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Translational Repression of *C. elegans* p53 by GLD-1 Regulates DNA Damage-Induced Apoptosis

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Summary

p53 is a tumor suppressor gene whose regulation is crucial to maintaining genome stability and for the apoptotic elimination of abnormal, potentially cancer-predisposing cells. *C. elegans* contains a primordial p53 gene, *cep-1*, that acts as a transcription factor necessary for DNA damage-induced apoptosis. In a genetic screen for negative regulators of CEP-1, we identified a mutation in GLD-1, a translational repressor implicated in multiple *C. elegans* germ cell fate decisions and related to mammalian Quaking proteins. CEP-1-dependent transcription of proapoptotic genes is upregulated in the *gld-1(op236)* mutant and an elevation of p53-mediated germ cell apoptosis in response to DNA damage is observed. Further, we demonstrate that GLD-1 mediates its repressive effect by directly binding to the 3′UTR of *cep-1/p53* mRNA and repressing its translation. This study reveals that the regulation of *cep-1/p53* translation influences DNA damage-induced apoptosis and demonstrates the physiological importance of this mechanism.

Introduction

The central role of p53 as a tumor suppressor is demonstrated by the fact that most human cancers evolve ways to evade p53 tumor suppressor activity, particularly its transcriptional activation function (Roemer, 1999; Pierotti and Dragani, 1992; Vogelstein et al., 2000). Although human cancers commonly contain mutations in the p53 gene itself, many of the remaining tumors have defects in upstream signaling components of the p53 pathway such as inactivation of the positive regulators ARF or CHK2 (Sharpless and DePinho, 1999; Bartek and Lukas, 2003), or overexpression of the negative regulator Mdm2 (Freedman et al., 1999). For those tumors that retain functional p53 but have amplification of Mdm2, therapeutic strategies have been developed to inhibit Mdm2, whereby increased p53 protein levels make tumor cells more susceptible to p53-mediated apoptosis (Chene, 2003; Lain and Lane, 2003; Vassilev et al., 2004). Such a therapeutic approach highlights the need to uncover additional pathways and mechanisms that negatively regulate p53 levels or activity.

Most studies on p53 signaling have been conducted in cell culture-based systems, and their translation into mouse models is often hampered by the fact that some regulatory mechanisms which exist in tissues and organisms are not present in cell culture and that some p53 regulators are likely to be essential for organismal viability. *C. elegans* contains a primordial p53 gene, *cep-1* (*C. elegans* p53), that is necessary for DNA damage-induced apoptosis and acts as a transcription factor (Derry et al., 2001, Schumacher et al., 2001). The apparent absence of an Mdm2 homolog (WormBase website, http://www.wormbase.org), leads to the hypothesis that novel, possibly evolutionarily conserved mechanisms for negatively regulating *cep-1*/p53 exist in *C. elegans*. Therefore, we have taken a forward genetic approach, an unbiased genetic screen to identify negative regulators of *cep-1*/p53, to isolate mutants with increased apoptosis and upregulated p53 signaling.

In adult hermaphrodite worms, the germline resides in two U-shaped gonads where different germ cell types are spatially arranged in a gradient of maturation, which includes a distal proliferative stem cell compartment, entry into meiotic prophase that coincides with the early stages of meiotic chromosome pairing (transition zone), and the various subsequent stages of meiotic prophase, early and late pachytene, as well as diplotene and diakinesis that go hand in hand with oocyte growth and differentiation (Figure 5A, top panel; Hubbard and Greenstein, 2000; Seydoux and Schedl, 2001). The organization of the hermaphrodite germline is reminiscent of mammalian male germline development and may involve similar regulatory mechanisms (Tunquist and Maller, 2003), and pachytene cells can undergo apoptotic demise that often involves p53 signaling (Cohen and Pollard, 2001; Cooke and Saunders, 2002; Matzuk and Lamb, 2002).

In *C. elegans*, several pathways can lead to germ cell apoptosis during meiotic development (Hofmann et al., 2000). Physiological germ cell apoptosis is thought to control germ cell number homeostasis whereas DNA damage-induced apoptosis involves a conserved set of

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upstream checkpoint proteins needed to eliminate cells that received DNA damage (Gumienny et al., 1999; Gartner et al., 2000). Both of these germ cell apoptosis pathways use the same apoptotic core machinery as somatic cell death occurring during embryogenesis (Figure 2, top panel; Gumienny et al., 1999; Gartner et al., 2000). In mitotically dividing germ cells, however, checkpoint signaling, which requires the same upstream DNA damage checkpoint proteins as DNA damage-induced apoptosis, leads to transient *cep-1*/p53-independent cell cycle arrest without apoptosis (Gartner et al., 2000; Derry et al., 2001; Schumacher et al., 2001). By contrast in pachytene cells, upon ionizing radiation (IR) CEP-1/p53 transcriptionally induces the BH3 domain-only protein EGL-1 (Hofmann et al., 2002), analogous to mammalian p53 induction of BH3 domain-only proteins (Villunger et al., 2003).

To identify new components of CEP-1/p53 regulation, we conducted a genetic screen for mutations that enhance p53 signaling. One such mutation, *op236*, affects the conserved GLD-1 protein, leading to the elevation of p53-mediated germ cell apoptosis in response to DNA damage while multiple other developmental functions of GLD-1 remain unaffected. We show that GLD-1 mediates its repressive effect by directly binding to the 3′ UTR of *cep-1*/p53 mRNA and repressing translation.

