentalized CLUH-dependent post-transcriptional mechanism that controls both mTORC1 and G3BP signaling and ensures survival.

Keywords CLUH; G3BP; mitochondria; mTORC1; RNA metabolism

Introduction

Traditionally considered as the powerhouse of the cell, mitochondria contribute in several ways to cell and tissue metabolism, by producing biosynthetic intermediates, hosting catabolic reactions, and participating in signaling pathways (Chandel, 2014; Spinelli & Haigis, 2018). To adapt their metabolic function to cellular needs, mitochondria change shape, fuse or divide, interact with other organelles, and are replaced by balanced biogenesis and turnover (Eisner et al., 2018). When nutrients are abundant, the expression of a subset of mitochondrial proteins involved in oxidative phosphorylation (OXPHOS) and mitochondrial translation is promoted in a mTORC1-dependent manner to enable the production of ATP necessary for protein synthesis, which is an energetically costly process (Morita et al., 2013; Saxton & Sabatini, 2017). Turnover of actively respiring mitochondria via mitophagy ensures the maintenance of a healthy organellar population (Melser et al., 2013). Rewiring of mitochondrial metabolism is crucial to survive transitions from nutrient sufficiency to nutrient deprivation. During starvation, mitochondria are mainly catabolic organelles that use amino acids and lipids released by autophagy and convert them into keto bodies and ATP to promote survival (Spinelli & Haigis, 2018). Inhibition of mTORC1 suppresses energy-consuming anabolic pathways and leads to mitochondrial hyperfusion as a mechanism to transiently protect mitochondria from autophagy and to suppress apoptotic cell death (Rambold et al., 2011; Morita et al., 2017). Prolonged starvation ultimately induces removal of mitochondria (Kristensen et al., 2008). Elucidating mechanisms that control the dynamic changes of mitochondrial metabolism and turnover to adapt them to energy needs is paramount to understand how organisms survive upon stress and starvation.

An important feature of a successful mitochondrial adaptive response is to be fast, flexible, and reversible. Coordination of post-transcriptional events by ribonucleoproteins (RNP) plays a fundamental role in living systems to respond in a quick and dynamic manner to environmental signals and stress (Keene, 2007; Gehring et al., 2017). RNA-binding proteins (RBPs) can control each step of the mRNA life cycle, determining stability or degradation, localization, and translation efficiency of mRNAs (Hentze et al., 2018). RBPs...
often assemble together with target transcripts in specific membrane-less subcellular compartments, such as stress granules (SGs), P-bodies, or other types of granules (Protter & Parker, 2016; Gomes & Shorter, 2019). These phase separations not only confer spatial regulation to the expression of groups of mRNAs with a common function, but also integrate it with signaling pathways and allow sensing environmental changes (Kedersha et al., 2013; Yoo et al., 2019). Whether membrane-less organelles regulate mitochondrial function is currently unknown.

CLUH (clustered mitochondria homolog) is an RBP that specifically binds several transcripts encoding mitochondrial proteins (Gao et al., 2014; Schatton et al., 2017). At least for a subset of these, CLUH promotes their stability and translation (Schatton et al., 2017). Mitochondrial proteins whose expression depends on CLUH belong to several pathways, including OXPHOS, tricarboxylic acid (TCA) cycle, amino acid degradation, fatty acid oxidation, and ketogenesis (Schatton et al., 2017). In the absence of CLUH, the mitochondrial proteome is severely depleted of polypeptides encoded by mRNAs under CLUH regulation (Gao et al., 2014; Schatton et al., 2017). Mitochondria appear abnormal in ultrastructure and display a characteristic clustering next to the nucleus. This phenotype, which has given the name to the gene, is extremely conserved upon deletion of CLUH orthologues in evolutionary distant species (Fields et al., 1998, 2002; Logan et al., 2003; Cox & Spradling, 2009; Gao et al., 2014; Schatton et al., 2017). CLUH-deficient cells show metabolic abnormalities characterized by respiratory deficiency, a shift toward a glycolytic metabolism, and impairment of the TCA cycle and β-oxidation (Schatton et al., 2017; Wakim et al., 2017). In vivo, CLUH plays a key role to allow survival during the fetal to neonatal transition, which is characterized by acute starvation and a shift to OXPHOS metabolism (Schatton et al., 2017). In the adult liver, CLUH is required to reach maximal respiratory capacity under nutrient sufficiency, but also to produce ketone bodies upon starvation (Schatton et al., 2017).

Despite its crucial role for mitochondrial function in the liver, it is unclear whether CLUH is a general regulator of mitochondrial gene expression or whether it has a specific role during the metabolic switches in response to physiological nutrient fluctuations. Here, we show that in primary hepatocytes, CLUH assembles with its bound mRNAs in specific RNP particles that function not only as compartments that coordinate the translation of target mRNAs, but also as signaling hubs that control the dynamics of mTORC1 activation and modulate the function of other RBPs, such as Ras-GTPase-activating protein SH3 domain-binding proteins 1 and 2 (G3BPs).

Through this mechanism, CLUH promotes turnover of mitochondria activating protein SH3 domain-binding proteins 1 and 2 (G3BPs). We previously showed that CLUH plays a physiological role in the adult mouse liver upon starvation to allow amino acid catabolism, to produce ketone bodies, and to maintain glucose levels (Schatton et al., 2017). Intriguingly, CLUH subcellular localization in the mouse liver and in primary hepatocytes changed depending on the nutrient condition. CLUH displayed a cytosolic punctate localization in the liver of fed mice, but formed bigger aggregates in the tissue of mice subjected to food deprivation (Fig EV1A and B). Similarly, when hepatocytes were cultured in basal glucose-rich medium, CLUH decorated small cytosolic puncta and a few bigger foci. In contrast, incubation of hepatocytes for 2 h in HBSS, a low-glucose medium devoid of serum and amino acids, increased CLUH redistribution to bigger structures, often located in the perinuclear region (Fig EV1A and C).

These results raised the possibility of CLUH assembly together with client mRNAs in RNP particles, which play a regulatory role in response to nutrient availability. To detect whether known CLUH target mRNAs localize to these granules, we combined immunofluorescence with in situ hybridization in primary hepatocytes. We selected two target transcripts highly expressed in the liver, the expression of which is reduced in the absence of CLUH (Schatton et al., 2017): Pcx (encoding pyruvate carboxylase involved in the carboxylation of pyruvate to oxaloacetate) and Hadha (encoding hydroxyacyl-CoA dehydrogenase that catalyzes the last three steps of β-oxidation of long fatty acids). As a negative control, we analyzed the distribution of Actb mRNA. Under basal conditions, very little colocalization was observed between CLUH and each mRNA species (Fig 1A–F). However, we noticed that Pcx and Hadha mRNA molecules colocalized with CLUH only within the few granules present (Fig 1A and B). After HBSS starvation, the pattern of Pcx and Hadha mRNA molecules became visibly more aggregated, and the colocalization with CLUH significantly increased (Fig 1A, B, D and E). In contrast, colocalization of CLUH with Actb mRNA was not enhanced by starvation and remained at background levels (Fig 1C and F).

To further investigate the nature of the CLUH particles, we examined whether they contained markers of well-characterized RNP granules, such as SGs and P-bodies. SGs form under conditions of stress and contain mRNAs stalled in translation initiation together with specific RBPs (Panas et al., 2016; Protter & Parker, 2016), while P-bodies are constitutively present and contain translationally repressed mRNAs (Hubstenberger et al., 2017). Upon HBSS treatment, CLUH colocalized with TIA-1 and G3BP1, two RBPs that are present in SGs (Fig 1G and H). However, classical G3BP1-positive SGs induced by arsenite treatment did not contain CLUH (Fig EV1D). Furthermore, CLUH granules were not positive for DCP1A, a marker of P-bodies, although they were closely located (Fig EV1E).

