AWARD NUMBER: W81XWH-18-1-0162

TITLE: Modeling the Impact of Radiation Protectors on Radiation-induced Sarcoma Risk

PRINCIPAL INVESTIGATOR: David G. Kirsch, MD, PhD

CONTRACTING ORGANIZATION: Duke University

REPORT DATE: November 2020

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
November 2020	Final	08/01/2018 - 07/31/2020

4. TITLE AND SUBTITLE

Modeling the Impact of Radiation Protectors on Radiation-Induced Sarcoma Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-18-1-0162

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S) David G. Kirsch, Andrea R. Daniel

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U. S. Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The threat of a radiation disaster, such as a nuclear accident or terrorist attack, is a growing concern for the military and warrants the development of medical countermeasures to prevent and mitigate acute and delayed radiation injury. We will evaluate the risks of delayed radiation-induced carcinogenesis after radiation alone or in a setting that models radiation protection strategies that block p53-dependent cell death pathways. To reduce radiation-induced cancer risk and to steer development of rationally designed pharmacological agents to mitigate radiation injury, we aim to elucidate the biological drivers of radiation-induced late effects. Understanding the link between improved survival of irradiated cells and cancer development will aid in selecting pharmacological strategies that not only prevent acute radiation injury, but also do not increase radiation carcinogenesis, thus improving outcomes for soldiers exposed to radiation.

15. SUBJECT TERMS

sarcoma, acute radiation syndrome, p53, satellite cells, radiation carcinogenesis, countermeasures, cancer, tumor, radiation injury, tumor suppressor

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU		USAMRMC

TABLE OF CONTENTS

Page

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

   The threat of a radiation disaster, such as a nuclear accident or terrorist attack, is a growing concern for the military and warrants the development of medical countermeasures to prevent and mitigate acute and delayed radiation injury. We will evaluate the risks of delayed radiation-induced carcinogenesis after radiation alone or in a setting that models radiation protection strategies that block p53-dependent cell death pathways. To reduce radiation-induced cancer risk and to steer development of rationally designed pharmacological agents to mitigate radiation injury, we aim to elucidate the biological drivers of radiation-induced late effects. Understanding the link between improved survival of irradiated cells and cancer development will aid in selecting pharmacological strategies that not only prevent acute radiation injury, but also do not increase radiation carcinogenesis, thus improving outcomes for soldiers exposed to radiation. We hypothesize that blocking cell death in irradiated tissues will increase survival of mice exposed to radiation, but damaged cells that would have died will develop into cancers like sarcomas. Late effects of radiation include life-threatening sarcomas or other solid tumors. The overall goal of this proposal is to evaluate whether strategies to prevent acute radiation injury by blocking the death of irradiated cells alter the risk of subsequently developing late effects of radiation including sarcomas. We utilize mice with inducible p53 shRNA expression to temporarily knockdown p53 during left hind leg irradiation compared to littermate controls. Mice are followed for tumor development and evaluated for mechanistic studies, which includes assessing cell fate of muscle satellite cells after radiation and p53 transcriptional programs.

2. **KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

   sarcoma, acute radiation syndrome, p53, satellite cells, radiation carcinogenesis, countermeasures, cancer, tumor, radiation injury, tumor suppressor

3. **ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

   What were the major goals of the project?
   List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

   Specific Aims

   1. Determine the effect of blocking p53-induced cell death on radiation-induced sarcoma.
      1.1 Evaluate the effect of blocking p53 on hind limb sarcoma development in irradiated mice
      1.2 Examine the genetic mechanisms by which radiation promotes sarcomagenesis

   2. Dissect the mechanisms of radiation-induced sarcoma development by examining p53-dependent satellite cell fate following irradiation.

2.1 Test whether transient knockdown of p53 protects muscle stem (satellite) cells from radiation induced death
2.2 Determine if radiation induces a selective advantage for the outgrowth of preexisting p53 mutant tumor-initiating satellite cells
2.3 Examine the specific p53 mediated transcriptional programs necessary for tumor suppression of radiation-induced sarcoma

Major Task 1 (Aim 1)

The first major goal for task 1 is evaluating the effect of blocking p53 on hind limb sarcoma development in irradiated mice (Aim 1.1). We followed cohorts of mice for sarcoma development for this task and the results are described below. We analyzed the radiation induced injury data from these mice and the results are described below.

The second major goal for task 1 was to examine the genetic mechanisms by which radiation promotes sarcomagenesis by using whole exome sequencing of tumor and paired normal tissues (Aim 1.2). We successfully completed this goal, which resulted in a publication (Lee, Mowery, Daniel, et al., *JCI Insight* 2019). Notably, we hypothesized that radiation-induced tumors would exhibit a common genetic signature and specific recurring mutations that drive sarcomagenesis. While we did identify a genetic signature for radiation-induced tumors, no specific driver mutations were found. Therefore, we hypothesize that radiation-induced sarcomas may be driven by transcriptional mechanisms regulating RNA expression rather than driver mutations in the DNA. Therefore, we submitted radiation-induced sarcoma samples for RNA sequencing to further explore this hypothesis. We are in the process of analyzing the RNA sequencing data.

