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To cite this article: G. Tettamanti, E. Carata, A. Montali, L. Dini & G. M. Fimia (2019) Autophagy in development and regeneration: role in tissue remodelling and cell survival, The European Zoological Journal, 86:1, 113-131, DOI: [10.1080/24750263.2019.1601271](https://doi.org/10.1080/24750263.2019.1601271)

To link to this article: <https://doi.org/10.1080/24750263.2019.1601271>



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Published online: 02 May 2019.



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Autophagy in development and regeneration: role in tissue remodelling and cell survival

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(Received 28 December 2018; accepted 23 March 2019)

Abstract

Morphogenetic events that occur during development and regeneration are energy demanding processes requiring profound rearrangements in cell architecture, which need to be coordinated in timely fashion with other cellular activities, such as proliferation, migration and differentiation. In the last 15 years, it has become evident that autophagy, an evolutionarily-conserved catabolic process that mediates the lysosomal turnover of organelles and macromolecules, is an essential “tool” to ensure remodelling events that occur at cellular and tissue levels. Indeed, studies in several model organisms have shown that the inactivation of autophagy genes has a significant impact on embryogenesis and tissue regeneration, leading to extensive cell death and persistence of unnecessary cell components. Interestingly, the increased understanding of the mechanisms that confers selectivity to the autophagic process has also contributed to identifying development-specific targets of autophagy across species. Moreover, alternative ways to deliver materials to the lysosome, such as microautophagy, are also emerging as key actors in these contexts, providing a more complete view of how the cell component repertoire is renovated. In this review, we discuss the role of different types of autophagy in development and regeneration of invertebrates and vertebrates, focusing in particular on its contribution in cnidarians, plathyhelminthes, nematodes, insects, zebrafish and mammals.

Keywords: *Autophagy, development, invertebrates, vertebrates, regeneration*

Abbreviations

Ambra1	activating molecule in Beclin1-regulated autophagy	20E	20-hydroxyecdysone
AMPK	AMP-activated protein kinase	Hif1 α	hypoxia-inducible factor
AMP	adenosine monophosphate	Hh	hedgehog
Atg	autophagy-related genes	Hop	Hsp70–Hsp90 organising protein
ATP	adenosine triphosphate	Hsc70	heat shock 70 kDa
Chk1	checkpoint kinase 1	Hsp40	heat shock protein 40
CMA	chaperone-mediated autophagy	IAP	inhibitor of apoptosis protein
DAP-1	death-associated protein 1	Lamp	lysosomal-associated membrane protein
Dvl	Dishevelled	T _{amp}	Amplitude of the ground surface temperature for the year (°C)
ESCRT	endosomal sorting complexes required for transport	mTor	mammalian target of rapamycin
EST	expressed sequence tag	mTorc1	mammalian target of rapamycin complex 1
FGFs	fibroblast growth factors	NF- κ B	nuclear factor- κ B
		PC	primary cilium

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PCD	programmed cell death
PE	phosphatidylethanolamine
PI3P	phosphatidylinositol-3-phosphate
Plekhm1	pleckstrin homology and RUN domain containing M1
SQSTM1	sequestosome 1
TEM	transmission electron microscopy
Ulk	Unc-51-like kinase
WIPI2	WD repeat domain phosphoinositide interacting 2

Introduction

The term autophagy (from the Greek *autòs* “himself” and *phagéin* “to eat”, i.e. eating itself) was first used in 1963 by the Belgian cytologist and biochemist Christian de Duve to indicate the presence of intracellular vesicles with cytoplasmic material trapped inside (Ohsumi 2014).

Autophagy is a key mechanism for the maintenance of cellular homeostasis, i.e. the balance between synthesis, degradation and recycling of the constituents of the cell (Mizushima 2018), which is rapidly induced under stress conditions (Galluzzi et al. 2014). For example, it intervenes when vital organelles, such as mitochondria, are severely damaged or become dysfunctional, as well as in the case of nutrient deficiency. In the latter case, the activation of autophagy represents a cellular response that provides basic elements, obtained from the degradation of non-essential components, for the production of energy or for novel synthesis of essential macromolecules to ensure cell survival.

In the last 25 years, our knowledge about the autophagic process has increased exponentially, as regards both the characterisation of the molecular mechanisms regulating this process and its relevance in physiological and pathological contexts. The decisive turning point in the autophagy field came from the use of the yeast *Saccharomyces cerevisiae* as a model organism (Ohsumi 2014; Mizushima 2018). In 1992, Yoshinori Ohsumi reported the presence of autophagosomes in yeast cells under nutrient deprivation, by electron microscopy, and the following year he identified the core genes that control the autophagy process, naming them *Atg* (Autophagy-related genes), through a genetic screening for yeast mutants unable to survive in starvation conditions (Takeshige et al. 1992; Tsukada & Ohsumi 1993). To date, the molecular mechanism that governs autophagy has been characterised in a very detailed manner. Homologous *Atgs* have been identified in many other organisms, revealing that autophagy is a ubiquitous catabolic process

highly conserved in eukaryotes. In 2016, Prof. Ohsumi was awarded the Nobel Prize for Medicine and Physiology for his pioneering discoveries of the autophagy machinery.

Autophagy plays a pivotal role in various developmental settings. It is also involved in reshaping tissues and organs, and contributes to regenerative programmes. In this review, after a survey on the autophagic process in mammals, we report current knowledge on autophagy in invertebrates and vertebrates, highlighting the role of this self-eating process during development and tissue remodelling, and discuss the peculiarities of its regulatory pathways in various animal models.

Different types of autophagy

In mammals, three different types of autophagy have been identified: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), as well as a series of selective subtypes.

They differ in: (1) the way the material to be degraded is conveyed to the lysosomes, (2) the type of material that is selected for degradation, and (3) the molecular mechanisms regulating the induction of these processes.

Macroautophagy

Macroautophagy, commonly referred to as autophagy, is based on a series of events that lead to the formation of the autophagosome, a double-membraned vesicle that seizes a portion of a cytoplasmic matrix (Mizushima & Komatsu 2011). Once closed, this structure fuses with the endo-lysosomal compartment, forming the autophagolysosome, which allows the degradation of cytoplasmic content by the lysosomal proteolytic enzymes. Elements obtained by the breakdown of macromolecules, such as amino acids, monosaccharides, nucleotides and fatty acids, are then transported to the cytoplasm to make them available for new synthesis or energy production.

Despite the initial assumption that cytoplasmic components were randomly sequestered in the autophagosomes, several experimental results point to a highly selective delivery of damaged or superfluous macromolecules/organelles to the autophagosome, such as aggregates of unfolded proteins and depolarised mitochondria (Khaminets et al. 2016). Macroautophagy occurs constitutively at low levels and is rapidly induced by various stress signals. It is primarily a cytoprotective mechanism, but excessive self-digestion can be detrimental to the cell itself, contributing to specific cell demise (Galluzzi et al. 2018).

Microautophagy

The term microautophagy was proposed by de Duve and Wattiaux more than 40 years ago, referring to the lysosomal degradation of a tiny portion of cytoplasm (de Duve & Wattiaux 1966). Unlike macroautophagy, microautophagy is not based on the formation of new membranes to isolate the target material. Instead, invaginations or protrusions of membranes of the endo-lysosomal compartments are used to sequester the target material.

