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

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9  
10 **Abstract (300 words)**

11 Emission of ionizing radiation (IR) in the environment is a natural phenomenon which can be  
12 enhanced by human activities. Ecosystems are then chronically exposed to IR. But environmental risk  
13 assessment of chronic exposure suffers from a lack of knowledge. Extrapolation of data from acute to  
14 chronic exposure is not always relevant, and can lead to uncertainties as effects could be different  
15 between the two irradiation modes, especially regarding reproduction endpoint, which is an  
16 ecologically relevant parameter. In the present study, we decided to refine the understanding of the  
17 molecular mechanisms involved in response to acute and chronic  $\gamma$ -irradiation by a global proteome  
18 label free LC-MS/MS analysis. *C. elegans* were exposed to 3 common cumulated radiation doses for  
19 acute or chronic exposure condition and global modification of the proteome was studied. This  
20 analysis of protein expression has demonstrated the modulation of proteins involved in regulatory  
21 biological processes such as lipid transport, DNA replication, germ cell development, apoptosis, ion  
22 transport, cuticle development, and aging at lower doses than those for which individual effects on  
23 reproduction have been previously observed. Thus, these proteins could constitute early and  
24 sensitive markers of radio-induced reprotoxicity; more specifically HAT-1, RPS-19 in acute and VIT-3  
25 for chronic conditions that are expressed in a dose-dependent manner. Finally, to focus on  
26 reproduction process, this analysis showed either repression or overexpression of 12 common  
27 proteins in organisms exposed to acute or chronic irradiation, respectively. These proteins include  
28 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.

33

34

35

36 **Title Page**

37 Protein expression differently modulated after acute and chronic exposure to  $\gamma$ -rays.

38 **Keywords**

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

40 **Introduction**

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

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

62 3 Gy) on mammal cell proteome and showed that modulation of protein expression could be a  
63 relevant biomarker to detect ionizing radiation exposure, to predict severity of associated lesions and  
64 ultimately to manage clinically these lesions [11-13]. However, these proteomic studies concern only  
65 acute irradiation of mammals. Data concerning the proteome sensitivity of non-human biota after  
66 chronic irradiation are scarce.

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

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

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

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

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

101

102

103 **Material and methods**

104 ***C. elegans* maintenance and age synchronization**

105 The wild-type N2 strain of *C. elegans* provided by CGC (*Caenorhabditis* Genetic Center) was used in  
106 this study. Populations were maintained at 19°C and 80% of humidity on 9cm petri dishes poured  
107 with NGM (Nematode Growth Medium) and seeded with *Escherichia Coli OP50* as food source.  
108 *E. coli* OP50 were grown in L-Browth medium at 37°C overnight. Petri dishes were seeded with 1mL  
109 of saturated culture of bacteria and UV killed (Bio-Link Crosslinker,  $\lambda = 254 \text{ nm}$ ; intensity =  $200 \text{ mWm}^{-2}$ )  
110 <sup>2</sup>) for 20 minutes to avoid food heterogeneity between dishes. 100 gravid worms were randomly  
111 selected from the stock population and placed on 9 cm petri dishes. 96 h later, eggs were separated  
112 from adult worms by a bleaching procedure and collected embryos were allowed to grow in a control  
113 incubator for 96 h. The gravid worms were separated from eggs already laid by a sucrose gradient (3  
114 – 7 %), and then re-synchronized by a bleaching procedure in order to collect the eggs *in utero*  
115 synchronized over 3 h.

116

117 **Irradiation**

118 Irradiations were performed in incubators (19 °C and 80 % humidity) in controlled conditions; data  
119 loggers were used in order to measure humidity and temperature during irradiation. Nematode  
120 plates were placed perpendicularly or parallelly (for acute and chronic exposure respectively) to the  
121 cesium-137 source to obtain a homogeneous dose rate at the surface of the plate. Radio Photo  
122 Luminescent dosimeters (RPL, GD-301 type, Chiyoda Technol Corporation, Japan) were placed on  
123 each experimental unit in order to measure the delivered cumulated dose received by organisms. At  
124 the end of each irradiation, worms were collected, rinsed with M9 medium (5 g.L<sup>-1</sup> NaCl, 25 mM KPO<sub>4</sub>  
125 buffer and 1 mM MgSO<sub>4</sub>) to ensure bacteria removal, centrifuged and pellets were snap frozen.

