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A New Type of Programmed Cell Death in C. Elegans

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A NEW TYPE OF PROGRAMMED CELL DEATH IN *C. ELEGANS*

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The most intensively studied form of programmed cell death (PCD) is apoptosis, which is characterized by stereotypical morphological features including chromatin compaction and by a requirement for the activity of caspase proteases, which are controlled by conserved gene pathways. Although non-apoptotic, caspase-independent programmed cell death pathways have been postulated, there is little evidence to convincingly prove their existence, and few insights regarding their molecular basis or possible *in vivo* functions. To investigate this question, we have studied the developmentally regulated PCD of the *Caenorhabditis elegans* linker cell.

We have carried out transmission electron microscopy studies of dying linker cells, which revealed non-apoptotic features, including nuclear crenellation in the absence of chromatin condensation, swelling of mitochondria and endoplasmic reticulum, and accumulation of cytoplasmic single- and multi-layered membrane-bound structures. Similar morphological changes occur during the normal developmental death of some vertebrate neurons in the spinal cord and ciliary ganglia, suggesting that this is a highly conserved cell death program.

Our genetic studies demonstrate that linker cell death is a non-apoptotic programmed cell death. This cell death is independent of the *ced-3* caspase, other *C. elegans* caspase homologs, and can occur even when a broad-spectrum caspase inhibitor is expressed. We have found that the engulfment of the linker cell is independent of the known *C. elegans* engulfment genes. We tested and found no evidence for the involvement of autophagic, necrotic, or Wallerian degeneration genes in linker cell death.

By ablating cells neighboring the linker cell, and by examining mutants in which the linker cell is abnormally positioned, we demonstrated that the linker cell employs a cell-autonomous program to promote its demise. Using a candidate gene approach, we showed that linker cell death is controlled by the microRNA *let-7* and by the zinc finger transcription factor *lin-29*, both components of the main developmental timing pathway in the animal.

Conducting a genome-wide RNAi screen, we have identified new candidate regulators of linker cell death. Characterization of these genes may uncover the molecular mechanism driving this new type of programmed cell death in *C. elegans*.

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Chapter One

INTRODUCTION: The Evolution and Control of Cell Death

We could probably not live without death. Regulated cell death gives shape to a developing embryo, is necessary for homeostasis, and when initiated in response to abnormalities can preserve an individual organism. Multifaceted death enlivens multicellular life.

Scientific Identification and Characterization of Cell Death

The first microscopy observations of cell deaths were made in the 1800s. For example, Carl Vogt observed neurons dying in toad embryos (Vogt, 1842). Some of the earliest work in the twentieth century came in the 1930s from the laboratory of Kallius in Heidelberg (Hamburger, 1992). He and his colleagues Ernst and Glücksmann published a few papers describing vertebrate cell death, detailing reproducible patterns of cell death. In 1949, studying chick embryos, Viktor Hamburger and Rita Levi-Montalcini published the first paper that revealed cell death to be a necessary process during normal development (Hamburger and Levi-Montalcini, 1949). With this paper, cell death was no longer a phenomenon that was just being described, it was now beginning to be understood. In 1965, Lockshin introduced the phrase programmed cell death (PCD) (Lockshin and Williams, 1965) for cell deaths that occur naturally during

development. In 1972, Kerr and colleagues invented the word apoptosis to define a type of programmed cell death with a characteristic morphology of cell death that included chromatin and cytoplasmic compaction (Kerr et al., 1972). Soon, genetic experiments in *C. elegans* (Ellis and Horvitz, 1986) and *Drosophila* (White et al., 1994) began to illuminate the genes, pathways, and mechanisms regulating programmed cell death. By two decades after the term apoptosis was coined, the molecular identity of the key executioner of apoptosis—namely the protease known as a caspase—was discovered, and a molecular criteria was added to the morphological criteria used to define apoptotic programmed cell death (Yuan et al., 1993).

Cell Death as a Vital Force for Life

Cell death plays many varied and essential roles during development. Cell death can remove unwanted or unnecessary structures, for example, removing the human tail during week seven of embryonic development (Sapunar et al., 2001). Cell death may be required to modify or remove primary transitional structures, cellular scaffolds that are necessary for the formation of a final structure. This can be seen when a tooth forms, as waves of apoptosis occur in spatially restricted regions to remove transitory structures like enamel knots (Matalova et al., 2004).

Cell death can carve the shape of an organism, which is beautifully outlined in the regulation of cell death to create digits or webbing, processes which have been observed and investigated across many species (Weatherbee

et al., 2006). A study of rat embryonic development revealed that apoptosis separates the upper and lower eyelids which are initially fused together (Mohamed et al., 2003).

Removal of cells can be used to separate or create boundaries between cellular regions, something that may have profound consequences for the action of developmental signals. In mammalian eye development, apoptosis at the boundary between the lens vesicle and the ectoderm has been suggested as a means of separating the two structures (Mohamed and Amemiya, 2003).

Cell death can edit the organism with precision at the level of a single specific cell, which represents the focus of research of this thesis. However, cellular doom can be unleashed on epic scales. It has been hypothesized that sometimes large amounts of cell death occur during development because more cells are formed than are needed, and these cells must compete to survive. If the fittest cells triumph, this will improve the fitness of the organism. One example of a cell type that is subject to extremely high attrition is female germ cells. In mice, rats, and humans, it has been estimated that two-thirds of all potential female germ cells die by the end of fetal development, and it has been calculated that for humans, greater than 99.9% of all female germ cells will die (Morita et al., 1999).

In post-embryonic life, cell death is an integral part of day-to-day life—it has been estimated that 10 billion cells die every day in a human being (Heemels, 2000). Cell death is an essential requirement for homeostasis of the immune system (Bidere et al., 2006), and the intestine (Edelblum et al., 2006)

where apoptosis removes cells at a rate that balances the constant proliferation there (Hall et al., 1994). The entire cellular surface of the intestine, covering an area of approximately 20 m^2, is renewed every 3-5 days in man (Hall et al., 1994).

A particularly vibrant area of current research is a body of work illuminating the relationship and direct connections between cell death and cell proliferation at the tissue level, which can be necessary both for development and for homeostasis. In some normal human systems, a balance between cell proliferation and cell death may be critical for normal cellular homeostasis—for example, in healthy bone and bone marrow (Weinstein et al., 2000). Studies in *Drosophila* have shown that apoptotic cells can induce compensatory cell proliferation (Ryoo et al., 2004), and many genes have been identified in flies that affect both proliferation and cell death. In *Xenopus,* it has been recently shown that apoptosis appears to be an important requirement for tail regeneration following lesion (Tseng et al., 2007), and apoptosis has also been observed in regenerating planarians (Hwang et al., 2004). Planarians shrink when they are starved and can regrow again when their feeding conditions improve. As they change size, how they maintain their organ systems in functional proportion by regulating the balance between proliferating and dying cells is not understood (Oviedo et al., 2003).

Defective cell death can lead to diseases. Faulty cell death can allow precancerous or cancerous cells to survive (Green and Evan, 2002),

inappropriate cell death may contribute to some neurodegenerative disorders (Culmsee and Landshammer, 2006), and if self-reactive immune cells are not destroyed, the result is autoimmunity (Navratil et al., 2006).

The Morphology of Apoptotic Programmed Cell Death

The earliest studies of cell death mainly focused on the classification of cell death based on morphological criteria, particularly using histological and transmission electron microscopy (TEM) imaging. In 1972, Kerr et al. coined the term apoptosis for a type of programmed cell death defined by morphological criteria seen by TEM. Hallmarks of apoptosis included chromatin compaction (dark regions of heterochromatin begin to condense at the periphery of the nuclear envelope, and eventually the entire nucleus succumbs to chromatin compaction), dramatic loss of cytoplasmic volume, and features like cell blebbing (Kerr et al., 1972). Phagocytosis of the cell corpse—complete ingestion of the corpse by another cell—was another characteristic of this type of cell death (Kerr et al., 1972), which seemed to frequently occur in isolated dying cells. Apoptosis could be observed in many different organisms and in many different cell types.

The Morphology of Autophagic Programmed Cell Death

Autophagy, a process of lysosomal mediated cellular destruction, is a part of normal cellular homeostasis, a process by which the cell can recycle cytoplasmic and organelle contents. Autophagy can be upregulated in response to cellular

stress, and extensive autophagy can sometimes be seen associated with cell death. Whether autophagy is initiated as a last resort to try to maintain cellular survival or is initiated to kill the cell, remains a matter of debate (Debnath et al., 2005). In development, some cell deaths are seen associated with extensive autophagy. The best known example is the *Drosophila* larval salivary gland (Jiang et al., 1997), where the simultaneous destruction of many cells occurs in response to a hormonal cue.

Visual recognition of autophagic cell death is by the formation of many autophagosomes—membrane-bound vesicles en route to the lysosome (Schweichel and Merker, 1973). They are formed when cytoplasmic material is enveloped in a vesicle with a characteristic double membrane (Arstila and Trump, 1968). After fusion with the lysosome, dark material is visible inside the vesicle as destruction proceeds.

When autophagic cell death was observed in large scale cell deaths in developmental contexts such as larval salivary gland cell death, such cell deaths were not always observed associated with phagocytosis, although recent data shows that a marker of phagocytosis is expressed at the time of *Drosophila* salivary gland cell death (Lee and Baehrecke, 2001).

The Morphology of Type III Programmed Cell Death

In Schweichel and Merker's morphological characterization of cell death, they also mentioned a third category of cell death (Schweichel and Merker, 1973).

This type of cell death was described as a cellular disintegration or fragmentation associated with organelle swelling that was non-lysosomal in nature, and led to the formation of empty spaces that connected with extracellular material. This death did not involve phagocytosis by neighboring cells. The *in vivo* examples given of this death were in regions of cartilage during mineralization, and also sometimes in mesenchymal tissues (Schweichel and Merker, 1973).

Later analysis by other scientists began to add to the description and examples of this category, and the definition was subdivided to include what has been described as “cytoplasmic” or type IIIB cell death—a cell death associated with organelle swelling and rounding, nuclear envelope dilation, lack of early nuclear condensation, and clearance of the corpse by phagocytosis (Clarke, 1990). It has been noted that this type of cell death shares some features with necrosis such as organelle swelling, suggesting that these processes may have something in common (Clarke, 1990).

The Morphology of Necrotic Cell Death

Necrosis is generally thought of as a death by misadventure, a type of cellular collapse from an unendurable trauma. Electron microscopy studies of a *C. elegans* necrotic cell death model showed early stage events including involutions and membrane whorls derived from the plasma membrane. This was followed by cellular swelling, the nucleus became distorted and chromatin clumped, vacuoles formed, and eventually there was a complete breakdown of

cellular structures (Hall et al., 1997). Across other systems, the general characteristics of necrotic death that have been observed include mitochondrial abnormalities, ATP depletion, perturbations in calcium levels, lysosomal rupture, organelle clustering, and plasma membrane rupture (Golstein and Kroemer, 2007).

Bacterial Origins of Cell Death

About 4 billion years ago when unicellular life began to arise on this planet, the first cellular deaths were probably just chaotic collapses of cellular viability. However, it seems likely that once the first bacteria emerged with a capacity to regulate death, the useful potential of this property was enormous. Just as for multicellular creatures, bacteria could use death for growth or defense.

Today, we can observe some bacterially regulated forms of cell death associated with morphological or developmental changes of a colony. In *Myxococcus xanthus,* a so-called "differentiating bacteria," autolysis occurs in the fruiting body formation prior to spore formation (Wireman and Dworkin, 1997). Cell death can be observed in biofilm formation in *Pseudomonas aeruginosa* (Webb et al., 2003). In the biofilm formed by *Pseudoalteromonas tunicata*, the protein AlpP has been identified as responsible for the cell death of a fraction of the cellular population (Mai-Prochnow et al., 2004). A mutant of AlpP has a detrimental effect on dispersal of cells from the biofilm, and the cells that did disperse were less metabolically active than the wild-type, leading to the

hypothesis that the dead cells may be releasing nutrients that increase the fitness of the surviving neighboring cells (Mai-Prochnow et al., 2006).

Many examples soon emerged of the regulation of cell death as a means of defense or attack. Addiction modules, plasmid maintenance systems that comprise a toxin and an antidote (with a much shorter half-life than the toxin), can kill off cells that have lost the plasmid. These systems can be found in archae, in bacteria, and as well as functioning on plasmids, such genes have also been identified at chromosomal locations (Engelberg-Kulka et al., 2006). In response to phage infection, suicidal bacterial cell death could limit virus infection (Hazan and Engelberg-Kulka, 2004).

At a molecular level, bacterial cell death genes are not related to eukaryotic cell death genes (Koonin et al., 2002).

Yeast and Programmed Cell Death

Yeast lack a caspase homolog or homologs of the key intrinsic cell death genes. However, more distantly related paracaspases and metacaspases have been identified from whole genome sequence analysis (Uren et al., 2000), and a potential yeast metacaspase homolog was put forward (Madeo et al., 2002). Questions remain about whether the substrate specificity of this gene would be the same as a standard caspase, and whether yeast undergo a bona fide apoptosis remains controversial (Váchová and Palkova, 2007).

Plants and Programmed Cell Death

Cell death can be important for many aspects of plant development, for example the regulation of the death of a petal may be tightly controlled (Rogers, 2006). Many examples of programmed cell death in plants appear to be autophagic (van Doorn and Woltering, 2005). In response to pathogens, plant cells can die in what is termed the hypersensitive response. These deaths do not appear to be occurring via autophagy, and the dying cells do not get engulfed (van Doorn and Wolterling, 2005). It is possible that some of these deaths fall into a non-apoptotic, non-autophagic, type III category of cell death. Unfortunately, not a great deal of ultrastructural work has been completed on such dying cells (van Doorn and Woltering, 2005), and the genes regulating the hypersensitive response remain unknown (Patel et al., 2006).

Eukaryotic Origins of Cell Death

How did the first cell deaths arise in multicellular organisms? Considering the central role that some mitochondrial genes have in eukaryotic apoptotic programmed cell death, some commentators have suggested that endosymbiosis of mitochondria, perhaps coupled with a form of cellular homicide by secreted proteases from the endosymbionts, could have laid the primordial foundation of eukaryotic cell death (Frade and Michaelidis, 1997). Caspases and apoptosis have been identified in Hydra, one of the simplest multicellular creatures (Cikala et al., 1999). Nematodes, based on 18sRNA analysis were recently placed in a

clade Ecdysozoa with *Drosophila* (Aguinaldo et al., 1997), so Hydra appears to be a more primitive metazoan. A fascinating question is which type of eukaryotic cell death is the most ancient: apoptosis, autophagy, type III PCD, or necrosis?

Evolution of Cell Death and Limited Cellular Destruction

In the evolution of cell death, an interesting possibility to consider is that cells first evolved the capacity for partial and limited cellular degradation, before being able to regulate complete cellular destruction. This capacity could be used to remodel a cell, as occurs when cytoplasmic volume is lost during sperm formation (Arama et al., 2003). Another important example of selective cytoplasmic destruction is Wallerian degeneration, the process of axonal destruction distal to the site of lesion or injury (Waller, 1850). There are many examples of selective axon pruning occurring naturally during development (Luo and O'Leary, 2005). Not only cytoplasm can be selectively destroyed. Red blood cells can selectively lose organelles such as their nucleus, but leave other cellular components intact. This activity may represent a modified use of cell death genes (Testa, 2004).

The Genetic Pathway of Apoptotic Programmed Cell Death

The most profound contribution that has been made to the field of cell death was from the invertebrate systems of *Caenorhabditis elegans* and *Drosophila* in which the genetic basis of cell death was established. This work proved that cell death was not a random disintegration of cellular function from wear and tear, but rather

a controlled process, regulated by a highly conserved pathway of genes leading to caspase activation (Figure 1.1).

The first work seeking a genetic understanding of cell death used the model system of *C. elegans*. This transparent self-fertilizing hermaphrodite with a rapid lifecycle had been chosen as a model system because of its suitability for use in genetic screens (Brenner, 1974). The publication of the worm lineage analysis, a microscopy study detailing the stereotyped development of the worm, revealed that there was an invariant pattern of somatic cell death and division that was reproducible from worm to worm (Sulston and Horvitz, 1977; Sulston et al., 1983). In *C. elegans,* specific cells die at specific times in specific locations. During hermaphrodite development, 1090 somatic cells are born, and 131 of these cells undergo programmed cell death, with 113 of the programmed cell deaths occurring during embryonic development (Sulston and Horvitz, 1977; Sulston et al., 1983). In the *C. elegans* germline, over 300 germ cells undergo programmed cell death (Gumienny et al., 1999). Most *C. elegans* cells destined to die during development do so within 30 minutes after they are born (Sulston and Horvitz, 1977; Sulston et al., 1983).

Using a visual genetic screen, one of the first cell death genes to be identified was *ced-1* in *C. elegans* (Hedgecock et al., 1983). This gene did not block programmed cell death, but was required for engulfment of dying cells in the worm. It was identified as a mutant which had persistent unengulfed cell corpses in the larvae (Hedgecock et al., 1983). Using the *ced-1* mutant to screen

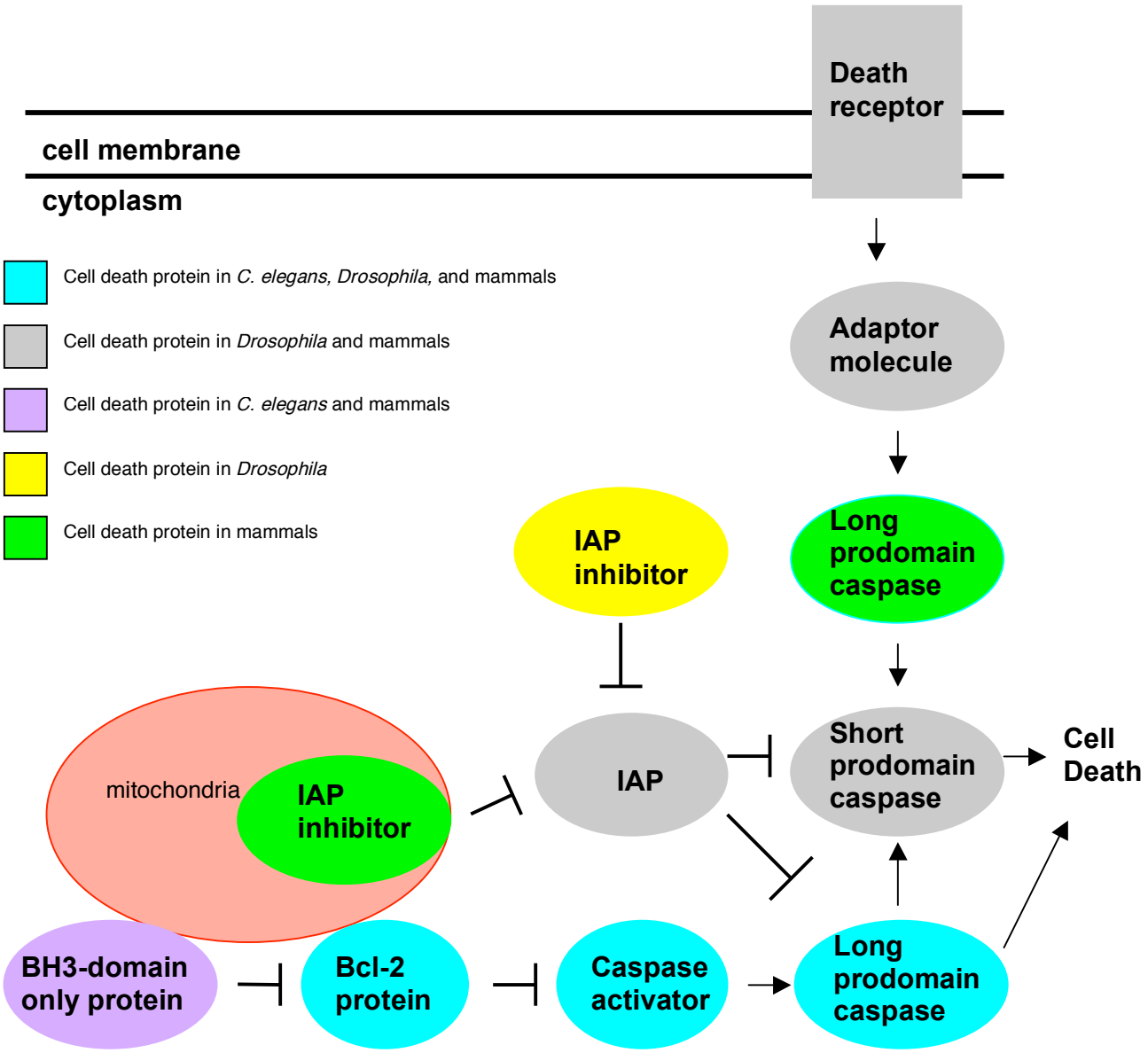


Figure 1.1 Pathways That Regulate Caspases in Apoptotic Cell Death
This figure summarizes three major pathways leading to caspase activation based on evidence from genetic epistasis and biochemical studies in mammals, *Drosophila,* and *C. elegans*. Membrane receptor complexes, such as Fas or TNF receptor complexes, can activate caspases directly following receptor aggregation. Mitochondrial proteins, including members of the Bcl-2 family, control caspase activity by regulating caspase activators such as the *C. elegans* protein CED-4 or its mammalian homolog Apaf-1. CED-4 and Apaf-1 promote caspase activation by acting as scaffolds, thereby allowing cross-activation of adjacent caspase zymogens. IAP (inhibitor of apoptosis) proteins inhibit apoptosis by binding to and inactivating mature caspases.

for animals that did not have persistent cell corpses, identified a mutant in which cell corpses were absent. In this mutant, *ced-3*, a caspase protease, virtually no programmed cell death occurred, and the first intrinsic or “core pathway” regulator of cell death was identified (Ellis and Horvitz, 1986; Yuan et al., 1993). Other screens at the time identified the genes *ced-4* and *egl-1* (Ellis and Horvitz, 1986), which act as part of the same pathway. The other key “core pathway” regulator of programmed cell death is *ced-9* which was identified as an allele with blockage of cell death in the NSM cells, and which was revealed to also cause a substantial block in programmed cell death (Hengartner et al., 1992). Subsequent extensive genetic and biochemical experiments are consistent with a general pathway (Figure 1.2) in which *ced-3* is the most downstream gene, the key executioner of cell death. *ced-3* is activated by *ced-4*. *ced-4* is prevented from activating *ced-3* by *ced-9*, and this repression of *ced-9* can be relieved by *egl-1* or *ced-13* (for a review of the data supporting this model see Lettre and Hengartner, 2006). This simple pathway is, of course, not always the full picture. For example: *ced-4* has a splice form that is anti-apoptotic (Shaham and Horvitz, 1996a); the death of the tail-spike cell is largely regulated by transcriptional

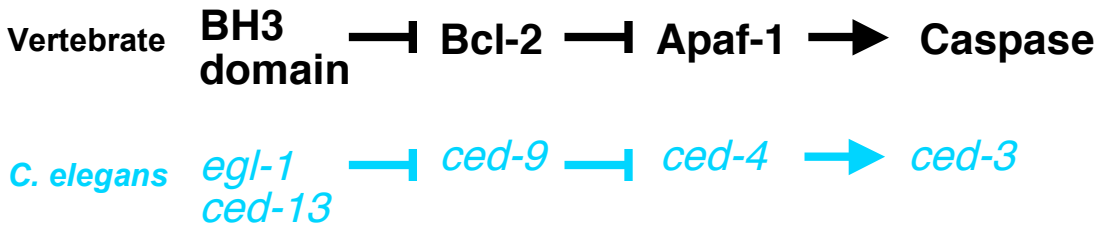


Figure 1.2 A Conserved Core Pathway of Genes Intrinsic to Apoptotic Programmed Cell Death

upregulation of *ced-3*, with only a minor role for *ced-9* or *egl-1* (Maurer et al., 2007); and Pn.p hypodermal cells can die in a *ced-3*-dependent, *ced-4*-independent manner (Joshi and Eisenmann, 2004). Some additional programmed cell death genes have been identified in *C. elegans* which act as upstream cell-specific regulators of the core pathway: for example, *ces-1* and *ces-2* are transcription factors that regulate *egl-1,* and thus the cell death of NSM sister neurons (Ellis and Horvitz, 1991), and the transcription factor *tra-1* regulates expression of *egl-1* in HSN neurons (Conradt and Horvitz, 1999).

Genetic screens in flies identified some additional regulators of the caspase-dependent apoptotic program including the genes *reaper* (White et al., 1994), *hid* (Grether et al., 1994), and *grim* (Chen et al., 1996), and inhibitors of apoptosis genes (IAPs) (Hay et al., 1995). It has been shown that *reaper*, *hid*, and *grim* act through inhibition of IAP function (Goyal et al., 2000). MicroRNAs can also regulate cell death in *Drosophila* (Leaman et al., 2005).

Following the identification of cell death genes, as their homologs in other systems were discovered, this led to the appreciation of the deep conservation of the cell death machinery in metazoans. *ced-3* was found to be homologous to a mammalian cysteine protease known as a caspase (Yuan et al., 1993), and caspases were soon found to be important for cell death in *Drosophila* (Chew et al., 2004; Daish et al., 2004), and mammals (Kuida et al., 1995). *ced-4* was homologous to mammalian Apaf-1 which participates in human cell death (Zou et al., 1997), and to a *Drosophila* gene that also plays a similar role in cell death

(Rodriguez et al., 1999; Zhou et al., 1999; Kanuka et al., 1999). *ced-9* was homologous to mammalian Bcl-2 (Hengartner and Horvitz, 1994), a gene which had previously been suggested might contribute to lymphoma by facilitating cell survival (Vaux et al., 1988). *egl-1* has several homologous mammalian pro-apoptotic counterparts such as Bad, Bim, and Puma (Willis and Adams, 2005). Smac/Diablo is the mammalian counterpart to *reaper*, *hid*, and *grim*, which also inhibits mammalian IAPs (Du et al., 2000; Verhagen et al., 2000).

The Fate of "Undead" Cells in Cell Death Mutant Backgrounds

ced-3 mutant animals in which almost all programmed cell death is blocked are generally healthy, of normal lifespan, but with low fertility (Ellis and Horvitz, 1986). An electron microscopy study of surviving "undead" cells in a *ced-3* mutant background showed that the cells were largely normal and had mainly adopted neuronal cell fates similar to those of their surviving siblings (White et al., 1991). It was shown that an "undead" cell could even take over the function of a cell that had been removed by ablation (Avery and Horvitz, 1987). The "undead" cells could form structures such as synapses or neuromuscular junctions: however, often, the fate of these cells could be variable, and there were some differences in synaptic structure compared with synapses in wild-type cells (White et al., 1991). Studies of extra "undead" cells in flies blocked in programmed cell death revealed that these extra cells could also have

differentiated cell fates, for example, forming extra abdominal neuroblasts (White et al., 1994).

Evidence for the Role of Caspases in Programmed Cell Death

In worms carrying a mutation in the *ced-3* gene, which encodes a caspase, virtually all cells normally fated to die by apoptosis survived (Ellis and Horvitz, 1986). Epistasis studies between mutations in *ced-3* and mutations in other genes encoding cell-death regulators suggested that *ced-3* was the most downstream regulator of apoptosis known in *C. elegans* (Shaham and Horvitz, 1996b).

Genetic studies in other organisms also suggest that caspases are at the heart of the apoptotic program. Mutations in several mammalian caspases disrupt apoptosis (Table 1.1). However, in contrast to *ced-3* mutants, in which virtually all apoptosis is prevented, mammalian caspase mutants often display tissue-specific defects in apoptosis, probably because of redundancy as mammals possess a greatly expanded repertoire of caspases (Table 1.1). For example, mice homozygous for knockout alleles of either *caspase-9* (Kuida et al., 1998; Hakem et al., 1998) or *caspase-3* (Kuida et al., 1998) died shortly after birth and had excess brain tissue, which appeared to be a consequence of defective apoptosis (Kuida et al., 1998; Hakem et al., 1998). Cell death in other major organs was, however, less prominently affected. In human beings, two different alterations in *caspase-10* are associated with type II autoimmune

Table 1.1 Known Mutations in Caspase Genes and Their Phenotypes

Organism	Caspase	Mutations Available?	Mutant Phenotype	Cell Death Defects or Other Roles	References
C. elegans	*ced-3*	yes	normal development	almost all somatic cell death blocked	Ellis and Horvitz, 1986
	csp-1	yes	normal development	none reported	Abraham et al., 2007
	csp-2	yes	normal development	none reported	Abraham et al., 2007
	csp-3	no			
Drosophila	*dcp-1*	yes	larval lethality + tumors	oogenesis	McCall et al.,1998
	dredd	yes	normal development	innate immune system function	Leulier et al., 2000
	dronc	yes	arrest as pupae	no damage induced cell death	Chew et al., 2004
			arrest as pupae	no damage induced cell death	Daish et al., 2004
	drice	yes	some morphological defects	resistant to damage induced cell death	Muro et al., 2006
				role in spermatid differentiation	Arama et al., 2003
	strica/dream	no			
	damm	no			
	decay	no			
Mouse	*caspase-1*	yes-knockout	normal development	defects in receptor mediated apoptosis	Kuida et al., 1995
				defects in production of IL-1α and IL-1β	Li et al., 1995
	caspase-2	yes-knockout	excess oocytes	oocytes reistant to cell death	Bergeron et al., 1998
	caspase-3	yes-knockout	perinatal lethality	defects in brain apoptosis	Kuida et al., 1996
				skeletal muscle differentiation	Fernando et al., 2002
	caspase-6	yes-knockout	normal development	none determined	Zheng et al., 1999
	caspase-7	yes-knockout	perinatal lethality	defects in cardiac development	Lakhani et al., 2006
				resistant to damage induced cell death	Lakhani et al., 2006
	caspase-8	yes-knockout	embryonic lethality	impaired heart development	Varfolomeev et al.,1998
				decreased hematopoietic precursors	Varfolomeev et al.,1998
				defects in receptor mediated cell death	Varfolomeev et al.,1998
				role in T cell function	Salmena et al., 2003
	caspase-9	yes-knockout	perinatal lethality	defects in brain apoptosis	Kuida et al., 1998
				resistant to damage induced cell death	Hakem et al., 1998
	caspase-11	yes-knockout	normal development	defects in production of IL-1α and IL-1β	Wang et al., 1998
				cell migration	Li et al., 2007
	caspase-12	yes-knockout	normal development	defects in ER stress induced apoptosis	Nakagawa et al., 2000
Human	*caspase-8*	familial mutation	immundeficiency	defects in receptor mediated cell death	Chun et al., 2002
	caspase-10	familial mutation	autoimmunity	defects in receptor mediated cell death	Wang et al., 1999
	caspase-14	no		terminal differentiation of keratinocytes	Eckhart et al., 2000
				terminal differentiation of keratinocytes	Lippens et al., 2000

lymphoproliferative syndrome—a condition characterized by defective apoptosis of lymphocytes (Wang et al., 1999). The altered Caspase-10 proteins exhibited decreased apoptotic and enzymatic activities, suggesting that the alterations might cause the disease (Wang et al., 1999). In *Drosophila*, mutants exist in only three of the seven genes encoding caspases. Neither *dcp-1* (Song et al., 1997) nor *dredd* mutants (Leulier et al., 2000) have an overt cell death phenotype. However, a mutation in *dronc* shows that it is required for programmed cell death during *Drosophila* development, and flies with mutations in *dronc* die during larval development (Chew et al., 2004; Daish et al., 2004).

Another approach taken to investigate the role of caspases in a mammalian system was the use of cell permeable caspase inhibitors. These were shown to be able to block mammalian cell death (Jacobson et al., 1996).

The fact that many viruses encode proteins such as p35, which directly inhibit caspases (Bump et al., 1995) and thus block cell death of an infected cell, underscores the centrality of caspases to apoptotic programmed cell death.

Biochemical and Structural Studies of Caspase Activity

A number of biochemical studies have elucidated how caspases carry out their function. Caspases are cysteine proteases that cleave substrates after specific aspartate residues. The specificity of target sites seems to be determined by a four-amino-acid recognition motif, as well as by other aspects of the three-dimensional structure of the target protein. Caspase are synthesized as

proenzymes that are activated through cleavage at internal aspartate residues by other caspases; however, caspases might also have weak catalytic activity in their unprocessed form. Proteins such as *C. elegans* CED-4 or its mammalian homolog Apaf-1 can bind to procaspases and can also multimerize. Multimerization might support cross-activation of adjacent caspase zymogens. Activated caspases consist of dimers of a large and a small subunit that, together, form the active site of the enzyme. Structures obtained by X-ray crystallography suggest that these heterodimers themselves dimerize to form an enzyme with two active sites. Procaspases are often divided into two classes; those with long N-terminal domains are termed initiator caspases, and those with short N-terminal domains are called executor caspases. Long prodomains can bind to activator molecules, such as Apaf-1, or adaptor molecules associated with membrane receptors, such as Fas. It is thought that long prodomain caspases activate short prodomain caspases; however, this assertion is only supported by a limited number of experiments.

The proteolytic activity of caspases has been shown *in vitro*, however, the key *in vivo* targets of caspases that irrevocably doom the cell, have not been determined (Timmer and Salvesen, 2007). Some proteomics approaches in *C. elegans* have suggested some candidates for important targets such as cytoskeletal genes like actin, tubulin, and the ER chaperone calreticulin (Taylor et al., 2007). Activated caspases can cleave multiple targets, and cell death may arise from destruction of a combination of essential cellular components, rather

than from just one or two key targets.

Non-Apoptotic Caspase Activity

Although caspases most frequently act as the chief executioners of cell death, sometimes caspase activation does not progress to cell death. Somewhat paradoxically, deadly caspases play an important role at the beginning of life itself, during sperm formation in *Drosophila*. Caspases aid the process of sperm individualization through which spermatids become separated from syncytia and lose the bulk of their cytoplasm (Arama et al., 2003). During individualization a cytoskeletal membrane complex, known as the individualization complex, translocates along spermatids, disconnecting cytoplasmic "bridges" between them and expelling spermatid cytoplasm and unnecessary organelles into a membrane bag called the cystic bulge. Immunostaining for the activated caspase *drice* marked the pre-individualized part of the spermatid and the cystic bulge (Arama et al., 2003). Synthetic and viral pan-caspase inhibitors severely impaired movement of the individualization complex and prevented removal of bulk cytoplasm from the spermatids (Arama et al., 2003). It is unclear whether caspases have a direct role in exclusion of cytoplasm from the spermatid, although it is reasonable to hypothesize that caspase activity might aid the degradation of expelled cytoplasm in the cystic bulge. The morphological defects and sterility of *Drosophila* sperm treated with caspase inhibitors strikingly resemble a common abnormality in human sperm (Hollanders and Carver-Ward,

1996); mouse knockouts of some apoptotic genes also cause male sterility. Thus, although these mammalian defects are largely uncharacterized, they might also point to a non-lethal role of caspases.

In red blood cells and lens fiber cells, caspase activation leads to a subset of apoptotic morphological changes without causing cell death. As embryonic erythroid cells differentiate into adult red blood cells, they show signs of apoptosis, including chromatin compaction, nuclear destruction, and caspase activation (Morioka et al., 1998). Lens fiber cells develop from epithelial cells that express caspases and degrade their organelles and nuclei during differentiation, presumably to allow cellular transparency (Ishizaki et al., 1998). In transgenic mice that overexpressed Bcl-xL, the lens fibers did not lose their nuclei (Wride et al., 1999). In both examples, it is unclear how caspase activity is controlled to trigger only non-lethal aspects of the apoptotic program.

There are also examples of caspase activation promoting differentiation in the absence of any morphological signs of apoptosis. For example, during infection, human monocytes differentiate to form macrophages. The differentiation process does not show morphological features of apoptosis; however, antibody staining showed caspase activation at the time of the switch and the monocyte to macrophage switch was blocked by caspase inhibitors (Sordet et al., 2002). Caspase-8 might also play a role in differentiation because *caspase-8* knockout mice exhibit defects in the development of heart muscle and also have a dramatically decreased pool of hematopoietic precursors

(Varfolomeev et al., 1998).

Immune functions are probably the best-characterized examples of non-lethal roles for caspases that do not involve apoptotic changes. Murine knockouts of *caspase-1* (Li et al., 1995; Kuida et al., 1995) and *caspase-11* (Wang et al., 1998) show defects in the production of IL-1α and IL-1β in response to the bacterial compound lipopolysaccharide. A human family pedigree showed that *caspase-8* mutations are linked to defects in the activation of T, B, and NK cells (Chun et al., 2002), and mice studies corroborated a role for *caspase-8* in T cell function (Salmena et al., 2003). It has been shown that Caspase-11 regulates cell migration in the immune system (Li et al., 2007). The link between caspases and the immune system also extends to *Drosophila*. A fly screen to identify mutants defective in innate immunity revealed that a mutant of the caspase *dredd* mounted a defective immune response when challenged with Gram-negative bacteria (Leulier et al., 2000).

Regulation of Apoptotic Programmed Cell Death

The genes responsible for cell death—also known as the "core pathway" genes—act cell-autonomously in the cells that will undergo programmed cell death. If *ced-3* is expressed in cells that normally don't undergo programmed cell death, such as the touch cell neurons, then these cells can be driven to undergo programmed cell death shown by electron microscopy to have a normal apoptotic

morphology (Shaham and Horvitz, 1996b). Work in other systems has confirmed this cell-autonomous model of action for core apoptotic genes.

