

Accepted Manuscript

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PII: S1046-2023(14)00397-1

DOI: <http://dx.doi.org/10.1016/j.ymeth.2014.12.004>

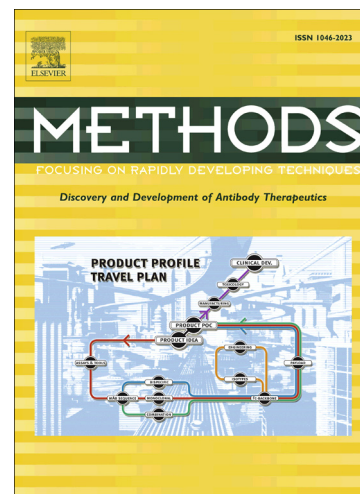
Reference: YMETH 3563

To appear in: *Methods*

Received Date: 1 October 2014

Revised Date: 30 November 2014

Accepted Date: 1 December 2014



Please cite this article as: M. Varga, E. Fodor, T. Vellai, Autophagy in zebrafish, *Methods* (2014), doi: <http://dx.doi.org/10.1016/j.ymeth.2014.12.004>

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Autophagy in zebrafish

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The authors declare no competing interest.

The number of Figures: 2

The number of Tables: 3

The number of words in the main text: 4441

Keywords: autophagy, zebrafish, morpholinos, reporter lines, small molecules, regeneration

Abstract

From a hitherto underappreciated phenomenon, autophagy has become one of the most intensively studied cellular processes in recent years. Its role in cellular homeostasis, development and disease is supported by a fast growing body of evidence. Surprisingly, only a small fraction of new observations regarding the physiological functions of cellular “self-digestion” comes from zebrafish, one of the most popular vertebrate model organisms. Here we review the existing information about autophagy reporter lines, genetic knock-down assays and small molecular reagents that have been tested in this system. As we argue, some of these tools have to be used carefully due to possible pleiotropic effects. However, when applied rigorously, in combination with novel mutant strains and genome editing techniques, they could also transform zebrafish into an important animal model of autophagy research.

Abbreviations:

3MA – 3-methyladenin, AC – adenylyl cyclase, AMPK – adenosine monophosphate-dependent kinase, Atg – Autophagy related gene, BafA1 – Bafilomycin A1, CMV – cytomegalovirus, CQ – chloroquine, dpa – days post amputation, dpf – days post fertilization, ERK – extracellular-signal-regulated kinase, FGFR – Fibroblast Growth Factor Receptor, GFP – green fluorescent protein, Gs α – G-stimulatory protein α , HD – Huntington’s Disease, I1R – Imidazolin-1 Receptor, IGF1R – Insulin-like Growth Factor-1 Receptor, IP3 – inositol 1,4,5-trisphosphate, LC3 – microtubule-associated protein Light Chain 3, LT – LysoTracker, MEK – mitogen activated protein kinase kinase, MO – morpholino, NAC – *N*-acetyl cysteine, PIP2 – phosphatidylinositol 4,5-bisphosphate, PLC- ϵ – Phospholipase C- ϵ , PPP – picropodophyllin, ROS – reactive oxygen species, TEM – transmission electron microscopy, TOR – kinase Target of Rapamycin

1. Introduction

Regulation of cellular homeostasis is of central importance for all eukaryotic organisms. This involves the continuous elimination of cellular damage including misfolded, oxidized and aggregated proteins, the remodeling of the cytoplasmic compartment according to the current needs of the cell, and provision of nutrients to support basic cellular functions. Autophagy, the process of “self-digestion”, is one of the central molecular mechanisms that maintain cellular homeostasis and ensure macromolecule turnover [1, 2]. During different forms of autophagy, parts of the cytoplasm are delivered to the lysosome for degradation. Macroautophagy (hereafter referred to as autophagy) involves the engulfment of cytoplasmic compartments into an intermediate, double membrane-bound organelle, the autophagosome, which later fuses with a lysosome, in which the cargo is eventually degraded by hydrolytic enzymes [1-3].

Advances in recent years have revealed the importance of autophagy in a wide range of physiological and pathological phenomena, from development to neurodegenerative diseases and cancer, from immunity to stem cell maintenance, regeneration and aging [4-16].

In parallel with the increased interest in autophagy, the past couple of years have also seen an explosion in zebrafish research [17]. Advantages of this vertebrate model system (such as small size, external fertilization, transparency, and great regeneration capabilities) have made it previously a prime subject of developmental studies, but recently it has been used with remarkable success to study human pathogenesis [18, 19]. This has been made possible by the fact that zebrafish is an ideal model organism for high throughput screening of chemical libraries (several compounds discovered this way are currently in clinical trials) [20], and by the availability of a wide range of transgenic and mutant strains [21]. Despite several successful forward genetic screens [22], for several years the sophistication of reverse genetic tools available in zebrafish was far inferior to the ones regularly used in other model organisms. However, in the past few years the gap has been closing fast. Highly efficient transgenesis techniques have been developed, and subsequently used for enhancer- and gene-trap screens [23, 24]. The wide array of enhancer trap Gal4 and CreERT2 lines created this way opened the possibility for intricate tissue and/or cell-type specific genetic manipulations [25-27]. Furthermore, the revolution in novel genome editing and targeted gene regulation techniques, based on Transcription Activator-Like Effectors Nucleases (TALENs) [28, 29] and the Clustered Regularly Interspaced Palindromic Repeats (CRISPR) system [30], was promptly implemented in zebrafish, too [31-37].

