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You Song, Brit Salbu, Hans-Christian Teien, Lene Sørlie Heier, Bjørn Olav Rosseland, Knut Erik Tollefsen. Dose-dependent hepatic transcriptional responses in Atlantic salmon (Salmo salar) exposed to sublethal doses of gamma radiation. Aquatic Toxicology. Volume 156, 2014, Pages 52-64, ISSN 0166-445X.

The article has been published in final form by Elsevier at

http://dx.doi.org/10.1016/j.aquatox.2014.07.021

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Dose-dependent hepatic transcriptional responses in Atlantic salmon (*Salmo salar*) exposed to sublethal doses of gamma radiation

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Abstract

Due to the production of free radicals, gamma radiation may pose a hazard to living organisms. The high-dose radiation effects have been extensively studied, whereas the ecotoxicity data on low-dose gamma radiation is still limited. The present study was therefore performed using Atlantic salmon (*Salmo salar*) to characterize effects of low-dose (15, 70 and 280 mGy) gamma radiation after short-term (48 h) exposure. Global transcriptional changes were studied using a combination of high-density oligonucleotide microarrays and quantitative real-time reverse transcription polymerase chain reaction (qPCR). Differentially expressed genes (DEGs; in this article the phrase gene expression is taken as a synonym of gene transcription, although it is acknowledged that gene expression can also be regulated, e.g., at protein stability and translational level) were determined and linked to their biological meanings predicted using both Gene Ontology (GO) and mammalian ortholog-based functional analyses. The plasma glucose level was also measured as a general stress biomarker at the organism level. Results from the microarray analysis revealed a dose-dependent pattern of global transcriptional responses, with 222, 495 and 909 DEGs regulated by 15, 70 and 280 mGy gamma radiation, respectively. Among these DEGs, only 34 were commonly regulated by all radiation doses, whereas the majority of differences was dose-specific. No GO functions were identified at low or medium doses, but repression of DEGs associated with GO functions such as DNA replication, cell cycle regulation and response to reactive oxygen species (ROS) were observed after 280 mGy gamma exposure. Ortholog-based toxicity pathway analysis further showed that 15 mGy radiation affected DEGs associated with cellular signaling and immune response; 70 mGy radiation affected cell cycle regulation and DNA damage repair, cellular energy production; and 280 mGy radiation affected pathways related to cell cycle regulation and DNA repair, mitochondrial dysfunction and immune functions. Twelve genes representative of key pathways found in this study were verified by qPCR. Potential common MoAs of low-dose gamma radiation may include induction of oxidative stress, DNA damage and disturbance of oxidative phosphorylation (OXPHOS). Although common MoAs were proposed, a number of DEGs and pathways were still found to be dose-specific, potentially indicating multiple mechanisms of action (MOAs) of low-dose gamma radiation in fish. In addition, plasma glucose displayed an apparent increase with increasing radiation doses, although the results were not significantly different from the control. These findings suggested that sublethal doses of gamma radiation may cause dose-dependent transcriptional changes in the liver of Atlantic salmon after short-term exposure. The

current study predicted multiple MoA for gamma radiation and may aid future impact assessment of environmental radioactivity in fish.

Key words: Gamma radiation, Atlantic salmon, gene expression, microarray, pathway, mode of action

1. Introduction

Due to the highly penetrative photons (gamma ray) and interactions (ionization and excitation) with water molecules, high-energy gamma radiation emitted from radionuclides such as cobalt-60 (^{60}Co) may induce the formation of reactive free radicals and pose risk to living organisms. While the majority of toxicological studies has been performed to understand the health effects of gamma radiation on man and other mammals, relatively little is known about other environmentally important species, such as fish. Moreover, most of the current toxicity data have been generated based on high-dose radiation studies, which raises a question on whether knowledge from high-dose studies can be extrapolated to low-dose, or vice versa. The answer is likely to be negative, as the dose-response relationship usually appears to be non-linear and difficult to establish for low-dose radiation, potentially due to the dynamics between the compensatory mechanisms against radiation-caused damages in an organism. Therefore, more mechanistic studies with focus on the molecular and cellular responses are needed to better understand the low-dose effects of gamma radiation on non-mammalian species.

Although not being as detailed for mammalian species, the biological effects of gamma radiation have been documented for fish species at different biological levels. At the individual level, gamma radiation may affect the survival, development, reproduction and behavior of fish (Sazykina and Kryshev, 2003; Geiger et al., 2006; Ocalewicz et al., 2009; Epperly et al., 2012; Rhee et al., 2012). At the cellular level, gamma radiation has been found to cause programmed cell death (Lyng et al., 2004), chromosomal aberrations and mutations (Ocalewicz et al., 2009; Ocalewicz et al., 2010). At the molecular level, gamma radiation has been widely recognized to induce free radical formation mainly through ionization and excitation of water molecules. Free radicals such

as reactive oxygen species (ROS) may subsequently lead to cellular oxidative stress, DNA damage and many downstream effects (Olsvik et al., 2010). Nuclear DNA damage has for a long time been thought as a major effect of gamma radiation, but it has now been realized that damage of mitochondrial DNA, disturbance of oxidative phosphorylation (OXPHOS) (O'Dowd et al., 2009; Kam and Banati, 2013), perturbations of genomic stability (Lorimore et al., 2003; Shimada and Shima, 2004) and bystander effects (O'Dowd et al., 2006; Mothersill et al., 2007; Salbu et al., 2008) are also highly relevant. Up to now, the underlying mechanisms of these effects have not been fully understood.

Advanced genomic tools have brought new insights into the hazard assessment of environmental radionuclides and radioactivity. Toxicogenomics (OMICS) approaches such as RNA-sequencing, genome-wide DNA microarrays, proteomics and metabolomics in combination with rapidly developed bioinformatics solutions allow more in-depth studies on global molecular responses in an organism after exposure to environmental stressors. Such broad-content approaches have greatly facilitated the characterization potential modes of action (MoA) of a stressor (Roy et al., 2009). The OMICS tools have been widely employed to study the effects of gamma radiation on humans and other mammalian species (Nose et al., 2013), but have not been used to the same degree in non-mammalian species such as fish (O'Dowd et al., 2009; Jaafar et al., 2013).

Being more focused on the environmental relevance, the present study used Atlantic salmon (*Salmo salar*), a widely spread and commercially important fish species across Europe, as a model to study the short-term effects of low-dose external gamma radiation. Atlantic salmon were exposed to sublethal doses (15, 70 and 280 mGy) of external gamma radiation emitted from a cobalt-60 (^{60}Co) source for 48 h. The hepatic responses in fish were studied using 60,000-feature (60 k) custom oligonucleotide salmonid microarrays supported by targeted biostatistical and bioinformatics analyses. The objectives of this study were to: 1) identify short-term hepatic effects of gamma radiation at the transcriptional level; 2) characterize potential MoAs of gamma radiation in Atlantic salmon to inform future radiation studies.

2 Materials and methods

2.1 Exposure and sampling

The gamma irradiation experiment was conducted at the FIGARO experimental facility (Norderås, Norway) of Norwegian University of Life Sciences (NMBU, Ås, Norway). Atlantic salmon juveniles (parr) were maintained at the Fish Laboratory of NMBU and transported to the FIGARO irradiation facility 7 days prior to the exposure studies to allow sufficient acclimatization. Feeding stopped two days before the exposure to avoid potential alteration of metabolic rate in fish and change of water variables. External gamma radiation was emitted from a ^{60}Co source. The radiation doses were controlled by distances from the ^{60}Co source. For each treatment, six fish were exposed in a dark exposure chamber (30 L, fish loading approx. 3 g/L) supplied with circulated lake water (Maridalsvannet, Norway) and air pumps. Three gamma doses were tested during one exposure experiment, their total doses were 15 mGy (approx. 0.3 mGy/h, referred to as Low), 70 mGy (approx. 1.5 mGy/h, referred to as Medium) and 280 mGy (approx. 5.8 mGy/h, referred to as High). These gamma doses were considered to be sublethal doses in fish and environmentally relevant at contamination sites (UNSCEAR, 1996; Benova et al., 2006; Jaafar et al., 2013).

After 48 h exposure, fish were immediately sacrificed by cephalic concussion. The morphology of test fish, such as skin color, mucus quality, gill shape and color were briefly checked. Blood samples were taken for measurement of plasma glucose levels using an i-STAT® portable analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) with EC8+ cassette (Abbot, East Windsor, USA). Tissue samples were collected and snap-frozen in liquid nitrogen and stored at -80 ^{o}C until further analysis. The fish experiment was approved by the local representative of the Norwegian Animal Research Authority (NARA ID: 3026). All operations strictly followed the Norwegian Welfare Act and Research Animal Legislation.

