Supporting Online Material for

Ceramide Biogenesis Is Required for Radiation-Induced Apoptosis in the Germ Line of *C. elegans*

Xinzhu Deng, Xianglei Yin, Richard Allan, Diane D. Lu, Carine W. Maurer, Adriana Haimovitz-Friedman, Zvi Fuks, Shai Shaham, Richard Kolesnick*

*To whom correspondence should be addressed. E-mail: r-kolesnick@ski.mskcc.org

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Supporting Online Material

Methods

Generation of mutant alleles in the ceramide pathway: Worms carrying homozygous deletion alleles *hyl-1(ok976), hyl-2(ok1766), lagr-1(gk327), lagr-1(gk331), sphk-1(ok1097), sptl-1(ok1693), sptl-3(ok1927)* and *T10B11.2(ok1252)* were isolated at the *C. elegans* Gene Knockout Consortium (Oklahoma). *tag-38(tm470), asm-3(tm2384)* and *T27F6.6(tm2178)* were isolated at the National Bioresource Project of Japan. *hyl-1(ok976), lagr-1(gk327)* and *tag-274(ok1097)* were backcrossed three times into the wild-type, and the deletion boundaries were determined by genomic DNA sequence analysis.

Nematode strains and strain construction: *C. elegans* worms were cultured as described by Brenner (*1*) at 20˚C. Wild-type N2, *egl-1(n1084n3082), ced-3(n717), ced-4(n1162), ced-9(n1950)* and *cep-1(gk138)* strains were provided by the Caenorhabditis Genetics Center. *abl-1(ok171)* was isolated as described previously (*2*). The CED-4::GFP line (*opIs219*) was provided by Dr. Michael Hengartner. Double mutants *lagr-1(gk327);hyl-1(ok976), hyl-1(ok976);abl-1(ok171)*, *lagr-1(gk327);abl-1(ok171), lagr-1(gk327);opIs219,* and *lagr-1(gk327);sphk-1 (ok1097),* and the triple mutants *lagr-1(gk327);hyl-1(ok976);abl-1(ok171)* and *lagr-1(gk327);hyl-1(ok976);ced-3(n717),* were generated and confirmed by single-worm polymerase chain reaction (PCR) as described (*2*).

Primers for strain isolation and identification:

	mutant allele (MT)	wild-type allele (WT)
hyl-1 (ok976)	5’-ccacgtcacttcgtacagag-3’	5’-ccacgtcacttcgtacagag-3’
	/5’-gccaaagaggaatatagaagg-3’	/5’-cttgttgtgagcatcatatcg-3’
lagr-1 (gk327)	5’-agatggacaaattaacggcg-3’	5’-acacacatgtggaagtagaat-3’
	/5’-gtcatggacccaacaacaca-3’	/5’- cattcccatcgtctatgatct-3’
sphk-1 (ok1097)	5’-ggcagttgatgagaaaacgg-3’	5’-ggcagttgatgagaaaacgg-3’
	/5’-gtgctttgcatatcgattttct-3’	/5’-gataatcagtcatatgcatcgt-3’
abl-1(ok171)	5’-atatgcctccctcctttgct-3’	5’-ttttgctttcaactcgcctt-3’

	/5'-ttttgctttcaactcgcctt-3'	/5'-gggaatctcttcattctcgg-3'
ced-3(n717)	5' -cggcttctttctccacacttgtta- 3'	/5' -cggcttctttctccacacttgcta- 3'
	5' -ggcgcacaccccatttgcattg- 3'	/5' -ggcgcacaccccatttgcattg- 3'

Phenotypic analysis of mutant animals: To measure somatic cell death, extra cells in the anterior pharynx were counted in larvae at L3 and L4 stages of development as previously described (*3*). Adult hermaphrodite germ cell nuclei were stained with DAPI and counted manually from 3-dimensional images acquired at 20X magnification. Reproductive/behavioral studies (embryo survival, male mating efficiency, and brood size) in wild-type, *hyl-1(ok976), lagr-1(gk327),* and the double mutant *lagr-1(gk327);hyl-1(ok976)* were performed as described in (*4*). Rates of germ cell corpse removal were determined as described previously (*2*). Significant differences were not detected between wild-type, and *hyl-1(ok976)* and *lagr-1(gk327)* worms with respect to gonad size, number of germ cell nuclei or brood size, excluding the possibility that gonadal developmental defects might have affected corpse number (Table S2). Germ cell apoptosis, induced by exposure to IR produced by a radioactive Cs^{137} source, was identified as in (*5*) and confirmed with SYTO 12 staining. Imaging was performed with a Zeiss Axioplan 2 microscope equipped with Nomarski optics and standard epifluorescence filters.