The key requirement for intrinsic execution of apoptosis is regulation of caspase activity, which can be achieved in a number of ways and in response to a number of different signals. For example, DNA damage can trigger up-regulation of p53, which can eventually lead to apoptosis, depending on the severity of the cellular perturbation, by engaging Bcl-2 or BH3-only proteins (Chipuk and Green, 2006). An interesting question is whether most cells are poised to die at any moment if trouble looms and the whole cell death machinery is ready on a hairline trigger, or if the death program has to be activated from dormancy or from scratch. Some studies in *C. elegans* (Maurer et al., 2007) and *Drosophila* (White et al., 1994) point to examples of transcriptional control of programmed cell death. A mammalian study showed that in response to a cell death stimulus, cells could die even when protein synthesis had been blocked, indicating that the cell death machinery was already present and poised to act in the cell prior to the death stimulus (Weil et al., 1996). In *C. elegans, ced-9* loss-of-function mutations have the phenotype of massive cell death and early embryonic lethality (Hengartner and Horvitz, 1994). *ced-9* loss-of-function could be causing widespread *ced-3* activation, suggesting that *ced-3* is expressed and ready to act in cells that don't normally die, and that in these cells it is normally held in check by *ced-9*. However, damage induced somatic apoptotic cell death has not been reported in *C. elegans*. In response to irradiation, increases in cell

death in the *C. elegans* germline can be seen, but irradiation does not affect somatic cell death when monitored by standard assessments like corpse formation in an engulfment mutant background or by counting cells in the anterior pharynx (Deng et al., 2004).

One early concept to emerge for a mechanism of regulation of programmed cell death came from pioneering studies on chick embryos illustrating the idea of cells requiring survival factors to keep them alive—more nerve cells are made than are needed, and only the cells that can migrate to the right place, make the right connections, and have access to survival factors, such as Nerve Growth Factor, will be able to avoid their demise (Hamburger and Levi-Montalcini, 1949). In oligodendrocytes it has been shown that cells may compete for growth factor for their survival, and increasing the growth factor can decrease oligodendrocyte cell death (Barres et al., 1992).

The best evidence to date of extrinsic signaling regulating apoptotic programmed cell death comes from mammalian systems, particularly in the immune system, where signaling through the Fas ligand (Suda et al., 1993) to the Fas receptor (Itoh et al., 1991) triggers apoptosis. It has been shown that a defect in Fas-mediated apoptosis was responsible for the lymphoproliferation associated with autoimmunity in the mouse lpr model, in which the animals suffer a lupus-like syndrome because they fail to remove autoreactive T cells (Watanabe-Fukunaga et al., 1992).

Extrinsic signaling through the Fas receptor can be used to spatially regulate immune system activity. A fascinating example occurs in the mammalian eye, which is a site of immune privilege because an immune response there could result in impaired vision or even blindness. The eye is protected from an immune response by expression of Fas ligand. Griffith and colleagues showed that when mice had herpes simplex virus injected into the anterior eye chamber, there was extensive apoptosis, detected by TUNEL staining of the recruited immune cells. However, in mice mutant for Fas receptor or ligand, this cell death did not occur (Griffith et al., 1995). The Fas ligand appeared to be required in the eye itself: expression of Fas ligand in the eye was shown by staining, and wild-type immune cells from a bone marrow transplant did not undergo PCD in a Fas ligand mutant background (Griffith et al., 1995).

Engulfment of Apoptotic Cells

In early studies of the morphology of cell death it was noted that dying apoptotic cells are usually engulfed by other cells. These may be cells whose primary role is engulfment—professional phagocytes like macrophages or dendritic cells which play an important role in immune system function—or sometimes less highly specialized cells can phagocytose dying corpses. A study in a mammalian system showed that professional phagocytes such as microglia could rapidly recognize and ingest dying cells, but non-professional phagocytes such as BHK cells will show signs of recognition of the dying cell, such as process extension,

but they do not ingest the cell until a much later stage in the death process (if pre-aged dying cells were presented to the non-professional phagocytes they were rapidly engulfed), perhaps because they only respond to more advanced signs of cell death from the dying cell (Parnaik et al., 2000).

In the case of *C. elegans,* which does not have professional phagocytes, cells are engulfed by neighboring cells. With the fixed lineage of *C. elegans*, the engulfing cell may always be the same, or it may be variable, as shown in 4D reconstructions of engulfment of dying cells during wild-type embryonic development (Hoeppner et al., 2001). In *C. elegans* development, some engulfments can happen very early in the cell death process, for example, in the death of certain hypodermal cells in which one of a pair of sibling cells die, engulfment of the dying cell can already begin before the cell division creating the cell has completed (Robertson and Thompson, 1982).

Engulfment Can Enhance Cell Death

Engulfment mutants in *C. elegans* do not block cell death, but instead result in persistent cell corpses (Hedgecock et al., 1983). However, there is some genetic evidence that engulfment can facilitate cell death. In a weak *ced-3* mutant background in which some cells that would usually die now survive, the number of surviving cells can be increased by adding in engulfment mutations (Reddien et al., 2001; Hoeppner et al., 2001). Various models have been proposed for why this might be case. Perhaps when the dying cell starts to express *ced-3*, it could

also signal to the neighboring engulfing cell, which in response could return some signal that will facilitate cell death.

In higher vertebrates, studies of macrophages have suggested that in some contexts these engulfing cells are required for programmed cell death to occur. In a mouse model in which subsets of macrophages were removed by transgenic expression of diptheria toxin, two transient structures in the eye—the hyaloid vasculature (capillaries that connect up with the lens) and the pupillary membrane (a single layer of cells in front of where the pupil will form)—were not removed and remodeled on schedule, but instead persisted (Lang and Bishop, 1993). Subsequent studies have shown that macrophages activate Wnt signaling to trigger apoptosis in the hyaloid vasculature (Kato et al., 2002; Lobov et al., 2005). There are human counterparts to these conditions, although the genes responsible have not been identified.

Caspase-independent Cell Death

Digit formation, the iconic image of the necessity of programmed cell death, may in some circumstances become an iconoclastic example of programmed cell death. In an Apaf-1 knockout mouse, digit formation is slightly delayed in about 25% of the animals, with delayed forelimb removal of interdigital webs in 4 out of 16 animals at E13, and delayed hindlimb webbing removal in 4 out of 12 animals at E14.5 (Yoshida et al., 1998). However, by E15.5 normal digit formation and removal of interdigital webbing was visible in all embryos (Yoshida et al., 1998).

Another study of removal of interdigital webbing in Apaf-1 knockout mice, characterized the process as caspase-independent with signs of necrotic morphology (Chautan et al., 1999).

Early cell death studies in *C. elegans* hinted that PCD could occur in the absence of caspases. Characterization of *C. elegans ced-3* mutants using light microscopy revealed that the migratory leader cell of the male gonad, the linker cell, underwent PCD even in the absence of *ced-3* (Ellis and Horvitz, 1986). Subsequent findings were consistent with the existence of *ced-3*-independent PCD, including reports that in the anterior pharynx some cells died even in animals harboring a deletion of the entire protease-encoding domain of *ced-3* (Shaham et al., 1999). The *C. elegans* genome contains three other caspase-related genes—*csp-1*, *csp-2*, and *csp-3* (Shaham, 1998). Investigation of these caspases by RNAi in either wild-type animals or animals homozygous for loss-of-function alleles of *ced-3* has failed to reveal a role for them in PCD (See Chapter Four). Thus, *ced-3* might be the only caspase associated with PCD in *C. elegans*. One hint about how a *ced-3*-independent pathway might promote PCD came from genetic studies. When CED-4 was overexpressed in the ALM neurons of *C. elegans*, it could kill these cells, however, killing was reduced but not completely blocked in a *ced-3* mutant background (Shaham and Horvitz, 1996b). This result suggests that *ced-4* can kill cells independently of *ced-3*; however, it is not clear whether the *ced-3* alleles used in these experiments completely eliminated *ced-3* activity or whether overexpressed CED-4 exhibited a novel toxicity. Consistent

with the ability of CED-4 to kill ALM neurons in the absence of CED-3, Rothman and colleagues (Bloss et al., 2003) showed that inactivation of a gene they termed *icd-1* (inhibitor of cell death) by RNAi promoted *ced-4*-dependent, but *ced-3*-independent death of a range of cell types including neurons and male ray cells (Bloss et al., 2003). ICD-1 protein is similar to both the *Drosophila* protein bicoid and the β subunit of the nascent polypeptide associated complex. TEM analysis of a dying cell in an *icd-1*(RNAi); *ced-3* double mutant revealed morphological changes characteristic of apoptosis, including compacted chromatin and membrane blebbing (Bloss et al., 2003). Furthermore, although overexpression of proteins rarely promotes cell survival, global overexpression of ICD-1 resulted in the survival of some embryonic cells in *C. elegans* that would have normally died by apoptosis (Bloss et al., 2003). Regardless of whether *icd-1* normally inhibits apoptosis in *C. elegans*, these studies suggest that the caspase CED-3 is not required for some types of cell death in this organism. The mechanism for these *ced-4*-dependent, *ced-3*-independent cell deaths is unknown, neither is it clear whether these deaths are truly caspase-independent. It would be interesting to know whether a broad-spectrum caspase inhibitor, such as baculovirus p35, could block the *icd-1*-dependent cell deaths or whether any of the other caspase homologs in *C. elegans* play a role there.

In mammals, there is evidence for caspase-independent PCD. Genetic studies on PCD induced by BH3-domain-only proteins, such as tBID, BIM and BAD showed that these proteins, which have been shown to promote caspase

activation and apoptosis, can also kill cells independently of *Apaf-1* and downstream caspases. Specifically, *Apaf-1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) could still die in response to the over-expression of BH3-domain-only proteins (Cheng et al., 2001). Although death was reported as apoptotic because dying cells stained with Annexin V, a marker for external exposure of the normally intracellular membrane lipid phosphatidylserine, such staining also might indicate non-apoptotic dying cells with membrane damage. Caspase activation was not detected in the dying cells when assayed with fluorogenic substrates for caspase-2, -3, -6, or -7, nor could cell death be blocked by the pan-caspase inhibitor zVAD.fmk (Cheng et al., 2001). Although it is possible that another known mammalian caspase that was not assayed for is the cell death executioner in these cells, the results are intriguing and suggestive of caspase-independent cell death. A clue to possible mediators of this potentially caspase-independent death came from studies of mice lacking the Bcl-2-related genes *bax* and *bak*. *bax* $^{-/-}$ *bak*$^{-/-}$ mice died just after birth and showed a brain overgrowth defect, lymphoid-cell accumulation, and retained interdigital webs—all defects associated with lack of PCD. Interestingly, MEFs derived from these mice and overexpressing BH3-domain-only proteins were almost completely resistant to death (Wei et al., 2001). These results suggest that the PCD pathway induced by BH3-domain-only proteins bifurcates downstream of Bax and Bak, with one branch being caspase- and Apaf-1-dependent and the other not. In mammary gland duct morphogenesis, recent work with a Bim (BH3-domain-only gene)

knockout revealed that cells in the luminal space were cleared, in a slightly delayed fashion, by a mechanism that did not show any signs of caspase activation (Mailleux et al., 2007). Activated caspase-3 was identified as being immunohistochemically localized in regions of apoptosis associated with mouse tooth formation (Shigemura et al., 2001), however, in caspase-3 knockout mice, although there were some defects in the primary enamel knot formation, the adult molar teeth formed normally (Matalova et al., 2006), and in caspase-9 or Apaf-1 knockout embryos, again tooth development appeared to have proceeded normally (Setkova et al., 2007). These results raise the question of what is the caspase replacement in these systems.

Other support for caspase-independent PCD comes from numerous *in vitro* studies demonstrating that cell death can proceed even in the presence of broad-spectrum caspase inhibitors.

It has been shown that the autophagic programmed cell death of the *Drosophila* salivary gland has some requirement for activity of apoptotic core pathway genes and caspases, even though morphologically the cell death doesn't resemble apoptosis (Martin and Baehrecke, 2004).

The *C. elegans* Linker Cell as a Model System to Study Programmed Cell Death

C. elegans with its known cell fate lineage offers an excellent system to study programmed cell death *in vivo*. No other current model organism offers the

possibility of following a programmed cell death at the level of an individual cell that is predictably fated to die. The linker cell, a cell that undergoes a developmentally regulated programmed cell death in the male, was chosen because it offered the potential to investigate many profound and fascinating questions about programmed cell death.

There was the indication in the literature that linker cell death might offer a model for a *ced-3,* and potentially caspase-independent programmed cell death (Ellis and Horvitz, 1986). Because of the redundancy of caspases in other systems, only in a simple genetic system like *C. elegans* can the question of whether caspase-independent programmed cell death occurs be satisfactorily addressed. And if caspase-independent programmed cell death does occur, *C. elegans* is a worthy system to try to quickly identify some of the unknown genes that facilitate the process.

Another intriguing anecdote about the linker cell was a report that it was killed (or more sensationally described as “murdered”) by its engulfing cell U.l/rp, because in the absence of U.l/rp, the linker cell survived (Sulston et al., 1980). This suggested that the linker cell might offer the first example of cell-to-cell extrinsic signaling regulating programmed cell death in *C. elegans*, and again the possibility of uncovering unknown genetic regulators of such a process.

Finally, as a large, differentiated cell that undergoes cell death after a long lifespan, the *C. elegans* linker cell could possibly offer some different challenges for programmed cell death that could lead to new discoveries and insights. Most

of the studied programmed cell deaths in *C. elegans* are the embryonic programmed cell deaths of small, undifferentiated cells that die after a very short lifespan. The fact that the linker cell is such a large cell means that it offers an excellent system for subtle observation of cell morphological changes during cell death. Most *C. elegans* cells destined to die during development do so within 30 minutes after they are born (Sulston and Horvitz, 1977; Sulston et al., 1983). Three *C. elegans* cells that die during normal development live much longer than most cells fated to die. Little is known about the function or death of one of these cells, MSpppaaa, the sister cell of the somatic gonad precursor cell, Z1. However, the other two long-lived cells, the tail-spike cell and the linker cell, exhibit obvious differentiated features (Sulston et al., 1980; Sulston et al., 1983). The onset of tail-spike cell death is regulated by transcriptional activation of the *ced-3* caspase, a previously unappreciated form of cell death timing control (Maurer et al., 2007). Our studies of linker cell death are presented here.

Chapter Two

Characterization of Wild-type Linker Cell Death

BACKGROUND

Birth of the Linker Cell

After the *C. elegans* embryo hatches, it progresses through four larval stages, known as L1, L2, L3, and L4, and on the final molt it becomes an adult. The life cycle takes about three days at 20°C.

The linker cell is born in the male at the second larval stage (L2) (Kimble and Hirsch, 1979). It arises from either Z1.paa or Z4.aaa, and the decision is made through a lateral signaling event. The cell that does not become the linker cell instead becomes a *vas deferens* precursor cell (Kimble and Hirsch, 1979). The lateral signaling acts through genes including *sys-1*, a novel gene with Armadillo repeats (Miskowski et al., 2001), *pop-1*, a TCF/LEF-1 transcription factor (Siegfried and Kimble, 2002), and there is also input via Wnt and MAPK pathways. A forkhead gene, *fkh-6*, is required for the linker cell to form (Chang et al., 2004). In the hermaphrodite, Z1.paa doesn't exist, and Z4.aaa can sometimes give rise to the anchor cell or divide further to form uterine cells (Kimble and Hirsch, 1979).

The Role of the Linker Cell

The linker cell plays an essential role as the migratory leader cell of the male gonad, since if it is ablated prior to completion of its migration there are severe defects in gonadal development and the gonad does not complete its normal migration to the posterior of the animal (Kimble and White, 1981). The linker cell undergoes a complex migration (Figure 2.1) that lasts for over 30 hours at 20 degrees. The genes regulating linker cell migration have not been extensively characterized. When the linker cell reaches the posterior of the worm at the end of the fourth larval stage (L4), around the time the animal is about to undergo the final molt to become an adult, the linker cell dies and is engulfed and removed by either the cell U.rp or the cell U.lp (hereafter in this thesis both these cells together will be referred to as U.l/rp)(Sulston et al., 1980). These U cell descendants are frequently fused with their anterior sibling cells (Sulston et al., 1980). Around the time the linker cell dies, the gonad is connected to the cloaca, and thus to the exterior (Sulston et al., 1980). Gonadal connection to the cloaca enables sperm to exit the animal (Figure 2.2). It has been hypothesized that linker cell death is required for fertility, since a surviving linker cell, positioned like a plug at the top of the *vas deferens* would presumably form a major obstruction preventing sperm exit (Figure 2.2).

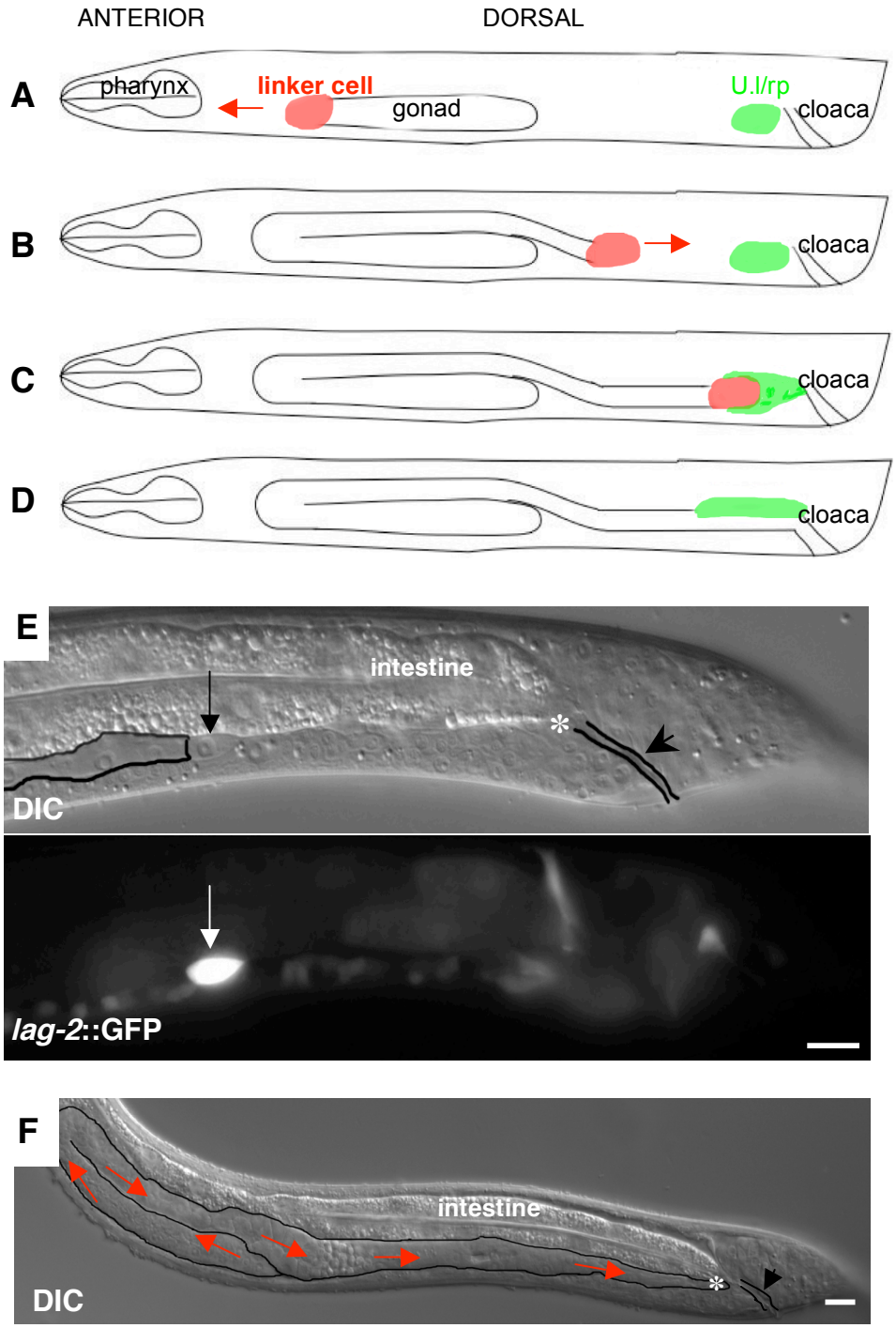


Figure 2.1 Stages of Linker Cell Migration and Death
(A-D) Diagrams depicting stages of linker cell migration and death.
(A) L2 animal. The linker cell has migrated anteriorly on the ventral side.
(B) Early L4 animal. The linker cell is migrating posteriorly on the dorsal side.
(C) Late L4 animal. The linker cell has reached the cloacal region, begins to die, and is engulfed by U.l/rp.

(D) Adult animal. The linker cell has died and disappeared. U.l/rp, which now has an extended morphology, connects to the *vas deferens*.
(E) An L4 male worm roughly corresponding to the stage B drawn above. DIC, top. Fluorescence, bottom. The linker cell (arrow) expresses a *lag-2*::GFP transgene. The *vas deferens* is outlined in black, as is the cloaca (arrowhead). The location of U.l/rp, the cells that engulf the linker cell, is indicated with an asterisk. Anterior, left. Dorsal, top. Scale bar, 10 μm.
(F) A late L4 male worm corresponding to the stage C drawn above. The gonad has been outlined in black and the red arrows trace the path of migration taken by the linker cell as the gonadal migratory leader cell. Asterisk denotes the location where the linker cell and U.l/rp are located. The cloaca, outlined in black, is indicated with an arrowhead. Scale bar, 10 μm.

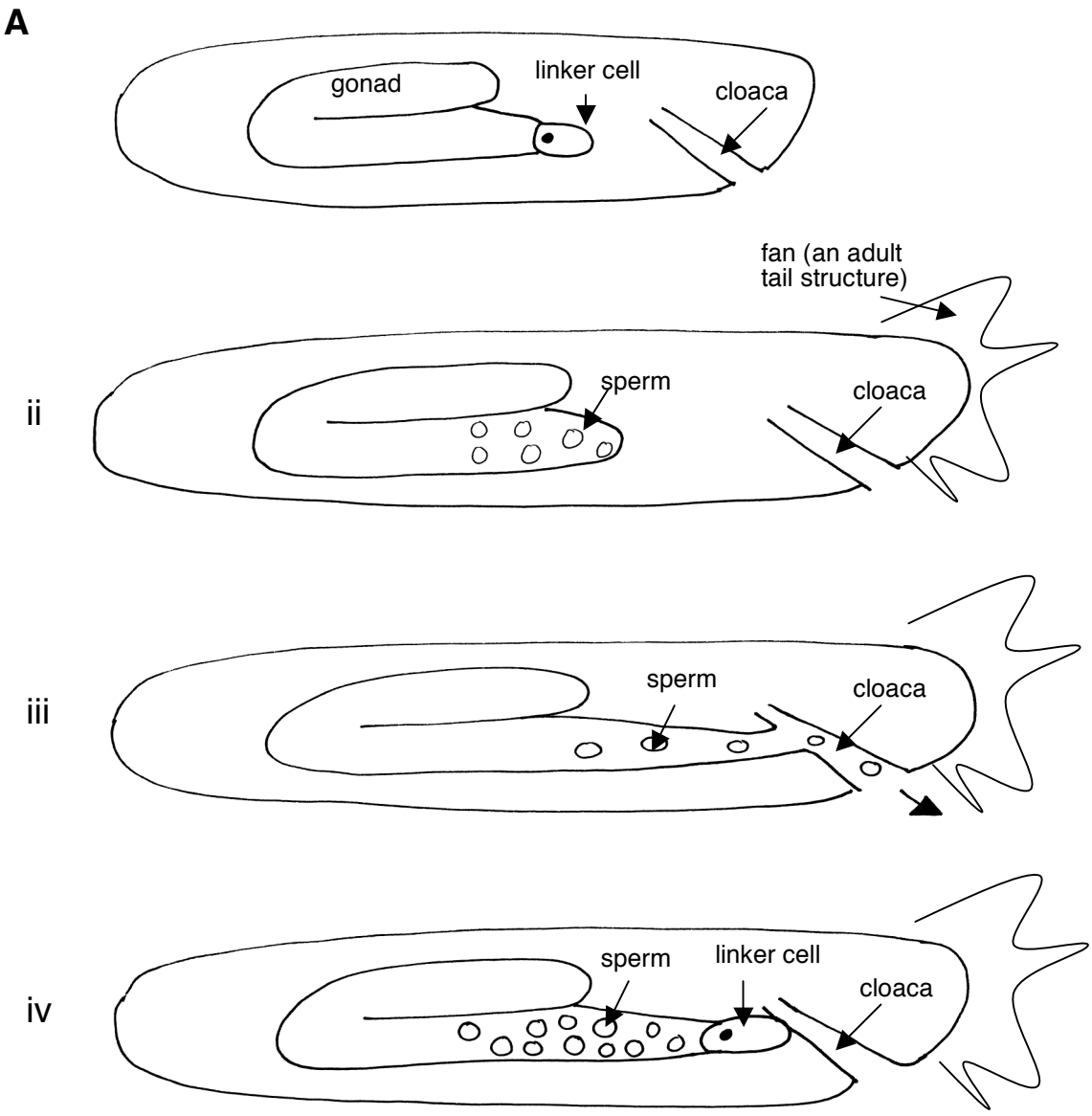

A
gonad
linker cell
cloaca
fan (an adult
tail structure)
ii
sperm
cloaca
iii
sperm
cloaca
iv
sperm
linker cell
cloaca
B
C
lin-29(n333)

Figure 2.2 The Importance of Linker Cell Death For Fertility
(A) Schematic showing the necessity of regulated linker cell death for fertility.
(i) Depicts a normal worm at the early L4 stage.
(ii) If the linker cell in (i) dies too early or is ablated, the gonad cannot migrate further, does not connect to the exterior, and sperm cannot exit the animal.
(iii) If the linker cell in (i) migrates to the normal location and dies, the gonad develops normally and is connected to the exterior allowing sperm exit in the adult.
(iv) If the linker cell in (i) migrates normally, but doesn't die, the linker cell sits at the end of the *vas deferens* like a plug preventing sperm exit.
(B) DIC image of an animal with a gonadal migration problem that could arise if the linker cell died too early. Note the sperm (arrowhead) trapped in the middle of the animal. Cloacal region indicated with asterisk. Anterior, left. Scale bar, 10 μm.
(C) DIC image of linker cell (arrow) surviving in the adult and sperm (arrowheads) building up behind the linker cell in the *vas deferens*. Cloaca (arrowhead) outlined in black. Anterior, left. Dorsal, top. Scale bar, 10 μm.

Previous Reports About the Linker Cell

A few anecdotal observations have been made in the published literature about linker cell death. Focusing a laser microbeam can be used to selectively destroy specific cells in *C. elegans* (Bargmann and Avery, 1995). Using this cell ablation technique at the L1 stage, Sulston removed U, the precursor of the cells (U.l/rp) that engulf the linker cell (Sulston, 1980). Sulston reported that in this background the linker cell then survived, and he cited anecdotal results that the linker cell survived in a migratory mutant (Sulston et al., 1980). This led to the model of linker cell "murder" via a signal from a U cell descendant.

In work from the Horvitz lab it had been reported that the linker cell could still die in a *ced-3* mutant background (Ellis and Horvitz, 1986) raising the possibility that this cell death was caspase-independent, although three other caspase homologs exist in the worm genome (Shaham, 1998).

RESULTS

Microscopy Imaging of Linker Cell Death

The advent of GFP technology (Chalfie et al., 1994) has been a particularly monumental advance for research in the transparent *C. elegans,* facilitating types of experimental approaches previously impossible. Fluorescent markers enable easy identification and observation of a single cell in a living animal. Using a cell-specific fluorescent marker vastly improves cell identification, which had

previously relied on cell identification based on cell positioning in animals that were mounted on glass slides and observed by Differential Interference Contrast (DIC). The ability to use cell-specific fluorescent markers, and thus to not be reliant on cell identification by DIC optics alone, has enabled marked cells in animals crawling on plates to be observed using epifluorescent dissecting microscopes, a major advance for rapid genetic screens.

A GFP marker—a *lag-2* promoter fused to GFP—had been previously reported as showing linker cell expression (Siegfried and Kimble, 2002). This tool was the starting point used to embark upon studies of linker cell death.

The *lag-2* promoter was found to be an excellent tool for observation of the linker cell in a living organism. The marker is expressed in young male animals as soon as the linker cell is born, and apart from expression in some readily distinguishable cells, such as small cells in the ventral nerve cord, the expression is specific enough, given the large size of the linker cell, to make the linker cell readily distinguishable (Figure 2.1E). Because GFP has a long half-life and persists for quite a while after it is made (Corish and Tyler-Smith, 1999), even if a cell is dying and has impaired transcription, the GFP already present will still be stable, making the fluorescent marker also useful for visualizing the linker cell corpse.

The Stages of Wild-type Linker Cell Death

The first study we carried out was an observation of wild-type linker cell death using an epifluorescent dissecting microscope. Doing population studies we could see that at the late L4 stage, the ovoid linker cell began to round up and divide into two parts, and that the cell had been completely removed or was a round corpse by the time that the animal had completed the molt to adult. These dissecting microscope studies suggested that the approximate timing of this process is about 2 to 4 hours from when the cell first begins to die until it is no longer visible.

Using DIC optics on a compound microscope, we observed at higher resolution the changes in cell morphology during wild-type linker cell death (Figure 2.3). A wild-type healthy migrating linker cell is ovoid in shape, with a high cytoplasmic to nuclear ratio, the nuclear envelope is round and smooth, and the cytoplasm has a characteristic mottled texture (Figure 2.3A). Among the early changes we notice are signs of shape changes to the nucleus such as indentation (or “crenellation”) of the nuclear envelope (Figure 2.3B) or partial nuclear envelope breakdown. Another early change is a loss of cytoplasmic volume and a marked reduction of the cytoplasmic to nuclear ratio (Figure 2.3C). This loss of volume is a consequence of a blebbing process, in which approximately half of the cytoplasmic volume of the cell is separated from the half of the cell still containing the nucleus (Figure 2.3D). Occasionally smaller blebs

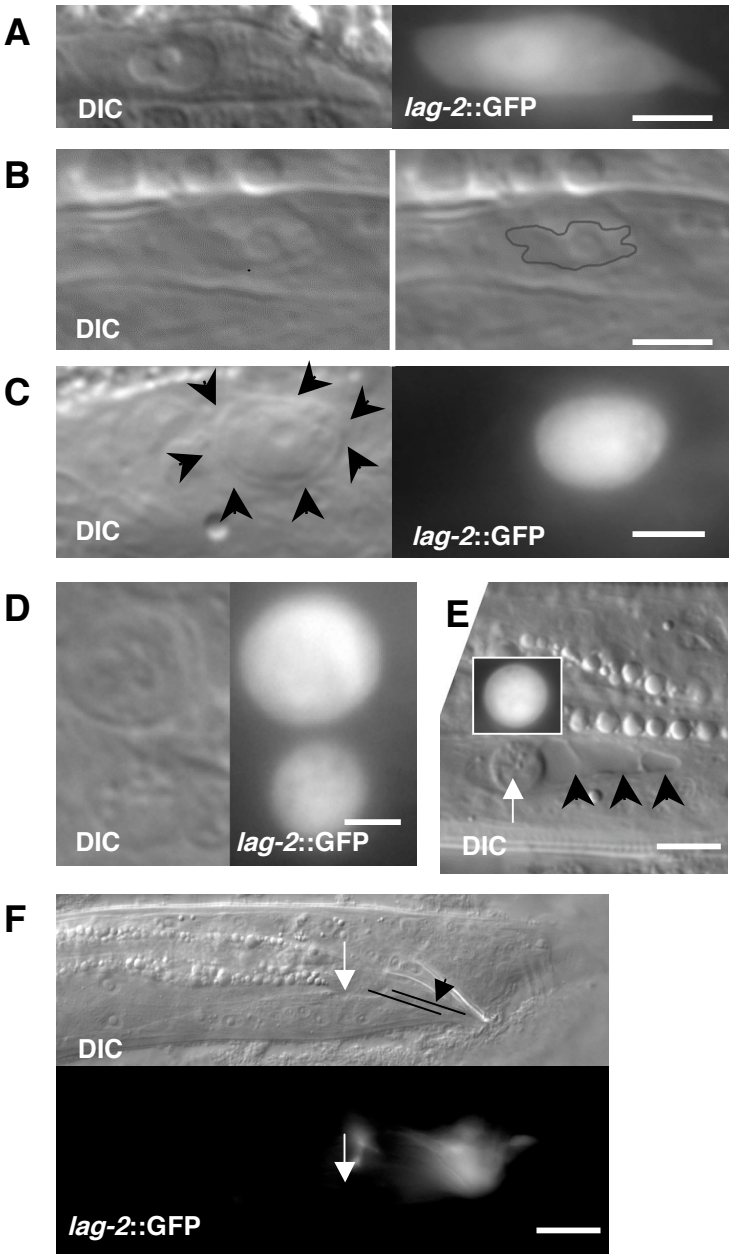


Figure 2.3 Stages of Wild-type Linker Cell Death

(A-F) Linker cell expresses a *lag-2*::GFP transgene. DIC and fluorescence images.
(A) A healthy migrating linker cell at the L3 larval stage. Note the smooth nuclear envelope and distinct nucleoplasm, prominent nucleolus, and large cytoplasmic to nuclear ratio. Scale bar, 2 μm.
(B) A linker cell beginning to die at the late L4 stage. The nuclear envelope has become indented or crenellated. The crenellated shape of the nuclear envelope has been outlined in the image on the right. Scale bar, 2 μm.
(C) A linker cell beginning to die at the late L4 stage. The cell membrane of the linker cell denoted with arrowheads. Note the loss of cytoplasmic to nuclear ratio. Scale bar, 2 μm.
(D) Image of linker cell blebbing into two parts at the late L4 stage. Scale bar, 2 μm.

(E) Image of the linker cell (arrow) forming a refractile button-like corpse in a late L4 animal. Vacuoles (arrowheads) are visible beside the linker cell corpse. Scale bar, 5 μm. (F) The linker cell has gone in a young adult worm, less than 2 hours after becoming an adult. Note the absence of GFP and absence of the linker cell in the region marked with an arrow. Cloaca (arrowhead) outlined in black. Anterior, left. Dorsal top. Scale bar, 10 μm.

can be observed, but the main and consistent form of blebbing is into two equal sized parts. At the stage when the cell has blebbed into two parts, initially the part of the cell containing the nucleus may still be of an ovoid shape. Cytoplasmic abnormalities or appearance of the cell membrane may also be apparent. The nuclear half of the linker cell then becomes completely round (Figure 2.3D), the nucleus continues to degrade and deform, and the nucleolus is often one of the last structures to disappear. When neither the nuclear envelope nor the nucleolus is visible, a later stage of degradation is when the linker cell forms a round refractile button-like corpse (Figure 2.3E). The round button-like corpse is similar to a classic apoptotic morphology seen during wild-type development (Sulston and Horvitz, 1977). Then the linker cell is completely removed (Figure 2.3F). Some population studies of wild-type linker cell death were undertaken to determine a normal time course of these changes (Table 2.1).

Linker Cell Blebbing Into Two Parts

Our initial characterization of the wild-type death revealed several unusual features. We observed the linker cell consistently blebbing into two parts when studying individual worms under the epifluorescent dissecting microscope and with population studies under the compound microscope (Figure 2.3D). In our TEM studies, detailed later in this chapter, we present an image (Figure 2.8H) which may represent the cytoplasmic bleb: adjacent to the linker cell corpse, a large circular structure was visible which lacked a nucleus in serial sections.

Table 2.1 DIC Study of Wild-type Morphology of Linker Cell Death

Animal Age	% Healthy Linker Cell (n)[a]	% Early Abnormal Linker Cell (n)[b]	% Late Abnormal Linker Cell (n)[c]	% Linker Cell Completely Gone (n)
Early L4	100(30)	0(30)	0(30)	0(30)
Late L4 Rays Visible	0(30)	3(30)	83(30)	13(30)
0-2-Hr-Old Adult	0(30)	0(30)	13(30)	87(30)
2-4-Hr-Old Adult	0(30)	0(30)	6(30)	94(30)
4-6-Hr-Old Adult	0(30)	0(30)	6(30)	94(30)
6-8-Hr-Old Adult	0(30)	0(30)	3(30)	97(30)

n, number of animals scored.
Males containing the *him-5(e1467)* mutation for high incidence of males and a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. In this population study each animal was scored once at one timepoint.
[a] A healthy linker cell morphology was scored if the linker cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example.
[b] An early abnormal linker cell corpse was recorded if the linker cell had lost substantial volume, or started large scale blebbing, or had nuclear envelope abnormalities such as abnormal indented (or crenellated) nuclear envelope shape or signs of nuclear envelope breakdown. See Figure 2.3B or 2.3C for an example.
[c] A late abnormal linker cell corpse was recorded if the linker cell had lost substantial volume and had assumed a completely round shape. Such linker cell corpses invariably had major nuclear abnormalities and in many cases the nucleus had completely broken down. See Figure 2.3D or 2.3E for an example.

