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REVIEW



# Autophagy and innate immunity: Insights from invertebrate model organisms

Cheng-Ju Kuo[a,b], Malene Hansen[c] and Emily Troemel[a]

[a]Division of Biological Sciences, University of California, San Diego, La Jolla, CA, USA; [b]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan; [c]Sanford Burnham Prebys Medical Discovery Institute, Program of Development, Aging and Regeneration, La Jolla, CA, USA

**ABSTRACT**

Macroautophagy/autophagy is a fundamental intracellular degradation process with multiple roles in immunity, including direct elimination of intracellular microorganisms via 'xenophagy.' In this review, we summarize studies from the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans* that highlight the roles of autophagy in innate immune responses to viral, bacterial, and fungal pathogens. Research from these genetically tractable invertebrates has uncovered several conserved immunological paradigms, such as direct targeting of intracellular pathogens by xenophagy and regulation of autophagy by pattern recognition receptors in *D. melanogaster*. Although *C. elegans* has no known pattern recognition receptors, this organism has been particularly useful in understanding many aspects of innate immunity. Indeed, work in *C. elegans* was the first to show xenophagic targeting of microsporidia, a fungal pathogen that infects all animals, and to identify TFEB/HLH-30, a helix-loop-helix transcription factor, as an evolutionarily conserved regulator of autophagy gene expression and host tolerance. Studies in *C. elegans* have also highlighted the more recently appreciated relationship between autophagy and tolerance to extracellular pathogens. Studies of simple, short-lived invertebrates such as flies and worms will continue to provide valuable insights into the molecular mechanisms by which autophagy and immunity pathways intersect and their contribution to organismal survival.

**Abbreviations:** Atg: autophagy related; BECN1: Beclin 1; CALCOCO2: calcium binding and coiled-coil domain 2; Cry5B: crystal toxin 5B; Daf: abnormal dauer formation; DKF-1: D kinase family-1; EPG-7: Ectopic P Granules-7; FuDR: fluorodeoxyuridine; GFP: green fluorescent protein; HLH-30: Helix Loop Helix-30; Imd: immune deficiency; *ins-18*: INSulin related-18; LET-363, LEThal-363; *lgg-1*: LC3, GABARAP and GATE-16 family-1; MAPK: mitogen-activated protein kinase; MATH: the meprin and TRAF homology; MTOR: mechanistic target of rapamycin; NBR1: neighbor of BRCA1 gene 1; NFKB: nuclear factor of kappa light polypeptide gene enhancer in B cells; NOD: nucleotide-binding oligomerization domain containing; OPTN: optineurin; PAMPs: pathogen-associated molecular patterns; *Park2*: Parkinson disease (autosomal recessive, juvenile) 2, parkin; *pdr-1*: Parkinson disease related; PFTs: pore-forming toxins; PGRP: peptidoglycan-recognition proteins; *PIK3C3*: phosphatidylinositol 3- kinase catalytic subunit type 3; *pink-1*: PINK (PTEN-I induced kinase) homolog; PRKD: protein kinase D; PLC, phospholipase C; PRKN: parkin RBR E3 ubiquitin protein ligase; PRRs: pattern-recognition receptors; PtdIns3P: phosphatidylinositol-3-phosphate; *rab-5*: RAB family-5; RB1CC1: RB1-inducible coiled-coil 1; RNAi: RNA interference; *sqst*: SeQueSTosome related; SQSTM1: sequestosome 1; TBK1: TANK-binding kinase 1; TFEB: transcription factor EB; TGFB/TGF-$\beta$: transforming growth factor beta; TLRs: toll-like receptors; *unc-51*: UNCoordinated-51; VPS: vacuolar protein sorting; VSV, vesicular stomatitis virus; VSV-G: VSV surface glycoprotein G; Wipi2: WD repeat domain, phosphoinositide interacting 2

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## Introduction to autophagy

Early studies of autophagy were mainly restricted to its role in nonselective recycling of intracellular material to the lysosome in yeast responding to starvation conditions.[1,2] However, it is now clear that autophagy has a range of specialized functions, including selective elimination of large endogenous material, such as damaged organelles (e.g., mitophagy), as well as exogenous material, such as invading pathogens (xenophagy). During autophagy, these 'cargo' are selectively recognized and sequestered within double-membrane vesicles called autophagosomes, which subsequently fuse with acidic lysosomes containing hydrolases used for degradation of cargo. This review will focus on macroautophagy (hereafter referred to as autophagy) in 2 invertebrate model organisms: the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*.

Autophagy proceeds through at least 5 sequential steps: (1) initiation, (2) double-membrane nucleation and formation of a pre-autophagosome or phagophore, (3) phagophore elongation and sequestration of cytoplasmic cargo, (4) autophagosome fusion with a lysosome to form an autolysosome, and (5) cargo degradation in the autolysosome (Fig. 1). Several conserved autophagy-related (Atg) proteins, many of which have clear homologs in *C. elegans* and *D. melanogaster* (Table 1),

**CONTACT** Malene Hansen mhansen@sbpdiscovery.org; Emily Troemel etroemel@ucsd.edu
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**Table 1.** Autophagy-related genes linked to immunity in *D. melanogaster* and *C. elegans*.