Microautophagy has been well characterised in yeasts, where both non-selective and selective forms have been described. The non-selective microautophagy degrades mainly portions of cytosol, while selective microautophagy targets specific organelles, such as mitochondria (named micromitophagy), portions of the nucleus (piecemeal microautophagy) and peroxisomes (micropexophagy) (Mijaljica et al. 2011). Because of the difficulty in identifying specific regulators of this process, the molecular characterisation of microautophagy and its physiological relevance in higher eukaryotes are still under investigation (Tekirdag & Cuervo 2018). Recently, endosomal microautophagy has been described in murine dendritic cells and *Drosophila melanogaster* synapses (Oku & Sakai 2018). In these systems, the ESCRT (endosomal sorting complexes required for transport) machinery and the chaperone Hsc70 play a key role in mediating the invagination of the sequestering membrane and in the selective recruitment of cytosolic proteins on the endosomal surface (Sahu et al. 2011; Uytterhoeven et al. 2015).

Chaperone-mediated autophagy

CMA differs from the other two forms of autophagy in that it does not use membrane structures to select material to be degraded (Kaushik & Cuervo 2018). In this process, the substrate proteins contain an identification signal consisting of the KFERQ-like pentapeptide, which is recognised by a chaperone complex including Hsc70 (heat shock protein 70 kDa), Chip (Hsc70-interacting protein), Hsp40 (heat shock protein 40) and Hop (Hsp70–Hsp90 organising protein). After being unfolded, the proteins are translocated to the lysosomes through a pore formed by multimers of Lamp2A, a specific isoform of the type 2 lysosomal membrane-associated protein (Agarraberes & Dice 2001).

CMA contributes to cellular homeostasis in normal and stress conditions, such as nutrient starvation, oxidative stress, genotoxic insults, lipid challenges, hypoxia and radiation, by regulating

the degradation of up to 20% of intracellular proteins. In particular, CMA has a well-characterised role in the regulation of glucose and lipid metabolism in nutrient limiting conditions, through selective degradation of key enzymes in these pathways, such as glycolytic enzymes, lipogenesis enzymes, lipid carriers and lipid droplet coat (Schneider et al. 2014). CMA also regulates transcriptional programmes, as in the case of the nuclear factor- κ B (NF- κ B), which is activated in nutrient starvation through the degradation of its inhibitor I κ B α (Cuervo et al. 1998). Moreover, CMA activity is involved in the regulation of the immune response by targeting negative regulators of T cell receptor signalling (Valdor et al. 2014), and cell cycle progression in stress conditions by targeting the protein kinase Chk1 (checkpoint kinase 1) during DNA damage recovery (Park et al. 2015) or the transcription regulator Hif1 α (hypoxia-inducible factor) in hypoxic conditions (Hubbi et al. 2014).

Regulation of macroautophagy

There are 37 Atg genes currently known to be involved in the autophagic process in yeast, most of which have homologues in higher eukaryotes (Ohsumi 2014). The functional characterisation of these genes has allowed the description of the molecular aspects that regulate autophagy (Figure 1). Four sequential steps are required for the accomplishment of the process: (1) induction, (2) nucleation of autophagosomal membrane, (3) elongation and closure, and (4) fusion of autophagosomes with the endo-lysosomal compartment and degradation of the content of autophagic vacuoles for recycling (Mizushima & Komatsu 2011; Antonoli et al. 2017).

Induction of autophagy. Stimuli that can trigger autophagy are numerous, including growth factors, hormones, intracellular calcium levels, levels of adenosine triphosphate (ATP), hypoxia, accumulation of misfolded proteins and infection by pathogens (Galluzzi et al. 2018). The best-characterised stimulus is nutrient deprivation, a condition in which the mammalian Target Of Rapamycin Complex 1 (mTorC1) protein complex is inhibited. The main component of mTorC1 is mTor, a serine/threonine protein kinase that positively regulates anabolic processes, such as protein synthesis, and inhibits catabolic ones, such as autophagy (Laplante & Sabatini 2012). In the presence of nutrients, mTorC1 phosphorylates and inhibits the upstream autophagy complex Atg1/Ulk (Kim et al. 2011). Conversely, in conditions of nutrient

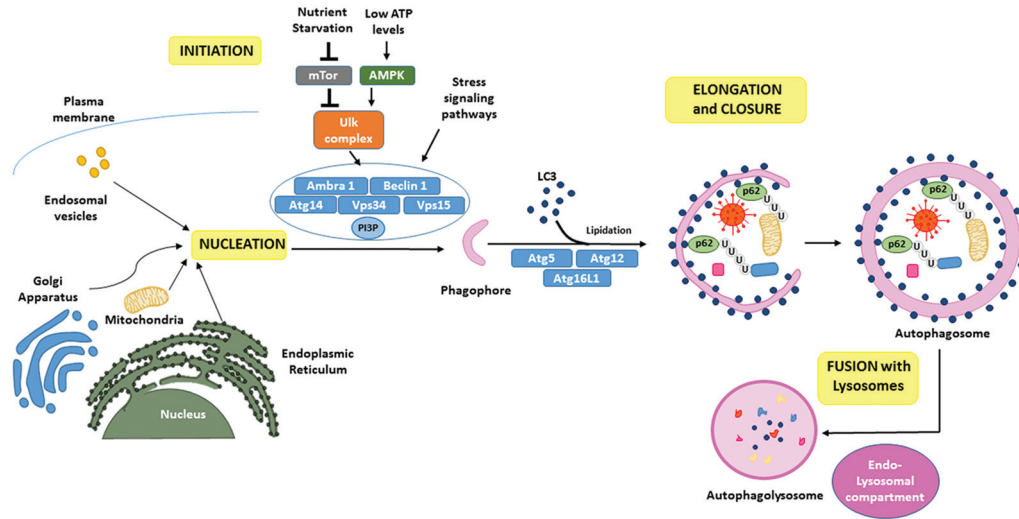


Figure 1. Regulation of the autophagic process. In the presence of nutrients, autophagy is negatively regulated by the serine/threonine kinase mTor. Nutrient starvation inhibits mTor and activates AMPK, a kinase that responds to low ATP levels, which results in the activation of the Ulk complex. A plethora of stress signalling pathways is also able to induce autophagy. Ulk1 phosphorylates various members of the Beclin 1 complex to stimulate the lipid kinase activity of Vps34. This complex is responsible for the production of phosphatidylinositol 3-phosphate (PI3P) by the Class III PI 3-kinase. PI3P allows the nucleation of an isolation membrane called a phagophore. PI3P recruits Atg12-5-16L1 to lipidate LC3, which is required for phagophore elongation and cargo recruitment. Then, the phagophore closes to form a double-membraned vesicle called an autophagosome. Finally, the autophagosome slides along cytoskeletal structures and fuses with the endo-lysosomal compartment to form the autophagolysosomes, where sequestered materials are degraded.

deficiency, mTorc1 is inactive and autophagy is induced.

Another stimulus that promotes autophagy is energy shortage, due either to excessive consumption or to a reduction in cellular energy reserves. High AMP/ATP ratios activate adenosine monophosphate (AMP)-activated protein kinase (AMPK), a main sensor of cellular energy levels (Herzig & Shaw 2018). AMPK acts as a positive regulator of autophagy, both indirectly by inhibiting the activity of mTorc1, and directly by promoting the activation through phosphorylation of the Ulk1 and Beclin-1 complexes (Kim et al. 2011).

