The *C. elegans* p53 pathway

Dissertation

der Fakultät für Biologie

der Ludwig-Maximilians-Universität

München

vorgelegt von

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München 2003

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Tag der mündlichen Prüfung: 29. März 2004

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Statement

I have written this thesis independently, without the help of others. The content of this thesis is solely based on experiments I performed myself, except indicated otherwise.
Part 1.1 to 1.3 of the introduction have been published previously in Gartner A, Alpi A, Schumacher B "Programmed cell death in *C. elegans*" in Genetics of Apoptosis, Grimm S (ed.), BIOS Scientific Publishers Limited, 2003, 155-175. Part 3.1 of the results section has been published in Schumacher B, Hofmann K, Boulton S, Gartner A "The *C. elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis" in *Curr Biol.* 2001 Oct 30;11(21):1722-7. Slight modifications were made where updates were available.

Abstract

The small nematode *C. elegans* has been a useful genetic model organism for studying programmed cell death. Recently, it has been shown that upon DNA damage a conserved checkpoint pathway halts the cell cycle to allow the damage to be repaired and initiates apoptosis in *C. elegans*. This checkpoint pathway has previously been characterized mainly through yeast genetics. Yeast, however, only invokes cell cycle arrest, as it does not have programmed cell death. The discovery of an apoptotic response to DNA damage in *C. elegans* allows using this genetic model system to study the link between checkpoint signaling and programmed cell death.

In human the tumor suppressor p53 is a major regulator of DNA damage response. p53 is a transcription factor that halts the cell cycle and induces cell death upon DNA damage. p53 is the most frequently mutated gene in human cancer. Here, we will show that *C. elegans* possesses a functional p53 homolog. *C. elegans* p53 (*cep-1*) is specifically required for the induction of apoptosis upon DNA damage but dispensable for cell cycle arrest. Furthermore, CEP-1 is a transcription factor that induces the BH3 only genes *egl-1* and *ced-13*. Both of these BH3 only proteins can trigger DNA damage induced cell death. We suggest that triggering programmed cell death through induction of BH3 only proteins defines an ancient p53 pathway that is conserved from worm to mammals.

To genetically identify novel factors involved in the p53 apoptotic pathway a genetic screen was performed. We screened for negative regulators of the p53 pathway. We identified a novel allele of the germ line tumor suppressor *gld-1* (germ line defective). GLD-1 has previously been shown to be an mRNA binding protein of the GSG/STAR family, which translationally represses target mRNAs. Here, we show that this novel allele *gld-1(op236)* leads to an enhancement of p53 dependent apoptotic signaling. Apoptosis in *gld-1(op236)* is dependent on the *cep-1* and *egl-1*. Furthermore, we show that GLD-1 directly binds to *cep-1* mRNA. In conclusion, we postulate that GLD-1 might regulate CEP-1 through translational repression, thus defining a novel regulatory mechanism of p53 signaling.

List of abbreviations

C. elegans: *Caenorhabditis elegans*

ced: cell death abnormality

cep: *C. elegans* p53 like

egl: egg laying defective

gld: germ line defective

RNAi: RNA interference

gf: gain-of-function

lf: loss-of-function

GSG/STAR family: GRP33/Sam68/GLD-1 / Signal Transduction and Activation of RNA family

KH motif: ribonucleotide K Homology motif

BH: Bcl-2 Homology

Gy: Gray

IR: ionizing radiation

DIC: differential interference contrast

HSN: hermaphrodite-specific neuron

NSM: neurosecretory motor neuron

NGM: nematode growth media

1 Introduction

First, a brief overview of the basic mechanisms of programmed cell death will be given. Secondly, we will elaborate on the role of the nematode worm *C. elegans* as a model system to study apoptosis. We then focus on aspects of DNA damage induced apoptosis in the model system. Finally, we will describe the regulation of the tumor suppressor p53 as the *C. elegans* p53 pathway is the main focus of this thesis.

1.1 Programmed cell death

Programmed cell death, or apoptosis, describes the process of genetically controlled cellular suicide. Upon induction of the apoptotic pathway cells activate caspases, which disassemble cellular components. Dying cells are then engulfed by neighboring cells or macrophages to avoid any harmful effect that the release of intracellular components might have on the organism. In humans programmed cell death is essential during development for shaping organs and tissues. In the adult organism apoptosis plays a major role in human diseases such as autoimmune diseases but also neurodegenerative diseases such as Parkinson and Alzheimer's where cells die that are not supposed to die, and cancer, where cells fail to die when they are supposed to die.

Programmed cell death in humans is an extraordinary complex process and only a brief outline of the basic principles will be given here. The effectors of cell death, the cysteinyl aspartate proteases (caspases) can be divided into effector caspases, which disassemble cellular components and activator caspases, which activate effector caspases through induced proximity (for review (Thornberry and Lazebnik, 1998)). There are more than a dozen caspases identified to date some of which are tissue specific and some of which function redundantly. Caspases can be activated essentially by two different mechanisms: either through a receptor mediated or through the mitochondrial pathway. The initiator Caspase-8 is activated by induced proximity when death receptors such as Fas oligomerize through their death domains and bind relay molecules through death effector domains (for review (Curtin and Cotter, 2003)). Caspase-8 then cleaves and thereby activates the effector Caspase-3. The second pathway acts through the mitochondria, where the multidomain Bcl-2 homology (BH) family proteins Bax and Bak release

cytochrome c from the mitochondria, which together with Apaf-1, leads to the activation of Caspase-9 (Li et al., 1997). Caspase-9 then activates effector caspases such as Caspase-3 (Zou et al., 1997). Bax and Bak are activated when their inhibitor, anti-apoptotic Bcl-2 is sequestered by the pro-apoptotic BH3 only family proteins such as Bad, Bim, Bid, Noxa or Puma (Yang et al., 1995; Wang et al., 1996; O'Connor et al., 1998; Wu and Deng, 2002; Nakano and Vousden, 2001; Yu et al., 2001). The BH3 only family of apoptotic triggers will be discussed in more detail later in this section. The receptor mediated pathway is linked to the mitochondria by the BH3 only protein Bid, which, when cleaved and activated by Caspase-8, induces the mitochondrial cell death pathway (Li et al., 1998; Luo et al., 1998; Zha et al., 2000)

1.2 Programmed cell death in *C. elegans*

The genetic model organism *C. elegans* has been crucial for understanding the apoptotic machinery. Apoptosis, as a genetically controlled process, has first been described in the nematode worm (Sulston and Horvitz, 1977). Genetic studies of programmed cell death in *C. elegans* take advantage of its highly reproducible and invariant somatic development. This reproducibility allowed the precise elucidation of the cell lineage that leads, within a time frame of approximately 3 days, to the generation of all of the 959 somatic cells that make up the adult hermaphrodite worm (Figure 1). Following embryonic and post-embryonic cell division patterns by lineage analysis revealed that during somatic development of the hermaphrodite worm, 131 out of the total of 1090 cells born undergo programmed cell death (Sulston and Horvitz, 1977; Sulston et al., 1983; Kimble and Hirsh, 1979). As is true for most other aspects of *C. elegans* development, these deaths show a high degree of uniformity with respect to the identity of dying cells as well as with respect to the timing of each of these cell deaths during development. Although the apoptotic fate is neither restricted to particular cell types nor to any particular cell lineage, the bulk of apoptotic events affect neuronal cells and to some extent also hypodermal cells (Sulston and Horvitz, 1977; Sulston et al., 1983; Kimble and Hirsh, 1979). The reproducibility of cell death provides unique advantages for genetic analysis: cell death can be studied on a single cell level and even mutations which cause only a very weak defect in programmed cell death or which affect only a small number of cell types can be identified. Programmed cell death of somatic cells is mostly used during embryonic development (113/131 deaths) which takes place within approximately 14 hours under standard growth conditions. During embryogenesis, apoptosis occurs mostly between 220 and 440 minutes after fertilization (Sulston et al., 1983). Programmed cell death further occurs, albeit to a lesser extent during the transition through the four larval stages (Sulston and Horvitz, 1977; Sulston et al., 1983; Kimble and Hirsh, 1979). Within the somatic tissues of the adult worm neither cell divisions nor programmed cell deaths can be observed. However, recent studies suggest that apoptosis is very prominent in adult hermaphrodites during female germ cell development (Gumienny et al., 1999).

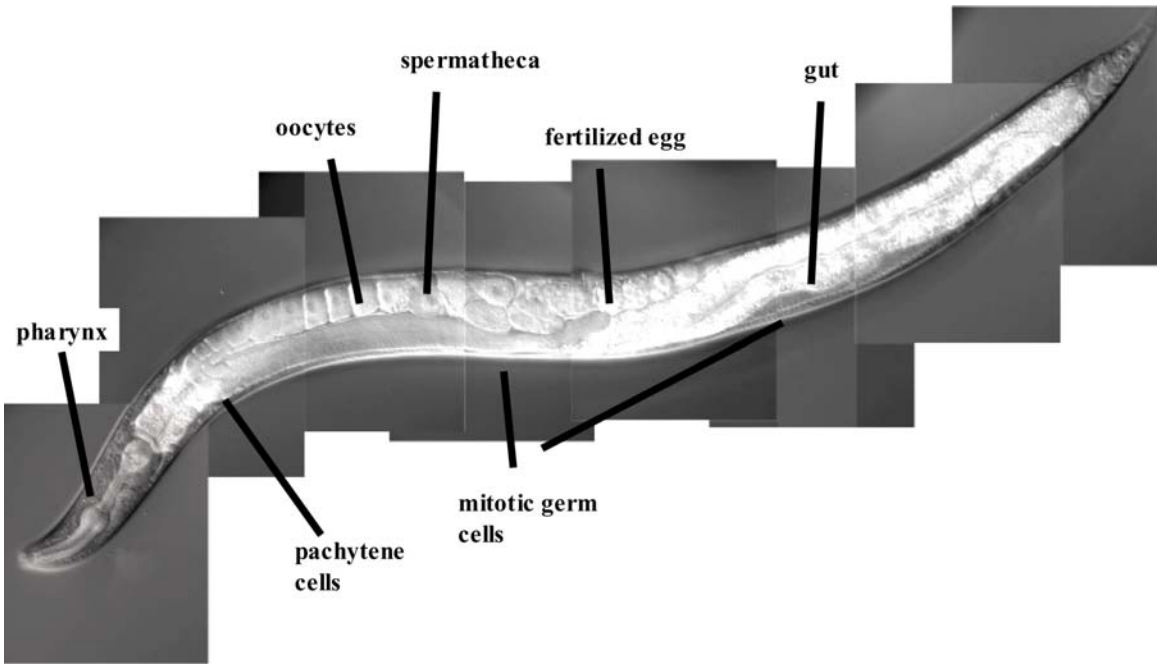


Figure 1 Adult *C. elegans* hermaphrodite. Adult hermaphrodite as observed under DIC optics. Adult worm measure about 1 mm in length. Various tissues are indicated.

Only quite recently, early observations suggesting that programmed cell death can occur in the *C. elegans* germ line were followed up (Gumienny et al., 1999). The *C. elegans* germ line proliferates both during larval development and in adult worms. Indeed, the germ line is the only proliferative tissue in the adult worm. In contrast to the almost invariant somatic *C. elegans* development, germ line development is much more malleable. As in mammalian tissues, populations of germ cells respond to growth factor regulation and are subject to stochastic events, including the elimination of many cells by programmed cell death, to maintain tissue homeostasis. Furthermore, as it occurs in mammalian tissues, *C. elegans* germ cells but not somatic cells are able to respond to environmental stress as genotoxic stress or to the stress conferred by bacterial infections with programmed cell death (see below). The only cell type within the germ line that are capable of undergoing programmed cell death are cells in the meiotic pachytene stage (Gumienny et al., 1999; Gartner et al., 2000). Within the adult *C. elegans* hermaphrodite ovotestis, germ cells are actually only partially surrounded by a plasma membrane and

are therefore part of a syncytium (Seydoux and Schedl, 2001; Hall et al., 1999). According to convention these partially enclosed nuclei are referred to as cells. Within the germ line, germ cells progress through various stages of differentiation (Figure 1). At the distal end of the U-shaped ovotestis mitotic precursor-cells are generated throughout the worm's life. Upon passage through the transition zone, germ cells cease to divide and begin meiosis. The most abundant population of meiotic cells is in pachytene stage of meiosis I and resides between the transition zone and the bend of the gonad. Upon exit from pachytene, germ cells progress into meiotic diplotene, cellularize, undergo the final stages of oogenesis, and finish meiosis after fertilization upon passage through the spermatheca (Seydoux and Schedl, 2001; Hall et al., 1999)(Figure 1). Under normal growth conditions up to 50 percent of female germ cells are fated to die by programmed cell death (Gumienny et al., 1999). These cells are eliminated rapidly by the corpse engulfment machinery resulting in a steady state level of 0 to 4 morphologically apoptotic cells (Gumienny et al., 1999).

Identification and quantification of programmed cell death in *C. elegans*.

Cell death in *C. elegans* can be readily observed in living animals using standard Normasky optics (Sulston and Horvitz, 1977). The first sign of impending death is a decrease in the refractivity of the cytoplasm that occurs concomitant with a slight increase in refractivity of the nucleus. Soon thereafter, both nucleus and cytoplasm become increasingly refractile until they resemble a flat round disk. After about 10-30 minutes this flat disk starts to disappear, the nucleus of the dying cell decreases in refractility, begins to appear crumpled and finally vanishes within less than 1 hour (Figure 2a) (Sulston and Horvitz, 1977). The morphology of corpses as well as the kinetics of their disappearance is similar between somatic and germ cell apoptosis (Figure 2a). However, as germ cells are only partially surrounded by a plasma membrane the first step in germ cell death is the full cellularization of the apoptotic cell (Gumienny et al., 1999).

Three distinct but related approaches have been used to quantify programmed cell death in *C. elegans*. The first, and most direct approach, takes advantage of the highly reproducible anatomy of the worm, which allows the unambiguous identification of each

cell in the body. The absence of programmed cell death can thus be scored indirectly by looking for the presence of extra "undead" cells (cells that should have died but instead survived). Undead cells cannot be distinguished from normal cells by their appearance under the microscope, but sometimes by their location (e.g., their presence at a position where no cell is normally found). To increase the reliability of the assay, scoring of undead cells is usually performed in the pharynx, the animal's feeding organ, which is separated from the rest of the body by a clearly visible basement membrane (Figure 1). Within the pharynx a large number of programmed cell deaths occurs, and thus a large number of deaths can be scored in a single animal, allowing the detection of even very weak effects on cell death (e.g., less than 2% extra cell survival) (Hengartner et al., 1992; Hengartner and Horvitz, 1994a). The drawback of this approach is that it scores the presence of cells that should not be there, rather than deaths *per se*. Consequently, care must be taken to confirm that extra cells are indeed the result of inhibition of death, rather than of extra cell divisions, or of aberrant cell migrations.

The second approach takes advantage of mutations in genes required for the efficient engulfment of apoptotic cells. In these mutants, cells still die, but many dying cells fail to be engulfed and removed from the animal. These persistent, un-degraded cell corpses are very obvious, even to the worm neophyte, and thus can be used as a simple assay for the extent of programmed cell death in the animal (Ellis et al., 1991) (Vaux et al., 1992). Elimination of programmed cell death results in the absence of persistent cell corpses in these mutants. The main advantage of this assay is its ease of scoring. However, the number of persistent cell corpses is more variable than the number of surviving cells, and weak effects on cell death cannot be detected with this method.

The third method to determine programmed cell death is to identify dying cells by their distinct morphology under DIC (differential interference contrast) optics (Figure 2a). For studying programmed cell death during somatic development this approach is tedious as only few animals can be followed at a certain time and only animals at the proper stage of development yield useful information. In contrast, cell death occurring in the hermaphrodite germ line can be readily followed under DIC optics (Gartner et al., 2000; Gumienny et al., 1999) (Figure 2a). Furthermore, apoptotic corpses can be visualized in living animals by staining with dyes like acridine-orange (Gumienny et al.,

1999). Indeed, this staining method allows for the detection of apoptotic corpses with standard fluorescence stereomicroscopes enabling a pair of trained eyes to screen for the presence of absence of apoptotic corpses in a population of hundreds of worms within a few minutes.

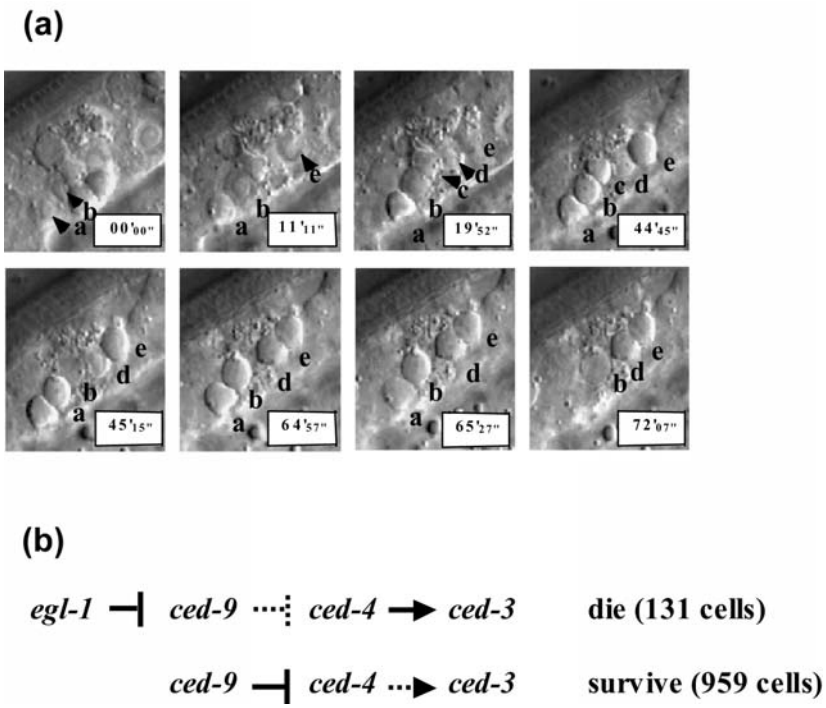


Figure 2 (a) Morphology of programmed cell deaths occurring in the germ line. Adapted from (Gartner et al., 2000). Various morphological phases of apoptosis under DIC optics are depicted. Note, "a" indicates a normal cell at 0 minutes, which becomes refractile at 11 minutes (the nucleus is still discernable at this stage). At 12 minutes the nuclear structure melts with the surrounding cytoplasm and this corpse like structure persists till ca. 65 minutes and disappears within less than a minute. Corpse morphology during somatic development resembles germ cell corpse morphology (Gumienny et al., 1999).
(b) Genetic pathway for programmed cell death. In the upper lane the status of cell death signaling as consequences of *egl-1* activation is shown. In the lower panel the status of signaling in surviving cells is indicated. Solid arrows and solid T-bars indicate activation and repression, respectively. Dotted arrows and T-bars indicate that activation and repression do not occur**.**

Mutants define four distinct steps in the core apoptotic pathway

Genetic analysis has lead to the identification of over 100 different mutations that affect programmed cell death. These mutations define more than 15 genes that affect all

programmed cell deaths and a smaller number of genes that are needed to commit specific cells to the apoptotic fate (Figures 2b, 3). Since mutants which are defective in all programmed cell deaths are viable and show no obvious defect in their development or adult behavior, it was easy to combine various double mutant combinations to build a genetic pathway for programmed cell death (Desai et al., 1988) (Ellis et al., 1991; Ellis and Horvitz, 1991; Hedgecock et al., 1983; Hengartner et al., 1992). Indeed, one of the future challenges in the *C. elegans* cell death field will be the identification of essential genes that also affect programmed cell death.

Apoptosis can be separated into four distinct steps, each of which is defined by the analysis of various mutants (Figure 3). 1) Initially, specific cell types are committed to the apoptotic fate. Subsequently, 2) the general apoptotic machinery, which is used in all dying cells, is activated. Later, 3) the recognition and engulfment of dying cells by a neighboring cell proceeds and finally 4) the remnants of engulfed cells are degraded. Genes of the first type generally affect very few cells or cell types, whereas the genes falling in to the subsequent classes affect all cell deaths. These genes will be described below.

Four genes, *egl-1*, *ced-3*, *ced-4* and *ced-9* define a genetic pathway needed for (almost) all programmed cell deaths

The original isolation of mutants defective in all cell deaths during nematode development by Ellis and Horvitz (1986) was successful because all cells that are fated to die use a common pathway to execute programmed cell death (Ellis and Horvitz, 1986) (Figure 2b, 3). Searching for the desired mutants was simplified by screening in a genetic background where dead cells are easily recognized because they cannot be engulfed by neighboring cells. Later screens also focused on the identification of cell death mutants with extra undead cells (Ellis and Horvitz, 1991; Hengartner et al., 1992). As a result these screens, initially numerous loss-of-function (lf) alleles of both *ced-3* (cell death abnormality), *ced-4* and a single gain-of-function (gf) allele of *ced-9* were identified (Figure 2b) (Ellis and Horvitz, 1991; Hengartner et al., 1992) The loss of function alleles of *ced-3* and *ced-4* can be classified into allelic series, the strongest alleles of which presumably result in a complete loss of gene function that leads to the complete inhibition

of all programmed cell death. “Undead cells” that fail to die tend to acquire a fate similar to their ancestors but interestingly they lose the capacity to further divide.

The isolation of *ced-9* loss-of-function mutants was accomplished by looking for intragenic revertants of a rare *ced-9* gain-of-function allele (Hengartner et al., 1992). The analysis of these revertants revealed extensive apoptosis of cells that normally do not die within homozygote *ced-9* (lf) animals. As a consequence of these extensive deaths the affected animals die during embryogenesis (Hengartner et al., 1992). These observations suggest that *ced-9* normally functions to prevent programmed cell death (Figures 2b,3). Genetically, *ced-9* acts upstream of both *ced-3* and *ced-4,* as loss-of-function alleles of these genes are able to suppress the extensive cell death phenotype of *ced-9* (lf) animals (Hengartner et al., 1992) (Figures 2b,3). Interestingly embryos resulting from homozygous strong *ced-9* (lf) mutants can die at a very early developmental stage without any overt morphological indication of programmed cell death suggesting that *ced-9* might have an essential function in addition to its anti-apoptotic role (Hengartner et al., 1992). The fourth gene that affects all developmental cell death but evaded discovery in the initial genetic screens is *egl-1* (egg laying defective) (Conradt and Horvitz, 1998)(Figure 3). Interestingly, it turned out that *egl-1* (lf) alleles are defective in all programmed cell deaths occurring during somatic development. *egl-1* function was determined to be upstream of the other known cell death components due to the suppression of programmed cell death caused by the ectopic overexpression of *egl-1* in *ced-3*, *ced-4* and *ced-9* (gain-of-function) mutants (Conradt and Horvitz, 1998; Conradt and Horvitz, 1999).

Mosaic analysis suggests that *ced-3* and *ced-4* are likely to act cell autonomously indicating that they are needed to act within the cells that die (Yuan and Horvitz, 1990). This conclusion is further supported by experiments showing that *ced-3* and *ced-4* overexpression, in neuronal cells that usually do not die, is sufficient to induce programmed cell deaths (Shaham and Horvitz, 1996). In addition, these studies indicated that *ced-3* is likely to function downstream of *ced-4* because the induction of apoptosis by the overexpression of *ced-3* does not require *ced-4*, whereas in the converse experiment the induction of programmed cell death by *ced-4* overexpression requires the presence of *ced-3* (Shaham and Horvitz, 1996) (Figure 2b).

Several paradigms of our general understanding of programmed cell death could be made based on the studies described above. 1) The execution of programmed cell death during development - which formally may also be considered as a somewhat unusual terminal differentiation program - is subject to precise genetic control. 2) The execution of the apoptotic fate is an active program that can be best described as cellular suicide. In other words, a cell that senses that it is to die actively participates in this process by inducing (or helping to induce) its own demise. 3) Finally, every single cell may have the potential to undergo programmed cell death.

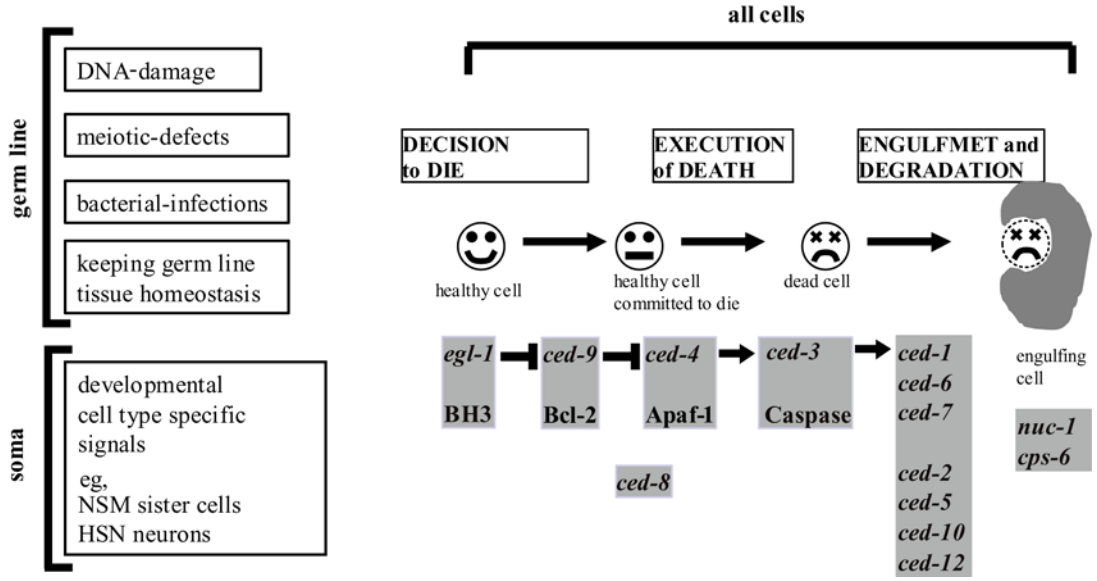


Figure 3 The genetic pathway of programmed cell death in *C. elegans*. Various developmental and environmental clues that can activate programmed cell death are indicated in the left panels. Arrows indicate activation, T-bars indicate repression

egl-1, ced-3, ced-4 and *ced-9* are functionally conserved throughout evolution

Positional cloning of the above-mentioned genes revealed that all these nematode cell death genes have homologs in mammals that perform similar functions in the control of apoptosis. c*ed-9* encodes a protein sharing 24% overall sequence identity with the mammalian bcl-2 protooncogene, which like *ced-9* negatively regulates cell death (Hengartner and Horvitz, 1994b). Similarly to *ced-9,* overexpression of bcl-2 protects cells from death, whereas bcl-2 (lf) mutations make cells hypersensitive to death inducing

signals (for reviews see (Ferri and Kroemer, 2001; Huang, 2000)). *ced-3* encodes a protein with similarity to a family of death inducing proteases called caspases (Yuan et al., 1993). CED-4 is related to mammalian Apaf-1, which in mammals acts together with Caspase-9, Cytochrome c and Caspase-3 in the core apoptotic program (Li et al., 1997; Zou et al., 1997; Yuan and Horvitz, 1992). Finally EGL-1 is related to mammalian BH3 only domain like for instance Bid, Bad, Bim or Puma all of which are implicated in proapoptotic signaling (Conradt and Horvitz, 1998; Yuan and Horvitz, 1992; Yang et al., 1995; Wang et al., 1996; O'Connor et al., 1998; Wu and Deng, 2002; Nakano and Vousden, 2001; Yu et al., 2001).

Interestingly, for all of the before mentioned *C. elegans* cell death genes there is only one functional family member encoded within the worm genome. *C. elegans* contains only one CED-9/Bcl-2 like protein and only one CED-4/Apaf-1 look like. In contrast, *C. elegans* contains besides CED-3, two additional caspases that do not seem to function in cell death regulation (The C.elegans Sequencing Consortium, 1998; Shaham, 1998). Besides EGL-1 there is one additional protein encoded in the worm genome containing a BH3 sequence motive (Genome Sequence of the nematode *C. elegans*, 1998). This protein CED-13 was subject of this thesis (see below). We can show that CED-13, in concert with EGL-1 triggers apoptosis following DNA damage. It is currently still unknown whether Cyctochrome c and its release from mitochondria may play a role in *C. elegans* cell death regulation. The fact that there are two Cyctochrome c copies encoded in the worm genome and the fact that Cyctochome c is an essential gene hindered genetic approaches to address the issue of a potential proapoptotic function of Cyctochrome c in worm cell death signaling. There has been a recent claim that a *C. elegans* homolog of mammalian AIF (apoptosis inducing factor) *wah-1* was implicated in worm programmed cell death (Wang et al., 2002). *C. elegans* also encodes two homologs of the IAP (inhibitor of apoptosis) protein family (Ishioka et al., 1993; Speliotes et al., 2000). These proteins that were initially found as baculovirus proteins able to prevent cell death of host cells are also implicated as anti-apoptotic proteins in *Drosphila*. However, the two *C. elegans* IAP proteins do not seem to play a role in the regulation of programmed cell death. Indeed *C. elegans* BIR-1 is involved in the regulation of cytokinesis and chromosome segregation similarly its human homolog survivin (Ishioka et al., 1993;

Speliotes et al., 2000; Kallio et al., 2001; Honda et al., 2003). Bloss et al. recently reported a CED-3-independent cell death pathway in *C. elegans* that requires CED-4 and is repressed by inhibitor of cell death-1 (ICD-1) (Bloss et al., 2003). In addition, the *C. elegans* genome does not encode any of the known components required for mammalian receptor mediated cell death signaling. In summary, based on the finding that *C. elegans* has only one component each of the core apoptotic pathway *C. elegans* might contain the evolutionarily most simple cell death pathway. Genome-sequencing and extensive EST projects of protozoa or sponges will reveal whether these organisms also contain those four components of the core apoptotic pathway. It is well possible that there are further, not-yet identified components of the core worm programmed cell death pathway. These putative genes might have eluded discovery because they might be essential or because they might act in redundant pathways.

