

# Death by necrosis

## Uncontrollable catastrophe, or is there order behind the chaos?

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Cells suffer necrotic death when exposed to extreme environmental conditions, adverse and excessive stimuli, or when deleterious mutations are encoded in their genetic material. Unlike apoptosis, which involves a highly regulated and elaborate network of biochemical events and cascades, necrosis has been considered generally to be a chaotic decadence process that effects the inexorable demise of cells otherwise not destined to die. This grim prospect is now slowly being overturned, mostly by exciting new findings in two simple model organisms, *Caenorhabditis elegans* and *Drosophila melanogaster*. Despite the wide spectrum of necrosis-initiating conditions, evidence is accumulating that execution of necrotic or neurodegenerative cell death may be carried out by a finite common set of mechanisms.

### Introduction

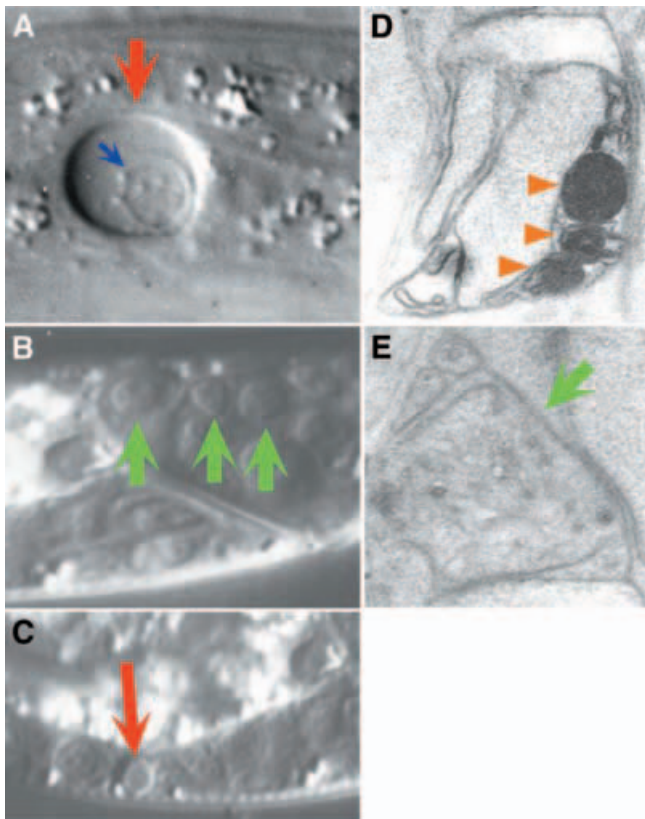
Early pioneering studies of cell death delineated two major, morphologically distinct types: apoptosis and necrosis (Walker *et al.*, 1988). Apoptosis, or programmed cell death, is an integral part of development and homeostasis, and is hardwired into the genetic material of cells that are destined to die. Often, under pathological circumstances, such as in some neurodegenerative diseases and in stroke, the apoptotic program can be inappropriately implemented, resulting in detrimental cellular destruction (Ferri and Kroemer, 2001; Leist and Jaattela, 2001). This process requires energy and often even *de novo* macromolecular synthesis, and the specific biochemical steps involved in triggering and executing apoptosis, as well as in removing the dead cell remnants generated by this process, have been described in great detail (Hengartner, 2000, 2001).

Necrosis, the second type of cell death, is radically different from apoptosis in almost every respect. The term derives from the Greek kernel 'necros', meaning 'dead' (with a sense of dismay), and refers to the accidental death of cells exposed to extreme environmental or genetically encoded insults (Walker *et al.*, 1988).

Injured cells undergoing necrosis display gross morphological and ultra structural features that contrast sharply with those exhibited by cells undergoing apoptosis. Death is accompanied by extensive swelling of the cell, distension of various cellular organelles, clumping and random degradation of nuclear DNA, extensive plasma membrane endocytosis and autophagy (Figure 1; Hall *et al.*, 1997; Ferri and Kroemer, 2001). Necrosis is generally considered to be a passive process because it does not require new protein synthesis, has only minimal energy requirements, and is not regulated by any homeostatic mechanism. In humans, necrotic cell death occurs generally in response to severe changes in physiological conditions, including hypoxia, ischemia, hypoglycemia, toxin exposure, exposure to reactive oxygen metabolites, extreme temperature changes and nutrient deprivation (Walker *et al.*, 1988; Nicotera *et al.*, 1999). Several neurodegenerative syndromes and diseases, such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis (Price *et al.*, 1998) and epilepsy, also involve necrosis.

