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Understanding The Roles Of Non-Homologous End Joining And P53 After Dna Damage

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A

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By

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DEDICATION

Trying to express in words my gratefulness to having such loving, understanding, and encouraging parents does not do justice to their contribution in my life, so I humbly dedicate this work to them—I would be nothing without you guys. To my sister, thank you for leading the way; you have always been my example of hard work, strength and excellence. To my best friend and twin brother, I am lucky to have such a role model; genius is often misused, but you are truly a genius. My favorite times have always been spent around the dinner table.

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ABSTRACT

The inability to maintain genomic stability and control proliferation are hallmarks of many cancers, which become exacerbated in the presence of unrepaired DNA damage. Such genotoxic stresses trigger the p53 tumor suppressor network to activate transient cell cycle arrest allowing for DNA repair; if the damage is excessive or irreparable, apoptosis or cellular senescence is triggered. One of the major DNA repair pathway that mends DNA double strand breaks is non-homologous end joining (NHEJ). Abrogating the NHEJ pathway leads to an accumulation of DNA damage in the lymphoid system that triggers p53-mediated apoptosis; complete deletion of p53 in this system leads to aggressive lymphomagenesis. Therefore, to study the effect of p53-dependent cell cycle arrest, we utilized a hypomorphic, separation-of-function mutant, $p53^{p/p}$, which completely abrogates apoptosis yet retains partial cell cycle arrest ability. We crossed DNA ligase IV deficiency, a downstream ligase crucial in mending breaks during NHEJ, into the $p53^{p/p}$ background ($Lig4^{-/-}p53^{p/p}$). The accumulation of DNA damage activated the p53/p21 axis to trigger cellular senescence in developing lymphoid cells, which absolutely suppressed tumorigenesis. Interestingly, these mice progressively succumb to severe diabetes. Mechanistic analysis revealed that spontaneous DNA damage accumulated in the pancreatic β-cells, a unique subset of endocrine cells solely responsible for insulin production to regulate glucose homeostasis. The genesis of adult β-cells predominantly occurs through self-replication, therefore modulating cellular proliferation is an essential component for renewal. The progressive accumulation of DNA damage, caused by $Lig4^{-/-}$, activated p53/p21-dependent cellular senescence in mutant pancreatic β-cells that lead to islet involution. Insulin levels subsequently

decreased, deregulating glucose homeostasis driving overt diabetes. Our $Lig4^{-/-}p53^{p/p}$ model aptly depicts the dichotomous role of cellular senescence—in the lymphoid system prevents tumorigenesis yet in the endocrine system leads to the decrease of insulin-producing cells causing diabetes.

To further delineate the function of NHEJ in pancreatic β-cells, we analyzed mice deficient in another component of the NHEJ pathway, Ku70. Although most notable for its role in DNA damage recognition and repair within the NHEJ pathway, Ku70 has NHEJ-independent functions in telomere maintenance, apoptosis, and transcriptional regulation/repression. To our surprise, $Ku70^{-/-}p53^{p/p}$ mutant mice displayed a stark increase in β-cell proliferation, resulting in islet expansion, heightened insulin levels and hypoglycemia. Augmented β-cell proliferation was accompanied with the stabilization of the canonical Wnt pathway, responsible for this phenotype. Interestingly, the progressive onset of cellular senescence prevented islet tumorigenesis. This study highlights Ku70 as an important modulator in not only maintaining genomic stability through NHEJ-dependent functions, but also reveals a novel NHEJ-independent function through regulation of pancreatic β-cell proliferation. Taken in aggregate, these studies underscore the importance for NHEJ to maintain genomic stability in β-cells as well as introduces a novel regulator for pancreatic β-cell proliferation.

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INTRODUCTION

1.1 The DNA Damage Response

Genomic instability occurs at all stages of cancers and is currently considered one of the hallmarks of cancers worth attempting to target therapeutically (Hanahan & Weinberg, 2011). Cells receive thousands of DNA lesions per day, which if not repaired, or repaired incorrectly, can lead to disastrous mutations that can compromise cell survival and proliferation rates (Jackson & Bartek, 2009). One of the most harmful types of genomic damage is DNA double strand breaks (DSBs) which can lead to chromosomal translocations and increase the propensity of cancers if left unrepaired. DNA double strand breaks can be caused by a variety of different genotoxic stresses such as gamma irradiation, oxidative stress, spontaneously, or through oncogenic activation. Briefly, DNA breaks are detected by DNA damage sensors, which in turn recruit the protein kinases Ataxia-telangiectasia mutated (ATM) or ATM and Rad3-related kinase (ATR). These kinases phosphorylate and activate certain DNA-damage mediators (examples include H2AX and 53bp1) as well as other downstream kinases together that can help further amplify this multifaceted signaling response as well as target downstream effectors like the tumor suppressor p53 ultimately stopping a damaged cell from cycling (Campisi, 2005; Shiloh, 2003). The purpose of halting the cell cycle, creating a checkpoint, is to allow time for DNA repair factors to fix the damage. These recruitment complexes bind to DNA and form foci, detectible by immunofluorescence, indicating an activated damage response (Lukas et al, 2003). If the damage is extensive or irreparable, the cell will initiate different cellular responses—apoptosis, autophagy, or cellular senescence, yet the factors involving the decision making to initiate such responses still remain unclear (d'Adda di Fagagna, 2008).

Pharmacological induction of DNA damage remains one of the pillar treatment for cancer therapy, unfortunately, cancer cells can adapt by increasing the activity of their DNA repair pathways (Bolderson et al, 2009). Collectively, this signaling cascade devised to prevent the cell from promoting proliferation of damaged or mutated DNA, is termed the DNA damage response (DDR). Additionally, the DDR directly halts transcription, activates autophagy for energy consumption, remodels chromatin, and repair DNA to combat these dangerous DNA lesions (Jackson & Bartek, 2009). The DNA damage response acts to repress tumorigenesis by activating downstream molecules which, when mutated or deleted directly cause human diseases and multiple cancer types (Meek, 2009). Due to the many different types of DNA damages, the cell has devised multiple DNA repair pathways—base excision repair corrects abnormal DNA bases; nucleotide excision repair fixes bulky adducts and ultraviolet-created lesions; mismatch repair senses and replaces DNA mismatches and insertions or deletions; and finally, both homologous recombination and non-homologous end joining function to repair DNA double strand breaks (Jackson & Bartek, 2009).

1.2 Non-homologous end joining

Hallmark to almost all human cancers is genomic instability, which is exacerbated in the presence of unrepaired DNA damage (Negrini et al, 2010). One of the major repair pathways for DNA double strand breaks is non-homologous end joining (NHEJ) functioning to fix open DNA ends (Mahaney et al, 2009). The main factors involved in the NHEJ pathway are the end recognition/ processing complex composed of a Ku70/80 heterodimer, DNA-PKcs, and repair factor Artemis, as well as the ligation

complex, functioning to join DSBs, consisting of DNA ligase IV (Lig4), XRCC4, and Cernunnos/XLF (Figure 1). In mice, inactivation of any non-homologous end joining factors besides Cernunnos/XLF result in severe combined immunodeficiency (SCID) due to failed lymphocyte development (Puebla-Osorio & Zhu, 2008). Interestingly, NHEJ deficient mice exhibit an accelerated aging phenotype and reports document the decline of NHEJ functionality with age suggesting a link between the inability to repair DNA upon age (Gorbunova et al, 2007). Notably, cells deficient in NHEJ factors lead to clastogen hypersensitivity (i.e., ionizing radiation), obviously due to the inability to repair breaks. Classically considered a caretaker tumorsuppressor, NHEJ has recently been proposed to modulate gatekeeper functions, like p53, to prevent the persistence of DNA DSBs that would otherwise lead to gross chromosomal rearrangements (Hasty, 2008). One example of this scenario is the deficiency of ligase IV or XRCC4, which present embryonic lethality due to excessive p53-mediated neuronal apoptosis, which is rescued by p53 deficiency (Frank et al, 1998; Frank et al, 2000; Gao et al, 2000). Although surviving prenatally, these double deficiencies lead to early, aggressive pro-B lymphomas due to chromosomal translocations predominantly involving chromosome 12 at the IgH locus and chromosome 15 near the proto-oncogene c-MYC (Gao et al, 1998; Zhu et al, 2002). It has been proposed, that targeting DNA repair pathways, such as NHEJ, in concert with DNA damaging agents will increase treatment efficacy and reduce off target effects because many tumors increase NHEJ factors. Cells deficient in NHEJ factors are sensitive to chemotherapeutic agents that induce DSBs, thus pharmacological inhibition of these molecules are showing promise and rapidly moving into the clinical setting (Bolderson et al, 2009; Martin et al, 2008; Zhao et al, 2006).

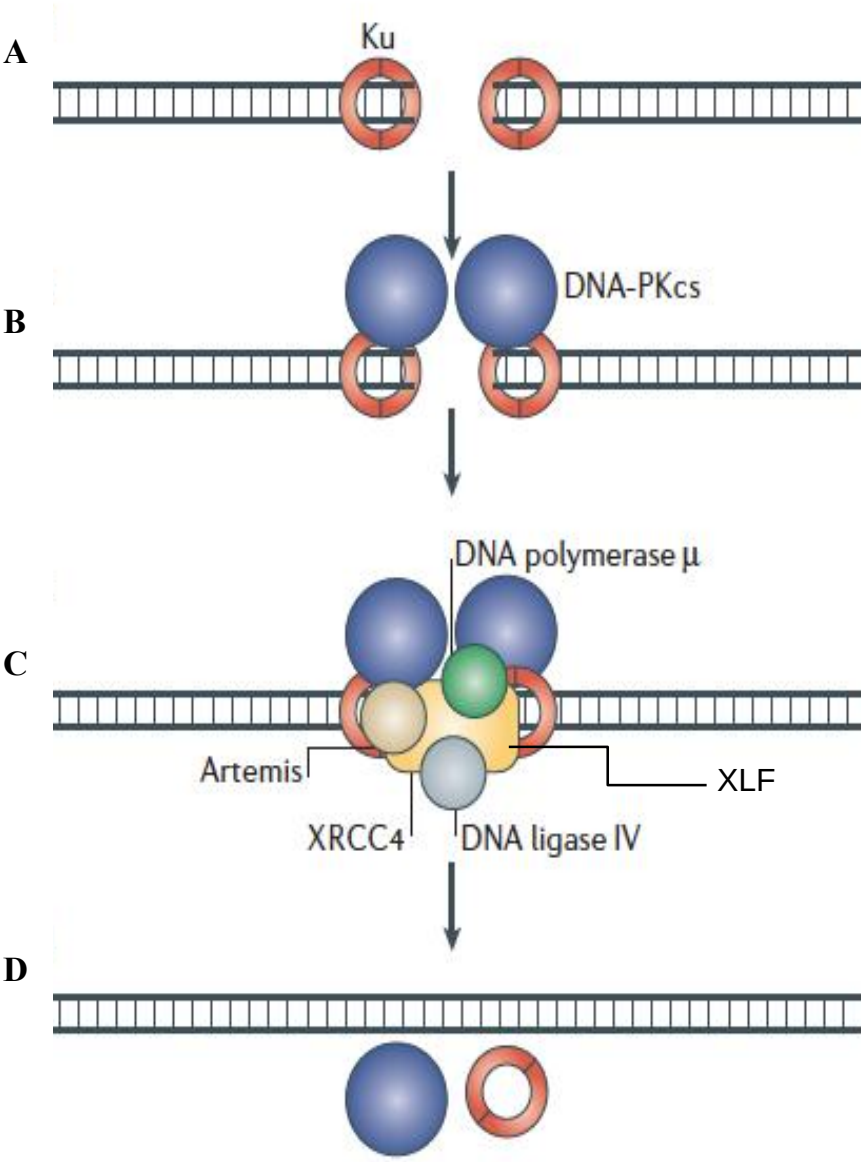

Figure 1. Non-homologous end joining steps.
(A) Ku70 and Ku80 heterodimer forms and binds DNA double-strand breaks. **(B)** DNA-PKcs is recruited and activated. **(C)** Repair factor Artemis is recruited and activated to blunt DNA ends. The ligase complex composed of XRCC4, XLF, and DNA Ligase IV is further recruited after DNA polymerase μ fills in gaps. **(D)** NHEJ factors dissociate from repaired DNA.
(Modified and reproduced from (Xu, 2006) with permission from Nature Reviews Immunology)

Classically, the factors involved in the non-homologous end joining pathway can be compiled in two groups: the DNA end recognition/processing complex consisting of the DNA-dependent kinase catalytic subunit (Ku70/80 heterodimer and DNA-PKcs) and the DNA repair factor Artemis along with the DNA ligation complex (DNA Ligase IV, XRCC4, Cernunnos/XLF)(Mahaney et al, 2009). Interestingly, viable NHEJ deficient mice exhibit an accelerated aging phenotype, when taken together with reports documenting the decline of NHEJ functionality with age, suggest a link between impaired DNA repair and age.(Gorbunova et al, 2007)

1.3 Tumor Suppressor p53

Termed the 'guardian of the genome', p53 is best characterized as a transcription factor that binds and transactivates genes to induce and balance a diverse network of cellular functions including autophagy, apoptosis and cellular senescence (Vousden & Prives, 2009). Such cellular mechanisms are crucial in protecting the cell against tumorigenesis in the presence of genotoxic stresses including DNA damage. Upon sensing DNA damage, the DDR will activate the stress responsive protein p53 to arrest cells from cycling, allowing time for DNA repair pathways to attempt the mend the lesions. Upon excessive and irreparable DNA damage, p53 can trigger a signaling cascade to induce apoptosis through transcription of different pro-apoptotic genes, including PUMA (p53-upregulated modulator of apoptosis) and Noxa (Meek, 2009; Vousden & Prives, 2009). Alternatively, p53 can induce irreversible cell-cycle arrest, termed cellular senescence, halting the proliferation of damaged cells despite stimulations with mitogenic factors (Campisi, 2005). Downstream target of p53 and

pivotal to the initiation of cell cycle arrest is the cyclin dependent kinase inhibitor p21 whose deletion leads to the bypass of senescence (Abbas & Dutta, 2009; Brown et al, 1997). By halting tumor proliferation, senescence acts in parallel to the apoptotic pathway as barriers to tumor development/progression (Figure 2).

Due to the high mutation rate of p53 in both familial and somatic cancers, p53 has become one of the most studied genes and tumor suppressors. Many cancers mutate or abolish p53 function, leaving cells vulnerable to replication after DNA damage which translates into a state of crisis (Lavin & Gueven, 2006). Complete abrogation of p53 gives rise to spontaneous tumors (Donehower et al, 1992), dubbing it as a tumor suppressor. The ability of p53 to execute and balance these cellular responses is crucial in preventing tumorigenesis; bypassing these functions with damaged or broken DNA leads to genomic instability and the beginning formation of a tumor. More specifically, deletion of p53 does not allow for the transactivation of putative downstream targets needed to initiate p53-mediated apoptosis or cell cycle arrest—two important arms in the network of p53 tumor suppression.

1.3.1 p53-mediated apoptosis

Apoptotic cell death is crucial for eliminating cells that have accumulated genomic instability and evaded cell cycle checkpoints allowing for improper and uncontrolled proliferation. Central to this mode of death is p53, as evidenced through the abrogation of radiation-induced apoptosis in thymocytes lacking endogenous p53 (Clarke et al, 1993; Lowe et al, 1993). Simplistically, apoptosis is triggered by two types of stimulation—extrinsic or intrinsic; the former being initiated through external cell surface receptors while the latter is activated by certain stresses which all converge

to the mitochondria. As mentioned above, p53 acts as a transcription factor, binding to the p53 consensus response elements in the promoters of proapoptotic target genes such as Bax, Puma, Noxa, and Bid (Fridman & Lowe, 2003). These Bcl-2 family members further initiate the release of cytochrome C from the mitochondria initiating an apoptotic-signaling cascade leading to cellular death.

1.3.2 p53 and the cell cycle

The eukaryotic cell cycle is a highly regulated succession of events responsible for cellular replication, important for the homeostasis of many organs. Simplistically, this cycle can be broken down into two stages: interphase, when the cell prepares for proper self duplication, and mitosis, when one cell physically divides into two genetically identical yet separate cells. Interphase can be further divided into the G_0 (Gap 0), G_1 (Gap 1), S (synthesis), and G_2 (Gap 2) phases—these phases are not bidirectional, once a cell advances phases, there is no reversal. The transitions from G_1 to S, S to G_2, and G_2 to M are tightly regulated and will not occur unless certain criteria have been reached—the barriers preventing progression into different stages is termed cell cycle checkpoints (Tavana & Zhu, 2011). The advancement through each phase is dependent on cyclins binding their respective cyclin-dependent kinases (CDKs), which in turn can phosphorylate downstream targets, initiating cell cycle progression. Alone, cyclins and CDKs are catalytically inactive. Further, cell cycle regulation comes in the form of cyclin/CDK complex inactivation and inhibition through cyclin-dependent kinase inhibitors (CDKIs) (For recent reviews, see refs. (Besson et al, 2008; Malumbres & Barbacid, 2009)). In general, overexpression of cyclins and CDKs or repression of

CDKIs should progress the cell cycle, while loss of cyclins and CDK expression or elevation of CDKI levels should prematurely prevent cycling.

Genotoxic stress compromising the stability and structure of DNA activates a damage response responsible for inhibiting cell proliferation. These genomic insults are dangerous if not repaired because they can lead to genomic instability, gene mutations and potentially tumorigenesis in the face of uncontrolled proliferation. The purpose of halting the cell cycle, creating a checkpoint, is to allow time for DNA repair factors to fix the damage. To prevent future complications, like chromosomal instability and tumor formation upon excessive or irreparable damage, the cell will initiate different terminal cellular responses—programmed cell death, termed apoptosis, or permanent cell cycle arrest, termed cellular senescence (Negrini et al, 2010). More specifically, cellular senescence is the process of irreversible cell-cycle arrest, halting the proliferation of damaged cells despite mitogenic signals. Telomere erosion, oncogenic stress, and DNA damage can all activate cellular senescence through different pathways, but similarly converge upon activation of the DNA damage response (DDR). This signal amplification cascade initiates the expression of key tumor suppressors which play an important role in the induction/maintenance of cellular senescence (Campisi, 2005). Although senescent cells undergo phenotypic changes in morphology, including hypertrophy and flattening/enlargement of cells, the most common and reliable method for detection and differentiation from a quiescence state (a temporary arrest of the cell cycle) is the expression of senescence-associated β-galactosidase (SA-βgal) (Dimri et al, 1995) (for a current protocol for detection of SA-βgal, see ref. (Debacq-Chainiaux et al, 2009). By permanently halting the proliferation of cells with unrepaired damage or

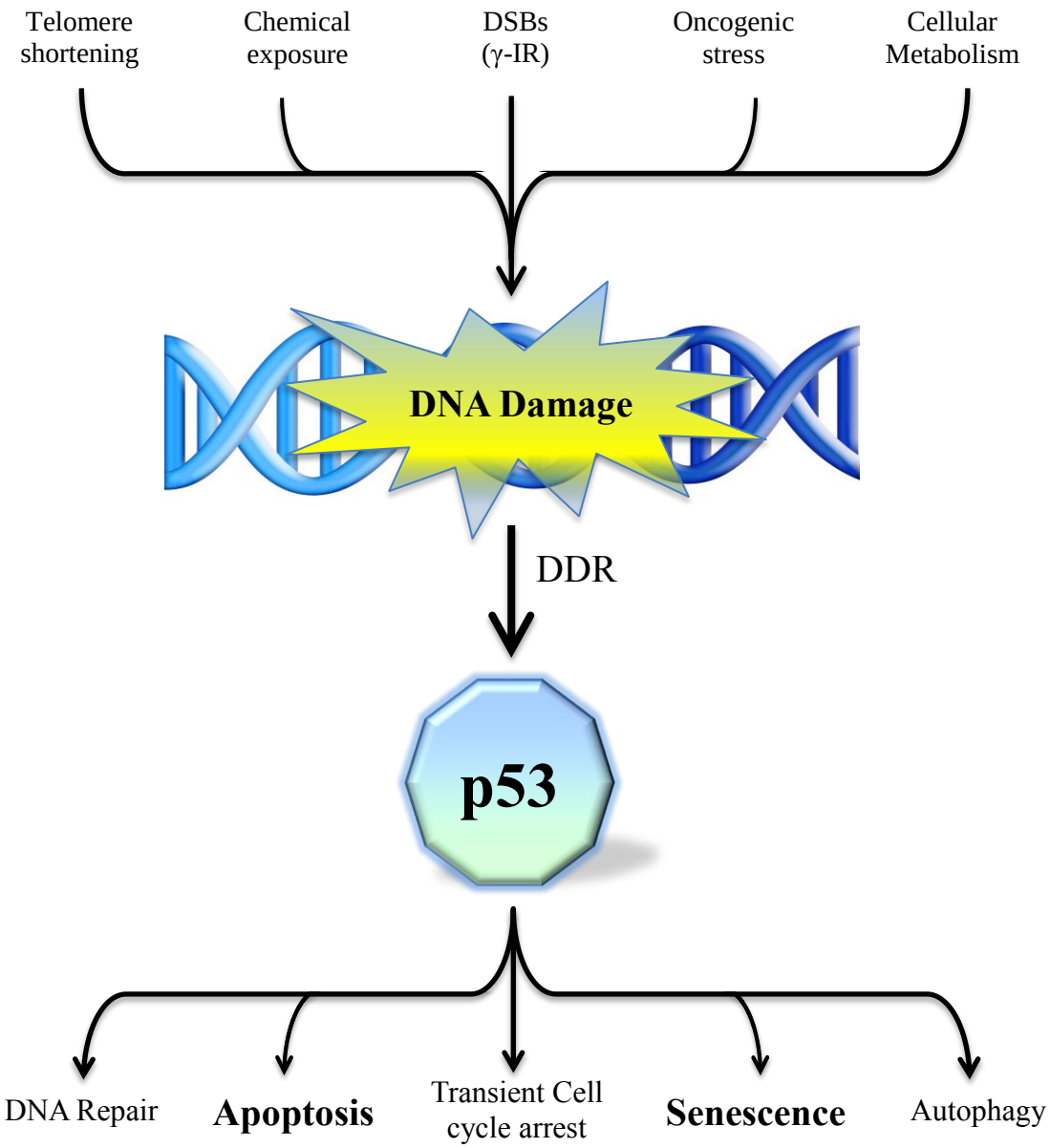

Figure 2. The p53 activation and signaling network.
Many different stress stimuli induce DNA damage and activate the DNA damage response (DDR), including telomere shortening, chemical exposure, DNA double strand breaks (DSBs), oncogenic stress and cellular metabolism. The DDR activates p53 to induce putative downstream targets, initiating DNA repair, transient cell cycle arrest, autophagy, apoptosis and cellular senescence. Bolded, are the responses known as barriers against tumorigenesis.

genomic instability, senescence acts in parallel to apoptosis by suppressing tumorigenesis (d'Adda di Fagagna, 2008). While this mechanism is beneficial for cancer prevention, increases in senescent cells can also be deleterious to tissue homeostasis and cell renewal, leading to premature aging and ageing-related disorders.

1.3.3 Cellular Senescence

The process of irreversible cell-cycle arrest, halting the proliferation of damaged cells despite stimulations with mitogenic factors is termed cellular senescence. Upon DNA damage, cells will initiate an amplification signal cascade, DNA damage response (DDR), activating key tumor suppressor pathways, either p53 or pRB, which play important roles in the induction of cellular senescence (Campisi, 2005). In fact, constitutive activation of the DNA damage response pathway, independent of actual DNA damage, is sufficient to induce cell cycle arrest and cellular senescence (Toledo et al, 2008). Although senescent cells permanently have arrested cycling, they remain metabolically active. Recently, much work has focused on identifying different secreted factors senescent cells expel which affect neighboring cells and the tumor microenvironment—senescence associated secretory phenotype (SASP). Of interest, IL6, IL8, and their respective receptors have been associated with the induction and maintenance of DNA damage/p53 mediated cellular senescence (Acosta et al, 2008; Kuilman et al, 2008; Rodier et al, 2009). Collectively, these articles reveal that the context-specific secretion of IL6 and IL8 lead to cellular arrest signals and created a positive feedback loop connecting the DDR to the propagation and maintenance of cellular senescence. These soluble factors have been proposed to play a significant role during tumor suppression by inducing senescence in neighboring cell types halting their

secretion patterns as well as initiating a pro-inflammatory response to initiate the removal of senescent cells (Kuilman & Peeper, 2009; Xue et al, 2007). In this context, senescence plays a parallel role to apoptosis in eliminating tumorigenic cells.

1.3.4 CDK inhibitor p21 and mutant $p53^{p/p}$

The putative downstream target of p53 playing a critical role in cell cycle checkpoint regulation and cell proliferation is p21, which inhibits the kinase activity of CDKs and prevents the cell from entering the S phase (Abbas & Dutta, 2009; Harper et al, 1993). As evidence that p21 plays a pivotal role in p53 mediated cellular senescence, its deletion led to the bypass of senescence and permitted replication after DNA damage(Brown et al, 1997). In fact, in the hypomorphic mutant $p53^{R172P}$ background, where p53 retains cell cycle checkpoint function but not apoptosis (hereafter referred to as $p53^{p/p}$) (Liu et al, 2004), p21 suppresses genomic instability; deletion of p21 led to loss of cell cycle control and accelerated tumorigenesis (Barboza et al, 2006). Additionally, p21 is elevated upon DNA damage due to nonhomologous end joining (NHEJ) deficiency, which led to tumor suppression in the $p53^{p/p}$ background (Van Nguyen et al, 2007). The loss of cell cycle checkpoint control is pivotal in allowing normal cells to transform into cancer cells. These studies highlight the importance of p21 and indicate a potential therapeutic target in cancer cells upon p53 loss.

1.4 Diabetes Overview

One of the most prevalent worldwide metabolic disorders is type 2 diabetes, often resulting from defects in maintaining sufficient numbers of insulin-secreting β-cells. This unique subset of endocrine cells controls glucose homeostasis in response to

the body's metabolic demands. Although proliferation occurs at an extremely slow rate, maintenance of β-cell number and islet mass is critical to sustain normoglycemia (Dor et al, 2004; Georgia & Bhushan, 2004). In fact, the production of these insulin-producing β-cells in adults does not occur through differentiation of their stem-cell progenitors but predominantly through self-duplication of mature cells (Dor et al, 2004; Georgia & Bhushan, 2004; Salpeter et al, 2010; Teta et al, 2007). However, the principle mechanism during embryogenesis remains as neogenesis, when precursor cells give rise to new β-cells. β-cell replication and subsequent islet mass expansion are regulated not only by extrinsic growth factors, but also in response to physiological demands (Ackermann & Gannon, 2007). Consequently, disrupting the self-replicating capability of β-cells leads to a decrease in islet mass, reducing the production of insulin. This in turn, deregulates glucose homeostasis, and ultimately, drives hyperglycemia leading to overt diabetes. Therefore analyzing the pathways that control islet mass and understanding the mechanisms that regulate β-cell regeneration will allow for novel therapeutic interventions for the prevention or treatment of diabetic patients (Tavana & Zhu, 2011). One strategy is to coax β-cells into continued proliferation without causing hyperplasia leading to insulinomas and hypoglycemia. To do this, we need a better understanding of the regulation during β-cell replication. It is currently well established that the regulation of β-cell proliferation and regeneration is controlled through cell cycle proteins and more recently, their upstream modulators. Additionally, recent reports have focused on DNA damage and the respective activated cellular response, which permanently halts the cell cycle of β-cells leading to diabetes. Ultimately, regardless of the stimuli used to foster β-cell regeneration, they must all converge on the

basic cell cycle replicative machinery. Many recent studies have focused on the cell cycle regulation of pancreatic β-cells, leading to disrupted glucose homeostasis and pathologically linking cellular senescence to Type 2 Diabetes Mellitus (Tavana & Zhu, 2011).

1.4.1 The role of Cyclins

Cyclins are named by their oscillating pattern in production and destruction during specific phases of the cell cycle; specifically, they regulate their partner cyclin dependent kinases, through protein-protein interactions, progressing the cell cycle (Malumbres & Barbacid, 2009). For example, the transition from G_1 to S phase in the murine islet is initiated by D-type cyclins, which bind and activate CDK4. Upon activation, retinoblastoma is hyperphosphorylated, relieving the inhibition of transcription factor E2F, whose targets are involved in DNA replication during S phase. Transcription of cyclin E is also initiated, allowing for binding to CDK2, pushing the cell into S phase (Malumbres & Barbacid, 2009).

In the murine islet, loss of specific cyclins or CDKs leads to cell cycle arrest, which under some stress- and cell-specific conditions can lead to cellular senescence (Campaner et al, 2010; Georgia & Bhushan, 2004; Kushner et al, 2005; Rane et al, 1999; Tsutsui et al, 1999) while aberrant expression of these proteins leads to increased proliferation and hyperplasia (Cozar-Castellano et al, 2004; He et al, 2009; Rane et al, 1999; Zhang et al, 2005) (Table 1). There are three D-type cyclins (D1, D2, D3), which bind to and activate CDK4 in the murine islet, promoting cell cycle progression. Cyclin D1 and D2 are expressed in islets, the latter in higher abundance (Cozar-Castellano et al, 2006c; Fatrai et al, 2006; Georgia & Bhushan, 2004; Kushner et al, 2005). As β-cell

replication decreases with age, so do the expression levels of cyclin D1 and D2 (Hinault et al, 2008). Cyclin D2, although not required for neonatal development, is critical for regulating β-cell growth and replication (Georgia & Bhushan, 2004; Kushner et al, 2005). Genetic ablation of cyclin D2 led to stark decreases in islet mass, attenuated β-cell proliferation rates, and glucose intolerance, eventually resulting in diabetes (independent of apoptosis) (Georgia & Bhushan, 2004; Kushner et al, 2005). Cyclin D1 partially compensates in the absence of cyclin D2, since the double mutant exacerbates this phenotype with uncontrollable diabetes driving death at an early age (Kushner et al, 2005). These studies indicate that D-type cyclins can control β-cell replication and mass, ultimately regulating glucose homeostasis (Tavana & Zhu, 2011). Mice expressing a single point mutation at site 280 of cyclin D2 (Cyclin $D2^{T280A}$), causing its constitutive activation and stabilization, present with a massive expansion of β-cells and improved glucose tolerance upon age. Interestingly, this was not caused by an increase in β-cell proliferation, rather β-cell turnover decreased at an early age, contributing to the accumulation of β-cells and increased islet mass over time (He et al, 2009). Many human endocrine tumors have increased levels of cyclin D1 (Chung et al, 2000). Researchers overexpressed cyclin D1 in the murine islet *in vivo* and found a drastic increase in islet mass without changes in apoptosis, suggesting β-cell replication as the culprit of the islet hyperplasia (Zhang et al, 2005). Excitingly, the β-cell specific overexpression of cyclin D1 did not lead to hypoglycemia; in fact glucose and insulin levels remained similar to wild-type (Zhang et al, 2005) suggesting a potential role for targeting cyclin D1 to increase β-cell proliferation. Collectively, these studies provide direct evidence that deletion of cyclin D molecules from the murine islet halts the cell

cycle, decreasing β-cell proliferation rates, while overexpressing cyclin Ds in β-cells lead to increased proliferation from overactive cell cycle progression (Tavana & Zhu, 2011).

1.4.2 The role of Cyclin dependent kinases

Both CDK2 and CDK4 are expressed in murine islets, with no significant change of CDK4 upon age, while CDK6 has not been detected (Cozar-Castellano et al, 2006a; Hinault et al, 2008; Martin et al, 2003). Presumably due to the lack of compensation from CDK6 expression in the islets (Cozar-Castellano et al, 2006c; Martin et al, 2003), the phenotype of CDK4 knockout mice presents a smaller body size with the testis, ovary, and pancreas showing replication defects (Rane et al, 1999; Tsutsui et al, 1999). Interestingly, mutant mice developed overt diabetes due to stark decreases in insulin production. This was attributed to the drastic decrease in islet mass, independent of apoptosis and autoimmunity, propelling the consequent dysregulation of glucose homeostasis (Mettus & Rane, 2003; Rane et al, 1999; Tsutsui et al, 1999). Collectively, these findings introduce CDK4 as the mainstay for postnatal β-cell proliferation (Tavana & Zhu, 2011). Seeing that CDK4 deletion leads to diminished β-cell proliferation, it would not be unexpected for CDK4 overexpression to increase β-cell replication. Indeed, Andrew Stewart's group showed that adenoviral delivery of CDK4 into human and rat β-cells did increase β-cell proliferation rates, which was elevated further with concomitant cyclin D1 infection (Cozar-Castellano et al, 2004). Interestingly, an Arg to Cys point mutation at amino acid 24 in the CDK4 protein ($CDK4^{R24C}$), initially identified from melanoma patients, renders CDK4 insensitive to p16 inhibition, becoming constitutively active (Wolfel et al, 1995; Zuo et al, 1996). A knock-in mouse was created and found to

have profound β-cell proliferation rates and islet hyperplasia (Rane et al, 1999). Confirming these results, a $CDK4^{R24C}$ β-cell specific knock-in was created (under the control of the insulin promoter) with similar results as above, although insulinomas were not observed. Taken together, these works indicate that regulation of CDK4 is central to the maintenance of β-cell replication (Tavana & Zhu, 2011).

1.4.3 The role of Cyclin Dependent Kinase Inhibitors

The inhibitors of active cyclin/CDK complexes make up two families: the INK4/ARF family composed of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, and $p19^{INK4d}$ and the CIP/KIP family composed of $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$. INK4 family members bind to both CDK4 and CDK6 blocking consequent activation by inhibiting D-type cyclin binding, while CIP family members bind both cyclins and CDKs (cyclin-D, -E, and -A and their respective CDKs) (Besson et al, 2008).

Persistent elevation of CDKI expression in murine islets either by negative regulation of upstream epigenetic modulators or ectopic expression leads to cell cycle arrest and in some conditions cellular senescence (Chen et al, 2009; Dhawan et al, 2009; Uchida et al, 2005; Yang et al, 2009; Zhong et al, 2007), while combinatorial deletion of CDKIs (due to overlapping compensation) or deletion of positive epigenetic regulators modulating CDKI loci lead to islet expansion and hyperplasia (Bertolino et al, 2003; Crabtree et al, 2003; Franklin et al, 2000; Georgia & Bhushan, 2006; Karnik et al, 2007; Karnik et al, 2005; Rachdi et al, 2006; Uchida et al, 2005) (Table 1).

1.4.3a p27 and epigenetic regulation

β-cells adapt to metabolic needs by rapidly increasing β-cell proliferation, leading to an expansion and rise in numbers (Georgia & Bhushan, 2004). Analysis of

CDKI's in terminally differentiated β-cells revealed marked increases in p27 levels. Deletion of p27 leads to a burst of β-cell proliferation and islet mass at early postnatal time periods, implicating its role in maintaining a transient cell cycle arrest, or state of quiescence in these cells (Georgia & Bhushan, 2006). p27 deficient mice showed improved glucose tolerance and increased insulin secretion (Rachdi et al, 2006). Interestingly, adult mice did not show islet hyperplasia, indicating some overlapping parallel CDKI functions to regulate β-cell proliferation (Georgia & Bhushan, 2006). Knocking out p27 in previously established diabetic models ($Irs2^{-/-}$ and $Lepr^{-/-}$) rescued the β-cell proliferation defects, pointing to therapeutic targeting of p27 (Uchida et al, 2005). Conversely, specifically overexpressing p27 in the murine islet (Rip-CDKn1b) resulted in a severe decrease in β-cell proliferation and smaller islet mass driving hyperglycemia and overt diabetes (Uchida et al, 2005). Collectively, these experiments, along with additional postmortem human diabetic sample analysis (which also showed increased nuclear p27 (Uchida et al, 2005)), emphasizes the notion that p27 is a key regulator during conditions that require increases in β-cell mass. Therefore negative targeting of this CDKI may be an important therapeutic strategy for facilitating β-cell regeneration (Tavana & Zhu, 2011).

