

The *C. elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis

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In mammals, one of the key regulators necessary for responding to genotoxic stress is the p53 transcription factor. p53 is the single most commonly mutated tumor suppressor gene in human cancers [1]. Here we report the identification of a *C. elegans* homolog of mammalian p53. Using RNAi and DNA cosuppression technology, we show that *C. elegans* p53 (*cep-1*) is required for DNA damage-induced apoptosis in the *C. elegans* germline. However, *cep-1* RNAi does not affect programmed cell death occurring during worm development and physiological (radiation-independent) germ cell death. The DNA binding domain of CEP-1 is related to vertebrate p53 members and possesses the conserved residues most frequently mutated in human tumors. Consistent with this, CEP-1 acts as a transcription factor and is able to activate a transcriptional reporter containing consensus human p53 binding sites. Our data support the notion that p53-mediated transcriptional regulation is part of an ancestral pathway mediating DNA damage-induced apoptosis and reveals *C. elegans* as a genetically tractable model organism for studying the p53 apoptotic pathway.

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Received: 30 August 2001
Revised: 25 September 2001
Accepted: 27 September 2001

Published: 30 October 2001

Current Biology 2001, 11:1722–1727

0960-9822/01/\$ – see front matter
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Results and discussion

Identification of a *C. elegans* p53 homolog

Studies focusing on programmed cell death during embryonic and larval development in *C. elegans* have been instrumental in elucidating the conserved core apoptotic pathway that involves the *egl-1* (BH-3 domain protein), *ced-9* (BCL-2 homolog), *ced-4* (Apaf-1 homolog), and *ced-3* (caspase) genes [2]. More recently, it was realized that pro-

grammed cell death also occurs in the *C. elegans* germline. Three different, genetically separable pathways leading to germ cell apoptosis have so far been described. Physiological germ cell death is independent of external stimuli and is needed for maintaining germline tissue homeostasis [3]. Furthermore, some bacterial pathogens trigger the activation of germ cell death [4]. Finally, genotoxic stress was shown to result in the induction of germ cell apoptosis and mitotic germ cell cycle arrest [5].

A specific effector of radiation-induced apoptosis has not been identified in *C. elegans*. The fact that the completion of the *C. elegans* genomic sequence failed to reveal any obvious worm p53 homolog led to the initial notion that p53 might be confined to the vertebrate lineage [6]. However, this notion was recently challenged by the discovery of a functional *Drosophila* p53 homolog [7, 8]. Assuming that the *C. elegans* p53 open reading frame (ORF) 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, we carefully searched for a worm p53 homolog. Sensitive database searches, such as those using generalized profiles [9], allow the reliable detection of more subtle sequence relationships [10]. 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, and 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 ORF 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. To confirm that this ORF 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 [11]. To identify the full-length *cep-1* ORF, 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 (aa) protein (Figure 1a; 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

1b). The detectable evolutionary conservation of *C. elegans* p53 is mostly limited to the regions involved in DNA binding (conserved regions II–V in Figure 1b). 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* [12]. 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 an asterisk in Figure 1b [13]). The two conserved amino acids R248 and R273 are by far the most frequently mutated residues and account for more than 20% of tumor-associated p53 mutations. [13]. Finally, all four residues implicated in Zn binding are conserved (indicated by “Z” in Figure 1b) [12]. Although they were weakly conserved, we found two potential phosphorylation sites (SQ) corresponding to serine 15 and serine 37 of human p53. These sites are implicated in DNA damage-dependent activation of p53 at the N terminus [14, 15]. 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 also found a small but distinct amino acid conservation at the tetramerization domain (indicated by “tet” in Figure 1b) [16]. However, there appears to be no obvious acidic domain characteristic for the human transactivation domain.

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 promoter containing Gal4 DNA binding sites. CEP-1 was found to possess an intrinsic transcriptional activity that maps to the N terminus and that is equivalent to that observed for the transcription factors BAR-1 and human p53, suggesting that CEP-1 can act as a transcription factor (Figure 2a). To determine whether CEP-1 could activate transcription from a promoter containing consensus human p53 DNA binding sites, we expressed CEP-1 in yeast containing pSS1, a p53 transcriptional reporter construct (Figure 3b; [17]). CEP-1, like its human counterpart, was found to activate transcription of the pSS1 reporter. Transcription of this reporter allowed growth on media lacking histidine (Figure 3c). 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.

