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Autophagy in zebrafish

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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.

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

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; Gsα, 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.

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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 on 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 (Gsα) 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 Gsα [51]. Activation of Gsα 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 IlR: 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 and B). It has been successfully