Since p27 prevents the activation of cyclin E/CDK2 and/or cyclin D/CDK4 complex formation, regulation of its turnover becomes critical to allow transitioning between cell cycle phases. The E3 ubiquitin ligase SKP2 (S-phase kinase-associated protein 2), which is part of the SKP-CUL1-F-box complex, ubiquitinates p27 for proteasome-mediated degradation; therefore SKP2-deficiency results in the accumulation of p27 levels. Similar to the RIP-p27 transgenic mouse (Uchida et al,

2005), this increased level of p27 leads to a decrease in β-cell mass and diabetes, which is rescued upon p27 deletion (Zhong et al, 2007).

Positive regulation by transcriptional promotion of p27 and other CDKI's (such as p18) is a function of the tumor suppressor multiple endocrine neoplasia type 1 (Men1) encoded by the gene *menin*. Men1 regulates gene transcription by functioning in a histone methyltransferase protein complex, promoting the transcription of different genes (similar to Bmi-1 and Ezh2 discussed below) (Karnik et al, 2005). Patients with mutations in *MENIN* develop multiple tumor types including pancreatic islet tumors. Since the $Men1^{-/-}$ mutation presents embryonic lethality, heterozygous (Crabtree et al, 2001) and conditional RIP-Men1 mice were created (Bertolino et al, 2003; Crabtree et al, 2003). Both models were characterized with severe pancreatic islet hyperplasia due to a massive increase in β-cell proliferation. Complete β-cell analysis of $Men1^{+/-}$ mice by Seung Kim's group linked the islet hyperplasia phenotype to concomitant downregulation of p27 and p18 (Karnik et al, 2007; Karnik et al, 2005). Conversely, *Menin* overexpression led to impaired cellular proliferation and growth (Karnik et al, 2005), which could be explained by the activation of chromosome modifications inducing premature cellular senescence (as seen with Bmi-1 and Ezh2 mutants) (Shin et al, 2010). Collectively, these studies all highlight the importance of p27 as a modulator of β-cell mass and islet homeostasis and present new therapeutic targets not only to repress p27 and other CDKI's in concert, but also to modulate their upstream regulators to increase islet mass without inducing insulinomas (Tavana & Zhu, 2011).

Table 1: β-cell replication models *in vivo*.

Category	Mouse Model	Whole body/ conditional	β-cell proliferation	Islet mass	β-cell apoptosis	Glucose tolerance	Diabetic	Other notes	Refs
Cyclin	Cyclin $D2^{-/-}$	Whole body	↓	↓	NC	Intolerant	Yes	Onset: 3m and 9-12m	(Georgia & Bhushan, 2004; Kushner et al, 2005)
	Cyclin $D1^{+/-}$ $D2^{-/-}$	Whole body	↓	↓	NC	Intolerant	Yes	die at 4m	(Kushner et al, 2005)
	Cyclin $D2^{-/-}$ $D3^{+/-}$	Whole body	ND (↓)	ND (↓)	ND	ND (intolerant)	Yes	similar to Cyclin $D2^{-/-}$	(Kushner et al, 2005)
	Cyclin $D2^{T280A}$	β-cells	NC	↑	↓	Improved	No	Islet tumors	(He et al, 2009)
	Cyclin D1 Tg	β-cells	↑	↑	NC	Normal	No	No islet tumors	(Zhang et al, 2005)
CDK	$CDK4^{-/-}$	Whole body	↓	↓	NC	Intolerant	Yes		(Rane et al, 1999; Tsutsui et al, 1999)
	$CDK4^{R24C}$	Whole body	↑	↑	NC	ND	No	Islet tumors	(Rane et al, 1999)
CDKi	$p27^{-/-}$	Whole body	↑	↑	ND	Improved	No	Rescues diabetes by increasing proliferation	(Georgia & Bhushan, 2006; Rachdi et al, 2006; Uchida et al, 2005)
	p27 Tg	β-cells	↓	↓	NC	Intolerant	Yes		(Georgia & Bhushan, 2006; Rachdi et al, 2006; Uchida et al, 2005)
	$p16^{-/-}$	Whole body	↑	ND	ND	Normal	No	No islet hyperplasia	(Krishnamurthy et al, 2006)
	p16 Tg	Whole body	↓	ND	ND	ND	No	similar to aged mice	(Krishnamurthy et al, 2006)
	$p21^{-/-}$	Whole body	NC	NC	NC	Intolerant	No	Lower insulin levels	(Cozar-Castellano et al, 2006b)
	p21 Tg	Islet specific	↓	↓	↑	ND in older mice	Yes		(Yang et al, 2009)

Category	Mouse Model	Whole body/ conditional	β-cell proliferation	Islet mass	β-cell apoptosis	Glucose tolerance	Diabetic	Other notes	Refs
CDKi modulators	SKP2$^{-/-}$	Whole body	↓	↓	ND	Intolerant	Yes	↑p27	(Zhong et al, 2007)
	Men1$^{-/-}$	β-cells	↑	↑	NC	ND	No	↓ p27 and p18; islet hyperplasia	(Bertolino et al, 2003; Crabtree et al, 2003)
	Bmi-1$^{-/-}$	Whole body	↓	↓	ND	Intolerant	Yes	↑p16/p19; islet senescence	(Dhawan et al, 2009)
	Ezh2$^{-/-}$	β-cells	↓	↓	ND	Intolerant	Yes	↑p16/p19	(Chen et al, 2009)
	p38$^{Af/+}$	Whole body	↑	ND	ND	Normal	No	↓p16/p15/p19/p21	(Wong et al, 2009)
	Wip1$^{-/-}$	Whole body	↓	ND	ND	Intolerant	Yes	↑p16/p19	(Wong et al, 2009)
	Ubc-Wip1	Whole body	↑	ND	ND	Normal	No	↓p16/p19	(Wong et al, 2009)
Damage	Terc$^{-/-}$ G4	Whole body	↓	↓	ND	Intolerant	Pre-		(Kuhlow et al, 2010; Minamino et al, 2009)
	Lig4$^{-/-}$p53$^{P/P}$	Whole body	↓	↓	NC	Intolerant	Yes	↑p53/p21; islet senescence	(Tavana et al, 2010c)
	PTTG$^{-/-}$	Whole body	↓	↓	Initial ↑	Intolerant	Yes	↑p21; islet senescence	(Chesnokova et al, 2009; Wang et al, 2003)
	ATM$^{-/-}$	Whole body	ND	ND	ND	Intolerant	Yes	Increased ATM improves glucose tolerance	(Miles et al, 2007; Schneider et al, 2006)
	p53$^{-/-}$	Whole body	ND	ND	ND	Normal	No	No change with HFD	(Razani et al, 2010)
	p53^{S18A}	Whole body	ND	ND	↓	Intolerant	Pre-	Increased ROS; intolerance improved with NAC	(Armata et al, 2010)
	Δ40p53	Whole body	↓	↓	NC	Intolerant	Yes	↑p53 stabilization/p21	(Hinault et al, 2011)

Key for Table 1: ↓, decrease; ↑, increase; ND, not described; NC, no change; Pre-, glucose intolerance but not diabetic; red font: hyperplastic models; black font: hypoplastic models; T, threonine; A, alanine; Tg, transgenic; CDK, cyclin dependent kinase; Sei, sertad; R, arginine; C, cysteine; ER, oestrogen receptor; TAM, tamoxifen; pIns, insulin promoter; Rip, rat insulin promoter; CDKI, cyclin dependent kinase inhibitor; SKP2, S-phase kinase-associated protein 2; Men1, multiple endocrine neoplasia type 1; Ezh-2, enhancer of zeste homology 2; AF, alanine phenylalanine substitutions; Ubc, ubiquitin C; Terc, RNA component of telomerase; G4, generation 4; Lig4, DNA ligase IV; P, proline; S, Serine; PTTG, pituitary tumor transforming gene; ATM, ataxia telangiectasia mutated; HFD, high fat diet; ROS, reactive oxygen species; NAC, N-Acetyl cysteine.

1.4.3b p21

While p21 levels are detected in pancreatic β-cells (Cozar-Castellano et al, 2006b; Cozar-Castellano et al, 2006c), islets can function normally when p21 is deleted. This suggests that other cell cycle regulators may compensate to function when p21 is deleted (Cozar-Castellano et al, 2006b). Upon potent mitogen overexpression using hepatocyte growth factor (HGF) or placental lacogene (PL), Andrew Stewart's group found a specific upregulation of p21 *in vitro.*(Cozar-Castellano et al, 2006c) However, *in vivo,* when analyzing p21 deficient mice crossed with RIP-PL mice, there were no observable differences compared to $p21^{+/+}$ RIP-PL mice,(Cozar-Castellano et al, 2006b) suggesting the dispensability of p21 during mitogenic stimulation, exposing an overall complexity for the role of p21 in β-cells. Prior to the finding that oxidative damage increases p21 levels (Minamino et al, 2009), researchers showed that increases in p21 are associated with the suppression of insulin expression and diabetes in Zucker diabetic fatty (ZDF) rats (Kaneto et al, 1999). Similar phenomena have been observed, that upon genomic damage, p21 is specifically elevated in diabetic mouse models(Chesnokova et al, 2009; Minamino et al, 2009; Sone & Kagawa, 2005; Tavana et al, 2010c); when deleting p21 in the $PTTG^{-/-}$ model (discussed below), hyperglycemia was delayed as diabetes was partially rescued probably due to decreasing β-cell senescence (Chesnokova et al, 2009).

Recently, a double transgenic mouse was devised to inducibly overexpress p21 in the islets (using the RIP-Cre mouse). Upon long-term p21 elevation, mice became overtly diabetic, surprisingly due to increased levels of apoptosis (Yang et al, 2009). Although p21 is predominantly thought to arrest the cell cycle, recent findings suggest

some interplay during apoptosis induction (Besson et al, 2008). Upon 70% partial pancreatectomy, a technique used to force β-cell replication, these p21 transgenic mice showed a dramatic decrease in proliferation compared to the wild-type controls(Yang et al, 2009), suggesting permanent cell cycle arrest or cellular senescence. Therefore, p21 is important in a stress-dependent context in the β-cells (Tavana & Zhu, 2011).

1.4.3c p16, epigenetic regulation

After examining a plethora of organs for cell cycle marker expression change in young and aging individuals, p16 emerged as a potential marker for aging, and a controller for balancing tissue renewal. Of particular interest is the discovery that aging murine islets accumulate p16 (Krishnamurthy et al, 2004). Norman Sharpless's group confirmed this notion through mouse models of p16 deletion and overexpression (Krishnamurthy et al, 2006). They found that whole genome deletion of p16 rescued the age-dependent decrease in islet proliferation rates, restoring the levels to that of younger individuals. To further confirm that p16 drives the age-dependent decline of β-cells, they created a mouse with an extra copy of p16. Indeed, the proliferation rate of these younger mutant mice correlated to that of aged mice, yet these mice did not develop diabetes nor demonstrate any metabolic defects. Additionally, p16 deletion did not develop islet neoplasia (Krishnamurthy et al, 2006), indicating possible compensation by other CDKI's and/or highlighting the importance of other islet regulators (Tavana & Zhu, 2011).

One mechanism that controls p16 regulation occurs at the transcriptional level through epigenetic changes on the INK/ARF locus, which is initiated through polycomb group proteins. One member of this protein family is Bmi-1, a negative regulator of p16

and p19. Intuitively, Anil Bhushan's group linked these findings to islet homeostasis. By knocking out Bmi-1, which was found highly expressed early in life, decreasing with age (and negatively correlating with p16), these mice express high p16 and p19 levels correlating with decreased islet proliferation (Dhawan et al, 2009). Unlike the mouse overexpressing p16 (Krishnamurthy et al, 2006), Bmi-1 deficiency reduced β-cell mass, driving glucose intolerance due to premature senescence, which drastically limited the expansion of β-cells (Dhawan et al, 2009). Another member of the polycomb family is the histone methyltransferase enhancer of zeste homology 2, Ezh2. The conditional $Ezh2^{-/-}$ mouse is basically a phenocopy of Bmi-$1^{-/-}$, developing glucose intolerance and diabetes due to decreased β-cell mass and proliferation over time (Chen et al, 2009). Interestingly, *Chen et al* showed increased H3K4 tri-methylation (correlating with active transcription) on aged β-cell DNA specifically at the p16/p19 locus and not on other CDKI loci (p15, p21, or p27) (Chen et al, 2009). This explains why other CDKI's are not highly expressed with age. $Ezh2^{-/-}p16^{-/-}$ double deficient mice rescued the diabetic phenotype, further highlighting the importance/dominance of p16 in β-cell biology (Chen et al, 2009).

Upstream of the aforementioned epigenetic modulators that regulate the p16/p19 locus lies the mitogen-activated protein kinase p38, which can negatively regulate Bmi1 through phosphorylation. Activation of p38 in certain cell lines leads to the upregulation of p16 and p19 (Bulavin et al, 2004). After investigation of aged islets, *Wong et al.* found increased levels of p38 as compared to young islets (Wong et al, 2009). Upon introduction of a dominant negative form of p38 abrogating its total function (since $p38^{-/-}$ presents embryonic lethality), all CDKI expression decreased with age in this mutant,

emphasizing the importance of p38 in CDKI regulation (Wong et al, 2009). The Wip1 phosphatase, considered an oncoprotein, is a negative regulator of p38 and decreases with age in islets (correlating with the concomitant increase of p38 and p16/p19). Deleting Wip1 results in p38 overexpression; with age, these mice become diabetic (which was reversed when crossed with the p38 mutant) due to a drastic decrease in β-cell proliferation and mass. Conversely, mild overexpression of Wip1 leads to a decrease in p16/p19 expression by decreasing p38 activity (Wong et al, 2009). These studies collectively emphasize the importance of p16 and upstream modulators in regulating the age-dependent decline of β-cells (Tavana & Zhu, 2011). Furthermore, analysis of islets from wild-type 12 month old mice fed a high fat diet showed glucose intolerance, decreased β-cell mass and plasma insulin levels, due to a lowered β-cell proliferation and islet senescence, which was associated with an increase in activated p38 (Sone & Kagawa, 2005), corroborating the previously mentioned findings. Taken together with the above studies, one can infer that the stress from the high fat diet and age activated p38, which in turn upregulates p16/p19, blocks β-cell proliferation and activates cellular senescence leading to a decrease in β-cell mass, resulting in glucose intolerance and onset of diabetes (Tavana & Zhu, 2011).

1.4.3d Aging

Recent back-to-back studies evaluated the ability of pancreatic β-cell proliferation with age (Rankin & Kushner, 2009; Tschen et al, 2009). Both groups coaxed β-cells to regenerate using multiple methods including a high fat diet, Exendin-4, STZ treatment, and partial pancreatectomy. They found that β-cells from older mice do not have the same capability to regenerate after damage as younger mice. Furthermore,

Bhushan's group correlated this trend with p16 regulation: older mice had higher levels of p16 and lower levels of Bmi-1 (Tschen et al, 2009). The plasticity of younger β-cells allows for adaptation to changes in metabolic demand as well as different types of damages, while aged islets are restricted in replication after such damages. This is in part due to the changes with age in the regulation of cell cycle components, negative regulators, as well as their upstream modulators. Indeed, Kushner's group took a genomic approach to study the global changes between young and old islets (Rankin & Kushner, 2010). They show that aged islets have different gene expression patterns in transcription factor expression, cell cycle regulation, and chromatin remodeling among other factors than young islets (Rankin & Kushner, 2010). These works collectively place aging as an additional limiting factor for adaptive β-cell replication. Interestingly, cellular senescence has been linked to some age-related diseases as well as accelerating the onset of other diseases, therefore it may be likely that β-cell senescence, a process involving chromatin reorganization, may play a larger role in β-cell homeostasis and age-related regeneration of β-cells than originally thought (Tavana & Zhu, 2011).

1.4.4 The role of DNA damage and downstream activated targets

Genotoxic stresses activate the DNA damage response cascade to upregulate the tumor suppressor p53 and putative downstream targets, halting the cell cycle, which allows for transient DNA repair or induction of apoptosis/cellular senescence when the damage is too extensive (Campisi, 2005; d'Adda di Fagagna, 2008). More recently, these stresses have been shown to cause progressive β-cell dysfunction and islet specific cellular senescence driving the onset of diabetes (Chesnokova et al, 2009; Dhawan et al, 2009; Halvorsen et al, 2000; Krishnamurthy et al, 2006; Kuhlow et al, 2010; Minamino

et al, 2009; Sone & Kagawa, 2005; Tavana et al, 2010c; Testa & Ceriello, 2007) (Table 1).

1.4.4a Telomeres

Briefly, regions of tandem repeating DNA segments that protect the ends of chromosomes from being recognized as DNA double strand breaks (DSBs) are termed telomeres. During each round of the cell cycle, telomeres lose these repeats, shortening in length. Upon reaching a certain threshold, short unprotected telomeres are perceived as DSBs (leading to genomic instability) and induce the DDR, which activates a p53-mediated checkpoint that can either induce apoptosis or p21-dependent cellular senescence (d'Adda di Fagagna, 2008; Herbig & Sedivy, 2006). To accelerate telomere shortening and mimic premature aging, either the reverse-transcriptase portion of telomerase (*Tert*) or the RNA component of telomerase (*Terc)*, the enzyme responsible for adding repetitive regions to the ends of chromosomes, can be deleted (Blasco et al, 1997; Lee et al, 1998). Successive back crossing of these mutant mice leads to shortened telomeres above the threshold for detection, activating the DNA damage cascade and replicative senescence. Upon analysis, generation 4 (G4) Terc deficient mice present impaired glucose clearing and impaired insulin secretion after glucose stimulation and defective gluconeogenesis (Kuhlow et al, 2010; Minamino et al, 2009; Sahin et al, 2011), which was partially alleviated by deleting p53 (Sahin et al, 2011). Furthermore, islet mass was significantly decreased in G4 mice from a lack in β-cell proliferation all due to shortened telomeres in the islets (Kuhlow et al, 2010). Increasing stress through a high fat diet, Minamino *et al.* elegantly showed G4 $Terc^{-/-}$ mice develop more prominent insulin resistance and glucose intolerance than age-matched wild-type

mice on a similar diet (Minamino et al, 2009). Although islets were not examined, DNA damage, p53 activation and cellular senescence in the fat pads of these mice were correlated with the insulin resistance. It is tempting to speculate these islets with shortened telomere length are undergoing cellular senescence, causing the diabetic phenotype since it is well accepted that telomere shortening, which resembles damaged DNA, activates p53-dependent cellular senescence (Tavana & Zhu, 2011).

1.4.4b DNA damage

Cells bearing unprotected or shortened telomeres (beyond a certain threshold) are genomically unstable, mimicking DSBs that initiate the DDR. Considering telomere dysfunction activates cellular senescence and insulin resistance, it is not inconceivable that islet-specific DSBs can also initiate cellular senescence and disruption of glucose homeostasis (Tavana & Zhu, 2011). In fact, our research team was the first to demonstrate this concept (described in below chapters). Utilizing a deficiency in the non-homologous end joining pathway, the major mammalian DNA repair pathway that fixes DSBs (Mahaney et al, 2009), combined with the absence of p53-mediated apoptosis (Liu et al, 2004), the persistence of DNA damage led to progressive diabetes (Tavana et al, 2010c). Specifically, the accumulation of unrepaired DNA damage, resulting from the deletion of DNA Ligase IV activated p53 and downstream cell cycle inhibitor p21 to initiate β-cell specific cellular senescence. Additionally, β-cell mass progressively decreased due to diminished β-cell replication, collectively lowering insulin levels and leading to overt diabetes. Interestingly, DNA Ligase IV levels are considerably high in isolated wild-type pancreatic β-cells potentially protecting these cells from excess DNA damage (Tavana et al, 2010c). Interestingly, diabetic patients

are reported to have increased levels of DNA damage coinciding with decreased DNA repair efficiencies (Blasiak et al, 2004; LeRoith et al, 2008; Pan et al, 2007; Sliwinska et al, 2010), highlighting the importance of not only studying the roles of the DNA damage response and different DNA repair factors in β-cell physiology, but also increasing the efficiency of these repair pathways during the treatment of diabetes (Tavana & Zhu, 2011). Recently, scientists reported a similar phenomenon in the islets to our model using mice deficient for Securin, also known as pituitary tumor transforming gene (PTTG) (Wang et al, 2003). PTTG deletion correlated with increased p53 and p21 due to higher levels of DNA damage, activating cellular senescence in different organs (Chesnokova & Melmed, 2010). Here, researchers show elevated p21 in β-cells is responsible for the decrease in proliferation and islet-specific cellular senescence observed leading to the diabetic phenotype, which was partially abrogated by deleting p21 (Chesnokova et al, 2009; Wang et al, 2003).

It is commonly hypothesized that aging occurs from an abundance of unrepaired DNA damage which recruits the DDR to initiate the network of responses (Campisi & Vijg, 2009). Furthermore, the increase of accumulated DNA damage and high levels of p53 have been correlated with mitochondrial dysfunction (Sahin et al, 2011), which may synergize with halted β-cell proliferation to induce diabetes. Consistent with this hypothesis, these collective studies show that DNA damage (either from DNA repair deficiencies or telomere shortening) and the downstream p53 responses directly alter the rate of proliferation as well as the overall function on pancreatic β-cells (Tavana & Zhu, 2011).

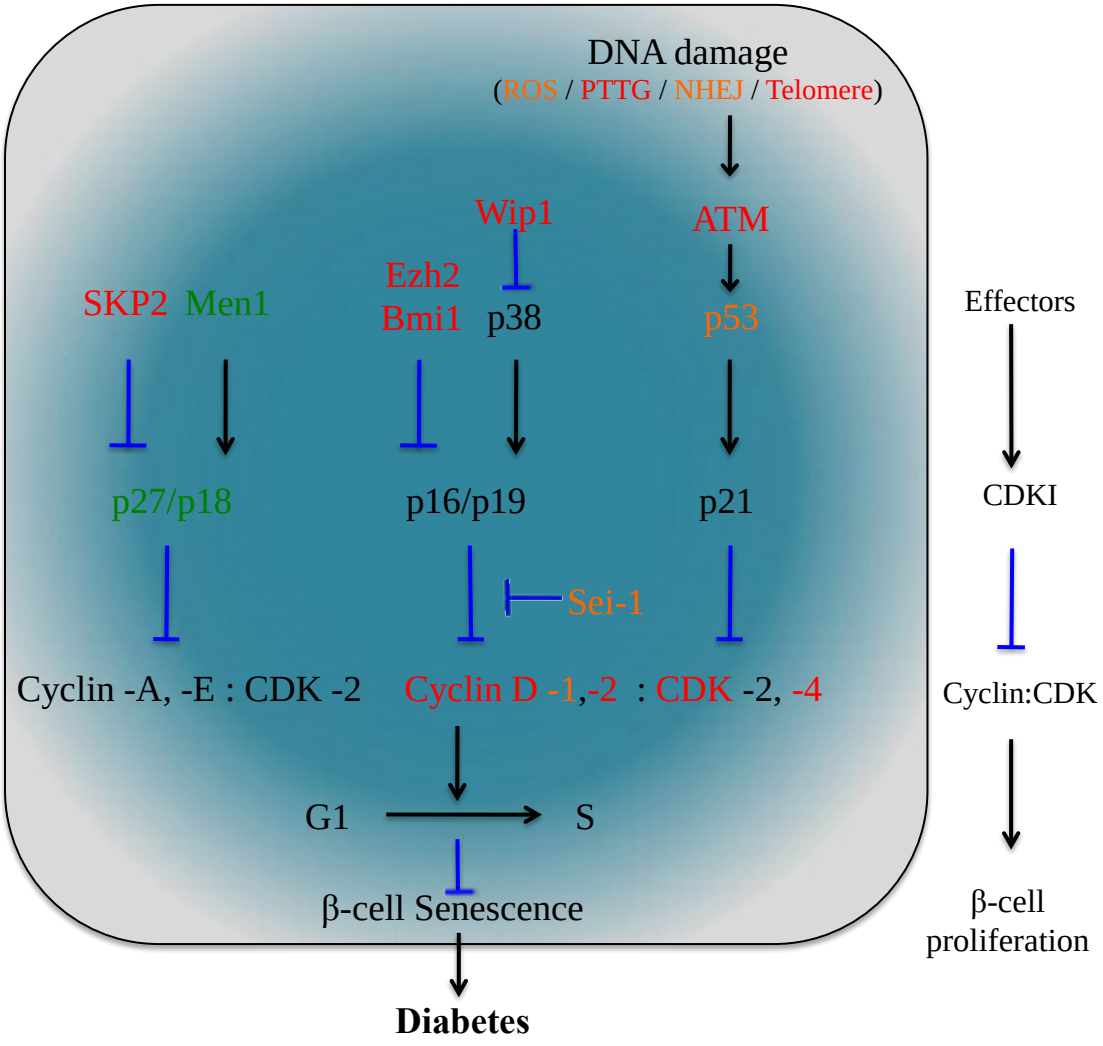

Figure 3. Schematic of the cell cycle regulation in pancreatic β-cells.
The cell cycle regulation of β-cells is complex. Upstream cell cycle effectors can transcriptionally regulate cell cycle kinase inhibitors. These inhibitors suppress the activation of cyclin:CDK complexes halting the cell cycle leading to β-cell senescence. Additionally, DNA damage can initiate cell cycle arrest protecting the cell from genomic instability. Red font, gene deletions that directly lead to diabetes; orange font, gene deletions that indirectly lead to diabetes; green font, gene deletions that lead to islet hyperplasia; NHEJ, non-homologous end joining.
(Reproduced from (Tavana, 2011) with permission from Cell Cycle)

1.4.4c ATM

The master cell cycle regulator, ataxia telangiectasia mutated is centered in the DNA damage response as a protein kinase phosphorylating many downstream targets upon activation, including p53 and recently found to be linked to metabolic disorders. Patients with this mutation (A-T) are predisposed to cancer, cereballar ataxia and skin telangiectasia, while some patients exhibit deregulated glucose homeostasis and insulin resistant diabetes, a poorly understood phenomenon (Bar et al, 1978; Blevins & Gebhart, 1996; Lavin, 2008; Schalch et al, 1970). Using aged, tumor-free ATM deficient mice, researchers linked this deficiency to increased non-fasting glucose levels coupled with glucose intolerance and overt diabetes (Miles et al, 2007). Apoptosis was not detected in young mice and was not analyzed in older murine islets. An independent study used a high fat diet to show that ATM haploinsufficiency was sufficient to drive glucose intolerance and insulin resistance compared to wild-type littermates (Schneider et al, 2006). Interestingly, administration of Chloroquine, an antimalarial drug that activates ATM independently of DNA damage (Bakkenist & Kastan, 2003), decreased both fasting and random glucose levels in two established models of insulin resistance. ATM was increased substantially in β-cells upon Chloroquine treatment, driving the restoration of glucose homeostasis (Schneider et al, 2006), which underscores a role for ATM in the regulation of β-cell maintenance (Tavana & Zhu, 2011). Furthermore, deleting p53 did not change glucose tolerance or insulin secretion compared to wild-type counterparts after Chloroquine administration and a high fat diet (Razani et al, 2010). This can be explained by stimuli and stress specific responses—the ATM-p53 axis may not be activated in the pancreas without specific stress, like excessive DNA damage.

These studies highlight an unique role for ATM in regulating glucose homeostasis (Tavana & Zhu, 2011). It would be interesting to analyze the morphometry of islets in aged ATM deficient diabetic mice as well as to further understand exactly what mechanistic role ATM deficiency plays in β-cells. ATM is also involved in DNA repair and its deficiency has been linked to increased levels of DNA damage (Shiloh, 2003) and premature senescence after genotoxic stress (Zhan et al, 2010) (similar to the NHEJ deficiencies). Therefore, we can interpret with caution that ATM deficiency might lead to increased levels of unrepaired DNA damage in the islets leading to the dysfunction of β-cells and ultimately diabetes; further investigation is needed (Tavana & Zhu, 2011).

1.4.4d p53

Stress prompts ATM to stabilize and activate p53 via phosphorylation—primarily on Serine 18 (Ser15 in humans) (Banin et al, 1998; Canman et al, 1998). Abrogating this phosphorylation site through mutation would theoretically abolish this ATM-p53 pathway, allowing further study of the impaired glucose homeostasis seen in ATM deficiency. Using the p53 mutant, $p53^{S18A}$, researchers indeed found metabolic dysregulation through impaired glucose tolerance and insulin resistance (Armata et al, 2010). Furthermore, this phenomenon was ablated upon antioxidant administration, demonstrating increased oxidative stress as the cause for this metabolic disorder. Combined with the previous studies utilizing ATM deficiency (Miles et al, 2007), these data highlight the importance of the ATM-p53 pathway in maintenance of glucose homeostasis. Yet the role of p53 during glucose homeostasis is still emerging (Tavana & Zhu, 2011). Recently, Minamino and colleagues unequivocally demonstrated a role for cellular aging in the influence of insulin resistance and type 2 diabetes through p53

(Minamino et al, 2009). Using a previously characterized obese diabetic model resulting from ectopic expression of the agouti peptide (Ay), they first showed that increased levels of oxidative damage in the adipose tissue activated p53/p21-dependent cellular senescence (independent of telomere-shortening). Decreasing p53 levels (using Ay $p53^{+/-}$ mice) drastically reduced the cellular senescence and normalized the increase in senescence-associated inflammation (associated with insulin resistance), significantly improving overall insulin sensitivity and glucose tolerance. Conversely, overexpressing p53 in adipose tissue led to increased p21 levels and higher circulating pro-inflammatory cytokines associated with glucose intolerance and insulin resistance. Further, analyzing fat pads from diabetic patients showed an increase in p53/p21, senescence, and senescence-associated inflammation (Minamino et al, 2009). Thus, the DNA damage from an increased caloric intake triggers activation of p53/p21 dependent senescence in adipose tissue, altering inflammation which collectively drives insulin resistance and diabetes (Tavana & Zhu, 2011). A proteomic study utilizing isolated human islets from patients with type 2 diabetes showed elevated p53 expression (Nyblom et al, 2009), while p53 has also been identified in cycling murine islets (Cozar-Castellano et al, 2006c). Yet when comparing young and aged β-cells, there was no significant difference in the p53 expression level as detected by immunostaining (Hinault et al, 2008). Recently, Rohit Kulkarni's group utilized a mouse model with an extra copy of the p53 isoform that presents with p53-dependent accelerated aging (Δ40p53 (Maier et al, 2004)) (Hinault et al, 2011). These mice have increased p53 stabilization with elevated levels of p21 and decreased cyclin D2 levels. Furthermore, these mice exhibited progressive diminishment of β-cell proliferation and mass, which was sufficient to drive

hypoinsulinemia, glucose intolerance and age-dependent diabetes (Hinault et al, 2011). In conjunction with the conclusions drawn from the previously mentioned work, p53 is becoming a more attractive link between aging and diabetes (Tavana & Zhu, 2011).

1.4.5 Diabetes Summary

Loss or dysfunction of pancreatic β-cells causes a progressive onset of diabetes, due to the unique function of these cells that cannot be compensated by any other cell type. Considering the predominant mechanism of β-cell generation is through self-renewal, it becomes essential to understand and manipulate this proliferative regulation for therapeutic interventions. Recent findings using animal models that underscore the complexity of cell cycle regulation and genomic damage response in the pancreatic β-cell are summarized in Figure 3 and Table 1. The commonality of many of these models converge on the notion that cell cycle dysregulation will halt the proliferation of β-cells, triggering cellular senescence in some cases, decreasing islet mass and insulin levels subsequently causing this metabolic disorder (Tavana & Zhu, 2011). To circumvent this problem, we need to find targets relieving the proliferative restriction placed on β-cells without driving neoplasia. However, many upstream transcriptional regulators of the cell cycle machinery that control β-cell proliferation are yet to be characterized. Additionally, the effects of genomic stresses, such as those induced by a high fat diet, as well as the role of DNA repair and the DNA damage response still remains elusive during the maintenance and regulation of β-cell homeostasis. In certain cases, targeting these pathways may help rescue glucose intolerance and avoid permanent cell cycle arrest. Interestingly, diabetic patients are reported to have increased levels of DNA damage coinciding with decreased DNA repair efficiencies (Blasiak et al, 2004; LeRoith

et al, 2008; Pan et al, 2007; Sliwinska et al, 2010), highlighting the importance of not only studying the roles of the DNA damage response and different DNA repair factors in β-cell physiology, but also increasing the efficiency of these repair pathways during the treatment of diabetes (Tavana & Zhu, 2011).

1.5 Dissertation Summary

In this dissertation, we provide the first direct evidence that the accumulation of DNA double strand breaks in the pancreatic islets, using the nonhomologous end-joining knockout Ligase IV, activates p53 to initiate cellular senescence (in the absence of apoptosis) and cause diabetes. This decrease in β-cell proliferation impacts glucose homeostasis through islet attrition (Tavana et al, 2010c). This study highlights two important features: the importance of NHEJ in mending spontaneous DNA double strand breaks, and the respective p53-activated response. Further, to delineate upstream NHEJ factors in the pancreatic β-cell, we utilized the $Ku70^{-/-}$ mouse model. Interestingly, we identified a novel role for Ku70 independent of the NHEJ pathway, which is to somehow modulate Wnt signaling. The stabilization of Wnt signaling, augments β-cell proliferation directly leading to islet propagation and increased insulin levels. This explains the hypoglycemia observed. Lastly, we analyze the impact of single strand breaks, using UVB radiation, in the absence of p53-mediated apoptosis (Tavana et al, 2010a). Here we show that low doses of UVB elevate inflammation, increase immunosuppression, and elevate skin damage in the absence of p53-mediated apoptosis *in vivo*. Further, mechanistically, we show both *in vivo* and *in vitro* that without p53-mediated apoptosis, in the presence of single-stranded DNA damage, cellular senescence

is initiated as a ‘fail safe’ response to prevent genomic instability. Taken in aggregate, these studies show that both single strand DNA damage as well as double strand DNA damage activates cellular responses that lead to aging related phenotypes—either photoaging or diabetes. Further, our models portray the importance of initiating cellular senescence as a mechanism to prevent tumorigenesis, although coming with a cost.

CHAPTER 2

MATERIALS AND METHODS

2.1 Generation of Mouse Models

Wild-type, $p53^{p/p}$ (Liu et al, 2004) and $p53^{-/-}$ (Donehower et al, 1992) mutant mice were bred into a mixed C57BL/6 and 129SV background. DNA Ligase $4^{+/-}$ (Frank et al, 1998) mutant mice were crossed to $p53^{p/p}$ or $p53^{-/-}$ mice to generate $Lig4^{-/-}p53^{p/p}$ (Van Nguyen et al, 2007) and $Lig4^{-/-}p53^{-/-}$ (Zhu et al, 2002) respectively. $Ku70^{+/-}$ (Gu et al, 1997) mice were crossed to each other to create $Ku70^{-/-}$ or with $p53^{p/p}$ to generate $Ku70^{-/-}p53^{p/p}$. The protocols were approved by the Institutional Animal Care and Use Committee at The University of Texas M.D. Anderson Cancer Center.

2.2 Acute Irradiation

For acute studies, mice were shaved on the dorsal area and irradiated once at indicated doses from a ^{137}Cs γ-ray source (JLS Shepard & Associates, Glendale, CA), or UV using four FS40 sunlamps (National Biological, Twinsburg, OH) (Nghiem et al, 2002; Sreevidya et al, 2010). The UVB output of the sunlamps was measured with an IL 1700 radiometer. At indicated time points post irradiation, the dorsal skin fold was measured with calipers and skin samples from areas of irradiation were frozen in liquid nitrogen and a portion fixed for further studies. DNA was extracted from the frozen samples. Radioimmunoassay was performed to determine the extent of cyclopyrimidine dimer and (6-4) photoproduct formation (Mitchell, 2006).

2.3 Immune Suppression

To measure the effect of UVB on the immune response, a delayed-type hypersensitivity reaction was used, as described (Nghiem et al, 2002). Mice were shaved

on the dorsal side and the following day they were given indicated amounts of UVB radiation. Five days later they were immunized with formalin-fixed *Candida albicans*. Delayed-type hypersensitivity, as determined by an increase in footpad swelling in response to a challenge with *C. albicans*-specific antigen (Alerchek, Portland, ME), was measured 10 days post immunization. The mean change in footpad thickness (left foot + right foot ÷ 2) was calculated for each animal in the group (N = 5). The change in thickness ± the SEM was then calculated for the group.

