Cell non-autonomous regulation of death in *C. elegans*

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Programmed cell death (PCD or apoptosis) is an evolutionarily conserved, genetically controlled suicide mechanism for cells, which when deregulated, can lead to developmental defects, cancers and degenerative diseases [1, 2]. In *C. elegans*, DNA damage induces germ cell death by signaling through *cep-1/p53* ultimately leading to the activation of the CED-3/caspase [3-13]. It has been hypothesized that the major regulatory events controlling cell death occur by cell autonomous mechanisms, that is within the dying cell. In support of this, genetic studies in *C. elegans* have shown that the core apoptosis pathway genes *ced-4/APAF1* and *ced-3/caspase* are required in cells fated to die [3]. However, it is not known whether the upstream signals that activate apoptosis function in a cell autonomous manner. Here I show that two genes, *kri-1*, an ortholog of KRIT1/CCM1 that is mutated in the human neurovascular disease cerebral cavernous malformations (CCMs) [14, 15] and *daf-2*, an insulin-like receptor [16-18], are required to activate DNA damage-dependent cell death independently of *cep-1/p53*. Interestingly, I found that both genes can regulate cell death in a non-autonomous manner, revealing a novel role for non-dying cells in eliciting death in response to DNA damage.
Acknowledgements

Graduate school is an incubation period of sorts for intellectual, personal and social development. The “science” student must become a student of how to interact with people and build relationships as well as acquire life skills to problem solve everyday issues. Unfortunately, few are able to teach these skills in a world so focused on publications, except for the following names and groups of people:

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This thesis was brought to you in part by a) blood, b) sweat, c) tears, d) less sleep, e) ...signal problems at...station... f) all of the above. It is dedicated to my grandfather, who was a geneticist and to anyone else brave enough to read through this work. Meaning, you survived graduate studies.

Probably.
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<thead>
<tr>
<th>C. elegans Genes</th>
<th>Other Genes</th>
</tr>
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<tbody>
<tr>
<td>age-1 ageing alteration</td>
<td>53BP1 tumour protein p53 binding protein 1</td>
</tr>
<tr>
<td>akt-1, 2 AKT kinase family</td>
<td>9-1-1 Rad9-Hus1-Rad1 DNA clamp complex</td>
</tr>
<tr>
<td>atl-1 ATM-like</td>
<td>A1 BCL2-related protein A1</td>
</tr>
<tr>
<td>atm-1 ATM family</td>
<td>APAF1 apoptotic peptidase activating factor 1</td>
</tr>
<tr>
<td>ced-1, 3, 4, 5, 7, 9, 13 cell death abnormality</td>
<td>ATM ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>cep-1 C. elegans p53-like protein</td>
<td>ATR ataxia telangiectasia and Rad3 related</td>
</tr>
<tr>
<td>chk-1, 2 checkpoint kinase</td>
<td>BAD BCL2-associated agonist of cell death</td>
</tr>
<tr>
<td>clk-2 clock (biological timing) abnormality</td>
<td>BAK BCL2-antagonist/killer 1</td>
</tr>
<tr>
<td>daf-1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 14, 16 abnormal dauer formation</td>
<td>BAX BCL2-associated X protein</td>
</tr>
<tr>
<td>dpy-30 dumpy</td>
<td>BCL-W BCL2-like 2</td>
</tr>
<tr>
<td>egl-1 egg laying defective</td>
<td>BCL2 B-cell lymphoma 2</td>
</tr>
<tr>
<td>hif-1 hypoxia inducible factor homolog</td>
<td>BCL2L1 BCL2-like 1</td>
</tr>
<tr>
<td>hpr-9 homolog of S. pombe Rad</td>
<td>BH3-only BCL homology domain 3</td>
</tr>
<tr>
<td>hus-1 human HUS1 related</td>
<td>BID BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>kri-1 human KRI1 homolog</td>
<td>BIM BCL2-like 11</td>
</tr>
<tr>
<td>lin-35 abnormal cell lineage</td>
<td>BMF BCL2 modifying factor</td>
</tr>
<tr>
<td>mrt-2 mortal germline</td>
<td>CASP3, 7, 9 apoptosis-related cysteine peptidase</td>
</tr>
<tr>
<td>myo-3 myosin heavy chain structural genes</td>
<td>CCM1, 2, 3 cerebral cavernous malformation 1, 2, 3</td>
</tr>
<tr>
<td>pdk-1 PDK-class protein kinase</td>
<td>CHEK1 CHK1 checkpoint homolog</td>
</tr>
<tr>
<td>pmk-3 p38 MAP kinase family</td>
<td>CHEK2 CHK2 checkpoint homolog</td>
</tr>
<tr>
<td>ppw-1 PAZ/PWI domain containing</td>
<td>FAK focal adhesion kinase</td>
</tr>
<tr>
<td>rol-6 roller</td>
<td>FRMD3 FERM domain containing protein 3</td>
</tr>
<tr>
<td>rrf-1 RNA-dependent RNA polymerase family</td>
<td>HEG1 heart of glass</td>
</tr>
<tr>
<td>sir-2.1 yeast SIR related</td>
<td>HIV1 human immunodeficiency virus type 1</td>
</tr>
</tbody>
</table>
tyr-2 tyrosinase
unc-54 uncoordinated

ICAP1 integrin cytoplasmic associated protein 1
KRIT1 Krev interaction trapped 1
MCL1 myeloid cell leukemia sequence 1
MDM2 mouse double minute 2
MYC cellular myelocytomatosis
PUMA p53 upregulated mediator of apoptosis
RAP1A RAS-related protein 1A
RB retinoblastoma
SOD1 superoxide dismutase 1
TEL2 telomere maintenance 2
TNF tumour necrosis factor

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>CCM</td>
<td>cerebral cavernous malformation</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DISC</td>
<td>death inducing signaling complex</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand break</td>
</tr>
<tr>
<td>DTC</td>
<td>distal tip cell</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitroso urea</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FERM</td>
<td>band 4.1, ezrin, radixin, moesin and merlin</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
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</table>
1. Introduction
Responses to cell stress

Suppose for a moment that you are a cell living inside a host human. Life is good, being surrounded by sibling cells and protected by a selective membrane that allows intake of nutrients necessary for growth but that excludes the rest of the external environment. That is until the host decides he needs to diet and stops eating. Without any new intake of food, you, the cell, are left to find other sources of energy or risk dying. Perhaps it is time to lift any inhibitions about cannibalism and start scavenging for nutrients left by your siblings who have already met an untimely end. Or perhaps it is best to breakdown some unneeded parts inside you to keep living. Just as you are about to make a life-altering ethical decision, the human host smartens up and decides that exercise, instead of a diet, is the way to achieve his goals. With a sudden influx of nutrients again, you acquire new siblings and are no longer forced to make hard, ethical decisions. Until another problem arises: low oxygen. As a couch potato host human, a five minute walk around the block has left him gasping for air; and you, the cell, with a backlog of byproducts from energy production that has lost its dumping ground. Every moment that passes, toxic byproducts accumulate and this time, you decide, is the end. Like your siblings before you, you push your internal self-destruct button and hope that by the time your luckier siblings have to make similar ethical decisions that things will be better again.

In this way, all cellular life on Earth face a multitude of external stresses such as nutrient deprivation, hypoxia, osmotic stress and DNA damage that must be balanced against signals to proliferate and divide. Accordingly, cells initiate a variety of genetic programs designed to respond to these stresses that enables their continued existence [19, 20]. These responses include a cell’s catabolic process of digesting its own subcellular components called autophagy [21], activation of stress specific signal cascades and transcription factors [22], cell cycle arrest, DNA repair and
programmed cell death [23]. Oftentimes, they are adaptive in nature: that is, the response helps a cell overcome various insults. However, when environmental stresses overpower a cell’s ability to cope in the environment, an irreversible programmed cell death (PCD or apoptosis) can occur. An imbalance of signals in favour of stress/death causes the cell to initiate a highly conserved and regulated genetic program to dismantle itself. Disrupting this regulation can have deadly consequences, as outlined below.

Worms under stress

As illustrated in the preceding example, a cell’s response to stress is governed not only by the cell in question but also its surrounding environment, including its surrounding neighbours. Therefore, it is difficult to determine a bona fide biological response to stress where cell-cell and cell-environment interactions are concerned without an intact whole organism. In this respect, the nematode *Caenorhabditis elegans* represents an organism in which responses to stress can be studied, especially since many of the pathways that respond to stress are conserved in humans. For example, the worm initiates autophagy in response to starvation conditions, similar to how mammalian cells also undergo autophagy when deprived of nutrients [24]. Transcription factors such as HIF (hypoxia inducible factor) are upregulated in low oxygen environments in both worms and humans [25]. Finally, in response to DNA damage, a subset of cells initiate cell cycle arrest to repair the damage; however, cell death can occur if the repair is unsuccessful, using a conserved genetic pathway [26]. The worm is particularly suited to studying the process of cell death, since dying cells can be visualized directly and quantified by microscopy (Figure 1.1A). Furthermore, the genetic pathway regulating the process is conserved but simplified compared to higher eukaryotes.
Combined with its ease of genetic manipulation, *C. elegans* is a useful tool for identifying genes and determining pathways important for cell death.

**Cell death in *C. elegans***

There are two major types of cell deaths that occur in *C. elegans*. The first occurs during development, where 131 out of the 1090 somatic cells that are generated die via a specific program [27, 28]. Cell deaths during development are regulated by cell specific transcription factors and occur invariably; that is, the same cell dies in approximately the same position at the same time in every worm (Figure 1.1B). Contrasting this developmental cell death are the stochastic cell deaths that occur in the germline of the adult hermaphrodite worm [6]. It is estimated that about 50% of germ cells undergo cell death in the absence of external insults, hypothesized to function as nurse cells in order to provide nutrients for surviving cells. This physiological form of germ cell death is dependent on MAPK (mitogen activated protein kinase) activity in germ cells (Figure 1.1C) [6]. In addition to the baseline death that occurs, external insults, including osmotic, oxidative, thermal, nutritional and genetic stress can cause germ cells to die [29]. Both developmental and germline cell death employ the same core machinery (discussed below), although the upstream signals that feed into it differs depending on the cell that is dying [30]. Of the many stress signals that cause cell death, one of the most potent inducers is DNA damage caused by a variety of sources such as ENU (N-ethyl-N-nitrosourea), a DNA alkylating agent and IR (ionizing radiation), which induces DSBs (double strand breaks) in the DNA. Repairing the DNA damage or killing the unrepairable cell is vital for maintaining organismal integrity. Fortunately, the worm is equipped with a well characterized genetic program that is able to deal with DNA damage stress.
Figure 1.1: *C. elegans* anatomy.

(A) Schematic diagram showing major tissues in a lateral cross section of an adult hermaphrodite worm. Image adapted from www.wormatlas.org.

(B) A DIC (differential interference contrast) image of a wild-type comma stage embryo corresponding to the small black rectangle in (A). Developmental apoptosis is indicated by the black arrow. Corpses appear as highly refractile, round, button-like objects in this view.

(C) A DIC image of the bend region (distal germline, top; proximal germline, bottom) of a wild-type adult hermaphrodite germline corresponding to the large black rectangle in (A). Cell deaths that have occurred in response to DNA damage are indicated by the black arrows. Germline corpses have a similar morphology to developmental corpses shown in (B).

(D) A transverse cross section of an adult hermaphrodite showing both the distal (top; rachis) and proximal (bottom; oocyte) germlines (left) and a schematic representation of distal germline organization (right). Image adapted from www.wormatlas.org.
DNA damage-dependent cell death

DNA damage is perhaps the most deleterious of all stresses due to its ability to change the characteristics of a cell by causing base pair changes or rearrangements of entire chromosomes. Of all of the various types of DNA lesions – DNA mismatch, base damage, helix structure damage, replication fork stalling – DSBs caused by IR are the most difficult to repair [23]. Damaged cells will try and repair the DNA; however, if they are unable to do so, a well characterized death signaling cascade will be initiated.

Sensors and transducers

Different types of DNA damage are sensed by different protein complexes [31]. The two best characterized protein complexes in mammals involve ATM (ataxia telangiectasia mutated), which responds specifically to DSBs and ATR (ataxia telangiectasia and Rad3 related), which is activated in response to replication fork stalling. ATM localizes to DSBs to initiate a cell cycle arrest so that the lesion can be repaired either by non-homologous end joining or homologous recombination [23]. However, if the damage cannot be repaired or removed, ATM recruits proteins such as 53BP1 (tumour protein p53 binding protein 1) and CHEK2 (CHK2 checkpoint homolog), which through a series of phosphorylation events transduce signals to activate p53, a tumour suppressor protein responsible for mediating cell death (Figure 1.2) [31]. On the other hand, ATR is responsible for mediating cell cycle arrest via signaling through the 9-1-1 DNA clamp complex, leading to the activation of CHEK1 (CHK1 checkpoint homolog) [31]. In worms, DNA damage is sensed by the homologs of ATM and ATR, atm-1 [32] and atl-1 [33, 34], respectively. Whereas mammalian ATM is solely responsible for responding to DSBs, both atm-1 and atl-1 are recruited to DSBs in worms and are required for cell death to occur [32, 35]. Downstream of atm-1 and atl-1, two partially redundant pathways, one mediated by genes encoding for the 9-1-1-like complex, hpr-9, hus-1 and
mrt-2 [7, 13] and the other by clk-2, the ortholog of TEL2 in yeast [10], responds to DNA damage. A signal transduction cascade is presumably activated in a manner similar to mammalian cells through the chk-1/CHEK1 [34, 36] and chk-2/CHEK2 [37, 38] kinases.