C_{16}-ceramide on germ cell apoptosis: Fresh stock solution of 5 mM C_{16}-ceramide (Biomol) was prepared by dissolving desiccated C_{16}-ceramide in ethanol containing 2% dodecane (vehicle) at 37°C. To induce germ cell apoptosis, the stock solution was diluted in M9 buffer (22 mM KH_2PO_4, 42 mM $NaHPO_4$, 85.6 mM NaCl, 1 mM $MgSO_4$) to the desired concentration and microinjected into the posterior gonad of young adult hermaphrodites. Controls received vehicle ($1x10^{-5}$-$5x10^{-2}$ dilution in M9 buffer). Germ cell corpses in the posterior gonad were counted at the indicated times after injection. For restoration of radiation-induced germ cell apoptosis, worms were irradiated immediately following ceramide injection. For these studies, sub-lethal doses of C_{16}-ceramide were titrated against restoration of the radiation response; while 0.001 μM gonadal ceramide (calculated as below) was ineffective, complete restoration was achieved at 0.005 μM gonadal ceramide.

To calculate gonadal ceramide concentration, we performed 1190 microinjections into wild-type worms using borosilicate glass capillaries (World Precision Instruments, IB100F-4, inner capillary diameter 0.58 μm). The microinjection capillary was mounted onto a Narishige MMN-1 coarse manipulator and connected to an Eppendorf Transjector 5246 (injection pressure: 1176hPa, compensation pressure: 276hPa, and injection duration: 0.4s). Images were taken of the fluid meniscus in the glass capillary before and after microinjection at 10X using a QIMAGING RETIGA 2000R FAST Mono Cooled Digital CCD Camera mounted onto an Axiovert S100 inverted microscope (Zeiss). Using QCapture Pro software to measure fluid displacement, we calculated each microinjection volume to be ~0.1 nL. Based on data from Brenner (*1*) that the dimensions of the adult hermaphrodite approximate a 1.0 mm x 0.08 mm cylinder, the volume of the entire adult was calculated as ~5.0 nL and of one gonadal arm as ~1.0 nL. Using this value and an injection volume of 0.1 nL, the gonadal ceramide concentration was calculated after C_{16}-ceramide microinjection.

Northern blot: Total and polyA-enriched RNA were isolated from wild-type and *hyl-1(ok976), lagr-1(gk327)* and *sphk-1(ok1097)* worms using the RNeasy kit (QIAGEN) and the MicroPoly(A)Purist mRNA purification kit, respectively (Ambion). The template DNA with T7 promoter was generated by PCR from cDNA of wild-type, *hyl-1(ok976), lagr-1(gk327)* and *sphk-1(ok1097)*. The anti-sense RNA probes were generated using T7 polymerases. Probes were labeled with [α-^{32}P]UTP using Strip-EZ RNA kit (Ambion) according to the manufacturer's instructions.

Quantitative RT-PCR: Synchronized wild-type, *hyl-1(ok976)* and *lagr-1(gk327)* hermaphrodites were irradiated with 120Gy at the young adult stage. Worms were collected at 0, 3 and 9 hours after radiation and total RNA was extracted using RNeasy Kit (QIAGEN) according to the manufacturer's instructions. 1 μg of total RNA was reverse-transcribed using the Thermoscript RT-PCR system (Invitrogen) at 52°C for 1 hour. 20 ng of resultant was used in a Q-PCR reaction using iCycler (Biorad) and the TaqMan Assay System for *egl-1* (probe: 5' AGCCGATCTCGTAGCCGATGCTGCT 3'; primer pairs: 5'CATGTTCTACTCCTCGTCTCAGG 3'/5'GTCAT CGCACATTGCTGCTAG 3'), and the control standard *mec-7* (probe: 5' AGCAGAAGCAATCAGCAGTATCGTGCCAT 3'; primer