We were curious about the fate of the cytoplasmic bleb that was detaching from the nuclear half of the cell. Using a *lin-48*::GFP marker for U.l/rp (Johnson et al., 2001) and an RFP marked linker cell, we observed that as the linker cell divided into two parts, each of the parts was taken up by one of the U cell descendants—so that both U.lp and U.rp each engulfed a part of the linker cell (Figure 2.4). It had not been reported that both U.lp and U.rp each took up part of the linker cell. This is probably because without a fluorescent marker, the non-nuclear bleb is difficult to follow. This large-scale division into two parts has not been previously described for any other cell deaths in *C. elegans* or for programmed cell deaths in other systems. The blebbing that is normally described associated with apoptosis occurs on a much smaller scale—tiny fragments of the cytoplasm bleb off from the dying cell. This blebbing process is not dependent on the U cell descendants, and may be a cell-intrinsic property of the linker cell, as it can also occur in a linker cell migratory mutant background such as *him-4* (Vogel and Hedgecock, 2001), in which the linker cell does not reach the vicinity of U.l/rp (Figure 2.5A). What appears to be an intrinsic linker cell blebbing process can also be consistently seen in *daf-12* animals (Antebi et al., 2000), in which the non-nuclear half of the bleb appears to be able to migrate a significant distance in the correct direction, even though it is not attached to the nuclear half, or attached through a long cytoplasmic process (Figure 2.5C). Perhaps because it is such a large cell, the linker cell divides into two parts to facilitate its engulfment.

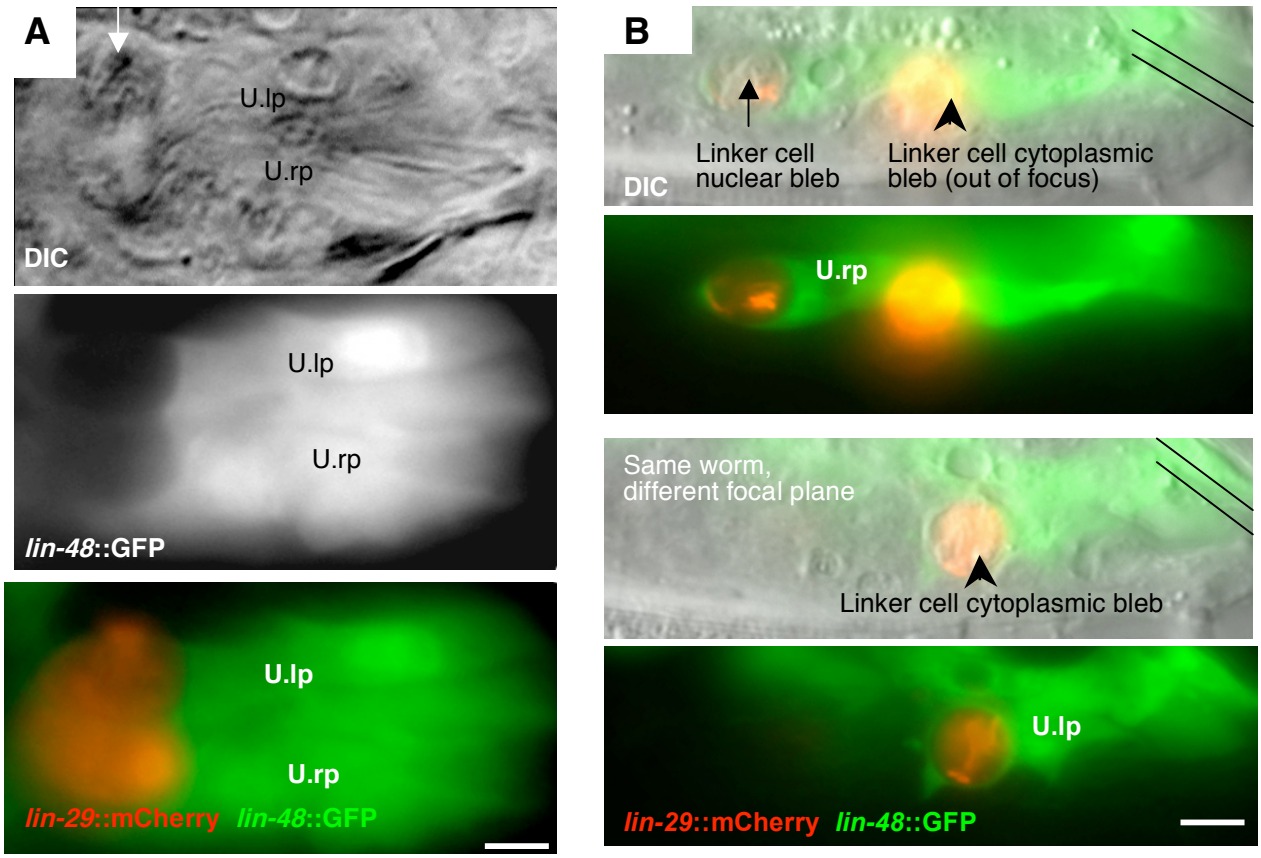


Figure 2.4 The Linker Cell Blebs into Two Parts, Each Part is Engulfed by Either U.lp or U.rp.
(A) DIC (top), GFP fluorescence (middle), and combined GFP + mCherry fluorescence (bottom) images of a linker cell (arrow) expressing a *lin-29* promoter::mCherry reporter transgene being divided into two parts as it is being engulfed by U.lp (top cell) and U.rp (bottom cell) expressing a *lin-48* promoter::GFP reporter transgene. Ventral view. Scale bar, 3 µm.
(B) Fluorescence and DIC merge (top), and fluorescence merge (bottom) of images taken at two different focal planes in the same animal in which the nuclear half of the linker cell bleb (arrow) is completely engulfed by U.rp, and the cytoplasmic half of the linker cell bleb (arrowhead) is completely engulfed by U.lp. The linker cell expresses a *lin-29* promoter::mCherry reporter transgene and U.l/rp expresses a *lin-48* promoter::GFP reporter transgene. Cloaca outlined in black. RFP bleed-through of the linker cell cytoplasmic half is visible in the image of the linker cell nuclear half. Anterior, left. Dorsal, top. Scale bar, 5 µm.

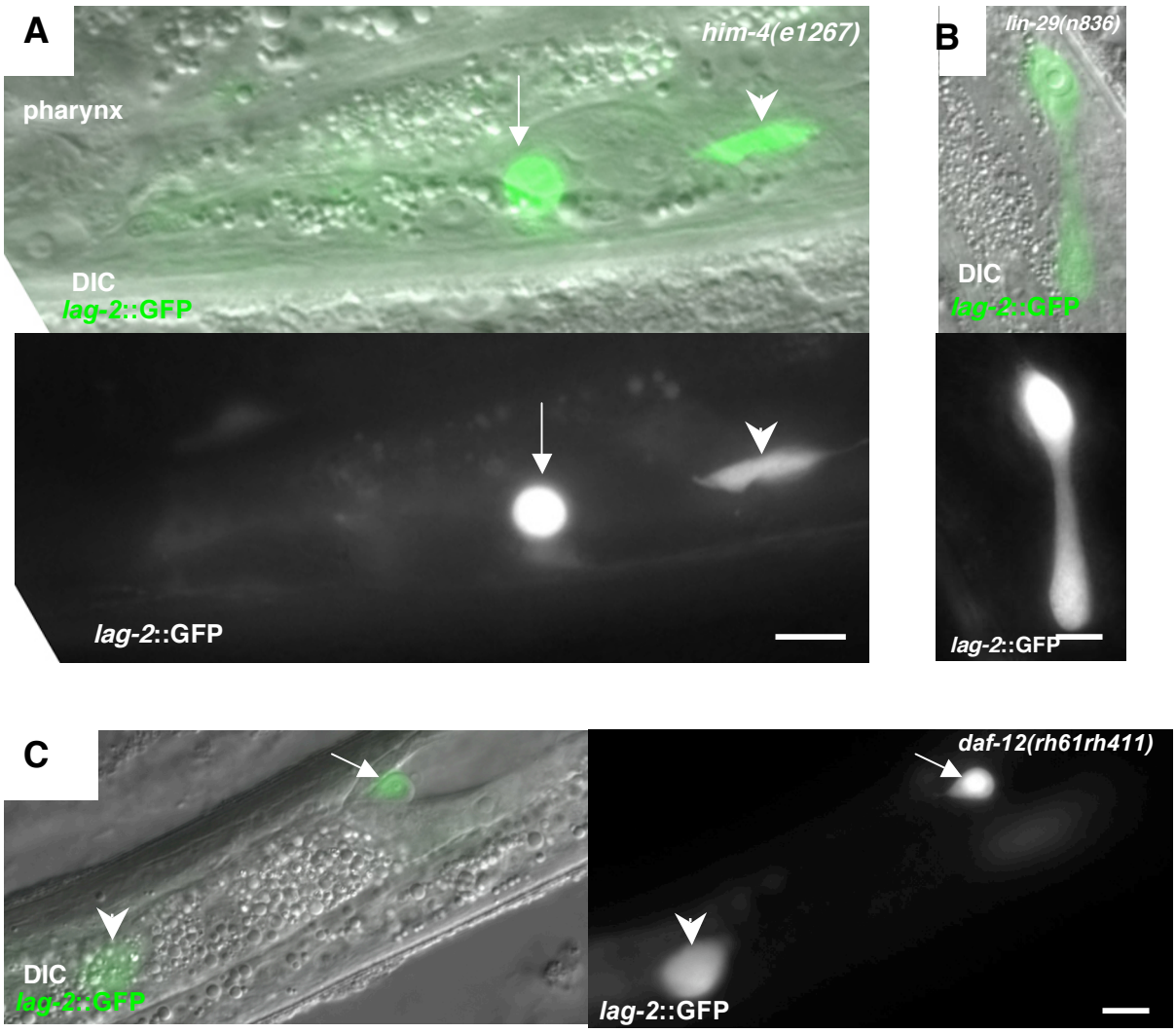


Figure 2.5 Linker Cell Blebbing into Two Does Not Require U.l/rp
(A) Combined GFP + DIC (top), and GFP fluorescence (bottom) images of a linker corpse (arrow) expressing a *lag-2*::GFP transgene, which has given off a bleb (arrowhead) in a *him-4(e1267)* migratory mutant in which the linker cell has migrated adjacent to the pharynx at the head of the worm. Anterior, left. Dorsal, top. Scale bar, 5 µm.
(B) An image of linker cell blebbing in progress. Scale bar, 5 µm.
(C) Combined GFP + DIC (left), and GFP fluorescence (right) images of a linker corpse (arrow) expressing a *lag-2*::GFP transgene, which has given off a bleb (arrowhead) in a *daf-12(rh61rh411)* migratory mutant. Note the considerable distance that the bleb has migrated away from the nuclear half of the cell. Scale bar, 5 µm.

Linker Cell Death and the Cell Cycle

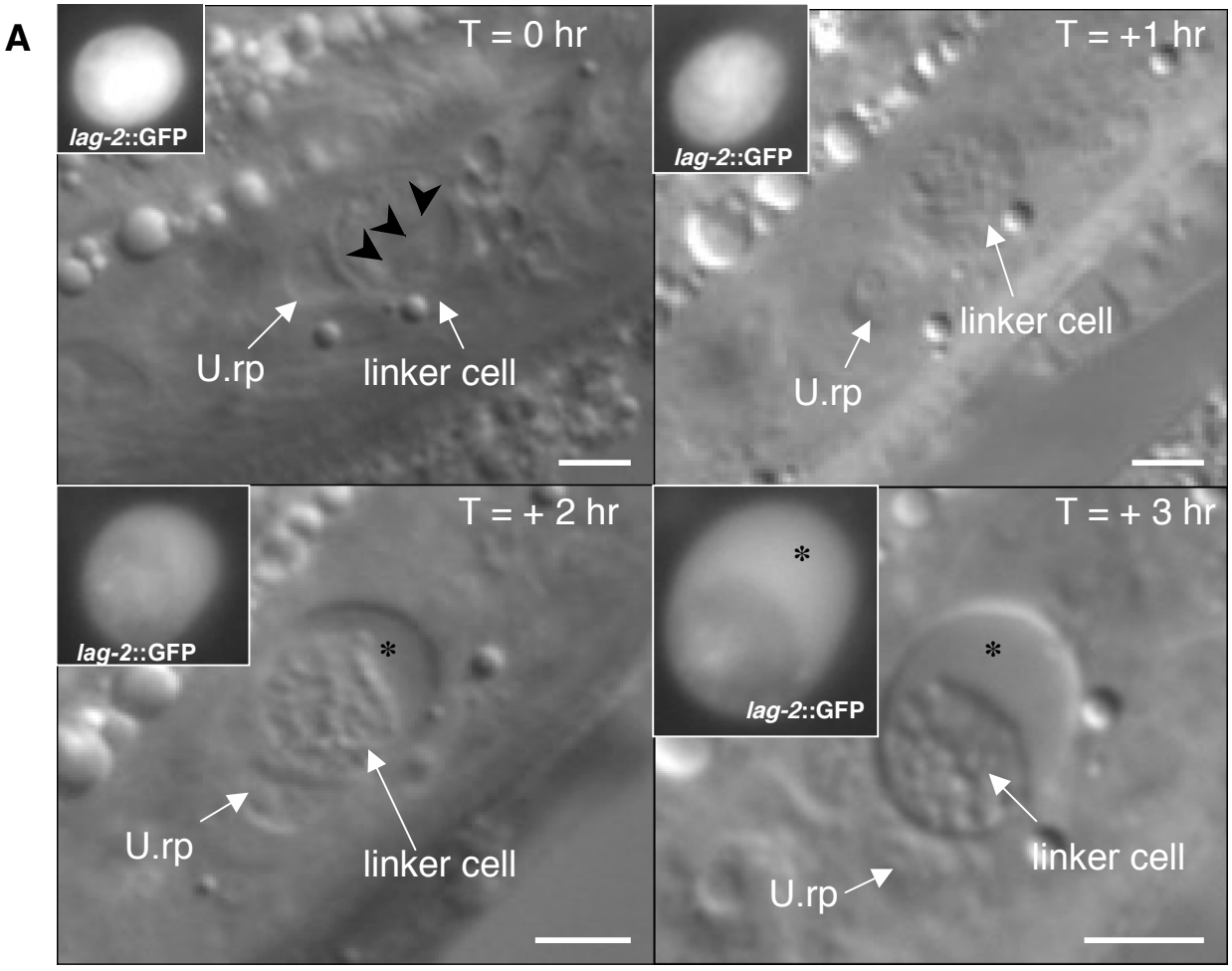
The observation of linker cell division into approximately two roughly equal sized parts led to the speculation that the cell death program might be utilizing aspects of the cell division machinery, for example, the cytokinesis apparatus. No fluorescent markers for cytokinesis have been described in *C. elegans*, however, we were able to check a fluorescent marker for cell differentiation—a promoter fusion of the gene *cki-1* (cyclin dependent kinase inhibitor) to GFP. *cki-1* is a G1 cyclin dependent kinase inhibitor whose expression is associated with post-mitotic or developmentally regulated G1 arrested cells (Hong et al., 1998). We could see *cki-1*::GFP expression in 44/45 linker cells at the late L4 stage when the linker cell had reached the region where it dies. We also checked for expression of the ribonucleotide reductase gene *rnr,* which is an S phase specific reporter (Hong et al., 1998). A transgenic strain with the *rnr* promoter fused to GFP (Hong et al., 1998) did not show fluorescent expression in the linker cell in 35/35 larvae examined around the time of linker cell death.

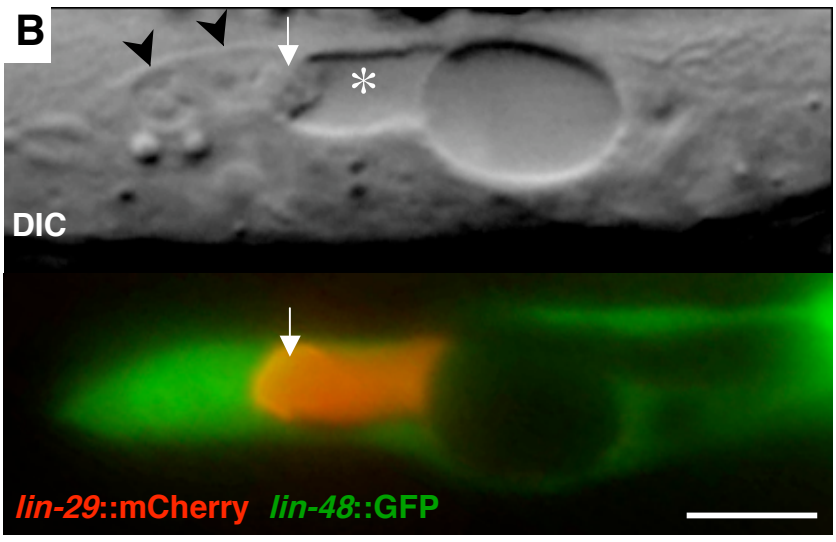
Linker Cell Death and Vacuolar Formation

Another striking feature of linker cell death was the appearance of vacuoles associated with the linker cell corpse as it degrades (Figure 2.3E). These structures were not a consequence of non-specific anesthetic damage, as they could be seen in linker cells mounted in simple buffer alone, such as S-Basal or M9. In a study in which linker cell death was observed in wild-type animals

anesthetized with levamisole, a muscle blocker, we could observe the vacuolar structures forming *in vivo* (Figure 2.6A). These vacuoles seem similar to structures that are observed by DIC optics during necrotic cell death in *C. elegans* (Hall et al., 1997). The vacuoles appear to be located inside the engulfing U cell descendants, and can be consistently observed during the process of linker cell death (Figure 2.6). With our fluorescently marked linker cells we can see that these vacuolar structures appear to be derived of material of linker cell origin (Figure 2.6). Sometimes by DIC optics we can see small unidentified particles moving rapidly about inside the vacuoles (Figure 2.6C). Interestingly, a study of wild-type aging *C. elegans* worms noted, "Necrotic cavities of various sizes appeared, often containing vibrating particles that appeared to display Brownian motion," which is a description that could be applied to linker cell associated vacuoles (Garigan et al., 2002).

We have also carried out transmission electron microscopy (TEM) studies of these vacuolar structures (TEM work by Yun Lu). The vacuoles appear to be empty apart from flocculent material, somewhat similar to vacuoles seen in late necrotic cells (Figure 2.7A). They may be possibly formed from swollen endoplasmic reticulum. In one animal, smaller swollen vesicles with similar content to the large vacuole could be seen (Figure 2.7B; Figure 2.7C). An image of medium-sized vacuoles apparently merging can be seen in the Appendix (Appendix Figure 3).


A
T = 0 hr
lag-2::GFP
U.rp
linker cell
T = +1 hr
lag-2::GFP
U.rp
linker cell
T = + 2 hr
lag-2::GFP
U.rp
linker cell
T = + 3 hr
lag-2::GFP
U.rp
linker cell


B
DIC
lin-29::mCherry lin-48::GFP

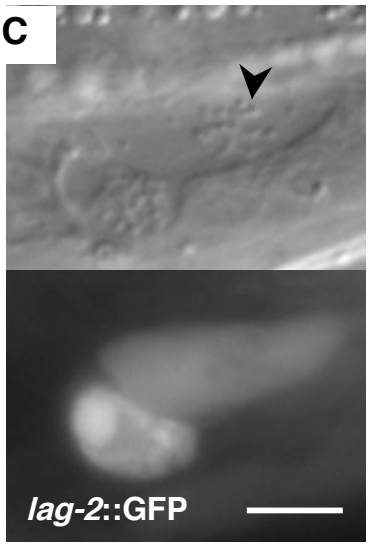

C
lag-2::GFP

Figure 2.6 Linker Cell Death is Associated With Vacuolar Formation
(A) Time course of vacuolar formation by the linker cell in a single wild-type late L4 animal observed at different timepoints. The worm expresses a *lag-2*::GFP reporter in the linker cell. DIC and fluorescent (inset) images. Vacuole formation indicated with asterisks. The worm was anesthetized with levamisole and kept on the slide between imaging. Under anesthetic treatment, development proceeds more slowly than normal, so the times between the different stages of cell death observed here are probably longer than in an unanesthetized wild-type animal. Scale bar, 3 µm.
At T=0, the linker cell (arrow) is completely round and its cell membrane is visible. The nuclear envelope of the linker cell nucleus is still partially visible (arrowheads), and very small vacuoles can be noted to the right of the linker cell corpse.
At T= + 1 hr, the linker cell nucleus is no longer visible and the cell has assumed a refractile button-like corpse morphology.
At T= +2 and +3 hr, vacuoles (asterisk) form which contain GFP derived from the linker cell.
(B) DIC (top) and fluorescence (bottom) images of a clear vacuole (asterisk) containing mCherry, in an animal expressing *lin-29* promoter::mCherry (arrow, linker cell corpse) and *lin-48*::GFP (U.l/rp) transgenes, late during linker cell death. Arrowheads, nuclei of engulfing cell. Scale bar, 5 µm.
(C) DIC (top) and fluorescence (bottom) images of a linker cell corpse in a wild-type animal expressing a *lag-2*::GFP reporter. Note the small particles visible in the vacuole (arrowhead). Scale bar, 5 µm.

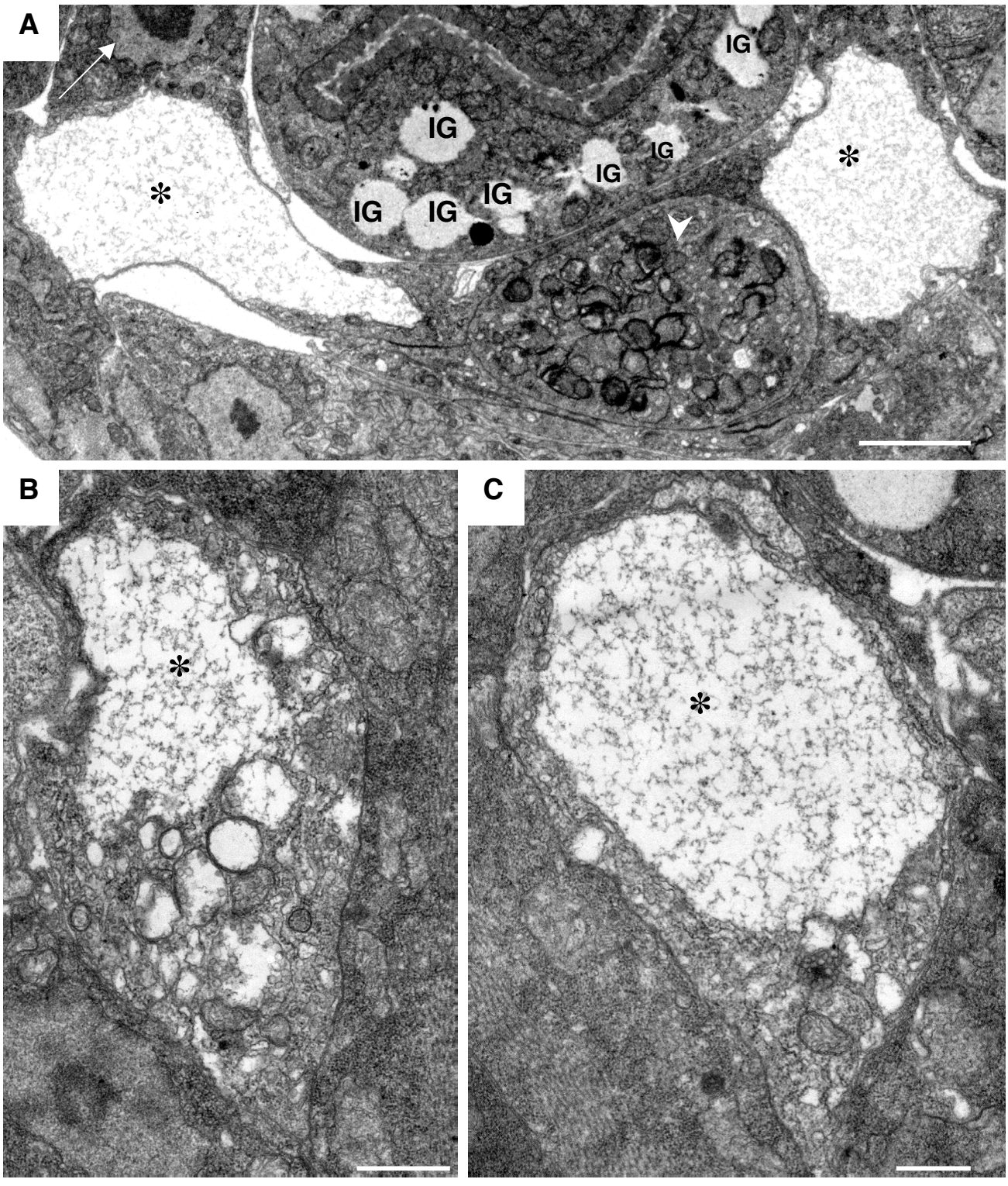


Figure 2.7 Electron Microscopy Studies of Vacuoles Associated With Linker Cell Death

(A) Two vacuoles containing flocculent material (asterisks) are shown within the U.l/rp cell. Arrow, U.l/rp nucleus. Such vacuoles may be engulfed linker cell components that have been degraded. Vacuoles flank a disintegrating linker cell (arrowhead) also engulfed by U.l/rp. IG, intestinal granule. Scale bar, 1 µm.
(B) In another animal, another example of a vacuole (asterisk). Smaller vesicles appear to be contributing material to the vacuole. Scale bar, 0.5 µm.
(C) Same animal as in (B), but a later section in which the vacuole (asterisk) is larger and there are substantially less smaller vesicles. Scale bar, 0.5 µm.

Electron Microscopy Studies of Wild-type Linker Cell Death

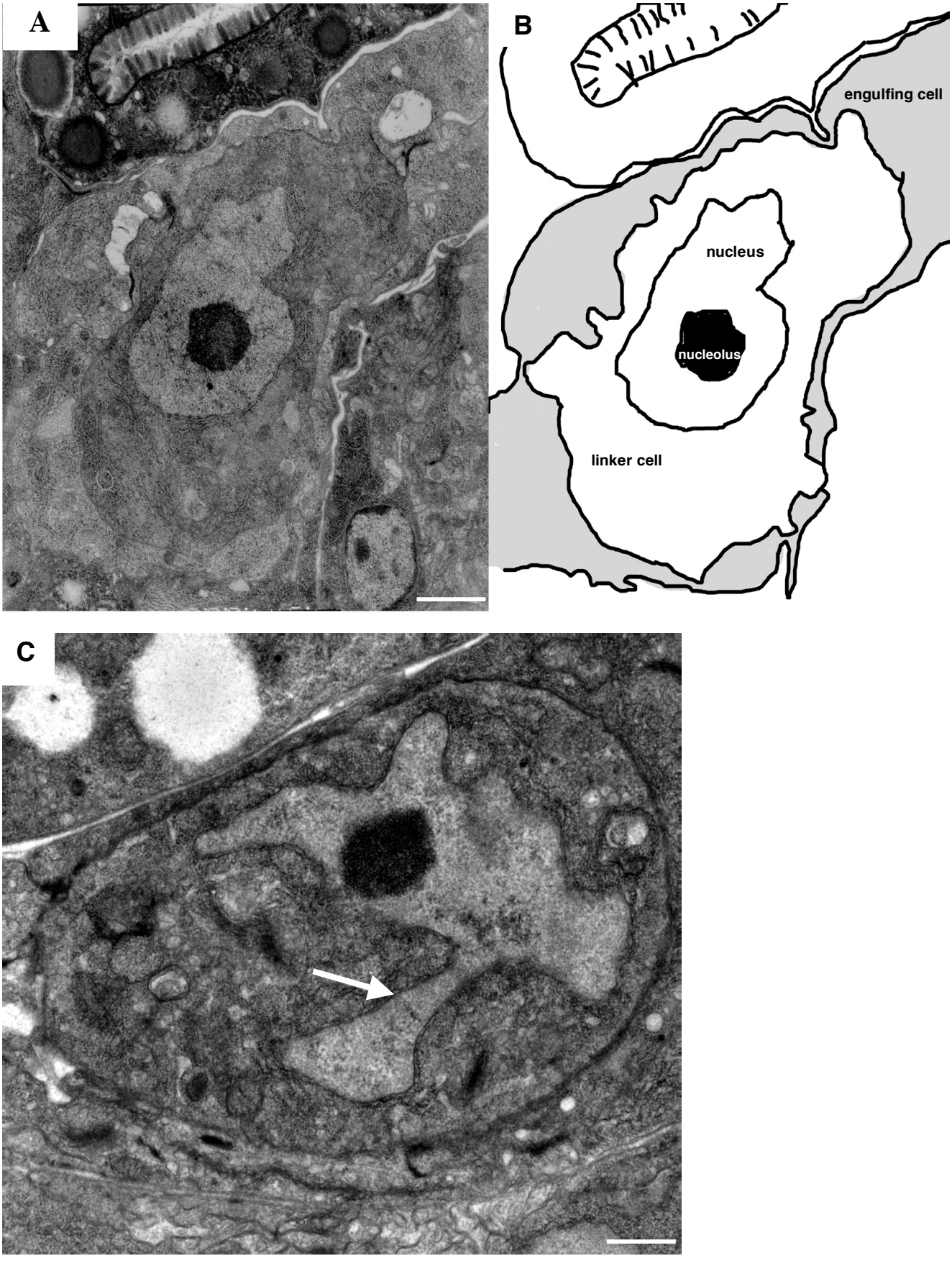
By DIC optics, we could see some morphologies of linker cell death that were characteristic of apoptotic programmed cell death—the button-like corpse (Figure 2.3E), and some morphologies that were novel, like large-scale blebbing (Figure 2.3D) or the necrotic-like vacuolar structures (Figure 2.3E). To try to determine the exact nature of linker cell death, in particular to try to discover if linker cell death showed the characteristic electron microscopy morphological features of apoptosis such as chromatin and cytoplasmic compaction or if it had different characteristics of cell death, we decided to conduct transmission electron microscopy studies of wild-type animals around the time of linker cell death (I started the TEM studies with initial help from Ken Nguyen, Gloria Stephney, and David Hall in the Hall laboratory, and then the vast majority of the TEM work was carried out by Yun Lu when she joined the Shaham laboratory).

The images we obtained of the earliest stage of linker cells undergoing programmed cell death showed comparatively healthy linker cells with normal nuclear architecture and chromatin, and normal cytoplasm. However, although the cell looked healthy, it was already being engulfed (Figure 2.8A).

Later stage images of cell death showed more dramatic changes to the nuclear architecture and much more extreme indentation of the nuclear envelope, also known as nuclear crenellation (Figure 2.8C). However, in the corpses where the nucleus was visible, we never saw any indications of dark regions of

chromatin compaction, a characteristic and defining hallmark of apoptotic programmed cell death.

The most dramatic phenotype visible in a dying linker cell was abnormalities in the cytoplasm, especially formation and aggregation of 200 nm wide single-membrane cytoplasmic vesicles (Figure 2.8F), some of which may be swollen mitochondria (Figure 2.8J). The accumulation of vesicles within the linker cell raised the possibility that these may be autophagosomes, however, the vesicles that we observed were morphologically distinct from previously described double membranous autophagosomes (Baehrecke, 2003; Baehrecke, 2005; Levine and Yuan, 2005) since they contained either single or multiple membranous layers, they did not appear to contain the dark material characteristic of lysosomal destruction, and they were substantially larger than typical autophagosomes. Swollen and degraded mitochondria within large multilayered membrane-bound structures could also be clearly seen (Figure 2.8J). Small electron-translucent “empty” membrane-bound cytoplasmic structures were abundant during linker cell death, and these structures may be derived from the endoplasmic reticulum (Figure 2.8H). These white swollen structures were also visible in a dying linker cell in a migratory mutant background of *him-4* (Vogel and Hedgecock, 2001), in which the linker cell migration is defective and the linker cell ends up at the head of the animal and is not engulfed by U.l/rp (Figure 2.8G).


A
B
engulfing cell
nucleus
nucleolus
linker cell
C

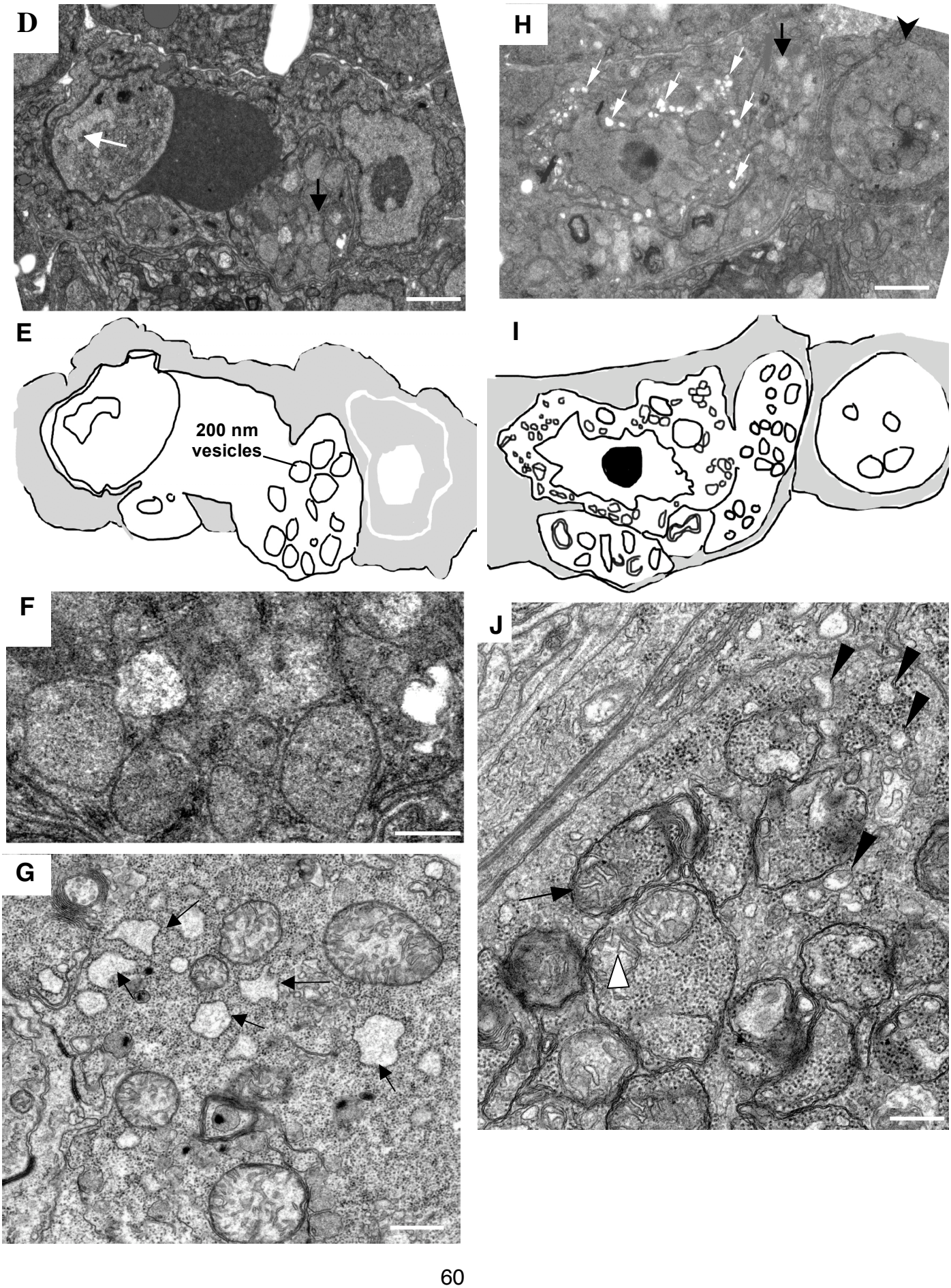

D
H
E
200 nm
vesicles
I
F
J
G

Figure 2.8 Linker Cell Death is Not Apoptotic by Electron Microscopy Analysis

(A) Image of a linker cell at the beginning of the cell death process before an obvious corpse structure is seen by DIC microscopy. Note that, although engulfed, the linker cell displays normal morphology, including a well-defined nucleus and a prominent nucleolus (black circular structure). Scale bar, 1 µm.
(B) A tracing of the image in (A).
(C) A highly invaginated ("crenellated") nuclear membrane (arrow) of a dying linker cell. Scale bar, 0.5µm.
(D) A linker cell at a later stage of death. Note the accumulation of 200 nm vesicles (black arrow), possibly degraded mitochondria, within the linker cell. Note absence of condensed chromatin in the nucleus (white arrow). The darkly staining material in the middle of the cell was only seen in 1/11 linker cells that we examined. Scale bar, 1 µm.
(E) A tracing of the image in (D).
(F) The vesicles shown in (D) at higher magnification. Scale bar, 100 nm.
(G) Dying linker cell cytoplasm in a *him-4(e1267)* male in which the linker cell migrates to the anterior of the animal and the linker cell is not engulfed by U.l/rp. Black arrows, swollen endoplasmic reticulum. Scale bar, 500 nm.
(H) Another linker cell at a late stage of death. The nuclear envelope is crenellated (indented). Black arrow indicates 200 nm vesicles. Arrowhead, a large membrane-bound structure that may correspond to the GFP-labeled bleb seen in Figure 2.3D. Note the "empty" clearings within the cytoplasm (white arrows). Scale bar, 1 µm.
(I) A tracing of the image in (H).
(J) A late stage dying linker cell. Note multilayered membrane structures surrounding mitochondria (arrow), the dilation of mitochondrial cristae (white arrowhead), and small clearings in the cytoplasm (black arrowheads). Scale bar, 200 nm.

