

Identifying Novel Mechanisms of Tp53-Mediated Tumor Suppression

by

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Duke University

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in
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ABSTRACT

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Abstract

Background: TP53 is the most commonly mutated gene in cancer. Canonical TP53 DNA damage response pathways are well characterized and classically thought to underlie the tumor suppressive effect of TP53. Challenging this dogma, mouse models have revealed that p53 driven apoptosis and cell cycle arrest are dispensable for tumor suppression. Here, we investigate two mouse models that seek to further elucidate the necessity of p53 pathways in tumor suppression. These mutants represent a paradoxical signaling of canonical targets and incidence in human cancer. One p53 mutation is deficient in signaling, but not found in human cancer and the other represents the inverse context of a p53 mutation predicted to drive the expression of canonical targets, but is detected in human cancer.

Methods: We established novel mouse models with single amino acid substitutions (GAG>GAC, mouse p53^{E221D} and GCC>GGG, mouse p53^{G259A}) in the DNA-binding domain. The first represents the mouse variant of a p53 mutant that has wild-type function in screening assays, but is paradoxically found in human cancer in Li Fraumeni syndrome. The second mutation has decreased transactivation in screening assays but is not widely found in human cancer. Using mouse models and the analogous human mutants, we evaluated expression, transcriptional activation, and tumor suppression in vitro and in vivo.

Results: Expression of human p53^{E224D} from cDNA translated to a fully functional p53 protein. However, mouse p53^{E221D/E221D} RNA transcribed from the endogenous locus is mis-spliced resulting in nonsense-mediated decay. Moreover, fibroblasts derived from p53^{E221D/E221D} mice do not express a detectable protein product. Mice homozygous for p53^{E221D/E221D} exhibited increased tumor penetrance and decreased life expectancy compared to p53^{WT/WT} animals. Expression of the human p53^{G262A} from cDNA translated into a functionally deficient p53 protein. The mouse p53^{G259A/G259A} mutation was transcribed in mouse embryonic fibroblasts, and hyper-stabilized in untreated cells. Signaling was modestly deficient and did not result in a significant tumor burden in p53^{G259A/G259A} mouse models.

Conclusions: Mouse p53^{E221D/E221D} and human p53^{E224D} mutations lead to splice variation and a biologically relevant p53 loss of function in vitro and in vivo. Mouse p53^{G259A/G259A} and human p53^{G262A} are deficient in transactivation, but maintenance of homeostasis is achieved through hyperstabilization of the mutant.

Dedication

I dedicate this my family, my partner, my dog and all of the mentors that I have had during my years of education. My parents are the kindest and most supportive people I could ask to have by my side. My siblings are resilient, brilliant, and constantly there for a smile. Thank you to my partner and our dogs, Odie and Luna, for many MANY nights of deep breaths and long walks to get us through the week. You all have made this possible over the last four years. I love you all immensely and look forward to many more years of celebrating your achievements as you have been here to celebrate mine.

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1. Introduction

1.1 P53 Overview

P53 is a sequence-specific transcription factor that exists at low levels under unstressed, or basal, conditions. This results in a decreased interaction with DNA and consequently low level expression of targets genes. Low levels of the P53 protein are maintained by the E3 ubiquitin ligase MDM2. Following cellular stress, negative regulation of P53 is disrupted primarily through post translational modification of MDM2 or P53 resulting in protein stabilization and accumulation. This stabilization drives homo-tetramerization, increasing the affinity of P53 for DNA and driving the transcription of downstream targets. P53 targets participate in a diverse array of downstream pathways including cell cycle arrest, apoptosis, senescence and metabolism. Indicative of P53s role as a modulator of these pathways critical for maintenance of homeostasis and prevention of tumorigenesis in the cell, P53 is the most frequently mutated gene in cancer.

1.1.1 P53 Structure

P53 is a 393 amino acid protein located on the short arm of chromosome 17, position 13 (Isobe et al. 1986b). This gene is translated into a 43.7kDa protein product that runs at 53kDa on a SDS-PAGE gel. The protein can be subdivided into six functional domains. The N-terminal 93 amino acids are defined as the transactivation (TAD) domain of the protein and the proline rich domain. The TAD is further subdivided into

two sub-transactivation domains at amino acids 1-40 (TAD1) and 40-61 (TAD2) (Joerger and Fersht 2008; Walker and Levine 1996; Chang et al. 1995). This segment is a partially intrinsically disordered region that can accommodate binding a large number of proteins specifically (Dunker et al. 2005; J. Liu et al. 2006). This binding event results in a disordered-to-ordered transition of small segments of the TAD resolving into small structured regions specific to binding partners like MDM2, RPA, and TFIIH (Kussie et al. 1996; Popowicz et al. 2007; Lello et al. 2006; Bochkareva et al. 2005; Vacic et al. 2007; Mohan et al. 2006).

Amino acids 64 to 92 make up the proline rich domain. The full function of this domain has yet to be elucidated, but studies have tied it to P53's ability drive cell cycle arrest, and suppress tumor formation, though the length and the structure of this repeated domain may contribute to function rather than specific intramolecular interactions (Toledo et al. 2007; Sakamuro et al. 1997; Venot et al. 1998).

The DNA binding domain (DBD) is defined as amino acids 94 to 292 whose confirmation confers specific binding to two copies of the DNA decamer motif 5'-RRRCWWGYYY-3' (R = A, G; W = A, T; Y = C, T), separated by 0-13 base pairs (El-Deiry et al. 1992; W. D. Funk et al. 1992). This domain resolves into a β sandwich that is constructed of a loop-sheet-helix architecture and two large loops stabilized by a zinc ion (Cho et al. 1994; Joerger and Fersht 2010; Lee et al. 1994). It is the loop-sheet-helix

structure that interacts with the major groove of DNA and the loops that interact with the minor group of the DNA structure during binding (Cho et al. 1994).

Amino acids 326 to 355 form the oligomerization or tetramerization domain (OD or TD). Prior to stabilization, P53 is primarily a dimer tightly controlled at low levels in the nucleus (Katz et al. 2018; Gaglia et al. 2013; Ma et al. 2005; Rajagopalan, Huang, and Fersht 2011). Stabilization results in formation of a tetramer, or a dimer of dimers, that increases the proteins DNA binding affinity 10 to 100 times.(Balagurumoorthy et al. 1995) This region also contains a nuclear export sequence that is masked after tetramerization therefore contributing to the subcellular localization.(Stommel et al. 1999) While the TD only represents approximately 2% of cancer associated somatic mutations in P53, up to 20% of patients with a germline mutation in P53, a disorder known as Li-Fraumeni syndrome, harbor mutations in this domain. (Malkin et al. 1990; Bouaoun et al. 2016; Petitjean et al. 2007) The final domain of P53, the C-Terminal Domain or CTD, is hypothesized to be responsible for select non-specific DNA binding activities as well as serving as a regulatory region for P53 through post-translational modifications. (Feng et al. 2005; Krummel et al. 2005; Tang et al. 2008; Hamard et al. 2013)

The above regions are as defined by the human P53 gene and the protein translated from it. Significant in the P53 field, however, is the use of mouse models to understand the development and evolution of cancer as it relates P53 altered function.

There are significant differences in sequences of subsections between the mouse Trp53 gene and the human TP53 gene, but a conserved overall function and structure of specific domains. The transactivation domain sequence is not highly conserved, but there is conservation of large numbers of acidic residues and a few key hydrophobic residues conferring conservation of function.(Isobe et al. 1986a; Raj and Attardi 2017) The DNA binding domain is highly similar in its sequence with an overall sequence homology of 89%.(Zhao et al. 2001) Similar to the TAD, the C terminal domain is not well conserved though generally hydrophilic with charged residues. (Isobe et al. 1986a)

1.2 P53 Activation in Response to Stress

P53 has an expanding array of stimuli that drive specific and varied responses. The most well defined P53 responses are to nutrient deprivation, hypoxia, oncogenic stress, ribosomal dysfunction, and DNA-damage.

In the instance of ionizing radiation induced DNA damage, double stranded breaks rapidly drive the activation of ATM which in turn rapidly phosphorylates downstream targets including Chk1, P53, and Brca1 (Gatei et al. 2003; Canman et al. 1998; Cortez et al. 1999). Activation of P53 results from stabilization of the P53 protein through phosphorylation of critical residues that mediate the interaction between P53 and its negative regulators. (Canman et al. 1998; Khanna et al. 1998; Banin et al. 1998; Sluss et al. 2004; Chehab et al. 1999) Stabilized P53 subsequently assembles into homotetramers and binds to specific P53 response elements across the genome

(Rajagopalan, Huang, and Fersht 2011; Gaglia et al. 2013; Weinberg, Veprintsev, and Fersht 2004). At these response elements, P53 recruits co-activators with a variety of functions including P300/CBP, mSin3a, and the transcription preinitiation complex to regulate the expression of the target genes (Barlev et al. 2001; Espinosa and Emerson 2001; Lin et al. 2005; Chang et al. 1995).

At each step of this cascade there are multiple pathways of diversification that result from different stress signals driving different responses. At the level of transcription and translation, there are 12 known P53 isoforms that arise from 9 different mRNA transcripts (Marcel et al. 2010; Courtois et al. 2002; Jorruiz and Bourdon 2016; Bourdon et al. 2005; Yin et al. 2003; Anbarasan 2019). This diversity results from multiple promoter regions, alternative splicing, and alternative translation start sites.

Transcription and translation of these isoforms is context specific and can modulate the function of the P53 protein as it drives a cell towards either cell cycle arrest or apoptosis pathways (Aoubala et al. 2011). After translation, there are nearly 50 positions on the protein that can be modified to alter the structure, function, and stability of the P53 protein by marks including phosphorylation, ubiquitination, acetylation, methylation, sumoylation, neddylation, O-GlcNAcylation, ADP-ribosylation, hydroxylation, and β -hydroxybutyrylation. (Y. Liu, Tavana, and Gu 2019)

1.3 Regulation of P53

MDM2 and its homolog, MDM4 or MDMX, are foremost among the barriers to aberrant P53 signaling. The ring domain E3 ubiquitin ligase MDM2 binds P53 in the TAD domain at residues 18 to 26 and the DNA binding domain at Ser269, and facilitates the ubiquitination of five lysines in the CTD (Gu, Shi, and Roeder 1997; Shimizu et al. 2002; Kussie et al. 1996; Böttger et al. 1997). MDM4, lacking the E3 ligase activity, is capable of interrupting P53 function through binding the TAD domain and the DBD (Huang et al. 2018; Hu et al. 2006). These essential functions underlie a model by which these proteins cooperate and antagonize each other to establish homeostasis in P53 prevalence and signaling. MDM2 primarily modulates P53 through ubiquitination and degradation via the proteasome, a function that is promoted and potentially necessitates heterodimerization with MDM4 (Xinjiang Wang, Wang, and Jiang 2011). MDM4, as previously stated, also acts independently to antagonize the P53 response to stress through binding of the TAD domain and the DBD (Francoz et al. 2006). In response to DNA damage, MDM2 facilitates the degradation of itself, through auto-ubiquitination, and MDM4, thereby allowing accumulation of the P53 protein and reducing barriers to a robust P53 signaling cascade. (Kawai et al. 2003; Pan and Chen 2003; C. Li, Chen, and Chen 2002) Alternatively, MDM2 can be sequestered and degraded through ARF binding and recruitment of E3 cofactors after oncogenic stress leading to p53 stabilization (Pan and Chen 2003). In addition to the negative regulatory functions, after

phosphorylation by ATM at Ser395, MDM2 can promote translation of P53 through binding and folding of the nascent P53 mRNA.(Yin et al. 2003; Malbert-Colas et al. 2014; Gajjar et al. 2012; Maya et al. 2001) Recently characterized and still to be fully elucidated ubiquitin ligases Pirh2, Cop1, and Arf-Bp1 have also been found to target P53. (Dornan et al. 2004; Leng et al. 2003; Chen et al. 2005)

P300/CBP are also potent potentiators of P53 function. Through the transactivation domain interaction P53 can bind P300/CBP to promote transcriptional activity as well as the DNA Damage repair process (Williams and Schumacher 2016). These proteins also acetylate P53s CTD, interfering with the MDM2 mediated degradation through poly-ubiquitination of this region, interrupt recruitment of MDM2 recruitment to P53 target genes, and disrupt SET binding of the CTD and the subsequent abrogation of P53 signaling (D. Wang et al. 2016; Tang et al. 2008).

1.4 P53 Cellular Pathways

1.4.1 DNA Damage Repair

DNA Damage repair is an essential system of human biology that overcomes errors in transcription and environmental stress to maintain integrity of the genome. There are three classical systems that drive repair in the genomes and each of influenced by P53 in different ways, two are highlighted here. The most broadly relevant system of repair is Nucleotide Excision Repair or NER. NER is facilitated by the XPC protein complex sensing of damage and recruitment of the TFIIH complex with cofactors XPB,

XPD, and XPA to facilitate the excision and repair of the damaged site. In the case of UV damage, the heterodimer of DDB1 and DDB2 can bind to DNA lesions and recruit XPC to expedite the repair process (Reviewed in 65,66). P53 has been shown to both interact with the NER proteins XPD and XPB as well as enhance transcription of DDB2 and XPC after DNA damage (Hwang et al. 1999; Adimoolam and Ford 2002; Hastak et al. 2012). The second version of the repair process is BER or base excision repair. Base excision repair is initialized by the removal of the flipped out base by a glycosylase. Downstream of the glycosylase, the dual Apurinic/apyrimidinic-endonuclease APE1 performs functions in multiple BER sub pathways, both as an endonuclease and exonuclease. P53 is influenced by the APE1 protein as redox reactions catalyzed by APE1 can increase the P53 DNA-binding capacity through tetramerization (Hanson, Kim, and Deppert 2005). Independent of transcription, P53 can influence the binding and repair capacity of BER repair enzyme Pol β as well as glycosylases APE1 and OGG1 (Achanta and Huang 2004; Zhou et al. 2001).

1.4.2 Cell Cycle Arrest and Senescence

P53 mediated cell cycle arrest is primarily driven by CDKN1A or p21. P21 was established as a target of p53 in the early 1990s and is significantly upregulated following DNA damage (El-Deiry et al. 1993; Wade Harper et al. 1993). After induction, p21 binds to cyclins E and D, Cdk2 and Cdk4 respectively (Xiong et al. 1993; Dulić et al. 1994). This prevents the phosphorylation of Retinoblastoma gene, Rb1 which in turn

binds to E2F1 and prevents transcription of genes related to cell cycle progression.

Prolonged expression of p21 can lead to the irreversible cell state known as senescence.

Cell cycle arrest, or the temporary halt of the cell cycle, is utilized by the cell to allow maintenance and repair of the genome to conclude before progressing in the cell cycle.

1.4.3 Apoptosis

Apoptosis is categorized through both intrinsic and extrinsic pathways and p53 participates in both through transactivation and transcriptionally independent mechanisms. The predominant p53 input is directed through the intrinsic pathway by p53 transcriptional upregulation of the BH3-domain proteins PUMA, NOXA, and BAX. (Villunger et al. 2003; Jeffers et al. 2003; Shibue et al. 2006; Yu and Zhang 2003; Chipuk et al. 2004). These proteins bind to the anti-BH3 protein BCL-2 and BCL-XL which frees BAX and BAK to translocate to the mitochondrial membrane to release cytochrome c through mitochondrial outer membrane permeabilization (MOMP). P53 can also translocate to the cytoplasm to form a complex with BCL-2 that similarly releases BAX to dimerize and induce MOMP (P. Li et al. 1997; Hakem et al. 1998; Kuida et al. 1998). When released into the cytoplasm cytochrome c can activate caspases 3 and 7 to initiate the apoptosome (Lakhani et al. 2006). The extrinsic pathway regulation by P53 is related to transcriptional upregulation of cell surface receptors FAS, DR5 and PERP that can mediate external apoptosis influences.

