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Hyperactive p53 Leads to Age-related Phenotypes in Mice Model

A thesis submitted in partial satisfaction of the
requirements for the degree of Master of Science

in

Biology

by

Linda Ye Ou

Committee in charge:

Professor Yang Xu, Chair
Professor Colin Jamora
Professor Cornelis Murre

2009

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The Thesis of Linda Ye Ou is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2009

DEDICATION

For my mother who's every effort in supporting me
through school and life has encrypted on each word in here.

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LIST OF ABBREVIATIONS

ATM…… Ataxia-Telangiectasia Mutated

ATR……. ATM- and Rad-3 Associated

CBP……. Creb-Binding Protein

CDK…… Cyclin-dependent Kinase

CHK…… Checkpoint Kinase

DAPI…… 4',6-diamidino-2-phenylindole

DNA-PK…….. DNA-activated Protein Kinase

DSB……. Double-Stranded Break

GADD…….. Growth Arrest and DNA Damage

IR……. Ionizing Radiation

MAPK……. Mitogen-Activated Protein Kinase

MDM…….. Mouse Double-Minute

MEFs…….. Mouse Embryonic Fibroblasts

NLS……. Nuclear Localization Signal

PCR……. Polymerase Chain Reaction

PGK……. Phosphoglycerol Kinase

PKC……. Protein Kinase C

TNF……. Tumor Necrosis Factor

UV…….. Ultraviolet

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ABSTRACT OF THE THESIS

Hyperactive p53 Leads to Age-related Phenotypes in Mice Model

by

Linda Ye Ou

Master of Science in Biology

University of California, San Diego, 2009

Professor Yang Xu, Chair

Previous work in determining the connection between p53 hyperactivity and premature aging has yielded conflicting results. By introducing aspartic acid mutations on sites Threonine 21 and Serine 23, hyperactivity of p53 was analyzed for its transcriptional activity of various target genes and subsequent physiological influences on cellular response. Target gene expressions show elevation in hyperactive heterozygous T21S23/D mutants in comparison to wild-type mice. Additionally, $p53^{T21S23D/-}$ mutant mice display signs of stem cell depletion, stagnant cell regeneration, organismal atrophy and degeneration as seen normally in aging. $P53^{T21S23D/-}$ mutant mice exhibit reduced lifespan surviving less than 2 months. Aging-inducing cellular responses such as apoptosis, cellular senescence and cell cycle arrest occurring only post to genotoxic

stresses were suggested taking place in these super-active p53 mice models even without DNA damage. Furthermore, knock-out of p53 target gene PAI-1 failed to rescue homozygous T21S23/D mutant embryonic lethality. Taken together, it is concluded that hyperactivity of p53 through T21S23/D mutation leads to age-related phenotypes in mice models.

INTRODUCTION

The p53 gene has become the most widely studied and best understood tumor suppressor known to date. In normal conditions, p53 protein is expressed at low level and has short half-life in cells. The tumor suppressor becomes activated and stabilized only in response to genotoxic stresses including DNA damage, hypoxia, oncogenes activation and nucleotide depletion (Giaccia, 1998). P53 encodes a protein of 393 amino acids that composes of four monomers, each of which can be divided into four domains: an N-terminal transactivation domain (TAD), a sequence specific DNA-binding domain (DBD), a homo-oligomerization domain, and a C-terminal regulatory domain (Levine, 1997). P53 serves its role as a "guardian of the genome" through two main pathways, cell cycle arrest and apoptosis. Delicate control of p53 expression by regulatory elements such as Mdm2 and ATM balances p53's intricate tumor suppression activity (El-Deiry, 1998).

As shown by previous studies, p53 deficient mice are prone to spontaneous tumorigenesis including an array of sarcomas and lymphomas at an early age (Donehower, 1992). More than half of human cancers contain mutations of this gene, suggesting that loss of normal p53 function induces malignant phenotypes (Levine, 1997). However, hyperactivity of this tumor suppressor gene also contributes to phenotypes such as accelerated aging, tissue loss, decreased body weight and osteoporosis (Tyner, 2002).

p53 Structure

P53 is a 393 amino acid long protein that becomes a tetramer when active. The tetramer is composed of four identical monomers, each of which can be divided into four domains: an N-terminal transactivation domain (TAD), a sequence specific DNA-binding domain (DBD), a homo-oligomerization domain, and a C-terminal regulatory domain (Levine, 1997). The acidic N-terminal region constitutes a hydrophobic interface that interacts with the transactivation machinery of the cell. This domain spanning approximately amino acids 1 to 96 activates multi-subunit transcription factor IID (TFIID) through site-specific interactions (Thut, 1995). Hydrophobic amino acids, such as Phe19, Leu22 and Trp23 located in this region take significant roles in transcription activation in vivo, as well as binding to adenovirus E1B-55Kd protein and MDM2 (Hdm2 in human) protein for negative regulation of p53 (Lin, 1994). Key p53 regulatory mechanisms are associated with the amino-terminal domain. Approximately 80% of target genes induced by full length p53 were not induced by p53 containing point mutations at all serine residues within two TADs located at the N-terminus (Ohki, 2007). Nuclear export signaling of p53 is also located within this region. Two serine residues within amino acids 11-27 phosphorylate upon DNA damage thus inducing p53 nuclear export, which in turn optimizes p53 activation in the cell (Zhang, 2001).

Among all p53 induced cancers, approximately 71% of full length protein mutations result from a single amino acid change in the DNA binding domain (Dearth, 2007). This core domain folds into a beta-sandwich scaffold containing four-stranded and five-stranded anti-parallel beta sheets. Two alpha helical loops and a loop-sheet-helix motif in this region are sufficient for p53's contact and binding to DNA. This protease resistant

region spans amino acids 97 to 292 and consists of Zinc ions at locations for DNA-binding activity and protein subunit interactions (Cho, 1994). There are two commonly found types of mutations in DBD. One type of mutation is derived from missense mutations in amino acid residues that disrupt direct DNA interaction. In consequence, DNA binding is lost and p53 loses its ability as a transcription factor (Levine, 1997). Residues Arg-175 and Arg-273 are examples of frequently mutated amino acids in the core domain that induce this type of mutations (Cho, 1994). Disruptions of the structural basis of beta sheets and alpha loops compose the second type of common mutations. Structural integrity in this region directly influences DNA interaction. P53 binding-induced DNA bending plays an important role in p53-DNA and p53-p53 interactions. Previous studies have shown that CATG is the most frequently interacted sequence for p53 at the center of the half sites (Wei, 2006). Furthermore, complexes with mutated central four base pairs CAAG, CTAG, and CTTG containing DNA sequences show partially dissociated and unstable interactions (Pan, 2008). While the extent and explanation determining the DNA bending remains unknown, the DNA binding central domain is an essential element for p53 function and stability.

Amino acids 320 to 360 make up the tetramerization domain. A dimer of a dimer resides in this region and is composed of two anti-parallel alpha helices and an anti-parallel beta-sheet. Each helix pair contains a hydrophobic surface which holds the two dimers together, forming an unusual four-helix bundle. The tetramerization domain associates with the DNA-binding core domain by a flexible linker of 37 residues. It functions in DNA-binding affinity and provides physical contact between p53 protein subunits (Jeffrey, 1995). The two closely associated domains connect in a tetrameric

conformation by having one dimer in contact with the first two quarter-sites and the other dimer to the last two quarter sites (G.McLure, 1998). Furthermore, this region participates in the conformational shift and propagation of intact tetrameric structure of p53 (Halazonetis, 1993).

The C-terminal domain is an open protease-sensitive region (amino acids 364-390) that nonspecifically binds to sites of DNA damage which implicates activation of p53 transcriptional activity. Damaged sites include dsDNA, ssDNA, internal loops, and any loose ends (Lee, 1995). There are several possible events in C-terminal that reactivate p53's central DBD. Deletion of domain, phosphorylation of essential lysine residues and PAb421 antibody binding could all lead to C-terminal's allosteric regulation of specific DNA-binding (Hupp, 1994). Furthermore, this domain contains several nuclear localization signals (NLSs) that direct p53 export into the cell nucleus. Out of the three NLS domains observed, NLSI appears to be the most active and highly conserved domain (Shaulsky, 1990). Study shows that acetylation of lysine residues at C-terminus regulates oligomerization-dependent p53 nuclear export. Mutations of minimal four lysine residues prevent p53 normal tetramerization, thus localizes the protein to the nucleus for degradation (Kawaguchi, 2005).

p53 Upstream Signal and Post-translational Modification

In normal cells, p53 protein exists in a latent, inactive form remaining at a low concentration (Schuler, 2005). This is mainly due to p53's fast turnover rate which involves ubiquitin-mediated proteolysis regulated by Mdm2 (Giaccia, 1998). Upon receiving signals from cellular stress events including DNA damage, hypoxia, oncogenes activation and nucleotide depletion, the tumor suppressor gene activates and turns on appropriate pathways resulting in cell arrest or apoptosis (Giaccia, 1998). By acting as a sensor for upstream stress signals, p53 is essential in many cellular pathways involving various regulatory and mediatory proteins. Different sites on p53 are modified to propagate different cellular response depending on sources and level of damage the cell experiences (Apella, 2001).

One of the most important upstream regulator is ATM protein, encoded by the gene responsible for the genetic disorder Ataxia-telangiectasia. ATM protein specifically respond to DNA double stranded breaks (DSBs) resulting from ionizing radiation, radiomimetic agents and nucleotide base changes caused by UV light exposure (Rotman, 1999). Being a nuclear kinase, ATM contains a protein kinase domain that phosphorylates Ser15 on p53 to induce p53 propagation. Activation of ATM and p53 by DNA damage has been widely studied. Studies show in addition to DSBs, single stranded DNA could also induce ATM/p53 depended cell cycle arrest and apoptosis (Nur-E-Kamal, 2003). Furthermore, p53's negative regulatory protein Mdm2 undergoes ATM dependent phosphorylation on Ser395, leading to p53 stabilization and accumulation. It was shown that in response to DNA damage, ATM phosphorylates Mdm2 in vivo and weakens Mdm2 and p53 interaction, thus attenuates p53 degradation and nuclear

localization (Maya, 2001). As a consequence, p53 level increases and is activated by upstream regulator ATM.

p53 is phosophorylated at multiple sites in both amino and carboxyl terminals post to genotoxic insult. Phosphorylation of several amino acids on the N-terminus, such as Ser18, Ser23, and Ser46, are critical to activate p53 dependent apoptosis (Xu, 2006). DNA-PK also phosphorylates p53's N terminal domain at site Ser37, which in turn assist in p53 DNA binding ability and its transactivation activity. Proteins such as PKC and CKII phosphorylate p53 at its C terminus in response to DNA damage. Activation and proliferation of p53 are achieved upon phosphorylation on sites Ser373 and Ser375 in response to ionizing radiation (Xu, 2006). Other upstream protein kinases that phosphorylate p53 include ATR, CHK1 and CHK2. While ATM and CHK2 induce p53 functions under IR stress, ATR and CHK1 promote p53 response upon UV damage (Apella, 2001). Variation on sites modified is mainly due to different stress signals received by the protein.

Besides phosphorylation, acetylation is another major cellular event that occurs in p53's response to upstream genotoxic stress signals. Histone acetyltransferases such as PCAF and CREB-binding proteins/p300 can acetylate p53s lysine residues at C-terminal domain through interaction with the N-terminus (Apella, 2001). As previously mentioned, various phosphorylation events follow cellular DNA damage. These modifications are believed to be in close association with p53 interaction to CBP/p300. Ser33 and Ser15 phosphorylation promote p53 binding to CBP/p300 (Xu, 2006). In addition, tumor suppressor PML (promyelocytic leukemia gene) under stress conditions colocalize with p53 and CBP into nuclear bodies which induce phosphorylation of p53 at

the N-terminus and acetylation at the C-terminus (Pearson, 2001) (Apella, 2001). In terms of p53 response, this event would trigger transcriptional activation of the protein and its role in apoptosis and senescence. Studies have shown that lysine residues at C-terminal domain are critical for promoting MDM2/p53 mediated ubiquitination degradation. For determining the interactions between acetylation of these lysine residues and ubiquitination, 6KR p53 mutants are generated with six Lys residues mutated to Arg at sites 370, 372, 373, 381, 382 and 386. Contrasting to wildtype p53, MDM2 depended p53 proteolysis is abolished in 6KR mutants (Rodriguez, 2000). Since p300/CBP modify these residues by acetylation post to genotoxic insult, acetyltransferase activities are then directly linked to preventing MDM2 ubiquitination and p53 stablization.

