Chapter 2
Caspase-Independent Cell Death Mechanisms in Simple Animal Models

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2.1 Introduction

Cells undergo cell death in many forms and due to different insults. Programmed cell death (PCD) is crucial for correct development of the organism and the clearance of harmful cells like tumor cells or autoreactive immune cells. PCD is initiated by the activation of cell death receptors and in most cases it is associated with the activation of the cysteine proteases caspases, which lead to apoptotic cell death; cells shrink, chromatin clumps and forms a large, sharply demacrated, crescent-shaped or round masses, the nucleus condenses, apoptotic bodies are formed and eventually dead cells are engulfed by a neighboring cell or cleared by phagocytosis (Kerr et al. 1972). Other insults can trigger this ordered disposal of a cell, such as radiation leading to DNA damage, via p53 which in turn activates the apoptotic pathway (Xiang et al. 1996). The classical caspase-dependent cell death pathway has been studied in great detail not only in mammalian cells, but also in model organisms *C. elegans* and *Drosophila* (Hengartner 2000; Danial and Korsmeyer 2004; Hay and Guo 2006; Lettre and Hengartner 2006).

The view of apoptosis as the only form of PCD, entirely dependent on caspases, is now challenged by several findings in both *C. elegans* and *Drosophila*. Several paradigms of cell death have been shown to be executed independently of caspases: autophagy, necrosis, and even apoptosis (Broker et al. 2005; Kroemer and Martin 2005; Stefanis 2005). Different cell organelles have been implicated in contributing to cell death in a caspase-independent manner with the mitochondrion playing the central role by releasing death executors from the intermembrane space to the cytosol, triggering the breakdown of the cell (Lorenzo and Susin 2004; Kim et al. 2006). Here, we review caspase-independent cell death mechanisms and relevant genes in the nematode and the fruit fly (Table 2.1). We discuss the roles of autophagy and necrosis and possible interplay between caspase-dependent and -independent

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Table 2.1 Proteins implicated in caspase-independent cell death mechanisms in mammals and their homologs in *C. elegans* and *Drosophila* (Proteins involved in autophagic cell death are discussed in Baehrecke 2003; Samara and Tavernarakis 2008)

Mitotic catastrophy				
Mammals	*C. elegans*	*D. melanogaster*	Protein function	Refs
AIF	WAH-1	fAIF	(FAD)-binding oxidoreductase	Wang et al. (2002)
endoG	CPS-6	CG8862	Sequence-unspecific DNase	Parrish et al. (2001)
Omi/HtrA2	–	dOmi	Serine protease	Challa et al. (2007)
HSpin1	C39E9.10, C13C4.5, CEF09A5	spin		Nakano et al. (2001)
WWOX	–	DmWWOX	Oxido-reductase	O'Keefe et al. (2005)
AMID	–	–	(FAD)-binding oxidoreductase	Wu et al. (2002)
PRG3	–	–	(FAD)-binding oxidoreductase	Ohiro et al. (2002)
Autophagy				
Yeast	*C. elegans*	*D. melanogaster*	Protein function	Refs
Induction of autophagy				
Atg1	UNC-51	Atg1	Ser/Thr protein kinase	Kamada et al. (2000)
TOR1/TOR2	LET-363	Tor	Rapamycin-sensitive Ser/Thr protein kinase	Scott et al. (2004)
Autophagosome nucleation				
Atg6	BEC-1	Atg6	Component of class III PI3-kinase complex	Furuya et al. (2005)
VPS34	VPS-34	Vps34	Class III PI3-kinase	Furuya et al. (2005)
Autophagosome maturation				
Atg3	Y55F3AM.4	Aut1	E2-like enzyme	
Atg4	Y87G2A.3	Atg4	Cys protease	
Atg5	ATGR-5	Atg5		Scott et al. (2004)
Atg7	ATGR-7	Atg7	E1-like enzyme	Juhasz et al. (2007a)
Atg8	LGG-1	–	Ubiquitin-like protein conjugated to PE	Juhasz et al. (2007b)
Atg10	D2085.2	–	E2-like enzyme	
Atg12	LGG-3	Atg12	Ubiquitin-like protein	
Atg16	K06A1.5	–		

AUT2/APG4	–	CG1694	Cysteine-type endopeptidase	Thumm and Kadowaki (2001)
AUT7/APG8/CVT5	–	Atg8a	Microtubule binding; cytoskeleton biogenesis	Simonsen et al. (2008)
–	–	Atg8b	Microtubule binding; cytoskeleton biogenesis	
Autophagic protein retrival				
Atg2	–	Atg2		Samara and Tavernarakis (2008)
Atg9	ATGR-9	Atg9	Integral membrane protein	Samara and Tavernarakis (2008)
Atg18	ATGR-18	Atg18		Samara and Tavernarakis (2008)
Necrosis				
Mammals	*C. elegans*		Protein function	Refs
Adenylyl cyclase	ACY-1		Adenylyl cyclase	Berger et al. (1998)
	ASP-3		Aspartyl protease	Syntichaki et al. (2002)
	ASP-4		Aspartyl protease	Syntichaki et al. (2002)
	CAD-1			Artal-Sanz et al. (2006)
	CLP-1		Calcium-activated cysteine protease	Syntichaki et al. (2002)
Calnexin	CNX-1		ER Ca^{2+} binding chaperone	Xu et al. (2001)
Calreticulin	CRT-1		ER Ca^{2+} binding-storing protein	Xu et al. (2001)
Mucolipin-1	CUP-5			Artal-Sanz et al. (2006)
	DAF-2		Receptor of insulin-like ligands	Scott et al. (2002)
	DAT-1		Dopamine transporter	Nass et al. (2002)
	ITR-1		Inositol triphosphate receptor ion channel	Xu et al. (2001)
	PQE-1		Q/P-rich protein	Faber et al. (2002)
	SGS-1		Adenylyl cyclase	Korswagen et al. (1998)
	SPE-5		Vacuolar H^+-ATPase B subunit	Syntichaki et al. (2005)
	TRA-3		Calcium-activated cysteine protease	Syntichaki et al. (2002)
Ryanodine receptor	UNC-68		ER Ca^{2+} release channel	Xu et al. (2001)
	UNC-32		Vacuolar H^+-ATPase a subunit	Syntichaki et al. (2005)
	VHA-2		Vacuolar H^+-ATPase c subunit	Syntichaki et al. (2005)
	VHA-10		Vacuolar H^+-ATPase G subunit	Syntichaki et al. (2005)
	VHA-12		Vacuolar H^+-ATPase B subunit	Syntichaki et al. (2005)

pathways leading to cell death, as well as their implication in disorders like neurodegenerative diseases. Recent research has also provided evidence for additional novel forms of cell death in *C. elegans* and *Drosophila*, indicating that current cell death classification may need to be revisited in the future.

