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Apoptosis is a major determinant in the ability of cells to become cancerous. The aim of this grant was to identify conserved regulators of apoptosis using the nematode C. elegans as a model system. Specifically, we searched for proteins that interact with CED-9, the nematode homolog of the oncoprotein Bcl-2. We found that CED-9 interacts with CED-4, the worm homolog of the apoptotic regulator Apaf-1, and characterised the regulation of CED-9 by EGL-1, a BH3 domain protein that also directly binds to CED-9. Finally, we identified a dozen additional *C. elegans* proteins that interacted with both CED-9 and Bcl-2 in a yeast two-hybrid system. Unfortunately, none of these proteins showed any significant effect on nematode apoptosis, either when overexpressed or inactivated by RNA interference.

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FOREWORD

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4. TABLE OF CONTENTS

5. INTRODUCTION

Apoptosis (programmed cell death) is intimately involved in the regulation of cell numbers during normal breast physiology, e.g., during involution of the lactating breast after weaning (Strange et al., 1995; Walker et al., 1989), and has been implicated in the development of breast tumors (Schulte-Hermann et al., 1995) and their response to chemotherapy (Lowe et al., 1994; Thompson, 1995). Our previous work has shown that the small nematode *C. elegans* can be successfully used as a experimental system for the genetic dissection of apoptosis: many of the cell death genes identified in *C. elegans* have functional homologues in mammals, that play similar roles in the regulation of mammalian apoptosis (Hengartner, 2001; Hengartner, 1998; Hengartner, 2000; Hengartner, 1997; Hengartner, 1999; Metzstein et al., 1998). Thus, knowledge gained about nematode programmed cell death can also be used to further our understanding of this phenomenon in humans. In our application, we postulated that the identification and characterisation of additional genes that function in breast cell apoptosis would allow us to identify additional diagnostic and prognostic markers, as well as provide new targets for therapy. We proposed to use the nematode *C. elegans* as a model system to isolate such novel cell death genes. Specifically, we proposed to identify proteins that interact with the CED-9 cell survival protein in the yeast two-hybrid system. We argued that because of its simplicity, the reproducibility of its development, and the powerful molecular genetic tools available in this species, *C. elegans* is an excellent choice for such an enterprise.

6. BODY

6.1 Research aims/Technical objectives

The central goal of our grant is to identify and characterise proteins that interact with the C. elegans cell death regulator CED-9. Our original research proposal contained five (5) technical objectives:

1. Identification of proteins that interact with *C. elegans* CED-9 in the yeast two-hybrid system.
2. Confirmation of the specificity and relevance of the interaction detected between CED-9 and the isolated clones by testing for interaction using an *in vitro* assay
3. Analysis and characterisation of the isolated interactors, and determination of their possible role in nematode apoptosis.
4. Identification and cloning of cDNAs encoding mammalian homologs of the nematode proteins found to function in cell death.
5. Investigation of the possible involvement of the homologs in mammalian apoptosis.

We are happy to report that technical objective 1 (TO1) was completed quite successfully. As previously mentioned in our interim report, we have identified a direct interaction between CED-9 – a Bcl-2 homolog – and CED-4, the nematode homolog of the adaptor protein Apaf-1 (Spector et al., 1997). Our work in this area is described in section 6.2. In section 6.3, we describe our analysis of the interactions between CED-9 and EGL-1, a BH3 domain protein that others have shown to directly interact with CED-9 (Conradt and Horvitz, 1998; Conradt and Horvitz, 1999; del Peso et al., 2000; del Peso et al., 1998).

Our general screen for additional CED-9 interacting proteins was also successful: we identified over a dozen of CED-9-interacting proteins. However, further analysis of these interacting clones in TO2 and TO3 failed to reveal any obvious involvement of these genes in *C. elegans* apoptosis. Thus, this particular line of research had to be stopped. The negative results that led to this conclusion are described in section 6.4. Because we failed to identify any novel cell death interactors in our study, we could not complete TO4 and TO5 successfully.

A more successful strategy for the future might be to use a genetic approach to identify either enhancers or suppressors of CED-9 function. However, such screens could not be designed in time to be performed during the tenure of this grant, and will have to be performed under the aegis of another grant.

6.2 CED-4

6.2.1 *Direct interaction between* C. elegans *cell death regulators CED-9 and CED-4*

As part of our efforts to identify proteins that interact with CED-9, we discovered that CED-9 binds very tightly to CED-4, a protein that had previously been shown to be required for apoptotic cell death in C. elegans. While CED-4 can not be considered to be a "novel cell death protein," we

felt that this interaction was of great interest, and decided to aggressively pursue this observation. We found that CED-9's ability to bind to CED-4 correlated with its ability to prevent death, suggesting that the major function of CED-9 might be to sequestered CED-4 in an inactive conformation. These observations were published in Nature (Spector et al., 1997). A reprint of the paper has been attached as Appendix II. This reprint contains all the relevant technical information and experimental details. We therefore only briefly summarize our findings below.

6.2.1.1 CED-9 interacts with CED-4

Genetic studies in *C. elegans* have indicated that CED-9 prevents cell death by antagonizing the death-promoting activities of CED-3 and CED-4 (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Hengartner and Horvitz, 1994; Shaham and Horvitz, 1996). In our search for proteins that interact with CED-9, we found that full-length CED-9 binds strongly to CED-4S, the death promoting form of CED-4 (Figure 1, Table 1). This interaction, which could be detected using either CED-9 or CED-4S as the bait, is specific, as neither LexA-CED-9 nor LexA-CED-4S interacted with GAL4AD-STE11 and GAL4AD-Lamin, two proteins used as negative controls (Spector et al., 1997).

To test whether the CED-9/CED-4 interaction that we observed in the yeast two-hybrid system was direct, we asked whether CED-9 and CED-4 could interact in vitro. We found that in vitro translated CED-4S protein readily bound to glutathione-S-transferase-CED-9 (GST-CED-9) fusion protein. There is little binding of CED-4S and GST-CED-9 to GST-p16 and CDK4 (two non-relevant proteins used as negative controls), respectively, confirming that the CED-9/CED-4S interaction is specific (Spector et al., 1997).

6.2.1.2 CED-9 prevents apoptotic cell death by binding to CED-4

Given the strong genetic link between *ced-9* and *ced-4*, we strongly suspected that the direct physical interaction between CED-9 and CED-4 would be biologically significant. To test this hypothesis, we looked for a correlation between the ability of CED-9 to interact with CED-4 and its ability to prevent cell death. To this end, we introduced point mutations into the *ced-9* open reading frame (ORF) to recreate three CED-9 mutant proteins whose effect on cell death we had previously characterized at the genetic level: CED-9(G169E), the product of the dominant gain-of-function allele *ced-9(n1950)*, CED-9(Y149N), which corresponds to the loss-of-function mutation *ced-9(n1653ts)*, and Q160stop, which results in the truncated protein CED-9(1-159) and corresponds to the loss-of-function mutation *ced-9(n2077)* (Hengartner et al., 1992; Hengartner and Horvitz, 1994; Hengartner and Horvitz, 1994).

The *n1950* gain-of-function mutation results in a glycine-to-glutamate (G169E) substitution in the conserved Bcl-2 homology (BH) domain BH1, which is present in and mediates interactions between most Bcl-2 family members (Farrow and Brown, 1996). Consistent with the observation that *n1950* does not eliminate *ced-9* function, we found that CED-9(G169E) interacted efficiently with CED-4S (Spector et al., 1997). Because the CED-9(G169E)/CED-4S interaction was not significantly stronger than the one we observed between CED-9(+) and CED-4S, we concluded that *n1950* was unlikely to cause a gain of function by simply increasing CED-9/CED-4 interaction

strength. Rather, we proposed that *n1950* affects the ability of CED-9 to interact with another protein- possibly the nematode equivalent of Bax.

This predicted proved to be almost exactly right. Recently, Conradt and Horvitz reported that *egl-1*, a negative regulator of *ced-9* function, encodes a small BH3 domain protein (Conradt and Horvitz, 1998). BH3 domain proteins in mammals act as pro-apoptotic agents, and can bind to full-fledged anti-apoptotic members of the Bcl-2 family and antagonise their activity (reviewed in Adams and Cory, 1998; Gross et al., 1999; Hengartner, 2000). True to its nature, EGL-1 also directly binds to CED-9 (Conradt and Horvitz, 1998). Interestingly, the G169>E mutation reduces CED-9's affinity for EGL-1 (del Peso et al., 2000; del Peso et al., 1998), thus providing a simple explanation for the ability of this mutation to confer resistance to apoptosis.

In contrast to the gain-of-function mutation, the Y149N and Q160stop loss-of-function mutations both abrogated interaction between CED-9 and CED-4 in the yeast two-hybrid system (Spector et al., 1997). Q160stop also eliminated the CED-9/CED-4 interaction in vitro, while Y149N only reduced it. The weak effect of Y149N on CED-9/CED-4 interaction in vitro is consistent with the temperature-sensitive nature of this mutation Hengartner et al., 1992, and the different temperatures at which our assays were performed (30 °C for yeast; 4 °C in vitro). In vivo, Y149N might prevent CED-9/CED-4 interaction only at high, but not at low temperatures (we have excluded trivial explanations, such as reduced protein stability for the *n1653* temperature sensitivity, as the Y149N protein is as stable as the wild-type protein under both permissive and restrictive temperatures; data not shown). The correlation between loss of *ced-9* function and loss of CED-4-binding is consistent with the hypothesis that direct interaction with CED-4 is important for the ability of CED-9 to prevent cell death.

6.2.1.3 Interaction of CED-9 with CED-4 requires most of the CED-9 protein, but not the hydrophobic C-terminal domain

To identify the domain(s) of CED-9 required for interaction with CED-4, we generated a number of CED-9 deletion constructs and tested their ability to interact with full-length CED-4S in vitro (Spector et al., 1997). Several Bcl-2 family members contain a C-terminal hydrophobic tail, which drives localization of these proteins to the outer surface of mitochondria, rough endoplasmic reticulum, and nuclear membranes. Truncations that remove this tail only slightly reduce the ability of Bcl-2 to prevent apoptosis (Hockenbery et al., 1993). Similarly, we found that CED-9(1-247), which lacks the hydrophobic tail, still prevents programmed cell death when overexpressed in *C. elegans*, albeit with reduced efficiency (data not shown). Thus, the C-terminal tail is not essential for Bcl-2 or CED-9 function. Consistent with these observations, CED-9(1-231), which lacks the C-terminal 49 amino acids, still interacted with CED-4S. However, elimination of a further 72 amino acids that includes the BH1 and BH2 domains [CED-9(1-159) vs. CED-9(1-231)] completely eliminated the ability of CED-9 to interact with CED-4S, both in vitro and in yeast (Spector et al., 1997), suggesting that the BH1 and BH2 domains, previously shown to be functionally important for several Bcl-2 family members, might be involved in CED-9/CED-4 interaction. Further support for this hypothesis comes from our observation that residues 97-231 (containing the BH1, BH2, and BH3 domains) were sufficient for interaction with CED-4S in vitro, albeit at a reduced level (Spector et al., 1997). Surprisingly, however, this truncated construct was inactive when expressed as a transgene in *C. elegans* (data not shown). We do not know the reason for this lack of in vivo

activity, but suspect that the truncated protein might not be folded properly in the animal. Alternatively, the reduced interaction detected in vitro might not be sufficient to confer biological activity in vivo.

6.2.1.4 CED-9 interacts with both splice forms of CED-4

In addition to the major CED-4 isoform, known as ced-4S, there exists a rare alternative splice variant, ced-4L, that is predicted to produce a protein containing 24 additional amino acids. In contrast to the major isoform (*ced-4*S), overexpression of *ced-4*L prevents death (Shaham, 1996 #673]). However, both forms of *ced-4* are antagonized by *ced-9*, possibly explaining why *ced-9* also possesses a minor death-promoting activity Hengartner and Horvitz, 1994. Because *ced-9* antagonizes both *ced-4*L and *ced-4*S, we suspected that CED-9 would also interact with CED-4L. Indeed, we found no qualitative differences between CED-4S and CED-4L in their ability to bind to CED-9, although the CED-9/CED-4L interaction was invariably weaker (Spector et al., 1997). Thus, the difference between *ced-4*S and *ced-4*L must lie in a differential ability to interact with or activate downstream targets, rather than in differential binding to CED-9.