Other modification that regulate p53 activity upon DNA damage include sumoylation , methylation and glycosylation. Upstream mediators that undergo post-translational methylation include oncogenes E2F-1, Ras, DMP1 and Myc, etc. (Jin, 2001). Studies have shown that methylation on lysine residues K372 play an important role in p53 transactivation ability. Together, acetylation and methylation on lysine residues function to sustain p53 stability and activity as a tumor suppressor upon DNA damage (Ivanov, 2007). A methyltransferase Set9 regulates p53's stability through methylating lysine residue specifically located in C-terminal domain (Chuikov, 2004).

Regulation of p53

Mdm2 (Murine Double Minute 2 or Hdm2 for human) is an oncoprotein that is ubiquitously expressed in cells to maintain normal tissue development and cell function. It acts as an E3 ubiquitin-protein ligase to down-regulate p53 level under normal physiological conditions (Chen, 2007; Ma, 2005). The ligase could either target itself or other substrate for degradation depending on post-translational modification such as sumoylation and phosphorylation (Fang, 2000). When cell experiences DNA damage, hypoxia or viral infection, sumoylation on Mdm2 is reduced and the protein prefers self-ubiquitination. As a result, Mdm2-dependent p53-ubiquitination is decreased allowing p53 level to increase in response to cellular stress (Buschmann, 2000). Similar effect is observed with p53 phosphorylation under stress triggering Mdm2 dissociation at binding site (Fuchs, 1998). Activated p53 controls its own activity through production of Mdm2 which forms a system of negative feedback loop (Wu, 1993). The critical role of Mdm2 in regulating p53 is demonstrated in studies where homologous deletion in Mdm2 with embryonic lethality is completely rescued by simultaneous deletion of p53 gene (Jones, 1995; Montes de Oca Luna, 1995). There are three mechanisms in which Mdm2 operates in the autoregulatory loop. One is through Mdm2 direct binding to p53 where it interferes with p53 transcriptional activity. As a consequence of this interference, other p53 dependent protein gene expression is blocked (Lev Bar-Or, 2000). Another mechanism is carried out by Mdm2's ability of nuclear localization and nuclear export of p53. By transporting p53 from the nucleus to the cytoplasm, the protein's transcriptional activity is abrogated and is subjected to proteolysis (Freedman, 1998). Lastly, Mdm2 as

E3 ligase attaches to p53's ubiquitin moieties and targets p53 to 26S proteasome for direct degradation (Oren, 2003).

Many proteins serve as regulators for p53 through their associations with Mdm2. In the case of ATM and DNA-PK, both stabilize p53 by phosphorylation on serine 15, which affect Mdm2 efficient association with p53 (Shieh, 1997). ATM also phosphorylates Mdm2 on residue serine 395 and affects Mdm2 nuclear export, therefore stabilizes p53 through a decrease in its degradation rate (Maya, 2001). AKT is another protein that can directly interact with Mdm2 in p53 regulation. Translocation of Mdm2 from cytoplasm to nucleus depends on phosphorylation on serines 166 and 186 by P13K/AKT kinases (Mayo, 2001). Mdm2 targets p53 for degradation in the nucleus, thus AKT acts as a negative regulator for p53 indirectly. MdmX (also known as Mdm4) is homologous to Mdm2 but does not carry abilities such as ubiquitination of p53. It is yet another important regulator of p53 (Brooks, 2006). Overexpressed MdmX is associated with the attenuation of Mdm2 ligase activity, which stabilizes p53 in return (Stad, 2001). ARF (Alternative Reading Frame also known as $p14^{ARF}$ in human and $p19^{ARF}$ in mouse) suppresses Mdm2 and ARF binding partner (ARF-bp1) to induce p53 activity upon receiving oncogenic signal (Chen, 2005).

With the fact that most of proteins regulate p53 through association to Mdm2, there are still many regulators functioning without involving the Mdm2/p53 feedback loop. One such proteins is the abovementioned MdmX. MdmX attenuates p53 transcriptional activity in vitro and suppresses acetylation of p53 by inhibiting p300/CBP (Kadakia, 2002; Danovi, 2004). JNK phosphorylates p53 to induce its transactivation cascade which involves non-Mdm2 regulation (Buschmann, 2001). H1F1α is another

important regulator for p53 where it affects p53 stabilization upon hypoxia stress (Carmeliet, 1998). Small ubiquitin-like proteins SUMO-1 and SUMO-2/3 all act as p53 regulators affecting transcriptional response and senescence pathways of p53 (Rodriguez, 1999; Li, 2006). SUMO proteins modify p53 at residue Lys-386 which could be considered as a possible therapeutic target for future experiments. Overexpression of SUMO-1 induces transcriptional activity of p53 in vivo (Rodreguez, 1999). Hyper-induced SUMO-2/3 in cells show premature senescence phenotypes in p53 knock-down mice models (Li, 2006). SUMO-2/3 overexpression elevates senescence pathway controlled by protein p21 expression in normal cells (Li, 2006). Other regulators for p53 that are commonly seen and do not involve Mdm2 include BRCA1 and p33ING1kl.

p53 and Cell Cycle

Cell cycle is a controlled loop in which events take place solely based on the successful completion of an earlier event, thus cycle checkpoints are critical for normal cell cycle progression. As aforementioned, p53 respond to DNA damage by several means including cell cycle arrest. p53 plays an important coordinating role in both G_1 and G_2/M cell cycle checkpoints, where it monitors cell DNA replication and segregation (Agarwal, 1995).

In response to genomic stress, p53 mediates cell cycle arrest through transcriptional activity on its immediate downstream target $p21^{WAF1/CIP1}$ (El-Deiry, 1993; Harper, 1993; Agarwal, 1995; Winters, 1998). $p21^{WAF1/CIP1}$ are cyclin-dependent kinase (CDK) inhibitors which inhibit CDK complex cyclinD/CDK4 in suspension of G_1 checkpoint (Deng, 1995). Studies show heat shock at 43°C could induce transient

accumulation of cellular p53 and p21 which triggers p53 dependent cell cycle arrest at G_1 (Nitta , 1997). Basic mechanism of p21's interruption of G_1 rests upon its ability in disrupting phosphorylation of retinoblastoma gene product (pRB), thus delaying dissociation of E2F-pRB complex and S-phase entry of the cycle (Weintraub, 1992). Similar concept is observed in G_2/M cycle arrest regulated by p53. $p21^{WAF1/CIP1}$ inhibits CDK Cdc2, which in turns prohibits its binding to cyclin B and interrupts activation of Cdc 25 in entry to mitosis (Winters, 1998).

There have been conflicting ideas regarding p53's role in G_2/M checkpoint regulation. Studies show mutant p53 or p53 nullizygous cells enter DNA-damaged or p53-independent G_2/M cycle arrest (Kanstan, 1991). Hypothesis suggested G_1 and G_2/M checkpoints are interrelated and intricately coordinated by p53 control system. Depending on the extend of cellular damage, cell under p53 regulation decides its fate in cell cycle arrest progression at different checkpoints (Pellegata, 1996). Furthermore, evidence support p53's significance in G_2/M checkpoint. By expressing p53 in tetracycline-regulated cells originally without endogenous p53, p21/WAF1 is induced while both G_1 and G_2/M are perturbed (Agarwal, 1995). A recent study revealed that human bronchial epithelial cells with high zinc status experience elevated p53 and p21 activity, which lead to G_2/M cell cycle arrest (Wong, 2008). Mdm2 and p53 negative feedback loop also serves an important regulating role in cell cycle arrest. This is mainly due to Mdm2's inhibition ability in p53's transcriptional activity (Kanstan, 1995). In all, p53 is significant in maintaining genomic integrity through regulating cell cycle checkpoints.

p53 and Apoptosis

There are multiple pathways p53 use to activate apoptotic response in prevention of furthering cell damage and maintenance of tissue homeostasis: transactivation of apoptotic factors, translocation to mitochondria, and redistribution of reactive oxygen species. In which mechanism p53 undertakes depends on the cellular context such as type of stress signal, cell type and p53 expression level. P53 promote apoptosis and caspase activations through both transcriptional regulation of target genes and transcription-independent mechanisms.

Despite the complexity of p53 regulated apoptosis network, two major pathways are presumably far more important. The extrinsic pathway is activated through induction of transmembrane cell surface receptor proteins such as Fas, DR5 and PERP upon binding to death ligands such as CD9/APO-1, TRAIL receptors and tumor necrosis factor receptors (Locksley, 2001). The cell surface receptors contain a death domain that is crucial in conducting external messages to intracellular mechanisms (Muppidi, 2004). DNA damage triggers ligation of death-receptor-family receptor DR5/KILLER for TNF-related apoptosis-inducing ligand (TRAIL) (Wu, 1997; Haupt, 2003). Ligation then induces formation of death-inducing signaling complex (DISC) employ elements including adapter molecule Fas-associated death domain (FADD) and caspase 8 (Boldin, 1996). Caspase 8 is activated by recruitment of DISC and following that, it cleaves and activates downstream caspases such as caspase 3 thus leading to intracellular apoptotic caspase cascade (Ashkenazi, 1998; Schuler, 2001). Data show inactivation of caspase 8 could significantly attenuate p53 induced cell death response (Varfolomeev, 1998). As aforementioned, apoptosis pathway is context specific in example of DR5 induction with

cell-type specificity. Although basal expression of DR5 does not depend on p53, p53-dependent DR5 mRNA expression is detected in spleen, small intestine and thymus upon gamma irradiation (Burns, 2001). PERP is another apoptotic gene that is induced in transformed MEFs by doxorubicin which correlates with p53 apoptosis pathway (Benchimol, 2001). PERP directs p53 downstream effectors and is a plasma membrane protein that belongs to the PMP-22/gas family of proteins which regulates cell growth (Haupt, 2003). Although overexpression of PERP is observed in apoptotic cells, its mechanistic association to p53 has yet to be defined.

The intrinsic pathway involves transactivation of Bcl-2 protein family which mediates mitochondrial release of cytochrome c (Kuwana, 2002). Cytochrome c release facilitates formation of apoptosome complex employ adapter molecules Apaf-1 and caspase 9 which triggers caspase activation to cell apoptosis (Zou, 1997). The Bcl-2 family could be divided into two categories based on structural similarity to Bcl-2 homology domain: anti-apoptotic and pro-apoptotic proteins (Haupt, 2003). Pro-apoptotic members include p53 target genes including such as Bax, Bak, Noxa and PUMA (Haupt, 2003). Bax forms a homodimer upon cell stress and similar to DR5 in extrinsic pathway, its expression is cell-type specific (Skulachev, 1998; Chong, 2000). Data show accelerated tumor growth and attenuated p53-dependent apoptosis in Bax-deficient mice indicating the significance of Bax in p53-induced cell death (Yin, 1997). Noxa and PUMA proteins are upregulated in p53 mediated apoptosis and both regulate the release of cytochrome c (Nakano, 2001). Noxa is activated in response to X-ray irradiation and acts in a similar manner as Bax in contributing to p53-dependent apoptotic pathway (Haupt, 2003). PUMA and its connection to p53 will be discussed in detail in

the next section. A more recent study shows p53 transcriptionally regulates antiapoptotic protein with caspase recruitment domain (ARC). ARC is down-regulated under cell stress conditions while p53 knockdown could attenuate ARC protein decreasing rate, thus proving p53's repression of ARC in initiating apoptotic response (Li, 2008).

Although the abovementioned two pathways are often distinguished separately, studies show convergence of the two through a pro-apoptotic BH3 protein Bid (Haupt, 2003). While the recruitment of DISC activates caspase 8 and an array of caspase activities, Bid is cleaved and activated (Luo, 1998). Through activation, a new site on Bid is post-translationally myrisoylated and leads Bid to mitochondrial translocation where it activates Bax and Bak for p53-dependent cell death program (Chou, 2004).