Major Task 2 (Aim 2)

The first major goal for task 2 is to test whether transient knockdown of p53 protects muscle stem (satellite) cells from radiation induced death (Aim 2.1). We established a reliable protocol for isolating and quantifying muscle stem cells. Our preliminary data suggests that temporary knockdown of p53 in mice does increase muscle stem cell survival after high dose irradiation. Experiments to further address this question are currently ongoing.

The second major goal for task 2 is to determine if radiation induces a selective advantage for the outgrowth of preexisting p53 mutant tumor-initiating satellite cells (Aim 2.2). These experiments were designed to address the hypothesis that the mechanism of radiation-induced sarcoma development in the setting of temporarily reduced p53 would be similar to our prior studies in radiation-induced thymic lymphoma development (Lee et al., Nature Communications 2015). In particular, we hypothesized that like thymic lymphomas, sarcomas would develop after radiation in a non-cell autonomous manner. In radiation-induced thymic lymphomas, irradiated bone marrow cells with decreased p53 survive radiation insult and function to prevent the development of lymphoma by competing with preexisting tumor initiating cells. In contrast, bone marrow cells with wild type p53 levels die by radiation-induced apoptosis, which allows preexisting tumor initiating cells to expand into the niche free of competition and form a lymphoma. Importantly, evidence from our studies in Task 1 indicates that radiation-induced sarcomas do not develop in a similar manner to radiation-induced thymic lymphomas. Instead, results from Task 1 support an alternative model of a cell autonomous mechanism for radiation-induced sarcomas. Specifically, while temporary p53

knockdown prevents radiation-induced thymic lymphoma development, we find that temporary p53 knockdown increases radiation-induced sarcoma development. Furthermore, using whole exome sequencing, we identified a strong oxidative mutation genetic signature in the radiation-induced sarcomas (Lee, Mowery, Daniel, et al., *JCI Insight* 2019). These data taken together indicate a cell autonomous mechanism whereby muscle stem cells with low p53 levels undergo radiation mediated genetic damage, but do not undergo apoptosis. Thus, our revised working model is that damaged muscle stem cells survive radiation insult and go on to form a sarcoma, which is strongly supported by data generated in Task 1. Thus, as this sub-aim was designed to test a model in Task 2 that is no longer supported by the available data, we did not initiate this experiment and removed this experiment from our statement of work and milestones on last year's annual report.

The third major goal of Task 2 is to examine the specific p53 mediated transcriptional programs necessary for tumor suppression of radiation-induced sarcoma (Aim 2.3). We proposed to use *Myf6Cre; p53$^{LSL\text{-}25,\ 26/FL}$* and *Myf6Cre; p53$^{LSL\text{-}25,26,\ 53,\ 54/FL}$* mice with littermate control mice for this sub-aim of Task 2 in the annual report last year. These experiments are ongoing.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1) Major activity (Aim 1)

We irradiated *Actin-rtTA; TRE-p53.1224* and littermate control mouse cohorts on a C3H and C57Bl/6J mixed genetic background. 3 to 4-month-old male and female mice were placed on a doxycycline (Dox) containing diet for 10 days to knockdown p53 prior to delivery of 30 Gy or 40 Gy to the left hind limb using a micro-irradiator (225 kVp X-rays). Following irradiation, mice were placed on standard chow. Mice were examined weekly for signs of tumors. Once tumors developed, they were harvested for histological and molecular characterization. In addition, normal (liver, muscle) tissues were banked for future whole exome and RNA sequencing experiments as a germline/normal comparison for the radiation-induced sarcomas. We also continue to follow the *CMV-rtTA; TRE-p53.1224* C3H background or littermate control cohorts that received a single dose of 30 Gy left hind limb irradiation. We are also following additional control cohorts of unirradiated *Actin-rtTA* or *CMV-rtTA; TRE-p53.1224* and littermate control mice (n=30 per sex per genotype) and irradiated mice that were not fed Dox diet. Log-rank test will be used to perform statistical analysis on the data as previously described.

To determine the histological subtypes of radiation-induced sarcomas that developed in the mice we used formalin fixed paraffin embedded samples. Sections from each tumor were stained with specific antibodies against known markers for tumor characterization.