The biological importance of the CED-4L isoform is still unclear, and its role in the regulation of *C. elegans* apoptosis would clearly be warranted. However, further investigation of this question was outside the scope of this grant, we refrained for any further investigations in this area.

6.2.1.5 Conclusions

In summary, the work described above, and largely published in Spector et al. (1997), demonstrates the presence of a direct physical interaction between the *C. elegans* death suppressor CED-9 and one of its known downstream targets, CED-4. This interaction is disrupted in two known *ced-9(lf)* mutants, and might be mediated through the conserved BH1, BH2, and BH3 domains, as constructs that delete these domains also loose the ability to interact with CED-4. While our data suggest that binding to CED-4 is important for CED-9 function, we do not know whether it is sufficient. Indeed, our preliminary results with our deletion constructs suggest that there might be additional determinants of CED-9 function in the N-terminal region of CED-9, possibly mediated by the protein's BH4 domain (see above; Hengartner, 1998).

6.2.2 *The apoptosome Model of programmed cell death in* C. elegans

Our work on CED-9, and the work of several other groups, have led to the emergence of a simple molecular model for the control of apoptosis in C. elegans, which we have developed and refined over time (Hengartner, 1997; Hengartner, 1998; Hengartner, 1998; Hengartner, 1998; Hengartner, 2000; Hengartner, 1999). This model is based on a number of protein-protein interactions that have been detected between the various *C. elegans* cell death proteins (Figure 1). Generation of a clear model of how apoptosis is controlled in *C. elegans* is not just of academic interest, as it might also shed light on the mechanism that underlies apoptosis in mammals, or at the very least will suggest experiments that will address this question. Thus, we have spent a significant amount of time determining the limits of our model, and confronting it with any new data that we – or others – generated over the last few years.

6.2.2.1 CED-9 and CED-4 are part of a multiprotein complex – the nematode apoptosome

In addition to the interaction between CED-9 and CED-4, which we and several other groups reported (Chinnaiyan et al., 1997; James et al., 1997; Spector et al., 1997; Wu et al., 1997), CED-4 can also interact with proCED-3, the inactive zymogen form of CED-3, and promote its proteolytic activation (Seshagiri et a.,1997; Chinnaiyan et al., 1997; Wu et al., 1997). Thus, CED-4 might act as a chaperonin or co-factor in the activation of CED-3 (Hengartner, 1997). As was the case with the CED-9/CED-4 interaction, the binding of CED-4 to CED-3 is crucial for its ability to promote CED-3 activation: point mutations that inactivate the proapoptotic activity of CED-4 also abolish both interaction with proCED-3 and stimulation of CED-3 activation.

As might be expected from the available genetic data, binding of CED-9 to CED-4 abolishes its ability to promote CED-3 activation (Seshagiri et a.,1997; Chinnaiyan et al., 1997; Wu et al., 1997). However, CED-9-bound CED-4 can still interact with CED-3. Thus, in normal *C. elegans* cells, all three key cell death proteins are likely to be associated together in a multiprotein complex, which has been termed the "apoptosome", that controls cell death (Figure 1). In cells that are fated to die, we propose that the complex is modified in some way, such that CED-4 becomes active. Activation appears to associated with a release of CED-4 from the complex and its relocalisation to a perinuclear compartment (Chen et al., 2000). During somatic development, dissociation of the complex is mediated by EGL-1, a BH3 domain-containing protein that acts genetically as an inhibitor of CED-9 and has been shown to interact directly with CED-9 (Conradt and Horvitz, 1998).

Once freed from the shackles of CED-9, how does CED-4 promote proCED-3 processing? Recently, Baltimore and colleagues reported that CED-4, in addition to its ability to interact with CED-9 and CED-3, is also able to bind to other CED-4 molecules, resulting in CED-4 oligomerization (Yang et al., 1998). Because CED-4 is able to bind at the same time to proCED-3 and to another CED-4 molecule, this oligomerization brings together many proCED-3 molecules, resulting in a high local concentration of proCED-3. Under these conditions, the low protease activity inherent to procaspases (Muzio et al., 1998) appears to be sufficient to allow the molecules of proCED-3 to cleave and activate one another. A similar mechanism of activation, know as "induced proximity," has recently been suggested to mediate activation of caspase-8 in mammals (Muzio et al., 1998). Induced proximity might thus be a general strategy used by cells to activate caspases (Budihardjo et al., 1999; Earnshaw et al., 1999; Salvesen and Dixit, 1999).

As might be expected, CED-9-bound CED-4 is unable to oligomerize, either because of steric hindrance or a CED-9-induced conformational change. These observations lend further support to the idea that CED-9 prevents cell death simply by binding to CED-4 and thereby keeping proCED-3 in a safe, monomeric state (Figure 1).

6.2.2.2 Experimental support for the apoptosome model

To directly test our model, we raised monoclonal antibodies against bacterially expressed CED-9, CED-4, and CED-3. The antibodies were be generated by the Cold Spring Harbor Laboratory Monoclonal Antibody Facility (CSHL MAb Facility), which is part of the CSH Cancer Center, of which we are member. The CSHL MAb facility has an outstanding track record of generating useful MAbs that can be used for western blotting, immunoprecipitation, and/or immunocytochemistry. I a previous report, we mentioned our preliminary characterisation of three distinct antibodies generated

by the facility. All three antibodies appear to specifically recognize CED-9 on a western blot (Figure 2). Using these antibodies on fixed wild-type animals, we could confirm that CED-9 is widely expressed in early embryos, and localises to mitochondria, based on its near-perfect colocalisation with the mitochondrial marker MitoTracker; Figure 3). Furthermore, biochemical subcellular fractionation showed that CED-9 localises to the mitochondrial fraction (Figure 4). Unfortunately, the monoclonal antibodies that we generated against CED-4 and CED-3, while successful at recognizing recombinant protein, did not show enough affinity to identify the respective proteins in worm extracts, and thus could not be used further (data not shown).

To bypass our lack of specific anti-CED-4 and anti-CED-3 antibodies, we also generated a number constructs that drive the expression of green fluorescent protein (GFP) fused to CED-9, CED-4, and CED-3. We hoped that these fusions would allow us to detect any subcellular redistribution of these proteins that might occur once a cell decides to die. We found that the CED-9::GFP expression pattern did not change during apoptosis (data not shown). Unfortunately, the CED-4::GFP fusion that we generated did not express well. However, a recent paper by Horvitz and colleagues shows that, indeed, CED-4 does colocalise with CED-9 in living cells, while it translocates to the perinuclear space in dying cells (Chen et al., 2000). These results strongly support our apoptosome model. Unfortunately, no data is yet available regarding the subcellular localisation of either the pro-form or the active form of the CED-3 caspase.

6.2.2.3 Is there a mammalian apoptosome?

Since all three components of the *C. elegans* apoptosome have mammalian homologs, it is worth asking whether a similar death complex might exist in mammalian cells. Work from other groups suggest that this is at least partially the case.

The most obvious similarity is at the level of the CED-4/CED-3 interaction. In a very elegant series of papers, Wang and his colleagues have purified Apaf-1, a mammalian homolog of CED-4, based on its activity to promote the activation of caspase-9 (Apaf-3), a mammalian homolog of CED-3 (Zou et al., 1997). Indeed, Apaf-1 and caspase-9 can interact directly, as do CED-4 and CED-3. However, unlike the situation in *C. elegans*, this interaction requires the presence of another protein, cytochrome c (Apaf-2). Furthermore, while Apaf-1, like CED-4, is capable of oligomerization (Srinivasula et al., 1998), activation of caspase-9 is not mediated by induced proximity, but rather through the generation of a Apaf-1/caspase-9 holoenzyme (Rodriguez and Lazebnik, 1999).

While these differences between nematodes and mammals do not invalidate the basic premise that CED-4/CED-3 interaction is important for the activation of apoptosis, it clearly underscores the important point that once a mammalian homolog of a worm protein is identified, it is crucial to move the study to the mammalian system, if one wants to get an exact picture of what is going on.

In contrast to the interaction with caspases, the interaction between CED-9 and CED-4 might well not be conserved at all in mammals. While a number of groups have reported that Apaf-1 can also interact with Bcl-xL, a Bcl-2 family member (Hu et al., 1998; Pan et al., 1998), these studies used cell lines in which both proteins were significantly overexpressed. Indeed, others have failed to find any such interactions under physiological conditions (Hausmann et al., 2000). This lack of

interaction, and the apparent lack of involvement of cytochrome c release in nematode cell death, suggest that at least this part of the death program might have significantly diverged since the separation of the ancestors leading to nematodes and vertebrates.

6.3 EGL-1

The identification of EGL-1 as a BH3 domain protein provided us with a second bona fide CED-9 interactor (Conradt and Horvitz, 1998). Because of our extensive prior experience with *egl-1* at the genetic level, we could rapidly capitalise on this discovery to further dissect the apoptotic pathway in *C. elegans*. Furthermore, we needed to establish whether EGL-1 was the only input into the *C. elegans* apoptosome, as had been proposed by others (Conradt and Horvitz, 1998). If that were the case, the rationale for our yeast two-hybrid screen (see section 6.4 below) would have been seriously undermined.

Our previous work with *egl-1* focused on a group of rare gain-of-function mutations, which result in inappropriate expression of this BH3 domain protein in a set of motorneurons known as the HSN neurons. Death of the HSN in hermaphrodites leads to an egg-laying defect (Trent et al., 1983). The gain-of-function (gf) mutations cluster in a regulatory region of the *egl-1* promoter, and they all block the ability of the negative regulator TRA-1 to bind to this region and silence *egl-1* transcription (Conradt and Horvitz, 1999).

6.3.1 ced-9(n1950gf) *cooperates with mutations in* ced-3 *and* ced-4 *to suppress* egl-1-*induced apoptosis of the HSNs*

The fact that the CED-9 point mutant G169E, which corresponds to the gain-of-function allele *ced-9(n1950)*, fails to bind efficiently to EGL-1, suggested that this gain-of-function allele might be particularly apt at protecting cells from *egl-1*-mediated apoptosis. Indeed, we had already previously reported that *ced-9(n1950)* could suppress HSN apoptosis induced in the weak gain-of-function allele *egl-1(n487)* (Hengartner et al., 1992). Because both ced-9 and egl-1 mutations are dominant, however, we also investigated the relative strengths of the two mutations. We found that even a single copy of *n1950* could efficiently, but not completely, rescue the HSN death conferred by inappropriate *egl-1* mutation (Table 1). Significantly, removal of even one copy of either *ced-3* or *ced-4* further enhanced HSN survival (haploinsufficient enhancement; Table 1), suggesting that the *ced-9/+; egl-1/+* double mutant provides a very sensitive background to measure changes in the extent of apoptosis. Such sensitised backgrounds have often proved useful as starting points for genetic screens. We thus hope to be able to use this background in the future to screen for new modulators of apoptosis.

6.3.2 *A screen for additional suppressors of* egl-1-*induced HSN apoptosis*

As mentioned above, the interaction between EGL-1 and CED-9 is conserved through evolution: similar interactions are found between BH3 domain proteins, such as Bad and Bid, and e.g., Bcl-2 and Bcl-xL (Adams and Cory, 1998; Gross et al., 1999; Hengartner, 2000). In mammals, such interactions are regulated at several levels, including via transcription, proteolytic activation, and phosphorylation. To better understand the mechanisms that regulate EGL-1/CED-9 interactions (and

thus apoptosis) in vivo, we performed a genetic screen for mutations that suppress *egl-1(gf)*-induced HSN death (Figure 5). Mutations that affect this process might identify the regulatory molecules that control EGL-1 activation.

From a screen of approximately 40,000 genomes, we isolated over 20 new mutations (Table 2). As expected, several of these were new alleles of *ced-3* and *ced-4*, two known *egl-1* suppressors (Ellis and Horvitz, 1986; Hengartner et al., 1992). However, almost half of them identified a new set of mutations, which specifically affect apoptosis of the HSN cell (Table 3 and data not shown).

6.3.3 *Mutations in* egs-1 *and* egs-2 *specifically rescue HSN cells from* egl-1*-induced apoptosis*

The suppressors that we identified in our screen varied greatly in their ability to suppress egl-1-induced apoptosis (Table 3). Because it would have been impossible to characterise all suppressors within the timeframe of this grant, we concentrated our attention on the three strongest mutations from this set. Genetic mapping and phenotypic characterisation of these mutations showed that they affect two previously unknown genes, which we have named *egs-1* and *egs-2* (*egs*: *egl-1* suppressor). *egs-1* maps to the central region of chromosome IV, whereas *egs-1* maps to the right arm of chromosome X (data not shown). Because of their strong effect on *egl-1*-induced HSN apoptosis, we surmise these two genes likely play an important function in regulating either *egl-1* expression or activity in the HSN cells. It is of particular interest to note that as far as we can tell, mutations in *egs-1* and *egs-2* do not affect any other cell deaths; thus these genes are tissue-specific regulators of apoptosis, rather than general cell death genes.