2.2 Advantages of Invertebrate Model Organisms

Simple model organisms are becoming increasingly important for investigating principal biochemical and molecular mechanisms. *Caenorhabditis elegans* (*C. elegans*) and *Drosophila melanogaster* (*Drosophila*) have been instrumental in deciphering the molecular underpinnings of cell death. Both animals are ideal for genetic and molecular studies and additionally they have proved to be highly relevant models for studying human disorders, such as neurodegenerative diseases (Driscoll and Gerstbrein 2003; Celotto and Palladino 2005).

Both are multi cellular organisms with a relatively simple anatomy. In the case of *C. elegans*, the total number of cells of the animal is 959, including 302 neurons that form a simple nervous system. The cell lineage in the development of the nematode is fully deciphered and a complete lineage tree is available (see http://www.wormatlas.org/; Sulston et al. 1983). During development, 131 cells undergo programmed cell death (Ellis and Horvitz 1986). This makes *C. elegans* a powerful tool for investigating developmental biology (Bargmann and Avery 1995).

An important advantage of the nematode is its transparency, which makes microscopy far easier, permitting every cell division throughout development to be tracked. The simple nervous system is well documented, all neurons are mapped, and an almost complete wiring diagram has been created (see http://www.wormatlas.org/; White et al. 1983; Hall and Russell 1991). Specific behaviors, such as locomotion, chemo- or thermotaxis, as well as learning and memory, can be experimentally associated with the relevant neuron(s) (Thomas and Lockery 2005). The detailed characterization of its nervous system renders *C. elegans* particularly suited for the study of neurodegeneration and aging (Murakami 2007). The nervous system of *Drosophila* is far more complex and includes an intricate brain structure. The fly has been utilized with resounding success to study programmed cell death, neurodevelopment, as well as neurodegenerative diseases (Tabata and Takei 2004; Carthew 2007; Leyssen and Hassan 2007; Li and Baker 2007).

Both organisms go through a short life cycle and likewise, have a short mean life span. *C. elegans* develops from the fertilized egg to a self-fertilizing adult hermaphrodite within 3.5 days by undergoing four larval stages (L1 to L4). Due to food starvation or harsh environmental conditions the developing larva can enter the so-called dauer stage before completing the L1 stage, which increases the mean life span for more than 5 months. Favorable food conditions allow the animal to reenter the normal life cycle as an L4 larva. After entering the adult stage an approximately 3 day reproductive period follows, during which the animal lays about 300 eggs. *C. elegans* lives around 20 days, of which the last 2 weeks are characterized by a

decline in locomotion, food pumping, and recognizable tissue degeneration, revealing typical symptoms of aging. A low percentage of male animals (about 0.1 % of the progeny) is generated by hermaphrodites during self fertilization. These males enable genetic crosses that allow easy construction of double or multiple mutants (Riddle et al. 1997).

Drosophila needs about 8.5 days to develop from the zygote to the adult stage. After hatching, the animal undergoes three instar larval stages (first to third), followed by a prepupa and pupa stage, finally giving rise to the reproductive animal, which is either male or female. Females store the sperm of the male after mating and thereafter lay about 400 eggs (Lawrence 1992). Due to their short life span, both the nematode and the fruit fly are particularly popular for studying the mechanisms of aging and senescent decline (Lim et al. 2006).

Another important advantage of both animals is the easy maintenance in the laboratory. *C. elegans* feeds on bacteria (usually *Escherichia coli* strain OP50), which are grown either on solid agar plates or in liquid culture medium, and grows best at a temperature of 20°C. Drosophila is simply cultured at room temperature (25°C) and can be fed on different media containing a sugar source, like malt medium (Lakovaara 1969; Brenner 1974). The culturing temperature affects development timing of both animals. For example, *C. elegans* grows about 30% slower at 16°C compared to 20°C, while *Drosophila* needs about twice the time to complete a life cycle when grown at 18°C instead of 25°C, making it convenient to time experimental procedures. Both organisms can be cultured on a large scale.

Both the *C. elegans* and *Drosophila* genomes have been fully sequenced and annotated (Waterston and Sulston 1995; Kornberg and Krasnow 2000). Physical maps of the genome for both organisms based on the use of cosmids and yeast artificial chromosomes (YACs) have been created (Coulson et al. 1988; Hartl et al. 1992). The *C. elegans* genome is organized in five autosomes plus the sex chromosome X (sequence database: http://www.wormbase.org/). *Drosophila* only carries three autosomes plus the sex chromosome (sequence database: http://flybase.bio.indiana.edu/). Approximately 20,000 open reading frames (ORFs) for the nematode and about 14,000 ORFs for the fruit fly have been predicted (Blumenthal et al. 2002; Halligan and Keightley 2006). Additionally detailed protein interaction networks have been modeled for both organisms (Walhout et al. 2000; Lin et al. 2006).

The availability of fully-charted genomes allows the implementation of large-scale, genome-wide genetic and molecular methodologies such as double-stranded RNA-mediated interference (dsRNAi; Mello and Conte 2004). In *C. elegans* high-throughput RNAi screens against all 20,000 ORFs have been published (Simmer et al. 2003). The use of RNAi in the nervous system of the nematode has been less successful so far, but can be offset by the use of special hypersensitive mutants or the introduction of double-stranded hairpin RNAs (dshRNAs) through microinjection (Tavernarakis et al. 2000; Schmitz et al. 2007).

Both organisms are genetically malleable (Lee et al. 2004; Venken and Bellen 2005). The most straightforward method of creating mutants in both cases is random mutagenesis through the use of the chemical ethyl methanesulfonate (EMS).

Mutants for almost every gene are available or can be ordered. Animals carrying multiple mutations can be constructed and efficient genetic mapping is possible, by utilizing precise single nucleotide polymorphism (SNP) maps available for both model organisms (Jakubowski and Kornfeld 1999; Berger et al. 2001).

In the case of Drosophila loss of function mutants can also be generated by the use of *P* transposable elements or introducing dshRNAs through the GAL4/upstream activating sequence (GAL4/UAS) expression system, which is broadly used for gene overexpression (Brand and Perrimon 1993; Spradling et al. 1995; Cauchi and van den Heuvel 2006). In the fruit fly, the flippase (Flp)/flippase recombinase target (FRT) genetic mosaic system is also used (Golic 1991; Cauchi and van den Heuvel 2006). Other genetic manipulation methods are additionally available in *Drosophila* (Greenspan 1997).