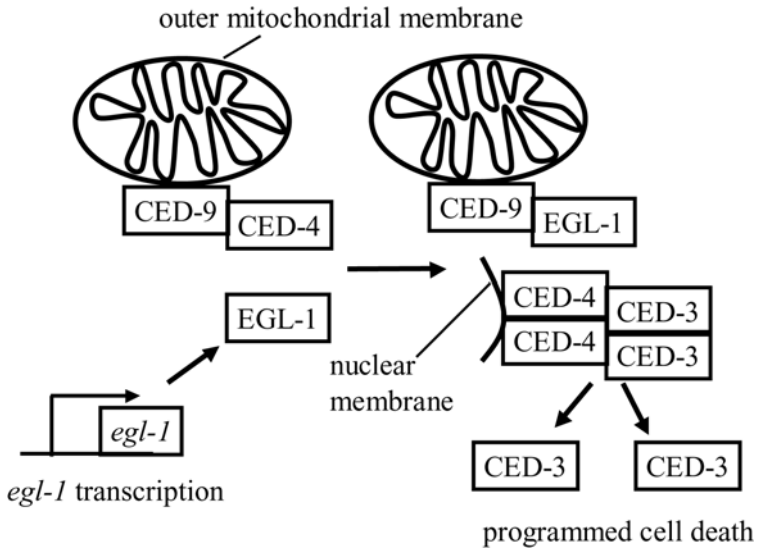


Figure 4 A molecular model for the activation of the core cell death program. The corresponding model is described within the text

EGL-1, CED-3, CED-4 and CED-9 are part of a molecular framework regulating programmed cell death

Biochemical studies indicate that the EGL-1, CED-9, CED-4 and CED-3 are part of a molecular pathway that regulates programmed cell death. The pattern of interactions between those proteins suggests a relatively simple molecular model (Figure 4). In cells that are supposed to survive the pro-apoptotic activity of CED-4 and CED-3 is kept at bay by the binding of CED-4 to CED-9. CED-9 is localized at the outer mitochondrial membrane (Chen et al., 2000). At present it is unclear whether CED-9 localization at the mitochondrial membrane is indeed necessary for the anti-apoptotic CED-9 activity. Likewise it is unclear whether permeabilization of the outer mitochondrial membrane is necessary for the induction of programmed cell death as it is the case in mammalian cells (Hengartner, 2000). According to the model apoptosis is initiated, at least during the execution of programmed cell death during development, by the transcriptional induction of the BH3-only domain protein EGL-1 (Conradt and Horvitz, 1998; Conradt and Horvitz, 1999). Upon transcriptional induction EGL-1 is proposed to bind to CED-9 and thereby releasing CED-9 bound CED-4 (Conradt and Horvitz, 1998; Parrish et al., 2000; del Peso et al., 2000). The above model is supported by the *in vitro* interaction patters of those proteins as well as by the finding that *egl-1* is transcriptionally induced in many cells that are destined to die (Conradt and Horvitz, 1999). It should be noted that models describing *C. elegans* and mammalian cell death activation differ with respect to CED-9 - CED-4 interaction as there is mounting evidence that the homologs of those two proteins Bcl-2 and Apaf-1 do not directly interact *in vivo* (Conus et al., 2000). In addition, the sequence of protein function is supported by the fact that both CED-3 as well as CED-4 overexpression is able to trigger programmed cell death in *C. elegans* as well as upon transfection into mammalian cells (Shaham and Horvitz, 1996; Chinnaiyan et al., 1997a; Chinnaiyan et al., 1997b; Wu et al., 1997). Upon being liberated from CED-9 CED-4 translocates to the nuclear membrane *in vivo* (Chen et al., 2000). This translocation can be induced by *egl-1* overexpression and precedes the activation of programmed cell death and might therefore be an integral part of cell death activation (Chen et al., 2000). Subsequently, CED-4 is thought to confer CED-3 activation by its association into a homo-polymeric complex which is needed to bring inactive CED-3 molecules into close proximity (via CED-4 binding) and thereby leading to the activation of programmed cell death via CED-3 caspase activation (Yang et al., 1998).

Modulators and downstream components of the cell death pathway

Very little is known about components that modulate the cell death pathway or about targets of the cell death pathway. *ced-8* causes an overall slowing down of the cell death process (Ellis et al., 1991; Stanfield and Horvitz, 2000). As compared to wild type an equal number of programmed cell deaths occur, and these deaths are initiated at the same time as compared to wild-type. When *ced-8* is combined with very weak alleles of *ced-3* or *ced-4* the weak cell death defect of these two mutations is enhanced. *ced-8* encodes a protein similar to the human XK transmembrane protein and worm *ced-8* indeed localizes to membranes (Stanfield and Horvitz, 2000). It is however unclear how *ced-8* impinges on the regulation of the core apoptotic pathway. Another, component that modulates programmed cell death was discovered via its interaction with *ced-4*. Upon over-expression of *mac-1* which encodes an AAA type ATPase, the level of programmed cell death is reduced (Wu et al., 1999). Unfortunately, it could not be addressed whether *mac-1* disruption leads to elevated levels of programmed cell death, as *mac-1* is essential for development. *nuc-1* encodes a worm DNase II ortholog, that was implicated in the efficient DNA fragmentation during programmed cell death (Lyon et al., 2000). *nuc-1* is also needed for the degradation of DNA in the digestive tract of *C. elegans* (Wu et al., 2000; Lyon et al., 2000). However, it seems that several other DNases must also be implicated in DNA degradation (see below). Corpses from *nuc-1* animals proceed with respect to DNA fragmentation to an intermediate stage that, in contrast to wild-type, allows for the detection of apoptotic corpses due to the accumulation of DNA 3´hydroxyl ends via the Tunnel method. *nuc-1* mediated DNA fragmentation is partially affected by corpse engulfment genes (see below) as Tunnel staining is reduced in *ced-1* and *ced-7* corpse engulfment defective mutants. In contrast to the situation in mammals no CPAN/CAP caspase activated nuclease/caspase activated DNase; DFF45/ICAD DNA fragmentation 45 kb/inhibitor of CAD like nuclease activity has been defined in *C. elegans*.

To find further targets of *ced-3* caspase a very elegant genetic screen was initiated. Assuming that there might be redundancy in the cell death pathway after the caspase step, Parrish et al., devised a sensitized genetic system allowing for the efficient

detection of mutations that enhance weak cell death defects (Parrish et al., 2001). As part of the experimental strategy an activated *ced-3* caspase as well as a GFP reporter is expressed in six nonessential mechanosensory neurons. Enhancement of cell death can be easily detected by a reduced number of surviving mechanosensory cells (reduced number of GFP dots). One mutant (*cps-6*) they identified is likely to encode the worm ortholog of endonuclease G (Parrish et al., 2001). Similarly to *ced-8* endonuclease G inactivation enhances the weak cell death defects of weak *ced-3* and *ced-4* mutations arguing that endunuclease G might act downstream of the *ced-3* caspase (Parrish et al., 2001). It is not clear whether endonuclease G is cleaved by *ced-3*. Interestingly worm endonuclease G localizes to mitochondria similar to its mammalian otholog that also has been implicated in programmed cell death (Parrish et al., 2001; Li et al., 2001). It will be interesting to see whether further downstream components of the cell death pathway will be uncovered via the above-described genetic screen.

1.3 DNA damage induced apoptosis

Germ line cell death

The finding that programmed cell death is not only occurring in the developmentally determined somatic cell lineage but also in the germ line of hermaphroditic worms has initiated a host of new discoveries. The *C. elegans* germ line is the only proliferating tissue in the adult animal. The distal tip cell generates germ cells and instructs them to mitotically divide until they reach the transition zone (Seydoux and Schedl, 2001). Here they initiate meiosis and proceed until diakinesis (Seydoux and Schedl, 2001). Upon fertilization in the spermatheca oozytes resume meiosis, giving rise to embryos (Figure 1). Gumienny et al. noticed the presence of about 0-4 germ cell corpses at any given time in the adult hermaphroditic germ line (Gumienny et al., 1999). Germ cell apoptosis is correlated with age as the number of corpses increases with the age of the worm (Gumienny et al., 1999). As a consequence up to 300 germ cells, corresponding to almost 50% of all germ cells produced, die through programmed cell death in an adult worm (Gumienny et al., 1999). These deaths only occur in the female gonade in cells which are in the pachytene phase of meiosis I. Since these deaths occur independent of environmental stimuli they were termed physiological germ cell deaths. Interestingly, physiological germ cell death is dependent on the worm Ras/MAP kinase pathway that is needed for the commitment of meiotic pachytene cells to enter the meiotic diplotene stage. In the absence of Ras/MAP kinase signaling germ cell death cannot occur. It is unclear whether this dependency is due to a direct connection of MAP kinase signaling with pro-apoptotic genes. Alternatively, the effect of MAP kinase signaling might be indirect as only cells committed to exit pachytene via the activation of MAP kinase signaling have the capacity to apoptose (Gumienny et al., 1999). Similar to somatic apoptosis physiological germ cell death is dependent on *ced-3* and *ced-4* and the engulfment of corpses requires the same set of genes as during somatic cell death. However, the somatic cell death trigger *egl-1* is not required for physiological germ cell death (Gumienny et al., 1999). Therefore, at least one additional, germ line specific apoptotic trigger has been postulated but its identity has remained elusive thus far.

Parasite induced cell death

In addition to physiological germ cell death, it was recently discovered that environmental stimuli can trigger programmed germ cell death. Infection with a virulent form of the bacterium *Salmonella typhimurium* induces apoptosis in the *C. elegans* germ line (Aballay and Ausubel, 2001). *C. elegans* is usually fed on a lawn of *E. coli*, but when Aballay and Ausubel left worms with *S. typhimurium* as their only food source, they observed elevated levels of germ cell death (Aballay and Ausubel, 2001). Bacterially induced programmed germ cell death requires all components of the core apoptotic pathway including *egl-1*. Interestingly, worms mutant for *egl-1, ced-3, ced-4*, or *ced-9* (gf) die earlier when fed on *S. typhimurium* as compared to their wild-type counterparts (Aballay and Ausubel, 2001). It will be interesting to define the specific pathways that lead to bacterially induced apoptosis.

DNA damage induced cell death

Genotoxic stress like ionizing irradiation can also lead to programmed cell death in *C. elegans* (Gartner et al., 2000). Upon irradiation, germ cells activate checkpoint pathways whose activation leads to a transient cell cycle arrest and to programmed cell death (Figure 5). Interestingly, those two DNA damage responses are spatially separated. Whereas mitotic germ cells halt cell cycle progression, meiotic pachytene cells undergo apoptosis (Gartner et al., 2000). Cells outside the germ line show neither of these responses (Gartner et al., 2000). As it is the case for *Salmonella* induced as well as for physiological germ cell death radiation induced apoptosis appears to be restricted to the hermaphroditic germ line and requires *ced-3* and *ced-4* and is also suppressed by *ced-9* gain-of-function mutations (Gartner et al., 2000). In addition, radiation induced apoptosis is partially dependent on *egl-1* (Gartner et al., 2000). Consistent with the notion that double strand breaks may be the cause of radiation induced programmed cell death, the level of apoptosis is dramatically enhanced by mutations that are defective in double strand break repair like for instance *mre-11* and *rad-51* (Gartner et al., 2000; Boulton et al., 2002).

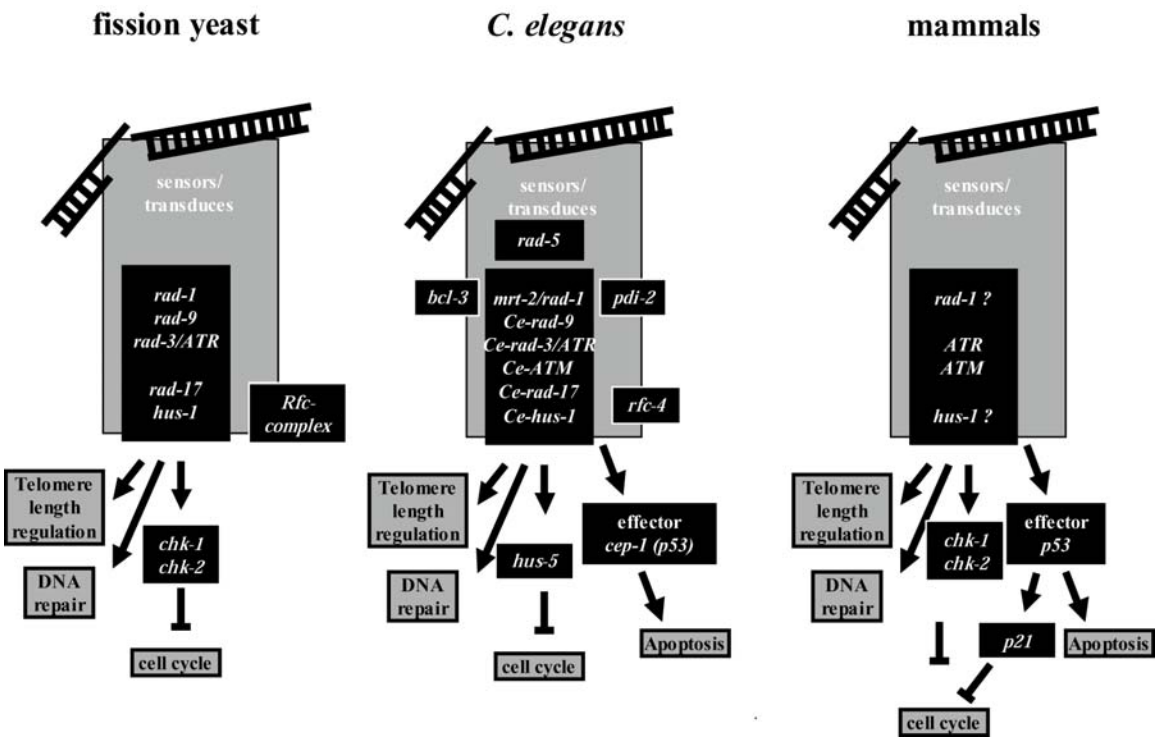


Figure 5 Simplified model of DNA damage checkpoint pathways in fission yeast, *C. elegans* and mammals. Simplified model of DNA damage checkpoint signaling in various organisms. Note that apoptosis does not occur in yeast. Worm p53 only affects programmed cell death but not cell cycle arrest. It has not been shown whether yeast or mammalian homologs of *rad-5* have a function in DNA damage checkpoint signaling.

Screens for mutants, which are defective in DNA damage induced apoptosis lead to the discovery of the *C. elegans* checkpoint genes *mrt-2*, *hus-1* and *rad-5* (Figure 5). These mutants are also defective in cell cycle arrest and render the worms hypersensitive to DNA damage (Gartner et al., 2000). *mrt-2* was found not only to be required for the DNA damage checkpoint but also for the regulation of telomere replication (Gartner et al., 2000; Ahmed and Hodgkin, 2000). When propagated over several generations, *mrt-2* homozygous worms accumulate progressively shorter telomeres leading to genomic instability and to the accumulation of chromosomal end-to-end fusion ultimately resulting in sterility (Ahmed and Hodgkin, 2000). Positional cloning of *mrt-2* revealed that this gene is the worm homolog of the *Schizosaccharomyces pombe rad1* and the *Saccharomyces cerevisiae rad17* checkpoint genes. *rad1*/*rad17* has previously been shown to be involved in yeast DNA damage checkpoints (Ahmed and Hodgkin, 2000). In

yeast checkpoint signaling involves a group of proteins needed for the recognition of DNA damage and for the transduction of the damage signal to effect cell cycle arrest (Figure 5) (for reviews see (Wahl and Carr, 2001; Abraham, 2001; Rhind and Russell, 2000; Walworth, 2000; Foiani et al., 2000; Murakami and Nurse, 2000; Caspari and Carr, 1999). *Schizosaccharomyces pombe* Rad9p, Rad1p and Hus1p form a complex that structurally resembles a PCNA sliding DNA clamp and has recently been shown to associate to DNA close to double-strand breaks *in vivo*, in a Rad17p dependent manner (Kondo et al., 2001; Melo et al., 2001). Analogously, *C. elegans* HUS-1 interacts with MRT-2 and a *hus-1* point mutation that is defective in checkpoint response is also defective in MRT-2 binding (Hofmann et al., 2002). In addition the *S. cerevisiae* homolog of mammalian ATM/ATR Mec1 also localizes to damaged DNA and its kinase activity is activated in response to DNA damage. The DNA damage signal is then relayed via the scMec1p/spRad3p/ATM/ATR kinases to CHK1 and CHK2 kinases, which cause cell cycle arrest via phosphorylation of key cell cycle proteins. Given that *mrt-2* is involved in radiation-induced apoptosis it was reasonable to assume that worm homologs of yeast checkpoint genes are also defective in DNA damage checkpoint responses. RNAi inactivation of these candidate worm checkpoint genes led to the inactivation of radiation induced cell cycle arrest (Boulton et al., 2002)(Figure 5). However, defects in radiation in induced programmed cell death could only be induced at a low penetrance, most likely because these checkpoint genes could only be partially inhibited in meiotic pachytene cells by RNAi (Boulton et al., 2002). Generation of true checkpoint gene mutation will therefore be necessary to unambiguously assign a function of to genes in radiation induced programmed cell death.

rad-5 was the first conserved checkpoint gene whose function in the DNA damage checkpoint was defined in the *C. elegans* system (Hartman and Herman, 1982). *rad-5* is an essential gene and the two known *rad-5* mutations are temperature sensitive lethals (Hartman and Herman, 1982). Cloning of the *C. elegans rad-5* revealed that this gene is related to *S. cerevisiae tel-2,* an essential gene shown to be involved in telomere length regulation (Ahmed et al., 2001). *rad-5* function in telomere regulation is less clear as telomere length in *rad-5* mutants fluctuates but does neither get progressively longer nor shorter (Ahmed et al., 2001). Interestingly the checkpoint gene *rad-5* was found to be

allelic with *clk-2* (Ahmed et al., 2001). *clk* genes were found to slow down the worm's development and to have an extension of their lifespan. (Benard et al., 2001; Lakowski and Hekimi, 1996; Lim et al., 2001). In *clk-2(qm37)* this lifespan extension is only weak and it is unclear if this effect is only an indirect consequence of the slow growth phenotype of *clk-2 (qm37)* mutation.

A recent functional genomics based approach lead to the identification of additional genes involved in worm checkpoint responses. Boulton et al. cloned all worm homologs of yeast checkpoint and DNA repair genes (Boulton et al., 2002). In a subsequent step all these genes were used for two-hybrid analysis to identify interaction partners (Boulton et al., 2002). The potential checkpoint function of these interaction partners was than assessed by RNAi (Figure 5). This approach lead to the identification of a worm homolog of the mammalian Bcl-3 oncogene needed for DNA damage signaling (Boulton et al., 2002). It will be interesting to see whether the mammalian homolog has a similar defect in DNA damage signaling.

The DNA damage checkpoint is not only activated after genotoxic insult but also upon meiotic defects as for instance the accumulation of unprocessed meiotic recombination intermediates. As part of a normal meiotic division double strand breaks, which are needed for meiotic recombination, are generated by the meiotic endonuclease SPO-11 (for review see (Roeder, 1997). At a following step of meiotic recombination double strand breaks are resected and the resulting single strand overhangs invade into the homologous chromosome via a strand exchange reaction that is mediated by the conserved RAD-51 strand exchange protein (Roeder, 1997). Worms lacking RAD-51 are thought to accumulate unprocessed SPO-11 induced double strand breaks (Takanami et al., 1998; Rinaldo et al., 1998). These breaks are recognized by the very same checkpoint proteins as the proteins sensing radiation-induced double strand breaks and result in elevated levels of germ cell apoptosis (Gartner et al., 2000). The observation that meiotic defects lead to increased levels of germ cell death can be employed to readily isolate mutations defective in meiotic chromosome pairing and recombination (Pawel Pasierbeck, Josef Loidl and Anton Gartner, personal communication).

Conclusively, the above-mentioned data indicate that the DNA damage checkpoint pathways are conserved throughout evolution (Figure 5). However, the apoptotic

response to DNA damage only exists in metazoans, since yeast does not have apoptosis. Although yeast can undergo cell death that involves at least one caspase related protease, none of the classical regulators of metazoan apoptosis are present in yeast (Madeo et al., 1997; Madeo et al., 2002). It is therefore conceivable that the conserved DNA damage checkpoint pathway that already exists in yeasts is linked to the metazoan specific apoptotic machinery. Additional genetic screens are likely to uncover novel potentially metazoan specific DNA damage checkpoint genes.

1.4 p53

Human p53 is the most frequently mutated tumor suppressor gene in cancer. About 50% of cancer patients show mutations in p53 (Roemer, 1999). Li-Fraumeni syndrome patients carry recessive mutations in p53 and are predestined to develop a variety of cancers (Pierotti and Dragani, 1992). p53 is a transcription factor that induces genes such as p21 to halt cell cycle progression or genes such as Puma and Noxa that trigger apoptosis (see below). Most of the tumor associated p53 mutations have been shown to interfere with p53's transcriptional activity emphasizing the significance of p53 target gene regulation in tumor suppression. In fact, the most frequently mutated p53 residues are those that directly bind to DNA (Hussain and Harris, 2000).

The list of p53 target genes is growing as microarray data are accumulating. p53 has been shown to induce the cyclin dependent kinase inhibitor p21, which induces G1/S cell cycle arrest. Additionally, p53 can induce 14-3-3 sigma expression, which was suggested to halt the cell cycle at G2/M phase (Hermeking et al., 1997). Furthermore, p53 has been shown to induce at least 16 genes, which are involved in programmed cell death (Vousden and Lu, 2002). p53 induces the BH3 only proteins Puma and Noxa as well as the multidomain cell death trigger Bax (Nakano and Vousden, 2001; Yu et al., 2001; Miyashita and Reed, 1995; Han et al., 2001; Oda et al., 2000a). p53 can also induce p53AIP1, p53DINP1, and Apaf-1 among others (Oda et al., 2000b; Okamura et al., 2001; Moroni et al., 2001; Fortin et al., 2001; Robles et al., 2001). The target gene induction appears to be dependent on the cell types used as well as on the level of p53 expression (Zhao et al., 2000). Whereas the induction of p21 is widely accepted as the major mechanism of p53 induced cell cycle arrest the situation for p53 induced cell death is less clear. This is likely to be due to cell type specific cell death regulation as well as functional redundancy among p53 target genes but also to the method used for the identification of particular target genes as many experiments used overexpressed p53 (Zhao et al., 2000). Based on recent mouse genetics experiments evaluating DNA damage induced cell death in thymocytes and fibroblasts a likely model for p53 dependent apoptosis mainly relies on induction of the BH3 only protein Puma (Villunger et al., 2003; Jeffers et al., 2003). Furthermore, Puma was also required for apoptosis

following various p53-independent cytotoxic insults such as cytokine deprivation, glucocorticoids, the kinase inhibitor staurosporine and phorbol ester. In some cell types such as fibroblasts a second BH3 only protein Noxa is required to trigger cell death upon DNA damage (Villunger et al., 2003; Shibue et al., 2003). These BH3 only proteins then inhibit Bcl-2 family proteins thereby inducing programmed cell death (Huang and Strasser, 2000). The role of other apoptotic p53 target genes in an organism is less clear as at least individually they appear to be dispensable (Rich et al., 2000) (Vousden and Lu, 2002).

1.5 BH3 only proteins in apoptosis

As BH3 only proteins are not only key inducers of the mitochondrial cell death pathway (Huang and Strasser, 2000) but are also likely to turn out to be major mediators of DNA damage induced cell death in worms (as shown in this thesis) and mammals (Villunger et al., 2003; Jeffers et al., 2003; Shibue et al., 2003) we will give a short overview of their mode of action.

The Bcl-2 homology (BH) domain is the defining domain of the Bcl-2 family of pro- and anti-apoptotic proteins (for review see (Adams and Cory, 1998)). The pro-survival branch, homologous to *C. elegans* CED-9, comprises Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1, Boo/Diva/Bcl-2-L10 and Bcl-B, all of which contain three to four BH domains. The pro-apoptotic Bcl-2 family members can be divided into multi-BH domain proteins and BH3 only proteins that play distinct roles in triggering cell death. The former ones, featuring two to three BH domains include Bax, Bak, Bcl-x_S, Bok/Mtd and Bcl-G_L, whereas the latter ones only have one BH3 domain. The *C. elegans* genome codes for two BH3 only proteins, whereas in mammals there are at least nine present (Bad, Bik/Nbk, Blk, Bid, Hrk/DP5, Bim/Bod, Bmf, Noxa, Puma/Bbc-3).

Similar to EGL-1 binding to CED-9, BH3 only proteins bind to anti-apoptotic Bcl-2 family proteins to trigger cell death (Huang and Strasser, 2000). Co-crystallization of Bcl-x_L with Bak peptides showed that the BH3 domain inserts into a hydrophobic cleft formed by the BH1, BH2 and BH3 domain of anti-apoptotic Bcl-2 proteins (Sattler et al., 1997).

EGL-1 is required for all developmental and the majority of DNA damage induced cell deaths (Conradt and Horvitz, 1999; Conradt and Horvitz, 1998; Gartner et al., 2000). As there are a number of different BH3 only proteins in mammals it is perhaps not surprising that some of them seem to be tissue specific or functionally redundant. Knock out mice have been generated for four of those BH3 only proteins. $bid^{-/-}$ mice show specific defects in anti-Fas antibody mediated apoptosis in hepatocytes (Yin et al., 1999), whereas Bim is required for hematopoietic homeostasis and protection from autoimmune disease (Bouillet et al., 2002; Bouillet et al., 1999). Furthermore, $bim^{-/-}$ lymphocytes were resistant to some cell death stimuli such as cytokine withdrawal, calcium influx and taxol but not to others such as ionizing irradiation or phorbol ester (Bouillet et al., 1999)As mentioned above, Puma is required for triggering cell death in response to DNA damage as well as other cytotoxic insults such as cytokine deprivation, glucocorticoids, the kinase inhibitor staurosporine and phorbol ester in thymocytes and fibroblast, whereas Noxa function seem to be restricted to DNA damage induced apoptosis in fibroblasts (Villunger et al., 2003; Shibue et al., 2003)

Similar to EGL-1, some mammalian BH3 only proteins are regulated through transcriptional induction. As described above Puma and Noxa both are transcriptionally induced by p53 (Han et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000a; Yu et al., 2001). Hrk/DP5 is transcriptionally induced through JNK signaling upon growth factor withdrawal (Harris and Johnson, Jr., 2001; Imaizumi et al., 1997; Putcha et al., 2001; Whitfield et al., 2001), whereas the forkhead transcription factor FKHR-L1 induces bim expression in hematopoietic cells upon cytokine withdrawal (Dijkers et al., 2000).

Besides transcriptional control BH3 only proteins are also regulated post-transcriptionally. Post-transcriptional modifications of BH3 only proteins determine cellular localization, which in turn determine their apoptotic activity. After cleavage by Caspase-8 the Bid fragment p15tBid is myristoylated and can bind to the mitochondrial membrane (Li et al., 1998; Luo et al., 1998; Zha et al., 2000). Casein kinase I and II can phosphorylate Bid, which then can no longer be cleaved by Caspase-8, thereby preventing Bid from inducing apoptosis (Desagher et al., 2001). Akt/PKB and mitochondrial PKA phosphorylate Bad in response to cytokine signaling (Datta et al., 1997; del Peso et al., 1997; Harada et al., 1999). Phosphorylated Bad is sequestered by

14-3-3ι in the cytosol and released upon cytokine withdrawal to then trigger cell death (Zha et al., 1996). Bim and Bmf are sequestered to microtubules by forming a complex with the dynein motor complex through the dynein light chain DLC-1/LC8 and DLC-2, respectively. Upon cytokine withdrawal, calcium influx or taxol treatment Bim, upon cell detachment Bmf then translocate to mitochondrial and nuclear envelope membranes to trigger cell death (Puthalakath et al., 2001; Puthalakath et al., 1999).

Gene dosage also plays a role in BH3 only protein mediated apoptosis as the loss of one copy of bim is sufficient to rescue the disorders of a bcl-$2^{-/-}$ mouse (Bouillet et al., 2001).