The Ulk complex is composed of Ulk1 or Ulk2 kinases (Unc-51-like kinase), associated with the regulatory proteins Atg13, Atg101 and Fip200 (also known as RB1CC1) (Lin & Hurley 2016). This complex is essential for the transmission of stress signals due to its ability to phosphorylate proteins essential for autophagosome formation (Mizushima & Komatsu 2011).

Nucleation of autophagosomal membrane. Nucleation of the autophagosomal membrane is triggered by the Atg6/Beclin1 complex. This complex consists of the class III phosphatidylinositol 3-kinase Vps34 and four autophagy specific cofactors, Atg6/Beclin 1, Atg14, Ambra1 and Vps15. Atg14 is required to recruit the Vps34/Beclin 1 complex to active sites

of autophagosome biogenesis (Matsunaga et al. 2010). Moreover, Atg14 is able to sense and maintain membrane curvature in the nascent autophagosome (Fan et al. 2011). Ambra1 is a WD40 protein required to stimulate Vps34 activity by favouring its interaction with Beclin1 (Fimia et al. 2007).

Once activated, Vps34 catalyses the production of phosphatidylinositol-3-phosphate (PI3P), an event that marks the membrane site where autophagosome membrane precursor is formed (Levine et al. 2015). Membrane structures rich in PI3P, called omegasomes because of their omega shape, act as a platform for the recruitment of machinery proteins that assemble the autophagosomes.

Beclin 1/Vps34 complex I is stimulated by Ulk1, which phosphorylates Beclin-1, Atg14 and Ambra1 subunits (Russell et al. 2013; Egan et al. 2015). Beclin activity is also stimulated by non-degradative ubiquitination mediated by various E3 ubiquitin ligases, such as Traf6 and Trim50 (Shi & Kehrl 2010; Fusco et al. 2018). Conversely, autophagosome formation mediated by the Beclin 1/Vps34 complex can be inhibited by different kinases, such as Akt and the epidermal growth factor receptor, which promote the binding of Beclin-1 with two types of negative regulators, Rubicon and members of the Bcl-2 family. Upon the occurrence of an autophagy stimulus, Beclin-1 dissociates from these

negative regulators through a variety of stress-activated kinases, such as Jnk, Dapk1 and Rock1 (Antonoli et al. 2017).

The cellular compartment from which the precursor of the autophagosome membrane, called the isolation membrane or phagophore, originates has been a matter of debate for a long time. The endoplasmic reticulum has been proposed as the main source of autophagosomal membranes, in particular in the contact sites between the endoplasmic reticulum and the mitochondrion or the plasma membrane. However, recent studies suggest a contribution of membrane vesicles from other organelles, such as the Golgi apparatus and late endosomes (Shibutani & Yoshimori 2014; Ge et al. 2015; Pavel & Rubinsztein 2017).

Elongation and closure. The autophagosome is a transient organelle characterised by a double membrane. This structure originates from the isolation membrane that expands, bends around the target material and seals to isolate the cargo to be delivered to the lysosomes. The extension and closure steps of autophagosome formation require members of the Atg8/LC3 family. Atg8 proteins are inserted into the phagophore through a covalent bond with phosphatidylethanolamine (PE). This modification of Atg8 proteins occurs through a cascade of events similar to those occurring during protein ubiquitination process. First, Atg8 proteins are cleaved at the C-terminus by the Atg4 proteases in order to expose a glycine residue. Once cleaved, the E1-like activating enzyme Atg7 and the E2-like conjugating enzyme Atg3 allow the covalent bind of Atg8 to a PE molecule at the terminal glycine residue. The E3-like ligase complex that facilitates the conjugation of PE to LC3 is composed of Atg12-Atg5/Atg16l1 proteins (Mizushima 2018).

The insertion site of LC3 to a specific membrane site is determined by WIPI2 (WD repeat domain phosphoinositide interacting 2), which binds to PI3P and Atg16l1 to recruit the E3-ligase-like complex on the phagophore (Dooley et al. 2014).

Atg8/LC3 proteins also play a major role in the recruitment of specific cargos within the autophagosomes by interacting directly with cargo receptors, such as sequestosome 1 (SQSTM1), better known as p62 (Katsuragi et al. 2015). p62 has both a LIR domain to interact with LC3 and a UBA domain to bind poly-ubiquitin chains, through which p62 recognises protein aggregates, damaged organelles and intracellular pathogens. p62 was the first identified member of a family of autophagy receptors, which also includes Nbr1, Ndp52 and Optineurin, which show some selectivity with respect to the ubiquitinated cargos (Lamark et al. 2017).

Autophagosome elongation also involves Atg9, a transmembrane protein that shuttles from the Golgi apparatus to the phagophore, which has been proposed to transport lipids for membrane expansion (Noda 2017).

Fusion of autophagosomes with the endo-lysosomal compartment and degradation. In the last step of the autophagic process, autophagosomes moving along microtubules fuse with the endolysosomal compartment and allow cargo degradation (Lamb et al. 2013). This process requires PI3P synthesis by a different Beclin 1/Vps34 complex, whose activity is stimulated by UVRAG, which replaces Atg14 in the complex, and is inhibited by Rubicon. In addition, UVRAG binds the homotypic fusion and protein sorting (HOPS) complex to stimulate Rab7 GTPase activity and autophagosome-lysosome fusion, an event that also requires the recruitment of the adaptor protein Plekhm1 (pleckstrin homology and RUN domain containing M1) and the Snare protein Syntaxin 17 (Amaya et al. 2015; Nakamura & Yoshimori 2017). Except for Syntaxin 17, which is an autophagosome-specific factor, the fusion machinery represents a common element between endocytosis and autophagy, indicating that both processes are altered when the function of these proteins is affected. Products generated by lysosomal degradation, such as amino acids, monosaccharides, nucleotides and fatty acids, are then released to the cytosol through specific membrane transporters, where they stimulate mTorc1 activity to reactivate anabolic processes and suppress autophagy in order to avoid excessive self-digestion, which could be harmful for the cells (Shen & Mizushima 2014).

Autophagy, development and tissue remodelling

Below, a survey of the main processes in which autophagy intervenes during development and regeneration, in both invertebrates (Figure 2) and vertebrates (Table I), is presented.

Role of autophagy during development and tissue remodelling in invertebrates

Autophagy as a regulator of tissue plasticity in cnidarians

Hydrozoans represent the most interesting cnidarian group for developmental biology studies. In particular, *Hydra* is a genus of small, freshwater organisms that, despite their relative anatomical simplicity, show a unique developmental plasticity among Metazoa. In fact, polyps can regenerate amputated parts of the body in a few days, they are able to





		Species	Process
Cnidarians		<ul style="list-style-type: none"> • <i>Hydra</i> spp. • <i>Hydractinia symbiolongicarpus</i> • <i>Nematostella vectensis</i> • <i>Porites</i> spp. 	<ul style="list-style-type: none"> • Regeneration • Nutrient starvation • Protection versus non-self
Platyhelminthes		<ul style="list-style-type: none"> • <i>Dugesia japonica</i> • <i>Girardia tigrina</i> • <i>Polycelis tenuis</i> 	<ul style="list-style-type: none"> • Regeneration • Nutrient starvation
Nematodes		<ul style="list-style-type: none"> • <i>Caenorhabditis elegans</i> 	<ul style="list-style-type: none"> • Dauer morphogenesis • Aging • Removal of cell components • Neural development
Insects		<ul style="list-style-type: none"> • <i>Bombyx mori</i> • <i>Drosophila melanogaster</i> • <i>Helicoverpa armigera</i> 	<ul style="list-style-type: none"> • Remodelling/removal of larval organs • Nutrient starvation

Figure 2. Examples of the role of autophagy in invertebrate development.

reproduce asexually through budding, and they can reaggregate if the body tissues are dissociated (Galliot et al. 2006).

