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Differential modification of the *C. elegans* proteome in response to acute and chronic gamma radiation: link with reproduction decline

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Abstract (300 words)

Emission of ionizing radiation (IR) in the environment is a natural phenomenon which can be enhanced by human activities. Ecosystems are then chronically exposed to IR. But environmental risk assessment of chronic exposure suffers from a lack of knowledge. Extrapolation of data from acute to chronic exposure is not always relevant, and can lead to uncertainties as effects could be different between the two irradiation modes, especially regarding reproduction endpoint, which is an ecologically relevant parameter. In the present study, we decided to refine the understanding of the molecular mechanisms involved in response to acute and chronic γ -irradiation by a global proteome label free LC-MS/MS analysis. *C. elegans* were exposed to 3 common cumulated radiation doses for acute or chronic exposure condition and global modification of the proteome was studied. This analysis of protein expression has demonstrated the modulation of proteins involved in regulatory biological processes such as lipid transport, DNA replication, germ cell development, apoptosis, ion transport, cuticle development, and aging at lower doses than those for which individual effects on reproduction have been previously observed. Thus, these proteins could constitute early and sensitive markers of radio-induced reprotoxicity; more specifically HAT-1, RPS-19 in acute and VIT-3 for chronic conditions that are expressed in a dose-dependent manner. Finally, to focus on reproduction process, this analysis showed either repression or overexpression of 12 common proteins in organisms exposed to acute or chronic irradiation, respectively. These proteins include the vitellogenin cluster notably involved in lipid transport and oocyte maturation and proteins

29 involved in cuticle development and molting *i.e* COL-14, GLF-1, NOAH-1, NOAH-2, ACN-1. This results
30 show that protein expression modulation is a sensitive and predictive marker of radio-induced
31 reproductive effects, but also highlight limitation of data extrapolation from acute to chronic
32 exposure for environmental risk assessment.

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Title Page

Protein expression differently modulated after acute and chronic exposure to γ -rays.

Keywords

Chronic vs acute exposure; gamma-rays; *C. elegans*; Proteome modulation; risk assessment

Introduction

Emission of ionizing radiation in the environment is a natural phenomenon which can be enhanced by human activities. Therefore, ecosystems are chronically exposed to ionizing radiations. In this context, ecologically relevant predictions of long-term biological effects induced by chronic doses of ionizing radiation on nonhuman biota are necessary. But environmental risk assessment of chronic exposure suffers from a lack of knowledge and a lack of sensitivity [1, 2]. Data extrapolation from acute to chronic exposure is not always relevant, and can lead to uncertainties. Indeed, for a same dose, radio-induced effects are often different between the two irradiation modes, especially regarding the reproduction endpoint, which is an ecologically relevant parameter directly influencing population dynamics [3-5].

Moreover one of the limitations of the risk assessment conducted on major physiological functions is their sensitivity. The use of molecular markers, usually more sensitive and modulated before individual-level effects, could be a solution. However, studies on cellular and molecular levels represent only 7 and 12%, respectively of the studies on environmental species [6]. In addition, the difficulty is then to be able to associate these molecular changes with the consequences on physiological functions [7, 8]. In this sense, scientific advances have been made in the understanding of the radiation-induced molecular and cellular mechanisms. However, to date, underlying molecular mechanisms governing the differences in the observed effects are poorly understood [9, 10]. While effects of ionizing radiation on DNA, have been extensively described and are now rather well-understood, the contribution of other radiation-induced molecular alterations, especially on proteins remains unclear. Proteins, which are the functional molecules of organisms, might be relevant biomarkers. Few studies have investigated the impact of an acute exposure to ionizing radiation (0.3-

3 Gy) on mammal cell proteome and showed that modulation of protein expression could be a relevant biomarker to detect ionizing radiation exposure, to predict severity of associated lesions and ultimately to manage clinically these lesions [11-13]. However, these proteomic studies concern only acute irradiation of mammals. Data concerning the proteome sensitivity of non-human biota after chronic irradiation are scarce.

The free living nematode *Caenorhabditis elegans* is a particularly convenient model organism to address this environmental risk assessment based proteomic issue [14, 15]. With its fully sequenced genome and its short life cycle, *C. elegans* has been successfully used to study acute and chronic irradiation effects and their consequences on germline development and hatching [4, 16-19].

Indeed our first results showed that a decrease of the number of progeny associated with a decrease of the embryo hatchability occurred from and above 30 Gy of acute irradiation [4]. In this paper, the decrease of the progeny number per individual have been hypothesized to be correlated to an increase of apoptosis whereas an explanation for the decrease of hatching success can be unrepaired DNA-damage then leading to non-viable eggs.

After chronic irradiation, a recent study of our team has shown that reproduction is the more sensitive macroscopic parameter regarding survival and growth [17]. Moreover, our first study has also highlighted that, contrary to acute irradiation, chronic irradiation from 3.3 Gy induced a decrease of the number of progeny without impacting the hatching success [4]. This could suggest that in such conditions, gametogenesis is more impacted than embryogenesis. However, mechanisms have not been fully elucidated yet; some of our team results coming from a multi-generation study revealed that, after three generations continuously irradiated, an increase of apoptosis, a decrease of the sperm cells number and an oocytes cell cycle arrest could explain this phenomenon[3]. As proteins are involved in key biological processes, including DNA repair, cell cycle control and apoptosis, as our first results have also shown that proteolytic response of cells are different between acute and chronic [4], it seems relevant to assess their global expression after both acute

and chronic exposure in order to better understand the toxicity mechanisms in response to ionizing radiations.

Therefore, in the present study, we decided to refine the understanding of the molecular mechanisms involved in response to acute and chronic irradiation, *i.e.* reprotoxicity effects demonstrated in our first publications [4, 17], by a global proteome analysis. *C. elegans* were exposed to 3 common cumulated radiation doses of acute or chronic exposure. Because the 3 radiation doses were either comparable or lower than the ones used in previous works showing an effect on the reproduction, we then expected to identify early and sensitive biomarkers of the impaired reproduction and improve risk assessment sensitivity. After radiation exposure, the global modification of the proteome was studied by using both a DIGE and a label free LC-MS/MS proteomic approach. Our objectives were to test the following hypotheses: (1) whether or not the proteome expression correlated both with the dose and the irradiation mode; (2) if the proteome expression modification was associated with effects on reproduction then leading to a direct link with an ecological risk assessment.

