The tumor suppressor LKB1 regulates starvation-induced autophagy under systemic metabolic stress

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Autophagy is an evolutionarily conserved process that degrades cellular components to restore energy homeostasis under limited nutrient conditions. How this starvation-induced autophagy is regulated at the whole-body level is not fully understood. Here, we show that the tumor suppressor Lkb1, which activates the key energy sensor AMPK, also regulates starvation-induced autophagy at the organismal level. Lkb1-deficient zebrafish larvae fail to activate autophagy in response to nutrient restriction upon yolk termination, shown by reduced levels of the autophagy-activating proteins Atg5, Lc3-II and Beclin1, and aberrant accumulation of the cargo receptor and autophagy substrate p62. We demonstrate that the autophagy defect in lkb1 mutants can be partially rescued by inhibiting mTOR signaling but not by inhibiting the PI3K pathway. Interestingly, mTOR-independent activation of autophagy restores degradation of the aberrantly accumulated p62 in lkb1 mutants and prolongs their survival. Our data uncover a novel critical role for Lkb1 in regulating starvation-induced autophagy at the organismal level, providing mechanistic insight into metabolic adaptation during development.
during *C. elegans* dauer (diapause) stage. However, the early embryonic lethality of both *Lkb1* mutant and *Ampkα1/a2* double mutant mice has precluded analysis of the in vivo role of LKB1/AMPK in physiological processes occurring at later developmental stages in vertebrates, such as during metabolic stress at birth.

The LKB1/AMPK axis is a negative regulator of mTOR signaling and mTOR signaling is a known inhibitor of autophagy. However, LKB1 also activates 12 other AMPK-related kinases, and many mTOR-dependent and mTOR-independent autophagy regulators exist.

LKB1/AMPK regulation of mTOR has been linked to the regulation of autophagy in different settings, for example in autophagy stimulated by fluid flow over the primary cilium of epithelial cells, and in cancer cells. Furthermore, AMPK directly stimulates autophagy via the ULK1/Atg1 phosphorylation. And LKB1 may also stimulate autophagy by stabilizing p27, thereby linking nutrient sensing to cell-cycle progression. However, whether and how LKB1 signaling regulates systemic starvation-induced autophagy in vertebrates is currently unknown.

The regulation of systemic metabolism and autophagy are often studied in zebrafish because of its small size and vertebrate physiology. Importantly, fundamental principles of energy homeostasis are highly conserved between humans and zebrafish. Autophagy has critical functions during zebrafish embryonic development, with autophagy-defective animals displaying abnormal heart development, which is also seen in mice. Zebrafish are also a valuable model for studying tissue regeneration, and autophagy has been shown to be required for the regeneration of amputated caudal fins. Like mammals, zebrafish also experience metabolic stress at birth, when the maternal nutrient supply (yolk) is depleted. The metabolic stress at birth in mammals is accompanied by induction of gluconeogenesis and of autophagy. While induction of gluconeogenesis also serves as a mechanism to restore energy homeostasis in zebrafish, a role for autophagy during this metabolic transition has not been investigated.

We previously used TILLING to generate zebrafish mutants that carried a point mutation leading to a stop codon in the kinase domain of *Lkb1* (*stk11hu1968*). These mutants survived gastrulation and early embryonic development but died prematurely from starvation at 7 to 8 days post-fertilization (dpf). Our experiments, impossible to conduct in mice due to the early embryonic lethality of *Lkb1* knock-out mice, established Lkb1 as a critical regulator of whole-body energy homeostasis. *Zebrafish lkb1* (*stk11hu1968*) mutants are unable to cope with the energetic stress induced upon yolk depletion and fail to adapt their metabolism to lower nutrient levels. *lkb1* mutants are indistinguishable from wild type (wt) siblings while their maternal nutrient supply is still present, until 5–6 dpf. However, they die within 1–2 days following yolk depletion whereas wt larvae can survive without food until 13–14 dpf.

The zebrafish *lkb1* mutant phenotype is reminiscent of the *Atg5* knock-out mice that appear normal until birth but die soon after, due to their inability to cope with the metabolic stress at birth. This resemblance prompted us to investigate the autophagy status in the *lkb1* mutants to study the role of Lkb1 in regulation of systemic starvation-induced autophagy.

We show that Lkb1-deficient larvae fail to activate autophagy in response to nutrient restriction. Furthermore, we demonstrate aberrant accumulation of the autophagy adaptor and substrate p62 in *lkb1* mutants, confirming impaired autophagy. Genetic or chemical induction of autophagy in *lkb1* mutants prolongs their survival, while suppression of autophagy shortens it. Survival prolongation only occurs when degradation of p62 is restored. We therefore show that autophagy is essential to survive the feeding-fasting transition in zebrafish, and identify Lkb1 as a critical regulator of whole-body starvation-induced autophagy in vertebrates.

**Results**

**lkb1** mutants fail to activate autophagy under nutrient limitation. To determine if autophagy initiation and maintenance is affected in the *lkb1* mutants we previously generated, we analyzed wt and *lkb1* larvae between 5–7 dpf during the metabolic transition following yolk depletion. *lkb1* mutants are indistinguishable from wt larvae up to day 5–6 dpf (while there is still yolk). We chose this time window also because the mor-tality between 5–7 dpf during the metabolic transition following yolk depletion.