126

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

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

132  
133 **Chronic** - For chronic irradiation, 3000 age synchronized nematodes were exposed to cesium-137  
134 source using the platforms MIRE (Mini Irradiator for Radio-Ecology) (1.6 GBq) from embryo stage to  
135 L4-YA adult stage to cover the complete lifecycle (65 h). Three dose rates (excluding controls): 7, 14,  
136  $50\text{mGy}\cdot\text{h}^{-1}$  corresponding to three cumulated doses (0.5, 1 and 3.3Gy) were tested.

137  
138 **Protein extraction and purification**

139 After irradiation, 3000 *C. elegans* per replicate were subjected to protein extraction. 300  $\mu\text{l}$  of 0.5-  
140 mm diameter zirconium beads and an equal amount of lysis buffer (30 mM Tris-HCl pH 7.4, 150mM  
141 NaCl, 1.0%(v/v) Igepal CA-630 (NP-40), 1%(v/v) TritonX-100, 0.5% (w/v) sodium deoxycholate,  
142 0.1%(w/v) sodium dodecyl sulfate (SDS), 2%(v/v) glycerol, 2 mM 1,4-dithiothreitol (DTT),  $1 \text{ mg}\cdot\text{ml}^{-1}$   
143 leupeptin,  $1 \text{ mg}\cdot\text{ml}^{-1}$  aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM  
144 ethylenediaminetetraacetic acid (EDTA)) were added on top of worm pellets and incubated for 15  
145 min on ice. *C. elegans* were then homogenized by three 6800-rpm cycles in the Precellys grinder  
146 system (Bertin Technologies, Montigny-le-Bretonneux). After 1 h incubation on ice, lysates were  
147 centrifuged (13500 g) at  $4^{\circ}\text{C}$  for 15 min. Supernatant was sampled, protein concentration was  
148 determined using the BCA kit (Thermo Scientific) using BSA as a standard, according to the  
149 manufacturer's instructions and the remaining volume quick frozen with liquid nitrogen.

150 20  $\mu\text{g}$  of proteins were precipitated on ice for 20 min by the addition of 10 % TCA (v/v). After washing  
151 steps, pellets were resuspended in UTC9231 (9 M urea, 2 M thiourea, 3% (w/v) CHAPS, 1% (w/v)  
152 ASB14, 20 mM Tris, pH 9.5) under stirring (1600 rpm) at  $30^{\circ}\text{C}$  in the dark for two hours.

153

154 **Label free sample preparation and mass spectrometry analysis**

155 15  $\mu\text{g}$  of proteins per replicate were loaded on a NuPAGE gel 4-12% (Life Technologies). Samples  
156 were then subjected to electrophoresis during 6min at 80V using a MOPS buffer (Thermo Fisher



157 Scientific) in order to stack proteins on the top of the gel before proceeding to coomassie blue  
158 staining. Protein bands were then excised with a scalpel and digested with trypsin.  
159 Each condition (0.5, 1 and 3.3 Gy) was injected in 3 biological replicates and 2 technical replicates in  
160 liquid chromatography (Ultimate 3000 RSLCnano chromatography system (Thermo Fisher Scientific))  
161 coupled with an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). First  
162 peptides were concentrated and purified on a pre-column from Dionex (C18 PepMap100, 2 cm × 100  
163 μm I.D, 100 Å pore size, 5 μm particle size) in solvent A (0.1% formic acid in 2% acetonitrile). In the  
164 second step, peptides were separated on a reverse phase LC EASY-Spray C18 column from Dionex  
165 (PepMap RSLC C18, 50 cm × 75 μm I.D, 100 Å pore size, 2 μm particle size) at 300 nL/min flow rate  
166 and 40°C. After column equilibration using 4% of solvent B (20% water - 80% acetonitrile - 0.1%  
167 formic acid), peptides were eluted from the analytical column by a two steps linear gradient (4-20%  
168 acetonitrile/H<sub>2</sub>O; 0.1 % formic acid for 220 min and 20-45% acetonitrile/H<sub>2</sub>O; 0.1 % formic acid for 20  
169 min). For peptide ionization in the EASY-Spray nanosource, spray voltage was set at 2.2 kV and the  
170 capillary temperature at 275 °C. The mass spectrometer was used in data dependent mode to switch  
171 consistently between MS and MS/MS. Time between Masters Scans was set to 3 seconds. MS spectra  
172 were acquired with the Orbitrap in the range of m/z 375-1500 at a FWHM resolution of 60 000  
173 measured at 200 m/z. AGC target was set at 4.0.10<sup>5</sup> with a 50 ms Maximum Injection Time. The more  
174 abundant precursor ions were selected and collision induced dissociation fragmentation at 35% was  
175 performed and analyzed in the ion trap using the “Inject Ions for All Available Parallelizable time”  
176 option with a maximum injection time of 105 ms and an AGC target of 1.0.10<sup>5</sup>. Charge state  
177 screening was enabled to include precursors with 2 and 7 charge states. Dynamic exclusion was  
178 enabled with a repeat count of 1 and a duration of 60s. These chromatographic conditions were  
179 previously optimized with a protein pool from all the samples.

