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USING MOUSE MODELS TO DEFINE HOW THE P53 R72P POLYMORPHISM IMPACTS THE ADVERSE EFFECTS OF DOXORUBICIN AND IONIZING RADIATION

Emily Dominguez

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A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Emily Domínguez
Smithville, Texas

December, 2016

Acknowledgements:

I would like to thank my Advisor, Dr. David Johnson, for agreeing to let me pursue my graduate education in his laboratory and for encouraging me to continue working toward my PhD despite the unprecedented number of difficulties my dissertation project presented. My Advisory Committee, Dr. Rick Finch, Dr. Mark Bedford, Dr. Donna Kusewitt and Dr. Kevin McBride, also provided much encouragement. Their sage advice and incredible patience made completing this project possible.

I would also like to thank Dr. Renier Velez-Cruz and Dr. Swarnalatha Manickavinayaham, who are among the few survivors of the dwindling Johnson lab, for their advice on the many technical issues my project faced.

I give a special thanks to Jen Orona, the long-time and soon-retiring lab manager of the Johnson lab, for her willingness to help me with so many experiments, for helping me track down supplies I ordered and forgot to order, and for having really good chocolate in her office. More importantly, I'd like to thank Jen for her friendship, which has made the best moments of my graduate career better and the darkest moments light enough to endure.

None of this would have been possible without the help and encouragement of our fearless administrator, Becky Brooks.

Finally, I'd like to acknowledge my family. Leonardo, my son, deserves a huge thank-you (and a lot of love) for never letting me give up on becoming "Dr. D" and for being such a good kid that I actually had the time to complete this program. I thank my (unofficial) husband Andy Esquivel for his unconditional love and emotional support (and for editing a hundred pages of scientific jargon when he'd rather be reading vampire stories).

USING MOUSE MODELS TO DEFINE HOW THE P53 R72P POLYMORPHISM IMPACTS THE ADVERSE EFFECTS OF DOXORUBICIN AND IONIZING RADIATION

Emily Domínguez, Ph.D.*

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The single nucleotide polymorphism (SNP) at codon 72 of the tumor suppressor gene *p53* codes for either an arginine (R) or proline (P) (*p53* R72P). This SNP may impact how cells respond to genotoxic insult. Studies in cell culture and in tissues from mouse models of the SNP indicate that, in response to genotoxic treatment, the two variants may differentially induce apoptosis and expression of *p53* target genes. In epidemiological studies, the P variant is associated with decreased cancer survival and increased risk of side-effects from genotoxic cancer treatment. Genotoxic therapy is still the mainstay of cancer treatment, and doxorubicin and/or ionizing radiation (IR) are used in many treatment regimens. In this project I employed our mouse models of the *p53* R72P polymorphism to test how this SNP modulates physiological effects of doxorubicin and IR. To test how the *p53* R72P polymorphism affects doxorubicin tolerance at a physiological level and in gene expression profiles, I performed blood counts and RNA sequencing of tissues from doxorubicin treated and untreated *p53* R72P mice. To test how the *p53* R72P polymorphism affects IR tolerance, I performed a survival study, immunohistochemical (IHC) staining, blood counts and used quantitative PCR to analyze *p53* target gene expression in IR treated and untreated *p53* R72P mice. In both studies I stringently controlled for background strain, age and sex. Due to an unexpected tolerance to doxorubicin in the FVB mouse strain employed, the doxorubicin studies were inconclusive. While IR did elicit a significant response, my findings did not support a role for the *p53* R72P polymorphism in modulating the adverse effect IR therapy, and indicate that personalization of these therapies based on this SNP could have limited clinical utility.

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Chapter 1:

Background and Introduction

1.1 The p53 tumor suppressor protein

1.1.1 Discovery of the p53 protein and its major functions

In 1979, four laboratories, all of which were screening transformed cell lines for potential tumor antigen candidates, independently published findings regarding an approximately 53kD protein. Three reports showed the protein could be detected by immunoprecipitation with anti-simian-virus 40 (SV40) tumor serum in SV40-transformed mouse, hamster, rat, monkey or human cultures or in uninfected embryonic carcinoma lines¹⁻³. A fourth report demonstrated this protein (sensibly baptized "p53") could also be immunoprecipitated by antisera produced in mice immunized against sarcomas in both murine sarcoma tissues and transformed murine cell lines, but not in primary mouse tissues or cultures⁴. Since p53 did not share features of viral proteins, these reports hypothesized that p53 was a cellular protein and was essential for and/or induced by cellular transformation/immortalization¹⁻⁴.

In accordance with these initial hypotheses, reports began to emerge showing that either p53 or very similar proteins were present in many human tumor cell lines. Other reports showed that some breast cancer patients had anti-p53 antibodies in their sera, and that colorectal and breast tumors had

detectable levels of p53⁵⁻⁷. Early experiments, which showed an inhibition of DNA synthesis in serum stimulated 3T3 cell culture following micro-injection of anti-p53 antibodies, suggested that p53 was a positive regulator of growth⁸. In 1984, three cell culture studies in *Nature* claimed that p53 expression in primary cells could induce immortality either independently or in co-operation with other tumorigenic genes⁹⁻¹¹. Considering these data, it initially seemed reasonable to conclude that p53 was an oncogene.

However, shortly thereafter, early p53 aficionados, such as Levine and Vogelstein, showed that vectors coding for wild-type (WT) p53 introduced in tumor cell cultures actually suppressed cell growth. Furthermore, they demonstrated that only mutated p53 allowed transformation of cell lines by oncogenes¹²⁻¹⁴. It was soon understood that mutated p53 co-operated with other oncogenes in a dominant negative fashion, and that a wide variety of neoplasia contained mutant p53 (reviewed in ¹⁵). Thus, p53 was not an oncogene, but an important tumor suppressor gene.

Several groups then began to reveal the cellular mechanisms responsible for p53's ability to suppress tumor growth. In 1991, Kastan et al. showed that p53 protein increased in response to DNA damaging agents, and this coincided with G1 growth arrest¹⁶. A few years later, p53's growth suppressive abilities in the context of DNA damage were shown to be contingent, in part, on

transcriptional activation of proteins involved in cell cycle arrest, including cyclin-dependent-kinase-inhibitor-1A (Cdkn1a/p21)^{17,18} and growth-arrest-and-DNA-damage-inducible-45-alpha (Gadd45a)^{19,20}. Concurrent with the discovery of p53's ability to induce growth arrest, other investigators were taking notice of p53's ability to induce apoptosis^{21,22}. It was soon shown that p53 induces apoptosis by up-regulation of the pro-apoptotic gene *BCL2-associated-X-protein (Bax)*²³. Other pro-apoptotic genes induced by p53, including *p53-upregulated-modulator-of-apoptosis (Puma/Bbc3)*²⁴ and *phorbol-12-myristate-13-acetate-induced-protein-1 (PMAIP1/Noxa)*²⁵, were discovered later. Furthermore, studies showed that p53 can also induce apoptosis via protein/protein interactions with Bcl-2 family pro- and anti-apoptotic proteins at the mitochondrial membrane (reviewed in²⁶).

Of course, the role of p53 as a transcription factor is not limited to the few genes listed above. In fact, a 2008 review found that WT p53 (but not mutant p53) either negatively or positively regulated the expression of 129 genes through p53's interaction with gene promoters. These 129 genes were those that met the stringent requirement of containing a response element for p53 and in which chromatin immunoprecipitation (ChIP) assays or gel shift assays showed p53 binding to promoter elements. Furthermore quantitative RNA analysis and protein level analysis had to show increased or decreased activity in

response to WT p53²⁷. More recent high-throughput sequencing and analysis techniques, unsurprisingly, have shown even more p53 transcriptional targets. A 2013 study used p53 WT and p53^{-/-} mouse embryonic fibroblasts (MEFS) exposed to the genotoxic agent doxorubicin to assess p53 target genes by both ChIP sequencing and RNA sequencing. This study found 423 genes bound by p53 at their promoters and differently regulated in a p53 dependent manner. Gene ontology analysis showed that there was a significant up-regulation of genes involved in cell cycle arrest and apoptosis. These data confirm the importance of these long-identified pathways as being central to the response of p53 to genotoxic insult. Interestingly, they also found p53 binding to, and p53 dependent up-regulation of autophagy-related genes and down-regulation of genes involved in growth ²⁸.

That p53 may be involved in autophagy is not surprising. For approximately a decade p53 has been implicated in regulating metabolism. Transcriptional activation of *AMP-activated-protein-kinase* (AMPK) by p53 suppresses mammalian-target-of rapamycin (mTOR) signaling. Such reduced m-Tor pathway signaling decreases cell growth (protein synthesis) and increases autophagy, although whether these effects are tumor-suppressing or tumor-promoting is subject to some debate (reviewed in ²⁹).

There are many other functions of p53 that could be important to cancer progression or cancer therapy research. For example, p53 transcriptional activation of *p21* is involved in senescence as well as cell cycle regulation. Other potentially important roles include reactive oxygen species (ROS) regulation, regulation of angiogenesis, roles immunity and roles in DNA damage repair (reviewed in³⁰). Exploring most of these functions in detail is beyond the scope of this dissertation, but the role of p53 in DNA repair should be mentioned.

The involvement of p53 in DNA repair can occur both through its role as a transcription factor and through non-transcription-dependent interactions. In cell culture studies and *in-vitro* assays of most types of DNA repair, WT p53 either increases or decreases efficiency when compared to the mutated p53 or p53 deficiency. In nucleotide excision repair (NER), p53 may transactivate genes involved in UV damage recognition as well as affect enzymatic activity of proteins involved in UV damage repair. In base excision repair (BER), WT p53 protein seems to enhance activity of some BER-associated-endonucleases and – DNA-polymerases and other BER associated enzymes, under certain conditions. Furthermore, p53 appears to associate, *in vitro*, with some of these enzymes. In mismatch repair (MMR), p53 transactivates the *MSH2* gene, and colocalizes with MSH2 and other proteins involved in MMR (reviewed in ³¹).

The role of p53 in DNA double strand breaks (DSB) is especially important to this thesis, as DSB repair is important in both the treatment efficacy and side effects of cancer chemotherapy and ionizing radiation treatment. DNA DSBs can be repaired by the more error-prone non-homologous-end-joining (NHEJ), or by or homologous recombination repair (HRR), a reduced-error method of DNA repair that relies on the presence of an intact homologous sister chromatid. The role of p53 in NHEJ is not clear. Studies using plasmid assays have given conflicting results as to whether p53 inhibits or enhances NHEJ. In HRR, p53 appears to be inhibitory, as p53 deficient mice show increased frequency of HRR. The inhibitory effects on HRR appear to be transactivation-independent. The binding of WT p53 to RAD51 is well established, and this binding may inhibit strand invasion and/or branch migration necessary for recombination. Furthermore, serine 15 (S15) phosphorylated p53 interacts with replication-protein-A (RPA) in earlier stages of HRR to suppress this method of repair (reviewed in^{31,32}).

1.1.2 Regulation of p53

The regulation of p53 has been the subject of intense investigation since its discovery. As early as 1984, Maltzman and Czyzyk showed that DNA damaging agents could induce increased levels of p53 which also coincided with cell cycle arrest³³. However, Kastan et al. showed in 1991 that this did not coincide with increased p53 mRNA production¹⁶, suggesting that regulation of protein levels was not by regulation of transcription. Further studies led to the current understanding of p53 regulation. Under normal cellular conditions, p53 levels are kept in check by a feedback loop in which p53 itself promotes transcription of its own negative regulator, mouse-double-minute-2-homolog (Mdm2)³⁴⁻³⁷. Mdm2 is a ubiquitin ligase which interacts with and ubiquitinates p53, targeting p53 for subsequent proteasome degradation³⁸⁻⁴⁰. However, under genotoxic stress, the ataxia-telangiectasia-mutated (ATM) kinase phosphorylates p53 at serine 15 and inhibits p53/Mdm2 interactions and therefore, its ubiquitination and degradation^{41,42}.

The regulation of p53 is actually much more complex: several other post-translational modifications and protein-protein interactions have been found to regulate p53 levels and activities in the Mdm2 pathway and myriad other pathways (reviewed in⁴³). It should also be noted that Wang et al. would show

that p53 mRNA *does* increase in response to some chemotherapeutic agents in cell culture, and that p53 itself is a transcription factor at least partially responsible for this increase⁴⁴. The general consensus remains that the Mdm2 pathway described above is the most important regulatory pathway for p53 activity^{45,46}.

1.1.3 Functional domains of the p53 tumor suppresser protein

The p53 protein has domains essential for most of its known functions. The transactivation domain, amino acid (AA) 1-60, is the site of phosphorylation events that stabilize p53 and prevent Mdm2 interaction under cellular stress and contains one of two regions essential for transactivation of target genes^{41,42,47}. Much of p53's transcriptional regulation is also contingent on the DNA binding domain of the protein AA 102-292⁴⁸⁻⁵⁰. Accordingly, many "hotspot" mutations of the *p53* gene in cancer occur in these domains, and these mutations can either be loss-of-function mutations which inhibit normal p53 transcriptional function or gain-of-function changes in which aberrant p53 induces transcription that increases oncogenic signaling, probably through interaction with other transcription factors (reviewed in^{51,52}). AA 311-363 comprises the tetramerization domain^{48,53}. The C-terminal regulatory domain (AA 363-393) is the site of many

post-translational modifications; these include ubiquitination by Mdm2 that leads to p53's degradation and other modifications that may affect transcription and interactions with DNA repair enzymes⁵⁴⁻⁵⁷.

Walker and Levine first characterized the proline rich domain (AA 61-94) in 1996. They found it contained five repeats of the sequence proline-two-other-amino-acids-proline (PxxP), and that elimination of this domain did not repress p53 mediated transcription (by reporter systems) or p53 stabilization. However, it did attenuate p53's growth arrest capabilities. Because PxxP domains bind src homology 3 (SH3) domains of other proteins and this binding activity is important in signal transduction pathways^{58,59}, they hypothesized that p53's PxxP domain may be essential for at least some downstream signaling⁶⁰. Another group did not find that elimination of the PxxP domain of the *p53* gene decreased growth arrest by p53, and they showed that growth arrest genes were not differently expressed (by reporter systems) when either *PxxP-deficient-p53* or *WT p53* expression vectors were reintroduced into p53 null cell lines. They did find differences in apoptosis, however, when comparing the two variants that they accounted for by *WT-p53* being much more proficient than *PxxP-deficient-p53* in inducing *p53-inducible-gene-3* (*Pig3/Tp5i3*) in reporter assays. They also noted decreased ROS production in cells with *PxxP-deficient-p53* as compared to *WT-p53*. As *Pig3* may be involved in ROS production,

which may influence apoptosis, they suggested that the decreased induction of *TP53* was the mechanism for decreased apoptosis in cells with *PxxP-deficient-p53*⁶¹. Another study found similar results. Although they saw decreased endogenous *p21* and *Mdm2* induction as a result of transfection with *PxxP-deficient-p53* as compared to *WT-p53* transfection, they did not find evidence of decreased growth arrest. However, as in the earlier study, they found decreased apoptosis and decreased induction of ROS producing genes with *PxxP-deficient-p53* vector transfection. They similarly suggested that this decreased ROS production ultimately lead to decreased apoptosis in cells harboring of *PxxP-deficient-p53* vectors⁶². Another study found increased affinity to *Mdm2* and increased susceptibility to *Mdm2* mediated ubiquitination and nuclear export with *PxxP-deficient p53* as compared to *WT p53*. This study also showed decreased apoptosis in cells transfected with *PxxP-deficient-p53* as compared to *WT-p53* transfected cells.

The general consensus, therefore, is that the proline rich domain of *p53* is probably dispensable for cell cycle arrest but necessary for inducing apoptosis when *p53* is re-introduced into *p53* deficient cell lines. However, the proposed cellular mechanisms for this effect vary by study⁶¹⁻⁶⁴. In addition to apoptosis induced by re-introduction of *p53* vectors in *p53* null cell lines, the PxxP domain appears to be necessary for apoptosis induced by most chemotherapeutics, at

least in cell culture⁶⁵, suggesting that, by extension, it is involved in apoptosis by other genotoxic insults.

The proline rich domain is contains the amino acids coded by the first discovered and by far most studied single nucleotide polymorphism (SNP) in the *p53* gene...

1.2 The p53 codon 72 polymorphism (rs1042522)

1.2.1 Discovery and early epidemiological studies of codon 72 polymorphism

Not long after the discovery of p53, several labs began noting that different human cell lines produced apparently different p53 protein species, as determined by polyacrylamide gel electrophoresis. Accordingly, cloning and sequencing revealed two different cDNA sequences at codon 72: CCC coding for proline and CGC coding for arginine^{5,66-70}. By 1990, PCR and enzyme digestion of DNA from 50 unrelated individuals provided evidence that these differences were (as had been previously suspected) not a mutation, but the first validated SNP in the *p53* gene⁷¹. This polymorphism is often referred to as the p53 R72P polymorphism.

An early report noted increased expression of the p53 R72P proline variant in ethnicities from equatorial regions as compared to ethnicities from northern latitudes⁷². Later, sequencing of the chimpanzee genome showed that only the proline variant is found in chimpanzees⁷³, suggesting that the proline variant is the ancestral form. Together these studies point to evolutionary survival advantages for the arginine variant in northern latitudes, and some

speculate this could be tied to cancer risk associated with environmental/climatic differences⁷⁴.

In the early 1990s, while the tumor suppressor role of p53 was still being established, attempts at understanding whether the p53 R72P polymorphism had any function in cancer incidence were already being published. That year the first epidemiological study attempting to associate the polymorphism with cancer incidence found no difference between polymorphic variants in colon cancer⁷⁵. Shortly thereafter, another study in acute myeloid leukemia also failed to find an association between either variant and cancer incidence⁷⁶. At least six early studies in lung cancer incidence found largely disparate results; however, when an increased risk was found, it tended to be associated with the proline allele⁷⁷⁻⁸². The Kawajiri et al. lung cancer study also looked at several other cancers and found that the arginine allele was associated with stomach cancers but found no association between p53 codon 72 genotypes and colorectal, bladder and breast cancers⁷⁸. In agreement with Kawajiri et al., another study found no associations between p53 R72P variants in urologic cancers⁸³. Unlike the Kawajiri et al. study, another report associated the proline variant with increased breast cancer incidence⁸⁴. Two other early reports also failed to conclusively link cancer incidence with p53 R72P polymorphic alleles. One showed there was no association between p53 R72P genotype and

nasopharyngeal carcinoma⁸⁵. Another showed that arginine homozygosity conveyed a survival advantage but also earlier age of onset in ovarian cancer⁸⁶. In summary, the mixed results of epidemiological studies from the 90s indicate an inability to definitively associate one p53 R72P variant with increased cancer susceptibility.

