Title: MMR deficiency does not sensitize or compromise the function of hematopoietic stem cells to low and high LET radiation.

Running title: Mlh1 null HSCs are not sensitized to radiation.

Authors: Rutulkumar Patel*[,1], Yulan Qing*[,2], Lucy Kennedy[3], Yan Yan[2], John Pink[2], Brittany Aguila[4], Amar Desai[2], Stanton L. Gerson[2,#], and Scott M. Welford[5,#]

Affiliations: [1]Department of Pharmacology, [2]Case Comprehensive Cancer Center, National Center for Regenerative Medicine, Seidman Cancer Center, University Hospitals Cleveland Medical center and Case Western Reserve University, Cleveland, OH 44106, USA

[3]Unit for Laboratory and Animal Medicine, University of Michigan, Ann Arbor, MI 48109 USA

[4]Department of Biochemistry, Case Western Reserve University, Cleveland, OH 44106, USA

[5]Department of Radiation Oncology, Sylvester Cancer Center, University of Miami, Miami, FL 33136 USA

*These authors contributed equally.

Author contributions:

- Rutulkumar Patel: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing
- Yulan Qing: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing
- Lucy Kennedy: Conception and design
- Yan Yan: Conception and design
- John Pink: Conception and design
- Brittany Aguila: Collection and/or assembly of data
- Amar Desai: Conception and design, Data analysis and interpretation
- Stanton L Gerson: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript
- Scott M Welford: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of record. Please cite this article as doi:10.1002/sctm.17-0295.

Corresponding Author: #Correspondence should be addressed to Scott M Welford, PhD; 1550 NW 10th Avenue, Pap 503, Miami, FL, 33136; phone 305-243-8337 scott.welford@miami.edu; or Stanton L. Gerson, MD, UH/Wearn 151 10900 Euclid Ave. Cleveland, Ohio 44106. Phone: (216) 844-8565, slg5@case.edu

Financial Support: This study was supported by NASA NNX14AC95G, and the Cytometry & Microscopy and Radiation Resources Shared Resources of the Case Comprehensive Cancer Center (P30CA043703).

Disclosures: The authors declared no conflict of interest.

Key Words: hematopoiesis, HSC, stem cells, ionizing radiation, DNA mismatch repair, *Mlh1*

Abstract:

One of the major health concerns on long-duration space missions will be radiation exposure to the astronauts. Outside the earth's magnetosphere, astronauts will be exposed to galactic cosmic rays (GCR) and solar particle events (SPE) that are principally composed of protons and He, Ca, O, Ne, Si, Ca, and Fe nuclei. Protons are by far the most common species, but the higher atomic number particles are thought to be more damaging to biological systems. Evaluation and amelioration of risks from GCR exposure will be important for deep space travel. The hematopoietic system is one of the most radiation-sensitive organ systems, and is highly dependent on functional DNA repair pathways for survival. Recent results from our group have demonstrated an acquired deficiency in mismatch repair (MMR) in human hematopoietic stem cells (HSCs) with age due to functional loss of the MLH1 protein, suggesting an additional risk to astronauts who may have significant numbers of MMR deficient HSCs at the time of space travel. In the present study, we investigated the effects gamma radiation, proton radiation, and ^{56}Fe radiation on HSC function in *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ marrow from mice in a variety of assays and have determined that while cosmic radiation is a major risk to the hematopoietic system, there is no dependence on MMR capacity.

Introduction:

Exposure to ionizing radiation (IR) is considered to be one of the major risk factors during space activities, especially long-duration space missions [1, 2]. Astronauts on missions to the International Space Station, the moon, or Mars will be exposed to IR with effective total doses in the range of 5 to 200 cGy (centigray) based on projected mission scenarios [3, 4].The primary components of space radiation are galactic cosmic rays (GCR) and radiation from solar particles [5]. This space radiation consists of 85% protons, 14% helium nuclei and 1% high-energy, high-charge (HZE) particles, including oxygen (^{16}O), carbon (^{12}C), silicon (^{28}Si), and iron (^{56}Fe) ions [6]. HZE radiation is of particular concern because it causes high linear-energy transfer (high-LET) damage in biological targets and induces repair-refractory clustered DNA damage in cells [2, 7-10]. These types of damage directly affect cell survival and genomic integrity in surviving cells [11]. In addition, it is estimated that for every cell traversed by a potentially lethal HZE nucleus (e.g. ^{56}Fe), another 32 cells are hit by δ rays of decreased energy that could induce non-lethal mutations [12]. ^{56}Fe has been thought to be the most biologically important HZE particles, since it may be the single largest naturally occurring particle [6].

The hematopoietic system is one of the most radiosensitive tissues of the body [13]. Hematopoietic stem cells (HSCs) reside in bone marrow (BM) and are responsible for generation and maintenance of multiple cell lineages in the blood supply [14]. It is well known that total body irradiation (TBI) affects both mature blood cells and hematopoietic stem/progenitor cells and causes both acute radiation hematopoietic syndrome and long-term BM injury [15, 16]. HZE particles like ^{56}Fe ions have been shown to be more toxic than γ rays with lethal doses (LD)50/30 (a radiation dose at which 50% lethality occurs at 30-days) of 5.8 Gy compared with 7.25 Gy for γ rays. Mice irradiated with a lethal dose of ^{56}Fe showed significantly lower white blood cell (WBC) recovery at 4 weeks post-IR, compared to γ-IR mice [17]. ^{56}Fe-

IR caused loss of hematopoietic stem/progenitor cells immediately after IR, which was maintained for up to 8 weeks [18]. In addition, protons, ^{28}Si ions and ^{56}Fe ions are also known to induce hematopoietic malignancies such as acute myeloid leukemia (AML), though not necessarily with greater efficiency than γ irradiation [19, 20].

Exposure of mice to 1 Gy ^{56}Fe results in highly complex chromosome aberrations, including dicentrics, as well as translocations, insertions and acentric fragments, which is unlike the damage from γ radiation. Cells exhibiting these aberrations disappear rapidly after exposure, probably as a result of death of heavily damaged cells. Cells with apparently simple exchanges as their only aberrations, appear to survive longer than heavily damaged cells. Eight weeks after exposure, the frequency of cells showing cytogenetic damage was reduced to less than 20% of the levels evident at 1 week. These results indicate that exposure to 1 Gy ^{56}Fe produces heavily damaged cells, a small fraction of which appear to be capable of surviving for relatively long periods [21, 22]. In addition, exposure to low doses of ^{56}Fe resulted in significant epigenetic alterations involving methylation of DNA, and expression of repetitive elements, which is also unique to high LET radiation [23]. Therefore, while damage from terrestrial γ radiation and X-rays forms the basis our understanding of cellular responses to DNA damage, GCR provides a unique cellular stress and highlights gaps in current models describing the response of biological systems to space radiation.

IR causes not only DNA strand breaks, but also nucleotide base and sugar damage [24]. Analyses of gamma ray and ^{56}Fe induced murine AML samples have identified similar molecular changes, including biallelic loss and/or mutation of *PU.1*. Microsatellite instability (MSI), a commonly observed marker of DNA mismatch repair (MMR) deficiency, has been observed in 42% of AML samples induced by gamma rays or ^{56}Fe [25]. MMR is an essential DNA repair pathway responsible for maintaining genomic integrity

primarily by removing base mismatches and small insertion/deletion loops (IDLs) introduced during replication or under genomic stress [26]. In humans, MMR gene defects (most notably in the *MLH1*, *MSH2*, *MSH6*, *PMS2* genes) and MSI have been most closely associated with Lynch Syndrome [27-29], but are also found in an increasing number of tumor types [30]. MMR deficiency has also been identified in primary and secondary hematopoietic malignancies and in leukemia and lymphoma cell lines [31-33]. Exposure of MMR-deficient mice to gamma radiation results in hypermutability compared to wild type mice, and much of this hypermutability can be attributed to induced instability of simple sequence repeats [34]. Interestingly, recent results from our laboratory have demonstrated that acquired MMR deficiency and increased MSI in human hematopoietic stem and progenitor cells is age-related, attributable to progressive loss of *Mlh1* through promoter hypermethylation [35, 36]. The data show that as many as 30% of HSCs in healthy individuals have lost MLH1 by 45 years of age, which is well within the age range of current and former astronauts. Therefore, TBI may pose an unappreciated risk to astronauts on deep space missions if they have significant numbers of MMR defective HSCs.