| *D. melanogaster* | *S. cerevisiae/H. sapiens* | Pathogen | Reference |
|---|---|---|---|
| *Atg1* | *Atg1/ULK1* | *Wolbachia*, VSV | 19,21 |
| *Atg6* | *VPS30/BECN1* | VSV | 21 |
| *Atg2* | *ATG2* | VSV | 21 |
| *Atg18* | *WIPI2* | VSV | 21 |
| *Atg9* | *ATG9* | VSV | 21 |
| *Atg12* | *ATG12* | *E. coli*, VSV | 16,21 |
| *Atg7* | *ATG7* | *E. coli*, VSV | 16,21 |
| *Atg4* | *ATG4* | VSV | 21 |
| *Atg5* | *ATG5* | *L. monocytogenes*, *E.coli* | 14,16 |
| *Atg8* | *MAP1LC3* | VSV | 21 |
| *Park* | *PRKN* | *M. marinum*, *S. enterica* | 18 |
| *C. elegans* | *S. cerevisiae/H. sapiens* | Pathogen | Reference |
| *unc-51* | *Atg1/ULK1* | *S. aureus* | 41 |
| *atg-13* | *ATG13* | *S. aureus* | 19,21 |
| *bec-1* | *Vps30/BECN1* | *S. enterica*, *P. aeruginosa*, Cry5B | 34,44,45 |
| *vps-34* | *Vps34/PIK3C3* | *S. aureus* | 41 |
| *atg-2* | *ATG2* | *S. aureus* | 19,21 |
| *atg-18* | *WIPI2* | *N. parisii*, Cry5B | 40,44 |
| *lgg-3* | *ATG12* | Cry5B | 44 |
| *atg-7* | *ATG7* | *S. enterica* | 34 |
| *atg-16.2* | *ATG16L1* | *S. aureus* | 19,21 |
| *atg-4.1/2* | *ATG4* | Cry5B | 44 |
| *lgg-1* | *MAP1LC3* | *S. aureus*, *S. enterica*, *P. aeruginosa*, *N. parisii*, Cry5B | 34,40,41,44,45 |
| *lgg-2* | *MAP1LC3* | *S. aureus* | 41 |
| *sqst-1* | *SQSTM1* | *N. parisii* | 40 |

Autophagy-related genes with reported roles in anti-viral, -bacterial, and -fungal immunity in *D. melanogaster* and *C. elegans* are shown, together with the yeast/human homologs. VSV, vesicular stomatitis virus; Cry5B, pore-forming toxin from *B. thuringiensis*. See Fig. 1 for a functional overview of autophagy genes, and the text for details.

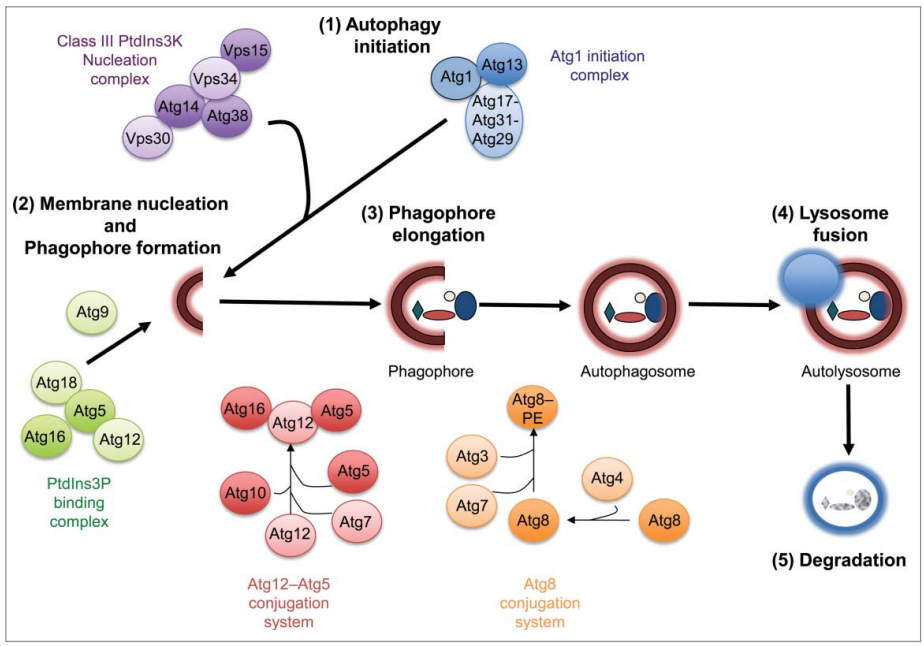


**Figure 1.** Overview of the macroautophagy process. Macroautophagy (referred to as autophagy) proceeds through at least 5 discrete steps: initiation, membrane nucleation and phagophore formation, phagophore elongation, lysosome fusion, and degradation. These steps are executed by at least 5 protein complexes: Atg1/ULK1 initiation complex, class III PtdIns 3-kinase nucleation complex, PtdIns3P-binding complex, Atg12 conjugation system, and Atg8/LC3 conjugation system. See text for details. Figure is modified from Gelino et al.[53]

function as macromolecular complexes at the different steps of the autophagy process (Fig. 1). Specifically, activation of the Atg1/ULK1 initiation complex, which contains Atg101 and Atg13 (and RB1CC1 in mammals, Atg17 in flies, and EPG-7 in worms), allows creation of a phagophore by the Vps34/PIK3C3-Vps30/BECN1 and phosphatidylinositol 3-phosphate (PtdIns3P)-binding complexes. Phagophore elongation is mediated by 2 ubiquitin-like conjugation systems. The first involves covalent conjugation of the ubiquitin-like protein Atg12 to Atg5 by the E1- and E2-like enzymes Atg7 and Atg10, respectively. The Atg12–Atg5 conjugate then promotes conjugation (possibly via its E3-like ligase activity) of phosphatidylethanolamine (PE) to cytosolic Atg8/LC3 (referred to as Atg8-I), which is formed by cleavage of the ubiquitin-like protein Atg8/LC3 by the protease Atg4. Processed and PE-conjugated Atg8/LC3 (referred to as Atg8-II/LC3-II) associates with the phagophore membrane, where it facilitates elongation and cargo recognition, and may regulate fusion with the lysosome (Fig. 1). Of note, some of the autophagy proteins mentioned above, including Atg8/LC3, also have nonautophagy roles.[3] A more detailed discussion of the different steps of autophagy can be found in Klionsky et al.[4]

The selectivity of autophagy is achieved by receptors that specifically recognize certain types of substrates (cargo), such as aggregated proteins or damaged organelles, and concomitantly interact with lipidated Atg8/LC3, thus bringing the cargo to the phagophore. Several cargo receptors recognize polyubiquitinated substrates. For example, SQSTM1/p62, CALCOCO2/NDP52 and OPTN/Optineurin recognize ubiquitinated mitochondria and facilitate their degradation by mitophagy.[5] Clearance of damaged mitochondria protects the cells from potentially toxic components, such as reactive oxygen species released by breakdown of mitochondrial membrane integrity. The ability of autophagic vesicles to engulf such bulky cargo several microns in diameter is also exploited by the cell to clear intracellular microbes, a process termed xenophagy. The involvement of autophagy in host defense is an exciting area of research that is addressing a number of key questions. For example, how are microbes selected for autophagic clearance? Does autophagy play roles in host immunity beyond xenophagic degradation of the pathogen? How does the host use this intracellular degradation pathway to fight infection by extracellular pathogens?