Interestingly, research in autophagy has been one of the few areas, where the full potential of the zebrafish model has not been yet exploited. This will probably change in the coming years: several people are advocating for such research [38, 39], and as zebrafish proteins involved in autophagy are highly similar to their human counterparts (~73% identical, and over 80% similar), it is likely the results obtained in zebrafish will be relevant in humans as well. Some recent results already

demonstrate [14, 40-42] that further studies of autophagy in zebrafish could result in profound novel insights into the physiological role of this fascinating cellular mechanism.

1.1 The core autophagic machinery

Autophagy-related (Atg) proteins and their cellular cofactors are involved in the induction of the phagophore, its expansion to an autophagosome, and finally the latter's fusion with the lysosome [1, 2, 8]. A detailed review of the molecular machinery driving autophagy is beyond the scope of this article (those interested in see [1, 2]), therefore we will discuss mainly those genes that have been targeted in zebrafish by genetic or pharmacological methods.

Upon induction, the serine-threonine kinase Ulk1 phosphorylates Beclin-1 and Ambra-1, two components of a Class III Phosphoinositide 3-kinase (PI3K) complex. The phosphorylation results in the translocation of the complex (which also contains Atg14L, Vps15/Pik3r4 and Vps34/Pik3c3) to the precursor of the phagophore, the isolation membrane. There, it will induce reactions essential for membrane elongation and closure: first the formation of an Atg5/Atg16/Atg12 complex which then regulates the covalent attachment of phosphatidylethanolamine (PE) to Lc3 and the orthologous Gabarap [1, 2, 43].

The ubiquitin-like Lc3 proteins are encoded by the *microtubule-associated light chain 3 (map1lc3)* orthologs, and are the vertebrate homologs of the yeast Atg8. They are synthesized in a precursor form (pro-Lc3), which is processed by Atg4 and Atg7 (Lc3-I), and after being covalently bound to PE (Lc3-II), it is attached to the phagophore membrane to catalyze membrane elongation [1, 2].

As functional disruption of these proteins often results in severe defect in the autophagic process, they are targeted in different research paradigms, aimed to understand the biological roles of the cellular self-digestion process. Furthermore, as Lc3 is an integral component of the autophagosome membrane, green fluorescent protein (GFP) fused with Lc3 is often used to investigate autophagy, using fluorescent microscopy (see below) [3].

It is important to note that, although for a long time autophagy was considered a non-selective process, recent results suggest that specific adaptor proteins, such as p62/Sqstm1, NBR1, NDP52 or optineurin, can specifically target polyubiquitinated cargo to the autophagosome [44-46]. The substrates of these adaptors can range from misfolded protein aggregates to cytosolic bacteria, such as *Shigella* or *Salmonella*, to damaged mitochondria, making these adaptor proteins important players in the regulation of cellular homeostasis and innate immunity.

After membrane closure, the double membrane-bound autophagosome is destined to fuse with the lysosome to form the autolysosome. In the autolysosome, degradation of both the inner membrane and luminal cargo occurs [1-3]. Finally, autolysosomal degradation products, e.g. amino acids and sugars,

are transported to the cytoplasm by lysosomal efflux transporters, such as Spinster homolog-1 (Spns1). This last step is essential for the correct regulation of autophagy, as if it is impaired, lysosome homeostasis cannot be restored, and reactivation of the Target of Rapamycin Complex 1 (TORC1) following starvation will also be delayed [47].

1.2 Regulation of autophagy by major signaling pathways

Autophagy is not only a degradation pathway, but in certain stress conditions increased autophagic activity helps the cells to counteract with stressors (external or internal agents that cause stress) and adapt to environmental changes. Throughout these functions, autophagy plays a role in various processes including development, cancer suppression, and antigen presentation. As so, autophagy is tightly regulated and balanced by distinct regulatory (genetic) pathways. Considering that autophagy is a highly conserved mechanism, it is likely that the main regulators are same in different model organisms. However, here we would give an overview of only those pathways that have been tested experimentally to play a role in the induction of autophagy in zebrafish.

One of the most intensely studied regulatory mechanisms of the autophagic process is the Target of Rapamycin (TOR) signaling pathway. The mammalian TOR (mTOR) kinase can form two distinct complexes, mTORC1 and mTORC2, with somewhat distinct cellular functions [48, 49]. The mTORC1 complex is known to be the main sensor of cellular nutrient status and a potent inhibitor of autophagy by directly targeting the Ulk1 kinase [2, 49, 50]. Several upstream pathways converge on mTORC1, transducing information from growth factor receptors (e.g. through the IGFR-PI3K-Akt signaling pathway) and the cells' actual energy/nutrient levels. As autophagy is blocked by mTORC1, inhibition of the complex quickly results in increased autophagic flux, allowing fast adaptation to stress signals. Blockage of the Ulk-complex's activity can be resolved by starvation (low ATP/AMP levels through AMPK signaling) or deprivation of amino acids. The same stimulatory effect on autophagy can also be triggered by the lack of growth factors.

