Genetic and molecular studies of cell-autonomous execution during programmed cell death in C. elegans

by

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Genetic and molecular studies of cell-autonomous execution
during programmed cell death in *C. elegans*

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Abstract

Apoptosis or programmed cell death was originally defined by evolutionarily conserved morphological characteristics that include shrinkage of cell volume and chromatin condensation. Apoptosis functions as a highly controlled mechanism for the elimination of unwanted or damaged cells and is essential for disease prevention. Apoptotic cell death is a cell-autonomous process driven by the caspase family of cysteine proteases. The discovery of the CED-3 caspase in *C. elegans* led to the paradigm that caspase cleavage of substrates drives cell death and promotes engulfment. While many caspase substrates have been identified, it is not well understood how caspase substrates act to promote cell death and engulfment. The control of caspase activation in *C. elegans* is conserved among metazoans and involves the interplay of pro and anti-apoptotic BCL-2 and BH3-only family proteins.

In *C. elegans* an increase in apoptotic cell refractility observed by Nomarski optics is one of the hallmark morphological characteristics of apoptosis. We found that the presumptive TRP channel CED-11 acts downstream of caspase activation in apoptotic cells to drive the increase in refractivity. We discovered that CED-11 is also required for a decrease in cell volume and increase in nuclear permeability of apoptotic cells. We showed that CED-11 is required for efficient degradation of apoptotic cells and facilitates the death process, suggesting that the decrease in cell volume and/or increase in nuclear permeability could promote the death and degradation of the cell. We conclude that CED-11 acts downstream of caspase activation to effect multiple observed changes to apoptotic cells and to facilitate death and degradation. In addition we investigated the anti-apoptotic function of the generally pro-apoptotic BCL-2 homolog CED-9.

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Chapter One

Introduction
Overview

Apoptosis, also known as programmed cell death, is critical to remove unwanted and damaged cells during development and in tissue homeostasis. Apoptosis can be thought of as three phases: the decision to die, the execution of the cell, and the engulfment of the dying cell. In this introduction I review the execution of the cell in *C. elegans* and mammals in two phases: first the activation of caspases and second the caspase-mediated death of the cell.
History: Programmed cell death in C. elegans

C. elegans have a nearly invariant cell lineage; during hermaphrodite development 1090 somatic cells are generated, of which 131 undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983). 113 of the programmed cell deaths occur during embryogenesis and an additional 18 occur during the L1 and L2 larval stages. The programmed cell deaths are considered cell fates as they occur reproducibly in fixed lineages at fixed developmental times. The dying cell corpses take on a highly refractile raised button-like appearance that is easy to differentiate from living cells when visualized by Nomarski differential interference contrast (DIC) microscopy. Screens for animals with altered numbers of the highly refractile cell corpses led to the identification of the cell-death execution pathway, which culminates in caspase activation.

I. The apoptotic execution pathway: regulation of caspase activation

Regulation of caspase activation in C. elegans

A series of genetic screens led to the isolation of alleles of four genes that block nearly all of the programmed cell deaths in C. elegans. Loss-of-function alleles of three of the genes (egl-1, ced-3, and ced-4) recessively inhibit cell death, indicating that these genes are required for cell death and are thus pro-apoptotic (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986). A gain-of-function allele of the fourth gene, ced-9, dominantly inhibits cell death, suggesting that this gene is anti-apoptotic (Hengartner et al., 1992). Indeed, loss-of-function alleles of ced-9 increase cell death and cause maternal effect lethality (i.e. homozygous ced-9 hermaphrodites from heterozygous hermaphrodite
parents are viable, but their progeny are not viable), confirming the anti-apoptotic function of ced-9. A combination of epistasis analysis and over-expression studies revealed a genetic pathway in which ced-4 acts upstream of or parallel to ced-3 and ced-9 negatively regulates ced-4 (Shaham and Horvitz, 1996). In dying cells EGL-1 inhibits CED-9, allowing CED-4 to activate CED-3 to promote apoptosis (Conradt and Horvitz, 1998).

All four of the cell-death execution genes have mammalian homologs, which are also involved in apoptosis. CED-9 is a BCL-2 family homolog (Hengartner and Horvitz, 1994). CED-9 and other BCL-2 proteins contain a transmembrane domain and 4 BH domains (BH1, BH2, BH3, and BH4); BH-1, BH-2, and BH-3 can fold to line a hydrophobic pocket that can bind BH3-only peptides (Woo et al., 2003). EGL-1 is a BH3-only family homolog; BH3-only proteins contain a BH3 domain that is often used for interaction with BCL-2 family proteins (Conradt and Horvitz, 1998; Zha et al., 1996). CED-4 is an APAF1 homolog; APAF1 and CED-4 share an N-terminal caspase recruitment domain (CARD), a AAA+ ATPase nucleotide binding and oligomerization domain (NB-ARC) and a winged-helix domain (WHD) (Yan et al., 2005; Zou et al., 1997). CED-3 is a caspase protease; caspases cleave substrates in a highly specific manner after an Asp residue in the tetra-peptide cleavage sequence (Yuan et al., 1993). Caspases, including CED-3, typically exist as inactive zymogens that require cleavage and dimerization for activation (MacKenzie and Clark, 2012).

A molecular model based on protein binding and localization studies suggests that in living cells CED-9 is localized to the outer mitochondrial membrane, where it binds to and sequesters CED-4 preventing CED-4 from binding to and activating CED-3
caspase (Chen et al., 2000; Chinnaiyan et al., 1997; Spector et al., 1997). In cells fated to die egl-1 is transcriptionally upregulated, EGL-1 binds to CED-9, causing a conformational change in CED-9 that leads to the release of CED-4 (Conradt and Horvitz, 1998; Peso et al., 2000). Released CED-4 moves from the mitochondria to the perinuclear membrane and is free to bind to and activate CED-3 caspase (Chen et al., 2000).

Crystal structures of CED-4/CED-9, CED-9/EGL-1, and CED-4/CED-3 complexes support the proposed model for the regulation of CED-3 activation. CED-9 binds one monomer of an asymmetric dimer of CED-4 (Yan et al., 2005). CED-4 and EGL-1 bind different structural elements of CED-9, but when bound to EGL-1 an alpha helix in CED-9 moves towards the CED-4 binding pocket, which is predicted to cause a steric clash that would cause the release of the CED-4 dimer (Yan et al., 2004). When free from CED-9, CED-4 can form an octameric ring composed of four asymmetric CED-4 homo-dimers with two units of CED-3 in the center (Qi et al., 2010). The proximity of the two CED-3 molecules leads to their dimerization and activation. CED-3 is translated as an inactive zymogen and requires autoproteolysis and dimerization for activation of its protease activity (Xue et al., 1996). Upon activation CED-3 cleaves many downstream targets leading to the death and engulfment of the cell (Taylor et al., 2007).

There is one discrepancy with the model that CED-9 inhibits CED-4 activity by sequestration of CED-4 at the mitochondria, which is that CED-4 localization to the perinuclear membrane does not ectopically activate death. For example, CED-4::GFP localizes to the perinuclear membrane in living cells and does not induce ectopic
apoptosis (Pourkarimi et al., 2012). CED-4::GFP is functional as it can rescue the cell death defect of cel-4 mutants. In addition, a dominant allele of cel-9, n3377, which causes a weak increase in cell survival also causes perinuclear localization of CED-4 (Bradley Hersh personal communication). Lastly, in animals with the cel-9 allele n1653ts, which recessively confers temperature sensitive lethality, some CED-4 protein is perinuclear at the permissive temperature (Chen et al., 2000). It is unclear why CED-4 perinuclear localization does not induce death in these cases, it could suggest that there is more to CED-4 activation than release from CED-9.

**Regulation of caspase activation in mammals**

There is stunning conservation between the nematode and mammals in the proteins required for apoptosis, to the extent that expression of the CED-9 homolog, BCL-2, in the nematode can rescue cel-9 loss-of-function lethality and cell-death defects and expression of CED-4 and CEL-3 in mammalian cells can induce apoptosis (Chinnaiyan et al., 1997; Hengartner and Horvitz, 1994; Vaux et al., 1992). However, despite the conservation of proteins involved in apoptotic activation, the molecular mechanism regulating caspase activation has diverged. In mammals the BCL-2 proteins do not directly inhibit APAF1 (CED-4). Instead APAF1 is auto-inhibited and requires release of cytochrome c from the mitochondria for activation (Li et al., 1997). The BCL-2 and BH3-only proteins act antagonistically to regulate mitochondrial outer membrane permeabilization (MOMP), which releases cytochrome c from the mitochondria (Chittenden et al., 1995; Oltvai et al., 1993; Vander Heiden et al., 1997; Vaux et al., 1988; Yang et al., 1995). APAF1 bound to cytochrome c can activate initiator caspase-9 (Li et al., 1997). Activated caspase-9 cleaves and activates effector caspases-3 and -7.
MOMP and cytochrome c release are not known to occur in *C. elegans* apoptosis and CED-4 lacks the WD40 repeats that bind to cytochrome c in APAF1 (Hu et al., 1998).

Two distinct pathways can activate caspases in mammals. The pathway involving MOMP, cytochrome c release and APAF1 activation is called the intrinsic pathway or mitochondrial pathway. The other pathway is known as the extrinsic pathway and is activated by the binding of ligands (i.e. FasL) to death domain receptors at the plasma membrane (i.e. Fas) (Nagata, 1997). The death domain receptors contain an intracellular death domain that is bound by FADD, which recruits and activates caspase-8 (Boldin et al., 1995; Muzio et al., 1996). Caspase-8 can activate the effector caspases without the BCL-2 family and mitochondrial involvement (type I). However, in some cells (type II) the extrinsic pathway has cross-talk with the intrinsic pathway through cleavage of the BH3-only protein BID, to tBID, which subsequently activates MOMP (Yin et al., 1999).

The intrinsic apoptotic pathway is activated by developmental cues or cytotoxic insults, such as DNA damage or viral infection. These signals regulate the balance of BCL-2 family and BH3-only family pro and anti-apoptotic activities. In mammals there are at least 12 proteins that share structural similarity to BCL-2 and an additional 8 proteins with only BH3 domains (Youle and Strasser, 2008). These proteins can roughly be broken into 3 functional classes. The first class is the anti-apoptotic BCL-2 like proteins, of which BCL-2, BCL-XL, BCL-W, and MCL1 are well characterized examples. The second class is the pro-apoptotic BCL-2 like effector proteins BAK and BAX, which activate MOMP and lack a BH4 domain. BAX and BAK are required for MOMP, as cells lacking both proteins fail to undergo MOMP and apoptosis (Wei et al., 2001). The third
class is the pro-apoptotic BH3 only proteins (i.e. BAD, BIM, NOXA, PUMA, and BID), which promote activation of BAK and BAX. There are two models for how the BH3-only proteins activate BAK and BAX. In one model the anti-apoptotic BCL-2 proteins directly inhibit BAK and BAX and the BH3-only proteins relieve this inhibition by binding to the BCL-2 proteins. In the other model the anti-apoptotic BCL-2 proteins inhibit BAK and BAX indirectly by inhibiting “direct activator” BH3-only proteins that are required for BAK/BAX activation; “sensitizer” BH3-only proteins bind to the BCL-2 proteins and relieve their inhibition of the “direct activator” BH3-only proteins, which then activate BAK/BAX. It is likely that aspects of both models are correct.

Once active, BAK and BAX form homo-oligomers, which are able to form higher order dimer-dimer oligomers in the mitochondrial membrane (Antonsson et al., 2001; Nechushtan et al., 2001). In single cell imaging studies it was estimated that over one hundred BAK and BAX molecules formed some of the complexes visualized at the mitochondria (Zhou and Chang, 2008). It is still unclear how BAK and BAX cause mitochondrial membrane permeabilization; the prevailing theory is that BAK and BAX form pores in the outer mitochondrial membrane that release cytochrome c and other molecules (Tait and Green, 2010). The one caveat to this theory is that many studies of BAK and BAX suggest that they only form pores that are large enough to release cytochrome c, but MOMP allows for the release of much larger proteins.

After MOMP, the released cytochrome c binds to APAF1, inducing a conformational change and oligomerization into a heptamer called the apoptosome that binds to and activates caspase-9 (Acehan et al., 2002; Li et al., 1997; Zou et al., 1997). The APAF1 C-terminus contains multiple WD-40 repeats that can bind cytochrome c
In living cells APAF1 exists as an auto-inhibited monomer with its C-terminal and N-terminal domain interacting (Reubold et al., 2011). When cytochrome c binds to the WD-40 repeats of the C-terminal, a conformational change occurs that coupled with ATP hydrolysis allows for APAF1 to oligomerize releasing the CARD domain (Kim et al., 2005). Once an APFA1 heptameric ring has been formed, then 2 molecules of caspase-9 can be recruited, their proximity causes dimerization and activation (Boatright et al., 2003). Active capsase-9 then cleaves the effector caspases, caspase-3 and caspase-7, beginning the death cascade (Li et al., 1997; Srinivasula et al., 1998).

II. The apoptotic execution pathway: caspase mediated death

The apoptotic cell undergoes a series of morphological changes downstream of caspase activation, which reflect the death and degradation of the cell. Here, I review the original morphological observations of apoptosis followed by our current understanding of the changes in the apoptotic cell downstream of caspase activation. I have divided this section into subsections based on these changes. While the events leading to caspase activation have been extensively studied in mammals and nematodes, less is known about the events downstream of caspase activation. Therefore, in the following review of apoptotic cell changes I will shift focus at times on the organism under study depending on our current level of understanding.

Historical morphological observations of apoptosis

The term apoptosis was first introduced in a study describing the ultrastructure of dying cells in mammalian tissue sections (Kerr et al., 1972). The apoptotic cells exhibit
cytoplasmic condensation, compact chromatin, nuclear fragmentation and budding of small membrane-bound cellular fragments. The membrane-bound cellular fragments, termed apoptotic bodies, are most often observed inside of other cells indicating that engulfment is rapid. Prior to this study, cells dying by apoptosis were identified and referred to as cells dying by “shrinkage-necrosis,” as the dying cells appear shrunken (Kerr, 1971, 1972). Apoptotic bodies were also previously observed and referred to as “Councilman bodies.” It was noted that “their most striking feature was increased cytoplasmic density, causing them to stand out as dark cells in contrast to adjacent tissue (Klion and Schaffner, 1966).”

