Two-generational study of biological effects from gamma radiation exposure during sensitive life stages in the zebrafish (*Danio rerio*)

Philosophiae Doctor (PhD) Thesis

Selma Hurem

Department of Food Safety and Infection biology
Faculty of Veterinary medicine and Biosciences
Norwegian University of Life Sciences

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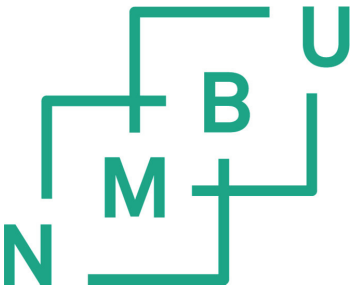

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Selma Hurem,
Oslo, September 2017.

Summary

There is growing concern about the harmful effects of ionizing radiation on animal populations, including potential effects on future generations. A number of life stages are particularly sensitive to radiation exposure including embryogenesis (embryonic development, earliest life stage) and gametogenesis (development of the germ cells). Furthermore, there is a possibility that adverse effects produced during these critical stages can be either inherited by progeny or carry consequences for the development and health later in life. These latent effects are often considered to be more important than acute toxic effects, since they can arise as a result of low dose chronic exposures, and thus are more relevant for the types of exposures seen in the environment.

The natural aquatic environment can be the recipient of radionuclides from waste discharges from nuclear power plants or other nuclear installations, as well as from nuclear accidents. Consequently, radiation exposure in this environment generally occurs in a chronic and continuous manner. Due to difficulties in mimicking environmental exposures under laboratory settings, data on chronic doses and dose rates of ionizing radiation, and their effects on sensitive life stages are still scarce. The present PhD project focused on external gamma radiation exposure related effects (macroscopic and subcellular) in zebrafish (*Danio rerio*) adults and embryos following exposure to a range of doses ranging from environmentally relevant low doses to high doses.

The overarching hypothesis was that observed effects would depend on the life stage at exposure, and that this would in turn influence the propagation of effects from irradiated parents to offspring. Developmental and reproductive effects, as well as oxidative stress, genotoxicity and gene expression were studied in controlled experiments where zebrafish were exposed during sensitive life stages: embryogenesis and the early larval stage, combined gametogenesis and embryogenesis in adults and their offspring, and gametogenesis alone. The common laboratory wild-type (AB) strain zebrafish was used for all studies.

First, the effects of low to moderate external dose rate (0.4 - 38 mGy/h) gamma radiation on the development and gene expression were studied. RNA-sequencing was performed to analyze early gene expression changes (2.5 to 5.5 hours post fertilization, “hpf”, corresponding to blastulation), while embryo development was observed until the early larval stage. The highest dose rate (38 mGy/h, total 3.6 Gy) caused increased

mortality, while exposure to the lower dose rate (0.4 mGy/h, total 19.2 mGy) resulted in the occurrence of deformities and differences in hatching. Gene expression analysis indicated that a 3-hour gamma radiation exposure to 0.54 mGy/h was able to cause gene expression changes, which were in accordance with the phenotype level adverse outcomes observed in 48 and 96 hpf stage larvae. Exposure to the lower dose resulted in the enrichment of genes and networks associated with antioxidant defense (including *RAR* activation, apoptosis and glutathione mediated detoxification), suggesting that low dose exposure induced reparatory mechanisms thereby preventing adverse effects at a later stage. In the higher radiation dose rates, an enrichment of genes and networks related to the cell cycle control (*tp53*), translation and cell survival (*eif2*, *mTOR*), and genes associated with disrupted development and cancer development (*myc*, *TGFb1*, *hnf4a*, *cebpa*) were found, which might have caused activation of pathways leading to observed adverse effects later in development.

One of the hypotheses of this study is that exposure of the parental gametes would result in adverse effects in offspring development and gene expression, and that these adverse effects could be connected to oxidative stress and genotoxicity. Therefore, the effects of gamma exposure was studied both in adult fish during gametogenesis, G (F0: 53 and 8.7 mGy/h for 27 days, total doses 31 and 5.2 Gy) and their offspring (first filial generation) during early embryonic development E (9.6 mGy/h for 65 hrs; total dose 0.62 Gy) consisting of G, GE, E and control F1 lines based on the stage of exposure. The results showed that irradiation of parents can result in adverse effects in their offspring as a consequence of damage caused to the parental germ cells (e.g. changes in hatching, deformity frequencies, and 100% embryo mortality at 8 hpf in the 53 mGy/h embryos). However, it was evident that in addition to the direct damage to the germline, latent and persistent inherited effects such as an increase in oxidative stress parameters (ROS formation and lipid peroxidation), increase in DNA damage and bystander effects are present up to one year after parental exposure in the F1 (GE and G) embryos.

Further, the study attempted to link the molecular background of the inherited effects with differences between gamma irradiation during embryogenesis and gametogenesis. Here, RNA sequencing was used to assess the short (one month after parental irradiation) and long-term effects (one year after parental irradiation) on the gene expression in the F1 G embryos. Networks and genes involved in cancer development, DNA damage responses and cell death, were similarly enriched at all time points and dose rates. Common modulated genes seen in all exposure groups are involved in mechanisms such as cell adhesion (*fn1a*), lipid peroxidation products metabolism (*aldh3a1*) and DNA

damage (*rrm2*), and ROS formation (*tfa*), which may explain the observed increase in embryo mortality and oxidative stress parameters. Parental gamma exposure also affected key upstream regulator genes (*tp53* and *hnf4a*) having a functional role in chronic inflammatory responses, which may be a mechanistic explanation for the persistently increased ROS formation observed in the offspring of exposed parents one month after the parental exposure. Gene expression analysis also revealed a differential expression of genes known to regulate the reproductive hormones, supporting the hypothesis that radiation exposure induced effects stem from the parental germ line.

In the adult zebrafish exposed continuously to gamma irradiation during gametogenesis, we assessed reproductive capacity, gonad histology and genotoxicity in erythrocytes. Increased DNA damage and micronuclei (MN) number were found in all the adults. Reproductive capacity was seriously impaired in fish exposed to 53 mGy/h dose rate, as shown by the lower rates of embryo production and the occurrence of sterility in almost all adult fish one year after exposure. Further, histopathological evaluation of the gonads in adults in this exposure group showed a regression of the reproductive organs, while in ovaries of zebrafish exposed to 8.7 mGy/h, an increase in pre-vitellogenic stage (non-mature) follicles was found. Previously, genes, which regulate reproductive hormones, were found to be dysregulated in offspring of these fish and these findings point to a parental exposure related effect. Together, these results suggest that chronic exposure of fish during early developmental stages can cause severe defects to the reproductive organs, resulting in impaired reproductive function, which potentially could have a deleterious effect at the population level. Although the dose rates used for adults in this study exceeded typical environmentally relevant dose rates, the total doses are relevant for short-term, accidental exposures to ionizing radiation and provide important insights into heritable effects. One of the main conclusions of this thesis is that any adverse effect as a consequence of radiation exposure to adults, particularly during gametogenesis and reproduction, could be inherited by their progeny.

This thesis work has increased our understanding of the mechanistic background of multigenerational inheritance of ionizing radiation effects from exposure during sensitive life stages. This new knowledge serves not only as an important indicator of possible outcomes relevant for radiation risk assessment from different life stage and chronic irradiation, but also as a foundation to further study mechanisms of inheritance, such as the transgenerational inheritance.

Sammendrag

Skadelige helseeffekter av ioniserende stråling, inkludert effekter i kommende generasjoner, er av økende interesse. En rekke livsstadier er spesielt følsomme for strålingseksponering, særlig embryogenesen (embryonisk utvikling som tidligste livsstil) og gametogenesen (utvikling av kimceller), og en negativ effekt som oppstår i disse kritiske stadiene kan henholdsvis bli nedarvet i avkom eller forårsake negative konsekvenser for utvikling og helse senere i livet. Slike latente effekter anses å være viktigere enn akutte toksiske effekter, da de kan oppstå fra lavere dose og kronisk eksponering som er miljømessig mer relevant.

Det akvatiske miljøet kan være mottaker av radionuklider fra atomulykker og utslipp av kjernefysiske anleggsavfall. Følgelig forekommer strålingseksponering i dette miljøet generelt på en kronisk og kontinuerlig måte. På grunn av vanskeligheter med å etterligne miljørelevante eksponeringer i eksperimenter, er data på kroniske doser av ioniserende stråling i sensitive livsstadier og effekter fortsatt begrenset. Dette doktorgradsprosjektet fokuserte på effekter (makroskopiske og subcellulære) relaterte til lave -og miljørelevante til høye doser med bruk av sebrafisk (*Danio rerio*) som modelldyr.

Den overordnede hypotesen var at effektene ville avhenge av livsstadie ved eksponering, og at dette i sin tur ville påvirke utbredelsen av effekter fra bestrålede foreldre til avkom. Utviklings -og reproduksjonseffekter, i tillegg til oksidativt stress, genotoksisitet og genekspresjon ble studert i ulike sensitive livsstadier: embryogenesen og tidlig larvestadium, kombinert gametogenese og embryogenese eksponering hos voksne og deres avkom, og gametogenese alene inkludert ikke-eksponerte kontroller i hvert eksperiment.

Initialt ble effekten av lavt til moderat ekstern doseringsgrad gammastråling på utvikling og genekspresjon studert. Den kjente sebrafiskmodellen wild-type (AB) ble anvendt. RNA-sekvensering ble utført for å analysere tidlige genekspresjonsendringer (2,5 til 5,5 timer etter befruktning, "teb", tilsvarende blastuleringen) og utvikling i embryoer frem til tidlig larvestadium. Den høyeste doseringshastigheten som ble brukt (38 mGy/h, totalt 3.6 Gy) forårsaket økt dødelighet, mens deformiteter og forskjeller i klekking ble funnet i grupper utsatt for lav dose (0.4 mGy/h, totalt 19.2 mGy) ved 48 teb. Genekspresjonsanalysen viste at en 3 timers gammastrålingseksponering kan forårsake genekspresjonsendringer. Dette er i samsvar med de uønskede fenotype effektene som ble observert på larvestadium 48 og 96 teb. Eksponering for lavere dose resulterte i anrikning av gener og nettverk assosiert med antioksidant forsvar (inkludert *RAR*-aktivering, apoptose

og glutation-mediert avgiftning). Dette kan være reparasjonsmekanismer som forårsaker manglende effekter på senere livsstadier. I de høyere stråledosene økte ekspresjonen av gener og nettverk relatert til kontroll av cellesyklus (*tp53*), translasjon og celleoverlevelse (*eif2*, *mTOR*). Forstyrret utvikling og kreft (*myc*, *TGFb1*, *hnf4a*, *cebpa*) ble også funnet, noe som kan ha forårsaket aktivering av baner som forklarer de observerte effektene på senere livsstadier.

Et av målene med denne oppgaven var å finne ut om eksponering av foreldregametene ville forårsake en lignende effekt på utvikling og genekspresjon i avkommene, og om de nevnte effektene kunne være forbundet med oksidativt stress og genotoksisitet. Effektene av gammaeksponering på voksen fisk under gametogenese, G, (F0: 53 og 8.7 mGy/h i 27 dager, totale doser 31 og 5.2 Gy) og avkom embryogenese, E, (9.6 mGy/h, total dose 0.62 Gy) ble studert i det første generasjon som danner G, GE, E og kontroll F1 linjer. Våre resultater viste at bestråling av foreldre kan gi utviklingseffekter fra direkte skade på foreldrekimceller (som endringer i klekking, deformitetsfrekvens og 100% embryo-dødelighet ved 8 teb i 53 mGy/h embryoene). I tillegg til den direkte skaden på kimlinjen, økte latente og vedvarende arvelige effekter som oksidative stressparametere (ROS-dannelse og lipidperoksidering), forsinket genomisk ustabilitet (økt DNA-skade) og bystander-effekter i F1 (GE og G) embryoer ett år etter foreldrenes eksponering.

Videre i denne oppgaven ble det også forsøkt å knytte den molekylære bakgrunnen for arvelige effekter sammen og å sammenligne forskjellene mellom gammastråling under embryogenese og gametogenese. Her ble RNA-sekvensering brukt til å vurdere kortsiktige (en måned etter foreldrenes bestråling) og langsiktige effekter (ett år etter foreldrenes bestråling) på genekspresjon i F1 G-embryoene. Nettverk og gener for molekylære mekanismer relatert til kreft, DNA-skaderespons og celledød ble oppregulert på alle tidspunkter og doser. Felles gener i alle eksponeringsgrupper var relaterte til celleadhesjon (*fn1a*), metabolisme av lipidperoksidasjonsprodukter (*aldh3a1*) og DNA-skade (*rrm2*), jernmetabolisme og sensibiliserende respons på stråling ved å øke ROS (*tfa*). Genekspresjonen var sammenlignbar med de tidligere observerte effektene i embryo-dødelighet og økning av oksidativt stressparametere. For å forklare den vedvarende økte ROS-dannelsen i embryoer en måned etter foreldrenes eksponering, har nettverkene av gener knyttet til kronisk inflammatorisk respons blitt foreslått. Tidligere har responsive oppstrømsregulatorer etter embryoeksponeringer, *tp53* og *hnf4a*, også blitt beriket som følge av foreldrenes eksponering. Genekspresjonsanalysen viste også en differensiell

ekspresjon av gener kjent for å regulere reproduktive hormoner, hvilket indikerer at de induserte strålingseffektene stammer fra foreldrenes kimlinje.

I arbeidet med å beskrive eksponeringsrelaterte effekter hos voksen sebrafisk etter kontinuerlig gammabestråling under gametogenesen, ble reproduksjon, gonade histologi og genotoksisitet i erytrocytter vurdert. Økt DNA-skade og mikronuklei ble funnet hos alle eksponerte voksne. Reproduksjonskapasiteten var alvorlig svekket i fisk utsatt for 53 mGy/h, som vist ved forekomsten av sterilitet i nesten alle voksne fisk ett år etter eksponering. Videre viste histopatologisk evaluering av gonadene hos voksne i denne eksponeringsgruppen en regresjon av reproduktive organer, mens i eggstokkene i sebrafisk eksponert for 8.7 mGy/h ble det funnet en økning i umodne follikler. Tidligere er det vist at gener for reproduktive hormoner dysreguleres i avkom fra denne fisken, og disse funnene viser en mulig effekt som er forårsaket av eksponering av foreldregenerasjonen. Tilsammen tyder disse resultatene på at kronisk eksponering av fisk under gametogenese kan forårsake alvorlige mangler i reproduktive organer, forringe overlevelsesevne og følgelig påvirke populasjonsbærekraftighet. Selv om doseringshastighetene som brukes for voksne i denne studien noen ganger overskred miljørelevante doser, de totale dosene er relevante for kortsiktige, utilsiktede eksponeringer for ioniserende stråling og gir viktig innsikt i arvelige effekter. En av hovedkonklusjonene i dette studiet er at eventuelle negative effekter av strålingseksponering hos voksne, spesielt i kimceller, kan arves av deres avkom.

Denne oppgaven har økt vår forståelse av den mekanistiske bakgrunnen for arvbarhet av ioniserende strålingseffekter over generasjoner fra eksponering under sensitive livsstadier. Denne nye kunnskapen er ikke bare en viktig indikator på mulige utfall som er relevante for risikovurderingen av stråling fra forskjellige livsstadier og kronisk bestråling, men er også et grunnlag for videre studier på mekanismer som nedarves over generasjoner.

Sažetak

Postoji sve veća zabrinutost zbog štetnog djelovanja jonizirajućeg zračenja na životinjsku populaciju, uključujući i potencijalne efekte na buduće generacije. Brojni životni stadiji posebno su osjetljivi na izlaganje zračenju, uključujući gametogenezu (razvoj zametnih ćelija) i embriogenezu (embrionalni razvoj, najraniji životni stadij). Štetni efekti nastali tokom ovih stadija mogu biti naslijeđeni od strane potomstva ili mogu imati negativne posljedice po razvoj i zdravlje kasnije u životu. Takvi latentni efekti često se smatraju važnijim od akutnih toksičnih efekata, budući da se mogu pojaviti kao posljedica niske doze, hronične izloženosti, a time su i relevantniji za eksponiranje zračenju s kojim se moguće susresti u okolini.

Vodeno okruženje može biti primatelj radionuklida uslijed ispuštanja otpada iz nuklearnih elektrana ili drugih nuklearnih postrojenja, kao i od nuklearnih incidenata. Posljedično tome, izloženost zračenju u ovom okruženju općenito se događa na hroničan i kontinuiran način. Zbog poteškoća u imitiranju okolinškog eksponiranja zračenju u laboratorijskim uslovima, podaci o hroničnim dozama i brzinama jonizirajućeg zračenja te njihovi učinci na osjetljive životne stadije još uvijek su nedostatni. Ovaj doktorski studij bio je usmjeren je na efekte (makroskopske i subcelularne) povezane s eksternim izlaganjem gama zračenju u rasponu od niskih i ekološki značajnih do visokih doza kod odraslih i embriona zebrice (*Danio rerio*).

Sveobuhvatna hipoteza bila je da štetni efekti ovise o životnom stadiju u toku kojeg je ozračivanje trajalo i da to zauzvrat utječe na prenošenje efekata od ozračenih roditelja na potomstvo. Razvojni i reproduktivni efekti, kao i oksidativni stres, genotoksičnost i ekspresija gena proučavani su u kontroliranim eksperimentima gdje su ribe bile izložene tokom osjetljivih razvojnih perioda: embriogeneze i ranog larvalnog stadija, kombinirano gametogeneze i embriogeneze odraslih i njihovih potomaka, i same gametogeneze. U svim ispitivanjima korištena je zebrica divljeg tipa (AB).

Najprije su proučavani efekti niskih do umjerenih brzina gama zračenja (0.4 - 38 mGy/h) na razvoj i gensku ekspresiju. Provedeno je RNA sekvenciranje kako bi se analizirale početne promjene gena (od 2,5 do 5,5 sati nakon oplodnje, "sno", što odgovara blastulaciji) i razvoj embriona do ranog larvalnog stadija. Najveća primijenjena doza (38 mGy/h, ukupno 3.6 Gy) uzrokovala je povećanu smrtnost, dok su deformacije i razlike u izlijeganju zabilježene u skupinama izloženim niskoj dozi (0.4 mGy/h, ukupno 19.2 mGy) u 48 sno. Analiza ekspresije gena pokazala je da je izlaganje gama zračenju od 0,54 mGy/h u

trajanju od tri sata uzrokovalo promjene u ekspresiji gena, koje su bile u skladu s ustanovljenim fenotipskim promjenama kod embriona i larvi u 48 i 96 sno. Izlaganje nižoj dozi rezultiralo je većom ekspresijom gena i mreža povezanih s antioksidativnom zaštitom, uključujući *RAR* aktivaciju, apoptozu i detoksikaciju posredovanu glutationom), što ukazuje na to da niske doze zračenja induciraju reparatorne mehanizme koji sprečavaju pojavu štetnih efekata u kasnijem razvoju. Pri višim dozama zračenja, pronađena je veća ekspresija gena i mreža povezanih sa kontrolom ćelijskog ciklusa (*tp53*), translacijom i preživljavanjem ćelija (*eif2*, *mTOR*) i gena povezanih s razvojnim poremećajima i nastankom raka (*myc*, *TGFb1*, *hnf4a*, *cebpa*), što je moglo uzrokovati aktivaciju genskih mreža koje su dovele do uočenih štetnih efekata kasnije u razvoju. Jedna od hipoteza ove studije bila je da će izlaganje roditeljskih gameta rezultirati štetnim djelovanjem na razvoj potomaka i gensku ekspresiju, te da bi štetni učinci mogli biti povezani s oksidativnim stresom i genotoksičnošću. Stoga su efekti izlaganja odraslih tokom gametogeneze, G (F0: 53 i 8.7 mGy/h za 27 dana, ukupne doze 31 i 5.2 Gy) i potomstva tokom embriogeneze, E (9.6 mGy/h za 65 sati, ukupna doza 0.62 Gy), proučavani u prvoj filijalnoj generaciji, koja se sastojala od G, GE, E i kontrolne F1 linije na temelju stadija izlaganja. Rezultati su pokazali da ozračivanje roditelja može prouzrokovati štetne efekte kod potomstva od neposrednih oštećenja uzrokovanih roditeljskim zametnim ćelijama (npr. promjene u izlijeganju, frekvenciji deformacija i 100%-tni mortalitet 8 sno kod embriona ozračenih s 53 mGy/h). Međutim, očigledno je da osim neposrednog oštećenja zametnih ćelija, latentni i perzistentni nasljedni efekti kao što su povećanje parametara oksidativnog stresa (formacije reaktivnih vrsta kisika - ROS, i lipidne peroksidacije), oštećenja DNA i "bystander" efekti prisutni su u F1 embrionima (GE i G) do jedne godine nakon ozračivanja roditelja.

Nadalje, ova studija pokušala je povezati molekularnu pozadinu nasljednih efekata s razlikama između gama zračenja tokom embriogeneze i gametogeneze. RNA sekvenciranje korišteno je za procjenu kratkoročnih (mjesec dana nakon zračenja roditelja) i dugoročnih efekata (godina nakon zračenja roditelja) na ekspresiju gena u F1 G embrionima. Mreže i geni uključeni u razvoj raka, oštećenja DNA i ćelijska smrt bili su na sličan način ekspresirani u svim vremenskim periodima i dozama. Zajednički modulirani geni koji se vide u svim grupama izloženim zračenju uključeni su u mehanizme kao što su ćelijska adhezija (*fn1a*), metabolizam lipidnih peroksidacijskih produkata (*aldh3a1*) i oštećenje DNA (*rrm2*), te ROS formacija (*tfa*), što može objasniti opaženo povećanje embrionalne smrtnosti i parametara oksidativnog stresa. Izlaganje roditelja gamma zračenju također je

djelovalo i na ključne regulatore (*tp53* i *hnf4a*) koji imaju funkcionalnu ulogu u hroničnim upalnim odgovorima, što može biti mehanističko objašnjenje za trajno povećanje stvaranja ROS-a, uočeno kod potomstva ozračenih roditelja mjesec dana nakon zračenja roditelja. Analiza genske ekspresije također je otkrila diferencijalnu ekspresiju gena koji reguliraju reproduktivne hormone, potkrepljujući hipotezu da efekti inducirani izlaganjem zračenju proizlaze iz roditeljskih zametnih ćelija.

Kod odraslih zebrica koje su kontinuirano izlagane gama zračenju tokom gametogeneze, također smo procijenili i reproduktivnu sposobnost, gonadnu histologiju i genotoksičnost u eritrocitima. Povećanje oštećenja DNA i mikronukleusi (MN) pronađeni su kod svih odraslih riba. Reproduktivna sposobnost bila je ozbiljno narušena kod riba izloženih brzini zračenja od 53 mGy/h, što je pokazalo postojanu nižu proizvodnju embriona i pojavu sterilnosti kod gotovo svih odraslih godinu dana nakon zračenja. Nadalje, histopatološka procjena u ovoj grupi pokazala je regresiju reproduktivnih organa, dok je u jajnicima zebrica ozračenih sa 8.7 mGy/h pronađeno povećanje folikula u predvitelogeničnim stadijima (nezreli folikuli). Prethodno je utvrđeno da su geni koji reguliraju reproduktivne hormone disregulirani kod potomaka ovih riba, a ovi nalazi zajedno upućuju na efekte uslijed ozračivanja roditelja. Ovi rezultati sugeriraju da hronično izlaganje riba tokom ranih razvojnih stadija može uzrokovati ozbiljne nedostatke u reproduktivnim organima, što može rezultirati oštećenjem reproduktivne funkcije, koja potencijalno može imati negativan efekat na nivou populacije. Iako su korištene brzine zračenja za odrasle ribe u ovoj studiji premašile tipične ekološki značajne brzine zračenja, ukupne doze su relevantne za kratkotrajna, slučajna izlaganja jonizirajućem zračenju i pružaju važne uvide u nasljedne efekte. Jedan od glavnih zaključaka ove teze je da bilo kakvi štetni efekti uslijed zračenja odraslih, naročito u zametnim ćelijama, mogu biti nasljedno preneseni na njihove potomke.

Ovaj rad poboljšava razumijevanje mehanističke pozadine multigeneracijskog nasljeđivanja efekata jonizirajućeg zračenja uslijed izlaganja zračenju tokom osjetljivih životnih stadija. Data nova saznanja služe ne samo kao važan pokazatelj mogućih ishoda relevantnih za procjenu rizika od zračenja u toku različitih životnih razdoblja i hroničnog ozračenja, već i kao temelj za dalje proučavanje mehanizama nasljeđivanja, kao što je transgeneracijsko nasljeđe.

Abbreviations and acronyms

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
Co	Cobalt (chemical element)
DCF	2',7'-dichlorofluorescein
DCRL	derived consideration reference levels
DEG	differentially expressed gene
DSB	double strand breaks
dpf	days post fertilization
FC	fold change
FDR	false discovery rate
FELASA	Federation of European Laboratory Animal Science Associations
FOTS	"Laboratory animal oversight and application system"
Gy	Gray (SI unit)
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HT_{50}	median hatching time; time when 50% of the population is hatched
hpf	hours post fertilization
IACUC	Institutional Animal Care and Use Committee
IPA	Ingenuity Pathway Analysis
ICRP	International Commission on Radiological Protection
LD_{50}	median lethal dose; radiation dose that is lethal for 50% of the population
$LD_{50/50}$	radiation dose that is lethal for 50% of the population within 50 days
LET	linear energy transfer
LNT	linear no threshold
LPO	lipid peroxidation
mpf	months post fertilization
MBT	Mid blastula transition
MN	Micronuclei
PNED(R)	predicted no observed effect dose (rate)
qPCR	quantitative real-time reverse transcription polymerase chain reaction
RAP	reference animals and plants
RNA seq	ribonucleic acid sequencing
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOP	standard operating procedures
SSB	single strand breaks
SW	system water
UNSCEAR	United Nations Scientific Committee on Effects of Atomic Radiation
wpf	weeks post fertilization
ypf	years post fertilization

ZF	Zebrafish
ZIRC	Zebrafish International Resource Center

List of genes and molecular pathways

aldh3a1	aldehyde dehydrogenase 3 family, member A1
apoA-IV	apolipoprotein A – IV
apoA1a	apolipoprotein A 1a
apoA1b	apolipoprotein A 1b
apoBb	apolipoprotein B b
cebpa	CCAAT enhancer binding protein C/EBP alpha
crabp2b	cellular retinoic acid binding protein 2 b
cyp2x6	cytochrome p450 2x6
eef1a	eukaryotic translation elongation factor 1a
eef2b	eukaryotic translation elongation factor 2b
eif2	eucariotic initiation factor 2
ESR1	estrogen receptor 1 signaling
fn1a	fibronectin 1a
GnRH	gonadotropin-releasing hormone signaling
FSH	follicle-stimulating hormone signaling
hmbs	hydroxymethylbilane synthase
hnf4a	hepatic nuclear factor 4 alpha
hprt1	hypoxanthine phosphoribosyltransferase 1
IGF2	Insulin-like growth factor
mTOR	mammalian target of rapamycin signaling
myc	avian myelocytomatosis viral oncogene homolog
pfkfb3	6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3
RARa	retinoic acid receptor alpha signaling
rps18	ribosomal protein S18
RRM2	ribonucleotide reductase subunit M2
shisa2	shisa family member 2
sox2	sex determining region Y-box 2
tfa	transferrin a
TGFb 1	transforming growth factor beta 1
TNFα	tumor necrosis factor alpha
tp53	tumor protein 53 signaling
usf1l	upstream transcription factor 1 like
vegfab	vascular endothelial growth factor alpha, b

List of papers

This thesis is based on the papers listed below, to which is referred to in the text by their Roman numerals.

I

Hurem, S., Martin, L.M., Brede, D.A., Skjerve, E., Nourizadeh-Lillabadi, R., Lind, O.C., Christensen, T., Berg, V., Teien, H.C., Salbu, B., Oughton, D.H., Aleström, P., Lyche, J.L. 2017.

Dose dependent effects of gamma-radiation on differentially expressed genes of early zebrafish embryo transcriptome. *PLoS ONE*, **12(6)**: e0179259.

II

Hurem, S., Gomes, T., Brede D.A., Lindbo-Hansen, E., Mutoloki, S., Fernandez, C., Mothersill, C., Salbu, B., Kassaye Y.A., Olsen A.K., Oughton D., Peter Aleström, P., Lyche J.L., 2017.

Parental gamma irradiation induces reprotoxic effects accompanied by genomic instability in zebrafish (*Danio rerio*) embryos. *Environmental Research*, **159:** 564-578

III

Hurem, S., Martin, L.M., Lindeman, L., Brede, D.A., Salbu, B., Lyche, J.L., Aleström, P., Kamstra, J. **Parental exposure to gamma radiation causes progressively altered transcriptomes linked to adverse effects in zebrafish offspring.** *Submitted manuscript* (August 2017)

IV

Hurem, S., Gomes, T., Brede D.A., Mayer, I., Lobert, V., Mutoloki, S., Gutzkow, K., Teien, H.C., Oughton, D., Aleström, P., Lyche J.L.

Gamma irradiation during gametogenesis in young adult zebrafish causes persistent genotoxicity and adverse reproductive effects. *Submitted manuscript* (September 2017)

1. Introduction – literature review

1.1 Background of the study

Living organisms are constantly exposed to low levels of radiation. The sources of ionizing radiation in the environment can be of natural origin (i.e. terrestrial and cosmic radiation) or the result of anthropogenic activities (e.g. nuclear waste disposal, weapon testing and atomic bombings). There is a clear interest in understanding the health effects of ionzing radiation, due to it being used for industrial purposes and medical interventions. Major nuclear incidents such as Chernobyl in 1986 and Fukushima Daiichi in 2011 raised concerns, not only on the harmful effects of direct exposure to both humans and wildlife, but also, possibly more importantly, the occurrence of adverse health effects in subsequent generations.

To date, information derived from animal and human studies states that environmental exposures to stressors such as ionizing radiation during sensitive life stages increase the possibility for developmental and reproductive abnormalities, the occurrence of adverse effects later in life (e.g. mutations and cancers) (UNSCEAR, 2006a), or the risk of a disease in the following generation (UNSCEAR, 2001). The harmful effects of radiation exposure are clearly related to the level of exposure, with high doses of radiation capable of causing direct damage to cellular components or to the DNA. The consequences of low dose exposure are less clear, although evidence now indicates long-term (i.e. chronic) exposure to ionizing radiation is able to induce adverse effects which can be transmitted over generations due to damaged germ cells (Slovinska et al., 2004; Barber et al., 2006).

Although there are increasing concerns over the harmful effects of radiation exposure, major knowledge gaps still remain in our understanding of the full consequences of radiation exposure, especially low dose and generation effects. In particular, experimental assessments of heritable biological effects of ionizing radiation are still scarce. UNSCEAR reports note that the sensitivity of an organism depends on the life stage at the time of radiation exposure, with earlier life stages generally being more sensitive than adults. However, exposure of adults during gametogenesis is also of particular concern as gamete quality (eggs and sperm) is essential for reproductive success. As such, a better understanding of the consequences of radiation exposure during gametogenesis is needed to determine possible future generation effects.

Given the growing concerns of the harmful effects of ionizing radiation, to both wildlife and humans, more information is needed to fully understand both the direct effects and the potential of long-term generation effects. This research will entail further studies, both involving high dose exposure and more importantly chronic low dose exposures, which are of greater environmental relevance. Only by improving our knowledge base on these exposure effects will we be able to formulate more accurate risk assessment paradigms, both to the environment and its biota.

1.2 Aim of the study

The aim of this study was to test the hypothesis that heritable effects, which can affect the development, oxidative stress response, DNA damage response or the gene expression are present in the first generation progeny (F1) from parents (F0) subjected to increasing external gamma radiation doses during gametogenesis. To do this, we compared the effects of gamma irradiation (^{60}Co source) in offspring exposed during embryogenesis with effects seen in the progeny of parents exposed during gametogenesis, whereby radiation induced effects were also determined in the parents. Accordingly, the following objectives were set:

I. To establish dose-response relationships, effects of gamma radiation on the development of embryos and larva including 5.5 hpf stage gene expression after exposure to increasing dose rates of gamma radiation (from low to high) starting from 2.5 hpf (Paper I).

II. To detect the potential transmissible effects of ionizing radiation on the germ cells; oxidative stress, genotoxicity and bystander effects were studied in directly exposed embryos, embryos of exposed parents during gametogenesis and directly exposed offspring of irradiated parents starting from 2.5 hpf (Paper II).

III. To compare differences between parental (indirect exposure) and offspring (direct exposure) effects, 5.5 hpf stage gene expression was tested in offspring of parents irradiated during gametogenesis (Paper III).

IV. To determine the potential adverse effects on the parental generation and their reproductive capacity, the reproduction and genotoxicity were tested in exposed adult fish (Paper IV).

1.3 Ionizing radiation

Ionizing radiation occurs as electromagnetic waves or atomic particles, which are potent enough to remove an electron from an atom with sufficient force, thereby creating an ion (unlike non-ionizing radiation). Gamma radiation originates from the decay of an atomic nucleus from a high-energy state to a lower energy state (gamma decay) (Harley et al. 2010). The radioactive decay of an unstable (radioactive) atom is expressed by the number of disintegrations per second as 1 Becquerel (1s-1, 1 Bq). Ionzing radiation is biologically hazardous, due to its interaction with molecules (excitation and ionization) and free radical generation, which can cause macromolecular damage.

The harmful effects of ionizing radiation on tissues depend on the energy transferred (i.e. absorbed dose). The rate of this energy transfer per unit distance is called "linear energy transfer" or LET, quantified as keV/mm. Alpha particles and neutrons are high-LET types of ionizing radiation. This means that they deposit greater amounts of energy in an absorbing medium, causing a relatively high proportion of DNA double strand breaks (DSBs) and misrepairs of DSBs can in turn cause mutagenicity (Rothkamm and Löbrich, 2002; Shibata, 2017).

Gamma radiation, along with X-rays and electrons (beta particles), is a low-LET ionizing radiation type, meaning that it produces ionizations diffusely and that they are homogeneously present in the entire cell. Low-LET gamma radiation at sufficiently high doses can cause DSBs, however, low doses tend to produce small amounts of ionizations in the DNA region, usually inducing single strand breaks (SSBs) (Harley, 2010), which are repaired quickly.

The radiation dose to tissue is expressed in Gray (Gy), quantified as absorbed energy per unit tissue mass (1 Joule per kg, J/kg = 100 rad). An absorbed dose of 1 Gy can generate about 2×10^3 ionizations in the DNA (Adams and Cox, 1997). As a measure of the health effect of low levels of ionizing radiation on the human body the weighted quantity (Sv) is used. Here, the radiation type and energy are taken in account by using the ratio of biological relative effectiveness (RBE) of one type of ionizing radiation to another, for the same amount of absorbed energy. The RBE is represented in regulations by the radiation weighting factor, (wR), which adjusts the dose average for the type of radiation and energy of radiation incident on the body.

For the purpose of gamma radiation exposures described in this PhD thesis, a ^{60}Co gamma radiation source was used. Different gamma radiation doses and dose rates were employed, ranging from low to high dose rates (0.4 - 53 mGy/h) and total doses from 1.6 mGy to 31 Gy. The range included doses known to cause DNA damage in order to test the effects on embryonic development and the health status of adult fish, as well as to assess transmission of potential adverse effects from parents to offspring.

1.4 Effects of ionizing radiation on biota

Generally, the adverse health effects of radiation are grouped into two categories: deterministic effects (tissue reactions), which are based on cell death and have a threshold dose, and stochastic effects (cancer and hereditary effects), where the probability of effect, not the severity increases with dose. Stochastic effects are most important for low level (mGy) and long-term (chronic) exposures, while deterministic effects dominate at high doses (in the order of Gy). The data on deterministic effects of radiation in humans is obtained from studies of radiotherapy patients, persons exposed in nuclear accidents, or atomic bomb survivors. They are characterized by a threshold dose that must be exceeded before the health effect occurs and are caused by extensive cell death or malfunction and examples are acute radiation syndrome, skin burns and sterility.

From experimental studies in the 1930s, H.J.Muller et al. and N.V. Timofeeff-Ressovsky et al. concluded that radiation induced mutation rates are directly proportional to the dose, which defines the linear no-threshold (LNT) model. In the LNT model, any dose of radiation can dose-proportionally cause detrimental effects such as cancer and heritable genetic mutations without a threshold dose. The LNT model does not distinguish between a total dose given in an acutely or chronically. This model is accepted to adopt policy for radiation protection in the world and recommendations for radiation protection are based on the theory. However, the LNT model for cancer occurrence is a matter of debate among scientists, particularly at low doses (e.g. below 0.1 Gy) (Zyuzikov et al. 2011; Selzer and Hebar 2012), as the direct relationship between DNA DSBs and radiation dose has been shown not to hold for low doses (Sorokina et al. 2011; Mothersill and Seymour 2012a). In addition, the threshold model states that no significant biological response is observed until exposure level reaches a threshold dose. Another possible dose-response, in the low dose area, represents the concept of radiation hormesis. This concept proposes that biological

systems can respond in a positive way, or be stimulated by exposure to low doses of radiation that would be detrimental at higher doses (Baldwin and Grantham, 2015).

Ionizing radiation damage to the cell can be caused both directly and indirectly. The indirect effects of exposure to ionizing radiation occur through the reaction with water, a process called radiolysis that results in the generation of free radicals, which induce reactive oxygen species (ROS) and reactive nitrogen species (RNS) that can attack cellular components (Fig 1) thereby causing oxidative stress in the cell/ tissue (Azzam et al., 2014) and in turn damaging the DNA.

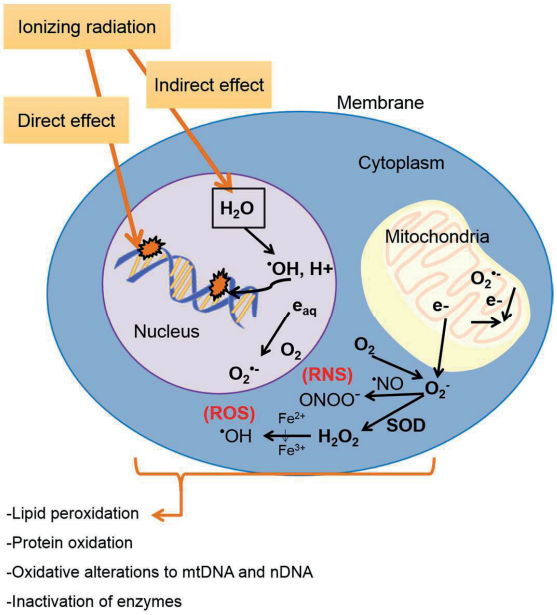

Fig 1. Direct and indirect cellular effects of ionizing radiation on macromolecules. Ionizing radiation can directly disrupt atomic structures, producing chemical and biological changes and indirectly, through radiolysis of cellular water and generation of ROS/RNS by stimulation of oxidases and nitric oxide synthases. Ionizing radiation may also disrupt mitochondrial functions, contributing to alterations in lipids, proteins, nuclear DNA and mitochondrial DNA.

The concentration of ROS is normally kept at a physiologically tolerable level by several interconnected enzymes: superoxide dismutase, catalase, glutathione peroxidase and peroxyredoxin (Reisz et al., 2014). Alternatively, direct damage from ionizing radiation

occurs following damage to the DNA molecule, resulting in disruption of the molecular structure and resulting in single strand breaks (SSB), double strand breaks (DSB) and DNA clusters (Desouky et al., 2015). Ionizing radiation induced DNA damage activates a number of response signaling cascades that control cell cycle arrest, DNA repair, and the cell's fate. At the cytological level, an extension of radiation induced DNA damage are chromosome breakages. If unrepaired, DNA damage has the potential to generate mutations in somatic or germline cells, which can alter phenotype and cause diseases.

As previously mentioned, the range of exposure in this study included doses that were able to cause genotoxicity in zebrafish. A multitude of assays exist for the testing of genotoxicity, such as the alkaline Comet assay (DNA SSBs, oxidized DNA as well as DNA-protein cross-links) used here; the gamma-H2AX assay (DNA DSBs; from nuclear foci formed by phosphorylation of the variant histone H2AX); and the micronucleus (MN) assay (chromosomal damage). It is essential to note that DNA damage in the low dose range can be quickly repaired (often before it can be detected), and different techniques can be used to detect the changes caused by such exposures. The major DNA repair mechanisms are Base excision repair (BER), which removes small, non-DNA-double helix-distorting base lesions from the genome; recombinational DNA repair (for repair of DNA DSBs) including homologous recombination (HR) and non-homologous end joining (NHEJ) and mismatch repair (MMR), which repairs replication errors during DNA replication and recombination (Alberts et al., 2002).

The radiation effects have been explained using target theory, according to which deleterious effects of ionizing radiation, such as mutation and carcinogenesis, are attributed to damage to a cellular target. This theory was challenged, and it is suggested that ionizing radiation can induce mutational events not only within targeted cells or nuclei but also within non-targeted intact nuclei and these responses at the cellular level are called non-targeted effects. They can result in the same type of damage as the targeted effects, but arise from different mechanisms of action and are divided in mainly two types. The first are radiation-induced bystander effects (RIBE), which arise in cells that receive no radiation exposure as a consequence of damage signals transmitted from neighboring irradiated cells or after they were immersed in the medium that was previously conditioned by the irradiated cells or organisms (Waldren, 2004; Bowler et al., 2006; Pereira et al., 2014). The second type of non-targeted effect is the radiation induced genomic instability (RIGI), when effects occur in the progeny of irradiated cells after many cell divisions (Ilnytskyy and Kovalchuk, 2011). Genomic instability can manifest as a persistently elevated frequency of

spontaneous mutations (Chang and Little, 1992) and was linked to many other mechanisms, including chromosomal aberrations (Plamadeala et al., 2015), micronuclei (Qian et al., 2016), gene expression changes (Snyder and Morgan, 2003), and epigenetic mechanisms (Aypar et al., 2011; Mothersill and Seymour, 2012b). Furthermore, genomic instability was also linked to differences in apoptosis, thereby allowing divisions of cells with unrepaired DNA DSBs (Lyng et al., 1996; Zhivotovsky and Kroemer, 2004).

Thus, depending on the energy and the cell type, radiation exposure can cause increased genomic instability and severe damage to tissues, whereby cells with a high proliferation and growth rate are considered more radiosensitive than others. For example, in the case of damage to embryonic cells, the development can be affected (Fig 2).

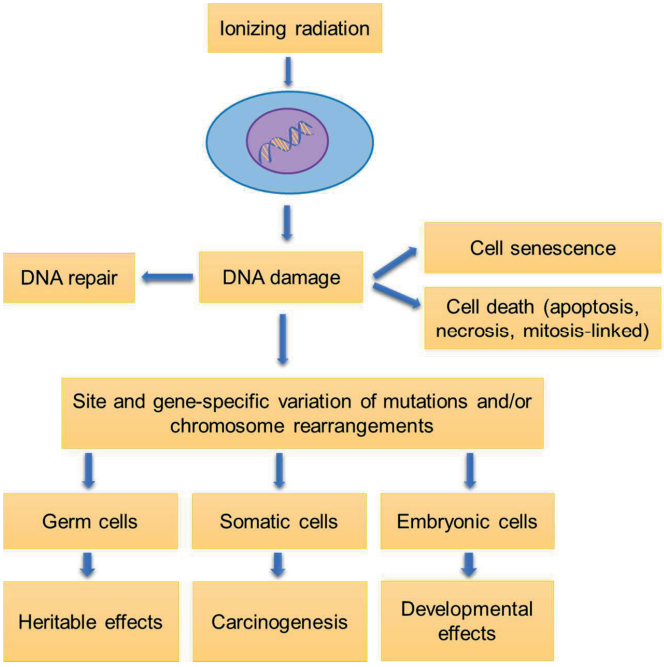

Fig 2. **Biological effects of cellular response to ionizing radiation.** Ionizing radiation deposits energy in the nucleus of the cell and induces DNA damage. The DNA damage can either be repaired or activate cell cycle checkpoint control and cause the damaged cell to go into a protracted senescent state. If the damage is substantial, cell death may occur via cellular pathways. If a damaged cell is not killed or inactivated, DNA mutations may be transmitted to the cell's progeny and result in gene mutations and/or chromosomal rearrangements. Depending upon the cell type, the insult may result

in carcinogenesis in somatic cells, heritable genetic effects in germ line cells and developmental defects in embryos (teratogenicity). Adopted from (UNSCEAR, 2006c).

In somatic cells, radiation induced mutations may lead to cancers, whereby cells that have a high mitotic future (such as erythroblasts and lymphocytes) are found to be more sensitive. And finally, damage to the reproductive tissue (i.e. germ cells) can result in infertility or mutations that can be passed on to the next generation, causing "heritable effects" (Fig 2). Often several mutated genes are the precursors of cancer, however, the disease in laboratory studies is rarely manifested, due to the time it takes to develop cancer and the relatively short life span of laboratory animals.

Radiation exposure can also result in distinct gene expression patterns, at a certain critical developmental stage (such as the gastrulation, *Section 1.7*), which represents the transcriptional response. Transcriptional responses to any given stressor will have sets of genes that show a dose dependent increase or decrease, and conversely gene sets that have sigmoidal dose responses and can further be detected by transcriptomics and other available methods (RNA sequencing, microarray, qPCR). In addition to inducing (DNA) damage, ionizing radiation may also lead to epigenetic changes, which do not alter the DNA sequence (described further in *Section 1.7*). Discovering such changes following irradiation, especially in earlier life stages can indicate the mechanisms causing the transcriptional changes (oxidative stress, bystander effects, epigenetic mechanisms), or indicate the adverse outcomes observed later in life, or potentially, in following generations.

1.5 Risk assessment and radiation protection

The aquatic environment can be a primary recipient of radioactive discharge. According to the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) report, the main exposure pathways to ionizing radiation in the marine aquatic environment are either external exposure from radionuclides in sediments (predominantly gamma emitters) or internal exposure from ingestion of radionuclides incorporated into the food chain (beta and alpha emitters) (UNSCEAR, 2008). Long-lived radioisotopes such as Caesium-137 (^{137}Cs) Strontium-90 (^{90}Sr) Tritium (^{3}H), Carbon-14 (^{14}C), and Iodine-129

(^{129}I), are of particular interest, although short-lived isotopes such as ^{131}I can be a significant dose contributor after nuclear accidents.

The environmental consequences of ionizing radiation from radioactive contaminations are still under debate with different conclusions from various studies (Beresford and Copplestone 2011). Some studies report harmful ecological effects (Garnier-Laplace et al. 2011; Galván et al. 2014; Mousseau et al. 2013), while others report no environmental effects (Murphy et al. 2011; Smith and Beresford 2005; Strand et al. 2014). Radiation risk represents the chance or probability that a living organism will be harmed, or experience an adverse health effect if exposed to radiation.

The International Atomic Energy Agency (IAEA), an organization that promotes peaceful use of nuclear energy, defines radiological protection as "the protection of people and the environment from harmful effects of ionizing radiation and for the safety of radiation sources" (IAEA, 2016). The recommendations and guidelines for radiological protection based on data on the biological effects are issued by the International Commission on Radiological Protection (ICRP). Although the main aim of radiation protection, according to the principles of the ICRP, was originally the protection of human health, there has been an increasing consensus that protection should also consider the impacts on ecosystems and their wildlife (ICRP, 2003b; ICRP, 2008). In line with the methodology currently used to protect human life, ICRP proposed a framework for protection based on a series of reference animals and plants (RAPs) (ICRP, 2012). A Reference Animal and Plant is defined as a hypothetical entity with assumed basic biological characteristics of a particular type of animal or plant, with defined anatomical, physiological and life stage properties, that can be used for the purposes of relating exposure to dose, and dose to effects, for that type of organism.

For all RAPs, the potential effects of radiation exposure have been consolidated from available literature data, and used to produce so-called derived consideration reference levels (DCRLs), which are a range of dose rates that are likely to produce adverse effects. In the reference animals and plants, fish are represented by trout (*Oncorhynchus mykiss*) and flatfish (*Pleuronectiformes*) (Table 1).

Table 1. Derived Consideration Reference Levels for fish represented in "reference animals and plants" (RAPs) of the International Commission on Radiological Protection (ICRP 2012).

Dose rate (mGy/d)	Reference Trout	Reference Flatfish
>1000	Mortality in embryos (0.3 to 19 Gy LD_{50}) depending on embryonic stage	Mortality in adults ($LD_{50/50}$ 30 Gy); mortality in eggs (LD_{50} 1 Gy)
100 - 1000	Potential for increased morbidity.	Some mortality expected in larvae. and hatchlings.
10 - 100	Some deleterious effects expected on young fish, e.g. reduction in resistance to infections. Reduced reproductive success.	Reduced reproductive success.
1 – 10	Possible reduced reproductive success.	Possible reduced reproductive success due to reduced fertility in males.
0.1 – 1	No information.	No information.
0.01 – 0.1	No information.	No information.
<0.01	Natural background.	Natural background.

Although the derived consideration levels stem from experimental studies, some studies have reported a discrepancy in radiosensitivity between wildlife populations and animals in controlled laboratory studies, with field effects postulated at lower dose rates than those needed to produce effects in the laboratory (Garnier-Laplace et al., 2010). Differences have been linked to confounding factors influencing wild populations, such as changes in food availability, predation, etc. (Garnier-Laplace et al., 2010). When it comes to acute radiation effects, fish and fish embryos are reported to be one of the most radiosensitive organisms to acute ionizing radiation (LD_{50} in the range of 10–25 Gy for assessment periods of up to 60 days following exposure) (UNSCEAR 2008), however chronic exposure is regarded as of higher importance for determining the environmental impact at the population level. Given that reproduction is one of the most relevant ecological endpoints for fish, information is needed on the effects of chronic radiation exposure over relevant life stages. Reproductive effects may be related not only to the sensitivity to radiation during gametogenesis and early development but also to the reproductive strategy, whereby in highly fecund species, the survival of early life stages may be very low, and the loss of abnormal embryos induced from radiation exposure may be masked by those lost from other confounding factors. The available data derived from previous reviews on chronic effects of ionizing radiation (where data from the Chernobyl

accident was also included), report 0.2 – 0.5 mGy/h (4.8 – 12 mGy/d) to be the first (known) dose rate to cause adverse effects in the germ line (Table 2).

In order to explore cumulative effects at the population level and in order to test the possible transmission of effects to the progeny and account for the germ line mediated transmission of factors that lead to mutations (i.e. DNA damage), studies in successive generations are warranted. To date, the predicted no-effect doses (PNED) for acute exposures are 900 mGy for marine ecosystems and 300 mGy for terrestrial and freshwater ecosystems, while 10 µGy/h (0.01 mGy/h) is the predicted no effect dose rate (PNEDR) used for screening purposes for chronic exposures – below which one could be confident that no adverse effects on populations would arise (UNSCEAR, 2008).

Table 2. Chronic effects of exposure to ionizing radiation on reproduction in fish table from the Framework for Assessment of Environmental Impact (FASSET) Radiation Effects Database — FRED (UNSCEAR, 2008) inherited by Environmental Risks from Ionizing Contaminants: Assessment and Management (ERICA).

Dose rate (µGy/h)	**Dose rate (mGy/d)**	**Reproductive effects**
0 – 99	0 – 2.4	The majority of of values obtained reflect control or background dose group, normal cell types, normal damage and normal mortality observed
100 – 199	2.4 – 4.8	No data available
200 – 499	4.8 – 12	Reduced spermatogonia and sperm in tissues
500 – 999	12 – 24	Delayed spawning, reduction in testis mass
1000 – 1999	24 – 48	Mean lifetime fecundity decreased, early onset of infertility
2000 – 4999	48 – 120	Reduced number of viable offspring; Increased number of embryos with abnormalities; Increased number of smolts in which sex was undifferentiated; Increased brood size; Increased embryo mortality.
5000 – 9999	120 – 240	Reduction in number of young fish surviving to 1 month of age; Increased vertebral abnormalities
> 10.000	> 240	Inter-brood time tends to decrease with increasing dose rate; Significant reduction in neonatal survival; Sterility in adult fish; Destruction of germ cells within 50 days in medaka (*Oryzias*

	latipes) fish; High mortality of fry, germ cells not evident; Significant decrease in number of male salmon (*Salmo salar*) returning to spawn; after 4 years, female salmon had significantly reduced fecundity

On more generic terms, UNSCEAR has defined a low dose rate of 6 mGy/h for a maximum of 1 hour of exposure (UNSCEAR, 2012).

To date, the studies performed in animal models and cells suggested that irradiation of parents to high doses (above 1 Gy) can lead to observable (genetic and epigenetic) effects in offspring. The studies of cancer incidence in children of survivors of the atomic bombings in Japan and of the children of patients treated with radiotherapy did not report convincing data on heritable (resulting from irradiation of germ cells) effects of radiation in humans (Little et al., 2013).

1.6 Sensitive life stages

Radiosensitivity is the probability of a cell, tissue, or organ suffering an effect per unit dose of radiation. The sensitivity of the organism to toxicants largely depends on the life stage at exposure. In order to address the objectives of this study mentioned in *Section 1.2*, exposure to gamma radiation has taken place during gametogenesis and embryogenesis and the early larval development.

1.6.1 Zebrafish gametogenesis

Gametogenesis (oogenesis and spermatogenesis) is a highly organized process that includes mitotic, meiotic and post-meiotic cells that ultimately lead to sperm and oocyte formation. Up to the period of 5 wpf (25 – 35 dpf), zebrafish gonads are not sex-specifically differentiated and gonad development for both females and males starts as juvenile ovary stage, which is known as juvenile hermaphroditism (Uchida et al, 2002). It is presumed that after this period, depending on fish size, and environmental factors, the morphological differentiation of the gonads in the zebrafish follows by apoptotic mechanisms (Uchida et al, 2002).

Oogenesis. In two weeks post fertilization (wpf) zebrafish, primordial germ cells can be seen in a dorsocaudal position. At 4 wpf, the majority of the fish possesses paired

gonads with primordial germ cells; these gonads represented presumptive ovaries (Maack and Segner, 2003). The primordial germ cells (primary oogonia, diploid, 2n), divide mitotically to produce secondary oogonia (2n). Secondary oogonia transform into primary oocytes. During maturation, primary oocytes undergo seven maturation stages, of which the first three stages are previtellogenic, i.e. no yolk formation, while the four last stages represent the vitellogenic stages, during which the oocytes increase in size as a consequence of the sequestration of the liver-derived yolk precursor vitellogenin (Lambert et al, 1970; Selman et al, 1993). At around 5 wpf, the ovaries have perinucleolar oocytes. Primary oocyes undergo meiosis I and unequal cytokinesis resulting in the first polar and the larger secondary oocyte (haploid, n). Secondary oocyte undergoes meiosis II to produce a second polar body and a larger cell which matures into an oocyte (Schulz et al., 2010). Finally, at about 7 wpf (~50 dpf), the ovaries increase in size.

In zebrafish (as well as other fish and in amphibians), germ cells in all stages of oogenesis are present in adult ovaries, while in mammals the mitotic divisions of oogonia finish before birth and only the meiotic germ cells are present in postnatal and adult ovaries (Nakamura et al., 2011).

Spermatogenesis. In zebrafish, the transition of hermaphroditic ovaries into early testes starts from 5 wpf and is presumed to be completed when primary spermatocytes are detected in addition to the gonial cells at ~7 wpf (~50 dpf) (Maack and Segner, 2003). At this time, cyst-like groups of gonial cells start appearing, which represent the primitive testis. From here, spermatogonia type A (diploid, 2n) start to appear and divide by mitosis to produce spermatogonia type B (2n), which transform into primary spermatocytes (2n). These further undergo meiosis I to produce secondary spermatocytes, which finally undergo meiosis II to produce spermatids (haploid, n) that differentiate into sperm. In more than half of 11 wpf zebrafish, spermatogonia, spermatocytes and spermatids can be found. In adult zebrafish testis, nine generations of spermatogonia (of type A and B) were found. In culture, the time of differentiation from spermatogonial stem cells to sperm lasted 17 days (Kawasaki et al., 2016). The meiotic and spermiogenic phases in adult zebrafish were found to last approximately 6 days (Leal et al., 2009).

1.6.2 Zebrafish embryogenesis and early development

Embryogenesis (embryo development) is the process of cell division and differentiation, which occurs in earliest stages of the development. Generally, embryogenesis starts with fertilization, and following the completion of meiosis, the

embryo undergoes rapid, synchronous cell divisions (Kane and Kimmel, 1993) and ends at hatching, which occurs from 48 – 72 hpf. The ZF embryogenesis is defined as "eleuthero", meaning it happens outside the womb, and is divided into seven developmental stages (Kimmel et al., 1995).

The first stage is zygote formation (1-2 cell) followed by the cleavage period (4 to 64-cell stage), during which the single cell formed after fertilization subsequently undergoes cell division of cells in the early embryo. These are followed by the blastulation (128-cell to 50% epiboly, < 5.25 hpf), which involves the formation of hollow sphere referred to as blastomere, surrounding an inner fluid-filled cavity. The next stage is the gastrulation, the developmental period during which the morphogenetic cell movements (e.g. epiboly) and the production of the three primary germ layers (ectoderm, mesoderm and endoderm) and the embryonic axis (> 5.25 hpf) occur. This stage is followed by segmentation (10 - 24 hpf). This stage encompasses the tailbud, which is considered a phylotypic period, when a high morphological resemblance of anatomical features in vertebrates occurs (Richardson et al., 1998). Segmentation is followed by the pharyngula stage (24 to 48 hpf) during which the development of the spinal cord is first visible. The pharyngula is followed by hatching, which occurs between 48 and 72 hpf, followed by the early larval stage (Fig 3).

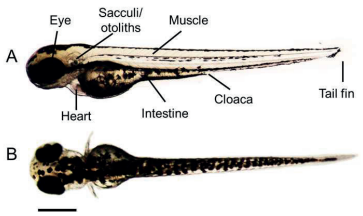

Fig 3. Zebrafish larva at 72 hpf with prominent anatomical features. (A) Lateral view. (B) Dorsal view. The scale bar indicates 500 μm.

The use of early life stage zebrafish embryos is considered to be the most sensitive to toxicants in the animal's life cycle (Scholz et al., 2008) and has been proposed as a relevant experimental bioassay to assess toxic effects of contaminants (OECD, 2004a; OECD, 2004b). Embryos are considered more sensitive to radiation than adults in terms of the probability for an adverse effects later in life (cancer) as these adverse effects reflect changes occurring at the cellular and molecular levels.

1.7 The genome, transcription and heritable effecs

The genome is the genetic material of an organism, while a gene is a region of the genome, which encodes a functional protein or RNA molecule (Gerstein et al., 2007). In both cases the DNA sequence information is first converted into RNA in a process known as transcription (White et al., 2009). If the gene in question is a protein coding gene, the transcribed RNA is called messenger RNA (mRNA) (Brenner et al., 1961), and is further converted into a sequence of amino acids, which folds into a functional protein (translation). Non-protein-coding genes give rise to non-coding RNAs (ncRNA), which carry out other functions in the cell such as ribosomal RNAs (rRNA) and various classes of ncRNAs: micro RNAs, short interfering RNAs and long non-coding RNAs (lncRNA) (Cech and Steitz, 2014; Bizuayehu and Babiak, 2014).

The zebrafish genome is fully sequenced and can present a great deal of information in terms of the development and heritable effects and their relation to the same effects seen in other animal models (Felsenfeld, 1996; Haffter et al., 1996; Haberle and Lenhard, 2012). Despite the evolutionary distance, the human and zebrafish genome exhibit considerable homology with the conservation of key genes involved in development, signal transduction, cell cycle progression and proliferation, and cell differentiation (about ~70 % of human gene orthologues and ~82 % of human cancer gene orthologues) (Amatruda and Zon, 1999; Trede et al., 2004; Otis et al., 2015; White et al., 2013; Howe et al., 2017).

The entire process of converting sequence information encoded within a gene into a precise amount of functional product is referred to as gene expression (Fig 4). This process is influenced by both internal and external stimuli and is tightly regulated by various mechanisms to ensure a correct amount of gene product is present in a particular cell at a particular point in time. Transcription is the initial critical step in gene expression (Fig 4), which copies the information from a particular portion of DNA nucleotide sequence (i.e. gene) into RNA.

A transcriptome contains all gene transcripts at a certain time point and may provide substantial information on global gene expression profile and biological pathways in response to an environmental stressor such as gamma radiation. Whereas the genome remains the same in all cells in an organism, with the exception of erythrocytes, which contain no DNA in mature state and immune cells, which rearrange their DNA sequences in response to antigens (Nossal, 2003), the transcriptome is a dynamic entity changing

between both different cell types and developmental stages, as well as in response to the environment.

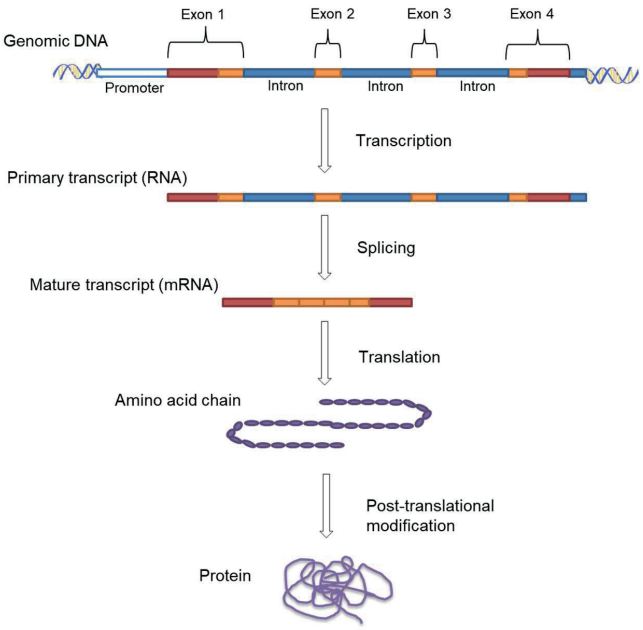

Fig 4. Simplified representation of gene expression, transcription and translation.

Transcriptome analysis allows for the registration of changes in gene expression and identification of differentially expressed genes (DEGs). The increased or decreased expression of one or more genes and consequently the protein(s) they encode, follows from up- and down-regulation, processes that occur within a cell triggered by an internal or external signal, respectively (Sherwood et al, 2012). This is related to biological or developmental processes and external stimuli and can be used to reveal potential mechanisms of toxicity, which may contribute to a better understanding of the biological effects of ionizing radiation.

At the 10^{th} cell cycle (512-cell stage, 2.75 hpf) in zebrafish maternal mRNAs begin to degrade and the zygotic transcription commences, known as the mid-blastula transition (MBT) (Aanes et al., 2011) (Fig 5). The degradation continues until 50% epiboly and this period marks the onset of cell differentiation and specification (Haberle et al., 2014).

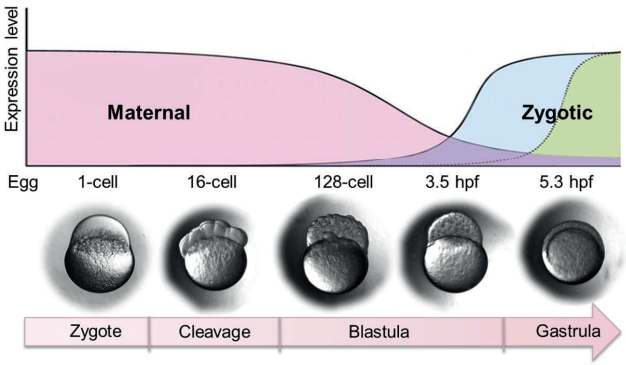

Fig 5. Mid blastula transition (MBT). Maternal transcripts degrade, while the zygotic genome transcription commences. Early gastrula (5.3 – 5.5 hpf) marks the timepoint when the zygotic genome is activated and most maternal transcripts are degraded. Modified after Aanes et al. (2014).

A basic assumption in biology until recently was that mutations in the DNA sequence were the only source of heritable phenotypic variability. Now it is known that effects which might lead to disease in following generations can also be transmitted via the parental germ line via heritable alterations in gene expression and without changes in underlying DNA sequence (epigenetic) (Koturbash et al., 2006; Merrifield and Kovalchuk, 2013; Vaiserman et al., 2017). These epigenetic mechanisms are DNA methylation, histone modifications and small RNA- mediated gene silencing. DNA methylation is the process by which methyl groups are added to a gene and can change its activity, without changing the sequence. Histone modifications impact gene expression by altering the chromatin structure and small RNA-mediated gene silencing by blocking the translation of mRNA (Ilnytskyy and Kovalchuk, 2011). These phenomena contribute to the theory of transgenerational inheritance. A transgenerational effect is by definition “an effect which is transmitted to the following generations by means of epigenetically mediated mechanisms, and not via the genetic sequence” (Koturbash et al., 2006; Shimada et al., 2005; Skinner, 2011), and such effects can cause genomic instability in prenatally exposed organisms, or in multiple generations (Fig 6).

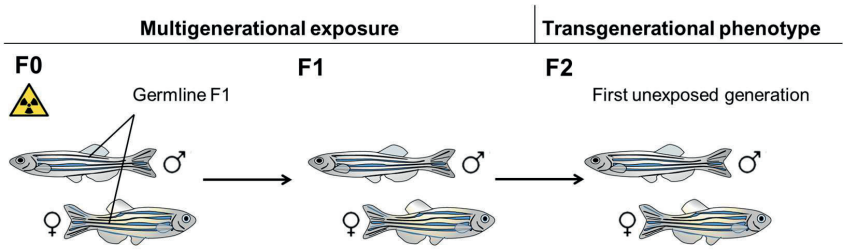

Fig 6. Schematic overview of multigenerational exposure and potential transgenerational effects. Adapted from (Skinner, 2011).