Though the effects of protons and HZE ions on normal mouse hematopoietic systems have been characterized at the effector cell level [17, 37], the impacts on HSC function are not fully known. Importantly, in humans demonstrating diminished MMR capacity, the potential for loss of HSC function and/or malignant transformation may be greater [35]. Therefore, the role of MMR in response to γ and GCR radiation damage needs to be carefully examined. In this study, we used *Mlh1*-deficient mice to investigate the importance of the MMR system on the response of HSCs to protons and ^{56}Fe, in comparison to γ radiation, with an aim to investigate the risk of hematopoietic failure in astronauts on deep space missions. In short, we find that while heavy ions are more damaging than gamma radiation to HSC functionality, no additional deficits in hematopoietic function were identified. The data suggest

that the greater risk of MMR deficient HSCs likely lies in malignant transformation rather than hematopoietic failure.

Materials and Methods:

Mice:

B6.129-*Mlh1*^{tm1Rak}/NCI heterozygous mice were obtained from NCI and then mated to produce *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice for the study. C57BL/6J mice were purchased from Jackson Laboratory, congenic strain B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyCrCrl (BoyJ, CD45.1) mice were obtained from Charles River Laboratory. All the mice were group-housed in ventilated microisolator cages in a specific pathogen-free facility. Mice had ad libitum access to food (Laboratory Rodent Diet 5LOD, Lab Diet, St. Louis, MO) and water. The animal housing room was maintained on a 12:12h light:dark cycle and constant temperature (72 ± 2˚ F). Male and female mice between 8 and 16 weeks of age were used for the study. All mouse studies were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University (Cleveland, OH), and Brookhaven National Laboratory (BNL) (Upton, NY).

Radiation:

Two to three month old, male and female, and *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice were used for the study. Proton and ^{56}Fe irradiation were performed at the NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratories. After 7 days of acclimation, the mice were exposed to TBI with 1 Gy of protons (1000 Me/V) or ^{56}Fe (600 MeV/n). Gamma irradiations were performed at BNL using a ^{137}Cs source for *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ animals, and at Case Western Reserve University for recipient mice irradiated with 11 Gy. Sham irradiated animals that traveled to BNL were used as controls. One set of sham irradiated *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice was used to compare ^{1}H ion and ^{56}Fe ion irradiated mice while second set of sham irradiated *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice were used to compare γ-ray irradiated mice because they were exposed on different trips to Brookhaven National Laboratory.

Peripheral Blood and BM Collection

Peripheral blood (PB) was collected from the submandibular vein using a heparinized hematocrit capillary tube. Complete blood count was measured by a Hemavet 950 FS that gives 5-part differentiation with 20 parameters. For BM collection, mice were sacrificed roughly three months after radiation exposure, the femur and tibia were harvested and immediately flushed with Phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) using 21 and 27-gauge needles and syringes to collect the BM. The number of BM cells was counted using a hemocytometer.

Colony-forming unit (CFU) assays

BM mononuclear cells (BM-MNCs) were cultured in methylcellulose medium containing cytokines, including mouse interleukin 3, human interleukin 6, mouse stem cell factor, and human erythropoietin, MethoCult GF M3434 (Stem Cell Technologies, Vancouver, BC). Total numbers of CFU colonies were scored on day 7, according to the manufacturer's protocol. Two separate experiments of three plates each per radiation exposure were performed, and combined results were shown in the results.

Flow cytometry

Flow cytometry was performed on a BD LSRII (BD Biosciences, San Jose, CA), and data were analyzed using FlowJo software (TreeStar, Ashland, OR). Antibodies include CD45.2 (clone 104), CD45.1 (clone A20), Ly-6G (Gr-1, clone RB6-8C5), CD11b (Mac-1, clone M1/70), CD45R/B220 (clone RA3-6B2), CD3 (clone 500A2), and Ter119/Ly76 (clone Ter-119), Sca1 (Ly-6A/E, clone D7), c-Kit (CD117, clone 2B8), CD48 (clone HM48-1, BD Bioscience) and CD150 (clone TC15-12F12.2, Biolegend, San Diego, CA).

Competitive repopulation assay

All mice were sacrificed roughly three months post radiation exposure and BM cells were collected for competitive repopulation assay. $2x10^6$ BM cells from mice (CD45.2) of each genotype were mixed with the same number of wild type (CD45.1) competitor BM and transplanted into lethally irradiated (11Gy) BoyJ recipients (CD45.1) through lateral tail veins. The recipients were monitored and analyzed for hematopoietic reconstitution and lymphoma development. Peripheral blood was collected 8 weeks and 16 weeks post BM transplant to measure percentage contribution of CD45.2 cells in circulation.

Statistical Analysis

Statistical analysis was performed using GraphPad 5.0. Student's t tests were used to determine the significance of pairwise comparisons; ANOVA were used for dose response analyses, and log-rank tests were used to analyze survival curves. $p<0.05$ was the measure of statistical significance.

Results:

High LET radiation induces similar long term damage to the bone marrow as γ radiation.

In order to begin to assess the effects of high LET radiation on MMR defective HSCs, we first sought to establish the effects of ^{56}Fe compared to γ radiation on stem cell function. Various studies in the literature have demonstrated that ^{56}Fe and other high LET sources can damage hematopoiesis with different relative biological effectiveness (RBE) [17, 38]. We thus assessed bone marrow cellularity; enumerated Sca1+, c-Kit+, Lin- (SKL) cells; measured proliferation; and determined repopulation capacity all at a latent time point. As seen in figure 1A, at three months after exposure to 1 Gy of γ or ^{56}Fe radiation, bone marrow cellularity was unchanged in the animals compared to control animals. In contrast, the SKL populations in the animals dropped by greater than 50% in both the γ and ^{56}Fe groups. Notably, there was no significant difference between the two radiation sources. HSCs are known to rapidly reenter the cell cycle from quiescence in response to hematopoietic stresses in order to regain homeostasis. By three months, however, both the irradiated cohorts displayed HSC proliferation that was identical to the non-irradiated animals, as evidenced by BrdU incorporation (Figure 1C). The behavior was similar with the broader SKL population as well as the more primitive CD48- SKL cells. Finally, we measured the functionality of the irradiated HSCs by performing a competitive transplantation assay using unirradiated marrow injected into lethally irradiated hosts. We observed that the irradiated marrow was functionally challenged and had grafting potential that was reduced nearly 50% in both γ and ^{56}Fe cohorts, indicating similar RBEs at the 1 Gy dose (Figure 1D). With these data in hand, we could now address the role of MMR function.

High LET radiation is more damaging to clonogenic capacity of stem cells than low LET radiation, but independent of MMR status.

Exposure of HSCs to multiple forms of ionizing radiation, including space radiation, is known to reduce clonogenic capacity [18]. To determine whether loss of MMR plays a role in BM vitality after exposure to γ, protons (1000 MeV/n) or ^{56}Fe radiation (600 MeV/n), clonogenic assays were performed on isolated bone marrow in a standard colony forming assay. As expected, high LET radiation effected a greater decrease in colony formation than γ or proton radiation across a range of doses from 0.1 to 2.5 Gy (Figure 2A-C). In addition, ^{56}Fe ion exposure clearly caused a greater decrease in the number of BFU-E and CFU-GM than low LET γ or proton radiation (supplementary figure 1A-1I). We did not, however observe significant differences between the wild type and *Mlh1* knockout marrow, suggesting that MMR function does not contribute to acute effects of IR on clonogenicity *in vitro*.