Ultrastructural study of *C. elegans* apoptotic cells revealed that many of the morphological changes are conserved including cytoplasmic condensation, chromatin compaction and dark staining in contrast to adjacent tissue (Robertson and Thomson, 1982). The *C. elegans* apoptotic cells are engulfed rapidly and cells fated to die can even be partially engulfed at their birth. These apoptotic morphological changes are observed in engulfment mutants and are thus independent of engulfment (Hedgecock et al., 1983). EM studies of apoptotic corpses at different times after death revealed that the corpses eventually have complete nuclear breakdown, lose the dark cytoplasmic staining and contain chaotic pieces of dark-staining material that are likely fragments of membrane and chromatin (Robertson and Thomson, 1982). Apoptotic bodies do not appear to form in *C. elegans.*

**An increase in cellular refractility**

In *C. elegans*, apoptotic cells have a distinctive highly refractile raised disc morphology when visualized by Nomarski DIC microscopy (Sulston and Horvitz, 1977;
Sulston et al., 1983). The highly refractile appearance allows the apoptotic cells to be distinguished from living cells. Maximal apoptotic cell refractility was used to record the time of cell death in the *C. elegans* lineage (Sulston and Horvitz, 1977). Cells fated to die undergo a series of morphological changes that can be divided into five stages when visualized by DIC: in the first stage the cell fated to die looks like other living cells; in the second stage the cytoplasm condenses becoming slightly more refractile around the granular nucleus; in the third stage there is a loss of distinction between the nucleus and cytoplasm; in the fourth stage the cell becomes highly refractile; in the fifth stage the cell shrinks and refractility decreases (Hoeppner et al., 2001; Robertson and Thomson, 1982). The fourth stage is generally considered to be the death of the cell. However, conclusive evidence supporting that the highly refractile cells are “dead,” as opposed to dying, is lacking. In addition, the biological cause of the increase in refractility is unknown.

The increase in refractility appears to correlate with CED-3 activity. In animals with a null mutation in *ced-3* the onset of morphological changes do not occur in living cells fated to die; however in animals with weak alleles of *ced-3*, cells fated to die can have a slight increase in refractility (stages 2 and 3) and then recover and survive, suggesting both that caspase is required for increased refractility and that if a threshold of caspase cleavage is not reached cells can recover (Hoeppner et al., 2001; Reddien et al., 2001). In addition, this indicates that the molecular mechanism of increased refractility is a reversible process. Furthermore, in animals with weak alleles of *ced-3*, cells that die have a delay in the onset of high refractility (stage four) by an average of 15 minutes (28 vs 40 minutes after the birth of the cell fated to die), suggesting that
increased refractility requires a threshold of caspase substrate cleavage (Hoeppner et al., 2001). Lastly, in animals with weak alleles of *ced-3* approximately 10% of cells are engulfed without reaching stage 4, indicating that high refractility is not essential to death. Engulfment is generally not required for the increase in apoptotic cell refractility, as mutations of genes required for engulfment cause the persistence of refractile apoptotic cells (Hedgecock et al., 1983).

In mammals, apoptotic cells increase in refractility, but this has not been studied in detail (Collins et al., 1997; Model and Schonbrun, 2013).

**A decrease in cell volume**

Cell shrinking is a hallmark of apoptosis, differentiating it from necrotic cell swelling and rupturing (Kerr, 1971). Multiple reports indicate that cell shrinking occurs both upstream of (Arrebola et al., 2006; Bortner et al., 1997; Dezaki et al., 2012; Ernest et al., 2008; Hasegawa et al., 2012; Hessler et al., 2005; Maeno et al., 2012) and downstream of caspase activation (Dallaporta et al., 1998; Kasim et al., 2013; McCarthy and Cotter, 1997; Nobel et al., 2000; Vu et al., 2001). In addition, there are reports that cell shrinking is required for apoptosis and others where it is not (Beauvais et al., 1995; Bortner and Cidlowski, 2003; Hortelano et al., 2002; Krumschnabel et al., 2007; Yurinskaya et al., 2005). These differences might reflect the different cell types and modes of apoptosis induction employed and perhaps reflect that there are two phases of cell shrinking: one before and one after caspase activation.

Cell volume is regulated by changes to the amount of intracellular water (Hoffmann et al., 2009). Animal cell membranes are permeable to water but are not
permeable to many osmolytes; thus, the cell water content is primarily regulated by the
difference between intracellular and extracellular concentrations of osmotically active
compounds. In mammals, intracellular and extracellular osmolarity can change due to a
number of physiological (i.e. transepithelial transport, accumulation of nutrients, and
neuronal, hormonal and autocrine activation of channels for ions or organic osmolytes)
and pathological conditions (i.e. hypoxia/ischemia, hypo/hypernatremia, hypothermia,
and hyperglycemia) (Hoffmann et al., 2009). In response to acute osmotic-induced
changes in cell volume, cells can activate regulatory volume increase (RVI) or
regulatory volume decrease (RVD) mechanisms to return to normal size. Cell volume
changes can be sensed by macromolecular concentration, the concentration of specific
ions, or by mechanical/chemical changes in the lipid bilayer (Pedersen et al., 2011).
TRP channels have been implicated in volume sensing and can be activated by
mechanical or chemical changes at the plasma membrane (Numata et al., 2007; Pan et
al., 2008). TRP channels are thought to act in cell volume regulation by increasing
intracellular calcium, which activates downstream effectors of volume regulation.

Regulatory volume increase (RVI) occurs in response to osmotic-induced cell
shrinking and returns the cell to a near physiological volume. RVI is mediated by an
influx of Na⁺, Cl⁻ and organic osmolytes into the cell followed by osmotically obliged
water. Many channels have been implicated in RVI, suggesting there are multiple
mechanisms to promote cell shrinking. Many Na⁺/H⁺ exchangers (NHEs) are activated
by cell shrinking and inhibited by cell swelling (Krump et al., 1997; Putney et al., 2002).
The mechanism of NHE activation by cell shrinking is not well understood, but might
involve phosphorylation by Ste20 related kinases or an increase in the levels of PIP2 in
the plasma membrane (L. K. Putney et al., 2002; Strange et al., 2006). It has been proposed that a Cl-/HCO3- transporter operates in parallel to NHEs to result in the net uptake of NaCl (Cala, 1980). The Na-K+-2Cl- transporters (NKCCs) are also activated by cell shrinking (Wehner and Tinel, 1998). NKCC1 is phosphorylated in response to osmotic shrinking, possibly by WNK and Ste20-related kinases. Organic osmolytes, like taurine, are also taken up in response to cell shrinking (Fugelli and Zachariassen, 1976).

Regulatory volume decrease (RVD) occurs in response to osmotic-induced cell swelling to return the cell to near its original volume. RVD is mediated by the efflux of K+ and Cl- from the cell followed by water. K+ and Cl- conductive pathways are activated in parallel resulting in an almost electroneutral KCl efflux (Hoffmann et al., 2009). The voltage regulated anion channel (VRAC) mediates the swelling activated Cl- current in many cell types (Jentsch, 2016). The molecular identity of VRAC is undefined, despite multiple proposed candidates. Both a reduction of intracellular ionic strength and a decrease in the cholesterol content of the plasma membrane can potentiate VRAC (Levitan et al., 2000; Voets et al., 1999). The calcium-activated bestrophin family of chloride channels is also activated during RVD in some cell types (Chien and Hartzell, 2007). A variety of K+ channels can be activated by cell swelling including: calcium-activates K+ channels in the BK, IK, and SK families; voltage-dependent K+ channels; KCNQ channels, and 4TM K+ channels. In addition, K+-Cl- cotransporters are activated by cell swelling in some cells (Hoffmann et al., 2009). The mechanism of activation varies and is not always fully understood, but can likely involve membrane stretch, protein kinases and phosphatases, and lipid mediators. The efflux of taurine from the
cell from an unidentified transporter also promotes regulatory volume decrease (Pedersen et al., 2006).

Cell shrinking during apoptosis is often referred to as apoptotic volume decrease (AVD) to differentiate it from osmotic induced cell shrinking or RVD (Maeno et al., 2000). AVD might occur by the efflux of potassium and chloride ions followed by water, similar to RVD, but under normotonic conditions. Consistent with a loss of intracellular water, apoptotic cells have an increase in buoyant density that is independent of transcription or translation (Wyllie and Morris, 1982; Yurinskaya et al., 2005). The concentration of K⁺ and Cl⁻ ions decreases in apoptotic cells and K⁺ and Cl⁻ channel blockers can reduce cell shrinkage in apoptotic cells (Arrebola et al., 2006; Beauvais et al., 1995; Wei et al., 2004). It is unknown which K⁺ and Cl⁻ channels are needed for AVD and how they are activated during apoptosis. It is thought that RVI is inhibited in shrunken apoptotic cells.

Although shrinkage of apoptotic cells by as much as 40-60% is reported, it is calculated that only 15-20% of AVD can be explained by K⁺ and Cl⁻ efflux due to a requirement for the balance of the electrochemical gradient, suggesting that other mechanisms might be involved in apoptotic cell shrinking (Model, 2014; Yurinskaya et al., 2005). In mammals the plasma membrane of apoptotic cells bleb and form apoptotic bodies that detach, which might account for some of the reported cell shrinking (Benson et al., 1996; Lane et al., 2005; Model and Schonbrun, 2013). Membrane blebbing is likely caused by activation of ROCK I kinase by cleavage of caspase-3; small molecule inhibitors of ROCK I reduce blebbing during apoptosis and expression of the caspase
cleaved form of ROCK I promotes blebbing in the absence of apoptosis (Coleman et al., 2001; Sebbagh et al., 2001).

Apoptotic cell shrinking is observed in *C. elegans*, but has not been extensively studied (Sulston and Horvitz, 1977). In *C. elegans* asymmetric cell divisions generate small cells fated to die and disruption of the asymmetry can lead to cell survival; survival is likely caused by the failure to deposit apoptotic signals in the daughter cell destined to die (Frank et al., 2005; Hatzold and Conradt, 2008). Nonetheless, it is interesting to speculate that the increased size of the daughter cell could also impact cell survival. Furthermore, in mammals thymocytes induced with glucocorticoid undergo apoptosis more quickly if they are smaller and denser to begin with (Wyllie and Morris, 1982). If there are two phases of AVD, perhaps the first phase contributes to caspase activation and the second phase is caspase dependent.

**An increase in nuclear membrane permeability and inactivation of active nucleocytoplasmic transport**

The nuclear membrane is a double membrane structure contiguous with the ER that is punctuated by nuclear pore complexes (Grossman et al., 2012). The nuclear pore complex is composed of about 30 proteins called Nups: membrane Nups anchor the nuclear pore to the membrane; barrier Nups contain FG-repeats that bind transporter proteins and act as a gel like sieve that sets the passive diffusion limit; scaffold Nups connect the membrane Nups to the barrier Nups. Molecules of up to 40-50 kD can passively diffuse through the nuclear pore. Active transport is required for larger molecules and is mediated by importins and exportins. Substrates bearing a nuclear localization sequence are imported into the nucleus and substrates bearing a
nuclear exclusion sequence are transported out of the nucleus. The active transport process is driven by a Ran gradient, with a high concentration of RanGTP and low concentration of RanGDP in the nucleus and a low concentration of RanGTP and high concentration of RanGDP in the cytoplasm.

Apoptotic mammalian cells have an increase in nuclear membrane permeability and a disruption of active nucleocytoplasmic transport; molecules in the 50-140 kD range (70 kD fluorescent dextran and 140 kD GFP5X) diffuse into the nucleus of apoptotic cells and NLS-GFP diffuses into the cytoplasm, whereas a 580 kD GFP-β-galactosidase tetramer remains restricted to the cytoplasm (Faleiro and Lazebnik, 2000; Ferrando-May et al., 2001). Caspases can cleave a number of nuclear pore proteins, suggesting a potential mechanism for the increase in diffusion and the inhibition of active transport (Kihlmark et al., 2004; Patre et al., 2006). Over-expression of BCL-2 at the nuclear envelope of living cells can also increase passive diffusion, suggesting the BCL-2 family might regulate nuclear membrane permeability (Strasser et al., 2012). It is proposed that the increase in passive diffusion facilitates the movement of death promoting molecules, including caspase-3 into the nucleus (Faleiro and Lazebnik, 2000).

In C. elegans an increase in nuclear membrane permeability and inactivation of active transport have been observed (This thesis and Holly Johnsen, personal communication).
Chromatin condensation and DNA cleavage

Chromatin condensation at the periphery of the nucleus was one of the first described morphological changes of apoptosis and internucleosomal cleavage of DNA was one of the first described biochemical changes (Williams et al., 1974; Wyllie, 1980). Apoptotic chromatin condensation in mammals is partially dependent on mammalian sterile-20 (MST1), which is activated by caspase-3 cleavage (Cheung et al., 2003; Ura et al., 2001). Caspase cleavage of MST1 permits shuttling of MST1 to the nucleus where it can phosphorylate histone 2B (H2B); phosphorylated H2B promotes chromatin condensation. Apoptotic internucleosomal DNA is cleaved by CAD, which is released from its inhibitor, ICAD, when ICAD is cleaved by caspase-3 (Enari et al., 1998; Sakahira et al., 1998). CAD mainly generates DNA fragments with blunt ends carrying a 5'-phosphate and 3'-hydroxyl group, which are substrates for the terminal transferase used in TUNEL reactions. Studies of CAD deficient mice led to the identification of a second mechanism of DNA degradation by DNase II in phagocytes (McIlroy et al., 2000). DNase II is located in lysosomes and can cleave apoptotic DNA after phagolysosome formation (Krieser et al., 2002). While DNA cleavage is not essential to cell death, incomplete DNA degradation can have harmful effects like autoimmune activation in mice (Jog et al., 2012; Krieser et al., 2002; Yoshida et al., 2005).

Some cell-autonomous residual endonuclease activity remains in the absence of CAD, indicating the existence of other apoptotic nucleases. Endonuclease G (EndoG) is released from mitochondria during MOMP and can induce nucleosomal DNA
fragmentation, but is not necessary for DNA fragmentation during apoptosis (David et al., 2005; Irvine et al., 2005; Li et al., 2001).

In *C. elegans* DNA degradation occurs in at least 3 steps: first, an unknown endonuclease cleaves the internucleosomal DNA and generates TUNEL-reactive DNA ends; second, the DNase II homolog NUC-1 cleaves the DNA and converts it to a TUNEL-unreactive state; third, DNA degradation is completed in the engulfing cell (Hedgecock et al., 1983; Sulston, 1976; Wu et al., 2000). The engulfing cell endonuclease is likely NUC-1, as NUC-1::mCherry containing lysosomes fuse to corpse containing phagosomes and NUC-1 is required for degradation of DNA within engulfing cells (Guo et al., 2010). Perhaps, the higher concentration of NUC-1 in the phagolysosome and the acidic environment allows for enhanced or altered NUC-1 activity and complete DNA degradation.

Other proteins that are partially required for the conversion of apoptotic DNA to a TUNEL-unreactive state include: the EndoG homolog CSP-6, the AIF homolog WAH-1, the cell-death related nucleases CRN1-6, and CYP-13 (Parrish and Xue, 2003; Parrish et al., 2001; Wang et al., 2002, 2007). Mutation of these genes causes the persistence of apoptotic cell corpses, which is not observed in nuc-1 mutants, indicating that these genes likely have additional functions.

CED-1, CED-7 and CED-8 are partially required for the generation of TUNEL reactive DNA (Stanfield and Horvitz, 2000; Wu et al., 2000). They are all transmembrane proteins involved in the recognition of the apoptotic cell by the engulfing cell: CED-8 acts in the dying cell; CED-1 acts in the engulfing cell; and CED-7 acts in both cells (Stanfield and Horvitz, 2000; Wu and Horvitz, 1998; Zhou et al., 2001). Their
role in promoting DNA cleavage is not understood. Other engulfment genes are not required for the generation of TUNEL reactive ends.