Exposure conditions, such as pH, temperature and conductivity of the test water were monitored throughout the experiment. Water samples were collected after the exposure to measure the concentrations of major cations (Ca, Mg, Na, K and Al), anions (NO^{3-}, SO_4^{2-} and Cl^-) and total organic carbon (TOC), as described previously (Song et al., 2012).

2.2 Microarray analysis

Total RNA was isolated from 20-30 mg frozen liver using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) as described previously (Song et al., 2012). The microarray gene expression analysis was performed according to Agilent's standard protocol "One-Color Microarray-Based Gene Expression Analysis, version 6.5" using a

custom salmonid oligonucleotide (60-mer) microarray (Gene Expression Omnibus, GEO platform accession number: GPL18864) manufactured by Agilent (Agilent Technologies, Santa Clara, CA, USA). Four biological replicates per exposure dose and control were used for microarray analysis.

Raw data extracted from microarray scan images (Feature Extraction software v10.7, Agilent Technologies) was corrected for background signal, flagged for low quality and missing features, normalized (quantile) and log2 transformed using GeneSpring GX v11.0 software (Agilent Technologies). Raw and normalized data have been deposited at GEO (accession number: GSE59035). Statistical determination of differentially expressed genes (DEGs) was performed using one-way analysis of variance (ANOVA). A fold change cut-off of 1.5 was applied to DEGs used for downstream analyses. Major patterns of global transcriptional response were visualized using k-means clustering implemented in GeneSpring (Agilent). To further differentiate dose-related DEGs, a Tukey HSD posthoc test was performed.

A Cytoscape v2.8 (Smoot et al., 2011) application Bingo v2.4 (Maere et al., 2005) was used for functional enrichment of Gene Ontology (GO) functions. Significant enrichment of GOs was determined using a hypergeometric test followed by Benjamini–Hochberg false discovery rate (FDR) correction. Furthermore, *Salmo salar* DEGs were mapped to mammalian orthologs by a modified Inparanoid algorithm (Ostlund et al., 2010) to perform gene network and pathway analyses in IPA (Ingenuity®Systems, www.ingenuity.com), as described previously (Song et al., in press). Venn diagram analysis was performed using Venny (bioinfogp.cnb.csic.es/tools/venny/index.html). Significant differences were considered at the $p<0.05$ level in all statistical tests.

2.3 Quantitative rtPCR verification

Twelve genes encoding proteins representative of important regulators in several pathways were verified by quantitative real-time reverse transcription polymerase chain reaction (qPCR). Briefly, a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California, USA) was used to synthesize complementary DNA (cDNA) from 2 μg total RNA (6 biological replicates). Five ng cDNA template per replicate was amplified in a 20 μL reaction containing 400 nM forward/reverse primers (Invitrogen™,Carlsbad, California, USA) and PerfeCTa® SYBR® Green FastMix® (Quanta BioSciencesTM, Gaithersburg, MD, USA) on a BioRad CFX 384 platform (Bio-Rad Laboratories, Hercules, CA, USA) with 2 technical replicates. A web-based Primer3

v0.4 program (frodo.wi.mit.edu/primer3) was used to design *Salmo salar* primer sequences (Table 1). Optimal annealing temperature was obtained prior to the formal analysis for each primer pair. Non-template controls (NTCs) and no-reverse-transcriptase controls (NRCs) were also included in the amplification and melting curves were determined for all samples. Standard curve was made based on 50, 10, 2 and 0.4 ng of pooled cDNA template from all samples. The relative starting quantity of cDNA template was calculated based on the standard curve, amplification efficiency and threshold cycle (Cq) value using the ΔCq implemented in CFX manager 3.1 data processing software (Bio-Rad). The calculated expression value was normalized to the geometric mean of two reference genes, 18S ribosomal RNA (18S) and translation elongation factor 1 alpha b (EF1Ab) using the ΔΔCq method. Fold change was calculated by comparing the normalized relative expression value to that in the control. All statistical analyses were then performed in Graphpad Prism v5.0 (Graphpad Software, Inc., San Diego, CA, USA). Outliers were removed using the ROUT test implemented in the Graphpad software. One-way ANOVA and Tukey's post-hoc tests were performed to determine differential gene expression between exposed and control groups. Data that failed to meet the assumption of normal distribution and equal variance were analysed using Kruskal-Wallis non-parametric test followed by a Dunn's post-hoc test. A p-value of 0.05 was applied to all tests.

Table 1. Primer sequences, Genbank accession numbers and annealing temperatures used for quantitative real-time rtPCR

Gene	**Genbank accession**	**Forward primer (5'-3')**	**Reverse primer (5'-3')**	**Annealing temp. (ºC)**
18S ribosomal RNA (18S)	AJ427629	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG	59.1
BCL2-associated X protein (BAX)	EG801847	ATTGGAAATGAGCTGGATGG	GCCGACAGGCAAAGTAGAAG	59.4
Caspase 6A (CASP6A)	DQ008068	TGAGCCACGGAGAGAACGA	CCCACCAGGCTCTTACACTTG	64.5
Cytochrome c oxidase subunit VIb polypeptide 1 (COX6B1)	BT125515	GACAATGCTTGGCACATACG	TGTCAGCAGATGCAGAGTCC	59.1
Cytochrome c-1 (CYC1)	BT057115	CAGGTGGTCTGTGCTGAAGA	TTGAGAAGACCCCCATCAAG	60
Translation elongation factor 1 alpha b	AF321836	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	59.1

(EF1Ab)				
Growth arrest and DNA-damage-inducible gamma (GADD45G)	EG905871	GAGAGCCGCAGTGTGTATGA	CCTCCACAGGGTAATCCAGA	59.4
Matrix metallopeptidase 23 (MMP23)	BT072316	GAGCACCATGATCTGGGAGT	AAGCCTGCCAAAAAGAGACA	60
Tumor protein TP53 (P53)	BT072559	ACAGGAGCGACTCGGACTAA	GTCCGTCCCTATTGACTCCA	65
Proliferating cell nuclear antigen (PCNA)	BT046966	CAGGGATCCATCCTGAAGAA	GTCCTCATTCCCAGCACACT	59.4
Peroxiredoxin 1 (PRDX1)	BT047146	TTCTTCTTCTACCCGCTGGA	CTGGTCCTCCTTCAGCACTC	60
RAD51 recombinase (RAD51)	NM_001140555	GGTCCAAAGAGCTGGACAAG	ACGTCACTCCCAACAAGTCC	63.4
Retinoblastoma 1 (RB1)	NM_001173615	CCATGTACGCCATATGCAAG	TGCAGGATGTTCGTCTTCAG	62.5
Copper-zinc superoxide dismutase (SOD)	BG936553	CCACGTCCATGCCTTTGG	TCAGCTGCTGCAGTCACGTT	61.6

3 Results

3.1 General exposure variables

The test media had temperature of 10.1±0.4 ºC, conductivity of 3.6±0.3 mS/m and pH of 7.1 throughout the experiment. No significant differences were found in TOC, ion or anion concentrations after exposure (Table 2). No mortality or visual morphological changes were observed in the test fish after 48 h irradiation. Test fish had an average length of 12±0.3 cm and a weight of 15.4±0.8 g. No significant differences were found between fish sizes. The plasma glucose levels were 6.4±4.8 mM (Control), 7.0±6.4 mM (Low), 7.3±4.2 mM (Medium) and 7.8±5.8 mM (High) after 48 h exposure. An apparent increase of glucose concentration was observed, however, the changes were not found to be significantly different from the control with the statistical cutoff used.

Table 2. Variables in the test water after 48 h gamma radiation exposure.

Treatment	TOC (mg/l)	Cl^- (mg/l)	NO^{3-} (mg/l)	SO_4^{2-} (mg/l)	Na (mg/l)	Mg (mg/l)	Al (µg/l)	K (mg/l)	Ca (mg/l)
Control	5.16	1.99	0.15	2.59	1.87	0.60	92	0.96	3.10
Low (15 mGy)	5.21	2.00	0.15	2.59	1.76	0.61	94	0.72	3.25
Medium (70 mGy)	5.16	1.99	0.15	2.59	1.73	0.61	92	0.80	3.28
High (280 mGy)	5.21	2.00	0.15	2.59	1.83	0.60	92	0.86	3.16

3.2 Global transcriptional changes

As many as 2241 gene transcripts were found to be differentially regulated (one-way ANOVA) across treatment groups compared to the control after exposure to gamma radiation. The DEGs were filtered by their expression level (fold change≥1.5) to minimize potential noise from marginally responsive genes. The remaining 1617 DEGs were then used for k-means clustering analysis to predict major response patterns. The k-means clustering revealed four major patterns of global transcriptional changes (Figure 1), in which the expression of approx. 38% DEGs increased with increasing the radiation dose (cluster 1), approx. 15% decreased with increasing the radiation dose (cluster 2). The remaining genes (cluster 3 and 4) had fluctuating response patterns from Low to High, representing roughly 15% and 32% of filtered DEGs, respectively.