2.4 Cell culture and UV irradiation

Murine embryonic fibroblasts ~day 13.5 were grown in Dulbecco's modified Eagle's medium (Mediatech) supplemented with 10% fetal bovine serum (SAFC Biosciences), and 100U/mL penicillin (Cambrex). Cells were plated in 10cm dishes in an incubator at 37ºC with 5% CO_2. An FS40 sunlamp was used for irradiation; UV output was measured with an IL 1700 radiometer. Upon subconfluency, cells were exposed to different UVB doses in a thin layer of PBS. Control cells were kept in the same culture conditions without UVB.

2.5 Radiation Sensitivity Assay

Briefly, MEFs were seeded onto 6cm dishes and later irradiated with differing dosages of ionizing radiation by using a ^{137}Cs γ-ray source. After irradiation, the medium was replaced and cultures were incubated for 7 days at 37°C with a change in media at day 3. At the end of the assay, MEFs were trypsinized, and viable cells were counted.

Additionally, MEFs were plated onto 10cm dishes and irradiated with UVB as described above and viability was assessed 3 days after irradiation.

2.6 Apoptosis and Cell Cycle Analysis by flow cytometry

MEFs were irradiated either with ionizing radiation or UVB as stated above and collected at indicated time points. Apoptosis was analyzed using a FITC-Annexin V Apoptosis detection kit (BD Pharmingen). Data were collected using a flow cytometer (FACScalibur, Becton Dickinson) and analyzed by FlowJo software (Tree Star, OR).

2.7 Skin histology and immunofluorescence

"Skin was fixed in Bouin (Ricca Chemical Company) solution in room temperature, overnight. Upon fixation, tissue was dehydrated by passing through ethanol followed by xylene. and embedded in paraffin for histological analysis. Sections (6μm) were incubated with antibodies against p53 (Cell Signaling), and p21 (Santa Cruz). Secondary antibodies were applied according to the manufacturer's recommendations and incubated with Vectashield Hard Set with DAPI (Vector). Slides were analyzed by fluorescent microscopy (Olympus BX41). Additionally, a transferase-mediated dUTP nick-end labeling (TUNEL) assay kit (Promega) was used to detect apoptosis with DNase-1 treated sections as positive controls" (Tavana et al, 2010b).

2.8 Cellular senescence

Senescence-associated β-galactosidase (SA-β-gal) activity was detected in the skin by a staining kit (Cell Signaling). SA-β-gal activity in MEFs was detected as described (Dimri et al, 1995). Slides were subjected to hematoxylin-eosin (H&E) staining and examined by light microscopy (Olympus BX41). MEFs positive for SA-β-gal activity were scored against total cells using ImageJ (National Institutes of Health, Bethesda, MD). For islet senescence, blocks were sectioned (6μm) and insulin was detected by immunohistochemical staining. Slides were counterstained with Nuclear Fast Red (Vector).

2.9 Western Blot Analysis

Cells were irradiated as stated above and collected in lysis buffer. 40μg of protein was loaded onto a 10% polyacrylamide gel. After transfer, the PVDF membrane (Biorad) was incubated with antibodies for p53, PUMA, and pChk1 (S345) (Cell Signaling), p21 (BD Pharmingen), PARP and Noxa (Abcam), Cdk4, Bcl-2 and Bax (Santa Cruz), Bak (Calbiochem), GAPDH (Epitomics), Ku70 (Abd Serotec), and β-Actin (Thermo Scientific). Horseradish peroxidase conjugated secondary antibodies were applied per the manufacturers' recommendations. Signals were detected with an enhanced chemiluminescence kit (Perkin Elmer).

2.10 Measurement of Blood Glucose and Insulin Levels

"For the glucose tolerance test, mice were fasted for 16 hours and injected intraperitoneally with glucose at 1.0g/kg body weight. For the insulin tolerance test, mice were fasted for 6 hours and injected intraperitoneally with insulin at 0.75U/kg (Sigma). Glucose levels were measured using a glucose analyzer (Bayer Contour). Blood insulin levels were measured by enzyme-linked immunosorbent assay (ELISA)" (Tavana et al, 2010b).

2.11 Pancreatic histology, immunohistochemistry and immunofluorescence

"Pancreata were fixed in Shandon Cryomatrix (Thermo) and placed at -20°C or Bouin's Solution (Ricca Chemical Company) overnight, dehydrated through ethanol and embedded in paraffin for histological analysis. Blocks were sectioned (6μm), deparaffinized, and rehydrated in decreasing concentrations of ethanol. Slides were quenched of endogenous peroxidase, permeabilized, and blocked with Superblock (Thermo). Slides were incubated with antibodies against Insulin, Glucagon, Cyclin D2, TCF4, and p53 (Cell Signaling), F480 (eBioscience), Cd11b, Nk1.1, β-catenin (BD Biosciences), p53, p21, and Cdk4 (Santa Cruz), PCNA (DAKO), Ku70 (Abd Serotec), or γH2AX (Abcam). BrdU was injected intraperitoneally at 100mg/kg. After 4 hours, pancreata were sectioned. Slides were incubated with anti-BrdU (Serotec) and anti-insulin (Cell Signaling) antibodies and analyzed. For immunohistochemistry, HRP-conjugated secondary antibodies were applied at manufacturers' recommendations. DAB-chromogen substrate mixture was applied or combined with AEC for double immunohistochemical detection (Vector). Slides were subjected to haematoxylin

and eosin (H&E) staining and examined by light microscopy (Olympus BX41). For immunofluorescence, secondary antibodies were applied accordingly at manufacturers' recommendations. Slides were analyzed by fluorescent microscopy" (Tavana et al, 2010b).

2.12 Analysis of pancreatic morphology

"Pancreata sections were prepared as described, insulin was detected by immunohistochemistry, counterstained with H&E. Each section was subjected to morphometric analysis using Image J (National Institutes of Health, Bethesda, MD). The area of the pancreas and of each islet in the tissue were measured. Raw data was statistical analyzed" (Tavana et al, 2010b).

2.13 Pancreatic Islet isolation

"Islets were isolated through collagenase XI (Sigma) perfusion, digested, filtered, and hand picked to purify islets as described in (Li et al, 2009). Islets from at least 3 mice per group were pooled together and 40μg of protein was loaded onto 6, 10, and 12% polyacrylamide gels depending on the protein of interest" (Tavana et al, 2010b).

2.14 TUNEL assay

A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay kit for immunofluorescent detection (Promega) or immunohistochemical deteciton (Millipore ApopTag) was used to detect apoptosis.

2.15 Statistical analysis

Results are presented as the mean ± SEM. Differences were determined using a two-tailed, unpaired Student *t* test with CI of 95%. A *P* value less than 0.05 was denoted as statistically significant (GraphPad Prism Software, GraphPad, San Diego, CA).

CHAPTER 3

ABSENCE OF P53-DEPENDENT APOPTOSIS LEADS TO UV RADIATION HYPERSENSITIVITY, ENHANCED IMMUNOSUPPRESSION AND CELLULAR SENESCENCE

3.1 Introduction

Fundamental to the initiation of many tumors is the persistence of DNA damage, which, if inappropriately repaired, can drive oncogene activation or deactivation of tumor suppressor genes that drive cell proliferation and/or survival even in the absence of physiological stimuli (Felsher, 2008; Negrini et al, 2010). A carcinogen that can directly damage DNA is ultraviolet radiation (Sancar et al, 2004; Sinha & Hader, 2002). Excessive exposure to UVR is potentially hazardous to our health, since evidence has linked UV radiation exposure to several conditions such as gene mutations, skin inflammation, immune-suppression, multiple types of cancers and accelerate the onset of aging (Halliday, 2005; Hussein, 2005; Yarosh, 2004). Upon UV radiation, p53 plays an essential role in mediating the cellular responses (Benjamin & Ananthaswamy, 2007; Latonen & Laiho, 2005). Previous studies revealed that in the absence of one or both alleles of p53, mice display extreme sensitivity to tumor induction by UVB and exhibit a drastic decrease of apoptosis in the dermis (Jiang et al, 2001; Ziegler et al, 1994). More recent studies have underscored UVB as an inducer of p53-mediated premature cellular senescence (Borlon et al, 2008; Chen et al, 2008) as well as p53-mediated apoptosis (Naik et al, 2007).

Cells can rapidly activate a network of pathways in response to genomic DNA damage. A central outcome of the DNA damage response pathway is the stabilization and activation of the tumor suppressor p53, which arrests the cell cycle to allow for DNA repair, apoptosis, or cellular senescence—the latter two occur when the DNA damage is too excessive or irreparable. p53 is best characterized as a transcription factor, which can transactivate many different genes to induce and regulate a diverse network of

cellular functions crucial in protecting the cell against genome instability as well as maintaining the homeostasis of cell growth and differentiation (Meek, 2009; Vousden & Prives, 2009). Initial genomic insults stabilize p53 to the nucleus where transient cell cycle arrest can be quickly activated, allowing for the repair of damaged DNA prior to replication. Upon excessive and irreparable damage, p53 can trigger a signaling cascade and induce programmed cell death through transcription of pro-apoptotic genes, most notably p53-upregulated modulator of apoptosis (PUMA) and Noxa (Roos & Kaina, 2006) as well as repress anti-apoptotic genes including Bcl-2 (Zuckerman et al, 2009). Apoptosis is a formidable barrier protecting cells against the accumulation of genomic instability leading to tumor formation.

Besides apoptosis, DNA damage may also induce an irreversible cell cycle arrest termed cellular senescence (Abbas & Dutta, 2009; Cheng & Chen, 2010; Morachis et al, 2010). A key p53 target playing an important function in cell cycle checkpoint regulation and induction of senescence is the cyclin-dependent kinase inhibitor p21. Upon activation, p21 induces G_1 arrest after DNA damage by inhibiting the cyclinE/CDK2 complex (Abbas & Dutta, 2009; Harper et al, 1993). Permanent cell cycle arrest can be induced through a variety of different stimuli including oncogenic signaling (oncogene-induced senescence), telomere shortening (replicative senescence), or stress/DNA damage independent of the previous two signals (premature senescence) (Campisi, 2005). Regardless of the stimuli, senescence plays a parallel function to apoptosis to prevent genomic instability thereby inhibiting tumorigenesis (Van Nguyen et al, 2007). The decision after DNA damage to either activate cellular senescence or apoptosis occurs via the p53 pathway, yet the triggers remain unclear—speculations

include different stimuli and strength/persistence of the signal, as well as cell type specific responses (Junttila & Evan, 2009).

To directly decipher between p53 functions after UVR, we took advantage of a separation-of-function, hypomorphic p53 point mutation, R172P, losing complete p53-mediated apoptosis, yet retaining partial cell cycle arrest capability (Liu et al, 2004). In this study, we found that mutant p53 mice, hereafter referred to as $p53^{p/p}$, are more susceptible to UV-induced inflammation, immunosuppression, and skin damage when compared to wild-type mice. At the cellular level, $p53^{p/p}$ mouse embryonic fibroblasts (MEFs) display UV hypersensitivity and cannot transactivate certain pro-apoptotic genes PUMA and Noxa. Upon UVB, these cells retain the ability to upregulate p21, which ultimately drives the premature cellular senescence and the sensitivity phenotype observed. Conversely, the wild-type MEFs exposed to UVB undergo apoptosis because of the ability to induce these pro-apoptotic genes. Collectively our research indicates the importance of p53-mediated apoptosis *in vivo* and further shows that in its absence, cellular senescence is triggered as a fail-safe mechanism protecting cells against genomic instability but comes at a cost (Tavana et al, 2010a).

3.2 Results

3.2.1 Mutant $p53^{p/p}$ mice are hypersensitive to UVB radiation.

The tumor suppressor p53 plays a central role in the response to UV radiation (Latonen & Laiho, 2005). Previous studies demonstrate that p53-deficient mice exposed to chronic UV radiation show increases in skin tumor formation (Benjamin &

Ananthaswamy, 2007). To investigate the cellular response to UV radiation (UVR), which causes inflammation and erythema in the skin, we exposed hypomorphic $p53^{p/p}$ mutant mice to UV radiation to analyze the acute cutaneous inflammatory response. Considering the difficulty to measure erythema in mice with pigmented skin, we decided to determine the macroscopic inflammation in skin by measuring the double-fold skin thickness after exposure to 4.5 kJ/m^2 of UVB radiation. At 24 and 48 hours post UVB exposure, the skin fold thickness marginally increased in wild-type mice by 6% and 27% from baseline measurements, while $p53^{p/p}$ mice presented an average of 27% and 74% increase, respectively (Figure 4a). Upon a second dose of UVB exposure, measurements taken 24 hours later showed a 33% increase from baseline in wild-type skin, while up to a 100% increase was noted in the $p53^{p/p}$ mice (Figure 4a). The skin fold swelling differences in the UVB-irradiated $p53^{p/+}$ mice were statistically indistinguishable from that observed in wild-type mice. To further investigate and visualize the inflammatory response, acutely-irradiated dorsal skin sections were counterstained with hematoxylin and eosin, to revealed that UVR not only increased the thickness of the epidermis, but also the dermal layer (Figure 4b). These data indicate that $p53^{p/p}$ mice are hypersensitive to UVB exposure.

3.2.2 $p53^{p/p}$ mice are more susceptible to UVR induced immunosuppression.

It is well appreciated that UV radiation increases the risk of skin cancer development as well as causes systemic immune suppression (Kripke, 1980; Kripke et al, 1977). Dr. Kripke's group further explored the severity of UV-induced DNA damage mediated immune suppression in wild-type mice (Jiang et al, 2001; Kripke et al, 1992). To determine whether $p53^{p/p}$ mice exhibit a difference in their immune status

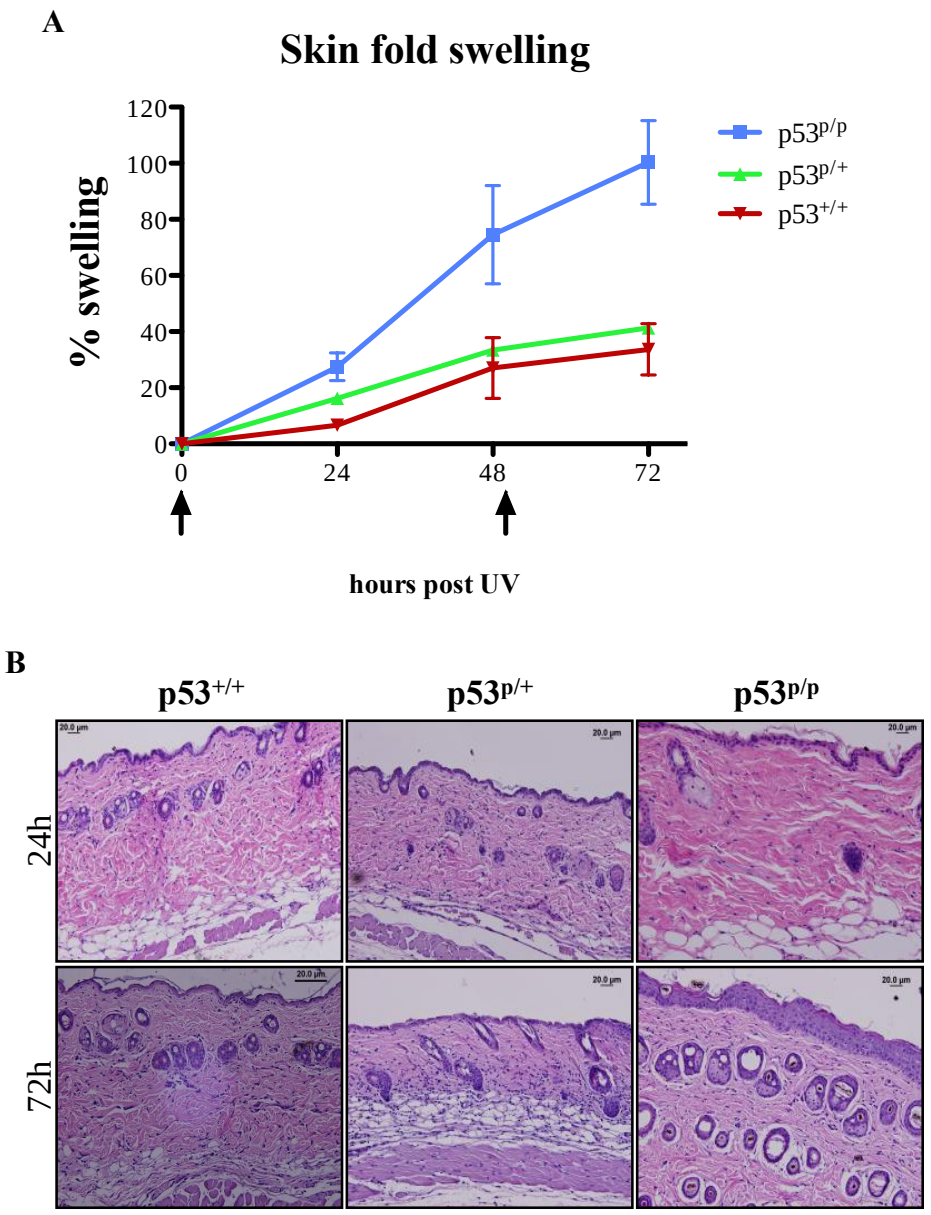

Figure 4. Mutant p53$^{p/p}$ mice are hypersensitive to UVB radiation.
(A) Mice were shaved and irradiated with 4.5kJ/m^2 of UVB at 0 and 48h. Skin swelling was determined by measuring double skin-fold thickness of the dorsal skin at indicated time points. N=5 per group. Data are the mean ± SEM, * indicates p<0.05.(**B)** Representative H&E dorsal skin sections after indicated time points. Magnification: 20X.
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upon UV exposure, we compared the extent of UVB-induced immune suppression among genotypes. A schematic of the delayed-type hypersensitivity assay (DTH) is shown in Figure 5a to measure the immune status, as described previously (Nghiem et al, 2002). Briefly, mice were exposed to UVB followed by an immunization with *C. albicans* 5 days later. 9 days following, a challenge to the hind footpads with *Candida* antigen was administered, and swelling (in mm^2) was measured 24 hours later. Results indicate that both wild-type and $p53^{p/p}$ non-irradiated mice mounted an effective DTH response (Figure 5b). Interestingly, $p53^{p/p}$ mice were significantly more sensitive to the immunosuppressive effects of UV radiation at relatively low UV doses, 0.5 and 2.5 kJ/m^2 ($p<0.005$) as compared to wild-type. At 5 kJ/m^2, which induced immune suppression in the wild-type mice, $p53^{p/p}$ mice demonstrated a significant increase in immune suppression ($p<0.005$). No significant difference in suppression was observed at the highest dose (10 kJ/m^2). Collectively, these results indicate that the increased susceptibility seen in $p53^{p/p}$ mice upon UV-induced immune suppression is in parallel with their hypersensitivity to UVB radiation.

3.2.3 UVR induces p53-p21 driven cellular senescence in the skin of $p53^{R172P}$ mice.

UVB exposure is known to induce a p53-dependent apoptotic response in wild-type mouse skin (Naik et al, 2007). To determine whether UVB can also induce apoptosis in $p53^{p/p}$ mice, we performed a TUNEL assay. Apoptotic cells were detected in wild-type samples receiving 5 and 10 kJ/m^2 of UVB radiation (Figure 6) while virtually no apoptotic cells were detected in $p53^{p/p}$ and non-irradiated wild-type samples. In contrast to UVR exposure, treatment with 10 Gy of ionizing irradiation induced slight

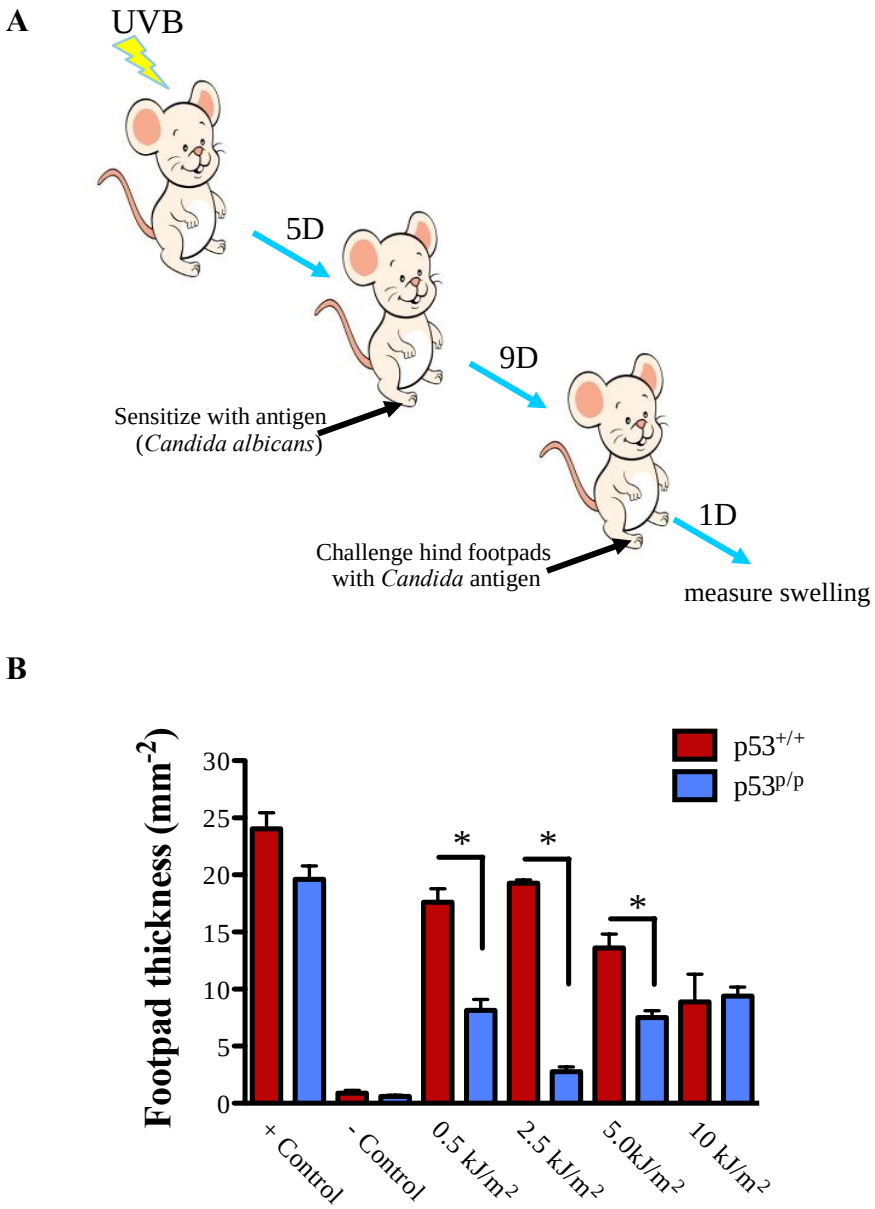

Figure 5. The role of mutant p53 in UVB-induced immune suppression.
(A) Schematic of the delayed type hypersensitivity (DTH) response to *C. albicans* devised to detect immune suppression after UVB. **(B)** Positive controls were obtained from non-irradiated, but immunized and challenged mice. Negative controls were measured in mice not immunized but challenged. N > 5 per group. Data are the mean ± SEM, * indicates $p<0.005$
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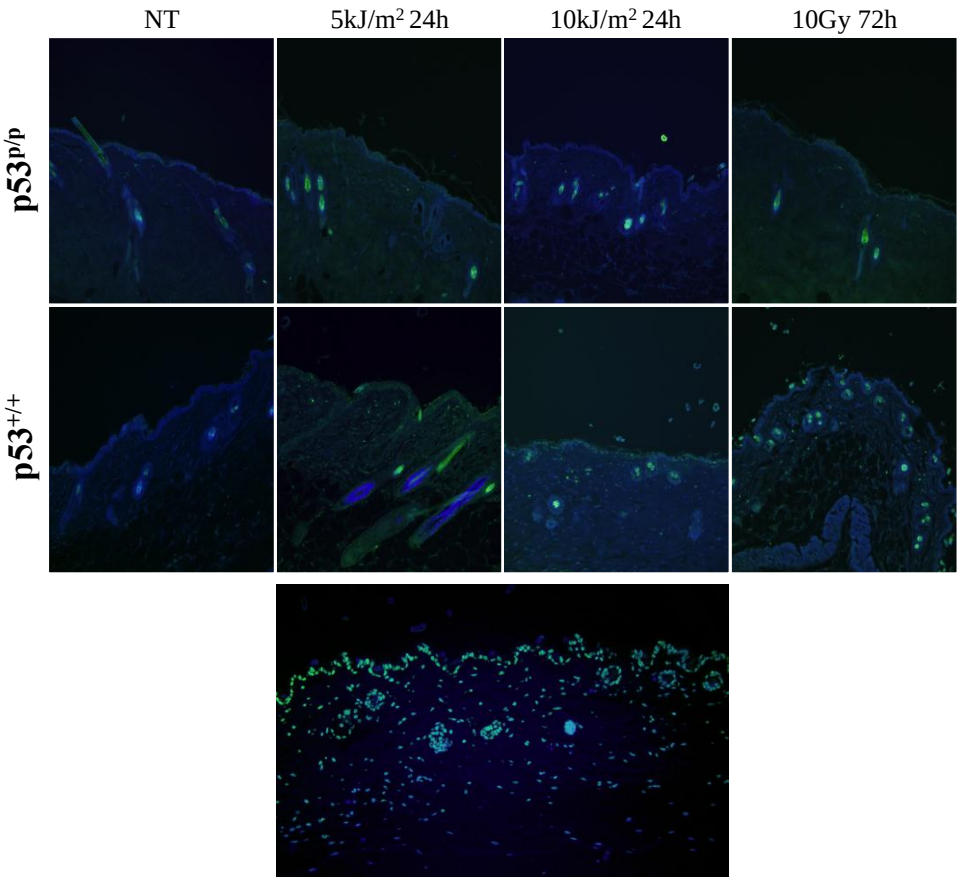

Figure 6. Absence of apoptosis in $p53^{p/p}$ skin sections.
Skin sections from $p53^{+/+}$ and $p53^{p/p}$ mice were analyzed with a TUNEL assay after indicated doses and time of UVB and γIR. Positive control treated with DNAse I. Magnification: 20X.
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apoptosis in wild-type skin, which was absent in $p53^{p/p}$ skin sections. This confirmed the apoptosis-deficient phenotype of $p53^{p/p}$ previously described (Liu et al, 2004).

To confirm if UVB activated a p53 response, we used immunofluorescent staining on skin sections to determine the expression of p53 and the downstream target, p21. Interestingly, skin sections from UV-irradiated $p53^{p/p}$ mice show an increased level of both p53 and p21 proteins, while the wild-type only modestly increased in p53 and p21 expression levels (Figure 7). The elevation of p53/p21 axis indicates cell cycle arrest and suggests cellular senescence. To identify whether UVB activates a permanent cell cycle arrest in skin sections, we stained for β-galactosidase (SA-β-gal), a marker indicative of cellular senescence, at 24 and 72 hours after UVR. As shown in Figure 8, senescent cells were observed specifically in the hair follicles of $p53^{p/p}$ mice 24 hours after 10 kJ/m^2 of UVB treatment; the number of these cells increased significantly 72 hours post UVB exposure. Low levels of cellular senescence were observed in skin sections of $p53^{p/+}$ mice, yet were absent in wild-type sections even upon exposure to ionizing radiation (IR). These results indicate that $p53^{p/p}$ skin cells undergo senescence in the absence of apoptosis as a response to UVB damage. Collectively, our results indicate that UVB induces a p53 and p21 response in both wild-type and mutant mice, yet the mutant skin undergoes senescence instead of apoptosis, possibly due to the high levels of p53 and nuclear p21, correlating with cell cycle inhibition activity.

3.2.4 $p53^{p/p}$ MEFs are specifically hypersensitive to UVR but not to γIR.

To determine the sensitivity of $p53^{p/p}$ cells after UVB exposure, we generated and exposed early passage $p53^{p/p}$ mouse embryonic fibroblasts (MEFs) to different doses

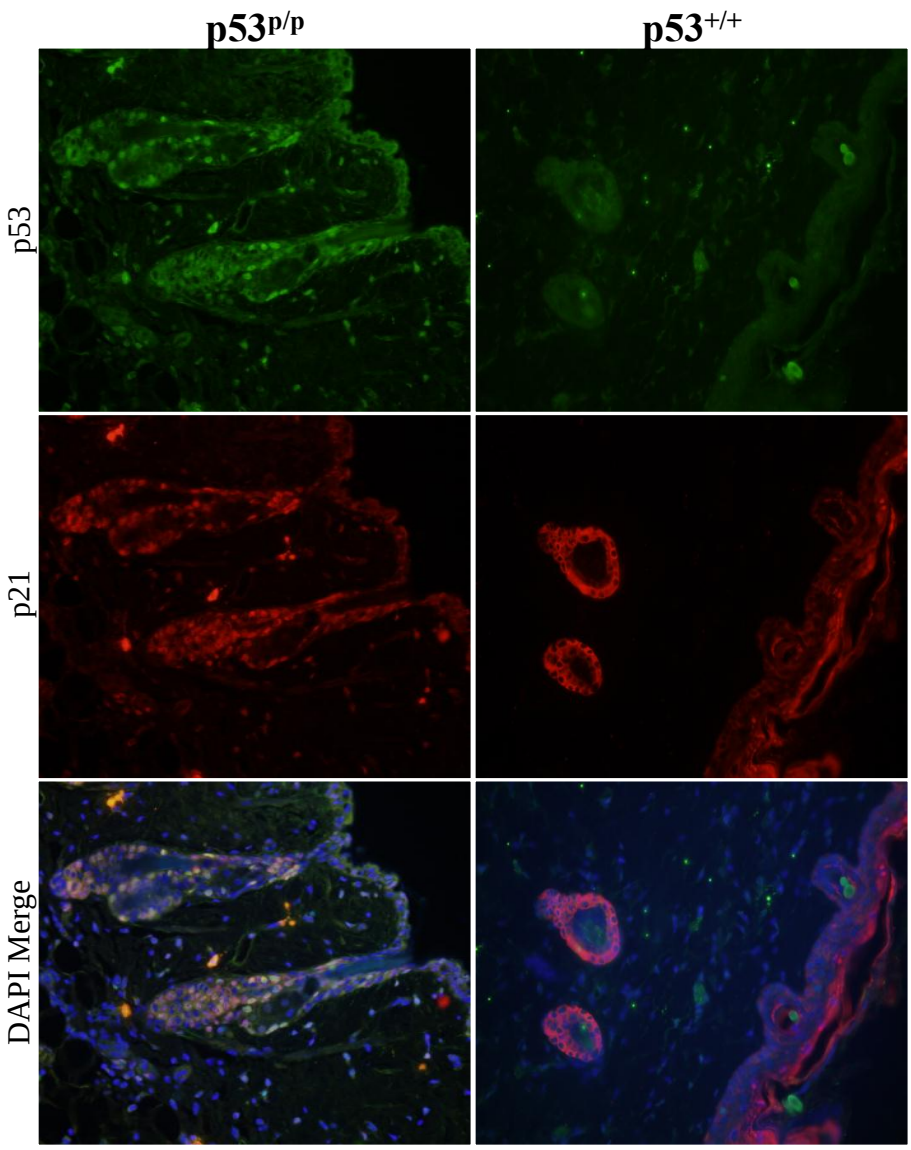

Figure 7. Elevation of p53 and p21 detected in p53$^{p/p}$ skin after UVB exposure. Representative immunofluorescent staining of skin sections from p53$^{p/p}$ and p53$^{+/+}$ mice; p53 (green), p21 (red) Dapi (blue). Magnification: 40X.
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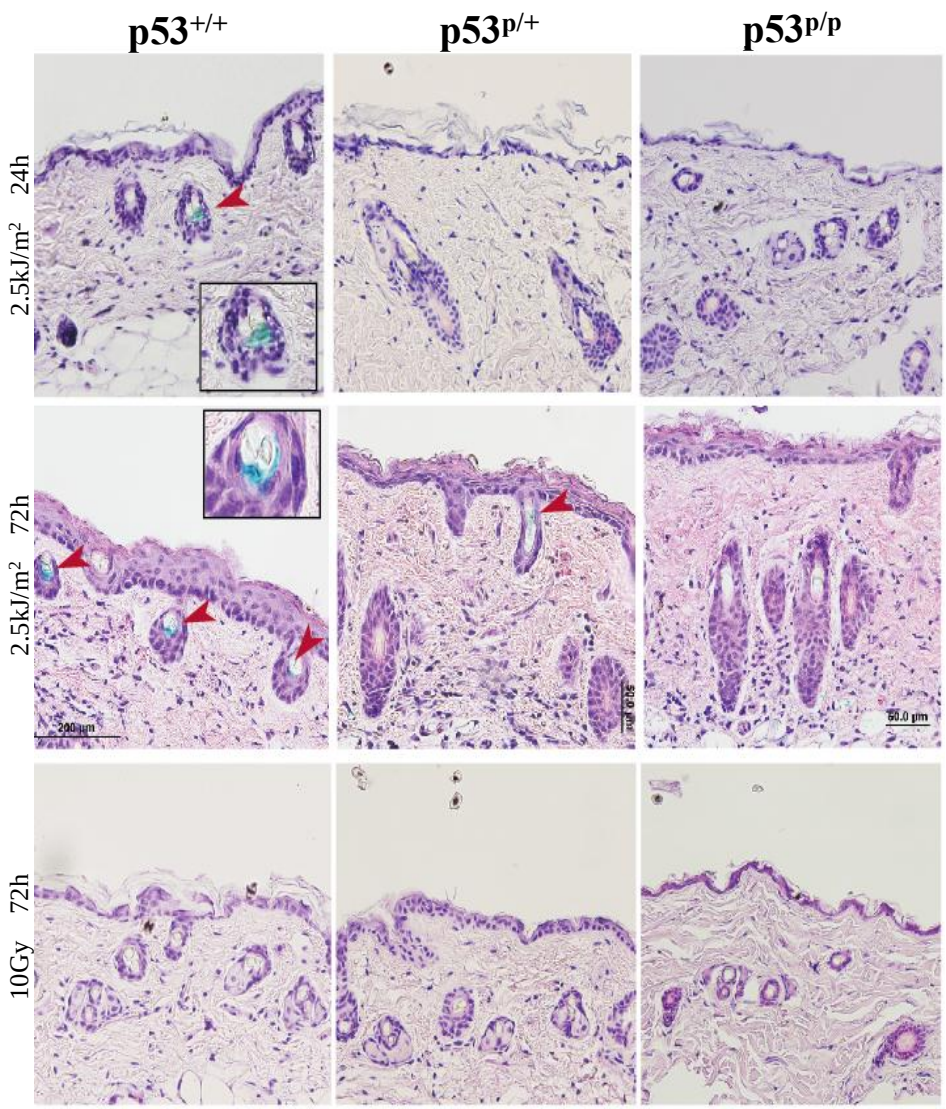

Figure 8. Cellular senescence detected in p53$^{p/p}$ skin sections.
Skin sections from p53$^{+/+}$, p53$^{p/+}$, and p53$^{p/p}$ mice after indicated radiation and recovery periods were analyzed for SA-β-gal, indicative of cellular senescence. Counter staining with H&E. Magnification: 40X.
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of UVB and evaluated the rate of cell survival when compared to wild-type and $p53^{-/-}$ MEFs. As shown in Figure 9a, UVB treatment at doses of 50, 100, and 250J/m^2 were slightly cytotoxic in wild-type cells (approximately 75, 55 and 20 percent survival respectively), but were not as cytotoxic to $p53^{-/-}$ MEFs. However, survival of $p53^{p/p}$ MEFs was significantly lowered when compared to wild-type and $p53^{-/-}$, with the greatest significant difference observed at 100J/m^2, which accounted for only a 27% survival rate. These results indicate that the hypomorphic $p53^{p/p}$ leads to MEF hypersensitivity after UVB treatment. Non-irradiated $p53^{p/p}$ MEFs grow at a similar rate when compared to wild-type cells (data not shown), ruling out the possibility that the observed sensitivity in $p53^{p/p}$ MEFs was due to a growth disadvantage in untreated MEFs (Barboza et al, 2006). To determine if this hypomorphic mutation is also important for MEF survival after IR, we exposed MEFs to different doses of treatment. As shown in Figure 9b, $p53^{p/p}$ and $p53^{-/-}$ show a relatively similar survival pattern in response to IR, while wild-type MEFs are more sensitive, which correlates to the p53-apoptotic response. A similar trend was observed after performing a colony formation assay with both UVR and IR, at similar doses (data not shown). Therefore, these results indicate that the hypomorphic p53 is selectively sensitive to UVB treatment and not IR.