**Mitochondrial fragmentation**

Mitochondria form elongated tubules in living cells that continually undergo fission and fusion (Chen et al., 2003; Lee et al., 2007). In apoptotic cells in mammals a dramatic increase in mitochondrial fission occurs that fragments the mitochondria immediately following cytochrome c release (Arnoult et al., 2005; Zhuang et al., 1998). Reducing the expression of the fission promoting protein DRP-1 causes an increase in mitochondrial fusion and a slight decrease in apoptosis, indicating that mitochondrial fragmentation might facilitate cell death (Lee et al., 2004). Apoptotic mitochondrial fragmentation might be mediated by BAX, which localizes to discrete puncta at the sites of mitochondrial fission with DRP-1 in apoptotic cells (Karbowski et al., 2002). In cells lacking both BAX and BAK mitochondria are shorter, suggesting BAX and BAK might promote mitochondrial fusion in living cells (Karbowski, 2006). BCL-XL overexpression causes shorter mitochondria in living cells, suggesting a potentially larger role for BCL-2 family proteins in regulation of mitochondrial fusion and fission (Sheridan et al., 2008).

The BCL-2 family might also regulate mitochondrial dynamics in *C. elegans*. While mutation of *ced-9* or *egl-1* does not alter global mitochondrial structure, overexpression of CED-9 can increase mitochondrial fusion and overexpression of CED-9 with EGL-1 can increase the presence of DRP-1 at mitochondria and mitochondrial fission, indicating that CED-9 and EGL-1 are not required for but can modulate mitochondrial dynamics (Breckenridge et al., 2009; Tan et al., 2008). *In vitro* CED-9 interacts with the mitofusin FZO-1 and CED-9 in complex with EGL-1 interacts
with DRP-1, suggesting a mechanism for how CED-9 can promote fusion in living cells and fission in dying cells (Lu et al., 2011). Lastly, overexpression of dominant negative DRP-1, results in a decrease in apoptotic fission and a weak increase in cell survival, suggesting mitochondrial fission might facilitate cell death (Jagasia et al., 2005).

**Golgi fragmentation**

The Golgi complex is composed of flattened cisternae linked together in a stacked formation by proteins that form filamentous cross bridges; the stacked morphology of the Golgi comes apart during mammalian apoptosis and the Golgi fragment into small tubular and vesicular structures (Sesso et al., 1999). The Golgi fragmentation is partially mediated by caspase cleavage of the Golgi stacking protein GRASP65 and vesicle tethering protein p115, as mutation of the caspase cleavage site of either protein delays Golgi breakdown during apoptosis (Chiu et al., 2002; Lane et al., 2002). In addition, caspases cleave other Golgi related proteins including: golgin-160, p150, cytoplasmic dynein intermediate chains and syntaxin-5.

**Nuclear fragmentation**

In mammals the nuclear envelope fragments during apoptosis and nuclear fragments can disperse into apoptotic bodies (Kerr et al., 1972). Actin and myosin contraction physically fragment the nucleus; caspase-cleavage of the Rho effector ROCK 1 and its subsequent phosphorylation of myosin light chain are required for this process (Croft et al., 2005). In addition, the nuclear lamins are a target of caspases and cleavage resistant lamins delay the onset of nuclear fragmentation, suggesting that cleavage of lamins weakens the nuclear membrane structure to facilitate actin-myosin
fragmentation (Lazebnik et al., 1995; Rao et al., 1996). The ER redistributes with nuclear fragmentation, likely through a similar mechanism of actin-myosin contraction (Lane et al., 2005). In C. elegans nuclear fragmentation during apoptosis has not been observed; complete disassembly of the nuclear membrane occurs post engulfment (Robertson and Thomson, 1982). Caspase cleavage of lamin is likely not required in C. elegans as lamin is not cleaved by CED-3 in vitro (Tzur et al., 2002).

**Dismantling cell adhesion complexes**

During apoptosis the cell detaches from the extra cellular matrix and takes on a more spherical shape, this is likely due to caspase-mediated demolition of cell-cell adhesion and cell-matrix focal adhesion complexes. In mammals, caspases cleave a number of proteins required for cell-cell adhesion (β-catenin, E-cadherin, desmoglein-3) and cell-matrix focal adhesion (pp125FAK and tensin) in vitro (Brancolini et al., 1997; Korsnes et al., 2007; Levkau et al., 1998; Steinhusen et al., 2001; Weiske et al., 2001). In vivo, these proteins change localization during apoptosis and can be shed from the cell or appear more diffuse, indicating that caspase cleavage of cell-adhesion proteins might dismantle cell-adhesion complexes.

**Loss of plasma membrane asymmetry**

The plasma membrane has an asymmetric distribution of lipids in living cells; phosphatidylcholine (PC) and sphingomyelin (SM) are mainly found on the outer leaflet, whereas phosphatidylserine (PS) and phosphatidylethanolamine (PE) localize exclusively to the inner leaflet (Clark, 2011). The distribution of lipids is regulated by transmembrane lipid transporters: flippases transport PS and PE from the outer leaflet to the inner leaflet;
floppases transport PC and SM from the inner leaflet to the outer leaflet; scramblases facilitate the movement of lipids along concentration gradients. The asymmetrical composition of the plasma membrane is disturbed during apoptosis and both PS and PE are exposed to the outer leaflet and the concentrations of PC and SM increase on the inner leaflet (Emoto et al., 1997; Fadok et al., 1992; Suzuki et al., 2013). PS exposure on the outer leaflet is the most well characterized example of an “eat me” signal to phagocytes. PS is exposed at early stages of apoptosis and promotes engulfment (Fadok et al., 1998; Ishimoto et al., 2000). Caspase cleavage of the flippase ATP11C inactivates its flippase activity and allows PS to accumulate on the outer leaflet (Segawa et al., 2014). Caspase cleavage of Xrk8, a transmembrane protein at the plasma membrane, facilitates PS and PE exposure and PC and SM internalization, suggesting it promotes a general scramblase activity. Reduction of Xrk8 expression reduces phagocytosis by macrophages. Other reported “eat-me” signals include: oxidation of PC, conformational changes to transmembrane glycoprotein ICAM3, and export of Anexin 1 and calreticulin to the outer leaflet of the plasma membrane (Arur et al., 2003; Chang et al., 2002; Gardai et al., 2005; Moffatt et al., 1999).

In mammals, apoptotic cells also release “find me” signals to professional phagocytes: such as phosphatidylcholine, sphingosine 1-phosphate, the fractalkine CX3CL1, and the nucleotides ATP and UTP (Elliott et al., 2009; Gude et al., 2008; Lauber et al., 2003; Truman et al., 2008).

In C. elegans, the Xkr8 homolog CED-8 promotes the externalization of PS on the outer leaflet of the plasma membrane of apoptotic cells (Suzuki et al., 2013). In ced-8 mutants there is a delay in the onset of corpse refractility, a delay in the first step of DNA
degradation and a delay in engulfment (Chen et al., 2013; Stanfield and Horvitz, 2000; Suzuki et al., 2013). CED-8 is a CED-3 caspase target and cleavage of CED-8 is required for the refractile appearance of corpses at normal developmental times (Chen et al., 2013). In addition, WAH-1, a homolog of AIF that is released from the mitochondria during apoptosis, activates the phospholipid scramblase SCRM-1 to promote PS externalization and engulfment (Wang et al., 2007). Mutation of wah-1 delays the second step of DNA degradation in C. elegans (Wang et al., 2002). There are conflicting reports on the role of CED-7 in promoting PS externalization during apoptosis: in one report somatic apoptotic cells lack PS externalization in ced-7 mutants; in the other report apoptotic cells in ced-7 mutants have an increased amount of externalized PS (Mapes et al., 2012; Venegas and Zhou, 2007). The increased amount of PS externalization in ced-7 mutants is proposed to be caused by a reduction in the transfer of PS from the outer leaflet of the dying cell to the outer leaflet of the engulfing cell. A model is proposed where CED-7 transfers PS from the outer leaflet of the dying cell to the outer leaflet of the engulfing cell by generating PS containing vesicles that require CED-1 for integration into the engulfing cell membrane (Mapes et al., 2012). It is unclear if there is a connection between the regulation of PS movement and DNA degradation or if it is merely coincidental that ced-8, ced-7, ced-1 and wah-1 are all implicated in both.

The exposure of "eat me" signals to engulfing cells is a critical step in apoptosis. Unengulfed apoptotic cells can undergo secondary necrosis and cause an inflammatory immune response. After engulfment the apoptotic cell-containing phagosomes mature and fuse with lysosomes; the dying cells are then completely degraded by lysosomal enzymes.
Conclusion

The cell-death execution pathway regulates caspase activation. Misregulation of caspase activation can cause too much or too little death and is associated with many diseases, including neurodegenerative disorders and cancers. Once activated, caspases cleave targets that promote the death, dismantling, and engulfment of the cell. Hundreds of caspase substrates have been identified and we are beginning to understand which targets are important for promoting changes in the dying cell, but there are still many open questions. In my thesis work, I have investigated the execution of the dying cell in *C. elegans*. In Chapter Two, I show that the presumptive TRP channel CED-11 acts downstream of CED-3 to promote multiple changes to the dying cell, including a decrease in cell volume. In Chapter Three, I investigate the previously made observation that CED-9 has a pro-apoptotic function in addition to its well characterized anti-apoptotic function.
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References


Chapter 2

The presumptive TRP channel CED-11 promotes cell-autonomous degradation of apoptotic cells in *C. elegans*

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Gillian isolated the *ced-11* alleles in a screen for suppressors of *ced-5* and identified the corresponding gene. She counted the number of apoptotic cells in wild-type and *ced-11* embryos at different embryonic stages and did the L1 EM. We both counted the number of apoptotic cells in 3-fold embryos with the different alleles of *ced-11*, examined extra cell survival in the anterior pharynx, and performed epistasis analysis with *ced-4*, *ced-3*, and *ced-11*. Rita did the EM. I did all other experiments.
Abstract:

Apoptotic cells undergo a series of cell-autonomous morphological changes that reflect the stages of cell death and degradation. These changes are dependent on caspase cleavage of downstream targets, but it is not well understood which targets are important for death and degradation. In C. elegans an increase in the refractility of the dying cell is caspase dependent and a hallmark of apoptotic cell morphology. We identify a presumptive TRP channel, CED-11, that acts in the dying cell to promote the increase in apoptotic cell refractility. CED-11 is required for multiple other morphological changes during apoptosis, including a decrease in cell volume and an increase in nuclear membrane permeability. In ced-11 mutants, apoptotic cells take longer to fully degrade. Mutation of ced-11 does not cause an increase in cell survival, but mutation of ced-11 can enhance cell survival of other cell-deaths mutants, indicating that ced-11 facilitates death. We anticipate our results to have impact on the understanding of the changes downstream of caspase activation that promote the death and degradation of the cell.
**Introduction:**

Apoptosis, also referred to as programmed cell death, is defined by a set of morphological and biochemical changes in the dying cell (Kerr et al., 1972; Wyllie, 1980). These changes not only reflect the death of the cell, but also prepare the cell for engulfment and degradation by phagocytes. Ineffective execution of apoptosis can lead to incomplete degradation and survival of unwanted cells, both of which are associated with a number of diseases including cancer and autoimmune disorders (Igney and Krammer, 2002; Poon et al., 2014). The morphological changes that occur during apoptosis are initiated by caspases, a family of cysteine proteases that cleave hundreds of proteins during apoptosis (Lüthi and Martin, 2007; Yuan et al., 1993). While proteomic studies have identified proteins cleaved by caspases, there are still many questions about how these proteins function during apoptosis and the significance of their contribution to the death process. However, it is clear that cumulatively caspase targets are responsible for many changes to the dying cell, including chromatin condensation, the fragmentation of organelles, DNA cleavage, and the exposure of “eat-me” signals to phagocytes (Chen et al., 2013; Enari et al., 1998; Lane et al., 2002; Suzuki et al., 2013; Ura et al., 2001).

Cell shrinking is a well-documented morphological change during apoptosis, originally used to differentiate apoptosis from necrosis, but the molecular mechanism and significance of apoptotic cell shrinking is unclear (Kerr, 1971; Model, 2014). Cell shrinking might be a two-step process, as cell shrinking has been observed to be upstream of caspase activation in some reports but has been dependent on caspase activation in others (Ernest et al., 2008; Porcelli et al., 2004; Saias et al., 2015; Vu et al., 2001). Regulatory volume decrease is a well-studied response to osmotic-induced cell swelling and is
regulated by the efflux of potassium and chloride ions through channels in the plasma membrane followed by an efflux of water molecules (Grinstein et al., 1984). It has been proposed that apoptotic volume decrease is facilitated by a similar mechanism (Okada et al., 2001).

Another cellular change during apoptosis is an increase in nuclear membrane permeability prior to the breakdown of the nuclear envelope (Faleiro and Lazebnik, 2000; Ferrando-May et al., 2001). Transport of proteins into the nucleus is either by passive diffusion or active transport. Proteins smaller than 40 kD can passively diffuse into the nucleus, but larger proteins and macromolecules require active transport (Raices and D'Angelo, 2012). During apoptosis proteins in the 40-150 kD range can passively diffuse into the nucleus, but much larger (580 kD) proteins cannot. Thus it was proposed that during apoptosis there is an increase in the nuclear pore passive diffusion limit. The increase in nuclear membrane permeability is dependent on caspase-9 (Faleiro and Lazebnik, 2000). It is unknown what regulates increased nuclear permeability downstream of caspases or how increased nuclear permeability helps to facilitate cell death.

Transient receptor potential (TRP) channels are non-selective cation channels often located at the plasma membrane that have been implicated in activating apoptosis by cytoplasmic calcium influx (Desai et al., 2012; Jaladanki N. Rao and Rao, 2006; Sappington et al., 2009; Xu et al., 2013a; Yamada et al., 2010; Zhang et al., 2006). TRP channels can also regulate cell volume decrease in times of osmotic stress, likely through an influx in cytoplasmic calcium which can stimulate calcium-activated potassium and chloride channels (Arniges et al., 2004; Numata et al., 2007).
In *C. elegans*, an increase in cell refractility observed by Nomarski microscopy is one of the defining apoptotic morphological changes (Sulston and Horvitz, 1977). The increase in refractility of apoptotic cells is dependent on CED-3 caspase (Ellis and Horvitz, 1986). In engulfment mutants the refractile apoptotic cells persist unengulfed (Hedgecock et al., 1983). In a screen for mutants that alter the accumulation of refractile apoptotic cells of a ced-5 engulfment mutant, we discovered a gene, *ced-11*, that is required for the refractile appearance of apoptotic cells (Wu and Horvitz, 1998). CED-11 is a presumptive TRP channel and can localize to the plasma membrane. We propose that CED-11 acts downstream of CED-3 activation in the dying cell to promote multiple changes, including a decrease in cell volume and an increase in nuclear membrane permeability. Without the changes induced by CED-11, the apoptotic cells takes longer to degrade and in a cell-death mutant background have an increased chance of survival.
Results:

**ced-11 is required for the highly-refractile morphology of apoptotic cells**

*C. elegans* apoptotic cells acquire a raised highly-refractile appearance when visualized by Nomarski differential interference contrast (DIC) microscopy (Figure 1A) (Sulston and Horvitz, 1977). We discovered that the gene *ced-11* (*cell death-11*) is required for the increase in refractility during apoptosis (Figure 1B). We identified *ced-11* in a screen for suppressors of the accumulation of refractile apoptotic cells observed in *ced-5* engulfment mutants (Figure 1C-1D). We recovered five alleles of *ced-11* from the screen and later recovered a sixth allele through deletion screening with a deletion at the start of *ced-11* (Figure 1E). Mutations in *ced-11* reduce the number of refractile apoptotic cells and result in the appearance of non-refractile apoptotic cells (see below) (Figure 1F-1G). We recovered one allele, *n2834*, that appears to have a partial loss of function as we see both refractile and non-refractile apoptotic cells in these embryos. The other alleles we recovered are likely null alleles as they create early stop codons and share the same phenotypic severity as the *ced-11* deletion.

