THE ROLE OF THE STRESS RESPONSE GENE GADD45B IN SENESCENCE

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ABSTRACT

THE ROLE OF THE STRESS RESPONSE GENE GADD45B IN SENESCENCE

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The Gadd45 family of proteins (Gadd45a, Gadd45b, and Gadd45g) has been shown to act as stress sensors in response to various physiological and environmental stressors, including oncogenic stress. However, the role of Gadd45b in senescence remained unclear.

Here, we show for the first time that primary mouse embryo fibroblasts (MEFs) from *Gadd45b* null mice proliferate slowly; accumulate increased levels of DNA damage, and senesce prematurely. Notably, this is in contrast with *Gadd45a* null MEFs that show enhanced growth rate and escape senescence. This difference in growth rate increases with increasing passage number, suggesting that senescence results from exposure to environmental stressors. The impaired proliferation and increased senescence in *Gadd45b* null MEFs can be partially reversed by culturing cells at physiological oxygen levels, indicating that in the absence of *Gadd45b*, primary MEFs are less able to cope with elevated levels of oxidative stress. Interestingly, in contrast to other senescent MEFs, which arrest at G1 phase of cell cycle, *Gadd45b* null MEFs arrest at the G2/M phase of cell cycle. Furthermore, FACS analysis of *Gadd45b* null MEFs showed less phospho-histone H3-positive cells compared to wild type MEFs indicating that *Gadd45b* null MEFs are arrested in G2 phase rather than M phase.

Interestingly, other stressors such as sub-lethal H_2O_2 and UV irradiation, that are known to increase oxidative stress, triggered increased premature senescence in *Gadd45b* null MEFs compared to wild type MEFs. By staining embryos for SA-β-gal gal, we also show that embryos from *Gadd45b* null mice exhibit increased SA-β-gal gal staining compared to wild type embryos, thus providing *in vivo* evidence for increased senescence in *Gadd45b* null mice. Finally, investigating the effect of loss of *Gadd45b* on senescence related diseases, we show that loss of *Gadd45b* promotes senescence and aging phenotypes in the skin as well as increased senescence and attenuated fibrotic response to CCl4 induced liver fibrosis. Together, these results highlight a novel and significant role for Gadd45b in the senescence response of cells to stress.

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TABLE OF CONTENTS

Page

ABSTRACT……………………………………………………………………………..…..ii

ACKNOWLEDGEMENTS……………………………………………………..........iv

LIST OF FIGURES…………………………………………………………………xi

LIST OF ABBREVIATIONS……………………………………………..…...……xiii

LIST OF TABLES …………………………………………………………………xv

CHAPTER 1. INTRODUCTION…......…………………………...………………………1

1.1 Overall Purpose: The Role of Gadd45b in Regulating Senescence…..…...…..………2

1.2 Senescence: Characteristics

1.2.1 Cell cycle arrest…..…...………….……………………………………………3

1.2.2 Morphological transformation…..……………………………………………..4

1.2.3 Activation of tumor suppressor networks….………………………………..4

1.2.4 Induction of Senescence associated beta galactosidase activity…………….5

1.2.5 Senescence-associated heterochromatic foci (SAHF) ……………………...6

1.3. Senescence: Causes

1.3.1 Telomere Shortening…..…...……………………………………………..7

1.3.2 Stress-induced senescence *in vitro* (Tissue Culture induced senescence)…..9

1.3.3 Mitogens and Proliferation-Associated Signals……………………………10

1.3.4 Epigenomic Damage…..…...………………………………………..…..12

1.3.5 Oncogene-induced senescence (OIS) *in vitro*……………………...…..…..12

1.3.6 DNA Damage and reactive oxygen species…..…...………………….………14

1.4. Senescence Related Diseases

1.4.1 Senescence in Ageing and Age-related disease…..…...…………….………..16

1.4.2 Beneficial effects of senescence in diseases

1.4.2.1 Attenuation of liver fibrosis…..…...……………………………20

1.4.2.2 Reduction of skin scarring and oral fibrosis……….….………….21

1.4.2.3 Limitation of cardiac fibrosis upon infarction………..……….21

1.4.2.4 Protection against atherosclerosis……….……….………………21

1.4.2.5 Protection against pulmonary hypertension……….…….….…….22

1.4.3 Detrimental effects of senescence in diseases

1.4.3.1 Senescence aggravates pulmonary fibrosis. ..……….…………...22

1.4.3.2 Adipocyte senescence is associated with obesity…………..…….23

1.4.3.3 Senescence contributes to Type 2 diabetes..….………...……….23

1.5. Gadd45 Family of Genes

1.5.1 Introduction: The Gadd45 Family of Genes..………………………………24

1.5.2 Gadd45 as sensors of oncogenic stress..…….……………….………………26

1.5.3 Gadd45 dis-regulation in cancer ..………………………….………….…….27

1.5.4 Gadd45 and cellular senescence..…….…………..…………………….…..28

1.5.5 Gadd45b..…….………….………………………….…………………….……..30

1.6. Gadd45, Senescence and Aging in the skin

1.6.1 Stress response and Aging..……………………….…………………………...35

1.6.2 Gadd45 in Aging..…….……………………………………….…………….36

1.6.2.1 Gadd45 in age-related pathologies: Cancer…….……………….….36

1.6.2.2 Gadd45 in age-related pathologies: Other age-related diseases…37

1.6.2.3 Gadd45 in age-related pathologies: Gadd45 and longevity…......38
1.6.3 Cellularity as an Aging phenotype in Skin………....................................39
1.6.4 g-H2AX……………………………………………..…………..........39
1.6.5 Senescence and Aging in Skin………………………………….............43
1.6.6 Gadd45 in Skin…………………………………..………......................46
1.7. Gadd45, Senescence and Liver Fibrosis
1.7.1. Gadd45 in Liver………………………………..………......................48
1.7.2 Senescence in CCl4 induced liver fibrosis……..……….............................50
1.8. Objectives and rationale……..………..53
CHAPTER 2. MATERIALS AND METHODS
2.1 Animals……..………..56
2.2 Mouse Embryonic Fibroblast Preparation and Growth..................................57
2.3 3T3 Cell Growth Protocol...57
2.4 UV irradiation and hydrogen peroxide treatment..58
2.5 Senescence-associated β-galactosidase assay...58
2.6 Protein Extraction and Immunoblotting..59
2.7 RNA Extraction and Quantitative Polymerase Chain Reaction......................60
2.8 Flow cytometric cell cycle analysis..61
2.9 Immunofluorescence and Mitotic index analysis..61
2.10 Alkaline comet assays...62
2.11 Histo-pathological Evaluation of skin samples...62
2.12 Immuno-histochemistry of skin samples..63
2.13 Senescence staining of skin samples...63

2.14 Hepatic-Fibrosis Model..63

2.15 Histopathological Evaluation of the Liver Tissue.......................................64

2.16 Statistical Analysis...64

CHAPTER 3. GADD45B DEFICIENCY IMPAIRS G2/M CELL CYCLE PROGRESSION LEADING TO PREMATURE SENESENCE

3.1 Introduction..66

3.2 Decreased proliferation and premature senescence in *Gadd45b* null MEFs...68

3.3 Premature senescence associated with Gadd45 deficiency can be rescued, in part, by culture at low oxygen..71

3.4 *Gadd45b* mRNA Expression is regulated by levels of Oxidative Stress........74

3.5 Growth arrest in *Gadd45b* null MEFs is associated with defective G2/M cell-cycle progression..74

3.6 Increased p19 Arf -p53-p21 signaling and impaired CDC2 expression in *Gadd45b* null MEFs..79

3.7 Loss of *Gadd45b* results in accumulation of spontaneous DNA damage.......82

3.8 *Gadd45b*$^{-/-}$ MEFs show increased senescence in response to environmental stresses…………………………………………………………………...83

3.9 Discussion..87

CHAPTER 4. GADD45B DEFICIENCY PROMOTES SENESCENCE AND AGING PHENOTYPES IN THE SKIN

4.1 Introduction..94

4.2 Loss of *Gadd45b* leads to increased DNA damage in skin...........................97

4.3 Loss of *Gadd45b* results in increased senescence in skin..............................98

4.4 Loss of *Gadd45b* results in decreased cellularity in the dermal section of the skin……………………………………………………………………………98

4.5 Discussion...102

CHAPTER 5. GADD45B DEFICIENCY PROMOTES SENESCENCE AND ATTENUATES CCL4 INDUCED LIVER FIBROSIS

5.1 Introduction...106

5.2 Loss of *Gadd45b* is protective against CCl4 induced chronic hepatic injury110

5.3 Loss of *Gadd45b* results in reduced collagen deposition in response to CCl4 induced chronic hepatic injury...110

5.4 Loss of *Gadd45b* results in increased senescence in response to CCl4 induced chronic hepatic injury...114

5.5 Discussion...114

REFERENCES CITED…………………………………………………………117

LIST OF FIGURES

Figure **Page**

Figure 1.1 Molecular pathways of senescence…………………………………..……..…6

Figure 1.2 Structures and organs undergoing pathological processes associated with damage-induced senescence (right) are depicted..………………………………………...9

Figure 1.3 Gadd45 function in stress signaling.………………………………………25

Figure 1.4 Map of molecular interactions involving GADD45 proteins and cell signaling pathways generated using Ingenuity Pathway Analysis software (IPA)…...……………34

Figure 1.5 Scheme illustrating the putative role of SOD2 in skin aging.………………..45

Figure 1.6 Hepatic stellate cell-derived myofibroblast fate in liver fibrosis. …………...52

Figure 3.1. Loss of *Gadd45b* results in impaired proliferation…………………………..69

Figure 3.2. Loss of *Gadd45b* results in premature and increased senescenc…...………..70

Figure 3.3. Impaired proliferation associated with *Gadd45b* deficiency can be rescued, in part, by culture at low oxygen …………………………………………………...……72

Figure 3.4. Premature senescence associated with *Gadd45b* deficiency can be rescued, in part, by culture at low oxygen ……………………………………………………….…...73

Figure 3.5. *Gadd45b* mRNA levels increase with increased exposure to oxidative stress………………………………………………………………………………...…75

Figure 3.6. Loss of *Gadd45b* results in an accumulation in G2/M………………………76

Figure 3.7. Loss of *Gadd45b* results in a reduced mitotic index (Defective G2/M cell-cycle progression)……………………………………………………………..………77

Figure 3.8. Loss of *Gadd45b* results in reduced number of phosphorylated Histone H3-positive cells (Defective G2/M cell-cycle progression)…………………………………78

Figure 3.9. Loss of *Gadd45b* results in increased expression of senescence markers – p16, p19ARF, p21, p53 & JNK phosphorylation and decreased expression of cdc2…………80

Figure 3.10. Premature senescence associated with *Gadd45b* deficiency can be rescued, in part, by inhibition of SAPK/JNK……….…………………………………...……..81

Figure 3.11. Increased DNA damage in MEFs lacking *Gadd45b*……………………….84

Figure 3.12. Increased senescence of *Gadd45b*$^{-/-}$ MEFs in response to environmental stress...85

Figure 3.13. Increased senescence staining in *Gadd45b*$^{-/-}$ embryos……………………..86

Figure 3.14. Schematic illustrating the role of *Gadd45b* in tissue culture induced senescence………………………………………………………………………..89

Figure 4.1. Loss of *Gadd45b* promotes DNA double-strand breaks in the ski………..…99

Figure 4.2. Loss of *Gadd45b* promotes senescence in the skin………………………...100

Figure 4.3. Loss of *Gadd45b* promotes aging phenotypes in the skin………………….101

Figure 4.4. Schematic illustrating the role of *Gadd45b* in skin aging………………….103

Figure 5.1 *Gadd45b* deficiency attenuates CCl4-induced liver fibrosis progression…..111

Figure 5.2. *Gadd45b* deficiency attenuates CCl4-induced collagen accumulation in liver……………………………………………………………………………..…112

Figure 5.3 Loss of *Gadd45b* results in increased senescence in response to CCl4 induced chronic hepatic injury…………………………………...……………………..…113

Figure 5.4. Schematic illustrating the role of *Gadd45b* in regulating CCl4 induced liver fibrosis……………….…………………………………………………………...…115

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated
BSA	Bovine serum albumin
Beta-gal	Beta Galactosidase
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CO2	Carbon Dioxide
DAPI	Diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
DDR	DNA damage response
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DSB	Double strand breaks
EDTA	Ethylene-Diamine-Tetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GADD45	Growth Arrest and DNA Damage
H&E	Hematoxylin & Eosin
H2O2	Hydrogen Peroxide
KDa	Kilo Daltons
KO	Knock-out
IACUC	Institutional Animal Care and Use Committee
JNK	cJun Nh2 Terminal Kinase
ml/mm/mM	Millilitre/millimetre/millimolar

MAPK	Mitogen-activated protein kinase
MEF	Mouse Embryonic Fribroblast
mRNA	Messenger ribonucleic acid
NaOH	Sodium Hydroxide
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
pRb	Retinoblastoma Protein
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT	Room temperature
SA β-gal	Senescence Associated β-galactosidase
SAFH	Senescence Associated Heterochromatin Foci
SIPS	Stress Induced Premature Senescence
SDS	Sodium Dodecyl Sulfate
TEMED	N, N, N, N-tetramethyl-1, 2 diaminoethane
μg/μl/μM	Microgram/microlitre/micromolar
UV	Ultraviolet
WT	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactoside

LIST OF TABLES

Table **Page**

Table 1. List of senescence-related diseases…………………………………………………..18

Table 2. Phenotypic characteristics of Gadd45 knockout mice………………………….30

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CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1.1 Overall Purpose: The Role of Gadd45b in Regulating Senescence

The Gadd45 family of genes (Gadd45a, Gadd45b, and Gadd45g) plays an important role in diverse cell functions including cell cycle control, survival and apoptosis. A growing body of evidence has highlighted the pivotal role these genes play as stress sensors that modulate the cellular response to a variety of stress conditions. Their cellular function is also dependent upon the physiological state of the target cell and the nature of the stress. The overall purpose of this research project was to examine the role of Gadd45b in response to various stress conditions that ultimately lead to senescence. We hypothesized that Gadd45b will play a critical role in the cellular response to tissue culture induced stress as well as senescence related diseases including aging phenotypes in the skin as well as CCl4 induced liver fibrosis. Elucidating the key signaling partners and the mechanism involved in Gadd45b related senescence response will provide vital information in understanding the impact of specific stress situations on a cell that may be utilized to design rational, novel therapies for treatment of disease states such as cancer and fibrosis.

1.2 Senescence: Characteristics

Cellular senescence was first described approximately five decades ago when Hayflick and colleague [63, 64] showed that, contrary to common belief, normal human fibroblasts did

not proliferate indefinitely in culture. They showed that cells have a finite replicative life span and undergo replicative or cellular senescence. The number of divisions that cells complete upon reaching the end of their replicative life span has been termed the Hayflick limit. This finite replicative life span of normal cells is in contrast to cancer cells, which possess an indefinite capacity to proliferate. In the last few years, a growing number of studies have shown that senescence can be induced by various stimuli and cellular contexts in multiple physiological and pathological processes [29, 93].

Senescent cells display a number of characteristics that allow their identification both *in vitro* and *in vivo*. While some of these biomarkers reflect the activation of signaling mechanisms, including the induction of tumor suppressor networks such as p53 and pRB that contribute to the senescence program, for other biomarkers like the increase in senescence associated β-galactosidase, their contribution to the senescence program is as yet unclear [36, 144].

1.2.1 Cell cycle arrest

One of the central and indispensable markers for the identification of cellular senescence both *in vitro* and *in vivo* is the long-term exit from the cell cycle. However, cell cycle exit cannot be used in isolation to identify senescent cells.

While early *in vitro* studies suggested that cellular senescence is strictly irreversible [80, 203], subsequent studies have shown that in certain scenarios, senescence can be reversed. In spite of the observation that stable RB-dependent heterochromatin structures and several other factors contribute to lock cells in their senescent state,

multiple strategies have been shown to successfully reverse the arrest, allowing cells to re-enter the cell cycle. These include, inactivation of the p53 pathway and inactivation of some interleukins [18, 50, 92]. In addition to the levels of certain tumor suppressor proteins, the strength of the oncogenic signal has also shown to contribute to the reversibility of senescence.

1.2.2 Morphological transformation

Cell senescence is generally accompanied by some striking morphological changes. Depending on the senescence trigger, cells can become large, flat, and multinucleated, or rather refractile. A flat cell phenotype is commonly seen in cells undergoing H-RASV12-induced senescence [46, 153], stress-induced senescence [135], or DNA damage-induced senescence [31]. Cells senescing due to BRAFE600 expression or the silencing of p400, however, acquire a more spindle-shaped morphology [117] [35]. Melanocytes undergoing RASV12-induced senescence display extensive vacuolization as a result of endoplasmic reticulum stress caused by the unfolded protein response [46].

1.2.3 Activation of tumor suppressor networks

The p53 and p16INK4A–RB signal transduction cascades commonly mediate the activation of the senescence program [108]. Consequently, components of these signaling pathways have been used as biomarkers to identify senescent cells. In human fibroblasts

undergoing replicative or premature senescence, RB accumulates in its active, hypophosphorylated form [159 153 103], and p53 displays increased activity and expression levels [9 25, 183]. The p53 protein has also shown to be phosphorylated on serine 15 by ATM as part of the DNA damage induced senescence response [26]. p53 serves as a node, mediating prosenescence signals from diverse stimuli including DNA damage, oncogene activation, telomere dysfunction, and reactive oxygen species (ROS). The pocket protein RB, in addition to p53, has a unique role in mediating senescence in human cells [33]. One of its primary activators, p16INK4A, is commonly induced in senescent cells in many contexts *in vitro* [153 27]. Induction of p16INK4A is commonly seen in senescent mouse and human lesions *in vivo* [166 35 117 47], and p53 and p21CIP1 induction is seen in senescent mouse prostate tumors [32].

1.2.4 Induction of senescence associated β-galactosidase activity

Senescence associated β-galactosidase activity is a commonly used senescence biomarker [45, 49]. Its increased activity in senescent cells derives from lysosomal b-D-galactosidase, which is encoded by the GLB1 gene. A major limitation with this biomarker is that its detection requires tissues to be snap-frozen to preserve enzymatic activity [45]. Also, it has been shown that non-senescent cells display β-galactosidase activity in the lysosomes at pH 4 [96]. Accordingly, the increase in senescence associated β-galactosidase activity in senescent cells is likely due to an expansion of the lysosomal compartment, giving rise to an increase in b-galactosidase activity that can be measured at suboptimal pH 6 (hence,

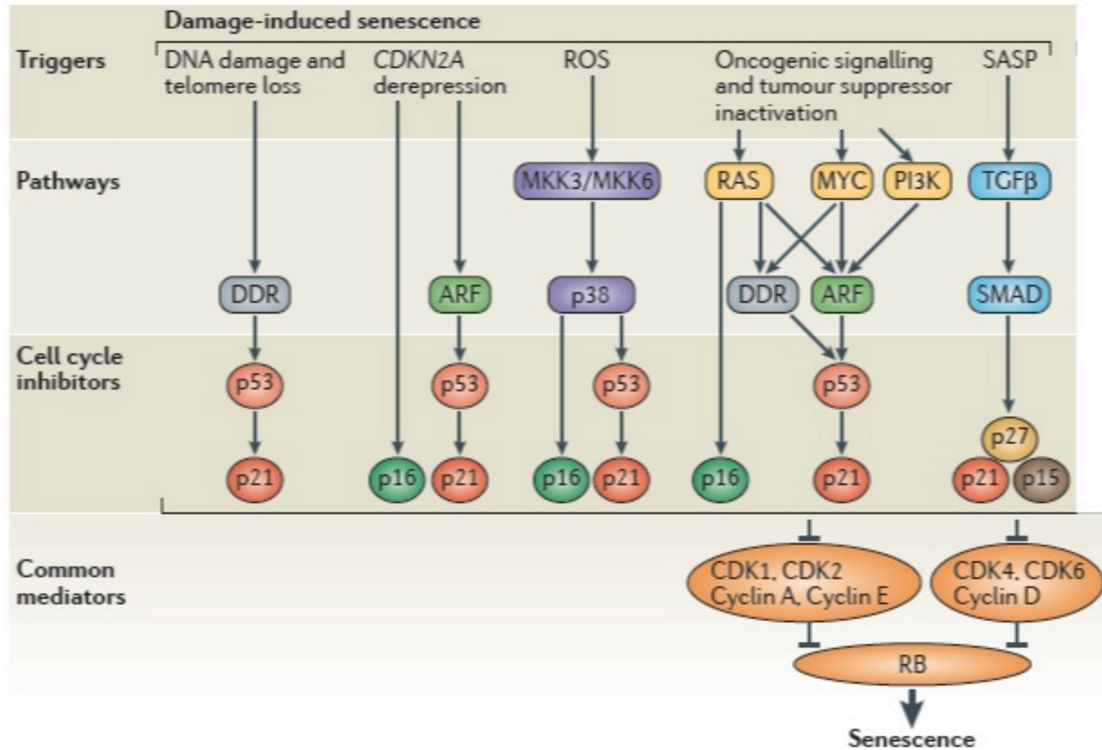

Figure 1.1 Molecular pathways of senescence.
Multiple stressors and damaging agents (triggers) activate signalling cascades (pathways) that converge on the activation of cell cycle inhibitors and the tumour suppressor RB. DNA damage agents and telomere loss activate the DNA-damage response (DDR), which directly activates p53 and its downstream transcriptional target p21. Many types of senescence are associated with the epigenetic derepression of the cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus (encoding the cell cycle inhibitor p16 and the p53 activator ARF). Reactive oxygen species (ROS) activate p16 and p53 through the kinases MKK3 (also known as MAPKK3) and MKK6 (also known as MAPKK6), and their downstream kinase effector p38. Oncogenic signalling or loss of tumour suppressors activates p16 and p53 with the participation of the DDR and ARF. (Munoz-Espin & Serrano, 2014)

senescence associated β-galactosidase) [94]. There is, however, as yet no evidence pointing to an actual involvement of this enzyme in the senescence response [192 96].

1.2.5 Senescence-associated heterochromatic foci (SAHF)

Cellular senescence, atleast *in vitro,* has been associated with an altered chromatin structure. While DNA dyes like DAPI display overall homogenous staining patterns in cycling or quiescent human cells, senescent cells often show strikingly different punctate staining patterns. These DNA Senescence-associated heterochromatic foci [127] are specifically enriched in methylated Lys 9 of histone H3 (a modification catalyzed by the histone methyltransferase Suv39h1), while euchromatin markers histone H3-Lys 9 acetylation and Lys 4 methylation are excluded from senescence-associated heterochromatic foci. While polycomb group proteins have been shown to repress the INK4A/ARF locus [59, 77], the histone H3-Lys 27 demethylase JMJD3 contributes to its transcriptional activation [4], thus regulating senescence. Disruption of p16INK4A–RB pathway leads to inhibition of senescence-associated heterochromatic foci formation leading to bypass of senescence.

1.3. Senescence: Causes

1.3.1 Telomere Shortening

The mechanism behind the finite replicative life span of normal cells first observed by Hayflick and colleague is now well understood [29]. Telomeres, the DNA-protein structures that cap the ends of linear chromosomes, shorten with each cell division [7]. Because

polymerases that copy DNA templates are unidirectional and require a labile primer, the ends of linear DNA molecules cannot be completely replicated [98], leading to telomere shortening. Telomere shortening does not occur in cells that express telomerase, the reverse transcriptase that can replenish the repetitive telomeric DNA de novo [115]. In mice, many cells in the adult animal are telomerase positive. In humans, however, such cells are rare. Telomerase positive human cells include most cancer cells, embryonic stem cells, certain adult stem cells, and a few somatic cells (for example, activated T cells). Functional telomeres prevent DNA repair machineries from recognizing chromosome ends as DNA double-strand breaks (DSBs) and attempt repair. During replication, repair followed by cell division will cause rampant genomic instability through cycles of chromosome fusion and breakage [143] a major risk factor for developing cancer. Thus, repeated cell division in the absence of telomerase eventually causes one or more telomeres to become critically short and dysfunctional. Dysfunctional telomeres elicit a DNA damage response (DDR) but suppress DNA repair [41, 57]. The DNA damage response, in turn, arrests cell division primarily through activation of the p53 tumor suppressor, thereby preventing genomic instability. Also, dysfunctional telomeres appear to be irreparable; consequently, cells with such telomeres experience persistent DDR signaling and p53 activation [185], which enforce the senescence growth arrest.

1.3.2 Stress-induced senescence *in vitro* (Tissue Culture induced senescence)

Premature senescence, *in vitro*, can result from tissue culture conditions. When cells are explanted from an organism and placed in culture, they are exposed to an artificial

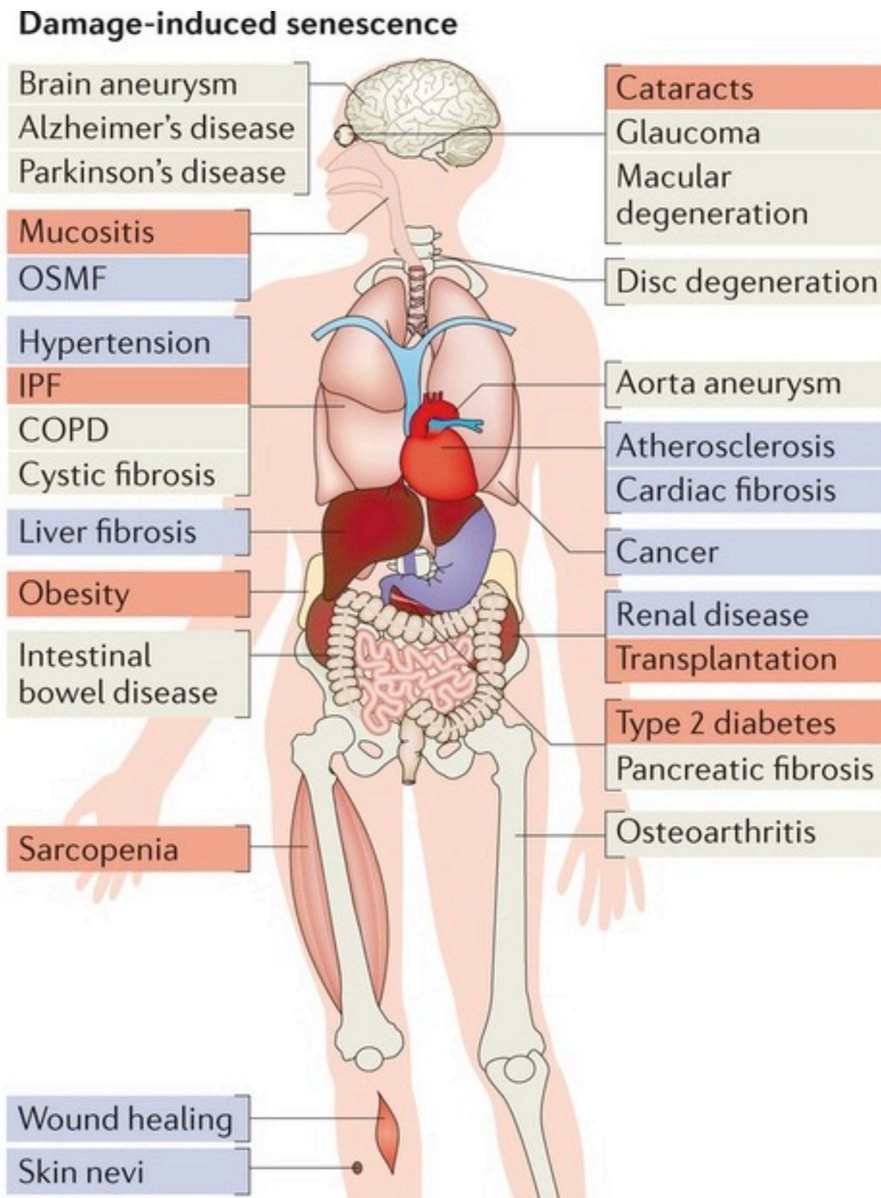

Figure 1.2 Structures and organs undergoing pathological processes associated with damage-induced senescence (right) are depicted. Diseases in which senescence has known beneficial (indicated in blue boxes) or detrimental (indicated in red boxes) roles are listed in addition to diseases in which a beneficial or detrimental role of senescence has not been established (indicated in beige boxes). COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; OSMF, oral submucous fibrosis. (Munoz-Espin & Serrano, 2014)

environment, characterized by abnormal concentrations of nutrients and growth factors and the presence of atmospheric O2 levels (21% versus a physiologically relevant level of 3%), as well as the absence of surrounding cell types and extracellular matrix components. One or more of these conditions induce a culture shock, resulting in stress-induced senescence [154]. This type of cell cycle arrest is independent of telomere length given the fact that it also occurs in mouse cells, which, in contrast to most human cells, express telomerase [142] and have long telomeres [85]. Mouse embryonic fibroblasts (MEFs) undergo senescence after a limited number of passages in culture, despite their retaining of long telomeres. Elongation of their life span can be achieved by culturing in serum-free medium supplemented with a number of defined growth factors [107] or by culturing under physiological oxygen conditions [135]. Consistent with this, oxidative stress induces a limited number of passages in culture in human cells as well ([131], where the replicative potential of human melanocytes and epithelial cells depends on the composition of the culture medium used, as well as on the use of feeder layers [20] . Senescence of MEFs can be bypassed also by inactivation of p53 or simultaneous ablation of RB family genes [165] [43, 146].

