

The SCF^{FSN-1} ubiquitin ligase controls germline apoptosis through CEP-1/p53 in *C. elegans*

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The nematode *Caenorhabditis elegans* contains a single ancestral p53 family member, *cep-1*, which is required to activate apoptosis of germ cells in response to DNA damage. To understand how the *cep-1*/p53 pathway is regulated in response to genotoxic stress, we performed an RNA interference screen and identified the neddylation pathway and components of an SCF (Skp1/cullin/F-box) E3 ubiquitin ligase as negative regulators of *cep-1*-dependent germ cell apoptosis. Here, we show that the cullin gene *cul-1*, the Skp1-related gene *skr-1*, and the ring box genes *rbx-1* and *rpm-1* all negatively regulate *cep-1*-dependent germ cell apoptosis in response to the DNA-alkylating agent *N*-ethyl-*N*-nitrosourea (ENU). We also identified the F-box protein FSN-1, previously shown to form an SCF ligase that regulates synapse development, as a negative regulator of *cep-1*-dependent germline apoptosis. The hypersensitivity of *fsn-1* mutants to ENU-induced germline apoptosis was completely suppressed by a *cep-1* loss-of-function allele. We further provide evidence that the transcriptional activity, phosphorylation status, and levels of endogenous CEP-1 are higher in *fsn-1* mutants compared with wild-type animals after ENU treatment. Our results uncover a novel role for the SCF^{FSN-1} E3 ubiquitin ligase in the regulation of *cep-1*-dependent germ cell apoptosis.

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Tumor suppressor p53 is a transcription factor that coordinates cellular responses to a variety of stresses, such as DNA damage, oncogene activation, nutrient depletion, and hypoxia.¹ Depending on the level of damage, p53 can either arrest proliferation and activate repair mechanisms, or trigger a cellular suicide program (apoptosis) that removes potentially hazardous cells from the organism.¹ Activation of apoptosis by p53 involves transcription-dependent and transcription-independent mechanisms that are crucial for preventing tumor formation.^{1,2} The activity of p53 is regulated by numerous post-translational modifications, including phosphorylation, ubiquitination, acetylation, and neddylation.^{1,3} Several ubiquitin ligases have been identified that control the stability of p53, including Mdm2, ARF-BP1, Pirh2, COP-1, and CHIP.³ The best-characterized p53-specific E3 ubiquitin ligase is Mdm2, which maintains low levels of p53 protein under non-stressed conditions through a negative feedback loop.^{4,5} Cellular stresses such as DNA damage stimulate the phosphorylation of p53 and Mdm2, which disrupts their association and stabilizes p53.^{1,3} As the cellular concentration of p53 rises, it can activate the transcription of genes such as PUMA and NOXA to trigger apoptosis.^{6,7}

The p53 family is comprised of three members: p53, p63, and p73. While it is currently unclear whether p63 and p73 are involved in tumorigenesis, they share many of the same properties as p53 in the control of cell survival.⁸ The stability of both p63 and p73 is regulated by the E3 ubiquitin ligase Itch, and recent work has shown that Mdm2-dependent neddylation of p73 antagonizes its transcriptional activity.^{9–11} The

nematode *Caenorhabditis elegans* contains a single ancestral p53 family member, *cep-1*, which is required to activate apoptosis of germ cells in response to DNA-damaging agents.^{12,13} We recently found that CEP-1 regulates the expression of many of the same genes as human p53 and p63, suggesting that it may represent a composite of the vertebrate paralogues.¹⁴ Structurally, the DNA binding domain of CEP-1 is similar to human p53 but its C terminus contains a SAM domain that resembles the C termini of vertebrate p63 and p73.^{15,16} Like its relatives, the apoptotic activity of CEP-1 is controlled by many of the same proteins, such as Akt/PKB, Brca1, and the p53-binding protein iASPP.^{17–19} Because *C. elegans* contains a single p53-like gene, genetic analysis is simplified in this organism and may reveal novel mechanisms by which the vertebrate paralogues are regulated. Here, we show that the neddylation pathway and the Skp1/cullin/F-box (SCF) E3 ubiquitin ligase SCF^{FSN-1} control the apoptotic activity of CEP-1 in response to genotoxic stress. We propose that neddylation and SCF^{FSN-1} negatively regulate the transcriptional activity, phosphorylation, and endogenous levels of CEP-1 protein in response to DNA damage through an intermediate protein.

Results

The neddylation pathway regulates *cep-1*-dependent germline apoptosis. From an RNA interference (RNAi) modifier screen, we found that *cep-1(gk138)* deletion

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Abbreviations: PCD, programmed cell death; ENU, *N*-ethyl-*N*-nitrosourea; SCF, Skp1/cullin/F-box; RNAi, RNA interference; NGM, nematode growth media

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mutants survived better on bacteria that express double-stranded RNA to the *ned-8* gene (Nedd8 in humans) compared with wild-type (N2) controls (data not shown). Recent studies in mammalian cells have implicated a key role for the neddylation pathway in suppressing p53- and p73-dependent apoptosis.^{9,20} To investigate how NED-8 modulates CEP-1 activity *in vivo*, we inhibited components of the neddylation pathway by RNAi and quantified DNA damage-induced apoptosis in the germ line, where *cep-1* has a well-established role.^{12,13,17,21} Ablation of *ned-8* caused significantly higher levels of *cep-1*-dependent germ cell apoptosis in response to the DNA-alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) compared with controls (Figure 1a–c). Inhibiting expression of the NED-8 E1-activating genes *ula-1* and *rfl-1*, as well as the NED-8 E2-conjugating gene *ubc-12*, also caused hypersensitivity to ENU-induced germ cell apoptosis (Figure 1d). In *rfl-1(or198ts)* mutants, there were elevated levels of germline apoptosis in the

absence and presence of ENU at the restrictive temperature (Supplementary Figure 1). Because physiological germline apoptosis was unaffected when the neddylation pathway was inhibited by RNAi, the *rfl-1(or198ts)* allele might be more potent than RNAi or activates the damage checkpoint in the absence of genotoxic stress.