Although the study of autophagy in cnidarian species is quite recent, significant evidence about the role of this self-eating process in this phylum has already been obtained. The genetic machinery that regulates autophagy in cnidarians has been studied in many species of the genera *Hydra* and *Nematostella*. In both cases, orthologous genes characterised as individual genes, or identified through expressed sequence tag (EST) projects, have demonstrated a high conservation of the main components of the autophagy machinery, as well as of the Tor pathway, a nutrient sensor that can mediate the activation of autophagy (Chera et al. 2009). This evidence, obtained in one of the simplest animal groups, cnidarians, strongly supports the view that the autophagy signalling pathway is well conserved across metazoans. Thanks to RNASeq analysis it has been possible to evaluate how these autophagy-related proteins are expressed in the *Hydra* body (Buzgariu et al. 2015). Transcripts of all members of the autophagic pathway are present in the epithelial cells, with high levels of expression for the kinase Atg1, a key regulator of starvation-dependent autophagy. Moreover, Dixit and colleagues recently identified and characterised the two core Atg genes that form the Atg12–Atg5 complex, which is required for Atg8 lipidation (Dixit et al. 2017). In situ

hybridisation demonstrated that the two genes are expressed in the body column of *Hydra*, especially in budding regions and growing buds, thus corroborating a possible role for autophagy in the growth and regeneration of the polyp.

In *Hydra*, autophagy plays different roles in at least two main settings: autophagy activated by nutrient starvation, and cytoprotective autophagy that intervenes in stress or damaged tissues. Once subjected to nutrient deprivation, the animal stops budding and progressively decreases in size. Autophagy is rapidly activated in starving polyps and the autophagic process reaches its maximal rate within 2 weeks of starvation (Buzgariu et al. 2008). In this case autophagy has a pro-survival role and the starving animal relies on this process to stay alive. A more complex situation occurs during regeneration of the polyp. *Hydra* is a bilayered organism characterised by two germ layers, the ectoderm and the endoderm, separated by an extracellular matrix called mesoglea. The digestive function requires cooperation between endodermal epithelial cells (i.e. digestive cells) and gland cells that release digestive enzymes in the gastric cavity. Chera et al. (2006) demonstrated that silencing the *Kazal1* gene, which codes for a serine-protease inhibitor, in *Hydra* through RNAi induces excessive autophagy in both digestive and gland cells, leading to cell death. Thus, under homeostatic conditions, *Kazal1* prevents

Table I. Examples of the role of autophagy in vertebrate development.

Organism	Role	Process	Type of autophagy	Reference
<i>Mus musculus</i>	Degradation of maternal materials to sustain cell division	Segmentation	Canonical macroautophagy	Kuma et al. 2004; Komatsu et al. 2005; Sou et al. 2008
<i>Mus musculus</i>	Degradation of non-essential materials in energy-limiting conditions	Survival of post-natal starvation	Canonical macroautophagy	Kuma et al. 2004; Komatsu et al. 2005; Sou et al. 2008
<i>Mus musculus</i>	Not defined	Organogenesis (brain, liver, erythrocyte and heart)	Non-canonical macroautophagy	Nishida et al. 2009; Dupont & Codogno 2013
<i>Mus musculus</i>	Delivery of ingested material to the apical vacuole	Gastrulation	Microautophagy	Tsukamoto et al. 2008
<i>Mus musculus</i>	Degradation of mitochondria	Erythroid differentiation	Mitophagy	Sandoval et al. 2008
<i>Mus musculus</i>	Degradation of paternal mitochondria	Removal of harmful organelle in fertilised oocytes	Mitophagy	Sato & Sato 2011
<i>Mus musculus</i>	Degradation of midbody	Regulation of stemness	Selective macroautophagy	Kuo et al. 2011
<i>Mus musculus</i>	Degradation of RhoA	Regulation of axonal growth in neurons	Selective macroautophagy	Ban et al. 2013
<i>Mus musculus</i>	Apoptosis inhibition	Survival of embryo inner-mass	Beclin 1-dependent macroautophagy	Yue et al. 2003
<i>Mus musculus</i>	Removal of dying cells	Embryonic cavitation	Beclin 1-dependent macroautophagy	Qu et al. 2007
<i>Mus musculus</i>	Regulation of proliferation and apoptosis	Neurulation	Ambra 1-dependent macroautophagy	Fimia et al. 2007
<i>Mus musculus</i>	Notch degradation	Neural, bone marrow and gut differentiation	Selective macroautophagy	Wu et al. 2016
<i>Mus musculus</i>	Regulation of autophagy gene expression	Hedgehog signalling	Canonical macroautophagy	Sanchez et al. 2012
<i>Danio rerio</i>	Apoptosis inhibition in blastema	Caudal fin regeneration	Canonical macroautophagy	Varga et al. 2014

excessive autophagy. Once *Hydra* is severed and the regenerative process is activated, Kazal1 expression is induced in the regenerating tips. Upon silencing of Kazal1, the amputation stress determines an alteration of the gland cells, vacuolisation of the digestive cells, and death. This evidence suggests that Kazal1 has a cytoprotective role and its activity is required to prevent excessive autophagy and allow the cells to recover from the amputation stress (Galliot 2006). Thus, this study clearly confirms the importance of maintaining a well-balanced autophagy flux to keep *Hydra* regeneration efficient (Galliot et al. 2018). A demonstration that autophagy occurs in a tightly controlled fashion comes from pharmacological modulation of autophagy. In fact, the administration of both activators (i.e. rapamycin) and inhibitors (i.e. wortmannin and bafilomycin) leads to a delay in the regeneration process (Chera et al. 2009).

Besides solitary polyps, at least three settings in which autophagy plays a key role have been reported in cnidarians: (1) in *Hydractinia symbiolongicarpus*, autophagy is activated in the contact zone between incompatible colonies and is necessary to block their fusion, thus demonstrating a role for the autophagic process in the hydroid allorecognition process (Buss et al. 2012); (2) using RNASeq analysis, Fuess et al. (2017) showed that the autophagic process is switched on in disease-tolerant *Porites* spp. in response to immune stress. Not only does autophagy allow the digestion of non-essential cell components to mobilise resources that can help to cope with the immune challenge, it also represents an ancient form of innate immune response in these organisms that can contribute to removing potential pathogens; (3) Downs et al. (2009) reported that during light and temperature stress, zooxanthellae are consumed in the host cell by symbiophagy, a peculiar process

derived from the innate intracellular protective pathway termed xenophagy. This mechanism, which leads to the loss of the symbiont, has been found to be involved in coral bleaching.