Strikingly similar morphologies have been ascribed to the little studied third class of programmed cell death, also known as type III—the non-apoptotic, non-autophagic programmed cell death (Clarke, 1990). This type of cell death, characterized predominantly by cytoplasmic abnormalities, involves nuclear crenellation (indentation) in the absence of chromatin compaction, accompanied by cytoplasmic changes characterized by "dilation of ER, nuclear envelope, Golgi and sometimes mitochondria, forming 'empty' spaces" (Clarke 1990). Several researchers have reported observing cell death with similar features in vertebrates. For example, Pilar and Landmesser (1976) described such morphological features during the normal death of neurons in developing chick ciliary ganglia, and this publication also contains images of vesicles strikingly similar to the 200nm vesicles we see in linker cell death. A type III morphology was described in chick retinal ganglion cells that have been axotomized (Borsello et al., 2002), and in motor neurons that normally die in the chick spinal cord (Chu-Wang and Oppenheim, 1978). Some cells have been reported to die with the type III morphology during human tail retraction (Sapunar et al., 2001). Intriguingly, spinal motor neurons in mice lacking caspase-3 or caspase-9 die as in normal development, and display mitochondrial swelling and electron-translucent cytoplasmic structures similar to those seen in dying linker cells, so perhaps the vertebrate pathway for type III PCD is caspase-independent (Oppenheim et al., 2001).

(For additional linker cell TEM images, and published images of similar cell deaths in vertebrates, see the Appendix.)

The Linker Cell and the Gonad

It had been previously reported that the linker cell is required as a migratory leader cell of the male gonad, and loss of the linker cell by ablation prevents the gonad from developing properly (Kimble and White, 1981). We repeated these experiments (n=2), and confirmed that when the linker cell is ablated there is abnormal gonadal development and the gonad does not migrate further or connect to the exterior of the animal. We also wanted to assess if the gonadal cells adjacent to the linker cell could be providing some positive signal that was necessary for linker cell survival. To assess this possibility, we carried out laser ablations (Bargmann and Avery, 1995) of the gonadal cells adjacent to the linker cell in animals in which the linker cell was still at the migratory stage of its life. We performed these ablations in an *egl-5* background in which most of the gonad does not migrate with the linker cell, making it easier to ablate the few gonadal cells that migrate adjacent to the linker cell (Chisholm, 1991). In 5/5 animals in which we had ablated the gonadal cells, the linker cell did not die, but appeared to be capable of migrating normally on its path. In rare instances in other mutant backgrounds, we have occasionally seen healthy linker cell cells migrating normally apparently in the absence of adjacent *vas deferens* cells, although in these cases we did not have a fluorescent marker to confirm absence of *vas deferens* cells, which were identified based only on DIC optics.

CONCLUSIONS

The biological importance of the correct regulation of linker cell death is underscored by linker cell ablation experiments, which confirmed that the linker cell plays an essential role as a migratory leader cell of the male gonad. When the linker cell is ablated, the gonad cannot migrate or connect to the exterior of the animal. So premature death of the linker cell would render a male sterile.

By light and fluorescence microscopy investigation we have been able to uncover some previously undescribed features of linker cell death. We have identified a range of morphological changes that characterize linker cell death, and identified two new features of linker cell death: a blebbing of the dying cell into two parts, each of which is taken up by either U.lp or U.rp, and a necrotic-like vacuolar formation in which a vacuole arises from linker cell derived material.

Transmission electron microscopy studies of dying linker cells reveal that the cell appears to die by a new type of programmed cell death in *C. elegans*; linker cell death is neither apoptotic, autophagic, nor necrotic, but most closely resembles type III PCD (Clarke, 1990). In TEM images, the nucleus of the dying linker cell undergoes shape changes such as indentation or crenellation of the nuclear envelope, but not chromatin compaction. The cell shows a range of cytoplasmic abnormalities including formation of many large vesicular structures that may be swollen organelles. Similar morphological features have been seen associated with a number of other vertebrate cell deaths, suggesting that this form of programmed cell death may be conserved.

Chapter Three

Engulfment and Linker Cell Death

BACKGROUND

Identification of the Process of Engulfment

In 1908, Ilya Metchnikov was awarded the Nobel Prize in Physiology or Medicine for his discovery of the process of phagocytosis and his subsequent work elucidating its importance in the functioning of the immune system. He and others had observed motile cells in organisms that appeared to have digestive capacity. His breakthrough came in 1882, when working on a transparent starfish larvae, he introduced a splinter of wood from a rose thorn and observed phagocytic cells swarming around the foreign object. He hypothesized that this was a defensive action by the cells (Tauber, 2003). He also recognized a role for phagocytosis in normal development, for example, citing the role of phagocytosis in removal of a tadpole's tail (Tauber, 2003), and he speculated that dysfunctional or aging cells could be targeted for removal by phagocytosis (Tauber, 2003). Metchnikov was interested in the theory that an organism is formed from competing groups of cells that have to somehow cooperate as a whole, and that perhaps phagocytes act as one of the mechanisms that can keep things in check, by removing cells that end up in the wrong place.

The Immune System and Phagocytosis of Dying Cells

Phagocytosis may be very important for correct immune system function, for example, in presentation of antigens by the phagocyte when an immune response needs to be triggered (Skoberne et al., 2005). What determines whether or not an immune response is launched is a major question and an active field of research. Usually phagocytosis of apoptotic cells is associated with an anti-inflammatory response, however, in cases of autoimmunity, through mechanisms unknown, an immune reaction may be triggered (Ren and Savill, 1998).

When a cell dies necrotically, this may be a consequence of disease or infection, circumstances where it may be advantageous to elicit an immune response. Some signals have recently been identified that may govern whether a necrotic cell triggers an immune response, such as the release of HMGB1, a nuclear protein associated with chromatin (Scaffidi et al., 2002). An *in vitro* study of human cells suggested that signals that triggered a necrotic form of cell death did not prevent the cells from being phagocytosed (Hirt et al., 2000), and in *C. elegans*, necrotic cells are engulfed using the same engulfment machinery used for apoptotic cells (Chung et al., 2000). In mammals, necrotic cells may not necessarily be taken up in the same way as apoptotic cells. In one study of an *in vitro* system of macrophage engulfment, apoptotic cells were taken up complete, whereas necrotic cells were taken up by a means of macropinocytosis (Krysko et al., 2006b).

Phagocytic Target Recognition—"Eat Me" Signals

Phosphatidylserine exposure is the classic example of a recognition signal given by a dying cell when it is engulfed by a macrophage (Fadok et al., 1992). However, the identity of the phosphatidylserine receptor remains to be conclusively determined (Bose et al., 2004). A number of ligands and receptors for engulfment have been identified in mammalian systems (Krysko et al., 2006a). For example, in a healthy cell CD31/PECAM-1 mediates detachment from macrophages, but this CD31-based detachment does not occur in an apoptotic cell, leaving the cell vulnerable to attachment by macrophages (Brown et al., 2002). SIRPα (a inhibitory receptor for phagocytosis on macrophages) and its ligand CD47 can interact and prevent phagocytosis—it has been shown that red blood cells lacking CD47 are rapidly cleared by macrophages (Oldenborg et al., 2000).

C. elegans Studies of Engulfment

Early studies of *C. elegans* development noted that dying cells could be engulfed by their sister cells during early development (Sulston et al., 1983), during later development by hypodermal cells (Robertson and Thompson, 1982), or by gonadal sheath cells in the germline (Gumienny et al., 1999). *C. elegans* does not have a dedicated class of specialized motile engulfing cells.

In vivo studies of engulfment are perhaps most advanced in *C. elegans*. Some of the first cell death genes isolated in *C. elegans* were the engulfment

genes *ced-1* and *ced-2* (Hedgecock et al., 1983). In these two mutant backgrounds one of the first observations made was that even though engulfment was impaired when observed by electron microscopy, this did not prevent cell death from occurring, and the main phenotype of the mutations was persistence of dead cell corpses (Hedgecock et al., 1983). In general, in *C. elegans* and in most studied systems, engulfment appears to be a process more important for clearance of corpses than being required for execution of cell death. Although there are indications that engulfment may have some role in facilitating cell death in *C. elegans* (Hoeppner et al., 2001; Reddien et al., 2001). A few examples have even been found where engulfment may be required for cell death to occur. This has been reported for the B.a(l/r)rapaav cell in the *C. elegans* male (Hedgecock et al., 1983), and a few such examples have also been given for vertebrates including macrophages being required for some cell deaths during eye development (Griffith et al., 1995).

In *C. elegans,* a total of seven engulfment genes have been cloned and identified, most of which act in the engulfing cell, and several of which appear to be cytoskeletal regulators (Table 3.1). Analysis of double engulfment mutants have shown that they appear to fall into two partially redundant pathways, one pathway defined by *ced-1, ced-6, ced-7,* and the other by *ced-2, ced-5, ced-10,* and *ced-12* (Ellis et al., 1991; Gumienny et al., 2001). Animals combining mutations in both pathways have a stronger engulfment block.

Table 3.1 Molecular Identity of Known *C. elegans* Engulfment Genes

Gene	Molecular Identity	Site of Action	Reference
ced-1	Transmembrane protein. Homologous to human CD91.	Engulfing cell	Zhou et al., 2001
ced-2	SH2 and SH3 containing adaptor protein. Homologous to human CrkII.	Engulfing cell	Reddien and Horvitz, 2000
ced-5	SH3 domain containing protein. Similar to human DOCK180.	Engulfing cell	Wu and Horvitz, 1998
ced-6	Contains SH3 and phosphotyrosine binding domain. Homologous to human CED-6/GULP.	Engulfing cell	Liu and Hengartner, 1998
ced-7	ABC transporter. Similar to human ABC transporters.	Dying cell and engulfing cell	Wu and Horvitz, 1998
ced-10	GTPase. Homologous to human RAC1.	Engulfing cell	Reddien and Horvitz, 2000
ced-12	PH domain protein. Similar to human ELMO.	Engulfing cell	Gumienny et al., 2001 Zhou et al., 2001

Drosophila Studies of Engulfment

In *Drosophila*, macrophages can ingest both invading bacteria and apoptotic cells. The receptor Croquemort, similar to CD36—a human scavenger receptor, which is expressed on macrophages, was identified as being important for engulfment of apoptotic cells (Franc et al., 1999). Ubiquitous expression of Croquemort did not permit cells to engulf that were not macrophages, so Croquemort is not sufficient for phagocytic behavior (Franc et al., 1999).

Engulfment and Human Disease

The autoimmune disease lupus has been associated with defective engulfment in mouse models of the disease (Potter et al., 2003), and macrophages from lupus patients have impaired engulfment capacity (Hermann et al., 1998). Impaired engulfment of apoptotic cells has been associated with some chronic inflammatory lung disease (Vandivier et al., 2006).

Engulfment, With a Limited Appetite

There are some suggestions from the literature that engulfment may also be regulated in some circumstances to devour only partial regions of a cell, for example, in axon pruning during development in *Drosophila* (Awasaki et al., 2006; MacDonald et al., 2006), or another fascinating process is the daily phagocytosis in the vertebrate eye of rod outer segments by the adjacent retinal epithelium (Nguyen-Legros and Hicks, 2000).

RESULTS

Microscopy Analysis of Wild-type Linker Cell Engulfment

Using the *lin-48*::GFP marker for U.l/rp (Johnson et al., 2001), and an RFP marker we created for the linker cell, we were able to observe linker cell engulfment *in vivo* (Figure 3.1). The linker cell approaches (Figure 3.1A), and then reaches U.l/rp (Figure 3.1B). It does not appear that U.l/rp reaches forward to contact the linker cell before the two cells meet. U.l/rp then begins to engulf the linker cell, forming a sort of "candy scoop" around it, even though the linker cell is of a healthy morphology (Figure 3.1C). The linker cell blebs into two parts, one nuclear and one non-nuclear (see Figure 2.3D; Figure 2.8H), which are engulfed by U.lp and U.rp and the linker cell is completely engulfed (Figure 3.1D). This process is accompanied by vacuole formation from linker cell derived material (see Figure 2.6; Figure 2.7). After engulfment and destruction of the linker cell, U.l/rp subsequently elongate and adhere to the *vas deferens* (Sulston et al., 1980).

Engulfment by U.l/rp is Not Required For Linker Cell Death

It has been reported that linker cell death may depend on a signal from its engulfing cell (Sulston et al., 1980). To test this hypothesis, at the L1 stage we used a laser microbeam to ablate U (Bargmann and Avery, 1995), the U.l/rp

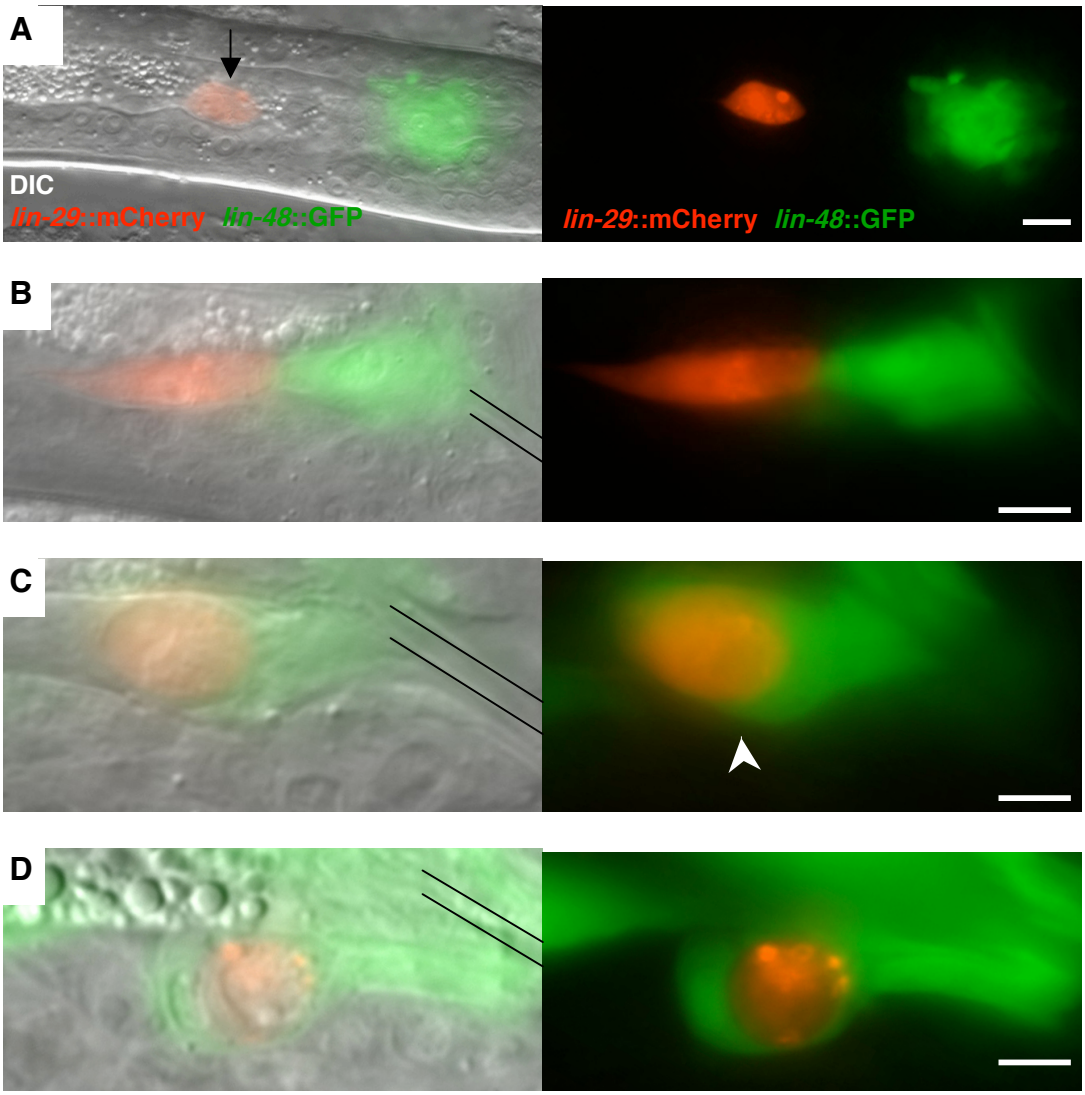


Figure 3.1 Wild-type Linker Cell Engulfment by U.l/rp
Animals express a *lin-29* promoter::mCherry in the linker cell and a lin-48 promoter::GFP in U.l/rp. Left, DIC and fluorescence merge. Right, fluorescence only. Scale bar, 5 μm.
(A) At late L4 stage, the linker cell (arrow) begins to approach U.l/rp. Ventral view.
(B) The linker cell reaches U.l/rp. Cloaca outlined in black. Anterior, left. Dorsal, top.
(C) The linker cell begins to be engulfed by U.l/rp. Note the processes (arrowhead) starting to envelope the linker cell. Cloaca outlined in black. Anterior, left, Dorsal, top.
(D) The linker cell is completely engulfed by U.l/rp. Cloaca outlined in black. Anterior, left. Dorsal, top.

grandparental precursor cell. Linker cell fate in operated animals containing a genomically integrated *lag-2* promoter::GFP transgene, expressed in the linker cell, was followed until 2 hr after the L4 to adult transition. By this time, linker cells in wild-type animals have already died (Table 2.1). In 18/18 operated animals and 17/18 mock-ablated animals, linker cell death occurred, as assessed by disappearance of GFP expression or appearance of a GFP-labeled cell corpse. This suggests that linker cell death does not require the U.l/rp cells.

Linker Cell Death Can Occur in a Migratory Mutant Background

To test whether other local cues instead of U.l/rp might promote linker cell death, we examined a *him-4(e1267)* background in which linker cell migration is abnormal and the cell often migrates only to the anterior of the animal (Vogel and Hedgecock, 2001). The linker cell died in most animals observed (Table 3.2), suggesting that linker cell death can occur cell autonomously and doesn't require any short-range extrinsic spatial cue from the cloacal region to initiate its death. Linker cell death also occurs in other migratory backgrounds. However, the cell did survive in 16% of *him-4* animals examined 6 hr after the L4 to adult molt. Furthermore, a dying linker cell corpse was still visible in 73% (n = 30) of *him-4* mutants 2 hr after the molt, while corpses at this stage were seen in only 13% (n = 30) of wild-type animals (Table 3.2; Table 2.1). These results suggest that a local signal from the cloacal area, or the engulfment capabilities of U.l/rp may increase the efficiency with which linker cell death or engulfment proceeds.

Table 3.2 DIC Study of Linker Cell Death Morphology in *him-4(e1267)*

Animal Age	% Healthy Linker Cell (n)[a]	% Early Abnormal Linker Cell (n)[b]	% Late Abnormal Linker Cell (n)[c]	% Linker Cell Completely Gone (n)
Early L4	100(15)	0(15)	0(15)	0(15)
Late L4 Rays Visible	20(25)	8(25)	68(25)	4(25)
0-2-Hr-Old Adult	13(30)	3(30)	70(30)	13(30)
2-4-Hr-Old Adult	7(30)	17(30)	46(30)	30(30)
4-6-Hr-Old Adult	16(32)	6(32)	31(32)	47(32)

n, number of animals scored.
Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. In this population study each animal was scored once at one timepoint.
[a] A healthy linker cell morphology was scored if the linker cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example.
[b] An early abnormal linker cell corpse was recorded if the linker cell had lost substantial volume or started large scale blebbing or had nuclear envelope abnormalities such as abnormal indented (or crenellated) nuclear envelope shape or signs of nuclear envelope breakdown. See Figure 2.3B or 2.3C for an example.
[c] A late abnormal linker cell corpse was recorded if the linker cell had lost substantial volume and had assumed a completely round shape. Such linker cell corpses invariably had major nuclear abnormalities and in many cases the nucleus had completely broken down. See Figure 2.3D or 2.3E for an example.

Linker Cell Death in an Animal With Two Linker Cells

The conclusion that death of the linker cell is more efficient in the normal location compared with a linker cell migratory mutant background, is consistent with experiments in which we observed linker cell death in animals with two linker cells. *mig-5*, a Dishevelled family member regulates cell fate decisions, and in a *mig-5* mutant background, the two cells with the potential to become the linker cell can both give rise to linker cells (Walston et al., 2006). When we carried out RNAi experiments targeting *mig-5*, we found that the two linker cells formed could have different migratory fates. Usually one linker cell would go to the normal cloacal region, and the other linker cell might also go to the cloacal region or could end up in the middle of the animal or in the head region. We examined 19 *mig-5* RNAi animals with two linker cells to determine the effect of linker cell location on linker cell death and corpse formation. We noted the approximate positions of the linker cells under the dissecting microscope, and when the animals were adults we assessed the linker cells using a compound microscope. In 8 of the animals (42%), the more anterior linker cell—the one further away from U.l/rp—was less advanced in the death program, for example, it was at an earlier stage of corpse formation compared with the linker cell that was beside U.l/rp. In 9 of the animals (47%), the stage of the death program seemed roughly equivalent for the linker cell beside U.l/rp and for the linker cell that was further away. In only two animals (11%), did the linker cell being engulfed by U.l/rp

appear less advanced in the death program compared with the linker cell dying elsewhere.

Linker Cell Death is Independent of Known Engulfment Genes

Two partially redundant pathways have been described in *C. elegans* for the phagocytosis of both apoptotic (Gumienny et al., 2001; Mangahas and Zhou, 2005) and necrotic (Chung et al., 2000) cells. One pathway consists of the genes *ced-1*, *ced-6*, and *ced-7*, and the other of the genes *ced-2*, *ced-5*, *ced-10*, and *ced-12*. Since it has been suggested that engulfment may facilitate programmed cell death (Reddien et al., 2001; Hoeppner et al., 2001), we assessed the effect of known engulfment genes on linker cell death and we found that these mutations had no effect on survival (Table 3.3). For example, within a 2 hr period after the L4 to adult transition, the linker cell died in 29/30 animals defective for both the *ced-1* and *ced-5* engulfment genes.

Linker Cell Engulfment Is Independent of Known Engulfment Genes

The result that engulfment genes do not block linker cell death suggests either that engulfment is not necessary for linker cell death, or that the known engulfment genes do not participate in linker cell engulfment, or both. To distinguish between these possibilities, we assessed the efficiency of linker cell engulfment by the U.l/rp in animals carrying mutations in known engulfment genes. The marker *lin-48* is expressed in the U.l/rp cells (Johnson et al., 2001), and using this marker we can visualize engulfment *in vivo*, which is not readily

Table 3.3 Role of Engulfment Genes in Linker Cell Death

Genotype[a]	% Linker Cell Survival in 0-2-Hr-Old Adults(n)[b]	% Linker Cell Survival in 4-8-Hr-Old Adults(n)	No. Extra Cells Anterior Pharynx (n) [c]
Wild-type	0(30)	0(30)	0.2±0.4(15)
ced-1(e1735)	7(30)	0(29)	0.1±0.3(15)
ced-2(e1752)	0(30)	3(30)	ND
ced-5(n1812)	3(30)	0(30)	0.3±0.5(15)
ced-6(n2095)	0(30)	3(30)	ND
ced-7(n1892)	3(30)	0(30)	0.1±0.3(15)
ced-10(n1993)	0(30)	3(30)	0.3±0.5(15)
ced-12(k149)	0(30)	0(30)	ND
ced-1(e1735); ced-5(n1812)	3(30)	3(30)	0.1±0.3(10)
ced-7(n1892); ced-10(n1993)	0(30)	3(30)	0.3±0.5(13)

n, number of animals scored. ND, not determined.

[a] Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. All strains described also contained either the *him-5(e1467)* or *him-8(e1489)* mutations for high incidence of males. In this population study each animal was scored once at one timepoint.

[b] Linker cell survival was scored if a healthy linker cell morphology was visible and the cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example of a healthy linker cell.

[c] Number of extra cells in the anterior pharynx of males of indicated genotype was assessed as described in the Materials and Methods section. Mean ± SD.

discernible without use of a fluorescent marker, since cell membranes in *C. elegans* are not usually visible by DIC optics. Specifically, GFP-labeled U.l/rp cells of males undergoing the L4 to adult molt were examined for cellular extensions completely surrounding the linker cell (Figure 3.1D). Surprisingly, we found that engulfment still proceeded at high efficiency in these mutants (Table 3.4). For example, 30/31 linker cells were still engulfed in *ced-1(e1735)*; *ced-5(n1812)* double mutants (Figure 3.2A). By comparison, the NSM sister cell that dies during development is not engulfed in 52% of *ced-1(e1735)*; *ced-5(n1812)* double mutants (Ellis et al., 1991), and the tail-spike cell is not engulfed in 94% of *ced-5(n1812)* mutants (Maurer et al., 2007). We also investigated any possible effect of *dyn-1,* a GTPase that is expressed and active in engulfing cells that are removing apoptotic corpses (Yu et al., 2006), but no effect was seen (Table 3.4).

The Linker Cell is Engulfed by a *ced-1*-independent Mechanism

In *C. elegans*, the CED-1 transmembrane protein, homologous to human scavenger receptors, is expressed in engulfing cells and clusters around dying cell corpses to promote engulfment (Zhou et al., 2001). A translational fusion of CED-1::GFP shows bright clustering around corpses being engulfed and this construct can rescue *ced-1* loss-of-function (Zhou et al., 2001). Consistent with our finding that known engulfment mutants do not block linker cell engulfment, we never detected CED-1 clustering around the linker cell in wild-type animals, despite expression of the transgene in U.l/rp cells (n = 30) (Figure 3.2B).

Table 3.4 Mutants of Known Engulfment Genes Do Not Block Linker Cell Engulfment

Genotype	% U.l/rp Cells Extending Processes to Surround the Linker Cell (n) [a]
Wild-type	96(27)
ced-1(e1735)	100(30)
ced-5(n1812)	100(30)
ced-7(n1892)	97(30)
ced-10(n1993)	93(30)
ced-10(n3417)[b]	92(13)
ced-1(e1735); ced-5(n1812)	97(31)
*dyn-1(ky51*ts)[c]	100(32)

n, number of animals observed.
[a] Animals were scored at the late L4 stage just prior to the molt to adult to provide enough time for complete engulfment, if it occurred, to become obvious.
[b] Homozygous mutants of this allele from a homozygous *ced-10* mother are embryonic lethal; therefore, we scored homozygous *ced-10* males from *ced-10*/+ mothers (homozygosity confirmed by PCR). Maternally rescued *ced-10* homozygotes are not embryonic lethal, but show engulfment defects at later stages of development (Kinchen et al., 2005).
[c] Animals shifted to the nonpermissive temperature (25°C) at least 12 hr prior to scoring.

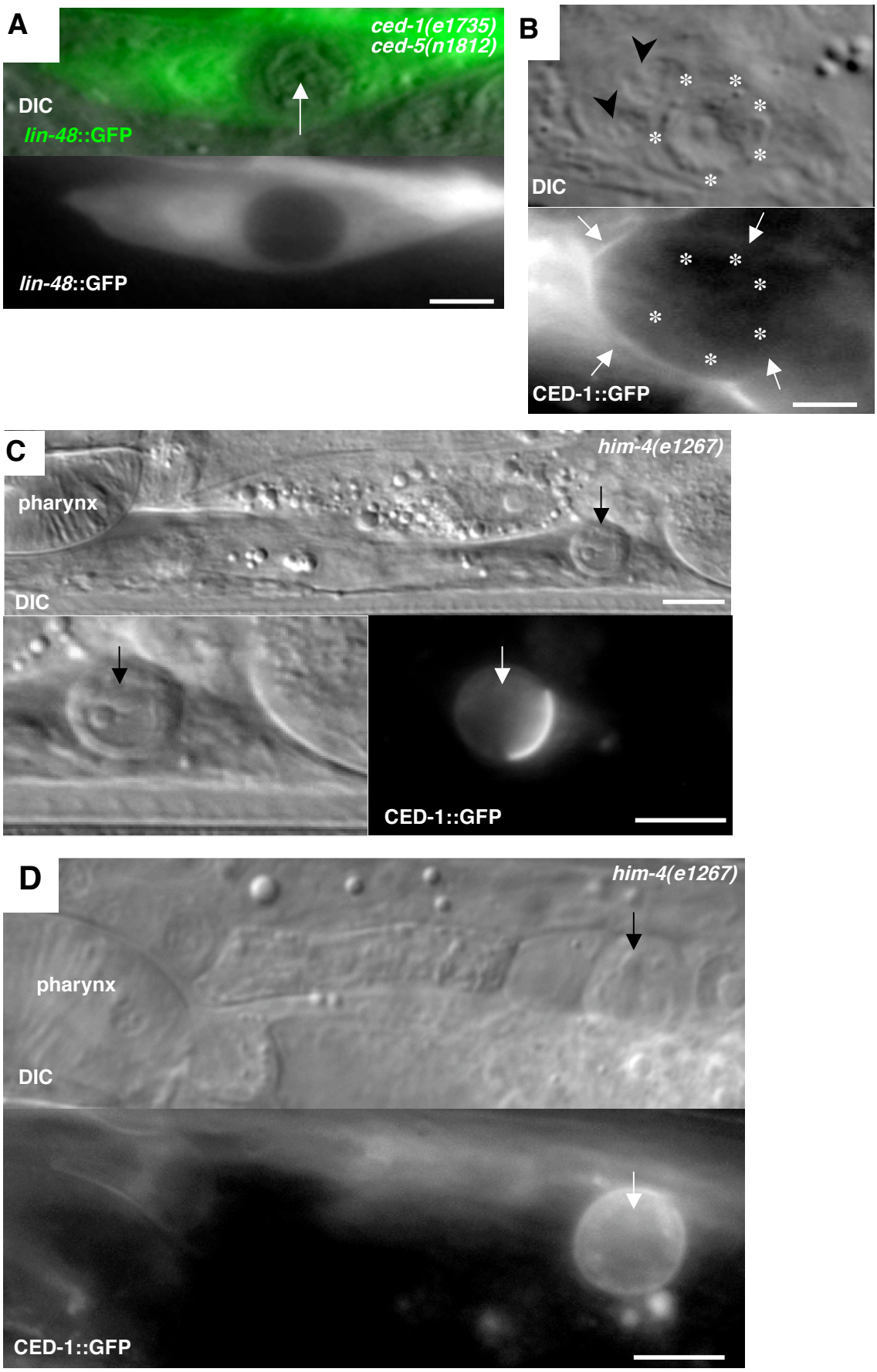

A
ced-1(e1735)
ced-5(n1812)
DIC
lin-48::GFP
lin-48::GFP
B
DIC
CED-1::GFP
C
him-4(e1267)
pharynx
DIC
CED-1::GFP
D
him-4(e1267)
pharynx
DIC
CED-1::GFP

Figure 3.2 Linker Cell Engulfment and the Role of CED-1
(A) A linker cell corpse (arrow) is completely engulfed by U.l/rp in a *ced-1(e1735); ced-5(n1812)* mutant. Scale bar, 5 µm.
(B) A linker cell (outlined with asterisks) dying in its normal position does not induce CED-1::GFP clustering, even though CED-1::GFP is expressed on the engulfing cell membrane (arrows). Arrowheads, nuclei of U.l/rp. DIC (top) and fluorescence (bottom) images. Scale bar, 5 µm.
(C) Top: DIC image of a dying linker cell (arrow) in a *him-4(e1267)* male. Note that the cell has failed to complete its normal migration, and ends up in an anterior ventral position beside the pharynx. Bottom: close-up DIC (left) and fluorescence (right) images of the linker cell showing linker cell engulfment by a neighboring cell expressing CED-1::GFP. Note GFP clustering around the linker cell appears brighter at one side suggesting that engulfment has just partially commenced. Anterior, left. Dorsal, top. Scale bar, 5 µm.
(D) Top: DIC image of a dying linker cell (arrow) in a *him-4(e1267)* male. Note that the cell has failed to complete its normal migration, and ends up in an anterior ventral position beside the pharynx. DIC (top) and fluorescence (bottom) images of the linker cell showing linker cell engulfment by a neighboring cell expressing CED-1::GFP. Note that the GFP clustering around the linker cell corpse appears more uniform than in (C), perhaps indicating the cell is more fully engulfed. Anterior, left. Dorsal, top. Scale bar, 5 µm.

Taken together, these results suggest that linker cell engulfment proceeds, at least in part, by a previously undescribed mechanism.

The fact that there might be an alternative engulfment pathway has already been hinted at from other published experiments. During embryogenesis and L1 larval development of a hermaphrodite, 93 cells die in the head region of the worm (Sulston et al., 1983). However, when the head of a *ced-1(e1735); ced-5 (n1812)* worm, with mutations in both of the partially redundant engulfment pathways, is observed at the L1 stage, only 44 persistent cell corpses are visible (Zhou et al., 2004). While some of the missing corpses could have been extruded into the fluid surrounding the developing embryo, it is also possible that some of the cells were engulfed by other mechanisms.

One possible explanation for why U.l/rp does not use the normal programmed cell death engulfment machinery is that it has to use a more efficient engulfment system to avoid possible linker cell engulfment by the *vas deferens*. In the hermaphrodite, gonadal sheath cells can engulf germline corpses (Gumienny et al., 1999). We have noticed that in migratory mutant backgrounds, the linker cell corpse frequently ends up engulfed inside the *vas deferens,* which appears to be fused together at its end and closed up upon itself (Figure 3.3A). It appears that the *vas deferens* expresses CED-1 in the region adjacent to the dying linker cell (Figure 3.3D). If the linker cell was taken up by the *vas deferens*, this would trap the linker cell corpse in a position that would

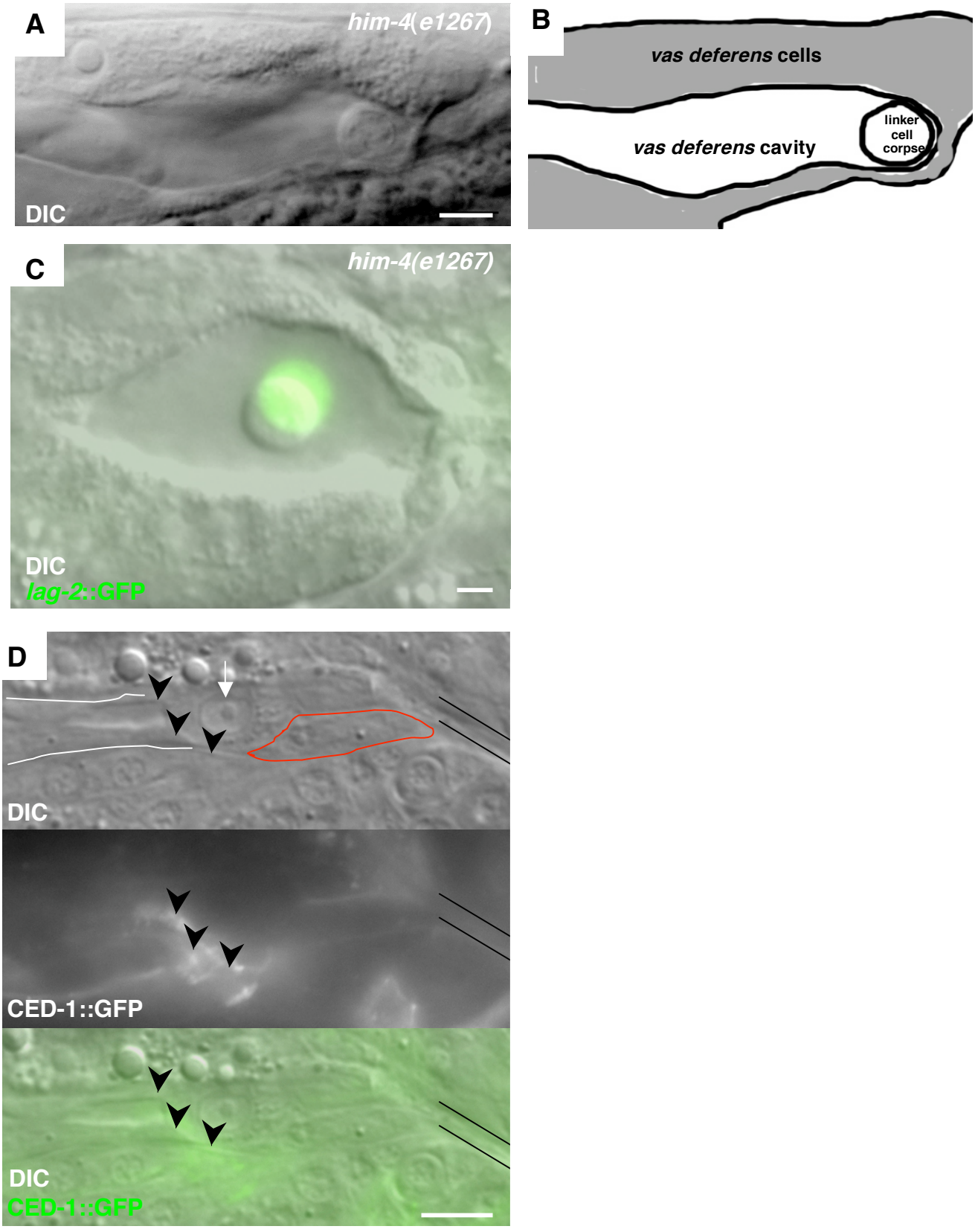

A
him-4(e1267)
DIC
B
vas deferens cells
linker cell corpse
vas deferens cavity
C
him-4(e1267)
DIC
lag-2::GFP
D
DIC
CED-1::GFP
DIC
CED-1::GFP

Figure 3.3 Linker Cell Engulfment by the *Vas Deferens*
(A) Image of a *him-4(e1267)* animal in which the linker cell migrated abnormally and was engulfed by the male gonad. Scale bar, 5 µm.
(B) Tracing of image in (A).
(C) Linker cell corpse apparently floating free in the *vas deferens.* Scale bar, 1 µm.
(D) CED-1 may be expressed on the *vas deferens* adjacent to the linker cell (arrow) when it reaches the cloacal region. The *vas deferens* is outlined in white. The engulfing cell U.l/rp is outlined in red. The cloaca is outlined in black. Arrowheads indicate the region of the *vas deferens* contacting the linker cell. Anterior, left. Dorsal, top. Scale bar, 5 µm.

likely block sperm exit, and the process of engulfment of the linker cell may fuse the *vas deferens* closed. Perhaps U.l/rp is better at engulfing than the *vas deferens,* and U.l/rp is like a professional mammalian phagocyte that can recognize earlier signs of cell death (Parnaik et al., 2000).

