Genome-wide RNAi identifies p53-dependent and -independent regulators of germ cell apoptosis in *C. elegans*

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Abstract

We used genome-wide RNA interference (RNAi) to identify genes that affect apoptosis in the *C. elegans* germ line. RNAi-mediated knockdown of 21 genes caused a moderate to strong increase in germ cell death. Genetic epistasis studies with these RNAi candidates showed that a large subset (16/21) requires p53 to activate germ cell apoptosis. Apoptosis following knockdown of the genes in the p53-dependent class also depended on a functional DNA damage response pathway, suggesting that these genes might function in DNA repair or to maintain genome integrity. As apoptotic pathways are conserved, orthologues of the worm germline apoptosis genes presented here could be involved in the maintenance of genomic stability, p53 activation, and fertility in mammals.

Introduction

Apoptosis is a physiological mechanism by which unneeded, harmful, or damaged cells commit suicide. It plays an important role in development and homeostasis, and its misregulation has been associated with various pathologies.[1–3] Although we understand the molecular events occurring during apoptosis relatively well, less is known about the regulatory signals that trigger this cell fate.

Here we describe a systematic approach to identify new genes that regulate germ cell apoptosis in *Caenorhabditis elegans*. RNA interference (RNAi) was used to knockdown expression of 16 757 worm genes to find antiapoptotic factors that prevent excess germ cell death. Knockdown of 21 genes reproducibly resulted in a moderate to strong increase in germ cell apoptosis in wild-type animals. By epistasis analysis, we show that a large subset of our RNAi candidates require p53 and a functional DNA damage response pathway to activate germ cell apoptosis. Furthermore, we characterize mutations in two genes identified in our screen, *pmk-3* and *bmk-1*, and show that these mutants indeed have more germ cell deaths than wild-type worms. The identification of new *C. elegans* *g*erm*l*ine *a*poptosis (*gla*) genes, many of which have mammalian homologues, should shed light on the different pathways that regulate apoptosis during metazoan development.

Results

C. elegans hermaphrodites possess two U-shaped gonads connected at a common uterus (Figure 1a). Each gonad has a distal-to-proximal polarity, the most distal part being capped by the somatic distal tip cell (DTC). The DTC expresses a growth factor, LAG-2, which promotes the mitotic proliferation of the adjacent germ cells. In the distal arms, germ cell nuclei are not completely enclosed by membrane, and thus form a large syncytium. Nevertheless, because each nucleus acts independently of its neighbour, we will refer to these syncytial nuclei as germ cells. Once germ cells escape the influence of the DTC, they enter meiosis (transition zone in Figure 1a) and progress into the pachytene stage of meiosis I; exit from pachytene requires the activation of the RAS/MAPK pathway.[4,5] Germ cells can then further differentiate into oocytes, or die by apoptosis. Germ cell apoptosis depends on the caspase CED-3 and the Apaf-1 homologue CED-4, and is blocked by the Bcl-2-like protein CED-9.[6] Animals with a conditional loss-of-function (*lf*) mutation in *ced-9* have five times more apoptotic corpses in their germ line than wild-type worms.[6] This result can readily be phenocopied by inactivating *ced-9* with RNAi (Figure 1b).[7]

To identify other antiapoptotic genes, we inactivated 86% of the ~19 500 predicted *C. elegans* protein-coding genes using a RNAi feeding library.[8] To visualize apoptotic germ cells by fluorescent microscopy, the vital dye acridine orange (AO) was used. Ingestion of AO-stained *Escherichia coli* by *C. elegans* specifically labels engulfed apoptotic corpses in the germ line (Figure 1c).[6] Using this assay combined with the RNAi library, we identified 21 gene inactivations that cause a Gla phenotype, that is, more germ cell apoptosis than

