

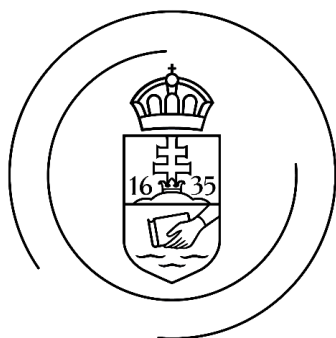
The effects of ionizing radiation on *tp53* deficient zebrafish embryos

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List of abbreviations

AB: One of the wild-type zebrafish strains

Axio Observer Z1: A model of Zeiss inverted fluorescent microscope

AxioCam MRm: A specific Zeiss camera module

DNA: Deoxyribonucleic acid

dpf: Days post-fertilization

dpi: Days post-irradiation

E3: Embryo medium

EBT3: Gafchromic *EBT3* self-developing dosimetry *film*

ELI-ALPS: Extreme Light Infrastructure - Attosecond Light Pulse Source

ELTE: Eötvös Loránd University

G1/S: A cell cycle restriction point

ggplot2: A graphical package for the programming language R

Gy: Gray, the unit of ionizing radiation

hpf: Hours post-fertilization

LD₅₀: The lethal dose required to kill 50% of the embryos

MDM2: An important negative regulator protein of the tp53

MIR-154-PA: A model of incubator

PBS: Phosphate buffered saline

PMMA: Polymethyl methacrylate

PTW TM30013: A model of Waterproof Farmer Chamber

qPCR: Quantitative polymerase chain reaction

R: A programming language

RecQ: A family of helicase enzymes

RS320: The model of the irradiation chamber

tp53: An important tumor suppressor gene

tp53^{M214K}: A specific mutation of the zebrafish *tp53* gene

Summary

Ionizing radiation is a well-documented source of DNA damage. Prolonged exposure to such radiation can have profound consequences on our health, therefore it is important to study the molecular consequences of short- and long-term radiation using model organisms. We have exposed embryos of two different strains of zebrafish (*Danio rerio*) to 250 keV X-ray irradiation and performed subsequent survival, and malformation analysis. We used the wild-type AB strain as a control and an already well-studied *tp53* deficient strain (*tp53^{M214K}*). Tp53 is one of the most important tumor suppressor proteins, frequently mutated in human cancers. It has a pivotal role in the regulation of the cell cycle and apoptosis, depending on the physiological state of the cell. Because of its small size, fast development and low associated cost zebrafish is an exceptionally good model to produce relatively high throughput data, therefore it is an ideal candidate for our assays. We observed wild-type and *tp53* mutant fish embryos and larvae for four days after being treated with different doses of X-ray source and recorded daily their survival rates and the different malformations (e.g. abnormal curvature of the spine and the appearance of pericardial edema) that arose as a result of the intervention. The experimental paradigm that we developed can be used later to study the effects of ionizing radiation on mutations of other genes, thought to be essential in genome maintenance and DNA repair as well.

Introduction

As a brief introduction, I would like to say a few words about the ionizing radiation used as a DNA-damaging factor in our research, the zebrafish model system we chose, and our gene of interest *tp53*. When we talk about ionizing radiation (Wavelength below 800 nm) we mean the flow of particles or photons of electromagnetic radiation whose energy is high enough to strip electrons from atoms in their paths (Havránková, 2020) in contrast to non-ionizing radiations, which is only capable to move the electrons of the atoms to a higher energy level. In terms of the cells these effects can cause different mutation events, which can lead to cancerous changes if, for example, various tumor suppressors are involved, or in more intense cases they can even result in cell death. Electromagnetic ionizing radiation is divided into two different categories, X-rays and gamma rays. Both are from the same part of the electromagnetic spectrum and differ only in their origin. While X-rays originate from the interactions of electrons in the electron shells of atoms, gamma rays are the result of nuclear reactions. Therefore, gamma radiation, as well as particle radiations such as alpha, beta, proton, neutron or heavy ion, is usually released when radioactive isotopes decay, in which their unstable nuclei undergo changes and strive to reach a more stable form (Hall & Giaccia, 2006; Prasad, 2020). 250 keV X-ray radiation was used during the tests we performed, which is a well-established clinical standard.

In recent times, the zebrafish has become a widely used model organism in various preclinical investigations, including genetic, pharmacological, and developmental studies. The first research that used this species as a model organism dates back to the 1960s, when George Streisinger proposed this species as an animal model for basic research based on its embryonic and genetic characteristics (Grunwald & Eisen, 2002). The zebrafish is a freshwater teleost fish native to Southeast Asia where it can be found in shallow lakes and rice fields (Spence et al., 2007). This species is characterized by rapid embryonic development, adults are able to reproduce just after 3 months of age and show well defined sexual dimorphism: males are torpedo-shaped, pinkish or yellowish in color, females are less pinkish with an enlarged abdomen due to the presence of the eggs (Singleman & Holtzman, 2014). Their relatively simple genetic modifiability, small size, easy sustainability, transportability, breeding, the associated low cost, as well as the optical transparency of the embryos and many other beneficial properties make the zebrafish a very useful model organism for medical biology research (Parker et al., 2012; Rico et al., 2011; Stewart et al.,

2015). The zebrafish is also an excellent alternative organism for modeling human diseases because it has a well-characterized, conserved genome (Barbazuk et al., 2000; Howe et al., 2013) and in key developmental processes, cell cycle progression, proliferation, and differentiation many of the genes are conserved between the two species. Approximately 70% of zebrafish genes have at least one human ortholog, and of these almost 50% have a one-to-one relationship with their human counterpart (Howe et al., 2013). Taking all these into account, it is not surprising that lately zebrafish has become a very popular vertebrate model species, as it has many advantages over traditional animal models, not to mention experiments can be carried out with them for 5 days after fertilization (until independent feeding begins) without any animal ethics approval. Due to their size (0.5-1 mm), embryos grow easily in 96-well plates, which facilitates their research use (Parng et al., 2002), and the applicability of a large number of embryos obtained during one hatching provides relevant statistical results. Most organs, such as the eyes, brain, heart, liver, muscles, bones and the gastrointestinal system, undergo rapid embryonic development which takes place completely in almost 48 hours (Rico et al., 2011). The zebrafish fully possesses the radiobiologically important organs specific to vertebrates, including the brain, eyes, spinal cord, vascular system, digestive system, and is therefore an effective and versatile model for evaluating the effects of ionizing radiations on viability, organ and tissue development and the detection of organ disorders (Geiger et al., 2006). Embryos do not require sterile conditions like cell cultures and are therefore more suitable for radiobiological studies performed at non-hospital research radiation sources.