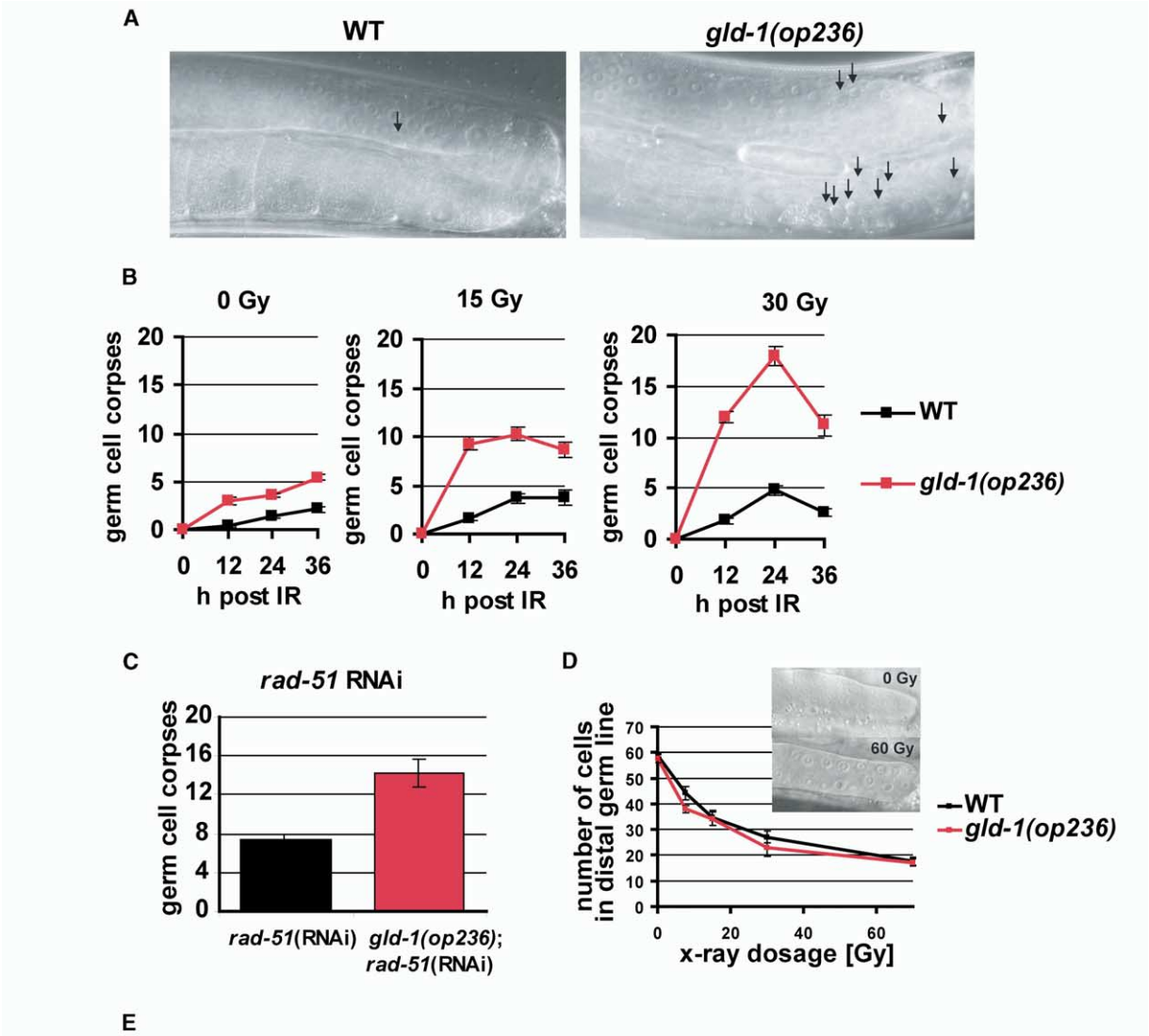
Results

A Genetic Screen for Negative Regulators of p53 Identifies a Novel Mutation in the *C. elegans* Germline Tumor Suppressor GLD-1

To identify genes that downregulate the p53 pathway in *C. elegans*, we conducted a genetic screen to find mutants that showed increased levels of apoptosis upon low doses of IR (Supplemental Note 1 at http://www.cell.com/cgi/content/full/120/3/357/DC1/). Two such mutants, *op236* and *op237*, showed a strong upregulation of apoptosis following IR (Figures 1A and 1B and data not shown) without a concomitant defect in DNA repair activity (see below). Both mutations are recessive and fail to complement each other, indicating that they are alleles of the same gene (data not shown). Positional cloning (Supplemental Data) and sequence analysis revealed that both mutants carry the same DNA alteration, a G to T transversion at nucleotide position 826 of the *C. elegans* germline tumor suppressor *gld-1* (T23G11.3), leading to a Valine to Phenylalanine substitution at amino acid 276, which lies in the GSG/STAR RNA binding domain (Jones and Schedl, 1995; Supplemental Figure S1a). Valine 276 is conserved in *Drosophila* and human GLD-1 homologs, Who/How and Quaking, respectively. *gld-1*-null mutant hermaphrodites have germline tumors as pachytene germ cells fail to maintain the oocyte differentiation pathway and re-enter the mitotic cell cycle. Other classes of *gld-1* alleles display feminization of the germline, masculinarization of the germline, or undifferentiated germline phenotypes (Francis et al., 1995a). However, none of the known *gld-1* alleles have been implicated in apoptosis. That the excess apoptosis phenotype of *op236* is due to a defect in *gld-1* function is indicated by the failure of *gld-1(null)* to complement the *op236* excess apoptosis phenotype and, conversely, a transgene expressing wild-type GLD-1 rescuing the excess apoptosis phenotype (Supplemental Note 2 and Supplemental Figure S1b). GLD-1 has been shown to bind and translationally repress a number of target mRNAs and is required for multiple aspects of germline development (Jan et al., 1999; Lee and Schedl, 2001, 2004; Marin and Evans, 2003; Xu et al., 2001; Mootz et al., 2004). To test whether *gld-1(op236)* has any of the developmental defects characteristic of other *gld-1* alleles, we looked at germlines of *gld-1(op236)* worms grown at 20°C (the standard temperature for propagating *C. elegans*) by Nomarski optics (not shown) as well as by DAPI staining. In both assays, the *gld-1(op236)* germlines resembled wild-type germlines (Supplemental Figure S2a). Furthermore, GLD-1 staining was not altered in wild-type and *gld-1(op236)* worms (Supplemental Figure S2a). Interestingly, the germlines of *gld-1(op236)/gld-1(null)* were feminized, indicating that *gld-1(op236)* is unable to rescue the sex-determination defect of *gld-1(null)* (data not shown). Because *gld-1(op236)* worms are essentially wild-type with the exception of their extra germ cell death phenotype, we wondered whether *gld-1(op236)* might display temperature-sensitive defects and examined mutants at 25°C. When grown at 25°C, *gld-1(op236)* animals indeed showed a dramatically increased level of germ cell apoptosis as compared to wild-type worms even in the absence of IR (Figure 3A). Affected germlines were smaller than wild-type and showed an extended pachytene region at the expense of oocytes (Figure 6C and Supplemental Figures S2b and S5a). The extended pachytene region appears to be a result of a delayed transition from pachytene to diakinesis with few or no diplotene oocytes at steady state, unlike wild-type germlines that have an ordered progression of oocytes from late pachytene through diplotene and diakinesis (Figure 6C and Supplemental Figures S2b and S5a). This phenotype is reminiscent of germlines where apoptosis is highly induced such as in *ced-9(lf)* or highly irradiated wild-type germlines where almost all pachytene cells die by apoptosis in late pachytene and hence, only very few progress further in oogenesis, resulting in a very low steady state level of diplotene and diakinesis oocytes (our unpublished data). We will refer to 25°C as the restrictive temperature for *gld-1(op236)* and 20°C as the semipermissive temperature.

The *gld-1(op236)* Mutation Affects *cep-1*/p53 Signaling upon DNA Damage

To assess whether *gld-1(op236)* might specifically affect the *cep-1*/p53 pathway, the following experiments were performed (at the semipermissive temperature). We evaluated whether the apoptotic phenotype of *gld-1(op236)* is due to DNA damage per se or due to a general stress response, such as oxidative stress that may also be caused by IR. To this end we generated unprocessed double-strand breaks in meiotic germ cells without IR by inactivation of *Ce-rad-51*, the functional homolog of bacterial recA, involved in strand invasion during meiotic recombination, which leads to unprocessed meiotic recombination intermediates in pachytene cells and *cep-1*/p53-dependent apoptosis



	0 Gy		30 Gy			60 Gy			120 Gy		
	egg laying	survival [%]	egg laying	relative egg laying [%]	survival [%]	egg laying	relative egg laying [%]	survival [%]	egg laying	relative egg laying [%]	survival [%]
WT	7.1 (±0.7)	99.5 (±0.3)	4.8 (±0.4)	68	84.0 (±2.7)	4.1 (±0.4)	58	58.6 (±1.78)	3.0 (±0.4)	42	35.5 (±4.3)
gld-1(op236)	4.7 (±1.1)	95.4 (±1.2)	2.4 (±0.3)	51	80.1 (±3.3)	2.0 (±0.3)	43	59.5 (±6.0)	0.6 (±0.3)	13	28.4 (±6.5)
cep-1	7.1 (±0.8)	98.4 (±0.7)	5.0 (±0.7)	70	72.9 (±5.0)	5.1 (±0.6)	72	54.0 (±2.9)	3.2 (±0.8)	45	21.9 (±4.7)
gld-1(op236) cep-1	5.5 (±0.5)	95.0 (±1.3)	3.7 (±0.3)	67	69.7 (±4.9)	2.9 (±0.2)	53	53.5 (±5.4)	2.2 (±0.3)	40	30.0 (±6.2)

Figure 1. *gld-1(op236)* Specifically Upregulates DNA Damage-Induced Apoptosis at the Semipermissive Temperature

Wild-type and *op236* mutant hermaphrodites (at 20°C) were irradiated at the L4 larval stage and apoptosis and cell cycle arrest was determined by Nomarski optics.

(A) Upon IR, *gld-1(op236)* shows an increased number of germ cell corpses (arrows) as seen by Nomarski optics.

(B) Quantification of germ cell corpses. Hermaphrodites (at 20°C) were irradiated at the L4 larval stage and the number of corpses was counted after the indicated time points. Error bars represent the standard error of the mean (SEM). For each dose and time point, 21 to 67 germlines were scored.

(C) Meiotic recombination intermediates hyper-induce apoptosis in *gld-1(op236)*. Wild-type (n = 16) and *gld-1(op236)* hermaphrodites (n = 28) were injected with double-strand *rad-51* RNA and progeny of *rad-51*-depleted animals were analyzed for germ cell apoptosis (Gartner et al., 2000, 2004).