In *C. elegans*, transgenic animals can be obtained by microinjection of engineered DNA samples into the gonad, where they generate inherited extrachromosomal arrays. This extrachromosomal array can further be integrated and stabilized in the genome through mutagenesis-induced integration (Mello and Fire 1995; Jin 2005; Rieckher et al. 2009).

In conclusion, both *C. elegans* and *Drosophila* are exceptionally powerful and convenient model organisms for investigating diverse biological phenomena, including cell death.

2.3 Cell Death by Mitotic Catastrophe

Compromised mitochondrial function irreversibly leads to cell death in both a caspase-dependent and -independent manner (Fig. 2.1). Mitochondrial breakdown is mainly the consequence of either extrinsic or intrinsic signals. Caspase-independent processes induce mitochondrial outer membrane permeabilization (MOMP). Although the precise mechanisms remain controversial, the involvement of Bcl-2 family proteins, among them the BH3-only proteins, is crucial in triggering MOMP (Green and Kroemer 2004). BH3-only proteins either activate or inhibit (Bcl-2 and Bcl-x_L) the proapoptotic Bcl-2 family members Bax and Bak. Oligomerization of these proteins generates pores in the outer membrane of mitochondria, releasing cell death factors from the intermembrane space to the cytoplasm (Kim et al. 2006). The process of MOMP initiated through Bax and Bak has been investigated intensively (Antignani and Youle 2006).

The *C. elegans* gene *ced-9* has been identified as an ortholog of the antiapoptotic members of the Bcl-2 family. CED-9 is anchored to the membrane of mitochondria and acts upstream of CED-3 and CED-4, negatively regulating the caspase-dependent cell death machinery (Igaki and Miura 2004). On the other hand, *egl-1* encodes a protein that belongs to the BH3-only protein subfamily also functioning upstream of CED-3 and CED-4, inducing cell death. CED-4 is released from the CED-4/CED-9 complex, which is localized to mitochondria by EGL-1, inducing caspase-dependent cell death (del Peso et al. 2000; Lettre and Hengartner 2006).

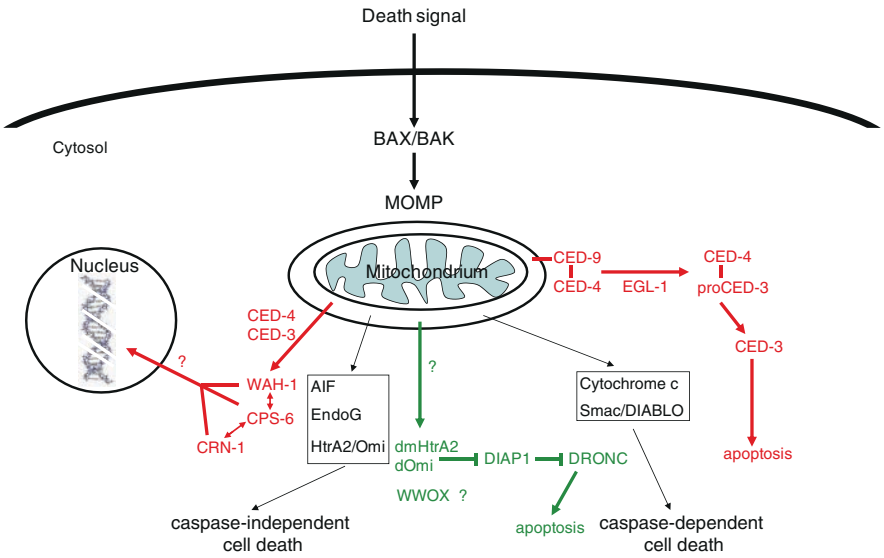

Fig. 2.1 Cell death by mitotic catastrophe. The induction of catastrophic breakdown of the mitochondrion derived from experimental results of mammalian cells (*black*) is shown. Various insults lead to the activation of BAX and BAK, which eventually trigger MOMP, resulting in the release of various enzymes. Depending on different factors, e.g., concentration, these enzymes cause cell death either in a caspase-dependent or -independent manner. *C. elegans* cell death pathways are shown in red and *Drosophila* mechanisms in green. Pathways for both animal models have also been shown to be dependent on caspases: The classical apoptotic pathway in *C. elegans* is based on the release of CED-4 from CED-9 leading to caspase-dependent cell death. The caspases CED-3 and CED-4 are also involved in the release of WAH-1. In *Drosophila*, the process of MOMP and the release of the HtrA2/Omi homologues, result in the downregulation of the caspase inhibitor DIAP1 and eventually the elevated activity of the caspase DRONC. It is not clear yet in which cell death pathway WWOX is involved. *DRONC* Drosophila Nedd2-like caspase; *MOMP* mitochondrial outer membrane permeabilization; *CED* cell death abnormality; *EGL* egg-laying deficiency; *WAH* worm AIF homolog; *CPS* CED-3 protease suppressor; *CRN* cell-death-related nuclease; *AIF* apoptosis inducing factor; *EndoG* endonuclease G; *DIABLO* direct inhibitor of apoptosis-binding protein with low p*I*; *WWOX* WW domain-containing oxidoreductase

In summary, although Bcl-2 family members have been shown to be involved in apoptosis and CED-9 is localized to the mitochondrial membrane, it is not known whether it is associated with MOMP (Estaquier and Arnoult 2006). In the fruit fly *Drosophila,* the involvement of mitochondria in cell death is less clear and they probably do not undergo MOMP (Varkey et al. 1999), although recent research findings may overturn this notion (see below; Challa et al. 2007; Igaki et al. 2007).

In mammalian cells the consequence of MOMP is the release of several factors (cytochrome c, Smac/Diablo, Omi/HtrA2, AIF and EndoG in mammals) from the intermembrane space into the cytosol, where they function as either caspase-dependent or -independent death executors (Kim et al. 2006). Cytochrome c release causes activation of Apaf-1 and leads to the classical apoptotic pathway, in which Smac/Diablo also plays a role as counteractor of IAPs (inhibitor of apoptosis

proteins; Hengartner 2000). The release of apoptosis inducing factor (AIF) HtrA2/Omi and EndoG have been shown to initiate caspase-independent mechanisms of cell death (Lorenzo and Susin 2004).