TP53 is one of the most important tumor suppressor proteins, frequently mutated in human cancers, so much so that more than half of solid tumors are harboring mutations in the *TP53* locus (Storer & Zon, 2010) and it also plays a particularly important role both in the regulation of the cell cycle and apoptosis. It is generally believed that the TP53 protein is initially activated by different stress sources before attaching to its consensus DNA-binding motif as a tetramer and activating its target genes transcriptionally. The TP53 tetramer's transactivation function is thought to be at least partially lost if just one of the four protein subunits is altered in the DNA binding domain. As a result, mutant TP53 proteins can limit the activity of wild-type proteins in a dominant-negative manner. In a healthy individual, TP53 undergoes constant formation and degradation. The breakdown is linked to MDM2. As part of a negative feedback loop, TP53 induces MDM2 function. Thus, mutant TP53 does not induce it, thereby enabling its high enrichment (Blagosklonny, 2002). Zebrafish *tp53* is orthologous to its mammalian counterpart in both structure and function. To be precise, 48%

of its amino acid sequence is identical to its human equivalent (Cheng et al., 1997). The strain we used in our research was *tp53*^{M214K} which is an already well-studied *tp53* allele (the fish we included in our experiments were homozygous to this mutation). Its deficiency results from the dysfunction of the DNA binding domain of the protein, which is impacted by a point mutation which causes an amino acid change at position 214 (methionine to lysine). Fish with this mutation develop tumors with a much higher incidence than their wild-type kinds, and in the event of a DNA-damaging stimulus, the rate of apoptosis was significantly reduced compared to what would normally be expected (Storer & Zon, 2010).

Aims

The experiments we conducted had two main goals. First, we wanted to gather more information about the role of Tp53 during repair mechanisms triggered by different levels of DNA damage caused by X-ray irradiation. The other cornerstone of our endeavors was to develop an experimental paradigm that can be used later to study the effects of ionizing radiation on mutations of other genes, thought to be essential in genome maintenance and DNA repair as well. Hence the main importance of this work is setting the stage for future experiments by creating a detailed experimental protocol, but the data collected during my experiment could also be informative in larger context due to the high degree of homology between the human *TP53* and zebrafish *tp53* genes. Due to TP53's important role in various cancerous diseases, the information obtained during the experiments could potentially even be of clinical importance. We have already started the preparations in order to repeat the previous experiments in the near future with different RecQ helicase mutant strains and with another new *tp53* deficient one that we created in-house. We also want to extract more quantitative information in the future from our experiments, such as body length, eye and yolk sac diameter with the help of automated image analyses.

Materials and methods

Embryo harvesting and maintenance

For our experiments, wild-type AB zebrafish (*Danio rerio*) embryos and an already well-studied *tp53* deficient strain (*tp53^{M214K}*) at the age of 24-hour post-fertilization (hpf) were used. All procedures were planned in compliance with EU Directive 2010/63/EU on the protection of animals used for scientific purposes, therefore every experiment was carried out up to five days post-fertilization (dpf), until this time point they are not able to feed independently, therefore no ethical approval is required for their use with experimental intent. Wild-type and *tp53* mutant fish lines were maintained in the animal facilities of ELTE Eötvös Loránd University according to standard protocols at 28.5°C on a 14 h light/10 h dark cycle. Two days before embryos were required for the planned experiments, the adults were sorted into embryo breeding tanks. Females and males were transferred into the tank separately with dividers a few hours before the end of their light period. At the beginning of the next light cycle, after the removal of the divider the fish bred. After breeding the adult fish were returned into their tanks. The embryos were collected with the help of a small strainer and an E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, 0.1% methylene blue) (Brand et al., 2002) filled plastic wash bottle from the bottom of the tank. Afterwards, the embryos were washed and sorted into E3 medium filled falcon tubes, then transported for irradiation between 21 and 22 hpf under the appropriate temperature conditions. For irradiation, the embryos were randomly allocated to the treatment groups and were pipetted in 96-well plates, which were filled with 200 µl E3 medium. The time point of final assessment was 120 hpf when the embryos were fixed in 4% paraformaldehyde containing PBS solution for subsequent histological analysis.

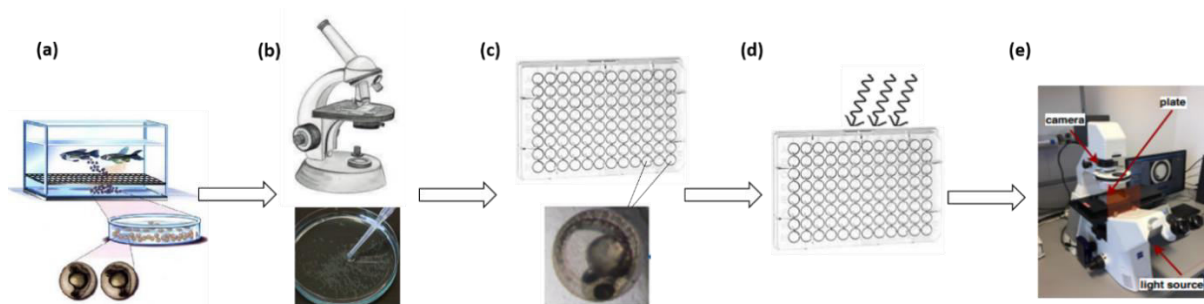


Figure 1. Experimental procedure of a zebrafish irradiation study. (a) Zebrafish adults spawning and embryo collection into petri dish. (b) Selection of the viable and unfertilized embryos under stereo microscope. (c) The different types of 24 hpf zebrafish embryos are sorted in 96 well plates (1 embryo/well) (d) and irradiated with X-ray. (e) The viability and the morphological abnormalities monitored daily using an inverted microscope. Representative photomicrographs taken with a camera (Lammer et al., 2009).

Radiation exposure

Wild-type and mutant embryos in the pharyngula period (24 hpf) (Kimmel et al., 1995) were exposed to doses of 0–20 Gy at increasing dose levels with 250 keV energy X-ray radiation beam (RS320, Xstrahl Limited) at the Extreme Light Infrastructure Attosecond Light Pulse Source (ELI-ALPS) Research Institute (Figure 2 (a)). Embryos in 96 well plates were placed into a special polymethyl methacrylate (PMMA) slab phantom (Figure 2(b, c)) (Polanek et al., 2020), to compensate for the built-up effect and to ensure the homogenous radiation exposure and the isocenter were positioned at the geometrical centers of the plates.

The delivered doses (0, 5, 10, 15, 20 Gy) were verified by performing dosimetry measurements using a calibrated ionization chamber (Farmer type ionization chamber–PTW TM30013), and film dosimetry (Gafchromic EBT3 self-developing dosimetry film). For each irradiated plate, dosimetry control measurements were performed using Gafchromic EBT3 films, which were positioned under the 96 well plate.