## 180 **Quantitative proteomics processing**

181 For data processing we used the free suite MaxQuant version 1.5.3.8[20]. The relative intensities  
182 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 17<sup>th</sup> 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 log<sub>2</sub> 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 log<sub>10</sub> 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](http://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

## 227 Results

### 228 Chronic exposure to gamma-rays

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

230 The analysis of differential protein expression by the 2D-DIGE methodology (Supplementary data)  
231 showed only one protein spot as variant after chronic irradiation of *C. elegans* from 0.5 to 3.3 Gy,  
232 compared to controls. A label-free approach was then performed on the same samples in order to  
233 increase the sensitivity of the proteome analysis. Indeed, the label free LC-MS/MS based proteomic  
234 approach was able to identify 2647 proteins. The most abundant identified proteins with high iBAQ  
235 (intensity-based absolute quantification) and the less abundant proteins with low iBAQ are presented  
236 in supplementary **Figure S1**. This figure shows that our proteomics analysis covers a dynamic range  
237 around 6 log of protein intensity (orders of magnitude). ANOVA analysis was performed on all  
238 conditions, 168 proteins were found significant (FDR<0.01) and their z-scored LFQ values were heat-  
239 mapped (**Figure 1** - Exhaustive data are given as supplementary excel file: Suppl-File1\_ANOVA  
240 conditionChronic 4clusters.xls) showing 4 clusters of proteins (A, B, C, D) within the whole targets, 2  
241 of them (A and D) making it possible to distinguish control and irradiated conditions whereas the two  
242 others tend to isolate the 1 Gy exposure condition. The cluster A encompassed proteins that were  
243 mainly decreased in the irradiation conditions group versus the controls. The cluster D included  
244 proteins which increased under irradiation versus control. Finally, cluster B and C contained proteins  
245 that were more or less upregulated in one specific irradiation condition 0.5 and 1 Gy respectively. As  
246 presented in Erreur ! Source du renvoi introuvable. (Exhaustive data are given as supplementary  
247 excel file: Suppl-File2\_Chronic-fdr001so01-pairwise full data.xls), the further pairwise analysis of the  
248 protein differential expression showed that among the 2647 proteins, 87 were significantly mis-  
249 regulated following chronic exposure (sum of the misregulated proteins after pairwise analysis of  
250 conditions - log<sub>2</sub> fold change > ±0.58 and log *p.value* > +/- 1.3). Finally, 51% of these 87 mis-regulated  
251 proteins were over-expressed compared to controls, while 49% of proteins were repressed. In  
252 addition, some of the misregulated proteins were common between the 3 doses (25.6 %). More

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

### 256 [Figure 1]

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

### 261 **Protein enrichment analysis makes possible to associate protein expression level and radio-** 262 **induced reproductive disturbance**

