Autophagy and its physiological relevance in arthropods

Current knowledge and perspectives

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The autophagic process is one of the best examples of a conserved mechanism of survival in eukaryotes. At the molecular level there are impressive similarities between unicellular and multicellular organisms, but there is increasing evidence that the same process may be used for different ends, i.e., survival or death, at least at the cellular level. Arthropods encompass a wide variety of invertebrates such as insects, crustaceans and spiders, and thus represent the taxon in which most of the investigations on autophagy in nonmammalian models are performed. The present review is focused on the genetic basis and the physiological meaning of the autophagic process in key models of arthropods. The involvement of autophagy in programmed cell death, especially during oogenesis and development, is also discussed.

Introduction

Arthropods represent an extremely fascinating group of metazoans including such different animals as insects, ticks, spiders and crustaceans. This wide taxon contains organisms that are of great interest for comparative biology, parasitology, zoology, ecology and agronomy, and therefore it is not surprising that numerous publications indicate animals from this taxon, particularly Drosophila melanogaster, as model organisms.1,2 Arthropod and vertebrate evolutionary history diverged more than 500 million years ago,3,4 but the molecular basis of several fundamental biological functions were already established in their common progenitor and have been conserved. As a consequence, much of the information derived from arthropod models proved important to gain deeper insights into human biology and pathology.5 Macroautophagy (henceforth autophagy), is a process involving intracellular membrane structures and lysosomes that developed in unicellular eukaryotes most probably to face starvation and for degrading obsolete proteins and organelles.6,7 The genetic and molecular bases of autophagy have been thoroughly investigated in the recent past6,8 and have been demonstrated to be conserved in metazoans.9 The conservation of basic autophagy mechanisms justifies the use of arthropod models (as they are much easier to manipulate than mammals) to obtain information on the role that autophagy has acquired in complex multicellular organisms.10 However, the conservation of the molecular basis of autophagy should not lead to an oversimplification or to the underestimation of the anatomical and physiological differences between arthropods and mammals. Despite the relevant similarity existing between autophagy-related genes (ATG), during metazoan diversification the autophagic process could have acquired different meaning and relevance in diverse taxa. For instance, the contribution of autophagy to programmed cell death (PCD) appears to be different in insects and mammals11,12 and also between different tissues of the same organism.13

This review will survey recent information on the genetics of autophagy in arthropods, then will focus its attention on the role of autophagy during insect development. In addition, the role of the midgut as a model organ to study autophagy in arthropods will be highlighted.

The Genetics of Autophagy in Arthropods

Tick ATG genes and the possible role of autophagy in ticks.
The ability to survive for prolonged periods without feeding is a hallmark of tick biology.14 Moreover, the survival strategy of ticks is important for the survival of pathogens they transmit. Because of their exceptional longevity, ticks can carry pathogens such as protozoa, rickettsiae, spirochaetes and viruses, over prolonged periods of time. Accordingly, ticks are not only vectors...
but also excellent reservoir hosts for the pathogens they bear.15 Since autophagy can be induced by starvation, it may be a process of remarkable importance to understand tick biology and tick-pathogen interactions during nonfeeding (fasting) periods. Only a few studies have examined ATG genes in ticks. Using EST databases of the hard tick *Haemaphysalis longicornis*, *HIATG12*, an ATG12 homologue, has been identified and characterized for the first time in ticks.16 Subsequently, three other ATG homologues, *HIATG3*, *HIATG4* and *HIATG8*, were isolated in *H. longicornis* (Umemiya-Shirafuji R, et al. unpublished data).

Recently, genomic analyses of hard ticks, *Amblyomma americanum*, *Amblyomma variegatum* [A. variegatum gene index (AvGl)],18 *Ixodes scapularis* (I. scapularis Genome Project),19 *Rhipicephalus appendiculatus* [R. appendiculatus gene index (RaGl)] and *Rhipicephalus (Boophilus) microplus* [B. microplus gene index (BmiGl)]20,21 were completed and the corresponding information is available (http://gs.jcvi.org/projects/msc/ixodes_scapularis/). In addition, ESTs from cDNA libraries of *Amblyomma cajennense*,25 *Dermacentor variabilis*24 and *Ixodes ricinus*26 were reported. Consequently, it has become possible to comprehensively search tick homologues of yeast or mammalian ATG genes. Tick ATG homologues obtained by screening the nucleotide/protein/gene databases publicly available, and the *H. longicornis* EST databases, are summarized in Table 1. Some ATG homologues were discovered in only the protozoan (*Babesia* and *Theileria*) parasitic/rickettsial vector *H. longicornis* and the borrelial vector *Amblyomma cajennense* [A. cajennense gene index (AcGl)]. At least seven putative ATG genes (*ATG3*, *ATG5*, *ATG6*, *ATG7*, *ATG8*, *ATG13* and *ATG16*) were found in *I. scapularis* and they all are involved in autophagosome formation (Table 1). Even if the identity between tick and yeast ATG genes is limited (for example, *HIATG4* shares 25% identity with yeast *ATG4*), amino acid residues that are essential for the function of yeast Atg proteins are highly conserved in *HIATG proteins* (Umemiya-Shirafuji R, et al. unpublished data).26 Factors involved in autophagosome formation, especially the ATG8 and ATG12 conjugation systems, are well conserved, suggesting a functional role of these conjugation systems in ticks.

No ATG homologues were found in tick databases except for *H. longicornis* and *I. scapularis*. However, conformational or functional homologues may exist in other tick species, even though the low degree of identity between amino acid sequences of yeast and the tick species did not allow their identification with common sequence alignment-based algorithms.

