CONTROL OF PROGRAMMED CELL DEATH BY GCN-1

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ABSTRACT

Apoptosis is a cell death phenomenon that is fundamental to the development of an organism and to the pathogenesis of disease states. Regulation of apoptosis occurs at various molecular steps, but its regulation at the translational level remains poorly understood. The phenomenon of germline apoptosis, either physiological (i.e., without stress) or stress-induced, occurs throughout eukaryotic organisms from worms to humans and may have a role in maintaining germline immortality by eliminating compromised germ cells. Therefore the ability to regulate germline apoptosis is intricately linked with the survival and fitness of species. GCN-1, a known translational regulator, has been traditionally associated with GCN-2 to activate the Integrated Stress Response in order to modulate protein synthesis in the context of stress.

In the present study/thesis, we have discovered potentially a novel translational control mechanism of programmed cell death by GCN-1, pending further molecular studies. Contrary to conventional wisdom, this control occurs in a GCN-2 and Integrated Stress Response pathway-independent manner, providing a non-canonical function to GCN-1. The present study also potentially provides further mechanistic understanding of the poorly understood biological phenomenon of germline programmed cell death; a biological process that initiates death to nurture life. The knowledge presented herein could help further understand human female reproductive physiology in order to potentially extend reproductive lifespan and mitigate oocyte loss and sterility caused by environmental stressors, such as radiation and chemotherapy.

Thesis Advisor: H. Robert Horvitz

Title: Professor of Biology

This thesis is dedicated to

Caenorhabditis elegans that have sacrificed

in the present study in the name of knowledge,

my family in Korea/Canada/USA,

and

my past, present, and future patients,

as well as to all those who had lived a life greater than themselves.

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une vie plus grande qu'eux-mêmes.

자신에게 주어진 삶을 초월한 분 들에게 바칩니다.

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Chapter 1

INTRODUCTION

Yoon Andrew Cho-Park

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OVERVIEW

Cell death is a fundamental biological process affecting all living organisms on this planet. Apoptosis is a programmed cell-death subtype that has been shown to be genetically controlled (Ellis and Horvitz, 1986) and of critical importance in development and in the pathogenesis of diseases. Germline apoptosis is a specialized form of apoptosis that occurs in the germline of all eukaryotic organisms from worms to humans and can be induced either physiologically/without stress or upon stress inputs.

In this introduction chapter, I will review the following concepts that will help the reader get acquainted to the background knowledge needed to fully grasp my thesis project:

1) The genetic and molecular basis of apoptosis in *Caenorhabditis elegans* and in the mammalian system,

2) Physiologic and stress-induced germline apoptosis and their molecular underpinnings in *Caenorhabditis elegans* and in other eukaryotic systems, and

3) Concepts in radiation-induced apoptosis and the integrated stress response.

A) <u>APOPTOSIS</u>

1. Morphological and cytological features of apoptosis and its early history

The regression of fetal structures had been observed by many, possibly even in Aristotle times in Antiquities, but the association of these developmental regression to cell death was established following the discovery of the microscope and the advent of cell theory (Clarke and Clarke, 1996). In fact, German scientist, Carl Vogt, wrote of cells being "resorbed" or "destroyed" when describing the regression of notochord in the midwife toad, *Alytes obstetricians* in 1842 (Clarke and Clarke, 1996; Vogt, 1842). Subsequent studies in the 19th century had associated cell death to various biological context, including the observation of cell death in ovarian follicles by German biologist Walther Flemming in 1885 (Flemming, 1885). Flemming coined the word *chromatolytic* to describe this cell death as he observed that the chromatin in the nucleus appeared to disintegrate and thus he argued that there must be chemical changes within the cells to cause cell death (Clarke and Clarke, 1996; Flemming, 1885). Following this observation by Flemming, other morphological features have been attributed to this cell death: nuclear and cytoplasmic condensation as well as fragmentation of the cell into many membrane-bound and well-preserved fragments (Glucksmann, 1951; Kerr et al., 1972).

The term Apoptosis was first coined in 1972 to name the biological process responsible for these morphological features (Kerr et al., 1972). Interestingly, the word Apoptosis in Greek describes of the "**falling off**" of petals from flowers (Kerr et al., 1972). It was observed that in addition to this cell death occurring in "physiological"/development context that apoptosis can be triggered in the presence of noxious agents (Kerr et al., 1972). Furthermore, it was hypothesized early on that apoptosis must be programmed, in that there are intrinsic "clocks" to trigger

apoptosis in susceptible areas at the right time (Saunders and Fallon, 1966). It will take the adaptation of *Caenorhabditis elegans* (*C. elegans*) as a genetic tool few years later to uncover this program in much greater detail.

2. Genetic and molecular basis of programmed cell death/apoptosis in *C. elegans*

Following Sydney Brenner's seminal paper on the promise of a small transparent nematode, *C. elegans*, to uncover biological processes using genetic approaches (Brenner, 1974), it has been noted that during *C.elegans'* hermaphrodite development, 131 cells undergo programmed cell death/apoptosis (Sulston and Horvitz, 1977; Sulston et al., 1983). This precise determination of the number of cells that undergo developmental apoptosis was aided by the unique feature of worm biology with its essentially invariant cell lineage, allowing one to ascertain the specific cell that die in addition to establishing when and where those cellular death events occur (Conradt et al., 2016; Sulston and Horvitz, 1977; Sulston et al., 1983). Initial genetic studies on these cell deaths occurring in development revealed that apoptosis was indeed programmed by genes (Ellis and Horvitz, 1986). Subsequent genetic analyses have uncovered a whole set of genes with roles in apoptosis. The characterization of these genes has led to the establishment of an evolutionarily conserved molecular genetic pathway that is set in motion in orderly phases to allow cells to die: the "specification phase", the "activation phase", and the "execution phase" (Conradt et al., 2016; Horvitz, 1999). We will briefly discuss each of these phases separately.

a) Cell death specification

Specification phase determines which cells die and which cells survive (Conradt et al., 2016). Important observation that aided in the identification of the genes involved in this phase was that most of the 131 cells that die are a result of asymmetric cell divisions that result in the larger daughter cells surviving and the smaller daughter cells being programmed to die (Sulston and White, 1980). In addition, the cells that die are often sisters of cells that adopt a neuronal fate and these sister cells could become neurons, if they are prevented from dying (Ellis and Horvitz, 1986, 1991). In this context, genes involved in the specification phase were identified through genetic screens that did not block general programmed cell death, but rather abrogate apoptosis in specific cells, such as the neurosecretory motorneuron (NSM) sister cells (Conradt et al., 2016; Ellis and Horvitz, 1986).

egl-1 is a gene critical in activating cell death and many of the genes that were isolated and found to have a role in the specification phase modulate transcription levels of *egl-1.* In fact, *egl-1* gene appears to be expressed at an increased level in cells that are undergoing apoptosis (Conradt and Horvitz, 1999; Hirose et al., 2010). Subsequent studies have uncovered that many of these "specification" genes are in fact transcriptional regulators that each act at specific cis-regulatory elements either upstream or downstream of *egl-1* gene to modulate *egl-1* transcription (Hatzold and Conradt, 2008; Hirose and Horvitz, 2013; Hirose et al., 2010; Thellmann et al., 2003). Furthermore, these transcriptional regulators appear to control *egl-1* transcription in select cells (Conradt et al., 2016). For instance, cell-death specification transcription factors, EYA-1 and CEH-34*,* physically interact with each other to bind to an upstream element of *egl-1* to increase *egl-1* transcription specifically in a pharyngeal motor neuron called M4 sister cell

(Hirose et al., 2010). This increased *egl-1* expression subsequently results in the death of the M4 sister cell through the activation of the core apoptotic pathway.

b) Cell death activation

This activation phase allows the apoptotic core program to become active in a cell that is fated to die. The isolation of *C. elegans* mutants that blocked apoptosis in general by causing inappropriate survival of the 131 cells that are normally fated to die in development yielded the first identification of these activation genes. The identification of *ced-3* and *ced-4* was found to be required for the initiation of apoptosis in *C. elegans* and was shown for the first time that a genetic program activated programmed cell death/apoptosis (Ellis and Horvitz, 1986). Subsequent genetic studies using *C. elegans* identified the *ced-9* gene, which in contrast to *ced-3* and *ced-4*, was shown to protect cells from dying (Hengartner et al., 1992). In fact, *ced-9(loss-of-function)* mutants are embryonically lethal in light of the excessive cell deaths that occur in these mutants. *egl-1*, which is a critical transcriptional target of cell-death specification genes as mentioned earlier, was subsequently found to also be required for most of the apoptosis occurring in somatic cells by acting as a negative regulator of *ced-9* (Conradt and Horvitz, 1998). These four activation genes, *egl-1*, *ced-9*, *ced-4*, and *ced-3,* would all act in a simple genetic program that make up the canonical cell-death activation pathway (Figure 1A). Multiple gene interaction studies would deduce that *egl-1* acts upstream of *ced-9* to activate cell death, *ced-9* acts upstream of *ced-4* to inhibit apoptosis, and finally that *ced-4* would act upstream of *ced-3* to cause death of the cell that is fated to die (Conradt and Horvitz, 1998; Hengartner et al., 1992; Metzstein et al., 1998).

This simple cell-death activation pathway in *C. elegans* is found to be evolutionarily conserved in mammals/humans. In fact, *ced-9* encodes for a protein that is similar to the human proto-oncogene *bcl-2* (Hengartner and Horvitz, 1994), which also acts to inhibit apoptosis in mammals (Hockenbery et al., 1990). *egl-1* encodes for a protein that contains the BH3 motif, which is important to bind to Bcl-2 (Bouillet and Strasser, 2002). The human homolog of *ced-3* gene are caspases, which consist of a family of cysteine proteases (Alnemri et al., 1996; Yuan et al., 1993). Finally, the human counterpart for CED-4 is APAF-1, which is found to activate human caspases (Yuan and Horvitz, 1992; Zou et al., 1997).

Subsequent molecular, structural, and cell biological studies have confirmed the intricate relationships between the genes/proteins in the canonical apoptosis activation pathway (Figure 1B) (Lettre and Hengartner, 2006). Protein-protein interaction studies have found that CED-4 and CED-9 interact physically *in vitro* and in cells (Chinnaiyan et al., 1997; Wu et al., 1997a). In addition, CED-4 has been found to physically interact with CED-3 *in vitro* and in cultured cells suggesting the critical importance of CED-4 as a mediator that molecularly activates the apoptosis pathway (Chinnaiyan et al., 1997; Yang et al., 1998). Cell biological experiments have shown that both endogenous CED-9 and CED-4 colocalize to the mitochondria in cells that do not die (Chen et al., 2000). In cells that had been induced to die, translocation of CED-4 to the perinuclear membranes was observed, which was dependent on EGL-1 (Chen et al., 2000). It was later discovered that the binding of EGL-1 to CED-9 appears to cause conformational changes in CED-9 protein, resulting in the release of the dimerized CED-4 protein from the CED-4/CED-9 complex (Yan et al., 2004, 2005). The free CED-4 dimers would then oligomerize to form a CED-4 octameric apoptosome in order to promote CED-3 zymogen

dimerization and autocatalytic maturation (Huang et al., 2013; Qi et al., 2010). CED-3 would then go on to activate the cell-death execution phase.

c) Cell death execution

Once activated, CED-3/caspases coordinate various cellular disassembly events that ultimately result in the killing of the cell fated to die (Conradt et al., 2016). This cell-death execution phase is marked by key cellular processes that include nuclear DNA fragmentation, presentation of an "eat me" signal through phosphatidylserine externalization, and clearance of apoptotic cells through engulfment by neighboring cells (Conradt et al., 2016; Steller, 1995). We will discuss each of the latter key cellular processes of the cell-death execution phase in further detail.

i) Nuclear DNA fragmentation

Fragmentation of nuclear DNA is a key morphological attribute of apoptosis. It is thought to facilitate apoptosis by abrogating DNA replication as well as transcription (Arends et al., 1990). Multiple genes encoding for nucleases have been identified in *C. elegans* with roles in DNA fragmentation during apoptosis, including *nuclease defective 1(nuc-1)* (Sulston, 1976). Either loss-of-function or reduction-of-function mutants in many of these nuclease genes have shown the delayed visualization of cell corpses or even the abrogation of cell death altogether, suggesting that these genes have a role in the progression of apoptosis and have a cell killing function (Nakagawa et al., 2010; Parrish and Xue, 2003, 2006). Subsequent studies have found

that these nuclear DNA fragmentation genes act in a sequential manner to cut through the DNA of the dying cell (Parrish and Xue, 2003).

The genes *cps-6* and *wah-1* encode for proteins residing in the mitochondria with its respective human homologs being human mitochondrial endonuclease G (EndoG) and apoptosis-inducing factor (AIF) (Parrish et al., 2001; Wang et al., 2002b). AIF and EndoG are known to be important in the nuclear DNA fragmentation in humans following their release from the mitochondria (Li et al., 2001; Susin et al., 1999). WAH-1 was found to interact physically with CPS-6 in order to increase the nuclease activity of CPS-6 (Wang et al., 2002b). These studies demonstrate a degree of conservation of the cell execution phase of apoptosis.

ii) Phosphatidylserine externalization

A dying cell undergoing apoptosis express "eat me" signal(s) in order to proceed in the clearance of that apoptotic cell (Savill and Fadok, 2000). One such "eat me" signal that has been discovered is the externalization of phosphatidylserine (PS) during apoptosis (Fadok et al., 1992; Martin et al., 1995). PS is normally located in the cytoplasmic side of the plasma membrane. This cellular event during apoptosis has been found to be evolutionarily conserved and serves an important role in order to remove apoptotic cells (Venegas and Zhou, 2007; Züllig et al., 2007).

WAH-1, which is the worm homolog of AIF, has a role in promoting PS exposure in apoptotic cells in addition to its role in nuclear DNA fragmentation (Wang et al., 2007), suggesting a possible coordination of these two cell-death execution events through WAH-1. WAH-1 has been demonstrated to bind to the *C. elegans* phospholipid scramblase, SCRM-1, to permit exposure of PS to the outer leaflet of the plasma membrane of the dying cell (Wang et al.,

2007). Interestingly, ectopically expressing PS on a living cell can lead to its phagocytosis as seen in *tat-1(loss-of-function)* mutants (Darland-Ransom et al., 2008). *tat-1* encodes for the aminophospholipid translocase transbilayer amphipath transporter 1 that promotes the maintenance of the asymmetric PS distribution across the plasma membrane (Darland-Ransom et al., 2008). This phagocytosis of living cells in *tat-1* mutants were found to be dependent on PSR-1 (PS receptor family 1) and CED-1, which are two receptors that recognize PS and act in their respective phagocytosis pathways (Darland-Ransom et al., 2008; Wang et al., 2003; Yang et al., 2015; Zhou et al., 2001a).

iii) Clearance of apoptotic cells through engulfment

Following PS externalization in a dying cell, this "eat me" signal is recognized by receptors on the engulfing cell. Subsequently through the process of phagocytosis, the apoptotic cells are cleared (Hochreiter-Hufford and Ravichandran, 2013). This clearance step is critical to mitigate any inflammatory/immune reaction that a cell-death process may trigger. In contrast to *Drosophila* or other higher eukaryotic organisms, *C. elegans* do not have dedicated engulfing cells such as macrophages, but instead the apoptotic cells are engulfed by neighboring cells (Sulston et al., 1983; Zhou et al., 2001a).

Through genetic analyses, we have increased our understanding of the biological mechanism behind apoptotic cell engulfment. In fact, genetic screens led to the identification of many of these "engulfment" genes and gene interaction studies allowed the recognition of two main redundant pathways mediating the engulfment biology (Ellis et al., 1991).

The CED-1/CED-6/CED-7 pathway is first engaged by the CED-1 protein, which encodes an engulfment receptor and is found to be only expressed in engulfing cells and not in dying cells (Zhou et al., 2001a). The use of CED-1::GFP fusion protein allowed the observation that CED-1 is found to cluster around apoptotic cells and that this is dependent on CED-7 (Zhou et al., 2001a). This clustering phenomena was also partially dependent on TTR-52, a transthyretin-like protein, which is thought to act as a bridging molecule between PS and CED-1 (Wang et al., 2010). Following the engagement of CED-1, the engulfment signal is transduced to CED-6, an adaptor protein, and DYN-1, a member of a large GTPase family that has a role in transport of vesicles (Clark et al., 1997; Liu and Hengartner, 1998; Yu et al., 2006). DYN-1 was found transiently accumulating on pseudopods during engulfment and that this was dependent on the presence of CED-1, CED-6, and CED-7 (Yu et al., 2006). It is thought that CED-6 may serve as the link of CED-1 signaling to DYN-1 (Conradt et al., 2016).

A second redundant engulfment pathway is characterized by the CED-2, CED-5, CED-10, and CED-12 proteins. There are three different receptors that initiate this second signaling pathway: PSR-1 (Wang et al., 2003), MOM-5, a worm Frizzled homolog (Cabello et al., 2010), and INA-1/PAT-3, members of the integrin family (Hsu and Wu, 2010). It is thought that the extracellular domains of both PSR-1 and INA-1 bind to PS on the surface of apoptotic cells (Hsu and Wu, 2010; Wang et al., 2003; Yang et al., 2015). In contrast, it is not clear how MOM-5 recognizes apoptotic cells. These three receptors all promote engulfment through the CED-5/CED-12 protein complex that subsequently modulate the activation of CED-10 GTPase (Brugnera et al., 2002; Reddien and Horvitz, 2000; Wang et al., 2003; Wu and Horvitz, 1998; Wu et al., 2001; Yang et al., 2015; Zhou et al., 2001b). The activation of CED-10 is thought to lead to cytoskeleton rearrangement for pseudopod formation/extension around a dying cell

(Kinchen et al., 2005). The receptors MOM-5 and INA-1 also require the adaptor protein, CED-2, to transduce their signaling activities (Cabello et al., 2010; Hsu and Wu, 2010), whereas PSR-1 is able to directly engage with CED-5/CED-12 complex without CED-2 (Wang et al., 2003).

Of note, it has been observed that the engulfment process actively contributes to the process of killing. In hypomorphs of cell killer gene, such as *ced-3*, survival of cells that normally are fated to die can be seen. In the latter genetic background, additional mutations in engulfment genes allowed a greater frequency of cell survival (Hoeppner et al., 2001; Reddien et al., 2001). Therefore, blocking engulfment alone was found to be sufficient to trigger survival and differentiation of cells that normally are supposed to die (Reddien et al., 2001). These observations suggest that the process of engulfment ensures that cells that are fated to die would indeed proceed to death. Subsequent study has denoted that one of the engulfment gene, CED-1, may promote apoptosis by allowing an asymmetric localization of apoptotic factors in the mothers of cells that are fated to die and this consequently allowed for unequal segregation of cell-death potential in the dying and surviving daughters (Chakraborty et al., 2015).

The biological basis of programmed cell death/apoptosis in *C. elegans* provided a framework to further understand apoptosis in higher eukaryotic organisms including humans.

3. Molecular basis of apoptosis in mammals/humans

In this section, we will discuss some of the key knowledge in the molecular basis of apoptosis in mammals/humans. In the mammalian system, the current understanding of the mechanism of apoptosis is divided into two main pathways: the intrinsic pathway and the extrinsic pathway. In the intrinsic pathway, the key player that mediates the apoptotic signal is

the mitochondria. This is in contrast to the extrinsic pathway, where the apoptotic signal is initiated through the binding of a ligand to a receptor in the extracellular space (Kesavardhana et al., 2020; Wallach and Kang, 2018).

a) Intrinsic pathway of apoptosis

Human homologs of genes discovered in *C. elegans* play essential roles in the intrinsic pathway of apoptosis in mammals/humans (Figure 2). The intrinsic pathway starts by the induction of members of the Bcl-2 protein family, which serve as key molecular players in the intrinsic pathway, in response to diverse apoptotic stimuli that change cellular homeostasis (Singh et al., 2019). This protein family is characterized by the presence of Bcl-2 homology (BH) domains, which is known to mediate protein-protein interactions between the members of this family (Adams and Cory, 2001). Various members of this family have different biological functions and key effectors of the apoptotic signal are the so-called BH3-only proteins, which contain a single BH3 domain (Singh et al., 2019). In fact, BH3-only proteins, such as the Bcl-2 interacting mediator of cell death (BIM), BH3-interacting domain death agonist (BID), and p53-upregulated modulator of apoptosis (PUMA) bind to the pro-survival Bcl-2 protein to inhibit its anti-apoptotic function all the while activating key pro-apoptotic mitochondria pore-forming proteins (Singh et al., 2019; Wallach and Kang, 2018). Indeed, the activation of Bcl-2 antagonist/killer (BAK) and Bcl-2-associated X (BAX) proteins allow their oligomerization and the formation of pores resulting in mitochondrial outer membrane permeabilization (MOMP) (Tait and Green, 2010). The initial induction of the intrinsic pathway highlights the critical

importance of the interaction between the diverse members of the Bcl-2 protein family, through its BH domains, to propagate the cellular death signal (Wallach and Kang, 2018).

The erosion of mitochondrial integrity as a consequence of MOMP results in the release of pro-apoptogenic mitochondrial proteins, such as cytochrome C and second mitochondria-derived activator of caspase (SMAC also named DIABLO) (Kesavardhana et al., 2020; Tait and Green, 2010). Subsequently, cytoplasmic cytochrome C binds to the scaffold protein called apoptotic protease-activating factor 1 (APAF-1), which results in the assembly of a multimeric complex called the apoptosome (Acehan et al., 2002; Jiang and Wang, 2004; Liu et al., 1996). The apoptosome activates an initiator caspase, caspase-9, which initiates the caspase cascade by driving activation of effector caspases like caspase-3/-6/-7 to trigger robust execution of programmed cell death (Kesavardhana et al., 2020; Wallach and Kang, 2018). This robust activation of caspases is further amplified by the release of SMAC to the cytoplasm through MOMP. In fact, SMAC inhibits the inhibitor of apoptosis proteins (IAPs) that act as caspase inhibitors (Riedl and Shi, 2004; Shiozaki and Shi, 2004).

b) Extrinsic pathway of apoptosis

In contrast to the intrinsic pathway, the extrinsic pathway of apoptosis is more straightforward with less molecular players involved (Figure 2). The central characteristic of the extrinsic pathway is the binding of an extracellular cognate ligand to a family of receptors called death receptors (DR), which include the tumor necrosis factor (TNF) receptors (Kesavardhana et al., 2020). The ligand-receptor binding results in receptor oligomerization and recruitment of adaptor proteins, such as FAS-associated death domain (FADD) or TNFR1-associated death

domain (TRADD) (Kesavardhana et al., 2020; Wallach and Kang, 2018). These adaptors further recruit an initiator caspase, caspase-8, along with other proteins to form a death-initiating signaling complex (DISC) (Kesavardhana et al., 2020; Ramirez and Salvesen, 2018). DISC allows the dimerization and activation of caspase-8, which subsequently activates effector caspases like caspase-3/-6/-7 (Kesavardhana et al., 2020; Wallach and Kang, 2018).

Caspase-8 has also been shown to activate the BH3-only protein BID by generating a cleaved version called tBID (Tait and Green, 2010; Wallach and Kang, 2018). This activated form of BID promotes mitochondrial pore formation through BAX and BAK resulting in MOMP, thus allowing a cooperative activation of both the intrinsic and extrinsic pathways of apoptosis (Kesavardhana et al., 2020).

B) GERMLINE APOPTOSIS

In addition to somatic cells, apoptosis occurs in the germline and some of its biological features are conserved throughout the animal kingdom. In comparison to our understanding of the mechanism of somatic apoptosis, apoptosis occurring in the germ cells are much less clearly understood. Germline apoptosis can occur in two different contexts: either in "physiological" condition (i.e., without stress inputs) or in the context of stress. I will briefly introduce the germline biology in *C. elegans* before discussing further both of these forms of germline apoptosis in worms. We will end this section by discussing this particular form of apoptosis in other organisms, such as in mammals/humans.

