P53 ISOFORMS AND CELLULAR SENESCENCE IN BRAIN CANCER AND RADIOTHERAPY

by

Jessica Beck

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THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Curtis Harris, Co-Chair

Laboratory of Human Carcinogenesis, National Cancer Institute

Dr. Margaret Miller, Co-Chair

Department of Comparative Pathobiology

Dr. Susan Mendrysa

Department of Basic Medical Sciences

Dr. Mark Simpson

Molecular Pathology Unit, National Cancer Institute

Dr. Jose Ramos-Vara

Department of Comparative Pathobiology

Approved by:

Dr. Sanjeev Narayanan

I dedicate this dissertation to my family.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
Gy	Gray
Hp1γ	Heterochromatin protein 1 gamma
HR	Homologous recombination
IGF-1	Insulin-like growth factor 1
IL	Interleukin
iPSC	induced pluripotent stem cell
NGF	Nerve growth factor
NHEJ	Non-homologous end joining
NOS2	Nitric oxide synthase 2
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PDGF	Platelet-derived growth factor
SA-β-gal	Senescence-associated β-galactosidase
SASP	Senescence-associated secretory phenotype
SRSF	Serine/arginine-rich protein-specific kinase
53BP1	p53-binding protein 1
γH2AX	Phosphorylated H2AX

ABSTRACT

In addition to the canonical full-length p53 (FLp53), the TP53 gene produces twelve protein isoforms through alternative RNA splicing or initiation of transcription and translation. Two of these isoforms, Δ133p53α and p53β, have been identified as endogenous regulators of cellular senescence. Cellular senescence is a durable cell cycle arrest that inhibits the continued replication of aged and DNA-damaged cells. This process is a critical mechanism of tumor suppression that prevents initiation and malignant progression and has been leveraged to treat cancers including glioblastoma. However, removal of senescent cells by macrophages is needed to restore tissue homeostasis. This process is impacted by a variety of factors. For example, senescent cells accumulate in aged individuals and can promote chronic inflammation and disease through the senescence-associated secretory phenotype (SASP).

As the global population ages, it will become more critical to understand the function of cellular senescence in disease. Targeting senescent cells, either through elimination (senolysis) or reprogramming, may have potential therapeutic value in individuals with a high senescent cell burden. Aged or DNA-damaged cells adopt a senescence-associated p53 isoform profile characterized by reduced expression of Δ133p53α and increased expression of p53β. Critically, restoration of Δ133p53α rescues cells from senescence and enhances DNA repair. Targeting p53 isoforms may represent a mechanism by which cells can be reprogrammed. A thorough understanding of the contexts in which senescent cells maintain beneficial or harmful roles is critical to developing senescence therapeutics in cancer and aging.

CHAPTER 1. CELLULAR SENESCENCE IN AGING AND CANCER: ROLE OF P53 ISOFORMS

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1.1 Introduction

In the 1960s, Hayflick found that normal human cells replicate for a finite number of cell doublings in culture with longer replicative lifespans observed in fetal, compared to adult, cells.[1,2] After repeated rounds of replication, critically shortened telomeres fail to interact with sufficient amounts of the telomere-binding protein complex 'shelterin' leading to destabilization of the protective t-loop configuration and exposure of telomeric DNA ends.[3-5] With deficiency in the 'shelterin' subunits, such as POT1 and TRF2, these telomere-associated DNA damage foci activate the DNA damage response leading to p53 activation, growth arrest, and induction of replicative senescence.[3,5-7] These initial experiments describe replicative senescence, a permanent or sustained cell cycle arrest in aged cells.[1-3] In addition to replicative senescence, stress-induced senescence is the premature induction of cellular senescence following the accumulation of non-telomeric DNA damage.[8-10] This mechanism also activates the DNA damage response to trigger cell cycle arrest and can be induced by a variety of physiologic and pathologic stressors including age-associated oxidative stress, genotoxic radiation, and aberrant activation of oncogenes such as Ras.[8-11]

By inhibiting the replication of aged and DNA-damaged cells, cellular senescence inhibits tumor initiation.[12-14] Cell cycle arrest is promoted by repression of proliferation-promoting genes, such as cyclin A and other E2F target genes, in senescence-associated heterochromatin foci (SAHF).[15-17] The formation of SAHF is considered an important step in the development of stable

cell cycle arrest in senescent cells.[17] In benign or premalignant tumors such as colon adenoma and melanocytic nevi, the induction of cellular senescence serves as a barrier to malignant progression.[14,18,19] Because of the intrinsic anti-tumor functions of cellular senescence, it has been used as an indicator of cancer therapy response.[20,21] In addition, senescence-inducing therapies have been developed that specifically leverage this anti-tumor mechanism to treat cancer patients.[20] However, senescent cells are resistant to apoptosis, have deficient DNA repair, and develop increased susceptibility to mutations suggesting that cellular senescence may be inferior to apoptosis as a mechanism of tumor suppression.[22-24]

In addition to undergoing cell cycle arrest, senescent cells produce an array of effects in neighboring cells.[25] These non-cell-autonomous functions are primarily mediated by the secretory factors produced as part of the senescence-associated secretory phenotype (SASP).[25-27] The SASP can vary by cell type and stressor and is characterized by increased secretion of proteins including inflammatory cytokines, chemokines, and matrix metalloproteinases (Figure 1.1).[25-41] The primary functions of SASP proteins are to promote the repair of damaged tissue and to recruit macrophages and lymphocytes, which remove senescent cells leading to restoration of normal tissue functions.[35,42-44] Unfortunately, inadequate removal of senescent cells over time may contribute to the physiology of aging or so-called organismal senescence.[45] Persistent senescent cells secrete SASP proteins to induce chronic inflammation, stimulate tissue fibrosis, and reduce viability of neighboring cells.[25,28,46,47] Because senescent cell burden is highest in aged individuals, these features are particularly relevant in age-related pathologies and underlie a variety of chronic diseases including renal disease, immune dysfunction, neurodegeneration, and pulmonary fibrosis.[48-53] Because the functions of cellular senescence vary by context, this review will focus on the roles of cellular senescence and SASP in development, cancer, and aging and will present

the potential therapeutic value of targeting senescent cells, such as through the modulation of p53 isoforms, to prevent, delay and treat senescence-associated disease.

1.2 Cellular senescence is critical for normal development and tissue repair

If the secretion of SASP proteins was always deleterious, why would the SASP phenotype persist through selection and evolution? Although many studies have identified the harmful effects of sustained cellular senescence and SASP, these mechanisms have critical and appropriate roles in normal physiologic functions such as embryogenesis and wound healing.[35,42,45] For example, cellular senescence is observed in the multinucleated syncytiotrophoblast layer of the human placenta but is reduced in pregnancies with intrauterine growth restriction suggesting that cellular senescence is important in promoting placental-dependent fetal growth and development.[54] The induction of cellular senescence in the placenta and fetal membranes may also be associated with the onset of parturition suggesting that the timing of cellular senescence is critical in pregnancy.[55,56]

During embryogenesis, cellular senescence is tightly regulated, initiated at specific times and locations, and has roles in morphogenesis, tissue remodeling, and cell population balance.[35] For example, induction of cellular senescence along the interdigital webs leads to the formation of individual fingers.[35] In the endolymphatic sac, cellular senescence is observed in specific cell types and is proposed to balance cell populations and ensure appropriate tissue morphology.[35] In both of these examples, senescent cells must be efficiently removed by macrophages to support appropriate tissue modeling and maturation.[35] Importantly, inhibition of cellular senescence in mice results in developmental defects underscoring the essential role of cellular senescence in normal embryogenesis.[35]

Later in life, induction of cellular senescence is observed in the initial stages of tissue repair, suggesting that wound healing and tissue regeneration rely on a similar mechanism of cellular senescence as observed during embryonic development.[35,42,57] Following injury, senescent fibroblasts and endothelial cells secrete a variety of proteins, including PDGF-AA.[42] PDGF-AA promotes myofibroblast differentiation and production of granulation tissue to initiate wound repair.[42] In addition, SASP-associated proteases help to control excess fibrosis.[42] Further, pharmacologic inhibition of cellular senescence *in vivo* inhibits wound healing.[58] This requirement for senescent cells and SASP during the initiation of tissue remodeling underscores the important albeit transient role of cellular senescence in development and repair.[35,42,45]

Interestingly, cellular senescence has been shown to have both beneficial and detrimental roles in the progression of tissue fibrosis.[45] In the lung, it is thought to promote pulmonary fibrosis.[28] However, it has also been suggested to attenuate fibrosis through the secretion of proteases during wound healing.[42] Additionally, inhibition of p53-mediated cellular senescence in hepatic stellate cells promotes fibrosis and tumorigenesis.[59] This is thought to be due to the loss of critical functions mediated by senescent stellate cells such as promotion of anti-tumor macrophages and inhibition of hepatocyte transformation.[59] This suggests that cellular senescence and SASP may limit fibrosis in specific contexts.[59,60] These findings in the liver also demonstrate the potential anti-tumor functions of cellular senescence.[59]

1.3 Cellular senescence is a critical barrier to carcinogenesis

Genomic instability increases during aging and is considered to be one of the major hallmarks of cancer cells.[61,62] By preventing the proliferation of aged and DNA-damaged cells, cellular senescence provides a critical tumor-suppressor function.[12-14] Similar to non-tumor cells, senescent cancer cells adopt SASP and develop modified receptor expression which alters their

signaling pathways.[63,64] SASP proteins produced by senescent cancer cells have both paracrine and autocrine functions. For example, secreted IL-8 recruits leukocytes and reinforces cancer cell senescence through their upregulated CXCR2 receptors, underscoring a tumor-suppressive role of SASP.[40,63,64]

Promoter hypermethylation or genetic mutation of senescence genes facilitates tumor initiation and underscores the role of cellular senescence in preventing tumorigenesis.[18,65-67] Cellular senescence is also a critical barrier to transformation implicated in the malignant progression of benign tumors including colonic adenoma to carcinoma and melanocytic nevi to melanoma.[14,18,19] In colonic carcinomas, dysregulation of p53 isoforms (discussed below) and p53 mutations are thought to inhibit the induction of p53-dependent cellular senescence and facilitate senescence escape.[18] This may suggest that adenoma cells are not completely committed to senescence programming, allowing their reversion to a pre-senescent proliferative state and transition to malignancy.[68] Based on these findings, the induction of cellular senescence has been identified as a critical barrier to carcinogenesis and may represent a therapeutic avenue to inhibit tumor initiation and malignant progression.

In contrast, similar DNA methylation patterns during senescence induction and tumorigenesis have led to another hypothesis that, rather than representing a barrier to carcinogenesis, senescence itself may contribute to tumorigenesis by priming cells for malignant transformation.[69-71] For instance, Milanovic and colleagues[71] found that chemotherapy-induced senescent lymphoma cells, upon escape from senescence, acquired stem cell-related properties and elevated tumor-initiating capacity *in vitro* and in animal models, indicating that senescence itself may prime cells to more malignant and aggressive phenotypes. In this way, escape from therapy-induced senescence has been proposed as a potential mechanism underlying cancer recurrence.[72]

However, others have identified distinct methylation of differentiation and metabolic genes in transformation and senescence, respectively, and shown that senescent cells actually resist malignant transformation.[73] Thus, the anti-tumor effect of cancer cell senescence remains controversial and contextual, depending primarily on whether cancer cell senescence is considered permanent or reversible.

1.4 SASP can mediate the pro-tumorigenic effects of cellular senescence

Senescent cells promote tumor growth, recurrence and metastasis and contribute to the development of cancer therapy-associated side effects through SASP.[11,25,47] Induction of cellular senescence within the tumor microenvironment may occur through several mechanisms including cancer therapy-induced senescence of tumor and non-tumor cells[8,11,47], via the SASP-associated spread of cellular senescence programming[29,38], or following successive rounds of replication in non-tumor cells responding to the tumor or tumor-associated tissue injury.[8,25,52] The outcomes of cellular senescence depend on the senescent cell type. For example, senescent resident brain cells and their secreted factors have been suggested to mediate an array of effects in the contexts of cancer and neurodegenerative disease (Figure 1.2).[8,11,25,46,51,74-82] The disruption of tissue homeostasis that occurs following the induction of cellular senescence in tumor and non-tumor cells brings into question whether inducing senescence remains the best method for cancer treatment.

In addition to tumor cell-specific effects, anti-cancer therapies can induce senescence in non-tumor cells, such as stromal cells, which may contribute to poor anti-tumor immune responses.[83] For example, in the skin, senescent cells facilitate recruitment of immunosuppressive myeloid cells which inhibit anti-tumor T cell responses.[84] In the tumor microenvironment, cancer cells and regulatory T cells (T-reg) have also been shown to induce cellular senescence of

responder T cells and represent important barriers to efficient cancer immunotherapy.[78,85,86] This is further exacerbated by aging which is associated with CD8+ T cell senescence and increased susceptibility to disease.[52,87,88] Critically, removal of senescent cells in aging mice delays tumorigenesis further underscoring the tumor-promoting functions of persistent cellular senescence and SASP.[58] However, it remains to be investigated whether clearance of persistent senescent cells also delays or inhibits tumor progression and recurrence in humans.

In addition, the development of senescent non-tumor cells may promote the long-term side-effects of cancer therapies.[8,47,53,89] This is particularly well-characterized in patients receiving radiotherapy.[90,91] For example, radiation-induced pneumocyte senescence activates fibroblasts to promote pulmonary fibrosis, a late effect of radiation treatment.[28] Radiation therapy also has been shown to induce endothelial cell senescence, which may contribute to cardiovascular disease in cancer survivors.[75,91] In pediatric leukemia patients, increased expression of p16 in non-tumor cells has been suggested as a potential biomarker for radiation-induced cellular senescence that may be associated with the late effects of cancer therapy.[92] These findings leave open the possibility that persistent senescent cells and SASP-associated inflammation are treatment-induced and can exacerbate chronic disease in cancer survivors.

1.5 Increased senescent cell burden contributes to disease in physiologic and premature aging

Pathologic changes induced by the persistence of senescent cells may not be limited to cancer- and cancer treatment-associated effects, but may also be promoted in tissues during physiological or accelerated aging.[45,58,93] This may be exacerbated by mechanisms that enhance accumulation or inhibit the resolution of cellular senescence. For example, the application of cellular stress, such as that induced by radiotherapy or traumatic brain injury, may induce DNA

damage, oxidative stress and inflammation, dramatically increase a tissue's senescent cell burden, and overwhelm the tissue's ability to target and remove senescent cells.[8,45,90,94,95] In tissues containing senescent cells, high levels of SASP-associated matrix metalloproteinases (MMPs) have been shown to induce autocrine ligand shedding, which renders senescent cells less vulnerable to immunosurveillance and clearance.[96] In aged individuals, increased HLA-E expression on senescent cell surfaces may inhibit NK and T cell responses allowing the persistence of senescent cells.[97] Moreover, cellular senescence has been described as contagious and is shown to mediate a 'bystander effect' by which senescence programming can spread from cell to cell through the paracrine effects of SASP secretory factors.[28,29,38] These mechanisms of rapid accumulation, persistence, and amplification may underlie the ability of senescent cells to impact organ function and lead to pathologic changes in aging.

During aging, replicative senescence is accompanied by alternative RNA splicing of multiple genes including lamin A (LMNA).[98,99] Alternative splicing of LMNA mRNA induces production of progerin (C-terminally truncated version of lamin A protein), initiates accumulation of progerin-induced DNA damage, and contributes to the induction of cellular senescence.[98-102] Senescent cells are also thought to play a role in premature aging syndromes such as Hutchinson-Gilford Progeria Syndrome (HGPS).[101,102] In children with HGPS, mutations in the LMNA gene result in progerin-producing alternative splicing, accelerated accumulations of progerin compared to normally aged individuals, and early onset of premature aging symptoms.[100,103] In contrast to fibroblasts from healthy donors, HGPS-derived fibroblasts have a shortened replicative lifespan, adopt an inflammatory SASP phenotype, and undergo premature cellular senescence.[101] Targeting senescent cells in both wild-type and progeroid mouse models delays age-related deterioration[58,93],

suggesting that the age-related accumulation of senescent cells contributes to cellular dysfunction in physiologic and premature aging.

1.6 Cellular senescence promotes age-related diseases including neurodegeneration

Cellular senescence and SASP contribute to organ dysfunction and tissue pathology in a variety of age-related diseases including pulmonary fibrosis[28,50], osteoarthritis[104,105], atherosclerosis[106-108], and Alzheimer's disease.[46,51,109] Studies of cellular senescence have identified increased numbers of senescent cells in aged human tissues, including the brain.[46,51,94,110] This elevated senescent cell burden in older patients has been suggested to contribute to disease. For example, increased numbers of senescent microglial cells are thought to contribute to poorer outcomes following traumatic brain injury in aged individuals due to an exaggerated microglial response and subsequent neuroinflammation.[51,94,95] Cellular senescence has also been observed in several neurodegenerative diseases.[51] The specific effects of cellular senescence vary based on the senescent brain cell type (Figure 1.2). For example, senescence of neural progenitor cells, as can occur in multiple sclerosis, may inhibit oligodendrocyte-mediated remyelination.[74] Increased numbers of senescent endothelial cells are thought to promote atherosclerosis and disrupt the blood-brain interface.[106-108,111] In patients with Parkinson's disease, increased numbers of senescent astrocytes may contribute to neurodegeneration.[112,113] Patients with Alzheimer's disease (AD) also have increased numbers of senescent astrocytes as well as aggregates of senescent oligodendrocyte precursor cells along amyloid plaques.[46,51,80] Consistent with the hypothesis that cellular senescence promotes neurodegeneration, senescent astrocytes induce neurotoxicity in co-culture[8,46,79], while targeting cellular senescence in AD model mice improves neurocognitive function.[80]

Recently, the p16-3MR and INK-ATTAC transgenic mouse models have facilitated the *in vivo* study of cellular senescence in disease progression.[42,93] In the p16-3MR mouse, the p16 promoter drives 3MR (trimodality reporter) expression containing monomeric red fluorescent protein and truncated herpes simplex virus 1 thymidine kinase (HSV-TK).[42] Intraperitoneal administration of ganciclovir selectively targets HSV-TK leading to apoptosis of senescent cells.[42] In contrast, the INK-ATTAC model takes advantage of the previously developed FAT-ATTAC model (fat apoptosis through targeted activation of caspase) by replacing the previously-developed promoter with that of p16 and adding the coding sequence for enhanced green fluorescent protein; this model uses AP20187 injection to induce apoptosis in senescent cells.[93] The identification and subsequent elimination of fluorescently-labeled, p16-positive senescent cells in these models has helped to characterize the functional roles of cellular senescence in disease.[42,93] For example, targeting senescent cells in a model of tau-dependent neurotoxicity reduces gliosis, inhibits neurodegeneration, and improves short-term memory.[109] Similarly, cognitive performance is improved following elimination of senescent cells in a mouse model of radiation-induced brain injury.[114] Although we have focused on senescence-associated neurodegeneration, cellular senescence has been implicated in a variety of age-related diseases.[45] For example, *in vivo* elimination of senescent cells reduces bone resorption and increases bone formation in age-associated osteoporosis, reduces glomerulosclerosis in the aged kidney, attenuates senile lipodystrophy, and improves cardiac stress response in aged mice[58,115] These mouse models demonstrate the potential value of therapeutic targeting of senescent cells in cancer- and age-related diseases.

1.7 Cellular senescence is regulated by p53 and its physiological isoforms

This review marks a significant milestone of 40 years of *Carcinogenesis* publication history and p53 research[116-118] and 15 years of p53 isoform research.[119] TP53 has been shown to regulate a variety of cellular functions including the induction of cellular senescence.[18,120] Senescent cells develop persistent p53-positive nuclear foci thought to maintain cellular senescence and referred to as "DNA segments with chromatin alterations reinforcing senescence" or DNA-SCARS.[121] Inhibition of p53 allows arrested cells with low expression of p16 to re-enter the cell cycle suggesting that cellular senescence is reversible at least in some contexts and is maintained by p53.[68,71,122] In addition to the canonical full-length p53 (FLp53 or p53α), the TP53 gene produces at least 12 truncated isoforms which can positively or negatively modulate FLp53 activity.[119,123] Of these p53 isoforms, Δ133p53α and p53β (Figure 1.3) are best characterized as endogenous regulators of cellular senescence.[18,124]

The production of p53β is increased in aged and DNA-damaged cells through alternative splicing regulated by serine- and arginine-rich splicing factors, namely decreased SRSF3 and increased SRSF7 activity, respectively.[125,126] Proteasomal degradation of FLp53 and p53β is mediated by the mouse double minute 2 homolog (MDM2) through direct binding to the trans-activating domain (TAD), a domain conserved in the alternatively spliced p53β isoform.[119,127,128] In contrast, Δ133p53α is initiated transcriptionally from an alternate promoter in intron 4 and translationally at a methionine codon in exon 5 (corresponding to the codon 133 in FLp53), leading to production of an N-terminus truncated protein lacking the TADs and proline-rich domain (PRD).[119] Thus, Δ133p53α has been shown to be only minimally affected by MDM2 overexpression and is instead regulated at the protein level through chaperone-assisted selective autophagy.[127,129] This process is inhibited by the E3 ubiquitin ligase STUB1 (also known as CHIP),

whose age-associated downregulation leads to increased autophagic degradation of Δ133p53α and induction of replicative senescence.[129]

Functionally, p53β cooperates with FLp53 to increase its transcriptional activity and induce cellular senescence.[18] In contrast, Δ133p53α functions as a physiological, senescence-selective, dominant-negative inhibitor of FLp53 that protects cells from cellular senescence and is diminished following accumulation of DNA damage or in senescent cells with reduced STUB1.[8,101,129,130] Consistent with these functions, senescent cells adopt a p53 isoform expression profile characterized by decreased Δ133p53α and increased p53β.[18] This senescence isoform profile is accompanied by diminished replicative lifespan, reduced homeostatic cellular functions, and increased secretion of SASP-associated proteins including inflammatory cytokines and chemokines.[18,46,87] Critically, modulation of this isoform profile, particularly through the restoration of the dominant-negative Δ133p53α, has been shown to rescue normal human cells from cellular senescence, restore homeostatic cellular functions, and inhibit the production and secretion of SASP proteins.[8,18,46,87,101]

1.8 p53 isoforms modulate cellular functions and SASP in normal human cells

After cellular senescence is induced, the primary cell-autonomous effect is the induction of cell cycle arrest. This reduction in replicative lifespan has been observed in a variety of normal human cells[18,87,101] and contributes to age-related disease and conditions.[45] For example, accumulation of senescent progenitor cells in the brain may predispose or worsen neurodegenerative diseases such as Alzheimer's disease or multiple sclerosis.[51,74,80] Modulation of the p53 isoform profile by reconstitution of Δ133p53α expression extends the replicative lifespan of normal human cells otherwise approaching senescence.[18] Importantly, this extension is not

indefinite and thus not analogous to the immortalization and unbridled proliferation observed in tumor cells.[87]

In addition to restoring replicative potential, p53 isoforms regulate normal cellular functions.[8,46] For example, senescent astrocytes disrupt normal tissue homeostasis and mediate neurotoxicity through reduced production of nerve growth factor.[46] Critically, homeostatic functions can be restored or enhanced through overexpression of Δ133p53α.[8,46] Following restoration of Δ133p53α, the neuroprotective functions in senescent astrocytes are restored leading to increased neuronal survival in co-culture.[8,46] Part of this neurotoxicity has been shown to be mediated by SASP-associated IL-6 secretion which can be ameliorated by IL-6 neutralizing antibodies.[46] These findings support a critical role for p53 isoforms in restoring or enhancing normal cellular functions in senescence-associated disease.

Another cell type that experiences dramatic loss of function is the CD8+ T cell.[52] During aging, senescent CD8+ T cells are associated with increased susceptibility to infectious disease and reduced anti-tumor functions underscoring an important barrier to cancer immunotherapy in aged patients.[52,87,88] Other inducers of T cell senescence include secreted proteins within the tumor microenvironment, cancer therapy, T-regs, and chronic infection.[52,78,84-88,131] Critically, reconstitution of Δ133p53α in senescent CD8+ T lymphocytes restores expression of the co-stimulating receptor CD28 and central memory markers (CD27 and CD62L) and decreases the expression of late-differentiated markers and immune checkpoint proteins LAG-3 and PD-1 (Figure 1.4).[87] Based on this research, the p53 isoforms have been identified as a potential mechanism to enhance T cell-based immunity via reprogramming or dedifferentiating senescent CD8+ T cells.[87]

Senescent CD8+ T cells are primarily defined by loss of CD28 expression.[52] In contrast, exhausted CD8+ T cells are defined by increased expression of immune checkpoint proteins, such as PD-1, TIM-3, CTLA-4 and LAG-3.[132] While T cell senescence and exhaustion are described as separate entities, these T cell states are not mutually exclusive and can coexist within the same tumor microenvironment.[78] Because Δ133p53α inhibits cellular senescence and downregulates immune checkpoint proteins associated with T cell exhaustion[87], it rescues both the senescent and exhausted states of CD8+ T cells back to their proliferative and functional state (Figure 1.4A). Δ133p53α thus has significant implications in cancer immunotherapy, including chimeric antigen receptor (CAR) T cell therapy (Figure 1.4B), where T cell senescence and exhaustion are major obstacles for improving therapy efficacy.[78,133,134]

The p53 isoforms also regulate DNA repair.[8,101,135] For example, although Δ133p53α overexpression does not protect normal human astrocytes from acute accumulation of DNA damage following radiation (i.e., γ-H2AX foci indicative of DNA double-strand breaks, DSB), the cells have fewer DNA damage foci by 24 hours post-radiation, suggesting that Δ133p53α facilitates DSB repair.[8] This is thought to be primarily regulated by RAD51, an essential factor for homologous recombination repair.[101,135] Although a mechanism has yet to be described, accelerated resolution of 53BP1 foci in Δ133p53α-overexpressing cells 24 hours post-radiation may also indicate a role for Δ133p53α in promoting non-homologous end joining repair.[8] In human pluripotent stem cells, remarkably high levels of Δ133p53α inhibit cellular senescence and promote DNA repair (Figure 1.5).[130,135,136] In this context, these functions are thought to support the self-renewing potential and genomic stability of stem cells.[130] Critically, while Δ133p53α inhibits the FLp53-inducible genes for cellular senescence, it does not inhibit those for DNA repair and apoptosis.[130,135,136] This further supports the role of Δ133p53α in stem cell function and

integrity through ensuring genome stability and apoptotic elimination of severely damaged cells thereby suppressing malignant transformation. These characteristics of Δ133p53α are in marked contrast to loss or mutation of TP53, which leads to genomic instability, cell immortalization, and cancer (Figure 1.5).[130] The selective nature of FLp53 inhibition by Δ133p53α facilitates the development of therapeutic applications of this non-mutagenic and non-oncogenic p53 isoform in senescence-associated diseases.