1.5 Using Mice to Model P53 and Cancer

1.5.1 Mutational Landscape of P53 Mutations

Nearly 50% of cancer cases harbor P53 mutations, though certain tumor subtypes vary significantly in the mutation rate (Olivier, Hollstein, and Hainaut 2010). Nearly 90% of all somatic mutations in cancer arising in P53 are in the DNA binding domain, and approximately 25% fall into a small group of DBD mutations called “hotspot mutations” (Olivier, Hollstein, and Hainaut 2010). These mutations are R175H, R248Q, R273H, R248W, R273C. More broadly, 30% of the somatic mutations are in the hot spot residues plus the R282W, G245S, R249S, Y220C, V157F. The R248Q, R273H, R248W, and R273C are categorized as DNA contact mutations (Baugh et al. 2018). These residues are key for binding of the P53 protein to the P53 response elements across the genome. Mutation of the arginine at residue 273 into a histidine results in 1000-fold decrease in its binding capacity for the gadd45a, and the resulting binding affinity is negligible for a P53 mediated response (Ang et al. 2006). The residues R175H, R282W, G245S, and R249S are structural mutants. As mentioned, one loop of the DNA binding domain structure interfaces with DNA. This interface is significantly perturbed after R245S and R249S mutations, reducing DNA binding and the cooperative binding of P53 molecules. The R175H, R282W, Y220C, and V157H significantly destabilize the P53 protein and result in very low binding affinity (Joerger, Ang, and Fersht 2006). The overall prevalence of missense mutations in P53, versus total loss of function nonsense mutations or large

indels, contrasts it with other tumor suppressor genes and suggests a specific etiology of these mutations that confers a survival benefit to their presence (Hainaut and Hollstein 1999). This benefit has been explored in numerous studies that hypothesize two primary functional benefits of the mutant protein. The first is these mutants are acting as a dominant negative. Though these mutations may have a severely limited DNA binding capacity, they still tetramerize within the nucleus with wild-type P53 transcribed from the other allele. This expands a partial loss of P53 from one mutated allele to a more penetrant loss of function as the mutant binds and diminishes transactivation of the wild-type P53 transcribed from the unaffected allele. The second hypothesis is that these mutations not only confer loss of function as it relates to critical barriers to transformation, but the mutant P53 protein has neomorphic characteristic that promote oncogenesis (Dearth et al. 2007; Boettcher et al. 2019; Stein, Aloni-Grinstein, and Rotter 2020; De Vries et al. 2002; Willis et al. 2004; Giacomelli et al. 2018; Bourdon et al. 2005; Y. Wang et al. 2011; Z. Wang, Strasser, and Kelly 2022; Monti et al. 2002).

The prevalence of cancer associated mutations gives clues about the sensitivity of other functional domains for mutations. This prevalence hints that while mutations in the DBD can significantly impact the functionality of the P53 protein, the N and C terminal regions are less sensitive to small alterations in sequence, potentially due to redundant mechanisms (Brady et al. 2011). The exception of this is the oligomerization

domain that harbors 2% of cancer associated mutations that do not lie within the DBD (Bouaoun et al. 2016).

1.5.2 Rationale of Mouse Models

Mouse models have contributed significantly to our understanding of the p53 pathway and its impact on the incidence of cancer. The first mouse models modulating p53 were developed by transgene introduction at the zygote stage, however these models are dissimilar to modern genetically engineered mouse models. The first knockout models of p53 generated through targeting of the endogenous gene in the mouse embryo were generated in the early 1990s, this knockout model was among the first genetically engineered mouse models (Donehower et al. 1992; Jacks et al. 1994). In the subsequent experimentation, mouse models have been used to characterize the impact of changes in the signaling cascade of p53 on tumor behavior and development. These observations are a result of models that alter the function of upstream regulators of P53 function and stability, models of dysregulation caused by mutation of p53 itself, and understanding the contribution of p53 to downstream processes by mutating critical targets of P53 function.

1.5.3 Mouse models of P53 regulation

Significant in our understanding of p53, is how the protein is regulated itself by the E3-ubiquitin ligases MDM2 and MDM4 and mice have played a significant role in the establishment of our working models. In a mouse model of homozygous knockout of

MDM2, the embryo does not survive out of early embryogenesis (Parant et al. 2001; Migliorini et al. 2002; Roberto, Daniel, and Guillermina 1995; Stephen et al. 1995). This is likely due to an unrestrained P53 apoptotic or ferroptotic response (L. Jiang et al. 2015; T. Li et al. 2012; Jennis et al. 2016). Interestingly, MDM4 knockout is also lethal during embryogenesis, though this happens later at embryonic day 10.5, E10.5 (Migliorini et al. 2002; Finch et al. 2002). This is contrasted with MDM2 knockout mice that do not survive E4.5 to E6.5. Supporting the hypothesis that this lethality is p53 mediated, mice that are deficient in MDM2 or MDM4 and p53 survive through embryogenesis.(Jackson and Berberich 2000; Shvarts et al. 1996; Parant et al. 2001; Migliorini et al. 2002; Finch et al. 2002; Stephen et al. 1995; Roberto, Daniel, and Guillermina 1995)

The role of MDM2 in controlling basal destabilization of the p53 protein, MDM2 is also present in 7% of cancers (Momand et al. 1998). Mouse models confirm, MDM2 amplifications suppress a normal p53 response, predisposing cells to transformation. MDM2 amplifications are also able to rescue the embryonic lethality of loss of MDM4, suggesting an overlapping but non-redundant regulatory purpose (Steinman et al. 2005).

1.5.4 Mouse Models of Mutant P53

1.5.4.1 Knockout Mouse Model

Mouse models of P53 mutations began with the knockout models developed in the early 1990's. These models deleted large sections of the DNA binding domain, inactivating P53. These models also established the tumor spectra that has become the

standard of comparison for subsequent studies on mutations in P53. These mice were developmentally normal and homozygous mice were significantly predisposed to lymphoma, 70%, and heterozygous mice were more predisposed to sarcomas. (Donehower et al. 1992; Jacks et al. 1994) These mice were a significant advancement in our understanding of p53 as well as our understanding of the utility of mouse models for modeling p53 loss in cancer. These mice, however have significant limitations in the use for study of p53 inactivation related to other disease sites.

The introduction of a cre recombinase mediated p53 conditional-knockout mouse allowed for the spatial and temporal restriction of loss of the p53 gene. (Marino et al. 2000) This drastically expanded the ability to restrict the loss of p53 to, with appropriate cre expression, different tissues and time points not hindered by the early tumor burden of the knockout mouse. These models however, are not biologically relevant to human disease as they eliminate large portions of the protein that make discernment of specific domain changes impossible.

1.5.4.2 Targeted Mutation in P53

Mouse models of targeted mutations in P53 are a continually expanding group of specific mutations to key residues or domains that allow for the discernment of specific facets of P53 mutations and the dynamics that underlie p53 differential function and subsequent tumor progression. The first functional domain of P53 is the transactivation domain. This domain is a significant interface of p53 with protein binding partners that

have various functions including modulating expression of P53 target genes and remodeling chromatin architecture. The first mutations in this domain modeled in mice were Ser18 (human Ser15) and Ser23 (human Ser20)(Sluss et al. 2004; MacPherson et al. 2004). As a phosphorylation target site of ATM after DNA damage, the Ser15 modification, a modification proposed to stabilize P53 through disruption of MDM2 binding, was hypothesized to affect P53 stability and simultaneously alter its capacity to drive downstream pathways. Stability of p53 and tumor suppression was found not altered though apoptosis was abrogated in assayed tissues (Chao et al. 2003; Sluss et al. 2004). In the Ser23 model, apoptosis of selected tissues was moderately affected and stability was slightly decreased. This model also found that, long term, mice developed B-cell lymphomas at a later stage than the T-cell lymphomas developed in the knockout model (MacPherson et al. 2004). When these mutations were combined, apoptosis is similar to p53KO levels and tumorigenesis is accelerated though still delayed from the p53KO (Chao et al. 2006). Subsequent models to dissect the function of the transactivation domain in signaling and tumor suppression were the p53^{25/26} and the p53^{53/54} mutants. The p53^{25/26} mutant is embryonic lethal. Under conditional expression, the p53^{25/26} mutant lacks apoptosis and cell cycle arrest capacity, a general loss of 90% transactivation capacity (Johnson et al. 2005; Brady et al. 2011; Jimenez et al. 2000). This translates to a lack of tumor suppression in a thymocyte restricted cre activation model, but this mutant is capable of suppressing transformation in a Kras^{G12D} induced lung

cancer model (Gaidarenko and Xu 2009; Brady et al. 2011). The p53^{53/54} model is similarly able to suppress tumor formation in the lung cancer model but the quad mutant p53^{25/26,53/54} is not able to suppress tumor formation, linking the ability of p53 to cooperate with protein partners with tumor suppression.

The p53^{S47P} mutation is found in 6% to 8% of certain African populations and to a lesser extent people of African ancestry in the US (Jennis et al. 2016). This mutation is adjacent to the Ser46 residue that is phosphorylated after significant DNA damage. When mutated, the resulting Ser47 mutated p53 protein is subtly deficient in driving apoptosis. The transgenic mouse model of the Ser47 mutation derived from the Hupki TP53 transgenic model is deficient in specific tissues for apoptosis but drives cell cycle arrest and senescence to a similar extent to that found in wild type. This mutant was not able to transactivate genes related to ferroptosis or metabolism proposed by the triple-lysine mutant model, discussed later in this section to be, important for tumor suppression. This mutant is predisposed to tumors with an onset between 12 and 18 months of age (Jennis et al. 2016). This suggests that the canonical targets are not sufficient to suppress tumor formation and that ferroptosis and metabolism may potentially play a significant role in the p53 mediated tumor suppression.

Three prime of the transactivation domain is the proline rich domain (PRD). Mutation of the PRD resulted in a target specific reduction in transactivation capacity and an increased susceptibility to MDM2/MDM4 dynamics. This mutant rescues

viability of embryos with homozygous MDM4 deletions, but fails to do so in MDM2 deleted embryos suggesting the PRD is important in managing the stability and interface with MDM2 and MDM4 proteins.

The DBD domain is the most mutated portion of the gene and the diverse models that have been developed and utilized to study p53 dynamics similarly reflect this representation. These models have been utilized to investigate fundamental questions regarding dominant negative, gain of function, and loss of function properties of these mutations.

In human disease, hotspot mutations are associated with an earlier onset of disease in patients with Li-Fraumeni syndrome as well as a worse overall prognosis and survival (Xiang Wang et al. 2014; Xu et al. 2014; Schulz-Heddergott et al. 2018; Cooks et al. 2018; Bougeard et al. 2008). Characteristics that match this increased malignant behavior have also been found in mouse models of p53 mutations. The R172H and R270H mutated mouse models, human R175H and R273H respectively, are associated with a more metastatic phenotype and a distinct tumor spectra relative to p53^{+/-}. (G. Liu et al. 2004; Lang et al. 2004; G. Liu et al. 2000; Olive et al. 2004). Additionally, mice harboring a transgenic R248Q mutation exhibit an earlier onset of disease and a shorter overall survival (Hanel et al. 2013). Each of these mutants conferred specific differences in target tissues and on the resulting tumors, but loss of the second allele still conferred a significant growth advantage. This presents a model where a hotspot mutation is

advantageous to disease onset and growth and loss of the residual activity from the wild-type allele further advantages progression. This is supported by the human disease data in which 91% of p53 mutant cancers have loss of the secondary allele through multiple mechanisms including copy-neutral loss of heterozygosity, mutation, or chromosomal deletion (Donehower et al. 2019).

In addition to the dynamics of the dominant negative or loss of function models, partial loss of function models has been used to discern the necessity of specific downstream functions for tumor suppression. A variant of the R175 residue, human R175P or mouse R172P, lead to criticism of the contribution of apoptosis to the overall tumors suppression capabilities of p53. These mice were predisposed to tumors, but with later onset compared to knockout models suggesting that other tenets of p53 function were important for p53 mediated tumors suppression.

The triple lysine mutant, p53^{K117R+K161R+K162R}, established soon after the p53^{25/26} mutant, represented another model that lacked canonical suppression of cell cycle arrest, senescence, and DNA damage and was not significantly predisposed to tumor formation. Mutation of the DNA binding domain residue 117, 161, and 162, three sites that are acetylated during the p53 response to stress, lacks classical canonical signaling, but retains control of metabolism related genes Gls2, Dram1, Glut3 and repression of the glucose transporter Slc7a11. This was theorized, and later further explored by the P47S

mutant, to be important for tumor suppression through control of a metabolic shift undergone during transformation (T. Li et al. 2012; L. Jiang et al. 2015).

The final category of mouse models that has shifted our understanding are the models that have stepwise mutated the downstream effectors of P53 function.

Consequential for the confirmation of downstream functions of P53 was the triple mutation of p53 target genes Cdkn1a, Puma, and Noxa. These genes are critical modulators of the P53 driven responses cell cycle arrest, Cdkn1a, apoptosis, Puma and Noxa (Valente et al. 2013). This model was not significantly tumor prone confirming the implications of the 90% loss of transactivation in the p53^{25/26} model with a complete loss of critical genes related to cell cycle arrest and apoptosis. The final model that I will discuss is the triple mutant of p53 effectors Cdkn1a, Puma, and Zmat3. This mouse combines the loss of apoptosis and cell cycle arrest with loss of a splicing factor that decreases time to tumor development significantly (Brennan et al., BioRxiv 2022).

Collectively these models suggest a tissue specific model of p53 regulation and tumor suppression that relies on multiple pathways to fully recapitulate the tumor suppressive phenotype. Continuing to expand our knowledge about the specificity of this response is critical to understanding how to address p53 loss in the treatment setting.

2. Mis-splicing Drives Loss of Function of p53E224D Point Mutation

2.1 Introduction

p53 is the most frequently altered gene in human cancer, as greater than 50% of cancers harbor p53 mutations. The primary p53 signaling axis is facilitated by protein stabilization through post-translational modification, homotetramerization and transcriptional upregulation of hundreds of target genes related to a diverse array of functions (Vousden and Lane 2007; Kasthuber and Lowe 2017; Freed-Pastor and Prives 2012). Classically, the most critical functions of p53 for tumor suppression were thought to be the canonical pathways leading to cell cycle arrest, apoptosis and senescence. Challenging this model, a triple knockout mouse with deletion of three critical p53 targets driving cell cycle arrest, Cdkn1a, and apoptosis, Puma and Noxa, did not exhibit a significant tumor burden (Yu and Zhang 2003; Shibue et al. 2003; Deng et al. 1995). This suggested that these effectors are insufficient or potentially unnecessary for p53 mediated tumor suppression in mice. In support of the insufficiency of these canonical pathways of tumor suppression, mice with p53 mutations in the transactivation domain (p53^{25,26}) or in three lysines (p53^{K117R+K161R+K162R}), which lack canonical signaling to initiate cell cycle arrest and apoptosis after DNA damage nevertheless retained tumor suppression (T. Li et al. 2012; D. Jiang et al. 2011).

In human cancers, 86% of mutations in p53 occur in the DNA binding domain and ~30% of these mutations are specific to the top six hotspot residues (Olivier,

Hollstein, and Hainaut 2010; C. Zhang et al. 2020). The impact of these hotspot mutations on the structure and function of p53 is well characterized. These mutations are classified as structural mutations that significantly affect the overall architecture and stability of the DNA binding domain or contact mutations that alter residues critical for DNA interaction (Bullock, Henckel, and Fersht 2000). Hotspot mutants are further characterized by their accumulation at the protein level due to disrupted negative feedback loops with potential neomorphic capabilities (Boettcher et al. 2019). These mutations also lead to loss of signaling through critical effectors of p53 function, a phenotype confirmed in mouse models (Monti et al. 2002; Boettcher et al. 2019; Giacomelli et al. 2018; Olive et al. 2004). For example, the p53^{R175P} and p53^{E177R} mouse models retain the ability to drive cell cycle arrest, but exhibit a loss of canonical p53 mediated apoptosis. The p53^{R175P/R175P} and the p53^{E177R/E177R} mice display a delayed onset of tumorigenesis relative to p53^{-/-} (G. Liu et al. 2004; Timofeev et al. 2013). Therefore, these mouse models of a DNA binding domain mutants only partially discriminate canonical signaling from tumor suppression.

Systematic characterization of the functional impact of point mutations in p53 on transactivation was first explored in yeast (Kato et al. 2003). Investigators generated single base pair amino acid substitutions in the previously uncharacterized portion of the DNA binding domain as well as the NH₂- and COOH- terminal domains. Using these mutants, investigators examined differential transactivation of eight critical

effectors of p53 function (Kato et al. 2003). As expected, loss of function mutations clustered in the DNA binding domain and the transactivation domain. This confirmed previous reports that other functional domains are insensitive to single amino acid substitutions (Sluss et al. 2004; MacPherson et al. 2004; Johnson et al. 2005; Brady et al. 2011; Krummel et al. 2005). Systematic studies in mammalian cell lines confirmed a distribution of transactivation functionality of p53 mutants (Kotler et al. 2018; Giacomelli et al. 2018).