2 to 4) Specific objective, results, and conclusions (Aim 1.1 Evaluate the effect of blocking p53 on hind limb sarcoma development in irradiated mice)

To address whether improving survival of irradiated cells by temporarily reducing p53 levels increases radiation-induced solid tumors, we examined radiation-induced sarcoma development in the setting of temporary p53 knockdown. Our data show that mice with all the genetic components to achieve temporary p53 knockdown while on the Dox diet exhibited an increased incidence of radiation-induced sarcoma development compared to control mice. Indeed, 20% of temporary p53 knockdown animals that received 30 Gy to the left hind limb developed a sarcoma in the radiation field compared to 0% of control animals (Figure 1A). Likewise, 25% of the temporary p53 knockdown animals that received 40 Gy developed an in-field sarcoma whereas only 4% of the control mice developed left hind limb sarcomas (Figure 1B). These data demonstrate that temporary p53 knockdown increases the risk of sarcoma development following single-dose irradiation. The histological subtype of the radiation-induced sarcomas was evaluated (Table 1).

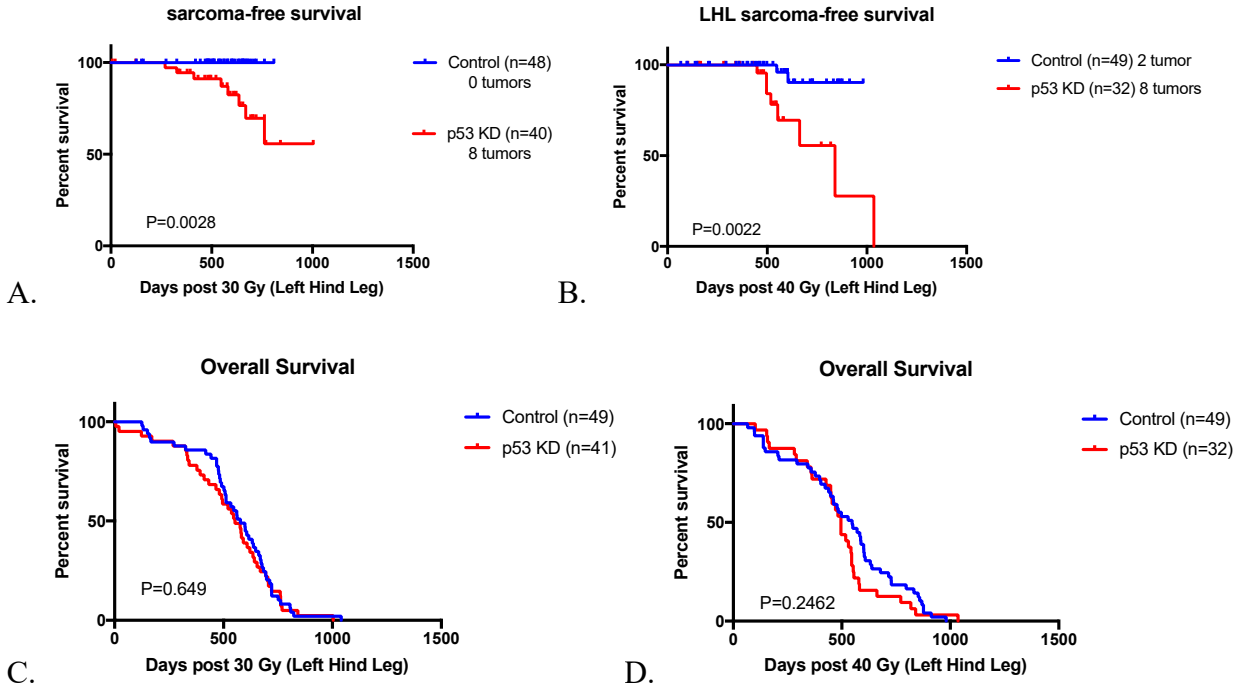

Figure 1. A. Kaplan-Meier curves showing left hind limb (LHL) sarcoma-free survival of *Actin-rtTA; TRE-p53.1224* (p53 knockdown or p53 KD) mice compared to littermate controls after 30 Gy left hind limb irradiation. B. Kaplan-Meier curves showing LHL sarcoma-free survival of *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 40 Gy irradiation. C. Kaplan-Meier curves showing overall survival of *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 30 Gy LHL irradiation. D. Kaplan-Meier curves showing overall survival of *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 40 Gy LHL irradiation. Log-rank test was used to generate p values.

Table 1

Mouse number	Genotype	Days at death	Injury score at death	Sex	IR Dose	Sarcoma subtype
320114	p53KD	411	4	F	30	Leiomyosarcoma or myofibrotic sarcoma
223181	p53KD	269	0.5	M	30	UPS
320006	p53KD	580	2.5	M	30	UPS
320239	p53KD	326	3.75	M	30	Pleomorphic myofibroblastic sarcoma
223178	p53KD	762	3.75	M	30	UPS
320258	p53KD	546	3.75	F	30	UPS
320085	p53KD	635	3.75	M	30	Angiosarcoma
320109	p53KD	669	4	M	30	UPS
223030	control	604	3.5	M	40	UPS
320177	control	548	4	F	40	UPS
222933	p53KD	496	3.5	M	40	UPS
320359	p53KD	518	3.75	M	40	UPS or leiomyosarcoma
320179	p53KD	662	4	M	40	Giant cells, pleomorphic sarcoma
223032	p53KD	496	3.5	M	40	Leiomyosarcoma or myofibrotic sarcoma
320132	p53KD	1035	4	F	40	UPS
320354	p53KD	840	4	F	40	Myxoid sarcoma, high grade spindle cell
320358	p53KD	553	3.5	M	40	UPS