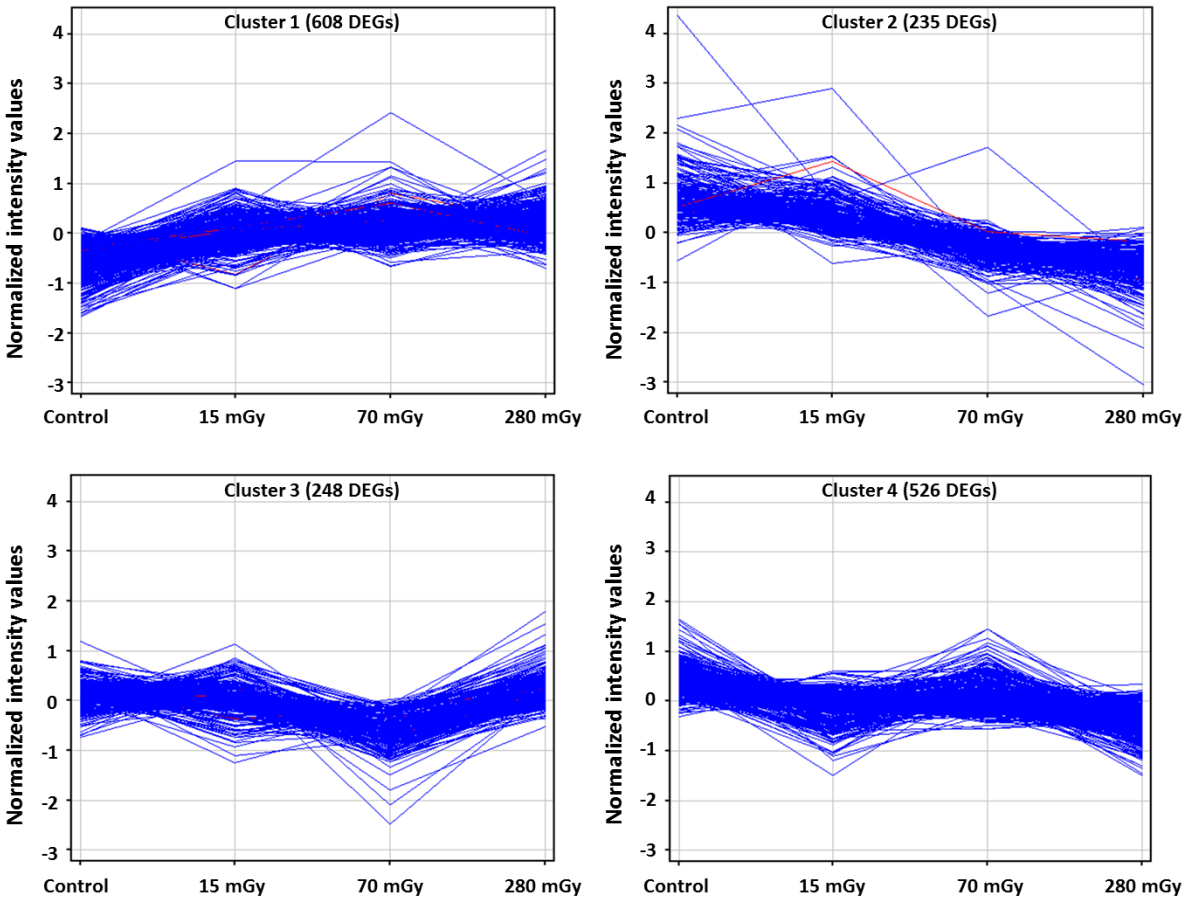

Figure 1. K-means clustering of hepatic differentially expressed genes (DEGs) in Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.

The ANOVA posthoc test identified that totally 222 (121 up-regulated, 101 down-regulated), 495 (259 up-, 236 down-) and 909 (403 up-, 506 down-) DEGs were regulated by Low, Medium and High gamma radiation exposure, respectively. The number of DEGs generally increased with increasing radiation dose. The Venn diagram analysis (Figure 2) showed that only 22 up- and 12 down-regulated DEGs were commonly regulated by all gamma doses, whereas high numbers of DEGs were uniquely regulated by different gamma doses. The complete DEG list can be found in Appendices (Table A1).

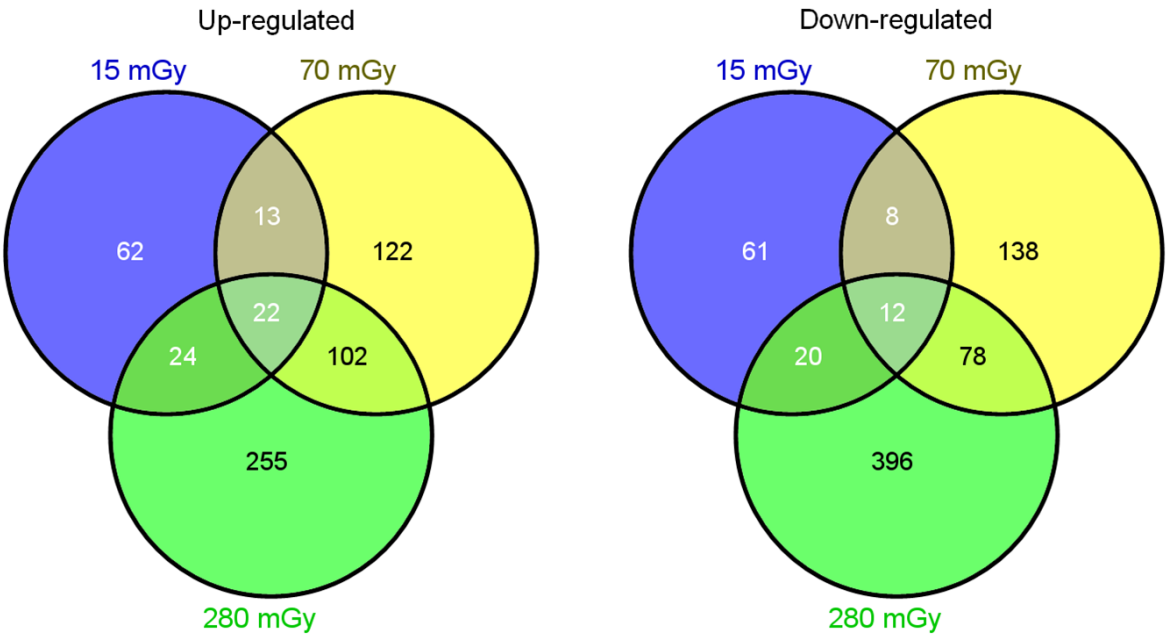

Figure 2. Venn diagram analysis of hepatic differentially expressed genes (DEGs) in Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.

3.3 Functional enrichment analysis

The biological functions of DEGs were predicted first by linking genes to associated GO terms. The GO functional enrichment analysis showed that no significant enrichment was identified in Low or Medium gamma treatment, whereas 56 GO terms were found to be significantly overrepresented in the High gamma exposure group. Among these, 8 GOs were associated with up-regulated DEGs and 48 were associated with down-regulated DEGs. The overrepresented GOs were mainly involved in 6 biological process (BP) categories (Figure 3). The majority of overrepresented biological processes were related to down-regulated genes. Up-regulated DEGs by High gamma were also related to molecular functions (MF) such as nucleoside kinase activity and ligase activity. Down-regulated DEGs were associated with molecular functions such as macromolecule binding, nuclease activity, transferase activity, structural molecular activity, hydrolase activity, and affected cellular components (CC) such as protein complex, cytoplasm, Ribosome, chromosome and extracellular part. Completed list of overrepresented GOs can be found in Appendices (Table A2).

