THE ROLE OF P53 SIGNALING PATHWAY IN AGING AND CANCER

by

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ABSTRACT OF THE DISSERTATION

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Part I:

p53 codon 72 SNP and aging

p53 has dual functions on longevity. p53 plays a crucial role in tumor suppression to prevent early death due to cancer. However, constitutively increased p53 activity accelerates the decline of stem/progenitor cells' self-renewal function, which leads to accelerated aging and a reduced lifespan. In humans, the role of p53 in aging and longevity has not been well established. As a haploinsufficient gene, p53 is under the tight regulation in cells. Attenuation of p53 function (even with only 2-fold change) contributes greatly to tumorigenesis. p53 codon 72 single nucleotide polymorphism (SNP) is a naturally occurring common SNP with a nucleotide change, which results in either an arginine (R72) or a proline (P72) at codon 72. p53 codon 72 SNPs can influence the activity of p53. The P72 allele is weaker than the R72 allele in inducing apoptosis and suppressing cellular transformation. However, it is unclear whether the change of p53 activity in humans by the functional SNP could impact longevity. A

perspective cohort study with an aging human population showed that P72 allele is associated with longer survival despite its increased risk for cancer development (van Heemst et al., 2005). These findings strongly suggest that p53 activity is reversely associated with aging, and functional SNPs in the p53 pathway could impact upon the lifespan in humans. In this study, we employed a mouse model system with knock-in of the human p53 gene (Hupki) carrying either R72 or P72 SNP to investigate the impact of p53 codon 72 SNP upon longevity and its underlying mechanism. Mice with p53 P72 allele showed weaker transcriptional activity than the R72 allele toward a subset of p53 target genes, suggesting that these mice retain the function of p53 codon 72 SNP in humans. We found that although mice with p53 P72 have increased cancer risk compared to mice with p53 R72, those mice with p53 P72 who escaped tumor development have longer lifespans compared to mice with p53 R72 that do not develop tumors. Mice with p53 P72 displayed a delayed aging process compared to mice with p53 R72, including less reduced bone density, less decreased dermal thickness and better wound healing ability. We further compared the effects of p53 codon 72 SNPs on stem cell population and function as a possible mechanism that contributes to their differences in longevity. Compared to mice with p53 R72, mice with p53 P72 allele have a smaller number of long-term stem/progenitor cells and better self-renewal function during the aging process. Consistent results were observed when long-term stem cell's ability of engraftment and repopulation was evaluated by

bone marrow transplantation assay. Taken together, results from this study demonstrate that p53 codon 72 SNP has a direct impact on aging and longevity *in vivo* in mouse models, and strongly support the role of p53 in the regulation of stem/progenitor cells' function and longevity.

Part II:

Mechanism of mutant p53 accumulation and gain-of-function (GOF) in tumors

p53 is the most frequently (~50%) mutated gene in human cancers. Besides the loss of tumor suppressive activity of wild-type p53 (wtp53), many tumor-associated mutant p53 (mutp53) proteins gain oncogenic functions to promote tumorigenesis, defined as gain-of-function (GOF). Mutp53 often accumulates to high levels in tumors, which is critical for mutp53 to exert GOF. The goal of this proposed research is to understand the mechanisms for mutp53 accumulation and GOF in tumors by identifying molecules that regulate mutp53 protein levels and functions, and furthermore, to develop potential intervention strategy targeting mutp53 accumulation and/or GOF. I searched for proteins interacting with mutp53 protein which may regulate mutp53 protein levels and/or GOF by immunoprecipitation combined with mass spectrometry screening in tumors from R172H mutp53 knock-in mice. Through this approach, we identified several novel mutp53- binding proteins, including Bcl-2-associated athanogene 2 (BAG2) and Pontin. BAG2 belongs to the BAG family and functions as a co-chaperone protein in cells. The expression level of BAG2 in cancers has been shown to associate

with prognosis in cancer patients, including breast cancer and colon cancer. Results from our study suggest that BAG2 overexpression promotes mutp53 protein accumulation, which in turn contributes to mutp53 GOF in tumorigenesis. BAG2, a member of BAG family proteins, can bind to mutp53 through its BAG domain to disrupt mutp53-MDM2 interaction. Therefore, BAG2 promotes mutp53 accumulation through inhibiting MDM2-mediated mutp53 ubiquitination and degradation. It is possible to develop novel treatment strategies that targeting BAG2 to treat cancers with mutp53. Pontin, a highly conserved AAA+ ATPase, plays an important role in many cellular activities, including regulation of transcription, telomerase activity, chromatin remodeling, metabolism, and DNA repair. We identified Pontin as a novel mutp53-binding protein. The mutp53-Pontin interaction promotes mutp53 GOF in migration and invasion. Pontin promotes mutp53 GOF through regulation of mutp53 transcriptional activity; knockdown of Pontin abolished the transcriptional regulation of mutp53 toward a group of genes. Blocking the ATPase activity of Pontin by a Pontin-specific ATPase inhibitor or an ATPase-deficient dominant-negative Pontin expression vector greatly diminished mutp53 GOF. Therefore, identification of novel targetable mutant p53 interacting proteins will shed light on potential cancer therapeutic strategies, especially to tumors containing mutp53.

ACKNOWLEDGMENTS

This work reflects major projects that I have been working over the years of my graduate training. Chapter I is a literature review and an introduction to this thesis. Chapter I-IV were adopted from my published works (Yue et al., 2015; Yue et al., 2017; Zhao et al., 2018; Zhao et al., 2015). The permission to re-use them in this thesis could be found at the following websites: https://s100.copyright.com/AppDispatchServlet; https: //elifesciences.org/terms; https://www.nature.com/cdd/authors-and-referees/authors.

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DEDICATIONS

I would like to dedicate this dissertation

To my grandfather,

Peiji Zhao

To my Parents,

Jianjun Zhao

&

Fengjun Wu

And to my husband,

Xiao Ding

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Chapter I: General introduction

1. Tumor suppressor p53

1.1 p53 is a tumor suppressor gene

p53 (encoded by TP53) is the most intensively studied gene in tumor biology. By now, ~90,000 p53-related publications have been listed in PubMed since the first discovery of p53 in 1979. In the first decade of p53 research, p53 was considered as an oncogene. p53 was discovered as a cellular partner of a tumor virus oncoprotein, simian virus 40 (SV40) large T-antigen (Lane & Crawford, 1979), multiple groups found this protein simultaneously (Kress, May, Cassingena, & May, 1979; Linzer & Levine, 1979; Melero, Stitt, Mangel, & Carroll, 1979). This estimated 53 kDa protein could directly interact with antibodies generated from animals immunized with SV40, in either tumor-derived cells or non-virally transformed cells, indicating this protein as a tumor antigen. In the first international p53 workshop held in Oxted, UK in 1983, this protein was named p53, based on its estimated molecular mass of 53 kDa according to its migration position in SDS-polyacrylamide gels. While the actual molecular weight of p53 is 43.7 kDa, p53 protein migrated slower in SDS-polyacrylamide gels due to its proline-rich region. Until now, p53 is one of the most thoroughly studied tumor suppressor genes.

In the early 1980s, p53 is considered as an oncogene, based on the facts that 1. The overexpression of p53 was positively associated with cellular transformation (Sarnow, Ho, Williams, & Levine, 1982). 2. Many tumors have increased p53 expression, which is not

observed in normal tissue under unstressed condition (Rotter, 1983). 3. Many tumor-associated p53 showed oncogenic activities (Eliyahu, Raz, Gruss, Givol, & Oren, 1984). 4. The tumor-associated p53 was found to cooperate with other oncogenic proteins to promote tumor initiation and progression in multiple experimental model systems (Jenkins, Rudge, & Currie, 1984; Parada, Land, Weinberg, Wolf, & Rotter, 1984). However, clues that p53 is a tumor suppressor gene accumulated in the late 1980s. p53 was found to be inactivated and/or lost in transformed cells (Ben David, Prideaux, Chow, Benchimol, & Bernstein, 1988; Mowat, Cheng, Kimura, Bernstein, & Benchimol, 1985; Wolf, Admon, Oren, & Rotter, 1984). The sequence of tumor-associated p53 clones was different from the sequence of p53 clones isolated from the normal mouse tissues, indicating that the tumor-associated p53 clones were mutant p53 (Eliyahu et al., 1988; Finlay et al., 1988; Halevy, Rodel, Peled, & Oren, 1991). According to the NCI Dictionary of Cancer Terms, the tumor suppressor gene (TSG) is a type of gene that makes a protein called a tumor suppressor protein that helps control cell growth. Mutations (changes in DNA) in tumor suppressor genes may lead to cancer. The TSG is also called anti-oncogene. The identification of frequent mutations of p53 in human tumors indicated that p53 might be a tumor suppressor gene. And later functional studies revealed that overexpression of wild-type p53 in transformed cells led to cell cycle arrest or cell death (Eliyahu, Michalovitz, Eliyahu, Pinhasi-Kimhi, & Oren, 1989; Finlay, Hinds, & Levine, 1989), further supporting the role of p53 as a tumor suppressor gene. In the experimental

animal model of the Trp53 knockout mice, Trp53 knockout mice are prone to develop spontaneous cancers (mostly lymphomas) by 2-6 month of age. The median survival of the Trp53 knockout mice is 5.5 months compared to the mice with wild-type p53 alleles with a median survival of 22 months (Donehower et al., 1992). Ample studies showed that the wild-type p53 alleles were frequently lost and/or inactivated by mutations, deletions or a combination of both in human tumor cells (Levine & Oren, 2009). About 50% of tumors harbor p53 mutations. A familial disease, namely Li-Fraumeni syndrome (LFS), which was characterized by the development of various types of cancers, particularly in children and young adults, was associated with germline p53 mutation (Malkin et al., 1990; Srivastava, Zou, Pirollo, Blattner, & Chang, 1990). In most LFS cases, patients carry a single mutant p53 allele inherited from their family, and the wild-type p53 allele is often lost during tumor initiation or development. The lifetime risk of tumors of LPS patients is ~93%. (Levine, Chan, Dudgeon, Puzio-Kuter, & Hainaut, 2015). All these data established p53 as a tumor suppressor.

1.2 The biological activities of p53

The p53 protein acts as a central hub in many biological processes. In response to stress, p53 can receive, integrate and transmit various signals to activate its downstream genes to ensure the cell and tissue homeostasis.

As the “ guardian of the genome”, p53 is activated when encountered with a wide variety of stress signals, including DNA damage, hypoxia, rNTP depletion, nutritional deprivation

as well as oncogene activation (J. Liu, Zhang, & Feng, 2014). p53 responses differently towards the stress signals with different intensities and duration (Kastenhuber & Lowe, 2017). The different functions of p53 are recognized/achieved through regulating its target genes. The classical responses of p53 activation are cell cycle arrest, DNA repair, apoptosis and senescence mediated by p21, bcl2, puma, noxa and p16INK4a, etc. Until recently, the triple knockout mouse model, which deficient of p21, puma and noxa, has been generated. Cells from these mice showed defects in DNA-damage induced cell cycle arrest, apoptosis, and senescence. However, these mice do not have increased risk in tumor development, indicating that those p53 canonical functions might be a partial explanation of p53's functions in tumor suppression (Valente et al., 2013).

Besides the above mentioned intensively studied canonical tumor suppressive function of p53, with the identification of novel p53 target genes, p53 has been found to play a very important role in other non-canonical programs that contribute to tumor suppression, including regulating metabolism, autophagy, tissue remodeling and pluripotency etc (Kastenhuber & Lowe, 2017). For example, p53 regulates metabolic pathways (Gnanapradeepan et al., 2018). p53 regulates mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK), two master regulators of cellular metabolism. In response to genotoxic stress, p53 transactivates its target genes sestrin 1/2, which activate AMPK. AMPK phosphorylates tuberous sclerosis protein 2 (TSC2), which inhibits mTOR complex 1 (mTORC1). p53 also transcriptional regulates a subset of genes in the

insulin-like growth factor- (IGF1)/AKT signaling pathway, including IGF-BP3, PTEN, TSC2, etc. in response to stress such as nutrient deprivation. All these genes are negative regulators of the IGF/AKT pathway, which is a critical pathway in regulation of cell proliferation, survival and energy metabolism (Budanov & Karin, 2008; Z. Feng, Hu, de Stanchina, et al., 2007; Z. Feng, Zhang, Levine, & Jin, 2005). p53 can also promote oxidative phosphorylation in mitochondria and reduce glycolysis through regulating a list of p53 target genes, including p53-induced glycolysis and apoptosis regulator (TIGAR), Cytochrome c Oxidase 2 (SCO2), glutaminase 2 (GLS2), Parkin, Ras-related associated with diabetes (RRAD), etc. (Bensaad et al., 2006; Hu, Zhang, et al., 2010; Matoba et al., 2006; Zhang et al., 2011; Zhang et al., 2014). p53 regulates lipid metabolism. p53 has been shown to promote fatty acid oxidation (FAO) and inhibit fatty acid synthesis. For example, p53 transcriptionally regulate carnitine palmitoyltransferase 1C (Cpt1c), which is a brain-specific member of mitochondria-associated enzymes mediating the transport of activated fatty acids to mitochondria for FAO (Sanchez-Macedo et al., 2013). Mice deficient of Cpt1c displayed delayed tumor development and increased survival rates. Some more p53 target genes have been identified, such as Lipin 1 (LPIN1), which modulates adipocyte development, and Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha (PGC-1α), which is a transcriptional coactivator that regulates the genes involved in energy metabolism such as gluconeogenesis and FAO (Assaily et al., 2011; Finck et al., 2006). In response to ROS, p53 transactivates LPIN1

and therefore suppresses fatty acid synthesis through negative regulating Sterol regulatory element binding protein 1 (SREBP1), a critical transcriptional factor in fatty acid synthesis (Peterson et al., 2011). In response to glucose starvation or serum starvation, p53 enhances the transcriptional activity of PGC-1α, which in turn binds to p53 and modulates p53's transcriptional regulation of its target genes, including TIGAR, SCO2, sestrin 2 (Sen, Satija, & Das, 2011). LPIN1 also interacts with PGC-1α, which amplifies the PGC-1α mediated lipid metabolism (Finck et al., 2006).

In addition to its role in metabolism regulation, p53 plays an important role in other biologic processes, including fertility and aging, etc. p53 regulates maternal reproduction through the transcriptional regulation of leukemia inhibitory factor (LIF), a cytokine critical for embryonic implantation (Hu, Feng, Teresky, & Levine, 2007). Administration of LIF to pregnant $p53^{-/-}$ mice with impaired implantation of embryo restored maternal reproduction. In human populations, a list of single nucleotide polymorphisms (SNPs) in the p53 pathway was investigated to analyze the impact of p53 on human fertility (Kang et al., 2009). Individuals with Proline at codon 72 of p53 (p53 P72), with decreased p53 transcriptional activities towards a subset of its target genes, were found to be vulnerable to infertility compared with individuals with Arginine at codon 72 of p53 (p53 R72). LIF levels are lower in cells with P72 than in cells with R72. What's more, a series of other functional SNPs in the p53 pathway, which can modify or regulate p53 functions and levels, including p53 target gene Hausp, MDM2, and MDM4, which are two major p53

negative regulators, are also associated with fertility in women. Selected alleles of SNPs of p53, Mdm2, Mdm4, and Hausp genes are enriched in *in vitro* fertilization patients younger than 35 years old, who are more likely have impaired implantation ability (Hu, 2009). All these data support that p53 plays an important role in reproduction ability. The role of p53 in the aging process is interesting but complex: loss of p53 leads to early development of various cancers, which shorten the lifespan; however, an excess amount of p53 leads to early aging phenotypes. It seems that p53 plays a dual role in aging, which will be further discussed in detail in section 1.5.

1.3 The molecular mechanisms of p53's function- p53 is a transcriptional factor.

The p53 protein is encoded by the TP53 gene, which is highly conserved throughout evolution and is located on human chromosome 17p13.1. Loss of heterozygosity (LOH) is frequently found on the chromosomal arms 17p in various types of human cancer. The p53 protein is composed of 393 amino acids, encoded by 11 exons. The human p53 protein can be divided into multiple regions, including the transactivation domains (TAD), proline-riched domain (PRD), DNA binding domain (DBD), tetramerization domain (TET) and regulatory domain (RD), each of these domains corresponding to a specific function (as shown in Figure 1).

1) The amino-terminus (amino acids, aa 1-63) contains two TAD domains (aa 1-42, aa 43-63), the mdm2 binding site (aa 13-29) and a nuclear exclusion signal (NES). The acidic amino-terminal of p53 is essential for the protein-protein interaction between p53

and its binding partners to trans-activate p53. The first highly conserved domain (HCD I) of p53 lies correspondence to the mdm2 binding site; deletion of HCD I abolished the p53-mdm2 interaction, however, retained the transcriptional activity of p53 (Marston, Crook, & Vousden, 1994).

2) The proline-rich domain (PRD, aa 64-92) contains a series of repeated proline residues. The proline shows restricted mobility according to its structure, is with a flat hydrophobic surface and serves as a strong hydrogen bond acceptor. The PRD is capable of binding rapidly and non-specifically to other proteins (Williamson, 1994). The PRD provides a protein-protein interaction platform and facilitates the transactivation of p53 (Soussi & May, 1996).

3) The central region of p53 (aa 101-300) contains the DBD. There are five HCDs within p53, four of them (HCD II to V) lie in this region. About 85% of single nucleotide mutations, including the six hotspot mutations, occur in the DBD. A critical function of the central region is specific DNA binding. In 1993, the HCD IV and V were first found to directly involved in DNA contact (Halazonetis, Davis, & Kandil, 1993). Wang et al. found that truncated p53 protein (aa 80-290) is necessary and sufficient for the specific DNA binding ability of p53 (Y. Wang et al., 1993). p53 binds to a specific DNA sequence, termed the p53-responsive element (RRRCWWGYYY-RRRCWWGYYY, where R is purine, A or G, Y is pyrimidine, T or C, and W is A or T), to regulate the expression of its target genes (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992).

4) The tetramerization domain (aa 307-355, TET) lies near the C-terminal region of p53. The wild-type p53 is normally found in the form of the tetramer, consisting of two dimers. It has been shown that aa 325-356 is sufficient to form a stable tetramer (Jeffrey, Gorina, & Pavletich, 1995). A beta-strand (aa 326-333) and an alpha-helix (aa 335-354) form a V-shaped structure. The dimer of p53 is formed via antiparallel interaction of their beta-strand, whereas the tetramer is formed through hydrophobic and electrostatic contact between their alpha-helices. A nuclear export signal (NES, aa 340-351) localizes in this domain, which is responsible for exporting p53 from nuclear to cytoplasm (Stommel et al., 1999).

5) The carboxyl-terminus of p53 (356-393) contains three nuclear localization signals (NLS) and a non-specific DBD. NLS I is highly conserved and the most active domain compared with the other two NLSs (Shaulsky, Goldfinger, Ben-Ze'ev, & Rotter, 1990). The interaction of p53 with other proteins, such as nuclear protein SV40 T-large antigen, also affects the ultimate localization of p53 in the cells as well as the function of p53. The 47 C-terminal amino acids are essential for p53 to bind non-specifically with genomic DNA. This region also plays an important role in downregulation of specific DNA binding ability of the central region of p53. Phosphorylation or blocking the C-terminus by monoclonal antibody Pab421 leads to activation of the specific DNA binding of p53 (Hupp, Meek, Midgley, & Lane, 1992). Small peptides derived from this negative regulatory domain of p53 have been shown to facilitate the allosteric activation of p53 (Hupp, Sparks, & Lane,

1995).

p53 is defined as a transcriptional factor based on the fact that it consists of TAD and DBD. Many p53 target genes were identified by the fact that the regulatory region of these genes contains the p53-binding element, which activated p53 can bind to and induce the expression of these genes. A recent meta-analysis with data from 13 high-throughput studies identified 3661 direct p53 target genes (Fischer, 2017). Majority of p53's tumor suppressor functions are conferred via these p53 target genes, which are involved in multiple cellular responses, including cell cycle arrest, DNA repair, apoptosis, metabolism, etc. Different cellular contexts and stimuli induce different sets of transcriptional targets, resulting in various cellular outcomes (Kastenhuber & Lowe, 2017).

1.4 p53 and MDM2

p53 is a haploinsufficient gene, a slight change in p53 levels and/or activities (e.g., 2-fold difference) will have a significant impact on tumorigenesis (Berger & Pandolfi, 2011; Bond et al., 2004; Sawada & Sunaga, 1975). p53 protein levels are normally under tight regulations in cells (Z. Feng, Hu, Rajagopal, & Levine, 2008; Vousden & Prives, 2009). p53 is a short-lived protein, with a half-life of about ~30min. The E3 ubiquitin ligase MDM2 is a key negative regulator of p53. Under unstressed condition, MDM2 binds to p53 through p53's TAD to block the transcriptional activity of p53, and further degrades p53 through the ubiquitination-proteasome system (UPS) (Y. Haupt, Maya, Kazaz, &

Oren, 1997; Kubbutat, Jones, & Vousden, 1997; Oliner et al., 1993). Meanwhile, MDM2 is a p53 target gene (Barak, Juven, Haffner, & Oren, 1993; X. Wu, Bayle, Olson, & Levine, 1993). p53 transcriptionally activates MDM2, which will negatively regulate p53's activities and levels. This negative feedback loop between p53 and MDM2 contributes to the maintenance of p53's level at a low basal state. Under the stressed condition, p53 is rapidly activated and stabilized. The extent and duration of the activated status of p53 are tightly regulated by its negative regulators, including MDM2. The p53-MDM2 auto-regulatory loop plays a significant role in guarding the appropriate function of p53. Since p53 is mainly negatively regulated by Mdm2, in a mdm2 knockout mouse model, p53 protein is liberated from the supervision of mdm2 and leads to massive apoptosis. Knockout of mdm2 in the mouse is embryonic lethal. The embryos are found dead at E3.5. However, this lethality can be rescued by p53 knockout (S. N. Jones, Roe, Donehower, & Bradley, 1995; Montes de Oca Luna, Wagner, & Lozano, 1995). The $p53^{-/-}$ $mdm2^{-/-}$ mouse is viable. A similar phenotype was observed in mdmx knockout mice. Mdmx is a family member of mdm2, which is also an E3 ubiquitin ligase and negatively regulates p53. The embryos of $mdmx^{-/-}$ mice are dead at E10.5. Simultaneously ablation of p53 can rescue the embryonic lethality (Parant et al., 2001). MDM2 and MDMX are not the only E3 ubiquitin ligases that negatively regulate p53. Several p53 regulators have been identified, including constitutively photomorphogenic 1 (COP1), p53 induced protein with a RING H2 domain (Pirh2) and TRIM32, etc. (Dornan et al., 2004; Leng et al., 2003;

J. Liu, Zhang, Wang, et al., 2014; Wade, Wang, & Wahl, 2010).

1.5 p53 and aging

Aging is a complex process of time-dependent series of progressive loss of function and structure of all systems, which leads to an increased vulnerability to death (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013). The molecular mechanisms under the aging phenotypes are the decline of healthy and functional cells which result from cumulative damages in cells and tissues. Since the main function of p53 is involved in the repair and genomic integrity maintenance, p53 could play an important role in the aging process. Under mild stress conditions, p53 will initiate cell cycle arrest to aid in the repair of the cell to promote cell survival. However, the repaired cells, including stem cell and progenitor cells, might continue to proliferate or fail to proliferate, which leads to prolonged or declined lifespan. Under severe, acute stress signals, p53 will directly eliminate the damaged cells through apoptosis, which prevents tumor formation by eliminating potential malignant transformed cells. However, p53 under this condition may also wipe out stem cells or progenitor cells which are important for self-renewal. The decline or exhaustion of stem cells population, as well as its self-renewal function, will eventually lead to aging (Lopez-Otin et al., 2013). Cells from aged individuals are found to have the declined function/activity of p53 (Z. Feng, Hu, Teresky, et al., 2007). This age-related reduced activity of p53 may contribute to the increased tumor incidence in the aged population. Early cancer development definitely leads to a shortened lifespan. In the

study of Li-Fraumeni patients, germline mutation of p53 leads to early onset of tumors. Interestingly some Li-Fraumeni patients who escaped from cancer, live longer than the general population (Levine et al., 2015). Consistent with this observation, a case study of a progeroid patient with a germline mutation in MDM2 is featured with accelerated aging phenotypes (Lessel et al., 2017). This patient carries a homozygous mutation in MDM2, which leads to increased p53 levels and activity. This aberrant regulation of p53 seems to be the driving cause of the premature aging phenotype. These observations indicate that a functional p53 can contribute to the human aging phenotype. To better understand the role of p53 in aging, several mouse models have been created and investigated to unravel the “two-sided sword” function of p53 in aging.

1.6 p53 and aging mouse models

To study the role of p53 in aging and longevity, results from ample studies using genetically engineered mice shed light on the role of p53 and p53 signaling pathway in aging.

1.6.1 Loss of p53 leads to shortened lifespan mainly due to cancers

The generation of p53 knockout mice provided crucial evidence in the role of p53 in tumor suppression. Cancer is one of the prominent aging-associated diseases. In humans, the cancer risk increases dramatically after 45 years old. Some tumor suppressor genes are considered longevity assurance genes since they can prevent early death from cancer. In mouse models, loss of one allele of p53 can lead to around 2-fold increased incidence of

tumors (Donehower, 1996, 2009; Donehower et al., 1992; Venkatachalam et al., 2001). Compared to mice with wild-type p53, loss of both alleles of p53 in mice can lead to dramatically shortened lifespan, from 38 months to 10 months. Majority of $p53^{-/-}$ mice develop thymus lymphoma, spleen lymphoma at the age of 4~6 months old and cancer is the major cause for their shortened lifespan.

1.6.2 p53 activation through proper regulation leads to cancer resistance, but the normal or prolonged lifespan

"Super p53" mice, carrying an extra p53 transgenic allele (p53-tg), showed increased tumor resistance and aged normally (Garcia-Cao et al., 2002). Instead of using the strategy of "classical" transgenes, which carries a short promoter region followed by p53 cDNA, the super p53 allele contains large DNA segment (130kb), which keeps p53 gene in its natural genomic context. p53-tg mice can rescue the p53 deficiency in both mouse embryonic fibroblasts (MEFs) and mice. In MEFs, the p53-tg allele is capable of respond to DNA damage to induce expression of p53 target gene p21, which leads to cell cycle arrest. What's more, $p53^{-/-;\ tg/-}$ MEFs showed the similar ability in resistance to oncogene RAS-induced neoplastic transformation compared with $p53^{+/-}$ MEFs. In mice, the average lifespan of $p53^{-/-;\ tg/-}$ mice is 9.5 months compared with the $p53^{-/-}$ mice with 4.5-month's average lifespan. Apparently, the p53-tg allele could rescue the tumorigenesis caused by loss of p53 function. However, the *in vivo* tumor suppressive function of p53-tg allele might be reduced compared with the endogenous p53 wild-type allele based on the fact

that the average lifespan of $p53^{+/-}$ mice is 14.0 months, which is 4.5 months longer than $p53^{-/-;\ tg/-}$ mice. In the chemical carcinogenesis models, super p53 ($p53^{+/+;\ tg/-}$) mice showed increased resistance to both fibro-sarcoma and urinary bladder carcinomas. For the induction of fibro-sarcoma, about 67% of the $p53^{+/+;\ tg/-}$ mice were tumor free compared with 8% of $p53^{+/+}$ mice. In the urinary bladder carcinoma cases, 57% $p53^{+/+}$ mice developed multifocal tumors while only 16% of $p53^{+/+;\ tg/-}$ mice did so. In both models, super p53 mice developed tumors with a longer latency. As for lifespan, there was no significant difference between $p53^{+/+}$ mice and $p53^{+/+;\ tg/-}$ mice (78% of $p53^{+/+}$ mice, n=40, survived over 2 years vs. 70% of $p53^{+/+;\ tg/-}$ mice, n=13 survived over 2 years).

In the following study based on the super p53 mice model, super Arf/p53 mice ($Arf^{+/+;tg/-}$ $p53^{+/+;tg/-}$) were generated. Arf is a positive regulator of p53; it could stabilize p53 and contribute to a more robust DNA damage response (Lowe, Cepero, & Evan, 2004). In $Arf^{+/+;tg/-}$ $p53^{+/+;tg/-}$ mice, the cancer protection was further enhanced compared to the cancer-resistance phenotype of super p53 mice. The incidences of malignant cancer were 53% (51/96) in p53 wild-type mice, 40% (31/77) in $p53^{+/+;\ tg/-}$ mice, 40% (17/42) in $Arf^{+/+;\ tg/-}$ mice and 6% (1/15) in $Arf^{+/+;tg/-}$ $p53^{+/+;tg/-}$ mice. The median lifespan of $Arf^{+/+;tg/-}$ $p53^{+/+;tg/-}$ mice increased ~16% compared with wild-type mice. These studies showed that elevated p53 activity protects the mice from cancers, whereas the maintenance of normal regulation of p53 ensures a normal lifespan. As we mentioned above, p53 levels and activity are under tight regulation. When p53 receives different signals, it is activated and

stabilized to exert its functions accordingly. While the effective p53 response is crucial to its tumor suppressive functions, excess p53 activation could lead to deleterious effects, including accelerating the exhaustion of the healthy stem cells, which is crucial for the maintenance of tissue self-renewal. Properly regulation of p53 leads to normal aging phenotypes in mice (D. Wu & Prives, 2018).

In another mouse model with mdm2 ablation, p53 activity was increased (Mendrysa et al., 2006). The mice were generated with a hypomorphic (puro) and a null (Δ7–12) mdm2 allele. The $mdm2^{puro/\Delta7-12}$ mice retained ~30% of Mdm2 levels compared with wild-type mice. The 70% decrease in Mdm2 resulted in the stimulation of p53 target genes, as well as the increased apoptotic function of p53 (Mendrysa et al., 2003). In the $Apc^{min/+}$ mice model, which spontaneously develops intestinal adenomas, the 3-fold decrease in Mdm2 greatly suppressed the tumor formation. The $Apc^{min/+}$; $mdm2^{+/+}$ mice were found to have an average of 48 adenomas in the intestine whereas the $Apc^{min/+}$; $mdm2^{puro/\Delta7-12}$ mice only developed three tumors per mice. This constitutive high p53 activity did not change the lifespan of those mice. 71% of wild-type mice lived over 2 years compared with 73% of $mdm2^{puro/\Delta7-12}$ mice lived over 2 years. In the aged mice, 50% of wild-type mice developed spontaneous hepatomas while 17% of $mdm2^{puro/\Delta7-12}$ mice did so. The increased p53 activity enhanced cancer resistance but with a normal lifespan, which implies those $mdm2^{puro/\Delta7-12}$ mice who escaped from cancer may have a shortened lifespan compared with wild-type mice.

1.6.3 Constitutive p53 activation with aberrant regulation is associated with decreased tumor incidence and accelerated aging

There are two mouse models with one wild-type p53 allele and one truncated p53 allele, which showed cancer resistance but premature aging phenotypes. The $p53^{+/m}$ mice were generated with one wild-type allele and a truncated C-terminal p53 mutant allele, which retains oligomerization domain for interacting with wild-type p53, but loses TAD and Mdm2 binding site (Tyner et al., 2002). These mice displayed hyperactive p53 activity and were tumor-resistant, but at the cost of accelerated aging and reduced lifespan. The $p53^{+/m}$ mice showed 6% of tumor incidence while $p53^{+/+}$ mice showed >45% tumor incidence. It would be expected that the lifespan will not change or be prolonged in $p53^{+/m}$ mice considering the low tumor incidence. However, the lifespan is shortened by 18.6% in $p53^{+/m}$ mice compared with $p53^{+/+}$ mice. Mechanically, the $p53^{+/m}$ mice had a faster decline of tissue stem cell self-renewal function during the aging process, suggesting that increased p53 activity leads to reduced stem cell self-renewal and tissue reconstitution ability (Dumble et al., 2007). Another mouse model expressed a naturally occurring short isoform of p53 (Maier et al., 2004). This truncated p53 protein p44, which lacks the N-terminal of the protein, has constitutively activated p53 function. The p44 mice started to show signs of aging from 4 months old. The median lifespan of p44 mice is 8 months compared with 24 months for the wild-type p53 mice (Maier et al., 2004). Similar results were obtained with the mouse model carrying knock-in of p53 with two

phosphorylation-site mutations (T21D, S23D), which mimics phosphorylated p53 in response to DNA damage (D. Liu et al., 2010). Homozygous $p53^{TSD/TSD}$ in mice leads to embryonic lethality. Heterozygous $p53^{TSD/-}$ mice with constitutively increased p53 activity exhibited accelerated aging phenotypes and died by 6 weeks old. The premature aging phenotypes of the $p53^{TSD/-}$ mice were accompanied with the depletion of adult stem cells in multiple tissues. All these results suggest that although constitutively activated p53 can ensure longer lifespan by eliminating cancer cells, it may promote aging possibly through negative regulation of the self-renewal functions of stem cells.

All these mouse models have provided some useful insights into how p53 might regulate the aging process. The role of p53 in assuring longevity through prevention of cancer is established. p53 can be pro-aging or pro-longevity depending on the context of its regulation and activity. The precise role of p53 in the intrinsic aging process, especially under the physiological conditions, remains unclear.

2. The role of p53 codon 72 SNP in cancers and aging process

2.1 p53 codon 72 SNP

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variations among people. Each SNP represents a difference in the nucleotide. Most SNPs have no effect, however, some SNPs have been found to affect the gene's function. p53 codon 72 SNP is one of the most intensively studied functional polymorphism in the p53 signaling pathway. Like other functional SNPs in p53 or p53 signaling pathway, p53 codon 72 SNP

alters the levels and the functions of p53. The p53 codon 72 SNP, rs1042522, locates in the exon 4 of the p53 gene (aa215 G to C) and results in an arginine (Arg, R) to proline (Pro, P) amino acid substitution in position 72 of the p53 protein. It was first identified in 1986; p53 R72 showed a faster-migrating band in the SDS PAGE gel compared to p53 P72 (N. Harris et al., 1986). This was mainly due to that p53 R72 is carrying a large side chain and Arg is positively charged, whereas p53 P72 is carrying a small, non-polar side chain and maintains a relative inert, closed circular structure. Because this SNP locates in the putative Src homology 3 (SH3) binding domain of p53, it influences binding capacity and thereby functional properties of p53. It has been shown that p53 codon 72 SNPs function differently. For example, p53 R72 is more efficient in inducing apoptosis, which is supported by the evidence that p53 R72 showed greater localization to mitochondria. The mitochondria localization is related to apoptotic potential. What's more, it has been shown that p53 R72 preferentially regulates a subset of p53 target genes in apoptosis, including noxa and puma. (Dumont, Leu, Della Pietra, George, & Murphy, 2003). The frequency of p53 R72 allele ranges from 70% among Western European Caucasians to 30% among South Africans (Beckman et al., 1994; Koushik, Platt, & Franco, 2004). p53 P72 is considered as the ancient allele, but the reason for the enrichment of R72 in Caucasians is not clear yet.