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 both cell cycle arrest of mitotic germ cells and apoptosis of meiotic pachytene cells in the *C. elegans* germline [5]. To assess whether *cep-1* functions in the DNA damage response, we inactivated *cep-1* by “RNAi feeding.” RNA interference (RNAi) leads to specific inhibition of targeted genes and has been used to inactivate genes on a genome-wide scale [18–20]. For inhibiting genes involved in post-embryonic processes, the RNAi feeding technique is most effective [18]. 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 generations [18, 20]. 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 3b, 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 3a,b). To determine whether this effect was specific to radiation-induced germ cell death, we also scored for the level of germ cell apoptosis in unirradiated *cep-1* RNAi-treated animals (Figure 3b). Physiological germ cell death is genetically distinct from radiation-induced cell death and manifests itself by the presence of 0–4 corpses per germline bend at any given time in unirradiated worms [3]. Because we did not observe a significant reduction of physiological germ cell death (0.4 ± 0.5 , $n = 14$ and 0.9 ± 1.0 , $n = 16$ corpses 36 hr post L4 larval stage in *cep-1* and *gfp* RNAi, respectively), we conclude that radiation-induced apoptosis is specifically affected in the germline.

The second response to the presence of DNA damage in the *C. elegans* germline is a transient cell cycle arrest. Mitotic cells in the distal arm of the *C. elegans* germline transiently halt cell proliferation after irradiation but continue to grow, as indicated by a decrease in cell number and an enlargement of cellular and nuclear size [5]. The checkpoint mutants *mrt-2(e2663)* and *rad-5(mn159)* are defective in this response [5]. Surprisingly, RNAi feeding of *cep-1* did not affect cell cycle arrest after irradiation, as demonstrated by the fact that mitotic germ cells from *cep-1* RNAi worms responded to irradiation comparably to the wild-type (*gfp* control RNAi; Figure S1a in the Supplementary material available with this article online). It is therefore conceivable that Cep-1 is dispensable for DNA damage-induced cell cycle arrest. However, since no cytological markers for various cell cycle phases are currently available, we cannot exclude the possibility that Cep-1 might only be required for a G1/S checkpoint. In the type of experiment performed here, cells defective for *cep-1* might still arrest the cell cycle at a G2/M checkpoint and appear to respond to DNA damage in a wild-type manner.

Figure 1

(a)

MEPDDSQLSDILKDARIPDSQDIGVNL TQNL SFDTVQK MIDGVFTPIFSQGTEDSLEKDILKTPG
 ISTIYNGILGNGETTKRTPKISDAFEPDLNTSGDVFDSDKSEDGLMND ESYLSNTTLSQVVLD S
 QKYEYLRV RTEEEQQLVIEKRARERFIRKSMKIAEETALS YENDG SRELSETMTQKVTQMDFT
 ETNV PFDGND ESSLAVRVQSDMNLNEDCEKWMEIDVLKQKVAKSSDMAFAISSEHEKYLW
 TKMGCLVPIQVKWKLDRHFNSNL SLRIRFVKYDKKENVEYAIRNPRSDVMKCRSHTEREQH
 PFDFSFFYIRNSEHEF

(b)

		II	D	III	*Z	Z
p53_caeel	213	QSDIQLREDCERKOMEIDILK	.. QKVAKSSDMAFAIS	SPHFKS	WTKHCLV	.. QKLDKRFH
p53_drome	77	AMIPKLENHMGVCFSMV	.. DEPPKSS	.. WMFSIP	LMKLYIRHMA	FMVDFQFKKMP
p53_loflo	102	QFVPSIKFPPGQYGDSEATP	SGCTKES	.. TATY	SKKIDKL	YORHMT
p53_myaa	123	IPVPSITNPPGQYGDSEATP	SGCTKES	.. TATY	SDILKGL	YORHMT
p53_xenopus	68	SCVPSITDDVAGAYELQDDEQ	.. NGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p53_chicken	79	SPVPSITDVGQYGDSEATP	.. AGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p53_human	94	SPVPSITDVGQYGDSEATP	.. AGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p53_mesau	97	SPVPSITDVGQYGDSEATP	.. AGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p53_mouse	88	SPVPSITDVGQYGDSEATP	.. AGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p53_trout	82	SPVPSITDVGQYGDSEATP	.. AGTAKSV	.. TCTY	SPDLMLK	LCGLRAT
p63_human	123	SPVPSITDVGQYGDSEATP	.. AGTAKSA	.. TCTY	SPDLMLK	LCGLRAT
p73_human	112	APVPSITDVGQYGDSEATP	.. AGTAKSA	.. TCTY	SPDLMLK	LCGLRAT

