REVIEW

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Cuts can kill: the roles of apoptotic nucleases in cell death and animal development

Abstract Chromosome fragmentation is one of the major biochemical hallmarks of apoptosis. However, until recently, its roles in apoptosis and mechanisms of action remained elusive. Recent biochemical and genetic studies have shown that chromosome fragmentation is a complex biochemical process that involves a plethora of conserved nucleases with distinct nuclease activities and substrate specificities. These apoptotic nucleases act cooperatively among themselves and with other nonnuclease cofactors to promote stepwise chromosome fragmentation and DNA degradation. Importantly, in addition to its direct contribution to the dismantling of the dying cell, apoptotic DNA degradation can facilitate cell killing and other apoptotic events such as clearance of apoptotic cells. Furthermore, some apoptotic nucleases apparently affect other aspects of animal development, including immune responses. The identification of new apoptotic nucleases and analysis of their functions in apoptosis and animal development should pave the way for future studies to uncover new functions for apoptotic nucleases and shed light on the hidden links between apoptotic DNA degradation and human diseases.

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Introduction

Programmed cell death, or apoptosis, is a highly regulated process necessary for the proper sculpting of structures during development, maintenance of appropriate cell number, and the elimination of unnecessary or damaged cells (Kerr et al. 1972; Danial and Korsmeyer 2004). Improper regulation of programmed cell death can lead to a variety of diseased states, including cellular transformations and degenerative disorders (Thompson 1995). Cells undergoing apoptosis display similar morphological changes in mammals and invertebrates, including *Drosophila melanogaster* and *Caenorhabditis elegans* (*C. elegans*), suggesting that evolutionarily conserved machineries may be employed to execute these characteristic changes in dying cells.

One of the hallmarks of apoptosis is the fragmentation of chromosomal DNA (Wyllie 1980). In dying cells, chromosomes condense and are cleaved at internucleosomal regions to generate approximately 180-bp DNA ladders, irreversibly compromising the ability of a cell to replicate its genome and to transcribe its genes, which are important for cell survival. This represents one of the most severe assaults levied against a dying cell and likely contributes directly to the demise of the cell. Despite the importance of this event, how it is activated and executed during apoptosis and whether it is a cause or simply a secondary event of apoptosis were, until recently, poorly understood. Furthermore, a variety of different nucleases have been implicated in apoptotic DNA degradation, but only recently were the identities of some of these nucleases revealed and their roles in apoptosis confirmed in vivo. A number of excellent reviews have focused on the discovery and mechanisms of action of some of these apoptotic nucleases (Nagata 2000; Zhang and Xu 2002; Samejima and Earnshaw 2005; Widlak and Garrard 2005). However, an equally important issue has received less attention, that is, what are the developmental consequences of disrupting apoptotic DNA degradation in vivo? In an attempt to address the roles of DNA degradation in apoptosis and normal animal development, we will review the findings from genetic studies of animals defective in apoptotic DNA

degradation, with a special emphasis on studies from *C. elegans*.

More than 20 years elapsed between the discovery of the first genetic mutant defective in apoptotic DNA degradation, the *C. elegans* nuclease defective (*nuc-1*) mutant, and the finding that *nuc-1* encodes a type II DNase that likely contributes directly to apoptotic DNA degradation (Sulston 1976; Wu et al. 2000). Why the delay? One reason is that programmed cell death proceeds normally in *nuc-1* mutants (discussed below), and the predominant view was that apoptotic DNA degradation was a late, dispensable event in apoptosis. Recent studies challenge this view by showing that apoptotic DNA degradation is important for activation and proper progression of cell death and can even affect other cell death execution events such as clearance of apoptotic cells.

NUC-1 is important for apoptotic DNA degradation in *C. elegans*

In *nuc-1* mutants, the DNA of dying cells persists and is detected as a dense mass in engulfing cells when stained with DNA labeling dyes or stains such as Feulgen or 4′6-diamidino-2-phenylindole·2HCl (DAPI) (Sulston 1976; Hedgecock et al. 1983; Wu et al. 2000). This persistent DNA most likely represents an intermediate of apoptotic DNA degradation since the chromosomal DNA from apoptotic cells in *nuc-1* mutants is stained by terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling (TUNEL) (Wu et al. 2000), an assay that labels 3′-hydroxyl DNA breaks generated during apoptosis (Gavrieli et al. 1992). However, the number of dying cells, the timing of cell death, or the engulfment of cell corpses is normal in *nuc-1* mutants (Hedgecock et al. 1983; Wu et al. 2000; Parrish et al. 2001; Parrish and Xue 2003). Furthermore, no genetic interaction has been observed between *nuc-1* and other cell death genes in affecting cell killing. Therefore, *nuc-1* is dispensable for cell killing in *C. elegans*.