I. PUMA. The PUMA (p53-upregulated modulator of apoptosis) gene is directly induced and regulated by activated p53 in response to DNA damage. Similar to Noxa, PUMA belongs to BH3 (Bcl-2 homology 3) only Bcl-2 family proteins, sharing only a short stretch of the family coding sequence (Yu, 2003). Characteristics that separate this proapoptotic protein from many other p53 downstream targets include its highly conserved coding and regulatory sequence and its effectiveness in inducing cell death (Yu, 2003). PUMA encodes two proteins PUMA-α and PUMA-β in humans (Nakano, 2001). Both proteins bind to Bcl-2 and induce mitochondrial release of cytochrome c upon p53 activation (Nakano, 2001). Factors influence permeability of mitochondrial membrane include Bax (Bcl-2-associated X protein) and Bak (Bcl-2-antogonist/killer) (Adams, 2007). Furthermore, release of cytochrome c promotes activities of caspase 3 and 9 in a p53-induced and cytochrome c/Apaf-1-dependent apoptotic pathway (Nakano,

2001). As seen in many data, PUMA is an essential player in cell death pathway and inhibition of cell growth. PUMA deficiency accelerates tumorigenesis and reduces apoptosis in $Puma^{-/-}$ mice (Danovi, 2008). Hematopoietic cells from $Puma^{-/-}$ mice under genotoxic stresses such as IR and deregulated c-Myc expression show cell death attenuation and lowered PUMA expression (Jeffers, 2003). Similar results were found where PUMA deficiency abolishes DNA damage and p53 induced cell death through inefficient transcription coactivator recruitment by PUMA promoter (Wang, 2007). While DNA damage independent apoptosis pathways were unaffected, the data show PUMA's essentiality in p53 regulated apoptosis (Wang, 2007). The balance between p21 and PUMA determines cell fate under stress conditions either to cell arrest or death (Yu, 2003). Studies show cell undergoes normal apoptosis when p21 gene is disrupted or annulled apoptosis when PUMA gene is disrupted (Yu, 2003). It is extremely important for cell to distinguish between p53 activation pathways in response to DNA damage. Future studies would focus on activation of PUMA through p53-independent mechanisms to serve purposes such as tumor suppression and cell regulation since PUMA is highly involved in many different pathways.

p53 is a Tumor Suppressor

P53's function as tumor suppressor is first identified through mutation and deletion of the gene in murine models. An array of cancer results from p53 deficiency which involves tumors of different cell types. Homozygous p53 deficient mice develop spontaneous tumors with frequency of 100% while heterozygous deficiency results in 90-95% tumor development (Donehower, 1992). Although tumor types vary based on allelicity of p53 gene, major tumors discovered in p53 deficient mice include lymphomas of various visceral organs, sarcomas and carcinomas (Donehower, 1992; Donehower, 1995). It is apparent that allelic specificity and genetic background are important effectors of cancer formation which varies between individuals.

P53 deficiency leads to Li-Fraumeni Syndrome in humans, a hereditary autosomal dominant disorder that increases host susceptibility to tumorigenesis (Iwakuma, 2005). Genetic inheritance of only one copy of functional p53 gene is similar to heterozygous p53 mutation in which both lead to reduced response to cell stress and an array of cancer. P53 has many direct and indirect links to tumor formation. It was discovered that level of p53 in skin cell determines phenotype of skin tumors indicating direct relationship between p53 dosage and cancer development (Kemp, 1993). Amplification of MDM2 ubiquitination and cytoplasm localization of p53 leads to many types of cancer, both due to down regulation of p53 level. Recent studies show p53's vital role in tumor suppression with restoration of endogenous p53 expression lead to tumor regression. Increased apoptosis in lymphomas and cell growth suppression in sarcomas are observed in restored functional p53 murine models (Ventura, 2007).

Through two ways p53 serves its function as a tumor suppressor. One is by p53's ability to arrest cell cycle progression and another is through its regulation in cell apoptosis. Several transcriptional target genes directly or indirectly regulated by p53 include p21, p16, p19, p27 and GADD45 all play important roles in cell cycle inhibition (Lukas, 1995; Agus, 1999). Regulation on genes such as PUMA, Bax, Perp, Killer/DR5 and Bcl-2 family protein allow p53 to function as a promoter for both transcription dependent and independent apoptosis pathways (Haupt, 2003).

p53 is a Transcription Factor

Crystallography analysis provided three dimensional structure of p53 to confirm that the protein's core domain is indeed involved in DNA binding (Cho, 1994; Jeffrey, 1995). Further structural analysis showed that p53 binds specifically to target DNA fragment that contains symmetrical 10bp palindromes PuPuPu(CA/TA/TG)PyPyPy separated by a spacer up to 13 nucleotides long (G.McLure, 1998). There are nearly 300 genes possible of being p53 transcription targets (Hoh, 2002). However, variability of the consensus sequence allows possibility of paired coding event which creates complexity in regulation and gene expression (Hoh, 2002). Due to the required stringent regulation and specificity of consensus sequence, p53 point mutations at DNA binding domain are the most commonly observed in cancers (Dearth, 2007). Loss of DNA binding ability directly effects p53's ability as a transcription factor (Levine, 1997).

Out of the many transcription targets of p53, cyclin-dependent kinase (CDK) inhibitor $p21^{WAF}$ is one of the most widely studied (El-Deiry, 1993). Two specific p53 DNA-binding response elements in p21 promoter directly regulate and control $p21^{WAF}$

expression (El-Deiry, 1998). $P21^{WAF}$ was isolated and recognized as the gene responsible for senescence (Noda, 1994). As a CDK inhibitor, it also plays important role in blocking G1/S transition in cell cycle progression following DNA damage (Waldman, 1995). Another p53 target gene, *GADD45* (GADD inducible gene#45) when overexpressed in cultured cells also induces G1 cell arrest (Kastan, 1992). It was suggested that *GADD45* with binding to proliferating cell nuclear antigen (PCNA) functions as a backup for p21 (Levine, 1997). *GADD45* was also identified to be responsible for G2 arrest through its inhibition on cyclin B/cdc2 (Zhan, 1998). Two indirect target genes of p53, p16 and p19 also play important roles in cell cycle control and senescence. P16 is indirectly regulated by p53 transactivity and in turn regulates G1/S transition phase (Lukas, 1995). When over-methylated, p16 loses its inhibition on cyclin D_1-Cdk4 which result in several types of tumors (Levine, 1997). MEFs undergo spontaneous immortalization when p19 expression is lost or when p53 experiences mutation (Sherr, 1998). Thus, p19 was suggested to serve critical functions in regulating cell senescence. Downstream mediators of p53-dependent apoptosis include Bax, Killer/DR5, Perp, Puma and PIGs (p53-induced genes) (El-Deiry, 1998). PIG3 (one of PIG gene) and Bax both appear to be directly regulated by p53 through p53 DNA-binding response elements located in the respective gene promoters (Polyak, 1997; Miyashita, 1995). Killer/DR5 is a death receptor gene that is induced by DNA damage through p53 regulated pathways (Wu, 1997).

One of the most important transcriptional target genes regulated by p53 is MDM2. MDM2 degrades p53 and forms a negative auto-regulatory loop with p53 (Fang, 2000). Several other p53-regulated target genes include Cyclin G, GML (GPI-anchored

molecule-like protein), and HIC-1 (Hypermethylated In Cancer #1), and many more (El-Deiry, 1998). GML and overexpression of cyclin G both lead to growth suppression and HIC-1 is a candidate for tumor suppressor (Okamoto, 1994; Furuhata, 1996; Issa, 1997). However, these genes and many other unidentified p53 target genes still remain unknown.

p53 and Senescence

Senescence is defined as cells, obtained from host, that are proliferating-capable achieves physical terminating state after a limited number of divisions in culture (Hayflick, 1961). Cultured murine cells experience cell cycle arrest at G1 phase induced by tumor suppressors ARF-p53 pathway to protect cells from oncogenic transformation (Hahn, 2002). Deletion of ARF, Rb proteins and p53 allows re-continuation in the proliferating ability of cells and bypass senescence pathways, thus providing apparent evidence for the connection between p53 and senescence (Lundberg, 2000). Overexpression of p53 in bladder carcinoma cells also triggers rapid cell cycle arrest in cells that express senescence morphology after 48-72 hours (Sugrue, 1997). More evidence is seen in experiments using antibody epitopes that are phosphorylation-sensitive to detect possible phosphorylation modifications on p53 in senescent cells. It is clear that replicative senescence is associated with increased phosphorylations on p53 residues Serine 15, Threonine-18 and Serine 376 and decreased phosphorylation on site serine 392 (Webley, 2000). The pathway and many regulating proteins have yet to be identified and understood completely. One protein, MORC3 (microrchidia3)-ATPase has recently been identified to be capable of regulating cellular senescence induction of p53

under stress conditions (Takahashi, 2007). Another important regulator of the p53 dependent senescence pathway is PAI-1.

I. PAI-1. Plasminogen activator inhibitor-1 (PAI-1) shows important function in several biological phenomena such as fibrin clot lysis, ovulation, embryogenesis, angiogenesis, inflammation, and metastasis (Carmeliet, 1993). PAI-1 is a downstream target of p53 and considered as a marker of replicative senescence due to its high expression during senescence (Serrano, 1997). Suppression of PAI-1 induces cell immortalization in both primary human and murine MEFs due to abrogated senescence (Kortlever, 2006). However, PAI-1 deficient mice showed normal viability, fertility and development compared to wild-type mice (Carmeliet, 1993). In cells experiencing senescence, PAI-1 and tumor suppressors p21 and p16 are all upregulated, while P13K-PKB is downregulated (Kotlever, 2006). Since p19 is an indirect transcriptional target gene of p53 which play important role in cell senescence, its level in PAI-$1^{-/-}$ was observed comparable to that of wild-type cells, thus conclusion that p19 expression was not lost with PAI-1 knockdown (Kotlever, 2006). Down regulation of PAI-1 causes activation of uPA (urakinase-type plasminogen activator), a protease that PAI-1 antagonizes. Cellular growth factor increases due to uPA activation, thus stimulating the proliferation and the repression of senescence (Kortlever, 2006). The pathway involved is the P13K-PKB route in stabilization of nuclear cyclin D1 (Kortlever 2006). Another study suggests PAI-1 knockdown cells or uPA overexpressing MEFs bypass senescence after a prolonged culture (Kortlever, 2006). The balance between uPA and PAI-1 becomes critical in p53 mediated proliferative senescence (Kortlever, 2006). Upregulated PAI-1 cells are

insensitive to mitogenic signals and senescence while applying no effect on other cells nearby. However, unbalanced uPA/PAI-1 cells could increase cell's sensitivity to mitogenic signals and cause cell and nearby cell to become immortal (Korlever, 2006). This explains the reason of PAI-1 suppression induces immortalization of both human and murine MEFs.

p53 and Aging

Studies show hyperactive or genetically-activated p53 have enhanced tumor-resistant ability. Thus leading to possible future cancer therapies, however, whether there is negative effect of premature aging through manipulating p53 activation is still controversially discussed (Tyner, 2002; Garcia-Cao, 2002; Donehower, 2002; Febeyre, 2002). Since correlation between p53 and aging was nearly impossible to investigate on p53-deficient murine models due to early onset of tumorigenesis, most of the studies are done through mutated p53 in vivo. A line of genetically modified mice exhibiting a truncated form of p53 with strengthened tumor suppressor activities showed accelerated aging phenotypes including reduced body weight, osteoporosis, decreased life span and organ atrophy (Tyner, 2002). Furthermore, mice containing over-expressed activated and partially mutated p53 also result in similar aging phenotypes (Tyner 2002). However, evidence showed mice generated with the truncated form of mutation contains a 24 genes deletion that could lead to possible explanation of aging phenotypes (Tyner, 2002; Gentry, 2005). Thus, the association between p53 and aging remains inconclusive.

Additionally, mice with several genomic transgenes of wild type p53 indicate no significant linkage between hyperactive p53 with aging (Garcia-Cao, 2002). These

"super p53" mice containing excessive copies of active p53 show rescue of p53 functionality and enhanced response to genotoxic stresses such as γ-irradiation and chemical carcinogenes (Garcia-Cao, 2002). Premature aging evidence was not detected in these "super p53" mice while they display normal life span, hair growth rate, degree of lordokyphosis, skin thickness and bone structure in comparison to wild-type p53 mice (Garcia-Cao, 2002). However, this model lacks full-support of increased p53 level associating with normal aging process since basal level of p53 activity shows wild type features and remains unaffected by the transgenic modifications (Garcia-Cao, 2002). A more recent study shows an increase in p53 activity through decreasing level of Mdm2 thus promoting p53 functionality while mice develop normal aging process (Mendrysa, 2006). Data from Mdm2 deficient mice indicate no change in body weight and bone thickness and statistical insignificant in skin thickness and subcutaneous fat measturement (Mendrysa, 2006). It becomes more apparent as evidences support that elevated p53 functionality do not lead to premature aging other than altered p53 such as resulted from the truncated forms.