observed in wild-type worms (Table 1). Sequence analysis of these candidates revealed that knockdown of a large variety of genes, belonging to several functional classes, induce germ cell apoptosis (Table 1; sequences can be retrieved at Wormbase; www.wormbase.org). Many of the genes identified in our screen encode proteins with known antiapoptotic functions in other systems, thereby validating our approach. For example, as has previously been shown,[9,10] we found that

knocking down RAD50 or the RecA-like protein RAD51, two proteins involved in homologous recombination and DNA double-strand break repair, results in a Gla phenotype (Table 1). Similarly, mice carrying a mutation in either Rad50 or Rad51 show increased apoptosis.[11,12] Moreover, it has recently been reported that inactivation of the p53 inhibitor iASPP, and its worm counterpart *ape-1*, induces apoptosis in a p53-dependent manner.[13] We independently identified *ape-1* in our RNAi screen, and confirmed that its inactivation requires p53 to cause a Gla phenotype (Table 1 and see below).

Why do so many different gene inactivations cause germ cell death? In an effort to address this issue, we performed epistasis analysis with our RNAi candidates. First, we showed that in all cases a strong *lf* mutation in the caspase *ced-3* completely suppresses the Gla phenotype, thus confirming the apoptotic nature of the cell deaths detected by AO and differential interference contrast (DIC) (Table 1). Apoptosis in the hermaphrodite *C. elegans* germ line can be triggered by genotoxic treatment, such as γ-irradiation.[10] It has been shown that these DNA damage-induced germ cell deaths are dependent on the worm p53 homologue *cep-1*[14,15] and the checkpoint gene *hus-1*.[16] To test if the gene candidates found in our screen activate the DNA damage response pathway, their expression was also inactivated by RNAi in *cep-1* and *hus-1* mutants. The basal level of germ cell death in these mutant backgrounds is lower than in wild-type worms (in Table 1, compare *control(RNAi)* for each mutant). The reason for this difference is unknown; one possibility is that in the absence