263 Biological processes associated to misregulated proteins found in the pairwise analysis were  
264 evaluated using gene ontology enrichment to find the possible disturbed pathways. A sum-up was  
265 done in **Figure 2**, the full results are in the excel file Suppl-File3\_Chronic ttest David Ease 01\_GO-  
266 BP.xls. We found that misregulated proteins are involved in lipid transport (9% in lipid transport, p.  
267 value =  $2.6 \times 10^{-5}$ ; 12.8% in lipid localization, p. value = 0.01; 3.8% in divalent inorganic cation  
268 transport, p. value = 0.04; 3.8% in divalent metal ion transport, p. value = 0.04; 17.9% in single-  
269 organism transport, p. value = 0.05), in cuticle development (7.7% in cuticle development, p. value =  
270 0.005; 3.8% in molting cycle process, p. value = 0.007; 11.5% in molting cycle, p. value = 0.01; 12.8%  
271 in germ cell development, p. value = 0.03), in DNA-dependent DNA replication (6.4% in DNA-  
272 dependent DNA replication, p. value =  $1.3 \times 10^{-4}$ ; 6.4% in DNA metabolic process, p. value = 0.03), in  
273 DNA unwinding (5.1 %, p. value = 0.002) and in cellular divalent inorganic cation homeostasis (3.8%,  
274 p. value = 0.01). In addition, protein enrichment was performed on ANOVA data (**Figure S2**) and  
275 particularly on clusters A and D to analyze the biological processes distinguishing control and  
276 irradiated conditions (**Table 1**; Suppl-File4\_Chronic ANOVA David Ease 01\_GO-BP.xls).

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

### 279 [Table 1]

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

282 (16%, p. value =  $8.4 \times 10^{-4}$ ), defense response (9.5%, p. value =  $3.7 \times 10^{-2}$ ) and locomotion (28%, p.  
283 value =  $1.5 \times 10^{-2}$ ). The cluster D corresponds to biological process such as embryo development  
284 ending in birth or hatching (61%, p. value =  $5 \times 10^{-9}$ ); DNA replication (15.9%, p. value =  $3.8 \times 10^{-7}$ ),  
285 nitrogen compound metabolism (41%, p. value =  $7.5 \times 10^{-4}$ ) and lipid transport (13.6%, p. value =  
286  $1.8 \times 10^{-6}$ ). For a simplified analysis, we chose to only analyze and describe biological processes that  
287 are potentially linked with reprotoxicity or radiotoxicity, *i.e.* biological process such as embryo  
288 development ending in birth or hatching, cell cycle processes and DNA replication processes. All of  
289 these proteins are overexpressed in at least one dose compared to control.

## 290 [Figure 2]

291 Figure 2 : Independent DAVID gene functional enrichment analysis based on results of all proteins identified as  
292 modulated after chronic irradiation (t-test, pairwise comparison). Significant GO-term biological processes (p. value  
293  $< 0.05$ ) were then summarized using REVIGO. The % of proteins involved in the process is written in each case. Tree maps  
294 show a two-level hierarchy of GO terms (main clusters and cluster members) ; the size of the rectangles is relative to the  
295  $\log_{10}$  (p. value) absolute. With a same color, biological processes belonging to one main head process: lipid transport  
296 (purple), cuticle development (blue), DNA-dependent DNA replication (yellow).

297

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

## 301 [Figure 3]

302 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,  
303 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  
304 Gy, C3: 3.3 Gy).

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

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

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

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

### 319 **[Figure 4]**

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

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

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

### 333 **[Table 2]**

334

### 335 **Acute exposure to gamma-rays**

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

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

340 3.3 Gy. Similarly to chronic exposure, 2D-DIGE methodology was used in the first instance  
341 (Supplementary data), refined by a label free LC-MS/MS based proteomic approach.  
342 The label free LC-MS/MS based proteomic approach was able to identified 2677 proteins. Similarly to  
343 chronic, the most abundant identified proteins with high iBAQ and the less abundant proteins with  
344 low iBAQ are presented in supplementary **Figure S1**. ANOVA analysis was performed on all  
345 conditions, 369 proteins were found significant (FDR<0.01) and their z-scored LFQ values were heat-  
346 mapped (**Figure 5** - Exhaustive data are given as supplementary excel file: Suppl-File5\_ANOVA  
347 condition Acute 2 clusters.xls) showing 2 clusters of proteins (Cluster A and Cluster B) within the  
348 whole targets. Both clusters show that the condition 0.5 Gy is very close to the control. The cluster A  
349 encompassed proteins that were mainly increased in the two higher irradiation conditions group  
350 versus the controls. Conversely, the cluster B included proteins which decreased in the two higher  
351 irradiation conditions versus control. As presented in **TableS3** (Exhaustive data are given as  
352 supplementary excel file: Suppl-File6\_Acute-fdr001so01 pairwise full data.xls), a further pairwise  
353 analysis of the protein differential expression, showed that among the 2677 proteins, 338 were  
354 significantly mis-regulated following acute exposure (sum of all misregulated proteins after pairwise  
355 analysis of conditions -  $\log_2$  fold change  $> \pm 0.58$  and  $\log p.value > \pm 1.1$ ). Most of these 338 mis-  
356 regulated proteins (70%) were over-expressed compared to controls, while 30% of proteins were  
357 repressed. In addition, some of them (2) were common between the 3 doses (0.6 %). More  
358 specifically, only 2 proteins (RPS-19 and HAT-1) were repressed at 0.5 Gy compared to controls. Then,  
359 at 1 Gy and 3.3 Gy, 32 (20 up-, 12 down-regulated) and 335 proteins (103 up-, 232 down-regulated)  
360 were mis-regulated, respectively. These results show a large deregulation of the proteome with  
361 increasing dose of radiation. Proteome modulation occurs as soon as 0.5Gy but only a small number  
362 of proteins is concerned.

