

Autophagy and Cell Death in *Caenorhabditis elegans*

Chrysanthi Samara and Nektarios Tavernarakis*

Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion 71110, Crete, Greece

Abstract: Cell death is a major component of developmental programs. Controlled killing of specific cells at appropriate time points is required for normal growth and shaping of organisms. However, cellular demolition can also result in a variety of pathologies that are frequently fatal, when implemented inappropriately. Delineation of cell death mechanisms has been greatly facilitated by the use of simple model organisms such as the nematode worm *Caenorhabditis elegans*. Research in *C. elegans* has proven instrumental for the elucidation of the molecular mechanisms underlying both apoptotic and necrotic cell death. Here, we introduce the *C. elegans* model and review the current understanding of cell death pathways in this organism. We further focus on recent studies implicating autophagy, the main cellular process for bulk protein and organelle recycling, in nematode cell death. These studies reveal that autophagic mechanisms have a prominent role in both apoptosis and necrosis. We survey the relevant findings in *C. elegans* and also consider the contribution of autophagy in cell death in other experimental systems. Comparative analysis suggests that the involvement of autophagy in cell death is evolutionary conserved in metazoans. Thus, interfering with the autophagic process may facilitate therapeutic intervention in human pathologies where aberrant cell death is a contributing factor.

Key Words: Apoptosis, Autophagy, Beclin, *Caenorhabditis elegans*, Excitotoxicity, Lysosome, Necrosis, Neurodegeneration.

INTRODUCTION: *C. elegans* BIOLOGY

Caenorhabditis elegans was introduced as an experimental model organism by Sydney Brenner more than half a century ago [1] and quickly became an established research platform, with seminal contributions in diverse fields of modern biology and medicine. *C. elegans* is a free-living small nematode worm; adults are approximately 1.3mm long with a diameter of 100µm. It naturally occupies water-filled micro-environmental loci in the soil, and in the laboratory it is grown either in Petri dishes or in liquid cultures, under an *Escherichia coli*-based diet. Its maintenance is inexpensive, not time-consuming, and it is further facilitated by the ability to store nematode larvae for some weeks or months at 15°C, and for several years at -80°C or in liquid nitrogen. The reproductive cycle of *C. elegans* takes about 2.5 days at 25°C, and animals can live for up to 2-3 weeks. After oocyte fertilization, development proceeds with embryonic growth and four larval stages (L1 to L4), which follow egg hatching and precede maturation into adulthood (Fig. (1)). Under adverse environmental conditions, such as food shortage, high temperature and overcrowding, L1 larvae cease growing, while L2 form dauers [2]. Dauers represent a stress-resistant, diapause type of larva that differs from normally developing animals in morphology and behavior. After detecting favorable conditions dauers re-enter lifecycle at the L4 stage. *C. elegans* animals are mainly hermaphroditic and can easily produce large, genotypically homogenous populations by self-fertilization. Males are generated by impaired chromosome segregation that results in the loss of one X sex chromosome. Males can be used for genetic manipulations designed to generate double and multiple mutants and transgenic animals.

Genetic and molecular studies are further facilitated by the fully sequenced and well-annotated genome of *C. elegans*. The genome encodes approximately 20,000 genes positioned on 5 autosomes and one X sex chromosome [3-5]. High-throughput, genome-wide methodologies have provided a wealth of data concerning protein-protein interactions, gene expression (microarray technology) and function (double-stranded RNA interference, dsRNAi) [6-12]. RNAi also enables rapid targeted downregulation of specific gene expression [13, 14]. The availability of RNAi-hypersensitive genetic backgrounds further assists this approach [15-18]. Reverse

genetics studies in worms are also facilitated by the ease of generating transgenic animals *via* DNA microinjection [19]. Appropriate nematode-specific vectors are available [20, 21]. Mapping of mutant alleles generated by classic forward genetics methodologies can be performed rapidly and with precision using the existing single nucleotide polymorphism map [22].

C. elegans has pioneered the investigation on the mechanisms of developmental cell death. Features, such as the transparency of worms and their invariable cell lineage have proved major advantages in this effort. The body of adult hermaphrodites consists of 959 post-mitotic cells and is formed through a specific series of cell divisions and elimination of exactly 131 cells during development [23, 24]. Dying cells are easily detectable by differential interference contrast (DIC) microscopy techniques. The nervous system of *C. elegans* has been fully mapped [25, 26]. In addition to the exact position and lineage history of each of the 302 neuron, the connections between them have been identified [27]. Thus, the complete “wiring diagram” of the worm nervous system is known. Ablation of individual cells is feasible through cell-specific expression of toxic proteins or by the use of a laser beam [28-30]. Furthermore, primary nematode-cell cultures have been developed recently, enabling *ex vivo* studies [31].

MECHANISMS OF AUTOPHAGY IN *C. elegans*

The term “autophagy” is used to describe the cellular process for degrading and recycling proteins and organelles [32]. There are three different types of autophagy; microautophagy, chaperone-mediated autophagy and macroautophagy (Fig. (2)) [33, 34]. Microautophagy is accomplished by the invagination of the lysosomal membrane and the concomitant internalization of cytoplasmic parts for degradation in the lysosome. The molecular mechanisms involved in microautophagy are unclear. In chaperone-mediated autophagy, soluble cytosolic proteins carrying the KFERQ motif are specifically targeted to the lysosome, where they undergo rapid proteolysis. Cytoplasmic and lysosomal chaperones and the function of the lysosomal membrane protein LAMP2 are required for this process. The lysosome is also the final destination of cargo delivered *via* macroautophagy, which is the best studied type of autophagy. In macroautophagy, cytoplasmic double-membrane vesicles are formed from a yet unknown membrane source [35]. These vesicles are called autophagosomes or autophagic vacuoles. Autophagosomes engulf large portions of cytoplasm and transfer them to lysosomes. The outer autophagosomal membrane fuses with the lysosomal membrane and the remaining autophagic body is re-

*Address correspondence to this author at the Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Vassilika Vouton, PO Box 1385, Heraklion 71110, Crete, Greece; Tel: +30 2810 39 1066; Fax: +30 2810 39 1067; E-mail: tavernarakis@imbb.forth.gr

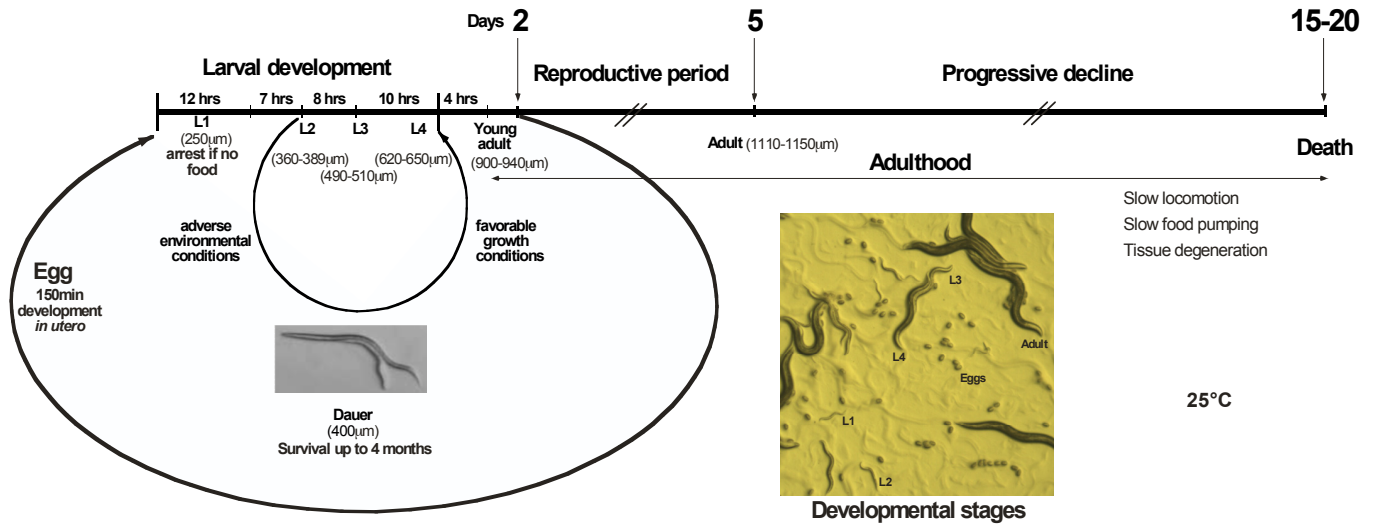


Fig. (1). The *C. elegans* life cycle at 25°C. Adult animals lay eggs, which hatch to release L1 larvae. Animals undergo four larval stages termed L1-L4 before they become sexually mature adults. Under adverse environmental conditions L1 larvae arrest ceasing their growth, while L2 larvae adopt an alternative stress-resistant developmental form termed “dauer”. When dauer larvae encounter favorable conditions, they re-enter the cycle at the L4 stage and continue to become adults with a normal lifespan.

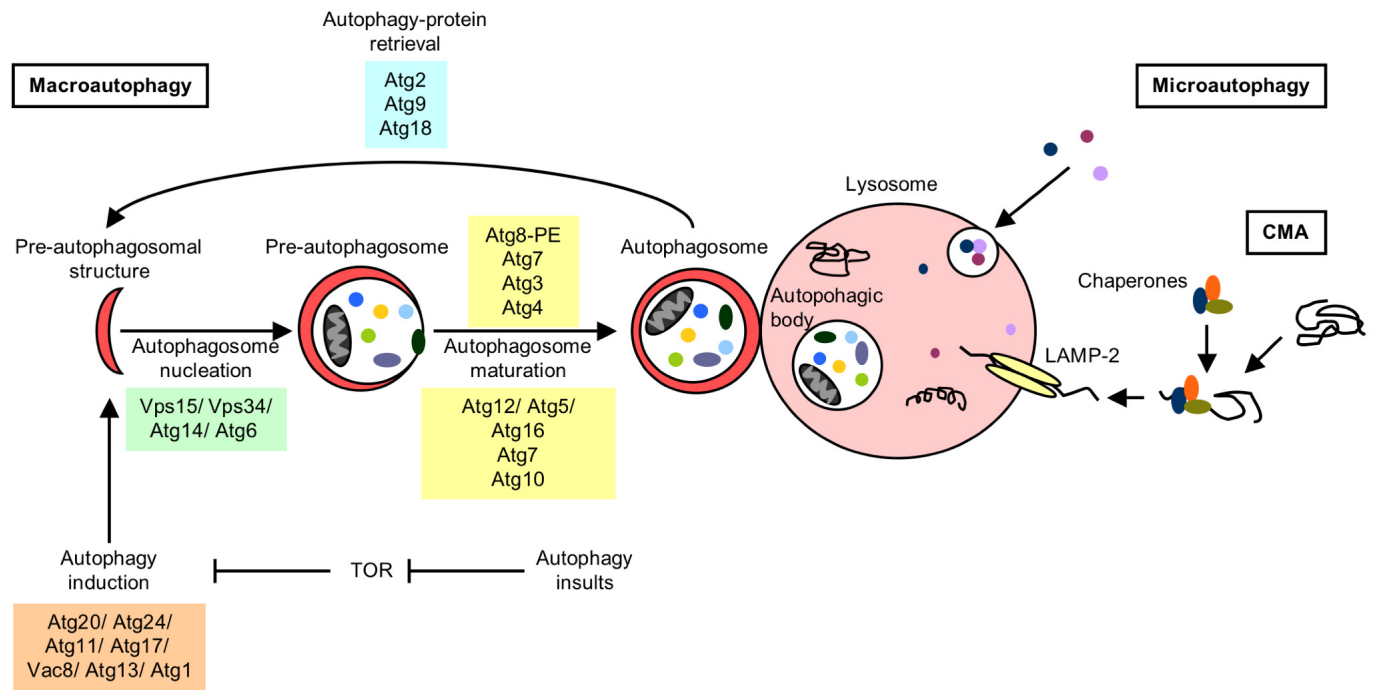


Fig. (2). The three types of autophagy. Macroautophagy proceeds with the formation of a double membrane autophagic vacuole that encloses portions of the cytoplasm containing molecules and cellular organelles, such as mitochondria. The outer membrane of the autophagosome eventually fuses with the lysosomal membrane, releasing an autophagic body inside the lysosome. During microautophagy, invagination of the lysosomal membrane leads to the internalization of cytoplasmic components. In chaperone-mediated autophagy (CMA) cytosolic proteins enter the lysosome by interacting with cytoplasmic chaperones, the lysosomal membrane protein LAMP2 and lysosomal chaperones.