1.3.3 Mitogens and Proliferation-Associated Signals

Strong, chronic, or unbalanced mitogenic signals can also induce cellular [21], consistent with its role in suppressing tumorigenesis. The best-studied evidence for the involvement of mitiogen signals in senescence includes cellular responses that are provoked by certain oncogenes. The first report describing oncogene-induced senescence showed that an oncogenic form of H-RAS (H-RASV12), which chronically stimulates the mitogen-

activated protein kinase (MAPK) signaling pathway, provokes senescence in normal cells [153]. Several other MAPK pathway components have since been shown to induce senescence when overexpressed or present in oncogenic forms [141]. Likewise, cells senesce in response to overexpressed growth factor receptors such as ERBB2 [175], chronic stimulation by cytokines such as interferon-β [121], loss of PTEN (which truncates growth factor signaling) [6], and several other forms of chronic or high-intensity mitogenic stimulation [21]. One of the mechanisms involved in mitogen signal-induced senescence involves the induction of DNA damage [15]. Some oncogenes and strong mitogenic stimuli cause DNA damage and persistent DNA damage response signaling, possibly as a consequence of DNA double strand breaks that are created by inappropriate replicon firing and replication fork collapse. However, this mechanism cannot explain all instances of mitogen-associated senescence. For example, hyperactivation of p38MAPK, a stress-responsive MAPK pathway component, induces senescence by a DDR-independent mechanism [56]. It can be concluded that, regardless of the initiating event, mitogenic signals ultimately engage the p53/p21 and/or p16INK4a/pRB pathways.

1.3.4 Epigenomic Damage

Cellular senescence is characterized by widespread changes in chromatin organization [3], including the formation of repressive heterochromatin at several loci that encode pro-proliferative genes [127]. Evidence has been obtained showing that perturbations to the epigenome can elicit a senescence response. For example, global chromatin relaxation (such as that caused by broad-acting histone deacetylase inhibitors) induces senescence, often by derepressing the p16INK4a tumor suppressor [125], which promotes the formation

of senescence-associated heterochromatin. Other inducers, for example, suboptimal c-MYC [61] or p300 histone acetyltransferase [13] activity, also appear to act by perturbing chromatin organization and inducing p16INK4a expression, a biomarker of aging and a tumor suppressor, which is expressed by many senescent cells [83]. Interestingly, under some circumstances, epigenomic changes can elicit a DNA damage response in the absence of physical DNA damage. For example, histone deacetylase inhibitors activate the DDR protein ATM (ataxia-telangiectasia-mutated), leading to DNA damage response signaling without DNA damage [12].

1.3.5 Oncogene-induced senescence (OIS) *in vitro*

Landmark studies on mutant HRAS (HRASV12) led to the discovery that, although it can cooperate with immortalizing genes in transforming primary cells and transform most immortal mammalian cell lines, it induces cell cycle arrest when it is introduced alone into primary cells (and at least one immortal rat fibroblast cell line) [153]. Serrano et al. noted the striking phenotypic resemblance of such non-proliferating cells to those in replicative senescence, and this phenomenon has eventually come to be known as Oncogene induced senescence. The list of oncogenes able to induce senescence has since increased to about 50 oncogenes [60]. However, unlike replicative senescence, Oncogene induced senescence cannot be bypassed by expression of hTERT, confirming its independence from telomere attrition [60]. One of the hallmarks shared by cells undergoing replicative senescence and Oncogene induced senescence is the critical involvement of the p53 and p16INK4A–RB pathways. In murine cells, functional inactivation of p53 or

its direct upstream regulator, p19ARF, is sufficient to bypass RASV12-induced senescence [153]. In human cells, p16INK4A seems to play a more prominent role than p53, as some cells depend solely on p16INK4A for Oncogene induced senescence [19]. While p19ARF is a critical sensor that is activated by oncogenic signals and mediates senescence in cultured murine cells, in human cells it does not seem to play a similarly dominant role ([117].

Interestingly, Oncogene induced senescence mechanisms do not seem to be universal across cell types and genetic contexts. While RASV12-induced senescence can be bypassed by abrogation of the p16INK4A–RB pathway [153], BRAFE600-triggered senescence cannot be bypassed by functional inactivation of p16INK4A, be it alone [117]. Interestingly, loss of tumor suppressors can also trigger senescence as exemplified by the losses of PTEN [6], neurofibromin (NF1) [39] or von Hippel-Lindau disease tumor suppressor (VHL) [194]. Importantly, it is well demonstrated that oncogene-induced senescence occurs *in vivo*, functioning as a brake during the early stages of tumorigenesis [37].

Importantly, recent evidence suggests the relevance of Oncogene induced senescence also in the context of induced pluripotency *in vitro*. At least two oncoproteins, c-MYC and KLF4, are required for the generation of induced pluripotent stem (iPS) cells. As the INK4A/ARF proteins and p53 limit the efficiency of iPS cell formation, recent reports have indicated that cellular senescence counteracts the induced conversion of primary cells into pluripotent stem cells [14 75, 99]. It is likely that cancer stem cells use a similar reprogramming process and so cellular senescence might suppress tumor formation not only by inducing a persistent cell cycle arrest, but also by limiting the generation of cancer stem reprogramming.

1.3.6 DNA Damage and reactive oxygen species

The role of DNA damage in the induction of replicative senescence by telomere shortening has been well established [42]. Many cells undergo senescence in response to severely damaged DNA, regardless of the genomic location [126]. DNA double strand breaks, such as those induced by topoisomerase inhibitors, ionizing radiation, and other agents, are especially potent senescence inducers [188]. Many types of cytotoxic chemotherapies have also been shown to be DNA-damaging agents that can induce senescence in both tumor cells and surrounding normal cells [149] both *in vitro* and *in vivo*, a phenomenon termed drug-induced senescence, which may be exploited for the treatment of cancer [149, 166].

Other DNA lesions—such as those caused by oxidative stress—have also shown to drive cells into senescence [135]. Oxidative stress and other DNA-damaging agents often cause DNA base damage and/or single-strand breaks that can be converted to double strand breaks during DNA replication or base excision repair [152]. Oxidative stress has also shown to accelerate telomere shortening, the G-rich telomeric DNA being particularly vulnerable to oxidative damage. Therefore, cells may senesce primarily in response to directly or indirectly generated DNA double strand breaks. An interesting study showed that double strand breaks are such potent senescence inducers that even a single unresolved DSB (using dose response experiments) could induce a senescence growth arrest [48]. Although the precise types of genomic lesions that induce senescence are unknown, effective lesions are known to generate persistent DNA damage response (DDR) signaling. This chronic DNA damage response is generally identified by the long-

term presence of nuclear DNA damage foci that contain a variety of activated DDR proteins, including activated p53 and contrasts sharply with the response to mild DNA damage, which generates a transient growth arrest and transient DDR signaling [57, 144].

Furthermore, activation of a DNA damage response has also been shown to contribute to Oncogene induced Senescence in several settings *in vitro* [42]. However, DNA damage response (DDR) activation is not a universal feature of Oncogene induced Senescence. For example, in the context of several types of senescent mouse adenomas, ATM is not required for Oncogene induced Senescence and no DNA damage response (DDR) is observed [51]. Furthermore, senescence triggered by RAF1 or BRAFE600 does not require p53 [93] and does not trigger DNA damage response (DDR) activation. Also, senescence induced upon genetic loss of Skp2 in the context of Pten heterozygosity is not associated with the emergence of DNA damage response (DDR) markers and does not depend on p53 signaling [104]. In an elegant study, Shamma and colleagues took advantage of the Rb loss-driven murine adenoma model to shed more light on the link between the DNA damage response and senescence. While early and late adenomas expressed similar levels of DNA damage response markers including H2AX phosphorylated at serine 139 (γH2AX) and p53 phosphorylated at serine 15 (p53pS15), senescence was apparent only in late (arrested) lesions, suggesting that, at least in this experimental system, it is primarily senescence and not DNA damage signaling that triggers cell cycle arrest [137]. Thus, DDR activation is involved in certain, but not all, OIS settings.

1.4. SENESCENCE RELATED DISEASES

1.4.1 Senescence in Ageing and Age-related disease

Several studies have provided convincing evidence that senescent cells indeed accumulate in tissues of humans, primates, and rodents with age [65 90]. Furthermore, senescent cells can also be found in the affected tissues of patients with age-related diseases such as osteoarthritis, pulmonary fibrosis, atherosclerosis, and Alzheimer's disease [128].

Two landmark studies in BubR1 progeroid mice, in which p16Ink4a-positive senescent cells were targeted in different ways provided the first direct evidence for Hayflick and Moorhead's early concept that senescent cells drive age-related pathologies [11, 128]. In the first study, genetic inactivation of p16Ink4a prevented the formation of senescent cells in skeletal muscle, eye and fat, significantly attenuating the onset of age-related pathologies in these tissues. In the second study, p16Ink4apositive senescent cells were allowed to accumulate but were consistently eliminated from weaning age onwards by the use of a transgene, termed INK-ATTAC, that selectively induced apoptosis in these cells upon administration of the synthetic drug AP20187[180]. Interestingly, late-life clearance of senescent cells slowed the progression of established age-related disorders in skeletal muscle and fat was unable to revert them.

Several explanations have been proposed to explain the mechanism by which senescence promotes age-related tissue dysfunction: Senescence contributes to the overall decline in tissue regenerative potential that occurs with ageing, which is supported by the observation that progenitor cell populations in both skeletal muscle and fat tissue of

BubR1 progeroid mice are highly prone to cellular senescence [180]. In addition to acting on stem cells by establishing a persistent growth arrest, senescence could also lead to the disruption of the local stem-cell niche through the Senescence Associate secretory Phenotype [23]. Exciting studies using parabiotic pairing have showed that the regenerative potential of old stem cells can be markedly improved when exposed to a young systemic environment thus highlighting the deleterious impact that the aged cellular microenvironment has on stem cell functionality.

Several transgene mouse models have helped investigate the role of senescence in aging. The phenotypic characterization of transgenic mice with hyperactive p53 provided compelling evidence that senescent cells can drive degenerative aging pathologies. Two landmark papers described mouse models in which constitutive expression of an artificially [176] or naturally [114] truncated p53 protein resulted in chronically elevated p53 activity. These mice were exceptionally cancer-free, which was not surprising, as p53 is a critical tumor suppressor. What was surprising was their shortened life span and premature aging. While

these mice did not completely phenocopy normal aging, similar to progeroid models, the mice showed premature degenerative changes, including loss of fertility, osteoporosis, dermal thinning, loss of subcutaneous fat, reduced hair growth, and retarded wound healing. Notably, cells from these mice underwent rapid senescence in culture [114]. Moreover, tissues from these mice rapidly accumulated senescent cells, and, in lymphoid tissue, the p53 response shifted from primarily apoptotic to primarily senescent *in vivo* [68] thus providing a strong correlation between excessive cellular senescence and premature aging phenotypes.

Table 1 Senescence-related diseases (Adapted from(Munoz-Espin & Serrano, 2014))

Diseases	Disease description and therapeutic strategies	Reference
Multiple pre-malignant tumour types	• Senescence is associated with the pathology and restricts tumour progression. • Cyclin-dependent kinase 4 (CDK4) inhibitors are pro-senescent and can induce tumour regression	(Collado & Serrano, 2010; Guha, 2013; Nardella, Clohessy, Alimonti, & Pandolfi, 2011)
Liver fibrosis	• Senescence is associated with the • Interleukin-22 (IL-22), CCN family member 1 (CCN1) and statins are pro-senescent and revert fibrosis	(Kong et al., 2012; Krizhanovsky et al., 2008)
Skin wound healing and oral submucous	• Senescence is associated with the pathology and restricts fibrosis • CCN1 is pro-senescent and limits fibrosis	(Jun & Lau, 2010a) (Jun & Lau, 2010b)
Renal fibrosis	• Senescence is associated with the pathology and restricts fibrosis upon urethral obstruction • A CDK4 inhibitor is pro-senescent and favours kidney repair upon ischaemic injury	(Wolstein et al., 2010) (DiRocco et al., 2014)
Myocardial infarction /cardiac fibrosis	Senescence is associated with the pathology and restricts fibrosis	(Minamino et al., 2002)
Atherosclerosis	Senescence is associated with the pathology and restricts atherosclerotic plaque formation	(Minamino et al., 2002)
Obesity	Senescence contributes to systemic inflammation and insulin resistance	(Baker et al., 2008)
Type 2 diabetes	Senescence is associated with the pathology and contributes to the disease	(Sone & Kagawa, 2005)
Alzheimer's disease	Senescence is associated with the pathology	(Bhat et al., 2012)
Macular degeneration	Senescence is associated with the pathology	(D. Zhu, Wu, Spee, Ryan, & Hinton, 2009)
Glaucoma	Senescence is associated with the pathology	(Liton et al., 2005)
Cataracts	Senescence is associated with cataracts and aggravates the pathology	(Baker et al., 2008)

Diseases	Disease description and therapeutic strategies	Reference
Pulmonary hypertension	• Senescence is associated with hypertension and restricts the pathology • Nutlin 3a is pro-senescent and reverts hypertension	(Noureddine et al., 2011) (Mouraret et al., 2013)
Chronic obstructive pulmonary disease	Senescence is associated with the pathology	(Tsuji, Aoshiba, & Nagai, 2004)
Renal transplantation	Senescence is associated with mucositis and decreases transplantation success	(Braun et al., 2012)

Likewise, a mouse model of Hutchinson- Gilford progeria syndrome (HGPS), a childhood premature aging syndrome caused by aberrant lamin A processing, developed phenotypes that overlap with those of HGPS children and do not include cancer; cells from these mice showed chronic DDR signaling, chronic p53 activation, and cellular senescence [182]. Further, administration of drugs such as statins and aminobisphosphonates reduced DDR signaling in the cells, and also alleviated some of the progeroid symptoms in the mice. Finally, mouse models lacking activated DDR signaling also suggest that senescent cells can drive aging phenotypes. Mice that lack CHIP (carboxy terminus of Hsp70-interacting protein), a chaperone/ubiquitin ligase that helps eliminate damaged proteins rapidly accumulate senescent cells, and rapidly develop age related phenotypes, including thin skin and loss of adiposity and bone density [118].

Senescent cells have been associated with multiple pathological processes, in which senescence can have both beneficial and deleterious effects.

1.4.2 Beneficial effects of senescence in diseases

At the pathological level, senescence has been detected in the benign stage of tumorigenesis, which depending on the tissue type is known as adenoma, tumour *in situ*, intraepithelial neoplasia and others. Several studies have shown that cells that undergo damage-induced senescence can be removed by immune-mediated clearance. Furthermore, there is evidence for the therapeutic effect of pro-senescent therapies in malignant tumors. For example, acute activation of p53 in hepatocellular carcinomas and sarcomas induces senescence and is followed by tumor elimination. Also, pharmacologic

CDK4 inhibitors have shown to induce senescence in many cancer cells and are showing promise in human clinical trials.

1.4.2.1 Attenuation of liver fibrosis

Liver fibrosis is the main precursor of cirrhosis and is characterized by the accumulation of fibrotic tissue and the concomitant loss of liver function. It can be triggered by chronic liver damage associated with hepatitis virus infection, alcohol abuse or liver steatosis (fatty liver disease). In human patients, SA β-gal-positive cells accumulate in the periphery of the fibrotic scar68. In rodents, chronic treatment with carbon tetrachloride (a liver-damaging agent) or bile duct ligation produces liver fibrosis and is characterized by positive SA β-gal staining [91] Evidence for the beneficial role of senescence in restricting liver fibrosis is demonstrated in senescence-deficient mice, where, upon liver damage, mice lacking *Trp53* and/or *Cdkn2a* have fibrotic areas that are larger than those in senescence-competent mice.

1.4.2.2 Reduction of skin scarring and oral fibrosis

Skin fibrosis is part of the process of wound healing and is very similar to liver fibrosis. Mice lacking the matricellular protein CCN1 do not present induction of senescence in dermal fibroblasts and do not express pro-inflammatory cytokines and antifibrotic MMPs [79]. Also, *Cyr61*-deficient mice do not activate senescence in the dermal fibroblasts that participate in cutaneous healing leading to an exacerbated fibrosis.

1.4.2.3 Limitation of cardiac fibrosis upon infarction

Cellular senescence has been shown to regulate cardiac fibrosis after myocardial infarction [202]. Myocardial infarction promotes the accumulation of senescent myofibroblasts in the heart and the expression of key senescence regulators, especially p53 but also p16, p21 and ARF, which decrease collagen production and cardiac fibrosis. Loss of *Trp53* significantly attenuates cardiac fibroblast senescence, inflammation, macrophage infiltration and MMP production, and increases collagen deposition at the fibrotic scar presenting an aggravated cardiac fibrosis phenotype.

1.4.2.4 Protection against atherosclerosis

Cellular senescence has been increasingly linked to the development of vascular pathologies including atherosclerosis. Mouse models with deficiencies in ARF, p53; p21 or p27 all show increased susceptibility to developing atherosclerosis and mice with increased *Trp53* gene expression are protected from atherosclerosis [119].

1.4.2.5 Protection against pulmonary hypertension

Pulmonary hypertension induces senescence in pulmonary artery smooth muscle cells from patients with chronic obstructive pulmonary disease (COPD) [129]. Mice lacking *Trp53* or *Cdkn1a show* aggravated pulmonary hypertension, whereas p53 stimulation with nutlin 3a ameliorates the disease thus indicating that pro-senescent therapies, such as nutlin 3a, could provide therapeutic benefit to patients with pulmonary hypertension [123].

1.4.3 Detrimental effects of senescence in diseases

At first glance, it might seem contradictory that a tumor suppressive mechanism, which is clearly beneficial, can also have deleterious effects. However, recent studies have highlighted the deleterious effects of senescence and its contribution to the pathogenesis of several diseases.

1.4.3.1 Senescence aggravates pulmonary

Cellular senescence has been implicated in idiopathic pulmonary fibrosis (IPF), a chronic and ultimately fatal disorder that is characterized by a progressive loss of lung function. The detrimental role of senescence in lung fibrosis has been highlighted by studies using *Cav1*-null mice that show an impaired senescence and SASP response and ultimately partial protection from bleomycin induced idiopathic pulmonary fibrosis [156].

1.4.3.2 Adipocyte senescence is associated with obesity

Nutritional excess and/or low energy expenditure leads to excess storage of energy in adipose tissue that eventually reaches a threshold that triggers a stress response and the recruitment of macrophages. This inflammatory response of the adipose tissue initiates a cascade of events with systemic pathological consequences, including liver steatosis and insulin resistance, hallmarks of metabolic syndrome. Interestingly, the adipose tissue of obese subjects' presents evidence of senescence, including senescence associated β-

galactosidase activity, senescence associated secretory phenotype, and up regulation of p53 and p21, in both mouse models and human patients [11]. Furthermore, whole-body deletion of the *Trp53* gene prevents adipose tissue senescence, and selective deletion of *Trp53* in adipose tissue protects mice from insulin resistance induced by chronic high-fat diet [120].

1.4.3.3 Senescence contributes to Type 2 diabetes

Insulin resistance due to obesity and ageing is initially compensated through an overproduction of insulin by pancreatic β-cells and an expansion of these cells, but this chronic challenge eventually leads to proliferative exhaustion and loss of β-cell mass [158]. Genetic evidence has highlighted the deleterious effectos of senescence in Type 2 diabetes. 12 months of high-fat diet triggers senescence associated β-galactosidase activity in the atrophic mouse β-cell. Moreover, type 2 diabetes induced by specific genetic manipulations such as *Pttg1* (pituitary tumour-transforming gene 1; encoding securin)-null mice [189], *Lig4* (ligase 4, DNA, ATP-dependent)-null mice and *Trp53*-hypomorphic mice also correlates with senescence associated β-galactosidase activity in the islets [189].

1.5. Gadd45 Family of Genes

1.5.1 Introduction: The Gadd45 Family of Genes.

The Gadd45 family of genes includes three genes, Gadd45a, Gadd45b, and Gadd45g,

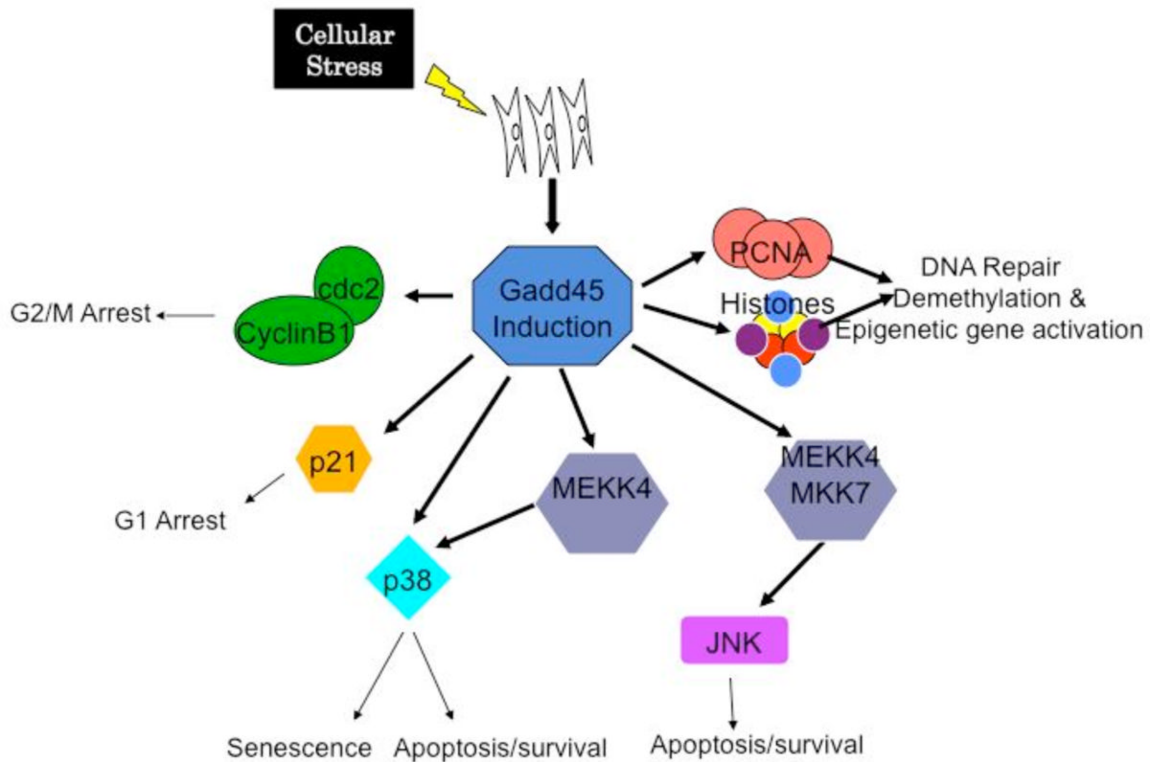

Figure 1.3 Gadd45 function in stress signaling.

Summary of the various functions of Gadd45 family members affecting cellular processes such as cell cycle arrest, DNA repair, survival, apoptosis, senescence as well as epigenetic gene activation. (Cretu, Sha, Tront, Hoffman, & Liebermann, 2009).

that encode for small (18 kDa), evolutionarily conserved proteins that are highly homologous to each other (55– 57% overall identity at the amino acid level), are highly acidic, and are localized within both the cell cytoplasm and nucleus [100, 200].

Gadd45a, originally termed gadd45, was cloned as one of many growth arrest and DNA damage-inducible (gadd) genes that are rapidly induced by UV radiation in Chinese hamster ovary (CHO) cells [198]. Gadd45b, originally termed MyD118, was cloned as one of many myeloid differentiation primary response (MyD) genes. Gadd45g, was cloned using an MyD118 (Gadd45b) cDNA probe and was subsequently found to encode for the murine homologue of human CR64, originally cloned as an immediate early response gene in T cells stimulated by interleukin-2[71]. Emerging evidence indicates that these proteins play pivotal roles as stress sensors and regulate the cellular response to a variety of environmental and physiological stressors [70, 72, 100] (TABLE 2)

As a protein with no known enzymatic activity, the GADD45 family of proteins exerts its function in response to diverse stress by interacting with distinct protein partners and modulating their activity to maintain cellular homeostasis.

1.5.2 Gadd45 as sensors of oncogenic stress

Recent studies have provided evidence, both *in vitro* and *in vivo*, for the critical role that Gadd45 proteins play as sensors of oncogenic stress. It is known that whereas primary mouse cells require introduction of two activated oncogenes for transformation, disruption of certain genes allows single oncogene transformation [111]. It was shown that, similar to certain key growth control genes, for MEFs obtained from *Gadd45a*$^{-/-}$ mice, H-

ras was sufficient for transformation [24, 73]. Evidence was also obtained that Gadd45 proteins play a role in modulating tumor development *in vivo*. *Gadd45a*$^{-/-}$ and *Gadd45b*$^{-/-}$ mice show increased mutation frequency, and susceptibility to ionizing radiation (IR) and chemical carcinogenesis [73].