		IV	Z	DZ	*	D*	V
p53_caeel	317	FPFDSFFYIRN	..	SEHFSYS	SAKCGST	.. FNI	..
p53_drome	169	NAKMESE	..	RSRMPNS	VVYCG
p53_loflo	197	IKPAPL	..	SEVRC
p53_myaa	228	NHPAPL	..	SEVRC
p53_xenopus	162	DAAPSL	..	HLIRVE
p53_chicken	173	GLAPPQ	..	HLIRVE
p53_human	187	GLAPPQ	..	HLIRVE
p53_mesau	190	GLAPPQ	..	HLIRVE
p53_mouse	181	CLAPPQ	..	HLIRVE
p53_trout	176	GLAPPQ	..	HLIRVE
p63_human	218	QAPPS	..	HLIRVE
p73_human	207	QAPPS	..	HLIRVE

		VI	VI	VI	VI	VI	VI
p53_caeel	413	REDARQK	..	D	FRPELP	AYK	..
p53_drome	274	LNSEKRRKS	..	VPEAA	AEDEP	SKV	..
p53_loflo	296	ASLUSK	..	P	SPK	NGFP	..
p53_myaa	327	CELPPMV
p53_xenopus	263	NVTKRGC
p53_chicken	274	NVTKRGC
p53_human	288	NVTKRGC
p53_mesau	291	NVTKRGC
p53_mouse	282	NVTKRGC
p53_trout	277	NVTKRGC
p63_human	319	SI
p73_human	308	NVTKRGC

(c)

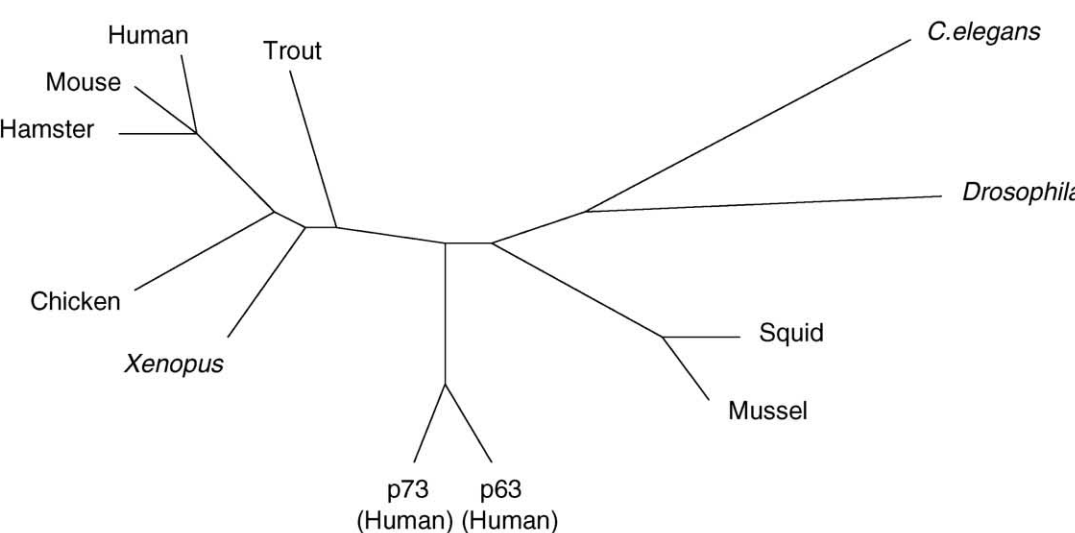
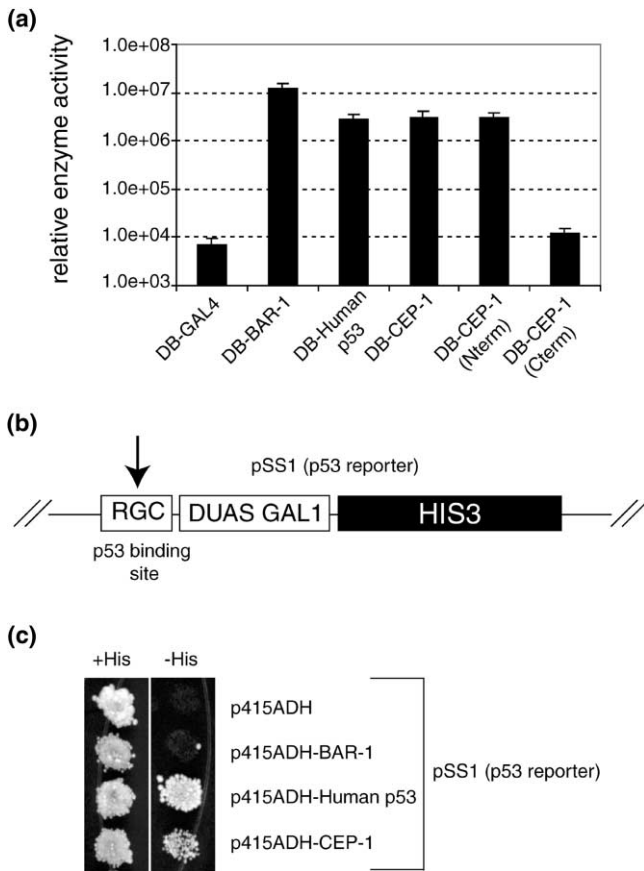