Such studies remained relatively unnoticed, however, when compared to the 1998 paper by Storey et al., which associated arginine coding homozygosity with increased cervical cancer risk. Storey showed greater p53 degradation of the arginine variant protein by E6 oncoproteins from the human papilloma virus, which he postulated as the mechanism for that difference⁸⁷. Perhaps because it was the first report to suggest a mechanism for cancer incidence differences and to employ cell culture models in addition to an epidemiological study, Storey's story catapulted the functional effects of p53 R72P polymorphism into the limelight⁷⁴. Since Storey's report, interest in the polymorphism's role in cancer has not waned even though the effect of the polymorphism on cervical cancer incidence has been inconsistent across many studies^{88,89}. This continued interest is evidenced by the fact that PubMed publications average over 36 per year between 1999 and 2015 using the search term "p53 codon 72 polymorphism cancer." Following the intensification of interest in the polymorphism after 1998, the functional effects of the polymorphism on cancer development and

response to therapy can generally be divided into studies that focus on cell culture, epidemiology or mouse models.

1.2.2 The p53 R72P polymorphism: cell culture models

In 1999, Thomas et al. was the first to show increased apoptosis in p53 null cells transfected with arginine variant p53 as compared to proline variant p53⁹⁰. Dumont et al. validated this observation in temperature-sensitive clone models of the two variants, but did not find that induction of p53 target genes differed between them. Their study attributed the increased apoptosis in the arginine variant to increased interaction with nuclear export factors, including Mdm2, and subsequent p53 mitochondrial localization⁹¹. Using estrogen-receptor-fusion-polymorphic-variant proteins in p53 null cells, Pim and Banks also noted an increase in apoptosis upon 4-hydroxy-tamoxifen induction in the arginine variant and increased G-1 cell cycle arrest in the proline variant⁹². Sullivan et al. showed that, in p53 null cell lines, transfection with arginine variant p53 vectors increased apoptosis in response to the chemotherapeutics doxorubicin and cisplatin when compared to proline variant transfection. This study further demonstrated that this effect was a result of increased arginine variant DNA interaction at the transcription start sites of (and subsequent transcription of) the pro-apoptotic genes *Puma*, *p53-effector-related-to-PMP-22* (*Perp*) and *actin-interacting-protein-1* (*Aip1/Pdcd6ip*)⁹³. Using temperature sensitive p53 clones and various DNA repair models, Siddique and Sabapathy

showed that the proline variant p53 was better able to induce DNA repair genes *p53R2* (*Rrm2b*), *gadd45* and *p48* (*Ddb2*) and more efficiently repair DNA damage. Incidentally, they also validated increased apoptosis when the arginine variant was re-introduced into *p53* null cells as compared to the proline variant, but did not find the up-regulated *Aip1* in the arginine variant noted in prior studies⁹⁴. Bergamaschi et al. used polymorphic variant vectors in *p53* null cells to show that the proline variant preferentially bound apoptosis-inhibiting-ankyrin-repeats-SH3-domain-proline-rich-region-protein (iASPP) and that iASPP decreased p53 R72P proline variant activity at the pro-apoptotic *Bax* promoter, which coincided with decreased apoptosis induced by that variant⁹⁵.

The major consensus in these studies is that p53 codon 72 arginine variant protein is better at inducing apoptosis, at least in cell culture models where *p53* is reintroduced into *p53* null cell lines. The conflicting mechanisms for the polymorphism's role in apoptosis underscore the fact that immortalized cell culture models have provided somewhat limited clarity into the role p53 R72P polymorphism may play in modulating human cancer incidence and survival. Yet even that role is highly debatable, considering that epidemiological studies have failed to clearly define the role of the polymorphism in human cancer incidence, survival and adverse effects of therapy.

1.2.3 Epidemiological literature on effect of the p53 R72P polymorphism on cancer incidence, survival and treatment side-effects

A 2009 review of p53 polymorphisms in cancer already highlighted the fact that the body of individual epidemiological studies on the effect of the p53 R72P polymorphism on cancer incidence was vast, complex and contradictory. The review also mentioned that many association studies were plagued with issues in study design⁷⁴; furthermore, others have suggested that the use of tumor tissue for genotyping may be introducing bias into these studies⁹⁶. A comprehensive meta-analysis of 302 case-controlled studies associated increased risk for thyroid, gastric, head and neck cancers and hepatocarcinoma with the p53^{P72/P72} genotype. Nevertheless, odds ratio (OR) indicated low risk. Furthermore, these associations were contingent on the selection criteria for studies included in the meta-analysis, and they differed when associations were analyzed in different ethnic groups⁹⁷. Other meta-analyses for individual cancer types often provided conflicting conclusions. For example, two recent breast cancer meta-analyses showed 1) no association⁹⁸, 2) and increased risk with the proline allele (except in Asian populations, in which the arginine variant showed increased risk)⁹⁹.

Epidemiological studies associating the p53 R72P polymorphism with side effects of therapy and cancer survival and are not nearly as numerous as incidence studies. For therapy side effects, a few studies have shown increased febrile neutropenia or neutropenia in $p53^{P72/P72}$ breast and small cell lung cancer patients, respectively, when compared to $p53^{P72/R72}$ and $p53^{R72/R72}$ patients^{100,101}. The trend in survival studies is for the R variant, in hetero- and/or homozygosity, to favor survival when compared to the P variant^{93,102-108}. Of these studies, Tommiska *et al.* found that $p53^{P72/P72}$ breast cancer patients exhibited decreased survival compared to $p53^{P72/R72}$ and $p53^{R72/R72}$ patients, and this held true for patients with tumors that had lost p53 expression¹⁰⁸. The latter suggests that the increased survival of $p53^{P72/R72}$ and $p53^{R72/R72}$ patients may result not from the tumor itself, but from tumor micro- and macro-environmental differences in the patient.

In short, the epidemiological literature is extremely inconsistent when the effect of the p53 R72P polymorphism on cancer incidence is studied. Some studies do point to the proline variant being more prone to increased side effects of therapy and less favorable for survival although the limited number of studies makes these results far from definitive.

1.2.4 Mouse models used in clarifying the functions of WT and mutant *p53* and mouse models of the *p53* R72P polymorphism

Genetically engineered mice have been essential to elucidating the role of *p53* in tumor suppression and other biological functions. The earliest *p53* mouse models were transgenic mice that expressed mutant *p53* and were more prone to a wide spectrum of tumor types. Later, knock-out (KO) mice, with large deletions of the *p53* gene were used to model the effects of *p53* inactivation in human cancer. The majority of *p53*^{-/-} mice succumb to early-onset lymphoma and soft tissue tumors while some develop other cancers. In mice heterozygous for the KO allele, tumor development is delayed compared to KO homozygous mice and accelerated compared to WT mice. The tumor spectrum in these mice tends toward lymphomas, soft tissue sarcomas and osteosarcomas. This reflects the tumor incidence in Li-Fraumeni syndrome, a familial cancer susceptibility syndrome associated with germline mutations in the *p53* gene, making *p53*^{+/-} mice an important model for this human genetic condition. These *p53* KO mice were also essential for showing the defects in apoptosis associated with *p53* loss. Furthermore, they were used (together with *Mdm2* KO mice) to confirm the importance of *Mdm2* in *p53* regulation. Humanized knock-in (KI) mouse models

of *p53*, in which portions of the mouse *p53* are replaced with either WT or mutant human *p53* have also been generated. These models provided important insights into the role of “hot-spot” mutations in the cancer development and progression (summarized from an excellent review by Donehower and Lozano ¹⁰⁹). We believe humanized *p53* knock-in (KI) mice can also be useful for understanding the role human SNPs in cancer development and treatment.

With over 80 million human SNPs currently known¹¹¹, it is very difficult to determine the effect of a single SNP in epidemiological studies of cancer patients cohort, especially considering the environmental differences as well as differences in treatment that these patients undergo. For these reasons, our laboratory, and others, has looked to mouse models of the polymorphism to address the role of the polymorphism in cancer *in-vivo* in a more controlled manner than epidemiological studies can

Since the *p53* R72P polymorphism is not conserved in mice (**Figure 1.1 a**), our laboratory created humanized mouse models of the two human SNP variants. In these mouse models, mouse exon 4 is replaced with the proline (codon 72 CCC) or arginine (codon 72 CGC) coding human exon 4 (**Figure 1.1 b**).

Zhu et al. used these models to study tissue-specific differences in apoptosis in $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated with ultraviolet and ionizing radiation (UV and IR), molecular pathways associated with apoptosis, and skin tumor induction in UV exposed $p53^{P72/P72}$ and $p53^{R72/R72}$ mice. In agreement with previous cell culture models, increased caspase-3 staining (an indicator of apoptosis) was noted by immunohistochemistry (IHC) in the intestine of IR-treated $p53^{R72/R72}$ when compared to tissues from $p53^{P72/P72}$ mice. However, p21 and p53 levels did not differ significantly between genotypes (**Figure 1.2**).

a

38	Q	A	M	D	D	L	M	L	S	P	D	D	I	E	Q	W	F	T	E	D	hTP53
39	H	C	M	D	D	L	L	L	-	P	Q	D	V	E	E	F	F	E	-	-	mTP53
<hr/>																					
58	P	G	P	D	E	A	P	R	M	P	E	A	A	P	P/R	V	A	P	A	P	hTP53
56	-	G	P	S	E	A	L	R	<u>V</u>	<u>S</u>	<u>G</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>A</u>	<u>Q</u>	<u>D</u>	<u>P</u>	<u>V</u>	<u>T</u>	mTP53
<hr/>																					
78	A	A	P	T	P	A	A	P	A	P	A	P	S	W	P	L	S	S	S	V	hTP53
75	<u>E</u>	<u>T</u>	<u>P</u>	<u>G</u>	<u>P</u>	<u>V</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>P</u>	<u>A</u>	<u>T</u>	<u>P</u>	<u>W</u>	<u>P</u>	<u>L</u>	<u>S</u>	<u>S</u>	<u>F</u>	<u>V</u>	mTP53

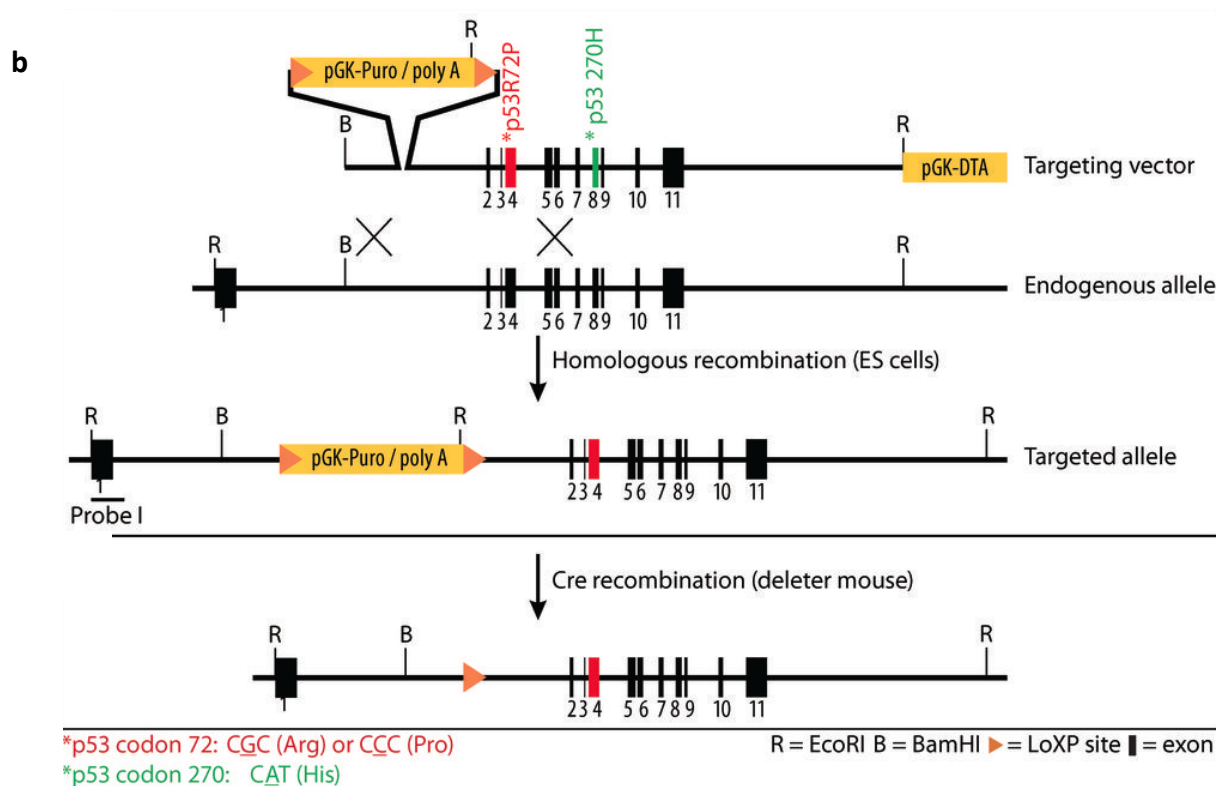


Figure 1.1 Mouse models employed. **a)** The p53 R72P polymorphism is in a region poorly conserved in mice. **b)** Schematic for constructs used to generate $p53^{P72/P72}$ and $p53^{R72/R72}$ mice as previously described.

Zhu, F., Dolle, M. E., Berton, T. R., Kuiper, R. V., Capps, C., Espejo, A., McArthur, M. J., Bedford, M. T., van Steeg, H., de Vries, A. & Johnson, D. G. Mouse models for the p53 R72P polymorphism mimic human phenotypes. *Cancer Res* **70**, 5851-5859, doi:10.1158/0008-5472.CAN-09-4646 (2010).

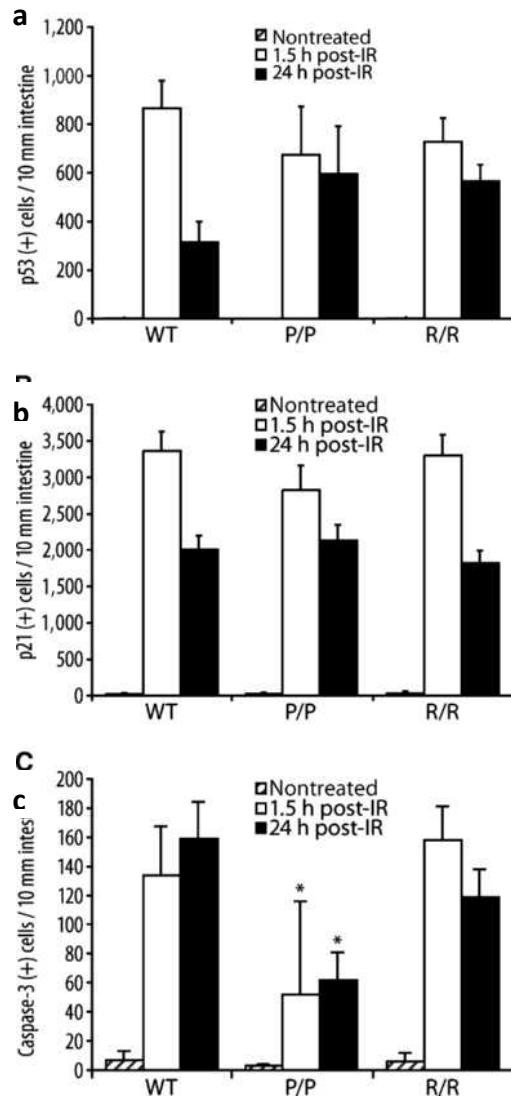


Figure 1.2 Apoptosis in p53 R72P mice. $p53^{P72/P72}$ and $p53^{R72/R72}$ mice were treated with 6 Gy IR, sacrificed at 1.5 or 24 hours after treatment and intestinal tissue was taken and immunostained with **a)** p53, **b)** p21 and caspase-3. Average number of positive cells for 10 mm of intestine are represented. $*p < 0.05$

Zhu, F., Dolle, M. E., Berton, T. R., Kuiper, R. V., Capps, C., Espejo, A., McArthur, M. J., Bedford, M. T., van Steeg, H., de Vries, A. & Johnson, D. G. Mouse models for the p53 R72P polymorphism mimic human phenotypes. *Cancer Res* **70**, 5851-5859, doi:10.1158/0008-5472.CAN-09-4646 (2010).

In our models, *Puma*, *Perp* and *Noxa* mRNA levels and mitochondrial localization of p53 were both increased in $p53^{R72/R72}$ mouse primary fibroblasts exposed to UV when compared to $p53^{P72/P72}$ fibroblasts exposed to UV, but *p21* mRNA levels were not affected. Surprisingly, there were no differences in skin tumor growth in UV exposed mice across genotypes, suggesting that differences in immediate apoptotic response to UV might not predict future tumor growth¹¹².

Our lab also generated bacterial artificial chromosome (BAC) models in which mice with the entire human p53 gene of either the P or R coding variant were crossed into p53 KO mice. In both variants, mice produced functional p53 that rescued them from the early-onset lymphoma seen in p53 KO mice without the BAC transgene. However, when the BAC models were used to study spontaneous tumor incidence, no statistically significant differences were found between R and P variants in overall survival and only a few differences in tumor spectrum were noted¹¹².

Maureen Murphy's laboratory generated and used other mouse models of the R72P polymorphism that replace exons four to nine of mouse *p53* with the codon 72 arginine or proline coding human variant exons^{113,114}. Employing these models, they found comparable levels of p53 protein, *Mdm2* and *Puma* mRNA, but increased levels of p21 protein and *p21* mRNA in $p53^{P72/P72}$ MEFs

exposed to genotoxic insult compared to $p53^{R72/R72}$ MEFs. Additionally, this study found IR treatment caused increased apoptosis in $p53^{P72/P72}$ mouse thymus compared to $p53^{R72/R72}$ mouse thymus by IHC. There was no up-regulation of the pro-apoptotic genes *Puma*, *Noxa*, *Bax* and *p53-induced-death-domain-protein-1 (Pidd)* in cultured $p53^{P72/P72}$ thymocytes exposed to IR compared to $p53^{R72/R72}$ thymocytes. However, there was an up-regulation of *p21* and several NF- κ B target genes in IR-exposed $p53^{P72/P72}$ thymocytes compared to $p53^{R72/R72}$ thymocytes. Most notably, *Caspase 4/11* which has functions in both pro-inflammatory and pro-apoptotic signaling was upregulated in the proline variant. Accordingly, this study also showed that NF- κ B subunit p65 RelA had increased interaction with $p53^{P72}$ protein when compared to the arginine variant. Furthermore, in a survival study, $p53^{P72/P72}$ mice appeared to be more susceptible to lipopolysaccharide (LPS) injection: a model for acute inflammatory response, such as that noted in septic shock. Taken together, this study suggests that the proline variant may be more likely to mount an inflammatory response than the arginine variant. Almost no difference in cancer incidence or latency was noted between the two variants in two separate genetic models of cancer induction: one using $E\mu$ -myc- $p53^{P72/P72}$ and $p53^{R72/R72}$ mice and another using $p53^{P72/-}$ and $p53^{R72/-}$ mice¹¹⁵. These data, like ours, suggest that the polymorphism is not a strong predictor of cancer risk. Also in agreement with our findings, the Murphy

laboratory later reported increased apoptosis in IR exposed $p53^{R72/R72}$ mouse intestine¹¹⁶.