Autophagy in planarian regeneration

Planarians are freshwater flatworms that belong to the phylum Platyhelminthes. Much attention has been devoted to these animals due to their ability to regenerate a full organism from a part of the body in a few days (Reddien & Sánchez Alvarado 2004; González-Estévez 2008; Elliott & Sánchez Alvarado 2013). Planarians are able to remodel their body as a consequence of asexual reproduction (fission), after amputation, or depending on food availability or the temperature of the environment (Brøndsted & Brøndsted 1956; Bowen et al. 1976; Hori & Kishida 1998). During starvation, for example, they decrease in size, reaching a size of less than 1 mm without any physiological impairment (shrinkage), but this process is reversible and their size increases as soon as food becomes available. This ability relies on the continuous proliferation of somatic stem cells, called neoblasts. Neoblasts are spread throughout the body of planarians, except for the head and the pharynx, and represent about 25–30% of total cells in the parenchyma (Baguña et al. 1989). They are the only dividing cells in the animal and are considered totipotent cells. Neoblasts proliferate to maintain physiological cell turnover or during the remodelling process; conversely, an interruption of cell proliferation and an increase in cell death processes occur during starvation, thus leading the animal to shrink (Baguña 1974; Baguña & Romero 1981; Romero & Baguña 1991). Planarians are an excellent model for studying programmed cell death (PCD), particularly the role of autophagy in cell death. Preliminary studies on autophagy were performed by Bowen et al. (1976, 1982) at the end of the last century. By using microscopical, autoradiographic, cytochemical and biochemical techniques, these authors highlighted the importance of autophagy in starving and regenerating adults of *Polycelis tenuis*. More recently, González-Estévez et al. (2007a,b) studied tissue remodelling during *Girardia tigrina* regeneration, a process that involves a combination of cell proliferation, apoptosis and autophagy. In particular, these authors investigated the function of Gtdap-1, the ortholog of human death-associated protein 1 (DAP-1), which is a positive mediator of programmed cell death originally identified in HeLa cells (Deiss et al. 1995). After cloning Gtdap-1, they analysed its expression pattern in planarians after amputation or starvation and demonstrated that the

gene was up-regulated in the region where remodelling occurred. Five days after amputation, Gtdap-1 became expressed in nearly half of the cells and in 40% of neoblast-like cells. By transmission electron microscopy (TEM) and in situ hybridisation they clearly demonstrated that this gene was expressed only in cells showing an autophagic morphology, while it was not associated with apoptotic cells. Although a co-presence of Gtdap-1 mRNA and cleaved caspase-3 was demonstrated in some cells, a colocalisation of Gtdap-1 with TUNEL positive cells was never observed. Gtdap-1 loss-of-function by RNAi showed a decrease in caspase-3 activity and an impairment of the remodelling process by reducing cell death and proliferation of neoblasts, corroborating the idea that autophagy and proliferation are tightly associated in planarians undergoing stress. Conversely, Gtdap-1 gain-of-function experiments increased cell death, demonstrating the involvement of Gtdap-1 in autophagy. Thus, this work provided evidence on the role of DAP-1 factor, a kinase that is usually involved in the apoptotic pathway and in the regulation of cell death, in the autophagic process. Some years ago, Conte et al. (2011) identified another important factor of the autophagy pathway in planarians. In particular, they cloned in *Dugesia japonica* a homologue of hsp90, named Djhsp90, a key component of chaperone-mediated autophagy. Due to the high expression of Djhsp90 in gastrodermal cells, the authors suggested a cytoprotective role for this gene in planarian gastrodermis (Conte et al. 2011). Since hsp90 is a key component of CMA, the authors hypothesised the existence of this type of autophagy in *D. japonica* and supposed that Djhsp90 expression in planarian cells could be associated with their capability to cope with starvation thanks to the recruitment of CMA. Unfortunately, to date the regulatory mechanism of CMA, as well as the correlation between Djhsp90 and cell death in planarians, has not yet been investigated. In 2018, three orthologues of the hsp90 gene (Djhsp90-1, 2 and 3), obtained from *D. japonica* transcriptome, were characterised (Dong et al. 2018). The authors demonstrated that these genes play different functions in planarians: in fact, they have a cytoprotective role in response to external stress, represent important regulators for maintaining the balance among proliferation, differentiation and cell death of neoblasts, and are also implicated in autophagy. Nevertheless, the precise mechanism of action of these genes remains unclear. Despite a growing interest in autophagy in planarians, information about the autophagic signalling pathway in these invertebrates remains limited. In this context, Ma et al. (2018) cloned the full-length cDNA of Atg7

in *D. japonica*. They showed that this gene is highly expressed in the intestinal branch and its expression gradually increases during regeneration. In addition, numerous autophagic structures have been observed in the cytoplasm of planarian tissues undergoing regeneration, providing, for the first time, morphological evidence of the autophagic cell death process during planarian regeneration. According to the results obtained, the authors hypothesise that autophagy provides the energy needed for the regeneration process.

The multifunctional role of autophagy in nematode development

Caenorhabditis elegans is a renowned model organism for the study of PCD. The life cycle of this nematode includes an embryonic phase, four larval stages and the adult stage. According to environmental conditions (i.e. temperature, food availability, and population density), newly hatched larvae can arrest their development and enter an alternative larval stage, namely dauer diapause, which involves a remodelling of different organs. This process was proven to be important for the survival of the worm under adverse conditions (Cassada & Russell 1975). Dauer larvae are subjected to morphological modifications: in this context an important role is played by hypodermal seam cells, which contribute to the constriction of the body and the formation of the alae, a cuticle ridge involved in nematode locomotion (Singh & Sulston 1978). Furthermore, dauer larvae show a different behaviour compared to normal worms (Cassada & Russell 1975; Albert & Riddle 1983). They can survive for several months without feeding but, when conditions are once again favourable, they return to normal development and proceed towards the adult stage. Autophagy is fundamental for normal dauer morphogenesis. Orthologs coding for different autophagy proteins have been found in the *C. elegans* genome (reviewed by Samara & Tavernarakis 2008; Palmisano & Meléndez 2018). In particular, autophagy factors such as Bec-1 (orthologue of Beclin1/Atg6), Atg1, Atg7, Atg8 and Atg18 are necessary for proper dauer morphogenesis (Meléndez et al. 2003) and, accordingly, RNAi silencing of these genes leads to a failure in the development of dauer larvae. Two homologues of Atg8, namely LGG-1 and LGG-2, were found in *C. elegans*, as well as in other nematode species (Alberti et al. 2010). It has been shown that GFP::LGG-1 positive structures and autophagosomes are abundant in hypodermal seam cells (Meléndez et al. 2003), and the expression pattern

of LGG-1 and LGG-2, which is normally overlapping, is modified during dauer formation (Alberti et al. 2010). At this stage, concomitantly to autophagy activation, the localisation of both GFP::LGG-1 and GFP::LGG-2 becomes more punctuated and less diffuse (Meléndez et al. 2003; Alberti et al. 2010). Although both genes are important for the formation of dauer larvae, more studies are needed to clarify the relation between them in this context.

In *C. elegans*, autophagy is activated not only during the larval stage-dauer transition, but also intervenes (1) after fertilisation, to remove paternal mitochondria from the early embryo (Al Rawi et al. 2011; Sato & Sato 2011); and (2) during the larval stage, where it plays a key role in neural development. In different neuron types (AIY and PVD) of *C. elegans*, autophagy intervenes in the control of the presynaptic assembly and the rate of axon outgrowth (Stavoe et al. 2016). Stavoe et al. (2016) showed that during nematode development, several components of the autophagy machinery (i.e. factors of the initiation, nucleation and elongation phases) are involved in synapse vesicle clustering in AIY neurons. In particular, the progression of neurodevelopment requires the correct localisation of Atg9 mediated by a kinesin in the presynaptic region and in the tip of the growing axons, where the protein regulates the biogenesis and the spatial distribution of autophagosomes. Furthermore, it has been demonstrated that autophagy controls neural development through a regulation of the cytoskeleton, and an impairment of the autophagic pathway in AIY neurons leads to cytoskeletal defects, with a consequent mislocalisation of synaptic vesicles (Stavoe et al. 2016).

