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REVIEW

Autophagy in Lepidoptera: more than old wine in new bottle

G Tettamanti[1], Y Cao[2], Q Feng[3], A Grimaldi[1], M de Eguileor[1]

[1]*Department of Biotechnology and Molecular Sciences, University of Insubria, 21100 Varese, Italy*
[2]*Laboratory of Insect Molecular Biology and Biotechnology, Guangdong Provincial Key Laboratory of Agro-animal Genomics and Molecular Breeding, College of Animal Science, South China Agricultural University, Guangzhou, 510642, China*
[3]*School of Life Sciences, South China Normal University, Guangzhou, 510631, China*

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Abstract

Autophagy is a cellular pathway that leads to the degradation of proteins and organelles. This process is usually involved in the maintenance of cell homeostasis when the organism experiences nutrient starvation, but in holometabolous insects autophagy also intervenes in the demolition of larval tissues and organs during metamorphosis. This review summarizes the current knowledge about autophagy research in Lepidoptera and discusses the use of moths and butterflies as models for studying the roles and regulation of autophagy. It also gives insights into the cooperation between autophagy and apoptosis in cell death events that occur in lepidopteran *in vivo* and *in vitro* systems.

Key Words: autophagy; Lepidoptera; *Bombyx mori*; *ATG* genes; apoptosis

Introduction

Autophagy is a cellular self-eating process involved in protein and organelle degradation. Although three types of autophagy (macroautophagy, microautophagy and chaperone-mediated autophagy) are known, the term "autophagy" usually refers to macroautophagy, which will be the focus of this review. In autophagy, a membrane, called phagophore or isolation membrane, is formed in the cell and progressively expands and grows to engulf a portion of cytoplasm. This double-membrane structure finally closes to become an autophagosome. Once the autophagosome membrane fuses with lysosomes, the content is degraded and the resulting macromolecules are recycled back into the cytosol (Mizushima *et al.*, 2008) (Fig. 1). All these steps are regulated by autophagy-related (*ATG*) genes, initially identified in yeast, but lately found in all eukaryotic organisms (see He and Klionsky, 2009; Inoue and Klionsky, 2010 for a complete description about the regulatory mechanisms of autophagy).

Although autophagy can potentially degrade cytoplasmic proteins and any organelles, selective organelle degradation has been described in yeast and other cell models. Pexophagy (Manjithaya *et al*., 2010), mitophagy (Narendra *et al*., 2008), nucleophagy (Park *et al.*, 2009) and reticulophagy (Bernales *et al*., 2007) are responsible for specific dismantling of peroxisomes, mitochondria, nucleus and endoplasmic reticulum, respectively.

A basal level of autophagy occurs in most tissues to allow an adequate turnover of the cell components. Notwithstanding, autophagy represents an adaptation of the cell to starvation. If cells experience nutrient deprivation, they break down a part of their content to stay alive. But in some circumstances, stress-induced autophagy exceeds the safe threshold and/or cooperates with apoptosis pathways, leading to a form of cell death characterized by the accumulation of autophagosomes, known as type II programmed cell death (type II PCD), where type I PCD is more classical apoptosis (Gozuacik and Kimchi, 2007). Type II PCD allows the elimination of large number of cells in tissues/organs and is usually associated with developmental, differentiation and tissue remodelling processes, such as in insect metamorphosis. Most evidence indicates that, at least in cells with intact apoptotic machinery, autophagy is primarily a pro-survival rather than a pro-death mechanism, but this issue is still under debate (Kroemer and Levine, 2008). Autophagy is also involved in some human diseases since it can work as a cytoprotective mechanism to prevent the insurgence of specific pathologies, or it can be deleterious allowing, for example, the proliferation of microbes that subvert autophagy for replication (Mizushima *et al.*, 2008). All these features, together

Corresponding author:
Gianluca Tettamanti
Department of Biotechnology and Molecular Sciences
University of Insubria
Via J.H. Dunant 3, 21100 Varese, Italy
E-mail: gianluca.tettamanti@uninsubria.it