of HUS-1 or CEP-1, endogenous DNA damage (e.g. aberrant meiotic intermediates) remains undetected and therefore cannot cause apoptosis. We found that *cep-1(lf)* and *hus-1(lf)* completely suppress the Gla phenotype of all but five genes, suggesting that p53 is a key sensor of germ cell stresses in *C. elegans* and that many of our RNAi candidates indirectly affect germ cell apoptosis through the activation of quality control checkpoint pathways (Table 1).[16,17] For the five gene knockdowns that are not suppressed by *cep-1* or *hus-1*, a reduction in the number of apoptotic germ cells was observed in comparison to wild-type worms (Table 1, p53-independent class). As already mentioned, these mutant strains have initially less germ cell deaths than the wild-type strain; this could explain the differences observed. Epistasis analysis with *ced-9(gf)*, a mutation that completely blocks *cep-1*-dependent germ cell death, but does not affect physiological germline apoptosis,[6,10] confirmed the above results (Table 1).
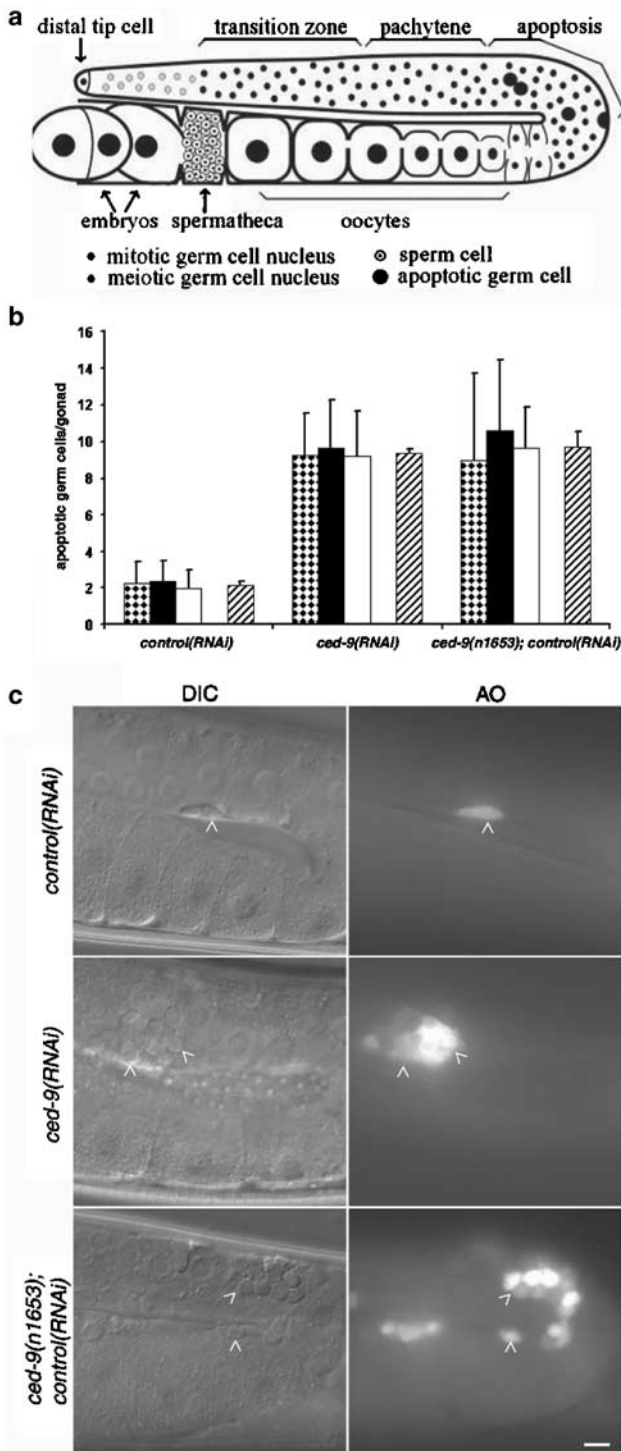
Figure 1 RNAi and acridine orange can be combined for a high-throughput, genome-wide screen for apoptotic germ cell death. (**a**) Schematic diagram of an adult hermaphrodite *C. elegans* gonad. Apoptotic cells are normally observed at and distal to the bend. (**b**) Knockdown of *ced-9* by RNAi can phenocopy a conditional loss-of-function mutation in *ced-9*. For each genotype, three independent experiments are shown; the checked, black, and white bars represent mean ± S.D. for each individual experiment ($n = 20$ gonads), whereas the dashed bars represent mean ± S.D. of the three experiments. See Materials and methods for a description of the statistics used in this study. (**c**) Left column shows representative pictures of a gonad after RNAi feeding observed by differential interference contrast (DIC) microscopy. Apoptotic germ cells can be seen as highly refractile disks (arrowheads). Right column shows that acridine orange (AO) stains specifically apoptotic corpses in the germ line. For all the pictures, anterior is to the left and dorsal to the top. Scale bar, 8 μm

