Coalescence of DNA double strand breaks induced by galactic cosmic radiation is modulated by genetics in 15 inbred strains of mice

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Abstract

In this manuscript we address the challenges associated with the ability to predict radiation sensitivity associated with exposure to either cosmic radiation or X-rays in a population study, by monitoring DNA damage sensing protein 53BP1 forming small nuclear radiation-induced foci (RIF) as a surrogate biomarker of DNA double strand breaks (DSB). 76 primary skin fibroblasts were isolated from 10 collaborative cross strains and five reference inbred mice (C57Bl/6, BALB/CByJ, B6C3, C3H and CBA/CaJ) and exposed to three different charged nuclei of increasing LET (350 MeV/n Si, 350 MeV/n Ar and 600 MeV/n Fe) and X-ray. Our data brings strong evidence against the classic "contact-first" model where DSBs are assumed to be immobile and repaired at the lesion site. In contrast, our model suggests nearby DSBs move into single repair unit characterized by large RIF before the repair machinery kicks in. Such model has the advantage of being much more efficient molecularly but is poorly suited to deal with cosmic radiation, where energy is concentrated along the particle trajectory, inducing a large density of DSBs along each particle track. In accordance with this model, RIF quantification after X-ray exposition showed a saturated dose response for early time points post-irradiation for all strains. Similarly, the high-LET response showed that RIF number matched the number of track per cell, not the number of expected DSB per cell (1). At the temporal level, we noted that the percentage of unrepaired high-LET tracks over a 48 hour time-course increased with LET, confirming that the DNA repair process becomes more difficult as more DSB coalesce into single RIF. There was also good

agreement between persistent RIF levels measured *in-vitro* in the primary skin cultures and survival levels of T-cells and B-cells collected in blood samples from 10 CC strains 24 hours after 0.1 Gy whole-body dose of X-ray. This suggests that persistent RIF 24 hour post-IR is a good surrogate *in-vitro* biomarker for *in-vivo* radiation toxicity. Finally, at the genomic level, large differences in repair rates between strains for high-LET allowed us to identify suggestive genetic loci associated with radiation sensitivity. Interestingly, the two highest LETs provided the most strain variation with a common locus on Chromosome 10 highly enriched for DNA repair associated genes we discussed in detail.

Introduction

The past few decades have witnessed a growing interest of space agencies for long-duration exploration missions beyond the low-earth orbital (LEO). One of the predominant concerns associated with these long-term missions, is the continuous exposure of astronauts to Galactic Cosmic Rays (GCR) (2, 3). The GCR spectrum is composed of 90% protons, 9% He ions and 1% heavier ions which are typically referred as HZE particles (i.e. particles with high charge Z and high energy E) with energies ranging mainly between 0.1 and 1 GeV/n (4). Despite their relative low abundance in the GCR spectrum, the cellular consequences of HZE particles exposition have important implications given their higher relative biological effectiveness (RBE) (5-7). Particles with LET above 10 keV/µm are typically referred to as high linear energy transfer (LET) particles. Their higher RBE is usually believed to be due to the greater complexity of damages generated at the cellular and molecular levels, as compared to low-LET radiation (8, 9).

DNA damage-sensing proteins have been known to concentrate at sites of DNA double-strand breaks (DSBs) after exposure to ionizing radiation (seconds to minutes). This accumulation of specific proteins allow the formation of immunofluorescently stainable nuclear domains, the so-called radiation-induced foci (RIF) (1, 10, 11). RIF measurements are routinely used to quantify DSB and evaluate repair kinetics after different treatments (12, 13). Previous results from our group have shown that RIF coalesce after exposure to high doses of X-rays (14) and such coalescing property enforces its deleterious effects by increasing the chances of cell death. Neumaier *et. al*. showed that coalescence can be characterized by evaluating the nonlinear dose dependency of RIF number which saturates for X-ray doses larger or equal to 1 Gy (15). Based on this finding, we established computer models which used RIF coalescence properties measured after exposure to X-rays to predict cell death dose dependency for any other LET, by simply simulating the spatial distribution of DSB using Monte Carlo simulations of high-LET radiation (16). However, most of our modeling work has been validated primarily with data from only one immortalized human cell line (breast line MCF10A). It remains to be seen whether RIF coalescence phenotype occurs in non-immortalized lines and whether the genetic architecture of an individual has a bearing on it. In this work, we addressed the challenge of understanding and predicting individual radiation sensitivities to ionizing radiation by associating RIF phenotype and genotype data.

Genome-wide association studies (GWAS) are a powerful and widely used tool for finding genetic variants that increase the risk of developing particular diseases (17, 18). In the last decade, several novel genes have been identified for complex human disorders such as diabetes, cancer and various cardiovascular diseases (19-21). However, given the high cost, time commitment and ethical barriers involved in human GWAS, association studies in mouse model have been used successfully where phenotype assessments in genetically identical organisms, i.e., inbred mice have been performed to reduce the potential confounding effects of environmental factors. The Collaborative Cross (CC) mice presents a multiparental recombinant inbred population of mice derived from generations of inbreeding from five classic inbred strains (A/J, C57BL/6, 129S1/SvImJ, NOD/ShiltJ and NZO/H1LtJ) and three wild-derived substrains (CAST/EiJ, PWK/PhJ) and WSB/EiJ) (22). We chose this panel of mice since their high level of genetic variation, distributed randomly across the CC genomes caused each line to be genetically independent and prone to fewer spurious associations in mapping studies (23). In addition, we used five reference strains that have been characterized for various radiation phenotypes in the past (i.e. C57Bl/6, BALB/CByJ, B6C3, C3H and CBA/CaJ). For our genome-wide analyses, we used a reformulated linear mixed model analysis, Factored Spectrally Transformed Linear Mixed Model (FaSTLmm), which performs linearly in run time and memory footprint to perform associations in large data sets (24). FASTLmm is based on efficient mixed model association (EMMA), which utilizes linear mixed model with single dimensional optimization and phylogenetic control based kinship analysis to account for population stratification (25).