2.2 p53 codon 72 SNP and cancers

In both human and mouse models, p53 exhibits haploinsufficient phenotypes: even with a

2-fold of p53 protein level change in cells results in the inability of the cell to execute its normal functions, which leads to accelerated tumorigenesis (Berger & Pandolfi, 2011; Bond et al., 2004; Venkatachalam et al., 2001). p53 P72 showed weaker p53 activities in inducing apoptosis, which is a critical function of p53 in suppressing tumorigenesis. To investigate whether p53 codon 72 SNP has different potential in tumor suppression, a large number of studies investigating the role of p53 codon 72 SNP in human populations in cancer have been carried out (Whibley, Pharoah, & Hollstein, 2009) (http://www.gwascentral.org). So far, there is no consensus in the literature. Some studies suggested that p53 R72 protects carriers from cancers; others showed that p53 R72 did not affect cancer incidence. Majority of studies support the protective effect of p53 R72 on cancer risk. In a study of squamous cell carcinoma of the head and neck (SCCHN) with 304 patients and 333 cancer-free controls, the p53 P72 carriers showed early onset of SCCHN (Shen, Zheng, Sturgis, Spitz, & Wei, 2002). The median ages of onset of SCCHN were 53 years for p53 P72 and 59 years for p53 R72 (p=0.057 P72/P72 and R72/P72 combined vs. R72/R72). The median age of onset of oral cancers was 51 years for p53 P72 and 62 years for p53 R72 (p=0.046). In a study of hereditary nonpolyposis colorectal cancer (HNPCC) in the Caucasian population, the median onset age of HNPCC was 13 years earlier in heterozygous carriers than homozygous p53 R72 carriers (J. S. Jones et al., 2004). Of the 92 participants, 47 developed colorectal cancer. The median onset age of cancer was 40 years for Arg/Pro (17/47, 36.2%) and 53 years for

Arg/Arg (29/47, 61.7%) and p=0.0311 in the log-rank test. In response to chemotherapy, cells with p53 P72 showed minor apoptosis at the drug concentrations that cause extensive apoptosis in cells with p53 R72 (Sullivan et al., 2004). In a cohort of 70 patients with advanced head and neck squamous cell carcinomas (HNSCC), p53 R72 carriers showed a better response with chemo-radiotherapy (including apoptosis-inducing agents cisplatin, taxol and 5-FU). 96% of p53 R72 carriers showed a complete response (n=28, p<0.04) and a better prognosis, with longer overall survival (OS, p=0.02) and progression-free survival (PFS, p=0.007). It seems that p53 P72 is associated with early onset of cancers and poorer prognosis. However, there are also lots of studies showed that p53 P72 is associated with decreased cancer risks, which leads to the discrepancies in the relationship of p53 codon 72 SNP and cancer risk (Murphy, 2006). It should be noted that the mutational status of p53 is not clear in many studies, which complicated the interpretation of the results obtained from these studies (Marin et al., 2000). A well-controlled study either in humans or mouse models should be carried out to better characterize the role the p53 codon 72 SNP in cancers.

2.3 p53 codon 72 SNP and aging

The most extensively studied role of p53 is in cancer prevention and tumor suppression. However, the anti-proliferative function of p53 can also exert its effect in depleting proliferation-competent progenitor or stem cells, which dampens the tissue self-renewal ability and leads to aging. P53 codon 72 SNPs, which show different potentials in

inducing different sets of p53 target genes, have been indicated to play a role in modulating the aging process. Using a formal meta-analysis of the published literature, Heemst et al. showed that p53 P72 carriers have an increased cancer risk compared to p53 R72 carriers. Interestingly, in this prospective study of aged population (>85 years, n=1226), p53 P72 carriers have a 41% increased survival albeit (p=0.032) with a 2.54-fold of increased mortality (p=0.007) from cancer (van Heemst et al., 2005). In another prospective study with the general population (20-95 years, n=9219), p53 P72 carriers have an increased median survival of 3 years compared to p53 R72 carriers (Bojesen & Nordestgaard, 2008). This study was carried out in Denmark, where the homogenous Danish populations have complete health registries. The morbidity and mortality of the general Danish populations were investigated. Overall, there was no significant association between p53 codon 72 SNP and risk of multiple cancers (including gastrointestinal, respiratory, urologic, female and male gender-specific cancers). However, p53 P72 carriers (8/662) showed a nearly 1.5-fold increase in the risk of hematologic cancer compared with p53 R72 carriers (42/4933). Regardless of morbidity from cancers, the overall 12-year survival of p53 P72 carriers was increased by 6% compared with the p53 R72 carriers (p=0.002). The median survival of p53 P72 carriers was 3 years longer than the p53 R72 carriers. The reduced mortality in p53 P72 carriers was explained by increased cell cycle arrest and decreased pro-apoptosis that protect a person from dying of critical illness, including cancers. A similar study with long-lived

individuals (n=131) in the Siberian region also showed the enrichment of p53 P72 alleles in these individuals (Smetannikova et al., 2004). All these studies in human populations suggest that the p53 P72 allele may ensure a longer lifespan despite a moderate increase in cancer risk. However, due to the complexity of the human population in genetic backgrounds, environmental variations, etc., the mechanism underlying this phenotype is not well elucidated.

2.4 Summary

Although the role of p53 in assuring longevity through prevention of cancers has been indicated in several mouse models, the role of p53 in regulating stem cell pool and self-renewal ability suggested that p53 also has pro-aging activity. p53 can be pro-aging or pro-longevity. So up until now, the precise role of p53 in the intrinsic aging process, especially under physiological condition, remains unclear.

p53 codon 72 SNP is a functional coding SNP and a common SNP. p53 R72 is more efficient in inducing apoptosis, whereas p53 P72 is more competent in inducing cell cycle arrest and repair pathways. Previous epidemiological human studies have shown that p53 codon 72 SNP may impact lifespan in humans. However, considering the complexity of genetic background variations of human populations and environmental factors in epidemiological studies, the precise role of p53 codon 72 SNP in aging and longevity remains elusive. So we aim to investigate the role of p53 codon 72 SNPs in aging and longevity in mouse models. Because mouse does not have codon 72, human codon 72

SNP was introduced into the mouse strain we used. The endogenous murine p53 sequence for exons 4-9 has been replaced with the homologous human p53 sequence. The transcription of this chimeric p53 gene is under the control of endogenous mouse promoters. The lifespan and aging phenotypes were monitored in mice carrying either p53 R72 or p53 P72. The results from this study will provide valuable evidence of the precise role of p53 in cancer and aging under a physiological condition with the isogenic genetic background.

3. Mutant p53 and mutant p53's Gain-Of-Function in tumorigenesis.

3.1 Introduction of mutant p53

Tumor suppressor p53 plays a central role in human cancer (Levine, Hu, & Feng, 2006; Levine & Oren, 2009; Vogelstein, Lane, & Levine, 2000; Vousden & Prives, 2009). p53 is a transcription factor and mainly exerts its role in tumor suppression through its transcriptional regulation of its downstream target genes. As the "guardian of the genome", p53 ensures the replication fidelity and genomic stability to prevent tumor initiation and progression. Loss of p53 function is often a prerequisite for tumor initiation and progression, which has been most clearly demonstrated by the increased cancer risk in Li–Fraumeni syndrome patients with germline p53 mutations and p53 knockout mouse models (Lang et al., 2004; Olive et al., 2004). Approximately 50% of Li–Fraumeni syndrome patients develop different types of cancers, including sarcoma, breast cancer, and brain cancer, by the age of 30, and almost 100% of p53 knockout mice develop

tumors, primarily lymphomas, and sarcomas, by the age of 10 months (Bougeard et al., 2008; Donehower et al., 1992; Jacks et al., 1994; Srivastava et al., 1990). In human cancer, p53 is the most frequently mutated gene. Around 50% of human cancers harbor p53 mutations (Freed-Pastor & Prives, 2012; C. C. Harris & Hollstein, 1993; P. A. Muller & Vousden, 2014; Olivier, Hussain, Caron de Fromentel, Hainaut, & Harris, 2004). In addition to p53 mutations, p53 function is frequently attenuated, and the p53 signaling is dysfunctional in human cancer through multiple mechanisms, including overexpression and/or amplification of different p53 negative regulators, such as MDM2, MDM4, Cop1, Pirh2, Trim32, and LIF (Danes et al., 2008; Dornan et al., 2004; S. L. Harris & Levine, 2005; Leng et al., 2003; Levine et al., 2006; J. Liu, Zhang, Wang, et al., 2014; Lu et al., 2007; Wade & Wahl, 2009; H. Yu et al., 2014).

While the critical role of wild-type p53 in tumor suppression has been firmly established, mounting evidence has demonstrated that many tumor-associated mutant p53 (mutp53) proteins not only lose the tumor-suppressive function of wild-type p53 but also gain tumor-promoting function through dominant-negative (DN) regulation of remaining wild-type p53 or independently of wild-type p53 (Brosh & Rotter, 2009; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013). While many other tumor suppressor genes are predominantly inactivated through deletion or truncating mutations in cancer, majority of p53 mutations in human cancer are missense mutations, which lead to the expression of full-length mutp53 proteins with the substitution of a single amino acid (Brosh & Rotter,

2009; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013). Furthermore, the majority of p53 mutations cluster in the DNA-binding domain (DBD), a region which is required for wild-type p53 to bind to its target genes and function as a transcription factor. Interestingly, more than 25% of p53 mutations in human cancers fall within 6 “hotspots”, including amino acid residues R175, G245, R248, R249, R273, and R282, although p53 mutations have been found in almost every codon within the p53 DBD in cancer. p53 mutations have two major categories: DNA contact mutations (e.g., R248 and R273) and conformational mutations (e.g., R175, G245, R282, and R249) (Brosh & Rotter, 2009; Cho, Gorina, Jeffrey, & Pavletich, 1994; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013). DNA contact mutations occur in the amino acid residues that make direct contact with p53 target DNA sequences and are critical for DNA binding, which impair the transcriptional activity of wild-type p53 without dramatically affecting the conformation of the p53 protein. Conformational mutations usually result in a more dramatic alteration of p53 protein structure compared with DNA contact mutants. These p53 mutations lead to the loss of affinity to the majority of p53 consensus DNA-binding elements in wild-type p53 target genes. Also, many mutp53 proteins exhibit DN effects on the remaining wild-type p53 allele. Mutant p53 proteins can form a tetramer with wild-type p53, which can block the function of the remaining wild-type p53 in tumor suppression (Milner & Medcalf, 1991; Milner, Medcalf, & Cook, 1991; Oren & Rotter, 2010; Sigal & Rotter, 2000). In addition to its DN effect on wild-type p53, mutp53 proteins often exhibit

oncogenic activity in cells lacking wild-type p53. p53 missense mutations in human cancer are usually followed by the loss of heterozygosity at the corresponding locus, suggesting that there is a selective advantage conferred by losing the remaining wild-type p53, even after one allele has been mutated (Baker et al., 1990; Brosh & Rotter, 2009; Freed-Pastor & Prives, 2012). The discovery of p53 as a “proto-oncogene” shortly after p53 discovery was in part due to the cloning of mutp53 cDNA from cancer cells; ectopic expression of mutp53 promoted cell transformation and increased tumorigenicity in p53-null cells (Eliyahu et al., 1984; Jenkins et al., 1984; Levine & Oren, 2009; Parada et al., 1984). While these early studies masked the true function of wild-type p53, they hinted the gain-of-function (GOF) of mutp53 in tumorigenesis.

3.2 Mutant p53 GOF

Mutant p53 GOF has been demonstrated by numerous cell-based experiments. This set of evidence has been mainly obtained through ectopically expressing mutp53 in p53-null human tumor cells or the knockdown of endogenous mutp53 in cells containing only one allele of mutp53 (Brosh & Rotter, 2009; Dittmer et al., 1993; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013). So far, many mutp53 GOFs have been identified, including promoting tumor cell proliferation, survival, migration, and invasion; enhancing chemoresistance; disrupting proper tissue architecture; and promoting cancer metabolism (both Warburg effect and lipid metabolism) (Brosh & Rotter, 2009; Dittmer et al., 1993; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013) (Blandino et al.,

2012; Freed-Pastor et al., 2012; P. A. Muller et al., 2009; Zhang et al., 2013) (Fig. 1). Mutant p53 GOF has also been demonstrated in mutp53 knock-in mouse models, engineered by introducing tumor-associated hotspot p53 mutations into the endogenous Trp53 locus in mice using homologous recombination (Hanel et al., 2013; Lang et al., 2004; Olive et al., 2004). These mice develop a different tumor spectrum, more aggressive tumors, or earlier tumors compared with p53-null mice (Alexandrova et al., 2015b; Lang et al., 2004; Olive et al., 2004). In human Li–Fraumeni syndrome patients, those with germline missense p53 mutations have an early age onset of cancer development compared with those with germline p53 deletion mutations (Bougeard et al., 2008). In many different types of human cancer, p53 mutations are frequently associated with more aggressive cancer, poorer response toward therapy, and prognosis (Elledge et al., 1993; P. A. Muller & Vousden, 2014; Olivier et al., 2006; Petitjean, Achatz, Borresen-Dale, Hainaut, & Olivier, 2007). It needs to point out that recent studies also showed that different p53 mutants could exhibit different GOF phenotypes or have the same GOF phenotypes but to different extents (Freed-Pastor & Prives, 2012; Hanel et al., 2013; Mello & Attardi, 2013; P. A. Muller & Vousden, 2014). Collectively, these results from cell-based experiments, mouse models, and human studies support the concept of mutp53 GOF.

Mutant p53, in general, can no longer recognize and bind to p53 DNA-binding elements and lose the transcriptional activity toward wild-type p53-regulated target genes (Brosh &

Rotter, 2009; Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013). However, many tumor-associated mutp53 proteins are mainly localized in the nucleus and can regulate the transcription of some genes through mechanisms different from wild-type p53. For example, mutp53 can bind to TAp63 and TAp73 and inhibit their transcriptional activities (Gaiddon, Lokshin, Ahn, Zhang, & Prives, 2001; Y. Li & Prives, 2007; P. A. Muller, Trinidad, Caswell, Norman, & Vousden, 2014; Stindt et al., 2015). TAp63 and TAp73 are two important p53 family members, which can regulate some of wild-type p53 target genes and compensate for some of the p53 tumor-suppressive functions (Belyi et al., 2010; Hu, 2009). Mutant p53 can also interact with other transcriptional factors and cofactors, including NF-Y, SREBP, VDR, Sp1, ETS2, and NRF2, and enhance or decrease their transcription activities to promote tumor progression (Di Agostino et al., 2006; Dupont et al., 2009; Freed-Pastor & Prives, 2012; Sampath et al., 2001; Stambolsky et al., 2010; Walerych et al., 2016). Mutant p53 can also participate in the regulation of chromatin structure to regulate the expression of some genes. For instance, mutp53 was reported to interact with chromatin remodeling complex SWI/SNF to cooperate their chromatin remodeling function (Pfister et al., 2015). Mutant p53 can also recognize and bind with high affinity to DNA regions of matrix attachment region DNA elements, which have a high potential for DNA base unpairing (Will, Warnecke, Wiesmuller, & Deppert, 1998). In addition to its nuclear function, mutp53 has a cytoplasmic function that contributes to its GOF. For example, mutp53 promotes the

translocation of glucose transporter 1 to the plasma membrane to stimulate the Warburg effect, a characterized metabolic change in tumor cells and a key contributor to tumor development (Zhang et al., 2013).

3.3 Mechanisms underlying mutant p53 GOF

The mechanism of mutp53 GOF is far from clear. There are several prevailing potential mechanisms: 1) mutp53 enhances or decreases transcription by interacting with transcription factors or cofactors, such as NF-Y, SREBP, p63 and p73 etc., and being recruited to the binding sites of their target genes to promote or inhibit their transcription(Adorno et al., 2009; Di Agostino et al., 2006; Freed-Pastor et al., 2012; Sampath et al., 2001); 2) mutp53 recognizes and interacts with DNA such as structure-specific DNA matrix attachment regions (MARs) to transcriptionally regulate their relevant promoters(Will et al., 1998); 3) mutp53 interacts with cellular proteins other than transcriptional factors to promotes its GOF in tumorigenesis, such as NRD1 and MRE11(Coffill et al., 2012; P. A. Muller & Vousden, 2013; Song, Hollstein, & Xu, 2007). These mechanisms are not mutually exclusive. Another important mechanism of the mutant p53 gain of function is through stabilization and accumulation of mutant p53. Mutant p53 proteins are found to be accumulated exclusively in tumor tissues. Stabilization of wtp53 enables the cells to either repair their damage or induce apoptosis to prevent cells from oncogenic transformation; whereas stabilization of mutp53 promotes its GOF in tumorigenesis. Stabilization of mutp53 can lead to mutp53 accumulation,

which augments the oncogenic properties of mutp53, a critical step for mutp53 to exert its GOF in tumorigenesis and tumor progression. We will further discuss in detail of recent studies on how mutant p53 is stabilized and accumulated.

3.4 Mutant p53 Protein Accumulation in Tumors

One unique feature of mutp53 is that mutp53 proteins often become stable and accumulate to very high levels in tumors (Freed-Pastor & Prives, 2012; J. Liu, Zhang, Hu, & Feng, 2015; P. A. Muller & Vousden, 2013; Oren & Rotter, 2010). Based on this characteristic of mutp53 protein, positive immunohistochemical staining of p53 in tumor tissues has been widely used as a surrogate for p53 mutation detection (Alsner et al., 2008; Bartek, Iggo, Gannon, & Lane, 1990; Freed-Pastor & Prives, 2012). Importantly, mutp53 accumulation in tumors is critical for mutp53 to exert its GOF in tumorigenesis and contributes to more advanced tumors (Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013; Terzian et al., 2008). Destabilizing mutp53 can greatly reduce mutp53 GOF in tumorigenesis, which is a promising strategy for cancer therapy that is currently under active investigation (Alexandrova et al., 2015a; D. Li, Marchenko, & Moll, 2011; P. A. Muller & Vousden, 2013).

The mechanism for mutp53 protein accumulation in tumors is not well-understood. Under non-stressed conditions, wild-type p53 protein levels are kept low in cells through efficient proteasomal degradation mainly mediated by E3 ubiquitin ligase MDM2 (Brooks & Gu, 2006; S. L. Harris & Levine, 2005; Hu, Feng, & Levine, 2012; Wade, Li, & Wahl, 2013).

MDM2 is a key negative regulator for wild-type p53; MDM2 binds to and degrades wild-type p53 through ubiquitination. Meanwhile, MDM2 itself is a p53-regulated gene. Thus, MDM2 forms a negative feedback loop with wild-type p53 to tightly regulate wild-type p53 protein levels and functions (Brooks & Gu, 2006; S. L. Harris & Levine, 2005; Wade et al., 2013). Mutant p53 can no longer induce MDM2. MDM2 interacts with multiple regions of wild-type p53. The conformation change of p53 protein due to mutations may decrease the interaction efficiency of MDM2 toward these regions. The overall efficiency of mutp53 ubiquitination is reduced compared with that of wild-type p53 (Lukashchuk & Vousden, 2007). The inability of mutp53 to induce MDM2 and the reduced ability of MDM2 to degrade mutp53 can disrupt the MDM2–p53 loop and had long been thought to be the main underlying mechanism for the accumulation of mutp53 protein in tumors (Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013; Oren & Rotter, 2010). However, this notion has been challenged by recent studies showing that mutp53 proteins are accumulated exclusively in tumor tissues but not normal tissues in mutp53 knock-in mice (Lang et al., 2004; Olive et al., 2004). Furthermore, MDM2 loss in mutp53 knock-in mice leads to mutp53 accumulation in normal tissues, which in turn promotes mutp53 GOF in tumor development and reduces survival (Terzian et al., 2008). These observations provide in vivo evidence that (1) MDM2 can negatively regulate mutp53 protein levels, and (2) mutp53 protein accumulation promotes mutp53 GOF. Consistently, recent studies showed that MDM2 could ubiquitinate and degrade mutp53 *in vitro*

(Lukashchuk & Vousden, 2007; Zheng et al., 2013). These results suggest that MDM2 maintains mutp53 protein levels low in normal tissues, whereas some changes in tumors disrupt MDM2-mediated mutp53 degradation, thereby leading to mutp53 protein accumulation in tumors.

3.5 Therapeutic Strategies to Target Mutant p53

Given that mutp53 proteins often accumulate to high levels and exert GOF to promote malignant progression in human cancer, targeting mutp53 has become an attractive therapeutic strategy for cancer containing mutp53 (Freed-Pastor & Prives, 2012; Gurpinar & Vousden, 2015; P. A. Muller & Vousden, 2013; Oren & Rotter, 2010). The main strategies to target mutp53 are restoring the wild-type p53 activity and depleting mutp53 protein in cancer. The strategies to target mutp53 in cancer include restoring wild-type p53 (wtp53) by PRIMA-1, APR-246, ZMC-1, etc. and depleting mutp53 by 17AAG, ganestespib, gambogic acid, etc., in cancer containing mutp53.

3.5.1 Restoring wild-type p53 activity

Mutant p53 proteins stabilize and accumulate only in tumors but not in normal tissues as shown by recent mutp53 knock-in mouse models (Alexandrova et al., 2015b; Lang et al., 2004; Olive et al., 2004). Therefore, restoring wild-type p53 activity can have the targeted effect on tumor cells through inducing cell apoptosis and inhibiting cell proliferation and migration in tumor cells containing mutp53 with limited side effects to normal tissues containing wild-type p53. This strategy is especially useful for the treatment of the

late-stage tumors, which often have one allele of mutp53 and lose the remaining wild-type p53 allele (Cavenee, Scrable, & James, 1991; Gonzalez et al., 1995). These tumors are usually more aggressive and resistant to treatment. The compounds that can restore wild-type p53 activity have been summarized in several recent reviews (Bykov et al., 2016; Oren, Tal, & Rotter, 2016). Among all the compounds, [2,2-bis (hydroxymethyl)-3-quinuclidinone] (PRIMA-1) is the most well-advanced small molecule (Bykov et al., 2002; Bykov et al., 2016). PRIMA-1 was identified from a screening showing a preferential inhibitory effect on proliferation of p53-null Saos2 cells with ectopic expression of mutp53 R175H compared with control Saos2 cells (Bykov et al., 2002). PRIMA-1 and its methyl analog APR-246 can restore wild-type p53 activity by inducing a conformational change of mutp53 proteins and refolding accumulated unfolded mutp53 proteins (Bykov et al., 2016). In addition, PRIMA-1 also restores unfolded wild-type p53, which can promote tumor invasion just like mutp53 proteins (Bykov et al., 2016) (Rieber & Strasberg-Rieber, 2012; Trinidad et al., 2013). It is, therefore, possible that PRIMA-1 will be beneficial to cancer patients with either mutp53 or wild-type p53 in tumors. Currently, PRIMA-1 has shown a favorable safety profile in phase I clinical trials, and APR-246 has entered a phase II clinical trial (Bykov et al., 2016) (B. D. Lehmann & Pietenpol, 2012; S. Lehmann et al., 2012).

Zinc metallochaperone-1 (ZMC-1) is another recently identified small molecule that restores the proper protein folding and transcriptional activity of mutp53 R175H (Blanden,

Yu, Loh, Levine, & Carpizo, 2015; X. Yu, Vazquez, Levine, & Carpizo, 2012). Zinc is required for the proper folding of wild-type p53 protein to ensure its stabilization and correct DBD structure. Some mutp53 proteins have the impaired zinc binding ability that prevents their proper protein folding and function. ZMC-1 buffers the intracellular free zinc levels to promote the binding of mutp53 to zinc and therefore facilitates the proper folding of mutp53 (Blanden et al., 2015; X. Yu et al., 2012). In addition, ZMC1 treatment generates Reactive oxygen species (ROS) and induces DNA oxidation, which can activate newly rescued mutp53 to induce the expression of wild-type p53 target genes. ZMC-1 exhibited strong toxicity to cells containing mutp53 R175H and a potent antitumor activity in p53 R175H tumors but showed limited effects on cells and tumors containing wild-type p53 or other mutp53 (R248Q and R273H). In addition to p53 R175H mutant, there are some other zinc-binding p53 mutants, including C176, C238, C242, H179, and M237. It is possible that ZMC-1 can reactivate these zinc-binding mutp53 like R175H, which should be elucidated by future studies.

3.5.2 Depleting mutant p53 protein

Growing evidence further suggests that many tumor cells become addicted to mutp53 protein and mutp53 GOF. Knockdown of mutp53 in cancer cells greatly reduces cell proliferation, tumor growth, chemo-response, and metastasis. For example, ablation of mutp53 in a conditional mouse model expressing R248Q mutp53 induces the regression of advanced tumors and greatly extends animal survival (Alexandrova et al., 2015b).

These observations on mutp53 addiction indicate that tumors containing mutp53 depend on the sustained expression of mutp53 for continued growth and strongly support depleting mutp53 proteins as a promising therapeutic strategy for tumors carrying mutp53.

Hsp90 can promote the stabilization and accumulation of mutp53 by inhibiting the degradation of mutp53 mediated by MDM2 and CHIP (Blagosklonny, Toretsky, Bohen, & Neckers, 1996; D. Li, Marchenko, Schulz, et al., 2011; P. Muller, Hrstka, Coomber, Lane, & Vojtesek, 2008; Whitesell, Sutphin, Pulcini, Martinez, & Cook, 1998). Inhibitors for Hsp90, including geldanamycin, 17AAG, and Ganetespib, have been tested as therapeutic agents for cancer containing mutp53. Treatment of cancer cells with 17AAG leads to the degradation of different mutp53, including R175H, L194F, R273H, and R280K, and the reduced viability of cancer cells containing these mutp53 (D. Li, Marchenko, Schulz, et al., 2011). Ganestespib, the highly potent synthetic HSP90 inhibitor, has a much higher efficacy (more than 50-fold) than 17AAG in degrading mutp53 (but not wild-type p53) and killing cancer cells containing mutp53 (Alexandrova et al., 2015b). Results from in vivo animal studies employing mutp53 knock-in mice and p53-null mice showed that ganestespib treatment suppresses tumor growth and increased survival in a mutp53-dependent manner (Alexandrova et al., 2015b). Ganestespib is currently being evaluated in the clinical trial, including a phase III lung cancer trial, and has demonstrated a favorable safety profile in cancer patients (Goyal et

al., 2015; Jhaveri et al., 2014; Ramalingam et al., 2015). In addition to Hsp90, inhibiting the activity of HDAC6, an essential positive regulator of Hsp90, has also been shown to degrade mutp53 (D. Li, Marchenko, & Moll, 2011). Histone deacetylase inhibitors (HDACi), specifically suberoylanilide hydroxamic acid (SAHA), can degrade mutp53 proteins and exhibit higher cytotoxicity to cells carrying mutp53 compared with cells containing wild-type p53 or cells deficient for p53. Gambogic acid, a natural product from Garcinia hanburyi tree, has been shown to promote mutp53 degradation (Gu et al., 2008). It has been suggested that gambogic acid promotes mutp53 degradation through preventing mutp53–Hsp90 interaction, enhancing mutp53–Hsp70 interaction, and promoting mutp53 nuclear exportation for degradation (J. Wang et al., 2011).

3.6 Summary/perspective

p53 is the most frequently (~50%) mutated genes in human cancers. Besides the loss of tumor-suppressive activity of wild-type p53, many tumor-associated mutant p53 (mutp53) proteins gain oncogenic functions to promote tumorigenesis, defined as gain of function (GOF). Mutp53 often accumulates to high levels in tumors, which is critical for mutp53 to exert GOF. Destabilizing mutp53 to inhibit mutp53 GOF in tumorigenesis is a promising novel strategy to target cancer carrying mutp53. The goal of my graduate study is to study the mechanisms for mutp53 accumulation and GOF, which are poorly understood currently. I searched for candidate proteins that regulate mutp53 protein levels and/or GOF by using an unbiased high throughput screening assays: immunoprecipitation (IP)

combined with mass spectrometry (MS) screening. This screening should disclose not only a single protein but also a group of proteins that synergistically work together to promote mutant p53 GOF. Through this approach, I identified several novel mutp53-binding proteins that regulate mutp53 protein levels and function, including co-chaperone proteins BAG2 and BAG5, which stabilize mutp53 and a potential helicase protein Pontin, which transcriptional regulate mutp53 target genes. Further understanding of the mechanism of mutant p53 accumulation and GOFs in tumors will help develop effective therapeutic strategies targeting tumors containing mutant p53.

Chapter II: p53 codon 72 SNP and aging in mouse models

Introduction

Aging is a complex process of time-dependent series of progressive loss of functions and structures of all systems, which leads to an increased vulnerability to death (Lopez-Otin et al., 2013). Cancer is an age-associated disease, which can lead to both premature death and age-associated increase in morbidity and mortality (Campisi & Yaswen, 2009). Tumor suppressor p53 plays a pivotal role in tumor prevention (Z. Feng et al., 2008; Vousden & Prives, 2009). Loss or disruption of p53 function is often a prerequisite for tumor initiation and development. In humans, more than 50% of all human tumors contain mutations in the p53 gene (Olivier et al., 2002). In mice, loss of both Trp53 alleles (p53-/-) leads to the development of tumors early in life and a reduced lifespan compared with wild-type mice (Donehower et al., 1992). Therefore, p53 ensures longevity by preventing cancer development early in life.

Longevity depends on the balance between tumor suppression and tissue renewal mechanisms (Campisi & Yaswen, 2009). While genomic instability is a hallmark of aging, stem cell exhaustion is another important hallmark of aging (Lopez-Otin et al., 2013). It has been indicated that the anti-proliferative function of p53 which is crucial for tumor suppression could affect self-renewal function of stem/progenitor cells and contribute to aging (Donehower, 2002; van Heemst et al., 2005). However, the precise role of p53 in aging process and longevity has not been clearly established. Inconsistent results on

aging and longevity have been reported in different mouse models in which the p53 activity has been manipulated through different strategies (Dumble et al., 2007; Garcia-Cao et al., 2002; D. Liu et al., 2010; Maier et al., 2004; Matheu et al., 2007; Mendrysa et al., 2006; Tyner et al., 2002). Specifically, transgenic mice with constitutively elevated p53 activity by expression of certain p53 mutants or a short p53 isoform showed increased cancer resistance but premature aging phenotypes (Dumble et al., 2007; D. Liu et al., 2010; Maier et al., 2004; Tyner et al., 2002). The ‘super p53’ mice with a regulated hyperactive p53 activity by having an extra copy of the wild-type Trp53 gene were resistant to cancer but did not exhibit signs of accelerated aging (Garcia-Cao et al., 2002; Mendrysa et al., 2006). Interestingly, the ‘super p53’ mice with an extra copy of Ink4/Arf showed extended longevity (Matheu et al., 2009; Matheu et al., 2007). It is worth noting that mouse models used in these studies did not reflect p53 activation under physiological conditions. It is therefore critical to address the role of p53 in the aging process and longevity using a proper mouse model reflecting the p53 activity under physiological conditions.

TP53 is a haploinsufficient gene, a little decrease in p53 levels or activity (e.g., 2-fold difference) significantly impacts tumorigenesis (Berger & Pandolfi, 2011; Bond et al., 2004; Venkatachalam et al., 2001). p53 protein levels and activity are under tight regulation in cells (Z. Feng et al., 2008; Vousden & Prives, 2009). In humans, naturally occurring single nucleotide polymorphisms (SNPs) in the p53 pathway, which modulate

the activity or levels of p53, have been found to significantly impact cancer risk (Basu & Murphy, 2016; Bond et al., 2004; Lin, Huang, Wu, Chang, & Lung, 2008; Whibley et al., 2009). p53 codon 72 SNP is a common coding SNP in the TP53 gene, which results in either an arginine (R72) or a proline (P72) residue at codon 72. We and others have reported that compared with the R72 allele, the P72 allele displays a weaker p53 transcriptional activity towards a group of its target genes, many of which are involved in apoptosis and suppressing cell transformation (Dumont et al., 2003; Jeong, Hu, Belyi, Rabadan, & Levine, 2010). Studies in human populations indicate that p53 codon 72 SNP may modify cancer risk, but currently, the consensus has not been reached on this in the literature (van Heemst et al., 2005; Whibley et al., 2009). Several studies of aged or general human populations indicate that the P72 carriers have an increased lifespan despite an increased mortality from cancer (Bojesen & Nordestgaard, 2008; Smetannikova et al., 2004; van Heemst et al., 2005). These epidemiological results support the dual functions of p53 in longevity, and suggest that codon 72 SNP may have an impact upon aging and longevity. Considering the genetic background variations of human populations and environmental factors in epidemiological studies, the precise role of p53 codon 72 SNP in aging and longevity remains elusive.

In this study, we employed a mouse model with knock-in of the human TP53 gene (Hupki) carrying codon 72 SNP to directly investigate the impact of p53 codon 72 SNP upon longevity and its underlying mechanism. The Hupki mice carrying codon 72 SNP

recapture the impacts of codon 72 SNP upon p53 transcriptional activity and function in tumor suppression, which is widely used for studies on p53 and codon 72 SNP (Azzam, Frank, Hollstein, & Murphy, 2011; Z. Feng et al., 2011; Frank et al., 2011; Kung et al., 2016; Leu, Murphy, & George, 2013; Reinbold et al., 2008). We found that despite the increased cancer risk, P72 mice that have escaped tumor development have a longer lifespan than R72 mice and display a delay of age-associated phenotypes compared with R72 mice. Mechanistically, P72 mice have a better ability to retain the self-renewal function of stem/progenitor cells compared with R72 mice during the aging process. Long-term stem cells from aging P72 mice have better engraftment and repopulation abilities than aging R72 mice. In turn, P72 mice have less expansion of long-term stem/progenitor cells than R72 mice during the aging process. Taken together, our study provides direct genetic evidence demonstrating that human p53 codon 72 SNP has a direct impact on aging and longevity in vivo, which supports the role of p53 in longevity.

Results

The lifespans of mice carrying human p53 Codon 72 SNP

To investigate the impact of human p53 codon 72 SNP upon aging and the lifespan, Hupki mice with knock-in of human TP53 gene carrying codon 72 SNP in place of the corresponding mouse Trp53 gene were employed (Frank et al., 2011; Kung et al., 2016; Leu et al., 2013). It has been reported that p53 protein levels in different tissues are comparable between R72 and P72 mice, which was confirmed in this study (Figure 2A)

(Frank et al., 2011; Kung et al., 2016; Leu et al., 2013). Previous studies including ours showed that the P72 allele in these mice has a weaker transcriptional activity towards a subset of p53 target genes than the R72 allele, suggesting that these mice retain the function of p53 codon 72 SNP in human (Azzam et al., 2011; Z. Feng et al., 2011; Kung et al., 2016). Because the lifespan of mice varies among different inbred strains, Hupki mice with p53 codon 72 SNP were backcrossed to mice with different genetic backgrounds, including 129SV^{sl} and C57BL/6J, for ten generations to establish p53 codon 72 SNP Hupki mice in 129SV^{sl} and C57BL/6J backgrounds, respectively. The lifespan of mice with p53 codon 72 SNP in 129SV^{sl} and C57BL/6J backgrounds was measured in a cohort of ~150 mice for each genotype. The median survival age was 740 days in 129SV^{sl} mice and 490 days in C57BL/6J mice, respectively (Figure 2B&C), which is consistent with previously reported lifespans of these two mouse strains (Storer, 1966).