In conclusion, CLUH forms granules with its target mRNAs in primary hepatocytes. These granules are positive for other RBPs and are more prominent upon starvation, but they are not induced by a classical SG triggering stimulus.

**Results**

**CLUH and its target mRNAs form G3BP1-positive RNA granules**

We previously showed that CLUH plays a physiological role in the adult mouse liver upon starvation to allow amino acid catabolism, to produce ketone bodies, and to maintain glucose levels (Schatton et al., 2017). Intriguingly, CLUH subcellular localization in the mouse liver and in primary hepatocytes changed depending on the nutrient condition. CLUH displayed a cytosolic punctate localization in the liver of fed mice, but formed bigger aggregates in the tissue of mice subjected to food deprivation (Fig EV1A and B). Similarly, when hepatocytes were cultured in basal glucose-rich medium, CLUH decorated small cytosolic puncta and a few bigger foci. In contrast, incubation of hepatocytes for 2 h in HBSS, a low-glucose medium devoid of serum and amino acids, increased CLUH redistribution to bigger structures, often located in the perinuclear region (Fig EV1A and C).

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In conclusion, CLUH forms granules with its target mRNAs in primary hepatocytes. These granules are positive for other RBPs and are more prominent upon starvation, but they are not induced by a classical SG triggering stimulus.

**CLUH-dependent granules temporally regulate translation of target mRNAs**

G3BP1 and TIA-1 are markers for SGs where mRNAs are stalled after recruitment of the translation initiation complex (Panas et al., 2016; Protter & Parker, 2016). Therefore, our first hypothesis was that the assembly of CLUH and its target mRNAs in these granules reflected translational arrest upon nutrient stress. To investigate the
Figure 1.

(A) *Hadha* (mRNA) with CLUH and Merge.

(B) *Pox* (mRNA) with CLUH and Merge.

(C) *Actb* (mRNA) with CLUH and Merge.

(D) *Hadha* mRNA - CLUH

(E) *Pox* mRNA - CLUH

(F) *Actb* mRNA - CLUH

Figure 1.
translational status of the CLUH-positive granules, we made use of the ribopuromycylation assay (David et al., 2012), which reveals the subcellular localization of protein translation, by detecting the incorporation of puromycin into translating polypeptides with a specific antibody (Appendix Fig S1A). Hepatocytes cultured in basal medium showed a diffuse puromycin signal in the cytosol, indicating pervasive translation (Fig 2A and B). As expected, this signal was suppressed by pre-incubation with both homoharringtonine (HHT) and arsenite. HHT causes ribosome stalling at the initiator codon, but leaves unaffected downstream ribosomes already engaged in elongation, while arsenite is a potent inducer of SGs (Fig 2A and B). Upon HBSS treatment, the puromycin signal showed a more granular pattern (Fig 2A). While the total fluorescent intensity of puromycin signal was dramatically reduced by incubation with HHT (Fig 2B), the number of puromycin granules was reduced but not completely abolished by HHT, possibly indicating translation stalled at the level of elongation (Fig 2C). We found that colocalization of puromycin signal with CLUH was higher in HBSS than in basal medium and was significantly reduced by HHT treatment (Fig 2D and E, and Appendix Fig S1B). Furthermore, the percentage of puromycin-positive granules also showing CLUH signal was reduced when samples were pre-treated with HHT (Fig 2F). These data suggest the existence of two types of puromycin-positive compartments in HBSS, one harboring active translation (which disappears upon HHT treatment) and another where translation might be stalled. Both types of granules contain CLUH.

We combined in situ hybridization and the ribopuromycylation assay to correlate the translational state of the granules with specific CLUH mRNA targets. Remarkably, CLUH granules containing Hadha or Pcx mRNAs incorporated puromycin when cells were cultured in glucose, but were mostly puromycin-negative after HBSS incubation (Fig 3A, B, D and E, Appendix Fig S2A and B). We hypothesized that the starvation-induced CLUH granules are dynamic and regulate translation of mRNAs depending on cellular requirements. We therefore probed the Hmgcs2 transcript, which encodes the mitochondrial enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2, implicated in the first reaction of ketogenesis. Defective production of β-hydroxybutyrate is the main metabolic defect of mice lacking CLUH specifically in the adult liver after starvation (Schaton et al., 2017). Hmgcs2 mRNA molecules were detected both in basal and in HBSS conditions. However, the colocalization of CLUH and puromycin was prominent only in HBSS medium (Fig 3C and F; Appendix Fig S2C). To test whether these granules reflect stalled translation in HBSS, we performed control experiments with HHT. Pre-incubation with HHT completely abrogated the detection of Hadha, Pcx, or Hmgcs2 mRNA molecules together with CLUH and puromycin (Appendix Fig S3A–C), demonstrating that CLUH granules are compartments where these mRNAs are translated.

CLUH granules form in the absence of G3BPs and are distinct from SGs

To test whether CLUH overexpression triggers granule formation in the absence of any stress, we transfected an untagged version in HeLa cells and examined overexpressing versus non-overexpressing cells in the same dish, using a specific antibody (Figs 4A and B, and EV2A and B). CLUH overexpression induced the formation of peripheral granules, positive for G3BP1 and the homolog protein G3BP2, in approximately 40% of transfected cells (Figs 4B and C, and EV2C and D). However, CLUH granules were not abrogated by cycloheximide (CHX) treatment, in contrast to arsenite-induced G3BP1-granules, indicating that they are not SGs (Fig EV2E–H). We downregulated G3BP1, G3BP2, or both RBPs and examined whether overexpressed CLUH retained the ability to induce granules (Fig 4A–C). The formation of CLUH granules was affected neither by G3BP1 downregulation nor by concomitant downregulation of both G3BPs (Fig 4B and C). Significantly less CLUH granules were seen in cells depleted for G3BP2, although the levels of overexpressed CLUH were decreased (Fig 4A). Thus, CLUH overexpression is sufficient for granule formation, independently from G3BP1 and G3BP2.

We then asked whether G3BP1 granules can still form in HeLa cells lacking CLUH (Wakim et al., 2017). To this purpose, we expressed G3BP1-GFP and used live imaging to follow the formation of granules upon HBSS incubation. We selected cells which did not show granules at the beginning of the imaging, to avoid analysis of SGs that are known to form upon G3BP1 overexpression (Protter & Parker, 2016). Strikingly, approximately 80% of WT cells formed G3BP1-positive granules in HBSS, while this rarely occurred in KO cells (Fig 4D and E). These granules were still formed upon CHX treatment, suggesting that most of them are not SGs (Fig 4D and E). In contrast, arsenite treatment induced classical CHX-sensitive SGs in both WT and KO cells (Fig EV2J and L). Therefore, CLUH is required for the efficient formation of starvation-induced G3BP1 granules in HeLa cells.

Lastly, we obtained hepatocytes from liver-specific Cluh knock-out (KO) mice (Li-Cluh<sup>KO</sup>) (Schaton et al., 2017) and used G3BP1 staining to detect granules. The number of cells showing G3BP1-positive granules was significantly lower in CLUH-deficient hepatocytes compared to control cells in both basal and HBSS conditions (Fig 4F and G). Intriguingly, the protein levels of G3BP1 were also lower in KO hepatocytes under basal conditions, but they increased similarly in HBSS in cells of both genotypes (Fig 4H and I). In contrast, G3BP1 mRNA levels were unaffected by lack of CLUH (Fig 4J). Thus, our data suggest that CLUH regulates G3BP1 abundance, independent from transcriptional regulation.
Figure 2. CLUH granules contain stalled and active translation sites.