Some of these questions are addressed in several excellent reviews on autophagy and infection.[6-8] Here, we summarize some of the insights gained from studies of *D. melanogaster* and *C. elegans*, 2 powerful model systems that have enabled important discoveries about how metazoans use autophagy to protect against microbial infections. We define 'microbe' as an entity that causes infection in *D. melanogaster* or *C. elegans*; specifically, viruses, bacteria, and eukaryotic single-celled organisms. Notably, *D. melanogaster* and *C. elegans* both lack an adaptive immune system, relying instead on innate immune responses mediated by dedicated 'professional' (*D. melanogaster*) or 'nonprofessional' (*C. elegans*) immune cells. In *D. melanogaster*, hemocytes are one type of professional immune cell, which are analogous to macrophages and can eliminate microbial pathogens by phagocytosis. In contrast, *C. elegans* appears to lack professional immune cells and instead relies on epithelial cells for immune defense. Interestingly, autophagy is also important in mammalian epithelial cells, although its role has been studied more extensively in professional immune cells.[9-11] Thus, *D. melanogaster* and *C. elegans* provide the opportunity to explore the contribution of autophagy to innate immunity, particularly epithelial defense, in the absence of confounding effects of adaptive immunity. Indeed, studies in *D. melanogaster* provided some of the earliest examples of how host pattern-recognition receptors (PRRs) promote autophagy, while studies in *C. elegans* provided the first example of xenophagy targeting microsporidia, and the first description of the conserved role of the transcription factor TFEB/HLH-30 in immunity. Several recent studies in *C. elegans* have begun to elucidate the involvement of autophagy in promoting host tolerance (i.e., the ability to limit detrimental impact on the host) rather than resistance (i.e., the ability to limit pathogen burden) to infection by extracellular pathogens. Both model organisms are genetically tractable at the whole organism and cell- and tissue-specific levels and additionally have short life cycles, which facilitate analysis of the role of autophagy on organismal survival. Within this context, we discuss how key components of innate immune signaling pathways in flies and worms interface with autophagy to regulate host defense against various types of microbial infection.

## Autophagy and immunity in *Drosophila melanogaster*

Studies in flies and mice in the 1990s uncovered the central paradigm that animals use PRRs to detect pathogen-associated molecular patterns (PAMPs), which are molecules produced specifically by microbes.[12] Most PAMPs may be more aptly named microbe-associated molecular patterns in that they are common to both pathogenic and non-pathogenic microbes. The 2 major immune detection systems in the fly, Toll and Imd pathways, recognize PAMPs derived from Gram-positive bacteria and fungi and from Gram-negative bacteria, respectively. Both the Toll and Imd pathways activate transcription factors orthologous to mammalian NFKB/NF-$\kappa$B, arguably the central transcription factor in mammalian immunity.[12] The Toll-NFKB pathway was first defined for its role in *D. melanogaster* embryonic development, and the subsequent discovery of its involvement in fly immunity occurred concurrent with the finding that a PAMP-toll-like receptor (TLR)-NFKB pathway is also involved in microbial defense in the mouse. PAMP-PRR signaling is not the only aspect of microbial immunity that is conserved in *D. melanogaster* and mice, as both species also use xenophagic turnover of pathogens to control intracellular infection. This topic was previously reviewed,[13] and, here, we summarize the most recent reports of how autophagy components and PRRs interface to detect and control intracellular pathogen infections in *D. melanogaster* (Fig. 2).

### *Xenophagy in* D. melanogaster

One of the first reports that xenophagy can control infection in *D. melanogaster* came from studies of the bacterium *Listeria monocytogenes*.[14] This pathogen causes food-borne illness in

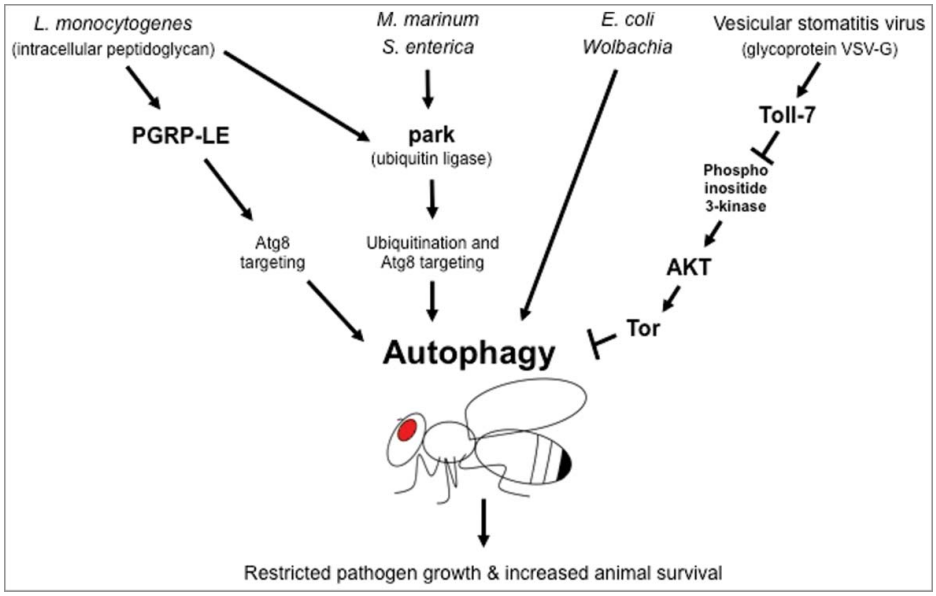


**Figure 2.** Pathogen responses linked to autophagy in *Drosophila melanogaster*. Autophagy is linked to defense against several intracellular pathogens in the fruit fly *D. melanogaster*. Intracellular peptidoglycan of the bacterium *Listeria monocytogenes* binds to the peptidoglycan recognition receptor PGRP-LE in hemocytes, which induces autophagy and clears the pathogen via Atg8 targeting. Infection by *Mycobacterium marinum, Salmonella enterica, Escherichia coli* and *Wolbachia*, a common insect pathogen, is also cleared by autophagy. In particular, clearance of *M. marinum* and *S. enterica* involves the ubiquitin ligase PARK2/park and Atg8 targeting. Clearance of viral infections has also been linked to autophagy. Specifically, binding of the vesicular stomatitis virus (VSV)-G glycoprotein to the pattern recognition receptor Toll-7 inhibits activation of Tor through the phosphoinositide 3-kinase-Akt pathway. Because Tor negatively regulates autophagy, its inhibition by VSV-G signaling results in induction of autophagy, which restricts viral replication and promotes organismal survival. See text for details and Table 1 for autophagy genes linked to immunity in *D. melanogaster*.