To the best of our knowledge, this study is one of the most extensive analysis for DNA damage response covering a large set of diverse strains of inbred mice for both low and high-LET radiation, with tens of millions of individual nuclei imaged and quantified for DNA damage. Fibroblast cells were extracted from 10 different CC strains and 5 reference mice strains. Cells were exposed to HZE particles (Si 350MeV/n, Ar 350MeV/n and Fe 600 MeV/n) and to 160 kVp X-ray. Individual animal's RIF coalescence and radiation sensitivity were investigated by X-ray RIF dose saturation and by evaluating the kinetic properties of RIF and high-LET track following exposure to various radiation qualities and doses. DNA repair kinetic measurements done this way were associated with the genotypes of the different strains of mice using FastLMM. We used FastLMM to identify chromosomal loci associated with radiation sensitivity phenotype. Our results show potential candidate DNA repair genes to be associated with HZE sensitivity.

Material & Method

Isolation of primary fibroblast cells from mice ears:

A total of 76 animals from 15 different strains were used in this study (10 CC strains - CC002, CC011, CC013, CC019, CC032, CC037, CC040, CC042, CC051, CC061 and 5 Reference strains - C57Bl/6, BALB/CByJ, B6C3, C3H and CBA/CaJ). Mice ears were decontaminated by soaking them in 70% ethanol for 15 seconds and were let dry. Ears were cut in small pieces with sterilized scissors and were transferred in a falcon tube containing 3 mL of 50 U/mL collagenase (Gibco 17101-015-1g) in HBSS (Gibco, Thermo Fisher Scientific). Tubes were placed at 37°C (waterbath) for 1 hour and were vortexed for 5 seconds every 15 minutes during this incubation. After incubation, the tubes were filled with Minimum Essential Media (MEM, Gibco, Thermo Fisher Scientific) supplemented with 10% v:v foetal bovine serum (FBS, Gibco, Thermo Fisher Scientific) and 1% v:v penicillin/streptomycine (Gibco, Thermo Fisher Scientific). Tubes were centrifuged at 1000 rpm for 5 minutes and the supernatant was discarded. The pellet was re-suspended in 2 mL of culture medium and transferred into a 6 well plate. Cells were incubated in a humidified incubator containing 5% CO_2 and 3% O_2. After 24 hours of incubation at 37°C, the wells were rinsed and 2 mL fresh medium was added. At passage 3, fibroblast cells were re-suspended in an appropriate freezing medium (90% FBS, 10% DMSO) at a concentration of 10^6 cells/mL and stored at -80°C in a cryo 1°C freezing container. The day after, cryo vials were transferred in liquid nitrogen tank until further use. This procedure was performed for each of the 76 different animals. On an average, cells from 3 males and 3 females for each strain were expanded and frozen for further characterization.

Genotyping:

Genomic DNA was extracted from each cell lines using Qiagen's AllPrep DNA/RNA mini kit. Purity of extracted DNA was assessed by measuring the 260 nm/280 nm and 260 nm/230 nm ratios using a nanodrop 2000 UV-vis spectrophotometer (ThermoFischer Scientific). DNA samples were thawed at a minimal concentration of 20 ng/µL and shipped to GeneSeek (Neogene, NE, USA) with cold pack. SNP analysis was performed using MegaMouse Universal Genotyping Array (MegaMUGA platform). MUGA was developed on Illumina Infinium platform in cooperation

with Neogene Inc. (Lincoln, NE) which contained 7851 SNP markers spaced uniformly about ~325 kb across the mouse reference genome. The MegaMUGA provided 10-fold higher marker density than MUGA (77,808 markers) with an additional 14,000 probes detecting variants segregating in wild-derived strains (26).

Irradiation procedure:

Fibroblast cells were thawed at BNL (Brookhaven National Laboratory; NY, USA) or LBNL (Lawrence Berkeley National Laboratory; CA, USA) and immediately aliquoted into 96 well plates, one well for each individual cell line (76 primary lines), with precise recording of each cell ID position in the plates. 10^4 cells were seeded in 96 well plates (IBIDI microplate, IBIDI®, Germany) and incubated at 37°C in a humidified incubator containing 5% CO_2 and 3% O_2. Medium was replaced two hours before exposure of each plate. Cells were exposed to three different LET (350 MeV/n Si, 350 MeV/n Ar and 600 MeV/n Fe with 63, 104 and 170 keV/µm LET respectively) and two ion fluences (1.1 and 3 particles per cell) at BNL. Since irradiation with each of the ions took place on different days, the confluency of the cells varied considerably. While for the Si run, 30% confluency was observed, the later experiments utilized higher cell plating density and 90% (Fe) and 80% (Ar) confluent cells were used for the Fe and Ar runs. For each run, two plates for each radiation condition (including control) were used, resulting in 64 plates for the high-LET experiments. **Supplementary Figure S1A** summarizes the experimental design. Two fluences were used for all three ions and plates were thus exposed to 6 different doses given their different LETs (i.e. 0.30 Gy and 0.82 Gy for 600 MeV/n Fe; 0.18 Gy and 0.50 Gy for 350 MeV/n Ar; 0.11 and 0.30 Gy). Also, each plates were exposed with the beam hitting below the plates, with the plate being angled at less than 5 degree, generating clear distinct track in the dish. Both for X-ray and ion exposure, dose rate was at 1Gy/min. X-ray experiments were conducted at LBNL on a 160 kVp Faxitron X-ray machine. After irradiation, cells were fixed at 4, 8, 24 and 48 hours post-exposure with 4% paraformaldehyde (Electron Microscopy Sciences, PA, USA) in PBS for 15 min at room temperature followed by extensive washing (three washes with PBS for 5 min each). Plates were then sealed with parafilm and kept at 4°C before being shipped to LBNL with cold pack.