3.2.5 Mutant $p53^{p/p}$ cells are able to remove UV-induced damaged bases normally.

UV irradiation induces lesions and distortions to DNA strands either by forming cyclobutane pyrimidine dimers (CPDs) or pyrimidine (6-4) pyrimidinone photoproduct [(6-4) photoproducts]. This type of DNA damage initiates the nucleotide excision repair (NER) pathway and activates cell cycle checkpoints (Nouspikel, 2009). To determine if the hypersensitivity observed in the $p53^{p/p}$ MEFs results from a defect in the ability to

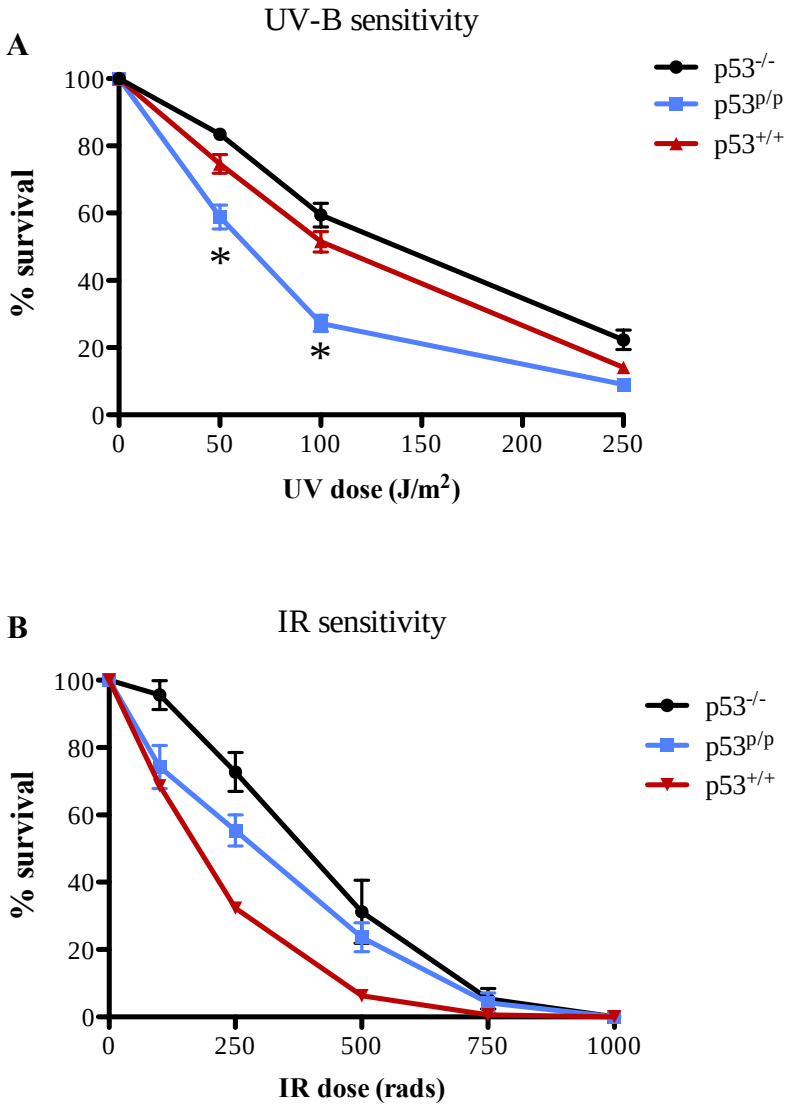

Figure 9. Mutant p53$^{p/p}$ MEFs are selectively sensitive to UVB and not IR exposure. (A) Growth curves of p53$^{+/+}$ (red triangles), p53$^{p/p}$ (blue squares) and p53$^{-/-}$ (black circles) MEFs after different doses of UVB. (B) Growth curve after different doses of IR. Survival is determined by the percentage of viable cells at each treated dose over the total viable cells in untreated conditions. Representative results are presented; each experiment was repeated independently more than 5 times. Data are the mean ± SEM, * indicates p<0.001 vs wild-type.
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repair UV-induced CPD and (6-4) photoproducts, we isolated DNA from UVB irradiated MEFs (200J/m^2) at 0, 6, 24, and 48 hours after irradiation and analyzed for remaining CPD and (6-4) photoproducts using a radioimmunoassay (Mitchell, 2006). The results in Figure 10a reveal that the rate of removal of both CPD and (6-4) photoproducts in the $p53^{p/p}$ background is as efficient as $p53^{p/+}$ and wild-type cells at 48 hours after UVR. This result suggests that the removal and repair of damaged bases *per se*, is not affected by the hypomorphic $p53^{p/p}$ mutation.

Next we analyzed if *in vivo* hypersensitivity observed in $p53^{p/p}$ mice occurs due to problems in repair of UV-induced CPD and (6-4) photoproducts, again by extracting DNA samples from mouse skin at 0, 6, and 24 hours post UVB. At 5.0 kJ/m^2 of UVB, a dose which showed significant immunosuppression and hypersensitivity, the (6-4) photoproducts were removed rapidly in both genotypes, with only 53-67% remaining at 6 hours and 19-43% still remaining after 24 hours; no significant changes in the rate of repair between genotypes was detected (Figure 10b). The removal of CPD was initially marginally slower with 51-71% remaining at 6 hours and 24-48% still remaining at 24 hours, yet the rate of repair was similar regardless of the genotype (Figure 10b). Taken in aggregate, these results indicate that the increased sensitivity of $p53^{p/p}$ mice by UV carcinogenesis is not caused by defects in the repair of UV-induced DNA damage.

3.2.6 Wild-type MEFs undergo apoptosis after exposure to UV radiation.

The characteristic cellular response for wild-type MEFs upon exposure to UV radiation is to initiate apoptosis, predominantly dependent upon p53 to mediate this death pathway (Naik et al, 2007; Zhang et al, 2008). Considering the $p53^{p/p}$ mutation

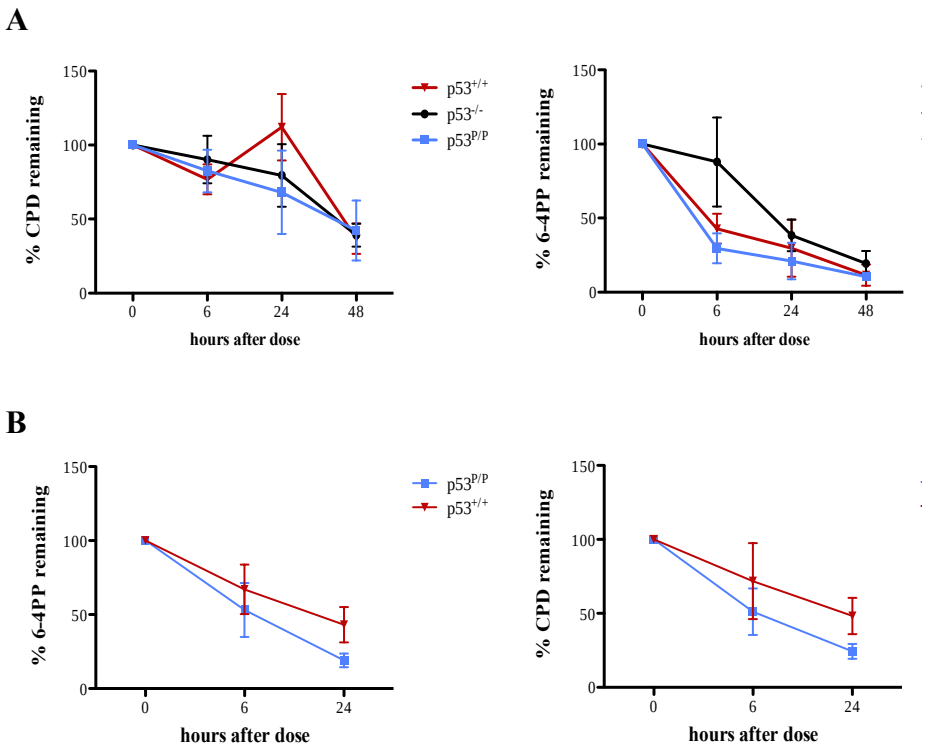

Figure 10. Mutant p53$^{p/p}$ cells remove UV-induced damaged bases normally.
(A) DNA was extracted from MEFs 0, 6, 24, and 48h post UVB exposure (200J/m^2). Photoproducts were measured by RIA using antibodies specific for CPDs or (6-4) photoproducts (6-4PP). Representative results are presented; each experiment was repeated independently more than 3 times. **(B)** DNA from dorsal skin of mice 0, 6, and 24h post UVB exposure (5 kJ/m^2); photoproducts were measured as described above. N=5 mice per time point.
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renders cells resistant to p53-dependent apoptosis (Liu et al, 2004; Rowan et al, 1996), we hypothesized that $p53^{p/p}$ MEFs would act similar to $p53^{-/-}$ MEFs in terms of initiating apoptosis. Therefore, to determine the extent of apoptosis in wild-type and mutant MEFs after UVB radiation, we stained irradiated MEFs with labeled AnnexinV and propidium iodide (PI), followed by analysis by flow cytometry (Vermes et al, 1995). MEFs were exposed to UVB (100 and 250J/m^2) and harvested 24 and 48 hours later. In response to 250J/m^2 of UVB, 20% of the wild-type MEFs underwent apoptosis after 24 hours of recovery (Figure 11a), correlating with previous reports (Peters et al, 2003). This percentage increased from 28% to 63% when cells were allowed to recover for 48h (Figure 11b). A typical histogram profile is shown in Figure 11c. The apoptosis observed is clearly dependent on p53, since a low level of apoptosis (under 10%) was detected in the p53-deficient MEFs; as hypothesized, $p53^{p/p}$ MEFs exhibited an almost identical apoptotic profile to $p53^{-/-}$ MEF, further indicating that $p53^{p/p}$ background is deficient in p53-mediated apoptosis.

3.2.7 In the absence of apoptosis, $p53^{R172P}$ MEFs undergo senescence after UV exposure.

Next, we asked if the initial DNA damage caused by UVB exposure could drive $p53^{p/p}$ MEFs into cellular senescence. Wild-type, $p53^{p/p}$ and $p53^{-/-}$ MEFs were treated with UVB radiation at doses of 100 and 250J/m^2, harvested 24 and 48 hours later, and scored for positively stained cells for senescence associated β-galactosidase. As shown in Figures 12a and 12b, both wild-type and $p53^{-/-}$ MEFs displayed a low percentage of senescent cells, while a significantly higher percentage of senescent cells were detected in $p53^{p/p}$ MEFs. The percentage of senescent cells exceeded 50% in $p53^{p/p}$ MEFs 48

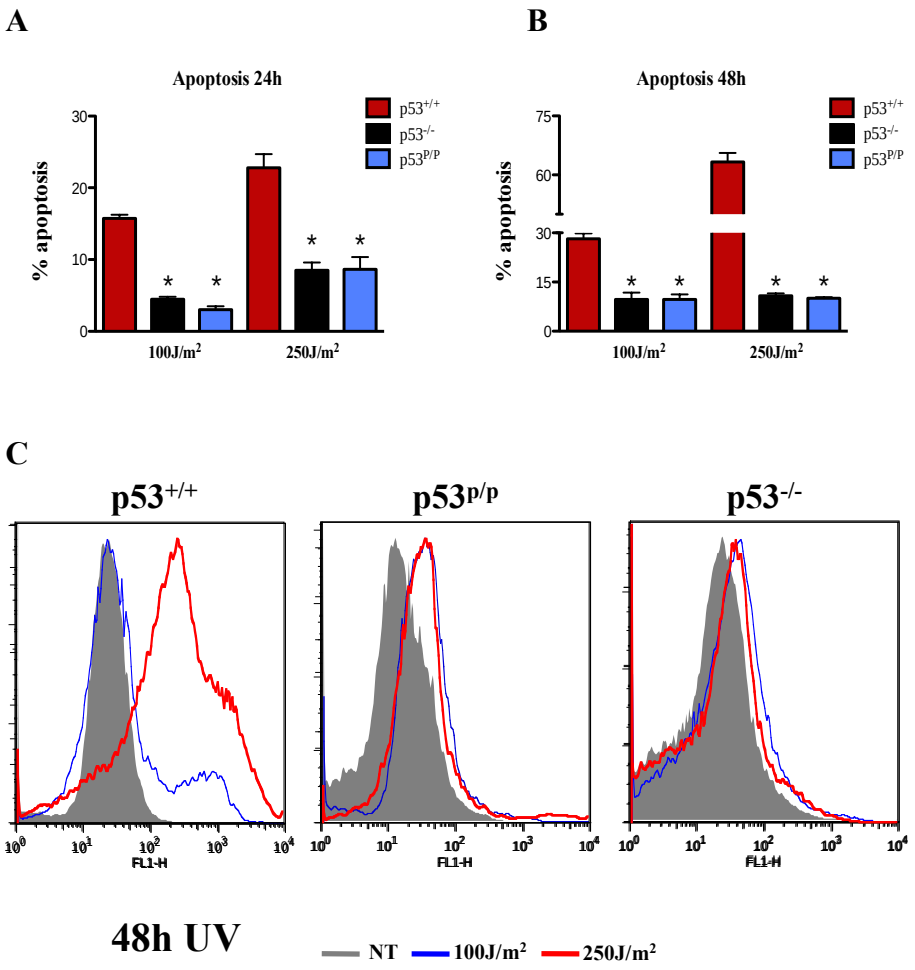

Figure 11. Wild-type MEFs undergo apoptosis after UVB unlike $p53^{p/p}$ and $p53^{-/-}$ MEFs. Apoptotic cells were measured using Annexin V and PI staining followed by FACS analysis after 24h **(A)** and 48h **(B)**. Error bars represent mean ± SEM. Differences determined using a two-tailed, unpaired student t-test. * indicates $p<0.001$. **(C)** Representative histogram profiles after Annexin V staining after 48h post UVB treatment. Experiments repeated a minimum of 3 times.
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hours after 250J/m^2 treatment compared to less than 20% found in wild-type $p53^{-/-}$ MEFs. Interestingly, although a significantly higher level of senescent cells were present in $p53^{p/p}$ MEFs after IR treatment when compared with wild-type and $p53^{-/-}$ MEFs, the overall number is much lower after IR treatment (only 3-6 %), indicating that MEFs do not rapidly undergo senescence after acute IR treatment (Figures 12c and 12d**)**. Representative fields of senescent $p53^{p/p}$ MEFs stained with SA-β-gal and counter-stained with H&E are shown in Figure 12e. Further, to analyze the activation of the p53/p21 axis, we utilized immunofluorescent staining on MEFs after 100 and 250J/m^2 of UVB. In both cases, an increase in both nuclear p53 and p21 was noted in the $p53^{p/p}$ mutant, indicative of activated cellular response—namely cell cycle arrest (Figure 13a and 13b); these results are similar to that observed in the skin staining. Taken in concert with *in vivo* skin data, these results indicate that the $p53^{p/p}$ mutation responds to UV-induced DNA damage by progressively driving cells into senescence in a time- and dose-dependent manner, which explains the lower expansion rate after UV treatment in $p53^{p/p}$ MEFs. Collectively, these results indicate hypomorphic $p53^{p/p}$ is activated upon UV-induced damage, but not gamma-irradiation, to drive cells into senescence.

3.2.8 Hypomorphic $p53^{p/p}$ is unable to transactivate proapoptotic genes upon UVB.

After exposure to DNA damage, p53 is stabilized and will transactivate certain downstream targets to induce a network of cellular programs. To determine the differences between wild-type and $p53^{p/p}$ in response to UVB irradiation, we analyzed the protein levels of known downstream p53-dependent apoptotic and cellular senescent markers 24, 48, and 72 hours after UVB irradiation (Figure 14a). Low levels of p53

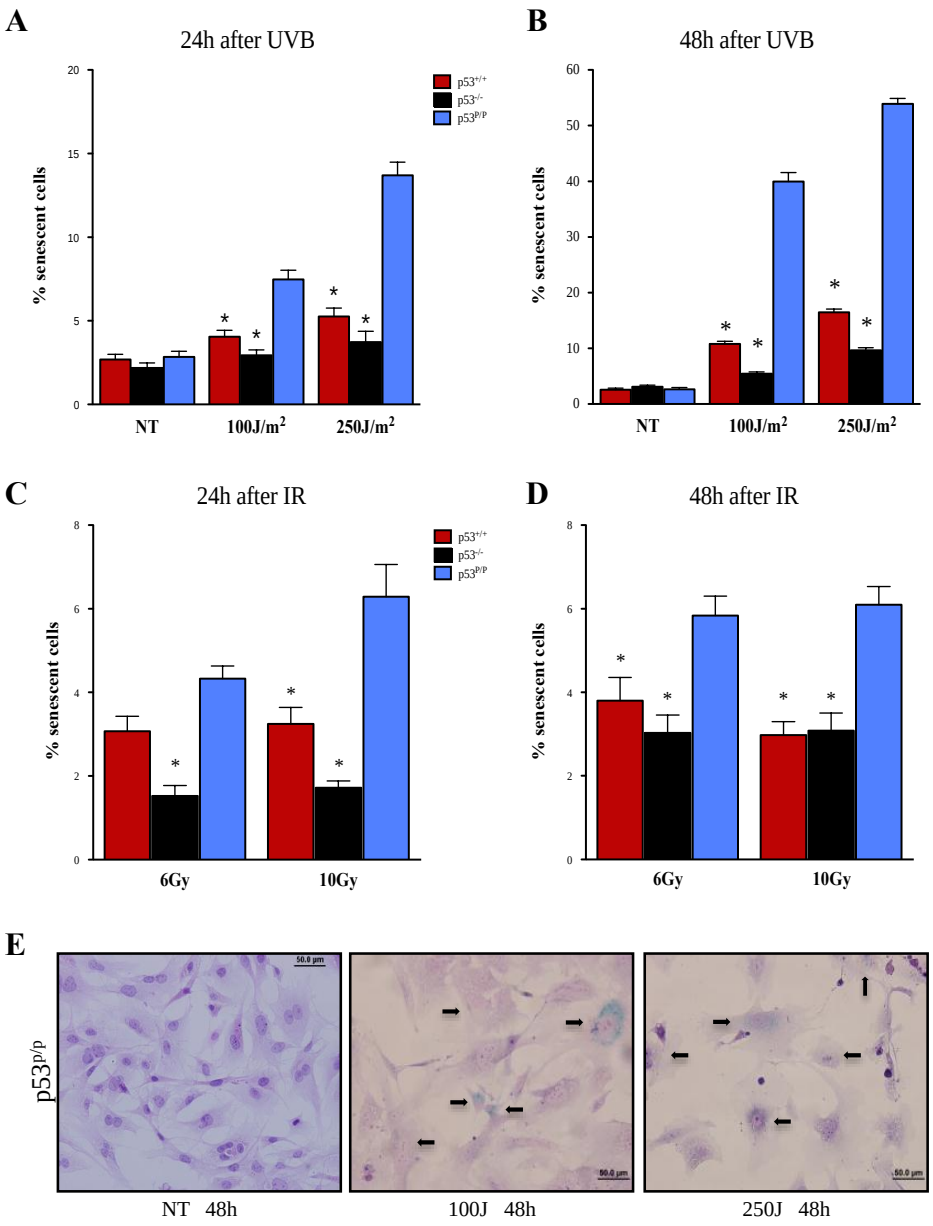

Figure 12. p53$^{p/p}$ MEFs undergo cellular senescence after UVB.
Quantification of SA-b-gal positive cells 24h **(A)** and 48h **(B)** after indicated doses of UVB. Quantification of SA-b-gal 24h **(C)** and 48h **(D)** after indicated doses of IR. Error bars represent mean ± SEM. * indicates $p<0.005$ vs wild-type. **(E)** Representative fields of p53$^{p/p}$ MEFs, untreated (left panel) and those exposed to 100 J/m^2 and 250J/m^2 of UVB radiation (middle and right panels) were stained with SA-b-gal and counterstained with H&E. Magnification: 40X
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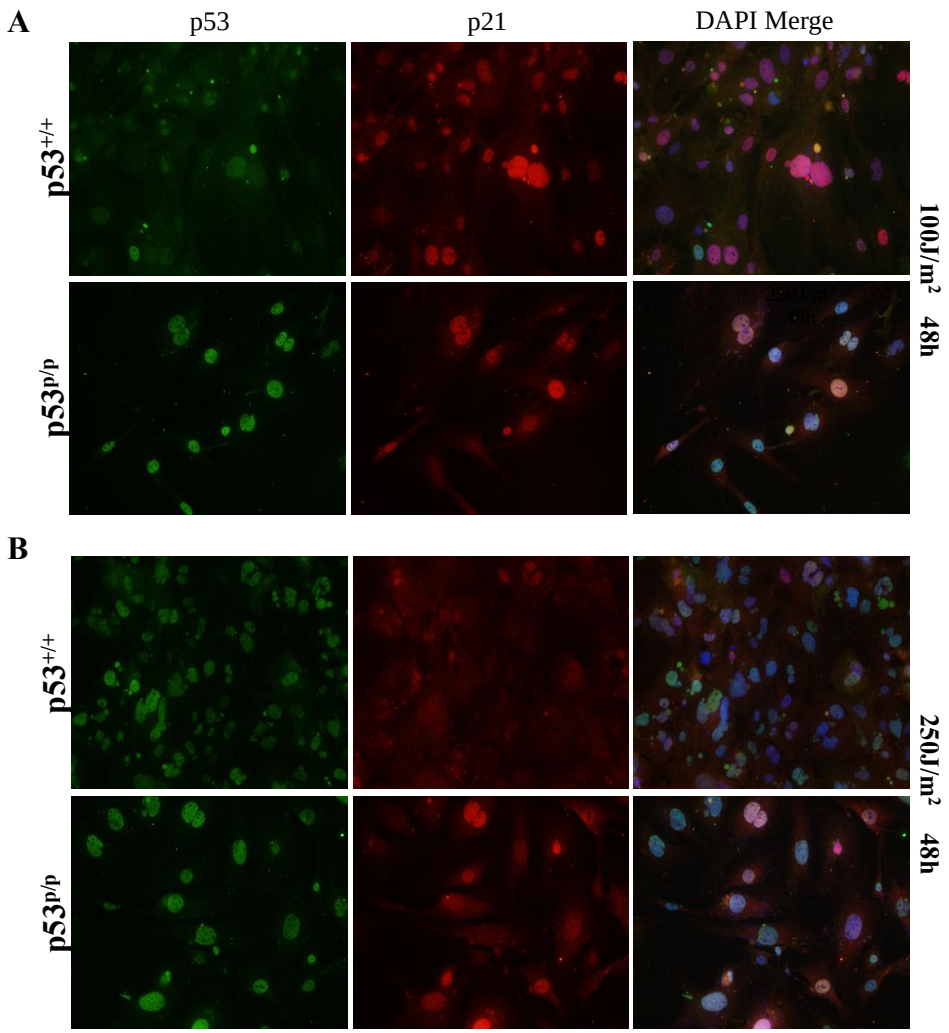

Figure 13. Immunofluorescent staining of p53 and p21 in MEFs after UVB.
Representative fields of $p53^{+/+}$ and $p53^{p/p}$ MEFs stained for p53 (green), p21 (red), and DAPI (blue) after 48 hours of 100J/m2 **(A)** or 250J/m2 **(B)** of UVB. Magnification: 40X.
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were detected in non-irradiated MEFs as well as in MEFs that received 100J/m^2 of UVB (data not shown). However, at 250J/m^2 the level of p53 in wild-type cells initially increased (24h), but began to decrease 48 and 72h post irradiation. In contrast, $p53^{p/p}$ levels constantly remained high. Increased p21 levels were noted in both wild-type and $p53^{p/p}$ MEFs after UVR at 250J/m^2 (Figure 14a). There is a slightly reduced level of p21 in the mutant MEFs compared to wild-type samples, which is consistent with previous reports that $p53^{p/p}$ mutant cells retain a partial ability to transactivate p21 (Barboza et al, 2006; Liu et al, 2004). Significantly, UVR upregulated the levels of proapoptotic protein PUMA (p53 upregulated modulator of apoptosis) in wild-type MEFs, while PUMA levels, as well as another p53-dependent proapoptotic protein NOXA, remained low in $p53^{p/p}$ cells. These results are consistent with the hypomorphic mutant being unable to transactivate p53-mediated apoptosis. Therefore, the lack of apoptosis after UVB seen in $p53^{p/p}$ is partially due to the inability of mutant p53 to transactivate proapoptotic factors that are important to induce apoptosis. Further, anti-apoptotic protein Bcl-2 levels decreased in wild-type after UVB treatment, which is in opposition to that seen in $p53^{p/p}$ MEFs where Bcl-2 levels remained high, and only slightly decreased at 72h in $p53^{p/p}$. Lastly, the kinetics of these proteins show an activation of PUMA, Noxa, and cleaved Parp upon 12 hours post UVB exposure in wild-type MEFs (Figure 14b). The involvement of additional apoptotic markers, Bax and Bak, were ruled out since these levels are not changed before or after the UVB treatment (Tavana et al, 2010a).

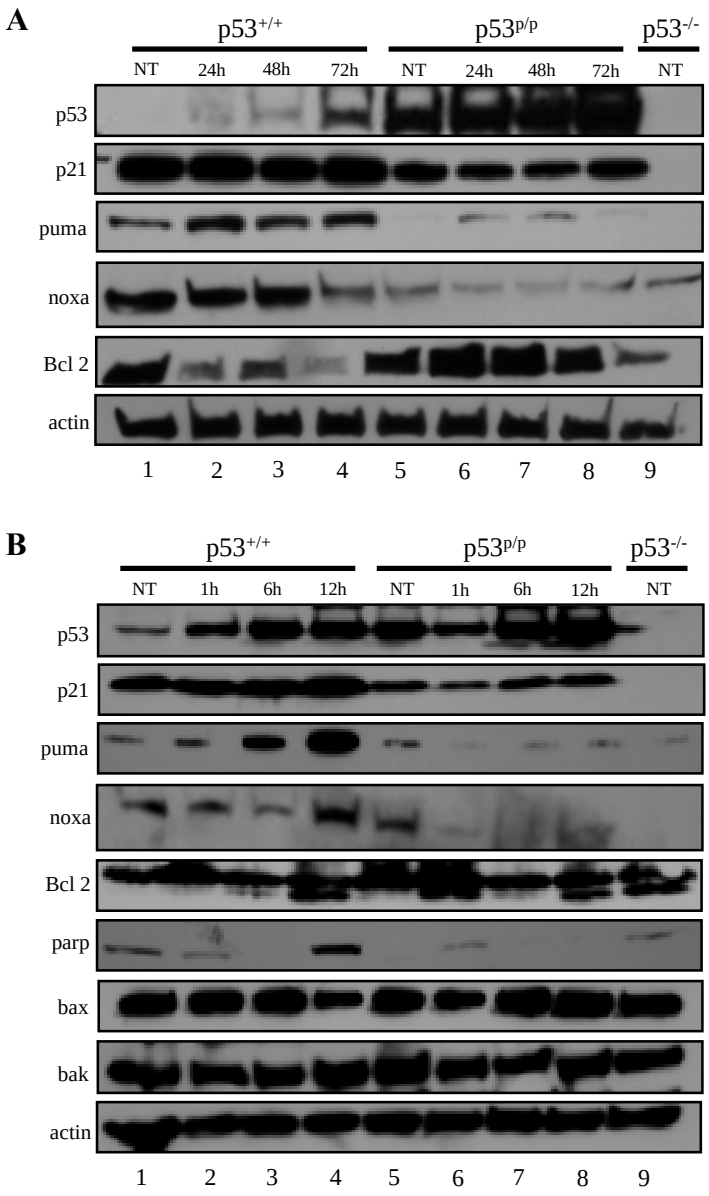

Figure 14. Western Blot Analysis of MEFs after UVB exposure.
Western Blot analysis of $p53^{+/+}$, $p53^{p/p}$, and $p53^{-/-}$ MEFs after 100J/m^2 **(A)** and 250J/m^2 **(B)** at indicated time points. Blots were probed for p53, p21, puma, noxa, Bcl-2, Parp, Bax, and Bak. b-actin is shown as a loading control.
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3.3 Discussion

By guarding the genome, p53 plays a central role in mediating cellular responses after UV radiation (Latonen & Laiho, 2005; Lavin & Gueven, 2006). This study demonstrates that the hypomorphic $p53^{p/p}$ mutation leads to UVB hypersensitivity both *in vitro* and *in vivo*. Abrogating p53-dependent apoptosis, mutant mice showed less tolerance to UVB radiation. Upon low doses, $p53^{p/p}$ mice suffered from severe skin swelling and higher immunosuppression compared to wild-type mice. However, this was independent to changes in repairing the UV-induced damage, indicating that the increased susceptibility results specifically from UV-induced cellular responses.

UV radiation has been shown to induce immunosuppression, identified as a major risk factor for cancer induction (Beissert & Loser, 2008). Further, UV radiation specifically causes immune suppression resulting in a susceptibility to UV-induced skin tumors (Kripke, 1981; Kripke & Morison, 1985). Interestingly, $p53^{p/p}$ mice were significantly more susceptible to the immunosuppressive effects of UV radiation, being observed at very low doses of UVR that did not suppress the delayed type hypersensitivity in wild-type mice. These results differ from $p53^{-/-}$ mice, which did not display increased susceptibility to immune suppression compared to wild-type mice (Jiang et al, 2001). Although different strains of mice exhibit different levels of UV-induced immune suppression (Hart et al, 1998; Noonan & Hoffman, 1994), it is possible that the strain utilized here (C57BL/6 129/Sv mix) could explain the discrepancy between the results observed in $p53^{-/-}$ and $p53^{p/p}$ mice. This is, however, unlikely because the littermate controls in our study did not exhibit the same sensitivity as $p53^{p/p}$ mice. Considering we did not observe defects in the repair of UV-induced DNA damage

or other genetic alterations in *p16*^{INK4a}, *p19*^{ARF}, and *RAS* genes in the p53$^{p/p}$ mice (data not shown), we concluded that the inability to repair DNA damage did not contribute to the increased susceptibility. Instead, the strong induction of permanently arresting the cell cycle may be the main contributor to the hypersensitivity after UVB treatment. Consistent with this notion, a recent study linked chronic UV exposure to the induction of premature senescence in human skin fibroblasts (Chen et al, 2008), potentially exacerbating UV-induced photoaging. Further research to investigate the role of p53-dependent apoptosis regarding UV-induced inflammation and immunosuppression is needed. Other p53 mutations may also be linked to immune suppression and possibly directly connected to tumorigenesis, an area not yet well explored.

The nucleotide excision repair pathways fixes UV-induced DNA damage (Nouspikel, 2009); as mentioned above, cells with severely damaged DNA undergo apoptosis—p53 has a central role in both processes (Chang et al, 2008). p53 can bind certain NER factors and directly participate in DNA damage repair (Ford et al, 1998; Ford & Hanawalt, 1995) as well as pause the cell cycle to provide ample time for cells to repair the damage. Further, p53 is necessary to induce programmed cell death after DNA damage. Unlike p53-deficient fibroblasts that are grossly defective in NER, our study showed that mutant p53$^{p/p}$ cells have a normal ability to repair DNA after UVR. This indicates that DNA damage *per se* is not the sole cause of sensitivity to UV radiation treatment. How p53 participates during the NER process is still not completely understood. Specifically, some studies show p53 may directly bind to NER helicase factors XPD or XPB (Wang et al, 1995), or function in NER by regulating gene expression, such as XPC or XPE genes (Adimoolam & Ford, 2002; Hwang et al, 1999).

Further, p53 can transactivate genes that encode for the initiation of global genome repair (Helton & Chen, 2007). Our study indicates that the hypomorphic mutant $p53^{p/p}$ retains at least a partial function in terms of repairing UV-induced DNA damage.

This study reveals that two p53-dependent events are important for the induction of apoptosis after UVB exposure: 1. upregulation of pro-apoptotic genes such as *PUMA* and *Noxa* and 2. the downregulation of anti-apoptotic gene *Bcl-2*, through the inactivation of the transcriptional factor E2F1 (Knezevic et al, 2007; Wikonkal et al, 2003). Our hypomorphic p53 mutant is defective in both events. These results are consistent with a previous study reporting that loss of Noxa or PUMA renders MEFs partially resistant to UV-induced cell death (Naik et al, 2007). Interestingly, overexpressing Bcl-2 also inhibits UV-induced apoptosis, which mimics the mutant $p53^{p/p}$ MEFs after UV radiation. The down regulation of Bcl-2 by RNA interference can restore the apoptotic response after UVB exposure in $p53^{-/-}$ fibroblasts (Knezevic et al, 2007), indicating that Bcl-2 plays a critical role in deciding this cell fate. It is interesting to observe that $p53^{p/p}$ fails to transactivate PUMA/Noxa but retains the ability to upregulate p21 to almost normal levels. Although we don't know the mechanism, a clue maybe lie the nature of promoters that p53 regulates. Rrecently, a study showed the kinetics of genes involved in different cellular responses are expressed differently—regulators of the cell cycle have "preloaded" promoters and can be activated much faster than the apoptotic molecules that need to assemble and activate the pre-initiation complex (Morachis et al, 2010). Therefore it is tempting to speculate, in regard to the inability of the mutant p53 to transactivate certain promoters could be due either to steric hindrance presented by the point mutation in the DNA binding domain or perhaps a lack

of consensus sequences needed to bind properly. Regardless, further studies are needed to understand exactly why the hypomorphic $p53^{p/p}$ cannot transactivate apoptotic molecules yet retains a partial ability to activate cell cycle regulators. Our data suggests this maybe why $p53^{p/p}$ cells undergo cellular senescence.

UV and IR exposure inflict different types of DNA damage, which trigger different responses upstream of p53; this may explain our observation that UVR and not IR exposure induces MEFs into apoptosis. UV treatment mainly causes modification of chemical bonds to the DNA bases. Modifications on pyrimidine bases form dimers or (6-4) photoproducts between C-T bases causing distortions in the chromatin structure different from the DNA double strand breaks caused by IR. Although they share some overlapping targets in cell cycle checkpoint activation, ATM and ATR are activated by different kinds of DNA damage. ATM senses double-strand ends and ATR senses single stranded DNA bound with RPA (Cimprich & Cortez, 2008). Further, ATR is activated slower when compared to ATM, which is mainly due to the DNA breaks in those cells being recognized during the S and G_2 phase of the cell cycle. UVB-induced genomic damage can be sensed via ATR (Unsal-Kacmaz et al, 2002), to activate a Chk1-dependent signaling pathway arresting the cell cycle, increasing DNA repair, or triggering senescence/apoptosis. Relevant to this study, UV and IR radiation leads to different p53 post-translational modifications. Specifically, UV leads to phosphorylation at Ser389, while IR does not, resulting in the activation of different downstream effectors. Interestingly, $p53^{S389A/\ S389A}$ mice are more susceptible to tumors after chronic UV exposure but not after IR exposure (Bruins et al, 2004). Therefore, different types of DNA damage can modify p53 at specific sites to trigger certain cellular responses.

The cellular response to UVR also differs amongst cell types (D'Errico et al, 2007). Recent studies show that the engagement of ATR-ATRIP with the TOPBP1 molecule triggers the DNA damage response and induces cells to undergo p53-dependent senescence (Toledo et al, 2008). This study specifically found that short transient signaling resulted in cell cycle arrest, whereas longer persistent signaling led to senescence. Our study shows that UVR treatment leads to apoptosis in wild-type mice in a dose- and time-dependent fashion, but induced senescence in the absence of apoptosis. Similar to our $p53^{p/p}$ mutant, where deletion of the proline rich domain of p53, important for the induction of apoptosis, eliminates the apoptosis pathway but not cell cycle arrest (Slatter et al, 2010). This mutation changes the tumor spectrum, where the mutant mice succumb to B-cell lymphoma in comparison with p53 null mice, which succumb to mostly thymic lymphomas. Most importantly, our work suggests that p53-dependent apoptosis, along with DNA repair and cell cycle arrest in response to UVR, work together to control the proliferation of cells and maintain the genomic integrity. These responses are necessary to ensure cell homeostasis. A deficiency in one component, in our case, the apoptotic pathway, may lead to unbalanced cell homeostasis that might propagate into pathogenic consequences if not suppressed (Tavana et al, 2010a). This study is a clear example of p53-dependent cellular responses, rather than DNA repair activity *per se*, which can play a dominant role in the regulation of cell growth and proliferation.