Bim, Bid, Bad and Noxa require at least one allele of the pro-apoptotic multi-BH domain proteins Bax or Bak to induce cell death in embryonic fibroblasts (Cheng et al., 2001; Zong et al., 2001). Bak and Bax function redundantly in triggering cell death (Lindsten et al., 2000) with the exception of neurons, where Bak is not required (Putcha et al., 2001). $bax^{-/-}/bak^{-/-}$ is epistatic in inducing progressive lymphoid hyperplasia with $bim^{-/-}$ and Bcl-2 overexpression suggesting a linear pathway (Bouillet et al., 1999; Lindsten et al., 2000; McDonnell et al., 1989; Sentman et al., 1991; Strasser et al., 1991a; Strasser et al., 1991b).

There is some controversy about the mechanism by which BH3 only protein binding to Bcl-2 family members leads to cell death. Bcl-2 was suggested to prevent the insertion of Bax and Bak into the outer mitochondrial membrane (Antonsson et al., 1997; Nechushtan et al., 2001). When Bcl-2 is sequestered by BH3 only proteins Bax and Bak might form a pore in the outer mitochondrial membrane through which Cytochrome c is released either directly (Antonsson et al., 1997; Eskes et al., 1998; Nechushtan et al., 2001; Pavlov et al., 2001) or through binding to VDAC and ANT (Marzo et al., 1998; Narita et al., 1998). Once released from the mitochondria Cytochrome c forms a complex with Apaf-1, which then binds to Caspase-9, leading to Caspase-9 and consequent Caspase-3 activation (Zou et al., 1997; Li et al., 1997). There are, however, some developmental cell deaths that are independent of Bax/Bak (Lindsten et al., 2000), Cytochrome c (Li et al., 2000), Apaf-1 (Cecconi et al., 1998), Caspase-9 (Hakem et al., 1998; Kuida et al., 1998)or Caspase-3 (Kuida et al., 1996) suggesting alternative mechanism of BH3 only protein and Bcl-2 family dependent cell deaths. It was proposed that such pathways might involve a direct

interaction of Bcl-2 with an as yet elusive CED-4 homolog, analogous to the *C. elegans* pathway (Bouillet and Strasser, 2002).

1.6 p53 regulation

As p53 is the central node in a signaling network controlling cell death, cell cycle arrest, and cellular senescence its tight regulation is essential for the survival of the organism (Vogelstein et al., 2000).

The major regulator of p53 activity is the Mdm2 oncoprotein (Michael and Oren, 2003). In unperturbed cells the p53 protein is low abundant as it is constantly targeted for degradation by the E3 ubiquitin ligase Mdm2 (Honda et al., 1997). Mdm2 shuttles p53 from the nucleus to the cytoplasm where p53 is degraded by the 26S proteasome. A well-characterized mechanism of p53 activation involves post-translational stabilization through alleviation of Mdm2 dependent p53 degradation (Michael and Oren, 2003).

Mdm2 has originally been described as a gene on the double minute chromosome that was amplified in spontaneously transformed BALB/c/3T3 cells (Cahilly-Snyder et al., 1987). In human the oncoprotein Mdm2 was found to be amplified in soft tissue tumors, osteosarcomas as well as in a variety of other tumors (Oliner et al., 1993).

In mitogenic signaling oncoproteins such as Ras and Myc activate transcription of the INK4a alternative reading frame ARF (Sherr, 1998). p14ARF in turn binds to Mdm2 in an allosteric fashion (Chene, 2003). Several mechanisms for ARF mediated Mdm2 inhibition have been suggested. ARF was reported to inhibit Mdm2 E3 ubiquitin ligase activity (Honda and Yasuda, 1999). Other groups showed that ARF sequesters Mdm2 in the nucleolus (Weber et al., 1999) (Tao and Levine, 1999), whereas Zhang and Xiong observed that ARF leaves the nucleolus and form a ternary complex with Mdm2 and p53 in the nucleus, preventing nuclear export (Zhang and Xiong, 1999).

Upon genotoxic insults p53 is stabilized through posttranslational modifications (Xu, 2003). The significance of the various posttranslational means of p53 modification, however, is not yet clear (Xu, 2003). p53 can be phosphorylated by a number of kinases at multiple sites at the N- and C-terminus *in vitro*. Upon ionizing irradiation checkpoint signaling activates the PI3-kinase Ataxia telangectasia mutated (Atm), which in turn activates the checkpoint kinase 2 (Chk2) (Matsuoka et al., 1998; Matsuoka et al., 2000).

Both, Atm and Chk2 can activate p53 (Hirao et al., 2000) (Kastan et al., 1992). Upon DNA damage Atm can phosphorylate p53 at Ser15 (Canman et al., 1998; Banin et al., 1998; Nakagawa et al., 1999), which was reported to lead to an alleviation of Mdm2 binding (Shieh et al., 1997). Knock-in experiments in mouse ES cells and differentiated ES cells with Alanine substitution of the corresponding mouse Ser18, however, showed a partial requirement of Ser18 phosphorylation for p53 dependent cell cycle arrest without any apparent effect on p53 stabilization (Chao et al., 2000). Claims that Ser15 phosphorylation was a prerequisite for C-terminal acetylation of p53 could not be supported either (Chao et al., 2000). Chk2 was reported to phosphorylate p53 at Ser20, which lies in the α-helix that directly interacts with Mdm2 (Shieh et al., 1999). Here again, an Alanine substitution of the human Ser20 equivalent in mice, Ser23, had no effect on p53 stability or p53 dependent cell cycle arrest in mouse knock-in experiments (Wu et al., 2002). One possibility to explain the discrepancies between experiments suggesting a role of individual Serine phosphorylations to have an effect on p53 stability could be a different mechanism of p53 regulation in human and mice. This, however seems unlikely, as the residues 13 to 27 comprising those Serines are identical between the species. The central core domain of human p53 can functionally substitute the one of mouse p53, suggesting a conserved mechanism of p53 regulation (Luo et al., 2001). Alternatively, some of the phosphorylation events might be functionally redundant, as Atm can also phosphorylate p53 at Ser9, Ser37 and Ser46 (Saito et al., 2003) and Thr18 phosphorylation was shown to interfere with Mdm2 interaction as well (Sakaguchi et al., 2000). In such a model a subset of phosphorylation events might be sufficient to alleviate Mdm2 binding and lead to p53 stabilization.

Atm has also been reported to phosphorylate Mdm2 suggesting another mechanism of interfering with p53 degradation (Khosravi et al., 1999). However, the regulation of p53 stabilization through alleviation of Mdm2 mediated degradation seems to be more complicated. Blattner et al. have recently reported that upon DNA damage Mdm2 gets hypophosphorylated in the conserved region II. Alanine substitutions in this region interfered indeed with p53 degradation, but not ubiquitination and also had no effect on p53 or ARF binding, suggesting additional requirements for p53 degradation besides Mdm2 binding and ubiquitination (Blattner et al., 2002).

In addition to p53 phosphorylation and ubiquitination, acetylation was suggested to play a role in p53 regulation. The acetyl transferases CBP/p300 and PCAF can acetylate p53 on Lys370/372/373/381/382 and Lys320, respectively (Gu and Roeder, 1997; Sakaguchi et al., 1998; Liu et al., 1999). Those Lysine residues are also acetylated upon ionizing irradiation and enhance sequence specific DNA binding of p53 (Gu and Roeder, 1997; Sakaguchi et al., 1998; Liu et al., 1999). Mutational analysis has revealed Lys370/372/373/381/382 to be the same residues that are ubiquitinated by Mdm2 suggesting a role of acetylation in p53 stabilization (Kubbutat et al., 1998; Nakamura et al., 2000; Rodriguez et al., 2000). p53 acetylation might be regulated through phosphorylation of Ser15/33/37 as those phosphorylations enhance binding to CBP/p300 (Sakaguchi et al., 1998).

Recently, p53 has also been reported to undergo sumoylation. The ubiquitin relative Sumo-1 is attached by PIAS family of E3-like SUMO ligases from the SUMO conjugating enzyme Ubc9 on lysine 386 (Schmidt and Muller, 2002). The effect of sumoylation on p53 function, however, is disputed. Two groups reported p53 activation upon SUMO attachment (Gostissa et al., 1999; Rodriguez et al., 1999) others failed to observe this effect (Kwek et al., 2001) or even reported a drop in p53 transactivation activity (Schmidt and Muller, 2002). In all experiments p53 as well as members of the sumoylation pathway were overexpressed. Therefore, the physiological effect of sumoylation on p53 remains to be evaluated.

Very little is known about post-transcriptional regulation of p53. It was shown that upon ionizing irradiation p53 mRNA levels remain constant while the p53 protein accumulates (Kastan et al., 1991). p53 accumulation was unaffected by the transcription inhibitor actinomycinD, put prevented by the translational inhibitor cyclohexamide (Kastan et al., 1991). Furthermore, it was shown that a translational repressor element in the 3'UTR of human p53 is necessary for translational control in OCI/AML-3 and OCI/AML-4 cells (Fu and Benchimol, 1997; Fu et al., 1999). In mouse Swiss 3T3 cells it was shown that p53 binds to the 5'UTR of its own mRNA, preventing it from translation (Mosner et al., 1995). There is no evidence for a role of the 5'UTR in human p53 regulation. However, the human p53 5'UTR is much shorter than the mouse one and is missing the sequence

that forms a stable secondary RNA structure predicted to be required for p53 mRNA binding in mouse (Mosner et al., 1995).

1.7 *C. elegans* p53

In the first part of this thesis we will show that *C. elegans* possesses a functional homolog of human p53. Although it initially evaded detection by conventional homology searches, using bioinformatics approaches based on generalized profiles, we and others were able to show that the worm genome encodes for a distant homolog of the mammalian p53 tumor suppressor gene termed *cep-1* (*C. elegans* p53 like, see below) (Schumacher et al., 2001; Derry et al., 2001). However, sequence alignments revealed that many of the p53 residues that are implicated either in DNA binding or in oncogenesis are conserved in *cep-1* (Schumacher et al., 2001). Unlike mammalian p53 but similar to Drosophila p53 *cep-1* is not required for DNA damage induced cell cycle arrest (Schumacher et al., 2001; Derry et al., 2001). In addition to its function in cell cycle arrest, Derry et al. could show an involvement of *cep-1* in stress response and some involvement in meiotic chromosome segregation as *cep-1* mutant animals showed weak defects in meiotic chromosome segregation (Derry et al., 2001).

The apparent complexity of p53 target induction in mammals prompted us to evaluate p53 target genes in *C. elegans*. We show here that CEP-1 induces both *C. elegans* BH3 only proteins EGL-1 and CED-13. Furthermore, we show that EGL-1 is the major trigger of DNA damage induced cell death. CED-13 only plays a minor role in inducing programmed cell death but enhances EGL-1. Therefore, we suggest that induction of BH3 only proteins define an ancient p53 apoptotic core pathway, which is conserved throughout evolution.

1.8 Perspectives on using p53 in tumor therapy

In a myc induced B cell lymphoma mouse model deletion of p53 has been shown to be epistatic with dominant negative Caspase-9 or Bcl-2 overexpression for tumor development (Schmitt et al., 2002a). This model suggests that the apoptotic function of p53 is necessary and sufficient for its ability to suppress tumor formation. Radiotherapy and many chemotherapeutical agents aim at inducing apoptosis through DNA damage.

Mutations in p53 correlate with drug resistance and poor prognosis, as p53 mutated tumor cells often do not respond by undergoing apoptosis or replicative senescence (Schmitt et al., 2002b). It is, therefore, of pivotal interest to understand the mechanisms that underlie p53 dependent apoptosis.

In tumors that have wild type p53 loci mutations in the above-described ARF locus have been found at a frequency that is approaching the one of tumor associated p53 mutations suggesting that most if not all tumors are defective in the p53 pathway (Sharpless and DePinho, 1999). As p53 acts downstream of ARF, it was suggested that in these tumors it might be possible to activate p53 by targeting Mdm2 with small peptides or small molecule compounds that mimic ARF and prevent Mdm2 from binding and downregulating p53 (Chene, 2003). The rationale of such an approach is to inhibit negative regulators of p53. Such an inhibition would then lead to p53 upregulation. Activation of p53 in turn would make cells prone to undergo apoptosis or senescence (Chene, 2003). For these reasons it would be highly desirable to identify negative regulators that in addition to Mdm2 control p53 activity.

1.9 Genetic screen for negative regulators of the p53 pathway

The *C. elegans* genome sequence did not reveal any evidence for a nematode Mdm2 homolog (The *C.elegans* Sequencing Consortium, 1998). We, therefore, hypothesized that a genetic screen for negative regulators of *cep-1* might reveal novel regulatory mechanisms of p53 activity that might potentially be masked by Mdm2 regulation in other organisms.

To identify novel components of CEP-1 regulation we conducted a genetic screen for mutations that inhibit the p53 pathway. We screened for enhanced levels of germ cell apoptosis upon low dosage of ionizing irradiation, as germ cell death is a consequence of CEP-1 activity. We identified a novel allele of the *C. elegans* germ line tumor suppressor *gld-1* (germ line defective), *gld-1(op236)*, which leads to a strong upregulation of apoptosis following DNA damage. GLD-1 has previously been characterized as an mRNA binding protein of the GSG/STAR (GRP33/Sam68/GLD-1/Signal Transduction and Activation of RNA) family of RNA binding proteins, which represses the translation of target mRNAs (Francis et al., 1995) (Jones and Schedl, 1995) (Lee and Schedl, 2001).

The number of apoptotic cells in the germ line of *gld-1(op236)* animals is increased two to three fold over levels in wild type worms, leading to a massive upregulation of apoptosis upon ionizing irradiation. This increase is dependent on *cep-1*, and its transcriptional targets, the BH3 only proteins EGL-1 and to a lesser extend CED-13. We show that GLD-1 binds *cep-1* mRNA suggesting a direct repression of *cep-1* mRNA by GLD-1.

2 Aim of this thesis

The aim of this thesis was to further characterize the apoptotic response to DNA damage. Most genes that are involved in DNA damage signaling have been identified through yeast genetics. Yeast, however, only responds with cell cycle arrest to DNA damage but does not undergo apoptosis. A genetic screening system has been unavailable to study DNA damage induced apoptosis thus far. The identification of a conserved DNA damage checkpoint pathway that induces apoptosis in *C. elegans* now allowed use of this genetic model organism to study DNA damage induced apoptosis.

2.1 *C. elegans* p53 (*cep-1*)

The tumor suppressor p53 is the most frequently mutated gene in human cancer (Roemer, 1999). Its apoptotic function is the most important tumor suppressor function (Schmitt et al., 2002a). In this thesis we identified a p53 homolog in *C. elegans*. We show that *C. elegans* p53 (*cep-1*) is specifically involved in DNA damage induced apoptosis but dispensable for cell cycle arrest. Inducing programmed cell death is likely to represent the ancient p53 function.

2.2 CEP-1 target genes

We next asked how CEP-1 induces the apoptotic core machinery, or more precisely, how CEP-1 confers CED-9 inhibition. We show that CEP-1 transcriptionally induces the BH3 only proteins EGL-1 and CED-13. Both of these proteins bind CED-9, which then releases CED-4 to activate CED-3, triggering cell death (Spector et al., 1997);(Chinnaiyan et al., 1997b; Wu et al., 1997; Conradt and Horvitz, 1998) (Parrish et al., 2000; del Peso et al., 2000); Shai Shaham personal communication). We thus propose a p53 core pathway, where p53 induces cell death through transcriptional induction of BH3 only proteins.

Furthermore, we conducted a genome wide gene expression analysis using Affymetrix gene chip technology. Among 22,629 analysed *C. elegans* genes we found *egl-1* to show

the strongest upregulation in dependence on DNA damage and CEP-1. Other target genes are currently being evaluated.

2.3 Genetic screen for negative regulators of CEP-1

The identification of a functional p53 homolog in *C. elegans* allowed us to use the genetic model system to genetically screen for novel components of the p53 pathway. We screened for repressors of the p53 pathway. Our screening output was upregulation of DNA damage induced apoptosis. We identified a novel allele of the tumor suppressor *gld-1* that specifically upregulates the *cep-1* pathway. GLD-1 is a member of the GSG/STAR family of RNA binding proteins and has previously been shown to translationally repress target mRNAs (Lee and Schedl, 2001; Jones and Schedl, 1995).

2.4 RNAi screen for regulators of the p53 pathway

Finally, we screened for regulators of the p53 pathway using RNA interference. We focused on genes that are specifically expressed in the *C. elegans* germ line, which is the only tissue that undergoes programmed cell death upon ionizing irradiation. This screen, however, failed to reveal any regulators of the p53 pathway.

2.5 Conclusion

In conclusion, we have established *C. elegans* as a genetic model for studying the apoptotic p53 pathway. We have identified a mutation in *gld-1* that leads to upregulation of the CEP-1 pathway. This point mutation revealed a novel function of the mRNA translation-regulating protein GLD-1 and leads us to propose a novel regulatory mechanism of p53 regulation at the level of translational control.

Our results emphasize the power of the genetic model organism *C. elegans* in the identification of apoptotic regulators. Further genetic screens for *cep-1* regulation in *C. elegans* might reveal additional novel components of the p53 pathway.

3 Results and Discussion

3.1 *C. elegans* p53 (*cep-1*)

3.1.1 Identification of a *C. elegans* homolog of the tumor suppressor p53

A specific effector of radiation-induced apoptosis has not been identified in *C. elegans*. The completion of the *C. elegans* genomic sequence failed to reveal any obvious worm p53 homolog leading to the initial notion that p53 might be confined to the vertebrate lineage (Genome Sequence of the nematode *C. elegans*, 1998). However, this notion was recently challenged by the discovery of a functional *Drosophila* p53 homolog (Brodsky et al., 2000; Ollmann et al., 2000). We carefully searched for a worm p53 homolog assuming that the *C. elegans* p53 open reading frame might have eluded previous BLAST searches due to an error in *C. elegans* p53 gene prediction or as a result of the gene being highly divergent from its vertebrate and *Drosophila* counterparts. Sensitive database searches, like those using generalized profiles (Bucher et al., 1996), allow the reliable detection of more subtle sequence relationships (Hofmann, 2000). In order to search the *C.elegans* genome for distant p53 family members, we constructed a series of profiles from a multiple alignment of accepted members of the p53 family (p53 of human, mouse, hamster, chicken, *Xenopus*, trout, squid, mussel, as well as human p63 and p73). Profiles constructed from the whole sequences, as well as those constructed from the DNA-binding region, identified a highly significant relationship (Error probability $p<0.01$) to the *C. elegans* predicted open reading frame F52B5.5 that possesses a region distinctly related to the DNA binding domain of p53 (see below). We did not find any other p53 family related sequences in the *C. elegans* genome (bioinformatics analysis done by Kay Hofmann). To confirm that this open reading frame is indeed expressed (there is no EST reported for F52B5.5) and to identify the correct cDNA sequence we wished to identify the full-length cDNA clone of F52B5.5. Most of *C. elegans* transcripts are trans-spliced to a 5´ leader SL-1 or SL-2 sequence (Hirsh and Huang, 1990). To identify the full-length *cep-1* open reading frame we designed oligonucleotides corresponding to either SL-1 or SL-2 sequences and internal F52B5.5 primers for PCR amplification from worm cDNA.

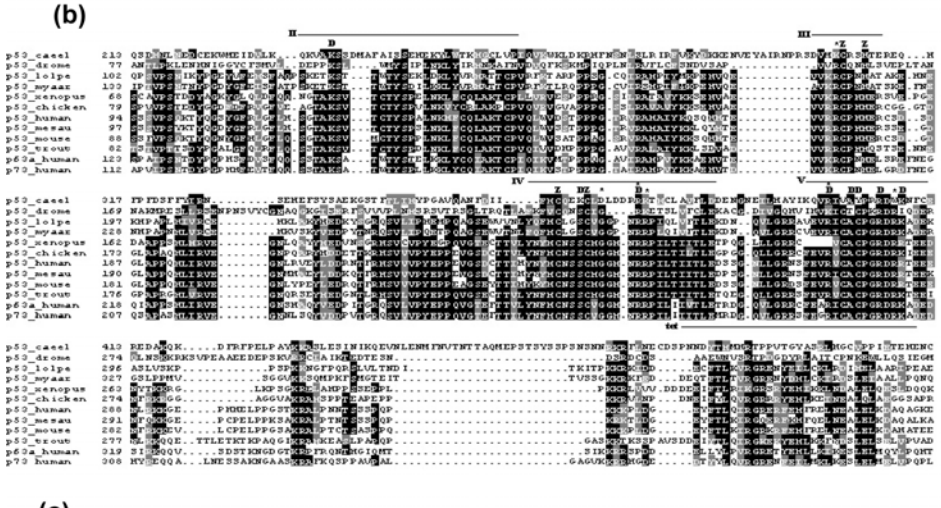
Using SL-1 primers we obtained a 1.9 kb cDNA that corresponds to the predicted genes F52B5.4 and F52B5.5 and gives rise to a putative 645 amino acid protein (Figure 6a) (We did not obtain any cDNA with SL-2 primers).

We used this putative 645 aa protein termed CEP-1 and aligned it with known members of the p53 family (Figure 6b). The detectable evolutionary conservation of *C. elegans* p53 is mostly limited to the regions involved in DNA binding (conserved regions II to V in Figure 6b). Comparison of CEP-1 with human p53 indicates that residues critical for DNA binding as revealed in the three-dimensional structure of p53 bound to DNA, are conserved in *C. elegans* (Cho et al., 1994). Five out of eight amino acids implicated in DNA binding are also conserved and an additional one is similar (indicated by D, in Figure 1b). Moreover, out of the six amino acid residues that are most frequently mutated in cancer two are conserved and two are substituted by similar amino acids (indicated by *, in Figure 6b (Roemer, 1999)). The two conserved amino acids R248 and R273 are by far the most frequently mutated residues and account for over 20% of tumor associated p53 mutations. (Roemer, 1999). Finally, all four residues implicated in Zn binding are conserved (indicated by Z, in Figure 6b) (Cho et al., 1994). Although weakly conserved we found two potential phosphorylation sites (SQ) corresponding to Serine 15 and Serine 37 of human p53, which are implicated in DNA damage dependent activation of p53, at the N-terminus (Shieh et al., 1997; Tibbetts et al., 1999). The one related to Serine 15 (CEP-1 S20) lies in a conserved region (PDSQ[D/E]) (not shown). At the C-terminus of CEP-1 we could also find a small but distinct amino acid conservation at the tetramerization domain (indicated by tet in Figure 6b)(Soussi and May, 1996). However, there appears to be no obvious acidic domain characteristic for the human transactivation domain.

(a)

MEPDDSQLSDILKDARIPDSQDIGVNLTQNLSFDTVQKMIDGVFTPIFSQGTEDSL
EKDILKTPGISTIYNGILGNGEETKKRTPKISDAFEPDLNTSGDVFDSDKSEDGLM
NDESYLSNTTLSQVVLDSQKYEYLRVRTEEEQQLVIEKRARERFIRKSMKIAEET
ALSYENDGSRELSETMTQKVTQMDFTETNVPFDGNDESSNLAVRVQSDMNLNE
DCEKWMEIDVLKQKVAKSSDMAFAISSEHEKYLWTKMGCLVPIQVKWKLDKR
HFNSNLSLRIRFVKYDKKENVEYAIRNPRSDVMKCRSHTEREQHFPFDSFFYIRNS
EHEFSYSAEKGSTFTLIMYPGAVQANFDIIFMCQEKCLDLDDRRKTMCLAVFLDD
ENGNEILHAYIKQVRIVAYPRRRDWKNFXEREDAKQKDFRFPELPAYKKASLESIN
IKQEVNLENMFNVTNTTAQMEPSTSYSSPSNSNNXKRFLNECDSPNNDYTMMHR
TXPVTGYASRLHGCVPPIETEHENCQSPSMKRSRCTNYSFRTLTLSTAEYTKVVE
FLAREAKVPRYTWVPTQVVSHILPTEGLERFLTAIKAGHDSVLFNANGIYTMGD
MIREFEKHNDIFERIGIDSSKLSKYYEAFLSFYRIQEAMKLPK

(b)



(c)

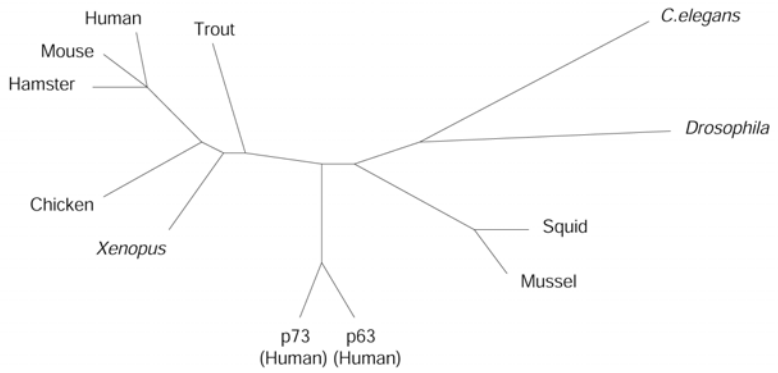


Figure 6 (a) Predicted amino acid sequence of CEP-1. **(b)** shows a profile-guided alignment of CEP-1 with other established members of the p53 family. Residues, which in human p53 interact with DNA are marked with D, those involved in Zn binding with Z and those most frequently mutated in cancers with a *. The evolutionary conserved regions II to IV and the tetramerization domain (tet) are indicated (Soussi and May, 1996).

(c) Neighbor-joining dendrogram of the p53 family. Overall, the dendrogram reflects the evolutionary relationships of the organisms involved. Obvious exceptions are p63 and p73, which have arisen from gene duplication events. CEP-1 robustly clusters with the Drosophila sequence (1000 out of 1000 bootstrap trials).