In another study from our laboratory, Sarkar et al. crossed $p53^{P72/P72}$ and $p53^{R72/R72}$ mice with K14-HPV16 mice –which express E6 and E7 oncoproteins from HPV viruses under the K-14 promoter – to test the effect of the polymorphism in a model of HPV infection. This allowed us to model carcinogen-induced (4-Nitroquinoline 1-oxide/4NQO) oral cavity and esophageal cancers in the context of HPV infection. Using this model, we showed esophageal cancer incidence and multiplicity were all greater in $p53^{P72/P72}$ -K14-HPV16 mice compared to $p53^{R72/R72}$ -K14-HPV16 mice exposed to 4NQO. There was no significant difference in incidence of cancer of the oral mucosa between genotypes but survival was attenuated in $p53^{P72/P72}$ -K14-HPV16 mice, suggesting shorter tumor latency. In addition, there were significantly increased neutrophil counts, white blood cell counts (WBC), serum globulin levels and inflammatory cell infiltrates in esophageal tumors in $p53^{P72/P72}$ -K14-HPV16 mice exposed to 4NQO when compared to $p53^{R72/R72}$ -K14-HPV16 mice and tumor tissues¹¹⁷. This study reinforced the Murphy laboratory's conclusion that genotoxic stress may result in increased inflammation associated with the proline allele in some tissues.

Unpublished data from a pilot study in our laboratory also showed what appeared to be increased sensitivity to IR in $p53^{P72/P72}$ mice compared to $p53^{R72/R72}$ mice, which suggested $p53^{P72/P72}$ mice might be more sensitive to adverse effects from ionizing radiation (IR), a commonly used genotoxic cancer therapy (**Figure 1.3**)

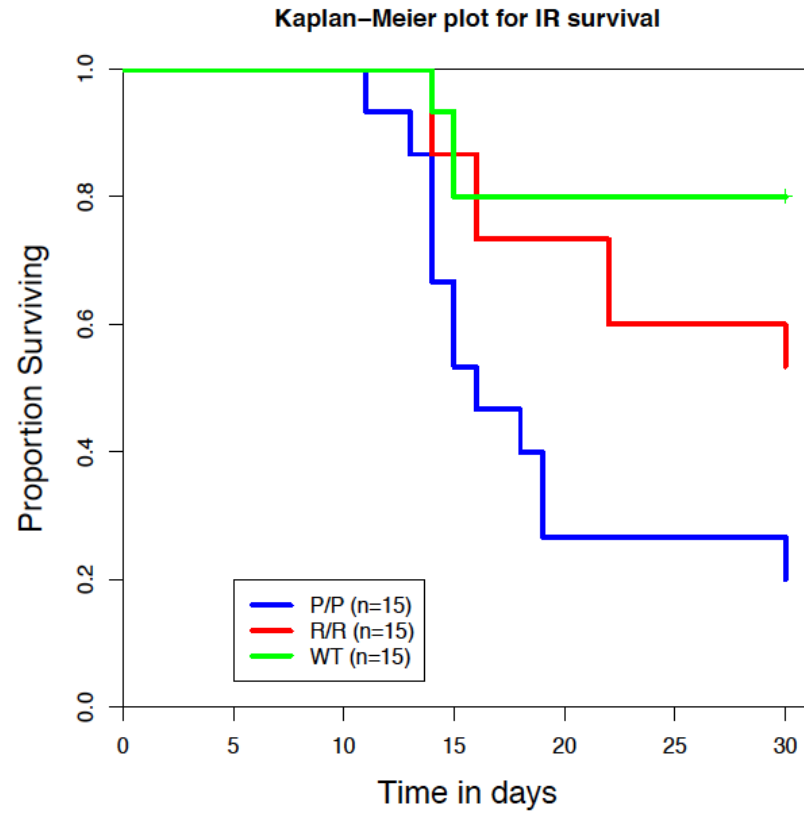


Figure 1.3 Different IR tolerance in p53 R72P variant mice. Young $p53^{P72/P72}$ and $p53^{R72/R72}$ and WT mice were treated with 6 Gy IR and followed to morbidity: $p53^{P72/P72}$ vs. $p53^{R72/R72}$ log rank test $p=0.03$

1.3 The importance of mouse strain background

Mouse background strain has long been known to affect IR sensitivity. Over 50 years ago it had been shown that, of the mouse strains employed at the time, the radio-resistant 129/J strain survived twice as long as the more radio-sensitive CBA/J following radiation exposure. Furthermore, the LD_{50.30} dose of radiation (i.e. the dose that killed 50% of mice by 30 days) was almost 2 Gy higher for 129/J mice compared to Balb/cJ mice^{118,119}.

Later studies showed that background strain may even alter radiation-induced apoptosis and target gene expression. These studies suggested that, upon exposure to equal doses of IR, the more radio-sensitive DBA/2 strain exhibited less IHC staining for markers of apoptosis in some tissues when compared to the more radio-resistant C57BL/6 strain. Furthermore following IR, the DBA/2 strain seemed to have decreased pro-apoptotic Bax protein, and increased p21 protein by western blot and IHC, at least in some tissues and at some time points¹²⁰⁻¹²².

For prior studies in our laboratory, we had backcrossed our p53 R72P KI mice for several generations to mice with the FVB and SKH (hairless) background. However, when we employed our Animal Genetics Core to perform marker assisted background characterization¹²³, we found that the

presumably FVB mice intended for future study still had mixed genetic backgrounds. We therefore collaborated with our Animal Genetics core to use marker assisted background characterization for maximizing the percentage of FVB background. In a process referred to as "speed congenics," $p53^{P72/P72}$ and $p53^{R72/R72}$ mice with the highest number of FVB genetic markers were crossed to WT FVB mice. The resulting $p53^{P72/WT}$ and $p53^{R72/WT}$ heterozygous progeny were chosen for further backcrossing by again choosing those mice with the most FVB markers. We continued this process until both $p53^{P72/WT}$ and $p53^{R72/WT}$ mice were greater than 99% FVB and then bred these mice to homozygosity for the purposes of this dissertation project. To prevent genetic drift in our population, homozygous $p53^{P72/P72}$ and $p53^{R72/R72}$ mice and/or heterozygous $p53^{P72/R72}$ mice were periodically outbred to recently acquired (from commercial facilities) FVB WT mice before being bred back to $p53^{P72/P72}$ and $p53^{R72/R72}$ homozygosity.

1.4 Hypothesis

The role of the p53 R72P polymorphism in human cancer is not well defined. Cell culture models suggest that the polymorphism affects apoptosis, with the cells expressing the R variant showing greater apoptosis in response to a wide variety of chemotherapeutics and IR. Nevertheless, epidemiological studies of cancer incidence fail to consistently show a major effect for the role of the p53 R72P polymorphism. Yet, limited epidemiological studies of survival differences and differential effects of cancer therapy consistently suggest that the proline variant negatively influences outcome and adversely affects therapy tolerance^{93,100-108}. Furthermore, this may not depend on the expression of p53 in the tumor¹⁰⁸ suggesting that non-tumor cell autonomous differences may account for this survival effect.

Two different mouse models of the p53 R72P SNP from two labs were able to show similar tissue specific induction of increased apoptosis in intestinal tissue in IR treated $p53^{R72/R72}$ mice when compared to IR treated $p53^{P72/P72}$ mice. Data showing that genotoxic insult may increase the inflammatory response in $p53^{P72/P72}$ mice compared to $p53^{R72/R72}$ mice^{115,117} provide preliminary evidence that differences in the tumor macro- and micro-environment may be mediating treatment and survival effects seen in human epidemiological studies. In

addition, our pilot study of IR sensitivity suggested that $p53^{P72/P72}$ mice are more sensitive to genotoxic insult, which supports the limited epidemiological evidence that patients with the proline variant of the polymorphism are more susceptible to adverse effects of cancer therapy. However, genes upregulated following genotoxic treatment were not always consistent across mouse studies or immortalized cell culture models, suggesting that mechanisms for any differences need to be further studied in primary cell cultures or *in-vivo*. In these mouse models, the evidence for differences between these polymorphic variants in tumor induction under oncogenic stress is much weaker than the evidence for differences in response to genotoxic insult.

Considering these data, it appeared that our mouse models of the polymorphism were best suited for studying tissue specific responses and tolerance of genotoxic cancer therapies. **For this project I hypothesized that the p53 R72P polymorphism modulates adverse effects of genotoxic cancer therapy by tissue specific regulation of pro-apoptotic and/or pro-inflammatory gene expression.** The novelty of this project is employing KI mouse models of a common human polymorphism as a pre-clinical assessment of how some adverse effects of genotoxic therapy may be modulated by the R72P polymorphism.

Chapter 2:

The Role of the p53 R72P Polymorphism

in the Adverse Effects of Doxorubicin

2.1 Introduction

Differences in cancer remission and survival following anthracycline-based chemotherapies are epidemiologically linked to differences in the p53 R72P genotypes^{102,124}. Although polymorphism-specific differences in p53-driven gene expression of pro-inflammatory and anti-apoptotic gene expression have been noted^{115,125}, a comprehensive *in vivo* study to identify mechanisms for these differences has not been performed.

This study sought to test the effects of anthracycline therapy in our mouse models of the p53 R72P SNP. By using mouse models, we could analyze systemic effects that cannot be studied in cell culture while still controlling for genetic and environmental differences found in epidemiological studies. We chose to focus on an RNA-sequencing experiment to analyze gene expression as the most unbiased and comprehensive approach to understanding differences in gene expression between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice. We chose doxorubicin exposure as a model chemotherapeutic as it is known to induce DNA damage and acute inflammation¹²⁶⁻¹²⁸ and is used in treatment regimens for many cancers¹²⁸. Thus, doxorubicin tolerance may be a factor in the differential survival and response to therapy patterns noted in human population

studies of the polymorphism. We used a dose of 5 mg/kg doxorubicin, which is considered a therapeutic dose in mouse models of tumor response¹²⁹.

RNA sequencing was used to investigate differences in gene expression between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice upon exposure to doxorubicin in two tissues: thymus and liver. We chose to analyze the thymus as data from a $p53^{R72P}$ mouse model showed differential gene expression patterns in thymocytes in response to genotoxic insult¹¹⁵. Furthermore, the thymus is important for re-establishing T-cell populations after lymphotoxic treatments in cancer patients¹³⁰. We chose the liver since it is the primary organ for doxorubicin metabolism^{131,132}. Blood tests were also performed to determine whether there would be differences in several white blood cell (WBC) numbers, a possible side effect of doxorubicin chemotherapy in humans¹³³.

Surprisingly, very few differences in gene expression between genotypes were found in treated and untreated mice. Additionally, we could not confirm that the doxorubicin dose we employed had elicited adverse effects on leukocyte numbers or body weight change, suggesting we needed to adjust the dose. Therefore, we later conducted a toxicology experiment on WT FVB mice to attempt to determine a dosing regimen that would elicit blood count or weight changes in FVB mice. This study also showed few quantifiable differences

in blood counts or weight at any dose between saline and doxorubicin treated mice, suggesting that FVB mice may be resistant to doxorubicin toxicity.

2.2 Materials and Methods

Mice and cell lines used:

Generation of $p53^{P72/P72}$ and $p53^{R72/R72}$ knock-in mice is described elsewhere¹¹². To avoid potential influence of mouse strain on phenotypes, we backcrossed to FVB mice until they were >99% FVB using marker-assisted background characterization¹²³. We generated mouse adult fibroblasts (MAFs) from adult $p53^{P72/P72}$ and $p53^{R72/R72}$ mice by mincing peritoneal tissue and subjecting the tissue to collagenase and trypsin digestion before growing the cells to confluence in DMEM with 10%-20% FBS. MAFs were passaged a maximum of five times.

Doxorubicin treatment:

For blood counts and RNA sequencing studies, at approximately six weeks of age, female $p53^{P72/P72}$ and $p53^{R72/R72}$ mice were injected intraperitoneally (IP) with either doxorubicin diluted in normal saline to 0.2 mg/ml, at a dose of 5mg/kg, or the equivalent volume of normal saline for control mice. Mice were weighed prior to treatment and seven days after the first treatment.

For the doxorubicin toxicity study, young female WT FVB mice were purchased (Harlan/Envigo, Indianapolis, IN, USA). When mice were approximately 100 days old, they were divided in to five treatment groups of eight mice for the different doxorubicin doses. These treatment groups were as follows:

Group	Treatment	Dose (mg/Kg)	Frequency	Duration
1	Saline	N/A	Every 48 hours	3 treatments
2	Doxorubicin	2.5	Every 48 hours	3 treatments
3	Doxorubicin	5.0	Every 48 hours	3 treatments
4	Doxorubicin	7.5	Every 48 hours	3 treatments
5	Doxorubicin	10.0	Once	1 treatment

Mice in groups 1-5 were weighed prior to the first injection and after the third injection. Mice that exhibited unexplained morbidity or death prior to the completion of the study were excluded.

RNA isolation and sequencing:

Mice were treated once weekly for two weeks with 5mg/kg doxorubicin or saline controls were sacrificed by CO₂ inhalation 24 hours following the final injection. Liver and thymus were extracted and immediately frozen in liquid nitrogen until RNA extraction. RNA was extracted with RNEASY kit with DNA digestion (Qiagen, Hilden, Germany) as per manufacturer's protocol. Synthesis

of cDNA and sequencing was performed in our Molecular Biology Core using the Illumina HiSeq 2000. Our Bioinformatics Core analyzed the data: briefly, they mapped reads to reference sequences using TopHat ¹³⁴, and quantified differential expression using the R/Bioconductor package ¹³⁵.

Doxorubicin toxicity, histopathology:

Two mice from each treatment group were sacrificed at 96 hours following the final treatment, necropsied and tissues were fixed in 10% buffered formalin. H & E staining was carried out using a Shandon Varistain Gemini automatic stainer (ThermoFischer Scientific, Waltham, MA, USA). Slides were read by our Veterinary Pathologist.

Blood counts:

For monitoring blood counts, 100 uL of blood was drawn from live approximately $p53^{P72/P72}$ and $p53^{R72/R72}$ mice by saphenous vein bleed prior to doxorubicin or saline injection and following treatment at the time-points indicated. Blood was collected in sodium EDTA micro-tubes and counted using the Hemavet 950 hematology system (Drew Scientific, Miami Lakes, FL, USA).

Reverse transcription and quantitative PCR (q-PCR)

cDNA was generated using the SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. Quantitative PCR was carried out using the following primer sets:

Cxd1: For. 5'TTAGGGTGAGGACATGTGTGG3' Rev. 5'TGCCCTACCAACTAGACACA3'

Inhbb: For. 5'AACAATCCTTCGAGTGGCCT3' Rev. 5'CAGCACACACCTTCACTCCA3'

Tgtp1: For. 5' AGGCATGTAAGCACCTCCAC3' Rev. 5'GGACAGAGAGGCAGGTTCAC3'

For *Xaf1*, PrimePCR™ SYBR® Green Assays qMmuCID0009205 and qMmuCED0049379 (Biorad, Hercules, CA, USA) were used.

2.3 Results

2.31 RNA sequencing in doxorubicin treated and untreated p53 R72P mice:

RNA sequencing was carried out on thymus and liver of six-week-old $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated with saline or doxorubicin once weekly for two weeks and sacrificed 24 hours following the final injection. For RNA sequencing data, we only considered genes with a false discovery rate (FDR) of 0.05 or less and a minimum of a two-fold difference between genotypes.

RNA sequencing of the thymic tissues revealed only one well-characterized gene differentially regulated per this criteria across genotypes: *X-inhibitor-of-apoptosis-associated-factor-1* (*Xaf1*). *Xaf1* was of interest to our study, as it is generally considered a negative regulator of inhibitor-of-apoptosis (IAP) protein family members, and has been implicated in modulating p53 apoptotic signaling upon some genotoxic insults¹³⁶. By RNA sequencing, *Xaf1* had 2.6-fold greater expression in doxorubicin treated $p53^{R72/R72}$ thymus as compared to doxorubicin treated $p53^{P72/P72}$ thymus. However, *Xaf1* was similarly up-regulated in both treated and untreated $p53^{R72/R72}$ thymus, compared to

untreated $p53^{P72/P72}$ thymus, suggesting that this allelic difference was not induced by doxorubicin treatment.

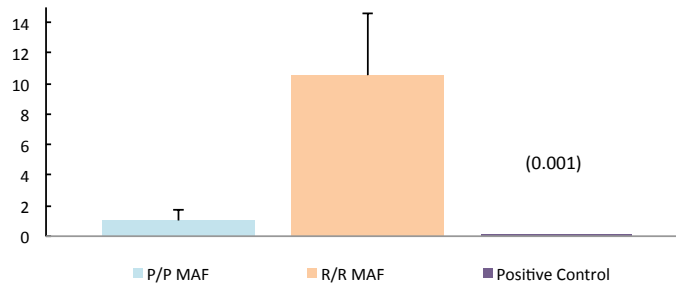
We attempted to use reverse transcription (RT) and q-PCR to confirm this difference in expression from the thymic RNA used in sequencing samples and a second biological replicate. In agreement with the RNA-Seq data, we found a highly significant increase in *Xaf1* expression in $p53^{R72/R72}$ mouse thymus compared to $p53^{P72/P72}$ thymus regardless of treatment, and this difference was not significant between treatment groups. We also found this difference in RNA expression in untreated and IR treated $p53^{P72/P72}$ and $p53^{R72/R72}$ MAFs.