What is the function of NUC-1? One important clue comes from the observation that the DNA of ingested bacteria remains undigested in the intestinal lumen of *nuc-1* mutants. Thus, NUC-1 likely functions generally in the digestion of undesirable or unnecessary DNA. Consistent with this hypothesis, NUC-1 is an acid-dependent nuclease similar to DNase II in mammals, which likely functions in lysosomes or other acidified compartments (Hevelone and Hartman 1988; Lyon et al. 2000). During apoptosis, NUC-1 most likely functions in engulfing cells, like mammalian DNase II, to degrade DNA of engulfed apoptotic cells (Kawane et al. 2003). Indeed, genomic DNA persists as a Feulgen- or DAPI-reactive mass in unengulfed apoptotic cells in *C. elegans* animals that are defective in cell corpse engulfment, indicating that engulfment by phagocytes may be a prerequisite for complete degradation of genomic DNA of dead cells (Hedgecock et al. 1983). However, it remains possible that NUC-1 can act cell-autonomously in dying cells because no mosaic analysis has been done to clearly define where NUC-1 acts to affect DNA degradation (Wu et al. 2000).

DFF40/CAD is an important apoptotic nuclease in mammals

The accumulation of TUNEL-reactive DNA breaks in *nuc-1* mutants suggests that at least one nuclease must function prior to NUC-1 to generate these TUNEL-reactive ends that are resolved by NUC-1. So far, no such nuclease has been found in *C. elegans*. However, a human nuclease, DFF40 [40-kd DNA fragmentation factor (DFF)] or CAD (caspase-activated deoxyribonuclease), appears to be a good candidate for such an activity. DFF40/CAD was first identified biochemically as a component of a DFF complex, which also contained an inhibitory subunit, DFF45 (also known as ICAD, inhibitor of CAD) (Liu et al. 1997; Enari et al. 1998; Liu et al. 1998; Sakahira et al. 1998). DFF45/ICAD serves both as a chaperone for the proper folding of DFF40/CAD and a cognate inhibitor that holds DFF40 activity in check in normal cells. During apoptosis, the cleavage of DFF45/ICAD by activated caspases such as caspase-3 and caspase-7 results in the release and activation of the DFF40/CAD endonuclease (Liu et al. 1997, 1998; Enari et al. 1998; Sakahira et al. 1998). The activated DFF40/CAD nuclease then associates with chromosomal proteins such as Histone H1, HMG proteins, and topoisomerase II to promote efficient cleavage of internucleosomal DNA (Liu et al. 1998; Durrieu et al. 2000). Importantly, cleavage of chromosomal DNA by DFF40/CAD creates 3′ hydroxyl DNA breaks (detected by TUNEL), leading to the generation of 50- to 300-kb cleavage products and subsequent internucleosomal DNA fragmentation (Widlak et al. 2000). Further degradation of chromosomal DNA fragments is likely achieved by other apoptotic nucleases that resolve the TUNEL-reactive DNA breaks or by DNase II from phagocytes once apoptotic cells are engulfed.

To study the in vivo functions of DFF40/CAD, DFF45-deficient mice, CAD-deficient mice, and mice expressing a caspase-resistant ICAD mutant were generated and examined (Zhang et al. 1998, 2000; McIlroy et al. 1999; Kawane et al. 2003). In all cases, cells from mutant mice show severe defects in chromatin condensation and fragmentation during apoptosis, demonstrating that DFF40/CAD activity is required for apoptotic DNA degradation. Importantly, primary thymocytes derived from DFF45 −/− mice display increased resistance to apoptosis induced by tumor necrosis factor (TNF) α or staurosporine, and DFF45 −/− mice are more resistant to kainic acid-induced neuronal cell death, suggesting that DFF40/DFF45-mediated DNA degradation can contribute to cell killing in vertebrates (Zhang et al. 1999). Interestingly, DFF45-deficient mice also exhibit enhanced spatial learning and memory and longer memory retention compared to wild-type control mice, which are possibly caused by an increase in the number of granule cells in the dentate gyrus of defective mice (Slane et al. 2000; Slane McQuade et al. 2002; Olariu et al. 2005).

Thus, DFF40/CAD not only affects apoptosis but also may affect other aspects of animal development and behavior. Although DFF40/CAD and DFF45/ICAD homologs have been identified in *Drosophila* and found to be important for apoptotic DNA degradation in vivo (Yokoyama et al. 2000; Mukae et al. 2002), no DFF40/CAD or DFF45/ICAD homolog has been identified in *C. elegans* or in yeast where cell death and nuclear DNA degradation also occur. Furthermore, DFF40-deficient mammalian cells display residual apoptotic DNA fragmentation (Li et al. 2001; Samejima et al. 2001; Zhang et al. 2001). Thus, other nucleases must be involved in cleaving chromosomal DNA during apoptosis in yeast, *C. elegans*, and mammals.