1.9 Eliminating and reprogramming of senescent cells as therapeutic strategies

With unprecedented population aging, it is critical to develop therapeutic strategies to improve and treat cancer- and age-associated disease.[48] Interventions to target senescent cells and increase healthspan are of great interest and are under investigation in human trials.[137-140] These anti-senescence therapies target specific features of cellular senescence, such as SASP-associated inflammation, or aim to eliminate or reprogram senescent cells to reduce senescent cell burden in aged individuals (Figure 1.6).

The non-cell-autonomous effects of cellular senescence are mediated through the inflammatory SASP.[25] Therapeutics that specifically target the morphology and functions of senescent cells, such as SASP, are referred to as senomorphics.[139] These treatments may prevent a number of SASP-associated effects on the microenvironment including tumor promotion.[25,30] Because NF-kB regulates SASP, inhibitors of NF-kB represent a method to modulate SASP and improve aging phenotypes.[139,141] For example, IKK/NF-κB inhibition by metformin prevents the pro-tumorigenic effects of SASP *in vitro* and improves healthspan in an aging mouse model.[142,143] Additional SASP inhibiting therapeutics include glucocorticoids, HMGCR-inhibiting statins, and JAK1/2 inhibitors.[144-147]

In addition, characterizing the mechanisms by which persistent senescent cells resist apoptosis may provide effective treatment.[139] For example, upregulation of anti-apoptotic proteins such as BCL-2 aids in the persistence of senescent cells.[22] BCL inhibitors have been used to inhibit anti-apoptotic proteins and induce apoptosis in senescent cells.[148,149] Senolytic compounds dasatinib and quercetin are also effective and have been shown to reduce senescent cell burden in research models and in human clinical trials.[138,140,150] Additional methods of inducing apoptosis in senescent cell populations are also under investigation such as the FOXO4 interfering peptide which localizes to DNA-SCARS and disrupts FOXO4-p53 signaling to induce p53-dependent apoptosis and improve age-related renal dysfunction.[151]

Compared to their non-senescent counterparts, senescent cells are reported to have multiple metabolic pathway alterations including increased glycolysis and mTOR activity.[152] Leveraging these metabolic differences may provide additional opportunities to target senescent cells. For example, rapamycin, an mTOR inhibitor, reduces secretion of SASP proteins and prevents tumor promotion by senescent fibroblasts in a mouse model of prostate cancer.[153] In a lymphoma model, therapeutic induction of cancer cell senescence identified increased glycolysis and a reliance on autophagy as potential therapeutic targets in cancer.[154] The high metabolic activity reported in senescent cells has been suggested to be related to SASP factor production and secretion.[152,154] As such, the targeting of these senescence-associated metabolic pathways may provide relief from the non-cell-autonomous effects of SASP.

Because p53 and p53 isoforms have been shown to have critical roles in maintaining and inhibiting cellular senescence, current research also aims to identify compounds which modulate these proteins. The importance of p53 in maintaining cellular senescence is also supported by work with senescent human cells with low p16 expression, which have been shown to re-enter the cell

cycle upon inactivation of p53.[122] Experimental reversal of the senescence-associated p53 isoform profile in non-disease and progeroid primary human cells restores replicative lifespan, improves homeostatic cellular functions, and reduces secretion of SASP-associated proteins including inflammatory cytokines and chemokines.[18,46,87,101] In particular, as mentioned above, the senescence-selective, dominant-negative effect of Δ133p53α over FLp53 suggests that targeting FLp53-dependent senescence may hold promise in reprogramming senescent cells for therapeutic purposes.[130,136]

In addition to their use in cancer immunotherapy, CAR T cells can also be engineered to target a disease-specific antigen on non-tumor cells. Molecularly engineered CD8+ T cells expressing a CAR against such antigen may hold promise in targeting senescent cells or treating age-related disease.[155,156] For example, senescent cells have been shown to induce fibrosis through the activation of fibroblasts.[28,157,158] In the heart, activated cardiac fibroblasts were successfully targeted by CAR T cells resulting in reduced cardiac fibrosis and improved cardiac function.[155] Other immune-mediated strategies that are of interest in future studies include enhancing the immunogenicity or immune cell clearance of senescent cells such as through the identification of senescence-specific cell surface proteins or enhancement of T cell cytotoxicity, respectively. For example, CAR T cell-mediated senolysis may be accomplished through targeting of the senescence-specific urokinase-type plasminogen activator receptor (uPAR) and leads to improved function and reduced fibrosis in a hepatic injury model and prolonged survival in a lung tumor model of treatment-induced senescence.[156] In this scenario, the Δ133p53α-mediated functional reprogramming of senescent CD8+ T cells, as described above, may also represent an approach for enhancing senescence-eliminating strategies in aged individuals and patients with age-associated diseases.[87]

Finally, the identification of senolytics or senescence reprogramming therapeutics requires a thorough understanding of the contexts in which senescent cells maintain beneficial or harmful roles. For example, cellular senescence is critical for placental development, embryogenesis, and wound healing suggesting that senolytics could be detrimental in these processes.[35,42,54] In addition to biologic context, the treatment goal would be an important consideration for the use of senolytics in disease. For example, senescent cells promote tumor-associated angiogenesis suggesting that targeting senescent cells may limit tumor progression.[25,32] However, increased angiogenesis and endothelial activation may also improve vascular access to tumors for immunotherapy. This could be particularly important in tumors with abundant desmoplastic stroma and poor immune infiltration such as pancreatic carcinomas.[77] These examples underscore the importance of considering both the specific physiologic context and therapeutic goal when investigating the utility of senolytics in disease progression.

1.10 Future directions

With a growing body of evidence identifying the functional roles of cellular senescence and SASP in aging, cancer, and cancer treatment-associated diseases, it is now time to translate accumulated knowledge into therapeutic approaches such as elimination or reprogramming of senescent cells. Cell-based studies suggest that the p53 isoforms are a promising target to be modulated for reprogramming senescent cells.[8,18,46,87,101] Extending these studies into animal models and accumulating *in vivo* functional data will be critical to the development of translational applications. However, the transcriptional and translational mechanism for generating Δ133p53α only exists in humans and primates[46]; therefore, these studies necessitate the development of transgenic mice humanized for this p53 isoform. Future studies using Δ133p53α-humanized mice will seek to recapitulate cancer- and age-associated human diseases such as neurocognitive

dysfunction in radiotherapy-induced brain injury or Alzheimer's disease and cardiovascular disease in HGPS models.

To support the therapeutic applications of p53 isoforms, repurposed drugs and small molecule compounds that modulate their expression and activity need to be identified via high-throughput screening. Previous data on the p53 isoform regulatory mechanisms can help to direct the screening strategies, for example, through the modulation of autophagy, STUB1 or chaperone functions (Figure 1.3C).[129] In addition, development of methods to inhibit the alternative RNA splicing generating p53β may be pursued based on the analysis of the regulatory splicing factors SRSF3 and SRSF7 and their binding cis-elements (Figure 1.3B).[125,126] Once potential compounds are identified, these screens may benefit from findings in previous studies including which cell types (e.g., astrocytes and CD8+ T cells) and phenotypes (e.g., senescence bypass and SASP expression/secretion) might be used to validate candidate compounds.[8,46,87]

To explore a wider range of therapeutic applications of the p53 isoforms, it is important to examine their *in vivo* expression profiles in various human tissues and organs of different ages, as well as different cancer types. A simultaneous *in situ* detection of multiple isoforms is also needed to dissect a whole picture of the complex and concerted regulations and functions of different p53 isoforms. Recently, a multiplex long amplicon digital PCR was developed to specifically and simultaneously quantify individual p53 transcripts.[159] In addition, RNAscope or BaseScope allows *in situ* detection of multiple isoform mRNA species on human tissue section slides.[160,161] These new assays should overcome the current difficulty of detecting low-abundance isoforms from large database repositories such as the Cancer Genome Atlas (TCGA).[162] Furthermore, the development of an efficient and high-resolution method for *in situ* detection of senescent human cells from donors of different ages and in various tissues types, including tumors and the tumor

microenvironment, will systemically identify cell types undergoing cellular senescence in human tissues. This type of application has the opportunity to further dissect critical hypotheses, such as the role of cellular senescence in dysfunction of tumor-associated T cells[78] or in the decline of adult tissue stem cell populations in the elderly[163], representing major advances in the field of cancer and aging research.

Finally, as discussed, one of the major remaining questions in cellular senescence research is whether cellular senescence is truly reversible or irreversible. Cellular senescence is primarily described as a permanent cell cycle arrest. However, we have described several studies where at least a population of apparently senescent cells regain pre-senescence functions.[18,68,71,122] Proponents of the reversibility of cellular senescence would suggest that these studies identify potential mechanisms by which senescent cells might be reprogrammed back to their pre-senescent, functional state. It is important to note that several studies that demonstrate reversibility of senescence use specific experimental interventions that may not commonly occur in the context of spontaneous senescence escape and may be more relevant to anti-senescence therapeutic applications. In addition, since a population of cells undergoing senescence is likely to be heterogeneous, it is possible that a small population of near-senescent or incompletely senescent cells are subject to reprogramming while those cells that have already committed to cellular senescence remain permanently senescent. It has also been suggested that not all pre-senescence features can be rescued but that there is a subset of functions, such as cellular proliferation, which can be restored in senescent cells underscoring the complexity surrounding the reversibility of cellular senescence. Further studies are necessary to clarify the identity of the cells that regain pre-senescent cell functions and compare the functional profile of these rescued cells to their fully senescent counterparts. Although it is not clear whether reprogramming of fully senescent cells is

achievable, it is likely that this type of intervention will reduce the spread of cellular senescence within a population by inhibiting the induction of cellular senescence in pre-senescent cells. Critically, this suggests a benefit for combining anti-senescence strategies, such as reprogramming therapies and senolytics, to enhance the overall therapeutic effect by inhibiting both the accumulation and persistence of senescent cells, respectively. These and other efforts to identify mechanisms to target, eliminate, or reprogram senescent cells and SASP in disease have wide-reaching implications for the treatment of cancer and aging.

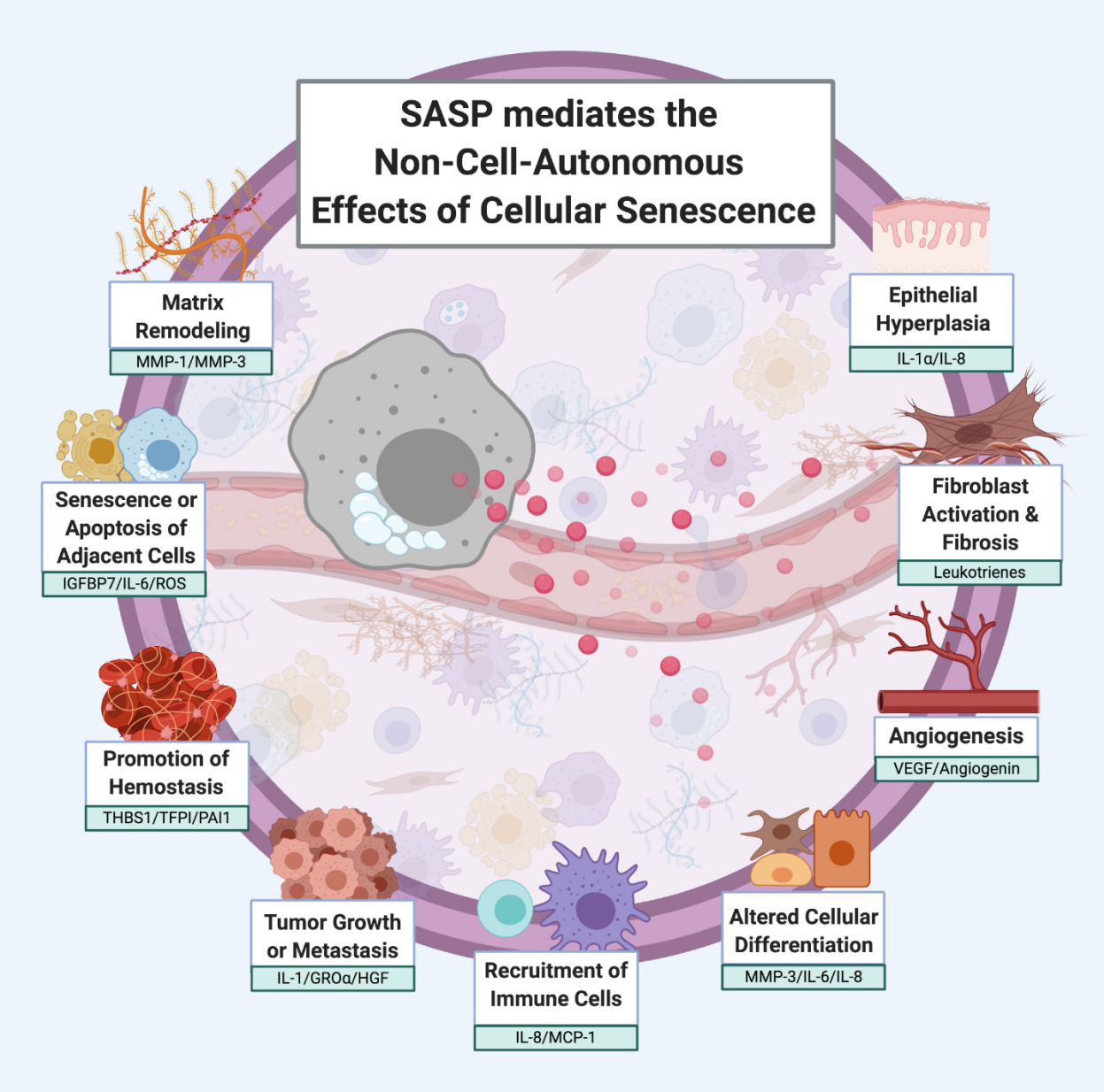

Figure 1.1. SASP mediates the Non-Cell-Autonomous Effects of Cellular Senescence. The factors secreted as part of SASP (senescence-associated secretory phenotype) mediate a variety of effects including epithelial hyperplasia, tumor growth, and extracellular matrix remodeling. These factors also serve to reinforce cellular senescence by inducing senescence programming in adjacent cells. Abbreviations: GROα, growth-regulated oncogene-alpha; HGF, hepatocyte growth factor; IGFBP7, insulin-like growth factor-binding protein 7; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP, matrix metalloproteinase; PAI1, plasminogen activator inhibitor-1; TFPI, tissue factor pathway inhibitor; THBS1, thrombospondin-1; VEGF, vascular endothelial growth factor.

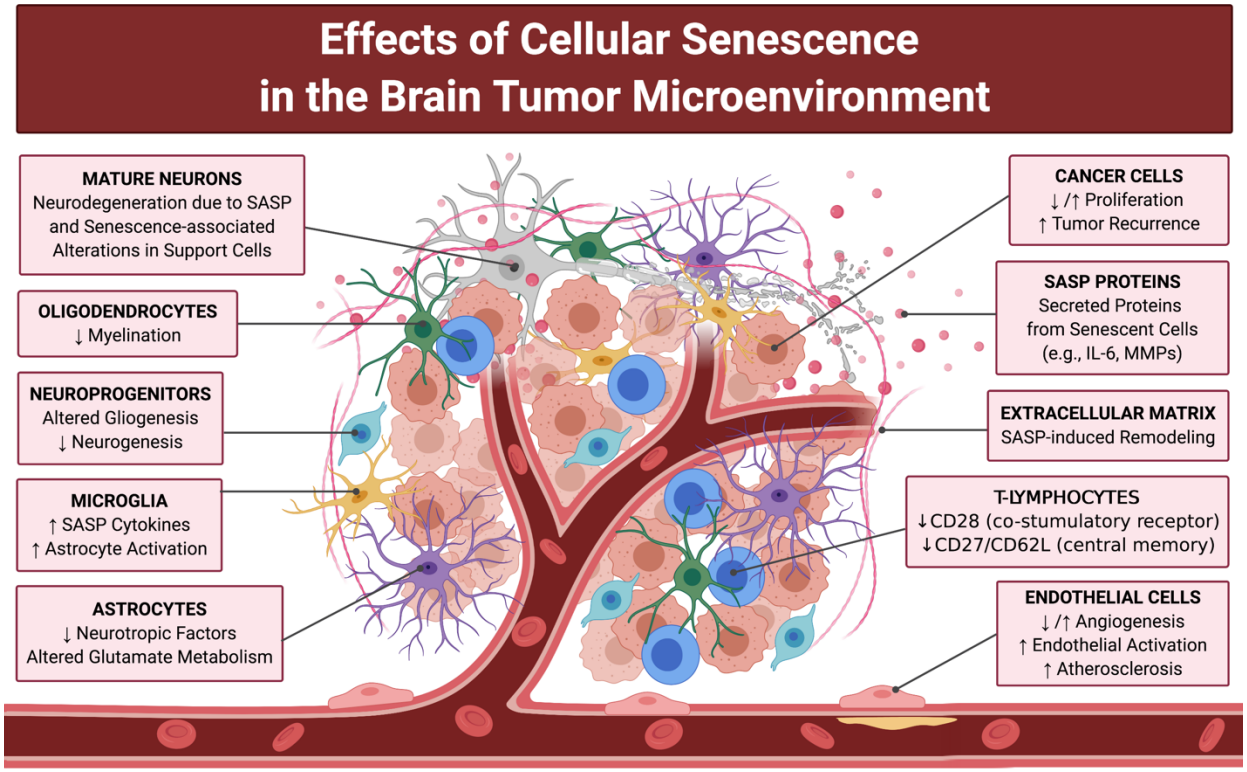

Figure 1.2. Effects of Cellular Senescence in the Brain Tumor Microenvironment. Cellular senescence represents a critical barrier to tumor initiation; however, SASP may induce tumor progression and recurrence underscoring the complex nature of cellular senescence and SASP. Further complicating the potential impact of cellular senescence in the brain tumor microenvironment, induction of replicative or stress-induced senescence in resident brain cells promotes the development of age-associated neurodegeneration and is suggested to mediate an array of effects including reduced proliferation of neural progenitor cells, poor anti-tumor immune response, and reduced myelination by oligodendrocytes. The non-cell-autonomous effects of cellular senescence are mediated by the altered homeostatic functions of senescent cells. For example, perturbed glutamate metabolism and SASP in senescent astrocytes is suggested to promote neuronal toxicity. Additional effects caused by exposure of non-senescent cells to senescent cell-derived SASP factors include induction of cancer cell proliferation, tumor recurrence, and angiogenesis.

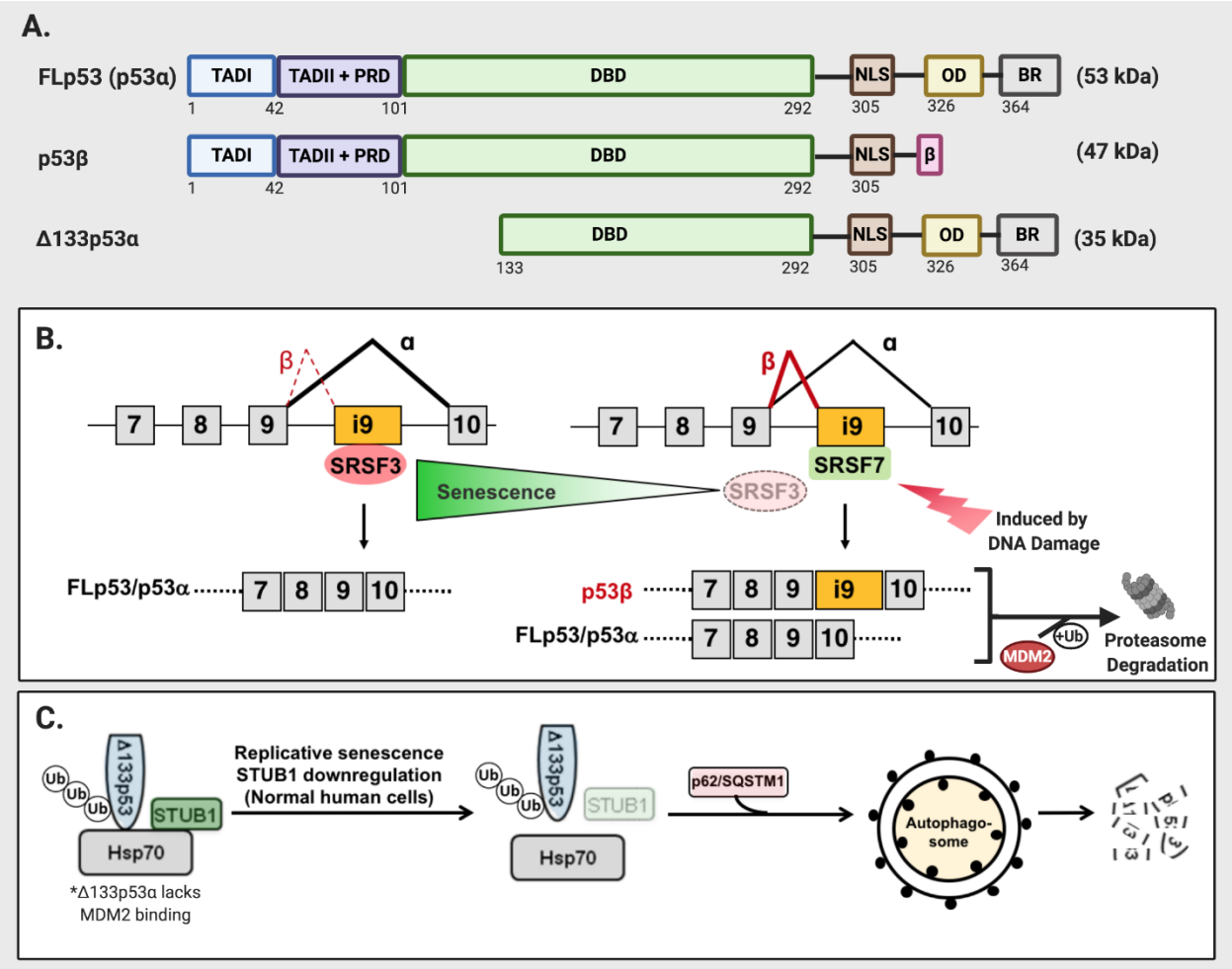

Figure 1.3. Regulation of FLp53 and its Senescence-modulating Isoforms, p53β and Δ133p53α. (A) The TP53 gene produces at least 12 isoforms including full-length p53 (FLp53; p53α), p53β, and Δ133p53α. (B) The expression of p53β is increased in aged and damaged cells due to an age-associated reduction in SRSF3 and DNA damage-induced enhancement of SRSF7 activity, respectively. Both FLp53 and p53β are subject to proteasomal degradation regulated by MDM2 binding to the trans-activating domain (TAD). (C) In contrast, Δ133p53α lacks this MDM2 binding domain and is instead regulated by STUB1-mediated, chaperone-assisted selective autophagy. Downregulation of STUB1 reduces Δ133p53α protein during replicative senescence.

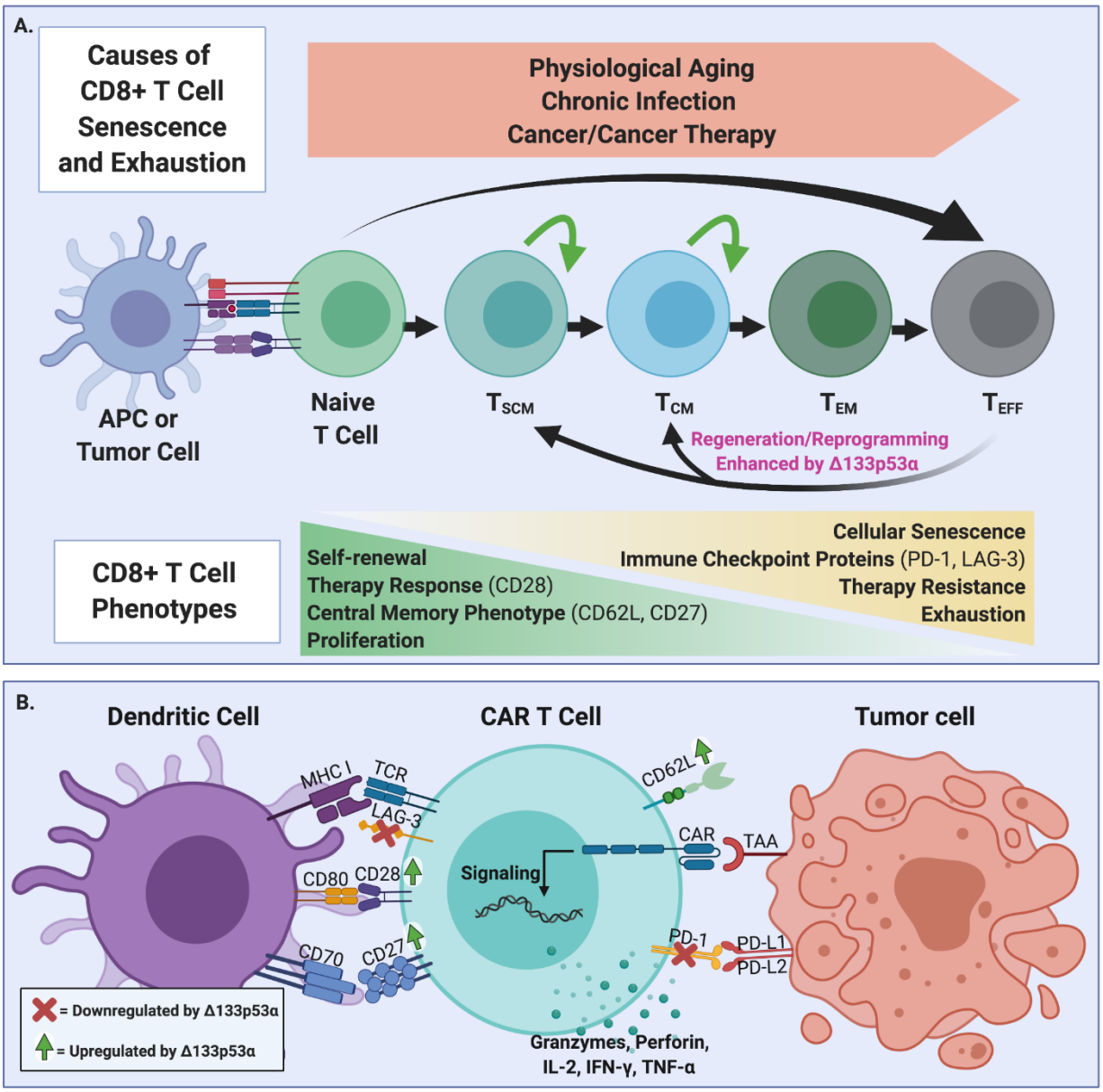

Figure 1.4. CD8+ T Cell Senescence is Rescued by Δ133p53α. (A) CD8+ T cells undergo cellular senescence and exhaustion during physiologic aging or when exposed to a variety of stressors including chronic infection or cancer. Differentiation of naïve T cells into effector T cells occurs after exposure to antigens and is accompanied by increased expression of immune checkpoint proteins, loss of the central memory phenotype, and reduced self-renewal. Restoration of Δ133p53α expression leads to reprogramming of T cells through enhanced expression of co-stimulatory receptor CD28, inhibition of cellular senescence, increased expression of central memory markers (CD62L, CD27), and downregulation of exhaustion-associated checkpoint proteins (e.g., PD-1, LAG-3). These findings suggest that Δ133p53α has critical functional implications for restoring immune function in the elderly and in individuals with chronic diseases. (B) Δ133p53α-regulated factors may also contribute to the improvement of cancer immunotherapies such as CAR T cell therapy.

