LEM-3 - A LEM domain containing nuclease involved in the DNA damage response in C. elegans

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Abstract: The small nematode Caenorhabditis elegans displays a spectrum of DNA damage responses similar to humans. In order to identify new DNA damage response genes, we isolated in a forward genetic screen 14 new mutations conferring hypersensitivity to ionizing radiation. We present here our characterization of lem-3, one of the genes identified in this screen. LEM-3 contains a LEM domain and a GIY nuclease domain. We confirm that LEM-3 has DNase activity in vitro. lem-3(If) mutants are hypersensitive to various types of DNA damage, including ionizing radiation, UV-C light and crosslinking agents. Embryos from irradiated lem-3 hermaphrodites displayed severe defects during cell division, including chromosome mis-segregation and anaphase bridges. The mitotic defects observed in irradiated lem-3 mutant embryos are similar to those found in baf-1 (barrier-to-autointegration factor) mutants. The baf-1 gene codes for an essential and highly conserved protein known to interact with the other two C. elegans LEM domain proteins, LEM-2 and EMR-1. We show that baf-1, lem-2, and emr-1 mutants are also hypersensitive to DNA damage and that loss of lem-3 sensitizes baf-1 mutants even in the absence of DNA damage. Our data suggest that BAF-1, together with the LEM domain proteins, plays an important role following DNA damage - possibly by promoting the reorganization of damaged chromatin.

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Introduction

Living organisms are continuously exposed to genotoxic stress, caused both endogenously by cellular metabolism and exogenously by e.g. UV light. To maintain genome integrity, cells possess a number of DNA damage response pathways, which ensure that lesions are removed from the DNA or that cells with irreparable damage are removed by apoptosis. Defects in DNA damage responses can lead to a number of diseases, including predisposition to cancer (for review see [1]).

Forward genetic screens are powerful tools for identifying new DNA damage response genes. Indeed, many DNA damage response genes, such as those found in Drosophila melanogaster (barrier-to-autointegration factor) mutants. The baf-1 gene codes for an essential and highly conserved protein known to interact with the other two LEM domain proteins, LEM-2 and EMR-1. We show that baf-1, lem-2, and emr-1 mutants are also hypersensitive to DNA damage and that loss of lem-3 sensitizes baf-1 mutants even in the absence of DNA damage. Our data suggest that BAF-1, together with the LEM domain proteins, plays an important role following DNA damage – possibly by promoting the reorganization of damaged chromatin.

Abstract

The small nematode Caenorhabditis elegans displays a spectrum of DNA damage responses similar to humans. In order to identify new DNA damage response genes, we isolated in a forward genetic screen 14 new mutations conferring hypersensitivity to ionizing radiation. We present here our characterization of lem-3, one of the genes identified in this screen. LEM-3 contains a LEM domain and a GIY nuclease domain. We confirm that LEM-3 has DNase activity in vitro. lem-3(II) mutants are hypersensitive to various types of DNA damage, including ionizing radiation, UV-C light and crosslinking agents. Embryos from irradiated lem-3 hermaphrodites displayed severe defects during cell division, including chromosome mis-segregation and anaphase bridges. The mitotic defects observed in irradiated lem-3 mutant embryos are similar to those found in baf-1 (barrier-to-autointegration factor) mutants. The baf-1 gene codes for an essential and highly conserved protein known to interact with the other two C. elegans LEM domain proteins, LEM-2 and EMR-1. We show that baf-1, lem-2, and emr-1 mutants are also hypersensitive to DNA damage and that loss of lem-3 sensitizes baf-1 mutants even in the absence of DNA damage. Our data suggest that BAF-1, together with the LEM domain proteins, plays an important role following DNA damage – possibly by promoting the reorganization of damaged chromatin.


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the germ line but leads to high embryonic lethality following treatment with X-rays, UV-C light and cisplatin. Following DNA damage lem-3 embryos display severe defects during cell division, including chromosome mis-segregation and anaphase bridges. These mitotic defects are similar to those found in baf-1 (barrier-to-autointegration factor) mutants [13]. The baf-1 gene encodes an essential and highly conserved protein known to interact with the other two C. elegans LEM domain proteins, LEM-2 and EMR-1 [14–18]. Here we show that baf-1, lem-2, and emr-1 mutants are also hypersensitive to DNA damage and that loss of lem-3 sensitizes baf-1 mutants even in the absence of DNA damage. Our data uncover a novel function for baf-1 following DNA damage, which is dependent on the LEM domain proteins.