Recent work has highlighted the role of Gadd45a as sensors of oncogenic stress in breast carcinogenesis [172-174]. Generation and analysis of MMTV-Myc versus MMTV-Ras mice strains that differ in their status of *Gadd45a*, have highlighted a unique role for Gadd45a as either a suppressor or promoter of breast cancer development by engaging with different signaling pathways, depending on the molecular nature of the activated oncogene. Gadd45a shows tumor suppressor function in response to Ras oncogenic stress by regulating the activation of JNK and p38 stress kinases. In contrast, Gadd45a shows tumor promotor function in response to Myc oncogenic stress by regulating MMP10 expression via the GSK3b / b-catenin signaling cascade.

1.5.3 Gadd45 dis-regulation in cancer

A growing body of evidence has shown that reduced expression of the three Gadd45 family members due to promoter methylation has been observed in several types of human cancers. The Gadd45a promoter is methylated in the majority of breast cancers, resulting in reduced expression when compared with normal breast epithelium [196]. In pituitary adenomas, silencing of the Gadd45g gene is seen in 67% of patients. This downregulation is primarily associated with methylation of the Gadd45g gene and

reversal of this epigenetic change results in re-expression of the protein [10]. Gadd45g is also down- regulated in anaplastic thyroid cancer and in 65% of hepatocellular carcinomas due to hypermethylation of the Gadd45g promoter [162]. Interestingly, the Gadd45b gene has also been shown to be methylated and silenced in hepatocellular carcinoma as well.

A recent study analyzed the methylation status of two regions in the Gadd45g promoter in a total of 75 cell lines as well as primary tissues and tumors [193]. They show that Gadd45g promoter hypermethylation is frequently detected in tumors cell lines, including 85% of non-Hodgkin, 50% of Hodgkin lymphoma, 73% of nasopharyngeal, 50% of cervical, 29% of esophageal, and 40% of lung carcinoma but not in immortalized normal epithelial cell lines, normal tissues, or peripheral blood mononuclear cells. Other observations showing that activated NF-ϰB leads to repression of GADD45a and GADD45g in various types of cancer [196] (Zerbini and Libermann, 2005) together with the frequent constitutive activation of NF-ϰB in cancers suggest that there are at least two different mechanisms whereby Gadd45 genes become repressed in cancer [40].

A recent study investigated the methylation-mediated repression of Gadd45a in prostate cancer. Interestingly, Gadd45a expression is elevated in several pancreatic ductal adenocarinoma cell lines, and loss of Gadd45a expression limits growth and survival of one cell line in culture. Also, ectopic expression of Gadd45a in the PANC1 pancreatic cancer cell line resulted in apoptosis and cell cycle arrest [150].

Analysis of clinical hepatocellular carcinoma samples showed that GADD45B expression is downreguated in 83.35% cases. In addition, GADD45A mRNA level is approximately 10 times lower in non-small cell lung carcinoma (NSCLC) compared to

normal lung tissues, and this low intratumoral GADD45A expression is significantly associated with a poorer histological grading.

A detailed study of Non-small-cell lung carcinoma (NSCLC) samples showed that the methylation frequencies of GADD45A, GADD45B, and GADD45G are 1.4%, 7.2%, and 31.6% respectively [66 164].

1.5.4 Gadd45 and cellular senescence

Evidence from the senescence-accelerated mice (SAMP1) model has revealed that GADD45B exhibits a higher expression in the aging articular cartilage of SAMP1 mice compared to that in control mice [164]. In normal human diploid fibroblasts undergoing replicative senescence, p53 preferentially occupies the promoters of growth arrest genes p21 and GADD45A. Furthermore, H2O2 stress induced senescence triggers an increase in GADD45A expression [100]. *Gadd45a* null MEFs do not undergo senescence in response to oncogenic H-ras [24]. Interestingly, in a mouse model of mammary

tumorigenesis, loss of Gadd45a in the presence of myc leads to increased senescence whereas loss of Gadd45a in the presence of Ras leads to decreased senescence [172, 173].

Furthermore, long-term activation of the checkpoint gene CDKN1A (p21) induces mitochondrial dysfunction and production of reactive oxygen species through serial signaling through GADD45A-p38MAPK-GRB2-TGFBR2-TGFbeta leading to the establishment of the senescent phenotype. [136].

Table 2. Phenotypic characteristics of Gadd45 knockout (Adapted (Moskalev et al., 2012))

Gadd45 member	Observed phenotype	Reference
Gadd45α$^{-/-}$	Genomic instability, abnormal nucleotide- and base-excision DNA repair, sensitivity to carcinogenesis Increased sensitivity to the induction of apoptosis	(Gupta, Gupta, Hoffman, & Liebermann, 2006; Hollander et al., 1999)
	Increased sensitivity to skin irradiation, Premature death, auto-immunity, Abnormal hematopoiesis Depletion of stem cell pool; decreased clonogenic potential of myeloid progenitors	(Gupta et al., 2005; Hildesheim et al., 2002; Hoffman & Liebermann, 2009)
Double KO: Gadd45α$^{-/-}$, XPC$^{-/-}$	Premature death and increased tumorigenesis	(Hollander et al., 2005)
Double KO: Gadd45α$^{-/-}$, Cdkn1a$^{-/-}$	Premature death, auto-immunity, abnormal hematopoiesis, impaired homeostasis, renal disorders	(Salvador et al., 2002)
Double KO: Gadd45α$^{-/-}$, Brca1$^{-/-}$	Exencephaly at embryonic days 9.5–10.5, complete prenatal lethality	(X. Wang et al., 2004)
Gadd45β$^{-/-}$	Impaired immunity and anti-tumor immune response; increased susceptibility to ionizing radiation and chemical carcinogens	(Ju et al., 2009)
	Autoimmune conditions	(Liu et al., 2005)
	Impaired ability for differentiation and increased sensitivity to the induction of apoptosis	(Gupta et al., 2006)
	Abnormal hematopoiesis	(Gupta et al., 2005)
	Depletion of stem cell pool; decreased clonogenic potential of myeloid progenitors	(Hoffman & Liebermann, 2007)

Gadd45 member	Observed phenotype	Reference
Gadd45$\gamma^{-/-}$	Impaired immunity Normal hematopoiesis, proliferative response to IL-2 Abnormal cell physiology	(Cai et al., 2006; Hoffmeyer, Piekorz, Moriggl, & Ihle, 2001)
Double KO:Gadd45$\beta^{-/-}$ Gadd45$\gamma^{-/-}$	Impaired immunity and anti-tumor immune response; Autoimmune conditions	(Ju et al., 2009; Liu et al., 2005)
Triple KO: Gadd45$\alpha^{-/-}$, Gadd45$\beta^{-/-}$,Gadd45 $\gamma^{-/-}$	Increased cell proliferation in the renal outer medullary cells	(Cai et al., 2006)

A recent study, using HCC Sk-Hep1, SMMC-7721, and Hep3B cells, demonstrated that ectopic GADD45G expression directly induces senescence. Notably, small interfere RNA (siRNA) mediated knockdown of GADD45G in Sk-Hep1 tumor cells attenuated MG132-induced senescence [164]. Furthermore, it was shown in breast cancer cells that the tumor suppressor function of GADD45G is mainly through the negative regulation of JAK/STAT3 signaling and induction of p53/p16/Rb-independent senescence. Restoration of STAT3 activity by either SHP2 inhibition or constitutive activation of STAT3 efficiently counteracts the GADD45G-induced senescence [164].

1.5.5 Gadd45b

Gadd45b (Gadd45β/MyD118) the 2nd member of a family of structurally and functionally related genes, that includes gadd45a and gadd45g, encode small (18kD), evolutionarily conserved proteins, sharing high homology (55-57%), high acidity (pI 4.0-4.2), and which are primarily localized to the nucleus. Gadd45b was first identified in 1991 as a primary response gene in the myeloid differentiation program, when leukemic myeloblasts (M1) were induced to differentiate with IL-6 [70].

As a protein with no known enzymatic activity, GADD45B exerts its function in response to diverse stressors by interacting with protein partners to modulate their activity and maintain cellular homeostasis. Specifically, GADD45B-protein partner interactions can influence cell cycle arrest at the G1/S and G2/M checkpoints, DNA repair, and paradoxically, both apoptosis and survival, depending on the specific cell type and/or context.

A growing number of studies have highlighted the critical role that Gadd45b plays

in regulating diverse cellular functions. In hematopoietic cells, GADD45B promotes survival following UV treatment by activating NF-κB signaling and blocking MKK4 activity to inhibit JNK- and p38-mediated apoptosis [62]. In stress- or TNFα- mediated apoptosis signaling, NF-κB blocks JNK induced apoptosis through GADD45B-mediated inactivation of MKK7/JNKK2 [132]. In B-lymphocytes, GADD45B mediates the protective effects of CD40 co-stimulation against FAS-induced apoptosis [195]. Most strikingly, endogenous Gadd45b over expression is associated with tumorigenesis and stress-resistant tumors in mice [52].

Molecular profiling studies on 14 gonadotrope tumors identified Gadd45b as a novel tumor suppressor in pituitary gonadotrope tumors. The authors also showed that overexpression of GADD45β in LβT2 mouse gonadotrope cells blocked tumor cell proliferation and increased apoptosis in response to growth factor withdrawal [116].

Pregnane X receptor (PXR), a transcription factor that induces hepatic drug metabolism by activating cytochrome P450 genes, activates Gadd45b, increasing p38 MAPK phosphorylation, and leading liver hepatocellular carcinoma (HepG2) cells to change morphology and migrate [87]. Induction of DNA damage-inducible gene GADD45B contributes to sorafenib-induced apoptosis in hepatocellular carcinoma cells [130]. Gadd45b mediates Fas-induced apoptosis by enhancing the interaction between p38 and retinoblastoma tumor suppressor [34]. Gadd45beta is induced through a constitutive androstane receptor (CAR)-dependent, TNF-independent pathway in murine liver hyperplasia [38]. Gadd45β is an inducible coactivator of transcription and facilitates rapid expansion of liver mass for protection against xenobiotic insults such as TCPOBOP (1,4-bis[2-(3,5)-dichoropyridyloxy] benzene) [168].

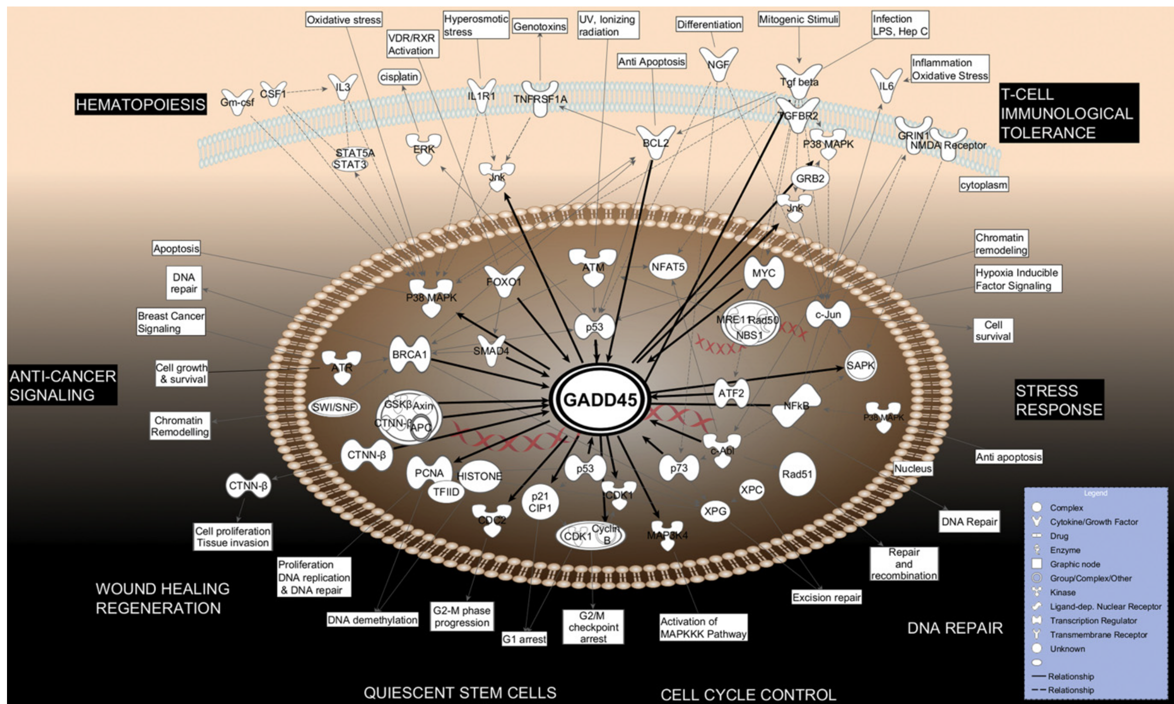

Figure 1.4 Map of molecular interactions involving GADD45 proteins and cell signaling pathways generated using Ingenuity Pathway Analysis software (IPA).

Top functions identified by IPA were Cell Death, Cell Cycle and Cell Growth and Proliferation. Each of the node shapes denotes the function of the interacting protein or group of proteins. Molecular interactions are indicated by the lines between nodes (Moskalev et al., 2012)

Gadd45b promotes hepatocyte survival during liver regeneration after partial hepatectomy in mice by modulating JNK signaling [134]. A recent study identified the interaction between the NF-κB-regulated antiapoptotic factor GADD45β and the JNK kinase MKK7 as a potential therapeutic target in multiple myeloma (MM). DTP3, a D-tripeptide, which disrupts the GADD45β/MKK7 complex, kills MM cells effectively, and, importantly, lacks toxicity to normal cells [171].

1.6. Gadd45, Senescence and Aging in the skin

1.6.1 Stress response and Aging

One of the hallmarks of aging is the decreased ability to cope with stress. Resistance to stress is a critical determinant of survival and longevity as evidenced in a wide variety of species, where individuals with longer life spans usually show a higher resistance to physiological and/or environmental stress [157].

A recent study used the ability to resist oxidative stress as a major criterion for identifying long-lived worm mutants in a genome-wide scale RNAi screen for new longevity regulators [84]. Mutations that contribute to a longer life span in yeast (Saccharomyces cerevisiae) and mice (Mus musculus) have been shown to accompany increased resistance to starvation, oxidative stress, and heat shock [139].

Importantly, overexpression of stress-related proteins (e.g., sirtuins, FOXO, HSP70, HSP22, superoxide dismutase, catalase) in certain tissues has been shown to result in increased life span. Taking advantage of the link between stress and longevity

many studies have shown that moderate exposures to harmful factors (or hormetic effects) such as thermal or oxidative stress, ionizing radiation, and hypergravity can stimulate protective mechanisms and eventually lead to increased longevity, a phenomenon called "longevity hormesis"[148].

The Gadd45 proteins, given their well-established role in regulating the cellular response to a wide variety of stressors ([100] will be ideal candidate proteins that regulate aging and longevity. At basal conditions, the Gadd45 family members have relatively low expression, but are rapidly and highly inducible in response to a wide plethora of stressful stimuli, both physiological and environmental.

Interestingly, the unusually short median half-life of the Gadd45 mRNA (less than 1 h) suggests a regulatory rather than metabolic function for Gadd45 proteins [122]. Expression of Gadd45a is stimulated by physical stress such as UVradiation, X-rays, g irradiation and oncogenic stress, low pH, cisplatinum, alkylating agent methyl methane sulfonate, ethanol, cigarette smoke condensate [71, 100, 164].

1.6.2 Gadd45 in Aging

The Gadd45 proteins have been implicated in several cellular functions that are intimately linked to aging and age-related diseases (ARDs), including DNA repair, maintaining genome stability, epigenetic regulation, cell cycle arrest, cellular senescence, apoptosis, inflammatory responses and immunity [100, 164]. Recently, it was shown that overexpression of the dGadd45 gene in the nervous system of Drosophila leads to the

extension of the maximum life span, without compromising such life quality factors as physical activity and female fertility [140]. Given that longevity-associated genes play a critical role in major age-related diseases and aging-associated conditions, studying the Gadd45 family of proteins may provide potential therapeutic targets for combating age-related diseases and promoting longevity [122].

1.6.2.1 Gadd45 in age-related pathologies: Cancer

Aging is a major risk factor for cancer. It has been shown that the incidence of cancer of the breast, prostate, colon, lung, stomach, bladder and skin dramatically increases with age [69]. Also, Aging-associated conditions such as oxidative stress, chronic inflammation and immunosenescence have been shown to predispose to tumorigenesis.

The anti-cancer activity of Gadd45a is well established by studies utilizing mouse knockout models. Mice with a *Gadd45a* gene deletion show genomic instability and increased sensitivity to carcinogenesis [73, 172]. *Gadd45a* knockout mice are also more prone to DMBA-induced ovarian tumors, hepatocellular tumors in males, and vascular tumors in both sexes. The authors also showed that the increased rate of tumorigenesis in these mice could be attributed to a very low efficiency of the thymidine dimer and nucleotide excision repair [74]. Mice with the *Gadd45b* gene knockout have been shown to be more susceptible to ionizing radiation and chemical carcinogens and display a lower immune response against implanted melanoma cells. Interestingly, in the *in vivo* model of Ras-overexpressing mice that differ in the levels of *Gadd45a* expression, it was shown

that Ras driven breast tumorigenesis is Gadd45a-dependent. The authors concluded that Gadd45a inhibits the onset and growth of mammary tumors via induction of JNK-activated apoptosis and p38-mediated cellular senescence[172, 173]. Importantly clinical observations have shown that Gadd45a is frequently deleted in breast cancer. Furthermore, disruption in Gadd45a expression was observed in multiple types of solid and hematopoietic malignancies including nasopharyngeal, breast, lung and prostate cancer, hepatocellular tumors, pituitary adenoma, and lymphoma [100, 122, 164].

The silencing of Gadd45 expression in cancers could be attributed to the epigenetic changes that typically occur with advanced age. In particular, promoters of many tumor suppressors were found to be hypermethylated in aging, with subsequent transcriptional gene silencing. Thus, this common epigenetic modification could be an important link between cancer and aging. It is therefore interesting to note that the methylation of the Gadd45a promoter was found to be significantly higher in different types of cancer than in normal tissues [201].

1.6.2.2 Gadd45 in age-related pathologies: Other age-related diseases

Gadd45a has been shown to be highly expressed in neurons of Alzheimer's disease patients and protect the neurons from apoptosis induced by extracellular accumulation of B-amyloid [122]. It was also shown that in human neuroblastoma cells, Gadd45a is up regulated in response to dopamine-induced neurotoxicity. Given the role of ROS and reactive quinones, produced during dopamine oxidation, in the pathogenesis of both Parkinson's disease and normal brain aging, an increased expression of Gadd45a in age-

related neuro-degeneration is likely a protective mechanism aimed at coping with the neurotoxic stress.

It was also shown in primary cultures of human endothelial cells derived from atherosclerotic aorta or coronary arteries, that activation of the lectin-like ox LDL receptor (LOX-1) leads to DNA damage and a 4-fold increase in Gadd45a expression. A comparable increase in Gadd45a transcription in aortic endothelial cells was also observed in a mouse model of atherosclerosis [167].

Immunosenescence, a phenomenon characterized by defective T cells is shown to occur in response to oxidative stress and chronic inflammation. Immunosenescence manifests in a decreased immune responsiveness to foreign and self-antigens, leading to an increased susceptibility to infection, cancer and autoimmune diseases. Mice with deficiency in *Gadd45b* and Gadd45g spontaneously develop signs of autoimmune lympho-proliferative syndrome and systemic lupus erythematosus. Given the key role that Gadd45b and Gadd45g play in maintaining immune tolerance, changes in the expression of Gadd45 proteins in immune cells could be a potential basis for the increased frequency of autoimmune conditions in aging [106].

Gadd45b has been shown to play a critical role in rheumatoid arthritis, whose incidence increases with advanced age [105]. Rheumatoid arthritis (RA) is characterized by persistent Th1 cell infiltration (resistance to apoptosis) and production of inflammatory cytokines in the location of joint lesion. A recent study reported that Th1 cells accumulated in patient synovial fluid showed high Gadd45b expression which further inhibited Th1 cell apoptosis. Furthermore *in vitro* culture of T cells with synovial fluid from Rheumatoid arthritis patients increased GADD45β expression in Th1 cells and

inhibited their apoptosis, while silencing of GADD45β by RNAi abolished the anti-apoptotic effects [122].

1.6.2.3 Gadd45 in age-related pathologies: Gadd45 and longevity

The potential role for Gadd45 proteins in regulating longevity was highlighted by a recent study that showed that Gadd45a is among the targets of a number of established longevity associated genes (LAGs) including SIRT1 and FOXO. Depletion of SIRT1 expression by RNA interference results in a marked inhibition of FOXO-mediated Gadd45a induction in response to oxidative stress [86].

It was also shown that in fruit flies, using a transgene model overexpressing dGadd45 in the nervous system, the constitutive or conditional overexpression of dGadd45 in the nervous system increases median and maximum Drosophila life span, without affecting fertility or physical activity. Importantly, the dGadd45-induced life span extension was accompanied by a considerable decrease (by approximately 25%) in the number of single-stranded breaks in DNA of Drosophila larvae neuroblast, as compared to their wildtype counterparts. Also, in wild-type fruit flies (Canton-S line), dGadd45 undergoes dramatic downregulation during the second half of life. Altogether, these finding suggests that the longevity-promoting effects of Gadd45a overexpression could be causatively related to a more efficient detection and correction of spontaneous DNA damage[140].

1.6.3 Cellularity as an Aging phenotype in Skin

Several studies have characterized the various aging phenotypes commonly seen in the skin. It is well established that naturally aged human skin had significantly reduced dermal cellularity and increased connective tissue abnormalities that can be partially rescued by treatment with retinol (vitamin A) for 7 d [181]. Furthermore, dermal thickness decreases with age thinning and is accompanied by a decrease in both vascularity and cellularity [53, 181]. Additionally, the general characteristics that are associated with skin aging include decreased cellularity, decreased elastic fibers, atrophy of the epidermis and decrease in the number of Langerhans cells. Interestingly, aged wild-type mice displayed reduced dermal cellularity and other aging phenotypes that were not seen in 11β-hydroxysteroid dehydrogenase type 1-null mice [169].

1.6.4 g-H2AX

One of the early events following Double strand breaks is the phosphorylation of serine 139 of histone H2AX (within minutes) to create g-H2AX which functions to recruit DNA damage response factors to sites of DNA damage [151]. Using a fluorescent antibody specific for the g-H2AX, discrete nuclear foci can be visualized at sites of DSBs. The phosphorylation is mediated by Ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR), and/or DNA dependent protein kinase (DNA-PK), and occurs (in a domain of about one mega base) at the break sites of Double strand breaks or single strand break [151]. For many years, the analysis of double strand breaks repair relied on pulsed-field gel electrophoresis (PFGE), which required high irradiation doses (50Gy). Recently, the g-H2AX foci have been used as an extraordinarily

sensitive technique to detect double strand break formation and repair even after very low dose irradiation [145]. DNA repair- and checkpoint associated proteins such as RAD50, RAD51 and BRCA1, as well as the 53BP1, co localize with g-H2AX. g-H2AX extends to mega base regions of DNA around the lesions and can be visualized by using immuno-fluororescence microscopy as discrete nuclear foci at the site of double strand breaks, either induced by exogenous agents such as radiation or generated endogenously during programmed DNA rearrangements [145].

Recent studies found that ATM and DNA-PK function redundantly to phosphorylate H2AX in response to double strand breaks. This overlapping function is observed in human, mouse, and vertebrate cells. ATM and DNA-PK both contribute to H2AX phosphorylation in response to double strand breaks. The precise biological function of the foci is still unclear but H2AX phosphorylation is required for the retention of several damage response proteins at the break site [54]. Initial studies observed a close correlation between the number of foci and the number of double strand breaks produced by decay of 125I incorporated into cellular DNA, suggesting that each focus may represent an individual DSB and that each break may form a focus. A recent study found that the correlation is close to a 1:1 ratio; strongly suggesting that the number of foci formed after ionizing radiation at low doses is similar to the predicted number of double strand breaks induction [145].

While the phosphorylation of H2AX occurs rapidly, additional recruitment and retention of g-H2AX at the site of DNA double strand break is dependent on another DNA damage-response protein: mediator of DNA damage checkpoint protein one (MDC1). The C-terminal tandem BRCT domain of MDC1 binds the C terminus of g-

H2AX, and without MDC1 the kinetics of formation and removal of g-H2AX is altered. Thus, g-H2AX serves as a recruitment factor for the assembly of multiple factors that collectively act to enhance the efficiency of DNA repair [161].

Immuno-histochemical analysis of g-H2Ax has been commonly used as a marker for DNA damage and double strand breaks in skin. Immuno-histochemical analysis of g-H2Ax was used to detect DNA damage response induced by UVB irradiation in mouse skin and naturally aged skin [199]. Furthermore, immuno-histochemical analysis of g-H2Ax was used to detect DNA damage response caused by SOD2 deficiency in mouse skin [184] and in mice with chronic activation of p53 (p53TSD/_ mice) .

1.6.5 Senescence and Aging in Skin

In 1995, Dimri and colleagues identified a novel biomarker for senescence - a beta galactosidase, histochemically detectable at pH 6. The group showed that in skin samples from human donors of different age, there was an age-dependent increase in senescence associated β-galactosidase activity in dermal fibroblasts and epidermal keratinocytes thus providing *in situ* evidence senescent cells may exist and accumulate with age *in vivo.*

To explore the idea that senescent cells accumulate *in vivo*, skin samples from 20 human donors, aged 20-90 yr, were sectioned and stained for senescence associated β-galactosidase. While none of the young donors (<40 yr) showed dermal staining, by contrast, all but one old (>69 yr) donor had positive dermal staining indicating that senescence staining in the dermis and epidermis increased with age in frequency and intensity [49].

It has been shown that mice lacking Mdm2 (the chief negative regulator of p53) in the epidermis showed an aging phenotype in the skin of mice, including increased cellular senescence, thinning of the epidermis, reduced wound healing, and a progressive loss of fur. The Mdm2Δ/Δ mice showed a premature aging phenotype in the skin that is highly similar to the accelerated aging phenotype that was observed previously in the skin of p53 m-allele mice, including a thinning of the epidermal layer, reduced wound healing, and a reduced capacity to re-grow fur. Given that the deletion of Mdm2 results in an increase in p53 levels and upregulation of select p53 target gene expression, these results corroborate that increased p53 activity leads to accelerated aging phenotypes observed previously in this model and in certain other p53 models [58].

It was also shown that the number of senescent cells, as well as impaired mitochondrial (complex II) activity increase in naturally aged mouse skin. They also showed, using a mouse model of genetic Sod2 deficiency, that loss of this important mitochondrial anti‑oxidant enzyme impairs mitochondrial complex II activity, causes nuclear DNA damage, and induces cellular senescence in the skin. They also showed that Sod2 deficiency resulted in reduced cellularity and thickness of the epidermis, thus indicating that mitochondrial oxidative stress and cellular senescence contribute to aging skin phenotypes *in vivo* [184].