Elderly population make up most members in cancer patients, thus relations between p53 and aging has long been suggested. Evidence show decreased p53 transcriptional activity, p53-dependent apoptosis, p53 protein post-IR damage, and ATM function in aging mice (Feng, 2007). p53's responses to DNA damage declines dramatically in different tissues of aged mice while the protein's stability decrease significantly after stress (Feng, 2007). Plausible evidence show strong correlation between aging and tumorigenesis due to p53 dependent activities and apoptosis (Feng 2007).

Significance of the Threonine-21 and Serine-23 (Human Thr18 and Ser20) Residues

Murine Threonine-21 and Serine-23 correspond to human Threonine-18 and Serine-20 respectively. These two sites are highly conserved in both mice and human, providing similar signaling cascade and regulation mechanisms. Both Thr21 and Ser23 contribute to p53 stability and activation through posttranslational modification. Structurally, Thr18 and Ser20 are both located in p53 N-terminus, which directly interact with Mdm2. Thr18's hydrogen bonding to neighboring residues helps to stabilize the amphipathic helix (Sakaguchi, 2000). Phosphorylation on this site would disrupt this bonding and in turn inhibit Mdm2 binding to p53. Studies using monocolonal antibodies that are highly specific for Thr18 and Ser20 in human indicates significant phosphorylation of p53 expression in human breast cancer. Evidence shows these two phosphorylation sites within p53 are important for p53 protein-protein interaction, Mdm2 binding and amplification of signaling cascades which modulates p53 activity in cancer (Craig, 1999). Defective phosphorylation of p53 at Thr18 causes aberrant interaction between p53 and Mdm2 (Craig, 1999). The role of phosphorylated Ser20 in proliferating cell is to decrease Mdm2 binding in order to lower Mdm2 dependent p53 degradation and increase p53 stability (Craig, 1999). Recent studies indicate correlation between phosphorylation of Thr18/Ser20 and p53 apoptosis (Nakamizo, 2008). Since wild type p53 cells are resistant to adenoviral-mediated p53 induced apoptosis, phosphorylation-specific antibodies were used to demonstrate that simultaneous phosphorylation at Thr18 and Ser20 induces p53-mediated apoptosis (Nakamizo, 2008). This finding would contribute greatly to clinical strategy of using Ad-p53 gene therapy in cancer treatment.

Many studies have analyzed mutations on Thr18 and Ser20 in order to deduce influences of these two sites in p53 functionality. Posttranslational modification of p53 is often regulated through a series of activities. For example, C-terminal acetylation is promoted by N-terminal phosphorylation on several sites including Thr18. It was found that point mutation of Thr18 or other codons such as Ser9, Ser15 to alanine reduced acetylation on Lys382 (Saito, 2002). However, changing Ser23 (murine Ser20) to alanine did not lead to significant change in p53 activity (Wu, 2002). Phosphorylation of Ser23 was found to be unnecessary for p53 response in UV and IR damage (Wu, 2002). In addition, alanine mutations to sites Ser15, Thr18 and Ser20 did not contribute to significant changes to p53's stability and nuclear localization (Yamauchi, 2004). These results contradict with the recent findings by Nakamizo et al which indicate clearly the essential role of Thr18 and Ser20 in p53 dependent apoptosis. Other contradictions were found through more studies of alanine mutation to Ser23. Apoptosis was nearly abolished in mouse thymocytes with Ser18/23A mutations under IR treatment (Chao, 2006). It was determined that Ser18 and Ser23 phosphorylation is critical for p53 induced apoptosis and tumor suppression (Chao, 2006). Apparent contradictory perspectives hold on this matter. To better understand the importance of Thr21 and Ser23 in relation to p53's activity, stability and regulation, further study on these two sites are necessary.

MATERIALS AND METHODS

Generation of p53 T18D/S21D knock-in mice. Explained mostly in Chao et al, 2006 and Chao et al, 2003. Insertion of a PGK-neomycin resistance selectable marker cassette flanked by two LoxP sites allowed PCR and Southern blotting confirmation producing wildtype band at 14kb and mutant band at 6.5 kb. The germline-transmitted mice were cross-bred with CMV-Cre mice to excise the PGK-neo^{R} gene from targeting alleles. However, mutant mice carrying PGK-neo^{R} gene were not seen due to embryonic lethality of Cre-deleted mutants. Crossbreeding of p53$^{T21S23D/+}$ and p53$^{+/-}$ mice were used to generate p53$^{T21S23D/-}$ mice as studying subjects for this research topic.

Generation and Treatment of Mouse Embryo Fibroblasts (MEFs). Embryos were isolated from pregnant mice at 12-15 days. Head and fetal livers were removed and the fetus was homogenized, trypsinized, and incubated at 37°C with 5% CO2. MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, antibiotics, and beta-mercaptoethanol.

Western blot analysis. Protein extracts from 2x 10^{4} MEFs were separated by 10% polyacrylamide gel through SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked with 5% dry non-fat milk in TBST and probed with a polyclonal antibody against p53 (CM-5; Vector Laboratories Inc.), a polyclonal antibody against p21 (Santa Cruz Biotechnology, Inc.). The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) developed with

ECL Plus (Amersham Biosciences). In order to determine the amount of expression is comparable in each lane, membranes were stripped and probed with a goat polyclonal antibody specific for β-actin (Santa Cruz Biotechnology). Kodak maximum resolution film was used to expose the blots.

Quantitative real-time PCR analysis. Total RNA was isolated from MEFs or mouse thymocytes with both the Trizol Reagent (Invitrogen Corporation) and RNeasy RNA cleanup (Qiagen). To remove genomic contamination, on-column DNase digestion was conducted (Qiagen). RNA was then reverse transcribed using Superscript II RT (Invitrogen). Real-time PCR was performed on a Prism 7000 Sequence Detection System (ABI) with SyBr Green PCR Master Mix (ABI). PCR conditions as follow: 10 minute hot-start at 95°C, followed by 40 cycles of 30 seconds at 95°C and 1 minute at 60°C. The average threshold cycle (C_t) for each gene was from triplicate reactions and normalized to expression of Glyceraldehyde-3-phosphate dehyrogenase for comparison. Primer sequences have been previously described by Chao et al, 2003.

Proliferation of MEFs. Proliferation of MEFs was analyzed with a standard NIH-3T3 protocol. $3x10^5$ MEFs were serially passaged in 6cm dishes and counted every three days up to 15-20 passages. Three plates of each genotype were counted for each passage.

Embedding and Cutting Frozen Section. Skin sample obtained from euthanized mice with body hair removed. Half of the skin sample removed was fixed in Bouin Fixative at 4°C overnight before switching to 70% Ethanol for further analysis. The other half of the

skin was immediately frozen in Optimal Cutting Temperature with template from Cryomold on dry CO_2.

Immunohistochemistry Sectioning. Deparaffinize with Xylenes followed with gradual hydration of sections from 100% Ethanol to 50% Ethanol. Three times of 0.01M Phosphate Buffered Saline (PBS) wash are conducted between buffers. Slides were heated in Sodium Citrate buffer in 98°C for 30 minutes. 0.3% H_2O_2/Methanol is used to quench endogenous peroxidase activity with concentration of the solution at 1 H_2O_2:100 Methanol. One hour incubation of slides in moistened box with addition of blocking buffer is followed by overnight incubation of slides with Primary Antibody (p63 for skin stem cell) in 4°C environment. Secondary antibody blocking buffer incubation for 1-2 hours in room temperature after brief 0.01M PBS wash. Overnight incubation for secondary antibody (Goat anti-mouse IgG HRP) in moistened box in 4°C room. Twice of 0.05M Tris-HCl washes are necessary after the incubation. 0.1% Diaminobenzidine (DAB) is used to react with peroxidase upon addition of hydrogen peroxidase and brown color was observed. Post to observance of immunofluoresence, Hematoxylin wash of slides for 5-8 minutes in room temperature. 1% HCl/Ethanol is used for washing off the color for future analysis. Drying of slides was done in room temperature overnight.

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RESULTS

Generation of $p53^{T21S23D/-}$ mice model for analyzing T21S23/D mutation function and expression. To begin with the construction, Thr21 and Ser23 codons were mutated to aspartic acid using site-directed mutagenesis technique. Endogenous alleles and ES cells that were transfected with the targeting vectors were homologously recombined. The knock-in strategy and locations of mutagenesis are shown in Figure 1 (Figure 1A-C). The PGK-NeoR gene insertion was made in intron 4, while mutations of T21S23/D are located in exon 2 (Figure 1B). As the targeting vector was transfected into ES cells, successful breeding of chimeric mice allowed the knock-in allele to transmit in the germline. Since T21S23/D heterozygous mice crossbreeding failed reproducing homozygous neo-inserted $p53^{T21S23/D}$ offspring, embryonic lethality was suggested for homozygous aspartic acid mutations in p53. In order to obtain mice carrying T21S23/D mutation, $p53^{T21S23D/+}$ mice were crossed with $p53^{+/-}$ mice to generate $p53^{T21S23D/-}$ offspring. $P53^{T21S23D/-}$ mice bear one T21S23/D mutated allele and one knock-out allele, in such expresses half of the homozygous mutant protein amount theoretically. The entire p53 cDNA from $p53^{T21S23D/-}$ MEFs was confirmed with sequencing to avoid other mutations to be selected. Although $p53^{T21S23D/-}$ protein expression differs from $p53^{T21S23/D}$ mice, $p53^{T21S23D/-}$ still serves as a better study model compare to $p53^{T21S23D/+}$ mice. Nonetheless, the number of $p53^{T21S23D/-}$ mutant offspring was still lower in comparison to other genotypes (Figure 1 E). Homozygous mutant $p53^{T21S23/D}$ mice were severely runted, lost body weight, developed motor and neural impairment and decreased dermal thickness (Lutske, 2006). Heterozygous $p53^{T21S23D/-}$ mice also developed similar phenotypes within 3 to 4 weeks that were typically observed only in 2 years or older

wild-type mice. All of the 19 studying subjects with the same living conditions as wild-type mice had a critically shortened lifespan (Figure 1F). While 100% of $p53^{T21S23D/-}$ mice (all 19) survived less than 50 days, $p53^{T21S23D/+}$ mice started to die around 180 days with some still surviving past 2 years (Figure 1F). Wild-type mice on the other hand generally survived with a lifespan longer than 2 years (Figure 1F). As the data indicates, a linkage between hyperactive p53 genotype and aging was suggested.

T21S23/D protein expression is confirmed in $p53^{T21S23D/-}$ MEFs, while p53 hyperactivity is found in $p53^{T21S23D/-}$ thymus. As aforementioned, $p53^{T21S23D/-}$ mice emerges as an appropriate study model for T21S23/D expression and function, thus p53 target gene expression in both $p53^{T21S23D/-}$ MEFs and thymus were analyzed. Western blot result showed a relatively clear band from $p53^{T21S23D/-}$ MEFs indicating T21S23/D protein expression (Figure 2A). Even though the expression level was not strong, the band's visibility was confirmative comparing to wild- type MEFs and the negative control of p53 knock-out mice (Figure 2A). Normally, p53 exists as inactive in cells and only increases expression upon cellular stress (Giaccia and Kastan, 1998). To test p53 transcriptional activity in MEFS before and after DNA damage in both wild-type and T21S23/D MEFs, the expression levels of a number of p53 target genes were analyzed by real-time PCR. As expected, wild-type MEFs showed apparent elevation in p53 target gene expression upon 10 hours of .25μM Doxorubicin treatment (Figure 2B). However, there was not any significant difference observed in treated and untreated T21S23/D mutant MEFs (Figure 2B). This could be explained by the hyperactive p53 allele carried by $p53^{T21S23D/-}$ mice. Since p53 activity has already reached its threshold in $p53^{T21S23D/-}$

MEFs before DNA stress occurred, thus Doxorubicin treatment was not able to provoke higher p53 activity. Furthermore, standard 3T3 proliferation assay was conducted on wild type, $p53^{+/-}$, $p53^{-/-}$ and $p53^{T21S23D/-}$ MEFs in order to analyze p53 proliferation rate and senescence progression (Figure 2C). As expected, $p53^{-/-}$ MEFs showed indefinite proliferation without cell senescence observed within 23 passages, while wild-type MEFs as a control showed normal proliferation progression with cell senescence appearing after eight passages (Chao, 2006). $p53^{T21S23D/+}$ MEFs were tested having normal proliferation rate as wild-type control MEFs (Lutske, 2006). As shown here, similar proliferation progression was observed between $p53^{T21S23D/-}$ and $p53^{+/-}$ MEFs suggesting that the T21S23/D allele in $p53^{T21S23D/-}$ MEFs proliferates in a similar fashion as the wild-type allele in p53 heterozygous MEFs (Figure 2C). Both reached immortalized state at the same time and consisted indefinite progression trend with a lower rate than that of wild-type MEFs (Figure 2C). Additionally, hyperactive p53 has a high target gene basal level expression in supporting the notion of aspartic acid mutations mimicking phosphorylation on sites Thr21 and Ser23 which in turn increases p53 activity. This was shown here with drastically increased thymus p53 activity in three weeks mutant mice in comparison to wild-type controls (Figure 2D). The overall trend of increasing p53 transcription activity showed super-active p53 expression in $p53^{T21S23D/-}$ thymus.