pairs: 5'CTTCTTCATGCCAGGATTCGC 3'/5'GTCAACCTCCTTCATGCTCATTC 3'). *ced-13* expression was evaluated in a SYBR Green reaction as described by Schumacher et al. (*6*). The expression level of each sample was normalized to a control standard. For studying C_{16}-ceramide effects on *egl-1* and *ced-13* transcription, young adult worms were soaked in C_{16}-ceramide for 6 hours, transferred to seeded NGM plates, and RNA isolated 5 hours after recovery.

Ceramide quantification and mitochondrial colocalization by confocal microscopy: Freeze-cracking and immunostaining of dissected gonads were carried out according to WormBook (*7*) with minor modifications. Staining was performed on poly-L-lysine coated slides. Gonads were fixed in 4% formaldehyde (Ted Pella) in 0.1M phosphate buffer for 1 h on ice. After fixation, slides were incubated in antibody solution (PBS containing 0.1% BSA, 0.5% Triton X-100, 1mM EDTA and 0.05% sodium azide) for 5 min on ice, then in block solution (antibody solution containing 2% BSA) for 2 h at room temperature, washed twice with PBS and incubated for 30 min with PBSB (PBS containing 0.5% BSA). Thereafter, gonads were stained overnight at 4°C with anti-ceramide mouse monoclonal IgM antibody [MID15B4, Alexis Biochemicals diluted 1:30 (v/v)] in antibody solution, followed by Cy-3 anti-mouse IgM secondary antibody (1:200 dilution) for 1 hour at room temperature. While there is one report that MID15B4 is not specific for ceramide as measured by dot blot (*8*), we have performed both immune thin layer chromatography and an ELISA assay and find a high level of specificity as it does not detect other sphingolipids including the ceramide precursor dihydroceramide (Yin and Kolesnick, unpublished). For evaluation of mitochondrial ceramide, Alexa Fluor 488 conjugated monoclonal mouse IgG anti-human OxPhos Complex IV subunit 1 (Invitrogen) antibody at a 1:180 dilution was co-incubated with primary ceramide antibody, and co-localization detected using a Leica TCS SP AOBS scanning confocal microscope (63x objective, 2.5x digital zoom) at the same instrument settings for each sample. A region containing >20 cells just distal to the loop was selected for evaluation of ceramide intensity. Average pixel intensity over the region was calculated with Volocity 4.1 software.

CED-4 translocation in germ line by confocal microscopy: For immunostaining, germ cells were released from dissected gonads by gently pressing the coverslip before freeze-cracking.

Germ cells were first stained with anti-CED-4 antibody (provided by Dr. Robert Horvitz) at a 1:15 dilution in PBSB at 4°C overnight, and then stained with an FITC-conjugated secondary antibody (ZYMED Laboratories Inc.) for 1 hour at room temperature. Germ cells were subsequently incubated with anti-Ce-lamin antibody (1:100 dilution) (provided by Dr. Yosef Gruenbaum) for 1 hour followed by a 1-hour incubation at room temperature with secondary antibody (Alexa Fluor 594 goat anti-rabbit IgG, Invitrogen). CED-4 and lamin staining were viewed using a Zeiss fluorescence microscope, and fields of interest were identified and images acquired using a Leica TCS SP AOBS scanning confocal microscope (100x objective, 4x digital zoom) at the same instrument settings for each sample. Quantitation of fluorescence intensity at the germ cell nuclear membrane was performed using Metamorph software (version 7). Specificity of the anti-CED-4 antibody was confirmed using *ced-4(n1162)* worms that lack CED-4 (Fig. S5). To visualize CED-4 and mitochondria co-localization in live worms, *opls219* L1 larvae were placed on NGM (Nematode Growth Medium) agar plates containing 1 μg/ml Rhodamine B (Invitrogen) allowing mitochondria staining. The young adult worms were treated with 120Gy and the distribution of GFP (CED-4) and Rhodamine B (mitochondria) was imaged at 36 hours post-irradiation. We acquired z-stack images (63x objective, 2x digital zoom) at 1 micron intervals over an area containing one-third of the length of the distal arm adjacent to the gonadal loop. Colocalization of CED-4 and mitochondria was calculated with Metamorph software (version 7).