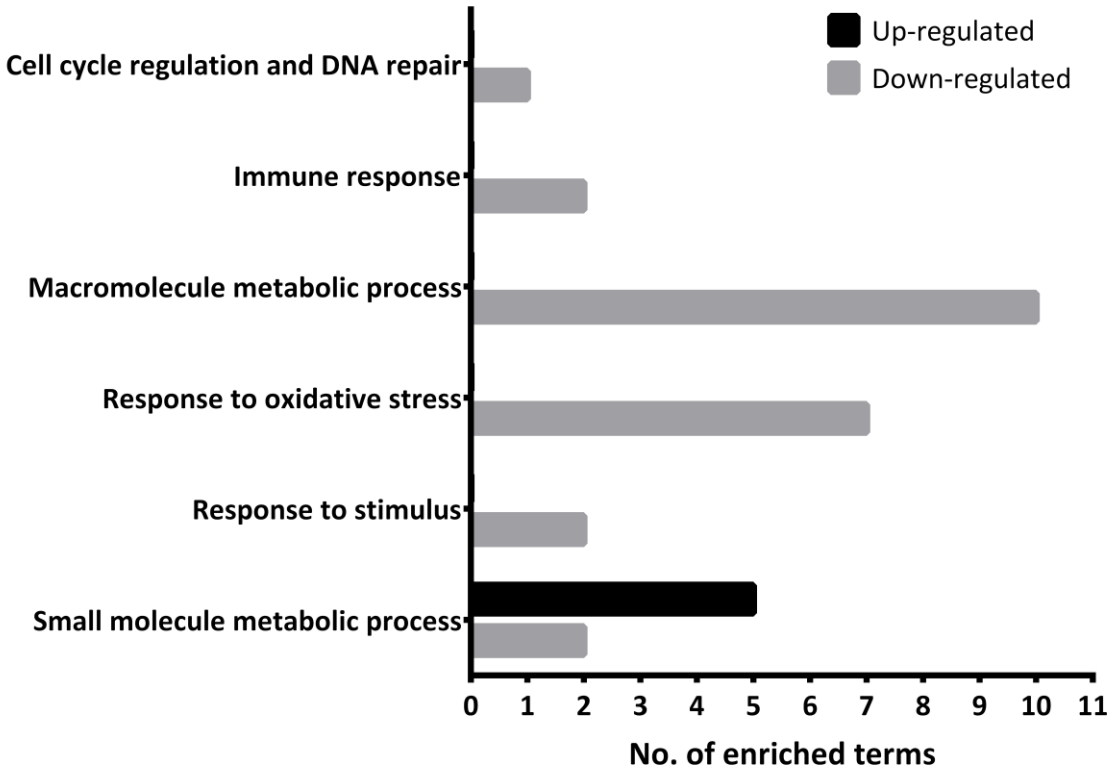

Figure 3. Major functional categories of hepatic overrepresented Gene Ontology (GO) biological processes that were regulated in Atlantic salmon (*Salmo salar*) after 48 h exposure to 280 mGy (total) gamma radiation.

To utilize existing information on well-curated mammalian protein-protein interactions and pathways as a reference to understand the fish data, *Salmo salar* DEGs were mapped to mammalian orthologs. Approximately 57.4% (Low gamma), 56.9% (Medium gamma) and 59.7% (High gamma) DEGs were successfully mapped (see Appendices Table A3 for full list). Gene network analysis was conducted first to obtain an overview of gene clusters according to their functions (Figure 4). The results showed that the number and complexity of gene networks increased with increasing the gamma dose. Fish exposed to the Low gamma resulted in differential regulation of gene networks related to transportation of molecules, protein-protein communications and development, disorders of connective tissues and carbohydrate metabolic processes. Four major gene networks related to development and morphology, carbohydrate and lipid metabolism, cell signaling, gene expression and posttranslational modifications and hematological system were identified in fish exposed to Medium gamma. High gamma regulated six predominant gene networks functioning in

development, hereditary processes, cellular function and maintenance, neurological functions, cell signaling, hematological system and various metabolic processes. Functions such as development, carbohydrate metabolism and cell signaling were predicted to be commonly affected by different radiation doses.

Figure 4. Top hepatic gene networks that were regulated in Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation. Major network functions (nodes) and common genes (next to edges) between different networks were presented.

Enrichment of toxicity and canonical pathways were then determined to obtain more in-depth understanding of DEGs predicted to be involved in specific toxicological and biological processes. Venn diagram analysis (Figure 5) clearly showed that most of the toxicity pathways regulated were dependent on the gamma doses, with Low gamma affected inorganic phosphate homeostasis, Medium gamma modulated DEGs associated with the mitochondrial membrane potential, fatty acid metabolism, nuclear receptor signaling and hypoxia responses, and High gamma affected DEGS associated with DNA damage response, antioxidant response,

mitochondrial dysfunction and cell death. Two pathways related to cell cycle regulation and cardiovascular disorders were common between Medium and High gamma. No toxicity pathway was found to be commonly regulated by all radiation doses. Complete lists of toxicity pathways regulated by different radiation doses can be found in Appendices (Table A4).

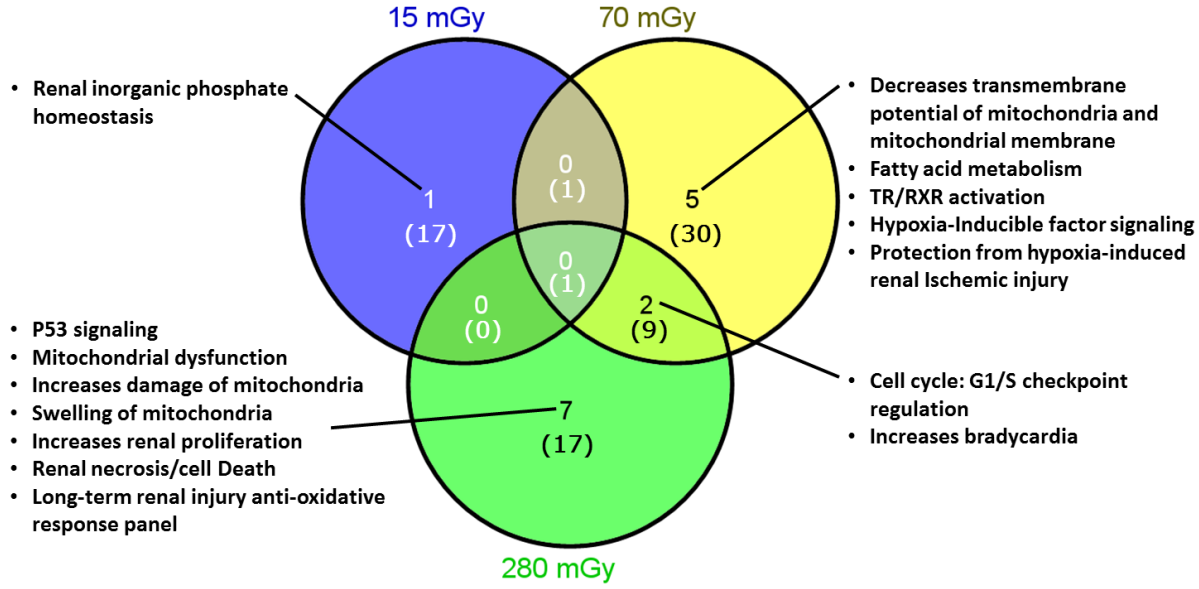

Figure 5. Venn diagram analysis of toxicity and canonical (in brackets) pathways that were affected in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation. Detailed lists of the toxicity pathways were also presented.

The majority of the enriched canonical pathways were affected in a dose-dependent manner (Figure 5). Totally 17, 30 and 17 canonical pathways were uniquely regulated by Low, Medium and High gamma, respectively. Only clathrin-mediated endocytosis signaling pathway was found to be common for all treatments. Medium and High gamma treatments tended to have more common pathways (9 pathways) compared to only one pathway between Low and Medium (4-aminobutyrate degradation I). Although few similar pathways were identified across different exposure doses, these pathways grouped into a few higher functional categories, such as protein metabolism, nuclear receptor signaling, neurotransmitters and other nervous system signaling, intracellular and second messenger signaling, immune response, hormones

biosynthesis, energy production, DNA metabolism, cellular growth, proliferation and development, cell cycle regulation and DNA repair and cancer signaling. Low gamma mainly regulated DEGs in canonical pathways involved in the immune functions (e.g. eicosanoid signaling and prostanoid biosynthesis), energy-associated metabolic processes (e.g. 3-phosphoinositide degradation) and protein metabolism (e.g. histidine degradation III). Medium gamma mainly affected pathways related to immune response (e.g. role of NFAT in regulation of the immune response), cancer signaling (e.g. glioma signaling) and cell cycle regulation and DNA repair (e.g. mitotic roles of polo-like kinase). High gamma dose regulated DEGs associated with functions such as cell cycle regulation and DNA repair (e.g. P53 signaling), energy production (e.g. mitochondrial dysfunction) and immune response (e.g. antigen presentation pathway). A selection of ecotoxicologically relevant canonical pathways and associated DEGs was shown in Table 3. Complete lists of canonical pathways regulated by different radiation doses can be found in Appendices (Table A5).