leased into the lysosomal lumen. Fusion with lysosomes is often the last step of consecutive fusions between autophagosomes and early endosomes, at least in mammalian cells [36]. Acidification of endocytic compartments is a critical prerequisite for fusion. Specific forms of macroautophagy also exist, such as pexophagy, mitophagy and endoplasmic reticulum (ER) autophagy (ER-phagy). These mediate specific degradation of peroxisomes, mitochondria and endoplasmic reticulum respectively. For simplicity, we adopt the term “autophagy” to denote macroautophagy for the rest of the text.

Autophagy was discovered in mammalian cells and has been extensively investigated in yeast [37]. These studies have identified many genes encoding proteins involved in autophagy (ATG proteins) [38]. ATG proteins participate in the induction of autophagy, the formation, expansion and maturation of autophagosomes, and in the retrieval of autophagic proteins from mature autophagosomes (Fig. (2)) [39]. Fusion processes occur *via* the t- and v-SNARE complexes, and other molecules, such as the Rab GTPases and components of the vacuolar protein-sorting (VPS) complex.

The autophagic process is highly conserved and most yeast Atg genes have homologs in higher organisms. Similarity searches have led to the identification of several homologs of Atg genes in *C. elegans* (Table 1). In addition to the sequence similarity, indirect experimental evidence has implicated specific nematode genes in autophagy. For example, the *C. elegans* beclin BEC-1 can functionally substitute for Atg6 in yeast autophagy but not for the cytoplasm-to-vacuole targeting (CVT) pathway [40]. Furthermore, dysfunction of *bec-1*, *lgg-1*, *atgr-7*, *atgr-18* or *unc-51* prevents upregulation of autophagy during dauer formation. Some of the nematode genes play additional roles apart from their participation in the autophagic process. For example, UNC-51, which is a Ser/Thr kinase with high similarity (52.7%) to yeast Atg1 protein, is expressed in most *C. elegans* neurons throughout development [41]. Elimination of Atg1 in yeast prevents autophagosome formation and compromises cell viability under starvation conditions [42]. In *C. elegans*, UNC-51 functions with UNC-14 to regulate sex myoblast migration and gonadal formation [43]. UNC-51 and UNC-14 also control the localization of the netrin receptor UNC-5 in neurons [44]. This function is not mediated by autophagy, since interference with other *C. elegans* autophagy genes does not affect UNC-5 localization, and UNC-5 is not detected in autophagosomes. UNC-51 also regulates axonal outgrowth and migration by interacting with and phosphorylating VAB-8 or UNC-14 [45]. Neuronal axons of *unc-51* mutant nematodes accumulate atypical membranous vesicles and cisternae-like structures. Whether these represent autophagosomes is not clear. The totality of the above observations indicates a general implication of UNC-51 in membrane dynamics.

The involvement of Atg1 homologs in endocytic membrane trafficking pathways is a common feature between nematodes and mammals. The mouse homolog Unc51.1 has been implicated in axonal extension by regulating the endocytic pathway [46]. In addition, the human homolog ULK1 interacts with GATE-16, which is involved in intra-Golgi transport, and with the γ -aminobutyric acid

(GABA)-receptor associated protein (GABARAP) [47]. Together with GABARAP, it colocalizes in intracellular compartments, such as the Golgi and the smooth ER, and may participate in GABA_A-receptor trafficking.

In both nematodes and mammals the autophagic process converges with endocytic pathways. Worms with impaired receptor-mediated endocytosis also show defects in the execution of autophagy. *CeVPS-27* is the ortholog of yeast endosomal membrane protein Vps27p, which regulates the formation of endosomal sorting complexes required for transport (ESCRT) [48]. Elimination of *CeVPS-27* causes endocytosis defects, such as accumulation of enlarged endosomes, abnormal LRP-1 receptor trafficking and cholesterol transport, and accumulation of autophagosomes in the lateral epidermis of worms.

Studies in *C. elegans* have provided novel insights on the physiological roles of autophagy in developmental processes and tissue remodeling. Mutant alleles of *bec-1* for example cause embryonic lethality or larval arrest [40]. Furthermore, autophagy is triggered as a general response to intracellular and extracellular stress such as starvation and accumulation of damaged cytoplasmic components [49]. Stress conditions promote dauer formation in *C. elegans*. This process requires autophagy upregulation, which is most probably essential for radial body constriction and dauer alae formation [40]. In addition, autophagy has been implicated in the regulation of inhibitory synapse strength and thus of the balance between excitatory and inhibitory inputs in nematodes [50, 51]. Loss of synaptic connections in dorsal muscles affects the stability of post-synaptic GABA_AR clustering, promoting its endocytosis and trafficking to autophagosomes for degradation. GABA_A-receptors also localize in autophagosomes in normally innervated muscles, although at a much lesser extent. By contrast, while absence of synaptic contact also affects the localization of acetylcholine receptors in post-synaptic clusters, it does not lead to their traf-

Table 1. Autophagy-Related Genes in *Caenorhabditis elegans*

Autophagy-Related Genes		Autophagy-Related Function	<i>C. elegans</i> Homolog
Yeast Gene	Mammalian Gene		
Regulation of Autophagy Induction			
<i>TOR1/TOR2</i>	<i>Hs FRAP1</i>	Rapamycin-sensitive Ser/ Thr protein kinase	<i>let-363</i>
<i>ATG1</i>	<i>Hs ULK2/ Mm Unc51.2</i>	Ser/ Thr protein kinase	<i>unc-51</i>
Autophagosome Nucleation			
<i>ATG6</i>	<i>Mm beclin 1</i>	Component of class III PI3-kinase complex	<i>bec-1</i>
<i>VPS34</i>	<i>Hs PI3-kinase, class III</i>	Class III PI3-kinase	<i>vps-34</i>
Autophagosome Expansion and Completion			
<i>ATG3</i>	<i>Hs ATG3/ Mm Apg3</i>	E2-like enzyme; conjugates Atg8 to PE	Y55F3AM.4
<i>ATG4</i>	<i>Hs ATG4/ Mm autophagin 1</i>	Cys protease; leaves C-terminal extension of PE from Atg8	Y87G2A.3
<i>ATG5</i>	<i>Hs ATG5/ Autophagy 5-like</i>	Conjugated to Atg12 through internal lysine	<i>atgr-5</i>
<i>ATG7</i>	<i>Hs ATG7/ Mm APG7</i>	E1-like enzyme; activates Atg8 and Atg12	<i>atgr-7</i>
<i>ATG8</i>	<i>Hs GABARAP/ Mm GABARAP-like 1</i>	Ubiquitin-like protein conjugated to PE	<i>lgg-1</i>
<i>ATG10</i>	<i>Hs/ Mm ATG10</i>	E2-like enzyme; conjugates Atg12 to Atg5	D2085.2
<i>ATG12</i>	<i>Hs APG12-like</i>	Ubiquitin-like protein conjugated to Atg5	<i>lgg-3</i>
<i>ATG16</i>	<i>Hs APG16-like isoform 2</i>	Component of Atg12-Atg5 complex	K06A1.5
Retrieval of Autophagic Proteins			
<i>ATG9</i>	<i>Hs/ Mm APG9-like 1</i>	Integral membrane protein	<i>atgr-9</i>
<i>ATG18</i>	<i>Sc ATG18/ Hs AAQ96867</i>	Localization of Atg2	<i>atgr-18</i>

Autophagy-related genes are classified according to the autophagic step, in which they participate. Yeast autophagy genes and their identified mammalian homologs are written in the two left subcolumns. "Hs" refers to *Homo sapiens* and "Mm" to *Mus musculus*. The middle column presents the contribution of each gene product specifically to the autophagic process. Identification of the nematode homologs written in the right column has been based on similarity BLAST searches conducted by our group.

ficking to autophagosomes. This suggests that the autophagic process may be selective for certain soluble cytosolic substrates.

Autophagy has also been linked to cell death pathways. Indeed, excess cytoplasmic vacuolation is the main feature of type II programmed cell death or autophagic cell death. Both protective and destructive contributions of autophagy during cell death have been reported [52-54]. In the following sections we consider the cell death pathways in *C. elegans* and the involvement of autophagic mechanisms in these pathways.

CELL DEATH PATHWAYS IN *C. elegans*

Three major types of cell death are distinguished based on morphological criteria, apoptotic, autophagic and necrotic [55]. Apoptosis or type I programmed cell death is characterized by chromatin condensation and fragmentation and overall cell shrinkage. Blebbing of the plasma membrane leads to the formation of apoptotic bodies, which are ingested by phagocytes. The basic feature of type II or autophagic cell death is the increased number of autophagosomes. By contrast, during the course of necrosis several intracellular organelles dilate and the plasma membrane breaks down, causing spillage of cytoplasmic material and inflammation.

Apoptotic Mechanisms in the Nematode

Apoptosis or type I programmed cell death is required for normal development and homeostasis. However, it is also aberrantly induced in pathological conditions. The main features of apoptosis are DNA fragmentation, chromatin condensation and loss of mitochondrial membrane potential. The apoptotic molecular pathway is highly conserved (Table 2) [56]. In *C. elegans* apoptotic cells acquire a button-like appearance under the DIC microscope and are easily tractable. During embryonic development and the L2 larval stage, 131 cells are eliminated by apoptosis in wild type animals [23, 24, 57]. They derive mainly from neuronal lineages but also include muscles, as well as cells of the hypodermis and the pharynx. In addition, about 50% of oocytes generated in the mature gonads are destroyed through apoptosis [58]. Apoptosis in the germline may serve to maintain homeostasis, limiting the number of oocytes competing for nutrients in the gonad. It is also activated as part of the nematode innate immunity response following invasion of pathogenic microorganisms, and in response to DNA-damaging agents and genotoxic stressors, such as ionizing radiation and specific cancer drugs [59].