3.1.2 CEP-1 is a transcription factor

Given that the transcriptional activity of p53 is essential for its function in vertebrates we wished to determine whether CEP-1 could act as a transcription factor. To test if CEP-1 can intrinsically activate transcription we cloned the full length CEP-1 ORF in frame with the Gal4 DNA binding domain. This fusion protein was scored for its ability to activate a LacZ transcriptional reporter construct behind a yeast promotor containing Gal4 DNA binding sites. CEP-1 was found to possess an intrinsic transcriptional activity that maps to the N-terminus equivalent to that observed for the transcription factors BAR-1 and human p53 suggesting that CEP-1 can act as a transcription factor (Figure 7a). To determine whether CEP-1 could activate transcription from a promotor containing consensus human p53 DNA binding sites we expressed CEP-1 in yeast containing pSS1, a p53 transcriptional reporter construct (Figure 7b; (Ishioka et al., 1993)). CEP-1, like its human counterpart, was found to activate transcription of the pSS1 reporter allowing growth on media lacking histidine (Figure 7c). These observations indicate that the DNA binding specificity of CEP-1 and human p53 are conserved and suggest that endogenous *C. elegans* promoters containing p53-binding elements may respond to CEP-1 *in vivo*. (experiment done by Simon Boulton)

3.1.3 *cep-1* is specifically required for DNA damage induced programmed cell death

To determine whether the sequence conservation of *C. elegans* p53 and human p53 reflects a conserved function we inactivated *cep-1* and analyzed the effect on programmed cell death. As shown previously, DNA damage activates a conserved DNA damage response pathway that induces cell cycle arrest of mitotic germ cells and

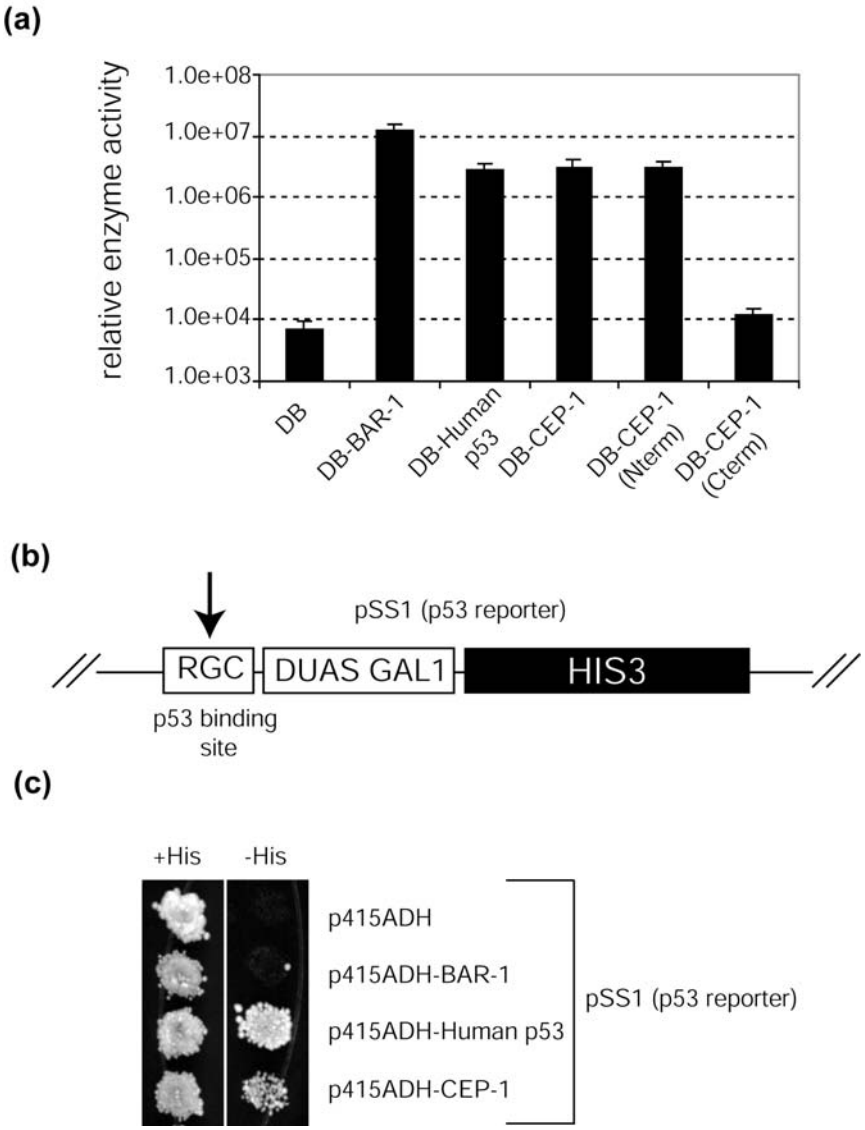
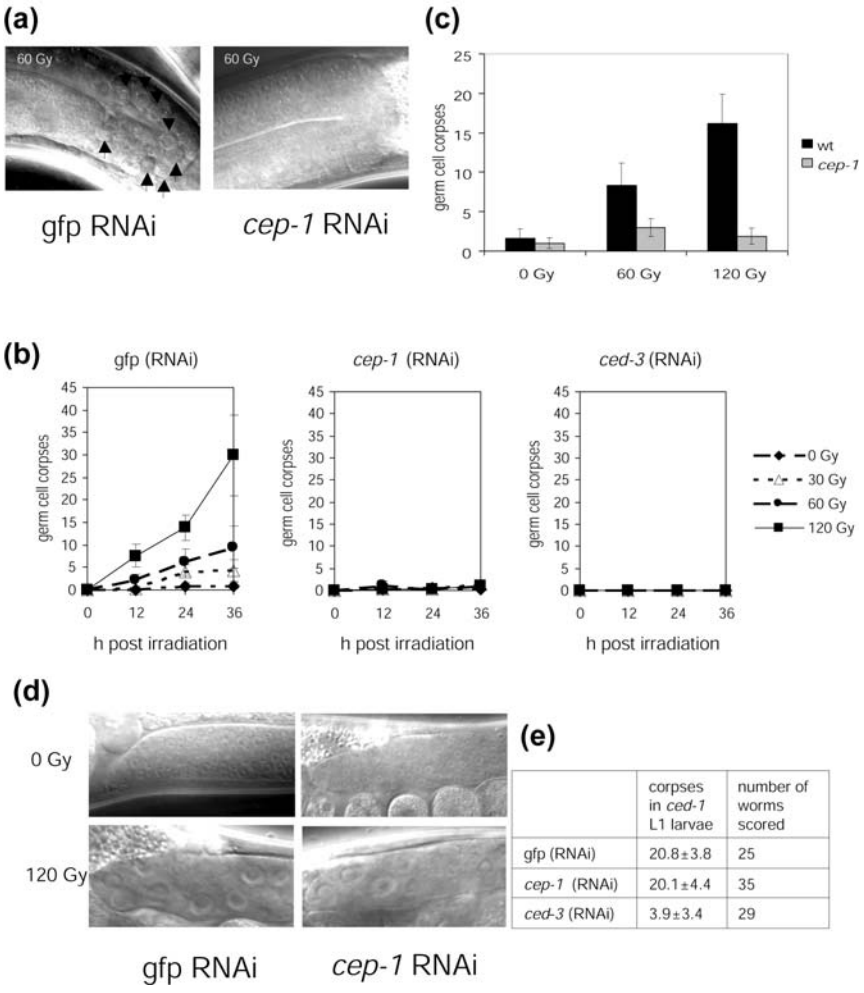


Figure 7 CEP-1 is a transcription factor (a) CEP-1 acts as a transcriptional activator. Various Gal-4 DNA binding domain fusions were introduced into yeast and transcriptional activation was measured by determining ß-Gal activity. **(b)** pSS1 p53 reporter construct. **(c)** Human and *C. elegans* p53 recognize the same consensus p53-binding site. p415ADH, p415ADH-BAR-1, p415ADH-Human p53 and p415ADH-CEP-1 were introduced into yeast strains containing the pSS1 reporter (human p53 binding sites). Transcriptional activation of the reporter was determined by the ability of yeast strains to grow in the absence of histidine. A derivate of the pSS1 reporter construct lacking the p53-binding site did not support growth in the absence of histidine (Simon Boulton, unpublished observation).

apoptosis of meiotic pachytene cells in the *C. elegans* germ line (Gartner et al., 2000). To assess whether *cep-1* functions in the DNA damage response we inactivated *cep-1* by "RNAi feeding". RNA interference (RNAi) leads to a specific inhibition of targeted

genes and has been used to inactivate genes on a genome wide scale (Fraser et al., 2000; Gonczy et al., 2000; Timmons et al., 2001). To inhibit genes involved in post-embryonic processes the RNAi feeding technique is most effective (Fraser et al., 2000). Worms are fed for several generations on bacteria that express double stranded RNA corresponding to a gene of choice and defects can be analyzed in the F1, F2 and F3 generation (Fraser et al., 2000; Timmons et al., 2001). We validated this approach by inactivating the cell death genes *ced-3* and *ced-4* by RNAi. In both cases germ cell death was completely abrogated (Figure 8b, data not shown). RNAi feeding with *cep-1* lead to a complete inactivation of radiation induced germ cell death in worms that were treated with increasing dosages of irradiation (Figure 8a and 8b). To determine whether this effect was specific to radiation induced germ cell death we also scored for the level of germ cell apoptosis in non-irradiated *cep-1* RNAi treated animals (Figure 8b). Physiological germ cell death is genetically distinct from radiation induced cell death and manifests itself by the presence of 0 to 4 corpses per germ line bend at any given time in non-irradiated worms (Gumienny et al., 1999). We did observe a slight but significant reduction of physiological germ cell death, (0.33 ± 0.13 n = 15 and 0.94 ± 0.27 n = 16 corpses 36 hours post L4 larval stage in *cep-1* and gfp RNAi respectively).

We conclude that CEP-1 is specifically required for DNA damage induced programmed cell death. The effect on germ cell apoptosis in non-irradiated worms is likely to be a consequence of either endogenous DNA damage or a result of meiotic recombination defects, which induce apoptosis. The latter possibility is supported by the observation that inhibition of RAD-51, a recA homolog that is required for strand invasion in meiotic recombination (see below), triggers the DNA damage checkpoint and leads to germ cell apoptosis (Gartner et al., 2000).



	corpses in *ced-1* L1 larvae	number of worms scored
gfp (RNAi)	20.8±3.8	25
cep-1 (RNAi)	20.1±4.4	35
ced-3 (RNAi)	3.9±3.4	29

Figure 8 *cep-1* is specifically required for DNA damage induced apoptosis. (a) Nomarski picture showing the pachytene section of the germ line taken 36h post 60 Gray irradiation. Corpses are indicated by arrows. Unrelated gfp sequence was used as control RNAi. **(b)** Germ cell corpses were counted at different time points after irradiation. **(c)** *cep-1* RNAi phenotype is mimicked by co-suppression. 50ng/µl *cep-1* was co-injected with 50ng/µl pRF-4 *rol-6* marker. Germ cell apoptosis was scored 36h post irradiation. **(d)** Mitotic cell cycle arrest is not affected by *cep-1* RNAi. Pictures showing the mitotic compartment of the germ line were taken 12h post irradiation. Upon irradiation mitotic cells arrest as cell number decreases and nuclei and cytoplasm enlarges. In contrast, checkpoint defective mutants such as *mrt-2(e2663)* show the same number of small mitotic cells as non-irradiated wildtype worms (Gartner et al., 2000). **(e)** Somatic programmed cell death is unaffected by *cep-1* in *ced-1(e1935)* engulfment defective L1 larvae as *cep-1* (RNAi) worms show as many unengulfed corpses as control RNAi whereas *ced-3* RNAi results in a 80% reduction of somatic programmed cell death in this assay. Corpses in the head were counted.

The second response to the presence of DNA damage in the *C. elegans* germ line is a transient cell cycle arrest. Mitotic cells in the distal arm of the *C. elegans* germ line transiently halt cell proliferation after irradiation but continue to grow as indicated by a decrease in cell number and enlargement of cellular and nuclear size (Gartner et al., 2000). The checkpoint mutants *mrt-2(e2663)* and *rad-5(mn159)* are defective in this response (Gartner et al., 2000). Surprisingly, RNAi feeding of *cep-1* did not affect cell cycle arrest after irradiation as mitotic germ cells from *cep-1* RNAi worms responded to irradiation comparably to WT (gfp control RNAi; Figure 8d). It is therefore conceivable that *cep-1* is dispensable for DNA damage induced cell cycle arrest. However, since there currently are no cytological markers for various cell cycle phases available we cannot exclude the possibility that c*ep-1* might only be required for a G1/S checkpoint. Cells defective for *cep-1* may in the type of experiment performed here still arrest the cell cycle at a G2/M checkpoint and will appear to respond to DNA damage in a wild type manner.

To confirm that inactivation of *cep-1* leads to an inhibition of radiation induced cell death we used a second, independent method to inactivate *cep-1* in the germ line. DNA co-suppression is based on the observation that a high copy number of a gene leads to a specific inactivation of this gene in the germ line (Ketting and Plasterk, 2000; Dernburg et al., 2000). This effect, which genetically partially overlaps with the RNAi phenomenon, is presumably due to the formation of ds RNAi due to transcription from copies of the transgene oriented in opposite directions (Ketting and Plasterk, 2000; Dernburg et al., 2000). We therefore generated transgenic worm lines that contained the pRF-4 roller marker as well as a high concentration of *cep-1* (promoter and first 3 exons). Upon irradiation of *cep-1* co-suppression lines we confirmed the results of our previous RNAi experiments: radiation induced cell cycle arrest was maintained whereas radiation induced pachytene cell apoptosis was completely abrogated (Figure 8c; data not shown).

We next wished to determine whether RNAi of *cep-1* would affect somatic cell death. In order to score for defects in somatic cell death we scored the number of corpses in the anterior part of young L1 larvae of a *ced-1(e1935)* corpse engulfment defective strain (Figure 8e). During embryonic and larval development 131 cells die by programmed cell

death and are consequently engulfed by neighboring cells. In a *ced-1(e1935)* mutant corpse engulfment is partially blocked and therefore some corpses persist in the L1 larvae (Hengartner et al., 1992; Zhou et al., 2001). *cep-1* RNAi resulted in the same number of persitant corpses in a *ced-1(e1935)* larvae whereas RNAi of *ced-3* lead to a 80% reduction of somatic programmed cell death in this assay (Figure 8e). We thus conclude that *cep-1* may specifically affect radiation-induced germ cell death consistent with p53 function in stress-induced apoptosis in mammals.

As RNA interference and co-suppression might only lead to partial reduction of a gene product we acquired a deletion mutant of *cep-1*, *cep-1(lg12501)*. *cep-1(lg12501)* carries a deletion of 1213bp effectively deleting exon 10, 11, and 12. We did not detect any *cep-1* mRNA in this mutant (data not shown), indicating that *cep-1(lg12501)* is a genetic null mutant of *cep-1*. The deletion mutant confirmed the results we obtained in the above-described RNAi and co-suppression experiments. The deletion mutant showed a slightly more dramatic effect on DNA damage induced apoptosis (Figure 10) and also supports our data on a slight but significant reduction on germ cell death in unperturbed worms (0.53 ±0.12 (n =40) and 1.71 ±0.22 (n=83) corpses 36 hours post L4 larval stage in *cep-1(lg12501)* and wild type worms, respectively).

3.1.2 Discussion: *C. elegans* p53

In conclusion, we have identified a functional *C. elegans* homolog of p53 that is required for radiation-induced apoptosis. While CEP-1 is most closely related to *Drosophila* p53, the sequence similarity is subtle (<20% identity) and is not revealed by conventional sequence comparison methods like BLAST (Altschul and Koonin, 1998). Functional conservation at such a low level of sequence similarity underscores the potential of the generalized profile method for the detection of homologs in distantly related model organisms (Bucher et al., 1996). Our experimental findings support the notion of an ancient function of p53 in DNA damage induced apoptosis (Brodsky et al., 2000; Ollmann et al., 2000). As is the case in *Drosophila, cep-1* function impinges on radiation induced programmed cell death but not on radiation induced cell cycle arrest. It is noteworthy that p53 is highly expressed in the germ line of flies, clams and mammals

(Ollmann et al., 2000; Hall and Lane, 1997). Here we report that *cep-1* function is required for DNA damage induced germ cell death in the *C. elegans* germ line. It is thus worth speculating about the selective advantage conferred by p53 expression in the germ line. In adult *C. elegans* hermaphrodites the germ line is the only proliferative tissue and approximately two thirds of embryonic cell division occur within the very first hours after fertilization apparently without any DNA damage checkpoints. To guard its progeny from acquiring deleterious mutations it would seem advantageous to install sensitive DNA damage checkpoints in the germ line. In *C. elegans* this is achieved by making only meiotic pachytene cells competent to die by DNA damage induced apoptosis. Given that meiotic recombination is being completed in the pachytene stage this checkpoint also guards from mistakes that may arise when SPO-11 induced double strand breaks, which are required to initiate meiotic recombination, are left unprocessed. In this light it is interesting that the absence of mouse p53 leads to a reduced amount of germ cell apoptosis resulting in a high frequency of abnormal sperm (Beumer et al., 1998; Yin et al., 1998). Thus, p53 may have an important and conserved role in maintaining the fidelity of germ cells by the elimination of compromised cells. In accordance to this we observed a significant reduction in germ cell death in the absence of ionizing irradiation. This fraction of germ cell death might result from failures in meiotic recombination, as those failures lead to checkpoint dependent apoptosis (Gartner et al., 2000). Many important cellular pathways have been identified by genetic methods in *C. elegans*. The study of the worm p53 pathway is likely to provide new insights into mammalian p53 regulation.

3.2 CEP-1 target genes

3.2.1 CEP-1 triggers apoptosis through transcriptional induction of the BH3 only proteins EGL-1 and CED-13

After we had shown that CEP-1 triggers apoptosis following DNA damage we next asked through which mechanism CEP-1 might activate the apoptotic program. Developmental cell death in *C. elegans* is triggered by the BH3 only protein EGL-1. In cells that are destined to die during embryonic and larval development EGL-1 is transcriptionally induced (Conradt and Horvitz, 1999) and binds to CED-9 (Conradt and Horvitz, 1998; Parrish et al., 2000; del Peso et al., 2000), which then releases CED-4 that in turn activates the caspase CED-3 (Spector et al., 1997; Chinnaiyan et al., 1997b; Wu et al., 1997). In physiological germ cell death, however, EGL-1 is not involved (Gumienny et al., 1999). It is not known yet which molecule inhibits CED-9 in these cell deaths. An *egl-1* loss-of-function mutant shows an attenuated apoptotic response to DNA damage (Gartner et al., 2000). This genetic evidence suggests that EGL-1 plays a role in irradiation induced cell death. As CEP-1 is a transcription factor (see above) we ask whether *egl-1* is transcriptionally induced upon ionizing irradiation and whether this induction would be dependent on CEP-1. CEP-1 is able to recognize the same promoter consensus site as human p53 (Figure 7c). We identified a sequence upstream of the *egl-1* open reading frame that closely resemble a p53 consensus site supporting the hypothesis that *egl-1* might be a CEP-1 target gene (Figure 9a). Using quantitative real time PCR, we observed a 5-fold induction of *egl-1* transcript following 120 Gy of irradiation (Figure 10a). We verified this level of upregulation by Northern blot (data not shown). We next looked at *egl-1* transcript in a *cep-1* deletion mutant. In *cep-1(lg12501) egl-1* transcript remained at the same level as in non-irradiated wild type worms (Figure 10a). Therefore, *egl-1* mRNA is upregulated upon DNA damage and this upregulation is dependent in *cep-1*.

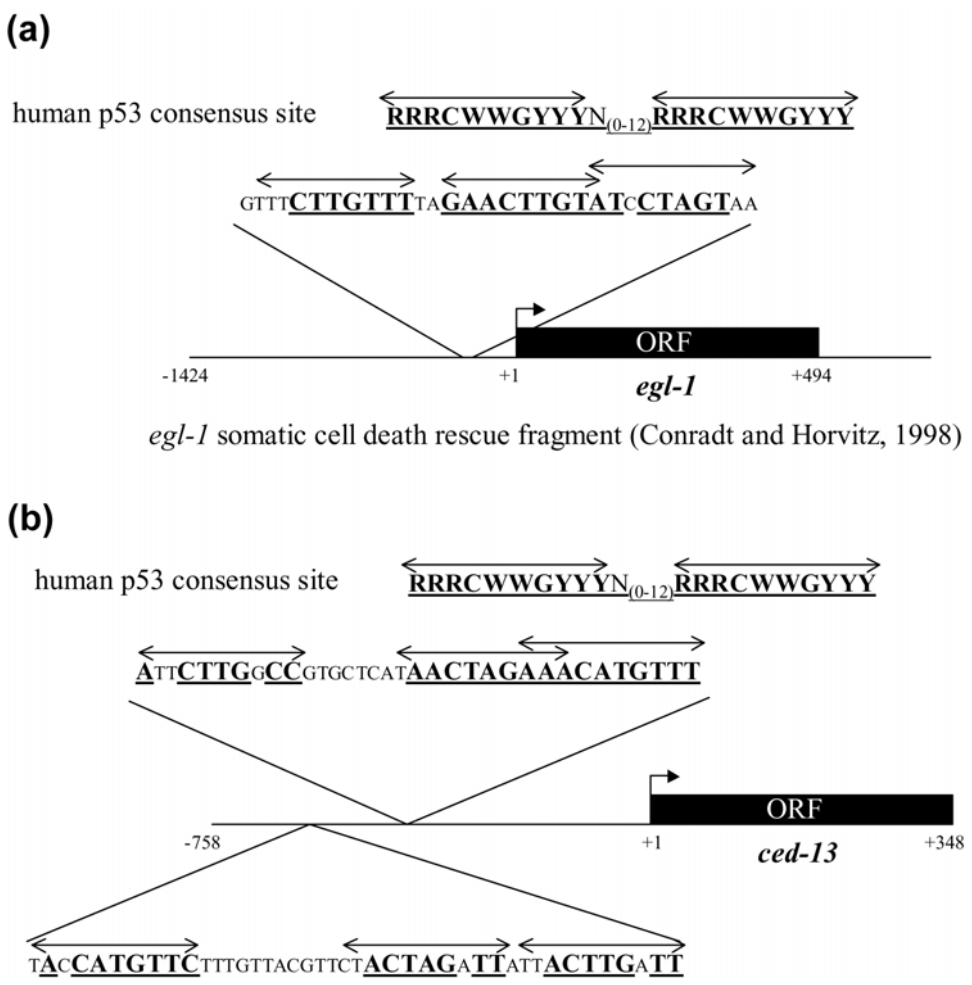


Figure 9 Putative CEP-1 binding sites upstream of the *egl-1* (a) and *ced-13* (b) open reading frames. p53 consensus site as defined by el-Deiry et al. 1992 (el Deiry et al., 1992)

As *cep-1* mutant worms are completely, but *egl-1* mutant worms only partially defective in DNA damage induced apoptosis (Figure 11) (Gartner et al., 2000; Schumacher et al., 2001) there is likely to be an additional cell death trigger acting in parallel to EGL-1 downstream of CEP-1. As *egl-1* is a BH3 only protein and the only known cell death trigger in *C. elegans* we hypothesized that such an additional trigger might be related to *egl-1*. The *C. elegans* genome contains one additional BH3 only gene, which we called *ced-13* (Figure 12)(Zhao et al., 2000).

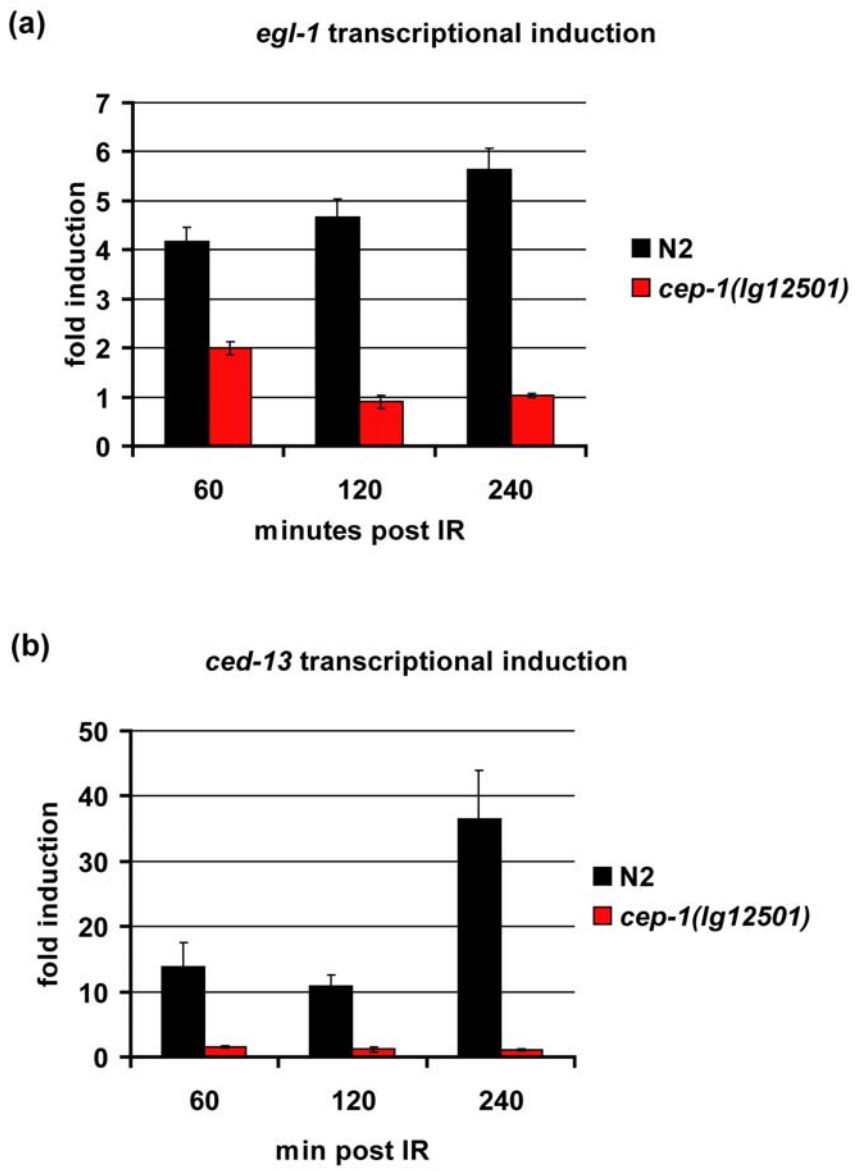


Figure 10 BH3 only genes *egl-1* (a) and *ced-13* (b) are CEP-1 dependently upregulated upon DNA damage. Worms were irradiated as young adults. Quantitative PCR shows *egl-1* and *ced-13* induction at indicated time points after 120 Gy of irradiation. Transcripts of irradiated versus unirradiated worms were normalized to gamma tubulin transcripts and compared.

Similar to the *egl-1* promotor we found two putative CEP-1 recognition sites upstream of the *ced-13* open reading frame (Figure 9b). To assess the function of *ced-13* we looked at the transcriptional level of *ced-13* following DNA damage. *ced-13* mRNA was virtually absent in non-treated animals as it was at the limit of detection by quantitative real time PCR. Upon 120Gy of ionizing irradiation we observed a 35-fold upregulation of *ced-13*

transcript (Figure 10b). In a *cep-1* deletion mutant *ced-13* mRNA remained at the levels of non-irradiated wild type worms (Figure 10b). *ced-13* and *egl-1* transcriptional induction upon DNA damage, therefore, depends on CEP-1.

As *ced-13* is induced upon DNA damage we wished to evaluate its function in DNA damage induced apoptosis. To this end we looked at germ cell apoptosis following ionizing irradiation in a *ced-13* deletion mutant. The deletion mutant *ced-13(tm536)* carries a deletion that comprises the entire *ced-13* open reading frame. As shown in Figure 11 *ced-13(tm536)* showed an apoptotic response comparable to wild type animals. Therefore, *ced-13* is not essential for DNA damage induced apoptosis. When we looked at *ced-13* function in a sensitized background of the below described mutant *gld-1(op236)*, however, we observed a significant reduction of DNA damage induced cell death, suggesting a minor role for *ced-13* as apoptotic trigger (Figure 19, see below).

If CED-13 was acting in parallel to EGL-1 in triggering cell death upon ionizing irradiation, we expected to see enhancement of *egl-1* loss-of-function by a deletion mutant of *ced-13*. When we looked at an *egl-1(n1084n3082);ced-13(tm536)* double mutant the apoptotic response to DNA damage was abrogated to the same level as in *cep-1(lg12501)* (Figure 11).

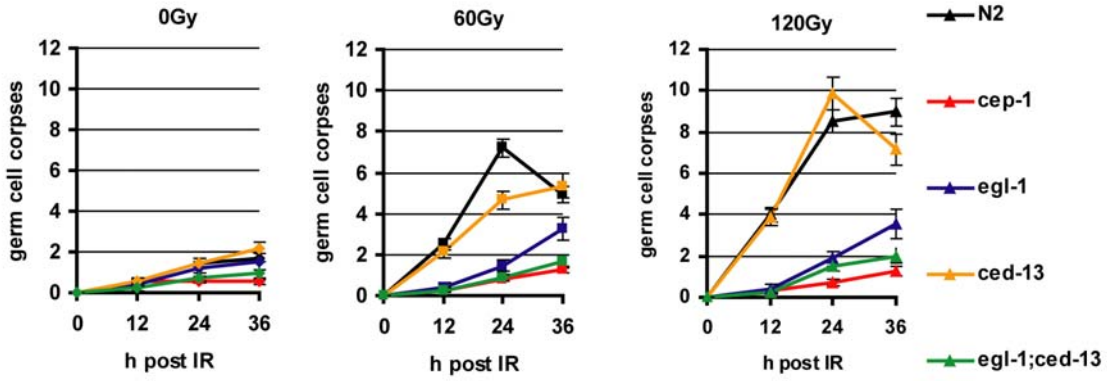


Figure 11 *ced-13* enhances *egl-1* in DNA damage induced apoptosis. Worms were irradiated with indicated dosages of ionizing irradiation at the L4 larval stage. Corpses were counted in Nomarski optics at indicated time points. Average of three independent experiments is shown. Error bars represent standard error of the mean (SEM).

We conclude that CEP-1 induces both *C. elegans* BH3 only proteins EGL-1 and CED-13, which in parallel act on CED-9 to trigger apoptosis (as CED-13 is able to bind CED-9 in the same fashion as EGL-1 (Shai Shaham, personal communication)).

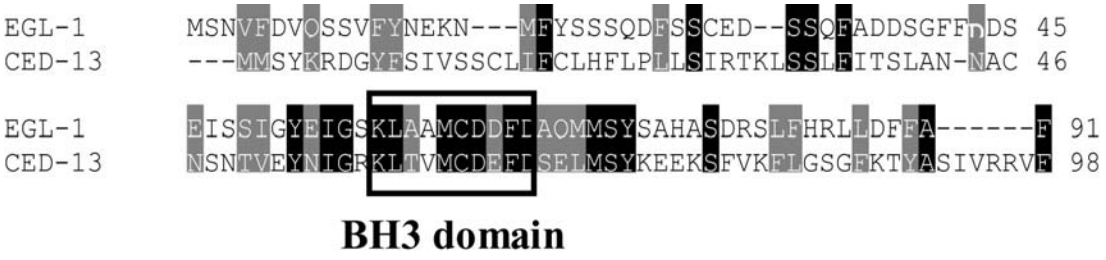


Figure 12 Sequence alignment of the two *C. elegans* BH3 only proteins. The BH3 motif is indicated.

