

Differential regulation of DNA damage response activation between somatic and germline cells in *Caenorhabditis elegans*

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The germline of *Caenorhabditis elegans* is a well-established model for DNA damage response (DDR) studies. However, the molecular basis of the observed cell death resistance in the soma of these animals remains unknown. We established a set of techniques to study ionizing radiation-induced DNA damage generation and DDR activation in a whole intact worm. Our single-cell analyses reveal that, although germline and somatic cells show similar levels of inflicted DNA damage, somatic cells, differently from germline cells, do not activate the crucial apical DDR kinase ataxia-telangiectasia mutated (ATM). We also show that DDR signaling proteins are undetectable in all somatic cells and this is due to transcriptional repression. However, DNA repair genes are expressed and somatic cells retain the ability to efficiently repair DNA damage. Finally, we demonstrate that germline cells, when induced to transdifferentiate into somatic cells within the gonad, lose the ability to activate ATM. Overall, these observations provide a molecular mechanism for the known, but hitherto unexplained, resistance to DNA damage-induced cell death in *C. elegans* somatic cells. We propose that the observed lack of signaling and cell death but retention of DNA repair functions in the soma is a *Caenorhabditis*-specific evolutionary-selected strategy to cope with its lack of adult somatic stem cell pools and regenerative capacity.

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DNA damage generation triggers a prompt set of cellular events known as the DNA damage response (DDR) to arrest proliferation of cycling cells and control cell death (checkpoint signaling function) and to repair DNA damage (DNA repair function). The DDR signaling cascade is highly conserved among species. ATM (ataxia-telangiectasia mutated) and ATR (ATM and Rad3-related; ATM-1 and ATL-1, respectively, in *Caenorhabditis elegans*) are apical kinases of the DDR pathway that phosphorylate a large number of substrates in response to DNA damage.¹ Activation of apical kinases depends on DNA damage sensors. The MRE11-RAD50-NBS1 (MRN; MRE-11, RAD-50 in *C. elegans*) complex localizes to sites of DNA double-strand breaks and it is necessary for ATM activation, while the sensing complex RAD9-HUS1-RAD1 (911; HUS-1, MRT-2 in *C. elegans*) and RPA (RPA-1 in *C. elegans*) are required for ATR activation. Through the activation of downstream kinases CHK1 and CHK2, signaling is amplified down to effectors molecules, such as p53 (CEP-1 in *C. elegans*). Although activation of adequate DNA damage signaling and repair systems depends on the type of lesion and the cell-cycle phase in which it occurs,² less clear is how DDR networks function in different cellular contexts. The small size of *C. elegans* allows the analysis of different cell types at once. Its adult body is made of a finite number (959) of somatic non-proliferating cells including neurons (302 cells), muscle (111 cells), intestine

(34 cells) and epidermis (213 cells) cells. Within the body built of somatic cells (Figure 1a) a large reproductive system is enclosed. The gonad of the reproductive system consists of two symmetrical U-shaped arms connected with a common uterus. Each gonad arm is organized in a distal to proximal manner. At the distal end of the gonad, germ cells undergo mitosis, they then pass through 'transition zone' (first stages of meiotic prophase), followed by pachytene and diplotene meiotic subphases, to give rise to oocytes arrested in diakinesis (Figure 1b). Pioneering studies in *C. elegans* reported that upon exposure to ionizing radiations (IR) checkpoint arrest is observed in the mitotic zone and apoptosis in the pachytene subphase of meiosis of the germline (Figure 1b), while no morphological signs of cell death or apoptosis in any cell of the somatic body of *C. elegans* are detectable.³

At present, the molecular mechanisms responsible for the observed lack of a DNA damage-induced cellular response and resistance to cell death in somatic cells are unknown. We monitored DNA damage generation and the signaling events that follow IR in individual cells of an entire animal.

Results

Phospho Serine/Threonine Glutamine (pS/TQ) signal is not detectable in the soma of *C. elegans*. ATM and ATR protein kinases phosphorylate their substrates preferentially

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Abbreviations: DDR, DNA damage response; DSB, double-strand break; IR, ionizing radiation; pS/TQ, phospho Serine/Threonine Glutamine; NHEJ, non-homologous end joining; NER, nucleotide-excision repair; synMuv, synthetic Multivulva; HDAC, histone deacetylase; VPA, valproic acid

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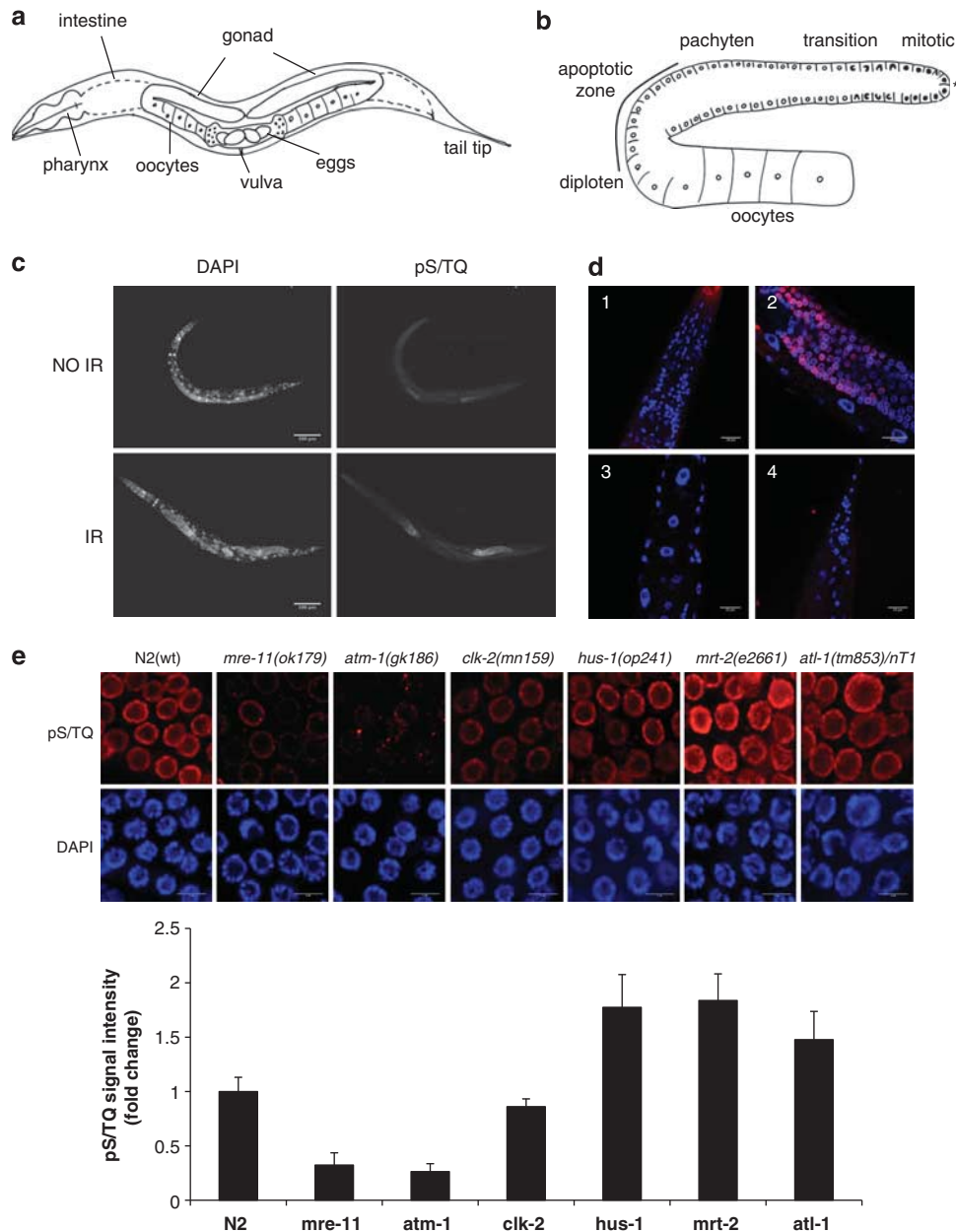