In 2012, Stoyanova and colleagues showed that loss of p53-induced genes DDB2 and p21 resulted in a severe Deficiency in UV-induced Premature Senescence. Senescence associated β-galactosidase staining of skin sections from mice irradiated with UV-B showed that while both p21-/- and Ddb2-/- mice exhibited deficiencies in

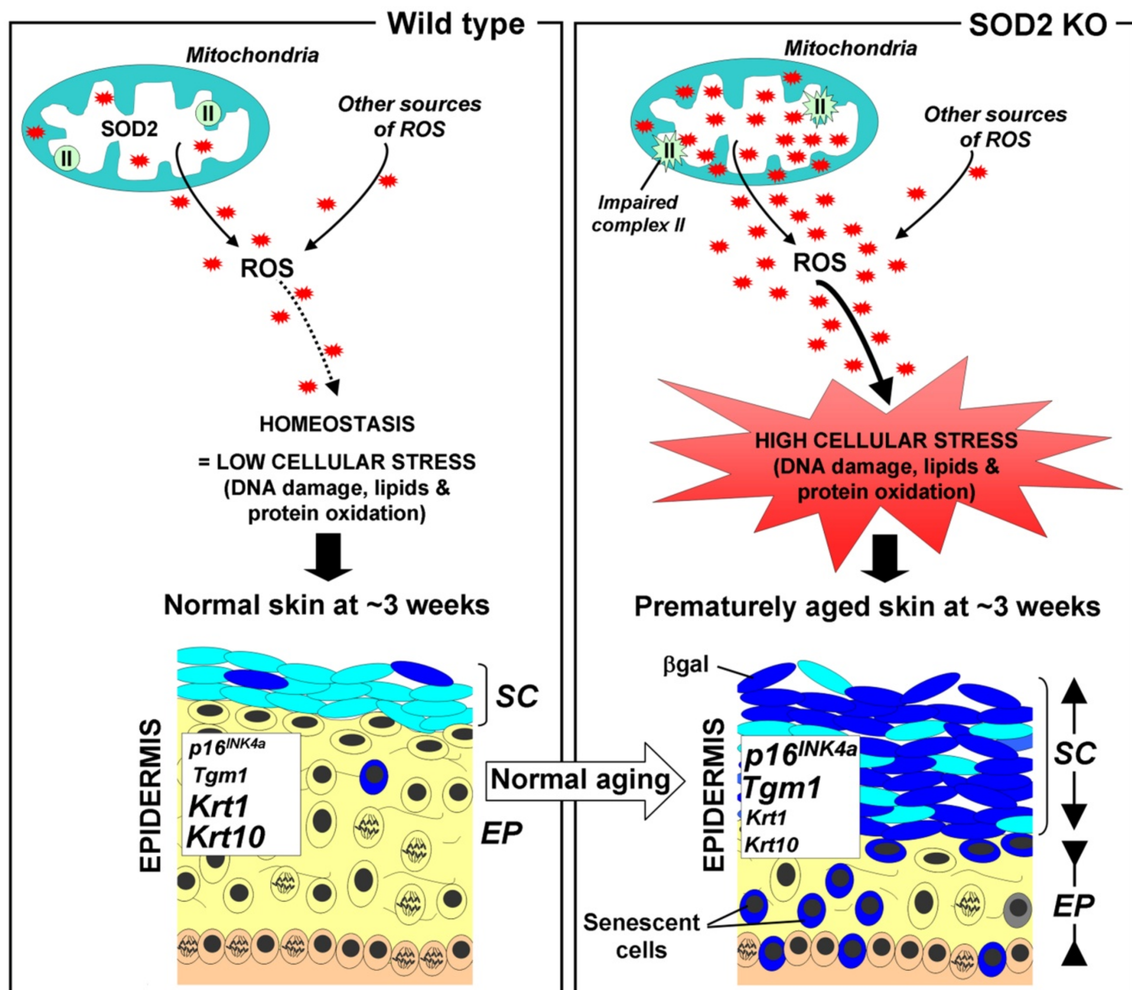

Figure 1.5 Scheme illustrating the putative role of SOD2 in skin aging.

(Left panel) In normal mice, SOD2 scavenges superoxide anions O2-, resulting in normal levels of reactive oxygen species (ROS, red stars). This homeostasis leads to minimal cellular stress (little damage to DNA, lipids and proteins), resulting in normal skin, characterized by a thin stratum corneum (SC) and a thick epidermis (EP). (Right panel) In contrast, SOD2 deficiency leads to elevated levels of ROS and oxidative stress (increased damage to DNA, proteins and lipids) as well as impaired mitochondrial (complex II) activity. This results in premature aging, characterized by increased thickness of the stratum corneum (SC) and thinning of the epidermis (EP). (Weyemi, Parekh, Redon, & Bonner, 2012).

senescence response in comparison with WT mice after UV damage, Interestingly, the Ddb2-/- p21-/- mice exhibited a more severe deficiency in senescence response compared with the Ddb2-/- or the p21-/- mice [160].

Studies using a transgenic mice model revealed that inducibly expressing p14ARF (human ortholog of murine p19ARF) led to the activation of endogenous Cdkn2a products p19ARF and p16Ink4a revealing a senescence promoting feed-forward loop in the skin. They also showed that p14ARF-induced epidermal cell senescence is dependent on p53. The authors hypothesized that, once initiated; the senescence program could be executed independently of the inducing signal. To test whether this is the case, they activated p14ARF for 4 days and then resilenced the transgene by doxycycline removal for another 3 days. While SA β-gal-positive cells appeared in mice in which the transgene was continuously expressed for 7 days, senescent cells were not observed in mice in which the transgene was silenced indicating that cells do not commit to senescence until at least 5 days of continuous high p14ARF expression levels, despite activating p53, p16Ink4a, and p19ARF, (i.e., persistent stress leading to continuous p14/19ARF expression is necessary for cells to commit to senescence) and are able to revert to a non-senescent state or be eliminated from the tissue [170].

1.6.6 Gadd45 in Skin

GADD45A is expressed in cultured skin keratinocytes and in human skin *in vivo* following UV irradiation. Interestingly, UV-induced GADD45a expression was inhibited by diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, and antioxidant, N-

acetyl-L-cysteine (NAC), indicating the involvement of reactive oxygen species in UV signaling. They also showed that PD168393, a potent EGF receptor inhibitor, blocked UV-induced GADD45a expression indicating that UV-induced GADD45a expression occurs via an EGF receptor-mediated oxidative pathway sensitive to antioxidant regulation [187].

Gadd45a is also a critical factor protecting the epidermis against UV radiation-induced tumorigenesis by promoting damaged keratinocytes to undergo apoptosis and/or cell cycle arrest. Using skin of *Gadd45a*-null mice targeted with UV radiation, the authors showed that the absence of *Gadd45a* results in loss of sustained p38/JNK MAPK activity beyond 15–30 min after UV radiation leading to inadequate p53 activation and loss of normal activation of G_1 and G_2 checkpoints. They also showed that *Gadd45a*-null mice are more prone to tumors and possess reduced number of UV-induced apoptotic keratinocytes [67].

It has also been reported that GADD45A promotes G2/M arrest via nuclear export and kinase activity of Cdc2, increases global genomic DNA repair, and inhibits cell death in keratinocytes exposed to ultraviolet B radiation. They showed that Gadd45a-deficient cells did not have a sustained G2M arrest, did not show Cdc2 sequestration in the cytoplasm and had a slower rate of nucleotide excision repair [113]. The authors continued their studies on *Gadd45a* in skin by using transgene mouse model deficient in *Gadd45a* and p21. They showed that while *Gadd45a*-deficient keratinocytes were defective in UV-induced NER, interestingly, *Gadd45a*/p21-null keratinocytes had normal NER in response to UV and had keratinocytes that were more resistant to UV-induced cell death

than Gadd45-deficient keratinocytes. These results support the hypothesis that Gadd45A enhances NER by negatively regulating basal p21 expression in keratinocytes.

Interestingly immune-histochemical analysis of Atypical fibroxanthoma (AFX), superficial leiomyosarcomas (S-LMS), and benign fibrous histiocytomas (BFH) skin samples revealed that Gadd45A is expressed in skin samples from these skin conditions [147].

1.7. Gadd45, Senescence and Liver Fibrosis

1.7.1. Gadd45 in Liver

Several studies have been published elucidating the role of Gadd45 proteins in liver. Gadd45b shows the strongest immediate-early induction common to two distinctive proliferation responses of the liver: (1) regeneration induced by surgical partial hepatectomy and (2) hyperplasia induced by the primary mitogen TCPOBOP, a ligand of the constitutive androstane receptor (CAR). Furthermore, Gadd45b Is induced through a CAR Dependent, TNF-Independent Pathway in murine liver hyperplasia [38, 168]. *Gadd45b* deficiency in mice led to decreased hepatocyte proliferation and increased program cell death during liver regeneration after partial hepatectomy. Notably, c-Jun N-terminal kinase (JNK) activity was markedly increased and sustained in livers of *Gadd45b*$^{-/-}$ mice compared with control animals after partial hepatectomy. Furthermore, loss of JNK2, by imposition of a Jnk2-null mutation, attenuated JNK activity, completely rescuing the regenerative response in *Gadd45b*$^{-/-}$ mice [134]. It was also shown that in murine hepatocyte

AML12 cells, Gadd45b mediates p38- induced Rb phosphorylation by enhancing the interaction between p38 and Rb during Fas-induced apoptosis.

Gadd45b$^{-/-}$ mice have also been shown to have intact proliferative responses, but marked growth delays following administration of a single dose of TCPOBOP. Moreover, early transcriptional stimulation of CAR target genes was weaker in *Gadd45b*$^{-/-}$ mice than in wild-type animals, and more genes were downregulated. Gadd45b was also found to directly regulate transcription by physically binding to CAR. Also, TCPOBOP treatment led to localization of both proteins to a regulatory element for the CAR target gene cytochrome P450 2b10 (Cyp2b10)[168].

Furthermore, Gadd45b induction facilitates increased transcription, allowing rapid expansion of liver mass for protection against xenobiotic insults such as TCPOBOP (1,4-bis[2-(3,5)-dichoropyridyloxy] benzene), a ligand of the nuclear receptor constitutive androstane receptor (CAR). A recent study showed that PPARα indirectly induces Gadd45b gene expession in liver through promoting degradation of the repressor STAT3 in response to elevated oxidative stress. Gadd45b mRNA was markedly induced by the PPARα agonist, Wy-14,643, in wild-type mice but not in Ppara-null mice. Also studies using liver-specific Stat3-null mice showed that STAT3 is a repressor of the Gadd45b gene through binding to upstream regulatory elements [81] .

1.7.2 Senescence in CCl4 induced liver fibrosis

More than five decades ago Leonard Hayflick and Paul Moorhead showed that normal human fibroblasts have a finite proliferative capacity in culture, a phenomenon that they named 'cellular senescence'. This property of normal cells was shown to be in contrast to cancer cells, which possess an indefinite capacity to proliferate. Findings in experimental mouse cancer models and with human tumor samples showed that precancerous tissues are composed of senescent cells leading to the notion that induction of cellular senescence provides an intrinsic barrier to cancer development and prevents the proliferation of cells that are damaged or at risk for neoplastic transformation [27, 29, 36, 138]. In 2008, Krizhanovsky et al. added a new and interesting dimension to the cellular senescence story by demonstrating that senescence is also required in the normal response to injury in the liver [91].

Fibrosis is a wound healing process that is characterized by the deposition of extracellular matrix components including collagens, leading to encapsulation of the injury site. Liver fibrosis, a pathological feature that is a precursor of cirrhosis, is characterized by the accumulation of fibrotic tissue and the concomitant loss of liver function. It can be triggered by chronic liver damage associated with hepatitis virus infection, alcohol abuse or liver steatosis (fatty liver disease). It has been shown that during chronic damage, hepatic stellate cells (HSCs) become activated and abnormally proliferate as myofibroblasts (damage-activated fibroblasts). Recent studies have shown that myofibroblasts become senescent and produce a stable fibrotic scar with abundant collagen and other extracellular matrix components. In human patients, SA β-gal-positive

cells accumulate in the periphery of the fibrotic scar [191]. In rodents, chronic treatment with carbon tetrachloride (a liver-damaging agent) or bile duct ligation produces liver fibrosis, which is characterized by positive SA β-gal-positive cells that are derived from activated HSCs and show increased p53, p21 and p16 and other senescence markers.

Senescence deficient mice have demonstrated the beneficial role of senescence in restricting liver fibrosis. In response to liver damage, mice lacking Trp53 and/or Cdkn2a present senescence negative fibrotic areas that are larger than those in senescence-competent mice. Similarly, the extracellular matrix protein CCN1 (also known as CYR61; a member of the CCN family) produced by damaged hepatocytes has been shown to be a key mediator of senescence induction in HSCs. Accordingly, mice with CCN1 deficient hepatocytes do not execute HSC senescence leading to an exacerbated fibrotic response [82]. Also, production of IL-22 by activated HSCs induces HSC senescence in association with p53 activation through STAT3 (signal transducer and activator of transcription 3)–SOCS3 (suppressor of cytokine signaling 3). Accordingly, transgenic mice over expressing IL-22 in the liver show a more efficient and faster resolution of fibrosis. These studies demonstrate that the induction of HSC senescence could be a potential therapeutic strategy to limit liver fibrosis. Recent studies have shown that treatment with recombinant protein CCN1 or IL-22 reverts already established hepatic fibrosis by promoting HSC senescence [89].

On the other hand, there are genes that limit senescence, and mice lacking these genes have been shown to present increased senescence and reduced fibrotic scars in response to liver toxins. The toll-like receptor-9 (TLR9) agonist cytosine phosphate

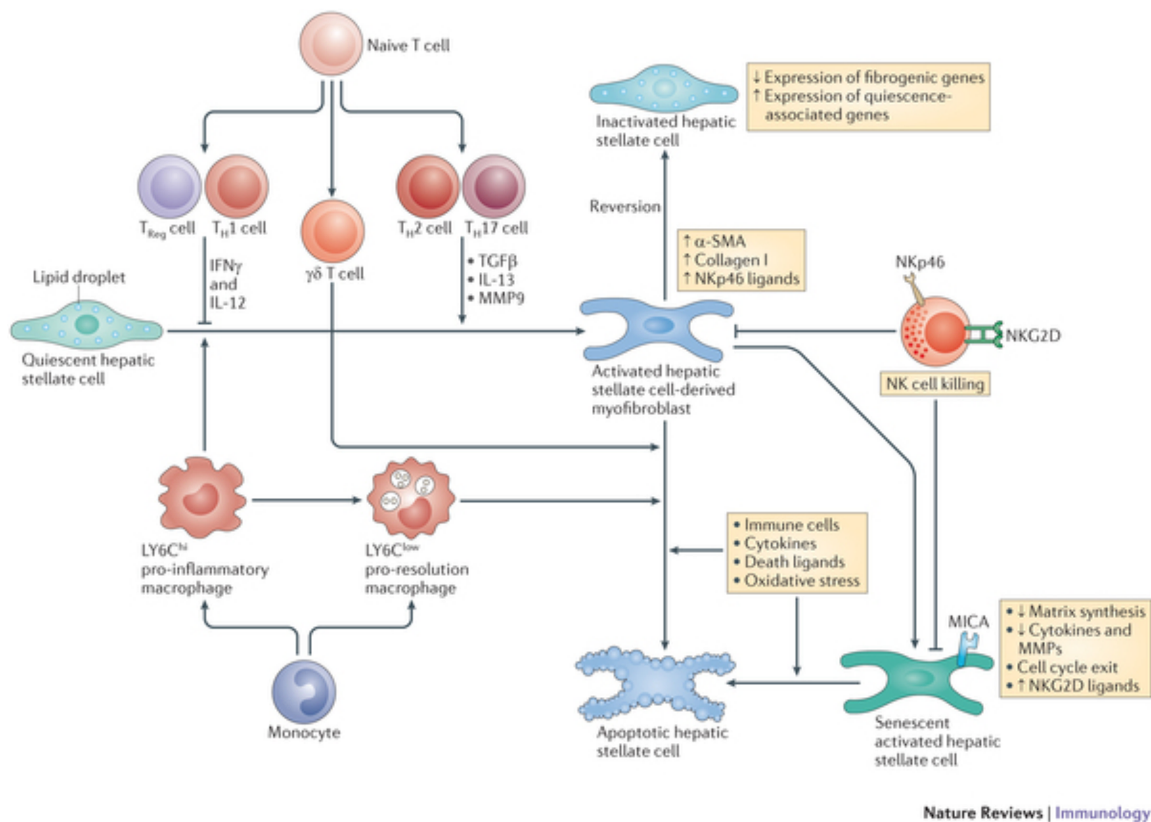

Figure 1.6 Hepatic stellate cell-derived myofibroblast fate in liver fibrosis.

Liver injury induces the production of pro-inflammatory cytokines, growth factors and reactive oxygen species, which leads to the transdifferentiation of quiescent hepatic stellate cells into myofibroblast-like cells, which are characterized by a loss of lipid droplets and an upregulation of α-smooth muscle actin (αSMA) and collagen I expression. Some hepatic stellate cell-derived myofibroblasts become senescent — a phenotype that is associated with decreased matrix and cytokine synthesis, with decreased matrix metalloproteinase (MMP) secretion and with gene expression profiles that is consistent with cell cycle exit and are subsequently deleted by natural killer (NK) cells (Pellicoro et al., 2014)

guanine (CpG) has been shown to activate hepatic stellate cells (HSCs) and mediate fibrosis. In TLR9$^{-/-}$ mice, HSCs become senescent shortly following activation leading to a decrease in the activated HSCs population and extracellular matrix deposition in fibrotic scars [2].

Loss of Smad3, a key mediator of the transforming growth factor (TGF)-β1 signaling pathway that plays central role in inflammation and fibrosis, led to reduced CCl4-induced liver fibrosis. Proteomic analysis revealed that proteins related to antioxidant activities such as senescence marker protein-30 (*SMP30*), selenium-binding proteins (SP56) and glutathione *S*-transferases (GSTs) were up-regulated in *Smad3*$^{-/-}$ mice indicating that that Smad3 deteriorate hepatic injury by inhibiting antioxidant proteins as well as mediators of TGF-β1 signaling. Hepatocyte-specific ablation of PP2Acα (PP2A catalytic subunit Cα) protects against CCl4-induced chronic hepatic injury and fibrogenesis through impaired TGF-β1/Smad signaling.[78, 109].

1.8. Objectives and rationale

Several studies have been carried out to investigate the role of Gadd45a in senescence. *Gadd45a* null MEFs do not undergo senescence in response to oncogenic H-ras [24]. Furthermore, long-term activation of the checkpoint gene CDKN1A (p21) induces mitochondrial dysfunction and production of reactive oxygen species through serial signaling through GADD45A-p38MAPK-GRB2-TGFBR2-TGFbeta leading to the establishment of the senescent phenotype. [136]. Interestingly, in a mouse model of mammary tumorigenesis, loss of Gadd45a in the presence of myc leads to increased senescence whereas loss of Gadd45a in the presence of Ras leads to decreased

senescence [172, 173]. Although Gadd45a has been shown to play a significant role in regulating cellular senescence to stress, the role of Gadd45b has not been studied. Given the similarities and diversity among the Gadd45 family of genes, it is of interest, therefore, to investigate the role of Gadd45b in senescence.

Our hypothesis was that Gadd45b regulates the cellular response to oxidative stress signals, ultimately resulting in a senescence phenotype. To test this hypothesis, we focused on the established tissue culture induced senescence model - 3T3 growth protocol using mouse embryonic fibroblasts that differed in their *Gadd45b* status. Furthermore, to test the role of *Gadd45b* in regulating senescence *in vivo,* we analyzed the effect of loss of *Gadd45b* on the development of aging phenotypes in the mouse skin as well as its effect on CCl4 induced liver fibrosis.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

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2.1 Animals

Mice were maintained in a temperature and humidity-controlled environment at Temple University's Health Science campus animal facilities following the guidelines of Institutional Animal Care and Use Committee (IACUC) of Temple University. *Gadd45b*$^{-/-}$ mice were maintained in a mixed (BL6/129VJ) genetic background and propagated using heterozygous breeding pairs. Mating between *Gadd45b* heterozygous mice gave the expected frequency of *Gadd45b* null (*Gadd45b*$^{-/-}$) mice and their wt (*Gadd45b*$^{+/+}$) littermates that were used as age-matched controls for all indicated experiments. Mice genotype was confirmed by PCR using specific primer sets for *Gadd45b* and the neomycin phosphotransferase reporter gene each time the litters were produced. At the time of weaning, a small piece of tail was cut from each animal that was used to isolate genomic DNA by standard procedures for PCR analysis. In order to determine the *Gadd45b* status, PCR reactions using three primers were allowed for simultaneous detection of the wildtype and mutant *Gadd45b* allele. A wt specific upstream primer (50 GCTGTGGAGCCAGGAGCAGCA 30) was located in the fourth exon, 50 prime to the SacII site, in the region ofthe *Gadd45b* gene that was replaced by the neo cassette. The neo, or null, specific primer (50 AAGCG CATGCTCCAGACTGCCTT 30) was located in the PGK promoter ofthe neo cassette. The common downstream primer (50 GCTGTGGAGCCAGGAGCAGCA 30) was located in the fourth exon, 30 prime to the

SacII site that formed the 30 prime junction between the neo cassette and the remainder of the fourth exon of the *Gadd45b* gene. Reactions were run for 37 cycles of 94°C for 1 min, 63°C for 14 sec, and 72°C for 12 sec. The wt *Gadd45b* PCR product was 310 bp long and the null PCR product was 190 bp in length.

2.2 Mouse Embryonic Fibroblast Preparation and Growth

13.5 days post coital embryos were collected and processed for cell culture. In summary, using a dissecting microscope, the developed organs are removed and discarded, such as the heart, lungs, liver, stomach and intestines. The remaining carcass is minced in trypson and passed through a cell strainer. The filtrate containing the cells is cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37° 10% C02. (Invitrogen, Carlsbad, CA).

2.3 3T3 Cell Growth Protocol

MEFs were grown in DMEM supplemented with 10% fetal bovine serum in 3% O_2/5% CO_2 for 2 days, then harvested, viably frozen, and labeled as passage 0. Seed 13.5d MEFs in 60mm dishes at a density of 3.0xl05. It is necessary to set up multiple plates per genotype in order to carry the protocol to late passages. For 3T3 assays, MEFs were seeded at $3X10^5$ cells per 10 cm plate. On day four, the medium was removed and the cells were washed two times with PBS. The cells were trypsinized and collected in fresh medium. The number of cells per plate was counted using a hemocytometer and 3.0xl05

cells were reseeded in 60mm dishes and incubated for three days. For growth at low oxygen levels, MEFs were grown in a humidified hypoxia chamber that was flooded with a gas mixture of 92% Nitrogen, 3% Oxygen, 5% Carbon Dioxide (2012 PlosOne TGFb). Cells were counted in triplicate. The number of cells obtained on day3 (N3) was divided by the initial cell number (N0 = $3X10^5$) and plotted as growth rate (N3/N0). The increase in the population doubling level (ΔPDL) was calculated according to the following formula: ΔPDL = log (Nf/N0)/log 2, where N0 is the initial number of cells ($3X10^5$) and Nf is the final number of cells.

2.4 UV irradiation and hydrogen peroxide treatment

UV irradiation (50 J/m2) of cells was carried out with a Stratalinker (Fisher Scientific) adjusted to UV-C irradiation. The cells were washed with phosphate-buffered saline before irradiation in the absence of any medium. Following irradiation, cells were supplemented with culture medium for 7 days and were analyzed for senescence associated β-galactosidase activity. Oxidative stress was induced by sub cytotoxic levels of hydrogen peroxide (150 μM for 4 hours) and then cells were recovered in normal medium for 7 days and analyzed for senescence associated β-galactosidase activity.

2.5 Senescence-associated β-galactosidase assay

Senescent cells were detected by staining for beta-gal using X-Gal (Cell Signaling, Danvers, MA) Stained cells were visualized using an Olympus inverted microscope with

digital imaging (Leica MZ16 stereomicroscope) and images were captured with a QImaging 5.0 RTV digital camera**.** A total of 300–400 cells were evaluated to assess the percentage of SA β-gal positive cells. Similarly, whole-mount embryo SA β-gal was also detected following overnight fixation and incubation with X-gal for 4–6 h.

2.6 Protein Extraction and Immunoblotting

Cells were collected and washed twice with PBS. The cell pellet was resuspended in 1X Cell Signaling Lysis buffer (Cell Signaling Technology, Boston, MA), supplemented with the protease inhibitor phenylmethlsulfonyl fluoride (1mM PMSF; Sigma), using equal volume of lysis buffer and cell pellet and incubated on ice for 30 minutes. The samples were then centrifuged at 14K rpm at 4°C for 10 minutes to remove cellular debris and protein containing supernatant was transferred to a fresh tube and stored at -80° until needed.

Protein quantification was performed using the BCA method using BioRad Bradford Reagent (Cat#500-0006), diluted 1:4 for working concentration. A standard curve was set up using the following concentrations, 0, 1, 5, 7.5, 10 ul of 10ug/ml Bovine Serum Albumin 9BSA) in order to determine unknown protein concentrations (BioRad Cat# 500-0007).

60-100 ug of each protein sample, depending on antibody being used to probe, was resolved on a SDS-Polyacyrlamide Gel at the appropriate percentage based on the size of the protein of interest. Following electrophoresis, the proteins were transferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA, USA) in 3-

[Cyclohexylamino]-1 propanesulfonic acid buffer (CAPS) plus 10% methanol at 65 volts 50 for 1 hour and 15 minutes on ice. The membrane was blocked for non-specific binding with 5% non-fat milk in 1X Tris Buffered Saline plus 0.01% Tween-2- (TBST) or 5% BSA in TBST, for phospho specific antibodies, for 10-45 minutes at room temperature. The membrane was incubated with primary antibody overnight at 4°C while rotating, followed by incubation with Horseradish Peroxidase (HRP) labeled secondary antibody at a concentration of 1:5000 for 1 hour at room temperature. The blot was developed using chemiluminescence kit (Cell Signaling Technology, MA).

Western blots were stripped with Restore Western Stripping Solution (Pierce, Rockford, IL, USA) before being probed with a new antibody. Westerns were run at least two times for each experiment. Equal loading was confirmed by probing with anti-Actin antibody.

The following antibodies were used. $p16^{Ink4A}$, $p19^{ARF}$, p21, Total-p53, cdc2 were from Abcam. Phospho-p53(Ser15), Cyclin B1, Phospho-SAPK/JNK, (Thr183/Tyr185),Total-SAPK/JNK and β-Actin were from Cell Signaling Technology.

2.7 RNA Extraction and Quantitative Polymerase Chain Reaction

RNA was extracted from samples using RNeasy kit (Qiagen) according to manufacturer's protocol. A reverse transcriptase polymerase chain reaction (RT-PCR) was performed to convert RNA to c-DNA using TaqMan Reverse Transcription reagents (Applied Bio systems) according to manufacturer's protocol. c-DNA was then used to run the real time polymerase chain reaction analysis (qRT-PCR) in a StepOne Real Time PCR machine (Applied Bio systems). Taqman probes used for the study are following, all

purchased from Life Technologies: - Mm00435123_m1 (mouse *Gadd45b*), and Mm04277571_S1, for 18s, used as an endogenous control.

