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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 γ -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 γ -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 mWm^{-2})
110 ²) 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⁻¹ NaCl, 25 mM KPO₄
125 buffer and 1 mM MgSO₄) 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 μl 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°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 μg 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°C in the dark for two hours.

153

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

155 15 μg 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/H₂O; 0.1 % formic acid for 220 min and 20-45% acetonitrile/H₂O; 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 4.0.10⁵ 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 1.0.10⁵. 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 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

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 - log₂ 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 = 2.6×10^{-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 = 1.3×10^{-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 = 1.3×10^{-3}), molting cycle (14% of proteins and p. value = 2×10^{-3}), regulation of growth

282 (16%, p. value = 8.4×10^{-4}), defense response (9.5%, p. value = 3.7×10^{-2}) and locomotion (28%, p.
283 value = 1.5×10^{-2}). The cluster D corresponds to biological process such as embryo development
284 ending in birth or hatching (61%, p. value = 5×10^{-9}); DNA replication (15.9%, p. value = 3.8×10^{-7}),
285 nitrogen compound metabolism (41%, p. value = 7.5×10^{-4}) and lipid transport (13.6%, p. value =
286 1.8×10^{-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 γ -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 log₁₀(*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 γ -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 γ -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 γ -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 γ -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 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

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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