Immunostaining and imaging procedure:

Cells were processed for immunostaining via programmed liquid handler (MultiFlo FX, BioTek, Winooksi, VT). Detailed protocols can be found in our previous publications (1, 15). Briefly, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS for 20 min and blocked for 1 hour with 3% Bovine Serum Albumin (BSA; Thermo Fisher Scientific) in PBS at room temperature. Cells were then incubated at room temperature with rabbit polyclonal anti-53BP1 primary antibody (IHC-00001, Bethyl Laboratories, Montgomery, TX - 1:300 dilution) for 1 hour, and subsequently incubated with 5 µg/mL Alexa 488 anti-rabbit secondary antibody (Thermo Fisher). Nuclei were stained with 5 µg/mL DAPI (Molecular Probes, Eugene, OR). Washing was performed between all incubations. Cells were subjected to high throughput automated imaging and quantification using proprietary microscope equipment. Immunofluorescence data was consolidated and analyzed using Exogen's automated foci quantification algorithm (Exogen Biotechnology Inc., CA, USA) as previously described (15)**.** Briefly, a wavelet morphological filter was applied to enhance foci peaks while reducing non-specific signal noise. Nuclear area was identified by applying a constant threshold on the wavelet-filtered image and foci were identified using a background subtraction method. Touching foci were separated using a watershed algorithm (15, 16). Each plate took one day for imaging and processing, for a total of 64 days of continuous imaging and quantification.

Statistical analysis and association analysis:

All statistical analyses were done using statistical package R and least-square fits were done using ggplot2 (Wickham H. ggplot2: elegant graphics for data analysis. Springer; 2016 Jun 8.) and ploynom (Venables B, Hornik K, Maechler M. polynom: A Collection of Functions to Implement a Class for Univariate Polynomial Manipulations, 2009. URL http://CRAN. R-project. org/package= polynom. R package version.;1:3-6.). Genome-wide association of data was performed using FaST-LMM which uses a linear mixed model to correct for population structure (24). Genotypes for all strains of mice were obtained from Neogene. FastLMM method is known to account for the population structure; however, it may be possible that segments of relatedness on some chromosomes might be presented as confounding. The SNPs used were filtered to have a minor allele frequency of >5% and missing genotype rate <10%. Significance threshold of 4.1 x 10^{-6}, determined through permutation and modeling has been widely used in case of inbred mice

(27). Linkage disequilibrium (LD) was determined by calculated pairwise r^2 SNP correlations for each chromosome ($r^2 > 0.8$) using PLINK.

In-vivo irradiation:

Animals were handled as previously described (28). Briefly, Collaborative Cross mice were obtained from UNC Chapel Hill. Genotyping data was obtained from the UNC Systems Genetics Core website (http://csbio.unc.edu/CCstatus/index.py), which used Mouse Universal Genotyping Arrays containing 77.8 K markers (megaMUGA). Mice were acclimatized at LBNL for eight weeks prior to the initiation of breeding. The study was carried out in strict accordance to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was approved by the Animal Welfare and Research Committee of the Lawrence Berkeley National Laboratory (Protocol File Number 271004). All mice were weaned at 21 days and group housed whenever possible. At 12 weeks of age, mice were exposed to a single acute dose of 10 cGy, 1 Gy or sham, using a Pantak 320 kVp X-ray machine, operated at 300 kV and 2 mA (18.5 cGy/min).

Results

Exposition to HZE particles:

76 primarily fibroblast cell lines from 15 different strains of mice were cultured from skin samples and cultivated as cell monolayers (see Material and Methods). All cell lines were grown in individual wells near confluence in 96 well plates before being exposed to three LETs: 350 MeV/n Si (63 keV/µm), 350 MeV/n Ar (104 keV/µm), 600 MeV/n Fe (170 keV/µm) and two ion fluences (1.1. and 3 tracks/100 μm^2) at the NASA Space Research Lab in Brookhaven National Laboratory (see detailed experimental setup in **Supplementary Figure S1 A, B**). Plates were fixed at various time point post-exposure and immunostained for DNA (DAPI) and p53 binding one protein (53BP1) as previously described (29). Nuclear detection software was gated to only consider nuclei of area ranging between 85 and 115 μm^2, with spherical shape factor less than 1.1 (1). Using automatic foci detection from Exogen Biotechnology Inc. (see Material and Methods), we reported the number of 53BP1 radiation induced foci (i.e. RIF/cell) for each individual primary cell-lines.