(D) *gld-1(op236)* does not affect the mitotic cell cycle arrest checkpoint response. Wild-type and *gld-1(op236)* mitotic cells similarly decrease in number, within a defined volume, but increase in size as they arrest upon DNA damage (n = 4 to 12) (Supplementary Note 3). In the right upper panel representative pictures of mitotic cells before and after irradiation are shown.

(E) *gld-1(op236)* is not hypersensitive to DNA damage. Hermaphrodites (n = 18) were irradiated at the L4 stage, transferred 24 hr post-irradiation, and allowed to lay eggs for 12 hr. Egg laying rates are indicated per animal and hour. Progeny survival was counted 36 hr later. Relative egg laying indicates the percentage of eggs laid in comparison to untreated worms (0 Gy) of the same genotype.

(Alpi et al., 2003; Gartner et al., 2000). Given that apoptosis is increased following *rad-51* RNAi in *gld-1(op236)* mutants at the semipermissive temperature as compared to wild-type, this is likely a specific response to damaged DNA (Figure 1C). The increased apoptosis in *gld-1(op236)* in response to IR and *Ce-rad-51* RNAi could be caused by defects in DNA repair or a specific upregulation of the p53 apoptotic signaling pathway which in *C. elegans* only affects IR-induced cell death and not DNA repair (Derry et al., 2001; Schumacher et al., 2001). DNA double-strand repair mutants, upon treatment with IR, display increased levels of germ cell apoptosis and progeny lethality due to unrepaired DNA damage (Boulton et al., 2002). To evaluate whether *gld-1(op236)* is deficient in repairing damaged DNA, we measured DNA damage sensitivity by scoring levels of progeny survival (at the semipermissive temperature) after IR (Gartner et al., 2000; Figure 1E). Following IR, the number of fertilized eggs drops more dramatically in *gld-1(op236)* as compared with wild-type, most likely as a result of increased germ cell death as the drop in the number of fertilized eggs can be largely rescued by a *p53/cep1(null)* mutant (also see below, Figure 1E). The progeny of *gld-1(op236)* animals, as well as the progeny of *gld-1(op236) cep-1(null)* double mutants, however, show the same survival rate as the progeny of wild-type worms and *cep-1(null)* worms. We next carefully analyzed the DNA damage-dependent cell cycle arrest phenotype, and consistent with the notion that *gld-1(op236)* and wild-type worms are equally sensitive to IR we found a similar drop in mitotic cell number in *gld-1(op236)* and wild-type with increasing dose of IR (Figure 1D), arguing that *gld-1(op236)* is not irradiation sensitive or affects upstream checkpoint signaling (Supplemental Note 3; Figure 1D) (Gartner et al., 2000, 2004). In summary, these results suggest that the *gld-1(op236)* mutation specifically affects the p53/*cep-1*pathway to upregulate the apoptotic response to DNA damage.

Genetic Epistasis Analysis with *gld-1(op236)*

We next evaluated whether the enhanced germ cell death in *gld-1(op236)* upon IR is dependent on the core apoptotic machinery (Figure 2A). We asked whether loss-of-function alleles of the *C. elegans* apoptosis genes, *ced-3* and *ced-4*, as well as a gain-of-function allele of *ced-9* would suppress the *gld-1(op236)* phenotype. Loss-of-function mutations *ced-3(n717)* and *ced-4(n1162)* completely suppressed and a gain-of-function allele of *ced-9(n1950)* very strongly suppressed apoptosis [*gld-1(op236); ced-3(n717)* 0 ± 0 germ cell corpses (n > 15), *gld-1(op236); ced-4(n1162)* 0 ± 0 germ cell corpses (n > 15), *gld-1(op236); ced-9(n1950)* 1.3 ± 0.3 germ cell corpses (n = 15), 24 hr post 60 Gy of IR, all at 20°C], suggesting that *gld-1(op236)* acts upstream of the core cell death pathway (Figure 2A).

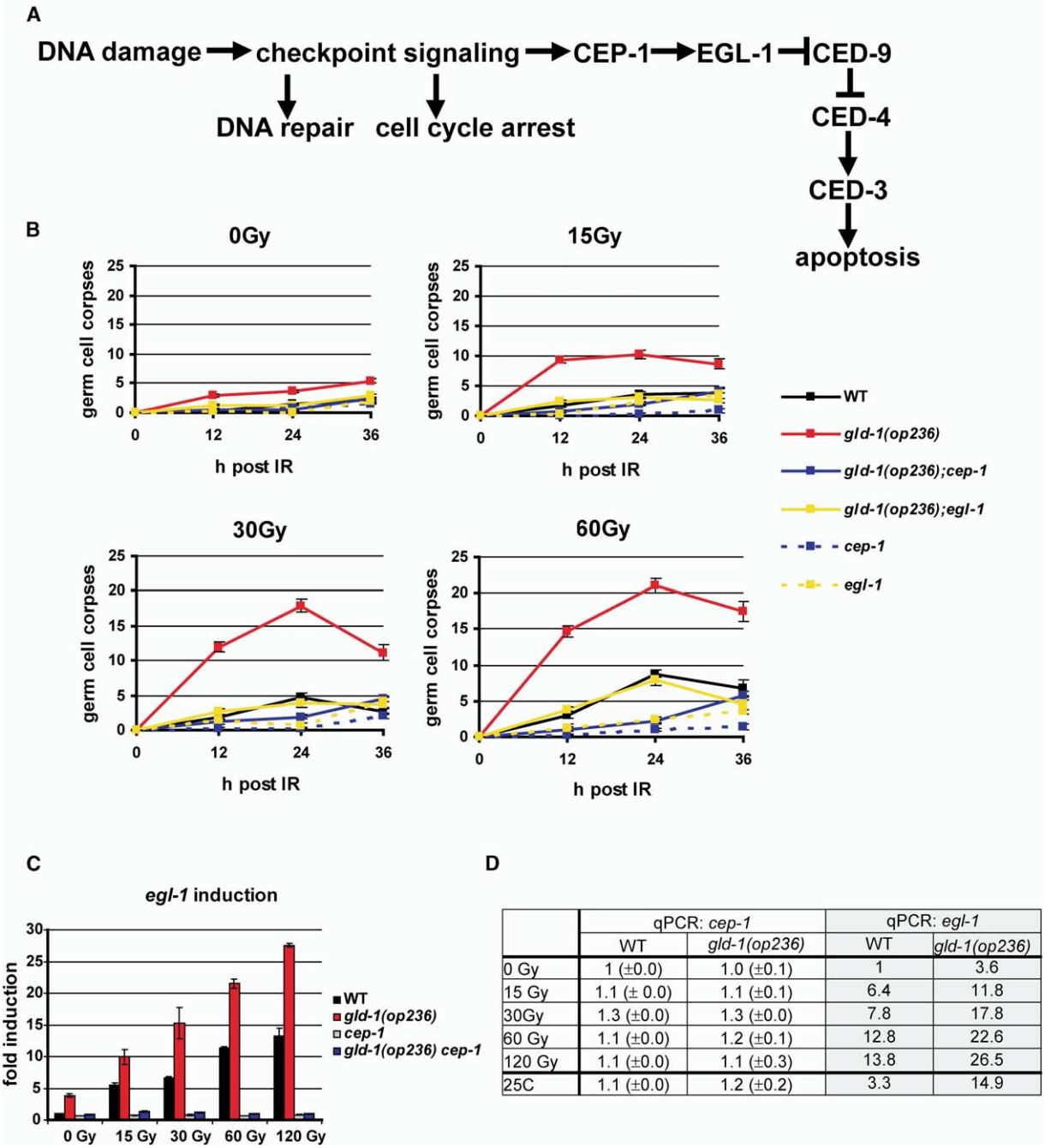
To further determine where *gld-1* acts in DNA damage-induced apoptosis, we performed genetic epistasis analysis with genes that act upstream of the core apoptotic machinery. A deletion mutant of *cep-1*/p53 almost completely suppressed *gld-1(op236)* IR-induced apoptosis while a null mutation in the CEP-1/p53 target gene *egl-1* strongly suppressed *gld-1(op236)* at the semipermissive temperature (Figure 2B). Furthermore, the increased cell death of *gld-1(op236)* at the restrictive temperature was also largely dependent on *cep-1*/p53 and *egl-1* as both mutants strongly suppressed the *gld-1(op236)* extra cell death phenotype at this temperature (Figure 3A). These results suggest that *gld-1(op236)* affects *cep-1*/p53 signaling at both the semipermissive and the restrictive temperatures and that it acts upstream or at the same level as *cep-1*/p53. Given that the *gld-1(op236)* extra cell death phenotype at 25°C is not completely suppressed by *cep-1*/p53, *gld-1* is likely to act on another, as of now uncharacterized gene(s), besides *cep-1.*