In 129SV^{sl} mice, P72 mice showed an overall longer lifespan compared with R72 mice; the median survival age was 759 days for P72 mice and 697 days for R72 mice, respectively (Log-rank test: $p<0.0001$) (Figure 3A). The causes of death included tumor, inflammation (including dermatitis), ocular lesion, urinary syndrome, nephropathy, etc., which are common causes of death in 129SV^{sl} mice as reported by previous studies (Brayton, Treuting, & Ward, 2012; Marx, Brice, Boston, & Smith, 2013; Radaelli et al., 2016) (Table 1). For those mice died from non-neoplastic events, P72 mice showed a significantly longer lifespan than R72 mice; the median survival was 768 days for P72

mice and 673 days for R72 mice, respectively (Log-rank test: $p<0.0001$) (Figure 3B). For those mice died from neoplastic diseases, R72 mice (with a median survival of 774 days) showed a longer lifespan than P72 mice (with a median survival of 756 days) (Log-rank test: $p=0.015$) (Figure 3C). Further analysis of mice older than 18 months, which are equivalent to humans older than 60 years (Dutta and Sengupta, 2016), showed that P72 mice had a longer lifespan (with a median survival of 780 days) than R72 mice (with a median survival of 715 days) (Log-rank test: $p<0.0001$) (Figure 3D).

Similar results were observed in C57BL/6J mice. P72 mice had an overall longer lifespan (with a median survival of 495.5 days) than R72 mice (with a median survival of 481 days) (Log-rank test: $p=0.015$) (Figure 4A). For those mice died from non-neoplastic events, P72 mice had a significantly longer lifespan (with a median survival of 564.5 days) than R72 mice (with a median survival of 438 days) (Log-rank test: $p<0.0001$) (Figure 4B). For those mice died from neoplastic diseases, R72 mice (with a median survival of 566 days) had a longer lifespan than P72 mice (with a median survival of 411 days) (Log-rank test: $p=0.0084$) (Figure 4C). Further analysis of mice older than 18 months showed that P72 mice had a longer lifespan (with a median survival of 693 days) than R72 mice (with a median survival of 657 days) (Log-rank test: $p<0.0001$) (Figure 4D).

P72 mice showed delayed aging-associated phenotypes

Our results that mice carrying different p53 codon 72 SNP have different lifespans suggest that p53 codon 72 SNP impacts the aging process. Therefore, several

aging-associated phenotypes were examined in R72 and P72 mice at different ages.

During the aging process, mice develop lordokyphosis which is characterized by an increased curvature of the spine (Lopez-Otin et al., 2013). In this study, skeleton structures of 129SV^{sl} mice at different ages were imaged and reconstructed by a micro-CT scan. A narrowing of the spine angle indicates an increase in lordokyphosis. In 6-month-old mice, lordokyphosis was not observed, and there was no significant difference in spinal curves between R72 and P72 mice (Figure 5A&B). In 18-month-old mice, lordokyphosis was observed. Notably, 18-month-old R72 mice developed more pronounced lordokyphosis compared with age-matched P72 mice (Figure 5B). A similar phenotype was observed in C57BL/6J mice (Figure 5G&H).

Another aging-related phenotype in both humans and mice is osteoporosis (Lopez-Otin et al., 2013). The mouse tibias bone structure and density were examined by a micro-CT scan followed by 3D reconstruction. The structure and density of the tibias bone between 6-month-old 129SV^{sl} R72 and P72 mice were morphologically identical, and showed no sign of osteoporosis (Figure 5C). Osteoporosis was observed in both R72 and P72 mice at the age of 18 months. Notably, R72 mice displayed a more obvious sign of osteoporosis than P72 mice (Figure 5C). Analysis of tibias bone structure and density of mice at different ages showed aging-related changes, including decreased bone volume/total volume (BV/TV), decreased the trabecular number and increased trabecular spacing during aging (Figure 5D–F). P72 mice showed delayed development of all these

aging-related changes compared with R72 mice, with the most obvious differences observed at the age of 18 months (Figure 5D–F). Similar results were obtained in C57BL/6J mice (Figure 5I–K).

Decreases in the skin dermal thickness and subcutaneous adipose tissues occur during the aging process (Lopez-Otin et al., 2013). Older 129SV^{sl} mice (12–18 month-old) had a thinner dermal layer and less subcutaneous adipose tissues than young mice (6-month-old) (Figure 6A–C). There was no obvious difference in the dermal thickness and the amount of subcutaneous adipose tissues between young R72 and P72 mice. Notably, in older mice, R72 mice showed more significant decreases in both skin dermal thickness and subcutaneous adipose thickness compared with P72 mice (Figure 6A–C).

One of the hallmarks of aging is the stem cell exhaustion, which leads to the reduced ability of tissue repair (Lopez-Otin et al., 2013). Under stress, such as skin wounds, epidermal stem cells exhibit a highly organized and complex self-renewal process to restore the integrity and function of the skin. This ability dampens down as both humans and mice age (Lopez-Otin et al., 2013). Therefore, the cutaneous repairability of mice was examined by measuring the wound healing process which reflects the function of the skin stem cell (Shaw & Martin, 2009). Three-mm wounds were introduced in the mouse skin by a punch, and the wound diameters were measured daily. Both R72 and P72 129SV^{sl} young mice (6-month-old) showed an efficient wound healing ability (Figure 6D). However, 12- and 18-month-old R72 mice showed a more pronounced decrease in the

wound healing ability than age-matched P72 mice (Figure 6D). Similar results were obtained in C57BL/6J mice (Figure 6E). These results demonstrate that P72 mice exhibited a delayed aging process compared with R72 mice.

The impact of p53 Codon 72 SNP upon hematopoietic stem cell self-renewal abilities during the aging process

Stem cell exhaustion is considered as a hallmark of the aging process (Lopez-Otin et al., 2013). During aging in both humans and mice, the regeneration ability of stem cells gradually diminishes (Lopez-Otin et al., 2013). Ample studies on the stem cell aging process have focused on hematopoietic stem cell (HSC) (Chambers et al., 2007; Seita & Weissman, 2010). Studies using mouse models demonstrated an HSC aging phenotype with the characteristic of the increase of the pool of stem/progenitor cells and the reduction of their self-renewal abilities during the aging process (Chambers et al., 2007; Dumble et al., 2007; Kaiser & Attardi, 2017). p53 has been indicated to play a critical role in regulating the function of stem/progenitor cells (Dumble et al., 2007; Kaiser & Attardi, 2018). Here, we investigated the impact of p53 codon 72 SNP upon HSC pool size and self-renewal function during aging.

To this end, the numbers of long-term-HSCs (LT-HSCs) as well as proliferating HSCs, which represent HSC pool size and self-renewal function, respectively, were measured in R72 and P72 mice at different ages. Bone marrow cells were isolated from mouse hind limb bones and stained with mature hematopoietic lineage markers. The numbers of

LT-HSCs (Lin-/low, Sca1+, c-kit+ and CD34-, Flk2-) were determined by FCM analysis (Figure 7). Consistent with previous reports (Akunuru et al., 2016; Morrison et al., 1996), the percentage of LT-HSCs in bone marrow cells clearly increased during the aging process in both 129SV^{sl} and C57BL/6J mice (Figure 8A–C). R72 mice showed a more rapid increase in the numbers of LT-HSCs than P72 mice during aging. While there was no significant difference in LT-HSC numbers between young 129SV^{sl} R72 and P72 mice, much higher LT-HSC numbers were observed in R72 mice than P72 mice at the age of both 12 and 18 months (Figure 8A&B). Similar results were obtained in C57BL/6J mice (Figure 8C). These results demonstrated that P72 mice showed a delayed HSC expansion during aging.

To determine the population of functional/proliferating HSCs in R72 and P72 mice at different ages, BrdU-labeled proliferating HSCs were quantified by FCM analysis. As shown in Figure 8D&E, the number of proliferating HSCs decreased during aging in both 129SV^{sl} and C57BL/6J mice, which is consistent with previous reports (Dumble et al., 2007; Chambers and Goodell, 2007). Notably, the decrease of proliferating HSC numbers was more rapid in R72 mice than P72 mice during aging. In 129SVsl mice, the percentage of proliferating HSCs in all HSCs in R72 mice decreased from 45% at the age of 6 months to 28% at the age of 22 months, whereas the decrease of proliferating HSCs in P72 mice was less pronounced: from 44% at the age of 6 months to 34% at the age of 22 months (Figure 8D). Similar results were obtained in C57BL/6J mice; the decrease of

proliferation HSCs was more rapid in R72 mice than P72 mice during aging (Figure 8E). To further evaluate the self-renewal and repopulation function of HSCs in mice with p53 codon 72 SNP during aging, the engraftment and repopulation abilities of HSCs of mice were determined by bone marrow transplantation assays. Bone marrow cells isolated from donor CD45.2 mice with different p53 codon 72 SNP at different ages were transplanted into lethally irradiated recipient CD45.1 mice along with CD45.1 bone marrow cells (Figure 9A). The long-term HSC engraftment and repopulation abilities were evaluated by analyzing CD45.1 or CD45.2 cell surface markers of peripheral blood cells at 16 weeks after transplantation. As shown in Figure 6B, while bone marrow cells from 6-month-old R72 and P72 mice showed similar abilities in engraftment and contribution to mature peripheral lymphocytes, bone marrow cells from 18-month-old P72 mice showed a significantly higher engraftment ability than R72 mice. For donors from 129SV^{sl} mice,~76% vs.~68% of lymphocytes were derived from 18-month-old P72 and R72 donors, respectively. Similar results were obtained in C57BL/6J mice;~74% vs. ~56% of lymphocytes were derived from 18-month-old P72 and R72 donors, respectively (Figure 9B). Taken together, these results demonstrated that P72 mice displayed a delayed aging process in HSC number and function compared with R72 mice, which contributes to the delayed aging phenotypes in P72 mice.

In response to stress, p53 transcriptionally regulates a group of target genes that can lead to different cell fates by inducing growth arrest or apoptosis, etc. Here, 129SV^{sl} mice

were employed to examine whether p53 codon 72 SNP differentially regulates the basal expression of its target genes involved in cell cycle arrest (p21) and apoptosis (Puma and Noxa), which in turn impacts the number and function of stem cells. As shown in Figure 9C, p21 mRNA expression levels in the bone marrow were slightly higher in P72 mice compared with R72 mice as determined by real-time PCR assays with this difference being more obvious in older mice than young mice. This difference in p21 expression levels was confirmed at the protein level as determined by Western-blot assays (Figure 9C). In contrast, the bone marrow from P72 mice displayed slightly lower expression levels of Puma and Noxa than that from R72 mice (Figure 9C). These results demonstrate the differential regulation of the basal expression levels of p21, Puma and Noxa by p53 codon 72 SNP in the bone marrow, which may contribute to the delayed aging process in HSC number and function observed in P72 mice.

Discussion

While the role of p53 in assuring longevity through prevention of early cancer development has been well established, its role in regulating aging and longevity aside from cancer prevention has not been well established. Divergent results have been obtained from different mouse models in which the p53 activity was manipulated through different strategies. The increased p53 activity was reported to lead to accelerated aging in some mouse models, but do not affect the lifespan or even prolong the lifespan in other mouse models (Dumble et al., 2007; Garcia-Cao et al., 2002; D. Liu et al., 2010; Maier et

al., 2004; Matheu et al., 2007; Mendrysa et al., 2006; Tyner et al., 2002). These results indicate that p53 can be pro-aging or pro-longevity depending on the context of its regulation and activity. The precise role of p53 in the intrinsic aging process, especially under the physiological condition, remains unclear.

Longevity depends on a balance between tumor suppression and tissue renewal mechanisms (Campisi, 2003a; Lopez-Otin et al., 2013). Declines in stem cells self-renewal and differentiation are critical components of aging(Campisi & Yaswen, 2009). The anti-proliferative function of p53, which is crucial for the suppression of cancer cells, plays a crucial role in eliminating damaged cells including stem cells (Shounan et al., 1996; TeKippe, Harrison, & Chen, 2003). The pleiotropic antagonism theory suggests that certain cellular processes that provide beneficial effects in youth may compromise organismal fitness later in life (Campisi, 2003b). Currently, it is unclear whether p53 has the antagonistic pleiotropy and how the balance of p53 for tumor surveillance and stem cell regulation is regulated.

In humans, functional SNPs have been identified in both p53 and its signaling pathways, such as p53 codon 72 SNP and SNP309 in p53 negative regulator MDM2. These SNPs alter the levels and/or function of p53. Some of these SNPs, including p53 codon 72 SNP, appear to have undergone the natural selection, which suggests that p53 has evolutionarily-conserved functions other than tumor suppression (Atwal et al., 2007; Atwal et al., 2009). It is possible that these SNPs modulate the function of p53 in maintaining

the balance between tumor surveillance and stem cell regulation, which are important genetic modifiers for human longevity. Up till now, majority studies on p53 codon 72 SNP have focused on its impact upon cancer risk. However, there is no consensus in the literature as to the impact of p53 codon 72 SNP upon cancer risk (van Heemst et al., 2005; Whibley et al., 2009). Further, the role of p53 codon 72 SNP in stem cell regulation and the aging process remains unclear. Several epidemiological studies of human populations indicate that p53 codon 72 SNP may influence human longevity. A perspective study with an aging human population (≥85 years, n = 1226) reported that individuals homozygous for the P72 allele have a 41% increased survival (p=0.032) despite a 2.54-fold increased mortality from cancer(van Heemst et al., 2005). Another perspective study of the Danish general population (ages 20–95, n = 9219) reported that the p53 P72 allele is associated with an increased overall survival rate (Bojesen & Nordestgaard, 2008). Similarly, a study of long-lived individuals in Novosibirsk and Tyumen Regions (n = 131) reported the enrichment of the p53 P72 allele in the long-lived group (Smetannikova et al., 2004). These findings suggest that p53 activity is reversely associated with aging, and p53 codon 72 SNP may impact the lifespan in humans.

In this study, we used a genetic approach to investigate whether p53 codon 72 SNP modulates longevity through regulating the balance of p53 functions in tumor surveillance and stem cell regulation by using Hupki mice carrying p53 codon 72 SNP. To exclude the effect of mixed mouse genetic backgrounds on the lifespan and the aging process, mice

carrying p53 codon 72 SNP were backcrossed with 129SV^{sl} mice and C57BL/6J mice, respectively, for 10 generations. As shown in Figure 1B&C and Table 1, the lifespan and causes of death of these two mouse strains are very different. p53 codon 72 SNP showed a clear impact upon the lifespan in both strains of mice; P72 mice have a longer lifespan compared with their R72 littermates, although P72 mice have a higher risk for tumor development. This result is consistent with the observation in human populations showing that P72 carriers have a longer lifespan (Bojesen & Nordestgaard, 2008; Smetannikova et al., 2004; van Heemst et al., 2005). Further, P72 mice display delayed aging-associated phenotypes compared with R72 mice. Results from this study further showed that P72 mice have a better self-renewal ability of stem cells and a delay of compensatory expansion of the stem cell pool compared with R72 mice. Stem cells from older P72 mice have better engraftment and repopulation ability compared with older R72 mice as determined by the bone marrow transplantation assays (Figure 10). It has been suggested that p53 codon 72 SNP can influence the basal expression levels of some p53 target genes in humans, including p21 and PAI-1 (Salvioli et al., 2005; Testa et al., 2009)(. For instance, it was reported that dermal fibroblasts from P72 carriers display a higher expression of p21 (Salvioli et al., 2005). In plasma samples from healthy populations, the P72 allele plays an important role in determining PAI-1 levels in aging populations (Testa et al., 2009). Results from this study showed that the bone marrow from P72 mice displays higher expression levels of p21 but lower expression levels of Puma and Noxa

compared with R72 mice. The differential expression of these target genes may modulate the p53 decision on cell fates towards survival or death, which may contribute to the delayed aging process in HSC function observed in P72 mice. In addition to p53 codon 72, a group of functional SNPs has been identified in the p53 gene and important genes in the p53 pathway, such as MDM2. A very recent study reported that a patient affected by a segmental progeroid syndrome has a germline mutation in the MDM2 gene (Lessel et al., 2017). This mutation abrogates the MDM2 function and leads to increased p53 levels and function, which might be the driving cause for the premature aging phenotype of this patient (Lessel et al., 2017). It will be of interest to study how these SNPs in the p53 pathway (individual or in combination) impact aging and longevity in future studies.

Taken together, results from this study provided the genetic evidence showing that functional p53 codon 72 SNP, which regulates the activity of p53, influences aging and longevity through the regulation of self-renewal function of stem cells. Results from this study strongly support a role of p53 in the regulation of stem/progenitor cell function and longevity.

Author contribution: Dr. Wenwei Hu designed experiments and analyzed the data, Yuhan Zhao, Lihua Wu, Xuetian Yue, Cen Zhang, Jun Li, and Jianming Wang carried out the experiments and analyzed the data, Yuhan Zhao and Dr. Wenwei Hu wrote the manuscript.

Chapter III: Role of Bag2 in mutant p53 gain-of-function

Abstract

Tumor suppressor p53 is the most frequently mutated gene in tumors. Many mutant p53 (mutp53) proteins promote tumorigenesis through the gain-of-function (GOF) mechanism. Mutp53 proteins often accumulate to high levels in tumors, which is critical for mutp53 GOF. Its underlying mechanism is poorly understood. Here, we found that BAG2, a protein of Bcl-2 associated athanogene (BAG) family, promotes mutp53 accumulation and GOF in tumors. Mechanistically, BAG2 binds to mutp53 and translocates to the nucleus to inhibit the MDM2-mutp53 interaction, and MDM2-mediated ubiquitination and degradation of mutp53. Thus, BAG2 promotes mutp53 accumulation and GOF in tumor growth, metastasis, and chemoresistance. BAG2 is frequently overexpressed in tumors. BAG2 overexpression is associated with poor prognosis in patients and mutp53 accumulation in tumors. These findings revealed a novel and important mechanism for mutp53 accumulation and GOF in tumors, and also uncovered an important role of BAG2 in tumorigenesis through promoting mutp53 accumulation and GOF.

Introduction

Tumor suppressor p53 plays a central role in tumor prevention (Levine et al., 2006; Levine & Oren, 2009; Vousden & Prives, 2009). Trp53 is the most frequently mutated gene in human tumors; it is mutated in over 50% of all tumors. Majority of Trp53 mutations are missense mutations that are localized in the p53 DNA binding domain

(DBD), including several mutational hotspots in tumors (e.g., R175, R248, and R273) (Freed-Pastor & Prives, 2012; C. C. Harris & Hollstein, 1993; P. A. Muller & Vousden, 2014). Many tumor-associated mutant p53 (mutp53) proteins not only lose the tumor suppressive function of wild-type p53 (wtp53), but also gain new oncogenic activities independently of wtp53, which is defined as mutp53 gain-of-function (GOF) (Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2014). So far, many mutp53 GOFs have been identified, including promoting tumor growth, metastasis, chemoresistance and metabolic changes (Blandino et al., 2012; Cooks et al., 2013; Freed-Pastor et al., 2012; Lang et al., 2004; P. A. Muller et al., 2009; Olive et al., 2004; Zhang et al., 2013).

Under the non-stressed condition, wtp53 protein levels are kept low in normal cells and tissues mainly through the proteasomal degradation mediated by E3 ubiquitin ligase MDM2, the most critical negative regulator for wtp53 (Brooks & Gu, 2006; Hu et al., 2012). At the same time, as a direct transcriptional target of p53, MDM2 is up-regulated by p53 under both non-stressed and stressed conditions. Thus, p53 and MDM2 form a negative feedback loop to tightly regulate p53 protein levels in cells. However, mutp53 proteins often become stable and accumulate to high levels in tumors, which is critical for mutp53 GOF in tumorigenesis and contributes greatly to tumor progression (Freed-Pastor & Prives, 2012; P. A. Muller & Vousden, 2013; Oren & Rotter, 2010). It had long been thought that the inability of MDM2 to degrade mutp53 was the main cause for mutp53 protein accumulation in tumors. However, recent studies from mice with knock-in of

R172H or R270H mutp53 (equivalent to human R175H and R273H mutp53, respectively) challenged this concept. Mutp53 protein is kept at low levels in normal tissues but accumulates to very high levels in tumors (Lang et al., 2004; Olive et al., 2004). Furthermore, loss of MDM2 in mutp53 knock-in mice leads to mutp53 protein accumulation in normal tissues, which in turn promotes tumor development (Terzian et al., 2008). Recent studies including ours also showed that MDM2 retains the ability to degrade mutp53 in *in vitro* cultured cells (Lukashchuk & Vousden, 2007; Zheng et al., 2013). These results strongly suggest that while MDM2 maintains mutp53 protein levels low in normal tissues, the disruption of MDM2-mediated mutp53 degradation in tumors could be the main cause for the frequently observed mutp53 protein accumulation in tumors. Currently, the mechanism underlying the disruption of MDM2-mediated mutp53 degradation in tumors is poorly understood. Destabilizing mutp53 to inhibit mutp53 GOF is being actively tested as a novel and promising strategy for cancer therapy. Understanding the underlying mechanism for mutp53 accumulation is critical for the development of novel targets and strategies for cancer therapy.

In this study, to investigate the mechanism underlying mutp53 accumulation in tumors, we screened for proteins interacting with mutp53 using liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays in tumors from R172H mutp53 knock-in mice, and identified BAG2 as a novel mutp53 binding protein that plays a critical role in promoting mutp53 accumulation in tumors. BAG2 belongs to the Bcl-2 associated

athanogene (BAG) family, which is characterized by the BAG domain. As a group of multifunctional proteins, BAG proteins interact with a variety of proteins and take part in diverse cellular processes, including cell division, cell death, and differentiation (Kabbage & Dickman, 2008; Takayama & Reed, 2001). Currently, the role of BAG2 in tumorigenesis and its underlying mechanism are poorly understood. We found that mutp53 binds to BAG2 and promotes the nuclear translocation of BAG2. The BAG2-mutp53 interaction in the nucleus inhibits the ubiquitination and degradation of mutp53 mediated by MDM2, and thereby promotes the mutp53 accumulation and mutp53 GOF in tumorigenesis. Knockdown of BAG2 greatly decreases mutp53 protein levels in tumors and compromises mutp53 GOF in tumorigenesis. BAG2 is frequently overexpressed in various types of human tumors. BAG2 overexpression is associated with poor prognosis in cancer patients and mutp53 accumulation in tumors. These results revealed a novel and critical mechanism for mutp53 protein accumulation in tumors, and strongly suggest that BAG2 is a potential target for therapy in tumors carrying mutp53. Our results also uncovered an important role of BAG2 in tumorigenesis and revealed that promoting mutp53 accumulation and GOF is a novel mechanism for BAG2 in tumorigenesis.

Results

BAG2 is a novel mutp53-interacting protein in Trp53$^{R172H/R172H}$ mouse tumors and human cells

R172H mutp53 knock-in (Trp53$^{R172H/R172H}$) mice mainly develop lymphomas in the spleen

and thymus (Lang et al., 2004; Olive et al., 2004). Mutp53 protein levels are drastically increased in the majority of tumors from Trp53$^{R172H/R172H}$ mice but are very low in normal tissues. To investigate the mechanism underlying mutp53 accumulation in tumors, we screened for proteins interacting with mutp53 in thymic lymphomas of Trp53$^{R172H/R172H}$ mice with drastic mutp53 accumulation (n = 3) using immunoprecipitation (IP) assays with an anti-p53 antibody followed by LC-MS/MS assays (Figure 11A). Normal tissues of Trp53$^{R172H/R172H}$ mice with low mutp53 levels were used as controls. LC-MS/MS assays identified a list of potential proteins binding to mutp53 in the thymic lymphomas of Trp53$^{R172H/R172H}$ mice (Figure 11B). Several known mutp53-binding proteins, including HSP90, Myosin, Cct8 and Pontin (Arjonen et al., 2014; P. Muller, Ceskova, & Vojtesek, 2005; Trinidad et al., 2013; Zhao et al., 2015)， were among the list of proteins identified in tumors of Trp53$^{R172H/R172H}$ mice, which validated our approach.

Interestingly, BAG2 was identified as a potential mutp53 binding protein (Figure 11B). The BAG2-mutp53 interaction in Trp53$^{R172H/R172H}$ tumors was confirmed by co-IP followed by Western blot assays (Figure 11C).

To investigate whether BAG2 specifically interacts with mutp53 in human cells, human p53-null lung cancer H1299 cells were transfected with human BAG2-HA expression vectors together with human wtp53 or mutp53 (R175H) expression vectors. Co-IP assays employing either anti-p53 or anti-HA antibodies showed that BAG2 preferentially bound to mutp53 compared with wtp53 (Figure 12A). In addition to R175H, the strong

BAG2-mutp53 interaction was observed in H1299 cells with ectopic expression of different mutp53 proteins, including R248W and R273H, respectively (Figure 12B). The interaction between the endogenous BAG2 and mutp53 proteins was also observed in several human cancer cell lines, including human colorectal cancer HCT116 $p53^{R248W/-}$, HT-29 and SW480 cell lines which contain a single copy of Trp53 gene with R248W and R273H mutation, respectively, human breast cancer SK-BR-3, MDA-MB-468 cell lines which contain a single copy of Trp53 gene with R175H and R273H mutation, respectively, and human hepatocellular carcinoma Huh-7 cell lines which contain a single copy of Trp53 gene with Y220C mutation (Figure 12C, Figure 13A). Together, these results demonstrate that BAG2 is a novel mutp53-specific binding partner, and this interaction is conserved in both mouse tumors and human cancer cells.

DBD of mutp53 and BAG domain of BAG2 are essential for the BAG2-mutp53 interaction

p53 protein contains two transcriptional activation domains (AD1 and AD2), a sequence-specific DBD, a tetramerization domain and a C-terminal domain (C-ter). To define the regions of mutp53 required for the BAG2-mutp53 interaction, expression vectors of fragments containing different mutp53 domains with HA-tag (Figure 12D, upper panel) and BAG2-Flag expression vectors were co-transfected into p53-null H1299 cells. Results of co-IP assays using an anti-Flag antibody showed that BAG2 interacted with all mutp53 (R175H) fragments containing the mutp53 DBD (P1-P5 in Figure 12D), but not

the fragment lacking the mutp53 DBD (P6 in Figure 12D). Furthermore, BAG2 preferentially bound to DBDs of different mutp53 (R175H, R248W, and R273H) but not wtp53 DBD (Figure 12E, 13B).

The regions of BAG2 required for the BAG2-mutp53 interaction was examined by co-transfecting cells with vectors expressing different Flag-tagged BAG2 deletion mutants (Figure 12F, left panel) and mutp53 (R175H) expression vectors followed by co-IP assays. BAG2 contains a BAG domain (amino acids 91–211) at the C-terminus (Dai et al., 2005). The fragments containing the BAG domain interacted with mutp53 while the N-terminus of BAG2 protein lacking the BAG domain did not interact with mutp53 (Figure 12F). Interestingly, the binding of mutp53 to the BAG2 fragment which lacks the N-terminus is much weaker compared with the full length (FL) BAG2 protein. It is possible that the N-terminus of BAG2 has an additional role for efficient BAG2-mutp53 complex formation although itself does not directly interact with mutp53. These results demonstrate that mutp53 DBD and BAG domain of BAG2 are essential for the BAG2-mutp53 interaction.

BAG2 promotes mutp53 protein accumulation in cancer cells

It was reported that BAG2 stabilizes some of its binding proteins, such as PINK1 and ataxin3-80Q (X. Che et al., 2013; X. Q. Che et al., 2015). To investigate whether BAG2 regulates mutp53 protein levels, endogenous BAG2 was knocked down by 2 different siRNA oligos, and its impact upon mutp53 protein levels was evaluated in HCT116

$p53^{R248W/-}$ cells and p53-null Saos2 cells with stable ectopic expression of different mutp53 (Saos2-R175H, Saos2-R248W, and Saos2-R273H). The knockdown of BAG2 was confirmed at both mRNA and protein levels by real-time PCR and Western blot assays, respectively (Figure 14A&B, 13D). While BAG2 knockdown showed no apparent effect on mutp53 mRNA levels, BAG2 knockdown greatly decreased the mutp53 protein levels in cells (Figure 14A). The effect of BAG2 overexpression on mutp53 protein levels was also determined in these cells. Ectopic BAG2 expression by vectors clearly increased mutp53 protein levels (Figure 14C), while had no clear effect on mutp53 mRNA levels in cells (Figure 13C). These results demonstrate that BAG2 increases mutp53 protein levels in cells.

BAG2 inhibits the degradation of mutp53 protein mediated by MDM2

BAG2 is a co-chaperone protein, which can regulate the ubiquitination and degradation of some proteins (X. Che et al., 2013; Dai et al., 2005). Here, we investigated whether BAG2 promotes mutp53 protein accumulation through the inhibition of mutp53 protein ubiquitination and degradation. Since endogenous BAG2 expression levels are relatively higher in Saos2 and HCT116 $p53^{R248W/-}$ cells compared with H1299 cells as determined at the RNA and protein levels (Figure 13E&F), experiments with knockdown of endogenous BAG2 were performed by using Saos2 and HCT116 $p53^{R248W/-}$ cells, and experiments with ectopic BAG2 expression were performed by using H1299 cells. We found that blocking proteasomal degradation by the proteasome inhibitor MG132 largely

abolished the effect of BAG2 knockdown on mutp53 protein levels in HCT116 $p53^{R248W/-}$, Saos2-R175H, Saos2-R248W and Saos2-R273H cells (Figure 14D). Ectopic expression of MDM2 clearly down-regulated mutp53 R175H in H1299 cells co-transfected with vectors expressing mutp53 R175H and MDM2 (Figure 14E), which is consistent with previous reports (Lukashchuk & Vousden, 2007; Zheng et al., 2013). Notably, co-expression of BAG2 largely reduced the degradation of mutp53 protein mediated by MDM2 (Figure 14E). Consistently, knockdown of endogenous MDM2 clearly increased mutp53 protein levels in Saoa2-R175H cells (Figure 14F). Notably, the effect of BAG2 knockdown on mutp53 protein levels was greatly reduced in cells with MDM2 knockdown, indicating that the effect of BAG2 knockdown on mutp53 protein levels is largely mediated by MDM2 (Figure 14F). MDM2 directly binds to mutp53 to negatively regulate mutp53. Co-expression of BAG2 clearly decreased the interaction of MDM2 with mutp53 in H1299 cells, which could be an important mechanism by which BAG2 inhibits MDM2-mediated mutp53 degradation (Figure 14G).

To investigate whether BAG2 regulates mutp53 protein through inhibiting mutp53 ubiquitination, in vivo ubiquitination assays were employed. Ectopic BAG2 expression reduced ubiquitination of mutp53 in H1299 cells (Figure 14H). Knockdown of endogenous BAG2 by siRNA increased ubiquitination of mutp53 in Saos2-R175H cells (Figure 14I). These results demonstrate that BAG2 interacts with mutp53, and inhibits MDM2 binding to and degradation of mutp53, which leads to the mutp53 accumulation in

cells.

Mutp53 promotes the nuclear translocation of BAG2

It has been reported that BAG2 proteins were mainly localized in the cytoplasm (Dai et al., 2005). Indeed, in H1299 cells with ectopic expression of BAG2 alone, BAG2 proteins were predominantly localized in the cytoplasm as determined by immunofluorescence (IF) staining (Figure 15A). Interestingly, we found that mutp53 promoted BAG2 nuclear translocation; ectopic expression of mutp53 (R175H, R248W, and R273H), which is mainly localized in the nucleus, clearly increased the translocation of BAG2 from the cytoplasm to the nucleus in cells transfected with vectors expressing BAG2 together with mutp53. Furthermore, BAG2 was largely co-localized with mutp53 in the nucleus (Figure 15A). In contrast, ectopic expression of wtp53, which is also mainly localized in the nucleus, did not have an obvious effect on BAG2 nuclear translocation in cells (Figure 15A). The effect of mutp53 on BAG2 nuclear translocation was also confirmed by Western blot assays using whole cell lysates and nuclear extracts isolated from H1299 cells transfected with BAG2 vectors alone or together with mutp53 vectors (Figure 15B). Both mutp53 and MDM2 proteins contain a nuclear localization signal (NLS) and are mainly localized in the nucleus, where MDM2 binds to and ubiquitinates mutp53 protein. The translocation of BAG2 to the nucleus where it interacts with mutp53 may play an important role in blocking MDM2 to bind to and degrade mutp53. To test this possibility, we constructed the vector expressing the NLS mutant of mutp53 R175H (mutp53^{NLS}) by

mutating Lys305, Arg306, Lys319, Lys320 and Lys321 to Ala as reported (O'Keefe, Li, & Zhang, 2003). Unlike mutp53 proteins which were mainly localized in the nucleus, mutp53^{NLS} proteins were mainly localized in the cytoplasm as shown by IF staining (Figure 15A). While mutp53^{NLS} readily interacted with BAG2 as determined by co-IP assays (Figure 15C), mutp53^{NLS} could not promote the nuclear translocation of BAG2. BAG2 was mainly localized in the cytoplasm in H1299 co-transfected with vectors expressing BAG2 and mutp53^{NLS} (Figure 15A). Notably, ectopic expression of MDM2 showed a limited effect on the degradation of mutp53^{NLS} protein compared with mutp53 (R175H) (Figure 15D). Furthermore, co-expression of BAG2 had no obvious effect on mutp53^{NLS} protein levels in cells co-transfected with expression vectors of BAG2, mutp53^{NLS}, and MDM2 (Figure 15D). These results strongly suggest that mutp53 promotes BAG2 nuclear localization and the BAG2-mutp53 interaction in the nucleus inhibits MDM2-mediated mutp53 protein degradation.

BAG2 promotes mutp53 GOF in chemoresistance

The accumulation of mutp53 proteins is critical for mutp53 GOF in tumorigenesis (Blandino et al., 2012; P. A. Muller & Vousden, 2014). Chemoresistance is one of the most important mutp53 GOFs (Masciarelli et al., 2014; Napoli, Girardini, & Del Sal, 2012). 5-fluorouracil (5-FU), which can induce apoptosis in cells, is one of the most commonly used chemotherapeutic agents for a wide variety of human cancers. 5-FU induced less apoptosis in Saos2-R175H, Saos2-R248W and Saos2-R273H cells compared with

Saos2-Con cells as determined by Annexin V staining and the levels of cleaved Caspase 3 protein, demonstrating that mutp53 promotes chemoresistance, which is consistent with previous reports (Masciarelli et al., 2014; Napoli et al., 2012) (Figure 16A, B). Notably, knockdown of BAG2 increased 5-FU-induced apoptosis in Saos2-R175H, Saos2-R248W and Saos2-R273H cells but showed a very limited effect in Saos2-Con cells (Figure 16A, B). Consistently, 5-FU induced less apoptosis in HCT116 $p53^{R248W/-}$ cells compared with HCT116 $p53^{-/-}$cells. Knockdown of BAG2 increased 5-FU-induced apoptosis in HCT116 $p53^{R248W/-}$but not HCT116 $p53^{-/-}$ cells (Figure 16C, D). These results demonstrate that BAG2, which promotes mutp53 protein accumulation, promotes mutp53 GOF in chemoresistance.

