The Nuclease CPS-6 Overcomes Autoinhibition by Interacting with *wah-1* During Apoptosis in *C. elegans*

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Abstract

Apoptosis depends on multiple pathways to execute changes in the biochemistry and structure of the dying cell. Apoptotic DNA degradation is one such pathway that fragments the chromosomes, allowing them to be cleared from the debris of the dead cell. In *C. elegans*, apoptotic DNA degradation is also important to the highly reproducible pattern of cell killing that occurs during normal development. Regulation of apoptotic DNA degradation occurs by multiple mechanisms. My thesis recapitulates the finding that interaction between nucleases is important for their killing activity. Additionally, I will show that mutation of the glutamine 130 residue of CPS-6 to alanine or lysine activates CPS-6. *cps-6* normally requires *wah-1* to promote the cell killing and the proper timing of apoptosis. These CPS-6 Q130 mutants, however, can function independently of *wah-1*. These results also suggest that, during apoptosis, the primary function of *wah-1* is to activate CPS-6.

Introduction

As the state of an organism changes over time cells become unneeded or potentially harmful. Such cells can be removed through apoptosis, a process of cell death. Apoptosis is conserved throughout metazoa (Vaux & Korsmeyer, 1999) with importance both in development and in the context of disease. For example, apoptosis patterns the nervous system during development, but the death of neurons that should survive contributes to Alzheimer's and Parkinson's diseases (Okouchi, Ekshyyan, Maracine, & Aw, 2007). Furthermore, some cancer cells contain mutations that suppress apoptosis (Vaux, Cory, & Adams, 1988). Apoptosis involves the coordination of many biochemical and cell biological processes. Cell death need not be entirely blocked to cause disease. Mutation of a single gene that degrades DNA in the dying cell has been associated with an autoimmune disease (Lehtinen, Harvy, Mulcahy, Hollis, & Perrino, 2008) (Chowdhury, et al., 2006). In order to treat these diseases it is important to know the mechanisms of apoptosis and its regulation.

The caspase family of aspartate-specific cysteine proteases plays a central role in the prototypical model of cell death. Caspase activation represents a critical transition from regulatory interactions to the execution of distinct cellular changes characteristic of apoptosis. These cellular changes include shrinkage of the cytoplasm, compaction and fragmentation of the chromosomes, and blebbing of membrane bound cellular debris. Signals for a cell to undergo apoptosis are transduced through an evolutionarily conserved set of proteins. These proteins include EGL-1[1] the homolog of mammalian BH3-only proteins, CED-9 the homolog of Bcl-2 family proteins and CED-4 the homolog of mammalian Apaf-1 (Metzstein, Stanfield, & Horvitz, 1998). CED-4 and its homolog Apaf-1 bind and activate CED-3 and caspase-9 respectively (Zou, Li, & Wang, 1999). *ced-3* encodes the only caspase in *C. elegans* and is required for all cell-death events that occur as part of embryogenesis (Ellis & Horvitz, 1986). In humans, however, there are

[1] By convention, *C. elegans* proteins are denoted using capitol letters, the genes associated with these proteins are denoted using italics

multiple caspases that are subject to complex regulation; their activation is neither necessary nor sufficient for cell death in (Kroemer & Martin, 2005). Once active, caspases cleave specific sequences in numerous targets to activate downstream pathways. Some of the downstream pathways function to fragment chromosomes (Parrish, Li, Klotz, Ledwich, Wang, & Xue, 2001) (Ellis & Horvitz, 1986), eliminate mitochondria (Breckenridge, Kang, Kokel, Matani, Staehelin, & Xue, 2008), and allow the engulfment of debris from the dying cell (Ellis & Horvitz, 1986).

Degradation of chromosomal DNA is performed by a set of Deoxyribonuclease (DNase) enzymes. Apoptotic DNases vary in both their substrate specificity and in the degradation reaction that they catalyze, potentially acting stepwise to promote chromosome fragmentation (Fig 1). The progress of apoptotic DNA degradation can be monitored by observing DNA degradation intermediates which contain 3' hydroxyl ends. tDCR is an endonuclease that generates these 3'OH intermediates (Nakagawa, Shi, Kage-Nakadai, Matani, & Xue, 2010). The endonuclease CPS-6 (a homolog of mammalian EndoG) acts later in the process and is important for resolving the 3' OH intermediates (Parrish et al., 2001). CPS-6 nuclease activity is enhanced by a cofactor WAH-1 that binds to CPS-6 *in vitro* (Wang, Yang, Chai, Shi, & Xue, 2002). WAH-1 is a homolog of mammalian AIF (Wang et al., 2002). Degradation of 3' OH intermediates also involves the 5'-3' exonuclease and gap-dependent endonuclease activities of CRN-1 (Parrish, Yang, Shen, & Xue, 2003). CRN-1 also interacts with CPS-6 causing enhancement of the activities of both nucleases (Parrish et al., 2003b). *dcr-1*, *wah-1*, *cps-6* and *crn-1* are important for ensuring that cells fated to die undergo apoptosis and for the normal timing of this process (Nakagawa et al., 2010) (Wang et al., 2002) (Parrish et al., 2001) (Parrish et al., 2003b). Other nucleases, such as *nuc-1* and *crn-6*, likely function primarily to clear chromosomal debris from the dying cell (Parrish & Xue, 2003a).

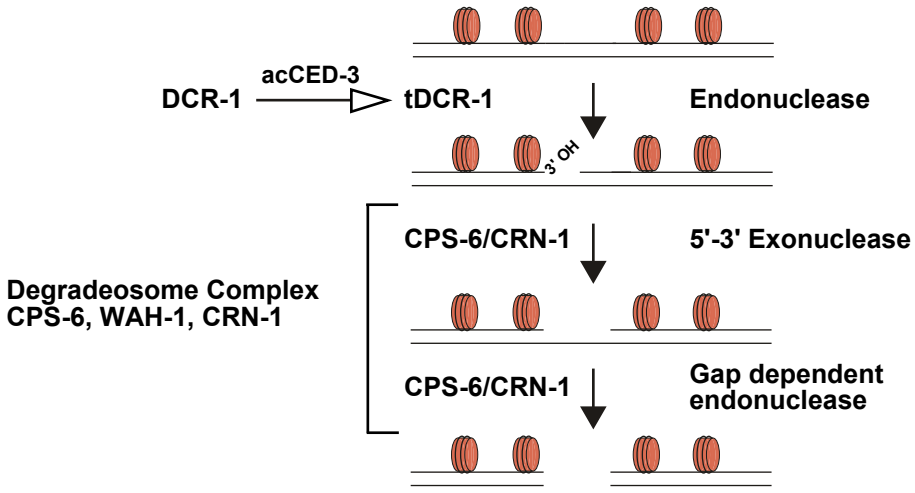


Figure 1. A model of apoptotic DNA degradation. tDCR-1, acts early in apoptotic DNA degradation by nicking DNA to generate TUNEL reactive 3'OH ends. The degradation of these intermediates proceeds through a pathway containing *wah-1, cps-6* and *crn-1*. CRN-1 has 5'-3' exonculease and gap-dependent endonuclease activities which may act sequentially to fragment chromosomes. CPS-6 also has endonuclease activity. WAH-1, CPS-6, CRN-1 and other proteins associate to form the degradeosome complex. Interactions within the degradasome are important for the activation of both CRN-1 and CPS-6 nuclease activities.