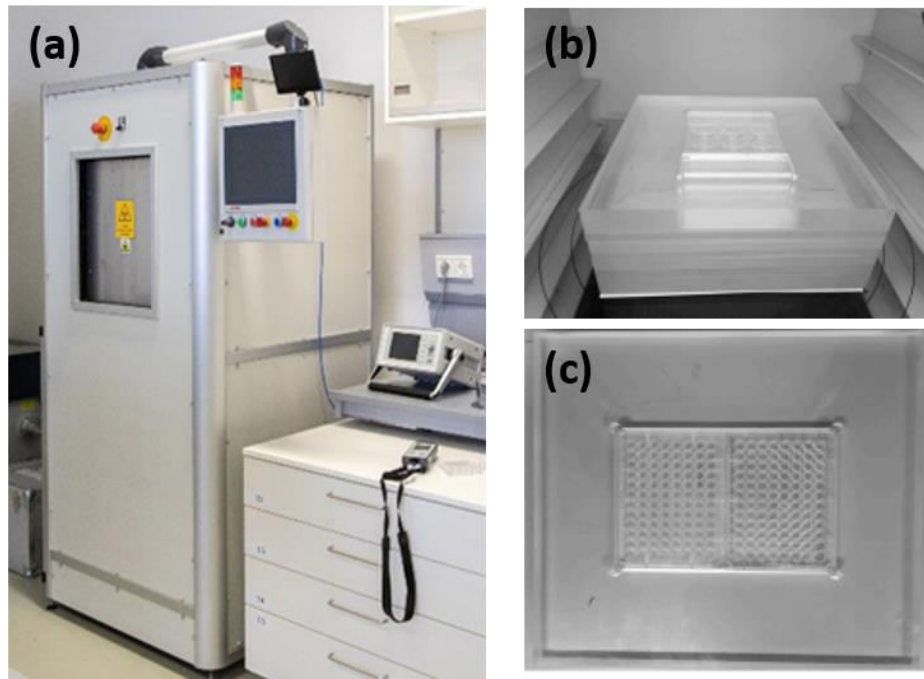


Figure 2. Setup and equipment for zebrafish embryo irradiation. (a) Xstrahl X-ray small animal irradiator system. (b) PMMA slab phantom with thickness of 2.5 cm with a 15 cm x 15 cm x 1.5 cm deep hollow in the middle. (c) PMMA sheet with 96 well plates inserted which contains the biological samples during the irradiation period (Polanek et al., 2020).

Survival and morphology analysis

Immediately after irradiation the viability of the embryos were checked using an inverted microscope (Axio Observer Z1, Zeiss), to evaluate the daily survival and morphology and afterwards they were maintained under normal conditions (28°C) in an incubator (MIR-154-PA, Panasonic). The embryo medium was changed every second day during the observational period, until 4 days post-irradiation (dpi).

The developmental status, the hatching rate and the survival of the embryos, were continually assessed in each experiment at 24-hour intervals from the point of fertilization until 120 hpf. Embryos were observed without any manipulation in place in the microplates and the morphological abnormalities, i.e., spine curvature and pericardial edema (Figure 3. (b)), were evaluated and monitored using a Zeiss Observer Z1 microscope (magnification: 25x). At 96 hpf and 120 hpf (3rd and 4th days of observation) representative images were acquired from the surviving and malformed embryos for quantitative morphological assessment using an AxioCam MRm at a Zeiss Observer Z1 microscope under a magnification of 25x. The criterion of embryonic survival was the presence of cardiac contractility and blood circulation.

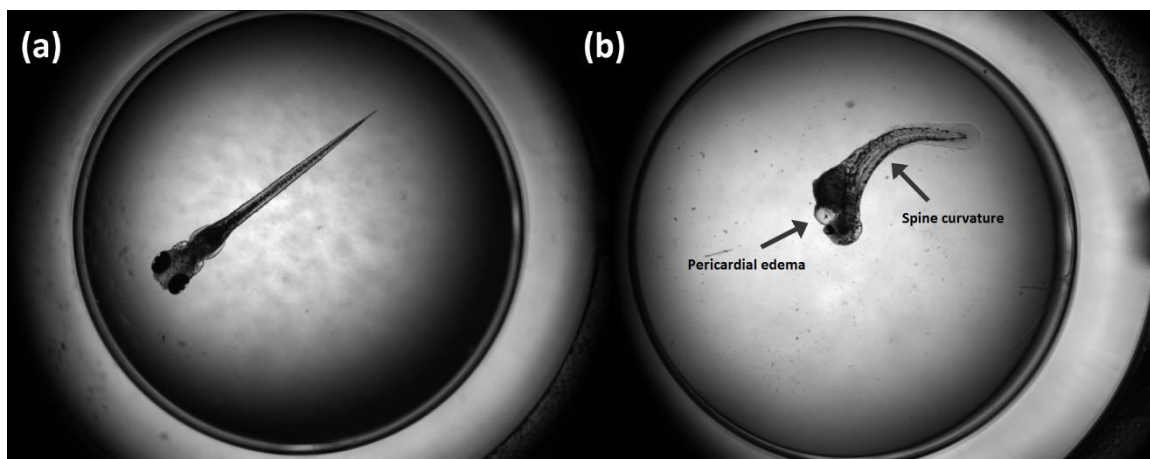


Figure 3. Malformations examined during the experiment. (a) 4 dpf control AB larva with no visible malformations. (b). Irradiation treated (20 Gy) 4 dpf AB larva, with pericardial edema and abnormal spine curvature.

Statistical analysis

The reported results represent the mean of at least two independent experiment replications i.e. embryos collected from different clutches on different days. Individual replications contained at least 50 embryos per dose group of 0 Gy, 5 Gy, 10 Gy, 15 Gy, 20 Gy. The hatching rate was detected on each day of the experiment series. It was calculated as a percentage of viable hatched embryos to the total number of embryos exposed in each treatment group over time. Survival rates were calculated similarly but for all viable embryos not just the already hatched ones. For graphical representation of the average survival rates, determined as mean value of the experiment replications, the ggplot2 package of the programming language R was applied. Individual survival levels, i.e. effects of different doses used on the same strains as well as the same doses but different zebrafish strains, were compared by using the Log-Rank test.

The morphological abnormalities like pericardial edema and spine curvature were evaluated daily in the proportion of the living embryos. The observed morphological abnormalities were quantified relating the number of malformed living embryos to the total number of living embryos at the corresponding day post irradiation. In doing so, the two abnormalities were considered individually, whereas the parallel appearance of both types of malformations was also recorded. Statistically significant differences are indicated for $p < 0.05$.

Visualization

After evaluating the data collected during the microscopic observations, we created spreadsheets out of them and then plotted them using the graphic package for the R programming language called ggplot2. For the line diagrams that represents our hatching data, and for the stacked bar plots that depicts our malformation data we used the tidy format and for the Kaplan-Meier survival plots we used the data structure type with the same name.

Results

Hatching analysis

To represent our hatching results, we used line diagrams (Figure 4). Based on the data, no statistically significant particular trend could be observed depending on the change in radiation dosage or between the different genotypes. For example, while small differences could be observed between different treatments, in the case of the controls we could not observe a clear trend (i.e. higher levels of radiation resulting in less hatching). Of note, in the case of the *tp53* mutants the largest level of radiation (20 Gy) resulted in somewhat delayed hatching. In general hatching was more or less complete by around 3 dpf.

