A ROLE FOR EPIGENETICS IN AGING AND THE AGE-DEPENDENT RESPONSE TO IONIZING RADIATION

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Dedicated to my parents – Irene and Juhani,

and my sisters – Nicole and Annikka,

for being there every step of the way!

ABSTRACT

Aging is associated with the functional decline of organs, and results in age-dependent differences in stress-sensitivity. Younger animals are more sensitive to mutagenic insults than adults, whereas in plants, the transition to reproductive growth is the most stress-sensitive.

Here, we show a role of reduced H3K9 trimethylation and corresponding genomic instability in the aging rat thymus and in senescence of human fibroblasts. A similar reduction of SUV39H1 expression was observed in response to ionizing radiation (IR), which correlated with the induction of senescence.

In plants exposed to IR during the transition to reproductive growth, histone methyltransferases were up-regulated, which correlated with reduced transcription of transposable elements.

This difference in response may be reflected in the differences in life cycles. Whereas plants rely on the survival and genome integrity of meristematic cells for their reproductive success, in mammals, limiting the cell division in cells that have incurred damage may be crucial.

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ABA	Abscisic acid
ACK lysis buffer	Ammonium-chloride-potassium lysis buffer
ANOVA	Analysis of variance
ATM	Ataxia telangiectasia mutated
BER	Base excision repair
BRCA	Breast cancer
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CCD	Charged couple device
CDKB2	Cyclin-dependent kinase B2
CD marker	Cluster of differentiation marker
cDNA	Complementary deoxyribonucleic acid
C_{12}FDG	5-Dodecanylaminofluorescein di-β-D-galactopyranoside
ChIP	Chromatin immunoprecipitation
CK	Cytokinin
CPM	Counts per minute
dCTP	Deoxycytidine triphosphate
dNTP	Deoxyribo nucleoside triphosphate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DSB	Double-strand break
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box class O
Fwd	Forward primer
GA	Gibberellin
Gbp	Gigabase pairs
gDNA	Genomic deoxyribonucleic acid
Gy	Gray
HR	Homologous recombination
HRF	Homologous recombination frequency
IFWB	Immunofluorescence wash buffer
IR	Ionizing radiation
JA	Jasmonic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
LRR	Leucine-rich repeat
LMP	Low melting point
Mbp	Megabase pairs
MeJA	Methyl jasmonate
MEM	Minimum essential medium
MMR	Mismatch repair

mRNA	Messenger ribonucleic acid
miRNA	MicroRNA
MS	Murashige and Skoog medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NBS	Nucleotide binding site
NER	Nucleotide excision repair
NHEJ	Non homologous end joining
nt	Nucleotide
ORF	Open reading frame
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing 0.1% Tween20
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD	Population doubling
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PFA	Paraformaldehyde
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis related gene
PRR	Pattern-recognition receptor
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
R gene	Resistance gene
RB	Retinoblastoma protein
rDNA	Ribosomal deoxyribonucleic acid
RE	End of reproductive growth stage
Rev	Reverse primer
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
SA	Salicylic acid
SA-β-GAL	Senescence associated β-Galactosidase
SADS	Senescence-associated distension of satellites
SAG	Senescence-associated gene
SAHF	Senescence-associated heterochromatin foci
SAM	Shoot apical meristem (in Chapter 3)
SAM	S-adenosylmethionine (in Chapter 6)
SAR	Systemic acquired resistance
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis
SIPS	Stress-induced premature senescence
siRNA	Small interfering RNA
SOD	Sodium dismutase
SOG1	Suppressor of gamma response 1
T6P	Trehalose-6-phosphate

TBE	Tris-borate-EDTA buffer
TFBS	Transcription factor binding site
TLR	Toll-like receptor
TOR	Target of rapamycin
TSS	Transcription start site
VE	End of vegetative growth phase

1. INTRODUCTION

Aging is a major risk factor for several morbidities, including cancer, cardiovascular disease and autoimmune disease. It is associated with an altered capacity to cope with internal stress induced by metabolism, and external stresses of biotic and abiotic nature. Thus, the understanding of the molecular mechanisms that advance aging will help us to understand why aging individuals are more prone to those diseases as well as why they may be less stress resistant.

Cells in the aging body exhibit extensive differences to cells in the juvenile organism, ranging from changes in gene expression and DNA methylation patterns (Heyn et al., 2012), shortened telomeres (Allsopp et al., 1992) to deteriorating genome maintenance mechanisms (Gorbunova et al., 2007). At the tissue, organ and systemic levels, cells are exposed to the altered stromal milieu caused by the altered secretory profiles of senescent cells (Rodier et al., 2009) and the deteriorating immune system which is not equally able to mount an immune response towards new antigens (Mackall et al., 1996). The combination of these factors may facilitate the malignant transformation of cells and prevent the efficient recognition and clearance of transformed cells, thus increasing the cancer incidence rate.

Most cancer patients are exposed to low doses of radiation during diagnostic procedures and to high doses of radiation for cancer treatment. While the precision of radiation treatments to target tumor tissues has been significantly improved over the years, in the course of these procedures, radiation exposure of some healthy tissues is inevitable (Paganetti, 2012). Taking into consideration the molecular differences present in cells of a young organism compared to an older one, the consequences of this stress are likely to differ between the two. In line with this, follow-up studies on radiation therapy patients have shown that younger patients suffer from more severe

and more frequent late effects than older patients (Krasin et al., 2010; Paulino et al., 2010). However, the knowledge on molecular mechanisms that underlie the differences in age-dependent radiation-sensitivity is limited.

In contrast to mammals, plants have a much higher radiation resistance – herbaceous plants can tolerate several hundreds of Gray of ionizing radiation compared to a maximum of 6 Gy delivered to humans during a whole body exposure. The molecular and cellular mechanisms underlying this difference are not understood. Possible reasons may include either a potential shielding effect of the plant cell wall or more efficient genome maintenance mechanisms mediated by the presence of multiple homologs of genes that function in DNA repair. A better understanding of the plant's response to ionizing radiation may, therefore, suggest ways to improve the outcome of radiation exposure in humans.

Another major difference between plants and mammals is the way they develop and age. Annual plants like *Arabidopsis thaliana* complete a whole life cycle from seed to seed within one to two months. In contrast to mammals, the germ line in plants is specified relatively late during development at the stage of flower formation (Meeks-Wagner et al., 1989). Thus, mutations and epigenetic modifications acquired throughout development are more readily passed on to the offspring, making efficient genome maintenance mechanisms a necessity in order to secure the success of offspring. The short life cycle of *Arabidopsis* further requires a very tight control of all developmental stages which all serve their specific purposes in ensuring the reproductive success of the plant. In line with this, senescence in plants is associated with the relocation of nutrients and resources from the leaves to the developing seed (Hensel et al., 1993). Although senescence results in a functional decline of primary

photosynthetic organs and eventually of the whole plant, it aids in increasing the overall fitness of the organism by improving seed quality (Leopold, 1961).

Those differences in the plant life cycle – the late determination of the germ line and a tight control over all developmental stages – suggest that the stress sensitivity in plants may not strictly depend on the biological age, but rather on the stage of development. In accordance with this, two studies described an increased stress sensitivity of plants during the reproductive stage (Kim et al., 2007; Zinn et al., 2010).

Based on the current state of knowledge, we hypothesized that aging in mammalian systems is associated with extensive changes to gene expression profiles, which may be mediated by changes in chromatin structure and altered transcriptional regulation. Those changes may further affect the nature of the response to radiation. Similarly, we hypothesized that changes in gene expression and epigenetic profiles throughout the plant's development may direct changes in radiation sensitivity and responses depending on the developmental stage.

In order to address these questions, we employed three different model systems: 1) Long Evans rats for the study of age-dependent changes to the chromatin structure and the transcriptome in primary and secondary immune organs; 2) the human foetal lung fibroblast cell strain WI-38 for the study of senescence-associated changes in gene expression and epigenetic profiles, as well as for the study of senescence-dependent changes in the radiation response; and 3) *Arabidopsis thaliana* (Columbia cultivar, line 15D8) for the study of development-dependent differences in the response to ionizing radiation.

In Chapter 4, we examined the role of changes in the epigenetic status during the aging process of immune organs in Long Evans rats. This showed that a decrease in global DNA methylation and histone H3K9 trimethylation in the thymus of old rats was associated with an increased genome instability. Aging further correlated with changing compositions of T cell populations in the thymus and spleen.

In Chapter 5, we investigated senescence-associated changes in gene expression patterns and the epigenetic status in WI-38 cells. This showed extensive changes in gene expression patterns with an increase in senescence of the culture, that affect cell cycle regulators, DNA repair genes, transcription factors and epigenetic regulators. In addition, we examined the role of SUV39H1 down-regulation in senescent cells and propose a role for it in the deregulation of gene expression and in the induction of genomic instability, which may ultimately contribute to the establishment of senescence.

In Chapter 6, we studied the role of DNA methylation in replicative senescence. This showed that the reduced DNMT1 expression correlated with global DNA hypomethylation and an extensively altered DNA methylation profile.

In Chapter 7, we examined senescence-dependent differences in response to ionizing radiation in WI-38 cells. This revealed that younger cell cultures were more prone to induce senescence in response to low (0.5 Gy) and high (5 Gy) doses of ionizing radiation, which may be associated with senescence-related changes in chromatin structure.

In Chapter 8, we investigated the development-dependent response to ionizing radiation in *Arabidopsis thaliana*. Plants were exposed to a low (10 Gy) or high (100 Gy) dose of X-ray at three different developmental stages. While plants that were irradiated early during development showed more severe aberrations in development

post-IR exposure and in the accumulation of biomass, and plants that were irradiated during the time of bolting induction showed differences in the efficiency of the induction of the expression of DNA repair genes and chromatin modifiers, plants that were irradiated late during development were relatively resistant to IR-dependent changes in the accumulation of biomass or in the regulation of gene expression.

2. LITERATURE REVIEW: GENETICS AND EPIGENETICS OF MAMMALIAN AGING AND AGE-DEPENDENT RADIATION RESPONSE

2.1 ABSTRACT

People have sought to determine a measure that extends life for centuries. Not only does increasing age come with increasing functional decline of organs and organ systems, but it also affects the body's capacity to respond to internal and external stressors, such as transformed cells, infections or DNA damaging agents, like ionizing radiation. Various model organisms have been studied in order to get a better understanding of what molecular mechanisms are driving this age-related functional decline and ultimately limit the lifespan of the given organism. The knowledge from those studies serves as a basis to investigate pharmacological ways to extend healthy lifespan, but also to determine reasons behind different stress sensitivities across the lifespan. In this review, we are discussing different model systems that are commonly used to study aging, some of the insights gained from those studies, as well as how the radiation sensitivity differs between old and young individuals and what might be possible reasons.

2.2 INTRODUCTION

The term "Aging" is generally used to describe the functional decline of cells, tissues, organs and organ systems, alongside the decreasing reproduction rate and increasing mortality rate of organisms with time (Rose, 1991). This is a very conserved phenomenon that occurs in unicellular organisms like budding yeast, multicellular eukaryotic organisms like *C. elegans*, and mammals alike. Therefore, scientists have been studying those various model systems for decades in an attempt

to discover the molecular reasons for aging. Understanding the mechanisms that direct the age-dependent functional decline of cells and organs could help in developing interventions that allow us to ultimately extend the maximum lifespan of an organism; or more interestingly to extend the functional lifespan or healthspan of an organism.

The various model systems offer different benefits to research on aging: short-lived species are useful for the study of genetic mutations or pharmacological interventions that modify their lifespan, whereas model systems that exhibit functional decline of specific organ systems are useful as models to study the causes and consequences of functional decline. Therefore, traditionally research on aging has followed those major directions. Model organisms like budding yeast and the roundworm *C. elegans* have extensively been used to determine genes that, when mutated or silenced, modify lifespan. Those studies have resulted in a vast amount of data supporting roles of insulin signalling, mitochondrial function, protein metabolism and other processes in limiting lifespan (Table 2.2). On the other hand, *Drosophila*, mice and rats have proven to be valuable model organisms to seek a better understanding of the age-dependent functional decline of the immune system, neurocognitive functions and the development of age-related pathologies.

Knowledge gained from both types of studies supports several theories that aim at explaining the causes of aging. Among those, the "Antagonistic Pleiotropy Theory" postulates that certain genes may affect the fitness of an individual differently depending on age, thus a given gene product may have beneficial effects early in life in ensuring reproductive success, but may have detrimental effects later on in life (Medawar, 1952). The "Mutation Accumulation Theory" is closely connected with this, as it proposes that the absence of a selective pressure on genes that do not affect

reproductive success or that have negative effects only after the reproductive age is reached allows for the accumulation of mutations that may negatively affect lifespan. While it is challenging to experimentally distinguish between the antagonistic pleiotropy and mutation accumulation theories, support for the mutation accumulation theory comes from the observation of increasing inbreeding depression with increasing age (Charlesworth and Hughes, 1996; Escobar et al., 2008). The "Mitochondrial Free Radical Theory" of aging on the other hand suggests that free radicals formed during mitochondrial oxidative phosphorylation direct cellular damage and thereby mediate gradual functional decline with increasing age (Harman, 1956). This theory has stimulated a tremendous amount of research that produced results in favour of the theory, such as the observed lifespan extension in animals with mutations in genes with functions in oxidative phosphorylation (Table 2.2), but have also shown that reactive oxygen species play a complex role in cellular physiology, as they not only induce damage to cellular macromolecules, but also play roles in cellular signalling.

While organismal aging is likely caused by the interplay of several processes, those and other theories have been tested, modified and have contributed to our current understanding of age-related functional decline and the arising of age-related pathologies along the way.

On the other hand, the organism's stress sensitivity is also affected by age-dependent functional changes. For instance, the reduced DNA repair capacity of senescent cells and the shortening of the telomeres interfere with the fidelity of repair of DNA damage induced by genotoxic agents, such as IR (Soler et al., 2009). Such changes in the response to IR critically affect radiation sensitivity of the organism and the likelihood of developing side effects from diagnostic and therapeutic radiation

exposures. A better understanding of causal relationships will help in controlling side effects of radiation exposures better in an age-dependent way.

Some of the models used for the study of aging, as well as some of the findings that were achieved and how they are thought to relate to the process of aging, will be discussed in this review. In addition, we discuss the current state of knowledge on age-dependent differences in radiation response and radiation sensitivity, as well as some underlying mechanisms.

2.3 AGING MODEL SYSTEMS

Aging is characterized by the functional decline of the organism and an increasing chance of death at any time. In order to determine molecular mechanisms that underlie aging, the field of aging research has pursued two major directions: the study of lifespan modifications, which allows us to determine effects of gene mutations on median and maximum lifespan of an organism, and the study of functional senescence, which investigates the age-related intrinsic functional decline in the functional status of cells, organs and organ systems (Grotewiel et al., 2005). While both approaches have significantly contributed to the current understanding of processes involved in aging, they also have limitations. For instance, it has been argued that some mutations that extend lifespan under laboratory conditions may represent better adaptation to this artificial environment rather than a change in aging *per se* (Gershon and Gershon, 2000). Indeed, a study by Walker *et al.* (2000) showed that *age-1* mutants of *C. elegans* in mixed *age-1*/wild-type cultures got eliminated from the population after only several generations when exposed to mild stress (Walker et al., 2000). This indicated that lifespan extension was associated with decreased fitness even under mild stress conditions. Similarly, *Drosophila* strains

selected for longevity performed weaker than control strains at locating food sources in a field experiment (Wit et al., 2013). On the other hand, when studying age-related functional decline the major challenge is that a given model system may be a good model for a certain process, but not for others. For instance, while increasing shortening of telomeres is associated with senescence in human cells (Harley, 1991; Harley et al., 1990), all chromosomes in mouse cells are flanked by much larger telomeres than in human cells, while shortening at the same rate, and in addition telomerase activity can be detected in cells of adult mice (Prowse and Greider, 1995).

Although there are inter-species and even inter-individual differences in what mechanisms drive the aging process, aging itself is very conserved and is observed in very simple eukaryotic organisms, such as the budding yeast (*Saccharomyces cerevisiae*), invertebrates like *C. elegans* as well as in mammalian species (Table 2.1). This suggests that some of the underlying molecular mechanisms may be conserved in eukaryotes. Therefore, most of the common laboratory organisms have been used for aging studies and they all come with their advantages and limitations as will be discussed in the following sections.

Saccharomyces cerevisiae (budding yeast)

The budding yeast is a unicellular, eukaryotic organism, which divides asymmetrically by forming a bud that eventually separates from the mother cell. It has a small genome, which has been sequenced, and a relatively short lifespan (Table 2.1). Those features make yeast a favourable model for the study of molecular mechanisms underlying various processes, such as cell cycle control (Hartwell et al., 1974), stress response (Glover and Lindquist, 1998) and aging.

The observation that a mother cell only gives rise to a limited number of buds (daughter cells) (Mortimer and Johnston, 1959) first indicated that yeast cells have a limited lifespan. This so-called budding lifespan or replicative lifespan resembles the limited *in vitro* replicative lifespan of human diploid fibroblasts (Hayflick, 1965). Another model for aging in yeast is based on measuring the survival time of cells in stationary phase and is also termed chronological lifespan (Werner-Washburne et al., 1996). The disadvantage of the stationary phase model is that it is connected with glucose starvation and possibly also accumulation of toxic metabolic products in the medium and may therefore resemble stress-induced senescence rather than age-related senescence. However, both models have been employed in order to determine mutations that modify the yeast lifespan.

Lifespan modification studies in yeast first indicated a role for silent information regulator (*SIR*) genes, particularly *Sir2*, in lifespan regulation (Delaney et al., 2011; Kaeberlein et al., 1999). Kaeberlein *et al.* (1999) showed that mutation of *Sir2* reduced yeast lifespan, whereas an extra copy of *Sir2* extended lifespan. Further, decreased expression of Sir2 was observed during aging and correlated with accumulation of acetylated H4K16 in subtelomeric and rDNA regions (Dang et al., 2009). Sirtuins have since been shown to delay age-associated pathologies and extend lifespan in worms, flies and mice as well (Table 2.2) (see also (Herranz and Serrano, 2010).

In summary, yeast aging may resemble the *in vitro* senescence of human diploid fibroblasts and may prove useful in finding candidate genes that modulate aging. However, despite the obvious advantages such as small genome size, straightforward genetic manipulations and its short lifespan, there are also shortcomings of yeast as a model organism for the study of aging. For instance, its unicellular lifestyle and lower

complexity of diverse regulatory mechanisms (e.g. absence of DNA methylation) make it impossible to study certain aspects of aging (Table 2.1).

Caenorhabditis elegans (roundworm)

C. elegans is a transparent nematode, which consists of less than a thousand cells. The lineage of its cells has been traced from zygote to adult stage and is highly invariant (Sulston and Horvitz, 1977; Sulston et al., 1983). *C. elegans* possesses distinct germ and somatic cell lineages and most of the cells in adult worms are postmitotic and highly specialized, similar to mammals. Its short generation time and the fact that the *C. elegans* genome has been sequenced, make this worm a favourable model for the study of molecular mechanisms of biological processes – including development and aging.

The development of *C. elegans* includes four larval stages (L1-L4) during which the worms are sensitive to environmental conditions. Under adverse conditions, they can enter the Dauer larva stage, in which they can survive for prolonged amounts of time without aging. Individuals that go through a Dauer stage during larval development have an equally long adult lifespan (3 to 4 weeks) as individuals that do not.

Studies focussing on functional senescence rely on comparing animals at several stages throughout adult life, based on survival rate and chronological age of the animals. These studies have indicated a decline in protein degradation resulting in the accumulation of non-functional proteins with age (Reznick and Gershon, 1979; Zeelon et al., 1973), altered mitochondrial function and accumulation of oxidative damage with age (Yasuda et al., 2006), as well as changes in gene expression (Golden and Melov, 2004) and miRNA expression profiles (Ibanez-Ventoso et al., 2006).

On the other hand, lifespan modification studies have been very prominent in the *C. elegans* model, due to the ease of down-regulation of gene expression using RNAi technology. This resulted in the identification of numerous potential regulators of aging that include genes involved in Dauer formation, clock (clk) genes and genes involved in food consumption and dietary restriction (Tables 2.2 and 2.3). Among those, *daf-2* mutants were recorded to have a twice longer lifespan when compared to wild-type worms (Kenyon et al., 1993) and *age-1* mutants exhibited 1.5 times the lifespan of wild-type worms (Friedman and Johnson, 1988). The lifespan extension in those mutants is partially due to induction of the Dauer larva stage. Both *daf-2* and *age-1* are part of an insulin-like signalling pathway, negatively regulating *daf-16* (Ogg et al., 1997). *daf-16* is a forkhead box transcription factor which regulates the expression of key metabolic and developmental control genes (Ogg et al., 1997). Mutations affecting the insulin signalling pathway were later on reported to extend the lifespan in mammals as well (Taguchi et al., 2007), thus supporting its relevance to mammalian aging (Table 2.2).

clk genes function as regulators of developmental timing during *C. elegans* development. Therefore, mutation of *clk* genes results in lifespan extension through prolongation of individual stages throughout development (Lakowski and Hekimi, 1996). This is thought to be at least partially due to the reduced metabolic activity observed in the mutant strains (Felkai et al., 1999).

In summary, *C. elegans* has been extensively used as a model system of aging and has helped to reveal some age-dependent molecular changes (accumulation of oxidative damage and non-functional proteins in aging animals) as well as potential regulatory pathways. Some of those seem to be conserved in mammals (e.g. accumulation of oxidative damage and non-functional proteins in aging organisms)

and may therefore help to unravel underlying molecular mechanisms of corresponding age-related changes. Other changes are not directly applicable to aging of higher eukaryotes (e.g. Dauer formation and lifespan extension through modified regulation of Dauer formation).

Drosophila melanogaster (fruit fly)

The fruit fly is another popular invertebrate model system. The *Drosophila* genome has been sequenced and is similar in size to the *C. elegans* genome, and considerably smaller than the human genome with 140 Mbp and approximately 15,400 genes (Adams et al., 2000). However, numerous human disease genes have homologs in the *Drosophila* genome, making it a valuable tool for studying molecular processes.

The life cycle of *Drosophila* includes three larval stages, during which the larvae feed on microorganisms, and a pupa stage, which involves a 4-day metamorphosis after which the adults emerge. In total fruit flies have a lifespan of approximately 80 days and experience functional decline, including decreased stress resistance (Bonilla et al., 2002), decreased learning capacity and altered innate immune function, in the later stages of their life cycle (as reviewed by (Grotewiel et al., 2005). Interestingly, not all functions deteriorate at the same rate, suggesting that certain organ systems senesce sooner than others.

Although, functional span and lifespan seem to be connected, it seems to be possible to extend lifespan without prolonging functional span and *vice versa* (Grotewiel et al., 2005), suggesting that some of the underlying mechanisms may be distinct.

The observation that many functions decline in aging fruit flies, which is similar to the functional decline during human aging, opens the possibility to study molecular

processes that direct functional decline at a much faster pace due to the short lifespan of *Drosophila*. Further, connections between lifespan and functional span can be studied in this model system.

Mus musculus (mouse)

The mouse is a very popular mammalian model organism for many biological processes, including various pathologies and aging. Compared to the invertebrate model systems discussed above, mice have a genome that is similar in size – 2.6 Gbp compared to 3 Gbp of the human genome – and content – about 99% of all mouse genes have homologs in the human genome (Mouse Genome Sequencing et al., 2002). In addition, mice have a relatively short lifespan (their median lifespan is around 2 years under laboratory conditions). Also, comprehensive genetic, genomic and epigenomic databases and a variety of molecular tools are available, which facilitates the experimental work and interpretation.

While genetic manipulations are more complicated than in lower organisms and homozygous mutants for genes that have important functions during the development are often lethal, some studies on lifespan modification have also been performed in mice. For instance, similar to observations in other model systems, caloric restriction also extended the lifespan of mice (Table 2.2) (Weindruch and Walford, 1982). However, two recent studies showed that this lifespan extension depended on the genetic background of the animals, and in some strains caloric restriction even reduced the lifespan (Liao et al., 2010; Rikke et al., 2010). Thus, in order to control for effects like the animals' environment and their genetic background on lifespan, the National Institute on Aging has designed an Interventions Testing Program (Miller et al., 2007). This program involves independent replication of experiments in three

different laboratories and the use of four-way crossed genetically heterogeneous animal populations. They mainly focus on testing the effect of various compounds like aspirin and rapamycin on aging and one of their major findings has been an increase in lifespan of rapamycin treated mice (Harrison et al., 2009).

In parallel, researchers have also worked towards a better understanding of functional senescence in mice. The Aging Center at the Jackson Laboratory conducted a large initiative by studying 30 mouse strains including 4 strains that were recently derived from the wild. They assessed blood borne traits – such as hormone levels, showing an inverse correlation of serum IGF1 levels with the median lifespan of the strain (Yuan et al., 2009), and immune cell lineages, showing high variability in strain-specific immune cell repertoires (Petkova et al., 2008), – as well as the function of several organs including the heart, showing that age-related changes in heart rate are strain-dependent (Xing et al., 2009), and body composition and an assessment of the development of diseases (Sundberg et al., 2011; Sundberg et al., 2008). These findings indicate that many age-related changes may have a genetic component. On the molecular side, OMICS studies have resulted in the accumulation of knowledge about metabolite status, which indicated several metabolites, including eicosatrienoic acid and oleic acid that showed predictive changes in abundance with increasing age (Tomas-Loba et al., 2013), and about mRNA expression profiles (Lee et al., 1999), which were associated with the down-regulation of biosynthetic enzymes and genes involved in protein turnover.

In summary, both lifespan modification and functional senescence studies have contributed significantly to the current understanding of pathways that may be involved in aging. While many pathways that are involved in aging seem to be conserved in mammalian species, mouse aging also exhibits differences to human

aging - mouse telomeres are substantially longer than their human counterparts (Kipling and Cooke, 1990).

Rattus norvegicus (rat)

Rats have been given considerably less attention than mice, when it comes to aging research. This is likely due to the longer lifespan of up to 4 years under laboratory conditions. Further, it proved to be difficult to find a rat strain appropriate for aging research (Sprott, 1991). Initially, Sprague-Dawley rats were used, followed by Fischer344, which was replaced with a Fischer344xBrown Norway hybrid due to frequent renal pathologies. In parallel to this, Long Evans rats were used to study cognitive functions in rats and have continued to be studied (Castellano et al., 2012; Meaney et al., 1988).

While some lifespan modification studies have been performed using rat model systems, including the first study that showed the effect of caloric restriction on lifespan (McCay et al., 1989), rats have been more commonly used for the study of functional senescence, in particular neurocognitive aging.

Cognitive functions rely on neural systems, which have homologous organization and function in the rat when compared to the human brain. Spatial cognition tasks, which assess hippocampus-dependent memory and learning, are performed more poorly by old when compared to young rats (Barnes et al., 1980). Interestingly, *de novo* hippocampal neurogenesis with increasing age correlated with decreased performance in cognition tasks (Bizon et al., 2004). In order to test for medial temporal lobe dependent memory, rats are assessed in tasks that require them to learn to recognize flavours or odours (Rowe et al., 1998). This was associated with the loss of synapses or the alteration of existing synapses (Geinisman et al., 1986). Changes in

the encoding of orbitofrontal cortex neurons were further associated with an impairment in reversal learning by aged rats, which may result in the reduced flexibility with age (Schoenbaum et al., 1999). The benefit of these studies is that they help in understanding changes in the aging brain that are associated with age-related mental deficits that resemble those that occur during human aging.

Thus, rats are considered to be useful models to study age-dependent functional decline of organs and organ systems (Hazzard, 1991). However, when it comes to modeling of specific age-related pathologies, specific strains of rats, mice or rodents in general may not suffice, as for example rodents do not suffer from atherosclerosis as they age.

Human diploid fibroblast cell strains

The study of molecular changes that occur with human aging is more difficult, due to the long lifespan of humans and ethical concerns. Two approaches to circumvent those issues have been to study molecular changes in blood cells from aged versus young donors on the one hand, or the use of human diploid fibroblasts – either established cell strains that are maintained by central suppliers or freshly isolated from skin biopsies of donors of different ages – on the other hand.

While studying blood cells from aging individuals may give insights into molecular processes involved in immunosenescence (Hadrup et al., 2006; Kuparinen et al., 2013) and may also aid in finding circulating biomarkers for aging (Mendelsohn and Larrick, 2013), human diploid fibroblasts have provided an important tool for studying mechanisms underlying senescence *in vitro*.

Human diploid fibroblasts were first studied by Hayflick and Moorhead (1961), who isolated 25 strains from human foetal tissue and serially subcultivated them until

the cell cultures started degenerating (Hayflick and Moorhead, 1961). The quantification of how many times the cells doubled in culture before reaching this degeneration phase showed that most of the human diploid lung fibroblast cell strains they had isolated doubled 50 ± 10 times (Hayflick, 1965). With this study they introduced the concept of the limited replicative lifespan of cell strains *in vitro* and provided a basis for cellular aging research. Since the studies done by Hayflick and Moorhead, several other cell types have been reported to exhibit finite lifespans *in vitro* – epidermal keratinocytes, vascular smooth muscle cells, lens epithelial cells, glial cells, endothelial cells, melanocytes, T cells and adrenocortical cells (as reviewed in (Cristofalo and Pignolo, 1993). While those cell strains have been valuable tools in the study of mechanisms underlying cell senescence, how senescence is thought to contribute to organismal aging will be discussed in the next section.

In summary, the use of all the individual aging model systems presented in this section has contributed significantly to the current understanding of age-related molecular changes and processes. Many of those seem to be surprisingly universal – lifespan extension through caloric restriction, the involvement of Sirtuins in lifespan regulation and accumulation of damage to macromolecules – to name a few. How those processes are thought to be involved in aging and how they mutually interact will be topic of the next section.

Table 2.1: Comparison of different aging model systems.

Genome size and gene numbers are based on the latest information from the NCBI Genome database (May 2014). Maximum lifespan numbers are based on information from the Animal Ageing and Longevity Database (AnAge).

	Yeast	***C. elegans***	***Drosophila***	**Mouse**	**Rat**	**Human fibroblasts**	**Human**
Genome size	12.16 Mb	100.29 Mb	139.49 Mb	2.8 Gb	2.9 Gb	3.2 Gb	3.2 Gb
Number of genes	6349	44867	15771	44492	38528	41507	41507
Max. lifespan	0.04 y	0.16 y	0.3 y	4 y	3.8 y	50 ± 10 PD	122.5 y
Advantages for being a model	short lifespan easy genetic mani-pulation	short lifespan RNAi well understood develop-ment most cells in adult are postmitotic	short lifespan useful for functional studies numerous homologs of human disease genes	short lifespan high similarity in genome 99% of mouse genes have human homologs	good model for neuro-cognitive aging	useful for the study of senescence	
Disadvantages	uni-cellular less complex	differences in develop-ment	differences in develop-ment and organ structures	differences in telomere biology not useful for all age-related pathologies	difficult to find adequate strain not useful for all age-related pathologies	removed from physio-logical environ-ment	

2.4 MOLECULAR THEORIES OF AGING

The decades of aging research using the various aging model systems that were discussed in the previous section have resulted in the accumulation of a considerable amount of information on potential regulatory mechanisms of aging.

Lifespan modification studies have indicated signalling pathways that may be involved in aging, whereas functional senescence studies have allowed the observation of age-related molecular changes, such as accumulation of damage and senescent cells as well as epigenetic changes. Thus, in the following sections those age-related changes and their potential role in human aging will be discussed.

Senescence

The role of senescence in aging is controversial. Several studies showed that senescent cells accumulate in aging tissues *in vivo* (Dimri et al., 1995; Herbig et al., 2006). Further, the *in vitro* replicative lifespan of fibroblasts weakly correlates with donor age and lifespan of the donor species (Martin et al., 1970; Rohme, 1981), and fibroblasts from progeria patients exhibit shortened replicative lifespans (Faragher et al., 1993), suggesting that cellular senescence is linked with organismal aging. In contrast, a study by Cristofalo *et al.* (1998) failed to reproduce the correlation of replicative lifespan of skin fibroblasts with donor age (Cristofalo et al., 1998).

When considering what role senescence may play in aging, this likely depends on the cells or cell types undergoing senescence. While senescence is a feature of the differentiated cells in most organs, senescence of the stem cells that are responsible for tissue renewal and repair is likely detrimental to the function of the tissue (Krishnamurthy et al., 2006). However, while a decreasing functionality of stem cells with age is observed, it is still unclear whether this is due to stem cell aging or whether it is a result of the changing environment for stem cells within the aging tissue (Rando, 2006). A recent study supports an active role of stem cell aging in the age-dependent functional decline, by showing that adult quiescent muscle stem cells switch to irreversible senescence in muscles of geriatric mice (Sousa-Victor et al., 2014).

Immune cells, on the other hand, rely on proliferation in order to perform their function. However, it was shown that after several rounds of clonal selection, T cells can become replicatively senescent *in vitro*, and T cells with senescence-like features

are also found *in vivo* (Effros, 2004). This is thought to contribute to immunosenescence – the age-related deterioration of the immune system.

To sum up, while senescent cells accumulate in tissues of aging individuals the involvement of senescence in aging seems to be dependent on the affected cell types and tissues.

Table 2.2: Effects of gene mutations, caloric restriction and telomere length on lifespan extension in aging model organisms.

This table shows genes that when mutated extend organismal lifespan. Additionally effects of caloric restriction and telomere length on lifespan extension are also indicated.