At present, the reverse genetic approach of RNA interference (RNAi) is the most widely used gene-silencing technique in ticks and it represents a valuable tool for studying tick gene function, characterizing the tick-pathogen interface, and screening/characterizing tick protective antigens.26 RNAi experiments were mainly performed in vivo on adult ticks. The effect of gene silencing on the double strand RNA-injected ticks is compared with control ticks during/after blood sucking. Most of the targets for RNAi are genes that are upregulated during blood feeding. A method for assessing ATG gene-silencing in ticks is controversial because these genes are downregulated during blood feeding. At present, K.F. and colleagues are trying to knock down tick ATG genes by RNAi and properly evaluate their function during nonfeeding periods.

The genetic regulation of autophagy in *D. melanogaster*. In a multicellular organism such as *D. melanogaster* the number of gene products and regulatory pathways involved in the functioning of the autophagic machinery may reasonably be assumed to be higher than in yeast. Drosophila represents one of the best genetic model systems, which is particularly attractive for studying molecular mechanisms underlying basic cellular processes such as autophagy. Its short generation time, the availability of hundreds of mutant lines, the relatively simple screening methods, and the powerful genetic and molecular techniques make Drosophila an

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**Table 1. List of the principal ATG identified in both mycetes (yeast) and arthropods**

<table>
<thead>
<tr>
<th>Yeast</th>
<th><em>H. longicornis</em></th>
<th><em>I. scapularis</em></th>
<th><em>D. melanogaster</em></th>
<th><em>B. mori</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>Gene (Accession no.)</td>
<td>Gene</td>
<td>Gene (Accession no.)</td>
<td>Gene (Accession no.)</td>
</tr>
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<td>ATG3</td>
<td>HIATG3p (ABS13349)</td>
<td>Autophagy protein, (putative) XM_0024022725</td>
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<td>-</td>
</tr>
<tr>
<td>ATG4</td>
<td>HIATG4p (ABS13350)</td>
<td>-</td>
<td>ATG4 NM_134719 BmAATG4 FJ416326</td>
<td></td>
</tr>
<tr>
<td>ATG5</td>
<td>-</td>
<td>Autophagy protein, (putative) XM_002414126 ATG5 NM_132162 BmAATG5 FJ418152</td>
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<td></td>
</tr>
<tr>
<td>ATG6</td>
<td>-</td>
<td>Beclin, putative XM_002414804 ATG6 NM_142952 BmAATG6 FJ416328</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATG7</td>
<td>-</td>
<td>Autophagy protein, (putative) XM_002406294 ATG7 NM_137506 BmAATG7 BGIBMGA001467</td>
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<td></td>
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<tr>
<td>ATG8</td>
<td>HIATG8b (ABS13351)</td>
<td>Gamma-aminobutyric acid receptor-associated protein (putative) XM_002408326 ATG8a ATG8b NM_167245 NM_142392 BmAATG8 FJ416330</td>
<td></td>
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<tr>
<td>ATG12</td>
<td>HIATG12</td>
<td>-</td>
<td>ATG12 NM_140294 BmAATG12 FJ416329</td>
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<tr>
<td>ATG16</td>
<td>-</td>
<td>WD domain and G-beta repeat containing protein (putative) XM_002403210 - - BmAATG16 BGIBMGA006504</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Genes homologous to yeast ATG that have been retrieved only in ticks or insects have been omitted. *Genes involved in the autophagosome formation.* R.U.-S., et al. unpublished data. *The accession numbers will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases in the future (R.U.-S., et al. unpublished data).* http://silkworm.genomics.org.cn/

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incisive tool for analyzing the regulation of autophagic processes at molecular and genetic levels. The discovery of new players in the regulatory network and molecular machinery of autophagy might provide new targets for drug design and might help us in fighting human pathologies such as neurodegenerative disorders, cell damage that follows cerebrovascular and myocardial strokes, viral infections and cancer. Moreover, since some ATG genes have a significant influence on longevity, a particularly interesting field of research will be the investigation of the relationship between autophagy and aging.

In order to identify several yet unknown genes that are involved in autophagy, the forward genetic approach can be used. The P-element (or EP-element), by causing loss-of-function mutations, allows the genetic dissection of the developmental autophagy and the observation of the direct effects of mutations. Some of the first results achieved by forward genetic approaches recently emerged in literature, and here the analysis will be focused on Blue cheese (bchs), snf4Aγ, Liquid facets (lfj), Paxillin (pax) and fbkbp39 genes.

Blue cheese is localized in the autophagosomes and its suggested function is the elimination of ubiquitinated proteins from neurons during aging. Using the candidate gene approach, a number of genetic interactions between bchs and known and novel ATG genes were identified. 27

In M.S.S laboratory, more than 300 late larval-lethal, P-element-induced mutant lines were tested. The original screening was carried out by light and electron microscopy methods and led to the identification of 21 potential lines in which the formation of autophagosomes and/or autolysosomes could not be induced by 20-hydroxyecdysone, by starvation or by oxidative stress. In these mutant lines the wild-type phenotype could be restored by remobilization of the P-element. One of the positive hits in that screening is SNF4Aγ, the Drosophila homologue of the AMP-activated Protein Kinase γ subunit. The life span of the snf4Aγ mutants is significantly reduced and in hormone-treated or starved snf4Aγ mutants, autophagy in the fat body is inhibited. Immunocytochemistry shows that SNF4Aγ translocates into the nuclei of fat body cells exactly at the onset of developmental autophagy confirming that SNF4Aγ has an essential role in autophagy of D. melanogaster, most probably via the regulation of TOR kinase activity. 28, 29