Statistics: Statistical differences between groups were calculated by Student's two-tailed *t* test.

Gene Product (s)	*C. elegans* Gene	WormBase Protein ID	Human ortholog		Mouse ortholog	
			Uniprot Accession Number	Identity	Uniprot Accession Number	Identity
Serine palmitoyltransferase 1	*sptl-1*	CE37749	O15269	50.7	O35704	51.6
Serine palmitoyltransferase 2	*sptl-3*	CE31996	O15270	48.6	P97363	48.6
Ceramide synthase	*hyl-1*	CE27675	Q96G23	32.7	Q924Z4	33.3
	hyl-2	CE34329	Q9HA82	29.3	Q9D6J1	29.9
	lagr-1	CE20184	P27544	30.0	P27545	29.4
Acid ceramidase	F27E5.1	CE01562	Q13510	34.2	Q9WV54	33.5
Sphingosine kinase	*sphk-1*	CE35833	Q9NYA1	29.6	O88886	30.2
Sphingosine phosphate lyase 1	*spl-1*	CE20348	O95470	37.8	Q8R0X7	38.3
	tag-38	CE06695	O96470	38.5	Q8R0X7	39.1
Ceramide kinase	T10B11.2	CE18241	Q8TCT0	25.8	Q8R0Q7	26.6
Acid sphingomyelinase	*asm-1*	CE30230	P17405	32.8	Q04519	32.0
	asm-2	CE31674	P17405	32.6	Q04519	34.3
	asm-3	CE39794	P17405	35.4	Q04519	35.5
Neutral sphingomyelinase	T27F6.6	CE34214	O60906	36.3	O70572	36.8

Table S1. Sequence comparison of proteins in the ceramide metabolic pathway.

Alignments for each class were performed using the ClustalW algorithm.

Table S2. Reproductive/behavioral control studies

	wild-type	*hyl-1(ok976)*	*lagr-1(gk327)*
Rate of germ cell corpse removal (min) ± SEM	31.2 ± 4.5 (10)	34.5 ± 2.8 (10)	40.6 ± 5.5 (9)
Germ cell nuclei ± SEM*	366.6 ±14.6 (10)	327.5 ±15.9 (10)	325.2 ±17.4 (10)
% Embryo survival ± SEM	97.5 ±4.2 (1019)	95.5 ±3.8 (1010)	95.8 ±4.0 (1259)
% Mating efficiency†	91.0	89.5	75.5
Brood size ± SEM	227.8 ±7.7 (22)	209.4 ±10.9 (20)	225.0 ±8.1 (25)

Data are presented as mean±SEM. (n) = number of animals. *The number of DAPI stained nuclei in one side of the distal gonad. †Assays for mating efficiency were performed in duplicate (*9*). For each strain, 16 males were mated with 16 late L4 stage *unc-51* hermaphrodites for 24 hours and then scored for cross progeny (non-*Dpy* progeny) and self progeny. Mating efficiency = cross progeny/total progeny.

Table S3. Loss-of-function mutations of ceramide synthases do not affect somatic cell death.

Genotype	Extra cells in the pharynx (n)
wild-type	0.05 ± 0.22 (20)
ced-3(n717)	12.1 ± 1.1 (15)
lagr-1(gk327)	0.1 ± 0.4 (30)
hyl-1(ok976)	0.17 ± 0.38 (30)
lagr-1(gk327); hyl-1(ok976)	0.07 ± 0.25 (30)
hyl-2 (ok1766)	0.25 ± 0.48 (57)

Extra cells in the anterior pharynx were counted in L3-L4 larvae. The apoptosis-defective *ced-3(n717)* was used as a positive control. Data are presented as mean±SD. (n) = number of animals.