	Yeast	***Drosophila***	***C. elegans***	**Mouse**	**Rat**	**Human**
Caloric restriction	(Lin et al., 2002)		(Klass, 1977)	(Liao et al., 2010; Rikke et al., 2010; Weindruch and Walford, 1982)*	(McCay et al., 1989)	(Li and Tollefsbol, 2011) [1]
Insulin signalling	Glucose signalling: *CDC25*, *TPK1, TPK2, TPK3, HXK2, GPR1, GPA2* (Lin et al., 2000)	*InR* (Tatar et al., 2001); *chico* (Yamamoto and Tatar, 2011); *Lnk* (Slack et al., 2010)	*daf-2* (Kenyon et al., 1993); *age-1* (Klass, 1983); *oga-1* (Rahman et al., 2010); *pdk-1* (Mansisidor et al., 2011); *akt-1* (Alam et al., 2010; Hamilton et al., 2005) [2,6]; *akt-2* (Alam et al., 2010)	*Igf1r* (Holzenberger et al., 2003)(Xu et al., 2014)[3]; *Papp-a* (Conover and Bale, 2007); *Irs1* (Selman et al., 2008); *Akt1* (Nojima et al., 2013) [3]		*IGF-1R* (Bonafe et al., 2003) [4]
Hormones		*EcR* (Simon et al., 2003) [3]	*daf-12* (Fisher and Lithgow, 2006)*; *nhr-154, nhr-14* (Hamilton et al., 2005) [2]	*Ghrh* (Sun et al., 2013); *Ghrhr* (Flurkey et al., 2001); *Ghr* (Coschigano et al., 2003)	*Gh* (Kuramoto et al., 2010)	*PPARG* (Barbieri et al., 2004) [4,*]
TOR signalling	*TOR1* (Kaeberlein et al., 2005)(Smith et al., 2008); *GLN3, LYS12, MEP3, AGP1, MEP2* (Powers et al., 2006); *SCH9* (Fabrizio et al., 2001)	*dTOR, dS6K* (Kapahi et al., 2004) [5]	*rsks-1* (Hansen et al., 2007; Pan et al., 2007) [2,6]; *let-363/CeTor* (Vellai et al., 2003)[6]; *daf-15/raptor* (Jia et al., 2004)[3]; *sinh-1* (Hansen et al., 2005)[2]	*mTor* (Harrison et al., 2009); *S6k1* (Selman et al., 2009)		
Translation	*RPL31A,*		*ife-2* (Hansen et			

	RPL6B (Kaeberlein et al., 2005; Steffen et al., 2008); *RPL9A, RPL19A* (Smith et al., 2008; Steffen et al., 2008); *TIF4631* (Managbanag et al., 2008; Smith et al., 2008); *TIF1, TIF2* (Laschober et al., 2010; Smith et al., 2008)*; *RPL20B* (Managbanag et al., 2008; Steffen et al., 2008); *RPL23A, RPL34B, RPL21B, RPP2B, RPL22A, RPL43B, RPL13A, RPL7A, RPL29* (Steffen et al., 2008); *RPL10, RPS18A, RPS18B, RPS6B* (Chiocchetti et al., 2007); *ELP4, TMA19, RPL37B* (Managbanag et al., 2008)		al., 2007; Syntichaki et al., 2007)[6]; *rps-15* (Hansen et al., 2007; Reis-Rodrigues et al., 2012)[2]; *rpl-6* (Hansen et al., 2007)[2]; *ifg-1* (Hansen et al., 2007; Pan et al., 2007)[2]; *eIF2Bδ* (Tohyama et al., 2008)[2]; *iftb-1* (Hansen et al., 2007)[2]; *rpl-28, rpl-P2, aars-2* (Stout et al., 2013)[2]; *rpl-17* (Reis-Rodrigues et al., 2012; Stout et al., 2013)[2]; *iff-1* (Hamilton et al., 2005)[2]; *drr-2* (Hansen et al., 2005)[2]; *Y39G10AR.8, D2085.3, eif-3.F, nol-5, rpl-19, nars-1, Y48G1A.4, tars-1* (Chen et al., 2007)[2]; *mars-1, F33D4.5* (Kim and Sun, 2007)[2]; *rps-23* (Kim and Sun, 2007; Reis-Rodrigues et al., 2012)[2]; *rps-5* (Kim and Sun, 2007; Reis-Rodrigues et al., 2012)[2]; *rps-16, inf-1, rpl-8, eef-1A.1, rps-0, eef-2, rla-0, rpl-23, rps-20, rpl-31, rpl-10a, rps-14, rars-1, rpl-10, rpl-7, rps-11, rpl-3* (Reis-Rodrigues et al., 2012)[2]; *gro-1* (Lakowski and Hekimi, 1996)	
Ubiquitin ligation/ proteolysis	*UBR2, MUB1* (Kruegel et al., 2011); *BRE5* (Kaeberlein et al., 2005); *TOM1* (Managbanag et al., 2008); *AFG3, HSE1* (Smith et al., 2008)		*vhl-1* (Mehta et al., 2009)[6]; *ubh-4, rnf-5, egl-3, nas-38, Y71H2AR.2* (Hamilton et al., 2005)[2]; *Y71H2 385.b, F49C12.9* (Kim and Sun, 2007)[2]; *rle-1* (Li et al., 2007b)	
Epigenetics	*YTA7, HMT1* (Laschober et al., 2010); *SAS2* (Dang et al., 2009); *SAM1* (Smith et al., 2008); *SET2* (Ryu et	*hop* (Larson et al., 2012)[3]	*ash-2, wdr-5, set-2, set-4* (Greer et al., 2010)[6]; *rbr-2* (Greer et al., 2010; Maures et al., 2011; Ni et al., 2012)[6,*]; *set-26, set-9, mes-2,*	*MIR-21* (Dellago et al., 2013)[1]

	al., 2014); *HHF1* (Li et al., 2011a)		*jmjd-2* (Ni et al., 2012)[2]; *lsd-1, jhdm-1* (Maures et al., 2011)[2,3,6];, *utx-1* (Maures et al., 2011; Ni et al., 2012)[2,3,6]; *sams-1* (Hansen et al., 2005)[2]; *epc-1* (Kim and Sun, 2007)[2]; *his-37* (Reis-Rodrigues et al., 2012)[2]; *miR-80* (Vora et al., 2013); *set-15, rbbp-5, cec-3* (Hamilton et al., 2005)[2]; *let-418* (De Vaux et al., 2013)		
Transcriptional regulation / transcript processing	*SPT4, DBP3, TIS11* (Smith et al., 2008); *GCN4, LOC1, SWI5* (Managbanag et al., 2008); *FZF1* (Yoshida et al., 2010); *LEU3* (Alvers et al., 2009a); *UGA3* (Kamei et al., 2011; Yoshida et al., 2010); *URE2* (Kaeberlein et al., 2005)		*lin-14* (Boehm and Slack, 2005)[6]; *ets-4* (Thyagarajan et al., 2010); *spr-3* (Yang et al., 2013a)[6,*]; *hif-1* (Chen et al., 2009; Mehta et al., 2009; Zhang et al., 2009)[6,*]; *spt-4, ceh-18, pup-2* (Hamilton et al., 2005)[2]; *rha-2* (Hansen et al., 2005)[2]; *C48E7.2, rpoa-2* (Chen et al., 2007)[2]; *F08B4.7* (Kim and Sun, 2007)[2]; *elt-6* (Budovskaya et al., 2008; Kim and Sun, 2007)[2]; *elt-5* (Budovskaya et al., 2008)[2]; *crtc-1, tax-6* (Mair et al., 2011)[2]; *hcf-1* (Li et al., 2008a)[6]; *exos-3* (Chen et al., 2007)[2]; *car-1, snr-2, cgh-1, pgl-1* (Stout et al., 2013)[2]; *pab-1* (Reis-Rodrigues et al., 2012; Stout et al., 2013)[2]	*Pit1 (=Pou1f1)* (Flurkey et al., 2001); *Prop1* (Brown-Borg et al., 1996)	
Cell cycle regulation / control of senescence	*MAD2, PCH2* (Laschober et al., 2010)	*Dmp53* (Bauer et al., 2007)[5]	*cid-1, chk-1, cdc-25* (Olsen et al., 2006) [6]	*p16ink4a, p19arf* (Carnero et al., 2000)[1,2]; *Cables* (Kirley et al., 2005) [1]	*p53* (Bond et al., 1996; Bond et al., 1994; Gollahon and Shay, 1996; Wei et al., 2003)[1,3,5]; *p16INK4A* (Fung et al., 2013; Noble et al., 1996)[1,2]; *p21, RB* (Wei et al., 2003)[1,3]; *BTG2* (Wheaton et al., 2010)[1,2]; *CHK2* (Gire et

					al., 2004)[1,5]
Oxidative stress	*PDX3* (Laschober et al., 2010); *CTA1* (Mesquita et al., 2010)		*sod-2* (Van Raamsdonk and Hekimi, 2009); *F53H1.3, T12E12.1, C03H5.3, lbp-6* (Ayyadevara et al., 2009)[2]	*p66Shc* (Migliaccio et al., 1999)	
Mitochondrial dysfunction	*MRG19* (Kharade et al., 2005); *AFO1* (Heeren et al., 2009); *SOY1* (Li et al., 2011a); *RIM1* (Managbanag et al., 2008); *MSW1* (Laschober et al., 2010; Managbanag et al., 2008)[*]; *YBR238C* (Kaeberlein et al., 2005)	*sbo* (Liu et al., 2011)[3]	*frh-1* (Vazquez-Manrique et al., 2006; Ventura et al., 2005) [2,*]; *clk-1* (Lakowski and Hekimi, 1996; Wong et al., 1995); *nuo-6* (Yang and Hekimi, 2010)[6]; *isp-1* (Feng et al., 2001; Yang and Hekimi, 2010)[6]; *mfn-1* (Ren et al., 2012)[2]; *lars-2, F13G3.7, K01C8.7, F28B3.5* (Lee et al., 2003)[2,6]; *cchl-1* (Hansen et al., 2005; Lee et al., 2003)[2]; *D2030.4, F26E4.6* (Hamilton et al., 2005; Lee et al., 2003)[2]; *W09C5.8* (Hamilton et al., 2005; Lee et al., 2003; Zuryn et al., 2010)[2]; *cco-1* (Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003; Zuryn et al., 2010)[2]; *C33F10.12, asg-2* (Hamilton et al., 2005)[2]; *T20H4.5* (Hamilton et al., 2005; Kim and Sun, 2007)[2]; *nuo-2* (Dillin et al., 2002; Hansen et al., 2005)[2]; *nuo-5, atp-4, atp-5, asb-2* (Hansen et al., 2005)[2]; *nuo-4* (Hamilton et al., 2005; Hansen et al., 2005; Kim and Sun, 2007)[2]; *lpd-5, F26E4.4, F59C6.5, sco-1, R53.4, mrpl-24, mrps-30, spg-7, mrpl-10* (Chen et al., 2007)[2]; *tag-99, mrps-9, mrpl-12, Y56A3A19, mrps-10, ant-1.1, Y119D3_463.b, Y53G8A_9248.c, Y53G8A_9248.d,*	*Mclk1 (Coq7)* (Liu et al., 2005)[3]; *Surf1* (Dell'agnello et al., 2007)	*UCP1* (Rose et al., 2011) [4]

	Y71H2_378.a, Y71H2_388.d, year-1, cyc-2.1, cyp-33E2, mrps-33, ZK809.3, C47E12.2, gars-5, Y55F3B_743.b (Kim and Sun, 2007)[2]; *F29C4.2* (Kim and Sun, 2007; Zuryn et al., 2010)[2]; *nuo-3* (Hansen et al., 2005; Kim and Sun, 2007; Zuryn et al., 2010)[2]; *C34B2.6, dlat-1, F01G4.6, B0250.5* (Reis-Rodrigues et al., 2012)[2]; *ucr-1* (Kim and Sun, 2007; Reis-Rodrigues et al., 2012; Zuryn et al., 2010)[2]; *mrps-5, nkcc-1, ttll-9, mrpl-1, mrpl-2, mrpl-37* (Houtkooper et al., 2013)[2]; *nuo-1* (Tsang et al., 2001; Zuryn et al., 2010)[2,6]; *atp-2* (Reis-Rodrigues et al., 2012; Tsang et al., 2001)[2,6]; *C33A12.1* (Kim and Sun, 2007; Zuryn et al., 2010)[2]; *C25H3.9, C18E9.4, ucr-2.3, F45H10.2, R07E4.3, T27E9.2, Y71H2AM.5, cco-3, cco-4* (Zuryn et al., 2010)[2]; *tag-174* (Kim and Sun, 2007; Zuryn et al., 2010)[2]; *cco-2* (Hansen et al., 2005; Zuryn et al., 2010)[2]; *phi-44* (Lee et al., 2003; Zuryn et al., 2010)[2]; *cyc-1* (Dillin et al., 2002; Hansen et al., 2005; Zuryn et al., 2010)[2]; *atp-3* (Dillin et al., 2002; Hansen et al., 2005)[2]; *hsp-6* (Ventura and Rea, 2007)[2]; *sft-1, oxa-1* (Maxwell et al., 2013)[2]; *mrpl-47* (Hamilton et al., 2005; Lee et al., 2003)[2]; *mics-1, atad-3*

			(Hoffmann et al., 2012) [6]		
DNA repair / genome integrity	*RNH201* (Laschober et al., 2010); *FOB1* (Kaeberlein et al., 2005)		*clk-2* (Lakowski and Hekimi, 1996)		
Telomere integrity/length	*TLC1* (Austriaco and Guarente, 1997)[5]		Long telomeres (Joeng et al., 2004)		Long telomeres (Wright et al., 1996) [1]
Other functions	*CYS4, DNF1, UBC12, PRS3, ALD4, PHO89, DIE2, ALG5, SSA3, LSB6* (Laschober et al., 2010); *GAD1* (Kamei et al., 2011); *UGA1* (Kamei et al., 2011; Laschober et al., 2010); *REI1, ROM2, MTC4, SLM6, IRC14* (Kaeberlein et al., 2005); *IDH2* (Kaeberlein et al., 2005; Smith et al., 2008); *ADH1, IDH1, PMR1, SIS2, YGR130C, YPT6, INP53* (Smith et al., 2008); *INP53* (Laschober et al., 2010; Smith et al., 2008)*; *SOK1, SPS1, BOI2, SIP2, SNF1* (Managbanag et al., 2008); *INP51* (Managbanag et al., 2008; Smith et al., 2008); *URH1* (Yoshida et al., 2010); *GUP1, HXT17, IPK1* (Li et al., 2011a); *PKH2* (Li et al., 2011a; Smith et al., 2008); *CYR1* (Fabrizio et al., 2004; Lin et al., 2000); *BST1, ERO1, EUG1, MPD1, HRD1, DER1, ALG3, OST3*	*snz* (Suh et al., 2008); *Dacer* (Yang et al., 2010); *ECP* (Wang et al., 2014)[2]; *Indy* (Rogina et al., 2000; Toivonen et al., 2007)*; *mth* (Lin et al., 1998b); *loco* (Lin et al., 2011)[3]; *ilk, mys* (Nishimura et al., 2014)[3]	*fgt-1a, fgt-1b* (Feng et al., 2013)[2]; *odr-2, odr-3, odr-7* (Shen et al., 2010b); *inx-14, pfn-2, K07H8.1, col-93, cdh-12, C35A11.3, clec-227, cwp-4, clec-186, T05A1.4, D1054.8, mecr-1, K08E3.5, idha-1, idh-1, F55B11.1, pghm-1, unc-83, nrfl-1, trim-9, F09F7.5, Y39H10A.6, Y54E5A.7, C36H8.1, scl-4, Y39F10C.1, cpna-3, gcy-29, C27B7.7, max-1, F35D2.3, scrm-8, K10B4.3, sru-17, srw-20, srh-254, F40F8.5, C09B7.2, C26B2.2, C32H11.1, E03H12.5, F49F1.12, H06H21.8, R05A10.5, R08E3.3, T06G6.4, T07A9.8, Y43F8B.12, laat-1, Y46H3C.6, Y53F4B.23, Y56A3A.9, Y75B8A.13, Y75B8A.33, sid-2* (Hamilton et al., 2005)[2]; *ttr-1, maoc-1, gpi-1, ddl-1, ddl2, ddl-3, pat-6, ril-1, ril-2, rab-10* (Hansen et al., 2005)[2]; *drr-1* (Hamilton et al., 2005; Hansen et al., 2005)[2]; *K11B4.1, abcx-1, B0491.5* (Chen et al., 2007)[2]; *pat-4* (Hansen et al., 2005; Kim and Sun, 2007; Kumsta et al., 2013)[2]; *ral-1, Y71H2_388.ct,*	*Adcy5* (Yan et al., 2007); *RIIbeta* (Enns et al., 2009)*; *RasGrf1* (Borras et al., 2011)	*NUAK1* (Humbert et al., 2010)[1,2]; *ADRB2* (Zhao et al., 2012)[4]; *NOTCH3* (Cui et al., 2013a)[1,2]; *MKP2, ERK2* (Tresini et al., 2007) [1,2,5]

	ALG12 (Labunskyy et al., 2014; Smith et al., 2008)	*hhat-2, asm-3, Y69A2A_2991.c, Y43F4B.7, Y119D3_446.d, Y53G8A_1734.g, grl-19, F42A6.1, aat-8, T05A1.5, amt-2, scl-8, cutl-28, tba-7, Y71H2_390.d, tes-1, F56D5.5, inx-8, inx-9, W03G1.5, acs-5, ogdh-1, acdh-13, tkt-1, dld-1, K12H4.5, T28D6.4, pod-1, B0546.3, spe-26, Y45F10D.8, ttr-5, Y66A7A1, T28A8.6, Y39A3C_82.a, Y53G8A_2702.a, Y53G8A_9248.b, F09C11.1, W07G9.1, C46G7.2, F13B6.1, K07H8.8, R10H10.7, C42C1.3, Y51H4A.m* (Kim and Sun, 2007)[2]; *pyc-1, mdh-1, tbb-2, F09F7.4, gdh-1, cpn-3, cct-1, acs-4, rack-1, cct-8* (Reis-Rodrigues et al., 2012)[2]; *sca-1* (Oh et al., 2006; Reis-Rodrigues et al., 2012)[2]; *ser-1, tph-1* (Murakami and Murakami, 2007); *sea-2* (Huang et al., 2011); *tatn-1* (Ferguson et al., 2013)[6]; *eak-7* (Alam et al., 2010; Ferguson et al., 2013)[6]; *slcf-1* (Mouchiroud et al., 2011)[6]; *glp-1* (Alam et al., 2010); *clk-3* (Lakowski and Hekimi, 1996); *ipgm-1* (Hamilton et al., 2005; Lee et al., 2003)[2]; *aco-2* (Hamilton et al., 2005; Reis-Rodrigues et al., 2012)[2]; *ndg-4, nrf-5, nrf-6, nrf-3* (Brejning et al., 2014); *ftn-1* (Quach et al., 2013)[2]; *arr-1, mpz-1*

			(Palmitessa and Benovic, 2010)[6]; *ocr-2, unc-31* (Lee and Ashrafi, 2008); *npp-16* (Stout et al., 2013)[2]			

[*] lifespan extension, shortening or no effects on lifespan have all been described, [1] studied in a cell culture model, [2] RNAi experiment, [3] animals heterozygous for the mutation were studied, [4] polymorphisms over-/underrepresented among long-lived populations, [5] over-expression of dominant-negative form, [6] RNAi and genetic mutant studied, [7] gain-of-function mutant

Table 2.3: Effects of gene mutations, caloric restriction and telomere length on lifespan shortening in aging model organisms.

This table lists gene mutations, as well as caloric restriction and telomere length changes, that negatively affect organismal lifespan.

	Yeast	***Drosophila***	***C. elegans***	**Mouse**	**Rat**	**Human**
Caloric restriction				(Liao et al., 2010; Rikke et al., 2010; Weindruch and Walford, 1982)[*]		
Insulin signalling	Glucose signalling: *PDE2* (Lin et al., 2000)	*foxo* (Yamamoto and Tatar, 2011)	*daf-18* (Mihaylova et al., 1999); *ogt-1* (Rahman et al., 2010)	*Irs2* (Selman et al., 2008); *Socs2* (Casellas and Medrano, 2008)		*FOXO3A* (Donlon et al., 2012; Pawlikowska et al., 2009; Willcox et al., 2008)[4,*]
Hormones			*daf-12* (Fisher and Lithgow, 2006)[*]; *CeKlotho* (Chateau et al., 2010)[2]	*Klotho* (Kuro-o et al., 1997); *Fgf23* (Razzaque et al., 2006)		
TOR signalling			*rict-1* (Soukas et al., 2009)			
Translation	*TIF1, TIF2* (Laschober et al., 2010; Smith et al., 2008)[*]; *RPL37A, SSF1* (Laschober et al., 2010)	*Aats-met* (Bayat et al., 2012)				
Ubiquitin ligation/ proteolysis	*RPT1S, RPT2RF, RPT3R, RPT5R, RPT6R, UMP1, UBP6, PRE9* (Kruegel et al., 2011); *UBC8, HRT3* (Laschober et al., 2010)	*Uba1* (Liu and Pfleger, 2013)	*pbs-5* (Stout et al., 2013)[2]; *aip-1* (Yun et al., 2008)[2]; *F42G10.1* (Oh et al., 2006)[2]	*Topors* (Marshall et al., 2010); *Herc1* (Mashimo et al., 2009)		*WWP1* (Cao et al., 2011)[1,2]; *SENP1* (Yates et al., 2008)[1,2]
Autophagy	*ATG1, ATG7* (Alvers et al., 2009a); *ATG18*	*Atg7* (Juhasz et al., 2007)	*atg-7, lgg-3* (Hars et al., 2007)[2]; *atg-3, atg-4.1, atg-4.2,*			*ATG7, ATG12, LAMP2* (Kang et al., 2011)[1,2]

	(Laschober et al., 2010)		*atg-5, lgg-1, atg-18* (Hashimoto et al., 2009)[2]; *bec-1* (Hashimoto et al., 2009; Melendez et al., 2003)[6]		
Sirtuins	*SIR2* (Kaeberlein et al., 1999); *SIR4* (Kaeberlein et al., 1999; Kennedy et al., 1995); *SIR3* (Kaeberlein et al., 1999)			*Sirt6* (Kanfi et al., 2012; Mostoslavsky et al., 2006)	
Epigenetics	*GCN5, HAT1, CHD1* (Laschober et al., 2010); *NSI1* (Ha et al., 2012); *RTT109, HST3, HST4* (Dang et al., 2009); *DOT1, SET1* (Ryu et al., 2014); *BRE1* (Walter et al., 2010)	*miR-34* (Liu et al., 2012); *Stat92E, Su(var)205* (Larson et al., 2012)[3]; *mir-14* (Xu et al., 2003)	*rbr-2* (Greer et al., 2010; Maures et al., 2011; Ni et al., 2012)[6,*]; *lin-4* (Boehm and Slack, 2005)[6]; *rde-4* (Mansisidor et al., 2011); *zfp-1* (Mansisidor et al., 2011; Oh et al., 2006)[2]	*Cbx7* (Gil et al., 2004)[1]	DNA hypomethylation (Fairweather et al., 1987; Holliday, 1986; Honda and Matsuo, 1987); *CBX7* ↓ [1] (Gil et al., 2004)
Transcriptional regulation / transcript processing	*RBP4, RBP9, PAT1, DHH1* (Duan et al., 2013); *RPN4* (Kruegel et al., 2011); *NPL3, MRT4, SSN3* (Laschober et al., 2010); *SFP1* (Heeren et al., 2009)		*nfi-1* (Lazakovitch et al., 2005); *spr-3* (Yang et al., 2013a)[6,*]; *skn-1* (An and Blackwell, 2003); *ttx-3, ceh-10* (Shen et al., 2010a); *ire-1* (Chen et al., 2009); *ldb-1, sup-37* (Oh et al., 2006)[2]	*Clock* (Dubrovsky et al., 2010); *Bmal1* (Kondratov et al., 2006)	*NRF2* (Kapeta et al., 2010)[1,2]; *NF1B* (Dellago et al., 2013)[1]
Cell cycle regulation / control of senescence	*CLB1, CLB2* (Laschober et al., 2010)	*D-p35* (Connell-Crowley et al., 2007); *CycD, Cdk4* (Icreverzi et al., 2012)		*BubR1* (Baker et al., 2004)	*CDC25A* (Dellago et al., 2013)[1]
Oxidative stress	*SOD1* (Barker et al., 1999; Unlu and Koc, 2007); *SOD2, CCS1* (Unlu and Koc, 2007); *CTT-1* (Van Zandycke et al., 2002); *TRX1* (Laschober et al., 2010)	*cSOD* (Phillips et al., 1989); *Trx-2* (Svensson and Larsson, 2007; Tsuda et al., 2010); *GLaz* (Sanchez et al., 2006)	*trx-1* (Miranda-Vizuete et al., 2006); *glod-4* (Morcos et al., 2008)[2]	*MsrA* (Moskovitz et al., 2001); *Sod2* (Li et al., 1995)	*TRX1* (Young et al., 2010)[1,2]
Mitochondrial dysfunction	*MGM1* (Scheckhuber et al., 2011); *PIM1* (Laschober et al., 2010); CYT1 (Li et al., 2011a); *MSW1*	*sdhB* (Walker et al., 2006); *dOpa1* (Tang et al., 2009)[3]	*frh-1* (Vazquez-Manrique et al., 2006; Ventura et al., 2005)[2,*]; *mev-1* (Ishii et al., 1998; Ishii et al., 1990); *gas-1* (Hartman et al., 2001; Kayser et	*Ucp2* (Andrews and Horvath, 2009); *Risp* (Hughes and Hekimi, 2011)[3,*]; *Bcs1l* (Leveen et al., 2011); *mSuv3* (Chen et al.,	

	(Laschober et al., 2010; Managbanag et al., 2008)*; *MEF2* (Callegari et al., 2011)		al., 2004); *nduf-2.2* (Kayser et al., 2004)	2013b)[3]	
DNA repair / genome integrity	*DNA2* (Hoopes et al., 2002); *SGS1* (Mankouri and Morgan, 2001); *SRS2* (Mankouri et al., 2002); *RAD27* (Laschober et al., 2010; Ringvoll et al., 2007); *MSH6, CHL1, MRE11, MSH2* (Laschober et al., 2010); *RAD52, RAD50, RAD51, RAD57* (Park et al., 1999)		*xpa-1* (Hyun et al., 2008)[6]	*Ercc1* (Weeda et al., 1997); *Ercc2* (Dolle et al., 2006); *Ku86 (=Ku80)* (Li et al., 2007a; Vogel et al., 1999); *Ku70* (Li et al., 2007a); *DNA-PKcs* (Espejel et al., 2004); *Top3beta* (Kwan and Wang, 2001)	*RECQL4* (Kitao et al., 1999); *WRN* (Yu et al., 1996)
Telomere integrity/length	Long telomeres, *RIF1* (Austria co and Guarente, 1997)[5]; *UTH4* (Kennedy et al., 1997)			Short telomeres (Vera et al., 2012)	Short telomeres (Counter et al., 1992)[1]
Other functions	*SHM1, LYS9, NCR1, KGD1, APM1, SYM1, IDP2, GSY2, ALT2, SIZ1, PTC5, RNR3, ERG24, NUP170, SSO1, TWF1, SGT2, LHS1, ARF1, DHP5, SPE2, DBP1* (Laschober et al., 2010); *INP53* (Laschober et al., 2010; Smith et al., 2008)*; *RAS2* (Hlavata et al., 2008); *ACH1* (Orlandi et al., 2012); *RGS2* (Lin et al., 2011); *SEL1, DIE2, KAR2* (Labunskyy et al., 2014)	*Hk* (Bushey et al., 2010); *Mgat1* (Sarkar et al., 2006); *Tpi* (Gnerer et al., 2006); *dnr1* (Cao et al., 2013); *Dcert* (Rao et al., 2007); *Ald* (Miller et al., 2012)	*smn-1* (Briese et al., 2009); *ptl-1* (Chew et al., 2013); *mod-5* (Murakami and Murakami, 2007); *jnk-1, jkk-1* (Oh et al., 2005); *sgk-1* (Alam et al., 2010; Chen et al., 2013a; Soukas et al., 2009); *sesn-1* (Yang et al., 2013b)[6]; *aak-2* (Apfeld et al., 2004); *nrf-4* (Brejning et al., 2014); *pnc-1* (van der Horst et al., 2007)[2]; *mnk-1* (Syntichaki et al., 2007)[2]	*eNos* (Li et al., 2004)*; *Fn* (Muro et al., 2003); *Ppm1d* (Nannenga et al., 2006)*; *Cdh1* (Li et al., 2008b)[1]; *Vimentin* (Tolstonog et al., 2001)[1]	*LMNA* (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003)

* both lifespan extension, shortening and no alteration of organismal lifespan have been described, [1] studied in a cell culture model, [2] RNAi experiment, [3] animals heterozygous for the mutation were studied, [4] polymorphisms over-/underrepresented among long-lived populations, [5] over-expression of dominant-negative form, [6] RNAi and genetic mutant studied, [7] gain-of-function mutant

Telomere shortening

Aside from the correlation of human fibroblast replicative lifespan with donor age, telomere lengths were shown to decrease with age and strongly correlate with the *in vitro* replicative lifespan of the fibroblast cell cultures (Allsopp et al., 1992; Harley et al., 1990).

Telomeres are structures at the ends of the chromatids that prevent the increasing loss of DNA during replication and end to end fusions of chromosomes (McClintock, 1941). Human telomeres consist of TTAGGG repeats protected by the shelterin complex, which is recruited by TRF1 and TRF2 (de Lange, 2009). This protection not only serves to prevent loss of DNA from the chromatid ends, but also prevents constant activation of DNA damage signalling and recombination at the unprotected ends (Sfeir and de Lange, 2012). When telomeres become critically short and exposed, this results in the activation of DNA damage signalling and growth arrest (Herbig et al., 2004).

However, during the replication of linear DNA, 50 to 200 bp are lost from the ends in each cycle. Thus in the absence of mechanisms that resynthesize them, telomeres will shorten with every cell division (Harley et al., 1990). There are two mechanisms in place to prevent progressive telomere shortening – telomerase dependent *de novo* synthesis of telomere repeats or a recombination-dependent process called alternative lengthening of telomeres (ALT). However, alternative lengthening has mostly been observed in cancer cells and telomerase activity in human cells decreases with age and potentially also after exposure to stress conditions (Entringer et al., 2011). As a result, leukocyte telomere length, which is considered a biomarker for aging, decreases over time in most people (Chen et al., 2011) and reduced telomere length

further correlates with the development of several age-related deficiencies, such as decreased cognitive function (Valdes et al., 2010), atherosclerosis and the risk for developing cardiovascular disease (Willeit et al., 2010).

While the knowledge on telomere biology indicates that it may serve as a biomarker for aging, it is debated whether telomere shortening is simply a by-product of other age-related cellular processes or whether it may be a causative agent in driving aging. When cancer resistant mice were engineered to constitutively express telomerase, they exhibited an increased median but not maximum lifespan (Tomas-Loba et al., 2008). Also, transient expression of human telomerase in human foreskin fibroblasts increased their replicative lifespan (Steinert et al., 2000), suggesting that telomere shortening does play a role in aging. Further, the immortalization of primary cells is often accompanied by the reactivation of telomerase expression (Counter et al., 1992).

As telomeres shorten with each cell division, cells that are highly proliferative were found to be more sensitive to telomere shortening (Lee et al., 1998). In line with this, critically short telomeres prevent senescent human mesenchymal stem cells from re-proliferating (He et al., 2014). Thus, telomere shortening may play a role in age-associated functional decline through inducing senescence in highly proliferative tissues.

Interestingly, when comparing different mammalian species, it becomes apparent that some species exhibit long telomeres and constitutively active telomerase, as observed in mice, while others have short telomeres and inactive telomerase, like humans (Gomes et al., 2011) (Tables 2.2 and 2.3). The phylogenetic study performed by Gomes *et al.* (2011) suggested that short telomeres and suppressed telomerase are the ancestral form, and longer telomeres and constitutive telomerase expression

evolved in several species with small body sizes independently. Therefore, while telomere shortening plays a role in replicative senescence in species with short telomeres and inactive telomerase, it is not a universal mechanism of aging.

Nutrient sensing and cellular signalling

The signalling pathways discussed in this section were all implicated to play a role in aging by their effect on lifespan (Tables 2.2 and 2.3). Interestingly, they all directly or indirectly respond to nutrient or metabolite levels and may therefore aid in understanding the effect of the metabolic rate on lifespan.

IGF1 signalling. Indications for the involvement of the insulin/IGF1 signalling pathway in aging mostly come from lifespan modification studies (Tables 2.2 and 2.3). For human aging, the IGF1-IGF1R signalling pathway seems to be more relevant than the insulin signalling pathway *per se*. IGF1 is secreted by the liver in response to growth hormone (GH), which is regulated by neurosecretory nuclei of the hypothalamus – it is induced by fasting and inhibited by circulating GH and IGF1 as well as hyperglycemia. The activation of IGF1R results in the activation of AKT and the exclusion of FOXO transcription factors from the nucleus (Fig. 2.1). FOXO transcription factors normally induce the expression of antioxidant enzymes, such as SOD2 (Kops et al., 2002) and catalase (Nemoto and Finkel, 2002), and also genes that induce cell cycle arrest, including p27 (Medema et al., 2000). Thus, alterations in this signalling pathway may affect the induction of senescence through modifying the gene expression profile. Similar to *daf-16* mutants of *C. elegans*, FOXO3A variants have been associated with longevity in humans (Pawlikowska et al., 2009; Willcox et al., 2008). However, a recent study showed that the detected sequence variations may

stem from misalignments of the genomic sequences and therefore FOXO3A variations may not be frequent enough to explain the longevity in the populations under study (Donlon et al., 2012).

On the other hand, some genes involved in the IGF1/IGF1R pathway are commonly found deregulated in senescent cells – *IGF1*, *IGF1R*, *IGFBP3* and *IGFBP5* (reviewed in (Fridman and Tainsky, 2008). *IGF1R* is expressed in most tissues and therefore cells in most tissues are susceptible to changes in this signalling pathway. However, its contribution to the human aging process needs further investigation.

IGF1 signalling intersects with numerous other signalling pathways inside the cell. Among those is the TOR signalling pathway, which is inhibited by AKT and is also implicated in lifespan regulation.

TOR signalling. The mammalian target of rapamycin (TOR) is part of two distinct complexes in the cell – the rapamycin sensitive TORC1 and the rapamycin insensitive TORC2. TORC1 integrates information from the insulin signalling pathway, energy sensing by AMPK, hypoxia, stress and nutrient levels and regulates translation, growth and metabolism (see also (Wullschleger et al., 2006)) and therefore plays a central role in cellular signalling (Fig. 2.1). Mutations in various genes in the TORC1 signalling pathway that lead to increased activity of the pathway are associated with diseases like cancer, autoimmune disease, cardiovascular disease and metabolic disorders like diabetes (Wullschleger et al., 2006). Thus, both mutation of TOR and inhibition of TOR using rapamycin were shown to increase the lifespan of various laboratory organisms independent of genetic background (Table 2.2) (Harrison et al., 2009). This may at least in part be due to a delay in the development of age-related pathologies (Anisimov et al., 2011). Further, TORC1 negatively regulates insulin

signalling by inducing a redistribution of IRS1 to favour its proteasomal degradation (Takano et al., 2001).

While the TORC1 has been more extensively studied, the TORC2 was also found to play a role in the regulation of longevity. The TORC2 was shown to be required for activating phosphorylation of AKT, which increased its activity towards phosphorylating a subset of its downstream targets, including FOXO (Guertin et al., 2006). Thus, the TORC2 likely affects the lifespan through its role in insulin signalling.

Despite this connection between TOR signalling and insulin signalling, the observation that *let-363* mutation in *C. elegans* does not require *daf-16* for lifespan extension (Vellai et al., 2003) suggests that TOR signalling may also affect lifespan through insulin signalling independent mechanisms. One of those mechanisms may be the inhibition of translation in TOR signalling mutants. This is in line with the observation that the inactivation of ribosomal proteins or translation initiation factors results in lifespan extension (Table 2.2), thereby suggesting that a lower rate of translation is beneficial to survival. Kaeberlein and Kennedy (2007) suggested that such a reduced rate of protein translation may be beneficial to protein homeostasis, by reducing the rate of the production of erroneous proteins on the one hand and by giving the cell time for coping with translation errors, misfolded, or damaged proteins on the other hand (Kaeberlein and Kennedy, 2007). Secondly, TOR signalling may affect lifespan through the inhibition of autophagy. This is supported by the observation that TOR inhibition induced autophagy in a chronological lifespan model (Alvers et al., 2009b), and is also in line with the findings that autophagy mutants exhibit reduced lifespans (Table 2.3). Thirdly, TOR inhibition was shown to induce mitochondrial respiration to result in extended chronological lifespan in yeast

(Bonawitz et al., 2007). A study in mice suggests that TORC1 inhibition in adipose tissue also induces mitochondrial respiration and results in lower body weight of the animals (Polak et al., 2008), suggesting that beneficial effects of TORC1 inhibition may be tissue-specific. Thus, the variety of the downstream targets of TOR signalling (Fig. 2.1) offers multiple ways of TOR to affect lifespan, which may be tissue-specific.