Endonuclease G and CPS-6 are conserved apoptotic nucleases

In an effort to identify the nuclease that mediates residual apoptotic DNA fragmentation in DFF45-deficient cells, Li et al. (2001) identified a mitochondrial nuclease, endonuclease G (endo G), as the second human nuclease involved in apoptotic DNA degradation. Independently, in a genetic screen in *C. elegans* for mutations that could suppress ectopic neuronal deaths induced by the CED-3 caspase, a new gene, *cps-6* (CED-3 protease suppressor), was identified and found to encode a *C. elegans* endo G homolog (Parrish et al. 2001). Consistent with its proposed role in nuclear DNA fragmentation, endo G is released from mitochondria during apoptosis and translocated into nuclei (Li et al. 2001). In a cell-free system with isolated HeLa cell nuclei, both recombinant endoG and CPS-6 proteins can induce generation of characteristic apoptotic DNA ladders, suggesting that they can serve as apoptotic nucleases (Li et al. 2001; Parrish et al. 2001).

The involvement of endo G/CPS-6 in apoptotic DNA degradation and apoptosis has been demonstrated by the analysis of *cps-6* mutant phenotypes in *C. elegans*. Similar to *nuc-1* mutants, reduction of *cps-6* activity by a loss-of-function mutation or RNAi causes accumulation of TUNEL-reactive cells in *C. elegans* embryos, indicating that *cps-6* is required for proper execution of apoptotic DNA degradation. In addition, reduction of *cps-6* activity delays the appearance of embryonic cell corpses and can inhibit cell killing in sensitized genetic backgrounds, suggesting that it is also important for normal progression of apoptosis, and it can promote cell killing (Parrish et al. 2001). Although the *cps-6* and *nuc-1* mutants display similar TUNEL phenotypes, they appear to have distinct functions in DNA degradation and apoptosis. First, *cps-6*; *nuc-1* double mutants have more TUNEL-positive cells than either single mutant, suggesting that *cps-6* and *nuc-1* act in different DNA degradation pathways (Parrish et al. 2001). Secondly, *cps-6* affects the timing and the activation of programmed cell death, whereas *nuc-1* is dispensable for apoptosis, suggesting that *cps-6* may act at an earlier stage of apoptosis than *nuc-1*.

To characterize the in vivo function of endo G in mammalian apoptosis, two groups have independently generated endo G knockout mice (Zhang et al. 2003; Irvine et al. 2005).

In one report, endo G deficiency causes embryonic lethality, and cells from endo G heterozygous mutant mice exhibit reduced apoptotic DNA fragmentation and increased resistance to cell death, which are consistent with endo G having an important role in mammalian apoptosis (Zhang et al. 2003). However, these studies are complicated by the observation that the targeting scheme used to disrupt the endo G gene likely affects an adjacent gene. A second group reports that disrupting a single exon of endo G does not result in embryonic lethality or obvious apoptotic defects (Irvine et al. 2005). Thus, it is likely that the embryonic lethality associated with the first endo G knockout may be due to disruption of the adjacent gene. However, since different apoptotic assays were used to examine the phenotypes of mutant mice in these two studies, and since endo G appears to play a secondary role to DFF40/CAD in mediating apoptotic DNA degradation in mammals, examination of DFF40−/− endo G−/− double knockout mice using more sensitive cell death assays may be needed to clearly define the roles of endo G in mammalian apoptosis.

Apoptosis-inducing factor functions in apoptotic DNA degradation

Apoptosis-inducing factor (AIF) was initially identified as a mitochondrial oxidoreductase that is released from mitochondria in response to apoptotic stimuli to induce various apoptotic events in mammals, including chromatin condensation and fragmentation (Susin et al. 1999). Overexpression of AIF or microinjection of the AIF protein into mammalian cells can induce chromatin condensation and high molecular weight chromosome fragmentation, suggesting that AIF may function at early stages of nuclear DNA degradation. However, since AIF does not have an obvious nuclease domain and has no documented nuclease activity (Susin et al. 1999), it seems likely that AIF may need to act through a nuclease effector to promote chromosome fragmentation. The importance of AIF in apoptosis has been confirmed by analysis of AIF-deficient mice, in which embryonic stem cells show resistance to cell death after serum deprivation (Joza et al. 2001; Klein et al. 2002). In addition, AIF is essential for programmed cell death during cavitation of embryoid bodies, which is an indispensable apoptotic event for mouse morphogenesis (Joza et al. 2001).