Blood counts demonstrate similar acute damage to the hematopoietic system across LET.

To assess the acute effects of IR on MMR competent and deficient marrow *in vivo*, we performed TBI with γ, protons or ^{56}Fe at 1 Gy, and performed regular blood counts for up to 30 days. The response of specific effector cells to IR has been well documented, with lymphocytes being the most sensitive cells. As seen in Figure 3A-C, total WBCs displayed significantly more sensitivity to any form of IR compared with red blood cells and platelets (Figure 3D-I), which here did not decrease substantially during the 30-day follow up. There were no significant differences between the different radiation sources, however, all three effected similar transient decreases in lymphocytes (supplementary figure 2A-2C). Again, no differences were observed between *Mlh1* competent and deficient marrow.

Long term effects on hematopoiesis by IR is independent of MMR status.

In order to assess latent or long term effects of high and low LET radiation on hematopoiesis, TBI was performed on cohorts of both wild type and *Mlh1*$^{-/-}$ mice with 1 or 2.5 Gy of γ radiation, 1 Gy of proton radiation, or 1 Gy of ^{56}Fe. Three months after exposure, we planned to analyze the frequencies and numbers of different hematopoietic cell populations in BM by flow cytometry, and measure HSC functionality by transplantation assays. We first, however, noted survival statistics of the cohorts. As previously published, *Mlh1*$^{-/-}$ animals are tumor prone, with lymphomas and gastrointestinal tumors being the most prevalent malignancies [39] and γ irradiation of *Mlh1* deficient mice is known to accelerate tumorigenesis [40]. With a three-month follow-up, we found that 43% of the null animals developed malignancy while none of the control, wild type mice developed any tumors. To break the numbers down by treatment group, 19% (4 of 21) unirradiated null mice, 64% (7 of 11) 1 Gy γ irradiated mice, 45% (5 of 11) 2.5 Gy γ irradiated mice, 33% (3 of 10) of 1 Gy proton irradiate mice, and 30% (3 of 9) 1 Gy ^{56}Fe irradiated mice were euthanized for morbidity due to tumor formation within three months after exposure (Figure 4A-C). The only statistically significant increase in malignancy was in the 1 Gy γ group (p=0.0155, Log-rank). Presumably, had the animals been given longer times to develop tumors, as has been done in other studies, our data would have shown increased tumorigenesis in the other radiation groups as well. Additionally, we found only lymphomas in our cohorts, also likely due to the short follow up, as found previously [40].

Our primary interest was in determining the function of the MMR-defective HSCs at extended times after radiation. Therefore, at three months after exposure, all surviving mice were euthanized, and marrow was harvested and assessed. We first quantified the SKL cells. As seen in Figure 5A-C, γ, proton, and ^{56}Fe irradiated animals demonstrated significant decreases in SKL cells compared to unirradiated control mice. The γ irradiated animals displayed dose dependent decreases in SKL cells of up to 56% of non-irradiated animals at 2.5 Gy ($p<0.0001$ for *Mlh1*$^{+/+}$, and $p=0.0002$ for *Mlh1*$^{-/-}$; one-way ANOVA). In

pairwise comparisons, we noted that unlike the wild type mice, the *Mlh1*$^{-/-}$ null mice did not show significantly reduced SKL cells in the marrow at the 1 Gy dose, potentially suggesting protection against IR ($p<0.0001$ for wild type, $p=0.25$ for *Mlh1*$^{-/-}$; student's t tests); but any protection was lost at the higher dose where both genotypes were similar. Likewise, proton radiation led to a significant decrease in SKL cells in the wild type ($p<0.0001$, student's t test), and *Mlh1*$^{-/-}$ animals ($p<0.0001$, student's t test). ^{56}Fe irradiation resulted in indistinguishable decreases of SKL cells in both genotypes ($p<0.05$ for both, student's t tests). In addition, BM cellularity was unchanged between *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice (supplementary figure 3). In summary, three months after IR, significant decreases can be observed in SKL cell numbers in the bone marrow of exposed mice, but *Mlh1* function does not appear to play a role in this response.

We next looked at hematopoietic progenitor cells (HPCs) by gating for Sca1-, c-Kit+, Lin− cells. Here we observed that there were no detectable differences in any of the radiation source groups, at any dose, and for either genotype (Figure 5D-F). These data suggest that the SKL cells that remain functional three months after IR are sufficient to produce the normal levels of progenitor cells that are required to maintain the hematopoietic system.

Finally, we tested the colony forming potential of the marrow in standard CFU assays, by embedding bone marrow derived cells in methylcellulose with a variety of stem cell cytokines and enumerating colonies after 7 days (Figure 5G-H). In agreement with our observations of SKL cells, we found that γ radiation led to statistically significant drops in CFU in a dose dependent manner for both genotypes ($p<0.0001$ for wild type and *Mlh1*$^{-/-}$, ANOVA). For proton radiation at 1 Gy, both genotypes displayed significant drops in CFU, 26.8% for wild type ($p<0.0001$, student's t test) and 31.6% for *Mlh1*$^{-/-}$, ($p<0.0001$ student's t test). Surprisingly, ^{56}Fe radiation did not cause any measureable defects in clonogenic

capacity, which is in contrast to the observed effects on SKL cells, and could be due to an iron specific hyperproliferation in the SKLs. Importantly, all of the observed effects were independent of *Mlh1* status.

Defects in Mlh1 function do not enhance decreased competitive repopulation caused by IR

As a long term, functional measure of hematopoiesis, we conducted competitive repopulation studies comparing irradiated marrow of each genotype to wild type, unirradiated marrow. The concept is that if long term functional defects exist, the irradiated and/or *Mlh1* defective marrow will contribute less efficiently to repopulation of the marrow after lethal IR, and can be demonstrated by flow cytometry of peripheral blood [41, 42]. We therefore mixed competitor bone marrow cells from CD45.1 mice at a 1:1 ratio with irradiated wild type, or *Mlh1*$^{-/-}$ marrow from each irradiation exposure, and transplanted the cells into lethally irradiated hosts. At 8 and 16 weeks after transplant, peripheral blood was analyzed and revealed marked decreases in competition of the irradiated marrow from all sources. Much like we observed with the SKL data, γ irradiation led to a dose dependent decrease in competition from 1 and 2.5 Gy ($p<0.0001$, ANOVA) treated mice of both genotypes, at both 8 and 16 weeks (Figure 6A, B). For proton irradiation, similar decreases were seen with 1 Gy in both genotypes, at both time points (Figure 6C, D), and no differences were observed between the genotypes. Finally, we tested the ^{56}Fe irradiated marrow and found reduced but consistent effects of IR on the marrow functionality (Figure 6E). Together the data confirm that long term damage occurs after low and high LET radiation, but that *Mlh1* status is inconsequential to the function of the marrow.

Discussion:

In the current study, we investigated the effects of low and high LET IR on HSCs *in vitro* and *in vivo* with the goal of evaluating the significance of MMR defects that are characteristic of aging individuals who

could be exposed during space travel. We found primarily that MMR, as a function of *Mlh1* gene presence or absence, is not a relevant factor for hematopoietic functionality after radiation. We assessed clonogenic capacity of bone marrow harvested immediately after irradiation, as well as clonogenic capacity in marrow harvested three months after irradiation with γ, proton or ^{56}Fe sources and found almost identical responses, regardless of *Mlh1* status. We assessed blood counts of the animals for up to 30 days post exposure and similarly found that *Mlh1* wild type and knock out animals responded indistinguishably. Finally, we assessed SKL cells and HPCs in the bone marrow at three months after exposure, and also measured transplantability of the bone marrow at three months and found notable decreases in stem cell number and function due to radiation, but not due to *Mlh1* status. Together the data support the conclusion that MMR status is not a relevant variable for function of the hematopoietic system after exposure to γ or space radiation, and thus does not contribute added risk to astronauts.