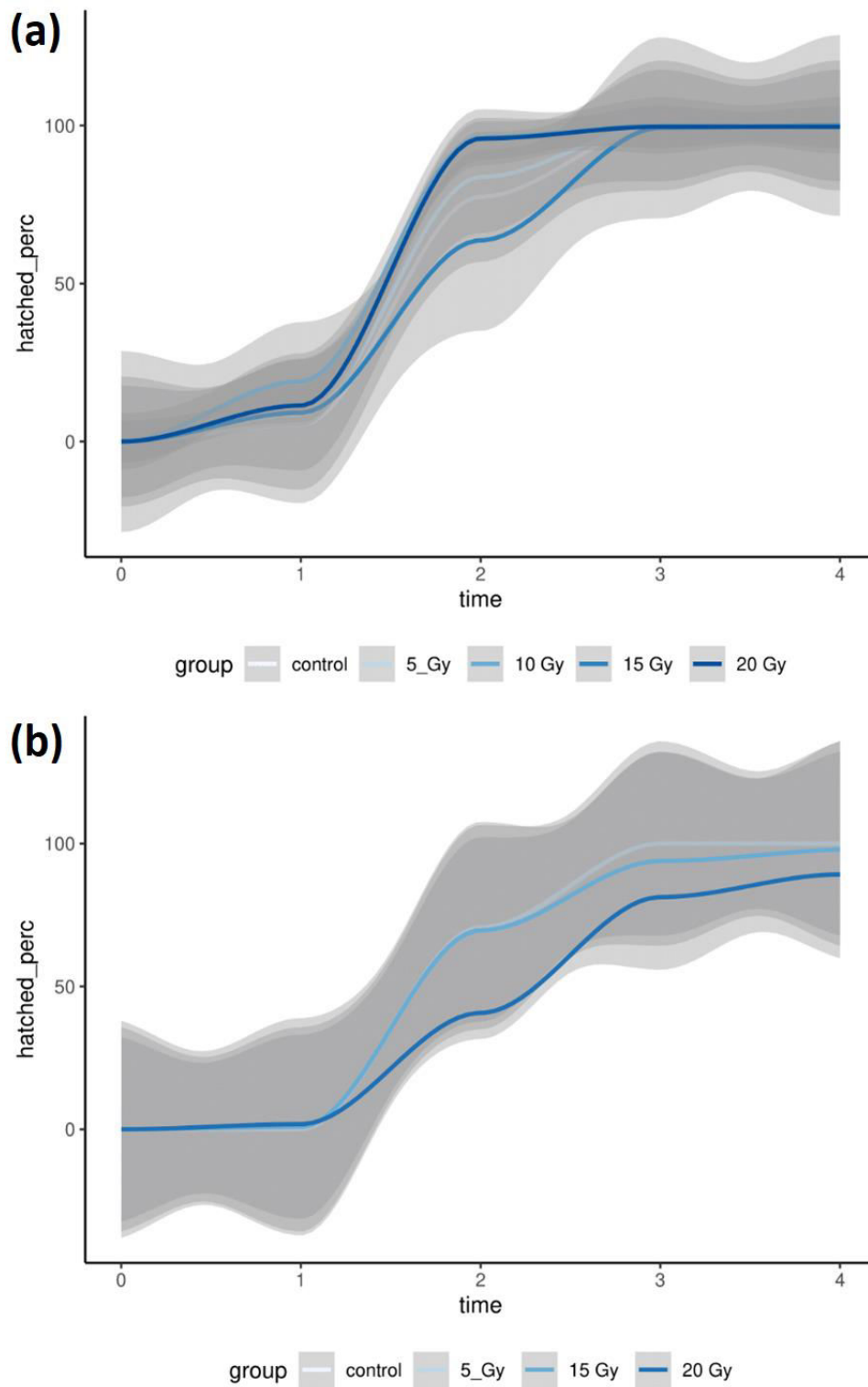


Figure 4. Line diagrams comparing the hatch rate of the different dosage groups by strains. The percentage hatch rates of different days and dosage groups for the wild-type AB strain are shown in graph (a), and for the Tp53 deficient *tp53*^{214K} strain in graph (b). The gray zones surrounding the lines represents the standard deviation of the data points.

Survival Analysis

We used Kaplan-Meier diagrams to represent our survival data (Figure 5 and 6). To statistically validate our data, we applied pairwise comparisons using the Log-Rank test. The number of surviving embryos was recorded daily for 4 subsequent days, starting immediately after irradiation. Dose dependent survival curves (Figure 5) were obtained correlating the number of living embryos of the respective day to the number of embryos irradiated. Based on the performed tests, we can clearly see that by increasing the dose, the death rate also increases (Table 1). When using the highest dose (20 Gy) we can detect a significant difference ($p = 0.012$) in survival between the two strains. It is also noteworthy that for 15 and 5 Gy a difference between the two strains is also apparent from the plots, with mortality being higher in the *tp53* mutant fish. In general, on embryonic survival for doses lower than 15 Gy no significant impact of irradiation was observed at the first two observational days. Survival rates declined from the 3rd dpi, and the survival was 90–100% for sham irradiated embryos.