***ned-8* regulates germline apoptosis through the core apoptosis pathway.** DNA damage-induced germ cell apoptosis in *C. elegans* is executed through the core programmed cell death (PCD) signaling pathway, which is composed of *egl-1*, *ced-9*, *ced-4*, and *ced-3*.^{22–24} To determine whether neddylation modulates apoptosis through the core PCD pathway, we inhibited *ned-8* in strains carrying the loss-of-function mutations *ced-3(n717)*, *ced-4(n1162)*, and *egl-1(n1084n3082)*, as well as a strain carrying the *ced-9(n1950)* gain-of-function mutation. In all of

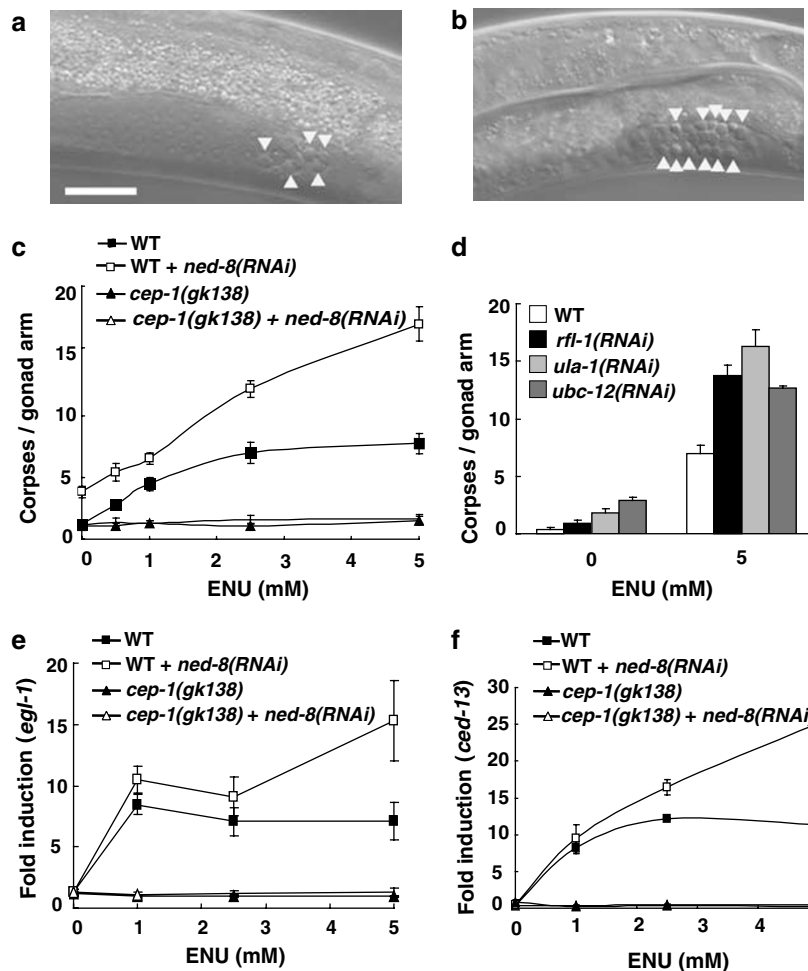


Figure 1 The neddylation pathway negatively regulates *cep-1*-dependent apoptosis via transcriptional activation of *egl-1* and *ced-13*. (a and b) Representative images of germline apoptotic corpses after 5 mM ENU treatment in the pachytene region of wild-type animals fed bacteria expressing the empty RNAi vector (L4440) (a) or RNAi to the *ned-8* gene (b). (a) Scale bar = 20 μ m. Germ cell corpses in panels a and b in one focal plane of the pachytene region are indicated by white arrowheads. (c) Effect of increasing concentrations of ENU on germ cell apoptosis in young adults depleted of *ned-8* 24 h after treatment. (d) Effect of 5 mM ENU on germ cell apoptosis in wild-type animals depleted of *rfl-1* and *ula-1*, the *ned-8* E1-activating genes, and *ubc-12*, which encodes the *ned-8* E2-conjugating gene. Between 20 and 35 germ lines were scored for each concentration of ENU in triplicate. Error bars represent the standard error of the mean (S.E.M.). (e and f) Transcript abundance of *egl-1* and *ced-13* was determined by qPCR 24 h after worms were treated with ENU. *egl-1* and *ced-13* transcript levels were normalized to γ -tubulin (*tbg-1*) mRNA. Fold induction was calculated relative to normalized transcript levels in untreated wild-type animals. Error bars = S.E.M.

these mutants, ENU-induced germ cell apoptosis was abolished (Supplementary Figure 2).

CEP-1 activates the core PCD pathway in the germ line by transactivating the BH3-only proteins EGL-1 and CED-13, which antagonize the antiapoptotic CED-9 protein.^{23–25} To assess whether NED-8 regulates the transcriptional activity of CEP-1 *in vivo*, we quantified *egl-1* and *ced-13* transcript levels by quantitative real-time PCR (qPCR). At 5 mM ENU, there was a two- to three-fold increase in both *egl-1* and *ced-13* transcript in wild-type animals depleted of *ned-8* compared with controls, but in *cep-1(gk138)* mutants the expression of these genes was not activated (Figure 1e and f). Together, these results indicate that NED-8 regulates germline apoptosis through the core PCD pathway by affecting the transcriptional activity of CEP-1.

Germline apoptosis is regulated by an SCF ubiquitin ligase. Because we could not detect neddylated CEP-1 from worm extracts (Figure 4, and data not shown), we reasoned that neddylation may regulate CEP-1 through an indirect mechanism. The best-characterized substrates for neddylation are proteins of the cullin family, which act as scaffolds for the assembly of multi-subunit E3 ubiquitin ligases.²⁶ Of the six cullin genes in *C. elegans*, only *cul-1* regulated ENU-induced germ cell apoptosis (Figure 2a).

Although *cul-1(e1756)/unc-69(e587)* heterozygous animals were partially hypersensitive to ENU-induced apoptosis (Supplementary Figure 3), this effect was not statistically significant. It was not possible to examine germline apoptosis in *cul-1(e1756)* homozygotes because they arrest during larval development.²⁷ Since CUL-1 is a component of the SCF complex, these results suggested that DNA damage-induced germ cell apoptosis is negatively regulated by an SCF E3 ubiquitin ligase. SCF complexes also contain a Skp1 protein, a RING domain protein, and an F-box protein.²⁶ Therefore, we next evaluated the two RING domain proteins, RBX-1 and RPM-1, and found that both of these proteins negatively regulate DNA damage-induced germ cell apoptosis (Figure 2b). Inhibition of *rbx-1* in *rpm-1(ju44)* mutants did not have an additive effect on ENU-induced apoptosis, suggesting that these RING proteins act redundantly in this process (Figure 2b).