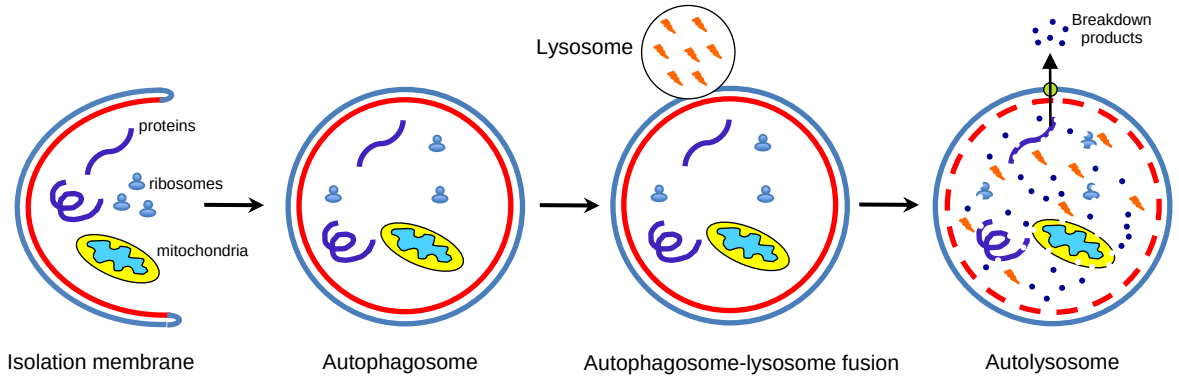

Fig. 1 Schematic model of macroautophagy. After induction, a portion of cytoplasm or specific organelles are surrounded by a growing membrane (isolation membrane or phagophore) that grows progressively, thus forming an autophagosome. The autophagosome fuses with a lysosome and in the resulting autolysosome, the hydrolytic enzymes break down the inner membrane of the autophagosome and its cargo. The macromolecules derived from the enzymatic digestion are released back to the cytoplasm through permeases and reused.

with the identification of autophagy genes in higher eukaryotes, have led to the reappraisal of some experimental models suitable for studying autophagy, and in the last ten years the number of papers published on this topic increased substantially (Klionsky, 2007).

This review presents our current knowledge on the autophagic processes that occur during growth, development and metamorphosis in Lepidoptera, highlighting why Lepidoptera can be an excellent model for studying autophagy, while also pointing out some issues that still need to be clarified.

Why study autophagy in insects?

In holometabolous insects, several organs are massively remodelled or even degenerate during metamorphosis and autophagic events occur extensively to eliminate those organs and tissues that are useful only during embryonic and larval stages (Tettamanti *et al.*, 2008b; Malagoli *et al.*, 2010) (Fig. 2).

Because of the short life cycle and the well-characterized genetics, *Drosophila melanogaster* has provided a useful model to dissect the molecular mechanisms and the physiological roles of autophagy. In *Drosophila* several *ATG* homologues and genes regulating autophagy have been identified and this has allowed the dissection of the different autophagic events occurring in this organism. Scott *et al.* (2004) clearly demonstrated that starvation induces an autophagic response in the fat body, a nutrient storage and mobilization organ analogous to the vertebrate liver. Moreover, development-associated autophagy, that is related to an increase in ecdysteroids, has been identified in the fly during oogenesis (Nezis *et al*., 2009) and in specific tissues, such as midgut and salivary glands (Rusten *et al*., 2004; Berry and Baehrecke, 2007; Denton *et al*., 2009). The effects of ecdysone are mediated through a heterodimeric nuclear receptor and hormone binding to the receptor complex not only activates a transcriptional regulatory hierarchy including early and late genes, but also influences the autophagic process through the PtdIns3K-AKT/PKB-TSC1/TSC2-Rheb-TOR signalling pathway (Malagoli *et al.*, 2010). The *ATG* genes identified up until now share evolutionary conservation and they have been proven to be essential for a correct initiation and completion of autophagy. Flies with mutation or inactivation of *ATG* genes including *ATG1*, *ATG2*, *ATG3*, *ATG5*, *ATG6*, *ATG7*, *ATG12* and *ATG18* are unable to carry out starvation-induced autophagy or fail to degrade larval organs at metamorphosis (Scott *et al*., 2004; Berry and Baehrecke, 2007). One of the most intriguing findings is that cell death events occurring in larval midgut and salivary glands are characterized by features typical of both apoptosis and autophagy (Jiang *et al.*, 1997; Martin and Baehrecke, 2004). In addition, PCD of the larval midgut has been demonstrated to be strictly dependent on autophagy, while the canonical apoptotic pathway is dispensable although caspases are active in the dying midgut (Denton *et al*., 2009). These observations make the whole story more complex and suggest an intricate relationship between autophagy and apoptosis.

Among insects, butterflies and moths have been widely used to study processes related to metamorphosis, because the larva is amenable to perform endocrinological, electrophysiological and developmental biology studies. Several lepidopteran species have been used throughout the years to analyze demolition of body tissues/organs through autophagy, as will be reported below. If we also take into consideration the establishment of a wide repertoire of new molecular tools for several species that belong to this taxon, Lepidoptera certainly represent an excellent model system for tackling a broad range of questions concerning autophagy. It is also worth noting that some of the most important