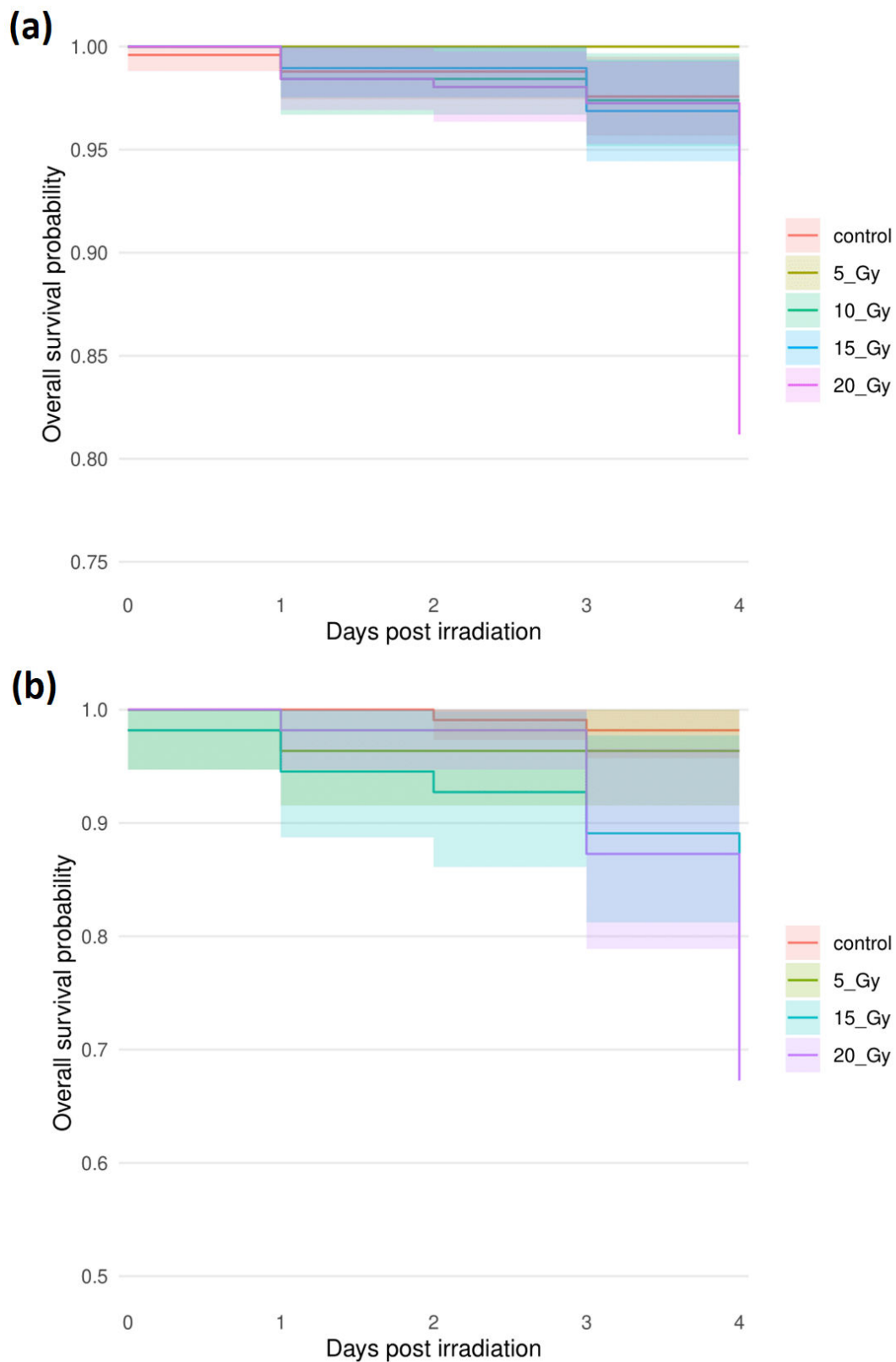


Figure 5. Kaplan-Meier graphs comparing the survival rate of the different dosage groups by strains. The survival rates of different days and dosage groups for the wild-type AB strain are shown in graph (a), and for the *tp53* deficient *tp53*^{214K} strain in graph (b) of the figure. The colored zones surrounding the lines represents the standard deviation of the data points.

Genotype	Doses	Control	5 Gy	10 Gy	15 Gy
AB	5 Gy	0.13928	-	-	-
	10 Gy	0.90309	0.13928	-	-
	15 Gy	0.07897	0.02557	0.12506	-
	20 Gy	0.000000061	0.000019	0.0000015	0.00044
<i>tp53</i>	5 Gy	0.4659	-	-	-
	15 Gy	0.0067	0.1059	-	-
	20 Gy	0.000000062	0.0004	-	0.0356

Table 1. P-values of the pairwise comparisons of the survival rates of the different dosage groups by strains using the Log-Rank test. The p values for the comparison of different wild-type dose group pairs are shown in the top part of the table, and for the *tp53* deficient *tp53*^{214K} strain in the bottom part of the table. P-values written in red are significant. The original output of the statistical software can be found in the supplementary (Supplementary table 1)

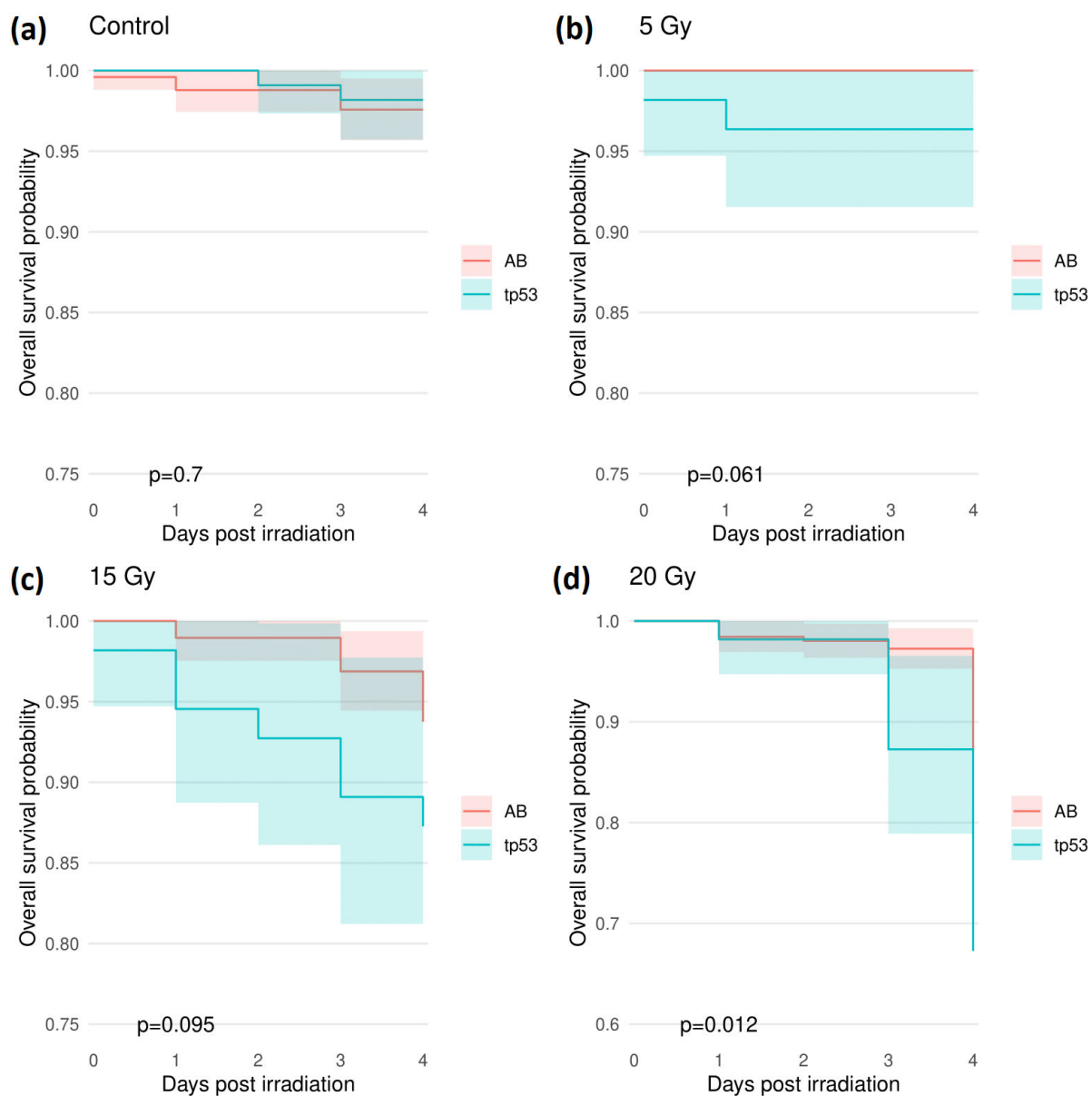


Figure 6. Kaplan-Meier graphs comparing the survival rate of the different strains by dose groups. The survival rates of different days and strains for the control group are shown in graph (a), for the 5 Gy group in graph (b), for the 15 Gy group in graph (c), and for the 20 Gy group in graph (d) of the figure. The colored zones surrounding the lines represents the standard deviation of the datapoints and at the bottom of each plot we can see the relevant p-values as well.