Figure 1 shows the dose dependency for each strain exposed to 600 MeV/n Fe particles (one strain per column), with each strain having multiple cell-lines quantified (up to 3 males and 3 females in

duplicate per strain, leading to up to 12 circles per dose point). Plots are distributed by row for each time point, with the first row for 4 hour time point and the last row for 48 hour time point. Overall, the number of RIF/cell for all strains show strong linear dependence with dose for all time points, which was fitted linearly by least square method including all cell-line data point for each strain (red lines in **Figure 1**). Note that similar good linear tendency was observed for the two other studied LET (**Supplementary Figures S2 and S3)**.

Assuming the average cross section of the nucleus in the fibroblasts was about 100 μm^2, the expected number of HZE tracks per cell should match the fluence: i.e. 1.1 and 3 tracks per cell. The expected number of tracks are shown as green lines for each time and strain panel in **Figure 1,** and **Supplementary Figures S2 and S3**. For both 4 and 8 hours following 600 MeV/n Fe, the majority of the strains show RIF number overlapping with the expected number of tracks per cell after irradiation (overlapping red and green lines in **Figure 1**). The dose responses for 350 MeV/n Ar show linear slopes which are parallel to the expected number of tracks/cell only for 4-hour post-IR (**Supplementary Figure S2**). The fitted slopes 8 hour after exposure to 350 MeV/n Ar is less steep than the expected number of tracks/cell. This lower slope is indicative of RIF resolution with all remaining DSBs along some Ar tracks being presumably fully repaired. In contrast the slope in RIF/Cell/Gy for 600 MeV/n Fe is unchanged at 8 hours still overlapping the theoretical number of track per cell clearly indicating repair is slower for this higher LET.

One must note here that even though the two fluences were constant for either Fe or Ar ions, the dose per cell was smaller for Ar due to its lower LET (i.e. 0.18 and 0.5 Gy versus 0.3 and 0.82 Gy for Ar and Fe respectively), leading to the same number of tracks per cell but more DSBs per track for Fe. The fact that RIF numbers are the same for both LET at the earliest time point clearly highlights the fact that nearby DSBs along track coalesce into single RIF and confirms that RIF are better surrogate markers of high-LET tracks than individual DSBs as previously hypothesized (1).

The 350 MeV/n Si experiment had cells which grew slower than expected (see Material and Method). Because of the delicate timing to access the beam line at the NSRL, these cells ended-up being in full growth phase instead of being confluent on our run-time. This led to a very high level of spontaneous foci (**Supplementary Figure S3**) as previously reported for actively dividing cells (1, 12). Even though, such high foci background complicated the interpretation of the dose response with strong RIF variability, one cans still observe here that the 4 hour dose response

shows a clear linear trend. Interestingly for this lowest LET experiment, the slope is less steep than the expected number of tracks/cell even for the earliest time point (i.e. 4 hours), indicating that some cells have repaired all damages induced by some tracks at 4 hours, confirming DNA repair is the fastest for the lowest LET.

Note that Si dataset illustrates nicely the usefulness of doing a linear fit on the dose dependency. First, the computed slope reflects the accurate number of foci induced by ionizing radiation, independently of the foci background. In addition, the y-intercept of the linear fit reflects the level of foci background for each strain and each time point (red line intercept at 0 Gy) and takes into account the variance of all dose points. It is therefore a more accurate approach than only computing the average number of foci at 0 Gy to evaluate foci background. In this latter more traditional approach, one only takes into account the variance of the sham group. This is further illustrated when plotting y-intercept values as a function of time post-IR for each dose response (**Supplementary Figure S4**). In this graph, 350 MeV/n Si experiment clearly shows a significant drop of foci background as time post-IR increases, illustrating the fact that the experiment started with cells in log-phase with 30% confluence and by 48 hours the cells were approaching confluence with lower cell divisions and therefore lower spontaneous foci. In contrast, this effect is less obvious for 350 MeV/n Ar and 600 MeV/n Fe experiments with earliest time point at 80% and 90% confluence respectively and much lower drops of foci background with time.

Another cautionary note for radiation biologists using the RIF assay can be brought to light here. If one reports the traditional RIF/cell/Gy for each LET as a surrogate metrics for DNA repair, interpretation about DNA repair kinetic would be wrong. For example, one would observe that there are 5.75 and 3.7 RIF/cell/Gy, 4 hours after exposure to 350 MeV/n Si and 600 MeV/n Fe respectively in B6C3 mice. However, if one assumes that there is a 1:1 correspondence between RIF and DSBs, and knowing that 1 Gy typically induces ~35 DSB/cell in G1 (12), one would then conclude wrongly that cells repair faster after exposure to 600 MeV/n Fe when the opposite has been shown to be true (30, 31). We thus propose to use a different metric here by normalizing the number of RIF/cell/Gy (slopes of red linear fits in **Figures 1, Supplementary Figure S2 and S3**) by the expected number of track per cell (slopes of green lines). This approach leads to the introduction of a new parameter "the proportion of unrepaired tracks". The expected number of tracks per nucleus assuming a cross section of 100 μm^2 leads to ~3.7 tracks/cell/Gy for 600 MeV/n Fe against 9.9 tracks/cell/Gy for 350 MeV/n Si: i.e. Si generates more tracks per cell for the same

dose, but less DSB per track due to its lower LET (**Supplementary Figure S5**). When comparing foci slopes to the expected number of track per cell, one can then conclude that 58% of all tracks are still unrepaired at 4 hours post Si exposition (i.e. 5.75 RIF/cell/Gy out of 9.9 tracks/Gy), whereas apparently none have repaired following Fe exposition (i.e. 3.7 RIF/cell/Gy out of 3.7 tracks/Gy). The faster repair of lower LET tracks is illustrated in **Figure 2A**, showing the average percentage of unrepaired tracks across all strains for each particle. **Figure 2B** illustrates repair of individual strains using the same metrics across three different strains, highlighting the biggest repair rate differences are more observable for late time-points.