Nevertheless, in experiments with a second *Xaf1* primer set, *Xaf1* q-PCR did not show significant differences between genotypes. This implied that the initial results might have been an artifact and not the actual *Xaf1* gene. Indeed, DNA sequencing of PCR fragments revealed what appeared to be an artifact using the first primer (that showed a large difference between genotypes) (**Figure 2.1a**). Sequencing also revealed that the second primer (which showed little difference between genotypes) produced a fragment of the correct size and sequence to be representative of actual *Xaf1* gene expression. Furthermore, q-PCR of proprietary Biorad positive control synthetic DNA did not show amplification of *Xaf1* using the first primer (that showed a difference in *Xaf1* expression between genotypes). On the other hand, there was strong

amplification of *Xaf1* using the positive control synthetic DNA with the second primer (that *did not* show a difference in *Xaf1* gene expression between p53 R72P genotypes) (**Figure 2.1b**). Taken together, these data suggested that an RNA artifact, not representative of actual *Xaf1* gene expression, was being differentially amplified in the initial RNA sequencing and in the q-QCR results (which had shown increased *Xaf1* expression in $p53^{R72/R72}$ MAFs). Considering this, we decided not to further pursue studying *Xaf1* expression in our mouse models of the p53 R72P polymorphism.

a

Untreated MAFS XAF1 Primer 1 (fold over P/P)



b

Untreated MAFS XAF1 Primer 2 (fold over P/P)

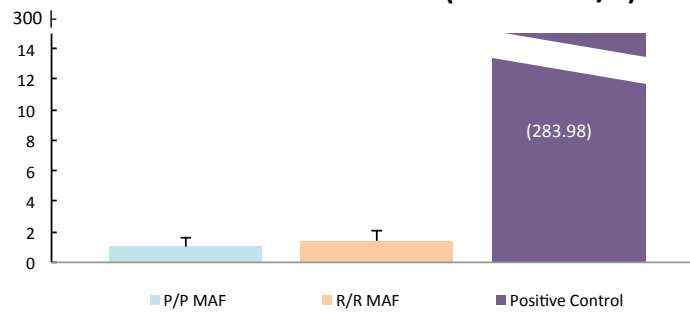


Figure 2.1 *Xaf1* expression in p53 R72P MAFs. q-PCR on cDNA from untreated MAFs using the *Xaf1* primers that **a)** amplified artifact cDNA, and **b)** amplified the actual *Xaf1* cDNA

In treated liver, only eight genes were found to have two fold or greater difference in expression between genotypes and an FDR of less than 0.05 and only five had well defined functions. Of those, three were involved in inflammation. Differences in transcriptional regulation of inflammatory genes have been seen in other tissues in another mouse model of the p53 R72P polymorphism¹¹⁵. Therefore, we attempted validation by RT and q-PCR on these genes. q-PCR on cDNA synthesized from the liver RNA used for the RNA-Seq experiment did not confirm the differences seen by RNA-Seq (**Table 2.1**). Due to the paucity of differences in gene expression by RNA-Seq between genotypes in thymus and liver, we decided not to further pursue differences in gene expression induced by doxorubicin.

Locus	Gene	Function	Expression increased in	Fold change	pvalue	FDR	Q-PCR supports RNA sequencing?
chr7:104768049-104777470	Usp17le	deubiquitylase	P/P	179.635427	1.64E-05	0.02	ND
chr5:90891245-90893115	Cxcl1	chemokine, neutrophil attraction, angiogenesis, wound healing, infection, tumorigenesis	P/P	6.12892169	8.74E-06	0.02	No
chr1:119415465-119422248	Inhbb	normally FSH inhibitor, also related anemia of inflammation via activation of hepcidin in liver	P/P	2.8077116	3.18E-06	0.02	No
chr17:3326573-3519397	Tiam2	neural development	R/R	3.93065176	2.37E-05	0.03	ND
chr11:48985329-48992246	Tgtp1	interferon gamma induced gene	R/R	9.08815308	4.44E-05	0.05	No

Table 2.1 Summary of RNA sequencing and q-PCR validation. A list of genes differently regulated in the liver of $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated once weekly for two weeks with doxorubicin 5mg/kg and sacrificed 24 hours following the final treatment. Genes associated with inflammation are highlighted in red.

2.3.2 Blood counts and weights in doxorubicin treated and untreated p53 R72P mice

Treating mice once with 5mg/kg of doxorubicin resulted in little change in WBC, neutrophil or lymphocyte counts one week after treatment compared to saline treated mice. There were minimal differences between genotypes and between treatment groups *prior* to treatment, and no significant differences between genotypes or between treatment groups *after* treatment (**Figure 2.2**). Furthermore, body weights were not affected by this dose (**Figure 2.3**). These data suggested that the doxorubicin dose of 5 mg/kg caused minimal systemic adverse effects in FVB mice.

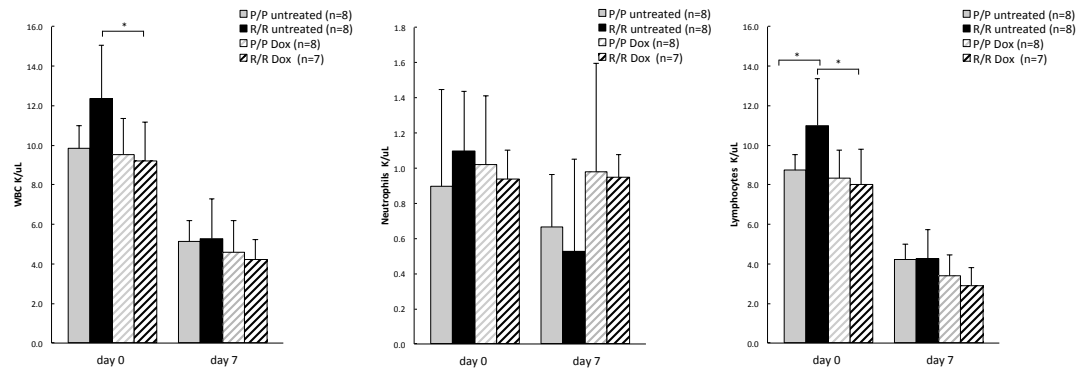


Figure 2.2 Hematology in p53 R72P mice untreated or treated with doxorubicin. Minimal differences between genotypes and treatment groups in hematology performed on $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated once with doxorubicin 5mg/kg or saline seven days after treatment

two-sided independent t-test

* $p < 0.05$.

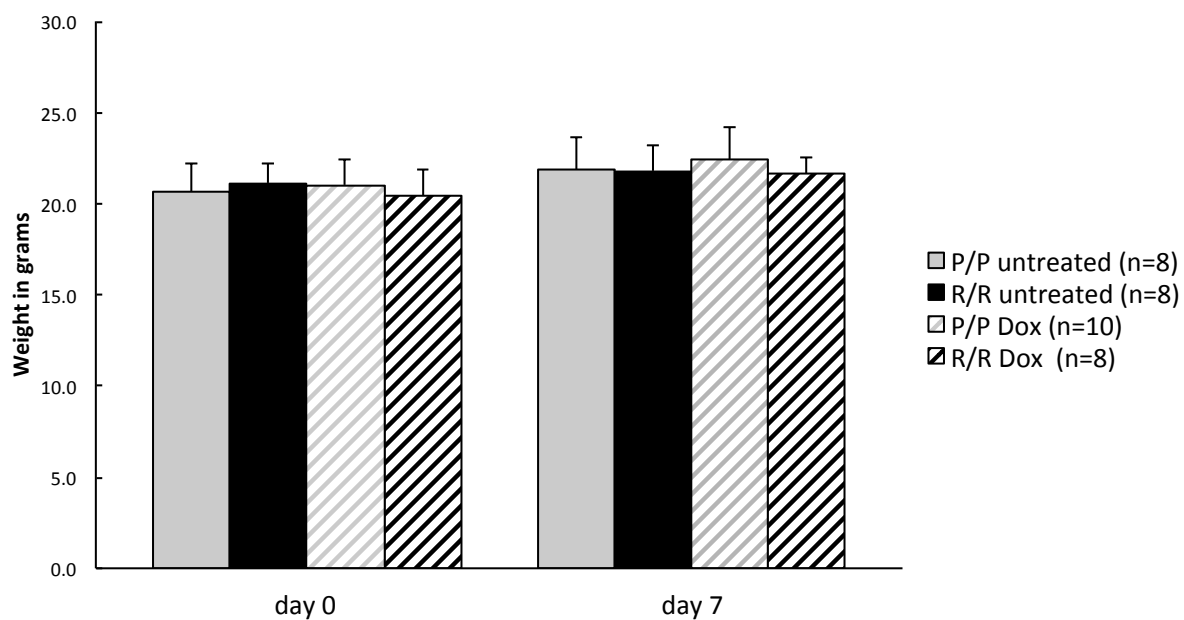


Figure 2.3 Weights of p53 R72P mice untreated or treated with doxorubicin. No differences between treatment groups or genotypes in weights of $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated once with doxorubicin 5 mg/kg or saline seven days after treatment.

2.3.3 Doxorubicin toxicity study in WT FVB mice

The above studies pointed to the lack of quantifiable adverse effects of doxorubicin at a dose of 5 mg/kg weekly, at least in FVB mice. Therefore, we decided to attempt to find a dosing regimen and time frame that would elicit measurable adverse effects in WT FVB mice prior to continuing study on the p53 R72P polymorphic mice. To this effect, same-aged, adult FVB WT female mice were treated with one of five dosing schedules: 1) saline only every 48 hours for three doses, 2) doxorubicin 2.5 mg/kg every 48 for three doses, 3) doxorubicin 5.0 mg/kg every 48 hours for three doses, 4) doxorubicin 7.5 mg/kg every 48 hours for three doses, or 5) doxorubicin 10.0 mg/kg for a single dose. Blood counts were performed in all treated mice at 72 hours following the final treatment. Weight changes and histopathological tissue changes were analyzed in a subset of mice.

Blood counts at 72 hours following the final dose failed to show significant differences between treatment groups, with the exception of an increase in neutrophil counts in mice treated at 5.0 and 7.5 mg/kg doses described above (**Figure 2.4**). A significant difference in weight was noted between the saline treated mice and those treated with 5.0 and 7.5 mg/kg

doxorubicin following the third treatment; however, there were also significant differences between groups prior to treatment (**Figure 2.5**).

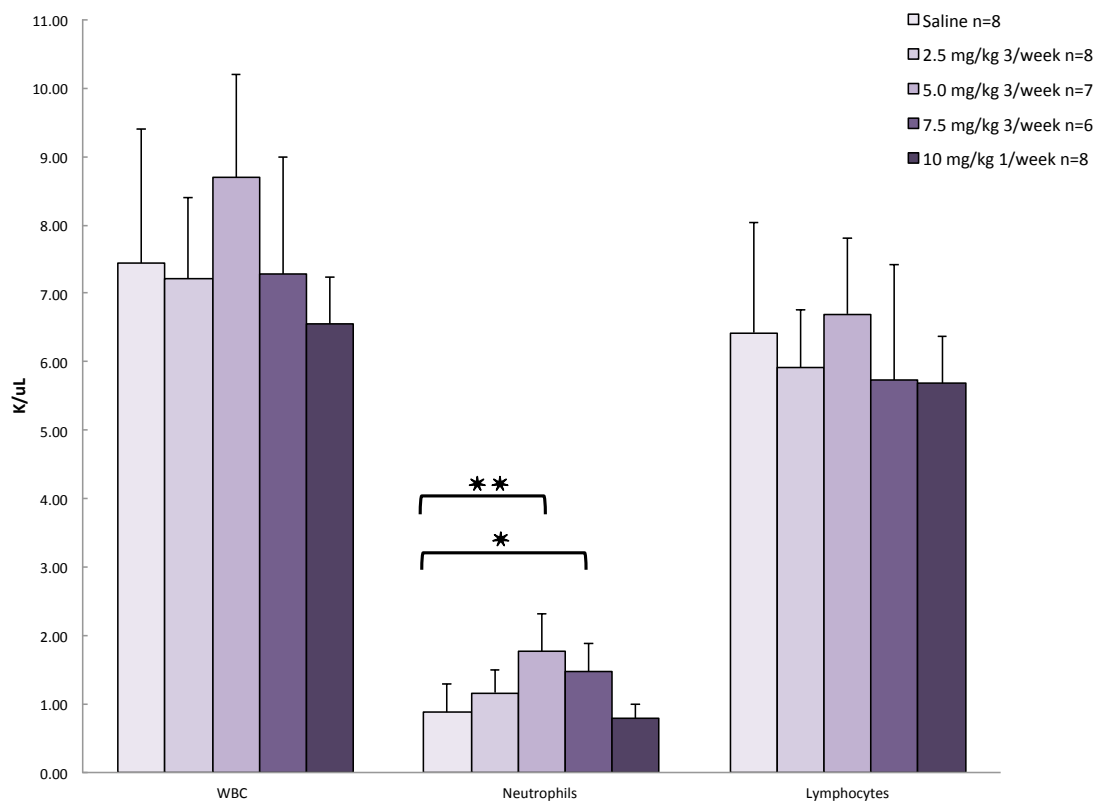


Figure 2.4 Hematology in WT FVB treated untreated or treated with doxorubicin. Minimal differences between treatment groups in hematology performed on WT FVB mice treated every 48 hours for three doses with saline, doxorubicin 2.5 mg/kg, 5.0 mg/kg, or 7.5 mg/kg or a single dose of doxorubicin 10 mg/kg at 72 hours after final treatment

two-sided independent t-test

* $p < 0.05$, ** $p < 0.005$

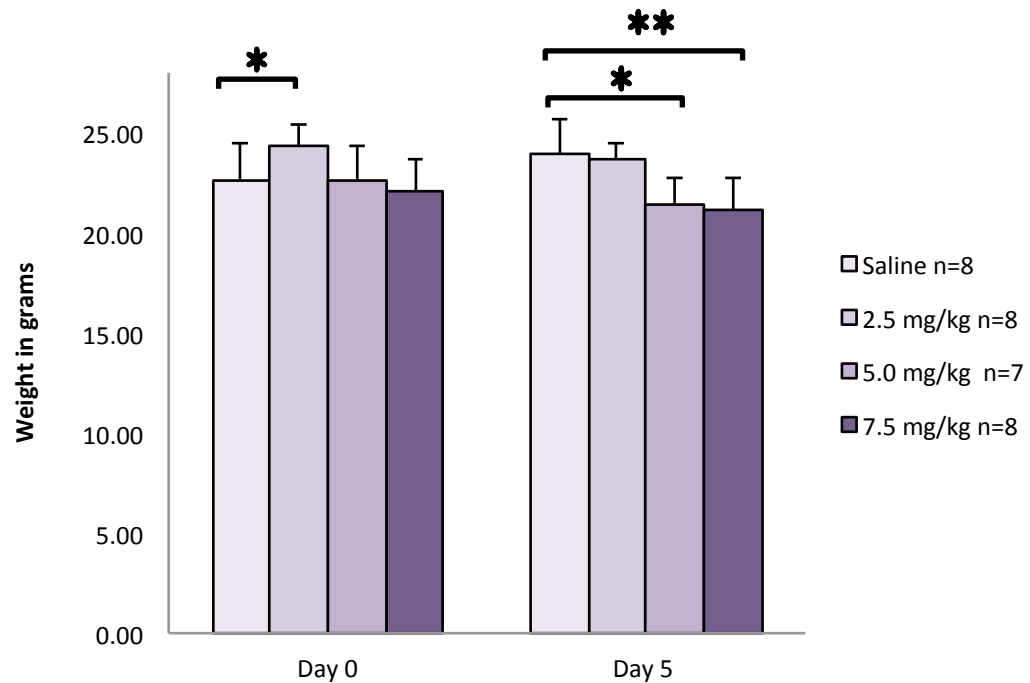


Figure 2.5 Weights of WT FVB mice untreated or treated with doxorubicin. Differences in weight in WT mice treated with saline or doxorubicin at 2.5, 5.0, or 7.5 mg/kg prior to initial treatment (Day 0) and prior to the third treatment (Day 5)

two-sided independent t-test

* $p < 0.05$, ** $p < 0.005$

Histological analysis of tissues by our veterinary pathologist from a subset of mice revealed some qualitative differences, especially in mice treated with higher doses of doxorubicin. Mice treated with 7.5 mg/kg doxorubicin for three doses and mice treated with 10 mg/kg doxorubicin for one dose showed mild cardiomyocyte degeneration, a well-established adverse effect of doxorubicin therapy¹³⁷. Mice treated with 7.5 mg/kg for three doses exhibited intestinal villous atrophy. Although cortical thymus atrophy was noted in all mice treated with doxorubicin, there was no evidence of increased apoptosis in splenic lymphocytes, suggesting that the thymic atrophy may have been a stress response and not a direct result of doxorubicin toxicity. Consistent with the minimal differences noted in blood counts, there was little histological evidence of bone marrow depletion in any treatment group.

Although the histopathology report and weight loss data did indicate some adverse effects of doxorubicin therapy at higher doses in FVB, these differences were not marked and were mainly qualitative. Furthermore, the lack of differences in blood counts between treatment groups, and limited indications of apoptosis in any tissue of doxorubicin treated FVB mice suggested that further study with doxorubicin would not generate quantifiable data. Therefore, we decided not to further pursue differences in adverse effects of doxorubicin therapy using our p53 R72P polymorphic mice.

2.4 Discussion

In this study we examined whether the p53 R72P polymorphism modulated the response to doxorubicin toxicity as a model of genotoxic cancer therapy. We sought to determine whether differences noted in gene expression upon doxorubicin therapy would correlate with physiological phenotypes of weight loss and reduced blood cell counts.

When mice were treated with doxorubicin at 5 mg/kg for two weekly treatments, RNA sequencing only revealed one gene differentially expressed in thymus of $p53^{P72/P72}$ and $p53^{R72/R72}$ mice, *Xaf1*. However, further investigation indicated that this difference was likely attributable to an artifact transcript, as only one primer (a primer which did not work for positive control synthetic DNA) was able to confirm any difference in *Xaf1* expression between genotypes. There were only five characterized genes differentially upregulated in the liver: none were associated with apoptosis, but three were associated with inflammation. Nevertheless, q-PCR failed to validate differences in their expression. The paucity of genes found precluded pathway analysis. Furthermore, it indicated that there were very few differences in gene expression between genotypes in our mouse models of the polymorphism when mice were exposed to the genotoxic agent doxorubicin.

Blood counts and weight were unaffected by this dose of doxorubicin, making comparison of physiological response between p53 R72P variants challenging. To attempt to find a dose of doxorubicin that would induce quantifiable differences in blood counts and weight, and qualitative differences in histology, we attempted a toxicity study in WT FVB mice. However, even at the highest doses, doxorubicin induced only minimal changes in weight and no significant decreases in blood cell counts. Furthermore, histological changes were minimal. Therefore, it seemed that using doxorubicin exposure in FVB mice was a poor model to assess the adverse effects of genotoxic cancer therapy. Since we had evidence that another genotoxic therapy, IR, produced much more quantifiable differences in gene expression and tissue damage¹¹², we chose to study how the p53 R72P polymorphism modulates adverse effects of cancer therapy using IR.

Chapter 3:

**The Role of the p53 R72P Polymorphism in
the Adverse Effects of Ionizing Radiation**

3.1 Introduction

Given that the above data suggested that doxorubicin was not eliciting quantifiable adverse effects in FVB mice, we decided that differential response to IR treatment may serve as a better model for how the p53 R72P polymorphism modulates therapy side effects. The genotoxic effects of IR are well established. At a cellular level, IR induces DNA double strand breaks (DSBs) in addition to other forms of DNA damage. This damage is done directly or through the generation of ROS. In tissues sensitive to ionizing radiation, such as intestinal epithelia and hematopoietic cells, and in tumors bearing WT p53, DSBs result in apoptosis, often through induction of pro-apoptotic genes by p53¹³⁸.

IR is widely used for cancer therapy, and at MD Anderson (mdanderson.org), it is used as a treatment of at least 50% of cancers. IR therapy can involve total body irradiation (TBI) or more targeted therapies. TBI is often used in hematologic malignancies as a myeloablative treatment before hematopoietic stem cell transplant¹³⁹. Localized treatment can sometimes induce systemic effects as well. Myeloblation can occur as an acute side effect of thoracic vertebrae IR therapy when large sections of bone marrow are exposed to IR. This can lead to neutropenia, leukopenia and thrombocytopenia¹⁴⁰. Other

acute side effects of high dose radiation include gastrointestinal disorders: TBI can induce nausea and vomiting¹³⁹, while IR that targets the pelvic region can induce mucositis with diarrhea and cramping¹⁴¹.