Figure 1.2: The mammalian and C. elegans cell death pathways.
The conserved genetic pathway regulating cell death in mammals and C. elegans. Sensors and transducers relay DNA damage signals to CEP-1/p53, which transcriptionally activates one (in C. elegans) or more (in mammals) BH3-only genes to promote apoptosis.

p53/cep-1
Once a threshold level of damage to activate apoptosis is achieved, CHEK2 can phosphorylate and activate the tumour suppressor protein p53 [39]. Often considered the master regulator of cell death, the p53 gene is mutated in over 50% of human cancers, which has spurred a plethora of research on its function and regulation [40, 41]. As a tetrameric transcription factor, p53 binds to cognate DNA binding sites to activate transcription of a number of genes including pro-apoptotic BH3-only genes (B-cell lymphoma homology domain 3) and its own negative regulator, MDM2 (mouse double minute 2 p53 binding protein homolog) [40]. The C. elegans ortholog of p53, cep-1, is presumably activated by similar upstream sensors and transducers, although this has not been biochemically validated [11, 12]. Upon activation, CEP-1 transcriptionally activates the BH3-only gene egl-1 (discussed below) and ced-13, although the physiological relevance of ced-13 activation
is disputable [42]. Finally, despite evidence for mammalian p53 also having transcription-independent cell death functions [43], such a role for *cep-1* has not been described.

**BH3-only/*egl-1**

BH3-only genes encode a subset of proteins belonging to the BCL2 (B-cell lymphoma 2) superfamily that contain anywhere from one to four BH (BCL2 homology) domains [44]. As the name implies, BH3-only proteins have a common BH3 domain that is used to promote cell death [45, 46]. The 9-16 amino acid long BH3 motif is crucial for the interaction of BH3-only proteins with other anti-apoptotic members of the BCL2 superfamily [45]. Studies have shown that the BH3 peptide alone can bind anti-apoptotic BCL2 proteins; furthermore, they have different binding affinities and specificities for anti-apoptotic BCL2 proteins (Figure 1.3) [47]. The two major ways that mammalian BH3-only proteins induce cell death are direct activation of other non-BH3-only pro-apoptotic members such as BAX (BCL2-associated X protein) and BAK (BCL2-antagonist/killer 1) and neutralization of anti-apoptotic proteins such as BCL2, BCL2L1 (BCL2-like 1) and MCL1 (myeloid cell leukemia sequence 1) (discussed below) [48]. The plethora of BH3-only proteins is regulated by a multitude of processes. This includes transcriptional regulation of NOXA [49] and PUMA (p53 upregulated mediator of apoptosis) [50] in response to DNA damage; cleavage of BID (BH3 interacting domain death agonist) by caspase 8 [51]; phosphorylation of BAD (BCL2-associated agonist of cell death) [52, 53] and BIM (BCL2-like 11) [54, 55] by various kinases; and finally, sequestration of BIM and BMF (BCL2 modifying factor) by the dynein motor complex [56] and the myosin V motor complex [57], respectively. As mentioned in the preceding section, *C. elegans* contains only two BH3-only genes, *egl-1* and *ced-13*, that are upregulated in a *cep-1/p53* dependent manner. Biochemical studies have revealed that the EGL-1 protein promotes apoptosis by binding to anti-apoptotic CED-9, thereby releasing the CED-4 adaptor protein and allowing it to
activate the downstream death inducing caspase, CED-3 [5]. None of the post-translational modifications that activate mammalian BH3-only proteins have been reported for EGL-1.

**Figure 1.3: Interaction of BH3-only proteins with the BCL2 family.**

Various BH3-only proteins (triangles) interact with different BCL2 family members (grey) or BAK/BAX in the indirect and direct models, respectively. Activated BAK/BAX form oligomers that act as channels for the release of cytochrome c from the mitochondrial intermembrane space.

BCL2/ced-9

Normal, healthy cells are kept alive by the action of anti-apoptotic BCL2 proteins keeping the mitochondrial outer membrane intact and preventing the release of death-inducing factors such as cytochrome c [44, 58]. Mammalian cells contain several anti-apoptotic BCL2 family members, including BCL2, MCL1, BCL-W (BCL2-like 2) and A1 (BCL2-related protein A1) [59], whereas worms contain only one BCL2 ortholog, ced-9 [4, 60]. Although the mitochondria has traditionally
been viewed as the site of action for BCL2 family members, other membranes including the ER (endoplasmic reticulum) and nuclear membranes also house BCL2 family members [44]. Inhibition of BCL2 family members at different membranes is important for cell death induction by various stimuli [61, 62]. Particularly, in response to DNA damage, BCL2 at the mitochondria must be inhibited by BH3-only proteins for death to occur [59]. Although immunofluorescence studies clearly show mitochondrial localization of CED-9 and its binding to CED-4 (a pro-apoptotic factor, discussed below) in *C. elegans*, which is essential for developmental cell death [8], the same requirement has not been shown for germ cell death in response to DNA damage.

### Apoptosome/ced-4

Upon induction of cell death factors such as cytochrome c escape from the mitochondrial intermembrane space into the cytosol to form a complex with APAF1 (apoptotic peptidase activating factor 1) and CASP9 (cysteiny1 aspartate proteinase caspase 9) [44, 58]. This complex, called the apoptosome, acts as a platform for the activation of downstream effector caspases through the catalytic activity of CASP9 [63-65]. In worms, the APAF1 ortholog, CED-4, is held in an inactive state by CED-9 until it is released when EGL-1 binds to CED-9 and displaces CED-4 [4, 5, 60]. A recent crystal structure of CED-4 revealed that the simplified *C. elegans* apoptosome (composed solely of CED-4 molecules) is an octameric structure [66]. The octameric apoptosome facilitates binding and activation of the CED-3 caspase.

### Caspases/ced-3

Most apoptotic signaling cascades end in the autocatalytic activation of cysteiny1 aspartate proteinases, generally called caspases [67]. Caspases are synthesized as inactive zymogens containing a prodomain, a large subunit (p20) and a small subunit (p10) that are separated by
proteolytic cleavage and reassociated without the prodomain when activated [67]. Mammalian caspases can be divided into two major classes: the initiator caspases and the effector caspases, the former responsible for activating the latter. Both classes of caspases recognize a consensus sequence XEXD and cleave C-terminal to the aspartate residue in the sequence [68]. In the pathway depicted in Figure 1.2, the initiator caspase (CASP9) is responsible for cleaving and activating the downstream effector caspases (CASP3 and CASP7). *C. elegans* encodes only one active caspase required for germ cell death, CED-3, which acts both as an initiator and effector caspase [3, 6, 69]. Once activated, caspases cleave downstream substrates including cytoskeletal proteins, proteins involved in ATP synthesis and RNA binding proteins that ultimately lead to cell death [70, 71].

The peculiarities of *C. elegans* cell death

As described above, all of the major genes that regulate cell death are conserved between *C. elegans* and higher eukaryotes. However, there are key mechanistic differences by which cell death is regulated in mammals and worms. For example, whereas the mitochondria plays an active role in providing death factors for mammalian cells to die [72], *C. elegans* mitochondria seems to play only a passive role keeping ced-9 anchored to its outer membrane [8]. Along the same lines, cytochrome c is not required for *C. elegans* cell death [73]. These observations raise the question of whether there are unidentified death factors that are being secreted from the mitochondria or other subcellular compartments in response to cell death signaling. Furthermore, germ cells are not fully enclosed by a plasma membrane and share a common cytoplasm as a syncytium (Figure 1.1D): they become fully enveloped only if they are dying or if they progress to become oocytes [74]. How certain germ cells are specified to die while others are allowed to live is an open question in the field. To add to the peculiarities of cell death in *C. elegans*, transcriptional reporter studies show
that the promoter for the BH3-only gene egl-1 is activated in response to DNA damage in all germ cells, irrespective of whether they live or die [13]. This suggests that egl-1 activation by cep-1/p53 alone and by inference cell autonomous signals, is insufficient to induce germ cell death and that other cep-1/p53-independent inputs are necessary to promote cell death. What these signals are and how they interact with the known pathway remains an enigma.

Morphological changes during death

One clue as to the identity of cep-1/p53-independent signals may lie in observations made almost 40 years ago that regardless of the species of origin, cells that are dying take on certain morphological characteristics [75]. Changes, such as condensation of the nucleus and cytoplasm, nuclear DNA fragmentation and membrane blebbing (balloon like protrusions on the surface of a cell) are defining features of dying cells (Figure 1.4). More recently, exposure of PS (phosphatidylserine) to the surface of apoptotic cells has been reported to be an early event in apoptosis [76]. This last change has been shown to be an “eat-me” signal for surrounding cells that engulf and ultimately degrade the dying cell in order to recycle cellular components. Presumably, the cell death pathway must be activated and substrates cleaved by caspases for the morphological changes to occur but reports of dying cells being engulfed before presentation of the canonical morphology exist [77]. Therefore, it would be premature to suggest a linear order of events: instead, it is possible that some changes (i.e. exposure of PS) occur concomitant with the activation of cell death, whereas others (i.e. nuclear condensation, membrane blebbing) occur only after specific substrates have been cleaved by the effector caspases. These changes may also lead to clues regarding how specific germ cells die and become engulfed, while others remain unharmed.
Figure 1.4: Morphological events during apoptosis.

When a cell receives a death inducing signal (zigzag), changes such as nuclear/cytoplasmic condensation, DNA fragmentation and PS exposure (green circles) occurs, followed by engulfment by neighbouring cells or specialized macrophages and finally degradation.

Suicide versus murder of cells

A second clue to the identity of *cep-1*/p53-independent signals lies in the fact that cells can respond to extracellular signals to initiate PCD, in addition to the cell autonomous mechanisms described in the preceding sections [78]. Some examples include the T-cell mediated killing ability of FAS ligand bound to FAS in the immune system and the pro-inflammatory cytokine TNF (tumour necrosis factor) bound to the TNF receptor [79]. In both cases, ligand binding to the receptor induces conformational changes to the receptor on the cytoplasmic side, allowing for the formation of a higher order protein complex called the DISC (death inducing signaling complex) [2]. The DISC mediates activation of downstream caspases that initiates the cell death program. Although the majority of cell deaths induced by extracellular signals and receptor signaling are FAS and TNF dependent, they are not the only extracellular signals that regulate cell death. Specifically, expression of the HIV1 (human immunodeficiency virus type 1) envelope protein can induce apoptosis in adjacent, uninfected cells independent of FAS and TNF signaling [80-83]. Studies in *D. melanogaster* have shown that cells expressing MYC (cellular myelocytomatosis) at high levels can...
induce apoptosis in surrounding cells with relatively lower levels of MYC in a phenomenon called cell competition [84, 85]. Cell non-autonomous death also occurs in *C. elegans*: two studies showed that cells responsible for engulfing and degrading dying cells can coax their target to die [86, 87]. Specifically, the researchers observed that some of the extra cells generated through weak mutations in *ced*-3 (i.e. making them unable to die) could be increased by mutations in genes mediating engulfment (i.e. that some deaths require the engulfment genes). They inferred that cell-cell communication must occur between the engulfing cell and indeed studies showed processes extending from the engulfing cell and surrounding the dying cell, mediated by the CED-1 receptor [88]. However, these studies focused on developmentally controlled cell death; therefore, the question remains whether similar mechanisms are at work when germ cells die. Finally, although these examples show that cell death can be induced by a variety of signals stemming from non-dying cells, such a genetic program, where death signals originate non-autonomously, has not been described for PCD in response to DNA damage in *C. elegans* or mammals.

Non-autonomous germline to soma signaling in *C. elegans*

Considering the peculiarities of germ cell death in *C. elegans* and the fact that death can be controlled from outside the dying cell, it is tempting to speculate a similar but novel non-autonomous mechanism for worm germ cell death. A possible conduit through which this type of signaling may occur are the gonadal sheath cells that envelop the dividing cells, physically separating germ cells from the rest of the body. The sheath cells in turn play an important role in regulating the development of its resident germ cells by providing proliferative signals or yolk proteins required for oocyte maturation [89, 90]. Several other developmental processes have been shown to rely on communication between the germline and soma [18, 90-94], although none of
these processes include cell death. Specifically, studies have shown that entry into the dauer stage, which is an alternative life stage that occurs in response to low nutrients, is controlled cell non-autonomously by the insulin-like growth factor receptor DAF-2 [92]. Interestingly, daf-2 has been shown to regulate germ cell death in the worm (Andrew Perrin, unpublished observations), although whether this occurs via germline to soma signaling is unknown. Worm lifespans are shortened by the presence of an intact germline that communicates with the somatic gonad (i.e. gonadal sheath cells); ablating germline precursor cells result in an increase in lifespan, whereas ablation of both germline and somatic gonad precursor cells has no effect on worm lifespan [95]. The DTC (distal tip cell) located at the distal end of the somatic gonad arm uses Notch signaling to keep germ cells proliferating mitotically; ablation of DTCs causes germ cells to enter meiosis ectopically leading to sterility [91, 96]. The spatial/temporal patterning of the germline (where/when mitotic germ cells enter meiosis, exit pachytene arrest, mature to oocytes and become fertilized) depends on interactions between the germline and the somatic gonad [90, 93, 94]. Finally, a physical link between the soma and germline was observed by electron microscopy studies that revealed processes extending from somatic sheath cells to oocytes in the germline and forming gap junctions between these two cell types, suggesting a possible passageway by which cross-tissue signaling occurs [89]. Together, these observations provide strong evidence for a soma-germline signaling pathway, which might also contribute cell death signals to the germline.