363 **[Figure 5]**

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



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

368

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

371 Based on gene ontology enrichment (GO) and associated *p. values*, biological processes associated to  
372 mis-regulated proteins found in the pairwise analysis of label free LC-MS/MS proteomic approach  
373 were evaluated using gene ontology enrichment to find the possible disturbed pathways (Exhaustive  
374 data are given as supplementary excel file: Suppl-File7\_Acute ttest David Ease 01\_GO-BP.xls). The  
375 sum-up of the analysis is showed in **Figure 6**. In addition, protein enrichment was performed on  
376 ANOVA clusters (**Figure S3; Table3**) to analyze the biological processes distinguishing control and the  
377 highest doses of irradiation conditions. Exhaustive list of biological processes found for each set of  
378 data are given in supplementary data (Suppl-File8\_Acute ANOVA David Ease 01\_GO-BP.xls). For a  
379 simplified analysis, we then chose to analyze and describe biological pathways that are potentially  
380 linked with reprotoxicity or radiotoxicity. Results on protein enrichment have been discussed mainly  
381 on the proteins found to be modulated after pairwise analysis.

382

### [Figure 6]

383 **Figure 6: Independent DAVID gene functional enrichment analysis on the basis of results of all proteins identified as**  
384 **modulated after acute irradiation (t-test, pairwise comparison). Significant GO-term of biological processes (*p.value***  
385 **<0.05) were then summarized using REVIGO. The tree maps show a two-level hierarchy of GO terms (main clusters and**  
386 **cluster members); the size of the rectangles is relative to absolute of log<sub>10</sub>(*p value*).**

387 The misregulated proteins were mainly involved in cuticle development process (with involvement in  
388 9 sub-processes: cuticle development, aging, embryo development ending in birth or egg hatching,  
389 embryo development, larval development, post-embryonic development, determination of adult  
390 lifespan, developmental growth, collagen and cuticulin-based cuticle development), alpha-amino acid  
391 metabolism (with involvement in 6 sub-processes: single-organism biosynthesis of organonitrogen  
392 compounds, organic substances and glycosyl compounds, metabolism of alpha amino acids and  
393 organonitrogen compounds), mitochondrion organization (mitochondrion organization, 4% proteins,

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

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

401 Interestingly, the 5 proteins involved in “embryonic development” are AIR-1, CGH-1, CIF-1, LAP-1 and  
402 MAG-1. These 5 proteins are repressed at 3.3 Gy compared to controls and belong more specifically  
403 to germ cell development. AIR-1 (aurora lpl1/related kinase) is involved in cytokinesis, CIF-1  
404 (COP9/Signalosome and eIF3 complex-shared subunit 1) and MAG-1 (Protein mago nashi homolog)  
405 are involved in oogenesis and LAP-1 (Leucine aminopeptidase 1) is involved in oviposition (**Figure**  
406 **7A**).