2. Methodology

2.1 Study design

The study design for the exposure of adult zebrafish used three different gamma radiation dose rates and total doses subchronically during gametogenesis (control, 8.7 and 53 mGy/h, total 4 μGy, 5.2 Gy and 31 Gy). According to the United States Environmental Protection Agency (US EPA 2003), subchronic exposure can be defined as a continuous exposure, which lasts for approximately ten percent of an experimental species lifetime. Furthermore, a range of dose rates (0.4 mGy/h to 38 mGy/h, lowest total 1.5 mGy and highest total 3.6 Gy) was used for the early embryogenesis and, the early larval exposure, respectively.

Four lines of offspring were created from the irradiated and control adult fish, named according to the periods of exposure in the parental (F0) or offspring (F1) generation as: F1 control, gametogenesis line (G), gametogenesis and embryogenesis exposed line (GE) and embryogenesis exposed line (E).

In Paper I, effects in embryos and early larva were observed after exposure to increasing doses of gamma radiation, which served as basis for the determination of the doses to be used in further experiments. The gene expression was tested in embryos exposed to 0.54, 5.4 and 10.9 mGy/h for 3 hours from 2.5 to 5.5 hpf. The survival and development, such as the deformity frequency and hatchability were assessed in embryos and larvae exposed to 0.4, 3.9, 15 and 38 mGy/h for 43.8 and 92 hours (until the 48 hpf and 96 hpf stage) and starting from 2.5 hpf to the 48 and 96 hpf stages. In Paper II, development, oxidative stress, genotoxicity and bystander effects were studied in directly exposed embryos, embryos of exposed parents during gametogenesis and directly exposed offspring starting from 2.5 hpf and lasting throughout the embryogenesis., while the 5.5 hpf gene expression described in Paper III was tested in offspring of exposed adult fish starting from 2.5 hpf. And finally, in Paper IV, reproduction and genotoxicity were tested in exposed adult fish during gametogenesis, but this study was completed last, due to sampling procedures and requirement of fish sacrifice. The time line of experiments of the present PhD project was planned in accordance with the life-timeline of the fish and is presented in Fig 7.

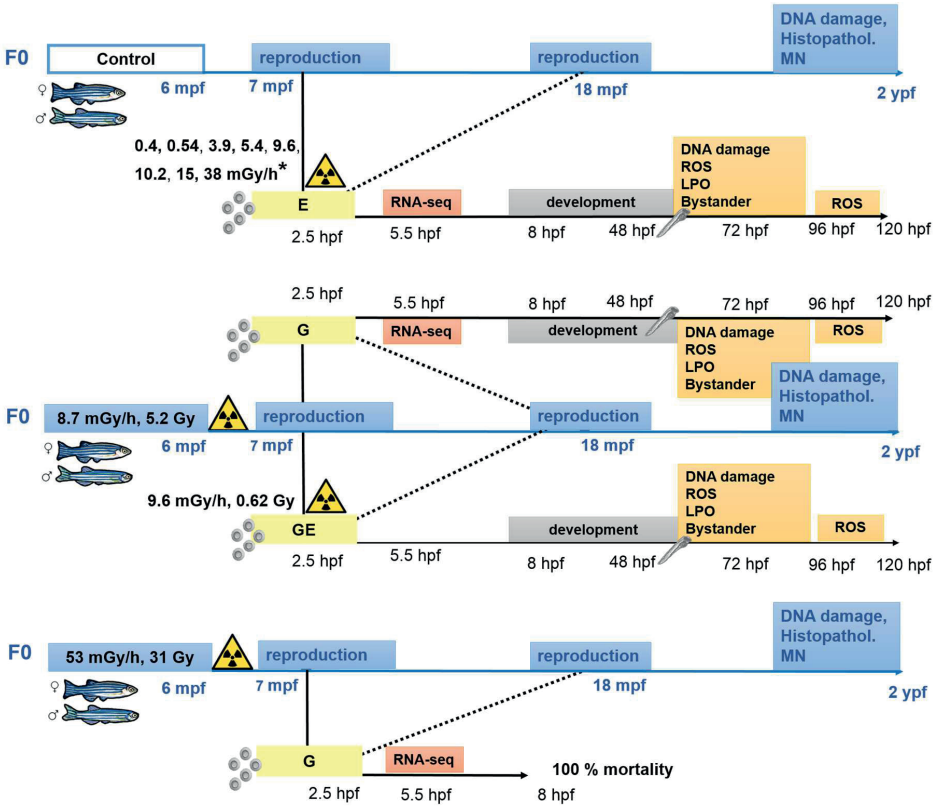

Fig 7. Two-generational study design. The timeline for assessment of relevant endpoints in adult zebrafish and offspring is separate. The dotted line represents production of new progeny at parental age. E -embryogenesis line of exposure; G - gametogenesis line of exposure; GE – exposed in both periods. For further information about total doses used in (*) exposures, see *Section 4,* Table 3.

2.2 Gamma irradiation

All studies involving gamma radiation were performed at the Figaro (NMBU) Experimental Radiation Facility (^{60}Co source, activity ~ 420 GBq) (Fig 8). At maximum activity, the source can provide a continuous dose rate field from approximately 3 Gy/h (at source) down to 0.4 mGy/h and allows simultaneous exposure over the dose-rate field. The irradiation room was thermostatically heated during all exposures and had the appropriate light cycle (explained in *Section 2.3*). For the embryo exposures, external gamma irradiation of zebrafish embryos commenced at 2.5 hpf with durations of 3 hours to 4 days (Paper I, II

and III), while for the adult fish, exposures commenced at 6 months of age, and lasted throughout gametogenesis (Paper II and IV).

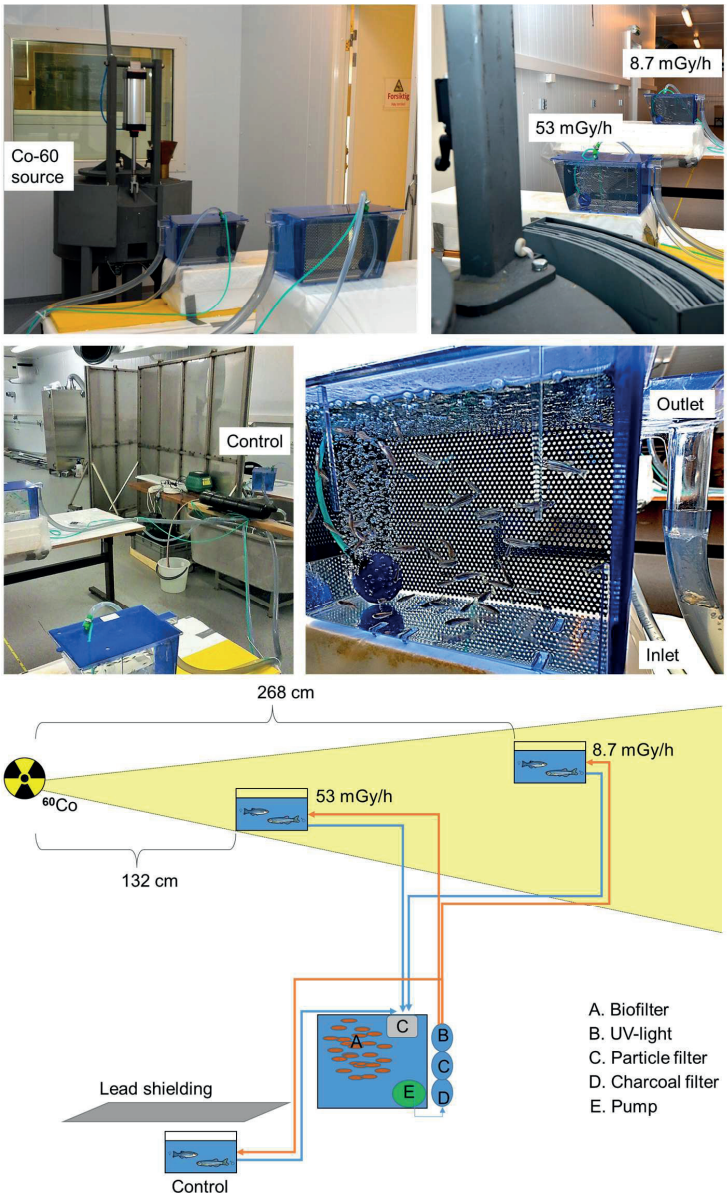

Fig 8. Gamma radiation exposure of adult zebrafish at the Figaro (NMBU) Experimental Radiation Facility.

Field dosimetry in Paper I and II (air kerma rates measured with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Norwegian Radiation Protection Authority, NRPA, Oslo, Norway) (Carlsson et al., 2014). For the embryo exposures in Paper I, average dose rates to water in the first and second rows of microplate wells were estimated according to technical document (Bjerke and Hetland, 2014) and used as a proxy for dose rates to the fish embryos. The estimated background (control) dose rate was ≤ 0.35 μGy/h (Thermo Eberline FHT6020).

The dosimetric measurements in Paper II for the adult fish were performed during exposures with optically stimulated luminescence dosimeters, called nanoDots (Landauer, Inc., Greenwood, IL) (see e.g. Hansen and Hetland, 2017). Average absorbed dose rates and doses to water in the aquaria were obtained based on their geometry, composition, locations in the field and weighted for modified swimming configurations. For well plates, conversion of air kerma rates to absorbed doses in air, ratio of mass enery absorption coeffiecients of water to air and tabulated peak scatter factors and depth dose curves were use to estimate the absorbed dose rates to water. The estimated background dose rate for exposures described in Papers II, III and IV was 4 μGy/h.

2.3 Model species

The laboratory Zebrafish (*Danio rerio*) of the wild-type (AB) strain was chosen as the test model. Among fish species, the most complete database on genomics, molecular genetics and embryology is available for the zebrafish, accessible through the Zebrafish Information Network (http://zfin.org/ZFIN).

Zebrafish have a relatively short generation interval and reach sexual maturity in at about 3-6 mpf depending on the laboratory conditions. Therefore, they are a good model for multigenerational studies. One of the main advantages of using zebrafish is that they are asynchronous spawners, meaning that they can ovulate on a regular basis, over a prolonged period, while their fecundity is typically high. Around 200-300 eggs can be produced per female in every alternating week for studies involving reproduction (Hoo et al., 2016). They are ovuliparous, meaning that eggs are externally fertilized (Lodé, 2012).

The development is very well studied and characterized (Kane and Kimmel, 1993; Kimmel et al., 1995). Zebrafish up to the 120 hpf earliest life-stages of zebrafish according to the EU Directive 2010/63/EU on the protection of animals used for scientific research are

considered an animal replacement strategy (Strähle et al., 2012). Standardized tests facilitate analysis of relevant endpoints related to radiation exposure, such as the zebrafish embryo toxicity test, ZFET (OECD/OCDE, 2013), while the small size of the animals allows employment of high throughput screening (HTS) methods, which can be used for environmental risk assessment (Scholz et al., 2008; Williams et al., 2014).

2.4 Fish husbandry

Adult fish used in the studies described in Paper II, III and IV were kept at 28 ± 2°C on a 14:10 hour light-dark cycle (250 – 320 lx) at a density of 5 - 10 fish/L, and a record of laboratory conditions was held during and between experimental periods. The system water (SW) was routinely prepared from particle and active charcoal filtered reverse osmosis (RO) deionized UV sterilized tap water with standard values for conductivity, general hardness and pH, which were measured daily and described in each paper. Adult fish were fed with Gemma Micro 300 (Skretting, Stavanger, Norway) dry feed twice a day and live artemia (Scanbur, Copenhagen, Denmark) once a day.

Embryos and early larvae (Paper I, II and III) were kept in autoclaved, temperate (28 ± 2°C) SW with daily water change. Health monitoring was performed by daily inspection. Adult fish were anesthetized using tricaine methanesulfonate (MS-222) (Sigma Aldrich, Oslo, Norway) in ddH_2O adjusted to pH 7.0 with 1.0M Tris (pH 9.5) combined with iced SW. Euthanasia of both larvae and adult fish was performed according to standard operating procedures and using an overdose of MS-222 in iced SW, whereby the fish were observed until no visible movement.

2.5 Ethics statement and animal welfare considerations

The research in the present PhD project was conducted in line with the Norwegian Animal Protection Act (implemented EU Directive 2010/63/EU) and approved on December 12, 2013 by IACUC at Norwegian University of Life Sciences, Faculty of Veterinary Medicine (VetBio), Oslo, Norway (Approval number (FOTS) ID 5793). Handling of fish was performed according to the SOP of the Zebrafish Facility at NMBU, which is accredited according to AAALAC standards (www.aaalac.org). The number of fish used depended on the endpoints of the study and in each case the number of experimental animals was kept to a minimum allowing statistically relevant data. Sentinel fish were sent

to ZIRC for pathology every six months and the microbiological analysis of water was undertaken at NMBU, VetBio, Oslo.

2.6 Methods

2.6.1 Gene expression analysis

The gene expression analysis is the determination of the pattern of genes expressed at the level of genetic transcription, in a specific cell or under a specific circumstance and is considered a highly sensitive study endpoint, which can report early modulatory effects of toxicants on a molecular level. In order to obtain homogeneous cell populations for the analysis of gene expression in zebrafish, embryos were exposed to gamma radiation from 2.5 to 5.5 hpf (corresponding to MBT mentioned in *Section 1.7*).

RNA sequencing

RNA sequencing is a revolutionary tool and high throughput method to measure the amount of RNA in a biological sample (Wang et al., 2010). The biological sample was a pooled batch of embryos at 5.5 hpf of development. Pooled embryos are often used in genomic studies, since they provide sufficient RNA for further analysis and (Fang et al., 2013, Aanes et al., 2014). Total mRNA was isolated from embryos (50 per sample in Paper I and 50 per sample in Paper III) according to methods in Paper I and Paper III. RNA purity and yield (A260/A280 > 1.8, A260/A230 > 2, yield > 200 ng/µl) was determined using NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality (RIN > 8.5 in Paper I, and RIN > 9 in Paper II, respectively) using RNA Nano LabChip Kit (Agilent Technologies) with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The mRNA sequencing for Paper I and Paper III was outsourced to BGI Tech Solutions Co., Ltd. (HiSeq 2000, Illumina, San Diego, CA) and to Novogene, (HiSeq 4000, Illumina), Hong Kong, China, respectively. Libraries were analyzed using 150 bp paired-end reads, with a depth of 20 million reads per sample.The DEGs were identified using tools and packages that count sequencing reads per gene and compare them between samples such as DESeq (Anders and Huber, 2010) and edgeR (Robinson et al., 2010), packages from Bioconductor (www.bioconductor.org), which use a model based on the negative binomial distribution.

Quantitative real-time PCR (qPCR) analysis

The quantitative real-time PCR (qPCR) is a low throughput method for the analysis of gene expression, meaning that a smaller number of genes were chosen for testing and in

this manner a verification of the RNA-sequencing results in Paper I and III was obtained. Eight (Paper I) and twenty (Paper III) differentially expressed genes (DEGs) from the respective RNA sequencing analyses were chosen for the qPCR. The genes which showed no differences following sequencing analysis (*rps18*, *hmbs*, *eef1a* and *hprt1* in Paper III) (Kamstra et al., 2017) were used as reference genes.

All qPCR experiments were conducted in accordance with the "Minimum information for publication of quantitative real-time PCR experiments" (MIQE) guidelines (Bustin et al., 2009). The methods between Paper I and Paper III differed slightly, for instance, the gene expression in Paper I was normalized to *hmbs*, while in Paper III, gene expression was normalized using the geometric average of 3 reference genes (rps18, hmbs, and *hprt1*).

2.6.2 ROS assay

The ROS assay used in this study is an *in vivo* technique for measuring intracellular ROS production in viable, hatched zebrafish embryos and larvae until 120 hpf. The method used for analysis is modified after Mugoni et al. (2014) and employs a fluorescent probe for detecting ROS. H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate, Invitrogen, Molecular Probes Inc., Eugene, OR, USA) is a non-polar and non-fluorescent probe that enters the cells freely and is hydrolysed by cellular esterases to non-fluorescent 2,7-dichlorodihydrofluorescein (H2DCF), which is retained in the cell. In the presence of of ROS, H2DCF is converted by oxidation to fluorescent 2,7-dichlorofluorescein (DCF), which is localized in the cytosol (Winterbourn, 2014). H2DCFDA is a marker of the general oxidative stress instead of indicator of specific ROS formation.

In Paper II, this assay was performed in embryos with no optically observable developmental defects one month and one year after irradiation of the parents and immediately after irradiation of progeny (F1). The outline of the method is presented in Fig 9.

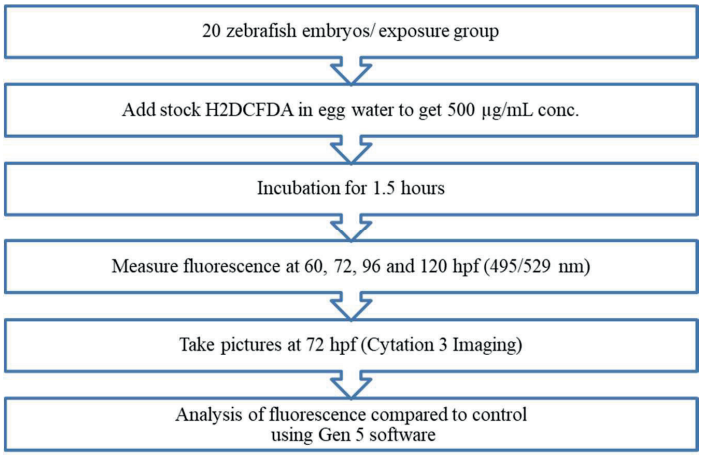

Fig 9. ROS assay outline used for detecting ROS formation in zebrafish embryos and larvae.

Natural fluorescence of irradiated egg water in combination with the probes (without presence of embryos) for each dose rate was analyzed and the resulting fluorescence was subtracted. The relative fluorescence obtained for each exposure group was expressed as fold induction from control. Further details can be found in Paper II.

2.6.3 Lipid peroxidation assay

The lipid peroxidation (LPO) assessed in Paper II using an *in vitro* colorimetric method and 72 hpf zebrafish larvae produced one year after irradiation of the parents and immediately after irradiation of embryos. The method was previously described by Erdelmeier et al. (1998) and is performed by determining by-products of malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides, following the, schematically depicted in Fig 10.

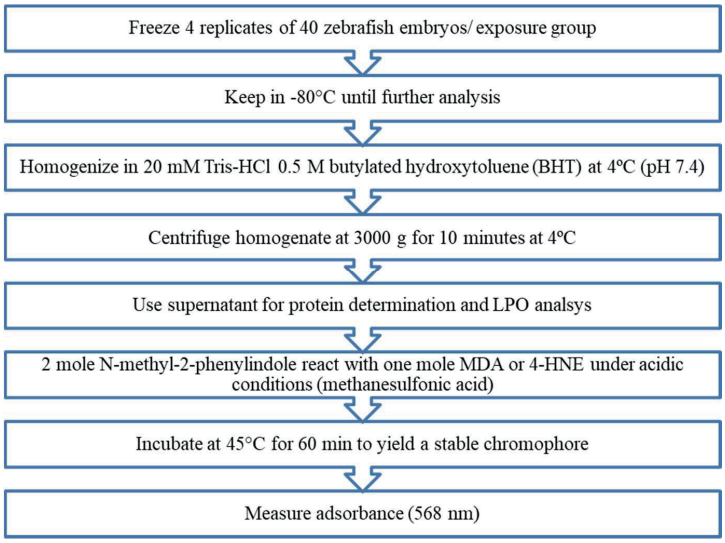

Fig 10. Lipid peroxidation (LPO) protocol outline used for analysis in zebrafish embryos at 72 hpf.

The protein content was determined using the method with Immunoglobulin G (IgG) as a standard (Bradford, 1976) and LPO was expressed as nmols of MDA and 4-HNE per gram of total protein concentration.

2.6.4 DNA damage

The alkaline high throughput screening (HTS) Comet assay developed by Gutzkow et al. (2013) was performed according to a slightly modified method previously described (Jarvis and Knowles, 2003) was used in Paper II and IV. The method detects single strand breaks and alkali-labile DNA lesions using GelBond®films (Hansen et al., 2010) for a HTS cell gel electrophoresis. The principle for detection of DNA damage is that as the frequency of DNA damage increases, the fraction of the DNA extending towards the anode increases, forming the Comet tail. For the Comet assay in Paper II, 10 ZF larvae which have previously not shown morphological defects were collected at 72 hpf as one biological replicate per exposure group and further processed according to Fig 11.

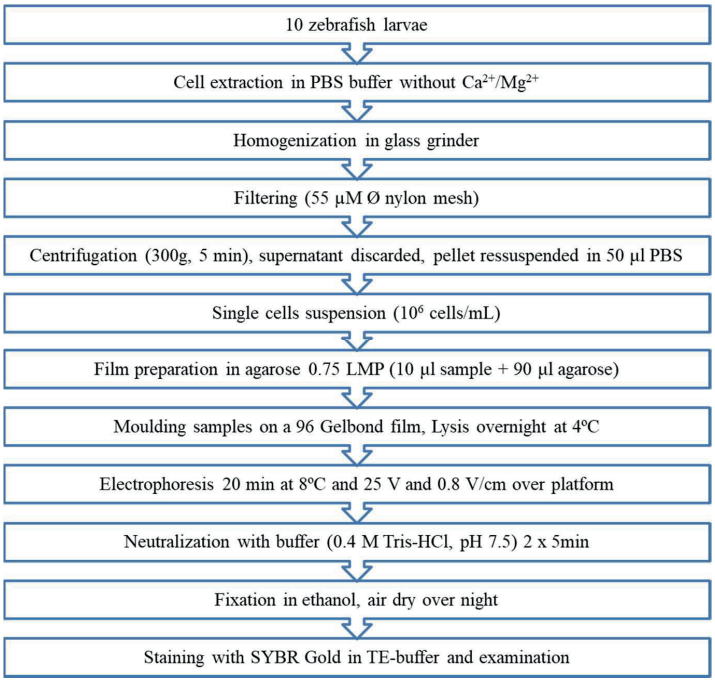

Fig 11. Comet assay protocol used for zebrafish at 72 hpf, after exposure to gamma radiation.

Three biological replicates were used and each subdivided in three technical replicates. Per each technical replicate, 50 "comets" were scored for tail intensity (% Tail DNA). Further details of the analysis are available in *Section 2.5.3* of Paper II.

In adult zebrafish, the Comet assay was performed 1.5 years after the gamma radiation exposure and post euthanasia in whole blood according to Fig 12. The analysis method was comparable to the method used for early larvae (*Section 2.7.1* of Paper IV).

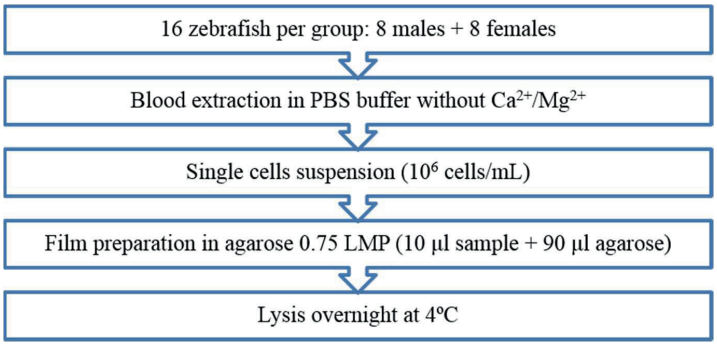

Fig 12. Comet assay protocol used for whole blood of ZF irradiated to specified gamma radiation doses.

2.6.5 Micronuclei analysis

Micronuclei were assessed in erythrocytes of adult fish in Paper IV. Blood samples were obtained from 8 to 11 males and females from each exposed and control group 1.5 years after irradiation. The blood smears were created in accordance with Fig 13.

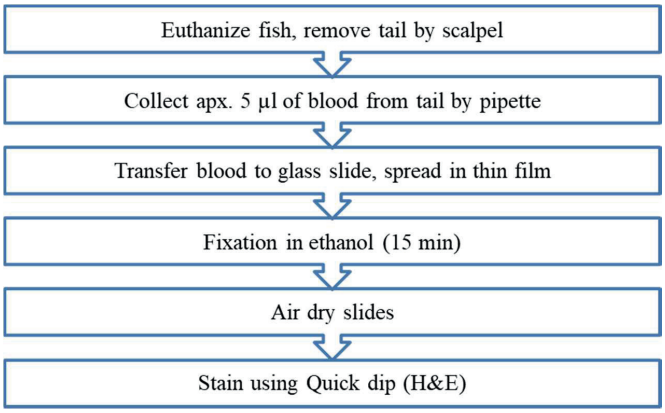

Fig 13. Outline for creation of whole blood smears for the analysis of MN in adult zebrafish exposed to gamma radiation.

The stained slides were viewed under a Zeiss Axioskop microscope with a digital camera (Leica SFC 420) and magnification 1000x, and between 1000-2000 erythrocytes scored per slide. The cells with one, two or three micronuclei (MN) were noted separately. The criteria for the scoring of micronuclei was previously described by Oliveira et al. (2009) and Song et al. (2012), whereby the MN should be a size from 1/10 to 1/30 of the main nucleus;

circular or ovoid chromatin body with the same staining pattern as the nucleus and MN must not touch the main nucleus.

2.6.6 Bystander effect analysis

The bystander effects in Paper II were measured by media transfer from irradiated to non-irradiated cells followed by a Ca-flux assay (Mothersill et al. 2014). The embryos used for the bystander effects were sampled one year after irradiation of the parents and immediately after irradiation of the F1 progeny at 72 hpf. The method used is briefly described in Fig 14.

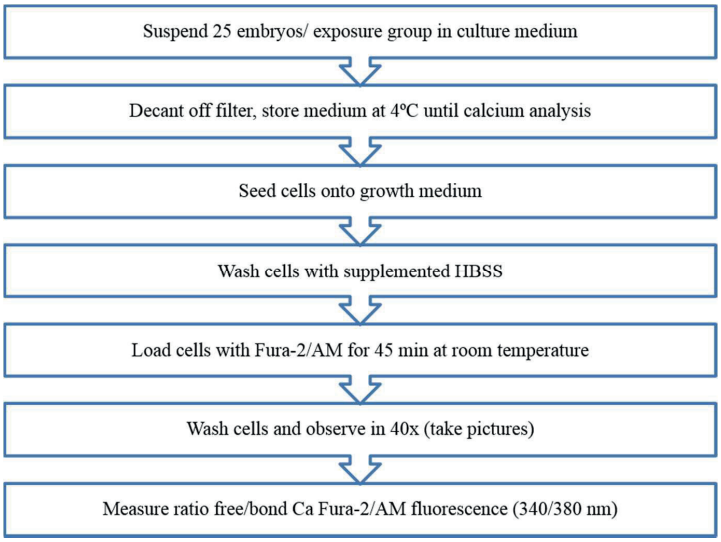

Fig 14. Outline for analysis of bystander effects in ZF embryos at 72 hpf after exposure to gamma radiation during embryogenesis and/or parental gametogenesis.

2.6.7 Developmental and histological examinations

The developmental effects (Paper I and II) of gamma irradiation in zebrafish (survival, hatching and deformities) from 48 hpf until end of the larval period (120 hpf) were manually or microscopically observed and analyzed according to the guidelines defined by the OECD zebrafish embryo toxicity test (OECD/OCDE, 2013). Minor modifications were made regarding the stage of development at time of assessment and exposure setups.

The histological examinations were performed post-euthanasia in embryos (Paper II) and adults (Paper IV), whereby groups were separately fixed in paraformaldehyde and

stored in 70 % ethanol until use. The samples were embedded in 1 % agarose mold for adequate positioning of the embryos (Tsao-Wu et al., 1998), transferred to paraffin, and there after 5 μm sections were processed according to standard procedures employed for staining with hematoxylin and eosin (H&E).

2.6.8 Reproduction

The reproduction was assessed in paper IV in adult fish, since it was proven to be a sensitive endpoint to ionizing radiation exposure (UNSCEAR, 1996) and it can be used as an indicator of population dynamics. The fish breeding was perfored using the "conservative" method and standard 1 L aquaria (Aquatic Habitats, Apopka, FL) breeding for 30 minutes. The same mating procedure was used to produce embryos for all endpoints. The mating experiments took place in six and five consecutive breeding weeks one month and one year after gamma irradiation, respectively. In each breeding trial, six 1L breeding tanks (n = 6 breeding pairs per group) were used. The breeding setup and male/female separation took place in the late afternoon and collection of embryos followed in the early morning.

2.7 Data processing

2.7.1 Bioinformatics tools

In Paper I, three single-end libraries (biological replicates), in the 5.4 and 10.9 mGy/h and a duplicate per 0.54 mGy/h exposure group were sequenced. Quality assessment of raw reads (49 nt long) and adapter trimming was performed using Trim Galore! v0.3.7, a wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files (Martin, 2011; Andrews). Only reads with Phred score > 20 were kept. Afterwards, using TopHat v2.0.9 (Trapnell et al., 2009) with bowtie1, reads were mapped to the ZF genome (version Zv9, release 76) downloaded from Ensembl (http://www.ensembl.org/Danio_rerio/Info/Index). Options -g (maximum multihits number) was modified from its default value to 1, --no-coverage-search was allowed, --library type was set to "fr-unstranded" and -p (number of threads) was restricted to 4. As for bowtie1 options, -q (fastq files), -v (report end to end), -k 20 (report up to 20 good alignments), -m 20 (suppress all alignments if > 20), -S (to use SAM format) were used. BAM files were uploaded into Seqmonk (Andrews, 2012) for visualization of aligned and mapped reads and read counting. Reads were counted as reads exactly overlapping with exons and the

resulting count table was analyzed for gene expression under edgeR v3.4.2 Bioconductor (Robinson et al, 2010). The RNA-seq experiment was deposited in SRA database (https://www.ncbi.nlm.nih.gov/) and is publically available under accession SRP096352 (presented in Paper I Supplementary material; S1 Fig.).

In Paper III, raw fastq files were adapter trimmed using trim_galore (v0.4.2, Babraham institute, UK) under standard parameters, with extra base clipping of 1 base at the 3' side of both reads and 12 bases at the 5' side of both reads. This was done since initial FastQC (v0.11.5, Babraham institute, UK) analysis revealed high K-mer content at the 5' side of the sequencing due to adapter contamination. To assess global insertions and deletions, the STAR aligner (v2.5.2b) (Dobin et al., 2013) was used to align and map sequences to the zebrafish genome (GRCz10, www.ensembl.org) with a recent release of the zebrafish transcriptome GTF (v85, www.ensembl.org). Since progeny of fish exposed to gamma radiation were assessed, the chimeric reads option in STAR was included, in order to assess the amount of chimeric genes possibly generated by DNA damage. After alignment, the generated SAM files were loaded into the SeqMonk sequence analysis tool (v1.35, Babraham institute, UK) and mRNAs were quantified using the built-in mRNA seq pipeline. Library quality was assessed by the RNA-seq QC plot and cumulative distribution plots within SeqMonk. A filter was used to only analyze mRNAs that had at least 30 reads in either of the replicate samples, in order to assure that for statistical analysis only mRNAs were included with enough reads.

2.7.2 Molecular pathway analysis (IPA)

The IPA® (Ingenuity® Pathway analysis Systems Inc., version 430520M, Qiagen, http://www.ingenuity.com, Redwood City, CA, U.S.) analysis and search tool was used to identify networks/pathways, and uncover the significance of transcriptomics data within the context of biological systems. Differentially expressed gene lists were imported into Ingenuity Pathway Analysis (IPA) and used with the ingenuity knowledge base as background since around 60% of the ZF genes were annotated as having a human orthologue by IPA. The differences between analyses in IPA for Paper I and III were minimal, although present. IPA calculates enrichment of genes, gene pathways, upstream regulators and diseases using Fisher's exact tests. In Paper I, the cut-ff p-value used was 0.001, while in Paper III the cut off p-value was set to 0.01. The nomenclature used for genes in paper one corresponds the nomenclature for zebrafish human orthologs where

applicable, while the original IPA nomenclature was kept in Paper III. For further detailed descriptions of the analyses, the reader is refered to Paper I and III.

2.7.3 Choice of statistical tests

In Paper I, statistical analyses were performed in Stata (MP/14 for Windows, StatCorp, College Station, TX). Confidence intervals were calculated using the proportion command for each of the outcomes survival, hatching and deformities at dose levels and the two exposure durations. Logistic regression reported as odds ratios (OR) was used to estimate the effect of the treatments on hatching, survival and deformities and standard methods were used to check model fit, whereby multiple comparisons were conducted using Tukey's or Dunnett's tests (Graphpad Prism 6, La Jolla, USA). Statistical significance was set to $p < 0.05$.

For analysis of gene expression, the dataset was TMM normalized (trimmed mean of M-values, edgeR v3.4.2 Bioconductor, Robinson, McCarthy, and Smyth 2010), followed by data exploration using the statistical package R v3.0.2 (R foundation for Statistical Computing, 2013). Data was explored for descriptive statistics such as: minimum, maximum, 1st quantile, 3rd quantile, median, mean, standard deviation, also the similarity among samples was determined by correlation analysis and hclust (ward method) analysis to determine the distance between samples. The statistical analysis of differentially expressed genes (DEGs) was based on pairwise comparison between treatment and control RNA-seq samples (biological replicates) with a cut off set to ± 0.40 log2 fold change (1.3 FC). The FDR (false discovery rate) was set up to a significance of $p \leq 0.05$. Venn diagram (Venny v2.1, Oliveros, (2007-2015) was used to explore overlapping differential expressed genes among radiation treatments. For qPCR, obtained mean relative gene expression values (exposed vs. control) were compared to mean relative gene expression values for the same genes from RNA-seq and a Pearson's correlation coefficient was calculated ($p < 0.05$) for all three exposure groups (Graphpad Prism 6, La Jolla, USA).

The statistical analyses in Paper II were performed using XLStat2016® (Addinsoft, Paris, France) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Results for all endpoints are presented as mean ± standard error (SEM) and compared between progeny lines. Significant differences between dose rates for all parameters were calculated using one-way analysis of variance (ANOVA) or Kruskal–Wallis One Way Analysis of Variance on Ranks. If significant, pairwise multiple comparison procedures were

conducted, using the Tukey test or the Dunnett's method. Statistical significance was set at $p < 0.05$.

In Paper III, differential gene expression was analyzed with the built-in Deseq2 filter in Seqmonk, using R (v3.3.1), on raw read counts. With this analysis mRNA isoforms were merged, since Deseq2 cannot assess differential expressed transcript isoforms. Deseq2 uses the negative binomial distribution to assess differential gene expression, with Benjamini Hochberg false discovery rate multiple comparisons adjustments (FDR) (Love et al., 2014). Following Deseq2 analysis, data was normalized by reads per million (RPM) in order to calculate fold change (FC) per gene averaging all replicates. Significant genes were reported as < 0.05 FDR with an absolute FC > 1.5, which was different from Paper I. The correlation of the expression between RNA-sequencing and qPCR results was calculated with non-parametric Spearman correlation in Graphpad (v. 7.1, La Jolla, CA) based on the fold-change of the differentially expressed genes.

In Paper IV, statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA, USA) and XLStat2016® (Addinsoft, Paris, France). Results are presented as mean ± standard error (SEM) for the reproduction, while analysis of biometric parameters and DNA damage were calculated using Kruskal–Wallis One Way Analysis of Variance on Ranks, whereby multiple comparisons were conducted using the Dunn's test. The cumulative embryo production and embryo production per breeding pair and MN were analyzed using a Two Way Analysis of Variance (with the dose rate and either time after irradiation or sex as independent variables). Multiple comparison procedures were conducted according to the Fisher's LSD, Dunnett's or the Tukey's test. Statistical significance was set at $p < 0.05$.

3. Results – summary of papers

3.1 Paper I

Dose-dependent effects of gamma radiation on the early zebrafish development and gene expression

Embryonic development and gene expression were assessed after continuous gamma radiation exposure starting at the blastula stage. The development was assessed after exposure of embryos to 0.4, 3.9, 15 and 38 mGy/h, from 2.5 hours post fertilization (hpf), and lasting through embryogenesis (until 48 or 96 hpf), while the gene expression was assessed in embryos exposed to 0.54, 5.4 and 10.9 mGy/h (from 2.5 – 5.5 hpf) (Fig 15). Mortality was higher at 96 hpf only in the 38 mGy/h group. The total hatching was significantly lower from controls in the 15 mGy/h group and a delay in hatching onset in the 0.4 mGy/h group was observed. The deformity frequency was significantly increased by prolonged exposure duration at dose rates ≥ 0.4 mGy/h.

The mRNA sequencing at onset of gastrulation (5.5 hpf transcriptome) revealed a dose-response in the numbers of differentially regulated genes (lowest total dose 1.6 mGy). The number of differentially expressed genes showed a dose-response, with 18 (2 down-regulated), 156 (27 down-regulated) and 556 (102 down-regulated) DEGs found in the 0.54, 5.4 and 10.9 mGy/h exposure groups, respectively.

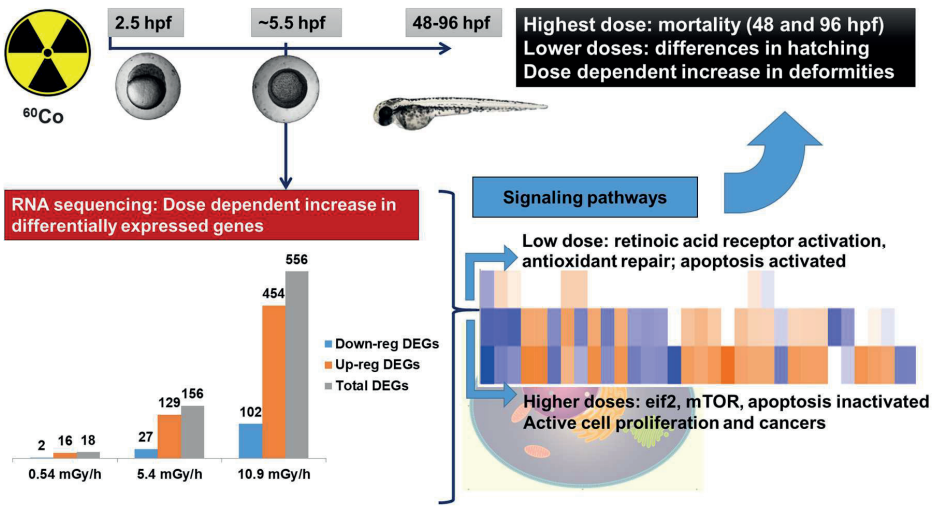

Fig 15. Graphical summary of main study endpoints and results in Paper I.

The most up-regulated gene in the higher doses was found to be transferrin a (*tfa*) and a number of apolipoprotein genes, while 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3 (*pfkfb3*) and *crabp2b* were differentially expressed in all groups. These genes were associated with various developmental processes (Paper I, Fig 6. for details).

The most significant upstream regulators were avian myelocytomatosis viral oncogene homolog (*myc*), tumor protein 53 (*tp53*), tumor necrosis factor alpha (*TNFa*), hepatic nuclear factor 4 alpha (*hnf4a*), transforming growth factor beta 1 (*TGFb1*) and CCAAT enhancer binding protein C/EBP alpha (*cebpa*), while cellular retinoic acid binding protein 2 b (*crabp2b*) and vascular endothelial growth factor alpha, b (*vegfab*) were identified as the most frequent downstream target genes.

In the lower dose, pathway analyses (IPA) identified retinoic acid receptor activation (*RARa*), apoptosis, and glutathione mediated detoxification signaling as the most affected pathways in the lower dose rate (0.54 mGy/h), while in the higher doses, eucariotic initiation factor 2 (*eif2*) and mammalian target of rapamycin (*mTOR*), i.e., genes involved in the modulation of angiogenesis, were most affected (Fig 5 of Paper I for details).

The study linked gene expression changes in the earliest life stage zebrafish embryos to developmental defects of exposure ranging from low to high dose rates of gamma radiation later in life, concluding differences in gene expression after exposure to low and higher doses of ionizing radiation.

3.2 Paper II

Parental gamma irradiation induces reprotoxic effects accompanied by genomic instability in zebrafish (*Danio rerio*) embryos

This study investigated the developmental effects, oxidative stress parameters, bystander effects and eye development in the first generation progeny of parents exposed to gamma radiation. These effects were assessed in F1 of irradiated F0 (creating the G and GE line irradiated embryos) and non-irradiated parental zebrafish during gametogenesis (creating the E line irradiated embryos and controls).

After administration of dose rates of 8.7 mGy/h and 53 mGy/h (27 days; total 5.2 and 31 Gy) to adults, and 9.6 mGy/h to offspring embryos (F1, total 0.62 Gy), 100% mortality occurred in all offspring of parents exposed to 31 Gy. The 8.7 mGy/h and control fish were used for production of embryos in order to create three progeny lines: G line, exposed only during parantel gametogenesis; GE line, exposed during gametogenesis and subsequent embryogenesis to 9.6 mGy/h (3 days) and E-line, exposed only during embryogenesis.

In all parentally exposed groups, levels of mortality and deformities, as well as hatching abnormalities were higher compared to controls, albeit not statistically significant in the G line.

The ROS formation was increased one month after parental irradiation in the G line (Fig 16), but not one year after irradiation, while the opposite was true for the E and GE lines. On the other hand, in both the G and GE lines, there was an increase in DNA damage one year after parental irradiation, while lipid peroxidation was increased only in the G line. Radiation-induced bystander effects and observed influx of Ca^{2+} ions through the cellular membrane of the reporter cells were increased in the G line (Fig 16) one month after irradiation of the parents. One year after parental irradiation, the bystander effects were increased in the E line compared to controls, but not in the progeny of irradiated parents (G and GE lines). Histopathological evaluation showed that irradiation during gametogenesis as well as during embryogenesis caused severe damage to eye development in embryos.

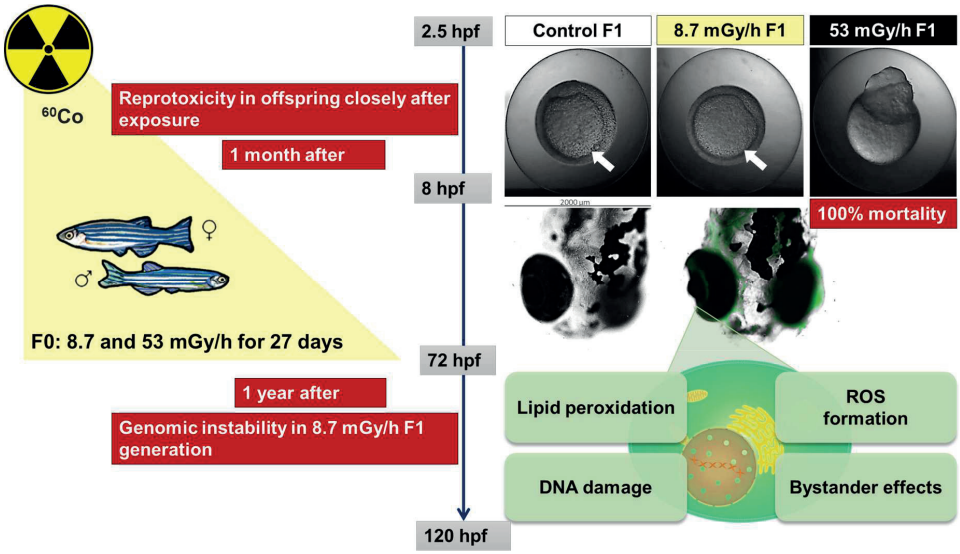

Fig 16. Graphical summary of main study endpoints and results (related to the G line) in Paper II.

The study demonstrated that subchronic exposure of parents ranging from a moderate to high gamma radiation dose rate results in transgenerational oxidative stress, genomic instability and disrupted development in irradiated (GE) and non-irradiated (G) progeny of irradiated parents, including increases in ROS formation, LPO, DNA damage and bystander effects.

3.3 Paper III

Parental exposure to gamma radiation causes progressively altered transcriptomes linked to adverse effects in zebrafish offspring

In this study mRNA sequencing was used to assess short and long-term gene expression changes (5.5 hpf) in offspring of exposed parents to doses mentioned in Section 2.2 (*Gamma irradiation*) both one month after parental irradiation, and one year after exposure (exclusively 8.7 mGy/h), respectively in order to investigate progressive effects of ionizing radiation (Fig 17).

One month after exposure, a global change in gene expression was observed in the 53 mGy/h group (5079 DEGs), which was manifested as embryonic death around 8 hpf, whereas the 8.7 mGy/h group was relatively unaffected (39 DEGs). The 8.7 mGy/h group one year after parental exposure exhibited a number of 2455 DEGs, and had a large overlap of genes and enriched pathways with the 53 mGy/h group one month after parental exposure (Fig 17). The pathways in the 8.7 mGy/h group one year after parental exposure were, however, oppositely regulated compared to the 53 mGy/h group (immediately after parental exposure). A number of differentially expressed histone methylases and demethylases in the 1 year 8.7 mGy/h group suggested a protective response against ionizing radiation via chromatin compactness. The difference in gene expression 1 month and 1 year in the 8.7 mGy/h group indicated a progressive change in gene expression and points to genomic instability in the parental germ line.

Gene pathways could be directly linked to the genotoxic and reproductive effects observed in adults and their offspring and therefore this paper concludes that latent effects following gamma radiation exposure of the parents can be transmitted to offspring. The by IPA predicted as most affected networks, were neurological disorders, malformation of the brain and degeneration of neurons (Paper III, Supplementary material), including pathways related to sex hormone homeostasis. Modulation of genes related to estrogen receptor 1 (*ESR1*), follicle-stimulating hormone (*FSH*), insulin-like growth factor 2 (*IGF2*) and gonadotropin-releasing hormone (*GnRH*) implied a disturbance in early stage embryos, which could be related to gonadal effects in exposed adult fish reported in Paper IV. Finally, five genes were mutually differentially expressed in all exposures, upstream transcription factor 1 like (*usf1l*), fibronectin 1a (*fn1a*), aldehyde dehydrogenase 3 family, member A1 (*aldh3a1*), ribonucleotide reductase subunit M2 (*RRM2*) and cytochrome p450

2x6 (*cyp2x6*) and these genes were connected to effects observed in Paper II (LPO, DNA damage).

Upstream regulators, previously shown to be responsive the direct embryonic exposures in Paper I, such as *tp53* and *hnf4* exhibited a clear correlation between the two exposure groups. Following validation, transferrin a (*tfa*) was found to be up-regulated in all exposures, a gene which was also consistently one of the most up-regulated genes in zebrafish embryos directly exposed to higher doses of gamma radiation (Paper I).

This study concluded that effects of parental exposure to sub-lethal gamma radiation dose rates can be passed on to offspring via aberrant gene expression and are related to pathways linked to observed developmental and reproductive effects. The progressive effect during one year in the lower dose rate in progeny could indicate a latent effect on the population.

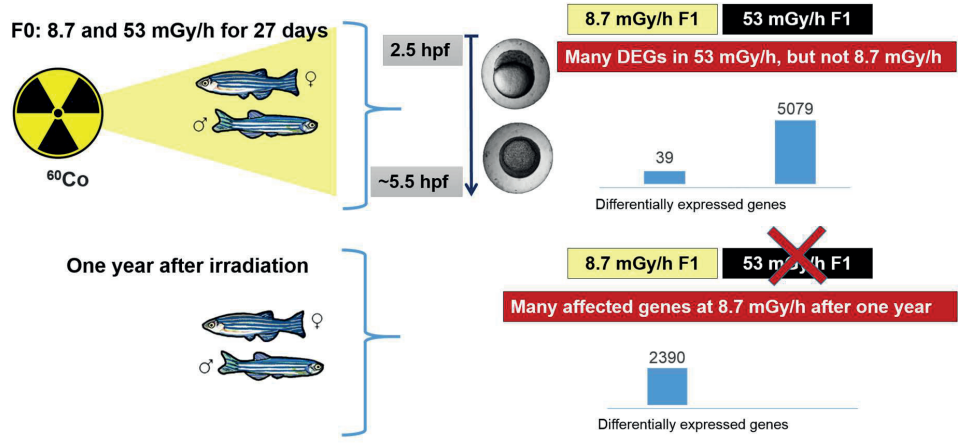

Fig 17. Graphical summary of main study endpoints and results in Paper III.

3.4 Paper IV

Gamma irradiation during gametogenesis causes adverse reproductive effects and sterility in zebrafish

In this study, the reproductive and genotoxic effects following subchronic exposure during the gametogenesis cycle (27 days to previously mentioned doses) were studied in adult zebrafish (Fig 18). Reduced embryo production was observed both one month and one year after exposure and sterility was observed one year post irradiation in the highest dose. A regression of reproductive organs was found in 20 % of the fish in the same group. A significantly lower condition factor, indicating poorer fitness what found in the 8.7 mGy/h group males compared to controls and 53 mGy/h. The ovaries of the fish exposed to 8.7 mGy/h demonstrated a higher number of previtellogenic follicles compared to controls.

Whole blood was used to determine the genotoxic effects of the exposure using the Comet assay, while blood smears were used to assess micronuclei (MN) in erythrocytes. Some differences between the sexes were observed in terms of DNA damage (which was higher in the 8.7 mGy/h males than in males exposed to 53 mGy/h), while the opposite was true for females and in terms of the histopathological examination in the gonads. Significant increase in MN frequency was found in both males and females of the 8.7 and 53 mGy/h groups compared to controls.

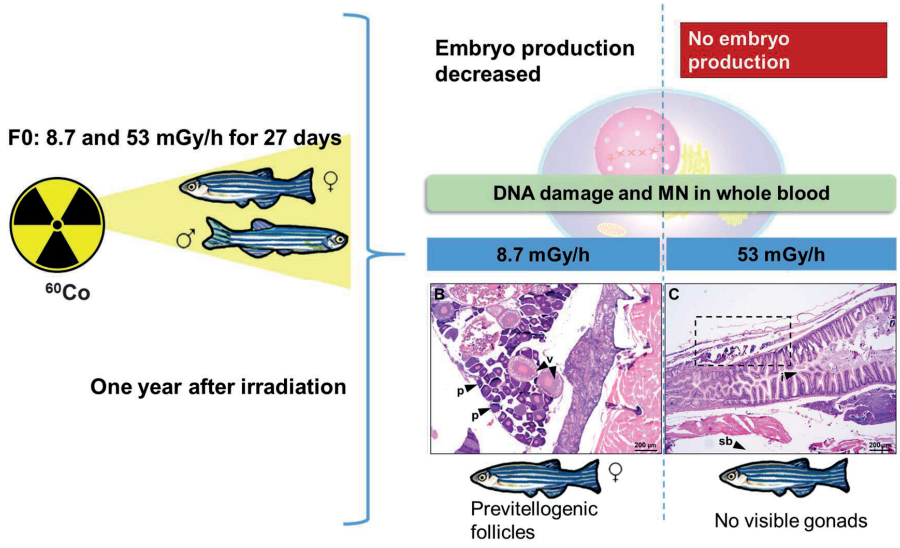

Fig. 18. Graphical summary of main study endpoints and results in Paper IV.

The study showed that subchronic exposure and sub-lethal gamma radiation doses caused adverse reproductive and genotoxic effects in adult zebrafish. Gametogenesis is concluded to be a very sensitive life stage to ionizing radiation exposure, as the adverse effects in the parental germ line were linked to effects observed in the first generation of offspring (Papers II and III).

4. Discussion

This project studied the developmental, reproductive, oxidative stress-related, genotoxic and gene expression related ionizing radiation exposure effects after irradiation of two subsequent generations during gametogenesis and embryogenesis.

Various methods for analyzing biological effects of radiation using zebrafish as model have already been validated, such as ROS or DNA damage analysis in both embryos and adults (Jarvis and Knowles, 2003; Lemos et al., 2017, Gagnaire et al., 2015; McAleer et al., 2005), bystander effects (Mothersill et al., 2007; Ryan et al., 2008, Choi et al., 2013, Pereira et al., 2014), but also genetic (Jaafar et al., 2013; Freeman et al., 2014) and epigenetic effects (Andersen et al., 2012; Andersen et al., 2013). With only a few exceptions, most of the mentioned studies used acute ionzing radiation doses and only one life stage of fish, therefore warranting studies with chronic low dose exposures and in successive generations.

The lack of data on the heritable radiation effects and delayed effects following parental exposure highlights the importance of this study. By using both adults and embryos after a continuous subchronic exposure, this thesis aims to give further insights into the population dynamics after radiation exposure during sensitive life stages. Thus, in order to find the missing links in the transmission of effects from parents to offspring, gamma radiation effects were assessed in: a) embryos after embryo exposure alone starting from 2.5 hpf; b) embryos after parental exposure during gametogenesis including or excluding offspring (embryogenesis) exposure starting from 2.5 hpf; and finally c) adult (parental) fish after gametogenesis exposure. The exposure regimes and main observations in from the three mentioned timepoints of assessment are summarized in Table 3.

Table 3. Dose rates, total doses and resulting effects of the two-generational study.

Paper	Exposure period	Dose rate (mGy/h)	Exposure duration (h)	Total dose (Gy)	Studied life stage	Studied endpoint	Observed effect(s)
I	Embryogenesis		43.8	0.017	embryos		Premature hatching
		0.4	92	0.04	larvae		No adverse effect observed
			43.8	0.17	embryos		No adverse effect observed
		3.9	92	0.36	larvae	Survival and development	Deformities
II III		9.6	65	0.62	embryos and early larvae (E line)	Survival and development; ROS; LPO; DNA damage; Bystander effects 1 year after parental exposure; 5.5 hpf gene expression; Eye development	-ROS increased immediately after irradiation; -Bystander effects increased -Impaired eye development
I			43.8	0.66	embryos		Deformities
		15	92	1.38	larvae		Lower total hatching;deformities
			43.8	1.67	embryos		Decreased survival; delayed hatching; deformities
		38	92	3.46	larvae	Survival and development	Decreased survival; delayed hatching; deformities
I		0.54		0.002	embryos		
		5.4		0.02	embryos	5.5 hpf gene expression	signicant increase in DEGs
		10.9	3	0.03	embryos		
I V	Gametogenesis	8.7	591.5	5.2	adult fish	Reproduction; Gonad histopathology; DNA damage; MN frequency in erythrocytes	-Reduced embry production 1 month after irradiation, but not 1 year after; -Reduced number of vitellogenic follicles in females; -Increased DNA damage in males and females; -Increased MN frequency in males and females.

IV			53	591.5	31	adult fish	Reproduction; Gonad histopathology; DNA damage; MN frequency in erythrocytes	-Lower embryo production 1 month after irradiation with 100% mortality in offspring; -Sterility 1 year after exposure; -No visible reproductive organs 1 year after irradiation; -Increased DNA damage in males and females; -Increased MN frequency in males and females.
II III		8.7	591.5	5.2		embryos and early larvae (G line)	Survival and development; ROS; LPO; DNA damage; Bystander effects; 5.5 hpf gene expression; Eye development	-Premature hatching; lower total hatching; Deformities -ROS increased one month after parental irradiation -DNA damage and LPO increased 1 year after parental irradiation -Bystander effects increased 1 month after parental irradiation; -Significant increase in DEGs -Impaired eye development
II III		53	591.5	31	embryo		Survival and development 5.5 hpf gene expression;	-100% mortality at 8 hpf. - significant increase in DEGs
II	Gametogenesis & embryogenesis	8.7 and 9.6	65	5.82		embryos and early larvae (GE line)	Survival and development; ROS; LPO; DNA damage; Bystander effects 1 year after arental exposure; Eye development	-Decreased survival; premature hatching; deformities -ROS increased immediately after irradiation; -Severely impaired eye development

4.1 The project in an environmental perspective

The gamma radiation doses used in this project for adult fish exposures exceed ionizing radiation levels recently observed in the environment around nuclear incident sites. As example, the highest reported radiological dose rate following the Fukushima accident to fish was 3.1 mGy/d in the greenling (*Hexagrammos Otakii*) from accumulation of ^{134,137}Cs (Johansen et al., 2015). The maximal dose rate for fish within the first month of the accident was estimated to 140 μGy/h, while terrestrial doses ranged from 10 – 300 μGy/h (Strand et al., 2014). The dose rates to adult fish in our study were also higher than the defined low dose rate of 6 mGy/h for a maximum of 1 hour (UNSCEAR, 2012).

Generally, the observed effects from adult fish exposures in offspring have shown that the used radiation doses are reprotoxic and genotoxic to zebrafish and are relevant for short-term exposure to high doses in accidents. This data, however, enables studies of the mechanistic background of heritable effects of radiation and shows how this type of exposure could affect future generations and the population.

In ecotoxicological research and risk assessment, molecular interactions and biomarker responses are often used as an early signal of environmental stress. In order to identify hazards, molecular data needs to reflect endpoints meaningful to ecological risk-effects, such as survival, development, and reproduction in individual organisms and populations. Therefore, the concept of adverse outcome pathways (AOPs) was developed and is a framework for organizing knowledge about the progression of toxicity events across scales of biological organization that lead to adverse outcomes relevant for risk assessment (OECD, 2016) (Ankley et al., 2010, Lee et al., 2015). The data obtained in this PhD project can be used for such a framework.

In Paper I, the lowest dose rate (0.4 mGy/h, Table 3) corresponded to recommended benchmarks (0.42 mGy/h, 2-10 mGy/day) at which adverse effects are not expected to occur in aquatic ecosystems (UNSCEAR 1996) and the derived consideration reference levels (DCRL) for fish (~0.42 mGy/h – 40 mGy/h), at which there are "likely to be some observable adverse effects occurring to individuals" (ICRP, 2012). In this group, some deleterious effects were found (premature hatching), supporting the DCRL in this dose rate range.

For exposures reported in Papers II, III and IV, the parental doses (8.7 mGy/h, total 5.2 Gy) and offspring doses (9.6 mGy/h, 0.62 Gy total) span the upper range of the DCRLs

for fish. Particularly interesting are the reproductive effects mentioned in Section *1.5 Risk assessment and radiation protection* (Table 2) where the dose rate reported to reduce the number of spermatogonia in male fish encompasses the doses that were used in this study. However, no exposure related effects were observed in male gonads of the 8.7 mGy/h exposure group in Paper IV, while effects were observed in female gonads in terms of increased previtellogenic oocytes, and correspondingly a reduction in the number of vitellogenic follicles. On the other hand, the higher dose rate used for adult fish (53 mGy/h, 31 Gy total) supports the mentioned dose effects in Table 2. Furthermore, all exposure dose rates for evaluating the phenotypically observable effects as well as subcellular effects were higher than the ERICA screening value of 10 µGy/h (0.24 mGy/d) (Garnier-Laplace et al., 2010). The doses and dose rates used for adult fish exposures are an order of magnitude higher than the levels (10 mGy/day; 0.24 mGy/h) described as not likely to have any detrimental effect to aquatic populations (UNSCEAR, 1996), and also three orders of magnitude higher than the suggested ERICA screening value of 10 µGy/h (predicted no effect dose rate, PNEDR for ecological effects) (Garnier-Laplace et al., 2010). The total dose from the adult fish exposure, 31 Gy (Paper II and III and IV) is close to the acute gamma radiation exposure dose of 40 Gy, which is reported to being the minimum lethal dose (MLD) for zebrafish resulting in death after 14 days, while 20 Gy was reported to be the sub-lethal dose (Traver et al., 2004).

Considering that no mortality was observed in the adult fish 1.5 year after irradiation, even at a dose of 53 mGy/h, the applied dose rates in these exposures are not likely to be life threatening for adult individuals, although the reduction in viable offspring could be detrimental to sustainability at the population levels.

4.2 The 5.5 hpf stage gene expression after embryogenesis and gametogenesis exposure

To determine the (whole transcriptome) difference in gene expression between the controls and exposed embryos or offspring of exposed parents, RNA-seq was conducted. The transcriptome analysis allowed registration of changes in gene expression related to various biological processes and revealed the regulation of radiotoxicity in the earliest stages of development from low dose gamma radiation exposure of 3 hours. Gene expression analysis was performed in both directly (Paper I) and parentally (Paper III)

exposed embryos, at the late blastula / early gastrula stage (~ 5.5 hpf). This is, as previously mentioned in *Section 1.7*, a critical stage of embryogenesis, characterized by intensive cell proliferation and specification and changes in transcriptome profile can be attributed to radiation effects on the transcriptional program of the embryo's own genome (Fig 5), while the inherited maternal transcript (synthesized during oogenesis and stored in the egg) is degraded.

4.2.1 Differentially expressed genes

During embryogenesis (Paper I), the embryos were irradiated at doses of 0.54, 5.4 and 10.9 mGy/h (approximate total 1.6, 16 and 33 mGy), while during gametogenesis (Paper III) parents were irradiated to 8.7 ad 53 mGy/h (total 5.2 and 31 Gy), which is three orders of magnitude higher. From here, it may be indicated that some hereditary effects might have played a large role in the number of expressed genes, which showed an overlap of 27.8 % (74 out of 268 DEGs, FC > 1.5, FDR < 0.05) between both studies. Considering that in zebrafish exposed during embryogenesis or at 16 weeks post fertilization (wpf) to short term radiation (0.1 and 1 Gy total dose) only a minimal overlap (<5%) was discovered in liver cells (Jaafar et al., 2013), this confirms that transcriptome analysis at the same developmental stage is essential for detecting similarities or differences between embryogenesis and gametogenesis exposure.

In Paper I, 18 (2 down-regulated), 156 (27 down-regulated) and 556 (102 down-regulated) DEGs were reported, respectively, in embryos exposed to increasing doses of gamma radiation (0.54, 5.4 and 10.9 mGy/h) from 2.5 to 5.5 hpf. On the other hand, 39 (19 down-regulated, 49.0%) and 5079 (2207 down-regulated, 43.5%) genes, for 8.7 and 53 mGy/h offspring of parents irradiated during gametogenesis were found immediately after irradiation of parents, while 2390 (1617 down-regulated, 67.7%) genes were found in the 8.7 mGy/h offspring 1 year after parental exposure.

Two genes (Paper I), *pfkfb3*, involved in promoting proliferation and survival in tumor cells and counteracting ionizing radiation generated ROS (Yalcin et al., 2009) and *crabp2b*, known to encode retinoic acid (a form of Vitamin A) binding protein 2b (Sharma et al., 2005) were found to be differentially expressed in all exposures in Paper I. This showed that exposure during embryogenesis might have triggered antioxidant mediated defense in response to radiation induced ROS formation, which resulted in no phenotypically observable adverse effects in the lowest dose. Further substantiating this could be the increased ROS formation observed in the embryogenesis group in Paper II.

In Paper III, 5 genes were mutually differentially expressed in all exposures: thy-1 cell surface antigen (*thy1*), fibronectin 1a (*fn1a*), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2 (*cited2*), ribonucleotide reductase subunit M2 (*RRM2*) and cytochrome p450 2x6 (*cyp2x6*). *Fn1a* and *RRM2* are genes involved in disintegration of cell structure (Pankov and Yamada, 2002) and DNA DSB repair in mammals (Niida et al., 2010), respectively. Modulation in these genes was connected to the effects observed in the offspring of parents irradiated during gametogenesis, as a mortality of 100% and increased DNA damage was also found in the 53 mGy/h offspring (Paper II).

Among the common genes modulated by 5.4 and 10.9 mGy/h exposures, the most significantly up-regulated gene is *tfa* (Paper I), and interestingly, the same gene was up-regulated in all gametogenesis exposure offspring groups (Paper III). This gene is critical for iron transport and iron regulated hormone expression (Fraenkel et al., 2009), and was altered in blood plasma of locally irradiated patients (Nylund et al., 2014). Additionally, highly up-regulated apolipoprotein genes in the two higher dose rate (5.4 and 10.9 mGy/h) embryogenesis exposure groups (*apoBb, apoA1a, apoA1b* and *apoA-IV*) were found and point to radiation affecting mechanisms related to lipid metabolism (Otis et al., 2015). Since we observed earlier hatching in the lower doses in Paper I and in the embryogenesis group in Paper II, and that hatching is attributed to higher metabolism (Gagnaire et al. 2015), it could be speculated that these genes might be a regulatory component of hatching in zebrafish.

Because the analysis of gene expression in embryos from parents exposed to 8.7 mGy/h was performed both one month and one year after exposure, we were able to show a temporal gene expression change in this group following the second principle component analysis (PCA), corresponding to aging of the parents. The temporal change in gene expression could be attributed to genomic instability in the parental germ line, and transmitted to progeny as has been previously suggested (Merrifield & Kovalchuk 2013), which was supported by results reported in Paper II.

A large overlap in DEGs between the 8.7 mGy/h group one year after parental exposure compared to the 53 mGy/h group one month after parental exposure was found (1514 genes, 63.3 % of all genes from the first group), however, the genes were found to be oppositely regulated and predominantly down-regulated in the 8.7 mGy/h group. This could potentially be caused by epigenetic changes and the hypothesis of a general induction in chromatin compactness supported by the histone methyltransferase DEGs discussed in

Paper III, and in turn could be hypothesized to be a protective function against ionizing radiation.

The results of the differential gene expression analyses (Paper I and III) provide valuable information regarding sensitive genetic biomarker endpoints for low to medium radiation dose exposures and AOPs for risk assessment.

4.2.2 Upstream regulators and molecular pathways

Two similar significant upstream regulators in both embryogenesis and gametogenesis exposed groups were found: *tp53* and *hnf4a*, which were previously associated with radiation exposure. *Tp53* is known to regulate apoptosis in response to DNA damage, (Meek et al., 2009) and is a critical factor for normal development and survival in zebrafish embryos after exposure to ionizing radiation (Duffy et al., 2007; Guo et al., 2012). *Hnf4a* is known to be a nuclear receptor involved in a variety of processes, and although it was not associated with radiation in zebrafish previously, it was found to be up-regulated in the blood of patients exposed to ionizing radiation (Savli et al., 2012) and was most prominent in a response to low dose in a human tissue model (Mezentsev and Amudsen, 2011).

The signaling pathways affected most significantly by exposure to 0.54 mGy/h during embryogenesis was *RARa* activation, which is involved in antioxidant mediated detoxification of ROS, DNA repair, apoptosis and immune functions (Feinendegen et al, 2004; Feinendegen, 2007). Interestingly, the same pathway was observed in response to gametogenesis exposure. This could mean that, considering that DNA damage was observed in the parents exposed during gametogenesis (Paper IV) and their offspring (Paper II), some reparatory mechanisms might have been transmitted from the parents to the offspring.