Expression level of PUMA and p21 generally elevates in T21S23D/- tissue samples while expression level of p16 and p19 remain arbitrary in comparison to wild-type samples. To understand the effect of T21S23/D p53 mutation on gene expression, four direct and indirect p53 transcriptionally regulated targets were analyzed. Tissue samples including brain, bone marrow, kidney, intestine, liver, lung, skin, spleen and thymus were taken from two weeks old mice post to euthanization. General trend shows elevation of PUMA and p21 mRNA expression when compared to wild-type expression levels in all tissue types (Figure 3). P21 especially shows dramatic increase in mRNA expression in the mutant T21S23D/- brain, kidney, liver, lung, spleen and thymus samples (Figure 3). The apparent difference in p21 gene expression indicates that it was highly transcribed as a target gene of p53 in mutant mice containing a hyperactive allele of p53. Level of PUMA expression was also observed with increasing trend in all the tissue types while most evidently observed in skin and thymus samples (Figure 3G, I). Taken together, elevated PUMA and p21 expression in T21S23D/- tissue samples explains mutant aberrant cell renewal and early senescence. Since both gene targets are directly influenced by p53 transcription activity, they might play important roles in p53 hyperactivity's correlation with aging. Ambiguous results were obtained for p16 and p19 mRNA expression in mutant tissue samples in comparison to wild-type samples. Expression level of p16 and p19 only increased in bone marrow, kidney and intestine tissue samples (Figure 3). Since p16 is an important regulator for G1/S phase in the cell cycle and p19 plays roles in cell senescence, these two target genes might also be significant in connections between p53 hyperactivity and aging.

Weaker signals are sensed in both K1 and K5 stained mutant p53$^{T21S23D/-}$ paw and skin samples comparing to wild-type mice. In addition to the severe degenerative phenotypes p53$^{T21S23D/-}$ mice bear, they also developed an interesting phenotype: black paw. An apparent dark color pigment deposition in p53$^{T21S23D/-}$ paw is observed when compared to wild-type mice (Figure 4A). This might be a result of hyperactive p53's effect on skin as phenotypes such as decreased skin density and delayed hair growth were also observed. Studies showed possible p53 interaction with skin tanning which led to our further analysis of p53$^{T21S23D/-}$ skin and paw tissues. Furthermore, it was well documented that p53 mutation was closely associated with skin cancer (Brash, 2006). As suggested, p53 might play important roles in maintaining normal skin function and development (Oren, 2007). Although p53$^{T21S23D/-}$ samples were not treated with UV radiation as done in studies by Brash et al, p53 as a well known "stress" protein might have triggered deposition of dark pigments through constant stress or irritation sensed. Possible factors leading to DNA stress could arise from abovementioned hyperactive p53 induced accelerated aging. To analyze dermal development more in depth, K1 and K5 staining were applied to p30 mice paw and skin cross sections (Figure 4 B-E). K1 is normally expressed in differentiating suprabasal cells while K5 is found in the basal keratinocytes. Weaker signals were observed in all mutant p53$^{T21S23D/-}$ tissues in comparison to wild-type samples (Figure 4 B-E). Obvious differences could be seen in paw K1 staining with p53$^{T21S23D/-}$ sample showing a much weaker signal (Figure 4 B & C). More obvious signal difference for K5 is observed in skin cross sections with brighter signal in wild-type sample (Figure 4 D & E). As the data indicates, it was suggested that p53$^{T21S23D/-}$ mice exhibit a slower rate of skin cells regeneration comparing

to wild-type mice. This again might have been a consequence of p53 hyperactivity and early aging. Hyperactive p53 expression in $p53^{T21S23D/-}$ mice might also cause slower turnover rate of dermal cells while dead cells deposit in a faster fashion than regeneration of new cells.

Lgr5 staining indicates lowered intestinal stem cell counts in $p53^{T21S23D/-}$ mice in comparison to wild-type mice. Lgr5 (leucine-rich-repeat-containing G-protein-coupled receptor 5), is also known as Gpr49 which is commonly found in small intestine and colon stem cells (Barker, 2007). However, Lgr5 was also found to express in rare cells in several tissues such as stomach glands (Barker, 2007). Moreover, Lgr5 was discovered to be highly expressed in hair follicle stem cells (Morris, 2004). Due to Lgr5's ability in marking stem cells in multiple tissues and cancers, it was commonly used to visualize and analyze stem cells differentiation. As shown here, Lgr5 serves as a marker for intestinal stem cells for visualizing stem cells behavior in both $p53^{T21S23D/-}$ and wild-type mice (Figure 5A, B). DAPI staining for the nucleus allowed visualization of Lgr5 in green color from stained cross sections. It is apparently observed that Lgr5 expression is weaker in $p53^{T21S23D/-}$ when compared with wild-type intestine sample (Figure 5A, B). A rough count of Lgr5 expressed cells also indicates that $p53^{T21S23D/-}$ only exhibits approximately half of the wild-type Lgr5 cell expression (Figure 5C). This implies that mutant $p53^{T21S23D/-}$ mice might have a slower rate of stem cell regeneration and differentiation rate. In consistent with previously stated hypothesis, degenerated stem cell renewal ability might have been caused by hyperactive p53 induced aging.

Plasminogen Activator Inhibitor-1 (PAI-1) deficiency does not rescue $p53^{T21S23/D}$ mutant embryonic lethality. PAI-1 is a downstream target gene of p53, which serves as a marker of replicative senescence due to its high expression during cell proliferation (Serrano, 1997). Since homozygous neo-inserted $p53^{T21S23/D}$ mutant mice were not observed during intercrossed breeding, PAI-1 knock-out mice were used to cross breed with $p53^{T21S23D/+}$ mice to test whether PAI-1 deficiency would rescue homozygous $p53^{T21S23/D}$ embryonic lethality. Original PAI-1 knock-out construct was proposed and created with homologous recombination strategy using both neomycin resistance gene (*neo*) and Herpes simplex virus thymidine kinase gene (*tk*) for double selection of the final recombinant allele (Figure 6A). Mice containing PAI-1 knock-out ($PAI\text{-}1^{-/-}$) gene was obtained from Jackson Laboratories and used to breed with $p53^{T21S23D/+}$ mice to generate PAI-1 deficient mice with hyperactive p53 allele. $P53^{T21S23D/+}/PAI\text{-}1^{-/-}$ mice were interbred to generate possible $p53^{T21S23/D}/PAI\text{-}1^{-/-}$ mice if the embryonic lethal phenotype was rescued. PAI-1 deficiency in mice obtained was identified by PCR analysis of tail tip genomic DNA (Figure 6A). PCR results indicate successful germline transmission of PAI-1 knock out gene along with hyperactive p53 allele (Figure 3B). However, there was no $p53^{T21S23/D}/PAI\text{-}1^{-/-}$ mice observed in either generation (Figure 6C). Among the 56 PAI-1 deficient mice in both generations, 14 homozygous $p53^{T21S23/D}$ mutant mice were expected from the Mendelian ratio, but none was observed (Figure 6C). From the second generation of interbreeding $p53^{T21S23D/+}/PAI\text{-}1^{-/-}$ mice, offspring with two other genotypes $p53^{T21S23D/+}/PAI\text{-}1^{-/-}$ mice and $p53^{+/+}/PAI\text{-}1^{-/-}$ mice both had a higher number count observed than the expected numbers (Figure 6C). Again, the failure of

producing $p53^{T21S23/D}$/PAI-$1^{-/-}$ mice indicated PAI-1 deficiency does not rescue p53 homozygous T21S23/D mutant's embryonic lethality.

DISCUSSION

Conflicting results were achieved in several studies on p53 hyperactivity and its association to aging. Super-active p53 enhances its tumor suppressive function although displaying early aging phenotypes such as weight loss, thin skin and osteoporosis (Tyner, 2002). However, the deletion of 24 genes accounting for nearly 20kb of DNA might have caused aging phenotypes in Tyner et al findings (Tyner, 2002). To balance p53 activity, delicate regulating elements and intricate upstream/downstream effectors play important roles. With a large scale deletion in Tyner et al studies, potential alterations in p53 activity could have been induced by absence of regulation and unknown functions. Contradicting results were brought up by Garcia-Cao et al, with data indicating no significant linkage between high p53 activity with aging (Garcia-Cao, 2002). Mice with multiple transgenes of p53 aged normally when expressed above threshold under genotoxic stresses (Garcia-Cao, 2002). Nevertheless, this model was argued to be inefficient due to the fact that these mice bear normal basal level of p53 activity (Garcia-Cao, 2002). In consistent with Tyner et al findings, it was found that transgenic mice carrying hyperactive truncated form of p53 also developed accelerated aging (Maier, 2004). The aging phenotypes were again considered as a result of decrease in p53 function due to transgene mutation. Overall, there was not much work done directly linking point mutation induced p53 hyperactivity with aging phenotypes. In order to analyze and confirm this phenomenon, hyperactive p53 was constructed through aspartic acid mutations on sites Threonine 21 and Serine 23, and subsequent with homologous recombinant technique and crossbreeding for germline-transmission. Since breeding of T21S23/D heterozygous mice failed to reproduce homozygous $p53^{T21S23/D}$ offspring,

embryonic lethality was suggested for mice carrying aspartic acid mutations on both of p53 alleles. To effectively analyze the influence of T21S23/D mutation on aging, heterozygous $p53^{T21S23D/-}$ mice with one T21S23/D mutated allele and one knock-out allele were selected for experimental use. It was observed that heterozygous $p53^{T21S23D/-}$ mice developed aging phenotypes such as reduced body mass, muscle and skin atrophy, and reduced cellularity within 3 to 4 weeks that were typically seen only in 2 years or older wild-type mice.

Another interesting phenotype observed was the development of black paw in $p53^{T21S23D/-}$ mice. Previous studies revealed correlation between p53 and skin tanning such that UV radiation induces p53 related hyperpigmentation process through over expression of p53 transcriptional targets POMC (pro-opiomelanocortin) and α-MSH (melanocyte-stimulating Hormone), which function together with melanocytes to produce skin-darkening pigment called melanin (Cui, 2007). It was also well documented that p53 mutation was closely associated with skin cancer (Brash, 2006). In all, p53 was suggested to be a key player in the maintenance of normal skin function and development including genome integrity of dermal keratinocytes and melanocytes (Brash, 2006; Oren, 2007). The abovementioned hyperpigmentation phenotype is often referred to as "age spot" which generally occurs in skin under constant stress or irritation (Cui, 2007). Although $p53^{T21S23D/-}$ samples were not treated with UV radiation as done in studies by Brash et al, p53 as a well known "stress" protein induces its activity when cells undergo stress related genetoxic damage. Possible factors leading to these stresses could arise from T21S23/D mutation and p53's hyperactivity effects on the mice. Further analysis of $p53^{T21S23D/-}$ paw and skin samples with K1 and K5 staining suggested that $p53^{T21S23D/-}$

mice exhibit a slower rate of cell regeneration in comparison to wild-type mice. Hyperactive p53 expression in $p53^{T21S23D/-}$ mice might be the reason for the slower turnover rate of dermal cells while dead cells deposit in a faster fashion than regeneration of newer cells.