Table 1 Gene candidates that increase germline apoptosis following RNAi

		Germ cell deaths per gonad[b]				
Gene(*locus*)	**Description[a]**	**wild-type (*t*-test)**	***ced-3(lf)***	***cep-1(lf) (t*-test)**	***hus-1(lf) (t*-test)**	***ced-9(gf) (t*-test)**
Control	Vector+*gfp*	2.2 ± 0.2	0	1.5 ± 0.2	1.0 ± 0.1	1.5 ± 0.2
p53-independent						
T07C4.8(*ced-9*)	Bcl-2 homologue	9.4 ± 0.2 (*P* = 1.2E-41)	0	5.1 ± 0.7 (*P* = 3.7E-11)	6.0 ± 1.8 (*P* = 2.4E-19)	5.9 ± 0.6 (*P* = 1.2E-13)
T02E1.3a	Zinc-finger domain, TIS11 family	9.1 ± 1.1 (*P* = 4.5E-26)	0	5.7 ± 0.6 (*P* = 3.4E-12)	4.6 ± 0.2 (*P* = 5.2E-22)	5.2 ± 0.7 (*P* = 1.7E-12)
B0414.5(*cpb-3*)	RNA-binding protein, CPEB family	8.7 ± 0.5 (*P* = 4.4E-28)	0	6.1 ± 0.4 (*P* = 3.6E-14)	6.2 ± 2.5 (*P* = 2.4E-21)	5.2 ± 1.5 (*P* = 1.0E-9)
R05D3.4	RING domain	8.0 ± 1.4 (*P* = 2.0E-18)	0	5.1 ± 1.9 (*P* = 6.1E-10)	4.8 ± 0.6 (*P* = 7.2E-26)	7.4 ± 1.7 (*P* = 1.5E-15)
F42G8.4(*pmk-3*)	p38 MAPK	4.1 ± 1.7 (*P* = 3.5E-6)	0	2.3 ± 0.2 (*P* = 0.003)	2.3 ± 0.3 (*P* = 1.2E-8)	3.0 ± 0.3 (*P* = 1.4E-5)
p53-dependent						
Y43C5A.6(*rad-51*)	DNA repair protein RAD51 homologue	7.9 ± 1.2 (*P* = 1.8E-30)	0	1.4 ± 0.2 (*P* = 0.24)	1.1 ± 0.1 (*P* = 0.28)	1.4 ± 0.3 (*P* = 0.43)
F23B12.8(*bmk-1*)	BimC kinesin-like homologue	4.4 ± 1.1 (*P* = 1.7E-11)	0	1.3 ± 0.1 (*P* = 0.24)	1.4 ± 0.6 (*P* = 0.04)	1.8 ± 0.8 (*P* = 0.20)
C16C8.14	Ubiquitin domain-containing protein	4.0 ± 0.9 (*P* = 3.8E-8)	0	1.5 ± 0.3 (*P* = 0.46)	1.4 ± 0.4 (*P* = 0.03)	2.1 ± 0.1 (*P* = 0.015)
Y38H6C.17	Amino acid/polyamine transporter	3.9 ± 0.6 (*P* = 2.0E-9)	0	1.6 ± 0.3 (*P* = 0.28)	1.4 ± 0.1 (*P* = 0.03)	2.4 ± 0.3 (*P* = 0.005)
K02F3.5	bZIP transcription factor	3.9 ± 0.8 (*P* = 6.9E-9)	0	1.3 ± 0.4 (*P* = 0.29)	1.6 ± 0.5 (*P* = 0.002)	2.0 ± 0.3 (*P* = 0.07)
T04H1.4(*rad-50*)	DNA repair protein RAD50 homologue	3.8 ± 0.1 (*P* = 1.1E-9)	0	1.4 ± 0.2 (*P* = 0.43)	1.0 ± 0.1 (*P* = 0.40)	1.5 ± 0.4 (*P* = 0.44)
F38H12.3	Nuclear hormone receptor	3.8 ± 0.4 (*P* = 5.2E-8)	0	1.7 ± 0.5 (*P* = 0.18)	1.1 ± 0.3 (*P* = 0.34)	1.4 ± 1.0 (*P* = 0.41)
Y23H5A.2	Novel	3.8 ± 0.9 (*P* = 8.0E-7)	0	1.4 ± 0.2 (*P* = 0.42)	1.2 ± 0.5 (*P* = 0.28)	2.4 ± 0.0 (*P* = 0.013)
F46F3.4(*ape-1*)	p53-binding protein (iASPP) like	3.7 ± 0.6 (*P* = 2.3E-7)	0	1.5 ± 0.0 (*P* = 0.47)	1.3 ± 0.4 (*P* = 0.09)	1.9 ± 0.3 (*P* = 0.11)
H06H21.9	PDZ/DHR/GLGF domain	3.5 ± 1.2 (*P* = 4.5E-6)	0	1.5 ± 0.3 (*P* = 0.46)	1.1 ± 0.6 (*P* = 0.30)	1.9 ± 0.1 (*P* = 0.15)
R11G11.7(*pqn-60*)	Glutamine/asparagines-rich domain, DUF148	3.5 ± 0.4 (*P* = 6.6E-7)	0	1.6 ± 0.7 (*P* = 0.32)	1.3 ± 0.1 (*P* = 0.08)	1.6 ± 0.6 (*P* = 0.35)
R03D7.6(*gst-5*)	Glutathione *S*-transferase	3.5 ± 0.7 (*P* = 4.8E-6)	0	1.1 ± 0.1 (*P* = 0.05)	1.2 ± 0.1 (*P* = 0.23)	1.4 ± 0.3 (*P* = 0.35)
F28A10.1	DUF13	3.5 ± 0.9 (*P* = 4.1E-5)	0	1.4 ± 0.1 (*P* = 0.42)	1.3 ± 0.5 (*P* = 0.05)	1.4 ± 0.2 (*P* = 0.39)
K02F2.6(*ser-3*)	Rhodopsin-like GPCR	3.4 ± 1.2 (*P* = 3.6E-5)	0	1.4 ± 0.0 (*P* = 0.46)	1.3 ± 0.0 (*P* = 0.05)	1.7 ± 0.0 (*P* = 0.24)
F29F11.2	UDP-glucoronosyltransferase	3.4 ± 0.6 (*P* = 6.7E-5)	0	1.9 ± 0.6 (*P* = 0.08)	1.4 ± 0.2 (*P* = 0.08)	1.8 ± 0.2 (*P* = 0.10)
K11D12.6	Trypsin inhibitor domain	3.4 ± 0.3 (*P* = 6.0E-5)	0	1.7 ± 0.4 (*P* = 0.21)	1.4 ± 0.7 (*P* = 0.02)	2.0 ± 0.6 (*P* = 0.05)