FGF23 signalling. In addition to IGF1, there are some implications that other systemic factors may affect the rate of aging. Klotho, a co-receptor protein for FGF23, has been shown to induce a phenotype similar to human aging in mice carrying a mutation in the *Klotho* gene (Kuro-o et al., 1997) and increase mouse lifespan when over-expressed (Kurosu et al., 2005). Further, *Fgf23* (-/-) mice exhibit a similar phenotype to *Klotho* mutants and are also characterized by a shorter lifespan (Razzaque et al., 2006). The cellular response to FGF23 is thought to include the activation of MAP kinase signalling pathways resulting in the activation of ERK and p38, as well as phosphorylation of AKT and thereby potential interaction with IGF1 and mTOR mediated signalling (Fig. 2.1) (Medici et al., 2008).

In contrast to TOR signalling, Klotho is expressed only in the kidney (Kuro-o et al., 1997) and in the parathyroid (Ben-Dov et al., 2007), while FGF23 is mainly secreted by bones (Liu et al., 2003). However, mutations in those genes affect endocrine functions that regulate Vitamin D and phosphate homeostasis (Consortium, 2000). The finding that phosphate deficient or vitamin-D deficient diets improved the survival in *Fgf23*-null mice (Stubbs et al., 2007) suggested that the effects on lifespan are mediated by hyperphosphatemia and Vitamin D intoxication. Thus, alterations in

FGF23/Klotho signalling may affect aging through disturbed homeostasis and promotion of age-related pathologies.

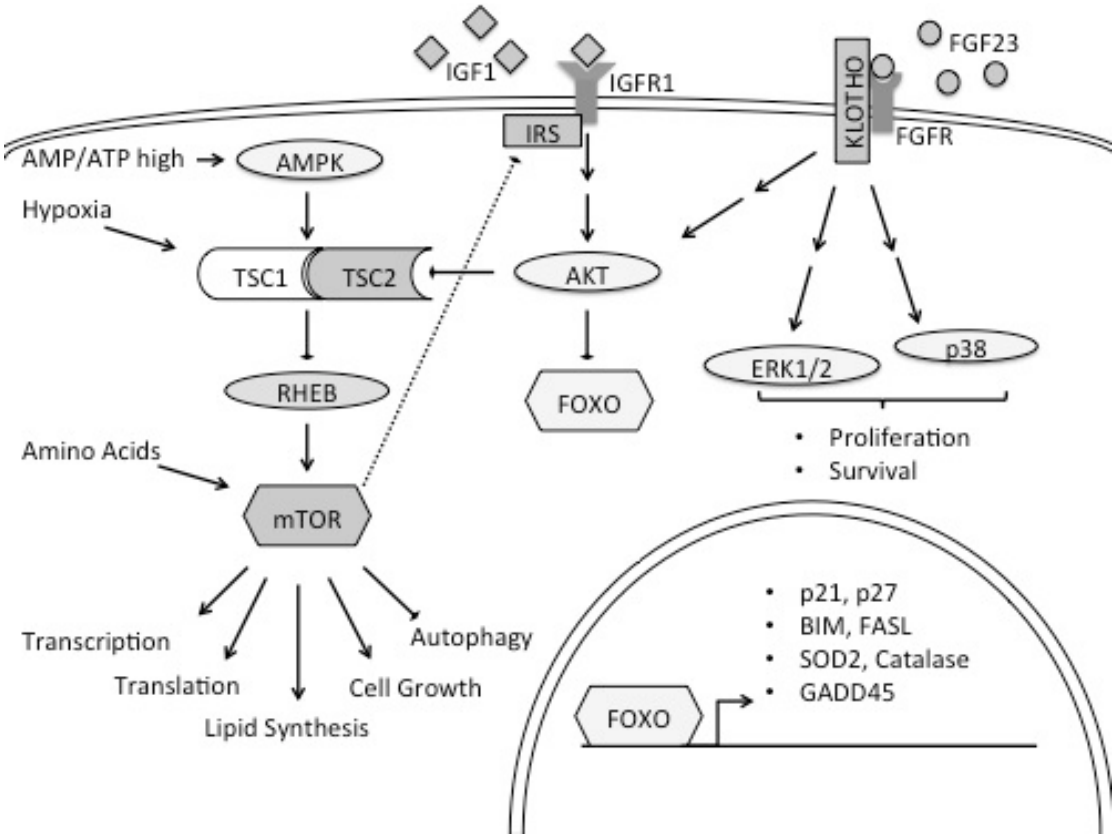

Figure 2.1: Cellular signalling pathways that may play various roles in aging.

Arrows with pointed ends indicate activation, whereas arrows with flat ends indicate inhibition.

In summary, while extensive information on mutations resulting in a lifespan increase or decrease has accumulated, their molecular role in affecting aging is only beginning to be understood. mTOR, AKT, IGF1 and FGF23 seem to be involved in a central pathway regulating aging. Interestingly, all of those signalling modules and pathways are sensitive to levels of different nutrients, like glucose, amino acids, phosphate and lipids, and in turn affect glucose, protein and phosphate homeostasis.

Therefore, understanding their combined function in aging may give hints as to how metabolism interacts with aging at a molecular level.

Oxidative stress

Metabolic activity not only affects cellular signalling, but also results in the accumulation of reactive oxygen species (ROS) and thus oxidative stress. This seems to be relevant to the aging process, since the lifespan of animals inversely correlates with their metabolic rate. However, animals with high metabolic rates but low levels of ROS production, such as birds and monkeys, have a longer than expected lifespan (Ku et al., 1993; Sohal and Weindruch, 1996).

Under normal physiological conditions there are several mechanisms in place to limit the damaging potential of ROS in the cell. On the one hand, there are enzymes for the detoxification of ROS – catalases, glutathione peroxidases and sodium dismutases – and on the other hand, the cell employs molecules with antioxidant properties, such as vitamins and glutathione. According to the "Oxidative Stress Hypothesis" the balance between the ROS generating and detoxifying mechanisms increasingly shifts towards ROS production with increasing age, thus resulting in accumulation of damaged macromolecules in the cell (Sohal and Weindruch, 1996).

Several lines of evidence support a role of oxidative damage in the aging process. First, several studies reported an accumulation of macromolecular oxidative damage with age, affecting DNA (Hamilton et al., 2001), proteins (Smith et al., 1991) and lipids (Rikans and Hornbrook, 1997). Second, mitochondrial ROS generation increases with age, due to increased respiratory rates (Sohal et al., 1994; Sohal and Sohal, 1991) or dysfunction of the electron transport chain (Boffoli et al., 1994; Sharma et al., 1998). Third, mutations in antioxidant enzymes reduce the lifespan in

various model organisms (Table 2.3). Lastly, the antioxidant systems may deteriorate with increasing age. This point is more controversial, since expression levels of antioxidant enzymes do not seem to change with age in most model systems and over-expression of SOD or catalase only extends the lifespan of some organisms (Orr and Sohal, 1994; Schriner et al., 2005).

However, ROS do not only cause damage to components of the cells, but they also play a role in cellular signalling through the oxidation of cysteines of proteins (de Keizer et al., 2011). This so-called redox signalling was shown to affect the activity of FOXO transcription factors (Dansen et al., 2009), but also various other signalling pathways within the cell.

Paradoxically, while in humans mutations in genes involved in mitochondrial function can have devastating effects, in lower organisms numerous such mutations have been described to have a positive effect on lifespan (Table 2.2). A study that employed serial dilutions of RNAi directed to several electron transport chain (ETC) genes showed that the lifespan extension associated with this down-regulation was independent of ROS levels, but correlated with slowed development and reduced adult size (Rea et al., 2007). On the other hand, ROS produced in dysfunctional mitochondria have been shown to induce a protective response by inducing the mitochondrial unfolded protein response (Baker et al., 2012). Further, there is evidence for the involvement of several transcriptional regulators in mediating the mitochondrial dysfunction associated lifespan extension, since mutation or knock-down of those transcription factors in mitochondrial mutants abrogated the lifespan extension (Khan et al., 2013; Lee et al., 2010; Ventura et al., 2009). Mild mitochondrial dysfunction has also been associated with the induction of autophagy (Schiavi et al., 2013), which is associated with beneficial effects on lifespan. In

summary, it seems that lifespan extension in lower organisms in response to mild mitochondrial dysfunction is a result of stress adaptation triggered by the chronic low levels of stress. This has been termed mitohormesis (Ristow and Zarse, 2010).

Thus, the role of ROS and mitochondrial function in aging is complex. Oxidative damage may affect tissues with higher metabolic rates more strongly, whereas changes in redox signalling may add to the already complex changes to cellular signalling with age that were described in the previous section.

Accumulation of damage

Damage to macromolecules within the cell is usually repaired or removed in order to prevent mutations and genomic instability or the aggregation of dysfunctional proteins. However, the corresponding coping mechanisms are subject to age-related changes.

DNA damage and repair. While the cumulative strain on the nuclear and mitochondrial DNA imposed by the exposure to reactive oxygen species, UV light and ionizing radiation accumulates throughout a lifetime, the DNA repair systems in place to repair the damage deteriorate with age (Fig. 2.2) (Gorbunova et al., 2007). This deterioration is characterized by a decreased repair capacity and an increased incidence of erroneous repair and has been described for most common DNA repair pathways – mismatch repair (MMR) (Krichevsky et al., 2004), nucleotide excision repair (NER) (Moriwaki et al., 1996), base excision repair (BER) (Cabelof et al., 2002) and non-homologous end joining (NHEJ) (Seluanov et al., 2004). The reduced repair capacity and fidelity results in an increased mutation rate (Szilard, 1959) and genomic instability, which includes microsatellite instability (Krichevsky et al.,

2004), loss of repetitive sequences and chromosomal aberrations (Martin et al., 1985; Tucker et al., 1999). The importance of functional DNA repair mechanisms is further underscored by the negative effect on lifespan observed in DNA repair mutants (Table 2.3).

While most DNA repair pathways are compromised with age, homologous recombination (HR) was shown to become more frequent with aging (Wiktor-Brown et al., 2006). However, this increase in HR seemed to be tissue-specific (Wiktor-Brown et al., 2008). Also, while HR is considered an error-free DNA repair mechanism, the recombination of misaligned sequences can result in chromosome rearrangement and copy number variants.

Further, unrepaired DNA damage and shortening telomeres induce a DNA damage response, which is characterized by the activation of ATM or ATR and subsequent phosphorylation of the histone variant H2AX. γH2AX then recruits DNA repair enzymes on the one hand, and relays a signal to p53 and p21 resulting either in apoptosis or in cell cycle arrest and possibly (premature) senescence. In line with this, both senescent human cells as well as senescent cells in mouse tissues have been shown to contain high levels of γH2AX foci (Sedelnikova et al., 2004). In some cell types senescence does not correlate with telomere shortening, but rather with the continuous activation of the DNA damage response pathway (Wang et al., 2009). Additionally, telomerase expression did not prevent human cells from entering stress-induced premature senescence (SIPS), which suggested that the activation of DNA damage signalling plays a role in establishing senescence (Gorbunova et al., 2002).

Thus deteriorating DNA repair systems coupled with increasing oxidative stress and continued exposure to stress from the environment, result in the accumulation of

point mutations and genomic instability. This further results in the activation of a DNA damage response leading to cellular senescence.

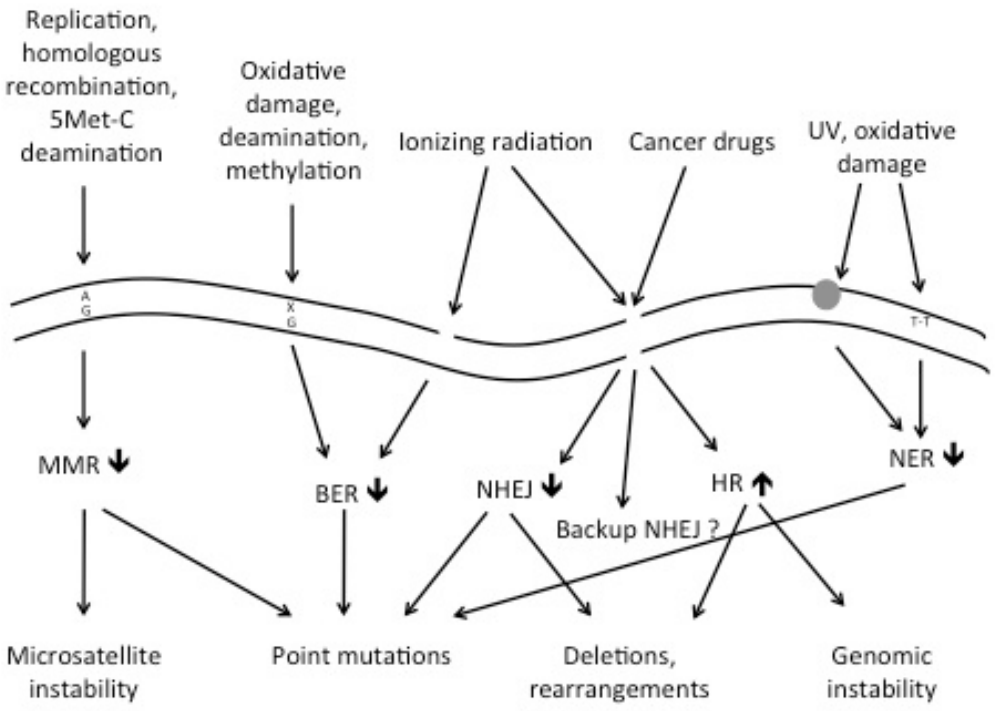

Figure 2.2: DNA damage and age-related changes in DNA repair.

Different DNA damaging agents that cause different types of DNA damage are listed on top, the repair pathways responsible to fix them in the middle and possible consequences of their dysfunction on the bottom. Arrows next to the repair pathways indicate functional decline with age. The grey circle indicates DNA damage in the form of bulky adducts. (modified from (Hoeijmakers, 2001))

Protein homeostasis. As previously mentioned, damaged proteins accumulate with age (Smith et al., 1991). In addition to reactive oxygen, other stress conditions, such as exposure to high temperature or osmotic stress, translation errors and protein misfolding contribute to the pool of damaged proteins.

Translation errors are estimated to cause missense mutations at a frequency of 10^{-3} to 10^{-4} per codon (Kramer and Farabaugh, 2007). Among those, mutations that disrupt the protein structure result in a loss of function (Guo et al., 2004). Such misfolded proteins are produced at a steady frequency throughout the lifespan (Harley et al., 1980). On the other hand, a decline in the protein folding efficiency due to reduced chaperone expression levels in the endoplasmic reticulum (ER) with age results in increased levels of un- or misfolded proteins (Erickson et al., 2006), which have a tendency to form potentially cytotoxic aggregates (Bucciantini et al., 2002).

In order to prevent this, cells possess several mechanisms that help protein folding or degradation of damaged proteins. The heat shock response involves chaperones that act by binding to unfolded and misfolded proteins and assisting them in refolding to their native structure or at least preventing them from aggregating (Freeman and Morimoto, 1996). In addition, some chaperones can release misfolded proteins from aggregates (Weibezahn et al., 2004). However, the heat shock response is down-regulated during aging, which is possibly mediated by the activated DNA damage signalling in senescent cells (Kim et al., 2012a). Further, the expression of heat shock proteins in response to stress is less efficient in old compared to young animals (Wu et al., 1993).

Proteins that fail to refold to their native structure or that carry unrepaired oxidative damage are targeted for ubiquitin-proteasome dependent degradation (Hershko et al., 1983). A decline in the activity of the ubiquitin-proteasome system has been shown to occur in several organs – heart, lung, kidney and liver – with increasing age (Keller et al., 2000), whereas this is still controversial for the brain (Cook et al., 2009; Keller et al., 2000).

Proteins that fail to be degraded or to refold to their native structure have an increased potential to form aggregates. While some proteins are known for aggregating – amyloid-β aggregates play a role in the pathogenesis of Alzheimer's disease and α-synuclein aggregation is involved in the pathogenesis of Parkinson's, most of the proteins have the potential to form aggregates (Goldschmidt et al., 2010). Stress in the form of a change in pH, temperature or protein concentration can induce the aggregation of metastable proteins. It was further shown that with increasing age, an increasing fraction of the cell's proteome becomes insoluble and experimental down-regulation of some of those genes induced lifespan extension in *C. elegans* (Reis-Rodrigues et al., 2012).

The role of the age-dependent protein aggregation in aging is not well understood. It has been hypothesized to contribute to the aggregation of disease protein leading to neurodegenerative disease by titrating the cell's chaperones (Gidalevitz et al., 2006). Further, the protein aggregates may sequester proteins and thereby leave the cell with a lower amount of functional protein and interfere with the function of the cell, or the aggregates may be toxic (David, 2012). However, it cannot be excluded that at least some of the aggregates have no adverse effect on the organism (Maji et al., 2009).

Further, other macromolecules and structures within the cell are subject to damage with aging. D'Angelo *et al.* (2009) showed that scaffold nucleoporins were down-regulated in postmitotic cells, resulting in the leakage of cytoplasmic proteins into the nucleus (D'Angelo et al., 2009). This in turn resulted in severe chromatin aberrations. The role of changes to the chromatin structure is the topic of the next section.

Epigenetics and aging

While Conrad Waddington coined the term of epigenetics as "the interactions of genes with their environment that bring the phenotype into being" in 1942 (Waddington, 2012), a role of epigenetics in establishing the aging phenotype has only been considered in recent years. In the meantime the definition of epigenetics has been modified to "heritable changes in gene expression that do not involve a change in DNA sequence". Instead, epigenetic mechanisms affect the overall chromatin structure through the methylation of DNA cytosines (Goll and Bestor, 2005), nucleosome occupancy and post-translational modifications to histone tails (Jenuwein and Allis, 2001), or the gene expression pattern through regulatory small RNAs (including small interfering RNAs and micro RNAs) (Bernstein and Allis, 2005; Goldberg et al., 2007).

Roughly, chromatin exists in two alternate conformations: euchromatin, which is loosely packed and therefore more accessible to regulatory factors and generally associated with active gene expression, but also more prone to damage; and heterochromatin, which is tightly packed and generally not transcriptionally active, but to some extent protected from damage. Thus, changes to the chromatin structure have been associated with the modulation of gene expression profiles and genome stability (Hennig, 1999). Since both changes to gene expression profiles and genome stability have been associated with aging, understanding the epigenetic changes that occur with age in more detail will help to clarify the role epigenetics plays in the regulation of aging.

DNA methylation. Bisulfite sequencing of the genomes from $CD4^+$ T cells from a newborn and a centenarian revealed a global loss of DNA methylation with age, with

differentially methylated sites being identified in promoter (~10%), exonic (~10%), intronic (~45%) and intergenic (~35%) regions (Heyn et al., 2012). When Heyn *et al.* (2012) compared the global methylation levels in the centenarian and newborn genomes to the level in the genome of a 26 year old, they found that the global DNA methylation level in the 26 year old was intermediate. Thus, they proposed a model, according to which cells accumulate stepwise changes in DNA methylation throughout the lifespan.

A decrease in genome methylation with age has also been observed in other aging model organisms - mice (Wilson et al., 1987) and rats (Vanyushin et al., 1973); and the inhibition of DNA methylation resulted in reduced replicative lifespan of cells (Table 2.3) (Holliday, 1986). However, the extent of global DNA hypomethylation and the CpG sites affected may vary between different tissues – DNA methylation levels in rat adipose tissues did not significantly change with age (Thompson et al., 2010).

While global DNA hypomethylation is observed in various tissues with age, the accumulation of domains of heterochromatin during the early steps leading to senescence has been described (Narita et al., 2003). These senescence associated heterochromatin foci (SAHF) were originally shown to coincide with E2F target promoters. However, while pRB and p53 were shown to play important roles in the establishment of SAHF, senescence in response to oncogene activation also involves the formation of SAHF (Ye et al., 2007). The observation that telomeres and subtelomeric regions become de-heterochromatinized in the absence of telomerase in aging mice (Benetti et al., 2007), lead Zhang and Adams to propose that during aging heterochromatin marks get redistributed from areas of constitutive heterochromatin,

such as telomeres and pericentromeres, to areas of facultative heterochromatin, such as SAHF (Zhang and Adams, 2007).

Thus, with a shift to hypomethylation of constitutive heterochromatin and hypermethylation of promoters of cell cycle promoting genes, age-dependent changes in DNA methylation may contribute to the increased genomic instability and to the establishment of a permanent cell cycle arrest. Further, the differential methylation of promoter CpGs may also contribute to the observed changes in senescence- and age-related gene expression profiles.

Post-translational modifications of histone tails and nucleosome occupancy. Post-translational modifications of the histone amino tails affect their affinity to DNA and to interacting proteins, and different combinations of such modification marks may have synergistic or antagonistic effects on those affinities. This indicates a role of histone modifications in the establishment of transcriptionally active or repressive chromatin states and has lead Jenuwein and Allis to the formulation of the "histone code" theory (Jenuwein and Allis, 2001). Generally, acetylation and phosphorylation of histone tails are considered to be euchromatin marks and methylation of histones is enriched in heterochromatin regions (Jenuwein, 2001). However, some exceptions are known too – H4K12 acetylation is a repressive mark, whereas H3K4 and H3R17 methylation are activating marks. The effect of histone modification marks is mediated by bromodomain containing proteins in the case of acetylation marks (Dhalluin et al., 1999) or by chromodomain containing proteins in the case of methylation marks (Bannister et al., 2001).

In addition to histone tail modifications, the nucleosome density affects the level of DNA packaging as well. Thus, DNA regions with a high density of nucleosomes are

likely transcriptionally inactive, while transcriptionally active DNA regions are characterized by more loose packaging and a low density of nucleosomes (Boeger et al., 2003).

Both nucleosome occupancy and histone tail modifications experience changes as cells age (Table 2.4). A decline in nucleosome occupancy with increasing age was first demonstrated in human skin fibroblasts, where the linker regions between nucleosomes were shown to become increasingly heterogeneous in length (Ishimi et al., 1987). The global loss of histones has been hypothesized to be associated with a reduced deposition of histones – which is mediated by ASF1 in a replication dependent and in a replication independent manner together with CAF1 and HIRA (Galvani et al., 2008; Groth et al., 2007), or by reduced expression, which is also regulated by ASF1 during replication (Sutton et al., 2001). The loss of function of ASF1 in yeast resulted in impaired heterochromatin formation and genomic instability (Tanae et al., 2012). The finding that ASF1 expression decreases with age in human cells (O'Sullivan et al., 2010) and that over-expression of histones increases lifespan (Feser et al., 2010), may indicate that declining nucleosome occupancy in aging human cells similarly results in a loss of heterochromatin paired with increased genomic instability and likely also in changes to the gene expression profile. However, in order to better understand the role of nucleosome loss in aging, a more detailed understanding of DNA regions affected by reduced nucleosome density as well as of the functional consequences is needed.

In addition to overall changes in nucleosome numbers, numerous changes in post-translational modifications of histone tails with age have been described (Table 2.4). However, their functional consequences are only beginning to be understood: H3K56ac promotes nucleosome assembly and transcriptional activation of histone

gene expression (Kaplan et al., 2008; Williams et al., 2008); H3K9me3 is involved in heterochromatin formation in telomeric, subtelomeric and pericentromeric regions in young cells (Benetti et al., 2007; Vaquero et al., 2007) and is also associated with the establishment of SAHF in senescing cells (Narita et al., 2003); H4K16ac, which is also involved in telomere silencing (Kozak et al., 2010).

Those modifications are regulated by various histone modifying enzymes – histone acetyltransferases and deacetylases (Kuo and Allis, 1998), histone methyltransferases and histone demethylases (Black et al., 2012). Thus, changes in the frequencies of different histone modifications may be associated with different expression or activity levels of histone modifying enzymes. In line with this, the redistribution of SIRT1 from heterochromatin regions to sites of DNA damage resulted in gene expression changes resembling those in aging tissues (Oberdoerffer et al., 2008) and the histone methyltransferase SUV39H1 is thought to play a key role in telomere maintenance through methylating H3K9 and inducing heterochromatin formation in the telomeric DNA regions (Uhlirova et al., 2010).

While several mutations in histone modifying enzymes have been shown to affect organismal lifespan (Tables 2.2 and 2.3), how their function interacts with the aging process is not clear. It seems likely that changes to histone metabolism and post-translational modifications, like changes in DNA methylation, may also affect aging through changes in the distribution of euchromatin and heterochromatin and thus changes in genome stability and gene expression. However, a more detailed understanding of the histone code, the roles of histone modifying enzymes in the establishment of senescence and aging as well as of the distribution of certain modifications across the genome is necessary in order to determine the role of histone biology in aging.

Table 2.4: Changes in histone modification pattern and nucleosome occupancy with increasing age and senescence.

Arrows indicate age-related up- (↑) or down-regulation (↓).

Modification	Yeast	Mouse	Rat	Human	
Global acetylation				↓	(Ryan and Cristofalo, 1972)
H4 acetylated			↓		(Pina et al., 1988)
H3K9me				↑	(O'Sullivan et al., 2010)
H3K9me2				↓	(O'Sullivan et al., 2010)
H3K9me3		↑		↓↑	(Braig et al., 2005; Di Micco et al., 2011; Larson et al., 2012; Narita et al., 2003; O'Sullivan et al., 2010; Scaffidi and Misteli, 2006)
H3K9ac		↑	↓	↑	(Kawakami et al., 2009; Michishita et al., 2008; Mostoslavsky et al., 2006; O'Sullivan et al., 2010; Scaffidi and Misteli, 2006)
H3S10phospho			↑	↓	(Kawakami et al., 2009; O'Sullivan et al., 2010)
H3K14ac		↑			(Huang et al., 2007)
H3K27me3		↓		↓	(Bracken et al., 2007; Dhawan et al., 2009)
H4K8ac		↑			(Huang et al., 2007)
H4K12ac		↑			(Huang et al., 2007; Peleg et al., 2010)
H3K56ac	↓			↓	(Dang et al., 2009; Feser et al., 2010; O'Sullivan et al., 2010)
H4K16ac	↑	↓		↑↓	(Dang et al., 2009; O'Sullivan et al., 2010)
H4K20me				↑	(O'Sullivan et al., 2010)
H4K20me2				↑	(O'Sullivan et al., 2010; Sarg et al., 2002)
H4K20me3			↑	↑↓	(O'Sullivan et al., 2010; Sarg et al., 2002)
H3.1	↓		↓	↓	(Feser et al., 2010; O'Sullivan et al., 2010; Pina and Suau, 1985; Rogakou and Sekeri-Pataryas, 1999)
H3.2	↓		↓	↓	(Feser et al., 2010; O'Sullivan et al., 2010; Pina and Suau, 1985; Rogakou and Sekeri-Pataryas, 1999)
H3.3			↑	↑	(Pina and Suau, 1985; Rogakou and Sekeri-Pataryas, 1999)
H4				↓	(O'Sullivan et al., 2010)
H2A.1	↓		↓	↓	(Feser et al., 2010; Pina and Suau, 1985; Rogakou and Sekeri-Pataryas, 1999)
H2A.2			↑	↑	(Pina and Suau, 1985; Rogakou and Sekeri-Pataryas, 1999)
γH2AX		↑		↑	(d'Adda di Fagagna et al., 2003; O'Sullivan et al., 2010)

miRNA expression profile. miRNAs are small (19 - 25 nt), single-stranded RNA molecules that are involved in post-transcriptional gene silencing in mammalian cells (Ambros, 2001). They are transcribed from miRNA genes by RNA Polymerase II, which results in a pri-miRNA transcript containing the hairpin loop that is the precursor of the final miRNA. This primary transcript undergoes a sequence of

maturation steps including the cropping mediated by Drosha, which isolates the hairpin loop, nuclear export mediated by Exportin 5, dicing by Dicer and strand selection (Kim, 2005). The selected strand is incorporated into the RISC complex which then mediates mRNA degradation in the case of extensive homology between miRNA and mRNA or translational repression in the case of partial homology between the miRNA and sequences in the 3'UTR of the mRNA (Bartel, 2004).

miRNAs are expressed from miRNA genes, which include promoter regions (Lee et al., 2004). Thus, miRNA expression may be subject to transcriptional regulation similar to protein coding genes. Indeed, many miRNAs are found to have tissue-specific (Liu et al., 2004) or development-dependent expression patterns (Miska et al., 2004). Accordingly, numerous miRNAs were found with altered expression levels in senescing cells (Table 2.5). Among those, miR-210, miR-376a*, miR-486-5p, miR-494 and miR-542-5p induced senescence-like phenotypes, including SAHF formation, senescence associated β-galactosidase activity, accumulation of DNA damage and ROS, when over-expressed in low passage IMR90 cells (Faraonio et al., 2012). The expression of all but miR-486-5p depended on the activity of the mTOR pathway. Further, the miR-17-92 cluster, which contains the first identified oncogenic miRNA, is commonly down-regulated in senescing cells and aging tissues. Expression of this cluster is induced by E2F, which becomes less active with age, and repressed by p53, which becomes more active with age (Grillari et al., 2010). On the other hand, different miRNAs modulate most of the processes that are thought to be involved in aging, as described above, including IGF1 signalling, mTOR signalling, the DNA damage response, stem cell depletion, oxidative stress (Grillari and Grillari-Voglauer, 2010) and mitochondrial function (Rippo et al., 2014).

In addition to roles of single miRNAs in the establishment of senescence, the down-regulation of miRNA biogenesis through the knock-down of DGCR8 resulted in the induction of senescence (Gomez-Cabello et al., 2013). This was associated with increased p21 protein levels due to the decreased expression of miRNAs targeting the p21 transcript.

Along with the de-heterochromatinization of the genome, the majority of deregulated miRNAs is up-regulated with age (Lanceta et al., 2010). Since miRNA-target recognition does not depend on perfect complementarity, one miRNA may regulate numerous targets (Friedman et al., 2009). Thus, changes in the expression of individual miRNAs can result in much broader changes in mRNA transcript profile. Therefore, a better understanding of the miRNA targets of senescence-associated miRNAs will help in better estimating the role of miRNAs in aging and senescence.

Table 2.5: Changes in miRNA expression profile associated with senescence.

miRNA	Change with age	Reference
let7 a/b/c/d	↑	(Uziel et al., 2010)
let7c	↓	(Bonifacio and Jarstfer, 2010)
miB134	↑	(Faraonio et al., 2012; Wang et al., 2011)
miR106a	↓	(Hackl et al., 2010; Wang et al., 2011)
miR106b	↓	(Faraonio et al., 2012; Wang et al., 2011)
miR10b	↑	(Bonifacio and Jarstfer, 2010)
miR125-5p	↓	(Uziel et al., 2010)
miR126	↑	(Faraonio et al., 2012)
miR128	↑	(Uziel et al., 2010)
miR130b	↑	(Rivetti di Val Cervo et al., 2012)
miR137	↑	(Shin et al., 2011)
miR138	↑	(Rivetti di Val Cervo et al., 2012; Uziel et al., 2010)
miR140-3p	↓	(Uziel et al., 2010)
miR141	↑	(Dimri et al., 2013)
miR141-3p	↑	(Yu et al., 2013)
miR143	↑	(Bonifacio and Jarstfer, 2010; Uziel et al., 2010)
miR145	↑	(Bonifacio and Jarstfer, 2010)
miR145	↓	(Wang et al., 2011)
miR146a	↓↑	(Bonifacio and Jarstfer, 2010; Rippo et al., 2014)
miR146b	↓	(Uziel et al., 2010)
miR148a	↑	(Uziel et al., 2010)
miR152	↑	(Mancini et al., 2012; Wang et al., 2011)
miR155	↓	(Bonifacio and Jarstfer, 2010; Faraonio et al., 2012; Wang et al., 2011)
miR15a	↓	(Wang et al., 2011)
miR15b	↓	(Faraonio et al., 2012; Wang et al., 2011)
miR17	↓	(Hackl et al., 2010; Uziel et al., 2010; Wang et al., 2011)
miR17-5p	↓	(Faraonio et al., 2012)

miR181a/b	↑	(Mancini et al., 2012; Overhoff et al., 2014; Rippo et al., 2014; Rivetti di Val Cervo et al., 2012; Uziel et al., 2010)
miR18a	↓	(Uziel et al., 2010)
miR191	↑	(Lena et al., 2012)
miR192	↓	(Uziel et al., 2010)
miR199a-3p	↑	(Bonifacio and Jarstfer, 2010; Uziel et al., 2010)
miR199b-5p	↓	(Faraonio et al., 2012)
miR19a	↓	(Bonifacio and Jarstfer, 2010; Uziel et al., 2010)
miR19b	↓	(Faraonio et al., 2012; Hackl et al., 2010)
miR20a	↓	(Faraonio et al., 2012; Hackl et al., 2010; Wang et al., 2011)
miR21	↓	(Bonifacio and Jarstfer, 2010; Uziel et al., 2010)
miR210	↑	(Faraonio et al., 2012; Overhoff et al., 2014)
miR217b	↓	(Wang et al., 2011)
miR23a	↑	(Bonifacio and Jarstfer, 2010)
miR23b	↑	(Faraonio et al., 2012)
miR25	↓	(Wang et al., 2011)
miR26a	↑	(Bonifacio and Jarstfer, 2010)
miR26b	↑	(Overhoff et al., 2014)
miR29	↑	(Martinez et al., 2011)
miR296	↑	(Wang et al., 2011)
miR296-5p	↓	(Faraonio et al., 2012)
miR30	↑	(Martinez et al., 2011)
miR30e-5p	↑	(Faraonio et al., 2012)
miR335	↑	(Tome et al., 2014)
miR34a	↑	(Bonifacio and Jarstfer, 2010; Rippo et al., 2014)
miR369-3p	↑	(Faraonio et al., 2012)
miR376a	↑	(Faraonio et al., 2012)
miR379	↑	(Faraonio et al., 2012)
miR410	↑	(Wang et al., 2011)
miR424	↑	(Overhoff et al., 2014)
miR424-2	↓	(Wang et al., 2011)
miR431	↑	(Wang et al., 2011)
miR432	↑	(Wang et al., 2011)
miR485-5p	↑	(Faraonio et al., 2012)
miR486-5p	↑	(Faraonio et al., 2012)
miR493	↑	(Wang et al., 2011)
miR494	↑	(Faraonio et al., 2012)
miR496	↑	(Wang et al., 2011)
miR542-5p	↑	(Bonifacio and Jarstfer, 2010; Faraonio et al., 2012)
miR654	↑	(Faraonio et al., 2012)
miR656	↑	(Faraonio et al., 2012)
miR668	↑	(Shin et al., 2011)
miR7	↓	(Wang et al., 2011)
miR92	↓	(Faraonio et al., 2012)
miR92-1b	↓	(Wang et al., 2011)
miR93	↓	(Faraonio et al., 2012)
miR93a	↓	(Wang et al., 2011)
let7 a/b/c/d	↑	(Uziel et al., 2010)

In summary, the exact mechanism, which causes organismal aging, is not yet understood. Vijg and Dolle (2002) have put forward a model, according to which aging is not a clonal phenomenon, but rather arises from increasing heterogeneity of the cells in a tissue (Vijg and Dolle, 2002). Thus, it is possible that the functional decline of a tissue is caused by various changes to the cells it is composed of and the functional decline of the organism by various changes to the tissues and organs it is

composed of. With the high specialization of all the different organs that make an organism, it is likely that the mechanisms that contribute to the functional decline of certain tissue- or cell types differ. Cells of the intestinal epithelium, which are constantly exposed to stress, may be more susceptible to senescence caused by activated DNA damage signalling (Wang et al., 2009), whereas T cells that in order to perform their immune function need to clonally expand may be more susceptible to senescence caused by telomere shortening (Bodnar et al., 1996).