GLD-1 Downregulates *cep-1*/p53-Dependent Transcription

As apoptosis in *gld-1(op236)* is dependent on the *cep-1*/p53 pathway, we first tested whether CEP-1/p53 activity is upregulated in *gld-1(op236)* worms grown at the semipermissive temperature. To assess CEP-1/p53 activity in vivo, we measured the transcript levels of *egl-1*, which was previously shown to be a CEP-1/p53 target, by quantitative real-time PCR (qPCR) (Figure 2C) (Hofmann et al., 2002). When wild-type and *gld-1(op236)* worms were compared, *egl-1* transcript levels were further increased by approximately 2- to 3-fold in *gld-1(op236)* worms (Figure 2C). We also tested whether the *cep-1*/p53 pathway was upregulated at the restrictive temperature, where *gld-1(op236)* showed a strong increase in apoptosis, even in the absence of IR (Figure 3A). *egl-1* mRNA levels in *gld-1(op236)* were indeed elevated at the restrictive temperature and this increase in *egl-1* mRNA levels following DNA damage was also dependent on *cep-1*/p53 (Figures 3B and 3C). Therefore, we conclude that the apoptotic induction in *gld-1(op236)* at the restrictive temperature is largely due to the upregulation of the p53 pathway. If the level of *egl-1* is upregulated in a partial loss-of-function *gld-1(op236)*, then the level of *egl-1* should also be upregulated in a *gld-1*-null allele. To test this, we measured *egl-1* mRNA levels in *gld-1(null)* and found that *egl-1* mRNA levels were indeed upregulated, 6.04 (±0.41)-fold over wild-type. We conclude that GLD-1 represses CEP-1 activity and this repressive effect is defective in *gld-1(op236)* mutants. To confirm that *egl-1* transcription generally reflects p53 activity, we measured mRNA levels of another transcriptional target of p53, *ced-13* (Schumacher et al., 2005), which indeed showed the same GLD-1 and irradiation dependency as *egl-1* (data not shown).

GLD-1 Binds to *cep-1* mRNA

Given that GLD-1 has previously been characterized as an mRNA binding protein that represses the translation of target mRNAs (Lee and Schedl, 2001, 2004), it seemed plausible that GLD-1 might directly bind the *cep-1*/p53 mRNA. To test this, we immunoprecipitated (IP) FLAG-tagged GLD-1 from cytosol extracts derived from adult hermaphrodites containing a *gld-1::flag* transgene and reverse transcribed the coprecipitated mRNAs, which were then subjected to semiquantitative PCR amplification using primers directed against candidate genes. Using this strategy, we found an enrich-



	qPCR: *cep-1*		qPCR: *egl-1*	
	WT	*gld-1(op236)*	WT	*gld-1(op236)*
0 Gy	1 (±0.0)	1.0 (±0.1)	1	3.6
15 Gy	1.1 (± 0.0)	1.1 (±0.1)	6.4	11.8
30Gy	1.3 (±0.0)	1.3 (±0.0)	7.8	17.8
60 Gy	1.1 (±0.0)	1.2 (±0.1)	12.8	22.6
120 Gy	1.1 (±0.0)	1.1 (±0.3)	13.8	26.5
25C	1.1 (±0.0)	1.2 (±0.2)	3.3	14.9

Figure 2. Genetic Analysis of *gld-1(op236)* Reveals that *gld-1* Acts Upstream or at the Same Level as *cep-1*/p53

(A) A diagram of the DNA damage checkpoint pathway is shown. Note that in *C. elegans* DNA damage-induced apoptosis but not cell cycle arrest or DNA repair is dependent on *cep-1/p53*.

(B) Apoptosis in *gld-1(op236)* is dependent on *cep-1*/p53 and *egl-1*. Hermaphrodites were irradiated and analyzed as in Figure 1 (n = 8 to 67 for each data point). *egl-1(n1084n3082)* null mutation is referred to as *egl-1* and the deletion mutant *cep-1(lg12501)* is referred to as *cep-1*.

(C) Quantitative real-time PCR (qPCR) to measure *egl-1* mRNA levels. L4 hermaphrodites treated with IR and total RNA were isolated after 20 hr. Levels were normalized to γ tubulin mRNA. Fold induction was calculated relative to levels in nontreated wild-type worms. In the representative experiment shown, qPCRs were done in duplicate; error bars represent SEM.

(D) Quantification of *cep-1* mRNA levels (*egl-1* qPCR was done as internal control).

ment of *cep-1*/p53 mRNA, similarly to the positive controls *rme-2* and *gna-2*, while there was no enrichment of *ced-9*, *ced-4*, *ced-3*, or *egl-1* mRNAs (Figure 4A). This indicates that GLD-1 preferentially binds to the *cep-1*/p53 mRNA. To confirm the interaction of GLD-1 with *cep-1*/p53 mRNA and to narrow down the GLD-1

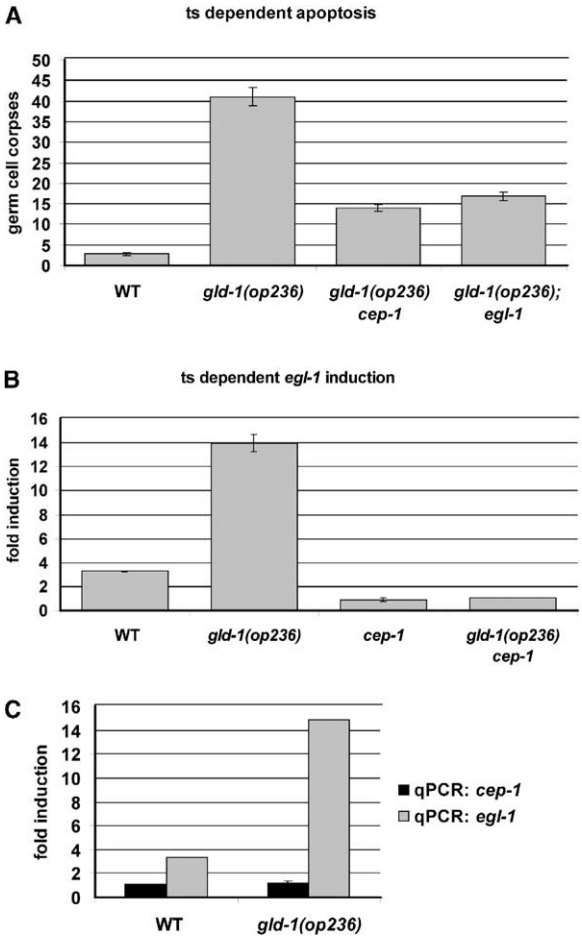


Figure 3. *gld-1(op236)* Is a Temperature-Sensitive Allele that Leads to the DNA Damage-Independent Induction of CEP-1/p53-Dependent *egl-1* Transcription and Apoptosis at the Restrictive Temperature

(A) Hermaphrodites at L4 larval stage were shifted to 25°C and germ cell corpses were quantified after 24 hr (n = 26 to 68). Error bars represent SEM.

(B) Hermaphrodites were treated as in (A), total RNA was isolated 20 hr post-temperature shift, and *egl-1* transcript levels were measured by qPCR. Fold induction was calculated relative to levels in nontreated wild-type hermaphrodites at 20°C.