In nematodes, autophagy also intervenes in regulating post-developmental events and, in particular, in controlling the *C. elegans* lifespan. Genetic studies have shown that various Atg genes (i.e. bec-1, Atg7 and Atg12) are involved in this process (Meléndez et al. 2003; Hars et al. 2007). An inactivation of the autophagy pathway results in a shorter lifespan of the worm, thus corroborating the role of autophagy in aging control (Tóth et al. 2008).

Autophagy is essential for insect metamorphosis

Cell death phenomena occur extensively during development and metamorphosis of holometabolous insects and are necessary to remove tissues and organs typical of the embryonic or larval life (Romanelli et al. 2014). Lepidoptera and *Drosophila melanogaster* are the main models that have been used to study autophagy in insect metamorphosis: lepidopteran larvae are in fact amenable to endocrinological studies, while the

fruit fly, thanks to well-characterised genetics, represents a useful system to dissect the molecular mechanisms of autophagy in insects (Tettamanti et al. 2011). In holometabolous insects, autophagy occurs in various larval organs that are remodelled at various degrees, or even degenerate, during the larva–pupa transition (Tettamanti et al. 2007, 2011; Franzetti et al. 2012; Tian et al. 2013; Romanelli et al. 2016; Montali et al. 2017) (Figure 3).

In eukaryotes, a coexistence of autophagy and apoptosis has been described in many biological settings, and it has been shown that the role of the two processes and the relationship between them is context-specific. In general, autophagy may have two main functions where it coexists with apoptosis: it can play a pro-survival role, for example by inhibiting apoptosis, or it can, directly (i.e. autophagic cell death) or indirectly (by activating apoptosis), lead to the death of the cell (Eisenberg-Lerner et al. 2009; Mariño et al. 2014). Unfortunately, although a co-occurrence of autophagy and apoptosis has been observed in many insect tissues, the regulation of the two processes in these organisms is still under debate, and the overlap with apoptosis makes the assessment of the role of autophagy in this setting difficult. In *Drosophila*, autophagy actively cooperates with apoptosis in the removal of salivary glands during metamorphosis. Accordingly, in Atg gene knockout or knockdown flies, an incomplete degradation of this organ is observed. Conversely, the overexpression of Atg1 determines a premature degradation of salivary glands without any involvement of caspases (Berry & Baehrecke 2007). Besides salivary glands, autophagy is also necessary for the degeneration of *Drosophila* larval midgut. Similarly to what is observed in salivary glands, loss-of-function Atg mutants or knockdown of Atg1 and Atg18 severely impair midgut removal. In addition, the high level of caspases observed in this context seems to be dispensable for midgut degeneration (Denton et al. 2009). A third tissue in which an interaction between autophagy and apoptosis has been

observed in the fruit fly is the larval fat body, which is considered analogous to the adipose tissue and liver of vertebrates. The overexpression of Atg1 in this tissue is sufficient to induce caspase-dependent cell death and the appearance of apoptotic features in degenerating adipocytes, thus substantiating the hypothesis that autophagy can induce apoptosis (Scott et al. 2007). Finally, the involvement of the autophagic process has been also observed in *Drosophila* oogenesis, where the degradation of the inhibitor of apoptosis protein (IAP) dBruce, mediated by autophagy, induces caspase activation and triggers apoptosis (Nezis et al. 2010).

Although the regulatory mechanisms underpinning the developmental processes in which autophagy and apoptosis cooperate were well known in *Drosophila*, the coexistence of autophagic and apoptotic features has been widely described also in Lepidoptera (Tettamanti et al. 2007; Franzetti et al. 2012; Tian et al. 2012, 2013). In these insects, homologues of various components of the autophagic pathway, including Atg proteins and other factors that are involved in the PI3K signal transduction pathway, were identified (Zhang et al. 2009; Romanelli et al. 2014). Autophagic features have been observed during metamorphosis in many lepidopteran organs, such as the midgut (Tettamanti et al. 2007; Franzetti et al. 2012; Li et al. 2018), the fat body (Muller et al. 2004; Casati et al. 2012; Tian et al. 2013) and the silk gland (Li et al. 2010, 2011; Montali et al. 2017). In the fat body of *Bombyx mori*, autophagy is activated by the 20-hydroxyecdysone (20E) commitment peak at larval–pupal transition (Tian et al. 2013). By using different experimental approaches, such as 20E injection and RNAi of genes coding for Atg proteins and ecdysone receptor, Tian et al. (2013) were able to modulate the occurrence of autophagy in the silkworm during metamorphosis. Their study clearly demonstrates that 20E, on one hand, inhibits the PI3K/Torc1 pathway switching autophagosome initiation and, on the other hand, activates Atg gene transcription contributing to autophagosome formation. In terms of the fat body, autophagy seems to be necessary

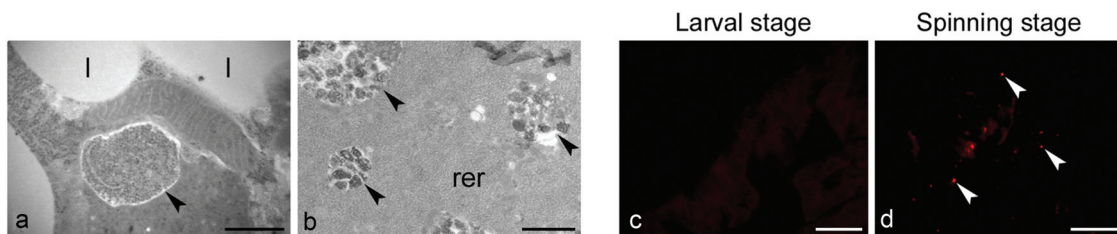


Figure 3. Autophagy in insect organs. During metamorphosis, the presence of autophagic compartments (arrowheads) can be observed in (a) the larval fat body and (b) the silk gland. The activation of the autophagic process in the midgut epithelium is confirmed by an increase in BmAtg8-positive puncta during the larva–pupa transition (arrowheads in c and d). l: lipid droplet; rer: rough endoplasmic reticulum. Scale bars: A = 0.5 µm; B = 2 µm; C, D = 200 µm.

to exploit nutrients that are stored in this adipose tissue to support the growth and differentiation of the adult organs (Tian et al. 2013). In Lepidoptera, the regulatory pathways of autophagy and apoptosis seem to be strictly linked and, probably, triggered by the same set of signals. In particular, it has been shown that 20E can mediate the developmental activation of both autophagy and apoptosis in many lepidopteran organs (Sekimoto et al. 2006; Matsui et al. 2012; Tian et al. 2012, 2013), as described for *Drosophila* (Romanelli et al. 2014). Moreover, it has been recently hypothesised that the induction of apoptosis in *B. mori* can be mediated by Atg proteins (Xie et al. 2016), thus corroborating the idea that the two mechanisms can interact also in Lepidoptera.