humans and is a facultative intracellular pathogen, meaning that it can replicate extracellularly or intracellularly. PRRs such as Toll receptors and TLRs are expressed both on the cell surface as well as intracellularly and are thus poised to detect both types of pathogens. Studies from Yano et al. demonstrated that PGRP-LE, an intracellular PRR in *D. melanogaster,* detects a peptidoglycan PAMP produced by *L. monocytogenes* and triggers LC3/Atg8 targeting to the bacteria followed by xenophagic engulfment.[14,15] Concurrently, a separate study indicated that autophagy components are important for *D. melanogaster* defense against infection with *Escherichia coli*. RNAi knockdown of autophagy components caused an increased pathogen load and decreased survival upon *E. coli* infection, although not a decreased lifespan overall.[16] Notably, studies of xenophagy against *L. monocytogenes* reported that xenophagy is induced independently of the Toll and Imd pathways, and the noncanonical pathway triggered by PGRP-LE has yet to be defined.[14] Specifically, these studies showed that flies defective in the autophagy component *Atg5* or in PGRP-LE carry a higher *L. monocytogenes* load and survive for shorter times after infection as compared to wild-type flies. Interestingly, a pathway with parallels to the *D. melanogaster* PRGP-LE–xenophagy pathway was subsequently found in mammals. Mammalian NOD1 and NOD2, which are intracellular receptors distinct from TLRs, also recognize peptidoglycans and trigger xenophagy in defense against intracellular pathogens.[17] Although NOD2 directly recruits ATG16L1 to bacteria at the site of membrane entry, it is less clear how bacteria are captured for xenophagy after escape into the cytosol.[7,8] Of note, variants of the *NOD2* and *ATG16L1* genes are associated with Crohn disease, a serious inflammatory bowel disease in humans.[11]

Several studies have suggested that some of the same autophagy machinery involved in mitophagy may also be important for xenophagy. PRKN/PARK2/parkin is an E3 ubiquitin ligase with a well-characterized role in conjugating ubiquitin to the surface of damaged mitochondria, thereby recruiting the autophagy machinery via ubiquitin-binding receptor proteins such as BNIP3. The finding that park/PARK2-deficient flies and mice have defects in pathogen clearance was therefore particularly exciting.[18] These animals carry higher pathogen loads than wild-type flies or mice upon infection with several facultative intracellular pathogens, including *L. monocytogenes, Mycobacterium* spp. (*M. tuberculosis* in mice and *M. marinum* in flies), and *Salmonella enterica.* Moreover, park-mutant flies succumb earlier to infection than wild-type flies, highlighting the importance of park for long-term health. In mice, PARK2 is required for ubiquitination of *M. tuberculosis* and recruitment of ubiquitin-recognition receptors, including SQSTM1, NBR1, CALCOCO2, and phospho-TBK1, as well as the autophagy proteins Atg8/LC3 and Atg12. The park protein is also required for ultimate microbial targeting to lysosomes and subsequent degradation.[18] However, the mechanisms by which park and other ubiquitin ligases recognize pathogen substrates to be targeted for ubiquitination and subsequent autophagic degradation remain poorly understood.[8]

Autophagy plays a similarly protective role in flies infected with the obligate intracellular Gram-negative bacterium *Wolbachia*,[19] which is commonly harbored by insects and nematodes. Because *Wolbachia* is vertically transmitted (i.e., it is transmitted through the mother to progeny through the eggs, and undergoes its entire life cycle inside the fly), it has a very close association with its host. Given that mitochondria were thought to be derived from an internalized bacterium that became an obligate intracellular microbe and eventually an organelle,[20] it is interesting to note that an obligate, intracellular microbe like *Wolbachia* would be targeted for xenophagic clearance as well.

Autophagy is also required for *D. melanogaster* defense against viral infection. Shelly et al. found that the mammalian viral pathogen vesicular stomatitis virus (VSV), can also infect *D. melanogaster* and trigger an anti-viral response.[21] In this study, inhibition of the autophagy-related genes *Atg1/Ulk1, Atg5, Atg8a/Lc3,* and *Atg18/Wipi2* in *D. melanogaster* S2 cells was found to increase the VSV infection rate. Similarly, RNAi knockdown of *Atg18/Wipi2* in adult flies increases viral replication and decreases the survival of infected flies. Whereas earlier studies in other systems had been able to detect viruses within autophagic vesicles, this study in *D. melanogaster* was the first to demonstrate that autophagy plays an active role in antiviral immunity. Interestingly, Shelly et al. found that autophagy induction is independent of VSV replication and can be triggered solely by the VSV surface glycoprotein G (VSV-G), which activates autophagy via the nutrient-sensing phosphoinositide 3-kinase-Akt signaling pathway.[21] Subsequent studies by Nakamoto et al. clarified how infected flies recognize VSV-G. Surprisingly, they found that VSV-G is detected by a cell-surface Toll receptor, indicating that the virus is sensed extracellularly. This study was pivotal in shedding light on the function of another one of the 9 Toll receptors expressed in *D. melanogaster*. After the initial discovery that a Toll receptor senses Gram-positive bacterial and fungal infections, the role of the other 8 Toll receptors had remained unclear. Nakamoto et al. showed that VSV replication and mortality are higher in *Toll-7* mutants compared with wild-type flies, thus identifying Toll-7 as a VSV-G receptor capable of triggering antiviral autophagy.[22] Interestingly, Moy et al. showed that *D. melanogaster* Toll-7 also directs antiviral immunity against arthropod-borne viruses that are more pathogenic to humans and those regulate autophagy defense against these viruses via toll-like receptor signaling in mammalian cells as well.[23]

As summarized here, studies in *D. melanogaster* have been instrumental in establishing PGRP-LE and Toll-7 as PRRs important for autophagy induction in response to intracellular bacterial and viral infections and in discovering the conserved function of the ubiquitin ligase park in targeting intracellular bacterial pathogens.