The complexity of SCF ligases in *C. elegans* is highlighted by the presence of 21 Skp1-related (*skr*) and 520 predicted F-box genes.^{28–30} Inhibiting *skr-1* or *skr-7* by RNAi caused a significant increase in ENU-induced germ cell apoptosis similar to inactivating *cul-1* or *ned-8* (Figure 2c and Supplementary Table 1). The increased apoptosis caused by inhibiting *cul-1* or *skr-1* was abolished in *cep-1(gk138)* mutants (Figure 2d), suggesting that these genes also act

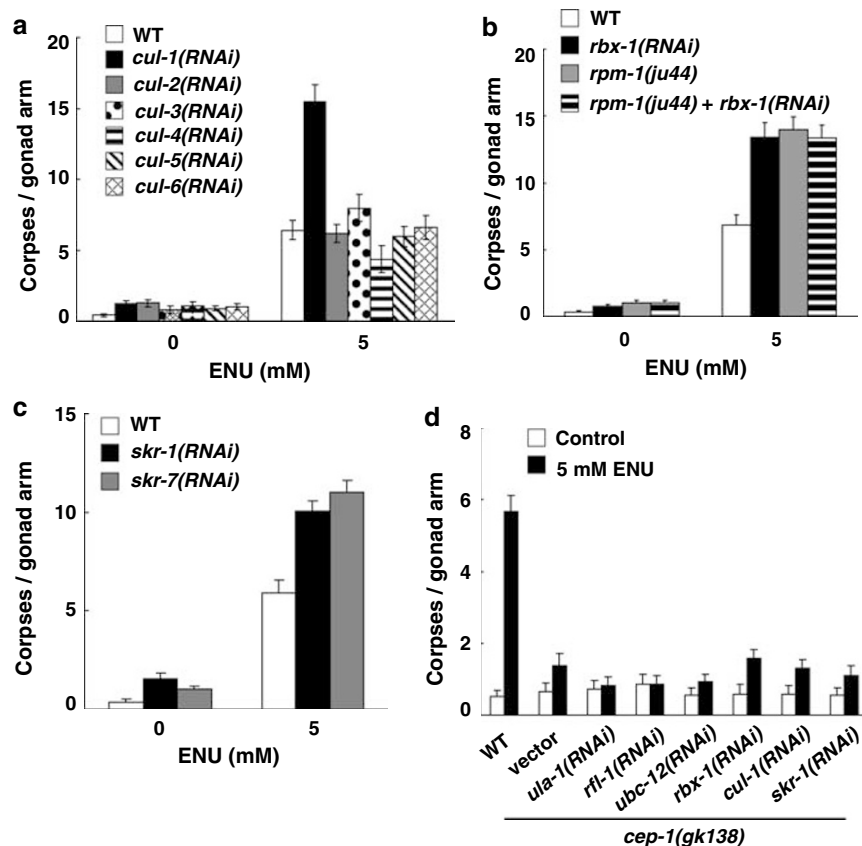


Figure 2 An SCF ubiquitin ligase complex antagonizes CEP-1/p53-mediated germ cell apoptosis in response to DNA damage. (a) Effect of 5 mM ENU on germ cell apoptosis in wild-type animals depleted of the six cullin genes. Only *cul-1(RNAi)* enhanced ENU-induced germ cell apoptosis. (b) Wild-type animals depleted of *rbx-1* by RNAi or *rpm-1(ju44)* mutants treated with 5 mM ENU exhibit hypersensitivity to germ cell apoptosis. No additive effects were observed when *rbx-1* was inhibited in *rpm-1(ju44)* mutants. (c) Inhibiting the Skp1-related genes *skr-1* and *skr-7* by RNAi sensitized worms to ENU-induced germ cell apoptosis. (d) The neddylation pathway and components of the SCF ligase regulates germ cell apoptosis in response to ENU through *cep-1*

upstream of *cep-1*. Because SKR-1 and SKR-7 bind to CUL-1,^{29,30} these results are consistent with the hypothesis that an SCF ligase regulates the apoptotic activity of CEP-1.

***fsn-1* regulates *cep-1*-dependent signaling in response to DNA damage.** The specificity of SCF ligases is controlled by F-box proteins, which bind to distinct substrate proteins that are targeted for ubiquitin-mediated proteolysis.²⁶ We systematically inhibited 328 of the 520 predicted F-box genes²⁸ by RNAi and identified FSN-1, an F-box protein conserved from worm to human that physically associates with RPM-1, SKR-1, and CUL-1 to regulate neuromuscular synapse formation.³¹ Using two different null alleles, *fsn-1(hp1)* and *fsn-1(gk429)*, we confirmed that FSN-1 is a potent negative regulator of ENU-induced germ cell apoptosis (Figure 3a). The increased apoptosis observed in *fsn-1* mutants was completely abolished by the *cep-1(gk138)* allele (Figure 3a), suggesting that *fsn-1* is upstream of *cep-1*. *fsn-1* mutants were also hypersensitive to *cep-1*-dependent apoptosis induced by ionizing radiation (Supplementary

Figure 4), indicating that it may have a general role in genotoxic stress response.

Since *fsn-1* functions in neurons to control synapse development,³¹ we asked whether expression of *fsn-1(+)* in neurons or muscle would rescue the sensitivity of *fsn-1(hp1)* mutants to ENU-induced germline apoptosis. *fsn-1(hp1)* mutants that express *fsn-1(+)* in neurons or muscle were treated with 0.5 mM ENU, which has minimal effects on germline apoptosis in wild-type animals but triggers widespread apoptosis in *fsn-1* mutants (Figure 3a). Expression of *fsn-1(+)* in these somatic tissues failed to rescue the sensitivity of *fsn-1(hp1)* mutants to germline apoptosis (Table 1). These results suggest that FSN-1 functions in a different tissue, such as the germ line, to regulate CEP-1-dependent apoptosis. To address the epistatic relationship between *ned-8* and *fsn-1*, we inhibited *ned-8* by RNAi in *fsn-1* mutants and quantified germline apoptosis in response to ENU. There was no difference in the levels of ENU-induced germ cell apoptosis when both genes were ablated (Supplementary Table 2), suggesting that *ned-8* and *fsn-1* act in the same pathway.

In addition to defects in activating *cep-1*-dependent apoptosis, *C. elegans* DNA repair mutants also produce a high fraction of dead progeny after treatment with DNA-damaging agents.^{18,32,33} To ascertain whether *fsn-1* mutants have defects in DNA repair, the survival of their progeny was quantified after ENU treatment. We found that *fsn-1(gk429)* mutants were as slightly sensitive to 2.5 mM ENU and *cep-1; fsn-1* double mutants were moderately sensitive to 1.0 mM ENU compared with *cep-1* single mutants. Therefore, we cannot rule out the possibility that *fsn-1* may have a minor role in DNA repair. Interestingly, *cep-1(gk138)* mutants were sensitive to 1.0 and 2.5 mM ENU (Table 2), suggesting that *cep-1* may have a role in the repair of damage caused by alkylating agents.