Exposition to X-rays:

A similar approach was taken at LBNL, where the 76 skin fibroblasts primarily lines were exposed to 160 kVp X-ray and RIF analyses were performed (see Material and Methods). In contrast with high-LET, the dose response profile was not the same depending on the time (**Figure 3**). For early time post-irradiation (4 hours), RIF asymptotic dependence was observed with RIF numbers increasing linearly with the dose before reaching a plateau passed 1 Gy (asymptotic fits shown as black lines), confirming the RIF number saturation we had previously reported for one human breast line (15). For later time points, we observe the expected linear dose dependency (linear fits shown as red lines). As done for high-LET, the change in the slope as a function of time can be used to interpret the repair kinetic of each individual strain, showing that RIF/cell/Gy is decreasing over time post-exposure for all strains. The "proportion of unrepaired DSB" could be computed by dividing the slope by the theoretical number of 35 DSB/cell/Gy (12).

Correlation between HZE particles and X-rays response:

In order to relate repair kinetics measured for both X-ray and high-LET, we propose to compare the "proportion of unrepaired track" obtained for each HZE with the "proportion of unrepaired DSB" for all 15 strains, for each X-ray dose and for all 3 common time-points between X-ray and particles (i.e. 4, 24 and 48h). The metric used for comparing the relative ranking of DNA repair efficiency across all strains between HZE and X-ray was the Pearson correlation. **Table 1** shows the correlation factors for the 15 CC strains at 4 hours, 24 hours and 48 hours post-exposure. When comparing the highest LET to X-ray, the proportion of unrepaired 600 MeV/n Fe tracks correlate best with the 4Gy X-ray response at 4 hour post-exposure with $R^2 = 0.635$ and with the 1 Gy X-

ray response at 24 and 48 hours post-exposure with $R^2 = 0.71$ and 0.177 respectively. Similarly, the proportion of unrepaired 350 MeV/n Ar tracks is best correlated with the 1 Gy X-ray response with $R^2 = 0.352$ and 0.572 for 4 and 48 hour respectively. In contrast, correlation between X-ray and 350 MeV/n Si is very low for all time points, probably reflecting the fact that there are fewer remaining DSBs in this experiment which is additionally confounded by the high noise level for this dataset. However, when focusing on the 4 hour time point where RIF signal was highest, the best correlation is with the lowest X-ray dose (0.1 Gy). To summarize, we observe better relative correlations when X-ray doses and particle LET increase together, suggesting high doses of X-ray reflect better high-LET DNA repair kinetic.

In-vivo irradiation:

In order to evaluate whether the persistent RIF measurements we are reporting in this study has any *in-vivo* relevance, we benefited from results gathered by the DOE low dose program conducted at LBNL with a large cohort of CC strains. In this project, our collaborators were measuring changes in T-cells and B-cells levels 24 hours after irradiation in a large CC cohort exposed to either 0.1 and 1 Gy whole-body X-rays. We therefore computed the Pearson correlation between the average fraction of T-cells or B-cells across all 10 common CC strains with the proportion of unrepaired DSBs measured in our earliest time point for X-ray. Our results are summarized in **Table 2**, showing the best correlation to be for 0.1 Gy *in-vivo* data with $R^2 = 0.61$ and 0.38 for T-cell fraction and B-cell fraction respectively. When repeating the same approach with the 1 Gy *in vivo* response, the correlation was strongly decreased when comparing with either the 0.1 Gy or the 1 Gy RIF levels (R^2 close to 0= 0.05 and 0.29 respectively for the T-cell and B-cell fractions). This later result potentially reflects the fact that 1 Gy whole-body dose may be inducing strong systemic changes in the animals, which would confound the level of radiation-induced cell deaths in blood-cells related to DNA repair efficiency.

Genome-wide association analysis of HZE particle induced RIF formation in inbred-mice skin derived fibroblasts

In order to isolate genes that participate in the DNA repair process, we sought to associate phenotype RIF/cell/Gy (normalized to background), averaged from males and female mice for each strain, at 48 hour time-point post radiation with the genotype of the CC and reference strains

of inbred mice. Since there was a higher variability in the unrepaired tracks after 48 hours of high and low LET irradiation (**Supplementary Figure S6)**, we used the RIF/cell/Gy phenotype. To identify genomic loci responsible for the variation in DNA damage response, we performed FASTLmm analysis with the genome-wide significance threshold of 4.1×10^{-6} and identified seven potential associations on chromosomes 2, 3, 7, 10, 11 and 19 corresponding to 350 Mev/n Ar (**Figure 4A**, **Table 3A**), three suggested associations on chromosomes 10 and 13, corresponding to 600 MeV/n Fe (**Figure 4B, Table 3B**) and two potential loci at chromosome 2 and 6, corresponding to 350 MeV/n Si (**Figure 4C, Table 3C**). For both the high LET radiations, Ar and Fe, a common locus on chromosome 10 was identified, with peak SNP as UNC18214722 (p value = 7.22×10^{-7}) along with fourteen genes affiliated with chromatin modification, DNA replication, transcription and double stranded break repair (Supplementary **Table 1, 2**). Among the high-LET radiation-induced repair associated genes that were identified from the skin fibroblasts, the functions of the following genes have been previously studied - *Sumo3*, *Dnmt3l*, *Mum1*, *Pias4*, *Btg1*, *Sirt6*, *Tdg*, *Hdac5*, *Brca1*, *Rdm1* and *Atxn7l3*. For low LET Si, one repair-associated gene, *Recql* at peak SNP JAX00629117 on chromosome 6 ($p = 1.2 \times 10^{-6}$) was identified (Supplementary **Table 3**).