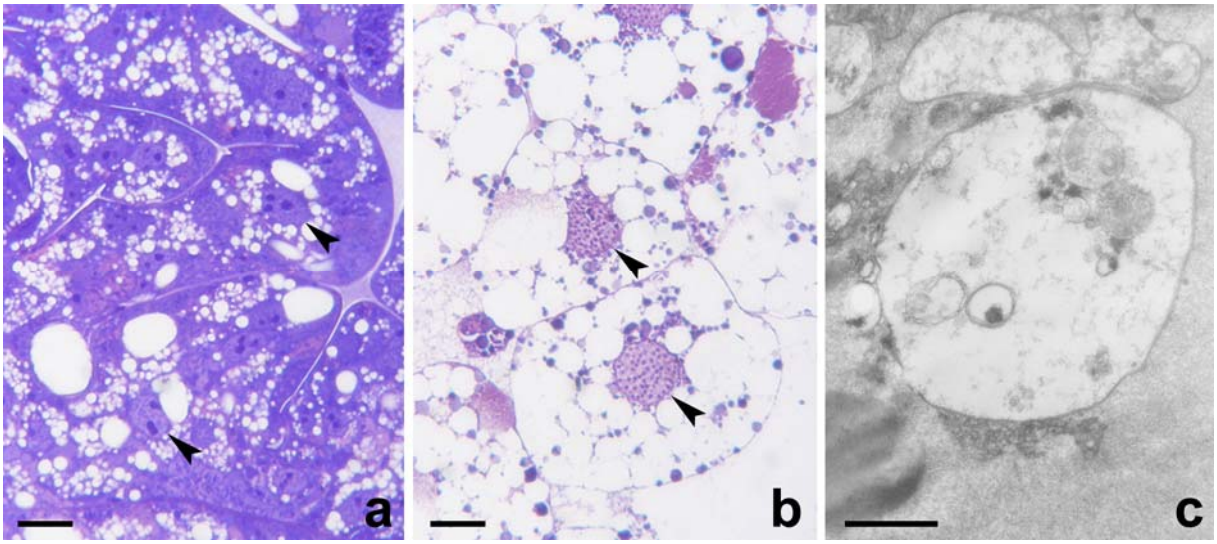

Fig. 2 Autophagy and organ degeneration in *Bombyx mori* larvae. During metamorphosis, the fat body is massively remodelled due to the intervention of autophagic processes: the pupal fat body (b) greatly differs from the larval one (a). (c) Autolysosomes containing organelle debris are visible in the cytoplasm of cells undergoing autophagy. Bars = 10 μm (a, b); 1 μm (c). Arrowheads indicate the nuclei.

species of high economic importance, such as those being bred for commercial purposes or phytophagous pests, are represented in this insect order. Therefore a deep knowledge of the processes regulating metamorphosis, and cell death in particular, in these organisms could contribute to ameliorate crop production and the textile industry.

The early age of autophagy in Lepidoptera…

The early studies of autophagy in Lepidoptera from the 1960s to the early 1980s were based on morphological analyses. A few years after the appearance of the term "autophagy" (de Duve and Wattiaux, 1966), Locke and Collins described in fat body of *Calpodes ethlius* larvae the isolation of cell organelles within paired membraned derived from Golgi (Locke and Collins, 1965, 1968). They not only described the complete sequence of autophagosome formation, but also suggested that the isolated compartment could be a region of massive lysis. A confirmation was obtained by using specific stainings for acid phosphatase, a lysosomal enzyme whose activity was detected in these "storage granules" (Larsen, 1970, 1976). This massive cellular autolysis in the larva caused a reduction of mitochondria in the pupal fat body and a deep rearrangement of this tissue before the emergence of the adult (Larsen, 1970, 1976). Beyond the fat body, detection of autophagic compartments and increases in lysosome number had been reported in other larval/pupal tissues/organs, including midgut (Misch, 1965), wing epithelium (Nardi *et al.*, 1991), silk gland (Matsuura and Tashiro, 1976; Tashiro *et al.*, 1976) and intersegmental muscles (Beaulaton and Lockshin, 1977; Lockshin and Beaulaton, 1979). All those early works established a close link between autophagy and its role in cell remodelling in insects undergoing complete metamorphosis.

These old papers tackled two important features of the autophagic process. The first is the endless search for the source of the isolation membrane. Although Golgi and endoplasmic reticulum seem to be the best candidates, recently new information has emerged suggesting that mitochondria may provide a membrane source as well (Hailey *et al*., 2010). This intriguing hypothesis could be the confirmation of what was previously seen in the *Bombyx mori* silk gland, where cup-shaped mitochondria function as a kind of autophagosome system, contributing to the autolytic process during the prepupal phase (Matsuura and Tashiro, 1976).