3.2.2 DNA damage and CEP-1 dependent genome wide expression analysis

We next asked if there might be transcriptional targets in CEP-1 mediated DNA damage induced apoptosis in addition to *egl-1* and *ced-13*. To this end we evaluated mRNA expression in dependence of ionizing irradiation and CEP-1 through a *C. elegans* Affymetrix gene expression chip. The Affymetrix chip contains oligonucleotides that cover 22,629 predicted open reading frames and was costumized for Aventis GmbH, which collaboratively perfomed the Affymetrix chip experiment. Results are summarized in Tables 1-6. We treated N2 worms and *cep-1* deletion mutants with 120Gy of ionizing radiation (IR) and compared expression profiles of IR treated vs. non-treated worms. The worms were irradiated as young adults (24h post L4 larval stage) and RNA was isolated 2 hours post treatment. We found 8 genes that showed IR and CEP-1 dependent regulation similar to *egl-1*. Remarkably, *egl-1* showed the strongest DNA damage dependent upregulation (2.4 fold) (Table1). In addition we found 88 genes that were induced upon DNA damage independently of *cep-1* (Table 2), 11 genes that were IR and *cep-1* dependently repressed (Table 3), 12 genes that were IR dependently but *cep-1* independently repressed (Table 4), 11 genes that were *cep-1* dependently but IR independently induced (Table 5), and finally 17 genes that showed *cep-1* dependent but IR independent repression (Table 6). Furthermore, we found that *ced-13* was five-fold upregulated in N2 worms but not in *cep-1* mutant animals. However, the signal for *ced-13* on the Affymetrix chip was considered "absent" by the Affymetrix software. Genes are scored as "absent" when the signal intensity on the Affymetrix chip is below a

threshold value. The threshold value is set by the absolute signal intensity of five different oligonucleotide probes. Roughly 50 percent of the 22,629 genes represented on the Affymetrix chip gave "absent" signals, which is a typical value for Affymtrix chip experiments. We found 339 genes that showed irradiation and *cep-1* dependent induction but were scored as "absent" by the Affymetrix software due to weak signal intensity (data not shown). We are currently confirming the expression data by quantitative real time PCR, particularly the data for those genes that were scored as "absent" on the Affymetrix chip. As a next step we will evaluate all IR and CEP-1 dependently regulated genes by RNA interference, as genome wide RNAi libraries are readily available (Kamath et al., 2003).

	Wormbase	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	F23B12.9	*egl-1*	**2.40**	0.80	**3.01**	3.73	1.24
2	F38H12.5	DUF40	**1.55**	0.55	**2.83**	2.67	0.95
3	F09A5.2	tyrosine-protein kinase	**1.55**	0.69	**2.23**	1.65	0.74
4	K10B4.2	DUF32	**1.96**	1.01	**1.93**	1.97	1.02
5	Y47G7B.2	n/a	**1.88**	0.99	**1.91**	2.91	1.53
6	C01B12.3	Putative membrane protein	**1.73**	0.96	**1.80**	1.74	0.97
7	Y54E2A.8	n/a	**1.54**	0.89	**1.72**	1.59	0.92
8	R09E12.5	n/a	**1.55**	1.03	**1.50**	2.08	1.39
9	F59F5.2	n/a	**1.61**	1.15	**1.41**	1.37	0.98

Table 1 IR and *cep-1* dependently upregulated genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2rad/N2crt>1.5, N2ind/*cep-1*ind>1.5 Wormbase=sequence name at www.wormbase.org , rad=worms treated with 120Gy of ionizing irradiation, crtl=non-irradiated control samples, ind= rad/crtl, DUF=domain of unkown function. Slight deviations from the criteria were allowed in some cases.

	WormbaseID	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	F49F1.6	Cytochrome c heme-binding site, Metridin-like ShK toxin domain	**240.19**	103.32	2.32	**1.01**	0.43
2	AU112775	n/a	**10.19**	8.72	1.17	**0.83**	0.71
3	F55G11.5	DUF141	**10.05**	7.80	1.29	**0.91**	0.71
4	F56D6.1	C-type lectin	**9.77**	6.51	1.50	**0.91**	0.61
5	F56D6.2	C-type lectin	**9.40**	6.51	1.44	**0.98**	0.68
6	C32H11.10	DUF141	**9.21**	7.20	1.28	**0.73**	0.57
7	K01D12.11	Glutathione S-transferase	**8.77**	7.63	1.15	**1.08**	0.94
8	C49G7.7	DUF141	**8.48**	1.98	4.28	**1.16**	0.27
9	C17H12.8	DUF141	**8.17**	8.69	0.94	**1.00**	1.07
10	C54D10.1	Glutathione S-transferases	**7.67**	5.37	1.43	**1.20**	0.84
11	K08D8.5	DUF141, CUB domain	**7.60**	6.23	1.22	**1.03**	0.85
12	Y41C4A.11	WD domain, G-beta repeat	**7.52**	5.45	1.38	**1.18**	0.86
13	T24B8.5	Metridin-like ShK toxin domain	**7.46**	5.76	1.29	**1.13**	0.87
14	F35E12.8	DUF141, CUB domain	**7.38**	6.51	1.13	**1.14**	1.00
15	C17H12.8	DUF141	**6.21**	6.77	0.92	**0.90**	0.98
16	K11H12.4	DUF274	**6.16**	5.84	1.06	**1.14**	1.08
17	T10B9.2	cytochrome P450	**6.15**	4.57	1.35	**1.43**	1.06
18	C31A11.5	Acyltransferase family, Nose resistant to fluoxetine-4	**6.08**	5.27	1.15	**0.97**	0.84
19	ZK1005.1	n/a	**5.66**	7.05	0.80	**0.68**	0.85
20	K08B4.3	glucuronosyltransferase	**5.52**	5.99	0.92	**1.11**	1.21
21	T24C4.4	n/a	**5.08**	4.54	1.12	**0.92**	0.82
22	R10D12.9	MtN3/saliva family	**5.04**	3.12	1.62	**1.01**	0.62
23	C10C5.2	Cyclin-like F-box	**4.50**	5.58	0.81	**0.83**	1.02
24	F35E8.8	glutathione S-transferase	**4.48**	3.06	1.46	**1.29**	0.88
25	C34H4.2	DUF274	**4.14**	3.36	1.23	**1.02**	0.83
26	Y38E10A.5	C-type lectin, CUB domain	**4.12**	5.77	0.71	**0.95**	1.33
27	C17H12.6	DUF141	**4.06**	3.28	1.24	**0.82**	0.66
28	M02F4.7	C-type lectin	**3.95**	3.63	1.09	**0.91**	0.84

Table 2 IR dependently, CEP-1 independently upregulated genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2rad/N2crt>2, N2rad/*cep-1*rad 0.67-1.5. Details as in legend of Table 1.

	WormbaseID	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	GB=C31052	n/a	0.32	**0.79**	**0.40**	0.32	0.80
2	F37B4.2	*icf-1*, intermediate filament protein	0.26	**0.61**	**0.43**	0.27	0.63
3	F14D7.7	n/a	0.58	**1.34**	**0.43**	0.42	0.98
4	Y94H6A.f	n/a	0.51	**1.09**	**0.47**	0.57	1.21
5	F48A11.6	n/a	0.62	**1.30**	**0.48**	0.72	1.51
6	Y105C5B.19	n/a	0.72	**1.48**	**0.49**	0.68	1.40
7	T27A8.2	forkhead domain	0.56	**1.15**	**0.49**	0.72	1.46
8	GB=D33902	n/a	0.67	**1.35**	**0.49**	0.77	1.56
9	R07B7.9	phospholipase	0.59	**1.17**	**0.50**	0.54	1.08
10	GB=T00014	n/a	0.37	**0.74**	**0.50**	0.53	1.06
11	C06G3.7	pyridine nucleotide-disulphide oxidoreductase	0.57	**1.13**	**0.50**	0.71	1.42

Table 3 IR and CEP-1 dependently repressed genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2ind/*cep-1*ind<0.5, *cep-1*rad/*cep-1*crtl 0.67-1.5. Details as in legend of Table 1.

	WormbaseID	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	C44C1.5	n/a	**0.43**	0.54	**0.79**	0.98	1.23
2	C05E11.5	*amt-4*, ammonium transporter	**0.44**	0.48	**0.91**	0.87	0.96
3	C17C3.12	n/a	**0.44**	0.97	**0.45**	0.82	1.81
4	Y48E1B.8	n/a	**0.45**	0.54	**0.82**	1.14	1.39
5	C03H5.1	n/a	**0.45**	0.70	**0.64**	0.66	1.02
6	ZC116.3	bone morphogenetic protein 1 like	**0.45**	0.58	**0.78**	0.80	1.02
7	C07E3.7	homeobox protein	**0.46**	0.86	**0.54**	0.81	1.50
8	D1025.2	n/a	**0.47**	0.57	**0.82**	1.00	1.23
9	T05E7.1	n/a	**0.47**	0.39	**1.20**	1.15	0.96
10	F08A8.2	acyl- A coenzyme oxidase peroxisomal	**0.49**	0.71	**0.69**	0.81	1.16
11	C43H6.1	n/a	**0.49**	0.71	**0.69**	0.76	1.11
12	F07H5.6	n/a	**0.49**	0.69	**0.72**	0.77	1.07

Table 4 IR dependently, CEP-1 independently repressed genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2rad/N2crt<0.5, N2ind/*cep-1*ind 0.67-1.5. Details as in legend of Table 1.

	WormbaseID	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	Y49E10.16	n/a	0.94	0.91	1.04	**14.46**	**13.87**
2	F52B5.5	*cep-1*	0.96	1.02	0.94	**8.50**	**9.04**
3	Y19D10A.7	n/a	1.04	1.06	0.98	**7.92**	**8.11**
4	Y110A2AL.9	n/a	0.76	2.23	0.34	**1.60**	**4.71**
5	F52B5.4	n/a	0.82	0.76	1.08	**3.44**	**3.18**
6	Y19D10A.C	n/a	1.04	1.14	0.91	**2.63**	**2.90**
7	ZK938.6	chitinase domains	0.64	1.19	0.53	**1.52**	**2.85**
8	C01B4.9	n/a	0.81	0.64	1.27	**3.58**	**2.83**
9	Y45G12C.2	*gst-10*, glutathione S-transferase	0.90	0.97	0.93	**2.55**	**2.75**
10	GB=AV180727	n/a	1.06	1.08	0.98	**2.01**	**2.05**
11	C01B4.6	aldose 1-epimerase	1.16	0.82	1.41	**2.85**	**2.02**

Table 5 CEP-1 dependently, IR independently upregulated genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2rad/*cep-1*rad>2, N2crtl/*cep-1*crtl>2 N2ind/*cep-1*ind 0.67-1.5. Details as in legend of

	WormbaseID	Description	N2rad/ N2ctrl	*cep-1*rad/ *cep-1*ctrl	N2ind/ *cep-1*ind	N2rad/ *cep-1*rad	N2ctrl/ *cep-1*ctrl
1	F09C6.10	n/a	0.83	**0.84**	0.99	0.27	**0.27**
2	K08B12.1	n/a	1.70	**1.06**	1.60	0.56	**0.35**
3	GB=C51741	n/a	0.92	**0.71**	1.31	0.50	**0.39**
4	C36H8.2	*inx-6*, UNC-7 like, Innexin	2.45	**1.16**	2.11	0.82	**0.39**
5	Y110A2AL.4	n/a	1.11	**0.77**	1.43	0.60	**0.42**
6	K12H6.9	n/a	1.54	**0.91**	1.68	0.71	**0.42**
7	g4204794	n/a	1.59	**1.33**	1.20	0.54	**0.45**
8	H42K12.3	PAN domain, Thrombospondin, type I	0.94	**1.00**	0.93	0.42	**0.46**
9	GB=AV179123	n/a	1.29	**1.26**	1.02	0.47	**0.46**
10	F38A3.2	collagen	1.45	**1.11**	1.31	0.61	**0.47**
11	W04A8.4	3'-5' exonuclease	1.19	**0.82**	1.45	0.68	**0.47**
12	C33C12.3	glucosylceramidase	1.36	**1.44**	0.94	0.45	**0.48**
13	T03F7.1	Na(+)/Cl(-)-dependent GABA transporter	1.32	**0.95**	1.40	0.67	**0.48**
14	F35D11.8	C-type lectin	1.35	**0.93**	1.45	0.70	**0.48**
15	GB=C56094	n/a	1.75	**0.82**	2.12	1.02	**0.48**
16	ZK180.5	n/a	1.29	**0.84**	1.54	0.76	**0.49**
17	C14C6.5	Metridin-like ShK toxin	1.31	**1.48**	0.88	0.44	**0.49**
18	Y46C8AL.b	n/a	1.19	**1.35**	0.88	0.44	**0.50**

Table 6 CEP-1 dependently, IR independently repressed genes. Ratio of signals of 3 independent Affymetrix chip experiments is given. Criteria were N2crtl/*cep-1*crtl>0.5, *cep-1*rad/*cep-1*crtl 0.67-1.5. Details as in legend of Table 1.

3.2.3 Discussion: CEP-1 mediated apoptosis

We have shown that CEP-1 induces the BH3 proteins EGL-1 and CED-13, which together trigger apoptosis. As suggested in the model in Figure 13 this pathway is likely to present the most ancient apoptosis p53 pathway as Villunger et al. could recently show that the functional homologs of EGL-1 and CED-13, mammalian Puma and Noxa, respectively, are induced by p53 and trigger cell death (Villunger et al., 2003; Jeffers et al., 2003; Shibue et al., 2003). Puma appears to be the major p53 target required for programmed cell death, whereas Noxa plays a more specialized role. This is very similar to our observations in *C. elegans*, where EGL-1 appears to be the major cell death trigger not only in response to DNA damage but also during developmental cell death. CED-13, however, does not function in any cell death other than upon DNA damage. *ced-13* mRNA is only detected upon ionizing irradiation and it is not strictly required to trigger cell death. CED-13 can bind CED-9 similarly as EGL-1 does (Shai Shaham, personal communication). It will be interesting to biochemically dissect the functional difference between the two BH3 only proteins and also evaluate potential other interactors. As these two proteins are likely to be functionally very similar to mammalian Puma and Noxa, further study of the *C. elegans* proteins might further our understanding of apoptotic mechanisms also in higher organisms.

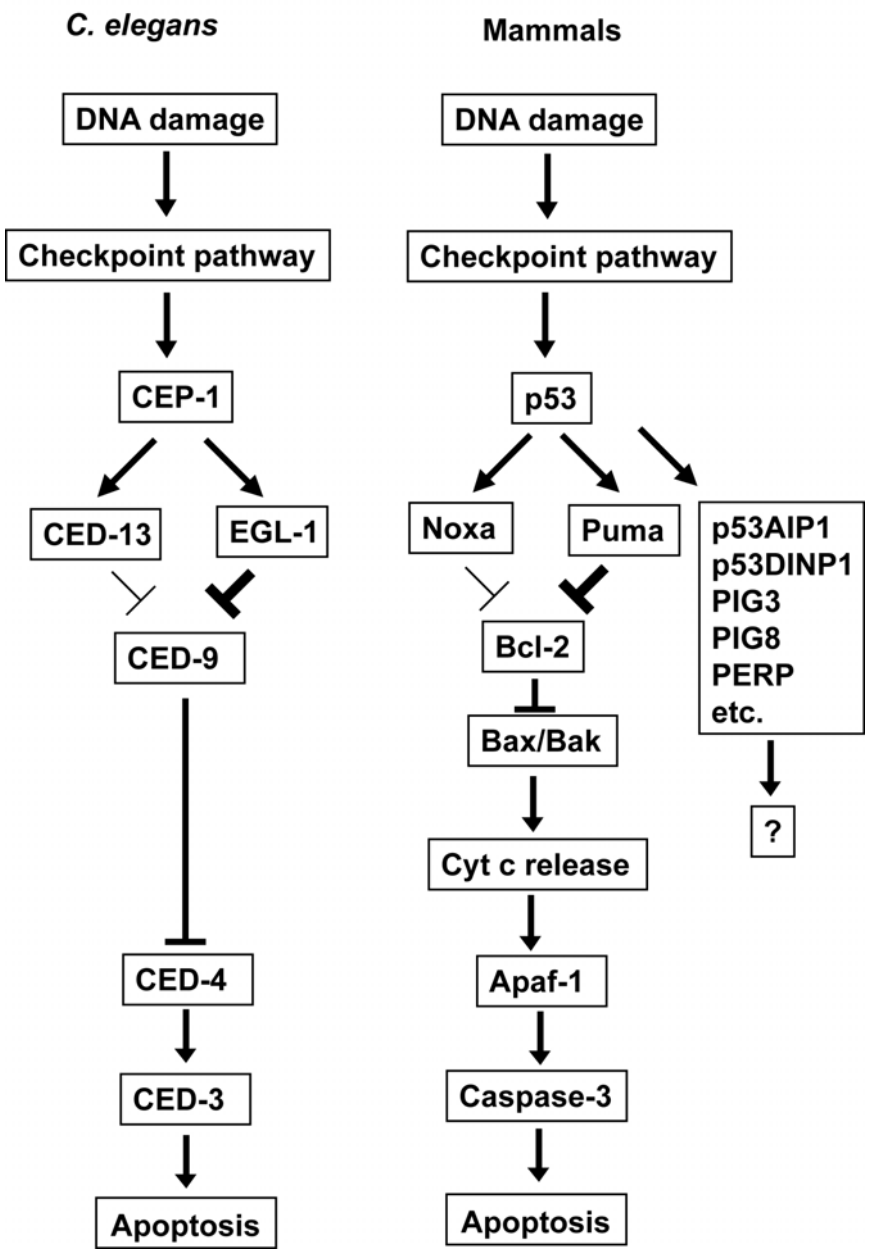


Figure 13 Model for a p53 core pathway. In its most ancient form CEP-1, which is regulated through checkpoint signaling upon DNA damage, transcriptionally induces the two BH3 only proteins EGL-1 and CED-13. This core pathway is conserved from worm to mammals as the role of EGL-1 and CED-13 is taken by Puma and Noxa, respectively. In mammals p53 has acquired a host of additional targets whose physiological relevance has yet to be determined.

3.3 Genetic screen for negative regulators of the *C. elegans* p53 pathway

To find genes that downregulate the p53 pathway in *C.* elegans we conducted a genetic screen for mutants that showed an increased level of programmed cell death upon ionizing irradiation. The screening procedure was facilitated using acridine orange (AO), a dye that specifically stains apoptotic cells during the course of engulfment (Gumienny et al., 1999). As outlined in Figure 14 we treated the F2 generation of mutagenized worms at the L4 larval stadium with 20Gy of ionizing irradiation and assessed levels of programmed cell death by AO staining between 28 and 32 hours post treatment. Wild type worms show only a very mild increase in apoptosis at that dosage. We screened approximately 20,000 genomes and selected mutants that showed a significant increase in acridine orange staining as compared to wild type worms. We identified four mutants that showed a high level of apoptosis following 20Gy of irradiation. One of the mutants showed high levels of cell death independently of DNA damage, and another one showed a high level of embryonic lethality, which most likely is a consequence of defects in meiotic recombination and DNA repair (data not shown). The remaining two mutants *op236* and *op237* we isolated both showed a strong upregulation of programmed cell death following irradiation (Figure 16a,b, data not shown) without concomitant effect on DNA repair activity (see below). We tested both mutants for genetic complementation. Cross-progeny of *op236* and *op237* worms showed similar levels of programmed cell death as parental animals (data not shown). This suggested that both mutations are alleles of the same gene. Sequence analysis revealed that both mutants carry the same point mutation.

Increased apoptosis in response to ionizing irradiation could have at least two causes: Defects in DNA repair or defects in downregulating the apoptotic signaling pathway. Mutations in DNA repair genes not only lead to increased levels of apoptosis, as apoptosis-triggering double strand breaks would accumulate, but also renders the progeny unable to survive, as damaged DNA persists into the embryo, which then ceases to grow (Boulton et al., 2002). To evaluate if *op236* was deficient in repair of damaged DNA we measured DNA damage sensitivity as the survival of progeny after ionizing irradiation

(Table 7). Upon ionizing irradiation the number of eggs laid drops as a result of germ cell death, which leads to a decrease in the number of oocytes that are generated. The progeny of *op236* animals, however, showed the same survival rate as wild type worms indicating that the sensitivity to DNA damage was not affected (Table 7). Therefore, the mutant *op236* showed increased levels of apoptosis without concomitant increased DNA damage sensitivity. We confirmed a normal DNA repair activity in *op236* by staining for the repair molecule RAD-51. RAD-51 forms foci at single-stranded DNA that results from DNA breaks. We did not see an elevated accumulation of RAD-51 foci in irradiated *(op236)* animals as compared to wild type worms. RAD-51 foci were comparable to the ones in wild type worms (data not shown). We conclude that the increased levels of apoptosis in *op236* upon ionizing irradiation are not caused by defects in DNA damage repair.

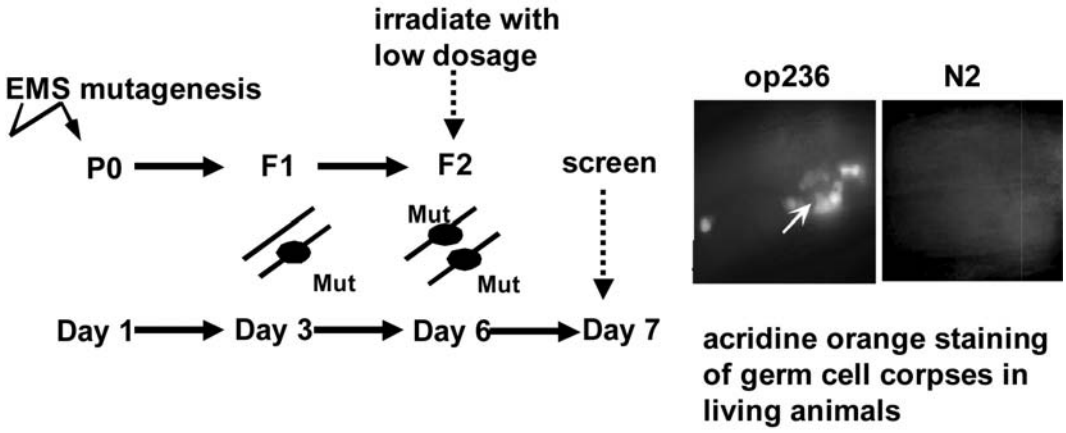


Figure 14 Outline of a genetic screen for negative regulators of the p53 pathway. P0 worms were mutagenized with 25mM EMS. F2 generation, which is homozygous for EMS induced mutations was irradiated with 20Gy of ionizing irradiation at L4 larval stage. 24h later germ cell corpses were visualized by AO staining. Worms with strong AO staining, indicative of elevated levels of cell death, were selected.

To evaluate whether the apoptotic phenotype of *(op236)* was due to DNA damage or simply a result of a general stress response we knocked down *rad-51* by RNA interference. *rad-51* is a functional homolog of bacterial recA and is involved in strand invasion in meiotic recombination (Alpi et al., 2003). RNAi of *rad-51* leads to unprocessed meiotic recombination intermediates in meiotic pachytene cells. These recombination intermediates are, similarly to DNA double strand breaks, recognized by

the DNA damage checkpoint that then invokes an apoptotic response (Gartner et al. 2000). *rad-51* RNAi in wild type worms increased the number of dying cells to 6.7 (±0.8) (Figure 15). RNAi of *rad-51* in *op236* animals, however, lead to an average of 14.8 (±1.4) dying cells in meiotic pachytene (Figure 15). Therefore, the increase in programmed cell death in *op236* is a specific response to damaged DNA

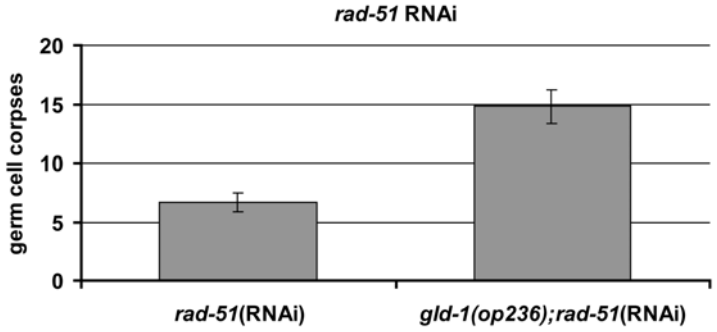


Figure 15 upregulation of apoptosis in *gld-1(op236)* is specific to DNA double strand breaks. *rad-51(RNAi)*, which leads to failures in meiotic recombination upregulates germ cell death more strongly in *gld-1(op236)* than in wild type worms. The F1 generation of RNAi treated animals was analyzed for apoptosis as young adults. Error bars indicate standard error of the mean.

Upregulation of the apoptotic response to DNA damage might be caused either by upregulation of checkpoint signaling in general or by specific upregulation of apoptotic signaling. To test whether this mutant upregulates checkpoint response in general or specifically induces programmed cell death we looked at the second output of checkpoint signaling besides apoptosis. Mitotic germ cells arrest the cell cycle progression in response to ionizing irradiation but continue to grow (Gartner et al., 2000). As a consequence the number of mitotic cells drops as they grow in size. We quantified the number of mitotic cells in mutant worms and found it equally decreased upon ionizing irradiation as in wild type worms (Figure 16c). Therefore, the cell cycle arrest response was unaffected by *op236*.

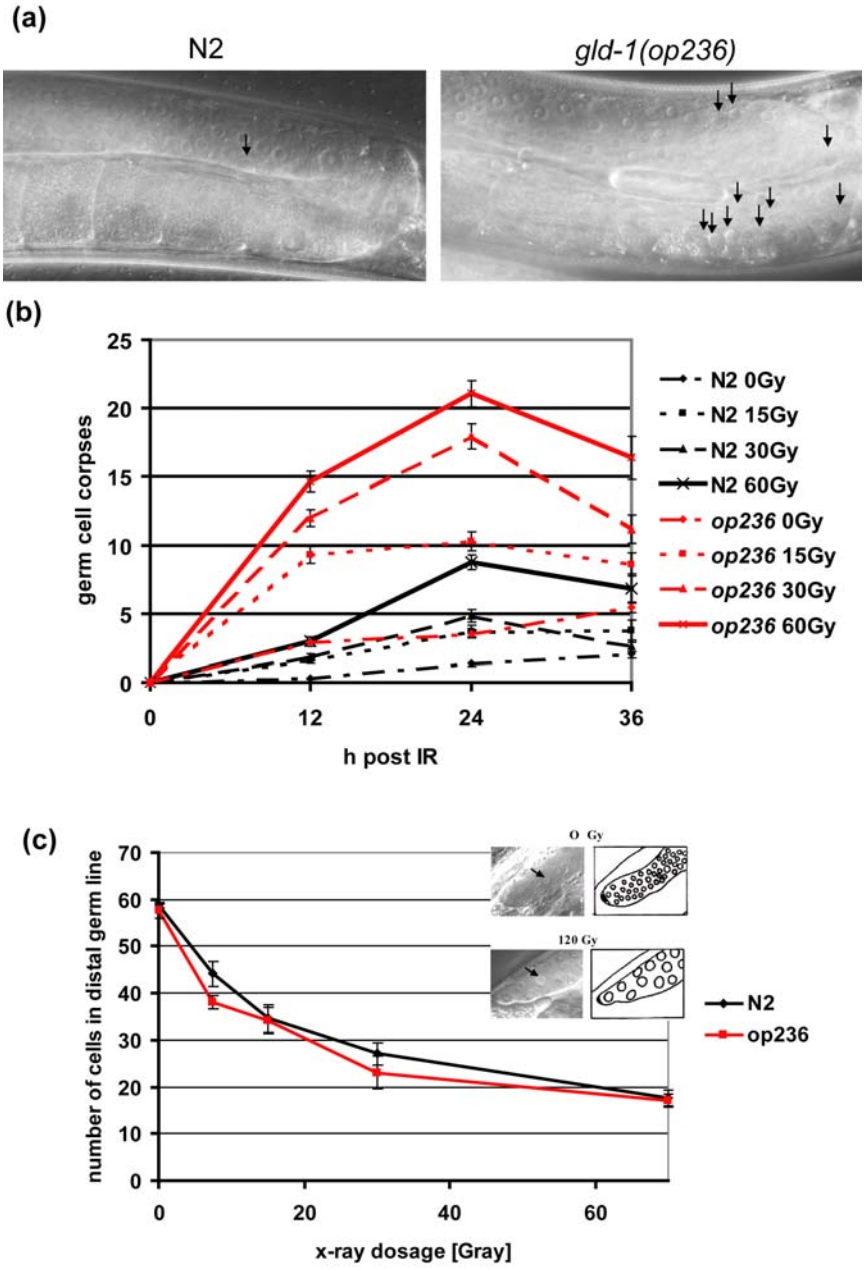


Figure 16 *op236* shows specific upregulation of DNA damage induced apoptosis. (a) Nomarski picture showing germ cell death in *op236* compared to wild type worms. Germ cell corpses are marked with arrows. **(b)** Worms were irradiated and corpses counted as in Figure 11. **(c)** *op236* does not affect checkpoint dependent cell cycle arrest upon DNA damage. Mitotic arrest was quantified by counting the number cell nuclei in the mitotic region of the germ line. Error bars indicate standard error of the mean.