We confirmed that the non-refractile cells observed in *ced-11* animals normally undergo apoptosis: null alleles of *ced-3* and *ced-4* prevent nearly all apoptotic cell deaths and prevented the appearance of non-refractile cells in *ced-11; ced-3* and *ced-4 ced-11* double mutant animals, indicating that the non-refractile cells in *ced-11* mutant are apoptotic (Table 1).

**CED-11 acts in the dying cell to promote increased refractility**
To determine if CED-11 acts in the dying cell, we expressed GFP::CED-11 in ced-11(n4666) animals using the egl-1 promoter, EGL-1 is expressed in dying cells. We found that GFP::CED-11 expressing apoptotic cells were refractile, suggesting that CED-11 functions in the dying cell to promote refractility (Figure 1H-1I). In addition, we occasionally observed engulfing cells that expressed GFP::CED-11 and contained non-refractile GFP-negative apoptotic cells, indicating that expression of CED-11 in the engulfing cell is not sufficient to rescue apoptotic cell refractility (data not shown).

Consistent with our results, engulfment mutant animals accumulate many highly refractile apoptotic cells, indicating that refractility is generally independent of engulfment (Figure 1C, Hedgecock et al., 1983). We conclude that CED-11 cell autonomously promotes an increase in refractility during apoptosis.

Engulfment is normal in ced-11 mutants

Next, we asked if ced-11 non-refractile apoptotic cells could be recognized and engulfed normally, despite their reduced refractility. CED-1 is a transmembrane receptor expressed in engulfing cells that clusters around apoptotic cells and mediates their engulfment (Zhou et al., 2001). We used CED-1::GFP to monitor engulfment of three cells (C1, C2, C3) that die during ventral enclosure, when the epidermal sheets meet at the ventral midline, in a series of time-lapse videos (Figure 2A-2D) (Yu et al., 2008). We observed that C1, C2, and C3 are engulfed during ventral enclosure in both wild-type and ced-11 mutant embryos, indicating that the onset of apoptosis and engulfment are generally normal (Figure 2A-2B). In addition, engulfment takes a normal amount of time in ced-11 mutant embryos, approximately 6 minutes, and CED-1::GFP is removed from the phagosomes at a normal rate, showing that both engulfment and the beginning of
the phagosome maturation process are normal (Figure 2E-2F). We conclude that ced-11 is required for the highly refractile morphology of apoptotic cells but is not required for the onset of apoptosis or engulfment.

**ced-11 mutants do not have extra surviving cells but mutation of ced-11 can enhance other cell-death mutations**

Can CED-11 contributed to the death of the apoptotic cells? We examined cell death in ced-11 animals using two assays: the first assay measures the survival of 16 cells in the anterior-pharynx that normally die during embryogenesis; the second assay measures the survival of five cells in the ventral nerve cord that normally die in the late L1/early L2 larval stage (Schwartz, 2007). We found no extra surviving cells in ced-11 mutant animals in either assay (Figure 3A-3B). We postulated that perhaps even if ced-11 promotes the death of apoptotic cells, if such cells are engulfed normally they will not survive. Therefore we assayed cell death in ced-11; engulfment double mutants. The C. elegans engulfment genes have a weak effect on cell death and in engulfment mutants, an average of 0.3-1.2 extra cells survive in the ventral nerve cord (Hoeppner et al., 2001; Reddien et al., 2001). We found that ced-11 mutation enhances the cell-death defect of engulfment mutants ced-1, ced-2, ced-5, ced-6, ced-10 and ced-12 in the ventral nerve cord, nearly doubling the average number of extra cells. Thus, ced-11 can facilitate cell killing (Figure 3B). Unexpectedly, ced-11 mutation appears to weakly suppress cell survival in ced-7 mutants.

In addition, we analyzed the ability of ced-11 mutation to enhance mutation in the cell-death execution genes (ced-3, ced-4, and egl-1), a caspase target (ced-8), and a gene required for phagosome maturation (unc-108) (Figure 3C). As expected, ced-11;
unc-108 double mutants did not cause extra cell survival, as unc-108 mutation does not alter engulfment or cell death and instead causes a delay in phagosome maturation (Mangahas et al., 2008). Mutation of ced-11 enhanced cell-survival caused by mutation of the execution genes ced-4 and egl-1, but did not enhance the allele of ced-3 that we tested (Figure 3C). It is unclear why ced-11 did not enhance ced-3. Mutation of ced-11 did not enhance ced-8: ced-8 mutants have a delay in apoptotic cell refractility, and CED-8 promotes phosphatidylserine exposure to the outer leaflet of dying cells (Chen et al., 2013; Stanfield and Horvitz, 2000; Suzuki et al., 2013). Perhaps ced-11 and ced-8 act in a partially redundant pathway to promote cell killing. Overall, it is clear that while mutation of ced-11 does not cause increased cell survival, it can enhance multiple other cell-death mutations and facilitate cell killing.

We observed when counting the number of apoptotic cells by DIC in embryos of different stages that there were fewer apoptotic cells in ced-11 embryos then in the wild type (Table 1, Figure 3 - Supplemental Figure 1). Given that there was no extra cell survival in ced-11 mutant animals, we conclude that the difference in apoptotic cell number is because some of the ced-11 non-refractile apoptotic cells are not easily discernable from living cells by DIC.

Apoptotic cells in ced-11 mutants have altered morphology by electron microscopy

We examined the morphology of apoptotic cells by electron microscopy (EM) in wild-type and ced-11 mutant embryos. Previous EM studies in C. elegans revealed some engulfed apoptotic cells have intact nuclei and some do not and this occurs sequentially (Robertson and Thomson, 1982). The apoptotic cells with intact nuclei stain
darkly compared to surrounding cells, have a condensed cytoplasm, and compact chromatin and will be referred to as “pre-breakdown” apoptotic cells; the apoptotic cells that do not have intact nuclei have lost the dark-staining cytoplasm and contain dark-staining material believed to be remnants of membranes and chromatin and will be referred to as “post-breakdown” corpses (Robertson and Thomson, 1982; Sulston et al., 1983). Dark staining as visualized in EM is caused by the binding of electron-dense dyes to lipids and proteins, and it has been proposed that the increased electron density of apoptotic cells is a result of an increase in density of macromolecules (Klion and Schaffner, 1966).

We classified apoptotic cells as any cells that appeared to be engulfed. As reported, in wild-type embryos we observed post-breakdown corpses with membranous whorls and bits of chromatin that did not stain darkly (Figure 4A). Post-breakdown corpses in ced-11 animals looked similar to wild-type, indicating that apoptotic cells in ced-11 animals reach late stages of degradation (Figure 4B-4C; Figure 4 – Supplemental Figure 1).

Again, as reported, in wild-type embryos we found pre-breakdown apoptotic cells that stained darker than living cells, with a shrunken cytoplasm, bloated organelles and condensed chromatin (Figure 4D). However, in ced-11 embryos the pre-breakdown apoptotic cells did not stain darkly and overall looked more similar to living cells, though they were still rounded and displayed some apoptotic cell features, including chromatin condensation and organelle bloating (Figure 4E-4F). To ensure that the lack of dark staining in pre-breakdown apoptotic cells of ced-11 embryos was not specific to
embryonic development we evaluated embryos at different ages, including before bean comma and post 1.5-fold (Figure 4 - Supplemental Figure 2).

In addition, we examined the apoptotic cells that died in the posterior ventral cord in late L1/early L2 larvae by EM (Figure 4 - Supplemental Figure 3). So that multiple apoptotic cells from a single animal could be examined, we used a ced-1 mutation, which prevents the rapid engulfment of dying cells without affecting the morphology of apoptotic cells by DIC or EM (Hedgecock et al., 1983). We identified apoptotic cells in wild-type, ced-1(e1735) and ced-1(e1735); ced-11(n2744) larvae by DIC and fixed them for EM. The wild-type and ced-1(e1735) apoptotic cells were indistinguishable and similar to the embryonic pre-breakdown apoptotic cells. The ced-11 apoptotic cells did not stain darkly and looked similar to the embryonic ced-11 pre-breakdown apoptotic cells. We determined that ced-11 is required for an increase in electron-dense staining by EM during apoptosis. Perhaps ced-11 is required for an increase in the density of proteins and other macromolecules in the cytoplasm of apoptotic cells.

In addition to the difference in electron-dense staining, we noticed that the ced-11 pre-breakdown apoptotic cells appeared to have a difference in nuclear architecture. The pre-breakdown apoptotic cells in wild-type embryos often had wrinkled nuclei (40%) and separation between the nucleus and cytoplasm (60%), though nuclear pore junctions could still be seen (Figure 4G-4H, Figure 4 - Supplemental Figure 1). We did not see wrinkled nuclei in the pre-breakdown apoptotic cells of ced-11 embryos and rarely saw nuclear-cytoplasm separation (9-19%) (Figure 4H, Figure 4 - Supplemental Figure 1). It is not known what causes the nuclear wrinkling and separation of the nucleus and cytoplasm seen in electron micrographs of apoptotic cells. The separation
of nucleus and cytoplasm might reflect changes in the structural integrity of the nuclear membrane.

**ced-11 is required for an increase in nuclear membrane permeability during apoptosis**

While using the genetically encoded calcium indicator GCaMP3 to monitor calcium dynamics during apoptosis in wild-type and *ced-11* embryos, we noticed that the majority of apoptotic cells in wild-type embryos had GCaMP3 fluorescence throughout the apoptotic cell, whereas many apoptotic cells in *ced-11* embryos had a reduction of fluorescence in the center of the cell, corresponding to the nucleus (Figure 5A-5B). We observed in the wild-type apoptotic cells that the GCaMP3 fluorescence was restricted to the cytoplasm of the cells prior to cell death but present in both the cytoplasm and the nucleus at the time of the onset of increased refractility (Figure 5C). Since *ced-11* seems to be required for changes to the nuclear membrane during apoptosis, we hypothesized that the reduction of GCaMP3 fluorescence in apoptotic cells in *ced-11* embryos was because of a difference in nuclear membrane integrity. We expressed a GFP dimer, GFP trimer and mCherry::GcAMP3 in apoptotic cells and found that these proteins are less likely to be found in the nucleus of apoptotic cells in *ced-11* embryos than wild-type embryos, confirming that *ced-11* is important for nuclear membrane permeability during apoptosis (Figure 5D-5E). We believe that the nuclear membrane is still grossly intact in wild-type apoptotic cells, because we have observed normal localization of mCherry::Ce-emerin, a nuclear lamina associated protein, in apoptotic cells with nuclear localized GFP trimer and nuclear membrane breakdown is a later event in *C. elegans* apoptosis (Figure 5F, Tzur et al., 2002). In addition, during
mammalian apoptosis an increase in nuclear permeability can occur when the nuclear membrane is still intact (Faleiro and Lazebnik, 2000). Thus, ced-11 might be required for an increase in nuclear membrane permeability during apoptosis, prior to the complete breakdown of the nuclear membrane.

**Apoptotic cells take longer to degrade in ced-11 mutant embryos**

We next tested if the apoptotic cells in ced-11 embryos take a longer time to degrade, by measuring the duration of GFP::RAB-7 phagosomes (Li et al., 2013). RAB-7 is recruited to apoptotic cell-containing phagosomes shortly after engulfment and persists on the phagosomes until degradation is complete. We found that the apoptotic cell-containing phagosomes in ced-11 embryos take longer to degrade (60 min) than do wild-type apoptotic cell-containing phagosomes (45 min) (Figure 6A). We conclude that ced-11 promotes degradation of apoptotic cells.

**Apoptotic cells are larger in ced-11 mutant embryos at the time of engulfment**

We had observed in multiple experiments that the apoptotic cells in ced-11 embryos look larger than the apoptotic cells in wild-type embryos. To quantify this difference we measured the sizes of apoptotic cell-containing phagosomes at the time of engulfment, using the same time-lapse videos of CED-1::GFP we used to analyze engulfment. We found that newly engulfed apoptotic cells in ced-11 embryos are larger than apoptotic cells in wild-type embryos, with an average diameter of 2.7 µm versus 2.3 µm (Figure 6B). Thus, ced-11 is required for apoptotic cell shrinking. Cell shrinking occurs as a result of the loss of intracellular water, which could cause an increase in
intracellular density consistent with the increase in refractility and the increase in dark staining by EM of apoptotic cells (Jentsch, 2016).

CED-11 is a presumptive TRP channel and can localize to the plasma membrane

CED-11 shares sequence similarity with mammalian TRP family channels (Figure 7 - Supplemental Figure 1). TRP channels are non-selective cation channels, generally located to the plasma membrane and permeable to calcium and magnesium (Montell et al., 2002). TRP channels have six membrane-spanning domains and a pore-forming region between transmembrane domains five and six. CED-11 has the highest local sequence alignment with members of the TRPM subfamily in the transmembrane and pore forming regions, sharing approximately 20% identity and 40% similarity with TRPM1 and TRPM3. There are three other C. elegans TRPM proteins (GTL-1, GTL-2, and GON-2), which are each more similar to the mammalian TRPM proteins than to CED-11, so CED-11 is the most divergent member of this group of proteins (Harteneck et al., 2000; Teramoto et al., 2005).

We made a translational GFP::CED-11 reporter to determine the expression pattern of CED-11. GFP::CED-11 rescued the non-refractile apoptotic cell phenotype of ced-11(n4666) mutant animals, indicating GFP::CED-11 is functional. We first detected GFP::CED-11 in pre-bean stage embryos, in which it was expressed broadly and has a diffuse cytoplasmic localization (Figure 7 – Supplemental Figure 2). By the 1.5-fold embryonic stage, GFP::CED-11 had a more restricted pattern inside the cell and also could be seen on the plasma membrane (Figure 7A-7D). GFP::CED-11 was expressed in a few cells in the head and tail of L1 larvae, where it was primarily localized to the plasma membrane (Figure 7E-7L). GFP::CED-11 is expressed in both living and dying
cells, as is also true for other apoptotic cell-death proteins (Chen et al., 2000). We detected GFP::CED-11 in puncta at the perimeter of apoptotic cells, consistent with plasma membrane localization, and within the cytosol. We do not know where CED-11 is acting to promote apoptotic cell refractility. Consistent with the potential function of CED-11 as a TRP channel, we find that CED-11 can localize to the plasma membrane.