Recently, handful of new autophagy inhibitors were tested in zebrafish, targeting the TOR independent cAMP-PLC- ϵ -inositol 1,4,5-trisphosphate (IP3) pathway and the Ca^{2+} -calpain-G-stimulatory protein α (G α) pathway [51-53]. The most upstream candidates of these loop-forming pathways are the Imidazolin-1 (I1R) receptor and L-type Ca^{2+} channels. Increased Ca^{2+} influx through the latter will activate the protease Calpain, which can modulate autophagic activity at least in two different ways regarding Ca^{2+} /Calpain/cAMP signaling: first, it can cleave Atg5, thereby directly inhibiting phagophore formation [54]; second, it can also activate G α [51]. Activation of G α leads to elevated IP3 levels through the adenylyl cyclase (AC)-cAMP - PLC- ϵ pathway. It is not yet fully known how IP3 conducts an inhibitory effect on autophagy: however, one possibility could be that it

can elevate intracellular Ca^{2+} levels by increasing its release from the endoplasmatic reticulum. This cycle can be modulated by the IIR: by lowering cAMP levels, activation of this receptor has a stimulating effect on autophagy [51].

A less-studied, but important activator of autophagy is the Ras/Raf/MAP-ERK pathway. This pathway can have various inputs, among others from AMPK, ROS and FGF signaling ([55]). Experimentally manipulating activity of upstream members of the pathway showed that ERK might modulate autophagy at the maturation step [56], but links to Bcn1 and G-protein-type regulators have also been proposed [57, 58]. However, the direct link between ERK and autophagosome formation remains to be elucidated. Importantly, through FGF signaling this pathway could be one of the links between autophagy and development/regeneration [14].

2. Detection of autophagy in zebrafish

2.1 Transmission electron microscopy (TEM)

TEM is the classical method to detect autophagic structures within eukaryotic cells [3] (Fig. 1A, B). It has been successfully used in zebrafish too, detecting such structures in diverse tissues: in embryos as early as 10 somite stage [59], in the yolk of 5 dpf old embryos [60], in the photoreceptors of *synj1* mutants [61] and the skeletal muscle of *mtmr14/mtm1* double morphants [62], in the intestinal epithelium of *titania* (*titi*) mutant larvae [63], or in the regenerating caudal fin [14]. Ultrastructural analysis also demonstrated the autophagic clearance of the pathogens *Mycobacterium marinum* and *Shigella flexneri* in infected embryos [41, 42, 64].

2.2 Lc3-positive vesicle counting

Although TEM studies are considered the most reliable demonstration of autophagic activity, correct interpretation of TEM data can be difficult and occasionally TEM can produce methodological artefact. Therefore, TEM is often used in combination with other detection methods. One widely used approach in the field is the quantification of Lc3 positive punctae [3]. Immunostaining can be useful for these studies [41, 63], but fluorescently-tagged versions of Lc3 are more popular as they allow for *in vivo* observations. In these chimeric proteins, GFP or mCherry coding sequence is attached usually to the N-terminal of Lc3 (Fig. 1C, D). As increase in autophagy is usually marked by an increase in Lc3-positive vesicles, a significant change in the number of fluorescent punctae is usually interpreted as a change in autophagic activity.

In zebrafish, several transient expression methods and stable transgenic lines have been recently developed to observe Lc3-positive punctae (Table 1). For example, in fish cell lines stably transfected with pGFP-MAP1-LC3 plasmids, a marked increase of green punctae can be observed upon starvation [65]. Similarly, in line with previous results in other animals, in muscle fibers of embryos injected

with a *hsp70l:RFP-Lc3* reporter, a decrease in fluorescent punctae can be seen when *ambra1a* or *ambra1b* function is impaired [66]. Injection of mRNA encoding mCherry-Lc3 was also successfully used to demonstrate the defect of the autophagic pathway in *tti* mutants [63].

A number of recently created stable transgenic lines have been also widely used to detect autophagy. The Tg(*CMV:GFP-Lc3*) and Tg(*CMV:GFP-GABARAP*) transgenic lines were the first specifically developed to study cellular self-digestion in zebrafish [67]. Tg(*CMV:GFP-Lc3*) has been used successfully in a number of other studies [14, 40, 41, 59] (Fig. 1D) and remains one of the most widely used reporter lines in the field. Recently, transgenics with tissue specific GFP-Lc3 expression have also been developed and used to study the role of autophagy in liver (Tg(*fabp10:GFP-Lc3*) [68]) or photoreceptor (Tg(*TaCP:GFP-Lc3*) [61]) function.

To ensure that punctate Lc3 expression does not result only from protein aggregation, several of these reporter lines have been used in combination with lysosomal dyes (e.g. LysoTracker; LT) that accumulate in acidic organelles of living cells, such as the autolysosomes (Fig. 1C). In such assays changes in GFP-Lc3 – LT colocalization can be used to estimate changes in the level of autophagy [40, 61, 67].

These transgenic approaches have also been used to study the autophagosomal targeting of invading bacteria in zebrafish embryos [41, 64], and an increase in Lc3-positive punctae was also observed in zebrafish cell lines infected with rhabdoviruses [69].

Recently, it was observed that GFP is less stable in acidic environments than mRFP or mCherry, therefore tandem mRFP/mCherry-GFP-LC3 fusion proteins can be readily used to estimate autophagic flux in cells (colocalization of GFP and RFP indicates an autophagic compartment that has not fused yet with a lysosome, whereas the loss of GFP signal suggests the transition to autolysosome) [3]. Although transgenic lines carrying similar tandem constructs have not yet been described in zebrafish, double transgenic Tg(*CMV:eGFP-Lc3;mCherry-Lc3*) and Tg(*CMV:eGFP-Gabarap;mCherry-Lc3*) lines have been recently used to demonstrate impaired autophagosome – lysosome fusion in *spns1* mutant larvae [40].