A novel regulator of cell death: kri-1

Recent work in *C. elegans* longevity signaling revealed a gene called *kri-1*, the ortholog of human KRIT1 (Krev interaction trapped 1), to be responsible for integrating germline signals in the soma (i.e. cell non-autonomous effects) to ultimately control the nuclear localization of the forkhead
transcription factor DAF-16 [97]. Furthermore, an RNAi (RNA interference) screen revealed a genetic interaction between the worm ortholog of p53, *cep-1*, and *kri-1* (W. Brent Derry, unpublished results). This suggested a possible role for *kri-1/KRIT1* in regulating cell death via a novel pathway involving germline to soma communications. Interestingly, a genetic interaction between KRIT1 and p53 was also observed in mice, further supporting this hypothesis [98]. In humans, KRIT1 is frequently mutated in the familial form of the neurovascular disease CCMs (cerebral cavernous malformations) [14, 15]. In addition, two other CCM genes, CCM2 and CCM3, are also mutated in inherited CCMs [99-103]. CCMs can also occur sporadically without evidence for germline mutations in one of these three genes [104]. The disease is characterized by vessel walls, primarily in the central nervous system, that lack supportive tissue such as smooth muscles. This causes the vessel walls to weaken, which can rupture, causing haemorrhage, stroke and death [105].

As alluded to above, a mouse model of this disease showed that although heterozygous KRIT1 (KRIT1+/−) mice do not develop vascular lesions associated with CCMs, heterozygous KRIT1 mice in a p53 null mutant background (p53−/−) develop CCMs [98]. Loss-of-heterozygosity was suspected as a cause for this observation; however, the authors could not find evidence for it when they sequenced the tissues of the lesions, suggesting a genetic interaction between KRIT1 and p53. Since p53 and its worm counterpart *cep-1* are central mediators of cell death specifically in response to DNA damage, these studies and observations implicate both KRIT1 and *kri-1* in novel, cell death regulating roles.

Known roles of KRIT1 and *kri-1*

KRIT1 was originally identified as a RAP1A (RAS-related protein 1A) interacting protein in a yeast two-hybrid screen [106]. Several other interacting partners are known for KRIT1, including ICAP1
(integrin cytoplasmic associated protein 1) [107, 108], microtubules [109] and the HEG1 (heart of glass) receptor [110]. In vivo, these interactions are important for proper cardiac development in zebrafish [111] as well as mice [112]. Molecularly, KRIT1 has been shown to bind to CCM2 and shuttle in and out of the nucleus to participate in the MAPK (mitogen activated protein kinase) signaling pathway [113]. In C. elegans, kri-1 is required for relocalization of the forkhead transcription factor DAF-16 to the nucleus when the longevity signaling pathway is active [97]. It is also involved in fat metabolism, by regulating the transcript levels of a triglyceride lipase gene K04A8.5 [114]. However, neither KRIT1 nor kri-1 has been linked to PCD.

Regulation of KRIT1 and kri-1

Both KRIT1 and kri-1 encode proteins containing N-terminal NPxY/F motifs, reserved for binding to PTB (phosphotyrosine binding) domains, an ankyrin repeat domain for protein-protein interactions and a FERM (band 4.1, ezrin, radixin, moesin and merlin) domain for interacting with cytoskeletal proteins (Figure 1.5) [115-117]. Structural homology studies suggest KRIT1 adopts open and closed conformations that may be a method for regulating its function [118]. The FERM domain, which can be subdivided into the F1 lobe that resembles an ubiquitin-like fold, the F2 lobe that folds into a subdomain resembling acyl-CoA binding proteins and the F3 lobe that has high structural similarity to PTB domains, plays an important role in the proposed open and closed conformations. Specifically, the F3 subdomain can interact with the second and third NPxF motifs in the N-terminus of KRIT1. This intramolecular interaction may prevent KRIT1 from interacting with other proteins by masking the ankyrin repeat domains typically used for protein-protein interactions, until post-translational modifications restructure KRIT1 into an open conformation. Similarly, other FERM domain proteins such as ezrin [119, 120], moesin [121, 122], radixin [123, 124],
merlin [125, 126] and talin [116] have all been reported to undergo head-to-tail intermolecular interactions that regulate their function. Despite the clues that KRIT1 may be regulated post-translationally in an open and closed conformation, it is not known whether C. elegans kri-1 is regulated in a similar manner. The only known functional characteristic of kri-1 is the fact that GFP (green fluorescent protein) tagged KRI-1 is expressed in somatic intestinal and pharyngeal cells of C. elegans [97], an observation supported by microarray data that excludes kri-1 expression from the germline [127].

Figure 1.5: Protein domain organization of KRIT1 and KRI-1.
The known protein domains of human KRIT1 (left) and C. elegans KRI-1 (right) are shown. An uncharacterized N-terminal domain is followed by three NPxY/F motifs (dark green), an ankyrin repeat domain (orange) and a C-terminal FERM domain (light green). The amino acid positions of the NPxY/F motifs are shown with their respective sequences.
Other FERM domains in apoptosis

Although neither KRIT1 nor kri-1 have been implicated in regulating PCD, other FERM domain proteins have been shown to be involved in the cell death process. Notably, FAK (focal adhesion kinase) can regulate downstream signaling cascades that direct cell proliferation and survival through its indirect interactions with integrins [128]. The FERM domain of FAK plays a key role in regulating its kinase activity, which can initiate the cell death program through p53. However, FAK has also been shown to interact directly with p53 [129] and enhance its degradation [130] providing evidence for a contradictory anti-apoptotic role for FAK. Clearly, further research is required to resolve these apparent contradictions. Another FERM domain protein, FRMD3 (FERM domain containing protein 3), was recently shown to be downregulated in non-small cell lung carcinomas and its re-expression in cell models lead to an increase in caspase-dependent cell death [131]. Finally, studies show that the FERM domain proteins Merlin and Expanded can regulate the hippo signaling pathway, which controls cell proliferation and apoptosis in D. melanogaster [132-134], by modulating the activity of the downstream transcriptional coactivator Yorkie [135, 136]. In addition, Merlin can inhibit HDM2-dependent (human murine double minute 2) degradation of p53, thereby stabilizing p53; however, this study was done using a single cell line and the question remains whether this is relevant in vivo [137]. Despite the contradictions in the functions of different FERM domain proteins, the fact that they are important for the regulation of cell death in certain contexts is difficult to dispute.

Suicide and murder of cells in disease

Why is it so important to determine the intricacies of cell death regulation? During the course of the development and life cycle of an organism, certain cells become either obsolete or damaged,
which must be removed lest they proliferate and release toxic molecules or spread potentially deleterious mutations. Otherwise, the persistence of damaged or unneeded cells can lead to a wide variety of developmental defects and diseases [1]. For example, during development of some metazoan limbs, the spaces between the digits are initially filled with cells that are later eliminated by PCD [138]. Disrupting PCD can lead to webbed digits in metazoans whose digits are normally separated [138]. In another example, studies in the fruit fly *Drosophila melanogaster* have shown that cell death controlled by the *hippo* pathway plays a key role in regulating organ size and that misregulation of this process can lead to oversized organs [134, 139]. Coupling the failure to undergo PCD with over-proliferation of cells leads to cancers such as B-cell lymphomas [140]. Conversely, abnormal increases in PCD is one of the outcomes of degenerative diseases such as Alzheimer's disease [141]. Interestingly, many neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, spinocerebellar ataxias and prion diseases have all been demonstrated to include cell death by non-autonomous mechanisms in their pathologies [142]. Specifically, studies by various researchers has shown that mutations in genes known to cause disease – SOD1 (superoxide dismutase 1) in ALS, α-synuclein in Parkinson's disease, huntingtin in Huntington's disease – can cause disease even when the mutations occur in cells surrounding the primary target neuron such as astrocytes, microglial cells and oligodendrocytes [142]. Although the exact mechanism of PCD induction varies depending on the stress response, these studies nonetheless demonstrate the importance of considering not just the cell that is dying but also the surrounding cells that may contribute to the death program.
The unanswered questions

Cell death in response to DNA damage has traditionally been thought to occur via cell autonomous mechanisms, controlled by the p53 master regulator. However, mounting evidence in mammals and worms suggest the existence of p53-independent pathways and a cell non-autonomous mechanism of cell death. By using *C. elegans* as an *in vivo* model for cell death, the intricate interplay of different cell types can be studied in a genetically simplified background. Specifically, I asked whether cell death caused by DNA damage is regulated solely by p53 and whether it occurs cell autonomously. The answers to these questions will shed light on the intricacies of cell death regulation in *C. elegans* and may eventually provide insight into how cells in the tumour microenvironment can be better targeted for therapies.
2. *kri-1* regulates germ cell death
kri-1 is required for germ cell death

In an RNAi screen unrelated to apoptosis, we serendipitously uncovered a cep-1/p53 interacting gene, kri-1, the ortholog of human KRIT1/CCM1 which is frequently mutated in the neurovascular disease CCMs [14, 15]. Because this gene had been previously shown to integrate signals from reproductive tissues (germ cells) to elicit longevity effects in non-reproductive (somatic) tissues [97] and it interacted with cep-1, an important mediator of germ cell death (Figure 1.2) [11, 12], we asked whether kri-1 is involved in a novel, cell non-autonomous mechanism to regulate germ cell death.

To test this, we first investigated whether kri-1 regulated cell death like cep-1, by quantifying the number of germ cell corpses in wild-type animals fed bacteria producing double stranded RNA against a control gene or kri-1 exposed to IR (ionizing radiation) (Figure 2.1A). We found that knockdown of kri-1 by RNAi significantly reduced the number of germ cell corpses after DNA damage (IR) compared to animals fed control(RNAi) (p=0.01), suggesting that kri-1 is required for germ cell death. We verified this initial observation by performing a dose response analysis of the kri-1(ok1251) deletion mutant. In contrast to wild-type animals, kri-1(ok1251) deletion mutants did not exhibit an increase in germ cell apoptosis after exposure to increasing doses of IR (Figure 2.1B, C). This was reminiscent of cep-1(lf) (loss of function) mutants that are also resistant to IR-induced apoptosis.
Figure 2.1: kri-1 is required for germ cell death.

(A) Wild-type animals fed control(RNAi) (black) or kri-1(RNAi) (white) were subjected to ionizing radiation (IR) at 20°C and germ cell apoptosis was quantified 24h later. Data represent mean ± SEM (standard error of the mean) of three independent experiments and at least 55 germlines in total per strain per condition. The asterisk (*) represents statistically significant results (p<0.05) when compared to wild-type animals. See Table 6.3 for a full list of p-values.

(B) Synchronized wild-type (black), cep-1(lf) (grey) and kri-1(ak1251) (white) young adult animals were treated with increasing doses of IR and germ cell apoptosis was scored as above. Data represent mean ± SEM of at least three independent experiments and at least 50 germlines in total per strain per condition. *p<0.05 versus wild-type.

(C) To increase the sensitivity of the apoptosis assay, kri-1(ak1251) mutants were crossed into the engulfment defective mutant ced-5(lf) strain in which germ cell corpses persist and consequently exhibit increased numbers of corpses. Germ cell death was quantified as above. Data represent mean ± SEM of three independent experiments and at least 40 germlines in total per strain per condition. *p<0.05 versus wild-type.
Therefore, we examined whether *kri-1* regulates germ cell death specifically, like *cep-1*, or whether *kri-1* regulates cell death in all cells, like *ced-3*, by quantifying apoptosis in developing embryos of wild-type, *cep-1(lf)* mutants and *kri-1(ok1251)* mutants. We found that developmental cell death was unaffected in *kri-1(ok1251)* mutants, suggesting that the regulation of cell death by *kri-1* is specific to germ cells, like *cep-1* (Figure 2.2).

Figure 2.2: *kri-1* does not regulate developmental cell death.
Embryos at the indicated developmental stages were scored for apoptosis using Nomarski optics in wild-type (black), *cep-1(lf)* (grey) and *kri-1(ok1251)* (white) animals. Data represent mean ± SEM of three independent experiments and at least 45 embryos in total per strain per condition.

To determine whether the *ok1251* allele behaved as a null, we performed a deficiency analysis by crossing *ok1251* into a strain containing the *hDf9* deficiency that removes the *kri-1* locus and quantified the number of germ cell corpses after DNA damage (Figure 2.3). Strains containing the *ok1251* allele in *trans* to *hDf9* were as resistant to damage-induced germ cell apoptosis as *ok1251* homozygotes, suggesting that *ok1251* is a null allele. Collectively, these and further observations
(see below) indicate that *kri-1* is specifically required for germ cell death in response to DNA damage.

**Figure 2.3: *kri-1(ok1251)* is a null mutant.**

Apoptosis was scored in wild-type animals (black), a strain with a wild-type copy of *kri-1* in *trans* to the *hDf9* deficiency (grey) and *ok1251* in *trans* to *hDf9* (white) treated with IR as above. Data represent mean ± SEM of at least four independent experiments and at least 25 germlines in total per strain per condition. The open circle (°) represents statistically significant results (p<0.01) when *kri-1(ok1251)/hDf9* was compared to +/hDf9.

*kri-1* functions downstream of the checkpoint and *cep-1*

Given that *kri-1* is required to promote germ cell death in response to DNA damage, we were interested to know at which step in the apoptosis pathway it might be functioning (Figure 1.2). In the *C. elegans* germline, the DNA damage checkpoint genes (*hpr-9, mrt-2, hus-1* and *clk-2*) are required to both transiently arrest mitotic proliferation and activate *cep-1*-dependent apoptosis of damaged germ cells [7, 10, 13]. To ascertain if *kri-1* was functioning in an analogous manner (i.e.
upstream of *cep-1*), we tested whether *kri-1(0)* (null) mutants mimic the germline phenotypes of checkpoint gene mutants. In contrast to *clk-2* mutants that are defective in cell cycle arrest, we found that *kri-1* was not required for IR-induced arrest of mitotically proliferating cells (Figure 2.4), implying that *kri-1* acts downstream or independently of the DNA damage checkpoint.