A Confocal images of primary hepatocytes grown under indicated conditions and treatments after ribopuromycylation assay stained with anti-puromycin antibody. Scale bar, 10 μm.

B Quantification of fluorescence intensity per cell of experiment shown in (A). AU, arbitrary units (n = 90–110 cells per treatment isolated from 4 mice).

C Quantification of the number of puromycin granules under indicated conditions (n ≥ 50 cells isolated from 4 mice).

D Confocal images of primary hepatocytes stained with anti-puromycin and anti-CLUH antibodies. The cells from which the enlarged areas (400 μm²) have been magnified are shown in Appendix Fig S1B for each individual channel. Scale bar, 4 μm. Cells analyzed were isolated from 4 different mice with similar results. Arrows point to colocalizing particles.

E Manders’ colocalization coefficient between puromycin and CLUH from experiment shown in (D) (n ≥ 50 cells isolated from 4 mice).

F Quantification of puromycin granules containing CLUH signal (n ≥ 80 cells isolated from 4 mice).

Data information: In (B, C, E, F), data are presented as boxplots showing the median, the first quartile, and the third quartile. Error bars show minimum and maximum values. (C, F) ***P ≤ 0.001; **P ≤ 0.01 (Student’s t-test). (E) ***P ≤ 0.001 (one-way ANOVA, Tukey’s multiple comparison test).
CLUH differentially regulates mRNAs involved in mitochondrial anabolic and catabolic pathways

To further understand the physiological role of CLUH granules, we performed RNA-seq and quantitative label-free mass spectrometry (MS) on wild-type (WT) and Cluh-deficient hepatocytes (Dataset EV1 and EV2). To systematically detect groups of mRNAs that participate in similar pathways and to reveal early proteome changes after the short starvation period, we performed one-dimensional (1D) pathway enrichment of transcriptome and proteome data (Dataset EV3), which allows the identification of even small shifts in pathways by summarizing the fold changes of all proteins.

Figure 3. CLUH granules are translationally active or dormant depending on the mRNA.
A–C Confocal images of primary hepatocytes after ribopuromycylation experiment combined with mRNA in situ hybridization for (A) Hadha, (B) Pcx, and (C) Hmgcs2. Scale bar, 4 μm.
D–F Fluorescence profile of 100-pixel line from the merged channel shown in (A–C). The cells from which the enlarged areas (400 μm²) have been magnified are shown in Appendix Fig S2 for each individual channel. Cells analyzed were isolated from 6 different mice with similar results.
Figure 4.
annotated with a specific term. Differently regulated terms between conditions were identified based on a false discovery rate, FDR, < 0.02.

To examine whether WT and KO cells respond in a similar manner to starvation, we first examined changes upon shifting WT or KO hepatocytes from basal glucose-rich medium to HBSS for 2 h. Starvation triggered a modest reshaping of the transcriptome and proteome in cells of both genotypes; however, analysis of pathways enriched or depleted specifically in WT or KO cells revealed differences (Appendix Fig S4A and B, and Dataset EV3). Terms associated with mitochondrial translation and mitochondrial respiratory complex I were enriched in transcriptomics of starved Cluh KO hepatocytes (Appendix Fig S4B and Dataset EV3), but not detected in starved control cells. At the proteome level, proteins involved in biosynthetic fatty acid processes were less represented in control cells, as expected upon starvation, while proteins involved in actin dynamics and ribonucleoprotein complexes were increased (Appendix Fig S4C and Dataset EV3). Instead, several terms linked with cap-dependent translation initiation were decreased in the absence of CLUH upon starvation, while terms related to mRNA decay and lysosomes were enriched (Appendix Fig S4D and Dataset EV3). These results suggest that WT and KO cells respond differently to starvation, and are consistent with a role of CLUH to protect target mRNAs from degradation and to promote a translation response under conditions of nutrient deprivation.

We then compared mRNA and protein changes between WT and KO hepatocytes (Fig 5A–D and Dataset EV1 and EV2). A subset of mRNAs and the corresponding mitochondrial proteins (Fig 5A and B) were significantly decreased in abundance. One-dimensional enrichment analysis of the data showed a global under-representation at both mRNA and protein level of several mitochondrial pathways, such as pyruvate metabolism, β-oxidation, and amino acid degradation, in both basal conditions and HBSS in CLUH-deficient hepatocytes (Fig 5C–E, blue, Dataset EV3), in agreement with our previous findings in embryonic livers (Schatton et al., 2017). However, pathways associated with mitochondrial translation and the mitochondrial large ribosomal subunit were enriched on the proteome level (Fig 5D, red, Dataset EV3), but decreased on the transcriptome level (Fig 5E, red, Dataset EV3) when CLUH was absent. We then compared the protein fold changes between WT and KO specifically for those proteins whose mRNA was previously identified in CLUH immunoprecipitations in HeLa cells (Gao et al., 2014). As expected, in KO hepatocytes the majority of these proteins showed a significant downregulation (Fig 5F). However, proteins encoded by a subset of target mRNAs were not affected or slightly upregulated (Fig 5F, inset box). A closer inspection of these proteins revealed that they comprised several mitochondrial ribosomal proteins and the mitochondrial transcription factor TFAM. We conclude that CLUH positively regulates mRNA and protein levels of mRNAs involved in catabolic pathways, such as amino acid degradation, fatty acid oxidation, and the TCA cycle, but may have a translationally inhibitory effect on another group of transcripts, involved in mitochondrial transcription and translation.

CLUH ensures mTORC1 inhibition upon starvation

The expression of TFAM and of mitochondrial ribosomal proteins is known to be regulated at the translational level by mTORC1, specifically via inhibition of the eukaryotic translation initiation factor eIF-4E-binding proteins (4E-BPs), to sustain ATP production during growth (Thoreen et al., 2012; Morita et al., 2013). Therefore, we investigated the level of mTORC1 activation in primary hepatocytes during HBSS starvation, by assessing the phosphorylation of downstream substrates. The phosphorylation of 4E-BP1 was higher in KO hepatocytes in both basal conditions and at different time points after HBSS starvation, while the phosphorylation of ribosomal protein S6 (RPS6) was not significantly increased (Fig 6A–C). We also examined whether Li-CluhKO mice could efficiently suppress mTORC1 activation during starvation. Similar to hepatocytes, the levels of phosphorylated 4E-BPs were higher in the KO livers after 24-h starvation than in controls (Fig 6D and E). The increase in the amount of phosphorylated RPS6 was less consistently observed (Fig 6D and F).

mTORC1 suppression during nutrient deprivation is fundamental to protect cells from apoptosis. One of the most upregulated proteins in KO hepatocytes compared to control cells in starvation was the pro-apoptotic protein Diablo (Fig 5B). In agreement with this result, we found increased caspase-3 cleavage in CLUH-deficient...
Figure 5. Transcriptomic and proteomic profile of WT and KO hepatocytes.

A, B Volcano plots showing significantly changed proteins in KO hepatocytes relative to WT in basal (A) and HBSS (B) conditions.

C 2D score plots of enriched pathways in transcriptomics analysis of KO and WT hepatocytes under basal condition and upon HBSS (2 h).

D 2D score plots of enriched pathways in proteomics analysis of KO and WT hepatocytes under basal condition and upon HBSS (2 h).

E 2D score plots of enriched pathways in proteomics versus transcriptomics analysis of KO and WT hepatocytes under basal condition.