Apoptosis-inducing factor is a highly conserved mitochondrial protein that is found in diverse organisms from yeast, *C. elegans*, *Drosophila*, to humans (Daugas et al. 2000) and appears to be generally important for apoptosis and DNA degradation (Susin et al. 1999; Wang et al. 2002; Cande et al. 2004; Vahsen et al. 2004; Wissing et al. 2004). For example, reduction of activity by RNAi of the *C. elegans* *wah-1* gene, a worm AIF homolog, causes cell death defects that are very similar to those of the *cps-6* mutant, including accumulation of TUNEL-positive cells, delay of embryonic cell corpse appearance, and inhibition of cell killing in sensitized genetic backgrounds (Wang et al. 2002). These observations suggest that WAH-1, like CPS-6, affects both

apoptosis and DNA degradation. Furthermore, *wah-1* RNAi does not enhance the cell death defects of the *cps-6* mutant, suggesting that *wah-1* and *cps-6* may function in the same pathway to promote DNA degradation and apoptosis. Indeed, AIF can physically associate with CPS-6 and enhance CPS-6 endonuclease activity in vitro and synergize with CPS-6 to promote ectopic cell killing in vivo (Wang et al. 2002). Thus, CPS-6 is likely a downstream nuclease effector of WAH-1, which defines a new mitochondria-initiated apoptotic DNA degradation pathway. Interestingly, although human AIF has been shown to promote apoptosis in a caspase-independent manner (Susin et al. 1999; Joza et al. 2001), some recent studies, including the study in *C. elegans*, indicate that the release of AIF from mitochondria during apotosis is partially dependent on caspase activity (Wang et al. 2002).

Additional cell-death-related nucleases function in multiple pathways or stages to promote apoptotic DNA degradation in *C. elegans*

In an attempt to identify all the nucleases involved in apoptotic DNA degradation in *C. elegans*, an RNAi-based, functional genomic screen was carried out to examine the functions of 77 nuclease (endo- and exonuclease) or nuclease-related genes present in the *C. elegans* genome (Parrish and Xue 2003). Seven nucleases in addition to CPS-6 and NUC-1 were found to be involved in apoptotic DNA degradation and have been referred to collectively as cell-death-related nucleases (CRNs). Reducing *crn* gene activity via RNAi treatment of any of these seven new genes, *crn-1*, *crn-2*, *crn-3*, *crn-4*, *crn-5*, *crn-6*, and *cyp-13* (cyclophilin), leads to an accumulation of TUNEL-positive cells in *C. elegans* embryos, indicating that each of these genes is required for normal apoptotic DNA degradation. In addition, reduction of *crn* gene activity in all cases but one (*crn-6*) causes cell death defects similar to those of the *cps-6* mutant, including a delay of cell death phenotype and inhibition of cell killing in sensitized genetic backgrounds. Furthermore, RNAi of either *crn-2* or *crn-3*, but not any other *crn* genes, significantly enhances the TUNEL phenotype of the *cps-6* mutant, indicating that *crn-2* and *crn-3* likely function in a DNA degradation pathway different from that of *cps-6* and four other *crn* genes (*crn-1*, *crn-4*, *crn-5*, and *cyp-13*). Unlike the other *crn* genes, *crn-6* is dispensable for apoptosis and encodes a DNase II homolog like NUC-1. CRN-6 may thus act in a later stage of apoptotic DNA degradation like NUC-1 (Fig. 1). However, CRN-6 appears to be more specialized for apoptosis and is not required for degradation of other DNA such as DNA from ingested bacteria (Parrish and Xue 2003).

CPS-6, WAH-1, and several CRN nucleases may function together to form a multinuclease complex

Genetic studies suggest that *wah-1* and several *crn* genes function in the same pathway as *cps-6* to promote DNA degradation. Consistent with the genetic observations, biochemical studies have shown that CPS-6, CRN-1, CRN-4, CRN-5, CYP-13, and the nonnuclease cofactor WAH-1 can interact with one another and may form a multinuclease complex, the degradeosome, to promote apoptotic DNA degradation (Parrish and Xue 2003). Why do multiple nucleases need to work together in a complex to promote apoptotic DNA degradation? Genetic and biochemical characterization of the interaction between CPS-6 and CRN-1 has provided important insights into this important question (Parrish et al. 2003). CRN-1 is homologous to human Flap endonuclease 1 (Fen-1), a nuclease critical for DNA replication and damage repair (Li et al. 1995; Bambara et al. 1997; Lieber 1997), and like Fen-1, possesses two unique structure-specific endonuclease activities (Parrish et al. 2003). First, both CRN-1 and Fen-1 cleave DNA flaps (bifurcated structures composed of double-stranded DNA and a displaced single strand), and this activity is important for DNA replication and damage repair (Li et al. 1995; Bambara et al. 1997; Lieber 1997; Parrish et al. 2003). Second, both CRN-1 and Fen-1 can cleave DNA gaps (double-stranded DNA with a single-stranded gap), and this activity appears to be involved in resolving stalled DNA replication forks (Parrish et al. 2003; Zheng et al. 2005). In addition, both Fen-1 and CRN-1 have 5′–3′ exonuclease activity. Interestingly, CRN-1 can potentiate the single-strand DNA nicking activity of CPS-6 via both nuclease-dependent and nuclease-independent mechanisms (Widlak et al. 2001; Parrish et al. 2003). On the other hand, CPS-6 can significantly enhance the gap-dependent endonuclease activity and the 5′–3′ exonulcease activity of CRN-1, but CPS-6 has no effect on its flap endonuclease activity. In both cases, physical interaction between CPS-6 and CRN-1 is required for the enhancement of mutual nuclease activities. Furthermore, CRN-1 can induce ectopic cell deaths in *C. elegans* in a CPS-6-dependent manner, providing important confirmation that these two nucleases need to act together in vivo (Parrish et al. 2003). Based on these studies, a molecular model has been proposed to illustrate how CPS-6, CRN-1, and other CRN nucleases may act cooperatively to promote stepwise chromosome fragmentation during apoptosis, starting from generating DNA nicks, then gaps, and subsequently double-strand DNA breaks (Fig. 2).