BAG2 promotes mutp53 GOF in metastasis and tumor growth

A critical GOF of mutp53 is to promote metastasis (Lang et al., 2004; Olive et al., 2004). We found that BAG2 promotes mutp53 GOF in metastasis. Migration is a critical step of metastasis. Compared with p53-null cells (Saos2-Con and HCT116 $p53^{-/-}$ cells), mutp53 (R175H, R248W, and R273H in Saos2 cells and R248W in HCT116 $p53^{R248W/-}$ cells) promoted migration of cells as determined by transwell assays (Figure 17A, B). Notably, knockdown of BAG2 by either siRNA oligos or shRNA vectors largely abolished the promoting effect of mutp53 on migration in these cells (Figure 17A, B). The effect of BAG2 on mutp53 GOF in metastasis was further examined in vivo. HCT116 $p53^{R248W/-}$ and HCT116 $p53^{-/-}$ cells stably transduced with shRNA vectors against BAG2

and control cells transduced with control shRNA vectors were injected into the tail vein of nude mice to evaluate the formation of lung metastatic tumors. Mutp53 (R248W) greatly promoted lung metastatic tumor formation in nude mice; HCT116 $p53^{R248W/-}$ cells formed significantly higher number and larger size of tumors compared with HCT116 $p53^{-/-}$ cells (Figure 17C). Notably, this effect was greatly abolished by knockdown of BAG2 (Figure 17C). These results demonstrate that BAG2 promotes mutp53 GOF in metastasis.

The mutp53 GOFs also include the abilities to promote proliferation of tumor cells and anchorage-independent cell growth (Zhang et al., 2013). As shown in Figure 17D, mutp53 (R248W) promoted proliferation and anchorage-independent growth of HCT116 cells. Notably, knockdown of BAG2 clearly inhibited the rates of cell proliferation and anchorage-independent growth in HCT116 $p53^{R248W/-}$ but not HCT116 $p53^{-/-}$ cells. The xenograft tumorigenesis assays were further performed to investigate whether BAG2 knockdown reduced mutp53 GOF in promoting tumor growth in vivo. As shown in Figure 17E, knockdown of BAG2 in HCT116 $p53^{R248W/-}$ cells significantly inhibited the growth of xenograft tumors, whereas knockdown of BAG2 in HCT116 $p53^{-/-}$ had much less effect on the growth of xenograft tumors. Furthermore, knockdown of endogenous BAG2 clearly decreased mutp53 protein levels in HCT116 $p53^{R248W/-}$ tumors as determined by Western blot assays (Figure 17F), which is consistent with the results obtained from in vitro cultured cells. These results demonstrate that BAG2 promotes mutp53 GOFs in tumor cell growth.

BAG2 is overexpressed in human tumors, and high levels of BAG2 are associated with poor prognosis in cancer patients and mutp53 protein accumulation in human tumors

Results from our study have demonstrated that BAG2 interacts with mutp53 and inhibits mutp53 degradation, which in turn promotes mutp53 protein accumulation and enhances mutp53 GOF in tumorigenesis. BAG2 expression was found elevated in many types of human tumors, including colorectal cancers, lung cancers, breast cancers and sarcomas, compared with normal tissues as analyzed in 4 databases from Oncomine (GSE20842, (Gaedcke et al., 2010); GSE10072, (Landi et al., 2008); GSE3744, (Richardson et al., 2006); GSE21122, (Barretina et al., 2010)) (Figure 18A). The amplification of BAG2 was observed in many types of human tumors as analyzed by employing the cBioportal for Cancer Genomics (Figure 19), suggesting that gene amplification is an important mechanism for BAG2 overexpression in tumors. We further investigated whether BAG2 overexpression is associated with poor prognosis in cancer patients by using the PrognoScan database. PrognoScan, which has a large collection of the publicly available database with microarray data and clinical information, can assess the prognostic power of gene expression levels (Mizuno et al., 2009). As shown in Figure 18B–E, BAG2 overexpression is associated with poor disease-free survival in colorectal cancer patients (HR = 1.40, p = 0.022), poor disease-specific survival in lung cancer patients (HR = 2.4, p = 0.00001), poor relapse-free survival in breast cancer patients (HR = 1.3, p = 0.00014)

and poor distant recurrence-free survival in soft tissue cancer patients (HR = 1.67, p = 0.00001). These results suggest the significant prognostic value of BAG2 expression levels for patients with various types of cancer.

The correlation between BAG2 overexpression and mutp53 accumulation was further investigated in a cohort of human colorectal cancer samples with known p53 mutation status and p53 protein levels (n = 100) (Zheng et al., 2013). p53 mutation status was determined by direct sequencing of exons 2–11 of p53 and the p53 protein levels were determined by IHC staining as previously described (Zheng et al., 2013). All tumors carrying mutp53 and a small percentage of tumors with wtp53 showed positive staining for p53 (>10% cells are stained). Tumors were divided into 2 groups according to median BAG2 expression levels as determined by TaqMan real-time PCR assays. There is a clear correlation between high BAG2 expression and mutp53 accumulation (Figure 18F). In tumors with mutp53, 66.7% of tumors (18 out of 27) with high BAG2 expression displayed high p53 staining (>30% cells are stained) while only 40.9% tumors (9 out of 22) with low BAG2 expression had high p53 staining (p = 0.035). In contrast, in tumors with wtp53, there is no correlation between BAG2 expression and p53 accumulation. Among these tumors, 21.7% of tumors (5 out of 23) with high BAG2 expression and 17.9% of tumors (5 out of 28) with low BAG2 expression displayed low p53 staining (10–30% cells are stained), respectively (p = 0.36) (Figure 18F). These results demonstrate that BAG2 overexpression is significantly correlated with accumulation of mutp53 protein in

colorectal cancers.

Discussion

Many tumor-associated mutp53 proteins gain new oncogenic activities independently of wtp53, which is critical for mutp53 to promote tumorigenesis. While wtp53 proteins are kept at low levels in normal tissues under normal conditions, mutp53 proteins often accumulate to high levels in tumors, which is critical for mutp53 GOF in tumorigenesis (J. Liu et al., 2015; P. A. Muller & Vousden, 2013; Oren & Rotter, 2010; Terzian et al., 2008). Currently, the mechanism for mutp53 accumulation in tumors is poorly understood. Results from mouse genetic experiments with knockout of MDM2 in mutp53 mice and cell-based in vitro experiments suggest that MDM2 maintains mutp53 protein levels low in normal tissues, whereas some changes occurred in tumors disrupt MDM2-mediated mutp53 degradation, thereby leading to mutp53 accumulation. Recent studies have reported that HSP90 can bind to mutp53 and reduce mutp53 degradation mediated by MDM2. Knockdown of HSP90 by siRNA or blocking HDAC6-HSP90 axis by SAHA induced destabilization of mutp53 and inhibited its GOF in tumorigenesis (D. Li, Marchenko, & Moll, 2011; D. Li, Marchenko, Schulz, et al., 2011). Our recent study showed that tumor-derived MDM2 short isoforms inhibited full-length MDM2-mediated mutp53 degradation, which promoted the mutp53 accumulation and enhanced GOF in tumorigenesis (Zheng et al., 2013). In this study, we searched for the changes that occurred in tumors to disrupt MDM2-mediated mutp53 degradation by screening for

mutp53 binding protein in tumors from mutp53 knock-in mice. Results from this study identified that BAG2 is a novel mutp53 binding protein that promotes mutp53 protein accumulation, which revealed a novel mechanism for mutp53 accumulation in tumor cells.

BAG2 belongs to the BAG family, which is characterized by the BAG domain. The BAG domain is a conserved region located at the C-terminus of the BAG-family proteins that bind to the ATPase domain of Hsc70 and has general nucleotide exchange activities towards Hsc70 (Kabbage & Dickman, 2008; Takayama & Reed, 2001). Therefore, proteins containing the BAG domain often functions as a co-chaperone protein. Aside from the formation of the BAG-Hsc70 interaction, BAG proteins functionally interact with many proteins to regulate cellular functions. The expression of BAG2 has been detected in many tissues. Recently, it was reported that BAG2 interacts with and stabilizes PINK1 and ataxin3-80Q, proteins involved in neurological diseases, through inhibiting their ubiquitination and degradation (X. Che et al., 2013; X. Q. Che et al., 2015). BAG2 can also deliver Tau to the proteasome for protein degradation independently of ubiquitination (Carrettiero, Hernandez, Neveu, Papagiannakopoulos, & Kosik, 2009). In this study, we found that BAG2 preferentially binds to mutp53 at the DBD domain. BAG2 can interact with many different mutp53, including several tumor-associated mutational hotspots. This BAG2-mutp53 interaction is conserved in both human tumor cells and mouse tissues. It is unclear how BAG2 can discriminate between the DBD of a diverse range of mutp53

proteins and wtp53. BAG2 is a co-chaperone protein. It is possible that conformational changes of mutp53 proteins lead to its association with a chaperone and co-chaperone proteins. It remains unclear whether BAG2 interacts with mutp53 directly or interacts with mutp53 through other protein, such as Hsc70, which will be of interest to investigate in future studies.

The role of BAG2 in the tumor is poorly understood. In this study, we found that mutp53 interacted with BAG2 and promoted the translocation of BAG2 from the cytoplasm to the nucleus, where BAG2 inhibited the binding of MDM2 to mutp53 and the ubiquitination and degradation of mutp53 protein mediated by MDM2. It is unclear how the BAG2-mutp53 interaction interferes with the MDM2-mutp53 interaction since MDM2 binds to the N-terminus of mutp53 whereas BAG2 binds to the DBD of mutp53. Future studies are needed to further understand its mechanism. Results from this study showed that BAG2 promoted mutp53 protein accumulation in tumor cells, which in turn promoted mutp53 GOF in tumorigenesis (Figure 18G). Knockdown of endogenous BAG2 significantly inhibited cell proliferation, migration, metastasis and chemoresistance of tumor cells in a largely mutp53-dependent manner in cultured cells and/or in mice. These results strongly suggest that targeting BAG2 could be developed as a novel strategy to destabilize mutp53 and inhibit its GOF in tumorigenesis.

Importantly, analysis of several databases from Oncomine showed that BAG2 is frequently overexpressed in many types of cancer (Figure 18A). Overexpression of BAG2

is significantly associated with poor prognosis in different types of cancer (Figure 18B–E). Furthermore, our results showed that BAG2 overexpression in colorectal tumors is significantly associated with mutp53 protein accumulation (Figure 18F). These results strongly suggest that BAG2 plays an important role in tumorigenesis and promoting mutp53 accumulation and GOF is a novel mechanism for BAG2 in tumorigenesis. It is unclear why not all tumors with BAG2 overexpression showed the accumulation of mutp53 protein. It is possible that additional mechanisms are involved in the regulation of mutp53 protein levels and/or the BAG2-mutp53 interaction. It will be interesting to examine whether BAG2 displays weak or no interaction with mutp53 protein in this subgroup of tumor samples in future studies. It is also worth noting that while normal tissues from Trp53$^{R172H/R172H}$ mice express a lot of BAG2, there is a limited amount of interacted BAG2-mutp53 protein complex and no clear accumulation of mutp53 proteins in the normal tissues (Figure 11C). These results suggest that some tumor-specific events might contribute to the effect of BAG2 on mutp53 accumulation.

Data from cBioportal showed amplification of the *BAG2* gene in many types of human tumors, suggesting that gene amplification is an important mechanism for BAG2 overexpression in human tumors. Considering that less than 10% of tumors had the amplification of BAG2 in the majority of tumor types, it is possible that additional mechanisms contribute to BAG2 overexpression in tumors, which needs further investigation in future studies.

Taken together, results from this study demonstrate that BAG2 interacts with mutp53 to prevent its degradation by MDM2, leading to mutp53 accumulation in tumor cells and enhanced mutp53 GOF in tumorigenesis. Knockdown of BAG2 greatly reduces mutp53 protein levels in tumor cells and greatly compromises mutp53 GOF in tumorigenesis, including tumor growth, metastasis, and chemoresistance. Considering that BAG2 is frequently overexpressed in cancer cells, our findings revealed a new and important mechanism for mutp53 protein accumulation in tumors. Trp53 is the most frequently mutated gene in tumors. Mutp53 protein is frequently accumulated in tumors, which is critical for mutp53 GOF in tumor development. Therefore, mutp53 has become an extremely attractive target for tumor therapy. Our findings that BAG2 promotes mutp53 protein accumulation and mutp53 GOF in tumorigenesis strongly suggest that BAG2 could be a potential target for cancer therapy in tumors containing mutp53.

Author contribution: Dr. Wenwei Hu designed experiments and analyzed the data, Xuetian Yue, Yuhan Zhao, Juan Liu, Cen Zhang, Haiyang Yu, Jiabei Wang, Tongsen Zheng, Jun Li carried out the experiments and analyzed the data, Xuetian Yue, Yuhan Zhao, and Wenwei Hu wrote the manuscript.

Chapter IV: The role of Pontin in gain-of-function of mutant p53

Abstract

Tumor-suppressor p53 is frequently mutated in human cancers. Many tumor-associated mutant p53 (mutp53) proteins gain new functions in promoting tumorigenesis, defined as gain-of-function (GOF). The mechanisms for mutp53 GOF are not well understood. Here, we report Pontin, a highly conserved AAA+ ATPase important for various cellular functions, as a new mutp53-binding protein. This Pontin–mutp53 interaction promotes mutp53 GOF in invasion, migration and anchorage-independent growth of tumor cells. The ATPase domain of Pontin is crucial for its promoting effect on mutp53 GOF; blocking the ATPase activity of Pontin by a Pontin-specific ATPase inhibitor or an ATPase-deficient dominant-negative Pontin expression vector greatly diminished mutp53 GOF. Pontin promotes mutp53 GOF through regulation of mutp53 transcriptional activity; knockdown of Pontin abolished the transcriptional regulation of mutp53 toward a group of genes. Furthermore, overexpression of Pontin in tumors is associated with the poor survival of cancer patients, especially those containing mutp53. Our results highlight an important role and mechanism for Pontin, a new mutp53 partner, in promoting mutp53 GOF in tumorigenesis.

Introduction

Tumor-suppressor p53 has a crucial role in tumor prevention (Levine & Oren, 2009). The p53 function is compromised in many human tumors, mostly due to p53 mutation (C. C. Harris & Hollstein, 1993). The majority of tumor-associated p53 mutations are point

mutations in p53 DNA-binding domain. Major categories of p53 mutations include DNA contact mutations (including R248Q and R273H) and conformational mutations (including R175H) (P. A. Muller & Vousden, 2013). Many tumor-associated mutant p53 (mutp53) proteins gain new properties to promote tumorigenesis in addition to the loss of tumor-suppressive activity of wild-type p53 (wtp53), which is defined as mutp53 gain-of-function (GOF). The mutp53 GOF includes the abilities to promote proliferation, metastasis, cell transformation and metabolic changes (Blandino et al., 2012; Freed-Pastor & Prives, 2012; Lang et al., 2004; P. A. Muller & Vousden, 2013; Olive et al., 2004). Mutp53 proteins often accumulate to high levels in tumors but not normal tissues, which is critical for mutp53 GOF in tumorigenesis (Lang et al., 2004; Olive et al., 2004). It has been suggested that chaperone protein Hsp90, which is frequently overexpressed in tumors, interacts with mutp53 to prevent its degradation, promoting mutp53 accumulation in tumors (P. Muller et al., 2008). Recently, we found that tumor-associated MDM2 isoforms inhibit MDM2-mediated mutp53 degradation and promote mutp53 accumulation in tumors (Zheng et al., 2013).

Although the concept of mutp53 GOF is well established, the underlying mechanisms for mutp53 GOF are not well understood. Mechanisms being proposed include protein interaction and the regulation of gene expression and microRNA expression(Freed-Pastor & Prives, 2012). In particular, the interaction of mutp53 with proteins to regulate their functions has been suggested as an important mechanism. For

example, mutp53 interacts with transcriptional factors p63, p73, SREBP and VDR to modulate their transcriptional activities (Freed-Pastor et al., 2012; Gaiddon et al., 2001; Stambolsky et al., 2010). Mutp53 forms protein complex with MRE11, and perturbs its function in DNA damage response (Song et al., 2007). Furthermore, the mutp53 function can be regulated by its interacting proteins. For example, PML interacts with mutp53 and enhances mutp53 transcriptional activity (S. Haupt et al., 2009). Pin1, which regulates protein conformation, binds to mutp53 and enhances mutp53 GOF (Girardini et al., 2011). Pontin is a highly conserved AAA+ ATPase, which is associated with several protein complexes and involved in multiple biological processes, including chromatin remodeling, transcriptional regulation, cell replication and telomerase activities (Jha, Shibata, & Dutta, 2008; Jin et al., 2005; Matias, Gorynia, Donner, & Carrondo, 2006; Venteicher, Meng, Mason, Veenstra, & Artandi, 2008). Some Pontin-binding proteins, including Tip60, c-Myc and β-catenin, have known functions in cancer (Y. Feng, Lee, & Fearon, 2003; Jha et al., 2008; Wood, McMahon, & Cole, 2000). Pontin is frequently overexpressed in many human cancers, suggesting the potential role of Pontin in tumorigenesis (Dehan et al., 2007; Lacombe et al., 2013; Lauscher et al., 2012). In this study, we identified Pontin as a novel mutp53-binding protein, which enhances mutp53 GOF in tumor cells. Results from this study showed an important role of Pontin in promoting mutp53 GOF and revealed an unidentified mechanism for mutp53 GOF in tumorigenesis.

Results

Pontin is a novel mutp53-interacting protein

Recently, Pontin was reported to bind to the p53 promoter and downregulate wtp53 levels and function in human colorectal cancer RKO cells (Taniue, Oda, Hayashi, Okuno, & Akiyama, 2011). This finding raised an interesting question about whether Pontin regulates mutp53 accumulation and GOF. We first confirmed that Pontin downregulates wtp53 levels in RKO cells; knockdown of Pontin increased both the mRNA and protein levels of wtp53 (Figure 20). However, this effect of Pontin on wtp53 appeared to be cell type specific because Pontin did not affect wtp53 at either mRNA or protein level in human colorectal cancer HCT116 $p53^{+/+}$ cells (Figure 20). Further, Pontin did not regulate mutp53 levels in cells. In human breast cancer SK-BR-3 and colorectal cancer HCT116 $p53^{R248W/-}$ cells, which contain a single copy of p53 gene with tumor hotspot mutations R175H and R248W, respectively, and human p53-null lung cancer H1299 cells with stable ectopic expression of mutp53 (R175H), knockdown of Pontin did not clearly affect mutp53 mRNA and protein levels (Figure 20).

Interestingly, we found that Pontin interacts with mutp53 protein. In H1299 cells transfected with human Pontin-Flag expression vectors together with wtp53 or mutp53 (R175H) expression vectors, Pontin preferentially bound to mutp53 compared with wtp53 as determined by co-immunoprecipitation (co-IP) assays (Figure 21A). The Pontin–mutp53 interaction was also observed in H1299 cells with ectopic expression of different

hotspot mutp53 proteins, including R248Q and R273H, in addition to R175H (Figure 21B). The endogenous Pontin–mutp53 interaction was also observed in SK-BR-3 cells (Figure 21C).

To determine the regions of mutp53 required for the Pontin–mutp53 interaction, expression vectors of different mutp53 domains (Figure 21D, left panel) were co-transfected with Pontin-Flag into H1299 cells. Expression vectors of wtp53 DNA-binding domain (DBD) were included as a control. Pontin interacted with all mutp53 fragments containing DBD, but not with mutp53 fragment lacking DBD or wtp53 DBD (Figure 21D). These results show that Pontin interacts with mutp53 through mutp53 DBD domain.

To investigate whether Pontin interacts with mouse mutp53, $p53^{R172H/R172H}$ mice with knock-in of mutp53 R172H (equivalent to human mutp53 R175H) and $p53^{-/-}$ mice, which mainly develop thymic lymphomas, were used. Thymic lymphomas and normal thymus from $p53^{R172H/R172H}$ mice, and thymic lymphomas from $p53^{-/-}$ mice were subjected to co-IP assays using an anti-p53 antibody. Consistent with previous reports (Lang et al., 2004; Olive et al., 2004), mutp53 protein accumulation was observed in thymic lymphomas but not normal tissues from $p53^{R172H/R172H}$ mice. The Pontin–mutp53 interaction was observed in thymic lymphomas from $p53^{R172H/R172H}$ mice but not normal thymus from $p53^{R172H/R172H}$ mice or thymic lymphomas from $p53^{-/-}$ mice (Figure 21E). Together, these results show that Pontin is a novel mutp53-binding protein, and this interaction is conserved in both

human tumor cells and mouse tissues.

Loss of the Pontin–mutp53 complex compromises mutp53 GOF

To investigate whether the Pontin–mutp53 interaction contributes to mutp53 GOF, endogenous Pontin was knocked down, and its impact upon mutp53 GOF was evaluated in p53-null H1299 and human osteosarcoma Saos2 cells with stable ectopic expression of mutp53 R175H (H1299-R175H and Saos2-R175H), respectively. Promoting tumor cell metastasis is an important mutp53 GOF (Lang et al., 2004; Olive et al., 2004). Migration and invasion are critical steps for tumor metastasis (Hanahan & Weinberg, 2011). As shown in Figures 22A&B and Figures 23A&B, H1299-R175H, and Saos2-R175H cells displayed increased migration and invasion abilities compared with H1299-Con and Saos2-Con cells transfected with control vectors as determined by transwell assays. Notably, knockdown of Pontin by siRNA decreased the migration and invasion abilities of H1299-R175H and Saos2-R175H but not H1299-Con and Saos2-Con cells (Figures 22A&B and Figures 23A&B). Similarly, in SK-BR-3 cells containing endogenous mutp53 R175H, Pontin knockdown significantly decreased the migration and invasion abilities of SK-BR-3 but not SK-BR-3-p53sh cells with stable mutp53 knockdown (Figures 22C&D). The Pontin knockdown was confirmed at both mRNA and protein levels by TaqMan real-time PCR and western blot assays, respectively (Figures 23C, D&E). Similar results were obtained by using two different siRNA oligos against Pontin. In addition to mutp53 R175H, Pontin knockdown decreased the migration ability of H1299 cells with stable

ectopic expression of mutp53 R248Q and R273H, respectively (Figure 23F). This effect of Pontin knockdown on mutp53 GOF in migration and invasion was not because of the changes in cell cycle distribution, cell viability or apoptosis as determined in H1299-Con and H1299-R175H cells with or without Pontin knockdown (Figure 24). The effect of Pontin knockdown on mutp53 GOF in migration was further confirmed by scratch motility assays. Motility of H1299-R175H cells was faster than that of H1299-Con cells (Figure 22E). Notably, Pontin knockdown significantly reduced the motility of H1299-R175H but not H1299-Con cells (Figure 22E). The effect of Pontin on mutp53 GOF in metastasis was further investigated in vivo. H1299-Con and H1299-R175H cells with or without knockdown of Pontin were injected into nude mice via the tail vein, and lung metastasis was analyzed. As shown in Figure 22F, mutp53-promoted tumor lung metastasis; H1299-R175H cells formed a significantly higher number of lung metastatic tumors compared with H1299-Con cells. Notably, Pontin knockdown greatly reduced lung tumor formation by H1299-R175H cells, and this effect was largely reduced in H1299-Con cells. Mutp53 promotes the anchorage-independent growth of tumor cells (Zhang et al., 2013). H1299-R175H, H1299-R248Q, and H1299-R273H cells displayed increased anchorage-independent cell growth on soft agar compared with H1299-Con cells (Figure 2g). Notably, Pontin knockdown by shRNA significantly reduced the anchorage-independent growth of H1299-R175H, H1299-R248Q, and H1299-R273H but not H1299-Con cells (Figure 22G). Together, these results show that Pontin knockdown

decreases mutp53 GOF in migration, invasion, metastasis and anchorage-independent cell growth of tumor cells.

The C-terminus of Pontin is required for the Pontin–mutp53 interaction and mutp53 GOF

Pontin has the following three distinct domains. Domain I (1–120 aa and 296–365 aa) contains Walker A and Walker B motifs that are responsible for binding and hydrolysis of ATP, respectively. Domain II (121–295 aa) is important for DNA/RNA binding. Domain III (368–456 aa) contains 4 α-helices and is important for ATP binding (Tucker & Sallai, 2007). To determine the regions of Pontin required for the Pontin–mutp53 interaction, expression vectors of different domains of Pontin (Figure 25A) were co-transfected with mutp53 (R175H) expression vectors into H1299 cells for co-IP assays. As shown in Figure 25B, mutp53 interacted with the Pontin fragments containing the C-terminus, including aa 292–456 and Δaa 125–291, which both contain Walker B motif in the C-terminus of domain I and domain III. Mutp53 did not interact with either the N terminus of Pontin (aa 1–124), which contains Walker A motif in domain I, or the Pontin domain II (aa 191–295). These results show that Pontin interacts with mutp53 through its C-terminus.

To determine the regions of Pontin protein required for mutp53 GOF, the effects of different Pontin fragments on mutp53 GOF in migration and invasion were examined. H1299-Con and H1299-R175H cells were first transfected with siRNA to knockdown

endogenous Pontin, and then transfected with the vectors expressing different Pontin fragments or the full-length siRNA-resistant Pontin (FL-Pontin-R) that are all resistant to Pontin siRNAs. Knockdown of endogenous Pontin significantly inhibited migration and invasion of H1299-R175H but not H1299-Con cells, which was fully reversed by the expression of FL-Pontin-R (Figures 25C&D). Interestingly, expression of the Pontin fragments containing the C-terminus (aa 292–456 and Δaa 125–291, respectively), which are the fragments that interact with mutp53, largely reversed the inhibitory effect of Pontin knockdown on mutp53 GOF in migration and invasion (Figures 25C&D). In contrast, expression of the Pontin fragments aa 1–124 and aa 121–295, which do not interact with mutp53, had no obvious effect on migration and invasion of H1299-R175H cells with knockdown of endogenous Pontin. These results suggest that the C-terminus of Pontin is required for Pontin's function in promoting mutp53 GOF.

The ATPase activity in Walker B motif is required for Pontin to promote mutp53 GOF

As both Pontin fragments (aa 292–456 and Δaa 125–291) that interact with mutp53 and promote mutp53 GOF contain the conserved Walker B motif, which is responsible for the ATPase activity, we investigated whether the ATPase activity is required for Pontin to promote mutp53 GOF. A single missense mutation in aa 302 in the Walker B motif (DEVH to NEVH) was reported to create an ATPase-deficient dominant-negative mutant Pontin-D302N (Mezard, Davies, Stasiak, & West, 1997). Pontin-D302N expression

vectors were co-transfected with mutp53-R175H into H1299 cells. As shown in Figure 26A, Pontin-D302N interacted with mutp53 to a similar degree compared with wild-type Pontin. Interestingly, Pontin-D302N greatly decreased the migration and invasion abilities of H1299-R175H but not H1299-Con cells, which is similar to the effect of Pontin knockdown (Figures 26B&C). Consistently, blocking Pontin's ATPase activity by Rottlerin, a Pontin-specific ATPase inhibitor (Elkaim et al., 2012), significantly decreased the migration ability of H1299-R175H but not H1299-Con cells (Figure 26D). To confirm that the inhibitory effect of Rottlerin on the migration ability of mutp53 was due to its inhibition of Pontin's ATPase activity but not nonspecific effects of Rottlerin, cells were simultaneously treated with Rottlerin and transfected with Pontin siRNA. Rottlerin treatment or Pontin knockdown decreased the migration ability of H1299-R175H cells to a similar extent (Figure 26E). Notably, combined treatments of Rottlerin and Pontin knockdown did not exhibit an additive or synergistic effect in H1299-R175H cells (Figure 26E). Furthermore, blocking Pontin's ATPase activity by Pontin-D302N significantly reduced the anchorage-independent cell growth in H1299-R175H but not H1299-Con cells (Figure 26F). These results show that the ATPase activity of the Walker B motif is important for Pontin's function in promoting mutp53 GOF.

Pontin promotes mutp53 GOF through regulation of mutp53 transcriptional activity

Pontin is associated with several chromatin-remodeling complexes, including Ino80, TIP60/NuA4, and SWR1 complexes, and has an important role in chromatin-remodeling

and transcriptional regulation (Jha et al., 2008; Jin et al., 2005). Mutp53 can transcriptionally regulate a group of genes involved in tumorigenesis (Brosh & Rotter, 2009). Here, we investigated whether Pontin promotes mutp53 GOF through regulating mutp53 transcriptional activity. First, immunofluorescence (IF) staining assays in H1299-R175H and SK-BR-3 cells showed that both Pontin and mutp53 proteins were predominantly localized in the nucleus. Furthermore, Pontin was largely colocalized with mutp53 in the nucleus (Figure 27A). The transcriptional regulation ability of mutp53 was then determined in SK-BR-3-Consh cells transduced with control shRNA and SK-BR-3-p53sh cells transfected with control siRNA or Pontin siRNA by Affymetrix microarray GeneChip analysis. The expression data were analyzed using the Affymetrix Microarray Suite version 5.0 (MAS 5.0, Santa Clara, CA, USA). Change of expression levels for each gene by mutp53 was calculated as expression levels in SK-BR-3-Consh compared with SK-BR-3-p53sh cells. As shown in Figure 27B, the expression levels of a group of genes were significantly altered ($P<0.01$) following mutp53 knockdown in SK-BR-3 cells; knockdown of mutp53 resulted in decreased expression (>2-fold) of 67 genes and increased expression (>2-fold) of 31 genes. Notably, Pontin knockdown reduced the transcriptional regulation of mutp53 toward a group of genes (Figure 27B). These genes that require Pontin for their transcriptional regulation by mutp53 (n=40 for genes upregulated by mutp53; n=12 for genes downregulated by mutp53) were involved in many important signaling pathways, including gap junction signaling, IGF-1 signaling,

EGF signaling, etc., as analyzed by ingenuity pathway analysis (IPA; Figure 27C). The dysregulation of these pathways has an important role in tumorigenesis and metastasis (Cronier, Crespin, Strale, Defamie, & Mesnil, 2009; Z. Feng & Levine, 2010; Tomas, Futter, & Eden, 2014). The requirement of Pontin in mutp53 function in transcriptional regulation was confirmed in a group of represented genes, including ADRB1, receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIPK1), inositol 1,4,5-triphosphate receptor, type I (ITPR1), P2RX5, stathmin 1 (STMN1) and SH3BGR by real-time PCR (Figure 27D). The gene products of ADRB1, RIPK1 belong to the gap junction signaling, which is important for cell motility (Bist et al., 2011; Guo et al., 2009; Xia et al., 2009). STMN1, which has a role in cell migration, was reported to be upregulated by mutp53 (Masciarelli et al., 2014). It is possible that some of these genes mediate the promoting effect of Pontin on mutp53 GOF. Mutp53 often transcriptionally regulates genes through interacting with other transcription factors, including SREBP, NF-Y, VDR, NF-κB, and binding to the binding sites of their regulated genes (Cooks et al., 2013; Di Agostino et al., 2006; Freed-Pastor & Prives, 2012; Stambolsky et al., 2010). Using the Champion ChiP Transcription Factor Search (SABiosciences, Valencia, CA, USA) to analyze the promoter and regulatory region of genes mentioned above, we identified an SREBP-binding site in the intron 1 of the STMN1 gene, and NF-κB-binding sites in the intron 1 of the RIPK1 and ITPR1 genes. Using chromatin IP (ChIP) assays, significant binding of mutp53 and Pontin was observed in the vicinity of the SREBP-binding site of

the STMN1 gene and the NF-κB-binding sites of the RIPK1 and ITPR1 genes in SK-BR-3 cells (Figure 27E). Pontin knockdown resulted in the loss of Pontin interaction with these regions, and largely reduced mutp53 interaction with these regions (Figure 27E). STMN1 was reported to promote cell migration (Byrne et al., 2014). We found that STMN1 partially mediates mutp53 GOF in migration in a Pontin-dependent manner (Figure 27F). Knockdown of STMN1 by siRNA reduced the migration ability of H1299-R175H but not H1299-Con cells (Figure 27F). Notably, the inhibitory effect of STMN1 knockdown on mutp53 GOF in migration was largely disappeared in H1299-R175H cells with knockdown of Pontin (Figure 27F). These results suggest that the Pontin–mutp53 interaction regulates mutp53 transcriptional activity, which in turn promotes mutp53 GOF.

High Pontin expression in tumors correlates with poor prognosis, especially for tumors carrying mutp53

Pontin is frequently overexpressed in many human cancers, including breast, lung and colorectal cancers (Lacombe et al., 2013; Lauscher et al., 2012). Here, we investigated the prognostic value of Pontin by using the KM Plotter, an online tool that incorporates microarray data with overall survival information of 1926 lung and 1115 breast cancer patients (Gyorffy et al., 2010). Patients were divided into two groups by auto-selection of the best cutoff. There was a significant association of high Pontin mRNA expression levels with poor prognosis in both lungs (HR=1.62, P=8.3e-14) and breast (HR=1.61, P=0.00066) cancer patients (Figures 28A&B), which suggests the significant prognostic

value of Pontin expression levels for lung and breast cancer patients. Pontin promotes mutp53 transcriptional activity toward a group of genes, including STMN1 and RIPK1. Notably, the prognostic value of Pontin together with STMN1 and RIPK1 was stronger than that of Pontin alone (Figures 28C&D). These results suggest that regulation of mutp53 transcriptional activity is an important mechanism by which Pontin promotes tumor progression.

To further investigate whether the promoting effect of Pontin on mutp53 GOF contributes to the poor survival associated with high Pontin expression levels, a cohort of 236 breast cancer patients (GSE3494) from the public available database with known p53 mutation status and survival information was used (Miller et al., 2005). Consistent with the results obtained from the KM plotter, there was a significant association of high Pontin mRNA levels with poor prognosis in these patients (n=234, HR=1.69, p=0.047; Figure 28E, left panel). The prognostic value of Pontin mRNA levels was further compared between patients carrying wtp53 or mutp53 in tumors. Although Pontin levels showed no significant effect on prognosis in patients with wtp53 (n=181, HR=1.27, P=0.35), high Pontin mRNA levels were significantly associated with poor prognosis in patients with mutp53 (n=53, HR=2.43, p=0.034; Figure 28E, middle and right panels). These results show that high Pontin expression levels are associated with poor prognosis, especially for tumors carrying mutp53.