Additionally, in offspring of parents exposed during gametogenesis, modulation of pathways related to reproduction hormones were found, such as estrogen receptor 1 (*ESR1*), follicle-stimulating hormone (*FSH*) signaling, insulin-like growth factor 2 (*IGF2*) and gonadotropin-releasing hormone (*GnRH*) signaling, which suggests a disturbance in offspring that could subsequently adversely affect the formation of the gonads later in life. Considering that differences in maturation of oocytes were observed in offspring of exposed parents, it could be speculated, whether the modulation of these genes might be a maternally transmitted effect.

4.3 Development after gamma irradiation in embryogenesis and gametogenesis

To determine adverse effects of gamma radiation and potential differences in sensitivity between various life stages, we exposed zebrafish during gametogenesis, embryogenesis, and during both stages. The developmental traits such as survival, deformities and hatching in the offspring of parents exposed during gametogenesis (27 days parental exposure) or in offspring exposed during embryogenesis (3 hpf to 4 dpf) without parental exposure were assessed in Paper I and II. The adverse effects on embryonic development after the continuous exposure during either gametogenesis or embryogenesis showed significant differences in responses, which are in line with the hypothesis that exposures to stressors such as ionizing radiation affect the physiological responses differentially depending on the life stage in which exposure occurs.

Following embryogenesis exposure, using low to high doses and dose rates (0.4 – 38 mGy/h, total 17.5 - 3496 mGy) in Paper I, embryo mortality was observed at 48 hpf, in the group exposed to the highest dose rate (38 mGy/h), with no significant further increase from 48 – 96 hpf. The lowest dose in the embryo exposures (0.4 mGy/h) corresponds doses (1-10 mGy/day) observed in the Chernobyl cooling pond from 1-60 days after the accident (UNSCEAR, 2008). This was also in line with the dose response study by Geiger et al. (2006), who determined the sensitivity of zebrafish to gamma radiation, albeit to higher doses (5-20 Gy), whereby exposure of the earlier life stages showed a larger effect size.

The exposure of parental fish during gametogenesis resulted in increased mortality for offspring of both the 8.7 mGy/h and 53 mGy/h (5.2 and 31 Gy) fish groups, as compared to control offspring. The offspring of parents exposed to 53 mGy/h showed 100% mortality at the gastrulation stage, between the onset and 75 % of epiboly (8 hpf), which is hypothesized to be a maternally encoded mechanism (Strähle and Jesuthasan, 1993). The stage at which mortality occurred corresponds to the movement and spreading of the blastoderm cells in order to cover the yolk and form a closure known as the blastopore and requires a coordination of microtubules, which contract with the cell movements (Kimmel et al., 1995; Warga and Kimmel, 1990). This suggests that parental irradiation caused damage to the microtubule system. In the offspring of the 8.7 mGy/h exposure group, mortality was increased only in the GE group, i.e. embryos that were also directly irradiated (9.6 mGy/h) following parental exposure, and amounted to 9.3%. Increased and dose-dependent embryonic mortality and deformities after parental exposure was previously

reported in other vertebrates (Nefyodova and Nefyodov, 2000), but not in fish. Mortality in directly irradiated zebrafish embryos was reported only for acute exposures from 1 to 24 hpf (1-10 Gy, X-rays) (McAleer et al., 2005) and 5 Gy (Geiger et al., 2006). However, no significant differences in embryo viability were found after receiving acute ionizing radiation doses ranging from 1 - 10 Gy (Freeman et al, 2014; Bladen et al, 2007), nor following continuous exposures up to 24 mGy/h (2280 mGy) (Gagnaire et al., 2015).

The lowest dose rate at which deformities were observed following direct embryogenesis exposure in the present study was 0.4 mGy/h during 96 hours (total dose 36.8 mGy), and we observed a dose dependent increase in deformities as reported in Paper I, with the highest deformities (11.6 %) in the group exposed to the 38 mGy/h dose rate. Nonetheless, our results from the two generational study reported in Paper II showed a significant increase in the parentally exposed offspring, i.e. G line, and the group exposed parentally including during embrygenesis, i.e. GE line (of 5.9 and 5.2%, respectively), while the embryogenesis exposed group showed no significant deformities (9.6 mGy/h, 620 mGy total). The reason for this difference between experiments is unknown. Yet, in line with the results presented in Paper I, multiple deformities were also found in a previous study after embryonic exposures to 24 mGy/h (2280 mGy) (Gagnaire et al., 2015).

Effects on eye development were found in both the embryogenesis and gametogenesis groups reported in Paper II. Eyes in zebrafish normally develop at 48 hpf (Geiger et al., 2006) and are functional within 3 days post fertilization (73-80 hpf) (Dahm et al., 2007; Jonasova and Kozmik, 2008). The difference in severity of the histological changes in the development of the eyes in directly and indirectly exposed embryos suggests direct damage to the exposed cells and a dependence on the exposure dose. It was previously reported that the eye diameter and head length decreased in embryos exposed to acute gamma radiation (10 Gy) (Freeman et al., 2014), which is two times higher than the parental dose for the fish lines (5.2 Gy).

The effects of embryogenesis and gametogenesis exposure on the total hatching at 96 hpf and the median hatching time varied dose-dependently (including the parental dose). As reported in Paper I and II, the onset of hatching in the groups irradiated during embryogenesis was premature in the 0.4 mGy/h group (17.5 mGy), accelerated during the entire hatching interval in the 9.6 mGy/h group (total 620 mGy/h), while it was significantly delayed in the 38 mGy/h group (1664 mGy total). Additionally, the total hatching in all embryogenesis groups, except for 15 mGy/h (657 mGy total), was unaffected. Similar results were reported following X-ray exposure during the blastula stage, earlier hatching

was associated with low dose (25 mGy at 0.43 Gy/min), while higher doses (250-500 mGy) delayed the onset of hatching, while exposure to 500 mGy reduced the total hatching (Miyachi et al., 2003).

In progeny of parents irradiated during gametogenesis (G-line), the hatching rate decreased after approaching 72 hpf, and subsequently, the total hatching was significantly decreased in these groups compared to controls (about 15 %). However, the calculated median hatching time (HT_{50}) has shown a decrease, and hatching was accelerated in all exposed groups compared to controls (Paper II). In addition, other studies suggest that both low and high doses had an accelerating effect on the hatching interval (Gagnaire et al., 2015; Pereira et al., 2014), partially supporting the results obtained in this study, since this was hypothesized to be a consequence of increase in global metabolism rates of the larva and earlier energy reserve consumption.

4.4 Genomic instability after embryogenesis and gametogenesis exposure

The developmental defects in offspring from parental irradiation and embryogenesis irradiation could have further implications at the cellular and metabolic level, particularly by ROS production and consequent oxidative stress effects. In order to determine the potential differences in oxidative stress parameters in directly and indirectly (parentally) exposed embryos, ROS formation, LPO and DNA damage were assessed one month and one year after irradiation of parents and immediately after irradiation of embryos in E, G, GE lines and controls as reported in Paper II. In addition, bystander effects were assessed in offspring one month and one year after irradiation of parents (Paper II).

4.4.1 ROS formation and LPO

The results obtained showed an increase in ROS formation, which depended on the dose (including parental exposure) and time of assessment after parental irradiation. In general, parental irradiation caused elevated ROS in G line one month post-parental irradiation. The mechanism behind this result cannot be attributed to radiolysis, but could be attributed to disrupted ROS metabolism in the embryos, caused by the parental exposure, which has been documented for cancer cells following radiation exposure (Kargalioglu et al., 2002). Consistently, the groups exposed during gametogenesis plus embryogenesis (GE) and embryogenesis (E) demonstrated an increase in ROS in embryos one year after

exposure of parents and immediately after exposure of embryos, suggesting that direct exposure during early life stages (embryo, early larval stage) induce irreversible changes in the ROS metabolism. However, the exact mechanism behind the disruption of ROS metabolism in embryos after parental exposure is still unknown. We speculate that elevated ROS might affect embryonic development at multiple levels, such as energy metabolism (mitochondria respiration), signaling (e.g. cell cycle, DNA repair, cell death) and ultimately cause oxidative damage. The ROS levels in the G group subsided one year after irradiation, suggesting that compensatory mechanisms might have been induced in the exposed parents.

This notion was substantiated by LPO measurements, known to be a sensitive marker of oxidative damage (Ayala et al., 2014), that showed a significant increase even one year after irradiation in the G group, while a decrease was observed in the GE and E groups, possibly attributing this to an adaptive response via antioxidant enzymatic system. This was previously shown in response to gamma radiation in zebrafish embryos exposed to a dose range from 0.1 to 1 Gy (Hu et al., 2016).

4.4.2. DNA damage and bystander effects

Gamma radiation exposure to high doses is known to result in cellular DNA damage, which, if unrepaired, can cause genetic alterations and lead to the development of cancer, whereby chronic versus acute oxidative stress may contribute to the development and/or maintenance of genomic instability (Limoli and Giedzinski, 2003). Studies with acute exposures report increased DNA damage in 5-6 dpf zebrafish larva, directly exposed to low dose gamma radiation (30 mGy) (Jarvis and Knowles, 2003) and in 24 and 48 hpf embryos after irradiation to 1-1000 mGy/d (Simon et al., 2011). The lowest dose rate in a chronic exposure of 20 days for a significant increase in DNA damage was 1 mGy/d for zebrafish embryos at 24 or 48 hpf (Simon et al., 2011). However, some studies using chronic exposures report DNA damage in directly irradiated embryos (570 mGy/d) compared to controls at 96 hpf (Gagnaire et al., 2015). As reported in Paper II, increased DNA damage levels were elevated in the G and GE line embryos one year after parental gamma irradiation (8.7 mGy/h) with 12.1 and 8.8 % tail DNA, respectively and could represent significant genomic instability in viable progeny, which showed no visible developmental defects. This is substantiated by the detected changes in gene expression associated with genomic instability as reported in Paper III, previously discussed in Section 4.2.

Bystander effects reflect the ability of irradiated cells to transmit stress signals, causing measurable radiation response signals (Ca flux) and effects, such as chromosomal breakage, sister chromatid exchange, gene mutations, apoptosis, and malignant transformations in unexposed neighboring cells (Streffer et al., 2004). The bystander effects reported in Paper II showed an increase in bystander effects in the G line one month after parental exposure, while one year after parental exposure, these were increased only in the directly exposed embryogenesis line. It appears that parental irradiation during gametogenesis can reduce the level of the calcium flux from progeny irradiated one year after parental exposure, compared to embryos which are exposed during embryogenesis only. This result also supports the notion that effects in cells of irradiated embryos and parents persist after irradiation, and that mechanisms behind the observed delayed effects seen in ROS, LPO and DNA damage could be different to those arising following direct irradiation. Epigenetic and non-targeted mechanisms (genomic instability, bystander effects, adaptive response) were found to predominate after low and chronic exposures and the results support other studies showing impacts of parental irradiation on non- and irradiated offspring (Parisot et al., 2015).

It has previously been shown that radiation causes inflammatory effects (Candéias et al., 2004; UNSCEAR, 2006b; Kusunoki and Hayashi, 2008) and modulates immune response, which is associated with increased ROS formation (Hekim et al., 2015), which in turn induces bystander effects and genomic instability (Georgakilas et al., 2015). The results presented in Paper II agree with these reported effects and show that parental radiological stress during gametogenesis leads to genomic instability as demonstrated by the compromised DNA integrity as well as increased LPO and bystander effects in irradiated and non-irradiated progeny. Elevated DNA damage was previously reported in non-irradiated progeny assessed immediately after parental exposure in ZF embryos, as comparable with the radiation dose (X-rays) of the parents (Lemos et al., 2017). The persistence of DNA damage in embryos of parents irradiated during gametogenesis point to the involvement of non-targeted mechanisms such as inflammation and bystander effects in addition to the established direct DNA damage following irradiation. Assuming that adverse effects in embryos are caused by the dose rate as well as the accumulated dose, it is necessary to mention that the dose rate administered to the fish embryos during embryogenesis is about one order of magnitude lower than the cumulative dose of the parents exposed during gametogenesis. Consequently, in the embryogenesis exposure groups (Papers I and II), only an immediate increase in ROS formation was observed, while

no DNA damage and bystander effects were detected, suggesting that the total dose in these exposures was too low. Another explanation may be that the sensitivity to radiation is higher during gametogenesis compared to embryogenesis thereby causing more severe effects (DNA damage).

We mention in Paper I that the relationship between the dose and survival of single cells constituting the embryo is generally expected to be proportional to the dose. Consequently, we speculate that oxidative stress and genomic instability are dependent on the type of DNA damage occurring, and are not expected to occur at low dose rates, because e.g. DNA SSBs can be repaired rapidly before another break has time to occur in order for the DNA structure to collapse (Chadwick and Leenhouts, 2005). Consistently, no significant increase was detected in the E line compared to controls (Paper II) and the lack of detectable increase of DNA damage in this group may be due to the lower total exposure dose of the embryos (0.62 Gy).

4.5 Effects in adult zebrafish after gametogenesis exposure

Separately from embryonic exposures and effects, the radiation exposure effects were also assessed in young adult zebrafish in order to elucidate the missing links involved in the transmission of effects from the parents to offspring. Thus, reproduction including overall fish health and genotoxicity (DNA damage and MN) were assessed after exposure during gametogenesis to doses mentioned in Table 3 and *Section 2.2*.

According to international reviews, the reproductive capacity in fish can be reduced at 1-10 mGy/d, which corresponds to 0.04 - 0.4 mGy/h (ICRP 2012), while X-ray doses as low as 0.3 Gy can impair gametogenesis in fish, with a 50 % reduction in germ cell (spermatogonia) production (UNSCEAR 2008). It has previously been discovered that differentiating spermatogonia are very sensitive to radiation and that, after exposure to a dose of 1 Gy, both their numbers and those of their daughter cells (spermatocytes) are severely reduced (Ash, 1980; Clifton and Brenner, 1983). High doses of radiation can result in permanent azoospermia, possibly due to the death of all the spermatogonial stem cells (Brauner et al, 1983; Leiper et al. 1983). In an acute exposure experiment with zebrafish, doses of 10, 20 and 40 Gy caused a decrease of spermatogonia in the testis, but the precursor cells regenerated to control levels in 20 days in the lower doses (Traver et al., 2004). However, there is still a paucity of information in chronic exposure scenarios and,

especially, lower dose rates of ionizing radiation that encompass the entire gametogenesis cycle.

Although reproduction studies in zebrafish following low or high doses ionizing radiation are scarce, studies in other fish species have indicated possible adverse outcomes of exposure to either lower doses or chronic exposures. For example, in medaka (*Oryzias latipes*), a reduction in egg number, egg viability, and hatchability was observed at a dose rate of 350 mGy/day (14.5 mGy/h), following 28 days exposure (Hinton et al. 2004). Effects on the maturation of oocytes were previously reported in adult loach (*Misgurnus anguillicaudatus*) (at 10 Gy, X-rays), which is approximately two times higher than the dose used by us (Egami and Aoki, 1966). Furthermore, lifetime fecundity was decreased in guppies (*Poecilia reticulata*) exposed to dose rates of 40, 96 and 305 mGy/d (1.6, 4 and 12.7 mGy/h) for 920 days (Woodhead 1977). Reduced fecundity and fertility were also reported after gamma irradiation of medaka eggs with an even higher dose rate of 8.7 Gy/day (362.5 mGy/h) (Hyodo-Taguchi and Etoh, 1986), while only temporary sterility was previously reported to be induced in medaka after 5 and 10 Gy gamma irradiation (Michibata et al, 1976). In addition, a dose of 4.7 Gy gamma radiation, which is close to the total dose used here, has been reported to cause accelerated spermatogenesis in fish (Kuwahara et al., 2003), however we have no data to support these findings.

In this study, reproduction was severely impaired in adult zebrafish of the 53 mGy/h (total 31 Gy) exposure group, and embryos of this group showed a 100 % mortality at the gastrulation stage (8 hpf) as reported in Paper II. Even though the fish produced non-viable embryos for several weeks, we found a complete regression of the gonads one year after exposure (Paper IV). These results indicate that exposed individuals should be followed up for extended periods of time in order to address potential latent or adaptive effects of episodes of radiation exposure.

The DNA damage in erythrocytes of adult zebrafish has previously only been reported after acute exposures to ionizing radiation (0.1-1 Gy, X-rays) (Lemos et al., 2017) and after exposure to 20 and 250 µg U/L for 20 days (Bourrachot et al., 2014). There are no previous data on MN formation in adult zebrafish following ionizing radiation, but the method has been used in other fish studies to assess DNA damage in blood cells after ionizing radiation (Russo et al., 2003, Pavlica et al., 2011; Song et al., 2012; Luzhna et al., 2013). As reported in Paper IV, gamma radiation caused a significant increase in DNA damage and micronuclei for both female and male zebrafish. Considering that these effects persisted up to one year after irradiation, the approach was validated.

4.6 Limitations and strengths of the study

The study was performed using approved test guidelines, and represents a controlled laboratory study. However, a number of limiting factors and methodological restrictions remained, and should be mentioned:

(a) It is challenging to extrapolate the data from this kind of controlled laboratory exposure to gamma radiation in the fish populations in the wild, because of different doses, but also due to keeping confounding factors under laboratory conditions at the minimum level.

(b) The total doses used in the study for observations of the developmental effects in embryos and reproduction in adults exceed the environmentally relevant levels, with exception of the lowest doses used for embryonic exposures. Limited time and other resources, as well as the laboratory settings, did not allow for testing a broader span of higher and lower dose rates in adult fish and embryos.

(c) There was a low level of replication for the adult fish exposures. Initial exposures used only one 9 L aquarium per treatment group (0, 5.2, and 31 Gy) and each contained 60 adult fish (30 males and 30 females). This raises the inquiry for occurrence of a potential aquarium to aquarium based variation in adult fish and subsequently the offspring. The width and height of the gamma radiation beam disabled the use of another setup. Efforts were however made to minimize a potential tank to tank effect, by keeping the fish in the same water system. Secondly, fish were randomly selected for each treatment and a setup that satisfies the NMBU Zebrafish facility SOP (approved by AAALAC) concerning fish density for the water volume used. Continuously throughout experiments, water quality parameters such as pH, temperature and conductivity were recorded daily, while nitrogen compounds (NH_3, NH_4^+, NO_2^- and NO_3^-) were recorded daily during the first 10 days, and twice a week for the remainder of the experiment in each aquarium. The measurements revealed no difference between aquaria, thus the variation was assumed to be negligible. Detailed dosimetry ensured the least varying conditions to fish in each treatment in terms of the radiation dose rate.

(d) The detailed consequences of exposure on embryogenesis could have been revealed by recording the early development in a 24-hour kinetic constant-record experiment in the imaging multi-mode reader (Cytation 3, Biotek, U.S.), nonetheless, this was not feasible

simultaneously with irradiation of embryos without greatly interrupting the exposure in the dose-response study (Paper I) or for F1 embryos (Paper II).

(e) It should be mentioned that analysis of the gene expression somewhat differed between studies. As reported in Paper I, the sequences were mapped to ZF genome (version Zv9, release 76), while in Paper III, the sequences were mapped to the ZF genome (GRCz10, www.ensembl.org). Other minor differences in gene expression analysis between studies are described in Sections *2.6.1*, *2.7.1* and *2.7.2*. However, it is accepted that both analyses are mutually comparable for describing differences in effect.

(f) The gene network and pathway analyses were compared to human orthologs and literature. While this might, on one hand, be beneficial for comparison to studies in human genes, where data is available, on the other hand, in terms of effects in fish, it might have contributed to loss of data and gene functions.

As a strength of the study, worth mentioning is that the length of the experiments enabled the assessment of latent radiation induced effects in both adults and embryos. The findings provided add new information regarding the radiosensitivity of different developmental stages of fish, but also on the heritable effects of ionizing radiation from parents to offspring. The exposure effects range from absence of effect towards the molecular, macroscopic and population level effect. Considering the length of the two-generational experiment, in order to account for environmental changes over a one year period and aging-related effects in adult fish, for analyses in embryos, age-appropriate controls were used at each time point.

Since phenotypic effects in the progeny of irradiated parents were observed, this would take in account potential genetic mutations occurring in the next generations (F2 onwards) and facilitate the follow up of potential transgenerational effects. Therefore, genome wide mutagenesis screening is warranted for further studies.

The exposed and non-exposed offspring of irradiated parental fish in this study were mated to F2 generation before euthanasia took place. DNA-methylation, histone modifications and miRNA analyses in this generation are subject of a couple other PhD projects at the Veterinary Faculty of the Norwegian University of Life Sciences and will provide insight into potential transgenerational effects of ionizing radiation from exposure of the F0 germ line.

5. Conclusions

The present PhD study used transcriptomics and oxidative stress analytical methods to analyze the effects of gamma radiation exposure in two generations of fish and two groups of progeny. The exposures were performed in embryos and adult fish in order to test the hypothesis that heritable radiation effects would depend on the dose and life stage: the first, gametogenesis exposure and offspring exposed indirectly via the parental germline; the second, exposed directly during embryogenesis. The following conclusions are based on objectives mentioned in *Section 1.2, Aim of the study*.

I. The dose-response study on effects of gamma radiation on the development of embryos and larva including 2.5-5.5 hpf gamma exposure gene expression concluded that continuous exposure to environmentally relevant dose rates (from 0.4 mGy/h, total dose 17.5 mGy) was able to cause biological harm when exposure is prolonged. However, as shown in Paper I, doses below or close to a certain level (0.4 mGy/h) (i.e. below the UNSCEAR recommended no effect dose), did not cause toxic effects such as deformities and mortality in the exposure period of 48 hpf. However, a similar dose (0.54 mGy/h, total 1.5 mGy) caused a significant transcriptional response, where ongoing antioxidant reparatory processes in response to radiation were indicated (*RAR*a pathway), in connection to formation of free radicals and DNA damage repair. The transcriptional responses at higher doses were connected to disrupted development and cancers (*myc*, *TGFb*, *hnf4a*). The results generally concluded that early stages of development are the most sensitive life stages to radiation, and point to the developmental defects, which would occur later in life.

II. The study of transmissible effects of ionizing radiation via the germ cells, in directly exposed embryos (E), embryos of exposed parents during gametogenesis (G) and directly exposed offspring of irradiated parents (GE) concluded that effects such as DNA damage, LPO and bystander effects are transmitted to offspring and are detectable one year after irradiation of parents. The delayed manifestation of these effects in offspring indicated that genomic instability and reprotoxicity are transmitted to offspring via the parental germline. The ROS formation was transmitted to offspring one month after irradiation of parents, but was only seen in directly exposed embryos one year after parental irradiation, attributing the ROS formation in embryos not only to be the consequence of radiolysis, but also to a disrupted ROS metabolism. The magnitudes of toxic effects on the embryonic development (survival, deformities, hatching, organ development) were proportional to the parental and embryonic radiation doses.

III. The comparison of differences between the effects of parental (indirect) exposure and embryo (direct) exposure on 5.5 hpf gene expression concluded that changes in gene expression point to the observed delayed genomic instability in parentally irradiated embryos (G). The gene expression changes in all offspring of irradiated parents, e.g. sex-hormone regulation, DNA strand break repair (*RRM2*), LPO (*aldh3a1*) were correlated to effects observed in the parental gonads and subcellular effects observed in offspring, and point to transmission of parental radiological stress to progeny. The fact that results of assessed effects were different immediately after exposure, compared to assessment longer time after exposure, contributes to the theory that the health status of parents at a certain time (i.e. the fitness of germ cells) might be behind observed effects in offspring.

IV. The study of adverse effects on the parents (young adults) and their reproductive capacity and genotoxicity (DNA damage) following gamma radiation exposure during gametogenesis concluded that reproduction is a very sensitive endpoint for radiation exposure. Since reproduction determines the survival and future of animal populations (life-time fitness), this endpoint proves to be an important indicator of offspring health. Although no significant differences in reproduction were observed at the lower dose (8.7 mGy/h, total 5.2 Gy), female gonads were affected by gamma radiation in terms of a large number of previtellogenic follicles, while a severe decline in reproductive capacity was observed at 53 mGy/h, including sterility one year after irradiation. The level of DNA damage was increased in whole blood in all exposed males and females.

Together, the results obtained in the project shed new light on the environmental impact of ionizing radiation and add important information on radiosensitivity for future multi- and transgenerational studies.

6. References

Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G.P., Lim, A.Y.M., Hajan, H.S., Collas, P., Bourque, G., Gong, Z., Korzh, V., Aleström, P., Mathavan, S., 2011. Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. Genome Res. 21, 1328–38. doi:10.1101/gr.116012.110

Aanes, H., Winata, C., Moen, L.F., Ostrup, O., Mathavan, S., Collas, P., Rognes, T., Aleström, P., 2014. Normalization of RNA-Sequencing Data from Samples with Varying mRNA Levels. PLoS One 9, e89158. doi:10.1371/journal.pone.0089158

Adams, G.E., Cox, R., 1997. Radiation Carcinogenesis, 3rd ed. Oxford University Press, Oxford.

Aisen, P., 2004. Transferrin receptor 1. Int. J. Biochem. Cell Biol. 36, 2137–2143. doi:10.1016/j.biocel.2004.02.007

Alberts B., Johnson A., Lewis J., Raff, M., Roberts, K., Walter, P., 2002. Molecular Biology of the Cell. 4th edition. Garland Science, New York. ISBN-10: 0-8153-3218-1

Amatruda, J.F., Zon, L.I., 1999. Dissecting hematopoiesis and disease using the zebrafish. Dev. Biol. 216, 1–15. doi:10.1006/dbio.1999.9462

Anders, S., Huber, W., 2010. Differential expression analysis for sequence count data. Genome Biol. 11, R106. doi:10.1186/gb-2010-11-10-r106

Andersen, I.S., Lindeman, L.C., Reiner, A.H., Østrup, O., Aanes, H., Aleström, P., Collas, P., 2013. Epigenetic marking of the zebrafish developmental program. Curr. Top. Dev. Biol. 104, 85–112. doi:10.1016/B978-0-12-416027-9.00003-6

Andersen, I.S., Ostrup, O., Lindeman, L.C., Aanes, H., Reiner, A.H., Mathavan, S., Aleström, P., Collas, P., 2012. Epigenetic complexity during the zebrafish mid-blastula transition. Biochem. Biophys. Res. Commun. 417, 1139–44. doi:10.1016/j.bbrc.2011.12.077

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741. doi:10.1002/etc.34

Aypar, U., Morgan, W.F., Baulch, J.E., 2011. Radiation-induced genomic instability: Are epigenetic mechanisms the missing link? Int. J. Radiat. Biol. 87, 179–191. doi:10.3109/09553002.2010.522686

Azzam, E.I., Jay-Gerin, J.-P., Pain, D., 2012. Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury. Cancer Lett. 327, 48–60. doi:10.1016/j.canlet.2011.12.012

Baldwin, J., Grantham, V., 2015. Radiation Hormesis: Historical and Current Perspectives. J. Nucl. Med. Technol. 43, 242–6. doi:10.2967/jnmt.115.166074

Barber R.C., Hickenbotham P., Hatch T., Kelly D., Topchiy N., Almeida G.M., Jones G.D., Johnson G.E., Parry J.M., Rothkamm K., Dubrova Y.E., 2006. Radiation-induced transgenerational

alterations in genome stability and DNA damage. Oncogene.; 25:7336–7342.

Beresford, N. A., & Copplestone, D. (2011). Effects of ionizing radiation on wildlife: What knowledge have we gained between the Chernobyl and Fukushima accidents? Integrated Environmental Assessment and Management, 7(3), 371–373. http://doi.org/10.1002/ieam.238

Bizuayehu, T.T., Babiak, I., 2014. MicroRNA in Teleost Fish. Genome Biol. Evol. 6, 1911–1937. doi:10.1093/gbe/evu151

Bjerke H, Hetland PO. Dosimetry at Figaro - gamma irradiation facility at NMBU, Aas; Dosimetri ved Figaro - gammaanlaget ved NMBU, Aas. Østerås; 2014.

Bladen, C.L., Flowers, M.A., Miyake, K., Podolsky, R.H., Barrett, J.T., Kozlowski, D.J., Dynan, W.S., 2007. Quantification of ionizing radiation-induced cell death in situ in a vertebrate embryo. Radiat. Res. 168, 149–57. doi:10.1667/RR0803.1

Bowler, D.A., Moore, S.R., Macdonald, D.A., Smyth, S.H., Clapham, P., Kadhim, M.A., 2006. Bystander-mediated genomic instability after high LET radiation in murine primary haemopoietic stem cells. Mutat. Res. Mol. Mech. Mutagen. 597, 50–61. doi:10.1016/j.mrfmmm.2005.04.025

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–54.

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., Stengel, D., Strecker, R., 2015. The fish embryo test (FET): origin, applications, and future. Environ. Sci. Pollut. Res. 22, 16247–16261. doi:10.1007/s11356-014-3814-7

Brenner S, Jacob F, Meselson M: An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature 1961, 190:576–581.

Buisset-Goussen, A., Goussen, B., Della-Vedova, C., Galas, S., Adam-Guillermin, C., Lecomte-Pradines, C., 2014. Effects of chronic gamma irradiation: a multigenerational study using Caenorhabditis elegans. J. Environ. Radioact. 137, 190–7. doi:10.1016/j.jenvrad.2014.07.014

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–22. doi:10.1373/clinchem.2008.112797

Calabrese, E.J., 2017. Flaws in the LNT single-hit model for cancer risk: An historical assessment. Environ. Res. 158, 773–788. doi:10.1016/j.envres.2017.07.030

Carlsson Tedgren Å, Bjerke H, Grindborg J-E, Hetland P-O, Kosunen A, Hellebust TP, et al. Comparison of high-dose-rate 192Ir source strength measurements using equipment with traceability to different standards. Brachytherapy. 2014;13: 420–423. http://dx.doi.org/10.1016/j.brachy.2014.01.002

Cech, T.R., Steitz, J.A., 2014. The Noncoding RNA Revolution—Trashing Old Rules to Forge New Ones. Cell 157, 77–94. doi:10.1016/j.cell.2014.03.008

Chadwick, K.H., Leenhouts, H.P., 2005. Radiation risk is linear with dose at low doses. Br. J. Radiol. 78, 8–10. doi:10.1259/bjr/51173413

Chang, W.P., Little, J.B., 1992. Evidence that DNA double-strand breaks initiate the phenotype of delayed reproductive death in Chinese hamster ovary cells , Radiat. Res. 131 53–59.

Choi, V.W.Y., Ng, C.Y.P., Kong, M.K.Y., Cheng, S.H., Yu, K.N., 2013. Adaptive response to ionising radiation induced by cadmium in zebrafish embryos. J. Radiol. Prot. 33, 101–12. doi:10.1088/0952-4746/33/1/101

Desouky, O. Ding, N., Zhou, Zhou G., Targeted and non-targeted effects of ionizing radiation, 2015. J. of Rad. Res. and Applied Sci. 8-2, 247-254. doi.org/10.1016/j.jrras.2015.03.003

Devi, P.U., Hossain, M., Bisht, K.S., 1999. Effect of late Fetal irradiation on adult behavior of mouse: Dose-response relationship. Neurotoxicol. Teratol. 21, 193–198. doi:10.1016/S0892-0362(98)00039-7

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. doi:10.1093/bioinformatics/bts635

Egami. N., Aoki K., 1966. Effects of x-irradiation of a part of the body on the ovary in the loach Misgurnus anguillicaudatus. Annot. Zool. Jpn. 39: 7-15

Erdelmeier, I., Gerard-Monnier, D., Yadan, J.-C., Chaudi?re, J., 1998. Reactions of N -Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Mechanistic Aspects of the Colorimetric Assay of Lipid Peroxidation. Chem. Res. Toxicol. 11, 1184–1194. doi:10.1021/tx970180z

Fang, X., Corrales, J., Thornton, C., Clerk, T., Scheffler, B.E., Willett, K.L., 2015. Transcriptomic Changes in Zebrafish Embryos and Larvae Following Benzo[a]pyrene Exposure. Toxicol. Sci. 146, 395–411. doi:10.1093/toxsci/kfv105

Felsenfeld, A.L., 1996. Defining the boundaries of zebrafish developmental genetics. Nat. Genet. 14, 258–263. doi:10.1038/ng1196-258

Fraenkel, P.G., Gibert, Y., Holzheimer, J.L., Lattanzi, V.J., Burnett, S.F., Dooley, K.A., Wingert, R.A., Zon, L.I., 2009. Transferrin-a modulates hepcidin expression in zebrafish embryos. Blood 113, 2843–50. doi:10.1182/blood-2008-06-165340

Freeman, J.L., Weber, G.J., Peterson, S.M., Nie, L.H., 2014a. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front. Genet. 5, 268. doi:10.3389/fgene.2014.00268

Freeman, J.L., Weber, G.J., Peterson, S.M., Nie, L.H., 2014b. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front. Genet. 5, 268. doi:10.3389/fgene.2014.00268

Gagnaire, B., Cavalié, I., Pereira, S., Floriani, M., Dubourg, N., Camilleri, V., Adam-Guillermin, C., 2015. External gamma irradiation-induced effects in early-life stages of zebrafish, Danio rerio. Aquat. Toxicol. 169, 69–78. doi:10.1016/j.aquatox.2015.10.005

Galván, I., Bonisoli-Alquati, A., Jenkinson, S., Ghanem, G., Wakamatsu, K., Mousseau, T. A., & Møller, A. P. (2014). Chronic exposure to low-dose radiation at Chernobyl favours adaptation to oxidative stress in birds. Functional Ecology, 28(6), 1387–1403. http://doi.org/10.1111/1365-2435.12283

Garnier-Laplace, J., Della-Vedova, C., Andersson, P., Copplestone, D., Cailes, C., Beresford, N.A., Howard, B.J., Howe, P., Whitehouse, P., 2010. A multi-criteria weight of evidence approach for deriving ecological benchmarks for radioactive substances. J. Radiol. Prot. 30, 215–33. doi:10.1088/0952-4746/30/2/S02

Geiger, G.A., Parker, S.E., Beothy, A.P., Tucker, J.A., Mullins, M.C., Kao, G.D., 2006. Zebrafish as a "biosensor"? Effects of ionizing radiation and amifostine on embryonic viability and development. Cancer Res. 66, 8172–81. doi:10.1158/0008-5472.CAN-06-0466

Gerstein MB, Bruce C, Rozowsky JS, Zheng D, Du J, Korbel JO, Emanuelsson O, Zhang ZD, Weissman S, Snyder M: What is a gene, post-ENCODE? History and updated definition. Genome Research 2007, 17:669–681.

Gutzkow, K.B., Langleite, T.M., Meier, S., Graupner, A., Collins, A.R., Brunborg, G., 2013. High-throughput comet assay using 96 minigels. Mutagenesis 28, 333–40. doi:10.1093/mutage/get012

Haberle, V., Lenhard, B., 2012. Dissecting genomic regulatory elements in vivo. Nat. Biotechnol. 30, 504–6. doi:10.1038/nbt.2266

Haberle, V., Li, N., Hadzhiev, Y., Plessy, C., Previti, C., Nepal, C., Gehrig, J., Dong, X., Akalin, A., Suzuki, A.M., van IJcken, W.F.J., Armant, O., Ferg, M., Strähle, U., Carninci, P., Müller, F., Lenhard, B., 2014. Two independent transcription initiation codes overlap on vertebrate core promoters. Nature 507, 381–385. doi:10.1038/nature12974

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nüsslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 123, 1–36.

Hall, J., Jeggo, P.A., West, C., Gomolka, M., Quintens, R., Badie, C., Laurent, O., Aerts, A., Anastasov, N., Azimzadeh, O., Azizova, T., Baatout, S., Baselet, B., Benotmane, M.A., Blanchardon, E., Guéguen, Y., Haghdoost, S., Harms-Ringhdahl, M., Hess, J., Kreuzer, M., Laurier, D., Macaeva, E., Manning, G., Pernot, E., Ravanat, J.-L., Sabatier, L., Tack, K., Tapio, S., Zitzelsberger, H., Cardis, E., 2017. Ionizing radiation biomarkers in epidemiological studies – An update. Mutat. Res. Mutat. Res. 771, 59–84. doi:10.1016/j.mrrev.2017.01.001

Han, W., Yu, K.N., 2012. Response of Cells to Ionizing Radiation, in: Advances in Biomedical Sciences and Engineering. Bentham science publishers, pp. 204–262. doi:10.2174/978160805040610901010204

Hansen, S.H., Olsen, A.K., Søderlund, E.J., Brunborg, G., 2010. In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes. Mutat. Res. 696, 55–61. doi:10.1016/j.mrgentox.2009.12.012

Harley, N., 2010. Toxic Effects of Radiation and Radioactive Materials | in Casarett & Doull's Essentials of Toxicology, 2ed. McGraw Hill, New York, p. 351/2.

Hoo, J.Y., Kumari, Y., Shaikh, M.F., Hue, S.M., Goh, B.H., 2016. Zebrafish: A Versatile Animal Model for Fertility Research. Biomed Res. Int. 2016, 9732780. doi:10.1155/2016/9732780

Hossain, M., Chetana, M., Uma Devi, P., 2005. Late effect of prenatal irradiation on the hippocampal histology and brain weight in adult mice. Int. J. Dev. Neurosci. 23, 307–313. doi:10.1016/j.ijdevneu.2005.01.006

Howe, D.G., Bradford, Y.M., Eagle, A., Fashena, D., Frazer, K., Kalita, P., Mani, P., Martin, R., Moxon, S.T., Paddock, H., Pich, C., Ramachandran, S., Ruzicka, L., Schaper, K., Shao, X., Singer, A., Toro, S., Van Slyke, C., Westerfield, M., 2017. The Zebrafish Model Organism Database: new support for human disease models, mutation details, gene expression phenotypes and searching. Nucleic Acids Res. 45, D758–D768. doi:10.1093/nar/gkw1116

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Eliott, D., Threadgold, G., Harden, G., Ware, D., Mortimore, B., Mortimer, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley,

C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Roest Crollius, H., Rogers, J., Stemple, D.L., Begum, S., Lloyd, C., Lanz, C., Raddatz, G., Schuster, S.C., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. doi:10.1038/nature12111

Hyodo-Taguchi Y, Etoh H (1986) Effects of tritiated water on germ cells in Medaka. II. Reproductive capacity following embryonic exposure. Radiat Res 106:321–330

IAEA 2016. Radiation Protection and Safety of Radiation Sources International Basic Safety Standards. IAEA Safety Standards Series No. GSR Part 3

ICRP, 2003a. Relative biological effectiveness (RBE), quality factor (Q), and radiation weighting factor (w(R)). ICRP Publication 92. Ann. ICRP 33 (4), 2003

ICRP, 2003b. Biological Effects after Prenatal Irradiation (Embryo and Fetus). ICRP Publication 90. Ann. ICRP 33 (1-2), 2003

ICRP, 2008. Environmental Protection - the Concept and Use of Reference Animals and Plants. ICRP Publication 108. Ann. ICRP 38 (4-6).

ICRP, 2012. ICRP 2012. Compend. Dose Coefficients based ICRP Publ. 60 0, 130. doi:10.1016/j.icrp.2006.06.001

Ilnytskyy, Y., Kovalchuk, O., 2011. Non-targeted radiation effects-an epigenetic connection. Mutat. Res. 714, 113–25. doi:10.1016/j.mrfmmm.2011.06.014

Jaafar, L., Podolsky, R.H., Dynan, W.S., 2013. Long-term effects of ionizing radiation on gene expression in a zebrafish model. PLoS One 8, e69445. doi:10.1371/journal.pone.0069445

Jarvis, R.., Knowles, J.., 2003. DNA damage in zebrafish larvae induced by exposure to low-dose rate γ-radiation: detection by the alkaline comet assay. Mutat. Res. Toxicol. Environ. Mutagen. 541, 63–69.

Johansen, M.P., Ruedig, E., Tagami, K., Uchida, S., Higley, K., Beresford, N.A., 2015. Radiological dose rates to marine fish from the fukushima daiichi accident: The first three years across the north pacific. Environ. Sci. Technol. 49, 1277–1285. doi:10.1021/es505064d

Kamstra, J.H., Sales, L.B., Aleström, P., Legler, J., 2017. Differential DNA methylation at conserved non-genic elements and evidence for transgenerational inheritance following developmental exposure to mono(2-ethylhexyl) phthalate and 5-azacytidine in zebrafish. Epigenetics Chromatin 10, 20. doi:10.1186/s13072-017-0126-4

Kane, D., Kimmel, C., 1993. The zebrafish midblastula transition. Development 119, 447–456.

Kaup, S., Grandjean, V., Mukherjee, R., Kapoor, A., Keyes, E., Seymour, C.B., Mothersill, C.E., Schofield, P.N., 2006. Radiation-induced genomic instability is associated with DNA

methylation changes in cultured human keratinocytes. Mutat. Res. Mol. Mech. Mutagen. 597, 87–97. doi:10.1016/j.mrfmmm.2005.06.032

Kawasaki, T., Siegfried, K.R., Sakai, N., 2016. Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture. Development 143, 566–574. doi:10.1242/dev.129643

Khalil, H., Tummala, H., Chakarov, S., Zhelev, N., Lane, D., 2012. Targeting ATM pathway for therapeutic intervention in cancer. Biodiscovery 1–13. doi:10.7750/BioDiscovery.2012.1.3

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. doi:10.1002/aja.1002030302

Koturbash, I., Baker, M., Loree, J., Kutanzi, K., Hudson, D., Pogribny, I., Sedelnikova, O., Bonner, W., Kovalchuk, O., 2006. Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo. Int. J. Radiat. Oncol. Biol. Phys. 66, 327–30. doi:10.1016/j.ijrobp.2006.06.012

Koturbash, I., Loree, J., Kutanzi, K., Koganow, C., Pogribny, I., Kovalchuk, O., 2008. In vivo bystander effect: cranial X-irradiation leads to elevated DNA damage, altered cellular proliferation and apoptosis, and increased p53 levels in shielded spleen. Int. J. Radiat. Oncol. Biol. Phys. 70, 554–62. doi:10.1016/j.ijrobp.2007.09.039

Kovalchuk, O., 2013. Environmental Epigenomics in Health and Disease. Epigenetics and Human Health 99–126. doi:10.1007/978-3-642-23380-7

Kuwahara, Y., Shimada, A., Mitani, H., Shima, A., 2003. Gamma-ray exposure accelerates spermatogenesis of medaka fish, Oryzias latipes. Mol. Reprod. Dev. 65, 204–11. doi:10.1002/mrd.10261

Lavelle, C., Foray, N., 2014. Chromatin structure and radiation-induced DNA damage: From structural biology to radiobiology. Int. J. Biochem. Cell Biol. 49, 84–97. doi:10.1016/j.biocel.2014.01.012

Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177–87. doi:10.1095/biolreprod.109.076299

Lee, J.W., Won, E.-J., Raisuddin, S., Lee, J.-S., 2015. Significance of adverse outcome pathways in biomarker-based environmental risk assessment in aquatic organisms. J. Environ. Sci. 35, 115–127. doi:10.1016/j.jes.2015.05.002

Lemos, J., Neuparth, T., Trigo, M., Costa, P., Vieira, D., Cunha, L., Ponte, F., Costa, P.S., Metello, L.F., Carvalho, A.P., 2017. Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny. Bull. Environ. Contam. Toxicol. 98, 190–195. doi:10.1007/s00128-016-2006-1

Little, M.P., Goodhead, D.T., Bridges, B.A., Bouffler, S.D., 2013. Evidence relevant to untargeted

and transgenerational effects in the offspring of irradiated parents. Mutat. Res. Mutat. Res. 753, 50–67. doi:10.1016/j.mrrev.2013.04.001

Lyng F.M., O'Reilly S., Cottell D.C., Seymour C.B., Mothersill C., 1996. Persistent expression of morphological abnormalities in the distant progeny of irradiated cells , Radiat. Environ. Biophys. 35 273–283.

Lourenço, J., Mendo, S., Pereira, R., 2016. Radioactively contaminated areas: Bioindicator species and biomarkers of effect in an early warning scheme for a preliminary risk assessment. J. Hazard. Mater. 317, 503–542. doi:10.1016/j.jhazmat.2016.06.020

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. doi:10.1186/s13059-014-0550-8

Luzhna, L., Kathiria, P., Kovalchuk, O., 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front. In Genetics 4, Art 131, 1-17 doi:10.3389/fgene.2013.00131

Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. J. Fish Biol. 62, 895–906. doi:10.1046/j.1095-8649.2003.00074.x

McAleer, M.F., Davidson, C., Davidson, W.R., Yentzer, B., Farber, S.A., Rodeck, U., Dicker, A.P., 2005. Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers. Int. J. Radiat. Oncol. 61, 10–13.

Merrifield, M., Kovalchuk, O., 2013. Epigenetics in radiation biology: a new research frontier. Front. Genet. 4, 40. doi:10.3389/fgene.2013.00040

Mezentsev, A., Amundson, S.A., 2011. Global gene expression responses to low- or high-dose radiation in a human three-dimensional tissue model. Radiat. Res. 175, 677–88. doi:10.1667/RR2483.1

Michibata, H., 1976. The role of spermatogonia in the recovery process from temporary sterility induced by gamma-ray irradiation in the telcost, Oorias lalipes. J. Radiat. Res. 17: 112-1 53 (1976).

MotherSill, C., Seymour, C., 2012a. Changing paradigms in radiobiology. Mutat. Res. Mutat. Res. 750, 85–95. doi:10.1016/j.mrrev.2011.12.007

Mothersill, C., Seymour, C., 2012b. Are epigenetic mechanisms involved in radiation-induced bystander effects? Front. Genet. 3, 74. doi:10.3389/fgene.2012.00074

Mothersill, C., Smith, R.W., Agnihotri, N., Seymour, C.B., 2007. Characterization of a Radiation-Induced Stress Response Communicated in Vivo between Zebrafish. Environ. Sci. Technol. 41, 3382–3387. doi:10.1021/es062978n

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., Laissue, J., Schroll, C., Seymour, C., 2014. Transmission of Signals from Rats Receiving High Doses of Microbeam Radiation to Cage Mates: An Inter-Mammal Bystander Effect. Dose-Response 12, 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mousseau TA, Møller AP (2013) Perspectives on Chernobyl and Fukushima health effects: what can be learned from Eastern European research? J Health & Pollut 3(5):2–6

Mugoni, V., Camporeale, A., Santoro, M.M., 2014. Analysis of oxidative stress in zebrafish embryos. J. Vis. Exp. doi:10.3791/51328

Murphy J.F., Nagorskaya, L.L., Smith, J.T., 2011. Abundance and diversity of aquatic macroinvertebrate communities in lakes exposed to Chernobyl-derived ionising radiation. Journal of Environmental Radioactivity, 102(7), 688–694. doi.org/10.1016/J.JENVRAD.2011.04.007

Nakamura, S., Kobayashi, K., Nishimura, T., Tanaka, M., 2011. Ovarian Germline Stem Cells in the Teleost Fish, Medaka (Oryzias latipes). Int. J. Biol. Sci. 7, 403–409. doi:10.7150/ijbs.7.403

Niida, H., Katsuno, Y., Sengoku, M., Niida, H., Katsuno, Y., Sengoku, M., Shimada, M., 2010. Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase service Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase 333–338. doi:10.1101/gad.1863810

Nossal, G.J. V., 2003. The double helix and immunology. Nature 421, 440–444. doi:10.1038/nature01409

Nylund, R., Lemola, E., Hartwig, S., Lehr, S., Acheva, A., Jahns, J., Hildebrandt, G., Lindholm, C., 2014. Profiling of low molecular weight proteins in plasma from locally irradiated individuals. J. Radiat. Res. 55, 674–82. doi:10.1093/jrr/rru007

OECD, 2004a. OECD guidelines for testing of chemicals, advance copy. Test 210 : Fish, early- life stage toxicity test.

OECD, 2004b. OECD guidelines for testing of chemicals, advance copy. Test 212 : Fish, short- term toxicity test on embryo and sac-fry stages.

OECD/OCDE, 2013. OECD GUIDELINES FOR THE TESTING OF CHEMICALS Fish Embryo Acute Toxicity (FET) Test. doi:10.1787/20745761

OECD, 2016. OECD series on adverse outcome pathways. OECD Publishing.

Oliveira, R., Domingues, I., Koppe Grisolia, C., Soares, A.M.V.M., 2009. Effects of triclosan on zebrafish early-life stages and adults. Environ. Sci. Pollut. Res. 16, 679–688. doi:10.1007/s11356-009-0119-3

Otis, J.P., Zeituni, E.M., Thierer, J.H., Anderson, J.L., Brown, A.C., Boehm, E.D., Cerchione, D.M., Ceasrine, A.M., Avraham-Davidi, I., Tempelhof, H., Yaniv, K., Farber, S.A., 2015. Zebrafish as a model for apolipoprotein biology: comprehensive expression analysis and a role for ApoA-IV in regulating food intake. Dis. Model. Mech. 8, 295–309. doi:10.1242/dmm.018754

Pankov, R., Yamada, K.M., 2002. Fibronectin at a glance. J. Cell Sci. 115, 3861–3863. doi:10.1242/jcs.00059

Pavlica, M., Štambuk, A., Malović, L., Mladinić, M., Klobučar, G.I. V., 2011. DNA integrity of

chub erythrocytes (Squalius cephalus L.) as an indicator of pollution-related genotoxicity in the River Sava. Environ. Monit. Assess. 177, 85–94. doi:10.1007/s10661-010-1620-3

Pedersen, M.T., Helin, K., 2010. Histone demethylases in development and disease. Trends Cell Biol. 20, 662–71. doi:10.1016/j.tcb.2010.08.011

Pereira, S., Malard, V., Ravanat, J.-L., Davin, A.-H., Armengaud, J., Foray, N., Adam-Guillermin, C., 2014. Low Doses of Gamma-Irradiation Induce an Early Bystander Effect in Zebrafish Cells Which Is Sufficient to Radioprotect Cells. PLoS One 9, e92974. doi:10.1371/journal.pone.0092974

Plamadeala, C., Wojcik, A., Creanga, D., 2015. Micronuclei versus Chromosomal Aberrations Induced by X-Ray in Radiosensitive Mammalian Cells. Iran. J. Public Health 44, 325–31.

Pogribny, I., Raiche, J., Slovack, M., Kovalchuk, O., 2004. Dose-dependence, sex- and tissue-specificity, and persistence of radiation-induced genomic DNA methylation changes. Biochem. Biophys. Res. Commun. 320, 1253–61. doi:10.1016/j.bbrc.2004.06.081

Qian, Q. Z., Cao, X. K., Shen, F. H., Wang, Q., 2016. Effects of ionising radiation on micronucleus formation and chromosomal aberrations in Chinese radiation workers. Radiat. Prot. Dosimetry 168, 197–203. doi:10.1093/rpd/ncv290

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. doi:10.1016/S0304-3940(02)01423-4

Rendic, S., Peter Guengerich, F., 2012. Summary of Information on the Effects of Ionizing and Non-ionizing Radiation on Cytochrome P450 and Other Drug Metabolizing Enzymes and Transporters. Curr. Drug Metab. 13, 787–814. doi:10.2174/138920012800840356

Reisz, J.A., Bansal, N., Qian, J., Zhao, W., Furdui, C.M., 2014. Effects of ionizing radiation on biological molecules--mechanisms of damage and emerging methods of detection. Antioxid. Redox Signal. 21, 260–92. doi:10.1089/ars.2013.5489

Richardson, M.K., Allen, S.P., Wright, G.M., Raynaud, A., Hanken, J., 1998. Somite number and vertebrate evolution. Development 125.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26, 139–40. doi:10.1093/bioinformatics/btp616

Rothkamm, K., Löbrich, M., 2002. Misrepair of radiation-induced DNA double-strand breaks and its relevance for tumorigenesis and cancer treatment (review). Int. J. Oncol. 21, 433–40.

Russo, C., Rocco, L., Morescalchi, M.A., Stingo, V., 2003. Assessment of environmental stress by the micronucleus test and the Comet assay on the genome of teleost populations from two natural environments. Ecotox. and Environ. Safety 57, 168–174. doi:10.1016/S0147-6513(03)00027-7

Ryan, L.A., Seymour, C.B., O'Neill-Mehlenbacher, A., Mothersill, C.E., 2008. Radiation-induced adaptive response in fish cell lines. J. Environ. Radioact. 99, 739–747.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Van Cruchten, S., 2016. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. J. Toxicol. Sci. 41, 1–11. doi:10.2131/jts.41.1

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. Int. 15, 394–404. doi:10.1007/s11356-008-0018-z

Schulz, R.W., de França, L.R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411. doi:10.1016/j.ygcen.2009.02.013

Selzer, E., Hebar, A., 2012. Basic principles of molecular effects of irradiation. Wiener Medizinische Wochenschrift 162, 47–54. doi:10.1007/s10354-012-0052-9

Sherwood, L., Klandorf, H., Yancey, P. (2012). Animal Physiology: From Genes to Organisms. Cengage Learning. ISBN 1133709516.

Shibata, A., 2017. Regulation of repair pathway choice at two-ended DNA double-strand breaks. Mutat. Res. Mol. Mech. Mutagen. doi:10.1016/j.mrfmmm.2017.07.011

Shimada, A., Eguchi, H., Yoshinaga, S., Shima, A., 2005. Dose rate effect on transgenerational mutation frequencies in spermatogonial stem cells of the medaka fish. Radiat. Res. 163, 112–4.

Skinner, M.K., 2011. Role of epigenetics in developmental biology and transgenerational inheritance. Birth Defects Res. C. Embryo Today 93, 51–5. doi:10.1002/bdrc.20199

Slovinska L., Elbertova A., Misurova E., 2004. Transmission of genome damage from irradiated male rats to their progeny. Mutat Res. 559:29–37.

Smith, J.T. Beresford, Nicholas A. Chernobyl - Catastrophe and Consequences, 2005. Praxis Publishing Ltd, Chichester, UK; Springer Praxis Books ISBN: 9783540280798

Snyder, A.R., Morgan, W.F., 2003. Persistent oxidative stress and gene expression changes in radiation-induced genomic instability. Int. Congr. Ser. 1258, 199–206. doi:10.1016/S0531-5131(03)01152-X

Song, Y., Salbu, B., Heier, L.S., Teien, H.-C., Lind, O.-C., Oughton, D., Petersen, K., Rosseland, B.O., Skipperud, L., Tollefsen, K.E., 2012. Early stress responses in Atlantic salmon (Salmo salar) exposed to environmentally relevant concentrations of uranium. Aquat. Toxicol. 112–113, 62–71. doi:10.1016/j.aquatox.2012.01.019

Sorokina, S.S., Zaichkina, S.I., Rozanova, O.M., Aptikaeva, G.F., Akhmadieva, A.K., Smirnova, E.N., Romanchenko, S.P., Vakhrusheva, O.A., Dyukina, A.R., Peleshko, V.N., 2011. Delayed effects of chronic low-dose high linear energy transfer (LET) radiation on mice in vivo. Radiat. Prot. Dosimetry 143, 305–310. doi:10.1093/rpd/ncq531

Soubry, A., Hoyo, C., Jirtle, R.L., Murphy, S.K., 2014. A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. Bioessays 36, 359–71. doi:10.1002/bies.201300113

Stevens, R.G., Kalkwarf, D.R., 1990. Iron, Radiation, and Cancer. Environ. Health Perspect. 87, 291. doi:10.2307/3431037

Stewart, F.A., Akleyev, A.V., Hauer-Jensen, M., Hendry, J.H., Kleiman, N.J., MacVittie, T.J., Aleman, B.M., Edgar, A.B., Mabuchi, K., Muirhead, C.R., Shore, R.E., Wallace, W.H., 2012. ICRP PUBLICATION 118: ICRP Statement on Tissue Reactions and Early and Late Effects of Radiation in Normal Tissues and Organs — Threshold Doses for Tissue Reactions in a Radiation Protection Context. Ann. ICRP 41, 1–322. doi:10.1016/j.icrp.2012.02.001

Strand, P., Aono, T., Brown, J.E., Garnier-Laplace, J., Hosseini, A., Sazykina, T., Steenhuisen, F., Vives i Batlle, J., 2014. Assessment of Fukushima-Derived Radiation Doses and Effects on Wildlife in Japan. Environ. Sci. Technol. Lett. 1, 198–203. doi:10.1021/ez500019j

Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., Schumacher, A., Selderslaghs, I., Weiss, C., Witters, H., Braunbeck, T., 2012. Zebrafish embryos as an alternative to animal experiments--a commentary on the definition of the onset of protected life stages in animal welfare regulations. Reprod. Toxicol. 33, 128–32. doi:10.1016/j.reprotox.2011.06.121

Timoféeff-Ressovsky, N. W., Zimmer, K. G. & Delbrück, M., 1935. Über die Natur der Genmutation und der Genkostruktur. Nachr. Ges. Wiss. Gottingen FG VI Biol. N.F. 1, 189–245

Traver, D., Winzeler, A., Stern, H.M., Mayhall, E.A., Langenau, D.M., Kutok, J.L., Look, A.T., Zon, L.I., 2004. Effects of lethal irradiation in zebrafish and rescue by hematopoietic cell transplantation. Blood 104, 1298–305. doi:10.1182/blood-2004-01-0100

Trede, N.S., Langenau, D.M., Traver, D., Look, A.T., Zon, L.I., 2004. The use of zebrafish to understand immunity. Immunity 20, 367–79.

Tsao-Wu, G.S., Weber, C.H., Budgeon, L.R., Cheng, K.C., 1998. Agarose-embedded tissue arrays for histologic and genetic analysis. Biotechniques 25, 614–8.

Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. J. Exp. Biol. 205, 711–8.

UNSCEAR, 1996. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation, Sources and effects of ionizing radiation. UNSCEAR 1996

UNSCEAR, 2001. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. Hereditary effects of radiation. UNSCEAR, 2001

UNSCEAR, 2006a. Annex A: Epidemiological studies of radiation and cancer. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. UNSCEAR, 2006

UNSCEAR, 2006b. Annex B: Epidemiological evaluation of cardiovascular disease and other non-cancer diseases following radiation exposure. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. UNSCEAR, 2006

UNSCEAR, 2006c. Annex C: Non-targeted and delayed effects of exposure to ionizing radiation. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. UNSCEAR, 2006

UNSCEAR, 2008. REPORT Vol. II Sources and effects of ionizing radiation. Scientific Annexes C, D and E. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation, UNSCEAR, 2008

UNSCEAR, 2012. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation, UNSCEAR, 2012

Vaiserman, A.M., Koliada, A.K., Jirtle, R.L., 2017. Non-genomic transmission of longevity between generations: potential mechanisms and evidence across species. Epigenetics Chromatin 10, 38. doi:10.1186/s13072-017-0145-1

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034.

Waldren, C.A., 2004. Classical radiation biology dogma, bystander effects and paradigm shifts. Hum. Exp. Toxicol. 23, 95–100. doi:10.1191/0960327104ht425oa

Wang, Z., Gerstein, M., Snyder, M., 2010. RNA-Seq : a revolutionary tool for transcriptomics 10, 57–63. doi:10.1038/nrg2484.RNA-Seq

White RJ: Gene Transcription. Oxford, UK: John Wiley & Sons; 2009.

White, R., Rose, K., Zon, L., 2013. Zebrafish cancer: the state of the art and the path forward. Nat. Rev. Cancer 13, 624–36. doi:10.1038/nrc3589

Williams, T.D., Mirbahai, L., Chipman, J.K., 2014. The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts. Brief. Funct. Genomics 13, 157–71. doi:10.1093/bfgp/elt053

Winterbourn, C.C., 2014. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. Biochim. Biophys. Acta - Gen. Subj. 1840, 730–738. doi:10.1016/j.bbagen.2013.05.004

Woodhead DS (1977) The effects of chronic irradiation on the breeding performance of the guppy, *Poecilia reticulata* (Osteichthyes: Teleostei). Int J Radiat Biol 32:1–22

Zhivotovsky, B., Kroemer, G., 2004. Apoptosis and genomic instability. Nat. Rev. Mol. Cell Biol. 5, 752–762. doi:10.1038/nrm1443

Zyuzikov, N.A., Coates, P.J., Parry, J.M., Lorimore, S.A., Wright, E.G., 2011. Lack of nontargeted effects in murine bone marrow after low-dose in vivo X irradiation. Radiat. Res. 175, 322–7. doi:10.1667/RR2386.1

Papers I – IV

I

PLOS ONE

RESEARCH ARTICLE

Dose-dependent effects of gamma radiation on the early zebrafish development and gene expression

Selma Hurem[1☯]*, Leonardo Martín Martín[1,2☯], Dag Anders Brede[3], Eystein Skjerve[1], Rasoul Nourizadeh-Lillabadi[1], Ole Christian Lind[3], Terje Christensen[4], Vidar Berg[1], Hans-Christian Teien[3], Brit Salbu[3], Deborah Helen Oughton[3], Peter Aleström[1], Jan Ludvig Lyche[1]

1 Norwegian University of Life Sciences (NMBU), CERAD CoE, Faculty of Veterinary Medicine and Biosciences, Oslo, Norway, **2** University of Camagüey Ignacio Agramonte y Loynaz (UC), Faculty of Agropecuary Sciences, Camagüey, Cuba, **3** Norwegian University of Life Sciences (NMBU), CERAD CoE, Faculty of Environmental Sciences and Natural Resource Management, 1433 Ås, Norway, **4** Norwegian Radiation Protection Authority (NRPA), CERAD CoE, Østerås, Norway

☯ These authors contributed equally to this work.
* selma.hurem@nmbu.no

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Abstract

Ionizing radiation from natural sources or anthropogenic activity has the potential to cause oxidative stress or genetic damage in living organisms, through the ionization and excitation of molecules and the subsequent production of free radicals and reactive oxygen species (ROS). The present work focuses on radiation-induced biological effects using the zebrafish (*Danio rerio*) vertebrate model. Changes in developmental traits and gene expression in zebrafish were assessed after continuous external gamma irradiation (0.4, 3.9, 15 and 38 mGy/h) with corresponding controls, starting at 2.5 hours post fertilization (hpf) and lasting through embryogenesis and the early larval stage. The lowest dose rate corresponded to recommended benchmarks at which adverse effects are not expected to occur in aquatic ecosystems (2–10 mGy/day). The survival observed at 96 hours post fertilization (hpf) in the 38 mGy/h group was significantly lower, while other groups showed no significant difference compared to controls. The total hatching was significantly lower from controls in the 15 mGy/h group and a delay in hatching onset in the 0.4 mGy/h group was observed. The deformity frequency was significantly increased by prolonged exposure duration at dose rates $\geq$ 0.4 mGy/h. Molecular responses analyzed by RNA-seq at gastrulation (5.5 hpf transcriptome) indicate that the radiation induced adverse effects occurred during the earliest stages of development. A dose-response relationship was found in the numbers of differentially regulated genes in exposure groups compared to controls at a total dose as low as 1.62 mGy. Ingenuity Pathway Analysis identified retinoic acid receptor activation, apoptosis, and glutathione mediated detoxification signaling as the most affected pathways in the lower dose rate (0.54 mGy/h), while *eif2* and *mTOR*, i.e., involved in the modulation of angiogenesis, were most affected in higher dose rates (5.4 and 10.9 mGy/h). By comparing gene expression data, *myc* was found to be the most significant upstream regulator, followed by *tp53*, *TNF*, *hnf4a*, *TGFb1* and *cebpa*, while *crabp2b* and *vegfab* were identified as

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most frequent downstream target genes. These genes are associated with various developmental processes. The present findings show that continuous gamma irradiation ($\geq$ 0.54 mGy/h) during early gastrula causes gene expression changes that are linked to developmental defects in zebrafish embryos.

Introduction

Living organisms are continuously exposed to ionizing radiation from naturally occurring radionuclides (e.g., radon daughters), cosmic radiation and from various anthropogenic activities (weapon testing, nuclear fuel reprocessing, nuclear accidents). Ionizing radiation interacts with matter by excitation and ionization of molecules, thereby producing free radicals and subsequently reactive oxygen species (ROS) and reactive nitrogen species (RNS) which can attack cell membranes or break chemical bonds in biological molecules, leading to oxidative stress or DNA damage [1]. Proliferating cells are specifically sensitive to radiation [2].

It is established that humans and animals are most vulnerable to ionizing radiation during early life stages such as gametogenesis, embryogenesis and organogenesis [3,4], due to the high rate of cell division, proliferation and differentiation. Ionizing radiation can affect all organs and biological systems, and can induce non-cancer effects as well as cancer [5]. Experimental studies have documented that exposure to ionizing radiation during critical periods of development may alter (reprogram) the differentiation signals leading to permanent toxic effects which can manifest later in life [5,6]. Permanent (irreversible) "developmental programming" is, among other mechanisms, attributed to epigenetic modification of gene transcription [7,8]. For aquatic organisms exposed to ionizing radiation, dose rates lower than 0.42 mGy/h (corresponding to 10 mGy/d) have been proposed as benchmark levels that are not likely to produce adverse effects at the population level [9]. Recently, a much lower predicted no effect dose rate (PNEDR) of 0.01 mGy/h (0.24 mGy/d) has been proposed as a risk assessment screening value below which one could be confident that exposures would not lead to adverse effects [10]. Protection criteria is based mostly on data from acute exposure experiments of adult organisms (IAEA), and the information on effects of ionizing radiation during sensitive life stages such as the embryonic and early larval development is scarce.

The zebrafish model offers many practical benefits, which may contribute to a better understanding of biological effects of radiation in both humans and non-human biota. Age-synchronized and optically transparent zebrafish embryos enable the visualization of major organ system development within all stages of the embryonic and early larval period. The available genomic resources in zebrafish, including a fully sequenced genome, have been proven valuable for providing various biological insights, including into human diseases [11]. Transcriptome analysis allows registration of changes in gene expression related to various biological processes and can be used to reveal potential mechanisms of radiotoxicity. The genome of the zebrafish is roughly half the size of the human genome and in comparison to it, shares about 70% of human gene orthologs and 82% of cancer gene orthologs [12,13].