Results

A forward genetic screen for mutants hypersensitive to DNA damage

To find novel components involved in DNA damage response pathways, we conducted a forward genetic screen for mutations that show increased embryonic lethality following exposure to sublethal doses of ionizing radiation. In a screen of approximately 2'000 genomes, we found 14 mutants with such a phenotype (Figure S1). Of these, we selected op444 for further analysis because it showed the highest increase in sensitivity to ionizing radiation but had no strong defect in the absence of exogenous DNA damage. Dose-response studies showed that op444 mutants were an order of magnitude more sensitive to ionizing radiation (IR) than wild type ($LD_{50}$ approx. 6 Gy vs. 60 Gy, Figure 1A).

op444 mutants are also sensitive to UV-C and cisplatin

We next tested whether op444 mutants showed sensitivity to other types of DNA damage. UV-C light (<280 nm) induces cyclobutane pyrimidine dimers and 6-4 photoproducts, which are mainly repaired by the nucleotide excision repair (NER) pathway. To determine whether op444 mutants are also sensitive to UV-C light, we exposed adult hermaphrodites to 60 J/m² and assessed embryonic lethality of their progeny (Figure 1B). We found that op444 mutants displayed a high lethality following UV-C exposure, similar to that observed in xpa-1(ok698) mutants, which carry a

Figure 1. lem-3(op444) phenotypes following DNA damage. Wild-type animals and lem-3(op444) mutants were treated 24 h post L4/adult molt with (A) X-rays (B) UV-C, or (C) as L4 larvae with cisplatin. F1 embryonic lethality was assessed as described in Materials and Methods. Data shown represent the average of three independent experiments ± S.D. The progeny of 10 worms (A, B) or 5 worms (C) were analysed for each experiment. (D) Late radiation phenotypes. L1 animals were irradiated with 60 Gy. The uncoordinated phenotype (Unc) and protruding vulva phenotype (P-vul) were scored only for those animals that reached adulthood (as described in Materials and Methods). Data shown represent the average of three independent experiments ± S.D. (n for wild type, lem-3(op444) and ckut-80(ok861) are 305, 236 and 373 respectively). doi:10.1371/journal.pone.0024555.g001
deletion in the C. elegans homolog of human XPA, a component of the nucleotide excision repair (NER) pathway [19].

We also treated op444 animals with cisplatin, a potent DNA damaging agent frequently used in chemotherapy, which generates intra- and interstrand DNA crosslinks. op444 mutants displayed increased embryonic lethality at both concentrations tested (1 mM and 5 mM, Figure 1C). Taken together, these data indicate that a variety of genotoxic insults can impair the survival of op444 mutant embryos.

**op444 is a mutation in lem-3**

We took advantage of fragment length polymorphisms (FLP) and single nucleotide polymorphisms (SNP) between the Hawaii isolate CB4856 and the Bristol wild-type strain N2 [20,21] to map op444 to a 20 kb interval containing six predicted genes on the right arm of LG I. Unfortunately, we could not phenocopy the radiation sensitivity of op444 mutants by RNAi knockdown of any of these six genes. However, by sequencing genomic DNA from op444 mutants, we identified a G to T point mutation at position +2363 in lem-3 (F42H11.2) (Figure 2A), a gene not previously implicated in any DNA damage response pathway. This mutation results in the change of an evolutionarily conserved leucine to a phenylalanine in the C-terminus of the protein (Figure 2B and D).

We used biologic transformation [22] to generate transgenic lines expressing lem-3 under the control of the promoter of the ubiquitously expressed gene agg-1. Three independent transgenic lines fully rescued the radiation sensitivity of op444 mutants; two of them are shown in Figure 2C. These results confirm that mutation of lem-3 is the cause of the hypersensitivity phenotype observed in op444 mutants. To determine the localisation of LEM-3, we built transgenic lines expressing N-terminally YFP tagged LEM-3. Two lines rescued the radiation sensitivity of lem-3(op444), one of them is shown in Figure S2A. YFP::LEM-3 was found to be expressed only in embryos. It localised in a foci-like pattern but we could not observe it inside the nucleus (Figure S2B). The localisation pattern does not change following irradiation (data not shown). The exact nature of the embryonic foci is currently unclear (see discussion).