Downstream of the checkpoint, *cep-1* initiates the apoptosis program by upregulating the pro-apoptotic *egl-1* gene. Because *kri-1* functions downstream of the checkpoint, we considered the possibility that *kri-1* might regulate *cep-1* activity. To delineate whether this is the case, we examined the activity of CEP-1 by quantifying the transcript levels *egl-1* in *kri-1(0)* mutants [42, 143]. Consistent with previous work, *egl-1* transcript levels as assessed by real-time qPCR (quantitative PCR), increased in response to DNA damage in wild-type animals but not in *cep-1(lf)* mutants (Figure 2.5). Interestingly, *egl-1* induction in *kri-1(0)* mutants was similar to that seen in wild-type animals, indicating that the transcriptional activity of CEP-1 is induced normally in the absence of *kri-1*. This is consistent with *kri-1* promoting damage-induced apoptosis downstream or independently of *cep-1*. 
Figure 2.4: kri-1 functions downstream of the checkpoint.

(A) Synchronized hermaphrodites at the L4 (fourth larval) stage were treated with IR and the number of nuclei per unit area in the mitotic region of the germline was quantified 24h later at 20°C. The mitotic region and nuclei have been outlined for clarity. Representative images from three independent experiments are shown.

(B) Quantification of nuclei shown in (A) in wild-type (black), clk-2(If) (dark grey), cep-1(If) (light grey) and kri-1(0) (white) animals. The partial cell cycle arrest in clk-2(If) mutants is likely due to contributions from an intact parallel pathway regulated by hpr-9/hus-1/mrt-2. Data represent mean ± SEM of at least three independent experiments and at least 35 germlines in total per strain per condition. *p<0.01 versus wild-type worms. °p<0.01 versus clk-2(If) worms.
Figure 2.5: *kri-1* does not affect *egl-1* mRNA levels.

RNA was isolated by TRIzol from synchronized wild-type (black), *cep-1(1f)* (grey) and *kri-1(0)* (white) mutants and *egl-1* transcript levels were measured by quantitative real-time PCR. Data represent mean ± SEM of three independent experiments.

Such a model raised the possibility that *cep-1* may regulate *kri-1* transcription or KRI-1 protein localization in response to DNA damage to promote germ cell death. However, neither *kri-1* transcript levels (Figure 2.6) nor GFP::KRI-1 localization (Figure 2.7) were significantly affected by IR or *cep-1* status.
**Figure 2.6: cep-1 does not affect kri-1 mRNA.**

RNA was extracted from synchronized wild-type (black) and cep-1(lf) (grey) young adult animals and kri-1 transcript levels were quantified as above. The primers were designed to amplify a region within the kri-1 deletion. Data represent mean ± SEM of three independent experiments.
Figure 2.7: cep-1 does not affect KRI-1 protein.

(A) kri-1(0); muEx353 worms were fed either control (RNAi) (top) or cep-1 (RNAi) (bottom) and subjected to 60 Gy of IR. The GFP::KRI-1 signal was visualized 24h post-IR. White boxes indicate areas that are enlarged in the right panel of each image. Arrowheads highlight apical membrane localization which does not change in response to IR or loss of cep-1 by RNAi. Representative images from three independent experiments are shown.

(B) Quantification of cell death in the germline of wild-type (black) and kri-1(0); muEx353 (white) worms fed control (RNAi) or cep-1 (RNAi) as in (A). Data represent mean ± SEM of three independent experiments and at least 35 germlines in total per strain per condition.
**kri-1 does not act on ced-3 or ced-4**

The data above suggests a model wherein *kri-1* functions downstream of or in parallel to the key decision making step in the cell death pathway and likely regulates components of the core death pathway (i.e. *egl-1, ced-9, ced-4* and *ced-3*). To investigate this further, we examined the epistatic relationship between *kri-1* and *ced-9*. Healthy cells require functional CED-9/BCL2 to prevent ectopic activation of CED-3/caspase by CED-4 (Figure 1.2). We reasoned that if *kri-1* functions downstream of *ced-9*, ablation of *kri-1* would suppress the increased cell death caused by *ced-9(lf)*; on the other hand, the converse would be true if *kri-1* acted upstream of *ced-9*. Knockdown of *ced-9* by RNAi (>50% knockdown, Figure 2.8A) caused a significant increase in apoptosis both before and after DNA damage, but this was unaffected by loss of *kri-1* (Figure 2.8B), which was confirmed in *kri-1(0); ced-9(lf)* double mutants (data not shown). This indicates that *kri-1* is not functioning strictly downstream of *ced-9* (i.e. in a manner similar to *ced-4* or *ced-3*). To be sure, we quantified the mRNA of both *ced-4* and *ced-3* by qPCR and found that their levels were not affected in *kri-1(0)* mutants in response to IR (Figure 2.8C, D); in addition, CED-4 protein expression and localization was not affected in *kri-1(0)* mutants (data not shown). Therefore, we infer from these results that *kri-1* acts upstream of, or parallel to *ced-9*. 


Figure 2.8: kri-1 does not act strictly downstream of ced-9.

(A) Immunoblot (top panel) and quantification (bottom panel) of CED-9 protein in wild-type fed control(RNAi) (lane 1), wild-type fed ced-9(RNAi) (lane 2), kri-1(0) fed control(RNAi) (lane 3) and kri-1(0) fed ced-9(RNAi) (lane 4). α-tubulin was used as an internal loading control. Quantification is relative to wild-type fed control(RNAi) (lane 1).

(B) Synchronized wild-type and kri-1(0) L4 animals fed control(RNAi) (black and white, respectively) or ced-9(RNAi) (dark grey and light grey, respectively) were subjected to IR and germ cell death was quantified. Data represent mean ± SEM of three independent experiments and at least 25 germlines in total per strain per condition. *p<0.01 versus wild-type worms. °p<0.01 versus kri-1(0); control(RNAi) worms.

(C-D) RNA was extracted from synchronized wild-type (black) and kri-1(0) (white) young adult animals and ced-3 (C) or ced-4 (D) transcript levels were quantified. Data represent mean ± SEM of four independent experiments.
**kri-1** does not act through known **cep-1**-independent pathways

Because **kri-1** functions independently of **cep-1** and impinges on the core death pathway, we were interested to know whether **kri-1** was cooperating with other genes known to regulate germ cell death independently or downstream of **cep-1**. In particular, the histone deacetylase **sir-2.1** [144], MAP kinase **pmk-3** [145] and RB (retinoblastoma) ortholog **lin-35** [146] have all been shown to regulate germ cell death independently of **cep-1**. In addition to activating cell death independently of **cep-1** in a manner similar to **kri-1**, the SIR-2.1 protein exits the nuclei of germ cells after DNA damage [144]. To determine whether the relocalization of SIR-2.1 is required for **kri-1**-mediated germ cell death, we immunostained **kri-1(0)** animals with SIR-2.1 antibodies to ascertain whether SIR-2.1 protein levels or localization were altered. We found that **kri-1** did not affect the SIR-2.1 protein staining pattern (Figure 2.9), which suggests that **kri-1** does not function through **sir-2.1**. To provide further evidence that these two genes were in different pathways, we created a double heterozygous mutant containing both the **kri-1(0)** and **sir-2.1(lf)** mutations (**kri-1(0)/+; sir-2.1(lf)/+**) and quantified the number of germ cell corpses after DNA damage. We reasoned that reducing signaling through a pathway by weak (i.e. heterozygous) mutations in two genes would have the same effect as completely ablating one gene, provided that the two genes are in the same pathway. However, we found wild-type levels of germ cell apoptosis in response to DNA damage in the double heterozygous mutants (data not shown), suggesting that these genes operate in different pathways.
Figure 2.9: *kri-1* does not act through SIR-2.1.

Wild-type and *kri-1(0)* animals were immunostained with DAPI or SIR-2.1 antibodies before and after IR. The images show the pachytene region of the germline. White arrowheads indicate nuclei that are positive for DAPI staining but that are negative for SIR-2.1 protein expression. Representative images of at least three independent experiments are shown. The images were provided by S. Greiss and A. Gartner.

In contrast to *sir-2.1* and *kri-1* that positively regulate germline apoptosis, the MAP kinase gene *pmk-3* inhibits germline apoptosis independently of *cep-1* [145]. Since the apoptotic phenotypes of *pmk-3(lf)* and *ced-9(lf)* mutants mimic each other, we employed the same reasoning (i.e. that an inability to suppress the increase in apoptosis in *pmk-3(lf)* mutants indicates that *kri-1* functions upstream of *pmk-3*) and created *kri-1(0); pmk-3(lf)* double mutants. We found that in the double mutants, germ cell death was suppressed to the same degree as *kri-1(0)* single mutants (Figure 2.10A), suggesting that *kri-1* is epistatic to *pmk-3* and that *kri-1* does not regulate cell death through *pmk-3*. The non-significant increase observed in the double mutant is likely a contribution from physiological cell death, which *pmk-3* has been shown to regulate negatively [145]. It is unlikely that *pmk-3* regulates *kri-1* because *kri-1* transcript levels and protein localization remain unchanged in *pmk-3(lf)* mutants (Figure 2.10B, C).
Figure 2.10: kri-1 functions independently of pmk-3.

(A) Wild-type (black), kri-1(0) (white), pmk-3(lf) (dark grey) and kri-1(0); pmk-3(lf) (light grey) animals were synchronized and scored for apoptosis as previously described. Data represent mean ± SEM of three independent experiments and at least 50 germlines in total per strain per condition. *p<0.05 versus wild-type worms. °p<0.05 versus pmk-3(lf) mutants.

(B) kri-1 transcript levels were quantified as previously described in synchronized wild-type (black), kri-1(0) (white) and pmk-3(lf) (grey) mutants. Data represent mean ± SEM of three independent experiments.

(C) The GFP::KRI-1 signal was visualized 24h post-IR in synchronized pmk-3(lf); gfp::kri-1(+) animals. Representative images from three independent experiments are shown.

Finally, since lin-35 positively regulates germ cell apoptosis by controlling the levels of the CED-9 protein (i.e. loss of lin-35 leads to an increase in CED-9 protein levels) [146], we tested whether kri-1 functions through lin-35/RB by quantifying CED-9 protein levels in kri-1(0) animals by
Western blot. We found that CED-9 protein levels were unaffected in kri-1(0) mutants (Ashley Ross, personal communication and data not shown), suggesting that kri-1 does not regulate germline apoptosis through this pathway.

Additionally, it has been shown that kri-1 influences the localization of the forkhead transcription factor DAF-16 in the intestine of worms by responding to signals from the germline and regulating worm lifespans [97] and that DAF-16 may negatively regulate IR-induced germ cell apoptosis [143]. These two pieces of evidence suggested kri-1 might function through daf-16 to regulate germ cell death. Animals fed daf-16(RNAi) exhibited wild-type levels of germ cell death in response to IR (Figure 2.11A), consistent with published results reporting that DAF-16 has a weak effect on germ cell death [143, 144]. We tested whether kri-1(0) could suppress apoptosis in animals fed daf-16(RNAi) and found that it did (Figure 2.11A), which we confirmed by creating kri-1(0) daf-16(lf) double mutants (data not shown). This suggests that kri-1 does not require daf-16 to mediate its apoptotic function. Alternatively, it was possible that daf-16 regulates kri-1 to mediate germ cell death; however, neither kri-1 transcript nor protein levels were significantly affected in daf-16(lf) mutants (Figure 2.11B, C). These observations suggest that kri-1 regulates germ cell death in a novel, previously uncharacterized pathway.
Figure 2.11: kri-1 does not function through daf-16.

(A) Germline apoptosis was quantified in synchronized wild-type and kri-1(0) mutants fed control(RNAi) (black and white, respectively) or daf-16(RNAi) (dark and light grey, respectively) treated with IR as previously described. Data represent mean ± SEM of four independent experiments and at least 25 germlines in total per strain per condition. *p<0.01 versus wild-type worms. °p<0.01 versus daf-16(RNAi) mutants.

(B) kri-1 transcript levels were quantified as described above in synchronized wild-type (black), kri-1(0) (white) and daf-16(lf) (grey) mutants. Data represent mean ± SEM of three independent experiments.

(C) The GFP::KRI-1 signal was visualized 24h post-IR in synchronized daf-16(lf); gfp::kri-1(+) animals. Representative images from three independent experiments are shown.

kri-1 functions cell non-autonomously to regulate death

There are two possible mechanisms by which kri-1 may promote germ cell death. The first is through a cell autonomous mechanism, where kri-1 regulates the core death pathway (EGL-1 or
CED-9) in germ cells to initiate cell death. Alternatively, it is possible that *kri-1* regulates cell death outside germ cells (i.e. from somatic cells) via a novel pathway. In support of the latter hypothesis, *kri-1* is required to extend the lifespan of worms through its effects on DAF-16 in the intestine, possibly by receiving signals from germ cells [97]; in addition, microarray data suggests that *kri-1* is not expressed in the germline [127]. To distinguish between these possibilities, we took advantage of tissue-specific RNAi in *C. elegans* and selectively knocked down *kri-1* in germ cells and the soma using *rrf-1(0f)* [147] and *ppw-1(0f)* [148] mutants, respectively, and quantified IR-induced germ cell apoptosis [146]. Wild-type, *rrf-1(0f)* and *ppw-1(0f)* mutants fed bacteria producing *control(RNAi)* had similar numbers of germ cell corpses after DNA damage (Figure 2.12; Figure 2.13). Ablation of *kri-1* by RNAi in wild-type animals inhibited DNA damage-induced germ cell apoptosis to the same extent as *kri-1(0f)* mutants (Figure 2.12).
Figure 2.12: kri-1(RNAi) causes resistance to apoptosis.