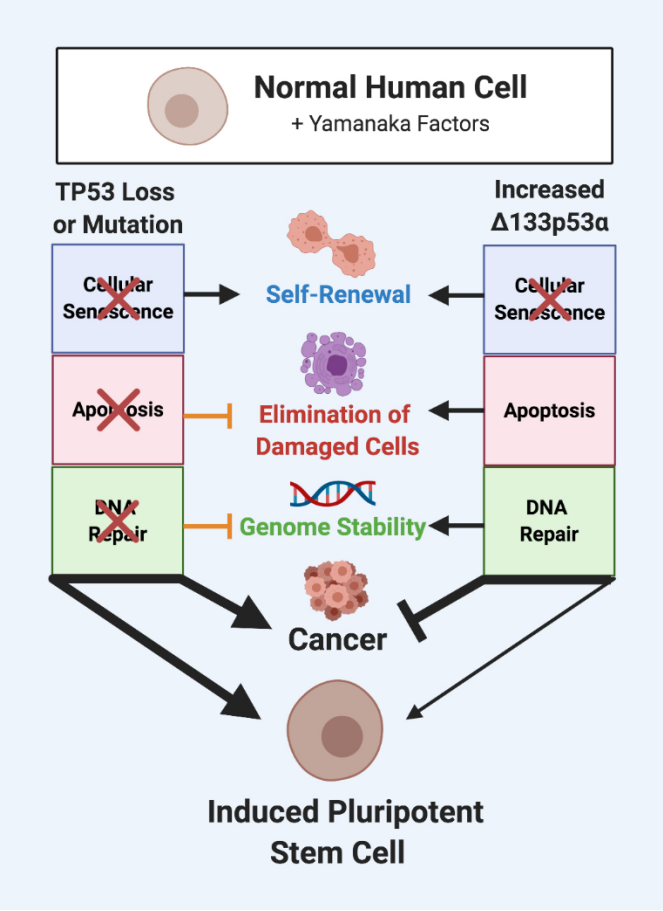

Figure 1.5. Functional Contrast Between p53 Deficiency and Increased Δ133p53α Expression in Human Induced Pluripotent Stem Cells. Loss or mutation of TP53 leads to inhibition of cellular senescence, apoptosis, and DNA repair which promotes self-renewal but inhibits the elimination of damaged cells and impairs genome stability. In contrast, increased Δ133p53α, either through overexpression or endogenous upregulation, inhibits cellular senescence but not apoptosis or DNA repair. This selective inhibition of FLp53 activity by Δ133p53α promotes self-renewal of stem cells while allowing elimination of damaged cells and promoting genomic stability. Although mutation or deletion of FLp53, with Yamanaka factors, leads to enhanced production of human induced pluripotent stems cells, disruption of all FLp53 activities is associated with tumorigenesis. In contrast, increased expression of Δ133p53α in pluripotent stem cells is neither mutagenic nor oncogenic.

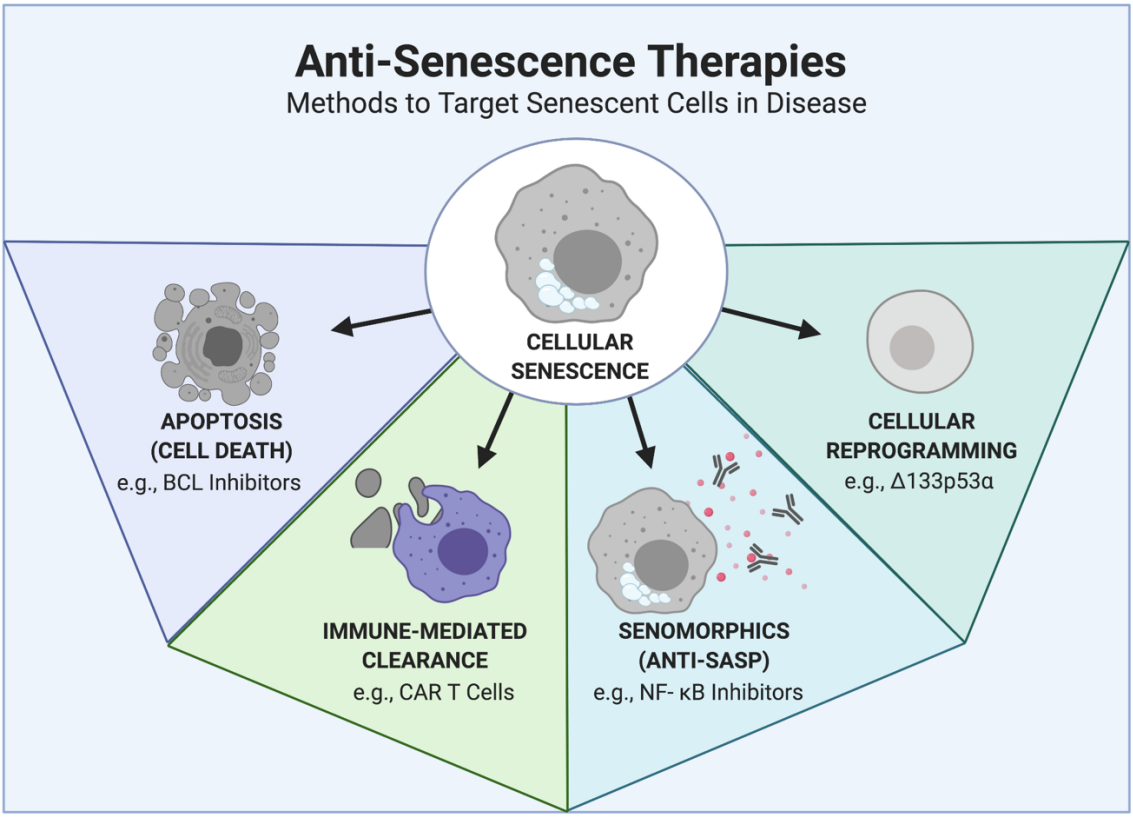

Figure 1.6. Anti-Senescence Therapies: Methods to Target Senescent Cells in Disease. Anti-senescence therapies have been developed to target senescent cells in age-associated diseases and have been shown to hold promise in cell culture studies, in mouse model experiments, and in human clinical trials. Methods that are being investigated include inducing apoptosis in senescent cells, enhancing immune-mediated senescent cell clearance, targeting of functional or morphologic senescence phenotypes such as SASP (senomorphics), and cellular reprogramming to inhibit cellular senescence and restore homeostatic cellular functions.

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CHAPTER 2. RADIATION-INDUCED ASTROCYTE SENESCENCE IS RESCUED BY Δ133P53

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2.1 Introduction

Cranial radiation therapy is used to effectively treat brain cancer in adult and pediatric patients.[1,2] Since its development, protocols have evolved to incorporate methods to reduce side effects such as shielding the hippocampus and fractioning the total radiation dose.[3-5] However, even with improvements over 40% of patients surviving greater than 6 months experience late side effects. In up to 5% of these patients, neurocognitive impairment progresses from decreased attention and problem-solving ability to memory loss, ataxia, and dementia.[6,7] Late effects may also develop in pediatric patients for whom radiation may be prescribed to treat the two most common cancer types: leukemia and glioma.[8-10] Side effects in these patients include deficits in social functioning, vocational difficulty, and poor performance in Intelligence Quotient (IQ) testing and are most severe in the youngest patients receiving the highest radiation doses.[4,9-13] As the number of cancer survivors increases, it becomes increasingly critical to understand the causes of these late effects and to develop strategies to prevent them.

Side effects of cancer therapy may be associated with injury to non-tumor cells.[14] Following radiation exposure and accumulation of DNA damage, cells may adopt one of several cell type-specific responses, including induction of cellular senescence.[5,15] Importantly, although senescent cells do not replicate, they may avoid clearance and persist in tissues while continuing to produce inflammatory factors that contribute to tissue injury.[16,17] In this way, radiation-induced

cellular senescence is being recognized as an important mediator of tissue dysfunction promoting chronic inflammation and contributing to radiation-induced side effects including pulmonary fibrosis and cerebrovascular dysfunction.[18,19]

To investigate the role of cellular senescence in cranial radiotherapy, this study examines brain tissue from patients who have undergone brain radiation treatment and identifies several senescent cell types including astrocytes. Astrocytes perform many neuroprotective functions including production of neurotrophic factors. However, astrocytes may also promote neurodegeneration in some diseases, including Alzheimer's disease, which is thought to be related to induction of a senescence-associated secretory phenotype, or SASP.[17,20] The role of astrocytes and astrocyte senescence in radiation-induced brain injury has not been previously characterized.[6]

After identifying senescent astrocytes in irradiated tissues, this study investigates the potential functions of astrocyte senescence and SASP in promoting brain injury. Based on previous studies[20] identifying regulation of replicative senescence by one of the p53 isoforms, Δ133p53, this study examines the role of Δ133p53 in regulating radiation-induced astrocyte senescence. These findings identify restoration of Δ133p53 as a potential therapeutic approach to inhibiting radiation-induced astrocyte senescence, promoting DNA repair in irradiated astrocytes, and preventing astrocyte-mediated neuroinflammation.

2.2 Methods

2.2.1 Human Patient Tissues

Case tissues were acquired with full IRB approval from the Georgetown Brain Bank, Histopathology Tissue Shared Resource at Georgetown University, and Johns Hopkins Brain Bank and included non-tumor brain tissue from cancer patients with a history of cranial radiation

treatment, with no history of treatment, or from non-disease, age-matched controls collected at autopsy (Table 2.1). Patients receiving chemotherapy or immunotherapy were excluded[14]. Tissues were anonymized, labeled with senescence-associated proteins (p16, Hp1γ; Appendix), and examined by three pathologists (J.B., B.H., I.O.). Each control and radiation-treated tissue was assigned an immunoreactivity score in a blinded manner based on the intensity of immunohistochemical labeling (Table 2.1, Figure 2.1). Quantification of $p16^{INK4A}$-positive astrocytes was completed in 20 microscopic fields (0.5 mm^2) from untreated cancer patients (n = 4) and cancer patients receiving cranial radiation treatment (n = 4). In addition, three patients received stereotactic radiotherapy allowing for comparison of irradiated and untreated regions within the same patient (Figure 2.1A, C-D); these case-matched tissues were further reviewed to identify Hp1γ-positive cell types (Figure 2.1H).

2.2.2 Cell Culture and Treatments

Primary human astrocytes were obtained from Sciencell (Carlsbad, CA, USA) and maintained in Astrocyte Medium supplemented with 2% fetal bovine serum,1% astrocyte growth supplement from Sciencell (Carlsbad, CA, USA), and 1% penicillin/streptomycin solution. Astrocytes expressed astrocyte-lineage marker (GFAP), were split at a ratio of 1:3 and continued to proliferate through passage 20. All experiments used proliferative, low passage astrocytes. SA-β-gal staining was performed with the Senescence Associated (SA)-β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA). Quantification of IL-6 in the cell culture media was performed with the Human IL-6 ELISA Kit (Sigma-Aldrich). Where indicated, human cells were exposed to ionizing radiation in an X-Rad 320 biologic irradiator (Precision X-ray, Inc.).

2.2.3 Lentiviral Vector Transduction

As described previously[20], Δ133p53 was cloned into the lentiviral vector pLOC-GFP-Blasticidin (Open Biosystem). Lentiviral constructs, together with the Trans-Lentiviral GIPZ packaging system (Open Biosystem), were transfected into 293T/17 Cells (ATCC, American Type Culture Collection) using Lipofectamine-2000 (Invitrogen), and the viral particles were collected after 48 hours.

2.2.4 Statistical analysis

Data are presented as mean and standard deviation of at least three independent experiments. Comparisons were made using two-sided, unpaired Student's t test. Differences were considered significant at a value of * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ or NS (not significant).

2.2.5 Western Blotting

Cells were lysed in radioimmunoprecipitation assay buffer (RIPA). Lysates were kept on ice for 30 minutes prior to sonication. Protein concentration was measured using the Bradford assay method. NuPAGE 4X loading buffer was added to all lysates and then boiled for 5 minutes. Then, 40 μg of protein was loaded onto a Tris-glycine gel (Novex) for electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 1:1 mixture of Superblock and Tris Buffered Saline (TBS, 125 mM Tris and 200 mM NaCl), containing 0.1% Tween-20. Membranes were incubated in the primary antibodies (Appendix) overnight at 4°C and washed 3 times in TBS-Tween-20. Membranes were then incubated in a mouse or rabbit HRP- conjugated secondary antibody (Pierce) 1 hour at RT and the signal visualized SuperSignal developing reagent and visualized using the Biorad imager. ImageJ software was used to quantify gel bands from immunoblots using densitometry.

2.2.6 Tissue Immunohistochemistry (IHC) and Immunofluorescence (IF)

Frozen and formalin-fixed paraffin embedded human tissue sections were washed in phosphate-buffered saline (PBS) before blocking for 1 hour in PBS containing 0.1% Triton X and 10% donkey serum (Sigma-Aldrich). Donkey serum is used to block non-specific binding sites before incubation with primary antibody (Appendix) overnight at 4°C. After overnight incubation they were washed in PBS 3 times for 10 minutes, before incubation with the appropriate conjugated secondary antibodies for 1 hour at room temperature (RT). The secondary antibody was conjugated to fluorophores: Alexa-488, -568 and -647 (Invitrogen, 1:400). After washing in PBS 3 times for 10 minutes, sections were incubated for 10 minutes in 4',6-diamidino-2-phenylindole (DAPI, 10ug/mL, Sigma- Aldrich) to counterstain the cell nuclei, and rinsed 3 times for 10 minutes in 0.1 M phosphate buffer (PB). Sections were mounted and slides coverslipped with FluorSave mounting medium (Chemicon). For IHC on paraffin sections, slides were heated to 65°C before immersion in histoclear and rehydration with graded alcohols. Sections were blocked in 1% H2O2 in PBS-Tween 20 (PBS-T) and then in 5% normal goat serum in PBS-T prior to application of primary antibodies (Appendix). Binding of the primary antibody was detected using a mouse or rabbit biotinylated secondary antibody (Pierce) with an ABC standard kit (Vector Laboratories). Visualization was enabled using a 0.05% diaminobenzene hydrochloride solution (DAB; Sigma-Aldrich).

2.2.7 Immunocytochemistry

Cells were washed with PBS and fixed for 10 minutes with 4% paraformaldehyde. Cells were permeabilized with 0.01% Triton-X for 4 minutes, washed with PBS and then blocked in 5% fetal bovine serum (FBS) for 1 hour at RT. Primary antibodies (Appendix) were applied overnight at 4◦C. Cells were washed with PBS before incubation with a secondary antibody conjugated to

fluorophores: Alexa-488, 568 and 647 at a dilution of 1:400 (Life Technologies) and DAPI for 1 hour. Coverslips were mounted on to slides with FluorSave mounting medium (Chemicon).

2.2.8 Quantitative Real-Time Polymerase Chain reaction (qRT-PCR)

Extraction of mRNA was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Cells were homogenized and lysate mixed 1:1 with 70% ethanol and centrifuged through the RNeasy Mini Spin column. RNA was eluted with RNase-free water. The abundance and quality of the resulting RNA was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). RNA samples were diluted so that 200 ng total RNA could be used for a 25 µl- reverse- transcription reaction. cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen).

For the quantitative analysis of mRNA expression, the Tecan Sunrise 7500 real time PCR system (Applied Biosystem) was employed with the DNA binding dye SYBR Green (Qiagen) or Taqman (Life Technologies) primers for detection of PCR products. Each reaction was performed in triplicate using 2 µL cDNA in a final volume of 20 µL. The following thermal cycle was used: 10 minutes-95°C; 40 cycles of 30 seconds-95°C, 40 seconds-primer specific annealing temperatures, 40 seconds-72°C. The expression level of each gene was analyzed using the ΔΔCt method and reported as relative expression normalized to the housekeeping gene. Taqman primers were purchased from Life Technologies (sequences available from Life Technologies).

2.2.9 Antibodies

Antibodies used in immunohistochemistry (IHC), Western blot (WB), and immunofluorescence (IF) are listed in Appendix.

2.3 Results

2.3.1 Astrocyte senescence is increased in irradiated patient tissues.

Radiation-induced cellular senescence is a stress-induced cell cycle arrest that may contribute to the development of radiotherapy side effects.[18,19] To characterize cellular senescence in the brain, tissue samples from patients with or without a history of radiation treatment were examined. Immunohistochemistry was performed using antibodies against senescence-associated proteins $p16^{INK4A}$ and $Hp1\gamma^{21-23}$ (Figure 2.1A) and scored based on the intensity of cellular labeling (Figure 2.1B). Tissue immunoexpression of senescence proteins was lowest in brain tissue from non-disease (ND), age-matched controls, was increased in untreated cancer patients, and was highest in irradiated tissues (Figure 2.1B). Similar results were observed in a subset of patients receiving stereotactic radiotherapy (Figure 2.1C-D) which in contrast to non-targeted whole brain radiotherapy, allows for comparison of irradiated to untreated brain regions within the same patient as an internal control.

We next aimed to characterize senescent cell types in irradiated patient tissues. HP1γ- and $p16^{INK4A}$-positive cells were identified by three independent pathologists (J.B., B.H., I. O.). The majority of senescence-associated markers co-localized with GFAP-positive astrocytes (Figure 2.1E) underscoring the potential importance of astrocyte senescence in the brain's response to radiation. The mild increase in cellular senescence in untreated cancer patient tissues compared to non-disease controls (Figure 2.1B) may indicate a role for the tumor microenvironment in promoting reactive astrocytosis and astrocyte senescence, which may be a general response of human astrocytes to injury. However, the number of $p16^{INK4A}$-positive astrocytes was higher in patients receiving radiation treatment compared to untreated cancer patients (Figure 2.1F-G), suggesting that radiotherapy may exacerbate this response. Astrocyte senescence is also increased

in Alzheimer's disease (Case 7, Figure 2.1C-D) and may promote neurotoxicity, highlighting the potential importance of astrocyte senescence in neurodegenerative diseases.[16,17,20] Finally, focal Hp1γ immunoreactivity was identified in several additional cell types, including microglia, which are important mediators of neuroinflammation[24]; however, this effect was less prominent than the described astrocyte senescence (Figure 2.1H).

2.3.2 Radiation induces cellular senescence in human astrocytes

Radiation can induce DNA damage either directly through ionization or indirectly through the production of free radicals.[5,14] Adult and pediatric patients with brain cancer may receive 30 to 60 Gy of radiation, which is administered in small doses or fractions of approximately 2 Gy per treatment until the total dose is achieved.[1-3] After a single 2 Gy fraction, primary human astrocytes irradiated *in vitro* have significant increases in DNA double-strand breaks indicated by γH2AX ($p = 0.013$) and 53BP1 ($p = 0.035$) (Figure 2.2A-B).[25]

Following accumulation of DNA damage, one of several cell-type specific responses may occur including induction of apoptosis, mitotic catastrophe, or cellular senescence.[5,15] Our study has identified astrocytes as the major senescent cell type in irradiated brain tissues. To further characterize this, we next investigated astrocytes irradiated *in vitro* for the induction of cellular senescence, a response that may promote side effects of cancer treatment.[18,19] Irradiated astrocytes experienced a significant increase in senescence-associated (SA)-β-gal staining beginning 2 days after irradiation ($p = 0.010$, 1.5-fold) and persisting for up to 1 week ($p = 0.03$, 2.3-fold) (Figure 2.2C-D). SASP-associated cytokines, including IL-1β and IL-6, are known to be upregulated in patients and animal models following radiation treatment.[20,26,27] To determine whether astrocytes may contribute to radiation-induced inflammation, we examined several cytokines implicated in neurodegeneration[20,28] and found a significant increase in IL-1β ($p = 0.016$), IL-6 ($p = 0.0005$),

and IL-8 ($p = 0.006$) (Figure 2.2E). The significant induction of SASP cytokines in irradiated astrocytes underscores their potential role in promoting neuroinflammation in radiation-induced brain injury. In addition, radiation-induced astrocyte senescence was accompanied by a significant loss of IGF-1 ($p = 0.015$, Figure 2.2F), a growth factor reported to promote astrocyte-mediated neuroprotection and improve neurocognitive function in mouse models of brain injury.[29,30] Irradiated astrocytes also demonstrated increased expression of senescence-associated $p16^{INK4A}$ ($p < 0.0001$) and p21 ($p = 0.009$) (Figure 2.2G-I). Finally, radiation-induced astrocyte senescence was found to be dose-dependent with high SA-β-gal activity and secretion of IL-6 in human astrocytes irradiated with radiosurgical doses (10 Gy, Figure 2.3). Taken together, these *in vitro* results indicate that irradiated astrocytes undergo senescence, which is consistent with our findings in patient tissues, and with animal models of radiation-induced brain injury.[27,28]

2.3.3 Δ133p53 is decreased in irradiated astrocytes and its overexpression protects astrocytes from radiation-induced cellular senescence

Senescent astrocytes are observed in patients with neurodegenerative diseases including Alzheimer's disease and amyotrophic lateral sclerosis and have been shown to have reduced expression of p53 isoform, Δ133p53.[20] To identify brain cells expressing Δ133p53 in human brain tissue, immunofluorescence was performed using a Δ133p53-specific antibody MAP4[20,31] and cell-type specific antibodies for astrocytes (GFAP-positive[20]) or neurons (NeuN-positive[32]). The majority of Δ133p53 expression co-localizes with GFAP-positive astrocytes (Figure 2.4A) indicating that astrocytes are the predominant source of Δ133p53. Following radiation exposure, primary human astrocytes have decreased Δ133p53 (Figure 2.4B), which is further diminished after exposure to a second 2 Gy fraction (4 Gy total dose) (Figure 2.4C), suggesting that loss of Δ133p53 may be associated with the induction of radiation-induced astrocyte senescence.

As Δ133p53 is diminished in irradiated senescent astrocytes, we investigated whether reconstitution of Δ133p53 expression would protect astrocytes from radiation-induced senescence. First, a lentiviral vector expressing Δ133p53 or pLOC control vector was transduced in primary human astrocytes three days after radiation exposure (Figure 2.4D). Irradiated astrocytes with reconstituted Δ133p53 had reduced SA-β-gal activity compared to control astrocytes ($p = 0.0006$) (Figure 2.4E-F), indicating that Δ133p53 can rescue astrocytes from radiation-induced senescence. Finally, we examined the impact of transducing astrocytes with lentiviral vectors expressing Δ133p53 or pLOC control prior to radiation exposure and found that astrocytes with Δ133p53 had no increase in SA-β-gal staining ($p = 0.483$) compared to an increase of approximately 55% in irradiated pLOC control astrocytes ($p < 0.0001$) (Figure 2.4G-H) demonstrating that increasing Δ133p53 protects astrocytes from radiation-induced senescence when induced either prior to or after radiation exposure.

2.3.4 Δ133p53 promotes DNA repair in irradiated astrocytes

Recently, Δ133p53 has been shown to promote DNA repair in fibroblasts from patients with Hutchinson-Gilford Progeria Syndrome through the promotion of homologous recombination (HR) DNA repair protein RAD51.[33] Following irradiation, RAD51 is significantly increased in astrocytes transduced with Δ133p53 ($p = 0.016$, Figure 2.5A). Although this increase may be due to accelerated cell proliferation[34], confluent human astrocytes transduced with Δ133p53 maintained a 2-to 3- fold increase in RAD51 (Figure 2.5B-C). The sustained increase in RAD51 at confluency, which is associated with G1 arrest[35], suggests that the effect of Δ133p53 on HR may be at least in part due to an increased baseline expression of RAD51, although this finding does not rule out a S/G2 phase-specific regulation of HR machinery. To further examine the role of Δ133p53 in HR, DNA double-stranded breaks were labeled with γH2AX. Six days after

irradiation, the percent of γH2AX-positive astrocytes was significantly reduced by Δ133p53 transduction after radiation exposure ($p < 0.0001$, Figure 2.5D-E). To examine DNA repair kinetics at earlier time points, astrocytes were transduced prior to irradiation and labeled at 4 and 24 hours post-irradiation with RAD51, γH2AX and 53BP1 (Figure 2.5F-H). After four hours, the number of DNA damage foci labeled by γH2AX and 53BP1 was not significantly different (Figure 3.5G-H), suggesting that both control and Δ133p53 transduced cells develop similar levels of radiation-induced DNA damage; however, after 24 hours, Δ133p53-transduced astrocytes had fewer γH2AX ($p = 0.00002$) and 53BP1 foci ($p = 0.0006$), suggesting that Δ133p53 promotes DNA repair in irradiated astrocytes.