The *lkb1* mutants are indistinguishable from wild type siblings after yolk depletion, which is also seen in mice. *Zebrafish lkb1* (*stk11hu1968*) mutants did not show such an increase, suggesting they fail to activate autophagy upon yolk depletion. *lkb1* mutants die at 8 dpf (Supplementary Fig. S1). Autophagic activity is commonly monitored by accumulation of the autophagy marker Lc3-II and Becn1 expression was also strongly reduced in the *lkb1* mutants, confirming autophagy upon yolk depletion.

In *lkb1* mutants, the expression of the common 56 KD complex was unaffected, the 47 KD complex, which is indicative of autophagy induction, was undetectable in the *lkb1* mutants (Fig. 1A). Finally, we also assessed the levels of Beclin 1 (*Becn1*), a protein involved in autophagosome nucleation, and also commonly used as an autophagy indicator. Beclin 1 expression was also strongly reduced in the *lkb1* mutants (Supplementary Fig. S2B). While *Lc3-I* and *Becn1* levels progressively increased in wt larvae between 5–7 dpf, indicating upregulation of autophagy upon yolk termination, *lkb1* mutants did not show such an increase, suggesting they fail to activate autophagy under nutrient limiting conditions (Fig. 1A, and Supplementary Fig. S2B).

To examine the spatial distribution of autophagy, we performed immunofluorescence analysis with antibodies against Lc3B on transverse liver and intestine sections of *lkb1* and wt larvae at 7 dpf. Lc3B expression was strongly reduced in *lkb1* intestines and livers as compared to their wt counterparts (Fig. 1B–E and Supplementary
Fig. S2E,F), confirming and supporting the immunoblotting results. Furthermore, immunohistochemistry (IHC) against Becn1 on transverse sections of wt and lkb1 mutants showed markedly reduced Becn1 staining in the lkb1 mutants compared to their wt counterparts (Supplementary Fig. S2C,D).

To further confirm that activation of autophagy is impaired in lkb1 mutants, we monitored expression levels of the p62 protein (also known as sequestosome 1, (SQSTM1). p62 is an adaptor protein that targets ubiquitinated proteins or organelles that bind to it for selective autophagy43. Accumulation of p62 has been observed in mouse AMPK-deficient fibroblasts 44, and is associated with liver toxicity in autophagy-deficient mouse liver 45. p62 itself is also an autophagy substrate, thus accumulation of p62 levels is a marker for impaired autophagy46. In agreement with our model that lkb1 mutants have impaired autophagy, Western blot analysis of p62 levels at 5, 6 and 7 dpf showed progressive accumulation of p62 specifically in the lkb1 mutants (Fig. 1A). IHC performed on transverse sections of lkb1 intestine and liver confirmed a marked accumulation of p62 in lkb1 larvae, whereas wt siblings were devoid of staining (Fig. 1F, G).

Collectively, these findings demonstrate that whole-body autophagy is impaired in lkb1 mutants during the feeding-fasting transition in zebrafish, which could contribute to their premature death.

Abrogation of autophagy further decreases survival of lkb1 mutants. To investigate the effect of inhibiting autophagy on lkb1 larvae survival prior to yolk depletion, we blocked autophagosome formation using an antisense morpholino oligonucleotide (MO), that targets the translational start-site of atg5 mRNA28, 47, atg5 MO. We confirmed that injection of atg5 MO abolishes Atg5 protein expression (Supplementary Fig. S3).

Atg5-knockdown led to a reduction in Lc3-II levels compared to the negative control in both wt and lkb1 mutants at 4 dpf, before yolk depletion, confirming autophagy suppression upon atg5 MO injection (Fig. 2A). While all un-injected wt and lkb1 larvae were alive at 4 dpf, a significant number of atg5 MO-injected embryos were found dead at that time point. Genotyping all larvae at 4 dpf revealed that 75% of atg5 MO-injected lkb1 larvae had died compared to only 25% of atg5 MO-injected wt larvae (Fig. 2B). Thus, lkb1 mutants, which fail to induce autophagy at the metabolic transition, are also more sensitive to autophagy inhibition at earlier embryonic stages.

The autophagy defect in lkb1 mutants can be ameliorated by mTOR-dependent and -independent mechanisms. We next investigated the mechanism behind the impaired autophagy observed in the lkb1 mutants. Signaling through mTOR is known to inhibit autophagy, and we and others have previously reported that mTOR activity is high in wt larvae between 2 and 5 dpf and is downregulated at later stages of larval development37, 47, 48. This suggests that at the time of yolk depletion, mTOR activity is switched off, enabling the activation of autophagy. It has also been shown in mice that suppression of mTOR activity at
birth enables activation of autophagy. We hypothesized that mTOR inactivation was defective in the absence of Lkb1. Therefore, we first assessed the status of mTOR signaling in lkb1 mutants at the metabolic transition (6 dpf) by analyzing phosphorylation of the mTOR-substrate ribosomal protein S6 (RS6) by Western blot. Total RS6 levels were almost undetectable in wt larvae at this stage, consistent with our previous report. However, both total and phospho- RS6 levels were high in lkb1 mutants (Fig. 3A), indicating active mTOR signaling, which was inhibited by rapamycin treatment. In comparison, in rapamycin-treated 6 dpf wt larvae, we observed increased phospho-RS6 expression (Fig. 3A). This could be explained by a known developmental delay caused by chronic mTOR inhibition during development. Consistent with this, rapamycin-treated wt larvae retained significant amounts of yolk at 7 dpf, demonstrating a delay in larval development (Supplementary Fig. S4C).