Figure 1 Somatic post-mitotic cells of *C. elegans* do not show IR-induced pS/TQ signals upon IR. **(a)** Schematic representation of *C. elegans* anatomy, outlining the main structures in the body: pharynx, intestine and components of the reproductive system. **(b)** Schematic representation of the germline. Distinct phases of germ cells differentiation into oocytes are shown. The asterisk indicates the distal end of the germline. Cell death occurs only in the outlined ('apoptotic zone') pachytene subphase of meiosis. **(c)** pS/TQ staining of intact wild-type (N2) worms treated with 180 Gy of IR and stained 1 h post treatment. Scale bar, 100 μ m. **(d)** Close up and merged (blue: DAPI; red: pS/TQ) images of different regions of worm body (1: head, 2: mid-body, 3: large nuclei of the hypodermis, 4: tail), showing the absence of pS/TQ signal in somatic cells. Scale bar, 20 μ m. **(e)** Representative images of pS/TQ staining of meiotic nuclei of the indicated genotypes after IR treatment. Histogram represents fold change in the mean intensity of the pS/TQ signal, normalized to the signal intensity of irradiated wild-type (N2) worms. At least 10 gonads per strain were analyzed. Error bars represent the mean \pm S.E.M. Scale bar, 5 μ m

on serine or threonine amino acidic residues preceding a glutamine.⁴ This substrate specificity is evolutionary conserved having been observed in a wide variety of organisms ranging from yeasts to mammals. We used a phospho-specific antibody raised against the phosphorylated consensus target site of ATM and ATR kinases (pS/TQ) to probe for the activation of the kinase activity of ATM and ATR in the whole body of a worm by immunofluorescence.

This tool, never reportedly used before in *C. elegans*, has been extensively validated in other systems.^{5–8}

We exposed wild-type adult animals to IR and 1 h later we immunostained whole animals with the anti-pS/TQ antibody. We observed an IR-induced pS/TQ signal in the germline, while such a signal was not detectable in any somatic cell, despite individual cells scrutiny (Figures 1c and d). Strikingly therefore, and differently from the germline, IR does not

induce activation of apical DDR kinases in the soma of *C. elegans*.

pS/TQ signal can be generated by the activity of either ATM or ATR kinase, or both. In order to distinguish their potential individual contribution to signal generation, we employed the germline to test a panel of worm mutants defective in either the ATM pathway (*atm-1*, *mre-11*)^{9–11} or the ATR pathway (*atl-1*, *mrt-2*, *hus-1*, *clk-2*)^{9,12–14}. We performed whole-worm immunofluorescence on checkpoint mutants and observed that pS/TQ signal in the germline is strongly reduced in worms impaired in the ATM pathway, in which either MRE-11 or ATM-1 is inactivated. It is only mildly changed in *clk-2* mutants, and it is robustly detectable in ATR pathway mutants (Figure 1e) – in these strains the observed increased pS/TQ signal may be the consequence of reduced competition for access to DNA lesions of the ATM complex. ATM-1 dependency of the pS/TQ signal was confirmed by RNAi against *atm-1* (Supplementary Figure S1A) and the observed radiosensitivity of germ, but not somatic, cells in *atm-1(gk186)* mutant worms demonstrates ATM-1's functional role in this organism (Supplementary Figure S1B). Though IR is the best known activator of ATM-1 signaling pathway, we exposed worms to additional DNA-damaging drugs throughout their development, to test whether prolonged and different forms of DNA damage could induce DDR in somatic cells. Wild-type worms exposed to either cisplatin or etoposide, did not show activation of DDR in their soma, while pS/TQ signal was observed in response to these drugs in the germline (Supplementary Figure S1C).

Interestingly, also other two *Caenorhabditis* species, *C. briggsae* and *C. brenneri*, do not show pS/TQ signal upon IR in their soma, but exclusively in the germline (Supplementary Figure S1D).

Overall, these results indicate that under these conditions, this immunological reagent in *C. elegans* preferentially recognizes ATM-1 dependent signals, which are detectable in the germline but not in the soma.