Activation of the BH3-only-domain, pro-apoptotic protein EGL-1 is critical for the induction of apoptosis [60]. How EGL-1 is activated is not well-understood. The proteins DPL-1, EFL-1, MCD-1, LIN-35, LIN-37 and LIN-52 are proposed to promote apoptosis by means of transcriptional regulation of *egl-1* [61]. *egl-1* transcription regulation has been studied extensively in the paradigm of the male-specific killing of the neurosecretory motor neurons (NSMs) and the hermaphrodite-specific neurons (HSNs) [62]. EGL-1 is activated in NSMs leading to their death by CES-2-mediated suppression of the CES-1 transcription factor [60]. CES-1 antagonizes the HLH-2/HLH-3 complex, which positively regulates *egl-1*, by binding at the same regulatory DNA sequence. The survival of HSNs in hermaphrodites requires the function of TRA-1A, which represses *egl-1* transcription [63]. Gain-of-function mutations in the *egl-41* gene lead to the inappropriate death of HSNs in hermaphrodites [64]. In males, the activity of EOR-1 and -2 mediates the elimination of HSNs [65].

Activation of EGL-1 has also been implicated in germ cell apoptosis induced by genotoxic stress. DNA damage activates CEP-1, the homolog of the mammalian apoptosis-inducer p53 [66, 67]. CEP-1 is a transcriptional activator of *egl-1*, and is negatively regulated by APE-1. However, under normal conditions oocyte elimination does not require EGL-1. Induction of apoptosis cascades is triggered by yet unknown developmental or other signals. Dysfunction of several molecules, such as the RNA helicase CGH-1, the

RNA-binding DAZ-1, as well as NOS-1 and NOS-2, leads to increased germ cell death [68]. An additional factor, GLA-3 has been identified in a screening for regulators of germ cell apoptosis [69]. GLA-3 is a TIS11-like zinc-finger protein, which acts through a mitogen-activated kinase (MAPK) signaling pathway to suppress germ line apoptosis. Recently, induction of apoptosis *via* direct transcriptional activation of *ced-3* by PAL-1 has been shown to mediate killing of the tail-spike cell [70].

Upon activation, EGL-1 binds the CED-9/CED-4 complex on the surface of mitochondria, releasing its components [60]. CED-9 is an anti-apoptotic protein similar to the mammalian Bcl-2 [71]. CED-4, which is orthologous to the human pro-apoptotic APAF-1, forms oligomers that translocate to the surface of the nucleus [60]. There, they interact with the nuclear envelope receptor protein matefin/SUN-1 and the proCED-3 caspase [72]. The concomitant formation of oligomeric complexes called apoptosomes mediates activation of CED-3, a member of the aspartyl cysteine protease family. Activation of EGL-1 also leads to the release of CPS-6 and WAH-1 from mitochondria [73, 74]. These proteins cooperate with other nucleases, such as NUC-1 and CRN-1, to promote DNA and RNA degradation [75, 76]. The contribution of mitochondria to the execution of apoptosis is not limited to providing the surface for interactions among EGL-1, CED-9 and CED-4, or for storing CPS-6 and WAH-1. Mitochondrial fission is important for the execution of cell death, and can even promote cellular destruction [77]. In addition, mitochondria contain ICD-1, which negatively regulates apoptosis in a CED-4-dependent, CED-3-independent manner [78].

Initiation of the apoptotic program also triggers the activation of neighboring cell pathways, leading to the engulfment and eventually degradation of the apoptotic corpses inside phagolysosomes [68]. Two separate molecular cascades are involved in this process; one including the proteins CED-7, CED-1, CED-6 and DYN-1, and a second that requires the function of UNC-73, MIG-2, CED-12, CED-5 and CED-2 [79, 80]. Both converge to the small GTPase CED-10, which promotes cytoskeletal rearrangements in the engulfing cell. During embryogenesis, cells localized next to a dying cell become engulfing cells. In larvae and adults, engulfment is mostly carried out by large epithelial cells. The engulfing process can be critical for cell destruction itself. In the male reproductive system the vas deferens is formed by one of two equivalent cells. The other cell dies by apoptosis, unless the engulfing cell has been ablated [81].

Other genes involved in *C. elegans* apoptosis include *nex-1* and *ced-8* [68]. *nex-1* is a homolog of the mammalian annexin I. Its knockdown by RNAi causes persistence of cell corpses [82]. *ced-8* codes for an integral membrane protein. CED-8 deficiency delays cell corpse appearance and removal, suggesting that it functions in cell killing or engulfment [83, 84].

The apoptotic program is highly conserved, and similar core mechanisms mediate apoptosis in higher organisms. Mammalian orthologs have been identified for most *C. elegans* apoptotic proteins (Table 2). Moreover, specific mammalian apoptotic molecules are functional in worms; the human Bcl-2 interacts with the pro-apoptotic nematode BH3-only protein EGL-1 and reduces developmental apoptosis [85]. However the apoptotic process in mammals is more complex, with higher level of redundancy, several additional factors involved and elaborate regulation. Nevertheless, *C. elegans* provides ground for investigating poorly understood aspects of apoptosis, such as caspase substrate specificity, the contribution of cell-corpse clearance to the execution of death, and the pathways that regulate apoptosis [83, 86]. Recent studies have shown that autophagy participates in the regulation of apoptosis. We discuss the relevant findings in the sections below.

The Interplay between Autophagy and Apoptosis

Interactions among components of autophagic and apoptotic processes indicate a crosstalk between the two pathways. ER-

Table 2. Nematode Proteins Involved in Apoptosis and their Mammalian Orthologs

<i>C. elegans</i> Protein	Function	Mammalian Ortholog
Apoptosis Regulation		
APE-1	Negative regulator of <i>cep-1</i>	iASPP
CED-8	Uncharacterized conserved protein	XK membrane transporter
CEP-1	Transcription factor	p53
CES-1	C2H2-type zinc finger transcription factor, member of the Snail family	SNAIL, SLUG
CES-2	bZIP transcription factor	NFIL3
DPL-1	E2F-heterodimerization partner-like protein	DP
EFL-1	E2F-like transcription factor	E2F
EOR-1	BTB/zinc-finger transcription factor	PLZF
EOR-2	Uncharacterized conserved protein	KIAA1205
GLA-3	TIS11-like protein with two CCCH-like zinc-finger domains	CATSPER1
HLH-2	Class I bHLH transcription factor	E2- α
HLH-3	bHLH transcription factor	HAND 1
LIN-35	Rb-like protein	Rb-like protein 2
LIN-37	Uncharacterized Mip40-like protein	-
LIN-52	Uncharacterized conserved protein	XP-040376
MCD-1	Uncharacterized protein with zing finger domain	ARID5B
PAL-1	Homeobox containing transcription factor	CDX-4
TRA-1	Transcriptional repressor, member of the GLI family	GLI2
Apoptosis Execution		
CED-3	Caspase	Caspase-2/ -3/ -9
CED-4	Adaptor protein, mediates CED-3 activation	APAF1
CED-9	Anti-apoptotic Bcl-2 family protein, prevents CED-3 activation	BCL2
CRN-1	5'-3' exonuclease	FEN1
CPS-6	Mitochondrial endonuclease G	EndoG
EGL-1	Pro-apoptotic BH3-only-protein	BH3-domain death activators
ICD-1	Mitochondrial protein	β NAC
NUC-1	Deoxyribonuclease II	DNaseII
WAH-1	Oxydoreductase homolog	AIF 1
Engulfment of Apoptotic Bodies		
CED-1	Transmembrane receptor	MEGF11
CED-2	SH2 and 3-containing adaptor protein	CrkII
CED-5	Signaling protein	DOCK180
CED-6	SH3 and PTB domain-containing adaptor protein, interacts with CED-1	CED-6/GULP
CED-7	ABC transporter, required for CED-1 localization	ABCA 3
CED-10	Ras-related small GTPase	RAC1
CED-12	Regulator of Rac1, activates CED-10	ELMO 1
DYN-1	Dynamin GTPase	Dynamin 2
MIG-2	Ras-related small GTPase, Rho type	RhoG
NEX-1	Annexin; member of a family of Ca ²⁺ -dependent phospholipid binding proteins	Annexin A13
PSR-1	Phosphatidylserine receptor	PTDSR
UNC-73	Guanine nucleotide exchange factor	TRIO

Apoptotic molecules are grouped according to their role in the apoptotic process. Regulatory proteins include mainly transcription factors involved in *egl-1* expression, but for PAL-1 that acts on *ced-3*, GLA-3 and CED-8. Members of the Bcl-2 family and hydrolytic enzymes, such as caspases and several nucleases, participate in the execution phase. Removal of apoptotic corpses requires the activity of two different molecular pathways (one containing CED-7, CED-1, CED-6, and the other including UNC-73, MIG-2, CED-12, CED-5 and CED-2) that synergize to activate CED-10. PSR-1 is implicated in the recognition of clearance-signals exposed on the surface of dying cells.

localized mammalian anti-apoptotic proteins Bcl-2 or Bcl-X_L suppress autophagy induced by Ca²⁺-mobilization in cultured cell lines [87, 88]. Suppression is likely due to interference with ER

Ca²⁺ levels. Bcl-2 and Bcl-X_L interact directly with the autophagy protein Beclin 1, the mammalian ortholog of yeast Atg6. Beclin 1 participates in autophagosome formation as a component of a class

III PI3-kinase complex together with Vps34. Beclin 1 interacts with Bcl-x_L via its highly conserved BH3 domain [89]. Similarly, the nematode protein BEC-1, which is orthologous to mammalian Beclin-1, forms separate complexes with the Bcl-2 homolog CED-9 and with LET-512/VPS34 [90]. *bec-1* is expressed ubiquitously in all tissues and at all developmental stages, most prominently during embryogenesis. It is essential for normal development, and its elimination causes embryonic lethality, larval arrest and sterility in *C. elegans* [40]. *bec-1*-deficient animals show increased number of apoptotic corpses in somatic tissues and in the germline as a result of induced apoptosis [90]. Although the interaction between BEC-1 and CED-9 may be involved in apoptosis regulation, it is not clear how loss of *bec-1* triggers apoptosis.