It is striking that despite the profound impact of necrotic cell death on human health, the molecular events that transpire during cellular necrosis remain obscure. The dominant concept that has permeated the field stipulated that necrotic death is merely the chaotic breakdown of a cell under intolerable conditions, involving execution mechanisms almost as diverse as the triggers initiating cell death. However, relatively recent observations in simple model organisms such as *Caenorhabditis elegans* and *Drosophila* challenge these views (Mutsuddi and Nambu, 1998; Tavernarakis and Driscoll, 2001a). Cells of different type and origin undergoing necrosis exhibit stereotyped morphological and ultrastructural features in response to injury, which points to underlying commonalities that may represent a conserved core execution program (Colbourne *et al.*, 1999). Understanding the intricacies of this process may facilitate the development of

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**Fig. 1.** 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. (Reproduced in part with permission from Hall *et al.*, 1997.)

novel, educated intervention methodologies for blocking or ameliorating necrotic cell death.

### Necrotic cell death in the realm of *C. elegans*

Several well-defined conditions are known to trigger necrotic cell death in *C. elegans*. The best-characterized case is that of unusual gain-of-function mutations, in several ion channel genes, which inflict a necrotic pattern of death on the neurons that express their protein products. For example, dominant mutations in the *deg-1* gene [*degenerin*; *deg-1(d)*] induce the death of specific interneurons and sensory neurons (Chalfie and

Wolinsky, 1990). Similar effects are seen with dominant mutations in the homologous *mec-4* gene [*mechanosensory*; *mec-4(d)*; Driscoll and Chalfie, 1991]. MEC-4 and DEG-1 are the founding members of the *C. elegans* degenerin ion channel family (Tavernarakis and Driscoll, 2001a), which also includes MEC-10, UNC-8 and UNC-105, all of which are mutable to forms that lead to degeneration or dysfunction in distinct groups of cells (Huang and Chalfie, 1994; Liu *et al.*, 1996; Tavernarakis *et al.*, 1997).

*Caenorhabditis elegans* degenerins share sequence similarity with *Drosophila* Ripped Pocket (RPK) and Pick Pocket (PPK), with subunits of the vertebrate amiloride-sensitive epithelial sodium channel (ENaC) and with other neuronally expressed ion channels. Together, these proteins define the DEG/ENaC protein superfamily (Tavernarakis and Driscoll, 2001b). 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 first detectable abnormality apparent in an ill-fated cell is the formation of small tightly wrapped membrane whorls that seem to originate at the plasma membrane and to coalesce into large electron-dense membranous structures upon internalization (Figure 1D; Hall *et al.*, 1997).

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  $\alpha$ -7 nicotinic acetylcholine receptor 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 *et al.*, 1998). In addition, mutant activated forms of the heterotrimeric G protein  $\alpha$  subunit ( $G\alpha$ , Q208L), from both *C. elegans* and rat, cause swelling and degeneration of many cell types when expressed in *C. elegans* (Korswagen *et al.*, 1997; Berger *et al.*, 1998).

### *Drosophila*: the flexible model

Many conditions that induce necrotic cell death have been characterized in *Drosophila* (Mutsuddi and Nambu, 1998), and recent findings have firmly established the fly as a potent model system for the study of several human neurodegenerative disorders. Investigations of expanded polyglutamine tract-induced neurodegeneration in *Drosophila* have been particularly successful in modeling the polyglutamine toxicity that characterizes at least eight inherited human neurodegenerative diseases (including Huntington's disease and spinocerebellar ataxias), through targeted expression of polyglutamine proteins (Bonini, 2001). As in the case of the human diseases, late-onset, progressive neurodegeneration is induced, indicating that mechanisms of cellular toxicity are conserved in flies (Warrick *et al.*, 1998).