CHAPTER 4

ABSENCE OF P53-DEPENDENT APOPTOSIS COMBINED WITH NON-HOMOLOGOUS END JOINING DEFICIENCY LEAD TO A SEVERE DIABETIC PHENOTYPE IN MICE

4.1 Introduction

"As mentioned above, classic non-homologous end joining is one of the two major pathways for the repair of DNA double strand breaks (DSB) (Mahaney et al, 2009). The factors involved form a DNA recognition complex composed of DNA-dependent kinase complex, DNA-PKcs, a Ku70/80 heterodimer, and repair factor Artemis. Once the DNA ends are processed, they are mended together using the DNA ligase complex composed of DNA ligase IV, XRCC4, and Cernunnos/XLF (Huang et al, 2009). Both DNA ligase IV and XRCC4 are absolutely required for the classic NHEJ pathway, as their deficiencies lead to embryonic lethality (Frank et al, 1998; Gao et al, 1998). Further, global NHEJ deficiencies render cells hypersensitive to DNA damaging agents, such as ionizing radiation, and premature senescence (Mills et al, 2004). During V(D)J recombination, programed double strand breaks are introduced purposely to allow for DNA segment rearrangement of the Variable, Diversity, and Joining regions—the NHEJ is extremely critical in mending these programed double strand breaks. In developing lymphocytes, the abrogation of NHEJ factors results in the accumulation of unrepaired DNA breaks, which reaches a threshold and activates p53-mediated apoptosis to eliminate genomic instability. Spontaneous DNA damage also occurs in other organs, such as the brain and CNS, and if too excessive, as in the case of DNA Ligase IV or XRCC4 deficiencies, can lead to an exorbitant amount of cells undergoing apoptosis and cause embryonic lethality (Frank et al, 1998; Gao et al, 1998). This embryonic lethality is rescued by p53 deficiency (Frank et al, 2000; Gao et al, 2000),

because the apoptotic ability of p53 is deleted. This comes at a cost, because the cell cycle arrest function of p53 is also deleted, driving aggressive and lethal pro-B lymphomas at an early age (Figure 15a) (Zhu et al, 2002). All these lymphomas carry a chromosomal translocation between the immunoglobulin and the proto-oncogene c-MYC loci, forming a complicon structure, which leads to amplification of the MYC genes (Figure 15a). Interestingly, the hypomorphic, separation-of-function mutant $p53^{p/p}$ (Liu et al, 2004), defective in apoptosis yet retains a partial cell cycle arrest function, not only rescues embryonic lethality but also entirely eliminates lymphomagenesis in the $Lig4^{-/-}$ (Figure 15b) (Van Nguyen et al, 2007). Analyses of $Lig4^{-/-}p53^{p/p}$ tissues revealed extensive cellular senescence in the developing lymphocytes with increased levels of p53 and p21 (Figure 15c). This indicates that Rag-mediated DSBs, which remain unrepaired due to the deficiency in end-joining, activate the hypomorphic p53 and putative downstream target p21, to ultimately drive these cells into cellular senescence. This p53-p21 driven senescence is proven to be an effective barrier in suppressing genomic instability and tumorigenesis. These studies are summarized in a schematic in Figure 16.

Cellular senescence is a mechanism, which prevents cells to undergo apoptosis and halts cell proliferation in tissues despite the potent tumor suppressor activity. These properties can accelerate ageing-related diseases (d'Adda di Fagagna, 2008). Although DNA damage-induced senescence can successfully prevent lymphomagenesis, all $Lig4^{-/-}p53^{p/p}$ mice have an ageing appearance and die before they reach 6 months of age (Figure 15b). After analysis of the glucose

levels in the urine of old Lig4$^{-/-}$p53$^{p/p}$, we predicted these mice were developing diabetes. Therefore, we hypothesized that spontaneous DNA damage, like that occurring in the brain, may also activate p53 and p21 to induce cellular senescence and decrease cellular proliferation and organ renewal capability. As a result, the insulin producing cells would not proliferate, and the maintenance of homeostasis would be compromised. Indeed, we describe the first animal model that progressively develops diabetes with a depletion of pancreatic β-cell mass due to accumulated DNA damage and a p53-dependent response that drives cells into senescence. Our model indicates that age-related DNA damage accumulation in the pancreatic β-cells, and its associated senescence, may be one cause of diabetes (Tavana et al, 2010c).

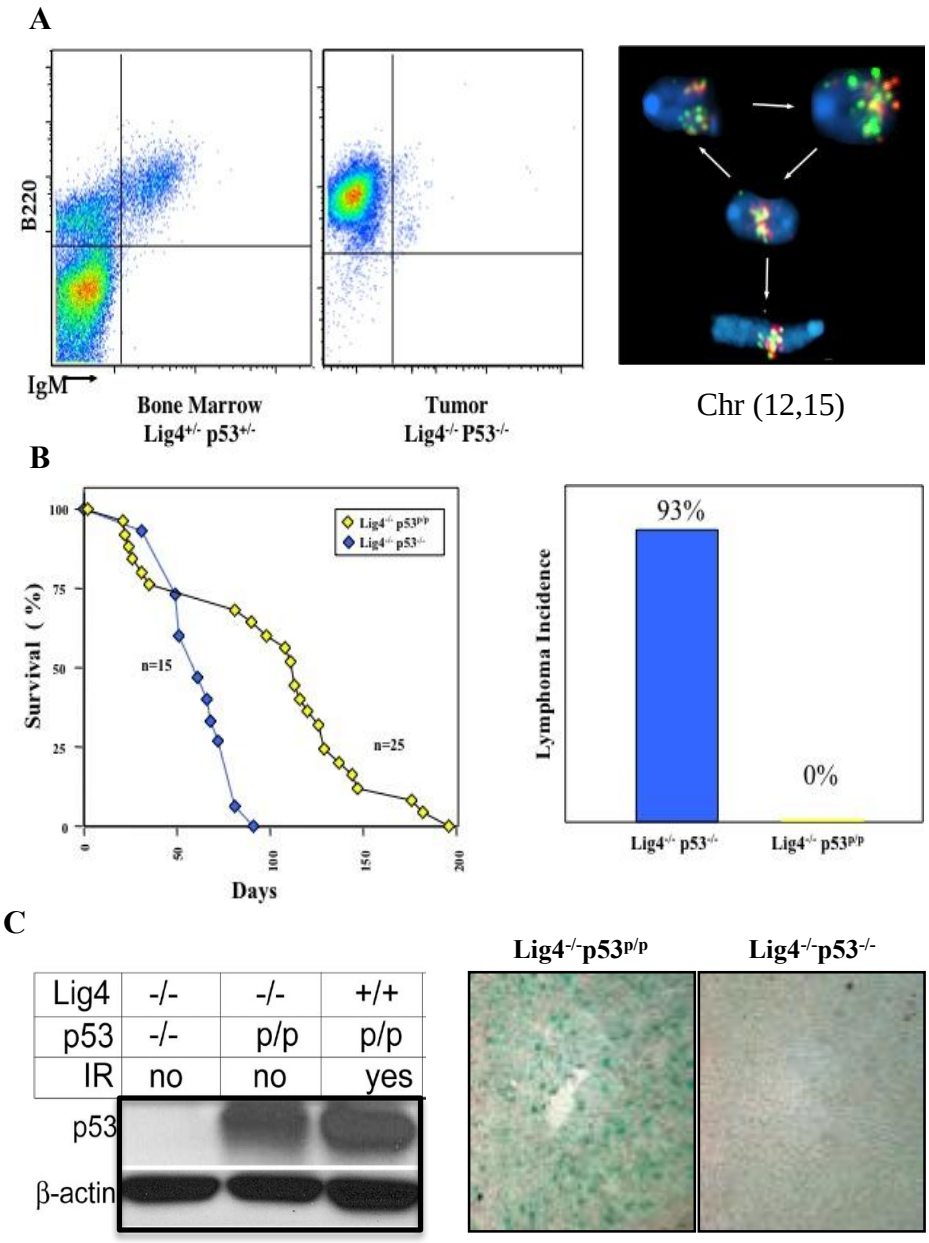

Figure 15. Ligase IV deficiency combined with either $p53^{-/-}$ or $p53^{p/p}$.
(A) Flow cytometric analysis of $Lig4^{+/-}p53^{+/-}$ B-cells or $Lig4^{-/-}p53^{-/-}$ pro-B cell lymphomas (left panels). FISH of IgH and c-MYC of $Lig4^{-/-}p53^{-/-}$ depicting the 'breakage-fusion-bridge' cycle (far right panel). **(B)** Kaplan-Meier curve or $Lig4^{-/-}p53^{p/p}$ (yellow) and $Lig4^{-/-}p53^{-/-}$ (blue). Percent lymphoma incidence in $Lig4^{-/-}p53^{-/-}$ and $Lig4^{-/-}p53^{p/p}$ mice (far right panel). **(C)** Western blot analysis of thymocytes detecting p53 levels. Representative staining of β-gal in $Lig4^{-/-}p53^{p/p}$ and $Lig4^{-/-}p53^{-/-}$ thymic sections (far right panel).
(Modified and reproduced from (Zhu, 2002) and (van Nguyen, 2007) with permission from Cell and Journal of Experimental Medicine, respectively)

4.2 Results

4.2.1 $Lig4^{-/-}p53^{p/p}$ mice exhibit glucose intolerance and impaired insulin production.

Previously, we showed that $Lig4^{-/-}p53^{p/p}$ mice do not develop lymphomas or any other type of tumors, yet die within 6 months of age (Figure 15b) (Van Nguyen et al, 2007). Aged mutant mice often appeared emaciated and lethargic prior to death. Analyzing the cause of death, the Veterinary Medicine Department of MDACC observed high levels of glucose in the urine of a 5-month old $Lig4^{-/-}$ $p53^{p/p}$ mouse. To confirm this data, we monitored glucose levels in the urine over time. As shown in Figure 17a, $Lig4^{-/-}p53^{p/p}$ mice presented increased glucose levels in the urine as early as 1 month, reaching up to 1000mg/dL as early as 2 months and increased beyond the limit of the assay at 4 months when compared to aged-matched wild-type and $p53^{p/p}$ controls. For a more accurate measurement, we monitored the blood glucose levels in non-fasting $Lig4^{-/-}p53^{p/p}$ mice at different ages and found a progressive increase in blood glucose levels, which was significantly different ($p<0.005$) from the levels detected in age-matched wild-type mice (Figure 17b). Significant hyperglycemia was recorded as early as 2-month of age, suggesting a time-delayed postnatal development of diabetes in the $Lig4^{-/-}p53^{p/p}$ mice.

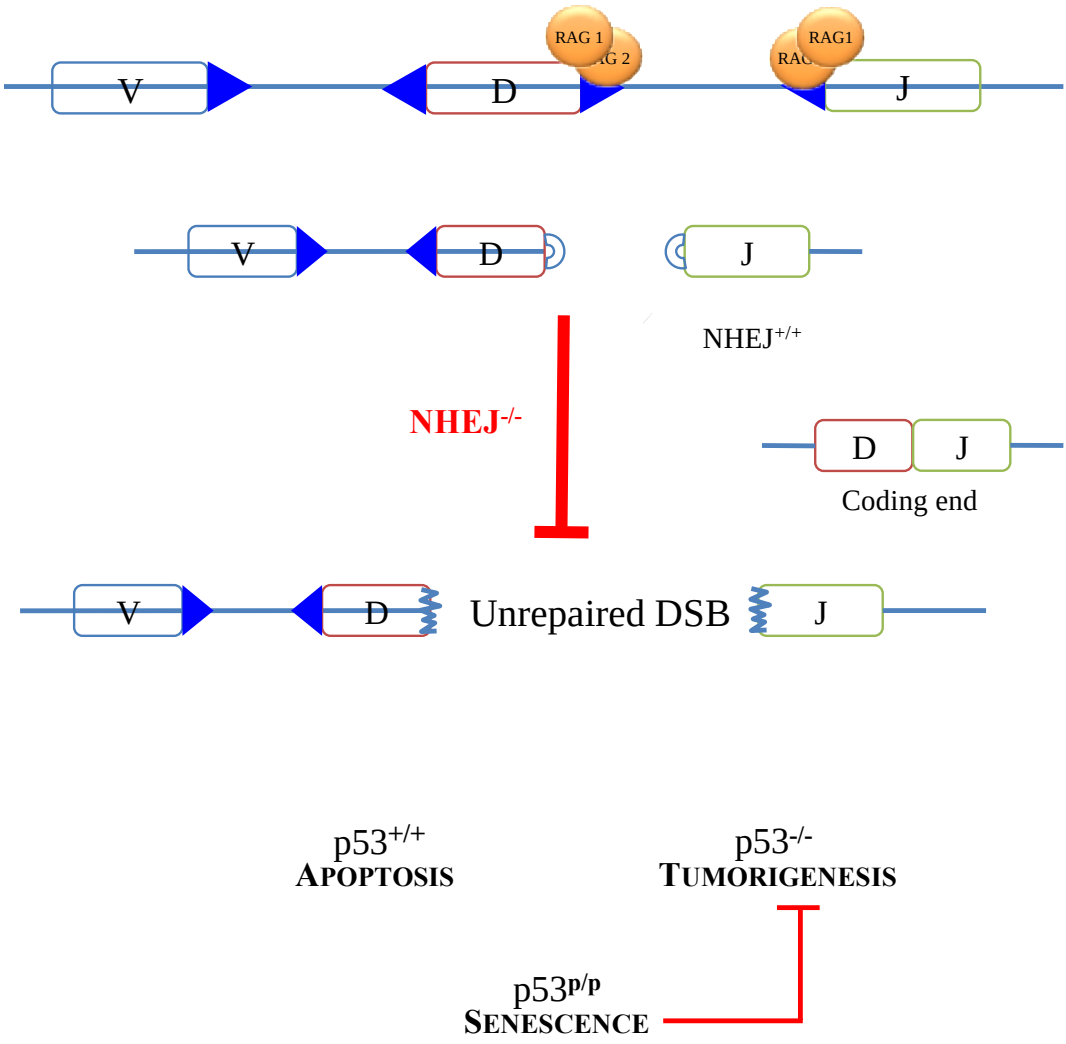

Figure 16. p53-dependent outcomes in the lymphoid system in the absence of NHEJ. During V(D)J recombination, Rag1/2 recognizes and cleaves DNA in double strand breaks (DSBs) at the recombination signal sequences (triangles). In a NHEJ-dependent manner, the hairpins are broken and merged together forming a coding end. In the absence of NHEJ, DNA DSBs remain and persist. The accumulation of unrepaired DSBs activates wild-type p53 ($p53^{+/+}$) to initiate apoptosis—a known mechanism to prevent tumorigenesis. In the absence of p53 ($p53^{-/-}$) cell cycle arrest and apoptotic functions are lost, and cells proliferate even with the persistent DNA damage. This leads to chromosomal translocations and tumorigenesis. Introducing a hypomorphic, separation-of-function mutant ($p53^{p/p}$), where apoptosis is abrogated yet cell cycle arrest remains intact, p53 triggers cellular senescence, which is in parallel to apoptosis, as a formidable barrier to tumorigenesis.

To determine the onset of diabetes, we performed a standard glucose tolerance test in both 1- and 3-month old mutant mice with age-matched wild-type mice as controls. The glucose tolerance test at 1-month of age did not exhibit a significant difference between the two groups (Figure 18a). Additionally, there was no significant difference in the secretion of insulin after glucose stimulation, as determined by ELISA (Figure 18b), depicting that 1-month old $Lig4^{-/-}p53^{p/p}$ pancreatic β-cells have no problems. However, 3-month old $Lig4^{-/-}p53^{p/p}$ mice showed hyperglycemia at the start point; after the glucose injection, the levels increased and were not readily cleared during the assay (Figure 18c). These results indicate a progressive impaired glucose tolerance and diabetes in the mutant mice. To determine the ability of pancreatic β-cells to secrete insulin upon glucose stimulation, we performed ELISA assays to determine the blood insulin levels in these mice. Unlike 1-month mutant mice, insulin levels in the 3-month old mutant mice were significantly lower ($p<0.005$) and were irresponsive to the glucose injection, suggesting insulin insufficiency in these mice (Figure 18d). Next, to determine if the overt diabetes was a cause of insulin resistance, we preformed an insulin tolerance test performed on 1-month and 3-month old mutant and wild-type mice, independently, which revealed no differences between the two genotypes, indicating normal insulin sensitivity prior to the onset of diabetes (Figure 18e and 18f). Collectively, these results indicate that $Lig4^{-/-}p53^{p/p}$ mice progressively develop diabetes due to an insufficient production of insulin, which results in impaired glucose tolerance.

A

	1wk	1month	2mon	3mon	4mon
WT	Negative	Negative	Negative	Negative	Negative
L4$^{+/+}$p53$^{P/P}$	Negative	Negative	Negative	Negative	Negative
L4$^{-/-}$p53$^{P/P}$	Negative	Neg-250	500-1000	1000-2000	>2000
number:	12	8	9	6	4

B

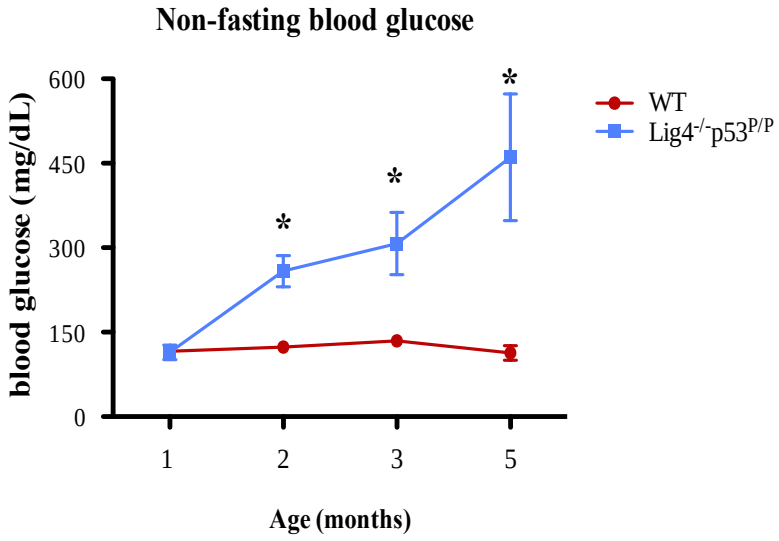

Figure 17. Lig4$^{-/-}$p53$^{p/p}$ mice progressively develop hyperglycemia
(A) Glucose levels in the urine were measured (mg/dL) over time in wild-type, Lig4$^{+/+}$p53$^{p/p}$ and Lig4$^{-/-}$p53$^{p/p}$ mice. **(B)** Glucose levels in the blood were measured (mg/dL) over time in wild-type (red circles) and Lig4$^{-/-}$p53$^{p/p}$ (blue squares) non-fasting mice. Each data point was an average of at least three animals. * represents $p<0.0001$ versus wild-type.
(Modified and reproduced from (Tavana, 2010) with permission from Diabetes)

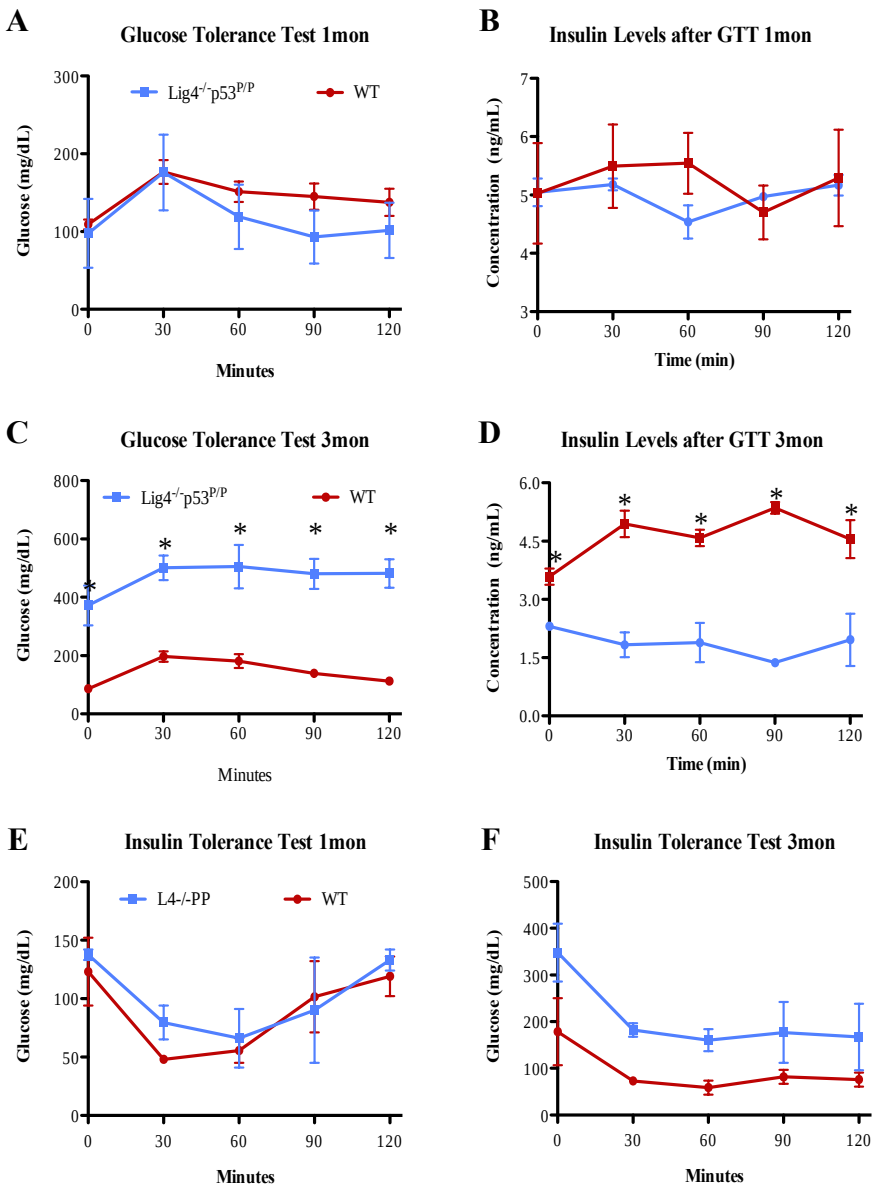

Figure 18. Lig4$^{-/-}$p53$^{p/p}$ mice are glucose intolerant but not insulin resistant. Blood glucose concentrations measured from a glucose tolerance test in wild-type and Lig4$^{-/-}$p53$^{p/p}$ mice at 1 **(A)** and 3 **(C)** months. Blood insulin concentrations were measured by ELISA at 1 **(B)** and 3 **(D)** months. Standard ITT test for insulin sensitivity in 1 **(E)** and 3 **(F)** month old wild-type and Lig4$^{-/-}$p53$^{p/p}$ mice. A minimum of three mice from each genotype were tested. * represents $p<0.005$ versus wild-type. (Modified and reproduced from (Tavana, 2010) with permission from Diabetes)

4.2.2 Progressive depletion of pancreatic β-cells in Lig4$^{-/-}$p53$^{p/p}$ mice.

Insulin insufficiency due to pancreatic β-cell attrition is a common characteristic in both types of diabetes. Type I diabetes results from the combined effects of environmental, genetic, and predominantly immunological factors that destroy pancreatic β-cells (Kukreja & Maclaren, 1999). Type 2 diabetes results from a progressive islet dysfunction leading to defective β-cell secretion and insulin resistance (DeFronzo, 1997; Karaca et al, 2009). In both cases, β-cell mass decreases. Although residual functional β-cells still exists, they are insufficient in number to maintain glucose tolerance. To determine whether the hyperglycemia and lowered insulin secretion observed in the Lig4$^{-/-}$ p53$^{p/p}$ mice was the result of a progressive decrease in β-cell mass, we examined the morphological changes in the pancreatic islets immunohistochemical staining for insulin. As shown in Figure 19a, mutant mice have a relatively normal size of pancreatic islets as compared to the wild-type at 1 week after birth. However, as mutant mice aged the size of the pancreatic islets gradually declined. A drastic involution in islet size was observed in the mutant mice as early as 1 month of age. At 5 months, an almost complete depletion of the pancreatic islets was observed. To quantitatively measure and assess the differences of β-cell mass in Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice, we determined the ratio of islet area to total pancreas area. Islet size was nearly normal at 1 week of age (p<0.25) (Figure 19b). In general, older mutant mice observed a drastic reduction in pancreatic islet area. A 46% reduction in islet area was observed in the mutant mice at 1 month (p<0.0001); by 5 months, a 73% decrease was observed in the

mutant mice (p<0.0001). Further immunohistochemical staining for glucagon, indicative of α-cells which signal the liver to breakdown glycogen and produce glucose upon hypoglycemic conditions, did not reveal any abnormalities in these mutant mice, yet still show islet attrition (Figure 20a). Lastly, although the measuring of islet area to total pancreatic area is a proportion, the possibility of $Lig4^{-/-}p53^{p/p}$ mice having a smaller pancreas was ruled out through analyzing the ratio of pancreas-to-body weight between mutant and wild-type mice (Figure 20b). Collectively, these results clearly indicate a severe diminishment of the pancreatic islet mass in $Lig4^{-/-}p53^{p/p}$ mice, which directly correlates with the progressive onset of diabetics observed.

4.2.3 The depletion of the pancreatic islets is not due to apoptosis or innate immunity.

The depletion of pancreatic islets in type I diabetes is mainly caused by activated immune cells specifically attacking β-cells. This is unlikely to happen in the $Lig4^{-/-}p53^{p/p}$ mice as the deficiency in NHEJ depletes all lymphocytes due to incomplete V(D)J recombination. Therefore, we examined whether the innate immunity branch has any involvement in β-cell depletion. Pancreatic sections from different ages were stained for a CD11b surface marker, to identify any infiltrated granulocytes, monocytes, dendritic cells or natural killer cells. Few cells were stained positive for CD11b in either $Lig4^{-/-}p53^{p/p}$ or wild-type pancreatic sections, while many cells were CD11b positive in the control spleen section (Figure 21).

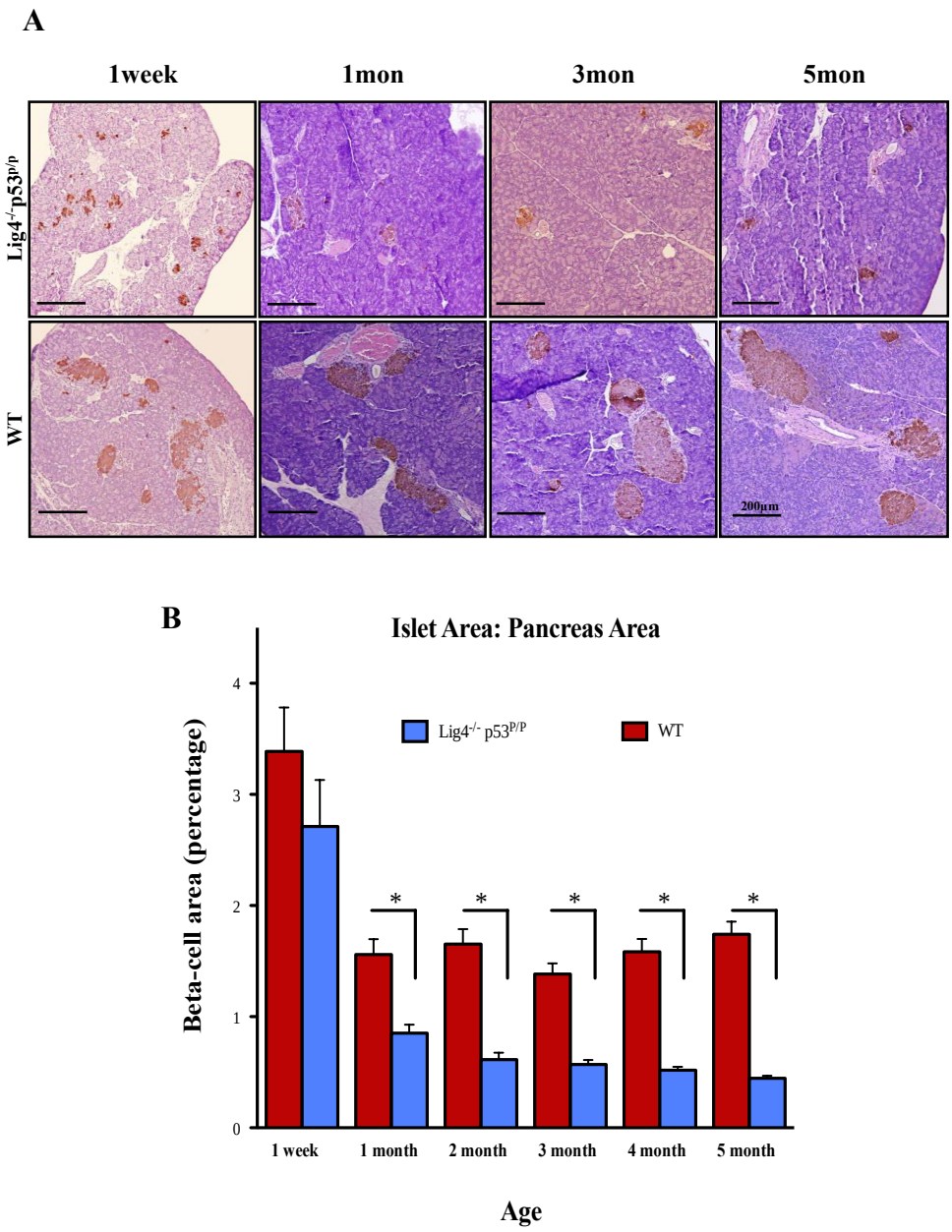

Figure 19. A progressive decrease is islet mass observed in Lig4$^{-/-}$p53$^{p/p}$ mice.
(A) Representative pancreatic sections from Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice stained by immunohistochemistry for insulin at indicated time points. Magnification: 40X. **(B)** Islet morphometric quantification of Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice. Multiple sections were analyzed for each pancreas, and 3-4 mice per group were analyzed, data represent the mean ± SEM; * represents p<0.0001 versus wild-type.
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We also stained for F4-80 and Toluidine blue, a marker of infiltrated macrophages and mast, respectively. Similar to CD11b staining, macrophage and Toluidine blue-positive cells were rarely detected in pancreatic sections in both genotypes (Figure 21). These results suggest that it is unlikely innate immunity is responsible for the destruction of the $Lig4^{-/-}p53^{p/p}$ pancreatic islets. Many diabetic models with resulting β-cell loss occur from an increase in islet-specific apoptosis (Butler et al, 2003; Scaglia et al, 1997). Although the $p53^{R172P}$ mutation prevents the induction of p53-mediated apoptosis, programmed cell death in pancreatic islets may occur independently of p53 expression (Nam et al, 2008). To test whether apoptosis is causing the pancreatic islet depletion observed in $Lig4^{-/-}p53^{p/p}$ mice, we performed a TUNEL assay in pancreatic sections. Apoptosis was not observed in 3-month mutant pancreatic sections while only a very low level of apoptosis was observed in wild-type as compared to a NHEJ deficient thymus section (Figure 22). Similar results were obtained with mice at different ages (results not shown). These data suggest that pancreatic islet depletion observed in the $Lig4^{-/-}p53^{p/p}$ mice is not due to apoptosis.

4.2.4 Decreased proliferation of β-cells in $Lig4^{-/-}p53^{p/p}$ mice.

The genesis of adult insulin-producing β-cells predominantly occurs through self-duplication of mature cells rather than through differentiation from their stem-cell progenitors (Dor et al, 2004; Georgia & Bhushan, 2004; Salpeter et al, 2010; Teta et al, 2007).

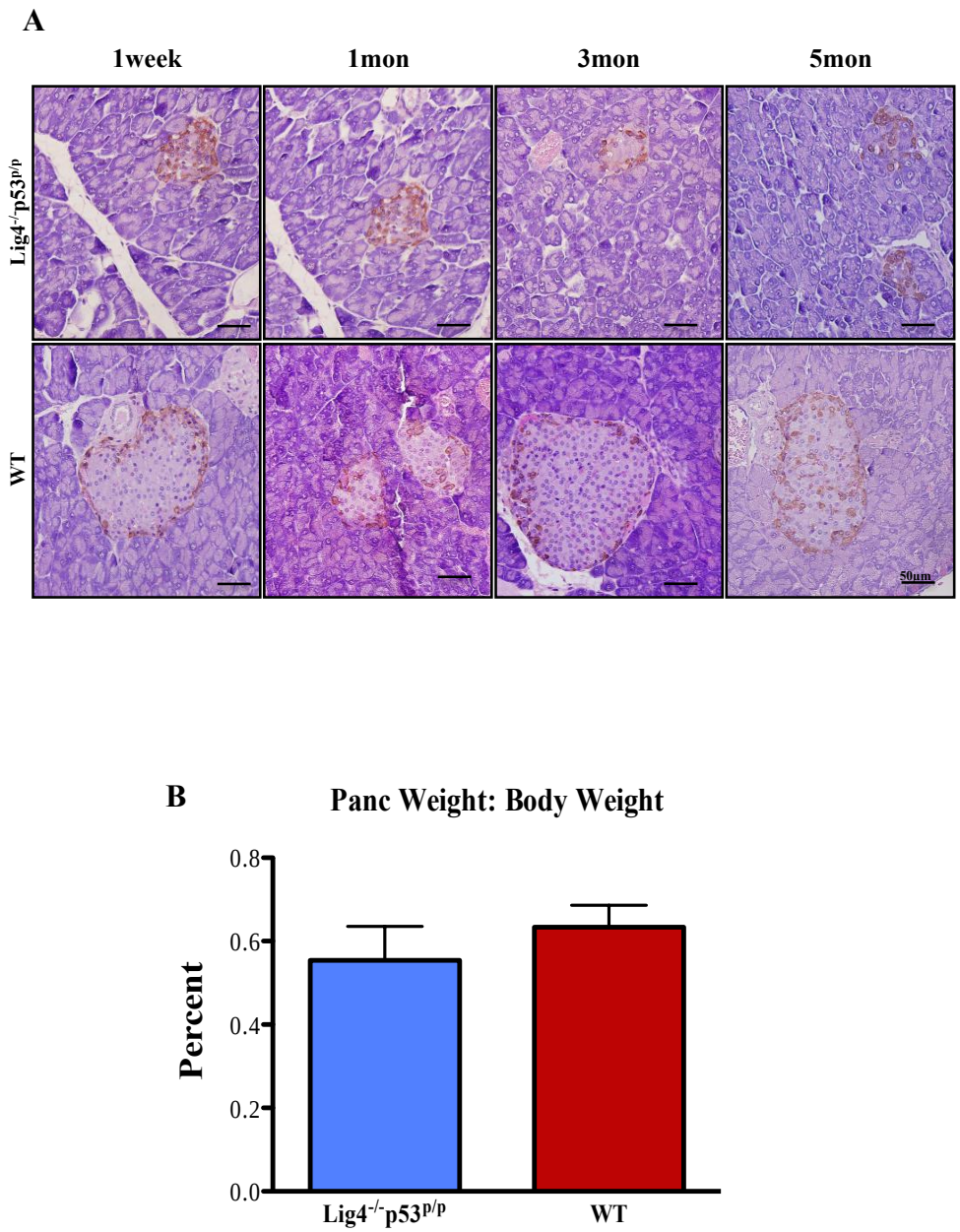

Figure 20. No decreases in alpha-cells or total pancreas size in Lig4$^{-/-}$p53$^{p/p}$ mice. **(A)** Representative pancreatic sections from Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice were immunohistochemically stained with anti-glucagon. Magnification: 40X. **(B)** The total weight of Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice were divided by the weight of their respective pancreata and the ratio was recorded. No statistical differences were noted.