**rad-1 mutants carry a mutation in lem-3**

Hartman and Herman (1982) had, in a screen similar to ours, isolated and genetically characterized nine rad (radiation hypersensitive) genes. One of these, rad-1(nm155), showed striking phenotypic similarities to lem-3(op444) and mapped within 2 cM of lem-3 [23], thereby prompting us to test whether rad-1(nm155) is allelic to lem-3. Indeed, rad-1(nm155) failed to complement the radiation sensitivity phenotype of lem-3(op444) (Figure S3A). Sequencing revealed a C to T point mutation at position 671, which leads to a premature stop codon (Figure 2A). This R190STOP mutation leads to a truncated protein lacking the LEM domain and the GIY-YIG domain (Figure 2A and B). This allele displays a weaker sensitivity to ionizing radiation (Figure S3B), possibly because the in-frame deletion does not affect any of the conserved domains of LEM-3.

LEM-3 has nuclease activity

As LEM-3 contains a GIY-YIG domain, we wanted to test whether LEM-3 also has nuclease activity. We cloned the cDNAs of wild-type lem-3 and lem-3(op444) and expressed the respective proteins in insect cells (Figure S4 and Materials & Methods). Incubation of supercoiled plasmid DNA with increasing amounts of wild-type LEM-3 protein converted the supercoiled plasmid to relaxed circular (nicked) and linear molecules (Figure 3B). This reaction was dependent on the concentration of protein added and was abolished in the presence of EDTA. In contrast, addition of the mutant protein only resulted in a minor, if at all, release of the supercoiled form. To further support these findings, we investigated the enzymatic activity of LEM-3 on DNA from PhiX174, which is rich in secondary structures that can be cleaved by many structure-specific endonucleases. Incubation of wild-type LEM-3 with PhiX174 resulted in DNA cleavage (Figure 3C). Again, this endonuclease activity was significantly reduced when mutant LEM-3 was used. In conclusion, these results indicate that LEM-3 has nuclease activity and that the L to F mutation corresponding to lem-3(op444) greatly diminishes this activity.

Cell cycle arrest is normal in op444 mutants

DNA damage induces a transient cell cycle arrest in the mitotic stem cell compartment of the adult C. elegans germ line (Figure 4A), resulting in a temporary reduction in cell numbers. Because DNA damage arrests cell division but not cell growth, arrested cells eventually become much larger than in control animals (Figure 4C) [5]. Unlike many DNA damage checkpoint mutants and DNA repair mutants which are defective for cell cycle arrest [2], lem-3(op444) mutants showed a normal proliferation arrest and recovery following exposure to ionizing radiation (Figure 4B and C). We conclude that the pathways that sense DNA damage and signal cell cycle arrest are not impaired in lem-3 mutants.

Apoptotic cell death is not impaired in op444 animals

During oocyte development (Figure 4A), approximately half of the germ cells die of apoptosis, through a developmental program called physiological germ cell death. Upon DNA damage, the number of apoptotic corpses increases significantly due to a CEP-1/p53-mediated up-regulation of the BH3 domain proteins EGL-1 and CED-13 [25–27]. Many DNA damage response mutants are defective in DNA damage induced germ cell apoptosis. By contrast, both basal and DNA damage induced apoptosis were normal in lem-3(op444) animals (Figure 4D). We conclude that regulation and execution of apoptosis is unimpaired in lem-3(op444) animals. Given that cell cycle arrest and apoptosis following DNA damage are both normal in lem-3(op444) mutants, we speculate that lem-3(op444) mutants are not impaired in DNA damage signalling but rather in another part of the DNA damage response system, most likely DNA repair.

Analysis of DNA damage response in lem-3(op444) mutants

Based on our data above, we next asked which repair pathway LEM-3 might participate in. We first investigated double strand break repair, as this process occurs extensively in the germ line as part
of meiotic recombination. Failure to resolve the double strand breaks induced by the SPO-11 endonuclease results in increased germ cell apoptosis due to activation of CEP-1/p53 and increased meiotic non-disjunction. The resulting aneuploidy leads to a high embryonic lethality and a high incidence of males. However, none of these phenotypes is apparent in lem-3(op444) mutants in the absence of exogenous DNA damage (Figure 1A, 4D, and data not shown). Thus, we conclude that lem-3 mutants are proficient in both meiosis and the resolution of dsDNA breaks induced during meiotic recombination.