Two additional examples of human proteins whose heterologous expression in *Drosophila* leads to neurodegeneration are those of  $\alpha$ -Synuclein and the microtubule-associated protein Tau. Mutations in the  *$\alpha$ -synuclein* gene have been linked to familial Parkinson's disease, a severe movement disorder in which aggregates of  $\alpha$ -Synuclein accumulate within specific dopaminergic neurons that degenerate progressively (Spillantini *et al.*, 1997). The microtubule-binding protein Tau has been implicated in the pathogenesis of Alzheimer's disease, with abnormally

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phosphorylated Tau accumulating and aggregating in degenerating neurons of patients. Inclusions known as neurofibrillary tangles are also formed, but it is unknown whether tangle formation is the cause or result of the neuronal degeneration (Braak and Braak, 1991). Expression of either the wild-type or mutant forms of these two proteins in *Drosophila* faithfully reproduces features of the human neurodegenerative conditions associated with them (Feany and Bender, 2000; Wittmann *et al.*, 2001; see Supplementary data available at *EMBO reports* Online).

Apart from the above examples of heterologous expression of disease genes, a plethora of mutations in *Drosophila* induce necrotic cell death in the nervous system and other tissues (Mutsuddi and Nambu, 1998). Specifically, three flavorsome fly mutants, *swiss cheese*, *spongecake* and *eggroll*, exhibit brain abnormalities reminiscent of many human neurodegenerative disorders. Another *Drosophila* mutant showing adult neurodegeneration is *bubblegum*. The *bubblegum*-encoded protein is similar to the vertebrate very long chain fatty acid (VLCFA) acyl coenzyme A synthetase, which promotes the  $\beta$ -oxidation of VLCFAs to thioester derivatives in peroxisomes. As a consequence, *bubblegum* flies show elevated levels of VLCFAs, as seen in human adrenoleukodystrophy (ALD). In patients with ALD, excess VLCFAs can be lowered by dietary treatment with 'Lorenzo's oil', a mixture of unsaturated fatty acids. Remarkably, feeding *bubblegum* flies one of the components of Lorenzo's oil, glycerylthioleate oil, blocks the accumulation of excess VLCFAs (Min and Benzer, 1999).

### A common denominator in necrotic cell death?

The classical viewpoint of necrotic cell death has been that once it is triggered, it is inevitable (Leist and Jaattela, 2001). But is it, after all, possible to survive without removing the trigger? Is there a set of downstream events common to the necrotic cell death that is inflicted by diverse and seemingly unrelated initiators? If so, the molecules enacting these events would constitute global effectors of necrosis and would make excellent therapeutic intervention targets. Genetic screens aimed at identifying modifiers (either suppressors or enhancers) of necrotic cell death induced by diverse stimuli in *C. elegans* suggest that this may indeed be the case.

Early work has established that specific mutations in the *mec-6* gene are general suppressors of degenerin-induced cell death in the nematode (Chalfie and Wolinsky, 1990). Although its biochemical role is not known, the MEC-6 protein is thought to be specifically required for degenerin channel function and is not needed for  $G\alpha_s$ -induced cell death (Korswagen *et al.*, 1997). Genetic screens for extragenic suppressors of activated  $G\alpha_s$ -induced cell death have identified *acy-1/sgs-1*, which encodes a member of the adenylyl cyclase family, transmembrane molecules that generate the second messenger cAMP and, in mammals, are stimulated by  $G\alpha_s$ . In *C. elegans*, *acy-1/sgs-1* is expressed broadly throughout the nervous system (Korswagen *et al.*, 1997; Berger *et al.*, 1998) including the touch receptor neurons, yet it is not required for the touch cell degeneration inflicted by mutant degenerins. This finding suggests that either distinct death-execution mechanisms are involved in necrosis induced by different initiating factors or the initiating events may feed into a common pathway at a point downstream of *acy-1/sgs-1*.