Lack of DDR activation in the soma despite demonstrated DNA damage generation. Lack of pS/TQ signal in the soma may be due to resistance of somatic cells to DNA damage generation by IR. TUNEL assay is routinely used in cell biology to detect the extensive DNA damage associated with the apoptotic process. This technique is based on the ability of terminal deoxynucleotidyl transferase (TdT) to elongate exposed 3' DNA ends by incorporating fluorescently-labeled nucleotides. DNA substrates for TdT are generated also upon exposure to DNA-damaging agents, including IR. We thus modified and exploited TUNEL assay to detect DNA damage in individual cells of intact adult worms. We exposed wild-type worms to IR or treated them with DNase I, as an *in vitro* control (Supplementary Figure S2A). Following TUNEL assays and their quantification, we observed that germline and somatic cells display very similar levels of detectable DNA damage upon IR exposure (Figure 2a, Supplementary Figure S2B). Thus, lack of DNA damage generation cannot be invoked to explain the observed profound lack of DDR signaling in the soma.

As an additional independent readout of checkpoint activation, we tested whether *egl-1*, a transcriptional target

of *cep-1* responsible for IR-induced apoptosis in the germline, is induced in the soma upon IR. To obtain RNA from somatic tissues only, we utilized the *glp-1(q224)* worm strain. *glp-1(q224)* mutants under restrictive temperature do not develop a germline and consists of somatic post-mitotic cells only¹⁵ (Supplementary Figure S2C). We performed quantitative RT-PCR (qRT-PCR) for *egl-1* transcript and observed that while *egl-1* is induced in wild-type worms upon IR, its induction is undetectable in germlineless *glp-1* worms (Figure 2b). We conclude that *C. elegans* is unable to activate DDR signaling in the adult soma despite the generation of DNA damage.

Next, we hypothesized that lack of DDR signaling may result from prompt and efficient DNA repair. We thus probed irradiated worms carrying mutations in *lig-4* and *cku-70*, the essential components of the non-homologous end joining (NHEJ) repair pathway. These irradiated mutants behaved like wild-type worms and did not show any pS/TQ signal in the soma (Figure 2c).

Alternatively, we considered that DDR activation might be slow in somatic cells. Therefore, we extended in time our analyses following irradiation. However, pS/TQ signal was not detected in somatic cells even 24 h post irradiation (Supplementary Figure S2D).

Finally, we asked whether worms undergoing development through the four larval stages (L1–L4) have the ability to activate DDR upon DNA damage. Surprisingly, upon IR exposure, we could not detect pS/TQ signal in any of the different larval stages (Figure 2d), aside from the already-described germline signal. Therefore, DDR activity is suppressed in somatic cells of developing worms too.

DDR factors are not expressed in the soma of *C. elegans*.

Lack of detectable DDR activity in the soma of adult worms could be due to an interruption in the signaling cascade. DDR requires the activities of DNA damage sensing factors.¹⁶ To study their expression in individual somatic cells, we used worm strains that stably express DDR sensors fused to a fluorescent protein under their endogenous transcriptional promoters. We examined the expression of DNA damage sensing proteins: HUS-1::GFP, RPA-1::YFP and MRE-11::YFP. We observed that while HUS-1::GFP and MRE-11::YFP are both expressed in the adult germline tissue, their expression is not detectable in any somatic cell (Figure 3a). Differently, when we studied the expression of RPA-1::YFP, we found it expressed in all worm cells. This is likely to reflect its known involvement also in nucleotide-excision repair in the soma.¹⁷

As DDR signaling pathways converge on p53, we analyzed the expression of *cep-1* gene fused to GFP under its endogenous promoter in the soma.¹⁰ CEP-1 expression was suppressed in the soma except a small subset of cells in the pharynx (Supplementary Figure S3A), as previously described.¹⁸ These CEP-1 expressing cells, nevertheless, did not show pS/TQ signal upon IR (Supplementary Figure S3A).

In sum, the available evidence suggests that the entire DDR signaling cascade, including DNA damage sensors (MRE-11, HUS-1), apical kinase (ATM-1) and effectors (CEP-1), is suppressed in the worm soma.

As key DDR proteins are not detectable in the soma, we tested if their repression was regulated at the transcriptional level. We performed qRT-PCR to monitor the