Effective DNA repair is an essential function for the fidelity of organisms. HSCs are no exception to this rule, and indeed it has been elegantly shown that HSCs that lack efficient repair as they age, through loss of Ligase IV or Ku70, and thus non-homologous end joining, display reduced hematopoietic stem cell functions [43, 44]. Studies of animals engineered to harbor defective nucleotide excision repair or telomere maintenance show similar age-associated defects [43]. However, defects in these major repair pathways, unlike MMR defects, are relatively low in frequency [35, 45]. Ionizing radiation poses a greater threat to HSC biology due to the relative facility of exposure in modern society, and the exquisite sensitivity of the HSCs to DNA damaging stress [46]. Clearly, the combination of DNA repair defects, in the presence of IR stress would be predicted to compromise HSC functions even further.

Tissue kinetics contribute significantly to the time of manifestation of radiation induced damage. The hematopoietic system is extremely sensitive to ionizing radiation, demonstrating depletion of functional cells within hours to days [47], and an associated proliferative response of progenitor cells in the same time frame [48]. Latent tissue damage, however, implies depletion of stem cells, or a continued source of cell stress that prolongs damage manifestation. In the current studies, both explanations are likely to contribute. We observed depletion of HSCs three months after exposure, as well as decreased repopulation capacity of the remaining stem cells in a competitive transplant assay. Previously, elevated levels of reactive oxygen species correlating with decreased HSCs have been observed at 22 weeks after exposure to proton radiation, in line with our observations here [49]. Added mutational load, which is known to occur with MMR defects and radiation exposure [34], however, did not exacerbate the latent phenotype, at least regarding HSC function. While we measured functional cells by complete blood counts and found no effect of Mlh1 status, it is possible that some lineage differences exist that were not detected. Most notably, though, decreased stem cell function at latent times could be due to stem cell exhaustion caused by excessive induced proliferation in the acute phase immediately after exposure, and remains a risk to hematopoiesis that is shown here to be independent of MMR function.

Therefore, the predominant additional risk to the hematopoietic system of MMR defective individuals is likely to remain in the development of malignancies. The identification of MMR defective cancers outside of the gastrointestinal family in which they were first appreciated continues to rise, highlighting the importance of MMR to tumor suppression [30, 50]. Further studies will be required to assess the role of *Mlh1* loss in the carcinogenic process in the hematopoietic lineages to most accurately assess risk for the purposes of deep space missions.

Figure Legends:

Figure 1: Low and high LET radiation have similar effects on hematopoiesis at 3 months post IR. Bone marrow cellularity (A), SKL numbers (B), and HSC proliferation (C) as measured by BrdU incorporation at three months post exposure to 1 Gy of γ or ^{56}Fe. (D) Competitive transplantation assay of marrow harvested from irradiated cohorts at three months against unirradiated healthy marrow.

Figure 2: MMR status does not contribute to clonogenic capacity of HSCs after radiation. γ (A), proton (B), and ^{56}Fe (C) irradiated wild type and *Mlh1* knockout BM cells in CFU assays after exposure to 0.1, 0.5, 1, or 2.5 Gy of radiation (n=6, number of plates used for each radiation exposure).

Figure 3: Blood counts of wild type and *Mlh1* knockout animals demonstrate radiation induced changes, but no *Mlh1* associated defects. Complete blood counts were performed at 10 days prior to, and 2, 10, 20, and 30 days after exposure to indicated doses of γ (A, D, G), proton (B, E, H) or ^{56}Fe (C, F, I) sources. White blood cells (WBC), Red blood cells (RBC), and Platelets, are shown (n=6, number of *Mlh1*$^{+/+}$ or *Mlh1*$^{-/-}$ mice used for each radiation exposure).

Figure 4: *Mlh1* knockout mice display enhanced sensitivity to ionizing radiation. Survival plots of cohorts of mice of wild type or *Mlh1* knockout genotypes exposed to 1 Gy of γ (A), proton (B), or ^{56}Fe (C) radiation (n=9-12, number of *Mlh1*$^{+/+}$ or *Mlh1*$^{-/-}$ mice used for each radiation exposure).

Figure 5: Latent effects of radiation are independent of MMR status. Quantification of SKL cells in wild type and *Mlh1* deficient animals three months after exposure to γ (A), proton (B), and ^{56}Fe (C) radiation at indicated doses. Quantification of HPC cells in wild type and *Mlh1* deficient animals three months after exposure to γ (D), proton (E), and ^{56}Fe (F) radiation at indicated doses. Quantification of colony formation of bone marrow preparations in wild type and *Mlh1* deficient animals three months after

exposure to γ (G), proton (H), and ^{56}Fe (I) radiation at indicated doses. Marrow was collected from between 4 and 12 animals.

Figure 6: Radiation, but not *Mlh1* deficiency, reduces competitive capacity of the bone marrow after exposure. Measurement of % contribution of CD45.2 positive cells to peripheral blood at 8 (A, C, and E) and 16 (B and D) weeks after competitive transplant of 1 Gy of γ (A and B), proton (C and D), and ^{56}Fe (E) irradiated marrow of indicated genotypes.

Acknowledgement:

This study was supported by NASA NNX14AC95G, and the Cytometry & Microscopy and Radiation Resources Shared Resources of the Case Comprehensive Cancer Center (P30CA043703). We would like to thank members of NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory for help and support of our studies. We further thank Thomas F. Peterson, Jr., for his generosity.

References:

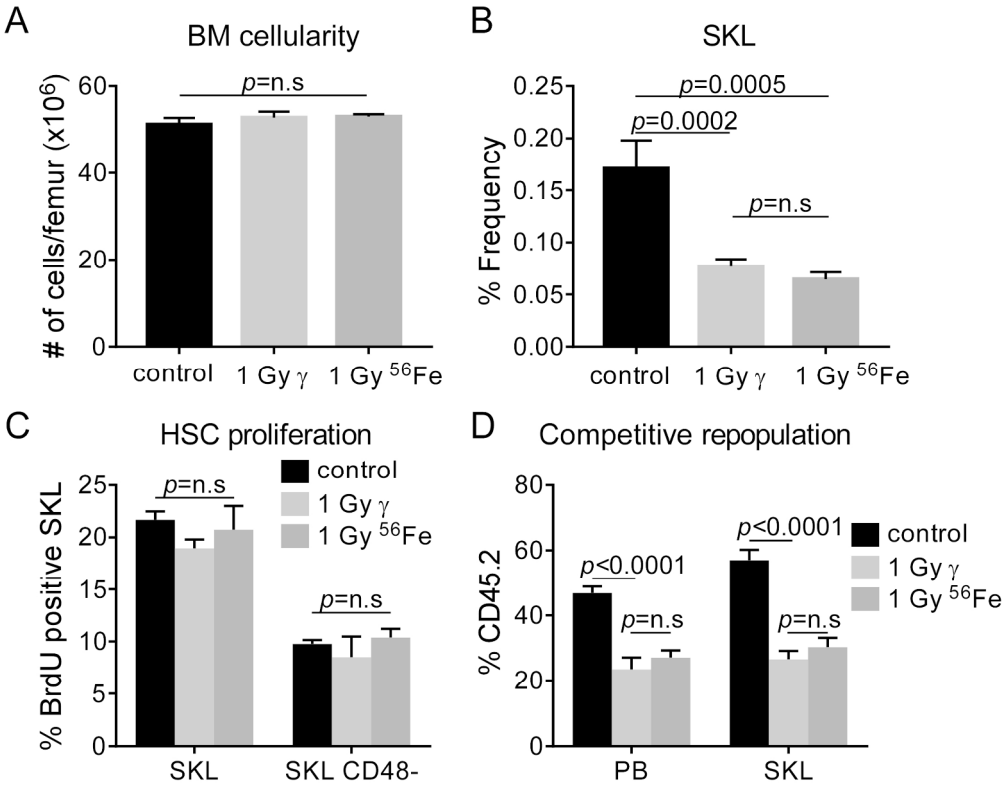
1. Kerr RA. Planetary exploration. Radiation will make astronauts' trip to Mars even riskier. **Science**. 2013;340:1031.
2. Durante M, Cucinotta FA. Heavy ion carcinogenesis and human space exploration. **Nat Rev Cancer**. 2008;8:465-472.
3. Cucinotta FA. Space radiation risks for astronauts on multiple International Space Station missions. **PLoS One**. 2014;9:e96099.
4. Zeitlin C, Hassler DM, Cucinotta FA et al. Measurements of energetic particle radiation in transit to Mars on the Mars Science Laboratory. **Science**. 2013;340:1080-1084.
5. Benton ER, Benton EV. Space radiation dosimetry in low-Earth orbit and beyond. **Nucl Instrum Methods Phys Res B**. 2001;184:255-294.
6. Cucinotta FA, Wu H, Shavers MR et al. Radiation dosimetry and biophysical models of space radiation effects. **Gravit Space Biol Bull**. 2003;16:11-18.
7. Sutherland BM, Bennett PV, Schenk H et al. Clustered DNA damages induced by high and low LET radiation, including heavy ions. **Phys Med**. 2001;17 Suppl 1:202-204.