(C) qPCR comparing the fold-induction of *cep-1* and *egl-1* mRNA levels at 25°C versus 20°C from wild-type and *gld-1(op236)* worms as also shown in Figure 2D.

binding region, we asked whether biotinylated *cep-1*/p53 mRNA subfragments can coprecipitate GLD-1 protein from cytosol extracts (Figure 4B). We found that only biotinylated *cep-1*/p53 mRNA subfragments that contained the 3′UTR of the *cep-1*/p53 mRNA coprecipitated GLD-1 protein from worm extracts (Figure 4B, lower panel). When the same concentration of *cep-1* mRNA and three previously characterized targets, *rme-2*, *tra-2*, and *gna-2* mRNAs (Lee and Schedl, 2001, 2004), were tested for GLD-1 binding, *cep-1* mRNA was bound less efficiently (Figure 4C). This suggests that the affinity of GLD-1 for the *cep-1* mRNA is relatively lower than for *rme-2*, *tra-2*, and *gna-2* mRNAs. When we used cytoplasmic extracts from *gld-1(op236)* adult hermaphrodites, the interaction of the *cep-1*/p53 3′UTR with GLD-1*(op236)* was dramatically reduced, indicating that GLD-1*(op236)* is defective in binding to *cep-1* mRNA (Figure 4C). In contrast, GLD-1(*op236*) binding to *rme-2, tra-2*, and *gna-2* mRNAs is relatively unaffected (Figure 4D). In summary, our data suggest that GLD-1 specifically binds to the *cep-1*/p53 3′UTR and that the interaction of GLD-1(*op236*) with *cep-1* mRNA is dramatically reduced while it retains sufficient binding to at least three other target mRNAs. This is consistent with the notion that GLD-1*(op236*) might be proficient in most, if not all, GLD-1 functions except its effect on *cep-1*-dependent apoptosis at the semipermissive temperature. Therefore, *gld-1(op236)* may be specifically defective in interaction with the *cep-1* mRNA while binding to other targets remains sufficiently strong.

GLD-1 Represses the Translation of *cep-1*/p53 mRNA

As GLD-1*(op236)* has specifically lost its interaction with *cep-1*/p53 mRNA, we next asked how GLD-1 downregulates *cep-1*/p53. We first addressed whether GLD-1 affects *cep-1* transcript levels. We found that *cep-1*/p53 transcript levels in *gld-1(op236)* worms at the semipermissive or the restrictive temperatures, following IR, or in *gld-1(null)* animals are similar to *cep-1*/p53 transcript levels in wild-type animals (Figures 2D and 3C and Supplemental Figure S3). Since GLD-1 has been shown to repress the translation of a number of target mRNAs (Jan et al., 1999; Lee and Schedl, 2001, 2004; Marin and Evans, 2003; Xu et al., 2001; Mootz et al., 2004), we asked whether GLD-1 represses *cep-1*/p53 translation. To assess CEP-1/p53 protein levels, we raised polyclonal antibodies to CEP-1/p53 and stained wild-type adult hermaphrodite germlines (Figure 5A and Supplemental Figure S4). CEP-1/p53 is abundant in mitotically dividing distal germ cells. Upon the entry into meiotic prophase in the transition zone, CEP-1/p53 protein is completely absent. CEP-1/p53 reappears in late meiotic pachytene cells and remains up to the diplotene/early diakinesis stage (Figure 5A and Supplemental Figure S4). Subcellularly, CEP-1/p53 is localized to the nucleoplasm (Figure 5A and Supplemental Figure S4) and the concentration and/or localization of CEP-1/p53 is apparently unaffected by IR (Supplemental Figures S6c and S6d). Furthermore, there is a reciprocal relationship between CEP-1/p53 and GLD-1 protein levels, as CEP-1/p53 is only high where GLD-1 levels are low, consistent with the hypothesis that GLD-1 might repress the translation of *cep-1* mRNA (Figure 6A).

We next asked whether CEP-1/p53 protein is misexpressed and/or whether its levels are increased in *gld-1* mutants. We found a dramatic misexpression and a dramatic increase in levels of CEP-1/p53 protein in early meiotic prophase germ cells in *gld-1(null)* as compared with wild-type animals (note the exposure time for the *gld-1(null)* germline is ~5 times shorter than the other pictures; Figure 5B versus 5A). Similarly, when we stained *gld-1(op236)* worms grown at the restrictive temperature, we observed increased levels of CEP-1/

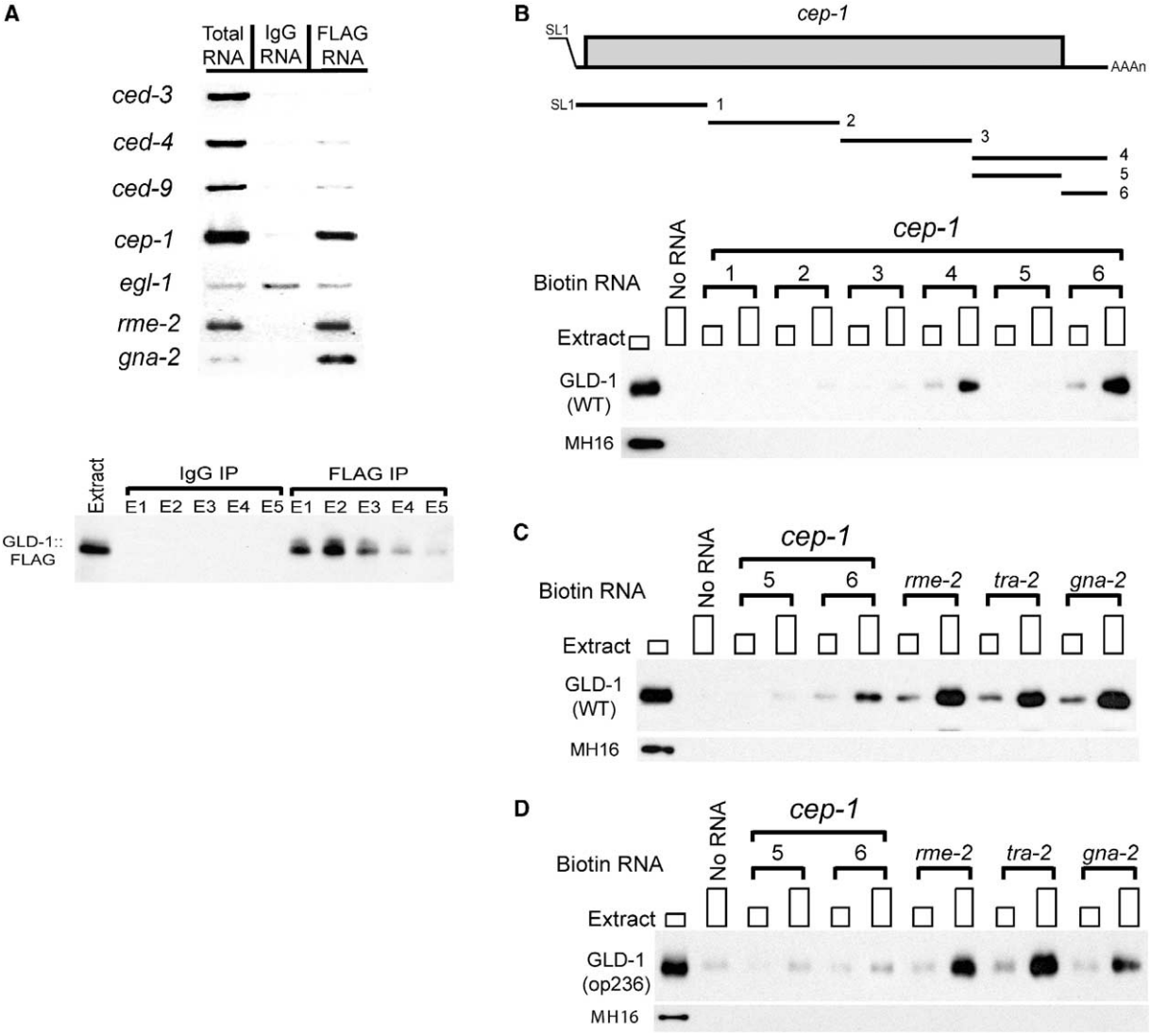


Figure 4. GLD-1 but Not GLD-1(*op236*) Binds to the 3′ UTR of *cep-1*/p53 mRNA

(A) Cytosol extracts from hermaphrodites containing a transgene expressing a GLD-1::FLAG fusion protein were used for coimmunoprecipitation of mRNAs, and the relative level of the precipitated mRNA for the indicated cell death genes, as wells as for the positive controls *rme-2* and *gna-2*, was assessed by cDNA synthesis and subsequent semiquantitative PCR reactions. In the left column, semiquantitative PCRs of total RNA; in the middle column, RNA from the control IgG IP; and in the right column, RNA from the FLAG IP are shown. In the lower panel, a Western blot indicating the specificity of the FLAG precipitation is shown. Upon precipitation with control IgG and anti-FLAG antibodies the immunoprecipitates were eluated with FLAG peptides and the eluates (E1 to E5) were subjected to Western blot analysis.