Rad54 has been implicated in several steps during homologous recombination and repair [28]. Down-regulation of rad-54 by RNAi results in increased apoptosis and radiation-induced embryonic lethality [29]. We have already shown that RAD-54 forms foci following IR, likely marking sites of active repair [30]. To get further insights into the ability of lem-3(op444) mutants to repair exogenously generated dsDNA breaks, we analyzed the subcellular localisation of YFP::RAD-54. In the absence of exogenous damage, YFP::RAD-54(opIs257) localized to distinct foci in the meiotic zone of the germ line, but showed a diffuse nuclear staining pattern in the mitotic zone (Figure 5A). This is in line with the known role of RAD-54 during homologous recombination. Upon irradiation, foci also appeared in the mitotic zone.

Figure 2. Characterization of the lem-3 locus. (A) Gene structure of lem-3. Boxes represent exons, lines represent introns. The position and nature of the mutations are indicated. (B) LEM-3 is a 704 AA protein with 2 ankyrin repeats (ANK), a LEM domain (LEM) and a GIY-YIG domain (GIY) of the COG3680 type. (C) Expression of a wild-type copy of lem-3 rescues the radiation hypersensitivity of lem-3(op444) mutants. Data represent the average of three experiments ± S.D. (two experiments for opIs309). The progeny of 10 worms were analysed for each experiment. (D) Alignment of the C-terminus of LEM-3 of different species starting with the GIY domain. The number of the first amino acid is indicated.

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zone (Figure 5B). Quantitative analysis revealed that the number of RAD-54 foci per nucleus were similar in wild type and lem-3(op444) (Figure 5D). After 17 hours, most cells in the mitotic zone had successfully removed RAD-54 in both wild type and lem-3(op444). Only a few, enlarged nuclei still showed RAD-54 foci (Figure 5C). These likely represent cells with persistent DNA damage that failed to re-enter the cell cycle. We conclude from these data that the repair of exogenously generated dsDNA breaks is normal lem-3(op444).

**lem-3 and xpa-1 act in separate pathways**

Since lem-3 mutants are also hypersensitive to UV-C light, we investigated a possible involvement of lem-3 in the NER pathway. We built and analyzed a double mutant with xpa-1(ok698), a factor

Figure 3. The LEM-3 endonuclease acts in parallel to the NER pathway. (A) Hermaphrodites were treated 24 h post L4/adult molt with 100 J/m² and F1 embryonic lethality scored as described in Materials and Methods. Data shown represent the average of three independent experiments ± S.D. The progeny of 10 worms were analysed for each experiment. (B) Non-specific endonuclease assay, in which supercoiled plasmid DNA was incubated with increasing amounts of SF9-expressed MBP-LEM-3 fusion protein. Endonucleolytic activity of LEM-3 (WT) leads to nicks in the DNA strands and converts the supercoiled (sc) plasmid DNA first to open-circular (oc) and then into linear (lin) DNA species. Endonucleolytic activity of LEM-3 can be inhibited by the addition of EDTA. The figure is a negative image of a 0.8% agarose gel stained with ethidium bromide. M: 1 kb marker; (−): no protein; WT: LEM-3 wild type; L−F: mutant corresponding to lem-3(op444). (C) PhiX174 single-stranded DNA was incubated with increasing amounts of SF9-expressed MBP-LEM-3 fusion protein. The single-stranded viral DNA of PhiX174 contains many secondary structures (hairpin loops, bulges etc.) that are cleaved by many structure-specific endonucleases. The figure is a negative image of a 0.8% agarose gel stained with SYBR Gold. (−): no protein; WT: LEM-3 wild type; L−F: mutant corresponding to lem-3(op444). EcExoIII, E. coli exonuclease III; HhaI, Hinfi, MboI, restriction endonucleases.

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Figure 4. *lem-3(op444)* mutants show normal cell cycle arrest and apoptotic responses. (A) Schematic representation of a *C. elegans* gonad. The most distal part is capped by the somatic distal tip cell. The cells close to the DTC are in mitosis. As they travel down the gonad, they enter the pachytene stage of meiosis I. Near the gonadal bend, some cells die by apoptosis. Apoptotic bodies become visible as "refractive disks". The mature oocytes are then pushed through the spermatheca and become fertilized. (B) Quantification of cell cycle arrest response following IR. The number of nuclei within 50 μm of the distant tip cell ± S.D was scored at various time points. n, number of germ lines analyzed. (C) Cell cycle arrest is normal in *lem-3*(op444) mutants. Representative DIC images of dissected gonads from wild-type and *lem-3*(op444) mutants are shown. Size bar: 10 μm. Black arrowheads indicate small (cycling) cell nuclei, white arrow heads indicate big (arrested) cells. (D) DNA damage-induced apoptosis is normal in *lem-3*(op444) mutants. Hermaphrodites were exposed to 60 Gy at L4/adult molt and apoptotic corpses were quantified at the indicated time points. Data shown represent the average of three independent experiments (10 germ lines per experiment) ± S.D.