In a screen designed to reveal genes required for necrotic cell death induced by *mec-4(d)*, suppressor mutations affecting one particular locus protected against necrosis induced not only by hyperactive degenerins such as *mec-4(d)* and *deg-1(d)*, but also by activated  $G\alpha_s$  (Xu *et al.*, 2001). This locus encodes calreticulin, a calcium-binding/storing protein that is found primarily in the lumen of the endoplasmic reticulum and serves as both a molecular chaperone and a central regulator of calcium homeostasis (Llewellyn *et al.*, 2000). Three additional endoplasmic reticulum proteins involved in the regulation of intracellular calcium are required for *mec-4(d)*-induced cell death: the calcium-binding chaperone calnexin, the inositol triphosphate receptor channel InsP3R and the ryanodine receptor channel RyR (the latter being calcium release channels). It is intriguing that elevated levels of three other chaperones, Hsp70, Hsp40 and Hsp104, can suppress polyglutamine toxicity in yeast, *C. elegans* and *Drosophila* (Krobitsch and Lindquist, 2000; Satyal *et al.*, 2000; Bonini, 2001; Parker *et al.*, 2001). Moreover, overexpression of Hsp70 ameliorates  $\alpha$ -Synuclein toxicity in *Drosophila* dopaminergic neurons (Auluck *et al.*, 2001). Thus, chaperones and the heat shock response appear to play a central role in necrosis (see Supplementary data).

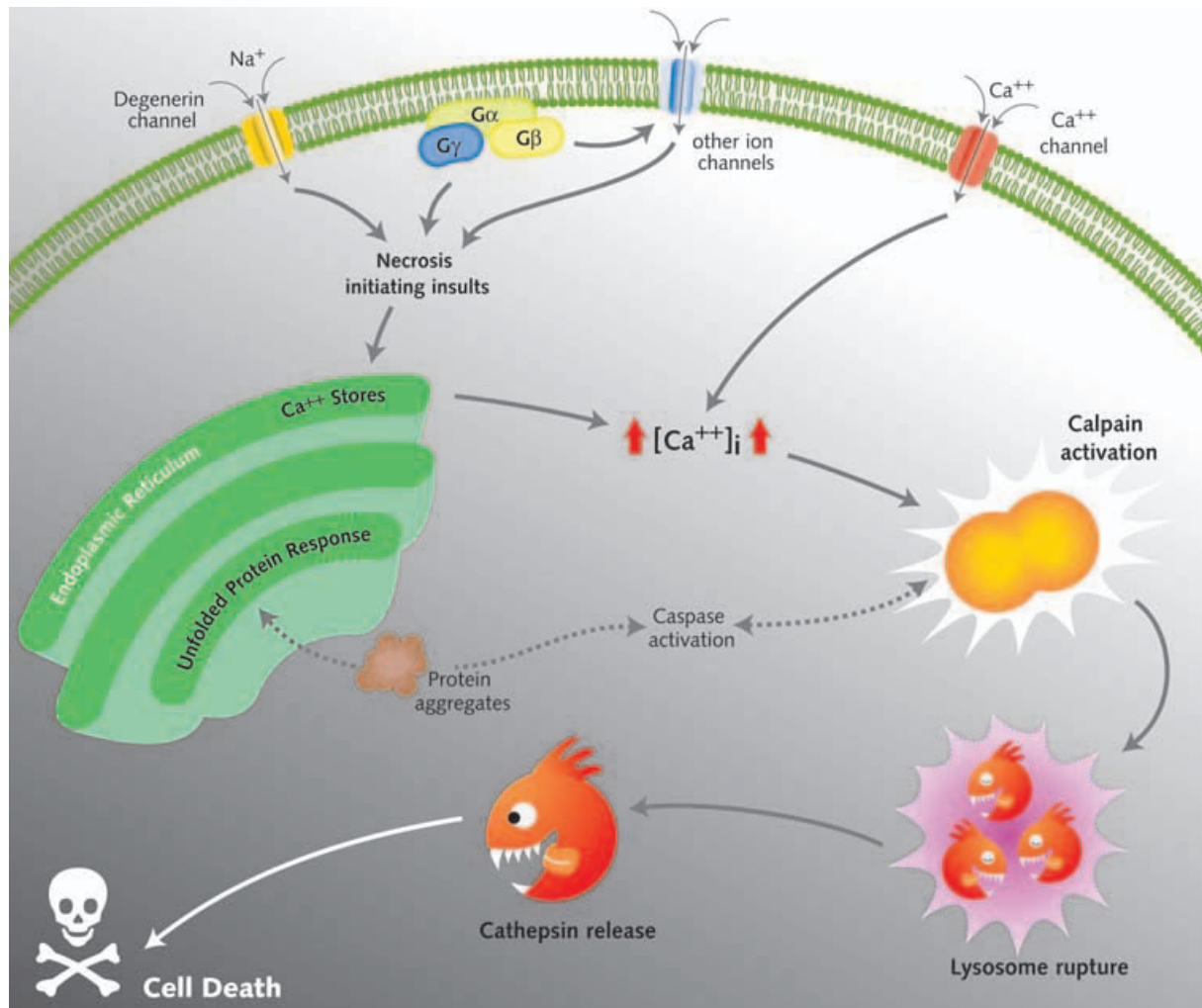
As is the case for necrosis in *C. elegans*, calcium homeostasis has also been incriminated in the morphologically and operationally related excitotoxic cell death in mammals (Choi, 1992). The glutamate-gated kainate, AMPA and NMDA receptor channels on post-synaptic neurons are hyper-activated by excess released glutamate, and cell death commences. Of these, NMDA conducts both sodium and calcium. Thus, directly, and maybe indirectly (through secondary activation of voltage-gated calcium channels), glutamate drives the perturbation of intracellular calcium levels, which might signal the initiation of necrosis. These findings highlight the role of calcium in necrotic cell death and demonstrate the relevance of research in simple model organisms to analogous phenomena in mammals.