To explore the link between transactivation and tumor suppression, here we compare the findings from the yeast screen study with available human p53 mutation data from cancers to correlate specific point mutations showing loss or retained transactivation function with the incidence of the same substitution in human cancer. We identified a subset of mutants that included p53^{E224D} as competent for transactivation in yeast, but paradoxically present in human cancers. Because the ability of a mutant of p53 to suppress cancer has historically been tightly linked with a specific capacity to drive the expression of transcriptional targets, we characterized the transactivation potential of cDNAs of these mutants in human cells and confirmed that p53^{E224D} retained transactivation. Therefore, we generated genetically engineered mice with an endogenous p53^{E221D} mutation (the mouse equivalent of p53^{E224D}). Using in-vitro experiments with mouse embryonic fibroblasts (MEFs) and human cancer cells harboring p53^{E224D}, and the in-vivo genetically engineered mice, we find that the p53^{E224D}

point mutation loses the ability to suppress tumor development because it causes a loss of p53 function as a result of mis-splicing of the p53 mRNA transcript.

2.2 Transactivation Mutants Co-Occur with Secondary DNA Binding Domain Mutations

To search for p53 mutants with intact transactivation in the yeast model system and present in human cancer, we searched the IARC, TCGA, and GENIE tumor databases for the relative frequency of p53 mutations in patient samples (Figure 1) (AACR Project GENIE Consortium 2017; Petitjean et al. 2007).

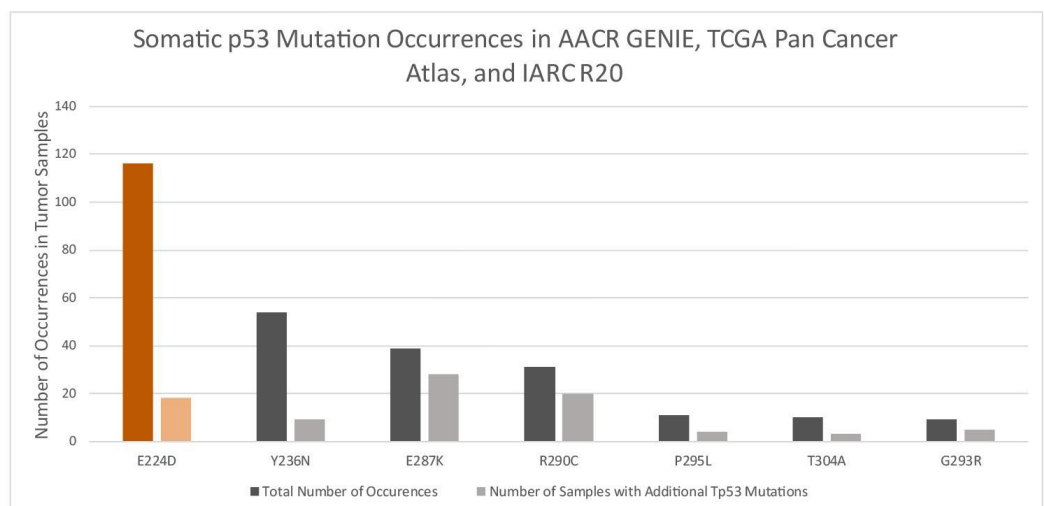


Figure 1: Somatic occurrences of select transactivation competent p53 mutants from IARC, GENIE, and TCGA databases. Samples containing protein variants accessed on public databases IARC, R20, TCGA, and AACR’s GENIE as of June 2023. Lighter adjacent bars represent the co-occurrence of non-synonymous p53 mutants in the same samples. Data and Analysis contributed IL and EM.

The yeast screening data identified 373 mutants classified as competent or wild-type for transactivation and simultaneously found in human tumor databases of p53 mutations (Kato et al. 2003). Co-occurrences, instances of a transactivation competent

mutant in a sample with a secondary non-synonymous p53 mutation, are noted because the overall loss of tumor suppressive capacity could potentially be attributed to either mutation if one mutant had dominant negative activity. Of note, the p53^{E224D} mutation occurs frequently compared to other mutants without a substantial number of co-occurring non-synonymous p53 mutations. In addition, the p53^{E224D} mutation was also found in the IARC database among germline, transactivation competent, mutants in the p53 gene (Figure 2).

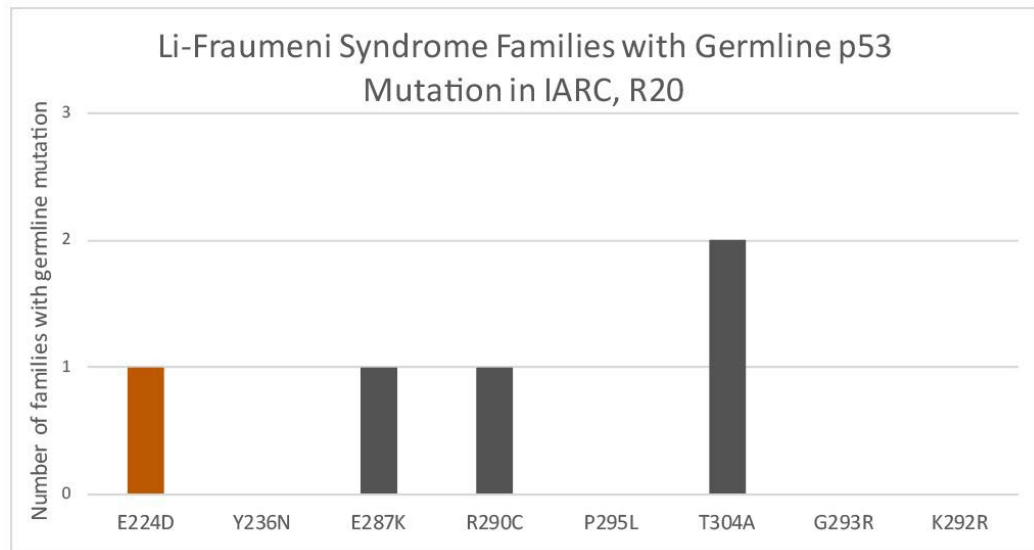


Figure 2: Li-Fraumeni Patient, or Germline, occurrences of p53 mutants from IARC, R20 database. Y-axis is the number of families that have reported protein variants on the x-axis. Data and Analysis contributed IL and EM.

These results suggest that p53^{E224D} is a primary event driving altered function of p53 in these tumors.

2.3 p53E224D cDNA Retains Transactivation in Human Cells

To evaluate the relationship of the yeast transactivation screen within a mammalian system, we used a tetracycline-inducible system to express various p53 mutants in the p53 null non-small cell lung cancer cell line H1299. H1299 cells have a homozygous deletion of a portion of the p53 gene and no protein expression to interfere with this re-expression assay (25). We transduced the H1299 cells with a tet-inducible backbone containing a doxycycline responsive cDNA that encodes p53 wild-type (WT) or various mutants identified in the yeast screen. Doxycycline addition was sufficient to overexpress p53 wild-type (WT) or mutant proteins and to assess their impacts on p53 targets MDM2 and CDKN1A by western blot (Figure 3).

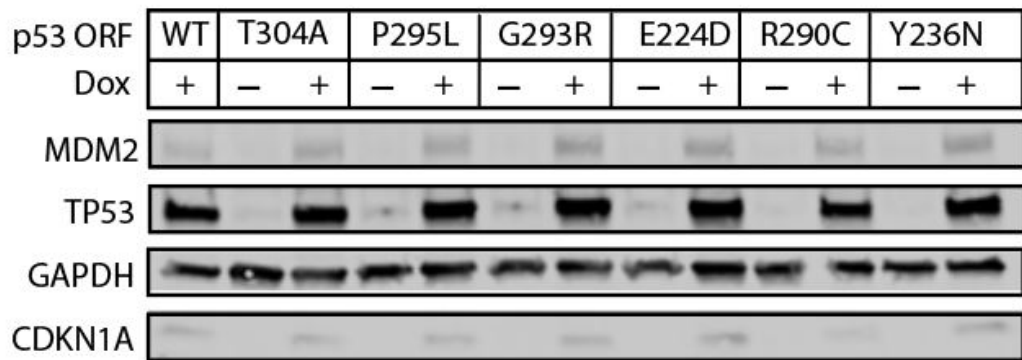


Figure 3: Expression of doxycycline inducible p53 mutant cDNA constructs in p53 null H1299 lung cancer cells. Expression validated by western blot using cell lysates probed for p53. Transactivation capacity assessed by MDM2 and CDKN1A expression using a GAPDH loading control 24 hours post doxycycline addition, 1µg/mL. Data and Analysis contributed WF, IL, and NL.

In this assay, human p53E224D expressed from cDNA retained the capacity to activate the key p53 target proteins MDM2 and CDKN1A.

The transactivation capacity of the p53E224D mutant was further assessed using qRT-PCR after doxycycline and irradiation treatments to induce a p53 transcriptional program. As expected, transactivation from the doxycycline induced p53E224D mutant was not significantly different from p53 WT for key transcriptional targets of p53 that regulate apoptosis (PUMA), cell-cycle arrest, and senescence (CDKN1A, 14-3-3 sigma, and GADD45A) as well as the E3 ubiquitin ligase that degrades p53 protein (MDM2) (Figure 2B). In contrast, the expression of these p53 target genes was not induced by a dominant negative R273H mutant (Figure 4).

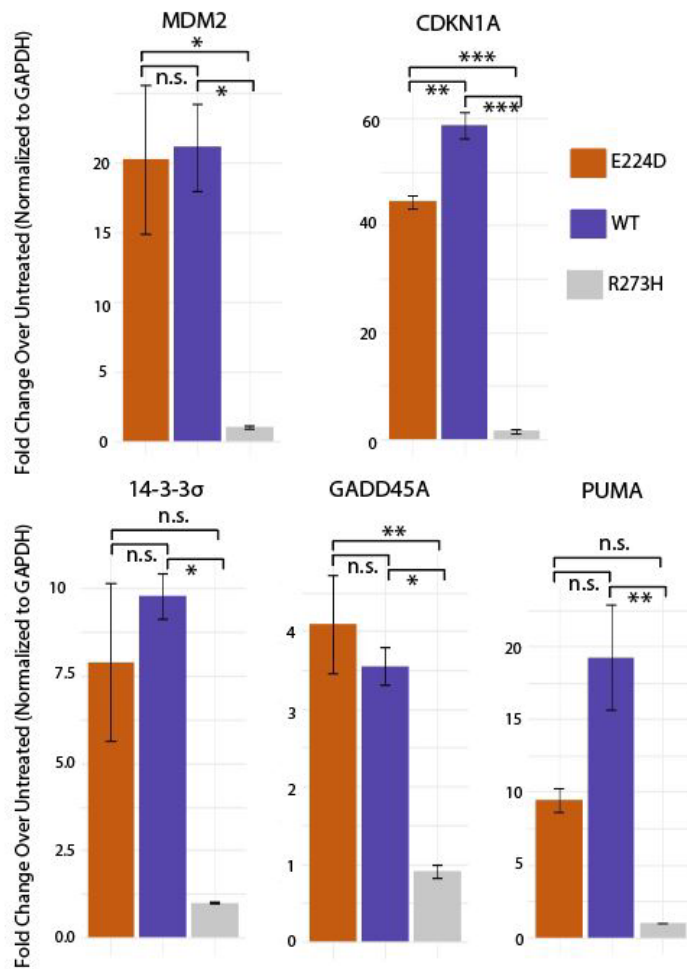


Figure 4: Transactivation of p53 targets by doxycycline induced p53E224D mutant (24 hours of 1μg/mL doxycycline, 1 hour after 10 Gy irradiation). Gene expression assessed by RT-qPCR. (p53E224D – Orange, WT – Purple, p53 Loss of Function (R273H) – Gray) (* = p<.05, ** = p<.01, * = p<.001) Average of three technical replicates analyzed by ANOVA. Data and Analysis contributed IL, WF, and NL.**

To test if transactivation of these p53 targets by p53E224D leads to cellular phenotypes, we compared the cell growth in H1299 cells harboring doxycycline-inducible p53WT, p53E224D, or p53R273H as well as the p53 null parental cells in the presence of doxycycline containing or control media. Growth was tracked via Incucyte

over a six-day period. Cumulative growth assessed on the final day is reported as average percent confluence (Figure 5).

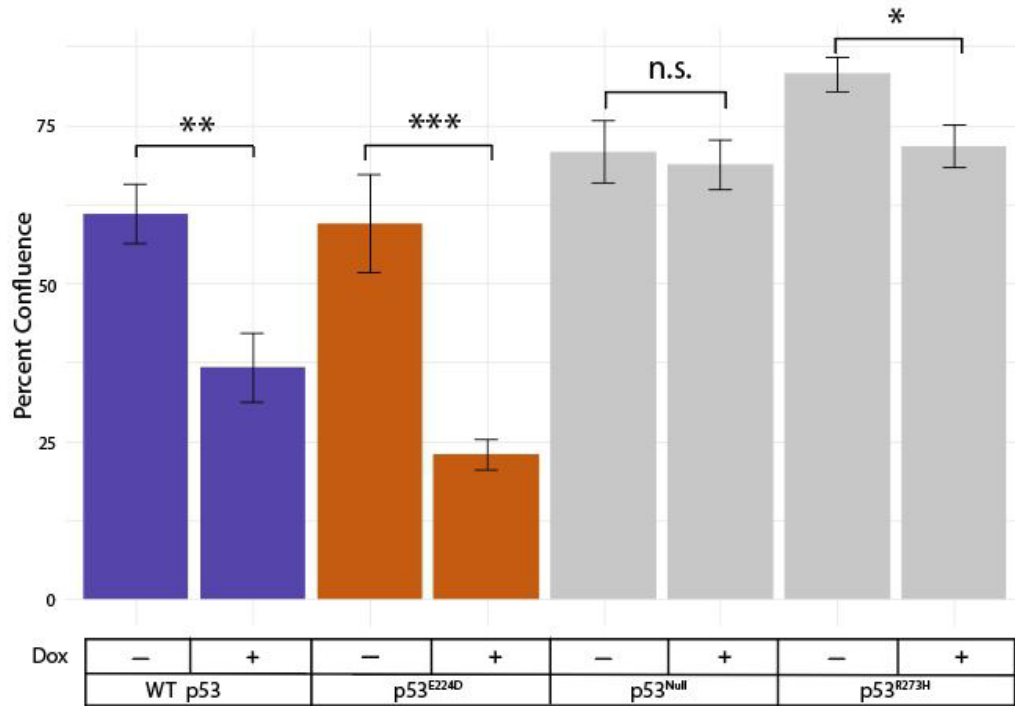


Figure 5: Incucyte measurement of growth arrest capacity of p53 mutants. Percent confluence of samples represented as an average after six days of growth in a 96 well plate. Compared to non-doxycycline containing media over the same time course (p53E224D – Orange, p53WT – Purple, p53 Loss of Function (Null or R273H) – Gray) (= $p < .01$, *** = $p < .001$, * = $p < .05$) Average of ten technical replicates and analyzed by ANOVA. Data and Analysis contributed IL, WF, and NL.**

p53 WT and p53E224D mutants induced by doxycycline retained growth suppression. Together, our results indicate that the cDNA for p53E224D retains the ability to transactivate downstream targets and suppress the growth of human cancer cells in vitro.

2.4 p53^{E221D} genetically engineered mouse model lacks p53 protein and RNA expression

Although the p53^{E224D} mutation does not significantly impair the function of p53 protein in the setting of cDNA expression, the impacts of the p53^{E224D} mutation on the expression and function of p53 protein from the endogenous p53 locus in-vivo remained unclear. Thus, we generated a novel genetically engineered mouse with a p53^{E221D} mutation (GAG>GAT, mouse equivalent of human p53^{E224D}) in the endogenous p53 gene through homology-directed CRISPR editing. The presence of the p53^{E221D} mutation was validated using targeted sequencing of the p53 gene (Figure 6).

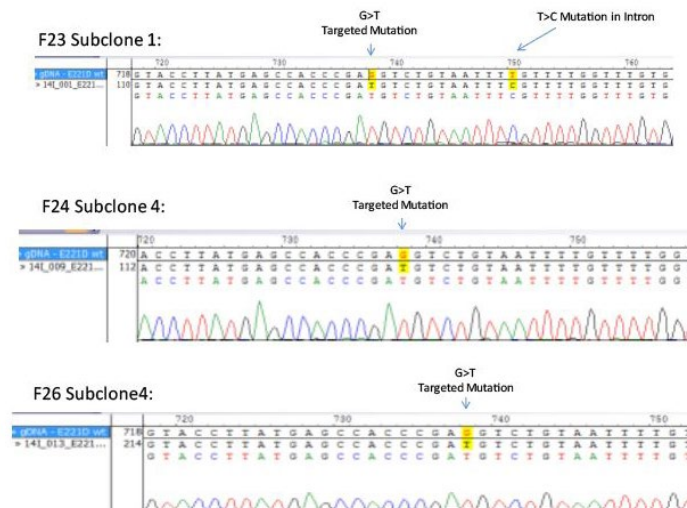


Figure 6: Sanger Validation of CRISPR Targeting in mouse p53E221D embryo. Product of PCR utilizing primers targeting the region of interest sequenced using sanger sequencing and visualized to validate on-target editing of the CRISPR/Cas9 construct. Primers can be found in Table 2. Data and Analysis contributed by Duke Transgenic Core.