Another mutant line identified bears the P-element in the laf gene, which encodes for an Epsin homologue of Drosophila. In laf mutants, autophagy is arrested during postembryonic development, and is a response to starvation or oxidative stress. As observed for snf4Aγ, laf mutants display a shortened life span. 28 GFP-tagged Lqf protein shows an exclusive colocalization with lysosomal markers or GFP-ATG8a-labeled autophagosomes and autolysosomes. Inhibition of TOR kinase does not restore autophagy and the normal development in laf mutant larvae, suggesting that Lqf acts downstream of TOR, the central kinase of the regulatory pathway of autophagy. Finally, it has also been observed that Lqf is essential not only for autophagy, but it fully inhibits receptor-mediated endocytosis of larval serum proteins by larval fat body cells. 30 According to the authors’ interpretation, Lqf might have the same function in the formation of curved membranes, as has been reported for the formation of curved membranes of primary endocytic vesicles. 31

By screening EP insertion lines, Chen et al. 32 identified a line mutated for pax. Paxillin belongs to the actin-binding proteins, which associate with the cytoplasmic domain of integrins, and in this way regulates cell migration, proliferation and survival. 33 In Drosophila pax (Dpax) mutants autophagy is inhibited or remains at a very low level, whereas overexpression of pax induces autophagy in Drosophila. In parallel with these observations, the expression level of ATG1 is strongly decreased in pax loss-of-function mutants. The results showed a strong genetic interaction between ATG1 and Pax. An ATG1-RNAi construct suppresses Pax-induced developmental autophagy. Mutations in the ecdysone receptor reduce both Pax and ATG1-mediated autophagy, and it is also observed that Pax could be a substrate of ATG1 kinase.

In terms of forward genetic approaches, a set of up or down-regulated genes were also identified by a microarray assay in the fat body cells of feeding and wandering D. melanogaster larvae. Among them, it is worth mentioning an autophagy inhibitor, the prolyl isomerase FKBP39, which inhibits the transcription factor FOXO, known as an inducer of autophagy. 34

Since the discovery of ATG genes in yeast more than a decade ago, 35, 36 very intensive research using the reverse genetic approach began in various model organisms. In D. melanogaster, the presence and the functions of the approximately 20 ATG genes have been tested so far in Drosophila using the available powerful genetic tools. Data and hypotheses concerning the physiological role of the best characterized ATG orthologues of the fruit fly (Table 1) are summarized below.

In Drosophila, null mutants for the ATG1 gene die during the late pupal period. 37 The mutation, generated by a deletion in the ATG1 locus, prevents starvation-induced autophagy in larval fat body cells, whereas overexpression of ATG1 generates the formation of autophagic structures. 38 A self-reinforcing feedback loop between TOR (target of rapamycin), a Ser-Thr kinase playing a key role in cell metabolism and growth, and ATG1 was reported. 39 Remarkably, the level of ATG1 mRNA declines in the cells during aging as has been observed also for ATG2, ATG5, ATG8a and ATG18. 40

The presence of an ATG4 homologue in Drosophila has been proven by several genome-wide analyses. 41, 42 In yeast and mammalian cells, the cysteine proteinase ATG4 cuts ATG8, which can then interact with phosphatidylethanolamine (PE). The ATG8-PE complex can be localized in the phagophore or the autophagosome. Later on, as the autophagosomes fuse with lysosomes, ATG8 on the surface of the autophagosomes is released because of a second cleavage by ATG4. 43 To our best knowledge, a detailed molecular analysis of Drosophila ATG4 has not been performed yet, but the same changes in the localization of ATG8 have been observed in Drosophila cells during different experiments analyzing autophagosome and autolysosome formation, suggesting that ATG4 plays the same role in the fruit fly and in mammals. Similarly, in Drosophila the protein ATG5 associates with the outer surface of the phagophore during its formation at the phagophore assembly site (PAS), and when the membrane of
the autophagosome is completed, ATG5 dissociates and is released back into the cytoplasm.44 In the cytoplasm, ATG5 is conjugated to ATG12 and also binds ATG16.45

Studies on viable ATG7 mutants reveal that autophagy has multiple functions in the adult stage. ATG7 mutant animals are more sensitive to stress conditions (starvation, CO₂ stress, hypothermal coma), their life span is shortened, and in their brain ubiquitinated proteins are accumulated in the form of occlusion bodies. This last observation makes it possible to speculate that in the absence of ATG7, the persisting autophagic activity observed in mutant cells presumably represents "alternative autophagy" that does not depend on the ubiquitin-like machinery. In this respect, it has been hypothesized that in neurons of wild-type animals the ubiquitinated proteins are degraded through autophagy.46,47 This hypothesis is strengthened also by the observations collected on another ATG gene, namely ATG8a. In the ATG8a mutant flies, the number of protein aggregates rapidly increases, the mutants have shorter life spans and increased sensitivity to stress conditions. In contrast, the overexpression of ATG8a extends life span by 56% and prevents the accumulation of the protein aggregates in the neurons of the CNS. These results indicate that autophagy, or at least the activity of ATG genes, is necessary for the survival of neurons.46,48

The results originating from forward and reverse genetic approach helped researchers recognize and understand the functions and role of evolutionarily conserved ATG genes in Drosophila. Moreover, these experiments demonstrate that the ATG genes, by regulating autophagy, have a deep impact in several very important biological functions such as cell growth, death and aging.

Insights into the Bombyx mori genome and autophagy. Lepidoptera are reliable organisms for studying not only the regulation of autophagy in a developmental setting, but also the relations between this self-eating process and apoptosis: In fact, autophagy intervenes massively during the larval-pupal phases of butterflies, and previous literature has provided evidence for a complex cross-talk between these two cell death mechanisms. Bombyx mori is a representative model among Lepidoptera, because of indisputable advantages such as a large amount of information gathered on its developmental biology, physiology and endocrinology, the availability of numerous genetic and molecular biology tools, and a completely sequenced genome. In particular, techniques for efficient gene transfer (stable germline transformation through transposon-based vectors or transient expression of genes by using virus vectors) or gene silencing (RNAi) have been developed for this species in the last ten years.49,50 Thus, silkworm can be an excellent nondrosophilid model system for tackling a broad range of questions concerning autophagy.