(B) Mapping the region of the *cep-1* mRNA that binds GLD-1. Each biotinylated *cep-1* mRNA subfragment, as indicated in the upper panel (400 ng or no RNA), was incubated with increasing amount of cytosol extract (50 ng and 150 ng total protein, small and large open bars, respectively) from adult wild-type (lower B panel and in [C]) or *gld-1(op236)* mutant hermaphrodites (D). GLD-1 was detected via Western analysis using anti-GLD-1 antibodies. MH16, an anti-Paramyosin antibody, was used for control Western blot.

(C) Comparison of *cep-1* binding with known GLD-1 targets. Biotinylated mRNAs (80 nM) of *cep-1*, *rme-2*, *tra-2*, and *gna-2* were used to assess their interaction with wild-type GLD-1.

(D) GLD-1(*op236*) specifically affects *cep-1* mRNA binding.

p53 protein that accumulated in more distal pachytene nuclei as compared with wild-type animals grown at the same temperature (Figure 5A and Supplemental Figure S5a). As expected from the phenotype and the RNA binding data, GLD-1(*op236*) was still able to repress the translation of another GLD-1 target, *rme-2* mRNA, at both temperatures (Supplemental Figures S5a and S5b). When *gld-1(op236)* hermaphrodites grown at the semipermissive temperature were examined, CEP-1/p53 protein levels as well as the number of CEP-1/p53-positive cells appeared slightly higher as compared to wild-type (Supplemental Figure S6a). This finding was supported by careful examination of many germlines where we found that the CEP-1/p53 accumulated in slightly but significantly more pachytene nuclei in *gld-1(op236)* than in wild-type (Figure 5C). As expected, we

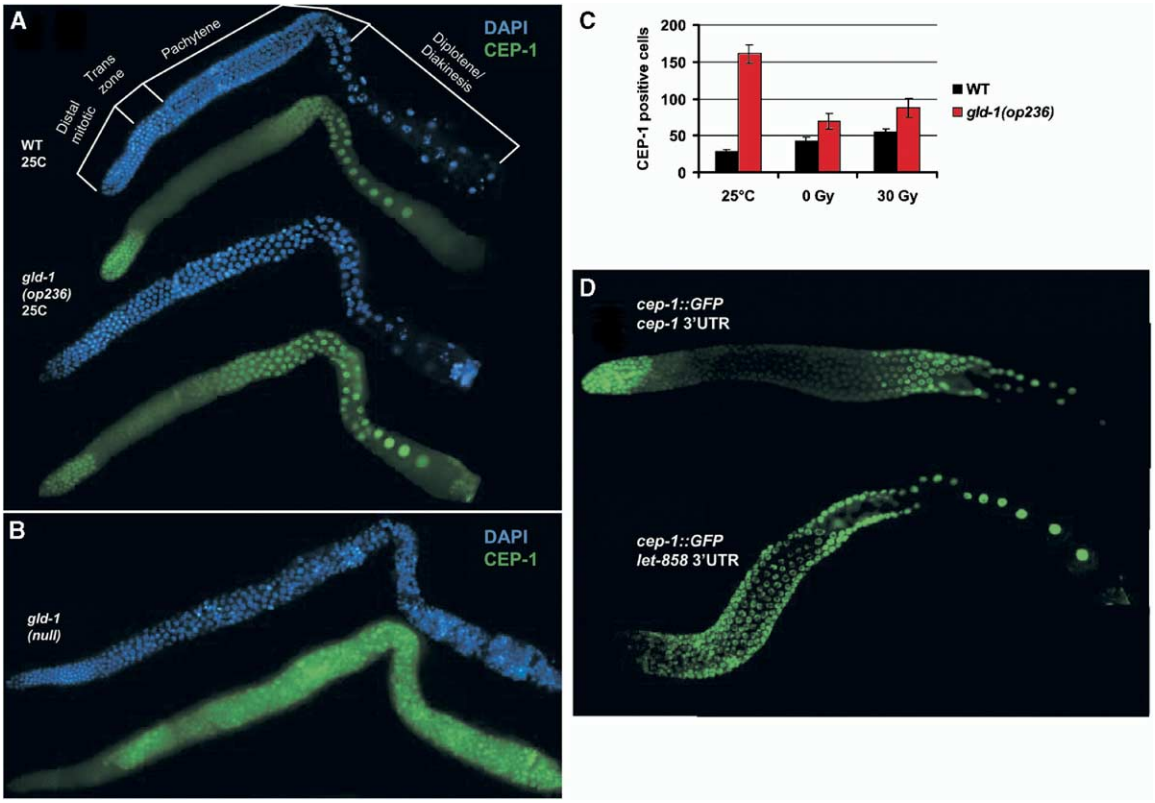


Figure 5. CEP-1/p53 Protein Levels Are Upregulated in *gld-1* Mutant Germlines

(A) Wild-type (upper) and *gld-1(op236)* (lower) germlines from hermaphrodites grown at the restrictive temperature (25°C) and dissected 16 hr post L4 larval stage and stained with anti-CEP-1 antibodies (green) and DAPI (blue). To detect quantitative differences in staining intensities, nonsaturating pictures were taken. The various stages of germ cells in the dissected gonad are indicated in the corresponding upper DAPI-stained germlines.

(B) *gld-1(null)* worms (grown at 20°C) were dissected 24 hr post L4 and treated similarly as in (A) but exposure time was ~5 times shorter, indicative of highly upregulated CEP-1/p53 levels.

(C) Quantification of the number of pachytene germline nuclei with CEP-1 staining in wild-type and *gld-1(op236)* at the restrictive temperature (25°C) as well as at the semipermissive temperature in the presence and absence of IR treatment (n = 11–15). The number of CEP-1-positive pachytene cells (as defined by their DAPI morphology) was identified by their distinct nuclear CEP-1 staining in dissected germline preparations.

(D) Dissected germline of a CEP-1::GFP fusions with the 3′UTR of *cep-1*/p53 (top panel) and with the 3′UTR of *let-858* (bottom panel). Note that the distal tip cell area of the germline in the bottom panel is out of focus. The CEP-1::GFP fusion transgene only leads to partial rescue of the IR-induced apoptosis phenotype of *cep-1(null)*, possibly because the GFP fusion, which is close to the CEP-1/p53 tetramerization domain, compromises its activity (R.H. and M.H., unpublished data).