Although the involvement of calcium in necrotic cell death is well known, it is not equally well understood (Lee *et al.*, 1999; Sattler and Tymianski, 2000; Mariol and Segalat, 2001). A likely mechanism is the misactivation of enzymes and biochemical cascades that eventually dismantle the cell. The obvious question is what these are.

### Proteolysis taking center stage?

Each cellular organelle has the ability to sense stressful and pathogenic alterations and to initiate local or global responses to stress. This can lead ultimately to adaptation or, once a critical threshold of insult or damage has been reached, cell death. When the extrinsic or intrinsic death cascades are induced to full throttle, most organelles of the dying cell manifest biochemical alterations, such as partial proteolysis and the permeabilization/rupture of membranes. Mitochondria, the endoplasmic reticulum and lysosomes have essential roles in the control of necrotic cell death (Ferri and Kroemer, 2001). Intriguingly, the lysosomal system exhibits prominent upregulation in the brain as a result of aging and Alzheimer's disease (Nixon *et al.*, 2000). Lysosomes, metaphorically also called the cell's 'suicide bag', contain over 80 types of hydrolytic enzymes, including the cathepsin class of non-specific proteases. Leakage of these enzymes into the cytoplasm due to lysosomal membrane injury or rupture has been





**Fig. 2.** A likely death scenario. Many diverse initiating conditions that trigger necrosis (such as hyperactive ion channels and  $G\alpha_s$  proteins, and protein aggregates formed in cases of neurodegenerative diseases) may provoke a net increase in the cytoplasmic calcium concentration ( $[Ca^{++}]_i$ ), either by stimulating uptake of extracellular calcium or by facilitating the release of calcium stores from the endoplasmic reticulum. Calcium could, in turn, signal the mobilization of executioner cathepsin proteases and other hydrolases through calpain activation. Calpains have also been implicated in the activation of pro-apoptotic caspase proteases (for a review, see Leist and Jaattela, 2001).

implicated in necrotic cell death after both heart and brain ischemic injuries (Adamec *et al.*, 2000). Lysosomal rupture might, in turn, be mediated by other cysteine proteases, the calpains, whose calmodulin-like, calcium binding domains sense calcium and activate their own protease moieties.