We previously showed that muscle tissue injury and wounds promote sarcoma development (Van Mater et al. *Cancer Research*, 2015). Radiation-induced chronic wounds occur due to an acute wound that fails to heal or arise months to years after radiation exposure in tissue that initially appears to have recovered from acute toxicity. Late persistent wounds are characterized by inflammation, ulceration, fibrosis, or necrosis of soft tissue, cartilage and bone. Damage to the vasculature of irradiated tissues may contribute to impaired wound healing due to a lack of neovascularization and thus insufficient perfusion. The cohorts of irradiated (and unirradiated control) mice were followed for the development of acute and late wounds by scoring the level of tissue injury on a weekly basis. Mice were evaluated based on a previously published rubric for skin injury that we adapted to comprehensively assess radiation-induced normal tissue toxicity of the skin, bone, and muscle (Douglas et al. *Radiation Researc*h, 1976). Mice exhibiting signs of injury (skin breakdown and/or swelling) were given a score of 1 and scoring increases with severity to a score of 4, or loss of the foot. Temporarily reducing p53 expression increases the number of mice that sustain a chronic wound following single high dose left hind limb irradiation at 30 Gy (Figure 2A-C). However, this difference was diminished in the 40Gy cohort, p53 KD resulted in increased numbers of mice that sustained a chronic injury score of 2 only. Our data revealed that mice that sustained an acute radiation-induced injury within the first 90 days post irradiation were more likely to develop a late chronic wound (Figure 4A). Notably, severe chronic radiation-induced injuries (score 3+) were associated with

sarcoma development (Figure 4B). The average final injury score (Table 1) for tumor bearing mice was 3.54 while the average final injury score for all irradiated mice was 2.28. These data suggest that radiation-induced injury plays a stimulatory role in sarcoma development.

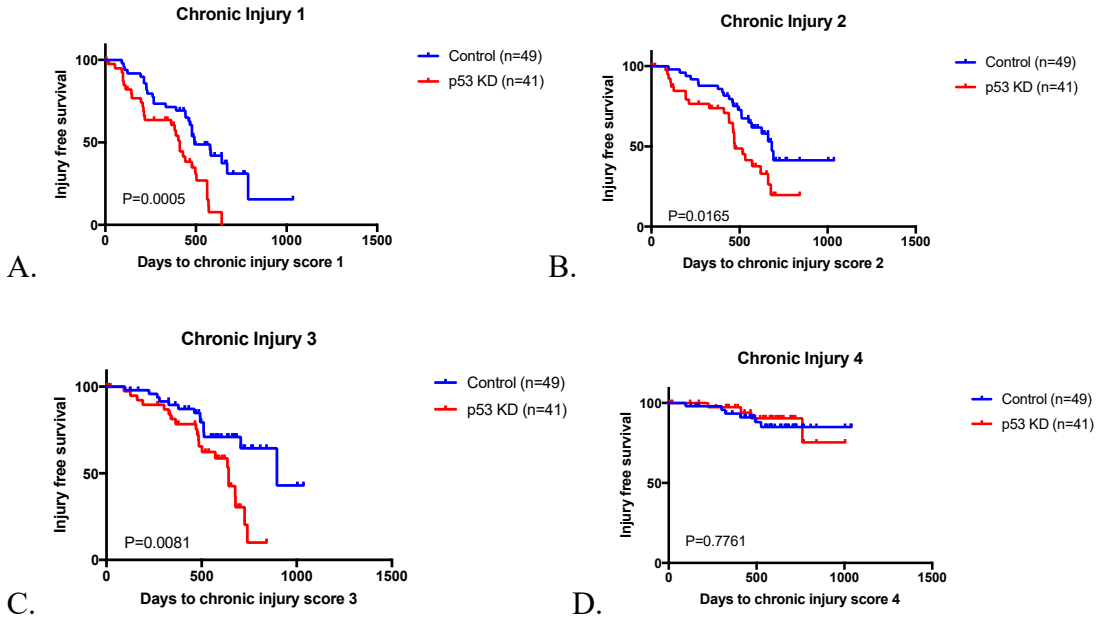

Figure 2. A. Kaplan-Meier curves showing injury free survival form a chronic injury score of 1in *Actin-rtTA; TRE-p53.1224* (p53 knockdown or p53 KD) mice compared to littermate controls after 30 Gy left hind limb irradiation. B. Kaplan-Meier curves showing injury free survival form a chronic injury score of 2 of *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 30 Gy irradiation. C. Kaplan-Meier curves showing injury free survival from a chronic injury score of 3in *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 30 Gy LHL irradiation. D. Kaplan-Meier curves showing injury free survival form a chronic injury score of 4 in *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 30 Gy LHL irradiation. Log-rank test was used to generate p values.