In addition to CRN-1, CPS-6, and WAH-1, three other degradeosome components are either exo- or endonucleases. CRN-4 is most similar to human histone mRNA 3′ end-specific exonuclease and is likely a 3′–5′ exonuclease (Dominski et al. 2003). CRN-5 is homologous to Rrp46, a 3′–5′ exonuclease component of the human exosome, which is a multiexonuclease complex important for processing of several types of RNA molecules (Mitchell et al. 1997). CYP-13 is similar to human cyclophilin E and is an endonuclease in vitro (Parrish and Xue 2003). How these three nucleases interact with other components of the degradeosome to promote apoptotic DNA degradation and whether additional components are required to form a functional degradeosome will be the focus of future studies.

Thus far, some of the degradeosome components have been shown to play conserved roles in mediating apoptotic

Fig. 1 Nucleases involved in apoptotic DNA degradation in *C. elegans*, their protein interaction map, and their potential acting sites in the dying or engulfing cell. A *double arrow* indicates an interaction detected between two proteins. CNR-X is a yet-to-be-identified nuclease that cleaves chromosomal DNA to create TUNEL-reactive DNA breaks. The apoptotic DNA degradation process may also generate an "eat me" signal (*red bar* marked with?) that is recognized by an unknown receptor (*pink fork-like structure* marked with?) in the phagocyte to induce phagocytosis
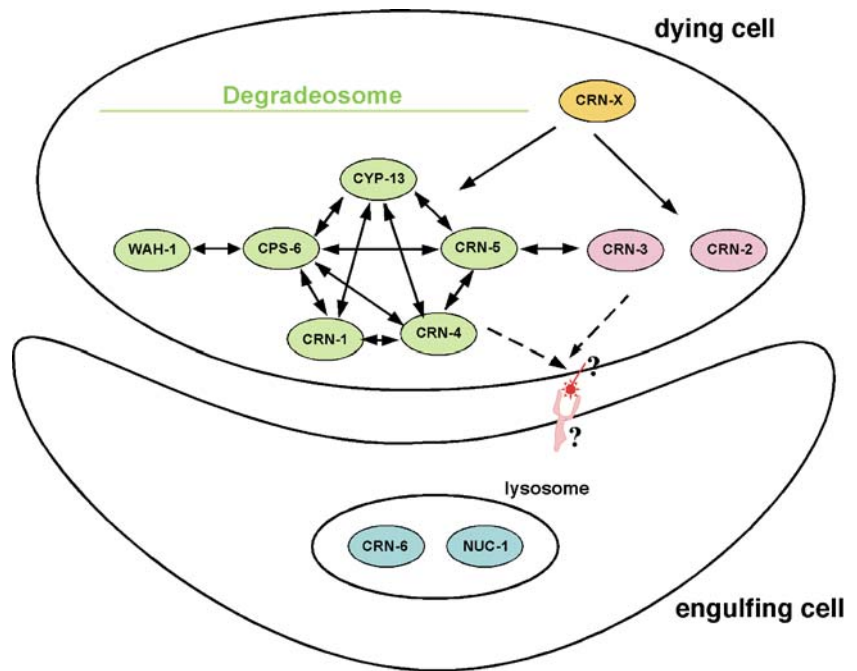
DNA degradation. For example, in both humans and yeast, AIF or its yeast homolog Aif1p physically associates with human or yeast cyclophilin A, respectively, and potentiates the endonuclease activity of cyclophilin A (Cande et al. 2004; Wissing et al. 2004). In vivo, the activity of cyclophilin A is important for AIF or Aif1p to induce apoptosis and apoptotic

DNA fragmentation. Thus, in yeast, *C. elegans*, and humans, cyclophilins are a conserved component of the degradeosome (Fig. 3). Furthermore, human AIF, Fen-1, and endo G have been shown to form a complex in vitro to promote DNA degradation, and Fen-1, a nuclear protein, is found to associate with endo G, a mitochondrial protein, only in apoptotic