Bioinformatics analysis revealed that homologues of most of the ATG genes identified in other insect species such as Drosophila are present in the B. mori genome.51 and more than 20 ATG genes are now identified in the silkworm genome. These include the BmAATG1, 3, 4, 5, 6, 7, 8, 9, 12, 16 and 18 genes as well as others, such as the genes involved in the TOR signal transduction pathway.51,52 Among ATG genes, seven are involved in the ubiquitin-like conjugation pathway, whereas 13 genes are involved in the PtdIns3K class I, and PtdIns3K class III signal transduction pathways and in formation of Cvt vesicles and autophagosomes. Some of these genes are actively expressed in different tissues during metamorphosis. High levels of expression of BmAATG1, 5, 6 and 8 were detected in peritracheal athrocytes by using real-time RT-PCR.52 Expression of the genes for the ubiquitin-like conjugation systems (including BmAATG3, BmAATG4, BmAATG8 and BmAATG12), for autophagosome formation (including BmAATG1, BmAATG6 and BmAATG18), and for upstream signal transduction of the autophagy pathway (including homologues of p70S6K, PKB and Rheb) were detected in the silk gland.53 Full-length cDNAs of BmAATG3, BmAATG4, BmAATG5, BmAATG6, BmAATG8 and BmAATG12 were cloned, and the encoded proteins are homogeneous to their orthologues in other eukaryotes. Lack of identification of ATG10 in silkworm, as previously assessed in flies and bees, has been suggested to be due to a possible compensation of ATG10 function by ATG3.51 Transcripts of the BmAATG5, BmAATG6 and BmAATG8 genes and BmAATG8 protein have also been detected in the remodeling midgut (Shi Yanxia, personal communication). The expression patterns and time course of these genes in the midgut were similar to those of BmAATG8 and BmAATG12 in the silk gland and they reached high levels at the 5th instar wandering stage. The existence of autophagy-specific genes in the silkworm genome and their expression in the degenerating silk gland, midgut and other tissues, as well as the increased expression of the lysosomal marker enzyme acidic phosphatase in the anterior silk gland during the prepupal stage,53 imply that the autophagy pathway takes place during metamorphosis in B. mori.

Autophagy and PCD in Arthropod Development

PCD is a genetically regulated and evolutionary conserved process occurring mainly during development in organisms, as a way of removing unwanted cells.54 PCD has been classified into three major subtypes based on morphological criteria. Apoptotic (or type 1) cell death is mainly characterized by caspase activation, chromatin condensation and DNA fragmentation. In autophagy-mediated cell death (type 2), the accumulation of autophagosomes and autolysosomes in the cytoplasm represents the most distinct morphological features of this type of cell death, whereas during necrotic cell death (type 3) the plasma membrane of the cell breaks down, causing inflammation.55,56 The role of autophagy in cell death has been controversial.57 Autophagy can promote both cell death and cell survival under certain circumstances.58,59 Dying cells possess autophagic features, but it is not clear whether autophagic activity causes cell death or acts in parallel to cell death.57

Autophagy and PCD in molting stages of H. longicornis. Studies in H. longicornis reveal that the mRNA expression of HlatG3, HlatG4, HlatG8 and HlatG12 showed higher levels during the nonfeeding period (fasting condition) than the feeding period (Umemiya-Shirafuji R, et al. unpublished data).16 Besides the fasting condition, molting stages also appear to be associated with the increased expression of HlatG genes, suggesting that some functions of HlatG genes may be associated
with metamorphosis (Umemiya-Shirafuji R, et al. unpublished data). Although two caspase-like genes categorized as apoptosis initiators have been identified in H. longicornis, the relationship between these putative caspases and the degradation of tick tissues remains uncertain. In ticks, the degradation of the salivary glands is an obligatory event that occurs after engorgement of female adult ticks, which is characterized by the appearance of autophagic vacuoles in the cytoplasm of the salivary gland cells. In contrast to autophagic PCD in insects, however, recent studies propose that in ticks the degradation of salivary glands during/after feeding may occur by apoptosis rather than by autophagy.

**Autophagy and PCD in regulating oogenesis and ovarian tissue physiology of D. melanogaster.** Studies during Drosophila development have demonstrated that autophagy plays a role in cell death during development. Autophagy promotes caspase-dependent elimination of an extra-embryonic tissue, known as amnioserosa (AS), during the final stages of Drosophila embryogenesis. Downregulation of autophagy results in persistent AS, whereas overexpression of ATG1 results in dissociation of AS, and this process is completely suppressed by co-expression of the caspase inhibitor p35. Likewise, ATG1 overexpression in the Drosophila larval fat body is capable of inducing autophagy and cell death in a caspase-dependent manner. Importantly, mutation of ATG7 results in an inhibition of DNA fragmentation in the mid gut. Taken together, these findings suggest that autophagy can promote cell death during Drosophila development.

A model developmental process for studying the interplay between autophagy and cell death is oogenesis. Oogenesis is a fundamental physiological process in insects and there are intriguing similarities between ovarian cell death in Drosophila and vertebrate species including fish, quail and mammals. Fertility disorders in humans are associated with excessive ovarian cell death. Thus, manipulating autophagy with targeted delivery of inhibitors of autophagy could be a way for treating reproduction disorders in humans.