In Linker Cell Migratory Mutants, Engulfment is by a *ced-1*-dependent Mechanism

We did find, however, that CED-1::GFP protein clusters around and encircles the linker cell when the cell died at an abnormal location in a *him-4* mutant (Figure 3.2C and 3.2D). In these abnormal positions the linker cell is probably engulfed by the nearest neighboring hypodermal cell.

Is Engulfment Required for Linker Cell Death?

Engulfment of the linker cell begins at a very early stage of linker cell death, when the linker cell still appears to have a healthy morphology (Figure 2.8A; Figure 3.1C). Since known engulfment mutants do not block the linker cell from being engulfed (Table 3.4), we cannot assess the role of engulfment in wild-type linker cell death. Does the linker cell require engulfment to die? We found that CED-1::GFP protein did cluster around and encircle the linker cell when the cell died at an abnormal location in a *him-4* mutant (Figure 3.2D). However, at this location, morphological changes similar to those observed during normal linker cell death may precede complete engulfment of the cell (Figure 3.2C), since we

can sometimes observe partial regions of CED-1 clustering around a corpse. In linker cell migratory mutants, we can occasionally observe a linker cell corpse apparently floating free in the *vas deferens* (Figure 3.3C), clearly not within another cell, although we can not rule out the fact that it may have been surrounded by another layer of membrane when it entered the *vas deferens*. These results may suggest that in some circumstances linker cell death can be uncoupled from engulfment.

CONCLUSIONS

We have developed a system where death and engulfment can be studied *in vivo* with fluorescent markers. This is the only such system to date in C. *elegans* where a predictable death and engulfment can be studied in such a way. By laser ablation we have found that U.l/rp, the usual engulfing cells of the linker cell, are not required for linker cell death. We have found that the engulfment of the linker cell surprisingly begins when the cell has a healthy morphology. Known *C. elegans* engulfment genes are not required for linker cell death, and they do not block linker cell engulfment by U.l/rp. U.l/rp does not use the transmembrane receptor CED-1 in linker cell engulfment. In a linker cell migratory mutant background, the linker cell still dies, suggesting that the death of the linker cell does not require a short range spatial cue from the location of its normal death. In a migratory mutant context, the linker cell corpse is engulfed using CED-1, and the corpse clearance is lower efficiency than in wild-type.

Chapter Four

Known Cell Death Genes and Linker Cell Death

BACKGROUND

Apoptotic Programmed Cell Death in *C. elegans*

The first gene identified that affected programmed cell death in *C. elegans* was *nuc-1,* which resulted in abnormalities in cell corpses in the ventral nerve cord of the worm (Sulston, 1976). When the gene was cloned, it was revealed to be homolog of mammalian DNase II, and worms carrying the mutation were unable to complete the normal process of DNA degradation during cell death (Wu et al., 2000). A number of genetic screens identified a core pathway of conserved genes (Figure 1.2) required for apoptosis in *C. elegans*. In mutants in *ced-3* (loss-of-function), *ced-4* (loss-of-function), *ced-9* (gain-of-function), or *egl-1* (loss-of-function) (Conradt and Horvitz, 1998), virtually all of the somatic programmed cell death that normally occurs is blocked (Ellis and Horvitz, 1986; Hengartner and Horvitz, 1994), and cells that would normally die will survive and can adopt a differentiated cell fate. However, Ellis and Horvitz (1986) reported that linker cell death occurred in 3/5 *ced-3* mutant males, 2/6 *ced-4* mutant males, and 1/6 *ced-4(n1162)*; *ced-3(n717)* males that they examined, suggesting that, while linker cell death was dependent on *ced-3* and *ced-4*, it could proceed in their absence.

In *C. elegans*, four caspase-related genes exist: *ced-3*, *csp-1*, *csp-2*, and *csp-3* (Shaham, 1998; Yuan et al., 1993); but only *ced-3* seems to be required for programmed cell death (Abraham and Shaham, 2004; Yuan et al., 1993), and only *ced-3* and *csp-1* are proteolytically active (Shaham, 1998). The CSP-2 caspase lacks key active-site residues, and *csp-3* encodes only a C-terminal caspase domain, entirely lacking the active site (Shaham, 1998). In somatic cells, CED-3 caspase activity is controlled by a conserved pathway consisting of three proteins: EGL-1 (BH3-only), CED-9 (Bcl-2-related), and CED-4 (Apaf-1). In living cells, CED-9 protein is thought to bind to and sequester CED-4, preventing activation of CED-3. EGL-1 is expressed in some cells destined to die, and can bind to CED-9, releasing CED-4 and allowing it to promote CED-3 activation (Metzstein et al, 1998). The EGL-1-related protein, CED-13, may act similarly in the *C. elegans* germline (Schumacher et al., 2005) (Figure 1.2).

The genes required for apoptotic programmed cell death in *C. elegans* appear to act in a cell-intrinsic manner in the dying cell. Transgenic expression of these core pathway genes in cells that don't normally die, such as the touch receptor neurons, can induce these cells to undergo programmed cell death with a normal apoptotic morphology as demonstrated by electron microscopy analysis (Shaham and Horvitz, 1996b). Mosaic studies of *ced-3* and *ced-4* mutant animals in which *ced-3* or *ced-4* was rescued by an extrachromosomal array also pointed towards their cell-autonomous action (Yuan and Horvitz, 1990).

Autophagic Cell Death in *C. elegans*

Autophagic programmed cell death has not been described in *C. elegans*, although it has been reported that the beclin homolog *bec-1* may regulate apoptotic cell death (Takacs-Vellai et al., 2005). Another study of *bec-1* in *C. elegans* suggested that it plays a role in dauer formation and lifespan extension (Melendez et al., 2003). There has been a recent report that autophagic genes may regulate necrotic cell death in *C. elegans* (Toth et al., 2007).

Necrotic Cell Death in *C. elegans*

A study of neurodegeneration of touch cells in *C. elegans*, identified dominant mutations in *mec-4,* a member of the degenerin gene family of ion channels, as being responsible for the degeneration of the cells in a manner similar to necrosis (Driscoll and Chalfie, 1991). *deg-1* also induces necrosis (Chalfie and Wolinsky, 1990). *mec-4* and *deg-1* are similar to the vertebrate amiloride-sensitive epithelial Na^+ channel (Canessa et al., 1993). The *mec-4* degeneration-inducing mutant is an amino acid substitution, and later work in a human system indicates that this mutation is likely to cause hyperactivity of the ion channel (Waldmann et al., 1996). Similar degenerating phenotypes have been found with a $G\alpha$ protein (Korswagen et al., 1997). The death of these and *mec-4* necrotic degenerating cells does not require the known apoptotic core pathway genes (Berger et al., 1998; Chung et al., 2000). Some genes that are required for the execution of necrosis in *C. elegans* have been identified, including aspartyl and calpain

proteases (Syntichaki et al., 2002), regulators of calcium release (Xu et al., 2001), components of the lysosomal pathway (Artal-Sanz et al., 2006), and the autophagic pathway (Toth et al., 2007).

RESULTS

Linker Cell Death Appears to Be Caspase-independent

Although a dying linker cell resembles other apoptotic dying cells in the animal by forming a button-like corpse seen with DIC optics when the cell dies (Figure 2.3E), the dependence of this death on known *C. elegans* cell death genes has not been extensively studied. Surprisingly, we found that the linker cell died just after the L4/adult transition in 30/30 *ced-3(n717)* loss-of-function (lf) mutant males examined, suggesting that linker cell death does not require *ced-3.* We could also see that the linker cell corpse was engulfed normally by U.l/rp in a *ced-3* mutant background, indicating that the linker cell was undergoing its normal process of cell death (Table 4.1; Figure 4.1). One possible reason for the stronger block in linker cell death compared with that reported previously for this allele, may be that in previous studies the linker cell was not followed during and after the L4 to adult molt (Ellis and Horvitz, 1986). Thus, scoring the state of the cell too early may have been misleading. Another possibility is that, after the transition to the adult, the linker cell becomes more difficult to distinguish from nearby cells. Therefore, we always used *lag-2*::GFP or *mig-24*::GFP reporter

Table 4.1 Role of Caspases in Linker Cell Death

Genotype[a]	% Linker Cell Survival in 0-2-Hr-Old Adults(n)[b]	% Linker Cell Survival in 4-8-Hr-Old Adults(n)	No. Extra Cells Anterior Pharynx (n) [c]
Wild-type	0(30)	0(30)	0.2±0.4(15)
ced-3(n717)	0(30)	0(30)	10.5±1.4(15)
ced-3(n2452)	5(18)	ND	ND
ced-3 (RNAi)	3(30)	3(30)	ND
csp-1(tm917)	7(30)	0(30)	0.1±0.4(15)
csp-1(RNAi)	4(26)	ND	ND
csp-2(tm1079)	3(30)	3(30)	0.1±0.3(15)
csp-3(RNAi)	7(30)	0(30)	0.1±0.4(15)
ced-3(717); csp-1(tm917)	2(50)	0(30)	10.8±1.7(15)
HS-p35[d]	0(31)	ND	3.9±4.2(15)

n, number of animals scored. ND, not determined.

[a] Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. All strains described also contained either the *him-5(e1467)* or *him-8(e1489)* mutations for high incidence of males. In this population study each animal was scored once at one timepoint.

[b] Linker cell survival was scored if a healthy linker cell morphology was visible and the cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example of a healthy linker cell.

[c] Number of extra cells in the anterior pharynx of males of indicated genotype was assessed as described in the Materials and Methods section. Mean ± SD.

[d] p35 expression was induced by applying a 45 minute heat shock to L4 males, or during early embryogenesis to assess pharyngeal cell survival.

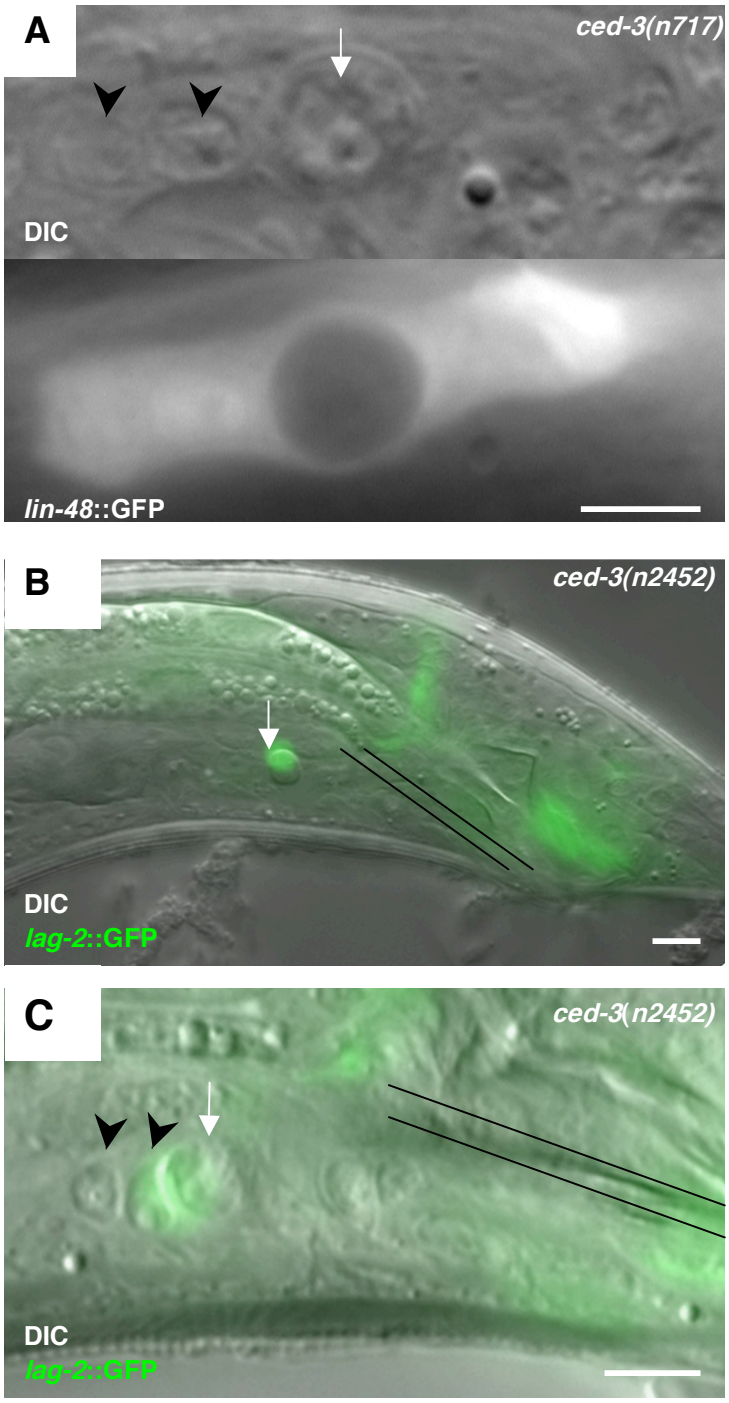


Figure 4.1 The Linker Cell Dies and is Engulfed Normally in a *ced-3* Mutant Background
(A) A dying and fully engulfed linker cell (arrow) in a *ced-3(n717)* male expressing a *lin-48* promoter::GFP reporter in the engulfing U.l/rp cell. Arrowheads, nuclei of the engulfing U.l/rp. Scale bar, 5 μm.
(B) A linker cell button-like corpse (arrow) in a 2-hour-old adult *ced-3(n2452)* animal lacking the entire protease domain of *ced-3*. The linker cell is marked with a *lag-2* promoter::GFP. Anterior, left. Dorsal, top.

Cloaca outlined in black. Scale bar, 5 μm.
(C) By DIC optics, a linker cell corpse (arrow) appears to be engulfed in a 2-hour-old adult *ced-3(2452)* animal. Arrowheads, nuclei of the engulfing U.l/rp. Cloaca outlined in black. Anterior, left. Dorsal, top. Scale bar, 5 μm.

transgenes to facilitate scoring linker cell presence. We observed linker cell death occurring in a *ced-3(n2452)* mutant with a deletion of the entire protease domain of the caspase (Table 4.1; Figure 4.1B), and in males subjected to *ced-3* RNA interference (RNAi) (Table 4.1).

To examine whether the other *C. elegans* caspases may be required for linker cell death, we assessed linker cell death in males with deletions in the *csp-1* and *csp-2* genes, or subjected to RNAi directed toward *csp-1* or *csp-3*. The *csp-1(tm917)* mutation is a 749 bp deletion in sequences encoding the N-terminal non-catalytic domain of the protein. Since the *csp-1* gene has an alternative start site downstream of the region targeted by the *tm917* deletion, we also tried to knockdown this gene's function using an RNAi construct targeting the C-terminal region of *csp-1* where the protease domain is located. The *csp-2(tm1079)* mutation consists of a 680 bp deletion in sequences encoding the N-terminal non-catalytic domain of the protein and a duplication of a small portion of this domain. As shown in Table 4.1, none of these mutants or RNAi experiments affected linker cell death. Furthermore, linker cell death still occurred in *csp-1*; *ced-3* double mutants, and in males in which the broad-spectrum caspase inhibitor, p35 (Clem et al., 1991), was overexpressed using a heat shock promoter (Sugimoto et al., 1994) (Table 4.1). Taken together, these data suggest that linker cell death is *ced-3*-independent, and likely caspase-independent.

Known Apoptotic Cell Death Genes Are Not Required for Linker Cell Death

ced-3-independent but *ced-4*-dependent cell death has been previously reported

in *C. elegans* embryos carrying mutations in the *icd-1* gene (Bloss et al., 2003). To determine whether linker cell death proceeded using a similar mechanism, we examined linker cell death in *ced-4* (loss-of-function) mutants. We found that linker cell death still occurred within 4–8 hr of the L4 to adult transition in 30/30 *ced-4(n1162*) males that we examined (Table 4.2). Likewise, linker cell death still occurred in males carrying an *egl-1* (loss-of-function) mutation or a *ced-9* (gain-of-function) mutation, both of which prevent the deaths of most somatic *C. elegans* cells destined to die (Conradt and Horvitz, 1998; Hengartner et al., 1992) (Table 4.2). Furthermore, linker cell death still occurred in the large majority of animals carrying combinations of mutations in *ced-3*, *ced-4*, *him-4*, and the engulfment-promoting genes *ced-1*, *ced-2*, *ced-5*, *ced-7*, and *ced-10*, demonstrating that, even in these highly sensitized genetic backgrounds, linker cell death could still proceed efficiently (Table 4.2). Although a slight delay in linker cell death occurred in *ced-4(n1162)* and *ced-9(n1950)* mutants, this may be attributable to a slight developmental delay in these mutants, rather than a direct effect on linker cell death per se. Taken together, the results presented here suggest that linker cell death must be regulated, at least in part, by a previously undescribed cell death program.

Table 4.2 Role of Apoptotic Programmed Cell Death Genes in Linker Cell Death

Genotype[a]	% Linker Cell Survival in 0-2-hr-Old Adults(n)[b]	% Linker Cell Survival in 4-8-hr-Old Adults(n)	No. Extra Cells Anterior Pharynx (n) [c]
Wild-type	0(30)	0(30)	0.2±0.4(15)
ced-3(n717)	0(30)	0(30)	10.5±1.4(15)
ced-4(n1162)	13(30)	0(30)	11.1±1.4(15)
ced-9(n1950)	16(32)	0(30)	10.9±1.6(10)
egl-1(n1084n3082)	3(30)	3(30)	10.6±1.9(15)
ced-13(sv32)	0(30)	0(30)	0.1±0.4(15)
ced-8(n1891)	0(30)	0(30)	ND
nuc-1(e1392)	0(30)	0(30)	ND
ced-1(e1735); ced-3(n717)	0(30)	0(30)	10.8±2.6(15)
ced-2(e1752); ced-3(n717)	0(30)	0(30)	10.3±1.4(15)
ced-7(n1892); ced-10(n1993); ced-3(n717)[d]	18(44)	15(33)	10.3±1.9(15)
ced-1(e1735); ced-4(n1162); ced-5(n1812)	7(30)	7(30)	10.2±1.3(10)
ced-3(n717); him-4(e1267)	16(32)	9(32)	11.0±1.4(10)
ced-4(n1162); him-4(e1267)	16(32)	3(29)	9.9±1.5(10)
ced-7(n1892); ced-10(n1993); ced-3(n717); him-4(RNAi)	38(29)	29(41)	10.2±2.0(15)
ced-1(e1735); ced-4(n1162); ced-5(n1812); him-4(RNAi)	28(29)	20(30)	9.9±1.7(10)

n, number of animals scored. ND, not determined.

[a] Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. All strains described also contained either the *him-4(e1267), him-5(e1267),* or *him-8(e1489)* mutations for high incidence of males. In this population study each animal was scored once at one timepoint.

[b] Linker cell survival was scored if a healthy linker cell morphology was visible and the cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example of a healthy linker cell.

[c] Number of extra cells in the anterior pharynx of males of indicated genotype was assessed as described in the Materials and Methods section. Mean ± SD.

[d] Unlike the other strains in this table, this strain was, for unknown reasons particularly unhealthy and grew very slowly. Therefore, any survival seen may reflect these developmental defects.

Known Apoptotic Genes May Facilitate Efficient Corpse Clearance In Linker Cell Death

We did make one observation concerning a possible role for core pathway genes in aiding efficient corpse clearance during linker cell death. In a wild-type worm within 2 hours of becoming an adult, the linker cell is a corpse in only about 13% of animals. We noticed that in some apoptotic mutant backgrounds, the linker cell would die normally, and show the same morphological characteristics that occur in wild-type linker cell death—loss of cytoplasmic volume, blebbing, nuclear envelope breakdown etc. However, in these backgrounds the corpses tended to persist longer, for example compare Table 4.3 showing *ced-3* corpse persistence with Table 2.1 for the wild-type. This could represent a weak late role for these genes in clearance of the corpse or could be some secondary consequence of the general developmental delay in these animals.

Known Autophagy, Necrosis, or Wallerian Degeneration Mutants Are Not Required for Linker Cell Death

To determine if autophagy could be playing a role in linker cell death we followed linker cell death in animals carrying mutations in the *bec-1* and *unc-51* genes, homologs of the autophagy genes beclin (Melendez et al., 2003; Takacs-Vellai et al., 2005) and APG1 (Matsuura et al., 1997), respectively. We saw no effects on linker cell survival in these mutants (Table 4.4). We also examined expression in the linker cell of an LGG-1::GFP protein, a marker for autophagosomal

Table 4.3 DIC Study of Linker Cell Death Morphology in *ced-3(n717)*

Animal Age	% Healthy Linker Cell (n)[a]	% Early Abnormal Linker Cell (n)[b]	% Late Abnormal Linker Cell (n)[c]	% Linker Cell Completely Gone (n)
Early L4	100(15)	0(15)	0(15)	0(15)
Late L4 Rays Visible	0(30)	10(30)	73(30)	17(30)
0-2-Hr-Old Adult	0(30)	23(30)	33(30)	43(30)
2-4-Hr-Old Adult	3(30)	13(30)	23(30)	60(30)
4-6-Hr-Old Adult	0(30)	20(30)	13(30)	67(30)
6-8-Hr-Old Adult	0(30)	3(30)	20(30)	77(30)

n, number of animals scored.
Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. In this population study each animal was scored once at one timepoint.
[a] A healthy linker cell morphology was scored if the linker cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example.
[b] An early abnormal linker cell corpse was recorded if the linker cell had lost substantial volume or started large scale blebbing or had nuclear envelope abnormalities such as abnormal indented (or crenellated) nuclear envelope shape or signs of nuclear envelope breakdown. See Figure 2.3B or 2.3C for an example.
[c] A late abnormal linker cell corpse was recorded if the linker cell had lost substantial volume and had assumed a completely round shape. Such linker cell corpses invariably had major nuclear abnormalities and in many cases the nucleus had completely broken down. See Figure 2.3D or 2.4E for an example.

Table 4.4 Autophagy, Necrosis, or Wallerian Degeneration Mutants Do Not Block Linker Cell Death

Genotype	% Linker Cell Survival (n)[a]
bec-1(ok700)	0(20)
unc-51(e369)	0(19)
clp-1(RNAi)	0(22)
tra-3(RNAi)	0(22)
asp-3(RNAi)	0(23)
asp-4(RNAi)	0(23)
Wld^s Expression[b]	0(60)

n, number of animals scored.
Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology.
[a] Animals were scored 2–4 hr after the L4 to adult molt, apart from the *bec-1* animals, which die at late L4, and were scored just around the molt to adult.
[b] Expression driven from the *mig-24* promoter, which is only expressed in the linker cell; results are combined data from three independent transgenic lines observed.

A

Linker Cell Stage	No. LGG-1 puncta (n)
Migrating	3.8 ± 2.8 (8)
Starting to be Engulfed	6.7 ± 2.1 (9)
Corpse	6.8 ± 4.5 (8)

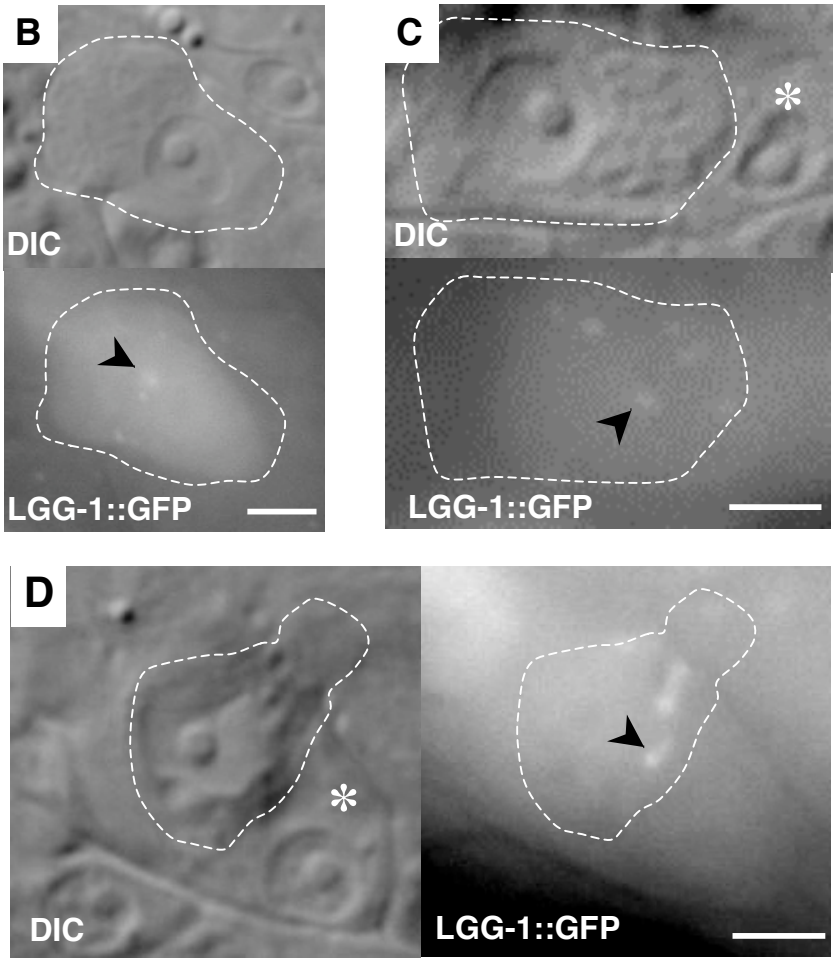


Figure 4.2 Linker Cell Death Does Not Appear to Be Autophagic
(A) Table showing the number of LGG-1::GFP puncta at different stages of linker cell development. n, number of animals scored. Mean ± SD
(B) A migrating linker cell (outlined) expressing a transgene consisting of the *lgg-1* promoter driving expression of a LGG-1::GFP fusion protein. Top, DIC image. Bottom, fluorescence image. Autophagosomal puncta marked with an arrowhead. Scale bar, 2 μm.
(C) A linker cell (outlined) beginning to be engulfed by U.l/rp (asterisk) in a late L4 stage animal expressing LGG1::GFP. Top, DIC image. Bottom, fluorescence image capturing most puncta (arrowhead) marking autophagosomes. Scale bar, 2 μm.
(D) Same as (C), except in a more completely engulfed linker cell. Asterisk marks U.l/rp. Scale bar, 2 μm.

membranes (Melendez et al., 2003; Roudier et al., 2005). LGG-1::GFP puncta could be seen in migrating linker cells; however, their abundance only increased by about 1.5-fold as the cells proceeded to die (Figure 4.2).

The vacuoles characteristic of necrosis that we could observe during linker cell death raised the question whether necrotic genes could be playing a role in linker cell death, even though in general necrosis is not considered a programmed cell death. However, reducing the functions of the proteases *clp-1*, *tra-3*, *asp-3*, or *asp-4*, which promote the necrotic morphology of *C. elegans* neurons expressing the constitutively open DEG/ENaC channel MEC-4 (Syntichaki et al., 2002; Xu et al., 2000), did not affect linker cell death (Table 4.4), suggesting that the program promoting linker cell death differs, at least in some respects, from MEC-4-induced necrosis.

It is of note that some of the conserved morphological features characterizing linker cell death, such as mitochondrial swelling and clustering, and the appearance of “empty” cytoplasmic membrane-bound structures, are also seen in Wallerian degeneration of axons (Griffin et al., 1996; Raff et al., 2002; Vial, 1958; Webster, 1962). This caspase-independent degenerative program (Finn et al., 2000) occurs after axon transection, and may be used for normal pruning that takes place in the developing nervous system. Thus, molecular components promoting this form of cytoplasmic degeneration may be similar to those promoting linker cell death. However, expression of the Wallerian degeneration inhibitor gene Wld^{s} (Mack et al., 2001) in the linker cell failed to

prevent linker cell death (Table 4.4). This suggests that linker cell death employs a program distinct, at least in part, from Wallerian degeneration, although we were unable to monitor directly the levels of Wld^s expression in this experiment.

CONCLUSIONS

Linker cell death appears to be a caspase-independent programmed cell death, since it can occur in mutant or RNAi backgrounds for *ced-3* or the other *C. elegans* caspases, and it can also occur even when a broad-spectrum caspase inhibitor is expressed. Linker cell death can still occur in backgrounds mutant for other core intrinsic apoptotic genes, and in mutants for the processes of autophagy, necrosis, and Wallerian degeneration.

Chapter Five

Linker Cell Death Requires Developmental Timing Genes

BACKGROUND

Timing of Cell Death

In 1962, Saunders and colleagues were studying the chick wing bud, and using grafting experiments they showed that even when cells were excised and moved elsewhere, regardless of the developmental age of the new host, the cells died on their own intrinsic schedule as if they had remained at their original location. Saunders described this process as the setting of the “death clock” (Saunders and Gasseling, 1962). This experiment suggests that in some contexts, cells may have intrinsic regulation determining when they die. However, very few examples have currently been unraveled of the mechanism that regulates when a cell will undergo programmed cell death during development (Maurer et al., 2007).

Temporal Control of Development

Temporal control of development is just as important as spatial control of development, and mutations affecting temporal patterns of development are a possible means of driving evolutionary change (Slack and Ruvkun, 1997).

Developmental timing mutants are also known as heterochronic mutants. In the nineteenth century, Ernst Haeckel proposed that the term heterochrony could be used to describe examples where developmental events appeared displaced from their expected time. A classic example of heterochrony is the Mexican Axolotl. This adult aquatic salamander resembles a salamander larvae in its somatic tissue, which remains in the gilled aquatic form, however, its germline reaches normal adult maturity. A simple model for how this creature reaches sexual maturity without undergoing metamorphosis to the land dwelling form is that the development of its somatic tissue is retarded, perhaps by altered expression of a steroid hormone (Slack and Ruvkun, 1997). It is known that some salamanders can be converted to the air breathing form by treatment with thyroid hormone (Huxley, 1920).

Genetic Studies of Heterochrony in *C. elegans*

The first genetic mutations in heterochronic genes were found in *C. elegans* in 1984 (Ambros and Horvitz, 1984). The fixed lineage and stereotyped development of *C. elegans,* in which characteristic cell divisions and cell fates are reproducibly associated with particular larval stages, makes this model system eminently suitable for investigation of this phenomenon. In developmental timing mutants, a subset of cells show cell fate transformations to cell fates characteristic of earlier or later times in development relative to other cells in the organism. The key point is that the change is relative to other cells or tissues in

the organism, which develop normally on cue. Heterochronic mutants in *C. elegans* do not block progression through each of the larval molts, which occur normally. A worm raised at 25 degrees will develop faster than one raised at 20 degrees. However, the animal at 25 degrees does not show a heterochronic phenotype because all the cells develop together at the same speed.

One classic example of developmental timing in *C. elegans* is regulation of the seam cells, which secrete the cuticle. Seam cells normally undergo rounds of cell division until the end of the L4 larval stage when they stop dividing, fuse together, and secrete an adult specific cuticle known as the alae. In heterochronic mutants, the seam cells can show either precocious or retarded development. In a precocious mutant the seam cells fuse together at an earlier larval stage than normal, and in a retarded mutant they undergo an extra round of cell division instead of fusing together. Both precocious and retarded developmental cell fate transformations can be observed for gain-of-function and loss-of-function mutants of the same heterochronic genes, for example, a *lin-14* gain-of-function mutant causes retarded seam cell development, a loss-of-function mutant in the same gene causes precocious seam cell development (Ambros and Horvitz, 1984). Not all heterochronic mutations govern cell division events, *lin-14* controls a neuronal rewiring event that occurs around the end of the L1 stage (Hallam and Jin, 1998).

The first *C. elegans* heterochronic mutants were identified as cell lineage mutants that affected egg laying behavior—*lin-14*, *lin-28*, and *lin-29* (Ambros and

Horvitz, 1984). These mutants showed many lineage defects including in the seam cells, however, they still reached adult maturity normally, the gonad developed and the first fertilized oocytes appeared on schedule (Ambros and Horvitz, 1984). To date, heterochronic mutants have not been identified in *C. elegans* that block the normal time course of gonadal development. A number of genes affecting heterochronic development in *C. elegans* have been identified and their activities ordered based on genetic or expression studies data.

Drosophila and Heterochrony

Genes equivalent to the heterochronic genes in *C. elegans* that affect cell lineage in development have not been identified in *Drosophila*. However, one study of *Drosophila* development showed changes in the timing of hairy expression in *D. melanogaster*, *D. simulans*, and *D. pseudoobscura,* which may be indicative of a heterochronic change between these species (Kim et al., 2000). The main genes identified that affect the normal progression of development in *Drosophila* have come from studies on the effect of ecdysone pulses on the progression of development (Thummel et al., 2001). For example, the E93 gene is responsive to ecdysone and required for larval salivary gland death (Lee et al., 2000). The *C. elegans* genome does not have an ecdysone receptor homolog, however, the *C. elegans* genome encodes 270 nuclear receptors, the vast majority of which are orphan receptors (Sluder and Maina, 2001).

C. elegans Heterochronic Genes Regulating the L4 to Adult Transition

Several heterochronic genes have been identified that affect the L4 to adult transition: *let-7*, a highly conserved (Pasquinelli et al., 2000) 21-nucleotide microRNA (Reinhart et al., 2000); *lin-41* an RBCC gene (Slack et al., 2000); *lin-29,* a zinc finger transcription factor (Ambros and Horvitz, 1984), and *dre-1*, an F box protein that may act as part of an SCF ubiquitin ligase complex (Fielenbach et al., 2007). From epistasis analysis, *lin-29* is the most downstream gene in the heterochronic pathway that acts at the L4 to adult transition. Its activity is held in check by *lin-41,* which may act by RNA binding or by protein degradation (Slack et al., 2000), and also by *dre-1*, which may act by targeting *lin-29* for protein degradation (Fielenbach et al., 2007). The microRNA *let-7* appears to negatively regulate the expression of *lin-41* through binding to the *lin-41* 3'UTR (Reinhart et al., 2000; Slack et al., 2000).

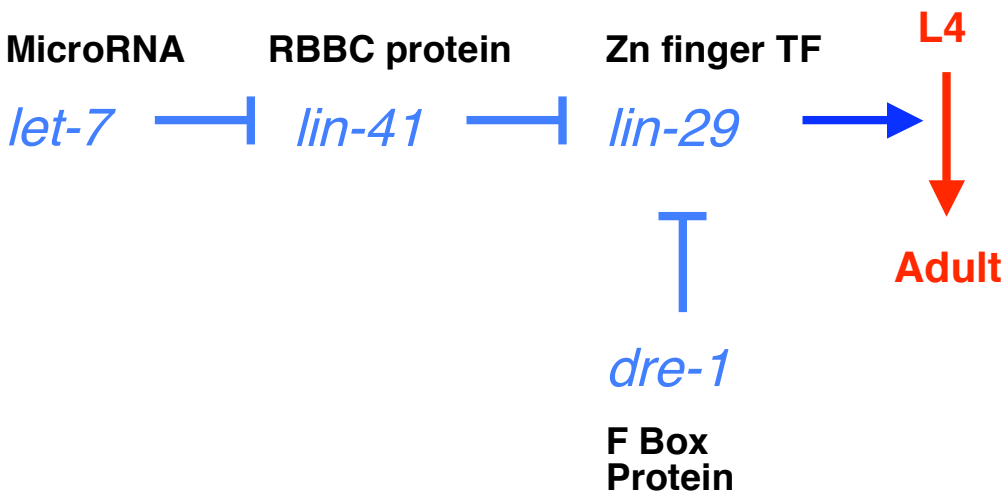


Figure 5.1 *C. elegans* Pathway of Heterochronic Genes Acting at the L4 to Adult Transition

lin-29

LIN-29 is required for the transition from L4 to adult. *lin-29* loss-of-function mutants show retarded development, in which even though the animal has completed the molt to adult and has formed adult-specific structures, some cells show cell fates associated with earlier larval stages. For example, *lin-29* is required for terminal differentiation of the hypodermal seam cells (Rougvie and Ambros, 1995) and has been shown to be required for vulval differentiation (Bettinger et al., 1997; Newman et al., 2000). *lin-29* is a zinc finger transcription factor belonging to the Cys2His2 family (Rougvie and Ambros, 1995). The only known transcriptional target of *lin-29* that has been demonstrated experimentally is a collagen gene, *col-19* (Rougvie and Ambros, 1995). Antibody studies of LIN-29 expression showed that it was expressed in seam cells, hypodermal nuclei in the head, in vulval cells, in ventral nerve cord nuclei, in the linker cell, probably in VA and DA motor neurons, and in B cell progeny that give rise to the male mating structures known as spicules (Bettinger et al., 1997; Euling et al., 1999).