2.8 Flow cytometric cell cycle analysis

Cells were collected at the indicated time points and fixed in methanol. Prior to analysis, cells were treated with a solution containing 10% propidium iodide (500 μg/ml), 5 mg/ml 10% RNase A and 80% 1× phosphate-buffered saline (PBS) plus 1% FBS, and incubated at 37°C for 30 min. The cells were analyzed with a FACSCalibur (BD) flow cytometer, and the data were analyzed using FlowJo analysis software (Tree Star). To determine cell cycle distributions in the G2 and M phases, cells were harvested with trypsin, stained using antiphospho-histone H3 - AlexaFluor® 488 antibody on ice for one hour in the dark, followed by staining using PI/RNase solution for 30 minutes at room temperature in the dark per the manufacturer's instructions (#FCCH025103, EMD Millipore Corporation, Ballerica, MA). The samples were analyzed by a FACScan flow cytometer (Becton-Dickinson), with FlowJo analysis software (Tree Star).

2.9 Immunofluorescence and Mitotic index analysis

Hoechst 33342 stock solution (Molecular Probes) was diluted 1:100 in H_2O. The medium was aspirated from cells grown on coverslips and the cells were rinsed three times with PBS. The cells were incubated in the diluted Hoechst solution for 15 min at room

temperature. After labeling, cells were washed with PBS and imaged under an EVOS™ Digital Inverted Fluorescence Microscope**.** The mitotic index, i.e., the percentage of cells in mitosis was determined by counting at least 300 cells in three independent experiments. . The mitotic index was determined by fluorescence microscopic analysis of Hoechst 33342 stained nuclear morphology.

2.10 Alkaline comet assays

Comet assays were performed using a Trevigen Comet Assay Kit (4250-050-K) as per the manufacturer's instructions. Cells (1000) were mixed with low melt agarose, spotted onto slides, lysed, and electrophoresed under denaturing conditions at 4 degree C. DNA was stained with SYBR green and images were captured using a 20 magnification on an EVOS™ Digital Inverted Fluorescence Microscope. Images were saved as bitmap files and olive tail moments were calculated using TriTek CometScoreTM Freeware v1.5.

2.11 Histo-pathological Evaluation of skin samples

Dorsal skin samples from euthanized mice were fixed in 10% buffered formalin for 24 h, transferred in 70% ethanol, embedded in paraffin, and sliced. The sections were stained with hematoxylin and eosin (H&E) for histological assessment.

2.12 Immuno-histochemistry of skin samples

OCT-embedded samples were cut into 10 μm sections. Sections were fixed in 10% buffered formalin, permeabilized with 0.5% triton-X, blocked with 4% donkey serum/1% BSA in PBS solution and incubated with anti-**γ**H2AX (NB100-79967, Novus Biologicals, 1:500) overnight at 4°C, followed by incubition with Alexa 555 donkey anti-rabbit (Invitrogen, 1:750) for 1 h at room temperature. Sections were mounted with Prolong Gold with DAPI (Invitrogen).

2.13 Senescence staining of skin samples

OCT-embedded skin sections were processed for SA β-gal staining using Senescence Detection Kit (BioVision, Mountain View, CA, USA). Sections were counterstained with nuclear fast red and visualized by brightfield microscopy.

2.14 Hepatic-Fibrosis Model

Hepatic fibrosis was induced in *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ animals using carbon tetrachloride (CCl4). Sub-lethal dosage of CCl4 (Sigma-Aldrich) (1 uL/g body weight of the 1:7 ratio CCl4:olive oil mix) or olive oil vehicle was introduced by intra-peritoneal injections twice a week, for 6 weeks. There were 10 animals included in each presented study group. All surgery was performed under anesthesia, and all efforts were made to minimize suffering. When sacrificed, livers were harvested 3 days after the final dose.

2.15 Histopathological Evaluation of the Liver Tissue

Pieces of liver were rapidly fixed in 10% neutral buffered formalin and embedded in paraffin. The sections were stained with hematoxylin and eosin (H & E) for histological assessment., or with Sirius Red for visualization of fibrotic deposition. Detection of SA β-gal activity was performed as described previously ([153]. Liver samples were embedded in frozen OCT medium and cut into 10 μm sections. Frozen sections of liver tissue were processed for SA β-gal staining using Senescence Detection Kit (BioVision, Mountain View, CA, USA). Sections were counterstained with nuclear fast red and visualized by brightfield microscopy.

2.16 Statistical Analysis

Unless indicated, significance for differences between experimental and control groups were determined using the two-tailed Student *t* test (GraphPad Prism v5.01 software, San Diego, CA) Error bars indicate s.e.m. *: p,0.05; **:p,0.01; ***:p,0.001.

CHAPTER 3

GADD45B DEFICIENCY IMPAIRS G2/M CELL CYCLE PROGRESSION LEADING TO PREMATURE SENESENCE

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3.1 Introduction.

Cellular senescence, first identified as a process that limits the proliferation or growth of human cells in culture [64] is now recognized as a crucial tumor suppressor mechanism and formidable barrier to malignant progression [28]. It was also shown to be induced by a variety of potentially oncogenic stimuli, such as telomere shortening, DNA damage, oxidative stress, and oncogene expression [19 36]. MEFs are primary cells with limited life-span, that senesce in culture [154] . The senescence observed in primary MEFs is at least in part due to the stress of them being placed in culture, particularly, hyperoxic culture conditions, which results in accumulation of DNA damage. [135 197 112].

Gadd45a, b and g constitute a family of genes, which encode for small (18 kDa) evolutionarily conserved proteins that are highly homologous to each other. Despite marked similarities, these genes are regulated in a differential manner and therefore exhibit functional diversity. They play a pivotal role in regulating diverse cellular functions such as cell cycle control, survival, and apoptosis and are regulated by the nature of the stress stimulus encountered, its magnitude, and the cell type. Gadd45a, Gadd45b and Gadd45g have been implicated in cell cycle arrest [55 17 1 200], DNA repair [177], apoptosis [178], innate immunity (Salerno 2012) and genomic stability [73]. Gadd45 proteins have been shown to stimulate the p38-c-Jun N-terminal kinase (JNK) mitogen-activated

protein (MAP) kinase pathways in response to stress and thereby sensitize cells to apoptosis, survival or growth arrest [163]. Gadd45 proteins are also shown to regulate cell cycle checkpoints in response to genotoxic stress, notably the G2/M checkpoint [179] [100]. Furthermore, Gadd45b has also been identified as a transcriptional target of NF-κB, encoding a potent and selective inhibitor of the JNK MAPK pathway and, therefore, of apoptosis [132, 133].

Several studies have highlighted the role of Gadd45a in senescence. *Gadd45a* null MEFs do not undergo senescence in response to oncogenic H-ras [24]. In a mouse model of mammary tumorigenesis, loss of *Gadd45a* in the presence of myc leads to increased senescence whereas loss of *Gadd45a* in the presence of Ras leads to decreased senescence [172, 173].

Furthermore, long-term activation of the checkpoint gene CDKN1A (p21) induces mitochondrial dysfunction and production of reactive oxygen species through serial signaling through GADD45A-p38MAPK-GRB2-TGFBR2-TGFbeta leading to the establishment of the senescent phenotype. [136]. Although Gadd45a has been shown to play a significant role in regulating cellular senescence to stress, the role of Gadd45b has not been studied. Given the similarities and diversity among the Gadd45 family of genes, it is of interest, therefore, to investigate the role of Gadd45b in senescence.

In order to achieve this goal, we have taken advantage of mouse embryonic fibroblasts that differ in the status of *Gadd45b* and show that mouse embryonic fibroblasts (MEFs) lacking *Gadd45b* have impaired proliferation, a G2/M cell-cycle arrest and premature senescence. We also show that *Gadd45b* null cells are more sensitive to hyperoxic stress and have higher levels of DNA damage than *Gadd45b*$^{+/+}$

cells. Furthermore, *Gadd45b* null MEFs arrest at the G2/M phase of cell cycle, with impaired G2/M cell-cycle progression, in contrast to other senescent MEFs, which arrest at G1. In addition to tissue culture-induced stress, other stressors such as sub-lethal H_2O_2 and UV irradiation that are known to increase oxidative stress increased premature senescence in *Gadd45b* null MEFs compared to *Gadd45b*$^{+/+}$ MEFs. Notably, we show that embryos from *Gadd45b*$^{-/-}$ mice exhibit increased SA β-gal staining compared to *Gadd45b*$^{+/+}$ embryos, thus providing *in vivo* evidence for increased senescence in *Gadd45b*$^{-/-}$ mice suggesting a hitherto unidentified role for *Gadd45b* in regulating stress-induced cellular senescence.

3.2 Decreased proliferation and premature senescence in *Gadd45b* null MEFs

Our working hypothesis was that Gadd45b is a stress sensor protein, which is up regulated by oxidative stress and functions to modulate senescence. To assess the validity of our hypothesis, *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs were subjected to serial passage using the 3T3 protocol under standard culture conditions, which included atmospheric (20%) oxygen. While the *Gadd45b*$^{+/+}$ MEFs exhibited characteristic biphasic growth kinetics seen in mouse embryonic fibroblasts, all *Gadd45b*$^{-/-}$ MEFs analyzed showed significantly reduced proliferation (Figure 3.1). This is in striking contrast with *Gadd45a*$^{-/-}$ MEFs, which show increased cell proliferation. Furthermore, in all MEF cell cultures analyzed, loss of *Gadd45b* resulted in reduced proliferation and premature

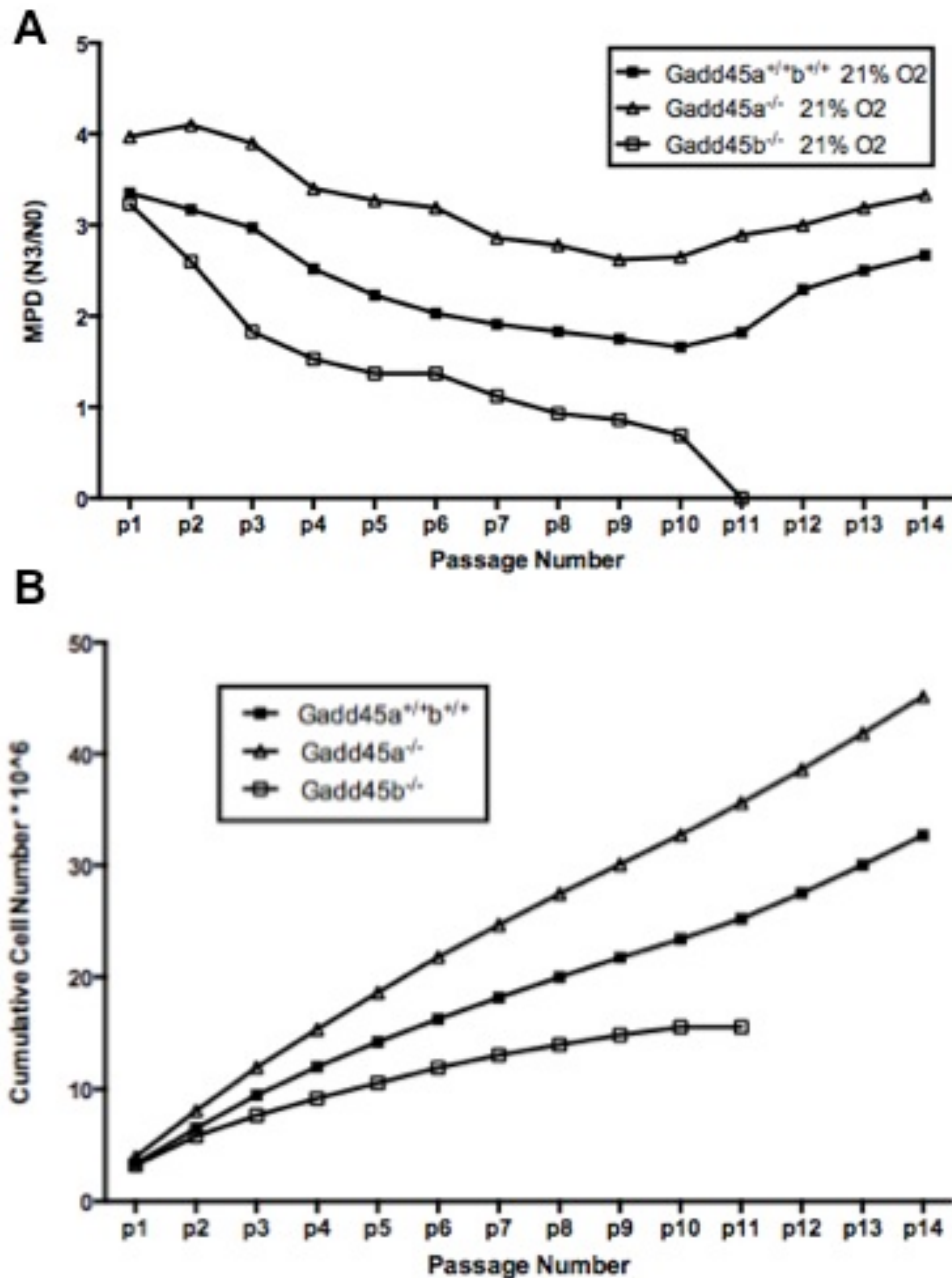


Figure 3.1. Loss of *Gadd45b* results in impaired proliferation

(a) *Gadd45b*$^{+/+}$ (filled squares), *Gadd45a*$^{-/-}$ (empty triangles) and *Gadd45b*$^{-/-}$ (empty squares) MEFs were cultured at 21% oxygen continuously for 14 passages. Cells were split every 3 days, and the total numbers of cells were counted and mean population doublings (MPD) were determined. (b) Cumulative cell number is plotted against passage number.

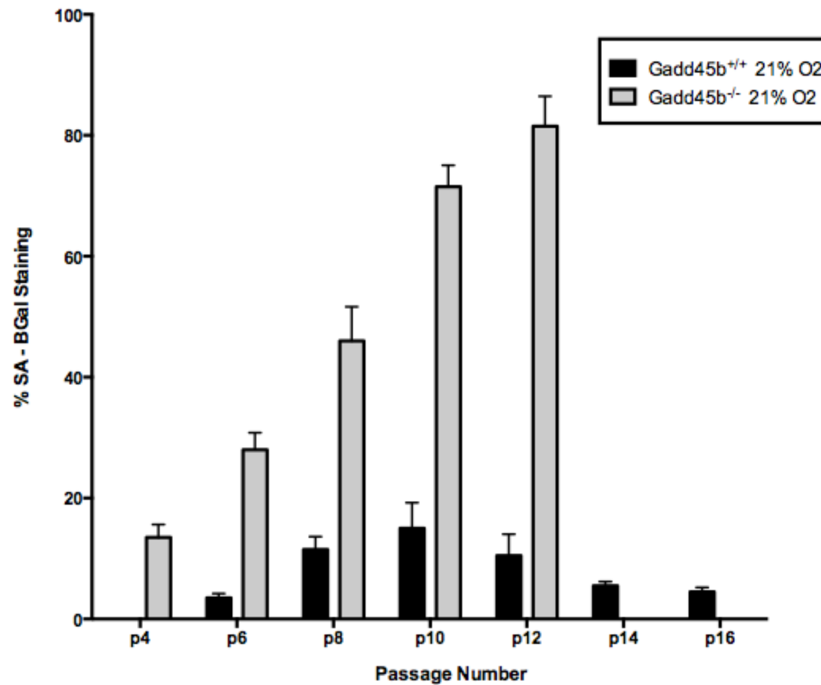


Figure 3.2. Loss of *Gadd45b* results in premature and increased senescence

Gadd45b$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs, cultured at 21% oxygen continuously for 16 passages, were stained for SA β-gal at each passage. SA β-gal positive cells were counted in at least 10 fields from triplicate plates. A quantification of SA β-gal positive *Gadd45b*$^{+/+}$ (solid black) and *Gadd45b*$^{-/-}$(solid grey) MEFs is shown for each passage.

senescence, as determined by an enlarged and flattened cell morphology and increased senescence-associated β-galactosidase (SA-β-gal) staining at earlier passages (Figure 3.2), both of which were established features of senescence in MEFs [49].

Taken together, these data support the hypothesis that *Gadd45b* regulates the senescence response to tissue culture stress.

3.3 Premature senescence associated with *Gadd45b* deficiency can be rescued, in part, by culture at low oxygen.

It is known that primary MEFs are sensitive to oxidative stress in culture [135]. Hence, to test whether exposure to hyperoxia might be a factor in the premature senescence of *Gadd45b*$^{-/-}$ MEFs, these MEFs were cultured in the presence of 3% oxygen, which is known to be similar to the physiologic oxygen condition *in vivo*. MEFs were prepared from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ embryos, and cultured under two different conditions, one at 21% O_2 and the other under physiologically relevant low oxygen (3% O_2) conditions. As shown in Figure 3.3, culturing cells under physiological oxygen conditions (3% O_2) resulted in a partial rescue of their ability to proliferate. It should be noted, however, that *Gadd45b*$^{+/+}$ MEFs also proliferated better in 3% O_2 compared to 21% O_2, yet the rate of proliferation was still much lower in *Gadd45b*$^{-/-}$ MEFs than *Gadd45b*$^{+/+}$ MEFs. Also, SA β-gal staining of *Gadd45b*$^{-/-}$ MEFs was less at 3% O_2 compared to staining at 21% O_2 (Figure 3.4). Taken together, these data indicate that *Gadd45b*$^{-/-}$ MEFs have increased sensitivity to high levels of oxidative stress as observed at 21% O_2 leading to increased senescence.

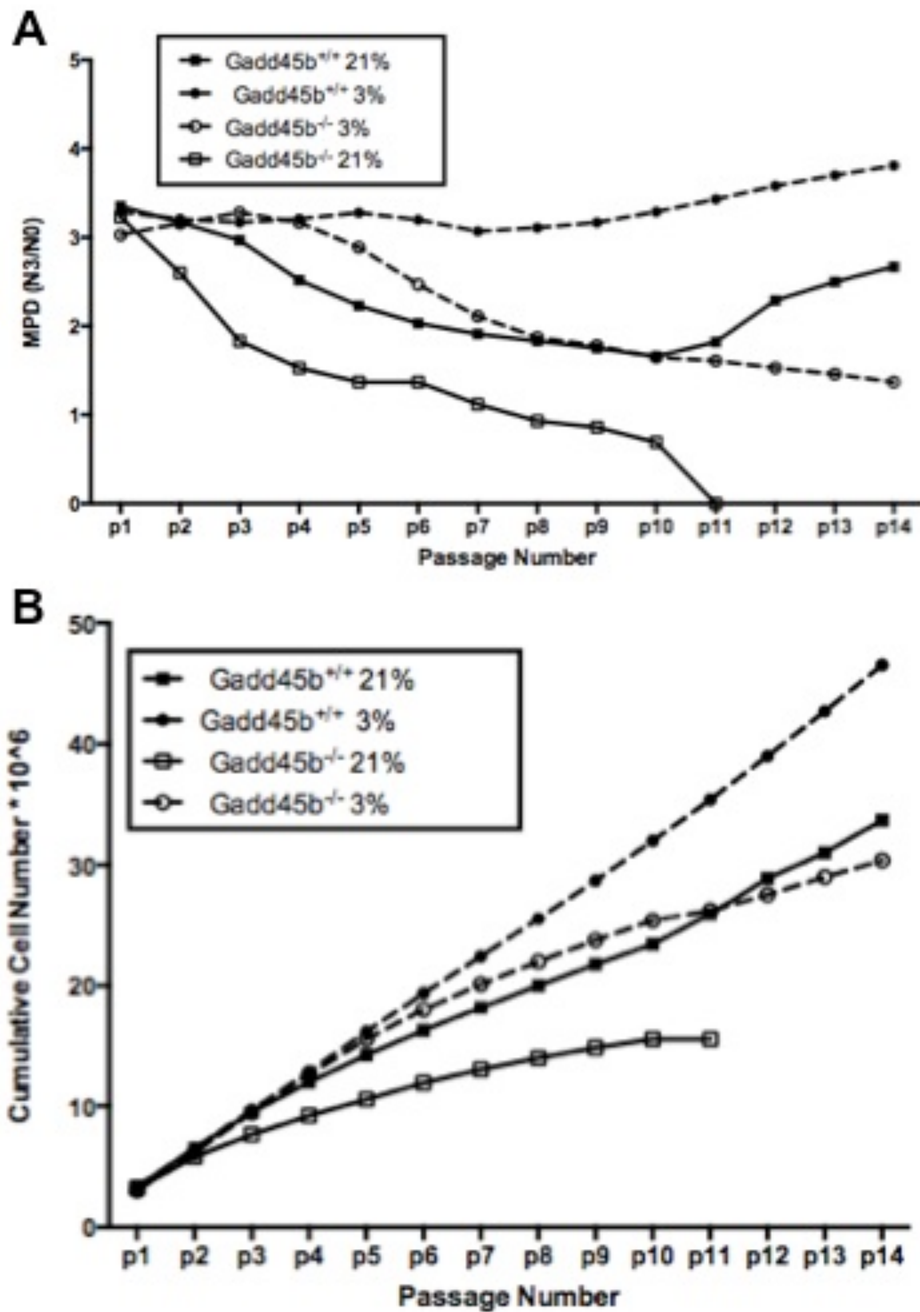


Figure 3.3. Impaired proliferation associated with *Gadd45b* deficiency can be rescued, in part, by culture at low oxygen

(a) *Gadd45b*$^{+/+}$ (filled), and *Gadd45b*$^{-/-}$ (empty) MEFs were cultured at either 21% oxygen (squares) or 3% oxygen (circles) continuously for 14 passages. Cells were split every 3 days, and the total numbers of cells were counted and mean population doublings (MPD) were determined. (b) Cumulative cell number is plotted against passage number.

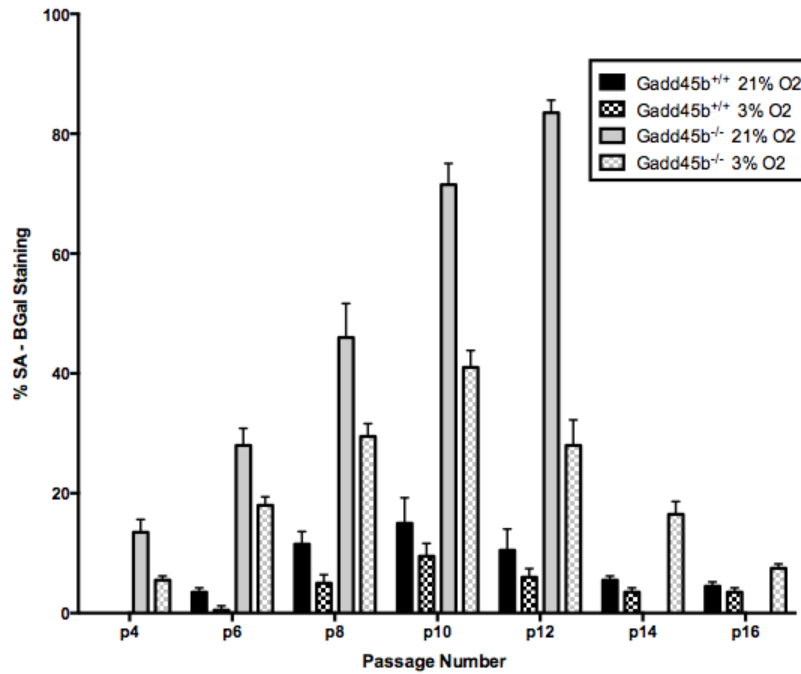


Figure 3.4. Premature senescence associated with *Gadd45b* deficiency can be rescued, in part, by culture at low oxygen

Gadd45b$^{+/+}$ (black) and *Gadd45b*$^{-/-}$ (grey) MEFs, cultured at either 21% oxygen (solid) or 3% oxygen (dotted) were stained for SA β-gal at each passage. SA β-gal positive cells were counted in at least 10 fields from triplicate plates. A quantification of SA β-gal positive *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs is shown for each passage.

3.4 *Gadd45b* mRNA Expression is regulated by the levels of Oxidative Stress

Loss of *Gadd45b* led to decreased proliferation and increased senescence in response to tissue culture and oxidative stress. Therefore, it was asked if oxidative stress could modulate *Gadd45b* mRNA expression. *Gadd45b* expression was examined in *Gadd45b*$^{+/+}$ MEFs from different passages; cultured at 3% and 21% oxygen by real time PCR analysis using *Gadd45b* specific taqman probes. Interestingly, we observed that the expression of *Gadd45b* in *Gadd45b*$^{+/+}$ MEFs progressively increased with passage numbers (Figure 3.5). Also, *Gadd45b* mRNA levels were consistently higher in MEFs cultured at 21% oxygen compared to cells cultured in 3 % oxygen. Taken together, these data indicate that *Gadd45b* expression is directly regulated by the level of oxidative stress.

3.5 Growth arrest in *Gadd45b* null MEFs is associated with defective G2/M cell-cycle progression

To determine whether changes in cell cycle control accompany the decline in growth of *Gadd45b*$^{-/-}$ MEFs, we compared the cell cycle profiles of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs. Surprisingly, *Gadd45b*$^{-/-}$ MEFs displayed a gradual increase in the proportion of G2/M cells with increased passage number compared to *Gadd45b*$^{+/+}$ MEFs (Figure 3.6). This finding is in striking contrast to other senescent MEFs, which normally arrest at G1. [155]. To delineate whether *Gadd45b*$^{-/-}$ deficient MEFs are arrested in G2 or M phase, we stained *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs with Hoechst 33342 solution and

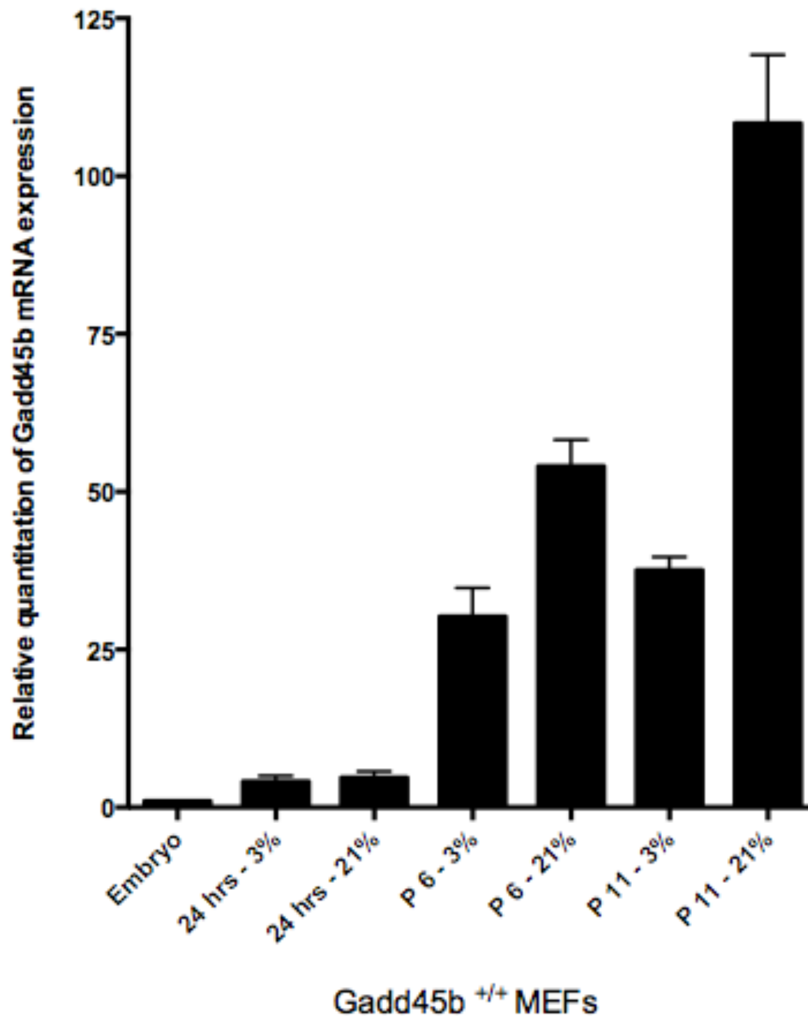


Figure 3.5. *Gadd45b* mRNA levels increase with increased exposure to oxidative stress

***Gadd45b* mRNA levels increase with increasing passage number and were consistently higher at 21% oxygen compared to 3% oxygen.** *Gadd45b*$^{+/+}$ MEFs were cultured at either 21% oxygen or 3% oxygen. Total RNA for each passage was analyzed by real time PCR for *Gadd45b* expression using taqman probe as described in Materials and methods. *18S* rRNA probe was used as an internal control.