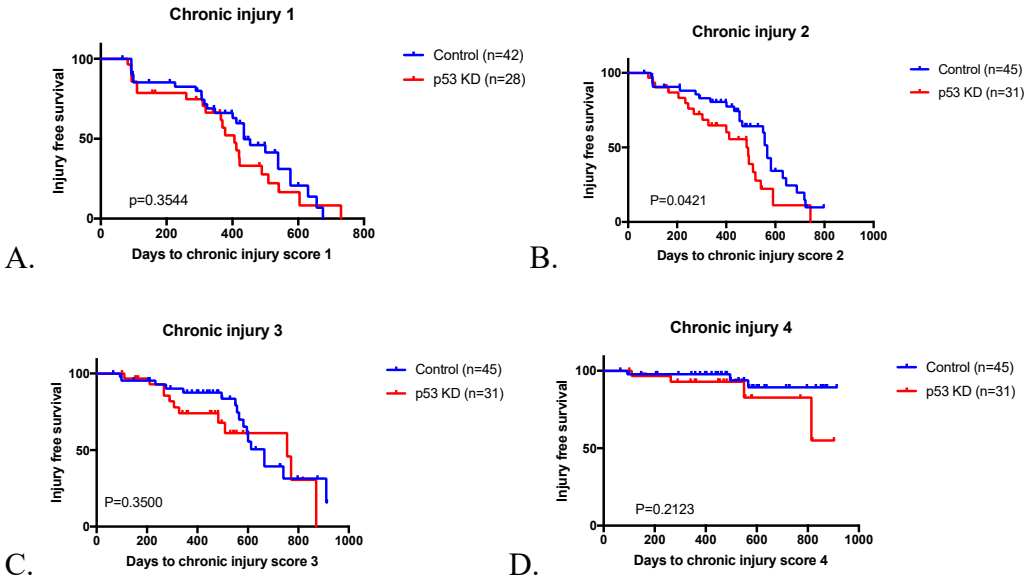

Figure 3. A. Kaplan-Meier curves showing injury free survival form a chronic injury score of 1in *Actin-rtTA; TRE-p53.1224* (p53 knockdown or p53 KD) mice compared to littermate controls after 40 Gy left hind limb irradiation. B. Kaplan-Meier curves showing injury free survival form a chronic injury score of 2 of *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 40 Gy irradiation. C. Kaplan-Meier curves showing injury free survival from a chronic injury score of 3in *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 40 Gy LHL irradiation. D. Kaplan-Meier curves showing injury free survival form a chronic injury score of 4 in *Actin-rtTA; TRE-p53.1224* (p53 KD) mice compared to littermate controls after 40 Gy LHL irradiation. Log-rank test was

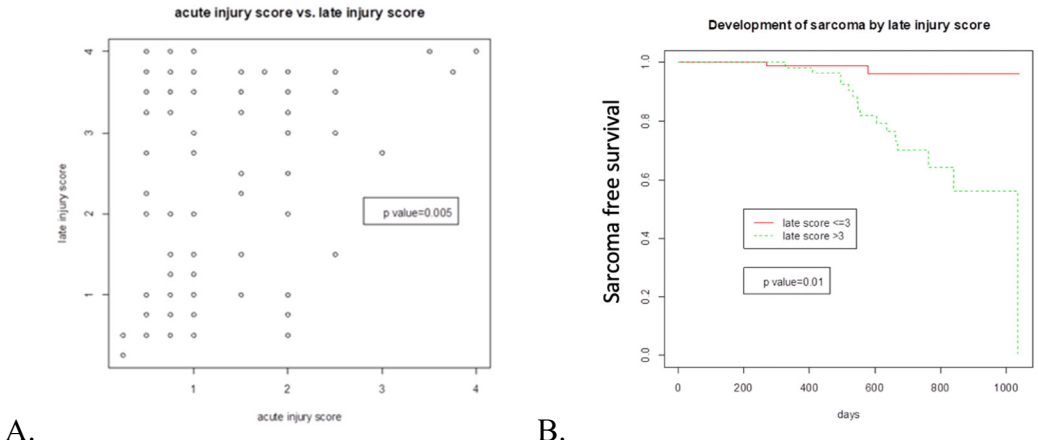

Figure 4. A. Correlation coefficient analysis showing high acute injury scores are associated with high chronic injury scores. B. Cox model using three co-variants for analysis (dose, genotype, injury) showing chronic radiation-induced injuries scoring 3 or above are associated with sarcoma development.