1. Introduction to the *C. elegans* germline

The germline tissue holds a special place in that it constitutes the area of an organism that allows gametogenesis and thus establishing a link from generation to generation (Hubbard, 2005). This is no different in *C. elegans*, where the germline is the third most abundant tissue type by volume following the intestine and the hypoderm (Froehlich et al., 2021) and contains half of all cell nuclei in the adult *C. elegans* (Seydoux and Schedl, 2001).

Following fertilization and during embryogenesis, the future germline tissue is specified early with the critical role of the protein PIE-1 (Mello et al., 1996; Seydoux et al., 1996). During larval development, the germline differentiates and proliferates to result in the easily recognizable two tubular gonadal arm structures observed in late larval and adult stages of the animal (Hubbard, 2005) (Figure 3). Germ cell proliferation occurs in the most distal portion of these gonadal arm structures through mitosis, close to where the somatic distal tip cells are located, whose cells play a critical role in germline proliferation and provide a stem cell niche (Hubbard, 2005).

C. elegans germline is often compared to an assembly line due to the orderly transition of these germ cells from mitosis through the different sequential stages of meiosis, as they move through the gonad from distal to proximal gonadal structures (Figure 3). In fact, each meiotic stages occur in spatially restricted regions of the gonad (Church et al., 1995). GLP-1 and the Notch signaling are thought to play a critical role in regulating the meiotic entry of germ cells (Austin and Kimble, 1987). Furthermore, other molecular players have been identified to regulate this meiotic entry, including GLD-1/2/3, FBF-1/2, and NOS-3, through post-transcriptional/translational regulation (Crittenden et al., 2002; Eckmann et al., 2004; Kraemer et

al., 1999; Wang et al., 2002a). Meiotic recombination occurs in the pachytene stage of meiosis, which physically happens around the gonadal loop region (Hillers, 2017; Hubbard, 2005). Subsequent pachytene exit and entry to the diplotene stage of meiosis requires the MAPK signaling pathway, including the critical role of MPK-1 (Church et al., 1995). Of note, germ cells are partially surrounded by plasma membranes and connect with other germ cells via cytoplasmic bridges onto a common cytoplasmic core, called rachis (Gartner et al., 2008) (Figure 3). Once they exit the pachytene stage, germ cells grow rapidly with significantly increased cytoplasmic volume to differentiate into cellularized individual oocytes in the proximal germline (Gartner et al., 2008). Gametogenesis occurs in the larval stage 4 (L4) and during adulthood; with spermatogenesis occurring in L4 stage and oogenesis during the adult stage (Hubbard, 2005).

2. Physiological germline apoptosis in *C. elegans*

In contrast to apoptosis occurring in somatic cells in *C. elegans*, apoptosis occurring in the germline is not linked with the invariant cell lineage, but is rather a part of normal oogenesis program. In fact, germline apoptosis does not occur in males nor is it seen in larvae. A paper published in 1999 detailed the first description of germline apoptosis in *C. elegans* hermaphrodite (Gumienny et al., 1999). It was found that a significant amount of germ cells are culled during this process. In fact, it is suggested that more than half of all female germ cells in a hermaphrodite animal die through physiological germline apoptosis (Gumienny et al., 1999). In the wild-type animals, most of these germ cell apoptosis occur as the germ cells exit the pachytene stage of meiosis and thus around the gonadal loop region (Gartner et al., 2008;

Gumienny et al., 1999). Ultrastructural analyses have denoted that when a germ cell dies, the dying cell "pinches off" from the common cytoplasmic core and thus cellularizes in order to form a discrete cell with little residual cytoplasm and a fully formed plasma membrane (Gartner et al., 2008; Gumienny et al., 1999) (Figure 3). Subsequently, the dying cell undergoes apoptosis to form a corpse, which is eventually engulfed by the neighboring somatic gonadal sheath cells (Gartner et al., 2008). It is thought that the engulfment mechanism in germline apoptosis utilizes similar molecular players as in somatic apoptosis.

It remains unclear why Nature would produce so many germ cells only to cull the majority of them during the process of physiological germline apoptosis in *C. elegans,* particularly since it is not required for oogenesis to proceed. Nevertheless, it was observed that blockage of physiological cell death reduces the number of offspring produced (Boag et al., 2005; Gumienny et al., 1999). This raises the question that perhaps the germ cells that die serve as "nurse cells" to contribute its cytoplasmic content to the surviving counterpart, which acquires significant cytoplasm to form the developing oocyte (Gartner et al., 2008; Gumienny et al., 1999). As we will see later in this section, the phenomenon of physiological germline apoptosis is widely observed across animal kingdom, including in humans. The "nurse cell" hypothesis has a wide acceptance in the scientific community to explain this conserved facet of biology.

Physiological germline apoptosis requires CED-3 and CED-4, but interestingly it has been noted that it can occur independent to EGL-1 activation (Gumienny et al., 1999). Furthermore, it is notable that *ced-9* gain-of-function mutation allele, *n1950,* does not appear to prevent physiological germline apoptosis (Gumienny et al., 1999). This is in contrast to somatic apoptosis, where the same mutation prevents completely its cell death (Hengartner et al., 1992).

These observations raised the question that germline apoptosis may be regulated through a different molecular mechanism than somatic cell death.

In addition to the critical role played by MPK-1/MAPK signaling pathway in determining germ cell exit from pachytene stage of meiosis (Church et al., 1995), early work on genetic control of physiological germline apoptosis suggested that the MAPK pathway activation is required for apoptosis to occur in the germline (Gumienny et al., 1999). Given that MAPK signaling is important in meiosis progression, the loss of physiological germline apoptosis in the MAP kinase mutants could be argued to be a developmental defect rather than a defect in apoptosis induction (Bailly and Gartner, 2013). Nevertheless, increased apoptosis was observed when analyzing the double mutants carrying loss-of-function mutations in both MAPK pathway and *ced-9*, suggesting that the lack of apoptosis seen in these MAP kinase mutants are due to genuine loss of apoptosis induction (Bailly and Gartner, 2013; Gumienny et al., 1999).

Transcriptional regulation of core apoptotic factors, CED-9, CED-4, and CED-3, were found to be important in influencing physiological germline cell death. In fact, *C. elegans* homolog of the human retinoblastoma (*Rb*) susceptibility gene, *lin-35,* was found to repress *ced-9* transcription to promote physiological germline apoptosis (Schertel and Conradt, 2007). RB protein binds to a protein heterodimer that is composed of a member of the DP protein family and a member of the E2F family (Schertel and Conradt, 2007). The worm homolog of DP protein, DPL-1, and the worm homologs of E2F proteins, EFL-1 and EFL-2, have also been found to promote physiological germline apoptosis by inducing *ced-3* and *ced-4* transcription in the germline (Schertel and Conradt, 2007).

Subsequent genetic studies have proposed other genes that modulate physiological germline apoptosis. In fact, a genome-wide RNAi and a targeted RNAi screens have identified some candidate genes that prevent excessive physiological germline apoptosis (Lackner et al., 2005; Lettre et al., 2004). Some in the field have commented on the modest number of genes that appear to have a role in preventing physiological germline cell death, thus suggesting that limited number of biological pathways may impact this form of germline cell death (Gartner et al., 2008). Two potential biological/cellular functional pathways that can be gathered amongst the few genes that have been identified are the endosomal-lysosomal trafficking pathway and genes that encode RNA-binding proteins.

RNA-binding proteins, CGH-1, CAR-1, and CPB-3, have each been found to have a role in physiological germline cell death in independent studies (Boag et al., 2005; Lettre et al., 2004; Navarro et al., 2001; Singh et al., 2017). Interestingly, across different species, these three RNA-binding proteins and its homologs are thought to physically and functionally interact with each other and have been shown in variety of studies to be implicated in translational regulation of mRNA (Audhya et al., 2005; Boag et al., 2005; Decker and Parker, 2006; Gartner et al., 2008; Rajyaguru and Parker, 2009; Squirrell et al., 2006; Weston and Sommerville, 2006; Wilczynska et al., 2005; Wilhelm et al., 2005). It is not understood how these RNA-binding proteins promote cell death. Nevertheless, we do know that each of these three proteins are found in a ribonucleoprotein (RNP) complex located in P granules, a cytoplasmic structure where mRNA translation are repressed and mRNA decay factors are localized (Boag et al., 2005; Gartner et al., 2008; Rajyaguru and Parker, 2009; Sengupta et al., 2013). This suggests a possible link between P granule biology and physiological germline cell death.

Recent studies have provided further evidence of this link in that loss of PGL-1 and PGL-3, two constitutive components of P granules, resulted in physiological germline cell death (Min et al., 2016). Specifically, PGL-1 and PGL-3 were found to be lost at the perinuclear region of germ cells prior to germline apoptosis and that this loss was further enhanced upon stress, such as genotoxic stress (Min et al., 2019). The latter observation suggests that P granules are also implicated in stress-induced germline apoptosis and thus may represent a source of common biology between the two forms of germline cell death.

3. Stress-induced germline apoptosis in *C. elegans*

Many different forms of stress can induce germline apoptosis in *C. elegans* (Salinas et al., 2006). Amongst the different forms of stress that can promote apoptosis, the one that we have greater biological understanding is related to DNA damage-induced germline apoptosis.

a) DNA damage-induced germline apoptosis

When confronted with genotoxic stress, an organism activates its DNA damage response pathways. The DNA damage response of an eukaryotic organism consists of three major components: cell cycle arrest, DNA repair, and apoptosis (Craig et al., 2012). In other words, upon genotoxic stress, there is activation of cell cycle arrest pathway in order to prevent propagation of compromised genetic material and allowing time to repair the DNA damage. If the latter is not successful and consequently the level of DNA damage is too elevated, programmed cell death can be activated to eliminate cells with compromised genome integrity.

This elimination of compromised cells is of particular importance in the germline, as there is a need to maintain accurate transmission of genetic material from generation to generation and thus maintaining germline immortality (Gartner et al., 2008). The latter was illustrated in *C. elegans*, whereby genotoxic agent such as ionizing radiation triggered the appearance of increased apoptotic cells in the germline (Gartner et al., 2000).

An advantage of studying *C. elegans* is that the competence of the biological processes of cell cycle arrest and apoptosis is spatially separated in the worm germline upon genotoxic stress exposure (Craig et al., 2012; Gartner et al., 2000). In fact, in wild-type animals, cell cycle arrest appears to occur only in the proliferating mitotic zone of the distal gonad, while apoptosis occurs in the late pachytene meiotic zone around the gonadal loop region, which is the same location seen in physiological germline cell death (Craig et al., 2012; Gartner et al., 2000). Morphologically, the appearance of apoptotic cells in DNA damage-induced cell death are indistinguishable from the ones associated with physiological germline cell death (Gartner et al., 2000). These observations set the stage for genetic dissection of the DNA damage-induced germline cell death.

Genetic analyses have revealed that this stress-induced cell death also requires the core apoptotic activation machinery in CED-9, CED-4, and CED-3 (Gartner et al., 2000). However, in contrast to physiological germline cell death, *ced-9* gain-of-function mutation allele, *n1950,* completely abrogated the DNA damage-induced germline cell death (Gartner et al., 2000). Furthermore, as opposed to physiological germline cell death, it was found that this stress-induced form is partly dependent on *egl-1* (Gartner et al., 2000). These results suggested that this form of cell death was likely mediated through a different pathway than physiological germline cell death.

i) DNA checkpoint genes/proteins

The first clue of the existence of this separate pathway dictating DNA damage-induced cell death came from the isolation of mutants that appeared to have a broad defect in all three components of DNA damage response: DNA repair, cell cycle arrest, and apoptosis (Craig et al., 2012). These mutants were subsequently mapped to *clk-2* or *rad-5*, *mrt-2*, and *hus-1* (Ahmed et al., 2001; Gartner et al., 2000; Hofmann et al., 2002) and these consist of DNA checkpoint genes. *mrt-2* and *hus-1* are conserved genes and its proteins encode part of the 9-1-1 PCNA (proliferating cell nuclear antigen) complex, which localizes to DNA double-strand breaks and are thought to act as sensors of DNA damage (Hofmann et al., 2002; Stergiou and Hengartner, 2004). *clk-2* or *rad-5*, which are allelic to each other, on the other hand is thought to function as DNA checkpoint gene in a parallel pathway to *mrt-2* and *hus-1* (Stergiou and Hengartner, 2004). It has been suggested that in *C. elegans*, CLK-2 senses misincorporation of uracil into the DNA, which is the most common endogenous DNA damage elicited (Dengg et al., 2006). CLK-2 is also a conserved protein from yeast to humans (Gartner et al., 2008). Human CLK-2 was shown to bind and stabilize ATR and ATM, which are two PI3 kinases with important signal transduction role in DNA damage response (Gartner et al., 2008; Stergiou and Hengartner, 2004; Takai et al., 2007).

As in humans, worm homologs of ATM and ATR kinases function in DNA checkpoint signaling. Worm homolog of ATR (ATL-1) is needed as a checkpoint protein in response to ionizing radiation, UV, and replication stress and has been shown to localize to damaged DNA resulting from irradiation or replication stress (Garcia-Muse and Boulton, 2005). In worms, the

homolog of ATM (ATM-1) is thought to play a relatively minor role in DNA damage response compared to ATL-1 (Bailly and Gartner, 2013; Garcia-Muse and Boulton, 2005). These two kinases are thought to phosphorylate various downstream substrates that are important in DNA damage response, such as CHK-1/CHK1, CHK-2/CHK2, and CEP-1/p53 in mammalian system. but this is less definite in *C. elegans* (Garcia-Muse and Boulton, 2005; Stergiou and Hengartner, 2004).

ii) CEP-1/p53 and CEP-1 regulation in *C. elegans*

Akin to the critical role played by the mammalian tumor suppressor protein p53 in response to genotoxic stress, its worm homolog, CEP-1, was found to be critically important for DNA damage-induced germline cell death (Derry et al., 2001; Schumacher et al., 2001). In contrast, CEP-1 does not seem to be involved in somatic cell death nor in physiological germline cell death (Schumacher et al., 2001). In fact, it is generally thought that physiological germline cell death is a CEP-1-independent process. Unlike mammalian *p53*, *cep-1* does not seem to be required for DNA damage-induced cell cycle arrest nor have a prominent DNA repair role upon ionizing radiation (Bailly and Gartner, 2013; Derry et al., 2001; Schumacher et al., 2001). Therefore, CEP-1 seems to have a specific role in promoting apoptosis in the DNA damage response. As a transcriptional regulator, CEP-1 promotes apoptosis through transcriptional activation of both *egl-1* and *ced-13*, which are both BH3-only genes (Schumacher et al., 2005a). Similar to EGL-1 binding to CED-9 to promote apoptosis, it was shown that CED-13 can also physically interact with CED-9 to induce programmed cell death (Schumacher et al., 2005a).

Given the critical role of CEP-1/p53 in mediating DNA damage-induced germline cell death, it is not surprising that this protein is highly regulated. In contrast to the mammalian system however, there appears to be no worm homolog of MDM2, a E3 ubiquitin ligase that functions as a critical negative regulator of p53 (Jones et al., 1995; Montes de Oca Luna et al., 1995). In *C. elegans*, it was demonstrated that ABL-1 negatively regulated CEP-1-mediated germline apoptosis after ionizing radiation and thus acting as an anti-apoptotic protein (Deng et al., 2004). It is worth denoting that its mammalian homolog, ABL1, is implicated in the BCR-ABL1 fusion protein encoded by the Philadelphia chromosome in Chronic Myeloid Leukemia (CML). Interestingly in the latter context, exposing wild-type worms to chemical inhibitors of ABL-1, including imatinib (Gleevec), which is used to treat CML, recapitulated the increased germline apoptosis phenotype of *abl-1(loss-of-function)* mutants (Deng et al., 2004). In addition to ABL-1, an arginine methyltransferase, PRMT-5, was found to negatively regulate CEP-1 transcriptional activity (Yang et al., 2009). This is thought to be achieved by methylating and thus silencing a CEP-1 cofactor called CBP-1, which is the worm homolog of human p300/CBP coactivator family (Yang et al., 2009).

GLD-1 is a RNA-binding protein with important role in controlling meiosis entry of germ cells as a translational repressor (Jan et al., 1999; Jones and Schedl, 1995). It was subsequently found that GLD-1 can also repress translation of *cep-1* mRNA by binding to its 3' UTR (Schumacher et al., 2005b). Since GLD-1 expression is elevated in early and mid-pachytene region of the gonad and starts to decline in late pachytene gonadal area, specifically around where germline apoptosis normally occurs, it is argued that the GLD-1-mediated translational repression of CEP-1 helps ensure that DNA damage-induced apoptosis does not occur in more distal portion of the gonad (Bailly and Gartner, 2013).

In addition to its critical role in physiological germline cell death, it was found that MPK-1/MAPK signaling also plays an important role in DNA damage-induced germline cell death (Eberhard et al., 2013; Rutkowski et al., 2011). In fact, MPK-1 was found to be phosphorylated and activated upon ionizing radiation, which led to enhanced CEP-1-dependent germline cell death (Rutkowski et al., 2011). The authors provide evidence that MPK-1 signaling impart the apoptotic competency of germ cells in late pachytene region by restricting CEP-1 expression in this region of the gonad (Rutkowski et al., 2011). This restriction of CEP-1 expression by MPK-1 is in turn thought to occur by regulating GLD-1 expression by MPK-1 (Rutkowski et al., 2011). Therefore, it is suggested that both MPK-1 and GLD-1 serve to set the spatial location of apoptotic competency of germ cells upon ionizing radiation.

iii) Role of ceramide in radiation-induced germline cell death in *C. elegans*

Ceramide is a molecule that arises from sphingolipid metabolism and is important for the integrity of plasma membrane (Bailly and Gartner, 2013). It was observed that cellular ceramide levels are increased following apoptotic stress inputs, such as ionizing radiation or UV exposure (Bailly and Gartner, 2013). It was in this context that a *C. elegans* study found that mutants blocking ceramide biogenesis abrogated ionizing radiation-induced germline cell death and that micro-injection of long-chain ceramide restored these mutants' ability to induce cell death upon radiation (Deng et al., 2008). Genetic analysis further suggested that the ceramide-induced germline cell death acted on a parallel pathway to *cep-1* pathway (Deng et al., 2008). In addition to its role in radiation-induced germline cell death, the data indicates a promoting role of ceramide in physiological germline cell death (Deng et al., 2008).

b) Other forms of stress-induced germline apoptosis in *C. elegans*

As denoted earlier in this section, other forms of stress, such as oxidative, osmotic, heat shock, starvation, and pathogenic bacteria, have all been demonstrated to be able to promote germline apoptosis (Aballay et al., 2003; Salinas et al., 2006). However, it is not clearly understood how these induce cell death in the germline.

Data presented by Fred Ausubel lab suggest that p38 MAPK pathway is important in pathogenic bacteria-induced germline apoptosis (Aballay et al., 2003). In fact, they have highlighted the requirement of *pmk-1* gene, a worm homolog of p38 MAP kinase, in promoting this form of stress-induced germline cell death.

In contrast to DNA damage-induced germline cell death, oxidative, osmotic, heat shock, and starvation stresses appear to trigger germline cell death independent of CEP-1 and EGL-1 and instead may rely on molecular players involved in the physiological germline cell apoptosis, including the MAPK pathway (Salinas et al., 2006). The latter observations suggest once more that common biology may yet be at the intersection of stress-induced and physiological germline cell death.

4. Germline apoptosis in other metazoans

Beyond *C. elegans*, the phenomenon of germline apoptosis can be observed across metazoans, from *hydra* to humans (Baum et al., 2005; Vaskivuo and Tapanainen, 2003). Furthermore, apoptosis occurs during both processes of spermatogenesis and oogenesis. In order

to focus our discussion and be reflective of germline apoptosis in *C. elegans* that only occurs in oogenesis, we will be discussing concepts of programmed cell death occurring in female germ cells in this section.

Common biological elements/themes can be discerned when observing germline apoptosis across worms to humans (Baum et al., 2005; Gartner et al., 2008; Pepling, 2006; Pepling and Spradling, 2001). First of all, following mitotic divisions that allow formation of significantly large number of female germ cells, there is a considerable amount of those germ cells that die through the process of apoptosis. In humans, the majority of the $7x10^6$ oocytes formed in early fetal life are culled to ultimately yield around 400 follicles being ovulated in a span of a woman's reproductive life before menopause sets in (Baker, 1963; Vaskivuo and Tapanainen, 2003). In fact, 99.9% of the total human female germ cells end up eliminated through the process of apoptosis leading up to menopause (Tilly, 2001). Apoptosis is thus suggested to be the mechanism that determines female reproductive lifespan and thus making the female biological clock tick (Vaskivuo and Tapanainen, 2003). This significant germ cell loss is also observed in female mice (Pepling and Spradling, 2001; Tilly, 2001).

It remains unclear why female germline tissues would produce so many germ cells only to eliminate the bulk of them through apoptosis. Few hypotheses have sprung over the years, but the most prevalent thinking about why these "physiological" germline apoptosis occurs across animal species relate to the "nurse-cell" theory or "death by self-sacrifice" (Baum et al., 2005; Tilly, 2001). Across many species, invertebrates and vertebrates, observations that germ cells are connected to each other via intercellular bridges and thus sharing a common cytoplasm have supported this "nurse-cell" theory. Prior to the death of these "nurse" cells, transfer of their cytoplasmic contents to the growing/"chosen" oocytes has been observed (Baum et al., 2005). In

a similar theme, mammalian germ cells are also connected to each other within cysts and are thought to transfer cytoplasmic contents amongst themselves prior to the death of these “nurse” germ cells via programmed cell death (Gartner et al., 2008; Pepling, 2006; Pepling and Spradling, 2001; Tilly, 2001).

Molecular mechanisms of how these physiological germline cell deaths occur in other organisms remain poorly understood. In mammals, similar players involved in the intrinsic apoptotic pathway, such as *Bcl-2* and *Bax* have been shown to be implicated (Hsu et al., 1996; Perez et al., 1999; Ratts et al., 1995).

Finally, akin to *C. elegans'* germline, mammalian germline is very sensitive to DNA damage-caused by radiation or other stressors. In fact, exposure to anti-cancer treatments in the form of chemotherapy or radiation can accelerate the rate of oocyte decline to cause infertility and premature menopause in young women treated with these modalities (Tilly, 2001). It is thought that approximately four to ten DNA double-stranded breaks are sufficient to induce apoptosis in mouse oocytes (Peters and Levy, 1964; Tuppi et al., 2018). We will explore in the next section how stress, specifically radiation, can induce apoptosis in both mammalian somatic and germline cells.

C) STRESS-INDUCED APOPTOSIS/INTEGRATED STRESS RESPONSE/GCN1

1. Stress-induced apoptosis (Radiation Stress)

In this section, I will be discussing concepts in stress-induced apoptosis in the context of radiation stress in the mammalian system. Similar to *C. elegans*, the critical molecular player responsible for radiation stress-induced apoptosis in mammalian system is the p53 protein. I will be separating our discussion in this section by discussing both p53-dependent radiation-induced apoptosis and p53-independent mechanisms.

a) p53-dependent radiation-induced apoptosis

p53 is widely referred to as the guardian of the genome (Efeyan and Serrano, 2007) and for good reason, as it is critical to maintain mammalian genome integrity. It is a transcription factor that mediates a variety of molecular functions in order to maintain this integrity: cell cycle arrest, DNA repair, and apoptosis. Therefore, as denoted previously, mammalian p53 is responsible for greater biological functions than CEP-1 in *C. elegans*. In this sub-section, I will focus on p53's role in apoptosis in the context of radiation stress.