## Autophagy and immunity in *Caenorhabditis elegans*

The microscopic nematode *C. elegans* is another common invertebrate model organism for studies of innate immunity, in part because of the extensive genetic toolbox available for this animal.[24,25] In contrast to work in *D. melanogaster*, which has concentrated on systemic immunity and signaling by professional immune cells, most studies in *C. elegans* have focused on intestinal epithelial immunity to oral infections. *C. elegans* is notable among models for studying immunity in that it is devoid of any specialized immune cells and instead relies on nonprofessional cells, such as epithelial cells, for defense. Fortunately, the transparent body plan of *C. elegans* facilitates direct observation of microbes and their interactions with host epithelial cells during infection, which has been useful for the study of both extracellular and intracellular pathogens. *C. elegans* contains 20 nonrenewing intestinal epithelial cells similar in structure and function to their mammalian counterparts.[26] In addition, *C. elegans* intestinal epithelial cells, like their mammalian counterparts, are nonphagocytic indicating that pathogen uptake occurs through a mechanism distinct from that of *D. melanogaster* hemocytes or mouse macrophages. Another intriguing mechanistic difference is that NFKB appears to have been lost from the evolutionary lineage that gave rise to *C. elegans*.[26] This feature provides the advantage that it permits dissection of the roles of transcription factors other than NFKB, which has been extensively studied in many species. Indeed, studies in *C. elegans* have identified several novel transcription factors with roles in immune defense, and it appears unlikely that a single factor plays the dominant role, as is the case for NFKB in flies and mammals. Instead, studies in *C. elegans* suggest that distinct transcription factors may be important for defense against different pathogens,[27,28] or even against different virulence factors from the same pathogen. For example, *C. elegans* displays several context-dependent modes of defense against the bacterial pathogen *Pseudomonas aeruginosa*, each involving distinct pathogen virulence factors and different host pathways.[29,30]

How does *C. elegans* detect pathogens? Surprisingly, no PRRs have yet been identified in *C. elegans*. Moreover, the single *C. elegans* Toll-like receptor, TOL-1, which was identified by sequence homology, does not appear to play a canonical role in pathogen defense. However, like its fly ortholog, *tol-1* does have a role in early development.[25] Intriguingly, *tol-1* also plays a role in the development of sensory neurons important for behavioral avoidance of pathogens.[25] Several reports suggest that *C. elegans* does not detect the pathogen or its products *per se*, but rather senses the physiological consequences of pathogenic attack, or 'patterns of pathogenesis'.[24,31] For example, *C. elegans* intestinal cells sense *P. aeruginosa* indirectly through the effects of the bacterial product exotoxin A, which is endocytosed by host cells and causes a block in mRNA translation.[24] Interestingly, it is the translational inhibition, not the toxin itself, that is sensed and leads to increased protein levels of the bZIP transcription factor ZIP-2 through an unknown mechanism. In turn, ZIP-2-mediated gene expression reduces pathogen load and increases host survival. Several *C. elegans* transcription factors have been shown to mediate defense by upregulating expression of large classes of predicted antimicrobial genes, including secreted C-type lectins, lysozymes, and lipases.[24,25] The molecular connections between the pathogenic triggers and host signaling pathways are still being defined, but the major pathway controlling expression of antimicrobial genes appears to be the conserved MAPK (mitogen-activated protein kinase)/p38 pathway. This pathway controls *C. elegans* resistance to most pathogens tested, although it appears to activate different transcription factors in response to different pathogens.[27,28,32] Another pathway that is involved in *C. elegans* immunity is the DAF-2/DAF-16 insulin-like signaling pathway, which also has roles in development, metabolism, and longevity.[33] How autophagy is involved in *C. elegans* defense via these signaling pathways is only beginning to be investigated. Below, we summarize the findings of several reports that not only suggest a key role for autophagy in *C. elegans* antiviral, -bacterial, and -fungal responses, but also highlight several novel autophagy regulators and immunological concepts, especially with respect to defense against extracellular pathogens (Fig. 3).