[a]As annotated in Wormbase. [b]Synchronized L1 worms were fed dsRNA-expressing bacteria until 24 h post-L4 stage, and then scored using DIC microscopy for a germline apoptosis phenotype. Data shown are mean number of apoptotic corpses per gonad ± S.D. of three independent experiments for wild-type, two experiments for *cep-1(lf)*, *hus-1(lf)*, and *ced-9(gf)*, and one experiment for *ced-3(lf)* (*n* = 20 gonads for each experiment). Wild-type: N2; *ced-3(lf)*: *ced-3(n717)*; *cep-1(lf)*: *cep-1(gk138)*; *hus-1(lf)*: *hus-1(op244)*; *ced-9(gf)*: *ced-9(n1950)*. *t*-test values were obtained by comparing the number of germ cell corpses in each experimental group with the respective *control(RNAi)* group

As RNAi does not always faithfully reproduce the known *lf* phenotypes, we sought to confirm our RNAi results with genetic mutants. As a first step towards this aim, we obtained and characterized deletion alleles for two of our RNAi candidates, *pmk-3* and *bmk-1*. Neither mutant has any obvious growth defects (data not shown),[18] but both *pmk-3(lf)* and *bmk-1(lf)* mutants displayed an increased number of germ cell corpses when compared with wild-type worms (Figure 2a). PMK-3 is one of the three p38 MAPK isoforms in *C. elegans*, which are all present on the same operon.[18] The