Discussion

Ample evidence has demonstrated that mutp53 can promote tumorigenesis through GOF mechanism (Freed-Pastor & Prives, 2012). However, its underlying mechanisms are not well understood. In this study, Pontin was identified as a new mutp53-binding protein. Pontin preferentially binds to mutp53, through its C-terminus. Pontin is an essential component of several protein complexes that regulate transcription, telomerase activities, chromatin remodeling, metabolism and DNA repair (Baek, 2008; Gallant, 2007; Gospodinov, Tsaneva, & Anachkova, 2009). Pontin is frequently overexpressed in tumors. Some of the Pontin-interacting proteins, including c-Myc and β-catenin, have pivotal roles in tumorigenesis, suggesting an important role of Pontin in tumorigenesis (Y. Feng et al., 2003; Wood et al., 2000). However, the mechanism of Pontin in tumorigenesis is poorly understood. This study shows that Pontin promotes mutp53 GOF in tumorigenesis, including increased migration, invasion, metastasis and anchorage-independent cell growth. Our results established an important role of Pontin in promoting mutp53 GOF, and also provided a new mechanism for Pontin in tumorigenesis.

As an ATPase, Pontin has Walker A and Walker B motifs that are involved in ATP binding and ATP hydrolysis, respectively. The ATPase activity of Pontin is important for many of its functions. Results in this study show that Pontin-D302N, an ATPase-deficient dominant-negative mutant, binds to mutp53 to a similar extent compared with wild-type Pontin, indicating that the ATPase activity is not required for the Pontin–mutp53

interaction. Notably, Pontin-D302N, which competes with endogenous wild-type Pontin to interact with mutp53, greatly inhibits mutp53 GOF. Similarly, Rottlerin, a Pontin-specific ATPase inhibitor, greatly inhibits mutp53 GOF. Furthermore, although knockdown of endogenous Pontin inhibits mutp53 GOF, ectopic expression of the Pontin fragments containing the Walker B motif restores mutp53 GOF in cells with knockdown of endogenous Pontin. These results show that its ATPase activity is required for Pontin to regulate mutp53 GOF.

Pontin is a component of several chromatin-remodeling complexes, including Ino80 complex, TIP60/NuA4 complex and SWR1 complex, and is essential for their chromatic-remodeling activities and transcriptional regulation (Jha et al., 2008; Jin et al., 2005). It is possible that the Pontin–mutp53 interaction recruits mutp53 to specific DNA-binding sites or chromatin landscapes, and facilitates mutp53 to transcriptionally regulate a group of genes directly or together with other transcription factors. Results from IF assays show that Pontin is predominantly localized in the nucleus and largely colocalized with mutp53. Knockdown of Pontin abolishes mutp53's transcriptional activity toward a group of genes. Some genes that require Pontin for their transcriptional regulation by mutp53, including STMN1, ADRB1, ITPR1, RIPK1, CtBP2, TPD52, and TOX3, have been suggested to have roles in tumorigenesis, especially for migration and metastasis (Bist et al., 2011; Chen, Paliwal, Draheim, Grossman, & Lewis, 2008; Goto et al., 2014; Guo et al., 2009; Messai et al., 2014; Shan et al., 2013). Results from ChIP

assays show that mutp53 interacts with some target genes at their regulatory regions in a Pontin-dependent manner. It remains unclear why Pontin selectively regulates mutp53 transcriptional activity toward certain genes. Future studies should further increase our understanding of the role and mechanisms of Pontin in regulating mutp53 GOF.

Pontin overexpression has been observed in many human cancers (Lauscher et al., 2012). Results from this study and previous reports show that Pontin overexpression in tumors is strongly associated with poor prognosis (Lauscher et al., 2012). Interestingly, Pontin expression levels have a much stronger prognostic value in breast cancers containing mutp53 than those without mutp53. This observation suggests that Pontin has an important role in mutp53-promoted tumorigenesis, and highlights the biological significance of Pontin overexpression in tumors, especially those containing mutp53.

In summary, this study established Pontin as a new mutp53-binding protein, and demonstrated that Pontin promotes mutp53 GOF in tumorigenesis in an ATPase-dependent manner. Results from this study raise interesting and intriguing possibilities to block mutp53 GOF in tumors through targeting Pontin or its ATPase activity.

Author contribution: Dr. Wenwei Hu and Dr. Zhaohui Feng designed experiments and analyzed the data, Yuhan Zhao, Cen Zhang, Xuetian Yue, Juan Liu, Haiyang Yu carried out the experiments and analyzed the data, Yuhan Zhao and Dr. Wenwei Hu wrote the manuscript.

Materials and methods

Mice

Hupki mice carrying either the P72 or R72 allele were generous gifts from Dr. Maureen Murphy (The Wistar Institute) (Kung et al., 2016). Hupki mice in 129SV^{sl} and C57BL/6J backgrounds were produced by backcrossing Hupki mice ten times to 129SV^{sl} and C57BL/6J, respectively. C57BL/6J CD45.1 mice (RRID:IMSR_JAX:002014) were purchased from The Jackson Laboratory (Bar Harbor, ME). p53$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME) and Trp53$^{R172H/R172H}$ mice were gifts from Dr. Gigi Lozano at MD Anderson Cancer Center. Expression vectors of BAG2-HA (pcDNA-HA-BAG2) were gifts from Dr. Cam Patterson at University of North Carolina.

All animal experiments were approved by the IACUC committee of Rutgers University.

Cell culture

Human lung cancer H1299, osteosarcoma Saos2, breast cancer SK-BR-3, MDA-MB-468, colorectal cancer HT29, SW480, RKO p53$^{+/+}$, HCT116 p53$^{+/+}$, and hepatocellular carcinoma Huh-7 cell lines were obtained from ATCC (Manassas, VA). Human HCT116 p53$^{R248W/-}$ cells were gifts from Dr. Bert Vogelstein at Johns Hopkins University. Stable cell lines expressing mutp53 R175H, R248Q and R273H were established as previously described (Zheng et al., 2013). SK-BR-3-p53sh cells with knockdown of endogenous mutp53 were established by stable transduction of retroviral shRNA vectors against p53 into SK-BR-3 cells.

Constructs and cell treatments

Expression vectors of mutp53 fragments containing aa 1–363 and aa 43–363 were obtained by using site-directed mutagenesis to introduce an R175H mutation into expression vectors of wtp53 fragments containing aa 1–363 and aa 43–363, respectively, which were generous gifts from Dr. Xinbin Chen at University of California, Davis. Expression vectors of mutp53 and wtp53 DBD were constructed by amplifying the fragments from pRC-mutp53-R175H and pRC-wtp53 vectors, respectively. Two-step overlap extension PCR was used to obtain mutp53 fragments without DBD. The final PCR products were inserted into pCMV-Flag vectors.R175H mutp53^{NLS} expression vectors were obtained by using site-directed mutagenesis. Primers used for site mutagenesis and cloning for mutp53 fragments, Flag-tagged FL BAG2 and BAG2 fragments are listed in Table 2. Expression vectors of Flag-tagged Pontin and Pontin fragments were gifts from Dr. Steve Artandi at Stanford University. Expression vectors of Flag-tagged ATPase-deficient dominant-negative mutant Pontin-D302N were gifts from Dr. Michael Cole at Dartmouth College. Site-directed mutagenesis was performed to produce the expression vectors of Pontin and Pontin fragments resistant to siRNA by using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Sequences of primers are listed in Table 2.

Retroviral shRNA vectors against human BAG2 and Pontin were purchased from Open Biosystems (Thermo Scientific, Waltham, MA, Cat#V2LHS-27769). Two different siRNA

oligos against MDM2 were purchased from Qiagen (Germantown, MD, Cat#SI00300846) and Dharmacon (Lafayette, CO, Cat#M-003279-01). The sequences of siRNA are listed in Table 3. Transfection of cells with expression vectors or siRNA was performed by using Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA).

5-FU and MG132 were purchased from Sigma (St. Louis, MO). For Rottlerin treatment, cells were treated with Rottlerin (Calbiochem, Billerica, MA, USA; 0.5 μM) for 48 h.

Micro-CT scan analysis

Mice were anesthetized for CT scanning of whole body skeletons using the INVEON PET/CT system (Siemens Healthcare). The images were reconstructed using INVEON Research Workplace software (Siemens Healthcare, Tarrytown, NY).

The bone microstructure measurement was carried out as previously described (Ell et al., 2013). In brief, mouse tibias were scanned by micro-CT. The images were reconstructed with Beam Hardening Correction, and Hounsfield calibrated before being analyzed using INVEON Research Workplace software. The 3D images were generated corresponding to the trabecular bone regions. CT scans were carried out at the Preclinical Imaging Shared Resource of Rutgers Cancer Institute of New Jersey.

Histology

Paraffin-embedded skin specimens were sectioned with 5 μm thickness and stained with hematoxylin and eosin (H&E). The thickness of the dermal and adipose layers from the skin samples were determined by taking three random measurements along the length of

each skin sample using ImageJ software.

Cutaneous wound healing assays

Cutaneous wound healing assays were carried out as previously described (Tyner et al., 2002). In brief, mice were anesthetized, and a full-thickness wound was generated in the mouse dorsal skin using a 3 mm biopsy punch (Integra, York, PA). Wound diameters were measured daily. Wound areas = 0.25 × π ×width × length.

Flow cytometry (FCM) assays

LT-HSC numbers were determined as previously described (Dumble et al., 2007). In brief, bone marrow cells were flushed out from mouse hind limb bones with PBS and stained with a cocktail of antibodies (BD Bioscience Pharmingen), including an anti-lineage-APC antibody, an anti-Sca-1-PE-Cy7 antibody, an anti-c-kit-PE-CF594 antibody, an anti-CD34-FITC antibody and an anti-Flk-2-PE antibody. LT-HSCs which were selected as Lin-/low, Sca1+, c-kit+ and CD34-, Flk2- cells were quantified by FCM analysis using a Beckman-Coulter Cytomics FC500 Flow Cytometer (Indianapolis, IN).

To determine the HSC proliferation ability, mice were injected intraperitoneally with 1 mg BrdU (BD Bioscience Pharmingen) at 16 hr before the collection of bone marrow. Bone marrow cells were stained with a cocktail of antibodies, including an anti-lineage-APC antibody, an anti-Sca-1-PE-Cy7 antibody, an anti-c-kit-PE-CF594 antibody, and an anti-Flk-2-PE antibody. After cell surface staining, a BrdU-FITC Flow Kit (BD Bioscience Pharmingen) was used to identify cycling cells according to the manufacturer’s

instructions. Proliferating HSCs were identified as Lin-/low, Sca1+, c-kit+, Flk2- and BrdU+ by FCM analysis.

Cell cycle analysis

Cell cycle distribution was analyzed as previously described (Hu, Chan, et al., 2010). In brief, cells were trypsinized, stained with propidium iodide (50 μg/ml) and then analyzed in flow cytometry.

Cell viability and cellular apoptosis assays

Cell viability and cellular apoptosis were determined as previously described (H. Yu et al., 2014). The Vi-CELL cell counter (Beckman Coulter, Brea, CA, USA) was used to determine cell viability. Apoptosis was measured by staining cells using Muse Annexin V and Dead Cell Assay Kit (Millipore) and analyzed in a bench flow cytometry, the Muse Cell Analyzer (Millipore, Billerica, MA).

Total bone marrow competitive transplantation assays

Bone marrow transplantation assays were carried out as previously described (Dumble et al., 2007). In brief, bone marrow cells from 6-month-old and 18-month-old ‘donor’ CD45.2 mice were mixed with bone marrow cells from 6-month-old ‘competitor’ CD45.1 mice at a ratio of 2:1. Recipient CD45.1 mice at the age of 6 to 12 week-old were irradiated with a lethal dose of 10 Gy the day before bone marrow transplantation. n = 6/group. The mixture of bone marrow cells was injected into recipient mice via the tail vein. Sixteen weeks after transplantation, peripheral white blood cells of recipient mice were analyzed

for CD45.1 and CD45.2 cell surface markers using an anti-CD45.1-PE antibody and an anti-CD45.2-FITC antibody, respectively (BD Biosciences Pharmingen).

Western-blot assays

Standard Western-blot assays were used to analyze protein expression in tissues and cells. Nuclear extracts were prepared by using the Qproteome Nuclear Protein Kit (Qiagen). The following antibodies were used for assays: anti-p53 (FL393, Santa Cruz Biotechnology), anti-p21 (Santa Cruz Biotechnology), and β-actin (Sigma), MDM2 (2A10), BAG2 (Aviva Systems Biology), HA (Roche), α-Tubulin (Santa Cruz), Lamin A/C (Santa Cruz), cleaved-caspase 3 (Cell Signaling), anti-Pontin (Sigma), anti-Flag (Sigma), anti-STMN1 (Cell Signaling, Billerica, MA, USA).

Quantitative real-time PCR

Total RNA was prepared by using an RNeasy kit (Qiagen, Valencia, CA, USA) and treated with DNase I to remove residual genomic DNA. RNA from formalin fixed and paraffin-embedded colorectal tumor sections was prepared with High Pure miRNA Isolation kit (Roche, Indianapolis, IN). The cDNA was prepared by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). All probes were purchased from Applied Biosystems. Real-time PCR was done in triplicate with TaqMan PCR mixture (Applied Biosystems) in the ABI Step-One Plus system. Primers for TaqMan real-time PCR assays were purchased from Applied Biosystems. The expression of genes was normalized to the β-actin gene.

IP assays and IP coupled with LC-MS/MS assays

To determine mutp53 binding partners in mouse tissues, mouse mutp53 protein complexes were purified from lysates from tumor and normal tissues of mutp53$^{R172H/R172H}$ mice by IP using anti-p53 (FL393) beads and eluted with 0.1 M Glycine solution. Eluted materials were separated in a 4–15% Tris SDS gel and visualized by silver staining using the silver staining kit (Invitrogen, Grand Island, NY) and Coomassie blue staining. Coomassie blue-stained protein bands were excised from the gel and subsequently analyzed by LC-MS/MS at the Biological MS facility of Rutgers University.

IP assays were performed as previously described (Zheng et al., 2013). In brief, 1 mg cell or tissue lysates in NP-40 buffer were used for IP using anti-p53 (DO-1 for human cells and FL393 for mouse tissues, Santa Cruz, Dallas, Texas), anti-HA, anti-Flag, and anti-Pontin antibodies (Sigma, St. Louis, MO, USA) to pull down mutp53, BAG2-HA, BAG2-Flag, Pontin-Flag, and Pontin protein, respectively.

In vivo ubiquitination of Mutp53

In vivo ubiquitination assays were performed as previously described (J. Liu, Zhang, Wang, et al., 2014). In brief, cells were transfected with different expression vectors, including mutp53 R175H, BAG2-HA, and His-ubiquitin, or transfected with siRNA against BAG2 together with His-ubiquitin expression vectors. At 24 hr after transfection, cells were treated with MG132 for 6 hr. The levels of mutp53 ubiquitination were determined by IP using DO-1 antibody followed by Western blot assays with an anti-ubiquitin antibody

(P4D1; Santa Cruz).

ChIP assays

ChIP assays were performed with an Upstate ChIP assay kit (Billerica, MA, USA) in accordance with the instructions of the manufacturer. SK-BR-3 cells were subjected to ChIP assays with antibodies against p53, Pontin, and IgG. DNA fragments pulled-down by antibodies were recovered and subjected to real-time PCR. The primer sets are list in Table 2.

IF staining assays

IF staining was performed as previously described (Zheng et al., 2013). In brief, cells grown on slides were fixed with 4% paraformaldehyde, treated with 0.5% TritonX-100 and then stained. Antibodies against p53 (FL393), Flag and Pontin were used to detect p53, BAG2-Flag and Pontin, respectively. Slides were then incubated with Alexa Fluor 555 Goat Anti-Rabbit IgG (H + L) and Alexa Fluor 488 Goat Anti-mouse IgG (H + L) (Invitrogen). Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA).

Cell migration and invasion assays

The transwell system (BD Biosciences, San Jose, CA, USA) was used for cell migration and invasion assays as previously described (X. Li et al., 2014; Zheng et al., 2013). In brief, cells in the FBS-free medium were seeded into upper chambers coated with or without matrigel (BD Biosciences) for invasion and migration assays, respectively. The

lower chamber was filled with medium supplemented with 10% FBS. For SK-BR-3 cells, the lower chamber was filled with 1:1 mix of medium supplemented with 10% FBS and NIH 3T3 cell-conditioned medium. Cells on the lower surface of upper chambers were counted after culturing at 37 °C for 24 h (NIH, Bethesda, MD, USA).

Scratch motility assays

Cells were scratched with 2 μl pipette tips and then cultured in the FBS-free medium. The scratched wound was monitored, and pictures were taken at the indicated time points. The distances between the two edges of the scratched wound were measured by using the ImageJ software (NIH, Bethesda, MD, USA).

In vivo lung metastasis analysis

In vivo lung metastasis assays were performed as previously described (Zheng et al., 2013). In brief, HCT116 $p53^{R248W/-}$ and HCT116 $p53^{-/-}$ cells with or without knockdown of BAG2 and H1299-Con and H1299-R175H cells with or without knockdown of Pontin (1×10^6 cells in 0.1 ml PBS) were injected into nude mice via the tail vein (n=8 mice per group). Mice were killed at 6 weeks after the inoculation. The number of lung tumor nodules were counted under a dissecting microscope and confirmed by histopathological analysis. The areas of tumor nodules were quantified in eight representative images taken at × 10 magnification by using the ImageJ software. Animal protocols were approved by the IACUC committee of Rutgers University.

Anchorage-independent growth assays

Anchorage-independent growth assays were performed as previously described (X. Li et al., 2014). In brief, cells were seeded in 6-well plates coated with media containing 0.6% agarose, and cultured in media containing 0.3% agarose. Colonies were stained and counted after 2–3 weeks. Each experiment was repeated at least three times with triplicate wells.

Xenograft tumorigenicity assays

Cells (5×10^6 in 0.2 ml PBS) were injected subcutaneously (s.c.) into 8-week-old BALB/c athymic nude mice (Taconic). Tumor volumes were measured every 2 days for 3 weeks. Tumor volume = 1/2 (length × width^2). Tumor samples were processed for routine histopathological examination.

Microarray analysis

SK-BR-3-Consh and SK-BR-3-p53sh cells with or without knockdown of Pontin were analyzed for genome-wide expression changes by Affymetrix GeneChip. Total RNA was reverse transcribed and hybridized to an Affymetrix GeneChip expression array (HGU133+2 microarrays) by Functions Genomics Core Facility of Rutgers Cancer Institute of New Jersey. Microarray data were deposited with GEO Accession Number: GSE58140. Data were analyzed by using Microarray Suite version 5.0 (MAS 5.0). Change of expression levels for each gene by mutp53 was calculated as expression levels in SK-BR-3-Consh cells compared with SK-BR-3-p53sh cells with the mutp53 knockdown. Heatmap of genes with significant expression change was visualized by

using Gene-E software (http://www.broadinstitute.org/cancer/software/GENE-E/index.html). Functional pathway and network analysis of genes with significant expression changes were performed by using the Ingenuity pathways analysis (IPA) software (Ingenuity Systems, http://www.ingenuity.com).

Database of cancer patients

PrognoScan (http://www.prognoscan.org/), which has a large collection of publicly available database with microarray data and clinical information (Mizuno, Kitada, Nakai, & Sarai, 2009), was used to analyze the prognostic power of BAG2 expression levels in colorectal cancer patients (GSE14333) (Jorissen et al., 2009), lung cancer patients (GSE14814) (Zhu et al., 2010), breast cancer patients (GSE12276) (Bos et al., 2009), and soft tissue cancer patients (GSE30929) (Gobble et al., 2011).

A cohort of the de-identified colorectal cancer tissues with known p53 mutation status and p53 protein levels was obtained from the database of the First Affiliated Hospital of Harbin Medical University (Harbin, China) with an IRB approval (Zheng et al., 2013). None of these patients received pre-surgical chemotherapy.

The KM plotter online survival database (http://www.kmplot.com) can assess the effect of 22 277 genes on overall survival in 1926 lung cancer and 1115 breast cancer patients (Gyorffy et al., 2010). Another cohort of the breast cancer patients with known p53 mutation status and survival information was obtained from a publically available database (GSE3494) (Miller et al., 2005). Genome-wide expression levels of all tumor

specimens were assessed on Affymetrix HGU133A and B arrays. p53 mutation status was identified by cDNA sequence analysis of exons 2–11 of the p53 gene (Miller et al., 2005). To analyze the prognostic value of Pontin expression levels, the patients were split into two groups by auto-selection of best cutoff in the KM plotter database and according to the median expression level of Pontin mRNA in the breast cancer database (GSE3494). The Affymetrix IDs used for analysis are: 201614_s_at for Pontin; 217714_x_at for STMN1 and 209941_at for RIPK1.

Statistical analysis

The data were present as mean ± SD. The lifespan of mice was summarized by Kaplan-Meier plots and compared using the log-rank test using GraphPad Prism software. Kaplan–Meier statistics and log-rank (one tail) test were performed to estimate the significance of differences in the survival of patients among different groups using the software in PrognoScan website. The differences in xenograft tumor growth among groups were analyzed for statistical significance by ANOVA, followed by Student's *t*-tests using GraphPad Prism software. All other p values were obtained using Student's *t*-test or $\chi 2$ test. Values of $p < 0.05$ were considered to be significant. Based on survival data of p53 codon 72 SNP mice, we hypothesized that the P72 mice have delayed development of aging-associated phenotypes. Therefore, one-tailed Student’s t-test was used for the majority of data analysis related to the development of aging-associated phenotypes. Values of $p<0.05$ were considered to be significant.

Figure 1. Human p53 protein's functional domains.

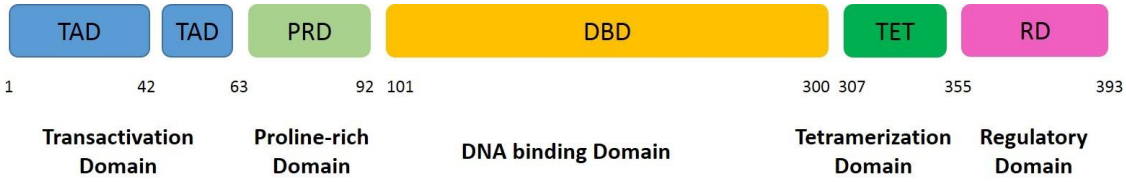
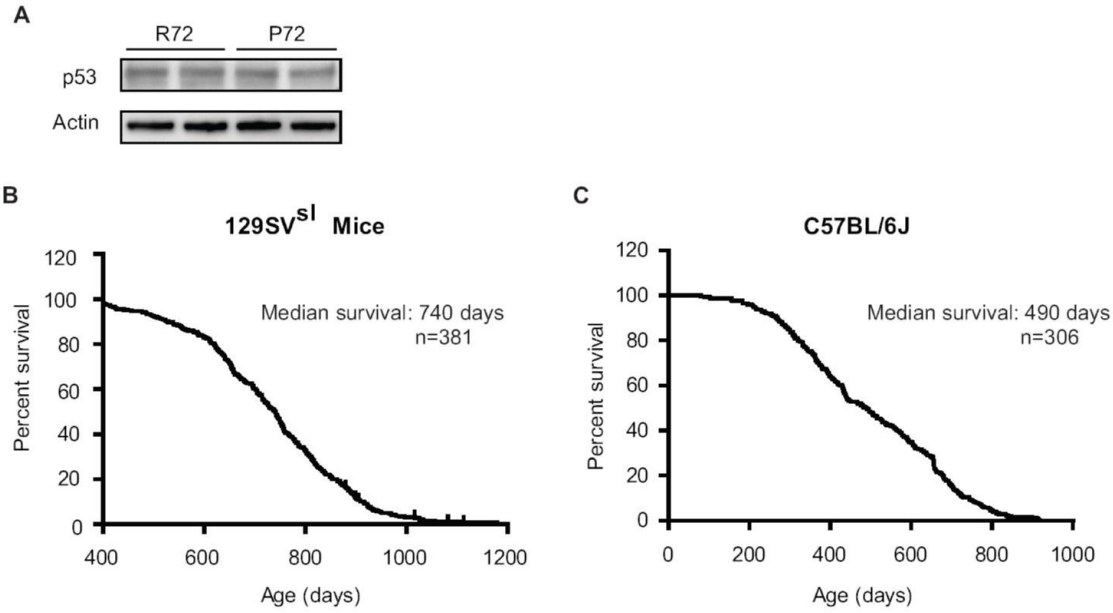
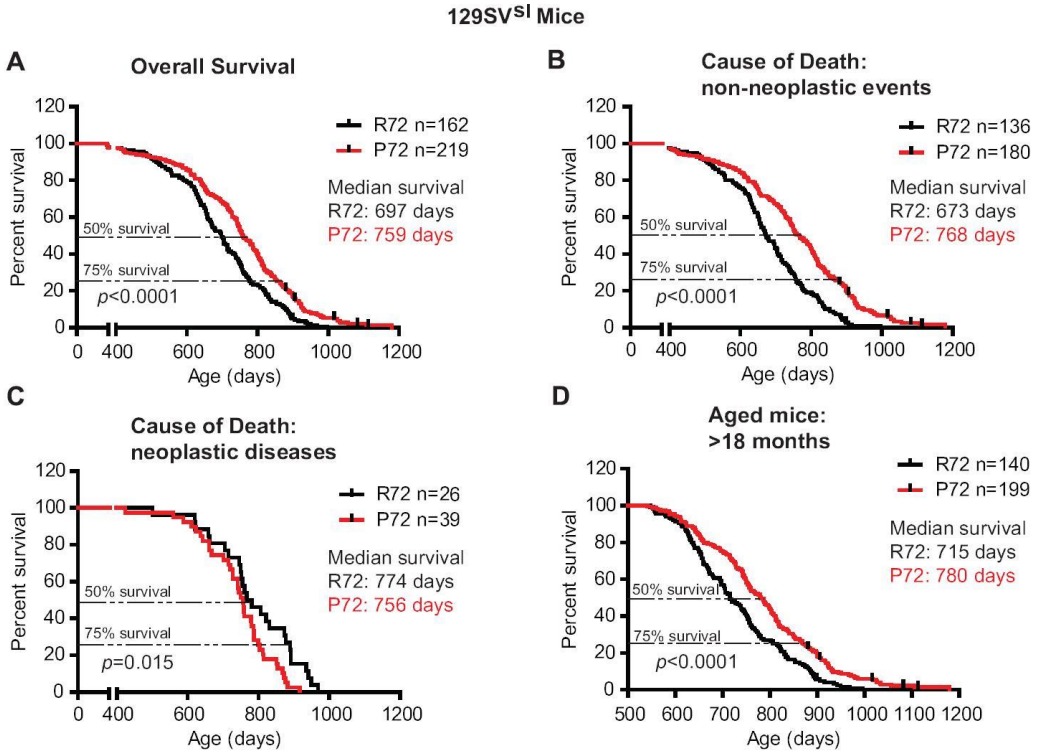


Figure 2. The p53 protein levels in the bone marrow from 129SV^{sl} p53 Hupki mice and the lifespans of 129SV^{sl} and C57BL/6J p53 Hupki mice.



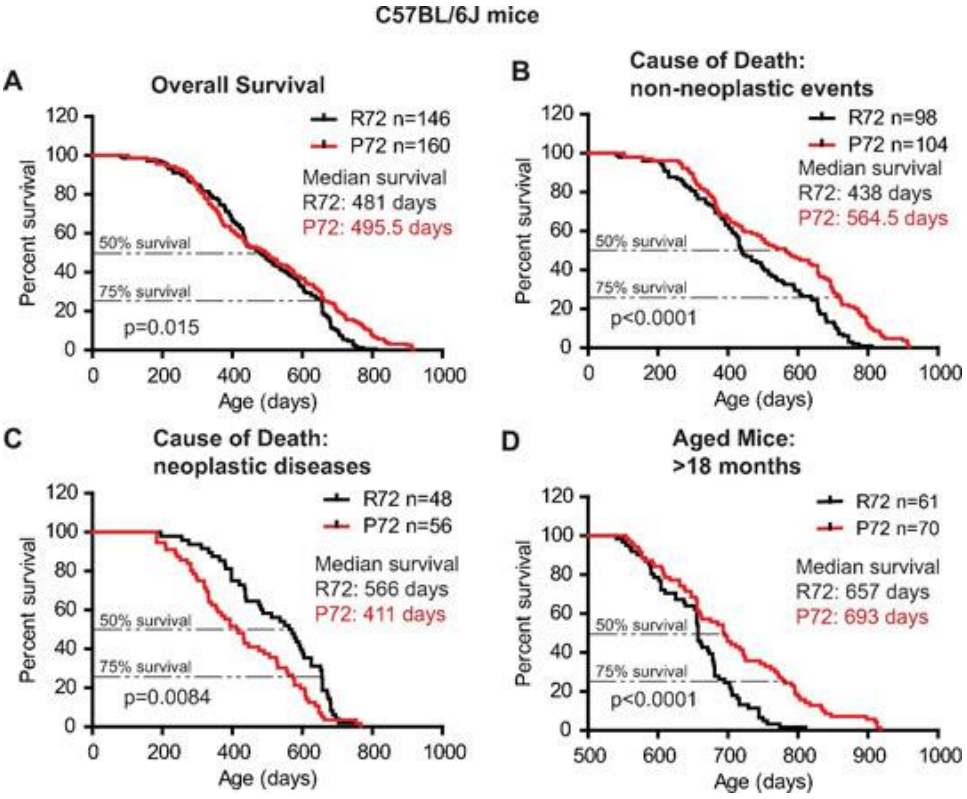
(A) The p53 protein levels in the bone marrow from 129SV^{sl} p53 P72 and R72 mice were determined by the Western-blot assays. (B) The lifespan of 129SV^{sl} p53 Hupki mice, including both p53 P72 and p53 R72 mice (median survival: 740 days; n = 381). (C) The lifespan of C57BL/6J Hupki mice, including both p53 P72 and p53 R72 mice (median survival: 490 days; n = 306).

Figure 3. The lifespans of 129SV^{sl} p53 R72 mice and p53 P72 mice.



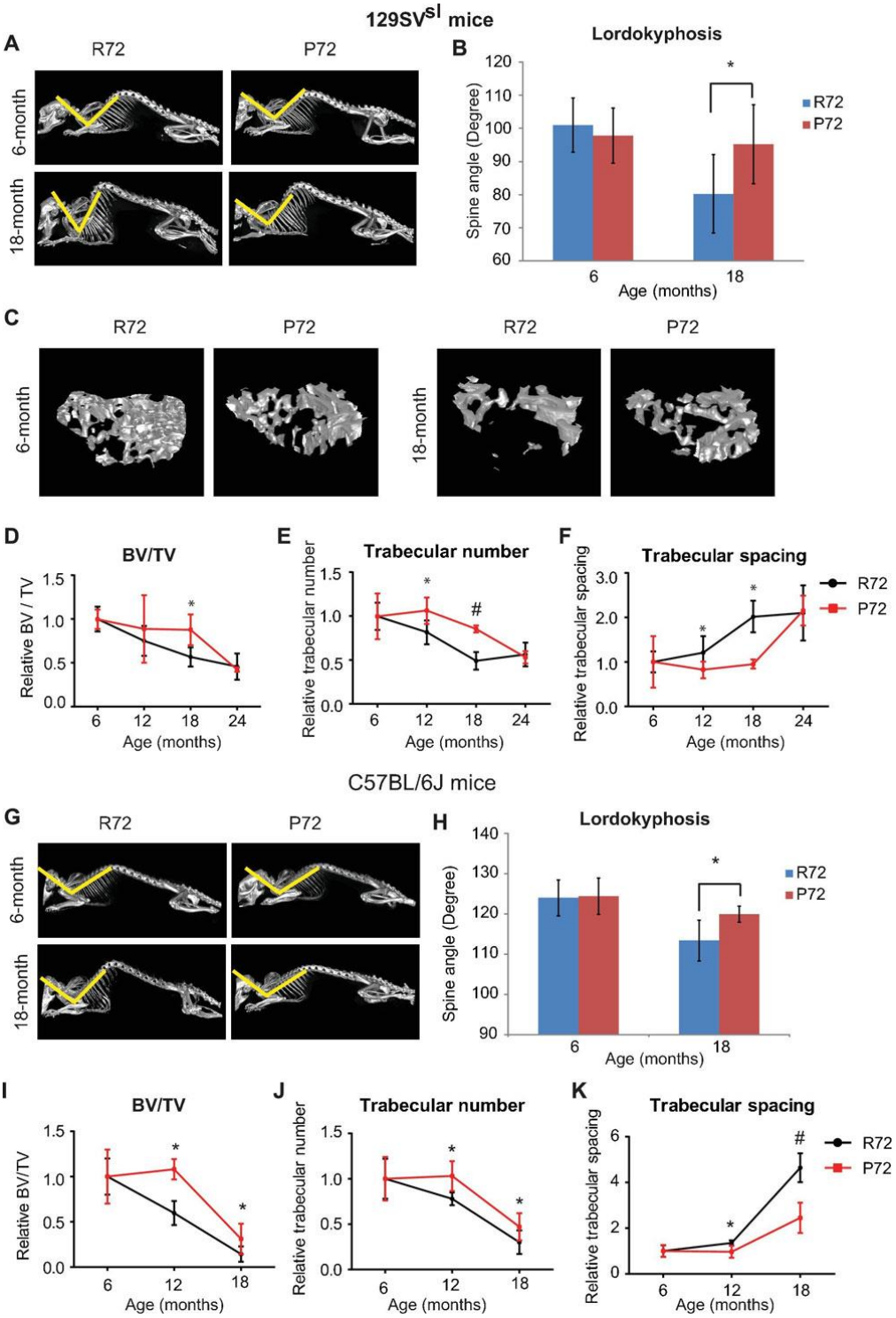
(A) The overall survival of 129SV^{sl} R72 mice (n = 162) and P72 mice (n = 219). The median survival is 697 days and 759 days for R72 and P72 mice, respectively. p<0.0001. (B) Survival of 129SVsl R72 mice (n = 136) and P72 mice (n = 180) died from non-neoplastic events. The median survival is 673 days and 768 days for R72 and P72 mice, respectively. p<0.0001. (C) Survival of 129SVsl R72 mice (n = 26) and P72 mice (n = 39) died from neoplastic diseases. The median survival is 774 days and 756 days for R72 mice and P72 mice, respectively. p=0.015. (D) Survival of 129SVsl R72 mice (n = 140) and P72 mice (n = 199) older than 18 months. The median survival is 715 days and 780 days for R72 mice and P72 mice, respectively. p<0.0001. The log-rank test was used to analyze the difference in survival.

Figure 4. The lifespans of C57BL/6J p53 R72 mice and p53 P72 mice.



(A) The overall survival of C57BL/6J R72 mice (n = 146) and P72 mice (n = 160). The median survival is 481 days and 495.5 days for R72 and P72 mice, respectively. p=0.015. (B) Survival of C57BL/6J R72 mice (n = 98) and P72 mice (n = 104) died from non-neoplastic events. The median survival is 438 days and 564.5 days for R72 and P72 mice, respectively. p<0.0001. (C) Survival of C57BL/6J R72 mice (n = 48) and P72 mice (n = 56) died from the neoplastic disease. The median survival is 566 days and 411 days for R72 mice and P72 mice, respectively. p=0.0084. (D) Survival of C57BL/6J R72 mice (n = 61) and P72 mice (n = 70) older than 18 months. The median survival is 657 days and 693 days for R72 mice and P72 mice, respectively. p<0.0001. The log-rank test was used to analyze the difference in survival.