Synchronized L4 hermaphrodites were fed either control(RNAi) (black) or kri-1(RNAi) (white) and treated with 60Gy of IR as described. The white colour in the schematic diagram of the adult worm (top) represents the absence of kri-1 expression in all tissues when fed kri-1(RNAi). Data represent mean ± SEM of three independent experiments and at least 35 germlines in total per strain per condition. *p<0.01 versus wild-type fed control(RNAi).

However, selective knockdown of kri-1 in germ cells using rrf-1(If) mutants did not inhibit IR-induced apoptosis, suggesting that kri-1 expression in germ cells is not required to promote apoptosis. Conversely, specific knockdown of kri-1 in the soma in ppw-1(If) mutants prevented germ cell death, suggesting that kri-1 is required in somatic tissue to regulate germ cell death (Figure 2.13).
**Figure 2.13: kri-1 expression is required in the soma to regulate cell death.**

Synchronized L4 hermaphrodites were fed either control(RNAi) (black) or kri-1(RNAi) (white) and treated with IR. Knockdown of kri-1 specifically in the germline in rrf-1(If) mutants (left) is represented by the absence of the green colour in the germline. Conversely, knockdown of kri-1 specifically in the soma in ppw-1(If) mutants (right) is represented by the presence of the green colour in the germline and its absence in the soma. Data represent mean ± SEM of three independent experiments and at least 35 germlines in total per strain per condition. *p<0.01 versus ppw-1(If) mutants fed control(RNAi).

In support of this contention, we were able to rescue damage-induced germ cell apoptosis to wild-type levels by expressing GFP::KRI-1 from a somatic extrachromosomal array (Figure 2.14). While it is possible that low-level expression of GFP::KRI-1 in the germline may account for this observation, the fact that extrachromosomal arrays are generally silenced in the *C. elegans* germline [149] strongly supports a model in which kri-1 is required in non-dying somatic cells to promote germ cell death.
**Figure 2.14: kri-1 regulates death cell non-autonomously.**

(A) GFP::KRI-1 expressed under the control of the endogenous kri-1 promoter is detectable in the pharynx (p) and intestine (i) of transgenic animals (top panel). GFP::KRI-1 is excluded from the germline in unirradiated animals (bottom left) and does not change localization after irradiation (bottom right). Representative images of at least three independent experiments are shown.

(B) Apoptotic germ cells were quantified in wild-type (black), kri-1(0) (white) and a kri-1(0) strain expressing a wild-type copy of GFP::KRI-1 in the soma (dark grey). Data represent mean ± SEM of three independent experiments and at least 40 germlines in total per strain per condition. *p<0.01 versus wild-type worms. °p<0.01 versus kri-1(0) mutants.
3. *daf-2* regulates germ cell death
From *kri-1* to insulin signaling via starvation

As discussed in previous chapters, non-autonomous signaling is important for many developmental processes including cell death not only in *C. elegans* but also in other multicellular eukaryotes. One of the most important uses for non-autonomous signaling is during a response to external conditions. In *C. elegans*, dauer formation occurs in response to three major environmental triggers – population density, nutrient availability and temperature – and is activated via a cell non-autonomous mechanism [150]. The well characterized insulin-like signaling pathway is one such pathways that can regulate dauer formation, specifically in response to nutrient availability [151]. The pathway is switched on under conditions of nutrient abundance when insulin-like molecules bind to the DAF-2 insulin-like receptor, which sets off a signaling cascade resulting in phosphorylation and activation of AGE-1, PDK-1 and ultimately AKT-1 (Figure 3.1) [152]. The activation of AKT-1 results in phosphorylation of many downstream targets including the transcription factor DAF-16, which when excluded from the nucleus is unable to transcribe starvation response genes such as those regulating dauer formation [153, 154]. When nutrients are low, however, the pathway is inactive leading to nuclear localization of DAF-16 and dauer formation. Based on the finding that *kri-1* can regulate DAF-16 nuclear localization [97], we wondered whether *kri-1* could also control starvation stress responses. Although *kri-1* mutants were not defective in dauer formation or recovery, they were sensitive to starvation conditions compared to wild-type animals (Figure 3.2). Interestingly, *akt-1* gain-of-function (gf) mutants showed a similar phenotype, suggesting a possible link between these two genes. To determine whether such a link existed, we constructed a *kri-1(0); akt-1(gf)* double mutant and assessed its ability to survive under starvation conditions. Surprisingly, the double mutant survived longer than
either single mutant alone, but shorter than wild-type animals, indicating a complex regulatory link between these two genes.

**Figure 3.1: The insulin-like signaling cascade.**
A series of phosphorylation events eventually leads to the phosphorylation of the DAF-16 transcription factor, which becomes excluded from the nucleus. This pathway can regulate processes including starvation response [153], lifespan [16] and cell death [143].

**Figure 3.2: kri-1(0) mutants are sensitive to starvation stress.**
Synchronized L1 wild-type (black), kri-1(0) (red), akt-1(gf) (blue) and kri-1(0); akt-1(gf) (green) worms were starved in buffer for 0-15 days at 20°C and removed to media containing bacteria (food) every three days. The numbers of adult worms that recovered from the starvation stress were quantified and these values were normalized to day 0 counts. Data represent mean ± SEM of at least six independent experiments with three technical repeats per data point per strain.
Insulin signaling and apoptosis

The link between kri-1 and akt-1 prompted us to determine whether these genes were involved in the same pathway to regulate cell death. We previously showed that AKT-1, a central component of the insulin-like signaling pathway in C. elegans, could regulate germ cell death by affecting the phosphorylation status of CEP-1/p53 [143]. Specifically, we showed that apoptosis in akt-1(lf) mutants was elevated compared to wild-type animals after IR. We hypothesized that if kri-1 was downstream of akt-1, then the elevated apoptosis in akt-1(lf) mutants would be suppressed; conversely, if kri-1 was upstream of akt-1, then the suppression would not occur. Quantification of cell death after IR revealed that cell death in kri-1(0); akt-1(lf) mutants were similar to kri-1(0) single mutants, suggesting that kri-1 is epistatic to akt-1 (Figure 3.3). We also assayed whether this was true for the redundant paralog, akt-2; indeed, the increased apoptosis of akt-2(lf) mutants was suppressed by mutations in kri-1 (Figure 3.4). Taken together, these results suggest a link between insulin signaling pathway components and apoptosis and that kri-1 can modulate apoptotic signaling downstream of or in parallel to akt-1 and akt-2.
Figure 3.3: *kri-1* regulates cell death downstream of *akt-1*.
Synchronized young adult wild-type (black), *kri-1(0)* (white), *akt-1(lf)* (light grey) and *kri-1(0); akt-1(lf)* (dark grey) worms were subjected to IR. Data represent mean ± SEM of two independent experiments and at least 55 germlines in total per strain per condition.

Figure 3.4: *kri-1* regulates cell death downstream of *akt-2*.
Synchronized young adult wild-type (black), *kri-1(0)* (white), *akt-2(lf)* (light grey) and *kri-1(0); akt-2(lf)* (dark grey) worms were subjected to IR. Data represent mean ± SEM of two independent experiments and at least 55 germlines in total per strain per condition.
Non-insulin pathway dauer genes do not regulate cell death

The findings above, that components of the insulin signaling regulates germ cell death in *C. elegans*, did not exclude the possibility that other dauer formation pathways could also regulate cell death. At least three other pathways regulate the formation of dauers, including the guanylyl cyclase pathway (*daf-1, 4, 7, 8, 11 and 14*), the TGF-β-like pathway (*daf-1, 3, 4, 5, 7, 8 and 14*) and the steroid hormone pathway (*daf-9 and daf-12*) [150]. Similar to *kri-1*, the steroid hormone pathway components *daf-9* and *daf-12* both regulate DAF-16 nuclear localization [97]. To test whether these genes (*daf-9*/*cytochrome P450* and *daf-12*/*steroid hormone receptor* [95]) also control germ cell death possibly through *kri-1*, we fed *kri-1*(RNAi) to *daf-9*(lf) and *daf-12*(lf) mutants and subjected them to IR. As shown in Figure 3.5, we found that neither mutant significantly regulated germ cell death in response to IR. Furthermore, both mutants were resistant to apoptosis when fed *kri-1*(RNAi), similar to wild-type worms fed *kri-1*(RNAi), suggesting that *kri-1* does not act through these two genes. These observations suggest that the regulation of germ cell death by dauer regulatory genes is specific to the insulin signaling pathway.
Figure 3.5: Neither daf-9 nor daf-12 regulates germ cell death.
Wild-type (black), daf-9(lf) (light grey) and daf-12(lf) (dark grey) animals were fed either control(RNAi) (left) or kri-1(RNAi) (right) and treated with IR as above. Data represent mean ± SEM of at least two independent experiments and at least 20 germlines in total per strain per condition.

daf-2 regulates germ cell death
Recent studies on the insulin signaling pathway have revealed a surprising role for other pathway components such as daf-2 in regulating C. elegans apoptosis (Figure 3.6; Andrew Perrin, personal communication). The insulin pathway regulates starvation stress or lifespan through a canonical, linear order of phosphorylation events. However, under genotoxic stress, this canonical pathway is drastically rearranged to regulate cell death. As described above, the DAF-16 transcription factor is an important downstream target of AKT-1 in the canonical pathway, but its role in cell death signaling is minimal, suggesting the existence of other AKT-1 targets. Furthermore, daf-2 appears to be epistatic to (i.e. downstream of) akt-1; whereas age-1 and pdk-1 may be in a separate, parallel pathway (Andrew Perrin, personal communication). Despite these differences between the canonical and cell death pathways, clues about how insulin signaling regulates cell death can be garnered from lifespan studies on daf-2 in the soma. Genetic mosaic analysis and tissue-specific
expression of *daf-2* revealed that it can regulate lifespan from neurons, weakly from the intestine but not from muscles [18, 92]. In light of these findings and similar to *kri-1*, *daf-2* may regulate germ cell death in *C. elegans* by a non-autonomous mechanism.

**Figure 3.6: *daf-2* regulates germ cell death in response to IR.**
Synchronized L4 wild-type worms were fed either control(RNAi) (black) or *daf-2*(RNAi) (white) and subjected to IR. Data represent mean ± SEM of three independent experiments and at least 40 germlines in total per strain per condition. The lack of colour in the schematic diagram of the worm (top) represents absence of *daf-2* expression. *p*<0.01 versus control(RNAi).

*daf-2* regulates cell death from both the soma and germline

Based on the fact that *daf-2* regulates cell death in response to DNA damage and that *daf-2* can rescue lifespan defects cell non-autonomously, we hypothesized that *daf-2* regulates cell death cell
non-autonomously, similar to kri-1. In order to test this hypothesis, we fed control(RNAi) or daf-2(RNAi) to ppw-1(lf) and rrf-1(lf) worms and tested whether germ cell death was affected in response to DNA damage (Figure 3.7). In both ppw-1(lf) and rrf-1(lf) worms fed daf-2(RNAi), the numbers of germ cell deaths were similar to each respective worm fed control(RNAi). This suggests that daf-2 expression in either the soma or the germline is sufficient to induce cell death.

**Figure 3.7: daf-2 regulates cell death from both the soma and germline.**
Synchronized L4 rrf-1(lf) (left) and ppw-1(lf) (right) worms were fed either control(RNAi) (black) or daf-2(RNAi) (white) and subjected to IR. The green colour represents expression of daf-2 in either the soma (left) or germline (right). Data represent mean ± SEM of three independent experiments and at least 40 germlines in total per strain per condition.

**daf-2 expression in muscles**

Although daf-2 expression in the soma was sufficient to rescue germ cell death, it is unknown whether expression is required in one or several tissues. Several groups have shown that daf-2 does not function solely in muscles to regulate lifespan [18, 92]. To test whether this was also true...
for germ cell death, cell death was quantified in synchronized L4 worms expressing daf-2 under the myo-3 muscle specific promoter subjected to IR. In contrast to wild-type animals that showed a normal apoptotic response to IR, worms expressing daf-2 specifically in muscle were resistant to apoptosis, similar to daf-2(If) mutants (Figure 3.8). This suggests that either daf-2 expression in muscle alone is insufficient to regulate cell death or that muscles do not play a role regulating germ cell death in C. elegans.