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Without compensatory β-cell replication, abrogating the β-cell cycle leads to a decrease in islet mass, consequentially reducing insulin production, which deregulates glucose homeostasis, and ultimately drives hyperglycemia and overt diabetes (reviewed in detail (Tavana & Zhu, 2011)). Therefore, we hypothesized that the diminishment of islets seen in the $Lig4^{-/-}p53^{p/p}$ islets could be due to decreased proliferation rates, thereby decreasing the production of other β-cells. To test this hypothesis, we compared the β-cell proliferation rates by BrdU incorporation. Proliferating pancreatic cells, dually positive for incorporated BrdU and insulin, were identified by immunostaining. This number was divided by cells positive for only insulin, to get the ratio of proliferating β-cells. As shown in Figures 23a and 23b, no differences in proliferation rates were observed at one week of age, but a discrete non-significant 28% decrease ($p<0.285$) in β-cell proliferation was observed in 1-month $Lig4^{-/-}p53^{p/p}$ pancreas. A significant decrease by 60% ($p<0.0001$) and 62% ($p<0.05$) in β-cell proliferation was observed in both 2- and 3-month old mutant mice, respectively, as opposed to their wild-type controls. These results strongly correlate with the time-dependent appearance of the diabetic phenotype, suggesting a direct relationship between the decline in the β-cell proliferation and the onset of diabetes.

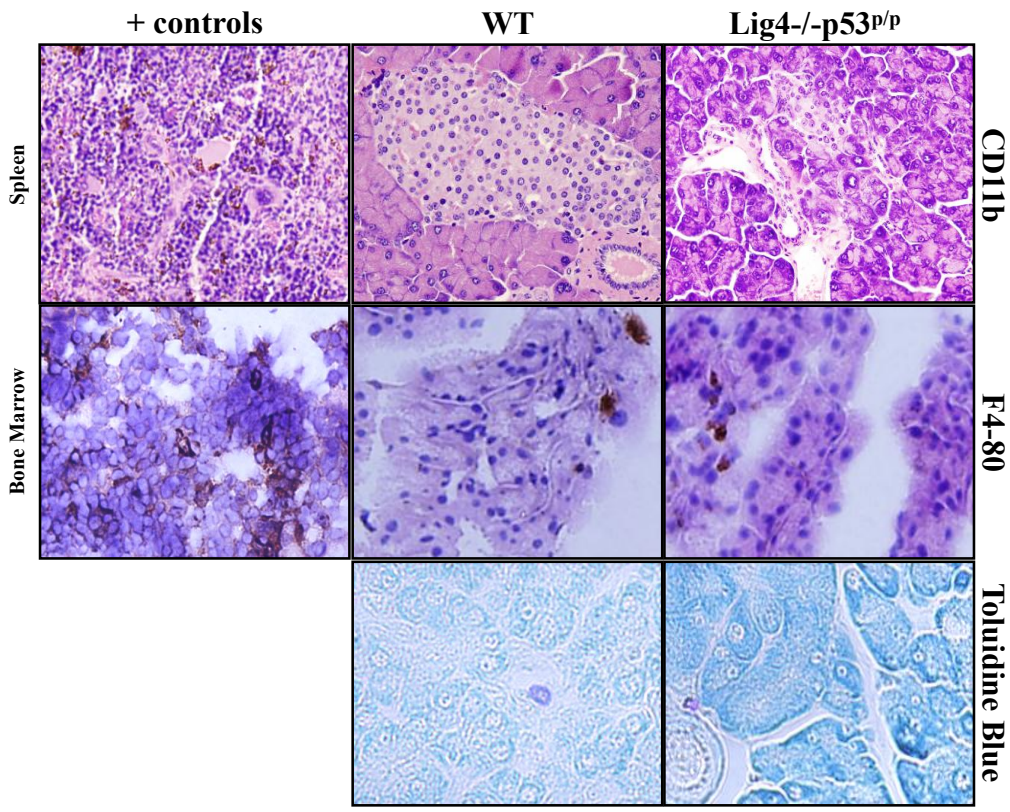

Figure 21. Observed islet depletion is not due to innate immunity infiltration. Pancreatic sections from Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice were stained for Cd11b (top panels) or F4-80 (middle panels) with wild-type spleen and bone marrow as respective controls. Toluidine blue staining was used to indicate mast cells (bottom panels). Magnification: 40X.
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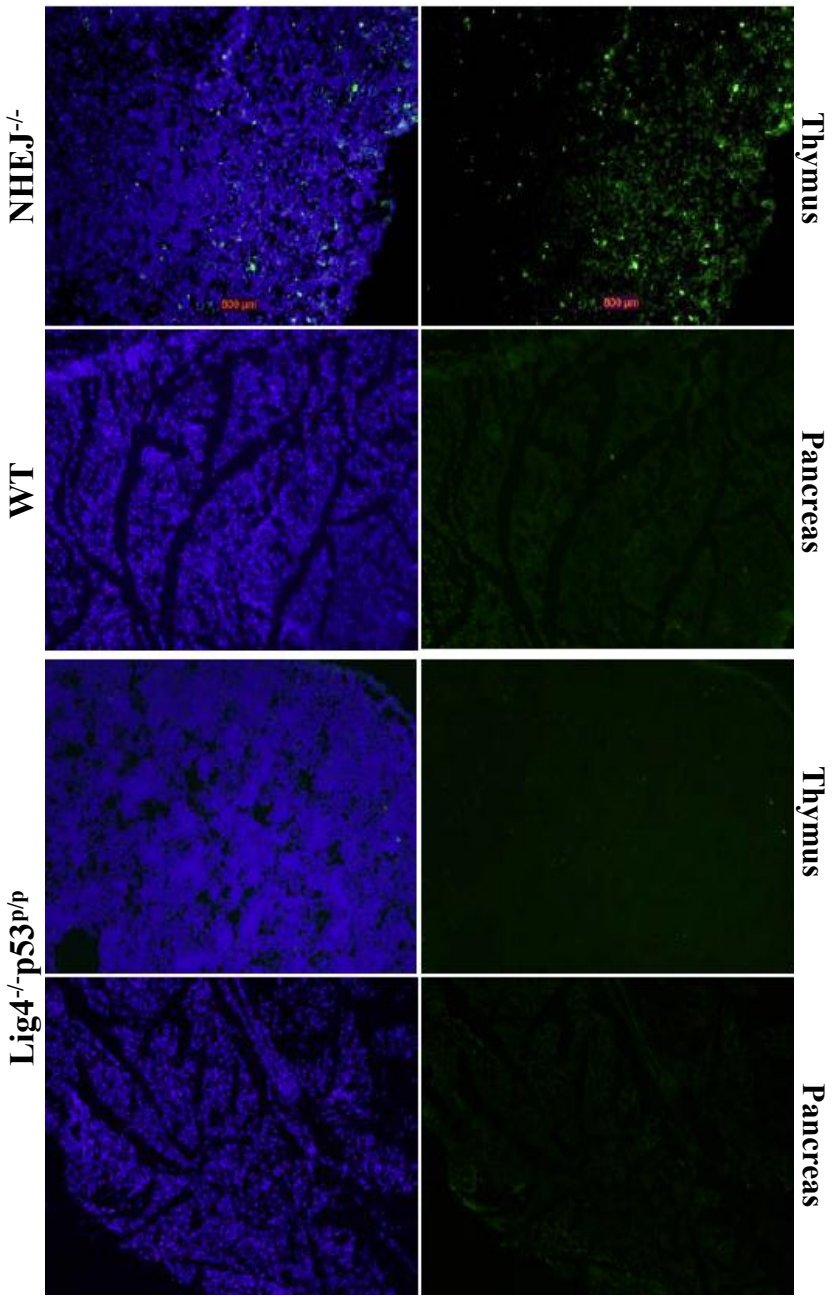

Figure 22. Observed islet depletion is not due to increased apoptosis.
Pancreatic sections from $Lig4^{-/-}p53^{p/p}$ and wild-type mice were analyzed with a TUNEL assay; no apoptotic cells detected in pancreata or thymic sections of $Lig4^{-/-}$ $p53^{p/p}$ mice. A $NHEJ^{-/-}$ (Artemis deficient) thymus was used as a positive control. Magnification: 10X
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4.2.5 Accumulated DNA damage in the pancreas of Lig4$^{-/-}$p53$^{p/p}$ mutant mice.

Due to high energy-consumption, pancreatic β-cells are particularly susceptible to DNA damages caused by intrinsic metabolic agents including reactive oxygen species (Lenzen, 2008). After sensing DNA damage, cells will arrest their cycling, in order to fix the broken DNA; therefore, it is absolutely vital to have an efficient DNA damage repair machinery to maintain the integrity of the genome. We hypothesized that in the absence of the NHEJ pathway, pancreatic β-cells will have an accumulation of unrepaired DNA damage. To determine whether γH2AX foci, a common marker indicative of DNA damage, accumulates in the mutant pancreas, we stained 2- and 4-month old pancreatic sections from Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice. γH2AX foci were detected throughout the 2-month old mutant but not in the wild-type sections. At 4 months, intense γH2AX foci was observed in most mutant pancreatic β-cells, indicating DNA damage was accumulating; this is in clear contrast to the absence of foci observed in the wild-type pancreas (Figure 24). These results indicate that in the absence of classic NHEJ, pancreatic islets suffer from gross accumulative DNA damage.

4.2.6 DNA damage-induced p53/p21 axis results in cellular senescence in the Lig4$^{-/-}$p53$^{p/p}$ islets.

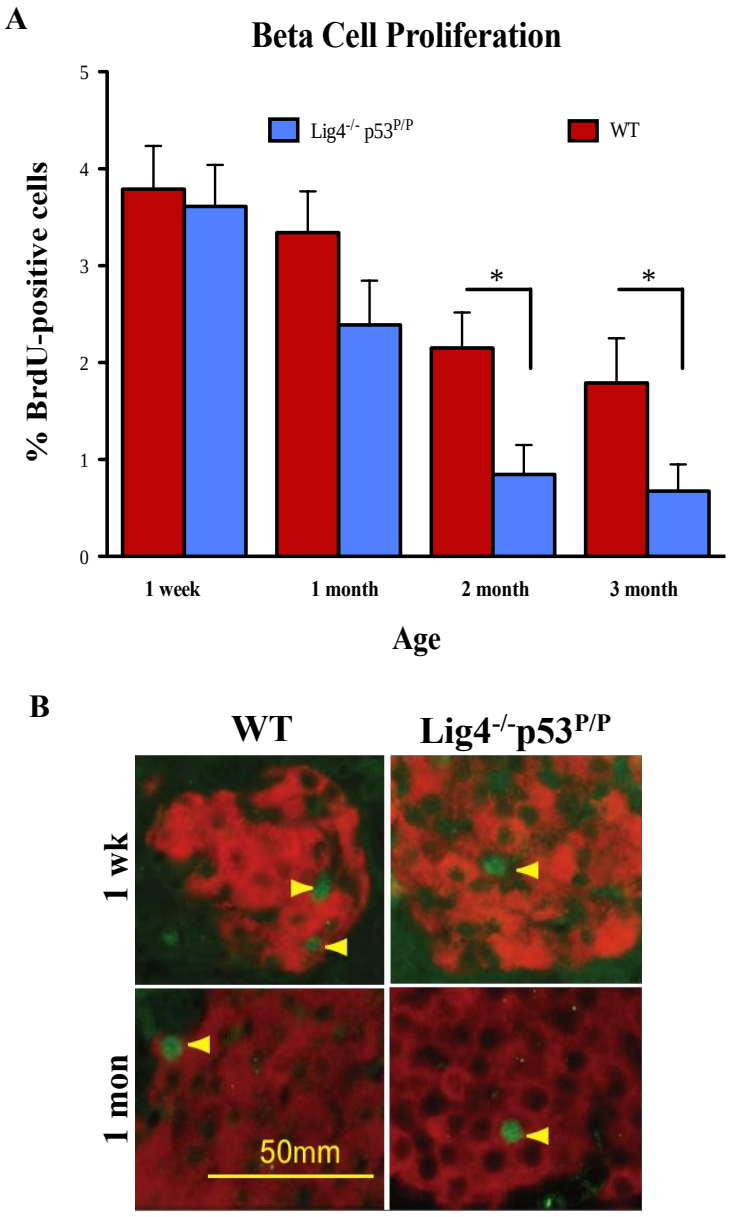

Figure 23. Decreased b-cells proliferation in Lig4$^{-/-}$p53$^{p/p}$ mice.
(A) Percent of BrdU incorporation in b-cells out of the total b-cells was measured in each age group of Lig4$^{-/-}$p53$^{p/p}$ mice, and plotted in comparison with age-matched wild-type mice. N≥2 mice per time point. Data are the mean ± SEM, * indicates $p<0.05$ versus wild-type. **(B)** Representative islet proliferation of pancreatic sections incubated with anti-BrdU antibody (green) and co-stained with anti-insulin antibody (red). Arrows indicated BrdU positive cells. Magnification 40X.
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Previously, we reported in the lymphoid system, programed DNA double strand breaks will accumulate, in the absence of NHEJ, which leads to the activation of p53 and p21 (Van Nguyen et al, 2007).

Therefore, to asses if the accumulation of DNA damage in pancreatic β-cells could stimulate p53 and p21 expression, we used immunostaining. Since DNA damage occurs randomly in the pancreas, as opposed to the programmed DNA damage in developing lymphocytes, we did not detect as strong of an expression of p53 and p21. However, the p53/p21 axis was activated in some pancreatic islets of $Lig4^{-/-}p53^{p/p}$ mice (Figure 25). Virtually neither p53 nor p21 were detected in age-matched wild-type pancreatic sections. These results indicated that accumulated DNA damage induces a p53-p21 dependent response in the pancreas of the $Lig4^{-/-}p53^{p/p}$ mice.

Activating the p53/p21 axis promotes cellular senescence as a mechanism to suppress genome instability and tumorigenesis, as show previously in the lymphoid system (Van Nguyen et al, 2007). Therefore, we asked whether the random accumulation of DNA damage, which induces p53-p21 activation, could render pancreatic β-cells to undergo cellular senescence. Pancreata from mutant and age-matched wild-type mice were stained for β-galactosidase (β-gal) activity, which indicates cellular senescence. Dual staining of β-gal and insulin demonstrated that at all three time points indicated in $Lig4^{-/-}p53^{p/p}$ mice, islet senescence was induced (Figure 26). Importantly, no senescent cells were detected in

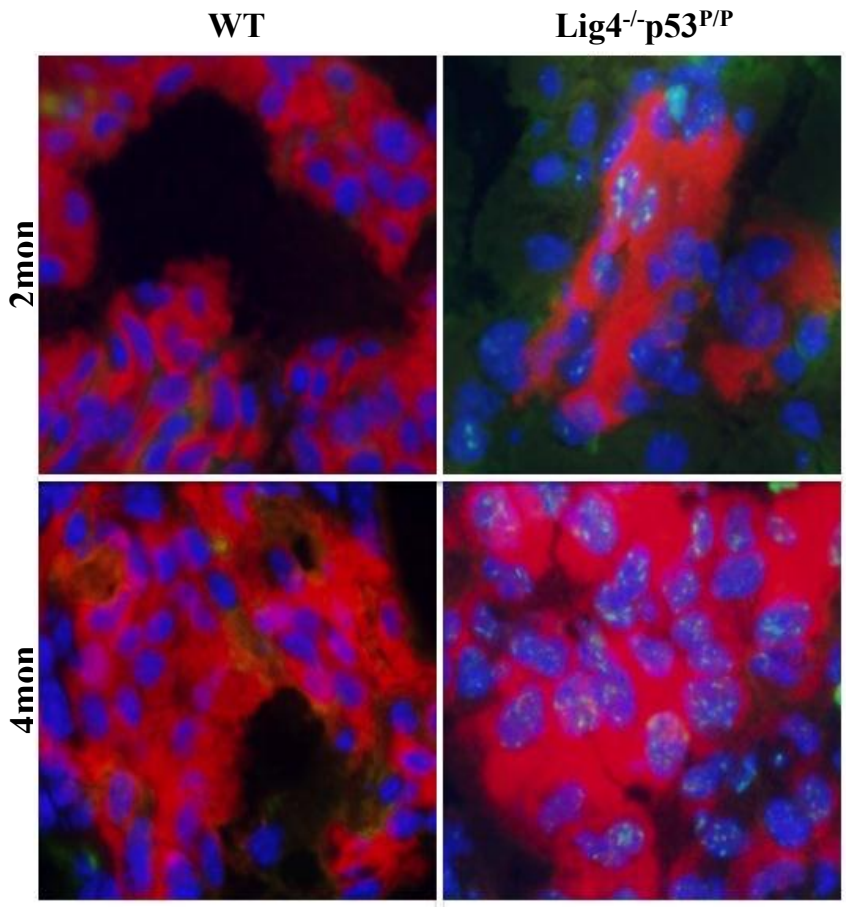

Figure 24. Accumulation of DNA damage in Lig4$^{-/-}$p53$^{p/p}$ mice.
Pancreatic sections immunofluorescent stained for insulin (red), γH2AX foci (green), and DAPI (blue) in 2- and 4 month Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice. Magnification 100X.
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age-matched wild-type pancreatic sections. Collectively, these results indicate that the accumulation of spontaneous DNA damage, caused from the DNA Ligase IV deficiency, was sufficient to activate p53/p21 and trigger cellular senescence in mutant β-cells. This permanent arrest lead to the decrease of insulin production and dysregulated glucose homeostatsis ultimately driving the diabetic phenotype observed (Tavana et al, 2010c). Remarkably, while DNA damage induction of p53-p21 and senescence is beneficial in the developing lymphocytes by suppressing genomic instability and preventing tumorigenesis, senescence in the pancreatic β-cells acts as a double-edged sword, leading to a depletion of islets and insulin insufficiency, resulting in diabetes.

4.3 Discussion

This study presents a unique animal model where accumulating DNA damage, due to a deficiency of end-joining (DNA Ligase IV), leads to up-regulation of p53-p21 and senescence in pancreatic β-cells. This p53 activation diminishes the self-replication capability of β-cells, resulting in a drastic loss in pancreatic islet number ultimately driving diabetes (Tavana et al, 2010c). Our animal model further emphasizes the importance of regulating cellular replication in already differentiated β-cells to maintain the homeostasis of islet mass and glucose metabolism. This is also a unique animal model that is the first to directly implicate the accumulation of DNA damage due to impaired DNA repair,

a typical phenotype of ageing, leading to the onset of diabetes. Therefore, this is significant for our understanding of diabetogenesis and offers potential insight into future therapies. Diabetes occurs when β-cells fail to produce sufficient insulin for metabolic demands, due to their dysfunction or simply a failure to maintain the islet cell mass which automatically will decrease the insulin production (Ackermann & Gannon, 2007; Masiello, 2006). In adult mice, β-cell mass is maintained by self-replication rather than differentiation from stem cells or neogenesis (Dor et al, 2004; Georgia & Bhushan, 2004). Therefore, factors mediating β-cell proliferation directly impact their maintenance. Mice deficient for these regulatory factors have defective β-cell replication directly leading to a reduction in cell number and the development of diabetes (reviewed in detail (Tavana & Zhu, 2011)). Such examples are CDK4 (Rane et al, 1999; Tsutsui et al, 1999) or Cyclin D2 deficient mice (Georgia & Bhushan, 2004; Kushner et al, 2005); both models have a progressive reduction of β-cell number and gross onset of diabetes with features of hyperglycemia and glucose intolerance. Conversely, overexpression of CDK4 (Rane et al, 1999) or Cyclin D1 (Zhang et al, 2005) in pancreatic β-cells leads to hyperplasia of pancreatic islet cells, indicating the importance of these key molecules, underscoring the critical role of cell proliferation in the maintenance of β-cells. Our results are inline with these models. Furthermore, it appears that adulthood and neonatal β-cell maintenance is dictated through different mechanisms. While both CDK4 and Cyclin D2 deficient newborn pups have relatively normal pancreatic islet mass, a progressive reduction in β-cell number occurs upon animal age. The $Lig4^{-/-}p53^{p/p}$

mice have a similar pattern of the progressive development of diabetes, strongly suggesting that reduction in β-cell proliferation directly leads to the decrease in β-cell numbers and resulting in severe diabetes (Tavana et al, 2010c). Additionally, Cyclin dependent kinase inhibitors, the Cip and Kip protein family along with the INK4/ARF group, play essential roles in directly regulating β-cell proliferation (Tavana & Zhu, 2011) and therefore impact β-cell maintenance. Overexpression of $p27^{kip1}$ (Uchida et al, 2005) or $p16^{INK4a}$ (Krishnamurthy et al, 2006) directly leads to a decrease in the rate of β-cell proliferation and a reduction in β-cell mass. Conversely, $p16^{INK4a}$ (Krishnamurthy et al, 2006), $p27^{kip1}$, or $p18^{INK4c}$ (Rachdi et al, 2006) deficiency results in pancreatic islet hyperplasia. These results indicate that CDK inhibitors are important regulators of cell proliferation in the pancreatic β-cells. While cell cycle inhibitor and putative p53 downstream target p21 is expressed in pancreatic β-cells (Cozar-Castellano et al, 2006b; Cozar-Castellano et al, 2006c), islets grow and function normally in the absence of p21, suggesting that other cell cycle inhibitors may function to compensate for the p21 deficiency. Interestingly, p21 is upregulated in murine pancreatic islets by overexpressing potent mitogens such as placental lactogene (PL) or hepatocyte growth factor (HGF), depicting the importance of p21 in inhibiting growth factor-driven proliferation in murine islets. Therefore, p21, although not vital for normal β-cell proliferation or function, plays an important role when β-cells exhibit stress.

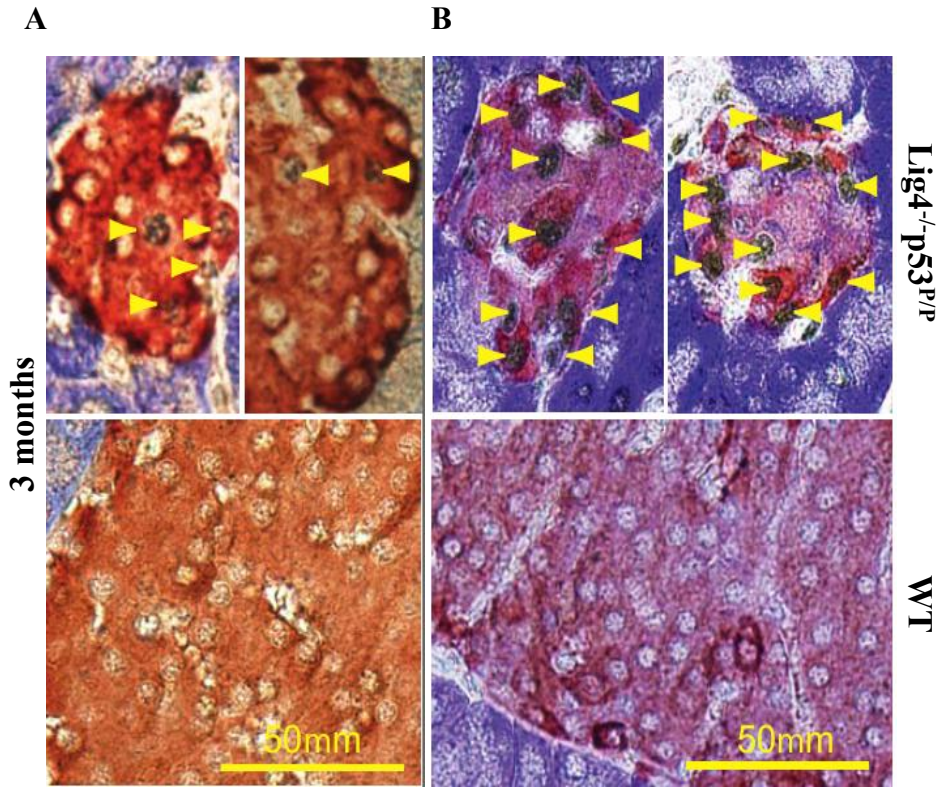

Figure 25. Elevated p53 and p21 in Lig4$^{-/-}$p53$^{p/p}$ islets.
(A) Double immunohistochemical staining of pancreatic sections from 3-month old Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice for p53 (black) and insulin (orange). **(B)** Double immunohistochemical staining of pancreatic sections from 3-month old Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice for p21 (black) in combination with insulin (red). Magnification 40X.
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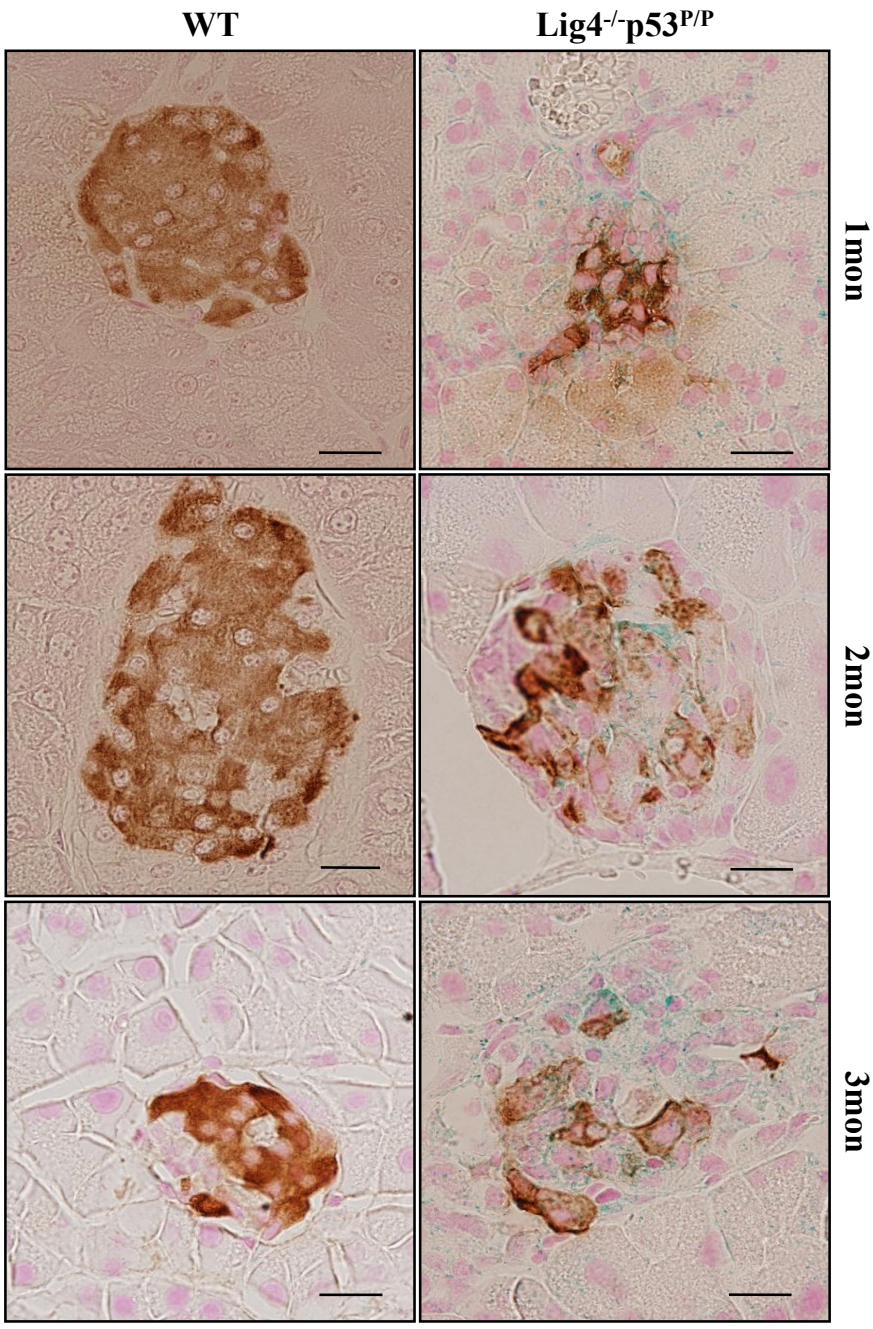

Figure 26. Increased cellular senescence in Lig4$^{-/-}$p53$^{p/p}$ islets.
Immunohistochemical staining for insulin (brown) and b-galactosidase (blue) staining of pancreatic sections from age-matched Lig4$^{-/-}$p53$^{p/p}$ and wild-type mice. Counterstaining with Nuclear Fast Red. Magnification 100X.
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Previously, our study demonstrated that in the response to genetically programmed DNA damage, p53 and its downstream target p21 are critical to drive cells into cell cycle arrest and senescence in the developing lymphocytes (Van Nguyen et al, 2007). Interestingly, in this study, the same DNA damage responsive pathway is activated, to also drive β-cells into senescence. Such a strong inhibitory mechanism of cell proliferation plays a pivotal role blocking β-cell proliferation. The maintenance of cellular homeostasis is dependent on strictly regulating cellular proliferation, and the tumor suppressor p53, which plays a key role during this regulation. The NHEJ deficient mice are unique models, which aptly demonstrate this concept. Deleting p53 combined with the deficiency of NHEJ leads to rapid tumorigenesis in developing lymphoid cells, due to the presence of unrepaired physiological DNA breaks in proliferating cells which lead to chromosomal translocation and subsequent oncogene activation (Difilippantonio et al, 2002; Zhu et al, 2002). The separation-of-function, hypomorphic $p53^{R172P}$ successfully rescued tumorigenesis because p53 activity is able to respond to physiological or other spontaneous DNA damages and block cellular proliferation to prevent genomic instability. But this potent tumor suppression mechanism comes with a price; this theory has been coined as 'antagonistic pleiotropy'(Kirkwood & Austad, 2000). Basically, cellular senescence can prevent tumorigenesis, but over time, in renewable organs, it leads to deleterious effects (Campisi, 2003), which is evident in our unique animal model. We propose that the persistent DNA damage due to NHEJ deficiency leads to p53-dependent senescence (in the absence of apoptosis), a

program that requires cells to undergo dramatic chromatin remodeling. Further, cells will shut down any DNA replication, preventing the cells to re-enter the cell cycle. Cellular senescence leads to the silencing of many genes related to cell growth and metabolism. This accumulative event exacerbates the ageing process, which is deleterious to those organs that require constant renewal through regeneration, such as in pancreatic islets. Further, we propose that $Lig4^{-/-}p53^{p/p}$ mice develop diabetes due to an incapability of β-cell proliferation and a failure to maintain β-cell mass to a certain threshold. Accumulative DNA damage and subsequent p53-induced senescence may be the main contribution to the inhibition of β-cell proliferation. However, we cannot rule out other effects accompanying senescence, such as the silencing of critical genes required for sensing growth signals, which can lead to β-cell irresponsiveness. Further investigation is required to address this matter. Additionally, there must be a mechanism to remove the senescence cells which caused the severe reduction of β-cell number in 3 month and older mutant mice by means other than apoptosis. Scott Lowe's group showed that senescent tumors are eradicated through the innate immune system (Xue et al, 2007). Because we did not see any infiltrating immune cells, this may not be the case for our model. Other studies have introduced autophagy to potentially play a role in clearing out senescent cells to recycle and reuse proteins/nutrients; further investigation is needed. Similar results have been recently reported on mice deficient for the pituitary tumor transforming gene (PTTG). Interestingly, deficiency of PTTG leads to a

progressive up-regulation of p21 that drives pancreatic beta cells into senescence that eventually results in insulinopenic diabetes (Chesnokova et al, 2009).

It has been hypothesized that the accumulation of DNA damage and the activated cellular response are both potential contributing factors for ageing (Campisi & Vijg, 2009). Consistent with this hypothesis, our study using this unique animal model showed that accumulation of DNA damage and its p53-dependent responses have a profound impact on the rate of proliferation in pancreatic β-cells. This is p53-dependent, as the $Lig4^{-/-}p53^{-/-}$ mice do not show an increase in blood glucose (Tavana unpublished data), even though they do succumb to rapid tumorigenesis around 2 months (Zhu et al, 2002). Deficiency in DNA damage repair factors obviously accelerates the accumulation of DNA damage. Spontaneous damage can occur frequently in pancreatic β-cells (Lenzen, 2008), making NHEJ activity important to repair the damage and to maintain the integrity of the genome. It is the mutation in the DNA damage response that gradually leads to the halting of the cellular proliferation. Diabetes incidence increases upon age, and the NHEJ pathway activity decreases among age. There may be a previously overlooked association that needs to be explored. Accumulating DNA damage and gene mutations, both require a long process to develop and could be a major ageing causative factor. Interestingly, this study highlights the importance of p21 in regulating β-cell proliferation upon excessive genomic stress. DNA damage-induced cellular senescence is a potent tumor suppressing mechanism, but also diminishes the regeneration and renewal capacity of certain organs. This can lead to ageing-related diseases, such as

diabetes with depletion of pancreatic islet mass causing lower insulin to regulate glucose homeostatsis" (Tavana et al, 2010b).

CHAPTER 5

ABSENCE OF KU70 LEADS TO INCREASED BETA CELL PROLIFERATION AND HYPOGLYCEMIA— A NOVEL NHEJ-INDEPENDENT FUNCTION.

5.1 Introduction

The inability to maintain genomic stability and control proliferation are hallmarks of many cancers, which are exacerbated in the presence of unrepaired DNA damage. One of the major pathways that repair DNA double strand breaks (DSBs) is non-homologous end joining (Mahaney et al, 2009). Classically, the NHEJ pathway mends DSBs in two steps: initially, broken DNA ends are recognized and processed, initiated by the Ku70/80 heterodimer, which recruits DNA-dependent protein kinase and repair factor Artemis; next, the DNA is ligated through a complex consisting of a DNA Ligase IV (Lig4), XRCC4, and Cernunnos/XLF (Mahaney et al, 2009). Previously, by utilizing a hypomorphic, separation-of-function p53 mutant, $p53^{R172P}$, which prevents p53-mediated apoptosis yet retains a partial cell cycle arrest function (Liu et al, 2004), combined with NHEJ deficiency ($Lig4^{-/-}p53^{p/p}$), we showed that mutant p53 not only rescues embryonic lethality but also entirely eliminated lymphomagenesis in the Ligase IV deficiency (Van Nguyen et al, 2007). Further analyzing developing lymphocytes revealed the broken DNA ends activated a permanent cell cycle arrest, termed cellular senescence, which acts in parallel to apoptosis in suppressing tumorigenesis (Van Nguyen et al, 2007).

Although completely free of tumors, $Lig4^{-/-}p53^{p/p}$ mice succumb to progressive and severe diabetes (Tavana et al, 2010c). Mechanistic analysis revealed that spontaneous DNA damage specifically accumulated in the insulin producing β-cells of the pancreas, activating the p53/p21 axis to trigger cellular senescence. This cascade halted the proliferation of β-cells, decreased islet mass, and compromised glucose homeostasis causing the severe diabetic phenotype (Tavana et al, 2010c). This study

highlighted a crucial role for the NHEJ pathway in preventing the accumulation of broken DNA and subsequent activation of cell cycle control in pancreatic β-cells.

The genesis of adult insulin-producing β-cells predominantly occurs through self-duplication of mature cells rather than through differentiation from their stem-cell progenitors (Dor et al, 2004; Georgia & Bhushan, 2004; Salpeter et al, 2010; Teta et al, 2007). Without compensatory β-cell replication, abrogating the β-cell cycle leads to a decrease in islet mass, consequentially reducing insulin production, which deregulates glucose homeostasis, and ultimately drives hyperglycemia and overt diabetes. Conversely, augmentation of the β-cell cycle increases islet area and insulin production, many times rescuing a diabetic phenotype, yet in some cases results in hypoglycemia and islet hyperplasia (reviewed in detail (Tavana & Zhu, 2011)). Components of cell cycle responsible for proliferation are vital in the maintenance of adult β-cells, but less is known about their upstream activators. One such pathway responsible for both pancreas development and control of postnatal β-cells is the canonical Wnt signaling pathway (Puri & Hebrok, 2010; Welters & Kulkarni, 2008). Activation of Wnt signaling, through the stabilization of β-catenin, has been recently shown to increase β-cell proliferation and elevated serum insulin levels (Heiser et al, 2006; Rulifson et al, 2007). Conversely, increasing β-catenin inhibitors lead to decreased β-cell proliferation levels (Dessimoz et al, 2005; Liu et al, 2008; Rulifson et al, 2007). Collectively, these studies, along with others, suggest that Wnt signaling plays a critical role in controlling β-cell proliferation.