The second issue concerns lysosomes. Although detection of lysosomes *per se* cannot be considered a sufficient evidence to demonstrate the activation of the autophagic pathway, it is undoubted that activation of the lysosomal compartment marks the onset of tissue lysis (He and Klionsky, 2009). Some papers show an increase of acid phosphatase granules in degenerating silk gland of *B. mori* (Matsuura *et al*., 1976; Tashiro *et al*., 1976). This enzyme is synthesized at the end of the last larval instar, stored mainly in the Golgi bodies and subsequently used (Matsuura *et al.*, 1976). The increase in enzyme activity is biphasic, with one peak at spinning/prepupal period and the other at early pupal phase (Matsuura *et al.*, 1976). This trend seems to explain the ultrastructural changes occurring in the silk gland and the role of the autophagic process: early "catabolic autophagy" provides energy and raw materials just after the

larva has ceased to feed, while subsequent autophagy is responsible for the demolition of the silk gland which is no longer necessary once the cocoon has been formed. The fat body differs slightly as elevated levels of acid phosphatase activity are visible later (6^{th} day of pupation) (Sumithra *et al.*, 2010). More recently *B. mori* cathepsin B and D have been cloned and characterized. Their activity is required for PCD of the fat body and midgut at metamorphosis (Gui *et al*., 2006; Lee *et al*., 2009). The expression patterns of these two lysosomal enzymes are similar to that of acid phosphatase and they are induced by 20-hydroxyecdysone (20E). Moreover, silencing through RNAi precludes correct pupation in the larvae. All these features suggest that the two proteinases could also contribute to autophagy in these two organs undergoing tissue remodelling (Gui *et al.*, 2006).

Along with the identification of the two main actors of the autophagic process, the autophagosome and lysosomes, during the period of the '70s and '80s, several research groups analyzed the signals and the signal transduction pathway that regulate autophagy in Lepidoptera. The onset of autophagy is triggered by 20E and the injection of this hormone in the body cavity of the larva induces an increase in the numbers of secondary lysosomes and mitophagy in midgut cells (Radford and Misch, 1971) and the fat body (de Priester *et al.*, 1979), while ligatures applied behind the brain-ring gland complex prevents the appearance of acid phosphatase-positive granules in degenerating cells (de Priester *et al.*, 1979). The sensitivity of the cells to ecdysone is age-dependent, since injection of the hormone in larvae at the penultimate instar does not induce the emergence of autophagic compartments in the fat body (Sass and Kovacs, 1977). Experiments performed *ex vivo* give additional information. Administration of 20E to the fat body isolated from fifth instar larvae before the programmed occurrence of autophagy (critical period) sets in motion the self-digestion process (Dean, 1978). Moreover, fat body taken soon after the critical period continues with the autophagic sequence in hormone-free medium (Dean, 1978). This not only confirms that autophagy is induced by ecdysone, but also demonstrates that, once the cells are commited to autophagy, the process does not require the continuing presence of the hormone for its completion.

Sass and colleagues (Sass *et al.*, 1983) identified cAMP as one of the possible mediators of the autophagic response induced by 20E in *Mamestra brassicae*. The first peak of cAMP content in fat body cells observed during mid-late fifth larval instar overlaps the beginning of the formation of autophagic vacuoles and both events can be prematurely induced by injecting ecdysone in larvae at the beginning of the instar (Sass *et al.*, 1983). A slight variation in cAMP levels was reported also in labial glands, but this change, as well as that of other secondary messengers such as cGMP and IP_3, occurs after the initial commitment to self-destruction (Halaby *et al.*, 1994), thus arguing against a true role of all these molecules on the signaling cascade induced by 20E. A second conundrum concerns protein synthesis. In fact, while an early drop in protein synthesis can be measured during PCD of this tissue (Halaby *et al*., 1994; Lockshin and Zakeri, 1994; Zakeri *et al*., 1996; Jochova *et al*., 1997), Komuves and colleagues demonstrated that 20E greatly enhances protein synthesis in the midgut and its inhibition by cycloheximide determines an impairment of the autophagic process (Komuves *et al.*, 1985).

…and the modern age

The beginning of twenty-first century witnessed the birth of a second age for the study of autophagy in Lepidoptera. The beginning of expressed sequence tag (ESTs) projects in various Lepidoptera species, the completion of genome sequencing in *B. mori*, the development of RNAi, stable germline transformation and viral vectors for transient gene expression (Daubnerova *et al*., 2009; Lee *et al*., 2009), have made it possible to analyze in depth the autophagic processes occurring in these insects and to manipulate the expression of autophagic genes that have now been identified in the silkworm.