Material and methods

C. elegans maintenance and age synchronization

The wild-type N2 strain of *C. elegans* provided by CGC (*Caenorhabditis* Genetic Center) was used in this study. Populations were maintained at 19°C and 80% of humidity on 9cm petri dishes poured with NGM (Nematode Growth Medium) and seeded with *Escherichia Coli OP50* as food source. *E. coli* OP50 were grown in L-Browth medium at 37°C overnight. Petri dishes were seeded with 1mL of saturated culture of bacteria and UV killed (Bio-Link Crosslinker, $\lambda = 254$ nm; intensity = 200 mWm⁻²) for 20 minutes to avoid food heterogeneity between dishes. 100 gravid worms were randomly selected from the stock population and placed on 9 cm petri dishes. 96 h later, eggs were separated from adult worms by a bleaching procedure and collected embryos were allowed to grow in a control incubator for 96 h. The gravid worms were separated from eggs already laid by a sucrose gradient (3 – 7 %), and then re-synchronized by a bleaching procedure in order to collect the eggs *in utero* synchronized over 3 h.

Irradiation

Irradiations were performed in incubators (19 °C and 80 % humidity) in controlled conditions; data loggers were used in order to measure humidity and temperature during irradiation. Nematode plates were placed perpendicularly or parallelly (for acute and chronic exposure respectively) to the cesium-137 source to obtain a homogeneous dose rate at the surface of the plate. Radio Photo Luminescent dosimeters (RPL, GD-301 type, Chiyoda Technol Corporation, Japan) were placed on each experimental unit in order to measure the delivered cumulated dose received by organisms. At the end of each irradiation, worms were collected, rinsed with M9 medium (5 g.L⁻¹ NaCl, 25 mM KPO₄ buffer and 1 mM MgSO₄) to ensure bacteria removal, centrifuged and pellets were snap frozen.

Acute - For acute irradiation, 3000 age-synchronized embryos were transferred to fresh 6 cm plates and allowed to reach L4-YA stage in a control incubator. Nematodes were then irradiated with a cesium-137 source (200 TBq) using the GSR-D1 apparatus from RadExpe platform (Curie Institute,

France). L4-YA *C. elegans* were irradiated at $1 \text{ Gy} \cdot \text{min}^{-1}$ during different times in order to test 3 cumulated doses (excluding control): 0.5, 1 and 3.3Gy.

Chronic - For chronic irradiation, 3000 age synchronized nematodes were exposed to cesium-137 source using the platforms MIRE (Mini Irradiator for Radio-Ecology) (1.6 GBq) from embryo stage to L4-YA adult stage to cover the complete lifecycle (65 h). Three dose rates (excluding controls): 7, 14, 50mGy.h⁻¹ corresponding to three cumulated doses (0.5, 1 and 3.3Gy) were tested.

Protein extraction and purification

After irradiation, 3000 *C. elegans* per replicate were subjected to protein extraction. 300 µl of 0.5-mm diameter zirconium beads and an equal amount of lysis buffer (30 mM Tris-HCl pH 7.4, 150mM NaCl, 1.0%(v/v) Igepal CA-630 (NP-40), 1%(v/v) TritonX-100, 0.5% (w/v) sodium deoxycholate, 0.1%(w/v) sodium dodecyl sulfate (SDS), 2%(v/v) glycerol, 2 mM 1,4-dithiothreitol (DTT), 1 mg.ml⁻¹ leupeptin, 1 mg.ml⁻¹ aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA)) were added on top of worm pellets and incubated for 15 min on ice. *C. elegans* were then homogenized by three 6800-rpm cycles in the Precellys grinder system (Bertin Technologies, Montigny-le-Bretonneux). After 1 h incubation on ice, lysates were centrifuged (13500 g) at 4°C for 15 min. Supernatant was sampled, protein concentration was determined using the BCA kit (Thermo Scientific) using BSA as a standard, according to the manufacturer's instructions and the remaining volume quick frozen with liquid nitrogen.

20 µg of proteins were precipitated on ice for 20 min by the addition of 10 % TCA (v/v). After washing steps, pellets were resuspended in UTC9231 (9 M urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (w/v) ASB14, 20 mM Tris, pH 9.5) under stirring (1600 rpm) at 30°C in the dark for two hours.

Label free sample preparation and mass spectrometry analysis

15 µg of proteins per replicate were loaded on a NuPAGE gel 4-12% (Life Technologies). Samples were then subjected to electrophoresis during 6min at 80V using a MOPS buffer (Thermo Fisher

Scientific) in order to stack proteins on the top of the gel before proceeding to coomassie blue staining. Protein bands were then excised with a scalpel and digested with trypsin. Each condition (0.5, 1 and 3.3 Gy) was injected in 3 biological replicates and 2 technical replicates in liquid chromatography (Ultimate 3000 RSLCnano chromatography system (Thermo Fisher Scientific)) coupled with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). First peptides were concentrated and purified on a pre-column from Dionex (C18 PepMap100, 2 cm × 100 µm I.D, 100 Å pore size, 5 µm particle size) in solvent A (0.1% formic acid in 2% acetonitrile). In the second step, peptides were separated on a reverse phase LC EASY-Spray C18 column from Dionex (PepMap RSLC C18, 50 cm × 75 µm I.D, 100 Å pore size, 2 µm particle size) at 300 nL/min flow rate and 40°C. After column equilibration using 4% of solvent B (20% water - 80% acetonitrile - 0.1% formic acid), peptides were eluted from the analytical column by a two steps linear gradient (4-20% acetonitrile/H₂O; 0.1 % formic acid for 220 min and 20-45% acetonitrile/H₂O; 0.1 % formic acid for 20 min). For peptide ionization in the EASY-Spray nanosource, spray voltage was set at 2.2 kV and the capillary temperature at 275 °C. The mass spectrometer was used in data dependent mode to switch consistently between MS and MS/MS. Time between Masters Scans was set to 3 seconds. MS spectra were acquired with the Orbitrap in the range of m/z 375-1500 at a FWHM resolution of 60 000 measured at 200 m/z. AGC target was set at 4.0.10⁵ with a 50 ms Maximum Injection Time. The more abundant precursor ions were selected and collision induced dissociation fragmentation at 35% was performed and analyzed in the ion trap using the “Inject Ions for All Available Parallelizable time” option with a maximum injection time of 105 ms and an AGC target of 1.0.10⁵. Charge state screening was enabled to include precursors with 2 and 7 charge states. Dynamic exclusion was enabled with a repeat count of 1 and a duration of 60s. These chromatographic conditions were previously optimized with a protein pool from all the samples.