2 to 4) Specific objective, results, and conclusions (Aim 1.2 Examine the genetic mechanisms by which radiation promotes sarcomagenesis)

To identify mutational signatures specific to radiation-induced tumors and to gain insight into how distinct cell-intrinsic and -extrinsic factors affect cancer development within the same tissue type, we performed genomic analysis across murine soft-tissue sarcomas induced by mutagen, MCA, oncogenic mutations, or ionizing radiation. Radiation-induced sarcomas were generated by focally irradiating the mouse hind limb using a single dose of 30 or 40 Gy. For comparison to radiation-induced sarcomas, we used an established genetically engineered mouse model of soft-tissue sarcoma in which localized delivery of Cre recombinase into the muscle of the hind limb activates oncogenic $Kras^{G12D}$ and deletes both alleles of *p53* (Kirsch et al. *Nature Medicine,* 2007). In addition, we generated MCA-induced sarcomas in the hind limb of either WT or $p53^{fl/fl}$ mice in which both copies of *p53* were deleted by Cre recombinase. Using these mouse models of oncogene-driven, chemical carcinogen–induced, or radiation-induced soft-tissue sarcoma, we performed whole-exome sequencing (WES) on paired tumor and normal tissue from each mouse and observed distinct facultative molecular signatures that are specific to each carcinogenic driver. Remarkably, ionizing radiation produced tumors with relatively low levels of nonsynonymous mutations, but a high frequency of somatic copy number alterations, with a preponderance of deletions and a tendency toward C-to-T and G-to-A transitions. The results from this study were published in *JCI Insight*.

Lee CL*, Mowery YM*, Daniel AR*, Zhang D, Sibley AB, Delaney JR, Wisdom AJ, Qin X, Wang X, Caraballo I, Gresham J, Luo L, Van Mater D, Owzar K, Kirsch DG. Mutational landscape in genetically engineered, carcinogen-induced, and radiation-induced mouse sarcoma. *JCI Insight*. 2019 Jul 11;4(13). pii: 128698. doi: 10.1172/jci.insight.128698. PMID: 31112524. *equal contribution.

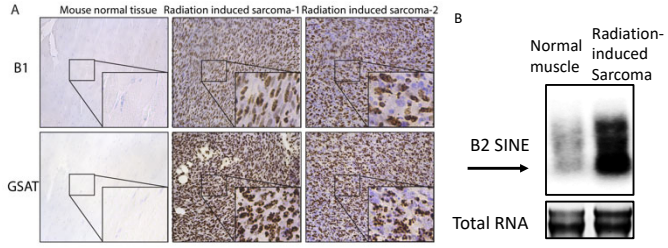

Figure 5. **Radiation-induced mouse sarcomas express high levels of repetitive elements.** A) RNA in situ hybridization of B1 SINE and GSAT in mouse normal muscle tissue (left) and radiation-induced sarcoma samples. B) Northern blot for B2 SINE RNA expression in normal muscle and radiation-induced sarcoma.

Our whole exome sequencing (WES) data defined distinguishing genetic features of radiation-induced sarcomas, such as C-to-T transitions, surprisingly there are no specific recurring mutations that drive radiation-induced sarcomas. Rather, our preliminary data shows that repetitive elements are highly expressed in radiation-induced sarcomas (Figure 5) raising the possibility that they may elements are de-repressed in a variety of human cancers. Approximately 50% of the mammalian genome consists of repetitive elements, the majority of which are retrotransposons. Classically, retrotransposon activation has been assumed to be a side effect of the epigenetic deregulation underlying the transformed state. Recent studies have revealed transposable elements as a rich source of cryptic regulatory sequences, which can be co-opted to drive oncogene expression in a process termed onco-exaptation. Onco-exaptation is widespread in cancer as more than 50% of human tumors in The Cancer Genome Atlas (TCGA) drive expression of at least one oncogene from a cryptic promoter in a repetitive element. Therefore, we **hypothesized** that radiation exposure can lead to expression of repetitive elements that were developmentally silenced, which can in turn lead to oncogene expression via onco-exaptation that drive sarcoma development.

We used RNA sequencing (150bp paired end reads, 300 million total reads per sample), to examine gene expression patterns, structural genetic alterations, repetitive element expression, and onco-exaptation events in normal muscle controls and in 18 radiation-induced mouse sarcomas. In collaboration with the Duke Center for Genomic and Computational Biology we developed a computational pipeline for evaluating onco-exaptation events from human RNAseq data based on published protocols and have adapted it for use with mouse RNAseq results. Preliminary analysis from a paired radiation-induced sarcoma and normal muscle revealed several unique oncogene exaptation events, including expression of a novel chimeric Raf1 transcripts driven by an intronic SINE B4. Raf1, a MAPK pathway member, is known to drive sarcoma cell proliferation. SINEB4-Raf1 transcripts lacking Exon 1, which encodes an autoinhibitory domain, account for 42% of the Raf1 expressed in the tumor tissue (Figure 6). Chimeric Raf1 transcripts were identified in 7 of 18 radiation-induced tumors and 0 of 5 normal muscles. We are currently continuing to analyze and validate the RNAseq data set.