2.3 Antibodies and Western blotting

Another popular method to examine autophagy is the detection of specific autophagic complexes, or estimating autophagic flux from changes in Lc3 levels, using Western blots (Fig. 1E, F). During the past couple of years several commercial antibodies raised against human or mice proteins important during autophagy have been tested and were shown to recognize their fish orthologs (Table 2). Using such antibodies it was shown that Lc3 conversion only occurs in zebrafish after 2 dpf (Fig. 1E), Atg5-specific Western blots were performed to examine the status of the Atg12-Atg5-Atg16 conjugation complex (Fig. 1F) [14, 59], and Bcn-1 levels were examined in *ambra1* knock-down embryos [70].

Observing changes in Lc3 levels can be also used to detect changes in autophagic activity [59, 63, 67, 68, 70-74]. However, it is important to emphasize that an increase in Lc3 II levels can be caused by both an increased flux or an impairment in autolysosome formation [3]. Increased flux is the hallmark of increased autophagy, and to unequivocally detect this it is necessary to follow Lc3 levels in the absence or presence of autophagy inhibitors [67, 68, 75]. A somewhat unrelated, but important use of antibodies in these studies is to verify the translation-blocking effect of ATG-targeting morpholinos (see below) [42, 59, 76].

2.4 Indirect analysis of autophagy by aggregate clearance assays

Autophagy is a major clearance mechanism of intracellular protein-aggregates and damaged organelles, thus it has an essential role in preempting neurodegenerative diseases, such as Huntington's disease (HD) [10] and Parkinson's disease (PD) [77]. Therefore, accumulation of mutant proteins and/or damaged mitochondria in certain neural tissues could be a sign of autophagy impairment.

A few years ago, a zebrafish model of HD was created where an eGFP tagged mutant form of the huntingtin protein (HDQ71) was expressed in photoreceptors [53]. This transgenic line (*Tg(rho:EGFP-HDQ71)*) was successfully used to search for novel HD targets in a Tor-independent autophagy pathway [53] to show the role of antioxidants in regulating basal levels of autophagy [73], and to show that surprisingly IGF-1R antagonism can inhibit autophagy [71].

In another recently published study the authors used a transiently expressed photoconvertible Dendra-tau fusion protein to study the modulatory effect of phosphatidylinositol binding clathrin assembly proteins (PICALM) on autophagic clearance [74]. They also created a transgenic model of tau-mediated neurodegeneration, *Tg(rho:GFP-tau)*, which overexpresses GFP-tagged tau in the photoreceptors [74].

3. Genetic manipulation of autophagy in zebrafish

A nice demonstration about the power of genetic studies comes from research done on two recently described mutants, *titania (tti)*, *rps7* and *spns1*, demonstrating that autophagy has a role in cell survival in zebrafish models of disrupted ribosome biogenesis [63, 78], and its impairment causes embryonic senescence [40]. However, as original forward genetic screens in zebrafish did not yield any mutations in the core autophagy genes, initial loss-of-function studies were performed by injecting synthetic anti-sense morpholino oligonucleotides (MOs) into early embryos. By binding to the ATG site of mature mRNAs, MOs can inhibit translation, or, alternatively, can mask the splice donor/acceptor site of the pre-mRNAs, leading to the formation of misspliced transcripts that will be degraded by nonsense mediated decay. This way, with well-designed MOs, almost perfect

phenocopies of known mutations could be created [79]. Furthermore, recent advances have made possible to covalently link MOs to special delivery moieties that can help the uptake of the MOs by the cells, thus such “vivo-MOs” can be used for targeted delivery into adults, too [80].

In the past few years several studies were published that aimed to unveil the role of autophagy during zebrafish development, regeneration and disease, mostly with the help of MOs. Independent knock-down of several different autophagy related genes resulted in similar phenocopies in early lethality and severe defects of the embryonic development, with shorter and bent trunk, smaller head, pericardial oedema [70], impaired neurogenesis [76], cardiac morphogenesis [59] and skeletal muscle formation [66] (Fig. 1G and Table 3). Using Atg5-vivo-MOs, a role for autophagy during adult caudal fin regeneration was also demonstrated [14], and p62 and Drpm1 depletion resulted in increased bacterial infections and decreased survival in larvae [41, 42]. Optineurin knock-downs have also been created and they show defects in axonal vesicle trafficking [81] and axonopathy [82].

Although most aforementioned embryonic morphant phenocopies seem concordant, they are also somewhat reminiscent of previously described off-target MO effects [83]. And while in most studies the authors have performed mRNA rescue experiments and/or coinjected p53MO to control for such aspecific phenotypes [83], it is important to mention that concerns over the use of MOs have been raised recently [84]. It is especially worrying that in several papers the authors use extremely high MO concentrations to achieve effective knockdowns as this causes a molar excess of MOs versus target mRNAs of several magnitudes (a standard injection of 1 ng MO per embryo results in a 2×10^4 -fold molar excess, in average) [84]. In the absence of *bona fide* mutant phenotypes, the specificity of MO effects cannot be assessed. The use of p53MO might also be problematic in these studies: p53 has several, well characterized, context-dependent roles in autophagy [85], thus its removal from the fish might impair the very process we want to study.