3.3.1 Positional cloning of *op236* reveals a novel mutation in the *C. elegans* tumor suppressor GLD-1

We mapped the mutation in *op236* by positional cloning and SNP (single nucleotide polymorphism) mapping. We finally sequenced all 14 predicted ORFs between T23G11.5 and F58H10.5. We only found one mutation in T23G11.3, which codes for *gld-1*. This mutation is a G to T transversion at position 826 leading to a Valine to Phenylalanine mutation at position 276, which lies in the GSG/STAR RNA binding domain in the germ line tumor suppressor *gld-1*(germ line defective) (Figure 17a). The Valine 276 within the GSG/STAR domain is conserved in the *Drosophila* and human GLD-1 homologs, Who/How and the quaking protein, respectively (Figure 17b).

There have been 31 *gld-1* alleles described previously (Francis et al., 1995). These alleles form distinct phenotypic classes. Complete loss-of-function mutations give rise to a germ line tumor as germ cells exit pachytene, enter mitosis and continue to divide mitotically. Partial loss-of-function mutations feminize the germ line or give rise to undifferentiated oocytes, whereas gain-of-function mutations masculinize the germ line (Francis et al., 1995). None of these alleles has been implicated in programmed cell death. The GLD-1 protein has been shown to bind and translationally repress target mRNAs (Lee and Schedl, 2001).

To evaluate if the mutation in *gld-1* was responsible for the *op236* phenotype we tested if a complete loss-of-function mutation of *gld-1* would fail to complement *op236*. The rationale here was that if the apoptotic phenotype of *op236* was indeed due to the mutation in *gld-1*, *op236* should rescue the tumorous phenotype of a complete loss-of-function mutation of *gld-1* but a complete loss-of-function mutation should fail to rescue the apoptotic phenotype of *op236*. Heterozygous *op236*/+ worms show wild type levels of apoptotic cells upon ionizing irradiation indicating that *op236* is a recessive loss-of-function mutation (data not shown). (Only at high dosages of ionizing irradiation *op236*/+ showed increased apoptotic levels as compared to wild type worms (data not shown), indicating that some degree of hypomorphism.) *op236* mutants heterozygous with a complete loss-of-function allele *gld-1(q485)*, however, showed the same apoptosis levels as *op236*. *op236*, on the other hand, rescued the tumorous germ line phenotype of *gld-1(q485)* (but not the sperm defects of *gld-1(q485)* (Figure 18, data not shown)). We next

tested whether exogenous GLD-1 protein could rescue *op236*. *op236* heterozygous with *gld-1(q485)*:GLD-1-GFP indeed suppressed the *op236* apoptotic phenotype (Figure 18). In conclusion, the above data show that *op236* is a novel allele of *gld-1*.

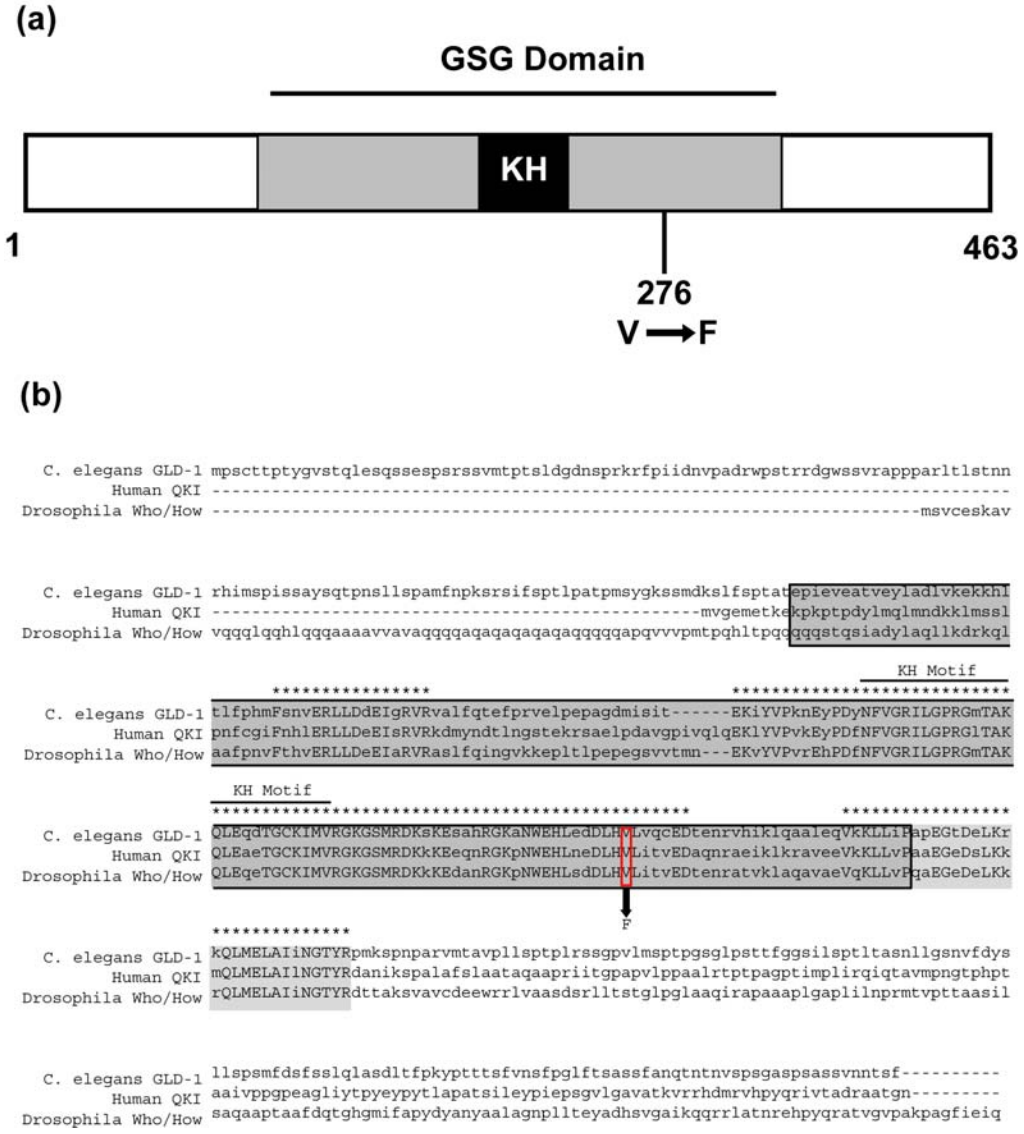


Figure 17 Domain structure of GLD-1. *gld-1(op236)* point mutation V276F lies in the GSG/STAR RNA binding domain **(a)**. **(b)** Alignment of GLD-1 with the human quaking protein (QKI) and *Drosophila* Who/How. KH motif is indicated. The GSG/STAR domain is marked with a box in dark gray, the CGA (Carboxy-terminal GSG domain associated), as defined by Jones and Schedl 1995 (Jones and Schedl, 1995) in light gray. V276F mutation in *gld-1(op236)* is marked in red. * indicate conserved aminoacids (identical or similar)

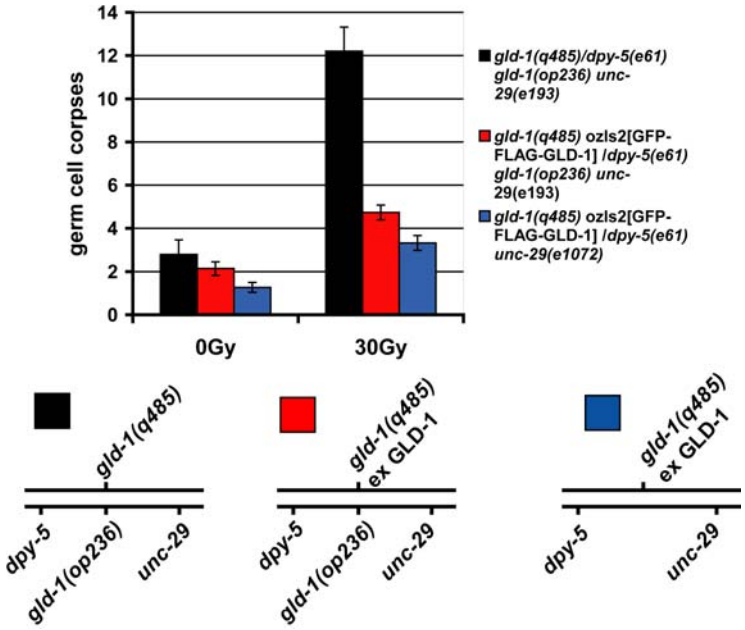


Figure 18 *op236* phenotype is caused by a mutation in *gld-1*. Genetic complementation was done by crossing a complete loss-of-function allele of *gld-1(q485)* with *gld-1(op236)* or *gld-1(q485)* carrying one copy of exogenous GLD-1. The indicated genotypes in the F1 generation were analyzed. *gld-1(q485)* did not complement *gld-1(op236)* (black bars). GLD-1 expression rescues *gld-1(op236)* (red bars). GLD-1 expression by itself has no effect on apoptosis (blue bars). Error bars indicate standard error of the mean.

3.3.2 Genetic characterization of *gld-1(op236)*

To test if *gld-1(op236)* showed any of the germ cell differentiation defects characteristic for the various other *gld-1* alleles we looked at germ cells by Nomarski optics as well as DAPI staining. In both assays the *gld-1(op236)* germ lines resembled wild type germ lines (data not shown).

We next evaluated if the cell death we saw in *gld-1(op236)* was dependent on the apoptotic core machinery. To this end we asked whether the *C. elegans* apoptosis genes *ced-3* and *ced-4* would suppress *gld-1(op236)*. Loss-of-function mutations *ced-3(n717)* and *ced-4(n1162)* completely and a gain-of-function allele of *ced-9(n1950)* strongly suppressed apoptosis in *gld-1(op236)* (data not shown). *gld-1(op236)*, however, enhanced the oocyte differentiation defects of *ced-3* and *ced-4* (Gumienny et al., 1999) mutants just as a loss-of-function mutation in *ced-9(n1653ts)* does (see below). Therefore, *gld-1* acts upstream of *ced-9*, *ced-4* and *ced-3* in apoptotic signaling.

To genetically characterize *gld-1* involvement in DNA damage induced apoptosis we did genetic epitasis analysis. DNA damage is sensed and relayed by a conserved checkpoint pathway that activates the *C. elegans* homolog of the tumor suppressor p53 *cep-1* (Boulton et al., 2002; Schumacher et al., 2001; Derry et al., 2001). CEP-1 in turn transcriptionally induces the BH3 only proteins EGL-1 and CED-13 ((Hofmann et al., 2002), see above). A deletion mutant of *cep-1(lg12501)* completely suppressed *gld-1(op236)* induced apoptosis (Figure 19). Also, mutations in the *cep-1* target gene *egl-1* strongly suppressed *gld-1(op236)*. The second known CEP-1 target gene, *ced-13* mildly suppressed *gld-1(op236)* consistent with the mild *ced-13(tm536)* apoptotic phenotype (Figure 11,19). An *egl-1(n1984n3082);ced-13(tm536)* double mutant completely suppressed *gld-1(op236)* as *cep-1(lg12501)* did (Figure 19).

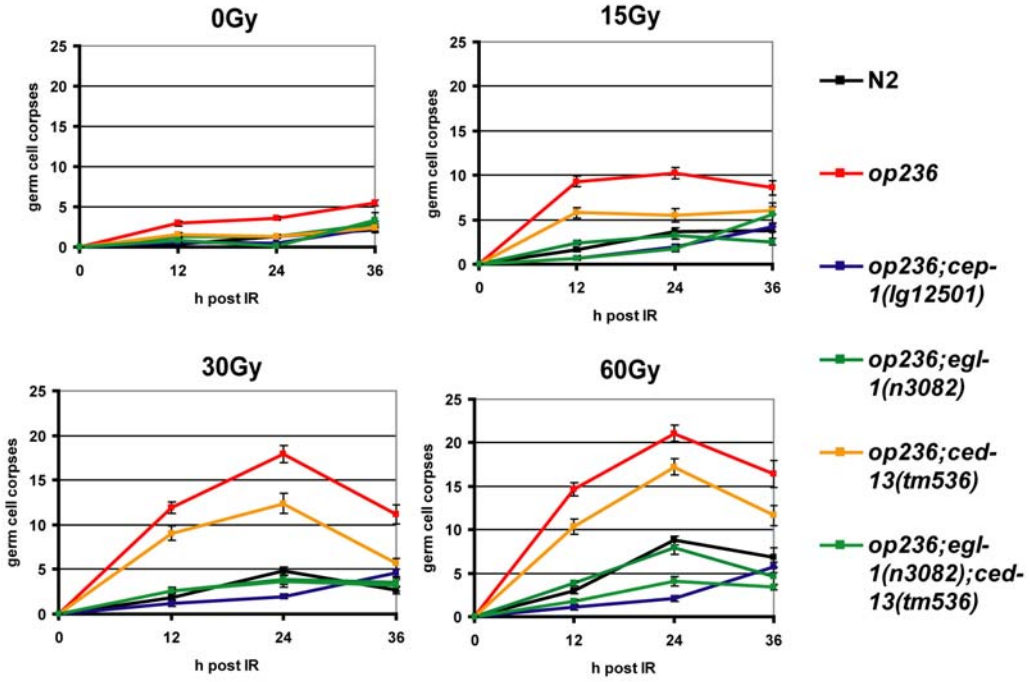


Figure 19 *gld-1(op236)* mediated apoptosis is dependent on the *C. elegans* p53 pathway. Double mutants were irradiated and cell corpses counted as in Figure 11.

As *gld-1(op236)* is a point mutation that only interferes with some of the GLD-1 functions we tested its temperature sensitivity. Worms normally are kept at 20C. When *gld-1(op236)* was shifted to 25C it showed increased levels of apoptosis comparable to high irradiation dosages (Figure 20). *gld-1(op236)* dependent apoptosis at 25C was also

dependent on *cep-1* as a *cep-1* deletion mutant strongly suppressed *gld-1(op236)* at 25C (Figure 20).

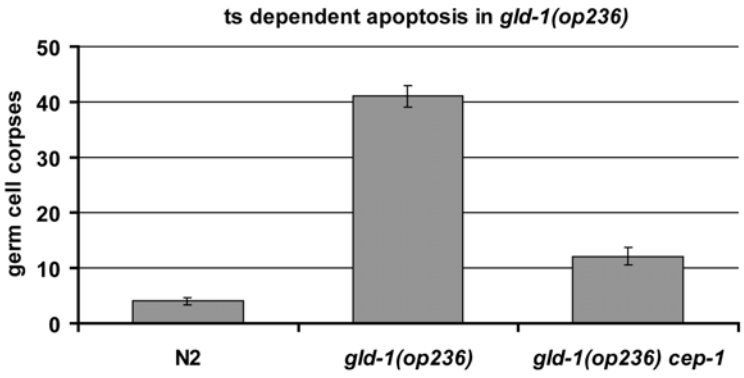


Figure 20 *gld-1(op236)* is a temperature sensitive mutant. Worms, normally held at 20°C were shifted to 25°C at L4 larval stage. Germ cell corpses were counted 20h post temperature shift.

Therefore, programmed cell death in *gld-1(op236)* is dependent on the CEP-1 and the downstream components of CEP-1 mediated apoptosis.

3.3.3 GLD-1 downregulates the CEP-1 pathway

As apoptosis in *gld-1(op236)* was dependent in the p53 pathway we tested if this pathway is upregulated in *gld-1(op236)*. We evaluated the levels of *egl-1* and *ced-13* mRNA by quantitative light cycler PCR (Figure 21a,b). Both, *egl-1* and *ced-13* mRNA levels were upregulated in *gld-1(op236)*. In non-irradiated worms *egl-1* and *ced-13* levels were two fold increased over levels in wild type animals. Upon irradiation both BH3 only genes were more strongly induced than in wild type worms. Interestingly, the level of *egl-1* and *ced-13* transcripts at e.g. 15Gy of irradiation corresponded to the levels in 60Gy irradiated wild type worms. This corresponds to the apoptotic levels of *gld-1(op236)* at 15Gy being about similar to the level of dying cells in 60Gy irradiated wild type worms, indicating that upregulation of CEP-1 target genes might sufficiently explain the apoptotic phenotype of *gld-1(op236)* (compare Figure 19 and Figure 21a). We also tested if the p53 pathway was upregulated at 25C, where *gld-1(op236)* showed an increase of apoptosis as at high dosages of ionizing irradiation. *egl*-1 mRNA levels were indeed

elevated over the level in wild type worms (Figure 22b). Therefore, we conclude that the apoptotic induction in *gld-1(op236)* is due to upregulation of the p53 pathway.

If *gld-1(op236)* is a partial loss-of-function allele of *gld-1* then a complete loss-of-function allele should also lead to an upregulation of *egl-1* mRNA. To test this we looked at *egl-1* mRNA levels in a complete loss-of-function mutation *gld-1(q485)*. *egl-1* mRNA was upregulated 6.5 fold over wild type levels (Figure 22b). (Note that *gld-1(q485)* animals do not form late stage pachytene cells that would be able to undergo apoptosis as pachytene cells in *gld-1(q485)* exit meiosis and resume mitosis (Francis et al., 1995)). Therefore, we conclude that GLD-1 downregulates *egl-1* expression.

CEP-1 induces *egl-1* transcription upon DNA damage (Hofmann et al., 2002) (Figure 10a). As *cep-1(lg12501)* suppressed the apoptotic phenotype of *gld-1(op236) cep-1* should also suppress *egl-1* upregulation in *gld-1(op236)*. *cep-1(lg12501)* indeed completely suppressed *egl-1* induction in wild type worms as well as in *gld-1(op236)* (Figure 22a). We conclude that GLD-1 represses CEP-1 activity and this repressive effect is alleviated in *gld-1(op236)* mutants.

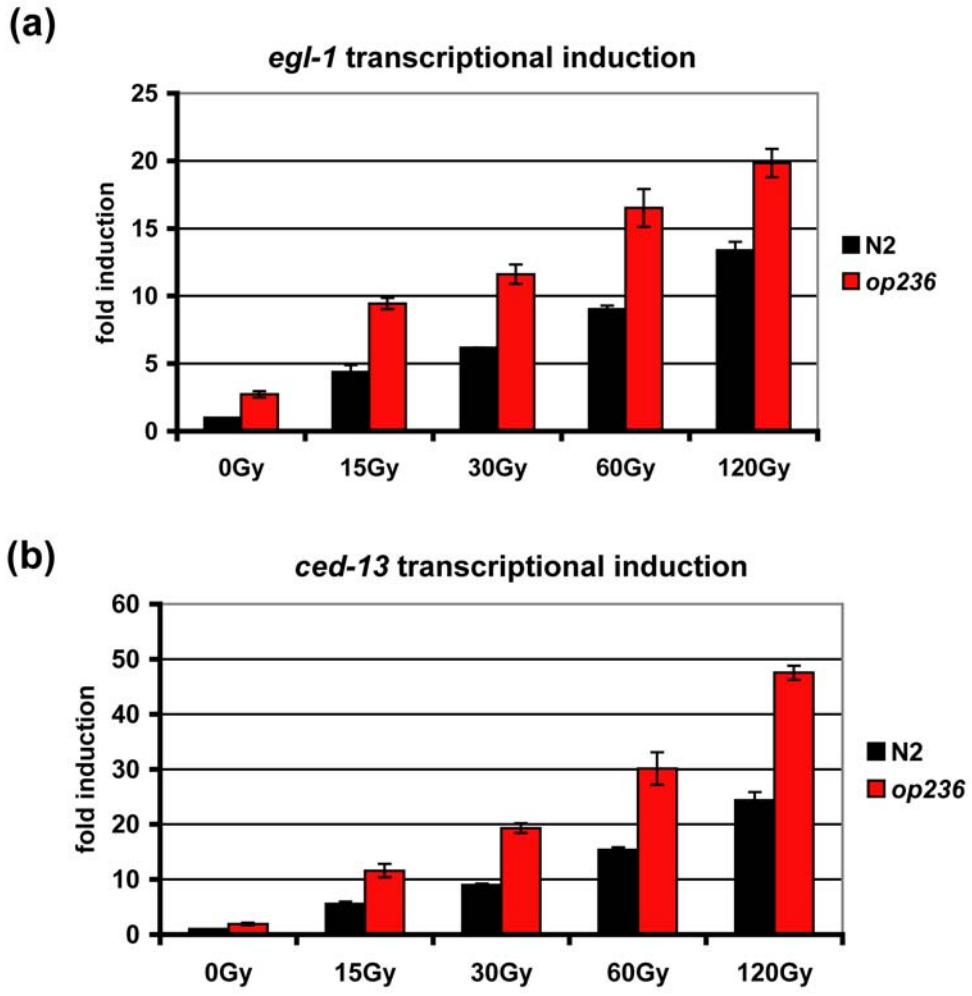


Figure 21 *gld-1(op236)* leads to upregulation of the CEP-1 target genes *egl-1* (a) and *ced-13* (b). Experiment was performed as described in Figure 10.

3.3.4 GLD-1 binds directly to *cep-1* mRNA

As GLD-1 has previously been characterized as an mRNA binding protein that represses translation of target mRNAs (Lee and Schedl, 2001) we tested if *cep-1* mRNA is a direct target of GLD-1. More than a dozen GLD-1 targets have previously been identified by co-immunoprecipitation of FLAG-GLD-1 in transgenic animals carrying a functional copy of tagged GLD-1 (Lee and Schedl, 2001). To test if GLD-1 binds *cep-1* mRNA we used RT-PCR to amplify *cep-1* mRNA in FLAG pull downs. We found a significant

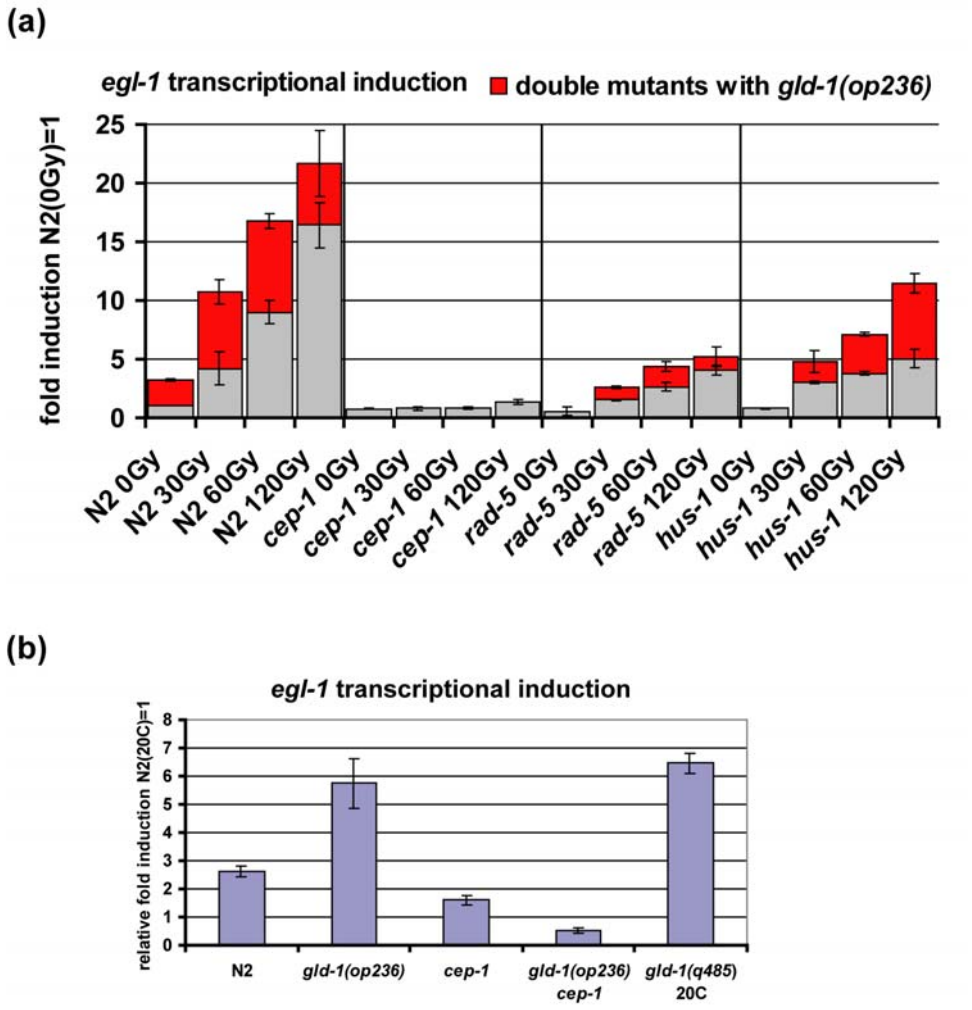


Figure 22 Induction of *egl-1* in *gld-1(op236)* is CEP-1 dependent. Experiment was performed as in Figure 10. Checkpoint mutants partially suppress *egl-1* upregulation in wild type and in *gld-1(op236)* animals. **(b) At 25C *egl-1* transcript is upregulated in *gld-1(op236)*, comparable to *egl-1* levels in a complete loss-of-function *gld-1(q485)* mutant.** ts dependent *egl-1* upregulation is also CEP-1 dependent.

increase in *cep-1* mRNA in GLD-1 pull-downs as compared to control pull-downs. This indicates that GLD-1 directly binds *cep-1* mRNA (experiment done by Min-Ho Lee and Tim Schedl, Figure 23a). We were, however, unable to show differences in CEP-1 protein levels as, despite extensive effort by our group as well as others, we could not generate anti-CEP-1 antibodies. We are currently proceeding with new immunizations to generate CEP-1 antibodies.

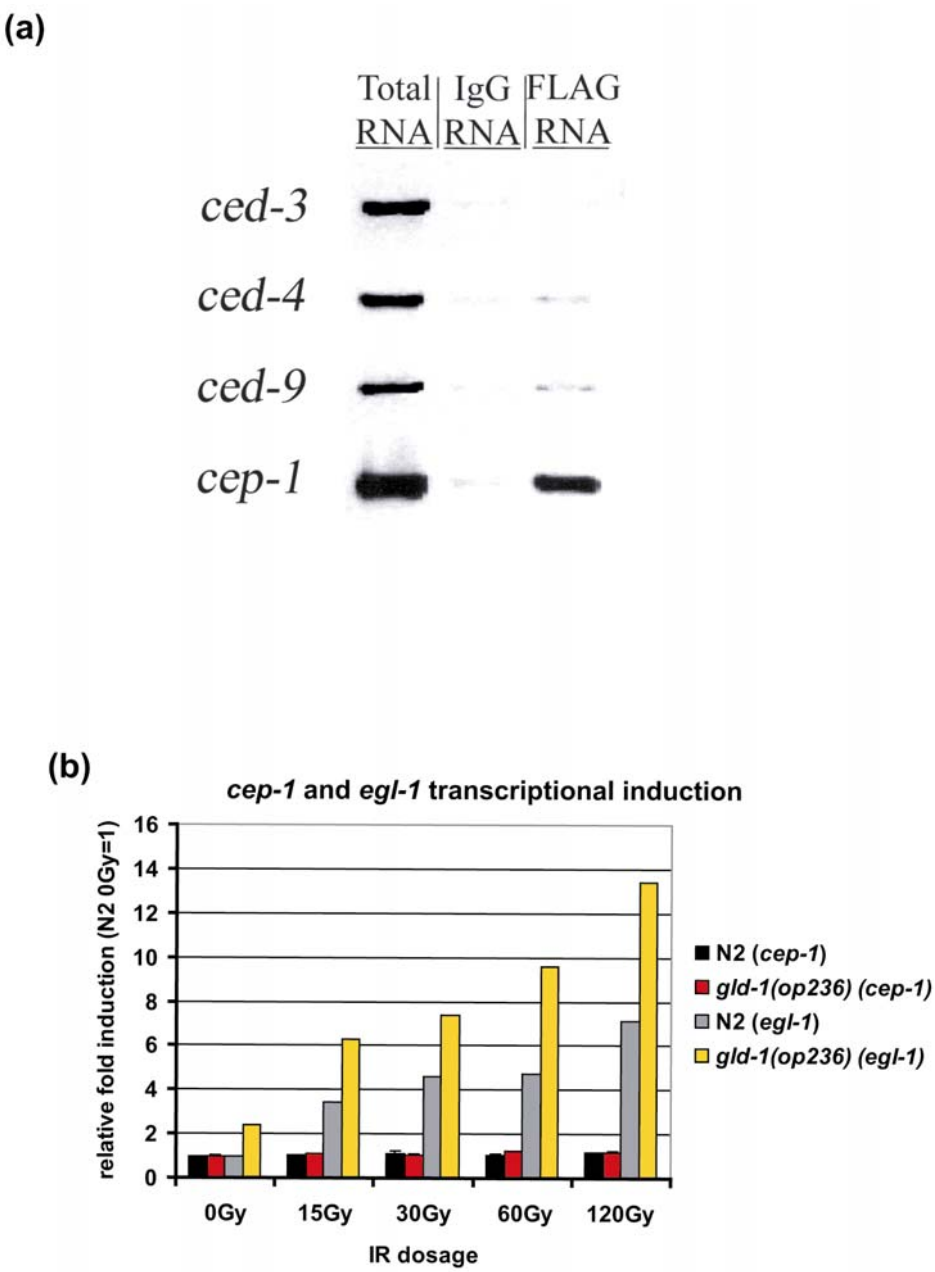


Figure 23 (a) GLD-1 specifically binds to *cep-1* mRNA. GLD-1 protein was co-immunoprecipitated from worms that carry a FLAG tagged GLD-1 copy. RT-PCR was performed for indicated genes on pull-downs. Only *cep-1* mRNA was found to bind GLD-1 but none of the control apoptotic genes (which only yielded background levels of RT-PCR amplification). **(b) *cep-1* mRNA level is not affected in *gld-1(op236)* or in response to ionizing irradiation.** Experiment was done as in Figure 10. *cep-1* induction is shown in N2 (black bars) and *gld-1(op236)* (red bars). *egl-1* induction was quantified as control in N2 (gray bars) and *gld-1(op236)* (orange bars).