2.3.5 Δ133p53 inhibits astrocyte-mediated neuroinflammation

Because radiation-induced brain injury is associated with neurocognitive dysfunction, many studies focus on the effects of radiation on neurons and NPCs[6,36,37]. Secretory factors derived from senescent astrocytes are known to impair astrocyte-mediated neuroprotection in animal models[38] and may promote the late effects of radiation injury by contributing to chronic neuroinflammation. Of the SASP cytokines, IL-6, is most frequently upregulated in neurodegeneration.[39] Following radiation exposure, human astrocytes produce significantly more IL-6 (Figure 2.2), similar to replicatively senescent astrocytes, which are neurotoxic via IL-6 in neuron-astrocyte co-culture experiments.[20] This was further examined through direct exposure of neural stem cells (NSCs) and mature neurons to IL-6 (5 ng/mL). After 24 hours, there was an approximately 10% increase in the percent of mature neurons expressing apoptotic marker, cleaved caspase-3 ($p = 0.013$; Figure 2.6A-B), and the viability of NSCs was reduced to less than 50% (p

= 0.0001; Figure 2.6C), suggesting that IL-6 plays a causative role in neuronal death mediated by radiation-induced senescent astrocytes.

Because Δ133p53 was found to rescue irradiated astrocytes from senescence (Figure 2.4), we next investigated whether Δ133p53 rescues astrocytes from radiation-induced production of neurotoxic IL-6. Irradiated control astrocytes experienced a significant 5-fold increase in IL-6 mRNA measured by qRT-PCR ($p = 0.0005$). In contrast, IL-6 mRNA was not significantly upregulated in irradiated astrocytes with restored Δ133p53 ($p = 0.389$, Figure 2.6D), indicating that astrocyte-mediated neuroinflammation is repressed by reconstitution of Δ133p53 after radiation treatment. Similar findings were also observed in astrocytes transduced prior to radiation including a significant reduction in secreted IL-6 ($p = 0.017$, Figure 2.6E). In addition, astrocytes transduced with Δ133p53 demonstrated a partial rescue of neurotrophic IGF-1 mRNA expression ($p = 0.015$, Figure 2.6F). Taken together, these findings suggest that radiation induces astrocyte senescence thereby promoting astrocyte-mediated neurotoxicity through the production of neurotoxic secretory factors. Critically, Δ133p53 has been identified as a potential therapeutic target for inhibiting radiation-induced astrocyte-mediated neurotoxicity (Figure 2.7).

2.4 Discussion

Radiation-induced brain injury may cause progressive cognitive deterioration, including dementia-like symptoms[6]. It shares pathological features with aging-associated neurodegeneration, including chronic oxidative stress, inflammation, and reduced neurogenesis.[6,40,41] Current understanding of the pathogenesis of radiation-induced brain injury focuses on the acute loss of NSCs and its effect on hippocampus-dependent functions such as learning and memory.[37,42] However, few studies have addressed the role of astrocytes. Our finding that astrocytes

preferentially undergo senescence, while NPCs undergo cell death, indicates that astrocyte SASP may underlie the chronic nature of radiation-induced brain injury.

Animal models of radiation-induced brain injury have identified hypertrophied astrocytes that persist for at least 12 months following radiation treatment.[43,44] Based on our findings in irradiated human tissues and our previous findings in Alzheimer's disease and ALS[20], many of these hypertrophied astrocytes are senescent, an important pathologic characterization that likely extends to other disease processes in the brain.

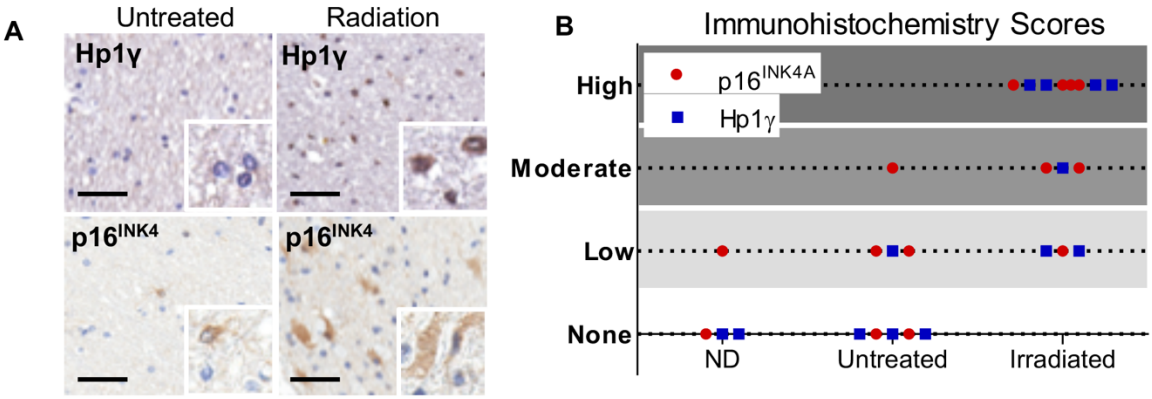
Following brain injury, astrocytes proliferate as part of reactive astrogliosis, which may lead to replicative senescence.[20,45,46] In addition, direct injury including DNA injury or oxidative damage may induce premature cellular senescence.[17,18,46] Both mechanisms of cellular senescence are controlled by p53 and its isoforms through p53-inducible cell cycle regulators, such as p21.[20,31] In humans, TP53 has at least 12 isoforms formed through alternative promoters or splicing that may promote or inhibit full-length p53 activities or have independent functions. Of these isoforms, Δ133p53 is the best characterized as an endogenous inhibitor of cellular senescence.[20,31,33] Based on this and previous studies[33], Δ133p53 enhances DNA repair in senescent cells by promoting HR; however, our study has also demonstrated that expression of Δ133p53 enhanced repair of foci positive for 53BP1, a component of non-homologous end-joining (NHEJ)[47], suggesting that Δ133p53 may also regulate NHEJ in radiation injury by a currently unknown mechanism.

In addition to accumulating DNA damage, senescent cells may promote inflammation through induction of SASP.[16,17] Increased release of SASP cytokines IL-6[48,49] and IL-1β[49] is reported in animal models of radiation-induced brain injury and may inhibit neurogenesis contributing to cognitive impairment.[26,50,51] Using anti-inflammatory drugs to target and reduce neuroinflammation in radiation injury improves neurogenesis[26] while IL-6 has been shown to

reinforce radiation-induced senescence in animal models[52], underscoring the role of chronic neuroinflammation in promoting radiation-induced brain injury. Based on the findings outlined in this study, astrocyte senescence and astrocyte-derived neuroinflammation have been identified as potential contributors to radiation-induced brain injury.

This and previous studies have demonstrated that Δ133p53, through the inhibition of full-length p53, regulates p21[31,33], RAD51[33], and IL-6[20,33], each of which has been shown to be important in radiation-induced injury and neurotoxicity. Although the regulatory interactions between these factors have yet to be elucidated, our findings suggest that induction of the p53 isoform, Δ133p53 may have potential therapeutic value by preventing astrocyte senescence and inhibiting astrocyte-mediated neuroinflammation (Figure 2.7). Critically, this endogenous isoform is produced in human cells and has not been shown to be mutagenic or oncogenic.[20,31,53] To study the role of Δ133p53 in other cell types and the tumor microenvironment *in vivo*, ongoing studies seek to establish an animal model and identify compounds which modulate Δ133p53. Future studies aim to reverse the senescence phenotype in diseases, such as radiation-induced brain injury, in which cellular senescence may initiate or worsen disease progression.[20,31,33]

Figure 2.1. Astrocyte Senescence is Increased in irradiated Patient Tissues. (A) Expression of senescence-associated proteins, Hp1γ and $p16^{INK4}$, in irradiated and untreated non-tumor brain tissues using immunohistochemistry. (B) Tissues were examined in a blinded-fashion by three pathologists and scored from 0 (none) to 3 (high) based on intensity of cell labeling. (C) Hp1γ and (D) $p16^{INK4A}$ immunohistochemical labeling in three patients receiving stereotactic radiation with comparison of irradiated to untreated tissue in the same patient as an internal control. *Case 7 is from a patient previously diagnosed with Alzheimer's disease in which astrocyte senescence is prominent and thought to promote neurodegeneration[1]. (E) Immunocytochemistry of irradiated brain tissue demonstrating co-localization of $p16^{INK4A}$ and GFAP in astrocytes. (F) $p16^{INK4A}$-positive astrocytes in irradiated human brain tissues using immunohistochemistry. (G) Quantification of $p16^{INK4A}$-positive astrocytes in twenty microscopic fields (0.5 mm^2) in non-tumor brain tissue from untreated cancer patients (n = 4) and cancer patients receiving radiation treatment (n = 4). (H) Representative images of cell types expressing senescence-associated Hp1γ in irradiated (stereotactic) and untreated brain tissue from the same patient, including endothelia, astrocytes, neurons, meninges, and microglia. Scale = 50μm.



C

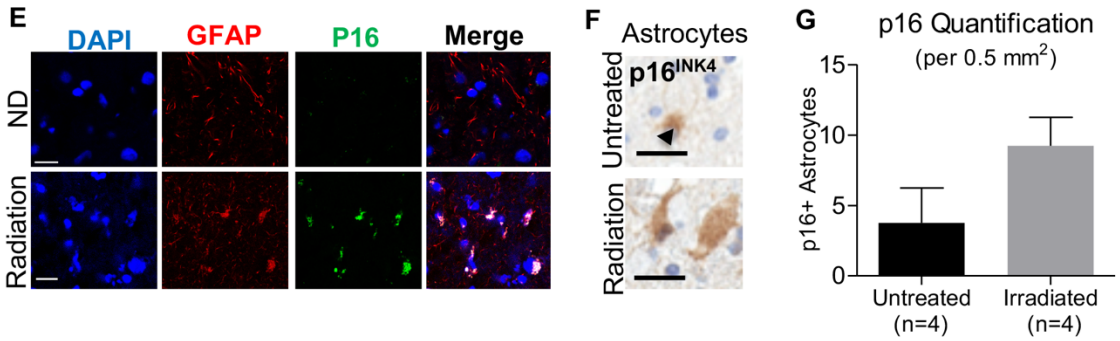
Hp1γ in Case-Matched Tissues

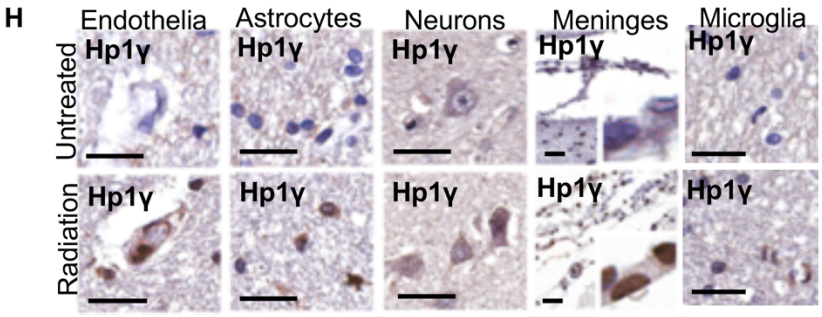
Case #	Untreated	Irradiated
Case 1	None	Low
Case 2	None	Moderate
Case 7	Low	Low

D

p16INK4A in Case-Matched Tissues

Case #	Untreated	Irradiated
Case 1	Low	High
Case 2	Low	High
Case 7	Moderate	High





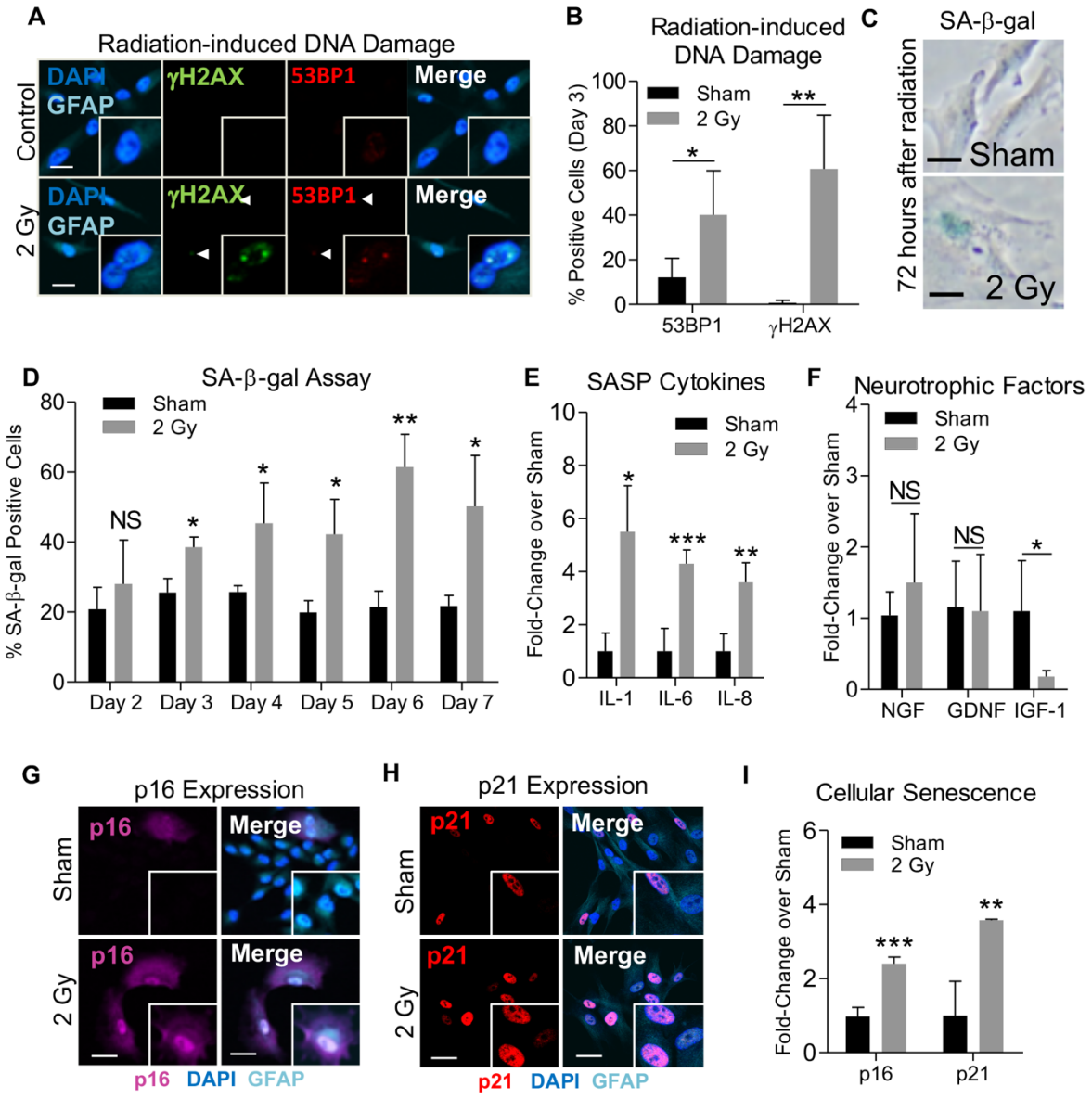


Figure 2.2. Radiation Induces Astrocyte Senescence and SASP. (A) Representative image and (B) quantification of radiation-induced DNA damage identified by immunolabeling of double-stranded DNA breaks by 53BP1 and γH2AX in primary human astrocytes 3 days after radiation exposure (2 Gy). (C) Representative images of SA-β-gal staining in human astrocytes on day 3 after exposure to radiation (2 Gy) (D) Quantitative summary of the percent of astrocytes with SA-β-gal staining from 2 to 7 days after radiation (2 Gy). (E) Production of SASP-associated cytokine mRNAs (IL-1β, IL-6 and IL-8 mRNA) and (F) neurotrophic factor mRNAs (NGF, GDNF, IGF-1) in irradiated or sham-treated primary human astrocytes measured by qRT-PCR (Taqman). Representative images of (G) $p16^{INK4}$ and (H) $p21^{WAF1}$ immunolabeling in irradiated and sham-treated human astrocytes (I) Quantitation of $p16^{INK4A}$ and $p21^{WAF1}$ immunoreactivity in irradiated (2 Gy) and sham-treated primary human astrocytes on day 6. NS indicates $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ by unpaired two-tailed Student's t test. Scale = 25μm

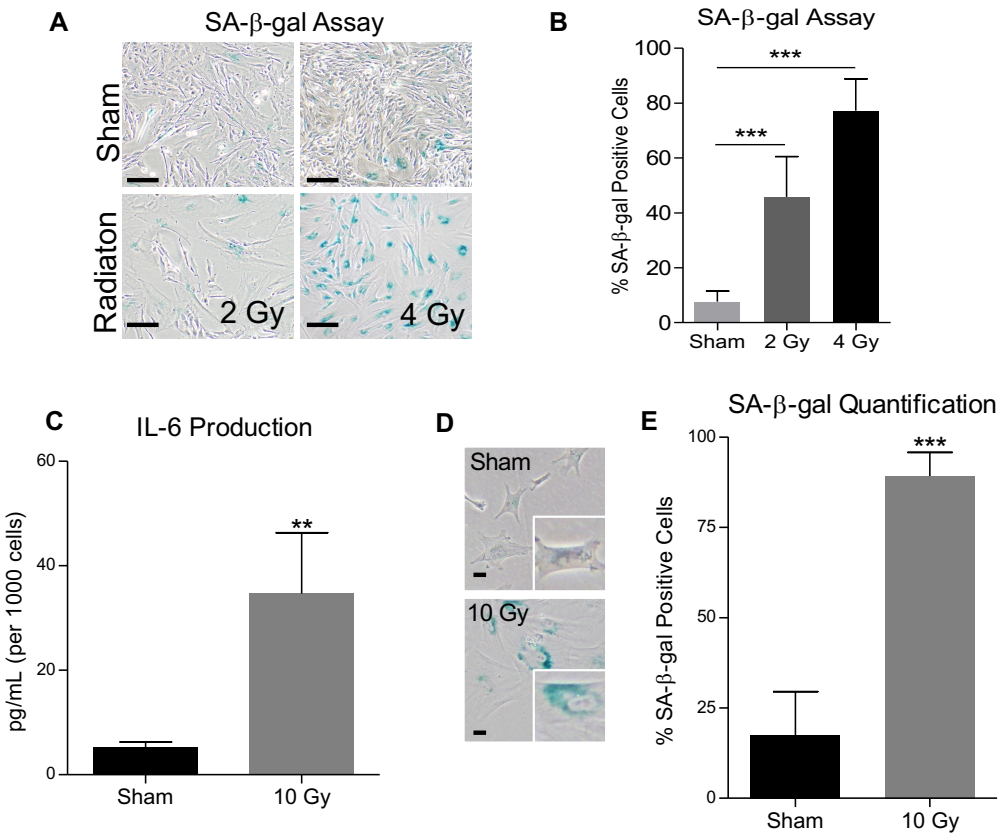
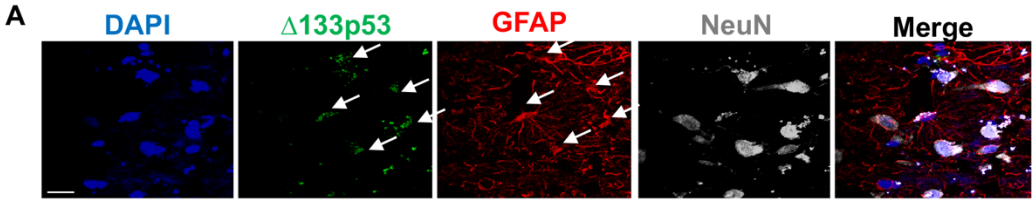
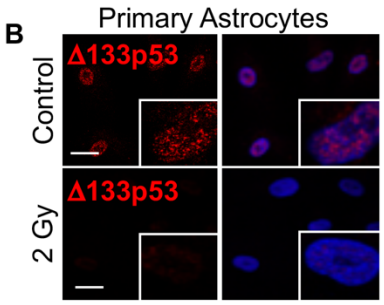


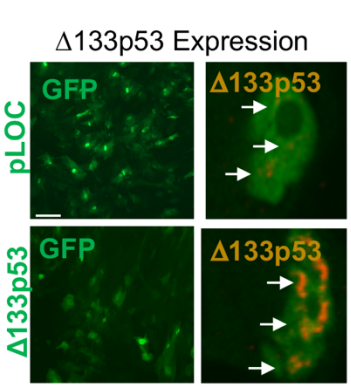
Figure 2.3. Radiation-induced Astrocyte Senescence is Dose-dependent. (A) Representative image and (B) Quantification of SA-β-gal staining in primary human astrocytes exposed to either 2 Gy or 4 Gy (2 Gy fraction given twice 24 hours apart) and analyzed on day 6 after radiation exposure. (C) IL-6 production and (D-E) SA-β-gal staining in astrocytes exposed to stereosurgical doses of radiation (10 Gy).

Figure 2.4. Δ133p53 is Decreased in Irradiated Astrocytes and its Overexpression Protects Astrocytes from Cellular Senescence. (A) Non-disease human brain tissue fluorescently-labeled with antibodies to Δ133p53, astrocytic glial fibrillary acidic protein (GFAP), and neuronal-specific nuclear protein (NeuN) to identify cellular sources of Δ133p53 (arrows). (B) Primary human astrocytes labeled with nuclear staining (DAPI) and Δ133p53 on day 6 following either sham or radiation treatment (2 Gy). (C) Western blot analysis of Δ133p53 on day 6 in sham-treated or irradiated primary human astrocytes irradiated one time at 2 Gy or twice at 2 Gy 24 hours apart (fractionated dose, 4 Gy total). (D) Nuclear Δ133p53 expression in human astrocytes transduced three days after radiation treatment (2 Gy) with either a GFP lentiviral vector driving Δ133p53 expression or its control vector (pLOC). (E) Representative image and (F) quantitative summary of SA-β-gal staining in primary human astrocytes with lentiviral pLOC and Δ133p53 transduced 3 days after radiation exposure (2 Gy). (G) Representative image and (H) quantification of SA-β-gal staining in primary human astrocytes transduced prior to radiation exposure (2 Gy). Scale = 25μm


A
DAPI
Δ133p53
GFAP
NeuN
Merge


B
Primary Astrocytes
Control
2 Gy
Δ133p53
Δ133p53


C
2 Gy
4 Gy
Sham
Gy
Sham
Gy
Δ133p53
GAPDH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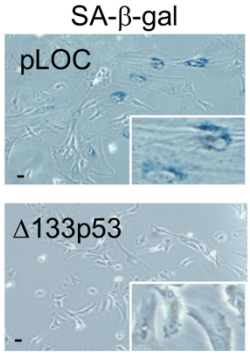
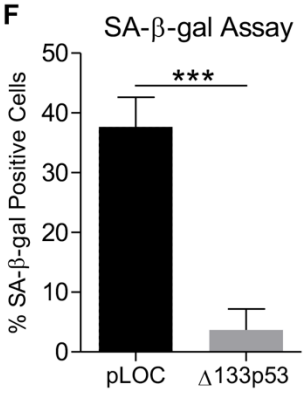
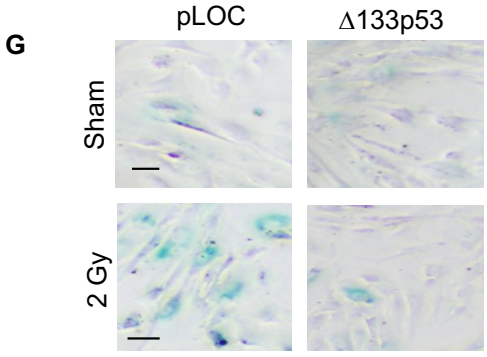
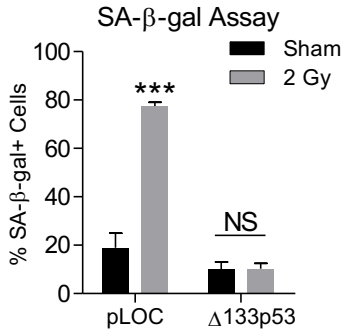
Δ133p53 Expression
pLOC
Δ133p53
GFP
Δ133p53
GFP
Δ133p53


E
SA-β-gal
pLOC
Δ133p53


F
SA-β-gal Assay
% SA-β-gal Positive Cells
50
40
30
20
10
0
***
pLOC
Δ133p53


G
pLOC
Δ133p53
Sham
2 Gy


H
SA-β-gal Assay
Sham
2 Gy
% SA-β-gal+ Cells
100
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NS
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Δ133p53