We generated mouse embryonic fibroblasts (MEFs) from the p53^{E221D} mice. Unexpectedly, we observed that p53 protein was not detected in p53^{E221D/E221D} MEFs from the knock-in mice even after treatment with either 10Gy irradiation or with the proteasomal degradation inhibitor MG132 (Figure 7).

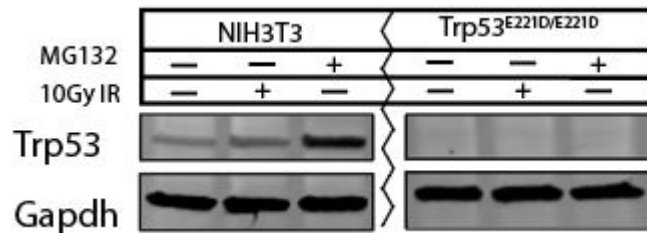


Figure 7: Mouse embryonic fibroblast (MEF) lysates were probed for p53. Visualization by western blot after stabilization 1 hour after 10 Gy irradiation or 6 hours after addition of the proteasomal degradation inhibitor MG132, 10μM. Data and Analysis contributed IL.

Similar to the results from our mouse model, the human colorectal cancer cell line NCI-H716 harboring a homozygous p53^{E224D} mutant also showed no detectable p53 protein after treatment (Figure 8).

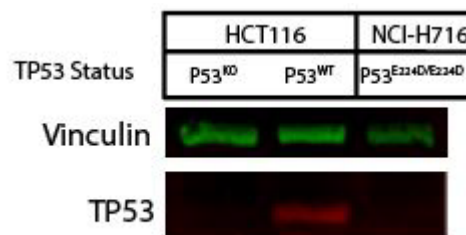


Figure 8: Human p53E224D expression. Human colorectal adenocarcinoma cell line, NCI-H716 which harbors the p53E224D mutation, and p53WT and p53KO human colorectal carcinoma, HCT116, probed for p53 protein after MG132 (6 hours, 10μg), G418 (48 hours, 400μg/mL), and Irradiation (1hr before extraction, 10Gy) treatment. Data and Analysis contributed IL.

Consistent with a loss of p53 function, we observed that both p53^{E221D/E221D} and p53^{E221D/WT} mice showed a significant decrease in overall survival compared to p53^{WT/WT} littermates (Figure 9).

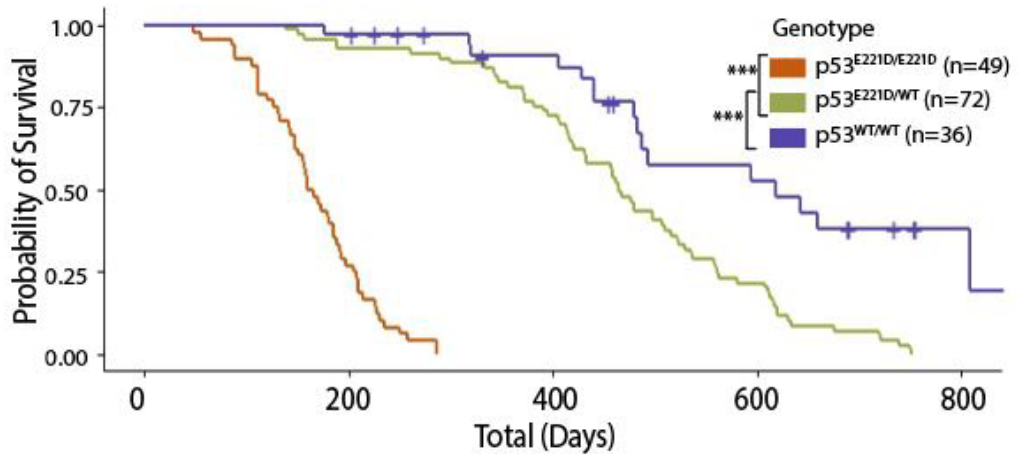


Figure 9: Survival Curves for p53 mutant mice. Mice monitored until ill health necessitated euthanasia or found dead. (p53^{E221D/E221D} – Orange, p53^{WT/WT} – Purple, p53^{E221D/WT} – Green) (*) = p<.001 using pairwise log-rank test). Data and Analysis contributed IL, LL, YM, and NW.**

These mice primarily developed sarcomas and thymic lymphomas, which are also the predominant types of tumors that occur in mice with germline deletion of p53 (Figure 10A-B) (Jacks et al. 1994; U. Kim et al. 2019; Donehower et al. 1992).

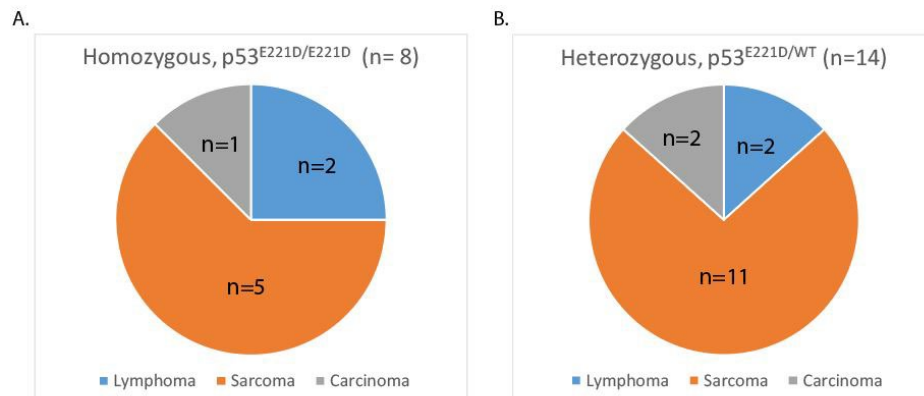


Figure 10: Venn Diagram of Cancer Subtypes in p53E221D Mouse Model separated by homozygous or heterozygous. Ascertained by H&E immunohistochemistry and assessed by sarcoma pathologist DC. Note: Due to the constraints of COVID19 procedures, there was a reduced capacity for consistent mouse observation. This resulted in a reduced capacity for collection of recently deceased and a likely bias towards gross morphological tumors reaching humane endpoints and subsequent collection. Therefore, we provide these data as a qualitative indication of a conserved tumor spectrum rather than a quantitative assessment of subtype prevalence. A. Pie chart of tumor spectrum observed in p53E221D/E221D littermate controls. B. Pie chart of tumor spectrum observed in p53E221D/WT littermate controls. Mouse 640847 presented multiple primary tumors, one lymphoma and one sarcoma and thus is counted in both categories. Data and Analysis contributed IL, DC, and NW.

The mean survival of p53^{E221D/E221D} is 167 ± 57 days, which is similar to the mean survival of p53^{-/-} mice (U. Kim et al. 2019; Lawler et al. 2001).

Compared to p53^{WT/WT} MEFs, the induction of transcriptional targets of p53 by ionizing radiation was markedly impaired in homozygous p53^{E1221D/E221D} MEFs or in a cell line derived from a homozygous p53^{E221D/E221D} sarcoma (Figure 11). Of note, the expression of p53 mRNA was absent in p53^{E221D/E221D} MEFs (Figure 11).

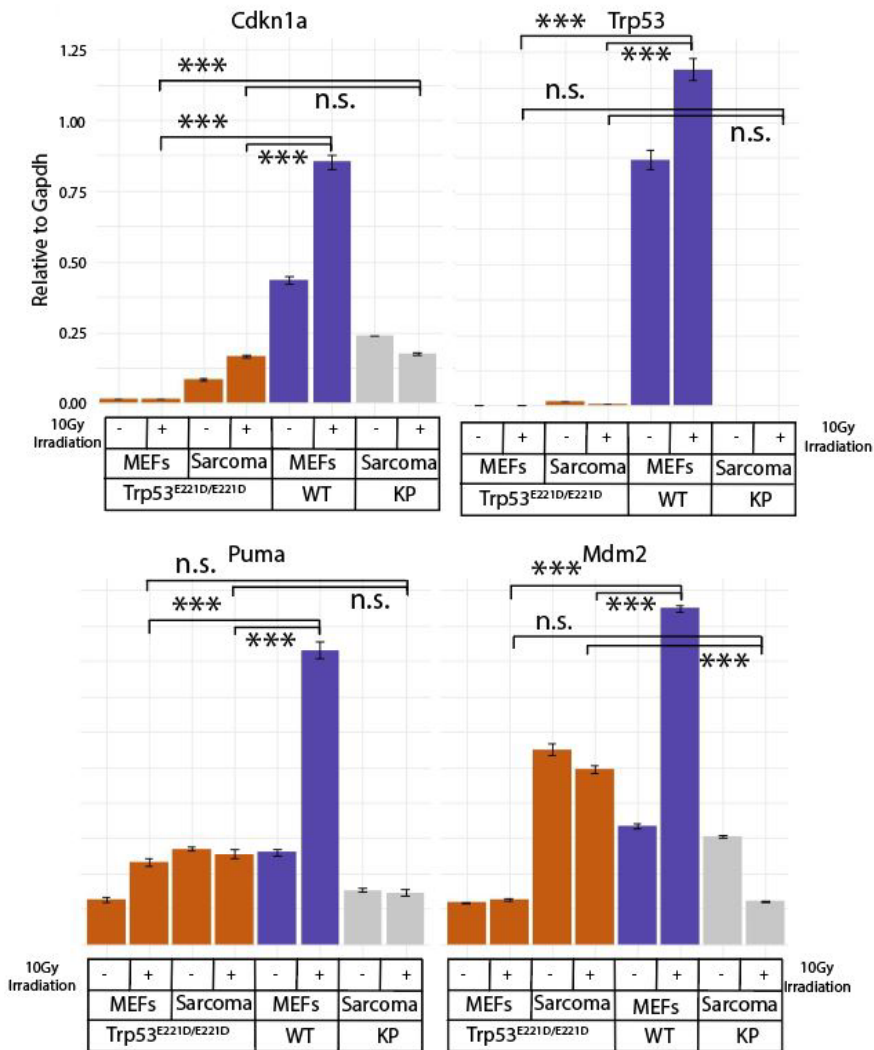


Figure 11: Expression of Trp53 and Downstream targets. p53 target gene expression changes, assessed by RT-qPCR, one hour after 10 Gy irradiation. (p53^{E221D/E221D} – Orange, p53^{WT/WT} – Purple, p53^{-/-} Kras^{G12D} - Gray) (*) = $p < .001$, ** = $p < .01$, * = $p < .05$) Average of three technical replicates. ANOVA used to assess statistical significance. Data and Analysis contributed IL.**

Collectively, our results from p53^{E221D/E221D} mice indicate that p53^{E221D} is a loss-of-function mutation that results from impaired expression of endogenous p53 mRNA in

vivo. These findings generated a hypothesis that mis-splicing of the E221D mutant leads to nonsense-mediated decay as a potential mechanism for this loss of function.

2.5 Mis-splicing drives loss of RNA expression

To investigate potential mis-splicing from the p53^{E221D} mutation, RNA-Seq was performed on p53^{E221D/E221D} MEFs and the p53^{E224D/E224D} human cell line. Transcript analysis of TopHat aligned RNA sequencing from homozygous p53^{E221D/E221D} and p53^{WT} MEFs supports a general loss of p53 transcripts at the RNA level (Figure 4A). Using stringtie, a bioinformatics tool for splice variation, four novel transcripts that include the exon 6 splice junction were identified in the mutant MEFs. Novel transcripts MSTRG.3218.1, MSTRG.3218.5, and MSTRG.3218.7 highlight exon skipping, and MSTRG.3218.8 retains intron 6 (Figure 12).

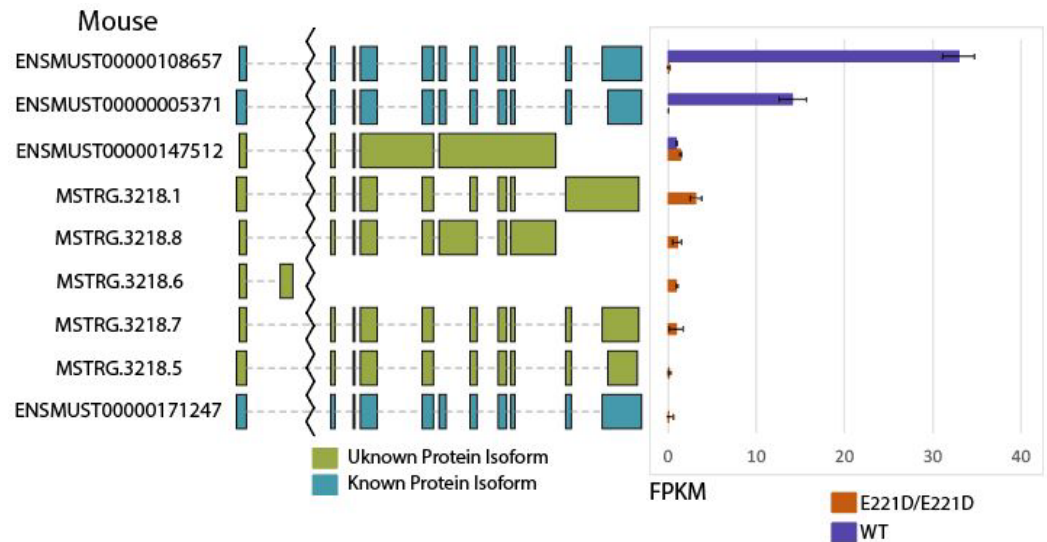


Figure 12: Visual reference for RNA transcript splicing adjacent to bar plot of relative abundance (FPKM) in p53^{E221D/E221D} and p53^{WT} MEFs. Known transcripts annotated with Ensembl identifier, unknown transcripts annotated with

unique Stringtie identifier. Color of the graphical representation of RNA transcript symbolizes predicted translation of protein of a known or unknown function. Sequencing done on three technical replicates. Data and Analysis contributed by IL.

The scale of the Sashimi plots from the Integrative Genomics Viewer (IGV) visualization of a representative sample confirms the reduction in read depth at this region of the p53^{E221D/E221D} mRNA. This plot also allows visualization of the 43 reads that confirm splicing of the Exon 5 donor to the Exon 7 splice acceptor and the relative increase in reads that map to Intron 6 in the p53^{E221D/E221D} MEFs. (Figure 13).

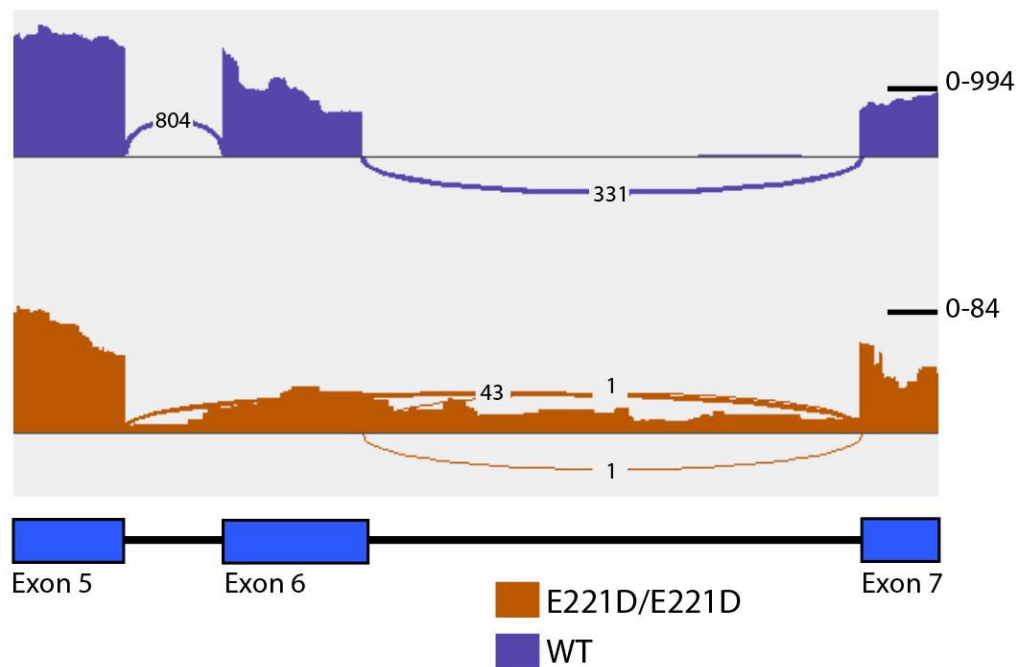


Figure 13: Representative sashimi plot visualization of read abundance from IGV. Linkages represent reads that span exon-exon junctions, i.e. alternative splice events in MEFs. Data and Analysis contributed IL.

