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Death-associated protein kinase (DAPK) and signal transduction: fine-tuning of autophagy in *Caenorhabditis elegans* homeostasis

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Abstract

Autophagy is an evolutionarily conserved lysosomal pathway used to degrade and recycle long-lived proteins and cytoplasmic organelles. This homeostatic ability makes autophagy an important pro-survival mechanism in response to several stresses, such as nutrient starvation, hypoxia, damaged mitochondria, protein aggregation and pathogens. However, several recent studies have highlighted that autophagy also acts as a pro-death mechanism. What on the surface seem like conflicting roles of autophagy may be explained by the fact that the decision between pro-survival and pro-death is determined by the level of activation. A better understanding of autophagy signaling pathways will be helpful to elucidate how the level of autophagy is precisely regulated under different conditions and eventually how the final outcome is decided. In this review, we briefly discuss the pro-survival and pro-death roles of autophagy, and then discuss the mechanism by which autophagy is regulated, mainly focusing on death-associated protein kinase in the nematode *Caenorhabditis elegans*.

Keywords

autophagy; *Caenorhabditis elegans*; cell death; death-associated protein kinase; starvation

Overview: autophagy

When food is not available and intracellular energy is depleted, multicellular as well as single-celled organisms start to break down their own components, generating metabolites to maintain nutrient and energy homeostasis. There are two major cellular degradation pathways, the ubiquitin-proteasome system, which is specialized for proteins, and autophagy, which is a lysosomal pathway responsible for degrading relatively diverse cellular constituents, even including entire organelles [1].

There are three main types of autophagy: chaperone-mediated autophagy, microautophagy and macroautophagy. This review will focus on the best-characterized type of autophagy, macroautophagy (herein referred to as autophagy). Autophagy is initiated by the formation of a double membrane vesicle, the autophagosome, which sequesters cytoplasmic material and subsequently fuses with lysosomes to form a single membrane vesicle called an autophagolysosome. The contents of the autophagolysosome are degraded by acidic lysosomal hydrolases, and the products of degradation are recycled to generate macromolecules and ATP so as to maintain cellular homeostasis [2-5]. It is generally believed that autophagy is a nonselective degradation pathway, but increasing evidence suggests that autophagy can be

selective for the degradation of cellular organelles and ubiquitinated protein aggregates in certain conditions [6]. Recently, Zhang *et al.* [7] showed that autophagy selectively removes several P granule components in somatic cells during *Caenorhabditis elegans* embryogenesis, providing another piece of evidence for selective autophagic degradation.

From yeast genetics more than 20 autophagy-related (*Atg*) genes were found to be necessary for the process of autophagy [8]. These *Atg* genes function in several distinctive steps of autophagy, including: (a) induction of autophagy (*Atg1*, *Atg13*, *Atg17*), (b) autophagosome nucleation (*Atg6*, *Atg14*, *Vps15*, *Vps34*), (c) autophagosome elongation (two ubiquitin-like conjugation systems: *Atg3*, *Atg4*, *Atg5*, *Atg7*, *Atg8*, *Atg10*, *Atg12*, *Atg16*), (d) retrieval of *Atg* proteins (*Atg2*, *Atg9*, *Atg18*) and (e) autophagic body breakdown (*Atg15*). Although some *Atg* genes are well characterized, the molecular functions of most *Atg* genes are still under investigation. Most *Atg* genes have been conserved during evolution and their orthologs have been isolated and functionally characterized in higher organisms, including *C. elegans* [2,9]. (For a more extensive review of the roles of *Atg* genes, see recent comprehensive reviews in [2,9].)

Because bulk degradation of cytoplasm is potentially harmful when it is dysregulated (too much destruction), autophagy must be strictly controlled. Autophagy can be induced by both environmental stress (e.g. nutrient starvation, oxidative stress, hypoxia, heat shock) and intracellular stress (e.g. damaged organelles, protein aggregates, infection). Once induced, autophagy plays a protective role to alleviate the harmful effects of intracellular and environmental stress, and once needs are adequately met, it returns to a basal level [3,5,10, 11]. Several recent studies have indicated that autophagy also functions as a pro-death mechanism in phenomena such as autophagic cell death [12-15]. Intuitively, an excess of autophagy above a threshold can be the mechanism by which autophagy functions as a pro-death factor. In fact, the situation is complicated by the fact that autophagy is tightly linked to other cellular death mechanisms, apoptosis and necrosis [16]. Furthermore, the interplay between autophagy and apoptosis or necrosis is somewhat paradoxical: autophagy inhibits apoptosis or necrosis in some instances, but in others promotes apoptosis or necrosis [12, 16-22]. Finally, autophagic cell death might have evolved as a pro-survival rather than a pro-death mechanism in multicellular organisms because it could be beneficial for animals to maintain whole body homeostasis by removing an irreversibly damaged part through autophagic cell death.