In mouse cells the endonuclease EndoG was identified as an apoptotic DNase that is released from mitochondria, subsequently localizing to the nucleus and fragmenting DNA independently of the activity of caspases. UV irradiation-induced DNA fragmentation mediated by EndoG still occurs in the presence of caspase inhibitors (Li et al. 2001). The function of the protein in mitochondria is the generation of RNA primers initiating DNA synthesis, a process important during mitochondrial replication (Cote and Ruiz-Carrillo 1993). The *C. elegans* homolog of mammalian EndoG, CPS-6 represents the first mitochondrial protein that has been identified to be involved in developmental programmed cell death in the nematode, indicating that an evolutionarily conserved family of nucleases plays an important role in apoptotic DNA degradation (Parrish et al. 2001). The activity of CPS-6 appears to be caspase-dependent, since down regulated CPS-6 function enhances cell survival in developing nematodes baring mutations in the caspases CED-3 and CED-4 (Parrish et al. 2001). Some interactors of CPS-6 have been identified: WAH-1, the *C. elegans* homolog of AIF (Wang et al. 2002), which is discussed below, and CRN-1, the homolog of human flap endonuclease-1 (FEN-1) (Parrish et al. 2003). CRN-1 possesses a 5′–3′ exonuclease and a structure-specific endonuclease activity. It acts as a co-factor of CPS-6, which is an endonuclease generating single-stranded nicks in DNA. Together they mediate stepwise DNA degradation (Parrish et al. 2003). Several more CRN nucleases might be involved in this process (Parrish and Xue 2003).

AIF was first identified in mammals as an effector of apoptotic cell death causing chromatin condensation and large-scale DNA fragmentation after localizing to the nucleus (Susin et al. 1999). Although it has been connected to the release of caspase-9 and therefore acting in the caspase-dependent pathway of cell death (Susin et al. 1999), AIF is also thought to be involved in a caspase-independent mechanism called “apoptosis-like” cell death (Leist and Jaattela 2001). The mechanism of releasing AIF to the cytosol is still under debate: The protein is embedded in the inner membrane of mitochondria and needs to be cleaved by proteases in order to be released. Cleavage occurs after the permeabilization of the outer membrane of mitochondria and is processed by the cysteine proteases cathepsins and calpains (Yuste et al. 2005). Such a scenario is supported by the fact that AIF is released to the cytosol through the same pore but much slower than cytochrome c, Smac/Diablo and Omi/HtrA2 (Munoz-Pinedo et al. 2006). However this notion contradicts earlier findings, where blocking caspase activity through zVAD-fmk prevents the release of AIF from mitochondria (Arnoult et al. 2003). Given that zVAD-fmk also blocks the activity of cysteine proteases these data need to be re-evaluated (Modjtahedi et al. 2006; Krantic et al. 2007).

The precise mechanism by which AIF promotes apoptosis-like cell death is not fully understood. Human AIF likely interacts with DNA since it shows a strong positive electrostatic potential (Ye et al. 2002) and most likely recruits potential partners such as nucleases to degrade DNA, triggering cell death (Lorenzo and Susin 2004). Indirectly, AIF may activate cell death via generation of free radicals after being

released to the cytosol. AIF exhibits NADH oxidase activity, reducing O_2 (Miramar et al. 2001). However, AIF also plays the role of a free radical scavenger, as shown in the Harlequin mouse (Klein et al. 2002). Thus, AIF might fulfill a dual role depending on its actual localization either to the cytosol (oxidase and cell death executor) or to the inner membrane of mitochondria (free radical scavenger involved in the mitochondrial respiratory chain; Porter and Urbano 2006).

Insight into the mode of AIF action has been obtained by studies of the *C. elegans* AIF homolog *wah-1* in developmental cell death. Wang and colleagues demonstrated that WAH-1 and the *C. elegans* EndoG (CPS-6) can be released from mitochondria by EGL-1 in a way similar to the release of cytochrome c and EndoG from mammalian mitochondria. Both proteins cooperate and act in the same pathway to promote apoptotic DNA degradation (Wang et al. 2002). Surprisingly, speed of WAH-1 release observed in a time-course study is at least partially dependent on caspase CED-3 activity, suggesting that *C. elegans* AIF and EndoG define a single, mitochondria-initiated apoptotic DNA degradation pathway that is conserved between *C. elegans* and mammals (Wang et al. 2002; Wang unpublished results). This assumption was recently confirmed by the discovery that WAH-1 promotes plasma membrane phosphatidylserine externalization and initiates cell engulfment typical for classical apoptosis in the nematode through activation of phospholipid scramblase 1 (SCRM-1; Wang et al. 2007).

The death effector Omi/HtrA2 was first identified in mammals as inhibitor of the X-chromosome linked inhibitor of apoptosis (XIAP) similar to Smac/Diablo. The same investigation showed the induction of a second mechanism of mediating cell death independent of caspases, probably due to its serine protease function (Suzuki et al. 2001). In mammals the protein is processed after import to the mitochondria and 133 of 458 residues are removed, leaving an active form of 36 kDa. The amino-terminus shares high homology with *Drosophila* pro-death proteins Grim, Hid, Reaper and mammalian Smac/Diablo proteins (Lorenzo and Susin 2004). Some evidence about the mechanism of Omi/HtrA2 action comes from studies in *Drosophila*: The mitochondrial proteins dOmi and dmHtrA2 were independently identified as highly homologous to the human HtrA2/Omi, particularly within the serine protease domain. During UV-irradiation-induced cell death, labeled dmHtrA2 or dOmi proteins and also cytochrome c, were observed outside mitochondria (Challa et al. 2007; Igaki et al. 2007). Release is both caspase-dependent and -independent (Challa et al. 2007). In the cytosol dOmi induces cell death in S2 cells and in the developing fly eye by proteolytically degrading DIAP1 (an IAP family caspase inhibitor), which finally displaces DRONC and acts in the classical apoptosis pathway (Challa et al. 2007; Igaki et al. 2007).

Another recently investigated gene involved in caspase-independent cell death is *hspin1*, a homolog of the *Drosophila spin* gene (Yanagisawa et al. 2003). Mutations in *spin* interfere with programmed cell death during the development of *Drosophila* nurse cells and neurons. Persistence of surviving cells leads to neurodegeneration and death of oocytes in the ovary (Nakano et al. 2001). In human cells HSpin1, which contains membrane spanning domains, causes necrotic cell death when overexpressed. HSpin1 binds to the antiapoptotic proteins Bcl-2 and Bcl-x_L and its

activity can be blocked by the necrosis inhibitor pyrrolidine dithiocarbamate (PDTC) but not by the caspase-inhibitors zVAD-fmk and p35. This indicates that HSpin1 titrates Bcl-2 and/or Bcl-x_L by localizing to the mitochondria and thereby promoting cell death in a caspase-independent way (Yanagisawa et al. 2003). Three homologs of the *spin* gene are encoded in the *C. elegans* genome and have not been characterized in detail (Nakano et al. 2001).