This study aimed to assess biological effects such as survival, hatching and the occurrence of deformities in zebrafish exposed to gamma radiation (dose rates 0.4, 3.9, 15, and 38 mGy/h) and controls during embryogenesis and larval development (2.5 to 96 hpf). In order to elucidate the changes in gene expression with accompanying functional network analyses, RNA sequencing of total RNA extracted from irradiated (0.54, 5.4 and 10.9 mGy/h) pooled embryo samples and controls was performed. The embryos were exposed during 2.5–5.5 hpf,

corresponding to the blastula period until the onset of the gastrula stage of development (256-cell stage until approximately 50% epiboly) [14]. This timeframe also includes the zygotic genome activation (ZGA) and onset of cell specification takes place [15–17]. The early molecular events initiated by a very low total dose of gamma radiation at 5.5 hpf and analyzed by transcriptomics were shown to be consistent with the observed developmental adversity in later stages.

Materials and methods

Ethics statement

The research was carried out according to the Norwegian Animal Protection Act (implemented EU Directive 2010/63/EU) and approved on December 12, 2013 by IACUC at Norwegian School of Veterinary Science (since 2014 Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, Oslo, Norway), under approval number FOTS ID 5793.

Zebrafish maintenance and embryo handling

Embryos from the AB wild type strain were obtained from the NMBU zebrafish facility (Norwegian Zebrafish Platform) and maintained according to standard operating procedures. Zebrafish were kept at 28 ± 1˚C on a 14–10 hour light-dark cycle at a density of 5–10 fish/L. The system water (SW) was prepared from particle and active charcoal filtered reverse osmosis (RO) deionized tap water, kept sterile by UV irradiation. To generate a conductivity of 500 μS/cm, general hardness (GH) of 4–5 and pH 7.5 (adjusted with 1M HCl), 155 mg synthetic sea salt (Instant Ocean, Blacksburg, USA), 53 mg sodium carbonate and 15 mg calcium chloride (Sigma-Aldrich) was added per liter RO water. Adult fish were fed with Gemma Micro 300 (Skretting, Stavanger, Norway) dry feed twice a day and live artemia (Scanbur, Copenhagen, Denmark) once a day. Health was monitored by daily inspection, sentinel fish were sent to ZIRC for pathology every six month and water sent for microbiology analysis (NMBU Vetbio, Oslo). Adult fish were allowed to mate for 30 min in standard 1 L breeding tanks (Aquatic Habitats, Apopka, FL). For gamma radiation experiments, embryos were collected immediately after breeding and individually placed in 2 first rows of replicate 96 well plates (Nunc™, Thermo-Fisher Scientific, Waltham, Massachusetts, USA) with 200 μl of egg water (temperate autoclaved system water). A second group of embryos was placed in 2.5 ml tubes (Thermo-Fisher Scientific, Waltham, Massachusetts, USA) (50 embryos/ tube) with 2 ml egg water.

Embryo irradiation and dosimetry

After collection, embryos were transported to the FIGARO experimental irradiation facility at NMBU, Ås, Norway (^{60}Co source, activity ~ 420 GBq). For both the toxicity endpoints and transcriptomic analyses, external gamma irradiation of zebrafish embryos commenced at 2.5 hpf with total doses to water ranging from 1.62 mGy– 3496 mGy during a 3 hour, 43.8 hour and 92 hour irradiation timespan (Table 1). Dose rates of 0.4, 3.9, 15 and 38 mGy/h were used for general toxicity analyses, and 0.54, 5.4 and 10.9 mGy/h for the transcriptomic analyses (Table 1). The experiments for both analyses were performed at separate time intervals. All exposures included corresponding controls. For the adverse effect observations and RNA-seq, 96-well plates and 2.5 ml tubes, respectively, were positioned at different distances from the gamma source corresponding to the dose rates to water (D_{Water}) presented in Table 1.

Field dosimetry (air kerma rates measured with an ionization chamber) was traceable to the Norwegian Secondary Standard Dosimetry Laboratory (Norwegian Radiation Protection

Table 1. Exposure groups and dosimetry. Total doses from measured dose rates during different time periods of exposure. **(A)**: 43.8 hours; **(B)**: 92 hours and **(C)**: 3 hours.

Developmental traits	**Dose rate D_{Water} (mGy/h)***		**0.4**	**3.9**	**15**	**38**
	Total dose D_{Water} (mGy)	**(A)**	17.5	171	657	1664
		(B)	36.8	359	1380	3496
RNA-seq	**Dose rate D_{Water} (mGy/h)***		**0.54**	**5.4**	**10.9**	
	Total dose D_{Water} (mGy)	**(C)**	1.62	16.2	32.7	

*Uncertainty (K = 2) for dose rate estimates is ~10% (Bjerke and Hetland, 2014).

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Authority, NRPA, Oslo, Norway) [18]. Average dose rates to water in the first and second rows of microplate wells were estimated according to established technical guidelines [19] and used as a proxy for dose rates to the fish embryos. Controls were placed in the same room, outside the beam cone and shielded by lead reducing the external (background) dose rate to $\leq$ 0.35 μGy/h (Thermo Eberline FHT6020). The irradiation room was thermostatically heated (28 ± 2˚C), and had a 14–10 hours light-dark cycle (250–320 lx). To minimize variation in temperature, 2 control groups were used for the transcriptomic analyses.

Sampling procedure and experimental analysis of general toxicity endpoints

At approximately 48 hpf, half of the 96-well plates were removed from exposure (Table 1, Group "A"), while the remaining embryos were irradiated until 96 hpf (Table 1, Group "B"), n $\geq$ 145/ group. To determine the general toxicity in terms of adverse effects on survival and hatching, the embryos and larva were manually observed in a stereo microscope (3.5–45x) at 48 and 96 hpf in group "A", and at 96 hpf in group in "B" (S1 Table). Additionally, the occurrence of deformities was observed at 96 hpf in both "A" and "B". Analysis of endpoints was performed according to observations guidelines [20]. After observations, the larva used in this study was euthanized (prior to independent feeding at 120 hpf) using Tricaine (MS-222) (Sigma-Aldrich) overdose followed by rapid freezing (-70˚C). For RNA extraction, embryos were sampled at 5.5 hpf (Table 1, Group "C") in 2.5 ml tubes (n = 50/ sample).

Transcriptome analysis at 5.5 hours post fertilization

RNA sequencing was conducted to compare gene expression profiles between the controls and the 0.54, 5.4 and 10.9 mGy/h exposed embryos. Total RNA was isolated from embryos exposed between 2.5 hours and 5.5 hpf with TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit (Qiagen) according to manufactures' instructions. Briefly, 1 ml TRIzol was added to each sample consisting of 50 embryos and homogenized using Magnalyser Beads (Roche Diagnostics). Isolated RNA was DNase I (Qiagen) treated for 20 min at 25˚C before further purification. Each sample was eluted in 50 μl RNase-free water and stored at – 80˚C until required. RNA purity and yield (A260/A280 > 1.8, A260/A230 > 2, yield > 200 ng/μl) was determined using NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality (RIN > 8.5) was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using RNA Nano LabChip Kit (Agilent Technologies). None of the samples showed any signs of degradation or impurities. Photometric parameters and RNA integrity number (Bioanalyzer; Agilent technologies, USA) determined the quality of RNA sequenced samples. The RNA was sequenced (Illumina HiSeq 2000) at BGI Tech Solutions Co., Ltd., Hong Kong. Three single-end libraries (biological replicates), in the 5.4 and 10.9 mGy/h groups and a duplicate per 0.54 exposure group were sequenced. The bioinformatics analysis pipeline of the

RNA sequencing data is presented in S1 Fig. Quality assessment of raw reads (49 nt long) and adapter trimming was performed using Trim Galore! v0.3.7, a wrapper tool around Cutadapt and FastQC to consistently apply quality and adapter trimming to FastQ files [21,22]. Only reads with Phred score > 20 were kept. Afterwards, using TopHat v2.0.9 [23] with bowtie1, reads were mapped to the ZF genome (version Zv9, release 76) downloaded from Ensembl (http://www.ensembl.org/Danio_rerio/Info/Index). Options -g (maximum multihits number) was modified from its default value to 1, - -no-coverage-search was allowed, - -library type was set to "fr-unstranded" and -p (number of threads) was restricted to 4. As for bowtie1 options, -q (fastq files), -v (report end to end), -k 20 (report up to 20 good alignments), -m 20 (suppress all alignments if > 20), -S (to use SAM format) were used. BAM files were uploaded into Seqmonk [24] for visualization of aligned and mapped reads and read counting. Reads were counted as reads exactly overlapping with exons and the resulting count table was analyzed for gene expression under edgeR v3.4.2 Bioconductor [25]. The RNA-seq experiment was deposited in SRA database (https://www.ncbi.nlm.nih.gov/) and is publically available under accession SRP096352.

Quantitative real-time PCR (qPCR) analysis

To verify the RNA-sequencing results, eight differently expressed genes were selected for qPCR analysis, based on their common differential expression in the exposure groups. The DNA Sequence information for each gene was retrieved from genebank (http://www.ncbi.nlm.nih.gov/Genbank). The Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/) was used to design primers. These primers were analyzed for oligo duplex and primer dimers. Amplicons which are shorter than 130 bp and spanned over different exons were selected (S1 Table). The cDNA was prepared from 1 μg of same total RNA used for RNA sequencing analyses (n = 3). For cDNA synthesis, Superscript III reverse transcriptase (Invitrogen, USA) and random hexamer primers were used according to product specifications. The qPCR was performed on a LightCycler® 96 Real-Time PCR system (Roche, Mannheim, Germany) using LightCycler® 480 SYBR Green I Master (Roche). Each cDNA was analyzed in a duplicate and composed of 5 μL mastermix, 2 μL primer mix (5 μM of each forward and reverse), and 3 μL of each 10× diluted cDNA sample in a total volume of 10 μL. The cycling parameters were 10 min pre-incubation at 95˚C, followed by 45 cycles of amplification at 95˚C for 10 sec, 60˚C for 10 sec and 72˚C for 8 sec, followed by a melting curve from 60˚C to 95˚C. The qPCR assay was performed in three biological replicates. RefFinder analysis tool (http://fulxie.0fees.us/) [26] was used to find the best candidate reference genes. Analyzed reference genes were *hmbs* (hydroxymethylbilane synthase), *b-actin* (beta-actin) and *rps18* (ribosomal protein S18). For all exposure groups, *hmbs* was found to be the most stable housekeeping gene. The expression of each target gene transcript was normalized to *hmbs* and the fold change was calculated using the ΔΔCT method.

Ingenuity pathway analysis

For predicted networks/pathways and biological function analyses of differently transcribed genes, IPA software (http://www.ingenuity.com, Ingenuity Systems Inc., Redwood City, CA) was used. The Core analysis and comparison sub analysis blocks were used to determine the interaction networks of up- and down-regulated genes, upstream regulators and biological states (diseases and bio functions) in each and across the three exposure doses. A right-tailed Fisher's exact test was used to determine the probability that each biological function is due to chance alone and the association identified as statistically significant and non-random ($p < 0.05$). The results in gene regulation are given as negative logarithms of the p-value

computed by numbers of genes participating in the process, number of genes from the reference dataset mapped to the network and the size of the entire network in the Ingenuity knowledge database. The upstream regulator analysis examines how many known targets of each transcription regulator are present in the dataset, comparing their expression to what is known from the literature. In the present study, ranking by overlap p-value (cutoff p-value ≤ 0.001) and filtering for genes, RNAs and proteins in order to predict the most relevant transcriptional regulators was used. For the predicted activation state of the transcription regulators, a z-score describing the quantity of activated (z-score > 0) or inhibited predictions (z-score < 0) was calculated. However, this prediction is not available for upstream regulators with less target genes in the datasets (i.e. in lower dose-rates), and could not be considered to determine the most likely relevant regulators where the value of the correction for the z-score was too high (bias > 0.25).

Statistical analyses

After establishing the database for the general toxicity observations, tabulating and checking for errors in Excel®, data were transferred to Stata (MP/14 for Windows, StatCorp, College Station, TX). Confidence intervals were calculated using the proportion command for each of the outcomes survival, hatching and deformities at dose levels and the two exposure durations. Further logistic regression reported as odds ratios (OR) was used to estimate the effect of the treatments on hatching, survival and deformities and standard methods were used to check model fit. If significant, multiple comparisons were conducted using Tukey's or Dunnett's tests (Graphpad Prism 6, La Jolla, USA). Statistical significance was set to $p < 0.05$.

For analysis of gene expression, the dataset was TMM normalized first (trimmed mean of M-values, edgeR v3.4.2 Bioconductor, Robinson, McCarthy, and Smyth 2010), followed by data exploration using the statistical package R v3.0.2 [27]. Data was explored for descriptive statistics such as: minimum, maximum, 1st quantile, 3rd quantile, median, mean, standard deviation, also the similarity among samples was determined by correlation analysis and hclust (ward method) analysis to determine the distance between samples. The statistical analysis of differentially expressed genes (DEGs) was based on pairwise comparison between treatment and control RNA-seq samples (biological replicates) with a cut off set to ± 0.40 log2 fold change (1.3 FC). The FDR (false discovery rate) was set up to a significance of $p \leq 0.05$. Venn diagram (Venny v2.1, Oliveros, (2007–2015)) was used to explore overlapping differential expressed genes among radiation treatments. For qPCR, obtained mean relative gene expression values (exposed vs. control) were compared to mean relative gene expression values for the same genes from RNA-seq and a Pearson's correlation coefficient was calculated ($p < 0.05$) for all three exposure groups (Graphpad Prism 6, La Jolla, USA).

Results

General toxicity

To determine the effects of gamma radiation on the embryonic and larval development, the survival, hatching rate and deformities were assessed at 48 and 96 hpf. Compared to controls, a decrease in survival was observed in all exposed groups, albeit only the 38 mGy/h group was statistically significant, both after a 43.8-hour and 92-hour exposure (Table 2, S2 Table). The timing of hatching was significantly affected by irradiation, as a premature onset of hatching in the 0.4 mGy/h group ($p < 0.0001$) and a delayed onset of hatching in the 38 mGy/h group ($p = 0.0072$), respectively, were observed (S2 Table). The total hatching was above 95% in all exposure groups, however, with significantly lower total hatching in fish exposed to 15 mGy/h compared to controls (Table 2, S2 Table). The deformity frequency at 96 hpf increased linearly

Table 2. Adverse effects. Adverse effects on total hatching, survival and deformities at 96 hpf, reported as Odds Ratios with 95% confidence intervals and related p-values compared to the base level (OR = 1). The OR describes the risk for occurrence of an adverse effect, given the two variables: dose rate and duration of exposure to the specified dose-rates. Significance denoted with (*).

Odds ratio (95% CI); p-values compared to base level				
Variables		**Hatching**	**Survival**	**Deformities**
Dose rate (mGy/h)	**Control**	1.00 (-)	1.00 (-)	1.00 (-)
	0.4	0.40 (0.08–2.10); 0.28	0.65 (0.41–1.04); 0.07	5.00 (1.09–23.0); 0.04*
	3.9	0.39 (0.08–2.03); 0.26	0.66 (0.42–1.06); 0.09	8.44 (1.93–37.0); 0.005*
	15	0.13 (0.03–0.59); 0.008*	0.75 (0.47–1.20); 0.23	13.43 (3.16–57.0); <0.001*
	38	0.26 (0.05–1.24); 0.09	0.46 (0.29–0.73); 0.001*	18.4 (4.37–77.6); <0.001*
Duration of exposure (hours)	**43.8**	1.00 (-)	1.00 (-)	1.00 (-)
	92	0.77 (0.45–1.33); 0.35	0.99 (0.78–1.27); 0.98	1.61 (1.09–2.37); 0.015*

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in response to dose for both the 43.8- and 92-hour exposure (linear regression, $R^2 = 0.93$ and $R^2 = 0.99$, respectively) and was significantly higher than in controls ($p < 0.05$) in all exposure groups, except from the 43.8-hour exposure to 0.4 mGy/h and 3.9 mGy/h (Fig 1, Table 2). The lowest dose rate (0.4 mGy/h) caused significant increase in deformities ($p = 0.049$) only in the group exposed for 92 hours (Fig 1, Table 2). The most frequently observed deformities were retardation in development manifested as failed hatching and absence of pigmentation, irregularities in formation of the head and eyes, as well as a short tail or lack of a tail (S15 Fig). In summary, a significant dose dependent response was observed for deformities and mortality, whereas hatching showed a non-monotonic dose-response.

Gene expression analysis

Transcriptional analysis was performed at the gastrula stage 5.5 hpf in order to identify potential changes to the transcriptional program induced by the gamma exposures. An average of 27 million reads (49 nt long) were obtained in both the treated and control groups. The mapping statistics showed a high grade of similarity among all samples (S2 Fig, S3 Table). The expression dataset analysis for replicability and distribution by means of multidimensional scaling

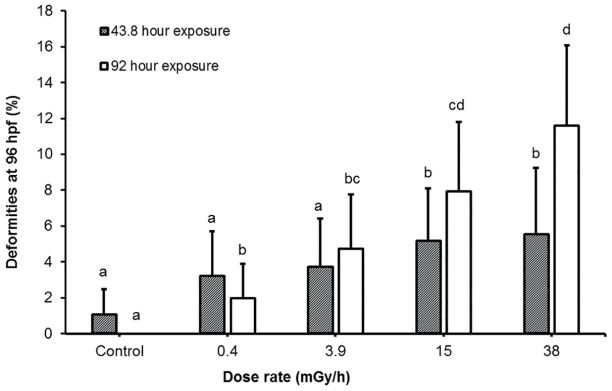

Fig 1. Deformities. Deformities observed at 96 hpf which occurred after a 43.8- and 92-hour exposure to the specified dose rates. The exposures had separate controls. Values presented as mean percentage ± 95% confidence interval ($p < 0.05$).

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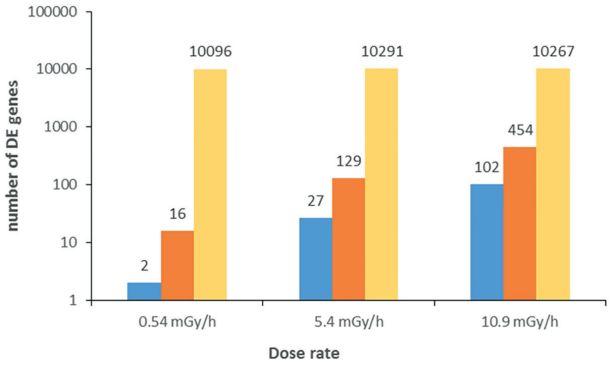

Fig 2. Expressed and differentially expressed genes in each exposure. Threshold set to FC ±1.3 FDR < 0.05; down-regulated genes (blue), up-regulated genes (red) and total number of expressed genes (yellow).

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plot (MDS) showed a clear difference between exposed and their respective controls (S3 Fig). A total number of ~10000 genes was expressed in all samples, while the number of differentially expressed genes (DEGs) showed a clear dose rate dependency (Fig 2 and S4 Fig and the full list of DEGs is available in S4 Table).

In the 0.54 mGy/h exposure group, 16 genes were up-regulated (FC from 1.3 to 2.2) and two genes down-regulated with FC from 1.3 to 1.7 (Fig 2, S4 Table). In the 5.4 mGy/h exposure group, 129 genes were up-regulated with FCs from 1.3 to 674, while 27 were down-regulated with FCs from 1.3 to 2 (Fig 2, S4 Table). In the 10.9 mGy/h exposure group 556 DEGs were split between 454 up-regulated with FCs from 1.3 to 3.2 and 102 down-regulated genes with FCs of 1.3 to 2.4 (Fig 2, S4 Table). Among the DEGs, two were found to be differentially expressed in all three exposure groups: *pfkfb3* (6-phosphofructo-2-kinase-fructose-2,6-biphosphatase 3) up-regulated in 0.54, but down-regulated in the 5.4 and 10.9 mGy/h; *crabp2b* (cellular retinoic acid binding protein 2b) which is similarly up-regulated in all exposure groups (Fig 3A, S4 Table).

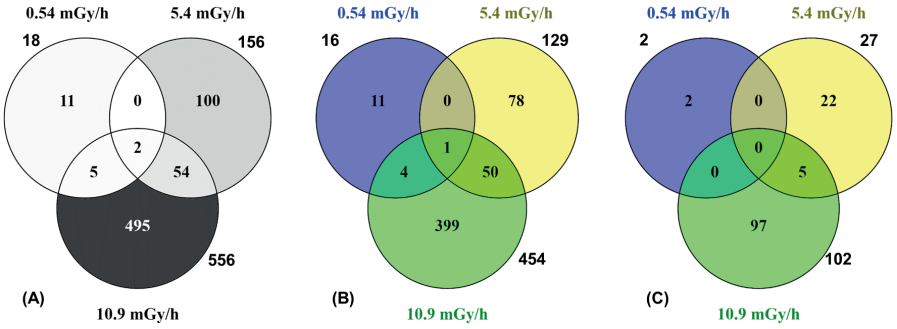

Fig 3. Venn diagram showing common and unique sets of differentially expressed genes between exposure treatments. Total number of **(A)** common genes between 0.54, 5.4 and 10.9 mGy/h after pairwise comparison to controls (FC ± 1.3, FDR < 0.05); **(B)** Up-regulated genes; **(C)** Down-regulated genes.

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In addition, five and 54 DEGs were overlapping between the 0.54/10.9 and 5.4/10.9 mGy/h groups, respectively (Fig 3A). As for the up-regulated DEGs, four genes were overlapping between the 0.54 and 10.9 mGy/h, while 50 genes were overlapping between the 5.4 and 10.9 mGy/h group (Fig 3B). Furthermore, down-regulated overlapping genes were found (five genes) only between the 5.4 and 10.9 mGy/h exposure (Fig 3C). The most up-regulated common gene in the 10.9 mGy/h group, *tfa* (transferrin-a), was also highly up-regulated in the 5.4 mGy/h group (S4 Table), although the FC values differed between the groups (S4 Fig). In addition, lipoprotein genes: *apoBb* (apolipoprotein Bb), *apoA1a* and *apoA1b* (apolipoprotein A-Ia/Ib), and common with the 10.9 mGy/h group, *apoA-IV* (apolipoprotein A-IV) were amid the top up-regulated in the 5.4 mGy/h group. The most down-regulated common gene between 5.4 and 10.9 mGy/h was *vegfab* (vascular endothelial factor Ab (S4 Table). The expression levels for up and down-regulated genes overlapping between the three dose rates are presented in Fig 4.

Pathway analysis

General pathways analysis. The core analysis IPA software tool was used to find the most significantly affected biological signaling (canonical) pathways by the DEGs in the three exposure groups. A statistically significant difference between the signaling pathways in the 0.54 mGy/h exposure group compared to the 5.4 and 10.9 mGy/h was found. In the lowest dose rate, the most affected signaling pathway was retinoic acid receptor activation *(RARa)*, followed by *RA* mediated apoptosis and glutathione mediated detoxification signaling (Fig 5).

Interestingly, compared to the signaling pathways in 0.54 mGy/h, the higher doses demonstrated some *RA* pathway activity, but this was below the significance threshold (Fig 5, S11 Fig). In the two higher dose rates, the most significantly affected signaling pathways were *eif2* (eukaryotic initiation factor 2) and *mTOR* (mechanistic target of rapamycin), which were not affected (p-value > 0.05) in the lowest dose rate group (Fig 5, S12 and S13 Figs).

Toxicological pathways. To identify the top diseases and biological functions of altered genes in each exposure group, the gene expression data sets were compared between all exposure groups in IPA. The DEGs in the datasets were shown to be involved in gene networks associated with various embryonic developmental processes and cell functions (Fig 6).

In the 0.54 mGy/h exposure group, gene networks associated with apoptosis and other cell death mechanisms were active, while gene networks associated with organismal death and proliferation of tumor cell lines (Fig 6) were inhibited. In contrast, in the 5.4 and 10.9 mGy/h groups, gene networks associated with apoptosis were inhibited and gene networks related to proliferation of tumor cell lines were active. Similarly to the lower dose rate exposure, the gene networks related to organismal death in these groups were inhibited. Comparison of expression of apoptosis genes showed that of total 129 DEGs found in the network, 5 were found in the 0.54 mGy/h group (all up-regulated), 40 in the 5.4 mGy/h group (34 up- and 6 down-regulated) and 101 in the 10.9 mGy/h (83 up- and 18 down-regulated) (S14 Fig). The one common and similarly expressed gene between all exposures in the apoptosis network was *crabp2b*, while expression levels of 16 common genes between 5.4 and 10.9 mG/h groups differed (S14 Fig). Additionally, networks associated with cell movement, growth, cardiovascular developmental processes and cancer development were significantly activated in the two higher dose rate exposure groups; albeit more significantly in the highest dose (Fig 6).

Key regulators. A transcription factor enrichment analysis was conducted to identify upstream regulators of transcriptional networks modulated by ionizing radiation. A total of 159, 632 and 939 transcription regulators in the 0.54, 5.4 and 10.9 mGy/h exposures were identified, respectively (S5 Table). *Myc* (v-myc avian myelocytomatosis viral oncogene derived

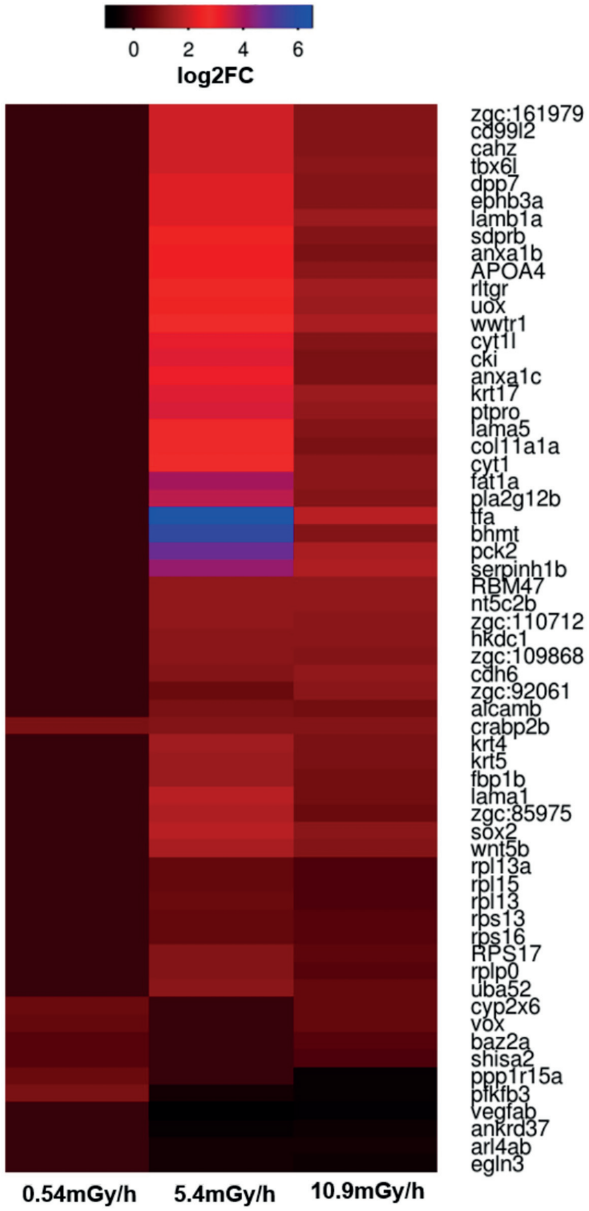

Fig 4. Expression levels for up and down-regulated overlapping genes between exposures. Expression levels in the 0.54, 5.4 and 10.9 mGy/h groups are given in log2 of the fold changes (FC).

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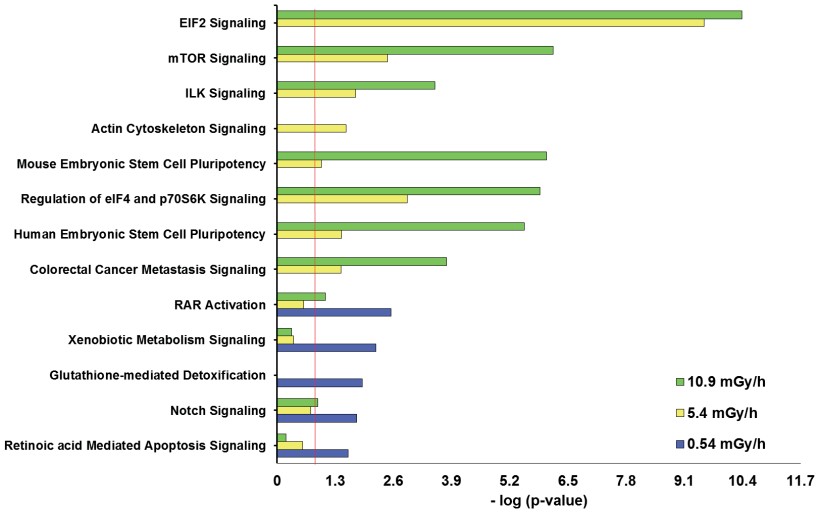

Fig 5. DEG functional analysis (IPA). Top signaling pathways in canonical pathway comparison between all exposure groups: 0.54 mGy/h, 5.4 mGy/h and 10.9 mGy/h. The rankings were based on Fisher's exact test and high-ranking categories are displayed along in a decreasing order of significance from top. The threshold line (red) denotes the cut-off for significance (p-value 0.05).

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homolog), *TNF* (tumor necrosis factor), *tp53* (tumor protein p53) and *hnf4a* (hepatic nuclear factor 4, alpha) were identified as upstream regulator genes in all exposure groups (S5 Table, S5–S8 Figs). In the two higher dose rates, *TGFb1* (transforming growth factor, beta 1) and *cebpa* (CCAAT/enhancer binding protein C/EBP, subunit alpha) were found to be significant upstream regulators (S5 Table, S9 and S10 Figs).

Validation by quantitative real-time PCR (qPCR)

In order to validate the RNA sequencing results, eight differently expressed genes were selected for quantitative real-time polymerase chain reaction (qPCR) analyses in the groups exposed to 0.54, 5.4, 10.9 mGy/h and controls. The selected genes and their respective fold changes are presented in Table 3. The data from real-time qPCR and the RNA-sequencing showed a good correlation (Pearson's linear correlation coefficient $r = 0.89$, $p < 0.0001$).

Two of the selected genes are common between all three exposure groups (*pfkfb3* and *crabp2b*). Three are common between 0.54 and 10.9 mGy/h groups (*vox*, *ppp1r15a* and *shisa2*) and between 5.4 and 10.9 mGy/h (*sox2*, *tfa* and *eef2b*). Only two genes were found to have an opposite regulation at one of the dose rates; *pfkfb3* in the 5.4 mGy/h group was up-regulated, while *shisa2* in the 10.9 mGy/h was down-regulated (Table 3).

Discussion

Previous studies in zebrafish reported underlying molecular mechanisms responsible for adverse biological effects such as DNA damage [28,29], ROS, oxidative stress, apoptosis, bystander effects [30–32] and also genetic [32–34] and epigenetic changes [8] following exposure to ionizing radiation. However, most of the genetic responses were studied following acute exposures.

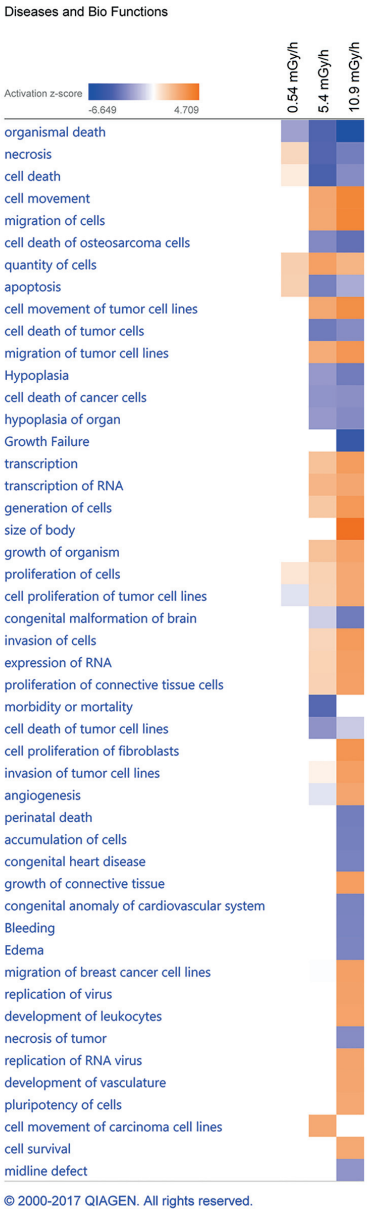

Fig 6. The most affected biological function and diseases networks. IPA predictions in comparison analysis between 0.54 (1) 5.4 (2) and 10.9 mGy/h (3) group. The heat map is based on the activation z-score, consistent with the particular activation state: "activated" (orange) or "inhibited" (blue). Z-score cut off set to ± 2.5 (arbitrary).

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Table 3. Real time qPCR verification of RNA sequencing. Results presented as fold change (FC) for eight genes. The (n.a.*) refers to not differentially expressed, while the fold change was not available for this gene in this group.

Gene ID		FC RNA-seq			FC RT qPCR		
	Dose rate (mGy/h)	0.54	5.4	10.9	0.54	5.4	10.9
pfkfb3		1.8	1.3	1.5	1.8	1.4	0.9
crabp2b		1.9	2.1	2.0	2.9	2.3	2.0
vox		1.5	1	1.5	1.6	2.2	0.7
ppp1r15a		1.6	0.8	1.5	1.8	1.3	0.6
shisa2		1.3	1	1.3	1.5	1.9	0.7
sox2		n.a.*	3.3	2.2	3.0	1.6	0.8
tfa		n.a.*	93.2	3.2	1.2	40.7	20.7
eef2b		1.1	1.3	1.2	0.6	1.6	1.4

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In this study, we focused on potential adverse effects on the embryonic development caused by low dose and dose rate ionizing radiation. To this end, we investigated the developmental and toxicological effects of continuous gamma irradiation (doses between 17.5–3496 mGy) during early blastula (2.5 hpf; 256-cell stage), through to the hatching period (48–72 hpf) and early larval development, i.e., life stages associated with numerous delicate morphological changes [35].

To investigate molecular initial events associated to effects of ionizing radiation later in development, analysis of the gastrula stage 5.5 hpf embryo transcriptome was carried out using RNA sequencing combined with a functional gene network analysis software.

Adverse effects of radiation in developing embryos

The results from the observations of survival, deformities and total hatching at 96 hpf showed that radiation caused a significant dose-dependent reduced survival, affected the total hatching and increased the number of deformities. (Table 2, Fig 1). The exposure dose rates for evaluating the phenotypic effects used in the present work (0.4, 3.9, 15, and 38 mGy/h) were higher than the ERICA screening value of 10 μGy/h (0.24 mGy/d) [10]. However, the dose-rates span the proposed level of 0.42 mGy/h (10 mGy/day), which is considered to be a level below which there is not likely to be any detrimental effect on aquatic populations (UNSCEAR Report, 1996) and the derived consideration reference levels (DCRL) for fish (~0.42 mGy/h– 40 mGy/h), at which there are "likely to be some observable adverse effects occurring to individuals" [36].

The lowest dose rate in the present work at which deformities were observed was 0.4 mGy/h (total dose 36.8 mGy). The onset of hatching was premature in the 0.4 mGy/h exposure group (17.5 mGy total dose, Table 1), and significantly delayed in the 38 mGy/h group (1664 mGy total) (S2 Table). The total hatching in these groups was unaffected (Table 2). Interestingly, in a previous study of hatching intervals following X-ray exposure during the blastula stage, earlier hatching was associated with low dose (25 mGy at 0.43 Gy/min), while higher doses (250–500 mGy) delayed the onset of hatching [37]. In addition, other studies report that both low and high doses had an accelerating effect on the hatching interval [28,32]. In the 15 mGy/h group, the total hatching was significantly decreased (Table 2). A similar result was reported after X-rays exposure to 500 mGy [37], which is close to the total dose (657 mGy) in the present 15 mGy/h exposure group (Table 1). The survival, although exceeding 82% in all groups (S2 Table), was significantly lower than control in the 38 mGy/h group (Table 2) after both 43.8 and 92 hours of exposure. Previously, mortality in zebrafish embryos was reported only for acute exposures from 1 to 24 hpf (1–10 Gy, X-rays) [38]. Although embryo mortality

from the 43.8 h exposure was observed at 48 hpf, no further increase was observed at 96 hpf (S2 Table). Collectively, these observations might indicate that the early developmental stages, prior to the hatching interval, are more sensitive to the effects of ionizing radiation, resulting in mortality. Other studies have reported no significant differences in embryo viability after receiving acute ionizing radiation doses ranging from 1–10 Gy [34,39], nor following continuous exposures up to 24 mGy/h (2280 mGy) [32], although the latter induced multiple deformities. Generally, the adverse effects on embryo development from the continuous exposure in the present study showed considerable variability in response to lower and higher doses, and in order to elucidate potential molecular mechanisms behind the observed effects, this variability was further studied by transcriptomics.

The 5.5 hours post fertilization embryo transcriptome

The gene expression analysis was performed at the late blastula / early gastrula stage (~ 5.5 hpf), a critical stage of embryogenesis, characterized by intensive cell proliferation and specification [17,35]. At this stage the zygotic genome is activated, while the inherited maternal transcript (synthesized during oogenesis and stored in the egg) is degraded [15]. Thus, changes in transcriptome profile can be attributed to radiation effects on the transcriptional program of the embryo's own genome. The choice of this stage served two major aims: early toxic effects and accompanying stress or defense mechanisms would be reflected at the transcriptional level, and deviation of the transcriptional program at this stage could be indicative or predictive to adverse outcome observed later during embryogenesis. The applied dose rates were selected to both encompass a toxic effects dose response and to be environmentally relevant. The RNAseq analysis was thus conducted on low total doses, which consequently should produce only low level of DNA damage. This strategy enables investigation of more subtle and less well-described molecular effects of ionizing radiation in addition to genotoxicity. The fact that significant transcriptional changes could be observed from a 3 hour exposure to total doses from 1.6 to 33 mGy corroborates the validity of the approach. Moreover, the observed responses were intelligibly connected to the adverse outcomes observed at the phenotype level. This correlation is important with respect to the level of dose rates and total doses that would be required to elicit changes at the molecular level.

The number of similarly and differently expressed genes, as well as overlapping DEGs, showed a clear dose-response effect in the gamma exposed embryos with the lowest number of modulated genes in the 0.54 mGy/h group and with an increasing number in the two highest exposure groups (5.4 and 10.9 mGy/h) (Fig 2). A considerable variation in FC between the 5.4 and 10.9 mGy/h groups was observed (S4 Fig), but a total of 56 DEGs were common in these exposure groups.

Two genes, *pfkfb3* and *crabp2b*, were found to be differentially expressed in all exposures. The *pfkfb3* gene is involved in regulating the expression of cyclin-dependent kinase 1, which promotes proliferation and survival in tumor cells [40] by protecting cancer cells against oxidative stress through S-glutathionylation and glucose metabolism switch to the pentose phosphate pathway [41,42], and thereby counteracting ionizing radiation generated ROS. The *crabp2b* gene is a one of the two zebrafish *crabp2* genes orthologous known to encode retinoic acid (*RA*) protein family and lipocalin/cytosolic fatty acid binding protein family. Interestingly, the *crabp2b* was found to be similarly up-regulated (FC ~ 2) in all three irradiation treatments in both the RNA-seq and the qPCR (Table 3, Fig 4). Retinoic acid is the biological active metabolite of Vitamin A and *crabp2* regulates the access of retinoic acid by binding with the nuclear retinoic acid receptor alpha (*RARa*) [43] and helps modulating the RA gradient, which is important for the development of vertebrates, including humans [43,44]. Deficient or excess

levels of vitamin A have induced malformations in experimental animals and humans, indicating that the concentration must be kept within a narrow range [45,46]. Furthermore, *crabp2b* is associated with regulation of the hindbrain anterior-posterior axis development [47], and is expressed in structures requiring the retinoic acid during embryonic development, such as the CNS, dorsal retina, branchial arches, epidermis, otic vesicle and pectoral fins [43]. Considering the increased number of malformations observed in irradiated fish, it could be hypothesized that this is in part induced by modification of the *crabp2b* gene.

Among the common genes modulated by 5.4 and 10.9 mGy/h exposures, the most significantly up-regulated gene is *tfa* (Fig 4). This gene is critical for iron transport and iron regulated hormone expression [48], and is involved in the immune response to bacterial infection [49]. A decrease in concentration of the transferrin protein was found in blood plasma of radiological accident victims compared to blood plasma from non-irradiated individuals, and reported as a possible mutagenic factor [50]. However, a protective role of the transferrin pathway for antioxidant repair and sequestering metals was also suggested [51]. Additionally, increased chromosomal damage combined with increased transferrin was demonstrated in lymphocyte cultures following exposure to 1 Gy of ionizing radiation, suggesting that transferrin is affected by radiation [52].

The highly up-regulated apolipoprotein genes in the two higher dose rate exposure groups, and notably the 5.4 mGy/h group (*apoBb*, *apoA1a*, *apoA1b* and *apoA-IV*), could point to radiation affecting mechanisms behind the lipid metabolism and transport from yolk cells to the embryo (S4 Table) [53]. Apolipoprotein genes play a role in reducing fat intake during embryonic development, as previously shown in zebrafish [53] and humans [54], causing malnutrition of the embryo, which may have disrupted normal development. In addition, apolipoprotein genes were reported to negatively regulate (*apoB*) [55], or even inhibit the angiogenesis (*apoA1*), in a *vegf* down-regulation dependent pathway [56]. Relatedly, among the common genes modulated by 5.4 and the 10.9 mGy/h treatments, the most significantly down-regulated gene in both data sets is *vegfab* (FC 1.6–2), an isoform of the human ortholog *VEGF-A* (Fig 4) [57]. At early life stages, this gene mediates differentiation of endothelial cells and early vascular development and angiogenesis (formation of new blood vessels) [58], including retinal angiogenesis [59]. In developed individuals, *vegf* stimulates the angiogenesis [60], either in a physiological (such as tissue repair processes) or pathological states (such as tumor growth), and *vegf* activity has been shown to be stimulated through an intracellular increase in ROS generated as a result of exposure to ionizing radiation [61]. In an experimental study of radiation effects in mice, *vegf* together with *eif2* was modulated in bladder tissue [62].

Molecular pathways—Potential mechanisms of radiotoxicity

A transcription factor enrichment analysis was performed to investigate whether gamma induced pathways or gene networks could be ascribed to master regulators. IPA analyzes of the datasets identified upstream regulator genes, which were not necessarily significantly affected, but may play key roles in the regulation of DEGs. The transcription factors *myc*, *TNF*, *tp53* and *hnf4a* were found to be in central positions of functional networks of modulated genes in comparison between the three exposure groups (S5 Table). Additionally, *TGFb1* and *cebpa* were identified as key regulators at the two higher dose-rates (5.4 and 10.9 mGy/h) (S5 Table).

Myc was found to be one of the top upstream regulators, in all three exposures (S5 Table, S5 Fig) and is implicated in the regulation of various processes in the cell, such as growth and proliferation, migration, differentiation and cell death. Up-regulation of the oncogenes *myc* and *mycn* is associated with poor outcomes of several cancers, such as aggressive neuroblastoma

[63], large B cell lymphoma [64], acute myeloid leukemia (AML) [65] and nephroblastoma (Wilms tumor) [66]. Combined *Myc* up-regulation with an altered retinoic acid (*RA*) pathway activity worsens the prognosis of such cancers [67]. Furthermore, *TNF* was found to regulate a high number of molecules in the datasets (S5 Table, S6 Fig). This cytokine was previously shown to be strongly protective at lower ionizing radiation doses for the hematopoietic stem cell system [68] and via selective destruction of blood vessels in T-cell tumors [69]. Interestingly, the activity of tumor necrosis factor-alpha (*TNF-α*) in cell lines was found to be antagonistic to the activity of *TGFb* [70]. Another identified upstream regulator, *tp53* (S5 Table, S7 Fig), is known to regulate apoptosis in response to DNA damage [71], but was also demonstrated to be a critical factor for normal development and survival in zebrafish embryos after exposure to ionizing radiation [72,73]. *Tp53* was found to decrease, but also to concomitantly regulate tumor suppressive *TGFb* responses through Smad2/3 DNA complexes [74]. Although not differentially expressed in the 0.54 and 5.4 mGy/h datasets, *hnf4a* is found to be a transcription regulator for a large number of DEGs in all exposure groups (S3 Table, S8 Fig). This transcription regulator was found to be up-regulated in the blood of patients exposed to ionizing radiation [75], and in a human tissue model exposed to low dose gamma radiation [76]. *Hnf4a* regulates the gastrulation [77], the developmental period during which the morphogenetic cell movements, along with production of the three primary germ layers (ectoderm, mesoderm and endoderm) and the embryonic axis (> 5.25 hpf) occur [78]. It is mainly expressed in the digestive system and in the brain. This data propose *hnf4a* as a factor involved in the induction of biological effects of radiation in humans as well as in other vertebrate species.

An activated predicted upstream regulator in both the 5.4 and 10.9 mGy/h, but not in the 0.54 mGy/h exposure was *TGFb1* (S3 Table, S9 Fig). The *TGFb1* cytokine regulates a variety of functions, and is known to be a mediator of the apoptosis, redox homeostasis and bystander effects in tissues and cells in response to radiation [69,79–81]. In addition, *TGFb* was found to co-regulate angiogenesis in tumors with *vegf* [82]. IPA also identified *cebpa* as a regulator gene among the common DEG in the two higher dose rates (5.4 and 10.9 mGy/h) (S10 Fig). In the study of B-cell chronic lymphocytic leukemia (B-CLL) patients in the post-Chernobyl period, similar key regulator genes, gene networks and signaling pathways were altered [83]. *Cebpa* is associated with regulation of hematopoiesis, hematopoietic stem cell migration, liver development and regulation of transcription [84]. It is predominantly found in mature myeloid cells and is required for the differentiation of myeloid cells in order to prevent the occurrence of myeloproliferative diseases [85]. Diseases associated with a down-regulation of *cebpa* include acute myeloid leukemia with *cebpa* somatic mutations [86]. Moreover, other studies have reported that ionizing radiation caused increased expression of *cebpa*, which was associated with a reduction of hematopoietic stem cells and the self-renewal of multipotent hematopoietic progenitor cells [87]. The similar regulation of these genes in mammals and zebrafish may suggest that similar mechanisms might be behind the molecular changes following exposure to radiation.

The signaling pathways affected most significantly by the 0.54 mGy/h exposure (*RAR* activation, *RA* mediated apoptosis and glutathione mediated detoxification seem to be consistent with the described repair mechanisms occurring at low doses. This adaptive response to low doses of ionizing radiation in biological systems is mainly characterized by antioxidant mediated detoxification of ROS, more rapid DNA repair, apoptosis signaling and stimulated immune response [88,89].

In the 5.4 and 10.9 mGy/h treatments, *eif2* and *mTOR* were the most significantly up-regulated signaling pathways. A significant role of the *eif2* signaling pathway is the adaptive response to stress by regulating the formation of translation initiation complexes, which leads

to reduced recognition of AUG start codons and therefore total translational inhibition and the induction of apoptosis [90]. The *mTOR* (mammalian target of rapamycin) signaling pathway is centrally involved in cell metabolism, growth, proliferation and survival via regulation of protein synthesis and mRNA stabilization [91]. Furthermore, it is activated during tumor formation and modulation of angiogenesis, development of diabetic retinopathy [92] and in radiation induced apoptosis [93]. A dysregulation of *mTOR* was reported to affect the premature aging of cells and destabilize the cytoskeletal structure after exposure to chronic ionizing radiation, in addition to changes in the *eif2* signaling pathway [94]. The *eif2* signaling pathway was in comparison to the present results found to be down-regulated in the blood of post Chernobyl leukemia patients [83]. The predicted top diseases and biological functions (IPA), suggest that the changes in signaling pathways and gene expression in the lower dose-rate (0.54 mGy/h) are activating gene networks associated with apoptosis and other cell death mechanisms in the embryos, while inhibiting proliferation of tumor cell lines (Fig 6). In the higher dose-rate exposure groups (5.4 and 10.9 mGy/h), gene networks involved in cell death and apoptosis were shown to be inhibited, while cell movement, cardiovascular development and tumor development were activated (Fig 6). The predictions from the gene expression suggest an early response of the developing embryos to continuous ionizing radiation and would be interesting to address in follow up studies using genetic, epigenetic and mutagenesis methods.

Conclusion

Continuous exposure to external gamma radiation at environmentally relevant dose-rates (from 0.4 mGy/h, total dose 17.5 mGy) resulted in severe consequences for the development and gene expression of zebrafish embryos and larva. Significant mortality compared to controls was observed in the groups exposed to the highest dose rate (38 mGy/h), while increased number of deformities and differences in the hatching was observed in groups exposed to lower doses $\geq$ 0.4 mGy/h (Tables 1 and 2 and S2 Table). Consistent with the observed adverse effects, the changes in gene transcription could be attributed to cell differentiation and morphological development. The results suggest that active repair mechanisms mediated by antioxidants could be the reason for the lack of phenotypic observable effects in the lower dose. The higher radiation dose rates instigate, among others, genes and networks involved in cell cycle control (tp53), translation and cell survival (eif2, mTOR), and disrupted development and cancer (myc, TGFb1, hnf4a, cebpa), which in sum increase the risk for an adverse effect. Thus, RNA sequencing enabled identification of molecular initiating events from a 3 hour gamma radiation exposure to 0.54, 5.4 and 10.9 mGy/h (total dose 1.6 to 33 mGy), which are consistent with the phenotype level adverse outcomes observed in 96 hpf stage larvae.

Supporting information

S1 Table. Real time qPCR primers.
(XLSX)

S2 Table. Survival and hatching. Survival and hatching after 43.8 and 92 hours exposure to specified dose rates. Survival at the 43.8 hours exposure did not differ at 48 and 96 hpf. All values presented as mean percentage ± 95% confidence interval (CI).
(XLSX)

S3 Table. Mapping statistics. Mapping statistics presented separately for each replicate (A) and each exposure (B) with their respective controls. Approximately 60% of the reads were mapped to the reference genome. Of the mapped reads, ~ 40% were mapped when allowing

no mismatch, while ~ 15% of the reads were mapped when ≤ 2 bp mismatches were allowed. On the other hand, ~ 56% out of the mapped reads were found to represent unique genome positions, with ~ 1.5% of reads mapping to multiple positions.
(XLSX)

S4 Table. Full DEGs list.
(XLSX)

S5 Table. IPA upstream regulators.
(XLSX)

S1 Fig. Bioinformatic analysis pipeline.
(TIF)

S2 Fig. RNA-seq mapping frequency of reads distribution. Differential expression threshold is FC ± 1.3. **A**, **B**, **C** and **D** show the distribution of mapped reads at 0.54, 5.4 and 10.9 mGy/h. **E** and **F** represent the distribution of mapped reads in control groups for the lowest (0.54 mGy/h) and for higher dose rates 5.4 and 10.9 mGy/h, respectively. All libraries were mapped to the ZF genome (Zv9).
(TIF)

S3 Fig. Multidimensional scaling (MDS) plot of RNA-seq libraries after trimmed mean of M-values (TMM) normalization. A) Group exposed at 0.54 mGy/h and the control group for the lowest dose. Two and three biological replicates of the exposed group and controls, respectively, were included in the analysis. **B)** and **C)** Groups exposed to 5.4 and 10.9 mGy/h and controls. Three replicates were included.
(TIF)

S4 Fig. Principal component analysis (PCA) of gene expression data. Analysis was conducted by pairwise comparison of exposed and their respective controls. **A)** 0.54 mGy/h, **B)** 5.4 mGy/h and **C)** 10.9 mGy/h. Expression values were log2 transformed. Black and red dots represent non-differential and differentially expressed genes respectively (FDR < 0.05) (edgeR v3.4.2 Bioconductor).
(TIF)

S5 Fig. *Myc* upstream regulator (IPA). *Myc* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S6 Fig. *TNF* upstream regulator (IPA). *TNF* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S7 Fig. *Tp53* upstream regulator (IPA). *Tp53* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S8 Fig. *Hnf4a* upstream regulator (IPA). *Hnf4a* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S9 Fig. *TGFb1* upstream regulator (IPA). *TGFB1* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S10 Fig. *Cebpa* upstream regulator (IPA). *Cebpa* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.
(TIF)

S11 Fig. Gene expression in *RARa* pathway (IPA).
(TIF)

S12 Fig. Gene expression in *eif2* pathway (IPA).
(TIF)

S13 Fig. Gene expression between in *mTOR* pathway (IPA).
(TIF)

S14 Fig. Gene expression in apoptosis network (IPA).
(TIF)

S15 Fig. Deformities in zebrafish larva exposed to gamma radiation. The observations were done at 96 hours post fertilization (hpf). **A.** Control larva showing normal development; **B-C.** Larvae exposed to 38 mGy/h for 92 hours (Group "B"), demonstrating general developmental defects and short-tails.
(TIF)

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Author Contributions

Formal analysis: SH LMM ES.

Investigation: HCT VB SH.

Project administration: JLL PA OCL.

Resources: RNL PA JLL.

Supervision: JLL.

Visualization: SH LMM.

Writing – original draft: SH LMM JLL DAB PA DHO TC OCL HCT BS RNL.

References

1. Jarvis R., Knowles J. DNA damage in zebrafish larvae induced by exposure to low-dose rate γ-radiation: detection by the alkaline comet assay. Mutat Res Toxicol Environ Mutagen. 2003; 541: 63–69.
2. Bergonié J, Tribondeau L. Interpretation of some results from radiotherapy and an attempt to determine a rational treatment technique. 1906. Yale J Biol Med. Yale Journal of Biology and Medicine; 2003; 76: 181–2. PMID: 15482657
3. Jacquet P. Sensitivity of germ cells and embryos to ionizing radiation. J Biol Regul Homeost Agents. 2004; 18: 106–14. PMID: 15471212
4. Streffer C. Bystander effects, adaptive response and genomic instability induced by prenatal irradiation. Mutat Res. 2004; 568: 79–87. https://doi.org/10.1016/j.mrfmmm.2004.07.014 PMID: 15530541
5. Li CI, Nishi N, McDougall JA, Semmens EO, Sugiyama H, Soda M, et al. Relationship between radiation exposure and risk of second primary cancers among atomic bomb survivors. Cancer Res. 2010; 70: 7187–98. https://doi.org/10.1158/0008-5472.CAN-10-0276 PMID: 20843820

6. Hudson D, Kovalchuk I, Koturbash I, Kolb B, Martin OA, Kovalchuk O. Induction and persistence of radiation-induced DNA damage is more pronounced in young animals than in old animals. Aging (Albany NY). 2011; 3: 609–20.
7. Skinner MK. Role of epigenetics in developmental biology and transgenerational inheritance. Birth Defects Res C Embryo Today. 2011; 93: 51–5. https://doi.org/10.1002/bdrc.20199 PMID: 21425441
8. Ilnytskyy Y, Kovalchuk O. Non-targeted radiation effects-an epigenetic connection. Mutat Res. 2011; 714: 113–25. https://doi.org/10.1016/j.mrfmmm.2011.06.014 PMID: 21784089
9. UNSCEAR. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) Report. New York; 1996.
10. Garnier-Laplace J, Della-Vedova C, Andersson P, Copplestone D, Cailes C, Beresford NA, et al. A multi-criteria weight of evidence approach for deriving ecological benchmarks for radioactive substances. J Radiol Prot. IOP Publishing; 2010; 30: 215–33. https://doi.org/10.1088/0952-4746/30/2/S02 PMID: 20530866
11. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 2007; 8: 353–67. https://doi.org/10.1038/nrg2091 PMID: 17440532
12. Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013; 496: 498–503. https://doi.org/10.1038/nature12111 PMID: 23594743
13. White R, Rose K, Zon L. Zebrafish cancer: the state of the art and the path forward. Nat Rev Cancer. 2013; 13: 624–36. https://doi.org/10.1038/nrc3589 PMID: 23969693
14. Kane D, Kimmel C. The zebrafish midblastula transition. Development. 1993; 119: 447–456. PMID: 8287796
15. Aanes H, Østrup O, Andersen IS, Moen LF, Mathavan S, Collas P, et al. Differential transcript isoform usage pre- and post-zygotic genome activation in zebrafish. BMC Genomics. 2013; 14: 331. https://doi.org/10.1186/1471-2164-14-331 PMID: 23676078
16. Lindeman LC, Andersen IS, Reiner AH, Li N, Aanes H, Østrup O, et al. Prepatterning of developmental gene expression by modified histones before zygotic genome activation. Dev Cell. 2011; 21: 993–1004. https://doi.org/10.1016/j.devcel.2011.10.008 PMID: 22137762
17. Haberle V, Li N, Hadzhiev Y, Plessy C, Previti C, Nepal C, et al. Two independent transcription initiation codes overlap on vertebrate core promoters. Nature. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2014; 507: 381–5. https://doi.org/10.1038/nature12974 PMID: 24531765
18. Carlsson Tedgren Å, Bjerke H, Grindborg J-E, Hetland P-O, Kosunen A, Hellebust TP, et al. Comparison of high-dose-rate 192Ir source strength measurements using equipment with traceability to different standards. Brachytherapy. 2014; 13: 420–423. https://doi.org/10.1016/j.brachy.2014.01.002 PMID: 24530343
19. Bjerke H, Hetland PO. Dosimetry at Figaro—gamma irradiation facility at NMBU, Aas; Dosimetri ved Figaro—gammaanlaget ved NMBU, Aas. Østerås; 2014.
20. OECD/OCDE. OECD GUIDELINES FOR THE TESTING OF CHEMICALS Fish Embryo Acute Toxicity (FET) Test. 2013. https://doi.org/10.1787/20745761
21. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal. 2011; 17: 10. https://doi.org/10.14806/ej.17.1.200
22. Andrews S. FastQC A Quality Control tool for High Throughput Sequence Data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
23. Trapnell C, Pachter L, Salzberg SL. TopHat: discovering splice junctions with RNA-Seq. Bioinformatics. 2009; 25: 1105–11. https://doi.org/10.1093/bioinformatics/btp120 PMID: 19289445
24. Andrews SS mapped sequence analysis tool. (2012). Babraham Bioinformatics—SeqMonk Mapped Sequence Analysis Tool [Internet]. http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/
25. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010; 26: 139–40. https://doi.org/10.1093/bioinformatics/btp616 PMID: 19910308
26. Xie F, Xiao P, Chen D, Xu L, Zhang B. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol. 2012; https://doi.org/10.1007/s11103-012-9885-2 PMID: 22290409
27. R Foundation for Statistical Computing, Vienna A (2013). R: The R Project for Statistical Computing [Internet]. 2014. https://www.r-project.org/
28. Pereira S, Bourrachot S, Cavalie I, Plaire D, Dutilleul M, Gilbin R, et al. Genotoxicity of acute and chronic gamma-irradiation on zebrafish cells and consequences for embryo development. Environ Toxicol Chem. 2011; 30: 2831–7. https://doi.org/10.1002/etc.695 PMID: 21954072

29. Simon O, Massarin S, Coppin F, Hinton TG, Gilbin R. Investigating the embryo/larval toxic and genotoxic effects of γ irradiation on zebrafish eggs. J Environ Radioact. Elsevier Ltd; 2011; 102: 1039–44. https://doi.org/10.1016/j.jenvrad.2011.06.004 PMID: 21741137

30. Bladen CL, Navarre S, Dynan WS, Kozlowski DJ. Expression of the Ku70 subunit (XRCC6) and protection from low dose ionizing radiation during zebrafish embryogenesis. Neurosci Lett. 2007; 422: 97–102. https://doi.org/10.1016/j.neulet.2007.05.045 PMID: 17630212

31. Pereira S, Malard V, Ravanat J-L, Davin A-H, Armengaud J, Foray N, et al. Low doses of gamma-irradiation induce an early bystander effect in zebrafish cells which is sufficient to radioprotect cells. PLoS One. 2014; 9: e92974. https://doi.org/10.1371/journal.pone.0092974 PMID: 24667817

32. Gagnaire B, Cavalié I, Pereira S, Floriani M, Dubourg N, Camilleri V, et al. External gamma irradiation-induced effects in early-life stages of zebrafish, Danio rerio. Aquat Toxicol. 2015; 169: 69–78. https://doi.org/10.1016/j.aquatox.2015.10.005 PMID: 26517177

33. Jaafar L, Podolsky RH, Dynan WS. Long-term effects of ionizing radiation on gene expression in a zebrafish model. PLoS One. 2013; 8: e69445. https://doi.org/10.1371/journal.pone.0069445 PMID: 23936019

34. Freeman JL, Weber GJ, Peterson SM, Nie LH. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front Genet. Frontiers; 2014; 5: 268. https://doi.org/10.3389/fgene.2014.00268 PMID: 25147559

35. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995; 203: 253–310. https://doi.org/10.1002/aja.1002030302 PMID: 8589427

36. ICRP. ICRP 2012. Compend Dose Coefficients based ICRP Publ 60. 2012; 0: 130. https://doi.org/10.1016/j.icrp.2006.06.001

37. Miyachi Y, Kanao T, Okamoto T. Marked depression of time interval between fertilization period and hatching period following exposure to low-dose X-rays in zebrafish. Environmental Research. 2003.

38. McAleer MF, Davidson C, Davidson WR, Yentzer B, Farber SA, Rodeck U, et al. Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers. Int J Radiat Oncol Biol Phys. 2005; 61: 10–3. https://doi.org/10.1016/j.ijrobp.2004.09.046 PMID: 15629588

39. Bladen CL, Kozlowski DJ, Dynan WS. Effects of low-dose ionizing radiation and menadione, an inducer of oxidative stress, alone and in combination in a vertebrate embryo model. Radiat Res. 2012; 178: 499–503. https://doi.org/10.1667/RR3042.2 PMID: 23092554

40. Yalcin A, Clem BF, Simmons A, Lane A, Nelson K, Clem AL, et al. Nuclear Targeting of 6-Phosphofructo-2-kinase (PFKFB3) Increases Proliferation via Cyclin-dependent Kinases. J Biol Chem. 2009; 284: 24223–24232. https://doi.org/10.1074/jbc.M109.016816 PMID: 19473963

41. Seo M, Lee Y-H. PFKFB3 Regulates Oxidative Stress Homeostasis via Its S-Glutathionylation in Cancer. J Mol Biol. 2014; 426: 830–842. https://doi.org/10.1016/j.jmb.2013.11.021 PMID: 24295899

42. Yamamoto T, Takano N, Ishiwata K, Ohmura M, Nagahata Y, Matsuura T, et al. Reduced methylation of PFKFB3 in cancer cells shunts glucose towards the pentose phosphate pathway. Nat Commun. 2014; 5. https://doi.org/10.1038/ncomms4480 PMID: 24633012

43. Sharma MK, Saxena V, Liu R-Z, Thisse C, Thisse B, Denovan-Wright EM, et al. Differential expression of the duplicated cellular retinoic acid-binding protein 2 genes (crabp2a and crabp2b) during zebrafish embryonic development. Gene Expr Patterns. 2005; 5: 371–9. https://doi.org/10.1016/j.modgep.2004.09.010 PMID: 15661643

44. Diez del Corral R, Storey KG. Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. Bioessays. 2004; 26: 857–69. https://doi.org/10.1002/bies.20080 PMID: 15273988

45. Blomhoff R, Beckman-Sundh, Brot C, Solvoll K, Steingrimsdóttir L C MH. Health risks related to high intake of preformed retinol (vitamin A) in the Nordic countries. Tema Nord 2003:502. Nordic Council of Ministers, Copenhagen; 2003.

46. Gutierrez-Mazariegos J, Theodosiou M, Campo-Paysaa F, Schubert M. Vitamin A: a multifunctional tool for development. Semin Cell Dev Biol. 2011; 22: 603–10. https://doi.org/10.1016/j.semcdb.2011.06.001 PMID: 21693195

47. Cai AQ, Radtke K, Linville A, Lander AD, Nie Q, Schilling TF. Cellular retinoic acid-binding proteins are essential for hindbrain patterning and signal robustness in zebrafish. Development. 2012; 139: 2150–5. https://doi.org/10.1242/dev.077065 PMID: 22619388

48. Fraenkel PG, Gibert Y, Holzheimer JL, Lattanzi VJ, Burnett SF, Dooley KA, et al. Transferrin-a modulates hepcidin expression in zebrafish embryos. Blood. 2009; 113: 2843–50. https://doi.org/10.1182/blood-2008-06-165340 PMID: 19047682

49. Rojo I, de Ilárduya OM, Estonba A, Pardo MA. Innate immune gene expression in individual zebrafish after Listonella anguillarum inoculation. Fish Shellfish Immunol. 2007; 23: 1285–93. https://doi.org/10.1016/j.fsi.2007.07.002 PMID: 17804254

50. Nylund R, Lemola E, Hartwig S, Lehr S, Acheva A, Jahns J, et al. Profiling of low molecular weight proteins in plasma from locally irradiated individuals. J Radiat Res. 2014; 55: 674–82. https://doi.org/10.1093/jrr/rru007 PMID: 24570173

51. Crichton RR, Wilmet S, Legssyer R, Ward RJ. Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells. J Inorg Biochem. 2002; 91: 9–18. https://doi.org/10.1016/S0162-0134(02)00461-0 PMID: 12121757

52. Beckman L, Nordenson I. Transferrin C2 and radiation-induced chromosomal damage. Hum Hered. 1988; 38: 56–8. PMID: 3350533

53. Otis JP, Zeituni EM, Thierer JH, Anderson JL, Brown AC, Boehm ED, et al. Zebrafish as a model for apolipoprotein biology: comprehensive expression analysis and a role for ApoA-IV in regulating food intake. Dis Model Mech. Company of Biologists; 2015; 8: 295–309. https://doi.org/10.1242/dmm.018754 PMID: 25633982

54. Shoji H, Murano Y, Mori M, Matsunaga N, Ohkawa N, Suganuma H, et al. Lipid profile and atherogenic indices soon after birth in Japanese preterm infants. Acta Paediatr. 2014; 103: 22–6. https://doi.org/10.1111/apa.12436 PMID: 24117869

55. Avraham-Davidi I, Ely Y, Pham VN, Castranova D, Grunspan M, Malkinson G, et al. ApoB-containing lipoproteins regulate angiogenesis by modulating expression of VEGF receptor 1. Nat Med. NIH Public Access; 2012; 18: 967–73. https://doi.org/10.1038/nm.2759 PMID: 22581286

56. Fang L, Choi S-H, Baek JS, Liu C, Almazan F, Ulrich F, et al. Control of angiogenesis by AIBP-mediated cholesterol efflux. Nature. Nature Research; 2013; 498: 118–122. https://doi.org/10.1038/nature12166 PMID: 23719382

57. Bahary N, Goishi K, Stuckenholz C, Weber G, Leblanc J, Schafer CA, et al. Duplicate VegfA genes and orthologues of the KDR receptor tyrosine kinase family mediate vascular development in the zebrafish. Blood. American Society of Hematology; 2007; 110: 3627–36. https://doi.org/10.1182/blood-2006-04-016378 PMID: 17698971

58. Goishi K, Klagsbrun M. Vascular endothelial growth factor and its receptors in embryonic zebrafish blood vessel development. Curr Top Dev Biol. 2004; 62: 127–52. https://doi.org/10.1016/S0070-2153(04)62005-9 PMID: 15522741

59. Stalmans I. Role of the vascular endothelial growth factor isoforms in retinal angiogenesis and DiGeorge syndrome. Verh—K Acad voor Geneeskd van België. 2005; 67: 229–76.