How cell-type-specific regulation of the ubiquitous BH3 domain proteins is achieved is one of the key issues currently in the apoptosis field. We are currently in the process of cloning and characterising these two genes at the genetic and molecular level. We hope that our future characterisation of *egs-1* and egs-2 will reveal a possibly novel mechanism of regulation of BH3 domain proteins.

6.3.4 egl-1 *is not required for physiological germ cell apoptosis*

In mammals, there are a large number of BH3 proteins, which are differentially used, depending on the cell type and the pro-apoptotic stimulus under scrutiny (Adams and Cory, 1998; Gross et al., 1999; Hengartner, 2000). In addition, some apoptotic signals appear to be independent of mitochondria– and thus also independent of BH3 domain proteins.

Loss of *egl-1* function results in survival of all 131 cells that normally die during hermaphrodite development, suggesting that egl-1 might be the main BH3 domain protein to regulate developmental apoptosis (Conradt and Horvitz, 1998). Might activation of EGL-1 be the only mechanism of death induction in *C. elegans*? To address this question, we initiated a search for situations other than developmental apoptosis in *C. elegans*, which might involve another death-inducing pathway.

As part of this search, we discovered that in the adult hermaphrodite, over half of all germ cells undergo apoptosis (Gumienny et al., 1999). While morphologically similar to the cell deaths that occur during somatic development, germ cell deaths were not as stereotyped: the extent of germ cell

death appeared to be sensitive to environmental conditions, suggesting that these cells might respond to a much richer spectrum of inputs (see discussion in Hengartner, 1997). Our analysis of the involvement of EGL-1 in germ cell apoptosis has been published (Gumienny et al., 1999). Only the most salient points will thus be reiterated here.

Interestingly, we found that while germ cell death is mediated by the same machinery that executes somatic cells – the CED-3/CED-4 apoptosome – it is subject to a different regulatory mechanism: Whereas loss of *egl-1* function or the gain-of-function mutation *ced-9(n1950)* completely abrogate developmental cell death (Conradt and Horvitz, 1998; Hengartner et al., 1992), neither had any effect on germ cell apoptosis (Figure 6).

6.3.5 egl-1 *participates in DNA damage-induced germ cell apoptosis*

One of the hallmarks of cancer progression is tumor cells sooner or later loose the ability to undergo programmed cell death in response to chemo- or radiotherapy. Thus, mutations in this pathway are important for tumor progression and/or resistance to treatment (Moll et al., 1995; Morgan and Kastan, 1997; Orr-Weaver and Weinberg, 1998; Weinert, 1997, but see also Waldman et al., 1997, for an alternative view). Our understanding of the mechanisms that promote maintenance of DNA integrity stems mostly from the analysis of unicellular organisms such as *E. coli* and *S. cerevisiae*. To counteract DNA damage, these organisms activate checkpoint controls that transiently arrest cell proliferation to allow DNA repair. In addition to DNA damage-induced cell cycle arrest, metazoans use a second output of checkpoint controls: activation of the apoptotic machinery, which results in the timely elimination of the damaged cell (Figure 7).

Because of its clear relevance to cancer, DNA damage has become one of the most intensely studied pro-apoptotic signals (Enoch and Norbury, 1995; Evan and Littlewood, 1998). Unfortunately, in contrast to the DNA repair pathway, very little is known about checkpoint-induced apoptosis, largely due to the absence of a good genetic model system (Morgan and Kastan, 1997) Rich et al., 2000; Weinert, 1998; Weinert, 1998; Zhou and Elledge, 2000.

To determine whether *C. elegans* can also respond to DNA damage, we exposed worms to increasing levels of gamma rays. We found that germ cells, but not somatic cells, responded to DNA damage by inducing cell cycle arrest, as well as apoptosis. Our preliminary analysis of this process has recently been published (Gartner et al., 2000). We will thus only briefly summarise the most relevant sections of our studies below.

Because the mitochondrial apoptotic pathway is clearly involved in apoptosis following DNA damage in mammals (Rich et al., 2000), we expected that *egl-1* might also be important for this process in *C. elegans*. Indeed, we found that *egl-1(lf)* mutants showed a reduced and delayed apoptotic response following DNA damage (Figure 8). However, *egl-1* mutants still showed a partial response, suggesting that at least one other pathway must act in parallel to *egl-1*. In contrast, the *ced-9(gf)* mutants were completely resistant to DNA damage-induced apoptosis(Figure 8) but not cell cycle arrest (data not shown), indicating that the additional pathway(s) must converge at the level of the CED-9 protein.

6.3.6 *Summary*

Our work on egl-1 showed that while this gene was a key regulator of developmental apoptosis, additional regulators of CED-9 must exist, as loss of egl-1 function only partially prevents DNA damage induced cell death (Figure 8) and does not alter physiological germ cell apoptosis at all (Figure 6). Thus, in addition to the more detailed study of *egl-1* regulation described in sections 6.3.2 and 6.3.3, we felt justified in continuing our yeast two-hybrid based screen for other proteins that might interact with, and thus regulate CED-9.

6.4 Other CED-9-interacting proteins

While the fact that CED-9 interacts with CED-4 and EGL-1 provided much insight into how apoptosis is regulated in *C. elegans*, both interactors were already known cell death regulators. To identify novel cell death regulators, we performed a screen for proteins that interact with CED-9 in the yeast two-hybrid screen.

In addition to CED-4 and EGL-1, we identified over a dozen proteins that interact with CED-9 in the yeast two-hybrid system. We present here our characterisation of these proteins. Unfortunately, further examination revealed that none of them showed any pro- or anti-apoptotic activity.

6.4.1 *Isolation of CED-9-interacting clones*

We screened two independent libraries for CED-9-interacting clones. Our detailed screening protocol and secondary screens are described in Spector et al. (1997), and will thus not be repeated here. Briefly, from a total of 4,500,000 primary transformants screened, we identified a total of 18 independent clones, representing 12 different genes (Table 4), that passed all the subsequent yeast tests (secondary screen, additional positive and negative screens). These 12 hits therefore represented good candidates for encoding *bona fide* CED-9-interacting proteins. Note that many of these clones were isolated only once (data not shown), suggesting that we had not saturated the screening of our libraries.

6.4.2 *The 12 CED-9-interacting clones also interact with CED-9G169E and with Bcl-2*

Because of the conserved nature of the programmed cell death pathway, we posited that a biologically relevant interaction between CED-9 and one of the clones that we identified in our screen might be conserved through evolution. We thus tested whether any of our clones could also interact with Bcl-2, one of the mammalian homologs of CED-9. To our great surprise, we found that all 12 clones strongly interacted with Bcl-2 (Table 4).

We mentioned above that the gain-of-function allele *ced-9(n1950)* completely protected *C. elegans* from developmental apoptosis, as well as from DNA damage-induced apoptosis. The point mutant protein corresponding to this allele, CED-9G169E, shows a reduced affinity to EGL-1 (del Peso et al., 2000), consistent with the position of this mutation in the BH1/2/3 hydrophobic groove into which BH3 domains are known to bind (Sattler et al., 1997). To test whether any of our CED-9 interactors might have a similar binding specificity as EGL-1 – as might be expected of the second

pathway that mediates response to DNA damage – we tested our hits for interaction with lexA-CED-9G169E. Based on our qualitative scale (intensity of blue of the colonies), we found no significant difference in the strength of interaction of our clones with CED-9G169E when compared to the wild-type CED-9 fusion (Table 4). We thus conclude that our hits likely interact with CED-9 on a surface distinct from the BH1/BH2/BH3 groove.

6.4.3 *Sequence analysis of CED-9-interacting clones*

Our next order of priority was to establish the nature of these clones. To this effect, we first sequenced both ends of the insert for each clone. Because the *C. elegans* genome has been completely sequenced (The C. elegans Sequencing Consortium, 1998), these short sequence tags were sufficient to uniquely identify the gene corresponding to the cDNA insert, as well as the extent of the gene present in the cDNA. Indeed, each one of our interacting clones corresponded to a previously-identified gene (Table 5).

Sequence analysis indicated that several of our candidate genes have homologs in other species (Table 5). However, none of them was homologous to previously-identified cell death genes. We considered this to be good news, as our goal was to identify novel cell death genes, not just nematode homologs of previously identified mammalian cell death genes! A detailed bioinformatic analysis suggested that three of the 12 genes (A. C, and O) contained potential BH3 domains. These three clones were thus of particular interest, given the results that we presented above on EGL-1 (see section 6.3). Nevertheless, we subjected all 12 clones to the same set of tests, to avoid any biases based on preconceived notions of what a cell death regulator should look like.

6.4.4 In vivo *loss-of-function analysis via RNA-mediated interference*

To determine whether the candidate genes identified by our clones function in programmed cell death, we used the recently described technique of RNA-mediated interference (RNAi) to generate a loss-of-function phenocopy for each of the 11 genes of interest. RNAi has recently been established as a rapid and powerful method to interfere with gene expression and generate loss-of-function phenocopies in *C. elegans* (Carthew, 2001; Fire et al., 1998; Kuwabara and Coulson, 2000). While the mechanism by which RNAi works is still under study, its efficacy is by now well known. RNAi by feeding is the most convenient approach; however, it is more variable than soaking or injection. Thus, we resolved to use RNAi by injection of in vitro synthesised dsRNA for our study. Briefly, the insert of interest is PCR amplified using Taq DNA polymerase and universal oligonucleotide primers based on the vector sequence flanking the insert. The primers contain at their respective 5' end a T7 RNA polymerase promoter. The PCR product is gel purified, and the Watson and Crick strands are simultaneously transcribed using an in vitro transcription kit (we use the RiboMax kit from Promega). For a more extensive description of our RNAi protocols, the reader can refer to a recent publication from my lab that is unrelated to this grant (Fraser et al., 1999).

We expected that if the gene is required for cell death, then its elimination should results in the presence of extra cells, which can readily be identified in the anterior pharynx (Hengartner et al., 1992). If the gene is required to protect cells from apoptosis, then its elimination would result in extra cell death, embryonic lethality, and possibly sterility of the mother (Hengartner et al., 1992). Weak effects could readily be identified through quantification of apoptotic cells in the embryo or in

the adult germ line. Indeed, mutations that weakly reduce *ced-9* function can readily be identified through both phenotypes.

To confirm that cell death genes can be indeed be inactivated by RNAi, we first tested *ced-3* and *ced-4*, two genes that are required for death, and *ced-9*, which is required to protect cells that should live (Hengartner, 1997). As expected, *ced-3(RNAi)* and *ced-4(RNAi)* F1 animals (i.e., the progeny of injected animals, respectively) had extra cells, whereas *ced-9(RNAi)* Po animals were either sterile or only generated dead embryos (Table 5). The *ced-9(RNAi)* also showed a conspicuous increase in germ cell corpses (Table 5 and Figure 9).

We also tried to eliminate *egl-1* function through RNAi. Surprisingly, we repeatedly failed to phenocopy the known *egl-1* phenotypes (e.g., extra cells in the pharynx). Our lack of success with *egl-1* was surprising, and might stem from the fact that its expression pattern is more restricted than that of the other three cell death genes that we tested. From these experiments, we concluded that RNAi can be used with success to determine whether our candidate genes have any role in programmed cell death, but that it might miss a fraction of bona fide cell death regulators due to limitations of this technique.

Our RNAi results are summarised in Table 5. Unfortunately, none of them showed any obvious cell death phenotype, be it extra cells or extra death (Table 5). We did detect some other defects, such as embryonic lethality. However, while these phenotypes are interesting in their own right, and might tell us something about the normal function of our candidate genes, we do not plan to pursue the characterization of these clones any further under this grant, as they have nothing to do with our stated objectives.