Additional proteins that are involved in mitochondrial caspase-independent cell death have been identified in mammalian cells: WWOX or FOR, the AIF homologue mitochondrion-associated inducer of death (AMID) and the p53 regulated gene 3 (PRG3). All these show sequence similarity to AIF (Lorenzo and Susin 2004). WWOX has a homolog in *Drosophila,* which has been shown to protect from ionizing radiation when overexpressed (O'Keefe et al. 2005).

2.4 Autophagic Cell Death

Although identified more than 50 years ago, the process of autophagy remained relatively mysterious until relatively recently. Interest in autophagy markedly increased within the last decade after it was shown to play a role in human pathophysiology (Klionsky 2007). Dual roles of autophagy in cell survival and death have been reported (Baehrecke 2005). Autophagy differs from apoptosis (type I programmed cell death) by the presence of autophagic vacuoles and autophagolysosomes which are involved in degradation of the dying cell.

Three different types of autophagy have been defined: microautophagy, chaperone-mediated autophagy and macroautophagy (Majeski and Dice 2004; Baehrecke 2005). Hereafter, we will refer to macroautophagy using the term autophagy for simplicity.

During autophagy, cytoplasmic double membrane vesicles, called autophagosomes or autophagic vacuoles are initially formed, primed from a yet unknown membrane source (Wang and Klionsky 2003). As autophagosomes form they engulf parts of the cytoplasm and/or organelles. Ultimately, their outer membrane fuses with lysosomes. The inner-single membrane vesicle (autophagic body) is released into the lumen, where it is digested, together with its content, by various enzymes (Yorimitsu and Klionsky 2007).

Most proteins involved in autophagy have been identified in the yeast *Saccharomyces cerevisiae* and are encoded by autophagy related genes (Atg; Klionsky et al. 2003; Yorimitsu and Klionsky 2007). These genes regulate every step of autophagy from induction, through cargo selection and packaging up to fusion with the lysosomes and degradation. Nevertheless, many unanswered questions about each phase of the process still remain (Klionsky 2005).

Cells use autophagy as a "regulated self-cannibalism" process. Cells degrade and recycle their contents in order to maintain viability in the absence of food. By sensing the presence of nutrients through the class I and class III phosphatidylinositol 3-kinase (PI3K) signaling pathways, the downstream acting target of rapamycin

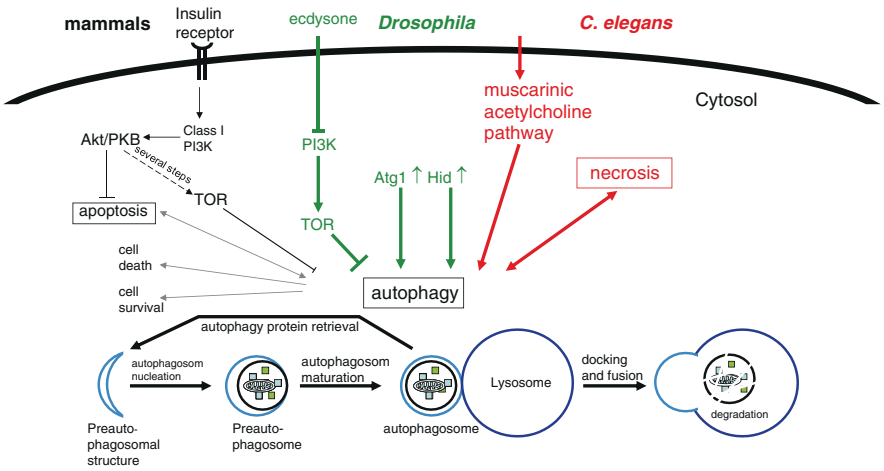

Fig. 2.2 Autophagy and cell death. Pathways leading to or interacting with autophagy that result in cell survival or cell death are shown. *C. elegans* mechanisms are shown in *red* and *Drosophila* in *green*. The main autophagic mechanism is also presented. Initially, a pre-autophagosomal structure arises from an unknown membrane source and develops into the preautophagosome. While developing the autophagosome engulfs parts of the cytosol, containing proteins and whole organelles. After completing maturation, the autophagosome docks to the lysosome and the outer membrane fuses with the lysosomal membrane. The inner autophagic body and its contents are degraded by lysosomal proteases. The genes involved in each step are presented in Table 1. *Atg* Autophagy-related gene; *PI3K* phosphoinositol 3 kinase; *TOR* target of rapamycin; *Akt/PKB* serine/threonine kinase/protein kinase B; *Hid* head involution defective

(TOR) kinase suppresses autophagy. In the absence of food, low insulin levels abrogate suppression (Klionsky 2004). Under harsh nutrient deprivation conditions excessive autophagy may lead to cell death (Fig. 2.2). Investigation of physiological as well as aberrant autophagy in invertebrate model organisms has provided new insight into the role of autophagy in cell survival and cell death. These studies in *Drosophila* and *C. elegans* point to the involvement of autophagy in caspase-independent cell death.

During the last larval stage of *Drosophila*, fat body cells, which are part of the fat body, a nutrient storage organ similar to the human liver, undergo programmed cell death and show induced autophagic vesicle formation in response to starvation. Cells appear to die in response to the hormone ecdysone, which down regulates PI3K signaling, resulting in pronounced induction of autophagy (Rusten et al. 2004). TOR was shown to be an important downstream effector in the pathway leading to the suppression of autophagy in the fruit fly (Scott et al. 2004). Additionally, the *Drosophila* homologue of the yeast protein Vps18, Deep Orange (Dor) has been shown to control programmed autophagy in fat body cells. Dor is required for ecdysone signaling and also mediates the fusion between autophagosome and lysosome (Lindmo et al. 2006; Lindmo and Stenmark 2006). Very recently, the importance of Atg1 in this process has been confirmed by overexpression studies in the fly, where it leads to suppression

of TOR and triggers autophagy, leading to caspase-dependent cell death in fat body cells (Neufeld 2007; Scott et al. 2007). Earlier overexpression studies suggest that the intracellular executor of programmed autophagy is the protein *head involution defective* (*hid* – counterpart of Smac/DIABLO), which kills cells in a caspase-independent way. However, this effect might be the result of overexpression (Juhasz and Sass 2005).