To determine whether mTOR signaling mediates the inhibition of autophagy seen in the lkb1 mutants at 6 dpf, we examined whether rapamycin treatment could restore autophagy in these mutants. We treated wt and lkb1 embryos with rapamycin from 1 dpf onwards. We have previously reported that rapamycin-treated lkb1 larvae survive until 9 dpf, but still have a considerable amount of yolk, demonstrating a developmental delay. Rapamycin treatment leads to increased Lc3-II levels in both wt and lkb1 larvae, while p62 accumulation remained high in rapamycin-treated lkb1 mutants. These results suggest that while mTOR inhibition can at least partially restore autophagy in lkb1 mutant larvae, it cannot entirely alleviate the observed phenotype.
We next analyzed the pro-survival PI3K pathway, which in response to external stimuli (growth factors, insulin) also suppresses autophagy, acting upstream of mTOR signaling. To this end, we used the small molecule AR-12, an inhibitor of phosphoinositide-dependent kinase (PDK)-1, a component of the PI3K pathway, which has been shown to activate autophagy in zebrafish. Treatment of wt and lkb1 siblings with AR-12 from 1 dpf onwards, led to accumulation of Lc3-II protein levels in wt larvae but not in lkb1 mutants. p62 levels remain high in AR-12-treated lkb1 larvae. Furthermore, while p62 protein expression was diminished in wt larvae upon AR-12-mediated activation of autophagy, accumulation of p62 remained unchanged in AR-12-treated lkb1 larvae. This indicates that inhibition of the PI3K pathway fails to induce autophagy in mutant larvae. We next assessed phosphorylation of the mTOR-substrate RS6 upon AR-12 treatment in wt and lkb1 larvae at 6 dpf. RS6 and phosphorylated RS6 (P-RS6) were not detectable in wt larvae at 6 dpf (Fig. 4C), consistent with downregulation of mTOR activity at later developmental stages. AR-12 treatment did not affect the high protein levels of RS6 or P-RS6 seen in the lkb1 mutants. Furthermore, while p62 protein expression was diminished in wt larvae upon AR-12-mediated activation of autophagy, accumulation of p62 remained unchanged in AR-12-treated lkb1 larvae. This indicates that inhibition of the PI3K pathway fails to induce autophagy in mutant larvae. We next assessed phosphorylation of the mTOR-substrate RS6 upon AR-12 treatment in wt and lkb1 larvae at 6 dpf. RS6 and phosphorylated RS6 (P-RS6) were not detectable in wt larvae at 6 dpf (Fig. 4C), consistent with downregulation of mTOR activity at later developmental stages (here and refs 37, 47 and 48). AR-12 treatment did not affect the high protein levels of RS6 or P-RS6 seen in the lkb1 mutants, indicating that they are unsusceptible to PI3K pathway inhibition. In line with the lack of autophagy induction, AR-12 treatment did not enhance lkb1 survival, as no statistically significant differences were observed in the percentage of AR-12-treated lkb1 larvae alive at 9 dpf compared to DMSO-treated controls (Fig. 4D).

Activation of autophagy by an mTOR-independent pathway can be achieved using calpeptin, which inhibits the autophagy inhibitors calpain proteases. Calpeptin treatment of lkb1 and wt embryos from 1 dpf onwards, in the presence or absence of chloroquine, enhanced Lc3-II levels in both wt and lkb1 larvae at 6 dpf (Fig. 5A and Supplementary Fig. S5) without any effects on development or yolk absorption. Calpeptin treatment also resulted in upregulation of the amounts of Atg5 and complexed Atg5 in wt larvae but not in lkb1 mutants. Uncropped images of the blots are shown in Supplementary Fig. S10A,B. Representative Western blot analysis of Ribosomal protein S6 (RS6), Phospho-RS6 and Tubulin (loading control). Protein levels of total RS6 and of P-RS6 were not affected by AR-12 treatment in lkb1 mutants. Uncropped images of the blots are shown in Supplementary Fig. S9A–C. Graph depicting survival percentage of lkb1 larvae alive at 9 dpf. Embryos were treated with 1 μM AR-12 or DMSO from 1 dpf, collected at 9 dpf, and genotyped for the lkb1 gene. Data represent the means ± standard errors of the means (SEM) and are pooled from three independent experiments. P value > 0.05, ns: not statistically significant.
Accumulation of p62 is an important regulator of autophagy in \textit{lkbi} mutants. Aberrant p62 accumulation appeared as a hallmark of impaired autophagy in \textit{lkbi} mutants, and strongly correlated with survival. While p62 is primarily thought of as a receptor delivering cargo proteins to autophagosomes for degradation, it has also been implicated in enhancing mTOR activity\textsuperscript{56}, thereby regulating autophagy as well. Loss of p62 function led to increased autophagy in mammalian cells and \textit{C. elegans}\textsuperscript{56}. We thus set out to determine whether reducing p62 levels in larvae would affect autophagy and survival. To this end, we injected a \textit{sqstm1/p62} MO, targeting splicing of \textit{sqstm1/p62} mRNA\textsuperscript{54}, into 1–2-cell stage embryos. RT-PCR confirmed that the \textit{sqstm1/p62} MO blocked \textit{sqstm1/p62} mRNA splicing until at least 5 dpf (Supplementary Fig. S6). Western blot analysis of 6 dpf larvae showed decreased p62 expression compared to un-injected controls in both wt and \textit{lkbi} lysates (Fig. 6A). This was coupled with increased Lc3-II protein levels, suggestive of autophagy induction. Knockdown of p62 significantly prolonged \textit{lkbi} survival up to 9 dpf: Approximately 70\% of \textit{sqstm1/p62} MO-injected \textit{lkbi} mutants survived to 9 dpf, whereas less than 5\% of un-injected \textit{lkbi} larvae were alive at this time-point (Fig. 6B). Therefore, depleting p62 is sufficient to activate impaired autophagy in \textit{lkbi} mutants and extend survival.