With regard to the close relationship between autophagy and apoptosis, the role of death-associated protein kinase (DAPK) in the regulation of autophagy is particularly interesting. DAPK is a serine/threonine kinase originally isolated by a genetic screening for positive mediators of interferon- γ -induced cell death [23]. However, increasing evidence suggests that DAPK modulates autophagy in several model systems, including *C. elegans* [24-26]. In this review, we emphasize the role of DAPK in the regulation of autophagy.

Although several aspects of autophagy regulation have been discovered, further examination of the various regulatory pathways is needed, including the complex network of multiple autophagy inducing – and inhibiting – signals, and, more importantly, the systemic regulation of autophagy responses in multicellular organisms, to resolve these seemingly paradoxical functions. In the following sections we discuss and speculate about the physiological roles of autophagy (Figs 1, 2) and the signaling pathways that regulate autophagy (Fig. 3), mainly in the genetically tractable model system, *C. elegans*.

Pro-survival roles of autophagy

Starvation response

The genetic screens in yeast that led to the identification of the *Atg* genes showed that *Atg* mutants grow normally in a rich medium, but they cannot survive long-term starvation, initially suggesting that autophagy is essential for adaptation to nutrient deprivation [8]. Subsequently, this autophagic response to starvation has been observed in other organisms, including worms, flies and mice. The loss of autophagy results in defects in dauer formation in *C. elegans*. The dauer is a specialized developmental stage for long-term starvation survival. The loss of autophagy causes hypersensitivity to starvation in *Drosophila* and early postnatal lethality in mice after termination of the placental nutrient supply [27-29]. Recently, we showed that inhibiting autophagy by *atg* gene RNAi decreases the survival of worms during starvation, and that the survival can be rescued by the addition of food. Autophagy-deficient worms have decreased activity of the pharynx, which is important for recovery from starvation. These data suggest that autophagy is required for the optimal survival of worms during starvation, providing an energy source or essential nutrients to maintain cellular activity [26].

Neurodegeneration

Protein quality control is especially critical for normal cellular function in postmitotic cells such as neurons [30]. It is frequently observed that abnormally aggregated proteins are associated with neurodegenerative disorders, including Alzheimer's disease, Huntington's disease and Parkinson's disease [3,5]. In *C. elegans*, the expression of aggregation-prone human A β peptides in muscles (an Alzheimer's disease model), mutant aggregation-prone, expanded polyQ proteins in neurons (a Huntington's disease model) and human α -synuclein in dopaminergic neurons (a Parkinson's disease model) results in paralysis and neurodegeneration, suggesting that these model systems can mimic the phenotype of the respective human diseases, and may be valuable for finding therapeutic targets through genetic screening [31-33]. These studies also found that the inhibition of autophagy by *atg* gene RNAi exacerbates paralysis and neurodegeneration, whereas an increase in autophagy using *daf-2* insulin receptor mutations promotes the degradation of aggregates and rescues paralysis and neurodegeneration. These data suggest that autophagy may play a protective role in diverse neurodegenerative diseases.

Aging

Damaged proteins and organelles accumulate with age in virtually all types of cell [30,34]. This fact, combined with the finding that autophagy decreases with age [35], leads to the intriguing possibility that autophagy plays an important role in the aging process. Indeed, autophagy is required for lifespan expansion by dietary restriction, the well-known intervention thought to slow down the aging process [36,37]. Furthermore, long-lived *daf-2* insulin receptor mutants or *cep-1* p53 mutants also require autophagy for their longevity [27,37-39]. These data suggest that autophagy may delay the aging process by degrading age-dependent damaged proteins and organelles and thus promote lifespan extension.

Hypoxia

Hypoxic injury is a condition in which the oxygen supply is inadequate. Autophagy is induced after a hypoxic insult in *C. elegans*, suggesting that autophagy might be required for recovery from hypoxia [40]. In fact, inhibition of autophagy in *C. elegans* decreases survival after a severe hypoxic insult, suggesting that autophagy plays a protective role against a hypoxic insult [40].