The insect ovary consists of two lobes, and each one contains linear arrays of developmentally ordered egg chambers, called ovarioles. The structural and functional unit of ovaries is the egg chamber (Fig. 1). Insect egg chambers consist of the oocyte (germline cell) being surrounded by an epithelial layer of somatically-derived follicle cells. In some insects, the oocyte is associated with nutritive cells called nurse cells (germline cells) (Fig. 1). Nurse cells provide the developing oocyte with RNA, proteins and organelles that are necessary for proper development. The follicle cells differentiate into distinct subpopulations, participate in oocyte polarity formation, and during the late stages of oogenesis secrete the complex eggshell that enhances survival of the embryo. Fourteen stages of oogenesis have been described based on morphological criteria in Drosophila, including egg chamber size, the proportion of the egg chamber occupied by the oocyte, the position of the follicle cells and the appearance of the eggshell coverings. Stage 1 represents the 16 cell syncytium immediately after encapsulation by the follicle cells, whereas stage 14 refers to the mature egg chamber where the nurse cells have degenerated and the eggshell is completed.

**Figure 1.** Morphology of an egg chamber in D. melanogaster. Confocal micrograph of a stage-10 egg chamber after propidium iodide (red) and phalloidin-FITC (green) staining to visualize the nuclei and filamentous actin (middle optical section), respectively. The egg chamber consists of the oocyte (OC) and nurse cells (NC) (germline cells), which are surrounded by the follicle cells (FC) (somatic cells). The germline cells are interconnected to each other via intercellular bridges called ring canals (RC). Scale bar: 50 µm.

PCD during oogenesis in insects occurs in the germarium (the anterior tip of each ovariole), and during the middle stages of oogenesis (developmental stages 7–9) and the late stages of oogenesis (developmental stages 12–14) (reviewed in ref. 81).

In D. melanogaster, cell death in the germarium region exhibits features of both apoptosis and autophagy being characterized by caspase activation, chromatin condensation, DNA fragmentation and the formation of autophagosomes and autolysosomes. Cell death in the germarium increases after nutrient deprivation and environmental stress, and therefore is thought to serve as a “checkpoint” mechanism to maintain the proper number of follicle cells that are needed to encapsulate the germline cyst during the beginning of oogenesis. Interestingly, genetic inhibition of autophagy by removing the function of autophagy genes ATG1 and ATG7 results in decreased levels of DNA fragmentation in region 2 of the germarium compared to wild type. These data suggest that autophagy can act upstream of apoptosis in the Drosophila gerarium.

The second “checkpoint” is during the middle stages of oogenesis, 7, 8 and 9. Egg chambers during stages 7–9 undergo cell death and contain degenerated nurse cells, characterized by caspase activation, chromatin condensation, DNA fragmentation, the formation of autophagosomes and autolysosomes and engulfment of nurse cell remnants by follicle cells. This phenotype has been observed in Drosophilidae (D. melanogaster and Drosophila viridis), in the Mediterranean fruit fly Ceratitis capitata, in the olive fruit fly Dacus oleae and in the mosquito.
**Culex pipiens pallens**, but mainly in the follicular epithelium.\(^{93}\)

Cell death during mid-oogenesis, known also as follicular atresia, is sporadically observed during normal development, but is significantly increased as a response to nutritional deprivation, ecdysone signaling inhibition, treatment with chemotherapeutic drugs, frequency of mating, temperature, modern hazards exposure and ectopic death of follicle cells in Drosophila (reviewed in refs. 81, 94 and 95). PCD during mid-oogenesis in *D. melanogaster* is not dependent on known and well established Drosophila cell death regulators *rpr*, *hid*, *grim*, *skl*, or on *ark*, *decb*, *p53*, *eiger* and *cyt-c-d*.\(^{81}\) This suggests that autophagy can be a key regulator of cell death during mid-oogenesis. Accordingly, degenerating mid-stage egg chambers in Drosophila and *C. capitata* are demonstrated to contain autophagosomes and autolysosomes.\(^{83,86,89-91}\) Furthermore, *ATG1* and *ATG7* mutants show a decrease in lysosomal staining and reduced levels of DNA fragmentation at mid-oogenesis, even though chromatin condensation in nurse cells still occurs normally.\(^{83,84,86}\) These data suggest that autophagy can act upstream of DNA fragmentation during PCD in mid-oogenesis and also indicate that chromatin condensation is regulated independently from DNA fragmentation, as was shown before for late oogenesis.\(^{96}\)

PCD of nurse cells and follicle cells is absolutely required for the normal maturation of the developing egg chambers during the late stages of *D. melanogaster*, *D. virilis*, *D. oleata*, *C. capitata* and *B. mori* oogenesis.\(^{88,97-109}\) Similar to mid-oogenesis cell death in *D. melanogaster*, the known cell death effectors and regulators *rpr*, *hid*, *grim*, *skl*, *decb*, *p53*, *eiger*, and *cyt-c-d* are not required for late oogenesis nurse cell death.\(^{83}\) Drosophila caspases Drice, Dcp-1, Dronc and Sirica play a minor role in nurse cell death during late oogenesis,\(^{106,107}\) suggesting that nonapoptotic forms of cell death also function in dying nurse cells during late oogenesis. Autophagy participates in this cell death process, functioning cooperatively with apoptosis for the most efficacious elimination of the degenerated nurse cells in several species.\(^{89-91,105,108}\) Autophagy is also required for the degeneration of the follicular epithelium in a caspase-independent manner.\(^{92,103}\) A very recent report by McCall’s group reports that dying nurse cells of Drosophila show hallmarks of necrosis together with a cell-autonomous requirement of lysosomal DNase II activity.\(^{109,110}\) These findings suggest that all the three forms of PCD participate in cell death of nurse cells during late oogenesis. Whether these types of cell death are distinct or interconnected, what molecular mechanisms control them, and how they are physiologically regulated in vivo are issues that need to be clarified in detail.