Discussion

Organisms adapt their metabolism in response to nutrient limitation to restore energy homeostasis and ensure survival. Here, we identify a novel link between metabolic adaptation during development and induction and maintenance of autophagy, mediated by the tumor suppressor Lkb1. Specifically, we use metabolically compromised \textit{lkbi}-deficient zebrafish larvae to show that Lkb1 is crucial in the induction of autophagy in response to the metabolic challenge accompanying depletion of the maternal nutrient supply. Our data therefore reveal an essential function for Lkb1 in controlling starvation-induced autophagy at the organismal level in vertebrates. Overall autophagy levels in \textit{lkbi} mutants are lower compared to those of wt siblings: while expression of autophagy–related proteins is progressively upregulated following yolk depletion in wt larvae, induction of autophagy in \textit{lkbi} mutants is strongly attenuated. Importantly, we demonstrate that genetic and chemical manipulation of autophagy levels significantly impacts \textit{lkbi} larvae survival: inducing autophagy by mTOR-dependent and – independent mechanisms prolongs survival, and suppressing autophagy by Atg5 depletion leads to premature death selectively of the mutants. The increased susceptibility of \textit{lkbi} larvae to Atg5 depletion during development...
occurred even while the yolk is not yet consumed, suggesting that even though the larvae do not show a morphological phenotype at this embryonic stage, the loss of Lkb1 appears to sensitize them to additional stress. This stress may be specifically autophagy inhibition, or related to alternative mechanisms, as autophagy-independent functions have been reported for several of the autophagy-related genes57, 58, including Atg559.

Various mechanisms, including mTOR and PI3K signaling, as well as calpains, are known to regulate autophagy54, and likely interact at multiple levels. Indeed, our results, together with published work, indicate that all these influence the energy-sensing defect we observe in \( lkb1 \) mutants. We show that activating autophagy by calpeptin, which inhibits the action of the general autophagy inhibitors calpains55, led to robust upregulation of Atg5 expression and restored degradation of p62 in \( lkb1 \) mutants. Thus, calpeptin fully rescued the autophagy defect of the \( lkb1 \) larvae and prolonged their survival. In contrast, while the mTOR-inhibitor rapamycin increased Lc3-II accumulation in \( lkb1 \) larvae, autophagy was not completely restored since p62 still accumulated. This may be due to the high mTOR activity in the mutants that could not be fully blocked by rapamycin treatment under these experimental conditions. In addition, although rapamycin treatment also prolonged \( lkb1 \) survival, we believe this was likely due to a generalized growth delay, evidenced by the presence of a considerable amount of yolk at 7 dpf (this study and refs 37 and 50), rather than due to partial restoration of autophagy. A developmental delay caused by rapamycin is further supported by the persistence of R56 expression in rapamycin-treated wt larvae at 7 dpf when mTOR would normally be suppressed (mTOR signaling is suppressed in wt larvae upon yolk depletion at 5–6 dpf57, 58).

The autophagy receptor and substrate p62 aberrantly accumulates in \( lkb1 \) mutants indicating deficient autophagy, p62 is also a regulator of autophagy, as it participates in a feed-forward loop in which p62 enhances mTOR activity resulting in reduced autophagy, in turn leading to higher p62 levels in mice60. Here we also show that depletion of p62 in \( lkb1 \) larvae leads to activation of autophagy and prolonged survival. This implies that as the amount of p62 decreases due to autophagosomal clearance, its effect on mTOR activity is also reduced, and thus autophagy can be maintained. Furthermore, the aberrant accumulation of p62 in \( lkb1 \) larvae may in itself contribute to their premature lethality, as it has been shown that increased levels of p62 in autophagy-deficient mouse livers cause hepatotoxicity (reviewed in ref. 60). Further supporting our hypothesis, in apoptosis-impaired tumor cells with deficient autophagy, p62 accumulation triggers a positive feedback loop for the generation of reactive oxygen species (ROS) leading to enhanced genomic instability and tumorogenesis61.