Histopathological changes evident in mice exposed to IR are in line with these patient symptoms. In mice, whole body irradiation is associated with histological changes such as decreased cellularity and accumulated nuclear debris in bone marrow, shortened villi in the small intestine and, in some cases, ulceration of the small intestine¹¹⁸. These data suggest that mice treated with whole body IR may serve as a model organism for the adverse effects of IR therapy in cancer patients.

Understanding the role of the p53 R72P polymorphism in adverse therapy effects could have implications for personalized medicine, especially in the field of radiogenomics. Radiogenomic studies focus on how naturally occurring polymorphisms influence tumor response to radiation and the adverse effects of IR therapy in patient populations. Current research in this field employs case/control studies and genome wide association studies (GWAS) to characterize the effects of gene variants on IR therapy (reviewed in ¹⁴²⁻¹⁴⁴). Radiogenomic studies of the p53 R72P polymorphism are limited to two studies. One study found an increase in late skin complications of patients harboring the proline allele ¹⁴⁵ although an earlier study had found the polymorphism did not

significantly affect *acute* skin damage following similar treatment ¹⁴⁶. Given this lack of epidemiological information and the fact that published and unpublished data from our laboratory (**Figures 1.2 & 1.3, Chapter 1**) and others suggested that there were differences not only in immediate apoptotic response, but also in longer-term survival between IR treated p53 R72P mice, we decided to use these mouse models in preclinical testing of how the p53 R72P polymorphism impacts IR toxicity. We tested the differential effects of IR therapy on adult $p53^{P72/P72}$ and $p53^{R72/R72}$ mice measuring blood counts, body and tissue weight changes, histomorphological changes and long-term survival studies.

3.2 Materials and Methods

Mice used:

Generation of $p53^{P72/P72}$ and $p53^{R72/R72}$ KI mice is described elsewhere ¹¹². To avoid potential influence of mouse strain on phenotypes, we backcrossed KI mice to FVB mice until they were determined to be >99% FVB, using marker-assisted background characterization ¹²³. To avoid the possibility that differences in development could account for phenotypes, we only used adult mice approximately 15 weeks old, and to control for differences between sexes, mice were sex-matched for each experiment, or an equal number of mice from each sex were employed.

IR survival studies:

Mice were subjected to X-ray IR at doses described below using an RS 2000 Biological Research Irradiator (RadSource, Suwanee, GA, USA). Approximately 15-week-old $p53^{P72/P72}$ and $p53^{R72/R72}$ mice were treated with 6.135 Gy IR. Mice were weighed, transferred to sterile housing and treated with prophylactic antibiotics (Clavamox: 50mg/ml amoxicillin and 12.5mg/ml clavulanic acid) in their drinking water. We monitored mice daily, weighed mice weekly and sacrificed mice exhibiting morbidity.

Western Blots:

Cells from spleen were forced through a cell filter into cold PBS and pelleted. The pellets were lysed with RIPA Lysis Buffer System (#SC-24948, Santa Cruz Biotechnology, CA, USA). Western blot was performed on lysates with primary anti-p53 antibody A-1 (#SC-393031, Santa Cruz Biotechnology, CA, USA, 1:500 dilution, 4°C overnight) and secondary antibody m-IgGκ BP-HRP (#SC-516102, Santa Cruz Biotechnology, CA, USA, 1:5000 dilution, room temperature (RT) 1 hour). Membranes were washed and re-probed with anti-GAPDH antibody (MAB374, Millipore Sigma, Darmstadt, Germany 1:5000 dilution, 4°C overnight) and rabbit anti-mouse secondary antibody (#SC-358914 Santa Cruz Biotechnology, CA, USA, 1:10,000 dilution, RT, 1 hour).

For MAF lysates, primary antibodies anti-p53 CM5 (#VP-P956, Vector Laboratories, Burlingame, CA, USA, 1:1000 dilution, 4°C overnight) or anti-phospho-p53 (Serine 15) (CST-#9284, Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution, 4°C overnight) and donkey anti-rabbit secondary antibody (#NA934, GE Healthcare UK Limited, Buckinghamshire, UK, 1:10,000, RT, 1 hour) were used. Membranes were stripped and re-probed with anti-β-actin clone AC-15 (#A1978, Sigma-Aldrich, Darmstadt, Germany, 1:10,000, room temperature, 30-60 minutes) and rabbit anti-mouse secondary (#SC-358914, Santa Cruz Biotechnology, CA, USA, 1:10,000 dilution, RT, 30-60 minutes).

Density of protein bands on film was determined using ImageJ software (<https://imagej.nih.gov/ij/>).

Reverse transcription (RT) and quantitative PCR (q-PCR):

Cells from thymus were forced through a cell filter into cold PBS and pelleted. RNeasy Plus Mini Kit (Hilden, Germany) was used, as per manufacturer's instructions to remove genomic DNA and extract RNA. cDNA was generated using the SuperScript® II Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. Quantitative PCR was carried out using the primers:

p21- 5'AATACCGTGGGTGTCAAAGC3' & 5'GTGTGAGGACTCGGGACAAT 3'
Mdm2-5'AGGAAGCGAAGGATAGCTTTG3' & 5'CACCGCCTCCTATTCAAATG3'
Noxa-5'TCAGCATGTGTATCCGTTTCA3' & 5'CCTTAATGCTGAGGCCCACT3',
Puma-5' TGTGACCACTGGCATTTCATT3' & 5'CCCAGACTCCTCCCTCTTCT3'.

PrimePCR™ SYBR® Green Assays qMmuCID0007961 was used for *Perp*, qMmuCID0006274 for *Bax* and qMmuCID0006264 for *p53* (Biorad, Hercules, CA, USA). All gene expression values were normalized to the average value of P/P untreated mice.

Post-IR blood counts and blood clinical chemistry:

For monitoring blood count, approximately 100 uL of blood was collected from the saphenous vein of live 14- to 17-week-old $p53^{P72/P72}$ and $p53^{R72/R72}$ mice before IR exposure. Mice were subjected to 6.135 Gy IR or left untreated, and blood sampling was repeated three days after exposure. Because other tissues were collected from mice on day seven following IR, for day seven blood counts and clinical chemistry, we sacrificed mice by CO₂ inhalation and immediately removed approximately 500 uL of blood by cardiac puncture.

For blood counts, blood was collected in sodium EDTA micro-tubes and counted using the Hemavet 950 hematology system (Drew Scientific, Miami Lakes, FL, USA). For clinical chemistry analysis, blood was collected in lithium heparin micro-tubes and analyzed using the Abaxis Vetscan VS2 clinical chemistry analyzer with Comprehensive Diagnostic Profile rotors #500-0038 (Union City, CA, USA).

Post IR spleen weight study:

Mice were weighed prior to treatment and at sacrifice, and the spleen was weighed after formalin fixation.

Post-IR IHC analysis:

Fourteen- to 17-week-old $p53^{P72/P72}$ and $p53^{R72/R72}$ mice were subjected to 6 Gy IR (four-hour study) or 6.135 Gy IR (24-hour and seven-day study) or left untreated and sacrificed at the indicated time-points. Tissues were fixed in 10% buffered formalin, embedded in paraffin and sectioned routinely. IHC staining was used to detect cleaved lamin-A (Cell Signaling #2035, Danvers, MA, USA, 1:50 dilution overnight at 4° C), γ H2AX (Cell Signaling #2577, Danvers, MA, 1:200 dilution, 2 hours at room temperature) and p53 (#SC-6243, Santa Cruz Biotechnology, CA, USA, 1:50 dilution one hour at room temperature). H & E staining was performed with Shandon Varistain Gemini automatic stainer (ThermoFischer Scientific, Waltham, MA, USA). Slides were digitized using a Scan Scope CS (Leica Biosystems, IL, USA) and analyzed with the manufacturer's GENIE software (Spectrum version 10.2.2.2315). The standard DAB nuclear analysis program was modified to improve its ability to recognize immunopositive nuclei and the modified analysis algorithm was applied to selected tissues.

Statistical testing:

Statistical significance for most studies was determined with either a one-way ANOVA followed by Tukey's multiple comparison test, or a two-sided

independent t-test (as indicated on figure legends). For survival curves, a Kaplan–Meier estimate was generated and analyzed for statistical significance with the log-rank test employing Survival Package R (<https://cran.r-project.org/>). In all cases, tests with a p value of 0.05 or less were considered statistically significant.

3.3 Results

3.3.1 The p53 R72P polymorphism in IR induced apoptosis in sensitive tissues

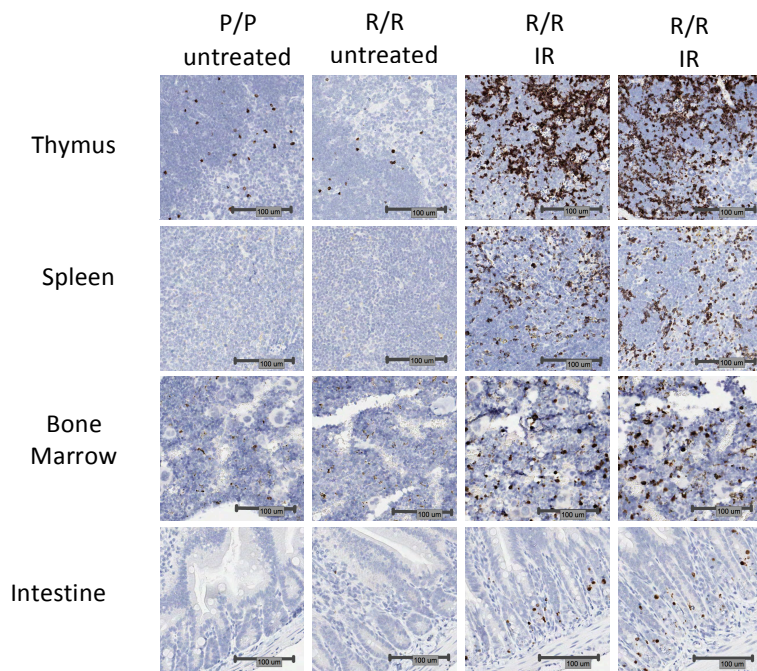
Prior studies by our laboratory and others (using distinct but similar knock-in models) indicated that the p53 R72P polymorphism modulates apoptotic response to IR in the intestine and the thymus^{112,116}. To confirm this finding in the current model we conducted two sets of experiments using age and sex-matched mice that were at least three months of age.

We treated FVB $p53^{P72/P72}$ (P/P) and (R/R) female mice, housed together to synchronize estrous cycles, with 6 Gy IR, and sacrificed these mice at four hours following treatment. Untreated cage-mates were sacrificed as controls. Radiosensitive tissues were formalin fixed for IHC staining. At four hours, cleaved lamin-A positive cells, a marker of apoptosis¹⁴⁷, increased in both genotypes in thymus, spleen, bone marrow and intestine when compared to tissues from untreated mice (**Figure 3.1a**). The percentage of cleaved lamin-A positive cells was determined with digitized slides and histomorphometry image analysis software. At four hours, a significant difference in percent positive cells was present for all tissues when IR treated tissues were compared to untreated tissues ($p < 0.001$ to $p < 0.05$). A few differences were noted between genotypes,

the most prominent being apparently reduced apoptosis in IR treated $p53^{P72/P72}$ bone marrow when compared to IR treated $p53^{R72/R72}$ bone marrow. However, in contrast to prior studies, there was lack of evidence for statistically significant differences between genotypes in any treatment group (**Figure 3.1 b**).

Next, we treated FVB $p53^{P72/P72}$ (P/P) and $p53^{R72/R72}$ (R/R) male mice with 6.135 Gy IR, and sacrificed these mice at 24 hours following treatment. Untreated cage-mates were sacrificed as controls. At 24 hours following IR, IHC staining for cleaved lamin-A was increased compared to control tissues. There was also markedly diminished cellularity and more cellular debris in thymus, spleen, and bone marrow of IR treated mice compared to controls. These observations suggested ongoing and completed apoptosis. As in the four-hour study, at 24 hours there was no qualitative difference between genotypes for any treatment group (**Figure 3.2a**). The percentage of cleaved lamin-A positive cells was determined with digitized slides and histomorphometry image analysis software. At 24 hours there was an increased percentage of positive cells in IR treated tissues were compared to untreated tissues; however, a significant difference between treatment groups was only found in $p53^{P72/P72}$ spleen and intestine ($p<0.001$). Although the percent cleaved lamin-A positive nuclei in IR treated $p53^{R72/R72}$ spleen and intestine was lower than the percent cleaved lamin-A positive nuclei in IR treated $p53^{P72/P72}$ spleen and intestine, there

was lack of evidence for statistically significant differences between genotypes in any treatment group (**Figure 3.2b**). Again, this is in contrast to prior studies.



b

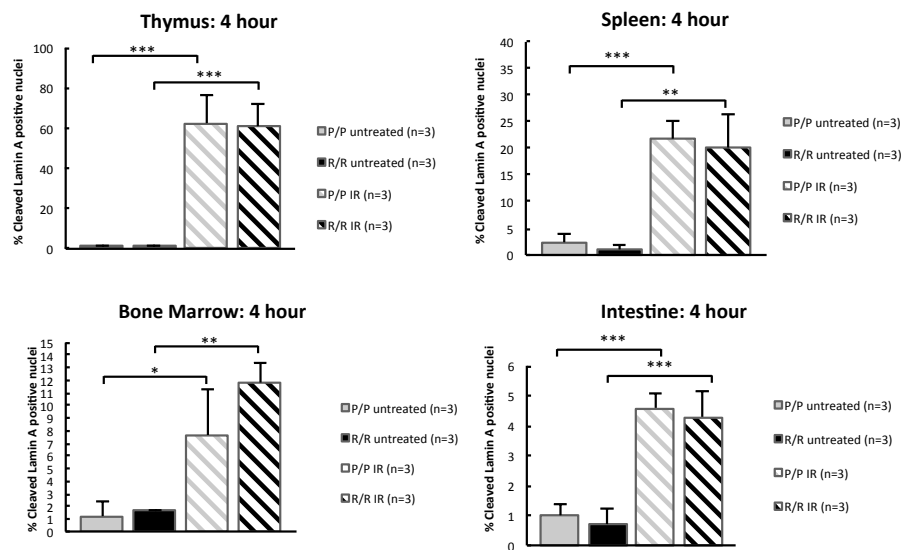
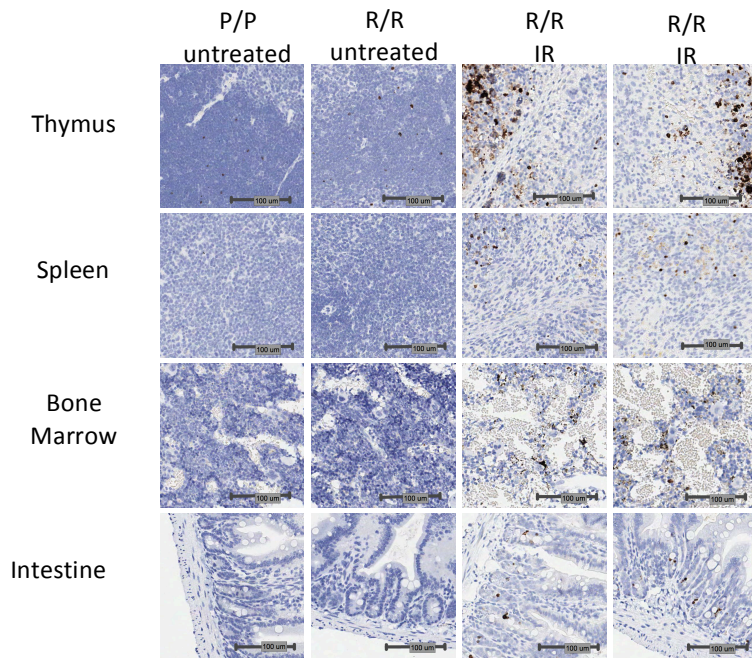


Figure 3.1 Cleaved lamin-A staining in untreated and IR treated p53 R72 mice at four hours following IR. a) Representative images of cleaved lamin-A IHC in p53 R72P female, adult mice untreated or treated untreated or treated with 6 Gy IR at 4 hours and **b)** percent cleaved lamin-A positive cells in thymus, spleen, bone marrow and intestine in these mice.