Therefore, it would be desirable to develop better genetic alternatives. Fortunately the list of reverse genetic methods that are available in zebrafish has been recently supplemented with the TALEN and CRISPR/Cas9 systems [25-27]. With such highly efficient and precise targeted genome editing methods, it will be relatively easy to create constitutive and conditional knock-outs in selected autophagy related genes (using the Cre/Lox system or similar technologies), with little or no off-target effects.

Furthermore, in the Zebrafish Mutation Project (ZMP) collection [21] hypomorphic and null-mutant alleles for several autophagy genes (e.g. *ambra1a*, *atg5*, *atg7*, *beclin1* and *ztor*) already exist, and these will be available for the wider community soon. A thorough characterization of these lines should be performed with urgency as it could validate previous MO studies, but it will also generate novel insights that can help us to get a better understanding about the role of autophagy in zebrafish biology. For example, it will be interesting to understand if embryonic lethality observed in specific

mouse *Atg* mutants [2] is an evolutionarily conserved feature of vertebrates, or there is species specific variation in *Atg*-related phenotypes. Also, even if larval lethal, these mutations could be used in assays that follow pathogen and aggregate clearance.

4. Pharmacological modulation of autophagy in zebrafish

The modulation of autophagy by small molecular reagents is currently to most widespread method in use. Broadly speaking, these reagents can be classified as 1.) reagents that induce/increase autophagy, 2.) reagents that decrease autophagy by modulating upstream regulatory processes, and 3.) reagents that impair autophagic function by blocking the autophagosome – lysosome fusion (Fig. 2 and Table 4).

Autophagy inducers can act by mimicking starvation, through blocking the function of TOR, such as rapamycin or torin-1, or through blocking the accumulation of cAMP. This latter can be achieved by L-type Ca^{2+} channel blockers (verapamil), calpain and adenylate cyclase antagonists (calpastatin, calpeptin and 2'5'ddA) or inducing IIR activity (clonidine, rilmenidine). Stress-induced autophagy can be triggered by the increase of reactive oxygen species (ROS), a consequence of AR-12 treatments. Treating zebrafish embryos and larvae with these inducers usually leads to increased autophagic flux and increased aggregate clearance (Table 4).

Autophagy-blocking reagents can act in the pathways that regulate phagophore formation and elongation (Fig. 2 and Table 4). TOR kinase activity can be modulated using antioxidants such as *N*-acetyl cysteine (NAC), that block the accumulation of ROS. High ROS concentrations can either block the Akt/TOR pathway or enhance the accumulation of Beclin 1, leading to the induction of autophagy [73]. L-type Ca^{2+} channels can be induced by (\pm)-BayK8644, leading to the intracellular accumulation of cAMP and IP3 which is inhibitory to phagophore formation [53]. MEK/Erk activity can be inhibited by U0126, leading to decreased number of autophagic structures [14].

Induction of autophagy can also be blocked by 3-methyladenine (3MA) and wortmannin, which impair the activity of the Beclin 1-containing PI3K complex or by pyrinium that leads to the transcriptional downregulation of *Atg* genes [72].

As the autophagosome – lysosome fusion depends on the pH in the lysosomal compartment [86], lysosmotropic agents, such as ammonium chloride (NH_4Cl) or chloroquine (CQ), or vacuolar ATP-ase (V-ATPase) antagonists, such as Bafilomycin A1 (BafA1), omeprazole, lansprazole or pantoprazole can act as blockers of the autophagic function [40, 87]. Finally, autolysosomal degradation can be attenuated using blockers of lysosomal proteases, such as E64d or pepstatin A (Fig. 2) [88].

Although these reagents have been all used with success in zebrafish studies (Table 4), it has to be emphasized that none of them is specific to autophagy. Furthermore, currently there is no consensus on the concentrations these reagents should be used in zebrafish in order to interfere with autophagy.

In studies reviewed here, working concentrations for most reagents vary by several orders of magnitude (Table 4), making it likely that some of the described effects are not specific for interfering with autophagy. Therefore when interpreting phenotypes, it has to be remembered that the use of these drugs in a complex system such as the living zebrafish can cause pleiotropic effects. For example, previous studies in cell cultures already demonstrated that 3MA only acts as an inhibitor of autophagy upon starvation [89, 90]. Otherwise, due to its effect on Class I PI3Ks, it can disrupt the anti-autophagic effect of TORC1 [89]. Due to the contents of their yolk-sac, in the first ~5 days of their life zebrafish larvae are in fully fed condition, thus autophagy cannot be induced by starvation in them. Therefore, to study the effect of 3MA, TOR-signaling was blocked by the use of rapamycin [67]. It is worth to note here that at least in one study basal autophagy was successfully blocked with 3MA in endothelial cells of 3 dpf larvae, without any other treatment [41].

V-ATPases can also have lysosome-independent functions, which will be affected by BafA1 treatment. For example, notochord formation in zebrafish depends on rapidly inflating intracellular vacuoles, and this developmental process is severely disrupted when BafA1 is added to the embryo medium [91].

ERK, IP3 and TORC1 also have several other targets than the other autophagic machinery. This is probably one of the reasons why, contrary to expectations, rapamycin treatment decreases the efficiency of anti-bacterial immune-response [41, 42]. The effects of rapamycin treatment can also be age-dependent in fish [92].

Finally, in some cases attenuation of the known autophagy-controlling pathways can have unexpected effects. Blocking IGF-1R receptor function with picropodophyllin (PPP) inhibited autophagosome formation, although based on our knowledge about the IGF-1R/Akt/TORC1 signaling pathway the opposite result was expected [71].