PI3K signaling is a nutrient-sensing pathway that is also implicated in starvation-induced autophagy. Inhibition of the PI3K pathway activated autophagy in wt larvae, but not in \( lkb1 \) mutants, and did not prolong their survival. This is consistent with our previous findings that PI3K signaling is compromised in \( lkb1 \) mutants37. We postulate that defective PI3K signaling may contribute to the autophagy defect seen in these mutants. While AMPK is considered a major regulator of metabolism and has an important role in induction of autophagy under energetic stress52, 53, it is not overtly activated in wt larvae at 7 dpf71; in agreement with these data, studies in mice have also reported that 24 hours of fasting did not lead to significant AMPK activation52, 63. Thus, the autophagy defect we describe in \( lkb1 \) mutants is unlikely to be solely attributable to impaired AMPK signaling, and deregulation of additional pathways, such as PI3K signaling and AMPK/mTOR-independent pathways may also be involved. Hence, nutrient-sensing pathways (like the PI3K pathway) and energy-sensing pathways (like the AMPK pathway) are likely in close cross-talk with each other, not only through their convergence on mTOR signaling but also through different, mTOR-independent mechanisms.

Together, our data indicate that Lkb1 plays an important role in the regulation of autophagy at the whole-organism level, and confirm that autophagy is critical for survival during the metabolic transition in development.
development. Since defects in autophagy are implicated in a plethora of diseases, a better understanding of the upstream regulatory pathways could provide new insights into their pathophysiology.

Materials and Methods
Zebrafish strains and Screening Methods. Zebrafish were handled in compliance with the local animal welfare regulations and were maintained according to standard protocols (zfin.org). Their culture was approved by the local animal welfare committee (DEC) of the University of Leiden and all protocols adhered to the international guidelines specified by the EU Animal Protection Directive 2010/63/EU. Genotype analysis for lkb1 mutants embryos was performed as previously described35.

Longitudinal analysis of survival of lkb1 mutants. Larvae obtained from single matings of heterozygous lkb1 adults were analyzed over time. 48–95 larvae were genotyped on 6, 7, 8, 9, 10 and 11 dpf to assess the numbers of lkb1 mutants alive.

Western Blot analysis. Approximately 20 larvae/sample were lysed (3 µl per larva) in cold lysis buffer (50 mM Hepes, pH 7.6, 50 mM KCl, 50 mM NaF, 5 mM NaPpi, 1 mM EGTA, 1 mM EDTA, 1 mM beta-Glycerophosphate, 1 mM DTT, 1 mM Vanadate, 1% NP40) containing phosphatase and proteinase inhibitors. Lysates were pelleted for 5 min, sonicated for 30 seconds at 30 seconds intervals for 5 min and centrifuged at 13,000 rpm for 15 min at 4 °C to pellet nuclei and cell debris. Protein lysates were boiled for 10 min and BCA assay was performed to measure protein concentration. Samples containing 12–30 µg of protein were heated at 95 °C for 5 min with 4 × Bolt LDS sample buffer (Thermofisher, #B0007), supplemented with 5% beta-mercaptoethanol, and loaded onto a 12% Bis-Tris plus gel (Thermofisher, #NW00172). The protein marker used was Precision Plus Protein® Dual Color Standards, #1610374 (BioRad). Proteins were transferred onto a nitrocellulose membrane (Thermofisher, #88018) using a wet transfer system (Bio-Rad) according to manufacturer’s instructions. Subsequent blocking and antibody incubation were performed in 5% skinned milk powder (#115363, Merck Millipore) in PBS containing 0.1% Tween-20. For the anti-p62 antibody, blocking was performed in 10% milk powder and antibody incubation in 1% milk powder in PBS containing 0.1% Tween-20. Antibodies used were: rabbit anti-LC3B (1:1000, Abcam, #ab51520), rabbit anti-p62 (1:1000, MBL, #PM045), rabbit anti-BECN1 (1:500, Santa Cruz, #sc-11427), rabbit anti-H3 (1:5000, Santa Cruz, #sc-10809), mouse anti-beta-actin (1:5000, Sigma, #A5441), mouse anti-Tubulin (1:500, Sigma, #T9026), rabbit anti-Atg5 (1:500, Novus, #NB110-53818). Secondary antibodies used were goat Anti-Mouse IgG (H + L)-HRP (1:10,000, BioRad, #1721011) and Goats Anti-Rabbit IgG (H + L)-HRP Conjugate (1:10,000, BioRad, #17210191). Membranes were developed using ECL (BioRad, #1705060), followed by chemiluminescence detection with a gel doc system (BioRad).