To determine whether the increased levels of apoptosis observed in *fsn-1* mutants was due to a defect in the engulfment of apoptotic corpses, we quantified the time germ cell corpses remained in the gonads of animals treated with ENU before they were engulfed.^{17,22} Germ cell corpses persisted for 27 ± 3 min ($n = 10$) in wild type and 26 ± 1 min in *fsn-1(hp1)* mutants ($n = 10$), indicating that *fsn-1* mutants do not have more germ cell corpses in response to ENU because of delayed engulfment.

FSN-1 downregulates *cep-1*-dependent transcription.

Because apoptosis in the *fsn-1*-null mutants was dependent on *cep-1*, we next evaluated whether CEP-1 activity was increased in *fsn-1* mutants. To measure CEP-1 activity *in vivo*, we quantified its transcriptional target gene *egl-1* by qPCR in wild type and *fsn-1(hp1)* and *fsn-1(gk429)* mutants treated with ENU (Figure 3b). In both *fsn-1* mutants, there was an ~2-fold increase in *egl-1* transcript over wild-type controls treated with 5 mM ENU (Figure 3b). The increased CEP-1 transcriptional activity in *fsn-1* mutants paralleled the higher levels of germ cell apoptosis observed in these mutants treated with ENU. Because apoptosis and CEP-1 transcriptional activity in the *fsn-1* mutants were suppressed by the *cep-1(gk138)* allele (Figure 3), we conclude that FSN-1

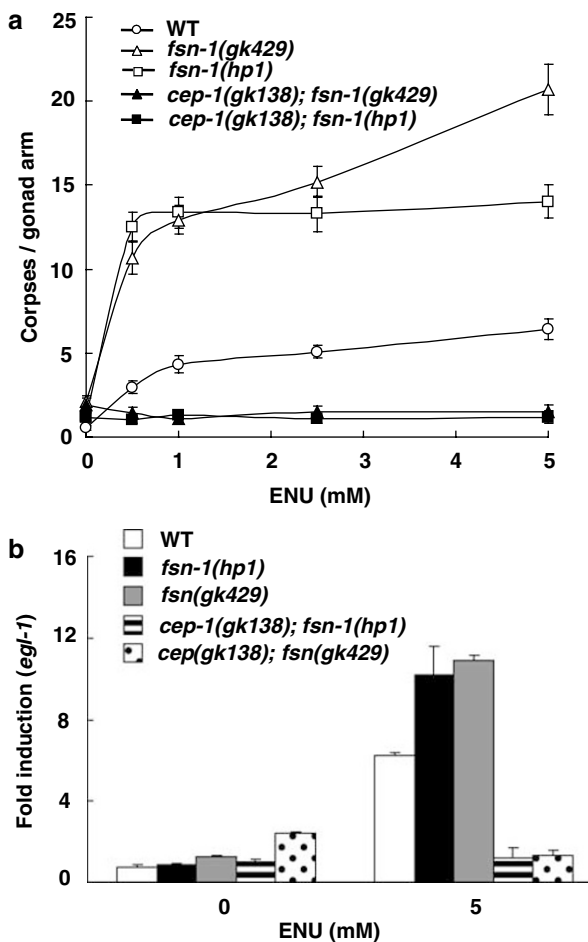


Figure 3 *fsn-1* regulates germline apoptosis through *cep-1*. (a) Hypersensitivity of *fsn-1(hp1)* and *fsn-1(gk429)* mutants to ENU-induced germ cell apoptosis is dependent on *cep-1*. Between 25 and 35 germ lines were scored at each dose of ENU in triplicate. (b) Effect of *fsn-1(hp1)* and *fsn-1(gk429)* mutants on *cep-1*-dependent transcription of *egl-1*. Fold induction of *egl-1* was calculated relative to levels in untreated wild-type hermaphrodites. Error bars = S.E.M.

Table 1 Expression of FSN-1 in somatic tissue does not rescue defects in germline apoptosis

| Strain | Genetic background | Transgene ^a (plasmid) | Mean \pm S.E.M. ^b | N |
|--------|--------------------------|--|--------------------------------|----|
| N2 | Wild type | | 2.6 \pm 0.4 | 16 |
| ZM876 | <i>fsn-1(hp1); juls1</i> | | 9.4 \pm 0.4 | 13 |
| ZM2140 | <i>fsn-1(hp1); juls1</i> | | 7.5 \pm 0.6 | 15 |
| ZM2140 | <i>fsn-1(hp1); juls1</i> | <i>P_{F25B3.3} fsn-1(+)</i> (pJH707) | 7.7 \pm 1.6 | 15 |
| ZM2139 | <i>fsn-1(hp1); juls1</i> | | 9.4 \pm 1.0 | 14 |
| ZM2139 | <i>fsn-1(hp1); juls1</i> | <i>P_{myo-3} fsn-1(+)</i> (pJH111) | 9.1 \pm 1.0 | 15 |
| ZM2135 | <i>fsn-1(hp1); juls1</i> | | 8.8 \pm 1.6 | 12 |
| ZM2135 | <i>fsn-1(hp1); juls1</i> | <i>P_{myo-3} fsn-1(+)</i> (pJH111) | 9.5 \pm 1.0 | 12 |

^aAll transgene arrays coinjected with pRF6 as a dominant marker. ^bGermline apoptosis values after treatment with 0.5 mM ENU

normally represses CEP-1 activity in the germ line in response to DNA damage.

Endogenous CEP-1 protein phosphorylation levels are regulated by FSN-1. We recently reported that endogenous CEP-1 is phosphorylated in response to ionizing radiation.¹⁷ To determine if CEP-1 protein is also affected by ENU treatment, we performed western analysis of worm extracts using an antibody against CEP-1.^{17,21} In wild-type extracts, we detected a band of \sim 75 kDa (the predicted size of full-length CEP-1) as well as a slower migrating band that was only detectable after ENU treatment (Figure 4a). The levels of modified CEP-1 reached a peak at 12 h and declined 24 h after ENU treatment. To assess whether this slower migrating band represents a phosphorylated form of CEP-1, we treated wild-type worm extracts with shrimp alkaline phosphatase and performed western analysis with anti-CEP-1. Phosphatase treatment caused the abundance of the upper band to dramatically decrease, but in the presence of phosphatase inhibitors this modified form of CEP-1 did not disappear (Figure 4b). Because the modified CEP-1 bands disappear after phosphatase treatment and were not present in extracts isolated from *cep-1(gk138)* deletion mutants (Figure 4c), we conclude that endogenous CEP-1 is phosphorylated in response to ENU. Since ENU caused the transcriptional activity of CEP-1 to increase in *fsn-1* mutants, we next asked whether CEP-1 protein was also augmented. In response to ENU, phosphorylated CEP-1 levels (upper band) increased by two- and three-fold in *fsn-1(hp1)* and *fsn-1(gk429)* mutants, respectively, over wild-type controls (Figure 4c and d). In non-stressed *fsn-1(gk429)* mutants, there were higher levels of unmodified CEP-1 (Figure 4d), suggesting that FSN-1 may also negatively regulate CEP-1 levels under normal conditions.