Robust regulation of the components of the apoptotic DNA degradation pathway prevents potential genome instability. The mechanisms of regulation commonly involve the transition of a nuclease from one function in the living cell to another function in the dying cell. In a living cell, DCR-1 functions as a ribonuclease (RNase), processing small RNA that are important for RNA interference (Ketting, Fischer, Bernstein, Sijen, Hannon, & Plasterk, 2001). CED-3 cleavage of DCR-1 converts its RNase activity to DNase activity thereby allowing it to degrade chromosomes (Nakagawa et al., 2010). In the living cell, CPS-6 and WAH-1 both localize to the mitochondria where CPS-6 may function in mitochondrial DNA replication (Cote & Ruiz-Carrillo, 1993) (Parrish, et al., 2001) (Wang et al., 2002). The impermeability of the mitochondrial membrane to these nucleases would preclude their translocation to the nucleus. Nonetheless, CPS-6 constructs lacking their putative mitochondrial localization signal have been observed to localize to the nucleus (Parrish et al., 2001). WAH-1 translocates to the nucleus in a CED-3

dependent manner where CPS-6 and WAH-1 act together to degrade the chromosomes (Wang et al., 2002). In addition to CPS-6 binding to WAH-1 and CRN-1, large numbers of other nucleases bind one another *in vitro* potentially forming a complex termed the "degradeosome" (Parrish & Xue, 2003a). The large number of binding interactions within this complex suggests the possibility of complex regulatory mechanisms, even after initial acCED-3 mediated activation. The remainder of this paper presents evidence that *wah-1* is important to overcoming CPS-6 autoinhibition, thereby allowing the nuclease to facilitate degradation of the chromosomes. A method for the observation of interactions between components of the apoptotic DNA degradation pathway *in vivo* is also presented and used to characterize the CPS-6/*wah-1* interaction.

Method

Strains and Culture Conditions

C. elegans strains were maintained using standard procedures (Brenner, 1974). Nematode growth media was prepared by dissolving 24g NaCl, 130g agar (Fisher), and 20 g peptone in 8 L distilled water. The solution was sterilized by autoclaving before adding the following solutions: 4 ml 10 mg/ml cholesterol dissolved in absolute ethanol, 8 ml 1M $CaCl_2$, 8 ml 1M $MgSO_4$ and 200 ml of pH6 potassium phosphate buffer. Plates were seeded with the OP50 strain of *E. coli*. Animals were cultured at room temperature (usually between 20 and 25°C). The following alleles were used in genetic analysis: LGI, *ced-1(e1735)*, *cps-6(sm116)*, *crn-1(tm5973)*; LGIII, *wah-1(tm1159)*, *dcr-1(ok247)*; LGIV *ced-3(n2433)*.

Quantification of Cell Corpses and Missing Cells

The number of cell corpses in living embryos and the number of cells missing from the anterior pharynx of L4-stage animals were observed using differential interference contrast (DIC) optics and quantified as described previously (Geng, et al., 2008). Specimens were mounted between a cover slip and a pad of nematode growth media on a glass slide. A drop (approximately 2μL) of 0.1mM sodium

azide was added to the pad to paralyze the worms. Observations were made using Zeiss AxioPlan-2 microscope. This microscope was equipped with DIC optics, including a Zeiss Plan-Neofluar 100x objective as well as optics to allow identification of transgenic animals using a GFP reporter.

Defects in the normal progression of apoptosis were identified based on a modified version of a previously described protocol (Ellis, Jacobson, & Horvitz, 1991). Mixed stage embryos were collected. Developmental stages were determined based on the shape of the embryos. Embryos with evidence of developmental defects were excluded from further observation. Cell corpses were quantified for embryos at comma, 1.5 fold, 2 fold, 3 fold and 4 fold stages. Under DIC optics, cell corpses show a distinctive raised disc-like and highly refractile morphology (Sulston & Horvitz, 1977).

Although specific cells are normally eliminated from the anterior pharynx by apoptosis, in cases where this process was defective, presence of these cells in L4 larva was identified using a previously developed method (Hengartner, Ellis, & Horvitz, 1992). Essentially, these extra cells are identified based on their position within the pharynx.

Nuclease Assay

DNase activity of CPS-6 on plasmid substrate was assayed as described previously (Wang et al., 2002). WAH-1 and CPS-6 were purified after expression in the BL21 *E. coli* strain. WAH-1 was expressed from a pET21b-WAH-1(214-700)::HIS construct and CPS-6 was expressed from pET30c-HIS::CPS-6(21-308)::FLAG construct that was either unmutated or carried a Q130 to A or Q130 to K mutation in CPS-6. Purified CPS-6 was incubated with 1μg of plasmid DNA either alone or in the presence of a given volume of the purified WAH-1 construct. The nuclease reaction proceeded for 1 hour in the previously described buffer and was visualized using a 1% agarose gel (Wang et al., 2002). When it was necessary, control of the quantity of different CPS-6 mutants was confirmed by anti-6xHis western blot.

Molecular Biology

Many of the constructs used in this study were generated and described as part of previous studies, including P_{dcr-1}tDCR-1 (Nakagawa et al., 2010), P_{dpy-30}CPS-6 and P_{hsp}CPS-6ΔN (Parrish et al., 2001), and P_{hsp}WAH-1ΔN (Wang et al., 2002). PCR-based site-directed mutagenesis was used to generate the P_{dpy-30}CPS-6(Q130A) and P_{hsp}CPS-6ΔN(Q130A). The primers for this PCR reaction were 5' TTC AAG CCA GAT ATC ACT TTC CCG AAG AAA TTC CTT TCT CAA AAT ACG 3' and 5' CGT ATT TTG AGA AAG GAA TTT CTT CGG GAA AGT GAT ATC TGG CTT GAA 3'. Similarly, PCR-based site-directed mutagenesis was used to generate P_{dpy-30}CPS-6(Q130K) and P_{hsp}CPS-6ΔN(Q130K) constructs. The primers used in this PCR reaction were 5' GCC AGA TAT CAC TTT CCC GGC CAA ATT CCT TTC TCA AAA TAC GG 3' and 5' CCG TAT TTT GAG AAA GGA ATT TGG CCG GGA AAG TGA TAT CTG GC 3'. P_{hsp}CRN-1 is a translational fusion of CRN-1 and RFP generated by Jay Parrish. Jay subcloned CRN-1 into pPD49.78 and pPD49.83 via EcoRV and Spe1 sites, but the exact sequence of CRN-1 used is not clear from his records. P_{hsp}WAH-1ΔN(R473E) was generated by modifying a pET21b WAH-1(R473E) construct made by Dr. Xiao Ge. The sequence encoding amino acids 214-700 of WAH-1 was amplified by PCR using the following primers, 5' TCC CCC GGG AGC ACT CTT CGC ATC GTC TT 3' and 5' CGG CTA GCA TGT CCG AAC AAC AAT CGA TG 3' then digested using Nhe1 and Sma1. This fragment was then ligated into both pPD49.78 and pPD49.83 via their Nhe1 and EcoRV sites.