Quantitative proteomics processing

For data processing we used the free suite MaxQuant version 1.5.3.8[20]. The relative intensities based on label-free quantification (LFQ) were calculated using the MaxLFQ algorithm[21]. The 48 LC-

183 MS raw acquisitions were processed by the Andromeda search engine integrated into MaxQuant[21].
184 The identification of the precursor ions present in the mass spectra was performed by comparison
185 with the protein database of *C. elegans* extracted from UniProt on the 17th of January, 2017 and
186 containing 28,794 entries. This database was supplemented with a set of 245 proteins that are
187 commonly found as contaminants. The following parameters were used for this search: (i) trypsin
188 cleavage authorization before prolines; (ii) authorization of two failed cleavages; (iii) fixed
189 modification of cysteines by carbamidomethylation (+57.02146 Da) and variable modification of
190 methionines by oxidation (+15.99491) and N-terminal proteins by acetylation (+42.0116); (iv)
191 authorization of 5 modifications per peptide; and (v) minimum peptides length of 7 amino acids and
192 a maximum mass of 4600 Da.

193 Spectra alignment was performed in two dimensions; the elution time of the precursor ions (min)
194 and the mass over charge (m/z ; amu). The "Match between runs" option has been enabled to allow
195 the transfer of identifications between LC-MS/MS based on the mass and the retention time using
196 the default settings. The false positive rate on identification was set at 1%. The statistical analysis was
197 carried out with the Perseus program (version 1.6.0.7) of the MaxQuant environment. The
198 normalized intensity LFQ was transformed by a base logarithm 2 to obtain a normal distribution.
199 Differential protein expressions were evidenced by the application of a multiple ANOVA t-test or
200 student t-test performed by controlling the false positive rate at 1% using 250 permutations. Proteins
201 differentially expressed between samples were analyzed based on the log2 difference of the LFQ
202 intensity of the protein between controls and the different doses (0.5Gy vs. control, 1Gy vs. control
203 and 3.3Gy vs. control) and log10 of the associated *p. value*. The differential proteomics analysis was
204 carried out on identified proteins after removal of proteins only identified with modified peptides,
205 peptides shared with other proteins, proteins from contaminant database and proteins which are
206 only represented in 2 replicates of 6 of the same condition. The mass spectrometry proteomics data,
207 including search result, have been deposited to the ProteomeXchange Consortium
208 (www.proteomexchange.org) via the PRIDE partner repository with datasets identifiers PXD011731.

209

210 **Enrichment analysis**

211 Following the differential identification of proteins expressed after irradiation in *C. elegans*, an
212 analysis of biological functions was performed using the DAVID gene interaction tool[22]. Indeed, the
213 proteins have been grouped according to the biological processes that they govern (GOTERM) for a
214 simplified analysis. After selection of the model organism (*C. elegans*), the UniProt accession number
215 of the differently expressed major proteins were implemented in the David gene tool, as well as the
216 background noise (all the majority proteins identified in our study). Then, the biological processes, in
217 which the variant proteins were involved between the different irradiation conditions, were searched
218 and classified according to their ease score (<0.1); excel files of each David gene analysis is given as
219 supplementary data: Suppl-File8_Acute ttest David Ease 01_GO-BP.xls, Suppl-File7_Acute ANOVA
220 David Ease 01_GO-BP, Suppl-File3_Chronic ttest David Ease 01_GO-BP, Suppl-File4_Chronic ANOVA
221 David Ease 01_GO-BP. Finally, biological processes were sorted and presented in this article
222 according to their *p. value* (<0.05), and results visualization with treemap was done using Revigo tool
223 to avoid redundancy between the different biological processes (medium similarity (0.7); semantic
224 similarity measure to use SimRel, and *C. elegans* database)[23].

225

226

Results

Chronic exposure to gamma-rays

Mis-regulation of protein expression from 0.5 Gy of chronic gamma radiation exposure.

The analysis of differential protein expression by the 2D-DIGE methodology (Supplementary data) showed only one protein spot as variant after chronic irradiation of *C. elegans* from 0.5 to 3.3 Gy, compared to controls. A label-free approach was then performed on the same samples in order to increase the sensitivity of the proteome analysis. Indeed, the label free LC-MS/MS based proteomic approach was able to identify 2647 proteins. The most abundant identified proteins with high iBAQ (intensity-based absolute quantification) and the less abundant proteins with low iBAQ are presented in supplementary **Figure S1**. This figure shows that our proteomics analysis covers a dynamic range around 6 log of protein intensity (orders of magnitude). ANOVA analysis was performed on all conditions, 168 proteins were found significant ($FDR < 0.01$) and their z-scored LFQ values were heat-mapped (**Figure 1** - Exhaustive data are given as supplementary excel file: Suppl-File1_ANOVA conditionChronic 4clusters.xls) showing 4 clusters of proteins (A, B, C, D) within the whole targets, 2 of them (A and D) making it possible to distinguish control and irradiated conditions whereas the two others tend to isolate the 1 Gy exposure condition. The cluster A encompassed proteins that were mainly decreased in the irradiation conditions group versus the controls. The cluster D included proteins which increased under irradiation versus control. Finally, cluster B and C contained proteins that were more or less upregulated in one specific irradiation condition 0.5 and 1 Gy respectively. As presented in **Erreur ! Source du renvoi introuvable.** (Exhaustive data are given as supplementary excel file: Suppl-File2_Chronic-fdr001so01-pairwise full data.xls), the further pairwise analysis of the protein differential expression showed that among the 2647 proteins, 87 were significantly mis-regulated following chronic exposure (sum of the misregulated proteins after pairwise analysis of conditions - \log_2 fold change $> \pm 0.58$ and $\log p.value > \pm 1.3$). Finally, 51% of these 87 mis-regulated proteins were over-expressed compared to controls, while 49% of proteins were repressed. In addition, some of the misregulated proteins were common between the 3 doses (25.6 %). More

specifically, 42 (23 up-, 19 down-), 52 (24 up-, 28 down-) and 39 (14 up-, 25 down-regulated) proteins were mis-regulated compared to controls at 0.5 Gy, 1 Gy, and 3.3 Gy, respectively. These results show that proteome modulation between control and irradiated organisms occurs as soon as 0.5 Gy.