GSG/STAR family members in other organisms have been reported to regulate mRNAs through mRNA stabilization or degradation. The two Drosophila isoforms of How, How(L) and How(S) have antagonistic effects on stripe mRNA degradation. The former How splice variant promotes mRNA degradation whereas the latter one elevates stripe levels (Nabel-Rosen et al., 2002). In fission yeast a GLD-1 homolog Rnc1 binds and stabilizes mRNA of the MAPK phosphatase Pmp1 (Sugiura et al., 2003). To test the possibility of such a regulatory mechanism of GLD-1 towards *cep-1* mRNA we compared *cep-1* mRNA levels between wild type worms and *gld-1(op236)* mutants by quantitative PCR. *cep-1* mRNA was equally abundant in wild type and *gld-1(op236)* worms (Figure 23b). *cep-1* mRNA levels remained constant at increasing dosage of ionizing irradiation. This indicates that *cep-1* mRNA stability is not regulated by GLD-1 or in response to DNA damage.

We suggest that, rather than regulating mRNA stability, GLD-1 is likely to act on CEP-1 translation as it does in case of other mRNAs (Lee and Schedl, 2001).

3.3.5 Survival and egg-laying activity

To further understand the physiological effects of *gld-1(op236)* we looked at egg-laying and DNA damage sensitivity in the double mutants. The rate of egg-laying is a result of germ line proliferation, fertilization of oocytes, and cell death of meiotic pachytene cells. *gld-1(op236)* showed a decreased egg-laying rate consistent with the increased levels of germ cell apoptosis (Table 7). Survival of progeny was similar to wild type animals also after irradiation (Table 7). Mutations in *cep-1*, *egl-1*, and *ced-13* have no influence on egg-laying rate or survival (Table 7, data not shown). Only at 120Gy of ionizing irradiation we observed a partial suppression of egg-laying defects of *gld-1(op236)* by *cep-1(lg12501)* (Table 7). This indicates that cells that fail to die in *gld-1(op236)*, when also defective in the p53 pathway still fail to go through normal germ cell development. Mutations in *ced-3*, however, lead to a dramatic decrease in egg-laying activity and survival of progeny (Table 7). *gld-1(op236);ced-3(n717)* animals indeed enhanced the oocyte differentiation defects seen in old *ced-3(n717)*animals (Gumienny et al., 1999). This differentiation defect is seen already in young *gld-1(op236);ced-3(n717)* animals, which renders germ cells unable to form oocytes and as a result decreases the egg-laying

rate (data not shown). This effect is also observed in *ced-9(n1653ts);ced-3(n717)* animals. In the temperature sensitive *ced-9(n1653ts)* mutant apoptosis in the germ line is highly induced at 25.5C as CED-4 is not inhibited by CED-9. *ced-3(n717)* completely suppresses the apoptotic phenotype but oocytes are unable to progress through oogenesis (data not shown). These observations are, therefore, consistent with the notion that, whenever strong pro-apoptotic signaling is blocked oocytes fail to differentiate. It is not known whether the effect of *ced-3* and *ced-4* on oocyte development is simply due to failure of germ cells to die through apoptosis, which would then lead to an accumulation of germ cells, or if it is a distinct effect on germ cell differentiation of *ced-3*.

	0Gy		60Gy		120Gy	
	egg laying	survival [%]	egg laying	survival [%]	egg laying	survival [%]
N2	7.7 (±0.8)	99.8 (±0.1)	4.8 (±0.4)	68.5 (±5.1)	4.0 (±0.3)	43.5 (±5.7)
gld-1(op236)	5.2 (±0.7)	99.6 (±0.2)	2.1 (±0.6)	65.5 (±1.2)	0.7 (±0.1)	46.4 (±6.8)
ced-3	4.3 (±0.1)	97.5 (±0.7)	4.2 (±0.1)	60.2 (±3.1)	4.0 (±0.1)	40.8 (±2.9)
gld-1(op236)ced-3	2.5 (±0.2)	61.3 (±8.6)	1.0 (±0.1)	35.1 (±6.8)	1.1 (±0.1)	23.1 (±3.1)
cep-1	6.0 (±0.7)	99.3 (±0.4)	5.3 (±0.5)	80.8 (±1.5)	4.4 (±0.8)	53.0 (±6.6)
gld-1(op236)cep-1	5.3 (±0.2)	97.7 (±0.5)	2.9 (±0.2)	68.8 (±5.2)	2.2 (±0.4)	52.4 (±6.4)

Table 7 *gld-1(op236)* worms are not hypersensitive to DNA damage. Mutations in *cep-1* have no effect on egg-laying or survival, whereas *ced-3* mutations lead to a reduced egg-laying rate. Egg-laying rate, expressed in eggs laid per hour and worm, and survival of embryos was assessed 24 to 36h post IR. Experiments were done in duplicate. Average of two independent experiments are shown. In brackets standard error of the mean is given.

3.3.6 Apoptosis in *gld-1(op236)* is dependent on checkpoint signaling

We next tested the relation of *gld-1(op236)* to the DNA damage checkpoint pathway. To this end we crossed *gld-1(op236)* to two characterized checkpoint mutants *hus-1* and *rad-5*. HUS-1 is homologous to human Hus1, which forms a PCNA like ring structure together with Rad1 and Rad9 on damaged DNA. *rad-5*, homologous to budding yeast tel2, is less well characterized. *rad-5* and *hus-1* mutants are defective in checkpoint signaling as in these mutants mitotic cells fail to halt the cell cycle and meiotic pachytene cells do not undergo cell death upon ionizing irradiation (Gartner et al., 2000; Hofmann et al., 2002; Ahmed et al., 2001). A point mutation in *rad-5* and a *hus-1* deletion suppressed *gld-1(op236)* induced apoptosis (Figure 24). Both mutations, however,

compromised the egg laying activity of *gld-1(op236)* as well as the survival of progeny upon irradiation (data not shown). *gld-1(op236)* mutants, when defective in either of those two checkpoint genes, show a dramatic decrease in egg-laying rate irrespective of irradiation (data not shown). We cannot exclude a synthetic effect on germ line development between *gld-1(op236)* and checkpoint mutants. To pin down the epistatic relations between checkpoint signaling and *gld-1(op236)* we evaluated the levels of p53 pathway activity. *egl-1* and *ced-13* mRNA were indeed upregulated in *gld-1(op236);rad-5* and *gld-1(op236) hus-1* double mutants albeit to a lesser extend than in *gld-1(op236)* (Figure 22a). Consistent with their effects on apoptosis *rad-5* and *hus-1* strongly suppressed *gld-1(op236)* dependent upregulation of *egl-1* and *ced-13*. Interestingly, *rad-5* and *hus-1* checkpoint mutants show DNA damage dependent upregulation of *egl-1* and *ced-13* although to a lesser extent than wild type worms (Figure 22a, data not shown). This indicates that defects in checkpoint signaling downregulate the *cep-1* pathway although there is likely to be a parallel pathway that is still able to relay DNA damage to CEP-1 activation. We suggest that checkpoint signaling at least in part acts as a threshold setting for CEP-1 activity, where checkpoint signaling antagonizes GLD-1 mediated downregulation of CEP-1 activity.

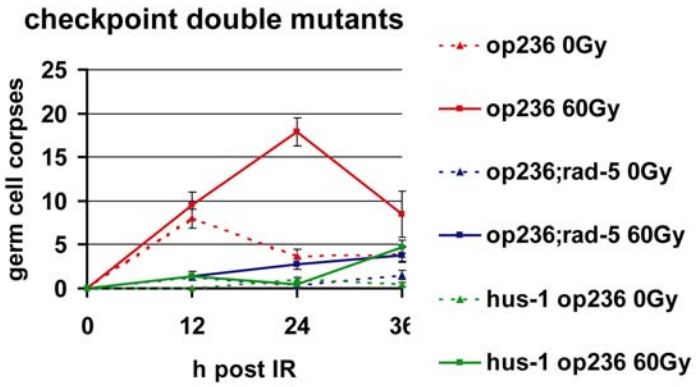


Figure 24 DNA damage induced apoptosis in *gld-1(op236)* is checkpoint dependent. Assay was performed as in Figure 11.

3.3.7 Discussion: GLD-1 regulation of CEP-1 activity

We undertook a genetic screen for regulators of the p53 pathway in *C. elegans*. We identified *gld-1* as a negative regulator of CEP-1 activity. *gld-1(op236)* specifically induces programmed cell death defining a novel class of *gld-1* alleles. *gld-1(op236)* upregulates germ cell death synergisticly with DNA damage. Furthermore, these cell deaths are dependent on the apoptosome CED-9, CED-4 and CED-3 as well as CEP-1 and the CEP-1 targets EGL-1 and, to a minor extent, CED-13. *gld-1(op236)* induced apoptosis is also dependent on checkpoint signaling through the checkpoint proteins RAD-5 and HUS-1. We have shown that in *gld-1(op236)* the CEP-1 target genes *egl-1* and *ced-13* are upregulated. The upregulation of these two BH3 only genes is dependent on CEP-1, indicating that *gld-1(op236)* leads to increased CEP-1 activity.

GLD-1 is a GSG/STAR family mRNA binding protein. It binds and translationally represses target mRNAs. More than a dozen mRNA targets have been identified so far (Lee and Schedl, 2001). Most of them have been shown to be involved in germ line development. GLD-1 has been shown to directly bind mRNA of the sex determination gene *tra-2* (via the 3'UTR) and the yolk receptor gene *rme-2* (via the 5' and the 3'UTRs) and represses their translation (Jan et al., 1999; Lee and Schedl, 2001). A *gld-1(q126)* point mutant G308E within the GSG/STAR domain leads to feminization of the germ line. Consistent with this partial loss-of-function phenotype GLD-1*(q126)* is deficient in *tra-2* mRNA binding but proficient in *rme-2* mRNA binding (Lee and Schedl, 2001). Our data suggest a direct repressive effect of GLD-1 on *cep-1* mRNA. We predict that, in analogy to the effect of GLD-1*(q126)* on *tra-2* mRNA binding, GLD-1*(op236)* might be deficient in *cep-1* mRNA binding but proficient in binding most if not all other GLD-1 target mRNAs, as germ line development is unaffected in *gld-1(op236)* mutants. According to this model, CEP-1 protein levels should be upregulated in *gld-1(op236)*. Further characterization of the interaction between GLD-1 and GLD-1*(op236)*, respectively, and *cep-1* mRNA will reveal details of the mechanism of this repression.

If GLD-1 regulates CEP-1 then the next interesting question will be what regulates GLD-1 repression towards CEP-1. *gld-1* mRNA is translationally repressed throughout the mitotic region of the germ line by FBF-1 and FBF-2 (*fem-3* binding factor), which can

bind to two FBF binding sites in the 3'UTR of *gld-1* mRNA (Crittenden et al., 2002). The FBF proteins are members of the PUF (Pumilio and FBF) family of RNA-binding proteins and are required for keeping germ cells in mitosis (Crittenden et al., 2002). When FBF is downregulated GLD-1 protein levels are induced and germ cells transit into meiosis. GLD-1 protein is expressed until cells exit meiotic pachytene (Jones et al., 1996). Previously identified GLD-1 targets have been shown to be translationally repressed precisely where GLD-1 protein is present (Lee and Schedl, 2001). This is unlikely to be the case for CEP-1 translation. Meiotic pachytene cells undergo programmed cell death upon DNA damage. These cells express GLD-1 and CEP-1, as CEP-1 is required for triggering the death of these cells. It is thus likely that GLD-1 activity towards *cep-1* mRNA is regulated not only by GLD-1 expression but also by specific mediators. Who might those mediators be? In fission yeast the KH motif protein Rnc1 has been shown to be regulated by the MAP kinase pathway (Sugiura et al., 2003). In this instance Rnc1 binds and stabilizes the mRNA of the MAPK phosphatase Pmp1. Pmp1 negatively regulates the MAPK Pmk1. Pmk1, in turn, phosphorylates Rnc1, thereby enhancing its ability to bind and stabilize Pmp1 mRNA, defining a negative-feedback regulation of MAPK signaling. Analogously, Sam68, a human GLD-1 homolog, has been shown to be regulated through ERK phosphorylation (Matter et al., 2002). In this instance ERK phosphorylation mediates Sam68 induced alternative splicing of CD44. Is such a regulation conceivable for GLD-1 in *C. elegans*? MAPK components have indeed been shown to be required for germ cell apoptosis (Gumienny et al., 1999). However, MAPK signaling is essential for progression into meiotic pachytene, which is the only germ cell type that undergoes programmed cell death. It is thus difficult to genetically assess the relation of MAPK signaling and programmed cell death.

Alternatively, it is conceivable that GLD-1 activity towards CEP-1 might be regulated by checkpoint signaling. Consistent with this idea, mutations in the checkpoint genes *rad-5*, and *hus-1* suppress *gld-1(op236)* mediated apoptosis. However, CEP-1 activity is not completely suppressed in *gld-1(op236)* checkpoint double mutants. There is also a synergistic effect on germ cell maturation, as all three double mutants show a decreased

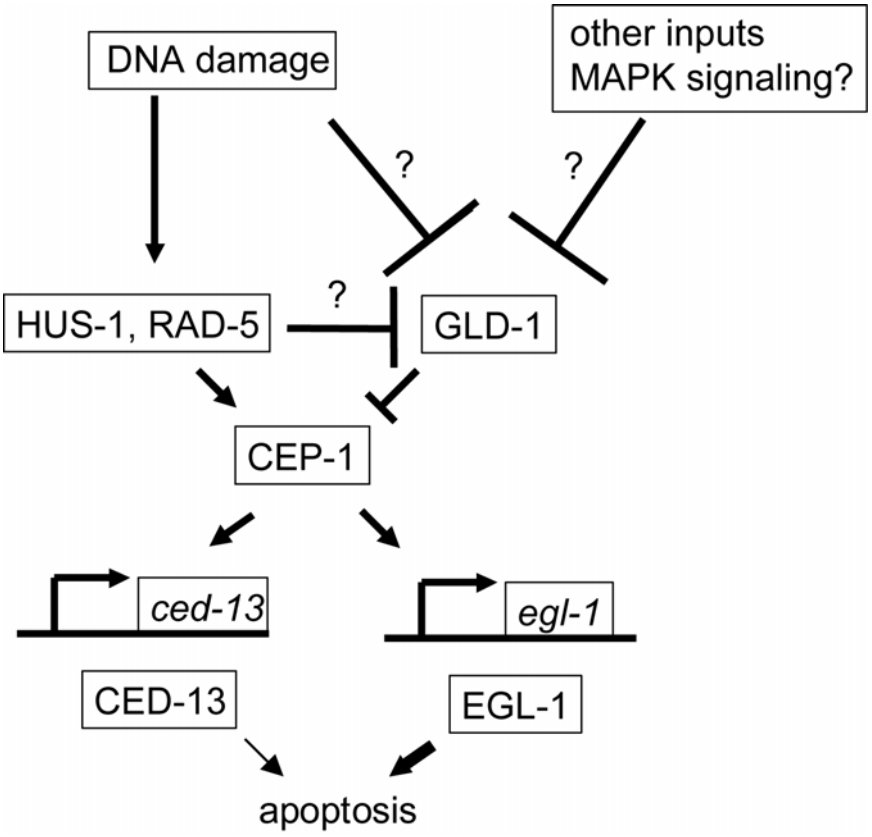


Figure 25 Model for GLD-1 regulation of CEP-1. GLD-1 downregulates CEP-1 activity presumable through translational repression of *cep-1* mRNA. It remains to be determined if GLD-1 in turn is subject to DNA damage dependent checkpoint regulation or other signaling pathways such as the MAPK pathway.

egg-laying rate. This argues for a parallel pathway of checkpoint signaling to upregulate CEP-1 activity on one side and GLD-1 repression of CEP-1 activity on the other. Consistent with this, mammalian Atm and Chk2 are known to upregulate p53 posttranslationally. We suggest, therefore, a model in which checkpoint signaling and GLD-1 act antagonistically to set a threshold of CEP-1 activity, which in turn determines the rate of programmed cell death in germ cells (Figure 25). Several observations support such a model. Firstly, in checkpoint mutants *egl-1* transcription is upregulated upon DNA damage, albeit to a lesser extent than in wild type worms. *egl-1* transcripts are less abundant in non-irradiated *rad-5* or *hus-1* animals as compared to wild type worms. The second evidence comes from *gld-1(op236)* mutant worms. Here, the effect on *egl-1* transcription is exactly the opposite of checkpoint defects. *egl-1* mRNA levels are higher than in wild type worms. Upon ionizing irradiation *egl-1* mRNA is upregulated in a

dosage dependent manner, indicating that CEP-1 is subject to checkpoint signaling in *gld-1(op236)*. The relative level of upregulation is comparable to that in wild type worms, just the threshold is higher in unperturbed *gld-1(op236)* than in wild type animals. An alternative interpretation could be that checkpoint signaling acts on both, posttranslationally through CEP-1 phosphorylation or alternative mechanisms, and posttranscriptionally through GLD-1. The precise mechanisms of GLD-1 regulation of CEP-1 activity, therefore, remain to be determined. It will be interesting to learn more about this aspect of GLD-1 regulation, as we might understand a novel mechanism of translational control of the p53 pathway.

gld-1 has previously been characterized as a germ line tumor suppressor. There are 31 alleles of *gld-1* to date, which form different phenotypic classes (Francis et al., 1995). Complete loss of *gld-1* function mutations lead to the formation of a germ line tumor, as cells transit into meiosis but then reverse into mitosis and continue to divide mitotically. Some point mutations feminize the hermaphroditic germ line, whereas others lead to differentiation defects in pachytene cells. Gain-of-function mutations, on the other hand, lead to masculinization of the hermaphroditic germ line (Francis et al., 1995). More than a dozen GLD-1 mRNA targets have been identified so far. For some of them GLD-1 has been shown to repress their translation. Furthermore, many of the GLD-1 target genes have been associated with functions in germ cell development (Lee and Schedl, 2001). All *gld-1* alleles are involved in cell fate decisions in meiotic pachytene cells. The fate of most cells is to develop into oocytes. The fate of some cells, however, is to undergo programmed cell death (Gumienny et al., 1999). *gld-1(op236)* leads to an upregulation of the p53 pathway. The p53 pathway in *C. elegans* specifically induces apoptosis upon DNA damage (Schumacher et al., 2001; Derry et al., 2001). However, also in non-irradiated worms *cep-1* contributes to some of the physiological cell death (see above). It is possible that this fraction of cell death is a result of failures in meiotic recombination. Under normal conditions GLD-1 might play a role in regulating germ cell death that is induced by meiotic recombination failures.

Homologs of GLD-1 in Drosophila include the Who/How gene, in mouse and human the quaking genes, which are alternatively spliced forms of the quaking (QKI) locus, and Sam68 (Src-associated in mitosis). Mouse knockouts and a number of ENU generated

mutations of the QKI locus confer embryonic lethality at day 9-10 gestation indicating a role in early development (Bode, 1984; Justice and Bode, 1988), (Shedlovsky et al., 1988). Two hypomorphic alleles show defects in myelination and in vascular development, respectively, resulting in embryonic lethality (SIDMAN et al., 1964; Samorajski et al., 1970). In Drosophila hypomorphic alleles of wings held out (Who) or held out wings (How) result in wing muscle defects. Complete loss-of-function alleles lead to defects in muscle development resulting in death prior to hatching (Zaffran et al., 1997; Baehrecke, 1997). Mechanistically, the How protein can act in two different ways on target mRNA. There are two splice forms How(L) and How(S). The first one resides in the nucleus and represses the stripe target mRNA, whereas the second one is found in the nucleus as well as in the cytoplasm and upregulates stripe protein levels (Nabel-Rosen et al., 2002).

It is likely that similarly to *C. elegans* GLD-1 GSG/STAR family proteins in other species regulate translation of a large number of different mRNAs. Subsets of functions, such as post-embryonic functions, can only be explored through the characterization of different alleles that affect binding to only a subset or even individual target mRNAs. We have identified a *gld-1* allele that only affects the apoptotic function of GLD-1. GLD-1 directly binds to *C. elegans* p53 mRNA. It is thus likely that GLD-1 represses apoptosis through translational repression of *cep-1*. *cep-1* translational control defines a novel regulatory mechanism of p53. There are strong indications that translation control also regulates p53 in humans (Kastan et al., 1991). It will be of great interest to evaluate if mammalian GSG/STAR family members also control p53 translation. The KH domain RNA-binding protein MCG10 has been reported to be a transcriptional target of human p53 (Zhu and Chen, 2000). Cell lines that overexpress MCG10 undergo apoptosis. The physiological role of MCG10, however, remains to be determined. As the GSG/STAR family has diverged during evolution it will be difficult to identify an analogous mechanism to GLD-1 regulation. We like to speculate that *C. elegans* genetics has once again shown to be crucial for elucidating a mechanism of apoptotic control that might give us insights into p53 regulation in mammals just as studies of the apoptotic pathway in *C. elegans* have provided the concept of a core pathway of apoptosis that has gained many variations during evolution.

Elucidating mechanisms of translational control of p53 might have implication for the development of new therapeutical approaches directed against tumor cells that have functional p53 but other mutations in the p53 pathway, such as ARF mutations (Sharpless and DePinho, 1999). Additionally, genetic screens for *gld-1(op236)* enhancers and suppressors might reveal genes that regulate GLD-1 mediated repression of CEP-1 activity, potentially adding to our understanding of translational control mechanisms.

3.4 RNAi screen for regulators of the p53 pathway

Large scale and even genome wide genetic screens using RNA interference have recently become feasible in *C. elegans* (Fraser et al., 2000; Gonczy et al., 2000; Timmons et al., 2001). We sought to use this method to screen through a large number of genes to identify novel components of the DNA damage induced cell death pathway. We hypothesized that candidate genes might exist among genes that are specifically expressed in the *C. elegans* germ line, as only germ cells undergo apoptosis upon DNA damage. Reinke et al. have recently identified 770 genes that are specifically expressed in the germ line by RNA microarray analysis (Reinke et al., 2000). We used the above-mentioned "feeding method" of RNA interference as this was reported to be the most efficient method to knock down gene expression in post-embyonic tissues (Fraser et al., 2000). We knocked down all 770 genes that Reinke et al. identified as germ line specific. We were able to reproduce the sterile and embryonic lethal phenotypes that were found in genome wide and ovary specific RNAi screens (Fraser et al., 2000; Piano et al., 2000) (data not shown). We found three genes that reproducibly attenuated the apoptotic response to ionising irradiation when inactivated by RNAi. One gene T05H10.5 is a homolog of *S. cerevisiae* ufd-2. ufd-2 has been shown to be an E4 ubiquitin ligase, which is required for poly-ubiquitination (Koegl et al., 1999). The second candidate gene T19A5.1 is a member of a previously unknown gene family that has homologs in metazoa such as Drosophila, mouse and human, but not in unicellular organisms. The third candidate gene F35C8.7 is a homolog of choline transporter-like genes that were identified as electric lobe suppressor of a yeast choline transport mutation (O'Regan et al., 2000). Furthermore, we found the putative cytoplasmic protein C16A11.6 to upregulate apoptosis when inactivated by RNAi independently of ionising irradiation. We could also identify 7 genes that reproducibly gave rise to a him (high incidence of males) phenotype, which is a result of meiotic non-disjunction. These genes are *him-3* (ZK381.1), the SUMO activating enzyme like C08B6.9, the G beta WD 40 repeat protein like C14B1.4, the worm specific gene C28C12.2, the phosphatase 2A regulatory subunit C25A1.9, the vaccinia related kinase like F28B12.3 and the FLYWCH zinc finger containing protein

family member Y11D7A.12. These meiotic genes will be followed up in future studies in collaboration with other laboratories.

To confirm the phenotypic observations we made by RNAi we isolated deletion mutants for T05H10.5 and T19A5.1. The first one showed strong defects in germ line proliferation, preventing any assessment of apoptotic phenotypes in the germ line (data not shown). For the latter candidate gene, we were unable to produce homozygous deletion mutants despite extensive genetic backcrossing. This indicates an essential function of this gene product. We cannot exclude the possibility that RNAi of those two genes resulted in a partial loss-of-function phenotype that might result in failure to undergo programmed cell death upon ionising irradiation even if complete loss-of-function alleles, as the deletion mutants are likely to be, interfere with an essential function. For the third candidate gene F35C8.7 we did not obtain a deletion mutant. It is, therefore, possible that this gene product might be involved in the regulation of DNA damage induced apoptosis.

We were, therefore, unable to identify any genes involved in DNA damage induced apoptosis by RNAi of germ line specific genes. This failure could have at least two reasons. First, RNAi work only in about 40% of genes involved in post-embryonic functions (Fraser et al., 2000). Secondly, genes such as *cep-1*, which are involved in DNA damage induced apoptosis, were not in the gene set identified by Reinke et al. (Reinke et al., 2000). It is thus possible that genes that are involved in this branch of the apoptotic pathway either evaded detection or that at least some of them are also expressed in other tissues. The latter possibility is interesting in so far as we have not observed any apoptotic DNA damage checkpoint response in other tissues than the germ line.

4 Conclusions and Future Perspectives

When I started this thesis work the nematode worm *C. elegans* had just become available as a genetic model system for studying DNA damage induced apoptosis. Gartner et al. had shown that in *C. elegans* DNA damage triggers cell cycle arrest and apoptosis through a conserved checkpoint pathway (Gartner et al., 2000). This conserved checkpoint pathway had been identified mostly through yeast genetics. With the metazoan model *C. elegans* at hand we now had a genetic system to study the apoptotic response to DNA damage, which does not occur in yeast. More precisely, the question was, what is the link between checkpoint signaling and CED-9 inhibition that would trigger cell death.

We were able to first identify a homolog of the tumor suppressor p53 and show that it specifically induces programmed cell death upon DNA damage. Secondly, we could show that CEP-1 triggers apoptosis through transcriptional induction of the two *C. elegans* BH3 only proteins EGL-1 and CED-13. This pathway defines the most ancient p53 apoptotic pathway known. These findings allowed us to use the nematode worm to conduct genetic screens aiming at identifying novel components of the p53 pathway. Using a forward genetic screen for upregulation of DNA damage induced cell death we identified GLD-1 as a negative regulator of CEP-1 activity. This defines a novel regulatory mechanism in the p53 pathway. In addition, we performed an RNAi based screen for regulators of the p53 pathway focusing on genes that are expressed in the *C. elegans* germ line, the only tissue where cells undergo apoptosis following DNA damage. Here, however, we failed to identify components of the DNA damage dependent apoptotic pathway.

We, therefore, have established *C. elegans* as a genetic model for the apoptotic p53 pathway. As *C. elegans* has been instrumental for understanding the mechanisms through which programmed cell death is executed we envision that the nematode worm could have a crucial role in the genetic identification of mechanisms of p53 dependent apoptosis. As the apoptotic function of p53 is a major guardian against cancer

development genetic studies of *C. elegans* p53 might not only further our understanding of tumor suppression but also aid the discovery of target genes for cancer therapy.

5 Materials and Methods

Growth media

Worms were grown on nematode growth media (NGM) at 20°C as described previously (Brenner, 1974) unless otherwise indicated. 1 l NGM contains 3 g NaCl, 17 g agar, 2.5 g peptone, 1 ml cholesterol (5 mg/ml in 95% EtOH), 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M potassium phosphate pH 6.

NGM plates were dried and *E. coli* strain OP50 was spread to serve as worm food. Seeded plates were incubated at 37°C overnight and used over a period of up to several weeks.

Liquid growth culture

Worms in liquid culture were grown in S- basal buffer containing *E. coli* strain OP50 under shaking at 20°C. 1 liter S-basal buffer contains: 5.8 g NaCl, 20 mM $KHPO_4$ (pH 6.0), 136.1 g KH_2PO_4 (pH 6.0), 5 ml cholesterol (5 mg/ml in 95% EtOH). After autoclaving sterile supplements were added: 3 ml/l 1 M $MgSO_4$, 6 ml/l 0.5 M $CaCl_2$, 10 ml 100X trace metals solution (0.692 g/l $FeSO_4.7H_20$, 1.86 g/l Na_2EDTA, 0.196 g/l $MnCl_2.4H_20$, 0.288 g/l $ZnS0_4.7H20$, 0.024 g/l $CuSO_4.5H20$), 10 ml/l 1M KCitrate, (pH 6.0, 210.1 g citric acid, monohydrate per liter), 1 ml/l Penicillin/Streptomycin (GibcoBRL; 10,000 units penicillin G sodium per ml, 10,000 μg streptomycin sulfate per ml in 0.85% saline), 1 ml/l Nystatin (GibcoBRL; 10,000 units nystatin per ml).