The molecular base of autophagy

Bioinformatics analysis performed by Zhang and colleagues (Zhang *et al.*, 2009) revealed that in the *B. mori* genome there are homologs of most of the *ATG* genes originally identified in yeast and subsequently in higher eukaryotes. Along with 11 *ATG* genes, they found genes involved in the PI3K I and PI3K III signal transduction pathway and in the formation of autophagosomes. In particular, most of these genes are involved in the two ubiquitin-like conjugation systems, Atg8-PE and Atg12-Atg5-Atg16. The number of genes regulating autophagy has been recently widened by the identification of two paralogous Target of rapamycin (TOR) genes, *BmTOR1* and *BmTOR2* (Zhou *et al.*, 2010) (Table 1). Several of these genes are actively transcribed in different tissues during development and metamorphosis, or are up-regulated by starvation. The expression levels of *BmATG8* and *BmATG12* in silk gland show a progressive increase, reaching a plateau at pre-pupal stage, when autophagic features become evident (Zhang *et al.*, 2009). A similar pattern for these genes is observable in larval midgut cells that die at metamorphosis (Cao Y, unpublished data). It is worth noting that four *ATG* genes (*BmATG1*, *BmATG5*, *BmATG6*, *BmATG8*) are expressed at high levels in *B. mori* peritracheal athrocytes (Owa *et al.*, 2008). These cells contribute to the regulation of the composition of hemolymph through the removal of certain molecules and therefore these genes could be involved in heterophagic lysosomal degradation, helping to maintain homeostasis during the development of the larva.

Both *BmTOR* genes are up-regulated by two autophagy-promoting signals, starvation and 20E, although with different sensitivity (He and Klionsky, 2009; Zhou *et al*., 2010). Since in higher eukaryotes the pathways through which hormones and nutrients regulate autophagy are different, but both converge

Table 1 List of the principal autophagy-related genes identified in *Bombyx mori*

Gene	Accession number	Protein function	Reference
Regulation of autophagy induction			
BmTOR1 *BmTOR2*	GU350772* GU350773*	Negative regulators of autophagy, rapamycin target	Zhou *et al*., 2010
BmATG1	BGIBMGA011986§	Ser/Thr protein kinase	Owa *et al*., 2008
Autophagosome nucleation			
BmATG6	FJ416328*	Component of class III PI3-kinase complex	Owa *et al*., 2008
Autophagosome expansion and completion			
BmATG3	FJ416327*	Conjugates phosphatidylethanolamine to Atg8	Zhang *et al*., 2009
BmATG4	FJ416326*	Cleaves Atg8 at C terminus	Zhang *et al*., 2009
BmATG5	FJ418152*	Conjugated to Atg12	Owa *et al*., 2008
BmATG7	BGIBMGA001467§	Activates Atg8 and Atg12	Zhang *et al*., 2009
BmATG8	FJ416330*	Ubiquitin-like protein conjugated to phosphatidylethanolamine	Owa *et al*., 2008
BmATG12	FJ416329*	Ubiquitin-like protein conjugated to Atg5	Zhang *et al*., 2009
BmATG16	BGIBMGA006504§	Component of Atg5-Atg12 complex	Zhang *et al*., 2009
Retrieval			Zhang *et al*., 2009
BmATG9	BGIBMGA012307§	Interacts with Atg2	Zhang *et al*., 2009
BmATG18	BGIBMGA007298§	Required for Atg2 localization	Zhang *et al*., 2009

Sequences from GeneBank are indicated with an asterisk, while those from SilkDataBase (http://silkworm.genomics.org.cn/) are indicated with §

on Tor (He and Klionsky, 2009), it is reasonable to hypothesize a key role for silkworm Tor in the signalling pathway that regulates autophagy.

Growing interest in *ATG* genes has led to the recent derivation of the crystal structure of BmAtg8 (Hu *et al.*, 2010). Atg8 is an ubiquitous protein among eukaryotes and, after its recruitment to the phagophore, is involved in the membrane-expansion step. BmAtg8 has several residues and an ubiquitin-fold domain at C-terminus conserved in different species, thus implicating a central role in the autophagic pathway. Although with small differences, such as the absence of an identifiable *BmATG10*, the identification, expression and structural characterization of the 25 autophagy-related genes in the silkworm confirm the existence of a well-organized autophagy pathway in this insect (Zhang *et al.*, 2009), while the exact mechanisms of action of the autophagy pathway remain to be elucidated.

The sequential gene activation triggered by 20E that leads to PCD in *B. mori* tissues has been characterized as well. The effects of 20E are mediated by a heterodimeric nuclear receptor formed by the ecdysone receptor (EcR) and Ultraspiracle (USP). Among the three isoforms of ecdysone receptor, EcR-B1 has been shown to actively take part in the onset of death processes in the silk gland. Its protein levels reach a maximum just before the larval to pupal transformation when autophagosomes appear (Goncu and Parlak, 2009). Although the main early and late genes involved in the *cis*-regulation downstream of EcR in *B. mori* are similar to that of *Drosophila* (Sekimoto *et al.*, 2006), the recruitment of these genes is different in the two insects. This difference in behaviour is particularly evident when the regulation of autophagic and apoptotic process within the same tissue is dissected. Accordingly, Li *et al.* (2010) demonstrated that in the silk gland the expression of *BmEcR*, *BmE74A*, *BmE75C* and *BmBR-C* peaks at the onset of the autophagic process, while *BmBFTZ-F1*, *BmHR39* and *BmE75B* are more likely involved in the initiation of apoptosis.