To address whether the increased levels of CEP-1 detected in *fsn-1* mutant extracts were due to ectopic expression outside the germ line, wild-type and *fsn-1* mutant worms were stained at various stages of development with CEP-1 antibodies. We observed CEP-1 staining in the same pharyngeal cells and in the germ line as previously reported,^{13,21} but did not detect ectopic CEP-1 staining in other somatic tissues in wild-type or *fsn-1* mutant worms treated with ENU (Supplementary Figure 5). In the germ line, there was a similar pattern of CEP-1 staining as described by Schumacher *et al.*,²¹ where levels were highest in the mitotic and meiotic regions and lower in the transition zone

(Supplementary Figure 5D). CEP-1 staining was more intense in the meiotic germ lines of *fsn-1* mutants treated with ENU compared to wild-type animals (Figure 5 and Supplementary Figure 6). In the absence of ENU, there was no detectable difference in CEP-1 staining in the germ lines of *fsn-1* mutants and wild-type animals, suggesting that CEP-1 levels in the germ line are regulated by FSN-1 in response to genotoxic stress. Interestingly, we also detected CEP-1 staining in the germ line at all stages of larval development and in the male germ line (Supplementary Figure 5) that was not previously reported.^{13,21} Therefore, we conclude that CEP-1 is expressed throughout germline development in both hermaphrodites and males, and that FSN-1 negatively regulates endogenous CEP-1 levels in the hermaphrodite germ line in response to DNA damage.

FSN-1 may regulate CEP-1 through an indirect mechanism. To determine if FSN-1 binds directly to CEP-1, we transiently transfected HEK293 cells with FLAG-tagged CEP-1 and H7-tagged FSN-1. Cell extracts were immunoprecipitated with anti-FLAG or anti-T7 and blots probed with anti-T7 or anti-FLAG, respectively. We were unable to detect FSN-1 binding to CEP-1 in cotransfected cells (Supplementary Figure 7), suggesting that FSN-1 may regulate CEP-1 by an indirect mechanism. Previously, FSN-1 was shown to regulate synaptogenesis by directly binding SCD-2, the worm orthologue of anaplastic lymphoma kinase.³¹ To determine if SCD-2 functions downstream of FSN-1 in the germ line, we treated *scd-2(ok565)* deletion mutants with ENU but observed similar levels of germ cell apoptosis as wild-type controls (Supplementary Table 3). The MAP kinase *dlk-1* regulates synapse formation downstream of *rpm-1* and *fsn-1* (unpublished data), but also does not affect ENU-induced germline apoptosis (Supplementary Table 3). To test if *scd-2* and *dlk-1* function redundantly downstream of *fsn-1* to regulate germ cell apoptosis, *scd-2* was inhibited by RNAi in *dlk-1(ju476)* mutants. As with the single mutants, there was no detectable effect on germ cell apoptosis when both genes were ablated (Supplementary Table 3), indicating that the SCF^{FSN-1} E3 ligase regulates CEP-1 activity in the germ line through a novel target.

Discussion

The *C. elegans* hermaphrodite germ line is an excellent model for studying the regulation of DNA damage-induced

Table 2 Survival of progeny from *cep-1* mutants is lower than that from *fsn-1* mutants treated with ENU

| Genotype | 0 mM | | 0.5 mM | | 1.0 mM | | 2.5 mM | | 5.0 mM | |
|-----------------------------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|------------|--------------|
| | Egg laying | Survival (%) | Egg laying | Survival (%) | Egg laying | Survival (%) | Egg laying | Survival (%) | Egg laying | Survival (%) |
| N2 | 7.2 ± 0.4 | 99.5 ± 0.3 | 5.7 ± 0.2 | 98.4 ± 0.6 | 4.3 ± 0.2 | 81.8 ± 1.4 | 2.4 ± 0.5 | 13.4 ± 2.5 | 1.2 ± 0.3 | 0 |
| <i>fsn-1(gk429)</i> | 6.8 ± 0.1 | 98.9 ± 0.4 | 5.6 ± 0.3 | 97.1 ± 0.8 | 4.4 ± 0.2 | 82.7 ± 1.2 | 1.1 ± 0.3 | 4.3 ± 3.8 | 0.7 ± 0.2 | 0 |
| <i>cep-1(gk138)</i> | 5.6 ± 0.3 | 99.0 ± 0.4 | 5.2 ± 0.2 | 91.4 ± 1.9 | 4.1 ± 0.1 | 50.7 ± 4.2 | 3.1 ± 0.2 | 0 | 1.9 ± 0.2 | 0 |
| <i>cep-1(gk138); fsn-1(gk429)</i> | 6.7 ± 0.3 | 98.5 ± 0.6 | 5.8 ± 0.3 | 95.4 ± 1.2 | 3.6 ± 0.2 | 38.7 ± 5.1 | 3.1 ± 0.5 | 0 | 1.5 ± 0.2 | 0 |

N2, *fsn-1(gk429)*, *cep-1(gk138)*, and *cep-1(gk138); fsn-1(gk429)* mutant worms at the L4 stage were treated with ENU for 4 h at 20°C. After ENU treatment, worms were allowed to recover on NGM plates seeded with OP50 bacteria for 20 h. Worms were then transferred to fresh plates and allowed to lay eggs for 12 h at 20°C. Unhatched eggs and surviving animals were counted 1 and 2 days later, respectively, to assess survival. Numbers shown represent the mean ± S.E.M. of the eggs laid per hour per adult hermaphrodite and the percent survival of embryos from six different animals per strain

apoptosis. Like its vertebrate relatives, the *C. elegans* p53-like gene *cep-1* rests at the hub of a complex signaling network to make life or death decisions in response to genotoxic stress.¹⁴ CEP-1 is tightly regulated by a number of conserved proteins, including the Akt/PKB serine/threonine kinase AKT-1, the p53-binding protein APE-1/iASPP, and the GLD-1 protein.^{17,19,21} Dysregulation of any of these inputs to CEP-1 leads to abnormal apoptosis in the germ line. Using the powerful genetics and functional genomics tools available in *C. elegans*, we identified the neddylation pathway and the E3 ubiquitin ligase SCF^{FSN-1} as important regulators of CEP-1-dependent apoptosis.