Transgenic Animals

Transgenic animals were generated by standard methods (Mello, Kramer, Stinchcomb, & Ambros, 1991). Combinations of P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}CPS-6ΔN(Q130A) , P_{hsp}CPS-6ΔN(Q130K), P_{hsp}WAH-1ΔN, P_{hsp}WAH-1ΔN(R473E), P_{hsp}CRN-1ΔN and pTG96 were injected into either *ced-1(e1735)* or *ced-1(e1735); ced-3(n2433)* animals at a concentration of 20 ng/µl. Either P_{dpy-30}CPS-6, P_{dpy-30}CPS-6(Q130A) or P_{dpy-30}CPS-6(Q130K), together with pTG96 were injected into *cps-*

6(sm116), crn-1(tm5973), wah-1(tm1159) or *dcr-1(ok247)* animals at 20 ng/µl. The pTG96 plasmid contains a *sur-5::gfp* translational fusion that is expressed in all cells at most developmental stages (Gu, Orita, & Han, 1998).

Heat-Shock Experiments

Plates containing mixed-stage animals were heat-shocked at 33°C for 45 minutes. Next, animals were returned to 20°C for 3 hours before being scored for either embryonic cell corpses or missing cells.

Results

Activated Apoptotic DNases Can Act Together to Induce Apoptosis

In addition to clearing chromosomal DNA from a dying cell, apoptotic nucleases are known to be important to the highly invariant killing of cells that are fated to die in *C. elegans*. Deficiencies in *dcr-1, wah-1, cps-6* or *crn-1* occasionally cause cells that would normally undergo apoptosis to persist throughout development; increased numbers of undead cells are observed in sensitized genetic backgrounds where the activation of apoptosis is compromised (Wang et al., 2002) (Nakagawa et al., 2010) (Parrish et al., 2001) (Parrish et al., 2003b). Pathway analysis has shown that deletion alleles of *dcr-1* or *crn-1(RNAi)* do not enhance the *cps-6(sm116)* extra-cell phenotype, suggesting that the *dcr-1* and *crn-1* promote cell killing in the *cps-6* pathway. (Nakagawa et al., 2010) (Parrish et al., 2003b) In contrast, *wah-1(RNAi)* of *cps-6(sm116)* resulted in a stronger extra-cell phenotype than either single mutant (Wang et al., 2002). The low proportion of extra cells in the *cps-6(sm116)* and *wah-1(RNAi)* animals make it difficult to test the significance of differences in these phenotypes without large samples. The role of *wah-1* in phosphatidylserine externalization in the dying cell, reported by (Wang, et al., 2007), may be responsible for this effect. Nevertheless, other experiments suggest that *cps-6* and *wah-1* function in the same pathway to resolve tunnel-reactive intermediates. Together, these results suggest that *dcr-1, wah-1* and *crn-1* all function in the same pathway as *cps-6* to degrade chromosomes during apoptosis.

The relationship of this pathway to other components of the cell-death machinery has been elucidated by epistasis experiments that place *cps-6* and *dcr-1* downstream of activated *ced-3*. These results show that apoptotic DNA degradation is important to the reproducible and irreversible killing of cells by apoptosis.

Just as loss-of-function mutations in apoptotic DNA-degradation pathway can prevent physiological cell death, gain-of-function mutations in this pathway can kill cells that would normally survive. To date, the apoptotic nuclease with the strongest cell-killing activity is tDCR-1, the activated form of DCR-1. Overexpression of tDCR-1 causes increased numbers of cell corpses at many stages of embryonic development (Nakagawa et al., 2010). Overexpression of other apoptotic nucleases has, thus far, not been shown to cause ectopic cell death during embryonic development. Nevertheless, cell-type specific overexpression of CPS-6, WAH-1, or CRN-1 in normally living PLM neurons can cause these neurons to die (Parrish et al., 2003b) (Wang et al., 2002). The strong loss-of-function allele, *ced-3(n2433)* was unable to block all of the ectopic PLM deaths (Parrish et al., 2003b) (Wang et al., 2002). This finding suggests that these deaths may have occurred by a mechanism other than apoptosis. Interpretation of these results in the context of physiological apoptosis is therefore problematic. Coexpression of CPS-6 and a truncated form of WAH-1 showed a synergistic enhancement of PLM cell killing, suggesting that these nucleases act together to kill the cell (Wang et al., 2002). Similarly, CRN-1 induced PLM killing is partially suppressed by *cps-6(sm116)* (Parrish et al., 2003b). Although apoptotic nucleases clearly have cell-killing activity, enhancement of this activity through interaction between components of the pathway also seems common. The highly regulated nature of the DNA degradation pathway makes observation of the cell-killing activity of its component nucleases difficult. Advances, in the understanding of the regulation of individual apoptotic nucleases, open the possibility of using multiple, activated nucleases to examine interactions within the pathway.