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common to transcription-coupled repair and global genomic repair, the two branches of the NER pathway. Interestingly, we found that the double mutant showed a significant increase in embryonic lethality compared to both single mutants (Figure 3A). Thus, we conclude that \textit{lem-3} and \textit{xpa-1} likely do not act in a linear pathway.

**LEM-3 is required for proper chromosome segregation following DNA damage**

As we could not find any clear defects in the germ line of \textit{lem-3(op444)} mutants, we investigated in more detail the cause of the lethality observed following DNA damage. To get a first insight into the nature of the defect, we stained chromat in early stage embryos derived from irradiated hermaphrodite worms. Whereas chromosome segregation was mostly normal in irradiated wild type embryos (91\%, \(n = 67\)), 77\% (\(n = 31\)) of irradiated \textit{lem-3(op444)} embryos showed chromosome mis-segregation starting at the second cell division, as evidenced by the generation of extra nuclear chromatin material and chromatin bridges following mitosis (Figure 6). We confirmed this result by using a GFP::H2B transgene to follow chromosome segregation in early embryos by time lapse microscopy (Movie S1 and Movie S2). To determine whether LEM-3 is also required for proper chromosome segregation during larval development, we irradiated freshly hatched L1 larvae and scored the animals as adults for locomotion defects or protruding vulvae – phenotypes arising from defective proliferation/division of the P.n.a neuroblast or P.n.p vulval precursor cells, respectively. These phenotypes can also be observed following irradiation of mutants of the non-homologous end joining pathway genes \textit{cku-70}, \textit{cku-80} and \textit{lig-4} [31]. Only 2\% of irradiated \textit{lem-3} mutants developed to adults without any developmental defects (96\% displayed an uncoordinated phenotype (Unc), 61\% had a protruding vulva (P-vul)). In contrast, 86\% of irradiated wild type L1 larvae developed to phenotypically normal adults (Figure 1D). These results suggest that \textit{lem-3} mutant embryos die after irradiation because of loss of genomic integrity due to chromosome segregation defects. Additionally, we conclude that \textit{lem-3} is also required during post-embryonic cell divisions after genotoxic stress to ensure normal cell proliferation.

**LEM-3 genetically interacts with BAF-1**

Three LEM-domain proteins, EMR-1 (Ce-emerin), LEM-2 (Ce-MAN1), and LEM-3 have been identified in \textit{C. elegans}. EMR-1 and LEM-2 have a transmembrane domain and an N-terminally located LEM domain [14], whereas LEM-3 lacks a transmembrane domain, and has a LEM-domain located in the middle of the protein [14]. EMR-1 and LEM-2 bind LMN-1, the \textit{C. elegans
lamin homologue, and BAF-1 (barrier-to-autointegration factor) [15]. BAF is an evolutionarily conserved and essential protein which was shown to interact with dsDNA, chromatin, nuclear lamina proteins, histones and transcription factors [32]. Down-regulation of either lmn-1 or baf-1 or co-depletion of emr-1 and lem-2 leads to severe defects in mitosis, including abnormal chromosome segregation and anaphase bridges, which ultimately leads to embryonic lethality [13,15,18,33]. BAF-1 is essential for nuclear envelope formation [34] and required to assemble LMN-1, EMR-1 and LEM-2 on the nuclear envelope [17].