F Correlation of protein fold changes in KO with respect to WT hepatocytes under basal condition and upon HBSS (2 h). Only genes previously found in CLUH RIP experiments in HeLa cells (Gao et al., 2014) are plotted. Inset shows magnification of upper part of the graph.
Figure 6.
hepatocytes cultured for 16 h in HBSS, which was rescued by treatment with the allosteric mTORC1 inhibitor rapamycin (Fig 6G and H). A higher number of apoptotic cells were also detected in the liver of Li-Clu^KO mice upon starvation (Fig 6I and J).

mTORC1 coordinates an integrated stress response in the presence of mitochondrial dysfunction (Khan et al., 2017), potentially explaining our findings. However, neither a significant change in the expression of the transcription factors Atf4 and Atf5, nor upregulation of the mRNA levels of Psat1, Flgdih, or Mthfd1L2, all markers of the mitochondrial integrated stress response, were observed in KO hepatocytes or livers (Appendix Fig SSA and B). mTORC1 can also be inhibited by dynamic recruitment of its components to RNA granules, for example, to SGs upon stress (Takahara & Maeda, 2012; Wippich et al., 2013), raising the possibility that CLUH granules may directly regulate the extent of the mTORC1 signaling response upon starvation. In agreement with this hypothesis, the G3BP1-positive granules which form in starved primary hepatocytes contained the mTOR kinase (Fig 6K and L).

These data highlight a crucial role of CLUH granules not only to coordinate the translation of nuclear-encoded mitochondrial proteins involved in amino acid and fatty acid catabolism and ketogenesis, but also to suppress the anabolic role of mTORC1 upon starvation, thereby protecting from cell death.

**Lack of CLUH is associated with increased bulk autophagy, but decreased mitophagy**

Our data indicate that CLUH granules may play signaling functions, by recruiting the kinase mTOR and other RBPs, like G3BP1 and G3BP2. mTORC1 controls autophagy at different steps: It inhibits not only the initiation of autophagy, but also late steps of autophagy, such as the fusion of autophagosomes and lysosomes, lysosomal biogenesis, and reformation (Yu et al., 2010; Zhou et al., 2013; Shen & Mizushima, 2014). G3BP1 and G3BP2 are well known for their role in SG formation; however, they play a plethora of other signaling functions (Alam & Kennedy, 2019). Of note, the yeast orthologue of G3BPs, Bre5, has been shown to promote ribophagy and other types of autophagy, and to inhibit mitophagy (Muller et al., 2015). Our data open up the possibility that CLUH RNP particles regulate autophagic pathways, which are essential to survive starvation. To analyze the impact of CLUH deficiency on general autophagy in primary hepatocytes and in the liver, we crossed Li-Clu^KO mice with transgenic mice expressing the LC3-GFP reporter (Mizushima et al., 2004) and analyzed them 4 weeks after complete CLUH deficiency was achieved (Schatton et al., 2017). In the absence of CLUH, LC3-GFP accumulated in larger aggregates in the liver and in primary hepatocytes (Fig 7A–E), indicating that autophagosomal formation was not impaired upon sustained CLUH deficiency. In addition, CLUH-deficient livers showed increased amount of LAMP1, a marker of lysosomes (Fig 7F and G). These results can reflect an increased autophagic flux, or a block in the consumption of autophagosomes. To distinguish between these possibilities, we analyzed the autophagic flux in primary hepatocytes, by measuring the conversion of LC3-I to LC3-II in the absence of the autophagic adaptor p62 (Fig 7H and K). Interestingly, the steady-state levels of CLUH were significantly reduced that the decrease in G3BP1 levels and G3BP1-positive granules in KO hepatocytes already in basal conditions could be caused by their increased removal by autophagy. We analyzed this possibility by measuring the conversion of LC3-I to LC3-II in the absence or presence of bafilomycin A, a potent inhibitor of the vacuolar H^+ ATPase. Surprisingly, lack of CLUH led to an increase in the autophagic flux under basal conditions (Fig 7H–J). The autophagic flux was increased in both WT and KO cells upon HBSS, as indicated by consumption of the autophagic adaptor p62 (Fig 7H and K). Interestingly, the steady-state levels of CLUH were significantly reduced upon starvation (Fig 7L). Together, our results indicate that autophagy is increased in hepatocytes lacking CLUH already under basal conditions, despite mTORC1 hyperactivation.

Granulophagy is a specialized form of autophagy that removes RNP particles, including SGs (Frankel et al., 2017). We hypothesized that the decrease in G3BP1 levels and G3BP1-positive granules in KO hepatocytes already in basal conditions could be caused by their increased removal by autophagy. We analyzed this possibility by assessing the colocalization of G3BP1 granules with lysosomes in basal conditions and after a short (30 min) or more prolonged (2 h) starvation in HBSS. In WT hepatocytes, G3BP1 granules showed colocalization with LAMP1 after 2 h of starvation, while when G3BP1 granules were observed in KO hepatocytes, they colocalized with LAMP1 after only 30 min in HBSS medium (Fig EV3A–C). We conclude that CLUH protects G3BP1-positive granules from premature autophagic degradation upon starvation.
Figure 7.
Surprisingly, p62 accumulated in CLUH-deficient livers (Fig 8A and B), despite functional autophagy. This prompted us to analyze mitophagy, a specialized form of autophagy that allows turnover of old or damaged mitochondria. We found that p62 and ubiquitin accumulated in liver mitochondrial lysates in the absence of CLUH (Fig 8C and D). Consistent with our previous findings, mitochondrial proteins encoded by CLUH target mRNAs analyzed in this study were decreased in purified mitochondria (Fig 8C and D). In further agreement with a mitophagy block, LC3 signal colocalized with mitochondria in liver mitochondrial lysates compared to controls (Fig 8E and F). Furthermore, more LC3-positive puncta localized with mitochondria in KO hepatocytes, and rapamycin treatment was effective in restoring this colocalization to WT levels (Fig 8G and H). To corroborate these findings, we transfected WT and Cluh-deficient mouse embryonic fibroblasts (MEFs) with a construct expressing a tandem mCherry-GFP fluorescent protein targeted to mitochondria (mCherry-GFP-FIS101). Upon engulfment of mitochondria in lysosomes, the GFP signal is quenched, while the red fluorescence is preserved, allowing it to distinguish and quantify mitochondria that have been successfully delivered to functional lysosomes (Allen et al., 2013). Mitophagy was induced by culturing cells in galactose, or by adding antimycin A and oligomycin (A/O) to the medium (Allen et al., 2013; Melser et al., 2013). Remarkably, turnover of mitochondria was impaired in cells depleted of CLUH in both conditions (Fig 8I and J) and rescued by rapamycin treatment (Fig 8K and L). Thus, constitutive lack of CLUH accelerates autophagy but inhibits mitophagy.

**Discussion**

We reveal a CLUH-dependent post-transcriptional mechanism that coordinates the mitochondrial catabolic response during starvation with signaling pathways that control bulk autophagy and the turnover of mitochondria. Central to this response is the formation of CLUH RNP particles, which act as spatial compartments to preserve mRNAs involved in mitochondrial catabolic pathways and allow their translation, but also as signaling hubs by recruiting the mTOR kinase and other RBPs, such as G3BP1 and G3BP2. Thus, CLUH plays a fundamental role to control the metabolic plasticity of hepatic mitochondria.

RNA granules are large, non-membrane-bound structures that reflect the coalescence of RBPs together with mRNAs via a phase transition from a soluble to a liquid droplet (Hyman et al., 2014). Several types of RNA granules, such as SGs, P-bodies, or neuronal RNA granules, have been shown to coordinately regulate the fate of
Figure 8. [Description of figure]
CLUH granules recruit mTOR and safeguard hepatocytes modulate the nutrient-sensing mTORC1 signaling (Saxton & Sabatini, 2017). CLUH granules recruit mTOR and safeguard hepatocytes modulate the nutrient-sensing mTORC1 signaling (Saxton & Sabatini, 2017).

Future studies will be required to fully understand what distinguishes a translationally silent, active, or stalled CLUH granule, and how the transition between these granules is mechanistically regulated.