One-way ANOVA Tukey's multiple comparisons test

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$



b

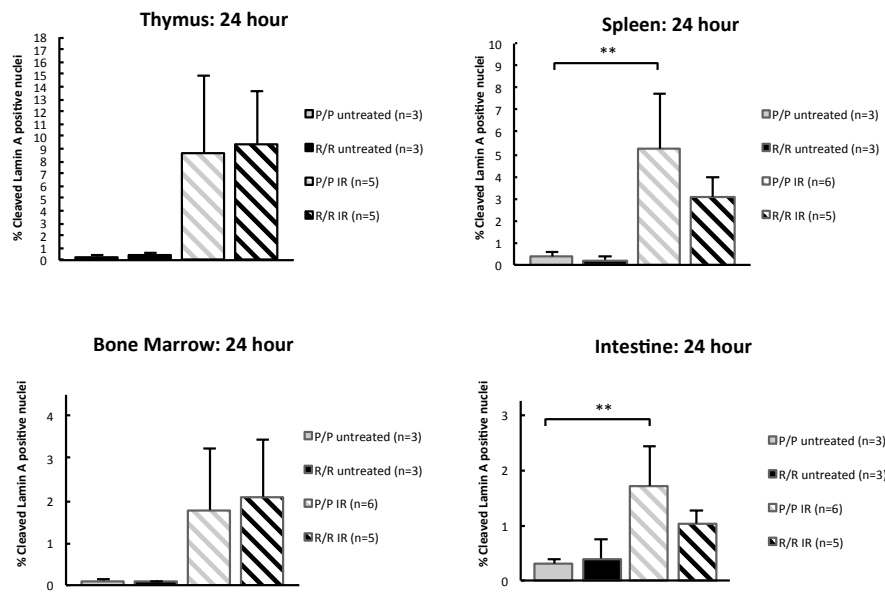


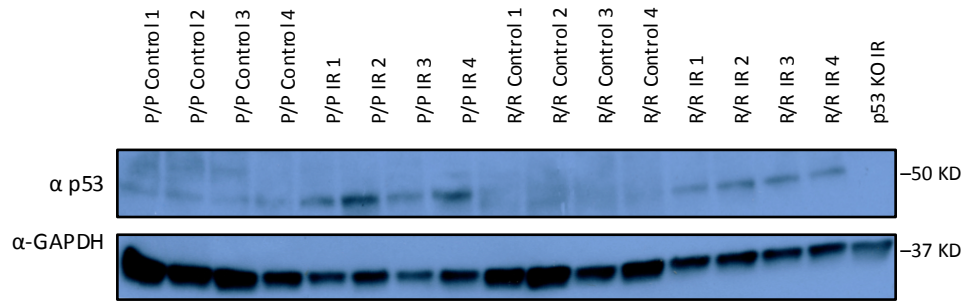
Figure 3.2 Cleaved lamin-A staining in untreated and IR treated p53 R72 mice at 24 hours following IR. a) Representative images of cleaved lamin-A IHC in male, adult p53 R72P mice untreated or treated with 6.135 Gy IR at 24 hours **b)** percent cleaved lamin-A positive cells in thymus, spleen, bone marrow and intestine of these mice

One-way ANOVA followed by Tukey's multiple comparisons test

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Although in prior studies we had noted no difference in p53 positive nuclei between IR treated or control $p53^{P72/P72}$ and $p53^{R72/R72}$ mice ¹¹², we sought to confirm this was true in the current study. Western blot analysis of four biological replicates for each genotype in splenocytes from untreated and IR treated mice (four hours post IR) was carried out and quantified by densitometry. This study revealed significantly more p53 protein in splenocytes of IR treated $p53^{P72/P72}$ (**Figure 3.3**).

a



b

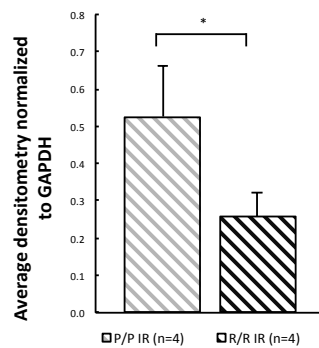


Figure 3.3 p53 protein expression in IR treated and untreated

p53 R72P splenocytes. p53 protein levels in splenocytes of adult male p53

R72P mice untreated or exposed to 6.135 Gy IR and sacrificed at four hours after

treatment **a)** western blot of four biological replicates of each

genotype/treatment group (p53 knockout mouse splenocytes used as negative

control for antibody) **b)** densitometry of p53 protein expression normalized to

GAPDH loading control

two-sided independent t-test

± refers to standard deviation

** p<0.05*

IHC staining for p53 was also performed on the tissues taken at four hours following IR and the control tissues for that treatment group. The percentage of p53 positive cells was determined with digitized slides and histomorphometry image analysis software. Unlike in prior studies from our lab¹¹², and in line with the western blot results in the current study, there appeared to be some increased p53 expression in IR treated $p53^{P72/P72}$ mouse thymus, spleen and intestine when compared to and $p53^{R72/R72}$ mouse tissues (**Figure 3.4**) although these differences were not significant. However, we cannot rule out that these differences may be due to increased affinity of the antibodies employed to proline variant p53. In support of this possibility, we have noted that at least one commercially available antibody could not recognize the R variant p53 at all (**Figure 3.5**)

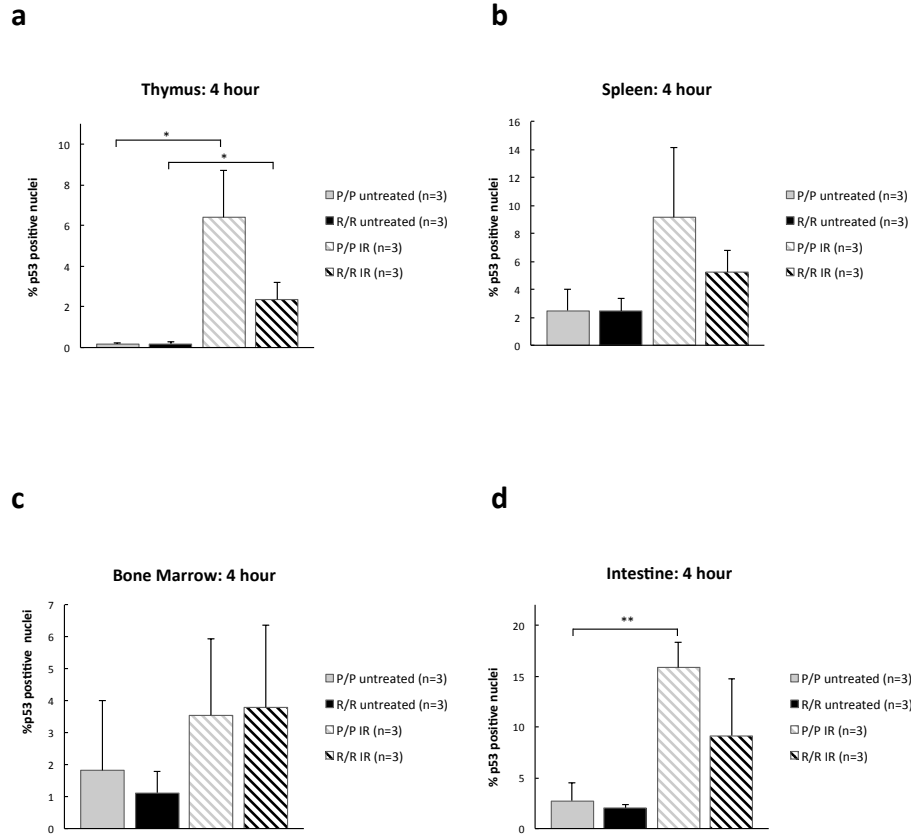


Figure 3.4 Quantification of IHC staining for p53 protein in untreated and IR treated p53 R72P mouse tissues. Percent p53 positive nuclei in adult p53 R72P female mice untreated or treated with 6 Gy IR at 4 hours in **a)** thymus, **b)** spleen, **c)** bone marrow and **d)** intestine

two-sided independent t-test

* $p < 0.05$, ** $p < 0.01$

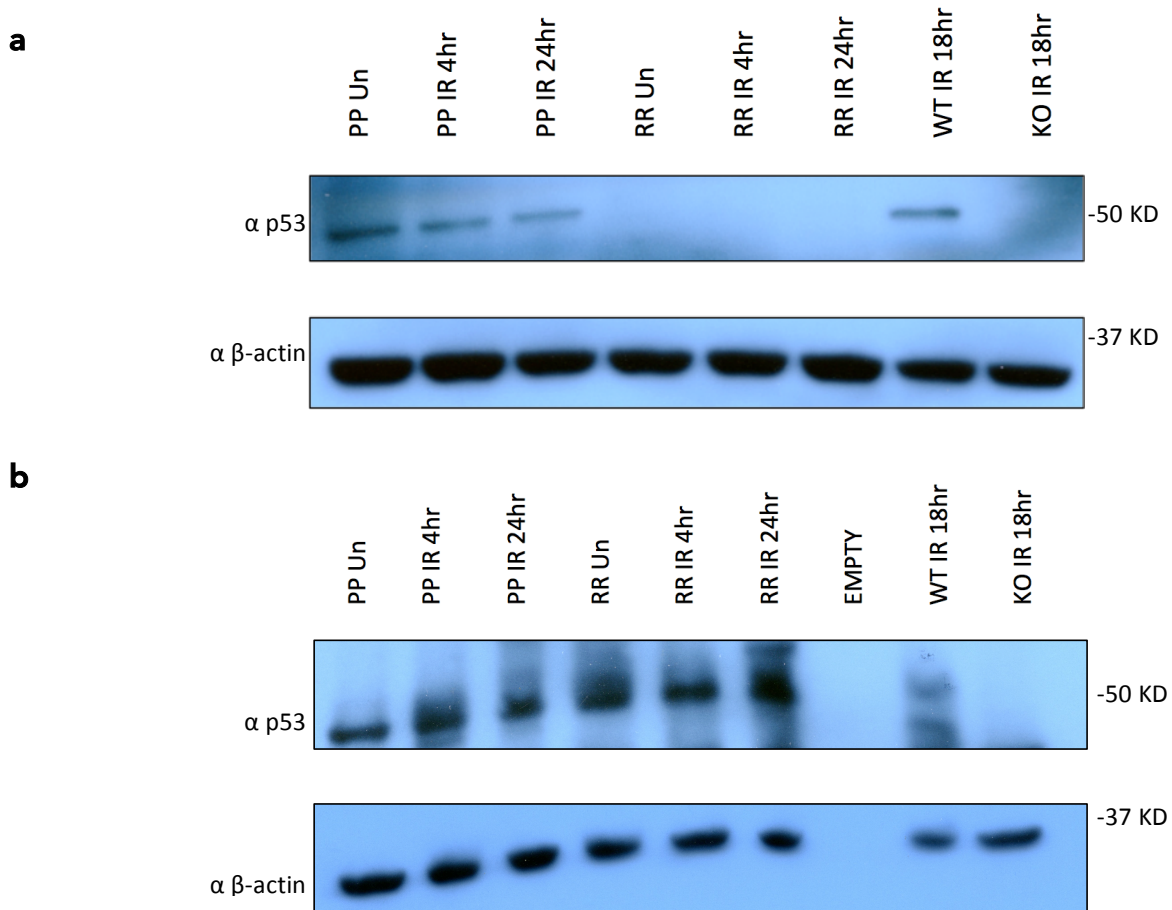


Figure 3.5 A commercially available antibody fails to recognize

p53 R72P R variant. p53 R72P mouse adult fibroblasts (MAFs) were treated with 10 Gy IR and lysed at four, 18 or 24 hours following IR. Lysates were probed with **a)** anti-p53 antibody (Vector CM5) or with **b)** anti-phospho-p53 antibody (Ser 15). p53 WT and p53 KO MAFs were used as positive and negative controls, respectively.

3.3.2 The p53 R72P polymorphism in the transcriptional regulation of p53 target genes following IR:

The p53 R72P polymorphism may influence p53 target gene expression upon exposure to genotoxic agents^{93,95,112,115,116}. To determine whether p53 target gene expression would differ between p53 R72P polymorphic variants following IR in this project, I extracted RNA from thymocytes of adult, male $p53^{P72/P72}$ and $p53^{R72/R72}$ mice four hours after exposure to 6.135 Gy IR and from the thymocytes of control mice. Quantitative PCR was performed to measure the expression levels of common p53 target genes. Expression of p53 was also measured, in light of the above noted increased expression of p53 protein in $p53^{P72/P72}$ mouse tissues as compared to $p53^{R72/R72}$ mouse tissue (**Figure 3.4** and **Figure 3.5**). Furthermore, some studies have shown p53 is involved in regulating its own transcription, and such transcription may be upregulated by exposure to genotoxic agents^{44,148}.

As expected, a significant increase was seen in gene expression of *p21*, *Bax*, *Noxa* and *Puma* in thymocytes from IR treated mice when compared to thymocytes from untreated mice. For these genes, this was true for both genotypes. For *Mdm2*, a statistically significant increase in IR treated thymocytes compared to untreated thymocytes was only noted in thymocytes from $p53^{P72/P72}$

mice. It also appeared that *Mdm2* was not induced in thymocytes from IR treated $p53^{R72/R72}$ mice, although the difference between *Mdm2* expression in IR treated thymocytes in $p53^{P72/P72}$ compared to $p53^{R72/R72}$ mice was not significant. In contrast to earlier studies^{112,115}, there were no differences in gene expression of *p21*, *Bax*, *Noxa*, *Perp* and *Puma* in thymocytes from either IR-treated or untreated $p53^{P72/P72}$ and $p53^{R72/R72}$ mice. IR treatment significantly reduced *p53* gene expression in thymocytes, but there were no differences between thymocytes from $p53^{P72/P72}$ and $p53^{R72/R72}$ mice (**Figure 3.6**). This is in contrast to prior studies suggesting that genotoxic insult increases *p53* transcription⁴⁴.

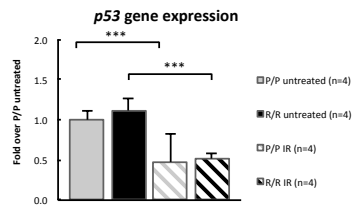
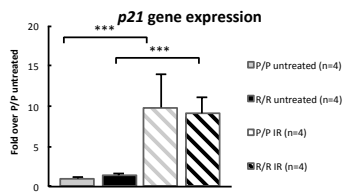
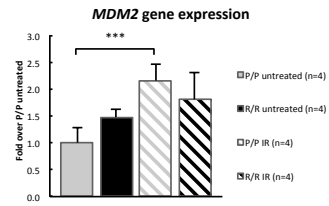
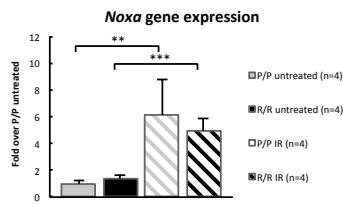
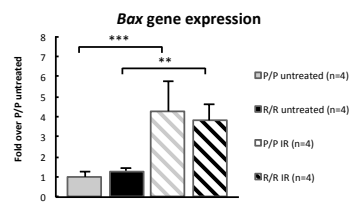
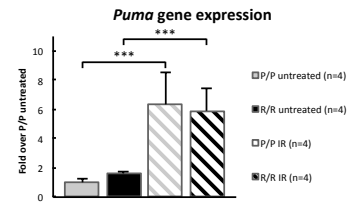
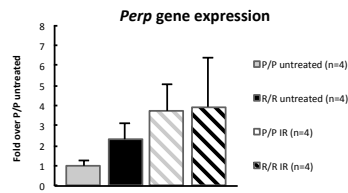
a**b****c****d****e****f****g**

Figure 3.6 p53 target gene expression in p53 R72P mice

untreated or treated with IR. p53 target gene expression in thymocytes of adult, male p53 R72P mice untreated or treated with 6.135 Gy IR and sacrificed at four hours following treatment, expressed as fold change over untreated P/P mice: **a) p53 b) p21 c) Mdm2 d) Noxa e) Bax f) Puma and g) Perp**

Perp

One-way ANOVA followed by Tukey's multiple comparisons test

± refers to standard deviation

ns=not significant

** $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$*

3.3.3 The p53 R72P polymorphism in DNA damage repair.

Studies in cell culture have shown that cells expressing the proline variant of p53 have improved ability to repair DNA after DNA damage⁹⁴. We sought to test whether the p53 R72P polymorphism would alter DNA double strand break (DSB) resolution. To test this, IHC staining for phosphorylated histone H2AX (γ H2AX) - a marker of DNA DSBs¹⁴⁹ - was performed on thymus and spleen from IR treated and untreated $p53^{P72/P72}$ and $p53^{R72/R72}$ mice. At four hours and 24 hours following IR there was increased staining for γ H2AX when compared to untreated mice. There was less γ H2AX staining in tissues of mice sacrificed at 24 hours following IR when compared to mice sacrificed at 4 hours following IR, but also decreased cellularity at 24 hours. This implies that there was irreparable DNA damage that led to apoptosis and perhaps DNA DSB resolution with γ H2AX clearance. There were no obvious differences between γ H2AX staining in tissues $p53^{P72/P72}$ and $p53^{R72/R72}$ mice at either time point. (**Figure 3.7**). We used digitized slides and histomorphometry image analysis software to quantify the percent of γ H2AX nuclei. IR treated mice displayed significantly higher percentages of γ H2AX stained cells when compared to untreated mice ($p < 0.0001$ to $p < 0.01$). However, there were no differences in the percent of γ H2AX positive cells between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice (**Figure 3.8**).

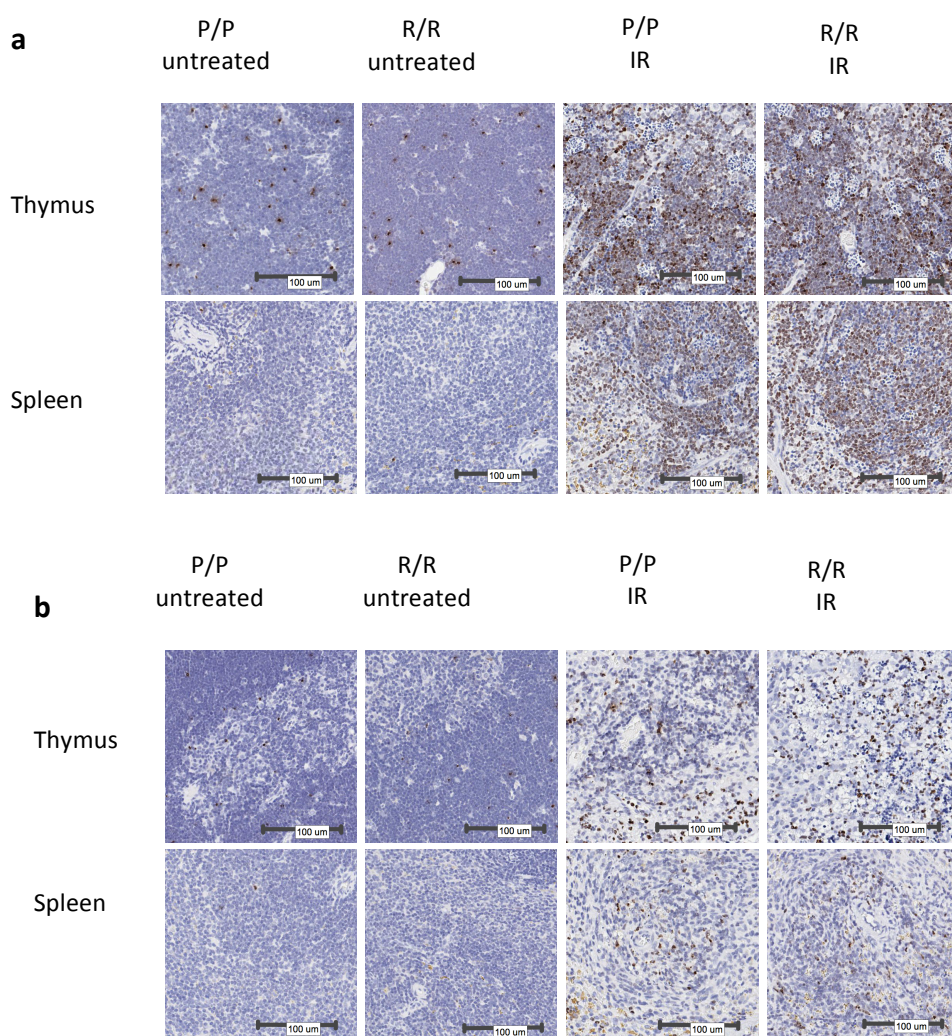


Figure 3.7 IHC staining for γ H2AX in p53 R72P mice untreated or treated with IR. Representative images of γ H2AX IHC in adult p53 R72P mice: **a)** females untreated or treated with 6 Gy IR at 4 hours **b)** males untreated or treated with 6.135 Gy IR at 24 hours