In these respects, an important issue is whether autophagy promotes the physiological function of the organ while triggering death in single cells. Ovaries in insects are extremely sensitive to the available nutritional and environmental status. Consequently, ovaries can be so small that they are difficult to dissect out, or they can occupy more than half of the body weight in females that are continuously provided with an excess of food (e.g., wet yeast paste).\(^{10}\) This means that the number of egg chambers is controlled by the available nutrients. Autophagy regulates cell death during the gerarium “checkpoint” and mid-oogenesis “checkpoint” in higher Dipteran, thus controlling the number of egg chambers.\(^{83,86,89-91}\) Although autophagy is suggested to promote cell death of individual cells in the egg chambers, the resources generated from cell death promote better conditions for the physiology of the ovary and the whole fly in general, finally resulting in cell survival. A similar example is the autophagic cell death of the salivary gland in Drosophila during metamorphosis, a life stage in which the fly does not eat and must develop adult structures in the absence of external nutrient resources.\(^{11,112}\) These findings suggest that in multicellular organisms we should always consider the role of autophagy in cell death in the context of the physiology of the cell, the tissue and the organism, and attention should be paid before making comparison with single-cell organisms or cultured cells.

**Autophagy and PCD in the bee ovary.** Bees are important in agriculture as producers of honey and as facilitators of pollination. In addition to their economic value, the honeybee is a model organism for studying social organization and behavioral traits and, for all these reasons, the honeybee *Apis mellifera* genome was sequenced. The *A. mellifera* genome was the first hymenopteran and the fifth insect genome to be sequenced and, although much remains to be done, the discovery of miRNAs and cis-regulatory elements\(^{111}\) and the availability of RNAi protocols, represent encouraging advances towards the elucidation of the molecular and genetic bases of many complex traits associated with honeybee sociality. Honeybee sociality is related to caste differentiation that is in turn connected with bee development, which includes selective and age-dependent activation of PCD according to the caste to which the insect will belong.

Honeybee ovary development is a good example for explaining the activation of a PCD process where features of autophagy and apoptosis are concomitant. The queen honeybee ovary is well developed, and this development seems to be independent from external stimuli only until sexual maturity, because if the queen is not fertilized, the ovary quickly displays signs of PCD and can be reabsorbed.\(^{112}\) Honeybee workers usually display poorly developed ovaries, but in the absence of the suppressive pheromones released by the queen, they also may develop functional ovaries and produce haploid eggs.\(^{113}\)

As reported for Drosophila, PCD has been observed in bee ovaries and accessory glands, and the cells may display features of apoptosis, necrosis and autophagy (Table 2). During pupal development, for example, the lateral oviducts of workers undergo an extensive cell death associated with cytoplasm vacuolization and dilatation of organelles such as mitochondria and rough endoplasmic reticulum.\(^{114,115}\)

Both queen and worker ovaries of Africanized *A. mellifera* display time-regulated features of cell death that are, however, linked to external stimuli.\(^{113}\) If the virgin queen does not mate after 15 days of emergence some features of cell death appear in the pre-follicular stage.\(^{116}\) Narcosis with CO\(_2\) could accelerate the general development of the virgin queen ovaries and prevented signals of PCD in ovaries, but it could not stop cell death if the virgin queen did not mate. Workers narcotized with CO\(_2\) or coming from queenless colonies developed their ovaries, but in both the populations, worker ovaries presented signs of cell death when bees were 15 days old. Commonly, forager
workers present more developed ovaries than nurse workers and it has been observed that the cell death program may revert naturally in worker ovaries. Workers fed with food supplements presented an abnormal development of their ovaries, reaching prefolicular and follicular stages also in the presence of the queen. However, even in this case, when these bees are 15 days old, signals of PCD in ovaries are observed suggesting that the age of the bee is the prevalent factor for execution of PCD (Abdalla FC, unpublished data). According to Colonello and Hartfelder, mating may represent a physiological stimulus for the queen ovaries of the Africanized *A. mellifera* to continue their development. Such stimulus may derive from the protein contents of the mucus gland of the males.

**Hormonal regulation of autophagy in insect metamorphosis.** Detailed light and electron microscopy studies demonstrated a long time ago that autophagy is an integral part of the morphogenetic changes during the metamorphosis of the larval organs of insects. In the larval tissues, i.e., in the fat body, salivary glands, midgut, prothoracic gland and Malpighian tubules, the onset of the formation of autophagic structures is finely time-regulated. The number and size of the autophagosomes and autolysosomes significantly increase in a very short time. Finally, most of the organelles are sequestered by the autolysosomes and only a small part of the cytoplasm remains in intact form.

Since the whole process of metamorphosis is under the control of the endocrine system, it was quite obvious to suppose that the autophagy is regulated also by hormonal stimuli. Indeed, hormones or other ligands can trigger a signaling pathway that is similar in holometabolous insects and results in the activation of autophagic activity (Fig. 2). Exogenous treatments by the molting hormone [20-hydroxyecdysone, (20E)] induce autophagic activity in larval fat body cells both in vivo and in vitro. Later on, the changes of the concentration of the molting hormone were determined in the hemolymph. The so-called “commitment peak” of 20E appears just at the beginning of the wandering period when the first autophagic structures are formed in the fat body cells. The physiological or developmental autophagy in the course of the normal postembryonic development is induced by this hormonal stimulus. However, the concentration of the juvenile hormone (JH) is very low or zero at the beginning of the wandering period. It has been demonstrated, that the topical application of exogenous JH inhibits autophagy in fat body cells. In addition, the concentration of the JH decreases at the very end of the penultimate larval stage for 6–8 hours before the last larval-larval molt. There is no detectable autophagy at the penultimate stage, since the concentration of 20E remains low in the hemolymph. However, at this stage it is possible to activate autophagy by exogenous 20E.

The molting hormone is a typical steroid hormone. After binding to its cytoplasmic receptor, the hormone-receptor complex is transported into the nucleus where it activates three sets of genes (very early, early and late genes). The protein products of the late genes are involved in the regulation of the metamorphic changes including autophagy. The idea that the hormonally-induced autophagy is regulated by some of the late genes is supported by the observations indicating that the inhibition of their transcription/translation blocks the formation of autophagic structures.