[Figure 1]

Figure 1: Chronic irradiation-heat map representing the Z-Scored LFQ intensity for each protein determined as significant after ANOVA analysis over the 3 groups. The top represents the different conditions including biological and analytical replicates. Four protein clusters annotated A, B, C and D are highlighted according to the ANOVA analysis between the 3 doses.

Protein enrichment analysis makes possible to associate protein expression level and radio-induced reproductive disturbance

Biological processes associated to misregulated proteins found in the pairwise analysis were evaluated using gene ontology enrichment to find the possible disturbed pathways. A sum-up was done in **Figure 2**, the full results are in the excel file Suppl-File3_Chronic ttest David Ease 01_GO-BP.xls. We found that misregulated proteins are involved in lipid transport (9% in lipid transport, p. value = 2.6×10^{-5} ; 12.8% in lipid localization, p. value = 0.01; 3.8% in divalent inorganic cation transport, p. value = 0.04; 3.8% in divalent metal ion transport, p. value = 0.04; 17.9% in single-organism transport, p. value = 0.05), in cuticle development (7.7% in cuticle development, p. value = 0.005; 3.8% in molting cycle process, p. value = 0.007; 11.5% in molting cycle, p. value = 0.01; 12.8% in germ cell development, p. value = 0.03), in DNA-dependent DNA replication (6.4% in DNA-dependent DNA replication, p. value = 1.3×10^{-4} ; 6.4% in DNA metabolic process, p. value = 0.03), in DNA unwinding (5.1 %, p. value = 0.002) and in cellular divalent inorganic cation homeostasis (3.8%, p. value = 0.01). In addition, protein enrichment was performed on ANOVA data (**Figure S2**) and particularly on clusters A and D to analyze the biological processes distinguishing control and irradiated conditions (**Table 1**; Suppl-File4_Chronic ANOVA David Ease 01_GO-BP.xls).

Table 1: Gene ontology enrichment based analysis of biological process associated to the 4 protein clusters found after ANOVA analysis between the 3 doses of chronic exposure with Z-scored value of LFQ, and their associated p. values.

[Table 1]

Cluster A corresponds mainly to biological processes such as cuticle development (9.5% of proteins and p. value = 1.3×10^{-3}), molting cycle (14% of proteins and p. value = 2×10^{-3}), regulation of growth

(16%, p. value = 8.4×10^{-4}), defense response (9.5%, p. value = 3.7×10^{-2}) and locomotion (28%, p. value = 1.5×10^{-2}). The cluster D corresponds to biological process such as embryo development ending in birth or hatching (61%, p. value = 5×10^{-9}); DNA replication (15.9%, p. value = 3.8×10^{-7}), nitrogen compound metabolism (41%, p. value = 7.5×10^{-4}) and lipid transport (13.6%, p. value = 1.8×10^{-6}). For a simplified analysis, we chose to only analyze and describe biological processes that are potentially linked with reprotoxicity or radiotoxicity, *i.e.* biological process such as embryo development ending in birth or hatching, cell cycle processes and DNA replication processes. All of these proteins are overexpressed in at least one dose compared to control.

[Figure 2]

Figure 2: Independent DAVID gene functional enrichment analysis based on results of all proteins identified as modulated after chronic irradiation (t-test, pairwise comparison). Significant GO-term biological processes (p. value <0.05) were then summarized using REVIGO. The % of proteins involved in the process is written in each case. Tree maps show a two-level hierarchy of GO terms (main clusters and cluster members); the size of the rectangles is relative to the \log_{10} (p. value) absolute. With a same color, biological processes belonging to one main head process: lipid transport (purple), cuticle development (blue), DNA-dependent DNA replication (yellow).

Interestingly, the ones involved in lipid transport but also in germcell development and particularly in oocyte maturation are VIT-1; VIT-2; VIT-3; VIT-4; VIT-5; VIT-6. These proteins are over-expressed from 0.5 Gy to 3.3 Gy and are yolk protein precursors (Figure 3A).

[Figure 3]

Figure 3: A/ Box plot of Vit-2 protein label free intensity for the three irradiation doses (C0: control, C1: 0.5 Gy, C2: 1 Gy, C3: 3.3 Gy). B/ Box plot of MCM-2 protein label free intensity for the three irradiation doses (C0: control, C1: 0.5 Gy, C2: 1 Gy, C3: 3.3 Gy).

In addition, the 2 proteins involved in the “embryo development ending in birth or hatching” biological process excepted vitellogenins are CPG-1 and CPG-2, two chondroitin proteoglycan protein that are over-expressed at 0.5 Gy compared to controls conditions, but there are not mis-regulated at higher doses tested. They are required for polar body extrusion during cytokinesis in embryo development and in meiotic chromosome segregation[24]. CPG-1 and CPG-2 are also involved in the cellular division process, mainly occurring in germ cells of *C. elegans*[24]. Moreover, the 4 proteins belonging to the “DNA replication pathway” process are MCM-2, MCM-3, MCM-6 and MCM-7

(**Figure 3B**), helicases involved in DNA replication after DNA repair for example that are overexpressed at 0.5 and 1 Gy. The MCM complex is expressed in all dividing cells during embryonic and postembryonic development, and is associated with chromatin[25].

Proteins involved in reproduction process are mis-regulated as soon as 0.5 Gy.

To answer our main question on the radio-induced toxicity mechanisms and determine relevant putative biomarkers of chronic exposure, we compared the mis-regulated proteins for the 3 radiation doses. The comparison highlighted 21 proteins in common. Results are presented in **Figure** .

[Figure 4]

Figure 4: Comparison of the differential proteins found in the 3 different conditions of chronic irradiation. A) Venn diagram of mis-regulated proteins (up or down-expressed) between 0.5, 1 and 3.3 Gy. The overlaps between conditions represent the amount of shared proteins within the conditions of interest. B) Heatmap of the Z-scored label free intensity of the 21 proteins in common between the three irradiation doses.

Among the 21 proteins in common between the three tested doses, 13 are annotated in UniProt database, and are presented in **Table** . Among them VIT-1, VIT-2, VIT-3, VIT-4, VIT-5 and VIT-6 have already been identified in biological processes of interest. As indicated in **Table** , these proteins are equivalently modulated between control and the three irradiated groups, *i.e.* at 0.5, 1 and 3.3 Gy. Chronic exposure has an effect on expression of proteins involved in reproduction as soon as 0.5 Gy.