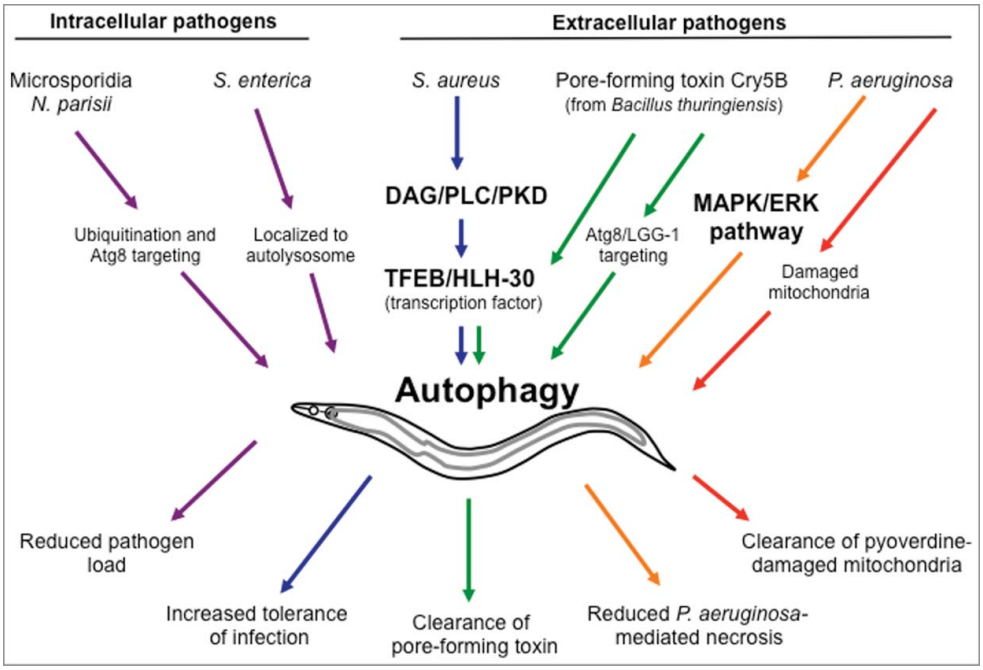


**Figure 3.** Pathogen responses linked to autophagy in *Caenorhabditis elegans*. Autophagy plays crucial roles in the defense against both intracellular and extracellular pathogens in the nematode *C. elegans*. The microsporidian pathogen *Nematocida parisii* is targeted for ubiquitination and recruitment of Atg8/LGG-1, which likely results in xenophagy. Replication of *Salmonella enterica* is restricted by localization to lysosomes. *Staphylococcus aureus* and the *Bacillus thuringiensis* pore-forming toxin Cry5B both induce transcription of autophagy-related genes via TFEB/HLH-30. *Pseudomonas aeruginosa*-induced necrosis of *C. elegans* is reduced by activation of autophagy through the MAPK/ERK signaling pathway. Autophagy (mitophagy) also clears mitochondria damaged by the *P. aeruginosa* virulence factor pyoverdine, thereby reducing mortality. See text for details and Table 1 for autophagy genes linked to immunity in *C. elegans*.

## *Xenophagy in* C. elegans

Most studies of bacterial infection in *C. elegans* have been performed by feeding worms with clinically relevant bacteria that accumulate in the intestinal lumen, inflict damage, and ultimately kill the host. All *C. elegans* bacterial pathogens characterized to date (including facultative intracellular bacteria such as *S. enterica* and *L. monocytogenes*) appear not to enter cells but remain in the intestinal lumen during the active stage of the infection (although for some bacterial pathogens, such as *P. aeruginosa*, invasion of intestinal cells in wild-type animals has been seen later in infection, when there is extensive intestinal tissue damage). The reason for this restriction to the lumen early during infection was proposed by Jia et al.[34] whose work suggested that autophagy may prevent *S. enterica* intracellular persistence and replication. Specifically, intact bacteria were not observed with electron microscopy within intestinal cells in control animals, whereas RNAi of the autophagy components *VPS30/Becn1/bec-1* and *ATG8/lgg-1* resulted in what appeared to be intracellular bacteria and an expanded bacterial population in the intestinal lumen. Notably, however, the electron microscopy lacked any specific markers to definititvely identify the bacteria, and the authors have been unable to observe fluorescently tagged *S. enterica* intracellularly in later work.[35] Irrespective of bacterial localization following infection, these studies suggest that RNAi-mediated inhibition of autophagy genes reduces the survival of infected *C. elegans*, indicating a role for this pathway in defense against *S. enterica* infection. The authors also examined *S. enterica* infection in *C. elegans daf-2*/IGF-1 receptor mutants, and showed that these mutants require autophagy genes for increased survival upon infection.[34] Taken together, these studies support a role for autophagy in limiting bacterial proliferation and reducing lumenal bacterial load. Although further details remain to be investigated, it is possible that autophagy exerts these effects via xenophagy, possibly in combination with regulating secretion of antimicrobial molecules by intestinal cells, similar to the behavior of Paneth cells in the mouse intestine.[36,37]

The first pathogen shown to normally reside and replicate in the intestinal cells of wild-type *C. elegans* is *Nematocida parisii*. *N. parisii* defines a new genus and species in the *Microsporidia* phylum, which contains diverse obligate intracellular pathogens that can infect many animals, including humans. Microsporidia have been isolated from wild-caught nematodes around the world,[38,39] suggesting that they exert evolutionary pressure on their hosts. Microsporidia deploy a unique invasion strategy involving an infection apparatus called a polar tube, which delivers a parasite cell called the sporoplasm directly into the host cell, where it replicates in the host cytoplasm. Not surprisingly, the cytoplasmic location makes the sporoplasms vulnerable to targeting by the autophagy machinery, as shown by Bakowski et al.[40] These authors performed transcriptional profiling studies of infected *C. elegans* and found that *N. parisii* induced a gene set distinct from that induced by extracellular pathogens such as *P. aeruginosa* and *Staphylococcus aureus* but nearly identical to that induced by Orsay virus, another natural obligate intracellular pathogen of *C. elegans* that also replicates in the intestinal cells.

The gene set commonly upregulated by Orsay virus and *N. parisii* infection is enriched for functional domains associated with ubiquitin-mediated proteolysis, including the ubiquitin

ligase adapter F-box and MATH domain genes. Following on this lead, the authors showed that ubiquitin and Atg8/LGG-1 are colocalized to *N. parisii* early during the parasite replication phase within the intestinal cells. Furthermore, knockdown of key autophagy components, such as *ATG8/lgg-1* and *Sqstm1/sqst-1*, increases the pathogen load; conversely, activation of autophagy by blocking the nutrient-sensor and negative autophagy regulator MTOR/LET-363 increases Atg8/LGG-1 targeting and reduces the pathogen load. Notably, the increase in pathogen load by knockdown of autophagy is relatively small, raising the possibility that *N. parisii* might actively suppress autophagy. Indeed, the authors showed that treatment of *C. elegans* with either the DNA synthesis inhibitor fluorodeoxyuridine (FuDR) or the antimicrosporidia drug fumagillin, both of which slow pathogen growth, increases the efficiency of ubiquitin targeting to parasite cells. Collectively, these results suggest that ubiquitination and autophagy play a role in controlling intestinal infection with *N. parisii* and provide the first demonstration of direct localization of autophagy machinery to intracellular pathogens in *C. elegans*.[40] These studies are also the first to demonstrate xenophagy of a microsporidia species, and this remains one of the few innate immune strategies known to defend against microsporidia infection.