5. Outlook

Overall, existing studies already establish zebrafish as a powerful vertebrate model to study autophagy. We have several reporter lines that can be used to monitor autophagy with high accuracy in the transparent embryos and a number of genetic tools can be used to disrupt autophagic activity in embryos and adults. Importantly, as most small molecular reagents can be easily dissolved in their medium, zebrafish embryos are ideally suited for the pharmacological manipulation of cellular self-digestion.

Yet, in order to exploit the full potential of this model organism further observations and the development of novel genetic tools will be necessary. For example it is controversial when exactly autophagy starts in the embryo. Ultrastructural studies have detected the presence of autophagosomes

as early as the 10 somite stage [62], yet autophagic flux was not observed before 48 hpf [67]. Also, existing lines often use the CMV promoter to drive the expression of GFP-Lc3 in fish, yet it is not clear if the activity of this promoter is affected by the different experimental manipulations or not.

Similarly to other vertebrates, the zebrafish genome contains three *map1lc3* orthologs [75]. As previous experiments suggested that these could have different, or, indeed, antagonistic functions [93], it will be important to create separate reporter lines for them to dissect their exact role in autophagy.

With the availability of novel genome editing tools, precise and/or conditional disruption of *Atg* genes and the creation of highly accurate knock-in reporters can be achieved. These novel tools will help us to exploit one of the great advantages of the zebrafish model, and screen molecular libraries for further drug-candidate molecules that affect autophagy, perhaps even more specifically than existing compounds [20].

Acknowledgements

This work has been supported by the OTKA grant K109349 to TV. MV is a János Bolyai Fellow of the Hungarian Academy of Sciences. We would like to thank our anonymous reviewers for their insightful comments and suggestions.

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Tables

Table 1: Transient and constitutive reporter constructs used to follow autophagic activity in zebrafish

Reporter	Expression	Reference
Tg(<i>CMV:EGFP-LC3</i>)	ubiquitous	[67]
Tg(<i>CMV:GABARAP-LC3</i>)	ubiquitous (not recommended)	[67]
Tg(<i>CMV:EGFP-LC3;mCherry</i>)	ubiquitous	[40]
Tg(<i>CMV:eGFP-Gabarap;mCherry-Lc3</i>)	ubiquitous	[40]
Tg(<i>fabp10: EGFP-Lc3</i>)	liver	[68]
Tg(<i>TaCP:GFP-LC3</i>)	cone photoreceptors	[61]
pEGFP-MAP1-LC3B	transient (in embryonic cells)	[65]
LC3-mCherry	transient	[63]
hsp70l:RFP-Lc3	transient	[66]

Table 2. Summary of antibodies used to detect autophagy-related proteins in zebrafish.
(Antibodies in italics have been used for immunostaining, too.)

Antibody	Company (product no.)	Reference
anti-LC3	<i>Abcam (ab51520)</i>	[63]
	<i>Thermo Scientific (PA1-46286)</i>	[41]
	Novus Biologicals (NB100-2331)	[67]
	Sigma (L7543)	[76]
	MBL (PD014)	[68]
	MBL (PM036)	[94]
	Novus Biologicals (NB100-2220)	[74, 94, 95]
	Cell Signaling (2775)	[70, 74]
anti-ATG5	Abcam (ab54033)	[76]
	Abgent (AP1812b)	
	Abgent (AP1812a)	
	Novus Biologicals (NB110-53818)	[14]
anti-BCN1	Santa Cruz (H-300 11427)	[59, 70]
anti-GABARAP	non-commercial	[67]
anti-p62	<i>Abcam (ab31545)</i>	[41]
	Cliniscience (PM045)	[42]
anti-TOR	Cell Signaling (2983)	[95]

Table 3. Summary of MOs used to silence autophagy-related genes in zebrafish. Sequences in italics indicate splice-blocking MOs. IEC = intestinal epithelial cells.

Targeted gene	MO sequence (5' to 3')	Observed phenocopies	Ref.
<i>ambra1a</i>	CTC CAA ACA CTC TTC CTC ACT C	smaller head, bent trunk axis, pericardial edema	[66, 70]
	<i>TGT AAT CAA AGT GGT CTT ACC TGT</i>	impaired skeletal muscle formation	
<i>ambra1b</i>	TTT TCC TCT TTA GTG CTC CAC GC		
	<i>TGA AAT TGA TTG TTA CCT ATC TGG</i>		
<i>atg5</i>	CAT CCT TGT CAT CTG CCA TTA TC	death of IECs in <i>titania</i> mutants	[63]
	CAT CCT TGT CAT CTG CCA TTA TC	bent axis, smaller head, cardiac oedema, impaired neurogenesis	[76]
	<i>GTG CCC TTA AAA CCA AAA ATA ACA</i>		
	CCT TGT CAT CTG CCA TTA TCA TC	defective caudal fin regeneration	[14]
	CAC ATC CTT GTC ATC TGC CAT TC	smaller head, bent trunk axis, abnormal development	[59]
	<i>ATT CCT TTA ACT TAC ATA GTA GGG</i>		
<i>atg7</i>	AGC TTC AGA CTG GAT TCC GCC A		
	<i>AGC TCG TTC TCC AAA CTC ACC GTT</i>		
<i>beclin1</i>	ACC TCA AAG TCT CCA TGC TTC TC		
	TGT TAT TGT GTG TTA CTG ACT TC		
	<i>CAT CCT GCA AAA CAC AAA TGG CT</i>	suppresses the Spns1 deficiency	[40]
<i>dram1</i>	AAG GCT GGA AAA CAA ACG TAC AG	decreased autophagic clearance of <i>Mycobacterium marinum</i>	[40]
	<i>GTC GTC TCC TGT AAC AAA ACA TGA</i>		
<i>optn</i>	CGA TGA TCC AGA TGC CAT GCT TC	causes motor axonopathy	[82]
	AAA <i>TTT CTC TCA CCT CAG CTC CAC</i>		
	TGT CCC CAT TCA TCA TCG ATG A	no change in gross morphology	[81]
	<i>TAA CCC GCA CCT TTC AGG TCT CG</i>		
<i>p62/sqstm1</i>	CAC TGT CAT CGA CAT CGT AGC C	decreased survival upon <i>Shigella</i> infection	[42]
	<i>CTT CAT CTA GAG ACA AAG TTC AGC</i>	decreased autophagic clearance of <i>Mycobacterium marinum</i>	[41]
<i>spns1</i>	ATC TGC TTG TGA CAT CAC TGC TC	smaller head, opaque yolk sac	[40]