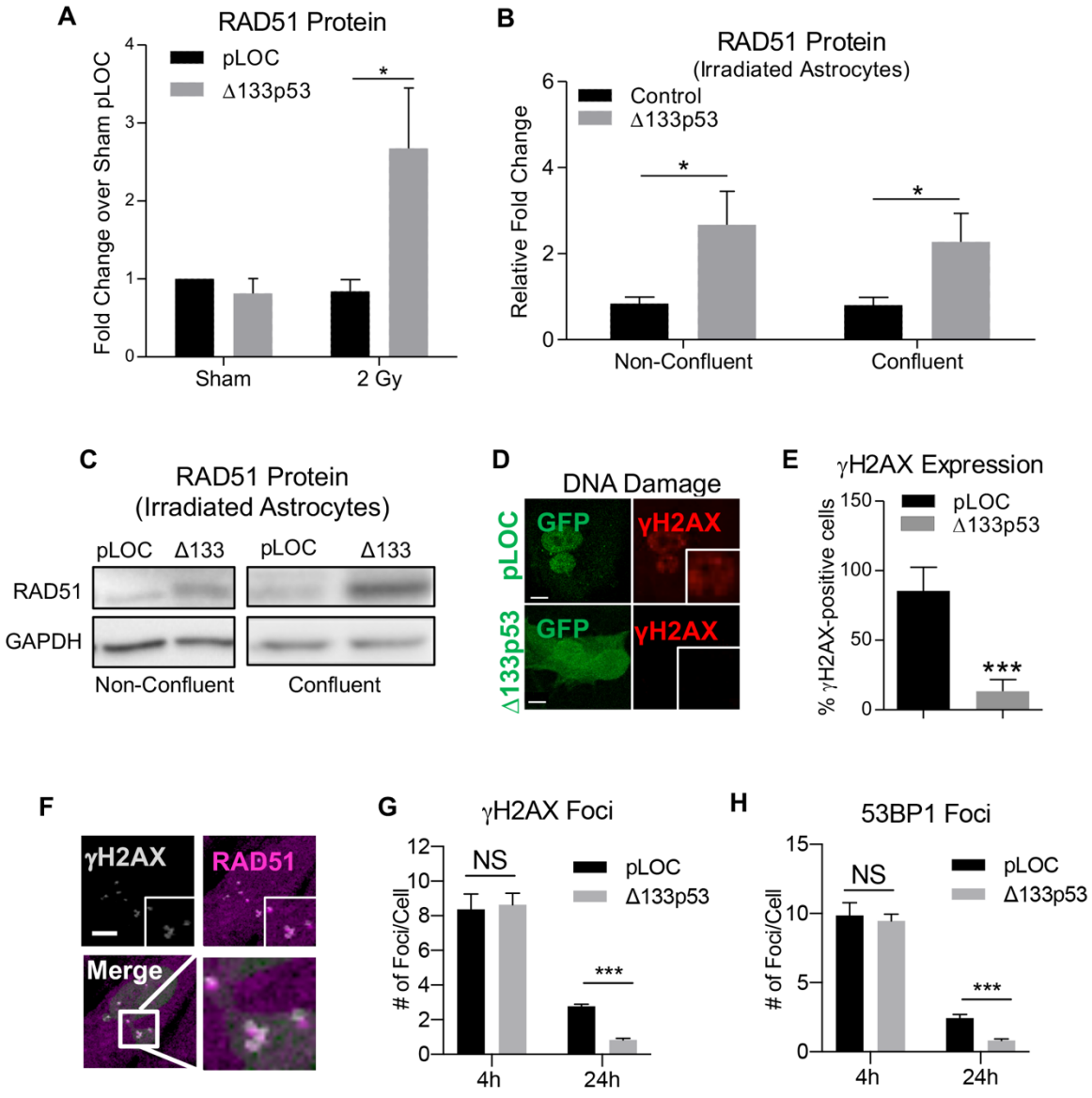

Figure 2.5. Δ133p53 Promotes DNA Repair. (A) RAD51 protein in sham and irradiated astrocytes expressing either control vector (pLOC) or Δ133p53 (B) Quantification and (C) representative western blots of RAD51 protein in irradiated astrocytes expressing pLOC or Δ133p53 at low and high confluency. (D) Labeling of DNA double-strand breaks with γH2AX in transduced, irradiated astrocytes. (E) Quantitative summary of γH2AX staining on day 6 in irradiated human astrocytes transduced with pLOC or Δ133p53 on day 3 after radiation exposure (2 Gy). (F) Representative image of RAD51 and γH2AX labeling 4 hours after radiation exposure. (G) Quantification of γH2AX- and (H) 53BP1-positive foci at 4 and 24 hours after radiation exposure in astrocytes transduced prior to radiation treatment. NS indicates $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ by unpaired two-tailed Student's t test. Scale = 5μm

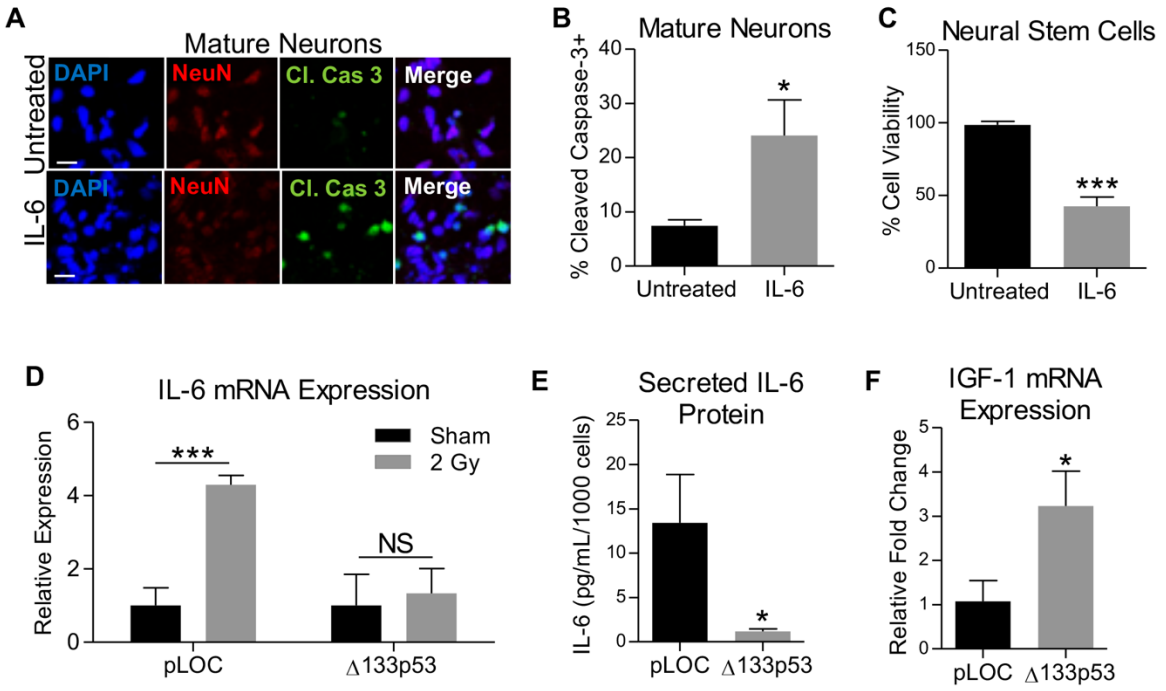

Figure 2.6. Δ133p53 Regulates Radiation-induced, Astrocyte-mediated Neurotoxicity. (A-B) Immunopositivity of cleaved caspase 3 in mature neurons and (C) viability of neural stem cells following 24-hour IL-6 exposure (5ng/mL). (D) IL-6 mRNA production in sham and irradiated astrocytes transduced with either Δ133p53 ($p = 0.389$) or the control vector on day three and examined on day 6 by qRT-PCR (taqman). (E) IL-6 protein secreted by astrocytes transduced prior to radiation and examined by ELISA (F) IGF-1 mRNA expression in irradiated astrocytes transduced with pLOC or Δ133p53 vector prior to radiation exposure ($p = 0.015$) and examined by qRT-PCR (taqman). NS indicates $p > 0.05$, *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ by unpaired two-tailed Student's t test. Scale = 25μm

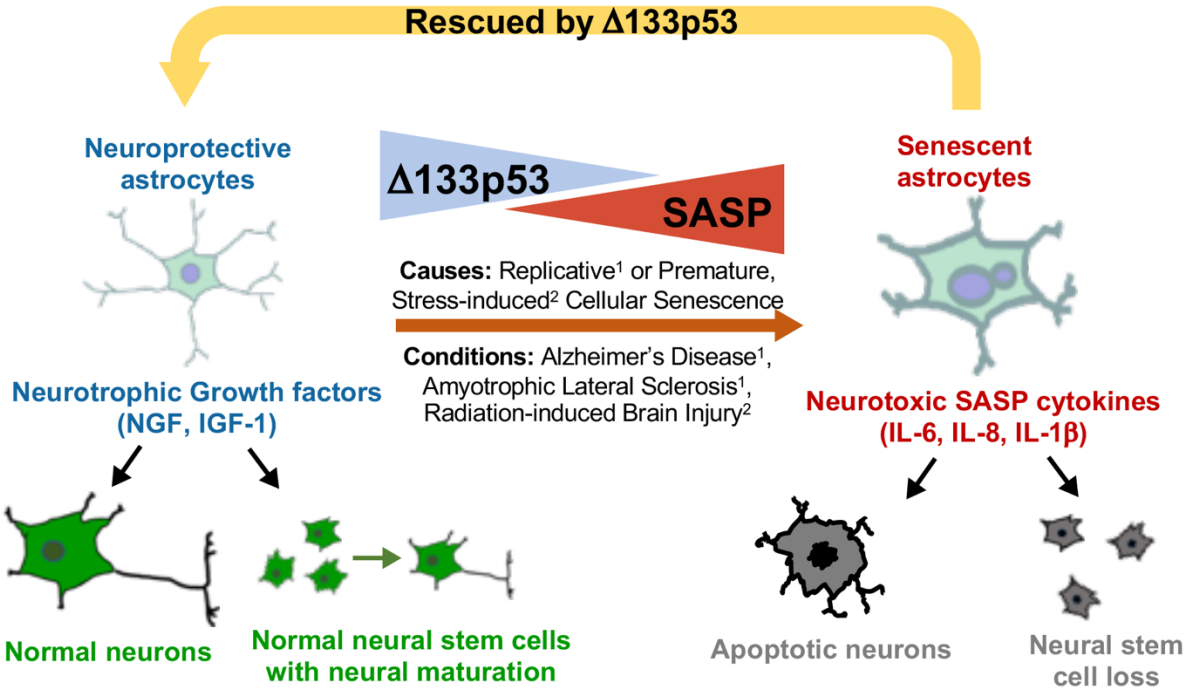

Figure 2.7. Proposed Model of Δ133p53 Regulation of Astrocyte-mediated Neuroprotection and Neuroinflammation. Senescent astrocytes are increased in neurodegenerative diseases, including Alzheimer's disease, and have diminished Δ133p53. Similarly, senescent astrocytes are observed in brain tissues from cancer patients receiving radiation treatment, suggesting that senescent astrocytes may contribute to chronic neuroinflammation in each of these pathologies. These findings are also reproduced *in vitro* where cellular senescence is induced in irradiated or replicatively exhausted astrocytes and is associated with loss of Δ133p53, adoption of the senescence-associated secretory phenotype (SASP), and diminished neurotrophic factor production, including insulin-like growth factor-1 (IGF-1), which can each be rescued by enhanced expression of Δ133p53. [1]Turnquist et al., 2016; [2]Current Study

Table 2.1 Patient tissue demographics and IHC scores. List of patient age and sex, radiation treatment including type and dose of radiation (whole brain, stereotactic) and $p16^{INK4A}$ and Hp1γ scores. Abbreviations: AD, Alzheimer's disease; CM, case-matched; G, gray; ND, Non-disease; N/A, additional brain tissue not available for evaluation; S, stereotactic radiation; WB, whole brain radiation

Group	Case #	Age/Sex	Gy/Radiotherapy	p16	Hp1γ
Brain radiation	1	68/M	40/WB	+3	+1
Brain radiation	2	49/M	30/S	+3	+2
Brain radiation	3	74/F	18/S	+1	+3
Brain radiation	4	74/M	15/S	+2	+3
Brain radiation	5	73/M	18/S	+2	+3
Brain radiation	6	64/M	30/WB+S	+3	+3
Brain radiation +AD	7	79/M	18S	+3	+1
Control(CM)	1	68/M	--	+1	0
Control(CM)	2	49/M	--	+1	0
Control(CM)	5	73/M	--	0	N/A
Control(CM+AD)	7	79/M	--	+2	+1
Control (ND)	8	72/M	--	0	N/A
Control (ND)	9	46/F	--	+1	N/A
Control (No radiation)	10	41/M	--	0	N/A
Control (ND)	11	72/M	--	N/A	0
Control (ND)	12	61/M	--	N/A	0
Control (No radiation)	13	97/F	--	N/A	0

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CHAPTER 3. TP53 ISOFORMS, Δ133P53 AND P53β, REGULATE CELLULAR SENESCENCE IN GLIOBLASTOMA MULTIFORME

Jessica A. Beck,[1,2] Sebastien Jouiz,[1] Izumi Horikawa,[1] Natalia von Muhlinen,[1] Akriti Trehan,[1] Chun Zhang Yang,[3] Mark R. Gilbert,[3] Curtis C. Harris[1]

[1]Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA (JAB, SJ, IH, NVM, AT, CCH)
[2]Department of Comparative Pathobiology, Purdue University, West Lafayette, IN, USA (JAB)
[3]Neuro-Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA (CZY, MRG)

3.1 Introduction

Glioblastomas, or Grade IV astrocytomas, are the most common malignant brain tumor and are characterized histologically by cellular atypia, regions of pseudo-palisading necrosis, and microvascular proliferation.[1,2] These tumors are difficult to treat with less than 50% of patients surviving one year after diagnosis.[2] In 2005, the addition of temozolomide therapy to glioblastoma treatment protocols extended the two year survival rate by 15%.[3] Temozolomide induces cellular senescence or permanent cell cycle arrest in glioblastoma cells.[4-6] Cellular senescence is a protective mechanism that prevents the continued replication of DNA-damaged cells; however, cancer cells may develop methods to overcome cellular senescence programming leading to uncontrolled proliferation of abnormal cells and treatment failure. For example, many tumor types harbor mutations in TP53[7] which have been shown to inhibit treatment-induced senescence.[8,9] In the case of temozolomide, the induction of senescence requires functional TP53 and sustained activation of the p53-target genes such as p21.[9] In addition, inhibition of p53 activity is critical to the development of tumors in experimental models.[10-12] Interestingly, the majority of glioblastomas lack p53 mutations.[13] This suggests that p53 pathway dysfunction occurs through alternative mechanisms in glioblastomas. Factors that contribute to p53 dysregulation in

glioblastomas include altered expression of p53 regulators and failed induction of p53 targets.[12,14-16]

Another potential mechanism by which cells modulate p53 activity is through the altered expression of p53 isoforms.[17] The p53 isoforms are truncated proteins physiologically expressed in normal human cells and produced through alternative RNA splicing or alternative initiation of transcription and translation.[4,18] Two of the isoforms, Δ133p53α and p53β, serve as endogenous regulators of cellular senescence.[4] The Δ133p53 isoform lacks the trans-activating domain (TAD) and dominant-negatively inhibits p53-mediated senescence in normal human cells. In contrast to Δ133p53, the p53β isoform is a C-terminally truncated protein that promotes cellular senescence.[4,17] Previous studies have identified aberrant expression of p53 isoforms in cancer tissues suggesting that they may have functional implications for cancer progression.[19-21]

In this study, we investigate the role of p53 isoforms, Δ133p53 and p53β, and the factors known to regulate their production, namely SRSF1, SRSF3, and SRSF7. Understanding the functions of p53 isoforms in tumor cells is critical to understanding the response of glioblastoma to senescence-inducing cancer therapies. This study identifies a role for p53 isoforms, Δ133p53 and p53β, in regulating glioblastoma senescence and identifies SRSF3 as a potential therapeutic target to enhance treatment response. These findings also underscore the critical need to consider aberrant p53 isoform function as a potential mechanism by which cancer cells, especially those without *TP53* mutations, disrupt the p53 pathway and promote tumor progression.

3.2 Methods

3.2.1 Cell Culture

Glioblastoma cells with wild-type p53 (U-87 MG, A172)[22] were obtained from the Neuro-Oncology Branch (C.Z.Y., M.G.). Glioblastoma cells with mutant p53 were obtained from the Division of Cancer Treatment & Diagnosis (DCTD) repository at the National Cancer Institute. Cells were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 1% L-glutamine. Where indicated, glioblastoma cells were exposed to temozolomide (50 μm for 5 days, Sigma). SA-β-gal staining was performed with the Senescence Associated (SA)-β-Galactosidase Staining Kit (Cell Signaling Technology, Danvers, MA, USA). Quantification of IL-6 in the cell culture media was performed using the Human IL-6 ELISA Kit (Sigma-Aldrich).

3.2.2 Transfection

siRNA oligonucleotides were transfected at a final concentration of 10 nM using Lipofectamine RNAiMAX (Invitrogen). The following oligonucleotides were obtained from Invitrogen targeting Δ133p53 (5′-GGAGGUGCUUACACAUGUU-3′), SRSF3 (SRSF3-A 5′-AGAGCUAGAUGGAAGAACATT-3′), and Stealth non-specific RNAi negative control (no. 12,935-100). SiRNA targeting beta (5’-GGACCAGACCAGCUUUCAA-3’) was purchased from Eurogentec and transfected at a final concentration of 15 nM using Lipofectamine RNAiMAX (Invitrogen). To examine Δ133p53 overexpression, U-87 glioblastoma cells were transfected with 1 ug of plasmid using TurboFect (Thermofisher Scientific) and selected using Geneticin (Gibco) as described previously.[19,23,24]

3.2.3 Western Blot

Cell samples were lysed in radioimmunoprecipitation assay buffer (RIPA) on ice. Protein concentration was measured using the Bradford assay method. Prior to running the western blot, all lysates were diluted with NuPAGE 4X loading buffer and boiled for 5 minutes. Tris-glycine gels (Novex) were loaded with 40 μg of protein for electrophoresis. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane at 4C. Membranes were blocked in 1:1 mixture of Superblock and Tris Buffered Saline (TBS, 125 mM Tris and 200 mM NaCl), containing 0.1% Tween-20. After blocking, membranes were incubated in the primary antibodies (Appendix) overnight at 4°C. The following day, membranes were washed 3 times in TBS-Tween-20 and incubated with mouse or rabbit HRP- conjugated secondary antibody (Pierce) for 1 hour at room temperature. Signal was visualized using SuperSignal developing reagent and the Biorad imager. To compare protein bands, densitometry was performed using ImageJ software. Patient sample lysates examined in Figure 3.9A were obtained from the Neuro-Oncology Branch at NCI (C.Z.Y. and M.R.G.)

3.2.4 Quantitative Real-Time Polymerase Chain reaction (qRT-PCR)

Extraction of mRNA from cell samples was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The abundance and quality of the resulting RNA was assessed using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). RNA samples were diluted in RNase-free water. A total of 2 μg of RNA was converted to single-stranded cDNA in a 20 μL reaction using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The Tecan Sunrise 7500 real time PCR system (Applied Biosystem) was used for the quantitative analysis of mRNA expression using DNA binding dye SYBR Green (Qiagen) or Taqman (Life Technologies) primers. Each reaction was performed in triplicate using 2 μL cDNA

in a final volume of 20 μL. The following thermal cycle was used: 10 minutes-95°C; 40 cycles of 30 seconds-95°C, 40 seconds-primer specific annealing temperatures, 40 seconds-72°C. The expression level of each gene was analyzed using the ΔΔCt method and reported as relative expression normalized to the housekeeping gene.

3.2.5 Immunocytochemistry

Cells were washed with PBS and fixed for 10-15 minutes with 4% paraformaldehyde. Following fixation, cells were permeabilized (0.01% Triton-X, 10 minutes), washed with PBS (3 washes, 10 minutes each), and blocked in 5% fetal bovine serum (FBS) for 1 hour at room temperature. Slides were incubated with primary antibodies (Appendix) overnight at 4°C. Secondary antibodies conjugated to fluorophores were applied at a dilution of 1:400 (Life Technologies). Coverslips were mounted on slides with Vectashield mounting medium with DAPI (VectorLabs).

3.2.6 Oncomine

Oncomine was used to examine publicly available microarray data from the TCGA glioblastoma cohort and the Sun et al., Cancer Cell cohort and represented as log2-median centered intensity. TCGA samples include tumor and non-tumor tissue (TCGA Research Network: https://www.cancer.gov/tcga). The Sun cohort (Sun et al., *Cancer Cell* 2006;9(4):287-300) contains non-tumor brain tissue, astrocytoma samples (grade II, grade III) and glioblastomas (grade IV astrocytomas).

3.2.7 Statistical Analysis

Data are presented as mean and standard deviation with comparisons made using two-sided, unpaired Student's t test unless otherwise stated. Differences are considered significant at a value of * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ or NS (not significant).

3.2.8 Antibodies

Antibodies used in Western blot (WB) and immunofluorescence (IF) are listed in Appendix.

3.3 Results

3.3.1 Temozolomide chemotherapy induces cellular senescence and is associated with reduced expression of Δ133p53 in glioblastoma cells

Glioblastoma therapies have been shown to induce cellular senescence in cancer cells.[5,6] The field of p53 research has expanded following the discovery of at least 12 isoforms produced from the TP53 gene that modulate full-length p53 (FLp53) activity.[17,25] Our previous studies identified a role for one of these isoforms, Δ133p53α, in inhibiting p53-mediated cellular senescence in non-neoplastic astrocytes.[26,27] To investigate whether Δ133p53 might also regulate senescence in cancer cells, we examined glioblastoma cells after exposure to temozolomide (TMZ) at the peak concentration reported in the serum (50μM).[28] Temozolomide induces DNA damage indicated by increased DNA breaks labeled by γH2AX in treated glioblastoma cells (Figure 3.1A). Senescent cells adopt the senescence-associated secretory phenotype (SASP) characterized by increased secretion of an array of factors including chemokines, cytokines, and metalloproteinases.[29,30] Temozolomide increases glioblastoma secretion of SASP-associated IL-6 (U-87MG, $p = 0.003$, Figure 3.1B) and induces glioblastoma cell senescence (U-87MG, $p = 0.0002$, Figure 3.1C). To determine whether p53 isoforms play a role in temozolomide-induced senescence,

we next examined temozolomide-exposed glioblastoma cells for the expression of the FLp53 and the senescence-inhibiting p53 isoform, Δ133p53α. Following temozolomide exposure, FLp53 is increased (U-87MG, p = 0.032, Figure 3.1D) and has been shown to be critical to the induction of temozolomide-induced senescence[9]; however, whether Δ133p53 isoforms also contribute to temozolomide-induced senescence was previously unknown. Five days after temozolomide treatment, glioblastoma cells have reduced expression of Δ133p53α protein (p = 0.018, Figure 3.1D). There is also a reduction in the expression of Δ133p53β/γ (p = 0.13, Figure 3.1D). While Δ133p53α has been identified as an endogenous regulator of cellular senescence in non-tumor cells,[4] it has not been thoroughly characterized in cancer cells. There are even fewer studies examining Δ133p53β or Δ133p53γ. These findings suggest that the Δ133p53 isoforms may contribute to temozolomide-induced senescence.

3.3.2 Δ133p53 regulates cellular senescence in glioblastoma cells

In addition to FLp53, the TP53 produces multiple truncated isoforms including Δ133p53α, Δ133p53β, and Δ133p53γ.[17] These isoforms are produced through translation at the methionine codon 133 and differ due to alternative splicing at the C-terminal domain producing either the α, β, or γ domain.[17] After identifying diminished Δ133p53 expression in temozolomide-treated glioblastoma cells (Figure 3.1), we next aimed to directly investigate the role of the Δ133p53 isoforms in glioblastoma senescence through siRNA knockdown. Following Δ133p53 knockdown (Figure 3.2A), U-87MG glioblastoma cells undergo cellular senescence (SA-β-gal activity, p < 0.001, Figure 3.2B) and upregulate SASP-associated inflammatory cytokines (Figure 2E) including IL-6 (5.5 fold, p = 0.008) and IL-8 (14.7-fold, p = 0.013). In addition, senescent glioblastoma cells have increased p21 mRNA (9.2-fold, p = 2.6E-5; Figure 3.2D) and protein (p =

0.01, Figure 2C) and demonstrate reduced expression of Ki-67[31] (p = 0.023, Figure 3.2D), a proliferation marker associated with poor prognosis.[31,32] Finally, senescent U-87MG glioblastoma cells upregulate p53-associated apoptosis genes BAX (2.4-fold, p = 0.033) and PUMA (7.8-fold, p = 0.0024, Figure 3.2F). Similar results were observed in A172 glioblastoma cells following Δ133p53 knockdown (Figure 3.3) including increased SA-β-gal activity (p = 0.0003), reduced Ki-67 (p = 0.007), and elevated p21 immunoreactivity (p = 0.01). Taken together, these findings identify Δ133p53 as a regulator of p53-mediated cell fate and identifies features of SASP in senescent glioblastoma cells.

3.3.3 SRSF3 and SRSF1 are overexpressed in glioblastoma

We next examined the known regulators of p53 isoforms to examine whether they could contribute to aberrant p53 isoform expression (Figure 3.4A).[17,18,36-38] Although SRSF7 was not found to be dysregulated in the TCGA cohort (-1.5-fold; p = 0.964), both SRSF1 (Figure 3.4B) and SRSF3 (Figure 3.4C) were increased in glioblastoma compared to non-tumor brain tissue. Downregulation of either of these factors leads to increased production of p53β (Figure 3.4A), suggesting that their overexpression may inhibit the production of the pro-senescent p53 isoform, p53β.[18,39] We also examined the expression of SRSF1 and SRSF3 in different grades of astrocytoma. This cohort also demonstrated elevated expression of SRSF1 (1.7-fold) and SRSF3 (4.1-fold) in glioblastoma cells compared to non-tumor brain tissue (Figure 3.4D-E). Interestingly, SRSF3 expression was elevated in increasing grades of astrocytoma (p trend = <0.0001, Figure 3.4E). A similar trend was not observed for SRSF1 (Figure 3.4D). Taken together, these findings identify high expression of SRSF1 and SRSF3 in glioblastoma tissues and suggest that overexpression of SRSF3 may be important to glioblastoma progression.