Clearly more research is needed to get a systemic understanding of underlying mechanisms that cause aging. Understanding the processes that drive aging at a cell and organ level is a first step and has already given insights into commonalities and differences in the aging process of different organs. In order to potentially increase the functional lifespan of humans, a systemic understanding of the aging process, including the identification of the very initial molecular changes that entail aging, is necessary. However, in order to reverse aging phenotypes caused by stress, such as radiation therapy, a better understanding of mechanisms underlying SIPS may help either adjust stress exposure (e.g. maximum doses of radiation delivered to healthy tissues during radiation therapy) or to develop therapeutics that can help prevent or reverse such phenotypes caused by the stress exposure.

2.5 AGING AND RESPONSE TO RADIATION IN MAMMALS

At the organismal level, those age-related molecular changes result in a higher risk for developing a variety of diseases, such as autoimmune disease, infectious disease, and cancer.

As ionizing radiation has long been known for its genotoxic potential (Muller, 1927), it has become a very commonly used tool for cancer treatment. In addition,

patients are also exposed to low doses of radiation during diagnostic procedures or in the form of scattered radiation. While the treatment strategies have been adjusted to focus the peak dose of radiation more and more precisely at the tumor tissue, the assessment of potential side effects of exposure to low dose ranges, exposure to scatter doses (around 0.1 Gy) received by distal organs and healthy tissue, and the age at exposure is still incomplete (Paganetti, 2012). In order to get a better understanding of this, more knowledge on the differences in the response of healthy cells of different age or senescence status to ionizing radiation is needed. In this section, we summarize the current knowledge on the response to IR, age-dependent differences in IR sensitivity and the current knowledge on mechanisms underlying those differences in IR sensitivity.

2.5.1 IR induced damage and damage response

Ionizing radiation has long been recognized for its mutagenic and genotoxic potential and for causing chromosomal aberrations, such as translocations, inversions and fragmentations (Muller, 1927). As molecular techniques became more sophisticated, it became established that the direct energy deposition on DNA results in DNA single- and double-strand breaks and crosslinks (Dizdaroglu, 1992; Gichner et al., 2000; Ptacek et al., 2001). Also, as γ-rays and X-rays pass through the aqueous cyto- and nucleoplasm of a cell, they can ionize water, leading to the formation of ROS (Calucci et al., 2003; Lee et al., 2009). The effects of short-lived hydroxyl radicals are neutralized by molecular scavengers. However, γ-irradiation also induces the formation of longer-lived radicals that are involved in the formation of inter-strand crosslinks and chromosomal aberrations (Koyama et al., 1998). DNA damage,

in particular double-strand breaks, is potentially lethal to cells. Thus, cells that have been exposed to ionizing radiation need to cope with this damage.

Exposure to ionizing radiation was found to prolong the time spent in several of the cell cycle phases very early on, most prominently the G2 phase (Terasima and Tolmach, 1963). Some cells irreversibly arrest in G2 and subsequently die, whereas others re-enter the cell cycle after a delay. This delay can be decreased by the treatment with agents that inhibit DNA repair (Lucke-Huhle et al., 1983), which suggests that the main purpose of the G2 arrest is to give the cells time to repair DNA damage.

At the center of the molecular response to radiation lies ATM, which rapidly becomes active in response to DNA double-strand breaks (Bakkenist and Kastan, 2003). The important role of ATM in the DNA damage response is underlined by the observation that *atm* mutants are markedly radiosensitive (Lees-Miller et al., 1995). Upon activation, ATM phosphorylates downstream targets like CHK2, which subsequently phosphorylates CDC25C, a CDK inhibitor (Matsuoka et al., 1998; Matsuoka et al., 2000); and p53 (Barlow et al., 1997). The activation of p53 can result either in the induction of cell cycle arrest or in the induction of apoptosis – through activating the expression of genes like p21 and 14-3-3σ on the one hand or PUMA, BAX and BAK on the other hand (Vogelstein et al., 2000).

DNA damage checkpoint recovery is achieved after the repair of the DNA damage and subsequent removal of γH2AX from the chromatin around the damage site and dephosphorylation of γH2AX (Bouquet et al., 2006; Keogh et al., 2006). Cells that fail to initiate the DNA damage checkpoint or recover from it without fully repairing the DNA enter mitosis and die by mitotic catastrophe (Castedo et al., 2004). On the other hand, long term G2 arrest has been shown to allow cells to slip into the G1

phase and senescence (Ye et al., 2013). Thus, the three major physiological outcomes for IR exposed cells are: 1) continued cell division after repair of the DNA damage, 2) death by necrosis, apoptosis or mitotic catastrophe due to an inability to repair the damage, and 3) prolonged growth arrest followed by stress-induced premature senescence. The response of an individual cell largely depends on its molecular and physiological status – cell- or tissue-specific gene expression profiles (Weichselbaum et al., 1988), as well as the cell cycle phase (Terasima and Tolmach, 1963) may affect the radiation sensitivity. As those parameters can differ extensively between cells in a young when compared to an old organism, as described in the previous section, they are likely to affect the organismal radiation sensitivity.

In addition to this physiological response to radiation, cells that are exposed to IR exhibit extensive changes to their molecular profiles:

Changes in gene expression profiles. The analysis of gene expression profiles of irradiated cells, showed changes in the expression of genes involved in the DNA damage response and repair, but more surprisingly also numerous genes involved in signal transduction, RNA and protein metabolism, energy metabolism, cell structure and cell adhesion, cellular transport and lysosome metabolism (Rieger and Chu, 2004). Further, a study that compared IR-effects on the transcriptomes of 63 different cancer cell lines revealed major differences in response depending on the presence or absence of functional p53 (Amundson et al., 2008), suggesting that p53 plays a major role in modulating the gene expression profile in response to radiation.

However, when DNA damage was caused by radioactive decay of a 125-Iodine nuclide incorporated in the DNA as 125-iododeoxyuridine, fewer genes were deregulated than in response to exposure to external ionizing radiation (Sokolov et al.,

2006), suggesting that the DNA damage response only in part contributes to the changes in gene expression profile. In addition, nitric oxide was also shown to affect the transcriptome following the exposure to ionizing radiation in normal human fibroblasts (Sokolov et al., 2012). However, it is unclear how age-related changes in gene expression profile and chromatin structure may affect those changes in gene expression in response to ionizing radiation and how those differences in transcript profiles could affect the functionality of the tissue they reside in.

Epigenetic changes. Exposure to ionizing radiation has been shown to result in a global loss of DNA methylation in the target tissues, resulting in increased genomic instability and aberrant gene expression (Koturbash et al., 2005; Pogribny et al., 2004). However, changes in methylation patterns in response to radiation depended on tissue, sex, dose and were not uniform across the genome (Kovalchuk et al., 2004b; Pogribny et al., 2004).

This observed loss of heterochromatin in response to ionizing radiation may initially be beneficial, by facilitating DNA repair (Zheng et al., 2014). However, if sustained past the initial DNA repair phase, it may result in genomic instability due to extrachromosomal recombination or reactivation of transposable elements, or in aberrant gene expression, including repression of tumor suppressors or induction of oncogenes. Thus, ultimately this could promote senescence, cell death or malignant transformation depending on the specific outcome.

Local changes in the combinations of post-translational histone modifications around the DNA damage site have also been observed and are thought to play important roles in the recruitment of proteins involved in the DNA damage checkpoint and DNA repair. This includes changes in histone methylation (Pei et al.,

2011), histone acetylation (Sharma et al., 2010) and histone ubiquitination (Huen et al., 2007). However, how histone modifications may be affected beyond the sites of DNA damage and how age-dependent changes in chromatin structure may affect the assembly of DNA damage foci remains subject of future study.

In addition to changes in DNA methylation and histone modification status, numerous miRNAs are deregulated upon exposure to ionizing radiation: The *let-7* family of miRNAs (Cha et al., 2009; Dickey et al., 2011; Nikiforova et al., 2011; Simone et al., 2009), which may sensitize cells to ionizing radiation through the induced expression of pro-apoptotic genes (Saleh et al., 2011); miR-34 (Nikiforova et al., 2011; Simone et al., 2009), which is thought to be involved in p53-dependent regulation of cell cycle arrest and apoptosis in response to radiation (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Kato et al., 2009); miR-21 (Cha et al., 2009; Dickey et al., 2011; Li et al., 2011b; Simone et al., 2009), which may also modulate the induction of the DNA damage checkpoint versus apoptosis in response to radiation (Anastasov et al., 2012; Li et al., 2011b); and the oncogenic miR-17-92 cluster (Chaudhry et al., 2012).

There are many overlaps in the changes in miRNA expression induced by radiation and as a result of senescence (see 2.4), which may be a result of similar molecular changes associated with both, such as the generation of ROS. Simone *et al*. (2009) showed that blocking oxidative stress induced by exposure to ionizing radiation suppressed changes in miRNA expression profile, suggesting that ROS are crucial mediators in modulating the miRNA expression profiles. Further, p53 has been shown to regulate the expression of some miRNAs, including the miR-17-92 cluster (Yan et al., 2009), and changes in p53 activation and stabilization may therefore also account for some of the changes in miRNA expression profiles associated with aging and

radiation response. However, further studies are needed to better understand differences in miRNA regulation in aging tissues and how such differences may affect the response to radiation.

2.5.2 Age-dependent differences in radiation sensitivity

While age is a risk factor for cancer, how the molecular changes that are observed with increasing age of the organism or as cells undergo replicative senescence, affect the response to radiation is not well understood.

Radiation sensitivity at the organismal level

Radiation sensitivity at the organismal level is judged by the frequency of side effects, cancer relapse or radiation related mortalities.

Therapeutic and diagnostic radiation exposures received during childhood have been associated with an increased risk of developing secondary cancers when compared to adult patients (Adams et al., 2010; Kleinerman, 2006). Further, studies on Japanese A-bomb survivors showed that their blood contained less naïve T cells when compared to an age-matched control cohort (Yamaoka et al., 2004), which may result in an increased susceptibility to infectious disease. In line with these observations, the main contributors to mortality within 30 years of the initial diagnosis are secondary tumors, tumor relapse or progression, cardiac and pulmonary death (Chaturvedi et al., 2007). Another concern is the stress induced premature senescence, which may interfere with growth and normal development, particularly in children (Krasin et al., 2010).

While radiation sensitivity does not seem to vary significantly with age throughout adulthood, it is thought that the elderly again become more radiation sensitive. This is

based on observations like the increased risk of developing dementia and brain atrophy after radiation treatment of brain tumors (Stylopoulos et al., 1988), which however may have resulted from the use of whole brain irradiation at that time (Walker et al., 1978). Indeed, three-dimensional (3D) irradiation strategies have allowed for more successful control of tumors in patients over 75 when compared to younger patients with no concurrent increase in side effects (Geinitz et al., 2005). Thus, radiation to healthy tissue in the elderly mainly results in side effects due to the inability to repair or replace the affected cells, while localized irradiation of the tumor is more efficient in controlling it.

It is noteworthy that most of the knowledge on late effects of radiation therapy is based on data from patients that were irradiated at a time when those treatments were much less precise than the current treatment modalities. As demonstrated by Geinitz *et al*. (2005) aiming the peak of the dose at the tumor tissue seems to be key to reducing the side effects. However, this is hard to achieve for malignancies that are not localized (Cella et al., 2013), and it is also still a challenge to estimate and control scatter doses received by distal organs (Joosten et al., 2013).

Radiation sensitivity at the level of the cell

As previously mentioned, how an individual cell responds to IR induced damage is dependent on its molecular and physiological status – cell- or tissue-specific gene expression profiles (Weichselbaum et al., 1988) as well as the cell cycle phase (Terasima and Tolmach, 1963) may affect the radiation sensitivity.

Cancer cells. The main goal of radiation therapy is to eliminate cancer cells. However, due to the high heterogeneity in genetic and epigenetic status of the cells in

different tumors (Vogelstein et al., 2013) and even between cells within the same tumor (Gerlinger et al., 2012), the response to different treatments is likely heterogeneous too (Weichselbaum et al., 1988). Whether tumor cells die in response to ionizing radiation seems to depend on their p53 status – while cells that express p53 are more prone to induce senescence, cells that lack p53 are more prone to undergo apoptosis (as reviewed in (Gewirtz et al., 2008).

Non-cancer dividing and senescent cells. Non-cancer cells exhibit a quick accumulation of DNA damage foci (as detected by γH2AX immunostaining) after exposure to ionizing radiation, which start disappearing within 1 hour of the exposure. However, each exposure causes the formation of a number of unrepairable double-strand breaks (Noda et al., 2012). This is thought to contribute to permanent growth arrest and premature senescence, as such an accumulation of unrepairable DNA damage is also observed in senescing cells and aging animals (Sedelnikova et al., 2004). The extent to which cells become senescent or die is dose dependent (Noda et al., 2012). However, they both may contribute to side effects of the radiation treatment. Stress-induced premature senescence results in changes to the secretory profile of the cells similar to the pro-inflammatory secretion profile of senescent cells. This may help reinforce the senescence status of the damaged cells (Rodier et al., 2009), but it may also induce tissue inflammation and fibrosis (Gallet et al., 2011) and stimulate proliferation or invasion in the neighbouring tumor cells (Krtolica et al., 2001). Further, senescence and apoptosis when induced in progenitor cells that are relevant to the development and growth of an organ can result in developmental deficits (Krasin et al., 2010).

The response of senescent cells to ionizing radiation is even less understood. Since senescent cells do not undergo active cell cycle, have already accumulated DNA damage, have an altered chromatin structure and show decreased expression of several DNA repair enzymes (see 2.4), they may respond with repair or tolerance of the DNA strand breaks or with cell death. While irradiated young cells mostly die by p53-dependent or –independent apoptosis, senescent cells fail to stabilize p53 and mostly die by necrosis (Seluanov et al., 2001). However, postmitotic cells in muscles and the brain seem to be more resistant to radiation-induced cell death than their dividing progenitors (Latella et al., 2004; Zou et al., 2012). Thus, further studies are needed to understand the response to radiation in senescent cells and how it may contribute to the radiation side effects observed at an organismal level.

2.5.3 Molecular mechanisms guiding side effects of radiation therapy

While differences in radiation sensitivity are observed between young and adult patients, there is only limited knowledge on what directs those differences at the molecular, cellular or systemic level. In the following paragraphs we will discuss some of the findings that indicate roles for altered hormonal regulation, alterations in cellular microenvironment, telomere shortening and genomic instability.

Hormones. Radiation therapy directed to the head and spine is commonly used to treat malignancies of the central nervous system (CNS) or for the prophylaxis of meningeal relapse of childhood leukemia (Pinkel and Woo, 1994). Such exposures often result in a lower final body height and weight of the patients in a dose-dependent way (Muller et al., 1998), and in early puberty (Lannering et al., 1997; Melin et al., 1998). Those changes correlate with altered hormone levels, such as

increased secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Lannering et al., 1997) or decreased growth hormone (GH) secretion (Melin et al., 1998). Similar changes were observed in a rat model of juvenile and infantile cranial irradiation (Roth et al., 2000). The authors suggested that the observed changes in hormone secretion profiles could be a result of unspecific neuronal lesions due to the exposure, which result in the premature activation of the secretion of Gonadotropin releasing hormone (GnRH), based on the finding that hypothalamic lesions had previously been shown to indirectly induce premature puberty in rats (Junier et al., 1991).

Loss of progenitor cells. Loss of progenitor cells in the brain due to radiation exposure has been addressed in a rat model of juvenile and infantile cranial irradiation (Fukuda et al., 2005). This study showed that a significantly higher number of cells in the subventricular zone of the lateral ventricles and in the subgranular zone of the dentate gyrus in the hippocampus, where progenitor cells are found in the brain, underwent apoptosis when comparing very young (9 day old) to young (23 day old) rats following cranial exposure to 8 Gy. This correlated with the activation of p53 and increased oxidative stress (Fukuda et al., 2005). Further, the radiation exposure compromised myelination in the corpus callosum, cortex and striatum more significantly when rats were exposed at the younger age (Fukuda et al., 2005). This loss of progenitor cells may explain for the observed deficits in intellectual and behavioural development (Duffner et al., 1985; Hall et al., 2004).

Telomere dysfunction and DNA repair deficiency. As previously mentioned, the repair of radiation-induced DNA damage is vital to the exposed cell's survival.

Therefore, cells with compromised DNA repair pathways are more susceptible to radiation-induced cell death (Taalman et al., 1983; Taylor et al., 1975). Since DNA repair is also compromised in senescent cells (see 2.4), this suggests that senescent cells may also be susceptible to radiation-induced cell death. In addition, senescence is associated with shortened telomeres in numerous cell types (see 2.4), and critically shortened telomeres can serve as an additional substrate during the repair of radiation-induced DNA damage (Soler et al., 2009). Soler *et al.* (2009) showed that critically short telomeres increasingly contributed to chromosomal rearrangements and genomic instability after radiation exposure in cell cultures with increasing senescence status. This enhanced genomic instability may render cells more sensitive to radiation-induced cell death or transformation. In line with this, the response of mice to an alkylating agent depended on the telomere length; while telomerase-null mice with critically short telomeres were prone to death, wild-type mice were prone to developing tumors, and animals with longer telomeres were less sensitive to tumor formation (Gonzalez-Suarez et al., 2003).

Altered microenvironment. Although direct damage to individual cells can affect their physiological response to IR, as discussed above, *in vivo* cells exist in a microenvironment that also affects their cell fate decisions. A recent study demonstrated that while hematopoietic stem cells in mice became more radioresistant with increasing age, they did not exhibit any age-dependent differences, when irradiated *in vitro* (Ariyoshi et al., 2014). The authors showed that the higher death rate among juvenile hematopoietic stem cells was associated with the absence of up-regulation of radioprotective cytokines in the bone marrow microenvironment. However, a fine balance in the expression of those cytokines is crucial, as the

increased survival of progenitor cells with increasing age at exposure also correlates with an increasing number of cells with chromosomal aberrations (Nakano et al., 2007). This could then contribute to the development of secondary malignancies.

Alterations in microenvironment can also be attributed to changing secretory profiles of exposed cells that undergo stress-induced premature senescence. Interestingly, the secretory profile of a senescent cell does not solely depend on the induction of senescence, but also on the cell type affected. In contrast to senescent vascular endothelial cells and retinal pigment epithelial cells, stromal fibroblasts adopt an inflammatory secretion profile (Shelton et al., 1999). Further, senescent fibroblasts express metalloproteinases (Millis et al., 1992) and collagenases (West et al., 1989) that degrade the extracellular matrix and can create a permissive environment for tumor metastasis (Tsai et al., 2005). In addition, breast carcinoma cells that were co-cultured with senescent stromal fibroblasts were shown to be more resistant to radiation (Tsai et al., 2009). Thus, the microenvironment of cells also seems to play a crucial role in the development of side effects, such as tumor relapse.

In summary, while the initial response to DNA damage (characterized by the activation of ATM and p53) has been studied extensively, a better understanding of how pathway choices towards apoptosis and senescence are made will help adjust treatment schedules to limit the potential side effects in an age- and tissue-dependent manner. Studies on age-dependent differences in the response to ionizing radiation indicate that cell death of progenitor cells affects the normal development and can induce secondary side effects in the future, while cell death of differentiated cells within organs can compromise their function. Alterations in hormonal regulation can further affect the development systemically, whereas alterations in microenvironment

can affect stem cell survival, as well as carcinogenesis. Further, more studies are needed to determine the differences in the molecular response to IR in senescent when compared to dividing cells, as this will allow a more in depth analysis of how molecular changes relate to the observed side effects and how those can be prevented.

2.6 CONCLUSIONS

While aging seems to be a very conserved phenomenon, the current knowledge on what causes it is still far behind. Most common laboratory organisms have been employed to study the molecular mechanisms of aging. Yeast and *C. elegans* have been particularly useful for the study of mutations that modify the organismal lifespan, due to the technically easy genetic manipulation in yeast and the availability of standardized RNAi protocols in *C. elegans*. Fruit flies and rodents on the other hand have extensively been used for the study of age-dependent functional decline.

Taken together, the wealth of all those findings has implicated roles for processes such as cellular signalling (most importantly insulin and TOR signalling), oxidative stress, accumulation of damaged DNA and proteins and changes in gene expression and epigenetic profiles in driving aging.

While those molecular changes affect the risk of developing different pathologies, like cancer, the risk that is imposed on the organism when exposing it to therapeutic or diagnostic radiation exposures is much less understood. Clinical follow-up studies have shown that children are more sensitive to radiation exposure than adults, this is likely caused by negative effects on hormonal regulation and stem cell survival. However, more studies are needed to determine the molecular differences in the response to ionizing radiation when comparing dividing and senescent cells, as well as young to old organisms.

3. LITERATURE REVIEW: GENETICS AND EPIGENETICS OF *ARABIDOPSIS THALIANA* DEVELOPMENT AND DEVELOPMENT-DEPENDENT STRESS RESPONSE

3.1 ABSTRACT

Arabidopsis thaliana reproduces only once in its lifetime, and thus its fitness solely depends on the success of this one reproductive event. Therefore, each stage of the development of the plant serves its very specific purpose, ranging from the accumulation of biomass and resources, to reproduction and remobilization of those resources to the maturing seed. Throughout their lifetime plants are also exposed to various biotic and abiotic stresses. As sessile organisms, they have evolved numerous mechanisms to cope with different stressors. While the stress response and leaf senescence involve some similar pathways, such as ROS signalling and altered phytohormone expression, how the age of the plant affects its response to stress is not well understood. In this review, we will discuss the major developmental stages of the *Arabidopsis* development, and some of the molecular mechanisms underlying developmental transitions. In addition, we will summarize the mechanisms that mediate the plant's response to stress, in particular to ionizing radiation, and the current knowledge on how this response depends on the developmental stage at exposure.

3.2 INTRODUCTION

Monocarpic plants, like *Arabidopsis thaliana*, only reproduce once during their lifetime. Thus, the overall fitness of the individual depends on its capacity to produce seeds that will give rise to the next generation. In order to achieve this, plants initiate

reproductive growth in a very tightly regulated way and use senescence as a means of redistributing resources from aging leaves to the maturing seed (Hensel et al., 1993). Unlike in mammals, the plant germ line is specified late during the reproductive growth stage and originates from floral meristems. Thus, the protection of the genome integrity of meristematic stem cells is crucial, in order to prevent the manifestation of chromosomal aberrations in the germ line.

How this late determination of the germ line and thus the dependence of the plant on the integrity of its meristematic stem cells may affect the stress sensitivity is still unclear. A study by Zinn *et al.* (2010) indicated that plants are the most sensitive to stress imposed by high or low temperatures during the reproductive stage, which also seems to be the most radiosensitive developmental stage (Kim et al., 2007).

While the environmental exposure of plants to increased levels of ionizing radiation (IR) is limited, studies that focus on the plant's response to IR are becoming increasingly important. For instance, the results of these studies may allow the use of plants for biomonitoring of radioactively contaminated areas, such as locations of nuclear accidents, including the Chernobyl nuclear power plant or the Fukushima reactors. In addition, plants are exposed to IR during phytosanitary procedures, and under laboratory conditions for mutagenesis or for the study of the response to DNA damage.

Thus, a better understanding of the radiation response and radiation sensitivity throughout the plant's development may help improve radiation-mediated mutagenesis protocols or determine biomarkers, which show a clear dose-response relationship that could then be used for biomonitoring. In addition, a better understanding of the differences in response to IR when comparing plants and

mammals may help determine ways to improve the radiation resistance and the outcomes for radiation therapy patients.

In this review, we will first discuss the development of *Arabidopsis thaliana* and what molecular mechanisms play roles in regulating it; and then outline the current state of knowledge on the development-dependent stress response and in particular the response to IR.

3.3 LIFE CYCLE OF *ARABIDOPSIS THALIANA*

Annual plants like *Arabidopsis* are faced with the challenge to develop all of their tissues and reproduce before they experience whole-plant senescence and death within one season. Therefore, the developmental processes need to be under very tight control and achieve defined goals: the synthesis of macromolecules as a source of energy for diverse cellular processes, the formation of an internal environment favourable to gamete formation and fertilization, and the seed development to ensure the survival to the next generation.

Arabidopsis serves as a useful model to study plant development due to its short generation time, availability of whole genome sequence information and mutant lines, as well as its relatively predictable chronological progression through the development (Boyes et al., 2001).

3.3.1 Germination and leaf development

Each seed contains a mature embryo with preformed hypocotyl and cotyledons along with shoot and root apical meristem systems. During germination, hypocotyl and cotyledons emerge. As the shoot becomes exposed to light, it undergoes photomorphogenesis, during which the cotyledons take up photosynthesis to provide

resources for the further development. Photomorphogenesis is driven by the detection of light and relies on the orchestrated activity of several light receptors, including cryptochromes (Ahmad and Cashmore, 1993; Lin et al., 1998a) and phytochromes (Butler et al., 1959; Sharrock and Quail, 1989). The activation of those receptors induces the expression of genes that are required for carotenoid synthesis (Apel, 1981; von Lintig et al., 1997), allowing the seedlings to perform photosynthesis. A recent study showed that this step further involves chromatin remodelling to induce the changes in gene expression that are necessary for the switch from heterotrophic to autotrophic metabolism (Jing et al., 2013).

In order to transition into a vegetative growth phase, the embryonic formation of the shoot apical meristem (SAM) is essential. Several genes are required for the determination of the SAM (Aida et al., 1999; Laux et al., 1996; Long et al., 1996), and induce downstream changes in gene expression profiles that guide the transition from embryonic development to vegetative growth (Lenhard et al., 2002). The initial vegetative growth phase comprises the formation of leaves by leaf primordia, which are derived from meristematic cells and are situated peripheral to the shoot apical meristem (Medford et al., 1992). The determination of cells within the SAM that form leaf primordia involves the suppression of transcriptional programs that maintain the cell's stem cell character (Byrne et al., 2000; Lin et al., 2003).

Leaves subsequently emerge in a pre-determined way, which is subject to hormonal regulation by auxin (Reinhardt et al., 2000), and is thought to be associated with the localized expression of expansins in the SAM (Pien et al., 2001). As the leaves mature, the mesophyll cells accumulate chloroplasts, reaching up to 120 per cell by maturity (Pyke and Leech, 1992). Thus, leaves reach their maximum photosynthetic

activity by maturity, which thereafter declines with increasing age of the leaf (Hensel et al., 1993).

3.3.2 Bolting and flowering

As the plant's cells perform photosynthesis they accumulate sugars, which induces the production of trehalose-6-phosphate (T6P) (Schluepmann et al., 2004). The increasing levels of T6P have been associated with the induction of flowering (Wahl et al., 2013) as well as leaf senescence (Wingler et al., 2012). Thus, the coupling of developmental processes to T6P levels may allow the plant to induce reproductive growth when there are enough resources. However, other environmental stimuli, such as the day length, which is detected by photoreceptors (Guo et al., 1998; Mockler et al., 1999), the temperature and the developmental stage of the plant, are also involved in the regulation of the shoot formation and flowering (Fornara et al., 2010). Once a flowering signal is produced, the plant is committed to flowering (Corbesier et al., 2007). The integration of signals from the various environmental stimuli results in the changed regulation of transcription factors (Liljegren et al., 1999; Yu et al., 2002), and thus the alteration of the gene expression profiles in cells of the SAM resulting in a switch of the meristem identity from vegetative to floral (Liljegren et al., 1999).

Thus, after the formation of six or more rosette leaves, plants progress to reproductive development, which is associated with morphological changes to the SAM (Medford et al., 1992). This transition is paralleled with the elongation of the shoot and the formation of side shoot meristems, before the inflorescence meristem generates floral meristems that eventually form the flowers.

The inflorescence meristem can induce the production of up to three flowers per day. The identities of the different flower organs are specified through distinct

expression patterns of homeotic genes (Bowman et al., 1989), which limits the number of cells with a potential to form the gametophytes. Thus, a limited number of meristematic cells eventually gives rise to the gametophytes and the germ cells are specified within the gametophytes, relatively late during the plant's development.

While the molecular mechanisms that guide the germ line specification are still under study, the transcription factor DUO1 was shown to play an essential role in the specification of the male germ line by activating the transcription of genes that are necessary for adequate germ cell division and sperm differentiation (Borg et al., 2011; Brownfield et al., 2009). Additionally, the observation that germ line cells show the incorporation of distinct Histone 3 variants into chromatin (Okada et al., 2005), suggests that epigenetic mechanisms may also play a role in shaping the gene expression profile or in affecting chromatin condensation in the male germ line.

On the other hand, the mechanisms that guide the specification of the female germ line are less understood. Advances in technology have allowed the isolation of single cells of the female gametophyte and the characterization of their gene expression profiles. This showed an important role of the up-regulation of DEAD/DEAH-box containing helicases in the specification of the megaspore mother cell (Schmidt et al., 2011), which subsequently gives rise to the germ line. On the other hand, the activity of siRNA production in somatic companion cells was shown to be important to restrict the gametogenesis of multiple somatic cells (Olmedo-Monfil et al., 2010). Further, findings from such studies have supported roles for epigenetic regulation, based on the up-regulation of genes like *DCL1*, *HYL1*, *AT4G00420*, *AGO1*, as well as PAZ and Piwi-domain containing genes (Wuest et al., 2010), and transcriptional regulation, based on the observed egg cell specific up-regulation of *WOX8* and *ABI4* (Wang et al., 2010), in the development of the female gamete. Additionally, several

mutants which exhibit mis-specifications of the egg cell have been described. Those include the *lachesis*, the *clotho* and the *atropos* mutants, in which the accessory cells also adopt a germ cell fate (Gross-Hardt et al., 2007; Moll et al., 2008). Interestingly, the products of the mutated genes all play a role in pre-mRNA splicing.

Once the gametes have been formed, *Arabidopsis* almost exclusively propagates through self-pollination (Abbott and Gomes, 1989). After fertilization and as the seeds mature, a signal from the maturing siliques leads to a terminal growth arrest, which suppresses the flower formation in all remaining floral meristems (Hensel et al., 1994), and the somatic tissues of the plant thereafter become increasingly senescent.

3.3.3 Leaf senescence

Senescence is the last stage of plant development. During the initial phase, this entails the breakdown of chloroplasts, which results in leaf yellowing at the organ level. At the molecular level, it results in a shift from anabolism to catabolism of chlorophyll and other macromolecules of the cell, which can then be mobilized and supplied to the maturing seeds (Hensel et al., 1993). This entails the programmed cell death (PCD) of mesophyll cells and is beneficial to plant reproduction, and therefore evolutionarily selected (Leopold, 1961).

The process of senescence is very tightly regulated on several levels – through distinct changes to gene expression profiles guided by several transcriptional programs, but also at the level of phytohormones, and is therefore responsive not only to developmental processes within the plant, but also to environmental stimuli including biotic and abiotic stress. Some of the senescence-related changes and

possible senescence-regulatory mechanisms will be discussed in the following sections.

3.4 MOLECULAR PATHWAYS UNDERLYING SENESCENCE

3.4.1 Phytohormone pathways

Most phytohormones have been shown to be involved in the regulation of leaf senescence (Fig. 3.1).

Cytokinins, which control cell proliferation, shoot formation and branching, have been shown to delay leaf senescence (Gan and Amasino, 1995). This is thought to be mediated by cytokinin reception by histidine kinase receptors AHK2 and AHK3 (Fig. 3.1) (Kim et al., 2006). Since cytokinins and auxins have synergistic and antagonistic effects on many processes during the development, including stem and root growth, it is not surprising that ***auxins*** were also found to affect leaf senescence. The findings that exogenously applied auxin resulted in the transcriptional repression of the senescence associated gene *SAG12* (Noh and Amasino, 1999) and that auxin biosynthetic genes were up-regulated in senescing leaves (van der Graaff et al., 2006) suggested that auxin, like cytokinin, may delay senescence. The repressive effect on senescence associated gene (SAG) expression in response to auxin may be mediated by auxin response factors (arf) (Fig. 3.1). In line with this, *arf2* mutants showed delayed leaf senescence as well as delayed flowering and silique formation (Ellis et al., 2005).

Ethylene has long been known to be involved in fruit ripening and senescence. *Arabidopsis* ethylene receptor mutants *etr1*, *etr2*, *ers1, ers2* and *ein4* were shown to convey ethylene insensitivity and failed to accelerate leaf senescence in response to ethylene (Bleecker et al., 1988; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998).

In the absence of ethylene, CTR1, a downstream serine/threonine kinase, is activated and inhibits the ethylene response, while *ctr1* mutant plants show a phenotype similar to ethylene-exposed plants (Kieber et al., 1993). The inactivation of CTR1 in response to ethylene leads to proteolytic cleavage and nuclear translocation of EIN2, which then activates a transcription factor, EIN3 that mediates changes in gene expression (Fig. 3.1) (Qiao et al., 2012).

Abscisic acid (ABA) synthesis is induced in response to numerous environmental stresses, but has also long been known for its involvement in developmental regulation, including seed dormancy (thus the original name dormin) and leaf abscission (Cracker and Abeles, 1969). Further, the exogenous application of ABA induced the expression of SAGs in *Arabidopsis* rosette leaves (Weaver et al., 1998). While the ABA response involves several interconnected pathways (see also (Cutler et al., 2010)), binding of ABA to PYR/PYL/RCAR receptors may initiate pathways that result in the modification of gene expression (Fig. 3.1) (Antoni et al., 2012; Park et al., 2009). Binding of ABA to the PYR/PYL/RCAR receptors leads to inhibition of PP2C, thereby relieving the inactivating phosphorylation of downstream kinases including SnRK2 (Umezawa et al., 2009). Among the targets of SnRK2 are several ABA response element binding factors (ABF), which modulate the transcription from ABA-responsive element (ABRE) containing promoters (Finkelstein and Lynch, 2000; Furihata et al., 2006; Hobo et al., 1999; Kobayashi et al., 2005).

Jasmonic acid (JA) and methyl jasmonate (MeJA) have been associated with leaf senescence, due to the finding that exogenously applied MeJA promoted leaf senescence (He et al., 2002). He *et al.* (2002) further showed that JA accumulated in senescing leaves and that this accumulation corresponded with the up-regulation of SAGs as well as genes involved in the biosynthesis of JA. Further, JA was also shown

to mediate floral organ abscission and meristematic arrest in *Arabidopsis* (Kim et al., 2013a). The response to JA depends on COI1 (Fig. 3.1) (Xie et al., 1998), which contains an F-box domain that leads to its association with Skp/Cullin/F-box (SCF)-type E3 ubiquitin ligase complexes (Devoto et al., 2002; Xu et al., 2002b). Upon JA binding, such SCF-E3 ubiquitin ligase complexes target the jasmonate-ZIM domain (JAZ) repressor family for proteasomal degradation, resulting in the de-repression of transcriptional activators, like MYC2 that mediate the transcriptional response to JA and MeJA (Chini et al., 2007; Thines et al., 2007). In addition to transcriptional regulation, the COI1-SCF-complexes may also directly target other proteins for proteasomal degradation, thereby contributing to the senescence associated proteome (Shan et al., 2011).