![Figure 3.8: Muscle-specific expression of daf-2 does not regulate apoptosis.](image)

Synchronized L4 wild-type (black), daf-2(If) (white) and a strain expressing a wild-type copy of daf-2 specifically from muscles in a loss of function background (light grey) were treated with IR and scored for apoptosis. Data represent mean ± SEM of three independent experiments and at least 25 germlines in total per strain per condition. *p<0.05 versus wild-type.
**daf-2 expression in the nervous system**

Lifespan assays have revealed that *daf-2* expression in the nervous system is sufficient to rescue the decreased lifespan of *daf-2(lf)* mutants [18]. We wondered whether a similar neuronal requirement for *daf-2* existed in cell death regulation. In order to determine whether neuronally restricted *daf-2* can restore apoptosis in the germline, *daf-2* was expressed under the pan-neuronal *F25B3.3* promoter (Figure 3.9). *daf-2(lf)* single mutants were resistant to apoptosis, similar to when worms were treated with *daf-2(RNAi)*. Neuronal expression of *daf-2* did not restore apoptosis in *daf-2(lf)* mutants, suggesting that either *daf-2* does not regulate cell death from neurons or that it is required in additional somatic tissues. A dauer formation assay (Table 3.1) confirmed that neuronally expressed *daf-2* was functional as it could rescue the constitutive dauer phenotype of *daf-2(lf)* worms, whereas muscle-specific *daf-2* could not, further supporting the conclusion that *daf-2* is required in multiple tissues to regulate cell death.
Figure 3.9: Neuronal-specific expression of daf-2 does not regulate cell death.
Synchronized L4 wild-type (black), daf-2(If) (white) and a strain expressing a wild-type copy of daf-2 specifically from neurons in a loss of function background (light grey) were treated with IR and scored for apoptosis. Data represent mean ± SEM of three independent experiments and at least 30 germlines in total per strain per condition. *p<0.01 versus wild-type.
Table 3.1: Dauer formation for muscle-specific and neuronally expressed *daf-2*

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*The remainder (52%) of worms were not found on the plate and were presumed to have died.*

†These adults were sterile.
4. Discussion
Cell non-autonomous regulation of cell death

Collectively, these data imply a novel mechanism whereby somatic cells communicate with germ cells to promote their death in response to DNA damage (Figure 4.1; Figure 4.2). Indeed, other genes and developmental processes have been shown to rely on soma-germline signaling [18, 89-97], confirming that these two tissues can signal to each other in response to certain stimuli. A recent study published in Nature confirmed our findings that cell non-autonomous inputs can control cell death in response to DNA damage [155]. The authors found that neuronally expressed tyr-2, a tyrosinase gene transcriptionally upregulated by the HIF-1 transcription factor, can negatively regulate IR-induced germ cell death. Observations such as these indicate that germ cell death in C. elegans is more complex than the previously accepted dogma. By inference, it also suggests that cell death in multicellular eukaryotes can be regulated through non-autonomous signaling. How this is accomplished remains to be determined, but model organisms such as C. elegans will be useful for elucidating the pathways that control these processes.

*kri-1 and daf-2 are novel regulators of cell death*

The fact that *kri-1* is required strictly in the soma to promote cell death suggests several mechanisms for how this is accomplished. First, although *kri-1* does not affect the transcript levels of the BH3-only gene *egl-1*, it is possible that *kri-1* is required to activate the EGL-1 protein. Similar to mammalian BH3-only proteins, EGL-1 may require other coactivating proteins or modifications in order to induce cell death in germ cells. For example, BID, BAD, BIM and BMF are mammalian pro-apoptotic proteins regulated post-translationally through proteolytic cleavage [51], phosphorylation [52-55] and sequestration by interacting proteins [56, 57], respectively. Therefore, it is formally possible that KRI-1 may facilitate the activation of EGL-1 by similar
transcription-independent mechanisms. Alternatively, KRI-1 may be involved in receiving signals from the germ cells, which results in the subsequent release of death-inducing factors (licence to kill factors). It is unlikely that KRI-1 itself is the licence to kill factor that is released from the soma since GFP tagged KRI-1 does not appear to change localization in response to DNA damage, despite *ex vivo* evidence in cells that suggest an analogous shuttling function for KRIT1 (although the shuttling occurs between the cytoplasm and the nucleus and not across tissues) [113]. Nevertheless, it remains possible that undetectable amounts of KRI-1 protein are transported to the germline from the soma.

![Diagram](image)

**Figure 4.1: Model of somatic requirement for *kri-1* function.**

*kri-1* is required in the soma to promote germ cell death. There are likely “license to kill” factors that are secreted from the soma into the germline to mediate cell death. Solid lines represent known regulatory interactions; dotted lines represent hypothetical interactions.

On the other hand, the observation that *daf-2* regulates cell death from the germline or soma suggests that *daf-2* expression in either tissue alone is sufficient to promote the process. Epistasis analysis of insulin signaling pathway components and the known cell death pathway place *daf-2*
downstream of *age-1, pdk-1* and *akt-1* and upstream of *ced-9* (Andrew Perrin, personal communication). Unlike *daf-2*, none of these genes appear to have similar equivalent requirements in the germline and soma. It is possible that there are two channels – one in somatic cells and one in germ cells – that *daf-2* transduces signals through. For example, if the model is that germ cells send signals to somatic cells once damaged to await further instructions on whether to die or not, then *daf-2* could play a role receiving signals in both somatic and germ cells (Figure 4.2).

Interestingly, one of the engulfment genes, the ABC transporter *CED-7*, is required in both dying and engulfing cells for proper removal of cell corpses [156]. Coupled with the observation that cell death can be influenced by engulfing cells (discussed below) and the cell non-autonomous, pleiotropic effects of *daf-2* on developmental processes ranging from lifespan to dauer arrest, it is not impossible that *daf-2* plays a similar role, regulating cell death from both tissues.

**Figure 4.2: Integrated model of *daf-2* and *kri-1* pathways.**

DNA damage sensed by the germ cell results in the production of death signals which are secreted and received by somatic cells. The DAF-2 receptor embedded in the somatic gonadal sheath cell responds to the signal and initiates a transduction cascade through KRI-1, resulting in the production of a license to kill factor. This factor is sent back to the dying germ cell where DAF-2 receives the signal to promote cell death.
However, *daf-2* does not act solely from neurons, where it has been shown previously to regulate worm lifespans and dauer formation [18, 92]. In contrast, as is the case for the PTEN-like (phosphatase and tensin homolog) phosphatase DAF-18 [157], *daf-2* regulation of cell death from the soma may occur from a combination of several different tissues, a hypothesis supported by the observation that muscle specific expression of *daf-2* also does not rescue the apoptosis defect of *daf-2(lf) mutants*. It is unlikely that *daf-2* is required in both the germline and the soma as tissue specific RNAi knockdown of *daf-2* in either tissue did not result in an apoptosis defect. Still, it is formally possible that incomplete knockdown of *daf-2* resulted in small amounts of death signals to reach and kill germ cells. Alternatively, it is possible that *daf-2* is required for germ cell death in other tissues that are not muscles or the nervous system.

**Passive cell death versus assisted cell death**

The germ cells that die in *C. elegans* are unique in that they are not fully enveloped by a plasma membrane when they are specified to die: cellularization presumably occurs after specification [6]. How does the worm keep death signals from leaking to other germ nuclei through the syncytium? The simple answer is that they are unable to: germ cells often die in clusters of four to five cells (unpublished observations). A small amount of the death factor may be taken up by a small number of germ cells as they cellularize before the signal spreads throughout the germline. One mechanism for circumventing such a bystander death effect is to ensure that only cells fated to die respond to the death factor (Figure 4.3A, B). Since p53 activation alone is insufficient to induce apoptosis (discussed below), it is possible that other changes occur in the germ cell to prime it for death, including the ability to respond appropriately to a secreted death signal. Reminiscent of the assisted suicide ritual of a dishonoured samurai in feudal Japan, where the dishonourable one
commits to death by plunging a blade into his midsection at which time a second would finish the deed by severing the head, KRI-1 may play a similar role to the second, by providing the finishing death touch to germ cells that have already committed to dying. In support of such a hypothesis, secondary changes have been observed on the membranous surface of dying germ cells, including the exposure of PS (phosphatidylserine) [158, 159] and the expression of CED-7 [156]. Furthermore, other changes such as the expression of annexin I [160] and the LPC (lysophosphatidylcholine) chemoattractant [161] have been noted in mammalian cells. Canonically, these changes are used by surrounding cells that engulf and digest the dying cell. Interestingly, these engulfing cells have been reported to play a role in assisting the dying cells that they envelope in C. elegans [86, 87], suggesting that assisted suicide can take place in some contexts. Other, as yet uncharacterized changes to the cell surface of dying cells may enable these cells to respond to death factors released by the somatic gonad. In addition, to help guide engulfing cells to the correct dying target, the LPC chemoattractant has been hypothesized to be secreted by apoptotic mammalian cells [161].

Although the engulfing somatic sheath cells in C. elegans are immobile, an analogous mechanism whereby engulfing cells secrete a chemoattractant instead of dying cells, is entirely possible (Figure 4.3C). This hypothesis also accounts for the observation that germ cells tend to die in clusters: if all dying germ cells are attracted to a limited number of gonadal sheath cells, then one would expect to find them as groups of dying cells.
Figure 4.3: Models for how germ cell death is specified by the somatic gonad.

(A) A schematic diagram representing the distal arm of a *C. elegans* germline with its surrounding sheath cells and the germ cells contained within. The diagram is oriented such that the distal (d) end is towards the left and the proximal (p) end is towards the right.

(B) DNA damage (represented by the lightening marks in (A)) can induce the production of a secreted death factor (squares) to which germ cells that have made the decision to die responds (black circles). Alternatively, this permissive death factor may be secreted constitutively and only those germ cells that have committed to die would respond to the factor.

(C) Chemoattractants (triangles) are released by the somatic gonad, to which germ cells respond (black circles with white triangles) by moving towards the source of the signal. Ultimately as they reach the source of the signal, either cell-cell contacts between the germ cell and the somatic gonad or threshold concentration of the chemoattractant will kill the germ cells.

(D) Direct contact between the somatic gonad and germ cells are necessary for germ cells to die (black circles).
p53-independent cell death and beyond

p53 and its worm counterpart cep-1 are considered master regulators of cell death as their activity is required for programmed cellular demise in response to DNA damage. The observation that mutations in kri-1 and daf-2 can suppress cell death even when cep-1 is active is intriguing as it suggests cep-1 activity is necessary but not sufficient to induce apoptosis. Indeed, several studies have identified genes that regulate germ cell death independently of cep-1, including pmk-3 [145], lin-35 [146] and sir-2.1 [144]. In addition, the fact that signaling is required between different tissues for cell death to occur may have important implications for how to target a tumour within the tumour microenvironment. Landmark studies of leukemia stem cells revealed that tumours can be separated into at least two distinct subpopulations – one that supports tumour formation and one that does not – with different cell surface antigen profiles, suggesting that a single therapeutic regimen, unless it targets the subpopulation that is able to reform tumours, is insufficient as a treatment option [162-165]. Furthermore, cancer stem cells are thought to be quiescent, making them insensitive to conventional therapies that mainly target actively dividing cells. Therefore, one viable therapeutic option is to target cancer stem cells indirectly through the non-stem cell population. These cells can be manipulated to secrete death factors that selectively affect cancer stem cells and induce death via a cell non-autonomous mechanism.

Worms as a model for CCMs

The finding that KRI-1 plays a cell non-autonomous role in promoting cell death may shed light on how the mammalian ortholog KRIT1 functions during the pathogenesis of CCMs. Although apoptosis has not been implicated in the pathogenesis of CCMs, mechanisms such as cross-tissue signaling may be at work through interactions of blood vessels and the surrounding, supportive
tissue. As described previously, CCMs occur when blood vessels in the brain become engorged with blood which hemorrhages due to a lack of supporting tissue [166]. The two main supportive tissues surrounding brain blood vessels are astrocytes that are thought to function in maintaining the blood-brain barrier and pericytes that express smooth muscle actin, which aid in the contractile functions of the blood vessel [167, 168]. Indeed, immunocytochemistry and electron microscopy revealed the absence of any pericytes or astrocyte foot processes surrounding the endothelial cells of the hemorrhaged blood vessel in CCM lesions [169]. How surrounding tissues maintain blood vessel integrity remains a mystery but studies in *C. elegans* may reveal similar pathways that interact with *kri-1/KRIT1*. One such pathway may act through RHO GTPases which regulates cell-cell junctions in conjunction with KRIT1 and other CCM proteins (CCM2 and CCM3) [110, 170, 171]. Specifically, KRIT1 interacts with the cytoplasmic side of the HEG1 transmembrane protein in endothelial cells along with CCM2; these interactions are important for maintaining the cell-cell junctions, as well as their permeability. It is thought that these proteins negatively regulate RHO GTPase signaling to maintain the integrity of endothelial cells, as loss of HEG1, KRIT1 or CCM2 results in phenotypes reminiscent of RHO activation, including actin stress fiber formation, MLC (myosin light chain) phosphorylation and increased permeability. These studies suggest that KRIT1 and its interacting partners play a cell autonomous role in regulating vessel integrity in zebrafish, mice and humans. Although this seems contradictory to the findings reported here, it is consistent with an evolutionarily conserved role for KRI-1/KRIT1 regulating various developmental processes from the endothelium. If we envisage the *C. elegans* germline (Figure 1.1) as a tube of gonadal sheath cells surrounding a population of dividing and maturing germ cells, the sheath cells can be considered as a primitive endothelium. Many of the functions ascribed to the endothelium including the ability to constrict to stimulate flow and act as a selective barrier are all present in the *C. elegans* gonad [172, 173]. The five pairs of sheath cells that surround the germline in *C.
*elegans* can be stained with anti-actin, anti-myosin or rhodamine-phalloidin antibodies to reveal a network of these structural proteins [89, 174, 175]. The contractile functions of these cells are required to extrude the developing oocyte into the spermatheca for fertilization. Mutating genes required for extrusion of oocytes in *C. elegans* leads to a defect in ovulation, as demonstrated by mutations in the *unc-54/myosin* gene [90]. The sheath cells and the surrounding basement membrane also act as a selective barrier and signaling module between the germline and the somatic cells surrounding it. Processes as diverse as aging and longevity [18, 92, 95], germline proliferation and maturation [90, 93, 94] and cell death have all been shown to signal through the somatic gonad. Electron microscopy of dissected worm gonads showed that yolk proteins which provide nutrients for the developing germ cells made their way from the intestine into the germline via pores found in the gonadal sheath cells [90]. The authors speculate that endocytosis is the major mode of transportation through these pores based on ultrastructural morphology, which suggests that the process is selective for yolk proteins. It will be interesting to try and develop the worm germline as a model for CCMs, perhaps by expressing human KRIPT1 in worms to determine whether it can substitute functionally for worm KRI-1. If so, KRI-1/KRIPT1 function can be studied taking advantage of the powerful genetic and high-throughput tools available to *C. elegans* and eventually translated to humans.