3.3.4 Loss of SRSF3 leads to increased p53β and induction of cellular senescence and apoptosis.

After demonstrating SRSF3 overexpression in glioblastoma cells, we further interrogated the role of SRSF3 by treating U-87MG glioblastoma cells with siRNA to induce its knockdown. SRSF3 inhibits the production of the pro-senescence p53 isoform, p53β.[18] SRSF3 is reduced in temozolomide-treated cells (Figure 3.5A). Knockdown of SRSF3 increases expression of p53β in glioblastoma cells (Figure 3.5B) and induces cellular senescence ($p < 0.0001$, Figure 3.5C). It is also associated with increased secretion of IL-6 ($p = 0.007$, Figure 3.5D) and elevated mRNA expression of p21 (8.9-fold, $p = 0.0006$; Figure 3.5E) and SASP cytokine IL-8 (114-fold, $p = 0.006$; Figure 3.5E). Finally, although the primary response of SRSF3 loss appears to be senescence, there is also increased expression of apoptosis genes, BAX (2.2-fold, $p = 0.03$) and PUMA (8.5-fold, $p = 0.004$) (Figure 3.5E), and increased cleaved caspase 3 immunoreactivity (Figure 3.5F). Similar expression of p53β and induction of cellular senescence ($p = <0.0001$) was observed following knockdown of SRSF3 in A172 glioblastoma cells (Figure 3.6).

3.3.5 Loss of SRSF3 induces mutant p53β and cellular senescence in glioblastoma cells with mutant p53

Secondary glioblastomas, which progress from lower grade astrocytomas, can be differentiated based on their molecular signature including the presence of IDH1 mutations.[40] In addition, although the majority of primary glioblastoma have wildtype p53, mutations in p53 are observed in approximately 65% of secondary glioblastomas.[13] Critically, the majority of TP53 mutations in cancer cells are observed in regions conserved in p53 isoforms (Figure 3.7). The increase in p53 mutations in secondary glioblastomas may suggest that the p53 pathway is important in malignant progression from lower grade astrocytoma to glioblastoma. In addition,

malignant transformation of benign tumors has been suggested to be at least partially regulated by p53 isoforms.[4] Because p53 has been shown to be important in SRSF3-induced senescence,[18] we next investigated this process in glioblastoma with mutant p53. Loss of SRSF3 was examined using SRSF3 siRNA in SF268 (R273H) and SF295 (R248Q) glioblastoma cells (SF268, $p = <0.001$; SF295, $p = <0.001$; Figure 3.8A). Loss of SRSF3 expression induces production of mutant p53β (SF268, $p = <0.001$; SF295, $p = <0.001$; Figure 3.8B-C) and is associated with induction of cellular senescence demonstrated by increased SA-β-gal activity (SF268, $p = 0.01$; SF295, $p = 0.005$; Figure 3.8D), increased secretion of IL-6 (SF268, $p = 0.04$; SF295, $p = 0.002$; Figure 3.8E), elevated expression of p21 (SF268, $p = <0.001$; SF295, $p = <0.001$; Figure 3.8F), reduced expression of Ki-67 (SF268, $p = 0.004$; SF295, $p = 0.001$; Figure 3.8G), and increased expression of cleaved-caspase 3 (SF268, $p = 0.030$; SF295, $p = 0.0014$; Figure 3.8H). These findings identify a role for SRSF3 in regulating cellular senescence in mutant p53 glioblastoma cells.

3.3.6 Δ133p53 isoforms regulate senescence in glioblastoma with mutant p53

To further examine the impact of p53 mutations, we next examined Δ133p53α, which was confirmed to be expressed in CD133+ tumor-initiating cells derived from patient glioblastoma samples with mutant p53 (Figure 3.9A). Knockdown of Δ133p53 isoforms in mutant glioblastoma cells (R273H; Figure 3.9B) increases secretion of SASP-associated cytokine IL-6 ($p = 0.011$, Figure 3.9C), induces cellular senescence ($p = 0.003$, Figure 3.9D) and reduces expression of proliferation marker, Ki-67 ($p = 0.002$, Figure 3.9E). These findings suggest that mutant Δ133p53 isoforms retain their ability to regulate cellular senescence in glioblastoma harboring *TP53* mutations.

3.4 Discussion

The TP53 gene is the most commonly mutated gene in cancer underscoring the importance of p53's anti-tumor activities. Because the isoforms modulate FLp53 activity[17], altered expression of p53 isoforms may represent a novel mechanism by which tumors inhibit p53 tumor suppressor activities.[20] In the current study, we examined the role of p53 isoforms, Δ133p53 and p53β, and their regulatory factors, SRSF1, SRSF3, and SRSF7, in cellular senescence. Critically, loss of Δ133p53 or SRSF3 induces cellular senescence in glioblastoma cells. These findings suggest that the p53 isoforms may have prognostic or therapeutic value in the treatment of glioblastoma. In addition, loss of Δ133p53 isoforms also induces senescence in tumors with mutant p53; however, it is not known whether high expression of mutant Δ133p53 isoforms contributes to temozolomide response.

Cellular senescence plays a complicated role in disease. For instance, the induction of cancer cell senescence and SASP may initially be associated with tumor regression and maintenance of cellular senescence.[9] However, the inflammatory milieu of senescent cells may also promote the side effects of cancer therapy and tumor recurrence.[41] In addition, premature senescence has been associated with neurodegenerative disease.[27] As such, modulating p53 isoforms may have conflicting outcomes depending on the target cell type. Another potential way to target p53 isoforms is through the factors known to regulate their production or degradation. In this study, we found that SRSF3 is highly expressed in glioblastoma cells. Loss of SRSF3 leads to increased p53β production, cellular senescence and apoptosis. While this has previously been shown to be dependent on p53,[18] the current study identified a similar response to SRSF3 loss in mutant p53 glioblastomas. Identifying how cancer cells modulate p53 isoforms and their regulatory proteins may facilitate the development of additional therapeutic opportunities. Further,

induction of apoptosis, as is observed in a subset of cells following SRSF3 knockdown, may represent a better therapeutic outcome as compared to cellular senescence. Examining p53 isoforms and their regulatory proteins in mouse models will be critical to understanding how targeting p53 isoforms may affect cells *in vivo*.

Finally, while the majority of primary glioblastoma harbor wildtype p53, TP53 is mutated in up to 65% of secondary glioblastomas which have progressed from lower grade astrocytomas.[13] Following temozolomide exposure, glioblastoma cells accumulate mutations and develop a specific temozolomide-associated mutation signature.[42] Mutations in p53 have been shown to reduce temozolomide efficacy while stabilizing the wildtype p53 conformation enhances cytotoxicity suggesting that an intact p53 pathway is essential to temozolomide treatment efficacy.[43] Many studies have focused on the various gain- or loss- of function for mutant FLp53.[44,45] In contrast, there is a relative paucity of mutant p53 isoform research. This is particularly important because most mutations in p53 occur in hotspots within the DNA-binding domain, a domain which is conserved within p53 isoforms (Figure 3.7).[17,46,47] In primary glioblastoma TCGA cases with mutant p53, 85% also have mutations in Δ133p53 isoforms while 100% have mutations in p53β. It is likely that, similar to the effects shown in FL-p53,[45] different types of p53 isoform mutations have distinct effects on p53 isoform functions. The current study underscores the need for p53 studies to consider the role of mutant p53 and p53 isoforms and the interplay between these mutated proteins in regulating cancer cell functions. Our ongoing studies aim to further interrogate the interplay of wildtype and mutant p53 isoforms in the regulation of p53-mediated cellular functions. Better characterization of mutant p53 isoform functions may elucidate additional mechanisms by which tumors disrupt the p53 pathway to promote progression,

better define the prognostic implications of p53 isoform expression, and identify novel therapeutic targets for the treatment of cancer.

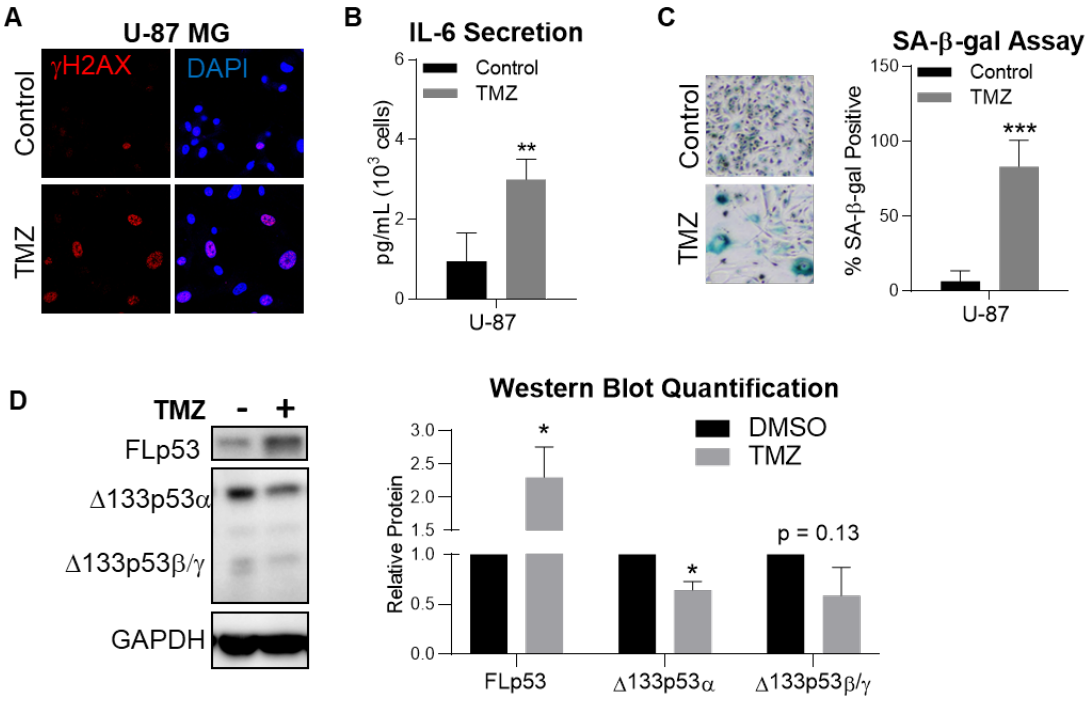

Figure 3.1. Temozolomide chemotherapy induces cellular senescence and is associated with reduced expression of Δ133p53α in glioblastoma cells. (A) Representative images of U-87 MG γH2AX immunoexpression following 5 days of temozolomide treatment (50 μM). (B) Quantification of IL-6 protein secreted in media after 5 days of temozolomide treatment (IL-6 ELISA). (C) Representative image and quantification of senescence-associated beta-galactosidase (SA-β-gal) staining in temozolomide-exposed glioblastoma cells. (D) Representative western blot and quantification of FLp53 and Δ133p53 in temozolomide-exposed glioblastoma cells.

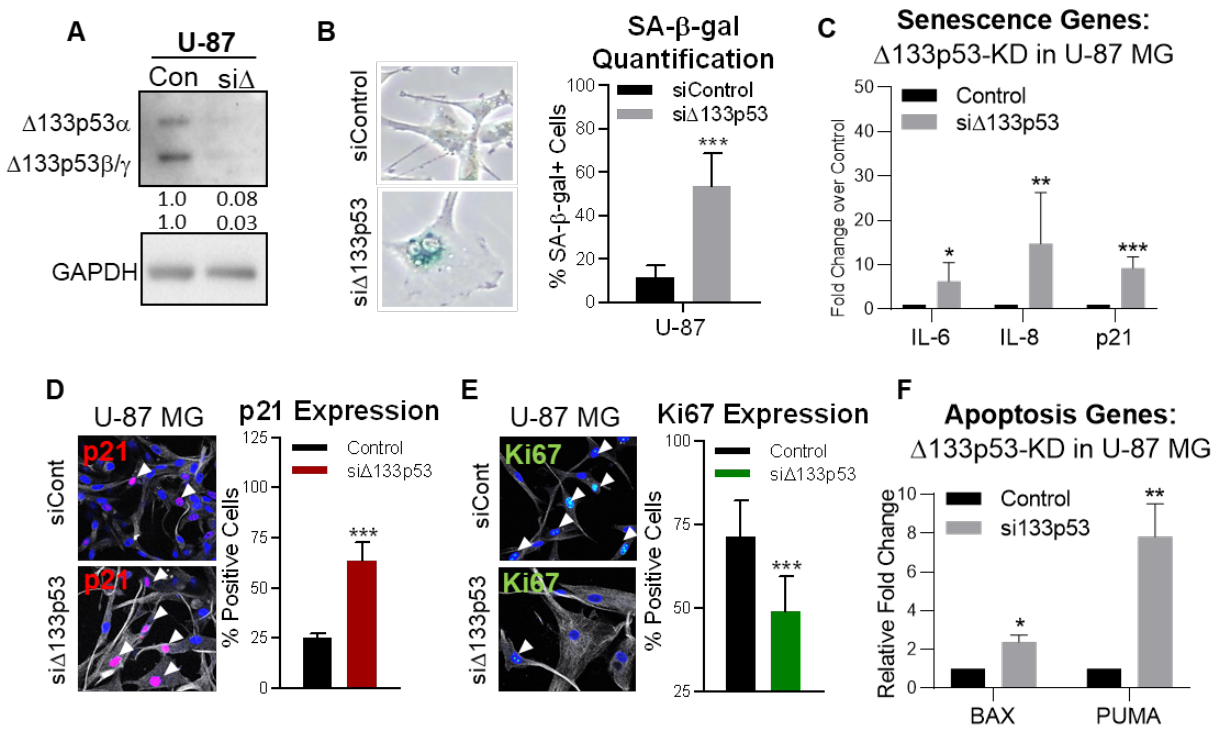

Figure 3.2. Δ133p53 regulates cellular senescence in glioblastoma. (A) Western blot demonstrating knockdown of Δ133p53 in U-87 MG glioblastoma cells. (B) Representative images and quantification of SA-β-gal activity following knockdown of Δ133p53 in U-87 MG glioblastoma cells. (C) Relative mRNA expression (fold-change) of p53 target genes following knockdown of Δ133p53 in U-87 MG glioblastoma cells (Taqman). (D) Representative image and quantification of p21 immunoexpression following knockdown of Δ133p53 in U-87 MG glioblastoma cells. (E) Representative image and quantification of Ki-67 immunoexpression following knockdown of Δ133p53 in U-87 MG glioblastoma cells. (F) Relative mRNA expression (fold-change) of p53 target genes following knockdown of Δ133p53 in U-87 MG glioblastoma cells (Taqman).

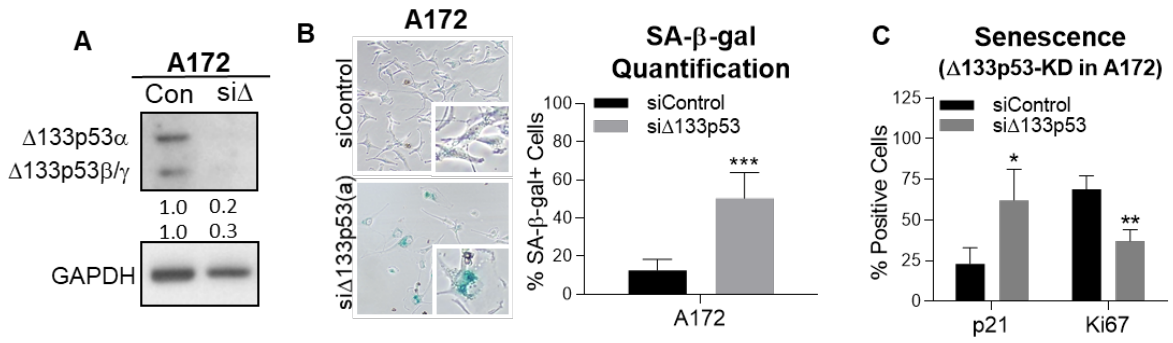

Figure 3.3. Δ133p53 regulates cellular senescence in glioblastoma. (A) Western blot demonstrating knockdown of Δ133p53 in A172 glioblastoma cells. (B) Representative images and quantification of SA-β-gal activity following knockdown of Δ133p53 in A172 glioblastoma cells. (C) Quantification of p21 and Ki-67 immunoexpression following knockdown of Δ133p53 in A172 glioblastoma cells.

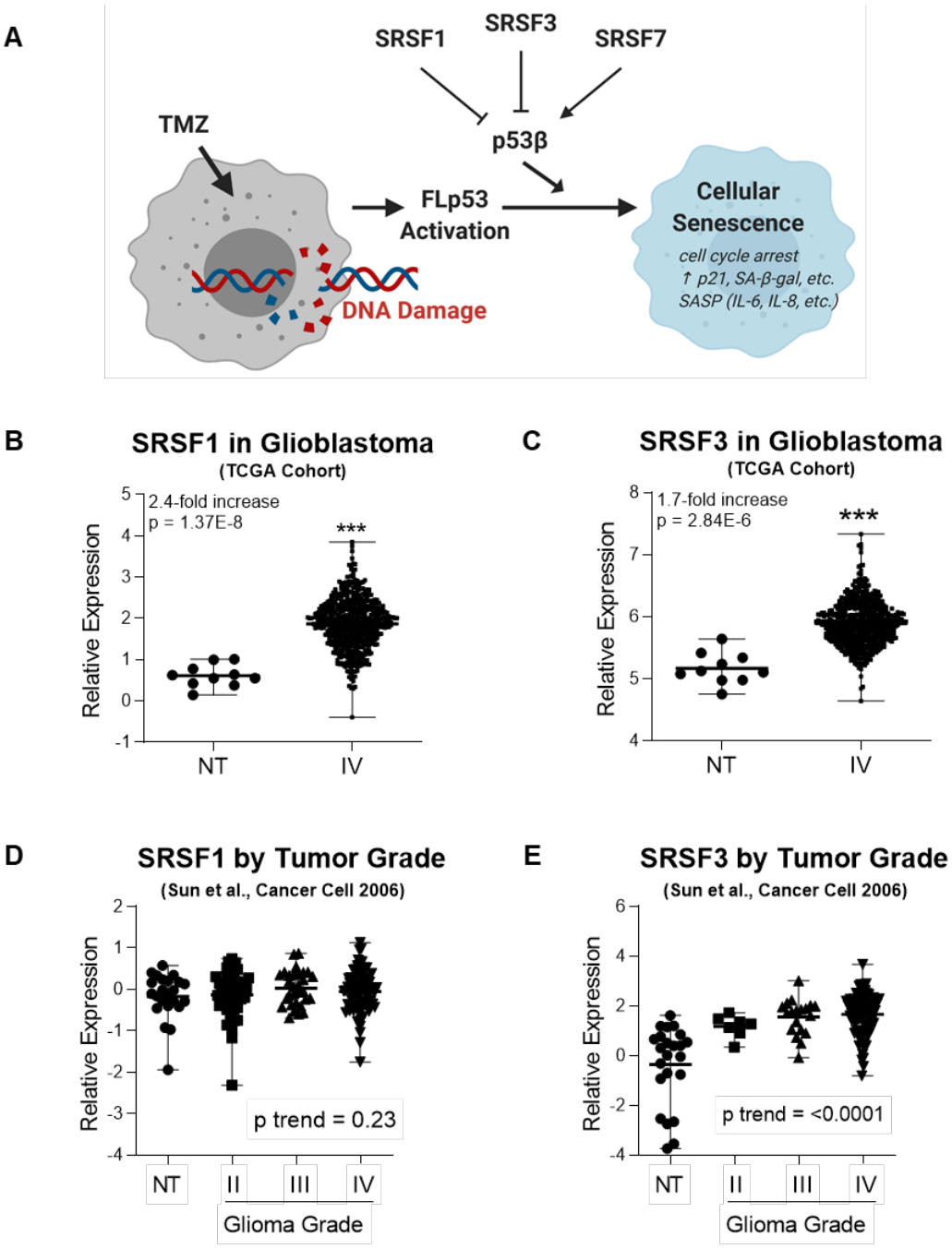

Figure 3.4. SRSF3 and SRSF1 are overexpressed in glioblastoma. (A) Cartoon depicting activities of SRSF1, SRSF3, and SRSF7 in the production of p53β. (B) SRSF1 and (C) SRSF3 expression in glioblastoma (IV) and non-tumor (NT) samples from TCGA. (D) SRSF1 and (E) SRSF3 expression in non-tumor (NT) tissue, astrocytoma (II, III) and in glioblastoma (IV) from the Sun cohort (Oncomine, log2).

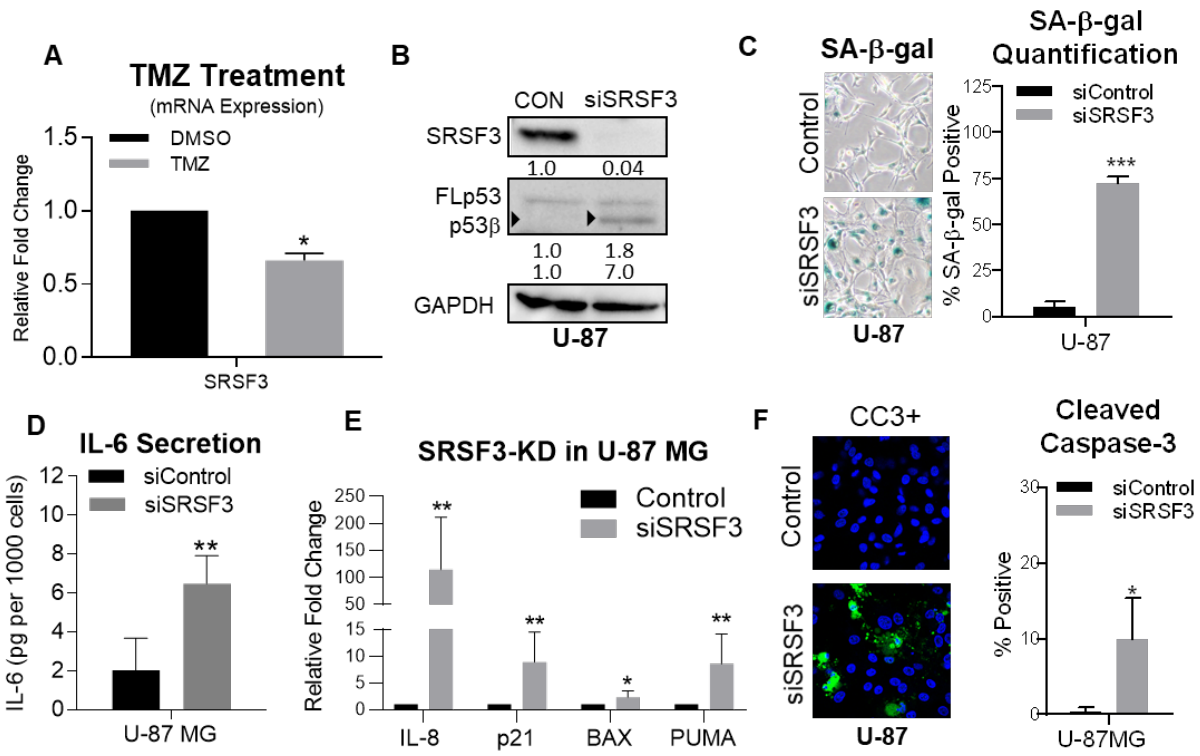

Figure 3.5. Loss of SRSF3 leads to increased expression of p53β and induction of cellular senescence. (A) mRNA expression of SRSF3 in DMSO and TMZ treated glioblastoma cells. (B) Western blot four days after SRSF3 knockdown demonstrating expression of SRSF3 and p53β in glioblastoma cells. (C) Representative image and quantification of SA-β-gal activity following SRSF3 knockdown in U-87MG glioblastoma cells. (D) Quantification of IL-6 secreted into media following SRSF3 knockdown. (E) Relative mRNA expression (fold-change) of p53 target genes following knockdown of p53β in U-87 MG glioblastoma cells (Taqman). (F) Representative image and quantification of cleaved caspase 3 (CC3) immunoexpression in U-87MG glioblastoma cells following SRSF3 knockdown.

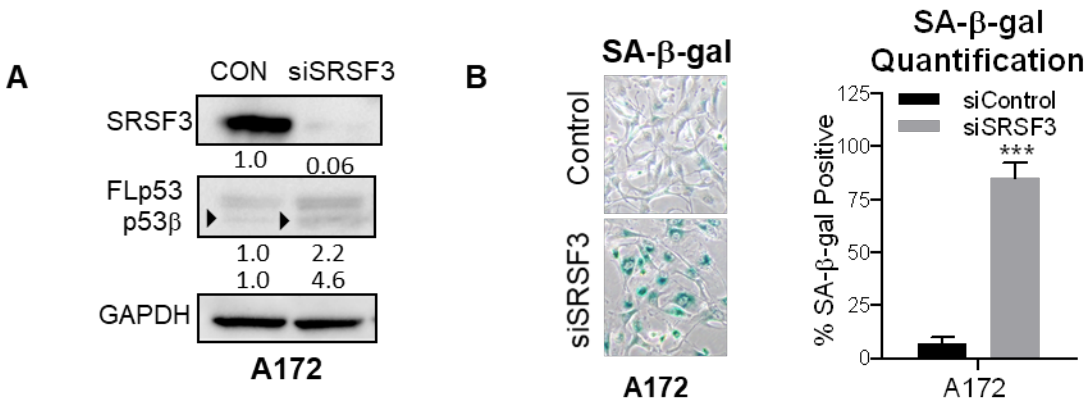

Figure 3.6. Loss of SRSF3 leads to increased expression of p53β and induction of cellular senescence. (A) Western blot four days after SRSF3 knockdown demonstrating expression of SRSF3 and p53β in glioblastoma cells. (C) Representative image and quantification of SA-β-gal activity following SRSF3 knockdown in A182 glioblastoma cells.