Table 2: List of the common annotated misregulated proteins after chronic exposure to γ -rays at 0.5, 1 and 3.3Gy; modulation of their associated expression for each of the conditions. Differences are given as the log2 of the protein intensity ratio between the control and irradiated worms (negative or positive values, means that the protein is over-expressed or repressed compared to control respectively).

[Table 2]

Acute exposure to gamma-rays

Misregulated proteins in response to 3 moderate doses of acute gamma radiation

In order to compare the global change of *C. elegans* proteome after acute vs. chronic exposure for a same cumulated dose and understand our radioinduced reprotoxicity results[4], we also performed a global analysis of the proteomic changes induced in *C. elegans* by acute gamma radiation from 0.5 to

3.3 Gy. Similarly to chronic exposure, 2D-DIGE methodology was used in the first instance (Supplementary data), refined by a label free LC-MS/MS based proteomic approach. The label free LC-MS/MS based proteomic approach was able to identified 2677 proteins. Similarly to chronic, the most abundant identified proteins with high iBAQ and the less abundant proteins with low iBAQ are presented in supplementary **Figure S1**. ANOVA analysis was performed on all conditions, 369 proteins were found significant (FDR<0.01) and their z-scored LFQ values were heat-mapped (**Figure 5** - Exhaustive data are given as supplementary excel file: Suppl-File5_ANOVA condition Acute 2 clusters.xls) showing 2 clusters of proteins (Cluster A and Cluster B) within the whole targets. Both clusters show that the condition 0.5 Gy is very close to the control. The cluster A encompassed proteins that were mainly increased in the two higher irradiation conditions group versus the controls. Conversely, the cluster B included proteins which decreased in the two higher irradiation conditions versus control. As presented in **TableS3** (Exhaustive data are given as supplementary excel file: Suppl-File6_Acute-fdr001so01 pairwise full data.xls), a further pairwise analysis of the protein differential expression, showed that among the 2677 proteins, 338 were significantly mis-regulated following acute exposure (sum of all misregulated proteins after pairwise analysis of conditions - log2 fold change > ± 0.58 and *log p.value* > ± 1.1). Most of these 338 mis-regulated proteins (70%) were over-expressed compared to controls, while 30% of proteins were repressed. In addition, some of them (2) were common between the 3 doses (0.6 %). More specifically, only 2 proteins (RPS-19 and HAT-1) were repressed at 0.5 Gy compared to controls. Then, at 1 Gy and 3.3 Gy, 32 (20 up-, 12 down-regulated) and 335 proteins (103 up-, 232 down-regulated) were mis-regulated, respectively. These results show a large deregulation of the proteome with increasing dose of radiation. Proteome modulation occurs as soon as 0.5Gy but only a small number of proteins is concerned.

[Figure 5]

Figure 5: Acute irradiation-heat map representing the Z-Scored LFQ intensity for each protein determined as significant after ANOVA analysis over the 3 groups. The top represents the different conditions including biological and analytical

replicates. Two protein clusters annotated A and B are highlighted according to the ANOVA analysis between the 3 doses.

Protein enrichment analysis after acute irradiation makes possible to associate protein expression level and radio-induced reproductive disturbance.

Based on gene ontology enrichment (GO) and associated *p. values*, biological processes associated to mis-regulated proteins found in the pairwise analysis of label free LC-MS/MS proteomic approach were evaluated using gene ontology enrichment to find the possible disturbed pathways (Exhaustive data are given as supplementary excel file: Suppl-File7_Acute ttest David Ease 01_GO-BP.xls). The sum-up of the analysis is showed in **Figure 6**. In addition, protein enrichment was performed on ANOVA clusters (**Figure S3; Table3**) to analyze the biological processes distinguishing control and the highest doses of irradiation conditions. Exhaustive list of biological processes found for each set of data are given in supplementary data (Suppl-File8_Acute ANOVA David Ease 01_GO-BP.xls). For a simplified analysis, we then chose to analyze and describe biological pathways that are potentially linked with reprotoxicity or radiotoxicity. Results on protein enrichment have been discussed mainly on the proteins found to be modulated after pairwise analysis.

[Figure 6]

Figure 6: Independent DAVID gene functional enrichment analysis on the basis of results of all proteins identified as modulated after acute irradiation (t-test, pairwise comparison). Significant GO-term of biological processes (*p.value* <0.05) were then summarized using REVIGO. The tree maps show a two-level hierarchy of GO terms (main clusters and cluster members); the size of the rectangles is relative to absolute of log10(*p value*).

The misregulated proteins were mainly involved in cuticle development process (with involvement in 9 sub-processes: cuticle development, aging, embryo development ending in birth or egg hatching, embryo development, larval development, post-embryonic development, determination of adult lifespan, developmental growth, collagen and cuticulin-based cuticle development), alpha-amino acid metabolism (with involvement in 6 sub-processes: single-organism biosynthesis of organonitrogen compounds, organic substances and glycosyl compounds, metabolism of alpha amino acids and organonitrogen compounds), mitochondrion organization (mitochondrion organization, 4% proteins,

p. value = 0.03; ribosome biogenesis, 6.1% proteins, *p. value* = 0.04), biosynthesis (26.4% proteins, *p. value* = 0.02) and defense response (5.5% proteins, *p. value* = 0.04).

Similarly to chronic, to have a deeper view on the radioinduced reprotoxicity mechanisms, a particular focus, *i.e.* analysis of misregulated proteins involved in each process of interest, was done on the biological processes linked to reproduction, *i.e.* “embryo development ending in birth or egg hatching”, “embryo development”, “defense response” and “reproduction”. All the proteins concerned are repressed in at least one dose compared to control.

Interestingly, the 5 proteins involved in “embryonic development” are AIR-1, CGH-1, CIF-1, LAP-1 and MAG-1. These 5 proteins are repressed at 3.3 Gy compared to controls and belong more specifically to germ cell development. AIR-1 (aurora lpl1/related kinase) is involved in cytokinesis, CIF-1 (COP9/Signalosome and eIF3 complex-shared subunit 1) and MAG-1 (Protein mago nashi homolog) are involved in oogenesis and LAP-1 (Leucine aminopeptidase 1) is involved in oviposition (**Figure 7A**).

[Figure 7]

Figure 7: A/ Box plot of LAP-1 protein label free intensity for the three irradiation doses (A0: control, A1: 0.5 Gy, A2: 1 Gy, A3: 3.3 Gy). B/ Boxplot CGH-1 protein label free intensity for the three irradiation doses (A0: control, A1: 0.5 Gy, A2: 1 Gy, A3: 3.3 Gy).