TRP channels have diverse functions and modes of activation. Some TRP channels regulate cell volume during osmotic stress (Numata et al., 2007; Pan et al., 2008). Perhaps CED-11 regulates cell volume by a similar mechanism, but in response to apoptosis activation rather than osmotic stress.
Discussion:

*ced-11* is required for many cellular changes that occur during apoptosis, including cell shrinking, increased refractility, increased electron density, nuclear membrane wrinkling and separation, and increased nuclear membrane permeability. *ced-11* does not appear to act upstream of *ced-3* caspase, as cells die in *ced-11* animals, and this cell death is suppressed by *ced-3* mutations. *ced-11* acts downstream of *ced-3*, because in strong *ced-3* mutants the onset of apoptotic cell refractility is never observed, suggesting that *ced-3* is required for the increase in apoptotic cell refractility, and hence *ced-11* activity (Reddien et al., 2001). We hypothesize that the increase in refractility and electron density during apoptosis are at least partially the result of an increased density of macromolecules caused by a loss of intracellular water and volume decrease and that in *ced-11* mutants, the dying cell volume does not change sufficiently to induce the increase in refractility and electron density. *ced-11* is required for increased nuclear membrane permeability during apoptosis, which might facilitate the entry of endonucleases into the nucleus to cleave DNA. *ced-11* mutations result in defects in apoptotic cell degradation and can lead to increased cell survival in cell-death mutant strains.

**CED-11 likely promotes a decrease in cell volume during apoptosis.**

Cell shrinking during apoptosis is referred to as apoptotic volume decrease (AVD), to differentiate it from osmotic-induced cell shrinking (Maeno et al., 2000). AVD occurs in normotonic cellular conditions and has been proposed to be induced by the opening of potassium and chloride channels in the plasma membrane causing an efflux of potassium and chloride ions from the cell followed by water (Barbiero et al., 1995;
Beauvais et al., 1995; Dezaki et al., 2012). The mechanism for activation of these channels during apoptosis is unknown. In living cells, regulatory volume increase occurs in response to osmotic induced cell shrinking; it has been suggested that regulatory volume increase is inhibited during apoptosis (Bortner and Cidlowski, 2007; Maeno et al., 2006). ced-11 likely regulates cell volume during apoptosis, as the ced-11 mutant apoptotic cells are larger at the time of engulfment. ced-11 might function to promote cell shrinking or to inhibit regulatory volume increase.

**CED-11 might function as a TRP channel to regulate apoptotic volume decrease**

TRP channels have been shown to act upstream of caspase activation in a number of models of mammalian cell death (Marasa et al., 2006; Xu et al., 2013). For example, overexpression of a TRP channel can potentiate increases in cytoplasmic calcium that lead to cytotoxicity and cell death (Hara et al., 2002; Sappington et al., 2009; Yamada et al., 2010). TRP channels can be caspase targets, in FasL-receptor induced apoptosis the TRPM7 C-terminus is cleaved by the initiator caspase-8 after Fas stimulation, increasing ion channel activity and stimulating Fas receptor internalization (Desai et al., 2012). Like TRPM7, CED-11 could be cleaved at its C-terminus by caspase. CED-11 has a possible caspase cleavage site near its C-terminus just beyond the presumptive sixth transmembrane domain.

Alternatively, CED-11 could be activated by changes in the lipid composition of the plasma membrane during apoptosis. TRP channels can be activated or inhibited by lipids and an individual lipid can act as both an inhibitor and activator depending on its plasma membrane leaflet localization (Senning et al., 2014). In apoptotic cells the lipid composition of the inner and outer leaflets of the plasma membrane is altered, such that
phosphatidylserine (PS) and phosphatidylethanolamine are exposed to the outer leaflet, while the concentration of phosphatidylcholine and sphingomyelin increases in the inner leaflet (Fadok et al., 1992; Suzuki et al., 2013). Interestingly ced-8, which is partially required for PS exposure on apoptotic cells (and the mammalian homolog of which, Xkr8, promotes the scrambling of multiple plasma membrane lipid species), has a delay in the onset of increased apoptotic cell refractility when mutated (Chen et al., 2013; Stanfield and Horvitz, 2000; Suzuki et al., 2013). A role of CED-8 in a plasma membrane lipid change that induced CED-11 activation could explain the delay of apoptotic cell refractility in ced-8 mutant animals. That ced-11 mutation did not enhance cell survival caused by ced-8 mutation is consistent with the hypothesis that CED-11 and CED-8 act in the same pathway to facilitate cell death.

TRP channels have been implicated in cell volume regulation. TRPM7 has been shown to regulate RVD by promoting calcium influx into the cell (Numata et al., 2007). Increased intracellular calcium can activate potassium and chloride channels, causing an efflux of potassium and chloride followed by water. It is possible that CED-11 activates apoptotic volume decrease by the same mechanism.

**Could the increase in apoptotic cell refractility be caused by cell shrinking?**

The defining morphological feature of apoptosis in *C. elegans* is the highly refractile appearance of the apoptotic cells as visualized by DIC optics (Sulston and Horvitz, 1977). The biological cause of this refractility is unknown. A linear relationship has been reported between increases in protein concentration and increases in refractive index (Barer and Tkaczyk, 1954). In addition, lipids, nucleic acids, and other non-protein macromolecules have a refractive index roughly similar to that of proteins.
The refractive index of protein solutions shows little to no change in response to changes in salt concentration, changes in pH, or denaturation of proteins or digestion of proteins to amino acids (Barer and Joseph, 1954). Thus, the increased refractility might be caused by an increased density of macromolecules in the dying cell.

Perhaps, CED-11 promotes a decrease in cell volume during apoptosis through a release of intracellular water, resulting in an increase in the density of proteins and other macromolecules and an increase in refractility. In ced-11 mutants the apoptotic cells would not have a sufficient change in volume and density and thus not display an increase in refractility. The overlap in size between some of the refractile and non-refractile apoptotic cells is not inconsistent with this hypothesis, as changes in volume rather than absolute volume would determine macromolecule density and refractility.

The refractility of mammalian cells also increases during apoptosis (Model and Schonbrun, 2013). The increase in refractility has been suggested to reflect a decrease in intracellular water and a corresponding increase in the density of proteins and other macromolecules in the cell. In addition, apoptotic thymocytes increase in buoyant density during apoptosis (Wyllie and Morris, 1982). These observations suggest that an increase in intracellular density and refractility might be a common event during apoptosis.

An increase in intracellular density of proteins and lipids might account for the increase in electron density of C. elegans apoptotic cells. The apoptotic cells in ced-11 embryos do not stain darkly, consistent with a failure to increase the density of macromolecules. Indeed, EM studies of mammalian cells in a hyperosmotic solution
that induced cell shrinking showed an increased electron density compared to cells in an iso-osmotic solution (Delpire et al., 1985).

**Increased nuclear membrane permeability during apoptosis might be independent of caspase cleavage of nucleoporins**

Nuclear membrane permeability increases during apoptosis in mammals, potentially by an increase in the nuclear pore passive diffusion limit (Faleiro and Lazebnik, 2000). Increased nuclear membrane permeability might promote the exchange of apoptotic factors, like endonucleases, between the cytoplasm and nucleus during the early stages of apoptosis. Caspases can cleave multiple nuclear pore proteins, suggesting this might be the mechanism of increased permeability (Kihlmark et al., 2004; Patre et al., 2006). We found that the presumptive TRP channel ced-11 is required for an increase in nuclear membrane permeability during apoptosis in C. elegans, suggesting that increased nuclear permeability is more complex than the caspase cleavage of nucleoporins. It is unclear how CED-11 regulates this increase in nuclear membrane permeability and if it is related to the decrease in apoptotic cell volume.

**ced-11 is required for efficient apoptotic cell death and degradation**

As increased refractility is often considered a sign of death in C. elegans apoptosis, there is a question about whether the non-refractile apoptotic cells in ced-11 embryos are actually “dead” when they are engulfed. As most of the cells still die in the ventral nerve cord of ced-11; engulfment double mutant strains it seems that the majority of ced-11 non-refractile apoptotic cells are indeed dead or dying. However, the
ability of a few extra cells to survive in *ced-11*; engulfment double mutant strains supports the idea that the non-refractile apoptotic cells in *ced-11* animals are not always dead at the time of engulfment, and given the chance to survive, can live. *ced-11* is required for a decrease in cell volume and an increase in nuclear permeability during apoptosis; it appears that these changes might aid the death of the apoptotic cell as the *ced-11* mutant apoptotic cells take longer to fully degrade and have an increased chance of survival in poised genetic backgrounds.
Future Directions:

To discover what acts downstream of ced-11 in apoptotic cells I performed a pilot screen with Josh Saul for suppressors of the ced-11 non-refractile apoptotic cell phenotype. We mutagenized sem-4(n1378) ced-1(e1735); ced-11(n4666) hermaphrodites and screened the F2 embryos inside of 1,000 F1 hermaphrodites for refractile apoptotic cells by Nomarksi optics. We recovered one isolate that retested, n5298. In this isolate we can observe what looks like refractile apoptotic cells in the heads of 3-fold embryos inside of heterozygous hermaphrodites. We have been unable to obtain homozygous n5298 mutant adults, suggesting that n5298 or a linked mutation cause lethality at some point after the 3-fold embryonic stage. The next steps are to determine if the refractile cells are apoptotic and if so to clone n5298. To determine if the refractile cells are apoptotic ced-3 RNAi can be used on sem-4(n1378) ced-1(e1735); ced-11(n4666); n5928, if ced-3 RNAi reduces the number of refractile cells than they are likely apoptotic. In addition finding a chromosomal balancer to maintain n5298 will greatly enhance the ability to work with this allele. This screen can be repeated to isolate more ced-11 suppressors.

To test if CED-11 is a CED-3 caspase target we can analyze GFP::CED-11 from embryo lysates by western blot to check if GFP::CED-11 is potentially cleaved. If so, we can analyze GFP::CED-11 in a ced-3 mutant to test is the potential cleavage is caspase dependent. The ability of CED-3 to cleave CED-11 can also be tested in vitro.
Materials and Methods:

C. elegans Strains

C. elegans strains were cultured at 20 °C as described previously (Brenner, 1974). N2 (Bristol) was the wild-type strain. The following mutations and arrays were used and have been described, except for those from this study:

LG I: unc-108(e713), ced-12(n3261), ced-1(e1735)

LG II: ced-4(n1162), ced-4(n3100), dpy-17(e164), lon-1(e185), ced-11(n4666), ced-11(n2834), ced-11(n2745 n6046), ced-11(n2832), ced-11(n2744), ced-11(n2833), ced-6(n1812), ced-7(n1892)

LG IV: ced-2(n1994), ced-10(n3426), ced-5(n2098), ced-5(n1812), ced-3(n717), ced-3(n2427)

LG V: egl-1(n4046)

LG X: ced-8(n1891), lin-15(n765tsAB), nls106 [P*lin-1::gfp*],

Unmapped: enls7[P*ced-1::ced-1::gfp*], enls38[P*ced-1::gfp::rab-7, P*ced-1::2xfyve::rfp*],
nls575[P*fp::gcamp3, lin-15(+)], nls790[P*ced-11::gfp::ced-11, lin-15(+)], nEx2344[P*egf-20::mcherry::ph, unc-76(+)], nEx2544[P*egl-1::gfp::ced-11, P*ced-1::2xfyve::rfp*],
nEx2546[P*egl-1::2xgfp, P*ced-1::2xfyve::rfp*], nEx2547[P*egl-1::3x::gfp, P*ced-1::2xfyve::rfp*],
nEx2550[P*egl-1::mcherry::gcamp3, lin-15(+)]

Screen for cell-death mutants

We performed our screen as described by Ellis and Horvitz (1986) with the following modifications. sem-4(n1378); ced-5(n2098) animals were mutagenized in 50 mM EMS as described (Brenner, 1974). We used Nomarski DIC microscopy to screen F3
embryos inside F2 mothers for animals with any alteration from the $\text{ced-5}(n2098)$ persistent apoptotic cell phenotype. We screened approximately 15,000 mutagenized haploid genomes for zygotically-acting genes and 6,000 haploid genomes for maternal-effect genes, and we isolated 12 mutations with specific effects on programmed cell death. Seven mutants had few or no cell apoptotic cells in embryos of any stage, consistent with a block in programmed cell death. We found that these mutants represented two alleles of $\text{ced-4}$ and five alleles of $\text{ced-3}$. The other five mutants ($n2744$, $n2745$, $n2832$, $n2833$ and $n2834$) had a similar novel cell-death phenotype and defined one complementation group, the gene $\text{ced-11}$.

**DIC Microscopy**

DIC images of 3-fold embryos with refractile and non-refractile apoptotic cells were obtained using an Axioskop II (Zeiss) compound microscope, an ORCA-ER CCD camera (Hamamatsu) and OpenLab software (Agilent) using a X100 objective. Embryos were mounted on a slide with a 4% agar pad in M9 solution. For quantification of apoptotic cells in the heads of 3-fold embryos, embryos were mounted in M9 solution with 20 mM sodium azide and examined after 30 minutes.

**Gene graphics and protein alignment**

The $\text{ced-11}$ exon-intron graphic was made with the WormWeb Exon-Intron Graphic Maker (http://wormweb.org/exonintron). Alignment of CED-11, TRPM1, and TRPM3 protein sequences was done with Clustal Omega and box shading was done with BoxShade.

**Cell-death assays**
The anterior pharynx and ventral cord cell-death assays were performed as described (Reddien et al., 2001; Schwartz, 2007). Student t-tests were performed using GraphPad Prism.

**Confocal Microscopy**

Embryos and larvae were mounted on 4% agarose pads in M9 solution and imaged using a X63 objective. Fluorescent images were analyzed using ImageJ. GFP::CED-11 and mCherry::PH images were taken on a Zeiss LSM800 laser scanning confocal, larvae were immobilized with 20 mM sodium azide. GFP multimer images were taken on a Zeiss LSM700 laser scanning confocal. Classification of apoptotic cells as having a cytoplasmic “ring” of fluorescence or homogenous nuclear and cytoplasmic fluorescence was done blindly by eye. Time-lapse imaging of fluorescent markers was performed as described, with a few modifications (Li et al., 2013). CED-1::GFP experiments were recorded on a Zeiss LSM800 laser scanning confocal; the embryos were imaged every 2 min at 15 Z-slices spaced 1 micron apart. The diameter of each CED-1::GFP phagosome was determined at the most central Z-section using the ImageJ measuring tool. GFP::RAB-7 and GCaMP3 time-lapse experiments were recorded on a Zeiss LSM510 laser scanning confocal; the embryos were imaged every 2 min at 10 Z-slices spaced 1 micron apart.