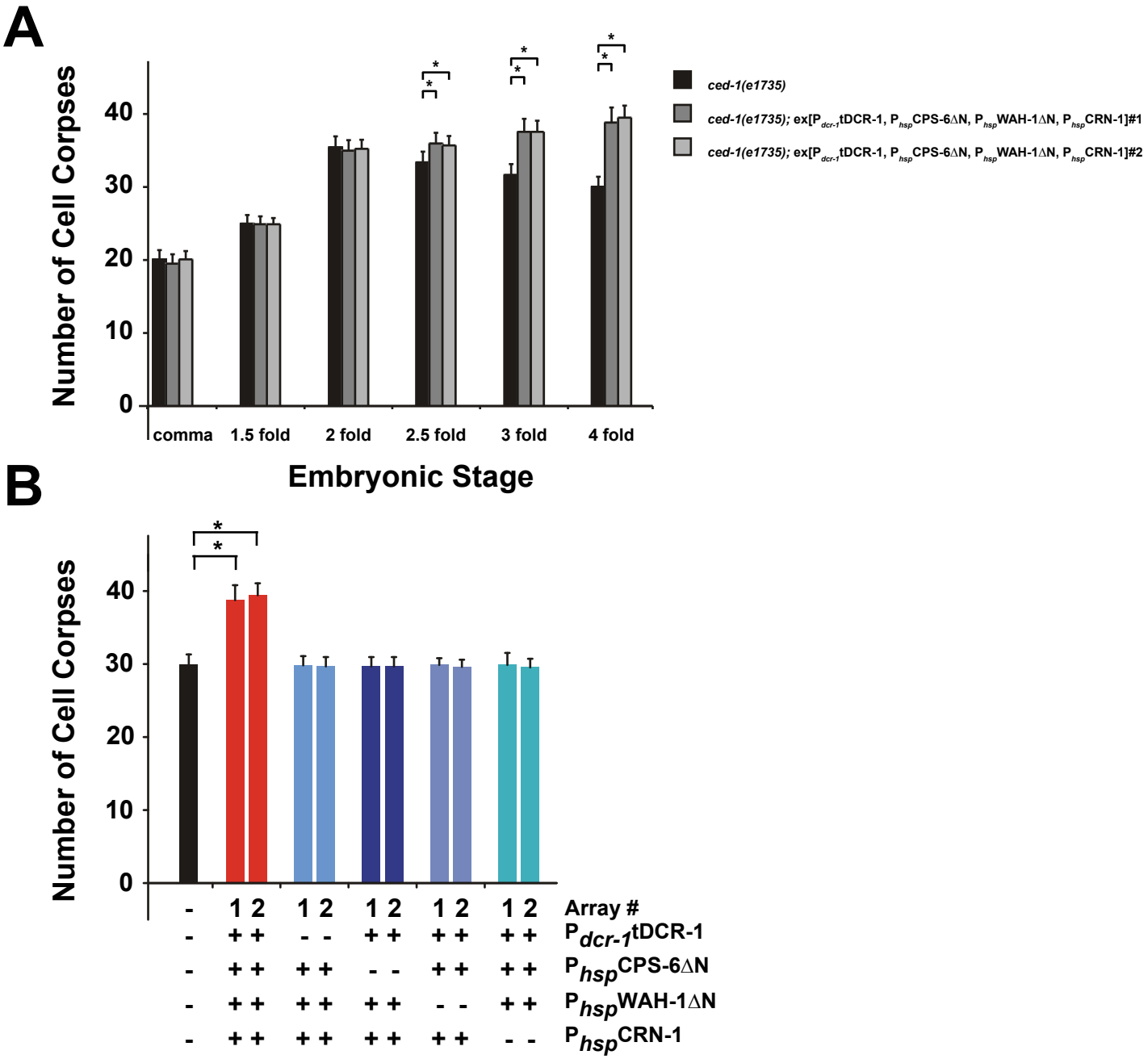


Figure 2. Ectopic cell death required multiple apoptotic nucleases. (A) Embryonic cell corpses were quantified, after heat shock, for the following animals: *ced-1(e1735)*, *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] arrays # 1-2. Embryonic stages observed were comma, 1.5 fold, 2 fold, 2.5 fold, 3 fold and 4 fold. (B) Cell corpses were scored in 4 fold stage embryos for the following animals: *ced-1(e1735)*, *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] arrays # 1-2, *ced-1(e1735);* ex[P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] arrays # 1-2, *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] arrays # 1-2, *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}CRN-1] arrays # 1-2 and *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN] arrays # 1-2. The y-axis represents the mean number of cell corpses of 15 embryos. Error bars represent standard deviation (SD). The significance of differences between different transgenic and non-transgenic backgrounds was determined by unpaired two-tailed t-test, *p<0.001, **p<0.05.

We tested whether multiple, activated nucleases could act together to cause ectopic embryonic cell death. Four nucleases were coexpressed: tDCR-1, CRN-1, CPS-6ΔN, and WAH-1ΔN. tDCR-1 was expressed under the endogenous *dcr-1* promoter. tDCR-1 is a truncated form of DCR-1 that does not require CED-3 cleavage in order to promote cell death. CRN-1 was expressed under heat-inducible promoters (P_{hsp}). The heat-shock promoters can drive transgene expression in most cells during embryonic development (Merritt, Gallo, Rasoloson, & Seydoux, 2010). The heat-shock promoter was also used to drive expression of CPS-6ΔN (containing amino acids 22-308) and WAH-1ΔN (containing amino acids 214-700). These proteins both lack N-terminal amino acid sequences predicted to encode mitochondrial localization signals (Parrish, et al., 2001) (Wang et al., 2002). Upon translocation of a protein into the mitochondria, the signal sequence can be proteolytically removed to generate a mature form of the protein. Without these targeting sequences, CPS-6ΔN and WAH-1ΔN mimic their mature form and may also mislocalize, thus bypassing their usual confinement to the mitochondrial matrix in living cells. An engulfment deficient mutant, *ced-1(e1730),* was used to facilitate the observation of small numbers of ectopic cell-death events. In *ced-1(e1730)*; ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] animals, increased numbers of cell corpses were observed in 2.5 fold, 3 fold and 4 fold stages of embryogenesis as compared to nontransgenic animals (Fig 2a). The increased number of cell corpses was suggestive of ectopic cell death, but further characterization of this phenotype was needed.

Cell corpse appearance is a complex phenomenon. Increased numbers of cell corpses could potentially result from increased cell death but also from changes in the progression of cell death, or the rate at which corpses are engulfed and removed. To better understand the increased numbers of cell corpses observed, Dr. Akihisa Nakagawa observed the pharynx of L4 stage animals expressing tDCR-1, WAH-1, CPS-6 and CRN-1. These nucleases caused missing cells of multiple cell types (Table 1). Next, we examined the requirements for tDCR-1, CPS-6, WAH-1 and CRN-1 to cause increased numbers of cell corpses during embryogenesis. The increased number of cell corpses caused by these nucleases was suppressed by *ced-3(n2433)*, a strong loss-of-function that blocks physiological apoptosis at all stages of embryogenesis

(Table 2). Requirement of *ced-3* is a hallmark of apoptotic cell death and suggests that these deaths did not occur by some alternative stress induced mechanism. To find the minimum set of these nucleases capable of inducing apoptosis, we expressed all combinations including 3 of the 4 original nucleases and checked for increased numbers of cell corpses in 4 fold stage embryos. No ectopic cell death was observed in the absence of any one of the four nucleases (Fig. 2b). Endogenous CRN-1 localizes to the nucleus of living cells (Parrish et al., 2003b). It is therefore surprising that this endogenous CRN-1 was not sufficient to promote ectopic cell death and that instead overexpression of CRN-1 was required. The synergistic interaction between these nucleases underscores the cooperative nature of the apoptotic DNA-degradation pathway's ability to promote cell death.