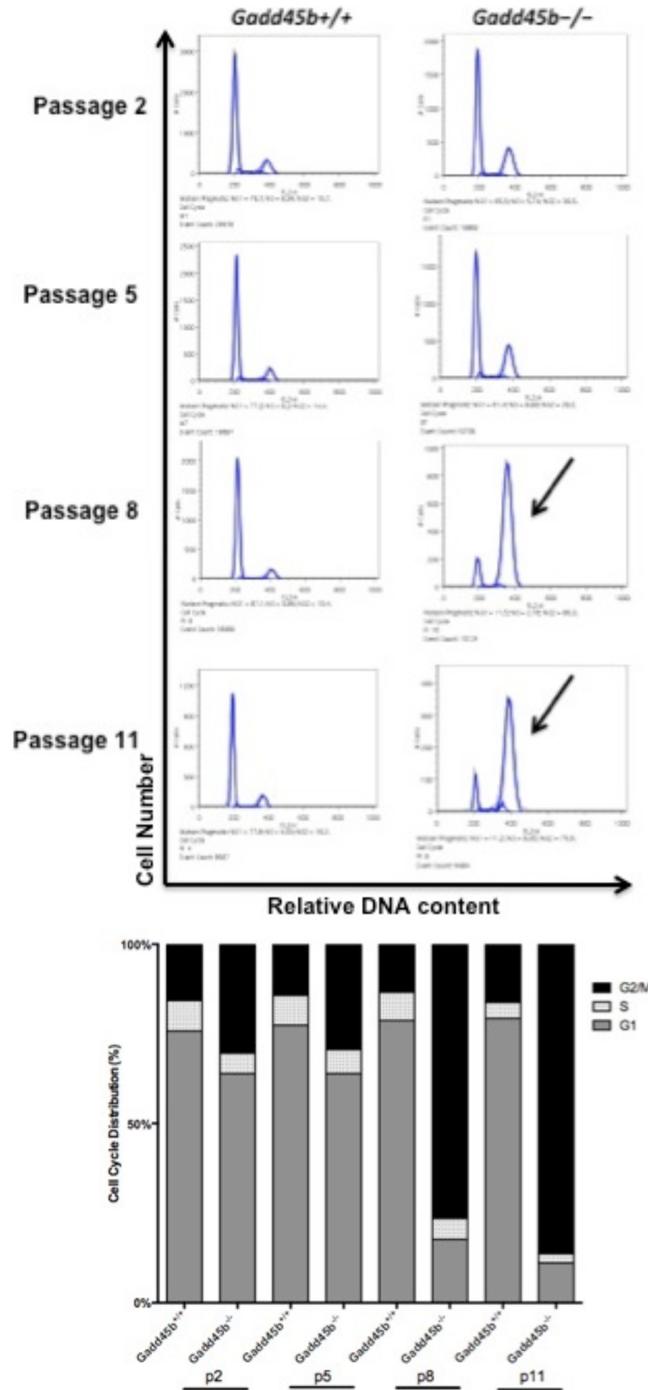


Figure 3.6. Loss of *Gadd45b* results in an accumulation in G2/M
Gadd45b$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs were cultured at 21% oxygen. Subconfluent cultures of cells were harvested at different passages and fixed prior to being stained with propidium iodide. DNA content was analyzed by flow cytometry. The arrow indicates enrichment of G2/M cells in late passage *Gadd45b*$^{-/-}$ cultures. A quantification of distribution of cells in G1 (dark grey), S (light grey) and G2/M (black) phase of the cell cycle is shown for different passages.

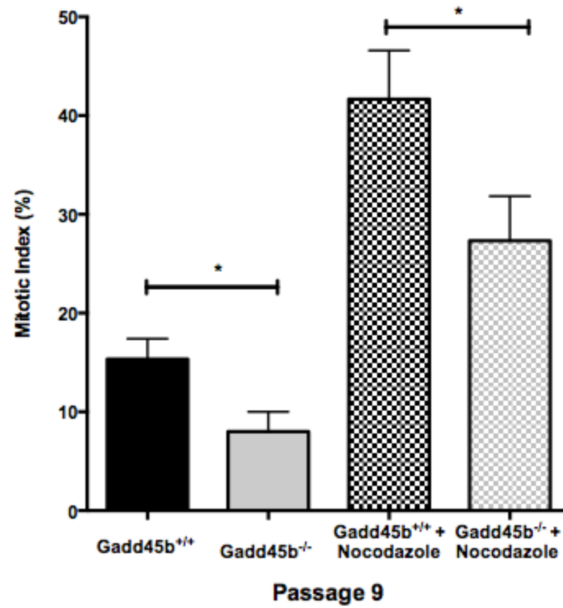
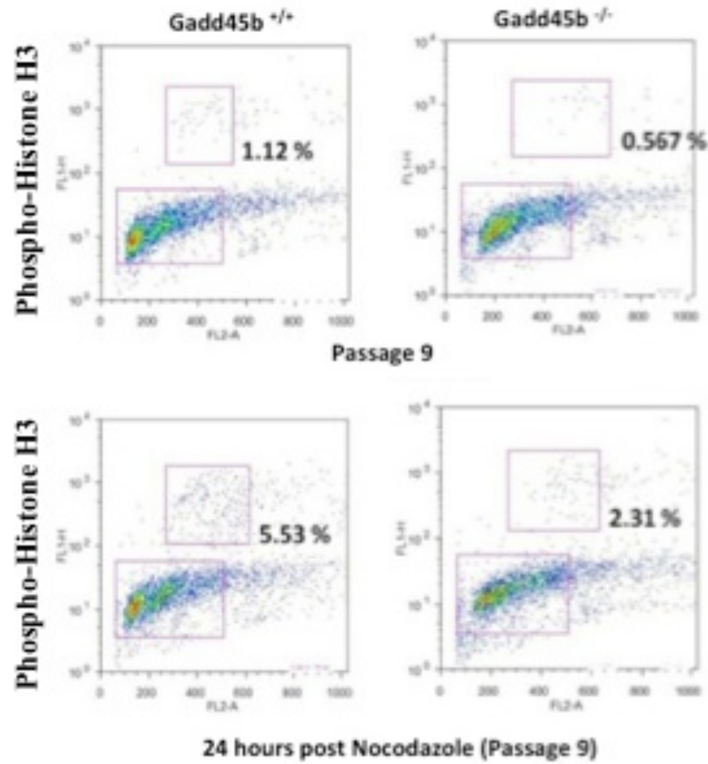


Figure 3.7. Loss of *Gadd45b* results in a reduced mitotic index (Defective G2/M cell-cycle progression)

The mitotic index was determined in *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs cultured at 21% oxygen at passage 9. Nocodazol treated cells were used as experimental control. *, $p < 0.05$.



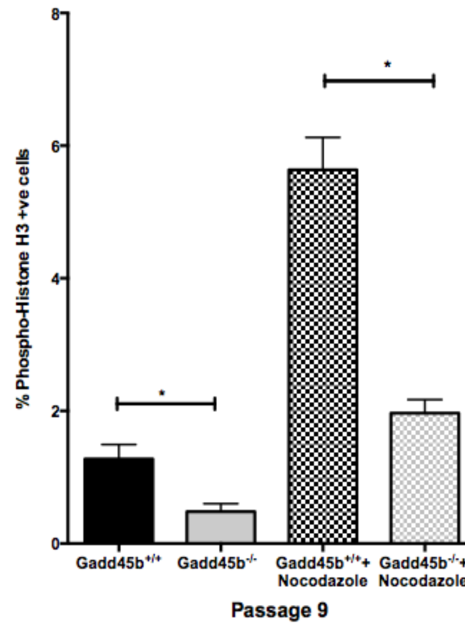


Figure 3.8. Loss of *Gadd45b* results in reduced number of phosphorylated Histone H3- positive cells (Defective G2/M cell-cycle progression)
Numbers of phosphorylated Histone H3- positive cells in $Gadd45b^{+/+}$ and $Gadd45b^{-/-}$ MEFs cultured at 21% oxygen (passage 9) using phosphospecific anti-Histone H3 (Ser10) antibodies. Mean values from three independent experiments are shown. (c) Scatter plots show phospho-histone H3 (y axis) plotted against PI (DNA content, x axis), gated to quantify mitotic (phospho-histone H3 positive) cells. *, $p < 0.05$.

scored for mitotic stage based on DNA morphology. The mitotic index of *Gadd45b*$^{-/-}$ MEFs was lower than that of *Gadd45b*$^{+/+}$ MEFs indicating that *Gadd45b*$^{-/-}$ MEFs are arrested in G2 phase rather than M phase (Figure 3.7). Nocodazole, a microtubule inhibitor used to arrest cells in mitosis, was used as an experimental control.

To further confirm this defect in G2/M cell cycle progression, phospho-Histone H3 staining was performed. Histone H3 is phosphorylated at Ser 10 during M phase, but not at G2 [95]. *Gadd45b*$^{-/-}$ MEFs showed less phospho-Histone H3-positive cells than *Gadd45b*$^{+/+}$ MEFs at passage 9 (Figure 3.8). Taken together, these data indicate that loss of *Gadd45b* in MEFs results in an unexpected defect in G2/M cell-cycle progression.

3.6 Increased p19 Arf -p53-p21 signaling and impaired CDC2 expression in *Gadd45b* null MEFs

Senescence in MEFs has been shown to be associated with increased levels of p53 and its downstream target p21 as well as increased levels of p16 and p19^{ARF} [93]. Consistent with our previous results where we observed reduced growth rate and increased SA-β-gal staining, we found that *Gadd45b*$^{-/-}$ MEFs showed increased levels of p16, p19^{ARF}, p21 and p53 proteins at earlier passages compared to *Gadd45b*$^{+/+}$ MEFs (Figure 3.9). Thus, loss of *Gadd45b* leads to significantly increased senescence, increased p16 and p19^{ARF} expression and activation of p53 function in turn leading to increased p21.

To identify the mechanism through which *Gadd45b* regulates proliferation and G2/M cell cycle progression, we examined the expression levels of the key G2/M

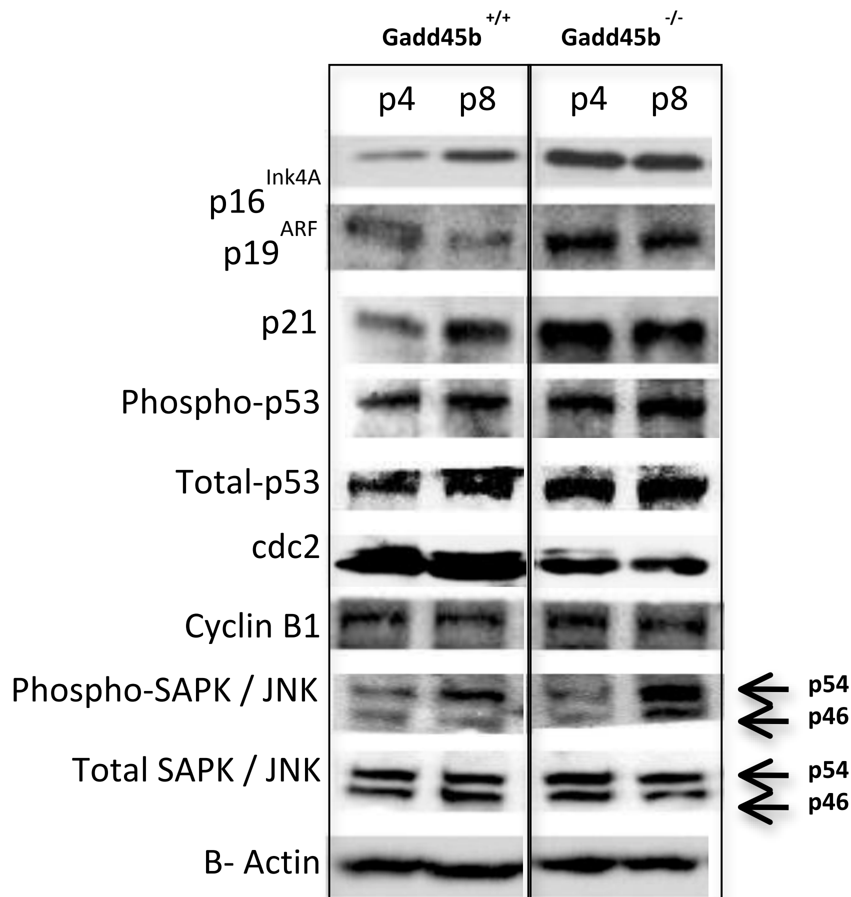


Figure 3.9. Loss of *Gadd45b* results in increased expression of senescence markers – p16, p19ARF, p21, p53 & JNK phosphorylation and decreased expression of cdc2

Western blotting analysis of $p16^{Ink4A}$, $p19^{ARF}$, p21, Phospho-p53 (Ser15), Total-p53, cdc2, Cyclin B1, Phospho-SAPK/JNK (Thr183/Tyr185) and Total-SAPK/JNK expression in cell extracts prepared from $Gadd45b^{+/+}$ and $Gadd45b^{-/-}$ MEFs at different passages cultured at 21% oxygen. β-Actin was used as a loading control.

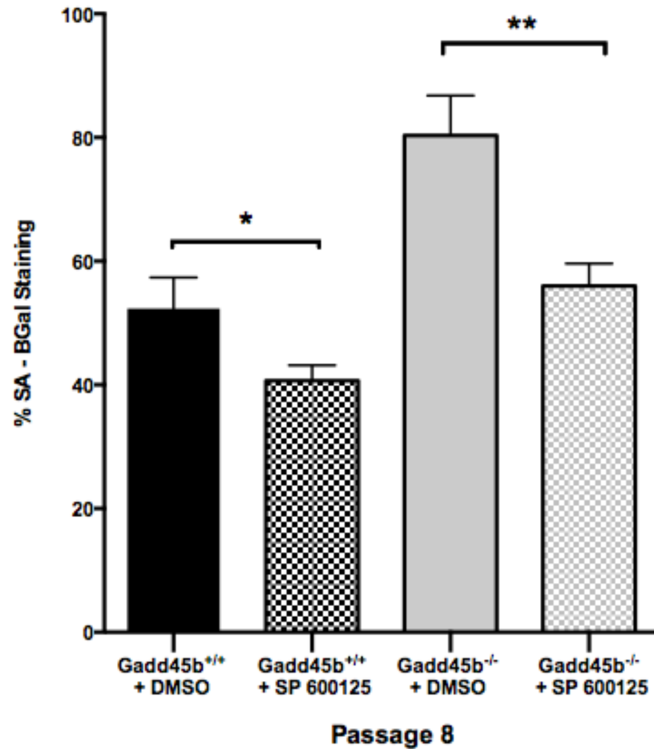


Figure 3.10. Premature senescence associated with *Gadd45b* deficiency can be rescued, in part, by inhibition of SAPK/JNK

Gadd45b$^{+/+}$ (black) and *Gadd45b*$^{-/-}$ (grey) MEFs cultured at 21% oxygen (passage 6) were treated with vehicle (0.1% DMSO) (solid) or J*NK inhibitor*, *SP600125* (dotted) for 48 hours and stained for SA β-gal. SA β-gal positive cells were counted in at least 10 fields from triplicate plates. A quantification of SA β-gal positive MEFs is shown. *, $p < 0.05$.

transition complex, Cyclin B1/Cdc2. While the expression of CYCLIN B1 was comparable between *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs, intriguingly, a marked reduction in the expression of CDC2 protein was observed in *Gadd45b*$^{-/-}$ MEFs (Figure 3.9). CDC2 has been shown to be essential for G2/M cell-cycle progression in multiple organisms [8]. These data suggest CDC2 as a potential molecular target for *Gadd45b* regulated stress response. Furthermore, increased phosphorylation of stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) was also observed in *Gadd45b*$^{-/-}$ MEFs at earlier passages compared to *Gadd45b*$^{+/+}$ MEFs (Figure 3.9) SAPK/JNK has been shown to regulate p53 dependent senescence [44]. To causally link increased activation of SAPK/JNK in *Gadd45b*$^{-/-}$ MEFs to increased tissue culture-induced senescence, *Gadd45b*$^{-/-}$ MEFs and *Gadd45b*$^{+/+}$ MEFs at passage 6 were treated with JNK specific inhibitor, SP600125 for 48 hours and then stained for senescence-associated β-galactosidase (SA-β-gal) 6 days later. As shown in Figure 3.10, inhibition of SAPK/JNK in *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs led to a decrease in SA-β-gal staining compared to untreated MEFs. Taken together, these data indicate that Gadd45b negatively regulates or limits tissue cultured induced senescence by modulating SAPK/JNK, CDC2 and senescence signaling.

3.7 Loss of *Gadd45b* results in accumulation of spontaneous DNA damage

Cells with defective mitogen signaling generally accumulate in the G1 phase of cell cycle, whereas arrest in the G2/M phase of cell cycle is frequently indicative of a DNA damage response [16]. The premature senescence induced by hyperoxic stress in

Gadd45b$^{-/-}$ MEFs was associated with accumulation of cells in the G2/M phase of the cell cycle (Figure 3.6), suggesting that this growth arrest might be associated with higher levels of endogenous DNA damage. To test whether there was increased DNA damage in *Gadd45b*$^{-/-}$ MEFs, cells were stained with an antibody that recognizes phosphorylated H2A.X (gH2AX) found specifically at repair foci. As shown in Figure 3.11, we observed an increase in phosphorylated gH2A.X positive cells in passage 8 *Gadd45b*$^{-/-}$ MEFs compared to *Gadd45b*$^{+/+}$ MEFs indicating that loss of Gadd45 leads to accumulation of DNA damage. Furthermore, *Gadd45b*$^{-/-}$ MEFs showed increased levels of serine 15 phosphorylation of p53 compared to *Gadd45b*$^{+/+}$ MEFs (Figure 3.10), a modification induced in response to certain forms of DNA damage.

To further determine whether the growth arrest of *Gadd45b*$^{-/-}$ MEFs was associated with accumulation of oxidative DNA damage and directly assess the level of DNA damage on a single-cell basis, we performed comet assay. The assay was done under denaturing conditions to detect both single and double strand breaks. As shown in Figure 3.11, we observed significantly more DNA damage in the *Gadd45b*$^{-/-}$ MEFs compared to *Gadd45b*$^{+/+}$ MEFs at passage 8. Taken together, these data indicate that loss of *Gadd45b* leads to an accumulation of DNA damage induced by oxidative stress.

3.8 *Gadd45b*$^{-/-}$ MEFs show increased senescence in response to environmental stresses

Given that the loss of *Gadd45b* led to increased senescence in response to tissue culture stress, it was of interest, therefore, to investigate the effect of loss of *Gadd45b* on

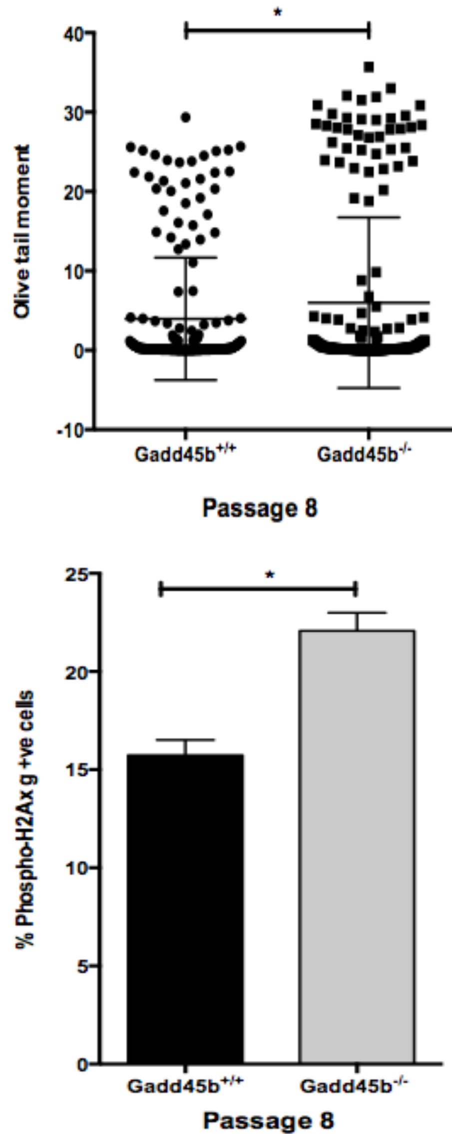


Figure 3.11. Increased DNA damage in MEFs lacking *Gadd45b*

Top: Dot plot showing DNA comet olive tail moment of 200 $Gadd45b^{+/+}$ and $Gadd45b^{-/-}$ MEFs cultured at 21% oxygen (passage 8). *, $p < 0.05$. Bottom: Numbers of phosphorylated Histone *H2AX* (Ser-139) positive cells in $Gadd45b^{+/+}$ and $Gadd45b^{-/-}$ MEFs cultured at 21% oxygen (passage 8) using phosphor-specific anti-Histone *H2AX* (Ser-139) antibodies. Mean values from three independent experiments are shown.

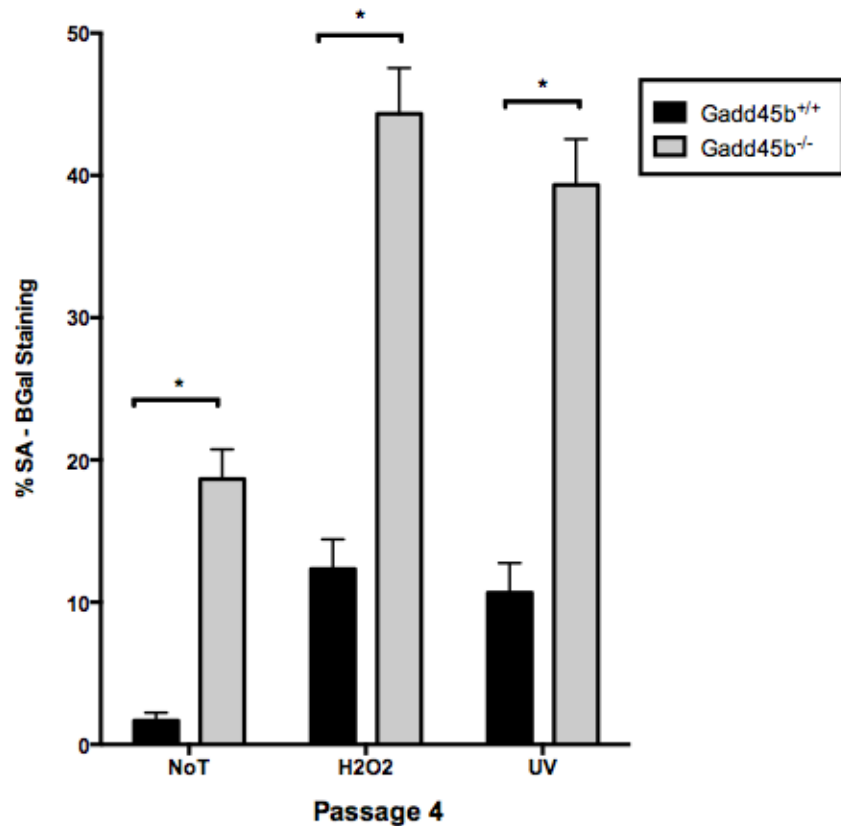


Figure 3.12. Increased senescence of *Gadd45b*$^{-/-}$ MEFs in response to environmental stress

Gadd45b$^{+/+}$ (black) and *Gadd45b*$^{-/-}$ (grey) MEFs cultured at 21% oxygen (passage 3) were treated with sub-lethal doses of UV-irradiation (30 J/m^2) or H2O2 (150 uM). Stress-induced senescence (mean numbers of SA β-gal -positive cells) was determined 7 days after stress. *, $p < 0.05$.

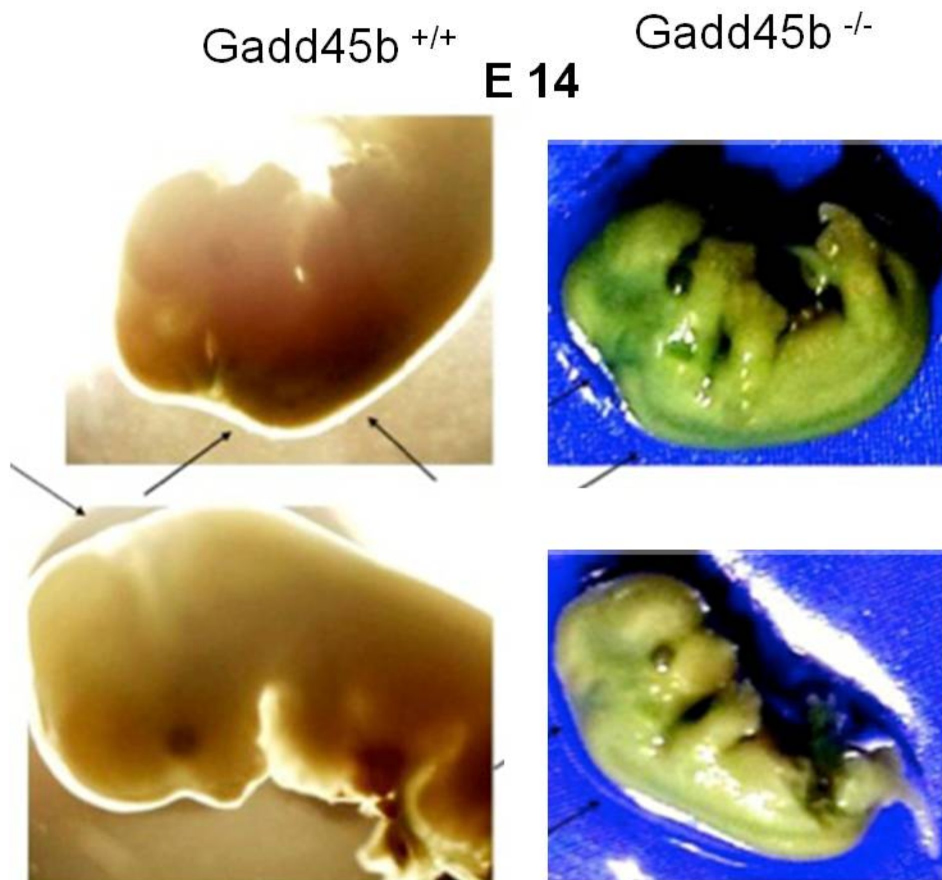


Figure 3.13. Increased senescence staining in *Gadd45b*$^{-/-}$ embryos

Photograph of SA β-gal staining of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ E14 embryos. Arrows indicate embryonic regions with strong senescence staining.

senescence triggered by environmental stressors such as UV irradiation and oxidative stress.