6.4.5 In vivo *overexpression analysis*

In addition to inactivation by RNAi, we also overexpressed several of our candidate genes. Two approaches were used for this. First, we ordered the cosmids containing each gene of interest, and generated transgenic lines that carried extrachromosomal arrays containing multiple copies of these cosmids. Previous work from many groups has shown that such extrachromosomal arrays result in overexpression of the genes present in multiple copies. Unfortunately, none of the arrays analysed showed any increase in cell survival (Table 5). We reasoned that maybe some of the genes of interest might be toxic to the animal (e.g., increased apoptosis can lead to embryonic lethality and adult sterility; Chen et al., 2000; Hengartner et al., 1992), and might thus have been selected against during the generation of our transgenic lines. We thus also subcloned some of our cDNAs under the control of the inducible heat-shock promoters *hsp-16.2* and *hsp-16.41* (Stringham et al., 1992). We then generated transgenic lines, and tested the transgenic animals for any change in cell death patterns following a mild heat shock (30 minutes at 30 °C). Again, we failed to find any change in cell death patterns.

6.4.6 *Summary*

While our yeast two-hybrid screen was successful in identifying proteins that interact physically with CED-9, we failed to find any in vivo evidence that these interactions were biologically relevant, because none of the genes showed any effect on apoptosis, neither when inactivated via RNAi nor

when overexpressed. Of course, the lack of effect on apoptosis does not necessarily mean that the interactions that we detected were spurious. Indeed, it is formally possible that these interactions, while not important for apoptosis, serve some other important, currently unrecognised function. Arguing against this hypothesis, however, weighs the fact that CED-9 appears to play no function outside of its involvement in the regulation of apoptosis (Hengartner et al., 1992).

Yeast two-hybrid screens are well-known to be susceptible to false positive hits (Bartel et al., 1993). Thus, based on the preponderance of negative evidence for any involvement in the regulation of apoptosis, we reluctantly concluded that the interactors that we identified in our screen most likely represented non-relevant interactions. Thus, we discontinued our characterisation of these genes, as any further work on these candidates was most unlikely to yield any new insights into the regulation of apoptosis.

6.5 Mammalian homologs of CED-9-interacting proteins

In the preceding sections, we expounded our analysis of CED-4 and EGL-1, two bona fide CED-9 interactors, and our identification – and subsequent elimination as likely false-positives – of a dozen additional candidate interacting proteins. The overarching aim of our project was to use *C. elegans* to identify, in an indirect fashion, mammalian regulators of apoptosis.

Unfortunately, the two biologically relevant interactors that we studied already have known mammalian homologs. Even more disappointing was our inability to find any evidence that the additional CED-9 interacting proteins that we identified in our yeast two-hybrid screen played any role in the regulation of apoptosis. As mentioned above, several of the new genes we identified in our screen have cognate mammalian homologs. However, because of the low probability of any one of these genes actually being involved in the apoptotic process, we decided against investing a significant amount of time and energy into cloning and characterising these mammalian homologs.

6.6 Discussion

Overall, our effort to identify and characterise CED-9-interactors can be considered a partial success. Importantly, we identified CED-4 as a CED-9-associated protein and as the key target of CED-9 regulation (Spector et al., 1997). This observation, which was published in a high profile journal, was important for the progression of the field. Surprisingly, this interaction appears not to be conserved in mammals (Hausmann et al., 2000), even though both CED-9 and CED-4 have clear functional human homologs. This unexpected finding highlights both the strengths and limitations of our approach: a search for interacting proteins can lead to the identification of important, conserved regulators, even though the interaction between the bait and these other proteins itself might not be conserved. Indeed, genetic pathways(order of gene function) appear to be more generally and deeply conserved than individual protein-protein interactions.

While we were not the first to identify the physical interaction between CED-9 and its regulator EGL-1, we could quickly capitalise on this finding to flesh out the regulation of the apoptotic machinery (the apoptosome) in *C. elegans*. Importantly, we were the first to show that *egl-1* was not

the only input into *ced-9* (Gumienny et al., 1999). We were also first to demonstrate the role that *egl-1* plays in the apoptotic response to DNA damage in *C. elegans* (Gartner et al., 2000). The interaction between CED-9 and EGL-1, unlike the CED-4/CED-9 interaction described above, is well conserved through evolution. Indeed, BH3 domain binding is probably the main mechanism of Bcl-2 regulation (the other probably being phosphorylation, as well as possibly proteolytic cleavage; Reed, 1997).

Our search for additional cell death regulator by screening for CED-9 interacting proteins in the yeast two-hybrid system was, on the other hand, rather unsuccessful. Early results strongly suggested that we were on the right track: not only did we identify a dozen new proteins that interacted with CED-9 in yeast, but each of these proteins also interacted with the mammalian CED-9 homolog Bcl-2. Because we and others had shown that Bcl-2 functions in *C. elegans* (Hengartner and Horvitz, 1994; Vaux et al., 1992), we took the positive interaction with Bcl-2 as a strong suggestion that the interactions that we had identified would be biologically relevant. With hindsight, we must admit that having over a dozen proteins all be "biologically relevant" was undoubtedly overly optimistic – particularly given they yeast two-hybrid's well known propensity to generate false positives. We do not know why all our clones interacted with Bcl-2. It is possible that all our clones interacted with a specific, conserved feature of CED-9 that is not completely crucial for its function. For example, the clones might have interacted with the hydrophobic C-terminal tail, which is shared by CED-9 and Bcl-2, but is not essential for CED-9 or Bcl-2 function (see section 6.2.1.3).

How airtight is our argument that the clones that we identified in our screen are not biologically significant? We have tried to do our best within the time constraints and manpower limitations imposed by the grant to test each clone as thoroughly as possible, testing the clones for both loss and gain of function phenotypes. While both approaches (RNAi and overexpression) have caveats associated with them, we feel confident that most of the clones that we pulled out indeed were "red herrings."

Does our failure to identify novel CED-9 interacting proteins in our yeast two-hybrid screen mean that the list of CED-9 interactors is limited to CED-4 andEGL-1? Most likely not. We presented strong evidence in section 6.3 that there must be at least one additional upstream regulatory input into CED-9. Why did we not identify this input in our screen? The most trivial explanation might be that the mysterious interacting protein that regulates CED-9 cannot readily be identified by yeast two-hybrid. Transient interactions, such as those found between kinases, proteases, or other modifying enzymes, and their substrates, are often not strong or persistent enough to generate a positive response in the yeast two-hybrid system. Another possibility is that the gene in question was not present in the two libraries that we screened. Both libraries are from whole animal, mixed developmental stages. Rare, cell type-specific or stage-specific messages might thus not be represented. Finally, maybe we just stopped screening too soon: while we screened almost 5 million transformants, we isolated most of our integrants only once, or a few times at most. Statistical arguments thus strongly suggest that we missed at least a few more clones that would have generated a positive response in this system. Whether these additional clones would only have added to our list of false positives is, of course, an open question.

Is there any other way to identify additional CED-9 regulators or targets? Our results with the *ced-9* gain-of-function allele n1950 (presented in section 6.3.1) suggest that we should be able to set up a very sensitive screen for modulators of CED-9 action by looking for enhancers or suppressors of apoptosis in a *ced-9/+; egl-1/+* genetic background. Thanks to the vast amount of information that we generated under this grant, we hope to be able to perform such a screen in the future, under the aegis of a different funding agency.

7. KEY RESEARCH ACCOMPLISHMENTS

- Identification CED-4 as a CED-9-interacting protein and the key target of CED-9
- Structure/function analysis of the CED-9/CED-4 interaction
- Development, refinement, and publication of the apoptosome model of apoptotic cell death regulation in *C. elegans*
- Identification of a genetic background sensitive to changes in apoptosis, which could be used successfully for a genetic screen for novel cell death regulators
- Demonstration that *egl-1* is not the only input into the *C. elegans* apoptosome
- Demonstration that *egl-1* is an important component of *ced-9* regulation following DNA damage in *C. elegans*
- Development and execution of a large genetic screen for regulators of egl-1 activity.
- Identification of over 20 suppressors of *egl-1*-induced apoptosis
- Genetic characterisation of *egs-1* and *egs-2*, two new regulators of apoptosis in *C. elegans*
- Progress towards the cloning of *egs-1* and *egs-2*
- Demonstration that RNAi inactivation is an efficient method to identify apoptotic regulators in *C. elegans*
- Setting up and execution of a large scale yeast two-hybrid screen for proteins that interact with CED-9
- Identification of 12 new proteins that interact with CED-9 in the yeast two-hybrid screen
- Additional testing of the 12 proteins for effect on apoptosis based on RNAi and overexpression studies

8. REPORTABLE OUTCOMES

8.1 Manuscripts

Spector MS, Desnoyers S, Hoeppner DJ, Hengartner MO: Direct interaction between *C. elegans* cell death regulators CED-9 and CED-4. *Nature* 1997, **385**:653-656.

Hengartner MO: Apoptosis: CED-4 is a stranger no more. *Nature* 1997, **388**:714-715.

Hengartner MO: Apoptosis: Death cycle and Swiss army knives. *Nature* 1998, **291**:441-442.

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Hengartner MO: Apoptosis: Death by crowd control. *Science* 1998, **281**:1298-1299.

Gumienny TL, Lambie E, Hartwieg E, Horvitz HR, Hengartner MO: Genetic control of programmed cell death in the *C. elegans* germ line. *Development* 1999, **126**:1011-1022.

Hengartner MO: The biochemistry of apoptosis. *Nature* 2000, **407**:770-776.

Gartner A, Milstein S, Ahmed S, Hodgkin J, Hengartner MO: A conserved checkpoint pathway mediates DNA damage–induced apoptosis and cell cycle arrest in *C. elegans*. *Mol Cell* 2000, **5**:435–443.

8.2 Abstracts

None, except as part of meeting abstracts (see below for list).

8.3 Presentations

Hengartner MO: Programmed cell death in the nematode *C. elegans*. AACR Special Conference on Molecular Mechanisms of Programmed Cell Death, Palm Springs, CA. January 9-13, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. University of Miami, Miami, FL. January 15, 1998. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. McGill University, Montreal, Canada. January 23, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Wilhelm Bernhard Workshop, Quebec, Canada. February 13, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. USGEB Annual Meeting, Lausanne, Switzerland. March 5-6, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Recent Progress in Hormone Research Meeting, Skamania Lodge, Stevensen, WA. August 3, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *Caenorhabditis elegans*. Third APOCB Meeting, Osaka, Japan. August 27, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *Caenorhabditis elegans*. University of Tokyo, Tokyo, Japan. August 31, 1998. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. National Institute of Genetics, Mishima, Japan. September 1, 1998. (Seminar)

Hengartner MO: Genetic control of programmed cell death in the nematode *C. elegans*. Annual Meeting of the Swiss Tissue Culture Society: Life and death of the cell. Fribourg, Switzerland. September 17, 1998 (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. First European Workshop on Cell Death, L'Aquila, Italy. October 23, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. SUNY at Stony Brook, Stony Brook, NY. December 10, 1998 (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. SEB Meeting on Apoptosis in Animals and Plants. Exeter University, Exeter, UK. December 14-17, 1998. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Division of reproductive biology. Johns Hopkins University School of Public Health, Baltimore, MD. January 7, 1999. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. ISREC meeting on cancer and the cell cycle, Lausanne, Switzerland. January 28, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Radiation Oncology Gordon Research Conference, Ventura, CA. February 3, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Department of Molecular Cell Biology, Harvard University, Cambridge, MA. February 12, 1999. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Genetical Society Spring Meeting, Warwick University, Warwick, UK. March 26, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Merck Frosst, Montreal, Canada. March 30, 1999. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. NRC Biotechnology Research Institute, Montreal, Canada. March 31, 1999. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Cancer Center, Washington University, St. Louis, MO. April 22, 1999. (Seminar)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Biochemistry and Molecular Biology '99 (Annual Meeting of the ASBMB), San Francisco, CA. May 17, 1999. (Talk)

Hoeppner DJ, Hengartner MO: Towards understanding cell type-specific cell death regulation. 12th international *C. elegans* meeting, Madison, WI. June 4, 1999. (Poster).

Hengartner MO: Signal transduction during cell death in the nematode, *C. elegans*. Gordon Research Conference on Second Messengers and Protein Phosphorylation, Kimball Union Academy, Meriden, NH. June 8, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Gordon Conference on Developmental Biology, Proctor Academy, Andover, NH. June 23, 1999. (Talk)
June 20-24, 1999

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Gordon Research Conference on Cell Death. NH. July 1, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Third Practical Workshop on Apoptosis, Cambridge University, Cambridge, UK. July 21, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. 7th KBF Symposium: Common Mechanisms in Development and Disease, Karlsruhe, Germany. September 9, 1999. (Talk)

Gartner A, Milstein S, Ahmet S, Hodgkin J, Hengartner MO: Genetic dissection of checkpoint-induced apoptosis and cell cycle arrest in C. elegans. CSHL Meeting on programmed cell death. Cold Spring Harbor, NY. September 29, 1999. (Talk)

Hengartner MO: Programmed cell death in the nematode *C. elegans*. University of Massachusetts at Amherst, Amherst, MA. October 5, 1999. (Seminar).