60. Ruhrberg C. Growing and shaping the vascular tree: multiple roles for VEGF. Bioessays. 2003; 25: 1052–60. https://doi.org/10.1002/bies.10351 PMID: 14579246

61. Kim Y-W, Byzova T V. Oxidative stress in angiogenesis and vascular disease. Blood. American Society of Hematology; 2014; 123: 625–31. https://doi.org/10.1182/blood-2013-09-512749 PMID: 24300855

62. Aktaş C, Kurtman C, Ozbilgin MK, Tek I, Toprak SK. An Experimental Study of Radiation Effect on Normal Tissue: Analysis of HIF-1α, VEGF, eIF2, TIA-1, and TSP-1 Expression. Turkish J Haematol Off J Turkish Soc Haematol. 2013; 30: 371–8. https://doi.org/10.4274/Tjh.2012.0142 PMID: 24385827

63. Huang R, Cheung N-K V, Vider J, Cheung IY, Gerald WL, Tickoo SK, et al. MYCN and MYC regulate tumor proliferation and tumorigenesis directly through BMI1 in human neuroblastomas. FASEB J. The Federation of American Societies for Experimental Biology; 2011; 25: 4138–49. https://doi.org/10.1096/fj.11-185033 PMID: 21856782

64. Schuhmacher B, Rengstl B, Döring C, Bein J, Newrzela S, Brunnberg U, et al. A strong host response and lack of MYC expression are characteristic for diffuse large B cell lymphoma transformed from nodular lymphocyte predominant Hodgkin lymphoma. Oncotarget. 2016; https://doi.org/10.18632/oncotarget.12363 PMID: 27708232

65. Shen L-J, Chen F-Y, Zhang Y, Cao L-F, Kuang Y, Zhong M, et al. MYCN transgenic zebrafish model with the characterization of acute myeloid leukemia and altered hematopoiesis. PLoS One. 2013; 8: e59070. https://doi.org/10.1371/journal.pone.0059070 PMID: 23554972

66. Williams RD, Chagtai T, Alcaide-German M, Apps J, Wegert J, Popov S, et al. Multiple mechanisms of MYCN dysregulation in Wilms tumour. Oncotarget. 2015; 6: 7232–43. https://doi.org/10.18632/oncotarget.3377 PMID: 25749049

67. Wegert J, Bausenwein S, Kneitz S, Roth S, Graf N, Geissinger E, et al. Retinoic acid pathway activity in Wilms tumors and characterization of biological responses in vitro. Mol Cancer. BioMed Central; 2011; 10: 136. https://doi.org/10.1186/1476-4598-10-136 PMID: 22067876

68. Karkanitsa L V., Komarovskaya ME, Krivenko SI. Abrogation of radiation injury to human hematopoietic stem cells with tumor necrosis factor-α. Stem Cells. 2009; 15: 95–102. https://doi.org/10.1002/stem.5530150714 PMID: 9368291

69. Miao L, Holley AK, Zhao Y, St Clair WH, St Clair DK. Redox-mediated and ionizing-radiation-induced inflammatory mediators in prostate cancer development and treatment. Antioxid Redox Signal. 2014; 20: 1481–500. https://doi.org/10.1089/ars.2013.5637 PMID: 24093432

70. Yamane K, Ihn H, Asano Y, Jinnin M, Tamaki K. Antagonistic effects of TNF-alpha on TGF-beta signaling through down-regulation of TGF-beta receptor type II in human dermal fibroblasts. J Immunol. 2003; 171: 3855–62. PMID: 14500687

71. Meek DW. Tumour suppression by p53: a role for the DNA damage response? Nat Rev Cancer. Nature Publishing Group; 2009; 9: 714. https://doi.org/10.1038/nrc2716 PMID: 19730431

72. Duffy KT, Wickstrom E. Zebrafish tp53 knockdown extends the survival of irradiated zebrafish embryos more effectively than the p53 inhibitor pifithrin-alpha. Cancer Biol Ther. 2007; 6: 675–8. PMID: 17426443

73. Guo L, Liew HP, Camus S, Goh AM, Chee LL, Lunny DP, et al. Ionizing radiation induces a dramatic persistence of p53 protein accumulation and DNA damage signaling in mutant p53 zebrafish. Oncogene. 2013; 32: 4009–16. https://doi.org/10.1038/onc.2012.409 PMID: 23069659

74. Cordenonsi M, Dupont S, Maretto S, Insinga A, Imbriano C, Piccolo S. Links between Tumor Suppressors: p53 Is Required for TGF-β Gene Responses by Cooperating with Smads. Cell. 2003; 113: 301–314. https://doi.org/10.1016/S0092-8674(03)00308-8 PMID: 12732139

75. Savli H, Sunnetci D, Cine N, Gluzman DF, Zavelevich MP, Sklyarenko LM, et al. Gene expression profiling of B-CLL in Ukrainian patients in post-Chernobyl period. Exp Oncol. 2012; 34: 57–63. PMID: 22453151

76. Mezentsev A, Amundson SA. Global gene expression responses to low- or high-dose radiation in a human three-dimensional tissue model. Radiat Res. 2011; 175: 677–88. https://doi.org/10.1667/RR2483.1 PMID: 21486161

77. Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, et al. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev. 1994; 8: 2466–77. PMID: 7958910

78. Warga RM, Kimmel CB. Cell movements during epiboly and gastrulation in zebrafish. Development. 1990; 108: 569–80. PMID: 2387236

79. Pardali K, Moustakas A. Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta. 2007; 1775: 21–62. https://doi.org/10.1016/j.bbcan.2006.06.004 PMID: 16904831

80. Dancea HC, Shareef MM, Ahmed MM. Role of Radiation-induced TGF-beta Signaling in Cancer Therapy. Mol Cell Pharmacol. 2009; 1: 44–56. PMID: 20336170

81. Hu W, Xu S, Yao B, Hong M, Wu X, Pei H, et al. MiR-663 inhibits radiation-induced bystander effects by targeting *TGFB1* in a feedback mode. RNA Biol. 2014; 11: 1189–1198. https://doi.org/10.4161/rna.34345 PMID: 25483041

82. Ferrari G, Cook BD, Terushkin V, Pintucci G, Mignatti P. Transforming growth factor-beta 1 (TGF-beta1) induces angiogenesis through vascular endothelial growth factor (VEGF)-mediated apoptosis. J Cell Physiol. NIH Public Access; 2009; 219: 449–58. https://doi.org/10.1002/jcp.21706 PMID: 19180561

83. Savlı H, Akkoyunlu RU, Çine N, Gluzman DF, Zavelevich MP, Sklyarenko LM, et al. Deregulated Levels of the NF-κB1, NF-κB2, and Rel Genes in Ukrainian Patients with Leukemia and Lymphoma in the Post-Chernobyl Period. Turkish J Haematol Off J Turkish Soc Haematol. 2016; 33: 8–14. https://doi.org/10.4274/tjh.2014.0190 PMID: 25912249

84. Yuan H, Wen B, Liu X, Gao C, Yang R, Wang L, et al. CCAAT/enhancer-binding protein α is required for hepatic outgrowth via the p53 pathway in zebrafish. Sci Rep. 2015; 5: 15838. https://doi.org/10.1038/srep15838 PMID: 26511037

85. Xu J, Patrick BA, Jaiswal AK. NRH:Quinone Oxidoreductase 2 (NQO2) Protein Competes with the 20 S Proteasome to Stabilize Transcription Factor CCAAT Enhancer-binding Protein (C/EBP), Leading to Protection against Radiation-induced Myeloproliferative Disease. J Biol Chem. 2013; 288: 34799–34808. https://doi.org/10.1074/jbc.M113.495580 PMID: 24142791

86. Tawana K, Fitzgibbon J. CEBPA-Associated Familial Acute Myeloid Leukemia (AML). University of Washington, Seattle; 2016.

87. Fleenor CJ, Rozhok AI, Zaberezhnyy V, Mathew D, Kim J, Tan A-C, et al. Contrasting roles for C/EBPα and Notch in irradiation-induced multipotent hematopoietic progenitor cell defects. Stem Cells. 2015; 33: 1345–58. https://doi.org/10.1002/stem.1936 PMID: 25546133

88. Feinendegen LE, Pollycove M, Sondhaus CA. Responses to low doses of ionizing radiation in biological systems. Nonlinearity Biol Toxicol Med. 2004; 2: 143–71. https://doi.org/10.1080/15401420490507431 PMID: 19330141

89. Feinendegen LE, Pollycove M, Neumann RD. Whole-body responses to low-level radiation exposure: new concepts in mammalian radiobiology. Exp Hematol. 2007; 35: 37–46. https://doi.org/10.1016/j.exphem.2007.01.011 PMID: 17379086

90. Rajesh K, Krishnamoorthy J, Kazimierczak U, Tenkerian C, Papadakis AI, Wang S, et al. Phosphorylation of the translation initiation factor eIF2α at serine 51 determines the cell fate decisions of Akt in response to oxidative stress. Cell Death Dis. Macmillan Publishers Limited; 2015; 6: e1591. https://doi.org/10.1038/cddis.2014.554 PMID: 25590801

91. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004; 18: 1926–45. https://doi.org/10.1101/gad.1212704 PMID: 15314020

92. Jacot JL, Sherris D. Potential Therapeutic Roles for Inhibition of the PI3K/Akt/mTOR Pathway in the Pathophysiology of Diabetic Retinopathy. J Ophthalmol. 2011; 2011: 589813. https://doi.org/10.1155/2011/589813 PMID: 22132311

93. Sharlow ER, Leimgruber S, Lira A, McConnell MJ, Norambuena A, Bloom GS, et al. A Small Molecule Screen Exposes mTOR Signaling Pathway Involvement in Radiation-Induced Apoptosis. ACS Chem Biol. American Chemical Society; 2016; https://doi.org/10.1021/acschembio.5b00909 PMID: 26938669

94. Yentrapalli R, Azimzadeh O, Sriharshan A, Malinowsky K, Merl J, Wojcik A, et al. The PI3K/Akt/mTOR pathway is implicated in the premature senescence of primary human endothelial cells exposed to chronic radiation. PLoS One. 2013; 8: e70024. https://doi.org/10.1371/journal.pone.0070024 PMID: 23936371

Supplementary material

Paper I

S1 Table. Real time qPCR primers.

Gene ID	Gene name	Accession no.	Forward primer (5'-3')	Reverse primer (5'-3')
pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_213397.1	TGTCCACACAGAACTGGCTT	ATAGGAAAAACCCAAGGGTCCAT
crabp2b	cellular retinoic acid binding protein 2, b	NM_001320394.1	GCACTCGGGTGTATGAACGA	CCACAAAATGACAGTTGAGTTGAGA
vox	ventral homeobox	AF255045.1	AACTTTTCCGTGGACTGGCT	TCTTGGCGTAACCTGAGCTG
ppp1r15a	protein phosphatase 1, regulatory subunit 15A	NM_001082921.1	TCCTCAATAATGGCGACCCG	CGCATAACTCCAGACCAGCA
shisa2	shisa family member 2	NM_001003631.1	TGGCATCAGTCGTTCTACCG	TCGTCTGTGCTTTCGGACTC
sox2	SRY (sex determining region Y)-box 2	NM_213118.1	TCAAGAGACCCATGAACGCC	AATGAGACGACGACGTGACC
tfa	transferrin-a	NM_001291499.1	ACTGCATGAGGAGCATAGCG	TATTGAAGCCAGAGCCAGCC
eef2b	eukaryotic translation elongation factor 2b	NM_200458.2	ACGAGCTGACGGAGAATGAC	CTGTCTCAGCACTGTCTCGG
hmbs	hydroxymethylbilane synthase	NM_201154.1	GTGTGTGGAATTGGACAACAAAGTG	CGAGGGCTGATGATGAGATATTGC

S2 Table. Survival and hatching.

Survival and hatching after 43.8 and 92 hours exposure to specified dose rates. Survival at the 43.8 hours exposure did not differ at 48 and 96 hpf. All values presented as mean percentage ± 95% confidence interval (CI).

Exposure (hours)	Analyzed at (hpf)	Endpoint	Dose rate (mGy/h)				
			Control	0.4	3.9	15	38
43.8	48	Hatching (%) ± CI	7.8 ± 3.82	29.8 ± 6.55	5.7 ± 3.28	7 ± 3.43	1.4 ± 1.9
	48 and 96	Survival (%) ± CI.	92.1 ± 1.95	88.3 ± 2.35	87.8 ± 2.38	87.9 ± 2.23	83.4 ± 3.1
	96	Hatching (%) ± CI.	99.0 ± 0.74	98.4 ± 0.92	98.9 ± 0.75	95.8 ± 1.38	99.3 ± 0.69
92	96	Survival (%) ± CI.	90.7 ± 2.1	86.6 ± 2.4	87.4 ± 2.42	89.9 ± 2.19	82.8 ± 2.69
		Hatching (%) ± CI.	100	99.0 ± 0.69	98.4 ± 0.91	96.8 ± 1.28	97.0 ± 1.22

S3 Table. Mapping statistics. Mapping statistics presented separately for each replicate **(A)** and each exposure **(B)** with their respective controls. Approximately 60% of the reads were mapped to the reference genome. Of the mapped reads, ~ 40% were mapped when allowing no mismatch, while ~ 15% of the reads were mapped when ≤ 2 bp mismatches were allowed. On the other hand, ~ 56% out of the mapped reads were found to represent unique genome positions, with ~ 1.5% of reads mapping to multiple positions.

(A)

Exposure	0.54 mGy/h			
Sample ID	nmbu-05-5		nmbu-05-7	
Total reads	26975612		25493626	
Total Basepair	1321804988		1249187674	
Total mapped reads	15590290	57.79%	14869476	58.33%
Perfect match	11848729	43.92%	11277618	44.24%
≤ 2bp mismatch	3741561	13.87%	3591858	14.09%
Unique match	15205009	56.37%	14510833	56.92%
Multi-position match	385281	1.43%	358643	1.41%
Total unmapped reads	11385322	42.21%	10624150	41.67%

Exposure	5.4 mGy/h						10.9 mGy/h					
Sample ID	nmbu-5-11		nmbu-5-1		nmbu-5-9		nmbu-10-14		nmbu-10-18		nmbu-10-4	
Total reads	26801069		27829476		27867678		26152386		27076349		27314971	
Total Basepair	1313252381		1363644324		1365516222		1281466914		1326741101		1.338E+09	
Total mapped reads	15854116	59.15%	16262774	58.44%	16425414	58.94%	15168837	58.00%	15607840	57.64%	15907380	58.24%
Perfect match	11850469	44.22%	12196817	43.83%	12285616	44.09%	11365013	43.46%	11722735	43.30%	11863735	43.43%
≤ 2bp mismatch	4003647	14.94%	4065957	14.61%	4139798	14.86%	3803824	14.54%	3885105	14.35%	4043645	14.80%
Unique match	15470983	57.73%	15860596	56.99%	15986815	57.37%	14789327	56.55%	15192532	56.11%	15497657	56.74%
Multi-position match	383133	1.43%	402178	1.45%	438599	1.57%	379510	1.45%	415308	1.53%	409723	1.50%
Total unmapped reads	10946953	40.85%	11566702	41.56%	11442264	41.06%	10983549	42.00%	11468509	42.36%	11407591	41.76%
Exposure	Control-1						Control-2					
Sample ID	nmbu-K1-12		nmbu-K1-15		nmbu-K1-17		nmbu-K2-13		nmbu-K2-20		nmbu-K2-9	
Total reads	25802353		27351132		27625642		27572514		26676120		27073316	
Total Basepair	1264315297		1340205468		1353656458		1351053186		1307129880		1.327E+09	
Total mapped reads	14985245	58.08%	15774364	57.67%	15913767	57.61%	15729959	57.05%	15271089	57.25%	15584381	57.56%
Perfect match	11211413	43.45%	11835487	43.27%	11844663	42.88%	11544856	41.87%	11449394	42.92%	11626755	42.95%
≤ 2bp mismatch	3773832	14.63%	3938877	14.40%	4069104	14.73%	4185103	15.18%	3821695	14.33%	3957626	14.62%
Unique match	14620547	56.66%	15402627	56.31%	15524523	56.20%	15337733	55.63%	14898847	55.85%	15202525	56.15%
Multi-position match	364698	1.41%	371737	1.36%	389244	1.41%	392226	1.42%	372242	1.40%	381856	1.41%
Total unmapped reads	10817108	41.92%	11576768	42.33%	11711875	42.39%	11842555	42.95%	11405031	42.75%	11488935	42.44%

(B)

	0.54 mGy/h	%	5.4 mGy/h	%	10.9 mGy/h	%	Control-1	%	Control-2	%
Total reads	17489746		27499408		26847902		26926376		27107316.67	
Total Basepair	856997554		1347470976		1315547198		1319392408		1328258517	
Total mapped reads	10153255	58.29	16180768	58.84	15561352	57.96	15557792	57.79	15528476.33	57.29
Perfect match	7708782	44.22	12110967	44.05	11650494	43.40	11630521	43.20	11540335	42.58
≤ 2bp mismatch	2444473	14.08	4069801	14.80	3910858	14.56	3927271	14.59	3988141.333	14.71
Unique match	9905281	56.87	15772798	57.36	15159839	56.47	15182566	56.39	15146368.33	55.88
Multi-position match	247975	1.42	407970	1.48	401514	1.49	375226	1.39	382108	1.41
Total unmapped reads	7336491	41.71	11318640	41.16	11286550	42.04	11368584	42.21	11578840.33	42.71

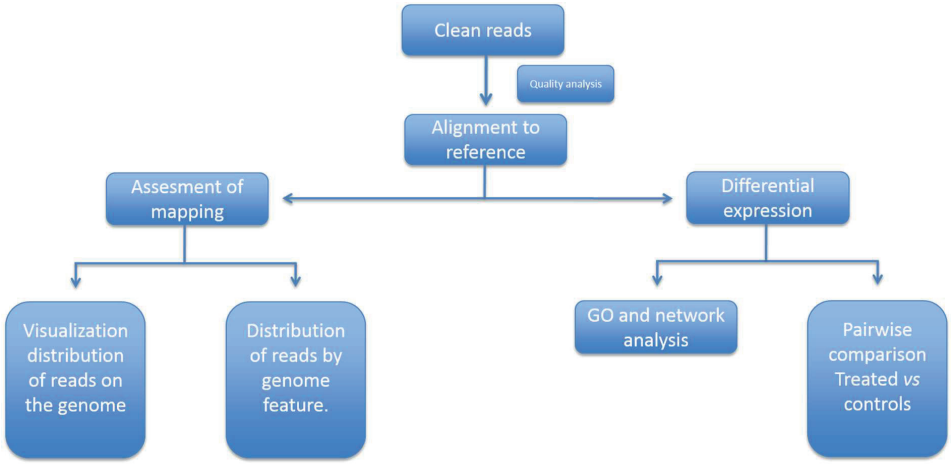
S4. Table. Full DEGs list.

File available in online version of the paper.

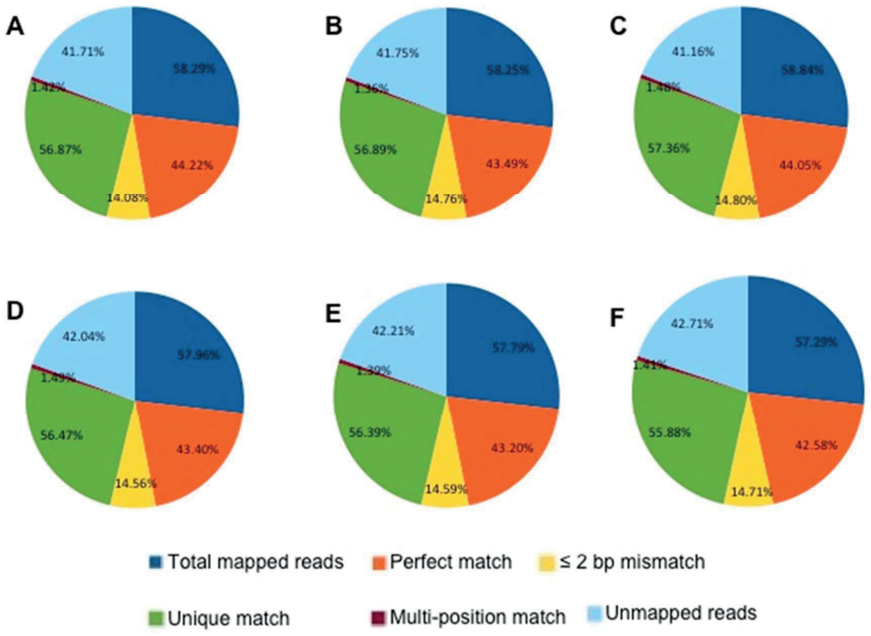
S5 Table. IPA upstream regulators.

File available in online version of the paper.

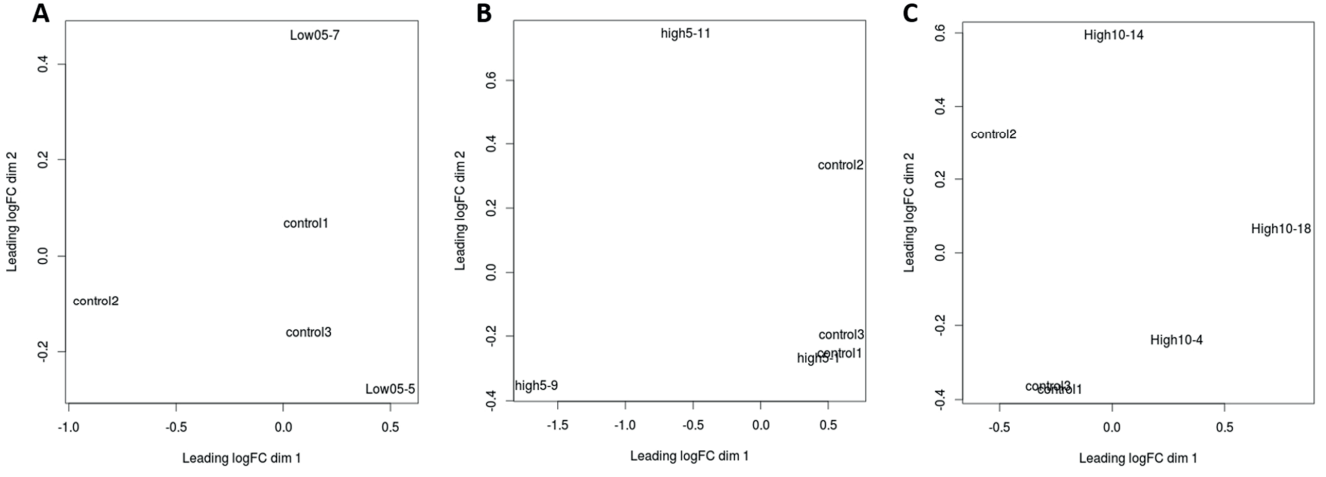
S1 Fig. Bioinformatic analysis pipeline.



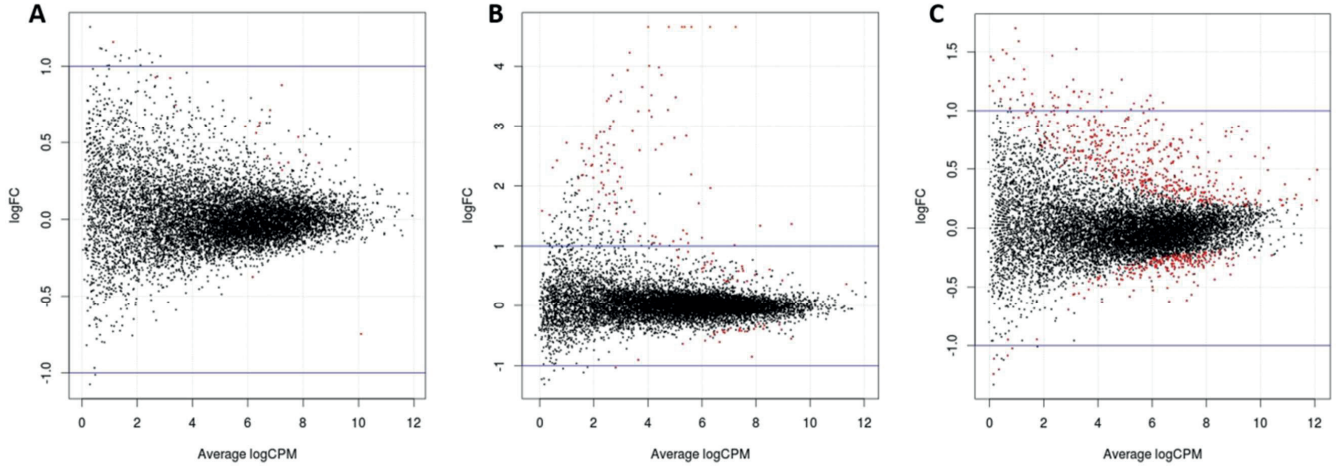
S2 Fig. RNA-seq mapping frequency of reads distribution. Differential expression threshold is FC ± 1.3. A, B, C and D show the distribution of mapped reads at 0.54, 5.4 and 10.9 mGy/h. E and F represent the distribution of mapped reads in control groups for the lowest (0.54 mGy/h) and for higher dose rates 5.4 and 10.9 mGy/h, respectively. All libraries were mapped to the ZF genome (Zv9).



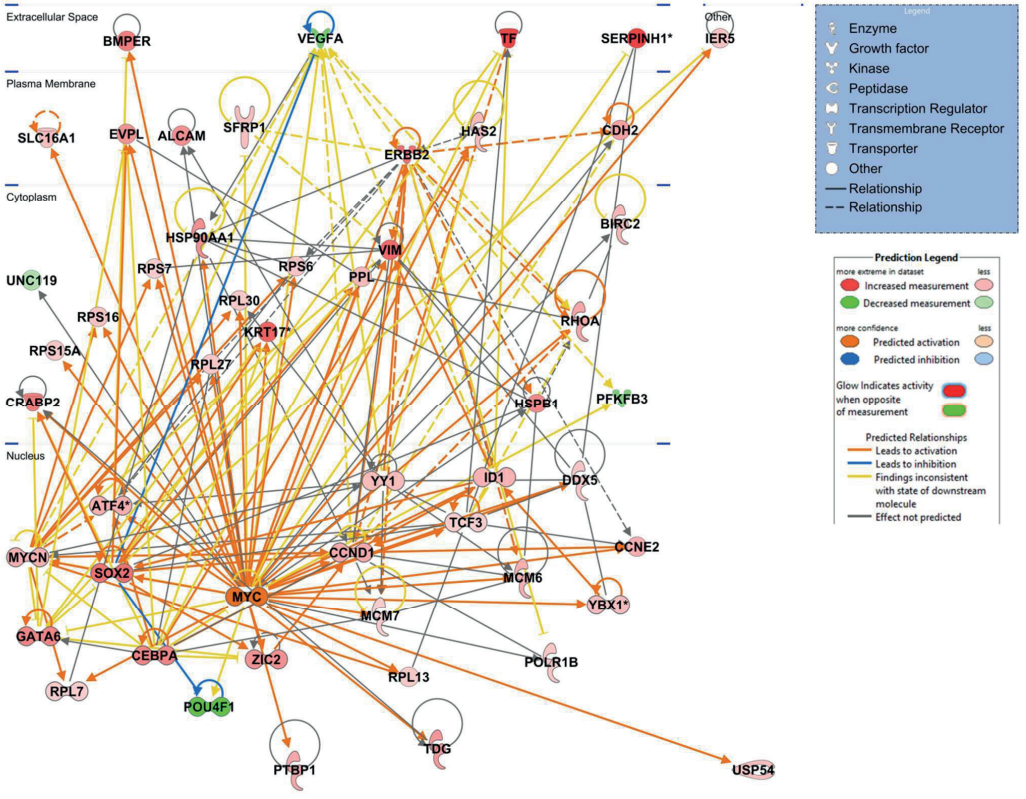
S3 Fig. Multidimensional scaling (MDS) plot of RNA-seq libraries after trimmed mean of M-values (TMM) normalization. **A)** Group exposed at 0.54 mGy/h and the control group for the lowest dose. Two and three biological replicates of the exposed group and controls, respectively, were included in the analysis. **B)** and **C)** Groups exposed to 5.4 and 10.9 mGy/h and controls. Three replicates were included.



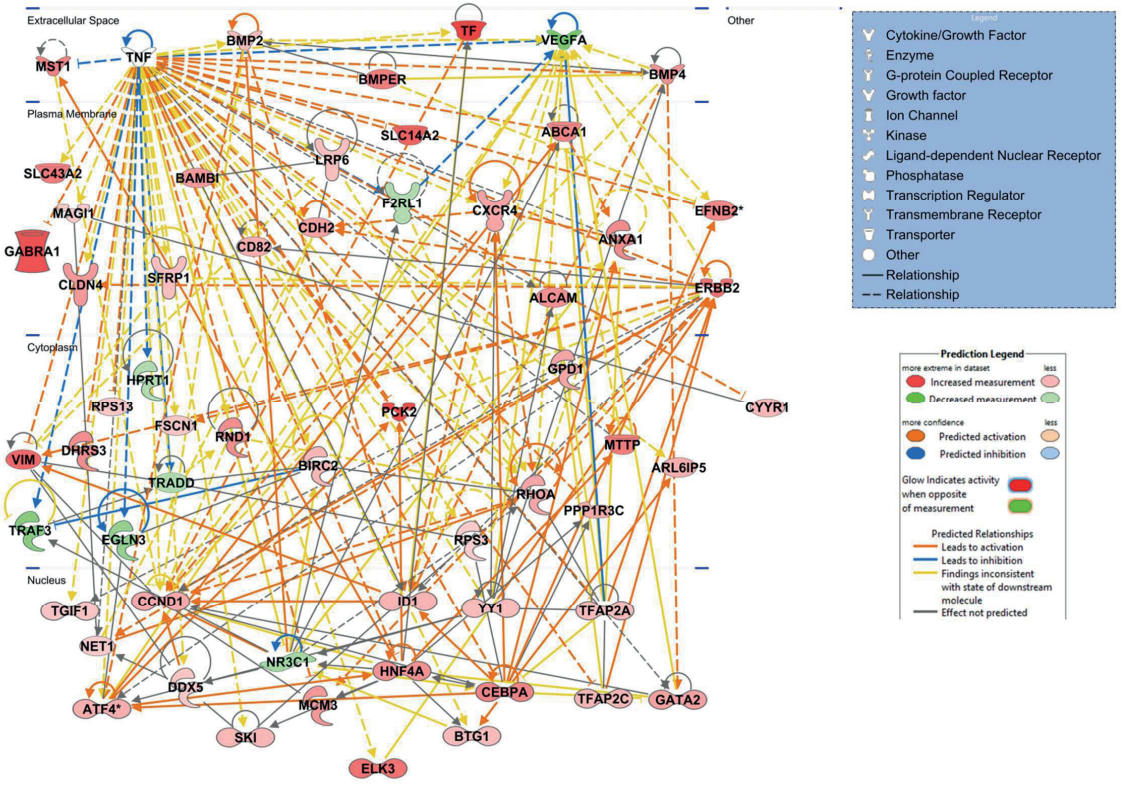
S4 Fig. Principal component analysis (PCA) of gene expression data. Analysis was conducted by pairwise comparison of exposed and their respective controls. A) 0.54 mGy/h, B) 5.4 mGy/h and C) 10.9 mGy/h. Expression values were log2 transformed. Black and red dots represent non-differential and differentially expressed genes respectively (FDR < 0.05) (edgeR v3.4.2 Bioconductor).



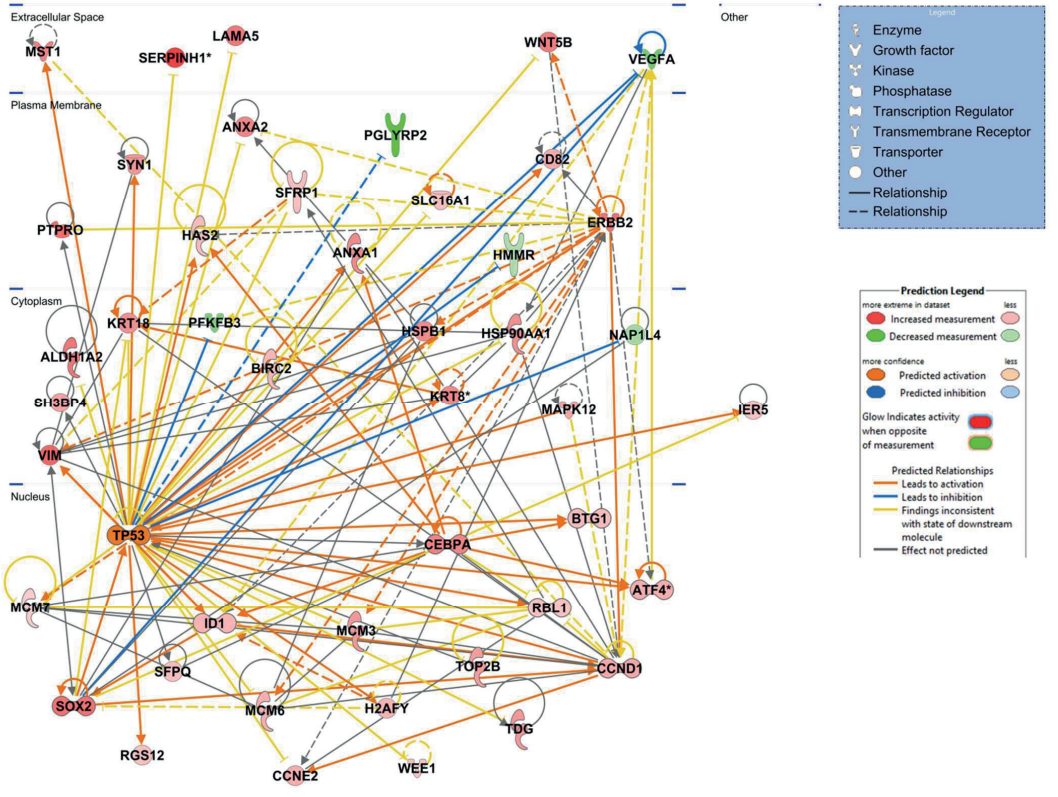
S5 Fig. *Myc* upstream regulator (IPA). *Myc* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



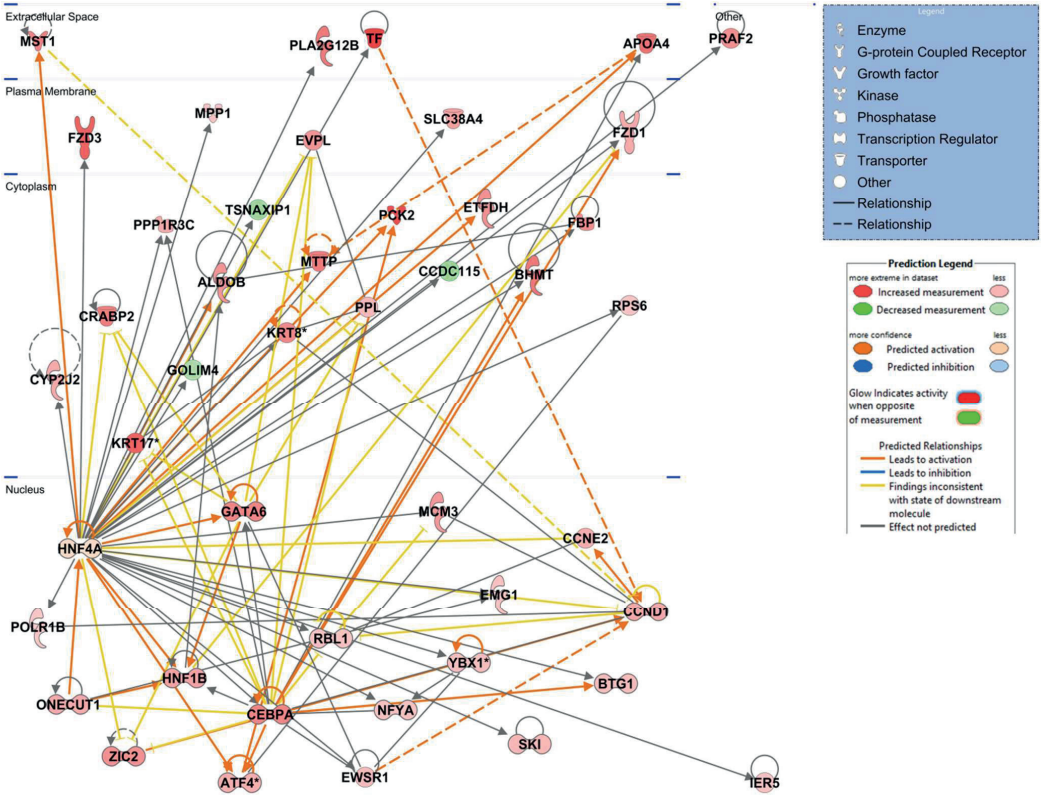
S6 Fig. *TNF* upstream regulator (IPA). *TNF* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



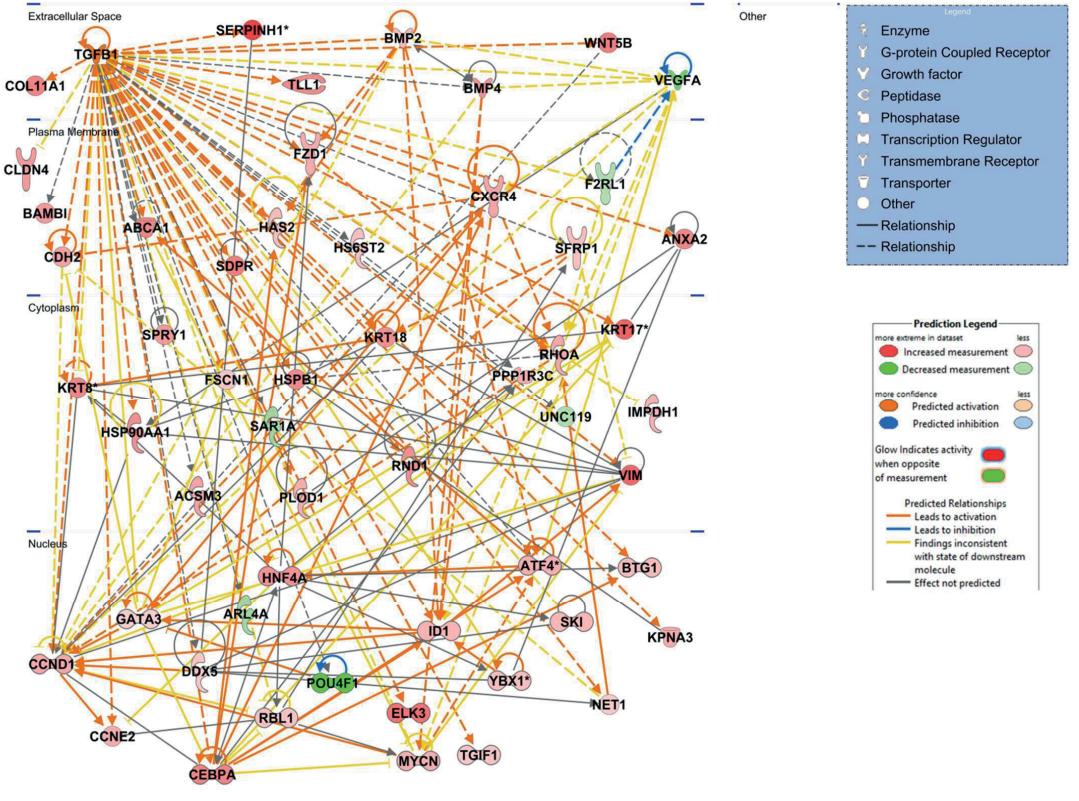
S7 Fig. *Tp53* upstream regulator (IPA). *Tp53* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



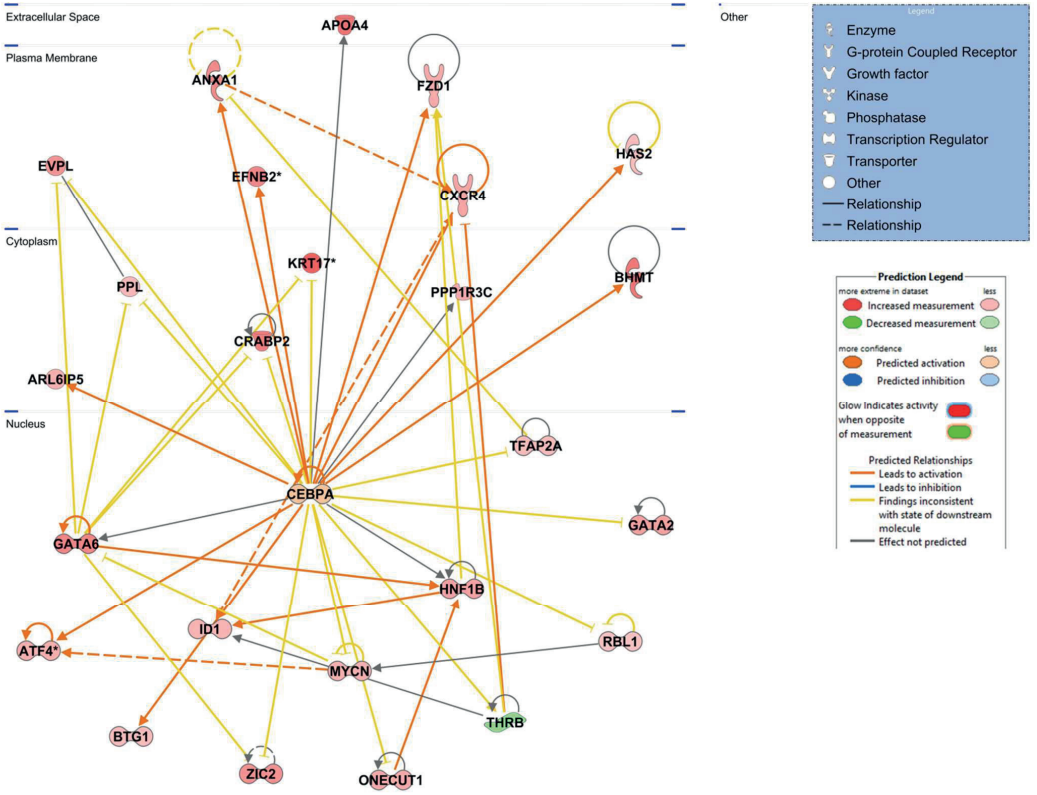
S8 Fig. *Hnf4a* upstream regulator (IPA). *Hnf4a* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



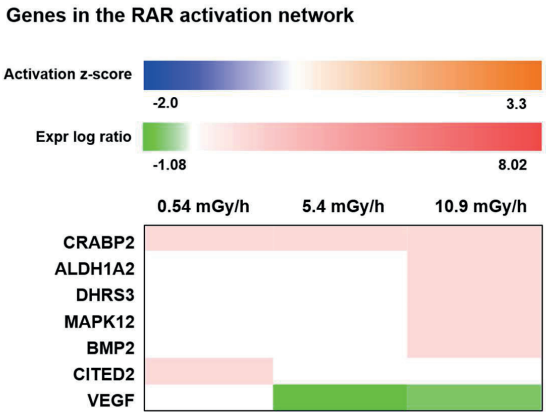
S9 Fig. *TGFb1* upstream regulator (IPA). *TGFB1* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



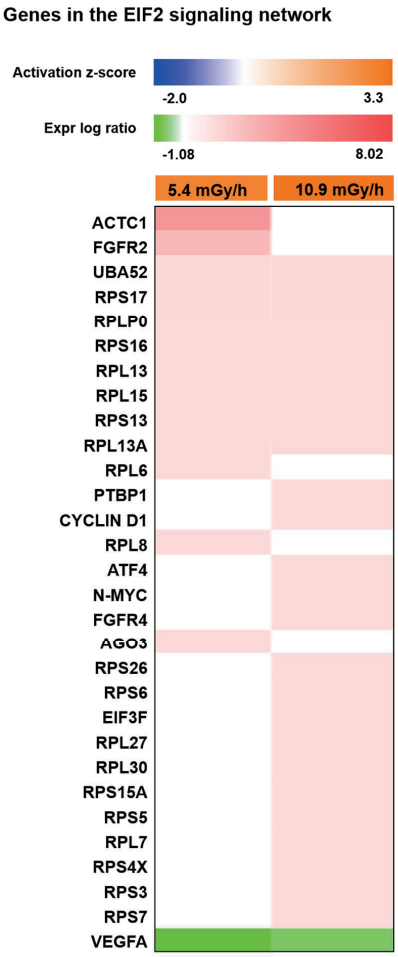
S10 Fig. *Cebpa* upstream regulator (IPA). *Cebpa* target gene networks and interactions, presented in a subcellular layout as part of the 10.9 mGy/h group.



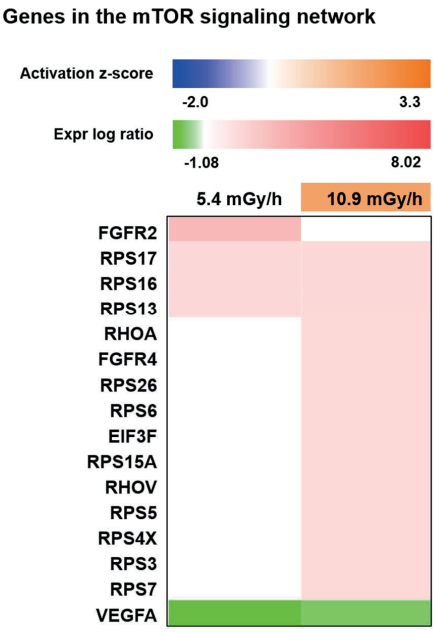
S11 Fig. Gene expression in *RARa* pathway (IPA).



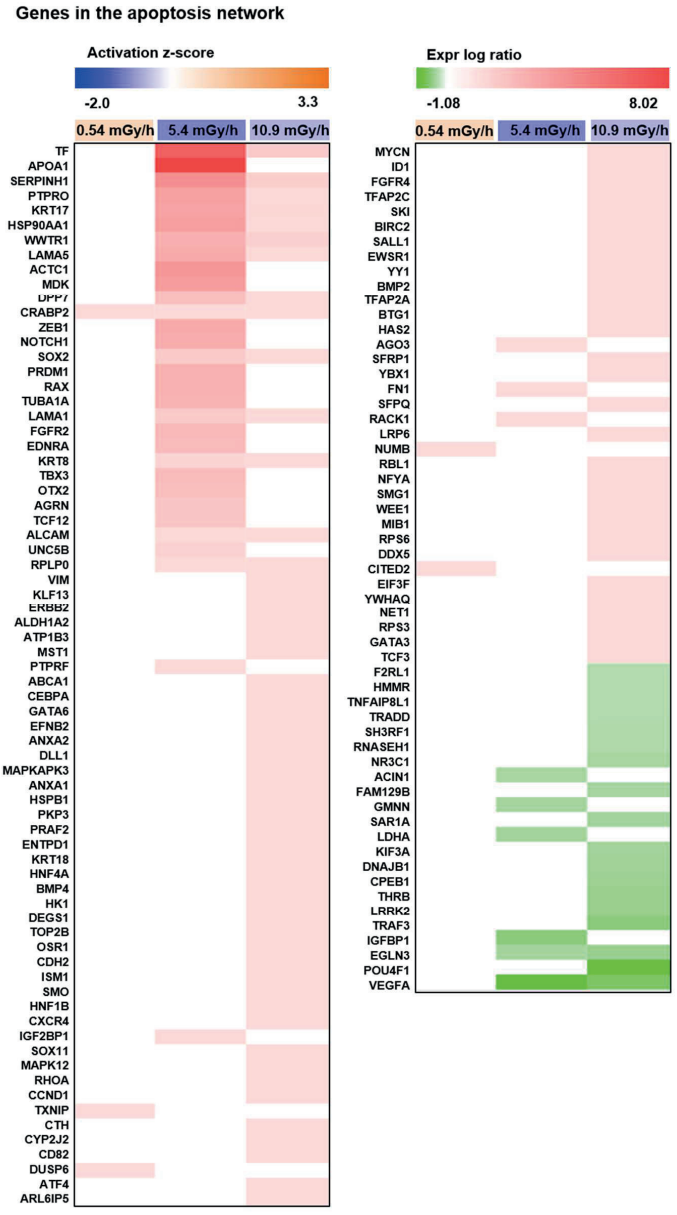
S12 Fig. Gene expression in *eif2* pathway (IPA).



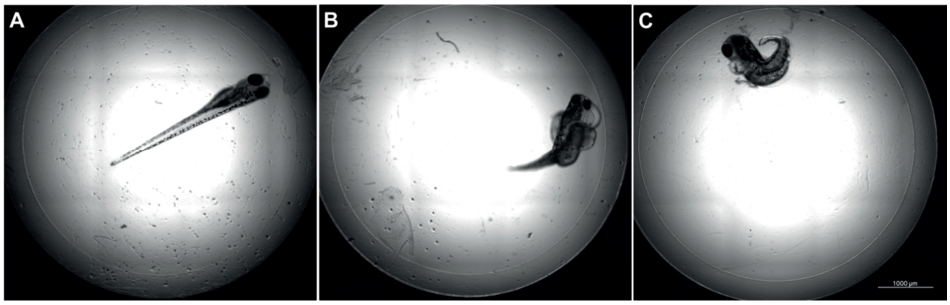
S13 Fig. Gene expression between in mTOR pathway (IPA).



S14 Fig. Gene expression in apoptosis network (IPA).



S15 Fig. Deformities in zebrafish larva exposed to gamma radiation. The observations were done at 96 hours post fertilization (hpf). **A.** Control larva showing normal development; **B-C.** Larvae exposed to 38 mGy/h for 92 hours (Group "B"), demonstrating general developmental defects and short-tails.



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Parental gamma irradiation induces reprotoxic effects accompanied by genomic instability in zebrafish (*Danio rerio*) embryos

Selma Hurem[a,b,*], Tânia Gomes[a,c], Dag A. Brede[a,d], Elisabeth Lindbo Hansen[a,e], Stephen Mutoloki[b], Cristian Fernandez[f], Carmel Mothersill[g], Brit Salbu[a,d], Yetneberk A. Kassaye[a,d], Ann-Karin Olsen[a,h], Deborah Oughton[a,d], Peter Alestrom[a,b], Jan L. Lyche[a,b]

[a] Centre for Environmental Radioactivity (CERAD CoE), NMBU, 1433 Ås, Norway
[b] Norwegian University of Life Sciences (NMBU), Faculty of Veterinary Medicine and Biosciences, P.O. Box 8146 Dep., 0033 Oslo, Norway
[c] Norwegian Institute for Water research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway
[d] Norwegian University of Life Sciences (NMBU), Faculty of Environmental Sciences and Natural Resource Management, 1433 Ås, Norway
[e] Norwegian Radiation Protection Authority (NRPA), Postboks 55, 1332 Østerås, Norway
[f] Institute of Anatomy, University of Bern, Baltzerstrasse 2, 3000 Bern, Switzerland
[g] McMaster University, Department of Biology, 1280 Main St. West Hamilton, Ontario, Canada
[h] Norwegian Institute of Public Health (NIPH), PO Box 4404 Nydalen, 0403 Oslo, Norway

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ABSTRACT

Gamma radiation represents a potential health risk to aquatic and terrestrial biota, due to its ability to ionize atoms and molecules in living tissues. The effects of exposure to ^{60}Co gamma radiation in zebrafish (*Danio rerio*) were studied during two sensitive life stages: gametogenesis (F0: 53 and 8.7 mGy/h for 27 days, total doses 31 and 5.2 Gy) and embryogenesis (9.6 mGy/h for 65 h; total dose 0.62 Gy). Progeny of F0 exposed to 53 mGy/h showed 100% mortality occurring at the gastrulation stage corresponding to 8 h post fertilization (hpf). Control and F0 fish exposed to 8.7 mGy/h were used to create four lines in the first filial generation (F1): control, G line (irradiated during parental gametogenesis), E line (irradiated during embryogenesis) and GE line (irradiated during parental gametogenesis and embryogenesis).

A statistically significant cumulative mortality of GE larva (9.3%) compared to controls was found at 96 hpf. E line embryos hatched significantly earlier compared to controls, G and GE (48–72 hpf). The deformity frequency was higher in G and GE, but not E line compared to controls at 72 hpf. One month after parental irradiation, the formation of reactive oxygen species (ROS) was increased in the G line, but did not significantly differ from controls one year after parental irradiation, while at the same time point it was significantly increased in the directly exposed E and GE lines from 60 to 120 hpf. Lipid peroxidation (LPO) was significantly increased in the G line one year after parental irradiation, while significant increase in DNA damage was detected in both the G and GE compared to controls and E line at 72 hpf. Radiation-induced bystander effects, triggered by culture media from tissue explants and observed as influx of Ca^{2+} ions through the cellular membrane of the reporter cells, were significantly increased in 72 hpf G line progeny one month after irradiation of the parents. One year after parental irradiation, the bystander effects were increased in the E line compared to controls, but not in progeny of irradiated parents (G and GE lines). Overall, this study showed that irradiation of parents can result in multigenerational oxidative stress and genomic instability in irradiated (GE) and non-irradiated (G) progeny of irradiated parents, including increases in ROS formation, LPO, DNA damage and bystander effects. The results therefore highlight the necessity for multi- and transgenerational studies to assess the environmental impact of gamma radiation.

1. Introduction

The release of radionuclides to the environment from nuclear accidents or other anthropogenic sources represents a long term health risk to aquatic and terrestrial biota (Sample, 2011; Vives i Batlle et al., 2007). It is well established that exposure to gamma radiation can cause

* Correspondence to: NMBU - School of Veterinary Science, Postboks 8146 Dep, 0033 Oslo, Norway.
E-mail address: selma.hurem@nmbu.no (S. Hurem).

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significant effects in fish in terms of survival, reproduction and development (Gagnaire et al., 2015; Geiger et al., 2006; Miyachi et al., 2003). Animal and in vitro studies suggest that the exposure of parents to doses ≥ 1 Gy might induce transgenerational biological effects in several subsequent generations (Buisset-Goussen et al., 2014; Soubry et al., 2014). Parental exposure can lead to hereditary effects in offspring as a result of DNA damage (Lemos et al., 2017), but also due to epigenetic mechanisms such as changes in DNA methylation patterns and non-coding RNA expression (Filkowski et al., 2010). From previous studies it is known that vertebrate embryos are particularly sensitive to ionizing radiation, due to a high rate of cell division and migration, whereby a disruption of processes involved in organogenesis and morphogenesis can lead to developmental defects (Jacquet, 2004; Simon et al., 2011; Streffer, 2004). However, the sensitivity to gamma radiation can vary on an individual level with different biological factors, such as stage of development, age, sex and overall health (Nascimento and Bradshaw, 2016; Won et al., 2015). Genes and proteins can be affected by gamma irradiation directly, through DNA damage (single and double strand breaks as well as DNA oxidation), or indirectly, via the induction of antioxidants as a response to excitation of water molecules and free-radical formation. Animal and cellular studies suggest that these effects are exacerbated by non-targeted effects, wherein the effects in irradiated cells can materialize either in non-irradiated neighboring cells (bystander effects), in the progeny of irradiated cells (genomic instability) (Streffer, 2004) or induce adaptive responses (Maguire et al., 2007; Ryan et al., 2009). The mechanisms are not yet fully understood, but the latest evidence links non-targeted effects to cell communication signals of a biological nature e.g. exosomes containing long non-coding RNA or DNA fragments (O'Leary et al., 2015; Xu et al., 2015). The underlying mechanisms behind both bystander effects and genomic instability have been linked to transgenerational effects. The concept of genomic instability describes delayed genetic alterations observed in the progeny of cells many generations after the initial insult caused either by direct ionizing radiation (Chang and Little, 1992; Kadhim et al., 1992; Seymour et al., 1986), or by bystander signals (Lorimore et al., 1998; Mothersill and Seymour, 1997).

Adult zebrafish are sexually mature at 3–5 months of age (depending on external factors) with an approximate 4-weeks gametogenesis cycle (Raz, 2003). Zebrafish embryos and larva have previously been shown to be sensitive to low dose gamma radiation, wherein adverse developmental effects and gene expression changes (Hurem et al., 2017) and DNA damage induction (Adam-Guillermin et al., 2012; Jarvis and Knowles, 2003; Simon et al., 2011) were observed during embryogenesis. A recent study reported a correlation between radiation-induced DNA damage in X-ray exposed parents and DNA damage in zebrafish progeny (Lemos et al., 2017). However, the potential heritability of radiation induced biological effects such as changes in ROS levels, LPO, DNA damage and bystander effects has not been well studied, and information on the relative sensitivity of different life stages, such as gametogenesis and embryogenesis to these types of effects is lacking.

The present study tests the hypothesis that heritable effects are present in progeny from parents subjected to external gamma radiation during gametogenesis. To do this we compared the effects of gamma radiation (^{60}Co source) in embryos that were directly exposed during embryogenesis (E line) with effects seen in the progeny of irradiated parents (G line).

2. Materials and methods

2.1. Fish husbandry

Zebrafish from the AB wild type strain were obtained from the NMBU zebrafish facility and maintained according to standard operating procedures. Adult fish (F0), aged 6 months (30 males and 30 females per exposure group), were kept at 28 ± 1 °C on a 14 − 10 h light-dark cycle (250 – 320 lx) at a density of 5 − 10 fish/L. The system water (SW) was prepared from particle and active charcoal filtered reverse osmosis (RO) deionized tap water kept sterile by UV irradiation. To generate a conductivity of 500 µS/cm, general hardness (GH) of 4 − 5 and pH 7.5 (adjusted with 1 M HCl), 155 mg synthetic sea salt (Instant Ocean, Blacksburg, USA), 53 mg sodium carbonate and 15 mg calcium chloride (Sigma-Aldrich, Norway AS) were added per liter of RO water. Adult fish were fed with Gemma Micro 300 (Skretting, Stavanger, Norway) dry feed twice a day and live artemia (Scanbur, Copenhagen, Denmark) once a day. Health monitoring was performed by daily inspection. Sentinel fish were sent to ZIRC for pathology every six months and water for microbiology analysis (NMBU Vetbio, Oslo).

2.2. Exposure experiment

2.2.1. Adult fish exposure, dosimetry and mating

The fish were exposed in 9 L plastic aquaria (Aquatic Habitats, Apopka, FL), with a total swimming space of 6 L. The external gamma radiation exposure took place at the FIGARO ^{60}Co irradiation facility (activity ~420 GBq) at NMBU. Adult zebrafish were exposed during a 27 day period of gametogenesis, with a total beam-on time of 591.5 h. A control aquarium was placed behind lead shielding, and two aquaria were placed with the front face at different distances to the source focus, resulting in calculated average absorbed dose rates to water of 8.7 mGy/h and 53 mGy/h, respectively. The corresponding average total absorbed doses to water were respectively 5.2 Gy and 31 Gy, further explained in Appendix A. (*Adult fish and embryo exposure and dosimetry*) and Table A1 and A2. To assure invariable conditions in exposure aquaria, the pH, temperature and conductivity were recorded daily, while nitrogen compounds (NH3, NH4+, NO2- and NO3-) were recorded daily during the first 10 days, and twice a week for the remainder of the experiment in each aquarium (Table A3). The adult and embryo exposure groups are schematically depicted in Fig. 1. After an acclimatization period of 6 days after irradiation, adult zebrafish were allowed to mate for 30 min in standard 1 L breeding aquaria (Aquatic Habitats, Apopka, FL). Each breeding aquarium had 3 males and 3 females and a total of 10 aquaria were used per group. The same mating procedure was used to produce embryos for all endpoints. Twelve days after F0 exposure, embryos were produced for the analysis of the survival and development. One month after F0 exposure, the early development, length, ROS and bystander effects were analyzed in the G line and controls. One year after irradiation of the adult fish, ROS, LPO, DNA damage and bystander effects as well as histological examinations were performed in all four lines.

2.2.2. Embryo exposure and dosimetry

Embryos (F1) were collected immediately after the breeding period and individually placed in first rows of replicate 12 well microtiter plates (Nunc™, Thermo Fisher Scientific, Pittsburgh, USA) with 3 mL of egg water (28 °C autoclaved system water). External gamma irradiation of the embryos from F0 control and irradiated zebrafish commenced from 2.5 to 3 and lasted until approximately 72 hpf, for a total of 65 h. The embryos were exposed to a dose-rate of 9.6 mGy/h with a total dose of 0.62 Gy (Table A2 and Fig. 1). Four progeny lines were created: control, F1-G line as non-irradiated progeny of F0 irradiated during gametogenesis, F1-E line irradiated only during embryogenesis and F1-GE line from F0 irradiated during gametogenesis and F1 embryogenesis (Fig. 1). Control plates were placed behind lead shielding, further information about embryo dosimetry can be found in Appendix A (*Adult fish and embryo exposure and dosimetry*).

2.3. Survival and development analyses

The survival and hatching of embryos (n ≥ 187 per exposure group) were manually observed and analyzed in all groups at 48, 54, 60, 72

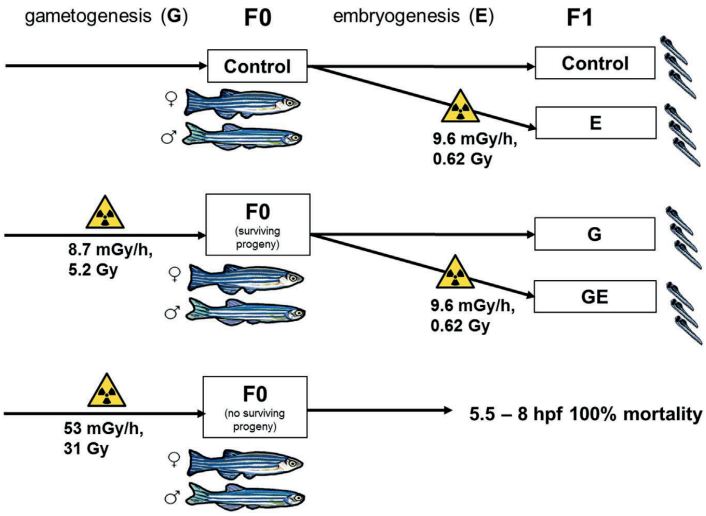

Fig. 1. Schematic representation of gamma radiation experiments. Parental generation (F0, adult fish) and progeny (F1 embryos) exposure during gametogenesis (27 days) or embryogenesis (2.5–3 to 72 hpf) and nomenclature of the fish lines.

and 96 hpf, according to the guidelines defined by the OECD zebrafish embryo toxicity test (OECD/OCDE, 2013) during and after offspring exposure and 12 days after end of parental exposure. Median hatching time (HT_{50}), which represents the time necessary for half of the eggs to hatch, was calculated for each exposure group using REGTOX®, as previously described (Gagnaire et al., 2015). One month after the irradiation of parents, the early development of G line embryos and controls (n = 24/group) was followed using combined Z-stack and 24-h kinetic experiment and final image stitching with Cytation 3 Cell Imaging Multi-Mode Reader and Gen 5 software (BioTek, Winooski, Vermont US). Length was measured in non-deformed offspring of irradiated parents (G line) and controls. Morphological observations and length measurement were performed at 72 hpf and representative embryos and larvae were photographed with a light microscope at 20× magnification with a Nikon Coolpix 8500 (Nikon SMZ1000) using NIS-Elements (Amsterdam, Netherlands) software for Windows. After analysis, larvae were anesthetized and subsequently euthanized according to standard operating procedures at 120 hpf using tricaine methanesulfonate (Sigma Aldrich, Oslo, Norway).

2.4. Histopathology

For histological examination, one year after irradiation of the parents and immediately after embryo irradiation, the embryos were fixed separately in 4% paraformaldehyde for 4 days, transferred and stored in 70% ethanol until use. The samples were embedded in 1% agarose mold for adequate positioning of the embryos (Tsao-Wu et al., 1998), transferred to paraffin, where after 5-µm sections were processed according to standard procedures employed for staining with hematoxylin and eosin (H & E). Three randomly selected embryos (72 hpf) per treatment (E, G, GE and control) were examined using a Zeiss Axioskop microscope equipped with a digital camera (Leica SFC 420).