Immunohistochemistry and Immunofluorescence. For transverse sections, larvae were fixed in 40% ethanol, 5% acetic acid and 10% formalin for 3 h at room temperature followed by three washes in 70% ethanol before being dehydrated following serial washes in Histoclear and reducing ethanol concentrations. Larvae were then sectioned at 5 µm intervals using a Reichert-Jung 2050 microtome (Leica). Sections were deparaffinized and hydrated following by 20 min of antigen retrieval in sodium citrate buffer pH 6.0 at 100 °C. Sections were blocked in 5% BSA in PBS – 0.1% Tween-20 for 1 h at room temperature and incubated overnight with sheep anti-p62 (1:200, Abcam, #ab31545) and rabbit anti-BECN1 (1:150, Santa Cruz, #sc-11427). Endogenous peroxidase activity was blocked in 0.3% H2O2, for 20 min at room temperature followed by incubation with rabbit anti-sheep antibody (1:800, Abcam, #ab6747) for 1 h at room temperature. Sections were incubated with 0.1 M imidazole prior to detection with 3,3′-diaminobenzidine (DAB) substrate and counterstaining with hematoxylin.

For immunofluorescence, larvae were fixed in 4% PFA overnight at 4 °C, embedded vertically in a 0.5% gelatin/30% albumin mixture and sectioned at 120 µm intervals using a VT1000S vibratome (Leica). Sections were transferred to the wells of a 24-well plate containing PBD (PBS + 0.1% Tween-20 and 0.5% Triton-X-100), which was then replaced with blocking solution (PBD + 1% BSA) for 1 h at RT. Sections were incubated with rabbit anti-LC3B (1:1000, Abcam, #ab51520) in blocking solution overnight at 4°C. Sections were washed three times for 15 min in PBS-0.1% Tween-20 and incubated with secondary anti-rabbit 488 green fluorescent antibody (1:100, Thermofisher, #A11008) for 2 h at room temperature. Sections were then washed three times for 15 min in PBS-0.1% Tween-20 prior to be incubated with phalloidin-Alexa 588 (1:25, Thermofisher, #A12380) and DAPI (1:200, Thermofisher, #62248) for 30 min at room temperature in the dark and rinsed three times for 5 min with dH2O. Sections were then imaged using the Zeiss LSM5 Exciter confocal laser-scanning microscope.

Equipment and settings. For immunohistochemistry, the sections were imaged on an upright compound Nikon Eclipse E800 microscope. The images were captured using a Nikon Digital Sight camera unit, equipped with a DS-Fi1 digital camera head and a DS-L2 camera controller. Pixel dimensions of the acquired images were W2584 × H1936 pixels, at 150 pixels/inch.

The magnification used was either 40 ×/0.75 magnification for anti-p62 staining (Fig. 1) or 100 ×/1.4 magnification for anti-Becn1 staining (Supplementary Fig. S2).

The images were processed using Photoshop CS6 software. The original images were scaled-down constraining proportions, and cropped to the area of interest. Adjustment of Image Levels was applied on whole images. Assembly of the composite figures and labeling was done on Illustrator CC2015.

Confocal images were obtained in a sequential manner using a Zeiss LSM5 Exciter Confocal Laser Scanning Microscope equipped with Argon (458, 488, 514 nm), and 405, 450 and 635 diode excitation lasers and a 40 × water immersion objective (C-APOCHROMAT 40 ×/1.2 Water). Emission ranges were set at 420–480, 505–550 and 560–615 nm in separate channels to prevent bleeding. Images were obtained using the Leica application X software (Leica, Wetzlar, Germany) and post-acquisition data analysis was performed using ImageJ software.
Morpholino injections. Translation-blocking morpholino (MO) directed against atg5 (CATCCCTGTCATCTGCGCATTATCAT) was obtained from Gene-Tools. The splice-blocking MO against Sgstm1/p62 (CTTCACTTAGAGGAAAGTTGCAGA) was a kind gift from Prof. AM Meijer. Splice efficiency of sgstm1 mRNA was tested in RT-PCR using a specific primer-set (Forward primer: 5′-ATTTGACGAAAAGTGCCTC-3′; Reverse primer: 5′-AGTGACGGGAAAACCCAGGAA-3′). Embryos were injected at the 1–2-cell stage with either 2 ng (atg5) or 4 ng (Sgstm1/p62) of MO.

Drug treatments. Wild type or lkb1 mutant zebrafish embryos were treated from 1 dpf in embryo-medium at 28°C with either of the following treatments: 50 μM calpeptin (Abcam, #ab120804), 1 μM AR-12 (Medkoo Biosciences, #200272), or 10 μM rapamycin (Sigma, #R0395). Stock solutions of AR-12, rapamycin and calpeptin were prepared in DMSO and diluted in embryo medium for treatment (final concentration of DMSO, 0.2%). Other treatments were prepared in embryo medium. All treatments were refreshed every 2–3 days, larvae collected at the specified time points and genotyped for the lkb1 gene. For Western Blotting, embryos were exposed to 2,5 μM chloroquine (Sigma, #C6628) for 14 h prior to lysing.

Statistics and quantification. Statistical significance was determined using Fisher’s exact test in GraphPad software. Error bars represent the means ± standard errors of the means (SEM) and are pooled from a minimum of two independent experiments. A p-value of <0.05 was used to define statistical significance.