407 **[Figure 7]**

408 **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,**  
409 **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**  
410 **Gy, A3: 3.3 Gy).**

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

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

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

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

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

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

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

#### 433 **[Table 4]**

434 The two differential proteins found in the 0.5 Gy conditions versus control were also found in the two  
435 others conditions. So the 3 doses comparison highlighted these 2 proteins repressed at 0.5; 1 and 3.3  
436 Gy compared to controls (**[Figure 8]**

437 **Figure** ). These proteins are RPS-19 (40S ribosomal protein S19) and HAT-1 (histone acetyltransferase  
438 1). In addition, 29 proteins are common between 1 Gy and 3.3 Gy, and are modulated in the same  
439 way between these conditions compared to controls.

#### 440 **[Figure 8]**

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

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

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

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

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

458 **Table 5: List of the 10 shared protein between acute and chronic exposure to  $\gamma$ -rays at 0.5, 1 and 3.3Gy and the**  
459 **modulation of their associated expression for each of the conditions. "↓↓" or "↑↑" means that the log2 of the protein**  
460 **intensity ratio between the exposure conditions and the controls is lower than -1 or higher than 1 respectively. "↓" or**  
461 **"↑" means that the log2 of the protein intensity ratio between the exposure conditions and the controls is lower**  
462 **than -0.58 or higher than 0.58 respectively.**

463 **[Table 5]**

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

466

467

468 **General discussion**

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

478 **1- Opposite modulation of some key proteins after acute vs. chronic exposure to  $\gamma$ -rays**

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

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

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

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

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

## 537 **2- Specificity of chronic gamma radiation: disturbance of lipid transport, DNA replication and germ** 538 **cell development processes.**

539 After chronic exposure, the most significant biological processes found after gene ontology  
540 enrichment analysis of the significantly modulated proteins are lipid transport, DNA replication, germ  
541 cell development, cellular chemical homeostasis, ion transport, cuticle development and locomotion.  
542 And the biological processes found after gene ontology enrichment analysis of the proteins found to  
543 be significant over all conditions (ANOVA analysis) are molting cycle, regulation of growth, defense  
544 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  $\gamma$ -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



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

572 **3- Specificity of acute gamma radiation: hatching success, embryo development and apoptotic**  
573 **processes.**

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

583 Only two proteins are common to the three doses which is less than after chronic exposure.  
584 Repressed at 0.5, 1 and 3.3 Gy compared to the controls, they could be putative markers of acute  
585 irradiation (RPS-19 and HAT-1). RPS-19 is linked to ribosomal activity and translation that are generic  
586 processes. Parallely, histone acetyltransferases allow the decompaction of chromatin, thus  
587 promoting the transcription of genes[40], but also enabling DNA repair by increasing the accessibility  
588 of the DNA[41]. Histone modification can therefore modify gene transcription by interacting with  
589 chromatin structure, allowing more or less the accessibility to the transcription initiating proteins, for  
590 example. This result is enhanced by the fact that histones 4, 11, 48, 41 and 39 are also found to be  
591 repressed at 3.3 Gy. This repression of histone cluster after acute exposure can also be associated to  
592 a modification of chromatin compaction and finally to a default of gene transcription. It is also  
593 possible that the repression of these proteins from 0.5 Gy leads to a decrease in the DNA repair  
594 activity, leading to an increase of apoptosis and therefore to a possible defect in reproduction [18,  
595 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].

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

## 624 **Conclusion**

625 This study provides a first comprehensive analysis of the gamma irradiation proteomic response in a  
626 model organism, *C. elegans*. It extends precedent findings on reprotoxicity of gamma irradiation by  
627 refining molecular mechanisms of gamma rays action after two different modes of exposure. This  
628 global analysis of protein expression has demonstrated the modulation of proteins involved in  
629 regulatory biological processes such as lipid transport, DNA replication, germ cell development,  
630 apoptosis, ion transport, cuticle development, and aging (including embryo development ending in  
631 birth or egg hatching and embryo development) at lower doses than those for which individual  
632 effects on reproduction have been previously observed, and these results are validated by the use of  
633 2 complementary differential proteomic analysis methodologies. Thus, these proteins could  
634 constitute early and sensitive markers of radio-induced reprotoxicity; more specifically HAT-1, RPS-19  
635 in acute and VIT-3 in chronic conditions that are expressed in a dose-dependent manner. Other  
636 target proteins seem equivalently modulated throughout all irradiated conditions and could  
637 constitute exposure markers. To better understand their role in this context, functional validation of  
638 these markers should now be done using GFP-transgenes or specific mutants.

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

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

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

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

658

659

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