8. Hada M, Sutherland BM. Spectrum of complex DNA damages depends on the incident radiation. **Radiat Res**. 2006;165:223-230.
9. Blakely EA, Kronenberg A. Heavy-ion radiobiology: new approaches to delineate mechanisms underlying enhanced biological effectiveness. **Radiat Res**. 1998;150:S126-145.
10. Datta K, Neumann RD, Winters TA. Characterization of complex apurinic/apyrimidinic-site clustering associated with an authentic site-specific radiation-induced DNA double-strand break. **Proc Natl Acad Sci U S A**. 2005;102:10569-10574.
11. Brooks AL, Bao S, Rithidech K et al. Induction and repair of HZE induced cytogenetic damage. **Phys Med**. 2001;17 Suppl 1:183-184.
12. Brooks A, Bao S, Rithidech K et al. Relative effectiveness of HZE iron-56 particles for the induction of cytogenetic damage in vivo. **Radiat Res**. 2001;155:353-359.
13. Waselenko JK, MacVittie TJ, Blakely WF et al. Medical management of the acute radiation syndrome: recommendations of the Strategic National Stockpile Radiation Working Group. **Ann Intern Med**. 2004;140:1037-1051.
14. Kondo M, Wagers AJ, Manz MG et al. Biology of hematopoietic stem cells and progenitors: implications for clinical application. **Annu Rev Immunol**. 2003;21:759-806.
15. Shao L, Luo Y, Zhou D. Hematopoietic stem cell injury induced by ionizing radiation. **Antioxid Redox Signal**. 2014;20:1447-1462.
16. Mauch P, Constine L, Greenberger J et al. Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. **Int J Radiat Oncol Biol Phys**. 1995;31:1319-1339.
17. Datta K, Suman S, Trani D et al. Accelerated hematopoietic toxicity by high energy (56)Fe radiation. **International journal of radiation biology**. 2012;88:213-222.
18. Muralidharan S, Sasi SP, Zuriaga MA et al. Ionizing Particle Radiation as a Modulator of Endogenous Bone Marrow Cell Reprogramming: Implications for Hematological Cancers. **Front Oncol**. 2015;5:231.
19. Weil MM, Bedford JS, Bielefeldt-Ohmann H et al. Incidence of acute myeloid leukemia and hepatocellular carcinoma in mice irradiated with 1 GeV/nucleon (56)Fe ions. **Radiat Res**. 2009;172:213-219.
20. Weil MM, Ray FA, Genik PC et al. Effects of 28Si ions, 56Fe ions, and protons on the induction of murine acute myeloid leukemia and hepatocellular carcinoma. **PLoS One**. 2014;9:e104819.
21. Tucker JD, Marples B, Ramsey MJ et al. Persistence of chromosome aberrations in mice acutely exposed to 56Fe+26 ions. **Radiat Res**. 2004;161:648-655.
22. Rithidech KN, Honikel L, Whorton EB. mFISH analysis of chromosomal damage in bone marrow cells collected from CBA/CaJ mice following whole body exposure to heavy ions (56Fe ions). **Radiat Environ Biophys**. 2007;46:137-145.
23. Miousse IR, Shao L, Chang J et al. Exposure to low-dose (56)Fe-ion radiation induces long-term epigenetic alterations in mouse bone marrow hematopoietic progenitor and stem cells. **Radiation research**. 2014;182:92-101.
24. Grosovsky AJ, de Boer JG, de Jong PJ et al. Base substitutions, frameshifts, and small deletions constitute ionizing radiation-induced point mutations in mammalian cells. **Proc Natl Acad Sci U S A**. 1988;85:185-188.
25. Steffen LS, Bacher JW, Peng Y et al. Molecular characterisation of murine acute myeloid leukaemia induced by 56Fe ion and 137Cs gamma ray irradiation. **Mutagenesis**. 2013;28:71-79.
26. Jiricny J. The multifaceted mismatch-repair system. **Nat Rev Mol Cell Biol**. 2006;7:335-346.
27. Fishel R, Lescoe MK, Rao MR et al. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. **Cell**. 1993;75:1027-1038.
28. Bronner CE, Baker SM, Morrison PT et al. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. **Nature**. 1994;368:258-261.

9. Liu B, Parsons R, Papadopoulos N et al. Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. **Nature medicine***.* 1996;2:169-174.
0. Hause RJ, Pritchard CC, Shendure J et al. Classification and characterization of microsatellite instability across 18 cancer types. **Nature medicine***.* 2016;22:1342-1350.
1. Wada C, Shionoya S, Fujino Y et al. Genomic instability of microsatellite repeats and its association with the evolution of chronic myelogenous leukemia. **Blood***.* 1994;83:3449-3456.
2. Zhu YM, Das-Gupta EP, Russell NH. Microsatellite instability and p53 mutations are associated with abnormal expression of the MSH2 gene in adult acute leukemia. **Blood***.* 1999;94:733-740.
3. Robledo M, Martinez B, Arranz E et al. Genetic instability of microsatellites in hematological neoplasms. **Leukemia***.* 1995;9:960-964.
4. Xu XS, Narayanan L, Dunklee B et al. Hypermutability to ionizing radiation in mismatch repair-deficient, Pms2 knockout mice. **Cancer research***.* 2001;61:3775-3780.
5. Kenyon J, Fu P, Lingas K et al. Humans accumulate microsatellite instability with acquired loss of MLH1 protein in hematopoietic stem and progenitor cells as a function of age. **Blood***.* 2012;120:3229-3236.
6. Kenyon J, Nickel-Meester G, Qing Y et al. Epigenetic Loss of MLH1 Expression in Normal Human Hematopoietic Stem Cell Clones is Defined by the Promoter CpG Methylation Pattern Observed by High-Throughput Methylation Specific Sequencing. **Int J Stem Cell Res Ther***.* 2016;3.
7. Gridley DS, Pecaut MJ, Nelson GA. Total-body irradiation with high-LET particles: acute and chronic effects on the immune system. **American journal of physiology Regulatory, integrative and comparative physiology***.* 2002;282:R677-688.
8. Ainsworth EJ. Early and late mammalian responses to heavy charged particles. **Adv Space Res***.* 1986;6:153-165.
9. Edelmann W, Yang K, Kuraguchi M et al. Tumorigenesis in Mlh1 and Mlh1/Apc1638N mutant mice. **Cancer research***.* 1999;59:1301-1307.
0. Tokairin Y, Kakinuma S, Arai M et al. Accelerated growth of intestinal tumours after radiation exposure in Mlh1-knockout mice: evaluation of the late effect of radiation on a mouse model of HNPCC. **International journal of experimental pathology***.* 2006;87:89-99.
1. Szilvassy SJ, Humphries RK, Lansdorp PM et al. Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. **Proc Natl Acad Sci U S A***.* 1990;87:8736-8740.
2. Szilvassy SJ, Nicolini FE, Eaves CJ et al. Quantitation of murine and human hematopoietic stem cells by limiting-dilution analysis in competitively repopulated hosts. **Methods Mol Med***.* 2002;63:167-187.
3. Rossi DJ, Bryder D, Seita J et al. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. **Nature***.* 2007;447:725-729.
4. Nijnik A, Woodbine L, Marchetti C et al. DNA repair is limiting for haematopoietic stem cells during ageing. **Nature***.* 2007;447:686-690.
5. Lynch HT, Lynch PM. Colorectal cancer: Update on the clinical management of Lynch syndrome. **Nat Rev Gastroenterol Hepatol***.* 2013;10:323-324.
6. Naka K, Hirao A. Maintenance of genomic integrity in hematopoietic stem cells. **Int J Hematol***.* 2011;93:434-439.
7. Hall EJ, Giaccia AJ. Radiobiology for the radiologist*.* Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2012:546.
8. Wang GJ, Cai L. Induction of cell-proliferation hormesis and cell-survival adaptive response in mouse hematopoietic cells by whole-body low-dose radiation. **Toxicological sciences : an official journal of the Society of Toxicology***.* 2000;53:369-376.