In order to investigate this, early passage *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs were treated with sub-lethal doses of hydrogen peroxide and UV light. As shown in Figure 3.12, an increase in SA-β-gal staining was observed in *Gadd45b*$^{-/-}$ MEFs compared to *Gadd45b*$^{+/+}$ MEFs further confirming that loss of *Gadd45b* leads to an increase in senescence in response to environmental stress. To investigate whether *Gadd45b* deficiency enhanced senescence *in vivo*, E14 embryos from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were stained with SA-β-gal stain. As shown in Figure 3.13, increased senescence staining was observed in *Gadd45b*$^{-/-}$ embryos compared to embryos from *Gadd45b*$^{+/+}$ mice, therefore providing conclusive *in vivo* evidence that *Gadd45b* regulates cellular senescence.

3.9 Discussion

The current study highlights a novel role for *Gadd45b* in the senescence response of mouse fibroblasts to oxidative stress, taking advantage of *Gadd45b*$^{-/-}$ mice. Our results show that *Gadd45b* is critical for MEF proliferation and G2/M cell-cycle progression. Interestingly, early passage *Gadd45b*$^{-/-}$ MEFs behave similarly to *Gadd45b*$^{+/+}$ cells, but after 4-5 passages, *Gadd45b*$^{-/-}$ MEFs display premature senescence, defective proliferation and reduced CDC2 expression. Thus, it seems that under conditions of environmental stress, *Gadd45b* might function to limit the senescence response and maintain the proliferative state of the cells.

As it is known that senescence in MEFs is associated with $p19^{Arf}$ -p53-p21 signaling, we next investigated the expression of these critical cell cycle regulators. Our results show that *Gadd45b*$^{-/-}$ MEFs have increased levels of p16, $p19^{ARF}$, p21 and p53 proteins at earlier passage compared to *Gadd45b*$^{+/+}$ MEFs. Interestingly, despite the increased expression of critical senescence proteins (p16, $p19^{ARF}$, p21 and p53) in *Gadd45b*$^{-/-}$ MEFs, these cells do not undergo G1 arrest which suggests that *Gadd45b*$^{-/-}$ MEFs accumulate DNA damage in the S phase, leading to activation of G2 checkpoint and cells arresting with G2 DNA content. Previous studies have shown that mkk7$^{-/-}$ MEFs and cJun$^{-/-}$ MEFs have impaired proliferation, premature senescence and a G/2M cell-cycle arrest [186 112]. They also identified that G2/M kinase CDC2 as a molecular target for the MKK7–JNK–cJun signaling pathway. As it is known that *Gadd45b* is associated with the MKK7–JNK–cJun pathway, we studied the expression levels of CDC2. Interestingly, similar to mkk7$^{-/-}$ MEFs and cJun$^{-/-}$ MEFs, *Gadd45b*$^{-/-}$ MEFs showed decreased CDC2 expression compared to *Gadd45b*$^{+/+}$ MEFs indicating that *Gadd45b* engages the MKK7–JNK–cJun pathway in regulating senescence. It is important to note that, given that there is a gradual increase in senescent cell population with increasing passage number (Fig 3.1), the modest decrease in SA-β-gal staining in MEFs treated with the JNK inhibitor SP600125 for 6 days suggests that, JNK inhibition leads to a decrease in newly senescing cell populations but has no effect on cell populations that are already senescent.

Similarities between the senescence phenotype of *Gadd45b*$^{-/-}$ MEFs and MEFs deficient for genes involved in DNA damage pathways suggested that growth arrest in *Gadd45b*$^{-/-}$ MEFs might be attributable to DNA damage accumulation. To test this, we

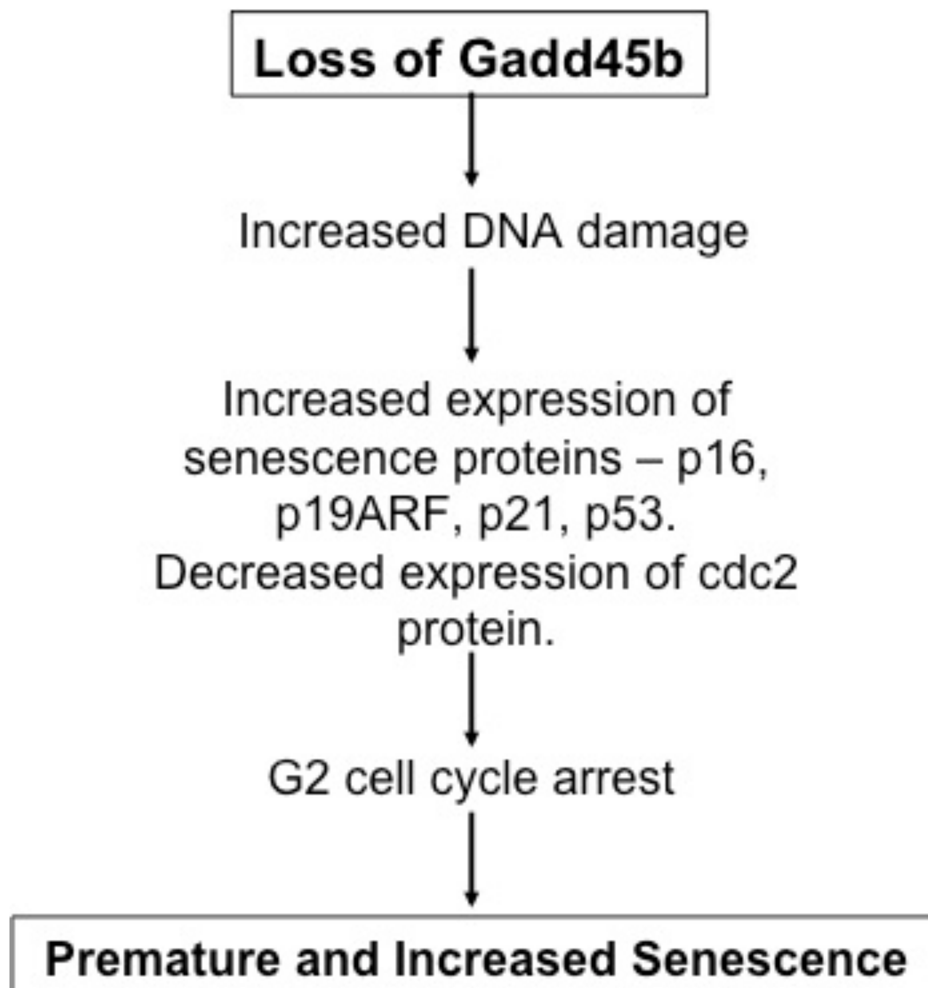


Figure 3.14. Schematic illustrating the role of *Gadd45b* in tissue culture induced senescence

Loss of *Gadd45b* leads to increased DNA damage, increased expression of senescence proteins, G2 celly cycle arrest and premature senescence.

cultured *Gadd45b*$^{-/-}$ MEFs under conditions which limit oxidative DNA damage (the major environmental insult cells experience during conventional culture) [135]. Our results show that culturing cells under more physiological oxygen conditions results in partial rescue of their ability to proliferate, thereby demonstrating that *Gadd45b*$^{-/-}$ MEFs have increased sensitivity to oxidative stress. It should be noted, however, that *Gadd45b*$^{+/+}$ MEFs also proliferated better in 3% oxygen. , such that there was still a difference between *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs. Both *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ MEFs had high levels of DNA damage and gH2AX-containing repair foci in their nuclei, but the number of repair foci and the amount of DNA damage were significantly higher in the *Gadd45b*$^{-/-}$ MEFs. Thus it appears that *Gadd45b*$^{-/-}$ MEFs have essentially the same defects such as high levels of DNA damage and premature senescence as later passage *Gadd45b*$^{+/+}$ MEFs but these defects occur earlier in cells lacking *Gadd45b*. Previous reports have shown that normal mouse cell senescence occurs as a consequence of the accumulation of DNA damage resulting from hyperoxic culture conditions [112] [197]. Our data provide an important extension of this notion, showing for the first time that *Gadd45b* plays a critical role in protecting MEFs from oxidative stress and limiting the senescence response. In addition to oxidative stress, other environmental stresses such as UV irradiation and hydrogen peroxide also trigger premature senescence in *Gadd45b*$^{-/-}$ MEFs compared to *Gadd45b*$^{+/+}$ MEFs.

Importantly, staining E14 embryos from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice with SA-β-gal staining provided *in vivo* evidence for increased senescence in *Gadd45b*$^{-/-}$ mice. This indicates that loss of *Gadd45b* modulates the senescence response to various stimulants involved in embryonic development. It will be interesting to further

characterize the senescence distribution among different organs and tissues in the embyos of *Gadd45b*$^{-/-}$ mice as well as analyze the senescence distribution during the various stages of embryonic development.

Finally, our findings raise several interesting questions that warrant further investigation: 1) How does *Gadd45b* regulate senescence driven by other stressors 2) How does *Gadd45b* interface with different signaling pathways in response to distinct stressors? 3) What role does *Gadd45b* expression or the lack of it play in the senescence response of human cells? and, 4) Do other Gadd45 genes (i.e., Gadd45a and Gadd45g), either separately or in combination with *Gadd45b*, regulate stress induced senescence? Several other genes including Mkk7 [186] , cJun [112] , Brca1[30], Id1 [5] , Hus1[97] , Jnk [44] , Ku80 [102] , Polmu [110] , Vhl [190], Dicer [124] and TGFb [197] have been observed to limit tissue culture-induced senescence similar to *Gadd45b*, where their loss resulted in premature senescence. Thus, it will be of interest to determine whether there is crosstalk between *Gadd45b* regulated molecular pathways that protect cells from undergoing tissue culture-induced senescence and molecular pathways regulated by these other proteins. Notably, the observation that *Gadd45b* loss results in premature senescence is in contrast to *Gadd45a* KO MEFs that were observed to escape senescence (Unpublished data). Thus, it will be also of interest to compare and contrast *Gadd45b* signaling to *Gadd45a* signaling in MEFs, where loss of *Gadd45a* results in escape from tissue cultured senescence. Furthermore, studies using transgene double knockout mouse model will help elucidate which function of Gadd45 proteins (senescence promoting versus senescence limiting) is dominant. Current research is targeted at addressing these interesting issues.

In conclusion, the results obtained indicate that Gadd45 proteins differentially modulate stress- and tissue culture-induced cellular senescence, providing the impetus to further investigate the role of Gadd45 proteins in senescence under normal physiological conditions as well as in various pathological conditions.

CHAPTER 4

GADD45B DEFICIENCY PROMOTES SENESCENCE AND AGING PHENOTYPES IN THE SKIN

CHAPTER 4

GADD45B DEFICIENCY PROMOTES SENESCENCE AND AGING PHENOTYPES IN THE SKIN

4.1 Introduction

The Gadd45 proteins have been implicated in several cellular functions that are intimately linked to aging and age-related diseases (ARDs), including DNA repair, maintaining genome stability, epigenetic regulation, cell cycle arrest, cellular senescence [71, 100, 164]. Given the critical role that Gadd45 proteins play in regulating senescence, studying the Gadd45 family of proteins may provide potential therapeutic targets for combating age-related diseases and promoting longevity.

In 1995, Dimri and colleagues identified a novel biomarker for senescence - a beta galactosidase, histochemically detectable at pH 6. The group showed that in skin samples from human donors of different age, there was an age-dependent increase in Senescence associated β-galactosidase activity in dermal fibroblasts and epidermal keratinocytes thus providing *in situ* evidence senescent cells may exist and accumulate with age *in vivo* [49]. To explore the idea that senescent cells accumulate in vivo, skin samples from 20 human donors, aged 20-90 yr, were sectioned and stained for Senescence associated β-galactosidase. While none of the young donors (<40 yr) showed dermal staining, by contrast, all but one old (>69 yr) donor had positive dermal staining indicating that senescence staining in the dermis and epidermis increased with age in frequency and intensity [49].

It has been shown that mice lacking Mdm2 (the chief negative regulator of p53) in the epidermis showed an aging phenotype in the skin of mice, including increased cellular senescence, thinning of the epidermis, reduced wound healing, and a progressive loss of fur. The Mdm2Δ/Δ mice showed a premature aging phenotype in the skin that is highly similar to the accelerated aging phenotype that was observed previously in the skin of p53 m-allele mice, including a thinning of the epidermal layer, reduced wound healing, and a reduced capacity to re-grow fur. Given that the deletion of Mdm2 results in an increase in p53 levels and upregulation of select p53 target gene expression, these results corroborate that increased p53 activity leads to accelerated aging phenotypes observed previously in this model and in certain other p53 models [58].

It was also shown that the number of senescent cells, as well as impaired mitochondrial (complex II) activity increase in naturally aged mouse skin. They also showed, using a mouse model of genetic Sod2 deficiency, that loss of this important mitochondrial anti-oxidant enzyme impairs mitochondrial complex II activity, causes nuclear DNA damage, and induces cellular senescence in the skin. They also showed that Sod2 deficiency resulted in reduced cellularity and thickness of the epidermis, thus indicating that mitochondrial oxidative stress and cellular senescence contribute to aging skin phenotypes *in vivo* [184].

In 2012, Stoyanova and colleagues showed that loss of p53-induced genes DDB2 and p21 resulted in a severe Deficiency in UV-induced Premature Senescence. SA- beta galactosidase staining of skin sections from mice irradiated with UV-B showed that while both p21-/- and Ddb2-/- mice exhibited deficiencies in senescence response in

comparison with WT mice after UV damage, Interestingly, the Ddb2-/- p21-/- mice exhibited a more severe deficiency in senescence response compared with the Ddb2-/- or the p21-/- mice [160].

Studies using a transgenic mice model revealed that inducibly expressing p14ARF (human ortholog of murine p19ARF) led to the activation of endogenous Cdkn2a products p19ARF and p16Ink4a revealing a senescence promoting feed-forward loop in the skin. They also showed that p14ARF-induced epidermal cell senescence is dependent on p53. The authors hypothesized that, once initiated; the senescence program could be executed independently of the inducing signal. To test whether this is the case, they activated p14ARF for 4 days and then resilenced the transgene by doxycycline removal for another 3 days. While SA β-gal-positive cells appeared in mice in which the transgene was continuously expressed for 7 days, senescent cells were not observed in mice in which the transgene was silenced indicating that cells do not commit to senescence until at least 5 days of continuous high p14ARF expression levels, despite activating p53, p16Ink4a, and p19ARF, (i.e., persistent stress leading to continuous p14/19ARF expression is necessary for cells to commit to senescence) and are able to revert to a non-senescent state or be eliminated from the tissue [170].

Given the critical role that senescence plays in aging, it was of interest, therefore, to extend our investigation of the role of *Gadd45b* in modulating senescence by expanding our research design to include aging phenotypes in the skin. By investigating the effects of aging in combination with *Gadd45b* deficiency in skin, we were able to examine how *Gadd45b* may modulate aging and senescence *in vivo* in a physiological context.

To investigate the role of *Gadd45b* in DNA damage and aging in skin, dorsal skin sections of 4 month and 11-month-old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were subjected to histo-pathological evaluation. We show that sections from 11-month-old *Gadd45b*$^{-/-}$ mice showed a significant increase in DNA damage, increase in Senescence associated β-galactosidase staining and a significant decrease in dermal cellularity compared with their wild type counterparts.

Taken together, these data indicate that *Gadd45b* deficiency promotes cellular senescence and aging phenotypes in the skin.

4.2 Loss of *Gadd45b* leads to increased DNA damage in skin

To investigate the role of *Gadd45b* in DNA damage and aging in skin, dorsal skin sections of 4 month and 11-month-old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were subjected to histo-pathological evaluation. Immuno-histochemical analysis of the well-established marker phosphorylated histone H2AX (γH2AX) was done to score skin sections for nuclear DNA double-strand breaks. While skin sections from 4-month-old *Gadd45b*$^{-/-}$ mice showed a slight increase in the number of phospho- histone H2AX (γH2AX) stained cells, skin sections from 11 month old *Gadd45b*$^{-/-}$ mice showed a significant increase in the number of phospho- histone H2AX (γH2AX) stained cells compared with their wild type counterparts (Figure 4.1). These data indicate that *Gadd45b* deficiency leads to increased DNA double-strand breaks in the skin *in vivo*.

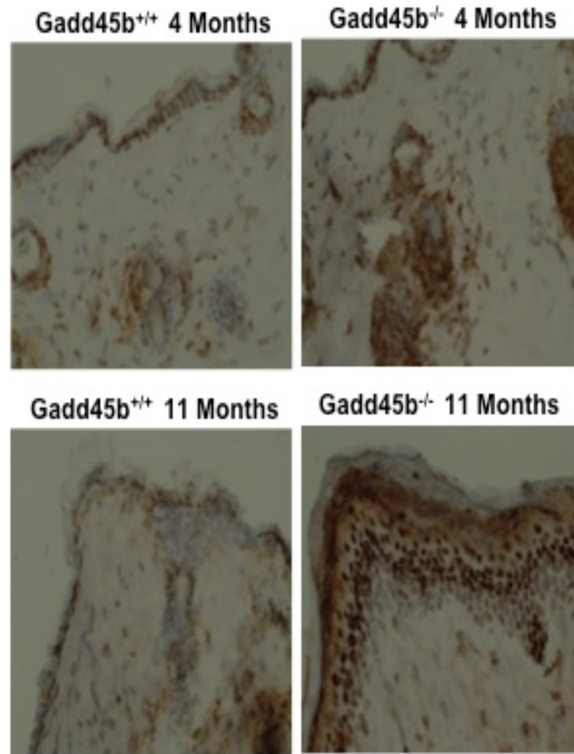
4.3 Loss of *Gadd45b* results in increased senescence in skin

In order to further characterize this aging phenotype and analyze the role of senescence in skin aging, Senescence associated β-galactosidase staining was carried out. Interestingly, while skin sections from 4-month-old *Gadd45b*$^{-/-}$ mice showed a slight increase in the number of Senescence associated β-galactosidase stained cells, skin sections from 11-month-old *Gadd45b*$^{-/-}$ mice showed a

significant increase in the number of Senescence associated β-galactosidase stained cells compared with their wild type counterparts (Figure 4.2). Furthermore, the senescence stained cells were mostly seen in the dermal section of the skin.

4.4 Loss of *Gadd45b* results in decreased cellularity in the dermal section of the skin

Given that DNA damage and senescence play a critical role in the aging of skin, the effect of *Gadd45b* deficiency on skin aging was investigated by histopathological analysis using H&E staining. While skin sections from 4-month-old *Gadd45b*$^{-/-}$ mice showed higher number of cells in the dermis, skin sections from 11-month-old *Gadd45b*$^{-/-}$ mice showed a significant decrease in the number of cells in the dermis, compared with their wild type counterparts (Figure 4.3). These data indicate that *Gadd45b* deficiency promotes aging phenotypes in the skin including increased DNA damage, increased senescence and decreased dermal cellularity.



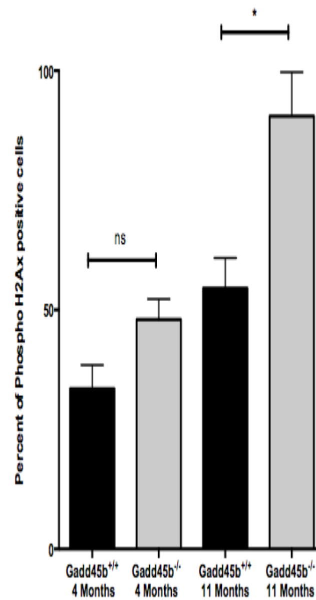
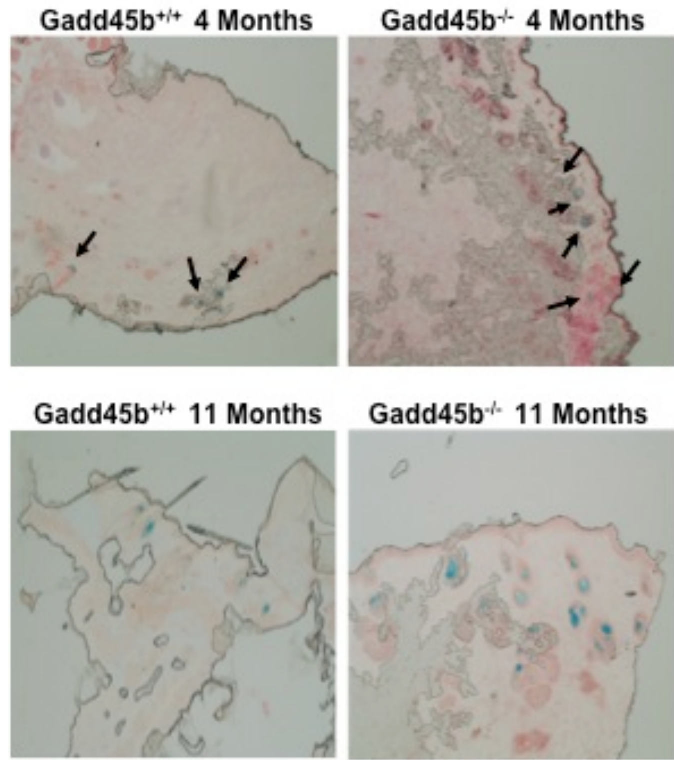


Figure 4.1. Loss of *Gadd45b* promotes DNA double-strand breaks in the skin. *Top:* Immuno-histochemical analysis of phosphorylated histone H2AX (γH2AX) of dorsal skin sections from 4 month and 11 month old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice showed a significant increase in the number of phospho- histone H2AX (γH2AX) stained cells in 11 month old *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts. *, $p < 0.05$. *Bottom:* A quantification of phosphorylated histone H2AX (γH2AX) cells from different mice is shown.



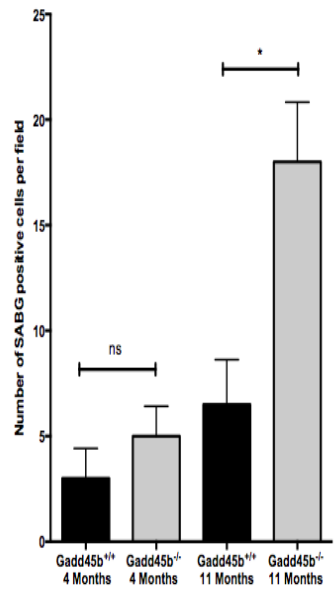
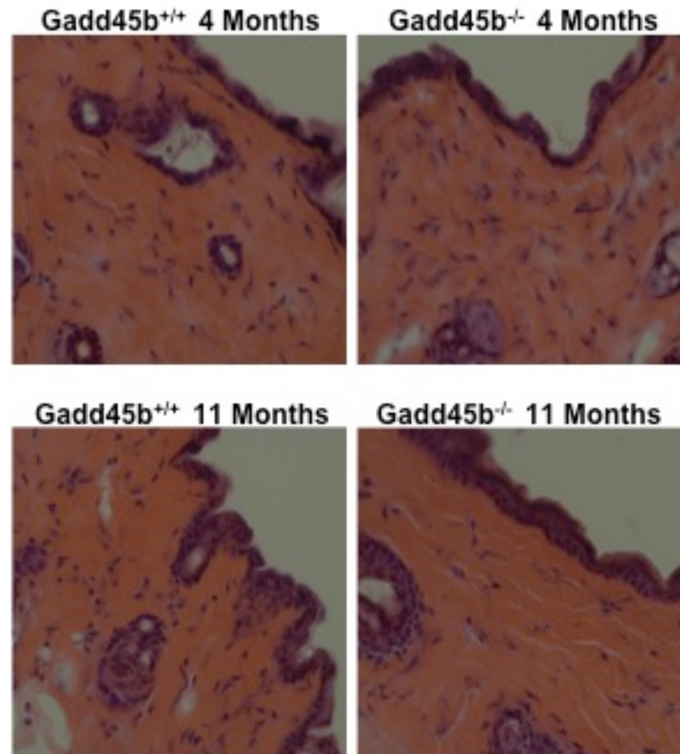


Figure 4.2. Loss of *Gadd45b* promotes senescence in the skin.

Top: SA β-gal staining of dorsal skin sections from 4 month and 11 month old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice showed a significant increase in the number of senescent cells in 11 month old *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts. *, $p < 0.05$. Arrows indicate skin sections with strong senescence staining. *Bottom:* A quantification of SA β-gal positive cells from different mice is shown.



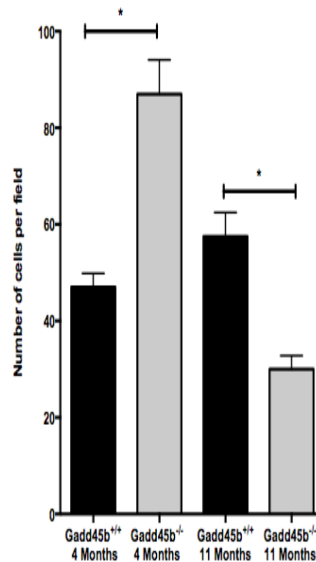


Figure 4.3. Loss of *Gadd45b* promotes aging phenotypes in the skin.

Top: Histopathological analysis using H&E staining of dorsal skin sections from 4 month and 11 month old *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice showed a significant decrease in dermal cellularity in 11 month old *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts, as indicated by decreased number of purple stained cells. *Bottom:* A quantification of purple stained cells in the dermis of different mice is shown. *, $p < 0.05$.

4.5 Discussion

Mouse models have proven invaluable to the investigation of senescence and DNA damage in aging. Given the critical role that senescence plays in aging, it was of interest, therefore, to extend our investigation of the role of *Gadd45b* in modulating senescence by expanding our research design to include aging phenotypes in the skin. By investigating the effects of aging in combination with *Gadd45b* deficiency in skin, we were able to examine how *Gadd45b* may modulate aging and senescence *in vivo* in a physiological context.

