Cell-Nonautonomous Regulation of *C. elegans* Germ Cell Death by *kri-1*

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**Summary**

Programmed cell death (or apoptosis) is an evolutionarily conserved, genetically controlled suicide mechanism for cells that, when deregulated, can lead to developmental defects, cancers, and degenerative diseases [1, 2]. In *C. elegans*, DNA damage induces germ cell death by signaling through cep-1/p53, ultimately leading to the activation of CED-3/caspase [3–13]. It has been hypothesized that the major regulatory events controlling cell death occur by cell-autonomous mechanisms, that is, within the dying cell. In support of this, genetic studies in *C. elegans* have shown that the core apoptosis pathway genes *ced-4*/*APAF-1* and *ced-3/caspase* are required in cells fated to die [9]. However, it is not known whether the upstream signals that activate apoptosis function in a cell-autonomous manner. Here we show that *kri-1*, an ortholog of KRIT1/CCM1, which is mutated in the human neurovascular disease cerebral cavernous malformation [14, 15], is required to activate DNA damage-dependent cell death independently of *cep-1/p53*. Interestingly, we find that *kri-1* regulates cell death in a cell-nonautonomous manner, revealing a novel regulatory role for non-dying cells in eliciting cell death in response to DNA damage.

**Results and Discussion**

In an RNA interference (RNAi) screen unrelated to apoptosis, we serendipitously uncovered a *cep-1/p53*-interacting gene, *kri-1*, the ortholog of human KRIT1/CCM1, which is frequently mutated in the neurovascular disease cerebral cavernous malformation [14, 15]. Because this gene had been previously shown to integrate signals from reproductive tissues (germ cells) to elicit longevity effects in nonreproductive (somatic) tissues [16] and interacts with *cep-1*, an important mediator of germ cell death (Figure 1A) [7, 8], we asked whether *kri-1* is involved in a novel, cell-nonautonomous mechanism to regulate germ cell death. To test this, we first investigated whether *kri-1* regulates cell death like *cep-1*, by quantifying the number of germ cell corpses in wild-type animals fed bacteria producing double-stranded RNA against a control gene or *kri-1* exposed to ionizing radiation (IR) (Figure 1B). We found that knockdown of *kri-1* by RNAi significantly reduced the number of germ cell corpses after DNA damage (IR) compared to animals fed control RNAi (p = 0.01), suggesting that *kri-1* is required for germ cell death. We verified this initial observation by performing a dose-response analysis of the *kri-1(ok1251)* deletion mutant. In contrast to wild-type animals, *kri-1(ok1251)* deletion mutants did not exhibit an increase in germ cell apoptosis after exposure to increasing doses of IR (Figure 1C; see also Figure S1 available online). This was reminiscent of *cep-1* loss-of-function (lf) mutants that are also resistant to IR-induced apoptosis. Therefore, we examined whether *kri-1* regulates germ cell death specifically, like *cep-1*, or whether it regulates cell death in all cells, like *ced-3*, by quantifying apoptosis in developing embryos of wild-type animals and *cep-1(0f)* and *kri-1(ok1251)* mutants. We found that developmental cell death was unaffected in *kri-1(ok1251)* mutants, suggesting that the regulation of cell death by *kri-1* is specific to germ cells, like *cep-1* (Figure 1D). Finally, to determine whether the *ok1251* allele is a null, we performed a deficiency analysis by crossing *ok1251* into a strain containing the *hd9* deficiency that removes the *kri-1* locus and quantified the number of germ cell corpses after DNA damage (Figure 1E). Strains containing the *ok1251* allele in trans to *hd9* were as resistant to damage-induced germ cell apoptosis as *ok1251* homozygotes, suggesting that *ok1251* is a null allele. Collectively, these and further observations (see below) indicate that *kri-1* is specifically required for germ cell death in response to DNA damage.