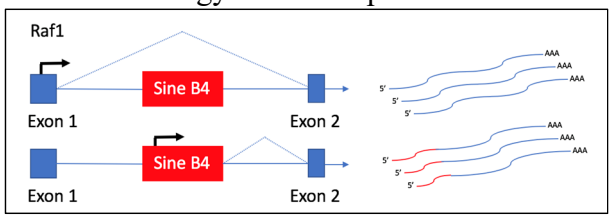

Figure 6. The Raf1 gene driven by the canonical promoter (top) or SINE-B4 to generate a novel oncogenic transcript (bottom).

1) Major activity (Aim 2)

To explore the mechanism by which radiation-induced sarcomagenesis is altered with temporary p53 knockdown, we are examining the fate of muscle stem/progenitor (satellite) cells following irradiation. We are using *Pax7-nGFP* mice to identify and isolate Pax7-expressing satellite cells in order to examine whether muscle stem/progenitor cells are protected from radiation-induced cell death by p53 knockdown and whether surviving satellite cells compete with or become tumor-initiating cells during sarcoma development. We have established a protocol to examine surviving satellite cell fractions by flow cytometry on muscle (irradiated and unirradiated hind legs) harvested from *Pax7-nGFP*; *Actin-rtTA; TRE-p53.1224* and *Pax7-nGFP* control mice. Three to four-month-old male and female mice were placed on a dox containing diet for 10 days prior to delivery of 30 Gy to the left hind limb using a micro-irradiator (225 kVp X-rays). Following irradiation, mice were placed on standard chow and 48 hours later the leg muscles were harvested for analysis. The satellite cell isolation protocol was adapted from (Lui et al. Nature Protocols, 2015). Flow cytometry was performed to quantify the percentage of Pax7 positive, GFP expressing (satellite cells). These experiments are currently ongoing.

2 to 4) Specific objective, results, and conclusions (Aim 2)

The experiments for Aim 2.1 and 2.3 are in progress. For Aim 2.3 we have irradiated *Myf6Cre; p53$^{LSL\text{-}25,\ 26/FL}$* and *Myf6Cre; p53$^{LSL\text{-}25,26,\ 53,\ 54/FL}$* mice with littermate control mice and we are following them for injury and tumor development.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training"

activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The data from the radiation-induced sarcoma studies were analyzed in conjunction with data from other graduate student and postdoc projects studying other mouse models of sarcoma in the lab, which were supported by other funding sources. The data were combined for the manuscript published in *JCI Insight*. Trainees that worked on the combined publication increased their skills through data analysis, manuscript preparation and publication.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Data from this project is the subject of an abstract accepted for an oral presentation by Andrea Daniel at the Radiation Research Society Annual meeting on Oct 18-21, 2020.

Data from this project was published in the following manuscript:

Lee CL*, Mowery YM*, Daniel AR*, Zhang D, Sibley AB, Delaney JR, Wisdom AJ, Qin X, Wang X, Caraballo I, Gresham J, Luo L, Van Mater D, Owzar K, Kirsch DG. Mutational landscape in genetically engineered, carcinogen-induced, and radiation-induced mouse sarcoma. *JCI Insight*. 2019 Jul 11;4(13). pii: 128698. doi: 10.1172/jci.insight.128698. PMID: 31112524. *equal contribution.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Nothing to Report

4. **IMPACT:** D*escribe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and

research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Our *JCI Insight* manuscript represents a rigorous comparison of the genetic landscape of mouse sarcomas generated as a result of irradiation (funded by this project) or other distinct tumor initiating events (funded by other sources) within the same tissue type. By comparing the whole exome sequencing of radiation-induced sarcomas with other sarcomas, we defined a radiation-induced tumor genetic signature. In addition, the raw sequencing data from these studies has been deposited in a public repository for other researchers to access.

Our studies are designed to elucidate the role that p53 plays in radiation-induced sarcoma. The results of these studies provide critical information relevant to designing therapies that could enhance tumor suppressor signals and prevent sarcoma development following radiation. In addition, a detailed understanding of the drivers of radiation-induced sarcoma will have an impact on the development of treatment approaches to improve the lives of patients with sarcoma.

Currently, a substantial drug development effort is underway to produce pharmaceuticals that prevent or mitigate acute radiation injuries. One strategy is to block cell death pathways and prevent cell loss in critical tissues after radiation while other strategies are aimed to induce tissue regeneration or cell replacement. The results of our studies indicate strategies aimed at preventing cell death may lead to increased risk of sarcoma development. Our studies have the potential to inform drug development strategies for radiation mitigators and for drugs designed to prevent radiation injury. In addition, these studies also inform the risk analysis and need for cancer screening for people with prior exposures to ionizing radiation and radiation-induced chronic wounds.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:
- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change
Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them
Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Aim 1.2: Because our initial hypothesis that radiation would cause recurrent mutations to cause sarcomas was not supported by the data in Task 1, we added an RNA sequencing experiment to Aim 1.2 to test an alternative model where changes in gene expression after radiation drive sarcomagenesis. Therefore, we extended the timeline for completion of this aim.