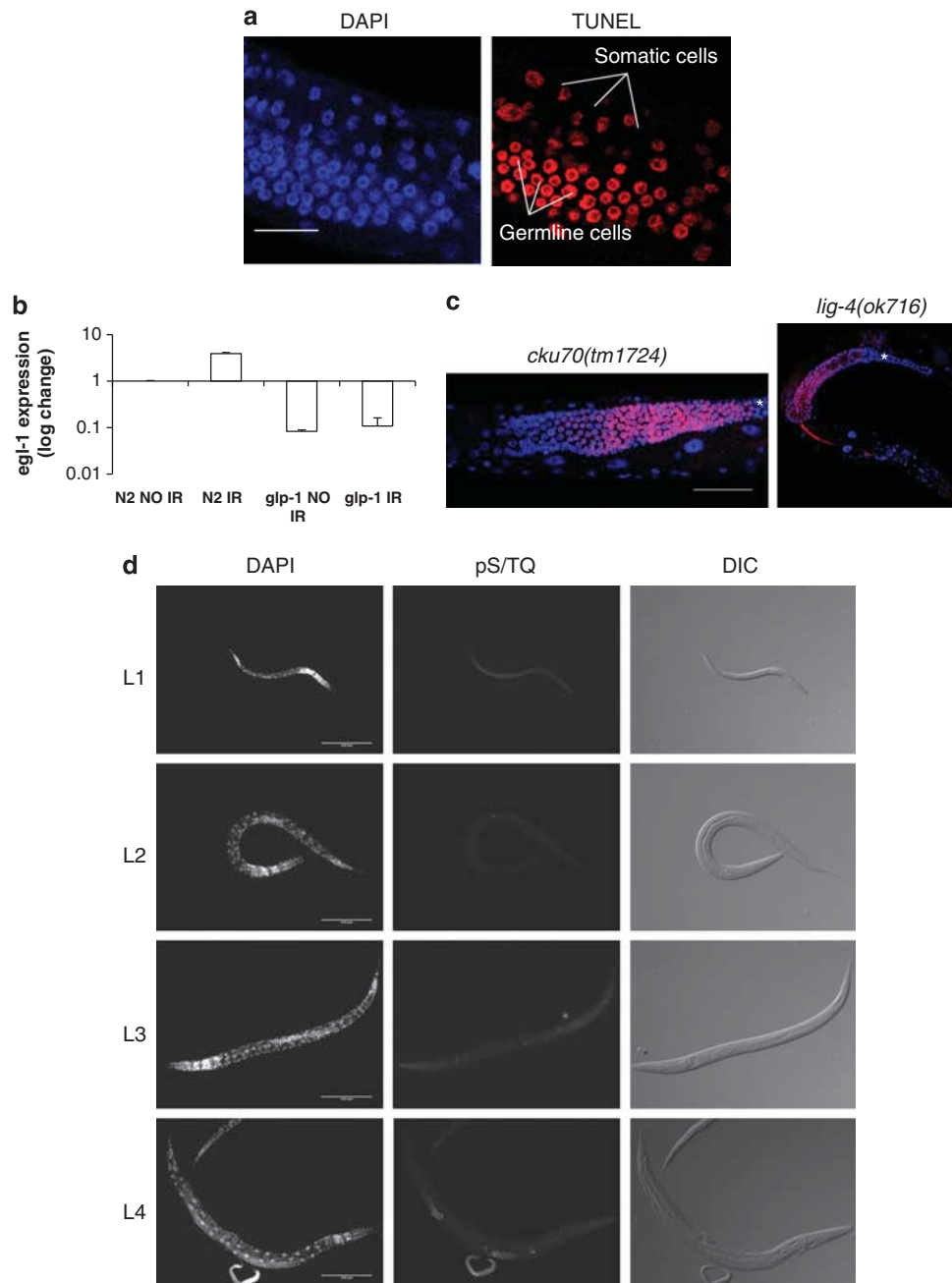


Figure 2 Both somatic and germline cells show the presence of DNA damage upon IR, but only germline cells show DDR activity. (a) Mid-body of wild-type (N2) worms showing both germline and somatic cells, stained by TUNEL assay for the presence of DNA breaks. Scale bar, 20 μ m. (b) qRT-PCR performed on wild type (N2) and (*glp-1*) IR-treated worms, with primers specific for *egl-1* gene, showing its induction only in the irradiated wild-type worms. mRNA levels were normalized to the value of wild type not irradiated worms. Error bars represent the mean \pm S.D. for a representative experiment performed in triplicate. (c) pS/TQ staining of *cku-70(tm1724)* and *lig-4(ok716)* IR-treated mutants show DDR signaling only in the germline. The asterisk indicates distal end of the germline. (d) DIC images and pS/TQ staining of four distinct developmental larval stages (L1–L4) of irradiated wild-type (N2) worms. Scale bar, 200 μ m

expression of different DDR genes (*hus-1*, *mre-11*, *atm-1*, *atl-1*, *clk-2*, *hsr-9*, *chk-1*, *chk-2* and *cep-1*) in wild type and *glp-1* mutant worms. *gld-1*, a gene expressed exclusively in the germline, was used as a control for the lack of germline. We discovered that DDR genes involved in DNA damage checkpoint signaling are transcriptionally silenced in the soma (Figure 3b). Differently, we found that DDR genes involved in

NHEJ DNA repair pathway (*lig-4* and *cku-70*) are not repressed in the soma, though some are detected at lower levels in germlineless than wild-type worms, as judged by qRT-PCR (Figure 3b).

Therefore, in the soma of *C. elegans* the expression of the DDR signaling apparatus is suppressed, while the expression of the DNA repair machinery is maintained.

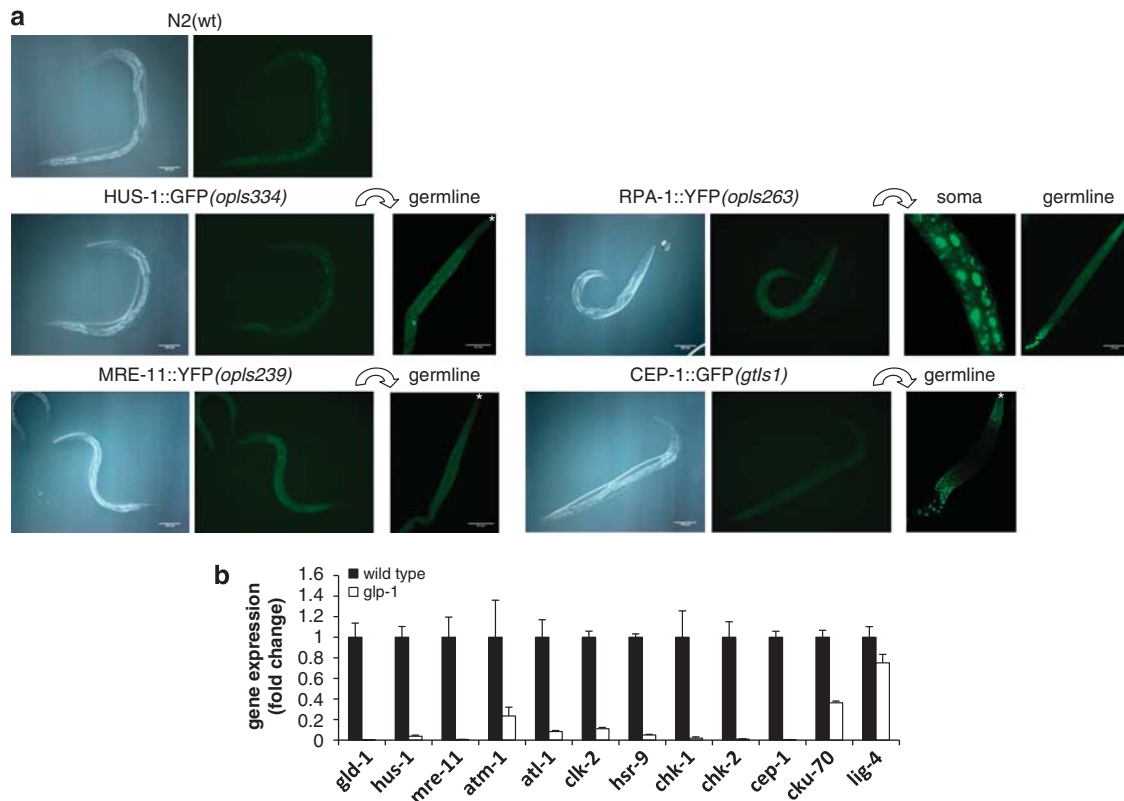


Figure 3 DDR genes are not expressed in the worm soma. (a) Differential interference contrast and fluorescent microscopy of wild type and transgenic animals expressing: HUS-1::GFP(*opls334*), RPA-1::YFP(*opls263*), MRE-11::YFP(*opls239*) and CEP-1::GFP(*gtls1*), RPA-1 is expressed in all worm cells, while MRE-11, HUS-1 and CEP-1 are expressed in germline cells only (close ups of the somatic cells and germline are indicated by a white arrow). The asterisk indicates distal end of the germline. Scale bar for whole-worm images 100 μ m, and 50 μ m for close ups. (b) qRT-PCR performed on wild type (N2) and (*glp-1*) worms with primers specific for the indicated DDR genes. Error bars represent the mean \pm S.D. for a representative experiment performed in triplicate