RESULTS

lin-29 is Required For Linker Cell Death

Linker cell death occurs at a specific place and time in developing *C. elegans* males. Given that spatial cues may only partially regulate linker cell death because we could observe linker cell death in a linker cell migration mutant

background (Table 3.2; Figure 3.2C; Figure 3.2D), we considered whether developmental timing genes might be used to control the initiation of linker cell death. We took a candidate approach, and since it had been previously reported that *lin-29* was expressed in the linker cell (Euling et al., 1999), we decided to investigate this gene. We examined linker cell survival in *lin-29(n333)* mutant males, and found that linker cell death was blocked in about half of these animals, even though the animals undergo a normal transition from L4 to adult and make adult specific structures including the spicules. Specifically, 16/30 *lin-29(n333)* males that we scored possessed a healthy looking linker cell at its normal position within 2 hr of the transition to the adult, and 12/30 animals that we scored possessed a healthy linker cell 4-8 hr after the transition (Table 5.1; Figure 5.2). Even after 24 hr, 7/20 linker cells still survived in *lin-29(n333)* animals. Similar effects were seen in *lin-29(n836)* males, and in males in which *lin-29* had been inactivated by RNAi (Table 5.1).

Several observations suggest that *lin-29* does not function as a general regulator of linker cell fate. In *lin-29(n333)* mutants the linker cell displays normal morphology, expresses the *lag-2* and *mig-24* transgenes appropriately, and can lead gonad migration. Although migration of the linker cell is defective in about 30% of *lin-29* animals (Euling et al., 1999), the linker cell death defect is still evident in normally migrating *lin-29* mutant linker cells (see Table 5.1 footnotes). Thus, *lin-29* is likely to have specific roles during linker cell development including, perhaps, transcription of genes promoting linker cell death.

Table 5.1. Role of Developmental Timing Genes in Linker Cell Death

Genotype[a]	% Early Linker Cell Death in L3/L4 Animals (n)	% Linker Cell Survival in 0-2-Hr-Old Adults(n)[b]	% Linker Cell Survival in 4-8-Hr-Old Adults(n)	No. Extra Cells Anterior Pharynx (n)[c]
Wild-type	0(30)	0(30)	0(30)	0.2±0.4(15)
Empty Vector (RNAi)	ND	0(30)	0(30)	ND
Wild-type (25°C)	ND	0(30)	0(30)	ND
lin-29(n333)[d]	ND	53(30)	40(30)	0.3±0.5(12)
lin-29(n836)[d]	ND	76(21)	54(13)	0.2±0.4(10)
lin-29 (RNAi)[d]	ND ND	50(30) 52(27)	24(29) 48(29)	0.2±0.6(15) 0.2±0.4(15)
*let-7(n2853*ts*)*[e]	ND	20(30)	17(30)	0.2±0.4(15)
lin-41(n2914)	3(179)	ND	ND	ND
lin-41(RNAi)	2(56)	ND	ND	ND
lin-42(ve11)	0(45)	ND	ND	ND

n, number of animals scored. ND, not determined.

[a] Males containing a genomically integrated *lag-2*::GFP reporter transgene were scored by DIC and fluorescence microscopy to assess linker cell morphology. All strains described also contained either the *him-5(e1467)* or *him-8(e1489)* mutations for high incidence of males. In this population study, each animal was scored once at one timepoint.

[b] Linker cell survival was scored if a healthy linker cell morphology was visible and the cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example of a healthy linker cell.

[c] Number of extra cells in the anterior pharynx of males of indicated genotype was assessed as described in the Materials and Methods section. Mean ± SD.

[d] In about 30% of *lin-29* mutants, the linker cell exhibited migration defects. We only scored males in which the linker cell reached U.l/rp on schedule.

[e] *let-7* animals were scored at 25°C.

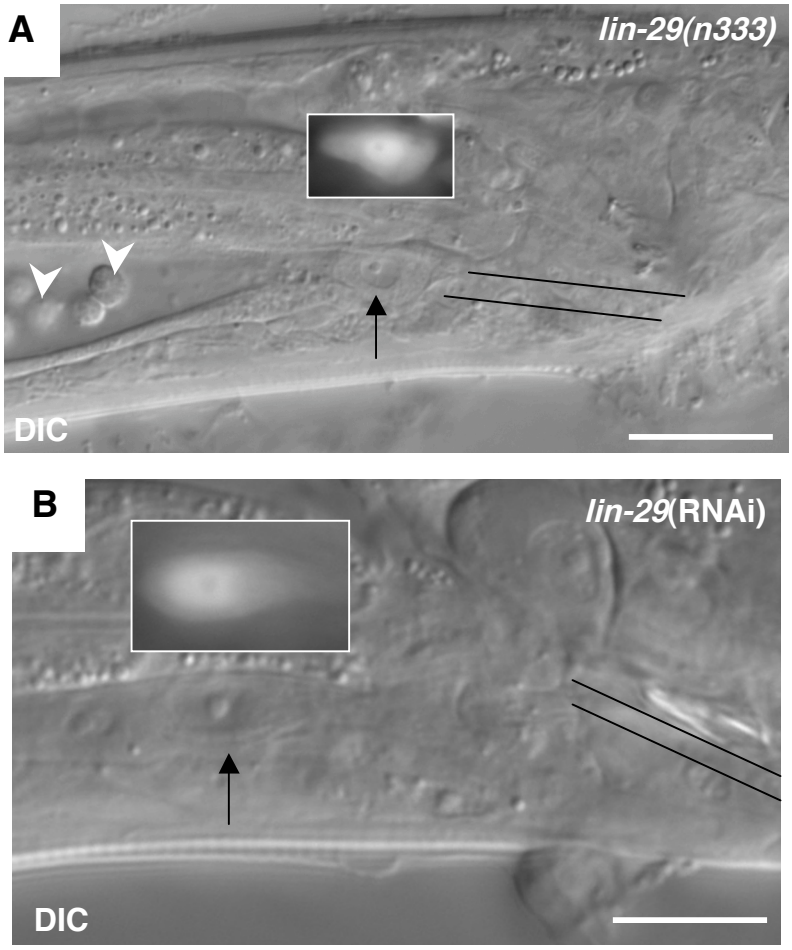


Figure 5.2 The Linker Cell Survives in a *lin-29* Mutant
(A) A surviving linker cell in an 8-hr-old *lin-29(n333)* mutant adult. The linker cell (arrow) is marked with a *lag-2* promoter::GFP reporter (see inset). Sperm cells (arrowheads) are visible in the *vas deferens*. Anterior, left. Dorsal, top. Cloaca outlined in black. Scale bar, 10 µm.
(B) A surviving linker cell (arrow) in a 6-hr-old *lin-29* RNAi treated adult. The linker cell is marked with a *lag-2* promoter::GFP reporter (see inset). Anterior, left. Dorsal, top. Cloaca outlined in black. Scale bar, 10 µm.

lin-29 is Expressed in the Linker Cell

To determine whether *lin-29* was required in the linker cell or in the engulfing cell to promote linker cell death, we examined GFP localization in males carrying a transgene containing 3.5 kb of *lin-29* promoter sequences fused to GFP. This transgene was strongly expressed in the linker cell and was not observed in any neighboring cells, including the U.l/rp cells (Figure 5.3A) consistent with previous studies of LIN-29 protein localization (Euling et al., 1999). We could also see expression in one unidentified neuron nearby. The linker cell expression of *lin-29* could be seen in early L3/L4 animals (Figure 5.3B), suggesting that *lin-29* was expressed earlier than the time of linker cell death, again consistent with previously published antibody staining (Euling et al., 1999). However, as the transgenic marker is a promoter driving GFP, the expression pattern may not mimic the protein expression *in vivo*, since *lin-29* may be regulated at the protein level (Slack et al., 2000; Fielenbach et al., 2007). These experiments suggest that *lin-29* may function within the linker cell to promote cell death, and support the model that at least part of the program leading to linker cell death is cell autonomous.

lin-29 is Required Cell Autonomously For Linker Cell Death

To try to determine the site of action of *lin-29* for linker cell death, we examined rescue of inappropriate linker cell survival in *lin-29(n836)* mutants carrying an

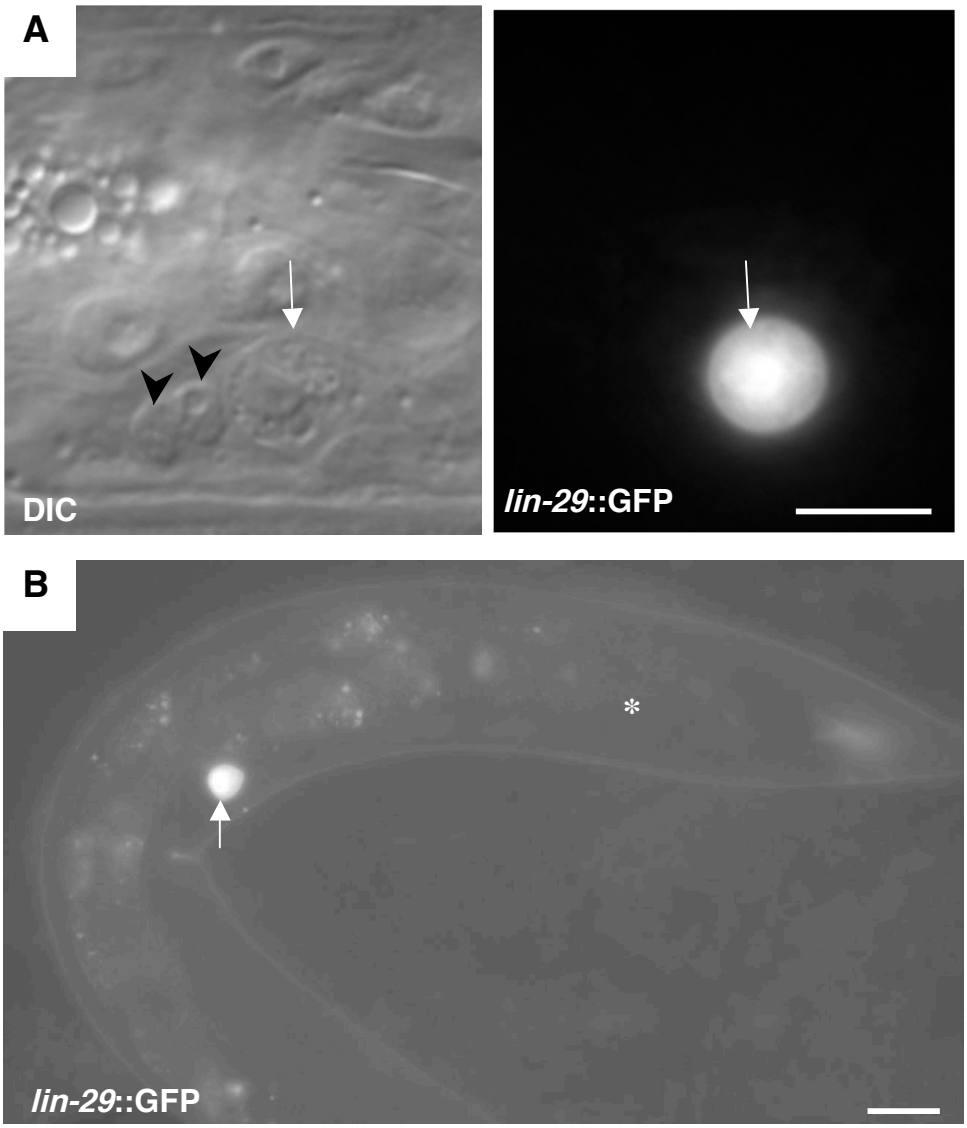


Figure 5.3 *lin-29* is Expressed in the Linker Cell
(A) A transgene containing a 3.5 kb *lin-29* promoter fragment fused to GFP is expressed in the linker cell (arrow) at the L4 stage, but not in neighboring cells. Left, DIC image. Right, fluorescence image. Arrowheads indicate engulfing U.l/rp nuclei. Scale bar, 5 µm.
(B) A transgene containing a 3.5 kb *lin-29* promoter fragment fused to GFP is expressed in the linker cell (arrow) at the L3/L4 stage when the linker cell is still migrating. An asterisk indicates the cloacal region where the linker cell will die. Anterior, left. Dorsal, top. Scale bar, 5 µm.

unstably transmitted extrachromosomal array of the wild-type *lin-29* gene. As expected, the linker cell died in 19/20 animals in which both the linker cell and the surrounding cells (including U.l/rp) received the wild-type *lin-29* gene. Similarly, the linker cell died in 9/10 animals in which the linker cell received the wild-type *lin-29* gene but surrounding cells did not, supporting the idea that *lin-29* functions cell autonomously to regulate linker cell death. In agreement with this, the linker cell survived in 14/19 animals in which neither the linker cell nor the surrounding cells received the wild-type *lin-29* gene, and in 5/10 animals in which the linker cell lacked the wild-type *lin-29* gene but the surrounding cells had the gene (Figure 5.4).

lin-29 Linker Cell Survivors Are Not Engulfed by U.l/rp

We also found that U.l/rp cells failed to extend phagocytic processes around the linker cell when the linker cell inappropriately survived in adult *lin-29* mutant males (Figure 5.5), even though U.l/rp still properly elongated. This suggests either that *lin-29* also regulates linker cell engulfment, or that initiation of *lin-29*-dependent linker cell death is a prerequisite for engulfment to occur.

let-7 Regulates Linker Cell Death

To determine whether *lin-29* functions within the linker cell in the context of the developmental timing program, we examined linker cell death in a *let-7(n2853)* temperature-sensitive mutant. As shown in Table 5.1, at 25°C the linker cell

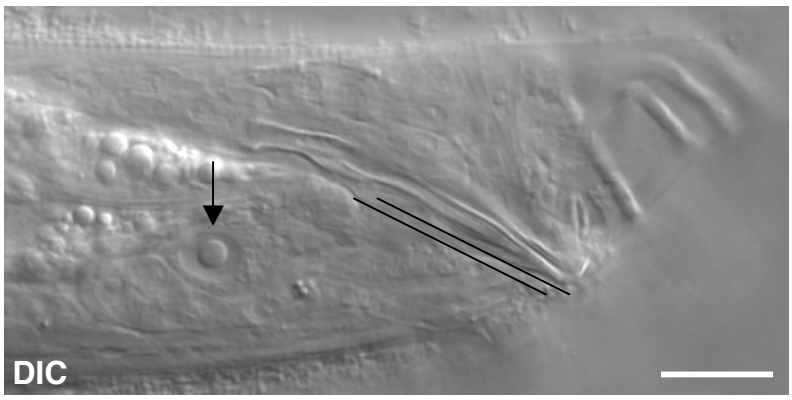


Figure 5.4 *lin-29* is Required Cell Autonomously For Linker Cell Death
A surviving linker cell (arrow) in an adult mosaic animal that lacks *lin-29* in the linker cell (hence the prominent nucleolus), but has *lin-29* in neighboring cells (which have normal nucleoli). Anterior, left. Dorsal, top. Cloaca outlined in black. Scale bar, 10 µm.

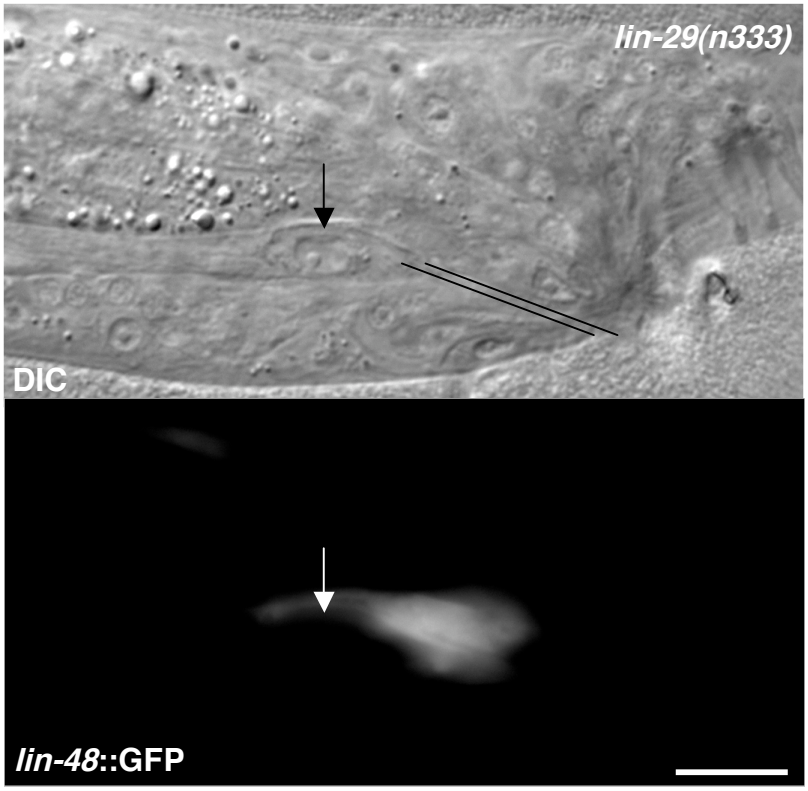


Figure 5.5 A *lin-29* Surviving Linker Cell is Not Engulfed
A surviving linker cell (arrow) in a *lin-29(n333)* adult male is not engulfed by the U.l/rp cell. DIC (top) and fluorescence (bottom) images. The engulfing cell is marked with a *lin-48* promoter::GFP transgene. Note that obvious extensions encircling the linker cell are absent. Anterior, left. Dorsal, top. Cloaca outlined in black. Scale bar, 10 µm.

inappropriately survived in these mutants. The extent of survival was lower than in *lin-29* mutants, presumably because *let-7(n2853)* mutants still retain some *let-7* function. This result supports the idea that linker cell death is regulated by components of the *C. elegans* developmental timing pathway.

Precocious Heterochronic Mutants Do Not Cause Precocious Linker Cell Death

lin-41 negatively regulates *lin-29*, and a *lin-41* loss-of-function mutant has a phenotype of precocious development. We therefore examined *lin-41* genetic mutants or animals subjected to RNAi against *lin-41* for precocious programmed cell death. We could not see any significant evidence for linker cell death prior to the normal time (Table 5.1). Another gene associated with a precocious phenotype is *lin-42,* a homolog of the period family of circadian rhythm genes, which can affect gonadal migration in the hermaphrodite and some stage-specific cell division events such as those of the seam cells (Abrahante et al., 1998; Tennessen et al., 2006). We could not see any evidence for precocious linker cell death in a *lin-42* mutant background (Table 5.1).

A *lin-41* mutant has been reported to cause precocious expression of LIN-29 (Slack et al., 2000). We do not know if the mutants tested here cause precocious *lin-29* expression in the linker cell. If *lin-29* expression is sufficient to drive linker cell death, precocious expression of *lin-29* should drive precocious linker cell death. If *lin-29* was precociously expressed in the linker cell in these

backgrounds, then it was not sufficient to drive linker cell death. This may not be surprising, considering the fact that many cells that express *lin-29* do not die. Either these cells all have some activity that represses the action of *lin-29*, or *lin-29* is required along with some other factor to cause cell death.

lin-29 Does Not Affect Embryonic Apoptotic Programmed Cell Death

As *lin-29* acts at the L4 to adult transition, it seemed unlikely that the gene would be required for embryonic apoptotic programmed cell death, however, we did check if it had a role by determining whether there were extra surviving cells in the anterior pharynx. *lin-29* did not have any effect on embryonic programmed cell death using this assay (Table 5.1).

CONCLUSIONS

We have shown that the zinc finger transcription factor *lin-29* is required in a cell-autonomous fashion for linker cell death. In *lin-29* mutants approximately half of the linker cells survive in adult worms, and these surviving linker cells have the morphology of a healthy linker cell and are not engulfed. We have shown that *lin-29* is expressed in the linker cell and not in the engulfing cell. *let-7* is also required for linker cell death. *lin-29* is probably required but not sufficient for linker cell death, since other cells that express *lin-29* do not die*,* and removing *lin-41,* which may cause precocious expression of *lin-29*, did not cause precocious linker cell death.

Chapter Six

Screening to Identify New Linker Cell Death Genes

BACKGROUND

Visual Screens to Find Linker Cell Death Genes

Using a transgenic strain with a GFP marked linker cell we can carry out a visual screen, with an epifluorescent dissecting microscope, to identify mutants with defects in linker cell death in which the cell does not undergo programmed cell death at the late L4 stage, but instead survives and is present in adult males. Adult males can be easily distinguished from L4 larvae because the adult male tail has a distinctive fan structure. In such a screen we could anticipate identifying mutants that prevent the initiation or execution of linker cell death, resulting in a healthy linker cell surviving into the adult. We might also identify mutants that affect more downstream events in the death of the cell, such as efficient linker cell corpse clearance.

Chemical Mutagenesis

One of the necessary triumphs of *C. elegans* as a model system is the ease with which one can create and study genetic mutants of a chosen biological pathway (Brenner, 1974). Ethylmethanesulfonate (EMS) is a chemical mutagen that

introduces point mutations or small deletions in DNA. At standard concentrations of use for *C. elegans* mutant screens it generates an average mutation frequency of 5×10^{-4} per gene (Anderson, 1995). Therefore, in a standard EMS screen in *C. elegans*, any given gene is theoretically predicted to be mutagenized once per 2,000 genomes screened. Once a mutant in a given process has been isolated, the nature and the location of the genetic lesion is typically identified by mapping techniques such as standard genetic two or three factor crosses or single nucleotide polymorphism mapping (Wicks et al., 2001). When the region of the mutation has been sufficiently narrowed down by mapping, the final stage of mutant identification usually comes from cosmid rescue experiments followed by sequencing of candidate genes. Determining the location of an unknown genetic mutation can take several weeks or even months.

RNAi

An alternative approach to chemical mutagenesis in *C. elegans* is the use of RNAi to knockdown gene expression. This technique avoids the necessity of mapping mutations, since the identity is already known of the gene used to produce the double-stranded RNA in any given experiment.

Identification of RNAi in *C. elegans*

In 1998, Fire and colleagues described the discovery of RNAi, a phenomenon in which introduction of exogenous double-stranded RNA results in specific targeted

depletion of endogenous mRNA which has the same sequence (Fire et al., 1998). The phenomenon was shown to have a systemic spreading effect; introduction of double-stranded RNA to one part of the animal could produce a potent effect in other tissues (Fire et al., 1998). Some genes facilitating systemic RNAi in *C. elegans* have been identified including *sid-1* (Tijsterman et al., 2004), which encodes a transmembrane receptor that enables cellular uptake of double-stranded RNA (Winston et al., 2002; Feinberg et al., 2003).

RNAi as a Widespread Phenomenon

RNAi is not unique to worms, it can occur in many organisms and has been shown to act on mammalian cells (Elbashir et al., 2001a). In the nine years since the discovery of RNAi, many aspects of how the mechanism works have been determined. The long double-stranded RNAi is cleaved by an enzyme known as Dicer into smaller 21-25 double-stranded RNAs, also known as small interfering RNAs (siRNAs) (Hutvagner et al., 2001; Bernstein et al., 2001; Elbashir et al., 2001b). Secondary siRNAs, created by an RNA-directed RNA polymerase, may contribute to the response (Pak et al., 2007; Sijen et al., 2007). siRNAs are bound by the RISC complex (Hammond et al., 2000), which contains members of the Argonaute family (Tabara et al., 1999), whose role is cleavage of the endogenous mRNAs being targeted by the siRNAs (Liu et al., 2004; Song et al., 2004).

It has been suggested that RNAi may represent an endogenous mechanism to silence transposons or viruses, both of which can potentially create double-stranded RNA, which is not a normal cellular product (Tabara et al., 1999; Ketting et al., 1999; Li et al., 2002). It has been shown in the *C. elegans* germline that a Tc1 transposon is normally silenced in a process that depends on RNAi (Sijen and Plasterk, 2003).

Practicalities of Using RNAi in *C. elegans*

Germline injection of double-stranded RNA was the technique first used to carry out RNAi in *C. elegans* (Fire et al., 1998). This method of RNA introduction is very labor intensive, however, it was discovered that *C. elegans* could ingest bacteria expressing double-stranded RNA under an IPTG inducible promoter, resulting in a potent RNAi effect (Timmons and Fire, 1998). A commercially available bacterial RNAi feeding library for *C. elegans* has been constructed with 16,757 clones, which offers 86% coverage of the predicted total genes in the *C. elegans* genome (Kamath et al., 2003). Some missing additional bacterial RNAi clones have been generated by another group, which brings the percentage of the *C. elegans* genome that can be targeted by RNAi up to 94% (Kim et al., 2005).

A study of the systemic effectiveness of RNAi to target all tissues, by observing loss of GFP expression in a strain expressing a transgenic somatic GFP reporter, revealed effective knockdown in all tissue types, although neuronal

cells appeared to be less effectively targeted (Kamath et al., 2001). The gene *eri-1*, may act as a negative regulator of RNAi in neurons (Kennedy et al., 2004). A mutation in *rrf-3*, a gene encoding an RNA-directed RNA polymerase, has also been shown to enhance general sensitivity to RNAi, including in the neurons (Simmer et al., 2002), and a *lin-35* mutant background has a similar RNAi enhancement phenotype (Lehner et al., 2006).

RNAi appears to be able to give a functional gene knockdown that can be of very high efficiency. In a study of 14 known maternal effect embryonic lethal genes, 13 of the RNAi clones showed 97 to 100% embryonic lethality and one clone showed 55% lethality (Kamath et al., 2001). A genome-wide screen using the RNAi feeding library on wild-type animals and scoring major categories of visible defects such as embryonic lethality, sterility, growth or motility defects, revealed phenotypes for 1,722 clones (10% of the total clones analyzed) (Kamath et al., 2003). 323 known loss-of-function mutations had a phenotype that should have been detected in this screen, 205 (63.5%) of the corresponding bacterial RNAi clones produced the same phenotype (Kamath et al., 2003). Some of the bacterial clones that did not reproduce the mutant phenotype may have been neuronal genes, which are more difficult to target by RNAi.

RESULTS

An EMS Screen to Isolate Linker Cell Death Mutants

Using a transgenic strain with a GFP marked linker cell we can carry out a visual screen using an epifluorescent dissecting microscope to identify mutants in which the linker cell does not undergo its normal programmed cell death at the late L4 stage, but instead survives and is present in adult males. Because an adult male with a surviving linker cell would presumably be sterile due to linker cell obstruction of sperm exit from the *vas deferens* (Figure 2.2C), any male mutants identified with an inappropriately surviving linker cell could not be used to propagate the mutation. Therefore the only way to recover such a mutation is if hermaphrodite siblings of the male can be isolated. One way to do this is to carry out an F1 clonal screen in which P_o parents are mutagenized with EMS, their F1 progeny are individually picked to single plates, and the F2 progeny are scored for linker cell survival in the adult male. Each F1 picked clonally has two sets of mutagenized chromosomes. For any given mutation at a particular locus in the F1 parent, only one quarter of the F2 progeny will be homozygous for the mutation and therefore should show a phenotype if it is a recessive mutation. If any plate of F2 worms shows a linker cell survivor phenotype for adult males, the hermaphrodite siblings on the same plate could be used to homozygose and propagate the mutation.

Pilot EMS Screen Results

A standard EMS mutagenesis (Sulston et al., 1988) was carried out on a strain with a *him* mutation for high incidence of males and a genomically integrated *lag-2*::GFP marker for the linker cell. A pilot F1 clonal screen was performed. In four rounds of mutagenesis, 1,100 mutagenized F1s were picked to individual plates and their F2 progeny scored, representing 2,200 mutagenized genomes.

From this screen we identified 5 mutants with linker cell survival phenotypes, and an image of one of the linker cell survivors identified is shown here (Figure 6.1). These genes all represent a new class of cell death mutants in *C. elegans* as they did not block embryonic apoptotic programmed cell death when scored by assessing survival of neurons in the anterior pharynx at the L4 stage (Table 6.1). However, these mutants appeared to have a low penetrance of linker cell survival, and they have not been mapped or further characterized.

Difficulties With the Pilot EMS Screen

A number of problems arose with the pilot EMS screen. An F1 clonal screen in which F1s have to be picked individually is highly labor intensive compared with a standard screen in which F2 hermaphrodites with a desired phenotype can be recovered directly. In a standard F2 screen, the genome can be screened several times in one week. Apart from the slower rate of progress with an F1 clonal screen, the greatest difficulty arising with this particular screen was the problem

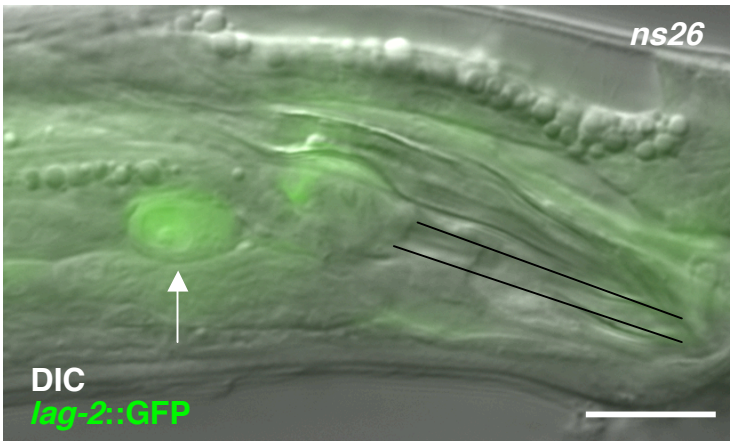


Figure 6.1 Linker Cell Survivor Mutant Identified From Chemical Mutagenesis
A surviving healthy linker cell of normal morphology (arrow) is shown here in an adult male with the *ns26* mutant allele. The linker cell expresses a *lag-2*::GFP transgene. Cloaca outlined in black. Anterior, left. Dorsal, top. Scale bar, 10 μm.

Table 6.1 Linker Cell Survival Mutants Do Not Block Embryonic Apoptotic Programmed Cell Death

Genotype[a]	No. Extra Cells in Anterior Pharynx(n)[b]
Wild-type	0.2±0.4(15)
ns26	0.1±0.3(10)
ns28	0.3±0.7(10)
ns33	0.1±0.3(10)
ns34	0.1±0.3(10)
ns36	0.1±0.3(10)

n, number of animals scored
[a]All strains described also contained the *him-5(e1467)* mutation for high incidence of males and a genomically integrated *lag-2*::GFP linker cell reporter transgene.
[b] Number of extra cells in the anterior pharynx of males of indicated genotype was assessed as described in the Materials and Methods section. Mean ± SD.

of decreased fertility resulting from the mutagenesis. The F2 plates frequently had insufficient males to confidently score the genome assessed on that particular plate. *C. elegans* hermaphrodites normally have about 300 offspring. Males normally represent less that 0.5% of the population, however this percentage can be increased to around 30% by use of a high incidence of males (*him*) mutation that usually creates more males by affecting the X chromosome during meiosis (Philips et al., 2005). A hermaphrodite with a standard *him* mutation will have roughly 90 male progeny. The *him* mutation itself can sometimes decrease fertility by a few percent, and a similar reduction can also occur due to transgene introduction. Of the F2 plates screened, a high percentage of the plates had a major reduction in fertility, resulting in substantially less than 90 males being present on the plate. For example, if less than 30 F2 males were present on a given plate, at best only one quarter of the worms—8 in total—would be homozygous for any given mutation. If a homozygous recessive mutation had incomplete penetrance, the number of worms with a phenotype would decrease further.

Another efficiency of screening issue was isolation of false positives. Using this visual screen, the goal is to note adult males with a GFP linker cell. Even in wild-type there is a low background level of GFP that can be visible in the adult from a few dead corpses that are still being degraded (Table 2.1). Sometimes these corpses can be distinguished under the dissecting microscope because they have rounded up. However, in the context of a screen, it is not

possible to take the time to carefully inspect each linker cell for signs of rounding—plates are simply scored as positive if they have above background levels of GFP linker cells in adult males. Therefore, the screen can also isolate mutations that affect corpse clearance but do not block initiation of linker cell death. Since the process of engulfment requires a good deal of cytoskeletal reorganization, any mutation that weakly perturbs general cellular or cytoskeletal function is likely to result in more GFP cells being visible in the adult. We saw a number of false positives in which plates were scored as positive for GFP linker cells in the adult, but the phenotype did not repeat in the next generation, and was probably due to minor perturbations in linker cell removal.

Advantages of Using a Genome-wide RNAi Screen

Because of the problems detailed above with the EMS screen, we decided to embark on a genome-wide RNAi screen instead. Since the creation of the genome-wide RNAi feeding library, many groups have already used the complete library to successfully identify new genes required for a range of biological processes as diverse as embryonic development (Labbe et al., 2006) and longevity (Hamilton et al., 2005). As detailed above, this technique has been shown to be capable of efficiently knocking down gene function to provide a similar phenotype to that obtained using a genetic mutant (Kamath et al., 2001; Kamath et al., 2003).

For our purposes, the RNAi screen offered several advantages over the EMS screen. Firstly, as we could add as many worms as we wanted to the RNAi feeding plate, the problem of having insufficient males to score a phenotype with confidence was removed. An RNAi screen would be a much faster way to assess the complete genome, compared with the length of time it would take to complete and map a clonal screen to near saturation. In an RNAi screen we could theoretically score nonessential embryonic lethal genes if we could subject larvae to RNAi and score the animal when it reached the adult stage. Unless an EMS screen was used with a temperature shift to find temperature sensitive mutations, all embryonic lethal genes—approximately 5% of the genome (Kamath et al., 2003)—could not be scored.

In an RNAi screen, the ability to add synchronized worms would improve our screening efficiency. In the EMS screen, F2 progeny are scored that represent a range of ages. Adding synchronized worms would allow adult males to be scored a few hours after the molt. This timepoint would decrease the false positives because the majority of corpse clearance would have occurred (Table 2.1), and it would also decrease the false negatives because scoring older adults is more challenging since they have increased background fluorescence from gut autofluorescence and more GFP expression in additional smaller cells, making the linker cell harder to distinguish.

The Linker Cell Can Be Targeted by RNAi

To determine if the linker cell can be effectively targeted by RNAi, we investigated if the fluorescence in a GFP marked linker cell could be effectively knocked down if the animal was fed RNAi bacteria targeting GFP. We found that GFP could be efficiently knocked down in the linker cell to a level at which no linker cell fluorescence was visible under the dissecting microscope in 18/18 males. We saw this effect in animals that had been added to RNAi feeding plates as L1 worms. Knockdown was apparent when plates were observed 11 hours after animals were added. If animals older than L1s were added, the RNAi appeared much less effective when the same animal was observed at a later stage. Fortunately, it is easy to obtain a large synchronous population of only L1 stage worms—if *C. elegans* embryos are collected by a bleaching procedure and allowed to hatch in the absence of food, all the worms will arrest at the L1 stage (Hope, 1999). We also tested *lin-29* RNAi feeding clones and found that they offered a similar effect on linker cell survival as seen for the *lin-29* genetic mutants, showing that in principle the screen should work (Table 5.1).

U.l/rp Are Less Effectively Targeted by RNAi

Although we know from ablation studies (see Chapter Three) that U.l/rp are not required for linker cell death, that does not mean that they are an uninteresting part of the system and unworthy of exploration. The communication between the morphologically healthy linker cell and the engulfing cell that leads to engulfment

is mysterious and does not use known engulfment genes (See Chapter Three). Therefore, it would be interesting to discover the nature of the signaling between the linker cell and its engulfing cell. To determine if we could target the U.l/rp by RNAi, a *lin-48*::GFP marked linker cell strain was subjected to RNAi against GFP. We found that fluorescence was still visible in U.l/rp under the dissecting microscope. The RNAi against GFP did have some effect in the U.l/rp, as we could see that the GFP was somewhat diminished by comparing images of control and GFP RNAi treated animals taken with the same exposure time under the compound microscope. Since U.l/rp seemed more resistant to RNAi than the linker cell, we decided to conduct the RNAi screen in a strain which included an *rrf-3* mutation that enhances sensitivity to RNAi (Simmer et al., 2002). However, even in this sensitized background, the knockdown of GFP expressed in U.l/rp is still only partial.

New Candidate Regulators of Linker Cell Death

In an equal collaboration with Elyse Blum, we have completed a genome-wide RNAi feeding screen to identify new regulators of linker cell death. Using a strain with an *rrf-3* mutation (Simmer et al., 2002) to enhance RNAi sensitivity, a genomically integrated transgene of *lag-2*::GFP to mark the linker cell, and a *him* mutation to increase the frequency of males, with an epifluorescent dissecting microscope we scored RNAi clones for linker cell survival in the adult. With the feeding libraries available from the Ahringer laboratory (Kamath et al., 2003) and

some additional clones that are available from the Vidal laboratory (Kim et al., 2005), we covered 94% of the genome, which is about 18,000 genes.

The screening procedure used is shown in schematic (Figure 6.2). We added synchronized L1 to plates with bacteria expressing double-stranded RNA and then scored the same animals two days later when they were adults. On each day of RNAi screening we also did positive controls of GFP and *lin-29* RNAi, which always gave a robust and reproducible phenotype. We also noticed that some of the RNAi clones tested had highly penetrant visible phenotypes such as lethality or extreme slow growth, suggesting that, at least for those particular clones, the RNAi procedure was working with high efficiency.

Although by adding L1 worms we could try to bypass problems in missing genes who play an essential role in embryonic development, there were still some clones that we could not assess for a role in linker cell death because they were either lethal or produced a larval growth arrest. For that reason, in the half of the total plates that I screened, 178 clones (approximately 2% of the total) were unscoreable for linker cell death. There were also many clones that were slower growing but not arrested, and these were scored on the next day when they had reached adulthood.