Aim 2.3: Because we proposed to use Myf6Cre mice instead of Pax7CreER mice, the studies in Aim 2.3 were delayed.

Changes that had a significant impact on expenditures
Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or

equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Not Applicable

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Lee CL*, Mowery YM*, Daniel AR*, Zhang D, Sibley AB, Delaney JR, Wisdom AJ, Qin X, Wang X, Caraballo I, Gresham J, Luo L, Van Mater D, Owzar K, Kirsch DG. Mutational landscape in genetically engineered, carcinogen-induced, and radiation-induced mouse sarcoma. *JCI Insight*. 2019 Jul 11;4(13). pii: 128698. doi: 10.1172/jci.insight.128698. PMID: 31112524. *equal contribution. Federal support was acknowledged (yes).

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report

  Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status*

of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Daniel AR, Lee CL, Williams N, Li Z, De Silva Campos L, Luo L, Ma Y, Cardona D, Owzar K, Kirsch DG. Temporary knockdown of p53 during irradiation increases the development of sarcomas and chronic injuries in mice. 2020 Radiation Research Society Annual Meeting. Invited Talk

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

The whole exome sequencing data along with the called mutations in vcf format have been deposited into the National Center for Biotechnology Information Sequence Read Archive under project ID PRJNA516973.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

  Nothing to Report

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

  Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*

- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name:	David Kirsch MD, PhD
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-2086-205X
Nearest person month worked:	1.2 CM
Contribution to Project:	Dr. Kirsch reviewed the design of all experiments and all of the data generated to complete the Aims of this proposal including histology, immunofluorescence, microscopy imaging, and flow cytometry.
Funding Support:	There is no additional funding support for this award.

Name:	Andrea Daniel, PhD
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.60 CM

Contribution to Project: Dr. Daniel analyzed the radiation-induced sarcomas and muscle satellite cells after radiation. She crossed mice to generate experimental cohorts and analyzed whole-exome sequencing and RNA sequencing data.

Funding Support: There is no additional funding support for this award.

Name: Yan Ma
Project Role: Lab Research Analyst
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2.28 CM

Contribution to Project: Ms. Ma processed mouse tissues with formalin fixation for paraffin embedding. She performed hematoxylin and eosin staining and immunohistochemistry on the tumor sections.

Funding Support: There is no additional funding support for this award.

Name: Nerissa Williams
Project Role: Lab Research Analyst
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 0.36 CM

Contribution to Project: Ms. Williams irradiated mice to induce sarcomas. She scored mice for acute and chronic injury and identified mice with radiation-induced sarcomas.

Funding Support: There is no additional funding support for this award.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Effort for the PI remained at 10% for this project as originally proposed and budgeted. For the other 90% effort, other active grants have started and ended, but these changes did not impact the effort on this project. Those projects are listed below.

AWARDED
7000000445/NNX16A069A (Fox)
Baylor College of Medicine/NASA
Mining biology's extremes for new space radiation resistance strategies

None assigned (Gersbach)
Gilbert Family Foundation
Genome Editing with Engineered Vectors to Correct Neurofibromatosis Type I

Goldman Sachs Philanthropy Fund
Emerson Collective
Dissecting the Role of Clonal Evolution in Tumor Response and Resistance to Radiation and Immunotherapy

None Assigned (Kirsch)
The Alan B. Slifka Foundation
Identifying the metabolic dependencies of primary sarcoma and sarcoma lung metastases

Connective Tissue Oncology Society (Kirsch)
Defining the Landscape and Function of Onco-exaptation and Repetitive Element-Derived Tumor-Specific Antigens in Undifferentiated Pleomorphic Sarcoma

Varian Medical Systems (Kirsch)
Radiation and Immunotherapy for Genetically Engineered Mouse Models of Soft Tissue Sarcoma

ENDED
5R01CA183811-04 (Alman)
NIH/NCI
Targeting Tumor Initiating Cells in Undifferentiated Pleomorphic Sarcoma

#18-03046 (Kirsch)
Levine Foundation

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to* ***https://ers.amedd.army.mil*** *for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.*

Not applicable.

9. APPENDICES: *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Lee CL*, Mowery YM*, Daniel AR*, Zhang D, Sibley AB, Delaney JR, Wisdom AJ, Qin X, Wang X, Caraballo I, Gresham J, Luo L, Van Mater D, Owzar K, Kirsch DG. Mutational landscape in

genetically engineered, carcinogen-induced, and radiation-induced mouse sarcoma. *JCI Insight*. 2019 Jul 11;4(13). pii: 128698. doi: 10.1172/jci.insight.128698. PMID: 31112524. *equal contribution.