Table 4. List of reagents previously used to interfere with autophagic activity in zebrafish. (LT-LysoTracker)

Reagent	Conc.	Context	Observed effect	Ref.
Reagents increasing autophagy				
2'5'DDA	100 μ M	autophagy modulates aggregate clearance in rod photoreceptors of HD model larvae	decrease in EGFP-LC3 aggregates	[53]
		validating autophagy modulation in zebrafish larvae	increased GFP-Lc3 colocalization and Lc3II/Lc3I ratio	[67]
AR-12	1 μ M	autophagic defense against mycobacterial infection	increased GFP-Lc3+ cells in epithelial cells, increased in	[41]
calpastatin	1 μ M	autophagy modulates aggregate clearance in rod photoreceptors of HD model larvae	decrease in EGFP-LC3 aggregates	[53]
calpeptin	50 μ M	validating autophagy modulation in larvae	increased GFP-Lc3 colocalization and Lc3II/Lc3I ratio	[67]
clonidine	3 μ M	autophagy modulates aggregate clearance in rod photoreceptors of HD model larvae	decrease in EGFP-LC3 aggregates	[53]
		validating autophagy modulation in zebrafish larvae	increased Lc3II/Lc3I ratio	[67]
		autophagy contributes to clearance in zebrafish larvae	increases Dendra-tau clearance in Tg(<i>rho:EGFP-tau</i>) larvae	[74]
	30 μ M	IGFR-1 mediated autophagy modulates aggregate clearance in rod photoreceptors of HD larvae	decrease in EGFP-LC3 aggregates	[71]
rapamycin	10 nM	induction of autophagy by amyloidogenic light chain (AL) proteins	autophagic flux restored, cell death decreased, but survival impaired AL-LC injected larvae	[96]
	50 nM	autophagic defense against <i>S. flexneri</i> infection	decreasing p62 and aggregates	[42]
	200 nM	autophagy attenuates DOX induced cardiomyopathy in adult fish	increased number of LC3 puncta	[95]
	400 nM	effects of autophagy on cardiomyocyte proliferation	inhibits cardiomyocyte proliferation	
	1 μ M	validating autophagy modulation in zebrafish larvae	increased GFP-Lc3 colocalization	[67]
		autophagic defense against mycobacterial infection	increased <i>Mycobacterium</i> in	[41]
		autophagic defense against <i>S. flexneri</i> infection	decreased survival upon <i>S. flexneri</i> infection	[42]
		expression analysis of zebrafish genes	increased <i>map1lc3a</i> and decreased <i>map1lc3b</i> expression at 72h	[75]
	10 μ M	autophagic activity in ribosomes	in combination with CQ in	[63]

		biogenesis mutants (<i>ttf^{ts450}</i>)	the number of Lc3+ puncta	
		zebrafish models of myofibrillar myopathy	decreases the percentage of phenotypic fibers in BA0 eGFP injected zebrafish	[97]
	30 μ M	autophagy contributes to clearance in zebrafish larvae	increases Dendra-tau clearance and the survival of photoreceptors in <i>Tg(rho:EGFP-tau)</i> embryos	[74]
rilmenidine	50 μ M	autophagy contributes to clearance in zebrafish larvae	increases Dendra-tau clearance	[74]
torin	500 nM	validating a novel transgenic <i>Tg(fabp10:EGFP-Lc3)</i>	increase in GFP-Lc3 puncta in carriers	[68]
verapamil	3 μ M	autophagy modulates aggregate clearance in rod photoreceptors HD model larvae	decrease in EGFP-Lc3 aggregates	[53]
		validating autophagy modulation in larvae	increased Lc3II/Lc3I ratio	[67]
Reagents decreasing autophagy				
3-MA	10 mM	validating autophagy modulation in larvae	decreased Lc3II/Lc3I ratio, GFP-LC3 – LT colocalisation, the presence of rapamycin, pepstatin	[67]
		autophagic defense against mycobacterial infection	increased <i>Mycobacterium</i> infection, decreased number of GFP-Lc3 puncta in endothelial cells	[41]
(\pm)BayK8644	1-3 μ M	autophagy modulates aggregate clearance in rod photoreceptors HD model larvae	increase in EGFP-Lc3 aggregates	[53]
NAC	300 μ M	antioxidants inhibit autophagy dependent aggregate clearance HD model larvae	increase in EGFP-Lc3 aggregates	[73]
	1 mM		decreased Lc3II/Lc3I ratio	
PPP	100 μ M	IGFR-1 mediated autophagy modulates aggregate clearance in rod photoreceptors of HD larvae	decrease in EGFP-Lc3 aggregates	[71]
pyrvinium	200 nM	pyrvinium inhibits autophagy <i>in vivo</i> in zebrafish larvae	decreased Lc3II/Lc3I ratio	[72]
U0126	25 μ M	role of autophagy in caudal fin regeneration	decrease in the number of autophagic structures in regenerating fin	[14]
wortmannin	100 nM	autophagy contributes to clearance in zebrafish larvae	decreases survival of photoreceptors in <i>Tg(rho:EGFP-tau)</i> larvae	[74]
Reagents decreasing autophagy by impairing autophagosome – lysosome fusions				
Baf1A	20 nM	role of autophagy in caudal fin regeneration	defects in caudal fin regeneration	[14]