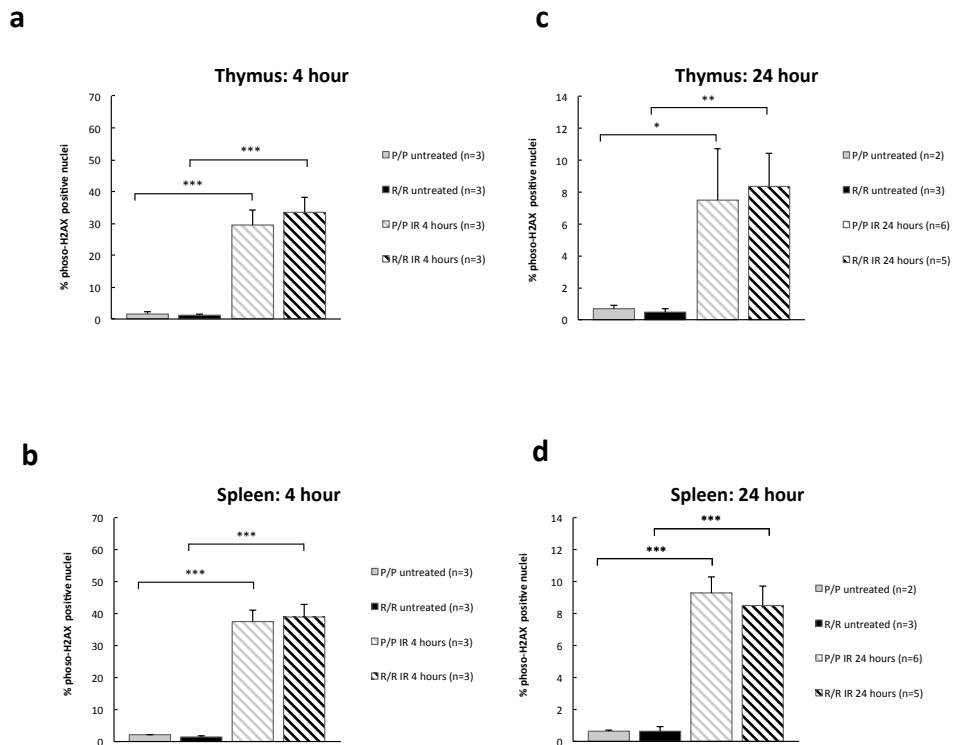


Figure 3.8 Quantification of γ H2AX staining in p53 R72P mice untreated or treated with IR. Percent γ H2AX positive nuclei in adult *p53* R72P female mice untreated or treated with 6 Gy IR at 4 hours in **a)** thymus **c)** spleen; *p53* R72P male mice untreated or treated with 6.135 Gy at 24 hours in **b)** thymus **d)** spleen

One-way ANOVA followed by Tukey's multiple comparisons test

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

3.3.4 The p53 R72P polymorphism in tissue homeostasis following IR

To examine how the p53 R72P polymorphism impacts the hematopoietic system during IR therapy, blood counts and blood clinical chemistry analysis were performed on adult $p53^{P72/P72}$ and $p53^{R72/R72}$ mice. Unlike our doxorubicin studies, in IR studies, white blood cell, neutrophil and lymphocyte counts for both genotypes decreased significantly at three and seven days following treatment. However, no statistically significant differences were noted between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice in the IR treated or untreated groups (**Figure 3.9**). Blood clinical chemistry analysis was also performed on adult male $p53^{P72/P72}$ and $p53^{R72/R72}$ mice treated with IR or untreated. However, few statistically significant changes were noted, even between treatment groups, suggesting this was a poor measure of the physiological effects of this treatment regimen, at least at seven days following IR (**Table 3.1**).

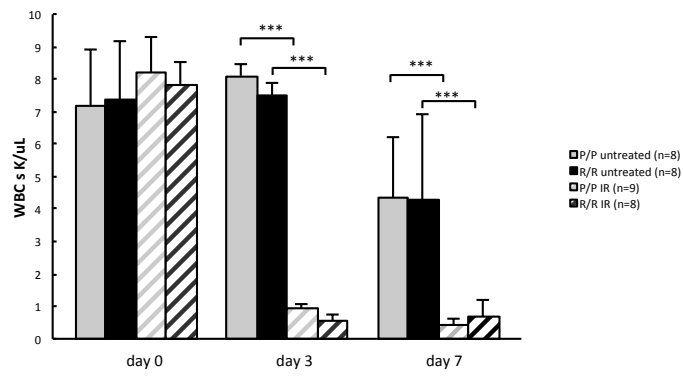
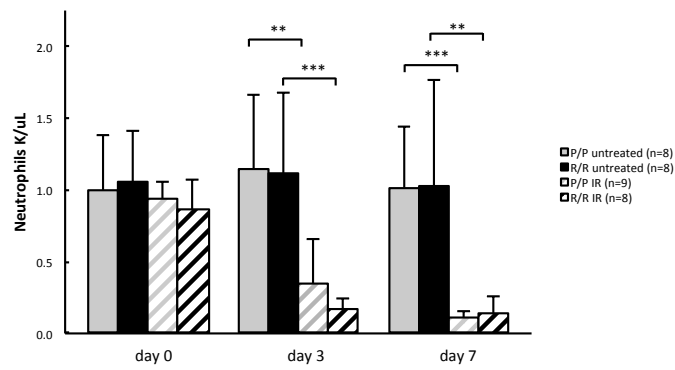
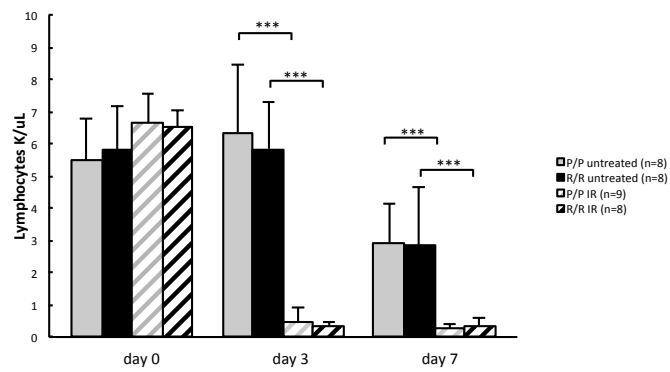
a**b****c**

Figure 3.9 Hematology in p53 R72P mice untreated or treated with IR. Hematology in adult p53 R72P mice untreated or treated with 6.135 Gy IR at 0, 3 and 7 days: **a)** white blood cell counts, **b)** neutrophil counts, **c)** lymphocyte counts

One-way ANOVA followed by Tukey's multiple comparisons test

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Genotype	IR treatment	Albumin	Alkaline phosphatase	Alanine aminotransferase	Amylase	Bilirubin	Blood urea nitrogen	Calcium	Phosphorous	Creatine*	Glucose	Sodium	Potassium**	Total protein	Globulin
P/P	N	3.7	65.0	763.0	1700.0	0.4	28.0	11.1	12.6	0.2	213.0	156.0	8.5	6.3	2.6
P/P	N	3.8	21.0	213.0	1291.0	ND	21.0	11.3	14.4	0.3	308.0	154.0	ND	5.7	1.9
P/P	N	3.8	88.0	39.0	1218.0	0.4	31.0	10.5	9.3	0.3	189.0	155.0	8.5	6.0	2.2
Average		3.8	58.0	338.3	1403.0	0.4	26.7	11.0	12.1	0.3	236.7	155.0	8.5	6.0	2.2
P/P	Y	3.8	63.0	43.0	1287.0	0.4	21.0	10.1	10.8	0.4	184.0	155.0	8.5	5.8	2.0
P/P	Y	4.1	83.0	100.0	1071.0	0.3	20.0	11.2	12.2	0.3	337.0	154.0	8.5	6.1	2.0
P/P	Y	4.0	34.0	440.0	1085.0	0.3	22.0	10.4	13.6	0.3	193.0	193.0	ND	5.6	1.5
Average		4.0	60.0	194.3	1147.7	0.3	21.0	10.6	12.2	0.3	238.0	167.3	8.5	5.8	1.8
R/R	N	3.5	73.0	40.0	1382.0	0.3	22.0	9.7	9.9	0.2	184.0	154.0	8.5	5.6	2.1
R/R	N	3.7	50.0	34.0	1259.0	0.3	27.0	10.7	10.7	0.4	193.0	159.0	8.5	5.7	2.0
R/R	N	3.8	42.0	1011.0	1178.0	0.3	23.0	11.1	12.2	0.5	293.0	157.0	8.5	6.1	2.3
R/R	N	3.8	66.0	12.0	1258.0	0.3	29.0	8.9	11.1	0.2	273.0	156.0	12.0	6.2	2.3
Average		3.7	57.8	274.3	1269.3	0.3	25.3	10.1	11.0	0.3	235.8	156.5	9.4	5.9	2.2
R/R	Y	3.9	76.0	44.0	1213.0	0.4	16.0	10.4	10.4	0.2	229.0	154.0	8.5	5.8	2.0
R/R	Y	4.2	71.0	113.0	1275.0	0.4	25.0	10.7	11.0	0.5	174.0	155.0	8.2	6.0	1.7
R/R	Y	4.0	59.0	48.0	1231.0	0.4	24.0	10.3	11.1	0.2	158.0	157.0	8.2	6.0	2.0
Average		4.0	68.7	68.3	1239.7	0.4	21.7	10.5	10.8	0.3	187.0	155.3	8.3	5.9	1.9
p value T-test P/P IR to untreated		0.139	0.939	0.604	0.226	NA	0.192	0.386	0.957	NA	0.984	0.438	NA	0.503	0.205
p value T-test R/R IR to untreated		0.039	0.267	0.464	0.554	0.000	0.349	0.520	0.801	NA	0.223	0.432	NA	0.846	0.092
p value T-Test P/P to R/R IR		0.621	0.613	0.417	0.317	0.184	0.838	0.797	0.228	NA	0.421	0.449	NA	0.579	0.752
p value T-Test P/P to R/R untreated		0.440	0.991	0.853	0.470	NA	0.702	0.188	0.536	NA	0.985	0.269	NA	0.681	0.808

Table 3.1 Clinical parameters in p53 R72P mice untreated of treated with IR. Blood clinical chemistry in adult male p53 R72P mice untreated or treated with 6.135 Gy IR at 7 days. * Creatine values quantified as <0.2 are included as 0.2, and ** Potassium values quantified as >8.5 and >12.0 are included as 8.5 and 12 on this table for general reference, ND= no data, NA=not applicable, p values from two sided independent t-test

Qualitative differences between IR sensitive tissues from $p53^{P72/P72}$ and $p53^{R72/R72}$ mice exposed to IR at seven days following treatment and from untreated mice were assessed by histopathology to determine if there were differences in tissue pathology and recovery. At this time point, there was still greatly reduced cellularity in the thymus, spleen and bone marrow of IR treated mice compared to controls (**Figure 3.10**). There was a slight increase in intestinal cellularity in IR treated mice, suggesting early recovery from treatment. Nevertheless, there was no notable difference between $p53^{P72/P72}$ and $p53^{R72/R72}$ IR-treated or control tissues (**Table 3.2**). The effect of IR on cellularity of the IR sensitive tissues was further evidenced by a marked decrease in the weight of fixed spleen as a percentage of body weight in IR treated mice at seven days following treatment, as compared to untreated mice in both genotypes. However, there was no difference in the weight of fixed spleen as a percent of body weight between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice at either time point or in each treatment group (**Figure 3.11**).

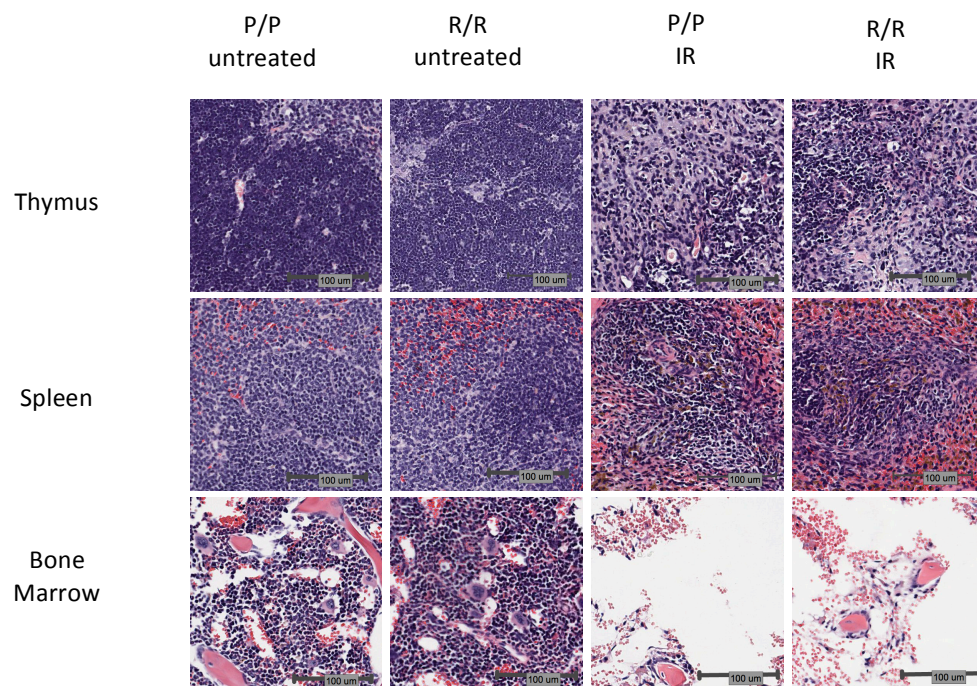


Figure 3.10 Histopathology in p53 R72P mice untreated or treated with IR. H & E staining showing representative histopathology in adult male p53 R72P mice untreated or treated with 6.135 Gy IR at seven days

Genotype	Treatment group	Thymus Cellularity	Spleen Cellularity	Bone Marrow Cellularity	Intestinal Crypt Hyperplasia
P/P	Untreated	Marked	Marked	Marked	None
P/P	Untreated	Marked	Marked	Marked	None
P/P	Untreated	Marked	Marked	Marked	Minimal
P/P	IR Treated	Minimal	Mild	Minimal	Mild
P/P	IR Treated	Minimal	Mild	Mild	Minimal
P/P	IR Treated	Mild	Mild	Minimal	Minimal
R/R	Untreated	Marked	Marked	Marked	None
R/R	Untreated	Moderate	Marked	Marked	None
R/R	Untreated	Marked	Marked	Marked	None
R/R	IR Treated	Minimal	Mild	Minimal	Minimal
R/R	IR Treated	Minimal	Mild	Minimal	Minimal
R/R	IR Treated	NP	Mild	Minimal	Minimal

Table 3.2 Summary of histopathological report of p53 R72P mice untreated or treated with IR. Histopathological changes in adult male p53 R72P mice untreated or treated with 6.135 Gy IR at 7 days. NP: Not present.

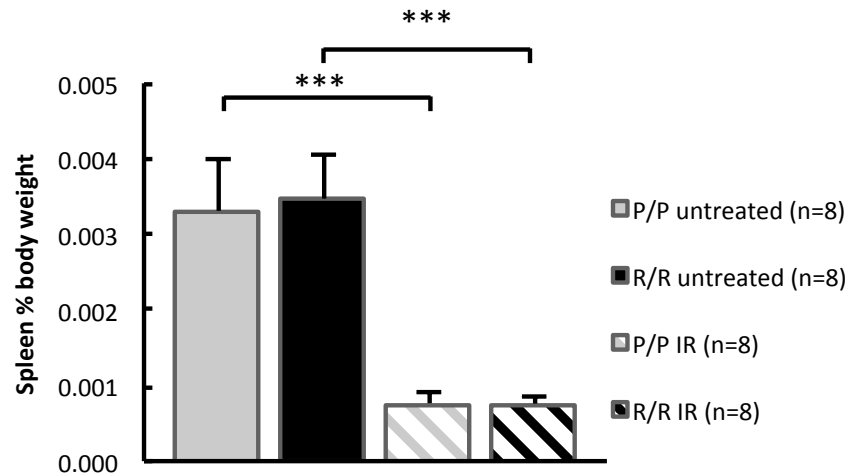


Figure 3.11 Spleen weights in p53 R72P mice untreated or treated with IR. The effects of IR on fixed spleen weight as a percent of body weight in adult p53 R72P mice untreated or treated with 6.135 Gy IR at 7 days.

One way ANOVA followed by Tukey's multiple comparisons test

*** $p < 0.001$

3.3.5 The p53 R72P polymorphism in long-term survival and weight loss following IR

To determine whether the R72P polymorphism affected mouse survival, FVB $p53^{P72/P72}$ and $p53^{R72/R72}$ mice were treated with 6.135 Gy IR and followed for 28 days or until they became moribund, at which point they were sacrificed. A mouse was judged to be moribund if it showed hunched posture, lethargy, greater than 20 percent weight loss, anorexia and rapid or labored breathing. To ensure that morbidity was not a result of opportunistic infection, mice were housed in sterile caging and treated with prophylactic antibiotics in their drinking water. Most mice became moribund before 25 days, and the majority were sacrificed between 10-20 days following treatment. All mice in this study were within five days of 15 weeks old and at least 20 male and 20 female mice of each genotype were used. A log-rank test was used to analyze survival curves, and the results showed that the p53 R72P polymorphism failed to modulate survival in adult male mice, female mice, or the both sexes as a combined group (**Figure 3.12**).

Since adverse effects of IR include gastrointestinal distress that may affect weight, mice in this study were weighed at day zero and day seven following IR treatment. Although body weight decreased for both genotypes and sexes

between five and 10 percent from day zero and day seven, and this change in weight was not significant and there was no significant difference in weight between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice at either time-point for either sex (**Figure 3.13**).

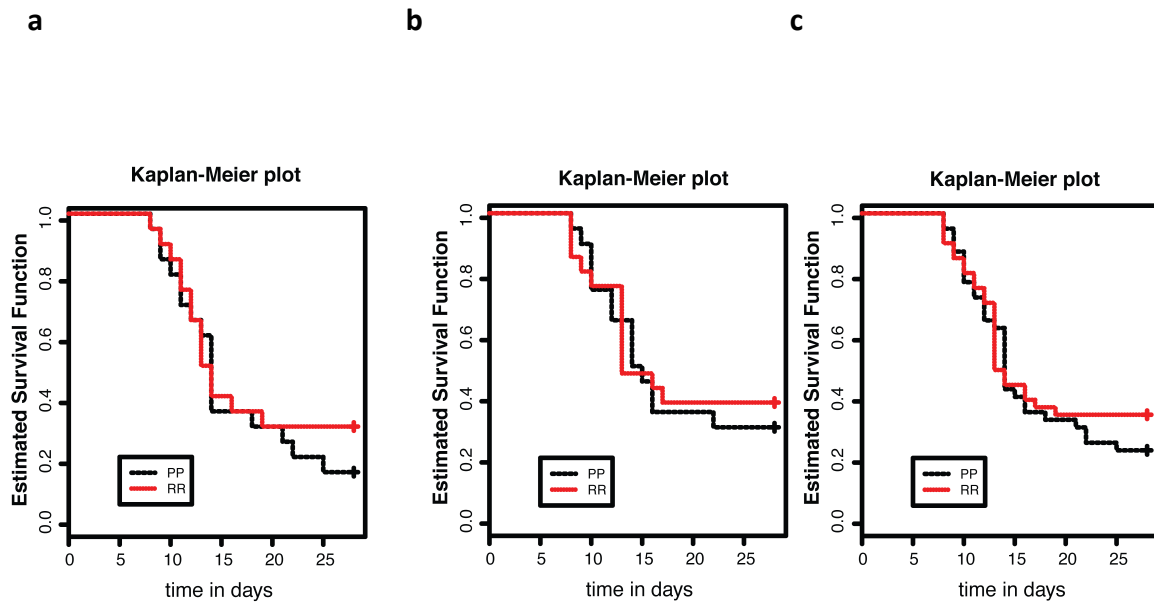


Figure 3.12 28-day survival in p53 R72P mice treated with IR.