While ***salicylic acid (SA)*** has traditionally been studied for its function in the response to pathogens, it was also shown to be involved in the establishment of senescence. *Arabidopsis* mutants that are deficient in SA signalling or biosynthesis (*npr1*, *pad4*, NahG) showed delayed leaf yellowing and failed to induce the expression of SAGs (Morris et al., 2000). Buchanan-Wollaston *et al.* (2005) showed that SA-mediated changes in gene expression partially overlap with senescence-associated gene expression profiles in wild-type plants. This seems to be mediated by the activation of WRKY transcription factors, including WRKY30, WRKY53, WRKY54 and WRKY70 (Besseau et al., 2012). In addition to modulating gene expression patterns, SA may also play a role in developmental PCD (Besseau et al., 2012).

Finally, while it has long been unclear whether ***gibberellins (GA)*** play a role in leaf senescence, a recent study showed that the accumulation of DELLA transcriptional repressors, which are normally degraded in response to gibberellin stimulation (Dill et

al., 2001), resulted in delayed leaf senescence, while the repression of DELLAs resulted in early senescence (Chen et al., 2014). The inactivation of DELLA dependent transcriptional repression resulted in the up-regulation of SAGs, genes involved in fatty acid β-oxidation and genes involved in the signalling pathways that respond to other phytohormones (Chen et al., 2014). This suggests a role for gibberellin-mediated DELLA degradation in de-repressing SAG expression and inducing leaf senescence.

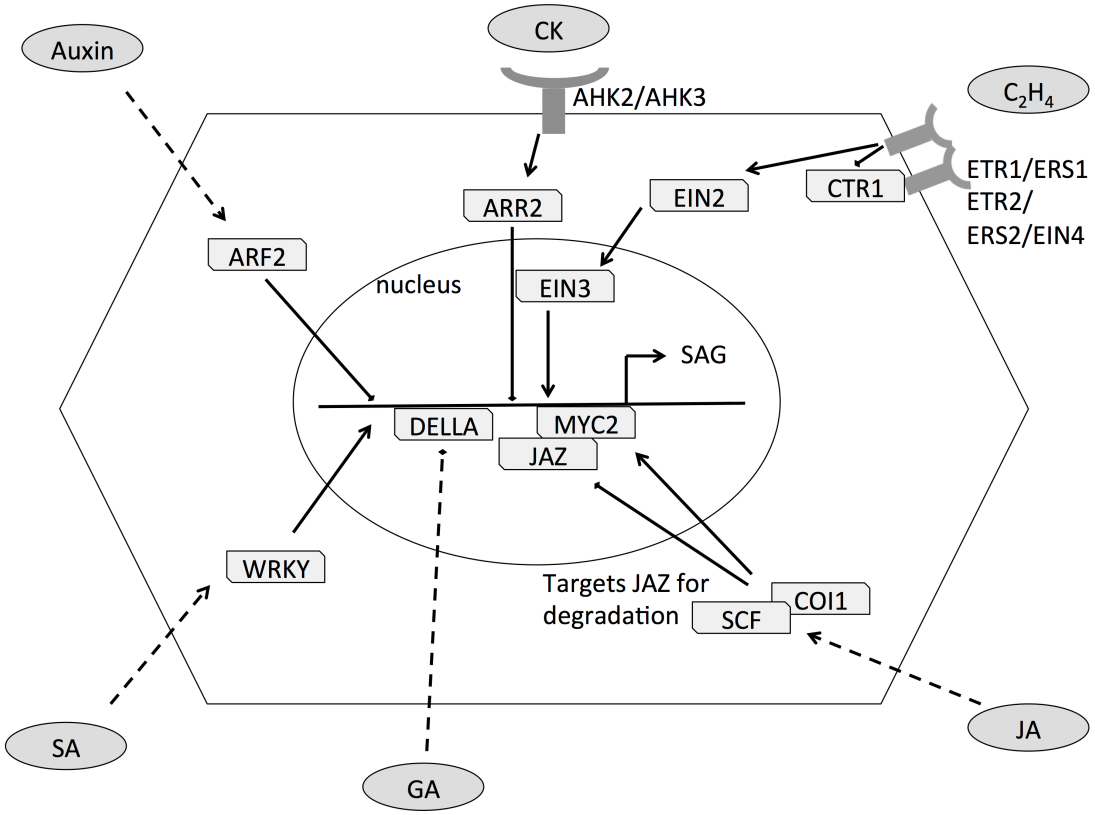

Figure 3.1: A role for phytohormones in regulating senescence.

Dashed lines indicate indirect interaction. Pointed arrowheads indicate activation and straight arrowheads indicate inhibition. For simplicity the effects of exposure to selected hormones on SAG expression are shown converged on one promoter,

however, *in vivo*, the gene expression patterns affected by different hormones are at least in part distinct.

In summary, there is evidence for the contribution of hormonal signalling to the changes in gene expression patterns observed during senescence. However, the regulation of senescence by hormones likely relies on a fine balance between senescence-promoting and senescence-inhibiting hormone action. Further, the secretion of senescence-promoting hormones – ethylene, ABA, SA and JA – is also induced in response to diverse environmental stresses, such as pathogen infection, drought and salinity. Thus, the response to stress may promote leaf senescence on the one hand, and on the other hand, senescing plants may be more resistant to exogenous stresses due to the endogenously induced production of stress hormones. However, to clarify this, further studies are required.

3.4.2 Sugar sensing

Another important decisive factor for the induction of senescence is the nutrient state. Dai *et al.* (1999) showed that plants over-expressing AtHXK1 (hexokinase), which is the first enzyme involved in the catabolic breakdown of hexose sugars, underwent rapid senescence (Dai et al., 1999), suggesting that hexokinase acts as a sugar sensor (Jang and Sheen, 1994). Exogenous application of glucose to wild-type or AtHXK1 over-expressing plants resulted in the down-regulation of photosynthetic genes in a glycolysis independent manner (Xiao et al., 2000), thus further supporting the sugar induced signalling function of hexokinase. Further, it was shown that the down-regulation of the photosynthetic activity precedes the activation of SAG expression, indicating that the sugar level-dependent down-regulation of

photosynthetic genes may be an early step in the establishment of leaf senescence (Hensel et al., 1993). Sugars were also shown to repress the expression of SAGs (Noh and Amasino, 1999), thus the reduction in sugar supply following the down-regulation of photosynthetic genes may contribute to the up-regulation of SAGs.

In addition to hexokinase, a role for trehalose-6-phosphate in the induction of leaf senescence has also been described (Wingler et al., 2012). While sugar signalling seems to affect the induction of leaf senescence, this may be dependent on other conditions, such as lighting (Weaver and Amasino, 2001) and developmental status.

3.4.3 Oxidative stress

Reactive oxygen species (ROS) play multiple roles during the induction of senescence. The observations that *Arabidopsis* mutants that are better able to tolerate oxidative stress also show delayed leaf senescence (Woo et al., 2004), while *Arabidopsis* mutants, such as the *cpr5* and *old5* mutants, exhibit increased oxidative stress and early senescence (Jing et al., 2008; Schippers et al., 2008), supports a causal role of ROS in establishing senescence. The formation of ROS may be induced in response to hormones, such as ABA (Hung and Kao, 2004) and MeJA (Zhang and Xing, 2008), development-specific down-regulation of antioxidant enzymes, such as SOD (Ye et al., 2000), or through changes in the cell metabolism with age. The main ways by which ROS affect the cell are by introducing damage to its macromolecules and through its involvement in cell signalling.

The generation of ROS within the cells results in oxidative damage to macromolecules, with lipids being one of the primary targets for oxidation during leaf senescence (Ye et al., 2000). Oxidative damage to proteins during wheat leaf senescence was shown to depend on the presence of reproductive organs and

contributed to the proteolytic degradation of proteins and the mobilization of nitrogen (Srivalli and Khanna-Chopra, 2004). Thus, oxidative stress may play an important role in the re-mobilization of nutrients from senescing leaves to support the seed development. Further, non-enzymatic lipid peroxidation during leaf senescence contributes to PCD (Berger et al., 2001). In addition, ROS are also involved in signalling pathways that mediate senescence-associated PCD directly (Cui et al., 2013b).

Further, the accumulation of ROS in senescing cells may also affect the gene expression patterns (Navabpour et al., 2003). In line with this, both the over-expression and the knock-down of the ROS-sensitive transcription factor JUB1 affect the timing of senescence by altering the expression levels of ROS-responsive genes (Wu et al., 2012).

Thus, changes in ROS levels within the cells can directly contribute to the functional decline associated with leaf senescence by inducing chloroplast destruction and PCD. However, through their involvement in cellular signalling they may also play an important role in integrating diverse internal and external stimuli and mediate downstream changes in gene expression in order to fine-tune the timing of senescence.

3.4.4 Accumulation of DNA damage

Changes in DNA repair and genomic stability throughout the development have also been described. At the molecular level, *KU70* expression has been described to increase with age, whereas *RAD51* expression decreased (Boyko et al., 2006). This correlated with the accumulation of unrepaired DNA strand breaks (Boyko et al., 2006), which may however be affected by additional factors. Further, the reduced

expression of mismatch repair genes (*MSH2* and *MSH7*) and changes in the expression of diverse DNA polymerases were observed and correlated with a reduced efficiency of the base excision and nucleotide excision repair pathways in aging plants (Golubov et al., 2010). Such developmental changes in the expression of DNA repair genes as well as the inherent accumulation of DNA strand breaks and microsatellite instability may affect the plants capacity to cope with additional stress.

In addition, while telomeres are thought to play a causal role in mammalian aging, plant telomeres seem to be more important for preventing genome instability and for contributing to the establishment of the chromosome architecture without playing a major role in senescence. Telomerase-deficient *Arabidopsis* can be propagated for ten generations, before the plants show severe growth defects and chromosomal abnormalities, like end-to-end fusions (Riha et al., 2001). However, the majority of cell divisions within plants occur in the meristem regions, which contain active telomerase (Oguchi et al., 1999). Thus, telomere length does not seem to affect senescence in *Arabidopsis*.

3.4.5 Gene expression changes

The highly coordinated nature of plant aging further becomes clear when studying transcriptomes of plants throughout development. Buchanan-Wollaston *et al.* (2005) described the more than 3-fold up-regulation of 827 genes specifically in senescent leaves (Buchanan-Wollaston et al., 2005). Those genes affected processes like the degradation of proteins, lipids, nucleic acids and chlorophyll, hormonal signalling and transcriptional regulation, autophagy, stress response and secondary metabolism. The comparison with expression profiles in plants that failed to synthesize JA, SA or

ethylene showed that all three pathways likely contribute to the senescence associated transcriptome (Buchanan-Wollaston et al., 2005).

3.4.6 A role of epigenetics in senescence

In addition to the numerous transcription factors with changed expression or activity during leaf senescence, a role for epigenetic changes in senescence has more recently been considered.

In particular, the deregulation of several histone-modifying enzymes resulted in lifespan modifications. The over-expression of a histone methyltransferase (SUVH2) in *Arabidopsis* prevented the induction of SAG expression in part through the methylation of H3K27 within the *WRKY53* promoter region (Ay et al., 2009). In addition, genome-wide changes in the H3K4 (associated with transcriptional activation) and H3K27 (associated with transcriptional repression) methylation patterns were described, and correlated with senescence-associated changes in gene expression (Brusslan et al., 2012). However, changes in histone methylation levels were not observed in the promoter regions of all SAGs, suggesting that epigenetic regulation of senescence mechanisms is locus-specific.

Moreover, the down-regulation of the histone deacetylase AtHD1 resulted in the accumulation of acetylated histones, aberrant gene expression and premature senescence (Tian and Chen, 2001). In contrast, the down-regulation of AtHDA6, another histone deacetylase, resulted in a longer leaf lifespan (Wu et al., 2008). Thus, the roles of distinct epigenetic modifications in the establishment of leaf senescence need further study.

Further, changes in chromatin remodelling may also affect SAG expression, since ORE7/ESC (an AT-hook containing chromatin remodelling protein) over-expressing

plants showed delayed senescence along with gene expression profiles that were more similar to those in younger leaves (Lim et al., 2007).

Senescence-associated changes in DNA methylation at the genome and gene levels have not been described in *Arabidopsis*. However, there are some reports on different species, including one that described a gradual loss of DNA methylation in the meristems of maturing *Pinus radiata* trees (Fraga et al., 2002).

Moreover, several small RNAs have been shown to be involved in the regulation of senescence. miR164, which is negatively regulated by EIN2 and thus increasingly down-regulated with age, targets *ORE1* mRNA for cleavage. Thus, the reduced expression of miR164 with age results in increased expression of ORE1, which mediates the transcription of a subset of SAGs that are predominantly involved in PCD (Kim et al., 2009). Other miRNAs were shown to modulate hormone-mediated senescence pathways – miR319 (Schommer et al., 2008) and miR390 (Fahlgren et al., 2006).

Thus, while epigenetic mechanisms seem to be involved in the regulation of senescence dependent changes in gene expression, they are just beginning to be understood.

3.5 PLANT STRESS RESPONSE

Plants are sessile organisms and as such had to evolve efficient coping mechanisms in order to adapt to the environmental conditions they are exposed to. The remarkable capacity of plants to adapt to their environment was already described by Lamarck in the early 19th century, while the molecular mechanisms underlying that adaptability have only started to be better understood over the last few decades.

3.5.1 Different types of stress

Plants are faced with two major different types of stress: one imposed by other organisms, such as predators or pathogens, which is termed *biotic stress*; and the second imposed by changes in the chemical or physical environment of the individual, such as changes in salinity, drought, temperature fluctuations and electromagnetic radiation, termed *abiotic stress*. While the initial stressors differ, the responses they trigger at least partially overlap.

Response to biotic stress

Plants are equipped with two major systems to recognize pathogens: surface receptors that recognize pathogen-associated molecular patterns (PAMPs), and resistance (R) proteins that recognize specific effector molecules present in the pathogen.

The recognition of general PAMPs, like flagellin or chitin, is mediated by leucine-rich repeat (LRR) receptor kinases. Receptor activation results in the rapid induction of the expression of defense-related genes, including additional pathogen receptors and *R* genes, antimicrobial genes, and genes involved in phytohormone signalling and transcriptional regulation (Zipfel et al., 2004).

R proteins recognize pathogen effector molecules inside infected cells via direct or indirect interaction and then trigger a complex response. R proteins are composed of different domains, including the nucleotide binding site (NBS) and the LRR domains, which are common to most *R* genes (Ellis et al., 2000). The LRR domain is mainly responsible for the recognition of pathogen effectors (Krasileva et al., 2010), while additional domains like the Toll/Interleukin 1 receptor (TIR) domain and the coiled coil (CC) domain mediate the induction of PCD (Rairdan et al., 2008; Swiderski et

al., 2009), which is the hallmark of the initial hypersensitive response. Further, some R proteins may translocate to the cell nucleus upon recognition and modulate gene expression through the interaction with transcription factors (Shen et al., 2007).

While the two modes of pathogen response are often considered separately, there is accumulating evidence that suggests overlapping functions of PAMP-mediated and pathogen effector-mediated pathogen resistance. R proteins can form complexes with pathogen-recognition receptors (PRRs) (Qi et al., 2011), the two modes of immunity were both shown to depend on intact phytohormone signalling (Tsuda et al., 2009) and the changes in gene expression profiles triggered in response to either stimulus show an extensive overlap (Navarro et al., 2004).

In response to the initial pathogen perception through PRRs or to the hypersensitive response triggered by R protein/pathogen effector interaction, systemic signals are produced that migrate to distant parts of the plants and trigger an adaptive response – the systemic acquired resistance (SAR). SAR has been extensively studied in connection with effector induced immunity, but was recently also reported to be induced in response to PRR activation (Mishina and Zeier, 2007). Several mobile immune signals have been identified, including SA (Gaffney et al., 1993), DIR1 and glycerol-3-phosphate (G3P) (Chanda et al., 2011), and azelaic acid (Jung et al., 2009). Those mobile signals trigger the accumulation of SA in distal tissues, which is associated with the induction of pathogenesis-related (PR) gene expression (Zhang et al., 1999), the accumulation of signalling components that may be activated during future infections (Beckers et al., 2009), DNA rearrangements through the induction of homologous recombination (Kovalchuk et al., 2003a) and changes in chromatin structure (Jaskiewicz et al., 2011). These changes may likely aid the infected plants in future protection against pathogen infections.

Response to abiotic stress

The response to abiotic stress starts with the stress perception, which involves receptor-like kinases (RLK) and ion channels (Orvar et al., 2000; Osakabe et al., 2010). This perception is followed by the production of secondary messengers, such as Ca^{2+}, ROS and IP3 (DeWald et al., 2001; Gleason et al., 2011), which relay the signal to downstream effectors. MAP kinase signalling is commonly involved in the transduction of stress signals and results in the regulation of numerous transcription factors, eventually resulting in changes to gene expression patterns (Ichimura et al., 2000; Teige et al., 2004). Genes with changed expression in response to abiotic stress include transcriptional regulators, antioxidants, specific defense genes, like ion transporters that re-establish ion homeostasis under salinity stress, repair genes and genes related to phytohormone biosynthesis and signalling (Kreps et al., 2002; Seki et al., 2002). These studies further showed that different stressors induce partially overlapping changes to expression profiles, while exposure to combinations of stresses induces distinct changes to expression profiles when compared to changes induced by single stressors, potentially resulting in better adaptation (Rasmussen et al., 2013). Further, there is an increasing amount of evidence supporting a role for epigenetic changes in mediating long-term adjustments of gene expression and thereby adaptation to stress, which can even span generations (Bilichak et al., 2012; Boyko et al., 2010; Kim et al., 2008; Kwon et al., 2009).

Response to ionizing radiation

The exposure of plants to ionizing radiation, confronts them with two different types of stress: on the one hand, the direct deposition of energy onto macromolecules

within the cells that were exposed can result in direct damage, including DNA strand breaks; on the other hand, the interaction of the high energy particles or rays with the aqueous environment of the cells can cause the formation of reactive oxygen species (ROS) and thus induce oxidative stress and oxidative damage to the macromolecules of the cell. Therefore, in order to cope with this stressor, the exposed plant needs to respond to both oxidative stress and DNA damage.

Response to oxidative stress. The exposure to IR results in the accumulation of ROS within cells in a dose-dependent way (Kim et al., 2011a), and consequently in increased levels of lipid peroxidation (Vanhoudt et al., 2010), or mitochondrial dysfunction and induction of PCD (Gao et al., 2008). The rapid accumulation of H_2O_2 and ROS upon the exposure to IR is followed by the up-regulation of radical scavengers and detoxifying enzymes, such as peroxidase, catalase, superoxide dismutase and glutathione reductase, in a wide range of species and in response to radiation doses ranging from a few Gray to several kilo-Gray (Croci et al., 1991; Kovalchuk et al., 2007; Ogawa and Uritani, 1970; Roy et al., 2006; Singh, 1974; Wada et al., 1998).

Further, this rapid accumulation of H_2O_2 and ROS interacts with cellular signalling and results in the differential expression of numerous genes, large fractions of which are involved in the stress response, plant metabolism and modification of transcription (Desikan et al., 2001). The role of ROS in mediating changes in gene expression is further supported by the observations that H_2O_2-induced expression changes partially overlap with IR-induced expression changes (Kim et al., 2013c).

In addition to inducing oxidative stress and changes in gene expression profiles, ROS have also been shown to play a role in mediating abscopal bystander effects (Li

et al., 2010; Yang et al., 2007), such as genomic instability in parts of the plant that were distant to the exposed tissue.

DNA damage and repair. The early response to IR depends on the function of AtATM. Consequently, *atm* mutants are radiation sensitive due to a failure to detect and/or repair the IR-induced DNA damage, and also accumulate chromosomal aberrations during meiosis, resulting in partial sterility (Garcia et al., 2003). Further, mutations in DNA repair genes, such as *AtKU80*, DNA ligase 4 and *AtRAD51*, caused increased radiation sensitivity in *Arabidopsis* (Abe et al., 2005; Friesner and Britt, 2003; Osakabe et al., 2005). Interestingly, AtATM is not only involved in initiating the DNA damage response, but also induces the expression of several DNA repair genes, like *BRCA1*, *RAD51A* and *PARP1* (Culligan et al., 2006). Culligan *et al.* (2006) further showed that the induction of most of the genes that were differentially expressed in response to IR was prevented in *atm* mutants. A putative transcription factor, suppressor of gamma response 1 (SOG1), was shown to mediate those changes in gene expression downstream of ATM and ATR (Yoshiyama et al., 2009).

Thus, the efficient and precise repair of DNA damage seems to be central to the radiation response. Exposure to high doses of IR has been shown to result in large regions of chromosomal deletions (Naito et al., 2005; Vizir and Mulligan, 1999), while the induction of homologous recombination at lower radiation doses may result in chromosomal aberrations (Kovalchuk et al., 2000). In order to prevent the transmission of those into the next generation, the DNA damage response is particularly important in meristematic cells. Indeed, the ATM-, ATR- and SOG1-dependent DNA damage response plays an essential role in DNA damage-mediated

cell death of stem cells (Fulcher and Sablowski, 2009; Furukawa et al., 2010), in order to prevent the manifestation and genetic transmission of genomic alterations.

Regulation of cell cycle and cell death. Not only does AtATM induce the repair of DNA strand breaks, but it also promotes cell cycle arrest through the activation of WEE1, a kinase that inhibits CDK (De Schutter et al., 2007). WEE1 was further shown to play a role in the response to replication stress, where it was important to inhibit premature differentiation (Cools et al., 2011). SOG1 has also been shown to play a role in the IR-dependent suppression of transcripts that are required for cell cycle progression, such as *KNOLLE* and *CDKB2;1* (Yoshiyama et al., 2009). Taken together, these findings suggest that plant cells can undergo DNA damage-induced cell cycle arrest. The DNA repair mutant *uvh1* undergoes IR-dependent G2 arrest, which is suppressed by a *sog1* mutation, however the IR-exposed double-mutants exhibit genomic instability (Preuss and Britt, 2003), suggesting that the major purpose of the G2 arrest in plant cells is DNA repair.

However, arresting the cell cycle comes with a high cost for a plant: delayed development of critical tissues, which may potentially jeopardize the reproductive success. Thus, cell cycle arrest seems to be restricted to meristematic cells, in which it is crucial to limit the accumulation of unrepaired DNA damage and potential mutations, and is virtually absent in somatic cells in the cotyledons and hypocotyl that undergo cell division (Hefner et al., 2006). Further, recently it was reported that plants may also sustain growth through the induction of endoreduplication in response to double-strand breaks (Adachi et al., 2011).

Meristematic cells are also prone to radiation-induced cell death through an autolytic process rather than apoptosis (Fulcher and Sablowski, 2009; Furukawa et al.,

2010). However, in order to secure the survival of the plant, a few surviving meristem cells are enough to organize a new meristem following the exposure to ionizing radiation (Clowes, 1961).

Thus, while the somatic tissue of *Arabidopsis* seems to be resistant to radiation-induced cell death or cell cycle arrest, multiple mechanisms are in place to protect the meristematic cells, in order to prevent the accumulation of mutations that may potentially get fixed in the germ line.

Changes in gene expression profiles. As previously mentioned, both the induction of ROS formation and of the DNA damage response result in changes to the cells' gene expression profiles. In order to better understand the molecular response to IR in *Arabidopsis*, several studies focused on the radiation-dependent changes in the transcriptome. While the conditions that were used varied between the different studies – the use of different cultivars (Columbia and Landsberg erecta), different doses ranging from 1 Gy to 2000 Gy, different ages of the plants ranging from 10 to 33 days post-germination – there were some genes or functional groups of genes that were commonly affected when comparing the results of two or more of the studies to each other (Gicquel et al., 2012; Kim et al., 2007; Kovalchuk et al., 2007; Nagata et al., 2005). Those similarities include the deregulation of ethylene responsive transcription factors, expansins, and the induction of the expression of *RAD51*, cytochrome P450 and glutathione-S-transferases. On the other hand, it was suggested that plants likely possess limited sensors and signal transduction systems to higher doses of IR than they are exposed to in nature (Kim et al., 2011a), suggesting that low doses of IR might trigger a more defined response than high doses.

In plants that were exposed to relatively low doses of X-ray (10 Gy or 40 Gy) 10 days post germination, a larger number of genes was found with changed expression when compared to control, including genes involved in both the light and the dark cycle of photosynthesis, heat shock proteins, cell cycle control, DNA replication, repair, histones, lipid and protein metabolism and senescence (Gicquel et al., 2012). This suggests that plants respond to radiation with significant adjustments in the regulation of cell growth and division as well as in metabolism.

In order to further differentiate short-term and long-term effects of radiation on plants, Kim *et al.* (2007, 2013c) studied transcriptomes at 2 h, 6 h, 24 h, 48 h, 5 d and 16 d after the exposure to 200 Gy. This work showed the rapid changes in the expression of 1,062 different genes by 6 h post-exposure, which decreased to 109 differentially expressed genes 24 h post-exposure, indicating that most of the differentially expressed genes are involved in the immediate response to the consequences of the radiation insult (Kim et al., 2013c). When they checked the long-term effects of radiation on gene expression, they found the deregulation of 3,899 genes 9 days post-exposure (Kim et al., 2007). This may partially reflect differences in development and physiology after the exposure - plants irradiated with 200 Gy may not be at the same developmental stage as control plants. However, they did find that 7 genes that were deregulated 48 h post-exposure, were still differentially expressed 16 days post-exposure (Kim et al., 2013c). Those 7 genes include 3 genes that are involved in double-strand break repair and the response to ionizing radiation (*PARP2*, *GMI1*, *XRI1*), 2 genes involved in dNTP production (*RNR1*, *TSO2*), 1 gene involved in hormonal signalling (*CIPK11*) and a gene encoding a nucleic acid binding zinc finger protein (At5g60250), suggesting that the plants that were exposed to ionizing

radiation were either still coping with unrepaired DNA damage or maintained a higher capacity to repair double-strand breaks.

Thus, IR-induced changes to the transcriptome are important both for the immediate response to IR-induced damage, as well as potentially for the long-term adaptation to IR.

Changes in epigenetic profiles. While some knowledge has accumulated on the role of epigenetic modifications in the response and adaptation to stress (see section Response to abiotic stress), their role in the response to IR is less understood. Chronic exposure to IR as experienced by the plants in the exclusion zone around the Chernobyl nuclear power plant, induced increased global DNA methylation levels in the early generations following the accident (Kovalchuk et al., 2004a; Kovalchuk et al., 2003b). This global hypermethylation may serve as an adaptive mechanism that protects the plant's genome against instability and rearrangement (Kovalchuk et al., 2004a; Kovalchuk et al., 2003b). A recent study showed that transcriptomes were affected differently in wild-type and *cmt3* mutant *Arabidopsis* plants exposed to 200 Gy of X-ray, suggesting a role of non-CG methylation in the response to IR (Kim et al., 2013c). The altered gene expression profile in wild-type *Arabidopsis* was associated with DNA CHH and CHG hypomethylation, due to high expression of the DNA demethylase ROS1 and low expression of CMT3 (Kim et al., 2013b). This suggests that IR-dependent changes in gene expression occur in a locus-specific way.

Further, while it was shown that the formation of γH2AX foci is induced in plants in response to 50 Gy of IR (Friesner et al., 2005), the effect of IR on other histone modifications has not been described until recently. Drury *et al.* (2012) showed that the chromatin of *Arabidopsis* exposed to 160 Gy of X-ray exhibits an increased

fraction of hyperacetylated Histone 3 and a decreased fraction of acetylated Histone 4 (Drury et al., 2012). Together with changes in DNA methylation, changes in the number and type of post-translational modifications to histone tails may alter the distribution of euchromatin and heterochromatin in exposed cells, and thus affect genome stability and gene expression profiles. Such changes to the chromatin structure have been shown to be essential to the DNA damage response (Shaked et al., 2006). In addition, the observation that genes that encode histones are subject to differential expression after exposure to 10 or 40 Gy of X-ray (Gicquel et al., 2012) suggests that nucleosome occupancy may also be affected by the exposure to IR.

While small RNAs, both siRNA and miRNA, have been associated with the response to diverse types of stress, the effect of IR on the expression of small RNAs has not been studied to date.

Physiological changes. The physiological consequences of the molecular changes induced by ionizing radiation are dose-dependent and include trichome formation (Nagata et al., 1999), radial expansion of root hairs (Nagata et al., 2004), changes in hormonal signalling (Nagata et al., 2004; Yao et al., 2011), developmental arrest (Haber et al., 1961) and induction of sterility (Mesken and Veen, 1968).

3.5.2 Developmental changes in the response to ionizing radiation

At the molecular level, there are numerous overlaps between the pathways that are active during the response to stress and during the establishment of leaf senescence, including the involvement of phytohormone and ROS signalling, and alterations in the regulation of gene expression. Consequently, there is opportunity for cross-talk,

during which the developmental status may affect the response to stress and *vice versa*.

While the analysis of molecular and physiological responses to IR was carried out in plants of different developmental stages in different studies, systematic analyses of the influence of the developmental stage on those responses are still rare. Kim *et al.* (2007) reported the phase between 4 and 5 weeks after seeds were sown to be the most radiation-sensitive time. The exposure of 4- to 5-week old plants to IR was associated with reduced stem growth, delayed leaf senescence and the accumulation of chlorophyll and carotenoid in plants (Kim et al., 2007). This is in line with the observation that the reproductive phase of *Arabidopsis* represents the most sensitive phase for responses to cold and hot temperatures (Zinn et al., 2010). Another study that compared the effects of a wide dose range (0.5 – 150 Gy) on plants of different ages (15, 20 or 25 days post germination), failed to find a significant correlation between the age and the physiological response to radiation other than the observation that plants irradiated at a younger age showed more severe effects on biomass than plants irradiated when they had already completed rosette growth (Kurimoto et al., 2010).

Two recent studies further compared the response to 200 Gy of IR delivered upon full rosette expansion (end of the vegetative growth phase, VE) or after the formation of the floral axis (late reproductive stage, RE) (Kim et al., 2011a; Kim et al., 2011b). While both studies reported a reduction in growth and final height of plants exposed at VE, plants irradiated during the RE showed an almost normal phenotype (Kim et al., 2011b), or reduced growth (Kim et al., 2011a). The plants exposed during VE further exhibited severely stunted shoot elongation (Kim et al., 2011b), and a decrease in silique formation (Kim et al., 2011a), while the plants exposed during RE showed

an increase in the number of siliques formed (Kim et al., 2011a). Both groups further analyzed the role of oxidative stress in the IR response. The formation of ROS was slightly reduced immediately after the exposure to 100 Gy of X-ray, but increased after the exposure to 800 Gy of X-ray in plants exposed at either developmental stage (Kim et al., 2011a). However, the alterations in gene expression, in particular the differential expression of ROS scavengers and signal transduction mediators, were distinct depending on the developmental stage at exposure (Kim et al., 2011a). When considering the long-term response, plants exposed to 200 Gy at VE showed a higher increase in ROS levels than plants exposed at RE, while those plants had higher basic levels of ROS due to their higher age at exposure (Kim et al., 2011b). This further correlated with an increase in oxidative damage to proteins and a reduced number of chloroplasts in plants exposed at VE. On the other hand, plants exposed at RE showed an increase in the number of chloroplasts when compared to controls. However, this may be associated with the onset of senescence in control plants indicating that the irradiated plants showed a delay in leaf senescence, and is in line with previous observations (Kim et al., 2007; Kim et al., 2011b).

Taken together, considering the susceptibility of meristematic stem cells to DNA damage dependent induction of cell cycle arrest or PCD, as described in the previous section, this may explain numerous observed differences in the IR response depending on the developmental stage. While the exposure to low doses of IR during the vegetative growth phase may induce cell cycle arrest and limited PCD in meristems and may thereby result in a delayed development and reduced accumulation of biomass, the exposure to higher doses of IR during this developmental stage may result in more extensive PCD within the meristem and more severely stunted growth. In contrast, when plants are exposed during the reproductive

growth phase, the overall biomass would be more severely affected in response to doses that induce extensive PCD in somatic cells. However, cell cycle arrest and PCD in cells of the inflorescence meristems may delay the production of flowers and thereby also the induction of leaf senescence. Of course, those conclusions are hypothetical and would need systematic testing.

While the interactions between the developmental stage at exposure and the physiological response that is triggered have been studied to some extent, the interactions between the molecular changes that are associated with different developmental stages and the response to X-ray are only beginning to be understood. For instance, the study by Kim *et al.* (2011b) showed that while plants exposed at the reproductive stage showed higher basic levels of ROS, the induction of ROS formation in response to IR was lower when compared to plants exposed during the vegetative growth phase (Kim et al., 2011b). This may affect the extent of the oxidative damage caused as well as alter the downstream signalling. However, the effects of other molecular changes have not been studied in plants exposed to IR at different developmental stages.

3.6 COMPARISON OF MAMMALS AND PLANTS

3.6.1 Similarities and differences in aging

While the precise sequence of events resulting in aging is not entirely understood in both mammalian and plant model systems, some similarities and differences become apparent. Aging is a very tightly controlled process in plants that serves the purpose of remobilizing resources from old, shaded and less productive leaves to younger leaves and during the reproductive development also to the maturing seed (Hensel et al., 1993). Thus, aging in plants helps ensure the reproductive success of the

individual and is therefore subject to natural selection. In contrast, reproduction occurs relatively early in the life of humans and most other mammals used as model organisms. Therefore, genes that have detrimental effects later in life but are beneficial to reproduction are positively selected, while molecular changes that occur after the reproductive age of the organism are likely not subject to selection (Medawar, 1952), suggesting that age-related molecular changes observed in mammalian model systems accumulate at least partially passively and may be more diverse than the ones observed in plants.

At the molecular level, aging involves vast changes in gene expression profiles that are regulated at various levels, including chromatin remodelling, transcription, translation as well as post-transcriptional and post-translational levels. Further, the reduced expression of DNA repair enzymes, increased genomic instability, increased oxidative stress and changes in cellular metabolism with increasing organ senescence or age have been described in both plant and mammalian model organisms (see 2.4 and 3.4). Those molecular changes are associated with the increasing functional decline of organs, such as during leaf senescence in *Arabidopsis* or affecting multiple organs and organ systems during human aging. While this decline is beneficial to the reproductive success in *Arabidopsis*, it does not affect the reproductive success in mammalian species, but contributes to their age-dependent increase in morbidity and mortality.

3.6.2 Similarities and differences in the response to radiation

One major difference between plants and mammals is the vastly different radiation sensitivity; while whole-body exposure to more than 6 Gy is lethal in 95 to 100% of

human cases (based on the Merck manual on radiation exposure and contamination[1]), *Arabidopsis* can tolerate several hundred Gy of X-ray (Kim et al., 2011a). However, what mediates this difference in radiation sensitivity at a cellular and molecular level is unclear.

Another difference between mammals and plants in terms of the response to radiation is that for mammals radiation induced malignancies are a big concern (Suit et al., 2007), as they may present a threat to the survival of the individual. In contrast, for plants the major challenge in dealing with a radiation exposure is to ensure genomic integrity of the DNA in meristematic cells in order to prevent the transmission of mutations to the next generation (see 3.5). Thus, mammals need to protect somatic and germ line cells equally, whereas in plants the protection of meristematic cells is crucial.