Observations in *cup-5* mutant animals further indicate an association between autophagy and apoptosis. The *C. elegans cup-5* gene is an ortholog of human mucolipin-1 [91]. Mucolipin-1 forms a non-selective cation channel, dysfunction of which results in the lysosomal storage disease Mucopolipidosis Type IV. *cup-5* is required for normal lysosomal biogenesis in worms [92]. *cup-5* null embryos experience starvation due to their inability to degrade yolk through lysosomes. Although normally starvation triggers autophagy, its completion depends on functional lysosomes. Thus, in *cup-5* mutants, autophagy cannot supply nutrients during embryogenesis. Consequently, starvation induces excess apoptosis leading to embryonic lethality. A similar effect is observed after blockage of autophagy in strains carrying the hypomorphic *cup-5(ar465)* allele. The *ar465* mutation alone causes only slightly increased autophagosome formation in coelomocytes, the scavenger cells in the worm body cavity [92]. Autophagy impairment in this genetic background activates apoptosis and results in embryonic death. Studies in cultured skin fibroblasts from patients with Mucopolipidosis type IV or other lysosomal storage diseases also link autophagy deficiency with induction of apoptosis [93]. Lysosomal dysfunction in these cells affects normal autophagic degradation of damaged mitochondria. Fragmented mitochondria incapable of buffering cytoplasmic Ca²⁺ persist, enhancing sensitivity of cells to Ca²⁺-mobilizing apoptotic stimuli. Concomitantly, fibroblasts die via pathways involving cytochrome c release and caspase-8 activation. The above observations in their totality indicate a protective role of autophagy against aberrant apoptosis.

Several studies show that both autophagy and apoptosis are activated under conditions of metabolic stress. NGF deprivation in isolated sympathetic neurons induces both autophagic and apoptotic (DNA fragmentation) features [94]. In specific cases of glucose deprivation, hypoxia, oncogene activation or genotoxic stress, activation of p53 mediates the parallel activation of apoptosis and autophagy [95, 96]. p53 acts by suppressing TOR and/or by targeting the lysosomal protein DRAM. Autophagy can precede apoptosis in cerebellar granule cells grown in media with low serum and potassium concentration [97]. Although the physiological importance of autophagy induction is not examined in these cases, many studies propose a protective role under conditions of nutrient shortage. Autophagy defective mammalian cells cannot tolerate starvation and die via apoptosis. Relevant studies include chemical autophagy inhibition, RNAi knockdown of essential autophagy genes and assays in calpain-deficient or LAMP2 depleted genetic backgrounds [98-101]. LAMP2 is a lysosomal protein required for autophagosome-lysosome fusion. LAMP2 deficiency has been associated with the development of cardiac and skeletal myopathies during Danon's disease [102]. Mammalian carcinoma cell survival under starvation requires the function of p27, a negative regulator of cyclin-cdk [103]. Metabolic stress activates the LKB1-AMPK pathway stabilizing p27, which sustains increased autophagy levels.

Autophagy-dependent destruction of cultured cells after prolonged amino acid starvation has been also reported [104]. Although cell death is characterized by caspase-3 upregulation, it is not dependent on caspase function and is reduced after addition of

3-methyladenine (3-MA) that inhibits autophagosome formation. In addition, overexpression of mutant forms of Beclin 1 that cannot bind to Bcl-2 leads to an Atg5-dependent cell death both under starvation and under normal growth conditions [105]. Interaction of Bcl-2 with Beclin 1 in the ER negatively regulates basal- and starvation-induced autophagy in several cell lines and in mice cardiac muscles. Starved cells may primarily activate autophagy as an alternative energy source. However, excess autophagy due to prolonged starvation, impaired autophagy-control, or overexpression of autophagy molecules (as described below for Atg1 and Atg5) can be detrimental.

In addition to starvation, cells also trigger autophagy in order to combat endoplasmic reticulum stress (ER-stress). Such a response is common from yeast to mammalian cells. Autophagy probably acts in concert with other degradation mechanisms, such as the proteasomal system, to remove misfolded proteins [106]. Thorough electron microscopic analysis of ER-stressed yeast cells have revealed the sequestration of ER portions into autophagosomes [107]. This process is essential for cell survival. In mammalian cells perturbation of either ER calcium homeostasis or ER function leads to increased number of autophagic vacuoles and apoptotic cell death [108]. The contribution of autophagy in the ER-stress response depends on the tissue type. In colon and prostate cancer cell lines impairment of autophagy exacerbates cellular distortion and death. In non-transformed but immortalized mouse embryonic fibroblasts (MEFs) and non-immortalized human colon cells autophagy dysfunction alleviates ER stress-induced apoptosis. Thus, autophagy is protective against ER-stress in transformed cancer cells.

The anti-apoptotic function of autophagy has also been demonstrated in paraquat-treated human neuroblastoma cells and during innate immunity responses in plants [109, 110]. However overexpression of specific autophagic factors induces apoptotic death. Overexpression of Beclin 1 further sensitizes the beclin 1 haploinsufficient human breast adenocarcinoma cell line to the toxic effects of the vitamin D analog EB1089 [111]. EB1089 effects include increased number of autophagic vacuoles and apoptotic nuclear characteristics (chromatin condensation and DNA fragmentation), and are suppressed after treatment with 3-MA. In *Drosophila melanogaster* overexpression of Atg1 induces autophagy and causes cell death [112]. Cellular destruction shows apoptotic characteristics and depends on autophagy factors and caspases. Overexpression of Atg5 increases sensitivity of tumor cells to apoptosis [113]. This effect is mediated by calpain cleavage of Atg5. The resulting N-terminal fragment interacts with Bcl-X_L in mitochondria, leading to cytochrome c release and apoptosis. Interestingly, autophagy has recently been shown to participate in the clearance of apoptotic bodies in embryos and during developmental apoptosis in mice [114]. This is probably attributed to low ATP levels in autophagy-defective cells, which prevent transmission of clearance-signals, such as phosphatidylserine exposure on the cell surface and secretion of lysophosphatidylcholine.

Apoptotic stimuli can promote autophagic cell death, especially when the apoptotic pathway is impaired. Suppression of caspase-8 activity causes non-apoptotic death in several mammalian cell lines and primary cell cultures [115]. Cells display loss of membrane integrity and accumulation of autophagosome-like vesicles in the cytoplasm. Execution of cell death requires activation of a pathway involving RIP, MKK7, JNK and c-Jun, and is reduced by down-regulation of the autophagy genes ATG7 and beclin 1. Autophagy-dependent death with concomitant RIP activation also follows zVAD administration in lipopolysaccharide (LPS)-sensitized macrophages [116]. Moreover, elimination of the pro-apoptotic proteins Bax and Bak sensitizes specific cell types to autophagic demolition. Bax and Bak-deprived MEFs treated with apoptotic stimuli (etoposide or staurosporine), or exposed to radiation, show increased levels of Beclin 1 and ATG5/ATG12 complexes, accumulate autophagic vacuoles and die [117, 118]. Death depends on the

anti-apoptotic proteins Bcl-2 or Bcl-xL and requires autophagy. Sensitization to non-apoptotic demise by Bax/Bak-deficiency is not merely due to apoptosis inhibition. Apaf-1 and caspase-9 depletion or zVad-fmk treatment do not cause autophagic death of MEFs. It is proposed that Bax/Bak act as negative regulators of autophagy. Autophagic death is also inflicted by histone deacetylase inhibitors in apoptosis-defective mammalian cells [119]. In murine leukaemia cells, treatment with Bcl-2, Bcl-X_L antagonists causes a rapid increase in autophagosome formation and apoptosis [120]. Further administration of the autophagy inhibitor wortmanin promotes apoptosis, whereas treatment with the caspase-3/7 suppressor zDEVD-fmk promotes autophagy. Thus, cells can compensate for potential deficiencies in programmed death mechanisms by activating alternative death pathways.

The autophagic process shares common stimuli and common denominators with apoptosis, such as starvation, ER-stress, anti-apoptotic factor antagonists and vitamin D analogs. In addition, ceramide administration in mammalian cells and dopamine injection into the substantia nigra of anaesthetized rats induce apoptotic and autophagic characteristics [121, 122]. In nematodes dysfunction of the isopentenyl-diphosphate isomerase IDI-1, required for the synthesis of lipophilic molecules, causes accumulation of autophagosomes and appearance of apoptotic corpses [123]. In addition, the DAPk and DRP-1 Ser/Thr kinases are common mediators of autophagy and apoptosis [124]. These kinases promote membrane blebbing and formation of autophagosomes in epithelial cell lines, in cases of apoptosis and autophagy induction respectively.

Necrotic Cell Death in *C. elegans*

Necrosis is usually triggered as a response to extreme insults, such as severe deprivation of oxygen and energy sources, toxin exposure and extreme temperatures [125]. Whether it can also be programmed is still under debate [126]. Necrotic-like destruction has been reported during development in chondrocytes and in intestinal epithelial cells. However, necrotic cell death mainly transpires during pathological conditions such as neurodegenerative disorders and stroke. Necrotic death has also been observed after activation of death receptors in cultured, primate neuronal cells exposed to hypoxic-ischemic conditions, in *Dictyostelium discoideum* and in *Caenorhabditis elegans* [126].

Several genetic and environmental insults trigger necrosis in nematodes. Mutations that result in the hyper-activation of degenerin (DEG) ion channels are among the most potent insults [127]. Substitution of small side chain amino acids located in the region forming the channel pore with larger amino acids deregulates gating of these channels, resulting in aberrant ionic influx that disrupts cellular ion homeostasis. For example, dominant, hyper-activating mutations in the *mec-4* gene, which encodes a subunit of a mechanosensitive ion channel expressed in the six touch receptor neurons, cause the death of these neurons in mutant animals [128]. Similarly, the gain-of function *u38* allele of the *deg-1* degenerin gene causes late-onset degeneration of posterior interneurons [129]. In addition, semi-dominant alleles of the *unc-8* and *unc-105* degenerins lead to dysfunction of ventral nerve cord and to muscle hypercontraction respectively [130]. Interference with the function of other ion channels not related to degenerins can also induce necrosis. Hyper-activation of the nicotinic acetylcholine receptor Ca²⁺ channel subunit DEG-3, expressed in several interneurons and sensory neurons, is one such example [131]. In addition, necrosis is inflicted by gain-of-function mutations in the α -subunit of trimeric plasma membrane G-proteins, which regulate ion channel function activity. Cell death depends on adenylyl cyclase ACY-1 and cAMP [132-134]. Necrotic cell death has also been reported in transgenic worms expressing a form of the ionotropic glutamate receptor GLR-2 that carries a glutamine (Q) to arginine (R) substitution at the pore loop region [135]. The formation of hybrid GLR-2(Q/R) channels with reduced Ca²⁺ permeability (compared to the normal

GLR-2(Q) channels) deregulates ion channel function and causes necrosis.