The overlap between autophagy and apoptosis, as well as the role of the autophagic process during tissue remodelling in Lepidoptera, has been well studied in the larval midgut of *B. mori* and *Helicoverpa armigera* (Franzetti et al. 2012; Li et al. 2016, 2018; Romanelli et al. 2016). Autophagy is activated in larval midgut cells at the beginning of metamorphosis to cope with nutrient starvation, and leads to the complete digestion of the cells. Apoptosis is activated later on and brings the cells to death (Romanelli et al. 2016). The cytoplasmic content, released by dead larval cells in the extracellular environment, can be absorbed by the new midgut epithelium that forms in the surrounding region. Thus, autophagy is needed to recycle products that derive from the degeneration of larval cells and can be used as nutrients by the moth (Franzetti et al. 2015). A pro-survival role of autophagy has been also described in the silk gland of *B. mori*. In this organ, autophagy is necessary to produce the energy that the gland cells need to survive until the spinning process of the silk cocoon is completed. In conclusion, the evidence collected in the larval midgut (Tettamanti et al. 2007; Franzetti et al. 2012, 2015; Romanelli et al. 2016), silk gland (Montali et al. 2017) and fat body (Tian et al. 2013) seems to indicate that the remodelling of larval organs in Lepidoptera occurs by cell death with autophagy, and calls into question the existence of autophagic cell death in these insects.

Role of autophagy during development and tissue remodelling in vertebrates

The majority of approaches used to evaluate the role of autophagy during vertebrate embryogenesis are based on knockout of Atg genes in mice and zebrafish (Wada et al. 2014). However, the observed phenotypes vary considerably depending on the

Atg gene that was inactivated. Two main reasons can explain these differences: (1) the lack of phenotype with respect to other Atg genes could be due to the fact that inactivation of a given Atg gene does not entirely abolish autophagic activity; (2) the presence of a phenotype associated only with a specific Atg gene could be due to the fact that this gene also plays roles in other processes in an autophagy-independent manner. In this section of the review, we will mainly focus on experimental evidence that directly links embryonic defects of Atg knockout animals to an impaired autophagy activity during development.

Degradation of intracellular deposits to support new synthesis in nutrient-limiting conditions

General and tissue-specific Atg knockout mice demonstrated the essential role of autophagy in early and late steps of vertebrate development. Autophagy is required in fertilised oocytes to allow the degradation of maternal mRNA, proteins and organelles to sustain the early stage proliferation (Tsukamoto et al. 2008; Wada et al. 2013). In these cells, autophagy is induced by a rise of Ca^{2+} after oocyte fertilisation and is sustained by maternal Atg proteins. The mutant phenotype was therefore observed in oocyte-specific knockout animals.

After the post-fertilisation steps described above, the Atg genes involved in LC3 lipidation, such as Atg5 and Atg7, appear not to be essential for embryogenesis, since their knockout embryos properly complete all developmental steps (Kuma et al. 2004; Komatsu et al. 2005; Sou et al. 2008). Atg5 and Atg7 expression is instead essential soon after birth. Autophagy is acutely induced in newborns where it plays a key role in the survival of animals during the physiological nutrient starvation that occurs before breastfeeding care (Kuma et al. 2004; Komatsu et al. 2005; Sou et al. 2008).

Whether autophagy is not required at all to support energy-consuming processes, such as cell growth and migration, during embryo development remains to be elucidated. In fact, at variance with Atg5 and Atg7, inactivation of early regulators of the autophagy process, such as components of Ulk1 and Beclin-1/Vps34 complexes (Yue et al. 2003; Zhou et al. 2011), shows severe embryonic phenotypes. The contribution of autophagy impairment to these early development phenotypes remains, however, to be determined. Of note, a so-called “non-canonical” form of macroautophagy, which requires only a subset of Atgs (Dupont & Codogno 2013), has been reported in brain, liver, erythrocytes and heart

of mouse embryos and is not affected by mutation in Atg5 or Atg7. In light of these findings, it remains to clarify whether non-canonical forms of autophagy may compensate for the inactivation of the LC3 lipidation pathway (Nishida et al. 2009).

Moreover, microautophagy also plays a key role during early development. In fact, it has been demonstrated that microautophagy is required in the visceral endoderm, an epithelial layer of polarised cells that governs morphogenetic signals and nutrient uptake at the onset of gastrulation. In particular, microautophagy cooperates with the endocytic pathway to guarantee the delivery of ingested material to the apical vacuole, which is essential to accomplish gastrulation (Kawamura et al. 2012).

Remodelling of organelles/cell structures during development

Autophagy is the only intracellular degradative process able to degrade entire organelles. Removal of unnecessary or potentially harmful organelles is observed at different stages of development. An interesting example is represented by maternal transmission of the mitochondrial genome, which is ensured by the rapid degradation of paternal mitochondria after fertilisation. This degradation is performed by the autophagic process and likely prevents the permanence of “heavily used” organelles in pluripotent cells, and has been observed in worms and in mammals (Sato & Sato 2011; Rojansky et al. 2016).

Mitochondria are also removed by autophagy at the end of erythrocyte differentiation. This selective removal is guaranteed by expression on the mitochondrial outer membrane of the protein Bnip3/Nix, which binds to LC3 and allow autophagosome engulfment (Schweers et al. 2007; Sandoval et al. 2008).

Besides membrane-enclosed organelles, other cell structures are targeted by autophagy during early development. Autophagy is required for the degradation of the midbody, a structure required for the separation between daughter cells during cytokinesis. It was observed that, in stem cells, midbody derivatives are inherited asymmetrically and are found in the daughter cell that contains the older centrosome, which maintains pluripotency. Conversely, in cell-differentiating cells, autophagy degrades midbody derivatives through the binding of the autophagic receptor Nbr1 to the midbody protein Cep55 (Pohl & Jentsch 2009; Kuo et al. 2011). Notably, autophagy inhibition results in

accumulation of midbody derivatives, which promotes stemness and inhibits differentiation.

Another example of how autophagy regulates cytoplasm remodelling is the control of the axonal growth in cultured postmitotic neurons (Tomoda et al. 2004). Autophagy inhibition, achieved by silencing the expression of Atg7, results in an increased axon length, while dendrite length and axon branching is unaffected. A possible mechanism through which autophagy may regulate the axonal growth is the degradation of RhoA, a small guanosine triphosphatase (GTPase) belonging to the Rho protein family that plays a key role in the modulation of axon morphology (Ban et al. 2013).

Role of autophagy in regulating proliferation and survival of precursor cells during tissue morphogenesis

Inactivation of autophagy initiation machinery by mutating Beclin 1 in mice results in early embryonic lethality at stages E7.5. Beclin 1 $-/-$ mice show a severe developmental delay, which causes embryonic lethality associated with a defective autophagy. Analysis of Beclin 1 $-/-$ stem cells, derived from embryo inner mass, reveals the inability of embryoid bodies to expand due to extensive cell death (Yue et al. 2003). Interestingly, a similar defect was observed *in vitro* in Atg5 $-/-$ stem cells.

Mice with knockout of Ambra1, a proautophagic protein essential for Ulk1 and Beclin 1 complex activity (Antonioli et al. 2014, 2017), show defects in the development of the central nervous system, the site where the gene is first expressed before becoming ubiquitously distributed at the late stages of embryogenesis and in adult mice (Fimia et al. 2007). Ambra1 mutant mice show impaired autophagy, accumulation of ubiquitinated protein, unbalanced cellular proliferation and excessive apoptotic cell death, which cause exencephaly and spina bifida and embryonic lethality between E10.5 and E13.5 (Fimia et al. 2007; Antonioli et al. 2015). Similarly, inhibition of the paralogous genes *ambra1a* and *ambra1b* in *Danio rerio* leads to incomplete development, with prelarvae surviving only for 3 and 4 days after fertilisation, which is associated with severe embryonic malformations, such as smaller head, reduced eyes and trunk, curved or twisted tail and delayed pigmentation, compared with controls (Benato et al. 2013). Mutated *D. rerio* embryos show low autophagic activity and increased cell death, further highlighting the functional relationship between autophagy and apoptosis during embryogenesis.