worm p38 PMK-1 has been implicated in stress responses and innate immunity, whereas the role of PMK-2 and PMK-3 is still unclear.[18–20] Contrary to the *pmk-3(lf)* Gla phenotype, knockdown of either *pmk-1* or *pmk-2* does not increase germ cell death (Figure 2b), suggesting a specific involvement of the PMK-3 isoform of p38 in the protection against germ cell apoptosis. *bmk-1* encodes a *C. elegans* homologue of the BimC kinesin-like motor protein involved in spindle forma-tion.[21] Double-mutant analysis revealed that *ced-3* is epistatic to both *pmk-3* and *bmk-1* (Figure 2b). In contrast, *cep-1(lf)*,
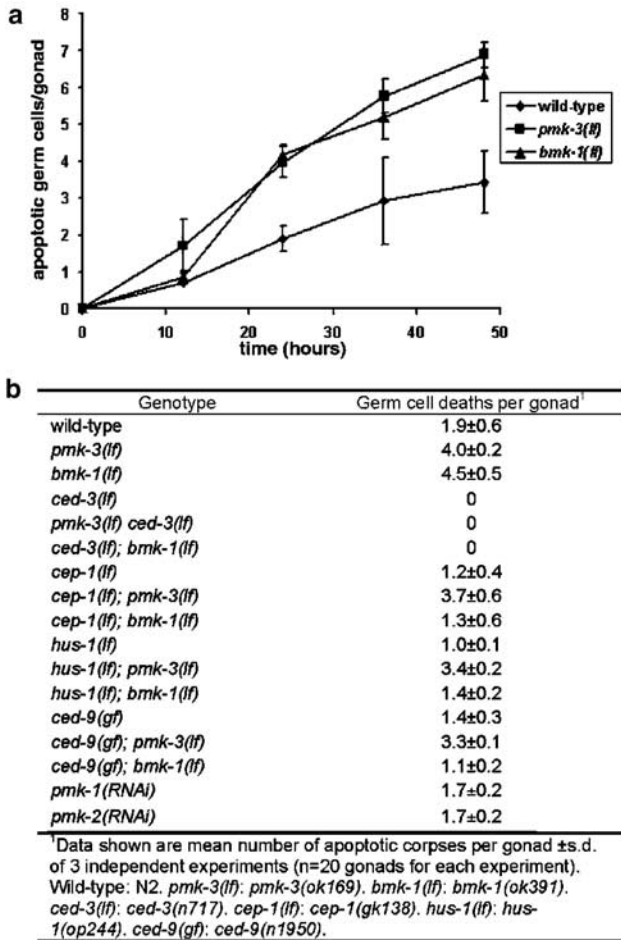
through activation of p53-dependent checkpoint or quality control mechanisms. The *C. elegans* p53 protein CEP-1 has been shown to participate in a conserved signalling pathway in response to DNA damage,[16] suggesting that at least some of these *gla* genes will be involved in the maintenance of genome stability. Consistent with this hypothesis, the p53-dependent class contains genes such as *rad-50* and *rad-51*, which are required for DNA repair and meiotic recombination, as well as genes predicted to protect from reactive oxygen species, such as *gst-5*.

We also identified in our screen *ape-1*, the worm orthologue of the p53-binding protein iASPP. Mammalian iASPP is the most phylogenetically conserved inhibitor of p53 identified so far.[13] It has been proposed that the primary function of iASPP is to inhibit the proapoptotic activity of p53 in the absence of genotoxic stress. As previously reported,[13] we found that *ape-1(RNAi)* induces germ cell apoptosis in a p53-dependent manner, consistent with the hypothesis that *C. elegans* iASPP APE-1 might also act as a p53 inhibitor by binding and regulating CEP-1's pro-apoptotic function. However, we found that *ape-1(RNAi)* resulted in *cep-1*-dependent apoptosis only in the presence of a functional upstream DNA damage response pathway (Table 1). This indicates that releasing CEP-1 from APE-1 inhibition is not sufficient by itself to trigger p53-dependent germ cell death. It is possible that effective activation of the apoptotic response might require not only release from APE-1, but also modification of CEP-1 by checkpoint regulators of the DNA damage response pathway. Further genetic and biochemical analysis is clearly required in order to understand the role of *C. elegans* iASPP in regulating p53-mediated apoptosis.