This led to analysis of stem cell behavior in both $p53^{T21S23D/-}$ and wild-type mice through Lgr5 staining of intestine samples. Due to Lgr5's ability in marking stem cells in multiple tissues and cancers, it is commonly used to visualize and analyze stem cells differentiation. It was found that Lgr5 expression is weaker in $p53^{T21S23D/-}$ when compared with wild-type samples. A rough count of Lgr5 expressed cells also indicated that $p53^{T21S23D/-}$ only exhibited approximately half of the wild-type Lgr5 stem cells. This implied that mutant $p53^{T21S23D/-}$ mice possibly have a slower rate of stem cell regeneration and differentiation rate. In consistence with previously stated hypothesis, degenerated stem cell renewal ability might have been caused by hyperactive p53 induced aging. It was found in earlier studies that p53 is a significant regulator in lymphoma cell line development and cell regeneration (Chen, 1999). Similar data have shown that aged organs from hyperactive p53 mice with premature aging characteristics display phenotypes including reduced cellularity and atrophy (Dumble, 2007). Furthermore, production of hematopoietic stem cells from this hyperactive mice model failed to keep up with aging, suggesting stem-cell regeneration deficiency (Dumble, 2007). Thus, it can be concluded that a connection exists between p53 hyperactivity induced aging and declined tissue stem cell regeneration.

To analyze T21S23/D mutation and its expression in $p53^{T21S23D/-}$ mice, several experiments were conducted. P53 target gene expression after 10 hours of .25μM

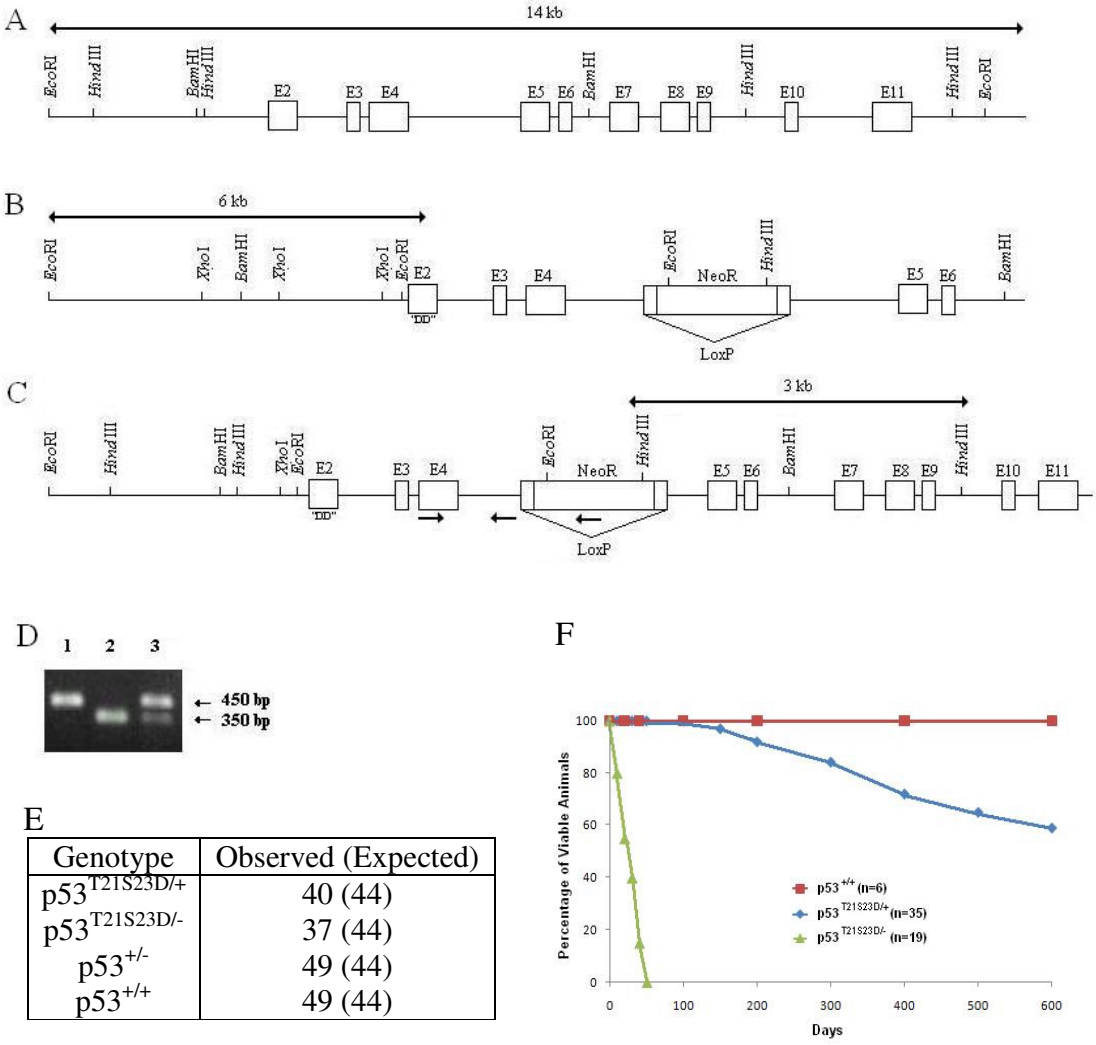
Doxorubicin treatment in both wild-type and T21S23/D mutant MEFs were analyzed for p53 transcriptional activity. There was no significant change observed after treatment in $p53^{T21S23D/-}$MEFs in comparison to an apparent elevation of gene expression in wild-type samples. Furthermore, standard 3T3 proliferation assay indicated similar proliferation progression for $p53^{T21S23D/-}$ and $p53^{+/-}$ MEFs suggesting that the T21S23/D allele in $p53^{T21S23D/-}$ MEFs proliferates in a similar manner as the wild-type allele in p53 heterozygous MEFs. Additionally, it was found that hyperactive p53 has a high target gene basal level in supporting the notion of aspartic acid mutations mimicking phosphorylation on sites Thr21 and Ser23 which in turn increases p53 activity. There was an overall increasing trend observed in p53 transcriptional activity in $p53^{T21S23D/-}$ thymus in consequence of hyperactive T21S23/D mutation. This could be explained by the hyperactive p53 allele function in $p53^{T21S23D/-}$ mice. Higher p53 transcriptional activity and target gene expression were able to be detected in $p53^{T21S23D/-}$ thymus without any DNA stress because p53 activity has already reached its threshold with presence of the mutant T21S23/D allele.

To further understand the effect of T21S23/D mutation on gene expression, four direct and indirect p53 target genes PUMA, p21, p16 and p19 were analyzed in various tissue samples. PUMA and p21 showed general increasing expression trend in $p53^{T21S23D/-}$ when compared to wild-type mice. In addition, levels of p16 and p19 were also seen to increase in $p53^{T21S23D/-}$ bone marrow, kidney and intestine tissue samples. Taken together, this data might help to explain the aberrant cell renewal and early senescence phenotypes observed in $p53^{T21S23D/-}$ mutant paw and intestine samples. PUMA is a critical regulator in p53 induced apoptosis pathway, while p19 serves

important function in cell senescence. Both p16 and p21 are important regulators for G1/S phase in cell cycle. Overall, these genes might provide substantial insight on T21S23/D mutation induced hyperactive p53 activity in relation to premature aging phenotypes. It was suggested that apoptosis and cellular senescence with tumor suppressing functions against genome stress might also induce aging phenotypes (Rodier, 2007). Apoptosis occurs when severe damage was done to the genome and is considered an irreversible tumor suppressor mechanism. Cell senescence establishes permanent cell growth arrest and accumulates dysfunctional senescent along with age. Both processes readily deplete stem cells and directly slowdown rate of cell regeneration, which result in generalized organ atrophy and degeneration as seen normally in aging (Rodier, 2007). Additional support came from Campisi's finding that cellular senescence is "antagonistically pleiotropic" and eventually cause tissue loss and degenerative function (Campisi, 2001). The connection between hyperactive T21S23/D mice and premature aging might comes from loss of stem cells and differentiated somatic cells normally induced by DNA damage. Only upon receiving signals from genotoxic damage, p53 proliferates and serves as a transcriptional activator for target genes such as PUMA, p21, p16 and p19 regulating responses including cell arrest, apoptosis or cellular senescence in protection of the genome. In the case of $p53^{T21S23D/-}$ mutant mice, with one p53 allele being hyperactivated, as shown with increased p53 target genes expression in various tissue samples, apoptosis regulated by PUMA and senescence responses controlled by p19 are observed even without DNA damage. This leads to an interesting phenomenon mentioned by Campisi that understanding aging is the key to understanding cancer (Campisi, 1997). $P53^{T21S23D/-}$ mice were probably free of cancer, since most of them died

within two months due to premature aging instead of cancer. Furthermore, mutations that cause abrogated apoptosis and cellular senescence have higher risk of developing cancer. However, over stimulated cellular responses also induce aging phenotypes as mentioned previously. In conjunction with this idea, connection between wild-type p53 and aging is suggested through p53 regulated protein expression. One of such proteins is p21, the cell cycle inhibitor. P21 could induce aging phenotypes through either p53-dependent or p53-independent pathways (Campisi, 2001). Thus, wild-type p53 might play a role in aging in the long run. In all, it is concluded that hyperactivity of p53 through T21S23/D mutation leads to age-related phenotypes in mice models.

As a downstream target gene of p53, PAI-1 serves as a marker of replicative senescence due to its high expression during cell proliferation (Serrano, 1997). Cellular senescence is delicately regulated by p53 in response to DNA damage. Since homozygous neo-inserted $p53^{T21S23/D}$ mutant mice were not observed during intercrossed breeding, PAI-1 knock-out mice were used to breed with $p53^{T21S23D/+}$ mice to test whether PAI-1 deficiency would rescue homozygous $p53^{T21S23/D}$ embryonic lethality. However, there was no successful reproduction of homozygous T21S23/D p53 mutant mice from over 100 offspring obtained. In spite of this result, hyperactive p53 from point mutations as seen in T21S23/D would be a probable candidate for cancer treatment, as it does not require stress signals or high expression level of p53 for activation. Future studies on other p53 target gene knock-outs that can rescue homozygous T21S23/D mutant embryonic lethality will be necessary to untwine the connections between p53 hyperactivity, aging and cancer with hopeful thoughts to uncouple tumor suppression and aging.



E

Genotype	Observed (Expected)
$p53^{T21S23D/+}$	40 (44)
$p53^{T21S23D/-}$	37 (44)
$p53^{+/-}$	49 (44)
$p53^{+/+}$	49 (44)

Figure 1: Generation of $p53^{T21S23/D}$ Knock-in mice. (A) The endogenous p53 allele. (B) The targeting vector. The PGK-NeoR gene insertion was made in intron 4. Mutations of T21S23/D are located in exon 2. (C) Homologous recombination of endogenous and targeting alleles producing knock-in p53 locus. Arrows indicate primers deduced for PCR tail genomic DNA analysis. (D) PCR results of tail genomic DNA from $p53^{T21S23/D}$ (lane 1), wild type (lane 2), and $p53^{T21S23D/+}$ (lane3) mice. However since no $p53^{T21S23/D}$ mice was observed at birth, $p53^{T21S23D/+}$ mice and $p53^{+/-}$ mice were crossbred to produce $p53^{T21S23D/-}$ mice that carry one mutant p53 allele. (E) The number of offspring observed from crossbreeding of $p53^{T21S23D/+}$ mice and $p53^{+/-}$ mice. Expected ratio is indicated in parentheses next to observed ratio. (F) Survival curve of wild-type, $p53^{T21S23D/+}$ and $p53^{T21S23D/-}$ mice. Mutant $p53^{T21S23D/-}$ mice do not live pass 50 days while wild-type mice could survive up to over two years. $p53^{T21S23D/+}$ mice started to die around 180 days.