It must be noted that Beclin1 function in membrane dynamics is not restricted to autophagy, taking part also in particular types of endocytosis and

phagocytosis. For instance, the activation of Toll-like receptor signalling by cell corpses in macrophages causes a rapid translocation of Beclin 1 to phagosomes, which mediates the translocation of LC3 to ensure the fusion with lysosomes (LC3-associated phagocytosis, LAP) (Sanjuan et al. 2007; Martinez et al. 2011). Consistently, Beclin 1 was described as essential to generate engulfment signals for the phagocytosis of dying cells during development (Qu et al. 2007).

Role of autophagy in the regulation of developmental signalling pathways

Recently, important functional crosstalks between the autophagic process and different morphogenetic signalling pathways, such as Notch, Wnt and Hedgehog, have been identified.

In stem cells, Notch signalling is important for the maintenance of pluripotency and is shut down when differentiation is triggered (Bray 2006). Autophagy contributes to the inhibition of this signalling by targeting Notch to the lysosome for degradation. In fact, Notch1 was found to colocalise with LC3 upon autophagic stimulation, and Beclin1 over-expression is sufficient to trigger Notch1 decrease. Notably, Atg7 or Atg16l1 knockdown *in vitro* and *in vivo* causes an increase in Notch levels, resulting in a delay of neural, bone marrow and gut differentiation (Wu et al. 2016).

Autophagy also targets components of the Wnt pathway, which plays an important role in the regulation of several aspects of development and tissue self-renewal. Gao and coworkers demonstrated that autophagy can negatively regulate Wnt signalling by promoting the degradation of the Dishevelled (Dvl) protein upon nutrient starvation in tumor cells. This degradation is mediated by the ubiquitination of Dvl by the E3 ubiquitin ligase Vhl (Von Hippel-Lindau) and its binding to the autophagy adaptor p62 (Gao et al. 2010). However, since these studies were mainly performed in tumor cells, the impact of autophagy-mediated degradation of Dvl during development remains to be assessed.

Moreover, several lines of evidence show that hedgehog (Hh), a key signal for neural tube formation and the induction of ventral neural fate, can act by regulating autophagy levels (Østerlund & Kogerman 2006). For example, Hh is able to inhibit autophagy throughout the transcription factors *Cubitus interruptus* in *Drosophila* and *Gli2* in mammals. The observation that *Gli2* knockout embryos show increased levels of LC3-II confirms that autophagy is indeed regulated by Hh during development (Sanchez et al. 2012). The

autophagy genes that are repressed by Hh have been partially characterised. An interesting example is represented by the protein kinase Perk, which controls protein translation and autophagy in response to endoplasmic reticulum stress. However, Perk down-regulation per se does not completely recapitulate the effect of Hh on autophagy.

An emerging mechanism by which autophagy may regulate morphogenetic pathways is by controlling the formation and activity of the primary cilium (PC). PC is a non-motile organelle of plasma membrane responsible for sensing both mechanical and chemical extracellular signals (Satir et al. 2010). Alterations in cilia function cause ciliopathies, a series of pathologies ranging from polycystic kidney disease to respiratory diseases, cognitive impairment disorders and developmental disorders. Interestingly, receptors and signalling molecules critical for vertebrate development and tissue homeostasis are enriched on the PC, including proteins involved in the Hh, Wnt and Notch pathways.

Autophagy has been shown to regulate ciliogenesis, both positively and negatively. In nutrient-rich conditions, basal autophagy limits PC growth by degrading the intraflagellar transport protein Ift20. Instead, autophagy induced by serum starvation results in the induction of ciliogenesis, by selectively targeting the PC growth repressor *Ofd1* for degradation (Pampliega et al. 2013; Tang et al. 2013).

Altogether, these findings highlight a complex crosstalk between autophagy and morphogenetic pathways, whose impact on development still requires a more in-depth characterisation.

Autophagy is required for zebrafish caudal fin regeneration

The role of autophagy in tissue regeneration remains largely uncharacterised. Important evidence on the contribution of autophagy to this process has been recently obtained in zebrafish, a vertebrate model in which tissue regeneration has been shown to occur in different districts, such as the cardiac ventricles after injury and the caudal fin after amputation.

In particular, the regeneration of caudal fin is based on the formation of the blastema, a highly proliferative tissue located in a distal region from the site of amputation, which is regulated by fibroblast growth factors (FGFs) and is generated via dedifferentiation of mature somatic cells, although a contribution of stem cells has been also reported.

By using LC3-GFP transgenic zebrafish, Varga et al. (2014) demonstrated that autophagy is induced early upon amputation in the blastema, 2

days after caudal fin amputation. In particular, the expression of LC3-GFP is high in the proliferating zone, suggesting that autophagy is active during the dedifferentiation–redifferentiation steps. The induction of autophagy is mediated by signalling pathways activated by FGFs. Notably, inhibition of autophagy by RNA interference or chemical approaches severely impairs regeneration due to a decrease in blastema proliferation and a massive induction of programmed cell death. Whether autophagy activity is required to sustain the metabolic requirements necessary for cell proliferation and differentiation, or to degrade cytoplasm components during the dedifferentiation step, is a key question that is expected to be elucidated soon using the zebrafish model system, which is easily manipulated through genetic and pharmacological approaches (Varga et al. 2014).

Conclusion and perspectives

Several studies in different model organisms have shown that the inhibition of autophagy genes has a significant impact on development. Different types of autophagy are required at different stages of development, mainly when energy stores are limiting or profound modifications in cell architecture are requested through a rapid renovation of intracellular components. Development-selective forms of autophagy appear to have evolved across species, depending on the specific characteristics of the organisms.

Many aspects still remain unclear. In this regard, two major issues are worthy to address in invertebrates: (1) given the difference in the role and regulation of autophagy in various insect organs during metamorphosis, it will be necessary to understand the regulatory mechanisms that underpin organ-specific occurrence of autophagy in these organisms; and (2) although KFERQ-targeting motifs of CMA substrates are conserved between *Drosophila* and mammals, thus supporting the existence of a CMA-like pathway in invertebrates, further studies are needed to address this point.

Similarly, the identification of new specific regulators of microautophagy, CMA and the emerging non-canonical types of macroautophagy in vertebrates is definitely required to better define the roles of these processes in development. Moreover, an in-depth characterisation of how morphogenetic pathways rely on autophagy to carry out their function at different stages of embryogenesis will provide in the near future a more comprehensive view of the molecular mechanisms by which autophagy controls development.

Acknowledgements

This work was supported by grants from the Italian Ministry of University and Research (PRIN2015 20152CB22L) and by FAR 2017 (University of Insubria). AM is a PhD student in the “Life Science and Biotechnology” course at the University of Insubria.

Funding

This work was supported by the FAR 2017 (University of Insubria) [FAR 2017]; and Ministero dell’Istruzione, dell’Università e della Ricerca [PRIN 20152CB22L].

Disclosure statement

No potential conflict of interest was reported by the authors.

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