References

1. Rabinowitz, J. D. & White, E. Autophagy and metabolism. Science 330, 1344–1348 (2010).
2. Kuma, A. et al. The role of autophagy during the early neonatal starvation period. Nature 432, 1032–1036 (2004).
3. Scott, R. C., Schuldmier, O. & Neufeld, T. P. Role and regulation of starvation-induced autophagy in the Drosophila fat body. Developmental cell 7, 167–179 (2004).
4. Kang, C., You, Y. J. & Avery, L. Dual roles of autophagy in the survival of Caenorhabditis elegans during starvation. Nature reviews. Molecular cell biology 13, 251–262 (2012).
5. Shaw, R. J. et al. The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell 6, 91–99 (2004).
6. Lagrange, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
7. Polak, P. & Hall, M. N. mTOR and the control of whole body metabolism. Nature reviews. Molecular cell biology 9, 1032–1036 (2004).
8. Rabinowitz, J. D. & White, E. Autophagy and metabolism. Science 330, 1344–1348 (2010).
9. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
10. Polak, P. & Hall, M. N. mTOR and the control of whole body metabolism. Current opinion in cell biology 21, 209–218 (2009).
11. Shaw, R. J. et al. The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci USA 101, 3329–3335 (2004).
12. Hawley, S. A. et al. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. Journal of biology 2, 28 (2003).
13. Liang, J. et al. The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nature cell biology 9, 218–224 (2007).
14. Narbonne, P. & Roy, R. Caenorhabditis elegans dauers need LKB1/AMPK to ration lipid reserves and ensure long-term survival. Nature 457, 210–214 (2009).
15. Ylikorkala, A. et al. Vascular abnormalities and deregulation of VEGF in Lkb1-deficient mice. Science 293, 1323–1326 (2001).
16. Laderoute, K. R. et al. S5′ AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol 26, 5336–5347 (2006).
17. Mizushima, N., Levine, B., Cuervo, A. M. & Klionsky, D. J. Autophagy fights disease through cellular self-digestion. Nature 451, 1069–1075 (2008).
18. Lizzano, J. M. et al. LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. The EMBO journal 23, 833–843 (2004).
19. Kroemer, G., Marino, G. & Levine, B. Autophagy and the integrated stress response. Molecular cell 40, 280–293 (2010).
20. Orhon, L. et al. Primary-cilium-dependent autophagy controls epithelial cell volume in response to fluid flow. Nature cell biology 18, 657–667 (2016).
21. Mairri, M. C. et al. Control of autophagy by oncogenes and tumor suppressor genes. Cell Death Differ 16, 87–93 (2009).
22. Sheng, D., Kim, J., Shaw, R. J. & Guan, K. L. The autophagy initiating kinase ULK1 is regulated via opposing phosphorylation by AMPK and mTOR. Autophagy 7, 643–644 (2011).
23. Kim, J., Kundu, M., Violett, B. & Guan, K. L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nature cell biology 13, 132–141 (2011).
24. Schlegel, A. & Stainier, D. Y. Lessons from “lower” organisms: what worms, flies, and zebrafish can teach us about human energy metabolism. PLoS Genet 3, e198 (2007).
25. Varga, M., Fodor, E. & Vellai, T. Autophagy in zebrafish. Methods 75, 172–180 (2015).
26. Schlegel, A. & Gurt, P. Metabolic insights from zebrafish genetics, physiology, and chemical biology. Cellular and molecular life sciences: CMLS (2015).
27. Seth, A., Steemple, D. L. & Barroso, I. The emerging use of zebrafish to model metabolic disease. Disease models & mechanisms 6, 1080–1088 (2013).
28. Hu, Z., Zhang, J. & Zhang, Q. Expression pattern and functions of autophagy-related gene atg5 in zebrafish organogenesis. Autophagy 7, 1514–1527 (2011).
29. Lee, E. et al. Autophagy is essential for cardiac morphogenesis during vertebrate development. Autophagy 10, 572–587 (2014).
30. Meyer, G. et al. The cellular autophagy markers Beclin-1 and LC3B-II are increased during reperfusion in fibrillated mouse hearts. Current pharmacological design 19, 6912–6918 (2013).
31. Varga, M. et al. Autophagy is required for zebrafish caudal fin regeneration. Cell Death Differ 21, 547–556 (2014).
32. Gustafsson, J. Neonatal energy substrate production. The Indian journal of medical research 130, 618–623 (2009).
33. Pilkis, S. J. & Ganner, D. K. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annual review of physiology 54, 885–909 (1992).
34. Heintz, N. Developmental biology: survival by self-digestion. Nature 432, 963 (2004).
35. Gut, P. et al. Whole-organism screening for gluconeogenesis identifies activators of fasting metabolism. Nature chemical biology 9, 97–104 (2013).
36. Wiendehols, E. et al. Efficient target-selected mutagenesis in zebrafish. Genome Res 13, 2700–2707 (2003).
37. van der Velden, Y. U. et al. The serine-threonine kinase LKB1 is essential for survival under energetic stress in zebrafish. Proc Natl Acad Sci USA 108, 4358–4363 (2011).