In normal physiological unstressed condition, p53 is negatively regulated by MDM2, which is a E3 ubiquitin ligase that degrades p53 protein and thus kept at a low level to prevent its inappropriate induction of apoptosis (Bailly and Gartner, 2013; Haupt et al., 1997; Jones et al., 1995; Kastenhuber and Lowe, 2017; Montes de Oca Luna et al., 1995). Upon radiation stress, p53 is stabilized through phosphorylation by kinases acting upstream in the DNA damage response pathway (Canman et al., 1998; Kastenhuber and Lowe, 2017). In fact, phosphorylation of p53 abrogates its interaction with MDM2 and thus inhibits MDM2-mediated p53 protein degradation (Shieh et al., 1997). A stable p53 protein then subsequently promotes transcription of variety of pro-apoptotic genes, most critical being the pro-apoptotic members of the Bcl-2

protein family (Eriksson and Stigbrand, 2010). Indeed, BH3-only members of the Bcl-2 family, *puma* (p53-upregulated modulator of apoptosis) and *noxa* are transcriptionally induced by p53 in response to radiation (Nakano and Vousden, 2001; Oda et al., 2000; Villunger et al., 2003). PUMA and NOXA subsequently inhibits Bcl-2 and as a result activates the intrinsic apoptotic pathway. Furthermore, it is thought that the extrinsic apoptotic pathway is also implicated in radiation-induced apoptosis in that there is upregulation of death receptor gene in a p53-dependent manner (Wu et al., 1997b).

In mammals, p53 is a member of a family of transcription factors that also consists of p63 and p73 (Bailly and Gartner, 2013). p63 is thought to be the most ancient of the three members and thus most closely related to CEP-1 in *C. elegans* (Bailly and Gartner, 2013). Interestingly and akin to the role played by CEP-1, p63 was demonstrated to be the p53 member that promotes DNA damage-induced cell death specifically in the mammalian germline (Suh et al., 2006). DNA damage checkpoint kinase CHK2 was determined to be an important player in the promotion of mammalian oocyte cell death (Bolcun-Filas et al., 2014). Similar to what occurs in somatic cells, recent studies have shown that activation of p63 requires its phosphorylation by upstream kinases, such as CHK2 and casein kinase 1(CK1) (Tuppi et al., 2018). Abrogating CK1 rescued oocytes from doxorubicin and cisplatin-induced apoptosis (Tuppi et al., 2018). Akin to the action of p53 in somatic cells, p63 is thought to induce transcription of both *puma* and *noxa,* which was shown to promote DNA damage-induced oocyte apoptosis and thus cause female infertility (Kerr et al., 2012).

b) p53-independent/ceramide-mediated radiation-induced apoptosis and radiation-induced bystander effects

p53 and its family of transcription factors are traditionally thought to be central in mediating the cell-death pathway resulting from DNA damage occurring in the nucleus. There have been discussions of parallel cell-death mechanism at play that is p53-independent in addition to not being mediated by DNA damage in the nucleus, but rather by radiation effects in the cell plasma membrane (Haimovitz-Friedman et al., 1994; Prise et al., 2005). The latter mechanism proposes that ionizing radiation activates acid sphingomyelinase, which hydrolyzes sphingomyelin located in plasma membranes to produce ceramide (Haimovitz-Friedman et al., 1994; Prise et al., 2005). Ceramide would then act as a second messenger to activate BAX-mediated mitochondrial outer membrane permeabilization (MOMP) in order to activate the intrinsic apoptosis pathway (Prise et al., 2005). It has also been suggested that ceramide may be produced and released from the mitochondria through activation of mitochondrial ceramide synthase upon radiation (Kolesnick and Fuks, 2003). As a feedback mechanism, this ceramide-mediated programmed cell death can be suppressed by a ceramide metabolite, sphingosine-1-phosphate (S1P) (Cuvillier et al., 1996).

As in our prior description of the role of ceramide/sphingomyelin metabolism in *C. elegans*' stress-induced germline cell death, it is interesting that ceramide/sphingomyelin metabolism was shown to be also important in mammalian oocyte apoptosis (Morita et al., 2000). In fact, application of S1P mitigated the appearance of oocyte apoptosis triggered by radiation (Morita et al., 2000).

In addition to apoptosis triggered by damages incurred in the nucleus or in the plasma membrane upon radiation, there has been recognition that unirradiated cells can be impacted biologically when their neighboring cells are irradiated. This is referred to radiation-induced bystander effect (RIBE). The molecular and cellular mechanism of how bystander effects occur remains poorly understood, but is thought to involve various cytokines, such as interleukins and tumor necrosis factor alpha, as well as oxidative stress mechanism (Prise et al., 2005). In fact, DNA damage is thought to be induced in bystander cells either via reactive oxygen or nitrogen species (Iyer et al., 2000; Prise et al., 2005; Shao et al., 2003). Intercellular communication via gap-junctions is also thought to be implicated in bystander responses (Azzam et al., 1998).

2. Integrated Stress Response (ISR) and ISR-induced apoptosis

An important and efficient way how a cell modulates gene expression in order to adapt to stress is through translation. In fact, translation is the final step in the central dogma of molecular biology and thus regulation at this step allows for a rapid response to changes in the environment (Holcik and Sonenberg, 2005). This is no different upon radiation stress. It is remarked that general translation output of a cell is reduced in response to most cellular stress inputs in exchange for selective increased translation of specific proteins that are important for the cell to adapt/survive under stress (Holcik and Sonenberg, 2005).

Translation has four distinct phases: initiation, elongation, termination, and recycling (Hershey et al., 2019). Each of these phases has their own regulatory mechanisms, but the most critical and rate-limiting step of translation that is regulated is the initiation step (Holcik and Sonenberg, 2005). Regulation at the initiation step probably evolved due to the fact that it is

effective and more energetically efficient to regulate at the onset of translation, instead of trying to halt the process in the middle of it, which in the case of translation may lead to undesired sequelae of aberrant protein synthetic products (Holcik and Sonenberg, 2005). A key pathway that a eukaryotic cell can activate to modulate the initiation step of translation upon stress is the evolutionary conserved pathway of Integrated Stress Response (ISR).

The Integrated Stress Response has its origins from the early work done on general amino acid control upon amino acid starvation stress in yeast (Hinnebusch, 2005). From the subsequent series of genetic and molecular studies, key genes/proteins such as GCN2, eIF2alpha and GCN4/ATF4 were discovered, characterized and help laid the foundation of what the Integrated Stress Response is today (Holcik and Sonenberg, 2005) (Figure 4).

The Integrated Stress Response pathway is characterized in the mammalian system by four distinct eIF2alpha kinases: GCN2, PKR, HRI, and PERK. Each of these kinases responds to particular stress inputs that allow them to phosphorylate eIF2alpha, which serves as a “gateway” for the Integrated Stress Response activation (Figure 4). GCN2 is activated upon amino acid starvation, but also have been found to be active under UV radiation stress (Deng et al., 2002; Lu et al., 2009). Although each of the eIF2alpha kinases responds to specific stress signal cues, it is now suggested that these kinases have some overlapping functions in order to either work cooperatively/synergistically with each other to tune the cellular response upon stress or to compensate for one’s loss of activity (Pakos-Zebrucka et al., 2016). For instance, GCN2 can respond to endoplasmic reticulum (ER) stress in PERK-deficient mouse embryonic fibroblasts (Hamanaka et al., 2005) and conversely PERK can compensate for the lack of GCN2 (Lehman et al., 2015).

The phosphorylation on eIF2alpha occurs on a conserved serine residue, which allows the latter to inactivate eIF2B. eIF2B, in normal physiological condition, serves as a guanine nucleotide exchange factor (GEF) allowing the exchange of GDP for GTP on eIF2 in order to form the ternary complex, eIF2/GTP/Methionyl-initiator tRNA (Met-tRNAi^{Met}) (Holcik and Sonenberg, 2005) (Figure 4). The formation of this ternary complex is critical for translation initiation to occur. Therefore, the result of the inactivation of eIF2B through phosphorylation of eIF2alpha is diminishment of general protein synthesis output in exchange for selective translation initiation of proteins critical for stress adaption. One such protein is GCN4 in yeast or ATF4 in other eukaryotes, including *C. elegans* and mammals (Dever et al., 1992; Pakos-Zebrucka et al., 2016). The selective translation initiation mechanism of GCN4/ATF4 resulting from ISR activation is mediated by a unique feature on the 5' UTR sequence of these mRNAs called upstream open reading frames (uORFs) (Mueller and Hinnebusch, 1986). As an ISR effector protein, GCN4/ATF4 serves as a critical transcription factor in charge of modulating the expression of variety of genes important for stress adaption.

Some of these genes that are activated by ATF4 play a role in inducing apoptosis, particularly when the stress adaptation response through ISR can not restore cellular homeostasis (Pakos-Zebrucka et al., 2016). One such gene/protein with important apoptotic function is C/EBP homologous protein (CHOP). CHOP is itself a transcription factor that can influence apoptosis in variety of ways, such as increasing gene expression of pro-apoptotic Bcl-2 family members (Galehdar et al., 2010; Puthalakath et al., 2007), as well as upregulating transcription of a death receptor, death receptor 5 (DR5) (Lu et al., 2014; Zou et al., 2008).

Another way of thinking about how the above apoptotic mechanism mediated by ATF4-CHOP gets engaged is when there is chronic ISR activation through persistent activity of the

upstream eIF2alpha kinases (Pakos-Zebrucka et al., 2016). In the case of GCN2, which is evolutionarily the most ancient of the four eIF2alpha kinases, its activation requires another gene/protein, which allows it to sense the upstream stress signals.

3. GCN1

GCN1 or GCN-1 in *C. elegans* is a large evolutionary conserved protein that is required for GCN2 to activate ISR (Marton et al., 1993). In fact, ever since their initial isolation from a common genetic screen in yeast (Penn et al., 1983; Skvirsky et al., 1986), the conventional wisdom dictates that GCN1 and GCN2 interacts together to activate the Integrated Stress Response pathway (Castilho et al., 2014; Marton et al., 1993). In yeast, GCN2 is implicated in a variety of stress responses and yeast GCN1 was found to be required for all of those GCN2-mediated responses (Castilho et al., 2014), highlighting the essential role of GCN1 for GCN2 action and the coupling of these two proteins to mediate variety of biological functions.

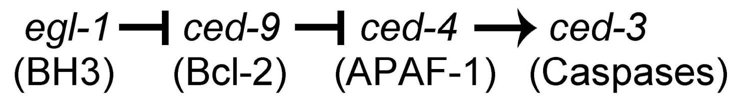
In addition to GCN2, GCN1 interacts with ABCF3 (GCN20 in yeast) and the 80S ribosome. In yeast, GCN1 forms a complex with GCN20 and this complex promotes GCN2 activation (Marton et al., 1997; Vazquez de Aldana et al., 1995). Like GCN1, GCN20 can also bind to the ribosome, but this is largely dependent on GCN1 (Marton et al., 1997). Reciprocally, it has also been suggested that GCN20 can modulate GCN1-ribosome interaction in the presence or absence of ATP such that in the absence of ATP, GCN20 can diminish the GCN1-ribosome interaction (Castilho et al., 2014; Marton et al., 1997). Structural details of GCN1 interaction with the ribosome was recently resolved by cryo-EM and further highlights GCN1's role as a translational regulator (Pochopien et al., 2021). Besides its interactions with GCN20 and the

ribosome, it is speculated that GCN1 mediates more protein-protein interactions given the unusually high number of HEAT repeat motifs present throughout its large protein (Castilho et al., 2014). HEAT repeat motifs are thought to facilitate protein-protein interactions (Andrade et al., 2001). Nevertheless, many of the functional biology of GCN1 remain unknown.

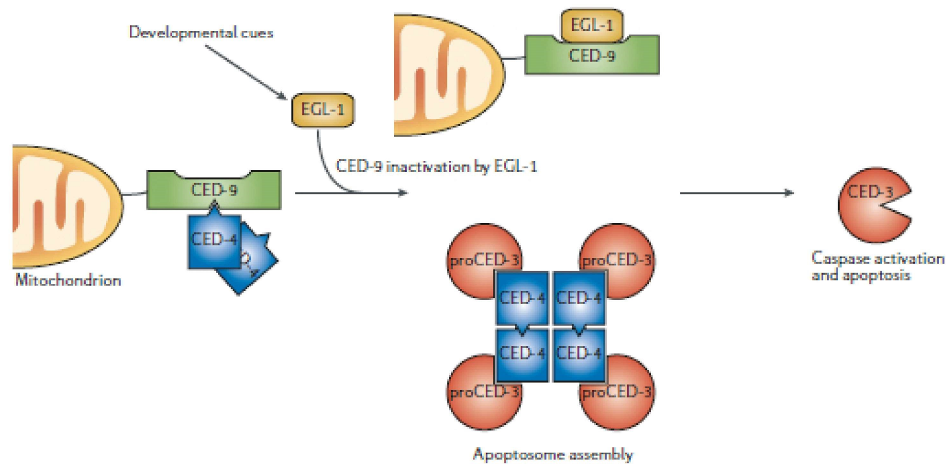
Amongst the scant descriptions of the biological function of GCN1, this large protein through its interaction with a viral envelope glycoprotein has been suggested to have a role in the pathogenesis of viral infections (Hirohata et al., 2015). This is thought to be achieved by allowing increased protein synthesis in the virus-infected host cell through GCN1's modulatory role on GCN2 and the Integrated Stress Response pathway. The latter is particularly of relevance in this COVID-19 pandemic age. Importantly, in the context of our topic of discussion on apoptosis, GCN-1 has been implicated in programmed cell death of neurons in *C. elegans* and may possibly have a role in ionizing radiation/stress-induced germline cell death (Hirose and Horvitz, 2014). It is unknown however how GCN-1 mediates programmed cell death.

Now that we have covered the important background knowledge of the research undertaken in this thesis, we will transition to the data chapter where I will further discuss our exploration of the nature of GCN-1's role in programmed cell death in addition to the exploration of how GCN-1 promotes cell-death mechanism.

A



B

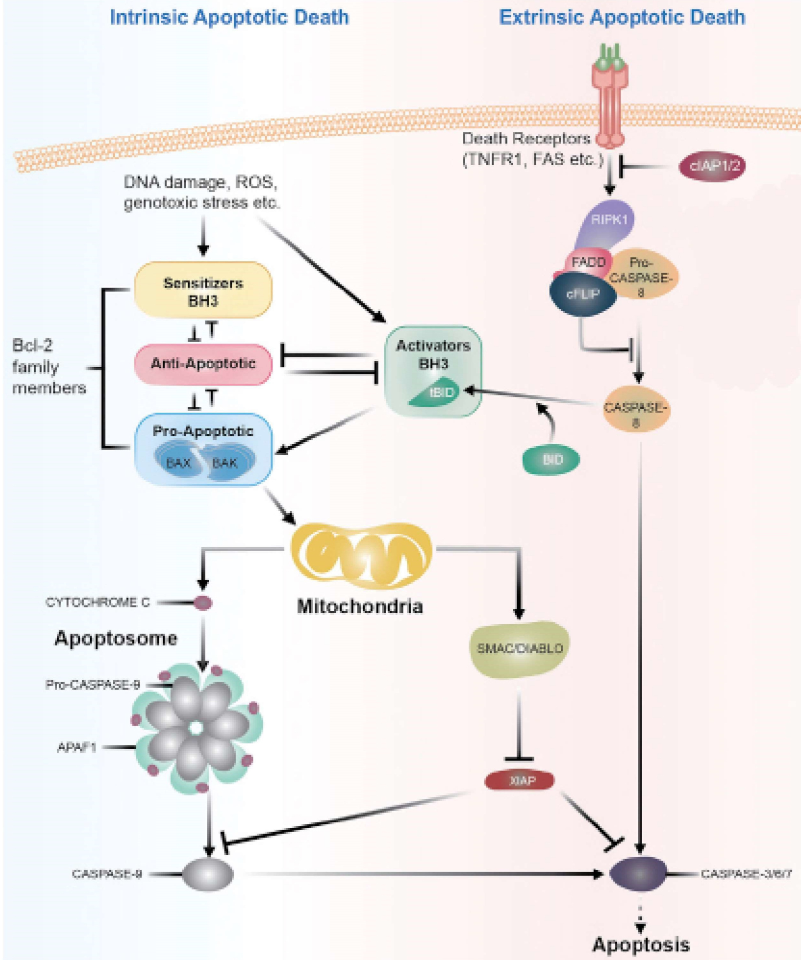


Adapted from Lettre, G., and Hengartner, M.O. (2006).
Nat. Rev. Molecular Cell Biology

Figure 1

Fig. 1| Genetic and molecular basis of apoptosis in *C. elegans*

a. Genetic pathway of cell-death activation program in *C. elegans*. Respective mammalian protein homologs are listed in parentheses. **b.** Molecular mechanism of cell-death activation program in *C. elegans*.

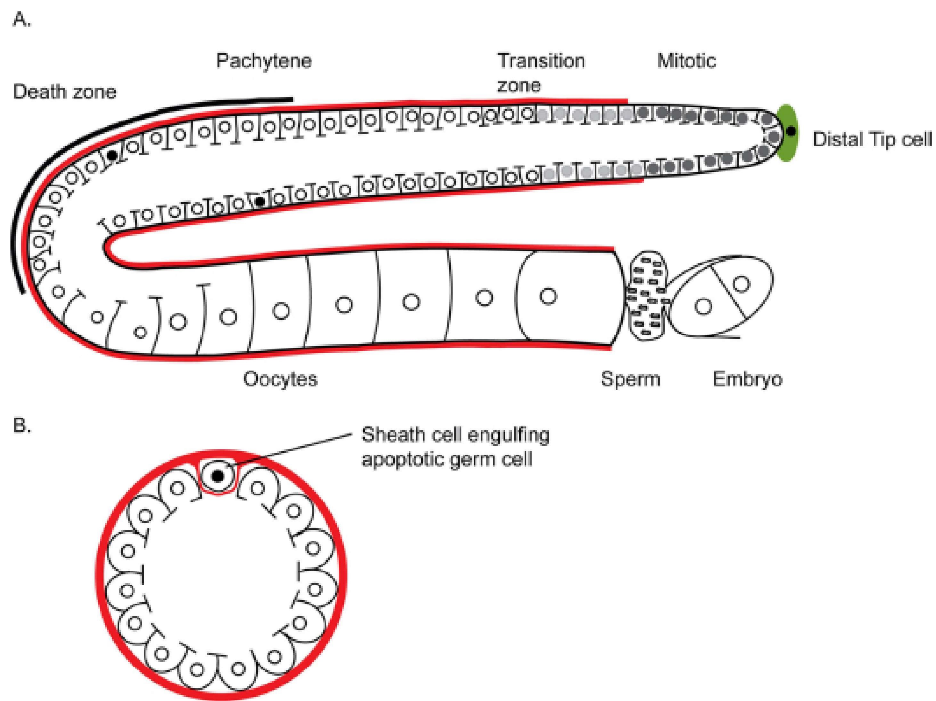


Adapted from Wallach, D., and Kang, T.B. (2018). Immunity

Figure 2

Fig. 2| Intrinsic and extrinsic apoptosis pathways in mammalian system

The intrinsic apoptosis pathway is notable due to the central role played by the mitochondria as well as the various Bcl-2 protein family members. In contrast, the extrinsic pathway is characterized by the central role played by a group of receptors called death receptors.

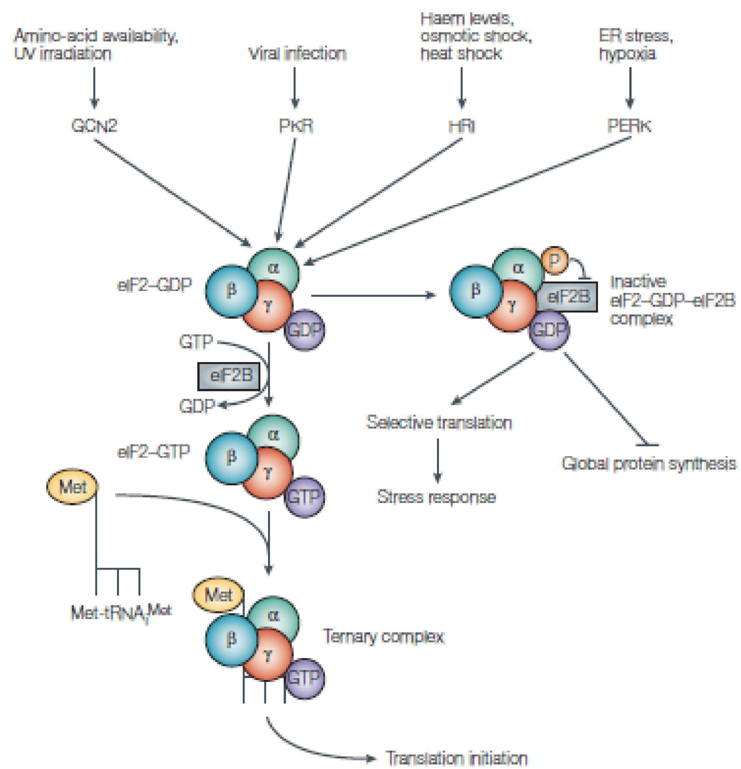


Adapted from Gartner, A. et al., (2008). Wormbook

Figure 3

Fig. 3| Anatomy of *C. elegans* germline

a. Representation of a gonad from an adult hermaphrodite. One of the two gonadal arm structures is shown. Germline apoptosis occurs predominantly within the loop region marked in black line. **b.** Cross section of the loop region is shown. A single germ cell undergoing apoptosis is demonstrated with its nucleus marked in black.



Adapted from Holcik, M., and Sonenberg, N. (2005).
Nat. Rev. Molecular Cell Biology

Figure 4

Fig. 4| Integrated stress response pathway in mammalian system

The integrated stress response is characterized by eIF2alpha kinases that phosphorylate eIF2alpha, the “gate keeper” of this pathway. Once activated, the integrated stress response pathway inhibits general protein synthesis. Instead, selective translation of proteins with roles in stress adaptation is promoted.

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Chapter 2

CONTROL OF PROGRAMMED CELL DEATH BY GCN-1

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ABSTRACT

Apoptosis is a cell death phenomenon that is fundamental to the development of an organism and to the pathogenesis of disease states. Regulation of apoptosis occurs at various molecular steps, but its regulation at the translational level remains poorly understood. The phenomenon of germline apoptosis, either physiological (i.e., without stress) or stress-induced, occurs throughout eukaryotic organisms from worms to humans and may have a role in maintaining germline immortality by eliminating compromised germ cells. Therefore the ability to regulate germline apoptosis is intricately linked with the survival and fitness of species. GCN-1, a known translational regulator, has been traditionally associated with GCN-2 to activate the Integrated Stress Response in order to modulate protein synthesis in the context of stress.

Here we show that GCN-1 promotes both physiological and stress-induced germline apoptosis through a GCN-2 and Integrated Stress Response-independent mechanism. Furthermore, in conditions of stress the role of GCN-1 in promoting apoptosis is at least in part independent of the action of CEP-1/p53. To determine how GCN-1 controls cell death, we identified a number of genes, which when abrogated/inhibited, suppressed the germline cell-death defect caused by loss-of-function of *gcn-1*. Many of the genes for which inhibition causes suppression encode for RNA-binding proteins with known roles in modulating translation. In addition, we have identified candidate translational targets of GCN-1. The present study proposes a potential novel translational control of apoptosis by GCN-1 with possible implications in further our understanding of germline apoptosis, as it relates to modulating female reproductive lifespan and safeguarding germ cells against environmental insults such as radiation and chemotherapy.

INTRODUCTION

To live or to die? Cells face life-and-death decisions during development and upon stress. The term apoptosis was first coined in 1972 (Kerr et al., 1972) to describe one of the most important cell death phenomenon in biology. Thanks to the unique features of *Caenorhabditis elegans* (*C. elegans*), genetic studies in this organism established that apoptosis involves intricate genetic control to modulate this programmed cell death (Ellis and Horvitz, 1986). Subsequent studies have established a canonical apoptosis activation pathway that proved to be evolutionarily conserved in many metazoans, including humans. The molecular players in this pathway consist of EGL-1/BH3, CED-9/Bcl-2, CED-4/APAF-1, and CED-3/caspases (Conradt et al., 2016; Metzstein et al., 1998). Regulation of the players in this apoptotic pathway occurs at several molecular steps, such as transcription, protein assembly, post-translational processing via proteolytic cleavage, protein degradation, and inhibition of enzymatic activity (Conradt et al., 2016; Subasic et al., 2016). However, translational regulation of apoptosis remains poorly understood (Conradt et al., 2016; Subasic et al., 2016).