Table 3. A selection of potential ecotoxicologically relevant canonical pathways and supporting hepatic differentially expressed gene transcripts (DEGs) in Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation. Up-regulated: ↑; down-regulated: ↓.

Functional category	Canonical Pathways	p-value	Ratio	Supporting DEGs[a]
15 mGy				
Cellular growth, proliferation and development	Inhibition of matrix metalloproteases	1.07E-03	7.50E-02	MMP23B↑, MMP2↑, LRP1↑
Immune response	Eicosanoid signaling	4.07E-02	2.47E-02	RARRES3↑, PTGDS↑
	Prostanoid biosynthesis	4.57E-02	6.67E-02	PTGDS↑
70 mGy				
Immune response	Role of NFAT in regulation of the immune response	1.51E-02	3.02E-02	BLNK↑, PIK3R1↓, GNG2↓, MS4A2↓, GNG3↑, RCAN2↓
	IL-8 signaling	2.24E-02	2.88E-02	ITGB2↓, PIK3R1↓, GNG2↓, RAC1↓, GNG3↑, IQGAP1↓
	CTLA4 signaling in cytotoxic T lymphocytes	2.29E-02	4.08E-02	PPP2R5D↑, PIK3R1↓, AP1B1↑, AP2A2↑
Neurotransmitters and other nervous system signaling	GABA receptor signaling	2.19E-03	7.14E-02	SLC6A11↑, GABARAP↑, AP1B1↑, AP2A2↑
Nuclear receptor signaling	TR/RXR activation	1.74E-02	4.17E-02	SLC2A1↑, PIK3R1↓, NCOA4↑, RCAN2↓
280 mGy				

Cell cycle regulation and DNA repair	P53 signaling	4.07E-03	7.29E-02	CASP6↑, RB1↓, CCND2↓, THBS1↑, RRM2B↓, CCND1↓, FAS↓
	Role of BRCA1 in DNA damage response	4.07E-02	6.15E-02	RB1↓, RBL2↓, FAM175A↓, BRCC3↑
Energy production	Mitochondrial dysfunction	1.95E-02	4.17E-02	NDUFA4↓, COX7A2↓, MAPK10↓, MT-CO3↓, PSENEN↓, NDUFB10↑, MAOA↓, COX6A2↓
Immune response	Leukocyte extravasation signaling	2.88E-02	4.35E-02	ITGB2↓, MMP23B↑, CRKL↑, ACTN2↓, ACTB↑, SIPA1↑, CD44↓, MAPK10↓, RAC1↓
	Antigen presentation pathway	4.57E-02	7.50E-02	HLA-B↓, HLA-DPB1↓, MR1↓
	CXCR4 signaling	4.90E-02	4.12E-02	GNG2↓, GNB5↑, MAPK10↓, RAC1↓, LYN↓, GNAZ↓, MYL1↑
Common between 70 & 280 mGy				
Cell cycle regulation and DNA repair	GADD45 signaling	8.71E-03	1.30E-01	CCND2↓, CCND1↓, CCNB1↓
	Cell cycle: G1/S checkpoint regulation	1.10E-02	7.46E-02	RB1↓, RBL2↓, CCND2↓, RPL5↓, CCND1↓
	Cyclins and cell cycle regulation	3.02E-02	5.56E-02	RB1↓, CCND2↓, PPP2R5D↑, CCND1↓, CCNB1↓
Energy production	Fatty acid β-oxidation I	2.69E-02	6.67E-02	SLC27A2↑, SDS↑, HADHA↓

[a] Full descriptions of gene symbols can be found in Appendices (Table A3).

[b] Data presented was from 280 mGy gamma radiation treatment.

3.4 Quantitative qrtPCR verification

A selection of genes encoding proteins involved in a few key toxicological functions, such as antioxidant defense (peroxiredoxin 1 (PRDX1) and copper-zinc superoxide dismutase (SOD)), cell cycle regulation and DNA repair (growth arrest and DNA-damage-inducible gamma (GADD45G), proliferating cell nuclear antigen (PCNA), retinoblastoma 1 (RB1), tumor protein TP53 (P53) and RAD51 recombinase (RAD51)), apoptosis (BCL2-associated X protein (BAX), caspase 6A (CASP6A)), mitochondrial electron transport chain (cytochrome c oxidase subunit VIb polypeptide 1 (COX6B1) and cytochrome c-1 (CYC1)) and extracellular matrix degradation (matrix metallopeptidase 23 (MMP23)). Among these, six DEGs (*CASP6A*, *SOD*, *PRDX1*,

MMP23, CYC1 and *RB1*) were verified to be significantly affected by different gamma doses using qPCR (Figure 6), although slight differences in both magnitude of expression and statistical significance were observed for some of the treatment groups (e.g. PRDX1 and MMP23 in 15 mGy gamma treatment). Four genes (*P53, GADD45G, COX6B1* and *BAX*) were identified by qPCR, but not in the microarray analysis, to be significantly affected. Some differences in response patterns were also observed between the two types of gene expression (of *P53* and *BAX*). Two genes (*PCNA* and *RAD51*) were not identified to be significantly expressed by either microarray or qPCR, but still had similar response patterns (i.e. magnitude and direction of regulation).

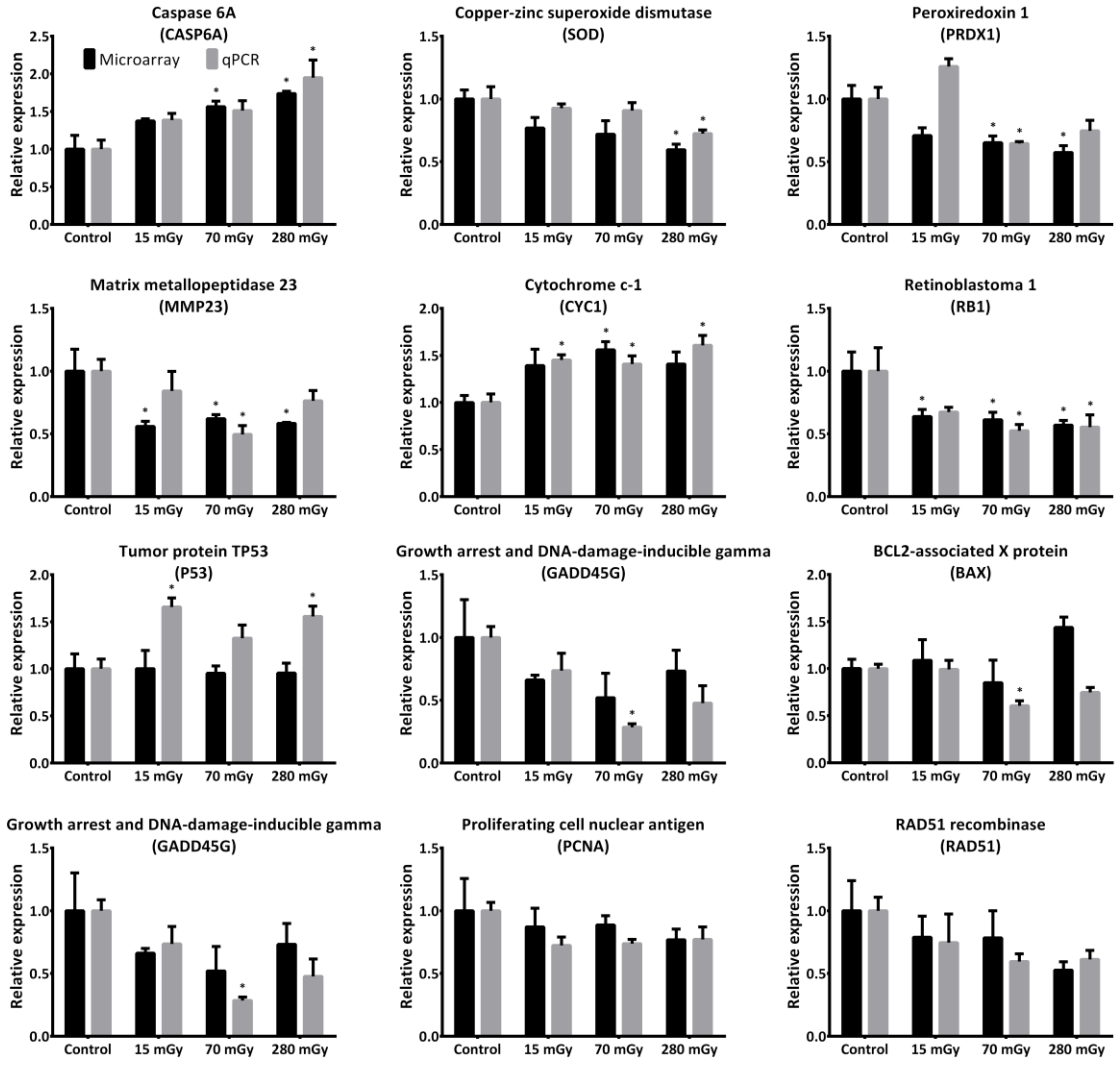

Figure 6. A comparison of hepatic gene expression responses in Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation measured by microarray (N=4) and quantitative qrtPCR (N=4-6). * Denotes significant difference from the corresponding control.