The p53-directed E3 ubiquitin ligase Mdm2 can conjugate Nedd8 to mammalian p53 and p73 to attenuate their transcriptional activity.^{9,20} Because there is no clear Mdm2 orthologue in *C. elegans* and we did not detect neddylation of CEP-1 from worm extracts, we asked if a cullin-based E3 ubiquitin ligase might regulate CEP-1. Previous work in *C. elegans* demonstrated that NED-8 regulates the mitosis to meiosis transition in early embryos by conjugating to CUL-3 to increase ubiquitin-mediated proteolysis of the microtubule severing protein MEI-1.³⁴ Thus, the regulation of cullin-based ligases by neddylation is well conserved in *C. elegans*. In mammals, the p53 family is regulated by several different E3 ubiquitin ligases, but it is not clear whether SCF ligases are involved in the regulation of these proteins. Here, we show that the neddylation pathway and the SCF^{FSN-1} E3 ligase regulate the apoptotic activity of CEP-1 in response to genotoxic stress. Our genetic epistasis results suggest that neddylation and SCF^{FSN-1} act in the same pathway (Supplementary Table 2), but we do not know the molecular mechanism by which these proteins regulate CEP-1 (Figure 6). It is possible that small amounts of CEP-1 are neddylation *in vivo*, which could not be detected with our antibody on western blots. Consistent with this possibility is the recent report demonstrating that p53 can be neddylation by the F-box protein FBX011.³⁵ If CEP-1 was directly regulated by neddylation and ubiquitin-mediated proteolysis through the SCF^{FSN-1} ligase, we might expect higher CEP-1 activity and increased germline apoptosis when these genes are ablated in the absence of genotoxic stress. For example, GLD-1 directly controls CEP-1 protein levels by binding to the 3-UTR of *cep-1* mRNA to repress its translation.²¹ In *gld-1(op236)* hypomorphic mutants, CEP-1 protein levels accumulate and trigger massive apoptosis of germ cells, which is further enhanced by DNA-damaging agents.²¹ Similarly, inhibiting the iASPP orthologue *ape-1* by RNAi leads to increased levels of physiological germ cell apoptosis (i.e., in the absence of genotoxic stress) that is dependent on *cep-1*.¹⁹ CEP-1 can also be induced to trigger germline apoptosis indirectly by inhibiting genes that normally prevent DNA damage under normal physiological conditions, such as the Brca1 orthologue *brc-1* or the Bloom's helicase *him-6*.³⁶ In contrast, *akt-1* negatively regulates DNA damage signals to *cep-1* but does not affect physiological germ cell apoptosis or CEP-1 activity in the absence of DNA-damaging agents.¹⁷ This is likely because AKT-1 functions to buffer damage signals to CEP-1 but has no role in the repair of endogenously (or exogenously) generated DNA damage. It is unlikely that SCF^{FSN-1} directly regulates AKT-1 because the levels of this kinase would be

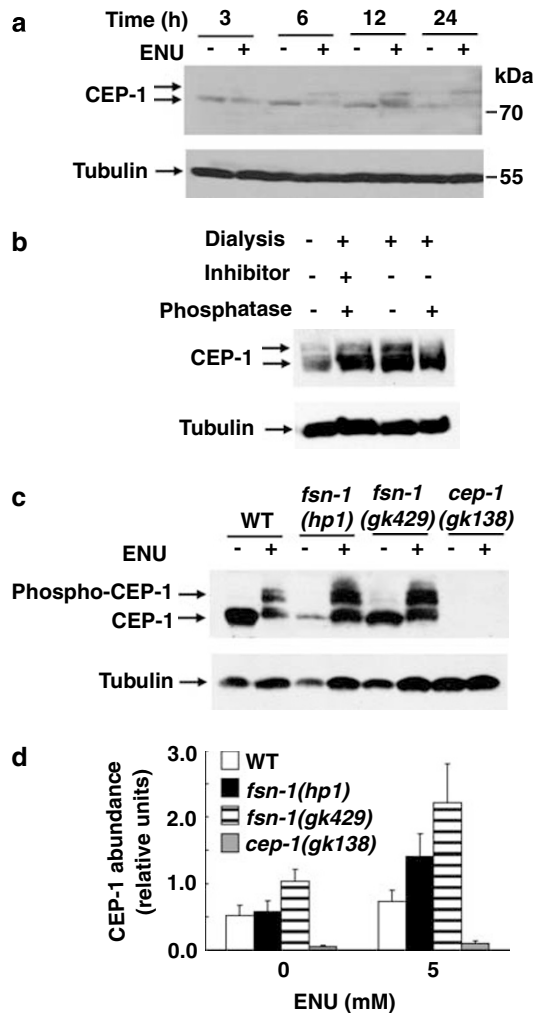


Figure 4 Endogenous CEP-1 is phosphorylated in response to ENU and its abundance is higher in *fsn-1* mutants. (a) The appearance of a slower migrating form of CEP-1 is detectable in wild-type animals at 6 h after ENU treatment. These bands were not present in *cep-1(gk138)* deletion mutants. Detection of α -tubulin on the same blot was used as a loading control. (b) The slower migrating form of CEP-1 in response to ENU is due to phosphorylation. Wild-type worm lysates were incubated in the presence or absence of shrimp alkaline phosphatase, then separated by SDS-PAGE and analyzed as above. (c) Wild-type, *fsn-1(hp1)*, *fsn-1(gk429)*, and *cep-1(gk138)* mutants were treated with ENU, 200 μ g of worm lysates was separated by SDS-PAGE and CEP-1 was detected as above. α -Tubulin was used as a loading control. (d) In response to ENU, there is more CEP-1 (unmodified + phosphorylated) in *fsn-1(hp1)* and *fsn-1(gk429)* mutants compared with wild-type controls. Relative densitometric units of total CEP-1 to α -tubulin are shown. Data represent three independent experiments. Error bars = S.E.M.

expected to increase in the absence of its negative regulator, and we have shown that activated AKT-1 suppresses damage-induced germ cell apoptosis.¹⁷

The SCF^{FSN-1} E3 ligase is conserved from nematodes to humans and was previously shown to regulate neuromuscular synapse development in *C. elegans*.³¹ Although the neddylation pathway was not examined in this study, we suspect that NED-8 activates the SCF^{FSN-1} ligase in the germ line because inhibiting *ned-8* in *fsn-1(hp1)* mutants did not enhance the levels of ENU-induced apoptosis compared with *fsn-1(hp1)*