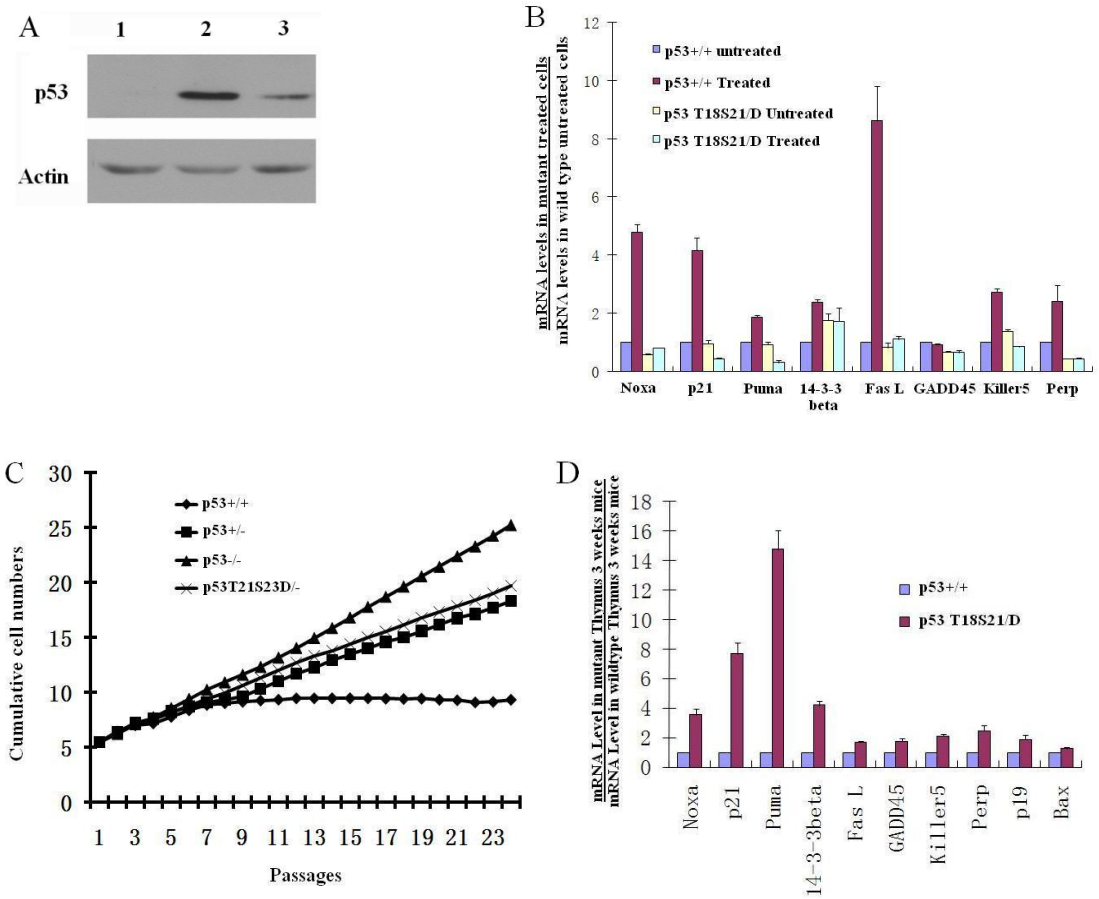


Figure 2: Analysis of T21S23/D transcription activity and protein expression in both MEFs and Thymus. (A) Western blot showing mutant protein level in MEFs from $p53^{-/-}$ (lane 1), wild type (lane 2), and $p53^{T21S23D/-}$ (lane 3) mice. Actin protein expression is used as a loading control. (B) Real-time PCR analysis of p53-dependent transcription after DNA damage. P53 target genes expression in wild-type and mutant MEFs after 10 hours of .25 μM Doxorubicin is shown relative to untreated wild type mRNA levels set equal to one. Untreated samples are included for comparison. Mean values from two independent experiments is shown with error bars. (C) 3T3 proliferation assay of wild-type, $p53^{+/-}$, $p53^{-/-}$ and $p53^{T21S23D/-}$ MEFs. MEFs were serially passaged and counted every three days at a density of $3x10^5$ cells per plate. Cumulative cell numbers are shown based on the average of triplicate plates from three sets of each genotype. (D) Real-time PCR analysis of p53-dependent transcription in thymus with mRNA expression level in wild-type was set equal to one. Mean values from two independent experiments is shown with error bar.

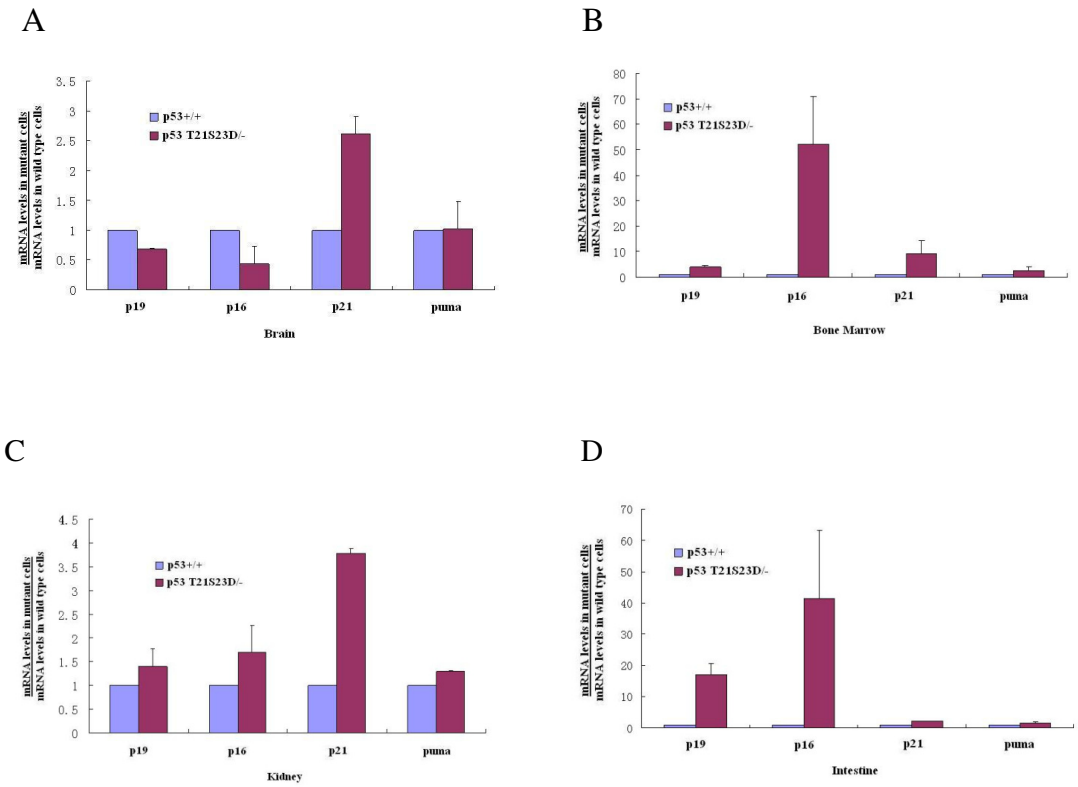


Figure 3: Analysis of p53 target gene mRNA expression in different tissue types. (A) to (I) show real-time PCR analysis of p53-dependent transcriptional activity from four different target genes. Mutant mRNA expression levels are shown relative to corresponding wild-type levels, which are set equal to one. Mean from two independent experiments is shown with error bars. Tissue samples including brain, bone marrow, kidney, intestine, liver, lung, skin, spleen and thymus were taken from two weeks old mice post to euthanization.

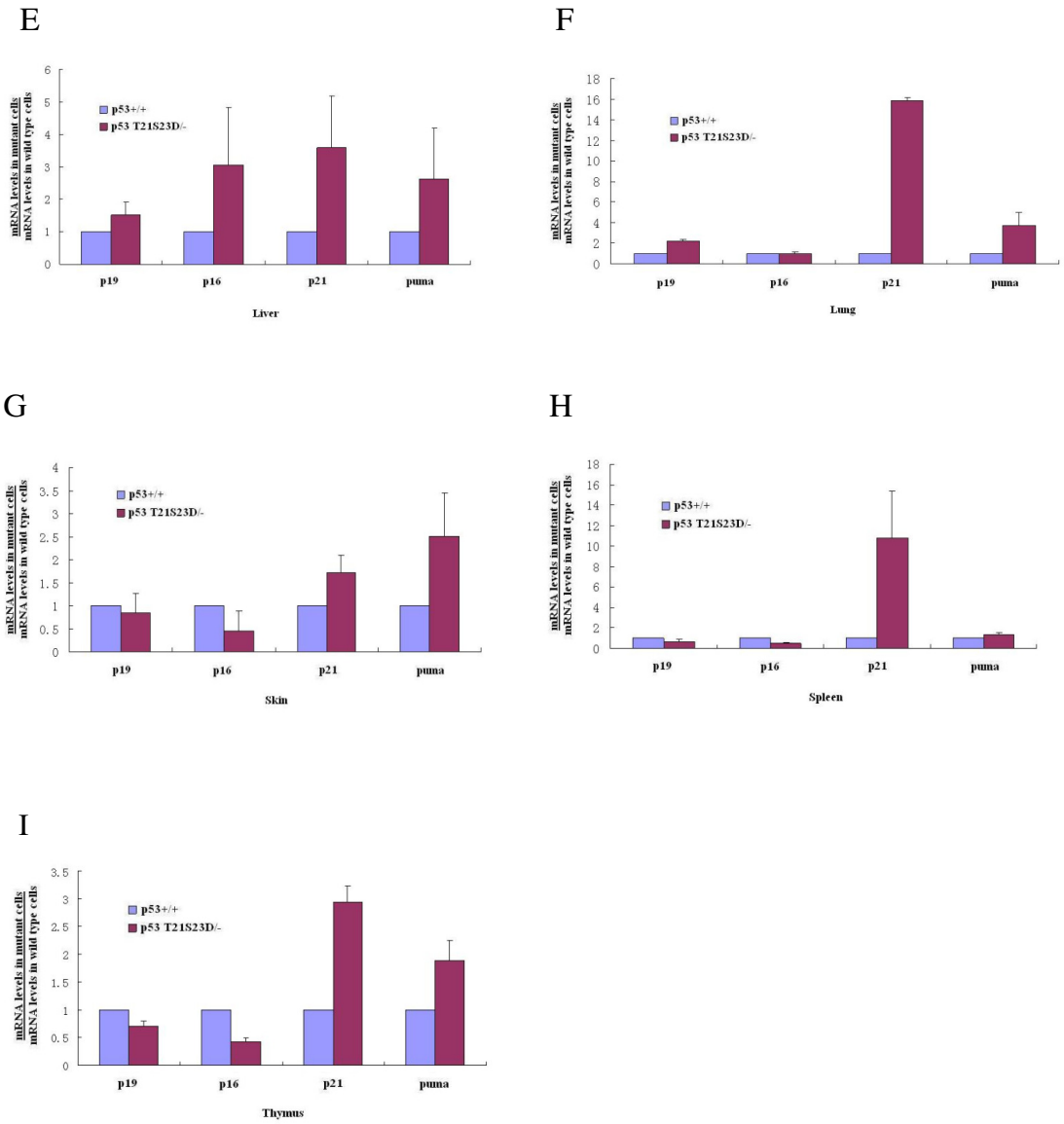


Figure 3 (Continued): Analysis of p53 mRNA expression in different tissue types. (A) to (I) show real-time PCR analysis of p53-dependent transcriptional activity. Mutant expression levels are shown relative to corresponding wild-type mRNA levels, which are set equal to one. Mean from two independent experiments is shown with error bars. Tissue samples were collected from two weeks old mice post euthanization.