Given that *kri-1* is required to promote germ cell death in response to DNA damage, we were interested to know at which step in the pathway it might be functioning (Figure 1A). In the *C. elegans* germline, the DNA damage checkpoint genes (*pdr-9, mrt-2, hup-1*, and *clk-2*) are required to both transiently arrest mitotic proliferation and activate *cep-1*-dependent apoptosis of damaged germ cells [5, 6]. To ascertain whether *kri-1* is functioning in an analogous manner (i.e., upstream of *cep-1*), we tested whether *kri-1* null (0) mutants mimic the germline phenotypes of checkpoint gene mutants. In contrast to *clk-2* mutants that are defective in cell-cycle arrest, we found that *kri-1* was not required for IR-induced arrest of mitotically proliferating cells (Figure 2A; Figure S2A), implying that *kri-1* acts downstream or independently of the DNA damage checkpoint. To delineate whether *kri-1* is required to transduce signals to the *cep-1* protein and therefore allow apoptosis to occur, we examined the activity of *cep-1* by quantifying the transcript levels *egl-1*, a proapoptotic target gene of *cep-1* [17, 18]. Consistent with previous work, *egl-1* transcript levels as assessed by real-time quantitative PCR (qPCR) increased in response to DNA damage in wild-type animals, but not in *cep-1(0f)* mutants (Figure 2B). Interestingly, *egl-1* induction in *kri-1(0)* mutants was similar to that seen in wild-type animals, indicating that the transcriptional activity of *cep-1* is induced normally in the absence of *kri-1*. This is consistent with *kri-1* promoting damage-induced apoptosis downstream or independently of *cep-1*. Such a model raised the possibility that *cep-1* might regulate *kri-1* transcription or KRI-1 protein localization in response to DNA damage and that this was required to promote germ cell death. However, neither *kri-1* transcript levels nor GFP::KRI-1 localization was significantly affected by IR or *cep-1* status (Figures S2B–S2D).
The data above suggest a model wherein \textit{kri-1} functions downstream of or in parallel to the key decision-making step in the cell death pathway and likely regulates components of the core death pathway (i.e., \textit{egl-1}, \textit{ced-9}, \textit{ced-4}, and \textit{ced-3}). To investigate this further, we examined the epistatic relationship between \textit{kri-1} and \textit{ced-9}. Healthy cells require functional CED-9/BCL2 to prevent ectopic activation of CED-3/caspase by CED-4 (Figure 1A). We reasoned that if \textit{kri-1} functions downstream of \textit{ced-9}, ablation of \textit{kri-1} would suppress the increased cell death caused by \textit{ced-9(\textit{lf})}; on the other hand, the converse would be true if \textit{kri-1} acts upstream of \textit{ced-9}. Knockdown of \textit{ced-9} by RNAi (>50% knockdown; Figure S2E) caused a significant increase in apoptosis both before and after DNA damage, but this was unaffected by loss of \textit{kri-1} (Figure 2C), which we confirmed in \textit{kri-1(\textit{ok1251})/hDf9} and +/hDf9 (white) treated with IR as above. Data represent mean ± SEM of at least four independent experiments and at least 25 germlines in total per strain per condition. *p < 0.05 versus wild-type; **p < 0.01 versus \textit{kri-1(\textit{ok1251})/hDf9} and +/hDf9. See also Figure S1.