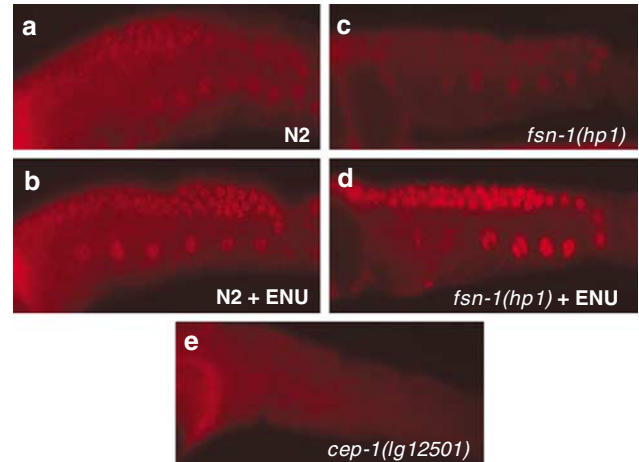


Figure 5 CEP-1 protein levels are higher in *fsn-1* mutants treated with ENU. To detect qualitative differences in CEP-1 germline staining intensities, photographs were taken at non-saturating intensities using identical exposure times for all samples. (a) Wild-type hermaphrodites stained with anti-CEP-1 antibodies in the absence of ENU. (b) Wild-type hermaphrodites treated with 5 mM ENU stained with anti-CEP-1 antibodies after 20 h. (c) *fsn-1(hp1)* mutant hermaphrodites stained with anti-CEP-1 antibodies in the absence of ENU. (d) *fsn-1(hp1)* mutant hermaphrodites + 5 mM ENU stained with anti-CEP-1 antibodies 20 h after ENU treatment. (e) *cep-1(lg12501)* mutant hermaphrodites do not stain with anti-CEP-1

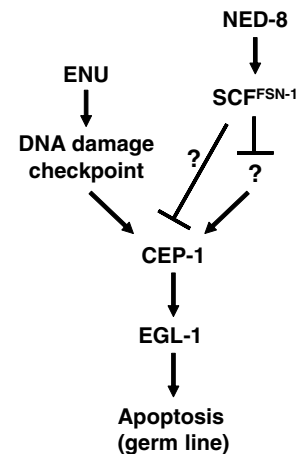


Figure 6 Model showing the regulation of CEP-1 by SCF^{FSN-1}. In the germ line, the SCF^{FSN-1} E3 ubiquitin ligase negatively regulates CEP-1. In response to ENU, the negative regulation of CEP-1 is disrupted, perhaps by inhibiting neddylation, and CEP-1 activates expression of the BH3-only protein EGL-1 and CED-13. SCF^{FSN-1} regulates CEP-1 independent of the kinases SCD-2 and DLK-1. Alternatively, SCF^{FSN-1} may directly bind and degrade phosphorylated CEP-1

single mutants (Supplementary Table 2). The role of *fsn-1* in the DNA damage response is not just restricted to alkylating agents because *fsn-1* mutants are also hypersensitive to ionizing radiation (Supplementary Figure 4). Expression of *fsn-1(+)* in neurons and muscle did not rescue the germline apoptosis defects of *fsn-1* mutants (Table 1), suggesting that it acts in a different tissue (perhaps the germ line) to modulate CEP-1 activity. Because we could not immunoprecipitate CEP-1 from worm extracts to determine if it interacts directly with FSN-1, we coexpressed tagged proteins in HEK293 cells and carried out coimmunoprecipitation experiments. Under

these conditions, we did not detect binding by CEP-1 and FSN-1 (Supplementary Figure 7). Taken together, this evidence suggests that CEP-1 is regulated by SCF^{FSN-1} through an intermediate protein.

At present, we do not know the identity of the germline SCF^{FSN-1} target protein(s). Because DNA damage-induced germline apoptosis was unaffected in *scd-2* or *dlk-1* mutants, we propose that FSN-1 targets a unique substrate in the germline that modulates damage signals to CEP-1 (Figure 6). Furthermore, RPM-1 may regulate distinct targets in different cellular processes.³⁷ Although the viability of progeny from *fsn-1* mutants treated with ENU is similar to wild type, we noticed a modest decrease in the survival of progeny from *fsn-1* mutants at 2.5 mM ENU (Table 2). Thus, it is possible that partial S-phase progression or chromosome segregation defects of mitotic germ cells could sensitize *fsn-1* mutants to ENU-dependent apoptosis. In this regard, SCF^{FSN-1} may target a protein in the germline that has a minor role in DNA repair, which feeds into CEP-1. However, F-box proteins typically interact with phosphorylated substrates,²⁶ and since we were unable to detect phosphorylated CEP-1 when it was expressed in HEK293 cells, it is possible that phospho-CEP-1 is the target of FSN-1 in the germline. It will be interesting to know whether the human FSN-1 orthologue, FBX045, has a conserved role in regulating the apoptotic activity of the p53 family. These studies may reveal conserved mechanisms by which the p53 family is regulated in response to genotoxic stress that could be exploited therapeutically for the treatment of human cancer.

Materials and Methods

Strains and genetics. *C. elegans* strains were cultured and maintained using standard procedures.³⁷ The following strains were kindly provided by the *Caenorhabditis* Genetics Center (University of Minnesota, St. Paul, MN, USA): wild-type var. Bristol (N2), *ced-3(n1717)*, *ced-4(n1162)*, *ced-9(n1950)*, *cep-1(gk138)*, *cep-1(lg12501)*, *cul-1(e1756)/unc-69(e587)*, *egl-1(n1084n3082)*, *fsn-1(gk429)*. Double mutant strains were generated using standard genetic methods.³⁸

RNAi. Gene expression was inhibited using the feeding method.³⁹ Bacteria expressing double-stranded RNA (dsRNA) to a specific *C. elegans* gene were grown on the nematode growth media (NGM) plates supplemented with antibiotics (50 µg/ml ampicillin and 12.5 µg/ml tetracycline) and 0.1 mM isopropyl-β-D-thiogalactopyranoside to induce dsRNA production.³⁹ Bacteria expressing an empty RNAi vector (L4440) were used as controls. Larval stage 2 (L2) hermaphrodites were placed on the RNAi-producing plates and allowed to develop until the young adult stage. We did not observe any morphological defects in the germlines of animals treated with RNAi to the neddylation pathway but observed penetrant and reproducible embryonic lethality in the F1 generation (data not shown), consistent with the essential role of this pathway in the embryo.³⁴ All RNAi clones were sequenced to confirm their identity.