Hengartner MO: Programmed cell death in the nematode *C. elegans*. Skirball Institute, New York University, New York, NY. October 25, 1999. (Seminar).

Hengartner MO: Genetic control of apoptosis in the nematode *C. elegans*. Molecular and Cell Biology Graduate Program, University of Maryland at Baltimore, Baltimore, MD. February 8, 2000. (Seminar)

Hengartner MO: Genetic control of apoptosis in the nematode *C. elegans*. AAAS Science and Innovation meeting, Washington, DC. February 18, 2000. (Talk)

Hengartner MO: Genetic control of apoptosis in the nematode *C. elegans*. AACR Special Conference on programmed cell death regulation: Basic mechanisms and therapeutic opportunities. Incline Village, NV. February 29, 2000. (Talk)

Hengartner MO: Genetic control of apoptosis in *C. elegans*. AIG, National Institutes of Health, Bethesda, MD. March 6, 2000. (Seminar)

Hengartner MO: A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell proliferation arrest in *C. elegans*. CSHL Symposium on quantitative biology LX: Biological responses to DNA damage. Cold Spring Harbor, NY. June 3, 2000. (Talk)

Hengartner MO: Genetic control of apoptosis in *C. elegans*. DOD Era of Hope Breast Cancer Meeting, Atlanta, GA. June 9, 2000. (Poster)

8.3 Degrees Obtained

Hoeppner DJ. Ph.D., SUNY at Stony Brook, 2000. Part of Dr. Hoeppner's work was supported by the DoD grant.

8.4 Development of resources

Work performed under this grant led to the generation and preliminary characterisation of over 20 new mutations that alter apoptosis patterns in the nematode *C. elegans*. Following publication, these strains will be deposited with the *Caenorhabditis* Genetics Center, which will distribute them free of charge to the *C. elegans* community.

8.5 Funding applied for based on work supported by this award

We plan to apply to various cancer foundations to continue the work that was previously funded by this grant. We are confident that such funding requests will be successful.

9. CONCLUSIONS

During the tenure of this grant, we have performed a genetic and molecular analysis of proteins that interact with CED-9, a nematode homolog of the Bcl-2 oncoprotein. We have shown that CED-9 interacts with and regulates CED-4, a key cell death protein homologous to mammalian Apaf-1. We have also studied in detail the genetic interactions between CED-9 and one of its upstream regulators, the BH3 domain protein EGL-1. Equally importantly, we have shown that EGL-1 is likely but one of several mechanisms of CED-9 regulation, and have started some work to elucidate what these other mechanisms might be. Finally, we have performed a yeast two-hybrid screen for novel CED-9-interacting proteins. We have identified a dozen such *C. elegans* proteins, and showed that they also interact with Bcl-2. Unfortunately, however, we could not find any clear *in vivo* evidence that these interacting proteins participate in apoptosis, casting serious doubt on the biological relevance of the interactions with CED-9 that we detected in the yeast system.

The nematode *C. elegans* has been used with great success for the identification of genes that function in apoptosis. Surprisingly, most of these genes have functional homologs in mammals (Metzstein et al., 1998). Our identification of new modulators of *egl-1*-mediated killing is thus most encouraging, as these new mutations might identify new regulators of BH3 domain-mediated killing. More generally, the development of a genome-wide protein interaction map in *C. elegans* (Walhout et al., 2000) promises to give a more systematic and thorough approach to the identification of novel candidate cell death regulators. Such approaches, in combination with powerful reverse genetic techniques such as RNAi, will allow the nematode system to continue to provide useful insight into conserved biological processes.

10. REFERENCES

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11. APPENDICES

11.1 Appendix I. Selected Reprints

1. Spector MS, Desnoyers S, Hoeppner DJ, Hengartner MO: Interaction between the *C. elegans* cell-death regulators CED-9 and CED-4. *Nature* 1997,**385**:653-656.

2. Gumienny TL, Lambie E, Hartwieg E, Horvitz HR, Hengartner MO: Genetic control of programmed cell death in the *C. elegans* germ line. *Development* 1999, **126**:1011-1022.

3. Gartner A, Milstein S, Ahmed S, Hodgkin J, Hengartner MO: A conserved checkpoint pathway mediates DNA damage–induced apoptosis and cell cycle arrest in *C. elegans*. *Mol Cell* 2000, **5**:435–443.

4. Hengartner MO: The apoptosome: molecular control of apoptosis in the nematode *C. elegans*. In: Pharmaceutical interventions in apoptotic pathways. Nagelkerke JF, van Dierendonck JH, Noteborn MHM, eds., North – Holland, Amsterdam, The Netherlands, 1998. pp. 45-51.

5. Hengartner MO: Programmed Cell Death in the Nematode *C. elegans*. *Recent Prog Hormone Res* 1999, **54**:213-224.

6. Hengartner MO: The biochemistry of apoptosis. *Nature* 2000, **407**:770-776.

11.2 Appendix II. Tables

11.2.1 *Table 1:* ced-9(n1950) *cooperates with* ced-3 *and* ced-4 *mutations in suppressing egl-1(gf)-induced HSN apoptosis and egg-laying deficiency.*

Genotype	Egg-laying defect (%)	n
Wild type	< 0,1	> 10
egl-1(n986) / +	92	16
ced-3(n717)/+; egl-1(n986)/+	85	76
ced-4(n1162)/+; egl-1(n986)/+	94	34
ced-4(n1162)/+; ced-3(n717); egl-1(n986)/+	88	148
ced-9(n1950)/+; egl-1(n986)/+	26	15
ced-4(n1162) ced-9(n1950)/+ +; egl-1(n986)/+	7	83
ced-9(n1950)/+; ced-3(n717)/+; egl-1(n986)/+	2	38
ced-4(n1162) ced-9(n1950)/+ +; ced-3(n717)/+; egl-1(n986)/+	0,6	64

Only relevant genotypes are shown. All strains were also heterozygous for the marker mutations *unc-79(e1068)* and *dpy-17(e164)*.

11.2.2 *Table 2: Phenotypic classes of* egl-1(n1084) *suppressors recovered.*

Phenotypic Class	Gene	Mutations isolated in screen
General Cell Death Suppressors	*ced-3*	*op149, op150, op157, op165, op168, op173, op175, op176*
	ced-4	*op151, op158*
Bypass Suppressor	ND	*op182*
Unstable Suppressors	ND	*op156, op159, op183, op184, op185, op186*
HSN-Specific Suppressors	*egs-1*	*op181*
	egs-2	*op166, op232*
	ND	*op153, op160, op167, op169, op170, op171, op172, op174, op177, op178, op179, op180*

egl-1(gf) suppressors were isolated from a screen of 54,000 haploid genomes in the F1 generation (underlined) and 39,000 haploid genomes in the F2 generation after EMS mutagenesis. 17,000 haploid genomes were screened for recessive suppressors in the F2 generation after ENU mutagenesis. Alleles isolated in this latter screen are indicated by an asterisk. General Cell Death Suppressors, such as mutations in *ced-3* or *ced-4*, block both HSN death and cell death in the anterior pharynx. The bypass suppressor can lay eggs but lacks visible HSN cell bodies in the expected positions. Unstable suppressors are mutations that cannot be maintained as stable non-Egl homozygous strains. HSN-Specific Suppressors are mutations that block *egl-1*-induced HSN death, but do not affect the normal survival of cells in the anterior pharynx. ND, not determined.

11.2.3 *Table 3: HSN-specific suppressors have varying penetrance.*

Class	Genotype	HSN survival (%)	n
Controls	Wild-type	99	90
	egl-1(n1084)	4	90
	ced-3(n2433); egl-1(n1084)	100	30
Strong	*egs-1(op181); egl-1(n1084)*	97	90
	egl-1(n1084); egs-2(op166)	98	90
	egl-1(n1084); egs-2(op232)	98	90
Weak	*op169; egl-1(n1084)*	63	90
	op170; egl-1(n1084)	50	90
	op178; egl-1(n1084)	50	90
	op167; egl-1(n1084)	43	20
	op172; egl-1(n1084)	40	20
	op179; egl-1(n1084)	31	16
	op153; egl-1(n1084)	25	24
	op180; egl-1(n1084)	25	20
	op174; egl-1(n1084)	23	30
	op171; egl-1(n1084)	15	13
	op177; egl-1(n1084)	13	30

The HSN-Specific *egl-1(n1084)* suppressors are arbitrarily divided into strong and weak groups based upon HSN survival. % HSN Survival reflects the frequency with which individual HSN nuclei were identified in each animal. n = the number of animals scored.

11.2.4 *Table 4: Twelve C. elegans clones interact with lexA-CED-9 and with lexA-Bcl-2.*

pGAL4$_{AD}$-Clone	Interaction with				
	lexA-CED-9	lexA-CED-9(gf)	lexA-Bcl-2	lexA-STE11	lexA-Lamin
STE11 (control)	-	-	-	+++	-
A (4x)	+++	+++	+++	-	-
C	+++	+++	+++	-	-
E	+++	+++	+++	-	-
F	++	++	++	-	-
G (2x)	+++	+++	+++	-	-
I (2x)	+++	+++	+++	-	-
J (2x)	+++	+++	+++	-	-
M	+++	+++	+++	-	-
O	++	++	++	-	-
Q	+++	+++	+++	-	-
T2	+++	+++	+++	-	-
T4	+++	+++	+++	-	-

Key: +++, strong signal (as strong as the STE11/STE11 positive control); ++, slightly weaker signal; -, signal not significantly over background. Number in parentheses indicate number of different clones that corresponded to the same gene. In these cases, only one clone from each group was kept for further analysis.

11.2.5 *Table 5: None of the identified CED-9 interactors alters cell death patterns in* C. elegans.

Clone	Gene	LG	RNAi phenotype	Overexpression phenotype	Function/Homology
Controls					
	ced-3	IV	Extra cells in pharynx	Embryonic lethality, increased cell death	Caspases
	ced-4	III	Extra cells in pharynx	Embryonic lethality, increased cell death	Apaf-1
	ced-9	III	Extra germ cell death, embryonic lethality, Po sterility	Extra cells in pharynx	Bcl-2
	egl-1	V	None	Embryonic lethality, increased cell death	BH3 domain protein
Interactors					
A	C05D11.7	III	Embryonic lethality, no change in cell death	None	GS2
C	Y47E8	V	Embryonic lethality	None	SEC23
E	F52B5.1	I	None	None	Anion exchange
F	T22B11.4	IV	None	None	Novel
G	B0034		None	None	Ribosomal protein
I	B0303.4	III	None	None	Novel
J	F56C9.7	III	None	None	Cation transporter
M	H21P03.a	IV	None	None	Novel
O	T07C4.3	III	DTC migration defect	None	Novel
Q	W06A7.3a	V	None	None	Novel
T2	*unc-40*	I	Extra pharyngeal cell	None	*DCC*
T4	C15B12.7	X	None	None	Cobalt uptake

LG, linkage group (chromosome). RNAi phenotype: Po young adults were injected with dsRNA, and replated every 12 hours. Both Po and their F1 progeny were analysed for any phenotype, in particular with respect to cell death. RNAi of our *unc-40* clone T2 resulted at a low frequency in a fate transformation that produced an extra pharyngeal cell. However, bona fide unc-40 mutants, including known null alleles of the gene, did not show this phenotype (data not shown). Overexpression phenotype: Either genomic clones, or in some cases (including all controls) cDNAs under the control of heat shock promoters, were injected into wild-type hermaphrodites with a dominant co-injection marker, and stable transgenic lines tested for any overexpression phenotype.

Overexpression data for *ced-3*, *ced-4*, *ced-9*, and *egl-1* was previously published (Chen et al., 2000; Hengartner and Horvitz, 1994; Shaham and Horvitz, 1996

11.3 Appendix III. Figures

11.3.1 *FIGURE 1 LEGEND*

The *C. elegans* apoptosome: a model for the mechanism of action of the cell death machinery.
Top: *C. elegans*. The cell death regulators CED-3, CED-4, and CED-9 are predicted to be stably associated in a multiprotein complex localised to the outer surface of mitochondria. This complex appears to be present in all cells – at least during embryonic development – but is inactive. In cells fated to die, The BH3 domain protein EGL-1 dislodges CED-4/CED-3 from its CED-9 anchor, leading to oligomerization of the complex and, through poorly characterised steps that might involve ATP hydrolysis, intermolecular processing of CED-3. The active protease then cleaves the relevant apoptotic substrates, bringing on the death of the cell.