When comparing the radiation response of *Arabidopsis* meristem cells and mammalian cultured cells, both induce DNA damage checkpoints and the expression of DNA repair enzymes to cope with the DNA damage and if the DNA cannot be repaired successfully, the affected cells may undergo PCD or enter senescence, although senescence is more predominant in mammalian cells. Further, this stress-response is associated with far-reaching changes to gene expression profiles, which may be enforced by changes in transcription factor expression or activity, and by changes in the chromatin structure.

However, how the response to radiation changes with age is not well understood in both plant and mammalian model systems. There are some indications that the stress response in *Arabidopsis* may vary with the developmental stage. Further, the risks

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1 www.merckmanuals.com/professional/injuries_poisoning/radiation_exposure_and_contamination/radiation_exposure_and_contamination.html

associated with radiation exposure are different for young when compared to older human cohorts. The IR response of stem cells or meristematic cells may play a crucial role in modulating the development- or age-dependent sensitivity to IR. Further studies are needed to determine the underlying molecular mechanisms.

3.7 CONCLUSIONS

Arabidopsis development is a very tightly regulated process, during which each step depends on the successful completion of the previous step and on appropriate environmental conditions. Unlike in mammals, the functional decline of *Arabidopsis* rosette leaves after reaching the reproductive stage is not a result of the absence of mechanisms that select against leaf senescence, but serves its own specific purpose by re-mobilizing nutrients and supplying them to the maturing seed. However, the processes that drive leaf senescence resemble the mechanisms that are associated with aging in mammals, and include the accumulation of DNA damage, oxidative damage, and changes in sugar sensing and hormone signalling.

The response to stress relies on similar processes, including an initial oxidative burst, the up-regulation of DNA damage repair genes, and changes in the regulation of cell cycle and PCD. The extent of overlap between the processes that underlie leaf senescence and the processes involved in the stress response suggests that the developmental stage at which a plant is exposed to stress, affects its response. There is some evidence for that at the level of plant physiology, but more studies are needed to determine how the development-dependent molecular changes interact with the stress response.

4. IMMUNOSENESCENCE IS ASSOCIATED WITH ALTERED GENE EXPRESSION AND EPIGENETIC REGULATION IN PRIMARY AND SECONDARY IMMUNE ORGANS[2]

4.1 ABSTRACT

Deterioration of the immune system (immunosenescence) with age is associated with an increased susceptibility to infection, autoimmune disease and cancer, and reduced responsiveness to vaccination. Immunosenescence entails a reduced supply of naïve T cells from the thymus and increased specialization of peripheral T cell clones. Both thymic involution and peripheral T cell homeostasis are thought to involve cellular senescence. In order to analyze this at the molecular level, we studied gene expression profiles, epigenetic status and genome stability in the thymus and spleen of 1-month, 4-month and 18-month old Long Evans rats. In the thymus, altered gene expression, DNA and histone H3K9 hypomethylation, increased genome instability and apoptosis were observed in 18-month old animals compared to 1- and 4-month old animals. In the spleen, alterations in gene expression and epigenetic regulation occurred already by the age of 4 months compared to 1 month and persisted in 18-month old compared to 1-month old rats. In both organs, these changes were accompanied by the altered composition of resident T cell populations. Our study suggests that both senescence and apoptosis may be involved in altered organ function.

[2] This chapter has been published in its entirety:
Sidler, C., Woycicki, R., Ilnytskyy, Y., Metz, G., Kovalchuk, I., Kovalchuk, O. (2013). Immunosenescence is associated with altered gene expression and epigenetic regulation in primary and secondary immune organs. Front Genet. *4*, 211.

4.2 INTRODUCTION

The adaptive immune system provides protection against a wide variety of pathogens and cancer. A fine balance between positive selection (T cell activation by antigen) and negative selection (limitation of autoreactive T cells) is in place to avoid autoimmune reactions. However, these selection processes are energy-demanding and inefficient – more than 95% of the initially produced T cells are subsequently eliminated (Surh and Sprent, 1994). Therefore, after peripheral lymphatic organs have been populated with T cells, the thymus starts decreasing in size, weight and cellularity initially by about 3% per year and after the 5th decade of life by about 1% per year in humans (Steinmann et al., 1985). This shrinkage of the thymus, also termed the age-associated thymic involution, is evolutionarily conserved in species with a thymus. It is accompanied by a change in thymus architecture resulting in the reduced thymic output of naïve T cells proportional to thymus size (Cunningham et al., 2001).

The decline in thymic T cell export along with a reduced proliferation of peripheral naïve T cells and altered composition of T cell populations contribute to the establishment of immunosenescence, which is linked with higher morbidity and mortality in the elderly (Ferguson et al., 1995).

The driving force of age-associated involution is thought to lie within the epithelial compartment of the thymus itself (Aw et al., 2009; Ortman et al., 2002; Zhu et al., 2007). Further, there is some evidence that apoptotic and senescent cells accumulate in the epithelial compartment during thymic involution (Aw et al., 2008), which results in the shrinkage of the epithelial compartment (Flores et al., 1999). Thus, thymic involution may be driven by similar molecular mechanisms as the aging process in other organs.

Despite decreasing thymic output, the T cell pool in the periphery is relatively constant in size, which can be explained by the expansion of peripheral T cell populations (Mackall et al., 1993). Mackall *et al.* (1993) showed that in the presence of a functional thymus, the peripheral expansion of T cells was suppressed. The peripheral expansion is driven by interactions with antigen and therefore leads to an increasing adaptation to the individual's environment and decreasing responses to new antigens (Mackall et al., 1996). After several rounds of clonal selection, T cells can become replicatively senescent *in vitro*, and T cells with senescence-like features are also found *in vivo* (Effros, 2004).

However, the molecular mechanisms that underlie those changes are only beginning to be understood. Altered expression and activity of several transcription factors are involved in thymic involution (Ortman et al., 2002; Trebilcock and Ponnappan, 1996). This suggests that transcriptional profiles in tissues of the aging immune system may be altered.

Further, senescence plays a role in thymic involution as well as in homeostasis of peripheral T cells. At a molecular level, cellular senescence is often linked with the accumulation of oxidative damage to macromolecules (including DNA as the genetic material and chromatin as the substrate for epigenetic regulation). While the accumulation of mutations has long been hypothesized to be a cause of aging, damage to chromatin has recently been suggested to be involved in aging as well (Sedivy et al., 2008).

Therefore, we hypothesized that if senescence plays a role in immunosenescence, gene expression and epigenetic profiles may be vastly altered in primary and peripheral immune organs of aging organisms. To assess this, we isolated thymus and spleen tissues from 1-month, 4-month (before or at an early stage of thymic

involution) and 18-month old (at a late stage of thymic involution) male Long Evans rats. Using the Illumina® Gene Expression BeadChip technology, we determined transcript levels in total RNA preparations from both organs. Here, we report that along with profoundly altered gene expression profiles, both in the thymus and spleen, transcriptional and epigenetic regulation are affected with increasing age. This is accompanied by altered expression of CD surface markers and the composition of T cell populations in both organs.

4.3 MATERIALS AND METHODS

4.3.1 Animal model and tissue sampling

20 male Long Evans rats, raised and bred at the local vivarium, were part of this study. Handling and care of animals were conducted in accordance with the recommendations of the Canadian Council for Animal Care and Use. The University of Lethbridge Animal Welfare Committee has approved all procedures. Animals were housed in a pathogen-free controlled facility at a 12 h light/dark cycle and given food and water *ad libitum*.

Animals were humanely sacrificed at the age of 1 month (6 animals), 4 months (6 animals) or 18 months (8 animals). Those time points were chosen as they represent different stages in the life of a rat: after weaning (1 month), early stage of thymic involution (4 months) and late stage of thymic involution (18 months).

Spleen and thymus cells were isolated as described for the comet and flow cytometer assays, and tissue samples were snap-frozen for RNA, protein and DNA extraction.

4.3.2 Gene expression profiling

RNA samples of the thymus of 2 animals and the spleen of 3 animals per group were prepared using TRIzol® Reagent (Invitrogen). Gene expression profiles were determined using Illumina® RatRef12 Gene Expression BeadChips. Differential expression analyses were performed by the Illumina® GenomeStudio software using an Illumina-custom model with an FDR applied. Only genes for which the differential expression analysis was significant based on $p < 0.05$ and a log2 fold change cut-off of -0.4/0.4 were considered for further analysis.

Functional classification of gene expression data

The bioinformatics analysis performed included sample clustering, which was performed using the Illumina® GenomeStudio Software, and functional classification of genes. The functional classification of genes was done using FunNet Transcriptional Networks Analysis[3] and DAVID Bioninformatics Resources 6.7 (Huang da et al., 2009b).

Analysis of promoters for transcription factor binding sites (TFBS)

Promoter regions of 1,000 bases upstream of the transcription start site (TSS) of differentially expressed RefSeq genes were extracted from the March 2012 (RGSC 5.0/rn5) rat genome assembly (Gibbs et al., 2004). TFBS were retrieved from Gencards[4] (Safran et al., 2010) or Bio-Base[5]. The promoter sequences were then searched for TFBS using the PatMatch v1.2 program (Yan et al., 2005), with allowing no mismatches.

[3] www.funnet.info
[4] www.genecards.org
[5] https://portal.biobase-international.com

The number of occurrences of a TFBS in the promoters of differentially expressed genes was compared to its occurrences in the promoters of all genes in the rat genome assembly. Only genes, for which the number of TFBS occurrences within their promoter was higher or lower than mean ± standard deviation number of occurrences in promoters of all the genes in the rat genome were considered for further analysis. Transcription factor – target gene correlation was performed to check whether expression changes in transcription factors coincided with expression changes in target genes. Target genes, for which such a correlation was found, were considered for further analysis.

4.3.3 Quantitative real-time PCR (qRT-PCR)

The validation of gene expression results was done by qRT-PCR. qRT-PCR reactions were set up using the SsoFast™ EvaGreen® Supermix (BioRad) and analyzed according to SSoFast™ guidelines with annealing temperatures as specified for specific primer pairs (Table 4.1).

Each experiment included three biological replicates with two technical replicates each. *B2m, Hprt, Ldha, Hspcb* and *RplP1* were used for the normalization, which was carried out using qbase^{PLUS} (Vandesompele et al., 2002).

Table 4.1: Primers used for qRT-PCR analysis of transcript levels.

Gene	Fwd/rev	Sequence	Annealing Temperature
Suv39h1	Fwd Rev	5'-ATGCTGGCTAATACTAAC-3' 5'-TCTACTTCCTGATGGTAA-3'	56.9°C
Lig4	Fwd Rev	5'-ATACAGAAGGTGAATGAA-3' 5'-TGGAAGATGGACAATATC-3'	56.9°C
Cidec	Fwd Rev	5'-CTGAACTGAATGGACATAG-3' 5'-TTACCGCAGACTCTAATG-3'	56.9°C
Hbxip	Fwd Rev	5'-GCCATTCACTTAATGTCCAAT-3' 5'-CCACCACACTCCTAACTG-3'	59.6°C
Bcl2l13	Fwd Rev	5'-TCTACGACTGTGCCTCTA-3' 5'-GGGACCTTGTTGTTTCTC-3'	59.6°C

Birc3	Fwd Rev	5'-TCAGAGCACAGGAGACAT-3' 5'-TCAGGTTAGAGACAGTGTATTG-3'	56.9°C
Tdg	Fwd Rev	5'-TTCTCTGACTTGATGGTA-3' 5'-TCATAGAACAGGCTACAT-3'	56.9°C
Rrm2	Fwd Rev	5'-TTAGCCAAGAAGTTCAAG-3' 5'-AATGTAAGTGTCAATAAGGA-3'	56.9°C
Cdc25b	Fwd Rev	5'-GTGAAGAAGATGACGGATT-3' 5'-TGGAGCACTAATGAGGTT-3'	56.9°C
Cd4	Fwd Rev	5'-AAGGTGAAGAGGTCAAGATG-3' 5'-CAGCCAGGAACATTGTCT-3'	56.9°C
Cd8a	Fwd Rev	5'-TATTGTCCTCTGTATTGTT-3' 5'-GACTATCTCTGGTGTTAC-3'	56.9°C
Ctgf	Fwd Rev	5'-TGTGCCTATTGTTCTTGT-3' 5'-CAGTCACTCAGGTTACAG-3'	59.6°C
Pot1a	Fwd Rev	5'-TAAGCCTCCATATCTCAG-3' 5'-GAACAATGTCTCCAACTT-3'	56.9°C
B2m	Fwd Rev	5'-TTAGCAGCCTAGCAGTTC-3' 5'-ACCACTTCACTTCACTCTG-3'	61.2°C
Hprt	Fwd Rev	5'-TGACTATAATGAGCACTTC-3' 5'-AGGACTCTTGTAGATTCA-3'	56.9°C
Hspcb	Fwd Rev	5'-TGAGAACAAGAAGAAGAAGAA-3' 5'-ACGGATGAAGTTGAGGTA-3'	56.9°C
RplP1	Fwd Rev	5'-GGTCACGGAGGATAAGAT-3' 5'-ATGAGGCTTCCAATGTTG-3'	56.9°C
Ldha	Fwd Rev	5'-CGAGAGCATAATGAAGAAC-3' 5'-TCCTTGATTCCATAGAGAC-3'	53.5°C

4.3.4 Western immunoblotting

Protein preparations were obtained by sonicating approximately 50 mg of spleen or thymus tissues in 100 µL of cold 1% SDS containing protease inhibitor (Roche). Western immunoblotting was performed as previously described (Pogribny et al., 2005) using the primary antibodies specified in Table 4.2. Chemiluminescence was detected using a FluorChem™ HD2 camera with FluorChem™ software (Cell Biosciences). To confirm equal loading, membranes were stained with Coomassie blue (BioRad). Signals were quantified using the NIH Image J64 software and normalized relative to Actin or Coomassie staining as indicated.

Table 4.2: Antibodies used for Western blots.

Target	Supplier, Cat No	Dilution
Mouse anti-CDK2	Santa Cruz, sc6248	1:500 in 5% milk (PBST)
Mouse anti-p16	Santa Cruz, sc1661	1:500 in 5% milk (PBST)
Mouse anti-PCNA	Santa Cruz, sc56	1:1000 in 5% milk (PBST)
Mouse anti-DNMT1	Abcam, ab13537	1:500 in 5% milk (PBST)
Rabbit anti-DNMT3A	Santa Cruz, sc20703	1:500 in 5% milk (PBST)
Rabbit anti-SUV39H1	Abcam, ab33056	1:500 in 5% milk (PBST)
Rabbit anti-SIRT2	Santa Cruz, sc20966	1:500 in 5% milk (PBST)
Rabbit anti-H3K9me3	Abcam, ab8898	1:500 in 5% milk (PBST)
Rabbit anti-H3	Cell Signaling, 9715	1:1000 in 5% milk (PBST)
Rabbit anti-H4K16ac	Cell Signaling, 8804	1:1000 in 5% milk (PBST)
Mouse anti-H4	Cell Signaling, 2935	1:1000 in 5% milk (PBST)
Rabbit anti-MSH2	Santa Cruz, sc494	1:500 in 5% milk (PBST)
Mouse anti-RAD51	Santa Cruz, sc56212	1:500 in 5% milk (PBST)
Rabbit anti-Cleaved CASP3	Cell Signaling, 9664	1:500 in 5% milk (PBST)
Mouse anti-ACTIN	Abcam, ab3280	1:1000 in 5% BSA (PBST)
Secondary Antibodies		
Donkey anti-Rabbit	Santa Cruz, sc2313	1:10000 in 5% milk (PBST)
Goat anti-Mouse	Santa Cruz, sc2005	1:5000 in 5% milk (PBST)

4.3.5 Cytosine extension assay

DNA was isolated from approximately 25 mg of spleen or thymus tissues using the DNeasy® Blood and Tissue Kit (Qiagen). A cytosine extension assay was performed as previously described (Boyko and Kovalchuk, 2010). Counts per minute (CPM) values were determined using a PerkinElmer® Liquid Scintillation Analyzer Tri-Carb 2910 TR.

4.3.6 DNA damage and repair assays

Comet assay

The isolation of cells from spleen and thymus tissues for comet assays was performed in mincing buffer (110 mM NaCl, 4 mM KCl, 0.35 mM KH_2PO_4, 0.27 mM Na_2HPO_4, 0.08% glucose, 3.33 mM $NaHCO_3$, 25.5 mM EDTA, 10% DMSO, pH 7-7.5) by grinding tissue pieces through 40 μm Nylon meshes. Comet assays were performed as described previously (Olive and Banath, 2006). For scoring comets, the slides were stained with 1:1,000 SYBR® Gold and viewed using a Zeiss Observer Z1

microscope and a Stingray CCD camera (Allied Vision Technologies). The comets were evaluated using the Comet Assay IV software (Perceptive Instruments Ltd.). The values of the Olive Tail moment were used as a measure of DNA damage. The experiment included three independent biological replicas, each including two technical repeats. In total, 300 to 400 comets were analyzed per experimental group.

γH2AX/PCNA immunofluorescence staining

Touch prints of spleen and thymus tissues were performed on positively charged microscope slides (VWR) and fixed in 4% PFA for 20 min. This was followed by washing in PBS for 20 min and storing in 70% ethanol.

Immediately before staining, the touch prints were washed in PBS and permeabilized in ice-cold methanol. This was followed by blocking 10% BSA/10% goat serum/0.1% Tween20 in PBS for 2 hours and incubation with primary antibody (rabbit anti-γH2AX (1:100, Cell Signaling) and mouse anti-PCNA (1:200, Santa Cruz) in blocking solution) overnight at 4°C. Antibody binding was detected by incubation with secondary antibodies AlexaFluor® 488 goat anti-rabbit and AlexaFluor® 546 goat anti-mouse (1:700, Invitrogen) in blocking solution. The slides were counterstained with Hoechst 33342. Following dehydration in increasing ethanol concentration, the slides were air-dried and mounted in 50% HARLECO® Krystalon™ Mounting Medium/50% ethanol and viewed using a Zeiss Observer Z1 epifluorescence microscope with AxioVision Rel 4.8 software.

4.3.7 Flow cytometer assays

Cell isolation

Cells from spleen and thymus were obtained by grinding tissue pieces through a 40 µm Nylon mesh in HyClone® Dulbecco's modified Eagle medium (DMEM) High Glucose (Fisher Scientific) containing 10% FBS (Gibco). The cells were washed and resuspended in 1 mL PBS in the case of cells isolated from thymus or in the case of spleen cells incubated in 500 µL ACK Lysis Buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2) for 10 min. Then the cells were supplemented with 9 mL of medium, pelleted and resuspended in 1 mL PBS.

Detection of apoptotic, necrotic and dead cells

In order to determine the ratios of apoptotic, necrotic and dead cells in thymus and spleen tissues, cells were stained using the FITC Annexin V Apoptosis Detection Kit (BD Pharmigen™) according to the manufacturer's instructions and analyzed using the BD FACS Canto II (BD Biosciences). 10,000 events were detected for each sample.

Detection of CD4 and CD8 cell surface markers

For the detection of CD4 and CD8 antigens, the cells were washed twice with IFWB (4% FBS/0.02% NaN_3 in PBS) followed by incubation with primary antibodies PE mouse anti-rat CD4 and PerCP mouse anti-rat CD8a (0.09 µg each, BD Pharmigen™) for 45 min. Cells were then washed twice in IFWB and fixed in 100 µL 4% PFA in PBS for 1 h at 4°C. The cells were washed in 500 µL of IFWB, resuspended in 500 µL PBS and analyzed on the BD FACS Canto II (BD Biosciences). 10,000 events were detected for each sample.

4.3.8 Statistical treatment of data

Results are presented as mean of at least three animals per group with standard deviation or 95% confidence interval as indicated. A statistical analysis of the results was performed using one-way ANOVA ($p < 0.05$) in the case of qRT-PCR experiments or two-tailed Student's t-test ($p < 0.05$) for the analysis of all other presented results as indicated.

4.4 RESULTS

4.4.1 Age-dependent gene expression changes do not occur simultaneously in different organs

To get an understanding of age-dependent changes that occur in primary and secondary immune organs, we profiled mRNA transcripts from thymus and spleen tissues extracted from 1-month (young), 4-month (mature) or 18-month old male Long Evans rats using Illumina® RatRef12 BeadChips (S1[6]). The number of genes affected by expression changes varied with age and tissue. In thymus, changes in the expression of 1,034 genes were detected between young and old animals, whereas only 86 genes were affected between 1-month and 4-month old animals. In spleen, high numbers of expression changes were observed when comparing old and mature animals to young animals (2,196 and 2,019 genes, respectively), whereas a low number of changes occurred between 4- and 18-month old animals (Fig. 4.1A). The cluster analysis based on all probes represented on the BeadChip further showed that for spleen, expression profiles of mature and old animals clustered more closely,

[6] http://journal.frontiersin.org/Journal/10.3389/fgene.2013.00211/abstract

whereas for thymus, profiles of young and mature animals clustered more closely (Fig. 4.1B).

When comparing the total gene expression changes that occur between young and old animals in spleen and thymus, 516 genes are differentially expressed in both tissues, whereas 1,591 are spleen-specific and 518 thymus-specific changes (Fig. 4.1C). The genes that were commonly differentially expressed in both spleen and thymus affected biological processes of cell cycle, DNA replication, immune response and epigenetics (Fig. 4.1C, S2[4]).

For better understanding of the functional implications of these expression changes, functional classification was performed. In both tissues, aging was associated with an increase in the number of differentially expressed genes involved in cell cycle regulation, DNA replication and senescence. Alongside those, genes involved in DNA repair, epigenetic regulation, apoptosis and immune response were also increasingly affected by age-dependent differential expression (Fig. 4.1D, S2[7]).

[7] http://journal.frontiersin.org/Journal/10.3389/fgene.2013.00211/abstract

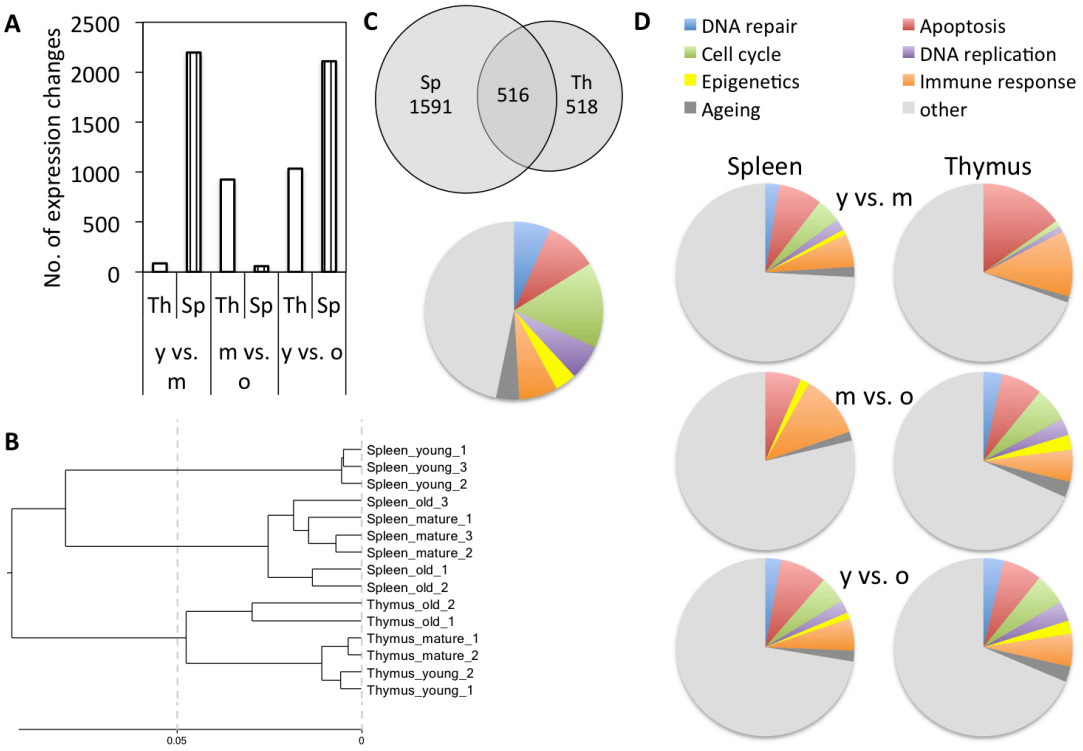

Figure 4.1: Cluster analysis and functional classification of gene expression results.

A) Number of differentially expressed genes when comparing different age groups; y is young, m is mature and o is old. B) Cluster analysis based on all probes represented on the BeadChip. C) Venn diagram showing the numbers of genes differentially expressed in spleen, thymus or both, when comparing old to young animals, and functional classification of the genes that were commonly differentially expressed. D) Functional classification of genes affected by differential expression.

4.4.2 Changes in cell cycle regulation and senescence gene expression indicate that older tissues contain more senescent cells

Functional classification showed increasing fractions of differentially expressed senescence-related genes in mature and old tissues in spleen, and old tissues in thymus, as compared to young tissues (Fig. 4.1D). This may be explained by an

accumulation of senescent cells in both tissues with age, which is in line with previous observations that senescent cells accumulate in the thymus of aging animals (Aw et al., 2008) and among peripheral T cells (Effros, 2004).

Key features of senescent cells include cell cycle arrest in G0/G1 phase (Sherwood et al., 1988) as well as distinct changes to gene expression profiles (de Magalhaes et al., 2009). In order to study cell cycle status of thymus and spleen samples, genes involved in cell cycle regulation were further sub-categorized into regulators of cell cycle progression, genes involved in the progression of M, G1, S and G2 phases of the cell cycle (Table 4.3). As a general trend, it was observed that a high number of genes involved in cell cycle regulation are down-regulated irrespective of whether the function of those genes is either to promote or inhibit cell cycle progression.

Among them, several cyclin genes (*Ccna2*, *Ccnb2* and *Ccne2*) were down-regulated in the spleen of mature and old animals and in the thymus of old animals, whereas *Ccnd1* was up-regulated in those tissues. Furthermore, *Cdc2*, which is important for G2/M transition and M progression, was down-regulated in the spleen of mature and old animals and in the thymus of old animals. In addition to those changes, in the spleen, *Cdk2* and *Cdk4* were down-regulated with increasing age. This indicates that in the spleen cells of mature and old animals and in the thymus cells of old animals, the potential for active cell cycling is limited.

Expression levels of several genes were confirmed at the RNA level by qRT-PCR or at the protein level by Western blot (Fig. 4.2A,B,C). The down-regulation of *Rrm2* at the RNA level and PCNA at the protein level supports that DNA replication occurs in a smaller fraction of cells in older tissues. The down-regulation of *Cdk2* and up-regulation of $p16^{INK4a}$ suggests that G1 progression occurs in a smaller fraction of cells in older tissues.

Table 4.3: Expression changes of cell cycle regulators.

Percentages of up- or down-regulated genes compared to the total number of genes with expression changes in the according sub-category. Arrows indicate whether the represented numbers reflect the fraction of up- or down-regulated genes.

Sub-category	Thymus	Spleen
Cell cycle progression		
Inhibitors ↑	0	14
Activators ↓	80	67
G1 progression		
Inhibitors ↑	25	36
Activators ↓	86	87
S phase progression ↓	100	93
DNA replication ↓	97	93
G2 progression ↓	75	50
Mitosis ↓	91	89

In respect to age-related gene expression changes, 18 out of 25 genes in the thymus and 35 out of 45 genes in the spleen showed expression changes consistent with previously reported age-related changes (de Magalhaes et al., 2009) (Table 4.4).

Transcript levels of *Ctgf* and *Pot1a* were determined using qRT-PCR. *Ctgf* showed a significantly increased expression in the spleen of old and mature animals and in the thymus of old animals, whereas *Pot1a* transcript levels were significantly decreased in the spleen of mature and old animals (Fig. 4.3).

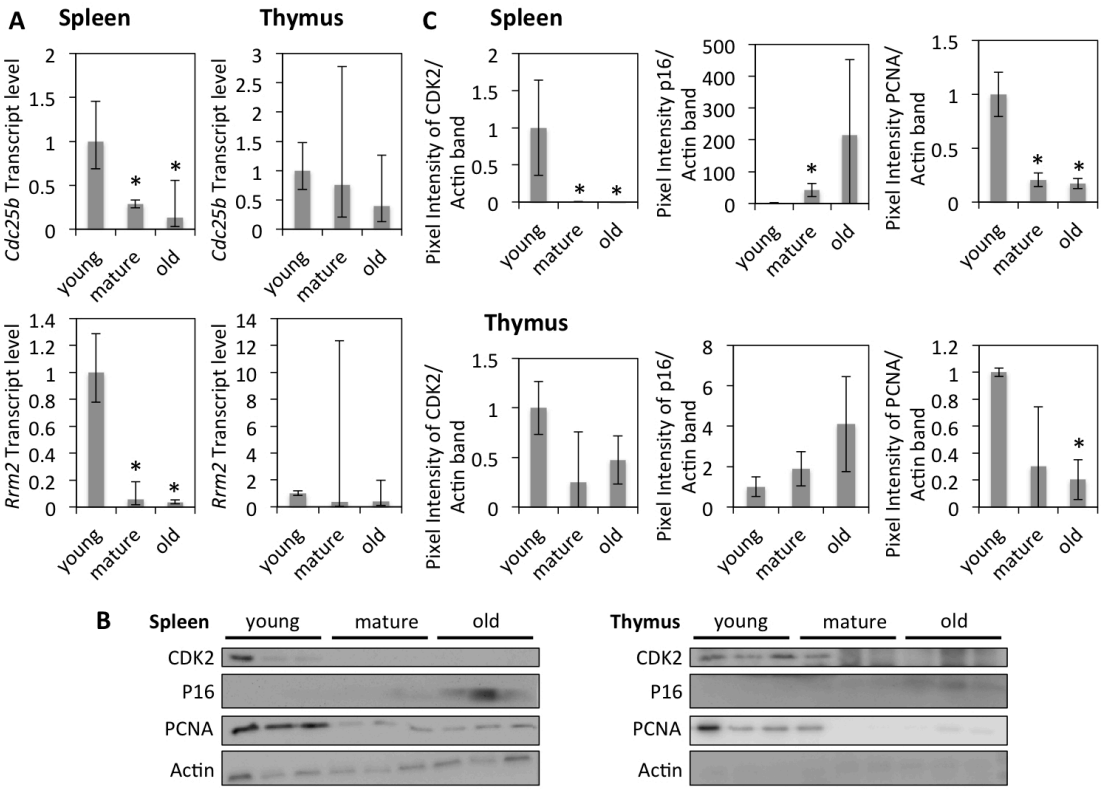

Figure 4.2: The effects of age on cell cycle and DNA replication in the spleen and thymus of rats.

A) Fold changes of mRNA expression as measured by qRT-PCR standardized to gene expression in young animals. The bars represent mean ± 95% confidence interval. B) Western blots showing protein levels of CDK2, p16 and PCNA. C) Protein expression levels of selected genes standardized to the expression in young animals and normalized to the expression of Actin. The error bars represent standard deviations.

Table 4.4: Senescence-associated gene expression.

Entries up- /down-regulated with age are based on microarray signatures from Human Ageing Genomic Resources (de Magalhaes et al., 2009). Other gene information is based on Genecards[8] (Safran et al., 2010). Values represent log2 fold changes in detected transcript levels; N.S. indicates a non-significant difference in transcript levels.

		Thymus			Spleen		
Gene	**Involvement in Aging**	**y-m**	**y-o**	**m-o**	**y-m**	**y-o**	**m-o**
Ada	modulates TERT activity	N.S.	-0.7	-0.6	0.6	0.9	N.S.
Adipoq	may prevent senescence	N.S.	N.S.	3.3	N.S.	N.S.	N.S.
Anxa3	up-regulated with age	N.S.	1.1	1.0	1.1	1.1	N.S.
Brd7	mediates p53-induced senescence	N.S.	-0.7	-0.6	-0.6	-0.7	N.S.
C1qa	up-regulated with age	N.S.	1.5	1.0	1.2	1.3	N.S.
C1qb	up-regulated with age	N.S.	1.6	1.3	1.0	0.9	N.S.
C1qc	up-regulated with age	0.6	2.0	1.4	1.3	1.3	N.S.
Cbx7	represses Cdkn2a, extends lifespan	N.S.	0.6	N.S.	0.8	0.8	N.S.
Ccl5	secreted by senescent fibroblasts	N.S.	N.S.	N.S.	1.4	1.0	N.S.
Cdkn1b	$=p27^{Kip1}$; up-regulated with age	N.S.	-0.6	N.S.	N.S.	-0.6	N.S.
Clu	up-regulated with age	N.S.	2.7	2.8	0.8	0.8	N.S.
Col1a1	down-regulated with age	N.S.	N.S.	N.S.	N.S.	-0.9	N.S.
Col3a1	down-regulated with age	-1.3	N.S.	N.S.	N.S.	-0.9	N.S.
Ctgf	up-regulated with age	N.S.	1.8	1.2	1.3	1.9	N.S.
Ctss	up-regulated with age	N.S.	1.4	1.0	1.5	1.7	N.S.
Cx3cl1	down-regulated with age	N.S.	N.S.	N.S.	N.S.	0.6	N.S.
Eif5a	up-regulated with senescence	N.S.	-0.6	N.S.	N.S.	N.S.	N.S.
Ets1	regulates genes involved in senescence	N.S.	-1.0	-0.9	1.3	0.9	N.S.
Fcgr2b	up-regulated with age	N.S.	0.9	N.S.	1.3	1.3	N.S.

[8] www.genecards.org

Foxo1a	involved in G1/G0 transition	N.S.	N.S.	N.S.	0.7	N.S.	N.S.
Gbp2	up-regulated with age	N.S.	0.8	0.7	1.9	1.8	N.S.
Gfap	up-regulated with age	N.S.	N.S.	N.S.	-2.0	-2.2	N.S.
Gns	up-regulated with age	N.S.	0.9	0.9	1.0	1.3	N.S.
Hcst	up-regulated with age	N.S.	N.S.	N.S.	1.1	1.2	N.S.
Id2	down-regulated with senescence	N.S.	N.S.	N.S.	0.9	1.0	N.S.
Igj	up-regulated with age	2.6	N.S.	N.S.	1.7	2.5	N.S.
Il15	involved in aging of immune system	N.S.	N.S.	N.S.	0.9	0.9	N.S.
Il1b	changed expression in senescent cells	N.S.	N.S.	N.S.	1.5	1.6	N.S.
Laptm5	up-regulated with age	N.S.	N.S.	N.S.	1.0	1.0	N.S.
Lgals3	up-regulated with age	N.S.	N.S.	N.S.	0.6	1.0	N.S.
Litaf	up-regulated with age	N.S.	N.S.	N.S.	0.8	0.6	N.S.
LOC500054	=Pot1a; involved in telomere maintenance	N.S.	-0.6	-0.7	N.S.	-0.7	N.S.
Lrp1	senescence-related gene expression changes	N.S.	N.S.	N.S.	N.S.	0.7	N.S.
Map2k1	is required for p53-induced senescence	N.S.	N.S.	N.S.	0.6	0.9	N.S.
Mgst1	up-regulated with age	N.S.	2.6	2.0	1.1	0.9	N.S.
Mmp9	regulates TERT and p16 expression	N.S.	N.S.	-0.7	N.S.	N.S.	N.S.
Mpeg1	up-regulated with age	N.S.	N.S.	N.S.	1.1	1.2	N.S.
Msn	up-regulated with age	N.S.	N.S.	N.S.	0.9	0.8	N.S.
Nbn	telomere maintenance	N.S.	N.S.	N.S.	-0.7	N.S.	N.S.
Ndrg1	up-regulated with age	N.S.	0.6	N.S.	0.7	N.S.	N.S.
Nfkb2	age-related gene expression changes	N.S.	N.S.	N.S.	1.0	1.0	N.S.
Npc2	up-regulated with age	N.S.	0.6	N.S.	0.9	0.7	N.S.
Pcsk6	up-regulated with age	N.S.	N.S.	N.S.	-0.6	N.S.	N.S.
Psmd11	up-regulated with age	N.S.	N.S.	N.S.	-0.6	-0.7	N.S.
Rad54l	telomere maintenance	N.S.	-0.8	-0.6	-1.3	-1.4	N.S.
RGD1305645	induces senescence	N.S.	N.S.	N.S.	2.0	2.1	N.S.
S100A4	up-regulated with age	N.S.	N.S.	N.S.	N.S.	0.6	0.6
Serping1	up-regulated with age	N.S.	N.S.	N.S.	1.0	1.2	N.S.
Sirt2	natural aging	N.S.	N.S.	N.S.	-0.6	-0.9	N.S.
Suv39h1	H3K9methylation dependent induction of senescence	N.S.	-0.9	-0.7	-1.2	-1.2	N.S.
Terc	telomere maintenance	N.S.	N.S.	-0.6	-1.8	N.S.	N.S.
Tfrc	down-regulated with age	N.S.	N.S.	N.S.	-2.2	-2.8	N.S.
Txnip	up-regulated with age	N.S.	0.6	0.6	0.8	0.6	N.S.
Vwf	up-regulated with age	N.S.	N.S.	N.S.	-1.4	-1.6	N.S.