Where do we go from here?

Aside from the fact that *kri-1* can regulate germ cell death from the soma, the molecular details of its function are unknown. Determining the tissue(s) of focus of *kri-1* by expressing *kri-1* under the control of various tissue specific promoters will provide clues as to which other genes are involved in this process; however, as *kri-1* appears to be toxic when expressed ubiquitously under the
control of the \textit{dpy-30} promoter (unpublished observations; see Appendix), it will be challenging to
definitively test the requirement of \textit{kri-1} in other tissues. The most likely candidates are the
somatic gonad (sheath cells) that surrounds the germline and the two tissues of known expression:
the pharynx and the intestine.

Likewise, the molecular details of \textit{daf-2} function in germ cell apoptosis remains to be investigated.
Do other components of the insulin signaling pathway (i.e. \textit{age-1} and \textit{pdk-1}) also act in germline
and somatic tissues? Or are they specific to one tissue like \textit{akt-1} is to the germline? Tissue-specific
expression of these components including \textit{daf-2} will provide definitive answers to some of these
questions. Also unanswered is the manner in which the insulin pathway and the cell death pathway
communicate with each other. It appears that in response to DNA damage, the downstream
effectors of cell death – \textit{CED-9}, \textit{CED-4} and \textit{CED-3} – are not upregulated transcriptionally or at the
protein level, pointing to \textit{EGL-1} as the possible point of integration for these two pathways. \textit{daf-2}
may influence some aspect of post-translational modification of \textit{EGL-1}, similarly proposed for \textit{kri-1}.
Antibodies to \textit{EGL-1} will be required to test the hypothesis that it undergoes post-translational
modifications and whether this is dependent on either \textit{daf-2} or \textit{kri-1}.

Another central question that remains unanswered in the field is how individual germ “cells” are
specified to die. If secreted death factors are indeed involved in specifying death, how are they
confined to only cells that are specified to die? Or do they die by another mechanism? Intriguingly,
electron microscopy of the germline reveals gap junctions between sheath cells and the proximal
oocytes and it is possible that such a connection with the distal germ cells (albeit a transient one)
might induce a more specific death signal (Figure 4.3D). A genetic screen that specifically identifies
gain of function mutations that cause sterility or increased germline apoptosis may reveal clues as
to what this death inducing factor may be. On the other hand a more interesting (and involved) experiment to directly test whether secreted factors play a role in specifying cell death is to isolate the syncytial cytoplasm from apoptosis-competent strains and inject it into the germlines of apoptosis-deficient mutant strains and assay whether this can restart the death program. This will also simultaneously answer whether specific cell-cell contacts are required for death to occur (i.e. between the somatic gonad sheath cells and the germ cells) or whether germ cells respond locally to a secreted diffusible factor.

Finally, the exact role of the soma during cell death is currently not known. Do germ cells fail to die if the somatic gonad (i.e. gonadal sheath cells) is absent? Laser ablation studies may provide an answer to this question, but will be hampered by the fact that the somatic gonad also contributes to the proliferation of the germline. Temporally controlled laser ablations – either just before irradiation or just after – will be required to dissect the role of somatic gonad during DNA damage dependent cell death. These studies will shed light on the mechanics of germ cell death in C. elegans: if apoptosis occurs when the somatic gonad is ablated before irradiation, then it would suggest that the surrounding sheath cells provide a death factor to germ cells regardless of whether they had been specified to die or not. Cells that were specified to die would only respond to the germline ubiquitous death factor(s) after undergoing appropriate secondary changes. On the other hand, if apoptosis is abrogated when the somatic gonad is removed after irradiation, then it would suggest that the “right” to die is specified in response to death-inducing stimuli. It would also suggest that dying germ cells and the gonadal sheath cells signal to each other, as opposed to a one way signaling mechanism.
Concluding remarks

This work has provided evidence for a novel finding that germ cell deaths in *C. elegans* are regulated by a cell non-autonomous mechanism, although the extent to which the soma plays a role in specifying or enabling cell death to occur remains a mystery. It also provides evidence for the existence of *cep-1/p53*-independent pathways for the regulation of cell death. More studies will be needed to fully understand and dissect the mechanism by which non-dying cells regulate cell death, but it is an exciting observation that has broad applicability not only in CCMs but also to apoptosis-related disease biology.
5. Materials and Methods
Strains and Maintenance

*C. elegans* strains were obtained from the Caenorhabditis Genetics Center (CGC) and maintained at 16°C on NGM agar plates according to standard protocols [176]. The following strains and alleles were used in the study: N2 (Bristol var. wild-type), cep-1(gk138)i, kri-1(ok1251)i, daf-16(mu86)i, rrf-1(pk1417)i, ppw-1(pk1425)i, dpy-5(e61) unc-13(e450) hDf9/szT1[lon-2(e678)]i; +/szT1 X, clk-2(qm37)III, daf-2(e1370)III, daf-2(e1370)III; hpEx791 [Pmyo-3::daf-2; rol-6(su1006)], daf-2(e1370)III; hpEx792 [Pmyo-3::daf-2; rol-6(su1006)], daf-2(e1370)III; hpEx791 [Pmyo-3::daf-2; rol-6(su1006)], daf-2(e1370)III; hpEx792 [Pmyo-3::daf-2; rol-6(su1006)], ced-5(n1812)IV, pmk-3(ok169)IV, sir-2.1(ok434)IV, akt-1(mg144)IV, akt-1(ok525)IV, akt-2(ok393)X, daf-9(m540)X and daf-12(rh61rh411). ok1251 was outcrossed six times to N2 for all the experiments that are described. Double and compound mutant strains were generated with kri-1(ok1251) and ced-5(n1812), pmk-3(ok169), daf-16(mu86), dpy-5(e61) unc-13(e450) hDf9/szT1[lon-2(e678)]i; +/szT1 X, akt-1(mg144), akt-1(ok525), akt-2(ok393) and muEx353 [Phec-1::gfp::kri-1] using standard genetic techniques. The presence of ced-5(n1812) was verified by PCR and restriction digest; both pmk-3(ok169) and daf-16(mu86) were verified by PCR (see below). muEx353 was obtained from Dr. Cynthia Kenyon and originally contained the glp-1(e2141) mutation, which was segregated away in the kri-1(ok1251); muEx353 strain. The absence of the e2141 allele was verified by PCR and restriction digest (see below). ok1251 was marked with dpy-5(e51) prior to introducing it in trans to hDf9. Unmarked sir-2.1(ok434) males were crossed into a marked kri-1(ok1251) strain to generate kri-1(ok1251) and sir-2.1(ok434) double heterozygous mutants. pmk-3(ok169) and daf-16(mu86) was crossed into the muEx353 strain using standard genetic techniques. hpEx791 and hpEx792 were obtained from Dr. Mei Zhen and originally contained the fsn-1(hp1) mutation and jul1 insertion which were segregated away using standard genetic techniques to create daf-2(e1370)III; hpEx791 and daf-2(e1370)III; hpEx792.
Molecular Biology

The following cycling conditions were used for all PCR reactions reported in this study: two minutes at 95°C; one minute at 95°C, 30 seconds annealing temperature, one minute per kilobase (kb) at 72°C, all repeated 34 times; 10 minutes at 72°C. All PCR reactions were done using TAQ polymerase from NEB (New England Biolabs).

The presence of *kri-1(ok1251)* in all strains were verified by using the first round PCR primers 5' ttggaacgatgtggattga 3' and 5' ttaggtccgcgacatttac 3' annealed at 56°C and extended for 2.5 minutes. This first round PCR product was used as template for a second round PCR using primers 5’ cttccggaattaacaatg 3’ and 5’ gccctattggagaatgtgtgaac 3’ annealed at 57°C and extended for two minutes. A poison PCR reaction using 5’ cttccggaattaacaatg 3’ and 5’ tcgtaattcgattttttttttttt 3’ annealed at 57°C and extended for two minutes was also performed in parallel.

The presence of *pmk-3(ok169)* was verified in double mutants by PCR using first round PCR primers 5' tcgccctttgtatgtcttcc 3' and 5' ttctccagggattaacggtg 3' annealed at 58°C and extended for 3.5 minutes. The first round PCR product was used as template for a second round PCR using primers 5' ttttcactgcgtctcaatcg 3' and 5' tttcaaatttgcaggtgtc 3' annealed at 56°C and extended for three minutes.

*daf-16(mu86)* was verified in double mutants by PCR with 5' caatagacgacgatttccgc 3' and 5’ gtttttgtgccgttcagctc 3’ annealed at 56°C and extended for two minutes. Two parallel reactions, one with the candidate DNA and another with candidate DNA spiked with wild-type DNA was used as template for the *daf-16(mu86)* PCR. The absence of a band in the candidate DNA reaction
combined with the presence of a wild-type band in the spiked reaction indicated the presence of the \textit{daf-16(mu86)} allele. The \textit{mu86} deletion is too large to be reliably amplified by PCR.

The presence of the \textit{ced-5(n1812)} mutation was verified using first round primers 5' ttgtagctgtgccgag 3' and 5' gcataactgtgcttgcttg 3' annealed at 56°C and extended for 3.5 minutes. The first round PCR product was used template for a second round PCR using primers 5' ttcgattgcagctgcttg 3' and 5' gggattttcttgatcttg 3' annealed at 56°C and extended for 2.5 minutes. \textit{n1812} introduces a RFLP which can be detected by digestion with \textit{Psil} (NEB).

For \textit{glp-1(e2141)}, first round PCR primers 5' atctgatgaaggatggtctcg 3' and 5' aaactctgtgagggaccagtg 3' were annealed at 56°C and extended for two minutes. A second round PCR using 5' atctaccagcctgcttg 3' and 5' cgactgtttccattcaggt 3' were annealed at 54°C and extended for two minutes. \textit{e2141} destroys a RFLP which can be detected by digestion with \textit{Cac8I} (NEB).

\textit{fsn-1(hp1)} was detected with PCR primers 5' ataactttccccttgaagttac 3' and 5' tgaattctagccaggaat 3' annealed at 58°C and extended for one minute. The \textit{hp1} mutation introduces a RFLP that can be detected by digestion with \textit{BspHI} (NEB).

**Cell Corpse Quantification**

Germ cell corpses were quantified in synchronized fourth larval stage (L4) or young adult animals irradiated with a $^{137}$Cs ionizing radiation (IR) source, 24 hours post-irradiation at 20°C by microscopy as previously described [143]. Developmental cell death was quantified by mounting mixed stage embryos on slides and counting embryos of the appropriate stage of development. At least five animals or embryos were scored per repeat in at least two independent experiments.
Mitotic Cell Cycle Arrest Assay

Synchronized L4 hermaphrodites were treated with 120Gy of IR and photomicrographs of the distal mitotic region of the germline were taken 24 hours post-irradiation. All of the nuclei in an approximately chosen area (about 20 germ nuclei width) were counted and expressed as a function of the area.

Quantitative RT-PCR

\textit{egl-1} (5’ tactctcgctcaggactt 3’ and 5’ catcgaagtcacgcacat 3’), \textit{kri-1} (5’ tgctggagagatacggaggt 3’ and 5’ gtcatttggtgcgaattca 3’), \textit{ced-3} (5’ ctttgagggagaagtc 3’ and 5’ tccacagtsgctgaactgc 3’) and \textit{ced-4} (5’ gcaacgtggagctcttctc 3’ and 5’ tggtatatgtcgtgctcgta 3’) transcript levels were quantified in wild-type, \textit{kri-1(ok1251)}, \textit{cep-1(gk138)}, \textit{pmk-3(ok169)} and \textit{daf-16(mu86)} worms as previously described using the listed primers and a Roche LightCycler 480 [143]. \textit{egl-1}, \textit{kri-1}, \textit{ced-3} and \textit{ced-4} transcript levels were normalized to an internal tubulin control (\textit{tbg-1}; 5’ cgctcatcgcaagtgaacagc 3’ and 5’ tgtgtatgtgcgtcggta 3’) and compared against the wild-type untreated (0Gy) sample, repeated in at least three independent experiments. The following cycling conditions were used for all qPCR reactions: five minutes at 95°C; 10 seconds at 95°C, 15 seconds at 60°C, 10 seconds at 72°C, all repeated 40 times; five seconds at 95°C; one minute at 65°C.

RNA Interference (RNAi)

Control (\textit{Y95B8A_84.g}, a non-expressed gene [177]), \textit{kri-1}, \textit{ced-9}, \textit{daf-16}, \textit{cep-1} and \textit{daf-2} double stranded RNA expressing bacteria were induced by IPTG on plates and fed to L4 or young adult animals as previously described [143] and their progeny synchronized and irradiated as described above for germ cell corpse quantification. All bacterial RNAi strains except for \textit{ced-9} and \textit{daf-2}
(which were created via cDNA amplification) were obtained from the Julie Ahringer RNAi library and verified by sequencing.