Bottom: Conservation of the pathway. Homologs of each key cell death regulator in *C. elegans* also function in the regulation of apoptosis in mammals. However, details of the biochemical interactions between the various proteins has diverged somewhat. In mammals, the apoptosome consists of a complex of Apaf-1, cytochrome c, and caspase-9. While activation of the caspase again brings on the apoptotic demise of the cell, regulation of the apoptosome by the Bcl-2 family is indirect. In mammals, the main function of the Bcl-2 family appears to be to regulate release of cytochrome c (and other apoptogenic factors) from mitochondria (Adams and Cory, 1998; Gross et al., 1999; Hengartner, 2000).

11.3.2 *FIGURE 2 LEGEND*

Monoclonal antibodies raised against CED-9.
Monoclonal antibodies generated by the CSHL cancer monoclonal shared resource were tested against whole-animal extracts of wild-type (WT) or *ced-9(n2812);ced-3(n717)* worms. The *n2812* mutation is a null allele that introduces a stop codon at residue 40 of the open reading frame. The *ced-3* mutation is present in the background of the *ced-9* strain to suppress the lethality conferred by *ced-9(lf)* mutations (Hengartner et al., 1992). The *ced-3* mutation has no effect on the levels of CED-9 proteins (data not shown). Note how each antibody recognizes a single major band, which is absent in the null mutant. Western blotting was performed using standard protocols (Harlow and Lane, 1988).

Bottom panel: Epitope map. the epitope recognized by the three MAbs were crudely mapped by probing various recombinant CED-9 truncation constructs by western blot.

11.3.3 *FIGURE 3 LEGEND*

CED-9 co-localises with the mitochondrial marker MitoTracker Red.
Left: Wild-type embryos were stained with a combination of the three anti-CED-9 MAbs, followed by labelled anti-mouse Abs. Note the punctate staining pattern.

Right: MitoTracker Red staining of the same embryo. Note similarity in staining patterns.

11.3.4 *FIGURE 4 LEGEND*

CED-9 co-fractionates with mitochondria in subcellular fractionation experiments.
Left: principle of the fractionation. Mixed stage worms were lysed using a French press. After an initial centrifugation to clear out excess debris, the extract was submitted to differential centrifugation at 16,000xg. Based on electron micrographic analysis, the pellet of this step contained mostly mitochondria and some nuclei, whereas the supernatant contained microsomes (and cytosol).

Right, western blot analysis of the crude extract and the various fraction. Note how CED-9 fractionates almost exclusively with the mitochondrial fraction. Loading was normalised such that the same amount of protein was loaded on each lane.

11.3.4 *FIGURE 4 LEGEND*

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Right, western blot analysis of the crude extract and the various fraction. Note how CED-9 fractionates almost exclusively with the mitochondrial fraction. Loading was normalised such that the same amount of protein was loaded on each lane.

11.3.5 *FIGURE 5 LEGEND*

A screen for mutations that suppress *egl-1*-induced HSN death.
egl-1(n1084) mutants were exposed to 50 mM EMS or 5 mM ENU, according to standard protocols (Anderson, 1995; De Stasio et al., 1997; Hengartner et al., 1992). L4 larvae and young adult hermaphrodites were allowed to recover, and then transferred to 100 mm plates seeded with *E. coli* strain OP50. The mutagenised Po animals were replated every 8 hours over the course of 3 days. Non-Egl F1 and F2 progeny were recovered, transferred individually to new plates and, if they bred true, kept for further analysis.

11.3.6 *FIGURE 6 LEGEND*

***ced-9(gf)* and *egl-1(lf)* mutations do not affect germ cell apoptosis**
A) *ced-3* and *ced-4* mutations prevent germ cell death. Whereas the number of germ cell corpses gradually increases with age in wild-type hermaphrodites, *ced-3* and *ced-4* mutant animals show almost no germline corpses at any age. Adult males also show no germ cell death at any age. Average corpse numbers are shown with the standard error of the mean (s.e.m.).

B) *ced-9(gf)* and *egl-1(lf)* do not prevent germ cell death. The *ced-1* background, by allowing corpses to persist, amplifies the difference between the wild type and *ced-3(n717)* animals; it does not affect the number of cell deaths (data not shown). Note the log scale of the Y-axis.

C) Cell-specific regulators of somatic cell deaths have no apparent effect in the germ line. Germ cell corpse counts in 24-hour adult *ces-1*, *ces-2*, and *egl-1(gf)* hermaphrodite gonads were similar to those of the wild type and significantly higher than those of *ced-3(n717)* animals. We studied the appearance and number of cell corpses in the germ line of nematodes by mounting animals in a drop of M9 salt solution containing 30 mM NaN_3 and observing the animals using Nomarski optics (Ellis et al., 1991). Germ cell corpses are cellularised and more refractive than syncytial nuclei or oocytes and can readily be identified under high magnification. Average corpse numbers with the standard error of the mean (s.e.m.) were determined by the Statview II program (Abacus Concepts, Incorporated, Berkeley, California).

11.3.7 *FIGURE 7 LEGEND*

DNA damage response pathways in mammals.
Recognition of the damage activates a damage response "checkpoint," which in turn activates DNA repair. In addition, the DNA damage checkpoint activates both cell cycle arrest and apoptosis. These events can be mediated by p53-dependent, as well a p53-independent pathways. During tumor development, mutations can arise at every one of these steps; each type of mutation may have different consequences in different cell types. Adapted from Evan and Littlewood (1998).

11.3.8 *FIGURE 8 LEGEND*

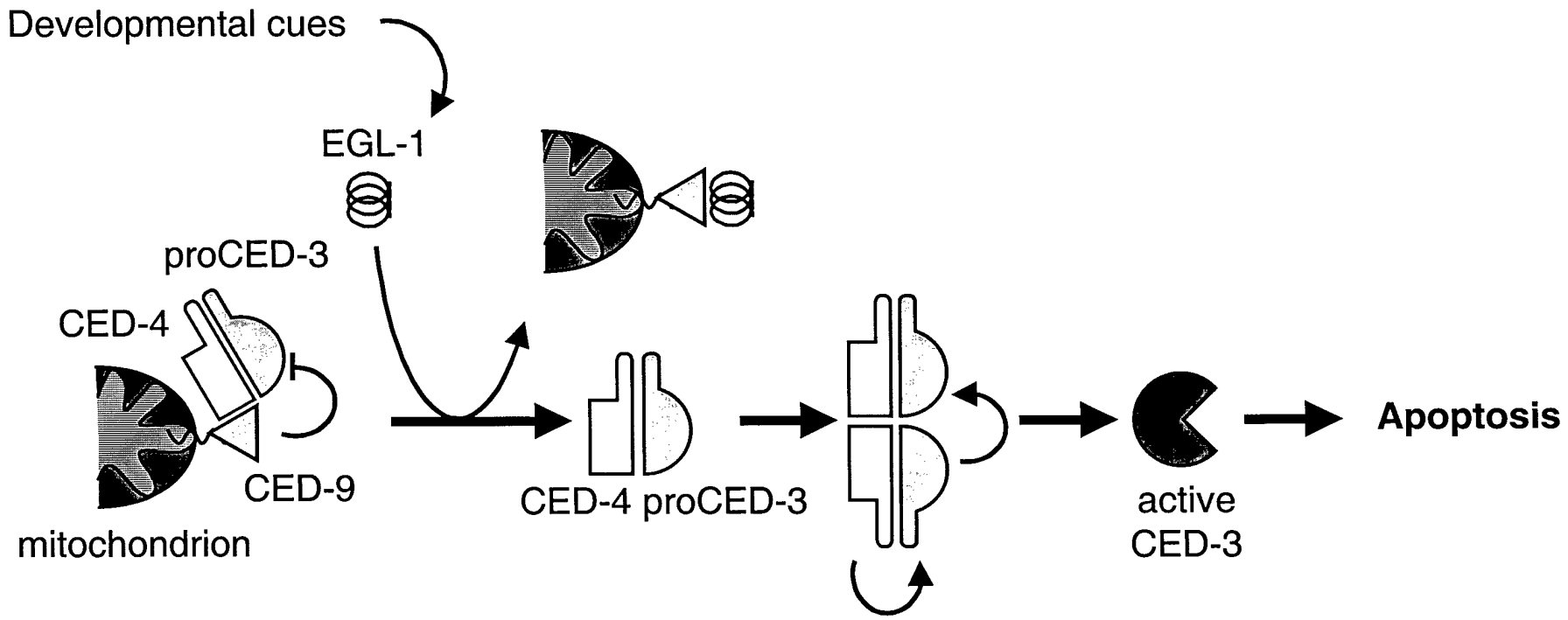
***egl-1* promotes, but is not essential for DNA damage-induced apoptosis.**
Late L4 stage hermaphrodite worms were irradiated with the indicated doses of gamma radiation (^{137}Cs) and scored after 0, 12, 24 and 36 hours. The extent of apoptosis was scored as described in Figure 6. For each time-point, 15 animals were scored for germ cell death in one gonad arm via direct observation using Nomarski optics. Data shown are mean +/- s.e.m. (standard error of the mean).

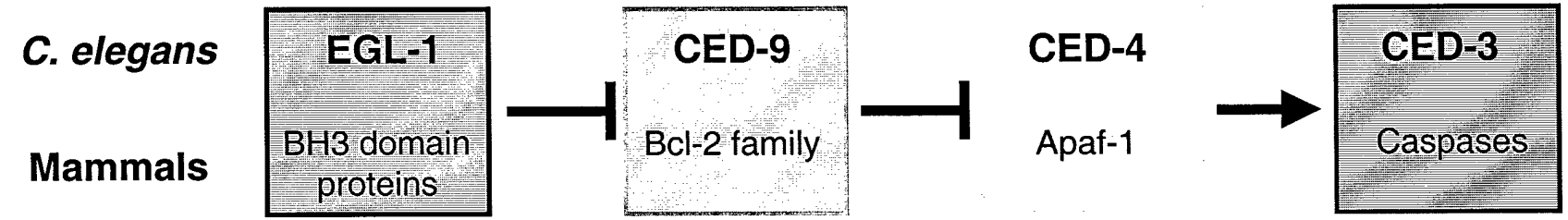
11.3.9 *FIGURE 9 LEGEND*

RNAi interference of *ced-9* results in increased apoptosis.
A) Injection of dsRNA corresponding to *ced-9* results in a large number of germ cell apoptosis (left), whereas injection of dsRNA to an unrelated gene (*bir-1*, right) has no effect on germ cell apoptosis.
B) The number of corpses observed in the germ line of young adult hermaphrodite worms were counted 28 hours after injection with dsRNA corresponding to *ced-9* or an irrelevant gene (*bir-1*), or with water as a control. Values are averages ± standard deviations for at least 10 animals.