could confirm elevated levels of CEP-1/p53 protein by Western blotting in *gld-1(null)* total worm extracts but not in *gld-1(op236)* worms grown at the restrictive or the semipermissive temperatures. This suggests that the elevated levels of CEP-1/p53 in *gld-1(op236)* pachytene cells are difficult to detect in total worm extracts, presumably due to the abundant CEP-1/p53 expression in many somatic tissues not regulated by *gld-1* (Derry et al., 2001), (B.S. and A.G., unpublished data; Supplemental Figure S6b). In conclusion, our results suggest that GLD-1 acts as a translational repressor of *cep-1*/p53 mRNA. Complete *gld-1* loss-of-function leads to the most dramatic de-repression of *cep-1*/p53 translational inhibition, while *gld-1(op236)* at the restrictive temperature leads to an intermediate effect, and at the semipermissive temperature leads to a weak de-repression of translational inhibition. These data indicate that translational repression of *cep-1*/p53 is likely mediated by the binding of GLD-1 to the 3′UTR of the *cep-1*/p53 mRNA. If correct, the exchange of the *cep-1* 3′UTR with an unrelated 3′UTR that does not confer GLD-1 regulation should lead to ectopic CEP-1/p53 accumulation similar to what is observed in *gld-1(null)*. To test this, we constructed a CEP-1::GFP fusion where the *cep-1*/p53 3′UTR is replaced with the 3′UTR of the *let-858* gene, which is expressed throughout the germline (Kelly et al., 1997) and assessed its expression in the presence of wild-type GLD-1. We found that this construct leads to ectopic accumulation of CEP-1/p53::GFP similar to the CEP-1 staining pattern observed in *gld-1(null)* germlines, whereas a CEP-1::GFP construct containing the *cep-1*/p53 3′UTR showed the wild-type pattern of CEP-1 staining (Figure 5D). Therefore, we conclude that GLD-1 translationally re-

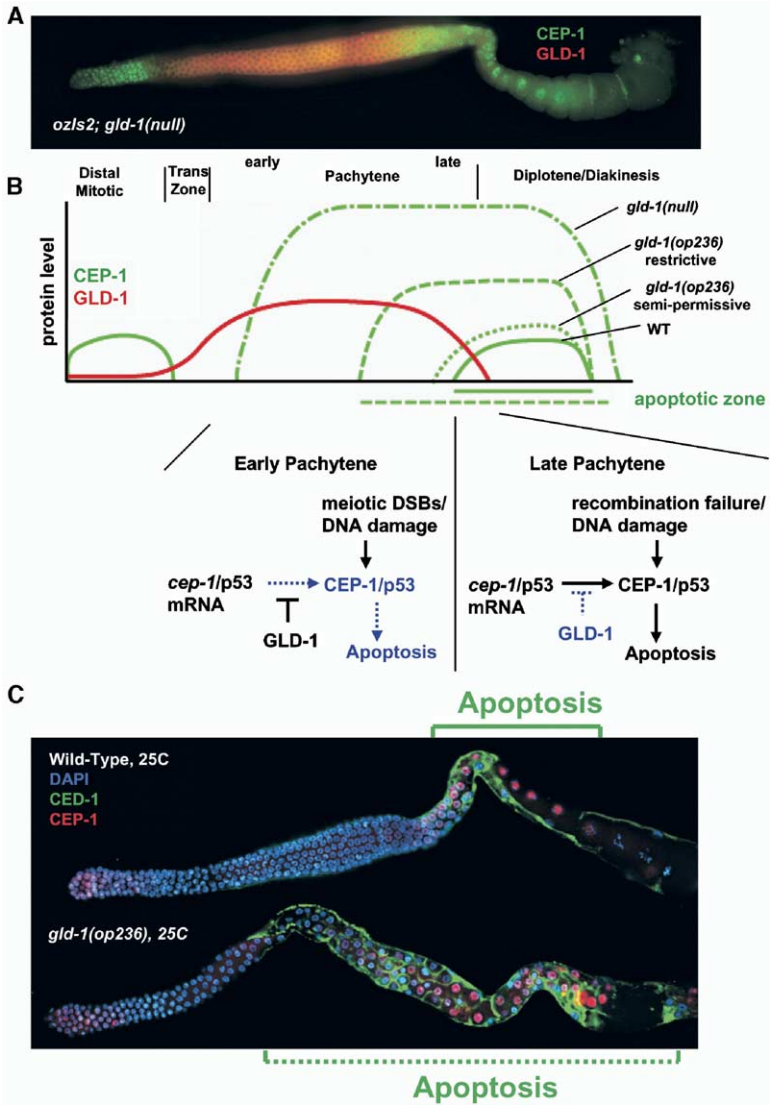


Figure 6. CEP-1/p53 Regulation in the *C. elegans* Germline

(A) Reciprocal relationship between CEP-1/p53 and GLD-1 protein levels. Double staining with anti-CEP-1 antibodies and anti-GFP antibodies of germlines from *gld-1(null); ozIs2* adult hermaphrodites expressing a GLD-1::GFP fusion protein (the GLD-1::GFP staining pattern resembles endogenous GLD-1 protein). To detect quantitative differences in staining intensities, nonsaturating pictures were taken and thus lower levels of GLD-1 in the mitotic region and distal transition zone are not observed under these conditions.

(B) Model for *cep-1*/p53 regulation. Upper panel: The reciprocal relationship between CEP-1 (green) and GLD-1 (red) protein levels is shown. CEP-1/p53 (green) levels in *gld-1* mutants are indicated by dotted and dashed curves. Furthermore, the zones of apoptosis in wild-type and *gld-1(op236)* hermaphrodites grown at the restrictive temperature are indicated by the solid and the dashed green lines, respectively. The various stages of germline development in the upper part of the panel are roughly aligned with the germline shown in (A). We note that in the transition zone an activity, in addition to GLD-1, is likely downregulating CEP-1/p53 levels. Lower panel: Model depicting antagonistic relationship between GLD-1 and CEP-1/p53. In early pachytene cells, high GLD-1 levels repress *cep-1*/p53 mRNA translation and DNA damage does not lead to apoptotic cell death due to the absence of CEP-1/p53. In late pachytene cells, where GLD-1 levels are falling, *cep-1*/p53 mRNA is translationally de-repressed and hence CEP-1/p53 can respond to DNA damage stimuli.

(C) Excess apoptosis in *gld-1(op236)* germlines at the restrictive temperature. Triple staining in wild-type and *gld-1(op236)* strains containing an integrated transgene expressing a CED-1::GFP fusion protein; DAPI (blue), anti-CEP-1 (red), and anti-GFP (green) were used to detect CED-1. CED-1 staining is found in somatic sheath cells that surround the germline. In apoptotic germ cells, which are engulfed by sheath cells, CED-1::GFP staining surrounds the corpse (Zhou et al., 2001). Both the number of germ cells and the distal-proximal region of the germline that contains CED-1::GFP that surrounds apoptotic germ cells is larger in *gld-1(op236)* than wild-type.

presses the *cep-1*/p53 mRNA by binding to the 3′UTR and alleviation of this repression leads to the accumulation of CEP-1/p53 protein.

Discussion

We undertook a genetic screen to identify regulators of the p53 pathway in *C. elegans* and discovered GLD-1 as a negative regulator of CEP-1. *gld-1(op236)* is a temperature-sensitive allele that at the semipermissive temperature leads to upregulation of *cep-1*/p53-dependent germ cell apoptosis synergistically with DNA damage signaling. At the restrictive temperature *cep-1*/p53-dependent germ cell apoptosis is upregulated even without DNA damage. In wild-type germlines, CEP-1/p53 protein levels are tightly regulated in the early stages of meiotic prophase by GLD-1. CEP-1/p53 levels are very low in the transition zone and early pachytene cells where GLD-1 levels are high in the cytoplasm. As GLD-1 levels decrease in late pachytene cells, CEP-1/p53 levels increase (Figures 6A and 6B, upper panel). In wild-type animals, the upregulation of CEP-1/p53 protein in late pachytene cells through alleviation of GLD-1-mediated translational repression is not sufficient to trigger apoptosis. We postulate that other events such as DNA damage-dependent phosphorylation of conserved CEP-1/p53 residues are likely required for its full function as a transcriptional activator of *egl-1* expression (Figure 6B). In *gld-1(op236)* worms grown at the semipermissive temperature, CEP-1 levels are only partially upregulated and we postulate that GLD-1(*op236*) is partially defective in binding to the *cep-1*/p53 3′UTR in vivo (Figure 6B). Under these conditions, CEP-1/p53-mediated apoptosis still requires

the DNA damage signal but is dramatically enhanced due to the elevated CEP-1/p53 levels. In *gld-1(op236)* worms grown at the restrictive temperature, GLD-1 binding to the *cep-1*/p53 3′UTR is likely further decreased, leading to further elevated levels of CEP-1/p53 protein as well as to the apparent misexpression of CEP-1/p53 in early pachytene cells (Figures 5A and 6C). Given that DNA damage-independent but *cep-1*/p53-dependent apoptosis occurs in *gld-1(op236)* mutants at the restrictive temperature, we think that CEP-1/p53 protein levels become sufficiently high to trigger apoptosis without further activation of CEP-1/p53 by the DNA damage pathways. At the restrictive temperature, however, other unknown GLD-1 targets that are involved in germline differentiation and apoptosis are likely misexpressed, which may explain the more severe germline phenotype and the relative increase in *cep-1*/p53-independent germ cell death (Figure 3A). In *gld-1(null)* germlines, the maximal de-repression of CEP-1/p53 translational inhibition occurs due to the absence of GLD-1 protein. In *gld-1(null)* germlines, however, we do not observe excessive apoptosis despite high CEP-1/p53 levels (data not shown); this is likely due to the very low number of late pachytene cells, which is the only germ cell type that undergoes apoptosis, since in *gld-1(null)* germlines early pachytene cells revert to mitotic proliferation (Francis et al., 1995a, 1995b).