The DNA repair machinery is functional in the soma of *C. elegans* despite the absence of detectable ATM signaling. As DDR signaling factors such as ATM, MRE-11 and p53 have been shown to have a role also in DNA repair,^{19–22} we tested whether DNA repair is effective in the somatic compartment by setting up a protocol for long PCR product amplification, to detect DNA damage in the worm. The probability that a DNA polymerase encounters a DNA break increases with the size of DNA amplicons, thus the reduction/absence of a PCR product reveals the presence of DNA breaks in a DNA template. We arbitrarily chose a genomic locus of approximately 11 kb as a template to monitor DNA damage, and we used a smaller 1 kb amplicon within the same genomic locus (thus less sensitive to DNA breaks), as a control for the quantity of DNA used (Supplementary Figure S4A). Worms exposed to IR, unlike controls, showed absence of 11 kb products, indicating the presence of DNA damage (Supplementary Figure S4B).

To investigate DNA repair in soma only, we utilized fully somatic adult *glp-1(q224)* worms. *glp-1(q224)* mutants were treated with increasing doses of IR and the presence of DNA damage was determined by PCR immediately after the treatment (T0) or 24 h later (T24). Increasing doses of IR lead to a decrease in the quantity of the 11 kb PCR product, indicating the generation of DNA damage. Twenty-four hours

after irradiation, however, the yield of the 11 kb PCR product had substantially recovered in all of the irradiated conditions (Figure 4a) as shown by the gel and histograms showing the quantifications.

Next, we asked whether NHEJ pathway, in many systems responsible predominantly for the repair of DNA breaks in G0/G1 cells, has a role in the repair of DNA damage in worm soma. We generated fully somatic *lig-4* deficient animals by crossing them into germlineless *glp-4* background. *glp-4(bn2) lig-4(ok716)* mutant was exposed to IR and the presence of DNA damage was determined by PCR immediately after the treatment (T0) or 24 h later (T24). *lig-4* deficient worms showed reduced, but not abolished, DNA repair ability (Figure 4b), indicating that *lig-4* has a role but other DNA repair mechanisms are also active in the soma of this strain.

Overall, these data indicate that worm somatic cells have the capacity to repair DNA damage independently of DDR checkpoint activation. This is, to our knowledge, the first example of functional DNA repair in the absence of known and characterized DDR signaling pathways observed in one organism, though we cannot exclude the existence of additional, yet uncharacterized, signaling pathways in somatic cells.

Somatic cells misexpressing germline genes do not reactivate DDR, while germ cells that transdifferentiate into somatic cell types lose DDR. It has been previously

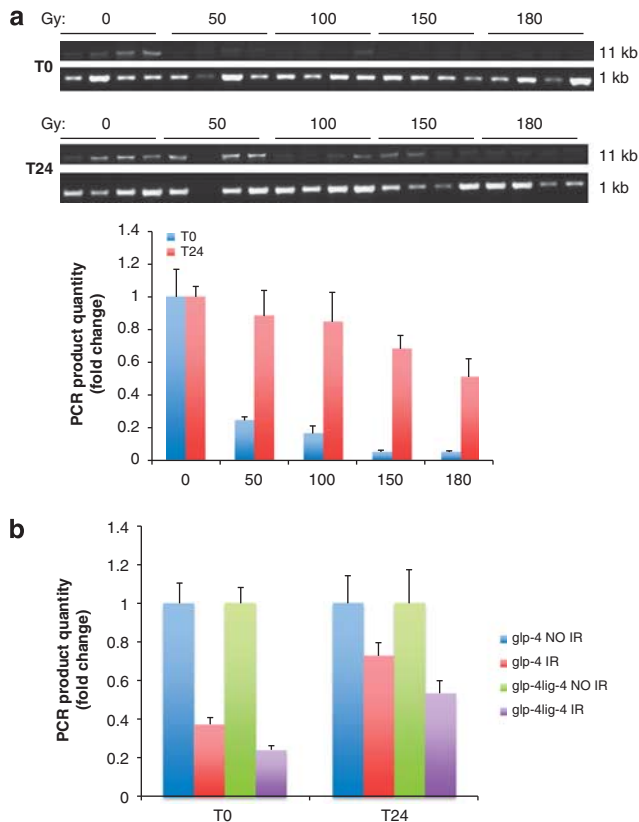


Figure 4 Worm somatic cells show DNA repair proficiency. (a) Fully somatic (*glp-1*) worms were irradiated with the indicated dose of IR and single-worm PCR was performed in quadruplicate, per irradiation point, either immediately after irradiation (T0) or 24 h after irradiation (T24). Bars on the histogram are mean values (for quadruplicate) of the 11 kb PCR product quantity normalized to the 1 kb PCR product quantity, and to the values of not irradiated wild-type worms. Error bars are mean \pm S.E.M. (b) *glp-4(bn2)* and *glp-4(bn2) lig-4(ok716)* mutants were irradiated with 100 Gy of X-rays and probed for DNA damage by PCR assay immediately (T0) or 24 h after irradiation (T24). Histogram represents quantification of PCR products for the biological triplicate

shown that loss of either components of the retinoblastoma (Rb) signaling pathway or insulin-like signaling receptor (*daf-2*) lead to misexpression of otherwise germline-restricted genes in the soma.^{23,24} We asked whether somatic cells that re-express germline genes could respond to IR by also re-activating DDR signaling pathways. Animals carrying mutations in *lin-35* and *lin-15*, two *C. elegans* homologs of the Rb tumor suppressor complex that belong to the group of synthetic multivulva (*synMuv*) genes, have been shown to misexpress germline genes in their soma.²³ We exposed *lin-35(n745)* and *lin-15b(n744)* mutants to IR, and stained them for germline marker PGL-1, previously shown to be misexpressed in the soma in these mutants,²³ and for pS/TQ. Although both *synMuv* mutants showed misexpression of germline genes *pgl-1* (Figure 5a) and *pie-1* (Supplementary Figure S5A), they did not display pS/TQ signals in any somatic cell, including PGL-1-positive ones (Figure 5a).