Fig. S1A.

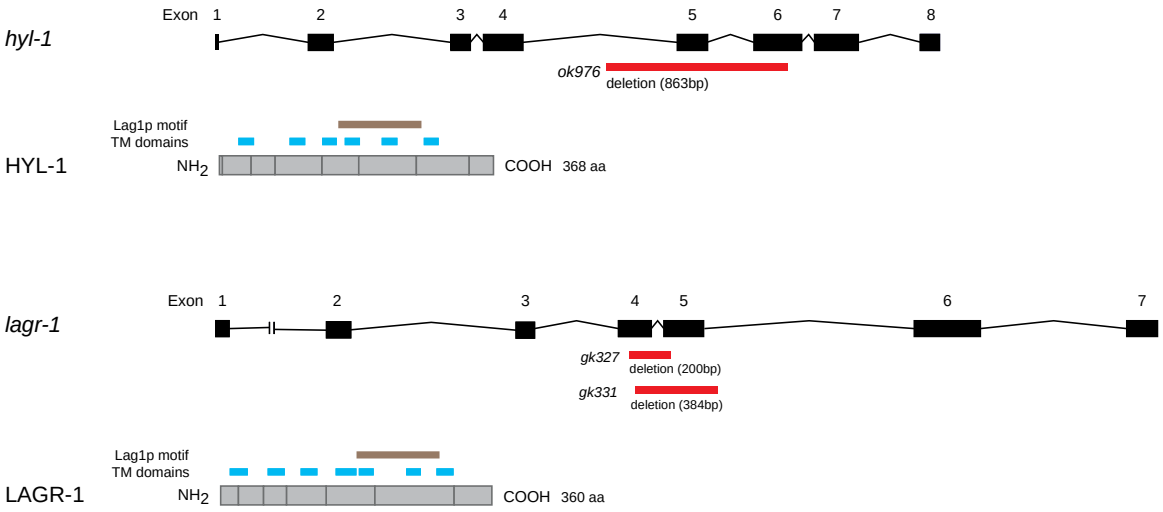

Fig. S1B.

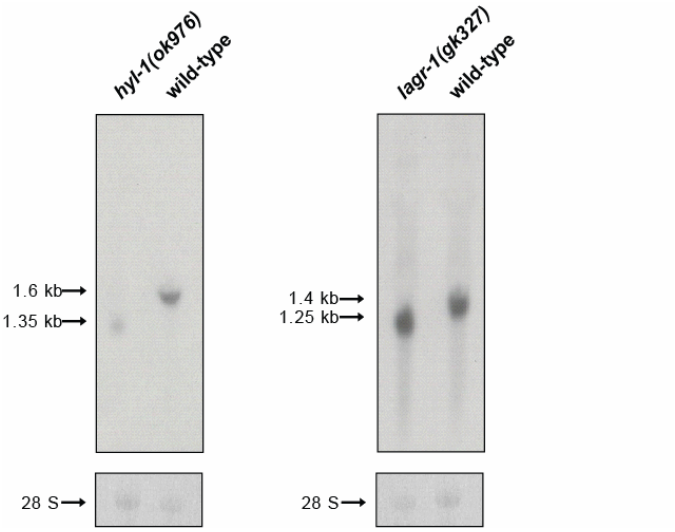

Figure S1C.