Fig. 2 Molecular model for how CRN nucleases (in particular CRN-1/CPS-6) act cooperatively to promote chromosome fragmentation during apoptosis. **a** Intact chromosomal DNA is likely nicked by CPS-6 (aided by CRN-1) and/or another nuclease (CRN-X?). **b** Following nicking, the 5′–3′ exonuclease activity of CRN-1 (aided by CPS-6) and, possibly, other 3′–5′ exonucleases (CRN-4 or CRN-5?) turn the nicks into gaps. **c** The resulting gapped substrates are cleaved by CRN-1 gap-dependent endonuclease activity (aided by CPS-6), resulting in fragmented substrates (**d**), which are either further processed by 3′–5′ exonucleases (CRN-4 or CRN-5?) (**e**) or can be directly processed (**f**) through similar steps (**a–d**, indicated by an *arrow* from **f–b**) to generate smaller DNA fragments
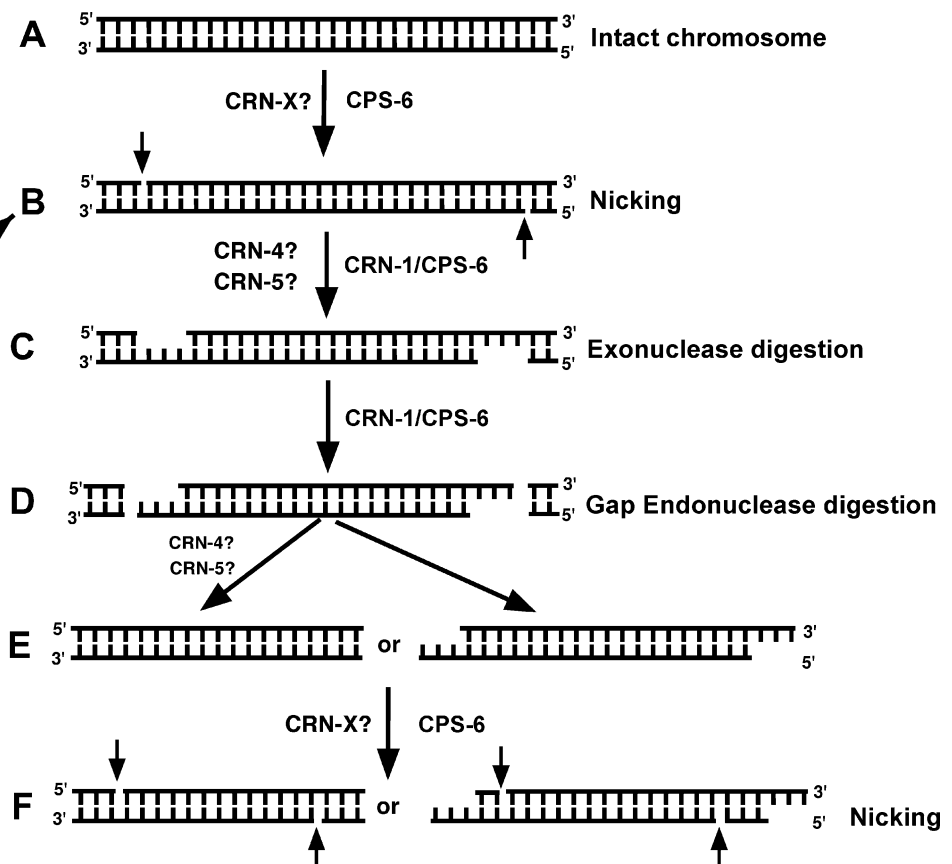
Fig. 3 Components of the apoptotic DNA degradation pathways are conserved among humans, *C. elegans*, and *S. cerevisiae*. Nucleases or proteins that play a conserved role in mediating apoptotic DNA degradation in different organisms are shown. − indicates that a homologous protein does not exist in a specific organism. +? indicates that a homolog exists, but its function in apoptosis has yet to be demonstrated. −? indicates that a homolog does not exist, but a protein with a similar function may exist
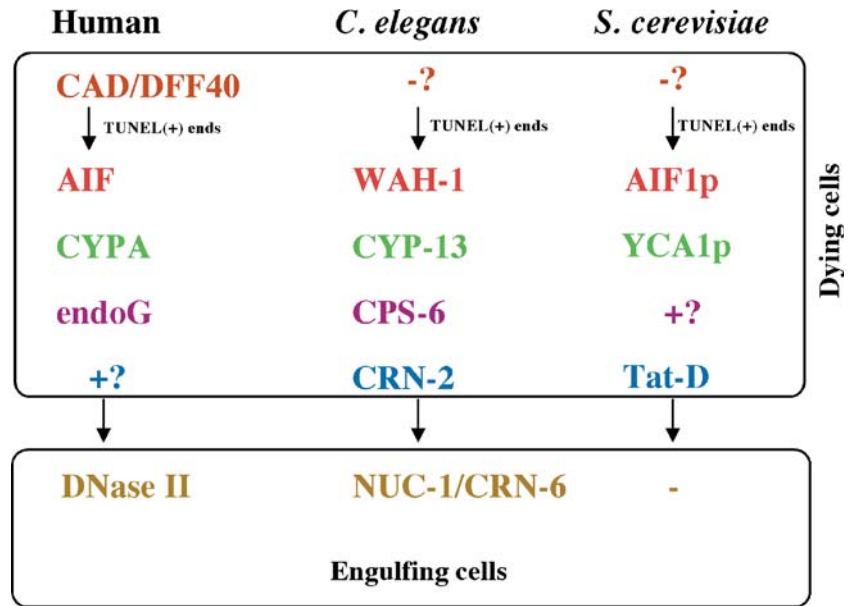
cells but not in normal cells (Kalinowska et al. 2005), suggesting that different components of the degradeosome may be assembled in nuclei from different cellular compartments in response to apoptotic stimuli. Importantly, some of these proteins also have normal cellular functions that are critical for cell survival. For example, CRN-1/FEN-1 is important for DNA repair and replication, cyclophilins for protein folding, AIF for mitochondrial respiration, and CRN-3 and CRN-5 for RNA processing (Parrish and Xue 2003; Vahsen et al. 2004; Joza et al. 2005). During apoptosis, these proteins are likely "hijacked" by the apoptotic machinery to become components of the degradeosome, and thus, these proteins play dual roles in both life and death.