Malformation Analysis

To represent our malformation results, we chose stacked bar charts (Figure 7). The presence of pericardial edema and spine deformations was investigated in parallel to the observation of embryonic mortality on each day post irradiation. At 1 dpi, there were just a few malformations which appeared in the group irradiated with 20 Gy. The number of malformations rose from day to day, but again, doses below 15 Gy were less efficient in inducing malformations within 4 dpi. At 2 dpi regardless of the type of the embryos irradiated at 15 Gy and higher dose level we detected that the spine curvature appeared earlier as one of the acute reactions after irradiation. We could only observe a smaller but significant amount of the cooccurrence of the two assessed malformations in the *tp53* 20 Gy group. In the first two days of the analysis the presence of the chorion made it somewhat difficult to evaluate the malformations, in contrast to 3 and 4 dpi when almost 100% of the embryos were hatched, and the spine as well as the pericardial region were clearly visible and could be observed properly. On the 3rd observational day, the number of individuals suffering from both disorders increased in the group irradiated with 20 Gy especially in case of the *tp53* strain. On the same day post treatment, this tendency could be observed in the *tp53* strain irradiated with 15 Gy, where in the case of the AB strain pericardial edema was observed in the majority of the malformed individuals. Compared to the third day, on the last observational day in the groups treated with 15 Gy the number of embryos suffering from some disorder has increased significantly. At the same observational time point, in the groups irradiated with 20 Gy depending on strains we could not observe any normal individual, almost all embryos exhibited a curved spine and also suffered from pericardial edema which also indicates the deterministic inflammation effect. Regarding the abundance of the assessed malformation types only dose dependent changes were observed (Table 2), we did not find any difference between the two examined genotypes during our experiments (Table 3).

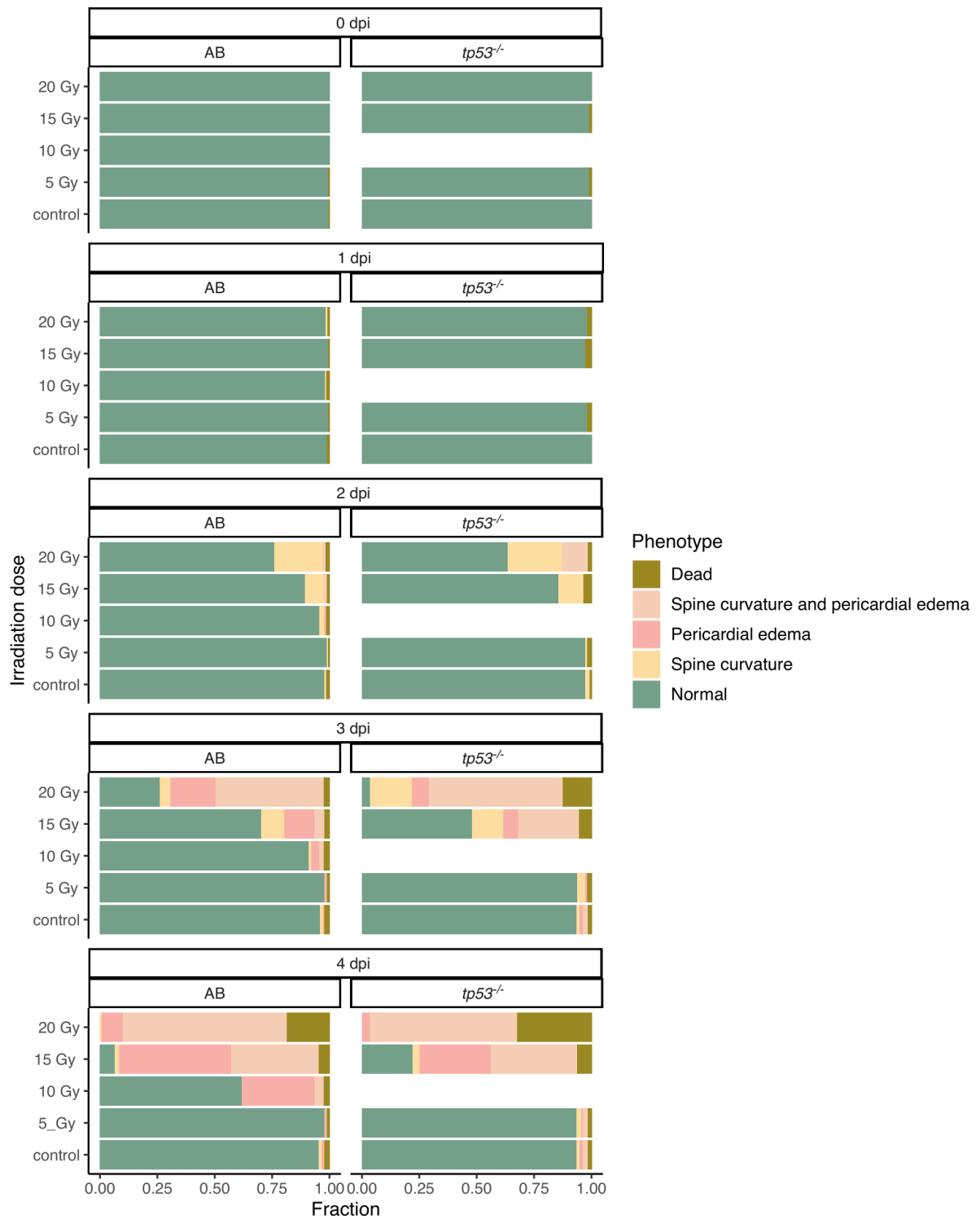


Figure 7. Stacked bar charts comparing the occurrence of the examined malformations of the different dosage groups by strains and days post irradiation. The percentage malformation rates of different days and dosage groups for the wild-type AB strain are shown on the left (dpi is marked with the number over the genotype), and for the *tp53*^{214K} strain on the right. The examined malformations were the pericardial edema, abnormal curvature of the spine, and the cooccurrence of the two.

	AB	<i>tp53</i>
0 dpi	NA	NA
1 dpi	NA	NA
2 dpi	0.00008372	NA
3 dpi	$< 2.2 \cdot 10^{16}$	$< 2.2 \cdot 10^{16}$
4 dpi	$< 2.2 \cdot 10^{16}$	$< 2.2 \cdot 10^{16}$

Table 2. Table containing the p-values obtained during the statistical analysis of the malformation data of the different dose groups. Here we also used Pearson's Chi-squared test to evaluate the likelihood of the possibility that any observed difference between the malformation data of the different dosage groups aroused by chance. In the AB column we can see the p-values from the statistical analysis of the wild-type fish, and in the *tp53* column the p-values from statistical analysis of the mutant fish. P-values written in red are significant. The original output of the statistical software can be found in the supplementary (Supplementary table 2)

	Control	5 Gy	15 Gy	20 Gy
0 dpi	NA	NA	NA	NA
1 dpi	NA	NA	NA	NA
2 dpi	NA	NA	NA	NA
3 dpi	0.6669	0.4672	0.001564	0.00005172
4 dpi	0.7416	0.6401	0.05881	NA

Table 3. Table containing the p-values obtained during the statistical analysis of the malformation data of the two examined genotypes. We used Pearson's Chi-squared test to evaluate the likelihood of the possibility that any observed difference between the malformation data of the two examined genotypes aroused by chance. P-values written in red are significant. The original output of the statistical software can be found in the supplementary (Supplementary table 3)