Table 1. Apoptotic nucleases cause missing cells

Genotype	Array #	Heat shock	Missing cell average	% of normal cells missing							
				m2	e1	I1	m1	I2	MC	NSM	M4
N2		-	0 ± 0	0	0	0	0	0	0	0	0
		+	0 ± 0	0	0	0	0	0	0	0	0
ced-1(e1735)		-	0 ± 0	0	0	0	0	0	0	0	0
		+	0 ± 0	0	0	0	0	0	0	0	0
ced-1(e1735); ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1]	1	-	0 ± 0	0	0	0	0	0	0	0	0
	1	+	0.24 ± 0.06	0.7	0.7	3.0	0.5	3.0	1.0	1.0	0
	2	-	0 ± 0	0	0	0	0	0	0	0	0
	2	+	0.28 ± 0.06	0.7	0	2.0	1.0	5.0	0	2.0	2.0

Table 1. Pharynxes of L4 hermaphrodites were checked for the absence of cells that would normally survive. The following strains were examined: N2, *ced-1(e1735),* and *ced-1(e1735);* ex[P_{dcr-1}tDCR-1 P_{hsp}CPS-6ΔN P_{hsp}WAH-1ΔN P_{hsp}CRN-1] arrays 1-2. Each strain was observed both with and without exposure to heat-shock conditions. Cell identity was determined using DIC optics. Cells that normally live, but were missing are reported as mean ± s.e.m. Normally, L4 hermaphrodites exhibit the following cells: 6 m2 muscle cells, 3 e1 epithelial cells, 2 I1 neurons, 4 m1 muscle cells in two ventral planes, 2 I2 neurons, 2 MC neurons, 2 NSM neurons, and one M4 neuron.

Table 2. ced-3(n2433) suppresses ectopic cell deaths caused by apoptotic nucleases

Genotype	Array #	Number of cell corpses		
		2.5 fold	3 fold	4 fold
ced-1(e1735)		33.3±1.4	31.5±1.5	29.9±1.3
ced-1(e1735); ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1]	1	35.9±1.5	37.5±1.5	38.7±2.1
	2	35.6±1.3	37.5±1.3	39.4±1.6
ced-3(n2433)		0±0	0±0	0±0
ced-1(e1735); ced-3(n2433); ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1]	1	0±0	0±0	0±0
	2	0±0	0±0	0±0
	3	0±0	0±0	0±0

Table 2. Embryonic cell corpses were quantified, after heat-shock, for the following animals: ced-1(e1735), ced-1(e1735); ex[P_{dcr-1}tDCR-1 P_{hsp}CPS-6ΔN P_{hsp}WAH-1ΔN P_{hsp}CRN-1] arrays 1-2, ced-3(n2433), ced-1(e1735); ced-3(n2433); ex[P_{dcr-1}tDCR-1 P_{hsp}CPS-6ΔN P_{hsp}WAH-1ΔN P_{hsp}CRN-1], arrays1-3. Embryonic stages observed were 2.5 fold, 3 fold, and 4 fold. For each strain and developmental stage, fifteen animals were observed. The number of cell corpses is reported as the sample mean ± SD.

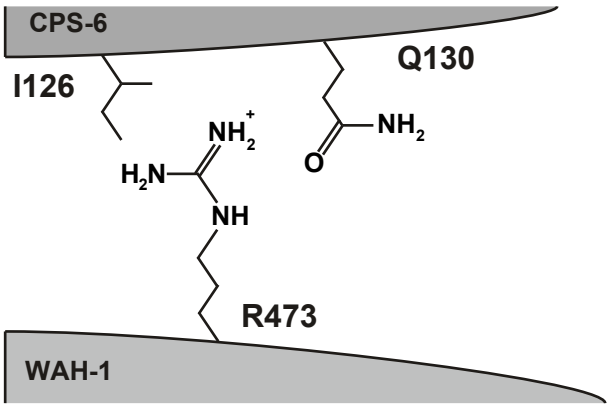
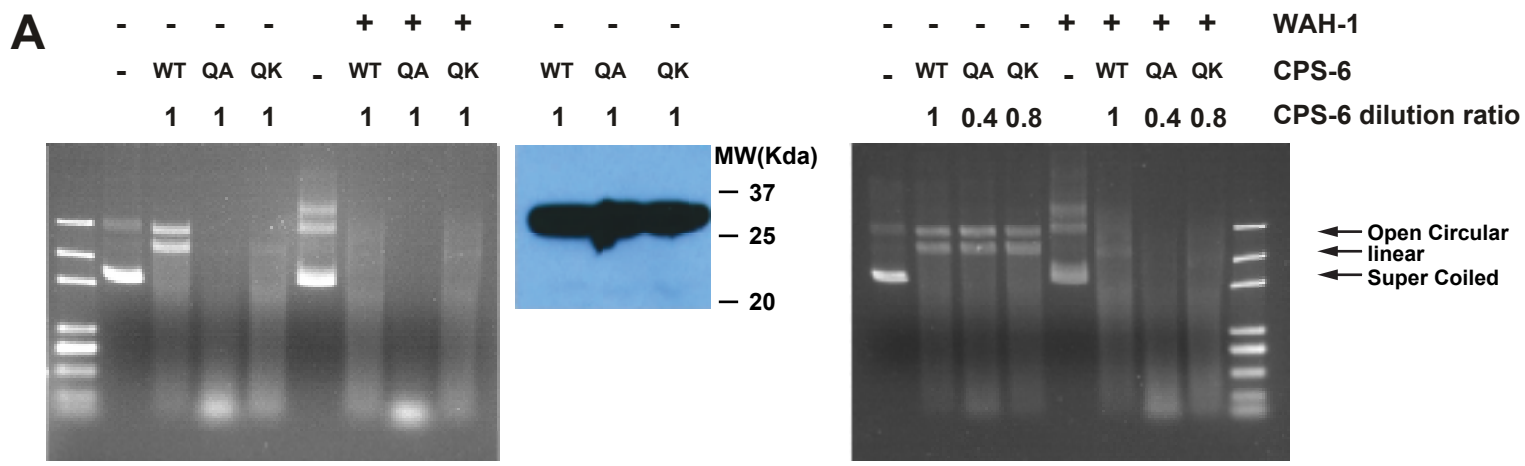


Figure 3. A model of the CPS-6/WAH-1 binding interface. Jason Lin and Hannah Yuan proposed that CPS-6 residues I136 and Q130 come into close proximity with WAH-1 residue R473 during binding.