The results of genetic interaction studies in Drosophila show that the endogenous and exogenous molting hormone inhibits PtdIns3K activity in the cell membrane of the fat body cells in parallel with the stimulation of the developmental autophagy. These results link the hormonal induction of autophagy to the known regulatory function of the PtdIns3K-AKT/PKB-TSC1/TSC2-Rheb-TOR signalling pathway. 20E regulates not only the formation of autophagic structures during the metamorphosis but also the synthesis and activity of various lysosomal enzymes.

Developmental autophagy has multiple functions during the metamorphosis of insects. As previously observed for fruitfly and bee ovaries, even if autophagy kills some larval cells, it is at the same time essential for the survival and for the normal development of the whole, metamorphosing insect. The autophagic structures begin to form in precise coincidence with the onset of the non-feeding (wandering) period of the last larval stage. Because there is no water and food uptake during the wandering, prepupal and pupal stages, the self-digestion in the larval cell types represents the only source of materials and energy. While providing a fundamental way to obtain energy, autophagy plays also the part of the remover of obsolete components. It would be probably impossible to eliminate all of the larval organs via phagocytosis of apoptotic bodies during metamorphosis. The number of the professional phagocytes is just not enough to take up and to digest the huge amount of polyploid larval cells. Therefore, most of the cytoplasm, the macromolecules and cellular organelles, are decomposed inside the autolysosomes, and the extreme autophagic reaction itself leads to the death of the larval

Table 2. Typologies of cell death observed in reproductive components of worker bees

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Cell death typology</th>
<th>Species</th>
<th>References</th>
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<tbody>
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<td>Germinative cells of larval ovariole</td>
<td>Apoptotic PCD</td>
<td>Africanized <em>A. mellifera</em></td>
<td>112</td>
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<tr>
<td>Somatic cells of larval ovariole</td>
<td>Apoptotic-like PCD</td>
<td>Africanized <em>A. mellifera</em></td>
<td>112</td>
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<tr>
<td>Cells of larval ovariole</td>
<td>Overlap of apoptotic, autophagic and necrotic features</td>
<td><em>Frieseomelitta varia</em></td>
<td>115</td>
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<tr>
<td>Stromatic cell of pupal ovarian</td>
<td>Cytoplasmic disintegration similar to necrosis</td>
<td>Africanized <em>A. mellifera</em></td>
<td>112</td>
</tr>
<tr>
<td>Capsular cells of pupal ovarian</td>
<td>Cytoplasmic disintegration similar to necrosis</td>
<td>Africanized <em>A. mellifera</em></td>
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<td>Columnar epithelial cells of lateral oviduct of pupa</td>
<td>Apoptotic PCD and necrosis</td>
<td><em>F. varia</em></td>
<td>114</td>
</tr>
<tr>
<td>Columnar epithelial cells of lateral oviduct of pupa</td>
<td>Apoptotic PCD and necrosis</td>
<td><em>M. quadri fasciata</em></td>
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cell types. The remnants of the degrading cells are probably taken up by the neighboring cells, i.e., nonprofessional phagocytes. From the perspective of specific larval cell types, developmental autophagy can therefore be considered as the mechanism leading to type II PCD in insects. In the salivary gland and proboscis gland, prolonged autophagy might be associated with some features characterizing the apoptotic process. In the fat body, the fragmentation of the DNA was described in parallel with the reduction of the cytoplasm/nucleus ratio followed by cell death. Unfortunately, in oligomycin A-treated cells it is impossible to understand if the activated autophagy is intended as a repairing process, or if it is finalized to cell demise, since oligomycin A directly damages IPLB-LdFB mitochondria. However, the medium conditioned by oligomycin A-treated cells is able to induce, in the absence of any drug, autophagic cell death in IPLB-LdFB cells that possess an intact apoptotic machinery and normal cytoplasmic ATP levels. This suggests that IPLB-LdFB fat body cells are able to synthesize mediators acting specifically as pro-death signals that stimulate autophagic, or type II, PCD. Proteomics analysis of the medium conditioned by oligomycin A-treated cells indicates a dramatic reduction in the secretion of an Imaginal Disk Growth Factor (IDGF)-like protein. IDGF family members are proteins that play a fundamental role in promoting the growth and survival of Mamestra brassicaceae fat body cells and hemocytes of the flesh fly Sarcophaga peregrina, and of imaginal disk cells in D. melanogaster. In the larvae of the silkworm B. mori, the synthesis of IDGF is modulated on the basis of food availability. Even if developmental autophagic cell death has been shown to occur in D. melanogaster, specific pro-autophagic and protein factors have not been identified yet in insects. Several experiments performed mainly in Lepidoptera have suggested the existence of factors promoting developmental cell death in insects. These factors should be reasonably conserved within holometabolous insects and, in accordance with this hypothesis, we have recently observed that the medium conditioned by oligomycin A-treated IPLB-LdFB cells can induce a significant reduction of the cytoplasm/nucleus ratio followed by cell death also in the Drosophila SL-2 hemocyte-derived cell line (Malagoli DM, unpublished data). The increasing information on ATG in lepidopteran molecular databases will conceivably represent the fundamental basis for the isolation of signals that promote autophagic cell death and whose presence has so far been postulated in insects, but not directly demonstrated.
of the midgut of fed ticks. In addition, amphisome-like structures\textsuperscript{161,162} are also seen in the midgut cells of unfed ticks,\textsuperscript{160} but their function(s) is still unknown.

In the salivary gland cells of ticks infected with the protozoan Theileria parva, the endoplasmic reticulum and associated ribosomes are eliminated in autophagic vacuoles.\textsuperscript{163} After two days from infection, the parasite-infected cells showed dismantling of their protein synthesizing equipment by selective autophagic degradation. This suggests that the parasite may stimulate the host cell to increase activities of protein synthesis for development. However, it remains largely unknown whether parasites may or may not induce autophagy in the host cell.