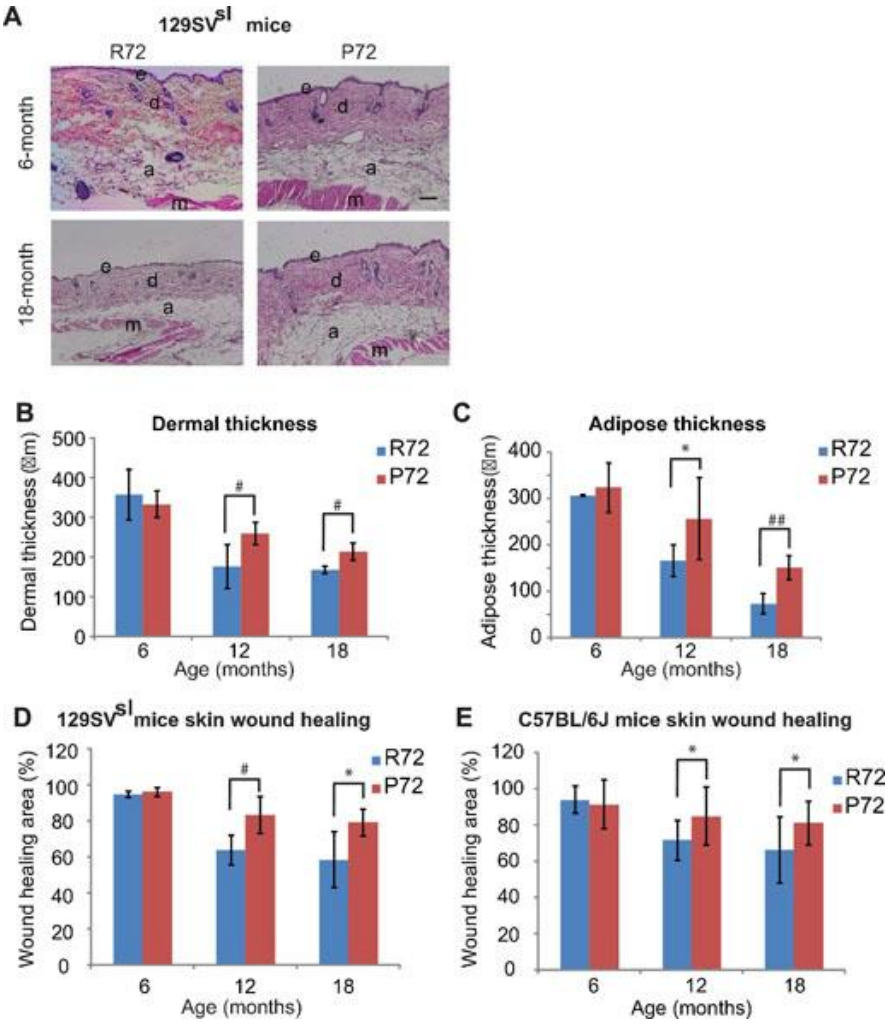
Figure 5. p53 P72 mice have a delay in developing aging-associated bone structure phenotypes compared with p53 R72 mice.



(A) Lordokyphosis in 129SV^{sl} R72 and P72 mice. Shown are representative images taken with the INVEON PET/CT system of mouse whole skeleton at different ages. (B) Average of spine angles from 6-month-old and 18-month-old 129SV^{sl} R72 and P72 mice. A narrowing of the angle

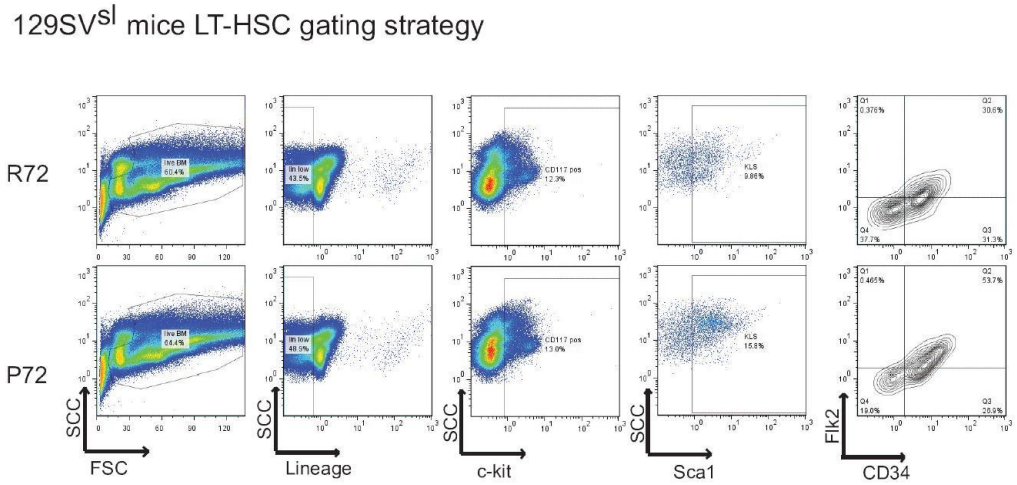
indicates an increase in lordokyphosis. n = 5/group, *: p<0.05; Student's t-test. (C) Representative micro-CT bone structure images taken with the INVEON PET/CT system of tibias from 129SV^{sl} R72 and P72 mice at different ages. (D–F) Quantification of bone volume relative to total volume (D), trabecular number (E) and trabecular spacing (F) from micro-CT scans of tibias from 129SV^{sl} R72 and P72 mice at different ages using INVEON Research Workplace software. Data were presented as mean ± SD. n = 3, *: p<0.05, #: p<0.01; Student's t-test. (G) Lordokyphosis in C57BL/6J R72 and P72 mice. Shown are representative images of whole mouse skeleton at different ages. (H) Average of spine angles from 6-month-old and 18-month-old C57BL/6J R72 or P72 mice. n = 5, *: p<0.05; Student's t-test. (I–K) Quantification of bone volume relative to total volume (I), trabecular number (J) and trabecular spacing (K) from micro-CT scans of tibias from C57BL/6J R72 and P72 mice at different ages using INVEON Research Workplace software. Data were presented as mean ± SD. n = 3, *: p<0.05, #: p<0.01; Student's t-test.

Figure 6. p53 P72 mice have a delay in developing aging-associated skin phenotypes compared with p53 R72 mice.



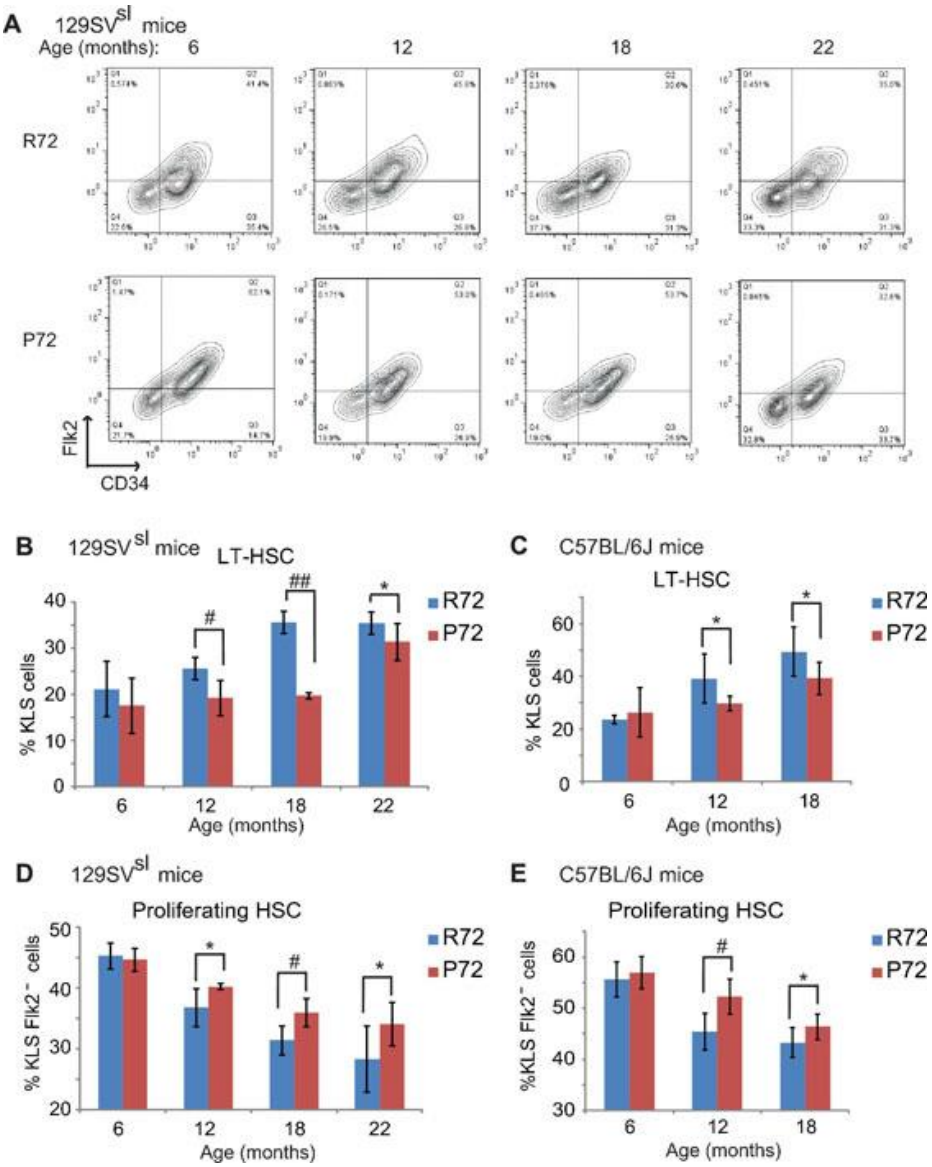
(A) H and E staining of cross-sections of dorsal skins from 129SV^{sl} R72 mice and P72 mice at different ages. e: epidermis; d: dermis; a: adipose; m: muscle. Scale bar: 100 μm. (B and C) Quantification of dermal thickness (B) and subcutaneous adipose layer thickness (C) of 129SVsl R72 mice and P72 mice at different ages. (D and E) Skin wound healing abilities in 129SVsl (D) and C57BL/6J (E) R72 and P72 mice at different ages. The skin wound area was quantified as 0.25 × π ×width × length. For (B–E), data were presented as mean ± SD, n ≥ 5/group, *: p<0.05, #:p<0.01, ##: p<0.001; Student's t-test.

Figure 7. The gating strategy to identify LT-HSCs.



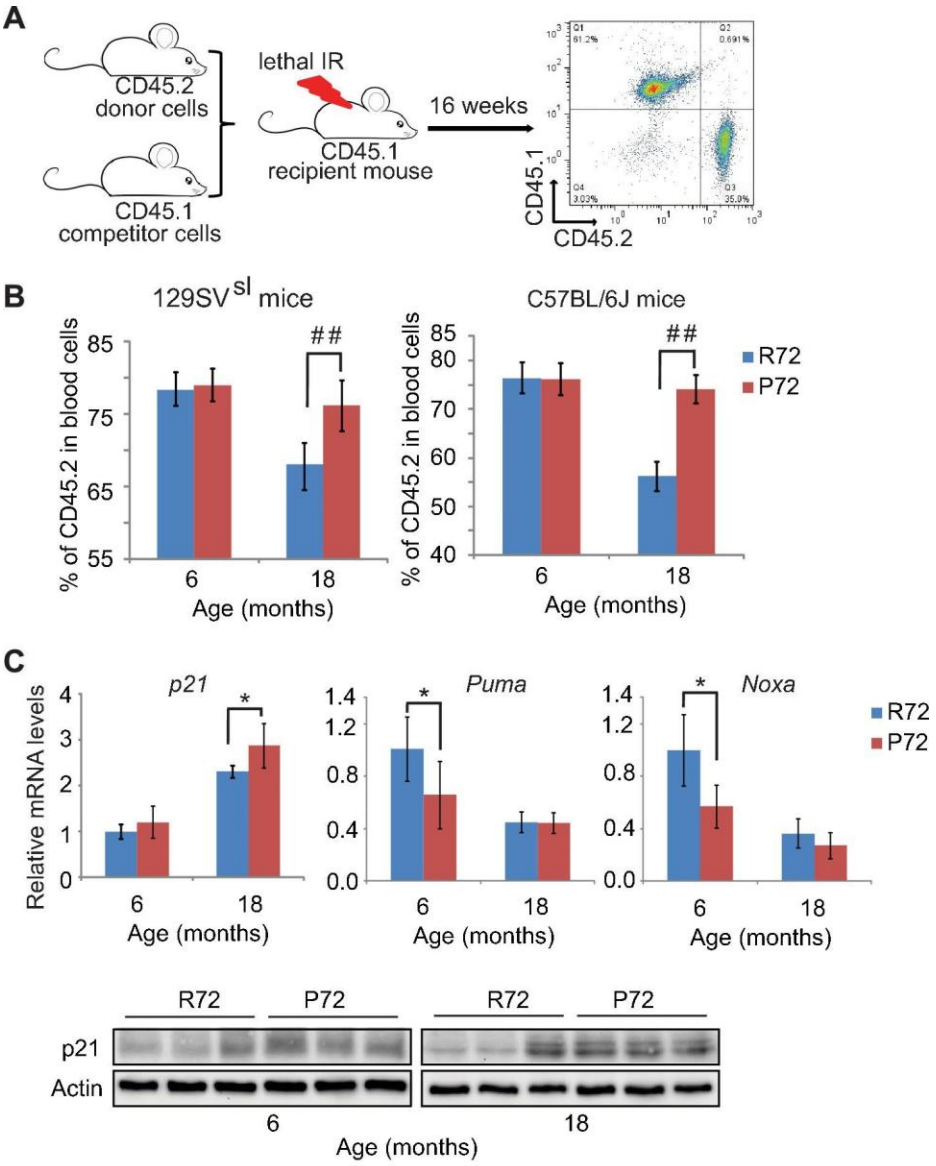
Live bone marrow cells from 18-month-old 129SV^{sl} p53 R72 mice (upper panels) and p53 P72 mice (lower panels) were gated to identify LT-HSCs. LT-HSCs were selected based on low or negative expression of mature lineage markers (Lin$^{-/low}$), dual positive staining of Sca-1 and c-kit, and double negative expression of Flk2 and CD34.

Figure 8. HSCs from p53 P72 mice exhibit a delayed aging phenotype compared with HSCs from p53 R72 mice.



(A) The representative gating of long-term HSCs (LT-HSCs) in 129SV^{sl} R72 mice and P72 mice. LT-HSCs were identified as Lin$^{-/low}$, Sca1^{+}, c-kit^{+} and CD34^{-}, Flk2^{-} cells from bone marrow by flow cytometry analysis. (B and C) The bar graph represents the percentage of LT-HSCs in the bone marrow of 129SV^{sl} (B) and C57BL/6J (C) R72 mice and P72 mice at different ages. Data were presented as mean ± SD. n ≥ 5/group, *: p<0.05, #: p<0.01, ##: p<0.001; Student's t-test. (D and E) The bar graph represents the percentage of proliferating HSCs in the bone marrow of 129SVsl (D) and C57BL/6J (E) R72 mice and P72 mice at different ages. Proliferating HSCs were labeled with BrdU for 16 hrs in vivo and then identified as Lin$^{-/low}$, Sca1^{+}, c-kit^{+}, Flk2^{-} and BrdU^{+} by flow cytometry analysis. Data were presented as mean ± SD. n = 5/group, *: p<0.05, #: p<0.01; Student's t-test.

Figure 9. Aged p53 P72 mice exhibit better hematopoietic stem cell self-renewal and repopulation abilities compared with aged p53 R72 mice.



(A) The experimental design for bone marrow transplantation assays to examine the engraftment and repopulation abilities of HSCs in mice. (B) Percentage of CD45.2 cells in peripheral blood at 16 weeks after engraftment. In both 129SV^{sl} mice (left) and C57BL/6J mice (right), aged P72 mice showed better stem cell abilities of engraftment and repopulation compared with aged R72 mice. n = 6/group, ##: $p<0.001$; Student's t-test. (C) The mRNA expression levels of p21, Puma and Noxa (upper panels) and the protein expression levels of p21 (lower panels) in bone marrow from 129SVsl p53 R72 and P72 mice at different ages as determined by real-time PCR assays and Western-blot assays, respectively. Data were presented as mean ± SD, n = 4/group, *$p<0.05$; Student's t-test.

Figure 10. A schematic model depicting the dual functions of p53 codon 72 SNP in regulating longevity.

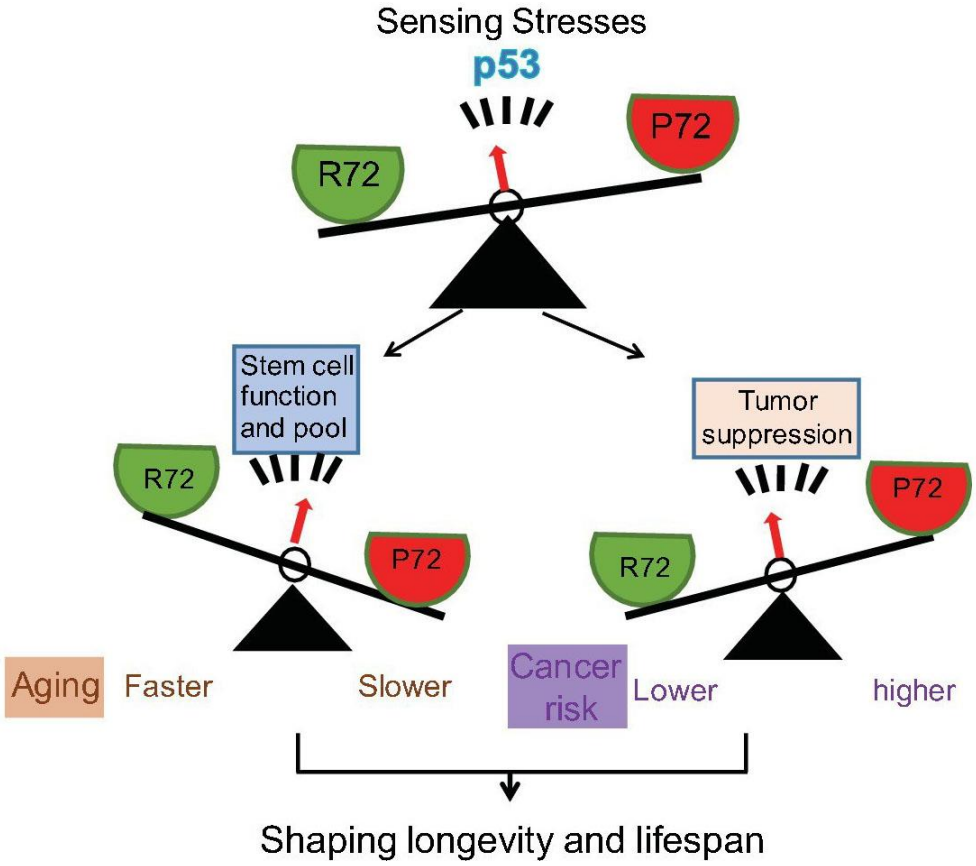
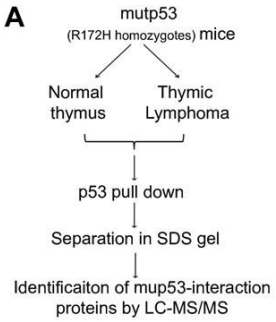


Figure 11. Identification of proteins interacting with mutant p53 (mutp53) protein in tumors from Trp53R172H/R172H mice.



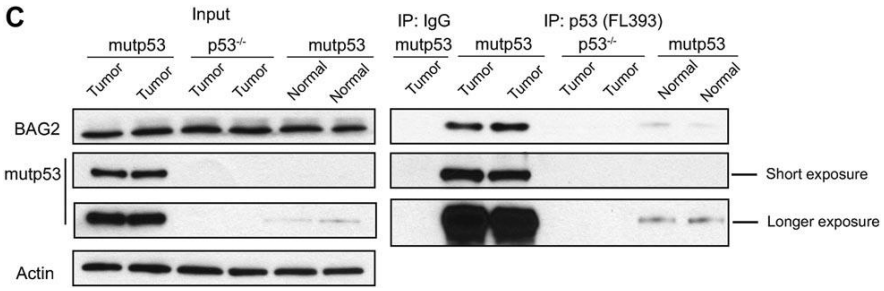
B A list of identified mutp53-interaction protein candidates

Detected only in p53 (R172H homozygotes) tumors

Gene names	Average counts: Normal thymus	Average counts: Thymic lymphoma	Major function
Ctsb	0	19	cysteine-type peptidase activity
BAG2	0	15	chaperone binding
Hyou1	0	14.5	molecular chaperone and protein folding
Hsp90b1	0	9	process and transport secreted proteins
Hspbp1	0	8.5	inhibits HSP70 chaperone activity
myosin	0	8	motor proteins

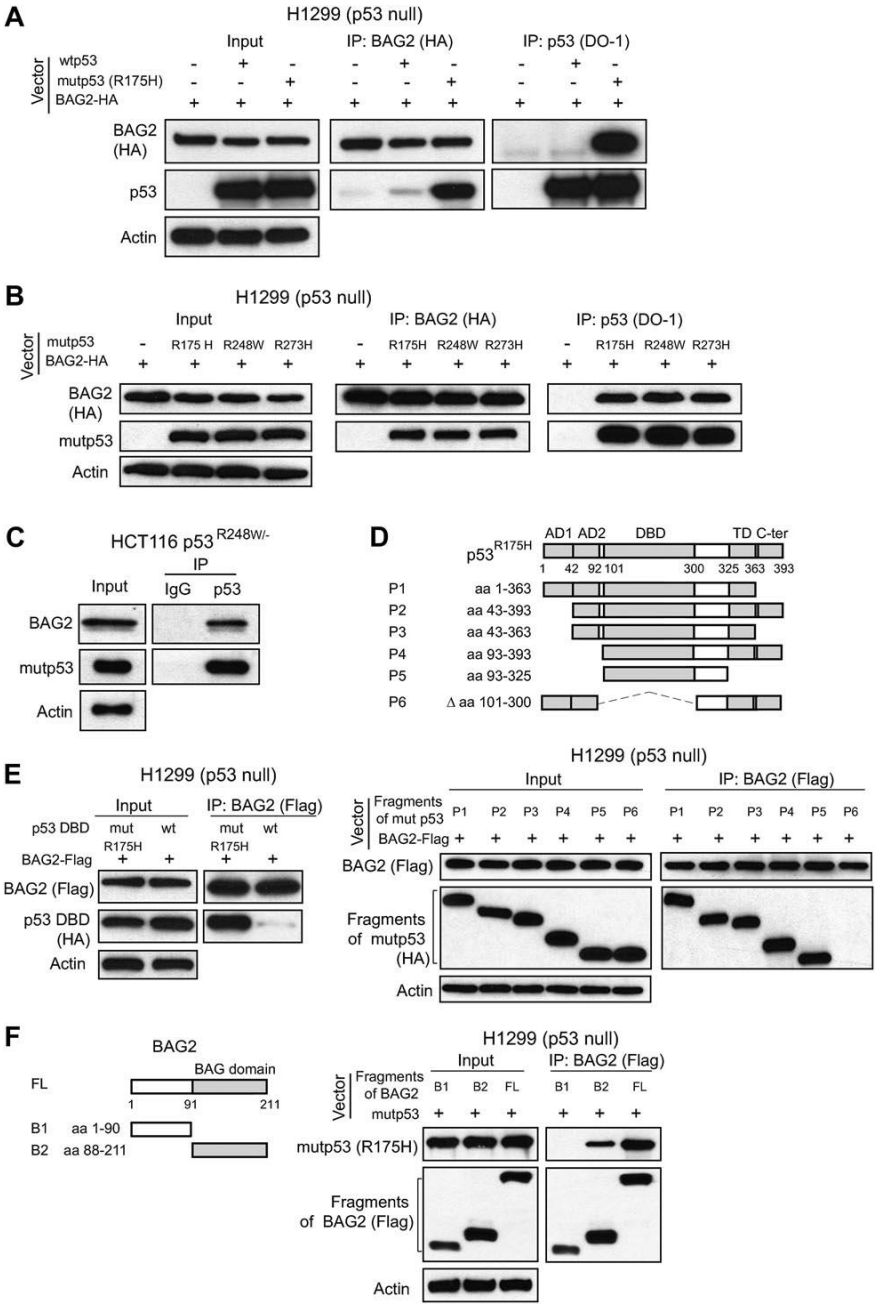
Detected in both p53 (R172H homozygous) tumor and normal tissues

Gene names	Average counts: Normal thymus	Average counts: Thymic lymphoma	Major function
Hsp70	1	66	molecular chaperone and protein folding
Hsp90	2	15.5	protein folding and quality control
Mcm7	4	11.5	initiation of eukaryotic genome replication
Pontin	4	11	DNA helicase and ATPase activities
Trim28	5	11	E3 ligase and transcription factor activities
Cct8	36	54	Chaperonin-mediated protein folding



(A) Workflow for identification of proteins interacting with the mutp53 protein. Lysate of thymic lymphomas and normal thymus from $Trp53^{R172H/R172H}$ mice were subjected to co-immunoprecipitation (co-IP) using anti-p53 (FL393) beads. Eluted proteins were separated in a 4–15% SDS PAGE gel and analyzed by LC-MS/MS. (B) The table of a list of protein candidates that interacted with mutp53 protein. (C) The interaction of mutp53 with BAG2 in thymic lymphomas of Trp53R172H/R172H mice was confirmed by co-IP assays followed by Western blot assays. Thymic lymphomas from $Trp53^{R172H/R172H}$ mice and $p53^{-/-}$ mice as well as normal thymic tissue from $Trp53^{R172H/R172H}$ mice were subjected to co-IP assays using an anti-p53 antibody.

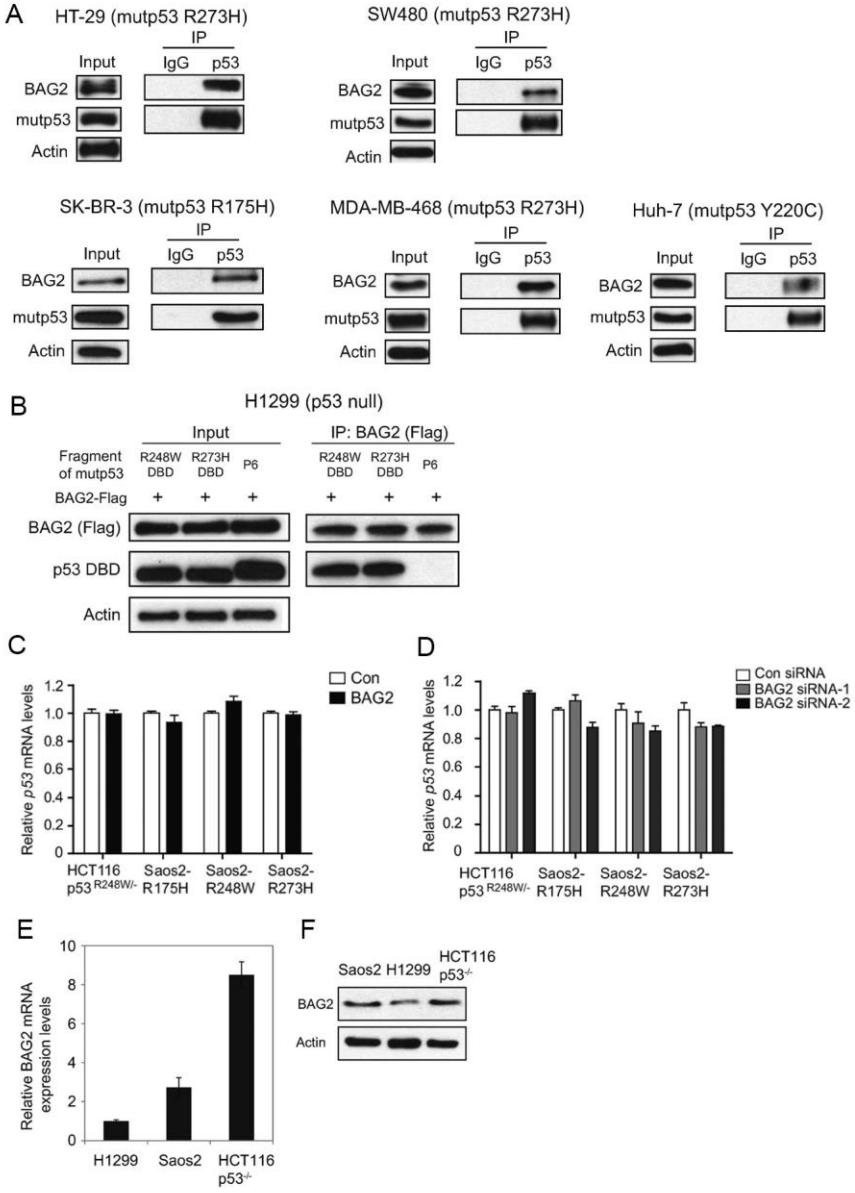
Figure 12. BAG2 is a mutp53-specific binding partner as determined by reciprocal co-IP assays in human cell lines.



(A) Ectopically expressed BAG2 preferentially interacted with mutp53 (R175H) protein compared with wild-type p53 (wtp53) protein in H1299 cells. H1299 cells were transiently transfected with vectors expressing mutp53 (R175H) or wtp53 together with HA-tagged BAG2 (BAG2-HA) expression vectors. Antibodies used for IP assays: HA for BAG2-HA and DO-1 for p53. (B) BAG2 interacted with several hotspot mutp53 proteins (R175H, R248W, and R273H) in H1299 cells. H1299 cells were transiently transfected with vectors expressing mutp53 (R175H, R248W or R273H) together with BAG2-HA expression vectors. (C) The interaction of endogenous BAG2 with

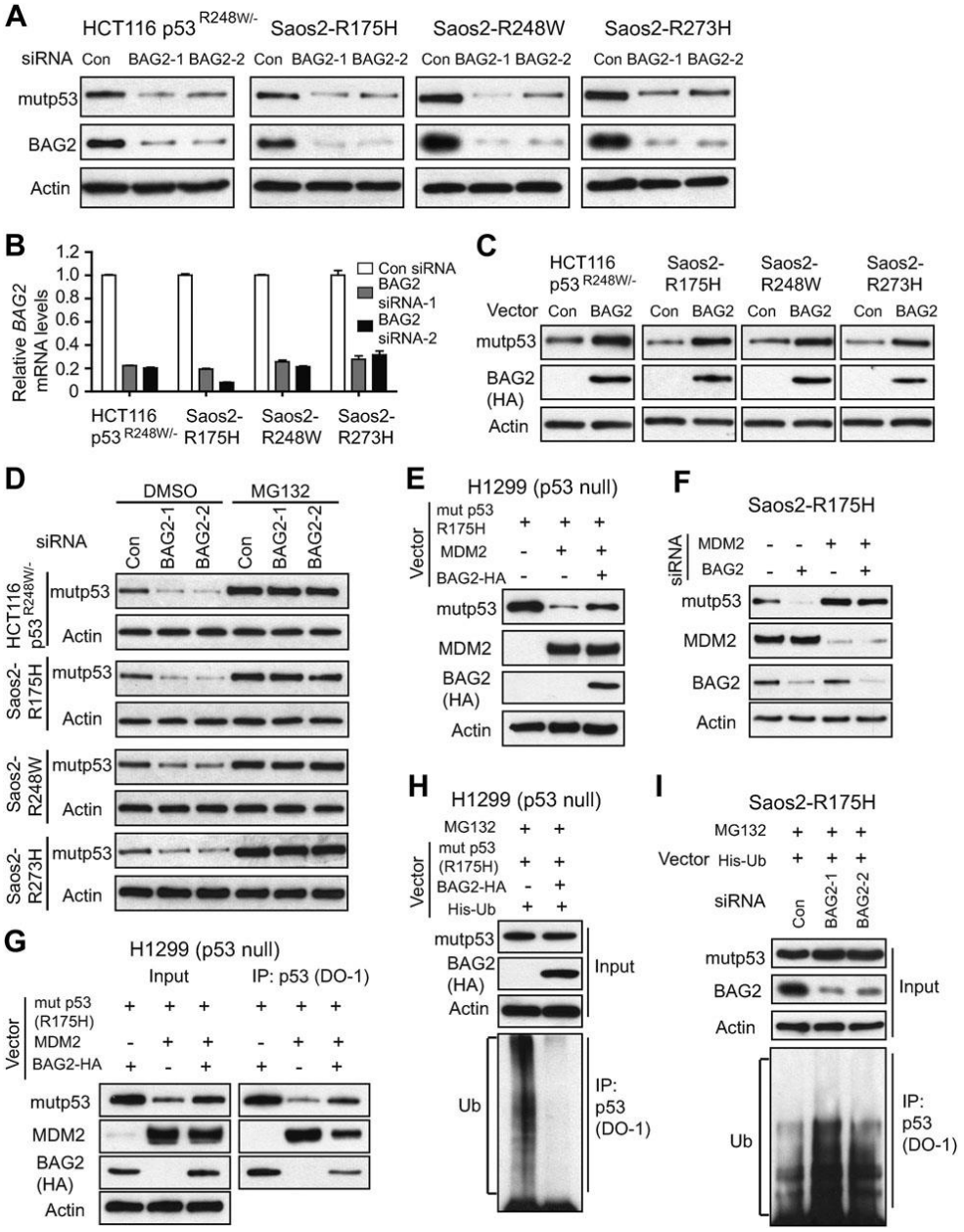
mutp53 (R248W) was observed in human colorectal cancer HCT116 $p53^{R248W/-}$ cells containing one allele of the mutant p53 gene (R248W). (D) BAG2 interacted with mutp53 DNA binding domain (DBD). Upper panel: Schematic diagram showing the domain structure of mutp53 (R175H). Lower Panel: H1299 cells were transiently transfected with expression vectors of HA-tagged mutp53 (R175H) fragments together with BAG2-Flag expression vectors. Antibodies used for IP: Flag for BAG2-Flag proteins. (E) BAG2 preferentially interacted with the DBD of mutp53 (R175H) but not wtp53 DBD. H1299 cells were transiently transfected with expression vectors of HA-tagged mutp53 (R175H) DBD or wtp53 DBD together with BAG2-Flag expression vectors. (F) Mutp53 interacted with the Bcl-2 associated athanogene (BAG) domain of BAG2. Left panel: Schematic diagram showing the domain structure of BAG2. Right panel: H1299 cells were transiently transfected with expression vectors of mutp53 (R175H) together with Flag-tagged BAG2 fragments.

Figure 13. The interaction of BAG2 with mutp53 in several human tumor cell lines.



(A) The interaction of endogenous BAG2 with mutp53 was observed in human colorectal cancer HT-29 and SW480 cell lines, human breast cancer SK-BR-3 and MDA-MB-468 cell lines and human hepatocellular carcinoma Huh-7 cells. (B) The interaction of BAG2 with mutp53 (R248W and R273H) DBD in H1299 cells. (C) Ectopic expression of BAG2 has no apparent effect on mutp53 mRNA expression levels in human cancer cells. (D) Knockdown of BAG2 has no apparent effect on mutp53 mRNA expression levels in human cancer cells. (E&F) The mRNA expression (E) and protein expression (F) levels of BAG2 in H1299, Saos2 and HCT116 $p53^{-/-}$ cells.