Much less is known about the DNA degradation pathway mediated by *crn-2* and *crn-3*. *crn-2* encodes a homolog of human Tat-D nuclease, which is conserved from yeast to mammals. A recent study from *Saccharomyces cerevisiae* demonstrates that yeast deficient in the Tat-D nuclease display rather similar cell death phenotypes to those of the worm *crn-2*(RNAi) animals, including increased TUNEL-positive staining and enhanced cell survival in response to hydrogen peroxide treatment (Qiu et al. 2005). In addition, overexpression of yeast Tat-D in *S. cerevisiae* facilitates cell death, suggesting that CRN-2/Tat-D is also a conserved apoptotic nuclease (Fig. 3). Interestingly, *crn-3* encodes a homolog of another human exosome component, the 100-kD polymyositis/scleroderma autoantigen (PM/Scl-100). It is intriguing that two homologs of the human exosome components are involved in apoptotic DNA degradation in *C. elegans*, but it is not yet clear if the exosome itself is involved in apoptosis.

Apoptotic DNA degradation may facilitate clearance of apoptotic cells

One intriguing observation that arose from the analyses of *C. elegans* CRN nucleases is that simultaneous disruption of the *crn-2* and *cps-6* DNA degradation pathways causes a synthetic cell corpse engulfment defect because more cell corpses are seen at every developmental stage in *cps-6* (*sm116*); *crn-2*(RNAi) or *cps-6(sm116)*; *crn-3*(RNAi) embryos than in *cps-6(sm116)*, control(RNAi) embryos. Lineage analysis using four-dimensional microscopy confirmed that compromising both DNA degradation pathways prolongs the persistence of embryonic cell corpses by more than 50% compared with wild-type embryos or embryos with a defect in one of two DNA degradation pathways (Parrish and Xue 2003). Therefore, the apoptotic DNA degradation process somehow facilitates the cell corpse engulfment process. Consistent with this observation, reduction of either *crn-2* or *cps-6* activity significantly enhances the defect of some cell corpse engulfment mutants (Aki Nakagawa and D. Xue, unpublished results). How does the apoptotic DNA degradation process affect clearance of apoptotic cells? One attractive hypothesis is that a certain "eat me" signal(s) is generated during apoptotic DNA fragmentation and exposed on the surface of apoptotic cells, which can trigger phagocytosis (Fig. 1). Indeed, two recent studies have shown that nucleosomes, products of chromosome fragmentation, are exposed on the surface of apoptotic cells and appear to enhance phagocytosis of apoptotic cells by macrophages (Radic et al. 2004; Frisoni et al. 2005). Thus, what eat me signal(s) is generated during apoptotic DNA degradation and how it is exposed on the surface of apoptotic cells and recognized by phagocytes will be an exciting question for future research.