Discussion

We recently showed by time lapse fluorescent microscopy of 53BP1 fused to GFP that RIF can coalesce with other nearby RIF when a large amount of them are generated simultaneously by high doses of X-ray (14). The spatial distribution of DSBs is specific to the type of radiation, with energy primarily deposited along linear track in the case of cosmic radiation exposure. Therefore, a cosmic particle traversing a cell always generates several close-by DSBs along its trajectory with the linear density of DSB increasing linearly with LET (29, 32). In contrast, RIF coalescence is difficult to observe for low doses of X-rays, mainly because such radiation generate DSB randomly in the nucleus. We and others have previously hypothesized (15, 16, 33) that RIF coalescence is the primary mechanism by which chromosomal rearrangement occurs, thereby explaining why induction of mutation, cell death and cancer is much more prevalent following cosmic radiation. Such hypothesis is in sharp contrast from the theory that hypersensitivity to high-LET can be explained only by the complexity of DSB with complex breaks primarily repaired by Homologous

Recombination (34). RIF coalescence is also an alternate hypothesis to the classic "contact-first" model where DSBs are assumed to be immobile and repaired at the lesion site (35, 36). In contrast, our model suggests nearby DSBs move into single repair unit characterized by large RIF before the repair machinery kicks in. Such model has the advantage of being much more efficient molecularly and was first discovered by Aten et al. (37).

In this work, we tested the universality of RIF coalescence by characterizing the spatiotemporal property of 53BP1 RIF in primary skin cells isolated from 15 different strains of CC and reference mice exposed to HZE particles and X-rays photons. This work represents one of the most extensive study done on such a large genetically diverse mice set. RIF quantification after X-ray exposition showed an asymptotic dependence to doses for early time points post-irradiation across all 15 strains, with clear difference between strains. Similarly, the high-LET response showed that RIF number matched the number of track per cell, not the number of expected DSB per cell as previously shown in human skin fibroblast (1). These results clearly illustrate that the RIF coalescence phenotype is "universal" but that the genotype of the animal modulates its amplitude. At the temporal level, we characterized DNA repair kinetic following both low- and high-LET. For high-LET characterization, we noted that the percentage of unrepaired tracks over a 48 hour time-course increased with LET, confirming that the repair process becomes more difficult for cells as RIF coalesce more. In addition, repair rates were modulated by genetic background, with animals from the same strain showing small variance while large repair rate differences were observed between the different strains. When comparing RIF repair kinetic across all strains, it is interesting to note that high-LET ranking correlated better with ranking from unrepaired levels following high doses of X-ray instead of low doses. In other words, DNA repair rates decrease when LET or dose increase. This suggests that increased spatial proximity of DSB is a clear alternate explanation for slower repair in contrast to the classical DNA damage complexity as the primary mechanism (31, 38).

This work is also one of the rare studies directly linking the DNA repair kinetic measured *in-vitro* with cell death *in-vivo* for a large number of strains. T-cells and B-cells survival levels collected in blood samples in the 10 CC strains 24 hours following a 0.1 Gy whole-body dose of X-ray correlated well with the remaining number of RIF measured in primary skin cell cultures of the same strains 4 hours after 0.1 Gy of X-ray. This suggests that the RIF number found in skin at early time post-irradiation is a surrogate marker for *in-vivo* radiation sensitivity in other tissue,

such as blood cells and that such response is modulated by genetic variability as well. In the past, researchers had only shown similarities for different *in-vitro* tests, such as residual level of unrepaired DSB 24 hours after irradiation and fraction of surviving cells (39-41).

One can also compare in the five reference mice strains used here our ranking for DNA repair sensitivity to *in-vivo* lethal doses 50 (LD_{50}) measured after X-ray exposition (42, 43). Doing so, we observe that mice with lower LD_{50} correspond to the radiosensitive strains in our ranking for X-ray, with BALB/C being the most sensitive and C3H being the most resistant for assays. They also corroborate the study of Nowosielska *et al.*, which evidenced differences in the stimulation of NK cells in BALB/c and C57BL/6 mice in response to X-ray exposition (44). They showed a stimulation of cytotoxic activity of NK cells which occurred between the first and fourth day, post-irradiation in BALB/c mice, while similar effects were detectable 3 to 7 days post-exposure in C57BL/6 mice. The authors attributed this faster stimulation of immune system in BALB/C mice to a more radiosensitive phenotype than for C57BL/6 mice. It is interesting to note that, there was a lack of similarity between the rankings of high-LET repair kinetic and X-ray LD_{50}, suggesting that different and more complex repair processes are at play. Growing number of evidences illustrate the importance of the non-homologous end joining (NHEJ) repair pathway for DSB induced by low-LET radiation compared to high-LET radiation (45). Nagasawa *et al.* showed that, cells deficient in the NHEJ pathway show higher sensitivity to x- and γ-rays with a dose-dependent increase in unrepaired DSBs (46). In the meantime, several reports indicated that high-LET particles induce DSB which cannot be repaired by NHEJ pathway (47, 48). Other mechanisms seem to be implicated in the case of HZE particles such as the homologous recombination pathway although the exact mechanism remains unclear (45). Furthermore, this difference in type of DNA repair pathway, after low and high-LET exposition, could explain the greater radiosensitivity of BALB/c mice to low-LET radiation as compared to high-LET particles. Indeed, Okayasu *et al.* attributed the increased sensitivity to the reduced expression of DNA-PKcs, an essential protein in NHEJ pathway, in BALB/C mice (49).