A complex intertwining between autophagy and apoptosis

A phenomenon that has been widely described in lepidopterans is that in many organs undergoing death during metamorphosis, autophagic and apoptotic features coexist: DNA fragmentation, apoptotic nuclei and caspase activation have been detected in silk gland (Goncu and Parlak, 2008; Li *et al.*, 2010), fat body (Muller *et al*., 2004; Sumithra *et al*., 2010), midgut (Tettamanti *et al*., 2007a, b; Vilaplana *et al*., 2007) and other tissues (Dai and Gilbert, 1999; Hoffman and Weeks, 2001; Kinch *et al.*, 2003; Mpakou *et al.*, 2006, 2008) where autophagy has been shown to play a key role in their degradation. Nuclear condensation and DNA cleavage, the presence of unusual apoptotic bodies and the absence of identifiable phagocytes that cleanse the tissue by removing cell debris in certain organs, are three elements that are not typically seen in autophagy-mediated cell death. Also the involvement of active caspases is under investigation, since it does not seem to be a feature exclusively linked to apoptosis. In fact, the larval midgut at the pupal stage exhibits positive immunostaining with an antibody specific for cleaved caspase-3 (Tettamanti *et al*., 2007a; Vilaplana *et al*., 2007), and ovarian nurse cells that degenerate during oogenesis are labelled by Red-VAD-FMK, a specific *in situ* assay for activated

caspases (Mpakou *et al.*, 2006, 2008). Expression of *BmCaspase-C* was also assessed by real-time PCR analysis in *B. mori* silk gland (Li *et al.*, 2010) and administration of a specific caspase inhibitor to dying motoneurons impairs the late phase of cell death which is autophagy-dependent (Hoffman and Weeks, 2001). The opposite situation has instead been described in *Manduca sexta* fat body, where no evidence of activity of executioner caspases, such as caspase-3 and 7, was found (Muller *et al.*, 2004).

An interesting alternative to the involvement of caspases in cell death processes mediated by autophagy has been provided by Lockshin and colleagues (Facey and Lockshin, 2010). In their attempt to ascertain if type II PCD really exists, they verified the possible occurrence of caspase action in *M. sexta* labial glands at metamorphosis. Although no caspase activity was detected, they demonstrated an increase in lysosomal proteolytic activity when the gland disintegrates. They suggest that lysosomal proteases, cathepsin B in particular, may play the major proteolytic role similar to the apoptotic caspase cascade in mammals, since they cleave PARP at late phases of the death process.

Summarizing these data, at least two settings that link autophagy and apoptosis in Lepidoptera can be outlined: i) Autophagy associated PCD, where autophagy is the driving force that promotes cell death and, although the autophagic machinery is functional, it does not involve precocious cytochrome c release, apoptosome formation and caspase recruitment (Facey and Lockshin, 2010); ii) Caspase-dependent autophagic cell death that involves activation of effector caspases after loss of mitochondrial function (Hoffman and Weeks, 2001; Kinch *et al.*, 2003). There is also a series of situations in which the borders are not clear, since autophagy can be accompanied by DNA fragmentation or nuclear condensation (Dai and Gilbert, 1999; Muller *et al*., 2004), but no activation of executioner caspases (Muller *et al*., 2004).

The overlap between autophagy and apoptosis and the evidence that some morphological, biochemical and molecular features are not exclusive to either autophagy or apoptosis (Berry and Baehrecke, 2007; Nezis *et al*., 2009) have led to a search for possible mediators common to these two processes. Thus, besides a role for caspases in autophagic cell death, other factors such as Inhibitor of Apoptosis Protein (IAP) are now hypothesized to intervene in this self-digesting process. Not only *IAP* expression modifies during midgut remodelling in Lepidoptera (Parthasarathy and Palli, 2007; Vilaplana *et al*., 2007), but the IAP protein Bruce in *Drosophila* is fundamental to autophagic processes (Hou *et al.*, 2008) and could provide a mechanistic link between autophagy and cell death (Nezis *et al.*, 2010). Among the autophagic genes, *ATG5* has been suggested to be a molecular link between autophagy and apoptosis in mammals, since it plays a role in autophagosome formation, and is also involved in a pro-apoptotic signalling pathway through cytochrome c release and caspase activation (Yousefi *et al.*, 2006). In the *B. mori* silk gland, the expression pattern of *BmATG5* suggests that it may function as a switch between autophagy and apoptosis during the prepupal stage as well (Li Q, personal communication).