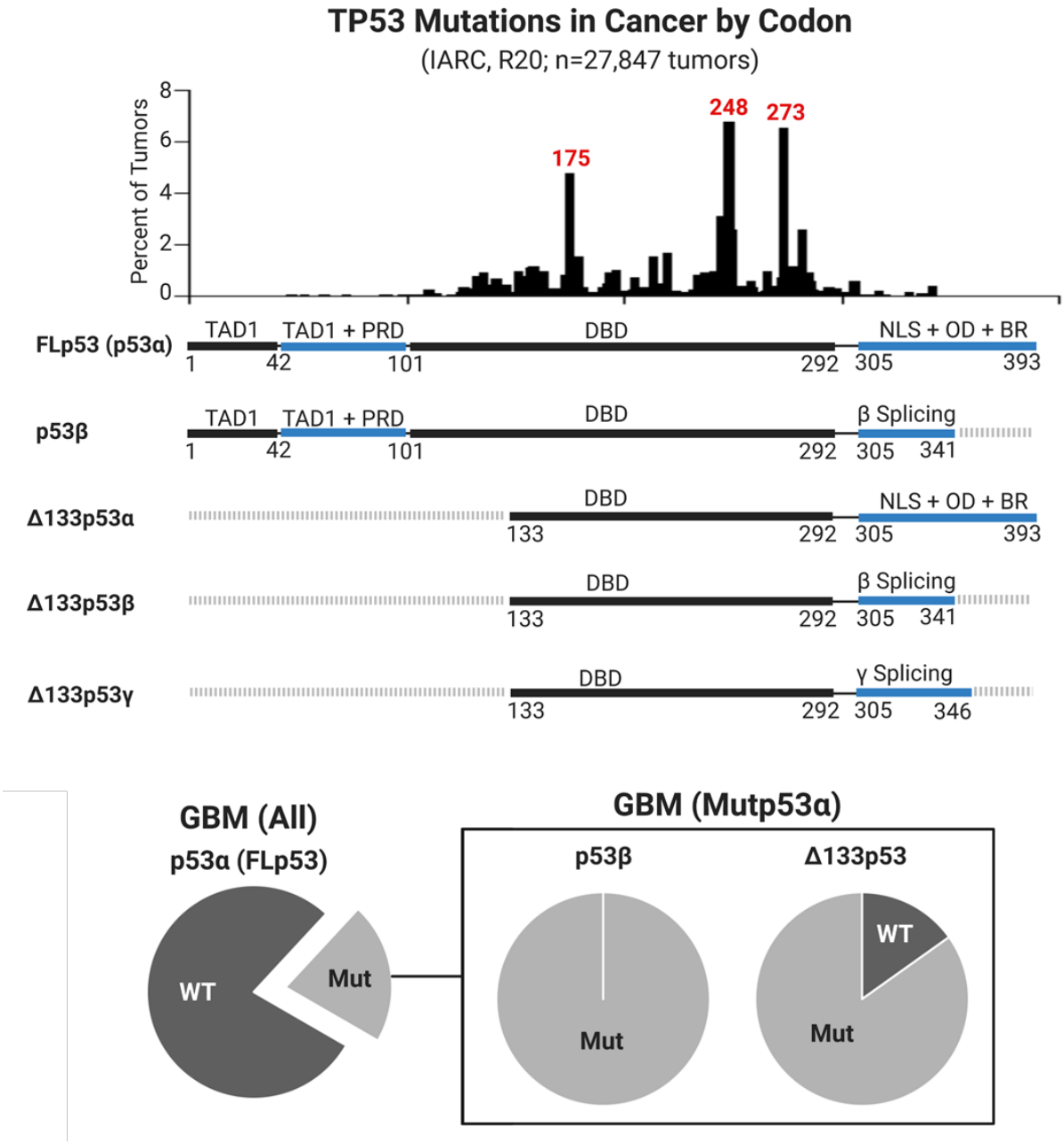

Figure 3.7. Mutations in FLp53 are conserved in p53 isoforms. The TP53 gene is the most frequently mutated gene in cancer (IARC database).[48] The majority of primary glioblastoma in the TCGA cohort have wildtype p53; however, those that have mutant p53 primarily harbor mutations in the DNA-binding domain which is conserved in p53 isoforms. As such, 100% have mutations in p53β while approximately 85% have mutations in Δ133p53. These findings underscore the critical need to evaluate p53 isoform mutations in the context of p53 functions in carcinogenesis and cancer treatment.

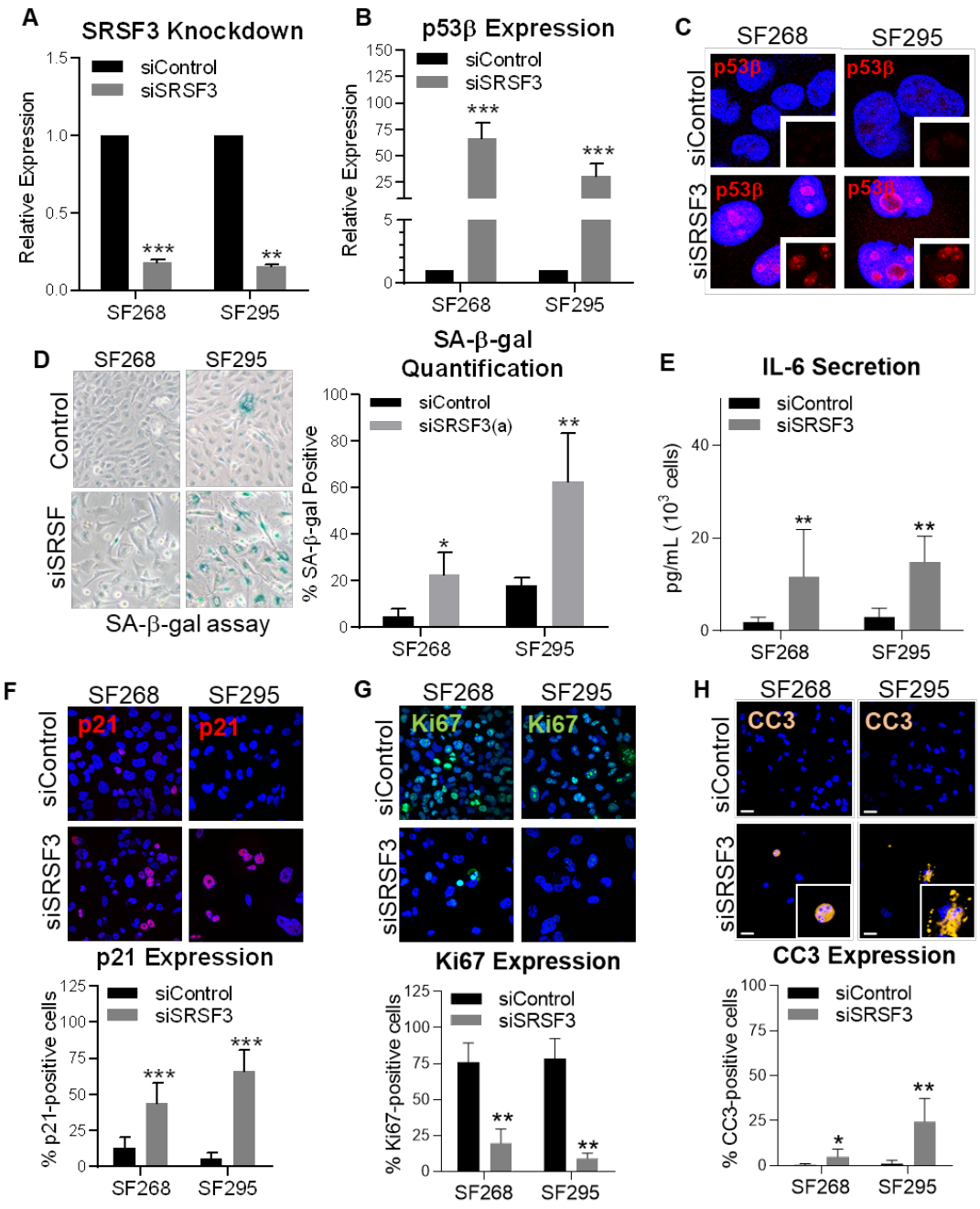

Figure 3.8. Loss of SRSF3 induces p53β and p53-mediated cell cycle arrest in glioblastoma cells with mutant p53. (A) SRSF3 and (B) p53β mRNA expression following treatment of mutant p53 glioblastoma cells (SF267, SF295) with siSRSF3. (C) p53β immunoexpression following SRSF3 knockdown in glioblastoma cells. (D) Representative image and quantification of SA-β-gal activity following SRSF3 knockdown. (E) Quantification of IL-6 protein secreted into the media after SRSF3 knockdown in mutant p53 glioblastoma cells (F) Representative image and quantification of p21, (G) Ki-67, and (H) Cleaved caspase 3 (CC3) expression after knockdown of SRSF3 in mutant p53 glioblastoma cells.

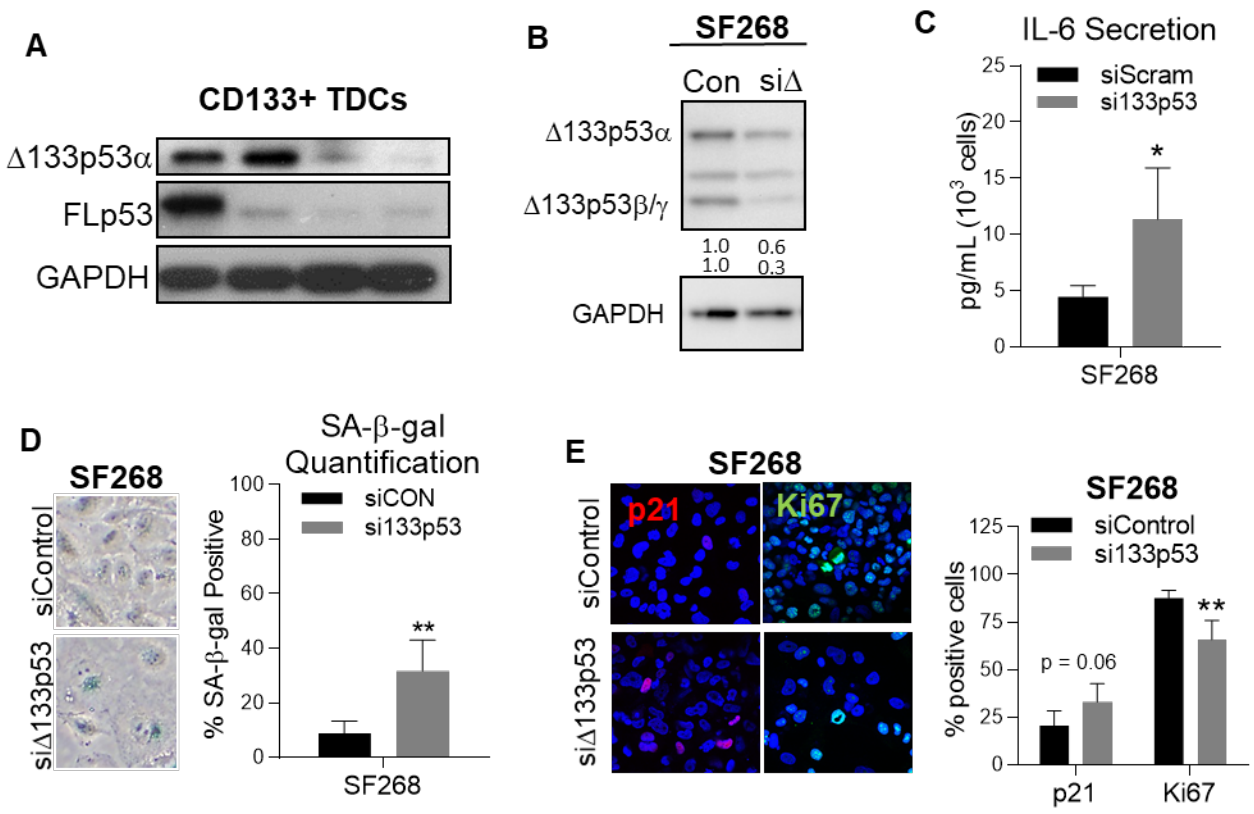

Figure 3.9. Δ133p53α regulates senescence in glioblastoma with mutant p53 . (A) Expression of FLp53 and Δ133p53 in CD133+ tumor cells isolated from four patients with mutant p53 GBM. (B) Western blot demonstrating Δ133p53 loss in SF268 glioblastoma cells. (C) Representative image and quantification of SA-β-gal activity in SF268 following Δ133p53 knockdown. (D) IL-6 secretion quantified by ELISA. (E) Immunoexpression of p21 and Ki67 following Δ133p53 knockdown. (F) Overexpression of Δ133p53α (R273H) in SF268. (G) Quantification of SA-β-gal activity in temozolomide-treated SF268 glioblastoma cells with or without overexpression of Δ133p53α (R273H).

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CHAPTER 4. CELLULAR SENESCENCE: MECHANISMS, MORPHOLOGY, AND MOUSE MODELS

Portions of this chapter are published in: Beck J, Horikawa I, Harris C. Cellular Senescence: Mechanisms, Morphology, and Mouse Models. Veterinary Pathology 2020;57(6):747-757.

4.1 Abstract

Cellular senescence is a cell cycle arrest in damaged or aged cells. Although this represents a critical mechanism of tumor suppression, persistence of senescent cells during aging induces chronic inflammation and tissue dysfunction through the adoption of senescence-associated secretory phenotype (SASP). This has been shown to promote the progression of age-associated diseases such as Alzheimer's disease, pulmonary fibrosis, and atherosclerosis. As the global population ages, the role of cellular senescence in disease is becoming a more critical area of research. In this review, mechanisms, biomarkers, and pathology of cellular senescence and SASP are described with a brief discussion of literature supporting a role for cellular senescence in veterinary diseases. Cell culture and mouse models used in senescence studies are also reviewed including the senescence-accelerated mouse-prone (SAMP), senescence pathway knockout mice (p53, p21 [CDKN1A] and p16 [CDKN2A]), and the more recently developed senolysis mice which allow for direct visualization and elimination (or lysis) of senescent cells in live mice (p16-3MR and INK-ATTAC). These and other mouse models have demonstrated the importance of cellular senescence in embryogenesis and wound healing but have also identified a therapeutic benefit for targeting persistent senescent cells in age-associated diseases including neurodegeneration, diabetes, and cardiac fibrosis.

4.2 Introduction

Cellular senescence is a highly durable mode of cell cycle arrest that occurs in aged or DNA-damaged cells.[1-4] Following the induction of cellular senescence, senescent cells adopt the senescence-associated secretory phenotype (SASP) and produce a variety of factors including cytokines, chemokines, and matrix metalloproteinases.[5-8] SASP proteins reinforce cellular senescence programming and mediate multiple tissue effects.[9-11] During development and healing, tightly regulated induction of cellular senescence promotes tissue reorganization and repair.[12,13] Secreted SASP chemokines recruit inflammatory cells, such as macrophages, which help to remove senescent cells and restore normal tissue functions.[6,13-15] However, in some cases, senescent cells are not effectively removed.[14,16,17] This may be caused by an imbalance of senescence induction and clearance due to aging,[18,19] immune dysfunction,[20-22] or an acute stressor such as radiation[23,24] or traumatic injury.[25,26] Regardless of the cause, senescent cells accumulate in aged humans and animals and continue to secrete proteins which promote chronic inflammation, disrupt tissue homeostasis, and induce organ dysfunction.[5,8,11,14,27] It is in this way that cellular senescence has been implicated as an important mechanism underlying age-associated degenerative disease (Figure 4.1). For example, senescent cells have been implicated in the progression of atherosclerosis,[6,28] cystic fibrosis,[29] age-related cataracts,[30] and radiation-induced pulmonary fibrosis.[24] In addition, mouse models have identified a therapeutic benefit for targeting and removing these senescent cells in disease including rescue of neurocognitive function in tau-induced neurodegeneration,[31] improved glucose metabolism in a model of type 2 diabetes,[32,33] and attenuated myocardial hypertrophy and fibrosis in cardiac aging.[34,35] Finally, although cellular senescence inhibits tumor initiation by preventing proliferation of damaged cells, it can also promote tumor progression and contribute to the late effects of cancer therapy through SASP.[11,36,37] Consistent with this, *in vivo* clearance of senescent cells has reduced tumor recurrence and

chemotherapy-associated side effects in a mouse model.[36] In this review, we discuss the current understanding of the mechanisms and functions of cellular senescence and outline several experimental models used to study cellular senescence and SASP in disease.

4.3 Mechanisms of Cellular Senescence

The two major pathways of cellular senescence are replicative and stress-induced senescence (Figure 4.2).[1-4] In replicative senescence, successive rounds of cellular replication and telomere shortening expose the chromosomal ends which are sensed by the cell as endogenous DNA damage.[38,39] In contrast, internal or external stressors such as traumatic injury,[25,26] oxidative stress,[3,8,40] radiation,[8,24] or chemotherapy[8,36] cause cells to rapidly accumulate DNA damage and undergo stress-induced senescence.[1] Both pathways trigger the DNA damage response mediated by p53, p21, and p16.[1,39,41,42] This leads to inhibition of cyclin-dependent kinases resulting in cell cycle arrest and induction of cellular senescence.[1,43] Critically, once cells undergo senescence, they secrete numerous proteins as part of the senescence-associated secretory phenotype (SASP).[5,7,8] Secreted chemokines recruit macrophages that assist in removing these stressed cells.[6,13] Secretory factors have also been shown to induce senescence re-programming in adjacent cells, often referred to as the "bystander effect".[24,44,45] This is particularly well described in the lung where senescent pneumocytes produce secretory proteins that induce senescence in adjacent pneumocytes.[24] Additional studies in mice have found significant increases in senescent cell burden following injection of senescent cells, further underscoring the importance of SASP in reinforcing cellular senescence.[44,45] Finally, senescent cells can also affect neighboring cells of different cell types to induce a range of cellular states, including apoptosis or activation.[11] For example, senescent astrocytes can induce cell death in neuronal cells[31,46-48] while senescent pneumocytes activate fibroblasts resulting in pulmonary fibrosis.[24,49] These findings highlight the

roles of cellular senescence in disease progression and identify cellular senescence as a potential therapeutic target in age-associated disease.[16,49,50]

4.4 Senescent Cell Morphology and Biomarkers

Researchers use several methods to identify senescent cells, including changes in cellular morphology and senescence-associated alterations in protein expression.[51-54] In culture, senescent cells are typically enlarged, flattened, multinucleated and vacuolated (Figure 4.3A-B).[1,7] Similar morphologic changes are also reported in tissues but may be restricted by the surrounding tissue architecture and thus more difficult to appreciate.[1,53] In these cases, it may be easier to identify histologic features of chronic inflammation associated with cellular senescence such as fibrosis and mononuclear cell inflammation.[6,14,24,49] Senescent cells also accumulate lipofuscin, a yellow-brown lipid-containing residue formed through lysosomal digestion, which can be visualized histologically or highlighted using histochemical stains such as Sudan Black B.[55,56] In addition, the senescence-associated β-galactosidase (SA-β-gal) assay is probably the best described and most commonly used staining technique.[52] It can be used *in vitro* or *in vivo* and highlights senescent cells with blue dye by taking advantage of increased beta galactosidase activity within the lysosomes of aged cells (Figure 4.3C-D).[57] Finally, altered protein expression in senescent cells can be detected using a variety of techniques including western blotting, quantitative real-time polymerase chain reaction or immunohistochemistry.[51,58] Double-strand DNA breaks can be identified by γH2AX or 53BP1 labelling in both replicative and stress-induced cellular senescence (Figure 4.3E-F).[51,59,60] Following activation of the DNA damage response pathway, early senescent cells have increased nuclear expression of p53 and p21 while late senescence is best characterized by elevated p16.[1,42,43] Activation of these pathways induces cell cycle arrest and formation of senescence-associated heterochromatin foci (SAHFs) identified by DAPI staining,

trimethylated H3K9, or heterochromatin protein 1.[7,61,62] These SAHF contribute to the stability of cellular senescence by repressing genes, such as those associated with cellular proliferation.[62] Functionally, senescent cells adopt SASP characterized by increased secretion of chemokines, growth factors, matrix metalloproteases and inflammatory cytokines.[5,8,11] Over 300 SASP proteins have been characterized and can vary by cell type, stressor and time course but frequently include cytokines, such as IL-1, IL-6 and IL-8. These SASP proteins can be identified by increased mRNA production within cell or tissue samples or through the detection of secreted proteins in media or serum.[51,63,64] Finally, senescent cells upregulate the expression of anti-apoptotic BCL-2 family proteins such as BCL-XL resulting in resistance to apoptosis and subsequent tissue persistence.[17,65,66]

4.5 Models of Cellular Senescence: Cell Culture

Primary cells proliferate in culture for a finite period of time.[2,4] Serial passaging of human cells has been used as an experimental method to study cellular senescence and aging *in vitro*.[4,46,67] Cells in culture may also accumulate DNA damage over time or following application of an acute stressor such as radiation,[8,68] chemotherapy,[8,69] or oxidative stress (hydrogen peroxide)[3,8,40] resulting in stress-induced senescence. These studies can be used to isolate the effect of specific stimuli on individual cells and to investigate the potential roles of cellular senescence in disease processes.[5,8,11] These types of experiments are also helpful to define the secretory proteins produced by senescent cell types. In addition, experiments involving transwells, co-culture or conditioned media exposure can be used to define the effects of senescent cells and their associated secretory factors on neighboring cells.[11,46,70]

4.6 Mouse Models: Senescence-accelerated Mouse-prone (SAMP)

While maintaining an inbred colony of AKR/J mice at Kyoto University, researchers identified an aging phenotype in a subset of litters.[71,72] Features of these mice included a poor hair coat, reduced activity, and decreased lifespan.[71] Aging features are thought to develop due to elevated oxidative stress, are inherited by subsequent generations, and have been further separated into several distinct SAMP phenotypes.[73-75] Litters of inbred AKR/J mice that did not experience an accelerated aging process are referred to as senescence-accelerated mouse-resistant (SAMR) and are typically used as controls for SAMP experimental studies.[71,72]

Organismal senescence, such as that observed in SAMP mice, is the process of biological aging accompanied by the gradual deterioration of functional characteristics.[19,76,77] Importantly, organismal senescence is a term applied to the entire organism and is not equivalent to cellular senescence which is a mechanism of cell cycle arrest within individual cells.[19,77] Although organismal senescence is associated with increased numbers of senescent cells, not all cell types within an aged individual undergo cellular senescence.[15,19] As such, cellular and organismal senescence are not mutually inclusive but do appear to maintain a critical interplay. In the case of aged SAMP mice, organismal senescence is associated with increased cellular senescence in a variety of cell types including astrocytes,[78,79] endothelial cells,[75,80] progenitor cells,[81] retinal epithelial cells,[82] and fibroblasts.[83]

The role of cellular senescence in promoting neurodegeneration is a key area of aging research.[47] SAMP8 mice experience age-dependent deficits in learning and memory and develop a variety of age-associated neuropathologic changes similar to those described in aging humans.[84] By as early as 5 months of age, neuropathologic changes include astrogliosis, microgliosis, and neurodegeneration.[85,86] SAMP8 mice also accumulate amyloid,[87,88] have increased nitric oxide synthase activity,[88,89] and demonstrate age-associated hyperphosphorylation of tau further

underscoring their utility as a model of brain aging and Alzheimer's disease.[84,90] Senescent SAMP8-derived astrocytes have been identified as a key contributor to neurodegeneration and demonstrate critical impairment of their normal neuroprotective functions.[78,79] A similar mechanism for astrocyte-mediated neurotoxicity in humans has also been shown to induce neuronal toxicity *in vitro* and may contribute to neurodegeneration in patients with Alzheimer's disease, amyotrophic lateral sclerosis, and radiotherapy-induced brain injury.[46-48,91]

In humans, cellular senescence has been suggested to promote a variety of musculoskeletal diseases including sarcopenia,[92,93] muscular dystrophy,[93,94] and osteoporosis.[81,93,95] Adult SAMP6 mice have a significantly higher incidence of spontaneous leg fractures attributed to osteoporosis.[81,96,97] Induction of cellular senescence in bone marrow progenitor cells disrupts their differentiation favoring adipogenesis over osteoblastogenesis.[81,98,99] This mechanism has been suggested to result in inefficient osteoblastic activity and osteoporosis in SAMP6 mice and in aged humans.[81,93,100]

Critically, although SAMP mice are separated into distinct phenotypes, organismal senescence may be associated with age-related dysfunction in multiple organs within the same mouse. For example, although most frequently used for neurodegeneration research, SAMP8 mice also develop age-associated vascular disease and have been used to study cellular senescence in endothelial cells.[75,101,102] In humans, increased numbers of senescent cells have been suggested to contribute to atherosclerosis,[6,28,103] impaired angiogenesis,[68,104] and cardiac fibrosis.[34] While the development of accelerated aging in multiple organs can complicate the interpretation at a cellular level, these models may be more representative of the physiologic effects of aging on the entire organism.

In addition to these neurologic and musculoskeletal alterations, SAMP6 and SAMP8 mice also develop a variety of other age-associated conditions including retinal degeneration,[82] testosterone deficiency,[75] myocardial fibrosis,[101] and hepatic lipidosis[105] (Table 4.1). Finally, there are more than 10 SAMP mice strains, each of which can develop a variety of age-related conditions such as renal fibrosis (contracted kidneys), immune dysfunction, and degenerative joint disease.[76,106,107] Although SAMP studies do not always examine cellular senescence in the development of age-related disease, each of these conditions has been associated with induction of cellular senescence in other experimental models suggesting that cellular senescence may also contribute to the development of these conditions in SAMP mice.[21,22,26,108-110]

4.7 Mouse Models: Targeting Senescence Pathways (p53/p21/p16)

Inhibiting senescence pathways is another way to investigate the role of cellular senescence in diseases, including targeting the induction (p53/p21) or persistence (p16) of senescence.[1] These knockout mice may also be crossed to disease models to study the roles of these pathways in the progression of age-associated disease.