Solutions

-M9 buffer: 3 g/l KH_2P0_4, 6 g/l $Na_2 HPO_4$, 5 g/l NaCl, 1 mM Mg $S0_4$ (added after sterilization), pH between 6.9 and 7.

-PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4

Worm stocks were frozen with 50% freezing solution in M9 buffer:

-Freezing solution (per l): 5.85 g NaCl (0.1M), 6.8 g KH2 PO4 (0.05M), 300 g Glycerol (0.3 g/ml), 5.6 ml 1 M NaOH (5.6 mM). 0.3 mM $MgSO_4$ was added after autoclaving.

-Lysis buffer for genomic DNA preparations: 0.1 M Tris pH 8.5, 0.1 M NaCl, 50 mM EDTA, 1% SDS, 40 µl proteinase K (20 mg/ml) to each 1 ml of lysis buffer.

BLAST Searches and Alignments

Profile searches and profile-guided multiple alignments were done with the PFTOOLS V2.1 package (P. Bucher, available by ftp from ftp.isrec.isb-sib.ch) as described previously (Weimbs et al., 1997). Dendrogram analysis with bootstrapping was done with the PHYLIP package (Felsenstein, 1989) using the neighbor-joining algorithm (Saitou and Nei, 1987). (Bioinformatics were done by Kay Hofmann, Memorex GmbH, Cologne)

Scoring for programmed cell death

Worms at L4 larval stage were irradiated with X-ray source Siemens "Stabilipan". Animals were paralyzed with 30 mM NaN_3 and mounted on 4% agar pads for observation under Nomarski optics as described (Gartner et al., 2000). Germ cell corpses, corpses in L1 animals, and the number of cells in the pharynx (pharynx assay) were scored as described previously (Hengartner et al., 1992). The DNA-damage checkpoint response during meiotic prophase was measured by scoring numbers of apoptotic germ cell corpses in the meiotic prophase region of the adult hermaphrodite germ line. *C. elegans* germ cell deaths are similar to the programmed cell deaths that occur during somatic development of the nematode and can be readily observed in living animals using standard Nomarski differential interference contrast (DIC) microscopy.

The first morphological sign of impending germ cell death is a decrease in the refractivity of the cytoplasm that occurs concomitant with an increase in refractivity of the nucleus (Figure 2a). In addition, a distinct boundary between dying cells and the surrounding germ line becomes visible (Figure 2a) soon thereafter, both nucleus and cytoplasm become increasingly refractile and start to blend with each other until they resemble a flat round, highly refractile disc (Figure 2a). After about 10-30 minutes, this flat disc often gets distorted and finally starts to disappear (Figure 2a). In late stage corpses, the nucleus of the dying cell decreases in refractility, begins to appear crumpled, and finally vanishes within less than 1 hour (Figure 2a). Late corpses often accumulate granular structures at their rim (Figure 2a). The morphology of corpses as well as the kinetics of their

disappearance is similar between somatic and germ cell apoptosis. However, as germ cells are only partially surrounded by a plasma membrane, the first step in germ cell death is the full cellularization of the apoptotic cell. Under conditions where massive germ cell death occurs, corpses tend to accumulate next to each other (Figure 2a). Sometimes, when massive germ cell death occurs, “late apoptotic corpses” also accumulate at more proximal positions in the germ line and tend to align next to developing oocyctes**.**

Mitotic Germ Cell Cycle Arrest Upon Ionizing Irradiation (IR)

To assess the proliferation arrest response to IR, worms were irradiated with 0 to 120 Gy at the late L4 larval stage as described previously (Gartner et al., 2000). 12 hours post-irradiation, worms were mounted on 4% agar pads and paralyzed with 30 mM NaN_3 for Nomarski DIC microscopy and the distal region of the germ line was scored for the presence of sparsely spaced, enlarged nuclei. For quantification the number of nuclei in all focal planes within a defined area of 3.125 μm x 6.25 μm in the most distal (premeiotic) region of the germ line (Figure 16c) were counted.

Genetic screen for increased apoptosis upon ionizing radiation

Chemical mutagenesis: Young adult worms were mutagenized with 50 mM EMS (methanesulfonate ethyl ester, Sigma #M0880). (EMS was deposed by adding KOH or NaOH pellets to the EMS solution). Worms were incubated at 20°C for 4 hours in EMS solution and washed in M9 buffer. Mutagenized worms were then allowed to recover overnight at 15°C. On the following day P0 worms were picked and allowed to lay eggs for 4h before they were moved to a fresh plate for a total period of 3 days. Each F1 individual has two sets of chromosomes that were exposed to the mutagen. If one of these chromosomes is mutated, one quarter of that individual's progeny will be mutant. Therefore, the number of mutagenized genomes corresponds to two times the number of F1 worms. F2 animals were treated with 20 Gy of ionizing radiation on day 6 after mutagenesis. Germ cell death was evaluated 28 to 32 hours post treatment by staining apoptotic corpses with acridine orange (AO). Candidate worms with strong AO staining,

which is indicative of elevated levels of germ cell death, were isolated, propagated and further analyzed by Nomarski DIC microscopy as described above.

Acridine orange (AO) staining

10 μg/μl of AO (Molecular Probes Inc. A3568) in M9 buffer was used as a staining solution. 0.5 ml of staining solution were added per 60 mm plate of worms and incubated in the dark for approximately 1 hour at room temperature (RT) and then washed and incubated for another 45 minutes in the dark at RT. Worms were analyzed with a fluorescence microscope within one hour.

Developmentally synchronizing worms

Worms were synchronized by bleaching. *C. elegans* eggs are able to survive treatment with bleach up to 5 minutes and more, whereas larvae and adult worms die. After bleaching of the P0 worms the surviving F1 eggs were collected. Worms were washed into 9 ml of M9 buffer and 4 ml commercial bleach (Danchlor) and 5 ml 2 M NaOH were added. After 5 minutes incubation under vigorous shaking, eggs were washed three times with M9 buffer and plated without food for 24 h to achieve maximum synchrony, as embryos have hatched at that time and arrested at L1 larval stage due to starvation conditions. Hatched L1 worms were then plated on seeded NGM plates.

Cloning of full length worm p53

Total RNA from whole worms was prepared with GibcoBRL Trizol according to the manufacturer's instructions. Approximately 2000 developmentally synchronized worms were treated with 1 ml Trizol. Poly (dT) primed cDNA was generated using Clontech "Advantage RT for PCR kit" according to the manufacturer's instructions. 1.5 μg total RNA was used per RT-PCR reaction. *cep-1* cDNA was amplified from this cDNA pool with Roche "expanded long template PCR system" with a primer 5'-ggccacgcgtcgactagtacgtttaattacccaagtttgag-3' containing splice leader (SL) 1 sequence at the 5' end and a *cep-1* 3' end specific primer 5'-ttactttggcagtttcatcgc-3'. PCR products were dATP-tailed for ten minutes at 72°C with Takara Ex Taq enzyme (Takara Bio Inc.) and 0.2 mM dATP before TA-cloning into Invitrogen TA cloning kit vector pCRII

according to the manufacturer's instructions. Sequencing reactions were done by Medigenomix and Sequiserve GmbH with M13 forward and reverse primers for pCRII as well as *cep-1* sequence specific primers.

RNAi and co-suppression of *cep-1*

A 1200 bp genomic fragment of *cep-1* was amplified with primers 5'-gttcgaattgttgcctatcca-3' and 5'-tcatcgcttcctggatgcggt-3' using Takara ExTaq (Takara Bio Inc.) and cloned into the L4440 RNAi feeding vector (Timmons et al., 2001) using TA cloning. PCR products were dATP tailed with 0.2 mM dATP for 10 minutes at 72°C as described above. L4440 RNAi feeding vector was adapted for TA cloning using Promega dTTP terminal transferase according to the manufacturer's instructions. For RNAi feeding, L4440 *cep-1* expressing HT115 *E. coli* strain was spread on 2-3 cm^2 of an LB ampicillin (100 μg/ml) plate overnight. The *E.coli* lawn was re-suspended in 200 μl LB and 50 μl were seeded on NGM plates containing 6 mM IPTG and 100 μg/ml ampicillin. After drying, approximately 3 P0 worms were added to the plate and incubated at 15°C for 72 h. Three single F1 worms were transferred each to a new, freshly seeded plate and allowed to lay eggs for approximately 20 hours. F1 worms were removed and F2 worms were allowed to grow up to the L4 stage, treated with ionizing radiation and analyzed for radiation induced cell cycle arrest and apoptosis as described above.

For *cep-1* co-suppression fragments containing the promoter sequence and the first 2 exons of the *cep-1* locus were amplified with primers 5'-ctctgatctcttcccataggttc-3' and 5'-cgacacttcatcacatcacttc-3' using Takara ExTaq polymerase according to the manufacturer's instructions. PCR products and plasmid containing the *rol-6* transformation marker were phenol chloroform extracted. 50 ng/μl PCR products were co-injected with an equal concentration of *rol-6* transformation marker. Worms in the F1 generation were selected according to appearance of the *rol-6* phenotype. *rad-51* RNAi was performed as described in Gartner et al. 2000 (Gartner et al., 2000). To inactivate *rad-51*, RNA was synthesized from both orientations (T3 and T7 primers) from the *rad-51* encoding EST clone yk241d12 using Promega "Ribomax" RNA synthesis kit according to the manufacturer's instructions. Single-stranded RNAs were annealed and

injected as described (Fire et al., 1998). Transformants were selected according to the *rol-6* phenotype as described above.

CEP-1 acts as a transcriptional activator

Full-length *cep-1* was cloned using Gateway recombination into pDB-Gent and pADH-DEST yeast expression vectors to generate Gal4-DNA binding domain fusion and untagged proteins, respectively. pDB-CEP-1, together with pDB, pDB-BAR-1 and controls were introduced into MAV103 yeast by transformation (Walhout and Vidal, 2001). The ability of DB-CEP-1 to function as a transcription factor was determined by analyzing the level of transcriptional activation of an integrated LacZ reporter driven by a promoter containing Gal4 binding sites. The level of transcriptional activation of the lacZ reporter was quantitatively determined by analyzing β-Gal activity, as previously described (Nevels et al., 1999). The ability of CEP-1 to bind to and activate a promoter through a consensus human p53-binding site was performed as previously described (Ishioka et al., 1993). p415ADH, p415ADH-BAR-1, p415ADH-Human p53 and p415ADH-CEP-1 were introduced into yeast strains containing the pSS1 reporter (human p53 binding sites). Transcriptional activation of the reporter was determined by the ability of yeast strains to grow in the absence of histidine as previously described (Ishioka et al., 1993).

***ced-13* and *egl-1* transcriptional regulation**

Approximately 2000 worms per sample were developmentally synchronized by bleaching and treated with ionizing radiation as decribed above. Total RNA was extracted with 1 ml GibcoBRL Trizol according to the manufacturer's instructions. cDNA synthesis was done with Clontech "Advantage RT for PCR kit" using 1.5 μg of total RNA per reaction according to the manufacturer's instructions. Quantitative PCR (qPCR) was performed with a Roche LightCycler and FastStart DNA Master SYBR Green 1 kit (Roche Applied Science) according to the manufacturer's instructions. For qPCR of cDNA, primer pairs (for *egl-1* 878 5'-tactcctcgtctcaggactt-3' and 880 5'-catcgaagtcatcgcacat-3'; for *ced-13* 706 5'-acggtgtttgagttgcaagc-3' and 707 5'-gtcgtacaagcgtgatggat-3'; for *tbg-1* 710 5'-cgtcatcagcctggtagaaca-3' and 711 5'-tgatgactgtccacgttgga-3') were designed to generate

intron-spanning products of 110-150bp. The generation of specific PCR products was confirmed by melting curve analysis (which measures product specificity by the decrease in fluorescence signal when the PCR product is denatured), gel electrophoresis (using Roche Agarose MS for analyzing small PCR products) and sequencing. Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve (crossing point (CP) plotted versus log of template concentration), which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). gamma tubulin (*tbg-1*) mRNA was used as an external standard. For data analysis, the second derivative maximum method was applied, and induction of egl-1 cDNA was calculated according to Pfaffl (Pfaffl, 2001):

$(E_{egl\text{-}1}{}^{\Delta CP(cDNA\ 0Gy\text{-}cDNA)egl\text{-}1})/ (E_{tbg\text{-}1}{}^{\Delta CP(cDNA\ 0Gy\text{-}cDNA)tbg\text{-}1})$

Affymetrix gene chip

Approximately 2000 synchronized N2 and *cep-1(lg12501)* animals were treated with 120 Gy of ionizing irradiation as young adults (24 h post L4 larval stage). Total RNA was isolated 2 h post IR treatment with Trizol reagent as described above. Affymetrix gene chip sample preparations, hybridization and data analysis was done as described on www.affymetrix.com (the affymetrix gene chip experiment was done by Kaj Gandien and Jonathan Rothblatt of Aventis Pharma Deutschland GmbH, Center for Functional Genomics, Martinsried).

Egg-laying rate and survival assays (radiation sensitivity)

Germ lines of worms defective either in checkpoint pathways or repair pathways typically exhibit hypersensitivity to IR (Gartner et al., 2000). One easily assayed manifestation of such sensitivity is a severe drop in the production of viable progeny following genotoxic insult; this can be reflected in a drop in the number of zygotes produced, the fractional viability of the zygotes produced, or both. To measure radiation sensitivity, worms were irradiated at L4 larval stage and transferred to fresh plates 24 h post treatment, when the germ line is already fully developed. Adult worms were removed from the plates after 12 h and the percentage of hatched embryos was determined 24 to 36 hours later as described previously (Gartner et al., 2000).

Strains

The N2 Bristol strain was used for this study (Brenner, 1974). Worms were maintained and raised at 20°C on NGM plates unless indicated otherwise.

All used strains are described by J. Hodgkin 1997 (Blumenthal, 1997), unless otherwise stated. *ced-3(n717)* is described by (Yuan et al., 1993) *ced-4(n1162)* by (Yuan and Horvitz, 1992), *ced-9(n1950)* (Hengartner et al., 1992), *egl-1(n1084n3082)* by Conradt and Horvitz 1998 (Conradt and Horvitz, 1998), *ced-1(e1935)* by Zhou et al. 2001 (Zhou et al., 2001), *rad-5(mn159)* by Ahmed et al. 2001 (Ahmed et al., 2001) *hus-1(op244)* by Hofmann et al. 2002 (Hofmann et al., 2002) and *mrt-2(e2664)* by Ahmed and Hodgkin 2000 (Ahmed and Hodgkin, 2000).

Deletion mutant *cep-1(lg12501)* carries a 1213 bp deletion corresponding to 30458-31670 on the cosmid sequence F52B5, comprising the *cep-1* sequence. We did not detect *cep-1* mRNA in *cep-1(lg12501)*. *ced-13(tm536)* deletion comprises the entire *ced-13* open reading frame.

Positional cloning of *gld-1(op236)*

Mutants were cleaned up genetically by outcrossing them versus the N2 wild type strain five times before they were crossed into and out of tightly linked visible genetic mutations, which had themselves been backcrossed against N2 wild type at least five times. Flanking markers used were *dpy-5(e61)* and *unc-29(e193)*. For SNP (single nucleotide polymorphism) cloning worms were crossed with the Hawaii strain. Recombinants between *dpy-5(e61) gld-1(op236) unc-29(e193)* and Hawaii worms were examined by PCR and restriction digest or DNA sequencing for the SNPs between N2 and Hawaii genotypes.

RNAi screen of germ line specific genes

RNAi feeding was performed as described for *cep-1* RNAi above. Clones in RNAi feeding vectors were provided my Marc Vidal of Dana Farber Cancer Center.

Deletion library construction

The deletion libraries were provided by Simon Boulton, Clare Hall Laboratories and Britta Leiers, Thorsten Hoppe and Ralf Baumeister, Department of Molecular Neurogenetics, Ludwig Maximilians University, Munich. The deletion libraries were constructed by 4,5',8-trimethylpsoralen (TMP) + UV (ultraviolet) mutagenesis as described presiously (Jansen et al., 1997). Developmentally synchronized young adult worms were incubated with 50 mg/ml TMP (1 mg/ml stock solution in acetone) for 1 hour under agitation and then UV-irradiated at 0.260 mW/cm^2 for 60 seconds at a wavelength of 360 nm. Worms were recovered in M9 buffer. The F1 generation was harvested after bleaching as described above. After being allowed to lay eggs, the F1 generation was bleached and F2 worms were aliquoted into 96 well plates for freezing in 50% freezing solution (see above) and an equivalent for genomic DNA preparations to allow PCR screening for desired deletion mutations.

For genomic DNA preparations 750 ml lysis buffer (see above) was added to 100 ml of worm pellet. Resuspended worms were incubated at 65°C for 1 hour and genomic DNA was purified with Phenol/$CHCl_3$/isoamylalcohol and then treated with 2 ml Roche RNAse A (100 mg/ml) at 37°C for 30 minutes followed by Phenol/$CHCl_3$ and $CHCl_3$ extraction, followed by a final $CHCl_3$ extraction. Genomic DNA was then precipitated with Ethanol and resolved in 10 mM Tris (pH 8.5).

PCR screening of deletion libraries

The deletion library was screened by PCR with Roche Taq polymerase according to the manufacturer's instructions. For detection of genomic deletion two consecutive rounds of PCR amplification were performed. In the first round a "poison primer" was used in addition to the flanking primers as described previously (Edgley et al., 2002). This additional primer anneals in a region between the two flanking primers and gives rise to a second, smaller PCR product in wildtype DNA but not in deletion mutant DNA. In the consecutive round of nested PCR this product, however, cannot serve as a template and, therefore, only deletion mutant DNA will be amplified.

The following primers were used:

For T19A5.1:

external primers (first round of PCR):

779 T19A5.1 17795 F 5’-gattctgacgtcacgcttag-3’

780 T19A5.1 21308 R 5’-tagttgtggtgatgcatccg-3’

“poison primer”:

827 T19A5.1 poison 19531 R 5’-caagagctgaatacagcctc-3’

internal primers (second round of PCR):

777 T19A5.1 17862 F 5’-acagagaaaaaggtccgtcg-3’

782 T19A5.1 21281 R 5’-cgatgactcaacgatgtagc-3’

For T05H10.5:

external primers (first round of PCR):

765 T05H10.5 20329 F 5’-cagacctctcaccattctct-3’

768 T05H10.5 23670 R 5’-aggaacgcgtgaaatttacag-3’

“poison primer”:

818 T05H10.5 poison 21974 F 5’-caagcataagatcaatgcgct-3’

internal primers (second round of PCR):

766 T05H10.5 20331 F 5’-gacctctcaccattctcttta-3’

769 T05H10.5 23640 R 5’-acatgtgcaatggtcaagtac-3’

For F35C8.7:

external primers (first round of PCR):

773 F35C8.7 11065 F 5’-tatctctcttacggaggtaaat-3’

776 F35C8.7 7581 R 5’-agcaatttttttcgcggtagtt-3’

“poison primer”:

823 F35C8.7 poison 9333 R 5’-agggttcagttggctgaagt-3’

internal primers (second round of PCR):

772 F35C8.7 10975 F 5’-caacatctttctcaaatcgtgt-3’

774 F35C8.7 7681 R 5’-tcatgaaacaatgctcgataga-3’

Candidate deletion products were subcloned by TA cloning using Invitrogen TA cloning vector pCRII kit (see above) and sequenced.

Plasmid and cosmid preparation

Bacterial plasmids and cosmids were isolated with Qiagen Mini- or Midikits according to the manufacturer’s instructions.

Cytosol extracts and immunoprecipitation (IP)

Functional GLD-1 for IP was obtained from a transgenic strain in which the *gld-1(q485)* null mutant was rescued by an extrachromosomal array (*ozEx40*) containing wild-type GLD-1 with the FLAG epitope placed at the C-terminus (GLD-1/FLAG), distant from the RNA binding domain as described by Lee and Schedl 2001 (Lee and Schedl, 2001).

Cytosol extracts were prepared from roughly synchronized young adult hermaphrodites from *gld-1(q485); ozEx40* worms grown in liquid culture. Bleach-synchronized cultures were harvested and washed two times with cold 100 mM NaCl. The floating worms were collected into cold H_2O after 30% sucrose cushion centrifugation and washed two times with homogenization buffer (HB, 15 mM Hepes pH 7.6, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM EGTA, 44 mM Sucrose), which was treated with 0.1% diethylpyrocarbonate. The worm pellet was resuspended in 4x volume HB with 1 mM DTT, 4 mM NaF, 3 mM Na_2VO_4, and protease inhibitors (Roche). Worms were then broken into several pieces after passing twice through a small chilled French press at 4000 psi. The carcasses were removed by centrifugation at 200 g for 2 min. The supernatant was transferred into a dounce homogenizer and stroked 25 times with a B pestle. The nuclei were spun down at 800 g for 5 min. The supernatant was collected and spun at 14,000 g for 20 min to clear the lysate. The cleared lysates were collected and adjusted for salt concentration by adding 1/10th volume of 15 mM Hepes pH 7.6, 1.0 M NaCl. This was the working cytosol extract. The cytosol extracts were subjected to immunoprecipitation immediately after isolation to minimize RNA degradation.

For each immunoprecipitation, 10 - 12 ml of cytosol extract (~10 mg/ml) from *gld-1(q485); ozEx40* was first preincubated with mouse IgG chemically coupled to protein G-Sepharose (Sigma) for 10 min at 4^{o}C to preabsorb nonspecific binders. The preabsorded extract was divided into two and incubated with either mouse IgG or with Anti-FLAG Ab M2 (Sigma) chemically coupled to protein G-Sepharose for 1 - 1.5 h at 4 C. The beads were washed 4 times with IP buffer (HB + 100 mM NaCl). After the final wash, the beads were eluted 5 times with the same bead volume of Elution buffer (IP buffer + 200 μ*g*/ml FLAG peptide). 1/20th of each elution volume (E1 to E5) was mixed and boiled with SDS sample buffer for western analysis with anti-GLD-1 Ab; the remaining material

from fractions that contain GLD-1/FLAG were pooled and extracted with phenol/chloroform to isolate RNAs. The typical yield of RNA was approximately 100 ng for both IgG IP and FLAG.

RT-PCR of FLAG-GLD-1 coimmunoprecipitated RNAs

The RNAs from IgG IP, FLAG IP, 1 μg of total RNA from *gld-1(q485); ozEx40* adult hermaphrodite worms were converted into first strand cDNAs using the 3'-RACE primer (GCGGGATCCTCGAGAAGCTTTTTTTTTTT) and Superscript II (Lifetech), extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 200 μl TE pH 8.0. 1-3 μl of the first strand cDNAs was used as a template for PCR using the following gene specific primers:

ced-3: 654 5'-gagcttgctagagaggaaca-3', 655 5'- ggtgacattggacactcgaa-3'

ced-4: 656 5'-tgctctgcgaaatcgaatgc-3', 657 5'- gcaatcactactggacgaag-3'

ced-9: 652 5'- atgctcaggacttgccatca-3', 653 5'- aatgagattctgggcactgc-3'

cep-1: 378 5'- aagttgtgctcgactcccaa-3', 384 5'-attgcaaatgccatatctgaac-3'

Another independent immunoprecipitation was performed and a second set of first strand cDNA was made to reconfirm the RT-PCR data, which was essentially identical between the first and the second sets.

6 List of Publications

Schumacher B, Hofmann K, Boulton S, Gartner A
The C. elegans homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis.
Curr Biol. 2001 Oct 30; 11(21):1722-7.

Gartner A, Alpi A, **Schumacher B**
Programmed cell death in *C. elegans*
Genetics of Apoptosis, Grimm S (ed.), BIOS Scientific Publishers Limited, 2003, 155-175

Schumacher B, Alpi A, Gartner
Cell Cycle: Check the Asynchrony
Curr Biol. 2003 Jul 15; 13(14):R560-2.

7 Curriculum Vitae

Date and place of birth: 18 April 1975, Hannover, Germany
Address: Am Klopferspitz 18a, D-82152 Martinsried
Telephone: 089-8578 3121
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Academic status: PhD student
Academic title: Master of Arts (MA)
Nationality: German
Marital status: single, no children

Scientific Education

Since October 2000 continuing PhD thesis work with Dr. Anton Gartner, Department of Cell Biology, Max Planck Institute for Biochemistry, Martinsried, Germany, presumable thesis defense in fall 2003

PhD project: 'DNA-damage Response in *Caenorhabditis elegans*'

May 2000-October 2000 Collaborative research with Dr. Julie Ahringer, Wellcome/CRC Institute, University of Cambridge, United Kingdom, on a 'Genome-wide RNAi screen in *C. elegans*' as part of my PhD thesis

1999-2000 starting of my PhD thesis work with Dr. Anton Gartner and Dr. Michael Hengartner, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, on 'DNA-damage Response in *Caenorhabditis elegans*' in affiliation with the PhD program in Molecular Genetics and Microbiology of the Department of Molecular Genetics and Microbiology, SUNY Stony Brook

1998-1999 Visiting scholar in the Master's program of the Department of Molecular Genetics and Microbiology, State University of New York (SUNY) at Stony Brook, USA

Master's Thesis with Dr. Dafna Bar-Sagi on 'Regulation of Nucleotide Exchange on Ras by Inter- and Intramolecular Interactions' at the Department of Molecular Genetics and Microbiology, SUNY Stony Brook, 30 July 1999

1995-1998: Studies in Biology (Diplomstudiengang Biologie) Department of Biology, University of Konstanz, Germany

Diploma examination, Department of Biology, University of Konstanz, grade:1.0

Intermediate Examination (Vordiplom) in Biology, University of Konstanz, 21 July 1997, grade: 1.6 (ranked 2nd)

University entrance qualification (Abitur), 14 June 1994, IGS Muehlenberg-Hannover, grade: 1.4 (ranked 1st)

Teaching Activities

Teaching assistant in Microbiology, Medical school, SUNY Stony Brook (Spring 2000)

Extracurricular Interests

Philosophy of Science with Prof. Dr. Paul Hoyningen-Huehne, University of Konstanz (1995-1997)

Meeting Abstracts

Schumacher B, Momoyo Hanazawa, Sudhir Nayak, Hengartner M, Schedl T, Gartner A

A genetic screen for enhanced DNA damage induced apoptosis in *C. elegans* reveals that the *gld-1* tumor suppressor acts as a negative regulator of p53 dependent apoptosis

Programmed Cell Death,

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

August 2003

Schumacher B, Wittenburg N, Boulton S, Hofmann K, Shaham S, Vidal M, Conradt B, Gartner A

The p53 pathway in *C. elegans*

APOPTOSIS 2003 From signaling pathways to therapeutic tools.

January/February 2003

European Parliament Conference Center (Luxembourg)

Schumacher B, Wittenburg N, Boulton S, Hofmann K, Shaham S, Vidal M, Conradt B, Gartner A

The p53 pathway in *C. elegans* (oral presentation)

Cancer Genetics and Tumor Suppressor Genes

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

August 2002

Academic Awards

Research stipend, Cold Spring Harbor Laboratory (August 1999-October 2000)

Tuition scholarship, SUNY Stony Brook (August 1999-October 2000)

DAAD scholarship for studying at the Department of Molecular Genetics and Microbiology at SUNY Stony Brook (August 1998-May 1999)

Nomination for the exchange program between the University of Konstanz, Germany, and SUNY Stony Brook, USA, by the University of Konstanz

8 Acknowledgements

First and foremost I would like to thank my advisor Toni Gartner not only for scientific guidance but also for friendship. It has been great fun to do science and discussing ideas together.

Furthermore, I would like to thank my fellow PhD student Arno Alpi and (former) members of the Cell Biology Department Ingvar Ferby, Ben Short, Giulia Guarguaglini, Catherine Regnard, Martina Casenghi for friendship and scientific discussions. I would also like to thank Erich Nigg for support.

I would like to thank my great collaborators Simon Boulton and Tim Schedl, for discussion as well as reagents. Simon Boulton did the transcription assays of CEP-1 in yeast (Figure 7). Min-Ho Lee in Tim Schedl's lab did the RT-PCRs on the FLAG tag purified GLD-1 extracts (Figure 22a). Furthermore, I would like to acknowledge Kay Hofmann who did the bioinformatics on p53 (Figure 6b,c) and also Barbara Conradt for providing the *ced-13(tm536)* mutant, Claudia Rudolph of Elegene for the *cep-1(lg12501)* deletion mutant, Marc Vidal's lab for the germ line specific RNAi clones. The deletion mutant of T05H10.5 was provided by Thorsten Hoppe, the deletion mutant of T19A5.1 was isolated from a deletion library of Ralf Baumeister's lab. The Affymetrix gene chip experiment was done by Kaj Grandien and Jonathan Rothblatt at the Aventis Center for Functional Genomics Aventis GmbH.

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