We show that CLUH granules play crucial signaling roles, and modulate the nutrient-sensing mTORC1 signaling (Saxton & Sabatini, 2017). CLUH granules recruit mTOR and safeguard hepatocytes against a premature reactivation of mTORC1 during starvation that poses the cells at risk of apoptosis if energy-consuming anabolic pathways are stimulated despite energy stress (Mikeladze-Dvali et al., 2005; Teleman et al., 2005; Kim et al., 2008; Choo et al., 2010; Demetriades et al., 2014). Importantly, genes involved in transcription (such as Tfam) and translation of mtDNA (mitoribosomal proteins) are known to be translationally regulated by mTORC1 via 4E-BPs (Morita et al., 2005; Teleman et al., 2005) - a key feature of the process.

CLUH granules are the first example of compartments for post-transcriptional regulation (Anderson & Kedersha, 2009; Protter & Parker, 2016; Gomes & Shorter, 2019). The CLUH granules observed in hepatocytes are the first example of compartments for post-transcriptional regulation (Anderson & Kedersha, 2009; Protter & Parker, 2016; Gomes & Shorter, 2019). CLUH granules are distinct from SGs: (i) They incorporate puromycin; (ii) they are resistant to CHX treatment; and (iii) they form in the absence of G3BP1 and G3BP2 upon CLUH overexpression.

Interestingly, CLUH granules are dynamic and display a transcript-specific temporal activation of translation: In fact, Pcx and Hadha mRNAs are detected in translationally proficient granules mainly upon nutrient-rich conditions, while Hmgcs2 mRNA upon starvation, in agreement with the prevalence of anaplerotic reactions or the need to synthesize ketone bodies in the respective condition. Thus, cycles of translation can engage different transcripts at different times in the CLUH granules, to match the transcriptional profile to the cellular needs depending on nutrient availability. In addition, we found that a subset of CLUH granules can incorporate puromycin despite inhibition of the translation between translational initiation and elongation by HHT, suggesting that they might contain translationally stalled mRNAs. So far, none of the tested mRNAs could be detected in these granules, opening the question whether they regulate a different group of transcripts. Future studies will be required to fully understand what distinguishes a translationally silent, active, or stalled CLUH granule, and how the transition between these granules is mechanistically regulated.

Thousands of mRNAs (so called RNA regulons), by defining distinct compartments for post-transcriptional regulation (Anderson & Kedersha, 2009; Protter & Parker, 2016; Gomes & Shorter, 2019). The CLUH granules observed in hepatocytes are the first example of RNP particles specifically regulating the translation of mRNAs encoding mitochondrial proteins (here for Pcx, Hadha, and Hmgcs2). Although they contain G3BP1 and G3BP2, several evidences indicate that the CLUH granules are distinct from SGs: (i) They incorporate puromycin; (ii) they are resistant to CHX treatment; and (iii) they form in the absence of G3BP1 and G3BP2 upon CLUH overexpression.

Besides controlling protein synthesis and anabolic pathways, mTORC1 activation inhibits autophagy. Thus, it was surprising to find that in the absence of CLUH, bulk autophagy was not impaired, but rather enhanced. Induction of bulk RNA degradation by autophagy has been previously observed in hepatocytes upon amino acid starvation (Lardeux et al., 1987; Heydrick et al., 1991). We speculate...
Figure 9.
that sorting of target mRNAs into CLUH granules has a crucial role not only for their translation, but also to protect them against premature decay and autophagic degradation. Consistently, pathways associated with mRNA decay and the lysosome were enriched in proteomics analysis of starved KO hepatocytes. Furthermore, G3BP1-positive granules colocalized with LAMP1-positive organelles upon HBSS starvation with faster dynamics in KO hepatocytes. Whether the decreased occurrence of G3BP1-positive granules in hepatocytes lacking CLUH is totally explained by their faster turnover by autophagy or is also caused by failure of nucleation of the granules is a question to be addressed in the future. Furthermore, it is still unclear whether the role of CLUH in stabilizing target mRNAs is dependent on granule formation.

While inhibiting autophagic degradation of RNP particles containing transcripts for mitochondrial proteins, CLUH positively regulates turnover of mitochondria both in vivo in the liver and in vitro in hepatocytes and MEFs. Our data are consistent with studies in the fly showing increased levels of p62, mitochondrial recruitment of parkin, increased parkin–Pink1 interaction, and a decreased mitochondrial clearance in the absence of clueless (Sen et al., 2015; Wang et al., 2016). Importantly, during starvation, mitochondria are protected from macroautophagic degradation for a longer time with respect to cytosolic components or other organelles, for example, ribosomes (Kristensen et al., 2008; Rambold et al., 2011). However, in the starved liver it may be important to allow a basal level of mitochondrial turnover to replace organelles with a metabolic profile suited for conditions of high nutrients with those specified to perform catabolic functions. Therefore, differential regulation of bulk autophagy and mitophagy may be advantageous in the liver when nutrients are low. In this situation, CLUH plays a central role by coupling mitochondrial biogenesis and mitochondrial turnover to maintain the quality of liver mitochondria and adapt it to cellular needs. A similar concept is currently emerging also from studies implicating PINK1 not only in mitophagy (Lazarou et al., 2015), but also in localized translation at the surface of mitochondria (Gehrke et al., 2015).

The apparent contrasting finding on autophagy and mitophagy observed in cells and tissues lacking CLUH recapitulates the role of Brp5p, the yeast G3BP orthologue, which is required together with the ubiquitin protease Ubp3 for promoting ribophagy and other forms of autophagy, while inhibiting mitophagy (Ossareh-Nazari et al., 2010; Muller et al., 2015). We speculate that by recruiting G3BPs to granules, CLUH restricts their function. Consistently, concomitant depletion of CLUH and G3BPs prevented the formation of mitochondrial clustering upon CLUH downregulation, assigning for the first time to mammalian G3BPs a role in mitophagy regulation. This potentially broadens up the signaling function of CLUH granules to regulation of deubiquitination processes required for specialized forms of autophagy, via G3BPs. G3BP1 and G3BP2 are widely studied for their role in RNA metabolism and SG formation, but they have been associated with multiple functions, including Ras signaling, NFκB activation, and interferon-mediated signaling (Alam & Kennedy, 2019). Unraveling the role of G3BP1 and G3BP2 in mitophagy and regulation of the translation of mRNAs for mitochondrial proteins will be necessary to fully understand the role of CLUH.

Finally, we show that rapamycin administration improved mitochondrial clustering and rescued lysosomal accumulation in Cluh-deficient livers, confirming the hypothesis that mitochondrial clustering is a secondary phenotype deriving from impaired turnover of dysfunctional mitochondria and perturbed signaling responses. Interestingly, promoting mitophagy by parkin overexpression also rescues the clustering phenotype in the fly muscle and ovary (Sen et al., 2015; Wang et al., 2016).

In conclusion, we unveil a complex CLUH-dependent post-transcriptional mechanism of physiological significance in hepatocytes. CLUH coordinately regulates mTORC1 signaling, the catabolic capacity of mitochondria and their turnover to efficiently program metabolism to cope with energy levels (Fig EV5). In the absence of CLUH, hepatocytes are susceptible to cell death upon starvation. In general terms, our data highlight the importance of compartmentalization for post-transcriptional regulation of mitochondrial RNA regulons.