Figure 2



CEP-1 is a transcription factor. **(a)** CEP-1 acts as a transcriptional activator. We introduced various Gal4 DNA binding domain fusions into yeast and measured transcriptional activation by determining β -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 derivative of the pSS1 reporter construct lacking the p53 binding site did not support growth in the absence of histidine (S.B., unpublished observation).

To confirm that the 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 germline. DNA cosuppression is based on the observation that high copy number expression of a gene leads to specific inactivation of this gene in the germline [21, 22]. This effect,

(a) Predicted amino acid sequence of *cep-1*. **(b)** A profile-guided alignment of CEP-1 with other established members of the p53 family. Residues that interact with DNA in human p53 are marked with "D," those involved in Zn binding are marked with "Z," and those most frequently mutated in cancers are marked with an asterisk. The evolutionarily conserved regions II–IV and the tetramerization domain (tet) are indicated [16]. Abbreviations used are as follows: lolfo, *Loligo*

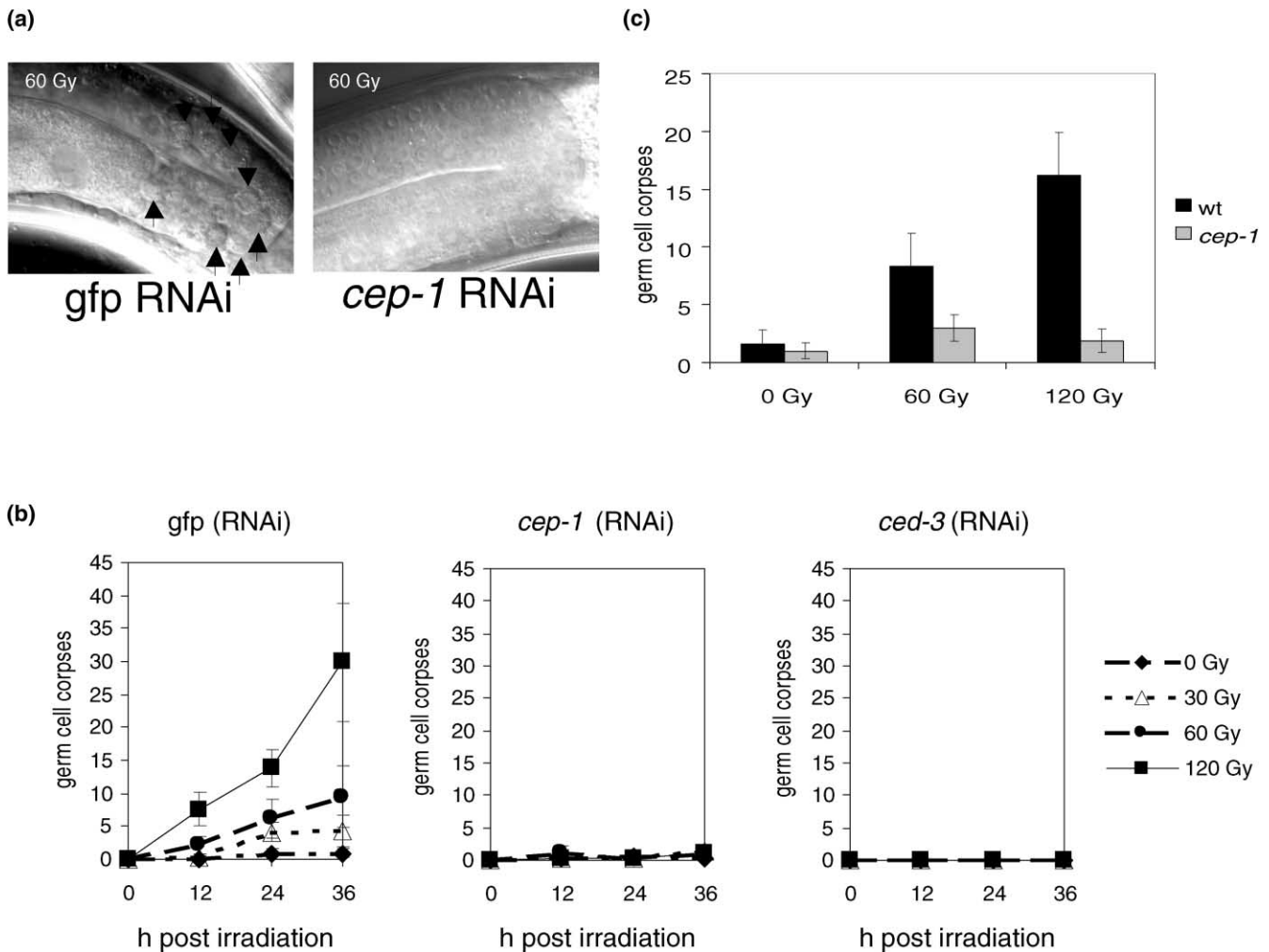
which genetically partially overlaps with the RNAi phenomenon, is presumably due to the formation of double-stranded RNAi due to transcription from copies of the transgene oriented in opposite directions [21, 22]. We therefore generated transgenic worm lines that contained the pRF-4 roller marker as well as a high concentration of *cep-1* (promoter and first three exons). Upon the irradiation of *cep-1* cosuppression 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 3c; 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 S1b in the Supplementary material). 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 [23, 24]. *cep-1* RNAi resulted in the same number of persistent corpses in *ced-1(e1935)* larvae, whereas RNAi of *ced-3* lead to an 80% reduction of somatic programmed cell death in this assay (Figure S1b in the Supplementary material). We thus conclude that, consistent with p53 function in stress-induced apoptosis in mammals, *cep-1* may specifically affect radiation-induced germ cell death.