Discussion

Zebrafish (*Dario rerio*) embryos nowadays are widely used to study the different aspects of ionizing radiation, like radio-adaptive responses (Freeman et al., 2014), and to test the effects of radiation modifying agents (McAleer et al., 2005; Szabó et al., 2016). Furthermore, the zebrafish embryo also represents a robust and simple alternative *in vivo* model for the assessment of radiation effects (Barriuso et al., 2015; Stern & Zon, 2003; Szabó et al., 2018) as well as it was utilized to demonstrate the applicability of the novel therapeutic approaches like FLASH radiotherapy, which is defined as a single ultra-high dose-rate (≥ 40 Gy/s) radiotherapy (Beyreuther et al., 2019). The previous studies demonstrate that this novel vertebrate model can be used to assess the biological effects of ionizing radiation, nevertheless the present work aimed to study the radiation sensitivity on different types of zebrafish embryos. Consistent with the previous observations, the hatching rates observed in this study after irradiation during pharyngula period show no significant dependence on radiation dose (Freeman et al., 2014), just a few exceptions were published where the authors observed lower hatching rates two days after irradiation of 24 hpf embryos with 1 Gy of ^{137}Cs photons (Hu et al., 2016; Li et al., 2018). In the previous experiments where the specimens were observed for a longer period of time, for example the 7 dpi study by Szabo et al. 2018, 20 Gy MV photon exposure was required to notice significantly higher lethality. In addition to the survival rates, the analysis of morphological abnormalities revealed that doses higher than 10 Gy are also required to trigger the appearance of pericardial edema and spinal curvature within the follow up time of four days. There are just a few studies that use *tp53* mutated *in vivo* strains to study the effects of ionizing radiation, consequently, by studying this area, we can potentially obtain useful information even from a clinical point of view. When the *tp53* gene is damaged, tumor suppression is significantly impaired. Individuals who inherit only one working copy of the *TP53* gene are more likely to develop a tumor. *TP53* can also be damaged in cells due to mutagenic effects, chemicals, radiation or viruses. Some form of mutation of *TP53* can be detected in more than 50% of human tumors. From the performed experiments, it is clear that with the increase of the irradiation dose, the death rate increases to a greater extent in the case of *tp53* deficient embryos compared to their wild-type counterparts. Despite extensive research into *tp53*, uncertainties about its role and associated downstream biochemical pathways still exist (Berghmans et al., 2005), but several mechanisms may be behind the observed trends. It is possible that the mortality of these

embryos increases to a greater extent with the increase of the dose, because the mutation blocks the *in vivo* Tp53-dependent apoptotic response and thus the cells are undergoing necrosis instead, which can be a potential reason for the lower survival. It is also conceivable that the background of the phenomenon is that, with the loss of Tp53, the cells are unable to stop at the G1/S cell cycle restriction point after DNA damage, so the cells do not have the opportunity to correct the accumulated mutations. Another possibility is that because of the fact that Tp53 promotes cell survival by inducing DNA repair, and other pro-survival pathways (Helton & Chen, 2007), embryos with a malfunctioning copy of the protein are hindered in these endeavors. In the near future, it would definitely be worthwhile to support these results by performing certain molecular experiments. For this reason, we have already stored samples on which we would like to perform apoptosis tests and qPCR reactions. With their help, we can potentially get closer to understanding what molecular processes may be behind the observed trends.

In conclusion, the present work validates the general applicability of different types of zebrafish embryos as an alternative small vertebrate model for studying the effects of ionizing radiation. The qualitative endpoints like hatching, survival and malformations can be used to describe the general impact of radiation on the whole organism, whereas more detailed endpoints are required to assess radiation damage to individual organs or on basis of molecular pathways.

Perhaps the use of other structured and comprehensive methods can help unravel the mechanisms underlying the indicated threshold dose of embryonic survival and the occurrence of morphological abnormalities. Extending the observation time is desirable and may produce different results in terms of dose dependence. To define the LD₅₀ (the lethal dose required to kill 50% of the embryos) a higher dose is required.

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Supplementary

(a) Pairwise comparisons using Log-Rank test

data: AB_rad_lifespan and dose

	control	5_Gy	10_Gy	15_Gy
5_Gy	0.13928	-	-	-
10_Gy	0.90309	0.13928	-	-
15_Gy	0.07897	0.02557	0.12506	-
20_Gy	6.1e-08	1.9e-05	1.5e-06	0.00044

P value adjustment method: BH

(b) Pairwise comparisons using Log-Rank test

data: tp53_rad_lifespan and dose

	control	5_Gy	15_Gy
5_Gy	0.4659	-	-
15_Gy	0.0067	0.1059	-
20_Gy	6.2e-08	0.0004	0.0356

P value adjustment method: BH

Supplementary table 1. The original output of the R statistical software from the pairwise comparisons of the survival rates of the different dosage groups by strains using the Log-Rank test. The p values for the comparison of different wild-type dose group pairs are shown in part (a) of the figure, and for the *tp53* deficient *tp53*^{214K} strain in part (b) of the figure.

(a)

```
[1] "Genotype: AB"
[1] "Time point: 0 days post irradiation"
      normal dead tg tp tpg
control    82 0.33 0 0 0
5_Gy       82 0.67 0 0 0
10 Gy      96 0.00 0 0 0
15 Gy       83 0.00 0 0 0
20 Gy       85 0.00 0 0 0

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = NaN, df = 16, p-value = NA

[1] "Genotype: AB"
[1] "Time point: 1 days post irradiation"
      normal dead tg tp tpg
control    82 1.00 0.00 0 0
5_Gy       82 0.67 0.00 0 0
10 Gy      94 1.50 0.50 0 0
15 Gy       82 0.67 0.00 0 0
20 Gy       84 1.00 0.33 0 0

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = NaN, df = 16, p-value = NA

[1] "Genotype: AB"
[1] "Time point: 2 days post irradiation"
      normal dead tg tp tpg
control    81 1.00 0.67 0.00 0.33
5_Gy       81 0.67 0.33 0.00 0.00
10 Gy      92 1.50 2.50 0.50 0.00
15 Gy       74 1.00 6.67 0.33 1.00
20 Gy       64 1.67 18.00 0.00 1.00

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = 46.423, df = 16, p-value = 8.372e-05

[1] "Genotype: AB"
[1] "Time point: 3 days post irradiation"
      normal dead tg tp tpg
control    79 2.0 1.3 0.33 0.00
5_Gy       81 1.0 0.0 0.33 0.33
10 Gy      87 2.5 1.5 3.00 2.00
15 Gy       58 2.0 8.3 10.67 3.67
20 Gy       22 2.3 4.0 16.67 40.00

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = 222.05, df = 16, p-value < 2.2e-16

[1] "Genotype: AB"
[1] "Time point: 4 days post irradiation"
      normal dead tg tp tpg
control   78.7 2.0 1.33 0.67 0.00
5_Gy      80.7 1.0 0.00 0.33 0.33
10 Gy     59.0 2.5 0.00 30.50 4.00
15 Gy      5.3 4.0 1.33 40.33 31.67
20 Gy      0.0 16.0 0.33 8.00 60.67

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = 407.08, df = 16, p-value < 2.2e-16
```