Of note, a small number of reads in each sample are correctly spliced, one in the representative sashimi visualization. These are visual confirmations of the transcript

analysis that discovered novel transcripts with complete intron 6 retention and exon 6 skipping. Transcript discovery was subsequently carried out using Star alignments (Figure 14A-B).

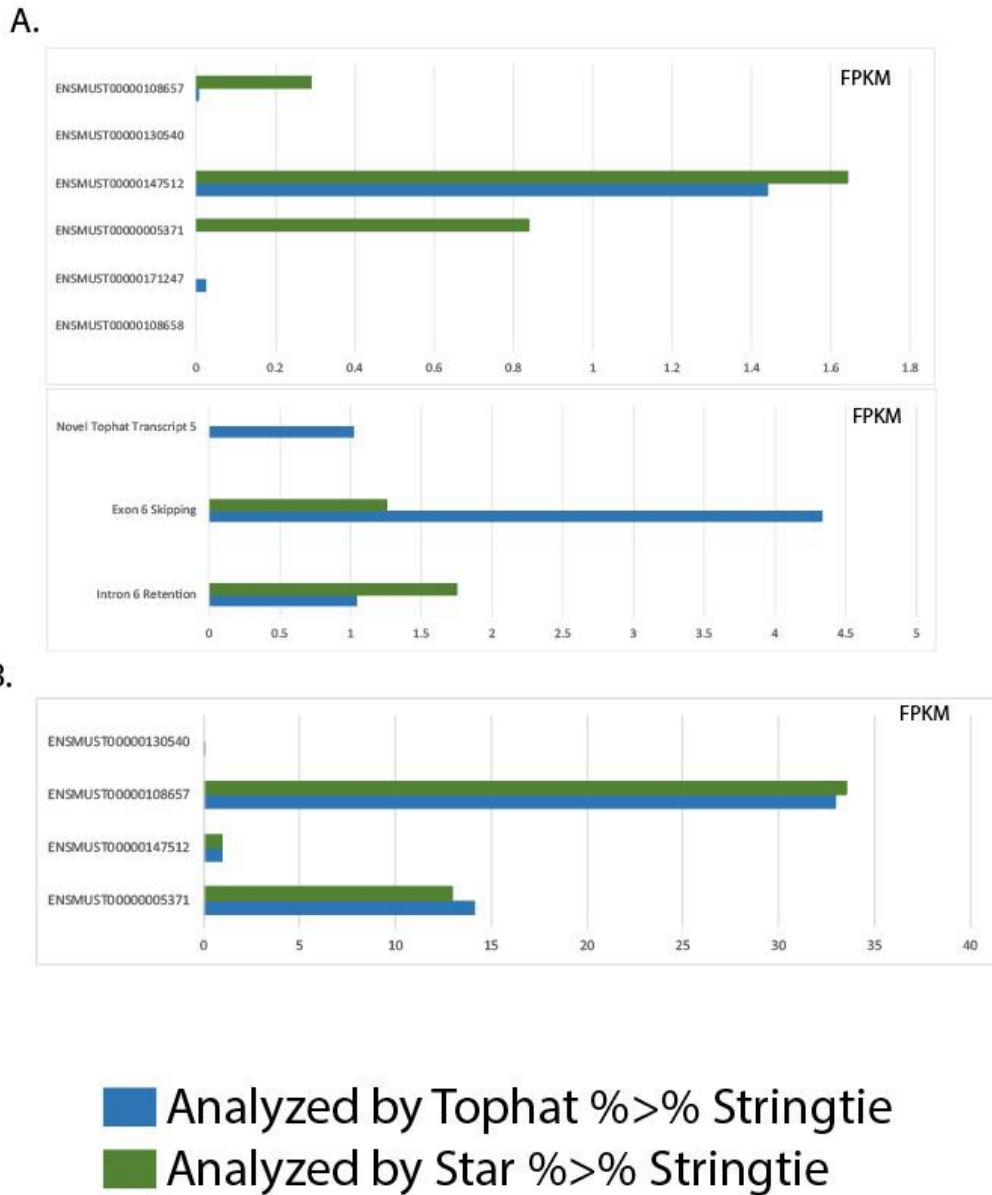


Figure 14: Transcript discovery by Stringtie in mouse samples. A. Bar chart comparison of known transcripts and novel splice events in Stringtie results of bam

files aligned with Tophat and Star from p53E221D/E221D MEF samples. (Blue = Tophat aligned, Green = Star aligned) B. Bar chart comparison of known transcripts in Stringtie results from Tophat and Star aligned reads from p53WT MEF samples. (Blue = Tophat aligned, Green = Star aligned) Data and Analysis contributed IL.

Transcript structure was broadly confirmed, but there was variability in the mutant transcript prevalence. The wild-type Trp53 transcripts were well conserved across alignment methods.

In contrast to the Tophat aligned novel transcript identification in the mouse model, when we performed RNAseq on human p53^{E224D/E224D} NCI-H716 compared to p53^{WT} HCT116 cells, there was one novel variant highlighted after Stringtie transcript identification (Figure 15).

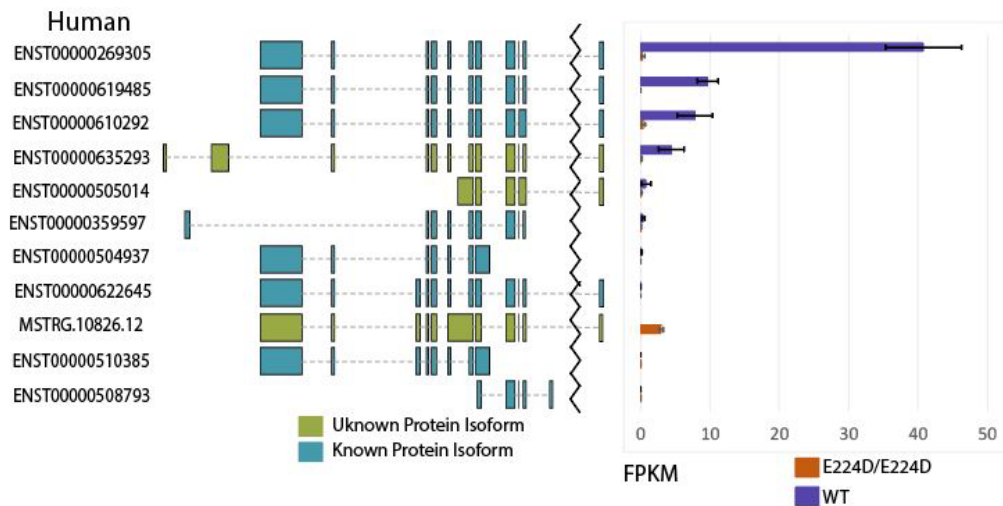


Figure 15: Visual reference for RNA transcript splicing adjacent to bar plot of relative abundance (FPKM) in p53E224D/E224D and p53WT human cancer cell line. NCI-H716 and HCT116, respectively. Known transcripts annotated with Ensembl identifier. Color of the graphical representation of RNA transcript symbolizes predicted translation of protein of a known or unknown function. Sequencing done on three technical replicates. Data and Analysis contributed IL.

This transcript variant, MSTRG.10826.12, exhibits intron 6 retention as the dominant transcript in the p53^{E224D/E224D} cell line (Figure 16).

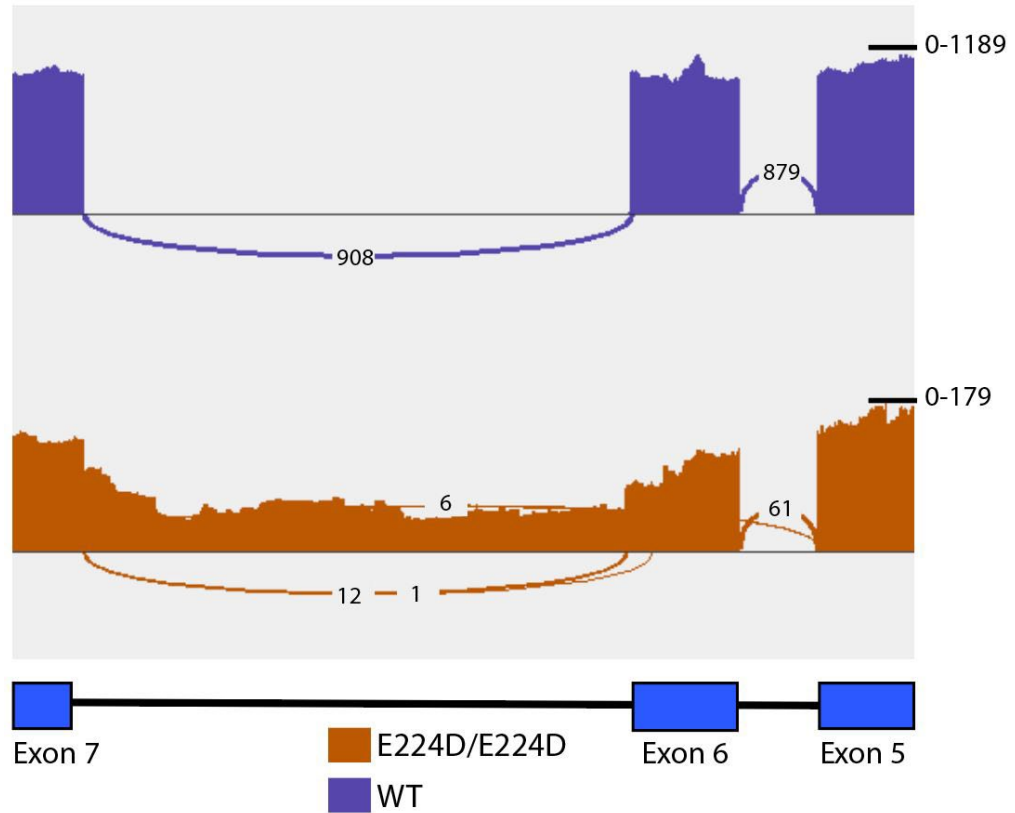


Figure 16: Representative sashimi plot visualization of read abundance from IGV. Linkages represent reads that span exon-exon junctions, i.e. splice events for human cell lines. Data and Analysis contributed IL.

In the Star aligned reads, however, Stringtie results suggest complete intron retention as the dominant transcript while a second novel transcript results from splicing at a cryptic splice site 5 base pairs downstream of the mutation. (Figure 17A-C).

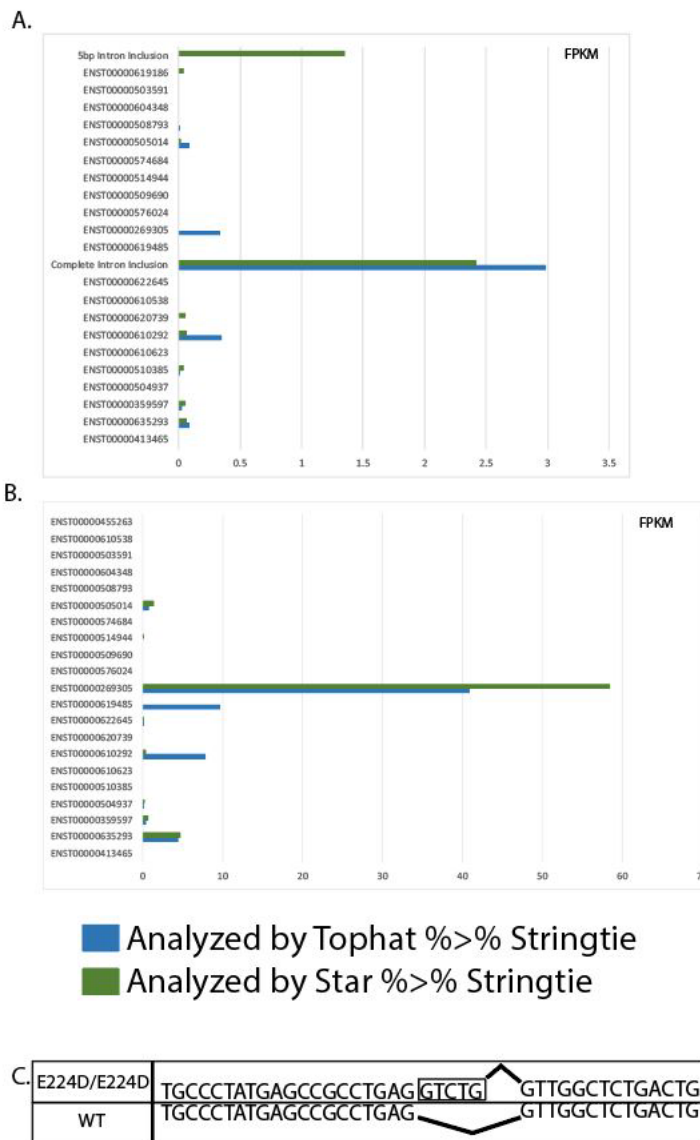


Figure 17: Novel Transcripts Identified in Human samples between methods.
A. Bar chart comparison of known transcripts and novel splice events in Stringtie results of bam files aligned with Tophat and Star from p53E224D/E224D NCI-H716 samples. Novel transcripts aggregated by the variation at the intron six 5' splice donor site. (Blue = Tophat aligned, Green = Star aligned) **B.** Bar chart comparison of known transcripts in Stringtie results from Tophat and Star aligned reads from p53WT HCT116 samples. (Blue = Tophat aligned, Green = Star aligned) **C.** Cryptic splice site in novel transcript, labeled 5bp intron inclusion in Figure 16A, called in Stringtie results of Star alignments includes five base pairs of the intron in human cancer harboring the p53E224D/E224D. Data and Analysis contributed IL.

Similar to the sequencing results in the MEFs, wild-type Tp53 transcripts are well conserved across sequence alignment methods. Together, these results show that the p53^{E221D} mutation in mice and the p53^{E224D} mutation in humans results in the loss of RNA expression through mis-splicing.

2.6 Inhibition of nonsense-mediated decay does not significantly alter transcript composition

One pharmacological strategy to rescue prematurely degraded p53 RNA is to inhibit nonsense-mediated decay and simultaneously encourage ribosomes to read through a premature stop codon. This could potentially result in the expression of a functional p53 protein that restores the ability of cells to activate p53-mediated tumor-suppressive pathways (Martin et al. 2014; M. Zhang et al. 2018; Wengrod et al. 2013; Floquet et al. 2011). To assess the p53^{E221D} mutant as a candidate for this treatment combination, cells were treated with G418 to promote ribosome read-through and the SMG7 inhibitor NMDI14 to inhibit non-sense mediated decay. We hypothesized that artificial increases in the transcript levels could increase correctly spliced transcripts to a physiologically relevant level. Using the combination described above, p53 RNA levels were significantly increased in both MEFs and a tumor derived sarcoma cell line from p53^{E221D/E221D} mice, however full length p53 RNA transcripts were not generated (Figure 18A-D).