Germline apoptosis and ENU sensitivity. Germline apoptosis was quantified by morphology as described.¹³ Young adult animals (12 h after the L4 stage) were treated with the DNA-alkylating agent ENU for 4 h at 20°C. After ENU treatment, worms were transferred to fresh plates and allowed to recover for 24 h. Germ cell corpses were quantified in the pachytene region of one gonad arm per animal at 12, 24 and 36 h after ENU treatment. Apoptotic nuclei were scored based on their refractile appearance at × 630 magnification using Nomarski optics. Germ cell corpses were confirmed using the fluorescent dye acridine orange, which is specifically retained in the nuclei of apoptosis germ cells.¹⁷

Preparation of RNA and qPCR. Approximately 2000–3000 young adult worms were treated with ENU and total RNA was isolated using GibcoBRL Trizol kit as described previously.¹⁷ RNA was dissolved in diethyl pyrocarbonate-treated

water and stored at –20°C. For all RNA samples, the ratio of the optical density at 260 nm to the optical density at 280 nm was > 1.50. Total RNA (4 µg) was converted to cDNA using SuperScript reverse transcriptase. qPCR was performed with a SYBR Green PCR Master Mix kit (Applied Biosystems), according to the manufacturer's instructions. The final PCR volume was 25 µl. Primer pairs (for *egl-1* 878 5'-tactctcgtctcaggact-3' and 880 5'-catcgaagtcacgcacat-3'; for *ced-13* 706 5'-acgggtgttgagttgcaagc-3' and 707 5'-gtctacaagcgtgatgat-3'; for *tbg-1* 710 5'-cgtcatcagcctgtagaaca-3' and 711 5'-tgatgactgtccacgttga-3') were designed to generate intron-spanning products of 110–150 bp.²⁴ A standard curve was prepared as a test for the sensitivity of the assay and as a reference for quantification. The standard curve was generated from six points, and each primer pair was tested with a logarithmic dilution of a cDNA mixture to generate a linear standard curve. For normalization of the quantitative results, γ-tubulin (*tbg-1*) mRNA was used as an external standard.

Western blot analysis. Approximately 2000–3000 adult worms were washed from plates with M9 buffer. After centrifugation at 5000 r.p.m. for 1 min, the supernatant was removed. The pelleted worms were resuspended and washed three times with M9 buffer and centrifuged as above. The worm pellet was resuspended in an equal volume of 2 × Laemmli sample buffer, sonicated for 2 min, and then boiled for 5 min. After a final centrifugation at 13 000 r.p.m. for 1 min, the extracts were stored at –80°C. 200 µg of worm lysate was separated by SDS-PAGE and then transferred to nitrocellulose membranes (Perkin-Elmer Life Science, Boston, MA, USA). The membrane was blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 and subsequently incubated with diluted (1:100) preabsorbed goat anti-CEP-1 antibody (a generous gift from Dr. A Gartner)²¹ or our own rabbit anti-CEP-1 antibody. After washing three times, the membrane was incubated with peroxidase-conjugated rabbit anti-goat immunoglobulin G antibody (Amersham Biosciences Co.), and the specific bands were detected using the Amersham ECL enhanced chemiluminescence reagent. An α-tubulin antibody (Sigma) was used as a loading control.

Phosphatase treatment. Approximately 2000–3000 adult worms were washed with M9 buffer as above. The worm pellet was resuspended in an equal volume of sample buffer, sonicated for 2 min, and then dialyzed overnight in shrimp alkaline phosphatase reaction buffer (0.01 M Tris-HCl, pH 7.5, 0.01 M MgCl₂, and 0.1 mg/ml BSA) in the presence or absence of inhibitors (0.8 mM Na₃VO₄ + 25 mM NaF) at 4°C. Next, 200 µg of dialyzed worm lysate was incubated with or without 15 U of shrimp alkaline phosphatase for 1 h at 37°C, and then separated by SDS-PAGE and analyzed as above.

Worm fixation. The fixation method was based on the protocol of Finney *et al.*⁴⁰ Worms were synchronized at the first larval (L1) stage on 100 mm NGM plates, grown to young adults at 20°C. Worms were washed off plates with M9 buffer into a 15 ml conical tube and pelleted at 3000 r.p.m. The supernatant was aspirated and pellets were washed with 10 ml water twice. Supernatant was removed and the worm suspension was quickly transferred to an Eppendorf tube. The worm suspension was washed two more times and pellets were resuspended in 300 µl water. Next, 500 µl of cold 2 × 'Witches Brew' buffer (160 mM KCl, 40 mM NaCl, 20 mM EGTA, 10 mM spermidine-HCl, 30 mM Na-Pipes, pH 7.4, 50% methanol) and 200 µl cold 20% paraformaldehyde were added to the worm suspension. Worms were then mixed by inversion and freeze-thawed in liquid nitrogen. Immediately after thawing, worm mixtures were put on ice for 2 h and inverted every 15 min. Worm mixtures were washed with Tris-Triton buffer twice (5 min for each wash) and then pelleted at 4000 r.p.m. for 1 min. Worms were then incubated in 1 ml B03 buffer (25 mM H₃BO₃, 12.5 mM NaOH, pH 9.3) + 10 µl β-mercaptoethanol for 2 h at 37°C on rotator (~280 r.p.m.). Next, worm suspensions were pelleted at 4000 r.p.m. for 1 min and washed in 1 ml of B03 buffer. Worm suspensions were pelleted after washing and incubated in 1 ml B03 buffer + 10 µl of 1 M DTT for 15 min at room temperature on rotator. Next, worm suspensions were pelleted as above, washed with 1 ml B03 buffer for 5 min, and then incubated in 1 ml B03 and 10 µl H₂O₂ for 15 min at room temperature (repeated twice). Worm suspensions were pelleted and incubated for 15 min in buffer B (1 × PBS, 0.1% BSA (Pentax Fraction V), 0.5% Triton X-100, 5 mM sodium azide, 1 mM EDTA) on rotator. Worms were pelleted, supernatant was removed, and worms were incubated in buffer A (same as buffer B, except 1% BSA) for ~ 1.5 h.

Antibody staining and immunofluorescence. To the fixed worms, 100 µl buffer A and 2 µl anti-CEP-1 antibody (1:50 dilution) were added and

incubated overnight at room temperature without agitation. Worms were pelleted and supernatant was removed by aspiration. Worms were washed with buffer B 4–5 times (10 min each) and pelleted at 3000 r.p.m. between washes. Secondary anti-rabbit Cy3 was added (1:400 dilution) and incubated for 3 h in a 37°C water bath with intermittent inversion. Worms were pelleted and washed 4–5 times in buffer B (10 min each). Worms were carefully pipetted onto poly-L-lysine-coated slides and sealed for immunofluorescence microscopy. *cep-1(g12501)* mutants were used for negative staining controls because there was less nonspecific staining in this strain using our CEP-1 antibody.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)