We cannot exclude the possibility that a genetic *cep-1* knockout would reveal additional phenotypes unrelated to DNA damage-induced apoptosis. However, several lines of evidence argue that, in our RNAi experiments, *cep-1* function was completely abrogated. We could confirm the results by using DNA cosuppression. Secondly, the inhibition of radiation-induced programmed cell death was complete and matched the reduction observed in the *mrt-2(e2663)* loss-of-function mutant [5].

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 such as BLAST [25]. Functional conservation at such a low level of sequence similarity underscores the

forbes (northern European squid); mesau, *Mesocricetus auratus* (golden hamster); and myaar, *Mya arenaria* (mussle). **(c)** Neighboring 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).

Figure 3

cep-1 is specifically required for DNA damage-induced apoptosis. **(a)** A Nomarski picture showing the pachytene section of the germline was taken 36 hr after 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 cosuppression. 50 ng/ μ l *cep-1* was coinjected with 50 ng/ μ l pRF-4 *rol-6* marker. Germ cell apoptosis was scored 36 hr post irradiation.

potential of the generalized-profile method for the detection of homologs in distantly related model organisms [9]. Our experimental findings support the notion of an ancient function for p53 in DNA damage-induced apoptosis [7, 8]. 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 likely that this ancient p53-dependent pro-apoptotic function depends on the transcriptional activation of target genes that act on the core apoptotic pathway. It will be interesting to determine those targets in *C. elegans*. It is noteworthy that p53 is highly expressed in the germlines of flies, clams, and mammals [8, 26]. Here we report that *cep-1* function is required for DNA damage-induced germ cell death in the *C. elegans* germline. It is thus worth speculating about the selective advantage conferred by p53 ex-

pression in the germline. In adult *C. elegans* hermaphrodites, the germline is the only proliferative tissue, and approximately two thirds of embryonic cell division occurs 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 germline. 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 required to initiate the meiotic recombination process are left unprocessed. In light of this, it is interesting that the absence of mouse p53 leads to a reduced amount of germ cell

apoptosis, which results in a high frequency of abnormal sperm [27, 28]. Thus, p53 may have an important and conserved role in maintaining the fidelity of germ cells by the elimination of compromised cells. 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.

Acknowledgements

We thank members of the Gartner and Vidal labs for helpful discussions. We thank Ludgar Hengst, Dan Hoepfner, David Lydall, Francis Barr, and Erich Nigg for helpful discussions and for comments concerning the manuscript. Work was supported by the Max Planck Society (Erich Nigg) and by a Deutsche Forschungsgemeinschaft grant 703/1-1 to A.G. We thank Erich Nigg (Director of the Department for Cell Biology) for providing generous start-up facilities.

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