4. Discussion

Fish were generally considered to be fairly resistant to radiation compared to other vertebrate species (Ulsh et al., 2003). Although little toxicity data on gamma radiation aree available for Atlantic salmon so far, reports on other fish species such as *Danio rerio* (Jaafar et al., 2013) or *Poecilia reticulate* (Benova et al., 2006) showed that the acute 50% lethal dose (LD50, 4-24 h) of gamma radiation could be much higher (10-31 Gy) than the test doses (0.015-0.28 Gy) used in the current study. The plasma glucose level, which is usually considered to be a general stress parameter, was slightly increased with increasing gamma doses. However, this increase was not statistically significant. The glucose level in salmon was generally within the upper range expected for non-stressed fish (Kroglund et al., 2001). Although all test gamma doses in the present study were sublethal and considered to be relatively low, dramatic dose-related stress responses at the transcriptional level were still observed. These changes indicated that gene expressions could be used as sensitive endpoints to evaluate the effect of radiation at environmentally realistic exposure levels. To better interpret the gene expression data for non-model fish species such as Atlantic salmon, the current study mainly employed an ortholog mapping approach to link salmon DEGs towards well-characterized human and mammalian toxicological knowledge. Although limitations such as loss of information during ortholog mapping and differences in toxicological functions between fish and mammals may limit the value of this approach for certain toxicological responses, information on several responses to gamma radiation may still be derived for generating hypothesis on which MoAs are the most relevant in fish. Since fish were exposed to radiation only for a short term (48 h), the current transcriptomic data likely reflected a balance between stress-induced negative signaling pathways and activated hepatic defense mechanisms in salmon.

4.1 Transcriptional responses to 15 mGy radiation

Based on the gene network analysis, as low as 15 mGy gamma radiation may affect genes encoding proteins involved in several transport, developmental, signal transduction and metabolic processes. Pathway analysis

further showed a few toxicologically relevant canonical pathways associated with extracellular communication and inflammatory response such as inhibition of matrix metalloproteases (MMPs) and eicosanoid signaling were differentially regulated. The matrix metalloproteases (MMPs), which can usually be activated in extracellular space by environmental stressors such as X-ray (150 Gy/h, Araya et al., 2001), gamma radiation (289 Gy/h, Park et al., 2006) and metals (Cammarota et al., 2006), may be involved in various signaling processes such as cell growth, differentiation, migration, proliferation, polarization, release of apoptotic ligands, chemokine/cytokine-induced inflammation and carcinogenesis in mammalian species (van Lint and Libert, 2007; Hojilla et al., 2008; Tsang et al., 2010; Naba et al., 2012). The regulation of MMP may be at the transcription level, enzyme activation level or dynamic inhibition of enzymatic activity by tissue inhibitors of metalloproteinases (TIMPs) (Mauviel et al., 1993; Ries and Petrides, 1995). Since no TIMPs were found to be induced (data not shown), it was likely that the MMP functions were activated after radiation exposure. This was supported by the transcriptional up-regulation of key genes such as *MMP23B*, *MMP2* and the gene enoding low density lipoprotein receptor-related protein 1 (*LRP1*) in this pathway. The toxicological role of MMP activation has not been well-characterized in fish yet, but it has been shown in several types of human and mammalian cells that the expression of MMP genes or enzymes such as *MMP2* was elevated after radiation exposure (Sawaya et al., 1994; Zhao et al., 1999; Zhao et al., 2000; Araya et al., 2001). The MOA for MMP activation by radiation was probably due to the ability of ROS to act as messenger molecules to influence the normal signal transduction (Belkhiri et al., 1997; Siwik et al., 2001; Zhao et al., 2004), or due to the radiation-induced chemokine signaling during acute inflammatory response (Chadzinska et al., 2008; Castillo-Briceno et al., 2010). The latter was also supported by the differential regulation of eicosanoid signaling pathway, which plays major roles in signal transductions for mediating complex biological functions such as inflammation, vascular permeability, allergic reactions and induction of carcinogenesis (Candido and Hagemann, 2013; Claudiano Gda et al., 2013).

4.2 Transcriptional responses to 70 mGy radiation

More complex gene networks were found in the 70 mGy gamma exposed fish compared to that in the 15 mGy gamma group. Clusters of DEGs co-functioning in the organ development, morphology, carbohydrate and lipid metabolism, transcriptional regulation and cellular signal transduction were successfully

characterized using mammalian protein-protein interactions as a reference. Specific biological functions affected by Medium gamma were further revealed by pathway analyses. The enriched pathways were mainly related to mitochondrial functions and DNA repair processes.

The mitochondrion has been considered as an important target of ionizing radiation toxicity. The causal relationship between radiation exposure and mitochondrial dysfunction has been well-documented for mammalian species (Kam and Banati, 2013). A number of pathways related to the reduction of mitochondrial membrane potential were affected by the 70 mGy gamma exposure in the present study. Supporting DEGs in these pathways such as oxidation regulator gene encoding CDGSH iron sulfur domain 1 protein (*CISD1*) and non-selective cation channel regulator gene transient receptor potential cation channel subfamily V member 1 (*TRPV1*) were found to be repressed after exposure to medium gamma. It has been widely accepted that dissipation of mitochondrial membrane potential and subsequent uncoupling of oxidative phosphorylation may lead to the loss of aerobic energy supply or even cell death (Joshi and Bakowska, 2011). Reduced mitochondrial membrane potential has also been reported in other fish species, such as rainbow trout (*Onchorhynchus mykiss*), after exposed to 0.5 and 5 Gy gamma radiation (O'Dowd et al., 2006). Two potential mechanisms may be involved in the disturbance of mitochondrial transmembrane potential by gamma radiation. It is likely that gamma radiation-induced ROS generation may cause damages to the mitochondrial inner membrane and ion channel proteins (Strassle et al., 1987; Leanza et al., 2014), thus leading to the loss of membrane functions and reduced membrane potential. Another mechanism may be a potential compensatory action to control the endogenous ROS production in the mitochondrial electron transport chain (ETC). Recent studies suggested that self-uncoupling (reduction of membrane potential) processes may be triggered by excess ROS in the mitochondrion in order to reduce further ROS formation through redox reactions in the ETC (Mailloux and Harper, 2011; Shabalina, 2011; Mailloux and Harper, 2012). In addition to the loss of mitochondrial membrane potential, the enrichment of a toxicity pathway related to the fatty acid metabolism may also provide some clue on the perturbation of mitochondrial energetic functions by gamma radiation. A few DEGs in this pathway were found to play key roles in the fatty acid beta-oxidation, potentially indicating elevated energy demand in fish in response to radiation stress.

Another type of transcriptional responses to 70 mGy gamma was predicted to be related to the cell cycle regulation and DNA repair. Canonical pathways that served in the cell cycle checkpoint functions, such as

mitotic roles of polo-like kinase, cyclins and cell cycle regulation, cell cycle: G1/S checkpoint and GADD45 signaling were found to be affected. A few down-regulated DEGs were found to be commonly present in these pathways, such as genes encoding cyclin-dependent kinase 1 (CDK1) and cyclin B1 (*CCNB1*). Provided that the transcriptional changes are reflected in the protein activity changes, this indicates hampered progression of mitosis and potential activation of DNA repair (Clarke and Allan, 2009; Zhang et al., 2011). Based on these findings, it can be deduced that DNA damage may have occurred after 48 h exposure to 70 mGy gamma radiation. DNA damage has been considered as a primary MoA of ionizing radiation. Previous studies have revealed that both direct DNA damage through ionization of DNA molecules and indirect damage through ROS may take place following radiation exposure (Jarvis and Knowles, 2003; Simon et al., 2011; Guo et al., 2013). It has also been suggested that ROS may also be involved in various biological processes as messenger molecules and directly stimulate the mitogenic pathways, regulate the CDKs and phosphorylation of RB in G1-phase, and control the S-phase entry directly in order to activate DNA repair mechanisms and maintain genomic stability (Burhans and Heintz, 2009).