Microscopic observations of dying *mec-4(d)* neurons reveal a series of morphological changes that resemble experimentally induced excitotoxicity in rats [136]. Cell death begins with general cellular distortion and accumulation of small wrapped membrane whorls near the plasma membrane [137]. At later stages these membrane structures coalesce and move towards the nucleus, while the cell swells to several times its initial size. Recent studies reveal fusion and gathering of intracellular organelles including lysosomes around a swollen nucleus [138]. Elevation of cytoplasmic Ca²⁺ concentration is a central event in the necrotic process (Fig. (3)). Calcium influx from the extracellular space through voltage-gated channels and Ca²⁺/Na⁺ exchangers, and efflux from intracellular stores, mainly the endoplasmic reticulum and mitochondria, contributes to this elevation [139]. ER-channels, such as the ryanodine receptors (RyR) and the inositol-1,4,5-triphosphate receptors (Ins(1,4,5)P₃R), are potential mediators of Ca²⁺ release. Their dysfunction as well as elimination of calcium-binding ER-chaperones like calnexin and calreticulin confers resistance to necrosis. By contrast, chemically induced ER-Ca²⁺ release is sufficient to trigger necrotic death. The vacuolar necrotic-like neuronal death induced by expression of GLR-2(R) in worms also depends on ER-Ca²⁺ efflux [135].

Execution of necrotic cell death requires calcium-dependent calpain protease and aspartyl protease activity. In nematodes calpains CLP-1 and TRA-3 are required upstream of cathepsins ASP-3 and -4 to carry out necrosis [140]. Calpains promote a further increase of cytoplasmic Ca²⁺ levels by cleaving specific glutamate receptors in mammalian models of excitotoxicity [141]. Aspartyl proteases involved in necrosis are either cytoplasmic or lysosomal that are released into the cytoplasm after lysosome rupture. Disruption of normal lysosomal biogenesis ameliorates cell death, indicating that lysosomes contribute to the execution of necrosis [138]. Indeed, lysosomal rupture releases hydrolytic enzymes that degrade cellular components and creates acidic cytoplasmic micro-environments, optimal for hydrolases activity. Acidification of the cytoplasm that occurs during necrosis is critical for cell death and depends on the function of the vacuolar H⁺-ATPase (V-ATPases) [142]. The V-ATPase is a proton pump that acidifies endocytic compartments [143]. Acidosis is also observed during acute hypoxic-ischemic injury in cultured astrocytes [144].

Necrotic characteristics, such as perturbation of Ca²⁺ homeostasis, perinuclear clustering of organelles, activation of calpains and cathepsins, lysosomal rupture and eventually plasma membrane rupture are common in most experimental models of necrosis [126]. The mechanisms described in nematodes are consistent with the "calpain-cathepsin hypothesis", initially based on observations of primate brain neurons subjected to global ischemia [145]. Elevation of intracellular Ca²⁺ levels in these cells stimulates μ -calpains. Calpains bind to lysosomal membranes and together with reactive oxygen species (ROS) elevation compromise lysosomal membrane integrity. The concomitant release of hydrolytic enzymes mediates cell destruction.

Other paradigms of necrotic-like cell death in *C. elegans* have also been described. Absence of EGF-like-signaling in four precursor-uterine cells participating in the vulva-uterine connection prevents their differentiation and causes their death by necrosis [146]. Furthermore, necrotic-like death has been recently observed in postmitotic vulval cells, after exposure of their precursors (VPCs) to radiation [147]. Vulval cells die in a stochastic manner *via* a non-apoptotic pathway. In addition, vacuoles appear in the gonads of irradiated animals, and radiosensitivity is significantly enhanced by interfering with DNA damage response pathways.

Similarly to vertebrates, adverse environmental conditions also initiate necrosis in worms. For example, hypoxia reduces animal

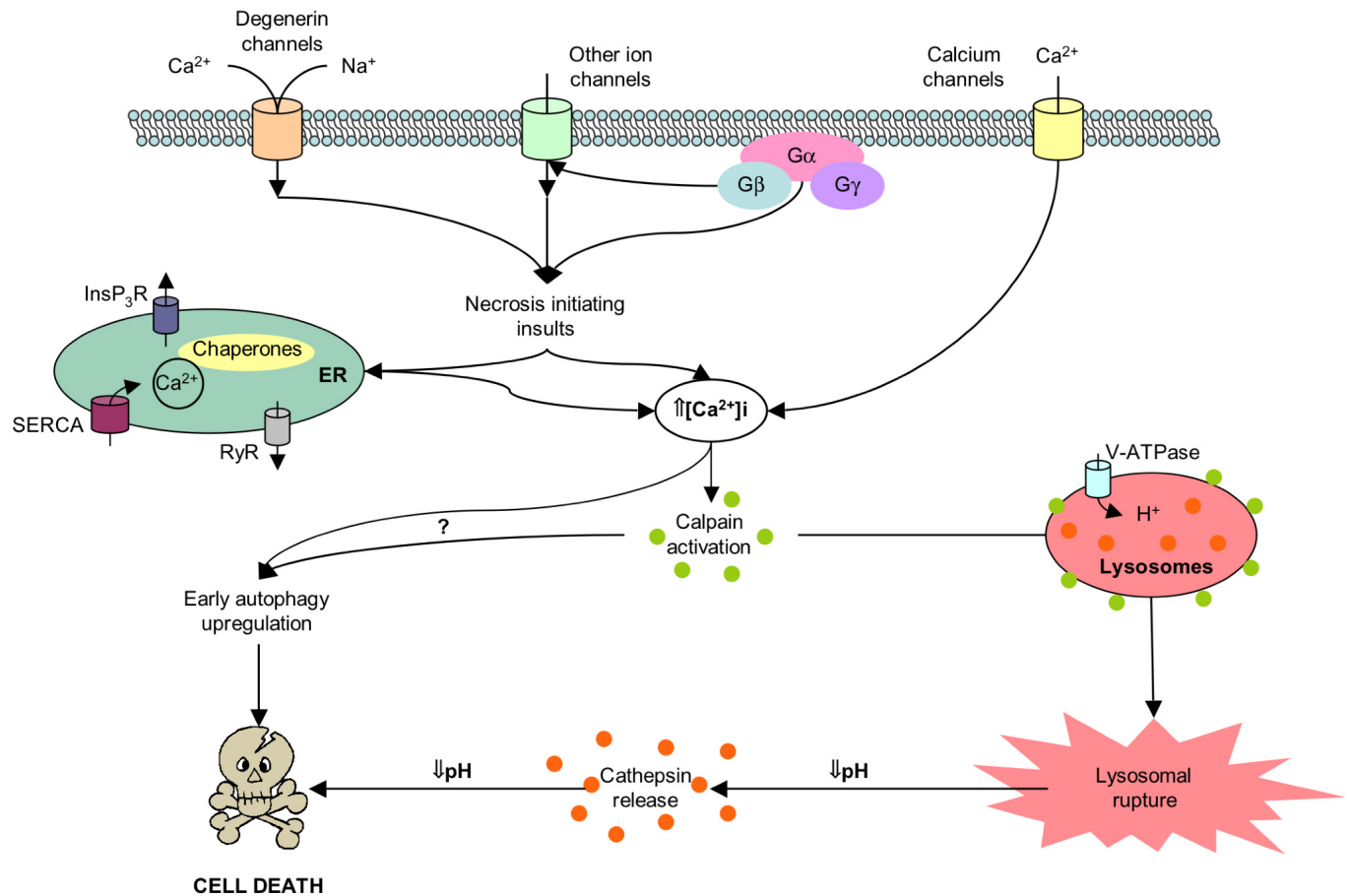


Fig. (3). A working model for necrotic cell death in *C. elegans*. Necrotic insults lead to increase of cytoplasmic calcium concentration. Consequently, calpain protease activation causes lysosomal membrane rupture. The concomitant release of hydrolytic enzymes such as cathepsin proteases, in combination with the development of acidic intracellular conditions due to lysosomal acidification by the V-ATPase, mediates cellular destruction. Elevated intracellular Ca^{2+} levels may also cause excessive autophagy induction, further contributing to cellular destruction.

viability and induces necrotic-like cell morphology in several tissues, such as the pharynx, body wall muscles and gonads [148]. The insulin/IGF signaling pathway has been implicated in cell death. Expression of the insulin-like receptor DAF-2 in neurons and muscles is critical, and specific alleles that reduce its function are hypoxia-resistant. The kinases AKT-1 and PDK-1 and the transcription factor DAF-16 mediate DAF-2 effects on the hypoxic response. Hypoxia-resistance in nematodes also depends on the activity of sodium-activated potassium channels, such as SLO-2. Mutations in *slo-2* gene increase sensitivity to hypoxia [149].

Autophagy Activation in Response to Necrotic Insults

Several studies have shown that autophagy is upregulated by necrosis-inducing stimuli. Traumatic brain injury in mice leads to elevation of Beclin 1 levels in neurons and in astrocytes [150, 151]. Dying brain cells of adult mice subjected to unilateral common carotid artery occlusion display cytoplasmic vacuolization, lysis of intracellular organelles and activated autophagy [152]. In addition, autophagy upregulation occurs in murine and chicken models of excitotoxicity [153-155]. While in some cases autophagosomes accumulate mostly in neuronal axons, in others they gather in the paranuclear region [153, 155]. Activation of RIP, a component of the Jun N-terminal kinase (JNK) pathway, in mammalian cells treated with caspase inhibitors also causes toxicity characterized by excess autophagosome formation [115]. The JNK pathway has also been associated with caspase-independent necrotic-like damage [156-158].

Although autophagy is upregulated in the above cases, it is not clear whether it protects cells or contributes to their destruction. Recent studies in *C. elegans* indicate a causative role of autophagy in necrotic degeneration [159]. Downregulation of the autophagy genes *bec-1*, *unc-51* and *lgg-1* confers partial protection against necrotic cell death inflicted by hyperactive MEC-4, DEG-1 and DEG-3 ion channels (our unpublished observations). By contrast, autophagy upregulation by knock down of the negative autophagy regulator *CeTOR* or under starvation exacerbates cellular destruction [159]. Thus, autophagy is required for necrosis in *C. elegans* (Fig. (3)).

In vivo and *in vitro* studies in mammals also indicate a positive correlation between autophagy and necrotic cellular destruction. In Lurcher mice carrying a gain-of-function allele of GluR δ 2 glutamate receptor, constant inward currents cause cell-autonomous excitotoxic-like death of Purkinje cells [160]. Lurcher Purkinje cells exhibit an excess number of double- and multi-membranous autophagic vacuoles [161]. GluR δ interacts with Beclin 1 and the protein nPIST in the cerebellum. Coexpression of Beclin 1 and a form of nPIST unable to interact with GluR δ induces autophagy. Expression of the mutated GluR δ ^{LC} further promotes autophagy-dependent cell death. In another experimental model, overexpression of the human protein Spin1 causes caspase-independent non-apoptotic death in cultured cells [162]. Spin1 is a transmembrane protein that interacts with the anti-apoptotic factors Bcl-x_L and Bcl-2 and colocalizes with them in mitochondria, most prominently after the application of apoptotic stimuli. Dying, Spin1-overex-

pressing cells display accumulation of large number of membrane structures and are protected by chemical blockers of either autophagy or necrosis. Thus, autophagy probably becomes detrimental under the extreme conditions that trigger necrosis.