	200 nM	autophagy inhibition rescues mutants (impaired in lys acidification)	rescues opaque yolk senescence phenotypes, EGFP-Lc3 – LT colocalizat	[40]
CQ	2.5 µM	autophagic activity in rib biogenesis mutants (<i>ttt^{s450}</i>)	in combination with rapa increases the number of positive punctae	[63]
	50 µM	expression analysis of the z <i>lc3</i> genes	increased Lc3II/Lc3I ratio presence of rapamycin	[75]
		validating a novel transgen <i>Tg(fabp10:EGFP-Lc3)</i>	increase in GFP-Lc3 pu carriers	[68]
	100 µM	increased autophagy, in <i>my</i> zebrafish tumors	more autophagosomes by T	[98]
		autophagic defense mycobacterial infection	increased infection	[41]
		zebrafish models of myofibrillar myopathy	increased percentage phenotypic fibers in BA0 eGFP injected zebrafish	[97]
	250 µM	role of autophagy in cau regeneration	impaired caudal fin regener	[14]
E64d	5 mM	validating autophagy modul zebrafish larvae	increased Lc3II/Lc3I ratio presence of pepstatin A	[67]
lansprazole	25 µM	autophagy inhibition rescues mutants (impaired in lys acidification)	rescues senescence phenot <i>spns1</i> -/- embryos	[40]
NH ₄ Cl	1 mM	autophagy contributes to clearance in zebrafish larvae	decreases Dendra-tau cleara	[74]
	10 mM		decreases survival photoreceptors in <i>Tg(rho. tau)</i> larvae	
	20 mM	pryvinium inhibits autoph <i>vivo</i> in zebrafish larvae	increased Lc3II/Lc3I ratio	[72]
	100 mM	IGFR-1 mediated aut modulates aggregate cleara rod photoreceptors of HD larvae	increased Lc3II/Lc3I ratio	[71]
omeprazole	25 µM	autophagy inhibition rescues mutants (impaired in lys acidification)	rescues senescence phenot <i>spns1</i> -/- embryos	[40]
pantoprazole	25 µM	autophagy inhibition rescues mutants (impaired in lys acidification)	rescues senescence phenot <i>spns1</i> -/- embryos	[40]
pepstatin A	10 µg/ml	validating autophagy modul zebrafish larvae	increased Lc3II/Lc3I rat GFP-Lc3 – LT colocaliza presence of E64d	[67]

Figure legends

Figure 1. Visualization of autophagic activity in zebrafish.

(A-B) Transmission electron microscopy pictures showing autophagic structures (arrows). (A, A') Lymphoblasts of Tg(*hsp70:Cre;rag2-LDL-EGFP-mMyc;rag2-EGFP-bcl-2*) fish overexpressing Myc and Bcl2 undergo autophagy (taken from Ref. [98] – permission needed). (B) In regenerating fin blastemas, autolysosomes can be observed in several cell types by transmission electron microscopy (black arrows) (courtesy of Miklós Sass, Eötvös University, Budapest, Hungary). (C) In 3 dpf *spns1*^{-/-};Tg(*CMV:EGFP-Lc3*) larvae, an increased number of EGFP-Lc3-positive punctae can be observed, compared to wild type. These punctae often colocalize with LysoTracker Red staining, suggesting the impairment of autophagic activity (taken from [40] – permission needed). (D) At 2 days post amputation (dpa), regenerating fin shows a marked increase in reporter activity in the blastemal region of a Tg(*CMV:EGFP-Lc3*) fish. (E) Autophagic activity, marked by Lc3 conversion, can be observed in 2 dpf zebrafish larvae, and can be modulated by small molecular autophagy agonists and antagonists (modified from Ref. [67] – permission needed). (3MA – 3-methyladenine, dpa – days post amputation, dpf – days post fertilization, P/E – pepstatinA and E64d, Rap – rapamycin). (F) Caudal fin regeneration results in an increase in the amount of Atg5-containing complexes and the appearance of a novel ~47 kDa complex (taken from Ref. [14] – permission needed). (G) Morpholino knock-down of different autophagy related genes results in characteristic phenotypes at 2 dpf (taken from Ref. [59] – permission needed).

Figure 2. A schematic overview of the core autophagy pathway and its regulation in zebrafish.

Discussed MOs are in bold red type, with their targets indicated. Compounds that enhance or inhibit autophagic activity are listed in blue and red boxes, respectively. (Due to its somewhat unexpected effects, PPP, although an autophagy inhibitor, is marked by both colors.) See the text for details.