Dying salivary glands have been intensively studied in Drosophila by serial analysis of gene expression (SAGE) which revealed the involvement of both autophagy, apoptosis and other genes (total: 1,244 transcripts) in cell death (Gorski et al. 2003). In another genome-wide analysis, the activation of genes involved in cell death of salivary glands caused by radiation (usually triggering apoptosis) and steroids (ecdysone) has been investigated by the use of microarrays. The response to the two different death triggers is radically different: ecdysone significantly increases the RNA levels of 932 gene transcripts, while in response to radiation only 34 genes were activated. Five genes were commonly activated in both cases, indicating a cross-talk between different cell death programs (Lee et al. 2003). Supporting this point, a recent study by Martin and Baehrecke suggests that salivary gland cells die via autophagy in a caspase-dependent manner (Martin and Baehrecke 2004). A recent shotgun proteome analysis of purified, dying (steroid-triggered) larval salivary glands, combined with whole-genome microarrays, revealed upregulation of proteins usually involved in apoptosis and autophagy. Known players, such as the ecdysone-response proteins, caspases and caspase-regulators were identified as well as proteins from caspase-independent acting mechanisms. Besides establishing a powerful screening method in *Drosophila* cells, this study confirmed earlier studies and strengthened the point that both caspase-independent (such as autophagy) and caspase-dependent mechanisms contribute to cell death in specific tissues (Martin et al. 2007).

Both *Drosophila* and *C. elegans* are particularly suited for investigating the role of autophagy in cell death associated with pathological conditions such as neurodegenerative diseases. For example, a *Drosophila* model of Huntington`s disease has been established (Ravikumar et al. 2004). The disease is associated with expanded polyglutamine repeats (polyQ) in the protein huntingtin, which causes aggregation of the protein and cytotoxicity (Lee and Kim 2006). Huntingtin aggregates are mostly cleared by the ubiquitin proteasome system (UPS). Autophagy also contributes to the clearance of aggregates when the UPS system becomes impaired. The *Drosophila* histone deacetylase HDAC6 appears to be involved in coordinating both mechanisms (Pandey et al. 2007). Autophagy is engaged in the turnover of polyQ and other aggregates by downregulation of TOR signaling. This has been shown in mammalian cells and also in flies expressing mutant huntingtin in the photoreceptor cells of the eye (Ravikumar et al. 2004). Rapamycin and its analog CCI-779 protect cells by inhibiting TOR and inducing autophagy, which clears aggregated huntingtin (Rubinsztein et al. 2007). In addition, small molecule enhancers (SMER) and inhibitors (SMIR) of rapamycin cytostatic effects have been studied in yeast. These molecules induce autophagy independent from rapamycin and enhance the clearance of huntingtin aggregates and also mutant α-synuclein aggregates associated with Parkinson's disease (Sarkar et al. 2007).

Autophagic cell death has also been studied in *C. elegans*. The physiological function of autophagy in the nematode is associated with dauer larva formation. L2 animals enter the arrested dauer developmental state due to unfavorable environmental conditions such as high temperature, absence of food or the presence of a pheromone, which indicates a highly dense population (Riddle 1988). Entering this state is negatively regulated by the insulin-like signaling pathway. *Bec-1*, the *C. elegans* ortholog of the yeast and mammalian autophagy gene *Atg6/Vps60/beclin1* plays an important role in dauer morphogenesis. Additionally, the orthologs of the yeast autophagy genes *Atg1*, *Atg7*, *Atg8,* and *Atg10* (which now are defined as *unc-51*, *atgr-7*, *lgg-1* and *atgr-18*; see Table 1) are involved in the process; their downregulation results in defect in dauer formation (Melendez et al. 2003; Riddle and Gorski 2003). *bec-1* forms a complex with CED-9/Bcl-2 and has also been found to play a role in apoptosis in *C. elegans*. Deletion of *bec-1* triggers CED-3/caspase-dependent cell death. BEC-1 is necessary for the function of the class III PI3 kinase LET-512/Vps34, which is involved in autophagy, membrane trafficking, and endocytosis (Takacs-Vellai et al. 2005). The muscarinic acetylcholine pathway signals induction of autophagy in pharyngeal muscles of *C. elegans* during starvation. Pumping rates are enhanced by activation of the muscarinic acetylcholine pathway and the energy needed for this process is likely provided by autophagy. Indeed, *bec-1* RNAi knockdown decreases autophagy and pumping rates during starvation (Kang et al. 2007). Autophagy is also associated with endocytosis in *C. elegans*. *Ce*VPS-27 is the ortholog of the yeast endosomal Vps27p, which regulates the formation of endosomal sorting complexes. *Ce*VPS-27 is important for larval development and inactivation of the corresponding gene leads to defects in endosome formation, as well as accumulation of autophagosomes, suggesting a role in autophagy (Roudier et al. 2005).

Recent findings in *C. elegans* indicate that autophagy also contributes to necrotic cell death. In an *unc-51* deficient background, necrotic cell death, triggered either by *deg-3(d)* or *mec-4(d)* or hypoxia (see the following section on necrosis for details) is significantly suppressed. This effect is also observed after downregulation of other autophagy-related genes such as *bec-1*, *lgg-1* and *atgr-18* by RNAi. In addition, increase of autophagosomes formation is observed under conditions of neurodegeneration. Calpain proteases and autophagy appear to act in the same pathway (Samara et al. 2008). *Ce*TOR signaling eventually prevents and starvation promotes neuronal cell death in a *mec-4(d)* background (Toth et al. 2007). Thus, autophagy appears to play a dual role in *C. elegans*, either by promoting survival (physiological autophagy) or causing death (insufficient or excessive autophagy; Samara et al. 2008; Samara and Tavernarakis 2008)

2.5 Necrotic Cell Death

Necrosis is considered to be one of the main caspase-independent cell death types and morphologically distinct from apoptosis. Among the major features of necrosis are the extensive swelling of the cell and various cellular organelles, the random

degradation and clumping of nuclear DNA, the formation of small, tightly wrapped membrane whorls, the rupture of the plasma membrane and the appearance of autophagosomes (Edinger and Thompson 2004). The word necrosis is derived from the Greek expression "necros," standing for "dead" and was traditionally considered as the chaotic breakdown of the cell. In humans, necrotic cell death accompanies prolonged hypoxia, ischemia, hypoglycemia, toxin exposure, exposure to reactive oxygen metabolites, extreme changes in temperature, and nutrient deprivation (Nicotera et al. 1999). Necrosis is also involved in neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis and epilepsy (Stefanis 2005). Necrotic cell death, unlike apoptosis, was thought to be a passive process, not requiring energy, synthesis of new proteins and specific regulatory mechanisms. However, recent findings in *Drosophila* and *C. elegans* have forced a shift of this simplistic view (Syntichaki and Tavernarakis 2002; Kourtis and Tavernarakis 2007).

In the nematode necrotic neuronal death can be triggered by a great variety of extrinsic and intrinsic signals, mainly by the expression of ion channels bearing a hyperactive mutation (Fig. 2.3; Syntichaki and Tavernarakis 2003). The most thoroughly studied case of necrotic cell death is the one induced by hyperactive

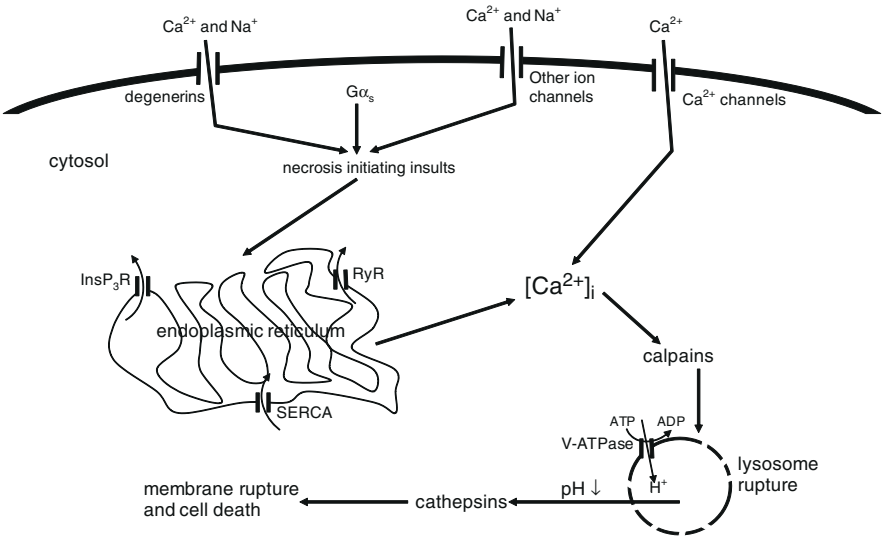

Fig. 2.3 Main necrotic pathways in *C. elegans*. Necrosis is triggered by mutant ion channels, as well as mutant $G\alpha_s$ (for more details see text). These insults stimulate Ca^{2+} release from the endoplasmic reticulum (ER) through the transporters RyR and $InsP_3R$, reuptake of calcium is facilitated through SERCA. Elevation of Ca^{2+} levels is also mediated directly by plasma membrane Ca^{2+} channels. Subsequently, calpain proteases become activated. Lysosomal rupture is the consequence of calpain activity, which leads to the release of lysosomal cathepsin proteases and a decrease in local pH, facilitated through the action of V-ATPase. *$G\alpha_s$* G-protein subunit; *$InsP_3R$* inositol triphosphate receptor; *RyR* ryanodin receptor; *SERCA* sarco-endoplasmic reticulum Ca^{2+}-ATPase; *V-ATPase* vacuolar H^+-ATPase)

deg-1(d) (*deg*enerin) and *mec-4*(d) (*mec*hanosensory) both carrying dominant mutations and causing necrosis in special neurons of *C. elegans*: Gain of function mutations in *deg-1* induce necrosis in a group of interneurons of the posterior touch sensory circuit (Chalfie and Wolinsky 1990). *mec-4* gain of function mutations cause similar effects in the six touch receptor neurons, which are required for the sensation of gentle touch of the body (Syntichaki and Tavernarakis 2004). Both genes belong to the family of degenerins, which induce cell degeneration when mutated to a hyperactive form. Dying cells exhibit the typical morphological characteristics of necrotic cell death. Degenerins are similar in sequence to the subunits of the amiloride-sensitive epithelial Na^+ channel (ENaC) in mammals (Tavernarakis and Driscoll 2001). Large side chain substitutions of amino acids close to the pore forming region of degenerins enhance sodium and calcium conductivity leading to necrotic cell death (Syntichaki and Tavernarakis 2004). Ultimately, extensive ion influx disrupts cellular Ca^{2+} homeostasis (Syntichaki and Tavernarakis 2003). Calcium imbalance caused by mutated ion channels triggers further release of Ca^{2+} from the endoplasmic reticulum (ER)via the ryanodine (RyR) and inositol-1,4,5-triphosphate receptors ($Ins(1,4,5)P_3PR$).

The ionic imbalance and subsequent cell death induced by mutant degenerins resembles excitotoxicity in vertebrates, where the collapse of presynaptic neuron membrane potential due to energy depletion results in the release of high amounts of the excitatory neurotransmitter glutamate into the synaptic cleft (Olney 1994). Accumulation of glutamate at the synapse causes hyper-excitation and necrotic cell death of postsynaptic neurons. Excitotoxicity is the prominent mechanism of neuronal loss during stroke, when nutrient and energy supply to neuronal cells is disrupted by blockage of the blood flow. Degenerin-induced neuronal death in *C. elegans* is an attractive model of excitotoxicity that renders the nematode a suitable and powerful tool for dissecting the molecular mechanisms of neurodegeneration.

In addition to mutant degenerins, several other triggers of nonprogrammed cell death in *C. elegans* have been described. Constitutive activation of the GTP-binding protein $G\alpha_s$, chemical inhibitors of the respiratory chain (e.g., NaN_3), hypoxic treatment, toxins, polyglutamine repeat proteins and macromolecular damage caused by radiation are potent inducers of cell death (Kourtis and Tavernarakis 2007). These inducers have been exploited in genetic and molecular studies that have elucidated key facets of necrotic cell death mechanisms (Artal-Sanz and Tavernarakis 2005).

Null mutations in calreticulin and knock-down of calnexin, which are calcium-binding chaperones, suppress necrotic cell death in *C. elegans* neurons triggered by *mec-4*(d). Also the blockage of Ca^{2+} release from the ER, either by mutations in the calcium release channels encoded by *unc-68* (RyR) and *itr-1* ($Ins(1,4,5)P_3PR$) or by pharmacological treatment results in similar suppression. These findings indicate that Ca^{2+} release from the ER plays an essential role in necrotic cell death (Xu et al. 2001).