In mammals, p53 has been shown to mediate apoptosis in response not only to DNA damage, but also other stresses, such as oncogene activation and hypoxia.[22] In contrast, we have been unable so far to identify a DNA damage-independent death pathway for CEP-1 in *C. elegans*: all the *cep-1*-dependent RNAi candidates that we report here also showed a significantly reduced Gla phenotype in animals mutant for the gene *hus-1* (Table 1), which acts early in the DNA damage response pathway. We suspect that the ability of p53 to respond to oncogene activation might be a more recent evolution than its ability to respond to DNA damage, as tumour development is not much of a threat for simple, short-lived invertebrates such as *C. elegans*. Alternatively, our screen might simply have been unable to generate stresses that activate other p53-dependent pathways.

Our screen led to the identification of a small number of genes that, upon knockdown, induce germ cell apoptosis in a p53-independent manner. One of these, *ced-9*, encodes the *C. elegans* homologue of mammalian Bcl-2.[23] CED-9 acts downstream of p53 in the regulation of germ cell apoptosis,[16] readily explaining the observed epistasis. The mechanism of action of the other four genes – two predicted RNA-binding proteins, a p38 MAPK, and the *C. elegans* homologue of the *S. cerevisiae* Ring-finger protein Bre1p– is more obscure. We have previously shown that about half of all potential oocytes undergo apoptosis in the adult *C. elegans* germ line, and have suggested that many of these deaths serve a homeostatic function: to eliminate nuclei that served transiently as nurse cells and thereby reduce the nuclear/cytoplasmic ratio of the

Figure 2 *pmk-3(ok169)* and *bmk-1(ok391)* mutants exhibit a Gla phenotype. (**a**) Time-course of germ cell death in *pmk-3(ok169)* and *bmk-1(ok391)* mutants. Worms were synchronized and scored for germ cell apoptosis by DIC every 12 h post-L4 stage. Data shown represent mean ± S.D. of three experiments (*n* = 20 gonads for each experiment). (**b**) *ced-3(lf)* prevents germ cell death in *pmk-3* and *bmk-1* mutants, whereas *cep-1(lf)*, *hus-1(lf)*, and *ced-9(gf)* block only *bmk-1(lf)*-induced germ cell death. Worms were scored 24 h post-L4 stage

hus-1(lf), and *ced-9(gf)* abrogated *bmk-1(lf)*-induced germ cell death, but not the Gla phenotype of *pmk-3(lf)* worms (Figure 2b). These results corroborate our RNAi data (Table 1), and imply that loss of *bmk-1* activates p53-dependent germ cell death, whereas *pmk-3* acts in a p53-independent germ cell apoptotic pathway.

Discussion

In this paper, we describe the results of a genome-wide RNAi screen for genes that affect apoptosis in the *C. elegans* adult hermaphrodite germ line. We identified 21 genes that reproducibly induce germ cell death when knocked down by RNAi, confirmed that the additional germ cell deaths were apoptotic in nature, and used various cell death mutants to determine which death-inducing pathways were activated in each case.

Interestingly, knockdown of most of the genes identified in our screen appears to cause germ cell death indirectly,

mature syncytial gonad.[6] It is tempting to suggest that these four genes function in the regulation of this physiological germ cell death process. Alternatively, knockdown of these four genes might simply activate another, p53-independent quality control pathway that results in germ cell apoptosis.

Did our screen identify all the genes that can induce germ cell death upon inactivation? Most certainly not. First, the RNAi collection we used contained only about 85% of all predicted *C. elegans* genes. Thus, about a seventh of the *C. elegans* genome was not tested in our screen. Second, because of the intrinsic variability and limited efficiency of RNAi,[24] we likely missed a significant number of positive genes in our primary and secondary screens due to false negative results. Third, the particular feeding protocol used in our screen (feeding of P_o L1 larvae, screening of P_o adults) would have precluded us from identifying any genes that are required not only for germ cell survival but also for larval growth, as well as genes that need to be fed for more than one generation for the RNAi phenotype to become evident.