Considering our previous work highlighting the importance of DNA Ligase IV in preventing genomic instability in pancreatic β-cells (Tavana et al, 2010c), we sought to examine the role of a different NHEJ factor, specifically Ku70, in both the wild-type and

mutant p53, $Ku70^{-/-}p53^{p/p}$, backgrounds in this study. Although most notable for its role in DNA damage recognition and repair within the NHEJ pathway, Ku70 has been implicated in many fundamental, yet different cellular networks (Downs & Jackson, 2004). Ku70 has been shown to play both direct and indirect roles in telomere maintenance (Bailey et al, 1999; Bertuch & Lundblad, 2003; Roy et al, 2004; Stellwagen et al, 2003), apoptosis (Amsel et al, 2008; De Zio et al, 2010; Sawada et al, 2003), and transcriptional regulation (Brenkman et al, 2010; De Zio et al, 2010; Giffin et al, 1996; Grote et al, 2006; Idogawa et al, 2007; Lebrun et al, 2005; Nolens et al, 2009). Through generation and analysis of both $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice, we surprisingly discovered that the deficiency for Ku70 progressively developed hypoglycemia. Further analysis revealed augmented β-cell replication and increased islet mass, which is in stark contrast to that observed in Lig4 deficiency. Mechanistic studies revealed a stabilization of islet-specific β-catenin with increased markers for cell cycle progression; tumorigenesis was prevented by the induction of islet-specific cellular senescence. Therefore, in addition to DNA damage repair, Ku70 has a previously undiscovered function independent of DNA end-joining, which has a direct impact on the proliferation of pancreatic β-cells.

5.2 Results

5.2.1 $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mutant mice exhibit hypoglycemia, increased insulin levels and glucose tolerance.

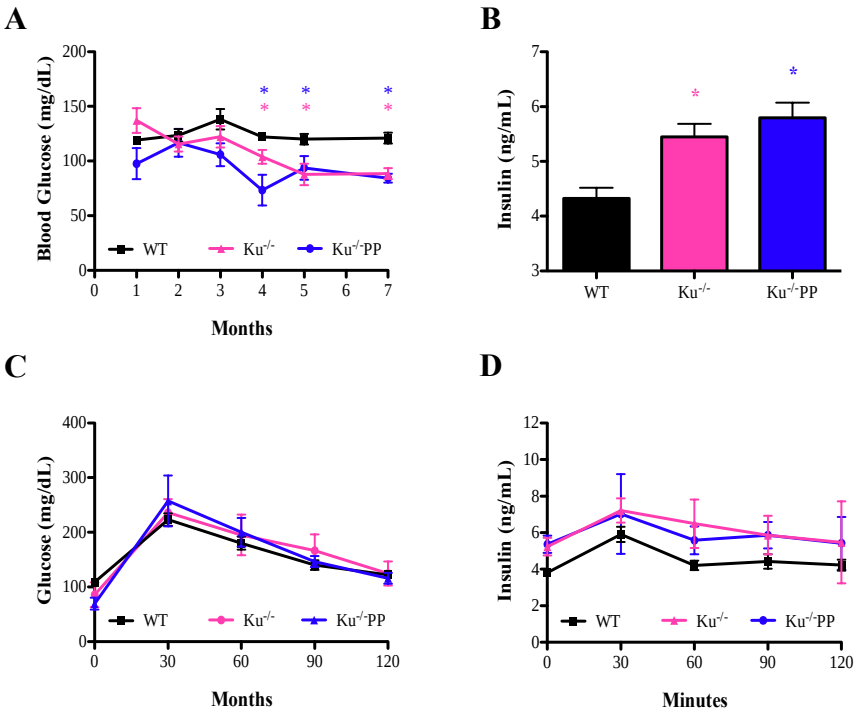

Figure 27. Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ mice are hypoglycemic and glucose tolerant. **(A)** Blood glucose concentrations from non-fasting animals at indicated ages. Glucose levels were significantly lower in mutant mice starting around 4 months of age. Each data point is an average of at least five animals. **(B)** Blood insulin concentrations were measured by ELISA at 7 months of age. Each column is an average of three animals ran in duplicates. **(C)** Blood glucose concentrations measured from a glucose tolerance test in old wild-type, Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ mice. Four mice from each genotype were tested. **(D)** Blood insulin concentrations were measured by ELISA from the GTT.

Examining the random non-fasting serum glucose levels in $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice revealed progressive hypoglycemia in aged mutant mice. The decrease in blood glucose levels became significant starting at 4 months in both mutant mice—more specifically, 7 month $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice displayed an 89% and 84% decrease, respectively, in blood glucose levels when compared to age-matched wild-type controls ($p<0.005$) (Figure 27a). To measure the non-fasting serum insulin levels, we performed ELISA assays in 7 month old mice and found significantly higher levels in both mutant mice ($p<0.005$) resulting in a 26-35% increase (Figure 27b), suggesting an imbalance in pancreatic β-cell regulation.

Routine glucose tolerance tests were preformed in young (data not shown) and older mice (6 months). Besides a lower fasting serum glucose level in mutant mice, no significant differences were seen between mutant mice when compared to wild-type controls after glucose injection (Figure 27c), indicating normal glucose tolerance. Interestingly, ELISA assays to determine blood insulin levels upon glucose stimulation showed higher levels of secreted serum insulin in both aged $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice (Figure 27d). Collectively, the increase in random serum insulin levels in the mutant mice, driving the hypoglycemia, suggested analyzing islet size and β-cell proliferation.

5.2.2 Ku70 deficiency results in β-cell mass expansion.

To determine whether the hypoglycemia and increased serum insulin concentrations observed in both $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice was the result of increased β-cell mass, we examined the morphological changes in the pancreatic islets

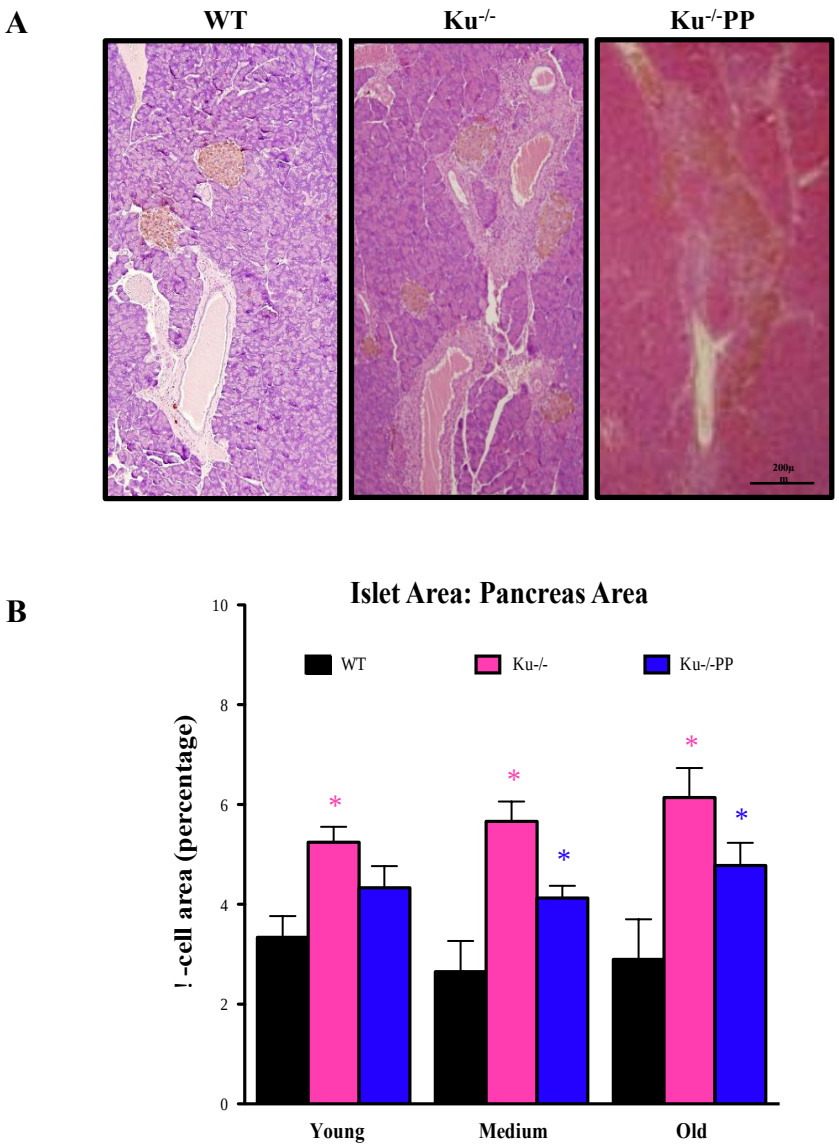

Figure 28. Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ mice present augmented pancreatic islet mass. (A) Representative pancreatic sections from medium aged mice stained by immunohistochemistry for insulin. Magnification: 10X. **(B)** Islet morphometric quantification of wild-type, Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ mice. Multiple sections were analyzed for each pancreas, and 3-4 mice per group were analyzed; data represent the mean ± SEM. * represents $p<0.05$-0.005 versus WT.

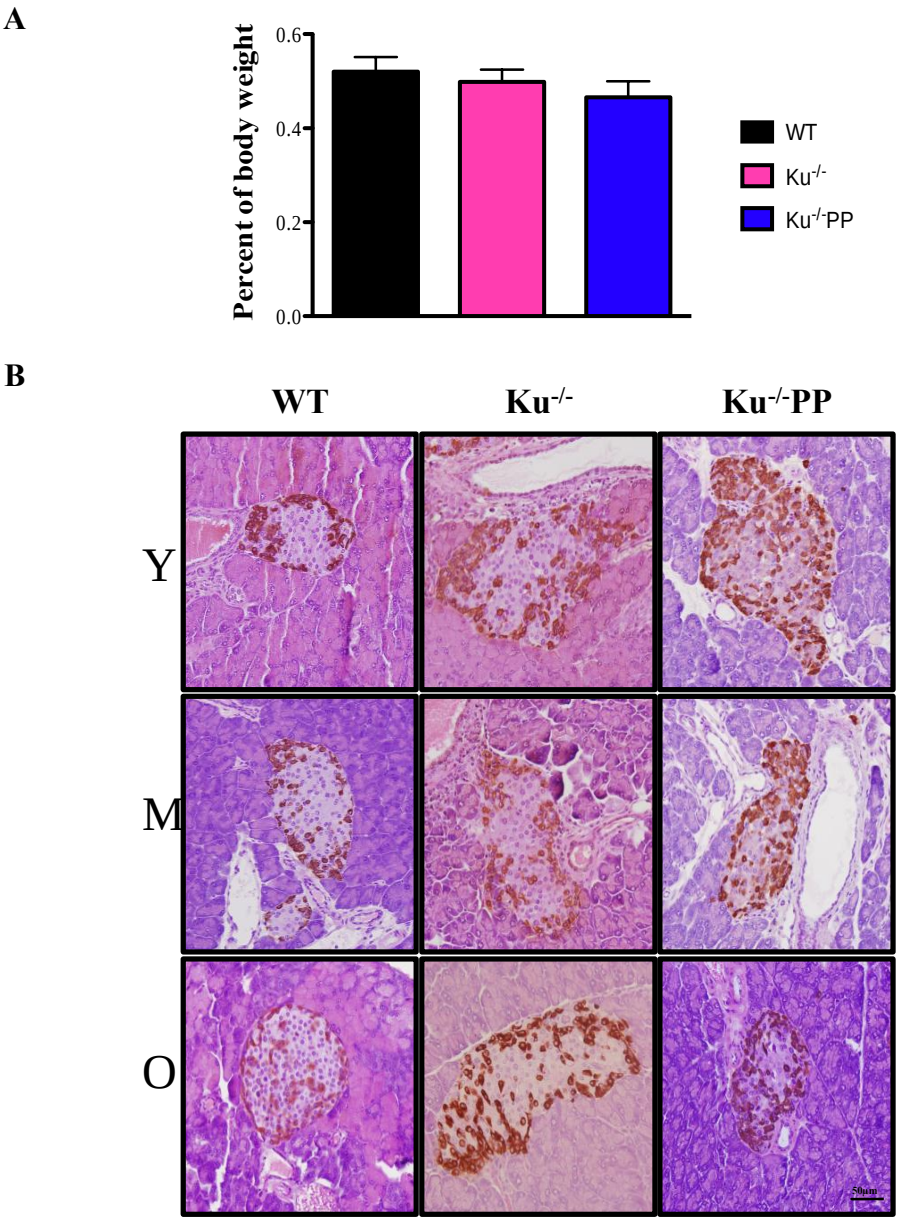

Figure 29. No decreases in alpha-cells or total pancreas size in the absence of Ku70. (A) The total weight of $Ku70^{-/-}$, $Ku70^{-/-}p53^{p/p}$ and wild-type mice were divided by the weight of their respective pancreata and the ratio was recorded. No statistical differences were noted. $n \geq 8$ per group. **(B)** Representative pancreatic sections from wild-type, $Ku70^{-/-}$, and $Ku70^{-/-}p53^{p/p}$ mice at different ages were immunohistochemically stained using an anti-glucagon antibody (brown). Magnification: 40X

by systematically measured the differences of β-cell mass in mutant and wild-type mice, by determining the ratio of islet area (immunohistochemical staining positive for insulin) divided by total pancreas area (Tavana et al, 2010c). For comparison purposes, we divided the mice into three age groups: young (2weeks-2.0 months), medium (2.5-4.0 months) and old (4.5-7.0 months).

As shown through representative pictures and quantification in Figures 28a and 28b, young mutant mice have a larger islet to total pancreas ratio as compared to the age-matched wild-type controls. As the mutant mice aged, the size of the pancreatic islets further increased, almost doubling the size of aged wild-type mice (p<0.05-0.005). The increase in mutant islets was independent of the total pancreas increasing since there was no significant difference in the ratio of total pancreas weight to body mass (Figure 29a).

Immunohistochemical staining for glucagon, indicative of α-cells that signal the liver to breakdown glycogen to produce glucose upon hypoglycemia, did not reveal any stark abnormalities in either mutant backgrounds at any time point when compared to age-matched wild-type controls (Figure 29b). These results clearly indicate a progressive augmentation of pancreatic islet mass in the absence of Ku70, which correlates with the increased insulin production and hypoglycemic phenotype.

5.2.3 Increased islet mass due to β-cell hyper-proliferation and increased Cdk4 levels in both $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice.

The production of adult β-cells predominantly occurs through self-duplication of mature cells giving rise to new β-cells rather than through differentiation of their stem-cell progenitors (Dor et al, 2004; Georgia & Bhushan, 2004; Teta et al, 2007). Therefore, we hypothesized that the increased islet mass observed in the mutant mice

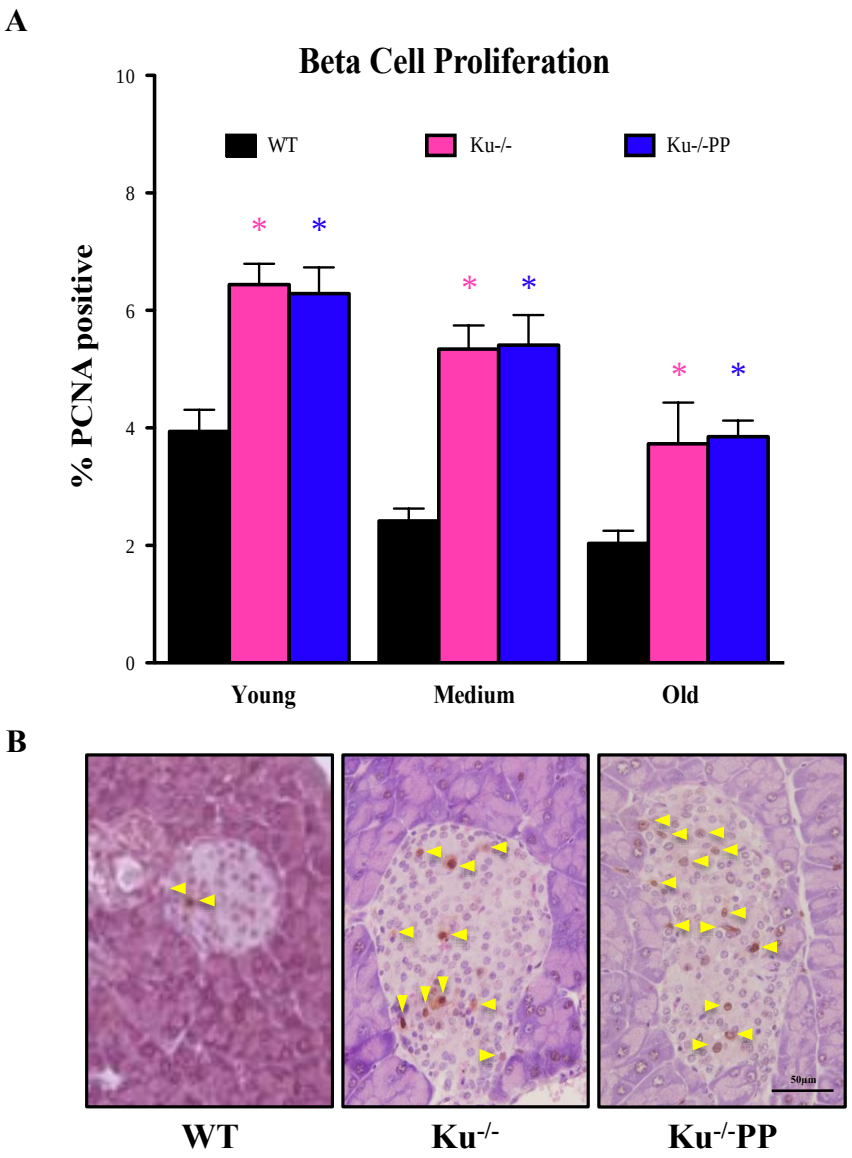

Figure 30. Increased b-cell proliferation in Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets.
(A) Percent of PCNA incorporation in b-cells out of the total b-cells was measured in each age group of Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets, and plotted in comparison with age-matched wild-type mice. Each column is an average of five mice. Data are the mean ± SEM, * indicates $p<0.005$ versus WT. **(B)** Representative sections of islet proliferation incubated with an anti-PCNA antibody (brown) and counterstained with H&E. Arrows indicate PCNA positive cells. Magnification 40X.

was attributed to heightened β-cell proliferation. By staining pancreatic sections for proliferating cell nuclear antigen (PCNA), counting the positive β-cells and dividing by the total number of β-cells, we can quantitatively assess the rate of β-cell proliferation. As shown in Figures 30a and 30b young mutant mice show a β-cell specific, hyper-proliferative phenotype presenting with 59-63% of an increase when compared with age-matched wild-type mice ($p<0.001$). Although showing a similar trend of decreased β-cell proliferation with age as in wild-type mice, β-cell proliferation was significantly higher in both aged $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice ($p<0.005$). Increased proliferation in young mutant mice was additionally confirmed through Ki67 staining (data not shown). Further, elevated PCNA staining was not seen in $Lig4^{-/-}p53^{p/p}$ mutant sections, indicating that the PCNA positive cells observed in the absence of Ku70 is not a result of accumulated DNA damage. These results strongly correlate with the hypoglycemic phenotype, indicating a relationship between the absence of Ku70 and increased β-cell proliferation and islet mass.

Considering the paramount role Cdk4 plays in regulating β-cell proliferation (Rane et al, 1999; Tsutsui et al, 1999), examining the expression pattern will further confirm the proliferative potential of $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ β-cells. Indeed, western blot analysis of isolated β-cells confirms increased Cdk4 protein expression in both mutant backgrounds at all time points when compared with age-matched wild-type controls (Figure 31a). Representative islet sections show increased nuclear localization of Cdk4 (Figure 31b) corroborating the western data. Further, Cyclin D2 levels were elevated in $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ islets as compared with age-matched wild-type controls (Figure 31c). Taken in concert with previous data, β-cells from $Ku70^{-/-}$ and

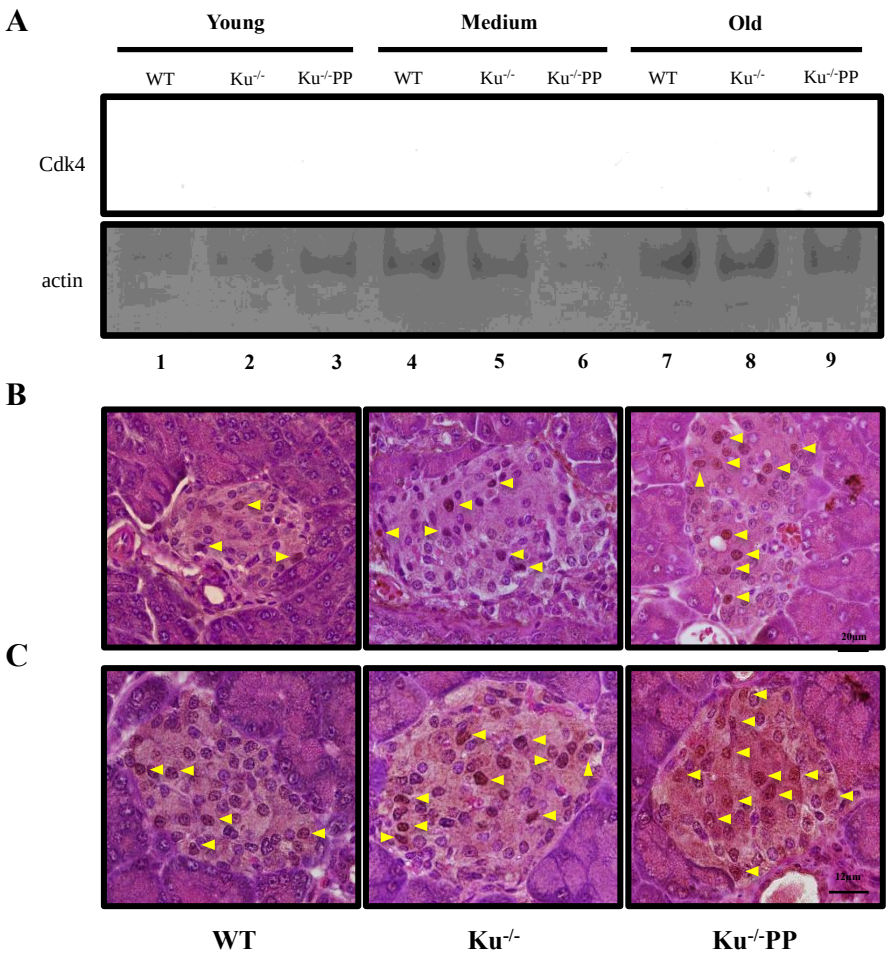

Figure 31. Increased Cdk4 and CyclinD2 expression in Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets. (A) Western blot analysis of Cdk4 using protein from pooled purified islets from three different mice per time point and genotype. β–actin is shown as a loading control. **(B)** Representative sections from medium aged mice incubated with an anti-Cdk4 antibody (brown) and counterstained with H&E. Arrows indicate Cdk4 positive cells. Magnification 100X. **(C)** Representative sections from medium aged mice incubated with an anti-CyclinD2 antibody (brown) and counterstained with H&E. Arrows indicate CyclinD2 positive cells. Magnification 60X.

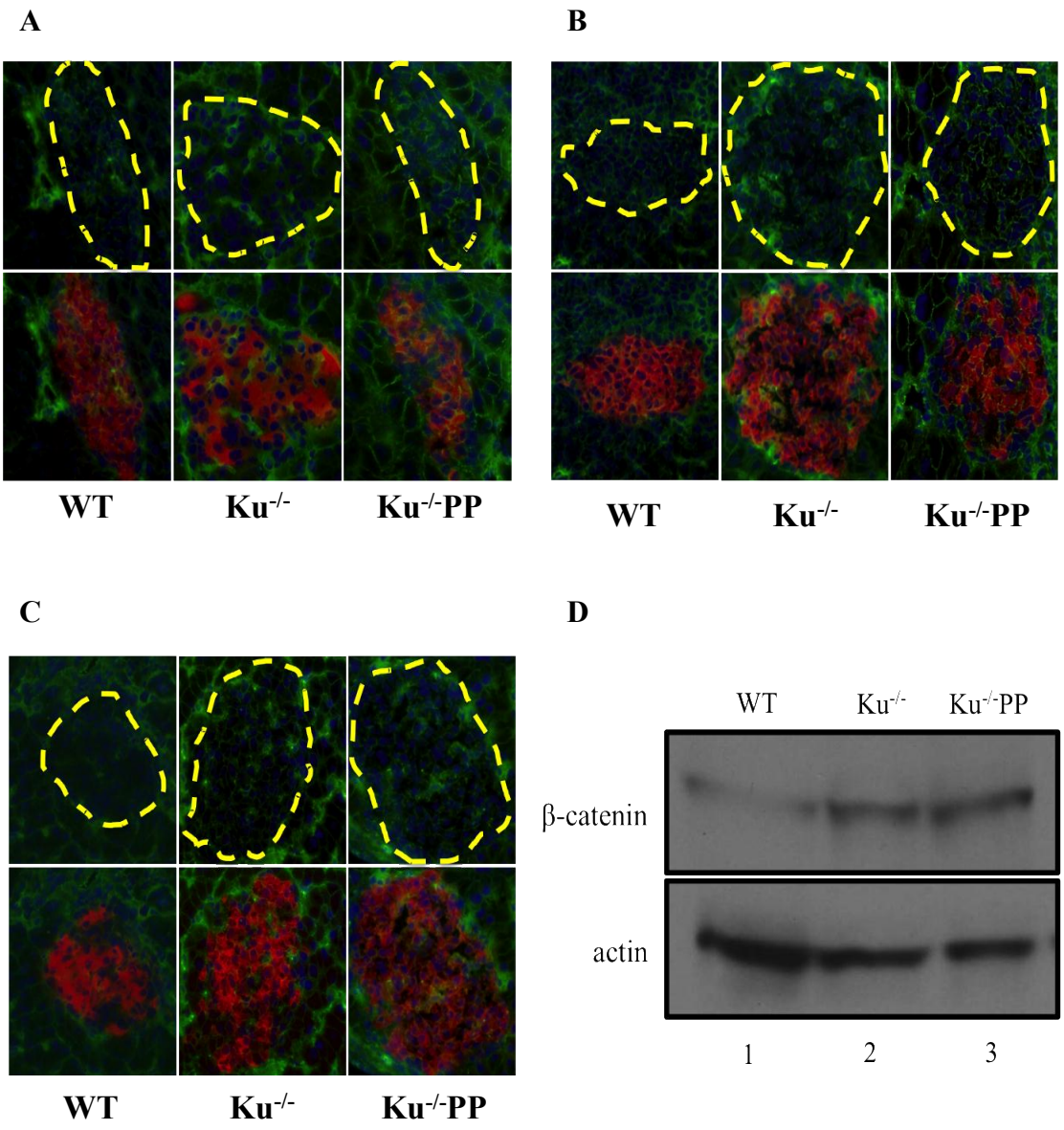

Figure 32. A progressive stabilization of β-catenin in the absence of Ku70.
Representative pancreatic sections from wild-type, Ku70$^{-/-}$, and Ku70$^{-/-}$p53$^{p/p}$ **(A)** young, **(B)** medium, **(C)** old mice were immunostained using an anti-β-catenin antibody (green), anti-insulin (red), DAPI (blue). Dashed yellow outline indicates islets. Magnification: 40X. **(D)** Western blot analysis of isolated islets from medium aged wild-type, Ku70$^{-/-}$, and Ku70$^{-/-}$p53$^{p/p}$ mice probing for β-catenin and β-actin as a loading control.

$Ku70^{-/-}p53^{p/p}$ show increased proliferation, increasing the islet area over time, resulting in the hypoglycemic phenotype observed.

5.2.4 The deficiency in Ku70 progressively leads to an increased stabilization of β-catenin

As mentioned above, canonical Wnt signaling regulates pancreatic β-cell proliferation. A hallmark for Wnt activation is the cytoplasmic accumulation of β-catenin, which eventually migrates into the nucleus to bind/activate the TCF/LEF transcriptional co-activators to upregulate target genes like Cyclin D1/2 and Cdk4 (Logan & Nusse, 2004). As shown in Figure 32, islet β-catenin levels are comprable in young mutant mice compared with age matched wild-type sections. Although some nuclear staining is evident, the increase in β-catenin levels becomes obvious upon age, where aged mutant mice (6-7months) show higher levels of both cytoplasmic and nuclear localization compared to age-matched wild type sections. This is consistent with reports indicating a normal downregulation of Wnt pathway being downregulated in wild-type adult pancreas (Murtaugh et al, 2005; Papadopoulou & Edlund, 2005). Corroborating the immunofluorescent data, we used purified islet protein extracts from medium wild-type, $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ islets and observed an increase in total β-catenin levels in the absence of Ku70 (Figure 32d). Further, early passage MEFs were generated and stained for β-catenin; while some nuclear localization was observed in wild-type MEFs, more cells showed stabilized β-catenin in the absence of $Ku70^{-/-}$ (Figure 33).

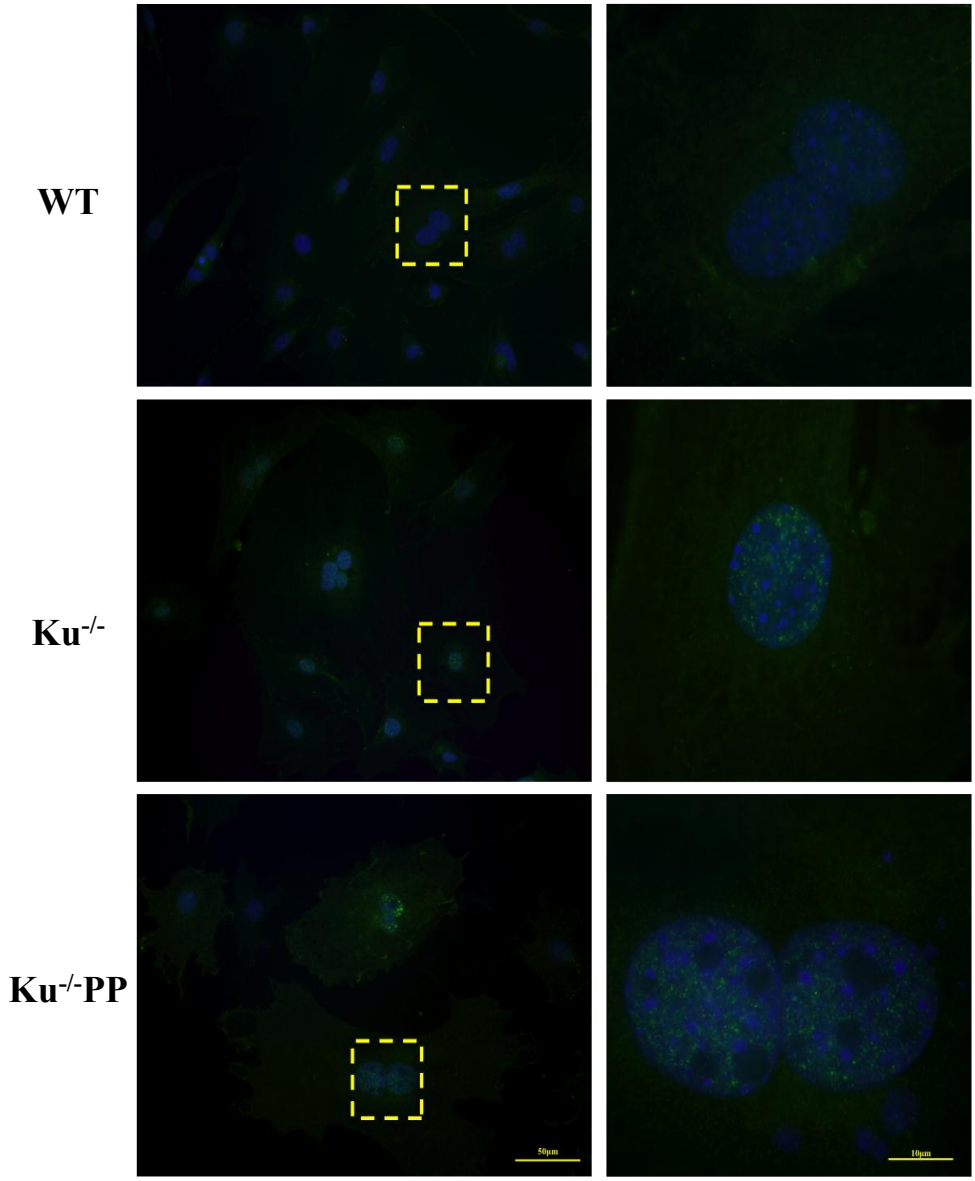

Figure 33. Increased nuclear β-catenin in early passage MEFs absent for Ku70. Representative fields from wild-type, Ku70$^{-/-}$, and Ku70$^{-/-}$p53$^{p/p}$ passage 2 MEFs immunostained using an anti-β-catenin antibody (green) and DAPI (blue). Left panels magnification 40X. Identified cells in dashed yellow outline in right panel; magnification 100X.

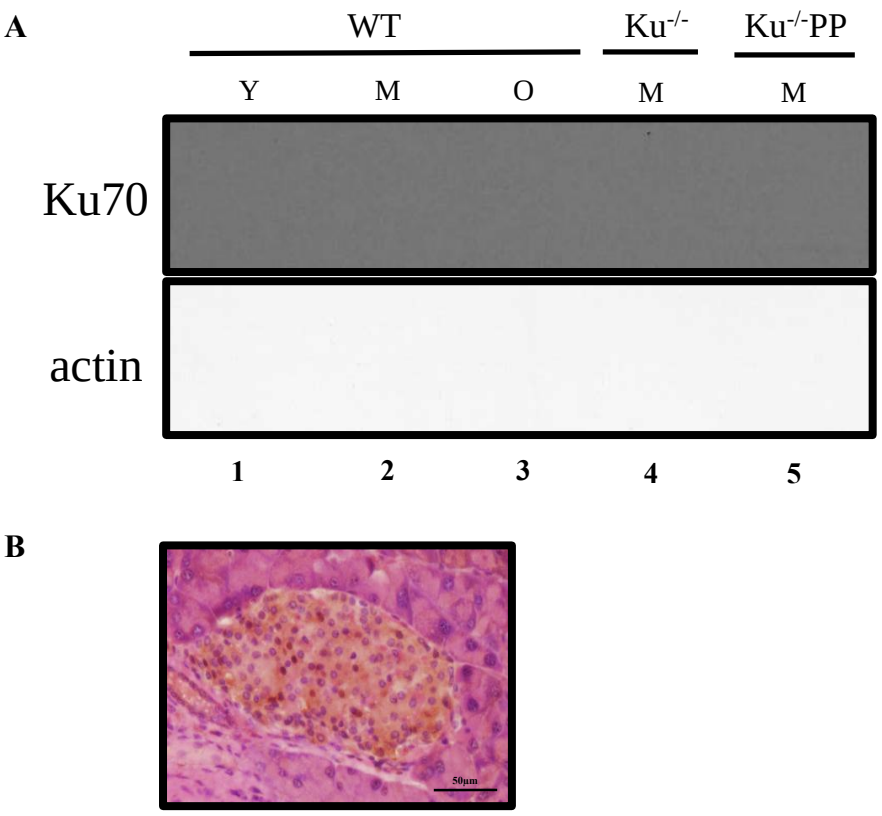

Figure 34. Ku70 expression in wild-type islets.
(A) Western blot analysis shows high levels of Ku70 in isolated wild-type islets which slightly increased with age while being completely absent in both $Ku70^{-/-}$ and $Ku70^{-/-}$ $p53^{p/p}$ mice. Samples n ≥ 3 mice islets pooled together. **(B)** Representative localization of Ku70 expression shows both nuclear and cytoplasmic in a medium aged WT mouse. Magnification: 40X.