As downregulation of either baf-1 or co-depletion of emr-1 and lem-2 leads to embryonic lethality [13,15,18] with phenotypes similar to those observed in lem-3 mutants following IR, we analyzed whether baf-1, lem-2 or emr-1 mutants might be sensitive to DNA damage. We found that emr-1 mutants were weakly sensitive, while lem-2 mutants displayed moderate sensitivity to IR (Figure 7B). The temperature sensitive baf-1(op1639ts) mutant at permissive temperature was also hypersensitive to irradiation treatment (Figure 7A). To test whether baf-1 and lem-3 interact genetically, we built double mutants with both lem-3 alleles and analyzed their sensitivity to irradiation. Interestingly, the complete loss ofLEM-3 function (lem-3(mn153)) greatly increased the lethality of the baf-1(op1639) mutation, even in the absence of exogenous DNA damage. By contrast, the double mutant with the point mutation (lem-3(op444)) showed only a minor increase in lethality, likely due to residual activity of the LEM-3 (L659F) protein. This result suggests that when BAF-1 function is limiting, LEM-3 function becomes important even in the absence of DNA damage. Taken together, the phenotypes described in irradiated lem-3 mutants and animals depleted of either lmn-1 or baf-1, or co-depleted of emr-1 and lem-2 suggest a possible common cause of the observed molecular defects.

**Discussion**

Here we report our identification of lem-3 as a new gene required for proper DNA damage response in C. elegans. We isolated the lem-3(op444) mutation in a forward genetic screen for animals hyper-sensitive to ionizing radiation. DNA damage response mutants are often defective in DNA lesion-induced cell cycle arrest or apoptosis. As cell cycle arrest (Figure 4B and C) and apoptosis (Figure 4D) were normal in lem-3 mutants, we conclude that, unlike HUS-1 [25], CLK-2/RAD-5 [5,10], or CEP-1/p53 [35,36], LEM-3 is likely not involved in the initial signalling process following DNA damage.

**LEM-3 belongs to the GIY-YIG superfamily of nucleases**

Our bioinformatic analysis revealed the presence of a GIY-YIG domain in LEM-3, which can be found in various enzymes involved in DNA repair and recombination [37]. When tested in two different types of assays in vitro, wild-type LEM-3 was able to cleave DNA, while the activity of the L659F (op444) mutant was largely reduced (Figure 3B and C). Given these results, we postulate that LEM-3 is a novel endonuclease involved in DNA damage repair. The exact repair pathway (if any) LEM-3 might participate in remains however to be identified.

**LEM-3 is conserved in humans**

LEM-3 is evolutionary conserved. A report by Brachner et al. (co-submitted) demonstrates that the human LEM-3 homologue Ankle1 is capable of inducing DNA cleavage when forced to localize to the nucleus. A leucine to phenylalanine mutation corresponding to the mutation in lem-3(op444), however, failed to cause DNA damage under the same conditions. These observations suggest that the molecular function of LEM-3/Ankle1 might be conserved through evolution.

**Subcellular localization of LEM-3**

We found that YFP::LEM-3 localizes in a distinct foci-like structure clearly outside of the nucleus (Figure S2B). To date, we have no definitive explanation for this localization pattern, which is independent of DNA damage. We speculate that LEM-3 is present at low concentrations in the nucleus where it fulfills its function. At the onset of anaphase, microtubules pull the chromosomes into a poleward direction. In between, non-kinetochore microtubules form a bundled structure and assemble as part of the central spindle complex, also referred to as the “midbody”. The observed foci could be remnants of the midbody – we were however unable to confirm this hypothesis.

**Interaction between LEM-3 and BAF-1**

Whereas lem-3 mutants do not show any defects in the adult germ line following DNA damage, embryos generated from irradiated germ lines show major defects in chromosome segregation starting at the second cell division. This phenotype is reminiscent of embryos depleted of lmn-1, baf-1 or co-depleted of lem-2 and emr-1. We found that a reduction of BAF-1 function in lem-3 mutants causes high embryonic lethality even in the absence of any genotoxic insult. Our data suggest a model in which LEM-3 is required to support BAF-1 function. We postulate that, for a currently unknown reason,
requirement for BAF-1 function increases following DNA damage. What exact function BAF-1 plays following DNA damage and how LEM domain proteins contribute to this function remains to be determined. Loss of lem-3 function uncovers this requirement. This model is supported by the fact that also the other C. elegans LEM domain protein mutants lem-2 and emr-1 show a moderate and a weak sensitivity to IR, respectively.

**Materials and Methods**

**Strains and general procedures**

N2 Bristol strain [38] was used as the wild-type strain in all experiments. For mapping experiments the polymorphic strain CB4856 was used. All strains were kept on NGM agar plates seeded with Escherichia coli OP50 at 20°C unless otherwise stated.
**Mutant alleles.** `lem-3(op444)` (this study), `rad-1(mn155)` [7], `lem-3(tm 3468)` (this study), `rad-5(mn159)` [7], `xpa-1(ok639)` [19], `chu-80(ok681)` [31], `lem-2(bn1582)` (this study), `ems-1(gk119)` [39], `baf-1(t563)` [40].