Materials and Methods

Animal experiments

The liver-specific Cluh-deficient mice (Li-Cluhko) used in this study were generated in a C57BL/6N congenic background and were described and characterized previously (Schatton et al., 2017). When
specified, mice were crossed with LC3-GFP transgenic mice (Mizushima et al., 2004) to visualize autophagosomes. All lines were maintained in a pure C57BL/6N background. In all experiments, both male and female mice at 8 weeks of age were used, unless stated otherwise. As controls, Clchfl/fl littermates from the same strain without the Cre allele were used (here named WT). Mice were maintained in individually ventilated cages with specified pathogen-free hygiene levels, kept under a 12-h/12-h dark/light cycle, given a regular chow diet ad libitum (Sniff V1554-300), and monitored regularly for signs of suffering. Starvation was induced by removing access to food but not water for 24 h. For tissue collections, mice were sacrificed by cervical dislocation. For histological studies, adult mice were anesthetized intraperitoneally with xylazine/ketanest (10 mg/100 mg per kilogram body weight) and perfused intracardially with 4% PFA/PBS.

Before the first injection of rapamycin or mock solution, mice were starved for synchronization. Animals were allocated randomly to treatments. Rapamycin was dissolved in DMSO to 100 mg/ml and then further in 5% PEG-1500 and TWEEN-20 in water until 1.2 mg/ml. Solution was filtered through a 0.2-μm filter. Rapamycin (7.5 mg/kg) or mock (5% PEG-1500, 5% TWEEN-20) was injected daily for 1 week. After this time, animals were sacrificed by cervical dislocation and tissues were collected for analysis. For immunostaining, tissues were kept in 4% PFA/PBS for 5 days before cutting in cryostat. All animal procedures were performed in accordance with European Union (EU directive 86/609/EEC), national (Tier schutzgesetz), and institutional guidelines and were approved by local authorities (Landesamt für Natur, Umwelt, und Verbraucherschutz Nordrhein-Westfalen, Germany).

Hepatocyte isolation

To isolate primary hepatocytes, adult mice at 8 weeks of age were anesthetized as specified above and perfused through the vena cava with EBSS w/o Ca²⁺ and Mg²⁺ supplemented with 0.5 mM EGTA, followed by EBSS including Ca²⁺ and Mg²⁺ containing 10 mM HEPES, 0.4 mg/ml collagenase type 2 (Worthington), and 0.04 mg/ml trypsin inhibitor (Sigma-Aldrich) at 37°C. Livers were dissected, and single-cell suspensions were generated through a 70-μm nylon filter. Cells were then mixed with Percoll/HBSS solution [9 vol Percoll (GE Healthcare) + 1 vol 10× HBSS buffer] and centrifuged at 1,000 × g for 7 min. Pellets were washed twice with 5% FBS/DMEM and plated on coverslips coated with fibronectin (Thermo Fisher Scientific). Primary hepatocytes were maintained in 4.5 g/l glucose DMEM including 5% FBS, 2 mM L-glutamine, and 2% penicillin/streptomycin (basal medium). Experiments in hepatocytes were performed 24 h after plating.

RNA in situ hybridization

Hepatocytes were fixed with 10% neutral buffered formalin for 15-30 min. Fixed cells were dehydrated in 5-min series of 50-70-100% ethanol and stored at −20°C until use. Single-molecule RNA in situ hybridization was performed using the RNAscope 2.5 HD Fluorescent Reagent Kit (Advanced Cell Diagnostics, Inc.). Coverslips were rehydrated in 5-min series 100-70-50% ethanol and a final 15-min incubation in PBST (PBS and 1% TWEEN-20), and treated with Protease III for 30 min at room temperature before hybridization with the corresponding probe. Target probes to detect murine Hadda (#459331), Pcx (#418831), Hmgcs2 (#437141), and Actb (#316741) were designed by the manufacturer. Pcx, Hadda, and Hmgcs2 were selected in C1 channel while Actb in C2 channel. Signal was amplified according to the manufacturer’s instructions and detected with an Amp4 Alt A-FL probe, which gives fluorescence in 488 nm for the probes in C1 or Amp4 Alt B-FL probe which gives fluorescence in 488 nm for probes in C2 (Advanced Cell Diagnostics, Inc.). When combined with immunostaining, coverslips were washed with PBST followed by blocking and incubation with primary antibodies. Nuclei were stained with DAPI. Samples were analyzed using a spinning-disk confocal microscope (UltraVIEW VoX, PerkinElmer) with a Plan Apochromat total internal reflection fluorescence 60 Å~1.49 oil DIC objective. Colocalization analyses were carried out using JACoP plugin (Bolte & Cordelieres, 2006). Manders’ coefficient was used to quantify colocalization (Manders et al., 1993).

Ribopuromycylation assay

Hepatocytes were incubated for 5 min at 37°C with 91 μM puromycin (Sigma-Aldrich) and 208 μM emetine (Sigma-Aldrich). Dishes were transferred to ice and washed twice with ice-cold PBS containing 355 μM CHX. Plates were incubated for 2 min on ice with ice-cold PB Buffer [50 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 25 mM KCl, 355 μM cycloheximide, 10 U/ml RNase OUT (Life Technologies), 0.007% digitonin with protease inhibitor EDTA-free (Roche)] and washed twice with ice-cold WB Buffer [50 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 25 mM KCl, 355 μM cycloheximide, 10 U/ml RNase OUT (Life Technologies), 20 mM sucrose with protease inhibitor EDTA-free (Roche)]. Hepatocytes were fixed with 10% formalin for 15 min. Immunofluorescence was performed as described previously, using an anti-puromycin antibody (#MABE343, clone 12D10, EMD Millipore) to detect active translation sites. When combined with RNA in situ hybridization, RNAscope protocol was performed prior to immunofluorescence. Samples incubated without puromycin were used as a control for antibody specificity. To inhibit translation, cells were treated with 500 μM arsenite for 30 min or with 2 μg/ml HHT for 15 min before ribopuromycylation was performed. Fluorescence intensity was visualized with mpl-liferno lut from ImageJ. Colocalization analyses were carried out using JACoP plugin (Bolte & Cordelieres, 2006). Manders’ coefficient was used to quantify colocalization (Manders et al., 1993). To quantify puromycin particles in HBSS, ImageJ was used first to remove nuclear signal from the images. Later, the puromycin channel was manually thresholded and the watershed plugin was used to separate particles. Puromycin particles bigger than 5 pixels were analyzed, using the “analyze particle” feature of ImageJ. To detect puromycin granules also positive for CLUH, the CLUH channel was first thresholded and the puromycin granules positive for CLUH were counted. The intensity profiles shown in Fig 3 were performed in the original images drawing a line of 100 pixel long and 10 pixels thick.

Immunofluorescence analysis

Cells were fixed in 4% PFA/PBS for 15 min and stored at 4°C in PBS. HeLa and COS-7 cells were permeabilized with 0.2% Triton
X-100 in PBS for 20 min, blocked for 30 min (5% milk, 2.5% BSA, 10% serum, 0.2% Triton X-100 in PBS), and incubated with the corresponding primary antibody overnight at 4°C or 2 h at room temperature. Antibodies used for immunofluorescence were polyclonal rabbit anti-CLUH (#ab178342, Abcam; # ARP70642_P050, Aviva), polyclonal rabbit anti-LAMP1 (#ab24170, Abcam), polyclonal rabbit anti-TOMM20 (#sc-11415, Santa Cruz Biotechnology), monoclonal mouse anti-G3BP1 (#sc-81940, clone TT-Y, Santa Cruz Biotechnology), monoclonal mouse anti-TIA-1 (#sc-166247, clone G3, Santa Cruz Biotechnology), monoclonal mouse anti-FLAG (#F3165, clone M2, Sigma-Aldrich), monoclonal mouse anti-puro-mycin (#MABE343, clone 12D10, EMD Millipore), polyclonal rabbit anti-mTOR (#2983, clone 7C10, Cell signaling), monoclonal mouse anti-DCP1A (#H00055802-M06, clone 3G4, Abnova), polyclonal rabbit anti-G3BP2 (#ab86135, Abcam), and polyclonal rabbit anti-CLUH (#NB100-93306, Novus Biologicals). Secondary antibodies were used in blocking buffer overnight at 4°C. Afterward, sections were washed with PBS and mounted with Fluorsave (EMD Millipore) and visualized with spinning-disk confocal microscope (UltraVIEW VoX, PerkinElmer) using a 60× objective. Microscopy chromatic aberration was tested regularly for spinning-disk microscope using standard samples with 100- and 500-nm beads. Images acquired with confocal microscope were deconvoluted using ImageJ (NIH) prior to analysis to increase resolution. Brightness was adjusted equally in the entire images. The amount, the relative size, and area of LC3 and LAMP1 particles were analyzed using a plugin developed by Dagda et al (2008).