CPS-6 Activity is Inhibited by its Q130 Residue

Genetic results provide valuable evidence that interactions within the apoptotic DNA degradation pathway are essential for regulating its cell-killing function. These experiments alone, however, do not reveal the molecular mechanism of the regulation. Previous experiments have shown that WAH-1 and CPS-6, two degradeosome components, not only bind, but also show enhanced nuclease activity in combination (Wang et al., 2002). In order to gain insight into the interaction between WAH-1 and CPS-6, Dr. Jason Lin and Dr. Hanna Yuan performed structural modeling (Yuan Lab unpublished data). Their model suggested that CPS-6 residues I126 and Q130 came into close proximity with WAH-1 residue R473 (Fig 3). Dr. Lin also found that, *in vitro*, CPS-6 binding to WAH-1 was not significantly affected by mutation of CPS-6 residue Q130 to lysine; however, mutation of WAH-1 residue R473 to glutamic acid completely abolished binding (Yuan Lab unpublished). Based on these results, Dr. Xiao Ge examined whether mutation of CPS-6 residue Q130 to lysine (QK) or alanine (QA) affected CPS-6 nuclease activity *in vitro*. Over the time interval of the reaction, wild-type CPS-6 degraded supercoiled plasmid DNA, forming many open circular and linear intermediates (Fig 4a). CPS-6 QA and QK mutants, however, showed greater activity, producing a ladder of small linear DNA fragments (Fig 4a). Dr. Xiao Ge also showed that CPS-6 QA and CPS-6 QK show grater enhancement of nuclease activity by WAH-1 than wild-type CPS-6 (Fig 4a). These results suggest that the CPS-6 Q130 residue inhibits CPS-6 nucleases activity and activation by WAH-1. Based on this result, Dr. Nakagawa and I tested whether mutation of the CPS-6 Q130 residue could affect the cell-killing activity of CPS-6. CPS-6ΔN QK and CPS-6ΔN QA did not require overexpression of WAH-1 in order to cause ectopic cell death (Fig 4b). *In vitro* and *in vivo* results suggest that CPS-6 QK and CPS-6 QA attain a level of nuclease activity similar to that of wild-type CPS-6 in the presence of WAH-1. Increased nuclease activity of CPS-6, however, does not necessarily cause a corresponding increase in its cell-killing activity