**Transgenes.** `opd-257 [P_rad-5::3’unUTR_rad-5:3’UTR_lem-3]` [30], `opd309 [P_app::lem-3:3’unUTR_lem-3]` (this study), `opd334 [P_app::lem-3:3’unUTR_lem-3]` (this study), `opd383 [P_app::YFP::lem-3:3’unUTR_lem-3]` (this study), `rad32 [unc-119(+)/pie-1::GFP::H2B]` [22].

**Ethyl methane sulfonate (EMS) based screen**

Synchronized L4-stage wild-type worms [38] were subjected to 50 mM EMS (M0880 Sigma-Aldrich) and incubated at 20°C for 4 hours. The screening was performed according to the second scheme previously described [7]. But instead of UV-C light, a dose of 60 Gy was used for inflicting DNA damage.

**Irradiation**

An Isovolt Titan 160 with Isovolt 160 M2/0.4–3.0 (Scifert) and a Stratalinker UV crosslinker, model 1800 (Stratagene) were used to deliver the indicated doses.

**Embryonic lethality (X-ray and UV-C)**

Animals were irradiated 24 h post L4/adult molt, individualized on plates and allowed to lay eggs for 4 hours. Eggs were quantified; unhatched eggs were counted 24 h later, and the percentage of embryonic lethality was calculated. All assays were performed on 20°C except otherwise stated.

**Late Rad phenotypes**

Animals were synchronized (freshly hatched L1s) and irradiated. Phenotypes of those animals that had reached adulthood were quantified 3 days later using a dissection microscope. The Uncordinated (Unc) phenotype was scored on the basis of sluggish movement.

**Cisplatin treatment**

For embryonic survival assays synchronized animals (L4/adult molt) were exposed to different doses of cisplatin (Sigma-Aldrich) on agar plates by evenly distributing 1.2 ml of the corresponding concentration of cisplatin on a 10 cm plate. Animals were transferred to fresh plates 24 h post-treatment. Animals were allowed to lay eggs for 12 hr. Adults were then removed and eggs were counted. Unhatched eggs were quantified 24 h later, and the percentage of embryonic lethality was calculated.

**Germline apoptosis**

Staged animals (12 h after L4/adult molt) were irradiated with 60 Gy. Apoptotic corpses were quantified in the meiotic zone of the germ line at indicated time points, as previously described [41].

**Cell cycle arrest**

Staged animals (L4/adult molt) were irradiated. Germ lines were dissected at the indicated time points. Images were taken using an ORCA-ER digital CCD camera. The diameters of 10 nuclei (in focus) were measured and the average diameter of nuclei per germ line was calculated using ImageJ 1.40 g software (Wayne Rasband, http://rsb.info.nih.gov/ij).

**FM4-64 staining**

Embryos were dissected from gravid adults in M9 containing 64 μM FM4-64 (Invitrogen). The eggshell was cracked as previously described [42].

**Time lapse microscopy**

For time lapse microscopy, an Olympus BX61 was used equipped with a Retiga 2000R camera. Dissected embryos were put on a 2% agarose pad. Images were taken every 20 seconds at 2× binning. Pictures were processed using Openlab software (Improvision).

**Alignments**

Alignments were ClustaW alignment done with the Jalview software (Version 11.0).

**Transgenic lines**

Transgenic lines were obtained by microparticle bombardment in a Biolistic PDS-1000 (Bio-Rad) transformation, as previously described [22].

**DNA constructs**

PCR amplification was done with Plusion High-Fidelity DNA polymerase (Finnzymes). Oligonucleotide primers were synthesized by Microsynth.