Indeed, we recovered in our screen only a fraction of the genes previously described to affect germ cell apoptosis. For example, we identified only two of the more than 12 DNA damage response genes previously shown to induce germ cell apoptosis upon RNAi-mediated knockdown.[9,10,25,26] However, many of these genes needed to be fed for several generations at high dsRNA concentrations for the apoptosis phenotype to become evident. We also failed to identify in our screen *daz-1* and the ste13/ME31B/RCK/p54 homologue *cgh-1*; both genes have been shown to induce germ cell apoptosis through as yet unknown mechanisms upon inactivation.[27,28] We did however identify in our screen CPB-3, one of four *C. elegans* CPEB family members. In clam and Xenopus oocytes, CPEB has been shown to interact functionally and physically with p47/p54, the orthologue of *C. elegans* CGH-1.[29] Whether CPB-3 and CGH-1 also interact in *C. elegans*, and whether they cooperate to control germ cell apoptosis, possibly by regulating the translation of mRNA targets, remains to be determined.

Previous genome-wide RNAi screens in *C. elegans* have been used to determine new gene functions[8,24] as well as to identify genes involved in body fat regulation[30] and genome stability.[31,32] Our results demonstrate that systematic RNAi screens can also successfully be used to identify genes that affect apoptosis. Indeed, based on our RNAi data, we identified two new genetic mutants that have excess germ cell death, confirming our reverse genomic approach. Further analysis of the apoptotic genes identified in our screen will provide useful genetic entry points into the study of mechanisms that control germ cell survival, genomic stability and of the genetic networks that regulate p53 activation.

Materials and Methods

Strains

C. elegans was cultivated using standard methods.[33] All strains were grown at 20°C. The strains used in this study were the following: wild-type N2 Bristol, *hus-1(op244)*, *cep-1(gk138)*, *ced-9(n1653)*, *ced-9(n1950gf)*, *pmk-3(ok169)*, *ced-3(n717)*, *bmk-1(ok391)*. *ok169* and *ok391* were backcrossed to the wild-type strain three times before phenotypical

analysis. The deletion alleles were detected by PCR using the following primer sequences: for *ok169* 5′-CCCATTTTTCACTGCGTCTCAATCG-3′ and 5′-TCTGCTTCTCCAGGGATTAACGGTG-3′, and for *ok391* 5′-ATTTGCTGCGAACCTTGACT-3′ and 5′-GCCGCGAATCATTGTATTTC-3′.

RNAi germ cell apoptosis screen

We carried out RNAi as described.[34] In total, 30–50 synchronized L1 worms were placed on NGM agarose plates seeded with *E. coli* producing double-stranded RNA (dsRNA). Worms were grown for 3 days, then stained for 1 h in the dark by adding 500 μl of M9 buffer containing AO (Molecular Probes; 0.02 mg/ml) to the plate. Worms were then transferred to fresh agar plates, and allowed to destain for 1 h in the dark. AO staining was assessed by fluorescent microscopy. RNAi candidates that induced a Gla phenotype were tested again in duplicate. The presence of increased germ cell corpses was confirmed by Nomarski optics 24 h post-L4 stage. For all clones that produced a Gla phenotype, plasmids were isolated and the DNA sequence encoding the dsRNA was sequenced to confirm the identity of the inactivated gene.

Statistical analysis

In Figure 1b, the checked, black, and white bars represent mean $\pm$ S.D. for each experiment ($n = 20$ gonads), to illustrate variation within each experiment. The dashed bar represent mean $\pm$ S.D. for the three experiments, to illustrate variation amongst experiments. This latter statistical representation is also used in Table 1 and Figure 2. Genes were considered to cause increased cell death only if they reproducibly caused significant increases in germ cell apoptosis (cutoff for inclusion in Table 1 was $P < 0.0001$ *versus* wild-type). In Table 1, the cutoff value to establish epistatic groups was chosen as $P < 0.001$ (RNAi *versus* respective mutant control). Although the P-value for *cep-1(gk138); pmk-3(RNAi)* ($P = 0.003$) is above the cutoff value, we decided to group *pmk-3* into the p53-independent class based on the clear phenotype of the double mutant (Figure 2b).

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