**Tissue histology, immunohistochemistry, and immunofluorescence**

Livers of perfused mice were post-fixed for 2 days in 4% PFA/PBS at 4°C. For cryostat sectioning, after fixation tissues were dehydrated in 15% sucrose for 6 h and 30% sucrose overnight. After this treatment, liver pieces were embedded in optimal cutting temperature mounting medium (Tissue-Tek) and stored at −80°C. Before cutting, samples were equilibrated to −20°C for 4 h and cut in 10-μm sections. Immunofluorescence was performed following a standard protocol. Sections were washed in PBS, blocked in 1% Western Blocking Reagent (Roche), and then incubated with indicated antibodies in blocking reagent overnight at 4°C. Afterward, sections were washed and incubated in secondary antibodies diluted in blocking buffer for 1 h at RT, washed again, stained with DAPI (Sigma-Aldrich), and mounted with Fluorsave (EMD Millipore). Antibodies used in tissue immunofluorescence were polyclonal rabbit anti-CLUH (# ARP70642_P050, Aviva), polyclonal rabbit anti-LAMP1 (#ab24170, Abcam), and polyclonal rabbit anti-TOMM20 (#sc-11415, Santa Cruz Biotechnology). For imaging, a spinning-disk confocal microscope (UltraView VoX; PerkinElmer) with a Plan Apochromat total internal reflection fluorescence 60×/1.49 NA oil DIC objective was used.

Analysis of apoptosis in liver was performed in paraffin-embedded samples. Paraffin liver sections were dehydrated, and antigen retrieval was performed by heating in 10 mM sodium citrate and 0.05% Tween 20, pH 6.2. Rabbit polyclonal anti-cleaved caspase 3 (Asp175) (#9661, Cell Signaling) was used to identify apoptotic cells. Biotinylated secondary antibodies were purchased from Vector Laboratories. Stainings were visualized with ABC kit Vectastain Elite (Vector Laboratories) and DAB substrate (Dako). The number of apoptotic cells was counted and shown relative to tissue area. Image analysis in tissues was performed on 4–5 animals. Four pictures from the same animal were taken, and the values were averaged.

**Cell lines**

Cell lines (COS-7, HeLa, and MEF) were cultured in 4.5 g/l glucose DMEM supplemented with 10% FetalClone III serum (Hyclone), 2% penicillin/streptomycin, and 2 mM L-glutamine (Invitrogen), and regularly checked for mycoplasma contamination. Club-deficient MEFs were previously described (Gao et al, 2014). HeLa cells lacking CLUH were generated by CRISPR-Cas9 technology (Wakim et al, 2017).

**RNA interference and mitochondrial clustering**

RNA interference of CLUH in COS-7 cells was done as described previously (Gao et al, 2014). To downregulate G3BP1 and G3BP2, HeLa and COS-7 cells were transfected with 100 nM siRNA (Ctrl siRNA: #D-001206-14-20; G3BP1 siRNA: #M-012099-02-0020; G3BP2 siRNA: #M-015329-00-0020; Dharmacon) using Lipofectamine 2000 (Life Technologies) on two consecutive days and experiments were performed 72 h after first transfection. Mitochondrial clustering recovery experiments were performed with 200 nM rapamycin or 200 nM torin for 6 h prior fixation. Mitochondrial morphology parameters were obtained using a previously described plugin (Dagda et al, 2009).

**CLUH granules and SG induction**

Canonical stress granules were induced in hepatocytes and HeLa cells by incubating with 500 μM arsenite for 30 min. To dissolve stress granules, 0.1 mg/ml cycloheximide was added to the cells for 30 min. To induce CLUH granules in HeLa cells, cells were transfected with constructs encoding full-length human untagged CLUH or CLUH-Flag for 48 h with Lipofectamine 2000 (Life Technologies). Full-length human CLUH was cloned into p3xFLAG-CMV-14 or into pcDNA3.1 using HindIII/EcoRI restriction sites. CLUH granules in hepatocytes were induced by HBSS treatment as stated.

**Live imaging expression of G3BP1-GFP**

For live imaging, 200,000 HeLa cells were seeded in glass bottom microwell dishes (#P356-1.5-14-C, Mat Tek Corporation) and transfected with 0.5 μg G3BP1-GFP plasmid (kindly provided by Dr. Jamal Tazi), using Lipofectamine 2000. Twenty-four h later, cells were incubated in HBSS or treated with arsenite (with or without CHX) and videos were recorded taking 1 picture/min using a spinning-disk confocal microscope (UltraVIEW VoX, PerkinElmer) at 37°C and 5% CO2. Only cells showing green fluorescence but not granules produced by G3BP1 overexpression were selected for recording.

**Autophagy and mitophagy experiments**

To assess autophagic flux, hepatocytes were treated with or without 100 nM bafilomycin A for 2 h in basal or HBSS medium. To assess
mitophagy, MEFs were transected with mCherry-GFP-FIS101–152 plasmid (Allen et al., 2013). After 24 h, mitophagy was induced either by replacing the medium with glucose-free DMEM (#11966-025, Invitrogen) supplemented with 10 mM galactose, 10% dialyzed antimycin A and 10 μM oligomycin for 16 h. When indicated, 200 nM rapamycin was added. Cells with mitophagosomes were counted when red signal was clearly recognizable (> 100 cells per replicate in 3–5 independent experiments).

Western blots and mitochondrial purification

Cell pellets were lysed in RIPA buffer [50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.25% deoxycholic acid, 1% Triton X-100, and protease inhibitor cocktail (Sigma-Aldrich)], while tissues were lysed in RIPA buffer including 0.1% SDS. Proteins were quantified with standard Bradford (Bio-Rad) assay prior to SDS–PAGE and blotting on PVDF membranes. To isolate mitochondria, tissues were lysed and homogenized in Mitochondria Isolation Buffer (MIB, 100 mM sucrose, 50 mM KCl, 1 mM EDTA, 20 mM TES, 0.2% free fatty acid–BSA) at 1,200 rpm until the solution was homogenous. The solution was centrifuged at 8,500 × g at 4°C for 5 min. Pellets were washed twice with MIB and centrifuged at 800 × g. Clean supernatants were then centrifuged at 8,200 × g to obtain pure mitochondria. Final pellets were resuspended in 500 µL MIB w/o BSA, and 10 µg of pure mitochondria were pelleted and resuspended in MIB w/o BSA. The following antibodies were used for Western blot: polyclonal rabbit anti-4E-BP1-pT37/46 (#2855, clone 2C11) from Abcam; monoclonal mouse anti-ubiquitin (#sc-8017, clone G-11) from Aviva; monoclonal mouse anti-p62 (#H00008878-M01, clone E10) from Abnova; monoclonal mouse anti-ubiquitin (#sc-8017, clone C4) from EMD Millipore; polyclonal rabbit anti-CLUH (for detection of human CLUH, #NB100-93306), polyclonal rabbit anti-RPSE (#NB100-1959), and polyclonal rabbit anti-LC3 (#NB100-2220) from Novus Biologicals; polyclonal rabbit anti-CLUH (for detection of murine CLUH, # ARP70642_P050) from Aviva; monoclonal mouse anti-p62 (#H00008878-M01, clone 2C11) from Abnova; monoclonal mouse anti-ubiquitin (#sc-8017, clone P4D1) and monoclonal mouse anti-HMGCS2 (#sc-376092, clone G-11) from Santa Cruz Biotechnology; and polyclonal rabbit anti-HADHA (#ab54477), monoclonal rabbit anti-PCX (#ab128952, clone EPR7365), monoclonal rabbit anti-G3BP1 (#ab181150, clone EPR13986(B)), polyclonal rabbit anti-G3BP2 (#ab86135), and polyclonal rabbit anti-LAMP1 (#ab24170) from Abcam.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