(b)

```
[1] "Genotype: tp53"
[1] "Time point: 0 days post irradiation"
      normal dead tg tp tpg
control    55 0.0 0 0 0
5_Gy       54 0.5 0 0 0
15 Gy       54 0.5 0 0 0
20 Gy       55 0.0 0 0 0

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = NaN, df = 12, p-value = NA

[1] "Genotype: tp53"
[1] "Time point: 1 days post irradiation"
      normal dead tg tp tpg
control    55 0.0 0 0 0
5_Gy       53 1.0 0 0 0
15 Gy       53 1.5 0 0 0
20 Gy       54 1.0 0 0 0

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = NaN, df = 12, p-value = NA

[1] "Genotype: tp53"
[1] "Time point: 2 days post irradiation"
      normal dead tg tp tpg
control    54 0.5 1.0 0 0
5_Gy       52 1.0 0.5 0 0
15 Gy       46 2.0 6.0 0 0
20 Gy       35 1.0 13.0 0 6

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = NaN, df = 12, p-value = NA

[1] "Genotype: tp53"
[1] "Time point: 3 days post irradiation"
      normal dead tg tp tpg
control    52 1 0.5 1.0 1
5_Gy       50 1 2.0 0.5 0
15 Gy       26 3 7.5 3.5 14
20 Gy        2 7 10.0 4.0 32

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = 131.34, df = 12, p-value < 2.2e-16

[1] "Genotype: tp53"
[1] "Time point: 4 days post irradiation"
      normal dead tg tp tpg
control    52 1.0 0.5 1.0 1
5_Gy       50 1.0 1.0 0.5 1
15 Gy       12 3.5 1.5 17.0 20
20 Gy        0 18.0 0.0 2.0 35

Pearson's Chi-squared test

data: irradi_malf_table
X-squared = 203.02, df = 12, p-value < 2.2e-16
```

Supplementary table 2. The original output of the R statistical software from the analysis of the malformation data of the different dosage groups. Here we also used Pearson's Chi-squared test to evaluate the likelihood of the possibility that any observed difference between the malformation data of the different dosage groups aroused by chance. In part (a) of the figure we can see the statistical analysis of the wild-type fish, and in part (b) the statistical analysis of the Tp53 deficient fish.

```

[1] "Time point: 0 days post irradiation" [1] "Time point: 1 days post irradiation" [1] "Time point: 3 days post irradiation"
[1] "Group: control" [1] "Group: 20 Gy" [1] "Group: 15 Gy "
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 82 0.33 0 0 0 AB 84 1 0.33 0 0 AB 58 2 8.3 10.7 3.7
tp53 55 0.00 0 0 0 tp53 54 1 0.00 0 0 tp53 26 3 7.5 3.5 14.5

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = NaN, df = 4, p-value = NA X-squared = 17.473, df = 4, p-value = 0.001564

[1] "Time point: 0 days post irradiation" [1] "Time point: 2 days post irradiation" [1] "Time point: 3 days post irradiation"
[1] "Group: 5 Gy " [1] "Group: control" [1] "Group: 20 Gy"
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 82 0.67 0 0 0 AB 81 1.0 0.67 0 0.33 AB 58 2 8.3 10.7 3.7
tp53 54 0.50 0 0 0 tp53 54 0.5 1.00 0 0.00 tp53 2 7.0 10 4 32

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = NaN, df = 4, p-value = NA X-squared = 24.94, df = 4, p-value = 5.172e-05

[1] "Time point: 0 days post irradiation" [1] "Time point: 2 days post irradiation" [1] "Time point: 4 days post irradiation"
[1] "Group: 15 Gy " [1] "Group: 5 Gy " [1] "Group: control"
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 83 0.0 0 0 0 AB 81 0.67 0.33 0 0 AB 79 2 1.3 0.67 0
tp53 54 0.5 0 0 0 tp53 52 1.00 0.50 0 0 tp53 52 1 0.5 1.00 1

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = NaN, df = 4, p-value = NA X-squared = 1.968, df = 4, p-value = 0.7416

[1] "Time point: 0 days post irradiation" [1] "Time point: 2 days post irradiation" [1] "Time point: 4 days post irradiation"
[1] "Group: 20 Gy" [1] "Group: 15 Gy " [1] "Group: 5 Gy "
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 85 0 0 0 0 AB 74 1 6.7 0.33 1 AB 81 1 0 0.33 0.33
tp53 55 0 0 0 0 tp53 46 2 6.0 0.00 0 tp53 50 1 1 0.50 1.00

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = 2.1502, df = 4, p-value = 0.7082 X-squared = 2.5253, df = 4, p-value = 0.6401

[1] "Time point: 1 days post irradiation" [1] "Time point: 2 days post irradiation" [1] "Time point: 4 days post irradiation"
[1] "Group: control" [1] "Group: 20 Gy" [1] "Group: 15 Gy "
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 82 1 0 0 0 AB 64 1.7 18 0 1 AB 5.3 4.0 1.3 40 32
tp53 55 0 0 0 0 tp53 35 1.0 13 0 6 tp53 12.0 3.5 1.5 17 20

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = NaN, df = 4, p-value = NA X-squared = 9.0932, df = 4, p-value = 0.05881

[1] "Time point: 1 days post irradiation" [1] "Time point: 3 days post irradiation" [1] "Time point: 4 days post irradiation"
[1] "Group: 5 Gy " [1] "Group: control" [1] "Group: 20 Gy"
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 82 0.67 0 0 0 AB 79 2 1.3 0.33 0 AB 0 16 0.33 8 61
tp53 53 1.00 0 0 0 tp53 52 1 0.5 1.00 1 tp53 0 18 0.00 2 35

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = 2.3763, df = 4, p-value = 0.6669 X-squared = NaN, df = 4, p-value = NA

[1] "Time point: 1 days post irradiation" [1] "Time point: 3 days post irradiation" [1] "Time point: 4 days post irradiation"
[1] "Group: 15 Gy " [1] "Group: 5 Gy " [1] "Group: 20 Gy"
normal dead tg tp tpg normal dead tg tp tpg normal dead tg tp tpg
AB 82 0.67 0 0 0 AB 81 1 0 0.33 0.33 AB 0 16 0.33 8 61
tp53 53 1.50 0 0 0 tp53 50 1 2 0.50 0.00 tp53 0 18 0.00 2 35

Pearson's Chi-squared test Pearson's Chi-squared test Pearson's Chi-squared test

data: irradi_malf_table data: irradi_malf_table data: irradi_malf_table
X-squared = NaN, df = 4, p-value = NA X-squared = 3.5705, df = 4, p-value = 0.4672

```

Supplementary table 3. The original output of the R statistical software from the analysis of the malformation data of the two examined genotypes. We used Pearson's Chi-squared test to evaluate the likelihood of the possibility that any observed difference between the malformation data of the two examined genotypes aroused by chance.