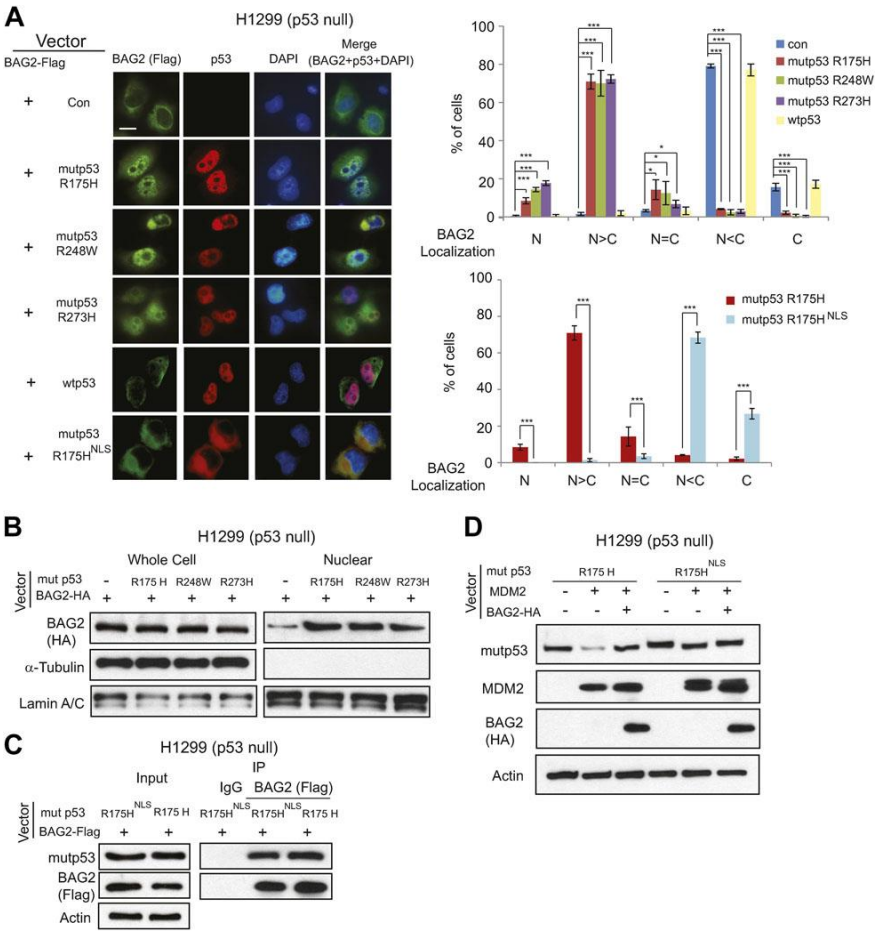
Figure 14. BAG2 promotes mutp53 protein accumulation in human cancer cells through the inhibition of the ubiquitination and degradation of mutp53 mediated by MDM2.



(A) Knockdown of endogenous BAG2 by 2 different siRNA oligos decreased the mutp53 protein levels in HCT116 p53$^{R248W/-}$ and Saos2 cells with stable ectopic expression of mutp53 (Saos2-R175H, Saos2-R248W, and Saos2-R273H). The knockdown of BAG2 by siRNA at the protein level was examined by Western blot assays. (B) The efficient knockdown of BAG2 by siRNA was confirmed at the mRNA level by real-time PCR. Data are present as mean ±SD (n = 3). (C) Ectopic expression of BAG2 by transfection of BAG2-HA expression vectors increased the mutp53 protein levels in cells. (D) Knockdown of endogenous BAG2 by siRNA decreased the

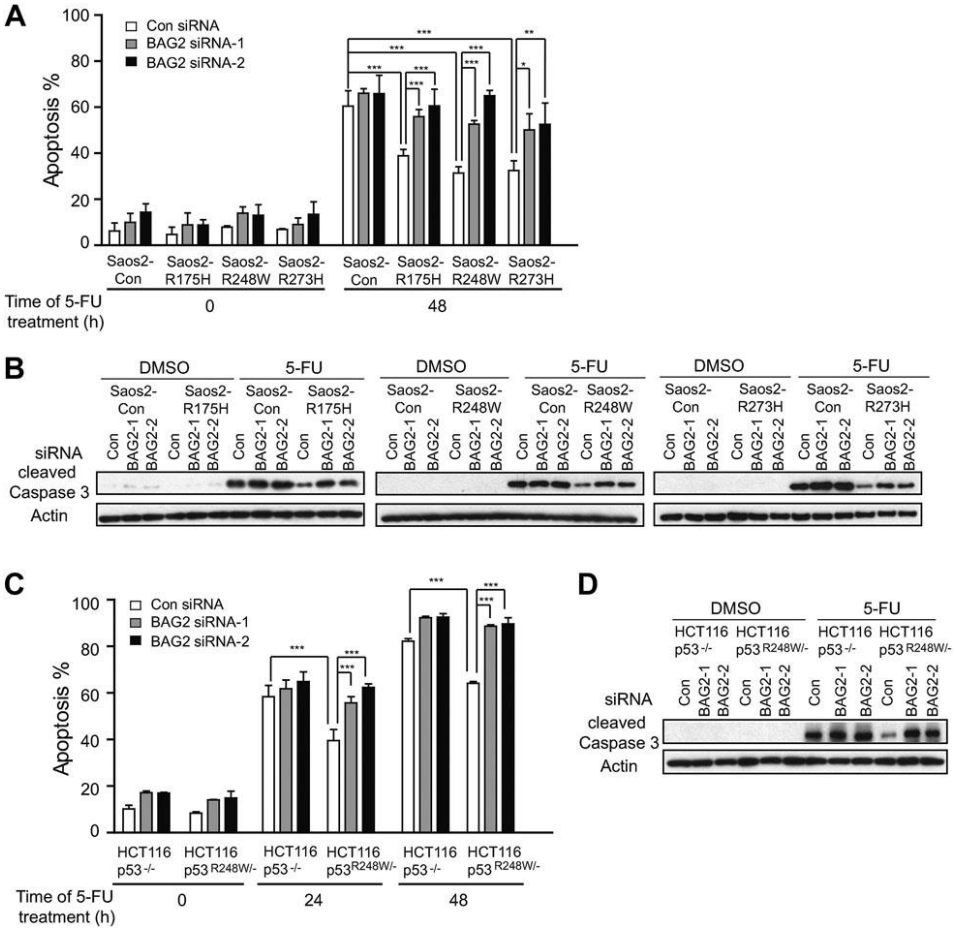
mutp53 protein levels in HCT116 $p53^{R248W/-}$, Saos2-R175H, Saos2-R248W and Saos2-R273H cells but not in these cells treated with the proteasome inhibitor MG132 (40 μM for 6 hr). (E) BAG2 inhibited the degradation of mutp53 (R175H) mediated by MDM2 in H1299 cells. Indicated combination of expression vectors of BAG2-HA, mutp53 (R175H), MDM2 were transfected into the cells. (F) Knockdown of MDM2 abolished the effect of BAG2 knockdown on the mutp53 protein level. Knockdown of endogenous BAG2 decreased mutp53 protein levels in Saos2-R175H cells but not in cells with knockdown of endogenous MDM2. (G) BAG2 reduced the interaction of mutp53 with MDM2 in H1299 cells as determined by IP assays. Indicated combination of expression vectors of BAG2-HA, mutp53 (R175H) and MDM2 were transfected into the cells. Antibodies used for IP: DO-1 for p53. (H) Ectopic BAG2 expression decreased the ubiquitination levels of mutp53 in H1299 cells. Cells were transfected with an indicated combination of expression vectors of BAG2-HA, mutp53 (R175H), His-ubiquitin (His-Ub), followed by MG132 treatment. Mutp53 ubiquitination was determined by IP using DO-1 antibody (for mutp53) followed by Western blot assays using an anti-Ub antibody. (I) Knockdown of endogenous BAG2 increased the ubiquitination levels of mutp53 in Saos2-R175H cells. Cells were transfected with an indicated combination of BAG2 siRNAs and expression vectors of His-Ub followed by MG132 treatment.

Figure 15. Mutp53 promotes the nuclear translocation of BAG2.



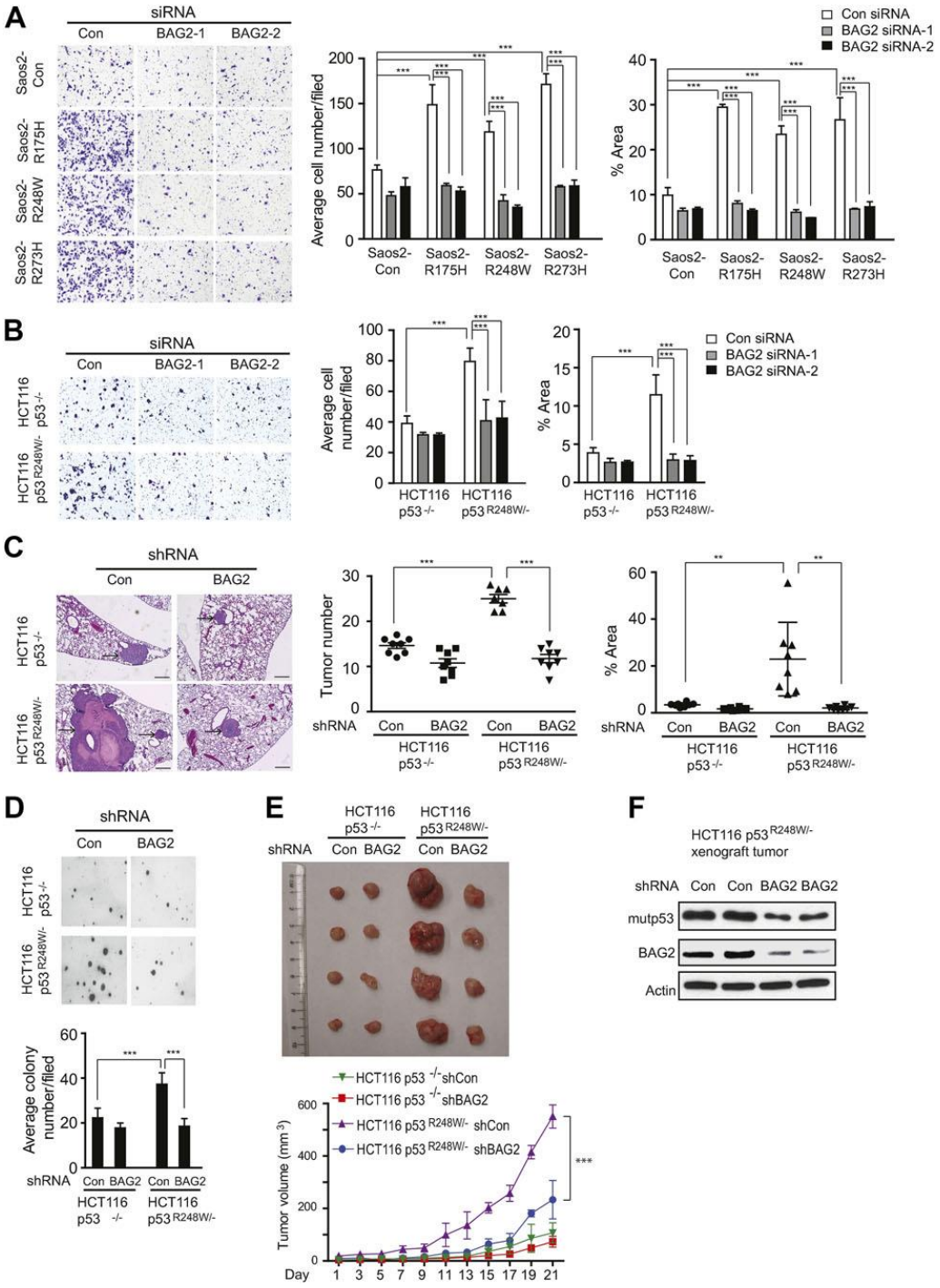
(A) H1299 cells were transiently transfected with vectors expressing BAG2-HA together with or without expression vectors of mutp53 (R175H, R248W, R273H, or R175HNLS) and wtp53. The protein localization of BAG2 and p53 in cells was determined by immunofluorescence (IF) staining. The antibody used for IF: Flag for BAG2-Flag and FL393 for p53. Nuclei were stained with DAPI. Left panels: representative IF images. Scale bar: 10 μm. Right panels: quantification of the subcellular distribution of BAG2 in 200 cells for each independent experiment. Data are present as mean ±SD (n = 4). *p < 0.05; ***p < 0.001. (B) Mutp53 promotes the nuclear translocation of BAG2 in H1299 cells as determined by Western blot assays. The protein levels of BAG2 were determined in whole cell lysates and nuclear extracts prepared from H1299 cells transfected with vectors expressing BAG2-HA together with or without mutp53 (R175H, R248W or R273H). (C) BAG2 interacted with mutp53NLS (R175HNLS) as determined by co-IP assays. H1299 cells were transfected with vectors expressing BAG2-Flag and mutp53 R175H or mutp53 R175HNLS. (D) MDM2 had a much reduced effect on degradation of mutp53NLS compared with mutp53 (R175H). While BAG2 inhibited the degradation of mutp53 (R175H) mediated by MDM2, it had no obvious effect on mutp53NLS protein levels in H1299 cells transfected with vectors expressing BAG2-HA, MDM2, and mutp53NLS.

Figure 16. BAG2 promotes mutp53 gain-of-function (GOF) in chemoresistance.



(A, B) BAG2 knockdown increased 5-FU-induced apoptosis in Saos2 cells in a largely mutp53-dependent manner. The endogenous BAG2 was knocked down by siRNA in Saos2-Con, Saos2-R175H, Saos2-R248W and Saos2-R273H cells followed by 5-FU treatment (4 mM) for 48 hr. In A, Annexin V assays were used to determine the percentage of apoptotic cells. Data are present as mean ±SD, n = 4. *p < 0.05; **p < 0.01; ***p < 0.001. In B, the levels of cleaved Caspase 3, which reflect the degree of apoptosis of cells, were determined by Western blot assays. (C, D) BAG2 knockdown increased 5-FU-induced apoptosis in HCT116 p53R248W/− cells but had a limited effect in HCT116 $p53^{-/-}$ cells as determined by Annexin V assays (C) and Western blot assays for the cleaved Caspase 3 protein levels (D).

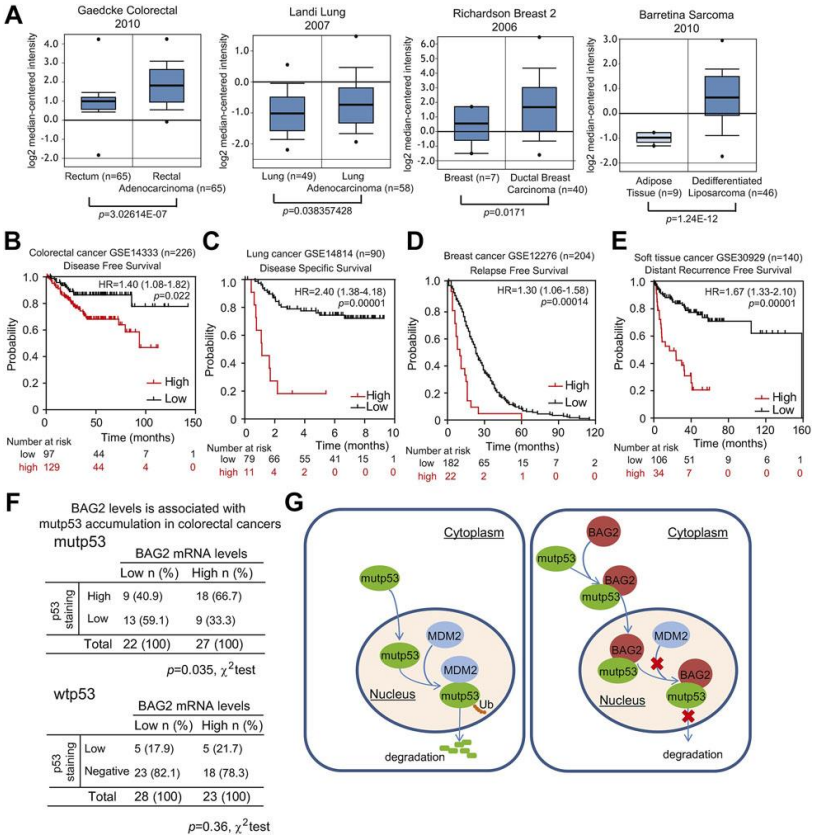
Figure 17. BAG2 promotes mutp53 GOF in promoting metastasis and tumor cell growth.



(A) Knockdown of endogenous BAG2 by 2 siRNA oligos preferentially inhibited the migration ability of Saos2-R175H, Saos2-R248W and Saos2-R273H cells compared with Saos2-Con cells as determined by transwell assays. Left panel: representative images form a portion of the field. Right panel: quantification of average number and area of migrated cells/field. (B) Knockdown of endogenous BAG2 preferentially inhibited the migration ability of HCT116 $p53^{R248W/-}$ cells

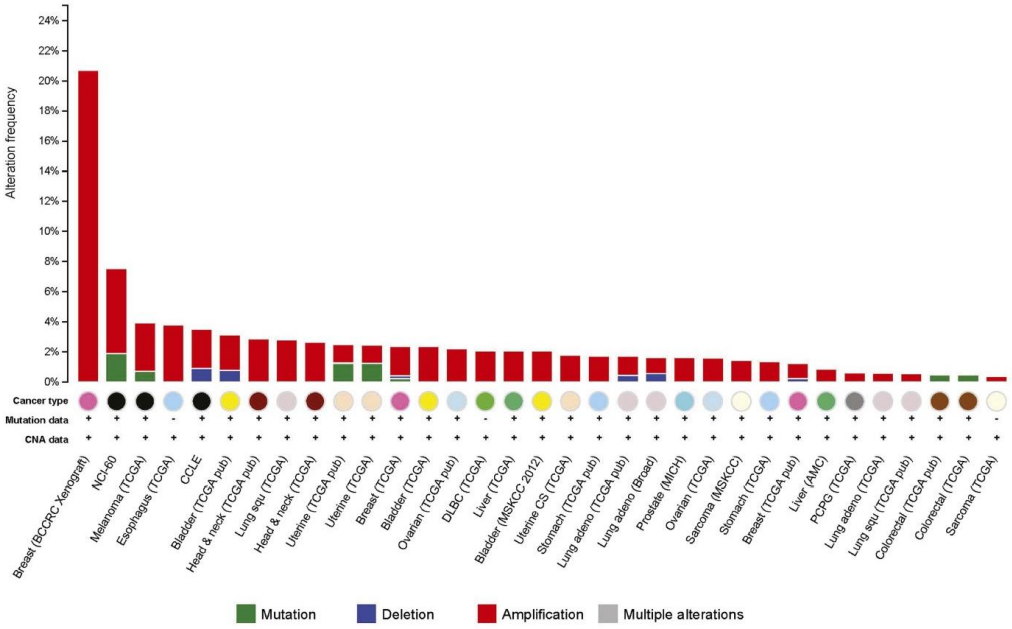
compared with HCT116 $p53^{-/-}$cells. For A, B, date are presented as mean ±SD, n = 4. ***p < 0.001. (C) BAG2 knockdown greatly inhibited lung metastasis of HCT116 $p53^{R248W/-}$ cells but had a limited effect on HCT116 $p53^{-/-}$ cells in vivo. HCT116 $p53^{R248W/-}$ and HCT116 $p53^{-/-}$ cells stably infected with shRNA against BAG2 and their control cells were injected into the nude mice via the tail vein. The number and size of lung metastatic tumors were determined at 6 weeks after inoculation. Left panel: representative H&E images of lung sections. Scale bar: 200 μm. Middle and Right panels: quantification of average number (middle panel) and area (right panel) of lung metastatic tumors, respectively. Date are presented as mean ±SD, n = 8/group. **p < 0.01; ***p < 0.001. (D) Knockdown of BAG2 by shRNA preferentially inhibited the anchorage-independent growth in HCT116$p53^{R248W/-}$ cells but not HCT116 $p53^{-/-}$ cells. Upper panel: representative images of cell colonies in soft agar. Lower panel: quantification of an average number of colonies/field. Date are presented as mean ±SD, n = 4. ***p < 0.001. (E) BAG2 knockdown inhibited the growth of HCT116 xenograft tumors in a largely mutp53-dependent manner. HCT116 $p53^{R248W/-}$ and HCT116 $p53^{-/-}$ cells stably infected with shRNA against BAG2 and their control cells were employed for xenograft tumor formation in nude mice. Upper panel: A representative image of xenograft tumors. Lower panel: growth curves of xenograft tumors. Tumor volumes are presented as mean ±SD, n = 6/group. ***p < 0.001. (F) BAG2 knockdown decreased mutp53 protein levels in HCT116 $p53^{R248w/-}$ xenograft tumors as determined by Western blot assays.

Figure 18. BAG2 is overexpressed in many human tumors and high levels of BAG2 are associated with mutp53 protein accumulation in human tumors.



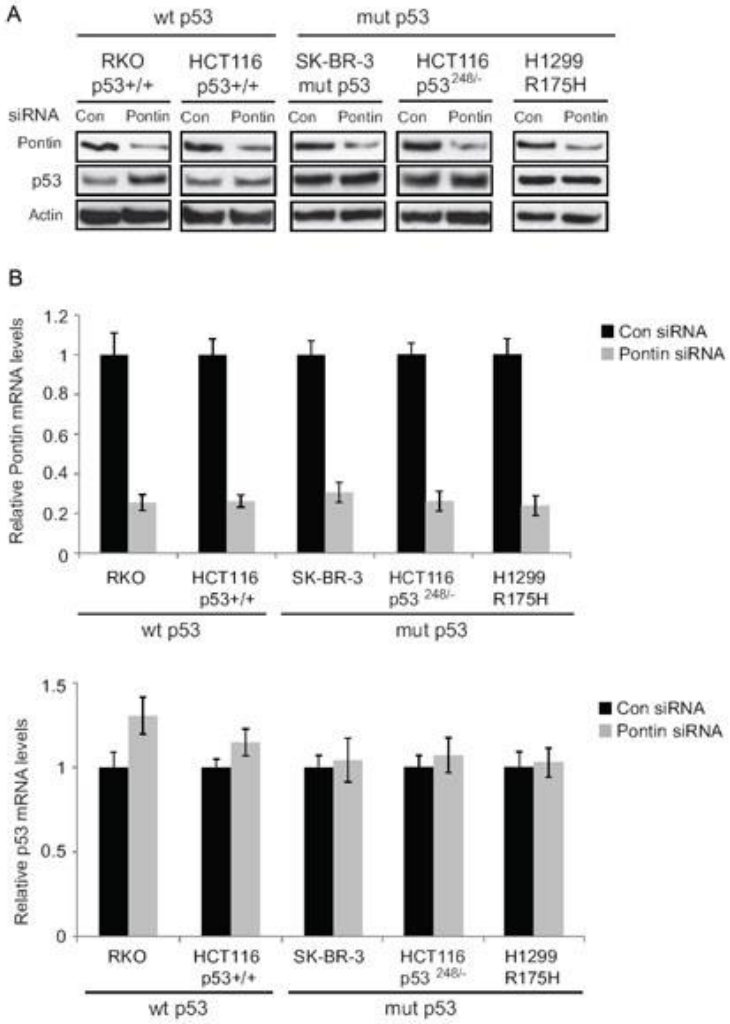
(A) BAG2 mRNA levels are elevated in human cancers, including colorectal cancers, lung cancers, breast cancers, and sarcomas. BAG2 mRNA levels in normal and cancer tissues are presented as box plots based on data in four different datasets obtained from the Oncomine database. The expression levels of BAG2 are expressed in terms of a log2 median-centered intensity which is calculated by normalizing the intensity of BAG2 probe to the median of the probe intensities across the entire array. (B–E) High levels of BAG2 are associated with poor prognosis in cancer patients. Kaplan–Meier curves indicate the disease-free survival of 226 colorectal cancer patients (B), the disease-specific survival of 90 lung cancer patients (C), the relapse-free survival of 204 breast cancer patients (D) and the distant recurrence-free survival of 140 soft tissue cancer patients (E). The survival information and expression levels of BAG2 were obtained from the public available databases (GSE14333 for B, GSE14814 for C, GSE12276 for D, and GSE30929 for E) and analyzed by PrognoScan, a web-based platform evaluating the prognostic power of gene expression levels. (F) BAG2 overexpression correlates with mutp53 protein accumulation (p = 0.036, χ2 test) but not wtp53 protein accumulation in human colorectal cancers. BAG2 mRNA levels were determined in human colorectal cancers and normalized with β-actin. (G) Schematic model depicting that mutp53 interacts with BAG2 and promotes BAG2 nuclear translocation to inhibit MDM2-mediated mutp53 protein degradation, which in turn promotes mutp53 protein accumulation and GOF in tumorigenesis.

Figure 19. Amplification of the BAG2 gene was observed in many human tumors.



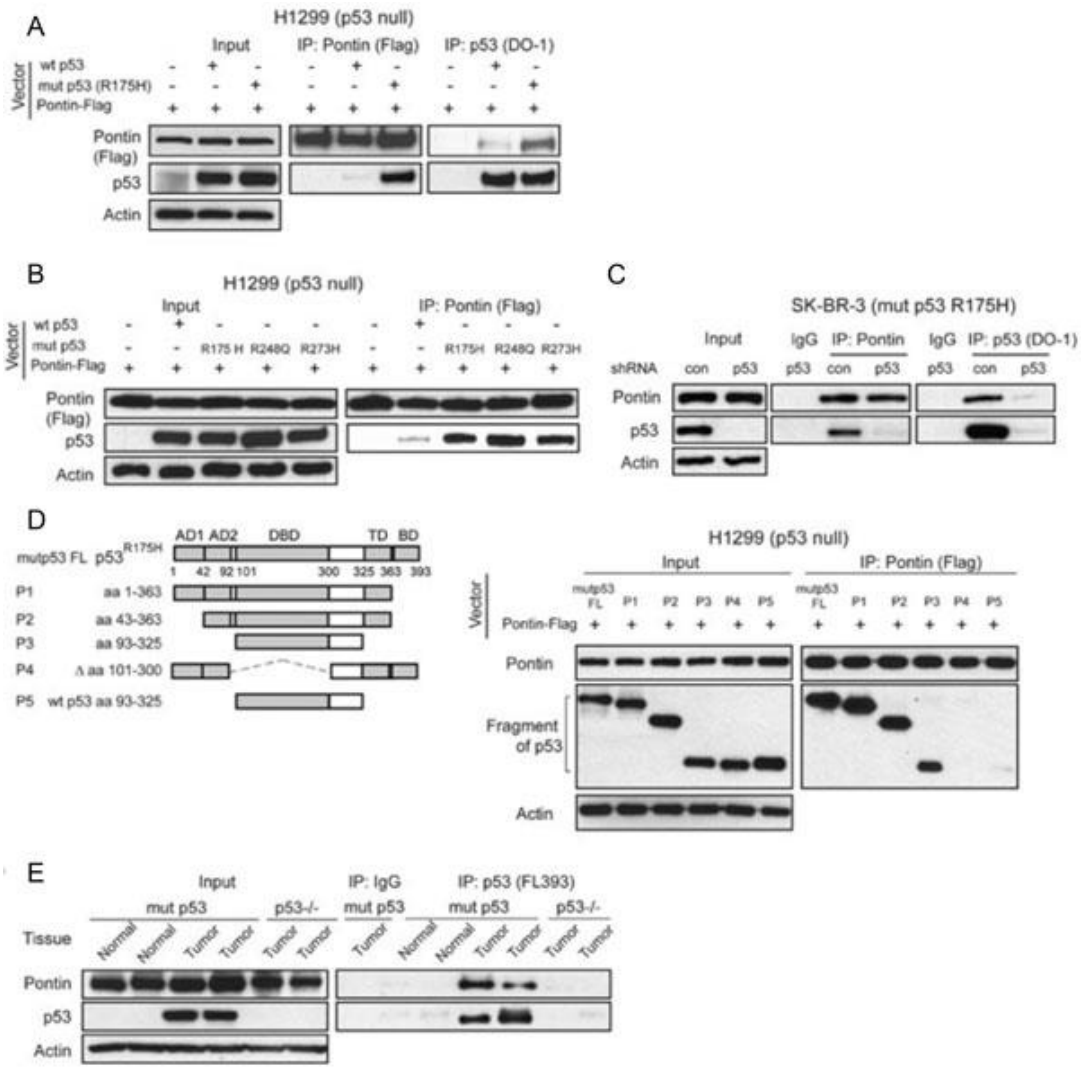
Percentage of tumors is showing genetic alterations, including amplification (red), mutation (green) and deletion (blue), in the BAG2 gene in different human tumors. Data were obtained from the cBioportal for Cancer Genomics (http://www.cbioportal.org).

Figure 20. Knockdown of Pontin has no apparent effect on mutp53 levels.



(A&B). Knockdown of Pontin by siRNA slightly increased wtp53 levels in RKO p53+/+ cells but had no obvious effect on wtp53 levels in HCT116 $p53^{+/+}$ cells, whereas knockdown of Pontin had no obvious effect on mutp53 levels in SK-BR-3, HCT116 p53R248W/- and H1299-R175H cells. The levels of p53 in these cells were determined at both protein (A) and mRNA (B, lower panel) levels by Western blot assays and TaqMan real-time PCR, respectively. The mRNA expression levels were normalized to actin. The knockdown of Pontin was confirmed at both protein (A) and mRNA (B, upper panel) levels. Data are presented as mean±SD (n=3).

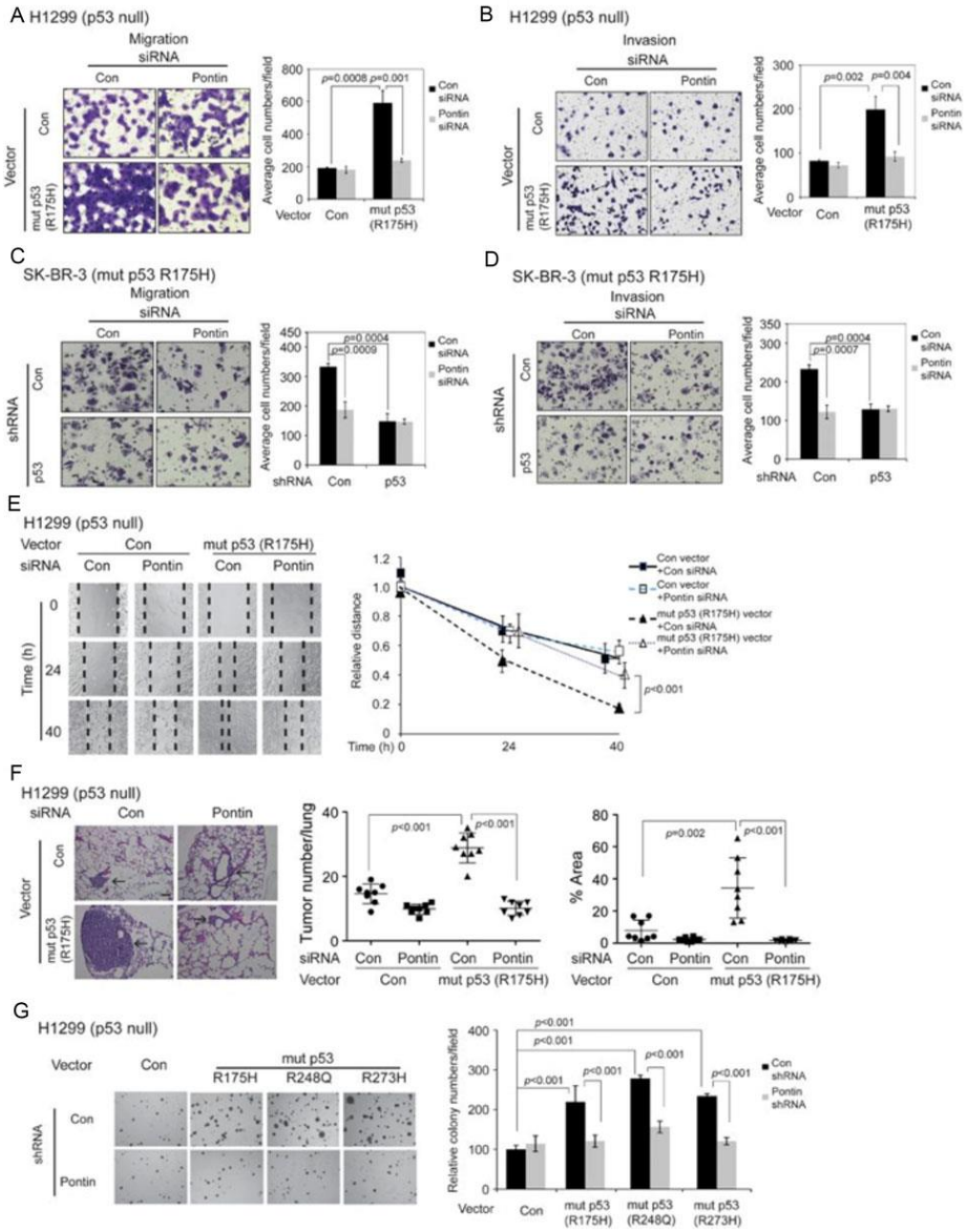
Figure 21. Pontin is a novel mutp53-interacting protein in both human cells and mutp53$^{R172H/R172H}$ tumors.



(A) Pontin preferentially bound to ectopically expressed mutp53 (R175H) protein compared with wtp53 in human p53-null H1299 cells. H1299 cells transiently transfected with expression vectors of wtp53 or mutp53 (R175H) along with Pontin-Flag expression vectors. Antibodies used for IP: Flag for Pontin-Flag and DO-1 for p53. (B) Pontin interacted with several hotspot mutp53 proteins. H1299 cells were transiently transfected with expression vectors of wtp53 or mutp53 (R175H, R248Q or R273H) along with Pontin-Flag expression vectors. (C) The interaction of endogenous Pontin with mutp53 (R175H) was observed in human SK-BR-3 cells but not SK-BR-3-p53sh cells with stable knockdown of endogenous mutp53 by shRNA. (D) Pontin interacted with mutp53 at its DBD. Left panel: schematic diagram showing the domain structure of mutp53 and the fragments of mutp53 and wtp53 proteins used for co-IP assays. Right panel: H1299 cells transiently transfected with expression vectors of different fragments of mutp53 and wtp53 along with Pontin-Flag expression vectors. Antibodies used for IP: Flag for Pontin-Flag. (E) The interaction of Pontin with mouse mutp53 protein was observed in thymic lymphomas from p53$^{R172H/R172H}$ mice expressing

high levels of mutp53 protein but not normal thymic tissues from $p53^{R172H/R172H}$ mice expressing very low levels of mutp53 protein or thymic lymphomas from $p53^{-/-}$ mice. Tumors and normal thymic tissues were subjected to co-IP assays using an anti-p53 (FL-393) antibody.