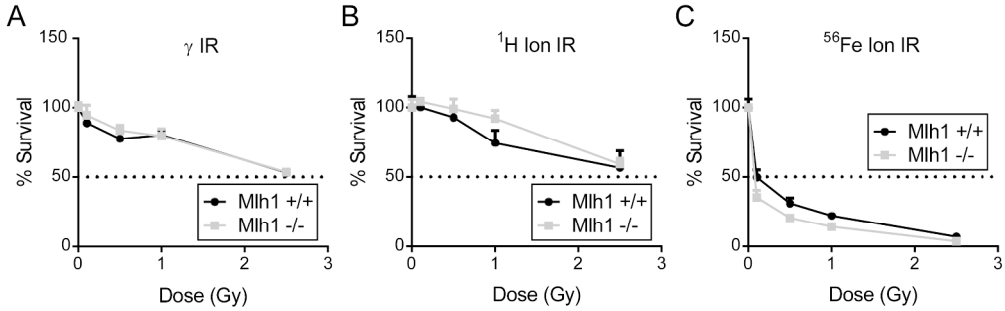
9. Chang J, Feng W, Wang Y et al. Whole-body proton irradiation causes long-term damage to hematopoietic stem cells in mice. **Radiation research**. 2015;183:240-248.
0. Davies H, Morganella S, Purdie CA et al. Whole-Genome Sequencing Reveals Breast Cancers with Mismatch Repair Deficiency. **Cancer research**. 2017;77:4755-4762.

Patel, Figure 1



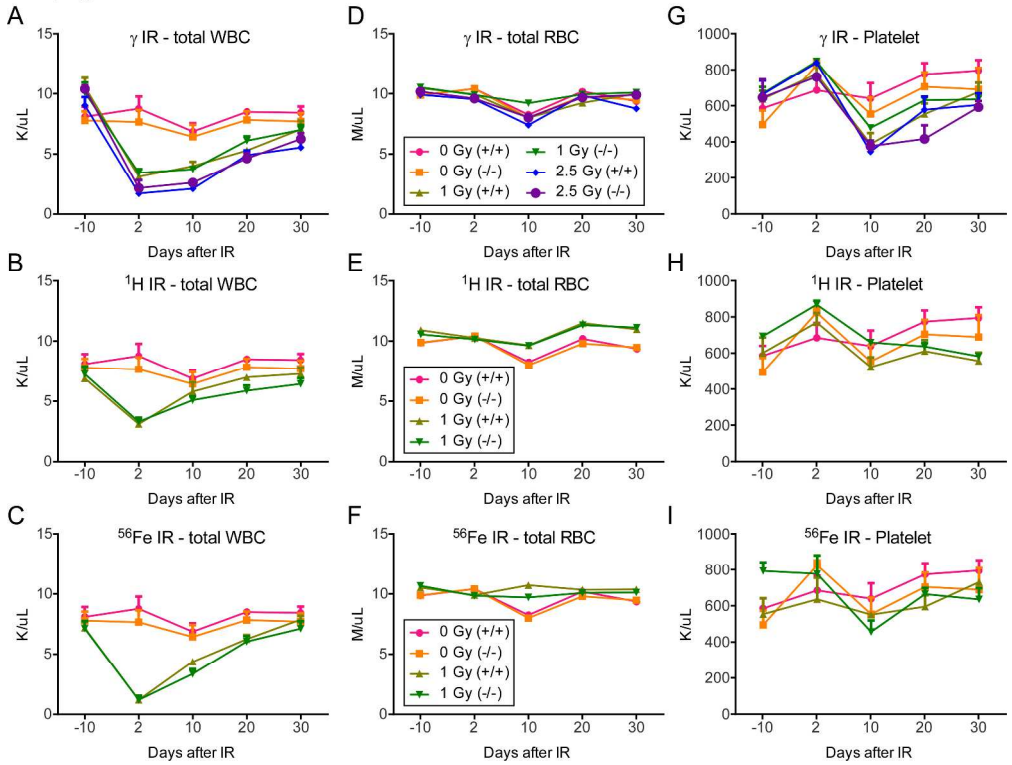
194x161mm (300 x 300 DPI)

Patel, Figure 2



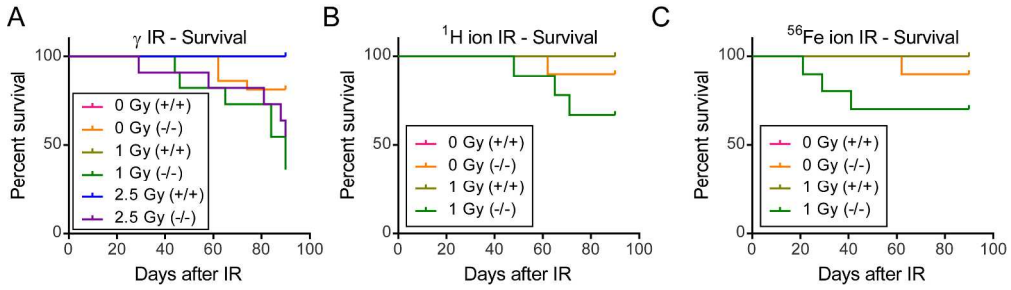
244x81mm (300 x 300 DPI)