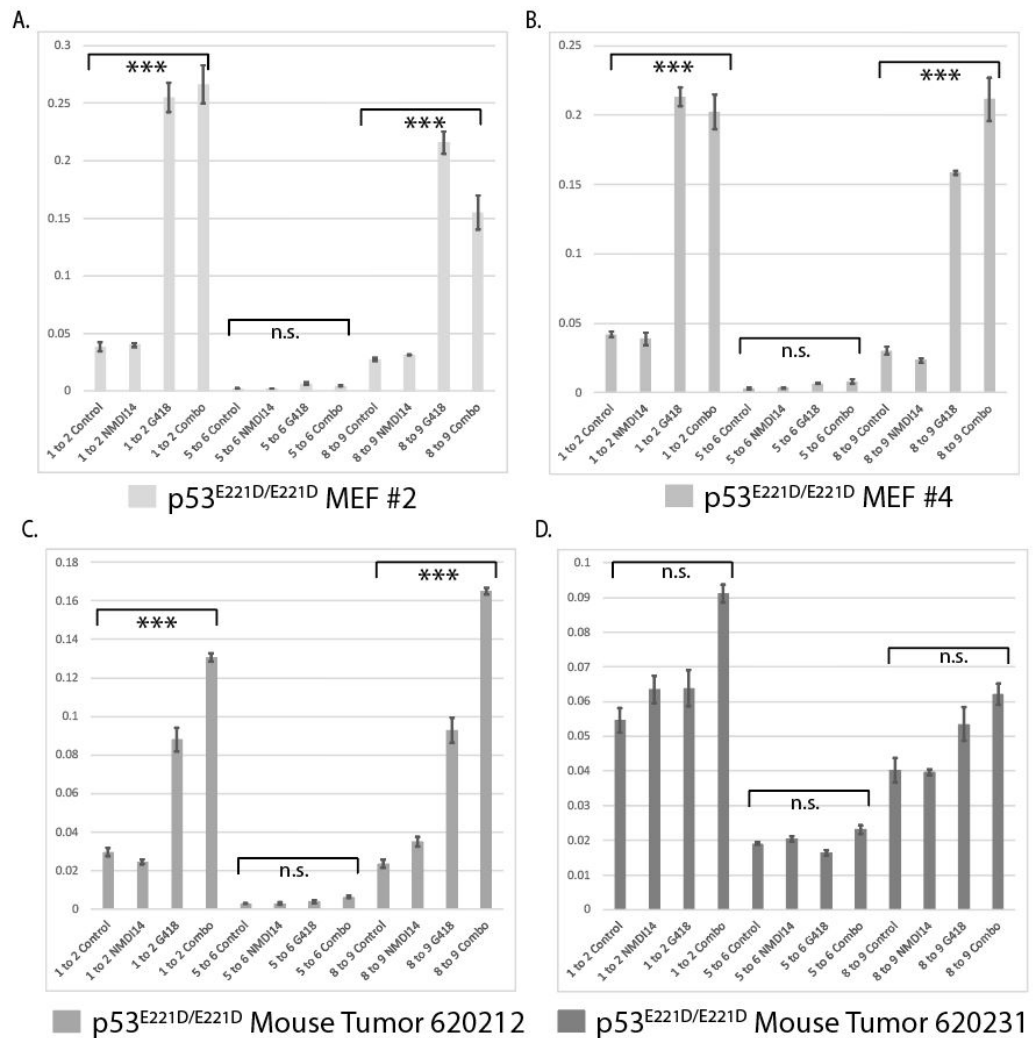


Figure 18: Nonsense-mediated Decay Inhibition in a Mouse Model. RNA was harvested from MEFs and mouse tumors homozygous for the P53E221D/E221D mutation. Probes spanning the p53 Exon1/Exon2, Exon 5/Exon6, and the Exon8/Exon9 junctions were used to assess any changes in the usage of these exons after treatment with G418, 400µg/mL, and the SMG7 inhibitor NMDI14, 5µM, for 48 hours. Changes in Transcript Abundance driven by NMD inhibition. A. MEF cell line #2, p53E221D/E221D Average of three technical replicates. B. MEF cell line #4, p53E221D/E221D Average of three technical replicates. C. Sarcoma cell line from p53E221D/E221D mouse 620212 Average of three technical replicates. D. Sarcoma cell line from p53E221D/E221D mouse 620231 Average of three technical replicates. One-Way ANOVA used to assess statistical significance. Data and Analysis contributed IL.

3. Maintenance of Pathway Homeostasis in p53^{G259A} Mouse Model

3.1 Transactivation Deficient Mutants Not Found in Cancer

To validate p53 mutants lacking intact transactivation in the yeast model system are not found in human cancer, we searched the IARC, R20 version, TCGA, and Genie databases (Table 1) (de Andrade et al. 2022). The yeast screening data identified 39 mutants not reported in human cancer and inactive for transactivation and a selection were chosen for validation (Kato et al. 2003).

Table 1: Abundance of Selected Variants in the Public Databases IARC, TCGA, and GENIE Samples containing protein variants accessed on public databases IARC, R20, TCGA, and AACR's GENIE as of June 2023. Data and Analysis contributed IL and EM.

	Occurrences in IARC	Occurrences in GENIE	Occurrences in TCGA
p.D7A	0	0	0
p.T118P	0	0	0
p.V122A	0	0	0
p.V122G	0	0	0
p.A138G	0	0	0
p.G262A	0	1	0
p.G293A	0	0	0
p.H297Q	0	1	0
p.P300T	0	0	0
p.K305Q	0	0	0
p.R306G	0	0	0
p.L308Q	0	0	0
p.G361W	0	0	0
p.S366T	0	0	0
p.Q375R	0	0	0

3.2 G262A cDNA has Impaired Transactivation In-Vitro

To evaluate the relationship of the yeast screening assay in a mammalian system, we introduced a tetracycline inducible cDNA of the p53 open reading frame in a p53 deleted cell line, H1299. After doxycycline induction of the cDNA, the relative levels of p53 and the critical targets of p53 for cell cycle arrest, CDKN1A, and the negative regulator of P53, MDM2 were assessed by western blot (Figure 19A). p53^{G259A} transactivation of these select targets is reduced relative to wild-type, supporting a decreased transactivation capacity of this mutant (Figure 19B).

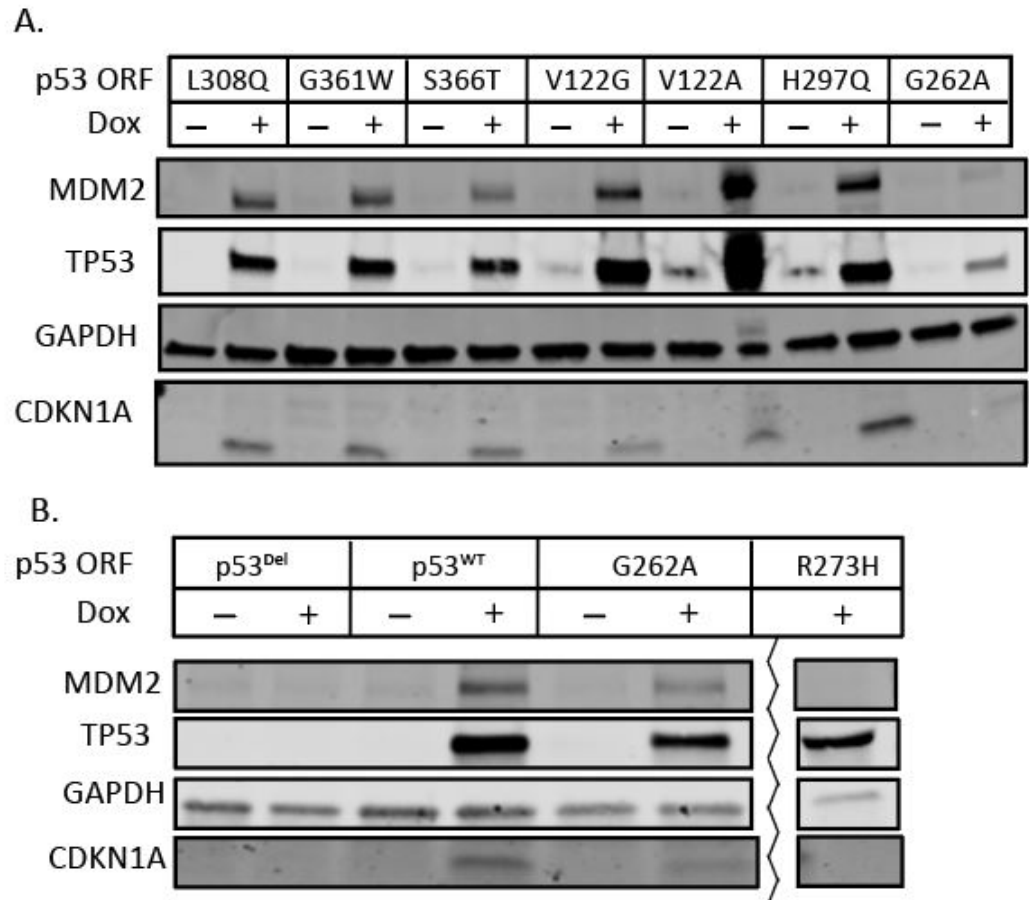


Figure 19: Expression of p53^{G262A} variant and targets CDKN1A and MDM2 in doxycycline expression model. Expression of doxycycline inducible p53 mutant cDNA constructs in p53 null H1299 lung cancer cells with expression validated by western blot using cell lysates probed for p53. Transactivation capacity assessed by MDM2 and CDKN1A expression using a GAPDH loading control 24 hours post doxycycline addition, 1 μ g/mL. A. Validation of a selection of non-transactivation mutants. B. Repeated transactivation of the p53^{G262A} variant in comparison to the p53 deleted (p53^{del}) and loss of function (p53^{R273H}) cell lines. Data and Analysis contributed by WF, NL, and IL.

The transactivation capacity of the p53^{G262A} variant was further evaluated using RT-qPCR after doxycycline induction and one hour after 10Gy irradiation to initiate the P53 signaling cascade. This mutant was deficient for transactivation of cell cycle arrest

targets, Cdkn1a and Gadd45a, targets mediating apoptosis, Puma, and a regulator of P53 stability, Mdm2 (Figure 20).

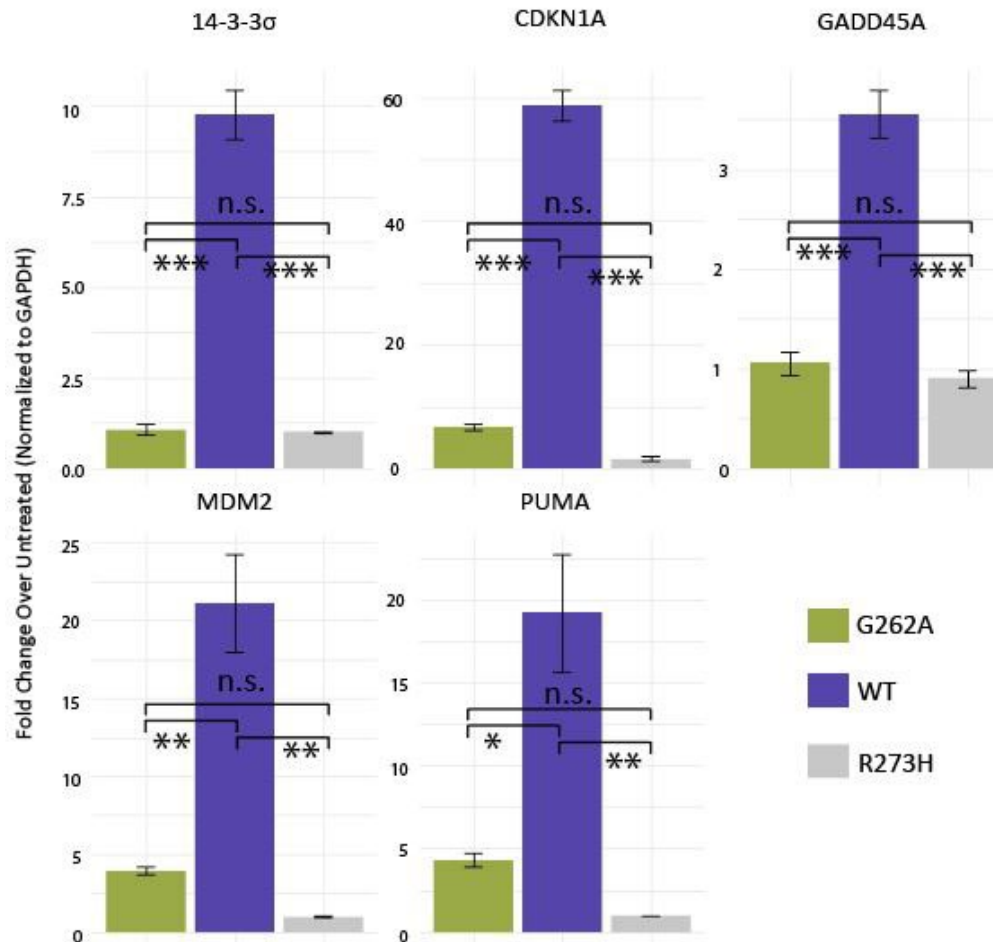


Figure 20: Transactivation of P53 targets in the doxycycline expression model. Transactivation of p53 targets by doxycycline (24 hours, 1 μ g/mL) induced p53^{G262A} mutant 1 hour after 10 Gy irradiation. Gene expression assessed by RT-qPCR. (p53^{G262A} – Green, WT – Purple, p53 Loss of Function (p53^{R273H}) – Gray) (***) = p<.001, ** = p<.01, * = p<.05.) Average of three technical replicates. ANOVA used to assess statistical significance. Data and Analysis contributed IL, WF, and NL.

The p53^{G262A} mutant signaling was indistinguishable from the negative control p53^{R273H} mutant cell line in this assay. To assess the ability of this mutant to facilitate p53

mediated growth arrest, cells were plated into doxycycline and control media for six days. The p53^{G262A} variant was unable to reduce the growth capacity after doxycycline induction when compared to the untreated control. (Figure 21)

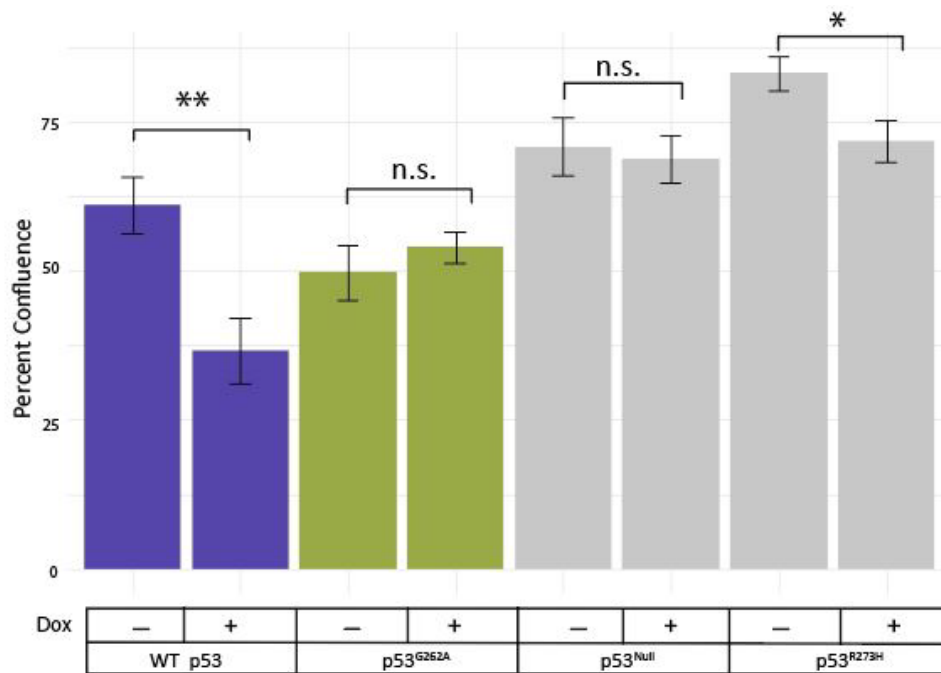


Figure 21: P53 mediated growth arrest in doxycycline and control media. Incucyte measurement of growth arrest capacity of p53 mutants. Percent confluence of samples represented as an average after six days of growth in a 96 well plate. Compared to non-doxycycline containing media over the same time course (p53^{G262A} – Green, WT – Purple, p53 Loss of Function (Null or R273H) – Gray) (= p<.01, * = p<.05) Average of ten technical replicates. ANOVA used to assess statistical significance. Data and Analysis contributed IL, WF, and NL.**

These data indicate this in-vitro expression of p53^{G262A} mutant does not significantly transactivate p53 target genes and is consequently deficient in promoting p53 mediated growth arrest. This deficiency supports the yeast screening assay conclusions and necessitates a further investigation of the in-vivo dynamics of p53^{G262A}

mediated tumor suppression given the absence of this mutation in a significant number of human tumors.

3.3 p53^{G259A} *genetically engineered mouse model is not tumor prone*

Using mechanisms of homologous repair and CRISPR/Cas9 gene editing, we engineered a novel mouse model with the p53^{G259A} (GCC>GTT, mouse equivalent of p53^{G262A}) mutation. The on-target editing of the p53 gene was validated using targeted sanger sequencing. Confirmatory of the relative frequency of the p53^{G262A} mutations in human cancer, the p53^{G259A} mouse model was not significantly predisposed to the development of tumors in comparison with heterozygous or wild-type littermate controls (Figure 22).