38. Daouk, T. et al. Long-term food-exposure of zebrafish to PCB mixtures mimicking some environmental situations induces ovary pathology and impairs reproduction ability. Aquatic toxicology 105, 270–278 (2011).
39. Sugawara, K. et al. The crystal structure of microtubule-associated protein light chain 3, a mammalian homologue of Saccharomyces cerevisiae Atg8. Genes Cells 9, 611–618 (2004).
40. Sintini, T. & Klionsky, D. J. Autophagy in health and disease: a double-edged sword. Science 306, 990–995 (2004).
41. Mizushima, N. & Yoshimori, T. How to interpret LC3 immunoblotting. Autophagy 3, 542–545 (2007).
42. Mizushima, N. & Klionsky, D. J. Protein turnover via autophagy: implications for metabolism. Annual review of nutrition 27, 19–40 (2007).
43. Lamark, T., Kirkin, V., Dikic, I. & Johansen, T. NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. Cell Cycle 8, 1986–1990 (2009).
44. Egan, D. F. et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456–461 (2011).
45. Komatsu, M. et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nature cell biology 12, 213–223 (2010).
46. Rusten, T. E. & Stenmark, H. p62, an autophagy hero or culprit? Nature cell biology 12, 207–209 (2010).
47. Boglev, Y. et al. Autophagy induction is a Tor- and Tp53-independent cell survival response in a zebrafish model of disrupted ribosome biogenesis. PLoS Genet 9, e1003279 (2013).
48. Marshall, K. E., Tomasini, A. J., Makky, K. S., N. K. & Mayer, A. N. Dynamic Lkb1-TORC1 signaling as a possible mechanism for changes in AMPK activity. American journal of physiology. Endocrinology and metabolism 298, E1032–1037 (2004).
49. Efeyan, A. Regulation of mTORC1 by PI3K signaling. Trends in cell biology 13, 713–723 (2013).
50. Makky, K., Tekiela, J. & Mayer, A. N. Target of rapamycin (TOR) signaling controls epithelial morphogenesis in the vertebrate intestine. Dev Biol 303, 501–513 (2007).
51. Chittaranjan, S., Bortnik, S. & Gorski, S. M. Monitoring Autophagic Flux by Using Lysosomal Inhibitors and Western Blotting of Endogenous MAP1LC3B. Cold Spring Harb Protoc 2015, 743–750 (2015).
52. Dibble, C. C. & Cantley, L. C. Regulation of mTORC1 by PI3K signaling. Trends in cell biology 25, 545–555 (2015).
53. Chiu, H. C. et al. Eradication of intracellular Francisella tularensis in THP-1 human macrophages with a novel autophagy inducing agent. Journal of biomedical science 16, 110 (2009).
54. van der Vaart, M. et al. The DNA damage-regulated autophagy modulator DRAM1 links mycobacterial recognition via TLP-MYD88 to autophagic defense. Cell host & microbe 15, 753–767 (2014).
55. Williams, A. et al. Novel targets for Huntington’s disease in an mTOR-independent autophagy pathway. Nature chemical biology 4, 295–305 (2008).
56. Makkly, K., Tekiela, J. & Mayer, A. N. Target of rapamycin (TOR) signaling controls epithelial morphogenesis in the vertebrate intestine. Dev Biol 303, 501–513 (2007).
57. Subramani, S. & Malhotra, V. Non-autophagic roles of autophagy-related proteins. Nature cell biology 15, 713–723 (2013).
58. Yousefi, S. et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nature cell biology 8, 1124–1132 (2006).
59. Moscat, J. & Diaz-Meco, M. T. Feedback on fat: p62-mTORC1-autophagy connections. Autophagy 3, 207–209 (2007).
60. Gonzalez, A. A. Regulation of mTORC1 by PI3K signaling. Trends in cell biology 25, 545–555 (2015).
61. Moscat, J. & Diaz-Meco, M. T. Feedback on fat: p62-mTORC1-autophagy connections. Autophagy 3, 207–209 (2007).
62. Williams, A. et al. Novel targets for Huntington’s disease in an mTOR-independent autophagy pathway. Nature chemical biology 4, 295–305 (2008).
63. Duran, A. et al. p62 is a key regulator of nutrient sensing in the mTORC1 pathway. Molecular cell 44, 134–146 (2011).
64. Subramani, S. & Malhotra, V. Non-autophagic roles of autophagy-related proteins. EMBO Rep 14, 143–151 (2013).
65. Boya, P., Randi, F. & Codogno, P. Emerging regulation and functions of autophagy. Nature cell biology 15, 713–723 (2013).
66. Youssef, S. et al. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. Nature cell biology 8, 1124–1132 (2006).
67. Moscat, J. & Diaz-Meco, M. T. Feedback on fat: p62-mTORC1-autophagy connections. Cell 147, 724–727 (2011).
68. Moscat, J. & Diaz-Meco, M. T. p62 at the crossroads of autophagy, apoptosis, and cancer. Cell 137, 1001–1004 (2009).
69. Gonzalez, A. A. et al. Metabolic adaptations to fasting and chronic caloric restriction in heart, muscle, and liver do not include changes in AMPK activity. American journal of physiology. Endocrinology and metabolism 287, E1032–1037 (2004).
70. Viollet, B. et al. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta physiologica 196, 81–98 (2009).