RNA isolation was performed with TRIzol reagent (Invitrogen) according to the manual instructions. 2 µg total RNA was retro-transcribed with the SuperScript First-Strand Synthesis System (Life Technologies) according to the instructions of the manual. Quantitative real-time PCR was performed with SYBR Green Master Mix (Applied Biosystems) using Quant Studio 12K Flex Real-Time PCR System thermocycler (Applied Biosystems). Each reaction was performed in duplicate. The following primers were used for amplification: G3bp1 forward: 5'–CCCCAGCGATGTGCAGAGAG–3'; G3bp1 reverse: 5'–CCCGAGAAACGTCCTCAAGTCG–3'; Rpl13 forward: 5'–CACTTCCTCAAGAG–3'; Rpl13 reverse: 5'–TTGGCCCTGCGGATCT–3'; Psa1 forward: 5'–AGTGAGCGCCAGAATAGAA–3'; Psa1 reverse: 5'–CTTGCGTGGTGCAAGCGTTA–3'; Phgdh forward: 5'–GACC CCCATCTCTCTCTCTGA–3'; Phgdh reverse: 5'–GCCACACTTCTCTTTCTGAC TCTGA–3'; Mthfd2 forward: 5'–CTGAAGTGGGAATACAGTGA–3'; Mthfd2 reverse: 5'–GTCAGGAGAAGCGGATGCG–3'; Hprt forward: 5'–TCTCCTCAGACCGGTGTTT–3'; and Hprt reverse: 5'–TATAACCTGGTGTACCG–3'. Rpl13 was used for normalization in hepatocytes and Hprt in tissues. Fold enrichment was calculated with the formula: 2^(-ΔΔCt).

RNA-seq

Libraries were prepared using the Illumina’s TruSeq® mRNA stranded sample preparation kit. Library preparation started with 1 µg total RNA. After poly-A selection (using poly-T oligo-attached magnetic beads), mRNA was purified and fragmented using divalent cations under elevated temperature. The RNA fragments underwent reverse transcription using random primers, followed by second-strand cDNA synthesis with DNA Polymerase I and RNase H. After end repair and A-tailing, indexing adapters were ligated. The products were then purified and amplified (14 PCR cycles) to create the final cDNA libraries. After library validation and quantification (Agilent 2100 Bioanalyzer), equimolar amounts of library were pooled. The pool was quantified by using the Pqlab KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced by using the Illumina novaSeq6000 instrument and a PE100 protocol. Data were analyzed using QuickNGS (Wagle et al., 2015). Reads were mapped to the human reference assembly, version GRCh38, using TopHat2 (Kim et al., 2013), and gene quantification was carried out using a combination of Cufflinks (Trapnell et al., 2010) and the DEseq2 package (Anders & Huber, 2010) with genomic annotation from the Ensembl database, version 80. The gene lists were filtered according to FC and P-value in comparison of the library-size normalized read counts between samples and controls. We used the DEseq2 package from the Bioconductor project to determine means as well as FC and P-values. Significance was determined using Wald’s test. In contrast, gene expression for the individual samples was calculated by the Cufflinks package and returned as FPKM (fragments per kilobase of transcript per million mapped reads) values.

LC-MS/MS analysis and bioinformatics

Proteins were extracted in 4% SDS followed by acetone precipitation overnight at −20°C. After resuspension in 6 M urea/2 M thiourea, proteins were reduced with DTT and carbamidomethylated with IAA. LysC (Wako) and trypsin (Promega) were added for overnight digestion. Samples were then desalted using SDB-RPS StageTips as previously described (Rappsilber et al., 2003). Proteomic analysis was performed using an Easy nLC 1000 UHPLC coupled to a QExactive Plus mass spectrometer (Thermo Fisher). Peptides were resuspended in Solvent A (0.1% FA) and spiked up with an autosampler, and loaded onto in-house made 50 cm fused silica columns (internal diameter (I.D.) 75 µm, C18 1.7 µm, Dr. Maisch beads) at a flow rate of 0.75 µl/min. A 240-min segmented gradient of 5–34% Solvent B (80% ACN in 0.1% FA) over 215 min, 34–55%
Solvent B over 5 min, and 55–90% Solvent B over 5 min at a flow rate of 250 nl/min was used to elute peptides. Eluted peptides were sprayed into the heated transfer capillary of the mass spectrometer using a nano-electrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in a data-dependent mode, where the Orbitrap acquired full MS scans (300–1,750 m/z) at a resolution (R) of 70,000 with an automated gain control (AGC) target of 3 × 10^6 ions collected within 20 ms. The dynamic exclusion time was set to 20 s. From the full MS scan, the 10 most intense peaks (z ≥ 2) were fragmented in the high-energy collision-induced dissociation (HCD) cell. The HCD normalized collision energy was set to 25%. MS/MS scans with an ion target of 5 × 10^5 ions were acquired with R = 17,500, with a maximal injection time of 60 ms and an isolation width of 2.1 m/z.

The raw files were processed using MaxQuant software and its implemented Andromeda search engine and LFQ algorithm (Cox et al., 2011). Parameters were set to default values. GO annotations, statistical analysis, and Welch t-test were performed using Perseus software (Tyanova et al., 2016). Significant protein fold changes were determined by permutation-based FDR approach (cutoff 0.05, number of permutations = 500, fudge factor [S0] = 0.1) (Tusher et al., 2001). One-dimensional enrichment of GO terms based on log2 fold changes was performed using the algorithm implemented in Perseus with a Benjamini–Hochberg FDR threshold of 0.02, and resulting scores were used for the 2D score plots. Plots were generated using Instant Clue (Nolte et al., 2018).

**Statistical analysis**

Data plotted as histograms correspond to the average values and standard error of the means of different animals or of independent experiments. Data from individual cells are presented as boxplot, showing the median, the first quartile, and the third quartile. To compare two groups, two-tailed paired or unpaired Student’s t-test was performed. When more than two groups were analyzed, one-way ANOVA was performed using Tukey’s post hoc test. To test statistical difference between genotypes at different time points, two-way ANOVA was performed. P-values were calculated using GraphPad Prism software (Version 6.02). Sample size was chosen according to our previous experience. No statistical test was used to predetermined sample size. At least three independent biological replicates were used unless specified otherwise. Values were considered statistically different when P < 0.05.

**Data availability**

The dataset produced in this study are available in the following databases: Proteomics: Data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD014098 (http://www.ebi.ac.uk/pride/archive/projects/PXD014098).

**Expanded View** for this article is available online.

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**Author contributions**

Funding Acquisition, EIR; Conceptualization, DP-M, DS, and EIR; Investigation and Formal Analysis, DP-M, DS, M-CM, and JLW; Resources, SK; Analysis of MS Data, JLW; Visualization, DP-M, DS, M-CM, and JLW; Writing—Original Draft, DP-M, DS, and EIR; Writing—Review & Editing, all authors; and Supervision, MK and EIR.

**Conflict of interest**

The authors declare that they have no conflict of interest.

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