Hengartner
Figure 1

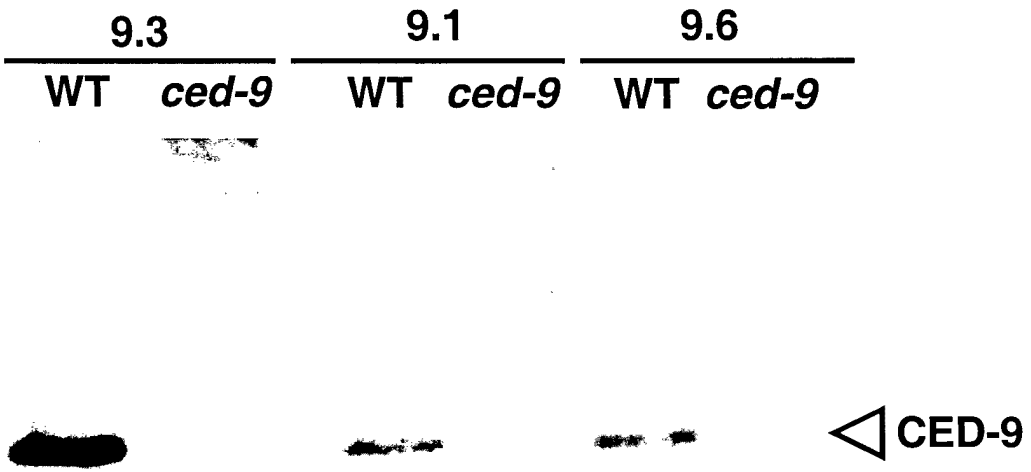
Activation of the cell death machinery during *C. elegans* development: A model





Hengartner
Figure 2

Three Mabs specifically recognize CED-9 on western blots of worm extracts



WT: wild type
***ced-9*:** *ced-9(n2812)* null mutant

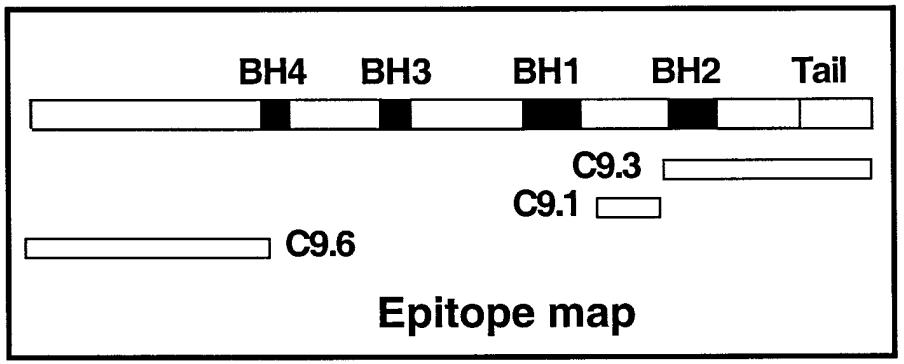


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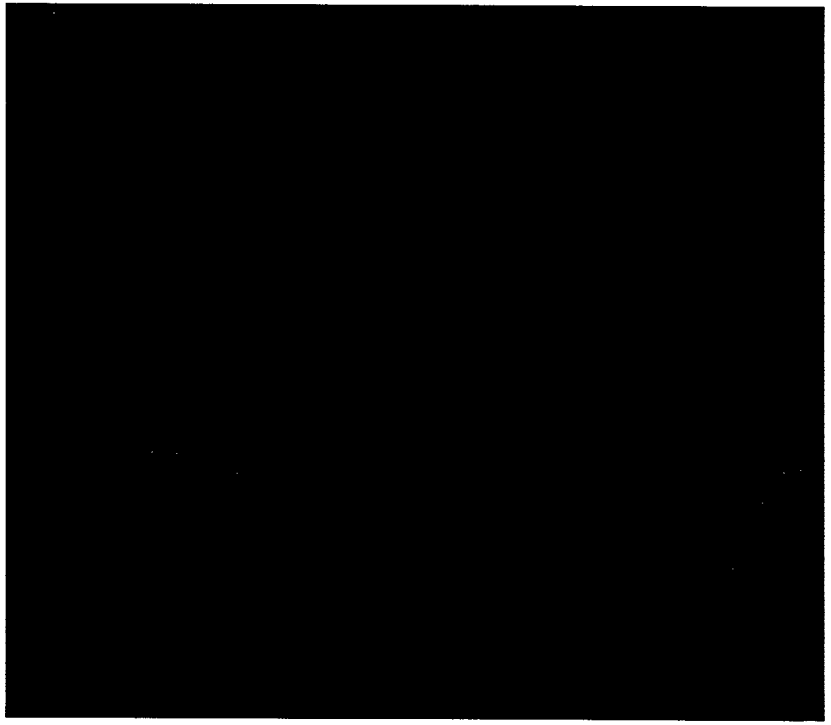
Figure 3

CED-9 localises to mitochondria

antiCED-9

MitoTracker Red
(mitochondrial marker)