**Primers**

- `npb-1` promoter
  - Pnpb-1Sbf_rev GATCCCTGCAAGTTATTGGTGTCATTT-CGGTTATTATGATTG
  - Pnpb-1Asc_rev CCGATCGCCGCGCCCTTGCGTGAACAAAAC-AAGGTTTATTAGAAATG
  - `lem-3` genomic plus 3’UTR
    - 3lem3Asc_hv GATCGGCCGCCCAGTGCTTCACCGAGCAATCAC-CAGCA
    - 3lem3Apal_hv GTGAGGCCCAGCAATTCAACCGACTAATAAGGT-
- `pmb-1` promoter
  - Ppmb-1Sbf_rev GATCCCTGCAAGTTATTGGTGTCATTT-CGGTTATTATGATTG
  - Ppmb-1Asc_rev CCGATCGCCGCGCCCTTGCGTGAACAAAAC-AAGGTTTATTAGAAATG
  - `lem-3` genomic plus 3’UTR
    - 3lem3Asc_hv GATCGGCCGCCCAGTGCTTCACCGAGCAATCAC-CAGCA
    - 3lem3Apal_hv GTGAGGCCCAGCAATTCAACCGACTAATAAGGT-

**Expression and enrichment of LEM-3**

The cDNA of LEM-3 WT and Δ→F mutant was cloned into a modified pFastBac1 vector (a kind gift of Petr Cejka) that contains an N-terminal MBP-tag. Baculovirus stocks were generated by a procedure described earlier and S99 cells (400 ml, 1×10^6 cells per ml) were infected following the User’s Manual (Gibco BRL). S99 cells expressing LEM-3 were harvested 48 h post infection by centrifugation, washed once in PBS and snap frozen. For enrichment, the pellet was resuspended in Column-buffer (25 mM Heps-KOH pH 7.4, 150 mM KCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA) containing 1 mM PMSF and a protease inhibitor cocktail (Roche), and sonicated. After centrifugation (18 000 g, 1 h, 4°C), the supernatant was incubated with Amylose resin (NEB). Subsequently, the column was washed with Column-buffer and MBP-LEM-3 was eluted (Elution buffer: 25 mM Heps-KOH pH 7.4, 150 mM KCl, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 10 mM Malto). Fractions containing MBP-LEM-3 were pooled, snap frozen and stored at −80°C.

**Non-specific endonuclease assay with supercoiled plasmid DNA**

80 ng of supercoiled pFastBac1 plasmid were incubated with increasing amounts of LEM-3 wild type or its op444 mutant in endonuclease buffer (25 mM Heps-KOH pH 7.4, 25 mM KCl, 1 mM MgCl₂). After incubation at 25°C for 1 h, the reaction was

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**ROLE OF C. ELEGANS LEM-3 IN DNA DAMAGE RESPONSE**

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terminated by the addition of 0.1% SDS, 14 mM EDTA and 0.1 mg/ml Proteinase K and incubation at 35°C for 10 min. 10% glycerol was added and the samples were separated on a 0.8% agarose gel for 45 min at 80 V. [43].

**PhiX174 non-specific endonuclease assay**

PhiX174 circular virion ssDNA (NEB) was incubated with LEM-3 wild type and L→F mutant in endonuclease buffer (25 mM Hepes-KOH pH 7.4, 60 mM KCl, 1 mM MgCl₂) for 1 h at 25°C (EcoRVIII, HhaI, HhaI and MboI) were incubated for 4 h at 25°C; The reaction was stopped by addition of 0.1% SDS, 14 mM EDTA and 0.1 mg/ml Proteinase K, and incubation at 35°C for 10 min. 10% glycerol was added and the DNA species were separated on a 0.8% agarose gel for 1 h at 70 V. Subsequently, the gel was stained with SYBR Gold and analyzed with a Imager (Typhoon).

**Supporting Information**

**Figure S1 Embryonic lethality of isolated mutants.**

Mutants were irradiated with the indicated doses and embryonic lethality was scored. Data shown represent the average number of dead embryos of five hermaphrodites ± S.D. (EPS)

**Figure S2 YFP::LEM-3 expression pattern.** (A) The transgene q6838 [popp-..:YFP::lem-3:3:UTR::lem-3] rescues the hypersensitivity of lem-3(op444) mutants. Data represent the average of three experiments ± S.D. The progeny of 10 worms were analysed for each experiment. (B) Representative DIC and fluorescence images of embryos expressing YFP::LEM-3: in green: YFP::LEM-3, in red: membrane staining with the dye FM4-64. (EPS)

**Figure S3 Complementation tests.** lem-3(op444) fails to complement (A) rad-1(tm155) and (B) lem-3(tm3468). F1 embryonic lethality after irradiation with 30 Gy was quantified. Data shown represent the average embryonic lethality of the progeny of 10 hermaphrodites. (EPS)

**References**