**Starvation Assay**

Eggs from fertile, gravid hermaphrodites were synchronized by a hypochlorite bleach solution treatment, followed by three washes in M9 buffer. The isolated eggs were incubated at 20°C overnight on a gentle rotator. The worm solutions were diluted as necessary so that each strain was at similar concentrations. 10μL aliquots were removed onto bacterial plates every three days for 15 days. The worms were allowed to recover for three days until they became adults, at which point the number of surviving worms were quantified.

**Dauer Formation Assay**

25 L1 animals were picked onto plates and allowed to grow at the permissive temperature (20°C) or restrictive temperature (25°C) for three days before the number of adults and dauer worms were quantified.

**Western Blotting**

An asynchronous population of wild-type and *kri-1(0)* mutants were grown on either control(RNAi) or *ced-9(RNAi)* for three days and lysed by sonication in RIPA buffer containing protease inhibitors (Roche). 20μg of total soluble protein was loaded onto an SDS-PAGE gel for each sample and probed with antibodies to tubulin (Sigma; used at 1:4000) or CED-9 (a generous gift from Dr. Barbara Conradt; used at 1:1000).
Plasmid Construction

The following five plasmids were constructed to express different domains of KRI-1 tagged to T7 and GFP, under the control of the endogenous kri-1 promoter: pSI34 [KRI-1(1-729)], pSI35 [KRI-1(1-290)], pSI36 [KRI-1(250-729)], pSI37 [KRI-1(415-729)] and pSI38 [KRI-1(1-415)]. The various KRI-1 truncation mutants were PCR amplified from a full length kri-1 cDNA plasmid (pSI15) using the following primers: KRI-1(1-729), 5' tttccggagatggcaagcatgactggtgga 3' and 5' gccactgttttaagatttcggaatitcgcg 3'; KRI-1(1-290), 5' tttccggagatggcaagcatgactggtgga 3' and 5' gttactagttttttgctcctacattgtt 3'; KRI-1(1-415), 5' tttccgggatggcaagcatgactggtgga 3' and 5' gttactagtttttacgagctgttggaagttc 3'; KRI-1(250-729), 5' tttccgggagatggcaagcatgactggtggaagttc 3'; KRI-1(415-729), 5' gccactagtttaagatttcggaatitcgcg 3'; KRI-1(1-415), 5' tttccgggatggcaagcatgactggtgga 3' and 5' gccactagtttaagatttcggaatitcgcg 3'. All constructs were designed with an N-terminal T7 tag and 5' SmaI and 3' SpeI sites, which were used to ligate the constructs downstream of the GFP tag in pPD95.02 (Andrew Fire Vector Kit 1995). GFP and T7 tagged KRI-1 clones were then excised using 5' KpnI and 3' SpeI sites and cloned downstream of the ubiquitous dpy-30 promoter in pCAW137.

The 1.5kb kri-1 promoter was amplified from wild-type genomic DNA using the primers 5' catgcatgcctttgtgttgcaaatctggtgc 3' and 5' atggtaacatgatcgttgagaatctggtgc 3' and cloned into the pDrive vector (Qiagen) to generate pSI33. A BamHI and Xbal fragment containing the promoter was excised from pSI33, which replaced the ubiquitous dpy-30 promoter in all of the constructs. The kri-1 cDNA was also cloned downstream of the hsp16-2 and hsp16-41 heat shock promoters (Bin Yu, personal communication).
Microinjections

pSI34, pSI35, pSI36, pSI37, pSI38 or pBR32 were microinjected into the distal gonad of adult wild-type hermaphrodites with the semi-dominant rol-6(su1006) coinjection marker (pRF4) at concentrations between 4ng/μL and 100ng/μL as previously described [178, 179]. Briefly, worms were immobilized on 2% agarose pads that were dried overnight and then immersed in halocarbon oil. A microinjection needle was then inserted at a 45° angle to the immobilized worm, in the distal gonad just before the pachytene region. Injected worms were quickly removed from the oil by picking and transferred to fresh NGM plates with OP50.

Statistical Analysis

All statistical tests were analyzed by Welch’s t-test (a variation of Student’s t-test) on the means of the independent repeats, assuming unequal variance.
6. Appendix
Possible toxicity effects of *kri-1*

In an effort to further define the domains of *kri-1* required for cell death to occur, four truncation mutants of *kri-1* were PCR amplified from a full length cDNA and cloned into various expression vectors (Table 6.1).

Table 6.1: KRI-1 constructs used for microinjections

<table>
<thead>
<tr>
<th>KRI-1 construct</th>
<th>Domains included</th>
<th>Promoters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-290</td>
<td>N-terminus</td>
<td><em>dpy-30</em> (ubiquitous), <em>kri-1</em> (endogenous)</td>
</tr>
<tr>
<td>1-415</td>
<td>N-terminus, ankyrin repeats</td>
<td><em>dpy-30</em> (ubiquitous), <em>kri-1</em> (endogenous)</td>
</tr>
<tr>
<td>250-729</td>
<td>ankyrin repeats, FERM</td>
<td><em>dpy-30</em> (ubiquitous), <em>kri-1</em> (endogenous)</td>
</tr>
<tr>
<td>415-729</td>
<td>FERM</td>
<td><em>dpy-30</em> (ubiquitous), <em>kri-1</em> (endogenous)</td>
</tr>
</tbody>
</table>

Specifically, each construct was fused to an N-terminal GFP tag under the control of the ubiquitous *dpy-30* promoter [18] or the endogenous *kri-1* promoter [97]. In addition, the full length construct was placed under the control of two heat shock promoters, *hsp16-2* and *hsp16-41* [180]. These constructs were microinjected into wild-type and *kri-1*(0) hermaphrodite gonads at ~100ng/μL, ~40ng/μL and ~4ng/μL concentrations with the *rol-6(su1006)* semi-dominant coinjection marker. Table 6.2 summarizes the various injection conditions attempted in order to generate transgenic strains.
Table 6.2: Conditions for microinjection

<table>
<thead>
<tr>
<th>Injection condition</th>
<th>Strain</th>
<th>KRI-1 construct*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1-729</td>
</tr>
<tr>
<td>$P_{dpy-30}::kri-1$ in TE</td>
<td>wild-type</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>kri-1(0)</td>
<td>20</td>
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<td>$P_{kri-1}::kri-1$ in gDNA (4ng/μL) [97] + pRF4 [rol-6(su1006)]</td>
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<td>wild-type</td>
<td>20**</td>
</tr>
</tbody>
</table>

*The number of injected worms are indicated in each cell.

^GFP-positive worms were isolated from this injection but failed to propagate beyond the second generation.

**Rolling worms were isolated from this injection but failed to propagate beyond the first generation.
After the initial injections using *kri-1(0)* mutants, it became apparent that they were not amenable to injections. Whereas wild-type worms readily produced transgenic lines when injected with the pRF4 [*rol-6(su1006)*] marker plasmid on its own, *kri-1(0)* mutants injected with the same plasmid did not produce any lines (data not shown). The *kri-1* constructs originally resuspended in TE buffer were resuspended in water to test the theory that the original DNA preparation itself was toxic. However, this did not appear to be the case since microinjections with the new DNA preparation did not yield transgenic lines. This pointed to the *kri-1* gene itself as the possible source toxicity. In order to simulate a genetic environment more similar to wild-type conditions, the ubiquitous *dpy-30* promoter was replaced with an upstream, 1.5kb promoter fragment of *kri-1*. The DNA was also diluted to 40ng/μL and 4ng/μL to address concerns about over-expression of *kri-1*, although none of these changes resulted in transmitting transgenic lines. To determine whether the toxicity effects were specific to these particular constructs, a second construct (pBR32) obtained from Dr. Cynthia Kenyon (University of California) [97] was used to inject wild-type and *kri-1(0)* mutants at two different concentrations and with the *rol-6(su1006)* marker plasmid. Although rolling worms were observed in the F1 generation indicating successful injection, they did not produce offspring that carried the transgene (i.e. F2 progeny were not rolling), suggesting that even this second, independent construct was either unstable or toxic.

Finally, pBR32 was diluted in genomic DNA (gDNA) to create complex arrays; however this failed to yield transgenic strains.

To directly address whether over-expression of *kri-1* could lead to lethality in *C. elegans*, full length *kri-1* cDNA was cloned downstream of the *hsp16-2* and *hsp16-41* heat shock promoters, which are activated upon shifting the worms from the normal culturing temperature of 20°C to 25°C. Both
constructs were diluted with gDNA and injected at two different concentrations: although rolling F1 progeny were observed, the transgene did not transmit beyond this generation.

Combined, these observations suggest that ectopically expressed \textit{kri-1} is toxic to the worm. Whether this is because of increased apoptosis or other pleiotropic effects remains to be determined. In the F1 generation of several independent injections, the GFP signal was found exclusively in dead worms, supporting the hypothesis that \textit{kri-1} is toxic at non-basal levels. One way to circumvent this issue is to specifically express \textit{kri-1} in the tissues that are believed to be the focus of action: namely the intestine and pharynx [97]. If transgenic lines can be obtained using these tissue specific promoters, it would strongly suggest that \textit{kri-1} expression in any other tissue is highly toxic. A second way to address the toxicity effects is to create single copy integrants by gene bombardment [181]. It is possible that multicopy extrachromosomal arrays are contributing to the toxicity effects of \textit{kri-1}, which may be ameliorated by creating single copy integrants. It is also possible that rearrangements have occurred such that the PCR reaction used to detect the transgene construct did not work. This would account for the observation that rolling transgenic worms are observed in the F1 generation but not the F2 generation.
List of p-values

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<th>Strain #1</th>
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<td>Strain #2</td>
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<td>Figure 3.5</td>
<td>control(RNAi)</td>
<td>kri-1(RNAi)</td>
<td>60 Gy</td>
<td><strong>5.34E-03</strong></td>
</tr>
<tr>
<td></td>
<td>control(RNAi)</td>
<td>daf-9(lf); control(RNAi)</td>
<td>60 Gy</td>
<td>4.54E-01</td>
</tr>
<tr>
<td></td>
<td>control(RNAi)</td>
<td>daf-12(lf); control(RNAi)</td>
<td>60 Gy</td>
<td>5.87E-01</td>
</tr>
<tr>
<td></td>
<td>kri-1(RNAi)</td>
<td>daf-9(lf); kri-1(RNAi)</td>
<td>60 Gy</td>
<td>7.19E-01</td>
</tr>
<tr>
<td></td>
<td>kri-1(RNAi)</td>
<td>daf-12(lf); kri-1(RNAi)</td>
<td>60 Gy</td>
<td>4.46E-01</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>control(RNAi)</td>
<td>daf-2(RNAi)</td>
<td>0 Gy</td>
<td>7.02E-01</td>
</tr>
<tr>
<td></td>
<td>control(RNAi)</td>
<td>daf-2(RNAi)</td>
<td>60 Gy</td>
<td><strong>3.64E-03</strong></td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>control(RNAi)</td>
<td>rrf-1(lf); control(RNAi)</td>
<td>0 Gy</td>
<td>4.63E-01</td>
</tr>
<tr>
<td></td>
<td>control(RNAi)</td>
<td>rrf-1(lf); control(RNAi)</td>
<td>0 Gy</td>
<td>9.62E-01</td>
</tr>
<tr>
<td></td>
<td>control(RNAi)</td>
<td>rrf-1(lf); control(RNAi)</td>
<td>60 Gy</td>
<td>6.21E-01</td>
</tr>
<tr>
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<td>control(RNAi)</td>
<td>rrf-1(lf); control(RNAi)</td>
<td>60 Gy</td>
<td>9.59E-01</td>
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<tr>
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<td>daf-2(RNAi)</td>
<td>rrf-1(lf); daf-2(RNAi)</td>
<td>0 Gy</td>
<td>3.99E-01</td>
</tr>
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<td>daf-2(RNAi)</td>
<td>ppw-1(lf); daf-2(RNAi)</td>
<td>0 Gy</td>
<td>9.74E-01</td>
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<tr>
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<td>daf-2(RNAi)</td>
<td>rrf-1(lf); daf-2(RNAi)</td>
<td>60 Gy</td>
<td><strong>1.40E-03</strong></td>
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<tr>
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<td>daf-2(RNAi)</td>
<td>ppw-1(lf); daf-2(RNAi)</td>
<td>60 Gy</td>
<td>1.22E-01</td>
</tr>
</tbody>
</table>
Figure | Strain #1 | Strain #2 | Treatment | p-value*
--- | --- | --- | --- | ---
Figure 3.8 | wild-type | *daf-2(lf)* | 0 Gy | 6.29E-02
wild-type | *daf-2(lf)* | 60 Gy | **1.49E-02**
wild-type | *daf-2(lf)*; *myo-3p::daf-2(+)* | 0 Gy | 8.39E-02
wild-type | *daf-2(lf)*; *myo-3p::daf-2(+)* | 60 Gy | **1.78E-02**
Figure 3.9 | wild-type | *daf-2(lf)* | 0 Gy | 7.45E-02
wild-type | *daf-2(lf)* | 60 Gy | **2.46E-03**
wild-type | *daf-2(lf)*; *F25B3.3p::daf-2(+)* | 0 Gy | 7.55E-02
wild-type | *daf-2(lf)*; *F25B3.3p::daf-2(+)* | 60 Gy | **7.78E-04**

*Significant values (p<0.05) are highlighted in bold.

**The comparison is between strain #1 at 0Gy and strain #2 at 60Gy.

^Although the p-value falls within the threshold value for significance, we believe that there is no biological significance between these values.
7. References


50. Nakano K and Vousden KH. **2001.** PUMA, a novel proapoptotic gene, is induced by p53.
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