2.5. Oxidative stress

2.5.1. ROS analysis 1 month and 1 year after parental fish irradiation

Intracellular ROS production (from 60 to 120 hpf) was determined in viable, hatched zebrafish with no visible developmental defects one month and one year after irradiation of the parents and immediately after irradiation of progeny (F1) using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen, Molecular Probes Inc., Eugene, OR, USA). H_2DCFDA is a non-polar, non-fluorescent probe that enters the cells freely and is hydrolysed by cellular esterases to non-fluorescent 2,7-dichlorodihydrofluorescein (H_2DCF), which is retained in the cell. In the presence of a variety of ROS, H_2DCF is converted by oxidation to fluorescent 2,7-dichlorofluorescein (DCF), which is localized in the cytosol (Winterbourn, 2014). A stock solution of 20 mM H_2DCFDA was made in DMSO and kept at −20 °C before use. On the day of the analysis, the H_2DCFDA stock solution was diluted in egg water to a final working solution of 500 µg/mL. Embryos were individually collected and incubated in a 96-well black microplate (Corning Costar, Cambridge, MA, USA) for 1.5 h with H_2DCFDA, with 20 replicate embryos per exposure group at 60 hpf. Fluorescence was recorded after the irradiation period at 72, 96 and 120 hpf in mean relative fluorescence units (RFU) using the Cytation 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski Vermont, USA) and analyzed using Gen5 Microplate Reader and Imager Software (Biotek, Winooski Vermont, USA). Natural fluorescence of irradiated egg water in combination with the probes (without presence of embryos) for each dose rate was also analyzed and the resulting fluorescence subtracted. The relative fluorescence obtained for each exposure group was expressed as fold induction comparative to the control.

2.5.2. Lipid peroxidation analysis 1 year after adult fish irradiation

LPO was assessed at 72 hpf zebrafish (F1) produced one year after irradiation of parental zebrafish and immediately after irradiation of embryos by determining malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) concentrations upon decomposition by polyunsaturated fatty acid peroxides, following the method previously described (Erdelmeier et al., 1998). Briefly, 4 groups of 40 zebrafish embryos were pooled, frozen in liquid nitrogen and stored at −80 °C until further analysis. Pooled zebrafish embryos (pool wet mass 5–25 mg) were homogenized using a Precellys 24 Lysis and Homogenization (Bertin Technologies, Montigny-le-Bretonneux, France) in 20 mM Tris-HCl (pH 7.4) containing 0.5 M butylated hydroxytoluene (BHT) at 4 °C. The resulting homogenate was centrifuged at 3000 g for 10 min at 4 °C and the supernatant used for protein determination and LPO analysis. LPO analysis was based on the reaction of two moles of N-methyl-2-phenylindole (3:1 mixture of acetonitrile/methanol), a chromogenic reagent, with one mole of either MDA or 4-HNE under acidic conditions (methanesulfonic acid) at 45 °C for 60 min to yield a stable chromophore that has maximum absorbance at 586 nm. Malondialdehyde bis-

(1,1,3,3-tetramethoxypropane) was used as a standard. Protein content was determined using the method with Immunoglobulin G (IgG) as a standard (Bradford, 1976). LPO was expressed as nmols of MDA and 4-HNE per gram of total protein concentration.

2.5.3. DNA damage analysis 1 year after adult fish irradiation

DNA damage was assessed in viable, hatched 72 hpf F1 zebrafish with no visible developmental defects, one year after irradiation of parents and immediately after irradiation of the progeny, using the alkaline comet assay. The method detects single strand breaks and alkali-labile DNA lesions using GelBond®films (Hansen et al., 2010) for a high throughput single cell gel electrophoresis (Gutzkow et al., 2013), and was adapted to the conditions in the present experiment. Pools of 10 embryos (3 biological replicates) were placed in PBS buffer without Ca^{2+}/Mg^{2+} (pH 7.4) and cells extracted by mechanical dissociation using a glass grinder. After extraction, the buffer containing the cells was filtered using a 55 μM nylon mesh and the resulting cell suspension centrifuged at 300*g* for 5 min (4 °C). The pellet was gently resuspended in PBS buffer without Ca^{2+}/Mg^{2+} (pH 7.4) and the final cell suspension adjusted to 1×10^6 cells/mL. Cell viability was checked by trypan blue exclusion assay. Cells were resuspended in 1:10 0.75% low melting point agarose at 37 °C and triplicates (3 × 4 μL) from each biological replicate were immediately applied on a cold GelBond®film. Lysis was performed overnight in lysis buffer at 4 °C (2.5 M NaCl, 0.1 M Na_2EDTA, 0.01 M Tris, 0.2 M NaOH, 0.034 M N-laurylsarcosine, 10% DMSO, 1% Triton X-100, pH 10). For unwinding, films were immersed in cold electrophoresis solution (0.3 M NaOH, 0.001 M Na_2EDTA, pH > 13) for 40 min. Electrophoresis was carried out in cold, fresh electrophoresis solution for 20 min at 8 °C, 25 V and 0.8 V/cm over the platform, with circulation of the electrophoresis solution. After electrophoresis, films were neutralized with a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 2 × 5 min, fixed in ethanol (> 90 min in 96% ethanol) and dried overnight. Films were stained with SYBR®Gold Nucleic Acid Gel Stain (Life Technologies, Paisley, UK) in TE-buffer (1 mM Na_2EDTA, 10 mM Tris–HCl, pH 8) before examination at a 20 × magnification under an Olympus BX51microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Fifty randomly chosen cells per replicate (150 cells per biological replicate, total 450 cells per dose rate) were scored using the Comet IV analysis software (Perceptive Instruments Ltd., Bury St. Edmunds, UK). Tail intensity (% Tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage induced by gamma radiation because it has been shown to be the most meaningful endpoint to assess genotoxicity (Kumaravel and Jha, 2006). Mean percentage (%) of DNA in the tail per exposure group was calculated using the median values of % tail DNA from the 50 comets from each technical replicate (total of 9 median values per exposure group).

2.6. Bystander effect analysis 1 month and 1 year after adult fish irradiation

The embryos used for the bystander effects were sampled one year after irradiation of parents and immediately after irradiation of F1 progeny at 72 hpf. The embryos were suspended in culture medium for 24 h, with 25 replicate embryos per exposure group, after which the medium was decanted off and filtered. The filtrate containing putative bystander signals was sterilized using 0.2 mm acrodisc filters and stored at 4 °C until calcium analysis. Details about the protocol used for ratiometric calcium measurements in cells can be found in previous studies (Mothersill et al., 2014). Briefly, 100,000 HaCaT cells were seeded onto Glass Bottom Dishes (MatTek Corporation) 24 h in advance using Roswell Park Memorial Institute growth medium (RPMI 1640, Gibco, Canada), supplemented with 10% FBS, 5 mL of L-glutamine (Gibco, Canada), 0.5 mg/mL of Hydrocortisone (Sigma-Aldrich, Canada), and 12.5 mL of 1 M HEPES buffer solution (Gibco, Canada). For calcium measurements, the culture medium was discarded and the cells were washed with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Cat#: 14025-092, Gibco, Oakville, Canada), supplemented with 25 mM of HEPES (Gibco, Oakville, Canada). The cells were loaded with 200 uL of 8.4 uM of Fura-2/AM (Sigma-Aldrich, Milwaukee, USA), for 45 min at room temperature. Cells were then washed and observed with a 40x oil objective using an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada) and images were captured with a CCD Cool-Snap HQ camera (Photometrics, Tucson, Arizona). For the calcium measurements, 100 μL of conditioned medium (from the Fish embryo) was added to the cells 60–90 s after the start of acquisition. The ratio of calcium-bound versus calcium-free Fura-2/AM was measured at 340 and 380 nm respectively, and that ratio correlates with the calcium flux through the cellular membrane (Lyng et al., 2000). Calcium concentrations were plotted as a function of time after the addition of the conditioned medium. Data was acquired from ten randomly selected cells and the area under the curve calculated for each cell, giving a mean value per sample.

2.7. Statistical analysis

Statistical analyses were performed using XLStat2016® (Addinsoft, Paris, France) and GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA). Results for all endpoints are presented as mean ± standard error (SEM) and compared between progeny lines. Significant differences between dose rates for all parameters were calculated using one-way analysis of variance (ANOVA) or Kruskal–Wallis One Way Analysis of Variance on Ranks. If significant, pairwise multiple comparison procedures were conducted, using the Tukey test or the Dunnett's method. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Parental irradiation causes massive impairment of progeny development

The early embryogenesis development of G-line embryos and controls was monitored one month after exposure of the parents. The fish exposed to 53 mGy/h during gametogenesis were fertile and produced functional gametes and live embryos, with mortality occurring during late epiboly. All progeny (F1) of F0 exposed to 53 mGy/h during gametogenesis showed 100% mortality at 7–8 hpf, and were excluded from further analysis (Fig. 2). This F0-group also failed to produce viable offspring 1.5 years after irradiation, at which time the F0 fish were sacrificed, suggesting no recovery of reproductive capacity.

The survival and hatching in E, G and GE line compared to controls were monitored at 48, 54, 60, 72 and 96 hpf, 12 days after irradiation of the parents (in embryos from the second spawning). At 96 hpf, a cumulative mortality of 9.3%, 5.2% and 3.1% was found in F1-GE, G and E, respectively, compared to 1.41% in controls (Fig. 3, Table A4). Only the GE line had a statistically significant increase in mortality compared to controls ($p < 0.0001$), and this group was significantly different from the E line ($p < 0.0001$). Mortality occurred before 48 hpf and did not further increase significantly at 96 hpf (Table A4).

The directly exposed progeny (GE and E lines) manifested a significantly increased hatching rate (HT_{50} of 53.5 and 53.7 hpf, respectively) compared to controls at 54 hpf ($p < 0.001$, HT_{50} = 57.4 hpf) (Fig. 4, Table A5). Additionally, significantly premature hatching in E line was seen, with more than 90% hatched at 60 hpf (HT_{50} = 53.7 hpf), compared to control (HT_{50} = 57.4 hpf) and progeny of irradiated parents. The total hatching was above 84% in all exposure groups. Although G line embryos showed significantly earlier hatching from controls at 54 hpf (HT_{50} = 54.3 hpf), the total hatching determined at 96 hpf in this group was significantly lower compared to controls (p = 0.046).

The highest numbers of deformities at 72 hpf were observed in

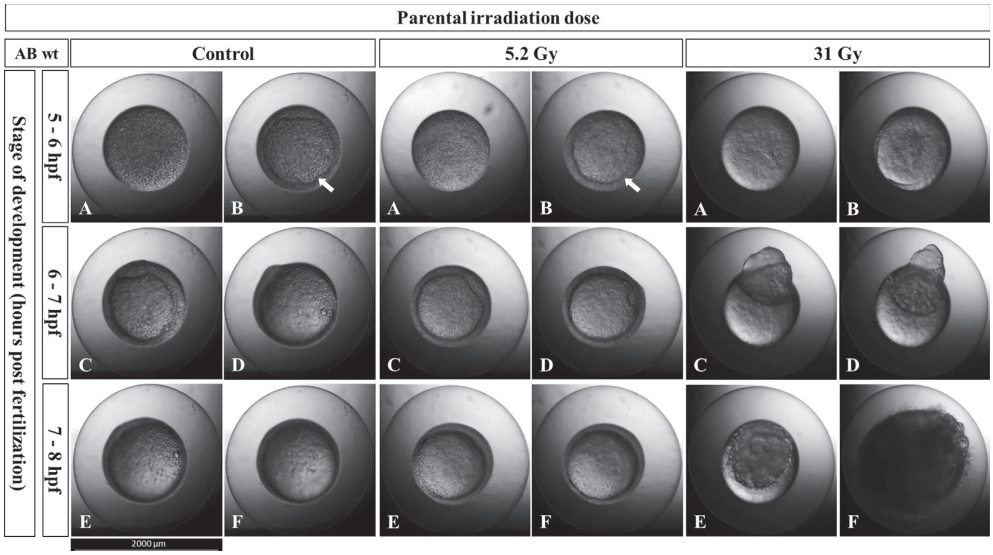

Fig. 2. Early development (5–8 hpf) in progeny of parental fish exposed to gamma radiation (Cytation 3 Imaging). A-B: 50% epiboly to shield stage, offspring of parents exposed to 53 mGy/h do not form a germ ring (arrows); **C-D:** shield stage; **E-F:** from shield stage to 75% epiboly, coagulation in progeny of parents exposed to 53 mGy/h dose rate.

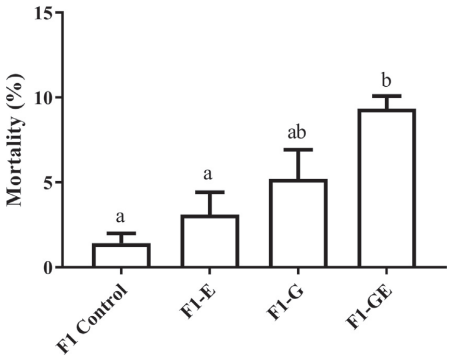

Fig. 3. Mortality observed at 96 hpf in progeny (F1) 12 days after exposure of parental zebrafish during gametogenesis to 8.7 mGy/h (total 5.2 Gy) and exposure of embryos to 9.6 mGy/h (total 0.62 Gy) compared to control. G line exposed during parental gametogenesis, GE during gametogenesis and embryogenesis and E during embryogenesis. Values are presented as mean percentage ± SEM. Letters represent statistical significance between exposure groups ($p < 0.05$).

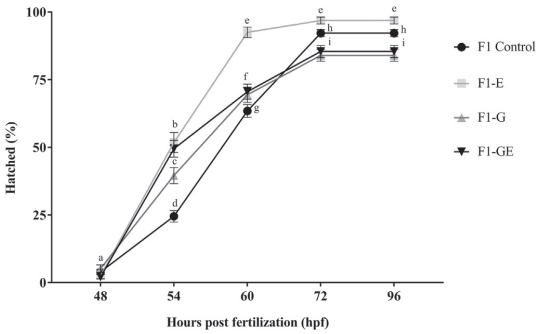

Fig. 4. Hatching rate observed from 48 hpf – 96 hpf in F1 progeny 12 days after the exposure of the parental zebrafish during gametogenesis to 8.7 mGy/h (total 5.2 Gy) and exposure of embryos to 9.6 mGy/h (total 0.62 Gy) compared to control. G line exposed during parental gametogenesis, GE during gametogenesis and embryogenesis and E during embryogenesis. Values are presented as mean percentage ± SEM. Letters represent statistical significance between exposure groups ($p < 0.05$).

progeny of irradiated parents, F1-GE and F1-G, 5.9% ($p = 0.0074$) and 5.2% ($p = 0.0009$), respectively (Fig. 5) that were significantly different from control. No significant difference in E line (1.55%) compared to controls (0.9%) was found ($p > 0.05$).

The most frequently observed deformities were retardation in development, manifested as failed hatching and absence of pigmentation, as well as short tail and spinal curvature (Fig. 6). No significant difference in whole body length was present in exposed lines (E, G and GE) compared to control.

3.2. Irradiation impairs eye development

Eye development is known to be radiosensitive to ionizing radiation (Stewart et al., 2012), particularly during the early stages of development (Geiger et al., 2006). Therefore the eye was selected as a marker of impact on the embryonic development at the organ level. Histologically, irregularities in formation of the eyes were found in higher frequencies in all lines compared to controls. In the E-line, cells were present in the lens (Fig. 7), while in non-irradiated progeny of irradiated parents, G line, a similar effect was observed in the lens and the cellular layers of the eye were not differentiated. In GE line embryos irradiated at both parental gametogenesis and embryogenesis, the effect ranged from undifferentiated cells in the lens to complete destruction of the eyes (Fig. 7).

3.3. Parental irradiation increases ROS formation in progeny

To assess the potential of gamma radiation to generate ROS in zebrafish, the formation of ROS using a fluorescent probe was measured in all progeny lines (Fig. 8). A significant production of ROS was observed in the F1-G group 1 month after parental irradiation to 8.7 mGy/

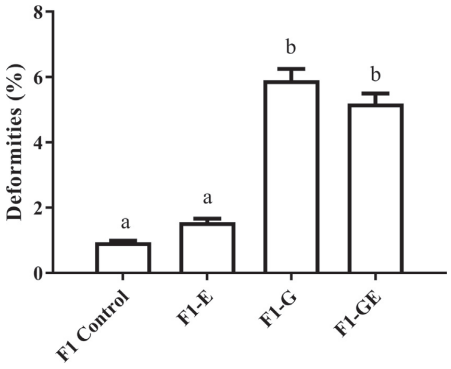

Fig. 5. Deformities observed at 72 hpf in F1 progeny spawned 12 days after exposure of parental zebrafish during gametogenesis to 8.7 mGy/h (total 5.2 Gy) and exposure of embryos to 9.6 mGy/h (total 0.62 Gy) compared to control. G line exposed during parental gametogenesis, GE during gametogenesis and embryogenesis and E line during embryogenesis. Values are presented as mean percentage ± SEM. Letters represent statistical significance between exposure groups ($p < 0.05$).

h during gametogenesis ($p < 0.05$); 1.5-, 2-, 1.6-fold at 75, 96 and 120 hpf respectively (Fig. 8A). This effect subsided and one year after parental irradiation no significant ROS production compared to the control was detected ($p > 0.05$, Fig. 8B). GE and E line (both exposed to 9.6 mGy/h during embryogenesis) showed a time-dependent generation of ROS ($p < 0.05$), more significant in the GE group (up to 5.3-fold increase at 96 hpf) compared to the E line (up to 2.7-fold increase at 120 hpf, Fig. 8B). ROS formation at 120 hpf was seen inside the larva (Fig. 8C).

3.4. Parental irradiation increases lipid peroxidation in progeny

LPO was assessed to investigate whether gamma irradiation caused oxidative damage in progeny produced one year after parental exposure. A significant increase in LPO was found only in the G line (1.3-fold, $p < 0.05$, Fig. 9). On the other hand, zebrafish embryos from the E and GE lines showed a similar significant decrease in LPO in comparison to the control ($p < 0.05$).

3.5. Parental irradiation causes genomic instability in progeny

DNA-damage was assessed in the progeny to investigate whether parental irradiation would affect the genomic integrity. Progeny of parents irradiated during gametogenesis groups showed higher DNA damage levels than controls ($p < 0.05$, Fig. 10): The increase was more pronounced in the G line (12.1% Tail DNA) compared to F1-GE line (8.8% Tail DNA). In the E line, DNA damage did not significantly differ compared to controls ($p > 0.05$).

3.6. Parental irradiation causes bystander effects in progeny

The ratiometric calcium assay in cells measures the calcium flux through the cellular membrane, and is the first sign that a bystander signal is present (Lyng et al., 2000). This triggers the induction of downstream bystander effects such as mitochondrial membrane depolarization and reproductive failure in reporter cells. One month after irradiation of the parents, a significantly higher calcium flux was induced by the cultured medium in the G line, while one year after parental exposure, higher calcium flux was seen in the E line compared to control (~ 25% and 30% increase; $p = 0.0019$ and 0.043, respectively) (Fig. 11A and B). Although prior parental irradiation during gametogenesis with 8.7 mGy/h (G and GE lines) induced a higher calcium flux than in controls one year after irradiation, it was not found to be

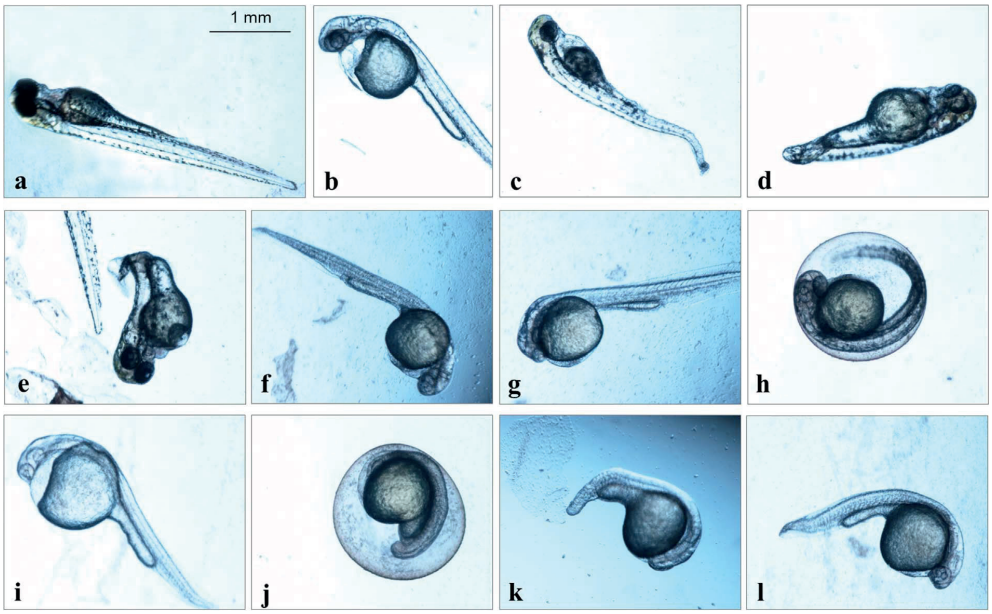

Fig. 6. Examples of most frequent deformities observed in zebrafish embryos at 72 hpf spawned 12 days after exposure of parents during gametogenesis to 8.7 mGy/h and exposure of embryos to 9.6 mGy/h. **(a) F1 Control**; **(b-d) E-line.** Pericardial edema (b), deformed tail (c) and short tailed, edematous larva (d); **(e-g) G-line.** The pictures show a hatched short-tailed larva with a spinal curvature (e), and larvae with no pigment and irregular head shape; **(h-l) GE-line.** The pictures show delayed hatching, lack of pigment, retardation in embryo development and short-tailed larvae with an overall retardation in development.

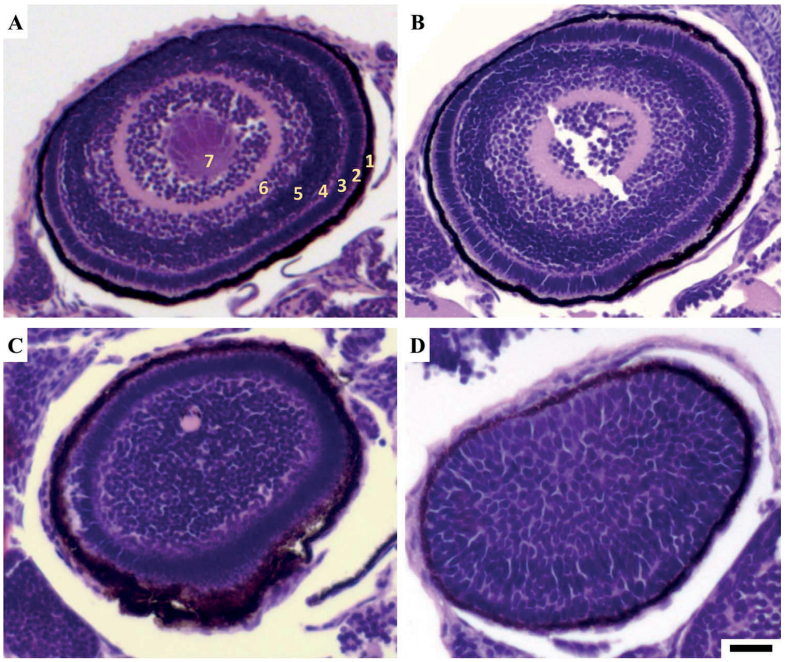

Fig. 7. Eye development in F1 progeny at 72 hpf one year after 27 day exposure of parental zebrafish during gametogenesis to 8.7 mGy/h and immediately after exposure of embryos compared to control. **A. F1 Control.** Normal eye morphology with clear lens (7) and clearly defined layers of cells: inner plexiform layer (6); inner nuclear layer (5); outer plexiform layer (4); outer nuclear layer (3); layer of rods and cones (2) surrounded by the retinal pigment epithelium (1). **B. E line.** Lens not clearly defined. **C. G line.** Poor differentiation of the retinal layers and lens not defined **D. GE line.** Undifferentiated cellular layer of the eye, lens not defined. Scale bar = 10 μm.

statistically significant ($p > 0.2$) (Fig. 11B).

4. Discussion

Gamma irradiation during zebrafish embryogenesis has been associated with effects such as DNA damage (Hudson et al., 2011; Jarvis and Knowles, 2003; Lemos et al., 2017), increased ROS, apoptosis, bystander effects (Gagnaire et al., 2015; Koturbash et al., 2008; Pereira et al., 2014) and also genetic (Freeman et al., 2014; Jaafar et al., 2013) and epigenetic changes (Ilnytskyy and Kovalchuk, 2011). However, very few studies have investigated the occurrence of adverse effects in progeny in the case of exposure of the parents during gametogenesis. The current study investigated the effects occurring in the progeny of gamma irradiated parents (F0) as well as the effects arising from irradiation of F1 embryos from the early blastula (2.5 hpf; 256-cell stage) corresponding the onset of cell specification (Haberle et al., 2014) and throughout the hatching period (48–72 hpf), a timeframe known to be associated with numerous morphological changes (Kimmel et al., 1995). The analysis of oxidative stress parameters such as ROS formation, DNA damage, LPO and bystander effects in embryos before hatching, identified molecular changes in progeny of irradiated parents and irradiated progeny one month after exposure of the parental fish to gamma radiation and one year after. But there were time dependent differences in effects between both the gametogenesis and embryogenesis exposure lines and controls. The dose rates for the parental fish (8.7 mGy/h) and offspring irradiation (9.6 mGy/h) span the upper range of the derived consideration reference levels (DCRL) for fish (~0.42 mGy/h – 40 mGy/h), at which there are "likely to be some observable adverse effects occurring to individuals" (ICRP, 2012). The doses are however an order of magnitude higher than the levels (10 mGy/day; 0.24 mGy/h) described as not likely to have any detrimental effect to aquatic populations (UNSCEAR, 1996) and also three orders of magnitude higher than the suggested ERICA screening value of 10 μGy/h (predicted no effect dose rate, PNEDR for ecological effects) (Garnier-Laplace et al., 2010). Considering that no mortality was observed in the adult fish 1.5 year after irradiation even at 53 mGy/h (results not shown), the applied doses seem to not be life threatening for adult individuals, but the reduction in viable offspring could be detrimental to sustainability at the population levels.

4.1. Development and histopathology

The results from the observations of survival, deformities and total hatching in progeny of irradiated parents showed statistically significant differences between the groups. Mortality and deformities were significantly increased in progeny of irradiated parents at both 8.7 mGy/h and 53 mGy/h compared to controls. The offspring of parents exposed to 53 mGy/h had 100% mortality at the gastrulation stage and between the onset and 50% of epiboly (Fig. 2). This stage of development plays a significant role in the embryonic morphogenesis, and includes movement and spreading of the blastoderm cells in order to cover the yolk and form a closure known as the blastopore and requires a coordination of microtubules which contract with the cell movements (Kimmel et al., 1995; Warga and Kimmel, 1990). Epiboly is hypothesized to be a maternally encoded mechanism and can be impaired by UV radiation (Strähle and Jesuthasan, 1993). Further studies are necessary to elucidate the mechanisms behind the impairment of epiboly following parental gamma radiation.

Although embryo mortality was observed at 48 hpf, no significant further increase was observed from 48 to 96 hpf (Fig. 3, Table A4). These observations suggest that the early developmental stages prior to the hatching interval may be more sensitive to the effects of ionizing radiation. Embryonic mortality after exposure of the parents to ionizing radiation during gametogenesis are still not available in zebrafish, however, increased and dose-dependent postnatal embryonic mortality and deformities after parental exposure was previously reported in other vertebrates (Nefyodova and Nefyodov, 2000). Regarding embryonic exposures, increased mortality in zebrafish embryos was reported only for acute exposures from 1 to 24 hpf, with maximal sensitivity after irradiation to ≥4 Gy before 4 hpf (X-rays) (McAleer et al.,

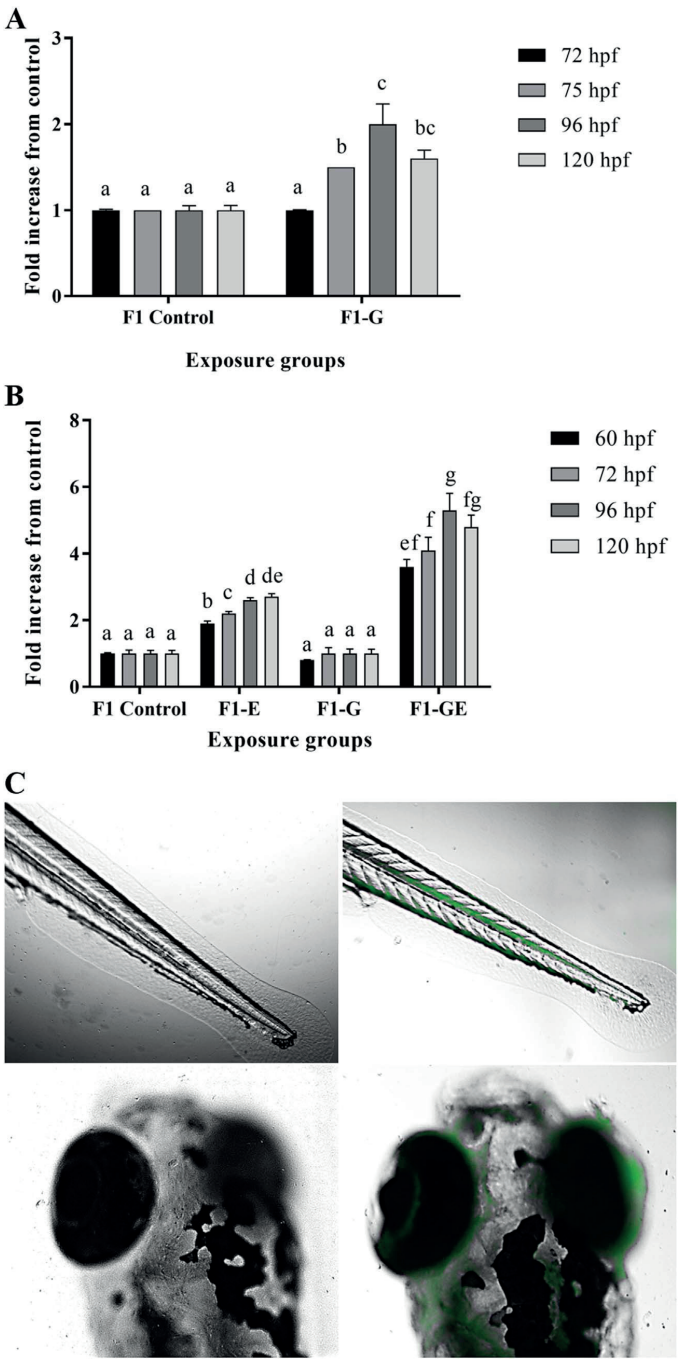

Fig. 8. Intracellular formation of reactive oxygen species (ROS) measured by 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) in F1 zebrafish (60–120 hpf). (**A**) ROS increase in G line one month after exposure of parents to 8.7 mGy/h during gametogenesis. (**B**) ROS increase in E and GE line one year after parental exposure and immediately after exposure of progeny during embryogenesis to 9.6 mGy/h. Letters represent statistical significance between exposure groups ($p < 0.05$), $n = 20$. (**C**) ROS formation measured as fluorescent 2,7-dichlorofluorescein (green) in control (left) and GE line (right) 120 hpf zebrafish. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2005) and from a chronic exposure to 38 mGy/h (1.66 Gy) (Hurem et al., 2017). Other studies, however, report no increase in embryo mortality after acute exposure to ionizing radiation at doses between 1 and 10 Gy (Freeman et al., 2014) or chronic exposures up to 24 mGy/h (2.28 Gy) (Gagnaire et al., 2015), although multiple deformities induced by continuous exposure have been shown.

In this study, premature hatching occurred in all exposed groups compared to controls at the start of the hatching interval (48 – 54 hpf)

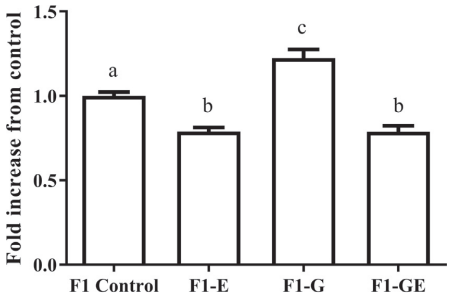

Fig. 9. Lipid peroxidation measured as malondialdehyde (MDA) and 4-hydroxyalkenals (4-HNE) in 72 hpf F1 zebrafish one year after parental exposure to 8.7 mGy/h during gametogenesis and immediately after progeny exposure to 9.6 mGy/h during embryogenesis in E, G and GE line compared to control. Letters represent statistical significance between exposure groups ($p < 0.05$) 1 year after exposure of parents, n = 4 pooled biological replicate samples of 40 embryos.

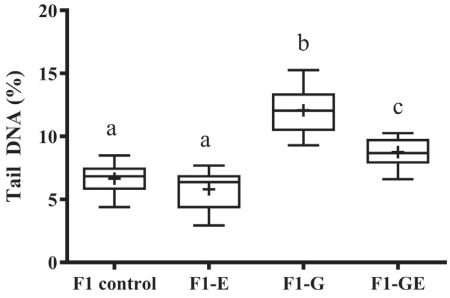

Fig. 10. DNA damage measured by the alkaline SCGE in F1 zebrafish one year after parental exposure to 8.7 mGy/h during gametogenesis and immediately after progeny (F1) exposure during embryogenesis to 9.6 mGy/h in E, G and GE line compared to control at 72 hpf. The box plot shows the median and 1st and 3rd quartile, and the minimum and maximum values obtained. Each cross represents the average Tail DNA (%) for each exposure group obtained from 3 biological replicate samples (10 pooled embryos in each sample), each biological sample with 3 technical replicates. Letters represent statistical significance between exposure groups ($p < 0.05$).

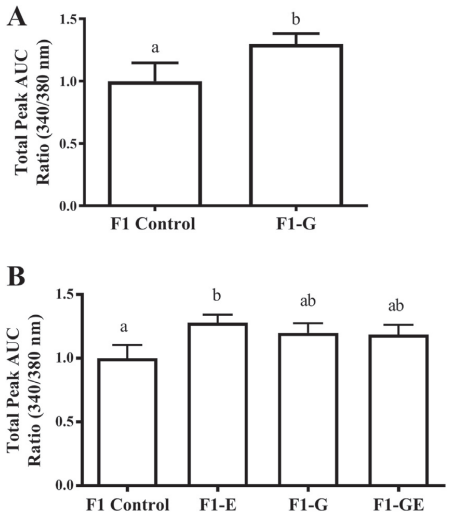

Fig. 11. Maximum Ca^{2+} flux through the cellular membrane of reporter HaCaT cells after exposure to cultured medium from F1 zebrafish (72 hpf). **(A)** Increase in G line one month after exposure of parents to 8.7 mGy/h during gametogenesis **(B)** Increase in E line one year after exposure of parents and immediately after exposure of progeny during embryogenesis to 9.6 mGy/h. The calcium concentrations are plotted as integrated area under the curve (AUC) for intracellular calcium after addition of the conditioned medium. Letters represent statistical significance between groups ($p < 0.05$), n = 25.

(Fig. 4, Table A5) and the HT_{50} was significantly decreased in all exposed lines compared to controls. The E-line embryos (0.62 Gy) showed accelerated hatching during the entire hatching interval, and no decrease in the % total hatching assessed at 96 hpf. In the G and GE lines, where the total doses (gametes and embryos) were 5.2 Gy and 5.82 Gy, respectively, the hatching rate decreased after approaching 72 hpf, and subsequently, the total hatching was significantly decreased in these groups compared to controls. Interestingly, in a study of hatching intervals after X-rays exposure during the blastula stage, earlier hatching was caused by low doses (25 mGy at 0.43 Gy/min), while higher doses (250–500 mGy) had a delaying effect on the hatching onset (Miyachi et al., 2003). In addition, other studies suggest that both low and high doses had an accelerating effect on the hatching interval (Gagnaire et al., 2015; Pereira et al., 2014), which the calculated HT_{50} obtained in this study agrees with, since this was hypothesized to be a consequence of increase in global metabolism rates of the larva and earlier energy reserve consumption. In terms of deformity occurrences, some studies previously report that the eye diameter and head length were found to be decreased in embryos exposed to acute gamma radiation (10 Gy) (Freeman et al., 2014), which is approximately two times higher than the total parental dose (5.2 Gy) in the present study (Fig. 6 and 7).

Eyes in zebrafish are normally developed at 48 hpf (Geiger et al., 2006) and are functional within 3 days post fertilization (73–80 hpf) (Dahm et al., 2007; Jonasova and Kozmik, 2008). The differences in the development of the eyes histologically determined in both directly irradiated progeny (E and GE), but also in non-irradiated progeny of irradiated parents (G), suggest direct damage to the exposed cells, however, the effects in progeny of irradiated parents are far more severe. In a study of eye development after X-ray irradiation (1–8 Gy at 8 hpf), significant ROS and apoptosis increase were found in 24–48 hpf zebrafish, while contrary to the present study, the ROS decreased to control level at 72 hpf and the only visible defect was a smaller eye diameter found at 144 hpf in the group exposed to 8 Gy (Zhou et al., 2014). However, more severe defects, such as poor definition of cellular layers of the eye, were found after exposure of embryos during the gastrulation stage (4 hpf) to 10 and 20 Gy gamma radiation, (Geiger et al., 2006), similar to what is found in the present study in progeny of irradiated zebrafish (G and GE line) at 72 hpf (Fig. 7). This suggests that not only direct embryonic irradiation with a high dose, but also irradiation during gametogenesis of the parents (5.2 Gy) can severely disrupt the embryonic developmental program as well as eye morphogenesis in zebrafish. The embryos irradiated only during embryogenesis (E line) with 0.62 Gy without prior parental irradiation revealed lack of lens clarity, but all cell layers were defined, suggesting delayed eye development. Overall, the adverse effects on embryonic development after the continuous exposure of parents and subsequently their progeny in the present study showed considerable difference in severity in response to the radiation dose as well as the developmental stage during which the exposure was carried out.

4.2. Inherited oxidative stress

We hypothesized that the observed embryonic developmental defects from parental irradiation could have further implications at the cellular and metabolic level, particularly by ROS production and consequent oxidative stress effects.

The results obtained for zebrafish embryos in the present study showed an increase in ROS formation, which depended on parental exposure, embryonic exposure, time of assessment post parental

irradiation and the embryo development from 60 to 120 hpf. In general, these results demonstrate that parental irradiation caused elevated ROS formation in progeny up to one month after parental irradiation (Fig. 8A). The ROS levels appeared to subside one year after irradiation, possibly suggesting that compensatory mechanisms had been induced in the exposed parents (Fig. 8B). We suspected that the elevated ROS might affect embryonic development at multiple levels, such as energy metabolism (e.g. disruption of mitochondria respiration), signalling (e.g. cell cycle, DNA repair, cell death) and ultimately cause oxidative damage to the cells. This notion was substantiated by LPO measurements that showed significant oxidative damage even one year after irradiation. LPO is a sensitive marker of oxidative damage involved in the toxicity process that can lead to cell death (Ayala et al., 2014). ROS in general, and oxyl-radicals in particular, are capable of initiating or enhancing the process of LPO, a chain reaction that results in the oxidative deterioration of polyunsaturated fatty acids present in cellular membranes. Oxidation of membrane proteins and lipids can result in the destabilization and disintegration of the cellular membrane and ultimately result in cell death (Halliwell and Gutteridge, 2007; Livingstone, 2001). Surprisingly, we observed a significant increase only in offspring of parents irradiated during gametogenesis (G line), while it was decreased in both groups irradiated during embryogenesis (E and GE line) compared to controls (Fig. 9). It could be speculated whether adaptive or hormetic responses to the observed oxidative stress in progeny after parental irradiation could lead to decreased LPO. The dose dependent activation of the antioxidant enzymatic system in response to gamma radiation has been shown previously in zebrafish embryos exposed to a dose range from 0.1 to 1 Gy (^{137}Cs gamma source) (Hu et al., 2016). Consistently, the groups exposed during embryogenesis demonstrated an increase in ROS in embryos produced one year after exposure, suggesting that direct exposure during early life stages (embryo, early larval stage) induces changes in the ROS metabolism. The fact that LPO was increased in the G line, while it decreased in both E and GE line compared to controls, further substantiates the notion that irradiation generates ROS both via radiolysis and altered metabolisms, to which the exposed embryos respond by activating antioxidant defence mechanisms. The parental exposure caused disrupted ROS metabolism in the developing embryos leading to oxidative damage. The results obtained in this study are in accordance to what has been documented in cancer cells exposed to low dose rate gamma radiation (Kargalioglu et al., 2002).

4.3. Inherited genomic instability and bystander effects

Exposure to environmental stressors such as gamma radiation can result in cellular DNA damage, which, if unrepaired, can cause genetic alterations and lead to the development of cancer. In addition, conditions of chronic versus acute oxidative stress may contribute to the development and/or maintenance of genomic instability (Limoli and Giedzinski, 2003). In fact, several studies have linked the formation of ROS and oxidative damage in the form of LPO and DNA damage to instability in several model systems after exposure to ionizing radiation (Limoli and Giedzinski, 2003; Snyder and Morgan, 2003).

The comet assay can detect a range of DNA lesions, including DNA single-strand breaks, double-strand breaks, oxidized DNA as well as DNA-protein cross-links. In the present study, increased DNA damage levels (here mostly single-strand breaks and alkali-labile sites) were seen in the G and GE lines one year after parental gamma irradiation (8.7 mGy/h) during gametogenesis. This suggests induction of genomic instability in viable progeny, originating from exposed stem cells to a rather high total dose, which showed no visible developmental defects (Fig. 10). No significant increase was detected in E line compared to controls which probably can be ascribed to the significantly lower total dose of the embryos (0.62 Gy) compared to the total dose given to parental gametes (5.2 Gy). Another contributing factor to the differences in response may be more efficient DNA repair mechanisms in the embryo than in the G and GE lines, although one year after exposure the gametes originate exclusively from stem cells believed to exhibit efficient repair. Further support for induction of genomic instability was obtained in a parallel study using embryos collected from this experiment, in which gene expression analyses showed enrichment of pathways related to cancer, DNA response and cell death in offspring persisting one month after parental irradiation (unpublished data). Contrary to the present findings, other studies report significantly increased DNA damage in zebrafish larvae after direct exposure to gamma irradiation, such as in the E-line in the present study. Although the analyzed life stages and duration of exposure differ from the present study, such increase was shown in 5–6 day old zebrafish larvae, directly exposed to low dose gamma radiation (30 mGy) (Jarvis and Knowles, 2003), and in 24 and 48 hpf embryos after irradiation to 1–1000 mGy/d (Simon et al., 2011). Furthermore, a reduction in DNA damage in directly irradiated embryos (570 mGy/d, total 2.2 Gy) compared to controls at 96 hpf was also reported (Gagnaire et al., 2015), albeit the total dose was higher from the embryonic dose in our study (0.62 Gy).

The results of the bystander studies support the observations that effects in the cells of irradiated embryos and parents can persist after irradiation, and that the mechanisms behind the observed delayed effects seen in ROS, LPO and DNA damage could be different to those arising following direct irradiation of cells. Epigenetic and non-targeted mechanisms seem to predominate after low and chronic exposures and the results showing impacts of parental irradiation on non- and irradiated offspring have been documented for daphnia as well as for zebrafish (Parisot et al., 2015). The results also show that parental irradiation during gametogenesis can increase the level of the calcium flux in embryos, although this decreases between one month and one year post irradiation. There is evidence from fathead minnow studies and cell culture experiments (Mothersill et al., 2013; Shi et al., 2016) that low dose and chronic exposures can lead to adaptive responses which are complex and require further experimentation to determine the precise mechanisms at play.

Ionizing radiation can also induce immunomodulatory effects, which include production of cytokines, chemokines and growth factors accompanied with inflammatory infiltration of macrophages and lymphocytes (Schaue et al., 2015). It is known that immunomodulation is an integral part of the healing effect of radiotherapy, but it also causes adverse proinflammatory effects (Candéias et al., 2004; UNSCEAR, 2006). Radiation may thus induce a prolonged inflammatory state, which is connected to disease (Kusunoki and Hayashi, 2008). At the molecular level, ionizing radiation related modulation of immune response is associated with oxidative burst and ROS formation (Hekim et al., 2015), which in turn induce bystander effects and genomic instability (Georgakilas et al., 2015). The results presented in our study are consistent with these reported effects. Our results show that parental radiological stress during gametogenesis leads to genomic instability as demonstrated by the compromised DNA integrity as well as increased LPO and bystander effects in progeny (F1). Similar findings have been reported previously with DNA damage levels in non-irradiated progeny assessed immediately after parental exposure in embryos, which was comparable with the radiation dose (X-rays) of the parents (Lemos et al., 2017). Moreover, the persistence of DNA damage in embryos of previously irradiated parents point to the involvement of non-targeted mechanisms such as inflammation and bystander effects in addition to the established direct DNA damage following irradiation.

5. Conclusions

This study showed reprotoxic and developmental effects of gamma radiation that could arise from the direct damage caused to cells (e.g. embryo mortality, changes in hatchability, and deformities) as well as delayed and persistent inherited effects caused by parental irradiation and expressed as an increase in ROS formation, DNA damage, bystander effects and LPO in offspring. The results reveal persistent DNA damage

in progeny (G and GE line) one year after exposure of parents to gamma radiation. This indicates heritability of oxidative damage in offspring of irradiated parents, and late onset genomic instability in F1, demonstrated in G and GE lines. Considering the present findings and observations of heritable effects in offspring of gamma-irradiated parents, further studies are necessary to provide insight into potential transgenerational effects and the environmental impact of ionizing radiation.

Funding

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Ethical statement

This research was performed in accordance with the Norwegian Animal Protection Act (implemented EU Directive 2010/63/EU). Approval number FOTS ID 5793 was issued on December 12, 2013 by IACUC of Norwegian School of Veterinary Science (since 2014 Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, Oslo, Norway).

Acknowledgements

We would like to thank Ole Christian Lind and Hans-Christian Teien for their help with the experiments.

Appendix A

See Tables A1–A5

Adult fish and embryo exposure and dosimetry

The exposures took place at the FIGARO low-dose Co-60 irradiation facility (NMBU, Ås). Adult zebrafish were exposed in two 9 L aquaria for a total beam-on time of 591.5 h. One aquarium was placed with its front face at a distance to the source focus of 132 cm near the central field axis (with its bottom edge 72.5 cm above the floor) and the other with its front face at a distance to the source focus of 268 cm in the upper half of the field (with its bottom edge 98 cm above the floor). A control aquarium was placed behind lead shielding where the air kerma rate was 4 μGy/h.

The exposure of embryos took place in the front four wells of 12 well microtiter plates (NuncTM, Thermo Fisher Scientific, Pittsburgh, USA) for a total beam-on time of 65 h. The wells had a diameter of 2.2 cm and were filled with 3 mL of water to a height of about 1 cm. The stack of plates was

Table A1
Measured and calculated dose quantities for the exposure of adult zebrafish and of zebrafish embryos. The measured quantities are the air kerma rates predicted from the reference measurements by the SSDL at the NRPA on the central field axis (Bjerke and Hetland, 2014) and air kerma rates measured with nanoDots (see e.g. Hansen and Hetland, 2017), also converted to absorbed dose rates to water. The simulated quantities were obtained from the Geant4 simulations (Agostinelli et al., 2003, Allison et al., 2006) and the calculated quantities from tabulated data (Aird et al., 1996). The average absorbed dose is the average absorbed dose rate multiplied with the exposure time. The relative standard uncertainty in the SSDL air kerma rates is 6% (coverage factor k = 2). The relative standard uncertainty in nanoDot measurements is estimated to 5% (coverage factor k = 2) and the reported values are means of repeated measurements. The simulation results are averages over n = 5 repeated runs, and the Type A statistical relative uncertainty in these results is at or below 1%.

	Measurement location	Air kerma rate [mGy/h]	Absorbed dose rate to water [mGy/h]	Absorbed dose to water [Gy]
Adults at 53 mGy/h				
SSDL nanoDots simulated	Aquarium front plane on central field axis	64		
	Average across aquarium front plane	61		
	Around center of aquarium plane at depth of dose maximum		73	
	Around center of aquarium plane at depth of 7.5 cm		54	
	Average across aquarium front plane	61		
	Average across aquarium plane at depth of dose maximum		73	
	Average across aquarium plane at depth of 7.5 cm		55	
	Average across aquarium plane at largest depth for 20 cm swimming configuration		27	
	Whole aquarium average for 20 cm swimming configuration		47	
	Whole aquarium average for 15 cm swimming configuration		54	
	Whole aquarium average weighted by time spent at each swimming configuration		53	31
Adults at 8.7 mGy/h				
SSDL simulated	Aquarium front plane on central field axis	15.5		
	Average across aquarium plane at depth of dose maximum		12	
	Average across aquarium plane at depth of 7.5 cm		9.4	
	Average across aquarium plane at largest depth for 20 cm swimming configuration		4.3	
	Whole aquarium average for 20 cm swimming configuration		7.9	
	Whole aquarium average for 15 cm swimming configuration		8.9	
	Whole aquarium average weighted by time spent at each swimming configuration		8.7	5.2
Embryos at 9.6 mGy/h				
SSDL nanoDots calculated	Microtiter plate front plane on central field axis	9.7		
	Microtiter plates front plane	8.8		
	Microtiter plates well average		9.6	0.62

Table A2
Dose rates and total doses used in the gamma radiation exposures with zebrafish embryos (including previous irradiation of parents).

Group	Parental exposure (during gametogenesis)			Offspring exposure (during embryogenesis)		
	Exposure time	Average absorbed		Exposure time	Average absorbed	
	[h]	Dose rate [mGy/h]	Dose [Gy]	[h]	Dose rate [mGy/h]	Dose [Gy]
F1 Control	-	-	-	-	-	-
F1-E	-	-	-	65	9.6	0.62
F1-G	591.5 h	8.7	5.2	-	-	-
F1-GE	591.5 h	8.7	5.2	65	9.6	0.62

Table A3
Water quality parameter values in each zebrafish aquarium during exposure of adult fish to gamma radiation for 27 days.

Water quality parameter	Aquarium exposure (mGy/h)	Mean ± SD	MIN	1st quartile	Median	3rd quartile	MAX
Temperature (°C)	Control	28.09 ± 0.49	26.7	27.85	28.2	28.4	28.8
	8.7	28.11 ± 0.49	26.7	27.9	28.2	28.33	28.9
	53	28.10 ± 0.48	26.7	27.88	28.2	28.33	28.8
pH	Control	7.59 ± 0.08	7.45	7.53	7.58	7.67	7.79
	8.7	7.61 ± 0.09	7.47	7.54	7.6	7.67	7.79
	53	7.61 ± 0.08	7.47	7.55	7.6	7.65	7.79
Conductivity µs/cm	Control	468.21 ± 31.86	412	443.5	463.5	487.5	556
	8.7	467.44 ± 32.03	412	442	463	488.5	555
	53	468.18 ± 32.01	412	442.75	462	488.5	554
Ammonia NH_3, NH_4+ (mg/l)	Control	0.13 ± 0.26	0	0.13	0.25	0.25	0.88
	8.7	0.13 ± 0.26	0	0.13	0.25	0.25	0.88
	53	0.13 ± 0.26	0	0.13	0.25	0.25	0.88
Nitrite NO_2- (mg/l)	Control	0.30 ± 0.08	0.15	0.15	0.3	0.3	0.3
	8.7	0.30 ± 0.08	0.15	0.15	0.3	0.3	0.3
	53	0.30 ± 0.08	0.15	0.15	0.3	0.3	0.3
Nitrate NO_3- (mg/l)	Control	16.25 ± 3.86	6.25	6.25	12.5	12.5	18.75
	8.7	16.25 ± 3.86	6.25	6.25	12.5	12.5	18.75
	53	16.25 ± 3.86	6.25	6.25	12.5	12.5	18.75

Table A4
Mortality rate in zebrafish lines from 48 to 96 hpf after exposure to gamma radiation during parental gametogenesis or during embryogenesis from 2.5 to 3 hpf to 72 hpf (see nomenclature). Data presented as mean ± SEM. Significance in comparison to control denoted with (*).

Group	Hours post fertilization (hpf)				
	48	54	60	72	96
F1 Control	1.4 ± 0.6	1.4 ± 0.6	1.4 ± 0.6	1.4 ± 0.6	1.4 ± 0.6
F1-E	3.1 ± 1.2	3.1 ± 1.2	3.1 ± 1.2	3.1 ± 1.2	3.1 ± 1.3
F1-G	4.5 ± 1.5	4.5 ± 1.5	4.5 ± 1.5	4.5 ± 1.5	5.2 ± 1.7
F1-GE	7.3 ± 0.7*	7.3 ± 0.7*	7.3 ± 0.7*	7.3 ± 0.7*	9.3 ± 1.7*

Table A5
Hatching rate and median hatching time (95% confidence intervals of HT_{50}) in zebrafish lines from 48 to 96 hpf after exposure to gamma radiation during parental gametogenesis or during embryogenesis from 2.5 to 3 hpf to 72 hpf (see nomenclature). Data presented as mean ± SEM. Significance in comparison to control denoted with (*).

Group	Hours post fertilization					
	48	54	60	72	96	HT_{50}
F1 Control	4 ± 0.96	24.5 ± 2.1	63.4 ± 2.4	92.2 ± 1.3	92.2 ± 1.3	57.4 (57.2 to 57.5)
F1-E	2.6 ± 1.2	51.8 ± 3.7*	92.5 ± 1.9*	96.9 ± 1.3	96.9 ± 1.3	53.7 (53.6 to 53.9)*
F1-G	5.2 ± 1.3	39.6 ± 3*	69.4 ± 2.8	84 ± 2.2*	84 ± 2.2*	54.6 (54.2 to 55.0)*
F1-GE	2.1 ± 0.9	49.5 ± 3.1*	70.6 ± 2.8*	85.5 ± 2.2*	85.5 ± 2.2*	53.5 (52.6 to 54.5)*

placed with its front row of wells at a distance to the source focus of 338 cm. Control plates were behind lead shielding where the air kerma rate was 4 μGy/h.

Reference measurements by the Secondary Standard Dosimetry Laboratory (SSDL) at the Norwegian Radiation Protection Authority (NRPA) predict air kerma rates on the central field axis at FIGARO with a relative standard uncertainty of 8% (coverage factor k = 2) (Bjerke and Hetland, 2014). Because the gamma fluence is lower higher in the field, the aquarium and microtiter plates placed farthest from the source and highest in the field received a lower dose than that predicted on the central field axis.

Measurements on the actual setups during exposures were made with optically stimulated luminescence dosimeters, so-called nanoDots (Landauer, Inc., Greenwood, IL) (see e.g. Hansen and Hetland, 2017). In addition, a newly developed Geant4 model of FIGARO was used to simulate average absorbed dose rates and doses to water in the aquaria (Table A1), based on their geometry, composition and locations in the field, and on the measured data (used for normalization at one dose level). Geant4 is a C++ code suit for Monte Carlo simulations of the passage of particles through matter (Agostinelli et al., 2003; Allison et al., 2006). The Geant4 model of FIGARO will be published elsewhere.

The exposures of adults took place with two different configurations of the swimming area in the aquaria. For the first week (for 123.2 h) zebrafish could swim to a depth of 20 cm while for the remaining weeks (for 468.3 h) the swimming area was limited to a depth of 15 cm (for practical concerns relating to fish husbandry). Average absorbed dose rates to water for the full exposure period were obtained by weighting the dose rates for the original and modified swimming configurations with the time spent at each configuration.

For the well plates, conversion of air kerma rates to absorbed doses to air, the ratio of the mass energy-absorption coefficients of water to air (Hubbell and Seltzer, 2004) plus tabulated peak scatter factors and depth dose curves (Aird et al., 1996) were used to estimate absorbed doses to water from the measured air kerma rates. The tabulated percentage depth dose data (for a 0 cm × 0 cm field because of the small amount of material in the beam) was calculated via conversion formula (Hubbell and Seltzer, 2004) to the actual source to surface distances in the setup. The mean of the percentage depth dose at the depth of dose maximum (0.5 cm, defined to 100%) and at a depth of 2 cm was used to estimate the average absorbed dose to water in the wells. Briefly, the ratio of the mass-energy absorption coefficients of water to air was 1.112 (tabulated for 1.25 MeV photons) and the peak scatter factor was set to 1.000 (again because of the small amount of material in the beam and its presence anyways during the air kerma measurements). The calculated percentage depth dose at a depth of 2 cm was respectively 94.5% (at 169 cm to the source focus) and 94.9% (at 338 cm to the source focus).

The resulting estimated average absorbed dose rates to water in the aquaria were 53 mGy/h and 8.7 mGy/h. The corresponding total absorbed doses were respectively 31 Gy and 5.2 Gy. For the well plates, the estimated average absorbed dose rate to water was 9.6 mGy/h. The total absorbed dose was 0.62 Gy. The absorbed dose rates and doses to fish swimming freely in the aquaria or to embryos in the well plates are estimated by the absorbed dose rates and doses to water.

References

Adam-Guillermin, C., Pereira, S., Della-Vedova, C., Hinton, T., Garnier-Laplace, J., 2012. Genotoxic and reprotoxic effects of tritium and external gamma irradiation on aquatic animals. Rev. Environ. Contam. Toxicol. 220, 67–103. http://dx.doi.org/10.1007/978-1-4614-3414-6_3.

Agostinelli, S., et al., 2003. Geant4—a simulation toolkit. Nucl. Instrum. Methods Phys. Res. Sect. A: Accel., Spectrom. Detect. Assoc. Equip. 506 (3), 250.

Aird, E.G.A., Burns, J.E., Day, M.J., Duane, S., Jordan, T.J., Kacperek, A., Klevenhagen, S.C., Harrison, R.M., Lillicrap, S.C., McKenzie, A.L., Pitchford, W.G., Shaw, J.E., Smith, C.W., 1996. British Journal of Radiology Supplement 25. Central Axis Depth Dose Data for Use in Radiotherapy. The British Institute of Radiology, London, UK.

Allison, J., et al., 2006. Geant4 developments and applications. IEEE Trans. Nucl. Sci. 53 (1), 270.

Ayala, A., Muñoz, M.F., Argüelles, S., 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid. Med. Cell. Longev. 2014, 360438. http://dx.doi.org/10.1155/2014/360438.

Bjerke, H., Hetland, P.O., 2014. Dosimetri ved FIGARO gammaanlegget ved NMBU, Ås. Målerapport fra oppmåling av doseraten i strålefeltet fra kobolt-60. NRPA Technical Document Series, 2.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buisset-Goussen, A., Goussen, B., Della-Vedova, C., Galas, S., Adam-Guillermin, C., Lecomte-Pradines, C., 2014. Effects of chronic gamma irradiation: a multi-generational study using Caenorhabditis elegans. J. Environ. Radioact. 137, 190–197. http://dx.doi.org/10.1016/j.jenvrad.2014.07.014.

Candéias, S.M., Mancini, S.J.C., Touvrey, C., Borel, E., Jouvin-Marche, E., Marche, P.N., 2004. p53-dependent and p53-independent pathways for radiation-induced immature thymocyte differentiation. Oncogene 23, 1922–1929. http://dx.doi.org/10.1038/sj.onc.1207320.

Chang, W.P., Little, J.B., 1992. Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving X-irradiation: association with delayed reproductive death phenotype. Mutat. Res. - Fundam. Mol. Mech. Mutagen. 270, 191–199. http://dx.doi.org/10.1016/0027-5107(92)90130-T.

Dahm, R., Schonthaler, H.B., Soehn, A.S., van Marle, J., Vrensen, G.F.J.M., 2007. Development and adult morphology of the eye lens in the zebrafish. Exp. Eye Res. 85, 74–89. http://dx.doi.org/10.1016/j.exer.2007.02.015.

Erdelmeier, I., Gérard-Monnier, D., Yadan, J.-C., Chaudi?re, J., 1998. Reactions of N -methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. mechanistic aspects of the colorimetric assay of lipid peroxidation. Chem. Res. Toxicol. 11, 1184–1194. http://dx.doi.org/10.1021/tx970180z.

Filkowski, J.N., Ilnytskyy, Y., Tamminga, J., Koturbash, I., Golubov, A., Bagnyukova, T., Pogribny, I.P., Kovalchuk, O., 2010. Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression. Carcinogenesis 31, 1110–1115. http://dx.doi.org/10.1093/carcin/bgp300.

Freeman, J.L., Weber, G.J., Peterson, S.M., Nie, L.H., 2014. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front. Genet. 5, 268. http://dx.doi.org/10.3389/fgene.2014.00268.

Gagnaire, B., Cavalié, I., Pereira, S., Floriani, M., Dubourg, N., Camilleri, V., Adam-Guillermin, C., 2015. External gamma irradiation-induced effects in early-life stages of zebrafish, Danio rerio. Aquat. Toxicol. 169, 69–78. http://dx.doi.org/10.1016/j.aquatox.2015.10.005.

Garnier-Laplace, J., Della-Vedova, C., Andersson, P., Copplestone, D., Cailes, C., Beresford, N.A., Howard, B.J., Howe, P., Whitehouse, P., 2010. A multi-criteria weight of evidence approach for deriving ecological benchmarks for radioactive substances. J. Radiol. Prot. 30, 215–233. http://dx.doi.org/10.1088/0952-4746/30/2/S02.

Geiger, G.A., Parker, S.E., Beothy, A.P., Tucker, J.A., Mullins, M.C., Kao, G.D., 2006. Zebrafish as a "biosensor"? Effects of ionizing radiation and amifostine on embryonic viability and development. Cancer Res. 66, 8172–8181. http://dx.doi.org/10.1158/0008-5472.CAN-06-0466.

Georgakilas, A.G., Pavlopoulou, A., Louka, M., Nikitaki, Z., Vorgias, C.E., Bagos, P.G., Michalopoulos, I., 2015. Emerging molecular networks common in ionizing radiation, immune and inflammatory responses by employing bioinformatics approaches. Cancer Lett. 368, 164–172. http://dx.doi.org/10.1016/j.canlet.2015.03.021.

Gutzkow, K.B., Langleite, T.M., Meier, S., Graupner, A., Collins, A.R., Brunborg, G., 2013. High-throughput comet assay using 96 minigels. Mutagenesis 28, 333–340. http://dx.doi.org/10.1093/mutage/get012.

Haberle, V., Li, N., Hadzhiev, Y., Plessy, C., Previti, C., Nepal, C., Gehrig, J., Dong, X., Akalin, A., Suzuki, A.M., van IJcken, W.F.J., Armant, O., Ferg, M., Strähle, U., Carninci, P., Müller, F., Lenhard, B., 2014. Two independent transcription initiation codes overlap on vertebrate core promoters. Nature 507, 381–385. http://dx.doi.org/10.1038/nature12974.

Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine, 4th. Oxford University Press, Oxford (ISBN-10: 019856869X).

Hansen, S.H., Olsen, A.K., Søderlund, E.J., Brunborg, G., 2010. In vitro investigations of glycidamide-induced DNA lesions in mouse male germ cells and in mouse and human lymphocytes. Mutat. Res. 696, 55–61. http://dx.doi.org/10.1016/j.mrgentox.2009.12.012.

Hansen, E.L., Hetland, P.O., 2017. Air kerma measurements with Landauer nanoDots in Cs-137 and Co-60 beams, Part I - SSDL exposures free in air. NRPA Technical Document Series, 8.

Hekim, N., Cetin, Z., Nikitaki, Z., Cort, A., Saygili, E.I., 2015. Radiation triggering immune response and inflammation. Cancer Lett. 368, 156–163. http://dx.doi.org/10.1016/j.canlet.2015.04.016.