5.2.5 Ku70 expression in pancreatic β-cells.

Previously we demonstrated that DNA ligase IV expression was high in isolated islets (Tavana et al, 2010c). We attributed the elevated expression of DNA Ligase IV to function in protecting against spontaneous genomic insults caused by intrinsic metabolic agents, highlighting the prominent role DNA damage repair machinery plays in protecting pancreatic β-cells. Seeing the high DNA ligase IV expression, we hypothesized that the NHEJ pathway may be very active in pancreatic β-cells. To analyze the expression of Ku70, we performed a Western blot in purified wild-type and mutant pancreatic islets. Our results showed that the Ku70 protein is expressed in pancreatic β-cells and expression level slightly increases with age (Figure 34a). Immunohistochemical staining reveals both cytoplasmic and nuclear localization of Ku70 correlating with its multiple functions both dependent and independent of NHEJ and DNA repair (Figure 34b).

5.2.6 Persistent DNA damage observed in the pancreas of $Ku70^{-/-}$ and $Ku70^{-/-}$ $p53^{p/p}$ mutant mice.

The high expression level of DNA ligase IV in pancreatic islets previously lead us to predict and confirm that DNA damage progressively accumulates in pancreatic islets of $Lig4^{-/-}p53^{p/p}$ mice (Tavana et al, 2010c). To determine whether γH2AX foci, a prominent marker representing DNA damage, is present in $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ islets, we stained mutant pancreatic sections and compared to age-matched wild-type controls. As shown in Figure 35, γH2AX foci and pan staining was abundantly present in aged mutant mice, but not in the wild-type mice sections. Additionally, young mutant pancreatic sections showed few γH2AX positive β-cells compared to none observed in

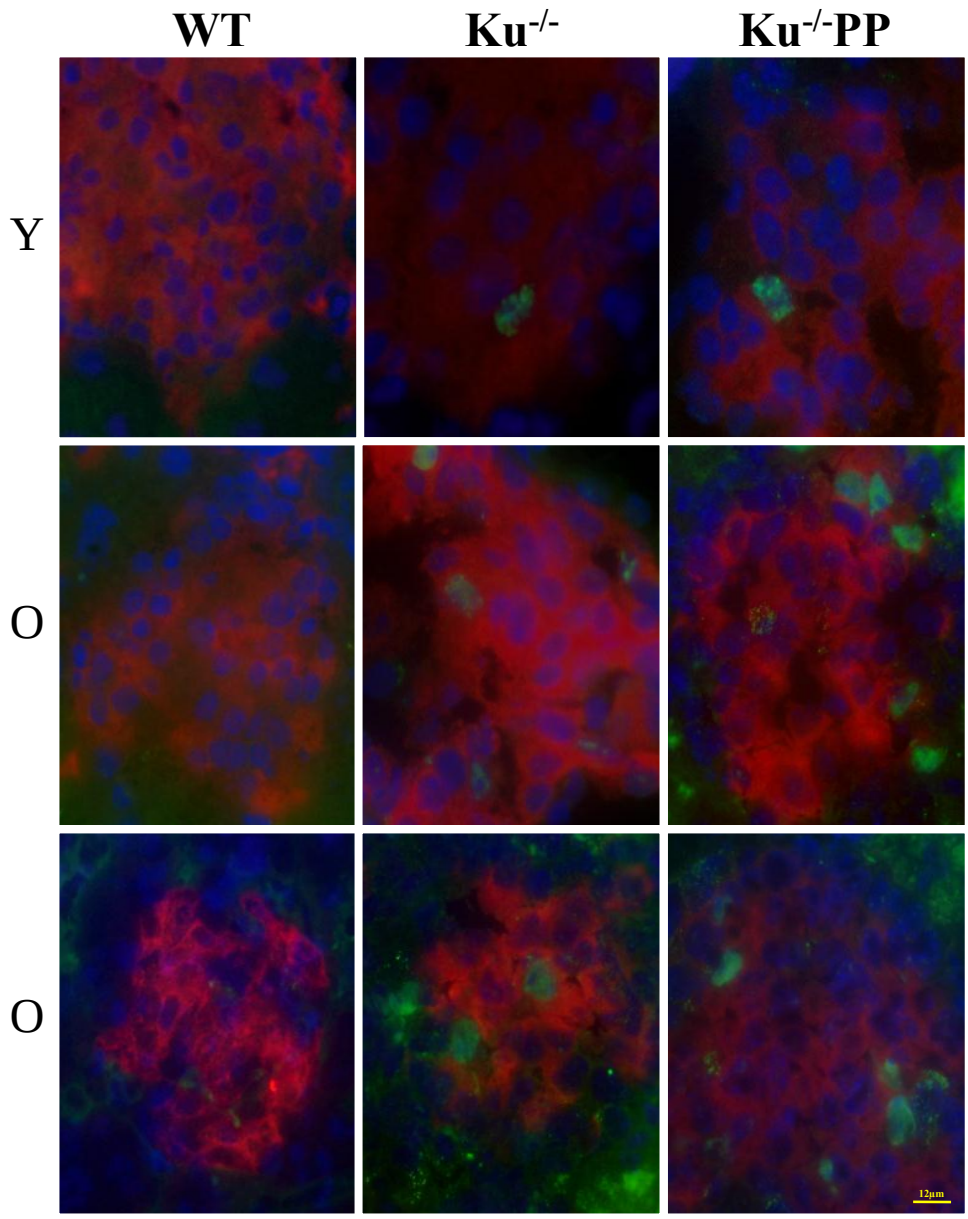

Figure 35. Accumulation of γH2AX in Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets.
Representative pancreatic sections from wild-type, Ku70$^{-/-}$, and Ku70$^{-/-}$p53$^{p/p}$ mice at different ages were stained using an anti-γH2AX antibody (green), anti-insulin antibody (red), and DAPI (blue). Magnification: 60X

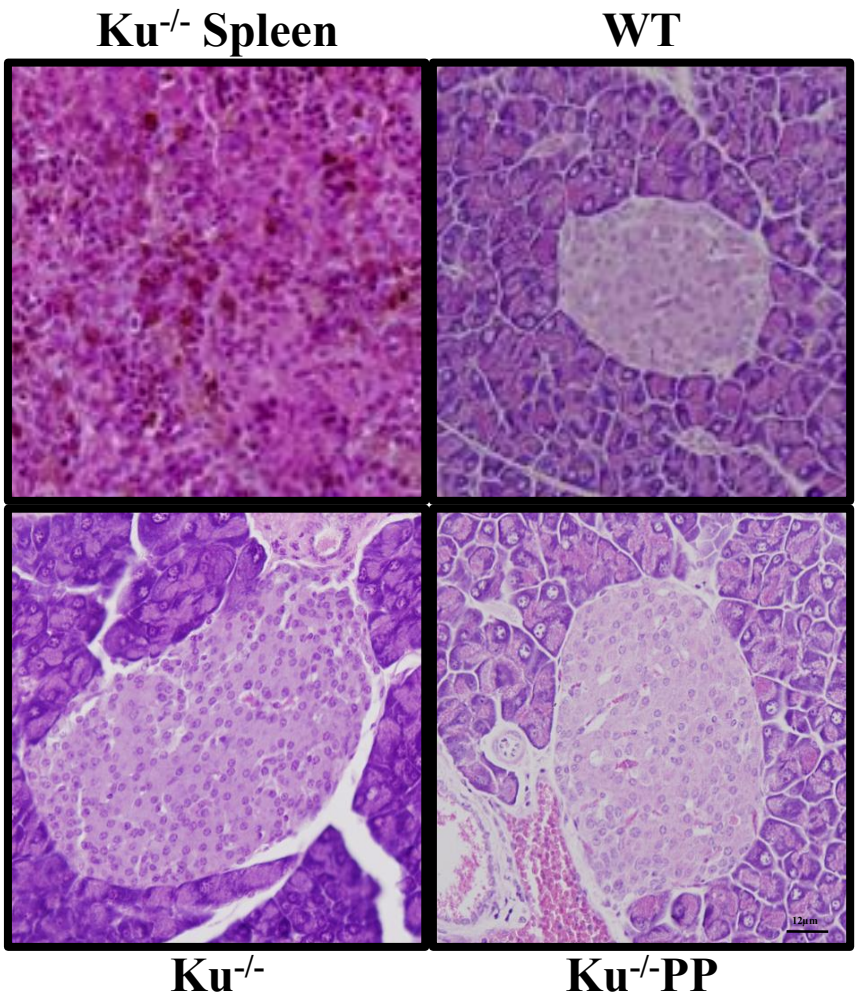

Figure 36. Absence of apoptosis in Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets.
Pancreatic sections from medium mutant and age-matched wild-type controls were analyzed with a TUNEL assay; no apoptotic cells were detected in the islets, while Ku70$^{-/-}$ spleen was used as a positive control. Magnification: 60X.

wild-type sections. These results indicate that, like the Lig4$^{-/-}$p53$^{p/p}$ mutant mice, in the absence of classical NHEJ, pancreatic islets suffer from the persistence of unrepaired DNA damage.

5.2.7 Increased expression of p53 and p21 triggers cellular senescence and not apoptosis in the pancreas of Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ mutant mice.

It is well appreciated that persistent DNA damage from NHEJ deficiency triggers p53-mediated apoptosis in the lymphoid system. We previously did not detect any apoptosis in the Lig4$^{-/-}$p53$^{p/p}$ mutant islets (Tavana et al, 2010c) ruling out p53-independent apoptosis, but since the Lig4 deficiency presents embryonic lethality (Frank et al, 1998), we could not accurately assess the role of p53-mediated apoptosis in β-cells after persistent DNA damage. Therefore, utilizing the Ku70 deficiency, we originally hypothesized to detect apoptosis in this mutant background while not detecting any apoptosis in the Ku70$^{-/-}$p53$^{p/p}$ mutant mice. Interestingly, using a TUNEL assay, no apoptosis was detected in either Ku70$^{-/-}$ or Ku70$^{-/-}$p53$^{p/p}$ islets, while very low levels were detected in wild-type sections as compared to a Ku70$^{-/-}$ spleen (Figure 36). Further, after 10 grays of gamma irradiation, neither wild-type nor Ku70 deficient islets showed elevated levels of apoptosis after 24 hours (data not shown). In aggregate, these data indicate that in the presence of DNA damage, pancreatic β-cells do not under apoptosis.

The accumulation of spontaneous unrepaired DNA damage in the Lig4$^{-/-}$p53$^{p/p}$ β-cells triggered p53 to transactivate p21 as shown through immunohistochemistry (Tavana et al, 2010c). To more quantitatively detect p53 and p21 levels in wild-type, Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets, Western blot analysis was performed on pooled isolated

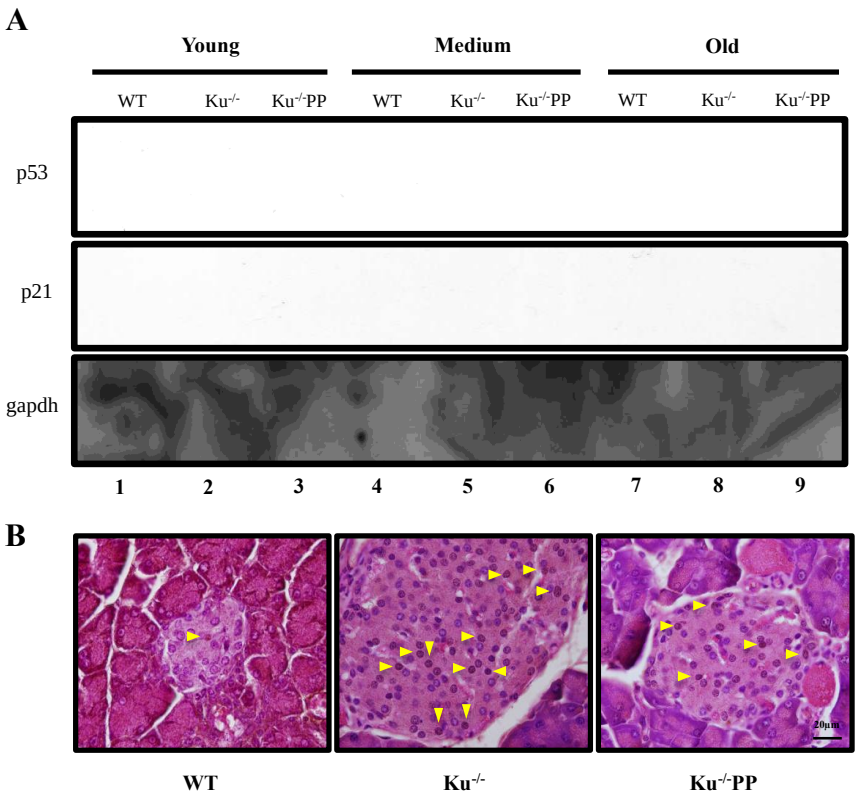

Figure 37. Elevation of p53 and p21 in both Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets.
(A) Western blot analysis of p53 and p21 using protein from pooled purified islets from three different mice per time point and genotype. GAPDH is shown as a loading control. **(B)** Representative sections from medium aged mice incubated with an anti-p53 antibody (brown) and counterstained with H&E to validate the western blot. Arrows indicate p53 positive cells. Magnification 100X.

islet samples. As show in Figure 37a, mutant mice expressed higher levels of p53 when compared to wild-type at all ages, and was predominantly localized to the nucleus Figure 37c. Interestingly, the expression of p21 initially was elevated in young mutant mice when compared against wild-type. Although still higher than wild-type, p21 levels in mutant mice decreased in medium aged mice and slightly increased with age Figure 37b.

Activated p53-p21 promotes cellular senescence in $Lig4^{-/-}p53^{p/p}$ mice as a mechanism to suppress tumorigenesis not only in the lymphoid system (Van Nguyen et al, 2007) but also in the pancreatic β-cells, which suppressed insulin production in many islets (Tavana et al, 2010c). We next asked if the DNA damage induced p53/p21 activation was also triggering cellular senescence in $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ islets. To better visualize the β-galactosidase expression, a common marker indicating cellular senescence, pancreatic sections were stained with glucagon after detection of β-galactosidase. As shown in Figure 38, few islets in young mutant mice showed cellular senescence; in contrast, many older mutant islets underwent cellular senescence. Interestingly, unlike $Lig4^{-/-}p53^{p/p}$ islets (Tavana et al, 2010c), insulin production was not affected in senescent islets as shown through immunohistochemical staining Figure 38. No senescent cells were detected in age-matched wild-type pancreatic sections. Remarkably, although the persistent DNA damage activates p53/p21-induced cellular senescence, preventing tumorigenesis, both $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice still present heightened β-cell proliferation, increased insulin production and hypoglycemia.

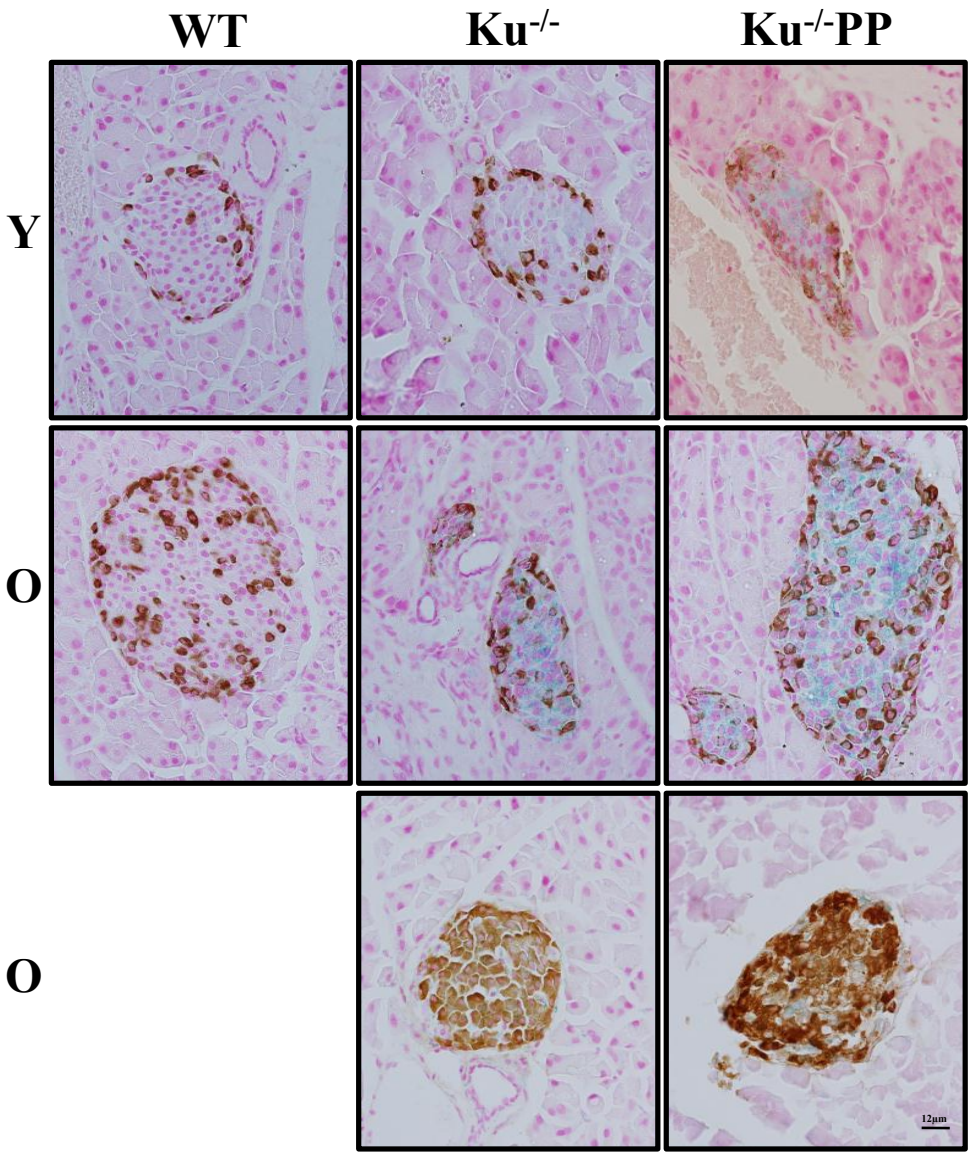

Figure 38. Presence of cellular senescence Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets. Pancreatic sections from young and old mutant and age-matched wild-type controls were stained for β–galactosidase, indicative of cellular senescence, then against glucagon (brown). Very little cellular senescence is detected in young islets while both old Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets show marked upregulation of senescence and very little detected in old wild-type islets. Bottom panels indicate that Ku70$^{-/-}$ and Ku70$^{-/-}$p53$^{p/p}$ islets do not lose the ability to produce insulin upon senescence. Magnification: 60X.

5.3 Discussion:

In this study, we have shown that Ku70, known best for its role in DNA end recognition during NHEJ DNA repair, has an important function in β-cell replication. More specifically, the deficiency of Ku70 results in augmented islet mass, which increases the insulin production driving progressive hypoglycemia. Mechanistic analysis revealed a progressive stabilization of β-catenin, and subsequent activation of TCF4. This was complimented with increased markers for β-cell proliferation—PCNA, cyclin D2 and CDK4. Interestingly, the DNA damage-induced cellular senescence prevented any islet hyperplasia or tumorigenesis, but failed to inhibit the hypoglycemia. This was in stark contrast to our previous findings in the $Lig4^{-/-}p53^{p/p}$ mice, where the DNA damage-induced islet senescence led to β-cell attrition and decreased insulin production, deregulating glucose homeostasis, resulting in the severe diabetic phenotype (Tavana et al, 2010a). The similarities of these phenotypes converge on the deficiency in NHEJ, where spontaneous DNA damage progressively accumulates inducing a p53-mediated response halting the β-cell cycle. Therefore, the most obvious difference between these phenotypes is present early in the absence of Ku70, where the β-cells show increased proliferation, escaping cell cycle control. These results allowed us to dissociate Ku70's function in the β-cell from solely a role in the conventional NHEJ-dependent DNA repair process as seen in the deficiency of Ligase IV.

Many reports have linked Ku70 to transcriptional regulation either by directly binding to DNA or indirectly through protein-protein interaction (Brenkman et al, 2010; De Zio et al, 2010; Giffin et al, 1996; Grote et al, 2006; Idogawa et al, 2007; Lebrun et al, 2005; Nolens et al, 2009). Of particular interest to the present study, researchers have

found that Ku70 may help regulate Wnt signaling upon DNA damage (Idogawa et al, 2007). Briefly, canonical Wnt signaling is centered around β-catenin-dependent transcription and activation of TCF/LEF target genes. In the absence of Wnt signaling, the destruction complex—composed of adenomatous polyposis coli protein (APC), Axin, casein kinase Iα (CKIα), and glycogen synthase kinase 3β (GSK-3β)—binds and targets β-catenin for degradation. Wnt signaling regulates many embryonic developmental events as well as processes to maintain tissue homeostasis, including controlling cellular proliferation (Logan & Nusse, 2004). Many reports have linked defective Wnt signaling, though genetic ablation of Wnt signaling components, to a lose in glucose homeostasis and diabetes while overactive Wnt signaling has been shown to drive β-cell proliferation and increased insulin secretion (reviewed in (Welters & Kulkarni, 2008)). Consistent with this notion, researchers constitutively activated β-catenin specifically in islets, and observed β-cell proliferation rates significantly increase along with elevated insulin levels (Rulifson et al, 2007). Further, inducing islet-specific β-catenin increased β-cell proliferation by 2.5 fold in adult mice (Heiser et al, 2006). Conversely, β-catenin inactivation resulted in islet hypoplasia (Dessimoz et al, 2005). Overexpressing Axin in islets, inhibiting β-catenin activity, greatly impaired β-cell proliferation (Rulifson et al, 2007). Additionally, perturbing β-catenin activity either by overexpression GSK-3β (Liu et al, 2008) or by deleting the Wnt co-receptor LRP5 (Fujino et al, 2003), led to decreased β-cell proliferation and glucose intolerance. Lastly, variants in TCF7L2 (also known as TCF4) have been identified as a strong risk factor for type 2 diabetes (Grant et al, 2006) while abolishing TCF7L2 *in vitro* leads to decreased β-cell proliferation (Shu et al, 2008). Collectively, these studies emphasize the importance of Wnt signaling in

controlling β-cell proliferation—activated Wnt signaling drives β-cell proliferation and increases insulin production, while attenuated Wnt signaling decreases β-cell proliferation.

Previously, researches identified Ku70 as a novel inhibitor of the β-catenin/TCF4 complex; more specifically, Ku70 physically binds to and inhibits TCF4 activation (Idogawa et al, 2007). Further, when Ku70 was abrogated, canonical Wnt signaling increased. Conversely, upon DNA damage induction, Ku70 levels increased, and TCF4 activation was ameliorated, evidenced by a decrease in putative downstream targets. Finally, this regulation was completely independent of Ku80 levels (Idogawa et al, 2007). These findings explain our data shown in Figures 32 and 33, where activation of Wnt signaling was observed in the absence of Ku70. It is commonly accepted that $Ku70^{-/-}$ MEFs prematurely senesce due to an accumulation of DNA damage and subsequent activation of the DNA damage response (Gu et al, 1997; Li et al, 1998). Yet in early passage MEFs, ameliorating Ku70 actually heightens proliferation compared to wild-type as well as rescues growth defects observed in telomere shelterin complex deficient mice. Further, this proliferation rescue was independent of p53 and ATM (Akhter et al, 2010; Lam et al, 2010). These data, combined with our findings, demonstrate that initial activation of Wnt signaling occurs in the absence of Ku70 increasing proliferation until the DNA damage accumulation becomes to excessive and prematurely activates p53-mediated cell cycle arrest preventing tumorigenesis. DNA damage-induced senescence has been previously shown to inhibit tumorigenesis in CKIα deficient gut (Elyada et al, 2011). As mentioned above, Ku70 is triggered upon DNA damage and can inhibit the activation of TCF4 (Idogawa et al, 2007); applied to our $Lig4^{-/-}p53^{p/p}$ model, therefore,

the accumulating islet-specific DNA damage should activate Ku70 to inhibit Wnt signaling in concert with cell cycle arrest to halt β-cell proliferation, leading to the diabetic phenotype observed. Our proposed model is depicted in Figure 39. Interestingly, similar to many models of over-activated Wnt signaling, both $Ku70^{-/-}$ and $Ku70^{-/-}p53^{p/p}$ mice show stabilized β-catenin in the columnar epithelial cells of the colon accompanied with high-grade dysplasia and adenocarcinoma (Puebla-Osorio and Zhu unpublished data). Further investigation is currently ongoing to elucidate the precise mechanism Ku70 functions in suppressing canonical Wnt signaling.

Complimenting the increased β-cell proliferation data, we observed an early and steady elevation in Cdk4 levels (Figure 31). This is not surprising, considering that Cdk4 regulation is paramount to controlling β-cell proliferation (Martin et al, 2003; Mettus & Rane, 2003; Rane et al, 1999; Tsutsui et al, 1999). Interestingly, a constitutively active Cdk4 mutant, $Cdk4^{R24C}$, presented a stark increase in β-cell proliferation and augmented islet mass leading to islet hyperplasia and in some cases, insulinomas (Hino et al, 2004; Rane et al, 1999). This increase in islet proliferation was enough to rescue previously established diabetic models (Miyawaki et al, 2008), collectively placing Cdk4 as a central β-cell regulator. Further, Sushil Rane's group recently revealed that Cdk4 not only promotes quiescent adult β-cells into proliferation, but also activates early β-cell progenitors in the ductal epithelium (Lee et al, 2010). Further characterization of elevated Cdk4 levels in the embryonic pancreas, using the $Cdk4^{R24C}$ mutant, showed increased proliferation and expansion of early mesenchymal endocrine precursors during pancreatic development (Kim & Rane, 2011). These studies underscore pivotal roles for Cdk4 both during embryogenesis by regulating pancreatic progenitors and adult

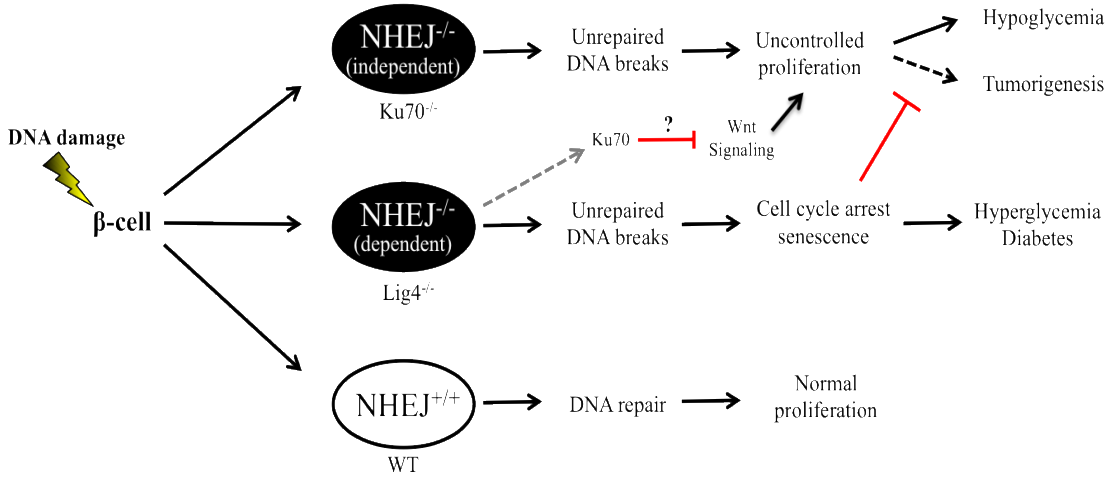

Figure 39. Proposed schematic depicting the outcome of β-cells after DNA damage. When pancreatic β-cells encounter spontaneous or induced DNA double strand breaks, the non-homologous end joining pathway is activated to repair the broken DNA. In the wild-type background, DNA is repaired and β-cell proliferation returns to normal. Deficiency of proteins solely responsible for DNA repair in the NHEJ pathway (as in $Lig4^{-/-}$), result in an accumulation of unrepaired DNA breaks which permanently halts the β-cell cycle decreasing islet mass and driving hyperglycemia/diabetes. Deletion of proteins involved in the NHEJ pathway that have additional roles outside of DNA repair (as in $Ku70^{-/-}$) result in an accumulation of unrepaired DNA breaks without initially arresting the β-cell cycle which results in uncontrolled β-cell proliferation and hypoglycemia. Cellular senescence is induced to suppress tumorigenesis.

β-cell regeneration by controlling the β-cell cycle. Taken together, it is therefore tempting to speculate that the elevated Cdk4 expression seen in both $Ku70^{-/-}$ and $Ku70^{-/-}$ $p53^{p/p}$ islets could be contributing not only to increasing adult β-cell proliferation, but also increasing the β-cell progenitor pools allowing for increased β-cell differentiation and augmented islet mass. Additionally, the presence of DNA damage and an activated DNA damage response protects against the formation of insulinomas, but not from hypoglycemia. Further mechanistic investigation must be preformed to test this theory, which is currently ongoing. Nevertheless, our study highlights the importance function of Ku70, independent of the DNA repair ability in the non-homologous end joining pathway, in the regulation of pancreatic β-cell proliferation.

CHAPTER 6

FUTURE DIRECTIONS

The insights gained from this dissertation suggest that cellular senescence is a formidable barrier against tumorigenesis, although it comes at a cost. The utilization of the hypomorphic, separation-of-function, mutant p53, $p53^{p/p}$, suggests that in the absence of p53-mediated apoptosis, cell cycle arrest can act in parallel to apoptosis in preventing genomic instability. Our study exposing mice to UVB radiation demonstrates that the hypomorphic $p53^{p/p}$ mutation leads to UVB hypersensitivity *in vivo* and *in vitro*. These mutant mice showed less tolerance to UVB radiation; low doses of radiation caused severe skin swelling and higher immunosuppression compared to wild-type mice. However, this was independent to changes in repairing the UV-induced damage, indicating that the increased susceptibility results specifically from UV-induced cellular responses (Chapter 3) (Tavana et al, 2010a). Interestingly, reduced immunosuppression has been identified as a major risk factor for the induction to cancers (Beissert & Loser, 2008). It would be interesting to test if the downstream effects of DNA damage, namely cellular senescence, cause this immunosuppression. We would need to generate $p53^{p/p}p21^{-/-}$ mice and analyze their response to low doses of UVB. This would directly reveal if cellular senescence is the cause of the immunosuppression and hypersensitivity seen in the $p53^{p/p}$ background, as $p53^{p/p}p21^{-/-}$ mice basically phenocopy $p53^{-/-}$ (Barboza et al, 2006). Interestingly, along with other reports, we have shown that the $p53^{p/p}$ mutant cannot transactivate critical downstream apoptotic markers yet retains the ability to partially upregulate p21, responsible for cell cycle arrest. This mechanism still remains unknown, and would be relevant to other p53 mutants that have this inability and directly drive cancer formation. A clue may lie in the nature of promoters that p53 directly regulates. A study showed that the kinetics of genes involved in cell cycle

regulation have "preloaded" promoters and can be activated much faster than the apoptotic molecules that need to assemble and activate the pre-initiation complex (Morachis et al, 2010). This may explain why $p53^{p/p}$ has the ability to partially transactivate p21 while apoptotic genes cannot be activated. Further, the introduction of a proline amino acid may slightly alter the DNA binding ability of certain genes that present much stricter protein stoichiometry than other sites. This can explain why the $p53^{h/h}$ mutant cannot even transactivate p21. Nevertheless, further studies are needed to understand exactly why the hypomorphic $p53^{p/p}$ cannot transactivate apoptotic molecules yet retains a partial ability to activate cell cycle regulators.

In regards to the presence of spontaneous double strand breaks in the $p53^{p/p}$ background, utilizing the deficiency in DNA ligase IV, we showed that the non-homologous end pathway has a previously overlooked function in protecting DNA damage in the pancreatic β-cells (Chapter 4). We have shown that the presence of endogenous DNA breaks can permanently halt the cell cycle in a p53/p21-dependent manner, which decreases the number of proliferating β-cells and decreases the mass of islets. Ultimately, islet involution lowers the levels of secreted insulin, because less cells have the ability to make insulin, and causes dysregulated glucose homeostasis and diabetes (Tavana et al, 2010c). We noted an increased level of DNA ligase IV in isolated pancreatic β-cells, yet the deficiency in DNA ligase IV leads to embryonic lethality. To further study the role of DNA ligase IV in the pancreatic β-cell, it would be interesting to generate an islet specific knock-out, using a RIP-Cre mouse; this would allow for the analysis of wild-type p53 function after severe DNA damage (more severe than the Ku70-/- observed in Chapter 5). Further, because the $Lig4^{-/-}p53^{-/-}$ mice

succumb to aggressive lymphomas around 2 months of age (Zhu et al, 2002), it would be interesting to take the conditional Lig4 mouse and cross it to a p53 deficiency to see if genomic instability occurs leading to islet tumorigenesis. Other than Ku70, we have not analyzed other levels of NHEJ factors in the islets, nor characterized their phenotypes.

In Chapter 5, we unexpectedly showed that the deficiency for Ku70 leads to increased β-cell proliferation and augmented islet mass. This caused stark elevations in secreted insulin levels, which drove the hypoglycemic phenotype observed. The driving force behind this augmented proliferation was the stabilization of the Wnt signaling pathway in the absence of Ku70. Unlike the $Lig4^{-/-}p53^{p/p}$ mutant, the induction of cellular senescence prevented β-cell neoplasia and islet tumorigenesis. The most interesting finding in this study is the relationship between Ku70 and Wnt signaling, underscoring a NHEJ-independent function of Ku70. Very few reports have identified any connection between these two pathways; one in particular has identified Ku70 as a novel competitive antagonist to TCF4 (Idogawa et al, 2007), the transcription factor responsible for activating many downstream targets involved in cellular proliferation as well as many other processes. Researchers further showed that DNA damage increases the levels of Ku70, which lead to a decrease in TCF targets, suggesting that Ku70 inhibits β-catenin/TCF4 binding. Our results indicate that in the absence of Ku70, Wnt signaling is stabilized—namely, β-catenin levels are elevated and eventually translocate into the nucleus. A few possible scenarios may explain the involvement of Ku70 in Wnt signaling: 1. Ku70 directly binds to β-catenin inhibiting the translocation into the nucleus, so in the absence, β-catenin can more readily enter the nucleus and activate TCF4; 2. Ku70 may facilitate the destruction complex responsible for degrading β-

catenin, where in the absence of Ku70 the destruction complex is less effective; 3. Ku70 either binds to the Wnt ligand or the receptor responsible for activating Wnt signaling. These are three possible hypotheses of direct Ku70 interaction with Wnt signaling; it is very likely that the relationship is indirect. Recently, researchers have identified Tert as a novel modulator of Wnt signaling (Park et al, 2009). This regulation occurs along side Smarcal, a gene involved in the DNA damage response. Therefore, it is plausible that Ku70 either interacts with Tert or Smarcal1 to negatively regulate Wnt signaling. Reports have already identified Ku70 to directly interact with Tert, although the interaction with Smarcal remains elusive. Nevertheless, it is important to mechanistically identify the role of Ku70 and canonical Wnt signaling. Lastly, we are proposing that Ku70 may be used as a therapeutic target to coax β-cells into proliferating, alleviating the major problem in some patients with type 2 diabetes mellitus. To strengthen this suggestion, we propose to cross Ku70 deficiency with other models that lead to type 2 diabetes due to a decrease in islet proliferation. One perfect model for this is our $Lig4^{-/-}p53^{p/p}$ model (Tavana et al, 2010c). Dr. Lieber's group has already generated $Lig4^{-/-}Ku70^{-/-}$ mice, and shown that the deficiency in Ku70 is sufficient enough to rescue the lethality from $Lig4^{-/-}$ (Karanjawala et al, 2002). Unfortunately, proliferation rates were not analyzed and this group speculated that the absence of Ku70 rendered any function of Lig4 ineffective because Ku70 is upstream in the NHEJ pathway. Since this group observed no changes in apoptosis levels, an alternative interpretation to their findings is that the deficiency of Ku70 increases proliferation of certain cell types, rescuing the lethality caused by the excessive apoptosis. Generating $Ku70^{-/-}Lig4^{-/-}p53^{p/p}$ mice will allow us to analyze the function of Ku70 in the recovery of

β-cell proliferation. Also, other models (described in (Tavana & Zhu, 2011)) may be used for further validation. Further work is needed to elucidate this puzzle. Taken in aggregate, this work portrays that the function of cell cycle arrest in the presence of DNA damage prevents tumorigenesis, but comes at a cost.

CHAPTER 7

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Vita

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