Every well where we thought we could see above background levels of linker cell GFP was recorded, along with a rough estimate of the number of linker cells visible. If the phenotype was striking, we would usually note if the linker cells looked mainly healthy (ovoid) or corpse (round). We had about 100 clones

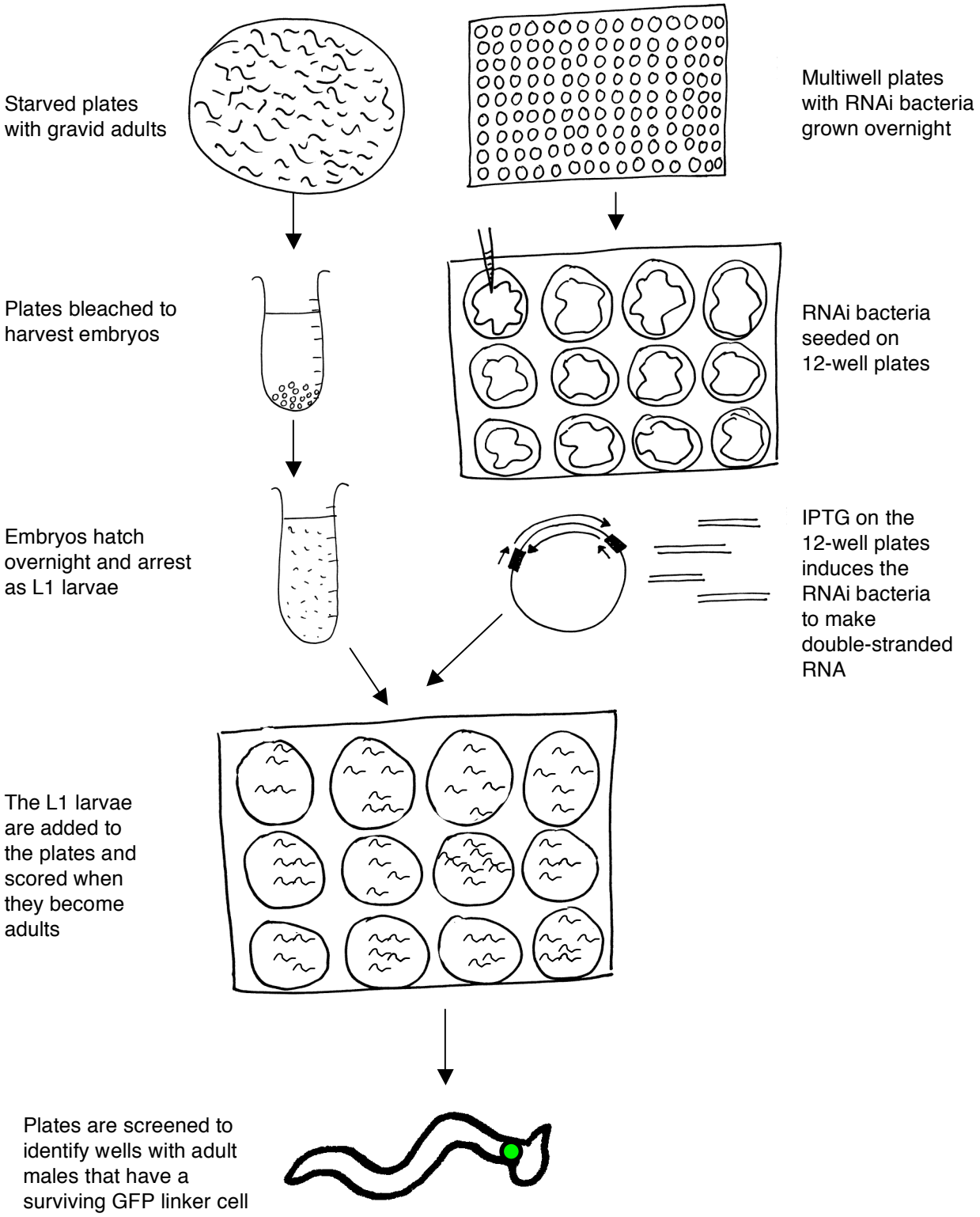


Figure 6.2 Schematic of RNAi Screening Procedure

that had looked most promising as worth retesting. Looking at the molecular identify of the genes that we retested, there was no particularly obvious pattern of any families of genes or genes associated with a particular function that appeared to be overrepresented (See Appendix for a list of genes retested). For each of the clones we wanted to investigate further, we did a first round of retests by repeating the RNAi under the dissecting microscope. This time the clone was assessed in three or four wells to determine if there was a consistent linker cell survival phenotype across each well of a given clone. If there appeared to be a consistent phenotype, the RNAi was repeated again and the next time the animals were used for observation under the compound microscope to determine if these clones were really producing a healthy surviving linker cell or if they were just producing corpse clearance defects. From these retests we have identified 5 clones that had promising levels of healthy linker cell survival under the compound microscope (Figure 6.3; Table 6.2). In 3 out of the 5 clones, the linker cell migrates normally to the cloaca. In the other two clones, the linker cell has a migratory defect and never reaches the cloaca.

pqn-41 is Expressed Specifically in a Dying Linker Cell Corpse

For one of the genes identified in the screen, *pqn-41*, a prion-like Q/N rich gene, a promoter GFP fusion transgene was created to assess its expression pattern. Interestingly the gene is not expressed in migrating linker cells (Figure 6.4A), but only appears to be expressed in linker cell corpses (Figure 6.4C). The expression

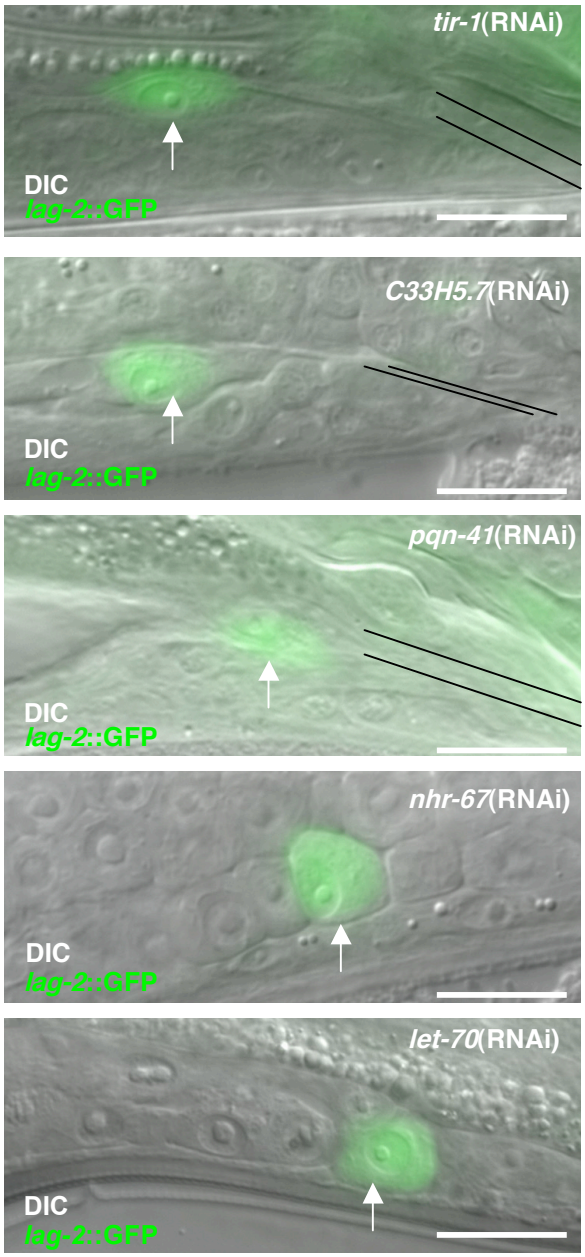


Figure 6.3 Linker Cell Survival in the Adult With Clones Identified From the Genome-wide RNAi Screen

A surviving healthy linker cell of normal morphology (arrow) is shown here in 2-hour-old-adult males subjected to RNAi against the genes indicated. The cloaca is outlined in black. *nhr-67* and *let-70* had migration defects and never reached the cloaca. The linker cell expresses a *lag-2*::GFP transgene. Anterior, left. Dorsal, top. Scale bar, 10 μm.

Table 6.2 Genes Identified in the RNAi Screen That Block Linker Cell Death

Clone Identity	Gene Description	% Linker Cell Survival in 0-3-Hr-Old Adults (n) [a]
Empty RNAi vector		0(30)
tir-1	TIR domain protein	27(30)
pqn-41	Q/N rich protein	20(30)
C33H5.7	Histone H3 lysine methyltransferase	23(30)
nhr-67[b]	Nuclear hormone receptor	67(30)
let-70[b]	Ubiquitin conjugating enzyme	67(30)

n, number of animals scored.
Males containing a genomically integrated *lag-2*::GFP reporter transgene were subjected to RNAi by feeding and scored by DIC and fluorescence microscopy to assess linker cell morphology.
[a] Linker cell survival was scored if a healthy linker cell morphology was visible and the cell was of normal shape and size and had normal nuclear architecture. See Figure 2.3A for an example of a healthy linker cell.
[b] In these clones the linker cell had a migration defect and did not reach U.l/rp.

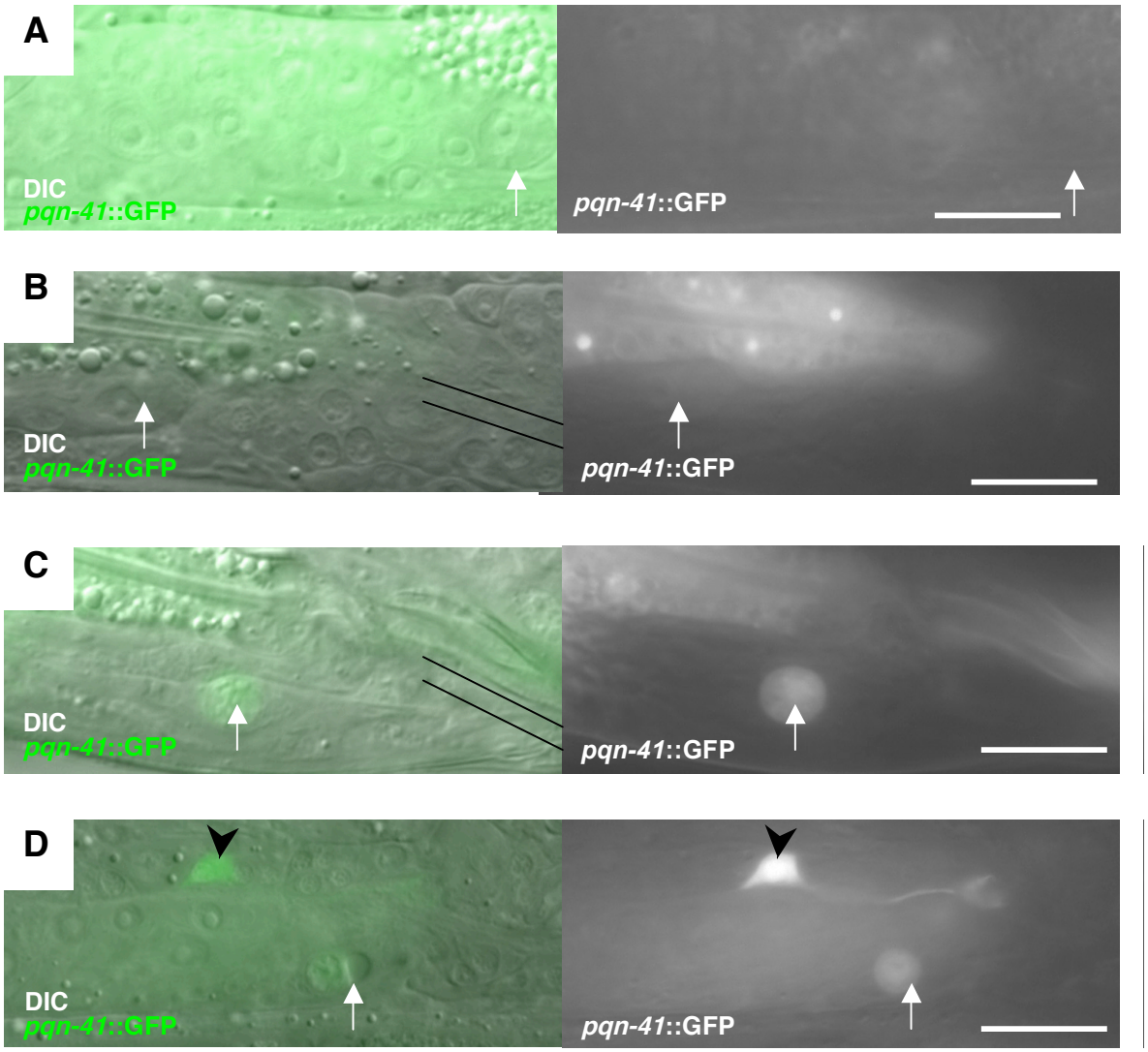


Figure 6.4 *pqn-41* is Expressed in a Dying Linker Cell Corpse
The animals shown all express a 2.5kb promoter of *pqn-41* fused to GFP. Scale bars, 10μm.
(A) In a migrating linker cell (arrow) at the L3 stage, *pqn-41* is not expressed.
(B) At the late L4 stage, as the linker (arrow) cell approaches the cloaca (outlined in black), *pqn-41* begins to be weakly expressed (The linker cell is faintly visible here). Cloaca outlined in black. Anterior, left. Dorsal, top.
(C) In the late L4 stage as the linker cell (arrow) becomes a rounded corpse, *pqn-41* shows stronger expression. (Images B and C taken with similar exposure times.) Anterior, left. Dorsal, top.
(D) *pqn-41* is also expressed in an unidentified neuron (arrowhead) in the male tail. The linker cell corpse (arrow) is also visible in this late L4 worm. Ventral view. Anterior, left.

pattern of this gene appears highly specific in the male, it is only expressed in the linker cell corpse as well as in one neuron in the vicinity of the male tail (Figure 6.4D).

CONCLUSIONS

Using EMS mutagenesis, 5 mutants have been isolated that can block linker cell death with a low penetrance. These mutants do not affect embryonic apoptotic programmed cell death. They have not been mapped or extensively characterized. Following completion of a genome-wide RNAi screen, we have identified and confirmed the role of 5 new genes in regulating linker cell death. The first promoter GFP study that we carried out of one of the genes identified from the RNAi screen, *pqn-41*, showed that it was specifically expressed in the linker cell as it starts to die.

Chapter Seven

Discussion

The Morphology of Linker Cell Death is Non-Apoptotic, but is Conserved in Vertebrates

Our characterization of wild-type linker cell death has revealed that this cell death has many unusual features not previously described for programmed cell death in *C. elegans*. The most striking finding was the observation that by transmission electron microscopy (Figure 2.8), the linker cell does not have an apoptotic morphology such as chromatin compaction, nor an autophagic morphology in which autophagosomal formation is the dominant theme. Instead, the features of linker cell programmed cell death that we observed were most similar to type III PCD (Clarke, 1990) in which the cellular perturbations associated with cell death appear to be initiated in the cytoplasm. The most striking changes we see associated with linker cell death are nuclear crenellation or indentation, formation of small white vesicles—apparently "empty spaces" in the cytoplasm, and formation of 200nm vesicles, of heterogeneous appearance which may represent swollen organelles. The "empty spaces" or white regions, sometimes around which a membrane is visible, might represent swollen endoplasmic reticulum. It is possible that these small white vesicles can connect together to form the large vacuoles associated with linker cell death (Figure 2.7).

The TEM morphological similarities between dying linker cells and the few published examples of type III PCD during normal development in some vertebrates are striking (Figure 2.8; Appendix Figure 4). This suggests that what we are studying is a widespread phenomenon. The fact that some morphologies characteristic of linker cell death could also be seen in dying vertebrate spinal cord motor neurons in a caspase-knockout background lends credence to the model that this cell death, which has a non-apoptotic morphology, is a caspase-independent cell death. No vertebrate genes have been identified to date which are required for type III PCD, the molecular mechanisms of which remain mysterious. The linker cell provides a tractable system to try to unravel the nematode pathway, and it is not an unreasonable hope that the genes and mechanisms uncovered may also be conserved in vertebrates.

What might be the role and significance of type III PCD? Circumstantial evidence is consistent with the existence of an alternative vertebrate developmental cell death program. For example, despite the pervasiveness of programmed cell death during early murine development, none of the mutations known to affect cell death in the mouse block the gross progression of development, and in many cases, mutant mice live to birth or beyond (Honarpour et al., 2000). Furthermore, mutations in caspase-3 and casapse-9, which play important roles in apoptotic cell deaths in mice, do not block motor-neuron death in the developing spinal cord (Oppenheim et al., 2001). In addition, none of the mutations affecting cell death in the mouse seem to affect the total number of

cells in the immune system to an extent that might be predicted given the large numbers of cells eliminated during normal T and B cell development. Finally, it has been previously demonstrated that the death of interdigital cells during murine development can proceed in the absence of Apaf-1, with a necrotic morphology (Chautan et al., 1999). Although all of these examples could be explained by feedback control of cell numbers, and/or redundancy within caspase and other cell death gene families, it is also possible that an entirely different pathway similar to the one regulating linker cell death regulates these cell deaths. If this is the case, this alternative mode of cell death must, therefore, play a major role during normal vertebrate development.

Linker cell death and type III PCD have some features that are reminiscent of necrotic cell death, for example, in the case of the linker cell—vacuolar formation and some organelle swelling. Necrosis is generally thought of as a more primitive and less sophisticated way to die, although it is clearly not simply cellular collapse, but a regulated process that has a pathway of genes required for its execution. As it seems less advanced, necrosis could perhaps represent the ancestral form of programmed cell death that got usurped by the more ruthlessly efficient program of apoptotic cell death. Where kinetics of an alternative type of non-apoptotic programmed death have been reported, the consensus is that it is less efficient than apoptotic programmed cell death. Linker cell death is also slower than embryonic apoptotic programmed cell death in

C. elegans, which can occur in around 30 minutes, while linker cell death takes at least roughly 2 hours to complete.

If type III PCD represents a regulated form of programmed cell death that has evolved from necrotic beginnings, it is easy to imagine advantages and disadvantages for this type of cell death. For example, a cell death that is substantially slower than apoptosis might make it easier for neighboring cells to adapt to the loss of the dying cell—if the dying cell played an important role in the tissue architecture or if the cell was highly connected to neighboring cells, such as a neuron. A slower and a more necrotic style of death might provide a better platform for the immune system to trigger a response, in the case of cell death caused by an outside agent. Retaining this type of cell death could also be a great advantage for places where the normal apoptotic cell death pathway has been blocked: for example, in viral infection if the virus is expressing a form of caspase inhibitor, or in cancer if the apoptotic cell machinery was disabled due to mutation, or to allow cell death in places where the apoptotic cell death machinery was deliberately inhibited, in circumstances in which caspases act in non-apoptotic roles. This type of cell death could also be important for killing cells that are large and more differentiated, two criteria that apply to the linker cell. One model that has been frequently put forth in favor of alternative death programs is that as cells get more differentiated, they are more difficult to kill. A study of conditional transgenic use of caspases for targeted cell deletion in *C. elegans* noted that the killing efficiency decreased as the animals got older, and

the killing efficiency of caspases in adults was only about half the level of efficiency observed in embryos (Chelur and Chalfie, 2007). This could have been because the heat shock promoter used was less efficient in adults, or because the adult cells were more resistant to caspase-mediated cell death.

Of course, there are many possible disadvantages to this type of cell death too. A slower cell death process could be highly detrimental during development because if cell corpses weren't cleared rapidly, their presence could obstruct and hinder normal cell migrations. The large vacuoles that form associated with linker cell death could also possibly be more challenging substrates for the phagocytic cell to neutralize.

Observing the morphology of type III PCD, it is interesting to speculate about the possible mechanism. In particular, the endoplasmic reticulum (ER) swelling is one early, and possibly primary, cellular pathology observed. An interesting question is whether this is related to normal pathways involved in ER stress response. If a cell begins to experience difficulties in effective protein folding in the ER—for example, if it is making a mutant protein, is having problems such as energy supply, or is experiencing calcium perturbation—this starts an ER stress response known as the unfolded protein response (UPR). This response enlists genes that can remedy the situation, such as ER folding chaperones or genes that will downregulate translation. However, if the cell is unable to regain its equilibrium despite the efforts of the UPR, it will undergo apoptosis (for a review of UPR, see Bernales et al., 2006). UPR has recently

been shown to be involved in a *Drosophila* neurodegenerative model of autosomal dominant retinitis pigmentosa (Ryoo et al., 2007).

As the linker cell migrates, it secretes hemicentin, an extremely large (5,198 amino acid) extracellular protein that has immunoglobulin superfamily repeats (Vogel and Hedgecock, 2001). The secreted hemicentin is believed to be deposited as a track that acts as a guidance cue for the gonad that is following behind it (Vogel and Hedgecock, 2001). One speculation to ponder is whether the secretion of hemicentin or other extracellular factors that act as guidance cues for the trailing gonad means that the linker cell has a comparatively high secretory output. If then, for example, the cell transcribed a gene that could interact and cause misfolding of the hemicentin or other secreted factors—possibly a prion-like gene—or if an inappropriately spliced version of one of these highly expressed genes was made, this could suddenly cause a major challenge for normal ER function, leading to catastrophic ER stress. This type of ER stress could also be a consequence of rapid engulfment of cell with a comparatively high secretory capacity—once a cell gets engulfed, the vesicles that would normally get secreted are now trapped and cause a backlog in the secretory system. However, swollen endoplasmic reticulum in the linker cell is not dependent on U.l/rp engulfment, as it also occurs in a linker cell migratory mutant where the cell is not engulfed by U.l/rp (Figure 2.8G).

Engulfment and Linker Cell Death

We have shown that the wild-type linker cell death shows some unusual engulfment characteristics. The linker cell is engulfed while it is still comparatively healthy, and it does not use the normal engulfment machinery that can work for both apoptotic and necrotic cells in *C. elegans*. It is likely that the linker cell signals to U.l/rp to engulf it, since U.l/rp do not engulf any other neighboring cells, suggesting that they are not constitutively activated for nonspecific engulfment. This unusually fast engulfment might need to occur to prevent the linker cell from being engulfed by the *vas deferens* (see Chapter Three for details). The cell probably also needs to be engulfed and taken out of the way swiftly to prevent it hindering the complex morphological changes that occur in the male tail at this time when the *vas deferens* is connected to the cloaca. Whatever signaling the linker cell is using may represent a very early or different signaling module from the normal signals used in apoptotic programmed cell death. This might also offer one explanation for why the linker cell doesn't show CED-1 clustering around the corpse in the wild-type location. The linker cell corpse might not be associated with this surface marker because it dies and is engulfed too swiftly. Another interesting possibility to consider is if this rapid engulfment of a healthy cell might be necessary for U.l/rp to take up or recycle some contents of the linker cell, for example, something that might facilitate U.l/rp at the post linker cell death stage when these cells connect up to the *vas deferens*. It is tempting to speculate whether some of the genes involved in linker cell engulfment could be similar to

those involved in the daily phagocytosis of portions of rod cells in the vertebrate eye, presumably a process in which the phagocyte is recognizing a target that is not dead.

Known Cell Death Genes and Linker Cell Death

Our transmission electron microscopy images of linker cell death look very different from apoptotic, autophagic, and necrotic cell death, a finding that is consistent with our genetic data showing that known cell death genes are not required for linker cell death, which therefore must represent a new pathway of programmed cell death. A very interesting question to consider is why the known cell death genes do not appear to be required for linker cell death. Apoptotic death appears to be swifter than linker cell death in its execution. However, in this case, the key may be not how fast the cell is cleared, but how fast the cell can get engulfed. If the linker cell can start to die in a way that can trigger an engulfment process that is faster than the apoptotic one, this could swiftly remove the cell from the top of the *vas deferens* and prevent linker cell interference of morphogenesis or inappropriate engulfment of the linker cell by the *vas deferens*. However, we do see a minor role for some of the core pathway genes in corpse clearance. Perhaps they can play a late role at the final stages of cell destruction. They could be activated specifically, or could be turned on in response to general cellular dysfunction, although the present thinking in the field is that only the germline in *C. elegans* shows damage or dysfunction induced programmed cell

death. Another possibility is that *ced-3* could play a role in linker cell migration and it could be expressed in the linker cell. In the mammalian system, caspase-11 has been shown to play a role in regulating cell migration in the immune system via regulation of Aip1-Cofilin mediated actin depolymerization (Li et al., 2007). In RNAi against *ced-3*, a low percentage of linker cell migratory mutants were seen. If *ced-3* was active in regulating the cytoskeleton for migration, *ced-3* might be otherwise engaged and unable to kill the cell. When migration stops and the cell starts to die, *ced-3* could redirect its energies towards its usual apoptotic targets. There are other examples of caspases playing a role in migration. In *Drosophila* ovarian border cell migration, it was found that *Drosophila* DIAP1 promotes cell migration. Clonal loss-of-function of this IAP gene did not produce apoptosis as might have been expected, and further experiments suggested that DIAP1 may act by inhibiting the caspase Dronc, since the cell migration is affected by mutants of Dark, the upstream activator of Dronc (Geisbrecht and Montell, 2004). It had been previously been shown that Rac can be a caspase target in lymphocytes (Zhang et al., 2003), and other studies have shown that caspases can target cytoskeletal components. In *C. elegans*, actin may be a target of CED-3 (Taylor et al., 2007). Caspase involvement in cell migration seems an elegant way to prevent inappropriate cell survival of a rogue migratory cell. A system could be envisioned in which during cell migration a cell uses a caspase activity for migration, and then if it stops migrating but ends up at an

inappropriate location, it will not receive the signals necessary to shut down the caspase and the cell would die.

Cell Migration and Cell Survival

There are several indications from the literature that there may be a relationship between cell migration and cell survival. For example, in mammalian systems Rho family GTPases are required for survival of primary cerebellar granule neurons (Linseman et al., 2001). FRL, a human formin related gene, binds Rac and regulates cell motility, and can block apoptosis (Yayoshi-Yamamoto et al., 2000). In *Drosophila,* germ cells undergo a migration pathway that has been well characterized. Only half of germ cells that form will make it to the correct gonadal location and eventually contribute to the germline (Underwood et al., 1980). Germ cells that migrate inappropriately will die, and this phenomenon of excess germ cell formation has been observed in a number of other species including mammals (Underwood et al., 1980). Interestingly, in the *Drosophila* study, some of the labeled germ cells that that migrated to inappropriate locations were observed to die with a necrotic morphology (Underwood et al., 1980). The cell death pathway that leads to the death of ectopic germ cells remains poorly understood. It is not known if these cells die in an apoptotic or autophagic fashion, however, since they show some necrotic features, this raises the possibility they could represent type III PCD. Some genes that regulate the cell

death and migration have been identified (Coffman et al., 2002; Zhang et al., 1996).

It is certainly possible that linker cell death could be initiated as a direct consequence of the fact that the linker cell stops migrating. At present, this idea is challenging to address experimentally, as genetic backgrounds that would enable manipulation of linker cell migration to allow it to continue beyond the end of the L4 stage have not been reported.

Developmental Timing Genes and Linker Cell Death

Linker cell death in *C. elegans* must be tightly regulated to ensure male fertility. Death of the linker cell prior to completion of its migration results in severe defects in gonadal elongation, as has been demonstrated by ablation of the linker cell during its migration (see Chapter 2; Kimble and White, 1981). It has been postulated that once the linker cell has completed its migration, cell death must ensue to allow fusion of the gonadal and cloacal tubes of the male. Our studies suggest that this may indeed be the case, since we have shown that in *lin-29* and *let-7* mutants, the connection between these tubes fails to form, and sperm accumulates within the male reproductive system (see Figure 2.2C which shows sperm build up in a *lin-29* animal).

The studies described here also suggest that even though linker cell death is cell intrinsic, both spatial and developmental inputs contribute to the cell's demise. Specifically, proximity to the cloaca seems to promote efficient linker cell

death, helping to ensure that the cell dies at the right place. The developmental timing pathway provides information ensuring that linker cell death occurs at the right developmental stage.

Although *lin-29* is required for linker cell death, it is probably not sufficient to promote cell death. The gene is normally expressed in other cells that do not die, and is expressed within the linker cell itself during the L3 and early L4 stages when the cell is still migrating (Euling et al., 1999; Figure 5.3B). These observations suggest that additional genes must exist that co-operate with *lin-29* to promote linker cell death.

Why might developmental timing genes be used as part of the pathway to regulate linker cell death? It is important that the linker cell dies at a specific time for normal fertility, which is one reason to enlist developmental timing genes in regulating the death (Figure 2.2). Or these genes might be enlisted to regulate linker cell death simply because they are expressed in the linker cell. Their linker cell expression might be because of an earlier role—they may guide linker cell migration, which executes specific turns in its migratory pathway at specific larval stages. In the distal tip cell, the hermaphrodite gonadal migratory leader cell, heterochronic mutations can regulate the migration of the cell (Tennessen et al., 2006), and *lin-29* animals have linker cell migration defects in approximately 30% of animals (Euling et al., 1999).

Many cell deaths must be regulated to occur at specific times during development, such as the hormonally triggered destruction of the larval salivary

gland during *Drosophila* development. The linker cell is the first described example that can link developmental timing genes to the regulation of programmed cell death. An interesting example of massive cell death regulated on the whole organismal level at a specific time in development is the death of Pacific salmon which die very rapidly after spawning, even though they are comparatively young and healthy. The morphology of cell death is described as a rapid degeneration of tissues and cells (Robertson and Wexler, 1960), and perhaps it could be type III PCD. This cell death appears to be under hormonal regulation from the gonad (Robertson and Wexler, 1962). There are some other examples of animals that die rapidly once they have completed their reproductive functions.

As *lin-29* is required, but probably not sufficient for linker cell death, and as the linker cell can die in a migratory mutant background, there is probably some additional long-range cue that is the key initiator of linker cell death. By analogy with other systems, such as Pacific salmon or *Drosophila* larval salivary glands, that cue may be hormonal. If we envision a model of a hormonal regulator expressed at the L4 to adult transition that works with *lin-29* to trigger linker cell death in *C. elegans*, the question still remains how this might specifically target the linker cell. It has recently been shown in *Drosophila* salivary glands that ecdysone mediated loss of the transcription factor forkhead is sufficient and required for cell death to proceed in the salivary gland (Cao et al., 2007). In *C. elegans* there are 15 forkhead transcription factors (Hope et al.,

2003), and *fkh-6* is required for the formation of the linker cell (Chang et al., 2004). Perhaps some linker cell specific gene like *fkh-6* is required as part of the pathway leading to linker cell death. Another possibility is that the linker cell could be singled out for death because it is a migrating cell, since there are no other *lin-29* expressing migrating cells in the male at that time.

Towards a Model For Linker Cell Death

Figure 7.1 illustrates our current model for linker cell death. The most important questions that remain to be addressed are what are the signals that initiate linker cell death and what are the executioner molecules for this death. Some of the missing genes may have been identified from the RNAi screen.

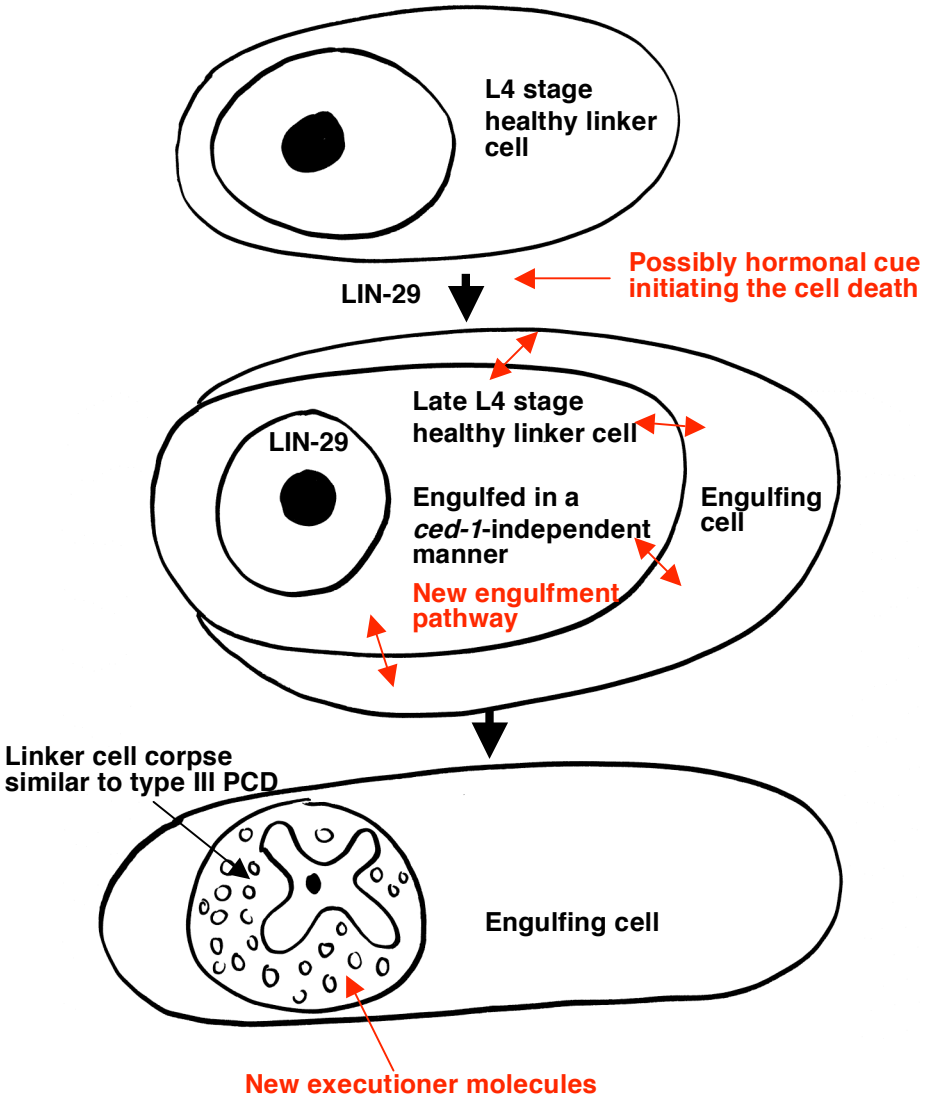


Figure 7.1 A Model for Linker Cell Death

Chapter Eight

Future Directions

Further Characterization of Positive Hits From the RNAi Screen

Having completed the screen, a number of experiments readily suggest themselves. The genes identified should be confirmed, since off-target effects can occur for RNAi, as has been shown in human cell studies (Jackson et al., 2003). A number of approaches are recommended to confirm an RNAi phenotype such as testing a scrambled RNAi construct, targeting another region of the RNA under investigation, or testing if there is an effect in loss-of-function genetic mutants (Echeverri et al., 2006). Promoter expression studies, such as the one for *pqn-41,* can be carried out to see where the genes are expressed. If the expression pattern looks particularly interesting, antibodies could be made against the gene of interest.

For a gene like *pqn-41*, which appears to be expressed specifically as the linker cell starts to die, there is the possibility that it could function as a killer gene. Therefore, it would be interesting to test whether expression of such a gene in other cells, for example in the touch cells under a touch cell specific promoter, could be sufficient to kill those cells with a morphology characteristic of linker cell death as determined by transmission electron microscopy. We can also start to investigate interactions between linker cell death genes to try to

determine if there are any hierarchies between them. For example, we could check in a *lin-29* mutant background to see if expression of any of the new genes identified are downregulated, indicating that these genes are potential targets of *lin-29.*

Some Additional Screening Approaches

If further characterization of the hits from the RNAi screen suggests that these genes are not the key regulators or executioners wanted, there are a range of alternative screening approaches available. Linker cell death represents a form of *ced-3*-independent programmed cell death in *C. elegans*, so one possibility to try to find other genes involved would be to screen directly for *ced-3*-independent cell death genes. For example, in a *ced-1; ced-3* background, any cells that die would be visible as persistent corpses. You would expect to see a few such corpses, since even in a strong *ced-3* mutant, not every single somatic cell death is blocked if you count additional surviving “undead” cells in the anterior pharynx. If these corpses represent the *ced-3*-independent pathway of cell death, then the genes involved could be identified if such a strain was mutagenized and screened for mutants in which the corpses were no longer present.

Another approach to try to find candidate genes involved in linker cell death is to look for mutations that cause precocious linker cell death, for example, by loss-of-function of a cell-specific inhibitor of linker cell death, if a such a hypothetical inhibitor of linker cell death exists. This is not beyond the

realms of possibility as apoptotic cell death pathways have many inhibitors that hold cell death in check. In the case of linker cell death where the correct timing of the death is likely to be so crucial for fertility, it does not seem unreasonable to suppose that the initiation of linker cell death may be poised on a hairline trigger. This screen would additionally pick up mutants where the cell doesn't form, due to a cell specification or lineage defect. Early linker cell loss could be easily recognized under a dissecting microscope as abnormalities in gonad development, as the clear patch visible if the gonad doesn't grow normally. If this negative regulator was already expressed in the linker cell as soon as it was born, this would be more difficult to tease out, as such as mutant would have a phenotype of no linker cell birth. Nonetheless, this might be an interesting and straightforward screen to undertake.