In addition to the genetic studies described above, we considered a chemical biology approach to make a wide

impact on the chromatin status of somatic cells. Heterochromatin formation and consequent gene silencing may be responsible for the transcriptional repression of DDR genes of terminally differentiated somatic cells. HDAC (histone deacetylases) are known regulators of chromatin structure and functions by controlling histones acetylation. We tested the impact of a well-characterized HDAC inhibitor, valproic acid (VPA), on potential DDR reactivation in the soma. Before exposure to IR, wild-type animals were treated with VPA and then stained for pS/TQ and histone acetylation. Although, we observed increased levels of acetylation of histone H4 in both germline and somatic cells, this was not accompanied by any somatic pS/TQ signal (Figure 5b). Thus, even a profound alteration of the chromatin status as that induced by VPA is not sufficient to allow DDR reactivation.

Finally, we tested a more global regulator of gene expression. Insulin-like signaling pathway has been shown to regulate diverse processes related to stress responses and aging. Animals carrying mutation in insulin-like receptor *daf-2*, show somatic misexpression of the FOXO transcription factor DAF-16, directly responsible for the regulation of stress response genes expression.²⁴ Given the pleiotropic roles of this pathway, we tested its potential involvement in DDR genes repression in the soma. As previously shown,²⁴ *daf-2(e1370)* mutant shows misexpression of germline-only gene *pie-1* in the soma (Supplementary Figure S5B). We exposed *daf-2(e1370)* worms to IR and stained them for pS/TQ. We observed that also in this mutant DDR was not activated in the soma (Supplementary Figure S5C). Therefore, the somatic compartment does not seem to be amenable to DDR reactivation despite its competence to the re-expression of some germline genes upon the manipulation of important and pleiotropic genetic pathways.

Germline and somatic cells derive from distinct cells that show a differential cell lineage commitment already at the two-cells stage during embryogenesis. We investigated if DDR signaling occurring only in the germline cells was the consequence of their cell lineage origin. Contrary to Rb and Insulin-like signaling mutants that reacquire germline characteristics in somatic cells, a *mex-3(or20) gld-1(q485)* mutant has been reported to lose stemness in the individual germ cells that undergo full transdifferentiation into various somatic cell types.²⁵ We wondered whether transdifferentiated cells of *mex-3(or20) gld-1(q485)* lose the ability to activate DDR. We stained the gonads of irradiated *mex-3(or20) gld-1(q485) unc-119::gfp* worms simultaneously with antibodies against pS/TQ and a muscle-specific marker (myosin), and neuronal cells were identified by the expression of UNC-119::GFP. Strikingly, we observed that transdifferentiation of germline cells into muscles or neurons is invariably associated with the loss of pS/TQ signal, while cells that have not undergone transdifferentiation remain signaling competent (Figure 5c). This result indicates that DDR signaling suppression is a cell-intrinsic mechanism that is not dependent on the microenvironment of the gonad, which instead supports DDR signaling in the germline cells. Combined, these results suggest that although DDR signaling can be suppressed upon somatic differentiation (*mex-3* mutants), such repressive mechanism once established may not be reversed (Rb and insulin mutants).