In *C. elegans*, as in many organisms, the germ cells can undergo programmed cell death (Gumienny et al., 1999). Germline cell deaths can be divided into two types. First, physiological germline cell death, where more than half of total germ cells of a hermaphrodite animal die by apoptosis during the course of oogenesis in the absence of any stress inputs (Gumienny et al., 1999). Second, stress-induced germline cell death, where environmental insults such as ionizing radiation induce apoptosis in the germ cells (Gartner et al., 2000). Germline apoptosis is of particular interest as it may have a role in maintaining germline immortality by eliminating compromised germ cells, particularly in the context of stress-induced germline cell death (Gartner et al., 2008), and thus is linked with the survival and fitness of species. Striking

similarities exist in germline apoptosis from worms to humans (Gartner et al., 2008; Pepling, 2006; Pepling and Spradling, 2001). Similar to worms, a large amount of germ cells are culled via apoptosis in human ovaries. In fact, in early fetal life, a human ovary contains approximately $7x10^6$ germ cells/oocytes. The majority of these will die via apoptosis during oogenesis, such that a total of only around 400 follicles will ovulate during the span of a woman's reproductive life (Baker, 1963; Vaskivuo and Tapanainen, 2003). Furthermore, mammalian germ cells are sensitive to dying via apoptosis when exposed to environmental stressors, such as radiation and chemotherapy (Hanoux et al., 2007; Matikainen et al., 2001; Meirow and Nugent, 2001; Perez et al., 1997; Vaskivuo and Tapanainen, 2003). The similarities in the biology and physiology of germ cell apoptosis across species suggest a broad conservation of the biological mechanism at play that may have an impact in determining the female reproductive lifespan and in safeguarding oocytes against environmental insults such as radiation and chemotherapy. In worms as in humans, the mechanism of the physiological and stress-induced germline cell death remains poorly understood, except for the subtype of stress-induced cell death mediated by p53 and the DNA damage response (Gartner et al., 2008). Broadly, further mechanistic understanding of stress-induced apoptosis is of particular relevance in medicine because of its fundamental contribution to the pathogenesis of disease states.

gcn1 was first identified in a genetic screen in yeast for mutants defective in the general control of amino acid biosynthesis in the context of amino acid starvation (Penn et al., 1983; Skvirsky et al., 1986). Through subsequent studies, GCN1 was found to interact with GCN2 to phosphorylate eIF2α in order to activate the Integrated Stress Response pathway, an evolutionary conserved stress response pathway that modulates protein synthesis in response to a variety of external stresses (Castilho et al., 2014; Holcik and Sonenberg, 2005; Marton et al., 1993;

Sattlegger and Hinnebusch, 2000). Biochemical and structural analyses have found that GCN1 interacts with the 80S ribosome and thus acts as a translation regulator (Castilho et al., 2014; Pochopien et al., 2021). Through its role in activating GCN2 and the Integrated Stress Response pathway, it has been shown to be implicated in the pathogenesis of viral infections by allowing increased protein synthesis in the virus-infected host cell (Hirohata et al., 2015). GCN-1 has also been implicated in programmed cell death of neurons in *C. elegans* and may possibly have a role in ionizing radiation/stress-induced germline cell death (Hirose and Horvitz, 2014).

In the present study, contrary to the conventional wisdom, we discovered that GCN-1 has a GCN-2 and Integrated Stress Response-independent role in promoting programmed cell death in the germline. In addition, we uncovered several suppressor and enhancer genes of *gcn-1* as well as some candidate target genes that may be subject to GCN-1-dependent translational control; pointing towards the discovery of a potentially novel translational control of programmed cell death.

RESULTS

GCN-1 promotes both physiological and stress-induced germline programmed cell death

A previous genetic screen designed to uncover new apoptotic genes using a marker of the M4 neuron and its sister, which normally dies, isolated *gcn-1(n4827)* mutants (Hirose and Horvitz, 2014). The *n4827* allele encodes for an early nonsense mutation and thus likely represents a null mutation (Figure 1 A). In addition to its role in M4 sister cell death, previous work had hinted of a possible role for GCN-1 in ionizing radiation stress-induced germline programmed cell death/apoptosis as determined using acridine orange labeling (Hirose and Horvitz, 2014). We utilized an integrated transgene expressing a translational GFP reporter *bcIs39[Plim-7::ced-1::gfp]*, which has been used to mark apoptotic cells in the germline (Lant and Derry, 2014a), to detect apoptotic cells in the non-irradiated 0 Gray (Gy) wild-type animals (Figure 1B, Figure 1G, Supplemental Figure 1A, and Supplemental Figure 1F). Apoptosis that occurs without any environmental stresses is described in the literature as "physiological" germline cell death (Lant and Derry, 2014b). In wild-type animals subjected to 60 Gy cumulated dose of gamma radiation, we observed a robust increase in the number of apoptotic cells (Figure 1B, Figure 1G, Supplemental Figure 1A, and Supplemental Figure 1F). Previous work indicated that in the context of abnormally elevated germ cell apoptosis, apoptotic cells are typically arranged in clusters (Gartner et al., 2008), which was readily observed in our animals (Figure 1B, Figure 1D, Supplemental Figure 1A, and Supplemental Figure 1C). For ease of scoring in this study, we used this phenomenon as another metric to quantify the increased presence of apoptotic cells (i.e., the presence of "apoptotic clusters") (Figure 1H). We found this latter metric particularly useful when screening for *gcn-1* suppressors in the stress condition. The germline

cell death that occurs in the context of gamma radiation exposure will henceforth be referred to as “stress-induced” germline cell death throughout this chapter.

In contrast to wild-type animals, *gcn-1(n4827)* mutants had a decreased number of apoptotic cells in both physiologic and stress-induced conditions (Figure 1C, Figure 1G, Figure 1H, Supplemental Figure 1B, and Supplemental Figure 1F). *gcn-1(nc40)*, an allele which represents a deletion of 6880 base pairs (Figure 1A), recapitulated the decreased apoptosis phenotype of *gcn-1(n4827)* (Figure 1E, Figure 1G, Figure 1H, and Supplemental Figure 1D). The decreased apoptotic cell number phenotype of *gcn-1(n4827)* was rescued by the introduction of a wild-type copy of *gcn-1* (Figure 1D, Figure 1G, Figure 1H, and Supplemental Figure 1C). These CED-1::GFP positive cells were not observed in the *ced-3(n717)* genetic background, confirming that they are indeed apoptotic cells (Figure 1F, Figure 1G, Figure 1H, and Supplemental Figure 1E). We also tested whether the decreased apoptotic cell phenotype in *gcn-1* loss-of-function mutants was a consequence of altered kinetics in apoptotic cell engulfment, such as faster engulfment leading to a decreased number of apoptotic cells visible without a defect in the actual number of dying cells. Using time-lapse video experiments, we determined that there were no changes in engulfment kinetics in *gcn-1(n4827); bcIs39* animals when compared to *bcIs39* animals (Supplemental Figure 2A and Supplemental Figure 2B). We also assessed whether the decreased number of apoptotic cells seen in *gcn-1(n4827)* mutants could be explained indirectly by a decreased number of germ cells in these mutant animals. Using an mCherry translational histone reporter, *ltIs37*, we found that there was no detectable difference in the amount of germ cell nuclei between the mutant and the wild-type animals (Supplemental Figure 2B and Supplemental Figure 2C).

Together, these results suggest that GCN-1 promotes both physiological and stress-induced germline cell death (Figure 1G, Figure 1H, and Supplemental Figure 1F).

The stress-induced cell-death-defective phenotype of *gcn-1* is independent of *gcn-2* and the Integrated Stress Response (ISR) activation

Conventional wisdom denotes that GCN-1 acts by promoting GCN-2 function, which then goes on to activate the Integrated Stress Response (Castilho et al., 2014; Holcik and Sonenberg, 2005). We tested whether stress-induced apoptosis promoted by GCN-1 involves GCN-2 and the Integrated Stress Response, as is predicted by the canonical model for GCN-1 function. This is particularly relevant, because GCN-2 has been shown to be activated upon UV radiation (Holcik and Sonenberg, 2005). In addition, in mammals, activation of the Integrated Stress Response pathway has been linked to apoptosis through the induction of C/EBP homologous protein (CHOP) (Pakos-Zebrucka et al., 2016) and Death Receptor 5 (Lu et al., 2014), amongst others. We were surprised to find that the stress-induced germline apoptosis phenotype was not abrogated in the presence of a loss-of-function mutation in *gcn-2* (Figure 2D and Figure 2E), suggesting that GCN-1 may have a GCN-2-independent biological role in cell death.

Next, we wanted to determine whether GCN-1 has a role that is independent to the Integrated Stress Response pathway itself, by taking into account the fact that eIF-2α serves as a "gateway" for the pathway activation through its phosphorylation (Holcik and Sonenberg, 2005; Kulalert and Kim, 2013). Specifically, we made use of the *eif-2α(qd338)* mutant strain that contains an S49F mutation, which renders eIF-2α unphosphorylatable (Kulalert et al., 2017) and

the Integrated Stress Response pathway inactive. We confirmed that the *eif-2α(qd338)* mutant indeed has an unphosphorylatable form of eIF-2α by utilizing an antibody that recognizes specifically the phosphoserine residue 49 of eIF-2α (P-eIF-2α) (Hirose and Horvitz, 2014). When immunoblotting against eIF-2α on worm extracts arising from both wild-type and *eif-2α(qd338)* animals cultured in physiological conditions, we detected similar band intensity in both genotypes (Figure 2A). In contrast, when immunoblotting against P-eIF-2α we were able to detect phosphorylated eIF-2α only in wild-type animals (Figure 2A).

We subsequently tested whether the unphosphorylated eIF-2α in *eif-2α(qd338)* animals rendered the Integrated Stress Response pathway inactive, by assessing a key output of this pathway: the upregulation of ATF-4 (Holcik and Sonenberg, 2005). We used a transcriptional GFP reporter, *Patf-4::gfp* (Ferraz et al., 2016), to address this question. In wild-type animals, we observed GFP induction upon 5mM DTT application in addition to a baseline level of GFP expression when exposed to only the solvent/vehicle (Figure 2B). By contrast, *eif-2α(qd338)* mutants had no GFP induction upon DTT exposure, let alone any baseline GFP expression (Figure 2B). This confirms that the unphosphorylated eIF-2α in *eif-2α(qd338)* mutants is unable to activate the Integrated Stress Response pathway.

Finally, we tested these *eif-2α(qd338)* mutants for germline cell-death phenotype and to our surprise, as was the case for *gcn-2* loss-of-function mutants, we did not observe any diminishment in the appearance of apoptotic cells, in stark contrast to *gcn-1* loss-of-function mutants (Figure 2C and Figure 2E). These experiments indicate that GCN-1 likely has an independent biological function to GCN-2 and the Integrated Stress Response pathway in germline cell death.

GCN-1 acts independently to CEP-1/p53 to promote germline apoptosis in gamma-irradiated animals

Given that ionizing radiation causes genotoxic stress, we sought to determine whether *gcn-1* acts in germline cell death together with or independently from *cep-1*, the worm homolog of *p53* mammalian gene (Derry et al., 2001; Schumacher et al., 2001). We tested the single mutant animals of *cep-1* containing a null mutation (Baruah et al., 2014), *cep-1(gk138)*, in the gamma radiation cell-death assay. As expected, there was a significant decrease in apoptotic cells in the 60 Gy stress condition in the *cep-1* mutants compared to the wild type (Figure 3A and Figure 3C). We then tested the double mutant animals of *cep-1(gk138)* and *gcn-1(n4827)* and found that there was greater defect in the number of apoptotic cells after radiation compared to the single mutants (Figure 3B and Figure 3C). Specifically, the total number of apoptotic cells seen in the *cep-1(gk138); gcn-1(n4827)* double mutants were lower than either of the single mutant animals alone. These results suggest that GCN-1 acts at least in part independently to CEP-1/p53 to promote germline cell death (Figure 3D).

GCN-1 may promote cell death independent of caspases and the canonical programmed cell-death pathway

To determine the relationship between GCN-1 and the canonical programmed cell-death pathway, we performed genetic epistasis analyses between *gcn-1* and *ced-9*. The latter gene usually functions as an anti-apoptotic gene and acts downstream of *egl-1* and upstream of *ced-4* and *ced-3* in the apoptosis pathway (Conradt et al., 2016; Metzstein et al., 1998). The *ced-9(n2812)* null allele causes lethality to the animal (Hengartner et al., 1992) and thus a partial

loss-of-function allele of *ced-3, n2427* allele, was used to suppress the *ced-9(n2812)* lethality. Consistent with the role of *ced-9* as an anti-apoptotic gene, the *ced-9(n2812); ced-3(n2427)* double mutant animals had a substantial increase of germline apoptotic cells in both physiological and radiation stress conditions (Figure 4B and Figure 4D), when compared to *ced-3(n2427)* animals (Figure 4A and Figure 4D). However, the amount of apoptotic cell generation did not significantly change when comparing the triple mutant *gcn-1(n4827) ced-9(n2812); ced-3(n2427)* animals to the double mutant *ced-9(n2812); ced-3(n2427)* animals (Figure 4C and Figure 4D) suggesting that the phenotype caused by the *ced-9* loss-of-function mutation is epistatic to that caused by *gcn-1* loss of function. Given this epistasis analyses, *gcn-1* could act upstream of *ced-9* in the canonical cell-death pathway or in parallel to the pathway (Figure 4E).

To further help discern between these latter two possibilities, we performed gene interaction studies between *gcn-1* loss-of-function allele and the partial loss-of-function allele of *ced-3*. We observed that the double mutant *gcn-1(n4827); ced-3(n2427)* animals showed an enhancement effect in diminishing the number of apoptotic cells when compared to the respective single mutant animals (Figure 4F and Figure 4G). In other words, the *gcn-1* loss-of-function allele enhances a partial loss-of-function/weak allele of *ced-3*. This gene interaction studies with *ced-3* combined with previous gene interaction studies between *gcn-1* and *ced-9* in the context of M4 sister cell death suggest that *gcn-1* is likely not acting upstream of *ced-9*. In fact, *gcn-1* loss-of-function allele enhanced the M4 sister cell-death defect of *ced-9* loss-of-function allele and thus indicating that *ced-9* is not required for *gcn-1* to promote programmed cell death (Hirose and Horvitz, 2014). The fact that *gcn-1* enhances a weak allele of *ced-3* raises the possibility of *gcn-1* possibly acting through *ced-3* or *ced-4* to promote its pro-apoptotic function. However the combination of the results of the epistasis analyses between *gcn-1* and

ced-9 both in the context of M4 sister cell death and germline cell death, as well as the gene interaction studies with *ced-3* suggest the possibility that *gcn-1* may promote cell death independently to *ced-3/caspases* and the canonical cell-death pathway (Figure 4H)

Amongst the four caspase genes in *C. elegans'* genome, *csp-1*, in addition to *ced-3*, has been previously shown to have pro-apoptotic function (Denning et al., 2013; Shaham, 1998). Therefore, we wanted to test whether GCN-1 acts through CSP-1 to promote apoptosis. The *csp-1(n4967)* mutant contains a deletion in the *csp-1* gene that abrogates its putative caspase active site (Denning et al., 2013). We found that *csp-1(n4967)* did diminish the number of apoptotic cells in the stress condition, but not to the level of *gcn-1* mutants (Supplemental Figure 3A and Supplemental Figure 3B). This suggests that CSP-1 is not required for GCN-1 to promote apoptosis.

The epistasis experiments with *ced-9(loss-of-function)* combined with gene interaction studies with *ced-3(reduction-of-function)* and *csp-1(loss-of-function)* suggest that GCN-1 may promote cell death independent of caspases and the canonical programmed cell-death pathway, but further molecular studies are needed to confirm this with certainty.

Identification of *gcn-1* suppressors and *gcn-1* enhancers in stress-induced cell death

We sought to investigate the mechanistic basis of how GCN-1 promotes cell death. We took two parallel approaches in order to answer this question.

First, we undertook a chemical mutagenesis (ethyl methanesulfonate (EMS)) genetic screen to isolate suppressor mutants of *gcn-1(n4827); bcIs39* animals in the context of stress-

induced cell death. This was done through an F2 non-clonal screen that was designed to isolate suppressor mutants that give rise to significant "apoptotic clusters" in *gcn-1(n4827)* mutants in the context of 60 Gy ionizing radiation stress (Figure 5A). We screened a total of about 42 680 mutagenized haploid genomes, which is two times the number of F1 animals involved in the genetic screen. All the F2 progenies that were grown from these F1 animals were subsequently screened. This was a particularly challenging genetic screen since the low resolution of the phenotype required a compound microscope for scoring and the ionizing radiation stress needed to be timed in order to be given to strictly L4 stage animals. We successfully isolated 22 suppressor mutants, which were subsequently retested to display the phenotype of suppression (Table 1). The mutants were also characterized for the mode of inheritance, presence of maternal effect, as well as the number of responsible loci that account for the suppressor phenotype. 5 out of the 22 suppressor mutants had a dominant inheritance pattern and 17 out of 22 suppressor mutants had a recessive inheritance pattern. 3 out of the 22 suppressor mutants displayed a maternal effect. Most of the suppressor mutants contained one single responsible locus.

Second, in light of the significant time commitment needed for the EMS genetic screen approach, in parallel, we undertook a candidate approach using RNA interference (RNAi) to identify suppressor genes (Fire et al., 1998; Kamath and Ahringer, 2003; Timmons and Fire, 1998). Candidates were selected based on previous work that identified anti-apoptotic genes via a genome-wide RNAi screen (Lettre et al., 2004). These genes, when inhibited through RNAi, would cause excess germline cell death in the wild-type genetic background. This prior work identified a total of 21 such genes that were further classified as either p53-dependent or p53-independent genes through their individual epistasis analysis with *cep-1/p53*. While knock down of those genes classified as p53-dependent genes in the *gcn-1(n4827); bcIs39* animals failed to

yield any suppressors (Figure 5B), RNAi against p53-independent genes on the other hand led to the identification of three suppressor genes (*gla-3*, *cpb-3*, and *pmk-3*) that when knocked down caused increased apoptosis (Figure 5C, Figure 5G, and Figure 5I). RNAi experiments in the non-mutant background (*bcIs39*) denoted that *gla-3(RNAi)* and *cpb-3(RNAi)* suppressing effects were not specific to *gcn-1(n4827); bcIs39* animals, as they also caused an increase in the levels of physiological germline apoptosis in *bcIs39* animals (Figure 5C and Supplemental Figure 4A). By contrast, *pmk-3(RNAi)* caused no significant increase in germline apoptosis compared to the control (Figure 5C and Supplemental Figure 4A).

CPB-3 is an evolutionarily conserved RNA-binding protein with roles in translation (Hasegawa et al., 2006; Singh et al., 2017) and can act in a protein complex with two other RNA-binding proteins in CAR-1 and CGH-1 (Boag et al., 2005; Gartner et al., 2008). Given that GCN-1 is a translational regulator (Castilho et al., 2014), we decided to inhibit both *car-1* and *cgh-1* to determine whether they can also suppress *gcn-1.* We were also keen in testing the rest of the p38 MAP kinase genes in *C. elegans* (*pmk-1* and *pmk-2*) given their roles in stress (Berman et al., 2001).

When *car-1* and *cgh-1* were individually inhibited by RNAi in the *gcn-1(n4827); bcIs39* genetic background, both displayed significant suppression (i.e., increased apoptosis) (Figure 5D, Figure 5E, and Figure 5F). Similarly, inhibition of *pmk-2* expression by RNAi also suppressed the *gcn-1(n4827); bcIs39* mutant effects (Figure 5D and Figure 5H). *cgh-1(RNAi)* and *pmk-2(RNAi)* effects appear to be specific to *gcn-1(n4827); bcIs39* animals, as there was no significant increase in apoptosis in *cgh-1(RNAi)* and *pmk-2(RNAi)*-treated *bcIs39* animals when compared to vector control (Supplemental Figure 4B). Intriguingly, we found that RNAi-mediated inhibition of *car-1* in the non-mutant background resulted in diminishment of apoptosis

(Supplemental Figure 4B, Supplemental Figure 4C, and Supplemental Figure 4D), suggesting that *car-1* is important for normal apoptotic levels when in the presence of wild-type *gcn-1*. This is in contrast to *car-1's* anti-apoptotic role in the presence of mutant/null *gcn-1* (Figure 5D and Figure 5E). These results suggest that CAR-1 potentially may engage in a special functional relationship with GCN-1 in relation to its impact on apoptosis levels.

For *cpb-3*, *car-1*, and *cgh-1*, we confirmed that increased CED-1::GFP positive cells were indeed apoptotic by repeating the respective RNAi treatments in the *ced-3(n717); bcIs39* genetic background (Supplemental Figures 5A-D).

Given that GCN-1 is a translational regulator, we also inhibited *ife-1*, the major germline isoform of the cap-binding translation initiation factor eIF4E (Eberhard et al., 2013), and *ife-4*, the worm homolog of translation inhibitor 4EHP (Cho et al., 2005; Mayya et al., 2021). Inhibition of these translational regulators by RNAi did not produce any significant suppressing or enhancing effects. *drg-2(RNAi)* was also tested in light of a recent work that suggested that it works in concert with *gcn-1* to modulate the expression of cell cycle genes (Yamazaki et al., 2020). No significant effects were seen (Figure 5D).

When inhibiting *mpk-1* by RNAi in the *gcn-1* mutant background, a possible enhancing effect was observed (Figure 5D). Given the established pro-apoptotic functions of *mpk-1* (Eberhard et al., 2013; Gumienny et al., 1999), together with the observation that *gcn-1* mutants have lower levels of "apoptotic clusters" to begin with, we were able to confirm the enhancing effect of *mpk-1* by utilizing the more sensitive metric of counting the individual apoptotic cells (Figure 6A and Figure 6B). In light of the observations that phosphatase LIP-1 functions as a negative regulator of the MAP kinase MPK-1 (Berset et al., 2001; Eberhard et al., 2013; Hajnal

and Berset, 2002), we also subjected *lip-1(zh15)* null mutants to our apoptosis assay. As expected, *lip-1(zh15)* mutants dramatically increased apoptosis, thereby confirming LIP-1's anti-apoptotic function (Figure 6C and Figure 6E). Since *gcn-1(n4827); lip-1(zh15)* double mutant effects were of similar magnitude as *lip-1(zh15)*, it is likely that *lip-1(loss-of-function)* is epistatic to *gcn-1(loss-of-function)* (Figure 6D and Figure 6E).

Many of the genes that we inhibited via RNAi are essential genes with no workable mutants, such as in the case of *mpk-1*, *car-1*, *cgh-1* and *pmk-2,* but we did test the mutants available for *pmk-3* as well as *pmk-1*. We were able to validate the *pmk-3(RNAi)* experiments by replicating the results in *pmk-3(ok269)* null mutants and the *gcn-1(n4827); pmk-3(ok269)* double mutants (Figure 6E). As for *pmk-1*, the last remaining p38 MAP kinase gene in *C. elegans* to be tested in our assay, we found that the gene may have a pro-apoptotic function, in contrast to *pmk-2* and *pmk-3*. In fact, a diminished stress-induced apoptosis phenotype was seen in the *pmk-1(km25)* single mutants (Figure 6F and Figure 6H). Furthermore, *pmk-1* appears to function as a *gcn-1* enhancer when analyzing the *gcn-1(n4827); pmk-1(km25)* double mutants for the stress-induced apoptosis phenotype (Figure 6G and Figure 6H).

Identification of potential translational targets of GCN-1

GCN-1 has been described as a translational regulator that physically interacts with 80S ribosome (Castilho et al., 2014; Pochopien et al., 2021). Three of the genes associated with suppression of the *gcn-1* phenotype following RNAi encode RNA-binding proteins, CAR-1, CGH-1, and CPB-3*,* that are thought to physically and functionally interact with each other to modulate the translation of target mRNAs (Audhya et al., 2005; Boag et al., 2005; Decker and

Parker, 2006; Gartner et al., 2008; Rajyaguru and Parker, 2009; Squirrell et al., 2006; Weston and Sommerville, 2006; Wilczynska et al., 2005; Wilhelm et al., 2005). We hypothesized that GCN-1 may potentially promote programmed cell death through translational control. To this end, we sought to identify the translational targets of GCN-1 by data-mining available Ribo-seq data (Hirose and Horvitz, 2014) in order to look for actively translated transcripts that were abrogated in *gcn-1(n4827)* mutants compared to the wild-type. Criteria used to identify potential candidates were for those with a fold change greater than 1.5 when comparing wild-type to the *gcn-1* mutant in addition to having a statistically significant p value of less than 0.05. From the list of candidates tested, we identified two genes for which loss-of-function mutants displayed diminished apoptosis in both physiological and stress-induced cell death, *inx-8* and *cpr-5* (Figure 7A-E).