Hu, M., Hu, N., Ding, D., Zhao, W., Feng, Y., Zhang, H., Li, G., Wang, Y., 2016. Developmental toxicity and oxidative stress induced by gamma irradiation in zebrafish embryos. Radiat. Environ. Biophys. 55, 441–450. http://dx.doi.org/10.1007/

s00411-016-0663-4.
Hubbell, J.H., Seltzer, S.M., 2004. Tables of X-Ray Mass Attenuation Coefficients and Mass Energy-Absorption Coefficients (version 1.4) [Online]. Available: ⟨http://www.nist.gov/pml/data/xraycoef/index.cfm⟩, National Institute of Standards and Technology, Gaithersburg, MD. Originally published as NISTIR 5632, National Institute of Standards and Technology, Gaithersburg, MD (1995).
Hudson, D., Kovalchuk, I., Koturbash, I., Kolb, B., Martin, O.A., Kovalchuk, O., 2011. Induction and persistence of radiation-induced DNA damage is more pronounced in young animals than in old animals. Aging (Albany NY). 3, 609–620. http://dx.doi.org/10.18632/aging.100340.
Hurem, S., Martín, L.M., Brede, D.A., Skjerve, E., Nourizadeh-Lillabadi, R., Lind, O.C., Christensen, T., Berg, V., Teien, H.C., Salbu, B., Oughton, D.H., Aleström, P., Lyche, J.L., 2017. Dose-dependent effects of gamma radiation on the early zebrafish development and gene expression. PLoS One 12, e0179259. http://dx.doi.org/10.1371/journal.pone.0179259.
ICRP, 2012. Compend. Dose Coefficients based ICRP Publ. 60 0, 130. doi: ⟨https://dx.doi.org/10.1016/j.icrp.2006.06.001⟩.
Ilnytskyy, Y., Kovalchuk, O., 2011. Non-targeted radiation effects-an epigenetic connection. Mutat. Res. 714, 113–125. http://dx.doi.org/10.1016/j.mrfmmm.2011.06.014.
Jaafar, L., Podolsky, R.H., Dynan, W.S., 2013. Long-term effects of ionizing radiation on gene expression in a zebrafish model. PLoS One 8, e69445. http://dx.doi.org/10.1371/journal.pone.0069445.
Jacquet, P., 2004. Sensitivity of germ cells and embryos to ionizing radiation. J. Biol. Regul. Homeost. Agents 18, 106–114.
Jarvis, R., Knowles, J., 2003. DNA damage in zebrafish larvae induced by exposure to low-dose rate γ-radiation: detection by the alkaline comet assay. Mutat. Res. Toxicol. Environ. Mutagen. 541, 63–69. http://dx.doi.org/10.1016/S1383-5718(03)00182-7.
Jonasova, K., Kozmik, Z., 2008. Eye evolution: lens and cornea as an upgrade of animal visual system. Semin. Cell Dev. Biol. 19, 71–81. http://dx.doi.org/10.1016/j.semcdb.2007.10.005.
Kadhim, M.A., Macdonald, D.A., Goodhead, D.T., Lorimore, S.A., Marsden, S.J., Wright, E.G., 1992. Transmission of chromosomal instability after plutonium alpha-particle irradiation. Nature 355, 738–740. http://dx.doi.org/10.1038/355738a0.
Kargalioglu, Y., McMillan, B.J., Minear, R.A., Plewa, M.J., 2002. Lipid peroxidation, DNA damage, and cellular morphology of R1 Rhabdomyosarcoma cell line irradiated in vitro by gamma-rays with different dose rates. Teratog. Carcinog. Mutagen. 22, 93–102. http://dx.doi.org/10.1002/tcm.10006.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. http://dx.doi.org/10.1002/aja.1002030302.
Koturbash, I., Kutanzi, K., Hendrickson, K., Rodriguez-Juarez, R., Kogosov, D., Kovalchuk, O., 2008. Radiation-induced bystander effects in vivo are sex specific. Mutat. Res. 642, 28–36. http://dx.doi.org/10.1016/j.mrfmmm.2008.04.002.
Kumaravel, T.S., Jha, A.N., 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat. Res. Toxicol. Environ. Mutagen. 605, 7–16. http://dx.doi.org/10.1016/j.mrgentox.2006.03.002.
Kusunoki, Y., Hayashi, T., 2008. Long-lasting alterations of the immune system by ionizing radiation exposure: implications for disease development among atomic bomb survivors. Int. J. Radiat. Biol. 84, 1–14. http://dx.doi.org/10.1080/09553000701616106.
Lemos, J., Neuparth, T., Trigo, M., Costa, P., Vieira, D., Cunha, L., Ponte, F., Costa, P.S., Metello, L.F., Carvalho, A.P., 2017. Single low-dose ionizing radiation induces genotoxicity in adult zebrafish and its non-irradiated progeny. Bull. Environ. Contam. Toxicol. 98, 190–195. http://dx.doi.org/10.1007/s00128-016-2006-1.
Limoli, C.L., Giedzinski, E., 2003. Induction of chromosomal instability by chronic oxidative stress. Neoplasia 5, 339–346.
Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656–666. http://dx.doi.org/10.1016/S0025-326X(01)00060-1.
Lorimore, S.A., Kadhim, M.A., Pocock, D.A., Papworth, D., Stevens, D.L., Goodhead, D.T., Wright, E.G., 1998. Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. Proc. Natl. Acad. Sci. USA 95, 5730–5733.
Lyng, F.M., Seymour, C.B., Mothersill, C., 2000. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. Br. J. Cancer 83, 1223–1230. http://dx.doi.org/10.1054/bjoc.2000.1433.
Maguire, P., Mothersill, C., McClean, B., Seymour, C., Lyng, F.M., 2007. Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. Radiat. Res. 167, 485–492. http://dx.doi.org/10.1667/RR0159.1.
McAleer, M.F., Davidson, C., Davidson, W.R., Yentzer, B., Farber, S.A., Rodeck, U., Dicker, A.P., 2005. Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers. Int. J. Radiat. Oncol. Biol. Phys. 61, 10–13. http://dx.doi.org/10.1016/j.ijrobp.2004.09.046.
Miyachi, Y., Kanao, T., Okamoto, T., 2003. Marked depression of time interval between fertilization period and hatching period following exposure to low-dose X-rays in zebrafish. Environ. Res. 93, 216–219. http://dx.doi.org/10.1016/S0013-9351(03)00042-2.
Mothersill, C., Seymour, C., 1997. Survival of human epithelial cells irradiated with cobalt 60 as microcolonies or single cells. Int. J. Radiat. Biol. 72, 597–606.
Mothersill, C., Smith, R., Lariviere, D., Seymour, C., 2013. Chronic exposure by ingestion of environmentally relevant doses of ^{226}Ra leads to transient growth perturbations in fathead minnow (*Pimephales promelas*, Rafinesque, 1820). Int. J. Radiat. Biol. 89, 950–964. http://dx.doi.org/10.3109/09553002.2013.809817.
Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., Laissue, J., Schroll, C., Seymour, C., 2014. Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. Dose-Response 12, 72–92. http://dx.doi.org/10.2203/dose-response.13-011.Mothersill.
Nascimento, F.J.A., Bradshaw, C., 2016. Direct and indirect effects of ionizing radiation on grazer–phytoplankton interactions. J. Environ. Radioact. 155–156, 63–70. http://dx.doi.org/10.1016/j.jenvrad.2016.02.007.
Nefyodova, I., Nefyodov, I., 2000. Hereditary radiation effects in offspring of the second and third generations after irradiation of both grandparents: experimental studies and hereditary radiation effects in offspring of the first generation after irradiation of one and both parents: exp. In: 10. International Congress of the International Radiation Protection Association. Hiroshima, pp. 2–79. ⟨https://inis.iaea.org/search/search.aspx?Orig_q = RN:32008612⟩.
O'Leary, V.B., Ovsepian, S.V., Carrascosa, L.G., Buske, F.A., Radulovic, V., Niyazi, M., Moertl, S., Trau, M., Atkinson, M.J., Anastasov, N., 2015. PARTICLE, a triplex-forming long ncRNA, regulates locus-specific methylation in response to low-dose irradiation. Cell Rep. 474–485. http://dx.doi.org/10.1016/j.celrep.2015.03.043.
OECD/OCDE, 2013. OECD Guidelines for the Testing of Chemicals Fish Embryo Acute Toxicity (FET) Test. http://dx.doi.org/10.1787/20745761.
Parisot, F., Bourdineaud, J.-P., Plaire, D., Adam-Guillermin, C., Alonzo, F., 2015. DNA alterations and effects on growth and reproduction in Daphnia magna during chronic exposure to gamma radiation over three successive generations. Aquat. Toxicol. 163, 27–36. http://dx.doi.org/10.1016/j.aquatox.2015.03.002.
Pereira, S., Malard, V., Ravanat, J.-L., Davin, A.-H., Armengaud, J., Foray, N., Adam-Guillermin, C., 2014. Low doses of gamma-irradiation induce an early bystander effect in zebrafish cells which is sufficient to radioprotect cells. PLoS One 9, e92974. http://dx.doi.org/10.1371/journal.pone.0092974.
Raz, E., 2003. Primordial germ-cell development: the zebrafish perspective. Nat. Rev. Genet. 4, 690–700. http://dx.doi.org/10.1038/nrg1154.
Ryan, L. a., Seymour, C.B., Joiner, M.C., Mothersill, C.E., 2009. Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. Int. J. Radiat. Biol. 85, 87–95. http://dx.doi.org/10.1080/09553000802635062.
Sample, B.E., 2011. Overview of exposure to and effects from radionuclides in terrestrial and marine environments. Integr. Environ. Assess. Manag. 7, 368–370. http://dx.doi.org/10.1002/ieam.239.
Schaue, D., Micewicz, E.D., Ratikan, J.A., Xie, M.W., Cheng, G., McBride, W.H., 2015. Radiation & inflammation. Semin Radiat. Oncol. 25 (1), 4–10. http://dx.doi.org/10.1016/j.semradonc.2014.07.007.
Seymour, C.B., Mothersill, C., Alper, T., 1986. High yields of lethal mutations in somatic mammalian cells that survive ionizing radiation. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 50, 167–179. http://dx.doi.org/10.1080/09553008614550541.
Shi, X., Seymour, C., Mothersill, C., 2016. The effects of chronic, low doses of Ra-226 on cultured fish and human cells. Environ. Res. 148, 303–309. http://dx.doi.org/10.1016/j.envres.2016.04.009.
Simon, O., Massarin, S., Coppin, F., Hinton, T.G., Gilbin, R., 2011. Investigating the embryo/larval toxic and genotoxic effects of γ irradiation on zebrafish eggs. J. Environ. Radioact. 102, 1039–1044. http://dx.doi.org/10.1016/j.jenvrad.2011.06.004.
Snyder, A.R., Morgan, W.F., 2003. Persistent oxidative stress and gene expression changes in radiation-induced genomic instability. Int. Congr. Ser. 1258, 199–206. http://dx.doi.org/10.1016/S0531-5131(03)01152-X.
Soubry, A., Hoyo, C., Jirtle, R.L., Murphy, S.K., 2014. A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. Bioessays 36, 359–371. http://dx.doi.org/10.1002/bies.201300113.
Stewart, F.A., Akleyev, A.V., Hauer-Jensen, M., Hendry, J.H., Kleiman, N.J., MacVittie, T.J., Aleman, B.M., Edgar, A.B., Mabuchi, K., Muirhead, C.R., Shore, R.E., Wallace, W.H., 2012. ICRP publication 118: ICRP Statement on tissue reactions and early and late effects of radiation in normal tissues and organs — threshold doses for tissue reactions in a radiation protection context. Ann. ICRP 41, 1–322. http://dx.doi.org/10.1016/j.icrp.2012.02.001.
Strähle, U., Jesuthasan, S., 1993. Ultraviolet irradiation impairs epiboly in zebrafish embryos: evidence for a microtubule-dependent mechanism of epiboly. Development 119, 909–919.
Streffer, C., 2004. Bystander effects, adaptive response and genomic instability induced by prenatal irradiation. Mutat. Res. 568, 79–87. http://dx.doi.org/10.1016/j.mrfmmm.2004.07.014.
Tsao-Wu, G.S., Weber, C.H., Budgeon, L.R., Cheng, K.C., 1998. Agarose-embedded tissue arrays for histologic and genetic analysis. Biotechniques 25, 614–618.
UNSCEAR, 1996. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) Report. New York. ⟨http://www.unscear.org/unscear/en/publications/1996.html⟩.
UNSCEAR, 2006. Effects of Ionizing Radiation. Annex C. New York. ⟨http://www.unscear.org/docs/publications/2006/UNSCEAR_2006_Annex-C.pdf⟩.
Vives i Batlle, J., Balonov, M., Beaugelin-Seiller, K., Beresford, N.A., Brown, J., Cheng, J.-J., Copplestone, D., Doi, M., Filistovic, V., Golikov, V., Horyna, J., Hosseini, A., Howard, B.J., Jones, S.R., Kamboj, S., Kryshev, A., Nedveckaite, T., Olyslaegers, G., Pröhl, G., Sazykina, T., Ulanovsky, A., Vives Lynch, S., Yankovich, T., Yu, C., 2007.

Inter-comparison of absorbed dose rates for non-human biota. Radiat. Environ. Biophys. 46, 349–373. http://dx.doi.org/10.1007/s00411-007-0124-1.
Warga, R.M., Kimmel, C.B., 1990. Cell movements during epiboly and gastrulation in zebrafish. Development 108, 569–580.
Winterbourn, C.C., 2014. The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. Biochim. Biophys. Acta - Gen. Subj. 1840, 730–738. http://dx.doi.org/10.1016/j.bbagen.2013.05.004.
Won, E.-J., Dahms, H.-U., Kumar, K.S., Shin, K.-H., Lee, J.-S., 2015. An integrated view of gamma radiation effects on marine fauna: from molecules to ecosystems. Environ. Sci. Pollut. Res. Int. 22, 17443–17452. http://dx.doi.org/10.1007/s11356-014-3797-4.
Xu, S., Ding, N., Pei, H., Hu, W., Wei, W., Zhang, X., Zhou, G., Wang, J., 2015. MiR-21 is involved in radiation-induced bystander effects. RNA Biol. 11, 1161–1170. http://dx.doi.org/10.4161/rna.34380.
Zhou, R., Si, J., Zhang, H., Wang, Z., Li, J., Zhou, X., Gan, L., Liu, Y., 2014. The effects of x-ray radiation on the eye development of zebrafish. Hum. Exp. Toxicol. 33, 1040–1050. http://dx.doi.org/10.1177/0960327114522278.

III

Parental exposure to gamma radiation causes progressively altered transcriptomes linked to adverse effects in zebrafish offspring

Authors:

Selma Hurem[a+], Leonardo Martín Martín[b,c,+], Leif Lindeman[b,d], Dag Anders Brede[d], Brit Salbu[d], Jan Ludvig Lyche[a], Peter Aleström[b], Jorke H Kamstra[b,*]

[+]Authors contributed equally

Affiliation:

[a]Faculty of Veterinary Medicine, Department of Food Safety and Infection Biology, CoE CERAD, Norwegian University of Life Sciences, P.O. Box 8146 Dep., 0033 Oslo, Norway.

[b]Faculty of Veterinary Medicine, Department of Basic Sciences and Aquatic Medicine, CoE CERAD, Norwegian University of Life Sciences, P.O. Box 8146 Dep., 0033 Oslo, Norway.

[c]Faculty of Agropecuary Sciences, Department of Morphophysiology, University of Camagüey, 74 650 Camagüey, Cuba.

[d]Faculty of Environmental Sciences and Natural Resource Management, Institute of environmental Sciences, Norwegian University of Life Sciences, 1433 Ås, Norway.

***Corresponding Author:** jorke.kamstra@nmbu.no

Faculty of Veterinary Medicine, Department of Basic Sciences and Aquatic Medicine, CoE CERAD, Norwegian University of Life Sciences, P.O. Box 8146 Dep., 0033 Oslo, Norway. Tel: +4767232089

Abstract

In zebrafish, parental exposure to ionizing radiation has been associated with effects in offspring, such as increased hatching rates, deformities, increased DNA damage and reactive oxygen species. Here, we assessed short (one month) and long term effects (one year) on gene expression in embryonic offspring (5.5 hours post fertilization) from zebrafish exposed during gametogenesis to gamma radiation (8.7 or 53 mGy/h for 27 days, total dose 5.2 or 31 Gy) using mRNA sequencing. One month after exposure, a global change in gene expression was observed in offspring from the 53 mGy/h group, followed by embryonic death at late gastrula, whereas offspring from the 8.7 mGy/h group was unaffected. Interestingly, one year after exposure newly derived embryos from the 8.7 mGy/h group exhibited 2455 (61.8% down-regulated) differentially expressed genes. Overlaps in differentially expressed genes and enriched biological pathways were evident between the 53 mGy/h group one month and 8.7 mGy/h one year after exposure, but were oppositely regulated. Pathways could be linked to effects in adults and offspring, such as DNA damage (via ATM signaling), lipid peroxidation (via *aldh3a1*) and reproduction (via Gnrh signaling). Comparison with gene expression analysis in directly exposed embryos indicate *transferrin a* and *cytochrome P450 2x6* as possible sensitive biomarkers for radiation response in zebrafish. Our results indicate latent effects following ionizing radiation exposure from the lower dose in parents that can be transmitted to offspring and warrants monitoring effects over subsequent generations.

Key words: **Ionizing radiation**, **mRNA sequencing**, **gene expression**, **zebrafish**, **radioecology**

Introduction

Gamma radiation, either anthropogenic or naturally occurring, can affect the genetic material directly, by induction of DNA single and double strand breaks and indirectly, via excitation of water molecules and formation of free radicals (Han and Yu, 2012). Exposure to gamma radiation is associated with a wide range of effects, such as genomic instability and tumor formation, as observed in animal models and human cohort studies (Unscear, 2010). Furthermore, studies in animal models provide evidence of effects in subsequent unexposed generations, due to affected germ cells exposed to radiation during gametogenesis (Buisset-Goussen et al., 2014; Soubry et al., 2014).

Recent studies show that zebrafish is a sensitive model in studying effects of ionizing radiation during embryogenesis (Choi and Yu, 2015). More specifically, embryos appear to be sensitive to effects of ionizing radiation at the transcriptional level, which may affect a diverse range of phenotypic outcomes, such as mortality rate, hatching time, embryo length, and malformation rate (Freeman et al., 2014b; Hurem et al., 2017b; Jaafar et al., 2013). However, the effects on gene expression and phenotypic traits in progeny following parental irradiation during gametogenesis are not well studied.

In a previous study we observed, a 100 % mortality in progeny around 8 hours post fertilization (hpf; 80 % epiboly, late gastrula), after irradiation of parental fish during gametogenesis to 53 mGy/h for 27 days (Hurem et al., 2017a). In the progeny of parents exposed to 8.7 mGy/h reactive oxygen species (ROS) were found to be increased in 72 hpf larvae one month after parental irradiation, but decreased one year after parental irradiation, while lipid peroxidation (LPO) and DNA damage were found to be significantly increased in embryos one year after parental exposure compared to controls (Hurem et al., 2017a). Similarly, a significant increase in DNA damage was reported in offspring of adult zebrafish exposed to 1 Gy of X-rays (Lemos et al., 2017). These results clearly indicate that biological effects of parental exposure to ionizing radiation may be transferred to their progeny.

In order to investigate the effects on the transcriptome, we produced embryos from exposed zebrafish one month and one year after exposure and performed mRNA sequencing. We sampled early gastrula stage embryos (5.5 hpf), a developmental stage where the zygotic genome has been activated, and most of the maternal mRNAs are degraded (Aanes et al., 2011; Haberle et al., 2014). Hence, this stage allows to measure expressed genes in a still relatively undifferentiated homogeneous cell population (Kimmel et al., 1995).

Here, we show a temporal progressive effect of gamma radiation on gene expression in progeny from exposed parents to 8.7 mGy/h for 27 days. At this dose only a few genes were differentially expressed one month after parental exposure, whereas many affected genes were observed one year after exposure. The global change in gene expression in offspring of fish exposed to 53 mGy/h indicates a global disruption in transcriptional processes, resulting in embryonic death.

Material and methods

Zebrafish husbandry and exposures

This study was approved by the institutional animal ethics committee (IACUC) and the Norwegian food inspection authority (NFIA), under permit number 5793. Zebrafish of the AB wild type strain were obtained from the Norwegian university of Life Sciences (NMBU) zebrafish facility and maintained according to standard operating procedures (Hurem et al., 2017a). The NMBU zebrafish facility is licensed by the NFIA and accredited by the association for assessment and accreditation of laboratory animal care (AAALAC, license number: 2014/225976). The exposures of fish, including mating and embryo production were done as described previously (Hurem et al., 2017a). In short, adult zebrafish (6 months of age) were exposed for 27 days to a ^{60}Co source at 8.7 and 53 mGy/h (Figure 1). Control fish were kept separately under similar environmental conditions. After exposures, fish were mated by family inbreeds per exposure. F1 embryos were pooled per exposure group, and were incubated in autoclaved system water (28 ± 2°C).

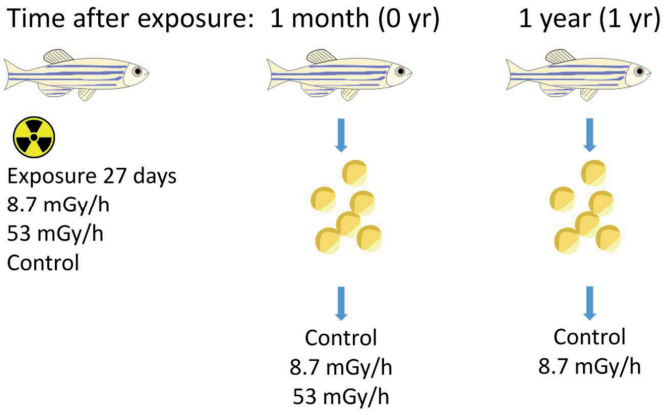


Figure 1: Experimental set-up. Zebrafish were exposed as indicated. Embryos were generated one month (0 yr) and one year after exposure (1 yr) for transcriptomics analysis.

Embryo sampling

F1 embryos were sampled in pools of 100 embryos (3 replicates per exposure), in 12 well plates in 3 mL temperature controlled autoclaved system water (28 ± 2°C), one month (0 yr) and

one year (1 yr) after exposure. This resulted in 5 groups; control 0 yr, 8.7 and 53 mGy/h 0 yr, control 1 yr and 8.7 mGy/h 1 yr (Figure 1). The 53 mGy/h 1 yr could not be generated due to sterility of parental fish. Unfertilized and poor quality eggs were excluded from analysis. At 50 % epiboly stage, embryos were transferred in 1.5 mL tubes (Thermo Fisher Scientific, Waltham, MA) and snap frozen in liquid nitrogen. Samples were stored at -80 °C until further analysis.

RNA purification

Total RNA was isolated with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA) according to manufactures' instructions. Briefly, 1 mL TRIzol was added to each sample consisting of 100 embryos and homogenized using Magnalyser Beads (Roche Diagnostics, Germany). Each sample was eluted in 40 µL RNase-free water and stored at −80 °C until further analysis. RNA purity and yield was determined using NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and RNA integrity number (RIN) was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) using RNA Nano LabChip Kit (Agilent Technologies, Santa Clara, CA), which were all of sufficient quality for sequencing (RIN > 9.0). One sample (control 0 yr) got lost during the RNA extraction and we proceeded with duplicate samples of the controls of 0 yr.

mRNA sequencing

Sequencing was outsourced to Novogene (Hong Kong, China). Per sample, a total of 1.5 µg total RNA was used for library preparation. Non-directional libraries were generated using the NEBNExt Ultra mRNA kit (New England Biolabs, Ipswich, MA) according to the manufacturers' recommendations. Total RNA was quality checked for integrity with the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA) and agarose gel electrophoresis. Concentration was determined with Qubit analysis (Thermo Fisher Scientific, Waltham, MA). After the QC procedures, mRNA was enriched using oligo(dT) beads, followed by fragmentation and first strand cDNA synthesis using random hexamers and M-MuLV reverse transcriptase. After first-strand synthesis, a second-strand synthesis buffer (Illumina, San Diego, CA) with dNTPs, RNase H and *Escherichia coli* polymerase I was added to generate the second strand. Subsequently, a cDNA library was generated after a round of purification, terminal repair, A-tailing, ligation of sequencing adapters, size selection and PCR enrichment. PCR products were

purified with the AMPure XP system (Beckman, US) and library quality was checked on the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). Libraries were analyzed using Hiseq 4000 (Illumina, San Diego, CA), using 150 bp paired-end reads, with a depth of 20 million reads per sample.

Bioinformatics

Raw fastq files were adapter trimmed using trim_galore (v0.4.2, Babraham institute, UK) under standard parameters, with extra base clipping of 1 base at the 3' side of both reads and 12 bases at the 5' side of both reads. This was done since initial FastQC (v0.11.5, Babraham institute, UK) analysis revealed high K-mer content at the 5' side of the sequencing due to adapter contamination. Since we also wanted to assess global insertions and deletions, the sequence quality needed to be as high as possible. We used the STAR aligner (v2.5.2b) (Dobin et al., 2013) to align and map sequences to the zebrafish genome (GRCz10, www.ensembl.org) with a recent release of the zebrafish transcriptome GTF (v85, www.ensembl.org). Since we were looking at progeny of fish exposed to gamma radiation, we included the chimeric reads option in STAR, in order to assess the amount of chimeric genes possibly generated by DNA damage. After alignment, the generated SAM files were loaded into the SeqMonk sequence analysis tool (v1.35, Babraham institute, UK) and mRNAs were quantified using the built-in mRNA seq pipeline. Library quality was assessed by the RNA-seq QC plot and cumulative distribution plots within SeqMonk. We used a filter to only analyze mRNAs that had at least 30 reads in either of the replicate samples, in order to assure that for statistical analysis only mRNAs were included with enough reads. Differential expression was analyzed with the built-in Deseq2 filter in Seqmonk, using R (v3.3.1), on raw read counts. With this analysis mRNA isoforms were merged, since Deseq2 cannot assess differential expressed transcript isoforms. Deseq2 uses the negative binomial distribution to assess differential gene expression, with Benjamini Hochberg false discovery rate multiple comparisons adjustments (FDR) (Love et al., 2014). Following Deseq2 analysis, data was normalized by reads per million (RPM) in order to calculate fold change (FC) per gene averaging all replicates. Significant genes were reported as < 0.05 FDR with an absolute $FC > 1.5$.

Pathway analysis

Differentially expressed gene lists were imported into Ingenuity Pathway Analysis (IPA) (version 430520M, Qiagen) and used with the ingenuity knowledge base as background. Around 60% of the genes were annotated as having a human orthologue by IPA, using homologene as the reference database, and this gene list was used for pathway analysis. If a gene was mapped twice (e.g. paralogues), the highest differential expression value is used for downstream analysis (default settings of IPA). Since IPA uses human orthologues for pathway analysis, we used the IPA nomenclature inside the context of pathways (e.g. *HNF4A*) and use official zebrafish gene nomenclature when referred to outside IPA context (e.g. *hnf4a*). IPA calculates over representation of genes and gene lists involved in known pathways, upstream regulators and diseases using Fisher's exact tests. Furthermore, it uses the direction of the differentially expressed genes to predict activation or inhibition of pathways and upstream regulators, by means of Z-scores. P values smaller than 0.01 were considered significant.

qPCR validation

Total RNA (similar batch from the sequencing analysis) (1 µg) was reverse transcribed using the high capacity cDNA kit (Thermo Fisher Scientific, Waltham, MA), according to the manufacturers' recommendations. cDNA was diluted 20x prior to QPCR reactions. Primers were designed with primerblast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) or taken from a previous publication (Supplemental data, Table S1) (Hurem et al., 2017b). The primers were checked for product length, dimers and specific products by means of melting curve analysis and gel electrophoresis (data not shown). QPCR reactions were performed in duplicate per sample in 10 µL, consisting of 5 µL FastStart Essential DNA Green Master mix (Roche, Norway), 0.25 µL forward and reverse primers (10 µM), 2 µL of 20x diluted cDNA template and 2.75 µL nuclease free water. Cycle conditions were 10 min hot start at 95 ºC followed by 45 cycles of 10 sec at 95 ºC and 30 sec at 60 ºC. A melting curve was generated at the end of each run from 60 to 90 ºC. We used 4 reference genes (Kamstra et al., 2017) which showed no differences following sequencing analysis (*rps18*, *hmbs*, *hprt1* and *eef1a*). The last, *eef1a*, which is often used as reference gene, was differentially regulated in the sequencing results. We used Linreg (v2017.0) (Ramakers et al., 2003) to determine Cq values and overall efficiency of the reactions and used these values for calculating normalized gene expression using the geometric average of 3 reference genes (*rps18*, *hmbs* and *hprt1*), taking into account the efficiency of each primer

(Vandesompele et al., 2002). Correlation was calculated with non-parametric Spearman correlation in Graphpad (v. 7.1, La Jolla, CA)

Results and Discussion

Sequencing Quality

Analysis by FastQC revealed high quality sequences with phred scores generally higher than 35 over all reads except for the paired-end read towards the 3' end where reads were still above the acceptable phred score of 20 (data not shown). Average mapping efficiency was over 87% unique reads (Table 1). In order to account for possible artifacts due to DNA damage, we monitored possible effects on mutations, insertion, deletion and chimeric reads, and did not observe notable differences, except for one sample showing relative high chimeric reads, however, taking into account other replicates no significant differences were observed by unpaired t-tests (Table 1).

Table 1: Mapping results of STAR alignment. Overview of total of reads, unique alignments and possible insertions and deletions and chimeric reads.

	Total reads (M)	% unique reads	% mismatches per base	% deletion rate per base	% insertion rate per base	% chimeric reads
control 1 0 yr	18.59	88.07	0.65	0.03	0.03	3.38
control 2 0 yr	21.26	88.11	0.69	0.03	0.03	3.26
8.7 mGy7h 1 0 yr	17.78	88.87	0.61	0.03	0.03	3.06
8.7 mGy7h 2 0 yr	20.20	88.82	0.59	0.03	0.03	3.29
8.7 mGy7h 3 0 yr	17.28	88.13	0.6	0.04	0.03	3.71
53 mGy/h 1 0 yr	19.79	88.35	0.59	0.03	0.03	4.44
53 mGy/h 2 0 yr	19.19	90.21	0.65	0.03	0.02	2.81
53 mGy/h 3 0 yr	18.28	88.84	0.53	0.03	0.03	3.51
control 1 1 yr	19.95	87.50	0.65	0.03	0.03	2.84
control 2 1 yr	16.45	87.41	0.64	0.03	0.03	3.14
control 3 1 yr	14.09	88.83	0.55	0.03	0.03	2.54
8.7 mGy7h 1 1 yr	16.46	81.82	0.54	0.03	0.02	8.47
8.7 mGy7h 2 1 yr	18.61	86.67	0.55	0.03	0.03	3.82
8.7 mGy7h 3 1 yr	18.73	87.91	0.53	0.03	0.03	3.27
average	18.33	87.82	0.60	0.03	0.03	3.68

Therefore, no global effects seem to be present on mutation rates, however site specific mutations cannot be ruled out.

Quality analysis of aligned reads show that nearly 100% of the sequence reads were located at genes and specifically at exons (Supplemental data, Figure S1). A low amount of reads were present at ribosomal RNA and mitochondrial RNA and about 70% of annotated genes in zebrafish were mapped (Supplemental data, Figure S1). The reads mapped equally to the sense and anti-sense strands. Since we used a non-stranded poly-A tail library preparation, these parameters matched the expected quality control outcome. A cumulative distribution analysis of expressed genes over all samples showed a consistent pattern over all levels of expression, which indicated highly similar sequencing libraries, and that further normalization using reads per million (RPM) was not biased (Supplemental data, Figure S2).

RNA sequencing reveals changes in gene expression in offspring from 53 mGy/h exposed parent and progressive changes over 1 yr at 8.7 mGy/h.

Deseq2 analysis revealed 39 (19 down-regulated, 49.0%) and 5079 (2207 down-regulated, 43.5%) genes, for 8.7 and 53 mGy/h 0 yr groups, respectively. Scatterplots indicate the low variation in differentially expressed transcripts in the 8.7 mGy/h (Figure 2a) versus the high variation in the 53 mGy/h group (Figure 2b) compared to controls, with Pearson correlations of 0.991 and 0.884 for the 8.7 and 53 mGy/h 0 yr groups, respectively. Since the 53 mGy/h group became sterile over time (Hurem et al., 2017a), the 1 yr samples only consisted of the 8.7 mGy/h group, which showed an increase in differentially expressed genes compared to 8.7 mGy/h 0 yr, with 2390 (1617 down-regulated, 67.7%) genes (Figure 2c). Also the correlation coefficient decreased to 0.975 compared to the 8.7 mGy/h 0 yr samples.

Taken into account all mutually measured genes, we observe a separation of the different groups following principle component analysis (PCA) (Figure 2d). The first principle component explained the majority of the variance (74.7 %), in which the exposures groups were separated. The second component separates the control groups from 0 yr and 1 yr from each other, indicating that with aging of parents a change in gene expression occurs in offspring. However, the second principle component explained only 12.4 % of the variance. Interestingly, the 1 yr 8.7 mGy/h group is oppositely located from the 0 yr 53 mGy/h group (Figure 2d). Although, both groups have a large overlap in mutually DEGs with an overlap of 1514 genes of the 2390 DEGs (63.3% of all genes from 1 yr 8.7 mGy/h, Figure 2e), PCA analysis indicates opposite gene regulation. Also, between the 0 yr 8.7 and 53 mGy/h groups 21 out of 39 genes were overlapping

(53.8%, Figure 2e). Finally, 5 genes were mutually differentially expressed in all exposures, thy-1 cell surface antigen (*thy1*), fibronectin 1a (*fn1a*), Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2 (*cited2*), ribonucleotide reductase subunit M2 (*rrm2*) and cytochrome p450 2x6 (*cyp2x6*) (Figure 2f). Comparing these results with a previous transcriptomics study where embryos were directly exposed to a comparable dose rate (10.9 mGy/h) for 3 hours revealed an overlap of 27.8 % (74 out of 268 DEGs, FC > 1.5, FDR < 0.05) (Hurem et al., 2017b). In contrast, a study in zebrafish exposed either during embryogenesis or at 16 weeks post fertilization to short term radiation (0.1 and 1 Gy total dose) showed minimal similarity in gene expression compared to our study (<5%, data not shown) (Jaafar et al., 2013). Similarly, zebrafish larvae irradiated to 1, 2, 5 or 10 Gy at 26 hpf did not show much overlap in gene expression with our study (Freeman et al., 2014b). Even though both studies exposed zebrafish to ionizing radiation, these datasets may be difficult to compare, since the exposures were performed differently compared to our study with respect to exposure, dose and timing and the gene expression analysis was performed at later life stages.

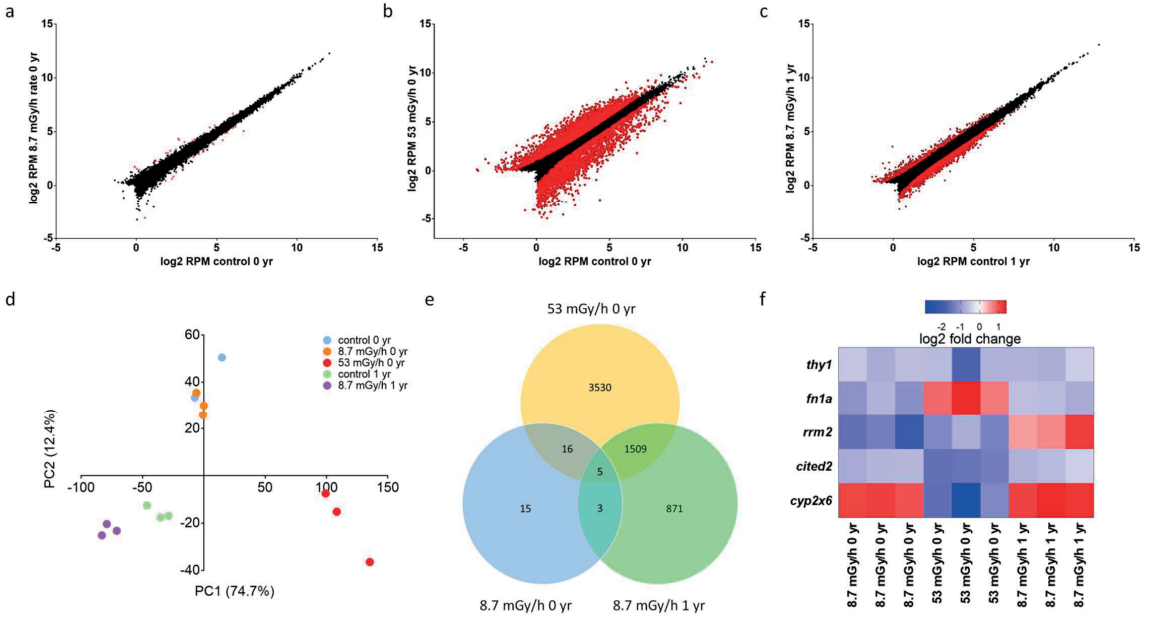


Figure 2: mRNA sequencing results. XY scatterplots of (a) 8.7 mGy/h and (b) 53 mGy/h dose group one month after exposure and (c) 8.7 mGy/h dose one year after exposure. Red dots are differentially expressed genes. (d) Principle component analysis of all expressed genes on all replicates. (e) Venn diagram showing the number of overlapping differentially expressed genes from each exposure scenario. (f) A heatmap of the five overlapping differentially expressed genes in all exposures.

IPA analysis reveals consistent enriched pathways, but predicts opposite regulation.

We imported the gene expression data into IPA, commonly used in zebrafish research to explore novel pathways and linkage to phenotype in ecotoxicology. Due to the limited amount of DEGs, relatively few significant pathways or upstream regulators were present in the 8.7 mGy/h 0 yr group (Supplemental File 1). In contrast, the amount of differentially expressed genes (DEGs) in the 53 mGy/h 0 yr group indicates an overall disruption of gene expression, resulting in many affected pathways involved in morbidity and organismal death (Supplemental File 1). These outcomes can be linked to embryonic death at late gastrula (Hurem et al., 2017a). When focused on the 53 mGy/h 0 yr and the 8.7 mGy/h 1 yr, many mutual pathways were enriched, generally associated to radiation response, such as molecular mechanisms of cancer, DNA damage response and cell death (Table 2 and Supplemental File 1).

Table 2: Top 20 pathways and upstream regulators derived from the IPA analysis

Pathways	-logP 53 mGy/h 0 yr	-logP 8.7 mGy/h 1 yr
Molecular Mechanisms of Cancer	13.3	4.8
NGF Signaling	9.6	7.1
Huntington's Disease Signaling	9.6	6.7
Hereditary Breast Cancer Signaling	11.7	3.7
Endothelin-1 Signaling	5.3	7.7
RAR Activation	7.7	3.7
GNRH Signaling	5.6	6.5
HGF Signaling	7.5	5.2
Breast Cancer Regulation by Stathmin1	5.9	6.0
HER-2 Signaling in Breast Cancer	8.1	4.4
Thrombin Signaling	5.8	5.5
B Cell Receptor Signaling	8.6	3.3
Production of NO and ROS in Macrophages	6.2	5.1
Pyridoxal 5'-phosphate Salvage Pathway	7.9	3.2
Role of NFAT in Cardiac Hypertrophy	3.8	6.5
RANK Signaling in Osteoclasts	8.0	3.1
ILK Signaling	8.1	2.2
PPARα/RXRα Activation	4.5	5.4
Type II Diabetes Mellitus Signaling	5.7	4.9
Small Cell Lung Cancer Signaling	7.9	2.6

Upstream regulators		
TP53	14.2	4.0
HNF4A	16.9	3.4
ESR1	10.1	4.2
camptothecin	10.8	3.3
YAP1	7.2	4.2
NUPR1	8.3	3.1
let-7	7.8	1.3
ERBB2	7.0	2.7
IGF2	6.9	2.0
cisplatin	5.5	3.5
FSH	5.0	5.3
FOXO1	5.4	1.8
methylprednisolone	3.2	2.9
INSR	6.9	1.9
L-dopa	5.2	3.5
SYVN1	6.6	2.1
HRAS	4.6	2.4
Sos	6.2	2.2
MYCN	3.5	2.2
RRP1B	6.5	1.8

However, pathways which have not previously been associated to effects of ionizing radiation, such as nerve growth factor (NGF) signaling and retinoic acid receptor activation and gonadotropin release hormone (GNRH) signaling were also significantly affected. Upstream regulators, previously shown to be responsive following radiation exposure in zebrafish, such as TP53 and HNF4a (Hurem et al., 2017b; Jaafar et al., 2013) (Table 2 and Supplemental File 1). In general, a correlation in significantly affected pathways was observed between 53 mGy/h 0 yr and 8.7 mGy/h 1 yr (Figure 3a, $r = 0.5955$, $P < 0.0001$). IPA can predict activation of pathways, by calculating Z-scores based on the direction of DEGs in the respective pathway. This analysis revealed an opposite regulation of significantly affected pathways between the 53 mGy/h 0 yr group and the 8.7 mGy/h 1 yr group, which is shown by the inversed correlation in Figure 3b ($r = -0.7339$, $P < 0.0001$).

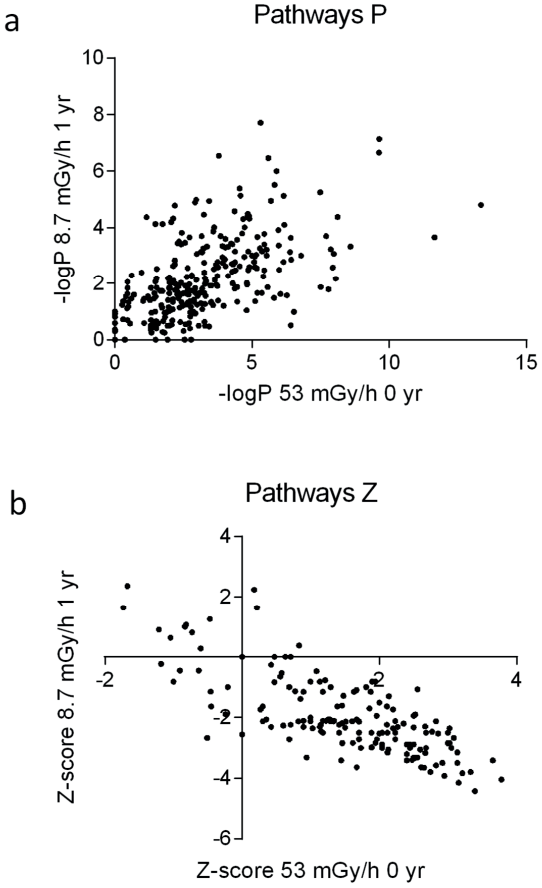
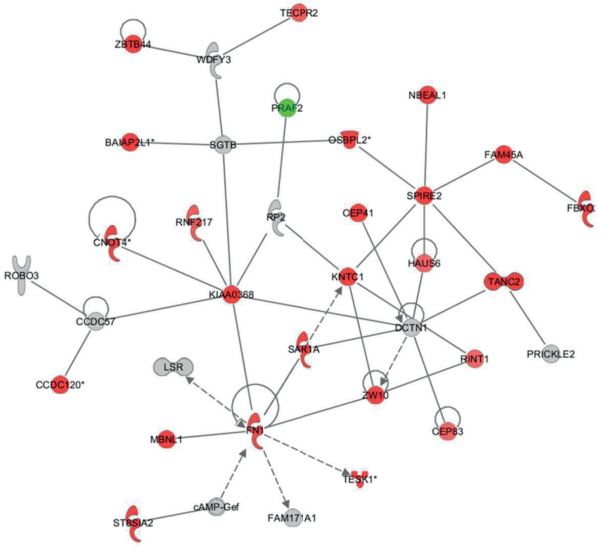


Figure 3: Results of Ingenuity Pathway Analysis. (a) Correlation plot of all overrepresented pathways based on –log(P) values ($r = 0.5955$, $P < 0.0001$). (b) Correlation plot of all overrepresented pathways based on activation Z scores ($r = -0.7339$, $P < 0.0001$).

A more clear visualization of this opposite regulation is demonstrated in Figure 4a and b, showing an overrepresented network involved in cell cycle, cellular compromise and developmental disorders, where many mutually differentially expressed genes are present between the two datasets, but show an opposite regulation.

a

53 mGy/h 0 yr



b

8.7 mGy/h 1 yr

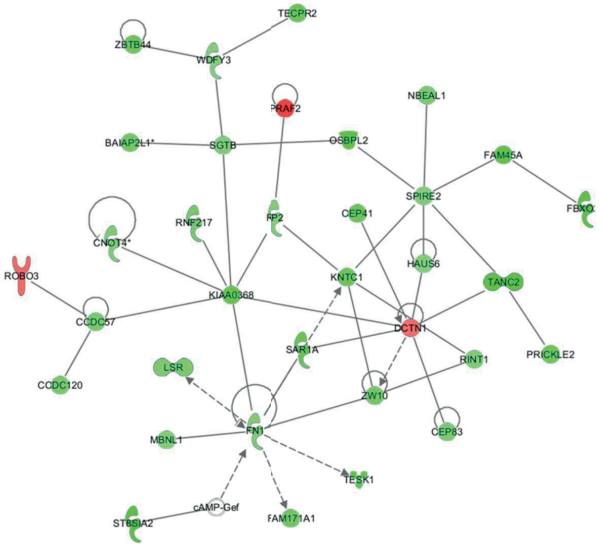


Figure 4: Predicted affected network of genes involved in cell cycle, cellular compromise and developmental disorders. (a) and (b) show overrepresented networks involved in neuronal development of 53 mGy/h 0 yr and 8.7 mGy/h 1 yr groups, respectively. Green is down-regulated and red is up-regulated.

Focused on the 8.7 mGy/h embryos one year after exposure, apart from gene networks involved in radiation response, genes involved in other pathways were overrepresented. IPA analyses identified significant gene networks with predicted involvement in neurological disorders (Supplemental File 1) such as congenital malformation of brain and degeneration of neurons (P values of 1.8E-8 and 1.17E-6, respectively), both showing a Z-score activation of more than 3 (Supplemental File 1). Although we used an early embryonic stage, the already differentially expressed genes involved in neurological disorders could indicate early effects that could potentially affect neuronal development later in life. These results corroborate with studies in mice, where offspring exposed to acute gamma radiation during gestation shows retarded brain development (Hossain et al., 2005), which is accompanied with neurobehavioral effects (Devi et al., 1999). The outcomes of these studies together with the obtained results here, warrant follow up research to cognitive endpoints of progeny from irradiated zebrafish.

IPA analysis also identified significant pathways related to sex hormone homeostasis. The modulation of genes related to estrogen receptor 1 (E*SR1*), follicle stimulating hormone (FSH) signaling, insulin growth factor 2 (*IGF2*) and gonadotropin releasing hormone (GNRH) signaling (Table 2) suggests a disturbance in early embryos, which could affect the formation of the gonads later in life. Interestingly, these results can also be related back to the exposed females where in ovaries of zebrafish exposed to 8.7 mGy/h, an increase in pre-vitellogenic follicles was found (unpublished data), suggesting adverse oocyte maturation, possibly via endocrine signaling.

In general, the pathways found to be affected in our study are comparable to other non-generational studies in zebrafish as well. The transcriptomics analysis in zebrafish embryos exposed to radiation which shows a large overlap in DEGs, also shows overlap in affected pathways, related to for instance HNF4a and TP53 (Hurem et al., 2017b). Notably, the studies in zebrafish that showed low overlap in DEGs (Freeman et al., 2014b; Jaafar et al., 2013), as described above, do exhibit similarly affected pathways. For instance, Jaafar et al. (2013)

reported involvement in the p53 signaling pathway and apoptosis pathways. Similarly, Freeman et al. (2014) found similar affected biological functions derived from IPA, such as psychological disorders and neurological diseases. These results indicate that when comparing results, pathway analysis is could reveal more similar effects than when looking at the individual gene itself.

Gene expression results indicate chromatin compactness in 8.7 mGy/h 1 yr embryos

We observed a change in gene expression in embryo offspring from 8.7 mGy/h exposed parents, when bred one month and one year after exposure. These progressive effects could be attributed to genomic instability in the parental germ line, and transmitted to progeny as has previously been suggested (Merrifield and Kovalchuk, 2013). Furthermore, we observed opposite differential gene regulation at the 8.7 mGy/h 1 yr group compared to the 53 mGy/h 0 year group. Notably, in the 8.7 mGy/h 1 year group more genes were down-regulated (67.7%) and most pathway Z-scores were below 0. This result could indicate a protective response against ionizing radiation via epigenetic changes and chromatin compactness. Indeed, many histone methylases and demethylases were differentially expressed (Table 3).

Table 3: Differentially expressed enzymes involved in post translational histone modifications from 8.7 mGy/h 1 yr samples modifications from 8.7 mGy/h 1 yr samples.

gene	description	enzym	FC
setd3	SET domain containing 3	methyltransferase	-1.83
setd4	SET domain containing 4	methyltransferase	-1.53
jmjd1cb	jumonji domain containing 1Cb	demethylase	-2.98
jmjd7	jumonji domain containing 7	demethylase	-2.32
kmt2a	lysine (K)-specific methyltransferase 2A	methyltransferase	-1.76
kmt2ca	lysine (K)-specific methyltransferase 2Ca	methyltransferase	-2.10
kdm2ba	lysine (K)-specific demethylase 2Ba	demethylase	1.64
kdm4aa	lysine (K)-specific demethylase 4A, genome duplicate a	demethylase	-1.52
kdm4ab	lysine (K)-specific demethylase 4A, genome duplicate b	demethylase	-1.92
kdm4c	lysine (K)-specific demethylase 4C	demethylase	-2.01
hdac11	histone deacetylase 11	deacytelase	-1.72
hdac12	histone deacetylase 12	deacytelase	-3.10
hdac4	histone deacetylase 4	deacytelase	-2.03
hdac5	histone deacetylase 5	deacytelase	-2.04
hdac6	histone deacetylase 6	deacytelase	1.84

For instance, the down-regulated *kmd4* gene

is involved in demethylation of histone H3 lysine 9 and 27 methylation, both related to compactness of chromatin (Pedersen and Helin, 2010). Together with the up-regulated gene *kmd2b*, involved in demethylation of active histone mark H3 lysine 4 trimethylation (Pedersen and Helin, 2010), these results indirectly suggest more histone compactness. Also the methyltransferase paralogues *setd3* and *4*, are down-regulated and are involved in methylation of H3 lysine 4. Many histone deacetylases are down-regulated, but no effects are found on histone acetylases. It has been observed by many that compactness of chromatin influences DNA damage (Lavelle and Foray, 2014). More compact chromatin structures make DNA less susceptible to ionizing radiation insults, but with the consequence that compact chromatin results in a repressive state of gene transcription, and therefore counteracts DNA repair mechanisms (Lavelle and Foray, 2014). Indeed, when focused on the double strand break responsive ATM pathway (Khalil et al., 2012), our RNA sequencing analysis showed that *atm, brca1* and *rad51,* genes directly involved in DNA repair, are down-regulated. Interestingly, increased DNA damage was only found one year, but not one month after exposure (Hurem et al., 2017a). These results indicate possible involvement in chromatin structure in the gene expression effects, and further research to chromatin compactness could aid in a more comprehensive understanding of the regulatory mechanisms that drives gene expression.

Validation with QPCR

We performed a validation of the sequencing results by measuring 11 genes that were differentially expressed in either of the 0 yr 53 mGy/h exposure group or the 1 yr 8.7 mGy/h exposure group and supplemented this list with eight genes that were differentially expressed in a study of directly exposed zebrafish embryos (Hurem et al., 2017b). We found a strong correlation between sequencing and qPCR data (Figure 5, $r = 0.8893$) (Supplemental data, Table S2), indicating that our sequencing data was properly analyzed.

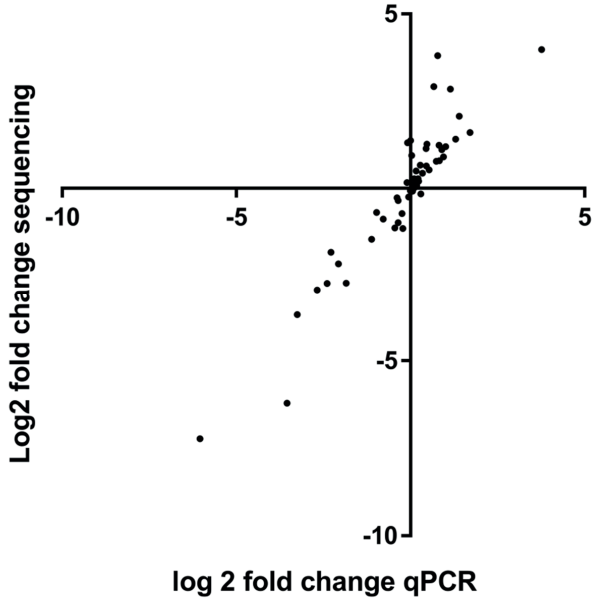


Figure 5: qPCR validation. A scatterplot showing the log2 fold changes from QPCR and sequencing data.

Mutually differentially expressed genes are associated with ionizing radiation response

We observed 5 genes that were differentially expressed in all different exposure scenarios, *fn1a, cited2, rrm2, thy1* and *cyp2x6* (Figure 2e and 2f). Fibronectin is a protein located in the extra cellular matrix, involved in, among others, cell adhesion (Pankov and Yamada, 2002). Interestingly, embryos from the 53 mGy/h dose rate group exhibited increased gene expression of *fn1a* and a complete disintegration of cell structure at 8 hpf (Hurem et al., 2017a). Ribonucleotide reductase subunit M2 (RRM2) has previously been associated with DNA damage in mammals, where RRM2 accumulates near sites with double strand breaks (Niida et al., 2010). Notably, the up-regulation of *rrm2* in the 8.7 mGy/h 1 yr group corresponds well with the increase in DNA damage found in the same group (Hurem et al., 2017a), and could indicate recruitment of Rrm2 to double strand breaks. The exact function of *cyp2x6* is currently unknown in zebrafish, but our results indicate the responsiveness of these gene to ionizing radiation. Cytochrome P450 enzymes are known to be affected by ionizing radiation (Rendic and Peter

Guengerich, 2012), and it is shown that the *cyp2x* family in other teleost species show responsiveness to environmental pollutants (Saad et al., 2016).

Following validation, we observed that transferrin a (*tfa*) was up-regulated in all exposures, a gene which was also among the highest and most consistent elevated genes in zebrafish embryos directly exposed gamma radiation (Hurem et al., 2017b). Transferrin is a ferric iron binding transporter protein which binds to the transferrin receptor on the cell surface, and is transported to the cytoplasm via endocytosis (Aisen, 2004). Knock down of *tfa* in zebrafish has been associated with anemia and lower iron stores, whereas overexpression partially restored iron stores (Fraenkel et al., 2009), indicating that the increased gene expression found in our study could be accompanied with increase iron stores. Increased cytoplasmic iron stores are suggested in sensitizing ionizing radiation response by increasing ROS (Stevens and Kalkwarf, 1990). Interestingly, transferrin protein levels in blood were altered in workers exposed to radiation following a radiological accident (Nylund et al., 2014). Together with *cyp2x6*, *tfa* was alters in all exposed samples from our study, as well in directly exposed embryos (Hurem et al., 2017b). These two genes could prove to be valuable biomarkers in response to ionizing radiation (Hall et al., 2017; Lourenço et al., 2016), and should be subject to further study in different exposures settings with a variety of taxa.

Genetic diversity and age related effects

The multiple doses used in our set-up allowed us to assess effects of genetic variation on gene expression. Following PCA, limited difference was observed between the 8.7 mGy/h group and the control at 0 yr (Figure 2d). This generally indicates that this dose of radiation did not exhibit effects, but also indicates that the heterogenic diversity of the fish was very low, and effects could be accounted to the parental radiation exposure. Additionally, the 0 yr and 1 yr control groups were separated by the 2nd principle component (Figure 2d), indicating a combination of effects related to minor fluctuations in environmental conditions over one year and/or parental age. Therefore, to account for environmental changes over a period of one year and related aging effects, for each time point appropriate controls were used which were compared to their respective exposure groups.

Relevance for environment and future perspectives

Our results indicate effects on gene expression in offspring from ionizing radiation exposed parents, related to pathways that can be linked to observed phenotypes. However, the dose rates used in this study exceed currently observed environmental relevant dose rates (Johansen et al., 2015; Strand et al., 2014), and are higher than the defined low dose rate by the United Nations Scientific Committee on the Effects of Atomic Radiation of 6 mGy/h for a maximum of 1 hour (Unscear, 2010). Nevertheless, the results presented here are a proof of principle that effects of exposure to non-acute moderate dose rates of ionizing radiation can be passed on to offspring via aberrant gene expression. Furthermore, the progressive effects over one year from the lower dose rate in progeny could potentially have a latent effect on ecosystems. Therefore, these results collectively contribute to the understanding to what extend the effects of ionizing radiation are inherited by offspring. Furthermore, these results warrant transgenerational studies with lower doses over longer periods, with focus on the mechanistic understanding of how these effects are inherited, and the possible impact on population dynamics.

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Author contributions

SH, JLL, PA, DAB and DO planned the exposures of the zebrafish. SH, LMM, JK and LL conducted the breeding and sampling of embryos. LMM performed the RNA extractions and quality control. JK and LMM performed the bioinformatics analysis. JK performed the RT qPCR validation, created the figures and wrote the manuscript. All coauthors contributed to revising the different sections of the manuscript prior to final submission.

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References

Aanes, H., Winata, C.L., Lin, C.H., Chen, J.P., Srinivasan, K.G., Lee, S.G.P., Lim, A.Y.M., Hajan, H.S., Collas, P., Bourque, G., Gong, Z., Korzh, V., Aleström, P., Mathavan, S., 2011. Zebrafish mRNA sequencing deciphers novelties in transcriptome dynamics during maternal to zygotic transition. Genome Res. 21, 1328–38. doi:10.1101/gr.116012.110

Adams, G.E., Cox, R., 1997. Radiation Carcinogenesis, 3rd ed. Oxford University Press, Oxford.

Aisen, P., 2004. Transferrin receptor 1. Int. J. Biochem. Cell Biol. 36, 2137–2143. doi:10.1016/j.biocel.2004.02.007

Amatruda, J.F., Patton, E.E., 2008. Genetic models of cancer in zebrafish. Int. Rev. Cell Mol. Biol. 271, 1–34. doi:10.1016/S1937-6448(08)01201-X

Amatruda, J.F., Zon, L.I., 1999. Dissecting hematopoiesis and disease using the zebrafish. Dev. Biol. 216, 1–15. doi:10.1006/dbio.1999.9462

Andersen, I.S., Lindeman, L.C., Reiner, A.H., Østrup, O., Aanes, H., Aleström, P., Collas, P., 2013. Epigenetic marking of the zebrafish developmental program. Curr. Top. Dev. Biol. 104, 85–112. doi:10.1016/B978-0-12-416027-9.00003-6

Andersen, I.S., Ostrup, O., Lindeman, L.C., Aanes, H., Reiner, A.H., Mathavan, S., Aleström, P., Collas, P., 2012. Epigenetic complexity during the zebrafish mid-blastula transition. Biochem. Biophys. Res. Commun. 417, 1139–44. doi:10.1016/j.bbrc.2011.12.077

Aypar, U., Morgan, W.F., Baulch, J.E., 2011. Radiation-induced genomic instability: Are epigenetic mechanisms the missing link?

Bladen, C.L., Flowers, M.A., Miyake, K., Podolsky, R.H., Barrett, J.T., Kozlowski, D.J., Dynan, W.S., 2007. Quantification of ionizing radiation-induced cell death in situ in a vertebrate embryo. Radiat. Res. 168, 149–57. doi:10.1667/RR0803.1

Buisset-Goussen, A., Goussen, B., Della-Vedova, C., Galas, S., Adam-Guillermin, C., Lecomte-Pradines, C., 2014. Effects of chronic gamma irradiation: a multigenerational study using Caenorhabditis elegans. J. Environ. Radioact. 137, 190–7. doi:10.1016/j.jenvrad.2014.07.014

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–22. doi:10.1373/clinchem.2008.112797

Calabrese, E.J., 2017. Flaws in the LNT single-hit model for cancer risk: An historical assessment. Environ. Res. 158, 773–788. doi:10.1016/j.envres.2017.07.030

Chadwick, K.H., Leenhouts, H.P., 2005. Radiation risk is linear with dose at low doses. Br. J. Radiol. 78, 8–10. doi:10.1259/bjr/51173413

Choi, V.W.Y., Yu, K.N., 2015. Embryos of the zebrafish Danio rerio in studies of non-targeted effects of ionizing radiation. Cancer Lett. 356, 91–104. doi:10.1016/j.canlet.2013.10.020

Choi, V.W.Y., Yu, K.N., 2013. Embryos of the zebrafish Danio rerio in studies of non-targeted effects of ionizing radiation. Cancer Lett.

Clegg, B., 2010. Armageddon science : the science of mass destruction. St. Martin's Press.

Devi, P.U., Hossain, M., Bisht, K.S., 1999. Effect of late Fetal irradiation on adult behavior of mouse: Dose-response relationship. Neurotoxicol. Teratol. 21, 193–198. doi:10.1016/S0892-0362(98)00039-7

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. doi:10.1093/bioinformatics/bts635

Erdelmeier, I., G?rard-Monnier, D., Yadan, J.-C., Chaudi?re, J., 1998. Reactions of *N* -Methyl-2-phenylindole with Malondialdehyde and 4-Hydroxyalkenals. Mechanistic Aspects of the Colorimetric Assay of Lipid Peroxidation. Chem. Res. Toxicol. 11, 1184–1194. doi:10.1021/tx970180z

Felsenfeld, A.L., 1996. Defining the boundaries of zebrafish developmental genetics. Nat. Genet. 14, 258–263. doi:10.1038/ng1196-258

Fraenkel, P.G., Gibert, Y., Holzheimer, J.L., Lattanzi, V.J., Burnett, S.F., Dooley, K.A., Wingert, R.A., Zon, L.I., 2009. Transferrin-a modulates hepcidin expression in zebrafish embryos. Blood 113, 2843–50. doi:10.1182/blood-2008-06-165340

Freeman, J.L., Weber, G.J., Peterson, S.M., Nie, L.H., 2014a. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front. Genet. 5, 268. doi:10.3389/fgene.2014.00268

Freeman, J.L., Weber, G.J., Peterson, S.M., Nie, L.H., 2014b. Embryonic ionizing radiation exposure results in expression alterations of genes associated with cardiovascular and neurological development, function, and disease and modified cardiovascular function in zebrafish. Front. Genet. 5, 268. doi:10.3389/fgene.2014.00268

Gagnaire, B., Cavalié, I., Pereira, S., Floriani, M., Dubourg, N., Camilleri, V., Adam-Guillermin, C., 2015. External gamma irradiation-induced effects in early-life stages of zebrafish, Danio rerio. Aquat. Toxicol. 169, 69–78. doi:10.1016/j.aquatox.2015.10.005

Garnier-Laplace, J., Della-Vedova, C., Andersson, P., Copplestone, D., Cailes, C., Beresford, N.A., Howard, B.J., Howe, P., Whitehouse, P., 2010. A multi-criteria weight of evidence approach for deriving ecological benchmarks for radioactive substances. J. Radiol. Prot. 30, 215–33.

doi:10.1088/0952-4746/30/2/S02

Haberle, V., Lenhard, B., 2012. Dissecting genomic regulatory elements in vivo. Nat. Biotechnol. 30, 504–6. doi:10.1038/nbt.2266

Haberle, V., Li, N., Hadzhiev, Y., Plessy, C., Previti, C., Nepal, C., Gehrig, J., Dong, X., Akalin, A., Suzuki, A.M., van IJcken, W.F.J., Armant, O., Ferg, M., Strähle, U., Carninci, P., Müller, F., Lenhard, B., 2014. Two independent transcription initiation codes overlap on vertebrate core promoters. Nature 507, 381–385. doi:10.1038/nature12974

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nüsslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 123, 1–36.

Hall, J., Jeggo, P.A., West, C., Gomolka, M., Quintens, R., Badie, C., Laurent, O., Aerts, A., Anastasov, N., Azimzadeh, O., Azizova, T., Baatout, S., Baselet, B., Benotmane, M.A., Blanchardon, E., Guéguen, Y., Haghdoost, S., Harms-Ringhdahl, M., Hess, J., Kreuzer, M., Laurier, D., Macaeva, E., Manning, G., Pernot, E., Ravanat, J.-L., Sabatier, L., Tack, K., Tapio, S., Zitzelsberger, H., Cardis, E., 2017. Ionizing radiation biomarkers in epidemiological studies – An update. Mutat. Res. Mutat. Res. 771, 59–84. doi:10.1016/j.mrrev.2017.01.001

Han, W., Yu, K.N., 2012. Response of Cells to Ionizing Radiation, in: Advances in Biomedical Sciences and Engineering. Bentham science publishers, pp. 204–262. doi:10.2174/978160805040610901010204

Hoo, J.Y., Kumari, Y., Shaikh, M.F., Hue, S.M., Goh, B.H., 2016. Zebrafish: A Versatile Animal Model for Fertility Research. Biomed Res. Int. 2016, 9732780. doi:10.1155/2016/9732780

Hossain, M., Chetana, M., Uma Devi, P., 2005. Late effect of prenatal irradiation on the hippocampal histology and brain weight in adult mice. Int. J. Dev. Neurosci. 23, 307–313. doi:10.1016/j.ijdevneu.2005.01.006

Howe, D.G., Bradford, Y.M., Eagle, A., Fashena, D., Frazer, K., Kalita, P., Mani, P., Martin, R., Moxon, S.T., Paddock, H., Pich, C., Ramachandran, S., Ruzicka, L., Schaper, K., Shao, X., Singer, A., Toro, S., Van Slyke, C., Westerfield, M., 2017. The Zebrafish Model Organism Database: new support for human disease models, mutation details, gene expression phenotypes and searching. Nucleic Acids Res. 45, D758–D768. doi:10.1093/nar/gkw1116

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.-J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assunção, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.-H., Eyre, T., Redmond, S., Banerjee, R., Chi, J.,

Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Eliott, D., Threadgold, G., Harden, G., Ware, D., Mortimore, B., Mortimer, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D., Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J.D., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Ürün, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberländer, M., Rudolph-Geiger, S., Teucke, M., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M.J., Enright, A., Geisler, R., Plasterk, R.H.A., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nüsslein-Volhard, C., Hubbard, T.J.P., Roest Crollius, H., Rogers, J., Stemple, D.L., Begum, S., Lloyd, C., Lanz, C., Raddatz, G., Schuster, S.C., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498–503. doi:10.1038/nature12111

Hurem, S., Gomes, T., Brede, D.A., Hansen, E.L., Mutoloki, S., Fernandez, C., Mothersill, C., Salbu, B., Kassaye, Y.A., Olsen, A.-K., Oughton, D., Aleström, P., Lyche, J.L., 2017a. Parental gamma irradiation induces reprotoxic effects accompanied by genomic instability in zebrafish (Danio rerio) embryos. Environ. Res. 159, 564–578. doi:10.1016/j.envres.2017.07.053

Hurem, S., Martin, L.M., Brede, D.A., Skjerve, E., Nourizadeh-Lillabadi, R., Lind, O.C., Christensen, T., Berg, V., Teien, H.-C., Salbu, B., Oughton, D.H., Alestr?m, P., Lyche, J.L., 2017b. Dose-dependent effects of gamma radiation on the early zebrafish development and gene expression. PLoS One 12, e0179259. doi:10.1371/journal.pone.0179259

ICRP, 2012. ICRP 2012. Compend. Dose Coefficients based ICRP Publ. 60 0, 130. doi:10.1016/j.icrp.2006.06.001

Ilnytskyy, Y., Kovalchuk, O., 2011a. Non-targeted radiation effects-an epigenetic connection. Mutat. Res. 714, 113–25. doi:10.1016/j.mrfmmm.2011.06.014

Ilnytskyy, Y., Kovalchuk, O., 2011b. Non-targeted radiation effects-an epigenetic connection. Mutat. Res. 714, 113–25. doi:10.1016/j.mrfmmm.2011.06.014

Jaafar, L., Podolsky, R.H., Dynan, W.S., 2013. Long-term effects of ionizing radiation on gene expression in a zebrafish model. PLoS One 8, e69445. doi:10.1371/journal.pone.0069445

Jarvis, R.., Knowles, J.., 2003. DNA damage in zebrafish larvae induced by exposure to low-dose rate γ-radiation: detection by the alkaline comet assay. Mutat. Res. Toxicol. Environ. Mutagen. 541, 63–69.