A pro-survival function for autophagy has also been proposed. Mice subjected to the N-methyl D-aspartate (NMDA) receptor agonist kainic acid (KA) suffer seizures and hippocampal excitotoxic death with both apoptotic and necrotic features [163]. Soon after KA administration, a transient increase in autophagosome number occurs in hippocampal neurons. Delayed neurodegeneration follows TOR and Akt kinase-mediated autophagy downregulation. Treatment with the autophagy-inducer rapamycin partially protects neurons, reduces microglia/macrophage activation and improves recovery in brain-injured mice [164]. The pro-survival effects of autophagy are also prominent in the myocardium upon exposure to ischemic insults. Autophagy upregulation has been observed in a porcine model of chronic ischemia [165]. Genetic and pharmaceutical manipulations that induce autophagy protect cultured cardiac myocytes after *in vitro* simulation of ischemia/reperfusion [166]. By contrast, treatment of isolated cardiomyocytes undergoing anoxia-reoxygenation with autophagy blockers exacerbates toxicity [167].

Activation of death-receptors in specific cell lines with or without simultaneous inhibition of caspases induces a type of death termed "necroptosis" [168]. Cells display necrotic features, such as organelle swelling and early loss of plasma membrane permeability. Nuclear condensation and an Atg5-dependent increase in the number of autophagic vacuoles are also observed. Autophagy impairment in this model has no effect on cell death. Thus, autophagy induction appears to be merely a secondary consequence of cell death. In addition, interference with known necrotic mediators, such as Ca^{2+} homeostasis and calpain proteases has no impact on necroptosis.

NEMATODE MODELS OF HUMAN PATHOLOGIES INDUCED BY AGGREGATION-PRONE PROTEINS

Amyotrophic laterar sclerosis (ALS), Alzheimer's disease (AD), Parkinson's and Huntington's disease (PD and HD respectively) are among the most frequent human neurodegenerative disorders. A common feature of these pathologies is the accumulation of proteins with altered conformation, which tend to form oligomers, fibers and aggregates [169]. Oligomers and fibers are considered the most toxic, while the formation of aggregates may initially serve a protective role. However, these aggregates eventually promote toxicity by sequestering useful molecules and/or perturbing intracellular trafficking and homeostasis. Post-mitotic neuronal cells are particularly sensitive. Because these cells do not divide, misfolded molecules accumulate continuously [169]. The symptoms of protein aggregation disorders include impairment of cognitive functions and death. Several cell and animal models have been used to elucidate the pathogenic mechanisms involved, in the effort to combat these diseases. Bellow, we discuss nematode-based models of neurodegenerative disorders.

Alzheimer's disease is a mainly sporadic disorder characterized by progressive loss of hippocampal neurons and dementia [170]. A few familial cases have been associated with dysfunction of genes that participate in amyloid- β ($A\beta$) peptide formation. $A\beta$ peptide is produced after proteolytic cleavage of the transmembrane amyloid precursor protein APP [171]. $A\beta$ is the main component of extracellular senile plaques. Accumulation of fibrillary tangles in the interior of neurons is another feature of AD. These are mainly composed of hyperphosphorylated tau aggregates. tau is a microtubule-associated protein implicated in a group of human pathologies called tauopathies [172]. Alzheimer's disease and FTDP-17 (frontotemporal dementia and parkinsonism-linked to chromosome 17) belong to this group.

Expression of the human $A\beta$ peptide in *C. elegans* muscles leads to progressive paralysis and intracellular deposition of amyloid-like fibrillar structures [173, 174]. Intracellular deposits form upon expression of a similar minigene in the nervous system. Studies in transgenic nematode lines suggest that such structures are not the main cause of toxicity and indicate involvement of $A\beta$ retrograde transport in the development of neuronal pathology. In addition, these studies reveal interactions between the $A\beta$ peptide and small HSP-16 proteins [175]. These proteins are homologous to α B-crystallin, and their transcription increases in $A\beta$ -expressing cells. α B-crystallin has been implicated in AD-associated supranuclear cataracts and in inclusion body myositis, a myopathy displaying intramuscular $A\beta$ accumulation [176, 177]. Proteomic studies have led to the identification of several molecules oxidized in worms expressing human $A\beta$ [178]. This finding supports the hypothesis that $A\beta$ -induced protein oxidation is implicated in the pathogenesis of AD [179].

Expression of wild type or mutant forms of human tau in *C. elegans* neurons also induces progressive neurodegeneration [180, 181]. The exogenously expressed protein is hyperphosphorylated, similarly to human tauopathies. Neuronal cells progressively accumulate insoluble tau-aggregates and animal movement becomes uncoordinated [180]. Neurites exhibit signs of deformation and neuronal loss occurs at late stages. Dying cells display axonal membrane infolding, formation of whorls and vacuolation. These effects are more pronounced after expression of mutated forms of tau. Neuronal death is not apoptotic and does not require functional *ced-3* or *ced-4*. Nevertheless, it is ameliorated by up-regulation of heat-shock proteins, such as HSP70 [181].

Familial cases of Alzheimer's disease have been associated with Presenilin, which mediates APP cleavage and $A\beta$ peptide formation [182]. The *C. elegans* genome encodes two homologs of presenilin, *sel-12* and *hop-1*. Loss of *sel-12* function leads to egg-laying defects, rescued by mutations in the genes *spr-1*, *-3*, *-4* and *-5* [183]. These genes code for orthologs of human factors that regulate gene expression. Their dysfunction results in the derepression of the other nematode presenilin gene *hop-1*, implying redundancy between SEL-12 and HOP-1 activities.

Nematodes have also been used to study protective effects of specific substances, such as EGb 761, against AD [184]. This chemical is extracted from *Ginkgo biloba* leaves and is extensively used to treat age-related cerebral dysfunctions and dementias. EGb 761 decreases ROS production and $A\beta$ oligomerization, and ameliorates paralysis in worms expressing $A\beta$ in muscles.

The term Parkinsonism is used to describe a group of disorders that involve loss of dopaminergic neurons and accumulation of Lewy bodies, which are intracellular inclusions. 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) or 1-methyl 4-phenylpyridinium (MPP⁺), 6-hydroxydopamine (6-OHDA) and the pesticide rotenone are potential contributors to the development of parkinsonism [125]. Rare familial cases have been associated with dysfunction of parkin, DJ-1 or α -synuclein [185]. The later is a pre-synaptic protein that constitutes the major component of Lewy bodies.

The toxicity of 6-OHDA and MPTP has been studied in nematodes. 6-OHDA is a neurotoxin that causes degeneration of dopamine neurons in *C. elegans* [186]. Cells exhibit morphological features that resemble apoptosis, such as condensed chromatin and shrunken cell bodies. However, *ced-3* and *ced-4* are not required for cell death. Expression of the human chaperone torsinA protects *C. elegans* neurons from 6-OHDA-toxicity by downregulating dopamine transporters [187]. TorsinA is a component of Lewy bodies that form in early-onset torsion dystonia. Degeneration of worm dopaminergic neurons also occurs after administration of MPTP or MPP⁺ [188]. These nematode models are suitable for high-throughput screenings aiming to identify potential therapeutic pharmaceutical agents.

Parkinsonism nematode-models also include genetically modified strains. Overexpression of wild type or mutant human α -synuclein in the nervous system causes selective degeneration of dopaminergic neurons [189]. Overexpressed α -synuclein accumulates specifically in cell bodies and neurites, promoting extensive degeneration and impairment of cellular function [190]. Exogenous addition of dopamine reverses behavioral defects. Genome-wide screenings have led to the identification of genes with altered expression in these transgenic animals [191]. These studies indicate involvement of histones in α -synuclein-mediated neurotoxicity and are also consistent with the previously reported implication of the lysosomal system and mitochondria in the process. Reduced expression of parkin and DJ-1 homologs affects mitochondria in worms and renders animals vulnerable to chemicals that perturb mitochondrial activity, such as the respiratory chain complex I blocker rotenone [185]. By contrast, administration of antioxidants, mitochondrial complex II activators and/or anti-apoptotic factors has a protective effect.

A variety of neurodegenerative disorders is associated with proteins bearing large, expanded tracts of glutamine residues. Huntington's disease develops due to autosomal dominant alterations that introduce long glutamine stretches in the N-terminus of Huntingtin (Htt) [169]. Expanded poly-glutamine (polyQ) repeat proteins form fibers, aggregates and intracellular inclusion bodies. Expression of an N-terminal human huntingtin fragment with 150 glutamine residues (Htt-Q150) in the ASH sensory neurons of *C. elegans* causes age-dependent progressive accumulation of protein aggregates [192]. Degeneration of these neurons depends on the function of the caspase CED-3. This experimental system has been used to screen for genetic and pharmaceutical suppressors of polyQ-neurotoxicity [193]. PQE-1, a protein containing a glutamine-proline-rich and a charged domain, confers neuronal protection [194]. CREB, CBP and histone deacetylases (HDACs) have also been implicated in polyQ toxicity [195].

Glutamine tract expansion in ataxin-3 causes spinocerebellar ataxia type 3 in humans. Expression of Ataxin-3 forms with different polyQ tract lengths in nematode neurons causes disruption of synaptic transmission, abnormal branching and swelling of axons [196].

Autophagy and Protein Aggregation Pathologies

Upregulation of the lysosomal system including autophagy is a common feature of most neurodegenerative diseases [197]. Autophagy appears to play a protective role by preventing aggregate deposition and by removing aggregate-prone and misfolded protein oligomers [197, 198]. While altered proteins are still in an unfolded state, their clearance occurs selectively *via* proteasomes and CMA [169]. Oligomers and fibrillar forms can only be degraded through lysosomal pathways. At late stages however, accumulating ROS and other factors impair the autophagic-lysosomal system [199]. The imbalance between production and removal of altered protein molecules results in neuronal damage by accumulation of toxic aggregates.

Upregulation of the endocytic-lysosomal system occurs very early in AD brains, before neurodegeneration takes over [200]. Downregulation of mTOR/p70S6k signaling reported in lymphocytes of AD patients is a potential means of autophagy induction [201]. Despite an initial protective role, there are also indications for autophagy contribution to the generation of toxic A β peptides. Autophagic vacuoles accumulate in distorted dendrites in a mouse model of β -amyloidosis [202]. These autophagosomes contain APP, A β peptides and enzymes needed for APP cleavage, such as presenilin and γ -secretase. Human neuroblastoma cells exposed to hyperoxic conditions also accumulate large lysosomes carrying A β [203]. This effect is blocked by administration of autophagy inhibitors.

Melanized neurons in the substantia nigra of PD patients display autophagic morphology [204]. Although proteasomes and

CMA degrade soluble forms of α -synuclein, insoluble toxic forms and aggregates are cleared by macro-autophagy [205, 206]. Chemically induced autophagy enhances clearance of mutant α -synuclein in cultured mammalian cells [207]. By contrast, autophagy exacerbates cell death that follows treatment with parkinsonism-inducing environmental toxins. Administration of MPP⁺ to cell cultures triggers autophagosome formation and degeneration, dependent on extracellular signal-regulated kinase (ERK) signaling and core Atg proteins, but not phosphoinositide 3-kinase (PI3K) or beclin 1 [208]. In *C. elegans*, loss of dopamine neurons after exposure to 6-OHDA requires the autophagy genes *bec-1* and *unc-51* [159].