Patel, Figure 3



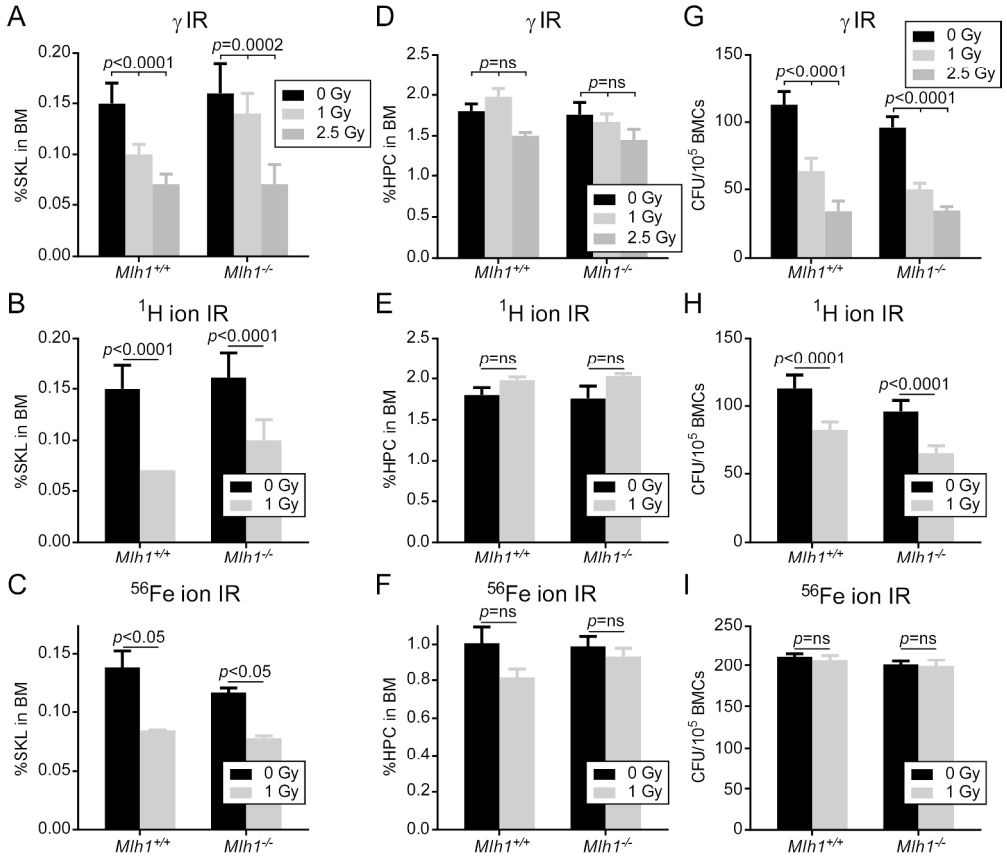
282x220mm (300 x 300 DPI)

Patel, Figure 4



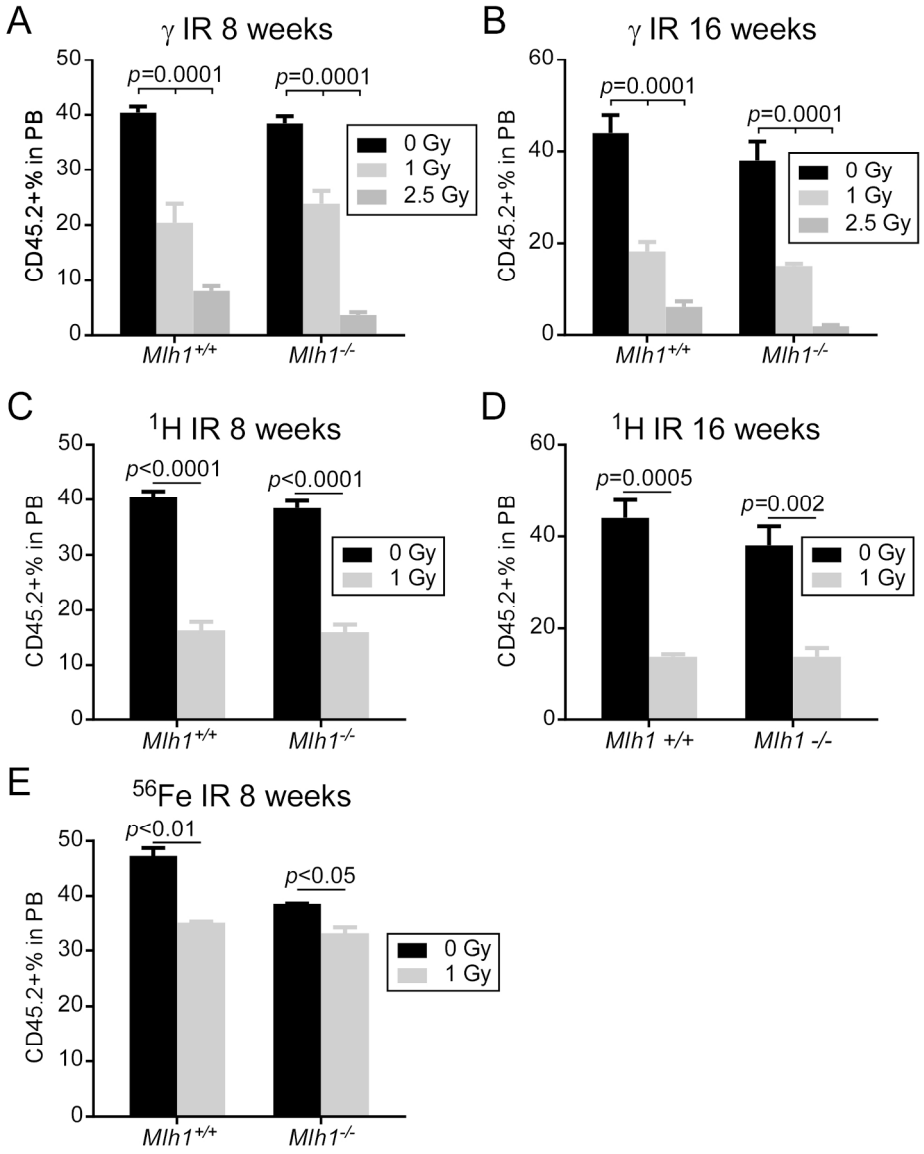
259x80mm (300 x 300 DPI)

Patel, Figure 5

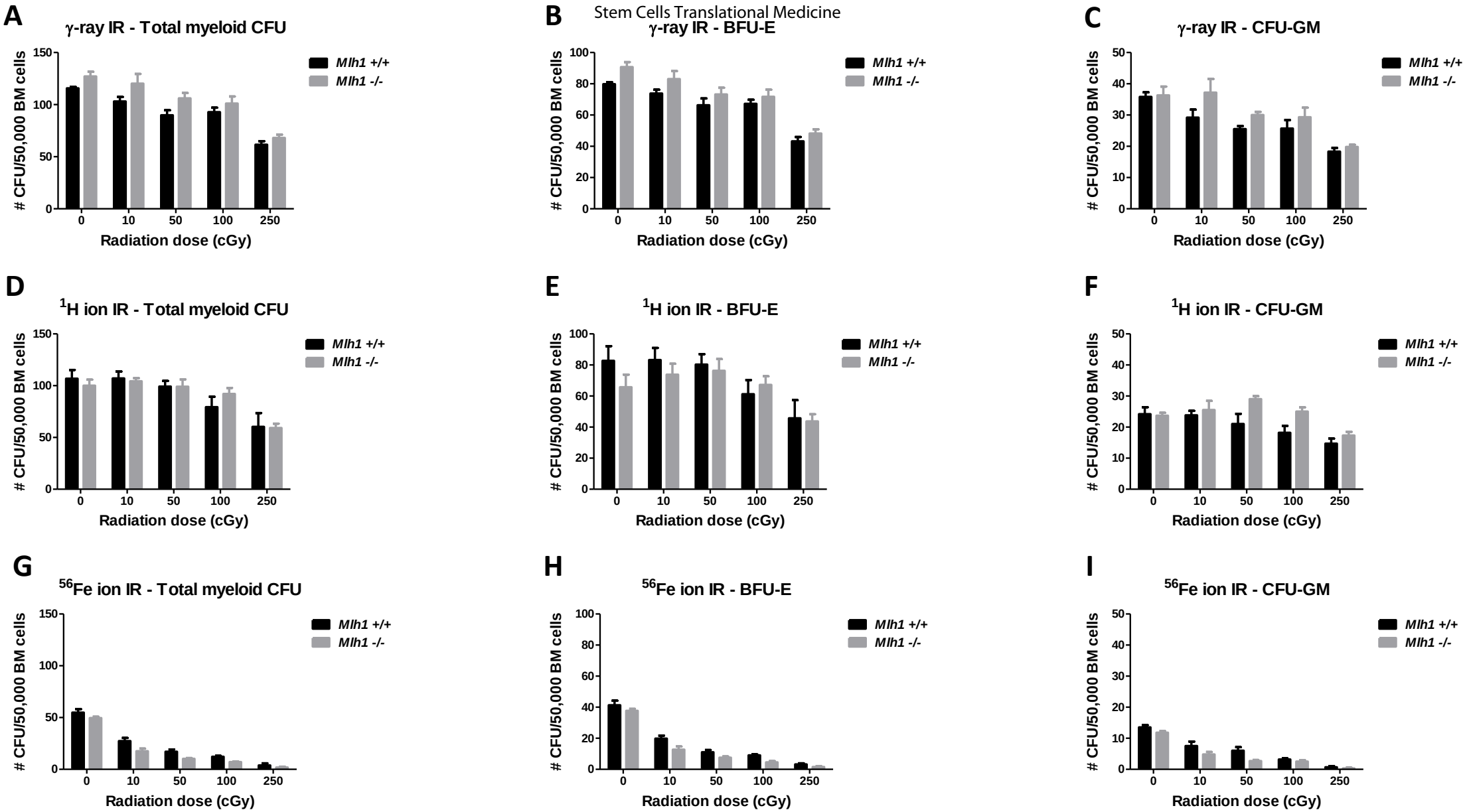


249x221mm (300 x 300 DPI)