## *HLH-30/TFEB and tolerance of* C. elegans *to infection*

Studies in *C. elegans* have been instrumental in identifying a new transcription factor, the helix-loop-helix transcription factor HLH-30 (ortholog of TFEB) as a key regulator of the innate immune response to the extracellular bacterium *S. aureus*.[41] TFEB had previously been shown to control expression of certain autophagy and lysosomal genes in response to nutritional stress in mammalian cells.[42] A search for transcription factors regulating expression of genes induced by infection of *C. elegans* with *S. aureus* identified HLH-30 as a candidate. Notably, *S. aureus* infection induces translocation of TFEB/HLH-30 to the nucleus, where it regulates ~80% of the transcriptional immune response to infection.[41] The affected genes included components of conserved signaling pathways (e.g., MAPK/JNK, MAPK/p38, TGFB/TGF-$\beta$, INS/INS-18), antimicrobial genes (e.g., lysozymes, C-type lectins), and autophagy and lysosomal genes. Consistent with these observations, Visvikis et al. demonstrated that TFEB/HLH-30 plays an important role in the immune response to *S. aureus* via upregulation of autophagy genes. They showed that expression of Atg8/LGG-1 is significantly induced in *C. elegans* intestinal cells by *S. aureus* infection, although—intriguingly—the bacteria remain extracellular. RNAi of *ATG8/lgg-1*, *ATG1/Ulk1/unc-51*, or *VPS34/pik3c3/vps-34* autophagy genes decrease the survival rate of infected wild-type animals, but not of *Tfeb/hlh-30* mutants, indicating a requirement for TFEB/HLH-30-regulated autophagy in the antibacterial defense. Similar observations have been made in murine macrophages infected with *S. aureus*, suggesting that TFEB is a conserved transcriptional regulator of immune responses.[41]

In mammals, phosphorylation of TFEB by MTOR and MAPK1/ERK2 inhibits TFEB nuclear translocation under basal conditions, but depletion of intracellular amino acids induces TFEB dephosphorylation and transport into the nucleus. To identify the relevant regulatory enzymes, Najibi et al. screened an RNAi library targeting most *C. elegans* protein kinases and phosphatases for genes capable of regulating nuclear localization of the reporter protein HLH-30::GFP. They found that a novel PRKD (protein kinase D), called DKF-1, is required for TFEB/HLH-30 nuclear localization in response to *S. aureus* infection.[43] Furthermore, knockdown of *dkf-1* increases the susceptibility to infection of wild-type animals but not of *Tfeb/hlh-30* mutants, suggesting that *dkf-1* and *Tfeb/hlh-30* act in the same pathway. Additional experiments placed phospholipase C (PLC) upstream of PRKD in the *C. elegans* pathway and also showed that the PLC-PRKD-TFEB pathway is conserved in murine macrophages.

Bacterial pathogens generate virulence factors known as pore-forming toxins (PFTs) that damage host cellular membranes. A recent report showed that the PFTs Cry5B and Cry21A, produced by the extracellular Gram-positive bacterium *Bacillus thuringiensis,* induce autophagy in *C. elegans* via TFEB/HLH-30.[44] Specifically, *C. elegans* fed with *E. coli* expressing Cry5B were examined by electron microscopy and by fluorescence microscopy to detect autophagy marker proteins. Cry5B feeding not only increased the abundance of autophagic vesicles but also induced the nuclear translocation of HLH-30::GFP in intestinal cells. Moreover, GFP-tagged Atg8/LGG-1 colocalizes with rhodamine-labeled Cry5B proteins within intestinal cells, suggesting that Cry5B is degraded by autophagy. Consistent with this observation, inhibition of *VPS30/Becn1/bec-1*, *ATG4/Atg4/atg-4.1/2*, *Atg8/lgg-1/2/3*, *Wipi2/atg-18,* and *Tfeb/hlh-30* decreases the survival of animals fed *E. coli* expressing Cry5B, but not animals fed *E. coli* expressing vector control. Thus, in contrast to the classical definition of xenophagy as an intracellular microbe-targeting pathway, targeting of Cry5B appears to be an example of xenophagy of intracellular toxins delivered by extracellular microbes. Interestingly, transcriptomic analysis of the Cry5B-fed *C. elegans* revealed that HLH-30/TFEB also regulates the transcription of membrane-repair genes (e.g., the small GTPase *rab-5*). Autophagy genes are required for the repair of membrane pores induced by Cry5B,[44] suggesting an additional function for autophagy in host defense to PFT-producing bacteria. Among the genes induced by Cry5B are a number related to C-type lectins, lysosomes, peroxisomes, and heat shock proteins, which is in agreement with the overall function of TFEB/HLH-30 in regulating expression of cytoprotective and antimicrobial genes in defense against *S. aureus* infection.[41] Taken together, these data demonstrate the essential role of transcriptional regulation of autophagy in the multi-step defense of *C. elegans* against Cry5B and *S. aureus*. Additionally, it appears that defense against extracellular bacteria such as *S. aureus* also involves xenophagic clearance of toxins delivered into the host cells. Further work will shed light on this important defense mechanism.

## *Nonxenophagic roles for autophagy-mediated defense in* C. elegans

As noted above, autophagy is required for the repair of membrane pores caused by Cry5B, highlighting the role of this pathway not only in clearing the pathogen/toxin but also in minimizing their deleterious effects and maintaining host

fitness during infection. In this regard, Zou et al.[45] showed that autophagy controlled *C. elegans* infection with the extracellular bacterium *P. aeruginosa* not by blocking intestinal accumulation but rather by inhibiting pathogen-induced necrosis of intestinal cells. Although autophagy interfaces with apoptosis,[2] relatively little is known about its connection to necrosis. Zou et al. found that autophagy is regulated via the MAPK/ERK pathway. Although *bec-1* RNAi reduces the survival of infected animals, concomitant knockdown of *bec-1* and the necrosis-related genes *asp-3* and *asp-4* rescue survival.[45] Thus, autophagy increases the host tolerance not by clearing the extracellular pathogen but by dampening its deleterious effects on the host.

Another nonxenophagic mechanism by which autophagy contributes to *C. elegans* immune defense is by clearing organelles damaged by pathogen products, as recently reported by Kirienko et al. In this study, the authors used a model of *P. aeruginosa* infection of *C. elegans* that involves different host and pathogen factors than the *P. aeruginosa* model described above. The *P. aeruginosa* virulence factor pyoverdine is an iron-chelating siderophore that disrupts iron metabolism and mitochondrial homeostasis in the host,[46] possibly by inducing iron scavenging resulting in a 'hypoxic crisis.' Exposure of *C. elegans* to *P. aeruginosa* or to partially purified pyoverdine causes the appearance of fragmented mitochondria with large, punctate bodies, in contrast to the long-branched tubular appearance of healthy mitochondria. Genetic mutation and RNAi experiments showed that knockdown of the autophagy-related genes *VPS30/Becn1/bec-1* and *lgg-1/ATG8* or of the mitophagy regulators *pink-1* and *Park2/pdr-1* reduces the survival of *P. aeruginosa*-infected *C. elegans*. Intriguingly, the pyoverdine-induced mitophagy response also promotes *C. elegans* resistance to *P. aeruginosa*. Taken together, these studies illustrate the essential nonxenophagic contributions of autophagy to the antimicrobial response of *C. elegans*.