Activation of the autophagic and endosomal-lysosomal system is also evident during polyQ-induced toxicity [209, 210]. Redistribution of the autophagy molecule Beclin 1 from trans-Golgi network (TGN) to polyQ aggregates has been reported in cultured cells, in murine brains expressing mutant Huntingtin and in striatal samples from HD patients [211]. Beclin 1 mediates the turnover of polyQ proteins *via* the autophagic pathway for degradation of long-lived proteins. These and other studies in mammalian cell cultures demonstrate the pro-survival function of autophagy [207, 212].

Animal models of neurodegenerative disorders also suggest a correlation between autophagy upregulation and protection from polyQ-induced toxicity. Transgenic worms expressing polyQ proteins in muscles or in sensory neurons display abnormal movement and neuronal degeneration respectively [213]. Autophagy impairment by knock down of *bec-1*, *atgr-7* or *atgr-18* enhances accumulation of polyQ-bearing aggregates and exacerbates toxicity. Increased autophagosome number accompanies polyQ aggregate formation in flies and mice [214]. The autophagy inhibitor mTOR promotes accumulation of mutant huntingtin aggregates and cell death. By contrast, autophagy induction reduces the size and number of aggregates, eliminates neurodegeneration in flies and ameliorates pathology symptoms in mouse models.

The exact mechanism of autophagy upregulation under neurodegenerative conditions remains unclear. Htt filaments have been proposed to inhibit proteasomal function [215]. Expression of Ataxin-3 forms with polyQ tracts impairs the ubiquitin-proteasome system in transgenic *C. elegans* lines [196]. Proteasome inhibition induces formation of ubiquitinated protein inclusions in cultured rat cortical neurons [216]. Concomitant activation of autophagy facilitates their dissolution. Thus, autophagy may be induced to compensate for the dysfunction of other cellular degradation systems. Aggregates appear to sequester the TOR kinase autophagy-suppressor among several other cell components [214]. TOR is a component of the insulin/IGF signaling pathway. Activation of this pathway facilitates clearance of polyQ aggregates in cell lines stably transfected with expanded polyQ sequences [217]. Clearance depends on the function of the hVps34 and Beclin 1 autophagy factors. Interestingly, the autophagic process is induced despite simultaneous activation of the negative regulator class I PI3K. Thus, findings in polyQ-toxicity models suggest that autophagy activation is the result of proteasomal and CMA impairment and/or sequestration of autophagic inhibitors during the aggregation process [169].

OTHER TYPES OF CELL DEATH IN *C. elegans*

Cell death modes that display neither apoptotic, nor autophagic or necrotic features have been described in *C. elegans*. The death of the linker cell in males is one such example. It occurs during or immediately after the L4/adult transition, when the male gonad is formed [218]. Although this type of cell death is developmentally programmed, it does not require caspases and does not display apoptotic morphological characteristics. Furthermore, it is not dependent on any of the proteins used for the execution of necrotic cell death in worms (calpains or aspartyl proteases). Electron microscopic studies reveal the formation of cytoplasmic vesicles and dilated mitochondria, often in the interior of multimembrane structures. Although these structures could represent autophagosomes,

mutations in the autophagy genes *unc-51* or *bec-1* do not affect death, and the number of autophagosomes increases only slightly in dying cells.

C. elegans has also been used as a model to study the pathogenesis of muscular dystrophies. Destruction of muscle cells occurs in some of these cases as a consequence of dysfunctional dystrophin. Dysfunction in the nematode dystrophin-coding gene *dys-1* in sensitized genetic background with mild mutations in MyoD, progressively impairs locomotory behavior in worms and induces extensive degeneration of body wall muscles [219]. Such *C. elegans* strains are useful for the screening of potential protective pharmaceutical agents [220]. Serotonin has been recently shown to confer protection and prevents muscle degeneration in these animals [219].

AUTOPHAGY AND AGEING IN *C. elegans*

C. elegans is widely appreciated as a powerful model organism for studying the mechanisms of ageing. Mortality rates in worms increase exponentially with age. Ageing is also accompanied by morphological deterioration, lower fertility and decrease in mobility, food uptake and defecation rhythms [221]. Several mutants affecting longevity have been identified, pointing to specific conserved molecular processes that affect lifespan. The insulin/IGF-1 signaling pathway is one of the most thoroughly studied and negatively regulates dauer formation, stress resistance and lifespan. Insulin-like ligands activate the nematode insulin-like receptor DAF-2 [222]. DAF-2 carries an intracellular tyrosine kinase domain that recruits the PI-3 kinase, consisting of AAP-1 and the catalytic subunit AGE-1 [223, 224]. The PI-3 kinase activates PDK-1 and subsequently the kinases AKT-1, AKT-2, SGK-1 and the FOXO transcription factor DAF-16 [221]. Phosphorylated DAF-16 translocates to the cytosol where it is inactive. DAF-18, a homolog of the mammalian PTEN, negatively regulates this pathway, by mediating DAF-16 dephosphorylation [225]. Other factors, such as SIR-2.1 and LIN-4, and the RAS- or JNK-signaling pathways also positively regulate DAF-16 to delay ageing [221].

DAF-16 cooperates with HSF-1 or SMK-1 to control the expression of several genes involved in metabolism, energy generation and the response to intra- and extra-cellular stressors [221]. It also exerts a positive feedback on the insulin/IGF-1 pathway through inhibition of the DAF-2 agonist INS-7 [226]. The insulin/IGF-1/DAF-16 signaling cascade influences *C. elegans* lifespan in a non-cell autonomous manner via specific neurons and/or the intestine during adulthood [227]. The intestine also receives signals from the germline. The genes *kri-1*, *daf-9* and *daf-12* promote DAF-16 function in germline nuclei [221].

The insulin/IGF-1 signaling pathway is also connected with the TOR kinase pathway. TOR functions in a complex with Raptor to sense cellular amino acid availability and control transcription and translation. The *C. elegans* TOR and Raptor proteins are encoded by *let-363* and *daf-15* respectively. Mutations in either *let-363* or *daf-15* cause an L3/dauer-like larval arrest phenotype (similar to Daf-constitutive, Daf-c) and increased fat storage [228, 229]. In addition, *daf-15* heterozygotes live longer than wild type or *daf-2* mutant animals [228]. This effect is reversed by *daf-16* elimination. Such a deficiency however does not affect Daf-c or fat deposition in *daf-15* homozygous mutants, suggesting negative regulation of *daf-15* by DAF-16. Elimination of *CeTOR* activity by RNAi or in homozygous *let-363* mutants results in significant lifespan extension in wild type but not in *daf-2(e1370)* background, indicating that *CeTOR* acts downstream of DAF-2 [230]. However, *CeTOR* does not appear to affect ageing through DAF-16. A role for TOR in ageing has also been reported in flies, where downregulation of dTOR similarly extends lifespan [231].

These observations, combined with the inhibition of autophagy by TOR in the presence of nutrients and growth factors implicate autophagy in the regulation of ageing. *CeTOR* deficiency not only increases lifespan, but also causes intestinal atrophy and subsequent

abnormality in nutrient absorption [229]. Caloric restriction promotes longevity in a variety of different organisms. Feeding-defective *C. elegans* mutants live longer, and this has been attributed to caloric restriction [232]. The seam cells of starved L3 larvae exhibit increased levels of autophagy, thus connecting extended longevity under caloric restriction with activated autophagy [233].

Additional studies associate autophagy impairment with shortening of lifespan. Knockdown of the autophagy genes *bec-1*, *atgr-7* and *atgr-12* compromises the longevity of *daf-2(e1370)* adult worms. Interference with *atgr-7* and *atgr-12* expression also affects lifespan in wild type animals [40, 234]. In addition, reduction of *bec-1* activity influences dauer survival. The lifespan of both dauer- and L1-arrested-larvae is also decreased in *pcm-1* mutants that are incapable of upregulating seam cell autophagy levels during dauer formation [235, 236]. *pcm-1* codes for the enzyme L-isospartyl-O-methyltransferase, which participates in the repair of damaged proteins.

The effects of autophagy on ageing may be exerted through the recycling of proteins and organelles. Indeed, a critical role of protein turnover in longevity control has recently been demonstrated in nematodes [237]. Protein turnover probably becomes more critical in old organisms, due to accumulation of damaged macromolecules as a result of oxidation by ROS. Oxidized products are better substrates for chaperone-mediated autophagy [238]. Moreover, macroautophagy degrades mitochondria, the cellular compartments wherein ROS are generated. Indeed, dysfunction of the mitochondrial electron transport chain components MEV-1 and GAS-1, results in short-lived nematodes [221]. By contrast, mitochondria impairment in *clk-1*, *isp-1* and *lsr-2* mutants increases nematode lifespan in a DAF-16-independent manner. Mice lacking one *mlk1* allele have been also reported to live longer [239]. These observations suggest that downregulation of mitochondrial activity is a conserved mechanism extending lifespan.

CONCLUSIONS: AUTOPHAGY IN SURVIVAL AND DEATH

Research in *C. elegans* has elucidated critical aspects of ageing and apoptotic mechanisms and ongoing efforts continue to provide missing pieces of the puzzle. A wealth of recent findings has shed light on death pathways that were previously poorly understood. For example, several novel contributors to necrotic cell death have been identified in *C. elegans*. In addition, features of neuromuscular degenerative disorders have been successfully reproduced in worms carrying mutations in related genes or expressing human toxic proteins, and in animals treated with environmental toxins. Thus, *C. elegans* is a powerful and versatile platform for the study of the cellular and molecular mechanisms underlying several human pathologies such as excitotoxicity following ischemic episodes.

The cellular demolition processes that do not exclusively display apoptotic or necrotic characteristics such as the linker cell death, which have been described in worms, indicate that there is no clear distinction between different death types. The mechanisms set in motion in each case likely depend on the type of tissue, the physiological conditions, the metabolic status of the cell and its surrounding environment, as well as on the type and the intensity of the insult. For example, mixed apoptotic and necrotic features underlie paraptosis in amyotrophic lateral sclerosis, where cytoplasmic vacuolation and absence of chromatin condensation are accompanied by the activation of apoptotic molecular pathways [145]. A similar combination of death characteristics has been observed in rats subjected to focal ischemia in the cerebral cortex induced by artery occlusion, and in cultured rat neurons exposed to excess glutamate or hypoxia in the presence or absence of excitotoxicity blockers [240-242]. Indeed, excitotoxic stimuli inflict a wide spectrum of cell death types. A continuum of apoptosis-necrosis and apoptosis-autophagocytosis responses occurs after administration of kainic acid in newborn rat forebrain, and also during glutamate-

induced excitotoxicity *in vivo* in adult rat striatum and *in vitro* in organotypic cultures of rat spinal cord motor neurons [243, 244]. Transient focal ischemia in the rat striatum induces necrosis in the striatal core, type II autophagic cell death in the middle part of the striatum and apoptosis in the head of the caudate putamen [245]. Other stimuli like oligomycin A, which inhibits the activity of the mitochondrial ATP-synthase, can also elicit various responses in cultured insect cells, such as excess autophagy, apoptotic death and oncosis (characterized by cell swelling, cytoplasmic vacuolization and increased plasma membrane permeability; thus, resembling necrotic cell death in other systems) [246]. In addition, there is extensive crosstalk between “apoptotic” and “necrotic” pathways and consequently, apoptotic and necrotic mechanisms intermix. Caspases contribute to the disruption of Ca^{2+} homeostasis by degrading membrane Ca^{2+} pumps, caspase-3 derepresses calpains by suppressing the calpain inhibitor calpastain, and cathepsins are able to activate caspase-3 [145]. Caspase-9, which is activated either directly *via* caspase-8-cleavage, or in apoptosomes after the induction of the mitochondrial apoptotic pathway, triggers lysosomal membrane permeability and consequent release of cysteine cathepsins in tumor necrosis factor (TNF)-treated MEFs [247]. Lysosomal rupture and the concomitant release of hydrolytic enzymes may result either in apoptosis or in necrosis depending on the insult intensity and the extent of the cellular damage.