4.3 Transcriptional responses to 280 mGy radiation

The global transcriptional changes after 280 mGy gamma radiation exposure seemed to be more complex compared to those in the lower gamma dose treatments. Gene Ontology-based functional analysis revealed a few cellular components related to DEGs, such as protein complex, ribosome and chromosome. Potential interactions of radiation with macromolecules were further shown by enriched GO molecular functions, such as macromolecule binding, nuclease activity, transferase activity, structural molecular activity and hydrolase activity. These transcriptional changes may be a consequence of direct effect of radiation on macromolecular structures which subsequently changes the functions of these molecules, or indirectly through ROS induced oxidative stress which damaged the molecules. Ortholog-based approaches further identified pathways related to DEGs regulated by 280 mGy gamma. Although multiple pathways were found to be enriched, they may still be grouped into a few functional categories, such as macromolecule metabolism, oxidative stress response, cell cycle regulation and DNA repair, energy production and metabolism and immune response. A few important functions such as oxidative stress response were identified by both GO and pathway analysis. Interestingly, DEGs associated with oxidative stress response were found to be mainly repressed by gamma radiation (e.g.

peroxiredoxin-1), indicating that antioxidant defense may be reduced after radiation exposure. The reason for this was not clear, but previous studies have suggested that excessive oxidative stress or cross-talks of signaling pathways may be linked to the reduction of antioxidant defense activities (Srinivasan et al., 2007; Liu et al., 2008; Ahmad et al., 2013). It has also been reported that apoptosis in murine hepatocytes may be involved in the cleavage of antioxidant enzymes (Franklin et al., 2003). These evidences may provide some hints on the potential repression of antioxidant genes after radiation exposure.

Besides pathways involved in the cell cycle control which has been observed in the 70 mGy gamma treatment, the predicted activation of tumor protein P53 signaling pathway by 280 mGy gamma radiation may provide another evidence to support the potential DNA damage caused by gamma radiation. The P53 gene has been considered as an exposure biomarker of ionizing radiation and may act as an upstream regulator for a number of downstream toxicological functions, such as cell cycle arrest, DNA single- and double-strand break repair, apoptosis and protein degradation (Schwartz and Rotter, 1998). Although P53 was not significantly regulated when considering the microarray data, it was found to be differentially regulated by both Low and High gamma treatments using qPCR. Similar findings on the induction of P53 gene has been observed in Atlantic salmon exposed to 75 mGy gamma radiation for 5 h (Olsvik et al., 2010). As direct downstream targets of P53, genes encoding proteins that regulate anti-angiogenesis (e.g. thrombospondin 1 (TSP1)) (Zhou et al., 2009), apoptosis (e.g. tumor necrosis factor receptor superfamily member 6 (FAS), BAX and CASP6) (Embree-Ku et al., 2002), cell cycle regulation (e.g. GADD45 and RB1) (Amundson et al., 1998) were also found to be differentially expressed (Figure 7-A). The repression of DEGs linked to cell cycle regulation may either indicate reduced cell progression or abnormal regulations of DNA damage checkpoints in the cell cycle, which may potentially lead to insufficient or even wrongly repaired DNA following DNA damage. When focusing on the P53-dependent apoptosis pathways, interesting patterns of response were observed. Genes that play important roles in the extrinsic (death receptor) apoptotic signaling (e.g. genes encoding Fas cell surface death receptor (*Fas*)) and intrinsic (mitochondrial) apoptosis signaling protein (*BAX*) were found to be down-regulated after High gamma dose exposure, whereas the downstream effector caspase, the caspase 6A gene was up-regulated. It seemed that apoptosis was potentially induced, however, not through major signaling pathways (i.e. intrinsic and extrinsic apoptosis). The transcriptional induction of the gene encoding caspase 6

by gamma radiation has also been observed previously in Atlantic salmon after 5 h exposure to 75 mGy gamma radiation (Olsvik et al., 2010).

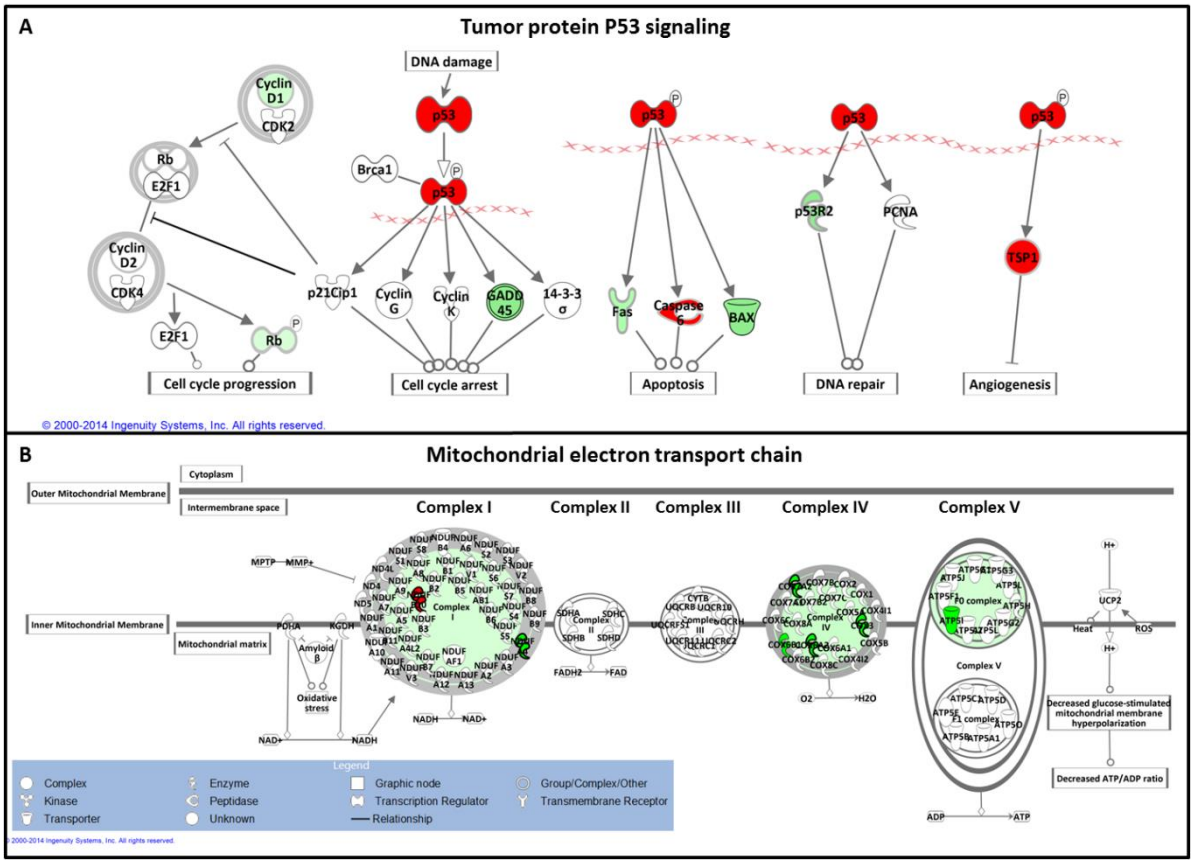

Figure 7. Canonical pathways that were affected by 280 mGy gamma radiation. A. Tumor protein P53 signaling pathway; B. Mitochondrial dysfunction pathway with focus on electron transport chain (ETC). Colored molecules are differentially expressed gene transcripts (DEGs) identified in the present study. Red: up-regulated; Green: down-regulated (Modified from Ingenuity Pathway Analysis www.ingenuity.com/products/ipa). Complete descriptions of gene symbols can be found in Appendices (Table A3).