## Conclusions and future directions

As summarized above, studies in the metazoans *D. melanogaster* and *C. elegans* have identified multiple links between the cellular recycling process of autophagy and the innate immune responses to diverse microbes. Seminal discoveries on PRR-mediated activation of autophagy were made in *D. melanogaster*, including the function of PGRP-LE in triggering xenophagy in response to intracellular bacteria. Somewhat surprisingly, viral glycoproteins appear to be sensed by the cell-surface receptor Toll-7 in flies, indicating that autophagy is triggered before viral invasion and replication. Ultimately, however, viral burden is likely lowered intracellularly through xenophagy. This strategy may be a form of 'priming,' allowing the host to upregulate defense mechanisms before extensive damage has been inflicted. Given recent findings about the complement system in *D. melanogaster* regulating autophagy in neighboring cells, perhaps antimicrobial autophagy responses might even be established in a cell nonautonomous fashion.[47]

*C. elegans* also uses xenophagic elimination of intracellular microbes, and autophagy machinery can be targeted to virulence factors secreted by extracellular microbes invading this organism. The *B. thuringiensis* Cry5B PFT disrupts host cell membrane integrity and is then endocytosed as part of the host membrane-repair process. In the cytoplasm, Cry5B colocalizes with the autophagy machinery and is subsequently cleared from the cell. These observations suggest that the definition of xenophagy could be expanded to include the targeting and degradation of intracellular microbial factors as well as the intact microbes themselves. In this case, the distinction between extracellular and intracellular pathogens is blurred, because the virulence factor is delivered into the host cell and targets core intracellular processes but the pathogen itself is extracellular. Given that hundreds of such virulence factors are deployed by pathogens to attack host cells,[48] it seems likely that autophagy may target a vast array of molecules in a similar manner. For example, *P. aeruginosa* appears to secrete many virulence factors in addition to pyoverdine and exotoxin A during *C. elegans* infection. It will be interesting to determine whether exotoxin A induces autophagy, as pyoverdine does.

The autophagy regulator TFEB/HLH-30 was first shown to be important for pathogen responses in *C. elegans* and then subsequently confirmed in mammals. While it is clear that TFEB/HLH-30 controls clearance of the *B. thuringiensis* Cry5B virulence factor via autophagy, it is not clear how it orchestrates *C. elegans* defense against the extracellular pathogen *S. aureus*. Loss of *hlh-30/Tfeb* does not appear to affect *S. aureus* pathogen load but does reduce host survival, suggesting that TFEB/HLH-30 affects host tolerance to infection. This model is supported by the observation that the survival advantage conferred by overexpression of *Tfeb/hlh-30* requires the conserved autophagy machinery. Perhaps autophagy clears an unidentified virulence factor produced by *S. aureus,* as with *B. thuringiensis* and Cry5B. Alternatively, or in addition, autophagy may degrade a damaged host factor or organelle to improve cellular health. This novel function for autophagy in controlling host tolerance has been demonstrated for several infectious agents in *C. elegans;* for example, pyoverdine-induced mitophagy during *P. aeruginosa* infection, as described above. Autophagy also appears to block necrosis induced by *P. aeruginosa*. Although the studies of *P. aeruginosa* mitophagy and necrosis used different models, it is tempting to speculate that the pathways could be linked, perhaps through triggering of necrosis by reactive oxygen species released from damaged mitochondria. Further work will be necessary to determine whether and how the various autophagic responses are linked, and whether they promote tolerance to infection by targeting host or pathogen factors.

*C. elegans* also provided the first example of xenophagic targeting of microsporidia, a phylum of fungal-like pathogens that infect virtually all animals. Unlike most intracellular bacteria, which replicate inside a specialized membrane-enclosed compartment inside the cell, microsporidia resemble viruses in that they replicate in the cytosol, where they can be directly targeted by the host xenophagic response. Studies in *C. elegans* have identified a novel role for xenophagy in resistance to *N. parisii* infection, although host defense is ultimately unsuccessful in controlling infection in the laboratory strain of *C. elegans*.

In closing, we note that evidence is accumulating for the involvement of different subsets of autophagy components in processes other than canonical autophagy. For example, Atg8/LC3-associated phagocytosis, first observed in murine macrophages, involves some, but not all, of the components involved

in canonical autophagy,[49] although this process has not been investigated extensively in *D. melanogaster* or *C. elegans*.[50] Likewise, a novel role for autophagy in unconventional secretion has recently been described in yeast and in mice, and it will be interesting to determine whether this also occurs in the model organisms discussed here.[51] Furthermore, a recent study in mammalian cells found that Atg8/LC3 can recruit innate immune proteins for a type of antiviral defense that appears to not involve fusion with the lysosome.[52] These new insights will likely stimulate further analysis of autophagy proteins also in *D. melanogaster* and *C. elegans*, to determine whether they act in canonical autophagy, noncanonical autophagy, or some other process to provide immune defense. Overall, it seems likely that different versions of autophagy may have evolved for different purposes. We speculate that this versatility may be especially important for defense against pathogens, which must evolve mechanisms to evade or suppress autophagy for their survival. Therefore, if one component of the host autophagy pathway is inhibited by a pathogen, alternative mechanisms may evolve to restore functional autophagy. In this way, new autophagy pathways could be built. Further work in model organisms such as *D. melanogaster* and *C. elegans* will help investigate this interesting idea.

## Note

Nomenclature: In the Introduction, yeast genes/proteins are stated first, followed by the mammalian name, if different. The nomenclature for other model organisms is used subsequently, where applicable.

## Disclosure of potential conflicts of interests

No potential conflicts of interest were disclosed.

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