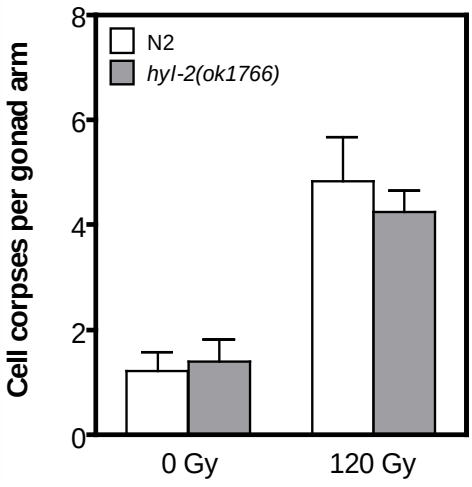

Fig. S1. Deletion alleles of *hyl-1(ok976), lagr-1(gk327)* and *hyl-2(ok1766)*. (**A**) Structure diagrams of *hyl-1* and *lagr-1*. Numbered boxes indicate exons, and introns are represented as lines. The genomic regions of each deletion are marked in red. In the diagram of HYL-1 and LAGR-1, transmembrane domains are marked in blue and Lag1p motifs are marked in brown. In *hyl-1(ok976)*, an 863 bp fragment from the *hyl-1* gene locus is deleted, eliminating exon 5 and 67% of exon 6, resulting in loss of the Lag1p coding sequence. In *lagr-1(gk327),* a 200 bp deletion from exon 4-5 corresponding to 150 bp of *lagr-1* cDNA results in a frame-shift that similarly disrupts the Lag1p motif. (**B**) Northern-blot analysis of *hyl-1(ok976)* and *lagr-1(gk327).* Northern blotting was performed using 3 μg of poly(A)-enriched RNA which were hybridized with RNA transcripts corresponding to cDNA bps 5-480 for *hyl-1* and bps 1-260 for *lagr-1*. 28 S rRNA was used as a loading control. In wild-type worms, a ~1.6 kb *hyl-1* transcript and a ~1.4 kb *lagr-1* transcript were detected, whereas a smaller ~1.35 kb transcript was observed in *hyl-1(ok976)* and a ~1.25 kb transcript in *lagr-1(gk327)*. (**C**) Germ cell corpses in *hyl-2(ok1766)* after 120Gy. Data (mean±SEM) are collated from 10-15 worms/group.

Fig. S2A.

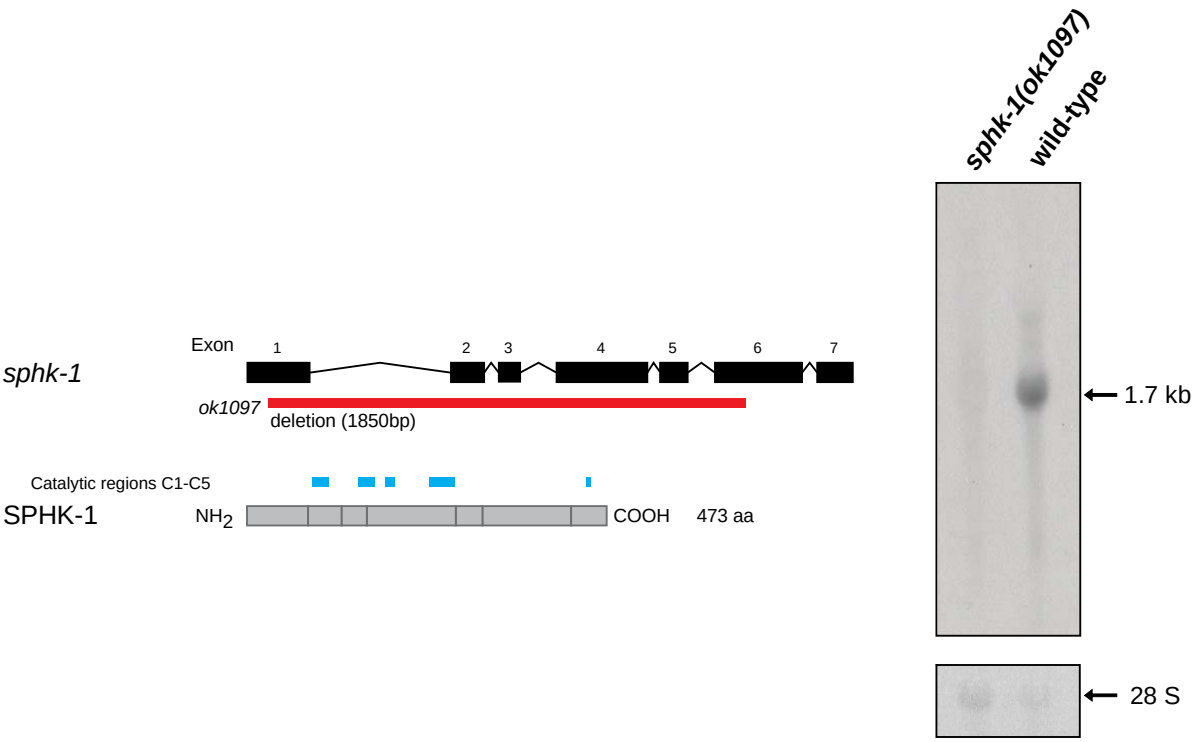

Fig. S2B.