At present we do not know the exact binding site(s) of GLD-1 in the *cep-1* 3′UTR. Recently, it has been shown that a hexanucleotide sequence in *tra-2* 3′UTR is important for GLD-1 binding in vitro and that this sequence is present in 3′UTRs of known GLD-1 targets (Ryder et al., 2004). Interestingly, the hexanucleotide sequence is present in the *cep-1* 3′UTR as well as in the 3′UTR of the *C. briggsae cep-1* orthologous gene, CBG04081. However, mutational alterations in this sequence in the *cep-1* 3′UTR did not affect GLD-1 binding while the same mutations in the GLD-1 binding regions of *rme-2* nearly abolished binding (M.-H.L. and T.S., unpublished data). Thus further studies will be necessary to define the GLD-1 binding sequences in the *cep-1* mRNA. Moreover, GLD-1 might act together with other proteins as a RNP complex to repress *cep-1* translation.

We propose that GLD-1 acts as a molecular rheostat to control CEP-1/p53 accumulation so that a threshold level is achieved in late pachytene that ensures responsiveness to DNA damage pathways (Figure 6B). Given the reciprocal relationship between CEP-1/p53 and GLD-1 protein levels during mid to late pachytene, GLD-1 regulation of CEP-1 protein levels is likely to be a part of the mechanism that ensures that only late pachytene cells have the potential to die in response to genotoxic insults. Indeed, it is a conserved feature that meiotic cells monitor various stages of recombination and a failure to complete recombination induces meiotic arrest in yeasts and apoptosis in mammals (Odorisio et al., 1998; Schwartz et al., 1999; Roeder and Bailis, 2000; Cohen and Pollard, 2001; Lydall et al., 1996). In *C. elegans* this checkpoint becomes dramatically manifested when meiotic double-strand breaks that are induced by SPO-11 are not properly processed due to inactivation of the conserved RAD-51 (RecA) single-strand exchange protein (Gartner et al., 2000; Alpi et al., 2003; Colaiacovo et al., 2003). Taken together, we suggest the following model: The translation of *cep-1*/p53 is completely repressed in transition zone nuclei where multiple double-strand breaks per chromosome are induced (Alpi et al., 2003; Bishop, 1994) and in early pachytene cells where double-strand breaks are processed and recombinational exchanges are restricted to a single site per chromosome (Hillers and Villeneuve, 2003). This might be part of a fail-safe mechanism to ensure that meiotic double-strand breaks or their intermediates do not mistakenly trigger the apoptotic demise of germ cells that are undergoing exchange between homologous chromosomes. CEP-1/p53 then becomes available again in late pachytene when meiotic recombination is supposed to be finished and cells harboring aberrant recombination intermediates can be eliminated through CEP-1/p53. Interestingly, mammalian p53 is similarly implicated in the apoptotic demise of meiotic pachytene cells upon DNA damage (Hasegawa et al., 1998; Sjoblom and Lahdetie, 1996; Odorisio et al., 1998), and presumably this activity must be blocked during the normal course of meiotic recombination. Therefore, it is possible that p53 might be similarly regulated at the translational level by mRNA binding proteins in mammalian gametogenesis. It has been shown previously that in cell culture-based systems, an element in the 3′UTR of human p53 is necessary for p53 translational control in OCI/AML-3 and OCI/AML-4 cells (Fu and Benchimol, 1997; Fu et al., 1999). Similarly, in mouse Swiss 3T3 cells it has been shown that the 5′UTR of p53 mediates translational repression (Mosner et al., 1995).

Translational repression by GLD-1 is important for cell fate choices—the proliferation versus initiation of meiotic development decision, the spermatogenesis versus oogenesis decision, and, as shown in this study, the pachytene progression versus apoptosis decision. The diverse germline functions of GLD-1 are a consequence of its regulation of multiple mRNA targets (Lee and Schedl, 2001, 2004; Jan et al., 1999; Marin and Evans, 2003; Xu et al., 2001; Mootz et al., 2004). Similar to *gld-1* genetics, mutations in GLD-1 homologs in other species show complex phenotypes. *Drosophila* WHO/HOW and mammalian Quaking are required for viability (Zaffran et al., 1997; Baehrecke, 1997; Bode, 1984; Justice and Bode, 1988; Shedlovsky et al., 1988), and hypomorphic alleles of mouse Quaking show defects in myelination and in vascular development (Sidman et al., 1964; Samorajski et al., 1970). This suggests that GLD-1 GSG/STAR protein family members in other species likewise regulate a number of different target mRNAs. Our results demonstrate the power of a forward genetic approach that revealed a specific process controlled by a multifunctional regulator. The identification of a separation-of-function mutation of *gld-1*, which appears to have lost its capacity to bind to a subset of mRNA targets, including the *cep-1* mRNA, reveals an unexpected link between the cell fate regulator GLD-1 and p53 DNA damage signaling. It will require further studies to establish the importance of translational repression of p53 or its family members p63 and p73, in intact mammalian tissues, which could potentially act through the mammalian GLD-1 homolog

Quaking or through other GSG/STAR family proteins. Such negative p53 regulation by translational repression might provide a novel target for tumor therapies aiming at upregulating p53 signaling.

Experimental Procedures

C. elegans DNA Damage Response Assays

The detailed experimental procedures for scoring DNA damage-induced apoptosis and mitotic cell cycle arrest, as well as for radiation survival (rad) assays and *egl-1* transcriptional assays are described by Gartner et al. (2004). For rad assays worms were irradiated at L4 larval stage and transferred to fresh plates 24 hr post-treatment and removed from the plates after 12 hr (Gartner et al., 2000). For irradiation an X-ray source Siemens "Stabilipan" was used. *rad-51* RNAi was performed as described in Gartner et al. (2004).

Genetic Screen for Increased Apoptosis upon Ionizing Radiation

Standard mutagensis conditions were used (Wood, 1996), and germ cell apoptosis was evaluated in F2 worms 28 to 32 hr post IR treatment by staining apoptotic corpses with acridine orange (AO) (Gartner et al., 2004).

Strains

Worms were maintained and raised at 20°C on NGM plates unless otherwise indicated. *ced-3(n717)* is described by Yuan et al. (1993), *ced-4(n1162)* by Yuan and Horvitz (1992), *ced-9(n1950)* by Hengartner et al. (1992), *egl-1(n1084n3082)*, referred to as *egl-1* in this manuscript, by Conradt and Horvitz (1998), *ced-1(e1935)* by Zhou et al. (2001), and *rad-5(mn159)* by Ahmed et al. (2001). The genetic null *gld-1(q485)* mutant referred to as *gld-1(null)* was described by Francis et al. (1995a). The deletion mutant *cep-1(lg12501)*, referred to as *cep-1*/p53, carries a 1213 bp deletion corresponding to 30458–31670 on cosmid F52B5 and takes out a large part of the *cep-1* open reading frame. The CED-1::GFP (bcIs39) V strain is a gift from Barbara Conradt.

Supplemental Data

Supplemental Data include six figures and are available with this article online at http://www.cell.com/cgi/content/full/120/3/357/DC1/.

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