Having demonstrated a concerted action between autophagy and apoptosis in the removal of larval tissues in Lepidoptera, the obvious question is what could be a possible explanation for this synergy? An interesting paper by Eisenberg-Lerner *et al.* (2009) analyzes this issue and concludes that the co-occurrence of autophagy and apoptosis within the same tissue does not seem to represent mere redundancy. Autophagy might cooperate with apoptosis to lead to cell death. In this way autophagy could work as a back-up system to ensure cell death if the apoptotic process failed, but it could also establish a partnership with apoptosis to maximize the death process. Alternatively, autophagy and apoptosis could have different goals and autophagy would act as a pro-survival mechanism that helps cells to maintain homeostasis until a point of no return, after which apoptosis is activated and the cell dies. A third possibility is that autophagy may enable apoptosis by participating in the regulation of some molecular mechanisms of the apoptotic machinery. Although all three of the hypotheses can be envisaged for Lepidoptera, the current lack of sufficient information about the molecular mechanisms underpinning the interconnection between apoptosis and autophagy, and the complexity of the phenotypic features evidenced in the dying tissues in the larvae, do not yet allow us to disentangle this problem. On the other hand, the observation of both processes in almost all larval tissues of butterflies and moths provides experimental models *ad hoc* to address this issue. The recent identification of a large series of apoptotic factors in silkworm (Zhang *et al*., 2010) could add new information about the connections between autophagy and apotosis in this animal model.

Two friendly models for studying autophagy

The larval midgut is one of most used organs for studying cell death processes and autophagy in Lepidoptera. The midgut represents the middle part of the alimentary canal of the insect and it is mainly involved in the digestion and absorption of nutrients. During metamorphosis, the epithelium formed during larval life is progressively displaced by a new epithelial layer that grows underneath and finally becomes the pupal midgut. The old tissue degenerates and forms the yellow body, a compact mass that is sloughed in the gut lumen, where the cells undergo cell death (Tettamanti *et al.*, 2007a; Hakim *et al*., 2010). Mitochondrial activity disappears in these cells (Tettamanti *et al*., 2007a), DNA fragmentation becomes visible (Tettamanti *et al*., 2007a; Vilaplana *et al*., 2007) and the expression levels for the anti-death factor *IAP* are reduced along with an increase in *Caspase-1* mRNA expression (Parthasarathy and Palli, 2007). Positivity for cleaved caspase-3 antibody can be detected in these cells at an early stage of degeneration (Tettamanti *et al*., 2007a; Vilaplana *et al*., 2007) and the mRNA of *ICE*, the homologue of mammalian caspase-3, peaks during the prepupal/pupal stage, when apoptosis occurs in the yellow body (Parthasarathy and Palli, 2007). The

action of ICE downstream of caspase-1 suggests that these caspases carry out various functions in the PCD machinery of larval midgut. Autophagy contributes actively to this scenario and seems to be essential for the death of midgut cells. Although dsRNA-mediated interference (RNAi) of *ATG* genes such as has been carried out in *Drosophila* (Denton *et al.*, 2009) has not yet been undertaken in Lepidoptera, several results strengthen this hypothesis. Autophagic compartments are visible in midgut cells during the whole pupal period (Tettamanti *et al*., 2007a), in addition to observed increases in lysosomal enzymes in the cytoplasm (Tettamanti *et al*., 2007a; Vilaplana *et al*., 2007). Moreover *BmATG5*, *BmATG6* and *BmATG8* are highly espressed at the prepupal stage, preceding the activation of autophagy in midgut cells (Cao Y, unpublished data). Thus the midgut represents a valuable model for studying the relationships between autophagy and apoptosis, for analyzing development-related autophagy regulated by ecdysone, and for investigating starvation-induced autophagy since these cells are directly responsible for nutrient digestion in the larva. The midgut is also the site of entry for many pathogens in the larva and is the first barrier towards exogenous agents. Since autophagy helps the cells to cope with stress that perturbs tissue homeostasis, this organ can also provide insights into the role of autophagy under pathological conditions. It must also be underlined that the midgut is more amenable to RNAi approaches than other organs (Huvenne and Smagghe, 2010), therefore a functional screening of *BmATG* and other autophagy-related genes in this tissue is certainly conceivable.

For *B. mori* and other lepidopterans, a large array of cell lines have been derived from the main tissues undergoing programmed cell death (Sudeep *et al.*, 2005; Zhong *et al.*, 2005). Along with the undisputed advantages of experimentation in cell lines, the efficacy of *in vitro* systems to disentangle the intricate network between autophagy and apoptosis has already been demonstrated in the study of cell death processes of two different lepidopteran species (Tettamanti *et al*., 2006; Liu *et al*., 2007).