Tumorigenesis

Despite the possibility that autophagy may contribute to tumor development by providing an alternative energy source to tumor cells located far from the blood supply [3,5], increasing evidence suggests that autophagy may also act as a tumor suppressor. Initially, autophagy was suggested to suppress tumors because monoallelic deletion of *beclin 1* is frequently associated with several human cancers, and because mice with heterozygous disruption of *beclin 1* are tumor prone [41]. This view is supported by recent findings that autophagy limits genome damage and chromosomal instability, potent tumorigenic factors [42,43]. In *C. elegans*, *gld-1* mutants are a model for germline tumors. Mutation of *gld-1* causes lethal germline tumors, shortening the lifespan. Recently, it has been shown that mutations of *daf-2* or *eat-2* confer resistance to these tumors [44]. These findings, together with the fact that mutations of *daf-2* or *eat-2* affect autophagy, lead to the intriguing possibility that autophagy may be involved in the effect of *daf-2* and *eat-2* mutations on *C. elegans* germline tumors. It will be interesting to test whether autophagy acts as a tumor suppressor in the *C. elegans* germline tumor model.

Pro-death roles of autophagy

Excessive levels of autophagy

Intuitively, autophagy above the physiological level might be expected to be deleterious. Pattingre *et al.* [45] elegantly showed that mutants of Beclin 1 that do not bind Bcl-2 demonstrate excessive levels of autophagy and promote autophagy-dependent cell death in MCF7 cells, supporting the hypothesis that excessive autophagy plays a pro-death role at a cellular level. Recently, we found in *C. elegans* that overactivated muscarinic acetylcholine signaling induces excessive autophagy and causes the death of worms during starvation, and that excessive autophagy causes defects in the pharyngeal muscles and eventually contributes to death after starvation [26,46,47]. These data provide *in vivo* evidence that excessive autophagy plays a pro-death role (Fig. 2).

Necrotic cell death

Type III or necrotic cell death is characterized by rapid loss of plasma membrane integrity, cellular swelling and subsequent release of internal contents [48]. In *C. elegans*, gain-of-function mutations of *mec-4*, *deg-1* or *deg-3*, which encode specific ion channel subunits, lead to necrotic-like degeneration of a subset of neurons [20,49]. The inhibition of autophagy by either *atg* gene RNAi or treatment with a chemical inhibitor suppresses necrotic-like degeneration, whereas the activation of autophagy by mutations in *C. elegans* target of rapamycin (CeTOR) or starvation exacerbates necrotic-like degeneration, suggesting that autophagy is required for necrotic cell death in *C. elegans* [20,49]. More importantly, excessive autophagy is induced by a hyperactive *mec-4* mutation [49], supporting the view that excessive levels of autophagy play a pro-death role (Fig. 2).

Autophagy signaling pathway

During the past decade, several signaling pathways involved in the regulation of autophagy have been discovered. These include: (a) mammalian target of rapamycin (mTOR) signaling, which is the key inhibitory signaling for autophagy, responding to growth factors and amino acid signaling, (b) AMP-activated protein kinase, which activates autophagy through the inhibition of mTOR signaling, (c) Bcl-2, which binds to Beclin 1 and inhibits autophagy, (d) BH3 only proteins, which release Beclin 1 from Bcl-2-dependent inhibition and thereby activate autophagy, (e) extracellular signal-regulated kinase, which phosphorylates and activates Gα interacting proteins and stimulates autophagy and (f) the eukaryotic initiation factor 2α, which regulates starvation- and virus-induced autophagy. As these autophagy

signaling pathways have been covered by comprehensive reviews [50,51], we focus here on recent findings about the forkhead transcription factor (FoxO), c-Jun N-terminal kinase (JNK), calcineurin and DAPK signaling pathways and the systemic regulation of autophagy (Fig. 3).