These results suggest that *inx-8/connexins-pannexins* and *cpr-5/cathepsin-b* may be pro-apoptotic target genes that undergo GCN-1 dependent translational control to promote apoptosis, but this needs further genetic and molecular validations.

DISCUSSION

Pending further mechanistic molecular studies, our work presented herein suggest a potential novel control of programmed cell death in the context of both "physiological" germline cell death and stress-induced cell death by GCN-1.

Unexpectedly, this control of programmed cell death by GCN-1 is independent of GCN-2 and the Integrated Stress Response pathway. This goes against the conventional wisdom of the biological function of GCN-1. In fact, ever since their initial isolation from a common genetic screen in yeast (Penn et al., 1983; Skvirsky et al., 1986), the conventional wisdom is that GCN1 and GCN2 interacts together to activate the Integrated Stress Response pathway (Marton et al., 1993). Little is known about a possible GCN-2-independent role for GCN-1. Intriguingly, there are some indications of a possible independent role of GCN1 from GCN2 and the Integrated Stress Response pathway in *Arabidopsis thaliana* and in mice (Faus et al., 2018; Yamazaki et al., 2020), raising the possibility that the findings described here might have general applicability to other biological contexts and organisms. In fact, GCN1 appears to have a cell proliferation function that is independent to GCN2 in mice (Yamazaki et al., 2020). Furthermore, observations in *Arabidopsis* suggest GCN1 having biological roles in root development and chloroplast biogenesis that are independent to GCN2 (Faus et al., 2018). Taking into account the structure of the GCN1 protein with its greater than 20 HEAT repeat motifs distributed throughout its length (Andrade and Bork, 1995; Castilho et al., 2014; Pochopien et al., 2021), it seems very plausible that GCN1 has a GCN2 and Integrated Stress Response-independent biological function, given that GCN1 interacts with GCN2 only in the C terminus portion of its protein (Sattlegger and Hinnebusch, 2000). HEAT repeat motifs are known protein-protein interaction sites (Andrade et

al., 2001) and thus the structure of GCN1 leaves many other HEAT repeats to potentially facilitate non-GCN2 protein-protein interactions.

The mechanism of control of programmed cell death by GCN-1 appears to be at least in part independent of the contributions of CEP-1/p53. Specifically, the defect in cell death in the *gcn-1* mutant was stronger than that caused by a *cep-1* null allele and the phenotype of the double mutant was stronger than that of the single mutants. The fact that GCN-1 promotes both physiological germline cell death (Figure 1G, Figure 1H, and Supplemental Figure 1F) and stress-induced cell death support this independent role, as CEP-1/p53 has not been implicated in physiological germline cell death and instead is involved only in stress-induced/DNA damage-induced cell death (Derry et al., 2001; Gartner et al., 2008; Schumacher et al., 2001). Previous work has also hinted on the existence of stress-induced germline cell death that does not involve CEP-1 in which the increased apoptosis in response to variety of stress conditions (oxidative, heat shock, starvation, and osmotic) may involve molecular players acting concurrently in the physiological germline cell death (Salinas et al., 2006). Furthermore, we found that the control of programmed cell death by GCN-1 may possibly be independent to caspases and the canonical cell-death pathway, but this needs to be further validated with molecular studies. Caspase-independent cell death/apoptosis pathways have been previously described (Denning et al., 2012, 2013; Hirose and Horvitz, 2013) and are of particular interest given their potential repercussions in development and diseases. As such, it will be interesting to further test whether GCN-1 promotes apoptosis in a caspase-independent context.

As denoted previously, GCN1 is a known translational regulator that interacts physically with the 80S ribosome (Castilho et al., 2014; Pochopien et al., 2021). The identification of multiple RNA-binding proteins, CAR-1, CGH-1, and CPB-3, as suppressors of GCN-1 in

programmed cell death, raises the possibility that the control of programmed cell death by GCN-1 may be mediated through translational control. However, it is unknown at present whether GCN-1 and these RNA-binding proteins regulate the same or different molecular processes. This will be an important target for future work. Across multiple species, these three evolutionary conserved RNA-binding proteins are thought to physically and functionally interact with one another in a ribonucleoprotein (RNP) complex with implications in translational regulation of target mRNAs (Audhya et al., 2005; Boag et al., 2005; Decker and Parker, 2006; Gartner et al., 2008; Rajyaguru and Parker, 2009; Squirrell et al., 2006; Weston and Sommerville, 2006; Wilczynska et al., 2005; Wilhelm et al., 2005). Additionally, a conserved feature of oogenesis involves regulation of gene expression predominantly at the post-transcriptional/translational level, given the significant reduction in transcriptional activities in developing oocytes and their reliance on maternally-contributed mRNA (Kisielnicka et al., 2018; Richter and Lasko, 2011; Von Stetina and Orr-Weaver, 2011). These aspects of oogenesis further raise the possibility that the cell death promoted by GCN-1 in the germline may indeed involve a translational control mechanism. In contrast to transcriptional control of programmed cell death as illustrated by the critical step of transcriptional regulation of *egl-1* as well as the role played by transcriptional factor such as CEP-1/p53, translational control of programmed cell death remains poorly understood. Intriguingly, we found that CAR-1 had a pro-apoptotic or an anti-apoptotic functions depending on the wild-type or mutant status of GCN-1. In addition to its function as a RNA-binding protein, CAR-1/LSM14 has been shown to be an mRNA decay factor through its association with DCAP-1, a protein that removes the 5’cap of mRNAs (Squirrell et al., 2006; Tang et al., 2020). On the other hand, CAR-1 and its homologs have also been suggested to have a role in the secretory/protein trafficking pathway through its association with the endoplasmic

reticulum (Decker and Parker, 2006; Wilhelm et al., 2005). These seemingly opposing functions of CAR-1/LSM14 have raised the possibility that it could act as both translational activator and repressor (Decker and Parker, 2006). Therefore, one wonders whether this potential dual role of CAR-1 may be in fact reflected on the dual pro-apoptotic or anti-apoptotic functions observed in the present study.

We also identified *mpk-1/erk* and *lip-1/mkp* as *gcn-1* enhancer and *gcn-1* suppressor respectively. MPK-1, acting through a Ras/MAPK signaling pathway, has a variety of biological functions in *C. elegans* (Arur et al., 2009). Amongst its pleiotropic functions, it is thought to be important for physiologic germline cell death, oocyte maturation, and meiosis progression (Arur et al., 2009; Eberhard et al., 2013; Gumienny et al., 1999). Interestingly, MPK-1 has also been implicated in promoting stress-induced cell death through its phosphorylation and subsequent activation upon ionizing radiation (Eberhard et al., 2013; Rutkowski et al., 2011), thereby acting as a stress signal transducer. LIP-1, on the other hand, has been characterized as a MAPK phosphatase with opposing biological function to MPK-1 (Berset et al., 2001; Hajnal and Berset, 2002; Lee et al., 2006). This includes apoptosis where LIP-1 acts to protect germ cells from programmed cell death (Lee et al., 2007). *gla-3* was identified in this work as another *gcn-1* suppressor. GLA-3 was previously shown to physically interact with MPK-1 to downregulate its signaling pathway (Kritikou et al., 2006). Given its putative RNA-binding domains, GLA-3 was also proposed to stabilize *lip-1* mRNA upon binding to MPK-1 and thus facilitating a negative feedback loop (Kritikou et al., 2006). In cancer cells, activated ERK can promote apoptosis (Frese et al., 2003). On the other hand, MKPs are frequently upregulated in cancer to protect cancerous cells from stress-induced apoptosis and thus critical for neoplastic progression (Lee et

al., 2007; Small et al., 2007; Vogt et al., 2005). These highlight the application/relevance of the biology presented in this study to more broadly into the field of cancer biology.

MPK-1/ERK has also been shown to phosphorylate the RNA-binding protein CBP-3/CPEB1 to target it for degradation through the ubiquitin-proteasomal system in order to promote meiotic progression (Kisielnicka et al., 2018). This mechanism appears to be evolutionarily conserved in that MAPK cascade also triggers degradation of CPEB1 to promote meiotic progression in mice (Sha et al., 2017). Based on work done in *Xenopus* and mice, CPEB1 is a known key oocyte factor that regulate maternal mRNA translation during oogenesis (Hake and Richter, 1994; Kalous et al., 2018).

p38 MAP kinases are other members of the MAPK superfamily that have been implicated in variety of stress responses (Berman et al., 2001; Kim et al., 2002; Pagano et al., 2015). In *C. elegans*, three p38 genes (*pmk-1*, *pmk-2*, and *pmk-3*) have been identified and all three reside in a single operon (Berman et al., 2001). Interestingly, we identified *pmk-1* as a *gcn-1* enhancer and thus a pro-apoptotic gene. This is in contrast to both *pmk-2* and *pmk-3* that were *gcn-1* suppressors and anti-apoptotic genes. The identification of PMK-1 as a pro-apoptotic factor is consistent with prior findings demonstrating that *Salmonella enterica* infection-induced germline apoptosis in *C. elegans* required the *pmk-1* gene (Aballay et al., 2003). Interestingly, it has been shown that ultraviolet radiation can phosphorylate and thus activate PMK-1 in *C. elegans* (Ma et al., 2020). In light of this evidence, one wonders whether PMK-1 functions as yet another radiation stress transducer in addition to MPK-1. Contextually, the mammalian p38 MAPK signaling pathway has been demonstrated to also be activated by ultraviolet radiation (Aballay et al., 2003). Human p38α was shown to inhibit tumor initiation by inducing apoptosis (Lee et al., 2007).

The discovery of *inx-8/connexins-pannexins* and *cpr-5/cathepsin B* as possible translation targets of GCN-1 to promote programmed cell death is interesting in that their homologs in other species have been associated with apoptosis. Innexins, which are gap junction proteins in invertebrates and their vertebrate counterparts, connexins and pannexins, have been implicated in facilitating the intercellular communication of apoptotic signals (Chekeni et al., 2010; Chen et al., 2016; Gilleron et al., 2018). *cpr-5* encodes for a lysosomal cysteine protease with its human homolog known as cathepsin B (Larminie and Johnstone, 1996). Cathepsin B is thought to play a role in apoptosis through its release to the cytosol from the lysosome (Stoka et al., 2007), although it could also be secreted from the cell to act as an extracellular protease (Buck et al., 1992; Poole et al., 1978). It has also been suggested that cathepsin B could be involved in caspase-independent cell death (Bröker et al., 2004; Turk and Stoka, 2007). In fact, these proteases have been linked to other forms of cell death, such as necrosis (Turk and Stoka, 2007) and pyroptosis, the latter through activation of the NLRP3 inflammasome (Orlowski et al., 2015; Xu et al., 2021). It is thus not surprising that they have been associated with numerous human diseases, ranging from cancer, epilepsy, ischemic diseases, traumatic brain injury, and neurodegenerative diseases (Downs et al., 2005; Hook et al., 2014, 2008; Houseweart et al., 2003; Yin et al., 2012; Yoshida et al., 2002). Cathepsin B has been associated intriguingly with radiation in that another worm homolog of cathepsin B, *cpr-4*, was shown to mediate radiation-induced bystander effect (RIBE) (Peng et al., 2017). Subsequent experiments demonstrated that localized UV radiation at the head of the worm triggered *cpr-4*-dependent DNA damage in distant unexposed germ cells of the same animal (Zheng et al., 2019). Furthermore, a previous study that irradiated the mouse testes at 600 rads resulted in increased cathepsin B activity through potentially a radiation-induced enhancement of protein synthesis (Pogany and Lewis,

1985). In addition to the male gonad, cathepsin B is also expressed in the female gonad/ovaries in various non-mammalian and mammalian species (Eykelbosh and Van Der Kraak, 2010; Hu et al., 2021; Oksjoki et al., 2001). In fact and of particular interest, cathepsin B has been found to be important in follicular apoptosis in mammalian ovaries (Carnevali et al., 2006; Eykelbosh and Van Der Kraak, 2010). It has also been shown that tumor necrosis factor-induced apoptosis of ovarian cancer cells were dependent on cathepsin B (Liu et al., 2006). Thus targeting cathepsin B may potentially be of therapeutic interest.

In addition to the possible conserved role of cathepsin B in promoting ovarian/germline apoptosis, there are other elements of the biology studied herein in *C. elegans* that may attest to the broader conserved biology/physiology of germline apoptosis. In fact, during early meiotic prophase, mammalian oocytes are connected to each other within cysts and are thought to transfer cytoplasmic contents amongst themselves (Gartner et al., 2008; Pepling, 2006; Pepling and Spradling, 2001; Tilly, 2001). In *C. elegans*, germ cells are organized in syncytium, partially enclosed by a plasma membrane and sharing a common cytoplasmic core (Gartner et al., 2008). Similar to *C. elegans*, mammalian germ cell apoptosis also occurs around or just after the pachytene stage when the cytoplasmic connections are lost and individual germ cells/oocytes cellularize (Gartner et al., 2008; Pepling and Spradling, 2001; Rutkowski et al., 2011). As denoted previously, more than half of all female germ cells in *C. elegans* die through physiological apoptosis (Gumienny et al., 1999). In humans, the majority of the $7x10^6$ oocytes formed in early fetal life are culled through apoptosis to ultimately result in around 400 follicles being ovulated in a span of a woman's reproductive life before menopause sets in (Baker, 1963; Vaskivuo and Tapanainen, 2003). Apoptosis is thus suggested to be the mechanism that determines the female reproductive lifespan and thus making the female biological clock tick

(Vaskivuo and Tapanainen, 2003). In this context, it will be interesting to see whether the control of programmed cell death by GCN-1/GCN1 suggested herein can be extended to help shed further light into our understanding of human ovarian physiology and to mitigate oocyte loss and sterility caused by environmental insults, such as radiation or chemotherapy (Matikainen et al., 2001; Perez et al., 1997; Vaskivuo and Tapanainen, 2003).

In conclusion, we have discovered potentially a novel translational control mechanism of programmed cell death by GCN-1, pending further molecular studies. Contrary to conventional wisdom, this control occurs in a GCN-2 and Integrated Stress Response pathway-independent manner, providing a non-canonical function to GCN-1. The present study also potentially provides further mechanistic understanding of the poorly understood biological phenomenon of germline programmed cell death; a biological process that initiates death to nurture life and is found ubiquitously across the animal kingdom, from worms to humans. The knowledge presented herein could help further understand human female reproductive physiology in order to potentially extend reproductive lifespan and mitigate oocyte loss and sterility caused by environmental stressors.

MATERIALS AND METHODS

***C. elegans* strains**

C. elegans were grown at 20 °C as previously described (Brenner, 1974). The Bristol strain N2 is the wild-type strain. The following mutations, integrations, and arrays have been used in this study:

LGI: *cep-1(gk138)*

LGII: *gcn-2(ok871)*, *eif-2α(qd338)*, *csp-1(n4967)*

LGIII: *gcn-1(n4827, nc40)*, *ced-9(n2812)*

LGIV: *ced-3(n717, n2427)*, *pmk-3(ok169)*, *lip-1(zh15)*, *pmk-1(km25)*, *inx-8(gk42), ItIs37 [pAA64 (Ppie-1::mCherry::his-58), unc-119(+)]*

LGV: *bcIs39 [Plim-7::ced-1::gfp, lin-15(+)]*, *cpr-5(ok2344)*

Linkage unknown: *nEx1818 [Pgcn-1::gcn-1 cDNA::gcn-1 3'UTR, Plin-44::gfp]*,

nIs882 [*Patf-4(uORF)::GFP::unc-54(3'UTR)*]

Molecular biology

gcn-1(+) rescue construct (*Pgcn-1::gcn-1 cDNA::gcn-1 3'UTR*) was made using 4.2kbp of the 5' promoter region of *gcn-1,* the entire *gcn-1* cDNA sequence, and 1.0kbp 3' from the stop codon of *gcn-1.* These were constructed using PCR and cloned into a pBlueScript II plasmid using the

In-Fusion cloning system (Takara). The construct was verified by Sanger sequencing (Hirose and Horvitz, 2014).

Germline transformation

Germline transformation is undertaken through standard procedures described previously (Mello et al., 1991). The *gcn-1(+)* rescue construct was injected at 20 µg/ml concentration in addition to 50 µg/ml of $P_{lin\text{-}44}$*::gfp*, latter used as a coinjection marker.

Gamma radiation assay

L4 animals of a genotype of interest are picked and transferred to a fresh NGM plate. The animals are then subjected to a total cumulative ionizing radiation absorbed dose of 60 Gray using a Cobalt-60 irradiator source (Gamma Cell 220; Atomic Energy of Canada Limited). As control, a separate L4 batch of the same genotype animals are picked and transferred to a plate that is not irradiated at every experiment. Following irradiation, the irradiated and non-irradiated plates are then cultured in 20 °C for 24 hours before they are assayed under the compound epifluorescence microscope. Apoptotic cluster is defined as a cluster of CED-1::GFP positive cells spanning the entire width of the gonad. Number of CED-1::GFP positive cells per animal were calculated by counting the individual CED-1::GFP positive cells throughout the Z plane of a gonadal arm.

Microscopy

All genetic crosses containing fluorescent transgenes were performed using a SMZ18 (Nikon) fluorescent dissecting microscope. Epifluorescence images related to the *atf-4::gfp* transgene have been obtained using the Axio Zoom.V16 (Zeiss) fluorescent dissecting microscope. For compound microscope experiments, young adult (24 hour post-L4 stage) animals were picked and mounted on a glass slide (Corning) prepared with either a 3% agarose pad or a 5% agarose pad. The 5% agarose pads were used for the time-lapse video experiments to measure the kinetics of apoptotic cell clearance. The 3% agarose pads were used for all other microscopy experiments. The animals were immobilized using 50mM sodium azide. For the time-lapse video experiments, animals were immobilized using 0.1 µm polysterene beads (Polysciences). Epifluorescence and DIC images/videos were taken either using the 40x or the 63x objective lenses (Zeiss) on the Axio Imager.Z2 (Zeiss) compound microscope fitted with a CMOS digital camera (Hamamatsu) and the Zen Blue software (Zeiss). The 40x objective lens was used for the gamma radiation assay and the 63x objective lens was used for the germ cell nuclei assay as well as the time-lapse video experiments. Images were further processed with Fiji (NIH), Photoshop 7.0 (Adobe), and Illustrator 9.0 (Adobe) softwares.

Germ cell nuclei assay

ItIs37 and *gcn-1(n4827); ItIs37* non-irradiated 24 hour post-L4 animals were visualized under the compound microscope. Epifluorescence images were taken with the visualizing plane providing the best parallel view of the upper or lower horizontal boundary of the gonadal tubular structure. From these images, all germ cell nuclei contained within 150 µm distal (towards the distal tip cell) to the gonadal loop region were counted.

Time-lapse video for kinetics of apoptotic cell clearance

bcIs39 and *gcn-1(n4827); bcIs39* non-irradiated 24 hour post-L4 animals were visualized under the compound microscope using DIC optics. Following the appearance of a germline apoptotic cell as described previously (Gumienny et al., 1999), it is tracked until its disappearance. Time of apoptotic cell clearance is determined by analyzing the time-lapse video.

Western blot

Protein extracts were prepared from N2 and *eif-2α(qd338)* animals. 50µg of protein was loaded onto a 10% SDS PAGE gel and subsequently transferred to nitrocellulose membranes. The membranes were incubated with anti-eIF2α (Hirose and Horvitz, 2014; Nukazuka et al., 2008) and anti-phospho-eIF2α antibodies (Cell Signaling Technology). HRP-conjugated Goat anti-rabbit IgG secondary antibodies (Bio-Rad Laboratories) were then used followed by chemiluminescence (Western Lightning ECL, PerkinElmer).

***atf-4::gfp* analysis**

For *atf-4* expression experiments, 30 gravid young adult worms were picked into fresh-seeded plates. Drops of either 5mM DTT (Sigma-Aldrich) or vehicle were applied to the animals and subsequently cultivated in 20°C for 3 hours before imaging (Ferraz et al., 2016).

RNAi

Gene-knockdown experiments were performed with RNAi by feeding as previously described (Kamath and Ahringer, 2003; Rual et al., 2004). Bacteria expressing double-stranded RNA were seeded on NGM agarose plates supplemented with 75 µg/ml ampicillin and 1mM IPTG and then subsequently incubated for 24 hours in 22°C. Plasmids were always Sanger sequenced to confirm correct target genes. Worms were synchronized by bleaching and freshly-hatched L1 larvae were transferred to the RNAi plates. An empty vector (L4440) RNAi clone as well as an *unc-22* RNAi clone served as controls in every RNAi experiment.

EMS mutagenesis and genetic screen

gcn-1(n4827); bcIs39 animals were mutagenized using ethyl methanesulfonate (EMS) as previously described (Brenner, 1974). Four mutagenized gravid adult P0 animals were picked onto a fresh plate for a total of 12 plates. When the F1 animals reached gravid adult stage, these worms were subsequently bleached and freshly-hatched synchronized L1 larvae of the F2 animals were transferred onto fresh NGM plates. When these F2 animals reached L4 stage, they were subjected to the gamma radiation assay as described above, except that these animals were not transferred to fresh new plates and the plates were all irradiated in hope to isolate suppressor mutants in the context of radiation stress. An F2 non-clonal genetic screen was performed isolating mutants with prominent germline apoptotic clusters. These candidate mutants were singled onto individual plates. The progeny of these singled isolate mutants were examined for penetrance of apoptotic clusters in both irradiated and non-irradiated conditions. The selected

mutants listed in Table 1 were further outcrossed 3 times into *gcn-1(n4827); bcIs39* animals and maintained as homozygotes for the carried causal mutation, *gcn-1(n4827)*, and *bcIs39*.

Statistical analyses

All statistical analyses were performed using Prism 7 (GraphPad) software. Statistical test and sample sizes are indicated in the figure legends.