Using this model, we demonstrate for the first time, that the loss of *Gadd45b* significantly promotes senescence and aging phenotypes in the skin. Furthermore, it is shown that loss of *Gadd45b* leads to increased DNA double strand breaks. This raises several interesting questions. 1) What is the expression status of senescence markers including the $p19^{Arf}$ -p53-p21 pathway in these skin samples? 2. What is the effect of loss of *Gadd45b* on aging in other organs and the entire life span? 3. What is the effect of loss of *Gadd45b* on aging at later time points? 4.Are the anti-aging effects of *Gadd45b* limited only to its role in regulating DNA damage? Or does it regulate other aging mechanisms including the endocrine system, immune system, free radical regulation, protein damage and maintenance? 5. What are the signaling pathways involved in *Gadd45b* mediated regulation of DNA damage and aging phenotypes in the skin? 6. What is the expression status of *Gadd45b* with increasing age? 7) Can the restoration of *Gadd45b* expression prevent or slow down aging? 8) What role does *Gadd45b* expression or the lack of it play in the senescence response of human skin? 9) Do other Gadd45

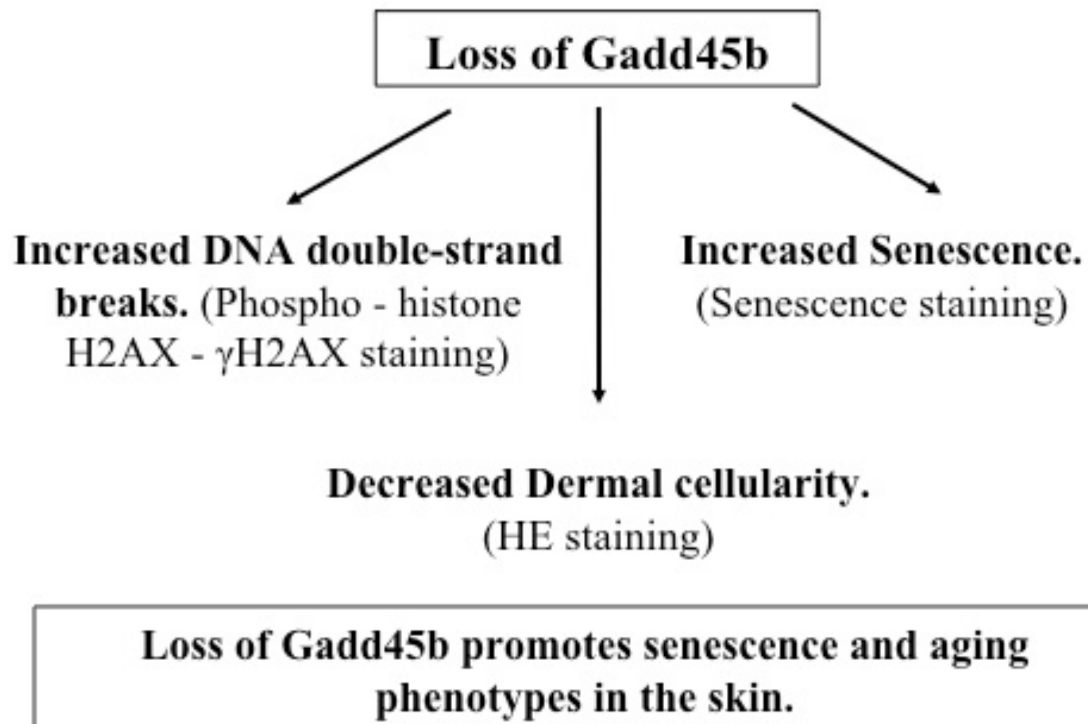


Figure 4.4. Schematic illustrating the role of *Gadd45b* in skin aging

Loss of *Gadd45b* leads to increased DNA damage and promotes senescence and aging phenotypes in the skin.

genes (i.e., Gadd45a and Gadd45g), either separately or in combination with *Gadd45b*, regulate aging in skin? 10) Several other genes including BMAL1 [88], XP-C [76], SOD2 [184], NRMT1 [22] have been observed to promote aging phenotypes in the skin. Thus, it will be of interest to determine whether there is a crosstalk between *Gadd45b* regulated molecular pathways and pathways regulated by these other proteins. Additional work is underway focused on addressing these interesting issues.

In conclusion, the results obtained highlight a novel and significant role for *Gadd45b* in regulating aging phenotypes in the skin providing the impetus to further investigate the role of Gadd45 proteins in physiological and pathological conditions that trigger senescence and aging in skin and other tissues.

CHAPTER 5

GADD45B DEFICIENCY PROMOTES SENESCENCE AND ATTENUATES CCL4 INDUCED LIVER FIBROSIS

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GADD45B DEFICIENCY PROMOTES SENESCENCE AND ATTENUATES CCL4 INDUCED LIVER FIBROSIS

5.1 Introduction

Fibrosis is a wound healing process that is characterized by the deposition of extracellular matrix components including collagens, leading to encapsulation of the injury site. Liver fibrosis, a pathological feature that is a precursor of cirrhosis, is characterized by the accumulation of fibrotic tissue and the concomitant loss of liver function. It can be triggered by chronic liver damage associated with hepatitis virus infection, alcohol abuse or liver steatosis (fatty liver disease). It has been shown that during chronic damage, hepatic stellate cells (HSCs) become activated and abnormally proliferate as myofibroblasts (damage-activated fibroblasts). Recent studies have shown that myofibroblasts become senescent and produce a stable fibrotic scar with abundant collagen and other extracellular matrix components. In human patients, SA β-gal-positive cells accumulate in the periphery of the fibrotic scar [191]. In rodents, chronic treatment with carbon tetrachloride (a liver-damaging agent) or bile duct ligation produces liver fibrosis, which is characterized by positive SA β-gal-positive cells that are derived from activated HSCs and show increased p53, p21 and p16 and other senescence markers.

Senescence deficient mice have demonstrated the beneficial role of senescence in restricting liver fibrosis. In response to liver damage, mice lacking Trp53 and/or Cdkn2a

present senescence negative fibrotic areas that are larger than those in senescence-competent mice. Similarly, the extracellular matrix protein CCN1 (also known as CYR61; a member of the CCN family) produced by damaged hepatocytes has been shown to be a key mediator of senescence induction in HSCs. Accordingly, mice with CCN1 deficient hepatocytes do not execute HSC senescence leading to an exacerbated fibrotic response [82]. Also, production of IL-22 by activated HSCs induces HSC senescence in association with p53 activation through STAT3 (signal transducer and activator of transcription 3)–SOCS3 (suppressor of cytokine signaling 3). Accordingly, transgenic mice over expressing IL-22 in the liver show a more efficient and faster resolution of fibrosis. These studies demonstrate that the induction of HSC senescence could be a potential therapeutic strategy to limit liver fibrosis. Recent studies have shown that treatment with recombinant protein CCN1 or IL-22 reverts already established hepatic fibrosis by promoting HSC senescence [89].

On the other hand, there are genes that limit senescence, and mice lacking these genes have been shown to present increased senescence and reduced fibrotic scars in response to liver toxins. The toll-like receptor-9 (TLR9) agonist cytosine phosphate guanine (CpG) has been shown to activate hepatic stellate cells (HSCs) and mediate fibrosis. In $TLR9^{-/-}$ mice, HSCs become senescent shortly following activation leading to a decrease in the activated HSCs population and extracellular matrix deposition in fibrotic scars [2].

Loss of Smad3, a key mediator of the transforming growth factor (TGF)-β1 signaling pathway that plays central role in inflammation and fibrosis, led to reduced CCl4-induced liver fibrosis. Proteomic analysis revealed that proteins related to

antioxidant activities such as senescence marker protein-30 (*SMP30*), selenium-binding proteins (SP56) and glutathione *S*-transferases (GSTs) were up-regulated in *Smad3*$^{-/-}$ mice indicating that that Smad3 deteriorate hepatic injury by inhibiting antioxidant proteins as well as mediators of TGF-β1 signaling. Hepatocyte-specific ablation of PP2Acα (PP2A catalytic subunit Cα) protects against CCl4-induced chronic hepatic injury and fibrogenesis through impaired TGF-β1/Smad signaling [78, 109].

Several studies have been published elucidating the role of Gadd45 proteins in liver. Gadd45b shows the strongest immediate-early induction common to two distinctive proliferation responses of the liver: (1) regeneration induced by surgical partial hepatectomy and (2) hyperplasia induced by the primary mitogen TCPOBOP, a ligand of the constitutive androstane receptor (CAR). Furthermore, *Gadd45b* is induced through a CAR Dependent, TNF-Independent Pathway in murine liver hyperplasia [38, 168]. *Gadd45b* deficiency in mice led to decreased hepatocyte proliferation and increased program cell death during liver regeneration after partial hepatectomy. Notably, c-Jun N-terminal kinase (JNK) activity was markedly increased and sustained in livers of *Gadd45b*$^{-/-}$ mice compared with control animals after partial hepatectomy. Furthermore, loss of JNK2, by imposition of a Jnk2-null mutation, attenuated JNK activity, completely rescuing the regenerative response in *Gadd45b*$^{-/-}$ mice [134]. It was also shown that in murine hepatocyte AML12 cells, *Gadd45b* mediates p38- induced Rb phosphorylation by enhancing the interaction between p38 and Rb during Fas-induced apoptosis.

Gadd45b$^{-/-}$ mice have also been shown to have intact proliferative responses, but marked growth delays following administration of a single dose of TCPOBOP. Moreover, early transcriptional stimulation of CAR target genes was weaker in *Gadd45b*$^{-}$

$^{/-}$ mice than in wild-type animals, and more genes were downregulated. Gadd45b was also found to directly regulate transcription by physically binding to CAR. Also, TCPOBOP treatment led to localization of both proteins to a regulatory element for the CAR target gene cytochrome P450 2b10 (Cyp2b10)[168].

Furthermore, Gadd45b induction facilitates increased transcription, allowing rapid expansion of liver mass for protection against xenobiotic insults such as TCPOBOP (1,4-bis[2-(3,5)-dichoropyridyloxy] benzene), a ligand of the nuclear receptor constitutive androstane receptor (CAR). A recent study showed that PPARα indirectly induces Gadd45b gene expession in liver through promoting degradation of the repressor STAT3 in response to elevated oxidative stress. Gadd45b mRNA was markedly induced by the PPARα agonist, Wy-14,643, in wild-type mice but not in Ppara-null mice. Also studies using liver-specific Stat3-null mice showed that STAT3 is a repressor of the Gadd45b gene through binding to upstream regulatory elements [81] .

Given the critical role that Gadd45b plays in liver regeneration and cellular senescence, it was of interest, therefore, to extend our investigation of the role of Gadd45b in modulating senescence by expanding our research design to include a toxin induced liver fibrosis model. By investigating the effects of chronic, sub lethal doses of CCL4 in combination with *Gadd45b* deficiency on liver fibrosis, we were able to examine how *Gadd45b* may modulate senescence *in vivo* in a disease context.

We show that liver sections from CCl4 treated *Gadd45b*$^{-/-}$ mice show reduced cyto-architectural damage (fibrotic scars), reduced collagen deposition and increased senescence. Taken together, these data indicate that *Gadd45b* deficiency promotes senescence and attenuates CCl4 induced liver fibrosis.

5.2 Loss of *Gadd45b* is protective against CCl4 induced chronic hepatic injury

To investigate the role of *Gadd45b* in liver fibrosis, *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were injected intra-peritoneally twice weekly for 6 weeks with sub-lethal doses of CCl4 in olive oil or an equal volume of olive oil alone as vehicle control. 3 days after the final injection, livers were harvested and processed for sectioning and histo-pathological evaluation. Interestingly, Histopathological analysis using H&E staining revealed reduced cyto-architectural damage (fibrotic scars) in *Gadd45b*$^{-/-}$ mice compared with *Gadd45b*$^{+/+}$ mice (Figure 5.1).

5.3 Loss of *Gadd45b* results in reduced collagen deposition in response to CCl4 induced chronic hepatic injury

In order to further characterize this fibrotic phenotype and analyze the collagen deposition, Sirius red staining was carried out. *Gadd45b*$^{-/-}$ mice showed reduced hepatic collagen accumulation compared with *Gadd45b*$^{+/+}$ mice after the chronic CCl4 challenge. These data indicate that loss of *Gadd45b* protected against CCl4 induced chronic hepatic injury (Figure 5.2).

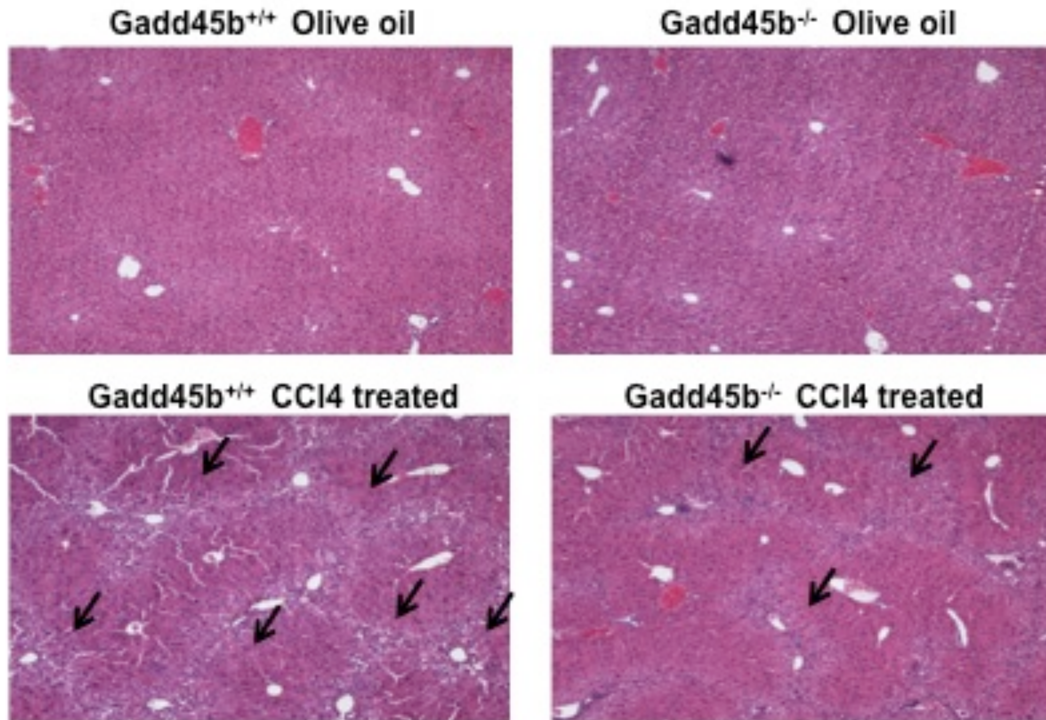

Figure 5.1 *Gadd45b* deficiency attenuates CCl4-induced liver fibrosis progression

Histopathological analysis using H&E staining of liver sections from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice treated with sub-lethal doses of CCl4 in olive oil or olive oil control showed a significant decrease in cyto-architectural damage (fibrotic scars) in CCl4 treated *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts. Arrows indicate liver sections with fibrotic scars.

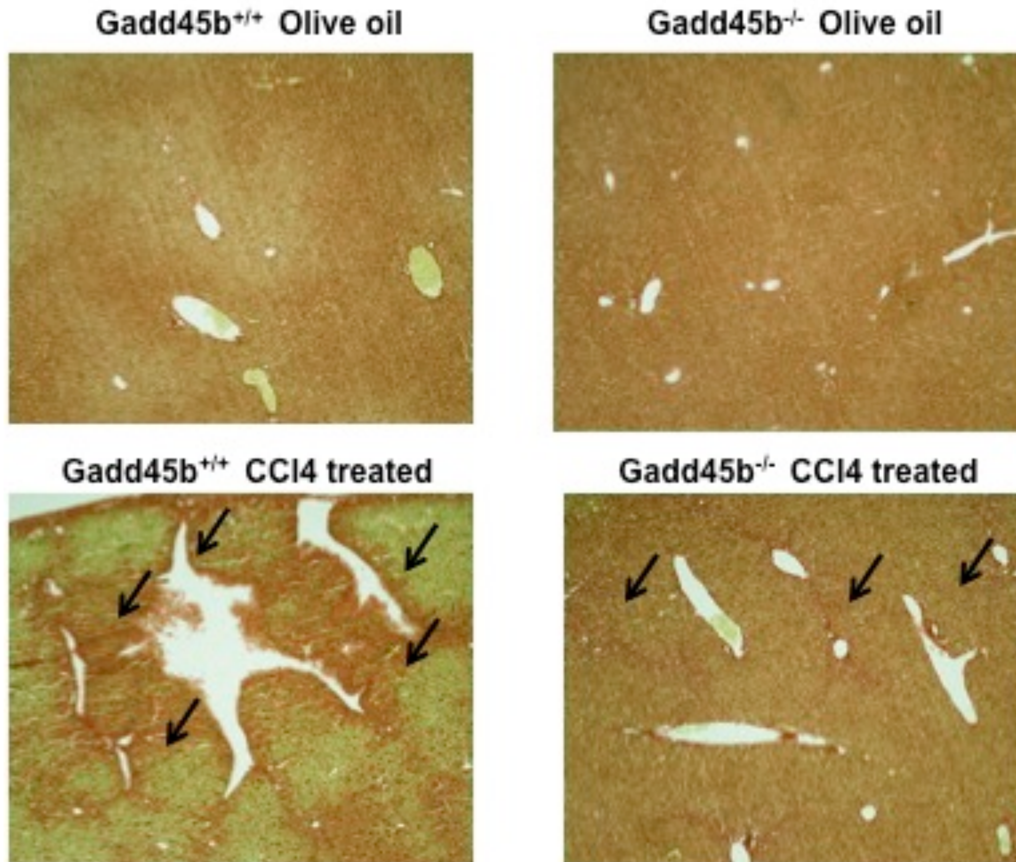

Figure 5.2. ***Gadd45b*** **deficiency attenuates CCl4-induced collagen accumulation in liver**

Histopathological analysis using Pico-Sirius Red staining of liver sections from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice treated with sub-lethal doses of CCl4 in olive oil or olive oil control showed a significant decrease in collagen accumulation in CCl4 treated *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts. Arrows indicate liver sections with collagen accumulation.

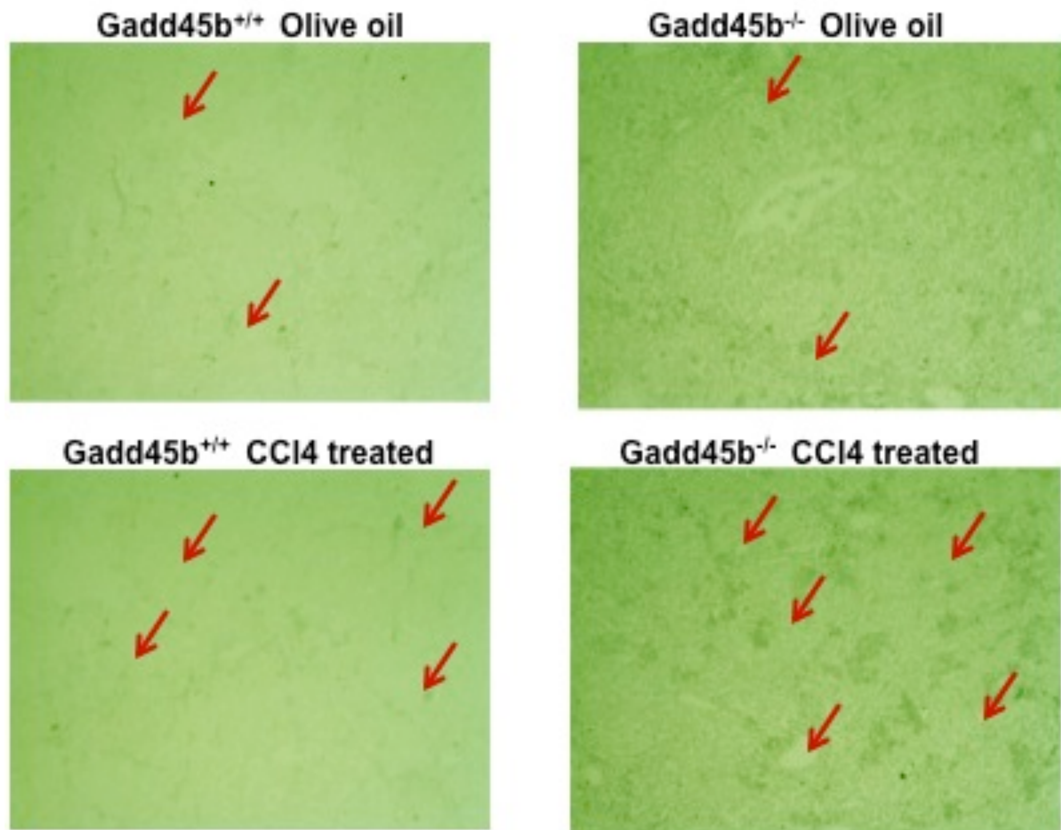

Figure 5.3 Loss of *Gadd45b* results in increased senescence in response to CCl4 induced chronic hepatic injury

SA β-gal staining of liver sections from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice treated with sub-lethal doses of CCl4 in olive oil or olive oil control showed a significant increase in senescence staining in CCl4 treated *Gadd45b*$^{-/-}$ mice compared with their wild type counterparts. Arrows indicate liver sections with senescence staining.

5.4 Loss of *Gadd45b* results in increased senescence in response to CCl4 induced chronic hepatic injury

Given that senescence has been shown to be a protective mechanism against liver fibrosis, and that *Gadd45b*$^{-/-}$ MEFs show increased senescence compared to *Gadd45b*$^{+/+}$ MEFs, it was of interest therefore to investigate the level of cellular senescence within the liver samples. To identify senescent cells in situ, liver sections from CCl4 and vehicle-treated mice were subjected to senescence associated β-galactosidase staining. As predicted, *Gadd45b*$^{-/-}$ mice showed increased senescence staining in livers of CCl4-treated mice compared with *Gadd45b*$^{+/+}$ mice. These data indicate that loss of *Gadd45b* leads to increased senescence, which is protective against CCl4 induced liver fibrosis leading to reading to reduced fibrotic scars and reduced collagen deposition (Figure 5.3).

5.5 Discussion

Mouse models of liver fibrosis have proven invaluable to the investigation of liver fibrogenesis and chronic hepatic injury. In order to examine the role of *Gadd45b* in liver fibrosis, a toxin induced liver fibrosis model, which involves the chronic injection of sub lethal doses of CCl4, was used. Using this model, we demonstrate for the first time, that the loss of *Gadd45b* significantly protects against liver fibrosis. Furthermore, it is shown that loss of *Gadd45b* leads to increased senescence in response to chronic liver injury. This raises several interesting questions. 1) What is the expression status of senescence markers including the p19^{Arf} -p53-p21 pathway? 2) Since activation of Hepatic Stellate

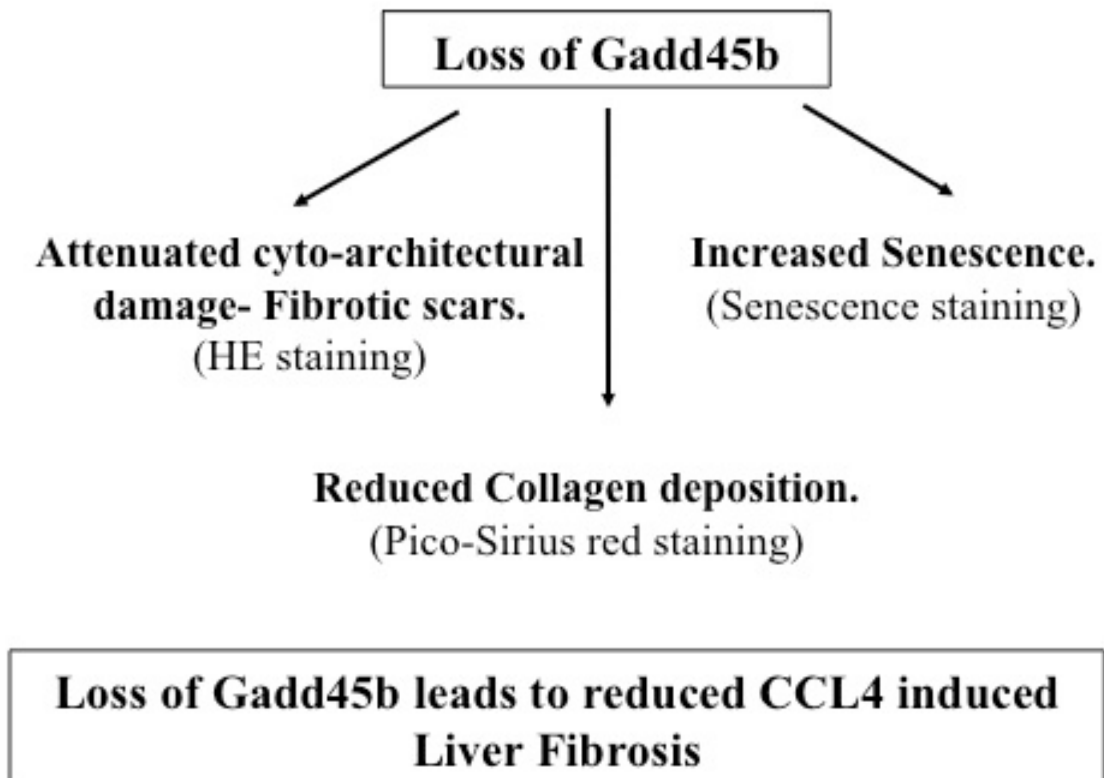

Figure 5.4. Schematic illustrating the role of *Gadd45b* in regulating CCl4 induced liver fibrosis

Loss of *Gadd45b* leads to increased senescence; reduced collagen deposition and an attenuated fibrotic response to CCl4 induced liver fibrosis.

Cells (HSCs) to overproduce extra cellular matrix is a key event in the pathophysiology of hepatic fibrosis and A -smooth muscle actin (A-SMA) is a marker for activated HSCs, what is the expression status of A-SMA in livers from control and CCl4 treated mice. 3) Is *Gadd45b* necessary for initiation alone or the progression of hepatic fibrogenesis? 4) Can novel drugs targeting Gadd45b – senescence pathways be used as candidate therapeutic agents in the treatment of chronic hepatic injury. 5) What are the signaling pathways involved in *Gadd45b* mediated regulation of CCl4 induced liver fibrosis. 6) Does *Gadd45b* regulate other chronic hepatic injury models including Cholestatic liver injury (bile duct ligation (BDL)), thioacetamide (TAA), Dimethylnitrosamine (DMN), Alpha-Naphthyl Isothiocyanate (ANIT), 3,5-Diethoxycarbonyl-1,4-dihydrocollidine (DDC) [101]? 7) What role does *Gadd45b* expression or the lack of it play in the senescence response of human cells? 8) Do other Gadd45 genes (i.e., Gadd45a and Gadd45g), either separately or in combination with *Gadd45b*, regulate liver fibrosis? 9) Several other genes including PP2Acα [109], TLR9 [2], SMAD3 [78] have been observed to promote liver fibrosis. Thus, it will be of interest to determine whether there is a crosstalk between *Gadd45b* regulated molecular pathways and pathways regulated by these other proteins. Additional work is underway focused on addressing these interesting issues.

In conclusion, the results obtained highlight a novel and significant role for *Gadd45b* in the senescence response to CCl4 induced liver injury providing the impetus to further investigate the role of Gadd45 proteins in physiological and pathological conditions that trigger senescence in liver.

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