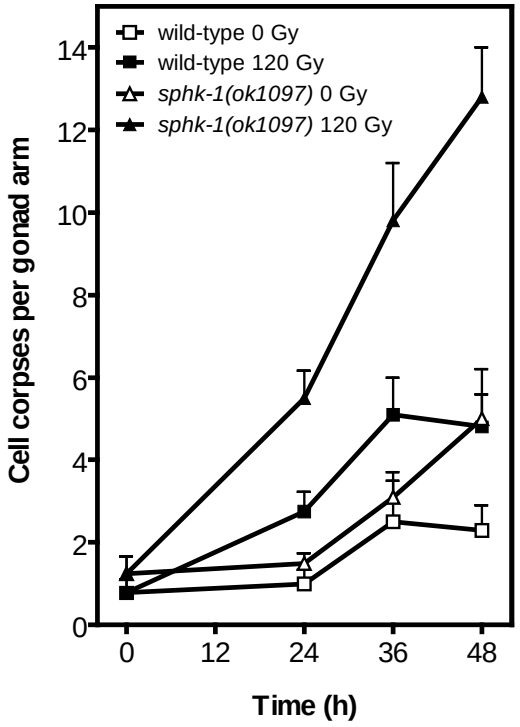

Fig. S2. *C. elegans* sphingosine kinase (SPHK) deletion allele *sphk-1(ok1097).* (**A**) *sphk-1* contains 7 exons. Four alternative splice variants of *C. elegans sphk* have been reported in Wormbase. *sphk-1* C34C6.5a is 1422 bp in length and *sphk-1* C34C6.5b1, b2 and b3 are 1272 bp in length with a shorter exon 1 (150bp). The region of deletion is marked in red. The catalytic regions of SPHK-1 are marked in blue. Northern blot analysis was performed using 3 µg of poly(A)-enriched RNA from wild-type and *sphk-1 (ok1097).* The blots were hybridized to an mRNA probe corresponding to bps 3-262 of *sphk-1* C34C6.5a coding sequence. 28 S rRNA was used as a loading control. While the wild-type SphK gene manifests a 1.7 kb transcript by northern blot analysis, *sphk-1(gk274)* manifests a 1.8 kb deletion from the gene locus corresponding to *sphk-1* exons 2-7, and displays no transcript. (**B**) Germ cell corpses in *sphk-1 (ok1097)* after 120Gy. Data (mean±SEM) are collated from 10-15 worms/group.

Fig. S3.

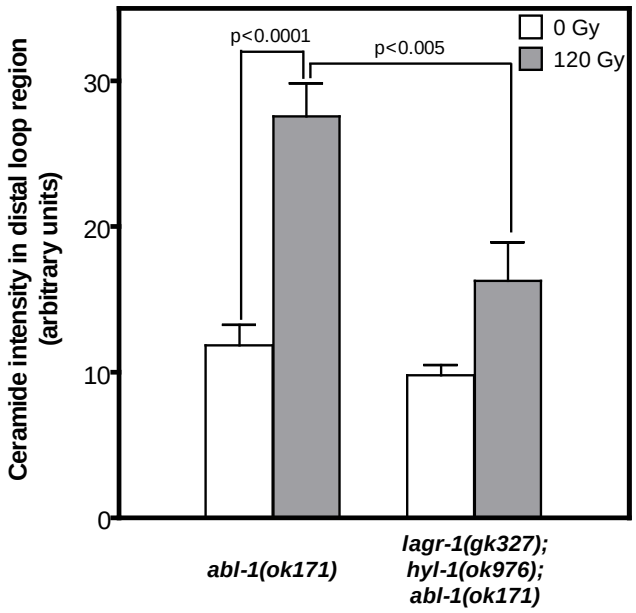

Fig. S3. Radiation-induced ceramide increase is abrogated in a CS mutant background. Gonads were dissected from young adult *abl-1(ok171)* and *lagr-1(gk327);hyl-1(ok976);abl-1(ok171)* at 24 hours after 120Gy and stained with anti-COX-IV antibody, anti-ceramide antibody and DAPI as in Fig. 3A. Gonadal ceramide intensity was quantified using Metamorph software. Data (mean±SEM) are from ≥14 gonads/group.