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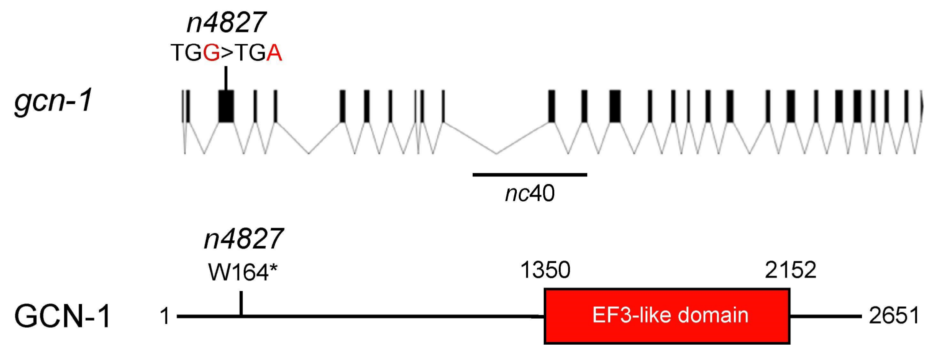
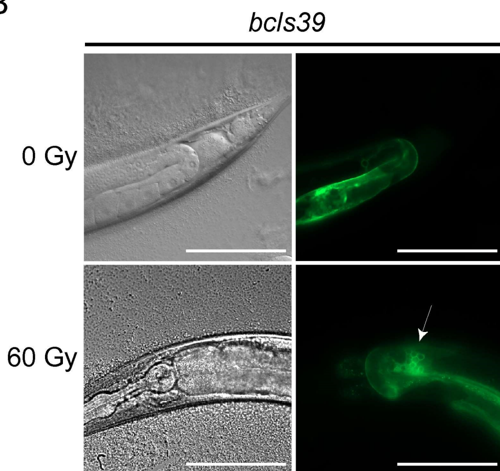
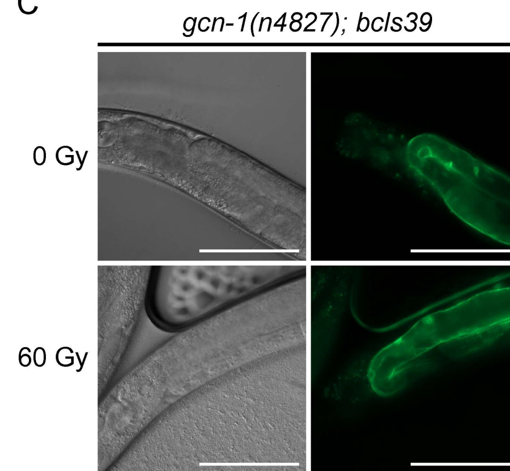
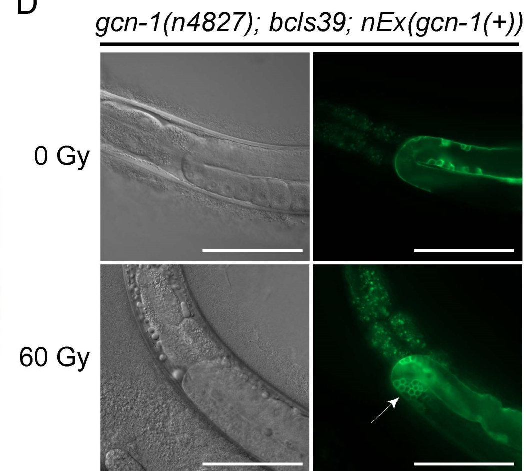
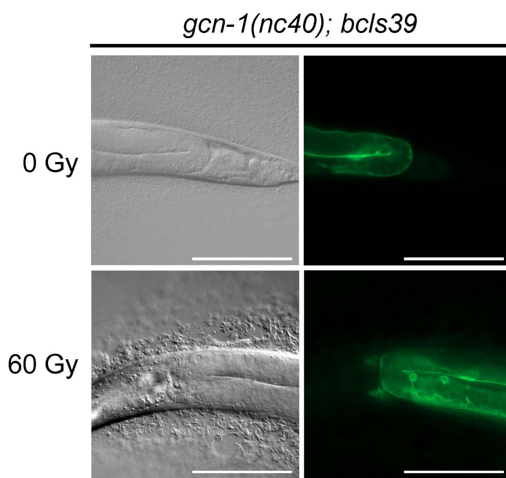
Competing Interests: The authors declare no competing interests.

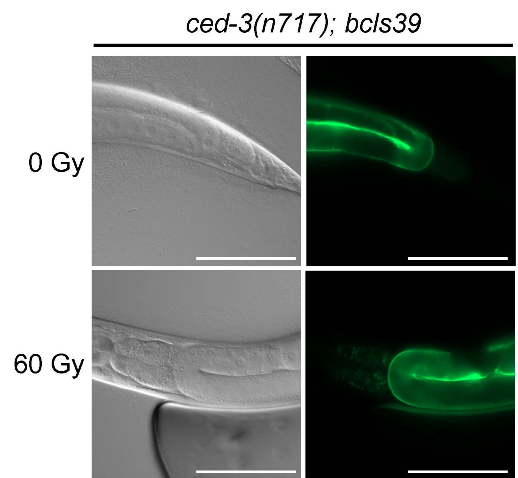


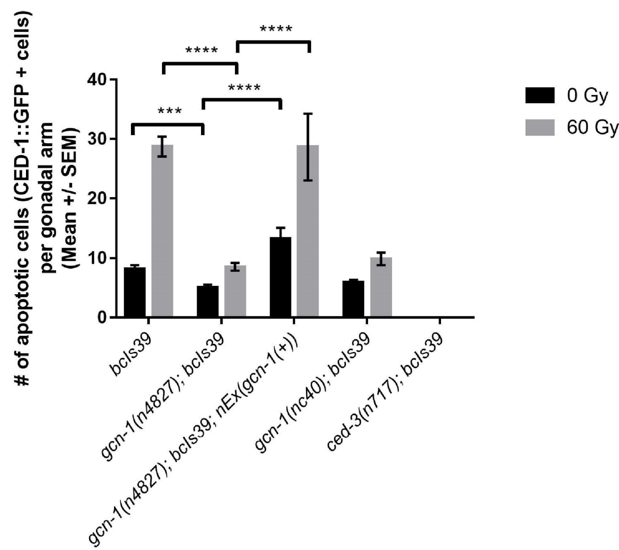












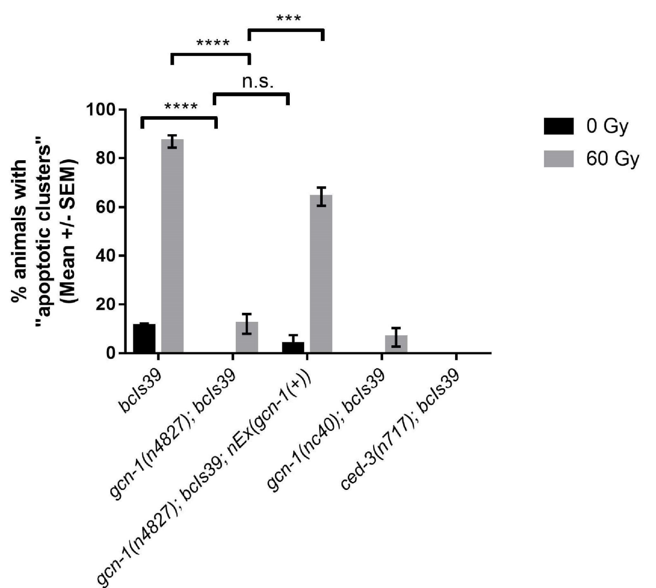


Figure 1

Fig. 1| GCN-1 promotes both physiological and stress-induced germline cell death

a. Gene and protein structures of *gcn-1*/GCN-1 and the molecular alterations in *gcn-1* mutants. **b-f.** DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Arrows represent presence of "apoptotic clusters". Scale bars represent 100 µm. **g.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 8-40 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. ***$p \leq 0.001$ and ****$p \leq 0.0001$. **h.** Quantification of percent animals with presence of "apoptotic clusters" for each of the genotype presented (n= 30-40 animals per genotype surveyed per replicate; 3 replicates). Statistical analyses were performed using an unpaired two-tailed t-test. ***$p \leq 0.001$ and ****$p \leq 0.0001$.

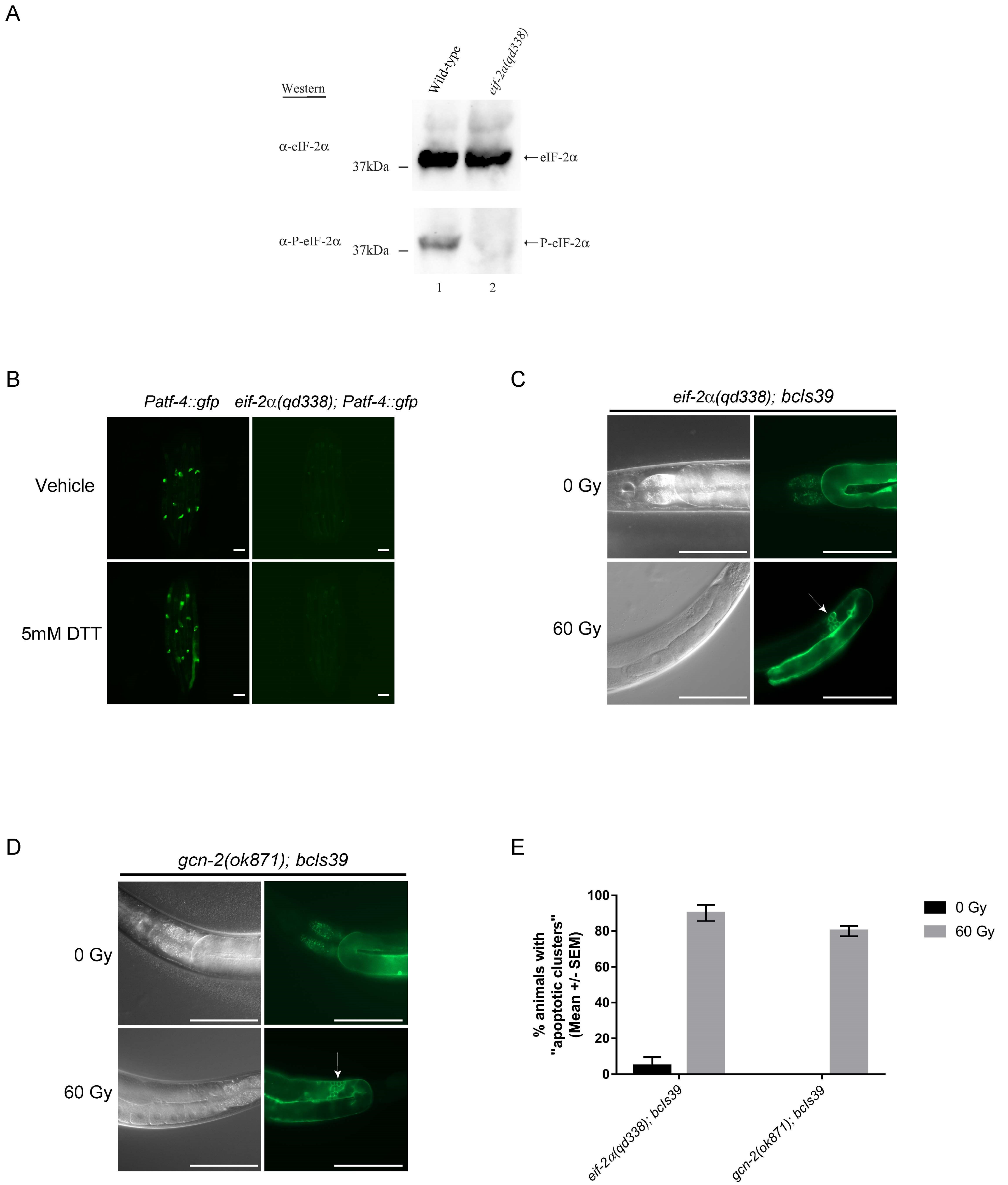
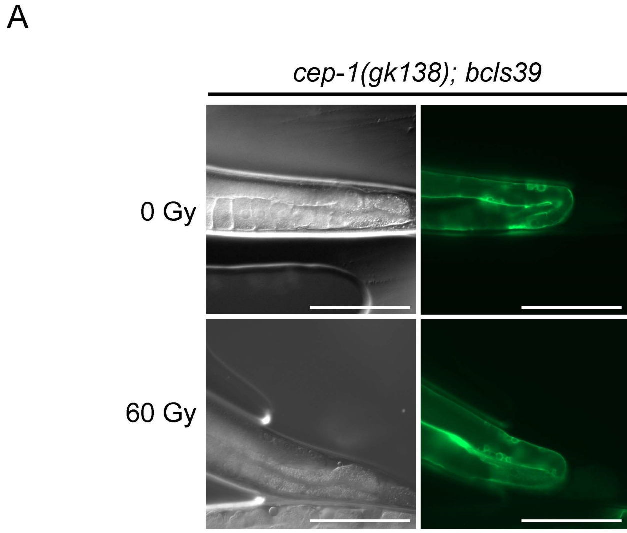
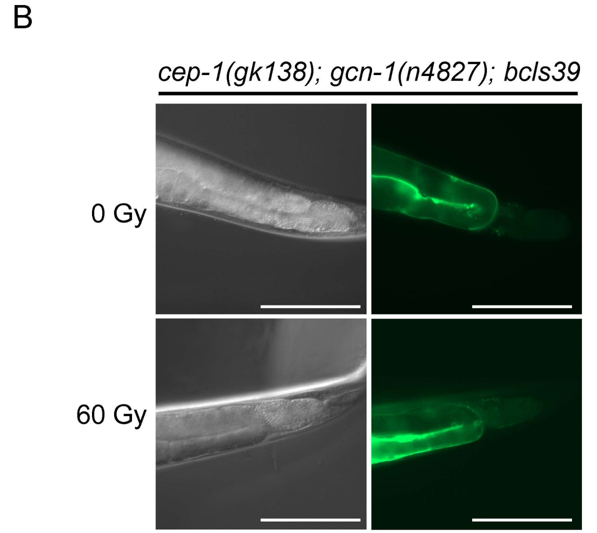


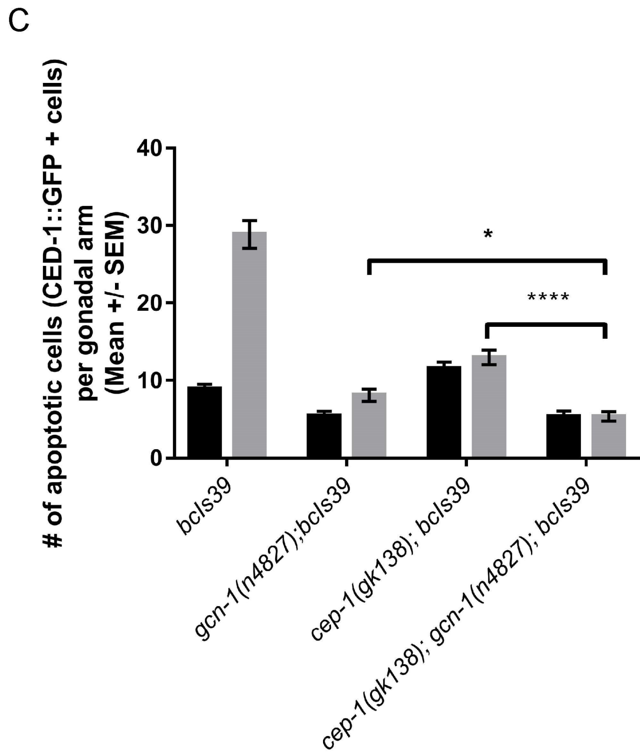
Figure 2

Fig. 2| Stress-induced cell-death phenotype of *gcn-1* is independent of *gcn-2* and the Integrated Stress Response (ISR) activation

a. Protein immunoblot demonstrating that *eif-2α(qd338)* mutants can not phosphorylate eIF-2α to activate ISR. **b.** GFP epifluorescence photomicrographs from a dissecting microscope denoting that *eif-2α(qd338)* mutants can not activate ISR using a downstream transgene readout of the ISR pathway activation ($P_{atf\text{-}4::gfp}$). Representative animals from a total of 3 replicates are presented. Scale bars represent 100 μm. **c-d.** DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Arrows represent presence of "apoptotic clusters". Scale bars represent 100 μm. **e.** Quantification of percent animals with presence of "apoptotic clusters" for each of the genotype presented (n= 16-33 animals per genotype surveyed per replicate; 2-3 replicates).







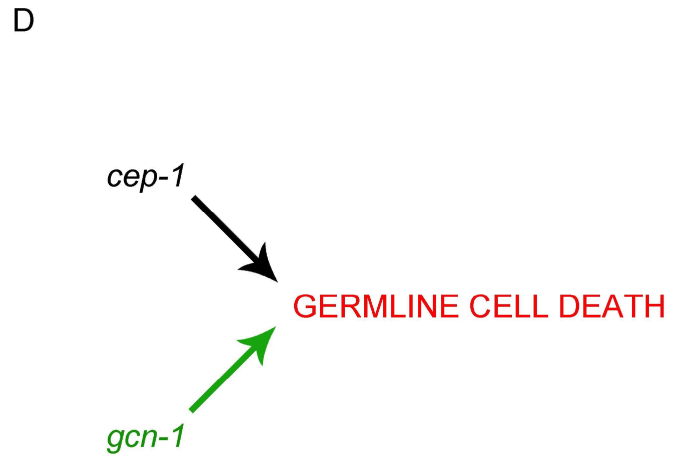


Figure 3

Fig. 3| GCN-1 promotes cell death in the stress condition independent of CEP-1/p53

a-b. DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Scale bars represent 100 µm. **c.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 18-39 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. $^{*}p \leq 0.05$ and $^{****}p \leq 0.0001$. **d.** *gcn-1* enhances *cep-1/p53* and thus acts independently to promote germline cell death.

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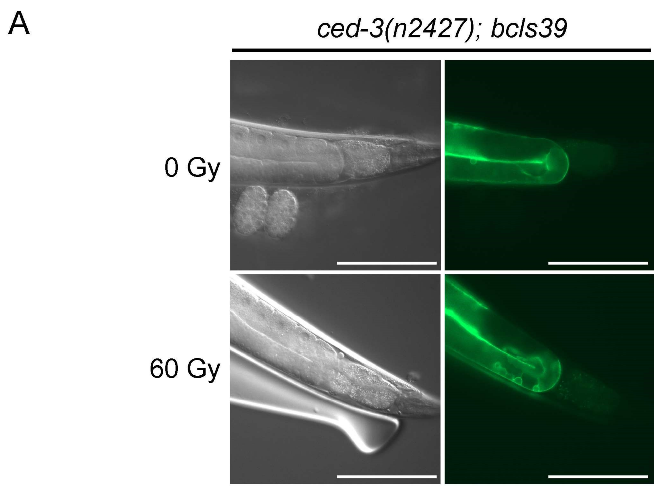

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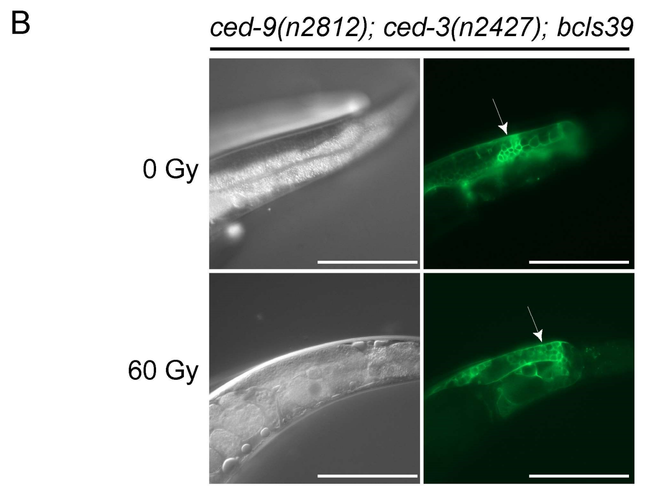

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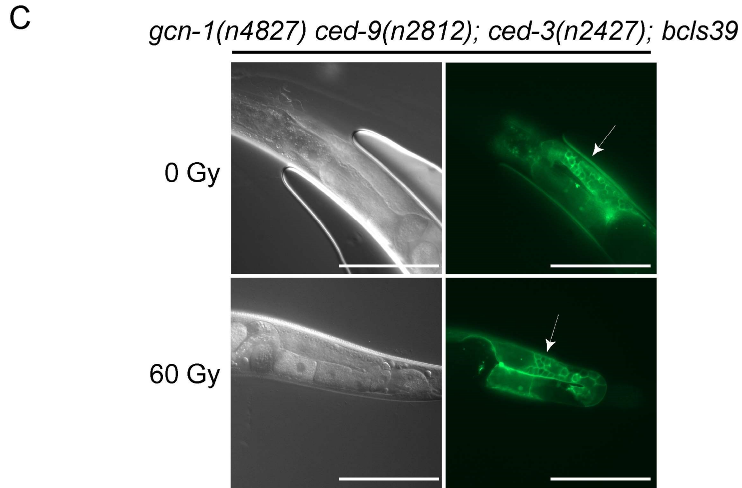
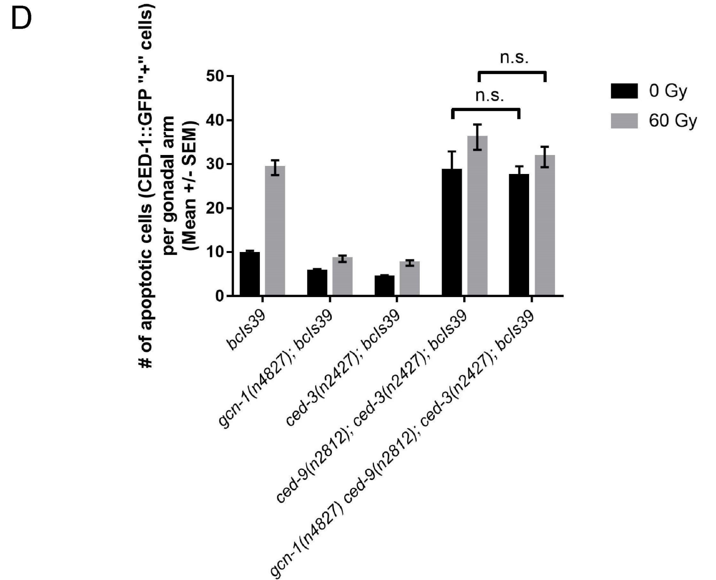


D



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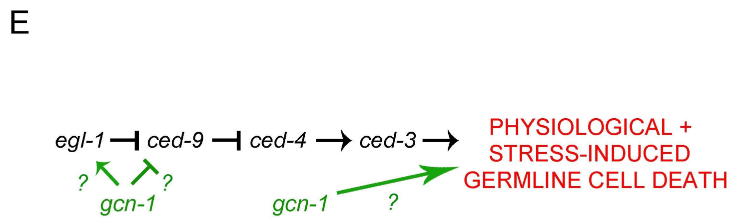

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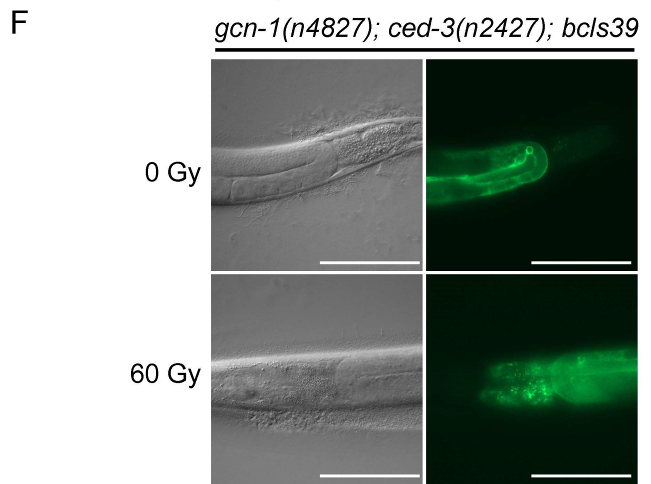

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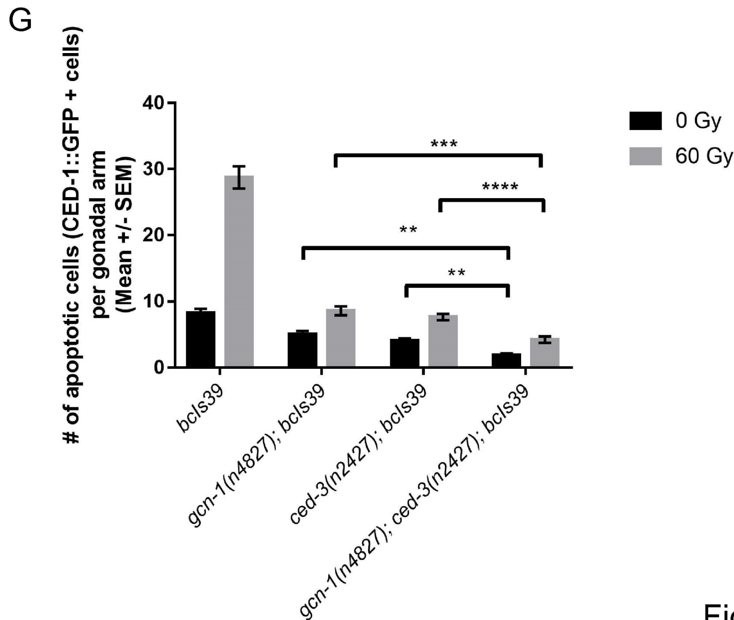

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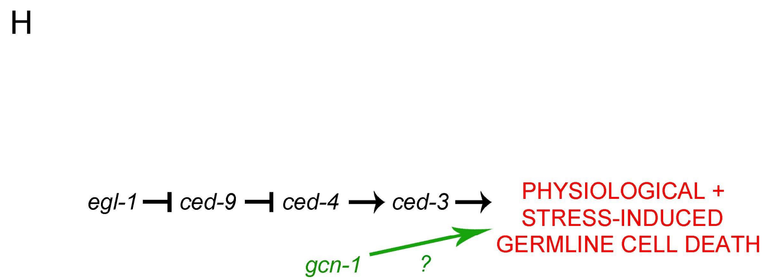


Figure 4

Fig. 4| GCN-1 may promote cell death independent of caspases and the canonical programmed cell-death pathway

a-c. DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Arrows represent presence of "apoptotic clusters". Scale bars represent 100 µm. **d.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 15-49 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. **e.** *ced-9(lf)* is epistatic to *gcn-1(lf)* and thus *gcn-1* could act upstream of *ced-9* or parallel to *ced-9* to promote physiological and stress-induced germline cell death. The possibilities of genetic action of *gcn-1* following epistasis experiments are indicated in green. **f.** DIC and GFP photomicrographs of representative genotype. Images obtained at 40x magnifications. Scale bars represent 100 µm. **g.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 10-55 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. **h.** *gcn-1* may possibly act in parallel to the canonical programmed cell-death pathway to promote physiological and stress-induced germline cell death, pending further molecular studies.