Finally, CGH-1 (ATP-dependent RNA helicase cgh-1) is involved in oocyte and spermatozoid function and is also known to prevent physiological apoptosis in *C. elegans* germline (**Figure 7B**).

Regarding “defense response” process, the proteins concerned are RPA-0 and SKR-1 and are repressed at 3.3 Gy compared to controls. RPA-0 (60S acidic ribosomal protein P0) is responsible for double strand break recognition and is required for the DNA repair and recombination after damage, while SKR-1 (skp1 related ubiquitin ligase compound) is involved in the negative modulation of the apoptosis response.

Moreover, concerning the process “embryo development ending in birth or egg hatching”, 158 proteins are involved in. Even if most of them are involved in several different biological processes,

some proteins have particular functions. Namely, vitellogenins 3, 4 and 5 are repressed at the two highest doses, and histones 4, 11, 48, 41 and 39 are repressed at 3.3 Gy compared to controls. The role of vitellogenins has already been previously described in the precedent section about chronic irradiation. Concerning histones, they are in eukaryotic cells nuclei the leading proteins in interaction with DNA which form the chromatin and pack the DNA into nucleosomes.

Mis-regulation of proteins involved in embryonic development from 1 Gy acute exposure

To determine relevant putative biomarkers of acute exposure, we compared the mis-regulated proteins for the 3 doses as well as for the 2 highest doses. Results of the annotated proteins in UniProt proteomic database are presented in **Table** .

Table 4: List of the common annotated misregulated proteins after acute exposure to γ -rays at 0.5, 1 and 3.3Gy; modulation of their associated expression for each of the conditions. Differences are given as the log2 of the protein intensity ratio between the control and irradiated worms (negative or positive values, means that the protein is over-expressed or repressed compared to control respectively).

[Table 4]

The two differential proteins found in the 0.5 Gy conditions versus control were also found in the two others conditions. So the 3 doses comparison highlighted these 2 proteins repressed at 0.5; 1 and 3.3 Gy compared to controls (**[Figure 8]** Figure). These proteins are RPS-19 (40S ribosomal protein S19) and HAT-1 (histone acetyltransferase 1). In addition, 29 proteins are common between 1 Gy and 3.3 Gy, and are modulated in the same way between these conditions compared to controls.

[Figure 8]

Figure 8: Comparison of the differential proteins found in the 3 different conditions of acute irradiation. A) Venn diagram of mis-regulated proteins (up or down-expressed) between 0.5, 1 and 3.3 Gy. The overlaps between conditions represent the amount of shared proteins within the conditions of interest. B) Box plot of label free intensity of the 2 proteins in common between the three irradiation doses.

Among the overexpressed proteins at 1 and 3.3 Gy, it is interesting to note SYM-1, needed for axogenesis and embryonic viability, and MUP-4 proteins which are essential for embryonic development. In addition, LYS-5 and LYS-6 have a lysozyme activity and ACN-1 is required for molting like NOAH-1, NOAH-2 and COL-14. In contrast, among the repressed proteins at 1 and 3.3 Gy, we find

again the vitellogenins 1, 3, 4 and 5, FAR-1 which is involved in lipid binding and CAT-4 which is involved in serotonin and dopamine biosynthesis that affects movement, mating behavior, foraging behavior, and cell migration.

Different mode of action between acute *versus* chronic exposure to gamma radiation.

Finally, to compare the mechanisms involved in the two irradiation modes, *i.e.* acute vs chronic, of exposure to gamma rays for the same final equivalent doses, we searched for common mis-regulated proteins between the two irradiation modes. We found that acute and chronic exposure share 12 common mis-regulated proteins. The list of the 10 annotated proteins among the 12 proteins are presented in **Table** .

Table 5: List of the 10 shared protein between acute and chronic exposure to γ -rays at 0.5, 1 and 3.3Gy and the modulation of their associated expression for each of the conditions. " $\downarrow\downarrow$ " or " $\uparrow\uparrow$ " means that the log2 of the protein intensity ratio between the exposure conditions and the controls is lower than -1 or higher than 1 respectively. " \downarrow " or " \uparrow " means that the log2 of the protein intensity ratio between the exposure conditions and the controls is lower than -0.58 or higher than 0.58 respectively.

[Table 5]

Among them, SYM-1 and vitellogenins, with a specificity of "vitellogenin 2" which was found only differentially repressed under acute 1Gy irradiation.

General discussion

Environmental risk assessment of ionizing radiations on non-human biota suffers from lack of knowledge on chronic exposure and from a lack of sensitivity. Thus, our objectives in this study were to improve the knowledge on (1) the proteome expression modulation after acute or chronic doses of γ -rays and (2) the possible link between proteome expression modification and effects on reproduction to explain our previous results on radio-induced reprotoxicity. We thus focused the analysis on proteins and biological processes i/ making it possible to distinguish irradiated from control conditions, to find putative biomarkers, ii/ enabling the distinction between acute and chronic modes of irradiation and also iii/ highlighting biological processes relative to reproduction, the key biological function which acts directly on population dynamics.

1- Opposite modulation of some key proteins after acute vs. chronic exposure to γ -rays

Proteomic analyzes in each condition were able to show differential protein expression variations between control organisms and organisms exposed to acute or chronic irradiation at 3 different cumulated doses, whereas an effect on reproduction function has been shown at higher doses for both acute and chronic exposure (*i.e* 30 Gy and 3.3 Gy respectively[4]), attesting of the sensitivity of the proteomic approach. We first focus on these mis-regulated targets. Some of the identified proteins are involved in the reproduction of *C. elegans i.e.* germ line development, embryonic development, and these are over-expressed after chronic exposure and repressed after acute exposure. 11 proteins have been found to be oppositely regulated; 5 of them are involved in cuticle development and molting (*i.e* COL-14, GLF-1, NOAH-1, NOAH-2, ACN-1), not directly linked to reproduction. Among the other targets, vitellogenins VIT-1; VIT-2; VIT-3; VIT-4; VIT-5; VIT-6 are over-expressed after chronic exposure whereas VIT-1, VIT-3, VIT-4 and VIT-5 are repressed at 1 and 3.3 Gy of acute exposure compared to controls. These proteins are yolk protein precursors; five closely related genes called vit-1 through vit-5 encode two polypeptides yp170A and yp170B, and vit-6 encodes two smaller proteins yp115 and yp88[26]. In nematodes, vitellogenins are expressed in the intestine and secreted into the pseudo-coelomic space before being internalized by maturing