At the genomic level, mouse genome wide association (GWA) analysis identified several potential candidate loci containing DNA repair associated genes. Association of normalized (to background) RIF/cell/dose obtained from irradiated fibroblasts to the 15 strains of inbred mice cannot resolve specific genes in the observed sensitivity. However, it may highlight potential genomic loci associated to high and low LET radiation. Interestingly, for the two high LET HZE particles, 350

MeV/n Ar and 600 MeV/n Fe, one common genetic locus on chromosome 10 was obtained that yielded fourteen DNA damage responsive genes. Among the genes identified in the 48 hour response to high LET radiation, SUMO3 (Small Ubiquitin-like Modifier) and PIAS4 (E3 ligase enzyme) have been known to accumulate at sites of DNA damage (50). SUMO3 acts in a mechanistically similar fashion to ubiquitin (50, 51) and Pias4 is required for the productive association of 53BP1and BRCA1 (52). BRCA1, also identified as one of the high LET response genes, has been studied extensively in relation to breast and ovarian cancer. Several lines of evidence have suggested recruitment of BRCA1 as a large complex into the sites of double stranded breaks and has been implicated in the maintenance of G1/S, S-phase and G2/M checkpoints. Interestingly, BRCA1 has been shown to participate in both DNA repair processes, the homologous recombination (HR) at G2 and S phase and non-homologous end joining (NHEJ) at G1 phase (53). HDAC5 was also identified and this class IIA lysine deacetylase is known to play a role in DNA repair involving base excision (BER), nucleotide excision and mismatch repair (54). TDG, also a gene candidate in this loci, is a thymine DNA glycosylase contributing to BER pathways through transcriptional regulation and demethylation (55). Of interest, DNMT3l which was also identifieds, acts as methylation regulator and interacts with HDAC1, repressing transcription during DNA damage response (56). Mammalian homologue of silent chromatin regulator 2 (Sir2), Sirtuin (SIRT) is a histone deacetylase, functioning in DNA repair through BER. SIRT6, as a histone deacetylase was found to activate PARP1 thus, confirming its role in both BER and DSB repair (57) . Yet another DDR protein, identified in the high LET associations is the EXPAND1/MUM1, which functions to support chromatin architecture by rapidly accumulating at the sites of DNA damage via its direct interaction with 53BP1 (58). Other candidate genes isolated for response to high LET radiation include BTG1, RDM1 and ATXN7l3.

We also associated the X-ray induced phenotype for high and low doses but no significant hits ($p > 4.1 \times 10^{-6}$) were obtained. This can be attributed to several factors such as trait prevalence, allele frequency and heritability (59). Radiation sensitivities in Caucasian cases in early onset of lung cancer with 4 Gy and 1 hour of repair time, was found to be highly heritable for basal damage (without irradiation) and decreased 20%-48% for remaining damage following different repair time-points (60). In several cancer states, various repair pathways coordinate in a spatiotemporal fashion to maintain genomic stability. Hence several population studies have reported the polygenic nature of DNA repair mechanisms in early breast cancer cases (61). Thus, a case control

study design with larger cohort of strains along with detailed analysis involving eQTL (cis and trans regulation) analyses and functional validation of identified genes for each category is warranted to further evaluate the multifactorial nature of in-vivo radiation induced DNA repair pathways in models organisms.

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Figure Legends

Figure 1: Quantification of 53BP1 radiation–induced foci observed after exposition to iron beam (600 MeV/n) based on the dose received by the cells. Each column represents each strain of mice and each row shows the results at 4, 8, 24 and 48 hours post-irradiation. In each graph, the circles represent skin fibroblasts from each animal (Values averaged from duplicate treatment of 3 females and 3 males) from each strain that was exposed to each dose of radiation. (. Green and red lines correspond to respectively the theoretical number of tracks per cell and a linear fit obtained from data from foci/ cell plotted versus the dose in Gy.

Figure 2: Proportion of unrepaired tracks at different time points computed in the fibroblasts after irradiation with Si 350MeV/n, Ar 350MeV/n or Fe 600MeV/n. (A) The number of unrepaired tracks followed by irradiation with high and low LET particles calculated in fibroblasts derived from all inbred strains of mice. Results presented as mean of all strains ± standard deviation. (B) Proportion of unrepaired tracks in fibroblasts derived from 3 strains B6C3, CC011 and CC061 at 4, 8, 24 and 48 hours post irradiation with Fe 600 MeV/n. The results presented mean from 3 males and 3 females? With no std deviation?

Figure 3: **Quantification of 53BP1 radiation–induced foci observed at 4 hours, 24 hours and 48 hours after X-ray photons exposition at various doses.** Each column represents each strain of mice and each row shows the results at 4, 8, 24 and 48 hours post-irradiation. In each graph, the circles represent skin fibroblasts from each animal (Values averaged from duplicate treatment of 3 females and 3 males) from each strain that was exposed to dose of radiation (ranging from 0 to 4 Gy). Green and red lines correspond to respectively the theoretical number of tracks per cell and a linear fit obtained from data from foci/ cell plotted versus the dose in Gy.