Fridman and Tainsky (2008) summarized the pathways affected by gene expression changes observed in several senescence and immortalization studies and reported that some of the key regulatory genes were involved in several pathways: pRB/p53, cytoskeletal, interferon-related, insulin growth factor-related, MAPK and oxidative stress pathways (Fridman and Tainsky, 2008). Our results are consistent with their findings: 16 genes involved in p53 signalling and 8 genes involved in MAPK signalling are differentially expressed in the spleen between young and old animals. Like Ning *et al.* in their study (Ning et al., 2003), in our data, we did not find an overrepresentation of genes that were located in proximity to telomeres, indicating that there was no location effect of telomeres on gene expression in this model system.

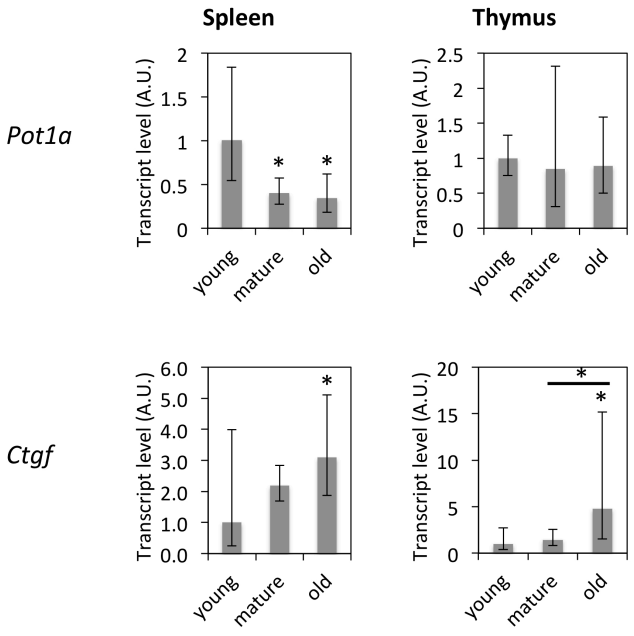

Figure 4.3: mRNA levels of senescence-related genes in aging spleen and thymus.

Bars represent averages from three animals and two technical repeats normalized to expression in young animals ± 95% confidence intervals.

In summary, age-related gene expression changes are observed in the spleen of mature and old animals and in the thymus of old animals. Further, gene expression changes affecting cell cycle progression are also observed in the same tissues, suggesting that higher ratios of cells are arrested in G1/G0 phase of the cell cycle. These findings support the assumption that there is a higher ratio of senescent cells present in the spleen of mature and old animals and in the thymus of old animals.

4.4.3 A potential role of transcription factors in regulating age-dependent gene expression profiles

The fact that high numbers of genes, including many transcription factors, were differentially expressed with increasing age in both tissues indicates a role of transcription factors in shaping the gene expression profiles observed. In order to address this, promoter regions of differentially expressed genes were screened for the occurrence of transcription factor binding sites (TFBS) of transcription factors with altered transcript levels.

Some transcription factors were differentially expressed in both tissues (Table 4.5), however, in opposite directions. The 4 transcription factors affected by gene expression changes in both organs – *Bcl6*, *Myc*, *Tcf7*, *Ets1* - were down-regulated in the thymus but up-regulated in the spleen. In addition, there were several transcription factors that were tissue-specific.

Genes that had specific TFBS and were regulated in a similar manner to these transcription factors (either up- or down-regulated) were further classified according to their function. The predicted targets of the BCL6 transcription factor in the spleen were involved in cell cycle regulation and DNA replication; the predicted targets of

SPIC and GATA1 were involved in lysosomal gene expression; and the predicted targets of HIF1α in chemokine signalling and cancer and insulin signalling pathways. For all other transcription factors considered, no overrepresentation of KEGG pathways among the target genes was detected by the DAVID software. Precise roles of diverse transcription factors in senescence and immunosenescence need further study.

Table 4.5: The correlation of transcription factor expression with gene expression pattern.

Number of target genes that correlate with transcription factor expression was examined. The transcription factors shaded in white are commonly deregulated in the thymus and spleen, light grey shading indicates the transcription factors with a thymus-specific expression pattern, and dark grey shading indicates transcription factors with a spleen-specific expression pattern.

	Thymus			**Spleen**		
	y - m	**m – o**	**y – o**	**y - m**	**m - o**	**y – o**
BCL6		14	16	38		43
MYC			9			13
TCF7		6	6	6		4
ETS1		9	5	21		21
AHR			6			
TCF12		21				
SFPQ		2				
NFYB				62		68
NFYC						68
SPIC				58		53
HIF1α				41		36
HHEX				19		21
FOXO3				12		16
JUNB				11		14
GATA1				6		10

SOX4		7
CSDA	3	2
FOXP1	2	2

4.4.4 Regulation of DNA methylation and histone modification is altered in an age-dependent manner

Another factor, which is known to affect the gene expression pattern is epigenetic regulation. The analysis of gene expression data revealed age-dependent down-regulation of DNA methyltransferases, histone modifying enzymes, chromatin remodelling enzymes and miRNA/siRNA processing factors, suggesting that epigenetic regulation in old tissues is altered compared to that in young tissues.

Down-regulation of DNMT1 and DNMT3a in the thymus and spleen of older animals was confirmed by Western blot analysis (Fig. 4.4A,E); lower levels of DNMT1 and DNMT3a correlated with genome hypomethylation in the thymus of older animals as determined by a cytosine extension assay (Fig. 4.4B). The difference in DNA methylation levels between spleens of young and old animals was not significant, whereas spleens of mature animals exhibited slight, but significant global DNA hypermethylation compared to tissues from young animals (Fig. 4.4B).

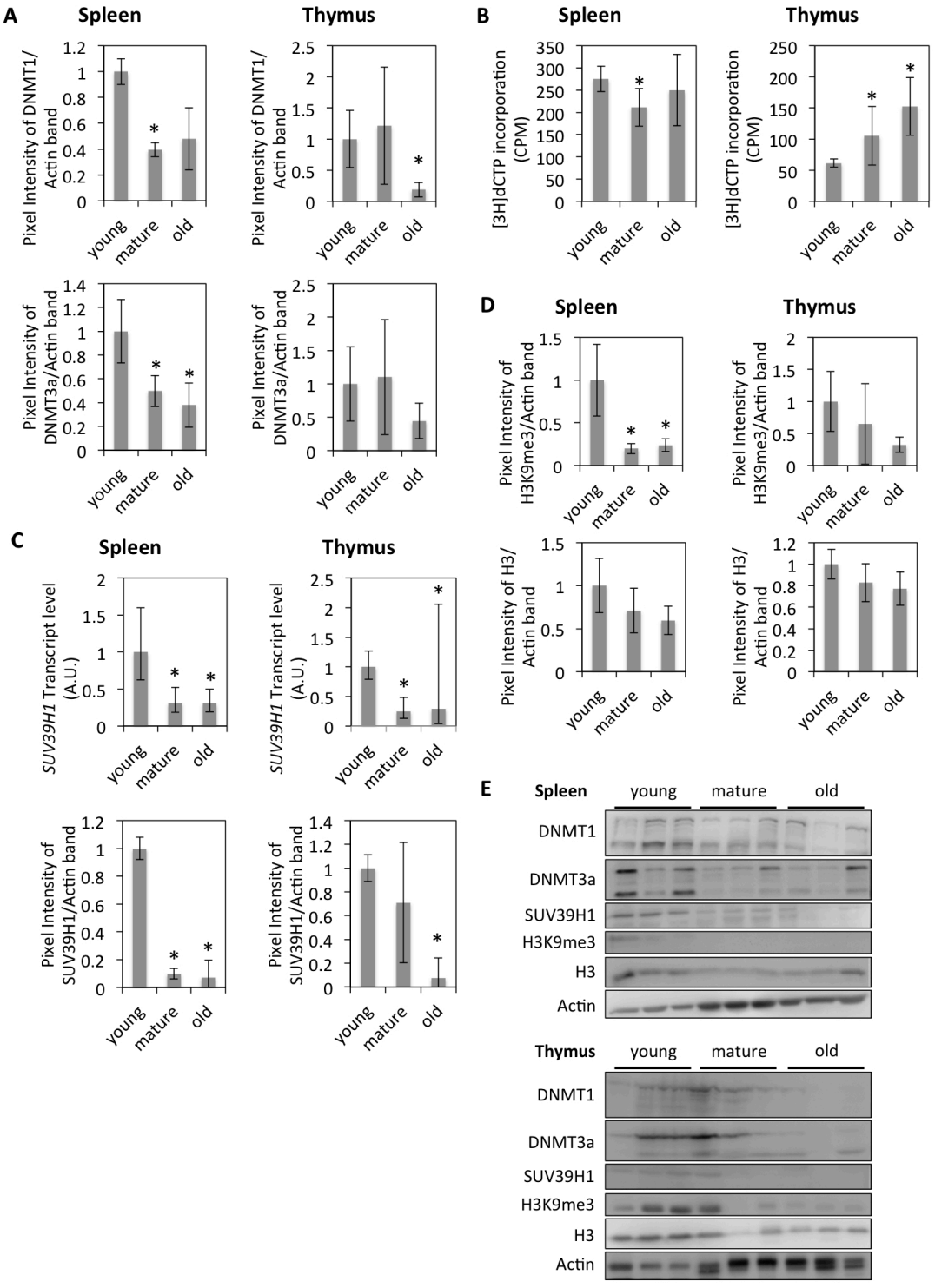

Figure 4.4: Changes in epigenetic regulation in the thymus and spleen of aging rats.

A) Protein levels of DNMT1 and DNMT3a. The bars represent means normalized to Actin expression and expression in young animals ± standard deviation. B) Global

DNA methylation levels as measured by the cytosine extension assay. The bars represent mean ± standard deviations. C) RNA and protein expression levels of histone modifying enzymes as measured by qRT-PCR or Western blot. For qRT-PCR graphs, the bars represent mean standardized to expression levels in young animals. The error bars represent 95% confidence intervals. For Western blots, the bars represent means normalized to Actin bands and expression in young animals ± standard deviation. D) Quantification of histones and histone modifications by Western blot. The bars represent means normalized to Actin bands or Coomassie loading controls and expression levels in young animals ± standard deviations. E) Western blot images.

It has been suggested elsewhere that with the onset of senescence, regions of constitutive heterochromatin become de-heterochromatinized, while senescence-associated heterochromatin foci are formed in regions of facultative heterochromatin (Zhang and Adams, 2007). Therefore, we hypothesized that global DNA hypomethylation in the thymus of old animals may be associated with the reactivation of transposable elements. Western blot analysis showed slightly increased expression levels of LINE1 ORF1 in the spleen and thymus of old animals; however, no increase in transcript levels could be detected (data not shown).

In addition to DNA methylation, post-translational modifications of core histone proteins play an important role in epigenetic control of gene expression. *Suv39h1* – a histone methyltransferase – was found to be down-regulated in the spleen and thymus of old animals, which was confirmed at the RNA level by qRT-PCR and at the protein level by Western blot (Fig. 4.4B,E). Lower expression levels of *Suv39h1* also correlated with reduced global levels of trimethylated Histone 3 (H3K9me3) which

was significant in spleen samples (Fig. 4.4B,E). SIRT2 – a histone deacetylase – showed lower expression levels in the spleen of older animals, which did not correlate with acetylation levels of H4K16 – a SIRT2 target (Fig. 4.5).

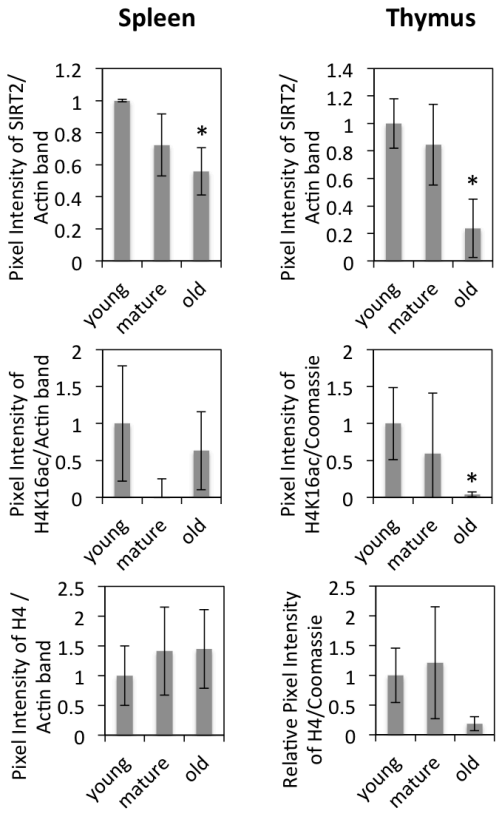

Figure 4.5: Reduced SIRT2 levels correlate with reduced H4K16ac levels in thymus.

Bar graphs show averages from three biological repeats and error bars represent standard deviations. Asterisks indicate significance based on Student's t-test ($p < 0.05$).

Both global DNA hypomethylation and histone hypomethylation indicate an increase in open chromatin and potentially genome instability, particularly in the thymus of mature and old animals.

4.4.5 Tissues in older animals exhibit decreased expression of DNA repair genes and DNA damage signalling genes

Since global DNA hypomethylation and histone hypomethylation were observed in the thymus of old animals and histone hypomethylation in the spleen of old animals, we hypothesized that tissues from old animals would exhibit an increased genomic instability. Gene expression data further indicated the down-regulation of genes involved in DNA damage signalling (*H2AX*, *Usp3*, *Bard1* and others), response to oxidative stress (*Gpx1*, *Gpx4*, *Prdx2*, *Prdx3*, *Sod1*, *Cat* in spleen only), base excision repair (*Fen1*, *Lig1*), nucleotide excision repair (*Rpa2*, *Rpa3*, *Rad23a*), double-strand break repair (*Lig4*, *Rad54l*, *Trip13*, *Rad21*) and mismatch repair (*Tdg*, *Exo1*, *Msh2* and *Msh6*). Expression levels of *Tdg* (MMR) and *Lig4* (NHEJ) were confirmed by qRT-PCR (Fig. 4.6A) at the RNA level, and expression levels of MSH2 (MMR) and RAD51 (HR) by Western blot (Fig. 4.6B,C) at the protein level. However, in the case of RAD51, while reduced transcript levels were detected in the spleen of old animals using the BeadChip technology, protein levels were found to increase with age.

The overall down-regulation of DNA repair enzymes at the transcript and protein levels suggested an increasing amount of unrepaired DNA damage in tissues of older animals. To study this, cells were isolated from the thymus and spleen of animals of different age groups and subjected to the comet assay. The results indicated a slightly, but significantly increased level of unrepaired DNA damage in the thymus of old animals compared to that of young animals, but a slightly, but significantly decreased level of unrepaired DNA damage in the spleen of mature and old animals as well as in the thymus of mature animals (Fig. 4.6D).

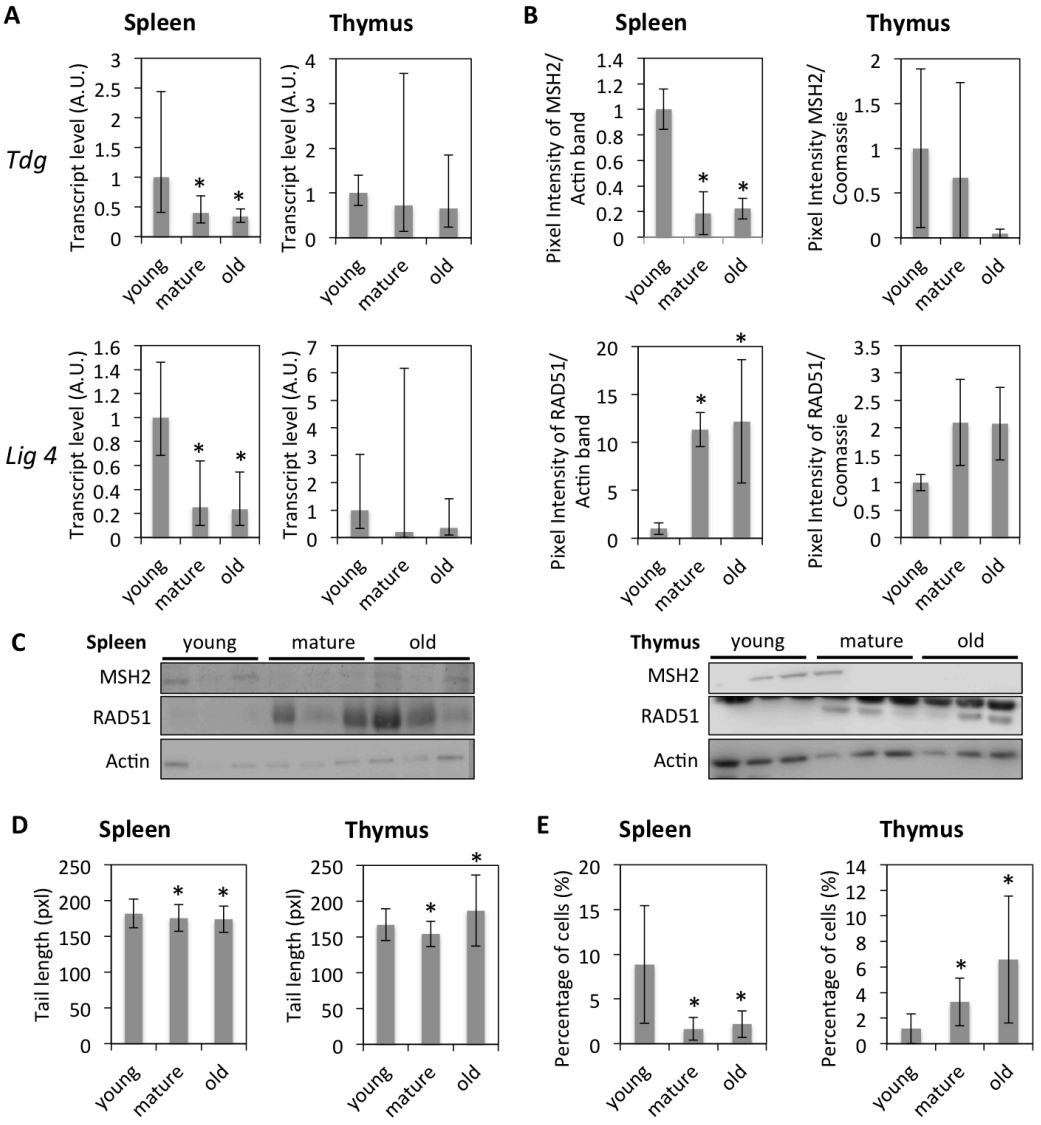

Figure 4.6: Age-dependent changes in DNA damage and repair.

A) mRNA fold expression changes normalized to a set of housekeeping genes and the expression in young animals as determined by qRT-PCR. The bars represent means ± 95% confidence intervals. B) Protein expression levels for selected genes measured by Western blot. The bars represent means normalized to the Actin expression levels and the expression in young animals ± standard deviation. C) Western blot images. D) Olive comet tail lengths determined by the comet assay. The data points represent averages of 300 – 400 comets per age, the error bars show

standard deviation. E) Percentage of cells positive for γH2AX. The bars represent averages of 300 to 400 cells per age and organ ± standard deviations.

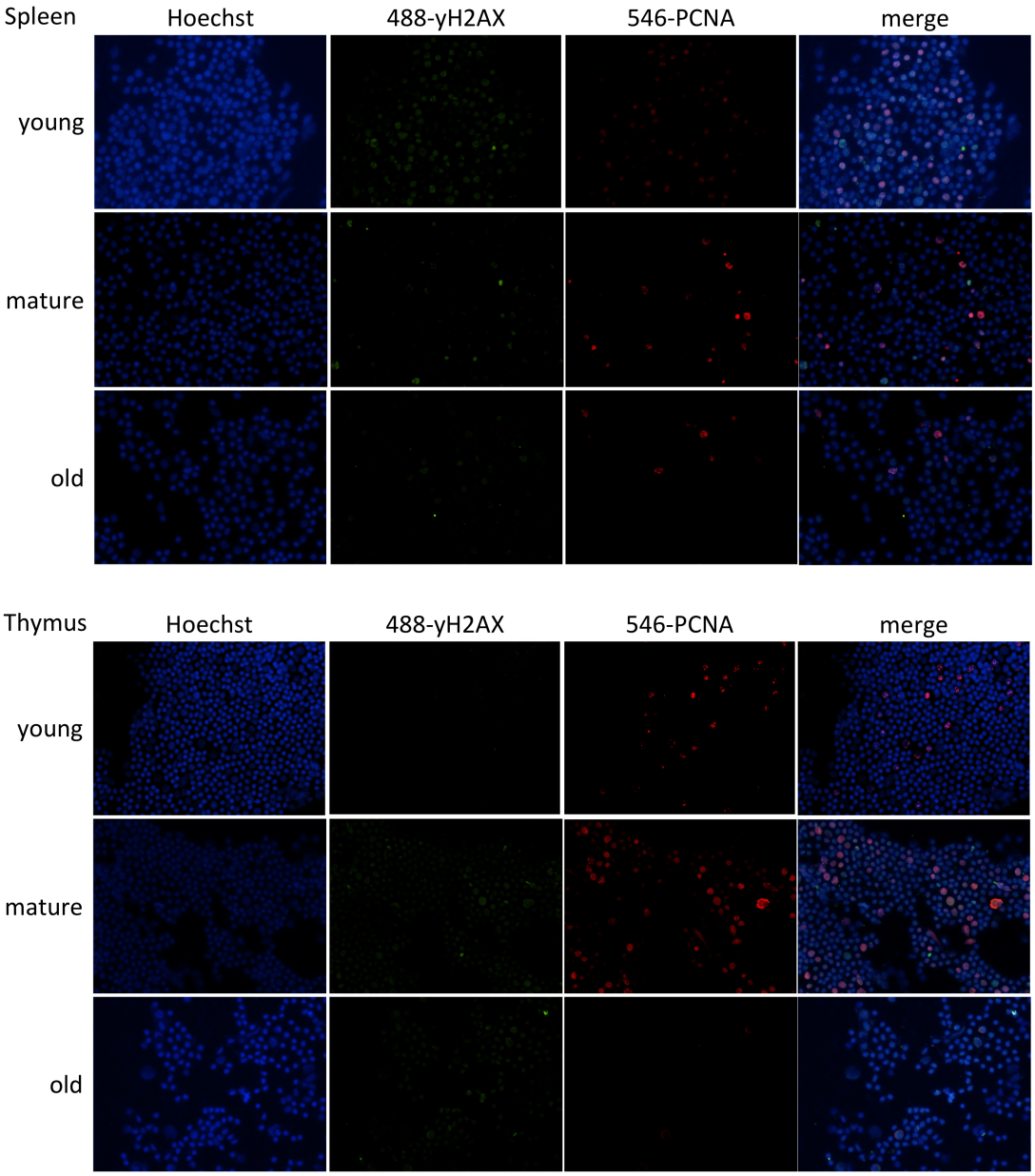

Figure 4.7: Representative images of γH2AX-PCNA immunofluorescence staining.

Images were taken at 40x magnification.

In order to study changes in DNA damage signalling, the occurrence of γH2AX foci was determined by immunofluorescence staining (Fig. 4.6E, Fig. 4.7). In line with the results of the comet assay, the percentage of cells with γH2AX foci increased with age in the thymus and decreased in the spleen, thus correlating with global genome hypomethylation in the thymus of old animals.

4.4.6 The thymus of older animals contains more apoptotic cells than in younger animals, but no clear trend is observed in the spleen

Changes in the expression of genes involved in apoptosis were different in thymus and spleen tissues, including both the induction and repression of both pro- and anti-apoptotic genes. In the thymus, 59% of pro-apoptotic genes were up-regulated, and 54% of anti-apoptotic genes were down-regulated, whereas in the spleen, 66% of pro-apoptotic genes were up-regulated and 50% of anti-apoptotic genes were down-regulated. Based on this, a slightly increased incidence of apoptotic cells in older tissues might be expected.

First, expression levels of some pro-apoptotic genes (*Bcl2l13*, *Cidec*) and anti-apoptotic genes (*Birc3*, *Hbxip*) were confirmed by qRT-PCR (Fig. 4.8A).
In order to study the effect of such gene expression changes on the function of encoded proteins, tissues were analyzed for their content of apoptotic cells by Western blot (cleaved Caspase 3) and Annexin V/PI staining followed by flow cytometer analysis. Western blot analysis for cleaved CASP3 revealed no significant differences among all three age groups either in the spleen or thymus, with a trend to an increased amount of cleaved CASP3 in the spleen of old animals and a decreased amount of that in the thymus (Fig. 4.8B).

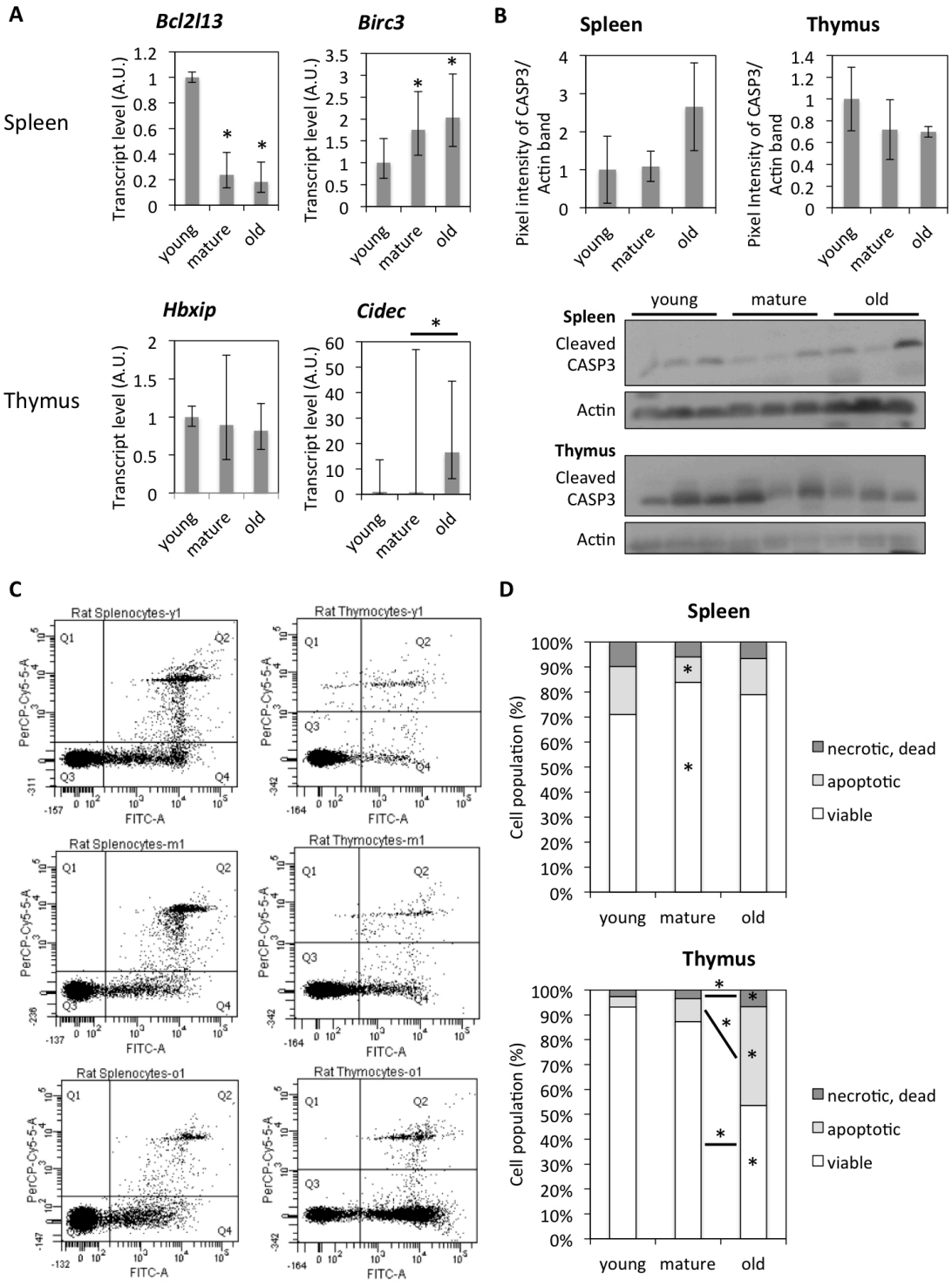

Figure 4.8: Age-dependent changes in the activity of the apoptotic pathway.

A) mRNA expression levels of genes involved in the apoptotic pathway by qRT-PCR. The data points represent means normalized to the expression in young animals ± 95% confidence intervals. B) Western blot images and quantification of cleaved

CASP3 band intensity normalized to Actin levels and protein levels detected in tissues from young animals. Each data point represents mean ± standard deviation. C) Plots of cell populations were measured by flow cytometry, the PerCP-Cy5 channel detected PI-positive cells, and the FITC channel detected Annexin V-FITC positive cells. D) Quantification of flow cytometer analysis of Annexin V/PI staining. The data points represent averages of six to eight animals with 10,000 events detected per animal. The asterisks on bars indicate significant differences between young animals, and the asterisks above lines indicate significant differences between the two samples.

Annexin V/PI staining of spleen cells indicated a significantly lower amount of apoptotic cells in mature compared to young tissues. Due to individual differences between animals, age-dependent differences were not significant (Fig. 4.8C,D). However, the thymus of old animals contained a significantly higher fraction of apoptotic and dead cells compared to that of young and mature animals (Fig. 4.8C,D).

4.4.7 The composition of T cell populations in the thymus and spleen changes with age

The gene expression analysis indicated that there was an increased expression of *Cd4* in the spleen and a reduced expression of *Cd8a* and *Cd8b* in the thymus of older animals (S1[9]). Changes in the expression of *Cd4*, *Cd8a* and *Cd8b* indicated that T cell populations in both tissues would have altered composition in aging individuals.

Analyzing immune cell populations by a flow cytometer-based assay, in the spleen, we found smaller populations of $CD4^-CD8^-$ cells but slightly larger populations of $CD4^+CD8^+$ cells and $CD4^+CD8^-$ cells (Fig. 4.9). In the thymus, individual differences

[9] http://journal.frontiersin.org/Journal/10.3389/fgene.2013.00211/abstract

were larger, but there was a significantly increased population of $CD4^+CD8^+$ cells in older animals along with a decrease in $CD4^+CD8^-$ populations (Fig. 4.9). Furthermore, those changes were observed in the spleen of mature and old animals, but only in the thymus of old animals.

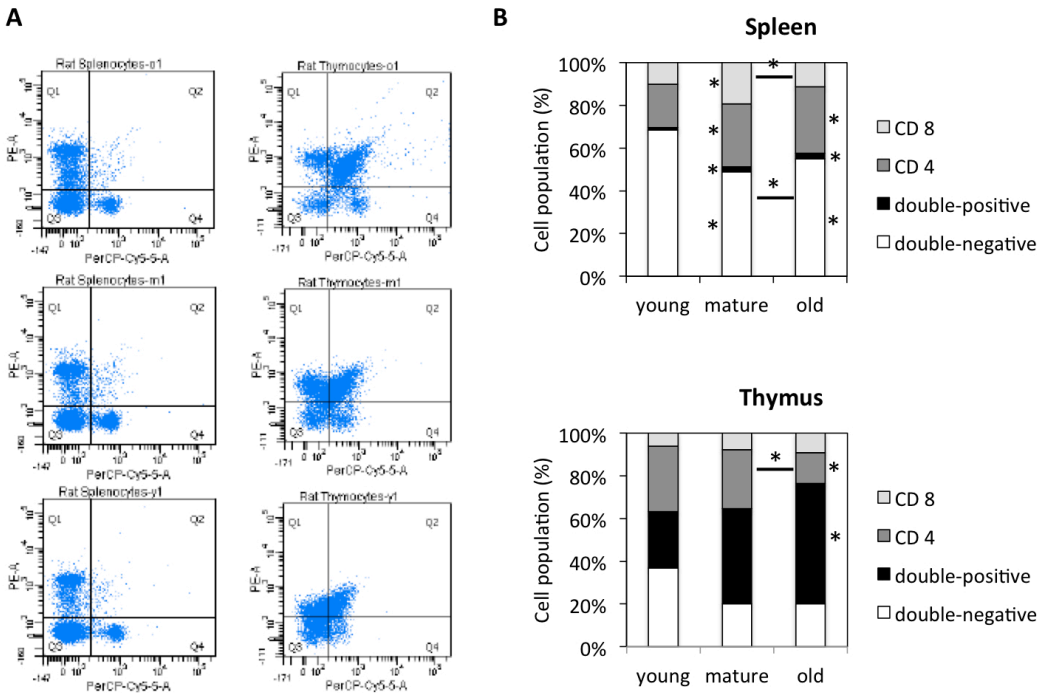

Figure 4.9: Changes in the composition of T cell populations in the spleen and thymus of aging animals.

A) Representative plots of PE-CD4 vs. PerCP-CD8A. B) The bar graphs represent the percentage of the total cell populations in four different quadrants. Each data point represents an average of six to eight samples with 10,000 events detected per animal. The asterisks next to the bars refer to significant differences to young animals, whereas the asterisks above lines indicate significant differences between mature and old animals.

4.5 DISCUSSION

Here, we show that the timing of aging in immune organs is organ-specific and is accompanied by profound changes in epigenetic and transcriptional regulation of gene expression and thus gene expression profiles.