In the IPLB-LdFB cell line, established from *Lymantria dispar* fat body, it is possible to selectively direct cells towards either apoptosis or autophagy (Malagoli, 2008; Tettamanti and Malagoli, 2008). The same cell line has allowed the analysis of the effects of energy deprivation induced by glucose starvation (Liu *et al.*, 2007), or obtained by treating the cells with oligomycin A, an inhibitor of the mitochondrial ATP synthase (Tettamanti *et al*., 2006, 2008a). When *Spodoptera litura* cells (Sl) are deprived of glucose, the appearance of autophagic vacuoles is observed. Autophagy can be reverted by readdition of glucose to the medium, but if starvation is prolonged for more that 48 hours, apoptosis initiates (Liu *et al.*, 2007). A quite different situation takes place in IPLB-LdFB cells. Although these cells have an apoptotic pathway that resembles that of mammals and apoptosis can be induced by suitable treatments (Malagoli, 2008), administration of oligomycin A to the cells for 2 hours followed by different retrieval periods in control medium, determines the insurgence of apoptosis, necrosis, oncosis or autophagy. Within 24 h, a small percentage of cells are positive for all four processes, but starting from 48 h autophagic cells become the predominant phenotype within the cell population (Tettamanti *et al.*, 2006). Mitochondria are the primary target of autophagy and the concomitant increase in reactive oxygen species (ROS) production generates a positive feedback loop that leads to cell death. Despite the modified configuration of the actin cytoskeleton to try to preserve the remaining undamaged mitochondria, a massive decrease in cell number can be observed (Tettamanti *et al*., 2008a). In this case, the dual role of autophagy can be easily assessed. The early pro-survival function of the autophagic process that enables the use of intracellular resources under starvation conditions and removes damaged mitochondria from the cytoplasm is rapidly overtaken, and autophagy becomes associated with cell death mechanisms. A proteomic analysis has been undertaken in these cells with the aim of identifying specific signals able to induce cell death (Malagoli *et al.*, 2009). In accordance with the autophagic mechanisms observed in dying cells, an up-regulation of proteins involved in cell metabolism, oxidative stress response and cytoplasm dynamics was found. But the most interesting result is a drastic reduction of imaginal disk growth factor (IDGF)-like secretion after oligomycin treatment. Thus, abolished release of this pro-survival factor could be associated with the activation of a signaling pathway leading to cell demise.

Unfortunately to date no lepidopteran cell lines in which ecdysone induces autophagy have been described. This could be mainly due to the fact that some lepidopteran cell lines are ecdysteroid-resistant, while some others show differential responsiveness to these hormones (Zhong *et al*., 2005; Swevers *et al.*, 2008). However, this is a gap that must be filled since this tool could be useful for studying developmental autophagy *in vitro*.

What next?

Although much knowledge regarding autophagy in Lepidoptera is available, many questions remain to be answered.

1) What is the role of programmed autophagy in larval tissues? Autophagy could be necessary to achieve cell death in organs and tissues not readily accessible to phagocytes (Dai and Gilbert, 1999). Alternatively, autophagy could help the apoptotic process by enhancing its efficacy or perhaps by offsetting its inefficiencies, but in any case is necessary to obtain large-scale histolysis (Mpakou *et al.*, 2006; Tettamanti *et al.*, 2007b).

2) Can starvation have a role in developmental autophagy? Programmed autophagy in larval tissues is switched on by ecdysteroids, but given that this self-digestion process is concomitant with the food starvation period that the larva experiences during metamorphosis, can nutrient deprivation contribute at least to maintain the autophagic process active, once started by the hormone signal? In other words, is it hormone signals or starvation

which plays the most important and direct role in the autophagic process in larval tissues/organs?

3) Does autophagy support the development of certain new tissues that originate in the pupa and form the adult organs? The evidence obtained in the midgut suggests that the molecules derived from the massive autophagic digestion of the larval cells are absorbed and recycled by the new forming pupal midgut (Tettamanti *et al.*, 2007a, b), but conclusive proof of this hypothesis is still lacking.

4) Is it possible to identify any factors that can switch autophagy from a pro-survival to a pro-death role as happens in IPLB-LdFB cells?

5) In biological systems where autophagy coexists or cooperates with apoptosis, which are the molecular signals specific for initiating autophagy and apoptosis, rather than the mediators that regulate this cross-talk?

6) Since in different tissues autophagy and apoptosis can intervene simultaneously or in an asynchronous manner, is it possible to define exactly the timing of their action in different tissues?

7) What about the evolution of the autophagic process in different organisms, *i.e.*, yeast, *Drosophila*, Lepidoptera and mammals? A phylogenetic analysis of the autophagy-related genes that includes a wide collection of invertebrate species is not yet available.

One of the most difficult issues is that in the published literature, the description of autophagic morphological characteristics is sometimes confused and autophagy in larval tissues/organs of Lepidoptera seems to have its own peculiar features that are slightly different from those already found in yeast, *Drosophila* and mammals. In order to have an accurate characterization and definition of the autophagic process in this model system, opportune biochemical and molecular markers are necessary.

The present and future work in Lepidoptera is and will be focused on the search for genes and proteins that initiate and regulate autophagy, as well as the identification of the complex interactions that relate autophagy and apoptosis. This will help us to understand what the roles are of this self-digesting process in different larval tissues both during the development of the animal and under physiological vs stress conditions.

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