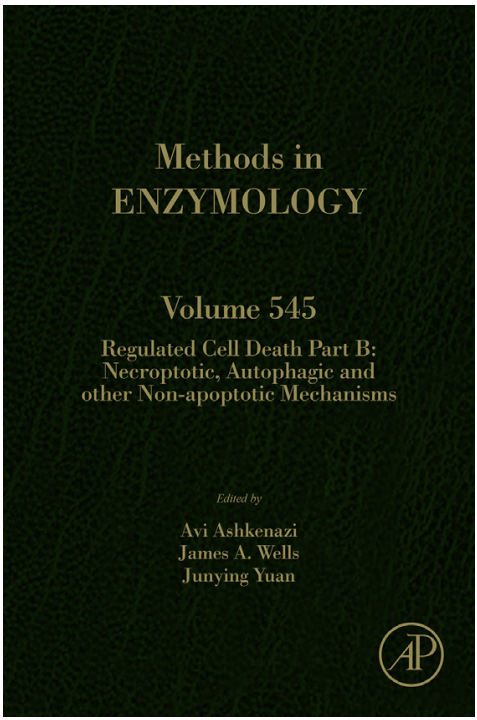

From Vassiliki Nikoletopoulou, Nektarios Tavernarakis, Necrotic Cell Death in *Caenorhabditis elegans*. In Avi Ashkenazi, James A.Wells, Junying Yuan editors: *Methods in Enzymology*, Vol. 545, Burlington: Academic Press, 2014, p. 127-155.
ISBN: 978-0-12-801430-1

Academic Press
Elsevier

CHAPTER SIX

Necrotic Cell Death in *Caenorhabditis elegans*

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Contents

Abstract

Similar to other organisms, necrotic cell death in the nematode *Caenorhabditis elegans* is manifested as the catastrophic collapse of cellular homeostasis, in response to overwhelming stress that is inflicted either in the form of extreme environmental stimuli or by intrinsic insults such as the expression of proteins carrying deleterious mutations. Remarkably, necrotic cell death in *C. elegans* and pathological cell death in humans share multiple fundamental features and mechanistic aspects. Therefore, mechanisms mediating necrosis are also conserved across the evolutionary spectrum and render the worm a versatile tool, with the capacity to facilitate studies of human pathologies. Here, we overview necrotic paradigms that have been characterized in the nematode and

Methods in Enzymology, Volume 545
ISSN 0076-6879
http://dx.doi.org/10.1016/B978-0-12-801430-1.00006-8

outline the cellular and molecular mechanisms that mediate this mode of cell demise. In addition, we discuss experimental approaches that utilize *C. elegans* to elucidate the molecular underpinnings of devastating human disorders that entail necrosis.

1. INTRODUCTION

1.1. Characteristics of necrotic cells

Early studies in the field of cell death described two major forms of cellular demise, apoptosis and necrosis, and contrasted them as being diametrically different in every aspect examined (Walker, Harmon, Gobe, & Kerr, 1988). Apoptosis, also known as caspase-dependent programmed cell death (PCD), was described as a controlled cell death process, proposed to function as a tissue homeostatic mechanism that is complementary and opposite to cell division (Kerr, Wyllie, & Currie, 1972). Necrosis was classically contrasted to apoptosis not only on grounds of context and mechanistic regulation or lack thereof but also based on notable morphological differences. The apoptotic cell profile is characterized by cell rounding, detachment from the basal membrane or cell culture substrate, chromatin condensation and nuclear fragmentation, blebbing of the plasma membrane, and shedding of vacuoles known as apoptotic bodies (Galluzzi et al., 2007). Necrotic cells were initially characterized in a negative fashion, exhibiting neither an apoptotic morphological profile nor an extensive vacuolization characteristic of autophagic cell death. However, specific morphological features were soon attributed to necrotic cells. These included an increasingly translucent cytoplasm, osmotic swelling of most organelles, increased cell volume, and finally rupture of the plasma membrane. The morphological profiles of apoptotic, necrotic, and autophagic cells are shown in Fig. 6.1. Notably, unlike apoptosis, necrosis does not feature major nuclear modifications but only minor ultrastructural changes. Moreover, necrotic cells do not fragment into distinct corpses as their apoptotic counterparts do (Galluzzi et al., 2007).

At the organismal level, a recent study demonstrated that necrotic death is accompanied by a burst of intense blue fluorescence immediately after the worms succumb to the necrotic stimuli. Such death fluorescence marks an anterior to posterior wave of intestinal cell death that is accompanied by cytosolic acidosis. This wave is propagated via the innexin INX-16, likely by calcium influx. Notably, inhibition of systemic necrosis can delay stress-induced death. Initially present in intestinal lysosome-related organelles (gut granules), the fluorescent substance was identified as anthranilic

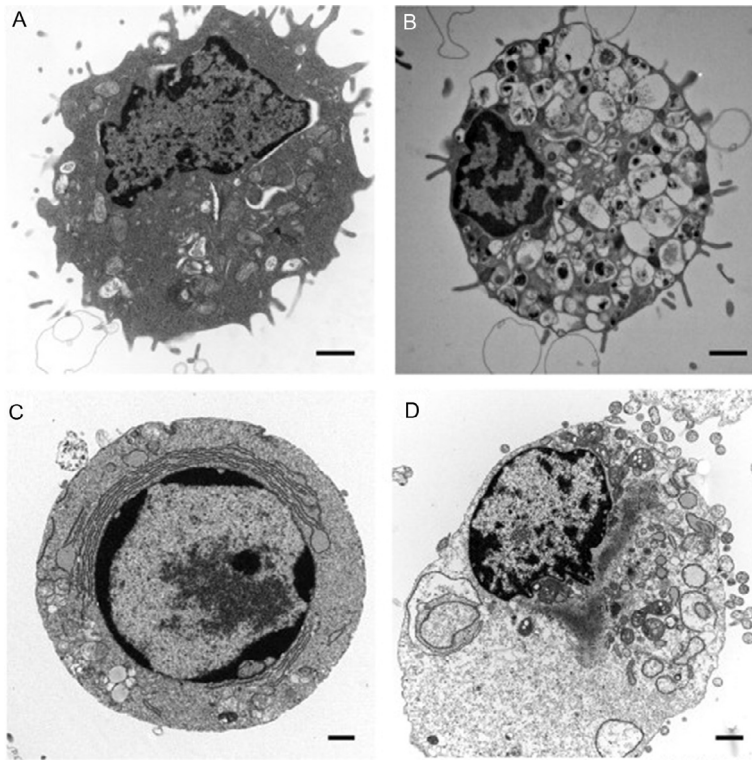

Figure 6.1 Morphological features of autophagic, apoptotic, and necrotic cells. (A) Normal, (B) autophagic, (C) apoptotic, (D) and necrotic cells. *Reprinted from Edinger and Thompson (2004), copyright (2004), with permission from Elsevier.*

acid glucosyl esters, derived from tryptophan by action of the kynurenine pathway (Coburn et al., 2013).