Findings in *C. elegans* implicate autophagy in cell death and provide evidence both for a protective and for a destructive role (Fig. (4)). Impairment of autophagy causes excessive apoptosis leading to embryonic death and accelerated ageing, while its activation facilitates clearance of toxic aggregate-prone proteins, thus promoting survival. By contrast, autophagy downregulation protects cells treated with the neurotoxin 6-OHDA and neurons expressing hyperactive ion channels [159]. Similar data have been obtained in other organisms. Transient upregulation of autophagy in specific tissues directly after birth is required for the survival of neonatal mice [248]. A basal autophagy level is also essential in the nervous system of adult mice to prevent the accumulation of abnormal proteins and non-degradable inclusion bodies implicated in neural degeneration [249, 250]. The autophagic process is also involved in innate and adaptive immune responses, by facilitating killing of invasive pathogens and participating in antigen presentation respectively. By contrast, an active role of autophagy in promoting cell death has been demonstrated in cells defective for apoptosis, after overexpression of Atg1 or Bcl-2 unbound Beclin 1, and in response to interferon- γ (IFN- γ), signals produced during human immunodeficiency virus (HIV) infections and several other apoptotic stimuli [251]. Atg5 appears to function as death-mediator in some of the cases *via* interactions with Fas-associated death domain (FADD) or the Bcl-xL anti-apoptotic protein [252]. The dual role of

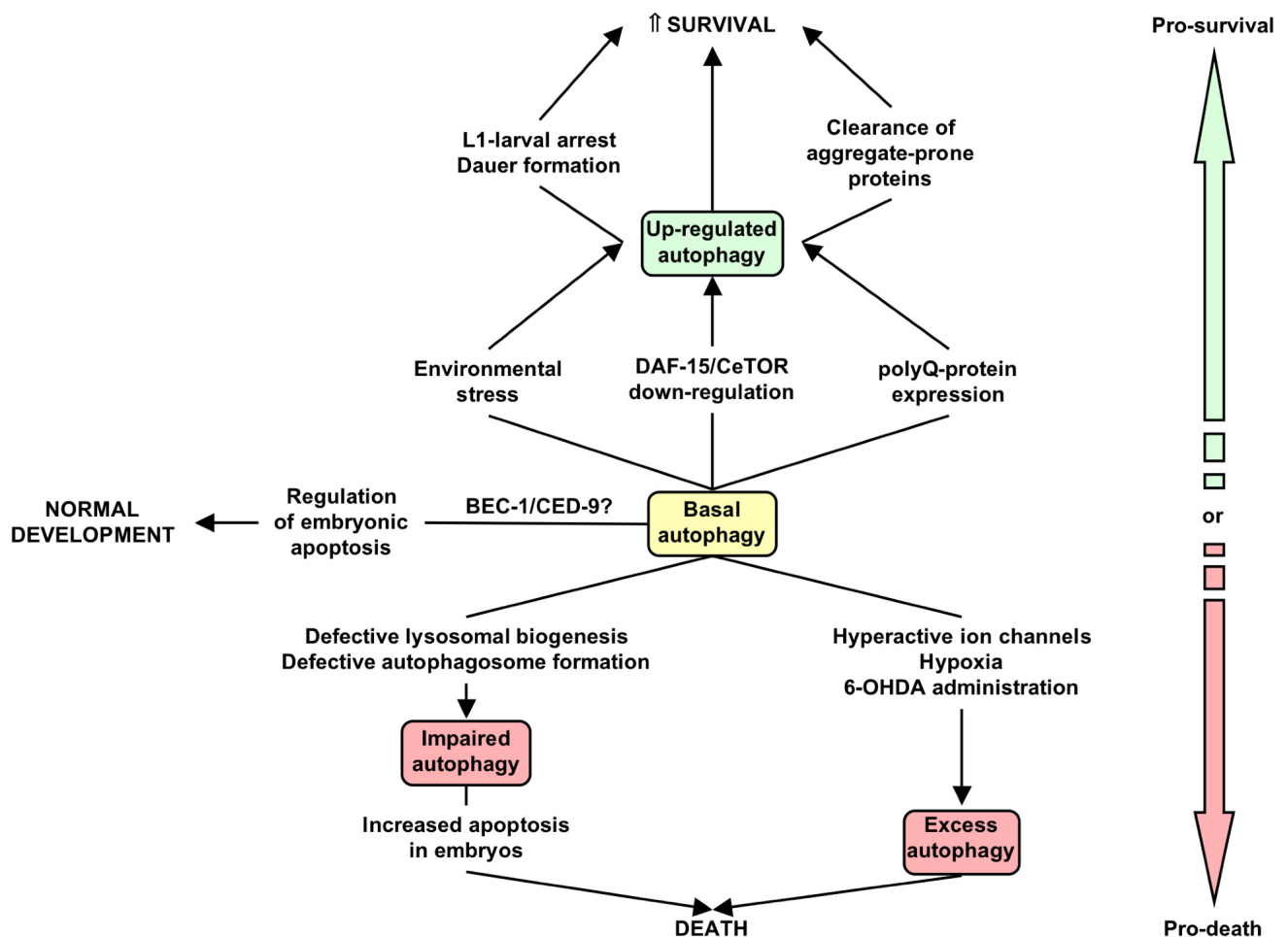


Fig. (4). Autophagy in survival and death. Autophagy is required for normal *C. elegans* development, and its impairment causes excess apoptosis in embryos and embryonic lethality. Autophagy also promotes survival under stress-conditions either by contributing to the formation of stress-resistant dauer larvae under conditions of nutrient shortage, high temperatures and overcrowding, or by facilitating degradation of aggregate-prone proteins. In addition, autophagy upregulation results in life span extension. By contrast, excessive autophagy, induced after exposure to necrotic stimuli such as the neurotoxin 6-OHDA, contributes to cellular demise.

autophagy becomes obvious when examining the function of Beclin 1 in cancer cells [111]. Although Beclin overexpression sensitizes breast cancer cells treated with the vitamin D analog EB1089 to undergo autophagic cell death, its depletion also has a detrimental effect in their survival, causing growth arrest and reduced DNA synthesis.

Autophagy appears to exert primarily a protective function by degrading damaged cellular components and/or by providing an energy source when energy availability is low. Thus, autophagy serves an adaptive function by facilitating survival under adverse conditions, without activating programmed death pathways. However, exposure to very intense stimuli, such as toxins and necrosis-triggering insults, boosts an extreme cellular reaction. Abrupt increase in cytoplasmic calcium levels triggers aberrant simultaneous activation of calcium-dependent molecules and signaling pathways with a detrimental consequence for cells. Under such extreme conditions, excessive autophagy induction would cause uncontrollable degradation or sequestration of cells contents. Thus, autophagy hyperactivation early during necrosis may promote cellular destruction before lysosomal rupture and concomitant release of hydrolytic enzymes.

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ABBREVIATIONS

A β	= Amyloid- β peptide
AD	= Alzheimer's disease
ALS	= Amyotrophic lateral sclerosis
APP	= Amyloid precursor protein
ATG	= Autophagy-related genes
BH3	= Bcl-2 homology region 3
CBP	= CREB binding protein
Ce	= <i>Caenorhabditis elegans</i>
CMA	= Chaperone-mediated autophagy
CREB	= cAMP response element-binding
CVT	= Cytoplasm-to-vacuole targeting
Daf-c	= Dauer-constitutive
DEG	= Degenerin ion channel family
DIC	= Differential interference contrast
dsRNAi	= Double-stranded RNA interference
ER	= Endoplasmic reticulum
ERK	= Extracellular signal-regulated kinase
ESCRT	= Endosomal sorting complex required for transport
FADD	= Fas-associated death domain
FOXO	= Forkhead box O forkhead members of the O class of transcription factors
FTDP-17	= Frontotemporal dementia and parkinsonism-linked to chromosome 17
GABA _A R	= γ -aminobutyric acid receptor
GABARAP	= GABA-receptor associated protein
GAS-1	= General anaesthetic sensitivity abnormal-1
GFP	= Green fluorescent protein
GLA-3	= Germ line apoptosis normal-3
GLR-2	= Glutamate receptor family (AMPA) family member-2
GTP	= Guanosine triphosphate

HAND 1	= Heart- and neural crest derivatives-expressed protein 1
HD	= Huntington's disease
HIV	= Human immunodeficiency virus
HDACs	= Histone deacetylases
Hs	= Homo sapiens
HSNs	= Hermaphrodite specific motoneurons
Htt	= Huntingtin
IFN- γ	= Interferon- γ
Ins(145)P ₃ R	= Inositol-145-triphosphate receptors
JNK	= Jun N-terminal kinase
KA	= Kainic acid
LPS	= Lipopolysaccharide
3-MA	= 3-Methyladenine
MAPK	= Mitogen-activated kinase
MEFs	= Mouse embryonic fibroblasts
MKK7	= Mitogen-activated protein kinase kinase 7
Mm	= Mus musculus
MPP+	= 1-Methyl 4-phenylpyridinium
MPTP	= 1-Methyl 4-phenyl 1236-tetrahydropyridine
NMDA	= N-methyl D-aspartic acid
NOS-1	= Nanos related-1
NSMs	= Pharyngeal neurosecretory motoneurons
6-OHDA	= 6-Hydroxydopamine
PD	= Parkinson's disease
PI3K	= Phosphoinositide 3-kinase
polyQ	= Poly-glutamine
PTEN	= Phosphatase and tensin homolog
Q	= Glutamine
R	= Arginine
RNAi	= RNA interference
ROS	= Reactive oxygen species
RyR	= Ryanodine receptors
SNARE	= SNAP receptors
TGN	= Trans-Golgi network
TNF	= Tumor necrosis factor
TOR	= Target of rapamycin
V-ATPase	= Vacuolar proton ATPase
VPCs	= Vulva precursor cells
VPS	= Vacuolar protein-sorting

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