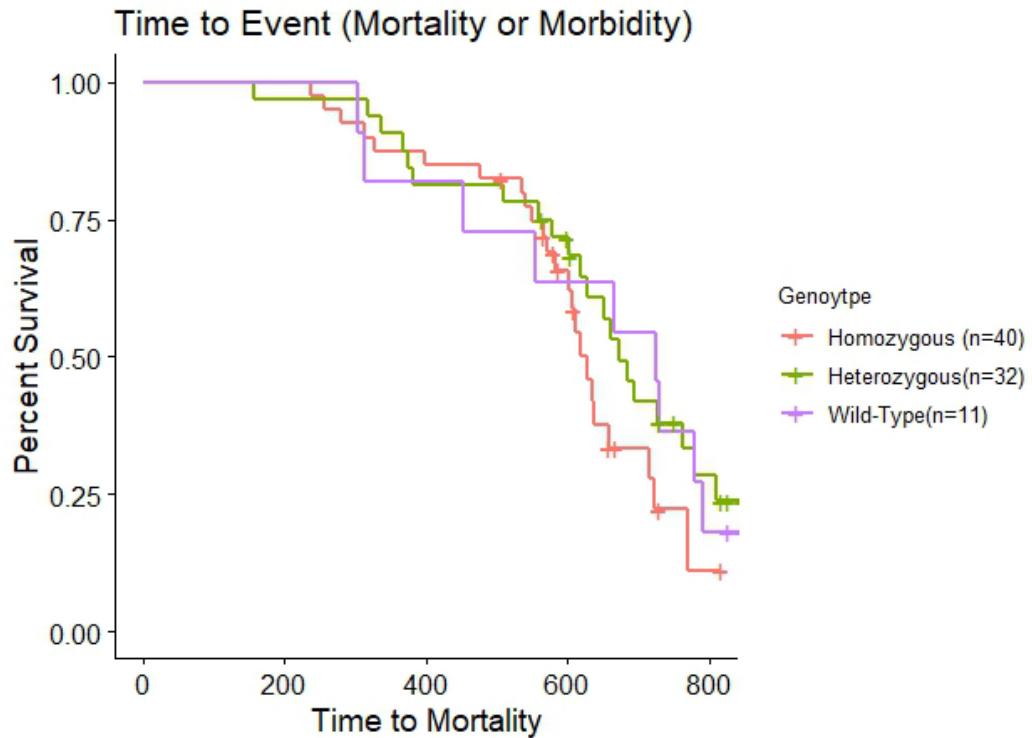


Figure 22: Time to morbidity or mortality for littermate controls of the $P53^{G259A}$ mouse. Survival Curves for $p53$ mutant mice. Mice monitored until ill health necessitated euthanasia or found dead. ($p53^{G259A/G259A}$ – Orange, $p53^{WT/WT}$ – Purple, $p53^{G259A/WT}$ – Green) Data and Analysis contributed IL, LL, NW, and YM.

Mouse embryonic fibroblasts derived from $p53^{G259A/G259A}$ mutant mice unexpectedly showed a significant increase in the stability of the $p53$ protein when compared to untreated wild-type controls. (Figure 23, A and C) Mutant $p53^{G259A/G259A}$ MEFs retained a stress induced stabilization response after doxorubicin treatment however the relative increase, increase over untreated, in protein abundance was reduced from wild type doxorubicin treated MEFs. (Figure 23, B and D)

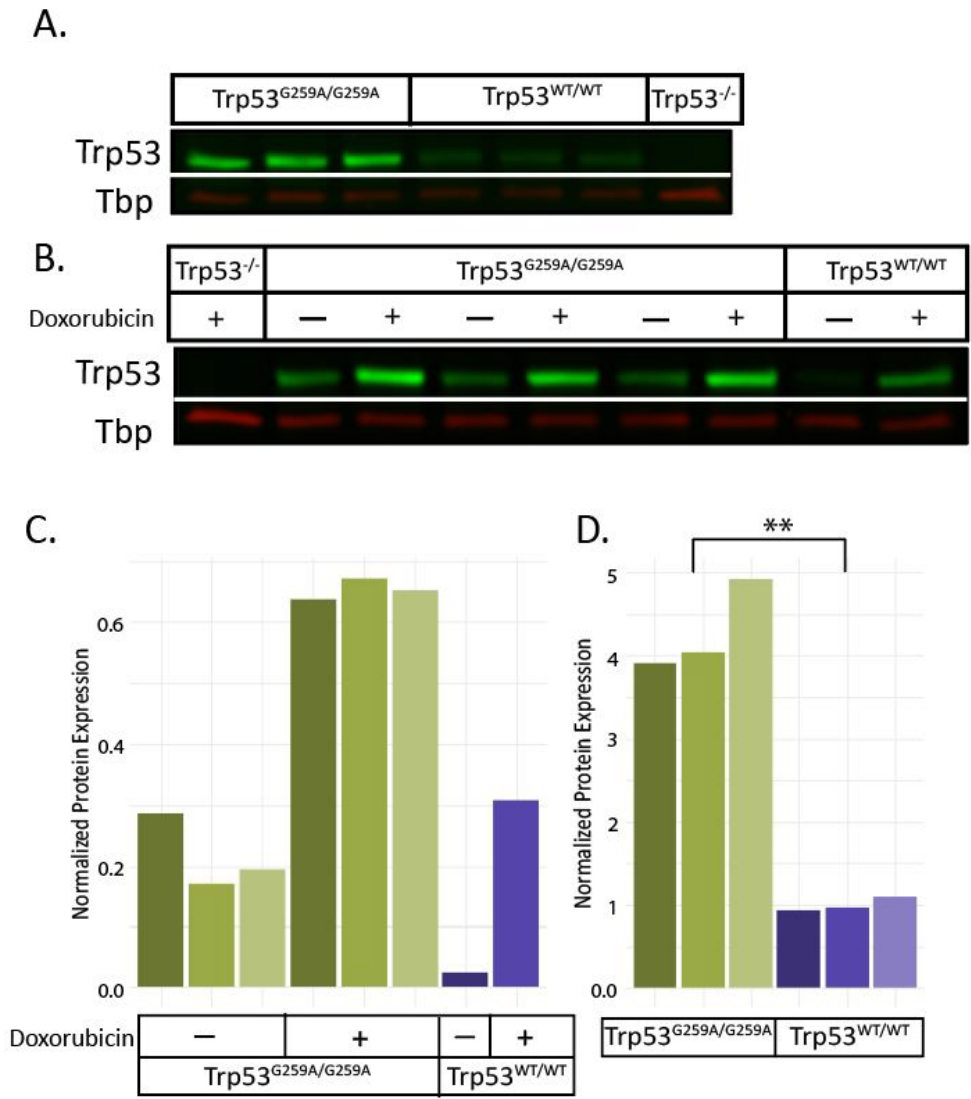


Figure 23: p53^{G259A} is Hyper-stabilized in MEFs. A. Untreated MEFs extracted from biological triplicates of p53^{G259A/G259A} and p53^{WT/WT} MEFs and protein visualized on a western blot with Tbp control B. MEFs exposed to .2µg/mL of doxorubicin for 8 hours and extracted for western blot visualization. C. Quantification of part B done using Tbp normalization of p53 fluorescence on Odyssey CLx Imager D. Quantification of Part A with Tbp normalization. Significance assessed using Student Paired T-Test (** = p<.01) Data and Analysis contributed by IL.

3.4 Target Gene Regulation is Promoter Specific

To elucidate the full scope of transactivation variability between the p53^{G259A/G259A} mutant and wild-type p53 samples we exposed MEFs to DNA damaging agent, doxorubicin, and interrogated the effects on transcription of established p53 target genes. Over stabilization of mutant p53 is observed in other loss of function mouse models that lack robust Mdm2 transactivation (G. Liu et al. 2004; D. Jiang et al. 2011). This is attributed to the loss of the negative feedback loops facilitated by p53 transactivation target Mdm2, even at basal p53 levels. The specific impact of a partial loss of function variant on transactivation of downstream targets of p53 is promoter specific. Data supports the p53 protein has selective binding affinity that is impacted both by the specific sequence of the response element, the distance from the transcription start site, and mutations that occur in the DNA binding domain (Fischer 2017; Verfaillie et al. 2016; Weinberg et al. 2005). These studies suggest Cell cycle arrest, DNA-Repair, and negative regulation have the lowest dissociation constant or strongest affinity (Weinberg et al. 2005). This is also consistent with mouse models of the DNA binding mutation R172P that exhibits loss a loss of apoptosis, but retains control of cell cycle arrest and DNA integrity related genes (G. Liu et al. 2004).

RNA sequencing of the p53^{G259A/G259A} MEFs revealed heterozygous and wild-type samples cluster closely with and without treatment in principal components analysis. Homozygous cluster together, but modestly apart from the wild-type and heterozygous

samples. Most significantly, there is a large differential in knockout samples from all other genotypes regardless of condition (Figure 24).

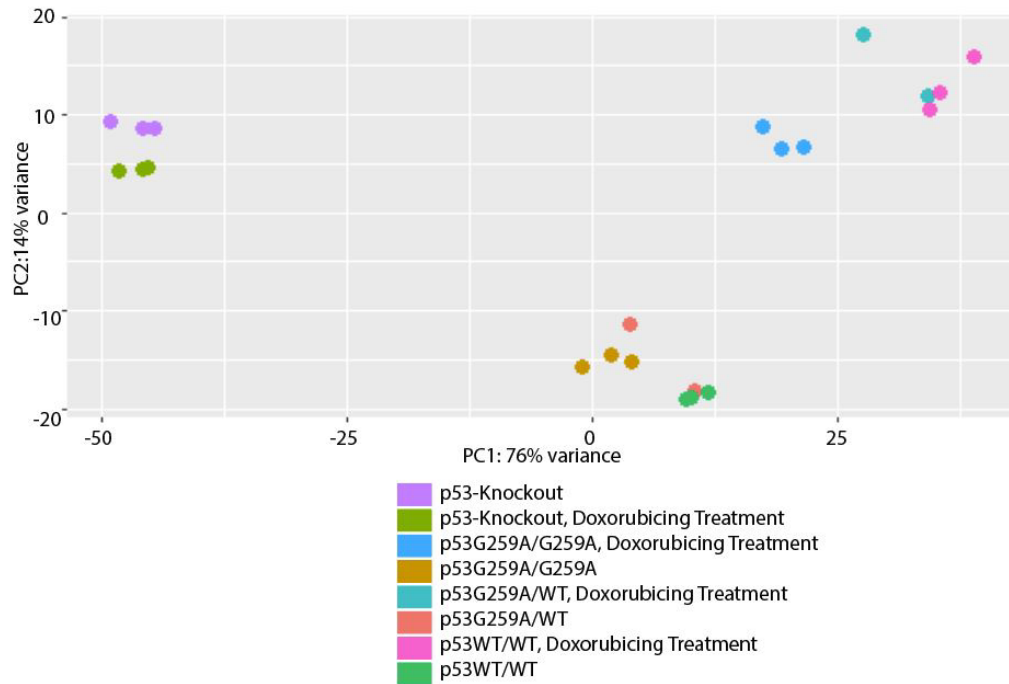


Figure 24: Principal Components Analysis for P53G259A/G259A, p53 G259A/WT,p53WT/WT. Samples treated with and without .2 $\mu\text{g}/\text{mL}$ of doxorubicin for 8 hours and extracted with Trizol. Paired-end reads aligned to mm39 with STAR. Normalized counts derived from DeSeq2. Data and Analysis contributed by IL.

Unexpectedly, while the transactivation capacity of the p53^{G262A} mutant expressed in H1299 cells was severely deficient for transactivation of canonical targets of p53. The impact on transactivation of the canonical targets in mouse embryonic fibroblasts was significantly, but subtly, different at the curated downstream targets. While treatment highlights a number of target gene expression differences that are significant, only one gene, Pmaip1 or Noxa, experienced a log2fold change greater than

one differential between treated wild-type and treated p53^{G259A/G259A}. The differential is especially limited in comparison to normalized counts for p53 knockout cells (Figure 24).

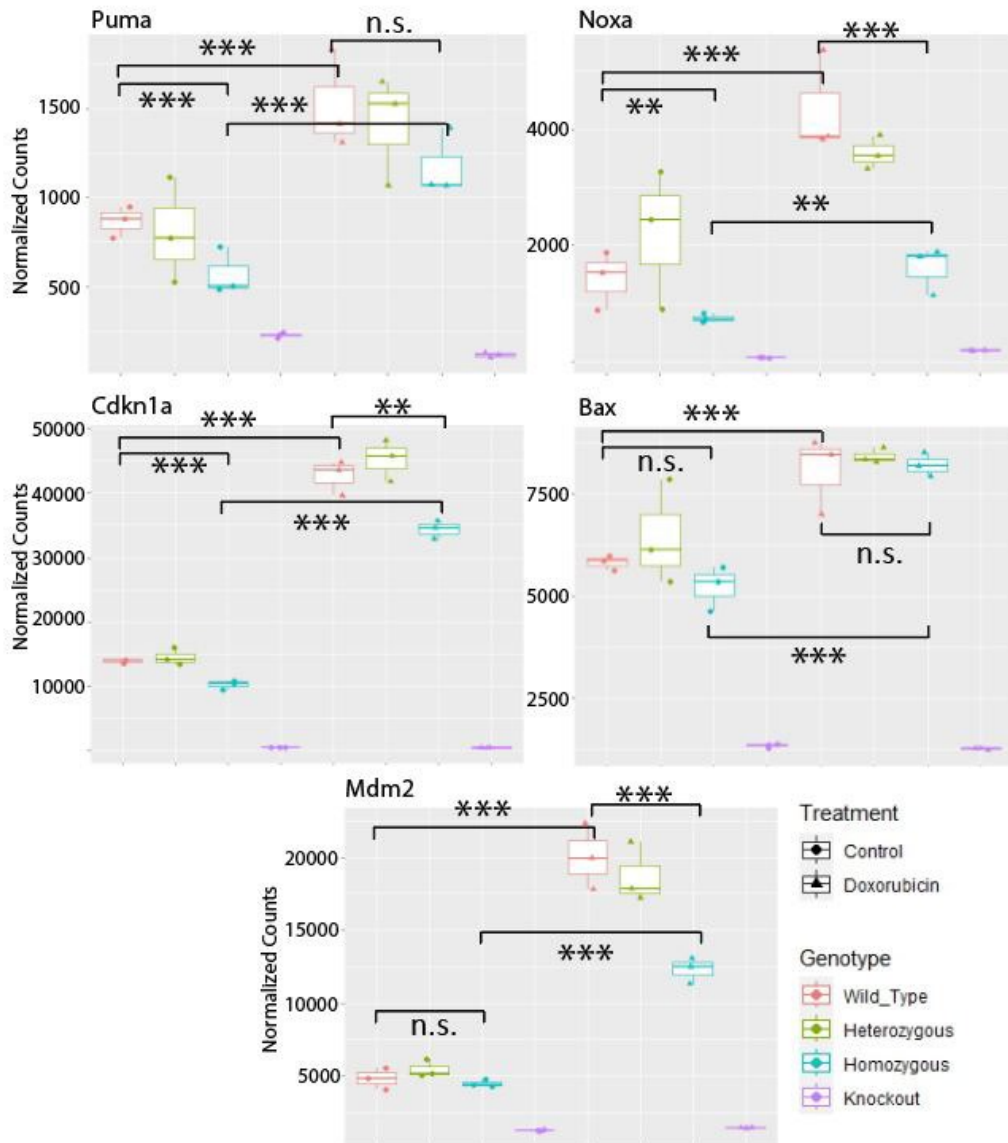


Figure 25: Transactivation of Canonical Targets of p53 is mixed and modest. Cells were treated with .2 $\mu\text{g}/\text{mL}$ of doxorubicin and RNA was extracted and sent for sequencing. (***) = $p < .001$, (**) = $p < .01$, (*) = $p < .05$) Boxplot visualizes the normalized counts of three biological replicates, each visualized within the graph as a point.

ANOVA with Tukey post-hoc analysis used to assess significance. Data and Analysis contributed by IL.

Filtering of all p53 pathway target genes, as defined by the molecular signatures database, differentially expressed in untreated samples results in 15 genes with a greater than a log2fold change of one between p53^{G259A} homozygous and wild-type MEF lines. (Liberzon et al. 2015) Filtering of transcripts of treated samples for p53 pathway genes reveals there are 26 genes, with a log2fold change greater than 1, that exhibit a significantly different expression after treatment between the p53^{G259A} MEFs and the wild type controls in the p53 pathway. Taken together, these results show an unexpected similarity between the P53^{G259A} MEFs and wild type MEFs insinuating that the overexpression of the p53 mutant overcomes a significant proportion of the signaling deficit conferred by the mutation to retain near normal function of the p53 pathway. Global analysis reveals a number of pathways that are significantly differentially regulated after doxorubicin treatments. Predominantly these pathways are related to mitosis and DNA repair functions, functions that are suppressed in cells that have wild-type transactivation capacity of p53.

3.5 Hyper-stabilization present in adult mouse tissues.

To assess if the p53^{G259A} mutant is hyper-stabilized in adult tissues after injury, p53^{G259A/G259A}, p53^{G259A/WT}, and p53^{WT/WT} mice were subjected to 14Gy abdominal irradiation and the intestines were harvested after 4 hours. P53 staining of the small intestine

indicates that adult tissues harboring the p53G259A mutation retain a hyper-stabilized protein (Figure 26).