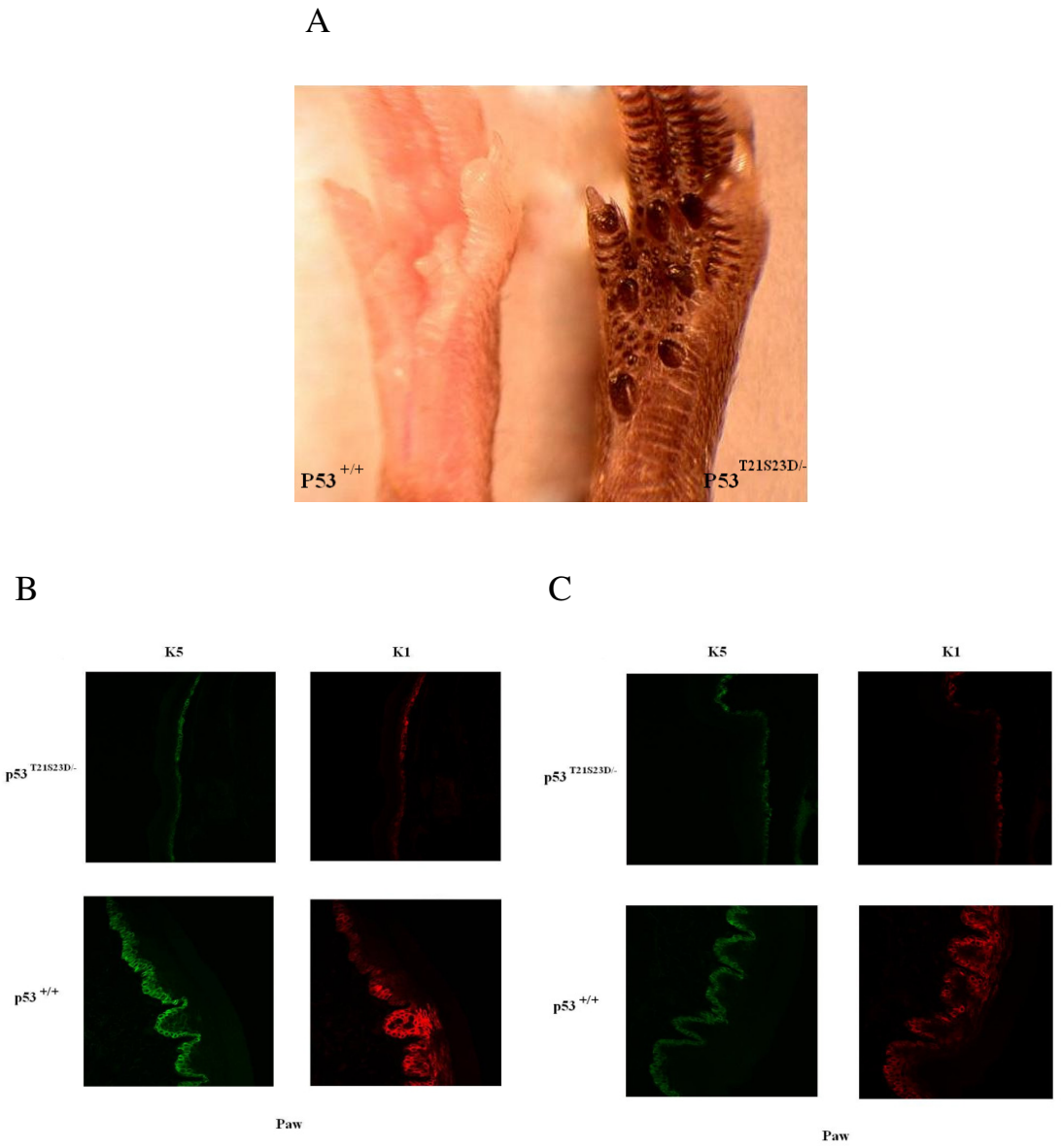


Figure 4: Analysis of paw and skin cell regeneration of p53$^{T21S23D/-}$ and wild type p30 mice. (A) Paw close-up picture of wild-type and p53$^{T21S23D/-}$ mice. (B-E) K1 and K5 stained paw and skin cross-section from p30 p53$^{T21S23D/-}$ and wild-type mice. K5 is normally expressed in the basal keratinocytes for indication of constant cell renewal by skin proliferation. K1 is expressed normally in differentiating suprabasal cells.

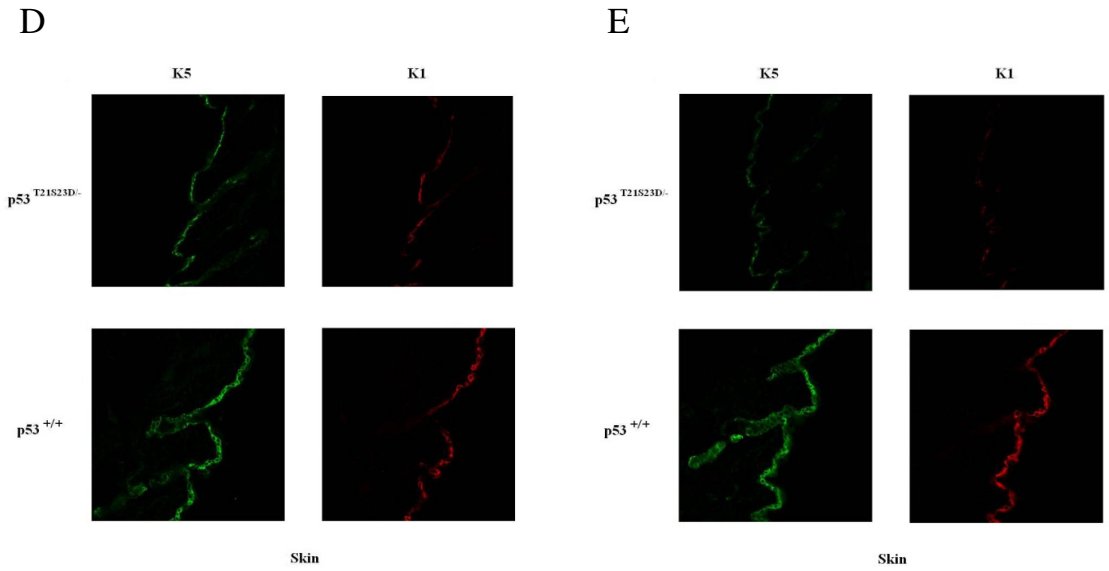


Figure 4 (Continued): Analysis of paw and skin cell regeneration of p53$^{T21S23D/-}$ and wild type p30 mice. (A) Paw close-up picture of wild-type and p53$^{T21S23D/-}$ mice. (B-E) K1 and K5 stained paw and skin cross-section from p30 p53$^{T21S23D/-}$ and wild-type mice. K5 is normally expressed in the basal keratinocytes for indication of constant cell renewal by skin proliferation. K1 is expressed normally in differentiating suprabasal cells.

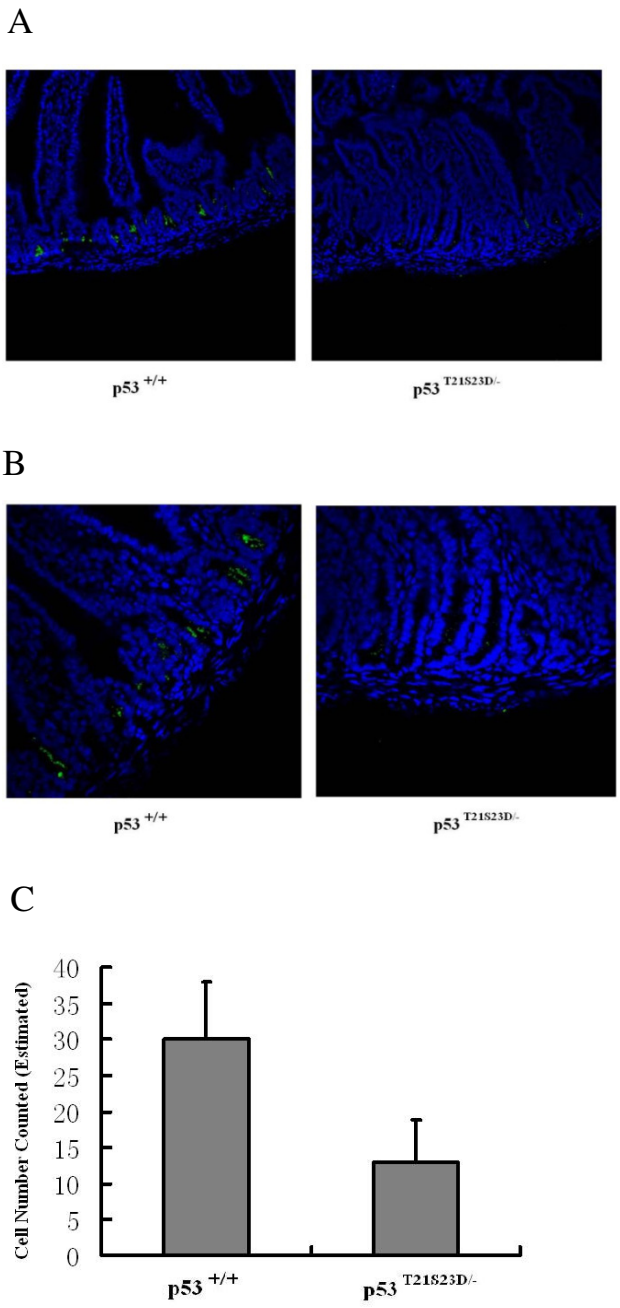
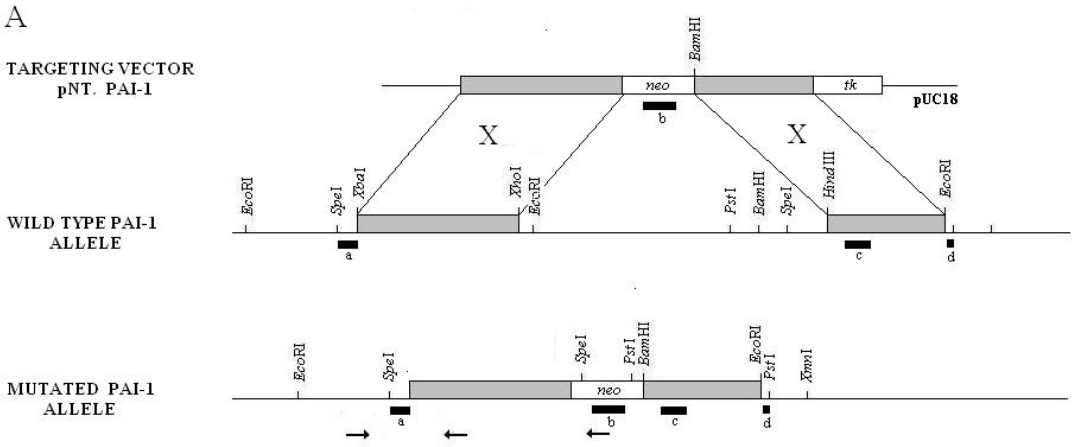
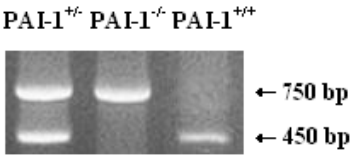


Figure 5: Lgr5 staining of intestine of wild-type and p53$^{T21S23D/-}$ mice. (A) and (B) Lgr5 serves as a marker for intestinal stem cells which express in several locations in small intestine and colon. DAPI was used to stain the nucleus while the green color indicates Lgr5 expression. (C) Roughly counted cell numbers based on the section shots.

A



B



C

Genotype	Observed (Expected)
$p53^{+/+}$ / PAI-$1^{+/+}$	15 (7)
$p53^{+/+}$ / PAI-$1^{+/-}$	27 (14)
$p53^{+/+}$ / PAI-$1^{-/-}$	4 (7)
$p53^{T21S23D/+}$ / PAI-$1^{+/+}$	22 (14)
$p53^{T21S23D/+}$ / PAI-$1^{+/-}$	25 (28)
$p53^{T21S23D/+}$ / PAI-$1^{-/-}$	21 (14)
$p53^{T21S23/D}$ / PAI-$1^{+/+}$	0 (7)
$p53^{T21S23/D}$ / PAI-$1^{+/-}$	0 (14)
$p53^{T21S23/D}$ / PAI-$1^{-/-}$	0 (7)
$p53^{+/+}$ / PAI-$1^{-/-}$	9 (7)
$p53^{T21S23D/+}$ / PAI-$1^{-/-}$	19 (14)
$p53^{T21S23/D}$ / PAI-$1^{-/-}$	0 (7)

Figure 6: Analysis of PAI-1 knock-out in rescue of p53 mutant mice. (A) Strategy used to generate homologously recombined PAI-1 allele with insertion of neomycin resistance gene (*neo*) and the Herpes simplex virus thymidine kinase gene (*tk*) as double selector markers (Carmeliet, 1993). Successful recombination results complete PAI-1 protein absence. (B) PCR analysis of genomic DNA from tails of PAI-$1^{+/-}$, PAI-$1^{-/-}$, and PAI-$1^{+/+}$ mice. Primers used are indicated by arrowheads in (A). (C) The number of offspring observed for each genotype of two generations. The expected frequency shown in parentheses is based on the expected Mendelian ratio.

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