Kaplan-Meier survival curves for adult p53 R72P mice treated with 6.135 Gy IR and followed for 28 days: **a)** males (P/P males $n=20$, R/R males $n=20$, log rank test: *ns*) **b)** females (P/P females $n=20$, R/R females $n=21$, log rank test: *ns*) and **c)** both sexes combined (P/P $n=40$, R/R $n=41$, log-rank test: *ns*)

ns: not significant

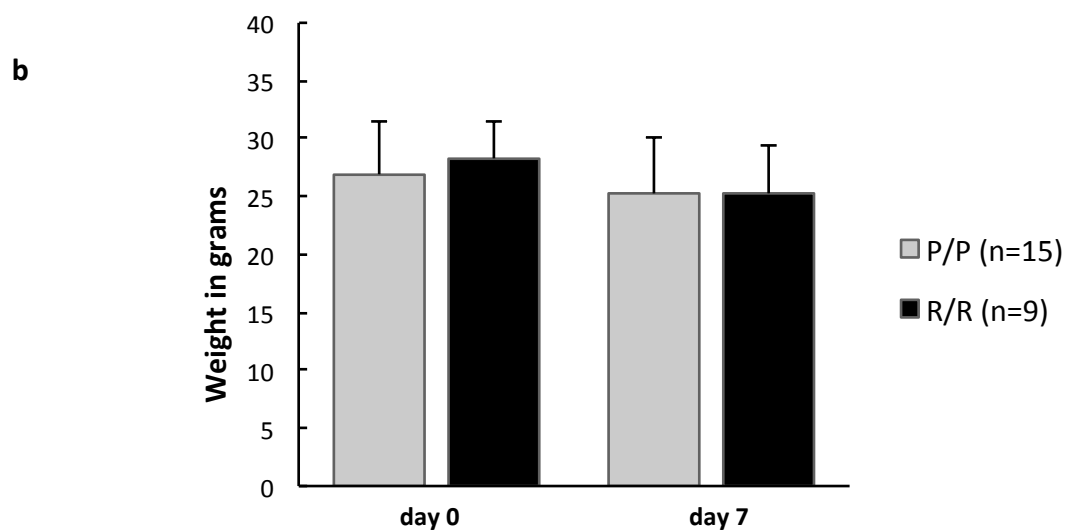
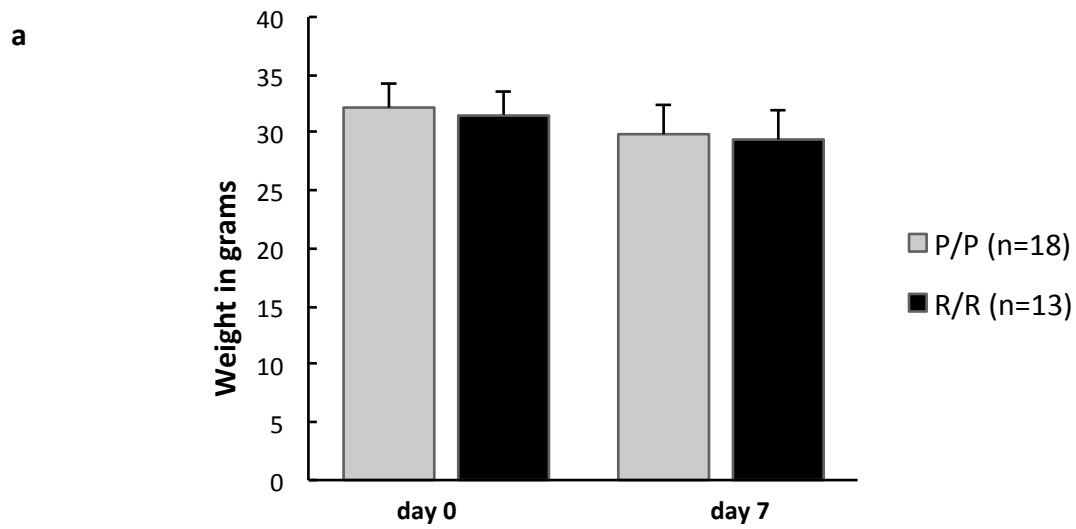


Figure 3.13 Weights in p53 R72P mice untreated or treated with IR. No significant change in weight of adult p53 R72P treated with 6.135 Gy IR at zero and seven days **a)** male mice **b)** female mice

One way ANOVA followed by Tukey's multiple comparisons test

ns= not significant

3.4 Discussion

In this Chapter, I describe using *p53* R72P mice to examine the effects of the R72P polymorphism on tissue damage, tissue recovery and long term survival following high dose IR treatment. There appeared to be some minor differences in levels of p53 protein in some tissues when mice were exposed to IR, with the proline variant generally showing higher levels of p53.

This minor difference did not seem to affect outcomes of IR exposure either at the cellular or physiological level. This is evidenced by several key results: 1) Expression of p53 target genes in IR-treated and untreated mouse thymocytes was very similar between p53 R72P genotypes. 2) Quantification of IHC staining suggested that the *p53* R72P polymorphism did not affect apoptosis in several radiosensitive tissues following IR exposure either at four or 24 hours. 3) There were no differences in nuclear γ -H2AX IHC staining at either four or 24 hours following IR treatment, suggesting no difference in the timing or vigor of DNA damage response or repair between *p53*^{P72/P72} and *p53*^{R72/R72} mice. 4) Thymus, spleen and bone marrow showed highly decreased cellularity at seven days following IR when compared to untreated tissue, but there were no apparent differences between *p53*^{P72/P72} and *p53*^{R72/R72} mice. These data suggest that neither immediate pathological response to IR nor short-term recovery are affected by the polymorphism. 5) Leukocyte numbers were

unaffected by the p53 R72P polymorphism at three and seven days following IR, with equivalent leukopenia in both genotypes. These data suggest that myelosuppression by IR is also equivalent between genotypes. 6) In a 28-day survival study, no difference was seen in survival between $p53^{P72/P72}$ and $p53^{R72/R72}$ mice exposed to high-dose IR. These data suggest that recovery from adverse effects of IR, such as bone marrow suppression and intestinal epithelial damage, were not affected by the polymorphism.

Previously, our laboratory and others found different levels of apoptosis when p53 R72P humanized mice were exposed to IR. For example, there was more nuclear IHC staining for markers of apoptosis in $p53^{R72/R72}$ mouse intestine as compared to $p53^{P72/P72}$ mouse intestine ^{112,116}. Furthermore, there was increased nuclear staining for apoptotic markers in $p53^{P72/P72}$ mouse thymus compared to $p53^{R72/R72}$ mouse thymus ¹¹⁵. We did not find such differences in nuclear staining for apoptosis in the current project. As one study suggested that there were differences between p53 R72P cell lines in DNA response and repair following genotoxic insult ⁹⁴, we tested this in mice by IHC staining for γ H2AX at four and 24 hours post IR in spleen and thymus. Although there was a large difference in γ H2AX in staining between treated and untreated mouse tissue, there was no difference between $p53^{P72/P72}$ and $p53^{R72/R72}$ in either treatment group.

Data from the current project do not support a role for the p53 R72P polymorphism in moderating the effects of high-dose TBI. However, this project did not include studying side effects of targeted IR treatment, or the adverse effects of fractionated IR dosing, so those may need to be investigated. Also, as this study focuses on the effect of the p53 R72P polymorphism as a single genetic variable, the effects of genetic interactions between this SNP and others in modifying IR-induced side effects cannot be excluded. Continued study of the p53 R72P polymorphism may be merited in these contexts.

Chapter 4:

Summary and Future Directions

There are over 80 million human SNPs documented in the human genome¹¹¹, so singling out the adverse effects of one SNP on cancer therapies can be challenging. Epidemiological studies of individual SNPs cannot always account for other genetic variances, environmental differences and differences in cancer treatments. Cell culture studies cannot often predict systemic/physiological differences in cancer therapy tolerance. We postulated that mouse models of SNPs could serve to bolster our understanding of pharmacogenomics and radiogenomics as they pertain to the adverse effects of genotoxic cancer therapies.

Investigators have extensively utilized genetically modified mice to understand the functions, regulation and cancer phenotypes of WT and mutant p53. These mouse models include those in which a portion of a mouse gene is replaced by the orthologous human section¹⁰⁹, such as the mice used in this dissertation project. Other such “genomically humanized mice” have been created to improve xenogeneic transplantation, to model non-cancer human diseases and to produce mice with more human-like cytochrome P450 activity for use in predicting drug-drug interactions in humans¹⁵⁰. Genomically humanized mice have also been used to determine how human SNPs in p53 pathway genes affect tumor latency and spectrum, and the molecular mechanisms involved. For example, Lozano et al. generated KI models of a

common human SNP in the *Mdm2* gene (G309T), which is not conserved in mice. Employing these models, they demonstrated that mice with the G variant express lower levels of *Mdm2*, and decreased apoptosis in some tissues as compared to mice with the T variant. Furthermore, in mice heterozygous for a hot-spot p53 mutation, mice with the G allele succumbed to tumors sooner and displayed an altered tumor spectrum when compared to mice with the T allele¹¹⁰.

The study of SNPs using genomically humanized mouse models in pharmacogenomics has also been established. Such models have been used to study how a human SNP in the *opioid-receptor-μ-1* gene modifies heroin addiction¹⁵¹. Additionally, they have been used to model a human SNP in the *brain-derived-neurotrophic-factor* gene which is associated with decreased hippocampal size and decreased memory in humans. Mice with one variant of this human SNP also showed an anxiety phenotype that has not been confirmed in humans with the same SNP variant. This allowed the researchers to test how the SNP affected the efficacy of fluoxetine, which is used to treat anxiety.¹⁵²

In spite of their utility in other fields of pharmacogenomics, genomically humanized mouse models of SNPs have not been used to characterize how human SNPs modulate side effects of therapeutic doses of chemotherapeutics. We also noted that no radiogenomic studies using genomically humanized

mouse models of SNPs have been published. Therefore, we sought to establish the utility of such models in the fields of pharmacogenomics and radiogenomics by using our mouse models of the p53 R72P polymorphism to characterize how this human SNP affects doxorubicin tolerance and to show the adverse effects of radiation therapy.

We found that a therapeutic dose of doxorubicin induced only minor cellular and systemic changes in FVB mice. These changes were, for the most part, not robust enough to be effectively quantified or validated. We then tested WT FVB at escalating doses of doxorubicin and found very little evidence of quantifiable adverse effects even at the highest doses. Whether FVB mice are particularly insensitive to doxorubicin compared to other mouse strains is not known. It would be advisable for any researcher considering a similar study to first test for measurable differences in systemic effects of doxorubicin in the WT mice of the mouse strain they intend to use prior to using genetically modified mouse models.

We do not know if other commonly used genotoxic chemotherapies, alone or in combination, would induce measurable differences in adverse effects or gene expression profiles in our mouse models of the p53 R72P polymorphism. Epidemiological studies have shown increased febrile neutropenia in patients homozygous for the proline allele in response to 5-

florouracil(5-FU)/epirubicin/cyclophosphamide treatments and increased neutropenia in response to etoposide/cisplatin treatment, compared to patients hetero- or homozygous for the arginine variant ^{100,101}. Future studies could use these chemotherapy combinations in p53 R72P polymorphic mice to determine if their response would model the epidemiological data. If they were good models, p53 R72P mice could serve as an *in vivo* pre-clinical proxy to test how changes in these regimens could reduce systemic effects in some patients.

As compared to the doxorubicin study, using our p53 R72P mouse models in IR studies provided much more robust differences between treatment and control groups. However, in contrast to earlier studies with IR, there was little to no difference between proline and arginine variants. Expression of common p53 target genes in thymocytes from untreated and IR treated mice was not affected by the p53 R72P polymorphism. Although there may have been slightly increased p53 protein levels in tissues from $p53^{P72/P72}$ mice when compared to tissues from $p53^{R72/R72}$ mice, these differences did not lead to changes at a physiological level. Neither early changes in cellular markers of apoptosis and DNA damage, nor short-term changes in tissue damage and recovery were noted between genotypes. Survival from high-dose IR to 28 days also did not differ between genotypes, suggesting similar long-term recovery in p53 R72P variants. Although no other published reports have tested systemic

effects and DNA damage markers in mice following IR treatment using p53 R72P polymorphic mouse models, several studies have tested tissue-specific differences in apoptosis and transcriptional activation of p53 targets in response to IR. As previously described in earlier studies, p53 R72P mouse models in our laboratory and the Murphy laboratory had shown some tissue specific differences in apoptosis and between genotypes and gene expression changes consistent with these changes^{112,115}.

In this dissertation project I saw few such differences. There are several possible reasons for this discrepancy. Although the age of the mice used in the Murphy laboratory experiments is not documented, in prior studies from our laboratory, young mice approximately eight weeks of age had been used. Early studies of the pathology of IR injury in mice have shown that radio-resistance follows a u-shaped pattern of high neonatal resistance, decreased resistance during maturation and a return to higher resistance with maturity¹¹⁸. Therefore, individual developmental differences between mice still reaching maturity could have accounted for changes in response to IR noted in our original studies.

Technological advances could also account for some differences. In prior studies, IHC quantification was performed with light microscope and apoptotic cells were counted manually by observation from small linear sections of tissues. In this study, slides were digitized and nuclear staining was quantified by

computer algorithm. This allowed us to gather information about much larger (and therefore more representative) sections of tissue and eliminated any unintentional bias associated with manual counts.

As previously discussed, background strain can influence gene expression patterns and radio-sensitivity¹²⁰⁻¹²². In the case of reports from our laboratory, prior studies had been unwittingly performed on mice with mixed background strains. Therefore, it is possible that the differences we noted were artifacts of background strain and not the polymorphism. We do not know to what extent the Murphy laboratory has characterized the genetic background of mice in their reports, but they used mice backcrossed into a C57BL/6 background. It is possible that this background strain has subtle genetic differences in p53 target genes that modulated the interaction of R and P humanized p53 with important transcriptional targets or nuclear and cytoplasmic proteins. Furthermore, different targeting vectors were used in the creation of p53 R72P mice between the two laboratories. The vector used in our laboratory replaced *p53* mouse exon four with human exon four coding for either the 72R or 72P variant. However, in the Murphy laboratory model vectors, several exons of mouse *p53* were replaced with human *R72P* coding variant exons. The effect of any of these differences could result in genetic interactions and protein/protein interactions

that enhanced p53 R72P polymorphism-driven differences in one model and muted them in another.

In this dissertation project I did not find significant differences in inflammation associated gene expression in the liver or thymus of mice exposed to doxorubicin. I did not test inflammatory gene expression in response to IR. However, as previously described, the p53 R72P polymorphism has been shown to modify inflammation in some tissues as shown by differential gene expression, tissue changes as well as systemic responses to genotoxic stimuli, tumor growth and endotoxin injection. One proposed mechanism for these differences is increased binding of the NF- κ B subunit p65 RelA with the proline variant of p53, leading to increased upregulation of inflammatory genes^{115,117}.

Considering this, it may be important to use p53 R72P polymorphic mouse models to test the how the polymorphism modifies both the adverse effects and the efficacy of immunological-based cancer therapies. For example, rituximab, an anti-CD-20 antibody used to treat B-cell lymphomas and leukemia, can cause severe cytokine release syndrome (CRS) with symptoms such as fever and hypotension that have been fatal^{153,154}. The mode of actions for its efficacy as a cancer treatment are mediated in part due to the inhibition NF- κ B signaling and increased Fas receptor/ligand signaling¹⁵⁵. How these signaling pathways are involved in CRS is not clear, although NF- κ B signaling is a well-known

inducer of cytokine activity and pro-survival signaling¹⁵⁶, and Fas signaling is generally known to be immunosuppressive via its activity in inducing caspase-8 mediated apoptosis in immune cells¹⁵⁷. Interestingly, the Fas receptor is a long-accepted p53 transcription target¹⁵⁸, but whether or not the p53 R72P polymorphism modulates Fas expression is unknown. Therefore, many avenues of study regarding the role of the p53 R72P polymorphism in CRS could be explored. This could be technically challenging as these anti-tumor-antibodies are designed for use in humans and do not elicit the same effects in mice, so our current models of the polymorphism may not be useful. However, humanizing severe combined immunodeficiency (SCID) immune deficient mice with human peripheral blood mononuclear cells (PBMCs) can be used to model CRS in the context of antibody based therapies¹⁵⁹. Therefore, it's possible to imagine using p53 72R and 72P PBMCs in SCID mice in a similar manner to test how the polymorphism affects CRS, and, if it does, examining the roles of NF- κ B and Fas signaling in any such differences. However, to date no epidemiological/pharmacogenomic studies have addressed whether the p53 R72P polymorphism (or any polymorphism of any gene) specifically affects CRS, so mouse model studies may be premature.

There are other polymorphisms in the *p53* gene and these may affect responses to cancer therapy and side effects of cancer therapy. For example,

another humanized mouse model from the Murphy laboratory of the p53 Pro47Ser polymorphism found that MEFs from $p53^{Ser47/Ser47}$ mice were much less sensitive to cisplatin than MEFs from $p53^{Pro47/Pro47}$ mice. However, when these MEFs were oncogene transformed, the reverse was true: $p53^{Ser47/Ser47}$ MEFs were more sensitive¹⁶⁰. In another report, the Murphy laboratory found that this difference in sensitivity to cisplatin was caused by a combination of decreased apoptosis and ferroptosis in $p53^{Ser47/Ser47}$ MEFs compared to $p53^{Pro47/Pro47}$ MEFs. They confirmed the decreased sensitivity of $p53^{Ser47/Ser47}$ MEFs to cisplatin and ferroptosis inducing agents in human $p53^{Ser47/Ser47}$ lymphoid cell lines¹⁶¹. Although they did not use their mice to model side-effects per se, these data suggest that polymorphisms can differently modulate sensitivity to genotoxic insult in healthy and transformed tissue and underscore the utility of mouse models in predicting such differences in human tissues.

The evidence in this dissertation does not support a major role for the p53 R72P polymorphism in moderating adverse effects of genotoxic therapy. However, pharmaco- and radiogenomics fields continue to elucidate the roles of subtle genetic changes in both tumor response and systemic effects of cancer therapies. The relevance and timeliness of radiogenomic studies, especially, is emphasized by a recent news article in *Science* addressing the need for increased study and novel methodology in this field¹⁶². Considering this, current

and improved humanized mouse models of SNPs may be increasingly important in bridging the gap between cell culture and epidemiological studies. This dissertation project shows the feasibility of using humanized mouse models to test the impact of a polymorphism on adverse effects of cancer therapy.

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