Johansen, M.P., Ruedig, E., Tagami, K., Uchida, S., Higley, K., Beresford, N.A., 2015. Radiological dose rates to marine fish from the fukushima daiichi accident: The first three years across the north pacific. Environ. Sci. Technol. 49, 1277–1285. doi:10.1021/es505064d

Kamstra, J.H., Sales, L.B., Aleström, P., Legler, J., 2017. Differential DNA methylation at conserved non-genic elements and evidence for transgenerational inheritance following developmental exposure to mono(2-ethylhexyl) phthalate and 5-azacytidine in zebrafish. Epigenetics Chromatin 10, 20. doi:10.1186/s13072-017-0126-4

Kane, D., Kimmel, C., 1993. The zebrafish midblastula transition. Development 119, 447–456.

Khalil, H., Tummala, H., Chakarov, S., Zhelev, N., Lane, D., 2012. Targeting ATM pathway for therapeutic intervention in cancer. Biodiscovery 1–13. doi:10.7750/BioDiscovery.2012.1.3

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310. doi:10.1002/aja.1002030302

Koç (Yön), N.D., Aytekin, Y., Yüce, R., 2008. Ovary maturatıon stages and histological investigation of ovary of the Zebrafish (Danio rerio). Brazilian Arch. Biol. Technol. 51, 513–522. doi:10.1590/S1516-89132008000300010

Koturbash, I., Baker, M., Loree, J., Kutanzi, K., Hudson, D., Pogribny, I., Sedelnikova, O., Bonner, W., Kovalchuk, O., 2006. Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo. Int. J. Radiat. Oncol. Biol. Phys. 66, 327–30. doi:10.1016/j.ijrobp.2006.06.012

Lavelle, C., Foray, N., 2014. Chromatin structure and radiation-induced DNA damage: From structural biology to radiobiology. Int. J. Biochem. Cell Biol. 49, 84–97. doi:10.1016/j.biocel.2014.01.012

Leal, M.C., Cardoso, E.R., Nóbrega, R.H., Batlouni, S.R., Bogerd, J., França, L.R., Schulz, R.W., 2009. Histological and stereological evaluation of zebrafish (Danio rerio) spermatogenesis with an emphasis on spermatogonial generations. Biol. Reprod. 81, 177–87. doi:10.1095/biolreprod.109.076299

Lemos, J., Neuparth, T., Trigo, M., Costa, P., Vieira, D., Cunha, L., Ponte, F., Costa, P.S., Metello, L.F., Carvalho, A.P., 2017. Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish

and its Non-Irradiated Progeny. Bull. Environ. Contam. Toxicol. 98, 190–195. doi:10.1007/s00128-016-2006-1

Lourenço, J., Mendo, S., Pereira, R., 2016. Radioactively contaminated areas: Bioindicator species and biomarkers of effect in an early warning scheme for a preliminary risk assessment. J. Hazard. Mater. 317, 503–542. doi:10.1016/j.jhazmat.2016.06.020

Love, M.I., Huber, W., Anders, S., 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. doi:10.1186/s13059-014-0550-8

McAleer, M.F., Davidson, C., Davidson, W.R., Yentzer, B., Farber, S.A., Rodeck, U., Dicker, A.P., 2005. Novel use of zebrafish as a vertebrate model to screen radiation protectors and sensitizers. Int. J. Radiat. Oncol. 61, 10–13.

Merrifield, M., Kovalchuk, O., 2013. Epigenetics in radiation biology: a new research frontier. Front. Genet. 4, 40. doi:10.3389/fgene.2013.00040

Mothersill, C., Smith, R.W., Agnihotri, N., Seymour, C.B., 2007. Characterization of a Radiation-Induced Stress Response Communicated in Vivo between Zebrafish. Environ. Sci. Technol. 41, 3382–3387. doi:10.1021/es062978n

Mugoni, V., Camporeale, A., Santoro, M.M., 2014. Analysis of oxidative stress in zebrafish embryos. J. Vis. Exp. doi:10.3791/51328

Niida, H., Katsuno, Y., Sengoku, M., Niida, H., Katsuno, Y., Sengoku, M., Shimada, M., 2010. Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase service Essential role of Tip60-dependent recruitment of ribonucleotide reductase at DNA damage sites in DNA repair during G1 phase 333–338. doi:10.1101/gad.1863810

Nylund, R., Lemola, E., Hartwig, S., Lehr, S., Acheva, A., Jahns, J., Hildebrandt, G., Lindholm, C., 2014. Profiling of low molecular weight proteins in plasma from locally irradiated individuals. J. Radiat. Res. 55, 674–82. doi:10.1093/jrr/rru007

OECD/OCDE, 2013. OECD GUIDELINES FOR THE TESTING OF CHEMICALS Fish Embryo Acute Toxicity (FET) Test. doi:10.1787/20745761

Pankov, R., Yamada, K.M., 2002. Fibronectin at a glance. J. Cell Sci. 115, 3861–3863. doi:10.1242/jcs.00059

Pedersen, M.T., Helin, K., 2010. Histone demethylases in development and disease. Trends Cell Biol. 20, 662–71. doi:10.1016/j.tcb.2010.08.011

Pogribny, I., Raiche, J., Slovack, M., Kovalchuk, O., 2004. Dose-dependence, sex- and tissue-specificity, and persistence of radiation-induced genomic DNA methylation changes. Biochem. Biophys. Res. Commun. 320, 1253–61. doi:10.1016/j.bbrc.2004.06.081

Ramakers, C., Ruijter, J.M., Lekanne Deprez, R.H., Moorman, A.F.M., 2003. Assumption-free analysis

of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. doi:10.1016/S0304-3940(02)01423-4

Rendic, S., Peter Guengerich, F., 2012. Summary of Information on the Effects of Ionizing and Non-ionizing Radiation on Cytochrome P450 and Other Drug Metabolizing Enzymes and Transporters. Curr. Drug Metab. 13, 787–814. doi:10.2174/138920012800840356

Rothkamm, K., Löbrich, M., 2002. Misrepair of radiation-induced DNA double-strand breaks and its relevance for tumorigenesis and cancer treatment (review). Int. J. Oncol. 21, 433–40.

Ryan, L.A., Seymour, C.B., O'Neill-Mehlenbacher, A., Mothersill, C.E., 2008. Radiation-induced adaptive response in fish cell lines. J. Environ. Radioact. 99, 739–747.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Van Cruchten, S., 2016. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. J. Toxicol. Sci. 41, 1–11. doi:10.2131/jts.41.1

Scholz, S., Fischer, S., Gündel, U., Küster, E., Luckenbach, T., Voelker, D., 2008. The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. Environ. Sci. Pollut. Res. Int. 15, 394–404. doi:10.1007/s11356-008-0018-z

Schulz, R.W., de França, L.R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411. doi:10.1016/j.ygcen.2009.02.013

Shibata, A., 2017. Regulation of repair pathway choice at two-ended DNA double-strand breaks. Mutat. Res. Mol. Mech. Mutagen. doi:10.1016/j.mrfmmm.2017.07.011

Shimada, A., Eguchi, H., Yoshinaga, S., Shima, A., 2005. Dose rateDose rate effect on transgenerational mutation frequencies in spermatogonial stem cells of the medaka fish. Radiat. Res. 163, 112–4.

Skinner, M.K., 2011. Role of epigenetics in developmental biology and transgenerational inheritance. Birth Defects Res. C. Embryo Today 93, 51–5. doi:10.1002/bdrc.20199

Soubry, A., Hoyo, C., Jirtle, R.L., Murphy, S.K., 2014. A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. Bioessays 36, 359–71. doi:10.1002/bies.201300113

Stevens, R.G., Kalkwarf, D.R., 1990. Iron, Radiation, and Cancer. Environ. Health Perspect. 87, 291. doi:10.2307/3431037

Stewart, F.A., Akleyev, A.V., Hauer-Jensen, M., Hendry, J.H., Kleiman, N.J., MacVittie, T.J., Aleman, B.M., Edgar, A.B., Mabuchi, K., Muirhead, C.R., Shore, R.E., Wallace, W.H., 2012. ICRP PUBLICATION 118: ICRP Statement on Tissue Reactions and Early and Late Effects of Radiation in Normal Tissues and Organs — Threshold Doses for Tissue Reactions in a Radiation Protection

Context. Ann. ICRP 41, 1–322. doi:10.1016/j.icrp.2012.02.001

Strand, P., Aono, T., Brown, J.E., Garnier-Laplace, J., Hosseini, A., Sazykina, T., Steenhuisen, F., Vives i Batlle, J., 2014. Assessment of Fukushima-Derived Radiation Doses and Effects on Wildlife in Japan. Environ. Sci. Technol. Lett. 1, 198–203. doi:10.1021/ez500019j

Unscear, 2010. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation 2010 UNSCEAR 2010 Report.

UNSCEAR, 1996. SOURCES AND EFFECTS OF IONIZING RADIATION. New York.

Vaiserman, A.M., Koliada, A.K., Jirtle, R.L., 2017. Non-genomic transmission of longevity between generations: potential mechanisms and evidence across species. Epigenetics Chromatin 10, 38. doi:10.1186/s13072-017-0145-1

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3, RESEARCH0034.

Wang, Z., Gerstein, M., Snyder, M., 2010. RNA-Seq : a revolutionary tool for transcriptomics 10, 57–63. doi:10.1038/nrg2484.RNA-Seq

White, R., Rose, K., Zon, L., 2013. Zebrafish cancer: the state of the art and the path forward. Nat. Rev. Cancer 13, 624–36. doi:10.1038/nrc3589

Williams, T.D., Mirbahai, L., Chipman, J.K., 2014. The toxicological application of transcriptomics and epigenomics in zebrafish and other teleosts. Brief. Funct. Genomics 13, 157–71. doi:10.1093/bfgp/elt053

Supplementary material

Paper III

Supporting information

Raw sequencing data and detailed data files (DEG lists) of all analyses can be found under Gene Expression Omnibus (GEO) number GSE98539.

Table S1. Primer sequences for validation with RT qPCR

Accession number	gene		Sequence (5'->3')
NM_131204.2	*serpinh1b*	Forward primer	CAAACAGCTGTCCAAGAGTGAA
		Reverse primer	ATGGAGGTTGCATGAGTGCT
NM_131668.3	*apt1b1a*	Forward primer	GCACAGGCTGTAGTTGGCTA
		Reverse primer	TGTGATAATCCTGGGGGTGC
NM_001291499.1	*tfa*	Forward primer	TGGGGCTTTCAGGTGTCTTG
		Reverse primer	ACCATCTGTGTAGTCCCCGA
NM_001128716.1	*samd7*	Forward primer	GGCATGTCTGCTGGAGAGTT
		Reverse primer	CGTTAGAGTTTGGGGGCAGT
NM_001128716.1	*ccna1*	Forward primer	AAGCGTGACGTCAGTACGTT
		Reverse primer	TCCATAAATGACCCTGAGCCAA
NM_001256780.1	*buc*	Forward primer	TGCAGGCCTTGTATTGAGCA
		Reverse primer	AACTTGCCTCCAAGACCTCG
NM_131702.1	*sox19b*	Forward primer	CGCCAGCTCTTACAGTCAAATG
		Reverse primer	GCGACAGGGGTTCTGGTTTA
NM_001024430.2	*spra*	Forward primer	ATTTGGGCTGTGAAGCAGGA
		Reverse primer	GAGAGAAGCGGCGTTATGGA
NM_214797.1	*zbtb*	Forward primer	TACCGTGGGGTAATGGCAAG
		Reverse primer	CACAGAGGAAACCCCACTCC
NM_181663	*szl*	Forward primer	CTGACGACTTGAGGACCCTG
		Reverse primer	GGATGAACCTGTCGAGGCAT
NM_001297547.1	*apela*	Forward primer	CACAACTGCCCGAAGAAACG
		Reverse primer	CCCGGAGCATCATAAAACCTCA
NM_212614.2	*mycn*	Forward primer	AACAAGAGGGAGAATGCCAGC
		Reverse primer	CCTCGTCCGGGTAGAAACAC
BC076032.1*	*apoa4b.1*	Forward primer	TGGGGCAGGACCTGATCAAT
		Reverse primer	CCTTCACACTCTGCTCCAGG
NM_213118.1*	*sox2*	Forward primer	CGAGTCTAGTTCGAGTCCGC
		Reverse primer	GTTAATCGTCGTACCGGGCA
NM_001320394.1*	*crabp2b*	Forward primer	GCACTCGGGTGTATGAACGA
		Reverse primer	CCACAAAATGACAGTTGAGTTGAGA
NM_001013565.1*	*cyp2x6*	Forward primer	CTCCACAGGTCTCCGTATGTG
		Reverse primer	AGCACTGTCAGCATCACGATT
NM_001082921.1*	*ppp1r15a*	Forward primer	TCCTCTGAGCTTCTCCTCGT
		Reverse primer	TGCTCTGTGTTCAGGCATCA
NM_001002332.1*	*uox*	Forward primer	TCAGCGGTGACTCCTGAAAC
		Reverse primer	GTGGACGGTGTTTTTGACGG
NM_001003631.1*	*shisa*	Forward primer	ATTGTGGTGAGTGCGTTCCT
		Reverse primer	GATCGCTTGCTTTGGGCTTC
NM_131263.1**	*eef1a*	Forward primer	TTGAGAAGAAAATCGGTGGTGCTG
		Reverse primer	GGAACGGTGTGATTGAGGGAAATTC
NM_173234.1**	*rps18*	Forward primer	CATCCCAGAGAAGTTTCAGCACATC
		Reverse primer	CGCCTTCCAACACCCTTAATAGC
NM_201154.1**	*hmbs*	Forward primer	GTGTGTGGAATTGGACAACAAAGTG
		Reverse primer	CGAGGGCTGATGATGAGATATTGC
NM_212986.1**	*hprt1*	Forward primer	CAGCGATGAGGAGCAAGGTTATG
		Reverse primer	GTCCATGATGAGCCCGTGAGG

*From Hurem et al. 2017 (ref: see main article)

**From Kamstra et al. 2017 (ref: see main article)

Table S2. qPCR results as shown in fold changes relative to respective controls

	8.7 mGy/h 0 yr	53 mGy/h 0 yr	8.7 mGy 1 yr
serpin1h1b	0.51	0.78	13.61
atp1b1	1.10	0.19	1.78
tfa	1.67	2.65	3.26
samd7	1.23	1.22	0.28
ccna1	1.04	1.39	0.85
buc	1.12	2.21	0.73
sox19b	1.08	1.59	0.78
szl	0.77	0.09	1.77
apela	1.00	0.02	1.45
apoa4b.1	<lod	<lod	1.93
sox2	<lod	<lod	2.02
crabp2b	0.93	0.16	1.37
cyp2x6	1.37	0.21	2.46
mycn	0.96	0.11	1.13
ppp1r15a	1.07	0.46	1.07
eef1a	1.16	0.58	1.27
spra	1.00	0.94	1.17
zbtb1	1.02	1.72	0.86
uox	<lod	<lod	1.87
shisa2	1.05	0.24	1.11

<lod: Cq values too high for accurate gene expression analysis

Fig S1. RNA quality control plot.

Shown are reads mapping to different feature from all individual samples.

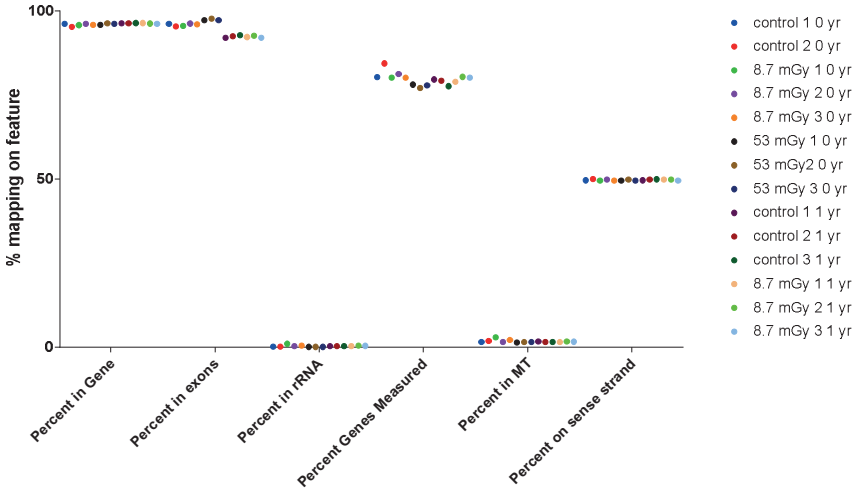
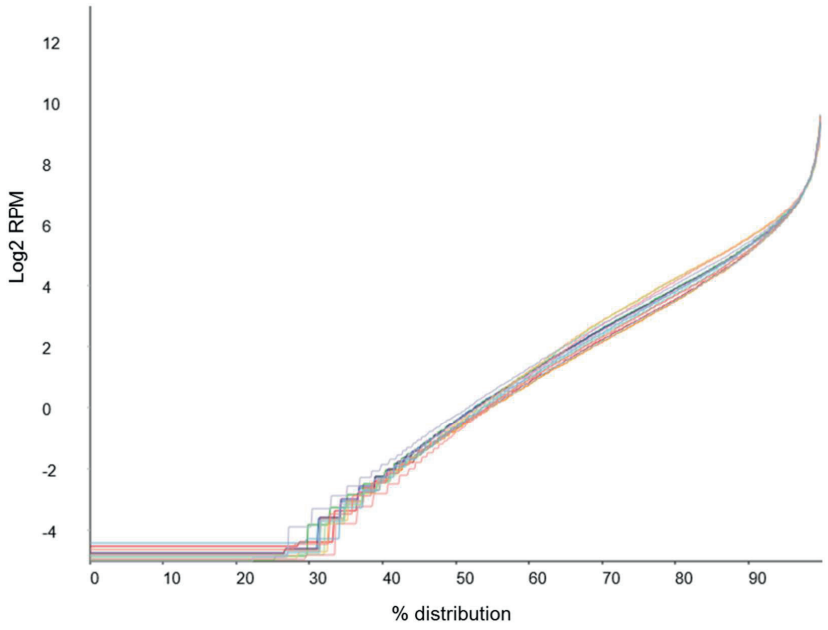


Fig S2. Cumulative distribution plot showing the cumulative distribution of log2 RPM values from low to highly expressed genes in all samples.



IV

Gamma irradiation during gametogenesis in young adult zebrafish causes persistent genotoxicity and adverse reproductive effects

Authors:

Selma Hurem[1,2*], Tânia Gomes[3], Dag A. Brede[1,4], Ian Mayer[1,2], Viola H. Lobert[2,5], Stephen Mutoloki[2], Kristine B. Gutzkow[6], Hans-Christian Teien[1,4], Deborah Oughton[1,4], Peter Alestrøm[1,2], Jan L. Lyche[1,2]

Affiliation:

[1]Centre for Environmental Radioactivity (CERAD CoE), NMBU, 1433 Ås, Norway

[2]Norwegian University of Life Sciences (NMBU), Faculty of Veterinary Medicine and Biosciences, P.O. Box 8146 Dep., 0033 Oslo, Norway.

[3]Norwegian Institute for Water research (NIVA), Gaustadalléen 21, NO-0349, Oslo, Norway

[4]Norwegian University of Life Sciences (NMBU), Faculty of Environmental Sciences and Natural Resource Management, 1433 Ås, Norway

[5]Oslo University Hospital, Institute for Cancer Research, Dept. of Molecular Cell Biology, Montebello, Oslo, Norway

[6]Norwegian Institute of Public Health, PO Box 4404 Nydalen, 0403 Oslo, Norway.

***Corresponding author:** Selma.hurem@nmbu.no

NMBU-School of Veterinary Science Postboks 8146 Dep, 0033 Oslo

Abstract

The biological effects of gamma radiation may exert damage beyond that of the individual through its deleterious effects on reproductive function. Impaired reproductive performance can result in reduced population size over consecutive generations. In a continued effort to investigate reproductive and heritable effects of ionizing radiation, we recently demonstrated adverse effects and genomic instability in progeny of parents exposed to gamma radiation. In the present study, genotoxicity and effects on the reproduction following subchronic exposure during a gametogenesis cycle to 60Co gamma radiation (27 days, 8.7 and 53 mGy/h, total doses 5.2 and 31 Gy) were investigated in the adult wild-type zebrafish (Danio rerio). A significant reduction in embryo production was observed one month after exposure in the 8.7 and 53 mGy/h exposure groups compared to control. One year later, embryo production was significantly different from controls only in the 53 mGy/h group, where sterility was observed, accompanied by a regression of reproductive organs in 100% at 1.5 years after exposure. Histopathological examinations revealed no significant changes in the testis in the 8.7 mGy/h group, while in 62.5 % of females exposed to this dose the oogenesis was found to be only at the early previtellogenic stage. The DNA damage determined in whole blood, 1.5 years after irradiation, using a high throughput Comet assay, was significantly higher in the exposed groups (1.2 and 3-fold increase in 8.7 and 53 mGy/h females respectively; 3-fold and 2-fold increase in 8.7 and 53 mGy/h males respectively) compared to controls. A significantly higher number of micronuclei (4-5 %) was found in erythrocytes of both the 8.7 and 53 mGy/h fish compared to controls. This study shows that gamma radiation at a dose of exposure ≥ 8.7 mGy/h during gametogenesis causes adverse reproductive effects and persistent genotoxicity (DNA damage and increased micronuclei) in adult zebrafish.

Key words: **zebrafish; gamma irradiation; reproduction; genotoxicity; DNA.**

1 Introduction

The aquatic environment is a primary recipient of ionizing radiation as the consequence of increasing amounts of gamma emitting radionuclides from various anthropogenic and non-anthropogenic activities (nuclear accidents, nuclear power plant waste discharge, cosmic radiation, naturally occurring primordial radionuclides). Gamma radiation is a potent agent for breaking bonds in the genetic material or causing cellular damage through the induction of oxidative stress, particularly in dividing cells having high active metabolism. As such, it

has the potential to induce reprotoxicity and genetic defects (Adam-Guillermin et al., 2012; Hurem et al., 2017a) and impair reproductive function in aquatic fauna (Won et al., 2015). Germ cells are the precursors of the gametes (oocytes and sperm), and due to their characteristics of rapid cell division and high active metabolism are particularly vulnerable to ionizing radiation. Ionizing radiation-induced cell damage can result in a variety of deleterious effects during the lifetime of an organism, and as germ cell damage has been found to be transmissible and inherited by future generations, such damage can also result in more long-term population effects (Kong et al., 2016).

To date, the effects of ionizing radiation on the reproductive performance in fish have only been studied following exposure to either acute (Michibata et al. 1976; Kuwahara et al., 2003) or very high chronic doses (Hyodo-Taguchi and Etoh, 1986). Studies of more environmentally realistic doses on reproduction in fish, encompassing both subchronic exposure and medium dose rates, especially covering the entire gametogenesis cycle, are currently lacking.

The zebrafish (Danio rerio) has proven to be a good vertebrate model to assess reproductive effects (Hoo et al., 2016; Laan et al., 2002) due to its developmental and physiological advantages such as a short reproduction cycle, high fecundity, transparent embryos and a high degree of similarity with other vertebrates. A pair of adult zebrafish can reproduce approximately two times per week over its breeding cycle, and yield 200 to 300 eggs at each spawning. In addition, the maximal reproductive capacity in zebrafish is known, and can be achieved by young sexually mature fish between three and six months of age (Skidmore, 1965). The United Nations Scientific Committee for the Effects of Atomic Radiation 1996 report stated that the reproductive organs in fish could be negatively affected by a dose rate of 100 μGy/h through a reduction in spermatogonia number (UNSCEAR, 1996). However, the span of dose rates known to inflict damage to the reproductive organs is quite broad as a total dose of 10 Gy caused minimal effects on the maturation of oocytes in fish (UNSCEAR 1996).

The present work assessed the effects of subchronic gamma radiation exposure (27 days, ^{60}Co, dose rates 8.7 and 53 mGy/h, total 5.2 and 31 Gy) in adult zebrafish during a gametogenesis cycle on the overall health, reproduction, and genotoxicity. In order to determine whether reproductive function is impaired in later life following radiation exposure, effects on reproduction were evaluated both one month and one year after irradiation. Histopathological examination of the gonads was performed in order to determine possible deleterious reproductive effects in irradiated adults, while the genotoxic

effects in the form of DNA damage and the number of micronuclei (MN) in red blood cells were assessed in both male and female zebrafish one year after gamma irradiation.

2 Materials and Methods

2.1 Fish husbandry

Adult zebrafish (ZF, aged 6 months) from the AB wild type strain (30 males and 30 females per exposure group) were obtained from the Zebrafish Facility at the Norwegian University of Life Sciences (NMBU). The exposure of ZF to external gamma radiation took place at the FIGARO Co-60 irradiation facility (activity ~420 GBq) at NMBU and is schematically depicted in Fig 1. Recirculating system water was prepared from particle and active charcoal filtered reverse osmosis kept sterile by UV irradiation water of pH 7.5 and temperature 28 ± 1 °C with regular weekly or daily water changes depending on the water quality described in Hurem et al. (2017b). The light regime of 10-14 light-dark cycle (250-320 lx) was used and fish were fed dry feed Gemma Micro 300 (Skretting, Stavanger, Norway) twice a day and live artemia (Scanbur, Copenhagen, Denmark) once a day, both during and after the experimental periods.

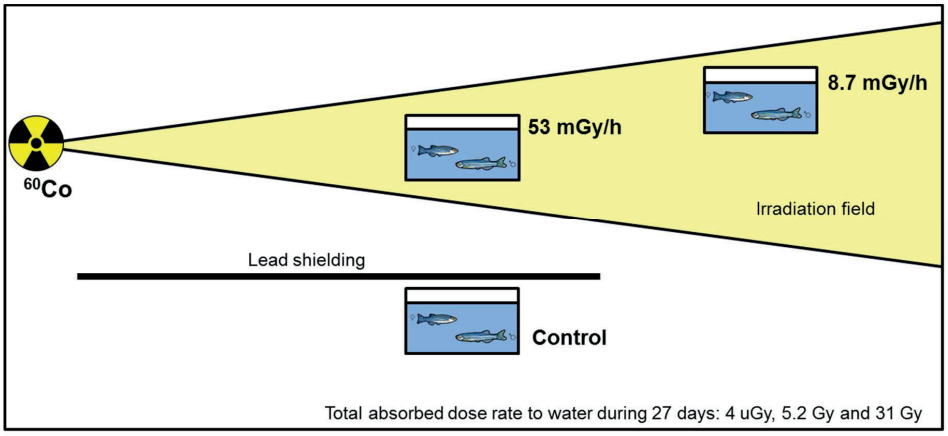


Fig 1. Schematic presentation of adult fish exposure at the FIGARO Co-60 irradiation facility at the Norwegian University of Life Sciences (NMBU). Fish were exposed in 9 L plastic aquaria, with 6 L swimming space (N = 30 males and 30 females per each aquarium). Exposure lasted for 27 days during gametogenesis, with total exposure time of 591.5 hours. A control aquarium was placed behind lead shielding, and two aquaria at different distances to the source focus, resulting in calculated average absorbed dose rates to

water of 8.7 mGy/h and 53 mGy/h, respectively, and total doses 5.2 Gy and 31 Gy, according to dosimetry described previously by Hurem et al., (2017b).

After exposure, fish were maintained according to standard operating procedures at the NMBU Zebrafish Facility until sampling for histopathology, genotoxic effects and measurement of weight and length.

2.2 Ethical statement

This research was performed in accordance with the Norwegian Animal Protection Act (implemented EU Directive 2010/63/EU). Approval number FOTS ID 5793 was issued on December 12, 2013 by IACUC of Norwegian School of Veterinary Science (since 2014 Norwegian University of Life Sciences, Faculty of Veterinary Medicine and Biosciences, Oslo, Norway).

2.3 Biometric parameters

Weight and length were measured 1.5 years after exposure, in 22 male and 22 female anesthetized fish from both the control and 8.7 mGy/h groups. In the 53 mGy/h group, weight and length were measured in 10 males and 10 females and in 24 fish of undetermined sex. The condition factor of unexposed and gamma irradiated fish was calculated according to the formula ($K = [\text{mass (g)} \times 100]/[\text{length (cm)}]3$) (Jones et al., 1999).

2.4 Reproduction assessment

Thirty adult irradiated male and female zebrafish of the AB wild type strain were used in the breeding trials. The mating experiments took place during six consecutive breeding weeks one month after gamma irradiation and during five consecutive breeding weeks one year after irradiation. For maintenance during the reproduction experiments, males and females from each exposure were divided into two groups, kept in 12 holding tanks of 2L volume, with 12 fish per tank and used intermittently over even and odd numbered breeding weeks. In each breeding trial, six standard (conservative) 1L breeding tanks with a meshed bottom for separation of eggs (Aquatic Habitats, Apopka, FL, USA) were used with one breeding pair per tank. The setup and male/female separation took place in the late afternoon and breeding pairs were formed using one male and female from the same exposure group. The morning after, barriers were removed and the breeding pairs were

allowed to mate for 30 minutes. Egg collection and counting was performed immediately after breeding, followed by the separation of sexes and transfer of fish to holding tanks.

2.5 Fish anesthesia and euthanasia

For anesthesia of the fish, 0.2% Tricaine Methanesulfonate (MS-222) (Sigma-Aldrich, Oslo, Norway) in dH2O adjusted to pH 7.0 with 1.0M Tris (pH 9.5) combined with iced system water was used. Briefly, fish remained in this solution until no visible movement was observed. For euthanasia, an overdose of tricaine was used in iced system water, and the fish were observed until failing to react to external stimuli and/or following cessation of opercular (gill) movement.

2.6 Histopathological analysis

Whole fish were fixed individually in 4% paraformaldehyde for a minimum of 4 days and then processed according to standard histological procedures using Hematoxylin and Eosin (H&E) stain. Histopathological examination was performed blindly using a Zeiss Axioskop microscope equipped with a digital camera (Leica SFC 420). Eight males and eight females from the two exposed groups and controls were processed, examined and analyzed 1.5 years after gamma exposure.

2.7 Genotoxicity analyses

2.7.1 Comet assay

For blood extraction, eight male and eight female fish were used from the two exposed groups and controls. The fish were euthanized 1.5 years after exposure, and a modified protocol similar to previous studies (Kovács et al., 2015) was used for blood collection for the Comet assay. Briefly, a 200 µl pipette was coated with 10 µl Heparin (5000 IE/ml, Leo®, Norway). After the tail was cut off, 5 µl of blood was collected with the coated pipette and transferred to a microtube containing 100 µl PBS without Ca2+/Mg2+ (pH 7.4). Samples were diluted 1:20 with PBS in order to obtain a cell concentration of 1x106 cells/mL. Cell viability was checked by trypan blue exclusion assay. Cells were resuspended 1:10 in 0.75 % low melting point agarose at 37 ºC, and triplicates (3 × 4 µL) from each biological replicate were immediately applied on a cold GelBond®film (as described in Gutzkow et al., 2013). Lysis was performed overnight in lysis buffer at 4 ºC (2.5 M NaCl, 0.1 M Na2EDTA, 0.01 M Tris, 0.2 M NaOH, 0.034 M N-laurylsarcosine, 10 % DMSO, 1 % Triton X-100, pH 10). For unwinding, films were immersed in cold

electrophoresis solution (0.3 M NaOH, 0.001 M Na2EDTA, pH > 13) for 40 min. Electrophoresis was carried out in cold, fresh electrophoresis solution at 25 V (0.8 V/cm across the platform) for 20 min at 8 ºC, with circulation of the electrophoresis solution. After electrophoresis, films were neutralized with a neutralization buffer (0.4 M Tris–HCl, pH 7.5) for 2×5 min, fixed in ethanol (> 90 min in 96 % ethanol) and dried overnight. Films were stained with SYBR®Gold Nucleic Acid Gel Stain (Life Technologies, Paisley, UK) diluted 1:10 000 in TE-buffer (1 mM Na2EDTA, 10 mM Tris–HCl, pH 8) before examination at a 20 × magnification under an Olympus BX51microscope (light source: Olympus BH2-RFL-T3, Olympus Optical Co., Ltd.; camera: A312f-VIS, BASLER, Ahrensburg, Germany). Fifty randomly chosen cells per replicate (150 cells per biological replicate, total 1200 cells per dose rate) were scored using the Comet IV analysis software (Perceptive Instruments Ltd., Bury St. Edmunds, UK). Tail intensity (% Tail DNA), defined as the percentage of DNA migrated from the head of the comet into the tail, was used as a measure of DNA damage to assess genotoxicity (Kumaravel and Jha, 2006). Blood cells were also categorized according to the grade of damage using the % of Tail DNA based on the previously mentioned criteria (Gomes et al., 2013): minimal 10% tail, low damage 10-25%, mid-damage 25-50%, high damage 50-75% and extreme damage >75%.

2.7.2 Blood slide examination

Peripheral blood was obtained from 8-11 males and females from the two exposed and control groups 1.5 years after irradiation. The tail of the euthanized fish was removed and approximately 5 µl of blood was collected by pipette from the severed tail of each euthanized fish, transferred to the frosted end of a glass slide, spread in a thin film and air-dried. After fixation in ethanol for 15 min, slides were left to air dry. The staining was performed using the Quick dip protocol (H&E). The stained slides were viewed under a Zeiss Axioskop microscope equipped with a digital camera (Leica SFC 420) and magnification 1000x, and between 1000-2000 erythrocytes scored per slide. The erythrocytes were also examined for the occurrence of two nuclei (binuclear cells) and for irregular shape (e.g. tear or sickle shaped erythrocytes). The cells with one, two or three micronuclei (MN) were noted separately. Criteria for the identification of fish micronuclei were previously described (Oliveira et al., 2009; Song et al., 2012): (a) MN should be a size smaller (1/10 to 1/30) than the main nucleus (b) MN should be a circular or ovoid chromatin body with the same staining characteristics as the nucleus; (c) MN must not touch the main nucleus.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc., La Jolla, CA, USA) and XLStat2016® (Addinsoft, Paris, France). Results are presented as mean ± standard error (SEM) for the reproduction assessments. Significant differences between dose rates for the biometric parameters and DNA damage were calculated using Kruskal–Wallis One Way Analysis of Variance on Ranks, whereby multiple comparisons were conducted using the Dunn's test. The cumulative embryo production and embryo production per breeding pair and MN were analyzed using a Two Way Analysis of Variance (with the dose rate and either time after irradiation or sex as independent variables). If significant, multiple comparison procedures were conducted according to the Fisher's LSD, Dunnett's or the Tukey's test. Statistical significance was set at $p < 0.05$.

3 Results

3.1 Biometric parameters in adult zebrafish

The weight and total length were measured in all fish 1.5 years after exposure in order to determine possible differences in size and condition factor (K) between exposed and control fish. Significant reduction of mean length and weight was observed in females of the 8.7 mGy/h exposure group, although there was no difference in condition factor (Table 1). In contrast, the length and weight of males in the 8.7 mGy/h were not significantly different compared to controls, however, the significant difference was found in the condition factor of these males compared to controls (Table 1). No significant differences were however found in fish in the 53 mGy/h group compared to controls (Table 1). For the 53 mGy/h exposure group, external sexual characteristics were non-distinguishable in 40 % of the fish 1.5 years after the exposure and this group was excluded from statistical analyses.

Table 1. Biometric parameters in male and female zebrafish measured 1.5 years after exposure to gamma radiation used for the reproduction, histopathology and MN assay. Data are presented as mean ± SD. Significance compared to corresponding controls denoted with (*) and significance compared to corresponding controls and the other exposed group denoted with (**), with respective p-value(s) given in parentheses according to Dunn's test.

Dose rate (mGy/h)	Sex	Length (cm)	Weight (g)	Condition factor (K)

Control [a]	male	3.39 ± 0.12	0.29 ± 0.05	0.74 ± 0.09
	female	3.67 ± 0.26	0.42 ± 0.08	0.85 ± 0.13
8.7 [b]	male	3.38 ± 0.17	0.26 ± 0.04	0.66 ± 0.10* (0.004)
	female	3.44 ± 0.18** (0.0001; 0.008)	0.32 ± 0.05** (0.004, 0.005)	0.79 ± 0.13
53 [c]	male	3.41 ± 0.07	0.27 ± 0.05	0.69 ± 0.10
	female	3.72 ± 0.20	0.41 ± 0.07	0.79 ± 0.09
	n.d	3.73 ± 0.11	0.33 ± 0.05	0.64 ± 0.08

K – ([mass (g)*100] /[length (cm)]^3)
[a]N = 22 males, 22 females
[b]N = 22 males, 22 females
[c]N = 10 males, 10 females and 24 fish of no determined (n.d) sex

3.2 Gamma radiation causes reproduction impairment and damage in gonads

The results of the breeding studies indicated a significant reduction in the reproductive capacity of fish exposed to gamma radiation, both at one month and one year after the exposure (Fig. 2). The cumulative embryo production per week in the 53 and the 8.7 mGy/h groups was significantly reduced one month after irradiation compared to controls (~ 80 % and ~ 35 %; p = 0.002 and 0.028, respectively). The reproductive capacity was further significantly reduced in both exposed groups one year after exposure within each group compared to one month after exposure (p = 0.01). During this breeding period, significant reduction in embryo production was found in the 53 mGy/h compared to controls (p = 0.04) (Fig 2) as only one breeding pair produced embryos.

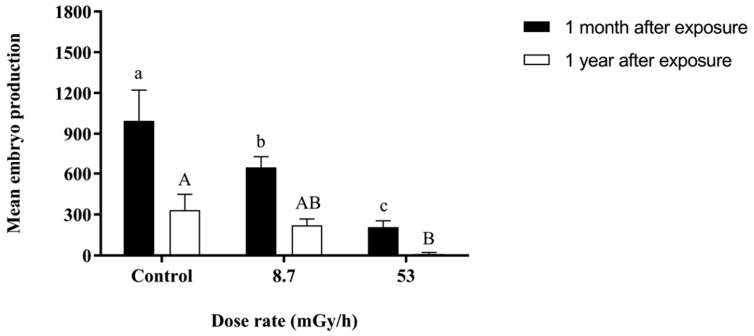


Fig 2. Cumulative embryo production in zebrafish per week one month and one year after exposure to gamma radiation during gametogenesis to either 8.7 or 53 mGy/h compared to

controls. Results presented as mean ± SEM ($p \leq 0.05$). Different lowercase letters (a, b, c) represent significant difference between groups one month after exposure, uppercase letters (A, AB, B) represent significant differences between groups one year after exposure, analyzed by the Fisher's LSD test (n = 6 breeding pairs per breeding week).

Similarly, embryo production per breeding pair in the 53 mGy/h group differed significantly from both the controls and the 8.7 mGy/h group one month and one year after the exposure (Table A1). In contrast, the embryo production per breeding pair in the 8.7 mGy/h group was significantly different from the controls one month after gamma irradiation, but not one year later (Table A1).

The histopathological examinations revealed significant effects in the gonads of the adult fish (2 years of age). Differences were found between controls and the 8.7 mGy/h females where 62.5 % of females of the latter group had ovaries containing predominantly previtellogenic oocytes (Fig 3B), whereas in the controls the ovaries had oocytes at all developmental stages (Fig 3A). In the 53 mGy/h group, the reproductive organs were massively regressed, which is consistent with the observed failed spawning and lack of embryo production (Fig. 3C).

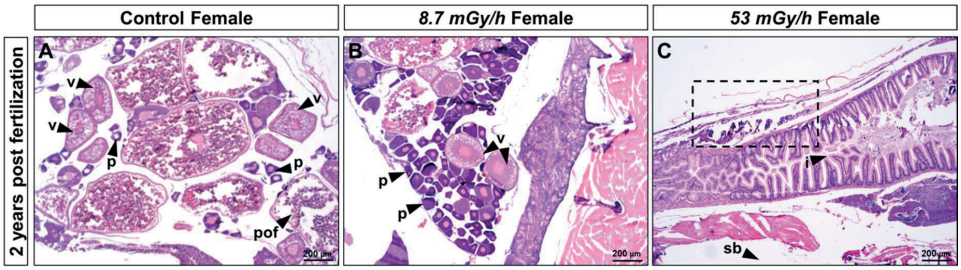


Fig 3. Histological sections of ovaries from (A) Control zebrafish with vitellogenic follicles (v), previtellogenic follicles (p) and postovulatory follicles (pof). (B) Female zebrafish exposed to 8.7 mGy/h during gametogenesis. Ovaries with a high number of previtellogenic follicules (p); (C) Female zebrafish exposed to 53 mGy/h during gametogenesis, showing no visible reproductive organs (dashed rectangle), i – intestine, sb – swimming bladder.

3.3 Persistent genotoxicity

3.3.1 Gamma radiation causes increased DNA damage

DNA damage assessed one year after gamma radiation exposure in whole blood of adult fish using the alkaline single-cell gel electrophoresis (SCGE) assay was significantly higher

in exposed groups compared to controls. Males in the 8.7 mGy/h and 53 mGy/h groups showed a 3-fold and 2-fold increase in DNA damage respectively, compared to controls (Fig 4A). Similarly, in females, a 1.2-fold and 3-fold increase in DNA damage was found in 8.7 and 53 mGy/h groups respectively, compared to controls (Fig 4B). The DNA damage was also significantly different between the 8.7 and the 53 mGy/h groups in both males and females.

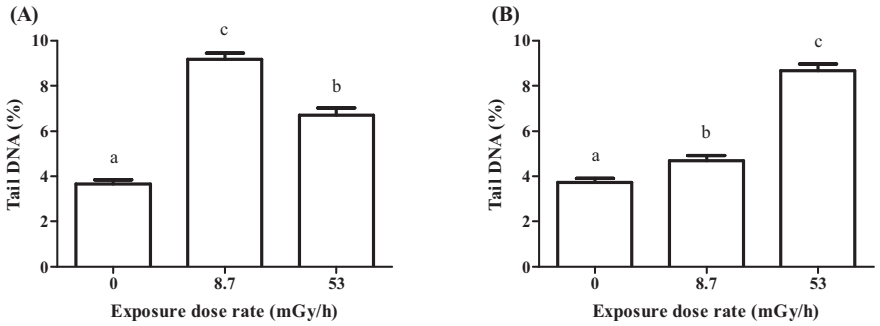


Fig. 4. DNA damage in adult zebrafish measured by the alkaline SCGE after exposure to gamma radiation expressed as percentage of tail DNA (average ± SEM, n=1200). Different lowercase letters (a, b, c) denote significant difference between groups. (A) Male zebrafish. (B) Female zebrafish.

The percentage of DNA in the tail seen in the Comet assay was used to categorize the grade of damage in unexposed and gamma irradiated zebrafish (Table A2). The majority of cells from both males and females from the control group showed minimal to low grade of damage (> 99% of the cells), characterized by zero or minimal DNA 'Comet-tail'. On the other hand, irradiated zebrafish presented a higher number of cells with low and mid damage compared with the control, reflecting an increase of DNA damage resulting from exposure to gamma radiation.

3.3.2 Gamma radiation causes persistent increase in mitotic malfunctions

Whole zebrafish blood slides were examined in order to determine possible abnormalities related to blood cell formation or renewal. Consequently, micronuclei (MN) were found in erythrocytes, and counts revealed a statistically significant increase in the frequency of one MN per cell in both males and females from the 8.7 and 53 mGy/h exposures, compared to controls ($p < 0.0001$) (Figure 5). Two and three MN per cell were found to be more frequent

in the 53 mGy/h males and females than in the controls, but this increase was not significant ($p > 0.9$). No significant differences were found in the increase of either micronuclei frequency or the number of MN per cell between the sexes ($p > 0.5$). Furthermore, the occurrence of irregular erythrocyte shape and binucleated cells in the exposed fish compared to controls was examined, without demonstrating any significant difference between the controls and the exposed zebrafish ($p > 0.9$).

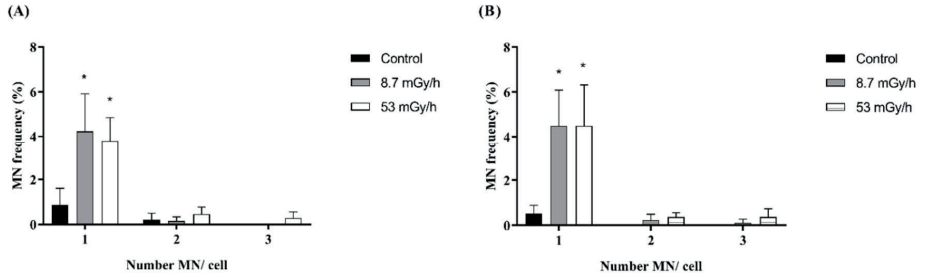


Fig 5. Frequency of micronucleated erythrocytes in zebrafish exposed to 8.7 and 53 mGy/h dose rates (total 5.2 and 31 Gy) of gamma radiation and controls; X-axis shows the number of micronuclei found per erythrocyte. Results are presented as mean percentage micronuclei ± 95% CI, significance denoted with (*) and compared to control according to two-way ANOVA and Tukey's tests (p-value < 0.0001). (A) Male zebrafish. (B) Female zebrafish. N =10.000 cells from 8-11 individuals.

4 Discussion

4.1 Fish condition and reproduction

This study has shown that exposure to gamma radiation (subchronic, 53 and 8.7 mGy/h, total 31 and 5.2 Gy) during the period of gametogenesis can severely affect the reproduction in fish. However, the dose rates used in this study are almost two orders of magnitude above the maximum dose rates (130-140 µGy/h) found in the aquatic environment following a nuclear fallout (Johansen et al., 2015; Strand et al., 2014). Although the fish survived the exposure, massive pathological changes in the gonads and reproductive failure were found, especially at the higher dose (31 Gy). Gametogenesis is the process in which cells undergo cell division and differentiation in order to form the mature male or female germ cells, which in zebrafish lasts for approximately four weeks between 3- 5 months of age (Koç et al., 2008; Laan et al., 2002). In fish, successful reproduction is dependent upon a good body condition and sufficient energy reserves. As such, condition factor (K) (Jakob et al., 1996;

Stevenson and Woods, 2006) was used as an indicator of overall health of fish populations, with heavier individuals of a certain length regarded as being in better breeding condition (Fulton, 1904; Bolger and Connolly, 1989). We found a slight, but significant difference in the condition factor in males exposed to 8.7 mGy/h gamma radiation compared to controls at 1.5 years after gamma irradiation. We also found that the females of the 8.7 mGy/h group were of smaller size, while the condition factor was not significantly different from the other groups. For using the described dose rates and the required number of biological replicates, the fish were randomly selected for each exposure tank, indicating that individual differences could have been present between fish in different exposures. Since the husbandry of the fish and water parameters did not differ significantly between exposure tanks (Hurem et al., 2017b), the reason behind these differences is unclear, but could reflect the balance between energy budget allocations between growth, repair of DNA damage and spermatogenesis.

A significant reduction in reproductive capacity, in terms of embryo production, was found in both the exposed groups compared to the controls one month after irradiation, this reduction being significantly greater in the 53 compared to the 8.7 mGy/h group. One year after gamma irradiation, this difference between 8.7 mGy/h group and controls was not significant, although oocyte maturation at 1.5 years after gamma irradiation was found to be severely disrupted with only non-mature previtellogenic oocytes predominating in the ovaries in more than half of the 8.7 mGy/h females. Similarly, reduced fecundity and fertility in fish were reported after gamma irradiation of medaka (*Oryzias latipes*) eggs with a dose of 5 Gy (362.5 mGy/h) (Hyodo-Taguchi and Etoh, 1986), while only temporary sterility was induced in medaka after 5 and 10 Gy gamma irradiation (Michibata et al, 1976). Effects on the maturation of oocytes has previously been reported after a whole body exposure of adult loach (*Misgurnus anguillicaudatus*) (10 Gy, x-rays), which is approximately two times higher the dose used in our study (Egami and Aoki, 1966). In addition, decreased vitellogenin concentration was found in zebrafish ovaries after exposure to alpha emitters (250 µg/L depleted U for 20 days) (Bourachot et al., 2014). It was earlier established that radiation at doses as low as 0.3 Gy (X-rays) can impair the gametogenesis in fish, with a 50 % reduction in spermatogonia (UNSCEAR 2008). However, in this study, no visible differences were observed in the testis of the 8.7 mGy/h (total 5.2 Gy) exposure group compared to control, indicating that female gonads are more susceptible to gamma radiation than male. Interestingly, a dose of 4.7 Gy gamma radiation, which is relatively close to the total dose used here, caused accelerated spermatogenesis in fish according to

Kuwahara and co-workers (Kuwahara et al., 2003). In the present study, however, reproduction was severely impaired in fish in the 53 mGy/h exposure group as they produced no embryos one year after the irradiation event, and showed complete regression in ovary and testis development. Additionally, in offspring of the 53 mGy/h exposed fish, modulation of gene pathways related to the endocrine regulation of reproduction was found. These pathways include estrogen receptor 1 (ESR1), follicle stimulating hormone (FSH) signalling, insulin growth factor 2 (IGF2) and gonadotropin releasing hormone (GnRH) signalling (unpublished data). Offspring of these fish (53 mGy/h) also showed 100 % mortality occurring at 8 hours post fertilization (hpf), corresponding to the gastrulation stage (Hurem et al., 2017b). This finding indicates that damaging signals that could lead to a modulation of reproduction hormone pathways, may have been transmitted to the progeny via parental germ cells.

4.2 Genotoxicity

Gamma radiation exposure to 8.7 – 53 mGy/h (total doses 5.2 and 31 Gy) caused a small but significant increase in DNA damage in both female and male zebrafish a considerable time after the irradiation ended (1.5 years), with the most prominent effect occurring in the 8.7 mGy/h exposed males and 53 mGy/h exposed females. The persistence of DNA damage may therefore point to genomic instability, which was observed in the progeny one year after exposure of the parents (Hurem et al., 2017b). Only a few studies have to date discussed sex-specific differences in sensitivity to ionizing radiation. A study in mice reported higher ionizing radiation induced (1 Gy, X-rays) DNA damage increase in males than in females (Koturbash et al., 2008), and attributed the effect to sex hormones and distinct cellular responses to whole body irradiation, considering that sterilization neutralized this difference. Therefore, it is conceivable that differences in endocrine signaling may contribute to higher susceptibility of male fish to DNA damage.

Although we found no studies on the effects of chronic gamma irradiation, DNA damage in whole blood of adult zebrafish was found to be significantly increased after an acute exposure to high doses of ionizing radiation (X-rays, 0.1 – 1 Gy), while DNA damage in the offspring was correlated with the DNA damage of the parents (Lemos et al., 2017). The DNA damage response was also examined after chronic exposure to depleted uranium (20 and 250 µg U/L for 20 days), and differences between males and females were observed (Bourrachot et al., 2014). Interestingly, in offspring of both the 8.7 and 53 mGy/h fish, a high expression of ribonucleotide reductase subunit 2 (rrm2) was found (unpublished data).

This gene is associated with DNA damage response in mammals and may perhaps have a role in the transmission of DNA damage to the offspring, in addition to non-targeted mechanisms such as inflammatory and bystander effects following radiation exposure (Hurem et al., 2017b).

Micronuclei originate from aberrant mitosis and are formed when intact chromosomes or their fragments are not properly segregated into the daughter cells nuclei after cell division and instead remain in the cytoplasm (Pernot et al., 2012; Sabharwal et al., 2015). The MN test is frequently used in fish as an indicator of environmental stress and correlates to increased DNA damage and mutation rate (Russo et al., 2003, Pavlica et al., 2011; Song et al., 2012; Luzhna et al., 2013). In the present study, the frequency of one MN per erythrocyte was significantly increased in the 8.7 and 53 mGy/h groups (males and females) compared to controls. The increase in MN demonstrates mitotic failure indicating a persistent genotoxic stress. It is worth noting that in male zebrafish, the frequency of one MN per cell was higher in the 8.7 mGy/h exposure group than in the 53 mGy/h, while in the females this frequency was higher in the 53 mGy/h than in the 8.7 mGy/h group (Figure 5). Although not statistically significant, the sex-difference in sensitivity in MN-formation resembles the difference in DNA damage increase in the different exposure groups for males and females (Fig 4, Table A2). This supports the fact that the micronucleus test in whole blood seems to be a good indicator of increased DNA damage in zebrafish (Luzhna et al., 2013). The differences in effects between the irradiated groups and control group suggest that genotoxic effects of gamma irradiation during the sensitive period of gametogenesis persist for up to one year after irradiation.

5 Conclusion

The present study demonstrated that subchronic gamma radiation (8.7 and 53 mGy/h) during the gametogenesis stage causes adverse reproductive and genotoxic effects such as increased MN formation in erythrocytes and DNA damage in whole blood persisting 1.5 years after gamma irradiation. Reduced embryo production and disrupted ovary development were found at dose rates $\geq$ 8.7 mGy/h one month and 1.5 years after the exposure, respectively, while sterility was observed in the highest dose rate (53 mGy/h) one year after exposure, including a total regression of the reproductive organs. Overall, while the doses used in this study did not cause increased mortality of irradiated fish, the observed adverse reproductive and genotoxic effects indicates that gametogenesis is a very sensitive

life stage to ionizing radiation exposure and that the difference in effects can be sex-dependent and transmissible to offspring.

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References

Adam-Guillermin, C., Pereira, S., Della-Vedova, C., Hinton, T., Garnier-Laplace, J., 2012. Genotoxic and reprotoxic effects of tritium and external gamma irradiation on aquatic animals. Rev. Environ. Contam. Toxicol. 220, 67–103. doi:10.1007/978-1-4614-3414-6_3

Bolger, T., Connolly, P. L., 1989. The selection of suitable indices for the measurement and analysis of fish condition, J. of Fish Biology, Vol 34-2 171–182. doi:10.1111/j.1095-8649.1989.tb03300.x

Bourrachot, S., Brion, F., Pereira, S., Floriani, M., Camilleri, V., Cavalié, I., Palluel, O., Adam-Guillermin, C., 2014. Effects of depleted uranium on the reproductive success and F1 generation survival of zebrafish (Danio rerio). Aquat. Tox. 154: 1–11 doi:10.1016/j.aquatox.2014.04.018

Egami. N., Aoki K., 1966. Effects of x-irradiation of a part of the body on the ovary in the loach Misgurnus anguillicaudatus. Annot. Zool. Jpn. 39: 7-15

Froese, R., 2006. Cube law, condition factor and weight-length relationships: history, meta-analysis and recommendations. J. Appl. Ichthyol. 22, 241–253. doi:10.1111/j.1439-0426.2006.00805.x

Fulton, T. W. 1904. The rate of growth of fishes. 22nd Annual Report of the Fishery Board of Scotland 1904 (3):141-241.

Gomes, T., Araújo, O., Pereira R., Almeida, A.C., Cravo, A., Bebianno, M.J. 2013. Genotoxicity of copper oxide and silver nanoparticles in the mussel Mytilus galloprovincialis. Marine Environ. Res.84: 51–59. doi: 10.1016/j.marenvres.2012.11.009

Gutzkow K.B., Langleite T.M., Meier S., Graupner A., Collins A.R., Brunborg G. High-throughput comet assay using 96 minigels. Mutagenesis. 2013 May;28(3):333-40. doi: 10.1093/mutage/get012.

Hoo, J.Y., Kumari, Y., Shaikh, M.F., Hue, S.M., Goh, B.H., 2016. Zebrafish: A Versatile Animal Model for Fertility Research. Biomed Res. Int. 2016, 9732780. doi:10.1155/2016/9732780

Hurem, S., Martin, L.M., Brede, D.A., Skjerve, E., Nourizadeh-Lillabadi, R., Lind, O.C.,

Christensen, T., Berg, V., Teien, H.-C., Salbu, B., Oughton, D.H., Alestr?m, P., Lyche, J.L., 2017a. Dose-dependent effects of gamma radiation on the early zebrafish development and gene expression. PLoS One 12, e0179259. doi:10.1371/journal.pone.0179259

Hurem, S., Gomes, T., Brede, D.A., Lindbo Hansen, E., Mutoloki, S., Fernandez, C., Mothersill, C., Salbu, B., Kassaye, Y.A., Olsen, A.K., Oughton, D., Alestrom, P., Lyche, J.L., 2017b. Parental gamma irradiation induces reprotoxic effects accompanied by genomic instability in zebrafish (Danio rerio) embryos. Environ. Res. 159, 564-578 doi:10.1016/j.envres.2017.07.053

Hyodo-Taguchi Y, Etoh H (1986) Effects of tritiated water on germ cells in Medaka. II. Reproductive capacity following embryonic exposure. Radiat Res 106:321–330

Jakob, E.M., Marshall, S.D., Uetz, G.W., 1996. Estimating Fitness: A Comparison of Body Condition Indices. Oikos 77, 61. doi:10.2307/3545585

Johansen, M.P., Ruedig, E., Tagami, K., Uchida, S., Higley, K., Beresford, N.A., 2015. Radiological dose rates to marine fish from the fukushima daiichi accident: The first three years across the north pacific. Environ. Sci. Technol. 49, 1277–1285. doi:10.1021/es505064d

Jones RE. Petrell RJ. Pauly D. Using modified length-weight relationships to assess the condition of fish. Aquac Eng. 1999;20:261–276.

Koç (Yön), N.D., Aytekin, Y., Yüce, R., 2008. Ovary maturatıon stages and histological investigation of ovary of the Zebrafish (Danio rerio). Brazilian Arch. Biol. Technol. 51, 513–522. doi:10.1590/S1516-89132008000300010

Kong, E.Y., Cheng, S., Yu, K.N., 2016. Zebrafish as an in vivo model to assess epigenetic effects of ionizing radiation. Int. J. Mol. Sci. 2016, 17, 2108; doi:10.3390/ijms17122108

Kovács, R., Csenki, Z., Bakos, K., Urbányi, B., Horváth, Á., Garaj-Vrhovac, V., Gajski, G., Gerić, M., Negreira, N., López de Alda, M., Barceló, D., Heath, E., Kosjek, T., Žegura, B., Novak, M., Zajc, I., Baebler, Š., Rotter, A., Ramšak, Ž., Filipič, M., 2015. Assessment of toxicity and genotoxicity of low doses of 5-fluorouracil in zebrafish (Danio rerio) two-generation study. Water Res. 77, 201–12. doi:10.1016/j.watres.2015.03.025

Koturbash, I., Kutanzi, K., Hendrickson, K., Rodriguez-Juarez, R., Kogosov, D., Kovalchuk, O., 2008. Radiation-induced bystander effects in vivo are sex specific. Mutat. Res. 642, 28-36. doi:10.1016/j.mrfmmm.2008.04.002

Kumaravel, T.S., Jha, A.N., 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. Mutat. Res. Toxicol. Environ. Mutagen. 605, 7–16. doi:10.1016/j.mrgentox.2006.03.002

Kuwahara, Y., Shimada, A., Mitani, H., Shima, A., 2003. Gamma-ray exposure accelerates spermatogenesis of medaka fish, Oryzias latipes. Mol. Reprod. Dev. 65, 204–11. doi:10.1002/mrd.10261

Laan, M., Richmond, H., He, C., Campbell, R.K., 2002. Zebrafish as a Model for Vertebrate Reproduction: Characterization of the First Functional Zebrafish (Danio rerio) Gonadotropin

Receptor. Gen. Comp. Endocrinol. 125, 349–364. doi:10.1006/gcen.2001.7738

Lemos, J., Neuparth, T., Trigo, M., Costa, P., Vieira, D., Cunha, L., Ponte, F., Costa, P.S., Metello, L.F., Carvalho, A.P., 2017. Single Low-Dose Ionizing Radiation Induces Genotoxicity in Adult Zebrafish and its Non-Irradiated Progeny. Bull. Environ. Contam. Toxicol. 98, 190–195. doi:10.1007/s00128-016-2006-1

Luzhna, L., Kathiria, P., Kovalchuk, O., 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front. In Genetics 4, Art 131, 1-17 doi:10.3389/fgene.2013.00131

Michibata, H., 1976. The role of spermatogonia in the recovery process from temporary sterility induced by gamma-ray irradiation in the telcost, Oorias lalipes. J. Radiat. Res. 17: 112-1 53 (1976).

Oliveira, R., Domingues, I., Koppe Grisolia, C., Soares, A. M. V. M., 2009. Effects of triclosan on zebrafish early-life stages and adults. Environ. Sci. and Pollut. Res. 16, 679–688. doi:10.1007/s11356-009-0119-3

Pavlica, M., Štambuk, A., Malović, L., Mladinić, M., Klobučar, G.I. V., 2011. DNA integrity of chub erythrocytes (Squalius cephalus L.) as an indicator of pollution-related genotoxicity in the River Sava. Environ. Monit. Assess. 177, 85–94. doi:10.1007/s10661-010-1620-3

Pernot, E., Hall, J., Baatout, S., Benotmane, M.A., Blanchardon, E., Bouffler, S., El Saghire, H., Gomolka, M., Guertler, A., Harms-Ringdahl, M., Jeggo, P., Kreuzer, M., Laurier, D., Lindholm, C., Mkacher, R., Quintens, R., Rothkamm, K., Sabatier, L., Tapio, S., de Vathaire, F., Cardis, E., 2012. Ionizing radiation biomarkers for potential use in epidemiological studies. Mutat. Res. 751, 258–286. doi:10.1016/j.mrrev.2012.05.003

Russo, C., Rocco, L., Morescalchi, M.A., Stingo, V., 2003. Assessment of environmental stress by the micronucleus test and the Comet assay on the genome of teleost populations from two natural environments. Ecotox. and Environ. Safety 57, 168–174. doi:10.1016/S0147-6513(03)00027-7

Sabharwal, R., Verma, P., Syed, M. A., Sharma, T. Subudhi, S. K., Mohanty, S., Gupta, S., 2015. Emergence of micronuclei as a genomic biomarker. Indian journal of medical and paediatric oncology : official journal of Indian Society of Medical & Paediatric Oncology, 36, 212-218. doi: 10.4103/0971-5851.171541

Skidmore, J.F., 1965. Resistance to zinc sulphate of the zebrafish (Brachydanio rerio Hamilton-Buchanan) at different phases of its life history. Ann Appl Biol. Aug; 56 (1): 47-53. doi:10.1111/j.1744-7348.1965.tb01214.x

Song, Y., Salbu, B., Heier, L.S., Teien, H.-C., Lind, O.-C., Oughton, D., Petersen, K., Rosseland, B.O., Skipperud, L., Tollefsen, K.E., 2012. Early stress responses in Atlantic salmon (Salmo salar) exposed to environmentally relevant concentrations of uranium. Aquat. Toxicol. 112, 62–71. doi:10.1016/j.aquatox.2012.01.019

Strand, P., Aono, T., Brown, J.E., Garnier-Laplace, J., Hosseini, A., Sazykina, T., Steenhuisen, F., Vives i Batlle, J., 2014. Assessment of Fukushima-Derived Radiation Doses and Effects on Wildlife in Japan. Environ. Sci. Technol. Lett. 1, 198–203. doi:10.1021/ez500019j

UNSCEAR, 1996. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) Report. United Nations, New York.

UNSCEAR, 2008. United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) Report to the General Assembly, with Scientific Annexes. United Nations, New York

Won, E.-J., Dahms, H.-U., Kumar, K.S., Shin, K.-H., Lee, J.-S., 2015. An integrated view of gamma radiation effects on marine fauna: from molecules to ecosystems. Environ. Sci. Pollut. Res. Int. 22, 17443–52. doi:10.1007/s11356-014-3797-4

Supplementary material

Paper IV

Table A1. Number of embryos produced per breeding pair in each breeding week and in all breeding weeks together in zebrafish exposed to 8.7 and 53 mGy/h gamma radiation for 27 days. Results presented as mean ± SEM.

Week	Number of embryos produced per breeding pair in the breeding weeks (mean ± SEM)					
	One month after exposure			One year after exposure		
	Control	8.7 mGy/h	53 mGy/h	Control	8.7 mGy/h	53 mGy/h
1	68.2 ± 62.8	131.3 ± 102.5*	24.5 ± 27.2	5.5 ± 5.5	6.2 ± 4.2	0
2	283 ± 218.7	162.8 ± 126.2*	42.3 ± 37.1*	6.5 ± 6.5	24.8 ± 12.2	0
3	226.8 ± 176.2	113.6 ± 87.8*	53.2 ± 44.9*	62.3 ± 24.6	57.3 ± 20.4	0*
4	196.8 ± 153.7	82 ± 63.4*	43.8 ± 36.4*	133.8 ± 32.8	49.8 ± 19.8*	0*
5	52.3 ± 41.1	81.3 ± 64.2	43 ± 35.7	61.5 ± 25.1	47.8 ± 16.3	11.6 ± 11.6
6	134.6 ± 104.1	74.3 ± 57.7*	0*			

(*) significant compared to corresponding controls according to Dunnett's test.

Table A2. Percentage of cells distributed by grade of DNA damage in zebrafish blood from controls and after exposure to gamma radiation (n=1200).

Dose rate (mGy/h)	Sex	DNA damage criteria				
		Minimal	**Low**	**Mid**	**High**	**Extreme**
	M	92.2	7.5	0.0	0.2	0.1
Control	**F**	91.8	7.3	0.6	0.1	0.2
	M	62.6	30.2	7.1	0.1	0.1
8.7	**F**	85.7	11.1	3.1	0.2	0.0
	M	75.1	18.5	5.9	0.3	0.2
53	**F**	70.3	24.6	4.4	0.3	0.4

Errata

Thesis title:	Two-generational study of biological effects from gamma radiation exposure during sensitive life stages in the zebrafish (*Danio rerio*)

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