oocytes[27]. These proteins constitute a stock of nutritive reserves (including lipids) for the oocytes, allowing the transport of cholesterol thus promoting oocyte maturation. Accumulation of cholesterol in the gonads is necessary for the nematode's spawning capacity by allowing the cell cycle progression and the exit of cells in maturation from the pachytene phase[28]. Therefore, increasing transporters after chronic exposure could mean an increase need of lipids and cellular energy. However, lipid content was analyzed in our study but no significant decrease has been observed before 6.8 Gy [29], that let us suppose that the excess of yolk protein is not the consequence of lipid catabolism but rather the trigger[30]. Yolk protein excess can also be the result of cellular fight against oxidative stress that has been shown to be partly orchestrated by SKN-1[31], also involved in lipid homeostasis and yolk accumulation[32] in opposite ways[30]. In germline stem cell ablated *C. elegans*, this phenomenon has already been seen[32] and the role of yolk proteins in response to chronic exposure has been suspected [29]. The link between reproduction, lipids and even lifespan has been evidenced through numerous studies[33] but still requires investigations. At the opposite, the repression of vitellogenins after acute irradiation could possibly yield a lack of oocyte maturation and constitute one explanation for the decline in the egg-laying observed from 30 Gy [4]. Nevertheless, reduced yolk proteins are not always a sign of reproduction defect[34, 35]. In addition, all vitellogenins don't have the same regulation and are not only involved in lipid transport. Indeed, in *C. elegans*, the transcription level of VIT-2 (protein only modulated after chronic exposure) and VIT-5 is controlled through a sperm-dependent signal[33]. Interestingly, a decrease of the sperm-cell number has been previously shown by Buisset-Goussen *et al.* after chronic exposure to gamma rays of three generation of *C. elegans*[3]. Our quantitative proteomic approach performed on whole worms did not enable the identification of proteins involved in spermatogenesis disturbance. This can be due to a lack of sensitivity as only 10% of the *C. elegans* proteome was identified or because of a whole worm study instead of a specific gonad one which could enable to access to deeper mechanisms. Anyway, even if discordance between vitellogenin transcripts and yolk proteins levels has already been observed[33], these results can constitute a cascade of events due to irradiation

exposure, in which the trigger needs to be elucidated. This could have an importance in terms of environmental risk assessment based on integrated approach at different biological organization levels.

SYM-1 protein involved in embryonic viability by helping the attachment of body muscle to the extracellular cuticle is also differentially modulated after acute or chronic exposure. This possibly suggests a perturbation of the embryo viability for the two irradiation modes but in a different manner and constitutes a specific marker of reproduction failure. The causal link, if any, remains to be investigated in both cases but, in literature, SYM-1 mutant present defects in the brood size but not in hatching success[36] similar to what have been found after chronic exposure.

This set of proteins could constitute sensitive markers of interest. Indeed, these molecular markers are modulated at a lower dose than the effects observed at the individual level and tend to confirm the differences observed at the individual scale at 3.3 Gy, notably on the spawning capacity of the nematode[4]. However, except VIT-3 of which the overexpression increases with dose, these proteins, in both conditions, are equivalently modulated throughout all irradiated conditions. That could presume a binary induction with irradiation and not a dose response relationship. This will be necessary to investigate in the perspective of finding markers for environmental risk assessment of ionizing radiations.

2- Specificity of chronic gamma radiation: disturbance of lipid transport, DNA replication and germ cell development processes.

After chronic exposure, the most significant biological processes found after gene ontology enrichment analysis of the significantly modulated proteins are lipid transport, DNA replication, germ cell development, cellular chemical homeostasis, ion transport, cuticle development and locomotion. And the biological processes found after gene ontology enrichment analysis of the proteins found to be significant over all conditions (ANOVA analysis) are molting cycle, regulation of growth, defense response, embryo development ending in birth or hatching and nitrogen compound metabolism.

545 Likely to be disturbed by chronic exposure, these processes illustrate the possible link between the
546 molecular responses, *i.e.* protein expression, and the individual parameters observed after chronic
547 exposure to γ -rays, notably the reproduction disturbance, *i.e.* decrease of total progeny. The
548 disturbance of defense response and proteolysis have already been observed after chronic exposure
549 with a proteasome analysis that showed activation from 1 Gy of its 20S form, notably corresponding
550 to oxidized protein proteolysis, and differential modulation of 26S and 30S proteasomes, ATP and
551 ubiquitin dependent forms[4]. This general process can constitute part of a response of organisms
552 fighting against oxidative stress.

553 Focus on proteins involved in these processes has been done to go further on mechanisms; particular
554 attention was given on proteins modulated over the three doses, or specifically dedicated to
555 reproduction. The role of vitellogenins (lipid transport process) and SYM-1 (embryonic development
556 process) has already been discussed and other proteins modulated over the three doses are more
557 involved in cuticle development and molting cycle than in reproduction. Interesting proteins
558 overexpressed at 0.5 and 1 Gy are helicase proteins from MCM complex (DNA replication process).
559 The overexpression of MCM complex proteins may be linked to the necessity to increase DNA
560 replication after DNA damage[37]. As this action can be concomitant with cell cycle arrest induced at
561 cellular control points in response to DNA damage[38], this result could be linked with the division
562 arrest of *C. elegans* germcell, *i.e.* oocyte precursors, already observed after chronic exposure at
563 2.5Gy[3]. This result can also constitute part of oxidative stress fighting response. Finally, CPG-1 and
564 CPG-2, overexpressed at 0.5 Gy, play essential roles in embryonic cell division in *C. elegans* and are
565 required for polar body extrusion during cytokinesis in embryo development[24].

566 Then evidence found in this paper, *i.e.* possible disturbance in lipid transport and axogenesis, can
567 argue both in gametogenesis and embryogenesis disturbance and oxidative stress response. This
568 needs to be investigated more deeply to find the cause of the reproduction defect. In addition, it is
569 interesting to see that developmental growth process is highlighted in this study whereas growth has

not been studied as endpoint; effect of gamma irradiation on growth has already been predicted and observed in Lecomte et al 2017 after chronic exposure[39].