FoxO3

FoxOs are evolutionally conserved and well-known downstream targets of the insulin signaling pathway. FoxOs are inhibited by acutely transforming retrovirus AKT8 in rodent T cell lymphoma (Akt)-dependent phosphorylation and subsequent nuclear export. FoxOs play an important role in metabolism, proliferation and stress responses [52]. Recently, it has been shown that activated FoxO3 stimulates lysosomal proteolysis in muscle by activating autophagy. FoxO3 induces the expression of autophagy-related genes, suggesting that decreased insulin signaling can activate autophagy, not only through the AKT–TSC–mTOR pathway, but also more slowly by FoxO3-dependent transcriptional regulation [53]. It was not known until very recently whether FoxOs stimulate autophagy in *C. elegans*. However, the fact that the *C. elegans* genome does not encode the tuberous sclerosis complex (TSC) complex, a critical negative regulator of mTOR, suggests the possibility that the insulin–AKT–FoxO3 signaling cascade may play more prominent roles in the insulin signaling-dependent regulation of autophagy in *C. elegans*. Indeed, Jia *et al.* [54] recently showed that overexpression of *daf-16*, a *C. elegans* homolog of FoxO, increases levels of autophagy and resistance to *Salmonella* infection, supporting the hypothesis.

JNK

JNK is a stress-activated mitogen-activated protein kinase. Recent studies have shown that JNK1 mediates starvation-induced Bcl-2 phosphorylation, which drives Bcl-2 dissociation from Beclin 1 and subsequent activation of autophagy [55]. JNK1 also activates ceramide-induced autophagy through the phosphorylation of Bcl-2 [56]. It is not known whether JNK stimulates autophagy in *C. elegans*. JNK-1, a *C. elegans* homolog of JNK, is known to activate DAF-16/FoxO to extend lifespan [57]. Furthermore, recent studies indicate that there is crosstalk between insulin and JNK signaling in *C. elegans* [58]. Taken together, these studies suggest the possibility that JNK-1 stimulates autophagy through DAF-16 in *C. elegans*.

Calcineurin

Calcineurin is a serine/threonine phosphatase that couples calcium signals to changes in gene transcription by regulating the nuclear factor of activated T cells family of transcription factors [59]. Recently, it has been shown that in *C. elegans* calcineurin mutants show an extended lifespan, dependent on two essential autophagy genes, *bec-1* and *atg-7* [60]. In addition, both *tax-6* and *cnb-1* calcineurin mutants exhibit high levels of autophagy, suggesting that calcineurin signaling may negatively regulate autophagy in *C. elegans*. However, because the gain-of-function mutation of *tax-6* does not further decrease basal levels of autophagy under normal conditions, it is possible that calcineurin signaling has a permissive role in the regulation of autophagy, rather than an instructive role [60]. Another possibility is that there is a ceiling effect of basal levels of autophagy under normal conditions. It will be interesting to test whether a gain-of-function mutation of *tax-6* can decrease high levels of autophagy under starvation conditions.

DAPK

DAPK-1 is a Ca^{2+} /calmodulin-regulated serine/threonine kinase that has cell death-associated functions. Activation of DAPK-1 leads to membrane blebbing, cell rounding, detachment from extracellular matrix and the formation of autophagic vesicles [24]. DAPK-1 binds to the microtubule-associated protein MAP1B during amino acid starvation, possibly promoting interaction with MAP1LC3B, and associating with autophagosomes. DAPK-1-induced

autophagy is reduced by knockdown of MAP1B, suggesting that a DAPK-1–MAP1B complex is required for the induction of autophagy [61]. Interestingly, DAPK-1 and MAP1B colocalize with α -tubulin and F-actin [61]. Because autophagosomes slide along cytoskeletal structures and fuse with lysosomes [62], DAPK-1 and MAP1B located to α -tubulin or F-actin may be involved in this sliding process.

Recently, it was shown that DAPK-1 phosphorylates and inhibits the TSC complex, leading to the activation of mTOR signaling. In addition, DAPK-1 also phosphorylates S6 and stimulates its activity [63]. These results seem inconsistent with the autophagy-stimulating role of DAPK-1, because mTOR signaling potentially inhibits autophagy. One possible explanation is that DAPK-1-induced mTOR activity may be responsible for the activation of S6K during starvation, which is required for the induction of autophagy. Three recent papers support this hypothesis: previous studies have shown that some level of S6K activity is required for starvation-induced autophagy in *Drosophila*, yet how S6K is somewhat activated under starvation conditions, where the insulin–Akt–mTOR signaling is inhibited, is unknown [28]. DAPK-1 stimulates mTOR signaling and S6 activity [63]. Our recent finding showed that starvation induces autophagy through DAPK-1 [26]. Together, these studies suggest the possibility that DAPK-1 may be an upstream activator of S6K signaling through stimulating mTOR signaling and S6 activity during starvation. DAPK-1 is also involved in endoplasmic reticulum stress-induced autophagic cell death [64].