**Electron microscopy**

Mixed-stage embryos were collected by gently washing larval and adult animals off plates and then vigorously washing off the remaining embryos and compacting them by centrifugation for 1 minute on a table-top centrifuge. Embryos were loaded into a
Type A carrier coated with 1-hexadecene and filled with a slush of OP50 E. coli, then covered with a Type B carrier. The sandwich was placed into the specimen holder and frozen using a high pressure freezer (Abra HPM010). Samples were substituted with 2% OsO₄, 0.1% uranyl acetate in 98% acetone, 2% water at -90 °C for 96 h, warmed to -20 °C over 14 h, held at -20 °C for 16 h and warmed to 0 °C over 3.3 h (RMC FS-2500). Samples were washed three times with acetone at 0 °C and three times at room temperature before stepwise infiltrated with Eponate 12 resin (Ted Pella) and polymerized at 65 °C. The resulting blocks were thin-sectioned at about 50 nm and imaged with a transmission electron microscope (JEOL JEM-1200 ExII) and CCD camera (AMT XR-41) at multiple magnifications.

We examined L2 larvae using Nomarski microscopy and chose animals containing visible apoptotic cell in the posterior ventral cord for ultrastructural study. We photographed and sketched the positions of living and dead cell nuclei in the posterior ventral cord region of selected larvae. We rescued the larvae from slides and prepared them for electron microscopy essentially as described (Bargmann et al., 1993). Serial sections were collected from the tail of the worm to the positions of the apoptotic cells. EM micrographs were taken periodically from sections in the ventral cord region, and the Nomarski micrographs and sketches of cell positions in the living animals were compared with these EM micrographs to reconstruct cell positions within the posterior ventral cord and identify the apoptotic cells. We then examined all serial sections containing apoptotic cells.

**Plasmid construction**
Pced-11::gfp::ced-11 was cloned into pRS426 using yeast homologous recombination as described (Ma et al., 1987). Genomic DNA spanning 5kb upstream of the ced-11 start codon and 5 kb downstream of the ced-11 stop codon was amplified from pGS31 and gfp was amplified from pPD95.75. The egl-1 promoter in Hirose et al. (2010) was used for all egl-1 constructs. The egl-1 promoter was cloned into the BamHI site of pNB14 to generate P_{egl-1}::gcamp3 and mcherry was added by Gibson cloning using pGH8 as a template to generate P_{egl-1}::mcherry::gcamp3. The egl-1 promoter was cloned into the BamHI site of pPD95.75 to generate P_{egl-1}::gfp. Gibson assembly was used to add extra gfp moieties or to add ced-11 cDNA to P_{egl-1}::gfp.
Table 1. Generation of non-refractile apoptotic cells in *ced-11* embryos is dependent on *ced-3* and *ced-4*.

### 1.5-fold embryos

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. refractile apoptotic cells</th>
<th>No. non-refractile apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>12.0 ± 1.8</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>ced-11</em>(n2744)</td>
<td>0.1 ± 0.3</td>
<td>4.8 ± 0.9</td>
</tr>
<tr>
<td><em>ced-3</em>(n717)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>ced-11</em>(n2744); <em>ced-3</em>(n717)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>ced-4</em>(n1162)*</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td><em>ced-4</em>(n1162) <em>ced-11</em>(n2744)*</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

### 3-fold embryos

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. refractile apoptotic cells</th>
<th>No. non-refractile apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ced-5</em>(n1812)</td>
<td>27.6 ± 3.1</td>
<td>1.1 ± 1.1</td>
</tr>
<tr>
<td><em>ced-11</em>(n2744); <em>ced-5</em>(n1812)</td>
<td>1.0 ± 1.1</td>
<td>24.3 ± 4.3</td>
</tr>
<tr>
<td><em>ced-5</em>(n1812) <em>ced-3</em>(n717)</td>
<td>0.3 ± 0.4</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td><em>ced-11</em>(n2744); <em>ced-5</em>(n1812) <em>ced-3</em>(n717)</td>
<td>0.6 ± 0.8</td>
<td>0.5 ± 0.8</td>
</tr>
</tbody>
</table>

Average number of refractile and non-refractile apoptotic cells with standard deviation. Apoptotic cells were identified by DIC. At least 20 embryos of each genotype were scored. * Animals contain *dpy-17*(e164).
Figure 1. *ced-11* is required for an increase in apoptotic cell refractility.

(A-D) DIC images of 3-fold stage embryos with arrowheads pointing to apoptotic cells. Red arrowheads point to apoptotic cells in insets. (A) wild-type embryos have refractile apoptotic cells, (B) *ced-11(n2744)* embryos have non-refractile apoptotic cells, (C) refractile apoptotic cells persist and accumulate in *ced-5(n2098)* embryos, (D) non-refractile apoptotic cells persist and accumulate in *ced-11(n2744); ced-5(n2098)* embryos. (E) Diagram of the *ced-11* gene. Arrowheads point to the location of *ced-11* alleles, † two alleles of *ced-11* were isolated in the same strain. Bracket indicates a deletion. (F-G) The average number of (F) refractile and (G) non-refractile apoptotic cells in the heads of 3-fold stage embryos of the indicated genotypes. *ced-5(n2098)* was used in all strains. * strain also includes *sem-4(n1378)*. Error bars, standard error of the mean. At least 20 embryos of each genotype were examined. (H-I) GFP::CED-11 can rescue apoptotic cell refractility when expressed in the apoptotic cell. *ced-11(n4666); nEx2544[P*egl-1::*gfp::*ced11]*.
Figure 2. Apoptotic cells in ced-11 embryos are engulfed normally.

(A-B) DIC and CED-1::GFP images of (A) wild-type and (B) ced-11 embryos at the onset of ventral enclosure when apoptotic cells C1, C2, and C3 have been engulfed. All strains contain en1ls7 [Pced-1::ced-1::gfp]. (C-D) CED-1::GFP clustering around apoptotic cells as they are engulfed in (C) wild-type and (D) ced-11 embryos with accompanying DIC images. Time 0 is the time point before CED-1::GFP begins to move around the apoptotic cell. (E) Average time to completion of apoptotic cell engulfment in wild-type and ced-11 embryos, starting at time 0 and ending when CED-1::GFP is completely around the apoptotic cell. At least 8 embryos and 25 apoptotic cells were analyzed for each genotype. Mean time with standard deviation. (F) Average time for CED-1::GFP to disappear from phagosomes after engulfment in wild-type and ced-11 embryos. At least 7 embryos and 21 apoptotic cells were analyzed for each genotype. Mean with standard deviation.
**A**

Wild-type

![Image of Wild-type CED-1::GFP](image)

**B**

ced-11

![Image of ced-11 CED-1::GFP](image)

**C**

Wild-type

![DIC images](image1)

**D**

ced-11

![DIC images](image2)

**E**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Engulfment (mins)</th>
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<tr>
<td>Wild-type</td>
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<tr>
<td>ced-11(n2744)</td>
<td>5.9 ± 1.7</td>
</tr>
<tr>
<td>ced-11(n4666)</td>
<td>5.9 ± 1.7</td>
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**F**

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<tr>
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<td>5.6 ± 3.4</td>
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<tr>
<td>ced-11(n4666)</td>
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Figure 3. Mutation of ced-11 does not cause cell survival on its own, but can enhance cell survival of other cell-death mutants.

(A) Average number of extra cells present in the anterior pharynx of L3-L4 hermaphrodites with standard error of the mean. At least 20 animals of each strain were scored. (B-C) Average number of extra cells in the ventral cord or L4 hermaphrodites of indicated genotypes. Error bars, standard error of the mean. At least 100 animals of each genotype were examined. All strains contain nls106. † this strain contains dpy-18. Unpaired students t-test, ** p < 0.01 *** p < 0.0001.
A

**Anterior Pharynx Cell Death Assay**

<table>
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<tr>
<th>Genotype</th>
<th>Number of extra cells</th>
<th>Range of extra cells</th>
</tr>
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<tr>
<td>wild-type</td>
<td>0.04 ± 0.03</td>
<td>0-1</td>
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<tr>
<td>ced-11(n2744)</td>
<td>0.09 ± 0.06</td>
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<tr>
<td>ced-11(n4666)</td>
<td>0.0 ± 0.0</td>
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</tbody>
</table>

B

No. extra cells (SEM)

C

No. extra cells (SEM)

---

87
Figure 4. Some apoptotic cells in *ced-11* animals have altered morphology by EM.

(A-F) Electron micrographs of apoptotic cells (outlined in red) and engulfing cells (outlined in blue) at 20,000X and 40,000X magnifications. Apoptotic corpses post-breakdown in (A) wild-type, (B) *ced-11*(n2744), and (C) *ced-11*(n4666) embryos contain membranous whorls and do not stain darkly. (D) A pre-breakdown apoptotic cell in a wild-type embryo stains darkly and looks distinct from the surrounding living cells. (E) A pre-breakdown apoptotic cell in a *ced-11*(n2744) embryo does not stain darkly and is less distinct from the surrounding living cells. (F) A pre-breakdown apoptotic cell in a *ced-11*(n4666) embryo looks similar to *ced-11*(n2744). (G) A pre-breakdown wild-type apoptotic cell with a wrinkled nucleus and separation of the nucleus and cytoplasm. (H) Summary table showing the percent of pre-breakdown apoptotic cells in wild-type and *ced-11* embryos with wrinkled nuclei and nuclear separation.
A Wild-type ced-11(n2744)  
B ced-11(n2744)  
C ced-11(n4666)  

D Wild-type  
E ced-11(n2744)  
F ced-11(n4666)  

Percent pre-breakdown with wrinkled nuclei  
Wild-type 40% (10/25) 0% (0/21) 0% (0/11)  

Percent pre-breakdown with nuclear separation  
Wild-type 60% (15/25) 19% (4/21) 9% (1/11)
Figure 5. *ced-11* regulates an increase in nuclear membrane permeability during apoptosis.

(A-B) GCaMP3 localization in apoptotic cells. Both strains contain *nls575 [Pflp-15::gcamp3]* (A) GCaMP3 localizes to the cytoplasm and nucleus of an apoptotic cell in a wild-type embryo. (B) GCaMP3 localizes only to the cytoplasm of an apoptotic cell in a *ced-11(n4666)* embryo. (C) Time lapse video of GCaMP3 fluorescence in a wild-type embryo. GCaMP3 is expressed in a dying cell and its neighboring cell. Arrow points to onset of apoptotic cell refractility (n = 3) (D) GFP-trimer expressed in a wild-type embryo can either have fluorescence throughout the apoptotic cell indicating cytoplasmic and nuclear localization or can have a ring of fluorescence indicating cytoplasmic localization. (E) Percent of apoptotic cells with fluorescence throughout in wild-type (black bars) and *ced-11(n4666)* (gray bars) embryos expressing the indicated fluorescent proteins under the *egl-1* promoter. At least 5 embryos and 16 apoptotic cells were scored for each genotype and protein. * p < .05, ** p < .01, *** p < .001 Fisher's exact test. (F) GFP-trimer and mCherry::Ce-emerin expressed in an apoptotic cell in a wild-type embryo.
A Wild-type GCaMP3 B ced-11 GCaMP3

C

t=0 t=2 t=4 t=6 t=8 t=10

GCaMP3

D GFP Trimer

Fluorescence throughout Fluorescent ring

F

GFP Trimer mCherry::Ce-emerin

E

% apoptotic cells with fluorescence throughout

<table>
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<tr>
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<th>Wild-type</th>
<th>ced-11</th>
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<tbody>
<tr>
<td>GFP2X (54 kDa)</td>
<td>100%</td>
<td>80%</td>
</tr>
<tr>
<td>GFP3X (81 kDa)</td>
<td>80%</td>
<td>60%</td>
</tr>
<tr>
<td>mCherry::GCaMP3 (77 kDa)</td>
<td>60%</td>
<td>40%</td>
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Figure 6. Apoptotic cells in ced-11 embryos are larger and take longer to degrade.

(A) Kaplan-Meier survival curve of apoptotic cell (C1, C2, and C3) degradation times in wild-type and ced-11(n2744) embryos. Apoptotic cells are occasionally lost because the embryos start moving around 60 minutes or due to stage drift. Dots and squares indicate apoptotic cells that were lost. 10 embryos and 30 apoptotic cells were evaluated for each genotype. p < .0001 Gehan-Breslow-Wilcoxon test.

(B) Diameter of apoptotic cells C1, C2, and C3 in wild-type, ced-11(n2744), and ced-11(n4666) embryos at the time of engulfment. We measured the diameter at the most central Z section of each apoptotic cell. Each dot, square, or triangle represents one apoptotic cell. Bars show the mean and standard deviation. At least 9 embryos and 26 apoptotic cells were measured for each genotype. *** p < .0001 One-way ANOVA with Dennett’s Correction.
A

Phagosome Persistence

Time (mins)

Wild-type

ced-11(n2744)

B

Apoptotic cell diameter (microns)

Wild-type  ced-11(n2744)  ced-11(n4666)

* * *

* * *
Figure 7. GFP::CED-11 can localize to the plasma membrane.

(A-L) Images of GFP::CED-11 and plasma membrane marker mCherry::PH ns790[P_ced-1::gfp::ced-11]; nex2344[P_evl-20::mcherry::ph]. (A-D) 1.5-fold embryo, with insets of an apoptotic cell (arrowhead). (E-H) Posterior pharynx of an L1 larva. (I-L) Tail of an L1 larva.
$P_{ced-11::gfp::ced-11}; P_{evi-20::mcherry::ph}$

1.5-fold embryo

L1 posterior pharynx

L1 tail
Figure 8: Model of CED-11 in apoptosis.

(A) CED-11 is directly or indirectly activated by the CED-3 caspase. CED-11 activation results in a decrease in cell volume and increases in cellular refractility, electron density and nuclear permeability. The decrease in cell volume might drive an increase in macromolecule density leading to the increases in refractility and electron density. The CED-11 mediated changes to the apoptotic cell promote its death and degradation. (B) CED-11 acts downstream of CED-3 to promote cell-autonomous death and degradation in parallel to engulfment and phagosome-mediated degradation.
Figure A: Caspase activated

- CED-11
  - Active CED-3 dimer
  - Cytosolic proteins
  - Nuclear proteins

- Dying
  - Cell volume down
  - Refractility up
  - Electron density up
  - Nuclear permeability up

- Dead

Figure B: Engulfment

- CED-3
  - Phagosome-mediated degradation
  - Complete degradation
  - Death and cell-autonomous degradation

- CED-11
  - Cell-shrinking and increased nuclear permeability
Figure 3 – Supplemental Figure 1. Number of apoptotic cells visible by DIC at different embryonic stages.

(A-B) Average number of refractile and non-refractile apoptotic cells identified by DIC in different staged embryos. (A) wild-type (B) *ced-11(n2744)* Stages are: B/C, bean and comma; 1.5X, 1.5-fold embryos; 2X, 2-fold embryos; 3XE, early 3-fold embryos; 3XL, late 3-fold embryos with a well-developed pharynx and grinder. Error bars, standard error of the mean.
A

Wild-type

B

ced-11

No. refractile apoptotic cells

No. non-refractile apoptotic cells

B/C 1.5X 2X 3XE 3XL
Figure 4 – Supplemental Figure 1. Electron micrographs of post-breakdown corpses in embryos.