Figure 4: Quantification of T-cells and B-cells fraction in the strains of mice, 24 hours after 10cGy and 100cGy total body X-rays exposition. Correlation factor measured between the "unrepaired DSB proportion" parameter obtained after 4h exposure of fibroblasts to X-ray and the decrease in T and B-cells fraction in mice after X-ray exposure.

Figure 5 : GWA results for RIF/cell/Gy at 48 hours post exposure to high and low LET radiation Manhattan plot showing the –log10 of the association p-values (-logp) for background normalized RIF/cell/Gy at 48 hours post irradiation with (A) 350MeV/n Ar (B) Fe 600MeV/n and (C) 350MeV/n Si in skin derived fibroblasts. The phenotype values are averaged from RIF/cell/Gy obtained from males and female mice from each of the 15 inbred strains of mice. Each chromosome is plotted on the x-axis in alternating light and dark colors. Genome-wide significance threshold line is shown in red (-logp = 5.39).

athways in human are depicted in brighter colors.

Tables:

Table 1: (A) Correlation factor measured between the "unrepaired track proportion" parameter from HZE particles experiment and the "unrepaired DSB proportion" parameter from X-ray experiment. (B) Correlation values normalized by the highest correlation value for each LET

Table 2: Calculated metric values for each strain derived fibroblasts irradiated by high-LET particles and X-rays. Mice strains were ranked in ascending order according to the metric value. The radiation resistant animals lie on the top of the table while radiosensitive mice lie at the bottom of the table.

Table 3: Table showing the association statistics and number of genes identified in the chromosomal locations for the different traits (A) 350 Mev/n Ar (B) 600 MeV/n Fe (C) 350 MeV/n Si

Supplementary Figure legends

Figure S1: Experimental design entailing dose determination and cell plating for irradiation. (A) Cells were exposed to three different LET (350 MeV/n Si, 350 MeV/n Ar and 600 MeV/n Fe with 63, 104 and 170 keV/µm LET respectively) and two ion fluences (1.1 and 3 particles per cell) at BNL. Thus 6 different doses given their different LETs (i.e. 0.30 Gy and 0.82 Gy for 600 MeV/n Fe; 0.18 Gy and 0.50 Gy for 350 MeV/n Ar; 0.11 and 0.30 Gy) were used. (B) Fibroblast cells were thawed at BNL (Brookhaven National Laboratory; NY, USA) or LBNL (Lawrence Berkeley National Laboratory; CA, USA) and immediately aliquoted into 96 well plates, one well for each individual cell line (76 primary lines), with precise recording of each cell ID position in the plates. 104 cells were seeded in 96 well IBIDI plates and incubated at 37°C in a humidified incubator

containing 5% CO2 and 3% O2. For each run, two plates for each radiation condition (including control) were used, resulting in 64 plates for the high-LET experiments.

Figure S2: Quantification of 53BP1 radiation–induced foci observed after exposition to argon beam (350 MeV/n) based on the dose received by the cells. Each column represents each strain of mice and each row shows the results at 4, 8, 24 and 48 hours post-irradiation. In each graph, the circles represent skin fibroblasts from each animal (Values averaged from duplicate treatment of 3 females and 3 males) from each strain that was exposed to each dose of radiation. Green and red lines correspond to respectively the theoretical number of tracks per cell and a linear fit obtained from data from foci/ cell plotted versus the dose in Gy.

Figure S3: Quantification of 53BP1 radiation–induced foci observed after exposition to silicon beam (350 MeV/n) based on the dose received by the cells. Each column represents each strain of mice and each row shows the results at 4, 8, 24 and 48 hours post-irradiation. In each graph, the circles represent skin fibroblasts from each animal (Values averaged from duplicate treatment of 3 females and 3 males) from each strain that was exposed to each dose of radiation. Green and red lines correspond to respectively the theoretical number of tracks per cell and a linear fit obtained from data from foci/ cell plotted versus the dose in Gy.

Figure S4: Quantification of 53BP1 radiation–induced foci of control non-irradiated samples. The foci/cell for each strain of mice is plotted in a separate color. Each circle represents one single fibroblast culture and control measurements were taken each irradiation with each beam.

Figure S5: Schematic representation of a cell nucleus exposed to a given dose of HZE particle (A) and photons (B). Each red dot represents an energy deposition by matter ionization. Both examples produce a same total number of ionizations evidencing a same dose deposited in the cell nucleus. However, due to their higher LET, HZE particles produces a more densely ionization in the nucleus leading to more complex DNA damages compared to photons.

Figure S6: Percentage of unrepaired tracks 24 and 48-hour post irradiation in 15 strains of inbred mice. A higher variation was observed in the percentage of unrepaired tracks after 48-hour post exposure to (A) 600MeV/n Fe (B) 350MeV/n Ar and (C) 350MeV/n Si in the strains of mice.

Supplementary Table 1: Gene list corresponding to UNC18214722 and JAX00021248 on Chromosome 10 and UNC20271233 on Chromosome 11, the peak SNPs associated with 48-hour response to DNA damage induced by 350 MeV/n Ar.

Supplementary Table 2: Gene list corresponding to UNC18214722 on Chromosome 10, the peak SNP associated with 48-hour response to DNA damage induced by 600 MeV/n Fe.

Supplementary Table 3: Gene list corresponding to JAX00629117 on Chromosome 6, the peak SNP associated with 48-hour response to DNA damage induced by 350 MeV/n Si