In *C. elegans*, we showed that starvation activates muscarinic acetylcholine signaling, which in turn activates extracellular signal-regulated kinase and induces autophagy through DAPK-1, the *C. elegans* homolog of DAPK. In starvation-hypersensitive mutants, muscarinic acetylcholine signaling is overactivated and induces excessive levels of autophagy, eventually leading to malfunction of the pharynx. Mutation of *dapk-1* rescues excessive levels of autophagy and improves survival of mutant worms, suggesting that DAPK-1 regulates the extent of starvation-induced autophagy [26,46]. The downstream target of DAPK-1 in starvation-induced autophagy in *C. elegans* remains elusive.

One possible downstream target of DAPK-1 is BEC-1. Recently, Zalckvar *et al.* [65] reported that DAPK phosphorylates beclin 1 on threonine 119 and promotes the dissociation of beclin 1 from Bcl-X_L and the induction of autophagy, similar to the mechanism by which JNK1 regulates autophagy by phosphorylation and dissociation of beclin 1 inhibitor, Bcl-2 [55]. Because this regulatory mechanism is probably evolutionary conserved in *C. elegans* [17,66], it could be the case that DAPK-1 modulates autophagy by regulating the interaction between BEC-1 and CED-9 in *C. elegans*. Furthermore, DAPK-1 and JNK-1 show a distinct expression pattern in *C. elegans* (<http://www.wormbase.org>). It is possible that DAPK-1 and JNK-1 modulate autophagy by regulating the interaction between BEC-1 and CED-9 in a tissue-specific manner.

Systemic regulation of autophagy

Because mTOR signaling, which is a downstream target of insulin–AKT signaling, is a major inhibitory signal for autophagy, it is generally assumed that autophagy may be regulated systemically (nonautonomously). However, it has not been directly shown that autophagy can be regulated systemically, especially in response to environmental change. We recently found that specific amino acids could suppress the excessive starvation-induced autophagy in the pharyngeal muscle of starvation-hypersensitive mutants, and that MGL-1 and MGL-2, *C. elegans* homologs of metabotropic glutamate receptors, were involved. MGL-1 and MGL-2 act in AIY and AIB neurons, respectively [67,68]. These data suggest that metabotropic glutamate receptor homologs in AIY and AIB neurons may sense amino acids (as antihunger signals) and subsequently modulate a systemic autophagy response, probably through

hormonal regulation (Fig. 3). Further experiments are needed to elucidate which hormones or neuropeptides regulate the systemic autophagy response downstream of AIY and AIB neurons.

Conclusion

Although significant advances have been made in our understanding of the physiological roles and molecular mechanisms of autophagy, many unanswered questions still remain: How might autophagy perform seemingly opposites roles as a pro-survival and a pro-death mechanism? How do multicellular organisms regulate autophagy in response to environmental change? How does autophagy interact with apoptosis or necrosis? Research in *C. elegans*, which has already been established as a genetically tractable model for cell death study and starvation studies, may help to answer these questions.

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Abbreviations

Akt	acutely transforming retrovirus AKT8 in rodent T cell lymphoma
DAPK	death-associated protein kinase
FoxO	forkhead transcription factor
JNK	c-Jun N-terminal kinase
MAP	microtubule-associated protein
mTOR	mammalian target of rapamycin
TSC	tuberous sclerosis complex

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Fig. 1. Pro-survival and pro-death roles of autophagy. Autophagy promotes starvation survival and inhibits neurodegeneration, aging process, hypoxia injury, and possibly germline tumors in *C. elegans*. Under different conditions, however, autophagy causes tissue malfunction and necrotic cell death. Dashed lines indicate possible regulatory effects. See the main text for details.

Fig. 2. Model of dual roles of autophagy in *C. elegans*. Physiological levels of autophagy are essential for cellular homeostasis, so as to promote survival. Insufficient autophagy causes defects in energy homeostasis and accumulation of damaged proteins and organelles, which is deleterious for survival. Excessive autophagy causes too much degradation and tissue malfunction, eventually leading to death.

Fig. 3. Schematic diagram of autophagy signaling pathway in *C. elegans*. Autophagy inducing- and inhibiting signaling pathway in *C. elegans*. Plain lines and dashed lines indicate known and possible regulatory pathways, respectively. See the main text for details.