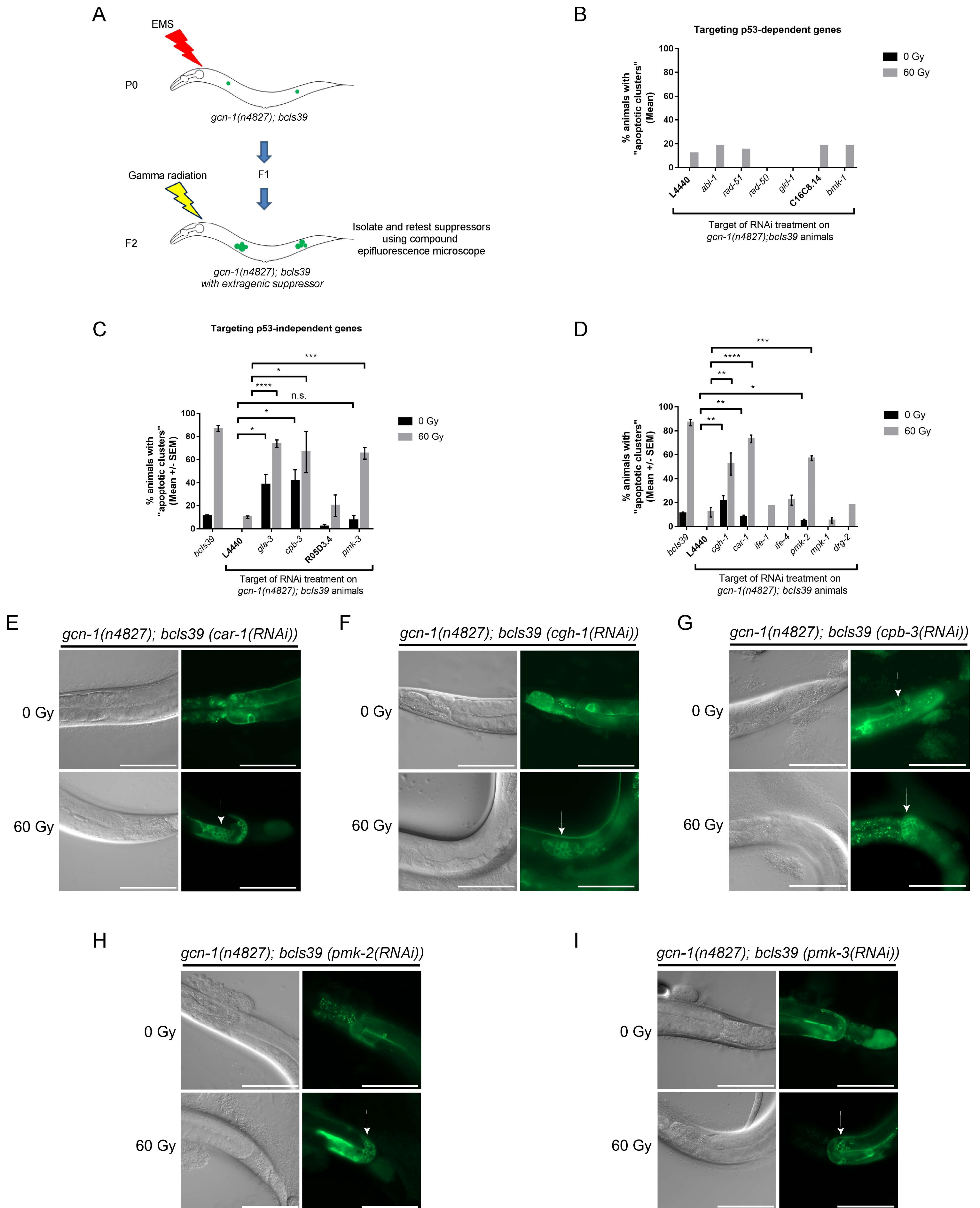


Figure 5

Fig. 5| Identification of extragenic suppressors of the stress-induced cell-death phenotype promoted by *gcn-1* through combination of EMS genetic screens and candidate RNAi screens

a. Schematic diagram of the EMS genetic screen undertaken to isolate genetic suppressors. **b.** Quantification of percent animals with presence of “apoptotic clusters” for each of the represented RNAi-treated *gcn-1(n4827); bcIs39* animals. (n= 20 animals per specific RNAi-treated animals). **c.** Quantification of percent animals with presence of “apoptotic clusters” for each of the represented RNAi-treated *gcn-1(n4827); bcIs39* animals (n= 40-50 animals per specific RNAi-treated animals per replicate; 3 replicates). Statistical analyses were performed using an unpaired two-tailed t-test. *$p \leq 0.05$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. **d.** Quantification of percent animals with presence of “apoptotic clusters” for each of the represented RNAi-treated *gcn-1(n4827); bcIs39* animals (n= 11-56 animals per specific RNAi-treated animals per replicate; 3 replicates, except for *ife-1(RNAi)* and *drg-2(RNAi)* experiments where 1 replicate was done given negative data). Statistical analyses were performed using an unpaired two-tailed t-test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. **e-i.** DIC and GFP photomicrographs of *gcn-1(n4827); bcIs39* animals treated with representative RNAi. Images obtained at 40x magnifications. Arrows represent presence of “apoptotic clusters”. Scale bars represent 100 µm.

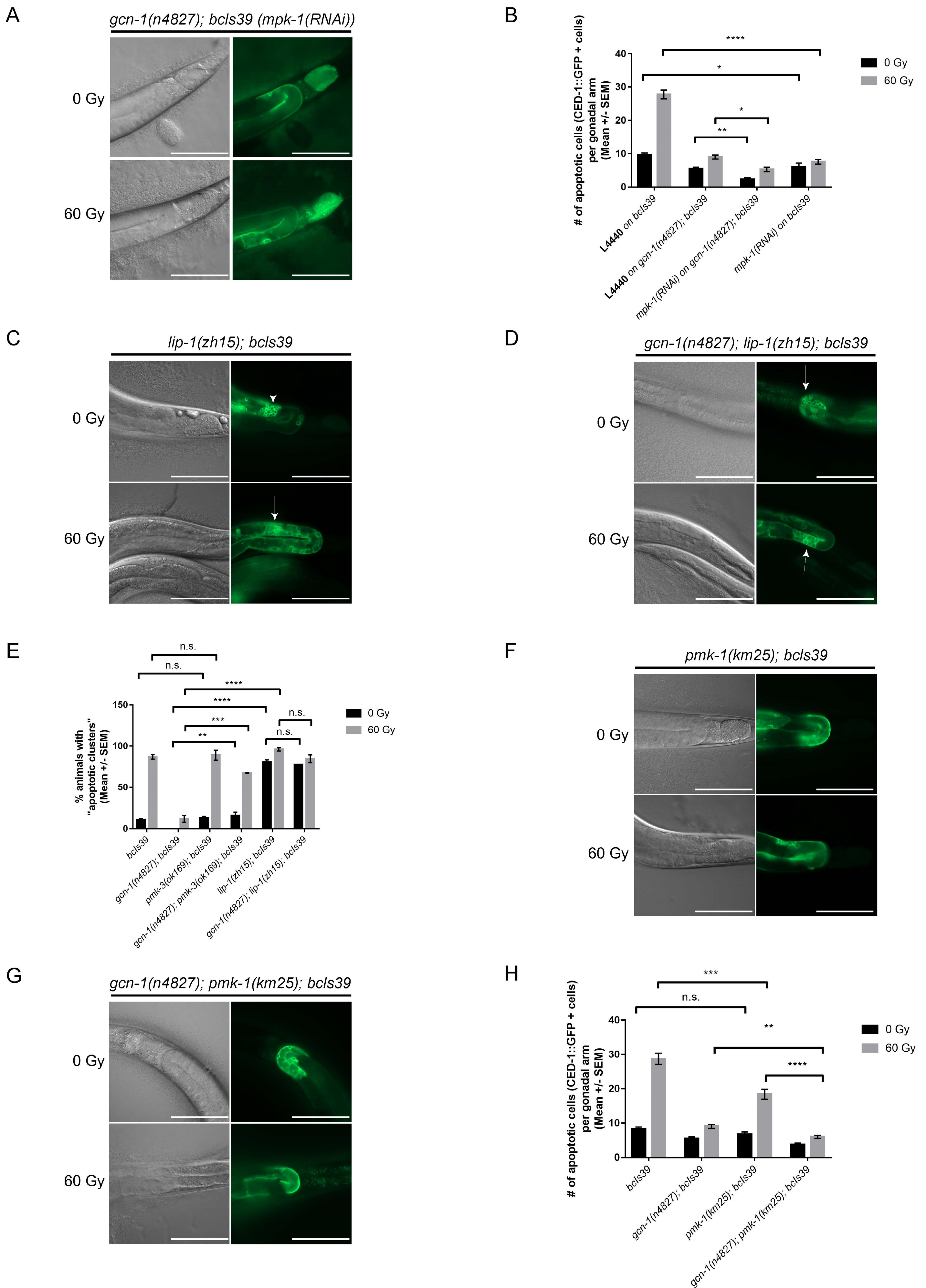
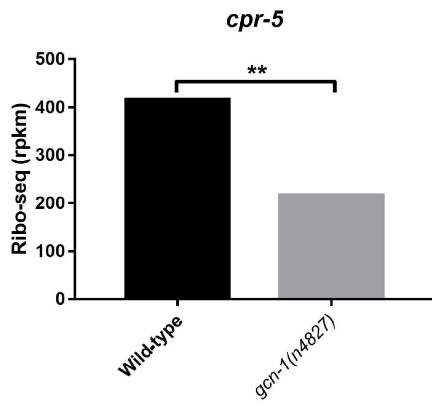


Figure 6

Fig. 6| MAPK/ERK and MAPK/p38 pathways modulate the cell-death phenotype promoted by *gcn-1*

a. DIC and GFP photomicrographs of *gcn-1(n4827); bcIs39* animals treated with *mpk-1(RNAi)*. Images obtained at 40x magnifications. Scale bars represent 100 µm. **b.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 7-14 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. *$p \leq 0.05$, **$p \leq 0.01$, and ****$p \leq 0.0001$. *mpk-1* appears to enhance *gcn-1* to promote the cell-death phenotype. **c-d.** DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Arrows represent presence of "apoptotic clusters". Scale bars represent 100 µm. **e.** Quantification of percent animals with presence of "apoptotic clusters" for each of the genotype presented (n= 10-23 animals per specific RNAi-treated animals per replicate; 2-3 replicates). Statistical analyses were performed using an unpaired two-tailed t-test. **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. *pmk-3(lf)* suppresses *gcn-1(lf) and lip-1(lf)* is epistastic to *gcn-1(lf).* **f-g.** DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Scale bars represent 100 µm. **h.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 18-30 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$. *pmk-1* promotes stress-induced cell death. *pmk-1(lf)* appears to enhance *gcn-1(lf)* to promote the stress-induced cell-death phenotype.

A



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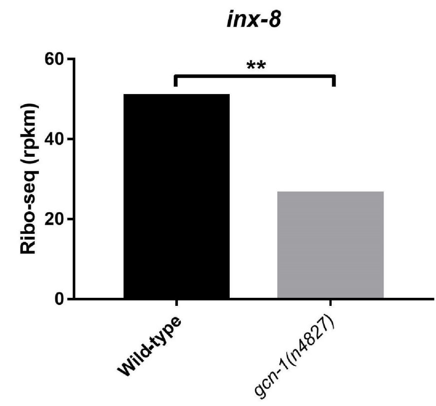

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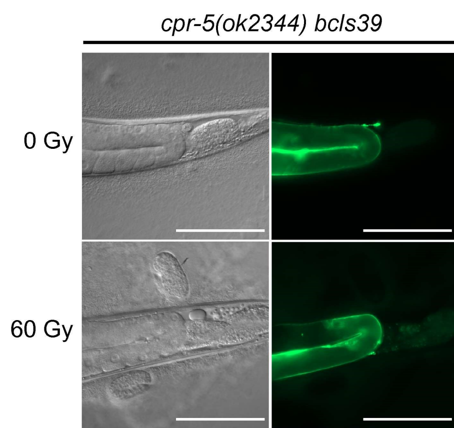

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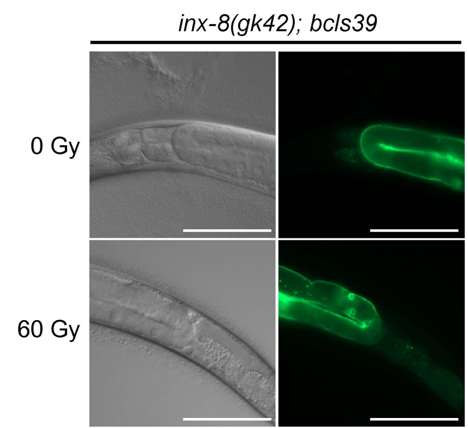

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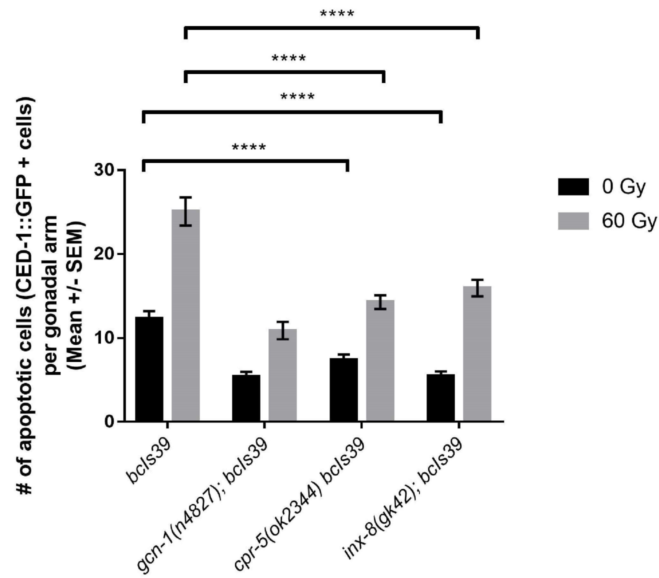


Figure 7

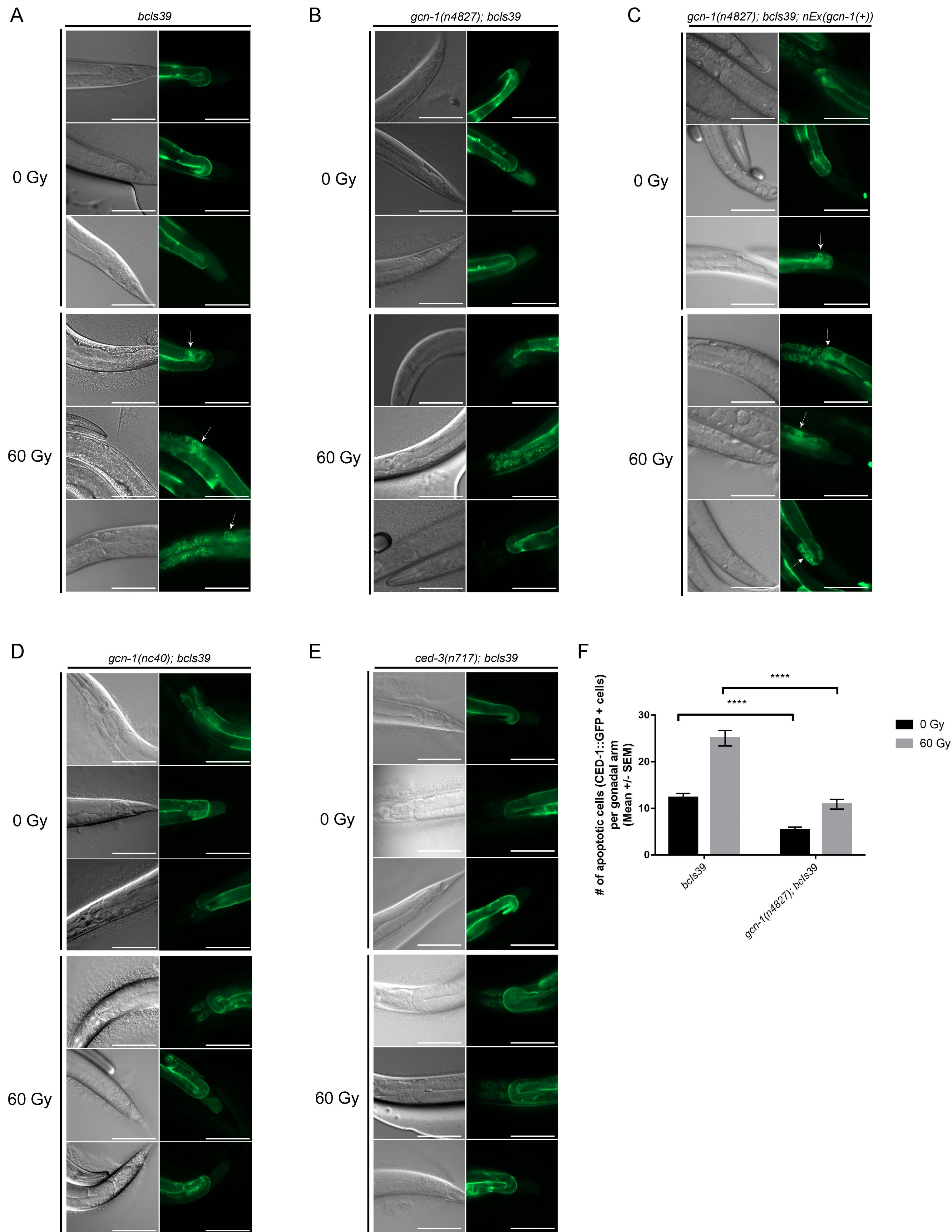
Fig. 7| Identification of possible translational pro-apoptotic targets of GCN-1

a. Ribo-seq data denoting actively translated *cpr-5* transcripts in the wild-type vs *gcn-1(lf)* animals, $**p \leq 0.01$. **b.** Ribo-seq data denoting actively translated *inx-8* transcripts in the wild-type vs *gcn-1(lf)* animals, $**p \leq 0.01$. **c-d.** DIC and GFP photomicrographs of representative genotypes. Images obtained at 40x magnifications. Scale bars represent 100 µm. **e.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 20 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. $****p \leq 0.0001$. Both *cpr-5* and *inx-8* appear to promote germline cell-death phenotype.

ISOLATE	% animals with presence of "apoptotic clusters" (IR-INDUCED APOPTOSIS)	% animals with presence of "apoptotic clusters" (PHYS. APOPTOSIS)	MODE OF INHERITANCE	MATERNAL EFFECT?	# OF LOCI?
bcls39	87%	11%			
gcn-1(n4827); bcls39	12%	0%			
5.1.3	100%	50%	Recessive	Yes	1
5.5.3	100%	71%	Recessive	Yes	1
9.3.4	100%	40%	Dominant		1
10.2.4	100%	25%	Recessive		2
7.10.2	92%	25%	Dominant		1
8.3.1	91%	29%	Recessive		1
6.5.1	90%	22%	Recessive		1
9.5.3	89%	60%	Recessive		2
6.2.5	89%	20%	Recessive		1
6.3.4	83%	50%	Recessive		1
7.1.5	83%	20%	Recessive		1
7.7.1	82%	50%	Dominant		1
5.2.2	80%	70%	Recessive		1
8.6.6	80%	67%	Recessive		1
7.4.1	85%	9%	Recessive		1
5.11.3	82%	5%	Dominant		?
5.5.2	78%	25%	Recessive		1
8.5.2	79%	22%	Dominant		?1
1.3.1	75%	21%	Recessive		1
6.1.1	73%	21%	Recessive		1
5.1.5	63%	9%	Recessive		1
7.7.4	60%	14%	Recessive	Yes	1

Table 1| List of *gcn-1* genetic suppressor isolates from the EMS genetic screen

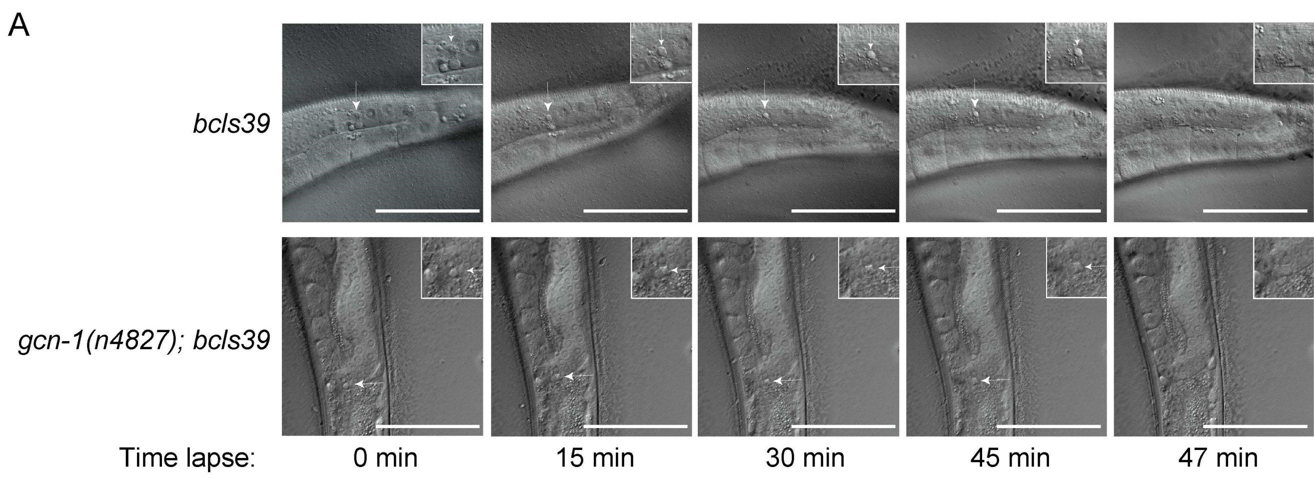
Listed in order of decreasing mean percent animals with presence of “apoptotic clusters” upon radiation stress.