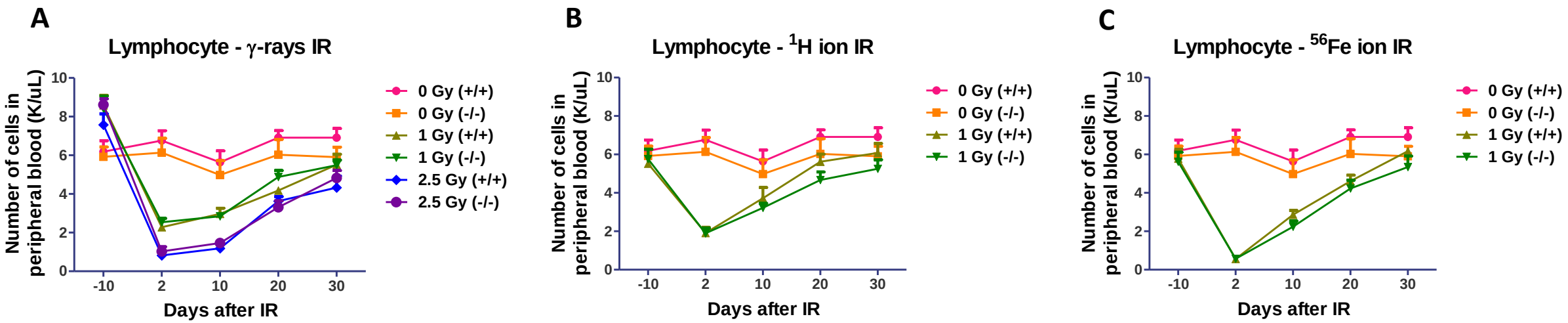
Patel, Figure 6



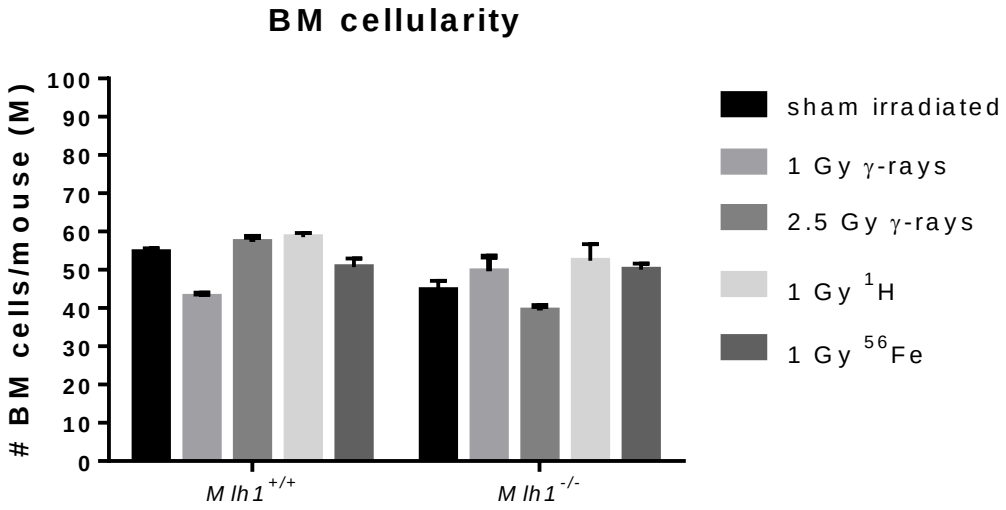
169x221mm (300 x 300 DPI)



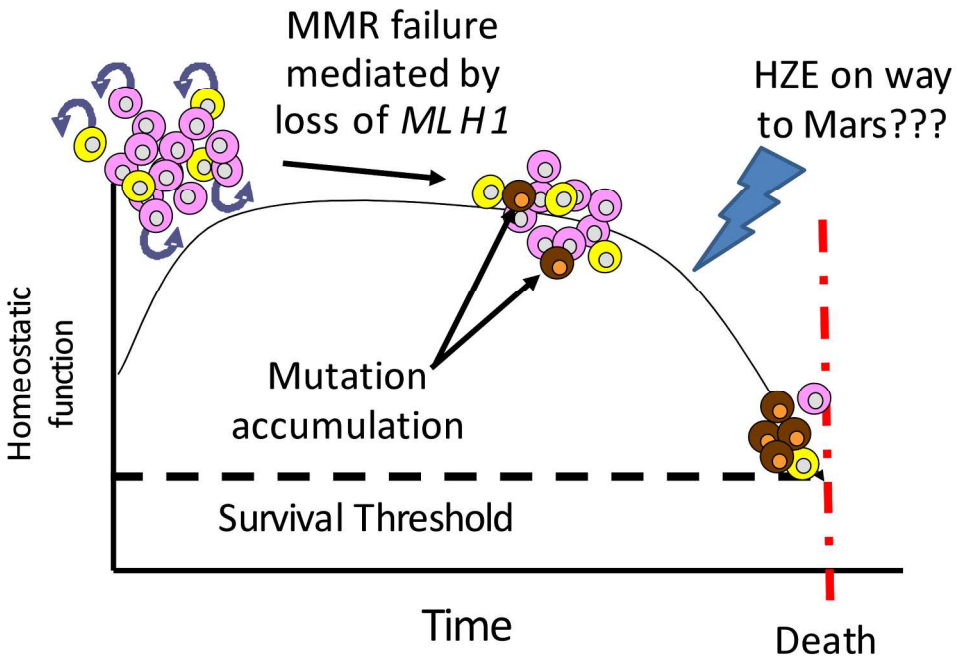
Supplementary figure 1: Myeloid CFU survival post radiation exposure. (A-C) Number of total myeloid CFU, BFU-E, and CFU-GM post different doses of gamma-rays IR. (D-F) Number of total myeloid CFU, BFU-E, and CFU-GM post different doses of proton ion IR. (G-I) Number of total myeloid CFU, BFU-E, and CFU-GM post different doses of ^{56}Fe ion IR.



Supplementary figure 2: Lymphocyte counts in *Mlh1*$^{+/+}$ and *Mlh1*$^{-/-}$ mice pre and post (A) γ-rays IR, (B) ^{1}H ion IR, and (C) ^{56}Fe ion IR.



Supplementary figure 3: Bone marrow cellularity three months after exposure to IR is unchanged in control and *Mlh1*$^{-/-}$ animals.



The hematopoietic system is essential for life, and normally has the capacity to sustain function for the duration of our lifetimes in spite of natural declination, which is associated with loss of DNA repair (including as DNA mismatch repair (MMR). Astronauts are exposed to ionizing radiation sources that are not commonly found on earth (such as HZE ions) and thus may display unforseen risks that need accounting in NASA risk models.

200x140mm (300 x 300 DPI)