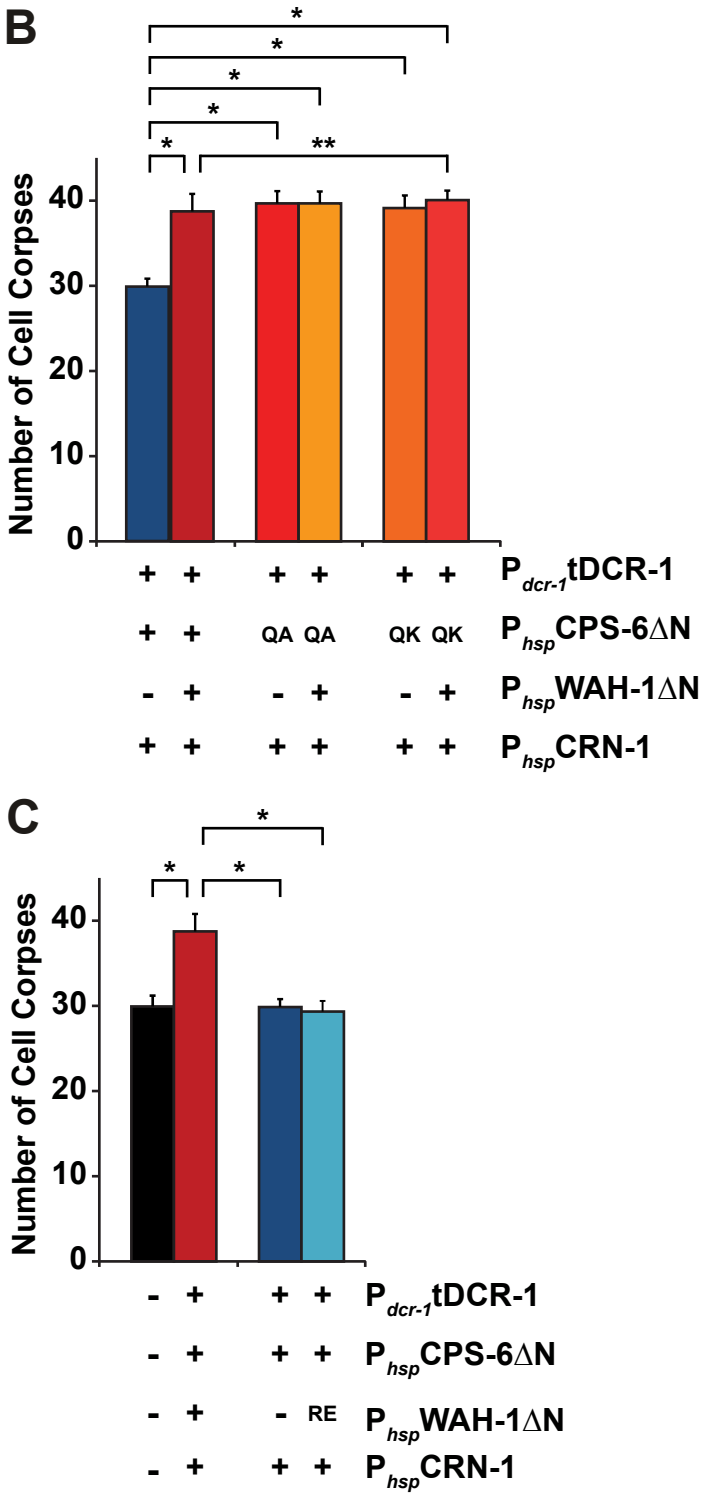


Figure 4. CPS-6 nuclease and cell-killing activities are inhibited by its Q130 residue. (A) Mutation of CPS-6 residue Q130 mimics WAH-1 enhancement of wild-type CPS-6. Plasmid was incubated with point mutants of CPS-6ΔN alone or in the presence of WAH-1ΔN and then resolved on an agarose gel. WT, wild-type CPS-6 ΔN; QA, CPS-6ΔN(QA); QK, CPS-6ΔN(QK). (Left panel) The quantity of each CPS-6ΔN mutant was held constant. (Middle panel) Input CPS-6 (37 Kda) was visualized by western blot. Dilution ratio indicates quantity of a CPS-6ΔN mutant used in a reaction divided quantity of the same CPS-6ΔN sample used in the western blot. (Right panel) CPS-6 mutants were diluted to show the same DNA degradation activity in the absence of WAH-1. (B) CPS-6 residue Q130 prevents CPS-6 from promoting ectopic cell death in the absence of WAH-1. Cell corpses were scored in the following animals: *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}CRN-1], *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1], *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN(QA), P_{hsp}CRN-1], *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN(QA), P_{hsp}WAH-1ΔN, P_{hsp}CRN-1], *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN(QK), P_{hsp}CRN-1], and *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN(QK), P_{hsp}WAH-1ΔN, P_{hsp}CRN-1].(C) WAH-1 activation of CPS-6 may require binding. Cell corpses were scored in the following animals: *ced-1(e1735)*, *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN, P_{hsp}CRN-1] , *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}CRN-1] , *ced-1(e1735);* ex[P_{dcr-1}tDCR-1, P_{hsp}CPS-6ΔN, P_{hsp}WAH-1ΔN(RE), P_{hsp}CRN-1], Each result is representative of multiple independent transgenic lines. The y-axis represents the mean number of corpses of 15 4-fold stage embryos. Error bars represent SD. The significance of differences between strains was determined by unpaired two-tailed Welch's t-test. * is p<0.001, ** is p<0.05, and other results are p>0.05.

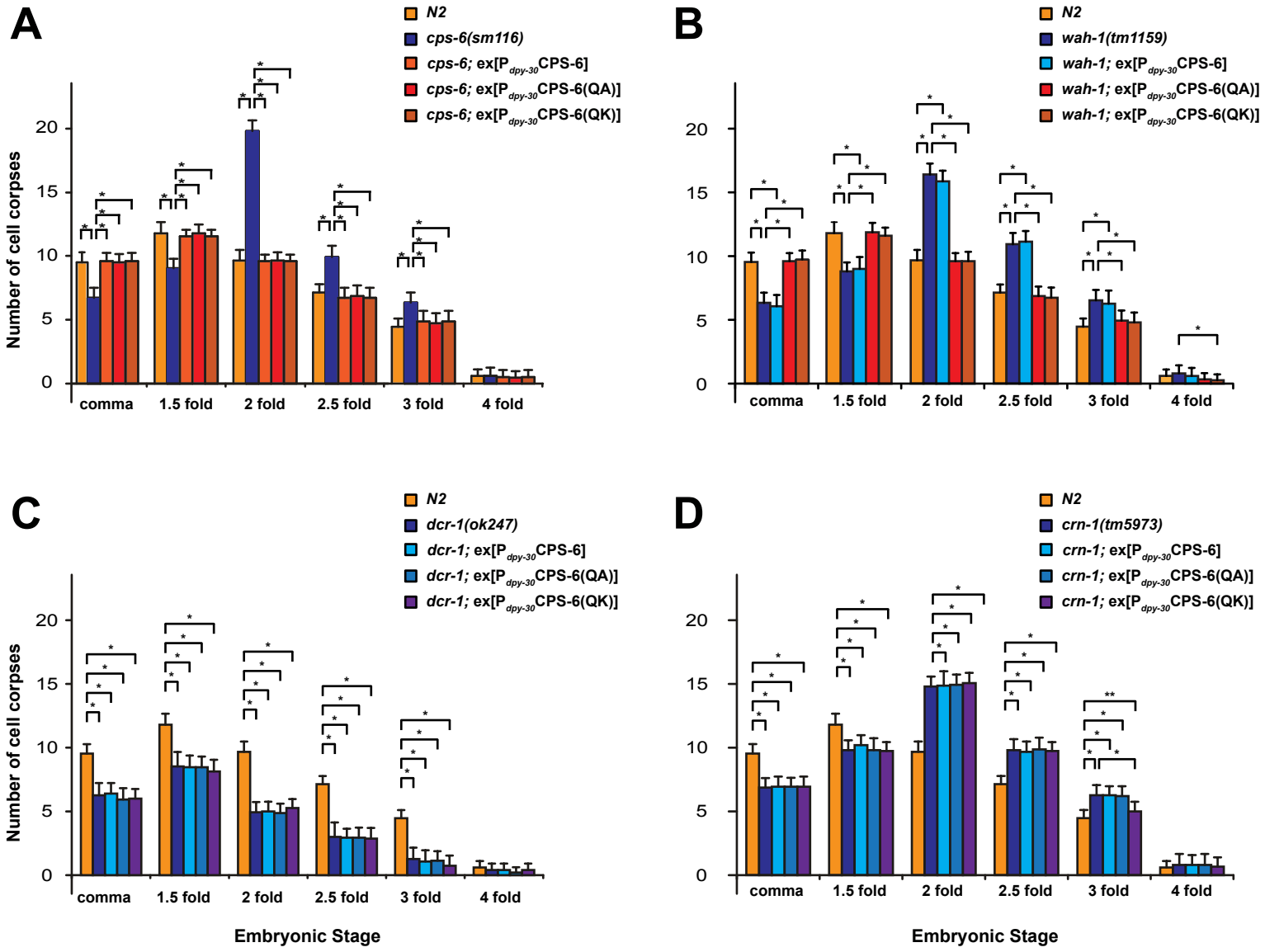


Figure 5. CPS-6 Q130 mutants acted independent of *wah-1* to promote physiological cell death. Cell corpses were scored for the following animals: (A) N2, *cps-6(sm116), cps-6(sm116);* ex[P_{dpy-30}CPS-6], *cps-6(sm116);* ex[P_{dpy-30}CPS-6 (QA)], *cps-6(sm116);* ex[P_{dpy-30}CPS-6 (QK)], (B) N2, *wah-1(tm1159), wah-1(tm1159);* ex[P_{dpy-30}CPS-6], *wah-1(tm1159);* ex[P_{dpy30}CPS-6 (QA)], *wah-1(tm1159);* ex[P_{dpy-30}CPS-6 (QK)], (C) N2, *dcr-1(ok247), dcr-1(ok247);* ex[P_{dpy-30}CPS-6], *dcr-1(ok247);* ex[P_{dpy-30}CPS-6 (QA)], *dcr-1(ok247);* ex[P_{dpy-30}CPS-6 (QK)],(D) N2, *crn-1(tm5973), crn-1(tm5973);* ex[P_{dpy-30}CPS-6], *cps-6(sm116); crn-1(tm5973)* ex[P_{dpy-30}CPS-6 (QA)], *crn-1(tm5973);* ex[P_{dpy-30}CPS-6 (QK)]. Transgenic lines are representative of observations made for three independent lines. Embryos were examined at the comma, 1.5 fold, 2 fold, 2.5 fold, 3 fold, and 4 fold stages of embryonic development. The y-axis represents the mean of 15 embryos observed. Error bars represent SD. Results for all strains were examined for significant differences from the sample mean of N2 animals and nontransgenic mutant animals using Welch's t-test (unpaired, two-tailed), *p<0.001, **p<0.05.