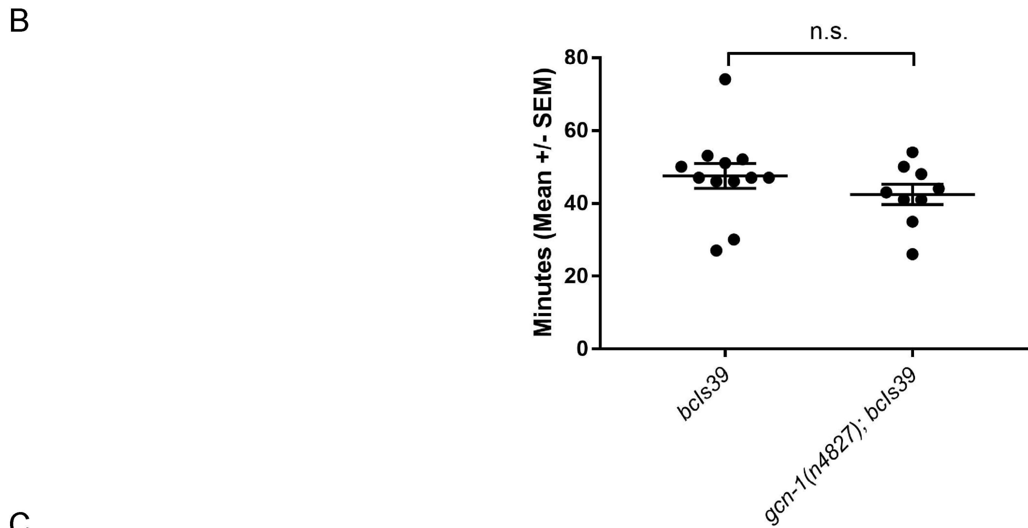


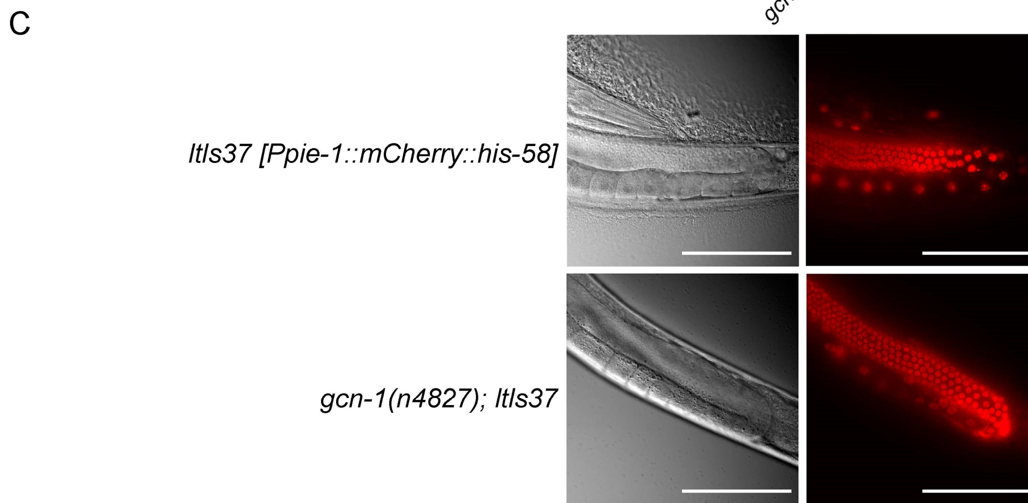
Supplemental Figure 1

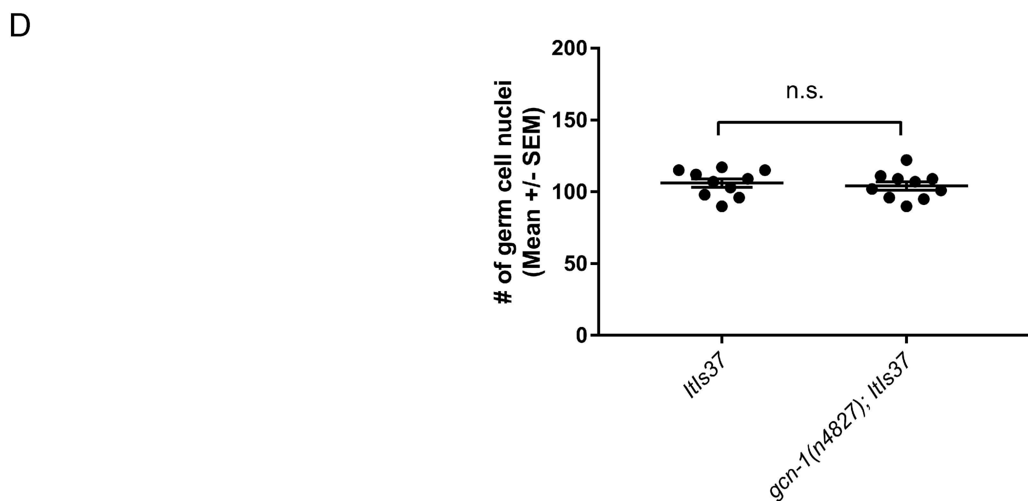
Supplemental Fig. 1| GCN-1 promotes both physiological and stress-induced germline cell death – Figure with more replicates

a-e. DIC and GFP photomicrographs of representative genotypes. Each set of one DIC and one GFP photomicrographs arranged in a row represent one animal. 3 representative animals are presented for the 0 Gy condition and another 3 animals for the 60 Gy condition for each genotype. Images obtained at 40x magnifications. Arrows represent presence of "apoptotic clusters". Scale bars represent 100 μm. **f.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented – representing more replicates (n= 15 to 20 animals per genotype). Statistical analyses were performed using an unpaired two-tailed t-test. ****$p \leq 0.0001$.







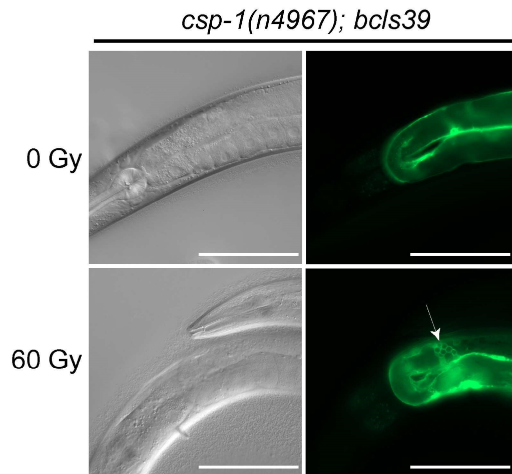


Supplemental Figure 2

Supplemental Fig. 2| GCN-1 does not influence the kinetics of apoptotic cell clearance nor have an impact in germ cell proliferation

a. Time-lapse DIC photomicrographs demonstrating apoptotic cell clearance kinetics in *bcIs39* and *gcn-1(n4827); bcIs39* animals. Arrows represent the refractile apoptotic cell that is being tracked across time. Note that at time = 0 minute, the arrows point at locations where an apoptotic corpse is about to appear. Insets are provided for magnified visualization of apoptotic corpses. Scale bars represent 100 µm. **b.** Quantification of clearance time of an apoptotic cell. Statistical analysis performed using an unpaired two-tailed t-test. **c.** DIC and mCherry photomicrographs of representative genotypes. Images obtained at 63x magnifications. Scale bars represent 100 µm. **d.** Quantification of number of germ cell nuclei. Statistical analysis performed using an unpaired two-tailed t-test.

A



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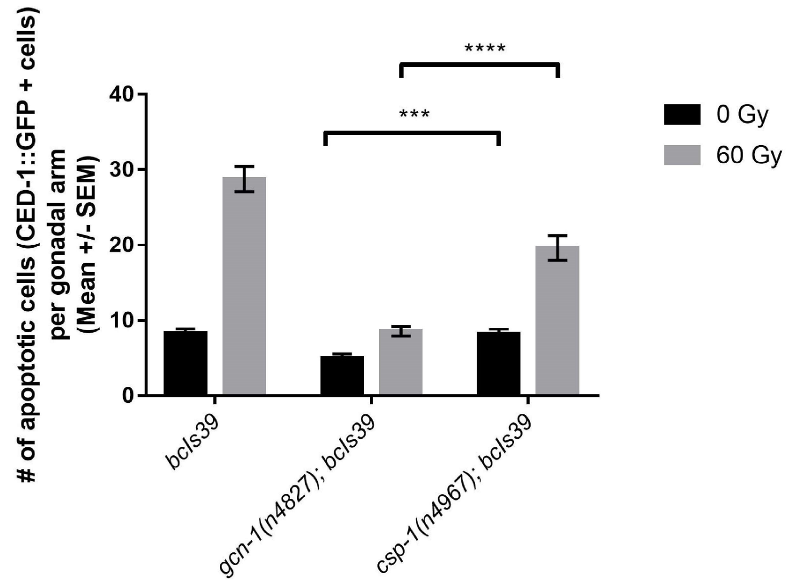
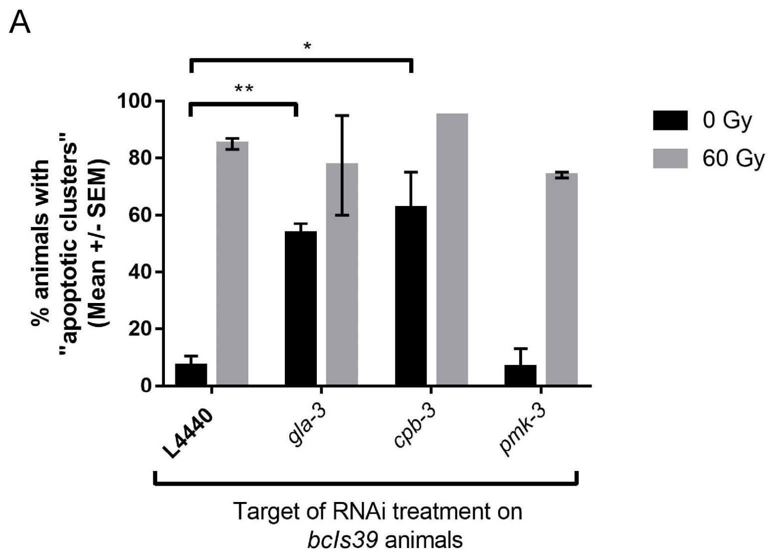
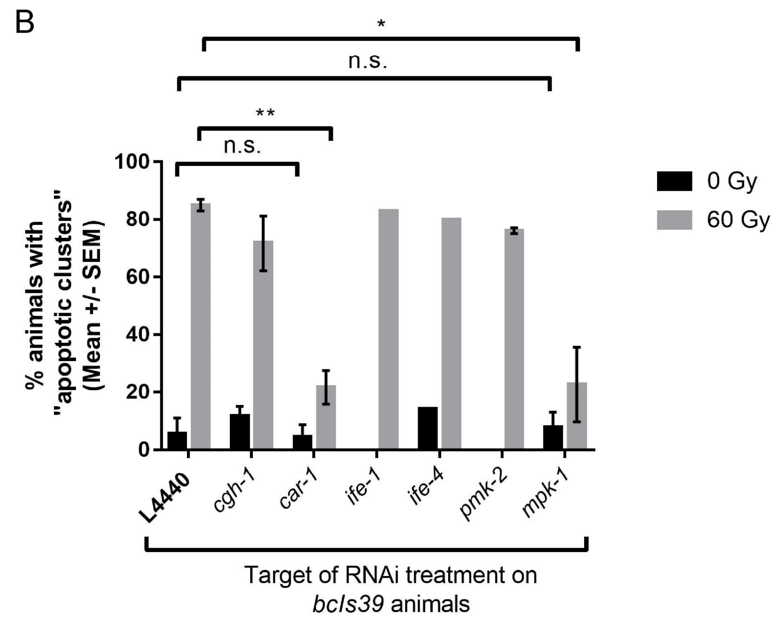


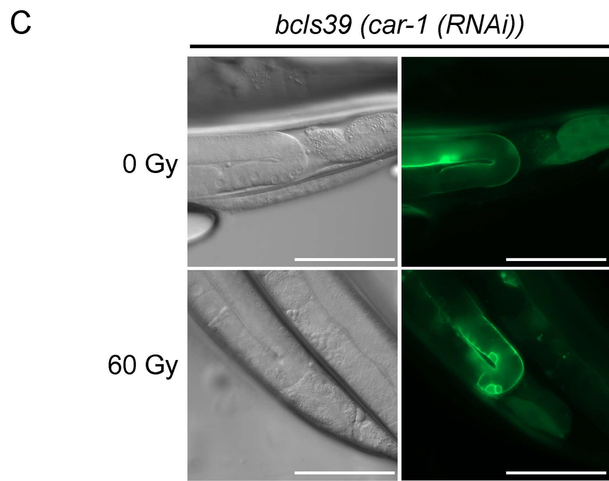
Supplemental Figure 3

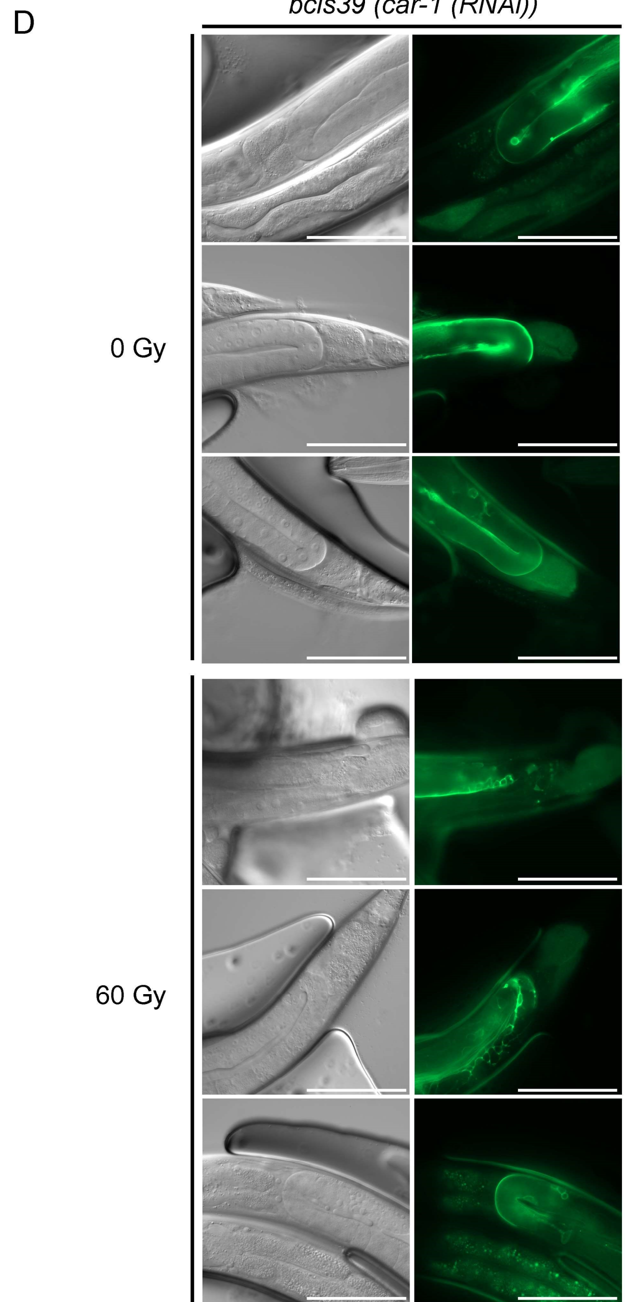
Supplemental Fig. 3| GCN-1 does not promote apoptosis through CSP-1 activation

a. DIC and GFP photomicrographs of representative genotype. *n4967* allele represents a 769 bp deletion that eliminates the putative protease active site of CSP-1, a pro-apoptotic caspase. Images obtained at 40x magnifications. Arrow represents presence of "apoptotic clusters". Scale bars represent 100 µm. **b.** Quantification of number of apoptotic cells (CED-1::GFP + cells) per gonadal arm for each of the genotype presented (n= 19-55 animals per genotype). ***$p \leq 0.001$ and ****$p \leq 0.0001$.







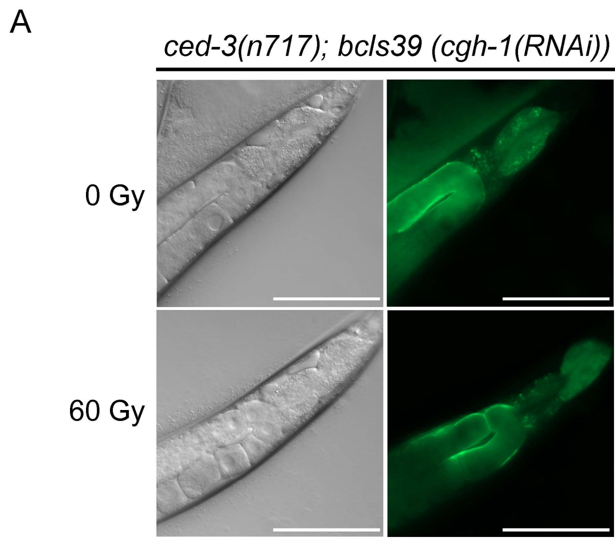


Supplemental Figure 4

Supplemental Fig. 4| RNAi experiments in *bcIs39* animals

a. Quantification of percent animals with presence of "apoptotic clusters" for each of the represented RNAi-treated *bcIs39* animals (n= 15-20 animals per specific RNAi-treated animals per replicate; 2 replicates). Statistical analyses were performed using an unpaired two-tailed t-test. $*p \leq 0.05$ and $**p \leq 0.01$. **b.** Quantification of percent animals with presence of "apoptotic clusters" for each of the represented RNAi-treated *bcIs39* animals (n= 11-30 animals per specific RNAi-treated animals per replicate; 3 replicates, except for *ife-1(RNAi)* and *ife-4(RNAi)* experiments where 1 replicate was done given negative data). Statistical analyses were performed using an unpaired two-tailed t-test. $*p \leq 0.05$ and $**p \leq 0.01$. **c.** DIC and GFP photomicrographs of *bcIs39* animals treated with *car-1(RNAi)*. Images obtained at 40x magnifications. Scale bars represent 100 µm. **d.** DIC and GFP photomicrographs of additional *bcIs39* animals treated with *car-1(RNAi)*. Each set of one DIC and one GFP photomicrographs arranged in a row represent one animal. 3 additional animals are presented for the 0 Gy condition and 3 additional animals for the 60 Gy condition. Images obtained at 40x magnifications. Scale bars represent 100 µm.

A



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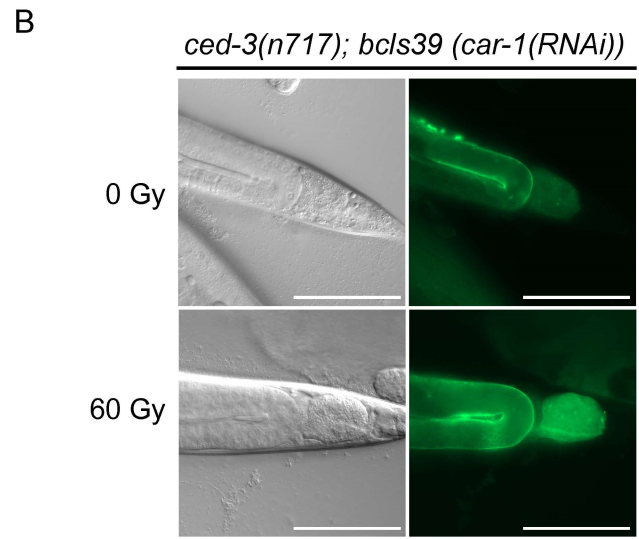

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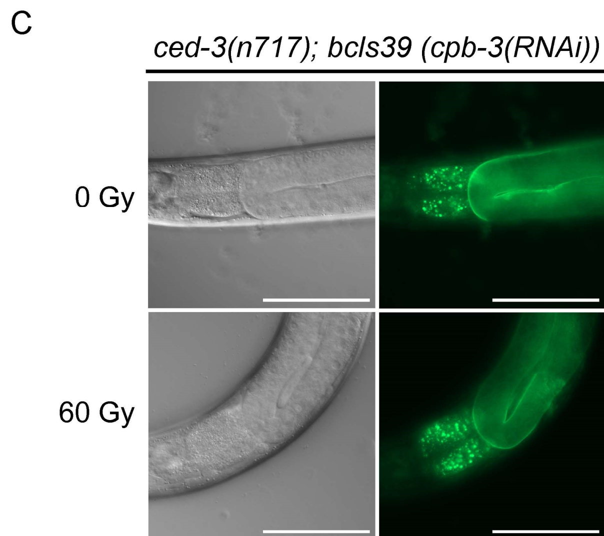

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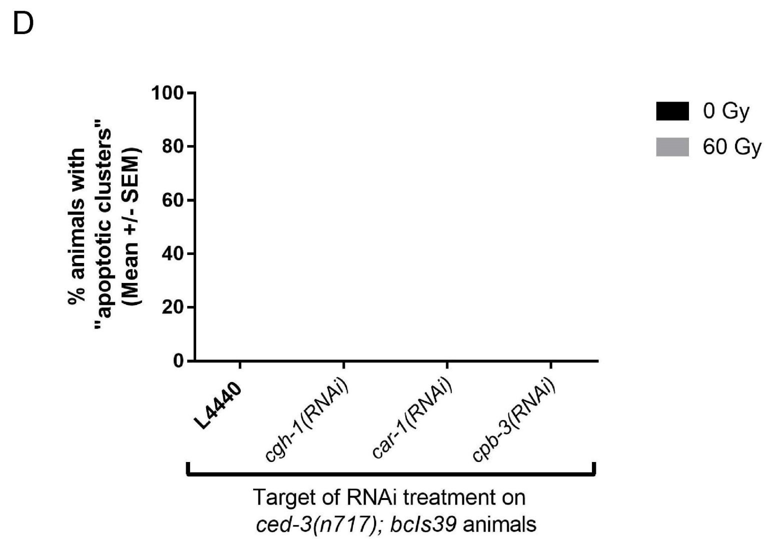

Supplemental Figure 5

Supplemental Fig. 5| *cgh-1(RNAi)*, *car-1(RNAi)*, and *cpb-3(RNAi)* experiments on *ced-3(n717); bcIs39* animals

a-c. DIC and GFP photomicrographs of *ced-3(n717); bcIs39* animals treated with respective RNAi. Images obtained at 40x magnifications. Scale bars represent 100 µm. **d.** Quantification of percent animals with presence of “apoptotic clusters” for each of the represented RNAi-treated *ced-3(n717); bcIs39* animals (n= 10-19 animals per specific RNAi-treated animals per replicate; 2 replicates).

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Chapter 3

FUTURE DIRECTIONS

Yoon Andrew Cho-Park

One future direction in the short term would be to provide a second genetic evidence of the pro-apoptotic phenotype seen in both *inx-8(loss-of-function)* and *cpr-5(loss-of-function)* mutants. This could be achieved by either using RNAi, creating a CRISPR null alleles of these two genes, or introducing a wild-type copy of *inx-8* and *cpr-5* to rescue the diminished apoptosis phenotype in the mutants.

Another future endeavor of this project would be to identify the suppressor genes in mutants isolated from the EMS mutagenesis screen. The identification of these genes could further validate my suppressors identified through RNAi and possibly shed light to other molecular players involved in GCN-1-mediated programmed cell death.

Given the identification of *car-1*, *cgh-1*, and *cpb-3* as *gcn-1* suppressor genes in our phenotype, a significant future endeavor would be to dissect this biology on a biochemical/molecular level. First of all, one could test the interesting relationship that possibly exists between *car-1* and *gcn-1* to determine whether they physically interact with each other by using assay such as co-immunoprecipitation. We could also test *in vivo* whether CAR-1 and GCN-1 interacts through a split-GFP system (Cabantous et al., 2013). Subsequently, we could extend the protein-protein interaction studies to the rest of the CAR-1/CGH-1/CPB-3 complex and determine whether GCN-1 can physically interact with the entire complex.

In light of the scant evidence in the literature hinting of an independent role of *gcn-1* to *gcn-2* and the Integrated Stress Response in *Arabidopsis thaliana* and mouse (Faus et al., 2018; Yamazaki et al., 2020), it will be interesting to examine whether one could extrapolate this *gcn-1*-independent role to other organisms. As such, it will be interesting to test this in human cell culture system to determine whether this independent function is conserved.

On the topic of conservation, many of the *gcn-1* suppressor genes identified in this project are conserved in other organisms, such as *car-1*, *cgh-1*, and *cpb-3* (Boag et al., 2005; Marnef et al., 2009; Singh et al., 2017; Tang et al., 2020). If confirmed that GCN-1 and these proteins are directly interacting with each other in *C. elegans*, it will be interesting to determine if these interactions are preserved in humans, using the human homologs of these proteins.

Given that GCN-1 is a translational regulator, a possible future direction would be to use the polysome profiling assay to compare the degree of translational activity either in the presence or absence of GCN-1 (Cattie et al., 2016; Pan et al., 2007). Subsequently the same assay could be used to observe the translational activity in *car-1(RNAi)*-treated wild-type and *gcn-1*(loss-of-function) animals. The latter experiment could be extrapolated for *cgh-1(RNAi)* and *cpb-3(RNAi)*.

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