Cellular senescence studies using p21- and p53- null mice have identified critical roles for cellular senescence in embryonic development and in the promotion of premature aging syndromes such as Hutchinson-Gilford progeria syndrome and ataxia telangiectasia.[13,111-113] However, studying these processes in p53- and p21-null mice is complicated by the loss of beneficial p53 pathway functions.[1,114,115] Critically, p53-mediated induction of cellular senescence is an important mechanism of tumor suppression.[42,116,117] Thus, mouse models that inhibit cellular senescence have an increased incidence of several neoplasms that can affect remarkably young mice (Table 4.2).[118-121] The p53-null mice develop tumors by 10 weeks of age with almost 75% of mice affected by 6 months of age.[120] As a result, mice are euthanized early making them difficult to study over time.[120]

The most common tumors in these mice include lymphoma (77%) and hemangiosarcoma (27%).[120] Because the majority of mice succumb to cancer, there are only rare cases of infectious or inflammatory diseases reported (e.g., abscesses, gastroenteritis, myocarditis).[120] An alternative method to target the p53 pathway is through deletion of its downstream effector, p21.[1,41] As expected, p21-null mice also develop tumors earlier than wild-type counterparts (16 months vs. >2 years).[119] Tumor types are similar to those reported in p53-null mice but with a longer latency period and include histiocytic sarcoma (52%), hemangioma/hemangiosarcoma (22%) and lymphoma (14%).[119,120] Although 55% of male p21-null mice succumb to their tumors, the most common cause of death in female p21-null mice is severe autoimmune glomerulonephritis which affects approximately 60% and is thought to be due to an abnormal proliferation of T cells.[119]

Finally, p16 has been characterized as the best *in vivo* marker for senescent cells and its knockout has produced another commonly used model to study cellular senescence.[118,122] Studies in p16-null mice have suggested that inhibiting cellular senescence in the kidney leads to enhanced recovery of renal function following ischemia-reperfusion injury and reduces interstitial fibrosis and tubular atrophy in renal transplant models.[108,123,124] Although these mice develop tumors later than p53 knockout mice, approximately 25% of p16-null mice develop tumors by 10 months of age.[118-120] Spontaneous tumors reported in these mice include malignant spindle cell neoplasms (29%), angiosarcoma (23%), osteosarcoma (18%), histiocytic lymphoma (18%) and melanoma (12%).[121] With loss of p16 function, these mice are also highly susceptible to carcinogen-induced tumors.[118,121] Other reported changes in p16-null mice include elevated T cell proliferation, absolute T cell counts, and thymic hyperplasia.[118]

Classically, the induction of cellular senescence in damaged cells is described as an important barrier to carcinogenesis. This theory is supported by the increased tumor burden

observed in these knockout mice; however, the role of cellular senescence in cancer progression may be more complicated (Figure 4.4).[11,36] For example, while induction of cellular senescence is initially associated with inhibition of tumor initiation or regression, persistence of senescent tumor and non-tumor cells has been shown to promote tumor recurrence through SASP.[11,36,37,125] In addition, some reports suggest that tumor cells can either escape or inhibit the induction of cellular senescence allowing them to re-enter the cell cycle.[115,126,127] The contrasting roles of cellular senescence in cancer are being further investigated in senolysis mice, which are used to study the roles of cellular senescence in many age-associated diseases including cancer and cancer recurrence.[36]

4.8 Mouse Models: Senolysis

Recently, several transgenic mouse models have been developed which allow for direct visualization and elimination of senescent, p16-positive cells including the p16-3MR (tri-modal reporter) and the INK-ATTAC (apoptosis through targeted activation of caspase 8) models.[12,128] These models identify p16-positive senescent cells through expression of red or green fluorescent protein (RFP/GFP).[12,128] This allows researchers to image and quantify fluorescent protein expression in live mice as they age or to study the accumulation of senescent cells following introduction of an acute stressor, such as chemotherapy.[36,129] Finally, these mouse models were designed to allow for specific targeting and removal of senescent cells.[12,128] This allows researchers to investigate the therapeutic value of senolysis in specific disease contexts.[12,128] Through the use of these models, the contributory roles of cellular senescence and the beneficial effects of senolysis have been demonstrated in several age-associated diseases including tau-related neuropathology and cognitive decline,[31] chemotherapy-induced fatigue,[36] cancer recurrence,[36] osteoporosis,[95] myocardial hypertrophy,[34] and cardiac fibrosis.[34]

Although fluorescent reporter genes offer a great opportunity to identify and study the spread of senescence, they are not without potential side effects.[130] For example, GFP has been reported to cause injury through production of reactive oxygen species, promotion of apoptosis, and induction of immunogenicity.[130,131] This is reported to be more severe in BALB/c than C57BL/6 mice suggesting that the background strain is important when evaluating mouse models for GFP immunogenicity.[131] In addition, transgenic mice using the INK-ATTAC model are injected with AP20187 while the p16-3MR model is controlled by ganciclovir administration.[12,128] Although side effects of either treatment are not well described in these models, any intraperitoneal injection has the potential to cause ileus and peritonitis.[132] Finally, inhibiting cellular senescence has been shown to impede wound healing which should be monitored in these mice.[12,129]

4.9 Evidence for Cellular Senescence in Domestic Animals

Although cellular senescence is not a newly discovered mechanism, researchers are still characterizing its potential role in human diseases,[14] including neurodegeneration,[31,47] pulmonary fibrosis,[24] wound healing,[12] and osteoarthritis.[26] As researchers aim to identify ways to modulate cellular senescence in disease, it will become important to understand the comparative role of cellular senescence in veterinary diseases. In addition to mouse model studies, there are reports of cellular senescence contributing to disease or cellular dysfunction in domestic animals. For example, dogs have increased numbers of senescent cells in the aged testis,[133] in chronic hepatitis,[134] and within the non-tumor tissue surrounding intraocular tumors.[135] In cats, cellular senescence is increased in feline chronic kidney disease and contributes to diminished self-renewal and reduced multipotency of mesenchymal stem cells *in vitro*.[136,137] Although classic markers of cellular senescence are not always examined, there is also good evidence that aging alters cellular functions in animals consistent with the induction of cellular senescence. Examples include

reduced proliferation, cellular dysfunction, and features of immunosenescence in aged horses,[138] cats,[139] and dogs,[139-141] in horses with asthma,[138] and in dogs with cancer.[140] In addition, senescence-associated histologic changes are reported in aged animals including fibrosis and mononuclear cell inflammation.[137,142,143] There is also evidence that SASP-associated cytokines such as IL-1, IL-6 and IL-8 are produced in animals with chronic inflammatory diseases that are attributed to cellular senescence in humans including pulmonary fibrosis, chronic kidney disease and arthritis.[144-146] In the future, further examination of these and other age-related diseases may better identify the potential roles for cellular senescence in promoting pathology in domestic species.

4.10 Conclusions

Cellular senescence is a normal process by which aged and damaged cells are inhibited from replicating.[116] It is important in normal embryonic development and serves as a critical tumor suppressor mechanism.[13,116] Secretion of SASP proteins is thought to induce removal of senescent cells through inflammatory cell recruitment, thereby promoting healing and restoring tissue homeostasis.[6,13,14] However, surviving senescent cells that are not cleared by the immune system continue to secrete SASP proteins and contribute to organ dysfunction.[14] Like other biologic processes, senescence programming is critical in specific contexts, but its persistence can lead to an exuberant and long-lasting inflammatory response which can contribute to further tissue injury.[12,14]

In this review, we discussed several mouse models used to investigate the roles of cellular senescence in aging and cancer; however, this list is not exhaustive. Importantly, cellular senescence may contribute to aging phenotypes in a variety of mouse models. For example, progeroid syndrome models, which have been reviewed previously, have increased numbers of

senescent cells that promote premature aging.[128,147] Sirtuin-deficient mouse models, such as SIRT6 null mice, also experience premature aging and have demonstrated a role for NF-κB signaling in regulating cellular senescence and SASP.[148-150] Finally, mouse models with deficient DNA damage repair mechanisms or models of traumatic injury, such as traumatic brain injury or post-traumatic osteoarthritis, may be used to investigate the roles of premature or stress-induced senescence in injured tissues.[18,25,26,151,152] These experimental models demonstrate the sudden increase in senescent cells following injury and, when incorporated into a senolysis mouse model, can be used to characterize the therapeutic benefit of senolysis on disease progression.[26]

Currently, there is intense interest in defining the roles of cellular senescence in human diseases.[14] This interest goes beyond simply characterizing senescent cells *in vitro* but rather identifying ways to restore or remove senescent cells to benefit patients.[16] One of the first methods by which this was pursued was through the use of therapeutics which target the production or secretion of SASP proteins such as cytokine-directed antibodies,[64,153] NF-κB inhibitors,[154] or sirtuin activators.[16,50,155] More recently, anti-SASP therapies have been augmented by senolytic drugs which reduce the organismal burden of senescent cells.[16,93,95,156] These senolytics have shown promise in preliminary clinical trials in humans.[157,158] As therapeutic interventions evolve, defining the roles of cellular senescence in veterinary diseases will be important not only for understanding chronic inflammation in the context of aging but also for facilitating the translation of anti-senescence therapies between humans and animals.

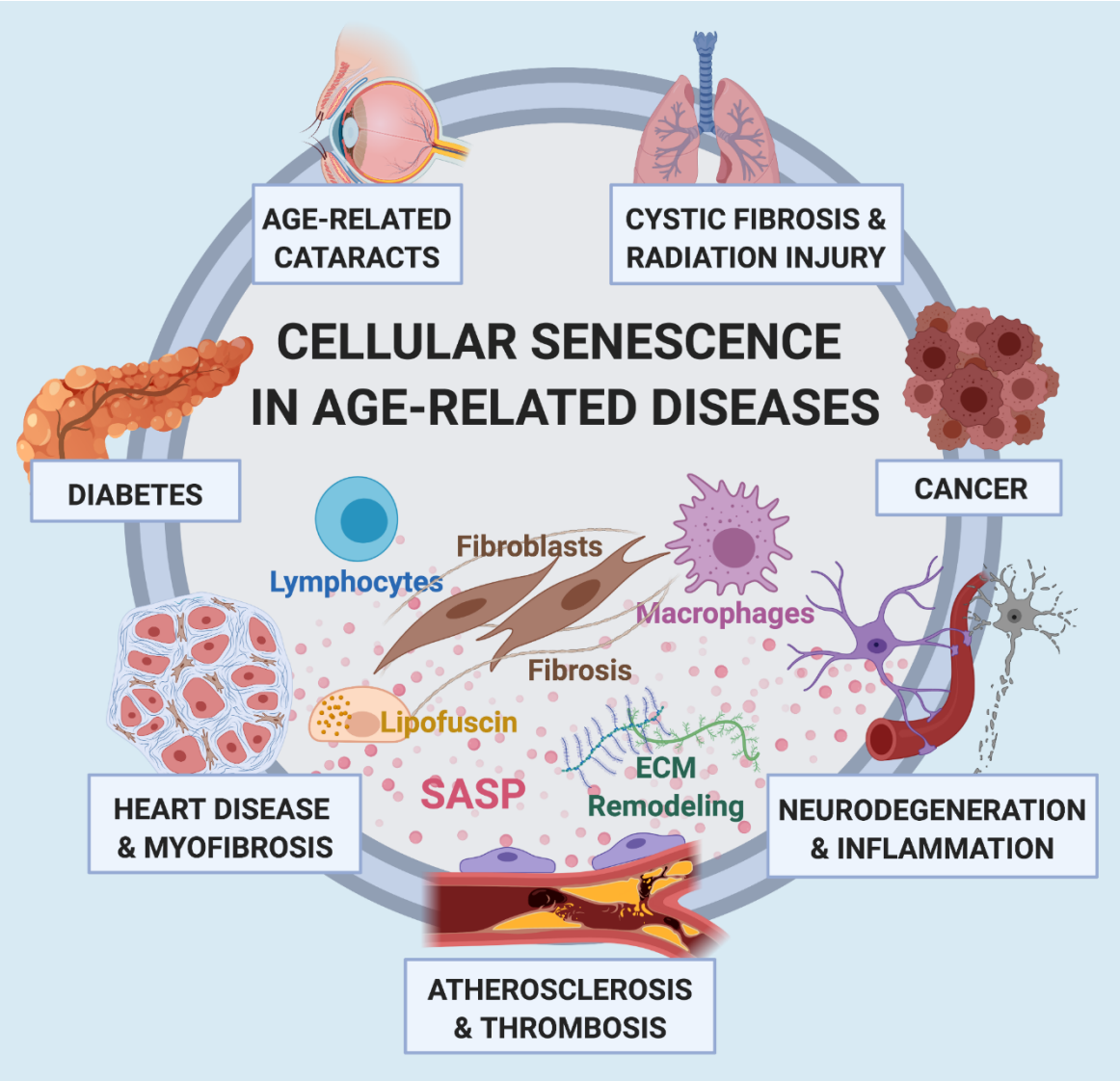

Figure 4.1. Cellular Senescence in Disease. Cellular senescence has been shown to promote or exacerbate age-associated diseases in humans through the induction of chronic inflammation and tissue dysfunction by the senescence-associated secretory phenotype (SASP). SASP proteins mediate an array of effects including mononuclear cell recruitment, fibroblast activation and degradation of the extracellular matrix. Senescent cells, such as endothelial cells, astrocytes, and myocardiocytes, contribute to the progression of age-related diseases including atherosclerosis, neurodegeneration, and myocardial fibrosis, respectively. In addition, although cellular senescence inhibits tumor initiation by preventing the proliferation of damaged cells, persistence of senescent cells within the tumor microenvironment promotes tumor recurrence. Thus, cellular senescence and SASP are potential therapeutic targets in the treatment of disease in aged individuals.

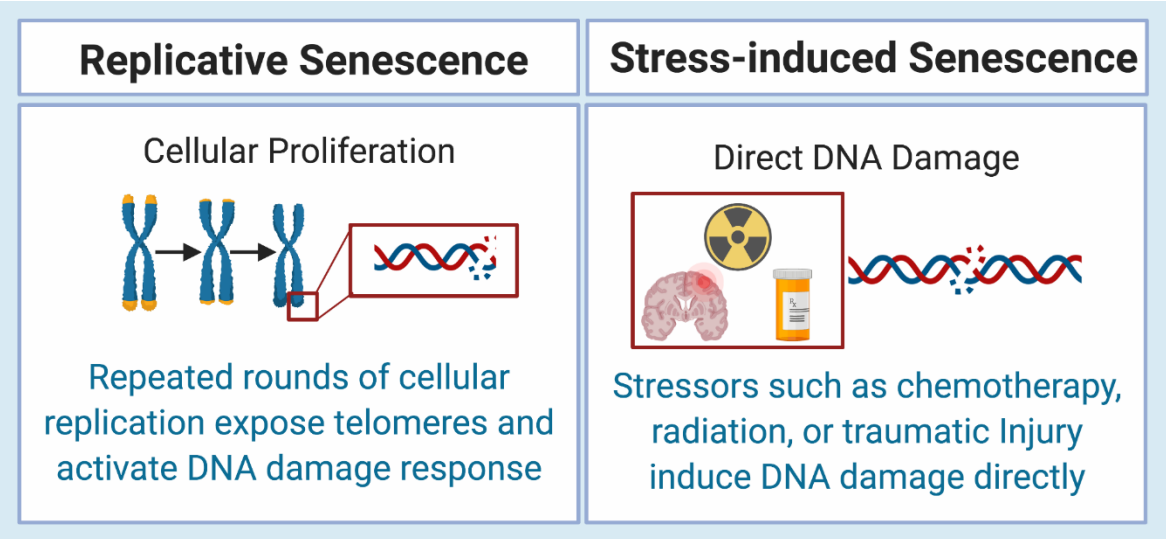

Figure 4.2. Mechanisms of Cellular Senescence. Cells undergo cellular senescence following successive rounds of replication leading to exposure of telomeric DNA and activation of the DNA repair pathway (replicative senescence). Cellular senescence may also be induced following the accumulation of non-telomeric DNA damage due to a variety of a stressors including radiation, chemotherapy, or traumatic injury (stress-induced or premature cellular senescence).

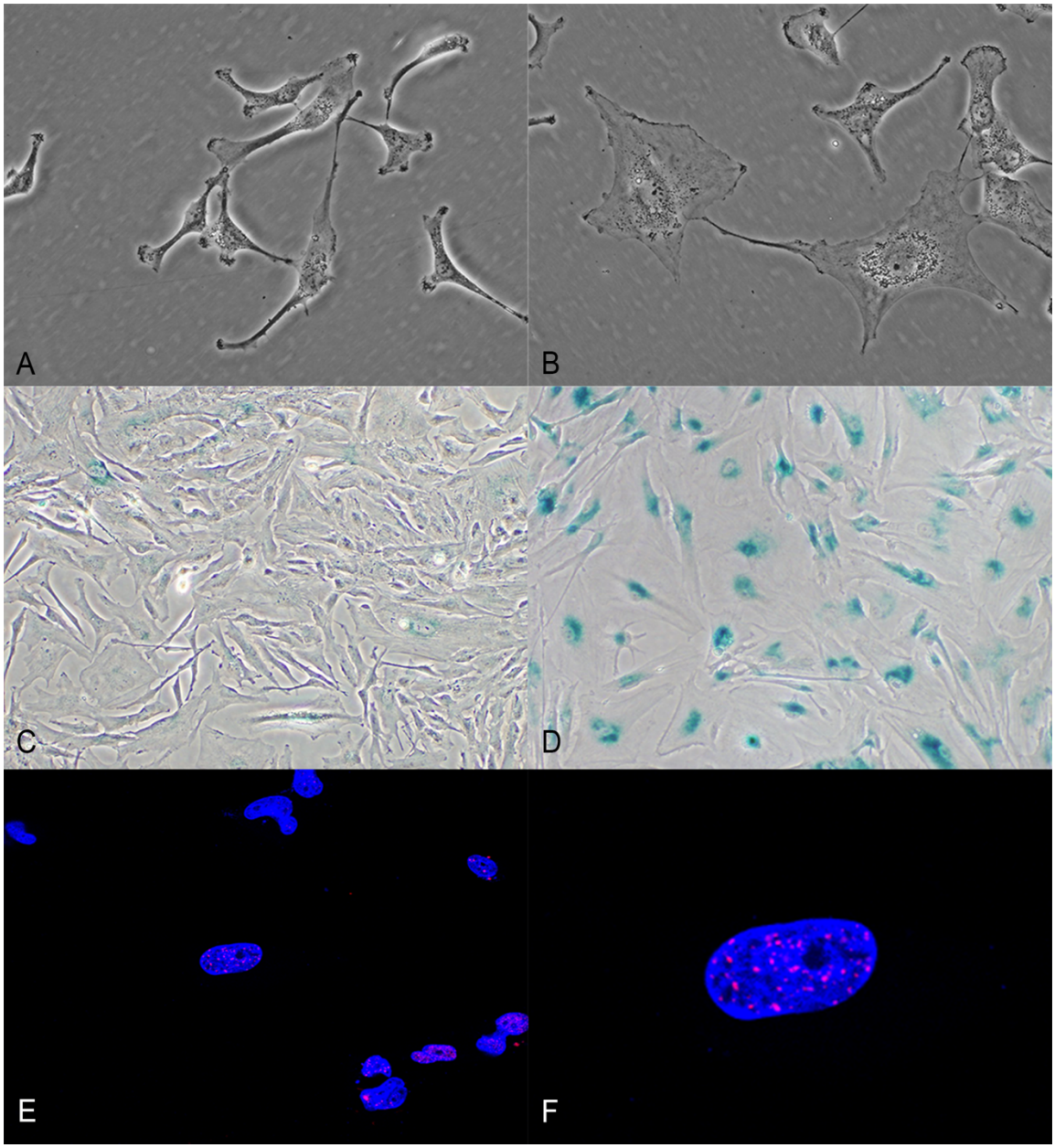

Figure 4.3. Senescent Cell Morphology and Biomarkers. (A) Morphology of control glioblastoma cells. (B) Senescent glioblastoma cells are flattened and have expanded cytoplasm. (C) The majority of control glioblastoma cells lack blue staining using the senescence-associated beta galactosidase assay (SA-β-gal staining, negative). (D) Senescent glioblastoma cells are SA-β-gal positive evidenced by perinuclear blue staining (SA-β-gal staining, positive). Scattered senescent glioblastoma cells are multinucleated. (E) Immunolabelling with γH2AX (red) and DAPI (blue) identifies DNA double-strand breaks in glioblastoma cells (immunofluorescence, confocal microscopy). (F) Higher magnification of nuclear γH2AX immunolabeling.

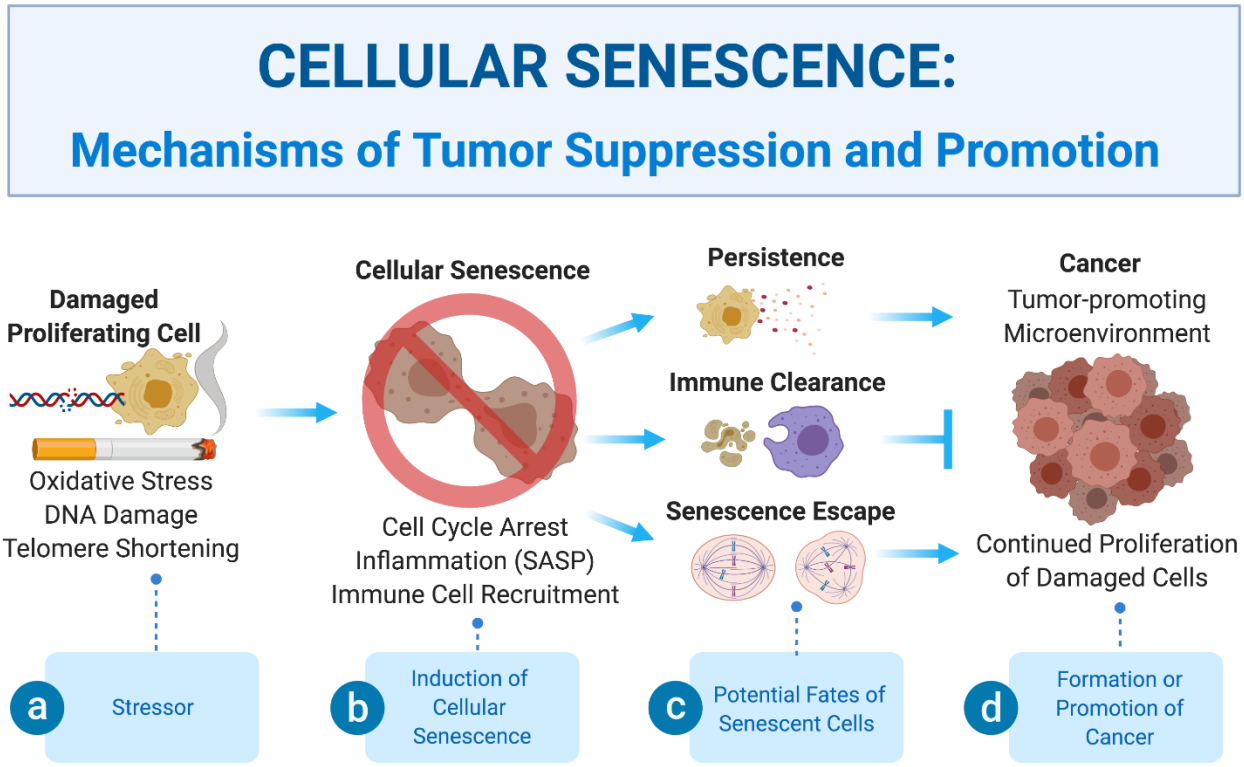

Figure 4.4. Mechanisms of Tumor Suppression and Promotion. (a) Induction of cellular senescence is an important mechanism of tumor suppression which prevents tumor initiation by inhibiting proliferation of damaged cells. (b) Senescent cells undergo a permanent cell cycle arrest and adopt the senescence-associated secretory phenotype (SASP) which recruits leukocytes to remove senescent cells. (c-d) However, there are several potential fates for cells undergoing senescence. First, normal tissue function can be restored if macrophages remove senescent cells; however, this process is not always efficient leading to the persistence of senescent cells which can promote tumor recurrence and the side effects of cancer therapy through SASP. Finally, some reports suggest that tumor cells can either escape or inhibit the induction of cellular senescence allowing them to re-enter the cell cycle leading to tumor initiation further underscoring the contrasting functions of cellular senescence in tumor suppression and promotion.

Table 4.1. Pathologic Findings in Senescence-accelerated Mouse-prone (SAMP). Description of conditions observed in specific tissues from SAMP6 and SAMP8 mice.

Strain	Tissue	Condition
SAMP6	Bone	*Osteoporosis, Increased Adipose Tissue*
	Liver	*Hepatic lipidosis*
SAMP8	Brain	*Amyloid Deposition, Astrogliosis, Microgliosis, Neurodegeneration*
	Eye	*Retinal Degeneration*
	Heart	*Fibrosis, Inflammation*
	Testis	*Senescent Leydig cells (low testosterone)*

Table 4.2. Mouse Models Targeting Senescence Pathways. List of tumor latency period, tumor type and the major contributing cause of death in p53-null, p21-null, and p16-null mice.

	p53-null	***p21-null***	***p16-null***
Tumor Latency	<6 months	~16 months	~18 months
Tumor Type	Lymphoma	Histiocytic Sarcoma	Spindle Cell Tumor
Cause of Death	Cancer	GN/Cancer[a]	Cancer

[a]*Contributing cause of death in p21-null mice varies by sex. Glomerulonephritis (GN) is the primary cause in 60% of females (males, 26%) while cancer is the cause in 55% of males (females, 26%).*

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APPENDIX

Antibodies

Antigen	Name/Product #	Source	Application
Cleaved Caspase 3	9661S	Cell Signaling	IF
GAPDH	MAB374	Millipore	WB
GFAP	AB5541	Millipore	IF
GFAP	MA5-12023	ThermoFisher	WB
HP1γ	AB56978	Abcam	IHC
NeuN	MAB377	Millipore	IF
NOS2	Ab129372	Abcam	IHC
$p16^{INK4A}$	550834	BD Pharmingen	IF, IHC
$p21^{WAF1}$	SC-6246	Santa Cruz	IF
p53	SC-126	Santa Cruz	WB
RAD51	Ab63801	Abcam	IF
RAD51	SC-8349	Santa Cruz	WB
53BP1	NB100-304	Novus	IF
53BP1	MAB3802	Sigma Aldrich	IF
β-Actin	Ab6276	Abcam	WB
γH2AX	05-636-1	Millipore	IF
Δ133p53	MAP4	Rabbit Serum	IF, WB

IF and IHC Antibodies were applied to formalin-fixed samples

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