Electron micrographs of post-breakdown corpses in embryos of indicated genotypes.
Figure 4 – Supplemental Figure 2. Electron micrographs of pre-breakdown apoptotic cells in embryos.

Electron micrographs of pre-breakdown apoptotic cells in embryos of indicated genotypes and stages. * indicates an apoptotic cell with a wrinkled nuclei. † indicates an apoptotic cell with separation of the nucleus and cytoplasm.
Wild-type pre-B/C

Wild-type post-1.5 fold

*Y54

ced-11(n4666) pre-B/C

ced-11(n4666) post-1.5 fold

ced-11(n2744)

Mixed Stage

1 μm
Figure 4 – Supplemental Figure 3. Electron micrographs of pre-breakdown apoptotic cells in L1 larvae.

DIC and electron micrographs of apoptotic cells. (A, B) Wild-type apoptotic cell. (C, D) ced-1 apoptotic cell. (E, F) ced-1; ced-11 apoptotic cells. In A, anterior is left; in C and E, anterior is right. In E, the posterior apoptotic cell is barely visible in this focal plane. Arrows, apoptotic cells in DIC images. Arrowheads, boundary of plasma membrane of dying cell. Scale bar for DIC images, 10 μm. Scale bar for electron micrographs (B, D and F), 500 nm.
Figure 7 – Supplemental Figure 1. CED-11 TRP Channel Alignment.

Multi-sequence alignment of *C. elegans* CED-11, human TRPM1, and human TRPM3. Black shaded boxes are identical residues and gray shaded boxes are similar residues.
Figure 7 – Supplemental Figure 2. $P_{ced-11}::gfp::ced-11$ expression.

(A-F) GFP::CED-11 expression pattern in embryos of indicated stages and an L1 larva. mCherry::PH localizes to the plasma membrane. mCherry is exposed to optimize detection in each image. (A-E) GFP is exposed with the same conditions to allow for comparison between embryos. Scale is the same for all embryos. (F) GFP is exposed to optimize detection.
Acknowledgments:
I thank Holly Johnsen, Anna Corrionero Saiz, and Christoph Engert for helpful comments about this chapter.

References:


TRPM7 releases the kinase domain from the ion channel and regulates its participation in Fas-induced apoptosis. Dev. Cell 22, 1149–1162.


Chapter 3

Investigating the killing function of CED-9

Kaitlin Driscoll and H. Robert Horvitz
Introduction:

Genetic screens of *C. elegans* for mutations that block cell death led to the identification of the four genes that comprise the core of the *C. elegans* cell-death pathway. Loss-of-function mutations in *egl-1*, *ced-4*, or *ced-3* and gain-of-function mutations in *ced-9* cause increased cell survival (Conradt and Horvitz, 1998; Ellis and Horvitz, 1986; Hengartner et al., 1992). These genes have well conserved orthologs among metazoans, and their identification directly informed our understanding of apoptosis in mammals. In living cells the anti-apoptotic protein CED-9, a homolog of the human proto-oncogene BCL-2, localizes to the mitochondrial outer membrane and binds an asymmetric homodimer of the pro-apoptotic protein CED-4, a homolog of human Apaf-1, to inhibit CED-4 from activating the caspase CED-3 (Chen et al., 2000; Yan et al., 2005). EGL-1, a BH3-only protein, is transcriptionally upregulated in cells destined to die (Conradt and Horvitz, 1998). EGL-1 binds to CED-9 and causes a conformational change that results in release of CED-4 (Chen et al., 2000; Peso et al., 2000). The released CED-4 localizes to the perinuclear membrane and forms an octamer of four asymmetric dimers that bind two molecules of pro-CED3 caspase to facilitate their autocatalytic activation and dimerization (Qi et al., 2010). Upon activation CED-3 cleaves downstream effectors of apoptosis that lead to the death and engulfment of the cell (Xue et al., 1996; Yuan et al., 1993b).

The *ced-9* gene has a well-established anti-apoptotic function, since *ced-9* null mutations cause ectopic cell death, whereas gain-of-function mutations prevent normal programmed cell deaths from occurring (Hengartner et al., 1992). However, *ced-9* also has a pro-apoptotic function, which was discovered because *ced-9* null mutations cause
the survival of cells that normally die in the anterior pharynx in sensitized genetic backgrounds containing a second mutation in the pro-apoptotic genes ced-4 or ced-3 (Figure 1A) (Hengartner and Horvitz, 1994b). While null alleles of ced-3 prevent nearly all programmed cell deaths, weak alleles of ced-3 only partially prevent cell deaths. If the sole function of ced-9 is to prevent cell death, a reduction of ced-9 function should decrease or not alter the number of extra cells that survive in animals with a weak allele of ced-3. Instead, ced-9 null mutation causes an increase in the number of extra cells that survive in the anterior pharynx of animals with a weak allele of ced-3, indicating that ced-9 can function to promote cell death and hence has a pro-apoptotic “killing” function. ced-9 can also enhance survival of the M4 sister cell in animals with a weak allele of ced-3 (Hirose and Horvitz, 2014). Certain mammalian BCL-2 family members have also been reported to have anti-and pro-apoptotic functions (Cheng et al., 1997; Lin et al., 2004).

There are two theories that have been proposed for CED-9 killing function. One theory is that CED-9 killing function is mediated by inhibition of CED-4L, an anti-apoptotic isoform of ced-4. The ced-4 transcript is alternatively spliced into two isoforms, the pro-apoptotic isoform ced-4S and the anti-apoptotic isoform ced-4L (Shaham and Horvitz, 1996). Both CED-4S and CED-4L can bind to CED-9, although CED-4S binds more strongly (Chinnaiyan et al., 1997). The other theory is that CED-9 promotes mitochondrial fission and fragmentation in apoptotic cells. CED-9 in complex with EGL-1 can interact with the mitochondrial fission promoting protein DRP-1 and over-expression of CED-9 with EGL-1 increases DRP-1 mitochondrial localization and mitochondrial fission (Lu et al., 2011) Overexpression of a dominant-negative DRP-1
reduces mitochondrial fission and causes a weak increase in cell survival in the anterior pharynx (Jagasia et al., 2005).

In the Horvitz laboratory an allele of ced-9, n3377, was identified in a screen for enhancers of a weak ced-3 allele (Figure 1B). ced-9(n3377) was crossed out of the ced-3 background and was found to recessively enhance cell survival on its own. ced-9(n3377) thus seems to act differently from dominant gain-of-function ced-9 alleles that increase cell survival or recessive loss-of-function alleles that increase cell death. Instead, ced-9(n3377) might have a loss of ced-9 killing function. It is also possible that n3377 has a dose-dependent gain-of-function. A dose-dependent gain-of-function allele might have similarities to a dominant gain-of-function allele. The ced-9 dominant gain-of-function allele n1950dm increases cell survival by altering the binding of EGL-1 and CED-9 so that CED-9 no longer releases CED-4 in the presence of EGL-1 (Hengartner and Horvitz, 1994b; Peso et al., 2000). In ced-9(n1950dm) embryos CED-4 is localized to the mitochondria even in cells that are overexpressing EGL-1, which would normally cause ectopic cell death and localization of CED-4 to the perinuclear membrane (Chen et al., 2000). Unlike the n1950dm allele, the n3377 allele causes a mislocalization of CED-4 that is similar to that of ced-9 null alleles (Figure 1 C-E). In ced-9(null); ced-3(lof) embryos CED-4 is localized to the perinuclear membrane (ced-3 mutations suppress the maternal-effect lethality of the ced-9(null) mutation, and ced-3 mutations do not affect CED-4 localization). In ced-9(n3377); ced-3(lof) embryos CED-4 is localized to the perinuclear membrane similar to the ced-9(null); ced-3(lof) embryos. Thus, the ced-9(n3377) allele does not act similar to the dominant gain-of-function allele and hence seems more likely to have an increase in cell survival caused by a loss-of-killing-
function rather than a dose dependent gain-of-function. The localization of CED-4 at the perinuclear membrane in ced-9(n3377) embryos suggests that CED-4 perinuclear localization is not sufficient to induce death.

We investigated the killing function of CED-9 using ced-9(n3377). We found that the n3377 mutation causes CED-9 to have reduced binding of EGL-1, CED-4S and CED-4L. We found that mitochondria still fragment in apoptotic cells of ced-9(n3377) animals. We propose further experiments to test the theories that CED-9 killing function is mediated through CED-4L or mitochondrial fragmentation.
Results:

Eliminating background mutations by backcrossing **ced-9(n3377)**

It is possible that **ced-9(n3377)** has a closely linked background mutation causing the increase in cell survival. I backcrossed the **n3377** allele 4 times using closely linked phenotypic markers to ensure that crossovers occurred on each side of **ced-9** within 6 cM (Figure 2A). I then confirmed that the backcrossed **n3377** allele confers a recessive cell-death defect that can enhance the **ced-3** cell death defect (Figure 2B-F). Thus, a background mutation is unlikely to cause the **ced-9(n3377)** cell-death defect.

A genetic screen for additional loss of **ced-9** killing function alleles

To investigate the role of **ced-9** killing it would be useful to have other alleles of **ced-9** in addition to **n3377** that lack the **ced-9** cell-killing function. Having mutant alleles is beneficial, as different mutations can alter the function of a protein in different ways and having alleles of different strengths can be used to optimize experiments. In addition, such mutations could be mapped onto the crystal structure of CED-9, indicating what protein interactions and/or domains are required for the killing function.

To seek additional loss of **ced-9** killing function alleles I performed a non-complementation screen with a **ced-9(null)** allele, as **ced-9(n3377)/ced-9(null)** animals have extra cells in the ventral cord and **ced-9(+)/ced-9(null)** animals do not (Figure 3A-B). In cell-death mutants up to 7 extra cells survive and differentiate into VC-like motor neurons in the ventral cord, 5 of which reliably express the reporter **P_{lin-11}::gfp** and are scored in the ventral-cord cell-death assay (Reddien et al., 2001). I screened 9,600 haploid genomes and isolated 43 animals with extra cells in the ventral cord. None of
these isolated retested. This screen was likely unsuccessful because not enough animals were screened. The mutagen ethyl methanesulfonate (EMS) induces loss-of-function mutations in a given C. elegans gene at an average frequency of 1: 2,000 haploid genomes (when used at a concentration of 50 mM), but such mutations are more likely to be null alleles than specific hypomorphic alleles like n3377 (Shaham, 2007). The screen could be redesigned to allow for isolation of lethal ced-9 null alleles, which would show the effectiveness of the screening process or with the advent of CRISPR-Cas9 technology, hypomorphic mutations of different domains of ced-9 could be made and tested to see if they behave like n3377.

A screen for ced-9(n3377) suppressors

To identify components of the ced-9 killing pathway I performed a screen for suppressors of the ced-9(n3377) cell-death defect in the ventral nerve cord. ced-9(n3377) mutants have an average of 2.1 extra cells in the ventral nerve cord and a range of 0-5 extra cells. Since 7% of ced-9(n3377) animals do not have extra cells, there would be a high false positive rate during such a screen. However, ced-9(n3377), ced-3(n2427) animals have a range of 4-5 extra cells in the ventral cord, yielding a more robust suppression screen. In addition suppressors of the ced-3 cell-death defect could also be interesting. I therefore EMS-mutagenized ced-9(n3377); ced-3(n2427); nls106 [Plin-11::gfp] L4 hermaphrodites and selected F2 progeny with fewer than three extra cells. I screened 30,000 haploid genomes and identified 180 candidates, 20 of which retested. None caused suppression of the extra cells in the anterior pharynx, indicating that the general defect in cell-death remained. The candidates appeared to have a partial silencing of the reporter rather than a reduction in cell killing. An F3 clonal screen
may be more effective, as it allows for isolation of lethal mutations and maternally contributed genes.

CED-9(E74K) interaction with cell-death proteins

*ced-9* killing function could be mediated through the canonical apoptosis pathway by the CED-9 interacting proteins CED-4 or EGL-1 after apoptosis is initiated and the protective function of CED-9 is no longer relevant. *ced-9* killing function could be mediated by CED-9 interaction with CED-4S downstream of apoptosis initiation, or CED-9 could act to inhibit the CED-4L anti-apoptotic function. The *ced-9(n3377)* mutation causes an amino acid change in the CED-4 binding pocket; CED-9 amino acid 74 changes from glutamate to lysine (Figure 4A). Alternatively, the E74K mutation might allosterically affect the interaction between CED-9 and EGL-1. EGL-1 might act with CED-9 to promote mitochondrial fission in dying cells. I tested the interaction of CED-9(E74K) with EGL-1, CED-4S, and CED-4L to see if changes in any of these interactions could shed light on the killing function of *ced-9*. I found that CED-9(E74K) pulled down half the amount of these proteins compared to CED-9 (Figure 4B). Thus it is possible that *ced-9* killing function is through one or multiple of these proteins, but the results do not help to differentiate among the different proposed mechanisms.

Mitochondrial fission in *ced-9(n3377)* apoptotic cells

If the killing function of *ced-9* is mediated through mitochondrial fission, then *ced-9(n3377)* animals might have a defect in fission during apoptosis. To test if *ced-9* killing function is through promoting fission during apoptosis I used a mitochondrial-localized GFP to determine if the mitochondria of apoptotic cells in *ced-9(n3377)* animals have
impaired fission. I found small round mitochondria in wild-type and ced-9(n3377) apoptotic corpses, suggesting fission is not impaired in ced-9(n3377). There are a few caveats to this experiment: 1) it is possible that the cells destined to die that survive in ced-9(n3377) animals do so because fission is impaired in those cells and, 2) there could be a fission defect that I am not seeing because of resolution or timing.

A screen for alleles of ced-4 that behave like a ced-9(null)

A ced-4 mutation that dominantly enhances ced-3 was previously isolated in a screen for enhancers of the ced-3(n2427) cell-death defect (Peter Reddien, personal communication). This isolate contained two mutations in ced-4: ced-4(n3392) alters R119, which is in the CED-9 binding pocket, to serine and ced-4(n5611) alters T28, which is in the caspase recruitment domain (CARD), to asparagine (Daniel Denning, personal communication). Many CARD domain missense mutations have strong cell-death effects. We proposed that n3392 causes a loss of the CED-4/CED-9 interaction and results in the dominant enhancement of cell survival in ced-3(n2427) animals, while n5611 causes a general recessive loss of CED-4 function. If ced-9 killing function is through its interaction with ced-4, a mutant of CED-4 that did not interact with CED-9 but retained the ability to activate CED-3 might behave as a ced-9 null allele and enhance cell survival in a ced-3 background. The ced-3 background would also inhibit the potential lethality of such an allele. To isolate an allele similar to n3392 without the linked n5611 CARD domain mutation, I performed a pilot screen of 15,000 haploid genomes for dominant enhancers of ced-3(n2424) and identified 8 candidates (Figure 5A-C). I sequenced the candidates for mutations in the ced-4 coding region and found that none of them had mutations in ced-4. Going forward it might be faster to use
CRISPR-Cas9 to generate n3392 without n5611 and other alleles of ced-4 that would be predicted to effect CED-4 interaction with CED-9 but not with CED-3.
Conclusions:

CED-9 primarily acts as an anti-apoptotic protein, but also has a pro-apoptotic function. Our two main hypotheses for CED-9 pro-apoptotic function concern the core cell-death proteins that CED-9 interacts with, CED-4 and EGL-1. CED-9 binds to both the pro- and anti-apoptotic isoforms of CED-4. While CED-9 functions in living cells to inhibit CED-4S, it could also act to prime CED-4S for apoptosome formation in dying cells. For example, CED-4 forms a dimer when bound to CED-9 and an octamer when bound to CED-3; perhaps CED-9 binding to the CED-4 dimer helps with the octamer formation in cells that have activated the cell death pathway. In addition CED-9 could bind to and inhibit the anti-apoptotic CED-4L and thus promote cell death. Alternatively CED-9 could promote death through its interaction with EGL-1 and promotion of mitochondrial fragmentation.