1.2. *Caenorhabditis elegans* as a model to study necrosis

Caenorhabditis elegans has been instrumental in deciphering both apoptotic and necrotic cellular programs. This can be largely attributed to the specific characteristics and well-described developmental stages of this nematode, which make it exceptionally well suited for the study of both normal and aberrant cell death at the cellular, genetic, and molecular level. Due to its transparency, the visualization and tracking of single cells as well as of individual nuclei is readily feasible by differential interference contrast optics, enabling researchers to follow somatic cell divisions from the fertilized egg all the way to the 959 cell adult hermaphrodite (Sulston & Horvitz,

1977; Sulston, Schierenberg, White, & Thomson, 1983). The resulting cell lineage map indicated early on that in certain lineages, particular divisions generate cells which are destined to die at specific times and locations that remain faithfully invariant from one animal to another. Exactly 131 somatic cells die every time the fertilized egg normally develops into the adult animal, by an apoptotic PCD process.

1.3. The apoptotic machinery in *C. elegans*

Genetic and molecular studies performed in *C. elegans* provided a fundamental insight into the mechanisms underlying this cell death process. In the 131 cells destined to die during development, the level of EGL-1, a BH3 domain protein, is increased. EGL-1 interacts with a protein complex composed of CED-9 (similar to the mammalian B-cell lymphoma protein 2) and CED-4 (similar to the mammalian apoptotic protease-activating factor 1), releasing CED-4 which in turn activates CED-3 (similar to human caspases) (Hengartner, 2000). In *C. elegans*, four caspase-related genes exist: *ced-3*, *csp-1*, *csp-2*, and *csp-3* (Shaham, 1998; Yuan, Shaham, Ledoux, Ellis, & Horvitz, 1993); however, only *ced-3* seems to be required for PCD (Abraham & Shaham, 2004; Yuan et al., 1993), and only ced-3 and *csp-1* are proteolytically active (Shaham, 1998). The CSP-2 caspase lacks key active-site residues, and *csp-3* encodes only a C-terminal caspase domain, entirely lacking the active site (Shaham, 1998). As it turns out, the genetic encoding for the regulation and execution of developmental apoptosis has been remarkably conserved between *C. elegans* and mammals.

2. NECROTIC CELL DEATH PARADIGMS DURING *C. ELEGANS* DEVELOPMENT

The identification of the caspase CED-3 as a key regulator of apoptosis has been a key contribution of *C. elegans* to the cell death field, as caspases also play crucial roles in the execution of PCD across many species. However, as it turns out, not quite all cell death events during *C. elegans* development follow the typical apoptotic pathway that involves CED-4 and CED-3. Below, we elaborate on some well-studied examples of developmental death in the nematode that follow a necrotic pathway.

2.1. Death of the linker cell

The *C. elegans* linker cell has been described as an example of death that occurs during development following a mode that is independent of

CED-4 and CED-3 (Horvitz, Sternberg, Greenwald, Fixsen, & Ellis, 1983). The linker cell is born during the second larval stage (L2) in the central region of the animal and follows a stereotypical path of migration. As the cell migrates, it leads the extension of the male gonad behind it (Kimble & Hirsh, 1979; Sulston, Albertson, & Thomson, 1980), and upon completion of its migratory route, it is positioned between the gonad (vas deferens) and the cloacal tube, serving as an exit channel for sperm in the adult. It is generally thought that the death and removal of the linker cell around the L4/adult transition facilitates the fusion between the vas deferens and cloaca, to connect the male reproductive system to the exterior.

Following up on early observations that the programmed death of the linker cell persists even in *ced-3* mutant animals, the fate of this cell was thoroughly studied by following a GFP-marked linker cell in animals harboring mutations in core genes of the apoptotic machinery, such as *ced-3* and *ced-4*, as well as in engulfment genes. These studies demonstrated that the linker cell dies in a cell autonomous manner that, unlike it was postulated by previous reports (Sulston et al., 1980), does not require extrinsic signals from engulfing or other cells. Moreover, they showed that this death event is independent of any known apoptotic genes, in line with the lack of apoptotic morphological features, such as chromatin condensation. Instead, there was a noted presence of swollen and degraded mitochondria within large multilayered membrane-bound structures, as well as small electron-translucent "empty" membrane-bound cytoplasmic structures that resembled vacuoles typically seen during necrotic cell death in *C. elegans* (Hall et al., 1997) (Fig. 6.2). Although linker cell death does not satisfy all classical criteria of necrotic death, it is even further away from classical apoptotic paradigms. Possibly, the death of the linker cells falls under the characteristics of more recently described programmed necrosis processes, also known as necroptosis. However, additional experiments would be required to test this hypothesis and to further characterize the precise mode of death of the linker cell.

2.2. Death of mis-specified uterine–vulval (uv1) cells

A robust example of a necrotic event during development is the demise of mis-specified uterine–vulval (uv1) cells that have an important role in egg laying. Egg laying in *C. elegans* requires a connection between the lumens of the uterus in the somatic gonad and the vulva in the extragonadal epithelium, facilitated by cell–cell interactions between gonadal and vulval cells. Two specialized cell types of the ventral uterine π lineage are integral

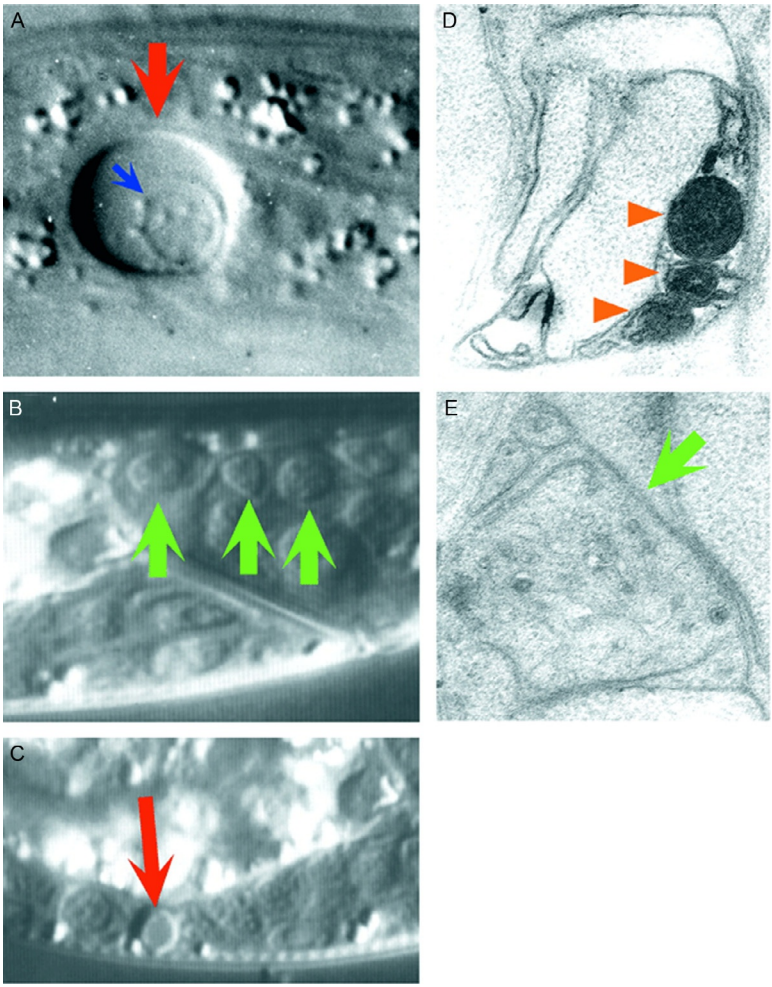

Figure 6.2 Necrotic cell death in *C. elegans*. The most prominent morphological characteristic of necrosis is the outstretched swelling of the cell to several times its normal diameter, which is manifested by a hollow, vacuole-like appearance under the optic microscope. For example, a dying PVM (posterior ventral microtubule) touch receptor is shown in (A) by a red arrow. This neuron is expressing a toxic variant of the degenerin MEC-4 (mechanosensory) protein that induces necrosis. The nucleus follows the cellular expansion (A; blue arrow). Healthy cells are indicated by green arrows for comparison (B). In sharp contrast, apoptosis, which normally occurs during nematode development, generates retractile cell corpses, compact in size, with a characteristic button-like appearance (C; red arrow). Under the electron microscope, the same degenerating neuron exhibits dark, electron-dense formations, most likely originating from plasma membrane-internalized material, arranged in onion-like concentric circles (D; arrowheads). At later stages of degeneration, the cytoplasm of the dying cell appears extensively depredated and fragmented. A normal neuron is shown in (E) by a green arrow. *Reprinted from Syntichaki and Tavernarakis (2002), copyright (2002), with permission from Nature Publishing Group.*

components of the uterine–vulval connection. These are the syncytial uterine seam (utse) cell, which overlies the vulval lumen, and the four uterine–vulval (uv1) cells, which directly contact the most dorsal vulval cell vulF (Newman, White, & Sternberg, 1996). The temporal and spatial specification of both these cell types largely relies on a specific signaling axis, where an inductive LIN-3 epidermal growth factor (EGF) signal derived from a single gonadal cell called the anchor cell activates the LET-23 EGF receptor on the receiving vulval precursor cells (Aroian, Koga, Mendel, Ohshima, & Sternberg, 1990; Hill & Sternberg, 1992). Mutations in genes of the LIN-3/LET-23/Ras signaling pathway compromise uv1 fate specification. A key study (Huang & Hanna-Rose, 2006) described the isolation of the *cog-3*(*ku212*) mutant, which uncouples gonadogenesis from its normal progression relative to the development of the vulva and shares phenotypes with heterochronic mutations that disturb the temporal coordination of vulval and uterine development. In *cog-3*(*ku212*) mutants, the entire uterus, including the pre-uv1 cells, is generated at a later stage of vulval development than is normal. Notably, the delayed pre-uv1 cells subsequently die by necrosis, leading to the absence of uv1 cells in the adult stage. Moreover, the study investigated if a LIN-3/LET-23/Ras signaling defect underlies the necrosis of uv1 defect in *cog-3*(*ku212*) mutants, by analyzing *cog-3*(*ku212*) double mutants with a gain-of-function allele of *let-23*. The results indicated that the *let-23*(*gf*) mutation rescued the mis-specification and death phenotype of uv1 cells, suggesting that the necrotic program is recruited during development in response to uncoordinated spatiotemporal development.

A recent study revealed the involvement of the *ku212* allele in uv1 cell necrosis, which maps to the *pnc-1* gene locus, encoding a nicotinamidase (van der Horst, Schavemaker, Pellis-van Berkel, & Burgering, 2007; Vrablik, Huang, Lange, & Hanna-Rose, 2009). Nicotinamidases are the first enzymes of the NAD^+ salvage pathway in invertebrates, using nicotinamide (NAM) as a substrate (Magni, Amici, Emanuelli, Raffaelli, & Ruggieri, 1999). Administration of high levels of NAM causes uv1 cells to die by necrosis at high frequency in wild-type animals. Thus, instead of compromised EGF signaling, the necrotic death of uv1 cells in *pnc-1* mutants may result from accumulation of the substrate NAM. In addition, the gonad-defective and uv1 cell death phenotypes are separable in *pnc-1* mutants. Constitutively active LET-23/EGF receptor prevents NAM-induced uv1 necrotic cell death, suggesting that EGF signaling may provide a survival cue that rescues uv1 cells from NAM-induced necrosis (reviewed in Vlachos & Tavernarakis, 2010).

3. NONDEVELOPMENTAL NECROTIC DEATH

In the adult nematode, necrotic cell death can be triggered by a wide variety of both extrinsic and intrinsic signals (Walker et al., 1988). Several well-defined conditions are known to trigger necrotic cell death in *C. elegans* and will be discussed below. The best-characterized case is the gain-of-function mutations in several ion channel genes, which result in an ionic imbalance and inflict a necrotic pattern of death on neurons. Other stimuli include extreme heat, hypo-osmotic shock, and bacterial infections. Cell demise in all these paradigms is accompanied by characteristic morphological features of necrosis, starting with the appearance of a distorted nucleus and cell body during the early phase of death. Gradually, the cell swells to several times its normal diameter and small, tightly wrapped membrane whorls form, originating from the plasma membrane and coalescing into large, electron-dense membranous structures (Hall et al., 1997). Interestingly enough, these membranous inclusions also represent characteristic hallmarks in mammalian neurodegenerative disorders, such as in neuronal ceroid lipofuscinosis (Batten's disease; the *mnd* mouse) as well as in the wobbler mouse, a model of amyotrophic lateral sclerosis (Blondet, Carpentier, Ait-Ikhlef, Murawsky, & Rieger, 2002; Cooper, Messer, Feng, Chua-Couzens, & Mobley, 1999).

3.1. Cell death induced by ionic imbalance

The most extensively characterized paradigm of non-PCD in adult *C. elegans* animals is the necrosis of cells expressing aberrant ion channels harboring unusual gain-of-function mutations (Syntichaki & Tavernarakis, 2003).

3.1.1 Degenerins

Dominant mutations in *deg-1* (degenerin; *deg-1(d)*) induce death of a group of interneurons of the nematode posterior touch sensory circuit (Chalfie & Wolinsky, 1990). Similarly, dominant mutations in the *mec-4* gene (mechanosensory; *mec-4(d)*) induce degeneration of six touch receptor neurons required for the sensation of gentle touch to the body (Syntichaki & Tavernarakis, 2004).

deg-1 and *mec-4* encode proteins that are very similar in sequence and were the first identified members of the *C. elegans* "degenerin" family, so named because several members can mutate to forms that induce cell degeneration (Chalfie, Driscoll, & Huang, 1993). Degenerins bear sequence

similarity to mammalian epithelial sodium channels (ENaCs). The time of degeneration onset correlates with the initiation of degenerin gene expression, and the severity of cell death is analogous to the dose of the toxic allele (Hall et al., 1997). Expression of mammalian homologous proteins, carrying amino acid substitutions analogous to those of toxic degenerins, leads to degeneration of cells in a manner reminiscent of necrotic cell death in *C. elegans*. Additional members of the degenerin family are *mec-10*, which can be engineered to encode toxic degeneration-inducing substitutions, *unc-8*, which can mutate to a semi-dominant form that induces swelling and dysfunction of ventral nerve cord and *unc-105*, which appears to be expressed in muscle and can mutate to a semi-dominant form that induces muscle hypercontraction (Syntichaki & Tavernarakis, 2004). Thus, a unifying feature of degenerin family members is that specific gain-of-function mutations have deleterious consequences for the cells in which they are expressed, which, at least in neurons, culminate into a necrotic cell death event.

C. elegans degenerins share sequence similarity with *Drosophila* ripped pocket and pickpocket, with subunits of the vertebrate amiloride-sensitive ENaC and with other neuronally expressed ion channels. Together, these proteins define the DEG/ENaC protein superfamily (Tavernarakis & Driscoll, 2001). Although mutant degenerins can kill different groups of neurons depending on their expression patterns, the morphological features of the cell death that they induce are the same and resemble those of mammalian cells undergoing necrotic cell death. The pattern of necrotic cell death inflicted by degenerins is not a peculiarity of this gene class. For example, *C. elegans deg-3*, whose product is related to the vertebrate α-7 nicotinic acetylcholine receptor (nAChr) and together with the related protein DES-2 forms a very efficient calcium channel, can mutate to induce necrotic cell death similar to that induced by degenerins (Treinin, Gillo, Liebman, & Chalfie, 1998). In addition, mutant-activated forms of the heterotrimeric G-protein α subunit ($G\alpha_s$ Q208L), from both *C. elegans* and rat, cause swelling and degeneration of many cell types when expressed in *C. elegans* (Berger, Hart, & Kaplan, 1998; Korswagen, Park, Ohshima, & Plasterk, 1997).

3.1.2 Other ion channels

In addition to degenerins, gain-of-function mutations in other ion channel genes such as deg-3 lead to vacuolar degeneration of various types of *C. elegans* neurons. deg-3 encodes an acetylcholine receptor ion channel,

related to the vertebrate nAChr that participates in the formation of a channel highly permeable to Ca^{2+} (Treinin & Chalfie, 1995). Moreover, expression of a constitutively active form of a heterotrimeric G-protein α subunit $G\alpha_s$ results in degeneration of a specific subset of neurons. Genetic suppressor analysis identified an adenyl cyclase as a downstream effector of $G\alpha_s$-induced neurodegeneration, indicating that cAMP signaling is critical for degeneration (Berger et al., 1998; Korswagen, van der Linden, & Plasterk, 1998).

Ionic imbalance and subsequent necrotic cell death induced by aberrant ion channel function in *C. elegans* is mechanistically and morphologically similar to excitotoxicity in vertebrates. Excitotoxic cell death is prevalent during stroke, where the energy required for sustaining ionic gradients and the resting potential of neurons is lost. Because membrane potential collapses, massive amounts of the excitatory neurotransmitter glutamate are released at synaptic clefts (Kauppinen, Enkvist, Holopainen, & Akerman, 1988; Kauppinen, McMahon, & Nicholls, 1988). Energy depletion also prevents reuptake of glutamate by dedicated transporters leading to accumulation of glutamate at synapses, hyperexcitation, and eventually necrotic death of downstream synaptic target neurons. Excitotoxicity is critically dependent on Ca^{2+} influx through glutamate-gated receptor ion channels (reviewed in Kourtis & Tavernarakis, 2007).

Malfunction of glutamate transporters and the resulting accumulation of glutamate are known to trigger excitotoxicity in several neurodegenerative diseases (Cleveland & Rothstein, 2001). However, the details on the cascade of events leading to neurodegeneration remain unclear. The molecular components of glutamatergic synapses assembled in *C. elegans* are highly conserved from nematodes to humans. A recent study describes a novel paradigm for nematode excitotoxicity, by investigating the *in vivo* effects of multiple mediators of glutamate-induced neuronal necrosis (Mano & Driscoll, 2009). Combined Δ*glt-3* glutamate transporter-null mutations and expression of a constitutively active form of the α subunit of the G-protein $G\alpha_s$ induces extensive neurodegeneration in head interneurons. Δ*glt-3*-dependent neurodegeneration acts through Ca^{2+}-permeable Glu receptors of the α-amino-3-hydroxyl-5-methyl-4-isoxazolepropionic acid subtype, requires calreticulin function, and is modulated by calcineurin and type-9 adenylyl cyclase (AC9). This glutamate-dependent toxicity defines a novel necrotic death paradigm in *C. elegans* that shares many basic features with excitotoxicity in mammalian neurons and may potentially be operative also in higher organisms.

3.2. Heat-induced necrotic death

Climate change has brought about a dramatic increase in the cases of heat stroke and related pathologies in humans. Causing core body temperature to reach over 40 °C, heat stroke inflicts immediate devastating tissue damage and inflammatory response that can be fatal, as well as long-term defects. To gain insight into the molecular mechanisms of heat cytotoxicity and to circumvent the confounding influence of secondary physiological and inflammatory responses, our laboratory developed and characterized a genetically tractable model of heat stroke in *C. elegans*. Widespread cell death across several tissues could be observed in animals exposed to hyperthermia, which in the nematode was simulated by a short exposure to 39 °C (Kourtis, Nikoletopoulou, & Tavernarakis, 2012). Dying cells displayed morphological features characteristic of necrosis, expressed markers of necrotic death, and became permeable to propidium iodide. Moreover, depletion of proteins required for necrosis strongly facilitated survival after heat stroke. In contrast, loss of key mediators and core components of the apoptotic or autophagic machineries did not suppress heat-stroke-induced cell death. Thus, heat stroke compromises viability by triggering extensive necrotic cell death and represents a newly added necrotic cell paradigm in the nematode.

Notably, we also observed that preconditioning animals at an intermediate, nonlethal temperature markedly enhanced their capacity to withstand a subsequent heat stroke. This protective effect is in line with the previously described phenomenon of hormesis (Calabrese, 2004), where preexposure to mild stress elicits increased resistance to subsequent severe stress. It is also worth noting that in addition to heat stroke, heat preconditioning conferred resistance against a wide range of necrotic death insults, including in particular ionic imbalance paradigms (discussed earlier), overexpression of aggregation-prone proteins (such as α-synuclein), and hypoxic conditions. In the case of hormesis by heat preconditioning, we found that cytoprotection is orchestrated at the molecular level by the hermetic induction of a single sHSP, HSP-16.1. sHSPs assemble into oligomeric complexes and serve as molecular chaperones, efficiently binding denatured proteins and/or preventing irreversible protein aggregation and insolubilization (Van Montfort, Slingsby, & Vierling, 2001). HSP-16.1 localizes in the Golgi, where it functions together with the PMR-1 pump to prevent cytoplasmic Ca^{2+} overload under extreme stress. We propose that HSP-16.1 contributes to stabilize and protect the stress-labile PMR-1 pump, allowing for efficient clearance of Ca^{2+} from the cytoplasm, after necrotic insult (Fig. 6.3).

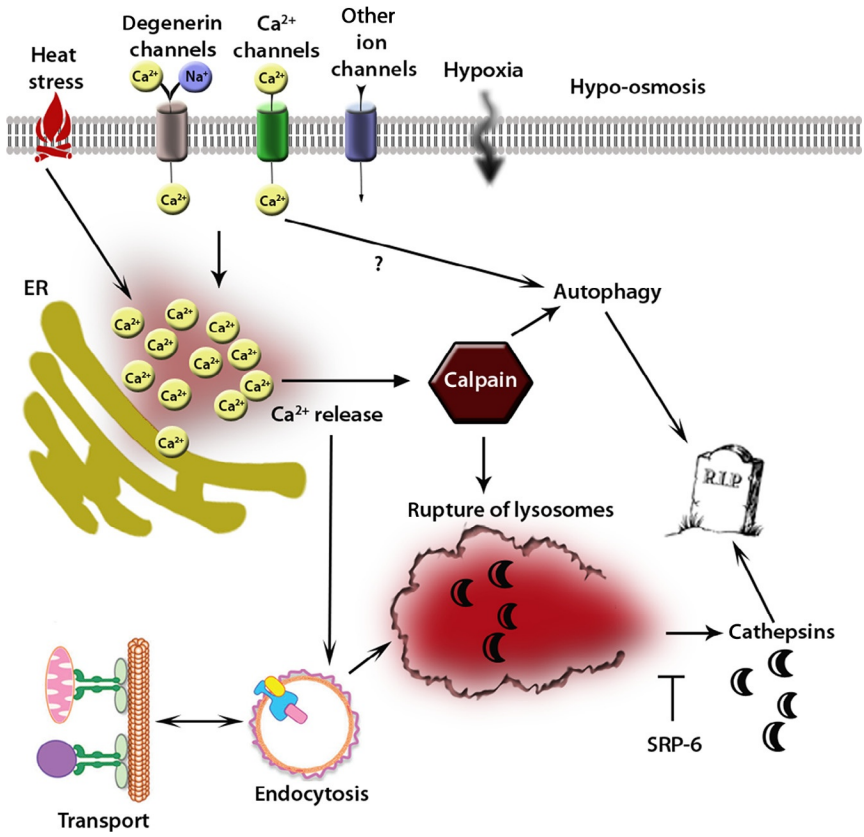

Figure 6.3 Necrotic cell death mechanisms. Various types of extrinsic necrotic insults converge on increased intracellular Ca^{2+} levels, caused by increased influx from extracellular pools through plasma membrane channels, or by Ca^{2+} efflux from intracellular stores, such as the endoplasmic reticulum (ER). Ca^{2+} then activates calpain proteases in the cytoplasm that attack lysosomal membrane proteins to compromise lysosomal integrity. Rupture of the lysosomes follows, and release of hydrolytic enzymes such as cathepsin proteases. In addition, autophagy is induced during necrosis, either directly by Ca^{2+} or via calpains and also contributes to cellular destruction. Moreover, both clathrin-mediated endocytosis and intracellular transport are required for necrotic death and are induced by necrosis-triggering insults.

Importantly, mammalian PMR-1 is selectively impaired during ischemic or reperfusion brain injury (Gidday, 2006; Lehotsky, Kaplan, Murin, & Raeymaekers, 2002; Pavlikova et al., 2009). Given the strong evolutionary conservation of the proteins involved, this mechanism is probably relevant to related human pathologies. Relevant to that, we also demonstrated that heat stroke induces widespread necrotic death in mammalian neurons,

which can be largely prevented by heat preconditioning. Moreover, hormesis in mammalian neurons in response to heat preconditioning also requires the function of PMR-1 and is mediated by the same molecular players as in the nematode.

3.3. Bacterial infection-induced necrosis

Infection of *C. elegans* with different bacterial pathogens has been shown to induce necrotic death of intestinal cells as part of a pathogen-shared response to infection (Wong, Bazopoulou, Pujol, Tavernarakis, & Ewbank, 2007). Using whole-genome microarrays representing 20,334 genes, this study analyzed the transcriptional response of *C. elegans* to four bacterial pathogens (*Serratia marcescens*; *Enterococcus faecalis*; *Erwinia carotovora*; *Photorhabdus luminescens*). Different bacteria provoked pathogen-specific signatures within the host, including genes that encode potential pathogen-recognition and antimicrobial proteins. Additionally, variance analysis also revealed a robust signature that was commonly elicited by the pathogens. This involved 22 genes associated with proteolysis, necrotic cell death, and stress responses. Necrosis aggravated the pathogenesis and accelerated the death of the host. At later stages of infection, necrotic vacuoles are also observed in epidermal and gonadal cells. Moreover, mutations in genes required for necrosis ameliorated the consequences of infection, suggesting that necrosis is an integral part of host–pathogen interaction that contributes to the pathology associated with infection in *C. elegans*. These results are the first indication that necrosis is important for disease susceptibility in *C. elegans*. Moreover, given the striking similarities between the innate immune systems of invertebrates and vertebrates, as well as the fact that necrosis has been implicated in infections of human tissues, these findings invite the possibility to employ *C. elegans* as a model for the study of innate immunity in humans in response to infections.

3.4. Hypo-osmotic shock-induced cell death

Lysosomal integrity and lysosomal proteolytic mechanisms are key factors modulating necrotic cell death in the nematode. Serpins are extracellular or intracellular regulators of proteolytic pathways and inhibitors of multiple peptidases (Silverman et al., 2001). One of the functions of intracellular serpins is the inhibition of lysosomal cysteine peptidases. SRP-6 is such an intracellular serpin in *C. elegans*. *srp-6* null mutants experiencing hypo-osmotic conditions die rapidly and display marked increase of necrotic cell death

of the intestinal epithelium (Luke et al., 2007). Ca^{2+} release from endoplasmic reticulum (ER) stores, together with other factors, induces calpain-mediated lysosomal rupture and massive release of lysosomal peptidases into the cytoplasm that mediate necrotic cell death. In addition to hypo-osmotic conditions, *srp-6* null mutants are susceptible to other stressors such as thermal and oxidative stress, hypoxia, and channel hyperactivity. SRP-6 appears to protect cells from lysosomal rupture and also ameliorates the deleterious consequences of lysosomal rupture triggered by various stressors. The protective function of SRP-6 may be adaptive by enhancing the degradation of misfolded proteins or by aiding cytoskeletal rearrangements through altering lysosomal membrane permeability and allowing the leakage of small amounts of peptidases. In the absence of SRP-6, the uncontrolled release of these peptidases leads to necrotic cell death.

4. EXECUTION OF NECROSIS

Intracellular calcium overload through different sources is considered as one of the leading steps in the necrotic pathway. Calcium may enter the cell through voltage-gated channels and the Na^+/Ca^{2+} exchanger and mutations that increase sodium influx augment calcium entry through these paths. The main intracellular compartment for calcium storage is the ER (Mattson et al., 2000; Paschen, 2001; Paschen & Frandsen, 2001), where calcium is sequestered by the sarcoendoplasmic reticulum Ca^{2+}-ATPase and is released back to the cytoplasm by ryanodine and inositol-1,4,5-triphosphate receptors. In *C. elegans*, extensive genetic screens for suppressors of *mec-4*(*d*)-induced necrosis have identified genes required for the execution of necrotic cell death. Two of these genes encode the calcium-binding chaperones calreticulin and calnexin, which were found to regulate intracellular calcium levels and to be required for necrotic cell death (Xu, Tavernarakis, & Driscoll, 2001). Moreover, treatment of animals with thapsigargin, a drug that induces release of calcium from the ER to the cytoplasm, triggers necrotic cell death, whereas pharmacological treatments or genetic mutations that inhibit calcium release from the ER have a strong protective effect against necrotic cell death.

Genetic studies in *C. elegans* have also shown that in addition to calcium homeostasis, intracellular pH is also an important modulator of necrotic cell death. Cytoplasmic acidification occurs during necrosis, whereas the vacuolar H^+-ATPase, which is a pump that acidifies lysosomes and other intracellular organelles, is required downstream of cytoplasmic calcium overload

to promote necrotic cell death (Syntichaki, Samara, & Tavernarakis, 2005). In line with this, reduced vacuolar H^+-ATPase activity or alkalization of acidic endosomal/lysosomal compartments by weak bases has a neuroprotective role against necrosis. Acidic conditions are required for full activity of cathepsins, aspartyl proteases that are primarily confined to lysosomes and other acidic endosomal compartments (Ishidoh & Kominami, 2002).

Lysosomal as well as cytoplasmic proteases have been implicated as downstream effectors of cellular destruction in necrosis. Calpains are cytoplasmic, papain-like cysteine proteases that depend on calcium for their activity. Under normal conditions, calpains function to mediate essential signaling and metabolic processes. However, during the course of necrotic cell death, these proteases localize onto lysosomal membranes and may compromise lysosomal integrity, thereby causing leakage of their acidic contents, including lysosomal proteases, into the cytoplasm (Yamashima, 2004). In primates, calpains rapidly localize to lysosomal membranes after the onset of ischemic episodes (Yamashima, 2000). In *C. elegans*, two specific calpains—TRA-3 and CLP-1—and two lysosomal cathepsin proteases—ASP-3 and ASP-4—are required for neurodegeneration (Syntichaki, Xu, Driscoll, & Tavernarakis, 2002). It is likely that ensuing cytoplasmic acidification, activation of the lysosomal, low-pH-dependent cathepsins and hydrolases contributes to cell demise. Mutations that interfere with lysosomal biogenesis and function influence necrotic cell death. For example, necrosis is exacerbated in mutants that accumulate abnormally large lysosomes, whereas impairment of lysosomal biogenesis protects from cell death (Artal-Sanz, Samara, Syntichaki, & Tavernarakis, 2006). Interestingly, lysosomes appear to coalesce around the nucleus and dramatically enlarge during early and intermediate stages of necrosis. In advanced stages of cell death, GFP-labeled lysosomal membranes fade, as lysosomes rupture.

In a recent study from our laboratory, we utilized well-characterized necrosis models in *C. elegans* to dissect the involvement of clathrin-mediated endocytosis and intracellular trafficking by kinesin motor proteins in cellular destruction during necrotic death (Troulinaki & Tavernarakis, 2012). Our findings revealed for the first time that both clathrin-mediated endocytosis and intracellular trafficking are required for the execution of necrosis in the nematode. Downregulation of endocytosis or kinesin-mediated trafficking by interfering with key proteins regulating these processes, including SNT-1, endophilin (UNC-57), AP180 (UNC-11), synaptojanin (UNC-26), heavy chain of kinesin 1 (UNC-116), and the monomeric kinesin UNC-104, significantly suppresses neurodegeneration induced by hyperactive ion

channels without affecting the expression, the localization, or the function of the toxic insults.

Moreover, using the same well-defined necrotic cell paradigm, we assayed animals that were deficient for both autophagy and endocytosis and observed significant synergistic protection against degeneration. These results suggest that autophagy and endocytosis function in parallel to contribute to necrotic cell death (Troulinaki & Tavernarakis, 2012).

5. *C. ELEGANS* AS A MODEL FOR HUMAN DISEASES ENTAILING NECROSIS

Nematode genes and major signaling pathways show significant conservation during evolution and more than 50% of the *C. elegans* genes have counterparts in humans. In addition to its contribution in elucidating developmental processes, the worm has also served as a platform to model many human pathological conditions such as neurodegenerative disorders, cancer, aging, and associated diseases (Baumeister & Ge, 2002; Lee, Goedert, & Trojanowski, 2001; Poulin, Nandakumar, & Ahringer, 2004). Systematic mapping of gene interactions and signaling pathways implicated in human disease using *C. elegans* has provided better understanding of complex pathologies (Bussey, Andrews, & Boone, 2006). The ability to produce "humanized" worms, which express human genes not present in the *C. elegans* genome, has further enhanced the experimental value of the nematode by allowing the dissection of molecular mechanisms relevant to human disorders. In addition, the ease of drug testing coupled with the efficiency of genetic screens in worms has made *C. elegans* a favorable tool for the identification and validation of novel drugs and drug targets, aiming to battle human pathological conditions (Kaletta & Hengartner, 2006). Here, we overview *C. elegans* models of human diseases that entail necrosis, focusing on hypoxia, Parkinson's disease, and tauopathies. Clearly, this list is only indicative of the applications of *C. elegans* in understanding complex human pathologies that involve necrotic death, and many more such diseases that are not mentioned here have been usefully modeled in the nematode.

5.1. Hypoxia

In humans, oxygen deprivation induces cell death in pathological conditions such as stroke and heart attack. In *C. elegans*, hypoxia inflicts necrotic death in a variety of cell types (Scott, Avidan, & Crowder, 2002). Interestingly, mutations in the *daf-2* gene, which encodes the *C. elegans* insulin/IGF receptor tyrosine kinase, confer resistance against hypoxic cell death.

DAF-2 is also known to regulate aging and dauer formation in *C. elegans* (Libina, Berman, & Kenyon, 2003). Related to this, many human neurodegenerative disorders show a late-onset pathogenesis, indicating that aging may alter the vulnerability of cells to various insults. However, while hypoxia resistance in *C. elegans* appears to be modulated by insulin signaling, other *daf-2* mutations that affect longevity and stress resistance do not affect hypoxic death. Selective expression of wild-type *daf-2* in neurons and muscles restores hypoxic death in *daf-2* hypoxia-resistant mutants, demonstrating a role of the insulin/IGF receptor in the protection of myocytes and neurons from hypoxic injury. Na^+-activated potassium (KNa) channels have been identified in cardiomyocytes and neurons as mediators of the protective mechanisms against hypoxic death (Bader, Bernheim, & Bertrand, 1985; Kameyama et al., 1984). In *C. elegans*, a KNa ion channel is encoded by the *slo-2* gene. *slo-2* mutants are hypersensitive to hypoxic death, suggesting that SLO-2 protects against hypoxia effects. Thus, molecular characterization of KNa channels may allow the development of specific agonists and antagonists, in an effort to combat hypoxia-caused pathologies (Yuan et al., 2003).

A recent study reported that SLO-2 channels, SLO-2a and a novel N-terminal variant isoform, SLO-2b, are activated by Ca^{2+} and voltage, but in contrast to previous reports, they do not exhibit Cl^- sensitivity. In contrast to SLO-1, SLO-2 loss-of-function mutants confer resistance to hypoxia in *C. elegans* (Zhang et al., 2013).

5.2. Parkinson's disease

α-Synuclein is a small protein expressed primarily at presynaptic terminals in the central nervous system. It is enriched at presynaptic terminals, where it promotes the assembly of the SNARE machinery, and is proposed to play a role in neurotransmitter release and in regulating membrane stability and neuronal plasticity (Kalia, Kalia, McLean, Lozano, & Lang, 2013; Recchia et al., 2004). Inclusions of α-synuclein represent a hallmark feature of pathology in both sporadic and familial cases of Parkinson's disease, and constitute the main component of Lewy bodies found in degenerating dopamine neurons (Spillantini et al., 1997). Mutations in the α-synuclein gene or multiplications of the α-synuclein locus have also been associated with some autosomal-dominant familial cases of Parkinson's disease (Chartier-Harlin et al., 2004; Polymeropoulos et al., 1997; Singleton et al., 2003). The A53T and A30P mutations have been shown to cause rare cases of autosomal-dominant heritable early-onset PD.

C. elegans models of wild-type or mutated human α-synuclein overexpression have been established, either pan-neuronally or specifically in dopaminergic neurons (Cao, Gelwix, Caldwell, & Caldwell, 2005; Cooper et al., 2006; Kuwahara et al., 2006; Lakso et al., 2003; Qiao et al., 2008), and result in significant motor deficits. No inclusion bodies or α-synuclein aggregation is observed, and intracellular inclusions are rarely observed in these transgenic animals. Overexpression of wild-type or mutant human α-synuclein specifically in worm dopaminergic neurons causes their degeneration, which becomes more pronounced as animal age (Cao et al., 2005; Cooper et al., 2006; Kuwahara et al., 2006).

One of the mechanisms implicated in the pathogenesis of Parkinson's disease is mitochondrial dysfunction (Schapira, 2008). Autosomal-dominant mutations in the leucine-rich repeat kinase 2 (LRRK2) have been associated with both familial and late-onset cases of PD, with G2019S being a prominent such mutation. *C elegans* engineered to express the human LRRK2 (G2019S) mutant form shows extensive loss of dopaminergic neurons (Saha et al., 2009), by increasing their vulnerability to mitochondrial stress. Expression of the wild-type LRRK2 has a milder effect on neuron loss. Similarly, loss-of-function mutations in the *lrk-1* gene, encoding the worm ortholog of LRRK2, also sensitize dopaminergic neurons to mitochondrial stress.

C. elegans models of α-synuclein-induced dopaminergic neurodegeneration have been used as a platform to identify suppressors of dopaminergic neuron loss with some success. For example, specific overexpression of human torsinA or the worm homolog TOR-2 protects dopamine neurons in these models (Cao et al., 2005). In addition, overexpression of the human lysosomal enzyme cathepsin D has a similar neuroprotective effect (Qiao et al., 2008). Several other molecules involved in autophagy, lysosomal function, trafficking, and G-protein signaling have also been identified in RNAi suppressor screenings (Hamamichi et al., 2008).

Moreover, a recent study (Ruan, Harrington, Caldwell, Caldwell, & Standaert, 2010) reported that the overexpression of hVPS41, which is the human ortholog of *vps-41*, could prevent dopamine neuron degeneration induced by α-synuclein overexpression in *C. elegans*. Furthermore, the neuroprotective effect of hVPS41 can enhance the clearance of misfolded and aggregated α-synuclein through the AP-3 (heterotetrameric adaptor protein complex) interaction domain and clathrin heavy-chain repeat domain (Harrington, Yacoubian, Slone, Caldwell, & Caldwell, 2012). These data reveal the critical role of lysosomal trafficking in maintaining cellular homeostasis in the presence of toxic proteins.

ATP13A2 (also known as PARK9) encodes a lysosomal protein that is linked with PD. Overexpression of ATP13A2 can rescue the loss of dopamine neuron caused by α-synuclein overexpression in *C. elegans*, while knockdown of the ATP13A2 ortholog in worms can enhance α-synuclein misfolding and toxicity (Gitler et al., 2009). A recent study performed a screen to identify the ATP13A2 interacting partners responsible for modifying α-synuclein aggregation and toxicity to dopamine neurons in *C. elegans* (Usenovic et al., 2012). The modifiers of α-syn misfolding and neurotoxicity belong to groups responsible for ER and Golgi transport (YIF1A), clathrin-mediated vesicular transport (AAK1), and lysosomal fusion and degradation of aggregated proteins (HDAC6), indicating the importance of these processes in α-synuclein-mediated toxicity.

Another study (Su et al., 2010) performed a high-throughput chemical screen in yeast and identified strong suppressors of α-synuclein toxicity. The compounds identified were also verified in *C. elegans*, where they also rescue α-synuclein-mediated dopaminergic neuron loss in the worm model of PD (Su et al., 2010). These results suggest that it is possible to develop novel therapeutic strategies to simultaneously target the multiple pathological features of PD.

5.3. Tau toxicity: Modeling Alzheimer's disease in *C. elegans*

Several neurodegenerative diseases (including, in particular, Alzheimer's disease, frontotemporal dementia and Parkinsonism linked to chromosome 17, FTDP-17) are characterized by neurofibrillary tangles consisting of hyperphosphorylated forms of the microtubule-associated protein *Tau*, encoded by the *mapt* gene (Lee et al., 2001). Although the exact role of *tau* in the pathogenesis of these diseases is not clear, the identification of autosomal-dominant mutations in the *mapt* gene indicates a crucial role for the altered *tau* protein in the neurodegenerative process (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini, Crowther, Kamphorst, Heutink, & van Swieten, 1998).

Two studies have investigated the effects of expressing different forms of human *tau* (wild-type *tau* or *tau* carrying FTDP-17 mutations) in the nervous system of *C. elegans*. These studies included pan-neuronal expression, under the control of the *aex-3* promoter (Kraemer et al., 2003), or specifically in touch receptor neurons of *C. elegans*, under the control of the *mec-7* promoter (Miyasaka et al., 2005). In the first study, expression of either wild-type or FTDP-17 human *tau* resulted in reduced lifespan, behavioral

abnormalities, progressive uncoordinated movement, accumulation of insoluble phosphorylated *tau*, defective cholinergic neurotransmission, and age-dependent axonal and neuronal degeneration. This degenerative phenotype was more severe in lines expressing FTDP-17 mutant tau compared to those expressing wild-type tau. Morphologically, neurodegeneration was manifested by axonal vacuolar clearing, collapsed membrane structure, and membranous infoldings and whorls (which are characteristic of necrotic cell death), with associated amorphous *tau* accumulations and abnormal *tau*-positive aggregates, without, however, tau filaments observed (Kraemer et al., 2003).

The second study analyzed transgenic worms expressing wild-type or mutant (P301L and R406W) tau specifically in the touch (mechanosensory) neurons. Whereas worms expressing wild-type tau showed a small decrease in the touch response across their lifespan, worms expressing mutant tau displayed a large and progressive decrease. Loss of touch neurons function was accompanied by prominent neuritic abnormalities and microtubular loss. A substantial fraction of degenerating neurons developed tau accumulation in the cell body and neuronal processes. Notably, this neuronal dysfunction was not related to the apoptotic process because little recovery from touch abnormality was observed in *ced-3* or *ced-4*-deficient backgrounds.

A recent study employed *C. elegans* to investigate the relationship between tau aggregation and toxicity by comparing transgenic worms expressing pro- or antiaggregative tau species in their nervous system. The findings indicated that animals expressing the highly amyloidogenic tau species showed accelerated aggregation and pathology manifested by severely impaired motility, impaired axonal transport of mitochondria, and evident neuronal dysfunction. By contrast, control animals expressing the antiaggregant combination had rather mild phenotype (Fatouros et al., 2012). Furthermore, this study used the transgenic worms to screen for compounds that act as inhibitors of tau aggregation. Treatment of the proaggregant transgenic strains with a novel tau aggregation inhibitor, a compound belonging to the aminothienopyridazine class, ameliorated the motility phenotype, reflected also by a reduced extent of the progressive accumulation of neuronal morphological abnormalities.

C. elegans was also recently used to identify factors that are required for tau-mediated neurotoxicity. *Sut-2* was identified as such as gene, as recessive loss-of-function mutations in the *sut-2* locus suppress the tau aggregation and neurodegenerative changes caused by human tau expression in worms (Guthrie, Schellenberg, & Kraemer, 2009). The *sut-2* gene encodes a novel

subtype of CCCH zinc finger protein conserved across animal phyla, sharing significant identity with the mammalian SUT-2 (MSUT-2). Notably, the involvement of sut-2 in tau-mediated toxicity was also verified in mammalian cells, both *in vitro* and in postmortem human tissues (Guthrie, Greenup, Leverenz, & Kraemer, 2011). Specifically, RNAi knockdown of MSUT-2 in cultured human cells overexpressing tau causes a marked decrease in tau aggregation. Both cell culture and postmortem tissue studies suggest that MSUT-2 levels may influence neuronal vulnerability to tau toxicity and aggregation. Thus, neuroprotective strategies targeting MSUT-2 may be of therapeutic interest for tauopathy disorders.

6. CONCLUDING REMARKS

In this chapter, we have attempted to provide a comprehensive overview of the necrotic cell death paradigms that have been established in *C. elegans* (see Table 6.1) and also to convey our current understanding

Table 6.1 Triggers and paradigms of necrotic death in *C. elegans*

Death initiator	Type of insult	Dying cells	References
mec-4(u231), referred to as *mec-4(d)*	Hyperactive degenerin ion channel	Touch receptor neurons	Driscoll and Chalfie (1991)
mec-10(A673V), referred to as *mec-10(d)*	Hyperactive degenerin ion channel	Touch receptor neurons	Huang and Chalfie (1994)
deg-1(u38), referred to as *deg-1(d)*	Hyperactive degenerin ion channel	Some polymodal neurons and specific interneurons	Chalfie and Wolinsky (1990)
unc-8(n491)	Hyperactive degenerin ion channel	Motor neurons	Shreffler, Magardino, Shekdar, & Wolinsky, (1995), Tavernarakis Shreffler, Wang, & Driscoll, (1997)
pnc-1(ku212) or *cog-3(ku212)* (as was initially named)	Excess nicotinamide levels	Uterine vulval 1 cells	Huang and Hanna-Rose (2006), Vrablik et al. (2009)

Continued

Table 6.1 Triggers and paradigms of necrotic death in *C. elegans*—cont'd

Death initiator	Type of insult	Dying cells	References
Nicotinamide	Excess nicotinamide levels	Uterine vulval 1 cells	Vrablik et al. (2009)
deg-3(*u662*), referred to as *deg-3*(*d*)	Hyperactive nicotinic acetylcholine receptor	Subset of sensory neurons and interneurons	Treinin and Chalfie (1995)
gsa-1(Q208L) and $G\alpha_s$(Q227L), referred to as α_s(*gf*)	Constitutively active GTP-binding protein $G\alpha_s$	Motor neurons, interneurons, head and tail ganglia neurons, and pharyngeal neurons or epithelial cells (unidentified)	Korswagen et al. (1997), Berger et al. (1998)
Thapsigargin	Elevation of intracellular Ca^{2+} levels	Random cells (including neuronal)	Xu et al. (2001)
Δ*glt-3*;α_s(*gf*)	Glutamate-dependent toxicity	Head neurons	Mano and Driscoll (2009)
Erwinia carotovora, Photorhabdus luminescens	Pathogen infection	Intestinal, epidermal, and gonadal cells	Wong et al. (2007)
Hypoxic treatment	Oxygen/energy limitation	Pharynx, gonad primordium, body wall muscles, and unidentified cells	Scott et al. (2002)
α-Synuclein	Stress induction	Dopaminergic neurons	Lakso et al. (2003), Cao et al. (2005), Cooper et al. (2006), Kuwahara et al. (2006), Qiao et al. (2008)
LRRK2 (leucine-rich repeat kinase 2)	Stress induction	Dopaminergic neurons	Saha et al. (2009)
Tau protein	Stress induction	Several neurons (including motor neurons)	Kraemer et al. (2003)

Summary of the necrotic stimuli and the cell populations they affect, as discussed in this chapter.

of the molecular mechanisms involved. The rich repertoire of necrotic cell death events that occur in *C. elegans* both during development, as well as, in the adult renders the nematode a particularly attractive platform for dissecting the mechanisms of pathological cell death in humans, which is typically mediated by necrotic processes.

The similarity of necrotic cell death triggered by hyperactive ion channels in *C. elegans* to excitotoxic cell death and neurodegeneration in mammals, both in terms of morphological characteristics and mechanistic aspects, reflects the extensive evolutionary conservation of necrosis-relevant genes between *C. elegans* and mammals. Moreover, conservation of the mechanisms that protect *C. elegans* and mammalian cells from necrotic death inflicted by diverse stimuli, as exhibited, for example, by the hormetic induction of HSF16.1 upon heat preconditioning, provides new prospects for employing the nematode in the battle against degeneration. Concomitantly, modeling of human degenerative disorders, such as Parkinson's disease and others, in *C. elegans* has already accelerated the pace of the molecular dissection of the underlying mechanisms and holds promise for the development and testing of innovative intervention strategies.

ACKNOWLEDGMENTS

Work in the authors' laboratory is funded by grants from the European Research Council (ERC), the European Commission Framework Programmes, and the Greek Ministry of Education. V. N. is supported by an EMBO long-term postdoctoral fellowship.

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