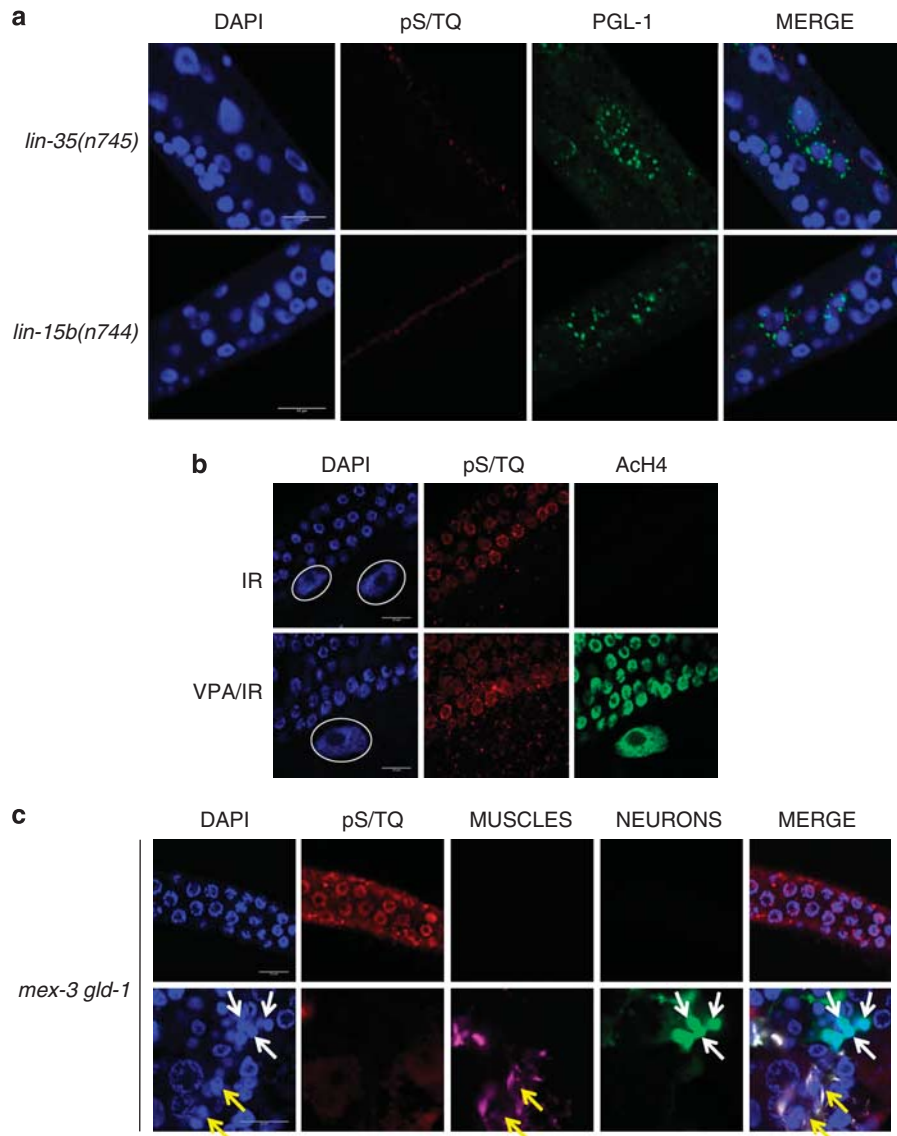


Figure 5 Somatic cells misexpressing germline genes do not activate DDR, while germline cells transdifferentiated into somatic cells lose DDR signaling. (a) *lin-35(n745)* and *lin-15b(n744)* mutants were exposed to IR and co-stained with pS/TQ and germline specific marker PGL-1. (b) Wild-type (N2) worms were treated with 10 mM VPA from L1 to the adult stage, exposed to IR and stained for pS/TQ and acetylated histone H4. Somatic cells that show the absence of pS/TQ signal are outlined with white circles. (c) *mex-3(or20) gld-1(q485) unc-119::gfp* mutant stained for pS/TQ (red), myosin (magenta) and neurons express *unc-119::GFP* (green). White arrows point to the nuclei of neuronal cells expressing *unc-119::GFP*, yellow arrows point to the nuclei of muscle cells stained with myosin. Scale bars on all images, 10 μ m

Discussion

In summary, we have discovered that somatic cells that compose the body of adult *C. elegans* do not activate detectable DDR upon exposure to IR. This provides a molecular basis for the stage-setting, but still unexplained, observation that, differently from germline cells; worm somatic cells do not show any apparent response to DNA-damaging agents and do not undergo cell death by apoptosis.³ Though both cell types show comparable levels of DNA damage upon genotoxic stress, only germline cells mount proficient DDR that eventually leads to the cell removal by apoptosis. We found that key DDR factors are not detectable in somatic cells at RNA or protein level. DDR inactivation, during the process

of terminal differentiation, is a cell-intrinsic mechanism, as we observed loss of DDR in neurons and muscles transdifferentiated from germ cells, within the germline. Interestingly, once the transcriptional inactivation of DDR genes is established in somatic cells, it cannot be overcome by modulation of evolutionary-conserved pathways such as Rb and insulin-like signaling pathway that were shown to be involved in the germline/soma cell commitment.^{23,24} Moreover, broad chromatin alterations induced by HDAC inhibitor were not sufficient to induce DDR activation in somatic cells.

The observed absence of checkpoint signaling in somatic post-mitotic cells of *C. elegans* and its retention in the germline suggest that, evolutionary, DDR signaling is maintained in this species but it is actively suppressed in the soma.

Adult *C. elegans* lacks somatic stem cells able to replenish damaged tissues. Considering the very-short reproductive cycle of the worms, it may be costly to invest energy in the renewal of damaged somatic components. Conceivably, therefore, DDR checkpoint signaling is tuned down in somatic cells to prevent apoptosis, which would be deleterious in a system unable to replenish the gaps left by the lost cells. However, as a minimal maintenance of the soma is necessary to support the germline, efficient DNA repair in the soma is preserved. DDR inactivation is an evolutionary strategy adopted by other nematodes as well, as DDR activity seems undetectable also in two other *Caenorhabditis* species analyzed: *briggsae* and *brenneri*. It would be interesting to understand, from an evolutionary point of view, whether or which other organisms have evolved similar mechanisms to evade cell loss and replacement.

It is possible that some of the features observed in worms may apply also to a restricted number of cell types in mammals too, as we have recently observed that terminally differentiated murine astrocytes, but not neurons or their precursor cells, show reduced DDR activation upon IR.²⁶

Taken together our results bring to light a significant difference in the way soma and immortal germline cope with DNA damage and may indicate the possibility that such distinction could be extended to other important cellular processes.

Materials and Methods

***C. elegans* strains and treatments.** Worms were handled according to standard procedures. The following strains were provided by the Caenorhabditis Genetics Centre (University of Minnesota, St. Paul, MN, USA): N2 (wt), *mre-11(ok179)*, *hus-1(op241)*, *smg-1(r861)*, *glp-1(q224)*, *daf-2(e1370)*, *glp-4(bn2)*, *C. briggsae* wild isolate, *C. brenneri*, *cku-70(tm1524)*, *lig-4(ok716)*, *mrt-2(e2661)*, *clk-2(mn159)* and *atm-1(gk186)* were a kind gift from Shawn Ahmed (The University of North Carolina at Chapel Hill, NC, USA). *atl-1(tm853)* was kindly provided by Shohei Mitani (National Bioresource Project, Tokyo, Japan). *atl-1(tm853)/VnT1(q1s50)* (IV;V) was a kind gift from Simon Bolton (London Research Institute, Clare Hall Laboratories, South Mimms, UK). Two *atl-1* strains showed consistent results. *mex-3(or20)* *gld-1(q485)* *unc-119::gfp* was a kind gift from Rafael Ciosk (FMI, Basel, Switzerland). *lin-35(n745)*; *lsbn1(pie-1p::gfp::pgl-1)* and *lin-15b(n744)*; *lsbn1(pie-1p::gfp::pgl-1)* are unpublished strains, kindly provided by Gary Ruvkun (MGH, Boston, MA, USA). Double mutant *glp-4(bn2)* *lig-4(ok716)* was generated in this study.

X-rays irradiation was induced by a high-voltage X-rays generator tube (Faxitron X-Ray Corporation, Faxitron RX-650 (Faxitron Bioptics, Lincolnshire, IL, USA)). Worms were irradiated with 180 Gy of X-rays and collected 1 h after irradiation, unless stated otherwise. DNA-damaging drugs were added to NGM plates seeded with OP50 *E. coli* bacteria to final concentrations: cisplatin-150 μ M, etoposide-50 μ M. Animals were placed on plates with DNA-damaging drugs at L1 larval stage and collected as adult worms. VPA was added to NGM agar to a final concentration of 10 mM. Plates were then seeded with concentrated OP50 bacteria and wild-type (N2) worms were grown on them until adulthood.

Transgenic worms. The following strains were kindly provided or generated in the laboratory of Michael Hengartner: HUS-1::GFP(*opls334*),¹³ RPA-1::YFP(*opls263*),²⁷ CEP-1::GFP(*gtl1*).²⁸ Generation of MRE-11::YFP(*opls239*): genomic fragments corresponding to *mre-11* promoter and ORF, as well as the 3'-UTR region were separately amplified by PCR from N2 genomic DNA using primers that added the appropriate restriction sites (the first two are for the promoter + ORF, the last two for the 3'-UTR) forward: SbfI-Fw-5'-*mre-11* GATCCTGCGAGGTGAGTTATCATTATATATTGCATATGTCG-3'; reverse: FseI-Rv-5'-*mre-11*GTACGGCCGGCCGAAGAACTAGATCCCTTTCTTGGAT-3'; forward: SspI-Fw-UTR5'-*mre-11*; reverse:GTACACTAGTATAATTGTATTTTCACTTA TCTCATTTACCG-3'; Apal-RvUTR5'-*mre-11*GTACGGGCCCAACGAAATGAAATG TTGAGACACAAGTAAT-3'. The amplified fragments were cloned into the

pLN022 expression vector upstream of *yfp* to generate pLS60 (MRE-11::YFP). Low copy transgenic worms were then generated by ballistic transformation.

Stainings. For whole-worm stainings, gravid hermaphrodites were subjected to a hypochlorite treatment (0.5% NaOCl, 0.25 M KOH) to obtain synchronized cultures. Worm immunostaining was performed using modified Finney/Ruvkun protocol for whole-mount animals²⁹ with addition of 1 μ M Microcystin (Alexis Biochemicals/Enzo Life Sciences, Vinci, Italy) phosphatase inhibitor to all buffers. Primary antibodies were incubated overnight at +4 °C, and after extensive washings worms were incubated at appropriate dilutions with secondary antibodies overnight at +4 °C. Nuclei were counterstained with DAPI (1 μ g/ml) and mounted with glycerol on an agarose pad.

To stain isolated gonads, gravid hermaphrodites were transferred to 1 mM levamisole on a poly-L-lysine coated slide. Extruded germlines were incubated with 2% para-formaldehyde for 10 min and permeabilized in PBT (PBS, 0.1% Triton-X) for 5 min. Primary antibody was diluted in PBSB (PBS, 1% BSA) and incubated overnight at 4 °C. After three washes in PBSB worms were incubated with secondary antibody for 1 h at room temperature and nuclei counterstained with DAPI (0.2 μ g/ml) before mounting on a slide with glycerol.

TUNEL assay was performed with *in situ* Cell Death detection kit (Roche Diagnostics S.p.A, Roche Applied Science, Monza, Italy) according to the manufacturer's instructions. Before being subjected to TUNEL labeling, synchronized worms were fixed and permeabilized as previously described in the protocol for immunostaining of whole-mount worms.

Antibodies: anti-pS/TQ antibody (Cell Signaling, Danvers, MA, USA) (1 : 50 for whole-worm stainings; 1 : 100 for isolated gonads), mAb5.6 (was a kind gift of Rafal Ciosk; FMI Basel, Switzerland) against muscle wall myosin (1 : 500 for isolated gonads), anti-GFP (Acris Antibodies GmbH, Herford, Germany) (1 : 100 for isolated gonads or whole-worm staining).

Anti-rabbit Cy3 (Jackson ImmunoResearch Europe Ltd, Suffolk, UK) (1 : 500 for whole-worm stainings; 1 : 1000 for isolated gonads); anti-mouse 647 (Invitrogen) (1 : 100 for isolated gonads); anti-mouse 488 (Invitrogen) (1 : 100 for isolated gonads).

Whole-worm images were acquired using wide field Olympus Biosystems Microscope (Olympus Italia Srl, Milano, Italy) and MetaVue software (Molecular Devices, Sunnyvale, CA, USA). Confocal images were obtained with a Leica TCS SP2 AOBS confocal laser microscope (Leica Microsystems S.r.l., Milano, Italy). Images were analyzed by ImageJ software (Research Services Branch, NIH, USA).

DNA damage detection by PCR. Synchronized young adult hermaphrodites were irradiated with increasing doses of IR. Five worms/sample were placed in 10 μ l of lysis buffer (25 mM KCl, 25 mM Tris-HCl (pH 8.2), 1.25 mM MgCl₂, 0.1% (w/v) NP-40, 0.1% (w/v) Tween20, 0.5% (w/v) gelatin and 0.25 mg/ml proteinase K), and frozen at -80 °C for at least 20 min. Samples were lysed at 65 °C for one hour. In all, 2 μ l of lysate (genomic equivalent of 1 worm) was used as a template for the PCR reaction. Eleven kilobase fragment of genomic DNA was amplified using the following primers: 5'-GATCGGCGGCCATGAATATCGATAA GGATGTTTCAGC-3' and 5'-GATCGGGCCCTCCGACGAGCTATACTATCAG-3', which spans the ORF of the *atl-1* gene and 1 kb fragment (within the C-terminus region of *atl-1*), was amplified using the following primers: 5'-GATCGGGCCCTT CCGACG AGCTACTACTATCAG-3' and 5'-GCGATAGACATACAAGAGACTTG ATGG TACA-3'. Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Vantaa, Finland) was utilized in the PCR reaction. Band intensities were quantified by ImageJ software.

Quantitative RT-PCR. Synchronized young adults were collected and frozen at -80 °C. Acid-washed glass beads (Sigma Aldrich, St. Louis, MO, USA) were added to TRIzol resuspended pellets and mechanically disrupted using a FastPrep machine (MP Biomedicals, LLC, Irvine, CA, USA). Total RNA was purified using standard TRIzol-chloroform preparation. complementary DNA (cDNA) was generated using the Superscript III Reverse Transcriptase (Invitrogen). The cDNA was used as template in real-time quantitative PCR reactions on a Roche LightCycler 480 Sequence Detection System (Roche Applied Science). The reactions were prepared using SyBR Green kit from Roche. In all, 18 S and *rpl-1* ribosomal genes were used as control genes for normalization.

RNAi. RNAi was performed by feeding as described previously.³⁰ The RNAi clone carrying dsRNA against *atm-1* gene was obtained from Ahringer RNAi library.³¹ L4440 empty vector was used as a control.

Conflict of Interest

The authors declare no conflict of interest.

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