Changes of gene expression in the mitochondrion were also observed in fish exposed to 280 mGy radiation (Figure 7B). Several DEGs involved in mitochondrial ETC complex I such as genes enoding NADH dehydrogenase 1 alpha subcomplex 4 (*NDUFA4*), complex IV such as, cytochrome c oxidase subunit 3 (*COX3*), cytochrome c oxidase subunit VIa (*COX6A*) and cytochrome c oxidase subunit VIIa (*COX7A*), and complex V such as *ATP5I* were found to be repressed by radiation. No DEGs related to ETC complex II and

III were identified. The suppressive action of ionizing radiation on ETC activity and associated ATP synthesis has been observed in a number of mammalian species (reviewed in Sweet et al., 2012), whereas knowledge on fish species is still limited. A recent study on rainbow trout reported the regulation of ETC complex II and III enzymes, and genes such as those encoding cytochrome c subunit Vb (*CCVb*), cytochrome c oxidase I (*COX1*), ATPase subunit 6 (*ATP6*) and NADH dehydrogenase subunit 1 (*ND1*) were affected by as low as 0.1 Gy gamma radiation (O'Dowd et al., 2009). The mechanisms underlying the down-regulation of ETC genes after High gamma exposure may be similar to that discussed earlier for Medium gamma treatment.

Immune functions may have also been affected by High gamma treatment in the present study, as 5 immune-related canonical pathways were found to be significantly enriched. Among these, the antigen presentation pathway may be an important one, as the antigen presentation is important for development of both innate and adaptive immunity (Watts, 2004). A few DEGs enriched in this pathway, including genes encoding major histocompatibility complex class I B (*HLA-B*), major histocompatibility complex class II DP beta 1 (*HLA-DPB1*), major histocompatibility complex class I-related (*MR1*) proteins were found to be down-regulated after the exposure. The repression of antigen presentation has been shown in Atlantic salmon under disease conditions (Fast et al., 2005; Young et al., 2008). In mammals, a study on nitric oxide radicals showed that the antigen presentation was inhibited similarly to that mediated by ROS in A20 mouse B lymphoma cells (Lemaire et al., 2009). Another study reported that ethanol-induced oxidative stress suppressed the generation of peptides for antigen presentation in human VL-17A cells (Osna et al., 2007). The repression of antigen presentation has also been considered to contribute to the immunodeficiency diseases in human (Hampton and Stanton, 2010).

The suppressive actions of gamma radiation on the immune functions have been observed in rainbow trout (Knowles, 1992) and zebrafish (Traver et al., 2004), however, the underlying mechanisms have not been fully understood yet. One possible mechanism may be that under radiation-induced oxidative stress, the immune activity was reduced by certain compensatory mechanisms, in order to control the production of excess ROS from the immune cells, such as the macrophages (Warnatsch et al., 2013). It has also been suggested that free radicals such as ROS and reactive nitrogen species (RNS) may regulate the immune response by acting as signal molecules (Pani et al., 2000; Al-Huseini et al., 2013; Gostner et al., 2013).

4.4 Potential toxicological mechanisms and future directions

Based on the hepatic transcriptional responses, the toxicological mechanisms of gamma radiation at sublethal doses may be deduced (Figure 8). Potential MoAs of gamma radiation may possibly include the induction of oxidative stress, DNA damage and uncoupling of oxidative phosphorylation. These MoAs may lead to diverse downstream responses such as macromolecular oxidation and degradation (e.g. protein ubiquitination pathway), immune responses, cell cycle regulation and DNA repair (e.g. p53 signaling pathway), reduction of ATP production and activation of apoptosis. Potential cancer signaling may also be activated as a result of DNA based on mammalian models, but whether this is also the case in fish still needs to be further investigated.

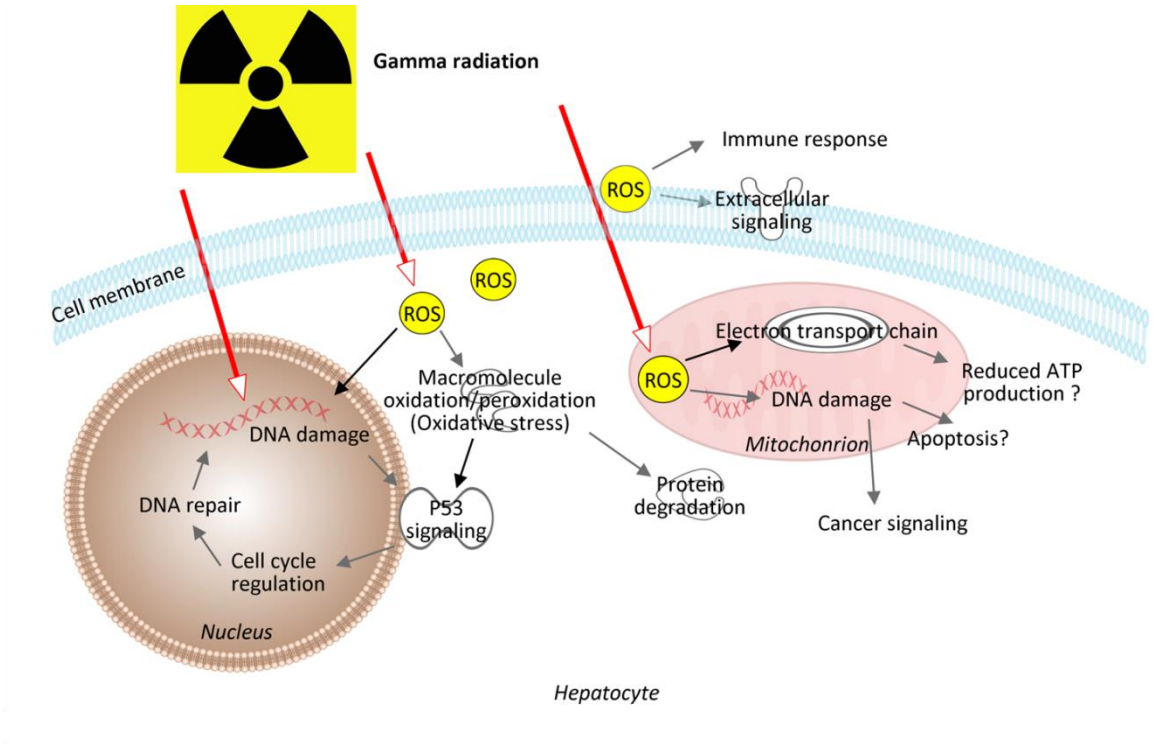

Figure 8. Putative toxicological mechanisms of low-dose gamma radiation in the liver of Atlantic salmon (*Salmo salar*) after short-term exposure.

5 Conclusions

To conclude, the present hepatic transcriptional study successfully characterized predicted key stress responses in Atlantic salmon at the molecular level after short-term exposure to sublethal doses of gamma radiation. Dose-dependent transcriptional changes were identified and their functions predicted. Potential modes of action of gamma radiation, such as induction of oxidative stress, DNA damage and uncoupling of oxidative phosphorylation were proposed and diverse downstream responses were linked to these MoAs. This study was not able to directly link the molecular responses to adversity at higher organismal levels due to the short exposure duration and low exposure levels. However, the current findings have substantially enriched the understanding of acute stress responses in fish exposed to environmentally relevant doses of gamma radiation and served as a preliminary approach for generating hypotheses. Further research will be focused on development of adverse outcome pathway (AOP) by linking the molecular initiating events caused by gamma radiation to adverse outcomes at the individual and population levels, Successful implementation of such conceptual framework is envisioned to facilitate the hazard and risk assessment for environmental radioactivity in the future.

Aknowledgement

The authors appreciate the support from Deborah Oughton, Ole-Christian Lind and Lindis Skipperud, Tove Loftaas, Marit Nandrup Pettersen and Merethe Kleiven (NMBU) for water sampling and fish dissection, Tore Høgåsen (NIVA) for the contribution of developing bioinformatics tools. The authors would also like to thank the funding from Norwegian University of Life Sciences (NMBU, PhD fellowship), the Research Council of Norway, RCN (Project No. 178621 MixTox), the RCN funded EU STAR Network of Excellence (NoE), NIVA institutional funding (Project No. 11033 SIS-MolPOP) and the Norwegian Research Council through its Centre of Excellence (CoE) funding scheme (Project No. 223268/F50).

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Appendices

Table A1. Differentially expressed genes (DEGs) that were regulated in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.

Table A2. Overrepresented Gene Ontology (GOs) functions that were regulated in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 280 mGy (total) gamma radiation.

Table A3. Mapped mammalian orthologs of differentially expressed genes (DEGs) that were regulated in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.

Table A4. Toxicity pathways that were significantly affected in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.

Table A5. Canonical pathways that were significantly affected in the liver of Atlantic salmon (*Salmo salar*) after 48 h exposure to 15, 70 and 280 mGy (total) gamma radiation.