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Because *kri-1* functions independently of *cep-1* and impinges on the core death pathway, we were interested to know whether *kri-1* is cooperating with other genes known to regulate germ cell death independently or downstream of *cep-1*. In particular, the histone deacetylase *sir-2.1* [19], the MAP kinase *pmk-3* [20], and the retinoblastoma (RB) ortholog *lin-35* [21] have all been shown to regulate germ cell death independently of *cep-1*. In addition to activating cell death independently of *cep-1* in a manner similar to *kri-1*, the SIR-2.1 protein exits the nuclei of germ cells after DNA damage [19]. To determine whether the relocation of SIR-2.1 is required for *kri-1*-mediated germ cell death, we immuno-stained *kri-1(0)* animals with SIR-2.1 antibodies to ascertain whether SIR-2.1 protein levels or localization was altered. Although we found that *kri-1* did not affect the SIR-2.1 protein staining pattern (Figure 3A), it still remained possible that *kri-1* and *sir-2.1* function in the same pathway. To address this, we created a double heterozygous mutant containing both the *kri-1(0)* and *sir-2.1(0)* mutations (*kri-1(0)/+; *sir-2.1(0)/+*), and we observed wild-type levels of germ cell apoptosis in response to DNA damage (data not shown), suggesting that these genes operate in different pathways. In contrast to *sir-2.1* and *kri-1*, which positively regulate germline apoptosis, the MAP kinase gene *pmk-3* inhibits germline apoptosis independently of *cep-1* [20]. Therefore, we tested whether *kri-1* was required for germ cell death caused by loss of function of *pmk-3*. We created *kri-1(0); pmk-3(0)* double mutants and found that germ cell death was suppressed to the same degree as *kri-1(0)* single mutants (Figure 3B), suggesting that *kri-1* is epistatic to *pmk-3* and does not regulate cell death through *pmk-3*. We do not believe that *pmk-3* regulates *kri-1* because *kri-1* transcript levels and protein localization remained unchanged in *pmk-3(0)* mutants (Figure S3). Finally, because *lin-35* positively regulates germ cell apoptosis by controlling the levels of the CED-9 protein (i.e., loss of *lin-35* lead to an increase in CED-9 protein levels) [21], we tested whether *kri-1* functions through *lin-35/RB* by quantifying CED-9 protein levels in *kri-1(0)* animals by western blot. We found that CED-9 protein levels were unaffected in *kri-1(0) daf-16(lf)* mutants (data not shown). This suggests that DAF-16 has a weak effect on germ cell death [17, 19] and we created a double heterozygous mutant containing both *sir-2.1(lf)* and *kri-1(0) daf-16(lf)*. We tested whether *kri-1* functions downstream of the checkpoint genes but upstream of *ced-9* (A) Synchronized hermaphrodites at the fourth larval stage (L4) were treated with IR, and the number of nuclei per unit area in the mitotic region of the germline was quantified 24 hr later at 20°C. The mitotic region and nuclei have been outlined for clarity. Representative images from three independent experiments are shown. (B) RNA was isolated by TRizol from synchronized wild-type (black), *cep-1(0ff)* (gray), and *kri-1(0)* (white) mutants, and *egl-1* transcript levels were measured by quantitative real-time PCR. Data represent mean ± SEM of three independent experiments. (C) Synchronized wild-type and *kri-1(0)* L4 animals fed control(RNAi) (Y95BBA.84.g, a non-expressed gene) (black and white, respectively) or *ced-9(RNAi)* (dark gray and light gray, respectively) were subjected to IR, and germ cell death was quantified as described above. Data represent mean ± SEM of three independent experiments and at least 25 germlines in total per strain per condition. *p < 0.01 versus wild-type; p < 0.05 versus *kri-1(0); control(RNAi)*. See also Figure S2.
There are two possible mechanisms by which kri-1 may promote germ cell death. The first is a cell-autonomous mechanism, in which kri-1 regulates the core death pathway (EGL-1 or CED-9) in germ cells to initiate cell death. Alternatively, it is possible that kri-1 regulates cell death outside of germ cells (i.e., from somatic cells) via a novel pathway. In support of the latter hypothesis, kri-1 is required to extend the life span of worms through its effects on DAF-16 in the intestine, possibly by receiving signals from germ cells [16]; in addition, microarray data suggest that kri-1 is not expressed in the germline [22]. To distinguish between these possibilities, we took advantage of tissue-specific RNAi in C. elegans and selectively knocked down kri-1 in germ cells and the soma in rrf-1(lf) [23] and ppw-1(lf) [24] mutants, respectively, and quantified IR-induced germ cell apoptosis [21] (Figure 4A). Wild-type, rrf-1(lf), and ppw-1(lf) mutants fed bacteria producing control(RNAi) had similar numbers of germ cell corpses after DNA damage. Ablation of kri-1 by RNAi in wild-type animals inhibited DNA damage-induced germ cell apoptosis to the same extent as kri-1(0) mutants. However, selective knockdown of kri-1 in kri-1(0) mutants caused an increase in IR-induced apoptosis, suggesting that kri-1 expression in germ cells is not required to promote apoptosis. Conversely, specific knockdown of kri-1 in the soma in ppw-1(lf) mutants prevented germ cell death, suggesting that kri-1 is required in somatic tissue to regulate germ cell death. In support of this contention, we were able to rescue damage-induced germ cell apoptosis to wild-type levels by expressing GFP::KRI-1 from a somatic extrachromosomal array (Figures 4B and 4C). Although it is possible that low-level expression of GFP::KRI-1 in the germline may account for this observation, the fact that extrachromosomal arrays are generally silenced in the C. elegans germline [25] strongly supports a model in which kri-1 is required in nondying somatic cells to promote germ cell death. Collectively, these data imply a novel mechanism whereby somatic cells communicate with germ cells to promote their death in response to DNA damage (Figure 4D). Indeed, other genes have been shown to participate in germline-soma signaling during proliferation and differentiation of the germline [26, 27], dauer formation, and life-span control [28, 29], confirming that these two tissues can signal to each other in response to certain stimuli. The fact that kri-1/KKRTT regulates germ cell death from cells outside of the germline independently of cep-1/p53 implies that cells not fated to die (somatic cells) in C. elegans can regulate the core death pathway in germ cells by novel, cell-nonautonomous mechanisms. In the case of kri-1, there are several ways in which it may be performing this function. First, we examined the possibility that kri-1 may be acting through daf-9 and daf-12 to promote apoptosis, in a manner analogous to its proposed role in life-span control [16], by feeding kri-1(RNAi) to daf-9(lf) and daf-12(lf) mutants. We found that both mutants exhibited a resistance to apoptosis when fed kri-1(RNAi), suggesting that kri-1 does not act through these two genes (Figure S4). Second, although we have shown that kri-1 does not affect the transcript levels of the BH3-only gene egl-1, it is possible that the kri-1 is required to activate the EGL-1 protein. Similar to mammalian BH3-only proteins, EGL-1 may require other coactivating proteins or modifications in order to induce cell death in germ cells. For example, BID, BAD, and BIM/BMF are proapoptotic BH3-only proteins regulated at the post-translational level through cleavage, phosphorylation, and sequestration by interacting proteins, respectively [30]. Therefore, it is formally possible that KRI-1 may facilitate the activation of EGL-1 by similar transcription-independent mechanisms. Alternatively, KRI-1 may be involved in receiving signals from the germ cells, which results in the subsequent release of death-inducing factors. Although further studies are required to resolve the biochemical mechanism by which KRI-1 dictates germ cell death from the soma, our
observations reveal a novel cross-tissue signaling mechanism whereby somatic tissue can promote germ cell death in response to DNA damage in C. elegans, which may have broader applicability to cell death in general.

Supplemental Information

Supplemental Information includes four figures, Supplemental Experimental Procedures, and one table and can be found with this article online at doi:10.1016/j.cub.2009.12.032.

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References