Fig. S4.

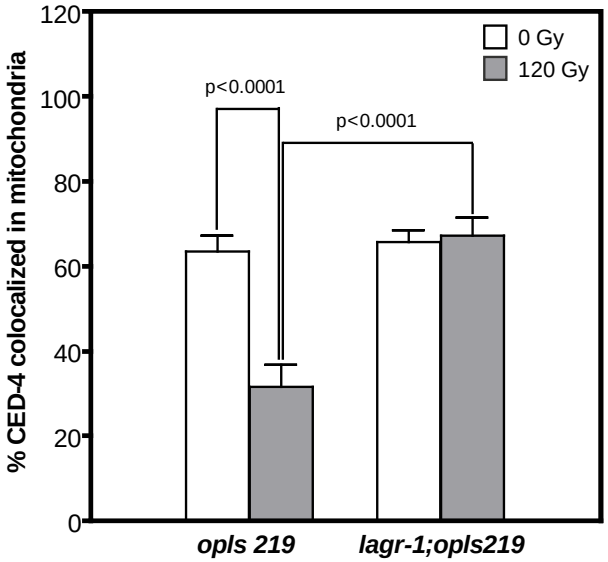

Fig. S4. *C. elegans* CS mutations block radiation-induced CED-4 reduction in mitochondria. L1 larvae of *opls219* and *lagr-1(gk327);opls219* were cultured on Rhodamine B-containing agar plates to the young adult stage, and then exposed to 120Gy. ~5 serials Z-stack images from each animal were evaluated for co-localization of CED-4 and mitochondria. Data (mean±SEM) are collated from ≥8 worms/group.

Fig. S5.

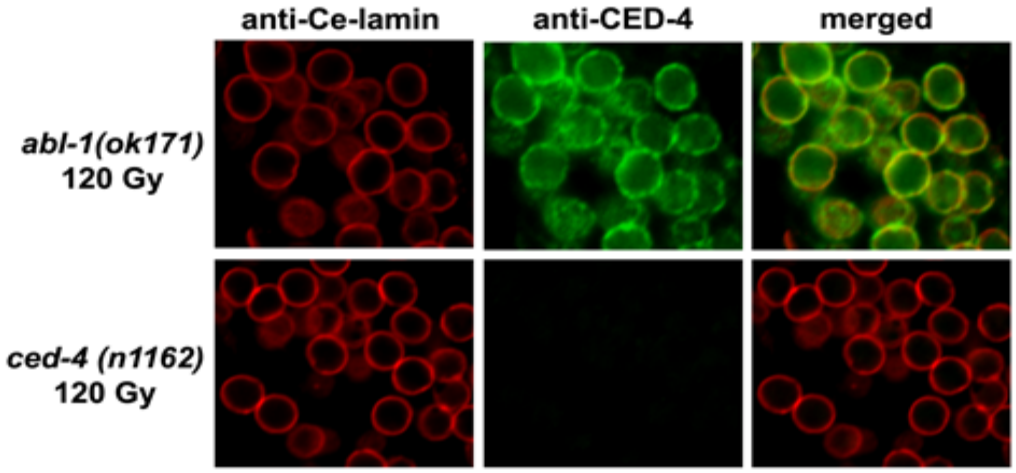

Fig. S5. Germ cells were released from gonads of young adult *abl-1(ok171)* and *ced-4(n1162)* at 24 hours after 120Gy, and stained with anti-Ce-lamin (red) and anti-CED-4 (green) as in Methods. CED-4 localizes to nuclear membranes in *abl-1(ok171*), but is absent in *ced-4(n1162)*.

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