Other possibilities for screening include screens for male steriles (due to blockage of linker cell death), or a repeat of the EMS screen for linker cell survival, this time carried out as an F2 clonal screen. This is more labor intensive than an F1 clonal screen, but would have the advantage that plates are screened in which all the animals are homozygous for particular mutations, thus preventing the problem of not having enough males to score to adequately assess a homozygous mutation that is only represented by one quarter of the animals on the plate.

What Does *lin-29* Transcribe in the Dying Linker Cell?

What is the target of *lin-29* required for linker cell death? Another possible way to uncover the linker cell death pathway is to try to determine what is expressed in a surviving linker cell by a Microarray study. The ideal system would be to isolate a *lin-29* surviving linker cell and compare it with a linker cell that has just begun to die in a wild-type animal. However, the worm's cuticle represents a substantial obstacle to removing a cell by laser capture microdissection (Emmert-Buck et al., 1996). To break open the worm and try to isolate fluorescent cells by FACS sorting for subsequent use by Microarray analysis has only been done for embryos (Colosimo et al., 2004). The simplest type of comparison that could be tried would be to Microarray profile a *lin-29* mutant worm and compare it with wild-type. However, as *lin-29* is expressed in many other cells in addition to the linker cell, this approach may not be entirely satisfactory in offering a good prospect of identifying linker cell specific *lin-29* targets. One possibility would be to compare *lin-29* mutant males with *lin-29* mutant hermaphrodites to find *lin-29* regulated male specific genes, and then also compare wild-type adult males with *lin-29* males. Any genes that were downregulated in *lin-29* males compared with wild-type males, but were not downregulated in *lin-29* mutant hermaphrodites are good candidates for *lin-29* male specific regulated genes. Another possibility would be to tag ribosomes in a linker cell specific fashion and then try to isolate the ribosomes and profile the genes being transcribed.

Investigating the Role of ER Stress in Linker Cell Death

ER swelling is a characteristic morphology of linker cell death by electron microscopy. A number of genes involved in UPR, the ER stress response, have homologs in *C. elegans* such as *ire-1, xbp-1, atf-6*, and *pek-1* (Shen et al., 2001; Shen et al., 2005) and construction of these mutant strains with a GFP marked linker cell is currently underway. Interestingly, gene expression profiling of worms with an *xbp-1* mutation, identified a class of genes that are upregulated in response to UPR and these genes were named *abu* genes for activated in blocked upr (Urano et al., 2002). They were subsequently identified as members of the *pqn* family.

Gonadal Cues and Linker Cell Death

It might be interesting to investigate if a signal from the gonad could be regulating linker cell death, since the gonad can provide a cue for death in salmon. Determining if linker cell death can occur in a background in which the male germline does not develop normally could assess this.

Why Does the Linker Cell Divide into Two Parts During Cell Death?

What is the nature and role of linker cell blebbing, which appears to be a cell-autonomous process, and how might it be regulated? Blebbing associated with cell death is not inhibited by caspase-inhibitors in cell culture, suggesting that this is a caspase-independent process (Xiang et al., 1996; McCarthy et al., 1997).

The linker cell blebbing appears strikingly reminiscent of a cell division, in which the cell is divided into roughly two equal halves. One possibility is that linker cell blebbing is regulated by some sort of cytokinesis. Interestingly, *bir-1,* the *C. elegans* IAP homolog, is associated with a cytokinetic defect, and this might be an interesting candidate gene to investigate for a role in linker cell blebbing.

Does the linker cell have a program to divide into two because as it is such a comparatively large cell in the animal, it could not physically be engulfed by just one cell on its own? In the absence of *mig-5,* two linker cells form instead of one. Our strain with an RFP marked linker cell and GFP marked U.l/rp cell strain could be used to investigate whether U.lp and U.rp can each take up one whole linker cell alone, when there are two linker cells present in a *mig-5* RNAi background.

Chapter Nine

Materials and Methods

Strains and Alleles

Strains were handled using standard methods (Brenner, 1974). All strains were maintained and scored at 20°C unless otherwise indicated. Most strains included mutations promoting a high incidence of males (*him-4(e1267)*, *him-5(e1467, e1490)*, *him-8(e1489)*) as indicated. Alleles used in this study were described in the indicated references (alleles first reported here are unreferenced). Linkage group (LG) I: *ced-1(e1735)* (Hedgecock et al., 1983), *ced-12(k149)* (Gumienny et al., 2001), *lin-41(n2914)* (Slack et al., 2000); LG II: *lin-29(n333*, *n836)* (Ambros et al., 1984), *csp-1(tm917)*, *lin-42(ve11)* (Tennessen et al., 2006), *rrf-3(pk1426)* (Simmer et al., 2002); LG III: *ced-4(n1162)* (Ellis and Horvitz, 1986) *ncl-1(e1865)* (Hedgecock and Herman, 1995), *unc-36(e251)* (Brenner, 1974), *ced-7(n1892)* (Ellis et al., 1991a), *ced-9(n1950)* (Hengartner et al., 1992), *ced-6(n2095)* (Liu and Hengartner, 1998), *egl-5(n486)* (Chisholm, 1991); LG IV: *ced-2(e1752)* (Ellis et al., 1991a), *ced-10(n1993)* (Ellis et al., 1991a), *ced-10(n3417)* (Lundquist et al., 2001), *bec-1(ok700)* (Takacs-Vellai et al., 2005), *csp-2(tm1079)*,

ced-5(n1812) (Ellis et al., 1991b), *him-8(e1489)* (Hodgkin and Brenner, 1977), *ced-3(n717)* (Ellis and Horvitz, 1986), *ced-3(n2452)* (Shaham, et al., 1999); LG V: *egl-1(n1084n3082)* (Conradt and Horvitz, 1988), *him-5(e1467*, *e1490)* (Hodgkin and Brenner, 1977), *unc-51(e369)* (Brenner, 1974); LG X: *ced-13(sv32)* (Schumacher et al., 2005), *him-4(e1267)* (Hodgkin and Brenner, 1977), *let-7(n2853)* (Reinhart et al., 2000), *dyn-1(ky51)* (Clark et al., 1997), *ced-8(n1891)* (Ellis et al, 1991a), *daf-12(rh61rh411)* (Antebi et al., 2000), *nuc-1(e1392)* (Sulston, 1976). *nT1 qIs51* (Belfiore et al., 2002) IV; V was used as a balancer for *bec-1(ok700)*. Integrated transgenes used were: *qIs56* [*lag-2* promoter::GFP] (Siegfried and Kimble, 2002), *enIs7* [*ced-1* promoter::*ced-1*::GFP + *unc-76*(+)] (gift from Z. Zhou), *nsIs1* [*lag-2* promoter::GFP], *nsIs65* [*mig-24* promoter::GFP], *adIs2122* [*lgg-1* promoter::*lgg-1*::GFP + *rol-6*(*su1006*)] (gift from L. Avery), and *saIs14* [*lin-48* promoter::GFP] (gift from H. Chamberlin). The following extrachromosomal arrays were used: *nEx1049* [*ced-1* promoter::*ced-1*::GFP + *unc-76*(+)] (gift from Z. Zhou), *nsEx1265* [*lin-29* promoter::mCherry + *rol-6(su1006)*], *nsEx905-908* [*lin-29* promoter::GFP + *rol-6(su1006)*], *nsEx2000-2002* [*mig-24* promoter::Wld^S + *rol-6(su1006)*], *nsEx1696-7* [*pqn-41* promoter::GFP + *rol-6(su1006)*], *wEx15* [Heat-shock promoter::p35 *+ rol-6(su1006)*] (Sugimoto et al., 1994), and *vEx112* [*lin-29*(*+*) *ncl-1*(*+*) *unc-36*(*+*)] (Euling et al., 1999).

Microscopy Analysis of Linker Cell Death

Animals were examined by epifluorescence using either a fluorescent dissecting microscope (Leica), or by epifluorescence or DIC on an Axioplan II compound microscope. When observed using the compound microscope, the animals were placed on a slide on an agar pad with a drop of 30 mM sodium azide to anesthetize the worm. Except for an experiment with levamisole to follow individual worms through time, all DIC population study results reported here represent studies in which individual worms were scored at one timepoint. Rescoring the same worm at later timepoints was avoided in case of possible effects of sodium azide on development. To have an approximately similarly aged group of worms for population studies, late L4 males were picked at the stage just prior to the molt to adult, in which the rays and the cuticle around the tail are visible under the dissecting microscope. These animals were then scored at specific times after they had become adults. The worms were not observed for linker cell fluorescence before they were scored under the compound microscope, to avoid any possible sampling bias.

To assess linker cell survival under the compound microscope, the GFP marked linker cell was identified using DIC and fluorescence, and characteristics of the cell noted: size, shape, nuclear architecture, blebbing etc. These observations can then be classified into four categories: healthy normal surviving linker cell, linker cell corpse with abnormalities characteristic of early stages of cell death (nuclear envelope crenellation or partial breakdown, blebbing, or loss

of cytoplasmic volume), linker cell corpse with abnormalities characteristic of later stages of cell death (a completely round linker cell, usually with severe nuclear abnormalities), and linker cell gone.

Cell Ablation Experiments

lin-48 promoter::GFP-marked U cells were ablated in L1 animals anesthetized in a drop of M9 on 5% agar pads containing 5 mM sodium azide using standard methods (Bargmann and Avery, 1995). Ablations were scored as successful if on the following day the U cell was absent, as observed by DIC and fluorescence microscopy. Mock-ablated animals provided controls. The strain used for U cell ablation contained a *lag-2* promoter::GFP-marked linker cell to facilitate scoring of linker cell fate.

For ablations of the gonadal cells in *egl-5* mutants, the gonadal cells were identified based on DIC optics and ablated in L3 or L4 worms. The GFP marked linker cell was then observed at least 4-6 hours later.

Plasmid Constructions and Germline Transformation

Germline injections were used to create extrachromosomal arrays (Mello et al. 1991); 3.5 kb of the *lin-29* promoter was fused to GFP or mCherry and injected at 20 ng/µl with the *rol-6(su1006)* coinjection marker; 1 kb of the *mig-24* promoter was fused to Wld^S and injected at 10 ng/µl with the *rol-6(su1006)* coinjection marker; 2.5 kb of *pqn-41* promoter was fused to GFP and injected at 20 ng/µl

with the *rol-6(su1006)* coinjection marker.

Scoring Anterior Pharynx Cell Survival

L4 stage males were mounted on a 5% agar pad in a drop of 30 mM sodium azide and extra cells in the anterior pharynx were scored using DIC (Ellis and Horvitz, 1991).

Transmission Electron Microscopy Analysis

12 animals with a linker cell GFP marker were observed under a fluorescence dissecting microscope or by DIC microscopy to determine the approximate stage of linker cell death. The animals observed were all late L4 or just around the molt to adult. For the *him-4* linker cell migratory mutant, the animals were all 0-2-hour-old adults. To obtain worms in which the linker cell vacuole was present, late L4 worms in which the rays were visible were mounted in a simple S-Basal buffer and observed by DIC. None of the animals used for electron microscopy were treated with anesthetic prior to fixation. Animals were fixed, stained, embedded in resin, and serially sectioned using standard methods (Lundquist et al., 2001). Photographs were taken with an FEI Tecnai G2 Spirit BioTwin transmission electron microscope equipped with a Gatan 4K × 4K digital camera.

Mosaic Analysis

Mosaic analysis was carried out by scoring animals of the genotype *lin-29(n836); ncl-1(e1865) unc-36(e251); qIs56 him-5(e1490)* for loss of the extrachromosomal array, *vEx112*, containing *lin-29*(+), *ncl-1*(+), and *unc-36*(+)(Euling et al., 1999). *unc-36* loss in the AB.p lineage, which gives rise to all the proctodeal cells that are in the vicinity of the linker cell at the time of its death (including the U.l/rp cells), confers an uncoordinated mobility phenotype. *unc-36* loss in the P1 lineage, giving rise to the linker cell, does not affect mobility. *ncl-1* loss results in large nucleoli. Mobility was assessed in L4 animals to determine whether array loss had occurred in the AB.p lineage. Animals were then mounted in S-Basal medium, and nucleolar size was used to confirm the presence or absence of the array in the U.l/rp and neighboring cells, and to determine if the array was present in the linker cell. Subsequent survival of the linker cell was followed in individual animals to determine whether the linker cell died, and whether death proceeded with normal kinetics. Only animals in which the linker cell migrated correctly were scored.

EMS Mutagenesis

qIs56(*lag-2*::GFP) *him-5(e1490)* animals were mutagenized with 30 mM ethylmethansulfonate (EMS) (Sulston and Hodgkin, 1988). 1,100 F1 mutagenized progeny were plated individually, and their F2 progeny scored under an epifluorescent dissecting microscope (Leica) for linker cell survival in the adult.

RNAi Assays

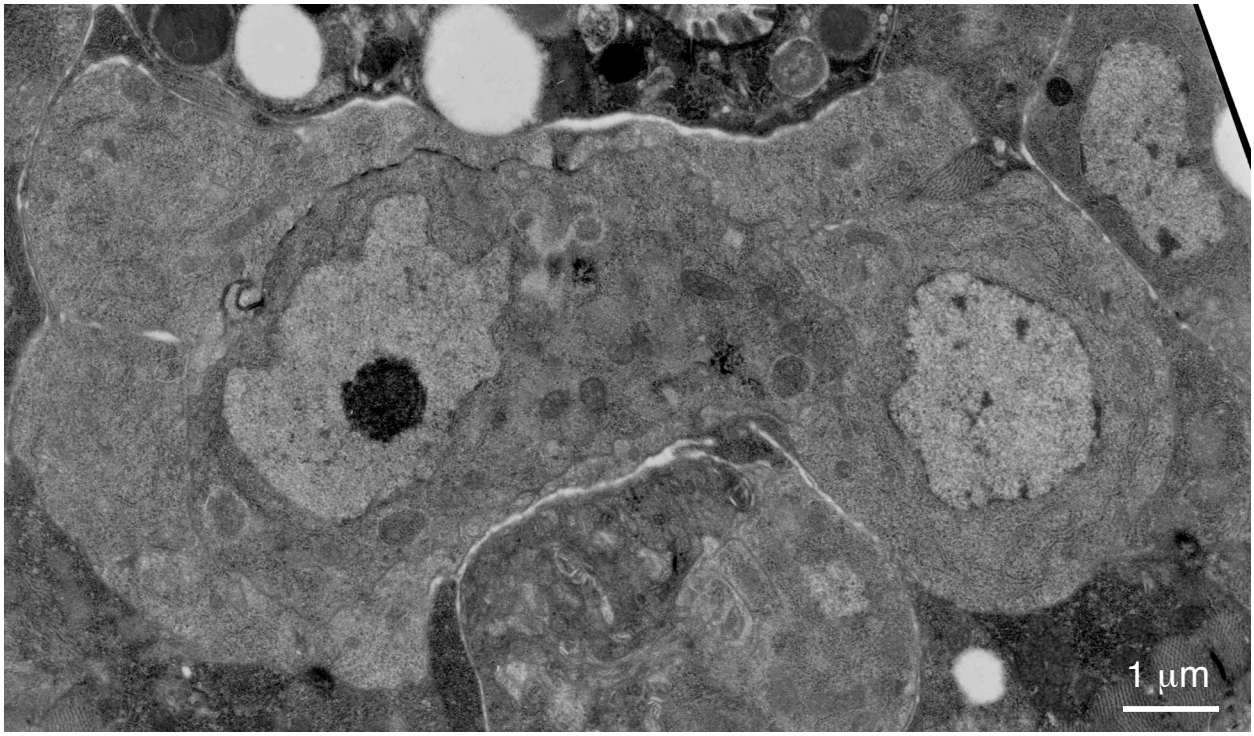
RNAi was carried out by feeding bacteria expressing double-stranded RNA (dsRNA) corresponding to the gene of interest to animals (Timmons and Fire, 1998). Plasmids used were obtained from the Ahringer library (Kamath et al., 2003), the Vidal library (Kim et al., 2005), and the *csp-1* plasmid was obtained from Open Biosystems. Bacterial RNAi clones were grown up overnight at 37°C for 12-17 hr and then seeded onto NGM plates with IPTG. Induction of dsRNA expression preceded the addition of animals to plates by 2–12 hr. A synchronous population of L1 larvae was obtained by hypochlorite treatment followed by growth in M9 for approximately 24 hr. The synchronized L1s were added to plates containing bacteria expressing dsRNA. An empty vector was used as a control, and positive controls for RNAi such as GFP or *lin-29* were also used to verify successful double-stranded RNA induction.

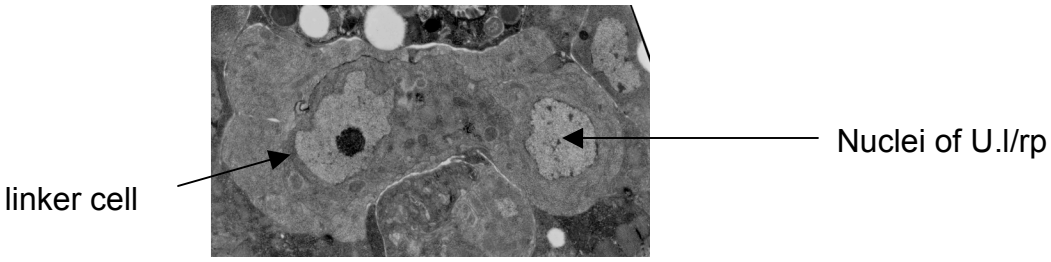
For the genome-wide library screen, bacteria grown overnight in 96-well plates were seeded onto 12-well NGM plates using a PerkinElmer Multiprobe machine. The plates were left to induce for 2 to 8 hours at room temperature and then 250 synchronized L1 worms were added per well from a strain with *rrf-3* (to enhance RNAi sensitivity), *lag-2*::GFP (to mark the linker cell), and *him-5* (to enhance the number of males in the population). The plates were scored using an epifluorescent dissecting microscope (Leica) about 48 hours after the L1 worms were added, when the males were approximately 2 to 4 hour-old adults.

Appendix Table 1. Additional Hits From the RNAi Screen Retested

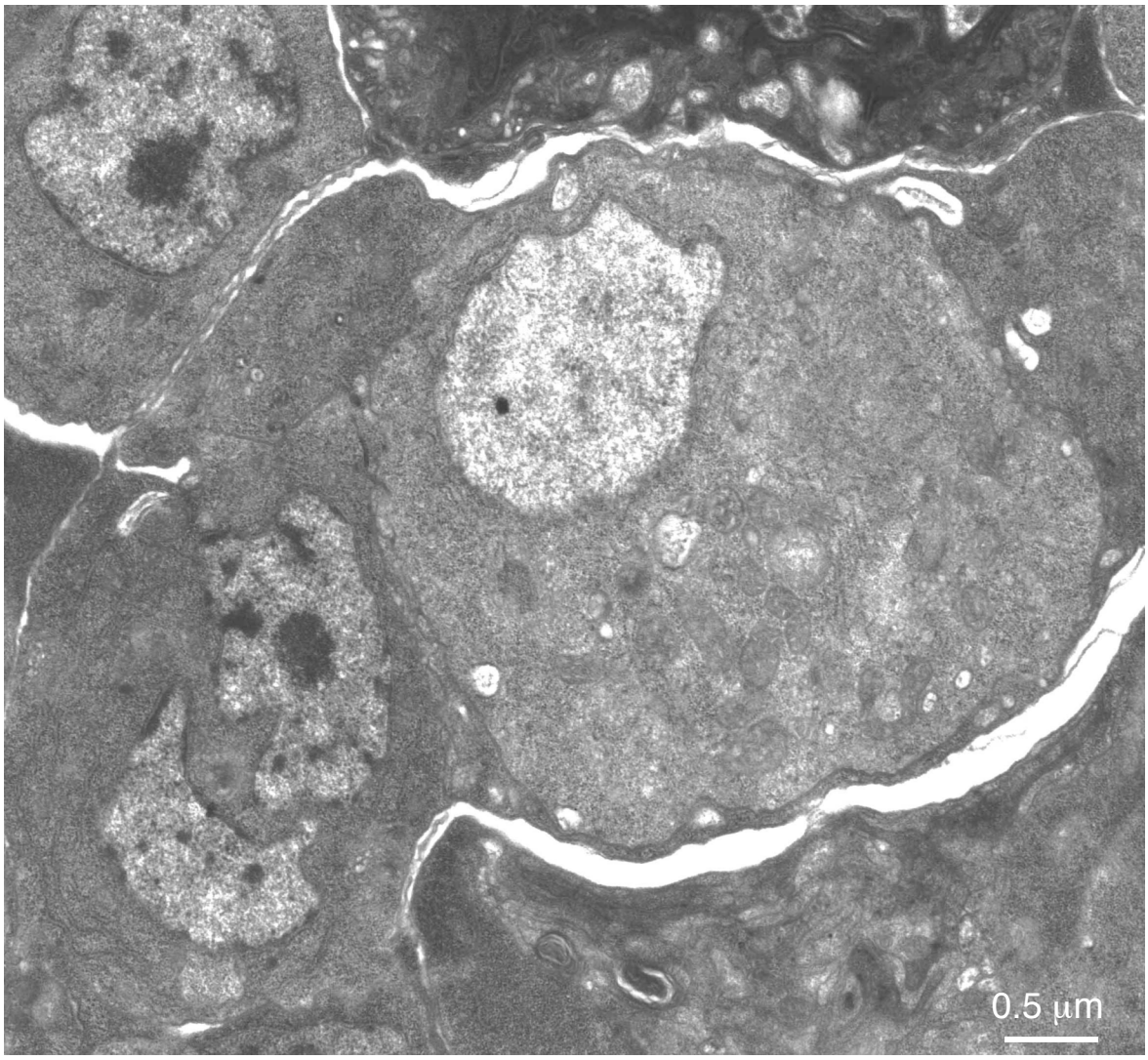
Gene	Gene name/identity if known
ZC123.2	homeboxznfinger
F47G6.1	dyb-1 (alpha dystobrevin homolog)
B0285.2	unknown gene
C45G9.2	tRNA-dihydrouridine synthase
ZK1098.4	Translation initiation factor 2B
F57B9.5	byn-1 (adhesion protein related)
C07A9.5	calcium binding, actin bundling motif
F54C8.2	cpar-1 (Histone)
Y79H2A.3	Q/N rich (pqn class)
Y75B8A.8	unknown gene
F58B3.2	lys-5 (lysozyme)
D2096.10	unknown gene
T09A12.2	Glutathione peroxidase
T26A8.4	Polyadenylation factor I complex,
W03G1.7	Acid sphingomyelinase and PHM5 phosphate metabolism protein
F48G7.10	Serine/threonine protein kinase
T21D12.4	pat-6 (ortholog of alpha parvin, required for muscle assembly)
Y24F12A.a	Predicted small GTPase involved in nuclear protein import
Y76B12C_66.c	mRNA cleavage and polyadenylation factor II complex, subunit CFT1
Y105C5A.m	unknown gene
W03F9.2	unknown gene
R11G11.3	proteinase inhibitor/serpin
W08A12.3	unknown gene
T27C4.4	egr-1 (homolog of human MTA1- acts in chromatin remodelling
F09F3.1	srx-130 (serpentine receptor)
F21C10.2	unknown gene
T23B12.8	unknown gene
ZC443.5	UDP-glucuronosyl and UDP-glucosyl transferase
F35E12.5	CUB like domain
F53F4.9	srd-11 (7TM receptor)
C34D1.5	zip-5 (Transcription factor)
F55B12.4	tRNA nucleotidyltransferase/poly(A) polymerase
C06B3.4	17 beta-hydroxysteroid dehydrogenase type 3
F55B12.8	srx-17 (serpentine receptor)
R10D12.8	unknown gene
F57A10.4	unknown gene
F11A1.3	daf-12 (steroid receptor hormone)
R12H7.3	skr-19 (ubiquitin ligase related)
F59C12.2	ser-1 (serotonin receptor)
F01G12.5a	let-2 (collagen)
T27A8.1	Zinc carboxypeptidase
H03G16.1	unknown gene
Y119D3B.17	pes-4 (KH and RNA binding domains)
Y39G10AL.3	cdk-7 (cyclin dependent kinase)
R06C1.2	Polyprenyl synthetase
Y62E10A.13	unknown gene
C45E5.1	p-Nitrophenyl phosphatase
K04F1.12	predicted receptor
C24G7.2	Non voltage-gated ion channels (DEG/ENaC family)
F40H3.4	fkh-8 (transcription factor)
D2013.2	wdfy-2 (WD40 and FYVE domains)
R166.4	pro-1 (ribosome biogenesis, + role in germline)
C09G5.3	col-79 (collagen)
Y119D3_465.o	unknown gene
Y55D5A_391.c	unknown gene
Y45F10C.4	unknown gene
Y47G6A.20	rnp-6 (splicing regulation)
F29G9.7	contains F box
C29A12.6	unknown gene
ZK836.3	unknown gene
F44G3.8	contains F box
C31A11.6	srxa-7 (serpentine receptor)
F21E9.3	uncharacterized gene with conserved cysteine
T16G1.1	pqn-67 (Q/N rich)
F48D6.1	Taf11.1 TFIID
T04F8.6	unknown gene
F08B12.2	prx-12 peroxisome organization
C30E1.8	unknown gene
C30G4.3	gcy-11 (guanylyl cyclase)
C48D1.2	ced-3
R1E3.6	eor-1 (transcription factor)
C02H7.1	microtubule binding protein
F52E1.3	unknown gene
F09F9.3	unknown gene
Y77E11A-3443.e	predicted kinase
C15A11.3	sol-1 (suppressor of lurcher)
F57A10.4	unknown gene
C01F6.1	unknown gene
C42C1.10	mitochondrial solute carrier protein
ZC142.2	7 transmembrane receptor
F28D1.11	Dolichol-phosphate mannosyltransferase
Y46H3C.e	DNA topoisomerase
Y44A6D.6	unknown gene
T23B12.3	mitochondrial ribosomal protein

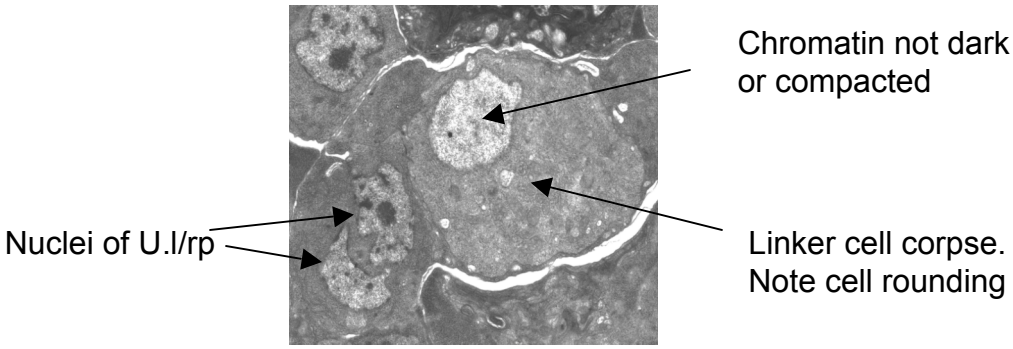
This is a list of the most promising clones (in addition to the 5 positive hits already identified), all of which have been retested again under the dissecting microscope. Four clones highlighted (arrows) may be worth testing under the compound microscope, as they appeared to have some extra linker cell GFP in the dissecting microscope retests. The remaining clones didn't repeat with a strong phenotype under the dissecting microscope, or if tested under the compound didn't give healthy linker cell survival.



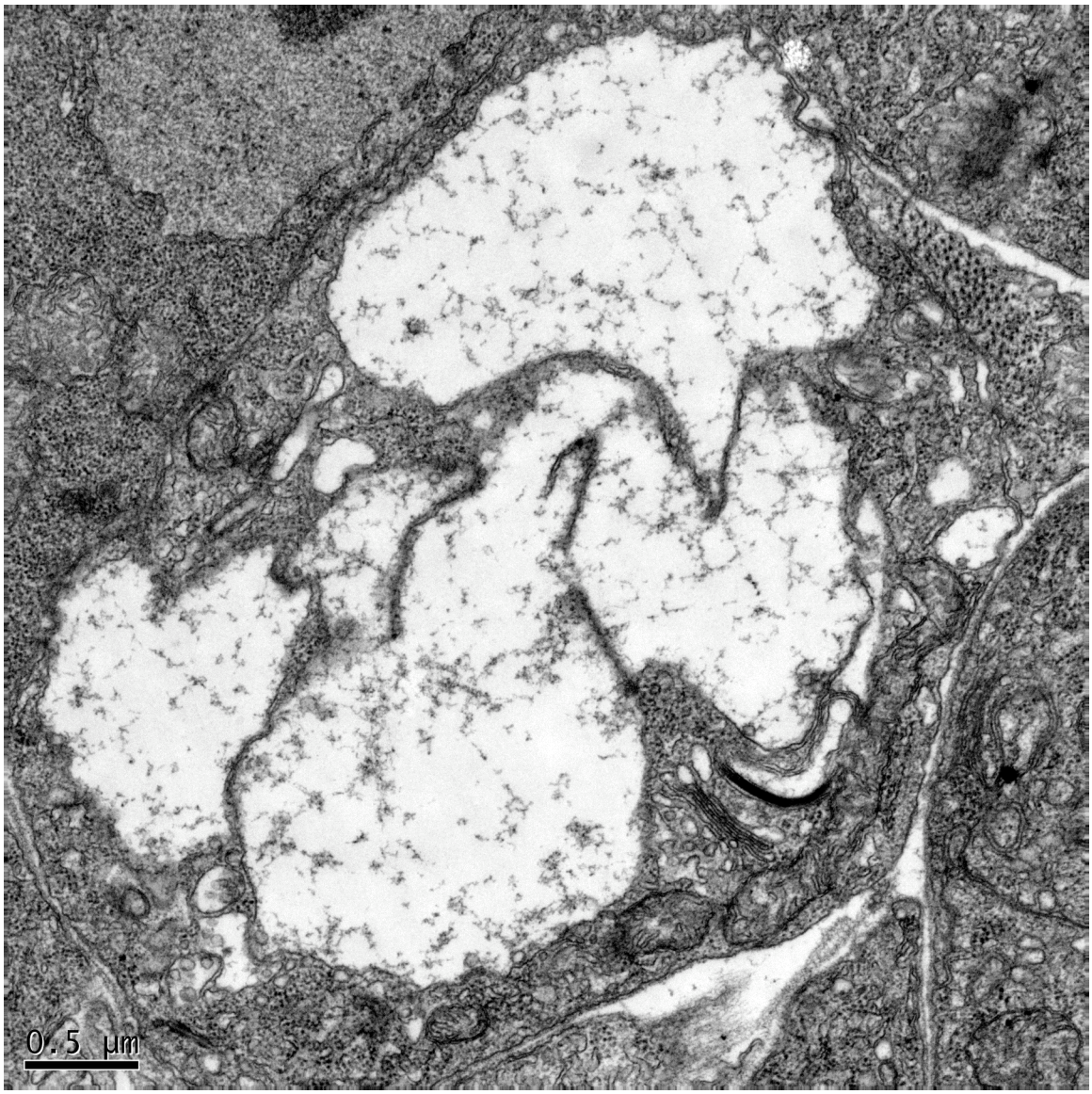


Appendix Figure 1.
TEM Image of a Healthy Linker Cell in the Process of Engulfment.

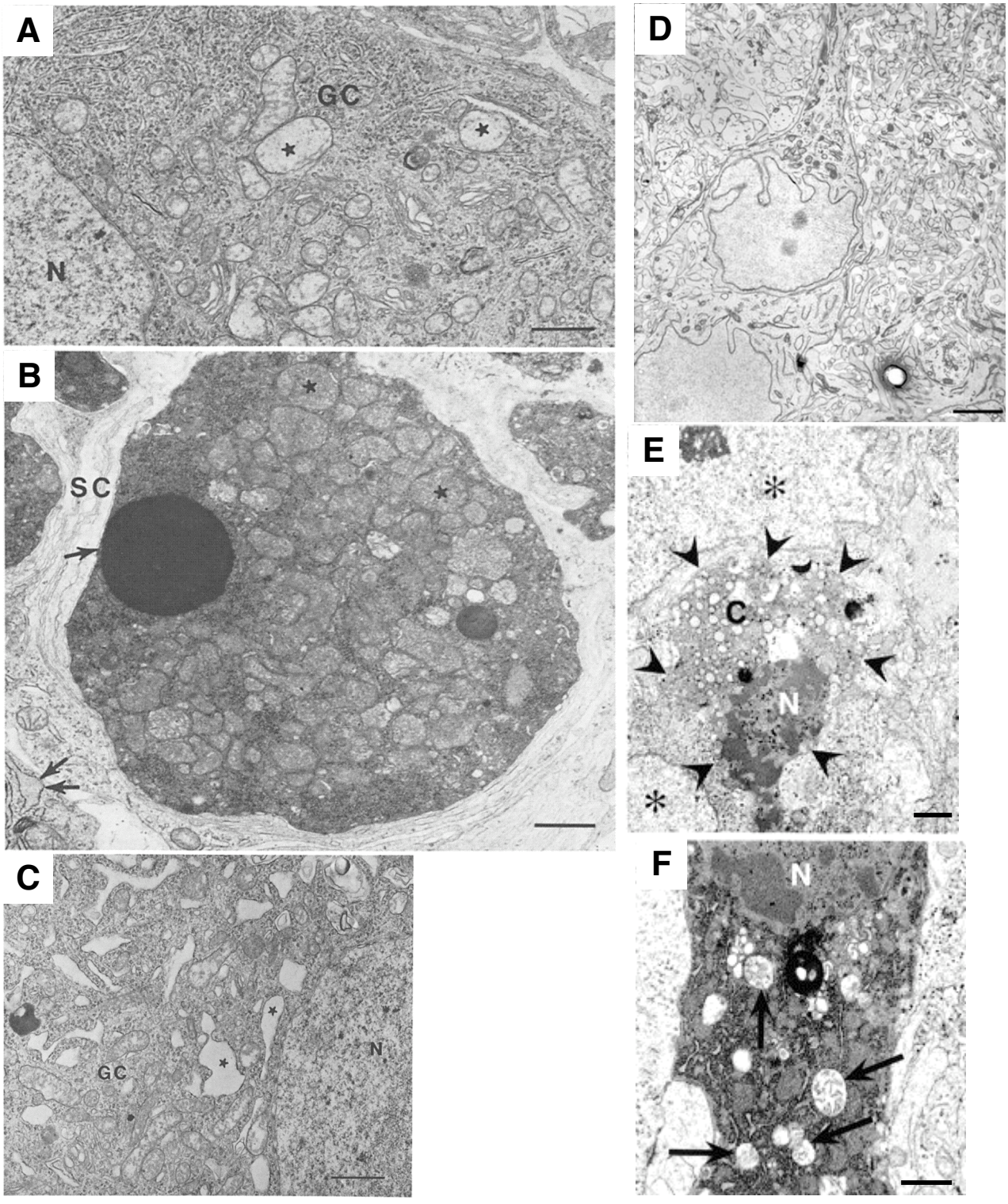




Appendix Figure 2.
TEM Image of a Round Linker Corpse Being Engulfed.



Appendix Figure 3.
TEM Image of Linker Cell Associated Vacuoles Merging Together.


A
GC
N
B
SC
C
GC
N
D
E
C
N
F
N

Appendix Figure 4. Published TEM Images of Vertebrate Cell Deaths with Morphological Features Similar to Linker Cell Death

(A) Figure 13 from Pilar and Landmesser (1976). Original legend reads: "Early stages of normally occurring cell death involving primarily swelling of mitochondria (asterisks). *GC*, ganglion cell; *N*, nucleus". Scale bar, 1μm.

(B) Figure 14 from Pilar and Landmesser (1976). Original legend reads: "Degenerating profile containing mostly greatly dilated mitochondria (asterisks) with a clump of chromatin (arrow). *SC*, satellite cell. Double arrow points to dilated cisternae of satellite cell RER (rough endoplasmic reticulum) containing floccular material". Notice similarity to the 200 nm vesicles seen in the linker cell (Figure 2.8F). Scale bar, 1μm.

(C) Figure 10 from Pilar and Landmesser (1976). Original legend reads: "The nucleus (*N*) retains a normal appearance in this ganglion cell (*GC*) undergoing degeneration during normal development, although the RER is extremely dilated (asterisks)." Notice similarity to "empty" cytoplasmic clearings seen in linker cell (Figure 2.8G, 2.8H and 2.8J). Scale bar, 1μm.

(D) Fig. 3C of Borsello et al. (2002). Original legend reads: "Invaginated nuclei in axotomized retinal ganglion cells at 3 days after an embryonic day 12 tectal lesion." Notice similarity to crenellated nucleus of the linker cell (Figure 2.8C). Scale bar, 2 μm.

(E & F) Figures 4D, F, respectively, of Oppenheim et al. (2001). Original legend reads: "Photomicrographs of degenerating spinal cord neurons from E14.5 caspase-3 KO -/- embryos showing the distinct morphology of dying neurons in the caspase KO. These cells exhibit reduced chromatin condensation and nuclear pyknosis, marked cytoplasmic vacuolization, and dilation of mitochondria and RER compared with neurons from the caspase-3 +/+ embryos...the arrows in F indicate mitochondria, and the arrowheads in D indicate the cell boundary of this degenerating motoneuron. Note the numerous vacuoles and abnormal organelles in the cytoplasm of the cells in D and F." Scale bar (E & F), 1 μm.

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