The cytoplasmic protease calpain, which is activated by calcium and functions in several signaling and metabolic pathways, also plays a role in necrosis. High levels of calcium activate calpains which then localize to lysosomes and cause

disintegration of the lysosomal membrane. Subsequent release of lysosomal aspartyl proteases and cathepsins into the cytoplasm causes the breakdown of the cell and rupturing of the plasma membrane. Detailed studies of cell death following brain ischemia in monkeys have led to the formulation of the "calpain–cathepsin" hypothesis for the execution of necrosis (Yamashima 2000, 2004). Genetic studies in *C. elegans* support the involvement of a calpain–cathepsin axis during neurodegeneration. Downregulation of the calpains CLP-1 and TRA-3 and cathepsins ASP-3 and ASP-4 by RNAi ameliorates neurodegeneration in the nematode (Syntichaki et al. 2002). The proteolytic action of cathepsins in the cytoplasm is further enhanced by the drop of pH in the cell, mediated by the vacuolar H^+-ATPase, which acidifies lysosomes and other cell organelles. Alkalization of those organelles prevents necrosis in *C. elegans*, supporting the involvement of cytoplasmic acidification in the process (Syntichaki et al. 2005).

The active involvement of lysosomes in necrotic, caspase-independent cell death mechanisms is corroborated by observations in mutant nematodes, defective in lysosomal function (Artal-Sanz et al. 2006). *cup-5(lf)* mutants, which show increased number of enlarged lysosomes (Hersh et al. 2002) are significantly more sensitive to necrotic cell death inducing insults. Visualization of lysosomal morphology during necrosis reveals aggregation of lysosomes around a swollen nucleus and ultimately lysosomal rupture, consistent with the calpain–cathepsin hypothesis (Artal-Sanz et al. 2006).

In *Drosophila*, a similar model of excitotoxicity has been utilized to gain insight into the mechanisms of neurodegeneration. The excitatory amino acid transporters (EAATs) are high-affinity transporters for L-glutamate (Glu) involved in clearing Glu from the synaptic cleft and preventing over-excitation of the postsynaptic neuron (Beart and O'Shea 2007). Downregulation of *Drosophila* dEAAT1, which is expressed in glia, reduces Glu uptake and clearing, which leads to degeneration of neuropil. Similarly to excitotoxicity, degeneration is accompanied by the formation of vacuoles, electron-dense material, and swollen mitochondria (Rival et al. 2004), which are typical features of necrotic cell death.

2.6 Novel Programs of Caspase-Independent Cell Death

During development and morphogenesis of multicellular organisms, programmed cell death controls cell number and also shapes organs (Vaux and Korsmeyer 1999). The most common type of cell death in this context is caspase-dependent apoptosis (Edinger and Thompson 2004). Recent research in *C. elegans* and *Drosophila* has revealed that specific cells also die in a caspase-independent manner (Kumar and Rothman 2007). Cell corpses are subsequently removed through engulfment by neighboring or specialized phagocytic cells in both *C. elegans* and *Drosophila*. The process of engulfment in the nematode assists apoptotic cell killing itself and is also involved in the clearance of necrotic cells (Zhou et al. 2004). In *Drosophila,* cells dying in a caspase-independent manner are removed by a similar mechanism

(Mergliano and Minden 2003). Interestingly, observations in cell-death deficient *H99 Drosophila* embryos revealed removal of cells in the epidermis via caspase-independent cell death that may involve engulfment of living cells (Mergliano and Minden 2003).

A nonapoptotic, caspase-independent cell death mechanism is involved in the removal of the linker cell of *C. elegans*, which is born during the second larval stage (L2) and is essential for male gonadal development in the nematode (Sulston et al. 1983). As the linker cell migrates it directs the extension of the male gonad and mediates the fusion of the vas deferens and cloaca. The linker cell finally dies at L4/adult stage. Death was thought to be dependent on the neighboring engulfing cells (Sulston et al. 1980). However, after laser ablation of the grandparental precursor engulfing cells the linker cell still dies, which hints at a linker cell intrinsic death program. Cell death is independent of genes typically involved in developmental timing, engulfment, and all types of cell death characterized in *C. elegans*, such as necrosis, apoptosis, and autophagy (Abraham et al. 2007). Some morphological features of the linker cell death such as nuclear crenellation, the absence of chromatin compaction and cytoplasmic changes such as dilation of cell organelles are reminiscent of caspase-independent cell death in other organisms (Clarke 1990). Thus, the linker cell death program in *C. elegans* may represent a conserved caspase-independent mode of cell death in diverse species (Abraham et al. 2007).

In *Drosophila,* 15 nurse cells assure the development of one growing oocyte each, by supplying it with essential macromolecules, such as proteins, mRNA, and organelles. Finally they die after extruding all their remaining cytoplasmic contents into the oocyte (McCall 2004). This type of cell death was thought to be classical, caspase-dependent apoptosis but recent research illuminated that nurse cells die in a caspase-independent manner (Mazzalupo and Cooley 2006). Visualization and inhibition of caspase activity demonstrates that caspases do not play a role during the death of the nurse cells. While the possibility of necrosis cannot be excluded, no signs of autophagic or apoptotic cell death have been detected (Mazzalupo and Cooley 2006). Similar to the linker cell death in *C. elegans*, a yet unidentified cell death mechanism likely underlies the demise of nurse cells.

2.7 Concluding Remarks

Several paradigms of caspase-independent cell death have been characterized in diverse species. Most can be grouped into three main types, mitotic catastrophe, autophagy, and necrosis. Proteins that normally serve physiological functions can be released from mitochondria after MOMP and once in the cytosol they act as death executors. The functions of some such proteins have been investigated in mammalian cells as well as in *C. elegan*s and *Drosophila*. These studies point towards conserved mechanisms of caspase-independent cell death. Interestingly, while some of these effectors trigger caspase-independent cell death in mammals, they preferentially engage caspase-dependent apoptotic cell death in invertebrates.

This indicates that caspase-independent cell death mechanisms may represent more recent additions to the cell death program.

Studies in yeast and in mammalian cells indicate that autophagy is a mediator of both cell survival and cell death. Starvation causes formation of autophagosomes, partial degradation of cell contents and recycling of the degraded components, which provides the cell with the energy required to overcome the shortage of nutrients. Nevertheless, abnormally high levels of autophagy may promote cellular destruction instead. Furthermore, the process of autophagy is intimately linked with both apoptosis and necrotic cell death. Necrosis was traditionally considered as merely the chaotic breakdown of cells. However, several recent studies in *C. elegans* indicate that specific molecular mechanisms are involved in the necrotic destruction of the cell. Because necrosis is implicated in many devastating human disorders, such as neurodegenerative diseases and stroke, elucidation of the biochemical events that transpire during necrosis has the potential to provide targets for effective pharmacological interventions.

In addition to the three major categories of caspase-independent cell death, novel cell death paradigms that do not involve caspase function are emerging. Genetic and molecular dissection of these examples of cell death in invertebrate models may reveal new mediators of cell death with relevance to human pathological conditions.

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