3- Specificity of acute gamma radiation: hatching success, embryo development and apoptotic processes.

Two protein clusters have been distinguished from ANOVA analysis of significant proteins and pairwise analysis; mainly, overexpressed proteins (at 1 and 3.3 Gy compared to control) were involved in i) molting cycle, cuticle development, developmental growth and lipoprotein biosynthesis, whereas repressed proteins (at 1 and 3.3 Gy compared to control) were involved in ii) adult lifespan, single organism metabolic process and aging, including embryo development ending in birth or egg hatching and embryo development. Indeed, similarly to chronic exposure, a focus was done on proteins found to be modulated by gamma acute exposure and belonging to the reproduction biological process and to other processes that could be correlated to a reproduction failure.

Only two proteins are common to the three doses which is less than after chronic exposure. Repressed at 0.5, 1 and 3.3 Gy compared to the controls, they could be putative markers of acute irradiation (RPS-19 and HAT-1). RPS-19 is linked to ribosomal activity and translation that are generic processes. Parallely, histone acetyltransferases allow the decompaction of chromatin, thus promoting the transcription of genes[40], but also enabling DNA repair by increasing the accessibility of the DNA[41]. Histone modification can therefore modify gene transcription by interacting with chromatin structure, allowing more or less the accessibility to the transcription initiating proteins, for example. This result is enhanced by the fact that histones 4, 11, 48, 41 and 39 are also found to be repressed at 3.3 Gy. This repression of histone cluster after acute exposure can also be associated to a modification of chromatin compaction and finally to a default of gene transcription. It is also possible that the repression of these proteins from 0.5 Gy leads to a decrease in the DNA repair activity, leading to an increase of apoptosis and therefore to a possible defect in reproduction [18, 42].

596 The role of vitellogenins (reproduction process) and SYM-1 (embryonic development process) has
597 already been discussed. Other repressed proteins, such as AIR-1, CGH-1, CIF-1, LAP-1, and MAG-1
598 belong to “hatching success” biological process and to biological processes such as embryonic
599 development. More specifically CGH-1 is a probable RNA helicase required for gametogenesis, but
600 also for embryonic cytokinesis[43, 44]; and CIF-1 is required for initiation of protein translation and
601 therefore has a role in embryogenesis[45]. In addition, these 5 proteins are involved in oogenesis,
602 suggesting that acute irradiation has an impact on gametogenesis. The fact that acute irradiation
603 could first lead to a gametogenesis default prior than an embryogenesis default supports the
604 hypothesis of a cumulative damage after acute exposure that cannot be repaired in developing
605 gametes and transmitted to the developing embryos, leading therefore to a hatching success
606 decline[34].

607 In addition, two proteins, *i.e.* RPA-0 and SKR-1, seen to be repressed at 3.3 Gy compared to controls
608 belong to the biological “apoptotic process”. RPA repression could suggest a modification or a
609 disturbance of DNA break recognition. In addition, it has recently been shown that the SKR-1 protein
610 i) has a negative regulation of the pro-apoptotic protein CEP-1 in *C. elegans*[46], and ii) is involved in
611 the ubiquitinylation of proteins to allow their degradation by the proteasome[47]. About this latter,
612 as previously demonstrated, proteasome activity is drastically inhibited after acute irradiation. More
613 specifically, the two ubiquitin dependent forms of the proteasome are inhibited at 0.5 Gy and 200 Gy
614 and from 50 Gy for the 30S and the 26S proteasomes respectively[4]. Down-regulation of SKR-1 could
615 therefore be a consequence of proteasome activity loss and associated to a repression of CIF-1,
616 already described and part of a complex involved in the regulation of ubiquitin. Finally, SKR-1
617 repression can also suggest an inhibition of the negative regulation of CEP-1 and thus an increased
618 apoptotic response after acute irradiation to eliminate damaged cells which is consistent with the
619 literature [34, 42].

Finally, our results tend to show a disturbance of the gametogenesis but also of embryo development and egg hatching biological pathways. Even if the trigger of the decline in total egg production per individual and in egg hatching has not been fully elucidated, our study highlighted some biological processes involved in this decay.

Conclusion

This study provides a first comprehensive analysis of the gamma irradiation proteomic response in a model organism, *C. elegans*. It extends precedent findings on reprotoxicity of gamma irradiation by refining molecular mechanisms of gamma rays action after two different modes of exposure. This global analysis of protein expression has demonstrated the modulation of proteins involved in regulatory biological processes such as lipid transport, DNA replication, germ cell development, apoptosis, ion transport, cuticle development, and aging (including embryo development ending in birth or egg hatching and embryo development) at lower doses than those for which individual effects on reproduction have been previously observed, and these results are validated by the use of 2 complementary differential proteomic analysis methodologies. Thus, these proteins could constitute early and sensitive markers of radio-induced reprotoxicity; more specifically HAT-1, RPS-19 in acute and VIT-3 in chronic conditions that are expressed in a dose-dependent manner. Other target proteins seem equivalently modulated throughout all irradiated conditions and could constitute exposure markers. To better understand their role in this context, functional validation of these markers should now be done using GFP-transgenes or specific mutants.

Similarly to phenotypic endpoints, our results confirm that the molecular mechanisms induced by chronic irradiation differ from those induced by acute irradiation, thus highlighting limitations of data extrapolation obtained for acute exposure in order to predict the effects of chronic exposure. Indeed, the risk assessment of chronic exposure should be based on specific data from chronic exposures, *i.e* exposure times that are representative of environmental conditions.

To focus on the reproduction process, the proteomic analysis showed either repression or overexpression of 12 proteins, including a vitellogenin cluster, in organisms exposed to acute or chronic irradiation, respectively.

Finally, our results seem showing more disturbance in proteins involved in oogenesis than in spermatogenesis after both acute and chronic exposure (except VIT-2). Further studies will be interesting to conduct on each gonad, *i.e* sperm-cell and oocytes, in order to understand their own sensitivity after acute vs. chronic exposure to gamma rays.

Future directions will be necessary to test the relevance of the proteomic markers found in this study at ecologically relevant doses rates such as for example $10 \mu\text{Gy.h}^{-1}$ which is considered as the no-effect dose rate for ecosystems[48]. Moreover, it will also be interesting to improve the understanding of the radio-induced molecular mechanisms after chronic exposure by adopting a comparative approach (multi-phylum) including environmental species which are more or less radiosensitive. This could help to define environmental thresholds to protect population in the long term.

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