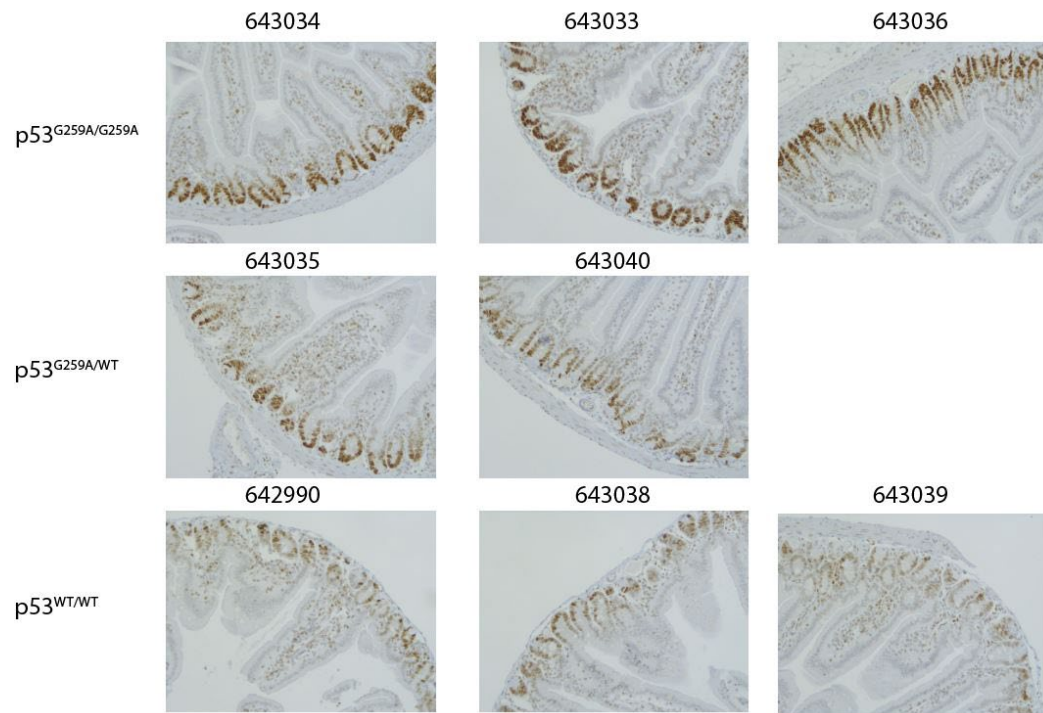


Figure 26: Immunohistochemistry staining of the small intestine with p53 antibody shows hyperstabilization of the p53G259A mutant. All eight mouse intestine samples extracted 4 hours after treatment with 14Gy to the abdomen. Intestine were extracted washes and fixed in formalin. Embedding, sectioning, and tissue staining done by Duke BioRepository & Precision Pathology Center. Data and Analysis contributed IL and BRPC.

4. Materials and Methods

4.1 Database Access

The TP53 Database (R20, July 2019): <https://tp53.isb-cgc.org> and GENIE (<https://genie.cbioportal.org/>) databases (AACR Project GENIE Consortium 2017). The results shown here are in whole or part based upon data generated by the TCGA Research Network: <https://www.cancer.gov/tcga>. These databases were searched and results curated for non-redundant studies of P53 mutations. All co-occurring non-synonymous mutations in TP53 found as of updates before June 2023 are counted.

4.2 Mouse Model Generation and Animal Use

P53^{E221D} and P53^{G259A} mice were generated by sgRNA, CAS9 (IDT cat# 1081059), and single stranded oligodeoxynucleotide, ssODN, electroporation of mouse embryo as previously described (45). Single guide RNA was synthesized via in-vitro transcription following the protocol described previously (Table 2) (45). Repair single stranded oligodeoxynucleotide repair oligo was purchased from Integrated DNA Technologies (Table 2). This process generated a cut site in the p53 locus at the desired mutation region and repair was guided using a homologous template for repair with the intended mutation (Figure). Chimeric mice were tested for successful targeting of the vector into the p53 locus and confirmed via PCR (Table 2). PCR products subcloned into pL253 via in-fusion cloning and sequenced for target validation.

Table 2: Repair and Guide Strategy for in-vivo CRISPR Editing of p53^{E221D} and p53^{G259A} mouse models

sgRNA-E221D	5' ggtaccttatgagccaccg 3'
sgRNA-G259A	5' ccactatccgggacaagaaa 3'
ssODN: 5'>3'	Ccccagcatcttatccgggtggaaggaaatttgatcccgagtatctggaagacaggcaga cttttcgccAcagcgtggtggtaccttatgagccaccgatgtctgtaattttgtttggtttgt gcttcttagagacagttgactccagcctagactgatgttgactttctagcaaccgttgctc accctcctga
E221D-253S:	5' ACCGCGGTGGCGGCCGCcttattcttgccttaggcctggc 3'
E221D-253AS	5'TAGAGGATCCACTAGTTGTAGTGGATGGTGGTATACTCA G 3'
G259A_g253S	5' caccatccactacaagtacatgtg 3'
G259A_g253AS	5' agataaagccactgaaaaagacct 3'

Mice were monitored weekly when possible (restricted during COVID19 lockdown procedures). Upon mortality or humane endpoint, tumors harvested and saved for histology and flash frozen if tumor size and condition allowed. All animal studies were performed in accordance with protocols approved by the Duke University Institutional Animal Care and Use Committee.

4.3 Genotyping

Mice were genotyped using tails collected from mouse pups. For early mouse generations tail genomic DNA was isolated and PCR was performed using primers listed in (Table 2). PCR products were visualized after electrophoresis in 1.5% agarose gels and product was further sent for sanger sequencing. Subsequent generations, genotyping was carried out by Gene Master LLC.

4.4 Immunohistochemistry

Tumors were fixed in formalin overnight, transferred to 70% ethanol, paraffin-embedded, and sectioned to 5 μ m thickness and stained with H&E. P53 antibody (NCL-L-p53-CM5p, Leica Biosystems) and Cleaved Caspase 3 antibody staining was performed by the Duke BioRepository & Precision Pathology Center. Tumor tissue sections were examined and interpreted by a sarcoma pathologist (D.M. Cardona).

4.5 Cell Lines and Reagents

HCT116 WT and HCT116 KO cells were a gift from Bert Vogelstein. NCI-716 and H1299 cells were obtained from ATCC and grown in DMEM media (Gibco), 10% FBS (Avantor), and 1X Antibiotic-Antimycotic (Gibco). H1299 cells expressing doxycycline inducible p53 WT and mutant constructs were generated by lentiviral infection in the Duke Functional Genomics Facility. Cells were cultured in 2 μ g/mL doxycycline for 24 hours before WB and qRTPCR experiments. Primary mouse embryonic fibroblasts were isolated from E12.5 to 13.5 mouse embryos and cultured in 37°C, 5% CO₂ conditions.

Cells were treated with G418 at 400 μ g/mL (Geneticin™ for 48 hours, ThermoFisher), NMDI14 was used at 5 μ M (Calbiochem) for 48 hours, and MG132 at 10 μ M (SelleckChem) for 6 hours. Cells were harvested following 1 hour following 10 Gy irradiation. Vehicles for G418 (water), NMDI14 (DMSO), and MG132 (DMSO) added to control conditions. Doxorubicin treatments were done at .2 μ g/mL and extracted after 8 hours of treatment as previously described (Brady et al. 2011).

4.6 Radiation Treatments

Cells were plated at least 24 hours prior to irradiation. The X-RAD 160 (Precision X-ray) cell irradiation system with 2 mm aluminum filter was used to deliver 10 Gy to cells. The Duke University Radiation Safety Division staff maintains and performs dosimetry for the irradiator.

4.7 Lentivirus Production

All lentiviruses for Tet-inducible expression of p53 mutants were prepared by cotransfecting HEK293T cells with the lentivector, psPAX2 (Addgene #12260) and pMD2.g (Addgen #122259) using TransIT-LT1 (Mirus) according to manufacturer instructions. Supernatant containing lentivirus was harvested at 48 hours, aliquoted and stored at -80 for transductions. H1299 cells were transduced with p53 mutant lentivirus as indicated at a multiplicity of infection (MOI) of <1 in the presence of 4 μ g/ml polybrene. Twenty-four hours post transduction, cells were selected with 2 μ g/ml puromycin for 72 hours and then expanded for use in experiments.

4.8 Immunoblots

Cells were lysed with RIPA (Thermo Scientific) supplemented with PhosStop Tablet (Roche), Aprotinin (Sigma Aldrich), Halt Protease and Phosphatase Inhibitor Cocktail and further lysis achieved by 10 second sonication (QSonica). 30µg of protein was subjected to SDS-PAGE and wet transferred to nitrocellulose membranes. Blots were blocked using Interceptor Blocking Buffer (LI-COR) for a minimum of one hour before probing with primary antibodies. Immunoblots were probed with antibodies against mouse p53 (Cell Signaling, 1C12), Gapdh (ProteinTech, 60004-1) human p53 (Invitrogen, PA5-27822), MDM2 (Santa Cruz Biotechnology, sc-965), and CDKN1A (Santa Cruz Biotechnology, sc-6246). Anti-rabbit IRDye® Secondary Antibodies, RDye® 680RD Donkey anti-Mouse IgG Secondary Antibody (LI-COR, 926-68072), or IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody (LI-COR, 926-32211) were used. The signal was detected using Odyssey imaging system (LI-COR Biosciences).

4.9 RNA Isolation, Preparation and Sequencing

RNA was isolated from cells using TriZol (Thermo Fisher) and purified using Zymo Direct-Zol RNA miniprep kit. DNase incubation was carried out for 15 minutes and each sample was quantified using a NanoDrop (Thermo Fisher). cDNA was generated from 1µg RNA using iScript cDNA Synthesis Kit (BioRad). Relative mRNA levels were determined using qPCR reactions performed on the QuantStudio™ 6 Flex Real-Time PCR System with TaqMan™ Fast Advanced Master Mix (Thermo Fisher

Scientific) and taqman probes designed to Cdkn1a (Mm04205640_g1, Hs00355782_m1), Mdm2 (Mm01233136_m1, Hs00540450_s1), p53 1-2 (Mm01731287_m1), p53 5-6 (Mm01731290_g1), p53 8-9 (Mm00441964_g1), Bbc3 (Mm00519268_m1), Gadd45a (Hs00169255_m1), 14-3-3 σ (Hs00968567_s1). Levels of mRNAs were normalized to the level of Gapdh mRNA. Relative mRNA levels were determined from experimentally determined standard curve (Applied Biosystems). Experiments were carried out in technical triplicates and analyzed using ANOVA with Tukey Post-Hoc Analysis.

Preparation of RNA library and 150bp paired end transcriptome sequencing was conducted by Novogene Co., LTD (Beijing, China). STAR and Tophat alignments were performed with default parameters to generate RNA-seq BAM files with the Genome Reference Consortium Mouse Build 39 genome and Human Build 38 (GRCm39 and GRCh38) (D. Kim et al. 2013; Dobin et al. 2013).p53^{E221D} Indexing with samtools and sashimi visualization was done in Integrative Genomics Viewer. Stringtie novel transcript discovery was carried out using default parameters and plotted using ballgown suite in R (D. Kim et al. 2013; Pertea et al. 2016)Differential expression was generated from STAR mapped reads to GRCm39. Counts were generated using feature counts and analyzed using DeSeq2 package. Subsequent comparisons and visualizations were done in rstudio using ggplot2.

4.10 Incucyte Growth Assay

100 cells were plated in a 96 well plate and placed in an incubator outfitted with the Sartorius Incucyte Live cell analyzer. Cells were plated in 2 μ g/mL doxycycline containing media or control media for six days and analyzed using companion Incucyte software. Experiment completed in ten technical replicates and analyzed by ANOVA.

5. Conclusion

5.1 Evaluating the impact of the mutant p53^{E224D} on tumor development

p53 DNA binding missense mutants continue to be an expanding area of research for designing new therapies targeted towards stabilization and reactivation of the proteins diverse critical pathways for tumor suppression (Reviewed in (Hoe, Verma, and Lane 2014; Joerger and Fersht 2016; Levine 2019; Bykov et al. 2018)). However, the impact of p53 mutations that lie outside of the most frequently occurring hotspot regions when expressed from the endogenous promoter in multiple cell lineages remains less well-investigated. In this study, we sought to understand the tumor suppressive dynamics of point mutations in the DNA binding domain of p53 that paradoxically retain transactivation of canonical targets, but are still found in human cancer. We narrowed a list of candidate mutations from a published screen in yeast where the mutants showed approximately wild-type transactivation for eight select promoters of p53 target genes and where the mutations are also found in human cancer. We used a human p53 null cancer cell line to validate transactivation of the cDNA of the p53^{E224D} in a mammalian system and selected the p53^{E224D} mutant for further study because it had been found in human cancers and in Li Fraumeni syndrome. Therefore, we generated a genetically engineered mouse model with the analogous p53^{E221D} mutation. Contrary to our expectation, however, the mouse model harboring the p53^{E221D} mutant did not express detectable p53 protein. This was further observed in a human p53^{E224D/E224D}

cancer cell line. Low p53 RNA levels in both the human and the mouse cell lines suggested that mis-splicing drives nonsense-mediated decay of the mutant p53 mRNA.

Our findings in the human samples are consistent with previous studies that did not observe detectable p53 protein in the NCI-H716 cell line as well as experiments that used available human data and in-vitro assays to investigate alternative splicing at this site (Carbonnier et al. 2020; Jung et al. 2015; Supek et al. 2014; J. Funk et al. 2023). These studies found partial intron retention at the 5' end of intron 6 was present after both the synonymous G>A mutation or the G>T/C mutations for p53^{E224D} as we observed (Carbonnier et al. 2020; Jung et al. 2015; Supek et al. 2014; J. Funk et al. 2023).

Our study contributes an expanded understanding of the intron retention in p53^{E224D} in human cells as well as developing a novel p53^{E221D} mouse model of mis-splicing of p53. Our sequencing suggests that there are multiple transcripts present following mis-splicing at this site. Consistent with previous studies on human data, the first 5 base pairs of intron 6 are retained in one transcript while larger portions of intron 6 are retained in other transcripts. To our knowledge, the p53^{E221D} variant that was generated in this study is the first mis-spliced variant of Trp53 to be explored in a mouse model. The variation in splicing that we observed in differential cryptic splice site usage and exon skipping provides a mouse model to investigate approaches to reactivate p53 by targeting nonsense-mediated decay. Moreover, this model functions as a p53

knockout model that can be used as a more biologically relevant model in studies investigating point mutant variability in the DNA binding domain.

5.2 Contextualizing the regulation of the p53^{G259A} mutant

The regulation of P53 is an area of interest both for the implications on predisposition or tumor development and treatment of cancer. Our study sought to understand the underlying consequences of loss of transactivation on tumor predisposition and development. Using doxycycline induction systems of p53 mutants in a human cancer cell line, we interrogated the limitations of a yeast model system for human mutations in the TP53 gene. Through this work we identified the p53G262A variant as deficient in transactivation of canonical TP53 targets. Consultation of databases of human data revealed this mutation is found in only a single reported human tumor. Using mouse embryonic fibroblasts we establish that the p53G259A mutant is highly stabilized under untreated and further stabilized under treated conditions. We also discovered that the mutant is abnormally stabilized in adult tissues. Upon interrogation of downstream targets of p53, the p53G259A mutant is modestly deficient for specific canonical targets in transactivation at baseline and after genotoxic stress. In support of a modest reduction of the function of the p53G259A mutant when overexpressed, the in-vivo mouse model is not significantly predisposed to cancer development in a long term study. These results suggest a system of regulation that maintains the p53 protein level relative to its transactivation characteristics in the

context of a partial loss of function mutation. Over stabilization and over expression in in-vitro models have described similar results that a loss of function variant can have wild-type function if overexpressed, but this is the first context that has potentially demonstrated the in-vivo regulation of this process.

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Biography

Ian Lock attended Macalester College in St. Paul Minnesota from 2013 to 2017 where he received a Bachelor's of Arts degree majoring in Biology and minoring in economics. During his undergraduate career Ian worked in the area of denovo and inherited p53 mutations in the lab of Logan Spector at the University of Minnesota. After Graduating from Macalester, Ian worked with Kevin B. Jones at the University of Utah on worked related to the BAF complex in Synovial sarcoma. He has attended Duke as a part of the Molecular Cancer Biology degree program in the Pharmacology and Cancer Biology department since 2019. Ian received the Chancellors fellowship at Duke University upon attending in 2019.

Ian contributed substantially to a number of manuscripts in the Jones and Kirsch Labs.

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Throughout his undergraduate, collegiate, and graduate career Ian has volunteered for the American Cancer Society and the American Cancer Society Cancer Action Network with a focus on promoting state and federal funding for research and prevention. This work significantly influenced his career interest and culminated in graduate school with an internship at Genentech in Regulatory Policy.