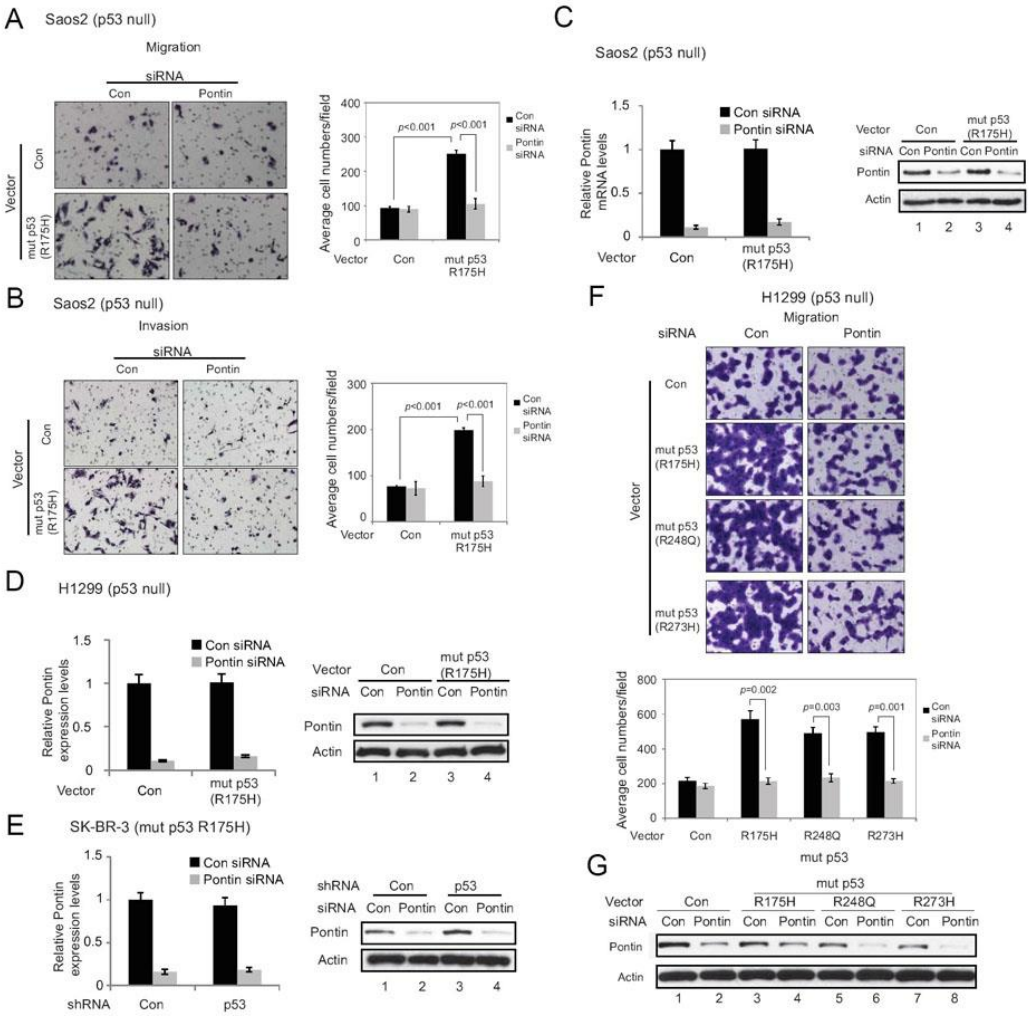
Figure 22. Knockdown of Pontin decreases mutp53 GOF in migration, invasion and anchorage-independent growth.



(A) Knockdown of Pontin by siRNA inhibited migration of H1299-R175H but not H1299-Con cells as determined by transwell assays. Left panel: representative images from a portion of the field; right panel: quantification of an average number of migrated cells/field (× 200 magnification). Two different siRNA oligos were used, and very similar results were obtained. For the sake of clarity, in (A–E), results from one siRNA were presented. (B) Knockdown of Pontin decreased invasion of H1299-R175H cells but not H1299-Con cells as determined by transwell assays in chambers coated with matrigel. (c and d) Pontin knockdown reduced the migration (C) and invasion (D)

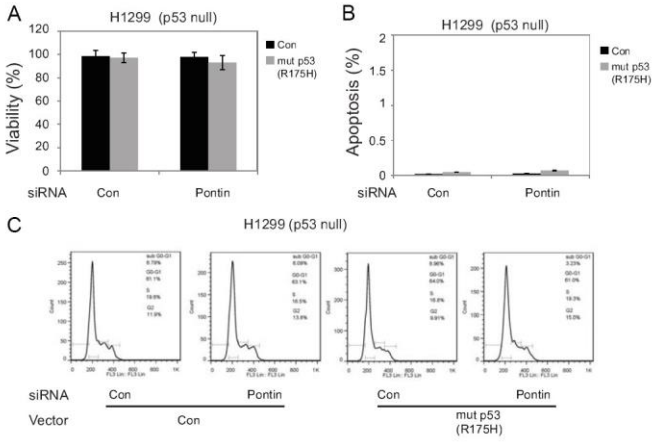
abilities of SK-BR-3-Consh cells but not SK-BR-3-p53sh cells. SK-BR-3 cells stably transfected with control shRNA (Consh) or shRNA against p53 (p53sh) were transiently transfected with control siRNA (Con) or siRNA against Pontin. (E) Pontin knockdown reduced the motility of H1299-R175H but not H1299-Con cells as determined by scratch motility assays. (F) Pontin knockdown significantly reduced lung tumors formed by H1299-R175H cells but not H1299-Con cells. H1299-Con and H1299-R175H cells with or without knockdown of Pontin were injected into nude mice via the tail vein. The number and area of lung tumors were determined after 6 weeks (n=8 per group). Left panel: representative H&E images of lung sections. Black arrows indicate tumors. Scale bar: 100 μm. (G) Pontin knockdown inhibited anchorage-independent cell growth on soft agar of H1299-mutp53 (R175H, R248Q, and R273H) cells but not H1299-Con cells. H1299-mutp53 and H1299-Con cells were transfected with shRNA against Pontin or control shRNA before assays. Left panel: representative images; right panel: relative colony numbers of cells on soft agar. For A–G, data are presented as mean±S.D. (n=3 for A–E, G; n=8 for F).

Figure 23. Knockdown of Pontin decreases mutp53 GOF in migration, invasion in cancer cells with mutp53.



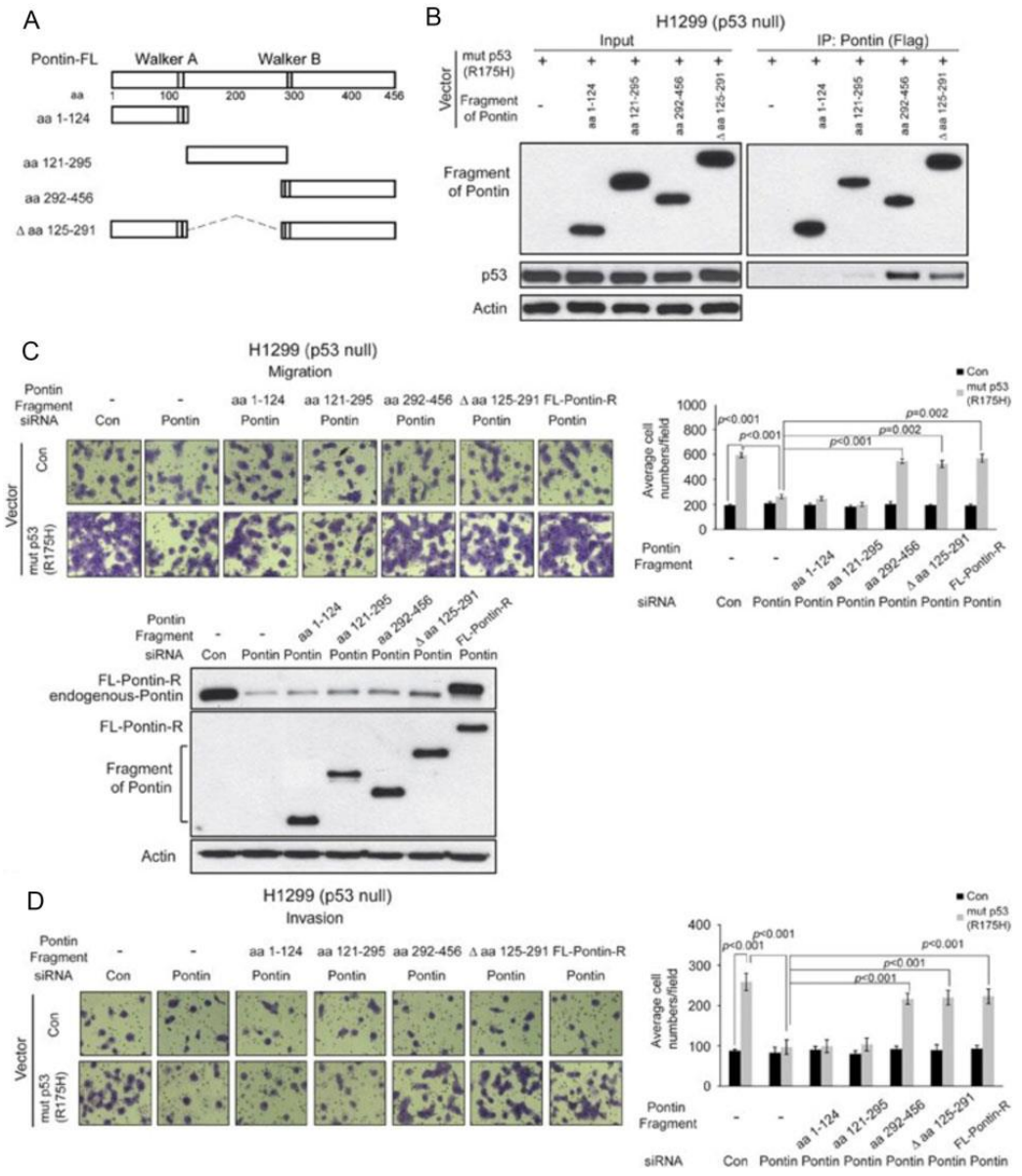
(A&B) Knockdown of Pontin by siRNA inhibited migration (A) and invasion (B) of Saos2-R175H but not p53-null Saos2-Con cells as determined by transwell assays. (C, D&E) The knockdown of Pontin in Soas2 (C), H1299 (D) and SK-BR-3 (E) cells was confirmed at both mRNA (left panel) and protein (right panel) levels by TaqMan real-time PCR and Western blot assays, respectively. (F) Knockdown of Pontin by siRNA inhibited migration of H1299 cells with expression of different mutations in the p53 gene (R175H, R248Q, and R273H) but not H1299-Con cell as determined by transwell assays. (G) The knockdown of Pontin was confirmed at the protein level by Western blot assays.

Figure 24. H1299-Con and H1299-R175H cells with or without knockdown of Pontin display similar cell viability, apoptosis, and cell cycle distribution.



(A) Cell viability was measured by the trypan blue exclusion method in a Vi-CELL cell counter. (B) Apoptosis was measured by Annexin V staining followed by the analysis in flow cytometry. (C) Cell cycle distribution was measured by propidium iodide staining in flow cytometry. Cells were transfected with control siRNA or siRNA against Pontin, and cell viability, apoptosis, and cell cycle distribution were analyzed at 24 h after transfection. No obvious difference in cell viability, apoptosis, and cell cycle distribution was observed among these cells. Data are presented as mean±SD (n=3).

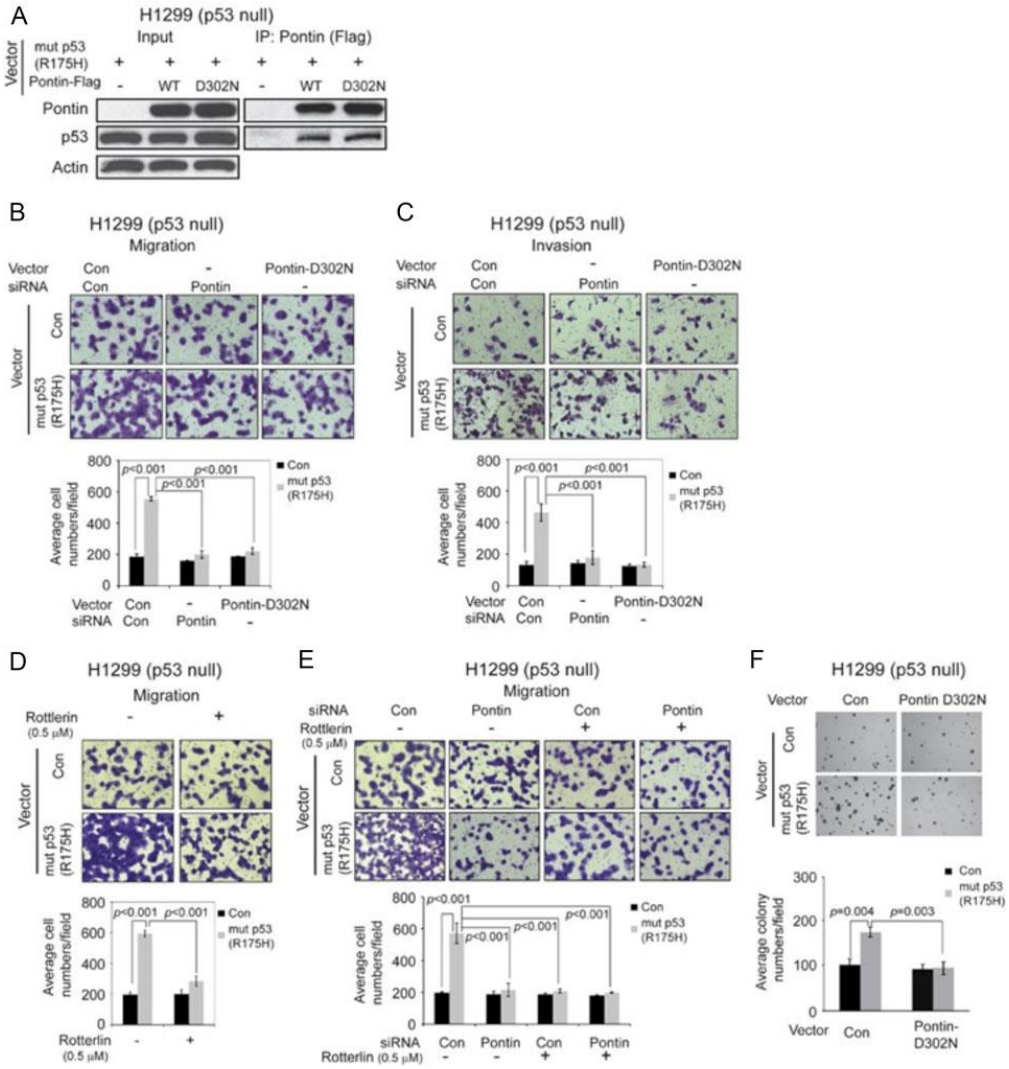
Figure 25. The C-terminus of Pontin is required for Pontin–mutp53 interaction and promotes mutp53 GOF.



(A) Schematic diagram showing the domain structure of Pontin and the fragments of Pontin protein used for co-IP assays. (B) Pontin fragments containing the C-terminus of Pontin bound to mutp53 (R175H) protein as determined by co-IP assays. H1299 cells transiently transfected with expression vectors of different fragments of Pontin-Flag protein along with mutp53 (R175H) expression vectors. Antibodies used for IP: Flag for Pontin fragment-Flag. (C and D) Ectopic expression of Pontin fragments containing the C-terminus of Pontin abolished the inhibitory effect of Pontin knockdown on mutp53 GOF in migration (C) and invasion (D). H1299-Con and H1299-R175H cells with knockdown of Pontin by siRNA were transfected with expression vectors of siRNA resistant Pontin-Flag fragments or full-length Pontin-Flag (FL-Pontin-R). The migration and invasion abilities of cells were determined by transwell assays in chambers coated without or

with matrigel, respectively. For (C and D), data are presented as mean±S.D. (n=3). The knockdown of endogenous Pontin, as well as the expression of Pontin fragments and FL-Pontin-R, were determined by western blot assays using anti-Pontin (for endogenous Pontin and FL-Pontin-R) and anti-Flag (for Pontin-Flag fragments and FL-Pontin-R) antibodies (C, lower panel). For (C and D), data are presented as mean±S.D. (n=3).

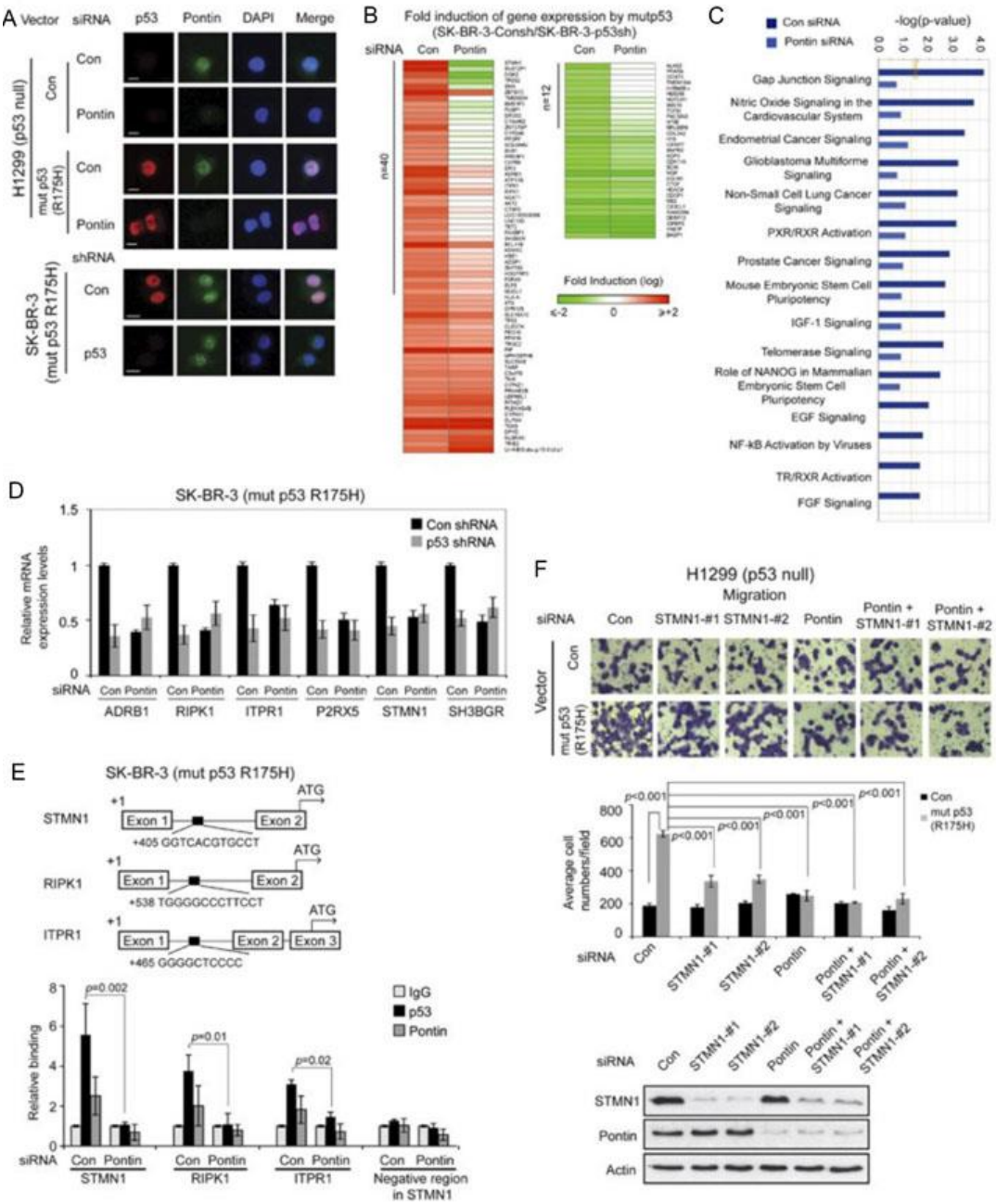
Figure 26. The ATPase activity in the Walker B motif is required for Pontin to promote mutp53 GOF in migration, invasion and anchorage-independent cell growth.



(A) The ATPase-deficient dominant-negative mutant Pontin-D302N bound to mutp53 protein. H1299 cells were co-transfected with expression vectors of mutp53 (R175H) along with wild-type Pontin or Pontin-D302N for co-IP assays. (B and C) Ectopic expression of Pontin-D302N largely blocked mutp53 GOF in migration (B) and invasion (C) as determined by transwell assays. H1299-Con and H1299-R175H cells were transfected with expression vectors of Pontin-D302N or control vectors. Cells were also transfected with siRNA against Pontin or control siRNA to compare the effect of Pontin-D302N with knockdown of endogenous Pontin on mutp53 GOF in migration and invasion. (D) Rottlerin, a Pontin-specific small molecular ATPase inhibitor, greatly compromised mutp53 GOF in migration. H1299-Con and H1299-R175H cells were treated with Rottlerin (0.5 μM) for 48 h, and their migration abilities were determined by transwell assays. (E) Combined treatments of Rottlerin and Pontin knockdown did not have an additive or synergistic effect on downregulation of mutp53 GOF. H1299-Con and H1299-R175H cells were subjected to

following treatments: transfection with siRNA against Pontin, treatment with Rottlerin or Pontin knockdown by siRNA followed by Rottlerin treatment as indicated. The migration abilities of cells were determined by transwell assays. (F) Ectopic expression of Pontin-D302N inhibited anchorage-independent cell growth on soft agar of H1299-R175H cells but not H1299-Con cells. H1299-R175H and H1299-Con cells were transfected with control or Pontin-D302N expression vectors. For (B–F), data are presented as mean±S.D. (n=3).

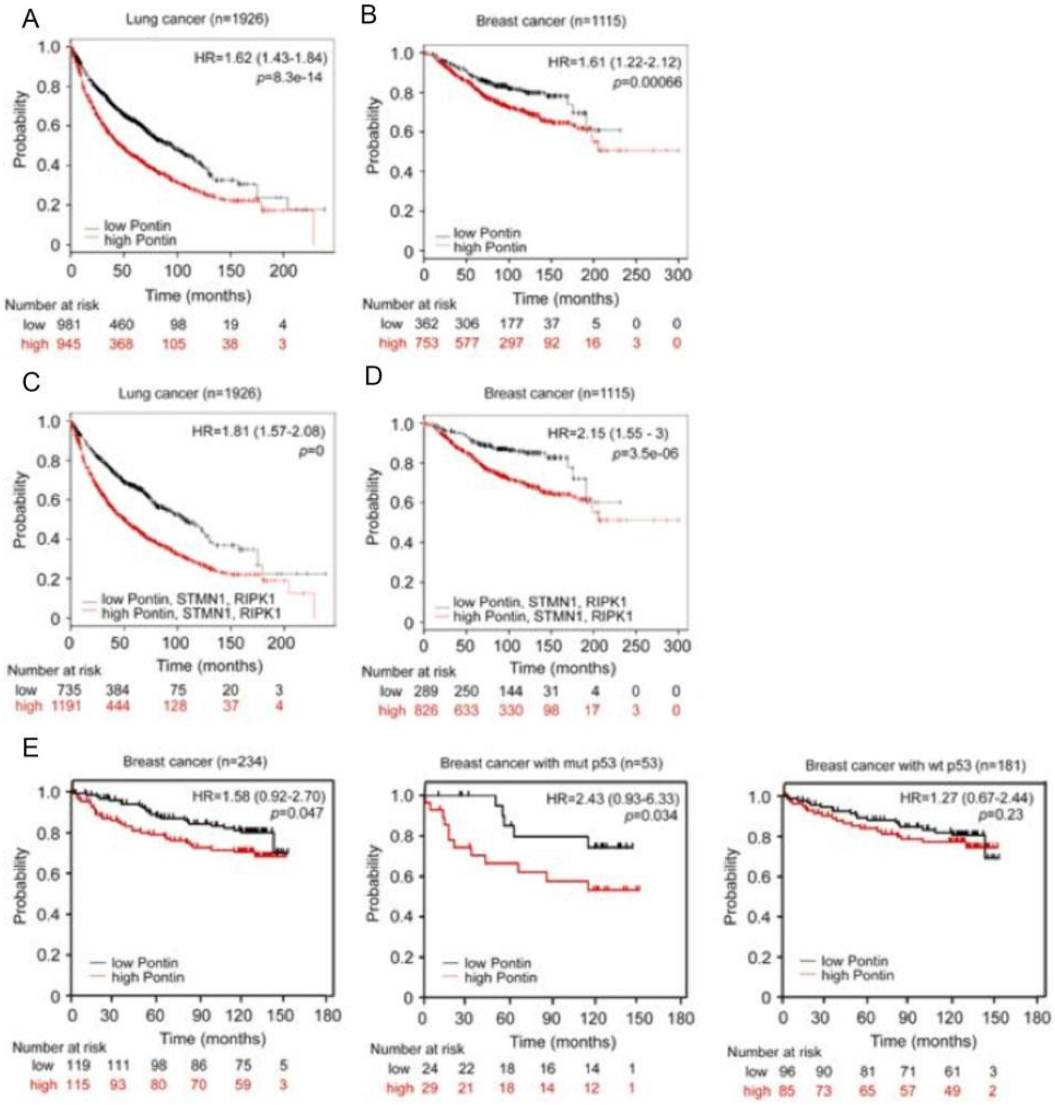
Figure 27. The interaction of Pontin with mutp53 is crucial for the transcriptional regulation of mutp53 toward a group of genes.



(A) Pontin was predominantly colocalized with mutp53 in the nucleus in H1299-R175H and SK-BR-3 cells as determined by IF staining. Anti-p53 and anti-Pontin antibodies were used for IF. Nuclei were stained with DAPI. Scale bar: 10 μM. (B) Knockdown of Pontin in SK-BR-3 cells reduced the transcriptional activity of mutp53 toward a subset of genes. Heatmap represents the change of gene expression levels by mutp53 obtained from microarray data of SK-BR-3-Consh and SK-BR-3-p53sh cells with or without knockdown of Pontin. The color scale represents the fold change of gene expression levels by mutp53 (R175H). The genes that were upregulated are

presented in red and genes that were downregulated are presented in green. (C) Blue bars represent top scoring pathways regulated by mutp53 that are significantly changed in SK-BR-3 cells with or without knockdown of Pontin. Data were analyzed by using of IPA. (D) Validation of microarray data by real-time PCR. The expression levels of a list of genes, including ADRB1, PIPK1, ITPR1, P2RX5, STMN1, and SH3BGR were determined by real-time PCR in SK-BR-3-Consh and SK-BR-3-p53sh cells with or without knockdown of Pontin by siRNA. All expression levels were normalized to actin. (E) Mutp53 and Pontin bound to the DNA regions in the vicinity of the SREBP-binding site in the STMN1 gene and the NF-κB-binding sites in the RIPK1 and ITPR1 genes in SK-BR-3 cells but not SK-BR-3 cells with knockdown of Pontin. Upper panel: the putative SREBP-binding site in the STMN1 gene and the NF-κB-binding sites in the RIPK1 and ITPR1 genes. Lower panel: SK-BR-3 cells were subjected to ChIP assays using antibodies against p53, Pontin and mouse IgG antibodies, respectively. A region of the coding sequence of the STMN1 gene was included for ChIP assays as a negative control. (F) STMN1 mediated Pontin's promoting effect on mutp53 GOF in migration. Knockdown of STMN1 by siRNA reduced the migration ability of H1299-R175H cells but not H1299-Con cells. This effect of STMN1 knockdown on mutp53 GOF in migration was largely disappeared in H1299-R175H cells with knockdown of Pontin. Upper panel: representative images from a portion of the field; middle panel: quantification of an average number of migrated cells/field (200 × magnification). The knockdown of endogenous STMN1 and Pontin was determined by western blot assays (lower panel). For (D–F), data are presented as mean±S.D. (n=3).

Figure 28. High levels of Pontin are associated with poor prognosis, especially in patients with mutp53 in tumors.



(A and B). Kaplan–Meier curves indicating the overall survival of 1926 lung cancer patients (A) and 1115 breast cancer patients (B) with different expression levels of Pontin. (C and D) Kaplan–Meier curves indicating the overall survival of 1926 lung cancer patients (C) and 1115 breast cancer patients (D) with different expression levels of Pontin, STMN1, and RIPK1. In (A–D), the overall survival information and expression levels of Pontin, STMN1, and RIPK1 of patients were obtained from the KM plotter (2015 version for lung cancer and 2014 version for breast cancer). The Affymetrix IDs used for analysis are: 201614_s_at for Pontin; 217714_x_at for STMN1 and 209941_at for RIPK1. Tumors with high levels of Pontin are associated with poor survival in both lung and breast cancer patients. Tumors with high levels of Pontin, STMN1, and RIPK1 are associated with poorer survival in both lung and breast cancer patients. (E) Kaplan–Meier curves indicating the overall survival of breast patients with different expression levels of Pontin in tumors containing mutp53 or wtp53. High levels of Pontin are associated with poor prognosis in breast

cancer patients containing mutp53 in tumors but not in patients containing wtp53 in tumors. The survival information, expression levels of Pontin and p53 mutation status were obtained from the public available database (GSE3494). Left panel: total patients in the cohort; middle panel: patients carrying mutp53 in tumors; right panel: patients carrying wtp53 in tumors.

Table 1. Major contributing causes of death in mice evaluated at the end of life.

	129SV^{sl}		C57BL6	
	R72	P72	R72	P72
	n (%)	n (%)	n (%)	n (%)
Neoplasm	26 (16.3)	39 (18.3)	48 (32.9)	56 (35)
Nonspecific systemic disease	30 (20.6)	47 (22.1)	21 (14.4)	31 (19.4)
Ocular lesion	38 (23.8)	53 (24.9)	7 (4.8)	5 (3.1)
Dermatitis	5 (3.1)	4 (1.9)	34 (23.3)	43 (26.9)
Megaesophagus	15 (9.4)	24 (11.3)		
Urinary syndrome/nephropathy	18 (11.3)	21 (9.9)	9 (6.2)	4 (2.5)
Neurological disease	5 (3.1)	7 (3.3)	4 (2.7)	3 (1.9)
Others	9 (5.6)	8 (3.8)	10 (6.8)	8 (5.0)
Unknown reason	11 (6.9)	10 (4.7)	13 (8.9)	10 (6.3)

Note:

Nonspecific systemic disease: age-related or -induced lesions including cardiac and respiratory failure; sepsis and DIC; female reproductive diseases or male urogenital diseases, etc.

Ocular lesion: includes corneal ulceration and chronic keratitis

Neurologic diseases: include head tilt, paresis, paralysis and ataxia

Others: include rectal prolapse, gastrointestinal bleedings

Table 2. List of sequences of the primer sets.

Name of Target	Primer sequence
Mutp53 R175H-HA P1-3 site mutagenesis	Forward: 5'-GAG GTT GTG AGG CAC TGC CCC CAC CAT-3'
	Reverse: 5'-ATG GTG GGG GCA GTG CCT CAC AAC CTC-3'
R175H mutp53^{NLS} site mutagenesis	Forward: 5'-GTT GGG CAG TGC TGC CGC AGT GCT CCC TGG GGG CAG-3'
	Reverse: 5'-CTG CCC CCA GGG AGC ACT GCG GCA GCA CTG CCC AAC-3'
Mutp53 R175H-HA P4	Forward: 5'-GCG AAT TCA CCA TGG GCT ACC CAT ACG ATG TTC CAG ATT ACG CTC TGT CAT CTT CTG TCC CTT-3'
	Reverse: 5'-GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT-3'
p53 DBD	Forward:5'-GCG AAT TCA CCA TGG GCT ACC CAT ACG ATG TTC CAG ATT ACG CTC TGT CAT CTT CTG TCC CTT-3'
	Reverse: 5'-GCG AAT TCT CAT CCA TCC AGT GGT TTC TT-3'
Mutp53 R175H-HA, P6 (Δaa 101–300)	Forward 1: 5'-GCG AAT TCA CCA TGG GCT ACC CAT ACG ATG TTC CAG ATT ACG CTG AGG AGC CGC AGT CAG ATC C-3′
	Reverse 1: 5'-CTT AGT GCT CCC TGG CTG GGA AGG GAC AGA-3'
	Forward 2: 5'-TCT GTC CCT TCC CAG CCA GGG AGC ACT AAG-3'
	Reverse 2: 5'-GAT CGA ATT CTC AGT CTG AGT CAG GCC CTT-3'
BAG2-Flag	Forward: 5'-CGG AAT TCA TGG CTC AGG CGA AGA-3'
	Reverse: 5'-CGG GAT CCA TTG AAT CTG CTT TCA GCA T-3'
BAG2 B1-Flag	Forward: 5'-CGG AAT TCA TGG CTC AGG CGA AGA-3'
	Reverse: 5'-CGG GAT CCT CTT CCC ATC AAA CGG TT-3'
BAG2 B2-Flag	Forward:5'-CGG AAT TCA CCA TGG GAA GAA CTC TCA CCG TT-3'
	Reverse: 5'-CGG GAT CCA TTG AAT CTG CTT TCA GCA T-3'
Pontin siRNA-R site mutagenesis	Forward: 5'-CCA AGA TGT GAC CTT GCA CGA TTT AGA CGT GGC TAA TGC GCG GCC C-3'
	Reverse: 5'-GGG CCG CGC ATT AGC CAC GTC TAA ATC GTG CAA GGT CAC ATC TTG G-3′
R175H mutation site mutagenesis	Forward: 5'-GAG GTT GTG AGG CAC TGC CCC CAC CAT-3'
	Reverse: 5'- ATG GTG GGG GCA GTG CCT CAC AAC CTC-3'
SREBP binding site in STMN1	Forward: 5′-GAGAATGGGGAGCTGGTTCG-3′
	Reverse: 5'-GACCACACTCTGAGCACCAA-3'
NF-κB-binding sites in RIPK1	Forward: 5'-ATCCGAGCACTCTTACCCATTCTA-3'
	Reverse: 5'-GTCACCTCTTCGCAAACTCCTG-3'
NF-κB-binding sites in RIPK1	Forward: 5'-CAGAAGTTTTTGCCCCGCTC-3'
	Reverse: 5'-GGAGCGCAAGAGAGGGATAC-3'

Table 3. List of siRNA oligos.

Si-BAG2	5′-GUU GGC UUU AGC GUU GAU CUU CGC CUG-3′
	5′-GUG UCA GUA GAA ACA AUU AGA AAC C-3′
Si-Pontin	5′-GCA UGA CUU GGA UGU GGC UAA UGC-3′
	5′-GGA CCU CCU GGA ACU GGC AAG ACA G-3′
Si-STMN1	5′-AGA ACC GAG AGG CAC AAA UGG CUG C-3′
	5′-GCA GAA GAA AGA CGC AAG UCC CAT G-3′

siRNA oligos against BAG2 were purchased from IDT (Coralville, Iowa).

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