Interestingly, in primate hippocampal neurons, degeneration following acute ischemia is accompanied by intracellular calcium elevation and concomitant calpain activation. Moreover, activated calpain appears to localize to disrupted lysosomal membranes (reviewed in Yamashima, 2000). These findings have led to the formulation of an attractive unifying proposal, the 'calpain–cathepsin' hypothesis, which involves three key players that mediate cellular destruction during necrosis (see Figure 2). First, intracellular calcium increases in concentration, either directly or indirectly, in response to many diverse necrosis-initiating stimuli. Secondly, calpains become activated in response to the elevated calcium concentrations. Thirdly,

killer cathepsins that dismantle the cell are liberated in the cytoplasm after activated calpains compromise the integrity of lysosomal membranes. This scenario, which is reminiscent of autophagy, summons cathepsins and other lysosomal hydrolases as the major executioners of the cell during necrosis. Activated calpains and also lysosomal hydrolases can consequently initiate a catastrophic chain reaction by spawning additional pro-death processes such as activation of effector caspases, blockage of energy metabolism in the cytoplasm and interference with mitochondrial function (Ferri and Kroemer, 2001; Leist and Jaattela, 2001). This overwhelming cataract of events, which results in rapid death, is in sharp contrast to the orderly destruction that takes place during apoptosis, and appears to be responsible for the characteristic differences in morphology between apoptotic and necrotic cell corpses (Walker *et al.*, 1988; Nicotera *et al.*, 1999).

## Open issues, caveats and limitations

Whereas the distinction between necrosis and apoptosis is obvious in certain situations, in others, including some human pathological conditions such as stroke, the dividing line between these two major types of death is blurring (Lipton and Nicotera, 1998). For example, alternative morphological death profiles such as paraptosis have been described (Clarke, 1990; Sperandio *et al.*, 2000) and certain dying cells are known to exhibit some, but not all, commonly distinctive features of either apoptosis or necrosis. Likewise, certain markers of death can be expressed by both apoptotic and necrotic cells (Fernandez *et al.*, 1994). Moreover, the same cell types can undergo either necrotic or apoptotic cell death in response to different stimuli. It is progressively becoming clear that what determines the cell fate is the intensity of an insult and the expression levels of the downstream signal transducers, as well as the extent of the calcium overload and the intracellular ATP levels (Yamashima, 2000). An additional factor to be considered is the temporal distribution of death-initiating conditions: insults delivered in an acute manner generate different responses than do similar insults delivered to the cell over prolonged periods of time (Choi, 1992).

The emerging theme is that, instead of the existence of distinct types of cell death, there is a continuum of responses that orchestrate cellular destruction, which is manifested in stereotyped macroscopic morphological patterns. Cells have evolved elaborate stress response mechanisms that, up to a certain level, will buffer exogenous or endogenous insults (Beere and Green, 2001). When these protective systems are overwhelmed, death is imminent. However, unlike the case for apoptosis, cells have not evolved to 'anticipate' necrosis. Execution of the latter type of death does not involve the mobilization of molecular mechanisms designed to specifically carry out such a task. Instead, cellular activities or processes that are not normally deleterious turn rogue and work against the cell itself. For example, inappropriate calpain activation and deregulation of the lysosomal degradation system appear to be common to necrotic cell death in both vertebrates and invertebrates (Yamashima, 2000). Noxious conditions that eventuate in necrosis essentially convert a specific cohort of normal, innocent cellular processes to destructive ones by tampering with their spatiotemporal confinement or by relaxing their regulation.

## Perspectives

Recent work has shed light on the many dimensions of necrotic cell death. The development of nematode and fly models that faithfully reproduce features of necrosis in mammals has facilitated the infusion of both forward- and reverse-genetics approaches into our efforts to obtain a detailed description of the molecular events underlying death. In combination with genome information and the high-throughput screening procedures that are now available (Fraser *et al.*, 2000; Kim *et al.*, 2001), these models should make comprehensive searches for the genes involved in necrosis possible. How relevant will the new findings be to human disease? Judging from the many parallels between necrotic cell death in invertebrates and neurodegeneration in higher organisms, we anticipate significant similarities between

the underlying molecular mechanisms, even if mammals possess a more elaborate repertoire of responses and cascades.

**Supplementary data.** Supplementary data are available at *EMBO reports* Online.

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