CPS-6 Q130 Mutants Promote Proper Timing of Cell-Death Events Independent of *wah-1*

In addition to being important in the decision of whether or not a cell will die, several apoptotic nucleases are important to the proper timing of cell-death events (Parrish & Xue, 2003a). As cells undergo apoptosis, they develop a highly retractile button-like morphology; this cell corpse morphology disappears as the cell is engulfed (Sulston & Horvitz, 1977). The transient appearance of this button-like morphology can be used to study subtle defects in the timing of apoptosis. Animals with a deficiency in *cps-6* show a delay of cell-death phenotype that is characterized by decreased numbers of cell corpses early in development (comma and 1.5 fold stages) and increased numbers of cell corpses later in development (2, 2.5 and 3 fold stages) (Fig 5a). We asked, whether expression of mutant forms of CPS-6 could rescue the *cps-6(sm116)* delay of cell-death phenotype. Transgenic constructs were expressed under control of the *dpy-30* promoter, which drives expression in all cells at most developmental stages (Hsu, Chuang, & Meyer, 1995). Expression of wild-type CPS-6, CPS-6 QA or CPS-6 QK fully rescued the *cps-6(sm116)* delay of cell-death phenotype (Fig 5a). This result suggests that all three transgenic constructs were capable of substituting for endogenous CPS-6, without causing ectopic cell death.

Based on previous observations of ectopic embryonic cell death, we hypothesized that CPS-6 QK and CPS-6 QA might function independently of *wah-1*. To test this, we used a deletion allele, *wah-1(tm1159),* which showed a delay of cell-death phenotype that was less severe than the Cps-6 phenotype. Wild-type CPS-6 failed to rescue the delay phenotype. CPS-6 QA and CPS-6 QK, however, both fully rescued the *wah-1(tm1159)* delay of cell-death phenotype (Fig 5b). This suggests that CPS-6 QA and QK can function independently of *wah-1* and that *wah-1* primarily promotes the timing of cell-death events by activating CPS-6.

It is possible that mutation of the CPS-6 Q130 residue results in a gain of function that allows the nuclease to degrade a new type of DNA substrate. Such a gain of function might bypass not only the requirement for cooperation with *wah-1*, but also other apoptotic nucleases. Based on the biochemical

model for apoptotic DNA degradation, we tested whether CPS-6 Q130 mutants could substitute for *dcr-1* or *crn-1*. *dcr-1(ok247)* is a deletion allele that shows reduction of cell-death phenotype characterized by lower numbers of cell corpses at all stages of embryonic development as compared to wild-type animals. Expression of either wild-type or mutant CPS-6 had no effect on the *dcr-1(ok247)* reduction of cell-death phenotype (Fig 5c). *crn-1(tm5973)* is a deletion allele that exhibits a delay of cell-death phenotype (Fig 5d). Expression of either wild-type or mutant CPS-6 had no effect on the *crn-1(tm5973)* delay of cell-death phenotype (Fig 5d). Together these results suggest that CPS-6 QA and CPS-6 QK do not require *wah-1* activation, but still cooperate with *dcr-1* and *crn-1* to promote the normal progression of apoptosis.

Discussion

The apoptotic DNA degradation pathway is composed of multiple nucleases that vary in their substrate specificity and the reaction that they catalyze. Alone these nucleases cannot degrade the chromosomes, but multiple nucleases can act together to accomplish this degradation. We performed experiments in an attempt to bridge the gap between biochemical understanding of these nucleases and their behavior *in vivo*. Observations of ectopic, embryonic cell death showed that interactions within the degradeosome may be more important to the cell-killing activity of the pathway than was suggested by previous results of Parrish, et al.(2003b) and Wang, et al. (2002). Our method represents an incremental advancement from assays of cell-killing activity that are cell type specific and observe both apoptotic and non-apoptotic mechanisms of cell death. Our results suggest that CPS-6 residue Q130 reduces CPS-6 nuclease activity. Mutation of CPS-6 Q130 allowed the nuclease to promote the execution and timing of cell death independently of *wah-1*. Additionally, we found that the primary function of *wah-1* during apoptosis is to activate CPS-6. This result is intriguing given that *wah-1* functions not only in the DNA degradation pathway, but also promotes PS externalization, which facilitates engulfment of the dying cell (Wang, et al., 2007).

An important link between biochemical and genetic understanding of apoptotic DNA degradation remains unknown. Apoptotic nucleases clearly cause cell death, but the mechanism that allows them to do so is unclear. One possibility is that the accumulation of double-stranded breaks in the chromosome during apoptotic DNA degradation causes the cell to die. Results published by Nakagawa, et al. (2010) suggest that high levels of tDCR-1 expression are capable of causing cell death. Conceivably, tDCR-1 can generate a high frequency of single-stranded nicks that produce double-stranded breaks in the chromosome. Similarly, cooperation between CPS-6 and CRN-1 to form double-stranded breaks could explain the correlation between CPS-6 nuclease activity and its cell killing activity. The importance of double-stranded breaks to cell killing could be tested by targeting restriction endonucleases (for example those used in molecular biology) to the nucleus to create double-stranded breaks.

Results of this project suggest several possible avenues of further investigation. The requirement of *ced-3* for nucleases to cause ectopic apoptosis is especially intriguing. One potential explanation of this result is a low level of CED-3 activity in the living cell. Another explanation is that the active apoptotic DNA-degradation pathway feeds back to activate CED-3. Either of these two cases would be quite interesting but distinguishing between them is difficult. Careful examination of these possibilities would require an assay of CED-3 activity, a tool not currently available in *C. elegans*. Another interesting possibility for future research would be a candidate-based genetic screen for other gain-of-function mutations within the degradeosome. The large number of potentially complex interactions makes screening an enticing possibility when compared with approaches based on structural modeling. The success of a screening procedure would depend on the proper selection of a mutagen and also a phenotype to be observed. The mutagen would consist of Cas-9 nuclease, targeted to sites flanking the coding region of the candidate gene, together with homologous repair template containing random mutations. Such a randomly mutated repair template could be generated by PCR techniques (such as Clonetech's "Diversify PCR Random Mutagenesis Kit."). Ectopic cell death is one potential phenotype that could be observed in the screen (i.e. Fig 2 and 4b). An easier phenotype to score would be embryonic lethality caused by

ectopic cell death; however, this would require development of a new combination of nuclease transgenes with higher cell-killing activity. Such phenotypes are not ideal when compared with screens for suppressors of lethality. Nevertheless, I believe that this strategy would identify important interactions within the degradeosome with greater efficiency, lower cost, and less bias compared to strategies that depend on structural modeling to generate hypotheses (i.e. Fig. 3). Regardless of the direction taken in future experiments, I believe that the improved understanding of the CPS-6/*wah-1* interaction will inform future investigation of interactions within the degradeosome.

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