Our lab previously isolated an allele of ced-9, n3377, that we hypothesize to have a ced-9 loss-of-killing function. CED-9(E74K), caused by the n3377 mutation, has reduced affinity for CED-4S, CED-4L and EGL-1 compared to CED-9. This finding is consistent with the observed localization of CED-4 to the perinuclear membrane in ced-9(n3377) mutants, which indicates that the CED-9/CED-4 interaction is disturbed. However, this result does not help to distinguish between the alternative hypotheses described.

We determined that mitochondria can fragment in apoptotic cells in ced-9(n3377) mutants; while this result would suggest that ced-9 killing function is not through mitochondrial fission there are caveats outlined above that make interpreting this result problematic.
Future Directions:

To determine if CED-9 is required for normal mitochondrial dynamics during apoptosis a next step would be to examine mitochondria using mitochondrial-localized GFP and time-lapse imaging in apoptotic cells of wild-type, ced-3(weak lof), and ced-9(null); ced-3(weak lof) animals. If in ced-9(null); ced-3(weak lof) animals a delay in or reduction of fragmentation is seen in apoptotic cells, the next step would be to test if increasing mitochondrial fragmentation could suppress ced-9(null) enhancement of ced-3(weak lof).

In support of the CED-4L hypothesis, mutations in spk-1, a gene thought to promote the splicing of ced-4 to generate ced-4L, are able to suppress ced-9(n3377), in addition to multiple alleles of ced-3 and ced-4 (Galvin et al., 2011). If ced-9(n3377) has a loss of ced-9 killing function and if loss of ced-9 killing function is through inhibiting ced-4L, in the ced-9(n3377) mutant there would be an increase in uninhibited CED-4L which would promote cell survival. In the ced-9(n3377); spk-1 double mutant, less CED-4L would be generated, so there would be less cell survival and ced-9(n3377) would be suppressed, as observed. To determine if ced-9 killing function is through ced-4, a next step would be to use CRISPR-Cas9 to generate alleles of ced-4 that alter its splicing and alleles of ced-4 and ced-9 that interrupt their interaction with each other but not with other proteins. Genetic and biochemical analyses of these mutants will help to determine if ced-9 killing function is through ced-4. For example, if CED-9 killing function is completely through inhibition of CED-4L, an allele of ced-4 defective in generating the ced-4L isoform would suppress ced-9(null) enhancement of ced-3(weak lof).
Materials and Methods:

C. elegans strains

C. elegans strains were cultured at 20 °C as described previously (Brenner, 1974b). N2 (Bristol) was the wild-type strain. The following mutations and integrants were used and have been described, except for those from this study:

LGIII: unc-69(e57am), ced-9(n3377), ced-9(n2812), ced-9(n1950dm n2077), dpy-18(e364am)

LGIV: ced-3(n2427)

LGX: nls106 [P<sub>lin-11::GFP</sub>]

Unmapped: n5686, n5687, n5688, n5689, n5690, n5691, n5692, n5693, bcIS49[P<sub>egl-1::mito::gfp</sub>]

Ventral cord cell-death assay

Survival of P2.aap and P9-12.aap were counted in L4 hermaphrodites using the nls106 reporter (Reddien et al., 2001).

Screens for cell-death mutants

Animals were mutagenized in 50 mM EMS as described (Brenner, 1974).

Pull-down assays

PGEGX-4T-3 plasmids containing ced-9 or ced-9(n3377) cDNA were transfected into Rosetta 2 bacterial competent cells and grown overnight at 37°C in 5ml LB + ampicillin + chloramphenicol. The next day cells were diluted 1:100 in 2xYT + ampicillin + chloramphenicol and grown to an OD600 of 1.0. Cultures were then induced with IPTG (80ul/100ml of 1M stock) and grown for 4 more hours at 30°C. Pelleted cells were
frozen at -80°C overnight and resuspended in 1X PBS + 0.1% Triton X-100 + Roche protease inhibitor complete (binding buffer). Cells were lysed by French press or sonication. The supernatant was incubated with GE Healthcare Glutathione Sepharose 4B beads and incubated for 1 hour at 4°C and washed 5 times with 1xPBS + 0.1% Triton X-100 (wash buffer). Beads were resuspended to a 50% slurry in wash buffer. The concentration of CED-9 protein was measured with the Bio-rad Bradford protein assay. 2 µg of GST-CED-9 was incubated with 40 µl of 35S-methionine labeled CED-4S, CED-4L or EGL-1 translated with TnT® Quick Coupled Transcription/Translation System Promega and brought to a total volume of 1 mL with binding buffer. Samples were incubated for 2 hours at 4°C and washed 5 times with wash buffer. The beads were boiled in 20 µl of 2XSDS and supernatant was run in an Any kD gel or a 15% gel (EGL-1) from Bio-Rad. GST-tagged proteins were detected by Coomassie staining and 35S labeled proteins were detected by Typhoon Phosphorimager. The amount of input pulled down was normalized to the amount of GST-fusion protein pulled down by glutathione resin beads in each reaction using Fiji to get the percentage of input pulled down (Lu et al., 2011). The percentage of input pulled down by GST::CED-9(E74K) was normalized to the percentage of input pulled down by GST::CED-9. Figure 4B is of one representative experiment. Experiments were performed twice with similar results.

Expression of mito::GFP in apoptotic cells

Wild-type and ced-9(n3377) embryos expressing mito::GFP in apoptotic cells were mounted on a slide with a 4% agar pad in M9 solution and examined on an Axioskop II (Zeiss) compound microscope using a X100 objective.

Plasmid Construction:
CED-9::GST: *ced-9* and *ced-9(n3377)* cDNAs were cloned into *BamHI* and *XhoI* sites in PGEX-4T-3.

**Primers:**
- ced-9F: cgccgctcggatccatgacacgctgcacggcgga
- ced-9R: cgccgctcggcttacttcaagctgaacatca

CED-4LS and EGL-1: *egl-1* cDNA was cloned into *HindIII* and *XbaI* sites of pSP64 and *ced-4* cDNAs were cloned into *PstI* and *SmaI* sites of pSP64 Promega vectors.

**Primers:**
- egl-1F: cgccgcgaagcttatgtccaacgtttttgacgt
- egl-1R: cgccgcgtctagattaaaaagcgaaaaagtcc
- ced-4F: cgccgcgtcagatgctctgctgcaatcgaatg
- ced-4R: cgccgcccgggttaacagcatgcaaaatttt
Figure 1. *ced-9* can promote cell death.

(A) Average number of extra surviving cells identified by DIC microscopy in the anterior pharynx of L4 animals. Mutations in the *ced-3* caspase gene cause the survival of cells that normally die in the anterior pharynx of *C. elegans*. *ced-9(n2812)*, a presumptive null allele, can enhance the cell survival of a weak *ced-3(n2427)* allele, indicating that *ced-9* can promote cell death. Data from (Hengartner and Horvitz, 1994b)). (B) *ced-9(n3377)* causes cell survival in the anterior pharynx and can enhance a weak *ced-3(n2427)* allele. Peter Reddien, personal communication. (C) CED-4 antibody staining in embryos of different *ced-9* mutant backgrounds. *ced-9(n1950)*, a dominant gain-of-function allele, causes CED-4 to remain at the mitochondrial membrane even after EGL-1 expression. *ced-9(n3400)*, a strong loss-of-function allele, causes CED-4 to localize to the perinuclear membrane (*ced-3* suppresses the *ced-9(lof)* lethality). *ced-9(n3377)* causes CED-4 to localize to the perinuclear membrane similar to the *ced-9* loss-of-function allele, suggesting that *ced-9(n3377)* is not causing increased cell survival by a gain of protective function but rather a loss of killing function. Bradley Hersh, personal communication.
A **ced-9(If)** mutations can increase the cell survival caused by weak **ced-3** mutations

<table>
<thead>
<tr>
<th>ced-3 genotype</th>
<th>Wild-type</th>
<th>ced-9(n2812)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ced-9 genotype</td>
<td>Extra cells</td>
<td>n</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.03 ± 0.05</td>
<td>50</td>
</tr>
<tr>
<td>Weak allele <strong>ced-3(n2427)</strong></td>
<td>2.8 ± 0.3</td>
<td>25</td>
</tr>
<tr>
<td>Strong allele <strong>ced-3(n717)</strong></td>
<td>12.5 ± 0.4</td>
<td>30</td>
</tr>
</tbody>
</table>

B **ced-9(n3377)** causes an increase in cell survival and can enhance cell survival of weak **ced-3** mutation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Extra cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>ced-9(n3377); unc-30</td>
<td>1.4 ± 0.3</td>
</tr>
<tr>
<td>ced-3(n2427)</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>ced-9(n3377); ced-3(n2427)</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>ced-9(n1812); unc-30 ced-3(n2427)</td>
<td>6.7 ± 0.4</td>
</tr>
</tbody>
</table>

C, D, E Images of CED-4 expression in various genotypes.
Figure 2. ced-9(n3377) cell-death defect is likely not caused by background mutations.

(A) Map of ced-9 genomic region with visual markers used to backcross ced-9(n3377). Crossovers occurred between ced-9 and dpy-19 on the left arm and between ced-9 and dpy-18 on the right arm. ced-9(n3377); nls106[Plin-11::gfp] males were mated with dpy-19(e125ts) unc-69(e587am) hermaphrodites, dpy-non-unc progeny (2 lines) were isolated in the F2. dpy19(e125ts) ced-9(n3377); nls106 hermaphrodites were mated with nls106 males and homozygous non-dpy progeny with extra cells were isolated in the F2. Males from the now 2x backcrossed ced-9(n3377); nls106 strain were mated with unc-49(e382) dpy-18(e364) hermaphrodites and the above strategy was repeated to obtain 4x backcrossed ced-9(n3377); nls106 animals. (B-E) Survival of extra cells in the ventral cord as detected by expression of Plin-11::gfp, n > 100. (B) wild-type (C) ced-3(n2427) (D) 4x backcrossed ced-9(n3377) (E) 4x backcrossed ced-9(n3377) with ced-3(n2427). (F) ced-9(n3377) heterozygotes from wild-type hermaphrodites mated with ced-9(n3377) males did not have extra cells, n = 37. All strains contained nls106.
A  
\begin{align*}
\text{dpy-19} & \quad \text{unc-69} & \quad \text{ced-9} & \quad \text{unc-49} & \quad \text{dpy-18} \\
-0.19 & \quad 2.31 & \quad 2.36 & \quad 3.35 & \quad 8.81
\end{align*}

B  
Wild-type  
\begin{itemize}
\item 0 1 2 3 4 5
\item 0 100 80 60 40 20
\end{itemize}

C  
\text{ced-3(n2427)}  
\begin{itemize}
\item 0 1 2 3 4 5
\item 0 20 40 60 80 100
\end{itemize}

D  
\text{ced-9(n3377)}  
\begin{itemize}
\item 0 1 2 3 4 5
\item 0 20 40 60 80 100
\end{itemize}

E  
\text{ced-9(n3377); ced-3(n2427)}  
\begin{itemize}
\item 0 1 2 3 4 5
\item 0 20 40 60 80 100
\end{itemize}

F  
\text{dpy-19 ced-9(n3377)/+}  
\begin{itemize}
\item 0 1 2 3 4 5
\item 0 50 100
\end{itemize}
Figure 3. A non-complementation screen for alleles of *ced-9* with a loss of killing function.

(A) *ced-9(n3377)/unc-69(e587am) ced-9(n1950dm, n2077) dpy-18(e364am); nls106* trans-heterozygotes have extra cells in the ventral cord, n = 52. (B) EMS-mutagenized *nls106* male worms were crossed with *unc-69(e587am) ced-9(n1950dm, n2077) dpy-18(e364am)/balancer(Myo-2::RFP); nls106* hermaphrodites. Rare cross progeny were sought with extra cells, which might be caused by a *ced-9* loss-of-killing function allele or by dominant cell-death mutations. Red is RFP expression in the pharynx. Green is GFP expression in VC neurons, extra surviving VC-like neurons, and vulval tissue (triangle).
A

$\text{ced-9(n3377)/}$

$\text{unc-69 ced-9(null) dpy-18}$

\begin{figure}
\centering
\begin{tikzpicture}
\begin{axis}[
width=\textwidth,
height=\textwidth,
axis x line=bottom,
axis y line=left,
xtick={0,1,2,3,4,5},
xticklabels={0,1,2,3,4,5},
ytick={0,20,40,60,80,100},
ylabel={\% animals},
xlabel=No. of extra cells,
]
\end{axis}
\end{tikzpicture}
\end{figure}

B

EMS-mutagenized male worms $\times$ $\text{unc-69 ced-9(null) dpy-18/}$

$\text{balancer (myo-2::RFP)}$ hermaphrodites

cross progeny

$\text{balancer/+}$

$\text{unc-69 ced-9(null) dpy-18/+}$

$\text{ced-9*/unc-69 ced-9(null) dpy-18}$

self progeny

$\text{unc-69 ced-9(null) dpy-18/balancer}$

$\text{unc-69 ced-9(null) dpy-18}$
Figure 4. CED-9(E74K) has reduced affinity for CED-4S, CED-4L, and EGL-1.

(A) CED-9 (red) binds a CED-4 heterodimer (blue and green). CED-9 E74 is magenta. ced-9(n3377) causes an E74K transition in the CED-4 binding interface. Graphic made with www.pymol.org. (B) The percentage of $^{35}$S-labeled EGL-1, CED-4S, and CED-4L pulled down by GST::CED-9E74K normalized to the percentage of EGL-1, CED-4S, and CED-4L pulled down by GST::CED-9.
Figure A shows a structural model of CED-9, CED-4a, and CED-4b. Figure B displays a bar graph comparing the relative pull-down of GST::CED-9 and GST::CED-9(E74K) with different proteins: EGL-1, CED-4S, and CED-4L.
Figure 5. A screen for dominant enhancers of *ced-3*.

(A) *ced-3(n2427); nls106* hermaphrodites have 0-1 extra cells in the ventral cord. (B) *ced-3(n2427); nls106* hermaphrodites were EMS-mutagenized and F1 progeny were screened for more than 2 extra cells. (C) Extra cells in animals homozygous for alleles isolated from the *ced-3* dominant enhancer screen in B, n > 100.
Acknowledgments:
I thank Cory Pender for helpful comments about this chapter.

References:


