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# A systems biology analysis of reproductive toxicity effects induced by multigenerational exposure to ionizing radiation in *C. elegans*

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## ARTICLE INFO

## ABSTRACT

Understanding the effects of chronic exposure to pollutants over generations is of primary importance for the protection of humans and the environment; however, to date, knowledge on the molecular mechanisms underlying multigenerational adverse effects is scarce. We employed a systems biology approach to analyze effects of chronic exposure to gamma radiation at molecular, tissue and individual levels in the nematode *Caenorhabditis elegans*. Our data show a decrease of 23% in the number of offspring on the first generation F0 and more than 40% in subsequent generations F1, F2 and F3. To unveil the impact on the germline, an in-depth analysis of reproductive processes involved in gametes formation was performed for all four generations. We measured a decrease in the number of mitotic germ cells accompanied by increased cell-cycle arrest in the distal part of the gonad. Further impact on the germline was manifested by decreased sperm quantity and quality. In order to obtain insight in the molecular mechanisms leading to decreased fecundity, gene expression was investigated via whole genome RNA sequencing. The transcriptomic analysis revealed modulation of transcription factors, as well as genes involved in stress response, unfolded protein response, lipid metabolism and reproduction. Furthermore, a drastic increase in the number of differentially expressed genes involved in defense response was measured in the last two generations, suggesting a cumulative stress effect of ionizing radiation exposure. Transcription factor binding site enrichment analysis and the use of transgenic strain identified daf-16/FOXO as a master regulator of genes differentially expressed in response to radiation. The presented data provide new knowledge with respect to the molecular mechanisms involved in reproductive toxic effects and accumulated stress resulting from multigenerational exposure to ionizing radiation.

## 1. Introduction

The study of multigenerational consequences of chronic exposure to environmental stressors is a growing field of interest for human and environmental protection. Both natural and anthropogenic sources of ionizing radiation (e.g medical, industrial or military sources, nuclear power plant accidents) can lead to the presence of radionuclides into the environment and long term/chronic exposure of organisms (UNSCEAR, 2008). Current knowledge on the effects of ionizing radiation mainly concerns short term/acute and single generation exposure, while the multigenerational effects of long-term exposure are still poorly understood. Emerging evidence suggests that the early stages of development

and reproductive mechanisms are particularly sensitive to chronic exposure to gamma radiation, even in tolerant species (Heyer et al., 2000; UNSCEAR, 2006; Honjo and Ichinohe, 2019). Understanding the overall toxic mechanisms induced by chronic low dose as well as generational effects is a major concern for improving prevention of toxicity (UNSCEAR, 2012). Some studies have highlighted that the phenotypical effects of ionizing radiation observed at the first exposed generation are not necessarily representative of the effects on the following generations (Adam-Guillermin et al., 2018; Buisset-Goussen et al., 2014). In order to provide a more holistic assessment of long-term impact of chronic exposure to ionizing radiation, it is necessary to carry out dedicated studies over several generations, including

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the investigation of molecular mechanisms to fundamentally understand the mode of toxic action through generations.

Ionizing radiation causes a wide range of effects in macromolecules by disruption of molecular structures, ionization of atoms, or indirectly through radiolysis of water and generation of oxidative stress (Azzam et al., 2012). Under exposure to ionizing radiation, cellular stress response pathways can be activated, including antioxidant defenses, DNA damage, or the unfolded protein response to promote survival (Fulda et al., 2010). If such response cannot counteract the harmful effects, the cell activates downstream pathways that can finally lead to programmed cell death (Greiss et al., 2008). In *C. elegans*, cell death apoptosis is controlled by activation of CEP-1 in the p53 pathway. CEP-1 modulates *egl-1* and *cep-13* transcription, which activates caspase induced germline apoptosis (Hoffman et al., 2014). Accumulation of damages and elimination of cells can lead to functional phenotypic consequences for the organism but can also impact subsequent generations if the gametes are affected. Thus, parental exposure to ionizing radiation can lead to genomic and epigenomic instability in subsequent generations (Kadhim et al., 2013).

In the present study, we investigated the impact of ionizing radiation on nematodes reproduction and the underlying molecular mechanisms induced by chronic exposure to gamma radiation over 4 generations. To address the current lack of knowledge concerning reproductive toxicity effects onto subsequent generations and the underlying molecular mechanisms, *C. elegans* was challenged over the entire lifecycle from the embryonic stage until sexual maturity. We employed a systems biology approach to assess alterations at the molecular, cellular and individual levels.

## 2. Materials and methods

### 2.1. C. elegans maintenance, strains and synchronization

The following *C. elegans* strains were used in the current study: Wild type Bristol (N2), CB4088 *him 5 (e1490)*, T356 *daf-16:GFP* (zIs356 (pDAF-16:DAF-16-GFP;rol-6)) provided by CGC (Caenorhabditis Genetic Center). Worms were cultured by standard techniques at 19 °C, 80% humidity, on petri dishes with NGM (Nematode Growth Medium) and seeded *Escherichia coli* OP50 as food source (Brenner, 1974).

### 2.2. Experimental design and gamma irradiation

Effects of ionizing radiation were evaluated on four generations of nematodes continuously exposed (F0, F1, F2, F3) from synchronized embryos to L4 young adult stage (65 h). Several irradiations were carried out, from 3 to 10 replicates with 1000–5000 worms. Chronic irradiation to 50 mGy h$^{-1}$ of gamma radiation was performed in incubators under controlled conditions using the platform MIRE (Mini irradiator for Radioecology) and MICADO irradiation facility (IRSN, Cadarache, France). Data loggers were used to measure humidity and temperature during irradiation. Dose rates were calculated based on the Monte-Carlo N-Particle model and validated during irradiation with radio-photo luminescent dosimeters (RPL, GD-301 type, Chiyoda Technol Corporation Japan) placed on each incubator to measure the delivered cumulative dose received from the Cesium-137 source. Samples for molecular and phenotypical analysis were collected after 65 h, to cover the entire lifecycle from embryo to L4 young adult stage. A part of the replicates was irradiated for additional 24 h in order to obtain sexually mature hermaphrodites and harvest the next generation.

### 2.3. Total brood size measurements

Brood size was determined by measuring the cumulative number of larvae produced by five nematodes continuously exposed for 65 h (5 biological replicates, n = 25 per condition), at each generation. Nematodes were transferred daily into new Petri dishes. The number of progenies was determined by counting the number of eggs produced over 8 days. Effects of gamma radiation through generation were assessed using a two-way ANOVA on the mean of each replicate. Residuals normality and homogeneity residuals assumption were checked using Shapiro-Wilk test and Levene test, respectively. Brood size of control and exposed groups were then compared at each generation using Wilcoxon test and Holm adjustment to take into account the multiple comparisons. P-value < 0.05 was considered as significant.

### 2.4. DIC and fluorescent microscopy

Transgenic strain T356 *daf-16:GFP* from 5 biological replicates was used to classify DAF-16 transcription factor sub-cellular localization on the basis of associated GFP fluorescence by using an inverted microscope (Zeiss Axio observer, Carl Zeiss, France) as previously described (Thabit et al., 2018). Sperm and mitotic cells were counted with a DAPI filter and apotome.2 after capturing images of more than 10 Z-sections. Sperm activation was measured via Nomarski Differential Interference Contrast (DIC) method. For more detailed examination, nematodes were immobilized on an agar pad, with 1 mM levamisole (Sigma) and images were collected with an LSM 780 confocal microscopy (Carl Zeiss, France).

### 2.5. Quantification of mitotic germ cells and spermatids

To measure the number of mitotic cells and spermatozoa, nematodes were dissected as previously described (Craig et al., 2012). Briefly, after 65 h of irradiation, worms were immersed into 10 μl of 2% PFA buffer on poly-lysine coated slide. A 27-gauge needle was used to cut each worm and allow the extrusion of the gonadal arms. The slide was freeze cracked in liquid nitrogen and stored at − 80 °C. Gonads were stained with 10 μl of DAPI mounting medium (Fluoroshield™ with Dapi, Sigma-Aldrich). Mitotic germ cells assay was performed by counting cells in the 50 μm Distal Tip Cell region, from at least 29 intact germlines and 3 biological replicates per condition. Replicates were analyzed with a (quasi) Poisson generalized linear model with multiple comparisons using Dunnett test approach. P-value < 0.05 was considered significant. For spermatozoa quantification, ten slides with 20 hermaphrodites per treatment were prepared. The number of spermatozoa was counted in 10 intact germlines from 10 biological replicates per condition. A total of ~25 Z-sections of ~6 μm were obtained with an inverted microscope (Zeiss Axio observer, Carl Zeiss, France) in order to assess the number of spermatids. A two-ways ANOVA and multiple comparison approach was used on the mean of each replicate, as described in Section 2.3.

### 2.6. Spermatozoa activation

In order to assess spermatozoa activation in response to gamma radiation, him-5 hermaphrodites were exposed for 65 h from embryonic stage. 20 males per replicates were isolated on fresh petri dishes and exposed from 66 h to 90 h to obtain a large number of spermatids. Celibate males were transferred with a pipette from droplet to droplet on slide with 1X sperm medium (50 mM HEPES, 25 mM KCl, 45 mM NaCl, 1 mM MgSO4, 5 mM CaCl2, 1 mM Dextrose, pH 7.6) in order to remove excess *E. coli*. Males were then transferred to a square traced with

Vaseline on a slide and dissected in 1X sperm medium containing pronase E (200 μg/ml) to release and activate spermatids. A coverslip was gently placed on the surface of the slide and spermatids/spermatozoa were counted after 10 min at 100X magnification with immersion oil. Several counts were made on 5 randomly selected areas per each replicate. To analyze this non-independent binary data, four independent replicates were analyzed with a general linear mixed model (GLMM) based on logistic regression with binomial errors. P-value < 0.05 was considered as significant.

### 2.7. Transcriptomic analysis

RNA sequencing analysis was performed in order to obtain a multigenerational gene expression profile of nematodes in response to chronic exposure to ionizing radiation. Total RNA was extracted from four generations of L4 young adult irradiated for 65 h from embryonic stage (n = 1000 per replicate). Total RNA was extracted using FastPrep with Direct-zol Reagent (Nordic Biosite) and purified with RNeasy Mini Kit (Zymo Research) according to manufacture instructions. RNA quality, integrity and concentration were assessed using Nanodrop (NanoDRop Technology, Wilmongton, DE) and Agilent 2100 Bioanalyzer (Agilent technologies) with RNA Nano Chip. cDNA synthesis and sequencing library were obtained using Strand-specific TruSeq RNA-seq pair-end libraries with 350 bp fragment size. Libraries were sequenced using Illumina HiSeq 3000 as paired-end 150 base reads with high quality score (Q30 higher than 98% for all samples) at the Norwegian High Throughput Sequencing Centre (Oslo, Norway). Reads were mapped to the *C. elegans* genome (Ensembl 91/WormBase235) using RNA-STAR. Hierarchical clustering of the variance stabilized data with Pearson's correlation and complete-linkage method was generated and showed a good correlation between biological replicates, except for one exposed and one control sample in F3 generation (Fig. S1), which were removed for the subsequent analysis. Deseq2 v1.10.2 and rlog R packages were used for normalization and differential expression analysis. Genes with a log2 fold change (Exp/Ctrl) > 0.5 and adjusted p-value < 0.05 (False Discovery Rate, FDR) were considered as significantly regulated.

### 2.8. Enrichment of transcriptomic data

In order to analyze biological processes impacted by gamma irradiation, Differentially Expressed Genes (DEGs) obtained from DESeq2 analysis were subjected to enrichment analysis. Gene Ontology (GO) enrichment analysis of biological functions was performed using the R package topGO and ViSEAGO. Enriched functions with p-value from Fisher's exact test < 0.1 were considered significant. Heatmap was generated using the R package ggplot2. Phenotype enrichment analysis was obtained from the DEGs from DESeq2. DEGs were analyzed using WormBase Phenotype Ontology database and modPhEA. Significantly enriched phenotypes were validated by Bonferroni-corrected p-value < 0.05 from Fisher's exact test. Network analysis was performed with a combination of Gene Ontology analysis and GeneMANIA 3.5.1 plug-in within the Cytoscape 3.8.1 software.

### 2.9. Transcription factor binding motif analysis

Promoter sequences of DEGs from four generations were analyzed in order to identify potential enrichment of transcription factor binding motifs after chronic exposure to gamma radiation. A set of 4500 randomly chosen promoters' sequences was selected as background. Promoter sequences are defined as DNA genomic sequences from 1500 bp upstream to 200 bp downstream of transcriptional start site and were retrieved via Regulatory Sequence Analysis Tools (RSAT) from Ensembl 91 release. Promoter sequences were analyzed with OPOSSUM v3 Single Site Analysis (SSA) and 43 non-redundant transcription factor binding profiles stored as position frequency matrices from JASPAR database. Binding sites with Z-score > 5 and Fisher-score > 2 were considered significantly enriched. The Z-score assessed whether the rate of occurrence of transcription binding site in the DEGs sequence differs significantly from the background sequence based on binomial distribution model. The Fisher-score assessed the probability of a non-random association between DEGs sequence and background.

### 2.10. qRT-PCR

RNA from four generations of irradiated nematodes was extracted with Qiagen mRNA kit and integrity was assessed with Bioanalyzer® (in conjunction with RNA 6000 Nano LabChip®). cDNAs were synthesized using the superscript affinity cDNA from Qiagen. Quantitative PCR (qPCR) analysis was performed in order to quantify the expression of *egl-1*. For this purpose, Mx4000 multiplex quantitative PCR system (Stratagene) with 1X MasterMix Agilent and 2 μM of primer set were used. The PCR reactions were amplified for 15 s at 95 °C and 1 min at 60 °C for 45 cycles. Transcript expression was normalized to PMP-3, CDC-42 and ACT-2 housekeeping genes. Primer sequences used are in Supplementary Data (Fig. S3). qRT-PCR were performed three times on three independent biological replicates. Statistical analysis was performed using dataassist software.

## 3. Results

### 3.1. Reproduction impairment is associated to effects on gamete production

In order to evaluate the impact of multigenerational exposure to ionizing radiation, phenotypical and molecular analyses were performed over four generations (F0, F1, F2 and F3) of *C. elegans* continuously exposed to 50 mGy h$^{-1}$ of gamma radiation. Each generation was irradiated for 65 h, from embryo to L4 young adult stage, and received a cumulative dose of 3.3 Gy. The dose rate and duration of exposure were previously defined as inducing significant effect on reproduction from the first generation (Buisset-Goussen et al., 2014; Dubois et al., 2018).

#### 3.1.1. Brood size reduction in C. elegans after chronic irradiation over four generations

*3.1.1.1. Number of larvae/progeny per individual.* The brood size decreased significantly in comparison with control (GLM- Dunnet test p-value < 0.001) when nematode populations were exposed from embryos to adult to 50 mGy h-1 of gamma radiation (Fig. 1b). The first exposed generation (F0) showed a brood size reduction of 23% (170 scored larvae) in comparison with control (221 scored larvae), while subsequent generations (F1 to F3) showed a brood size reduction of up to 40%. The hatching success was not affected, neither the embryonic mortality, the morbidity nor the lifespan of the progeny in any generation (data not shown). In the absence of detectable embryonic developmental failures, the effects on gamete formation were investigated in order to identify the mechanisms of reproductive toxicity. Therefore, a systematic investigation of the different stages of gamete development and performance was conducted. In the reproductive system of *C. elegans*, germ cells proliferate from the distal mitotic zone of the gonadal arm (Fig. 1a). The distal area of the gonad hosts the distal tip stem cells, from which proliferating cells migrate to the proximal part to
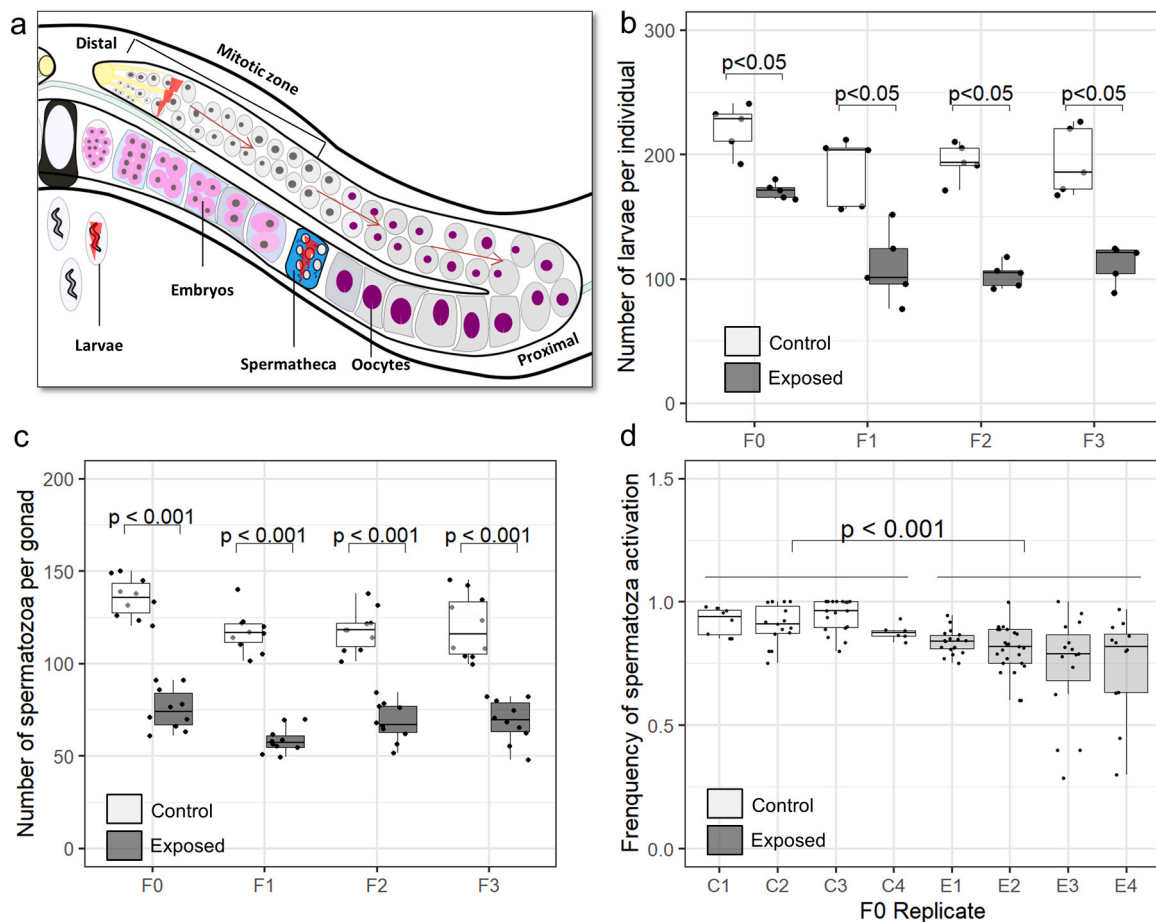
**Fig. 1.** (a) Diagram of a *C. elegans* gonadal arm. The red symbols indicate the processes investigated in the present study. (b,c) Box-plot of the number of larvae per individual (b) and spermatozoa per gonad arm at L4/YA stage (c) (p-value $\leq$ 0.5 from two ways ANOVA with multiple comparisons followed by Wilcoxon tests and holm p-values adjustment). (d) Box-plot of impacted spermatozoa activation in the first generation F0 from 4 biological replicates per conditions (p-value from generalized linear mode with logistic regression and binomial error). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the gonad and proceed to meiosis to produce the gametes. In order to specify a possible impact of the irradiation on the gamete production, we then assessed several endpoints including number of spermatozoa (Fig. 1c), spermatid activation (Fig. 1d), number of mitotic cells per gonadal arm and apoptosis (Fig. 2a, Fig. S2a.b, Fig. S3).

*3.1.2. Detrimental effect of gamma radiation on spermatids quantity and quality*

*3.1.2.1. Number of spermatids per gonad.* the potential multigenerational effect of gamma radiation on spermatogenesis was assessed by measuring the number of spermatozoa after 65 h of exposure from embryonic stage. The exposed F0 nematodes showed a 44% decrease in the number of spermatids (from 135 to 75 spermatozoa per gonad). The following generations (F1 to F3) presented a similar effect (between 42% and 50%) (Fig. 1c). Thus, all exposed generations showed a significant decrease in the number of spermatids, which followed a similar trend as the reduction in number of offspring compared to non-irradiated nematodes (GLM- Dunnet test p-value $<$ 0.001).

*3.1.2.2. Spermatids activation.* Because of the observed impact of gamma irradiation on the spermatids number, we then examined

whether the process of spermatids activation, by which fully functional spermatozoa are produced, was similarly affected. We observed a significant decrease (p-value $<$ 0.001) of spermatid activation frequency from controls (0.89) to exposed organisms (0.75) indicating that the chronic exposure to gamma radiation induced a significantly reduced quality by 14% less activation of spermatozoa from the produced spermatids (Fig. 1d). The effect was highly reproducible in all the independent replicates.

*3.1.3. Decreased number of mitotic germ cells and enhanced cell cycle arrest induced by chronic gamma irradiation*

*3.1.3.1. Number of mitotic germ cells.* We performed a standard germ-line proliferation assay on dissociated gonads from F0 and F3 generations (see Section 2.5). The distal part of the gonadal arm in F0 and F3 (Fig. 2a) showed a decrease in the number of mitotic cells by up to 25% (respectively 95.9 $\pm$ 3.13 and 95.9 $\pm$ 3.82 for control compared to 71.8 $\pm$ 3.52 and 71 $\pm$ 3.15 for exposed). A significant (p-value $<$ 0.01) increase of cell cycle arrest was also identified by cells size analysis and DNA staining (Fig. 2b, Fig. S2a.b). These results showed that chronic exposure to 50 mGy h$^{-1}$ of gamma radiation impaired the cell cycle, cell division and decreased the germ cell proliferation by ~25%.
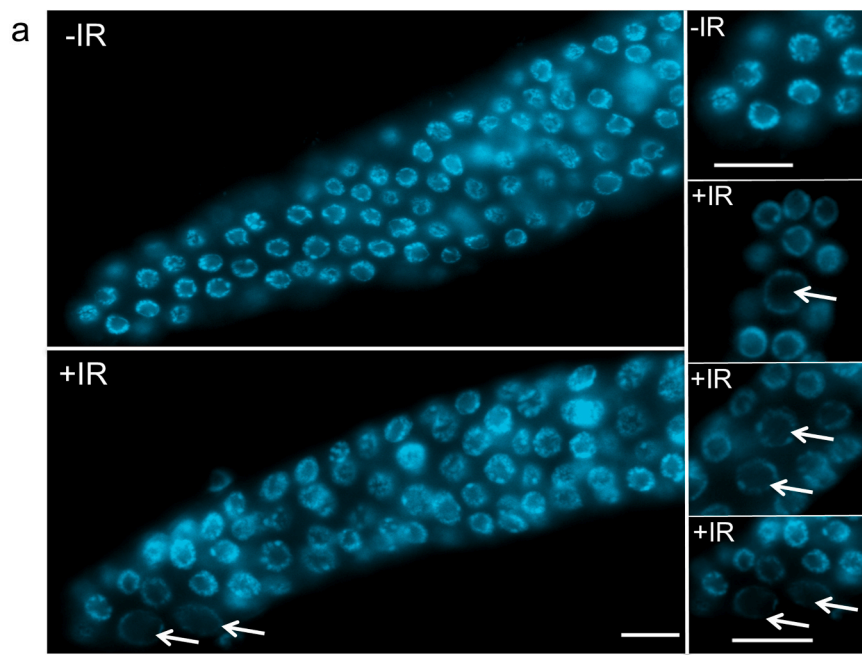
**Fig. 2.** (a) Distal germ cells from control (-IR) and exposed (+IR) *C. elegans* stained with DAPI. Mitotic germ cells under cell cycle arrest are indicated with white arrows. Scale bar = 10 μm. (b) Box plot of the number of mitotic germ cells in the first 50 μm of the gonad. Triple asterisks indicate a p-value < 0.001 from generalized linear model and multiple comparison using Dunnett's test approach (n indicates the number of different gonadal arms measured in the mitotic germ cells assays).
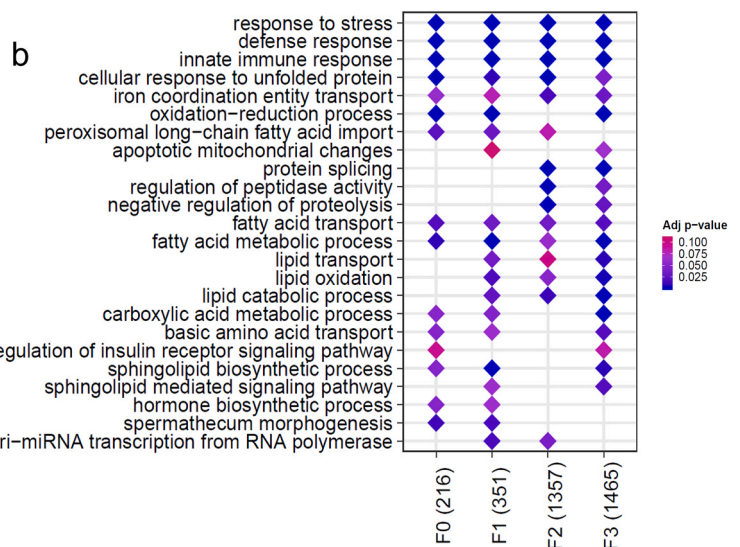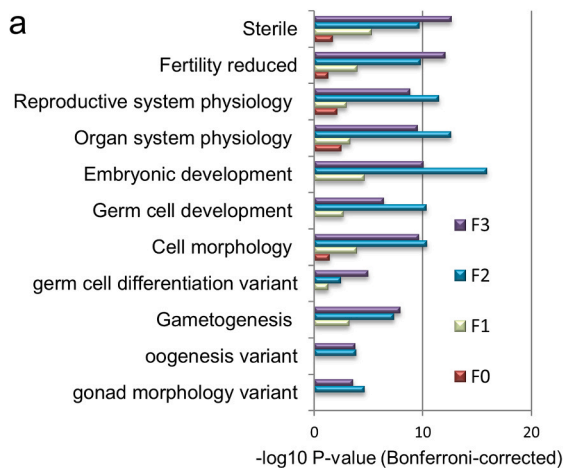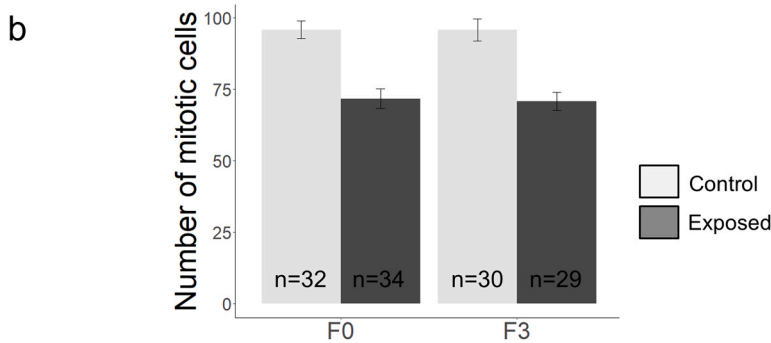


**Fig. 3.** (a) Bar plot of shared enriched phenotype terms from transcriptome analysis across generations exposed to chronic irradiation (Bonferroni corrected p-value ≤ 0.05). (b) Heatmap of shared GO terms modulated in response to chronic irradiation. Colors indicate the enrichment p-values from exact Fisher's test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

*3.1.3.2. Genotoxicity induced germ cell apoptosis.* egl-1 is required for *p53/cep-1* dependent germ cell apoptosis induced by genotoxic stress. The effect of chronic exposure to ionizing radiation was therefore assessed by measuring *egl-1* expression by qRT-PCR analysis in all generations (Fig. S3). A significant increase of *egl-1* expression from 1.5 to 4.2-fold change was observed over all exposed generations compared to control, thus substantiating increased apoptosis in response to chronic irradiation.

## 3.2. Transcriptomic profiling over four consecutive generations exposed to chronic gamma radiation

### 3.2.1. Stress response, metabolism and reproduction as the main enriched biological functions over multiple irradiated generations

In order to investigate the toxic mode of action (MoA) and the molecular response to germline damage induced by chronic irradiation, a genome wide transcriptome analysis was performed over four generations (F0 to F3) of *C. elegans* continuously exposed to 50 mGy h$^{-1}$. Hierarchical clustering of the variance revealed that irradiation modified the transcription profiles in all exposed generations compared to control conditions (Pearson's correlation and complete-linkage method). High correlation was shown for F1, F2 and F3 "control" populations whereas F0 generation clustered separately (Fig. S1). We therefore identified Differentially Expressed Genes (DEGs) by pairwise comparison between exposed and control populations. DEGs with a q-value < 0.05 and log2 fold change | ≥ 0.5 were considered significant. The analysis identified 272 genes differentially expressed in F0, with 216 (79%) up-regulated and 56 (21%) down-regulated genes respectively. The number of DEGs in F1 was comparable to F0 generation, with 225 (64.1%) up-regulated and 126 down-regulated (35.9%) genes (total of 351 DEGs).

An increase of total DEGs was observed between F1 and F2 generations. The F2 and F3 presented 1357 and 1465 DEGs, respectively, with 136 (10%) and 523 (36%) up-regulated and 1221(90%) and 942 (64%) down-regulated genes, respectively. Phenotype enrichment (Fig. 3a) and Gene Ontology (GO) biological processes (Fig. 3b, Table S1) were examined in order to identify biological functions impacted by the chronic exposure to gamma radiation over multiple generations.

Phenotype enrichment analysis of DEGs highlighted an impact of gamma radiation in all exposed generations on reproductive processes and cell functions (Fig. 3a). In line with the measured reproductive toxicity effects, the phenotypes related to sterility (p-value < 10–2), reproductive system physiology (p-value < 10–3) and reduced fertility (p-value < 10–2) were significantly enriched. Germ cell development (p-value < 10–2), embryonic development (p-value < 10–5) and gametogenesis (p-value < 10–4) were also significantly enriched phenotypes. Phenotype enrichment analysis was thus consistent with the identified phenotypic effects on the germline.

Next, we identified significantly enriched biological processes by GO terms shared by multiple generations (Fig. 3b). Consistent with the phenotype enrichment analysis the GO term biological process also showed an effect on spermatheca morphogenesis in F0 and F1. We observed a large proportion of differentially expressed genes linked to stress defense mechanisms like oxidation-reduction processes, innate immune response, defense response, apoptotic mitochondrial changes, and cellular response to unfolded protein, (Fig. 3). Effects on lipid metabolism and transport, including fatty acid metabolic process, lipid transport, sphingolipid biosynthetic processes and lipid oxidation were also observed. The GO terms analysis revealed modulation of signaling processes including insulin receptor signaling pathway, sphingolipid mediated signaling pathway which is required for radiation-induced
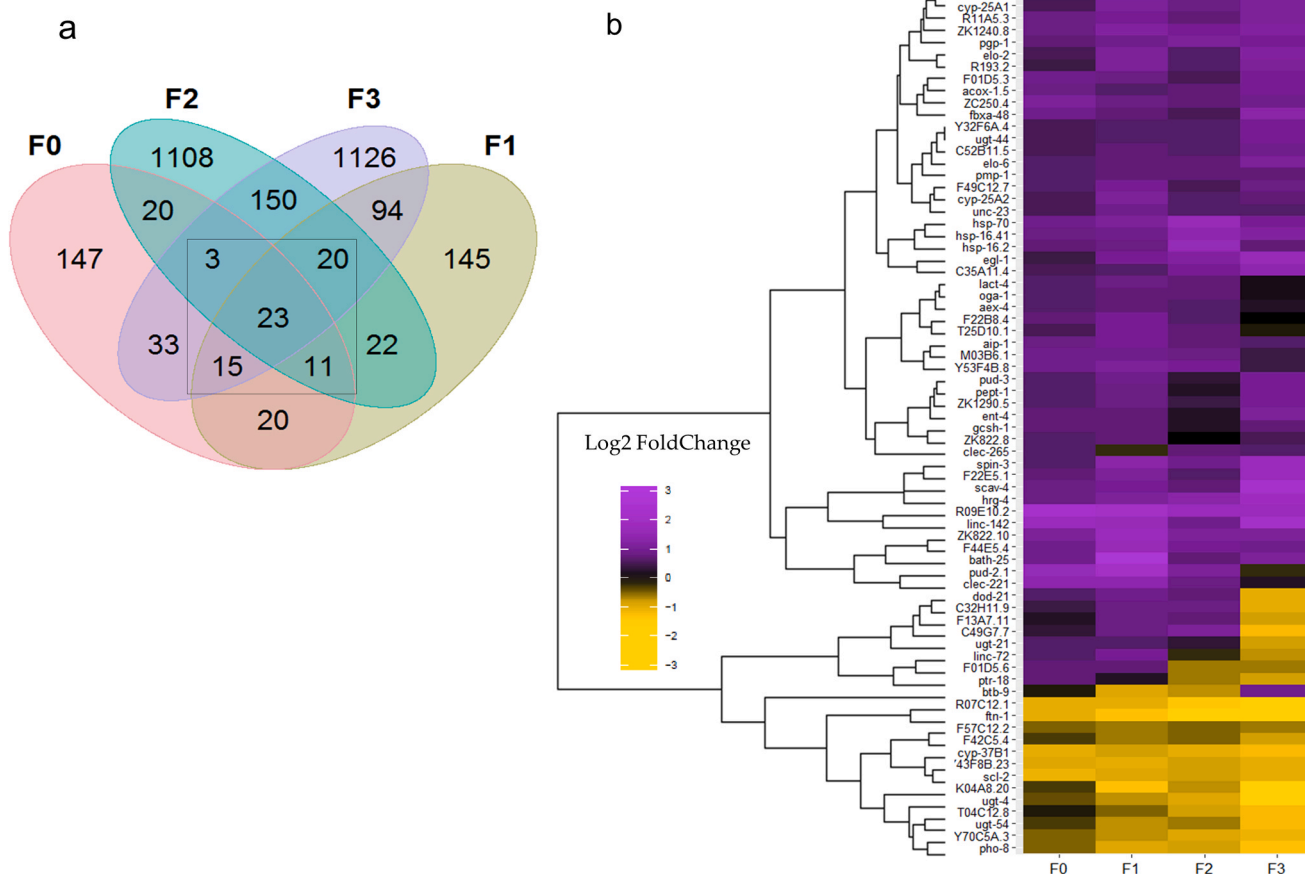


**Fig. 4.** (a) Venn diagram of differentially expressed genes in response to chronic irradiation over exposed generations (|Fold change| ≥ log2(0.5) and adjusted p-value ≤ 0.05. (b) Hierarchical clustering of differentially expressed genes after chronic exposure to 50 mGy h$^{-1}$ shared by at least three generations.

apoptosis in the germline through *elg-1*, hormone biosynthetic process and pri-miRNA transcription from RNA polymerase. In F2 and F3 generations, a modulation of biological processes related to protein synthesis and turnover was showed with terms related to regulation of peptidase activity, alternative protein splicing and negative regulation of proteolysis.

### 3.2.2. Consistent modulation of stress response genes across irradiated generations

In order to gain insights into the core transcriptomic response induced by radiation over multiple generations, a detailed analysis of common DEGs was performed. An overlap of 23 genes was found between the 4 exposed generations and a total of 72 genes was shared between 3 generations, suggesting that part of the transcriptomic response to chronic irradiation was stably maintained (Fig. 4a). The majority of the 72 shared DEGs was upregulated (Fig. 4b). Thirteen of the modulated genes were involved in terms related to stress response and to topologically incorrect protein, including *aip-1, hsp-16.41, hsp-16.2 hsp-70, pept-1* and *pgp-1*. In addition, seven genes were involved in oxidation-reduction processes: *ftn-1, cyp-25A1, cyp-25A2, acox-1.5, cyp-37B1, oga-1, ZK1290.5*. Seven genes were involved in lipid metabolic process and localization: *pept-1, acox-1.5, ugt-21, Y32F6A.4, pmp-1, elo-2* and *elo-6. elo-2* and *pept-1* are also involved in reproductive processes. In addition, the *egl-1* gene, regulated by CEP-1/P53 transcription factor, which is the hallmark of genotoxic stress induced germline apoptosis, was upregulated in all generations. It thus appeared that the DEGs shared by all generations were predominantly involved in stress response and apoptosis.

### 3.2.3. Transcription factor enrichment analysis identifies DAF-16 as key regulator of response to chronic ionizing radiation

An increase in the number of DEGs was observed between F0-F1 (respectively 272 and 351) and F2-F3 generations (respectively 1357 and 1465) (Fig. 4a). We hypothesized that this increase could be associated to regulatory pathways activated to counteract persistent stress, or repair needed to maintain fundamental biological functions. In order to identify potential pathways involved in the stress response, a binding motif enrichment test of 43 non-redundant transcription factors (TFBMs) was performed on differentially expressed genes promoter sequences ($-1500 +200$ bp from TSS). Twenty-three binding motifs were found to be significantly enriched in at least one of the four generations (Table 1, Table S3).

Two TFBMs of ELT family, ELT-2/GATA4-GATA5 and ELT-3, were enriched in all generations. ELT-2 is known to regulate gut

differentiation as well as its maintenance. Another TFBS enriched in F3 is DAF-16/FOXO, the main target of insulin/insulin-like growth factor signaling pathway (IIS) in response to stress (Table 1). DAF-16 interacts with many others TF to coordinate the stress response. Interestingly other TF involved in the regulation of the stress response PQM-1, were enriched in F0, F1 and F3 (Dowen et al., 2016). The upstream regulator of the core apoptotic cell death machinery, CES-2 was enriched in the F2 and F3 exposed generations. HLH-30/TFEB is involved in regulation of autophagy and lipolysis in stress response conditions (Lin et al., 2018). Moreover, HLH-30/TFEB forms a transcriptional regulatory module with DAF-16/FOXO to modulate oxidative stress response and protein homeostasis. This was in line with a significant enrichment of TF gene products under direct control of DAF-16 transcripts in all generations.

The simultaneous nuclear localization of the product of the DAF-16 and PQM-1 genes leads to antagonistic effects on the control of class I (DAF-16) and class II (PQM-1) target genes. The action of these two transcription factors allows switching between stress response (class I) and reproduction/growth functions (class II). The list of gene classes I and II comes from a robust consensus of transcriptomic and Chip-sequencing meta-analysis (Tepper et al., 2013). We found that 52% of the 72 shared DEGs were under control of DAF-16/PQM-1, of which 13 and 25 DEGs belonged to class I and to class II, respectively (Table 2). This notion was further corroborated by the significant enrichment of the DAF-16 binding motif in the promoter sequences of the 72 DEGs (Z-score = 8.9 and Fisher score = 3) (data not shown). These data strongly suggest that DAF-16 is a key regulator of the stress response imposed by chronic gamma radiation. Based on experimentally validated interactions, we built a network between the transcription factors and the shared DEGs. This showed the connection between DAF-16, PQM-1, ZTF-2, CES-2 and *egl-1*. A part of this network governs stress, apoptosis response and it was shared by four continuously exposed generations (Fig. S4).

To verify the responsiveness and activation by gamma radiation of DAF-16, the cellular localization was investigated in irradiated L4-young adult hermaphrodites using a *daf-16:gfp* reporter strain. Basal expression of DAF-16 is predominantly localized in the cytoplasm, while activation requires translocation to the nucleus. DAF-16 localization can thus be divided into three categories: cytoplasmic (inactivated state), intermediate or nuclear (activated state) (Fig. 5a). The nuclear localization shifted significantly, from 15% under control conditions to 47% in exposed nematodes, while the intermediate localization remained relatively stable (28.9–34.8%). The increased nuclear localization of DAF-16 in response to chronic irradiation was consistent with the transcriptomic data which demonstrated the activation of genes under the control of DAF-16.

## 4. Discussion

### 4.1. Chronic multigenerational exposure to ionizing radiation induces consistent reproductive toxicity

To address the existing knowledge gap in the field of chronic-low dose exposure to ionizing radiation and its impact over multiple generations, the present study investigated the progression of phenotypic and molecular toxic effects induced over four consecutive generations of *C. elegans* subjected to chronic gamma irradiation. The exposure condition used in this study was known to induce reproductive toxicity effects without affecting embryonic survival (Dubois et al., 2018). Moreover, since reproductive impairment has been recently linked to irradiation during the early stages of development (Maremonti et al., 2019), the exposure encompassed both embryonal and larval stages including gonadal development.

Total brood size was employed as the apical endpoint to assess reproductive toxicity, which revealed a persistent decrease in the number of offspring in all generations exposed to 50 mGy h$^{-1}$ (Fig. 1a). This result is consistent with the phenotypical effects previously

**Table 1**
Enrichment of transcription factor binding site in differentially expressed genes sequences ($-1500 +200$ bp from TSS). Binding sites with Z-score > 5 and Fisher-score > 2 were considered significantly enriched and annotated with x.

| TF name | F0E | F1E | F2E | F3E | Class |
|---|---|---|---|---|---|
| DAF-16/FOXO1, FOXO-4 | | | | x | Fork head/winged helix factors |
| CES-2/DBP and TEF | | | x | x | Basic leucine zipper factors (bZIP) |
| HLH-30/MITF | | | x | x | Basic helix-loop-helix factors (bHLH) |
| ELT-2/GATA4, GATA5 | x | x | x | x | Other C4 zinc finger-type factors |
| ELT-3 | x | x | x | x | Other C4 zinc finger-type factors |
| PQM-1 | x | x | | x | C2H2 zinc finger factors |
| SMA-4/SMAD4 | | x | | x | SMAD/NF-1 DNA-binding domain factors |
| CEBP-1 | | x | | x | Basic leucine zipper factors (bZIP) |
| ZIP-8 | | x | | x | Basic leucine zipper factors (bZIP) |
| CEH-10/VSX2 | x | | | | Homeo domain factors |

**Table 2**

Table of percentage of class I and class II genes and proteins regulated by DAF-16/PQM1 in response to chronic exposure to IR. The analysis includes DEGs resulting from four exposed generations as well as genes in common between three or four generations from our study. Other datasets include: Maremonti et al., 2019 (Maremonti et al., 2019), Maremonti et al., 2019 (Maremonti et al., 2020), Dubois et al., 2019 (Dubois et al., 2019).

| | This study | | | | | Other datasets | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | F0E | F1E | F2E | F3E | Genes shared | F0 (Maremonti et al., 2019) | | F0 (Maremonti et al., 2020) | | F0 (Dubois et al., 2019) |
| Type of analysis | Transcriptomic | | | | | | | | | Proteomic |
| Irradiation time (h) | 65 | | | | | 48 | 48 | 72 | 72 | 65 |
| Dose rate (mGy/h) | 50 | | | | | 10 | 100 | 10 | 100 | 50 |
| Class I % | 15.1 | 17.7 | 6.0 | 14.0 | 18.1 | 18.2 | 25.9 | 18.9 | 11.7 | 2.8 |
| Class II % | 22.8 | 22.6 | 6.5 | 17.3 | 34.7 | 10.0 | 8.3 | 32.1 | 5.9 | 19.4 |
| Total % | 37.9 | 40.3 | 12.5 | 31.2 | 52.8 | 28.2 | 34.3 | 50.9 | 17.6 | 22.2 |
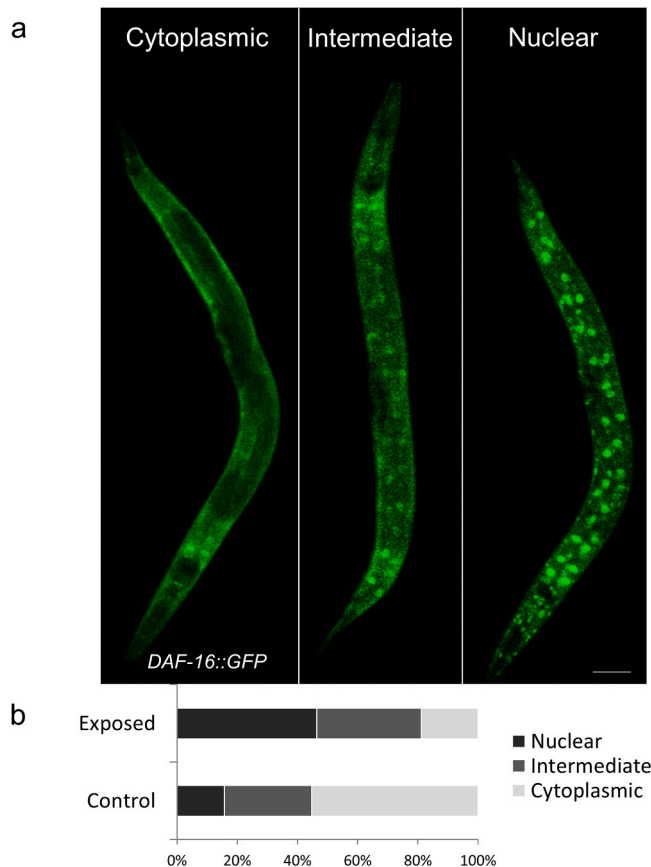


**Fig. 5.** (a) Representative picture of *DAF-16:GFP* localization in L4 YA hermaphrodites. DAF-16 localization is classified as cytoplasmic, intermediate or nuclear. Scale bar = 50 μm. (b) The proportion of *DAF-16:GFP* nuclear localization increased from 15% to 47% after chronic exposure to IR in the first generation F0. Standard deviation SD < 0.8 on each proportion.

observed in *C. elegans* upon exposure to chronic irradiation (Buisset-Goussen et al., 2014). A clear difference in terms of reproductive toxicity effect was found between F0 and the subsequent generations (23% to more than 40% decrease in number of offspring). This difference could be attributed to the fact that F0 nematodes were only exposed from the egg stage to young adults, whereas the following generations (F1 to F3) exposure included the gametogenesis event period during parental irradiation. This result showed no further effect of cumulative dose over the generations with respect to reproduction, but rather a steady state type of effect from F1 onward. This is in accordance with parental exposure to similar dose rates of gamma radiation that have recently shown to induce significant DNA damage in F1 embryos (Maremonti et al., 2019). The current study thus confirms that reproduction is a radiosensitive process in *C. elegans* (Buisset-Goussen et al., 2014;

Maremonti et al., 2019; Adam-Guillermin et al., 2012). However, the resulting offspring were viable and displayed no evident developmental defects, which is consistent with the fact that embryos upon exposure to stress display silenced cell checkpoints combined with a robust DNA repair that enables normal embryogenesis in spite of DNA-damage (Holway et al., 2006; Clejan et al., 2006). We therefore conducted a systematic investigation of central processes in germ cell maturation to unveil the cause of the reduced reproduction.

*4.2. Gametes and germ cell vulnerability to chronic gamma irradiation causes multigenerational reproductive toxicity effects in C. elegans*

In a previous study, irradiation during L1-L3 stages was demonstrated to be critical for reproduction (Maremonti et al., 2019). During these developmental stages, the precursors of the germline (Z1/Z4) and the somatic gonad (Z2/Z3) proliferate rapidly to form the gonad (Hubbard and Greenstein, 2005). In the current study, reduced number of mitotic cells in the distal gonad of irradiated nematodes in F0 and F3 generations, demonstrated a consistent impact on gamete production and germ line homeostasis (Fig. 1c.d, Fig. 2a.b), In line with these observations, Gene Ontology Phenotype analysis resulted in enrichment of terms related to "Germ cell development", "Germ cell differentiation variant" and "Gonad morphology variant" (Fig. 3a). The distal part of the gonad, containing germ cells and their niches are used to produce the germline and ensure the integrity of the subsequent generations. To further investigate the essential function of the distal gonad, radiation-induced cell proliferation arrest was assessed, which showed an increase in the frequency of enlarged cell nuclei and less condensed chromosomes (Fig. 2b; Fig. S2), indicating a potentially deleterious effect onto the germ line stem cell niches. When *C. elegans* is subjected to genotoxic stress, cell-cycle arrest and germline apoptosis are spatially separated. Cell-cycle arrest is restricted to the mitotic proliferation zone of the distal part, while apoptosis occurs in the meiotic pachytene zone, near to the bend (Craig et al., 2012). *Egl-1* acts in DNA damage-induced germline apoptosis whereas non-stress related germline apoptosis does not rely on this gene function (Gartner et al., 2018). Upregulation of *egl-1* was measured in all generations by both RNA-Seq and qRT-PCR (Fig. 4b, Fig. S3), in line with a recent study where chronic exposure to ionizing radiation was shown to induce DNA-damage germline apoptosis through *cep-1/p53* pathway (Maremonti et al., 2019). These data, thus, revealed an overall functional impact on the germ line caused by radiation-induced DNA damage consistent with the model where genetically damaged cells are removed to preserve genome integrity and progeny viability (Stergiou and Hengartner, 2004).

Further investigation of the impact on the germ line was performed by assessing the effects of ionizing radiation on spermatogenesis. In many species, the spermatozoa are among the most radiation vulnerable cells including crustaceans, mice and humans (Adam-Guillermin et al., 2018; Fukunaga et al., 2017). In *C. elegans* hermaphrodite, sperm is produced in limited amount and roughly 300 spermatids can be generated during L3-L4 stages of larval development (Chu and Shakes, 2013). Thus, the reproduction in *C. elegans* is limited by the number of sperm

produced and immediately stored in the spermatheca, which are then used to fertilize oocytes upon sexual maturation. The current study highlighted a significant, consistent and similar decrease of spermatids over the four exposed generations (Fig. 1c). The observed decrease was positively associated to the decreased number of offspring, suggesting that sperm toxicity is at the origin of the observed reproductive toxicity effects. Gene ontology term "spermathecum morphogenesis" was found significantly modulated for both F0 and F1 generations, as well as "Gametogenesis" in F1, F2 and F3 assessed by phenotype enrichment analysis (Fig. 3a,b). Consistent with the reduction of spermatids, a group of 68 genes linked to the sperm functions were downregulated, including 2 of the 5 genes required for sperm activation pathway in hermaphrodites (L'Hernault et al., 1988), three protamine sperm chromatin enriched genes, *spch-1, spch-2* and *spch-3,* histone genes and associated enzymes (Table S2), which are required for packaging of *C. elegans* sperm DNA in the transition from pachytene to mature sperm, and 46 Major Sperm Protein (MSP), required for sperm cytoskeleton dynamics, oocytes maturation and MAPK activation (Kuwabara, 2003; Yang et al., 2010) (Table S2). This indicated that both activation and sperm viability were affected. In order to test this hypothesis, sperm quality was assessed and showed a significant decrease (p-value < 0.001) in the activation of spermatids to spermatozoa in exposed nematodes (Fig. 1d). The spermatids DNA is compact chromatin transcriptionally silenced under the control of protamine and transmits epigenetic information to the embryos, which is required for proper spermatogenesis and oogenesis (Samson et al., 2014; Tabuchi et al., 2018). Collectively, these results indicate that radiation-induced decrease in sperm quality, *msp* and *spch* gene expression may influence reproduction by modification of the epigenetic information transmitted over the generations (Engert et al., 2018).

### 4.3. Molecular stress and defense mechanisms induced during multigenerational exposure to ionizing radiation

The data of the current study emphasize the difference between radiosensitive reproductive processes and the inherent robustness of somatic tissues in *C. elegans.* We therefore investigated the transcriptomic data to identify molecular mechanisms involved in defense and repair, and evidence of adaptation to chronic radiation. Response to stress, Innate immune response, Cellular response to unfolded protein and Iron transport were stress related GO terms shared between generations that involved an increasing number of DEGs over the 4 generations (Fig. 3b). A significant proportion of the increase in DEGs, observed between the first two (F0-F1) and the last two generations (F2-F3) (Fig. 4a), was related to stress response, lipid metabolism and signaling pathway.

Within the stress response categories, and consistent with increased ROS level and oxidative stress as molecular initiating events under exposure to ionizing radiation (Maremonti et al., 2020), we identified a significant up-regulation of oxidation-reduction processes in F0, F1 and F3 generations. In this regard, differential regulation of *ftn-1, cyp-25A1, cyp-25A2, acox-1.5, cyp-37B1, oga-1* and *ZK1290.5*.2, over the four exposed generations, indicated a persistent response to oxidative stress. Induction of oxidative stress by ionizing radiation leads to increased DNA damage and affects proteostasis (Maremonti et al., 2019; Dubois et al., 2019). In line with these findings, the *hsp-70 gene*, which is a member of a conserved chaperone gene family that plays a central role in stress response and clearance of protein aggregates (Kirstein et al., 2017) was found differentially regulated in all exposed generations (Fig. 4b). This gene was also found up-regulated in the crustacean model organism *Daphnia magna* after transgenerational chronic irradiation, suggesting a central role of this protein in maintaining proteostasis in response to chronic irradiation (Trijau et al., 2018). Interestingly, a recent study showed a modulation of the activity of the 20S, 26S, 30S subunit of the proteasome, in response to chronic gamma irradiation in *C. elegans* (Dubois et al., 2018).

Previous studies have shown that chronic exposure to ionizing radiation induces a modulation of lipid metabolism, via biosynthesis of unsaturated fatty acid, both in mice and nematode models [85]. Especially, it has also been shown that under persistent DNA damage caused by UV radiation, *C. elegans* adapts its metabolism by modulating fatty acids synthesis, via EGF, DAF-2/DAF-16 and AMPK like pathways (Edifizi et al., 2017). In addition, an increase in lipogenic processes after germline ablation has been shown to restore lipid homeostasis, by shifting from reproduction to longevity through DAF-16 and TCER-1 pathways (Amrit et al., 2016). In the present study, transcription factors and transcriptomic analysis showed significant up-regulation of *daf-16* as well as significant differential regulation of genes under control of insulin DAF-2/DAF-16 pathway, such as *pud-2.1, pud-3, pept-1* and *dod-21.* This was in line with the GO terms showing the influence on multiple signaling processes including regulation of insulin receptor signaling pathway, sphingolipid mediated signaling pathway, hormone biosynthetic process and pri-miRNA transcription from RNA polymerase.

Collectively, the observed modulation of protein and lipid metabolism, innate immune response, proteolysis, peptide activity and protein splicing, implies a cumulative stress that required activation of additional repair and signaling processes in the last two generations (F2-F3) (Fig. 3b).

### 4.4. Upstream transcription factors modulating gene expression over multiple irradiated generations

Transcription factor enrichment and gene networks analyses were performed to identify the key regulators involved in the radiation-induced transcriptional responses. Compiled DEGs resulting from the four exposed generations, identified transcription factors related to stress response and lipid and protein metabolism (Table 1). Specifically, ELT-2/GATA4-GATA5 and ELT-3 were among the most significantly enriched transcription factors in all exposed generations. Both are regulated during aging, causing the regulation of hundreds of genes (Budovskaya et al., 2008; Mann et al., 2016). ELT-2 is the main regulator of ageing through differentiation, maintenance and function of the intestine (Wiesenfahrt et al., 2016), essential to lipid storage and metabolism in *C. elegans* (Mullaney and Ashrafi, 2009). Overexpression of *elt-*2 significantly increases the lifespan, while the rate of gene expression changes that occur during normal aging is slowed (Mann et al., 2016), which implies that ionizing radiation affects *C. elegans* lipid metabolism, intestine, and ageing processes.

CES-2/DBP, TEF and HLH-30/TFEB were enriched in the 2 last generations (F2 and F3). CES-2 is a co-regulator of autophagy and apoptotic cell death pathways, which may indicate intensification of such toxicological responses over generations (Erdélyi et al., 2011). HLH-30 forms a transcriptional regulatory complex that translocates into the nucleus to promote stress resistance and longevity (Lin et al., 2018). Moreover, HLH-30 acts in peroxide stress response via activation of *ftn-1* (Quach et al., 2013), which was found significantly upregulated in all exposed generations (Fig. 4b). HLH-30 is also a regulator of both lipid metabolism and localization, through lipolysis and autophagy (O'Rourke and Ruvkun, 2013; Lapierre et al., 2013). At a certain threshold of persistent DNA damage, there is a switch from proteasome and chaperones expression towards autophagy (Edifizi et al., 2017), which also supports the notion that the last two generations experienced higher stress (Fig. 3b).

Furthermore, PQM-1, a central regulator of stress response, was enriched in 3 exposed generations (F0, F1 and F3). PQM-1 acts under control of DAF-2/DAF-16, mTOR and microRNAs to regulate fat storage through vitellogenin genes (Dowen et al., 2016). PQM-1 is also required for proteostasis and systemic chaperone signaling in order to reduce protein aggregates (O'Brien et al., 2018). PQM-1 and DAF-16 act as mutual antagonists on nuclear localization and transcription of Class I and II target genes (Tepper et al., 2013). Regulation of PQM-1 and

DAF-16/FOXO by the Insulin and IGF-1 signaling (IIS) pathways allows a rapid switch between stress response to reproduction, development, and metabolism (Zečić and Braeckman, 2020). In particular, the IIS pathway governs part of the development and extends lifespan by upregulating genes related to stress response and metabolism (class I), and by downregulating genes involved in development and reproduction (class II). DAF-16 regulates class I genes through the DAF-16 binding element (GTAAACA or TGTTTAC), whereas PQM-1 controls class II genes through DAF-16 associated elements (TGATAAG or CTTATCA). In relation to the enhanced transcriptomic stress response observed in the last generation, the well-characterized stress-induced transcription factor DAF-16/FOXO was found significantly enriched as well as the DAF-16 co-regulator PQM-1 and HLH-30 in the other generations (Table 1).

In the present study, consistent with the significant upregulation of class I genes, the translocation of DAF-16 transcription factor to the nucleus and hence its activation was shown in 47% of the exposed nematodes (Fig. 5b). This strongly suggests that, as environmental stressor, chronic exposure to ionizing radiation affects the transcriptional regulation over multiple generations, by inducing metabolic changes to modulate the allocation of resources, and a switch from reproduction and growth to cellular maintenance and repair (Henderson and Johnson, 2001; Mueller et al., 2014). However, the partial or cytoplasmic localization of DAF-16 in 45% of the exposed population as well as the proportion of class II genes indicated that other regulators were involved in radiation-induced stress response.

Thus, we further investigated the implication of DAF-16 by comparing the presence of class I and II genes and proteins under DAF-16/PQM-1 control with previously published omics datasets from chronic irradiation of *C. elegans*. In the current study, between 12.5% and 40.3% of class I and II genes were identified over the 4 exposed generations, where 52.8% were shared by 3 or 4 generations. In recent transcriptomic analysis carried out after 48 and 72 h of irradiation at 10 and 100 mGy h$^{-1}$ (Maremonti et al., 2019), similar rates from 17.6% to 50.9% were observed (Table 2). Moreover, a proteomic analysis on nematodes exposed to similar doses of chronic gamma radiation showed that 22% of proteins from class I and II genes were overexpressed (Dubois et al., 2019). Thus, these findings indicate that DAF-16 and PQM-1 constitute major regulators in response to single or multigenerational exposure to chronic irradiation.

Collectively, the transcriptomic and transcription factors enrichment analyses indicate that chronic irradiation elicits a cumulative effect manifested as a stronger transcriptional level response to stress in the last two generations (F2 and F3), which underlines the importance of performing multigenerational exposures. Non-targeted effects may also play a significant role in the effect induced by chronic irradiation. Indeed, microbeam irradiation of somatic cells increases DNA damage in the germ line as well as genomic instability in the F1 generation and affects several signaling pathways (Guo et al., 2013).

## 5. Conclusions

Multigenerational exposure to gamma radiation causes a significant and persistent reproductive toxicity effect in *C. elegans*, due to impact on gametes production. Specifically, radiation-induced cell cycle arrest and increased DNA damage-induced germ cell apoptosis, which caused a consistent reduction in the number of germ cells. The main cause of reproductive toxicity in all generations was decreased quality and quantity of sperm cells. The transcriptomic responses were overall directed towards counteracting stress and damage and increased sharply over the last two generations (F2-F3). This included defenses against oxidative stress and damage to DNA and proteins as well as a metabolic shift, and activation of DAF-16/FOXO stress resistance pathways. The adopted systems biology approach thus facilitated an improved understanding of the ionizing radiation toxic mode of action and linked phenotypic impact with underlying molecular mechanisms over

multiple exposed generations in *C. elegans*. Difference between generations underlines the importance of investigating transgenerational inherited effects from ionizing radiation exposure. This may involve heritable genetic or epigenetic effects of radiation onto subsequent generations which should be further investigated.

## CRediT authorship contribution statement

**Rémi Guédon**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Erica Maremonti**: Investigation, Methodology, Software, Writing – original draft, Review. **Olivier Armant**: Methodology, Software, Formal analysis, Review. **Simon Galas**: Conceptualization, Methodology, Resources, Review. **Dag Anders Brede**: Conceptualization, Project administration, Resources, Methodology, Writing – original draft, Review. **Catherines Lecomte-Pradines**: Conceptualization, Data curation, Project administration, Resources, Supervision, Methodology, Validation, Writing – original draft, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

## Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

## Data availability

All transcriptomics data are accessible in the GEO repository under the accession number GSE175918.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112793.

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