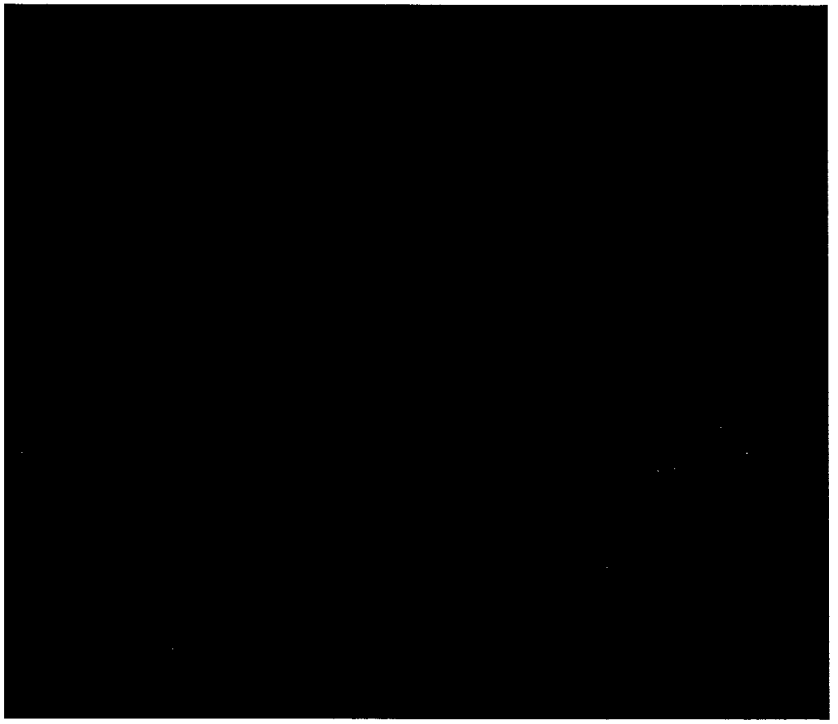


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Figure 4

CED-9 fractionates with the mitochondrial compartment

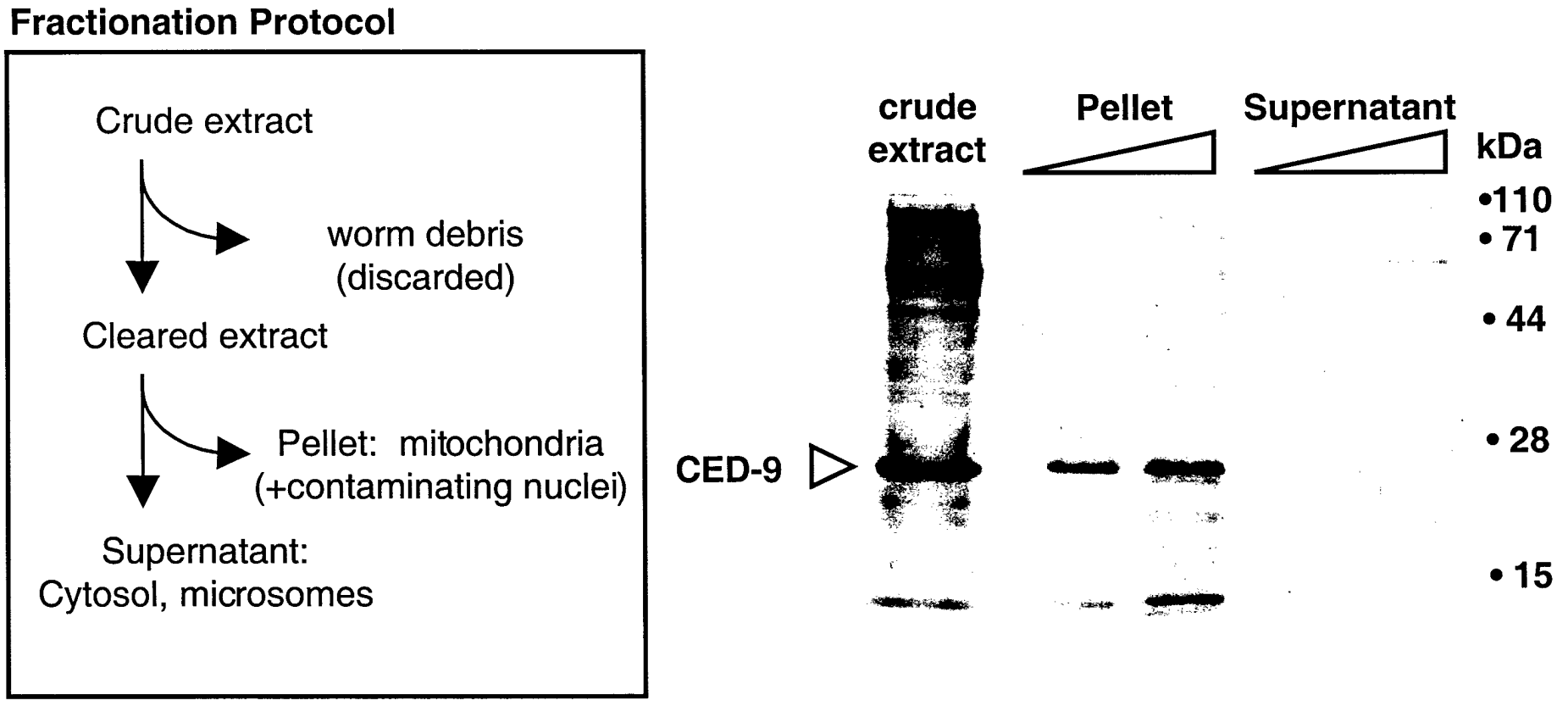

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Figure 5

A genetic screen for *egl-1(gf)* suppressors

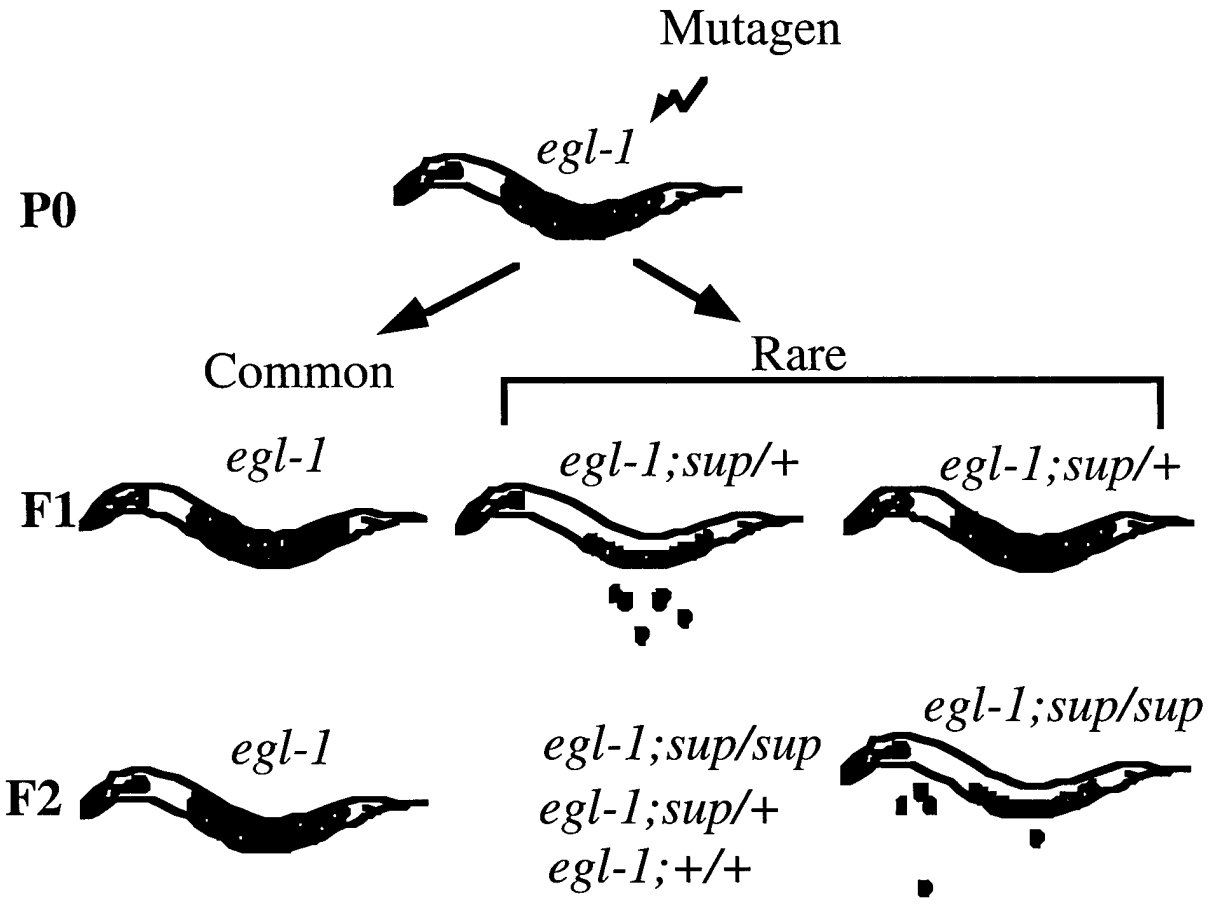

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Figure 6

ced-9(gf) and *egl-1* mutations do not affect germ cell apoptosis

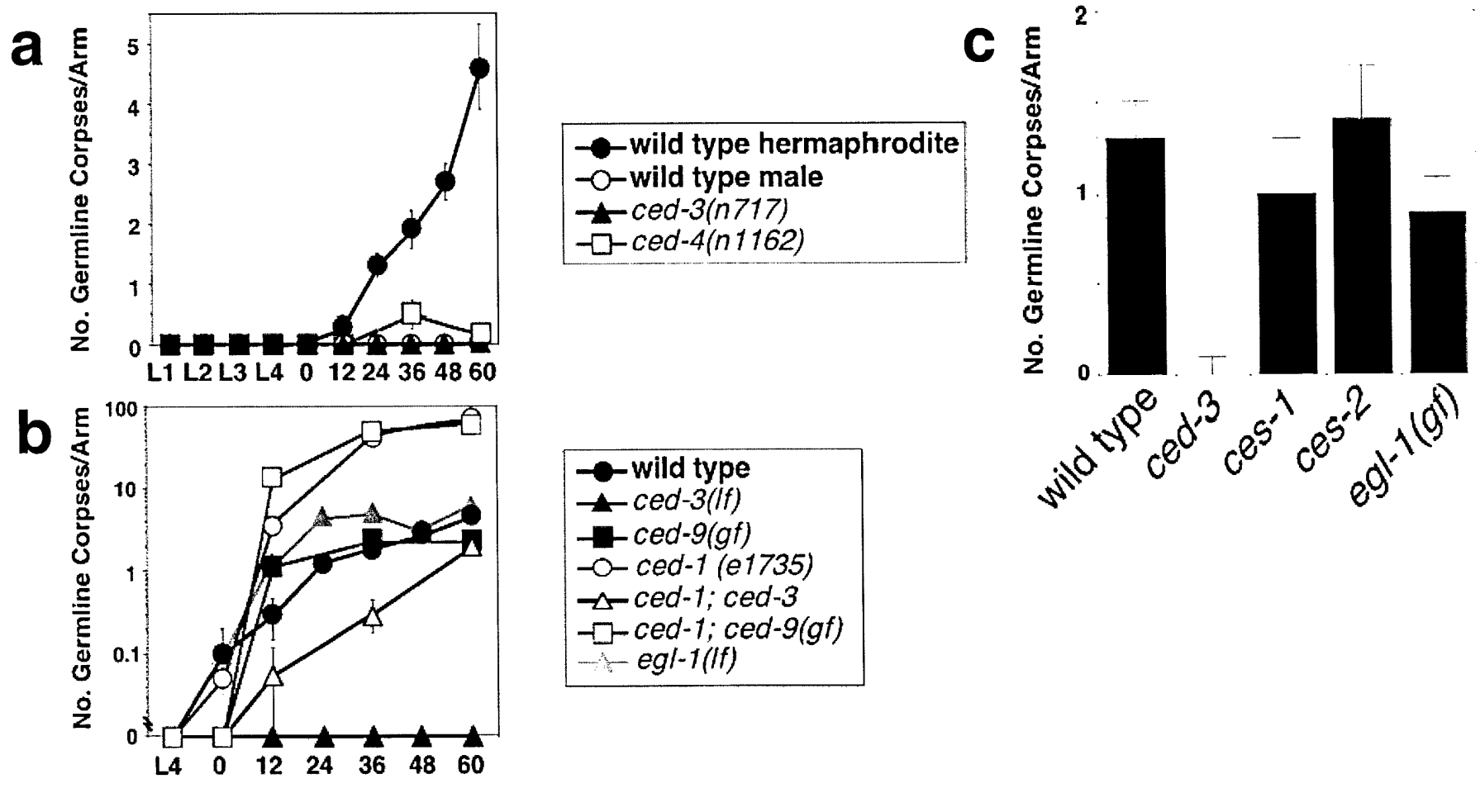

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Figure 7

DNA damage response pathways in mammals

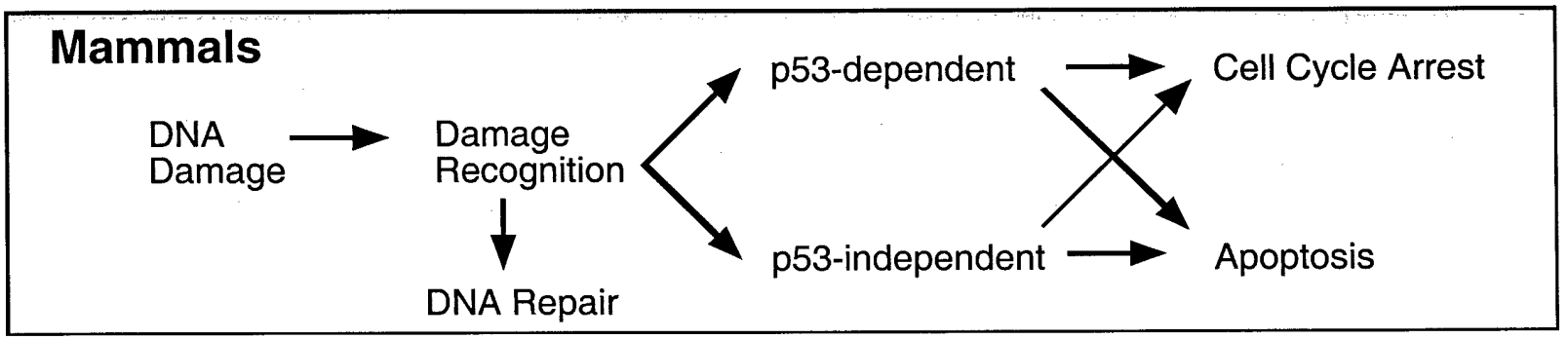

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Figure 8

egl-1 promotes, but is not essential for DNA damage-induced apoptosis

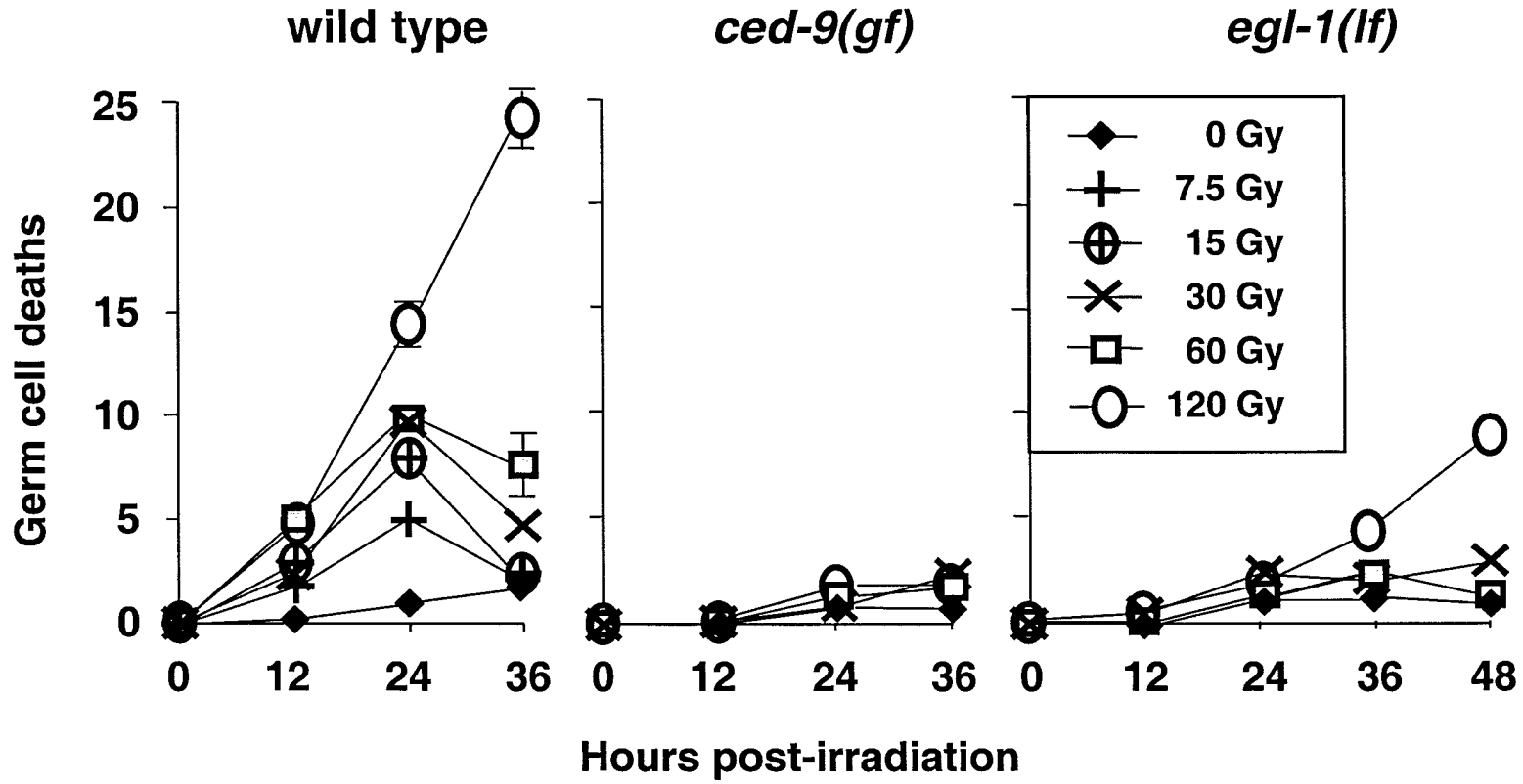

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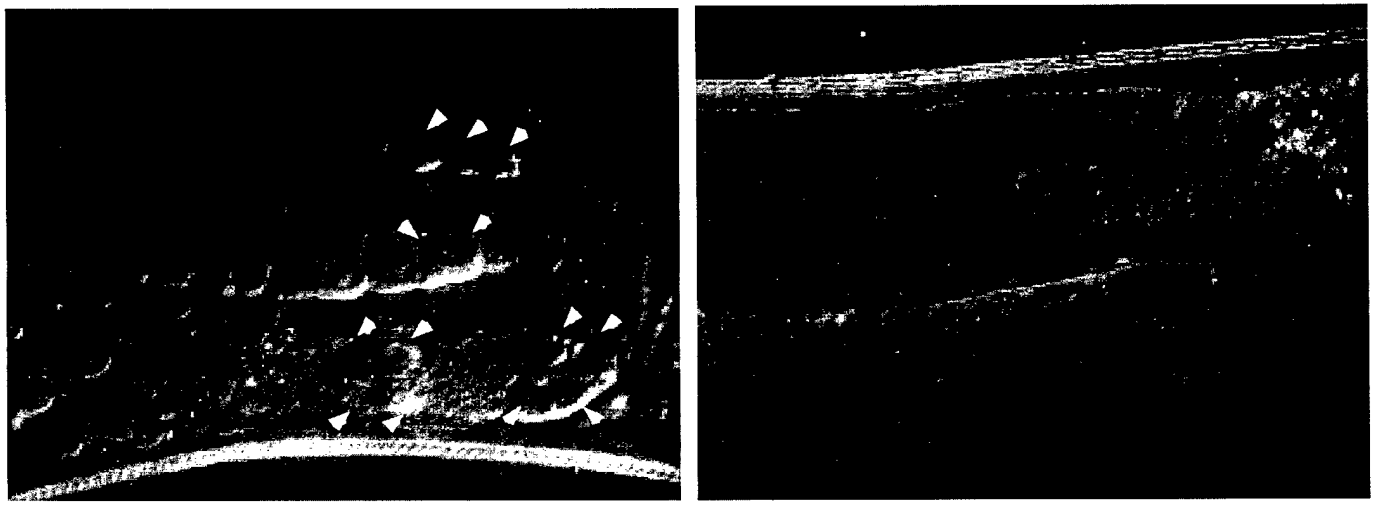
Figure 9

ced-9(RNAi) results in increased apoptosis

a



***ced-9* RNAi** ***bir-1* RNAi**

b