Autophagy and PCD in the midgut of A. mellifera and lepidopteran larvae. In insects, the function of epithelial cells of the midgut is secretion of digestive enzymes and absorption of nutritive components.\textsuperscript{164} During metamorphosis, the larval midgut epithelium degenerates and a new adult midgut epithelium is built during larval differentiation of regenerative cells.\textsuperscript{165,166} PCD in honeybee larvae was found during normal larval development using scanning electron microscopy\textsuperscript{169} and histochemical and immunohistochemical methods.\textsuperscript{166} Acid phosphatase (AP) activity can be used as a marker of lytic activity in the cell. When it is free in the cytoplasm, AP is an indicator of cellular autolysis.\textsuperscript{170} In A. mellifera, cell autolytic activity is histochemically visualized in several regions\textsuperscript{166} including the basal area of the epithelial cells in degenerating midgut.

Over the past several years apoptosis-\textsuperscript{171-174} and autophagy-related pathways have been suggested to be involved in the degeneration of larval tissues and organs during metamorphosis in Lepidoptera. In these insects, as in the largest part of holometabolous insects, larvae and adults are characterized by diverse food and feeding habits. This implies that during metamorphosis the larval midgut is progressively dismissed and shed into the gut lumen by the growing pupal epithelium (Fig. 3A and B).\textsuperscript{175} Multiple lines of evidence show the occurrence of apoptosis during this multistage process that leads to the disappearance of the larval midgut,\textsuperscript{175,176} whereas the involvement of autophagy has been assessed more recently. Besides the demonstration of an increase in lysosome number and lysosomal enzyme amount in midgut cells during their degeneration,\textsuperscript{175-177} ultrastructural analyses are also consistent with the intervention of an autophagic program. Studies performed on the moths Heliothis virescens\textsuperscript{175} and Alabama argillacea,\textsuperscript{178} and the butterfly Pieris brassicae,\textsuperscript{179} show autophagic compartments at different stages of development in the period preceding the pupa phase (Fig. 3C). At the molecular level, the increased expression of ATG-related genes has been recently assessed in the autophagic process occurring in midgut tissues of B. mori (Cao Y, unpublished data). In terms of physiological significance, since autophagy is detectable just after the larva ceases to feed, it could be a typical starvation-induced event that protects the larva while undergoing nutrient deprivation, as for other arthropods.\textsuperscript{160} However, the self-eating mechanism is most likely developmentally related since it starts after the

Figure 3. Autophagy and larval midgut degeneration in H. virescens. Larval midgut consists of a highly folded monolayered epithelium formed by columnar, goblet and stem cells. Stem cells set in motion the repair of damaged tissue, take part in the growth of the midgut epithelium during larval-larval molts and, during pupation, play a key role in the generation of the midgut of the adult. (A and B) Cross-sections of midgut at mid-late phase of the fifth larval instar. During the prepupal phase, the stem cells (arrows) progressively form the pupal midgut, while the old larval midgut epithelium (im) is sloughed into the lumen (l) and the cells die. Boxed area is shown at higher magnification in (B). (C) During degeneration of larval midgut cells, autophagic compartments are visible within the cytoplasm (arrowheads). The asterisk indicates mitochondria. Scale bars: 100 μm (A); 30 μm (B); 1 μm (C).
20E commitment peak, and Komuves et al. demonstrate that the appearance of autophagic vacuoles in midgut cells can be induced by administering 20E to the larvae.\textsuperscript{12} The presence of membrane transporters and hydrolytic enzymes in the newly formed pupal midgut epithelium suggests a recycling of the molecules broken down and released by the degraded midgut.\textsuperscript{13} This prosurvival pathway of autophagy would therefore provide metabolic support to the developing organism, which is without an external nutrient source for several days during the pupal period.\textsuperscript{180} Among the others, two fundamental, yet unresolved, issues need further investigation in Lepidoptera. First, the identification of autophagy in the midgut of some lepidopteran species is missing but this shortage of evidence might be simply related to the difficulty of detecting autophagy without appropriate methods of analysis.\textsuperscript{181} Second, the interplay between autophagy and apoptosis, as well as the timing and proportion of their occurrence in the midgut, are still poorly known and they are sometimes contradictory.\textsuperscript{182} The resolution of the intricate relationships between apoptosis and autophagy would also shed light on the real role of the latter and should permit verification of whether it accompanies or causes cell death. In fact, beyond making molecules available to the new pupal epithelium for recycling, it is reasonable to hypothesize that, as seen in Drosophila,\textsuperscript{12} autophagy may act as a key regulator of cell death or, alternatively, intervene in cleaning up cells committed to die by apoptosis since conventional phagocytes do not have ready access to the luminal environment.

\section*{Conclusion}

Despite the hundreds of millions of years of distance that separate arthropods from humans, basic mechanisms of fundamental cellular processes have been conserved during their diversification. For practical, financial, and ethical reasons arthropods in general, and insects in particular, represent models that are attracting increasing attention and resources. Genetic and molecular biology techniques led to the isolation of several ATG genes in arthropods, creating the basis for a deeper comprehension of autophagy realization and regulation. In the study of autophagy, arthropods have proved extremely interesting organisms from several points of view. For example, due to their particular developmental pattern that includes metamorphosis and the disposal of massive organs, holometabolous insects such as \textit{D. melanogaster}, \textit{A. mellifera} and \textit{B. mori} represent privileged models for studying type II PCD and the contribution that autophagy gives to cell demise. Intriguingly, it seems progressively evident in insects that autophagy, as usually indicated for apoptosis in mammals, may act as a pro-death process at the cellular/organ level, but its effects at the organismal level can still be considered as fundamental for survival.

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