DNase II in phagocytes contributes to apoptotic DNA degradation

In mice lacking DFF40/CAD activity, degradation of DNA from apoptotic cells appears to proceed normally in tissues (e.g., the immune system) where large numbers of cells

undergo apoptosis (McIlroy et al. 2000). Further studies have indicated that DNA from apoptotic cells appears to be degraded inside macrophages by DNase II, a lysosomal acidic DNase (McIlroy et al. 2000; Kawane et al. 2003), suggesting that phagocytes also contribute to the degradation of DNA from apoptotic cells. Indeed, many tissues from DNase II −/− deficient mice contain large DNA-containing bodies that likely result from engulfed but undigested apoptotic cells (Krieser et al. 2002; Kawane et al. 2003). In addition, these DNase II −/− deficient mice are defective in definitive erythropoiesis in the fetal liver and die at birth, suggesting that the DNase II activity is critical not only for digesting DNA of engulfed apoptotic cells but also for other cellular and developmental functions such as erythropoiesis (Kawane et al. 2001; Krieser et al. 2002).

DNase II −/− CAD −/− double-mutant mice display defects that are either similar to or stronger than those of the DNase II −/− mutant mice (Kawane et al. 2003). Notably, the number of thymocytes and the progression of thymocyte development are severely affected in the DNase II −/− CAD −/− mutant mice. In particular, expression of interferon-β is sharply up-regulated in the double-mutant mice. Therefore, it is possible that the undigested DNA accumulated in the double-mutant mice triggers an innate immune response, resulting in reduced numbers of thymocytes and impaired thymic development. Consistent with these observations in DNase II −/− CAD −/− mutant mice, *Drosophila* mutants that are defective in both *dicad* (*Drosophila icad)* and *dDNase II* (*Drosophila DNaseII*) or *dDNase II* alone also display inappropriate activation of innate immunity, as shown by the expression of a subset of antibacterial peptide genes (Mukae et al. 2002). Similar to DFF45/ICAD in mammals, dICAD is required for apoptotic DNA degradation in *Drosophila* embryos and ovaries. The role of *dDNase II* in apoptotic DNA degradation is less clear, but a large amount of undigested DNA accumulates in *dDNase II* mutant ovaries, which likely triggers constitutive expression of the antibacterial genes. These studies suggest that defects in apoptotic nucleases can have other physiological consequences in addition to affecting the proper activation and execution of apoptosis.

Apoptotic DNA degradation and autoimmune disorders

In addition to its roles in promoting cell killing, apoptotic DNA degradation may play an important role in higher organisms to remove highly antigenic DNA or nucleosomes from apoptotic cells to prevent them from eliciting autoimmune responses (Zhang and Xu 2002). In fact, a number of human autoimmune disorders, including lupus, are characterized by high concentrations of circulating DNA that may result from failure to properly execute apoptotic DNA degradation (Fournie 1988; Suzuki et al. 1997). For example, DNase I, a secreted nuclease present in serum, urine, and secreta, has been implicated in digesting extracellular DNA or chromatin released at sites of high cell turnover or apoptosis (Napirei et al. 2000). Reduction or loss of DNase I activity has been associated with systemic lupus erythema-

tosus (SLE), a multifactorial autoimmune disease characterized by the presence of autoantibodies against nucleosomal antigens (Napirei et al. 2000; Yasutomo et al. 2001). Additionally, autoantibodies against PM-Scl100 and Rrp46, mammalian homologs of CRN-3 and CRN-5, respectively, are often found in patients with scleroderma, polymyositis/scleroderma overlap syndrome, and idiopathic inflammatory myopathy (Brouwer et al. 2002). Given that CRN-3 and CRN-5 are important for apoptotic DNA degradation in *C. elegans*, inactivation of PM-Scl100 or Rrp46 by autoantibodies or genetic mutations in humans could compromise apoptotic DNA degradation, providing a source of undegraded DNA that either elicits or augments autoimmune responses. Therefore, it will be intriguing to determine whether inactivation by genetic mutations (or other means) of other apoptotic nucleases is associated with additional autoimmune disorders.

Concluding remarks

The last several years have witnessed major progress in dissecting the molecular components that activate and execute chromosome fragmentation in apoptotic cells. In particular, a plethora of apoptotic nucleases with different endo- and/or exo-nuclease activities and different substrate specificities have been identified and found to act cooperatively in multiple pathways and in both dying cells and phagocytes to promote stepwise chromosome fragmentation and DNA degradation. Importantly, apoptotic DNA degradation not only directly contributes to the disassembly of the dying cell but also can facilitate the cell-killing process and other apoptotic events such as clearance of apoptotic cells. Although defects in apoptotic DNA fragmentation have not been causally associated with defects in tissue homeostasis or diseases in humans, evidence has emerged that it can impact normal animal development, immune responses, and potentially, animal behavior. In particular, failure to degrade DNA of apoptotic cells can induce robust immune responses and may lead to the development of autoimmune disorders. Therefore, exciting future directions for researchers will be to understand the roles and functions of these apoptotic nucleases in animal development and tissue homeostasis and to uncover the hidden links between apoptotic DNA degradation and human diseases.

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