Increased expression levels of *p16*, along with decreased expression of *Cdk2*, *Pcna* and *Rrm2* in the spleen of mature and old animals and in the thymus of old animals (though mostly not significant in the thymus) indicate the accumulation of cells in G0/G1 phase of the cell cycle in those tissues. This, together with senescence-like gene expression patterns in the same tissues, suggests that senescent cells accumulate in the spleen of mature and old animals and in the thymus of old ones. Such age-dependent accumulation of senescent cells was previously observed in other species (Herbig et al., 2006) and has been associated with aging of the immune system (Aw et al., 2008).

Further, most cyclin genes were down-regulated in tissues of old animals (including *Ccna2*, *Ccnb2*, *Ccne1* and *Ccne2*) except *Ccnd1*, which is a G1-cyclin and has also been shown to play a role in transcriptional regulation independent of CDKs (Coqueret, 2002). Therefore, the up-regulation of *Ccnd1* in old tissues may play a role in the inhibition of cell division (Casimiro et al., 2012), but this requires further study.

Thymus involution has previously been linked to the deregulation of several transcription factors – E2A, FOXN1 (Ortman et al., 2002) and NF-κB (Trebilcock and Ponnappan, 1996). Considering the high number of age-dependent gene expression changes observed, it is likely that more than one transcriptional program is affected. Therefore, bioinformatics analysis was performed to detect TFBS in the promoter regions of genes with changed expression; only those transcription factors that had the same directionality of expression changes as targeted genes were taken into

consideration. Some limitations of this study were the limited availability of promoter sequences for genes represented on the expression BeadChip and of sequence information for TFBS. Nevertheless, this study identified several transcription factors correlating with the expression of their predicted targets. For some of those transcription factors, links with senescence, aging or lifespan variations have been made: BCL6 inhibits senescence (Shvarts et al., 2002); reduced MYC expression induces senescence (Guney et al., 2006); FOXO3 (Byun et al., 2013) and NF-Y are up-regulated with age (Matuoka and Chen, 2002). Among the transcription factors identified, BCL6, MYC, TCF7 and ETS1 may be interesting candidates for further studies in terms of immunosenescence because they exhibit opposing expression patterns in the thymus and spleen. Since decreased thymic output correlates with increased peripheral T cell proliferation (Mackall et al., 1993), the function of those transcription factors may give hints about the underlying mechanisms.

In addition to being controlled by transcription factors, expression profiles are modulated through epigenetic mechanisms. The gene expression analysis revealed the down-regulation of DNA methyltransferases in the spleen of mature and old rats and in the thymus of old rats, which was confirmed at the protein level and correlated with genome hypomethylation in the thymus but not spleen of mature and old animals. A decrease in genome methylation with age has been previously described in several species (Vanyushin et al., 1973; Wilson et al., 1987). Tissue-specific differences have been observed less frequently, but another study done on rats described non-significant DNA methylation changes in adipose tissues with age (Thompson et al., 2010).

Another heterochromatin mark – H3K9me3 – has also been previously observed to be decreased in aging humans (Scaffidi and Misteli, 2006) but increased in flies

(Wood et al., 2010) and mice (Braig et al., 2005). Braig *et al*. (2005) further determined H3K9me3 as a critical mark of senescence. However, in the spleen of mature and old rats and in the thymus of old rats, *Suv39h1* – a mediator of H3K9 methylation – was down-regulated at the RNA and protein levels and correlated with decreased levels of H3K9me3 in the spleen of mature and old rats and had a trend to low levels of H3K9me3 in the thymus of old rats. Therefore, in terms of H3K9 trimethylation, aging in rats is more similar to that in humans than in mice. The reduction in DNA methylation and H3K9me3 observed with increasing age suggests the loss of heterochromatin with age. However, no increase in euchromatin marks (histone acetylation) was detected. On the contrary, H4K16 acetylation levels were significantly reduced in the thymus of old compared to young animals. This is possibly due to the deregulation of both histone acetyltransferases (NuA4 complex) and histone deacetylases. Further, H4K16 is a target for deacetylation by *Sir2* in aging yeast (Dang et al., 2009), and therefore, acetylated H4K16 accumulates in aging yeast. However, we observed an opposite trend since mammalian genomes encode seven Sirtuin genes, and only *Sirt2* was down-regulated with age, which makes it possible that other histone deacetylases are responsible for the reduced levels of H4K16ac observed.

DNA hypomethylation and reduced levels of H3K9me3 may underlie some of the gene expression changes observed, but they might also affect silencing of repetitive sequences and miRNA expression. However, no significant induction of LINE1 ORF1 expression at either RNA or protein levels could be detected in the thymus and spleen of old rats. While several studies have found age-related changes in miRNA expression (reviewed in (Grillari and Grillari-Voglauer, 2010)), we did not study changes in miRNA expression profiles in aging rats. However, the results of gene

expression analysis pointed towards changes in siRNA/miRNA processing and function with increasing age, with several genes involved in those processes being deregulated: *Adar*, *Ddx6*, *Fus*, *Piwi4l*, *Rbm3*, *Tardbp*, *Zcchc11*.

The expression of numerous DNA repair genes confirmed for *Tdg*, *Lig4* and *Msh2* decreased with increasing age, suggesting a decreased efficiency/functionality of diverse repair pathways – BER, NER, MMR, HR and NHEJ. This is supported by similar findings in various species and tissues (as reviewed by (Gorbunova et al., 2007)).

Both reduced expression of DNA repair genes and genome de-heterochromatinization, may predispose cells to genomic instability. This was observed in the thymus of mature and old animals in the form of longer Olive comet tails that indicate a higher amount of DNA strand breaks and a higher percentage of cells with γH2AX foci that indicate sites of unrepaired DNA damage. On the other hand, in spleen tissues of the same animals, opposite trends were observed which correlate with a slight increase in DNA methylation detected in those tissues.

Those molecular changes are also reflected in changing functional characteristics of spleen and thymus of older animals. An increase in the number of apoptotic and dead cells in the thymus was significant after 4 months of age. This is in line with previous reports that showed that age-related thymus involution occurs in rats between the ages of 5 and 12 months (Brelinska et al., 2008) as well as a study that demonstrated the accumulation of apoptotic cells in the epithelial compartment of the thymus (Aw et al., 2008). In the spleen, the individual differences were larger than age-related differences in the ratio of apoptotic and dead cells.

Further, changes in the composition of T cell populations, which are considered to be a major feature of immunosenescence were observed in both tissues. In the

thymus, a slight decrease in the double-negative T cell population and a significant decrease in $CD8^+$ along with a prominent peak in the double-positive T cell populations were observed, while in the spleen, there was a significant decrease in the double-negative T cell populations along with an increase in the double-positive and $CD4^+$ T cell populations. Interestingly, changes in the thymus occurred after maturity during thymic involution, while changes in the spleen occurred prior to maturity and thymic involution.

In addition to the genes and processes discussed here, a large variety of other genes were affected by expression changes, including many genes involved in cell signalling and immune function. Therefore, further study of how diverse signalling pathways are affected by expression changes may give insight into external regulation of those molecular events. Modelling of combined effects of the deregulation of several transcription factors may help in dissecting the contributions of intrinsic factors; and on an epigenetic level, ChIP studies of DNA regions enriched/depleted in DNA methylation or histone methylation marks in old versus young tissues may elucidate the role of epigenetic regulation in immunosenescence.

In summary, our study shows that profound changes in mRNA expression profiles occur in the spleen and thymus of individuals at different chronological ages. Interestingly, these changes occur earlier in the spleen than in the thymus; and in the thymus, these changes seem to go along with thymic involution. This may be caused partially by altered expression of transcription factors and altered epigenetic profiles (Fig. 4.10). Further, in the thymus, DNA and histone hypomethylation correlate with genomic instability and increased incidence of apoptosis and cell death, thus supporting the hypothesis that the molecular mechanisms underlying thymic involution may resemble those ones associated with the aging process in other organs.

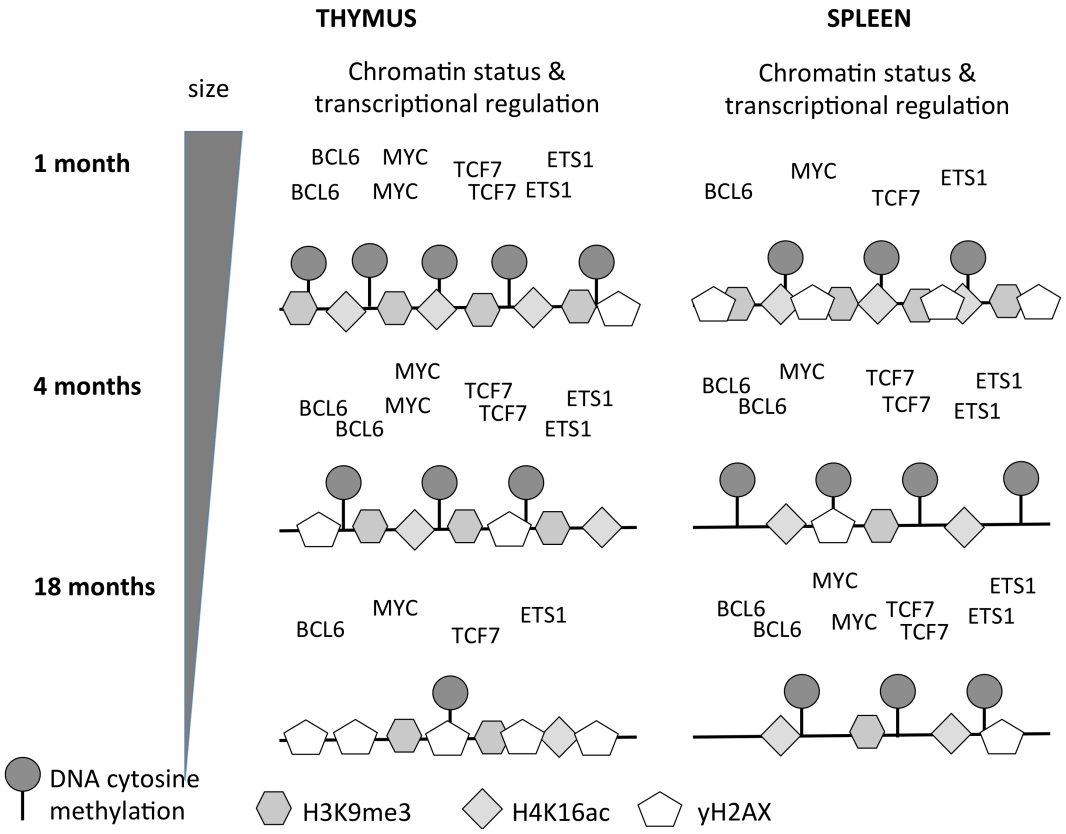

Figure 4.10: Changes in epigenetic and transcriptional regulation may underlie age-related changes in genome stability and organ function.

The black lines indicate DNA, the numbers of symbols correlate with the experimental data and are relative to the presence of the corresponding marks in young animals. Transcription factor names occur relative to expression levels and are also relative to the expression in young animals.

4.6 ACKNOWLEDGEMENTS

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5. A ROLE FOR SUV39H1-MEDIATED H3K9 TRIMETHYLATION IN THE CONTROL OF GENOME STABILITY AND SENESCENCE IN WI-38 HUMAN DIPLOID LUNG FIBROBLASTS[10]

5.1 ABSTRACT

Cellular senescence has been associated with the age-dependent decline in tissue repair and regeneration, the increasing deterioration of the immune system, and the age-dependent increase in the incidence of cancer. Here, we show that senescence of human lung fibroblast WI-38 cells is associated with extensive changes to the gene expression profile, including the differential expression of transcriptional and epigenetic regulators. Among those, *SUV39H1* was down-regulated in senescent cells, correlated with a decrease in global H3K9 trimethylation, reduced H3K9me3 levels in repetitive DNA sequence regions such as satellites and transposable elements, and increased transcription of these repetitive DNA sequences. This indicates that *SUV39H1* plays a role in limiting genomic instability in dividing cells and suggests that *SUV39H1* down-regulation may contribute to the establishment of senescence by increasing genomic instability. Additionally, the over-expression of *SUV39H1* in senescent cells induced cell division, whereas the inhibition of SUV39H1 in dividing cells slightly inhibited cell division, suggesting a causal role of *SUV39H1* in the establishment of cellular senescence. Thus, based on our findings and the results from previous reports, we propose a model in which *SUV39H1* down-regulation promotes the establishment of cellular senescence.

[10] This chapter has been published in its entirety:
Sidler, C., Woycicki, R., Li, D., Wang, B., Kovalchuk, I., and Kovalchuk, O. (2014). A role for SUV39H1-mediated H3K9 trimethylation in the control of genome stability and senescence in WI38 human diploid lung fibroblasts. Aging (Albany NY).

5.2 INTRODUCTION

Cellular senescence plays a multifaceted role in human aging and age-related pathologies; senescent cells exhibit altered secretory profiles, which may facilitate cell transformation and cancer progression (Coppe et al., 2008; Yang et al., 2006). The senescence of immune cells has been associated with the deterioration of the immune system (Effros, 2004), resulting in an increased susceptibility to infectious diseases as well as a reduced capacity to mount immune responses toward new antigens (Mackall et al., 1996) such as those associated with cancer cells or vaccinations (Goodwin et al., 2006). The senescence of stem cells limits the repair and regenerative capacity of aging tissues and organs and thus promotes age-associated functional decline (Krishnamurthy et al., 2006). On the other hand, the deregulation of senescence-associated genes is sufficient to induce the malignant transformation of human diploid cells (Hahn et al., 1999). Thus, a molecular understanding of the underlying mechanisms of senescence allows the development of strategies to prolong the human healthy lifespan and may point towards new potential drug targets for the treatment of cancer.

The study of senescence often employs human diploid fibroblasts, which exhibit a limited *in vitro* replicative lifespan of approximately 50 ± 10 population doublings (PD), after which the cell cultures progressively decline (Hayflick, 1965; Hayflick and Moorhead, 1961). Senescent cells have been shown, *in vivo*, to accumulate in tissues with increasing age (Dimri et al., 1995; Herbig et al., 2006) and the *in vitro* lifespan of primary cell cultures has been correlated with the age and maximum lifespan of the donor species (Martin et al., 1970; Rohme, 1981); thus, human diploid

fibroblasts have become a widely used model for age-related molecular and physiological changes.

There are numerous, not necessarily mutually exclusive, theories on what may be the driving cause of senescence, namely telomere shortening (Harley et al., 1990; Lundblad and Szostak, 1989) resulting in the exposure of the chromosome ends and in the permanent activation of a DNA damage signal leading to senescence (Sfeir and de Lange, 2012; Vaziri and Benchimol, 1996); changes in DNA repair efficiency and fidelity that result in the accumulation of unrepaired DNA damage and, thus, in DNA damage signalling-induced senescence (Sedelnikova et al., 2004; Seluanov et al., 2004); and oxidative stress associated with an increase in oxidative damage to DNA and other macromolecules of the cell (Sohal and Weindruch, 1996). While these different types of cellular stresses are well accepted for their roles in the establishment of senescence, the regulatory pathways that mediate it are not as well understood.

Several pathways have been implicated in the induction of senescence, including p53 activation (Vaziri and Benchimol, 1996) linking DNA damage response to the G1 cell cycle arrest, the transcriptional repression of E2F target genes through RB/E2F-directed targeting of heterochromatin formation (Narita et al., 2003), and the p38 MAPK-mediated increase in Lamin B1 expression resulting in changes to the nuclear shape and in senescence in response to oxidative stress (Barascu et al., 2012), among others. Further, changes in chromatin structure have been increasingly considered for their function in aging and senescence (as reviewed in (Feser and Tyler, 2011; O'Sullivan and Karlseder, 2012; Sedivy et al., 2008)). The decreased expression of histone genes and the reduction of heterochromatin marks, such as DNA methylation, and repressive histone marks, such as H3K9me3, H3K27me3, and H4K20me3, during senescence and aging suggest that aging is associated with the loss of

heterochromatin. This may ultimately result in changes in gene expression and genome integrity.

Here, we show that senescence in WI-38 human diploid lung fibroblasts is associated with extensive changes to the gene expression profile, including down-regulation of cell cycle genes and of genes with functions in transcriptional or epigenetic regulation. This included the reduced expression of *SUV39H1* and corresponding reduction in H3K9 trimethylation. Both the over-expression of *SUV39H1* and the inhibition of SUV39H1 interfered with the cell cycle distribution, suggesting a role of SUV39H1 in the control of senescence, which may be mediated through increased genomic instability and/or through the modification of the gene expression profile.

5.3 MATERIALS AND METHODS

5.3.1 Cell culture

Normal human foetal lung fibroblasts (WI-38) were obtained from the American Type Culture Collection (ATCC, CCL-75TM). The cell cultures were maintained in HyClone® minimum essential medium (MEM) Alpha Modification (Thermo Scientific, Waltham, MA, USA) containing 10% (v/v) foetal bovine serum (FBS) (Gibco/Invitrogen, Burlington, ON, Canada) in a humidified Forma Steri-Cycle CO_2 Incubator (Thermo Scientific) at 37°C and 6% CO_2. The cells were subcultured approximately every three days and the population doubling (PD) numbers were determined for each subculture as $\Delta PD = \log_2(n_f/n_i)$, where n_f is the final number of cells in a culture and n_i is the number of cells initially inoculated. The sum of all ΔPD up until a given subculture yielded the PD level of the given passage.

5.3.2 Senescence-associated β-galactosidase (SA-β-GAL) assay

Flow cytometry-based assay

A fluorescent SA-β-GAL staining assay was employed to quantify senescent cells by flow cytometry. This was performed according to a previous protocol involving the incubation of cells with 5-dodecanylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG, Sigma‑Aldrich Canada Ltd., Oakville, ON, Canada), a fluorogenic substrate for β-galactosidase, at standard culture conditions for 1.5 h (Debacq-Chainiaux et al., 2009). Following the staining procedure, cells were washed twice in Dulbecco's phosphate buffered saline (DPBS) and harvested by trypsinization. After centrifugation for 5 min at 200 *g* and 4°C, the cells were resuspended in 1 mL DPBS and analyzed on the BD FACS Canto II (BD Biosciences, Franklin Lakes, NJ). Three samples of each PD level were analyzed by detecting 10,000 events per sample.

Microscopy-based assay

For the microscopy-based analysis of SA-β-GAL activity, cells were fixed and stained using the SA-β-GAL Staining Kit (Cell Signaling, Bedford, MA). Images were taken using a Zeiss Observer Z1 epifluorescence microscope with AxioVision Rel 4.8 software.

5.3.3 Determination of cell cycle distribution of cell cultures

Cells from two 10-cm dishes were harvested for each sample, and three samples were collected for each PD level. Cells were harvested by trypsinization, centrifuged for 5 min at 200 *g* and 4°C, and resuspended in 1 mL PBS. The cells were fixed in 63% ethanol (final concentration) for 48 h at –20°C, washed in PBS, resuspended in staining solution (0.1% Triton-X-100, 20 μg/mL propidium iodide, and 20 μg/mL

ribonuclease A in PBS), and incubated for 15 min at 37°C. Samples were then analyzed on a BD FACS Canto II (BD Biosciences) and 10,000 events were detected per sample.

5.3.4 Gene expression profiling

For the determination of gene expression profiles, total RNA was extracted from three samples per PD level and cells from two 10-cm cell culture plates per sample. The RNA extraction was performed using TRIzol® Reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The RNA was quantified by NanoDrop2000c (Thermo Scientific) and the RNA Integrity was determined using 2100 BioAnalyzer (Agilent, Santa Clara, CA). Gene expression profiles were determined using Illumina® HumanHT12-v4 Gene Expression BeadChips according to the manufacturer's protocol. Differential expression analyses were performed using the Illumina® GenomeStudio software using an Illumina-custom model with an FDR of 0.05 applied; PD 38 was used as a reference. Only genes for which the differential expression analysis was significant based on $p < 0.05$ and the log2 fold change was smaller than –0.4 or larger than 0.4, were considered for further analysis.

5.3.5 Bioinformatics analyses

Sample clustering

Sample clustering was performed using the Illumina® GenomeStudio software.

Functional classification

Functional classification of genes was performed using FunNet Transcriptional Networks Analysis[11], g:Profiler (Reimand et al., 2011; Reimand et al., 2007), RENATO (Bleda et al., 2012), and DAVID Bioinformatics Resources 6.7 (Huang da et al., 2009a, b) software and compared with information on the genes available from the Genecards database[12] (Safran et al., 2010).

Transcription factor analysis

Promoter sequences of 1,000 bases upstream of the transcription start sites of the RefSeq genes from the RefSeq release 37 (November 2009, GRCh37/hg19) of the human genome assembly were downloaded from the UCSC Genome Bioinformatics website[13] (Dreszer et al., 2012; Flicek et al., 2010). The sequences of the transcription factor binding sites (TFBS) of transcription factors affected by differential expression were retrieved from Genecards[9] (Safran et al., 2010) or Bio-Base[14]. The promoter sequences were then searched for occurrences of TFBS using the PatMatch v1.2 program[15] (Yan et al., 2005) downloaded from the TAIR website (Lamesch et al., 2012) without allowing mismatches.

Only promoters which contained a higher or lower than the mean ± standard deviation (SD) number of occurrences of a specific TFBS when compared to all the promoters of all RefSeq genes were considered for further analysis. Additionally, a correlation analysis was performed to determine genes for which the change in gene expression correlated with the change in transcription factor expression. In order to

[11] www.funnet.info
[12] www.genecards.org
[13] http://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/upstream1000.fa.gz
[14] https://portal.biobase-international.com
[15] ftp://ftp.arabidopsis.org/home/tair/Software/Patmatch/patmatch_1.2.tar.gz

determine whether a given transcription factor may play an important role in senescence-dependent changes in gene expression, the enrichment of its TFBS within the differentially expressed genes was calculated in comparison to the occurrence of its TFBS in all promoter sequences.

5.3.6 Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed to confirm the results of gene expression profiling. In order to remove gDNA contamination, RNA samples were cleaned using the illustra RNAspin Mini Prep kit (GE Healthcare, Arlington Heights, IL); 500 ng RNA were used for cDNA synthesis using the iScript™ Select cDNA synthesis kit (BioRad, Hercules, CA). qRT-PCR reactions were set up using the SsoFast™ EvaGreen® Supermix (BioRad) and primers specific for target sequences of interest (Table 5.1). The reactions were analyzed on a C1000™ Thermo Cycler equipped with a CFX96™ Real-Time System (BioRad). The PCR programs were run according to the SSoFast™ guidelines with annealing temperatures as specified for the specific primer pairs (Table 5.1).

Each experiment included three biological replicates for each PD level and two technical replicates per sample. *HPRT1*, *RPL13A*, and *YWHAZ* were used for normalization, and the analysis of the qRT-PCR Ct results was carried out using qbase^{PLUS} (Vandesompele et al., 2002).

Table 5.1: Primers for qRT-PCR-based analysis of transcript levels.

Gene	Fwd/ Rev	Sequence	Annealing Temp	Reference
HPRT1	Fwd Rev	5'-TGACACTGGCAAAACAATGCA-3' 5'-GGTCCTTTTCACCAGCAAGCT-3'	59.5°C	(Vandesompele et al., 2002)
YWHAZ	Fwd Rev	5'-ACTTTTGGTACATTGTGGCTTCAA-3' 5'-CCGCCAGGACAAACCAGTAT-3'	59.5°C	(Vandesompele et

				al., 2002)
RPL13A	Fwd Rev	5'-CCTGGAGGAGAAGAGGAAAGAGA-3' 5'-TTGAGGACCTCTGTGTATTTGTCAA-3'	59.5°C	(Vandesompele et al., 2002)
COL3A1	Fwd Rev	5'-CGATGAGATTATGACTTC-3' 5'-ATTACAGAATACCTTGATAG-3'	53.0°C	
UBE2C	Fwd Rev	5'-ACATATGCCTGGACATCCTGA-3' 5'-GGTTCTCCTAGAAGGCTCTGG-3'	59.5°C	(Takahashi et al., 2006)
POT1	Fwd Rev	5'-GGGCAAAGCAGAAGTGGACGGAGCATC-3' 5'-ATTGACAGATAACATCTGAATGCTGATTGGCTGTC -3'	59.5°C	(Baumann et al., 2002)
FOXM1	Fwd Rev	5'-ACTTTAAGCACATTGCCAAGC-3' 5'-CGTGCAGGGAAAGGTTGT-3'	55.9°C	(Gemenetzidis et al., 2009)
E2F2	Fwd Rev	5'-CCAAGAATTACATCAGAGAA-3' 5'-GCTTACATTCCAGACTTC-3'	55.9°C	
SUV39H1	Fwd Rev	5'-CTACTATGGCAACATCTC-3' 5'-GTCAAGGTTGTCTATGAA-3'	55.9°C	
SAT2	Fwd Rev	5'-CATCGAATGGAAATGAAAGGAGTC-3' 5'-ACCATTGGATGATTGCAGTCAA-3'	59.5°C	(Wang et al., 2013)
majSAT	Fwd Rev	5'-GACGACTTGAAAAATGACGAAATC-3' 5'-CATATTCCAGGTCCTTCAGTGTGC-3'	55.9°C	(Wang et al., 2013)
alphaSAT	Fwd Rev	5'-CTGCACTACCTGAAGAGGAC-3' 5'-GATGGTTCAACACTCTTACA-3'	55.9°C	(Wang et al., 2013)

5.3.7 Western immunoblotting

For protein isolation, cells from four 10-cm cell culture plates were sonicated in 100 µL cold 1% sodium dodecyl sulphate (SDS) containing protease inhibitor (Roche, Basel, Switzerland). Protein concentrations were determined by the Bradford assay (BioRad) and by measuring the absorbance at 595 nm using NanoDrop 2000c (Thermo Scientific). Equal amounts of protein per lane (10–40 µg depending on the protein of interest) were separated by SDS-PAGE in slab gels of 10% to 15% polyacrylamide and transferred to Amersham Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Baie d'Urfé, Québec). The membranes were incubated with primary antibodies overnight at 4°C (Table 5.2). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies followed by ECL Plus immunoblotting detection system

(Amersham Biosciences). Chemiluminescence was detected using a FluorChem™ HD2 camera with the FluorChem™ software (Cell Biosciences, Santa Clara, CA). The PVDF membranes were stained with Coomassie blue G250 (BioRad) to confirm equal protein loading. The signals were quantified using NIH Image J64 software and normalized relative to GAPDH.

Table 5.2: Antibodies used for Western blots.

Target	Supplier, Cat No	Dilution
Mouse anti-SUV39H1	Abcam, ab12405	1:500 in 5% milk (PBST)
Rabbit anti-H3K9me3	Abcam, ab8898	1:500 in 5% milk (PBST)
Rabbit anti-H3K9ac	Abcam, ab10812	1:500 in 5% milk (PBST)
Rabbit anti-H3	Cell Signaling, 9715	1:1,000 in 5% milk (PBST)
Rabbit anti-CHK2	Abcam, ab8108	1:250 in 5% milk (PBST)
Rabbit anti-pT68CHK2	Abcam, ab3501	1:500 in 5% milk (PBST)
Mouse anti-CHK1	Cell Signaling, 2360	1:1,000 in 5% BSA (PBST)
Rabbit anti-pS345CHK1	Cell Signaling, 2341	1:1,000 in 5% milk (PBST)
Rabbit anti-p21	Abcam, ab7960	1:1,000 in 5% milk (PBST)
Mouse anti-GAPDH	Santa Cruz, sc47724	1:1,000 in 5% milk (PBST)
Mouse anti-ACTIN	Abcam, ab3280	1:1,000 in 5% BSA (PBST)
Secondary Antibodies		
Donkey anti-Rabbit	Santa Cruz, sc2313	1:10,000 in 5% milk (PBST)
Goat anti-Mouse	Santa Cruz, sc2005	1:5,000 in 5% milk (PBST)

5.3.8 MTT cytotoxicity assay

In order to determine the cell viability following treatment with various concentrations of chaetocin (Sigma), as indicated, approximately 5,000 cells were plated per well in a 96-well plate. Various concentrations of chaetocin, or DMSO as a control, were added to the cultures 24 h after plating. The cell viability was determined using the Cell Proliferation Kit I (Roche) according to the manufacturer's manual 48 h or 96 h after the addition of chaetocin.

5.3.9 SUV39H1 inhibition and over-expression

In order to inhibit SUV39H1, cells were treated with a 10 nM final concentration of chaetocin, which is a specific inhibitor of SUV39H1 (Greiner et al., 2005). For the

over-expression of SUV39H1, cells were transfected with the pCMV6-SUV39H1 expression plasmid (Origene, Rockville, MD) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's manual. The cells were harvested for RNA extraction or cell cycle analysis 48 h after drug treatment or transfection, as described above.

5.3.10 Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Nelson et al., 2006). Briefly, cross-linking was performed by treating cells with 1% formaldehyde for 15 min and quenching with 125 mM glycine for 5 min. The cells were sonicated in 500 µL immunoprecipitation (IP) buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1% Triton-X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin, proteinase inhibitor (Roche)) per 12 to 14 million cells in order to shear the DNA to fragments of 100 to 1,000 bp. Immunoprecipitation was performed by using antibodies targeted to rabbit anti-H3K9me3 (Abcam) or mouse anti-SUV39H1 (Abcam), or rabbit or mouse IgG as a negative control. Following immunoprecipitation, DNA fragments were isolated using 10% Chelex®100 (BioRad) and purified using a QIAquick® PCR purification kit (Qiagen).

ChIP-qRT-PCR was performed the same way as described above using the primers listed in Table 5.3.

Table 5.3: Primers used for ChIP-qRT-PCR.

Target	Fwd/ Rev	Sequence	Annealing Temp	Reference
IL6	Fwd Rev	5'-CTTCGTGCATGACTTCAGCTTT-3' 5'-CGTCCTTTAGCATCGCAAGAC-3'	62.5°C	
GAPDH	Fwd Rev	5'-TACTAGCGGTTTTACGGGCG-3' 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'	62.5°C	(Kawamoto et al., 2008)

ALU1	Fwd Rev	5'-ACGAGGTCAGGAGATCGAGA-3' 5'-CTCAGCCTCCCAAGTAGCTG-3'	51°C	(Zheng et al., 2014)
LINE1	Fwd Rev	5'-CAGAATCTCTGGGACGCATT-3' 5'-ATTGTGATGTTCGGGTGTCA-3'	64.1°C	(Zheng et al., 2014)
αSAT D5Z1	Fwd Rev	5'-TAGACAGAAATATTCTCACAATCGT-3' 5'-GCCCTCAAAGCGCTCCAAG-3'	62.5°C	(Zheng et al., 2014)
αSAT D5Z2	Fwd Rev	5'-TTTTTGTGCAATTGGCAAATGGAG-3' 5'-AGACTGTTTCCTCACTGCTCT-3'	62.5°C	(Zheng et al., 2014)

5.3.11 Statistical analysis

All experiments included three biological replications and statistically significant differences were determined by pairwise, two-tailed Student's t-test ($p < 0.05$).

5.4 RESULTS

5.4.1 Setup of the senescence model system WI-38

WI-38 human foetal lung fibroblasts have a limited *in vitro* lifespan of approximately 50 ± 10 PD (Hayflick, 1965; Hayflick and Moorhead, 1961). A cell stock obtained from ATCC at 19 PD was subcultured to reach three different passages (P10, P15 and P24). In order to determine the senescence status of those cells, we monitored their PD levels, their senescence ratios as determined by a flow cytometry-based SA-β-GAL assay which is commonly used as a biomarker for senescence (Dimri et al., 1995), and their cell cycle distribution, as senescence is characterized by the permanent cell cycle arrest in the G1/G0 phase of the cell cycle as determined by the DNA amount per cell (Sherwood et al., 1988).

Along with the increasing PD level of the cell cultures, the P15 and P24 cultures contained a significantly higher ratio of senescent cells than the P10 cultures (Table 5.4). On the other hand, the cell cycle analysis revealed an initial increase in the number of cells in the G0/G1 phase along with a decrease in cells in the S or G2/M phases of the cell cycle, when comparing P15 to P10 cells (Table 5.4). When

comparing P24 cells to P10 or P15 cells, a further decrease in S phase cells was observed, paralleled by a decrease in G1/G0 and an increase in G2/M cells (Table 5.4).

However, as the determination of the cell cycle profile relies on the detection of the amount of DNA – half the amount of DNA in G1 when compared to G2 phase – it does not allow a distinction between tetraploid cells and G2 cells. Such an accumulation of large cells with altered ploidy levels as cultures became senescent has been described (Sherwood et al., 1988), and therefore the most accurate measure to compare the level of cell division in the different cell cultures may be given by the comparison of the number of cells in the S phase at each PD level.

Based on the significant increase in the senescence ratio of the P15 and P24 cultures along with the reduced ratio of cells in the S phase, these three cultures were chosen for all further experiments and will be referred to by their PD numbers (P10 as PD 38, P15 as PD 47, and P24 as PD 54).

Table 5.4: Senescence properties of cell cultures.

Population doubling (PD). Numbers in the G0/G1, S, and G2/M columns represent percentages of cells in the given phase of the cell cycle and are averages of three samples with 10,000 events detected per sample ± standard deviation. SA-β-GAL pos (%) shows the percentage of SA-β-GAL positive cells shown as average ± standard deviation of three samples with 10,000 events detected per sample. Asterisks indicate significance based on Student's t-test ($p < 0.05$).

Passage	PD	G0/G1	S	G2/M	SA-β-GAL pos (%)
P10	38.3 ± 0.6	73.6 ± 0.4	5.8 ± 0.7	20.7 ± 0.7	56.4 ± 7.1
P15	46.8 ± 0.3 *	77.6 ± 1.1 *	4.1 ± 0.4 *	18.4 ± 0.7 *	72.7 ± 4.5 *
P24	54.2 ± 0.1 *	71.9 ± 0.2 *	3.1 ± 0.1 *	25 ± 0.3 *	74.5 ± 1.8 *

5.4.2 Senescence-related changes to gene expression profiles are accompanied by changes in expression of transcriptional and epigenetic regulators

To obtain an understanding of what molecular pathways may be involved in the establishment of senescence or may be regulated during senescence, the gene expression profiles of the three different cultures (PD 38, PD 47 and PD 54) were determined using Illumina® Gene Expression BeadChips (S1[16]). Forty genes were differentially expressed when comparing PD 38 and PD 47 cultures, whereas 1,137 genes were differentially expressed between PD 38 and PD 54 (Fig. 5.1A). Of those genes, the majority were up-regulated when comparing PD 47 to PD 38, whereas the majority were down-regulated when comparing PD 54 to PD 38 (Fig. 5.1B). To understand the functional implications that these gene expression changes may have, they were classified by function (Fig. 5.1C, S2[16]). Gene expression changes that occurred between PD 38 and PD 47 mainly affected apoptosis, but also transcriptional and epigenetic regulation. Changes that occurred between PD 38 and PD 54 mainly affected cell cycle regulation, but also DNA repair, apoptosis, and transcriptional and epigenetic regulation. Further, 80% of the genes that were differentially regulated between PD 38 and PD 47 were also differentially regulated between PD 38 and PD 54, which may indicate that these genes are involved early in the establishment of senescence and mainly play roles in cell signalling, apoptosis, and the modulation of the cytoskeleton and extracellular matrix (Fig. 5.1D). However, a few of the commonly deregulated genes are also involved in transcriptional or epigenetic regulation. This may indicate that changes in cellular signal transduction, as well as

[16] http://www.impactaging.com/papers/v6/n7/full/100678.html

changes in transcriptional regulation and in chromatin structure, may be involved during the onset of cellular senescence.

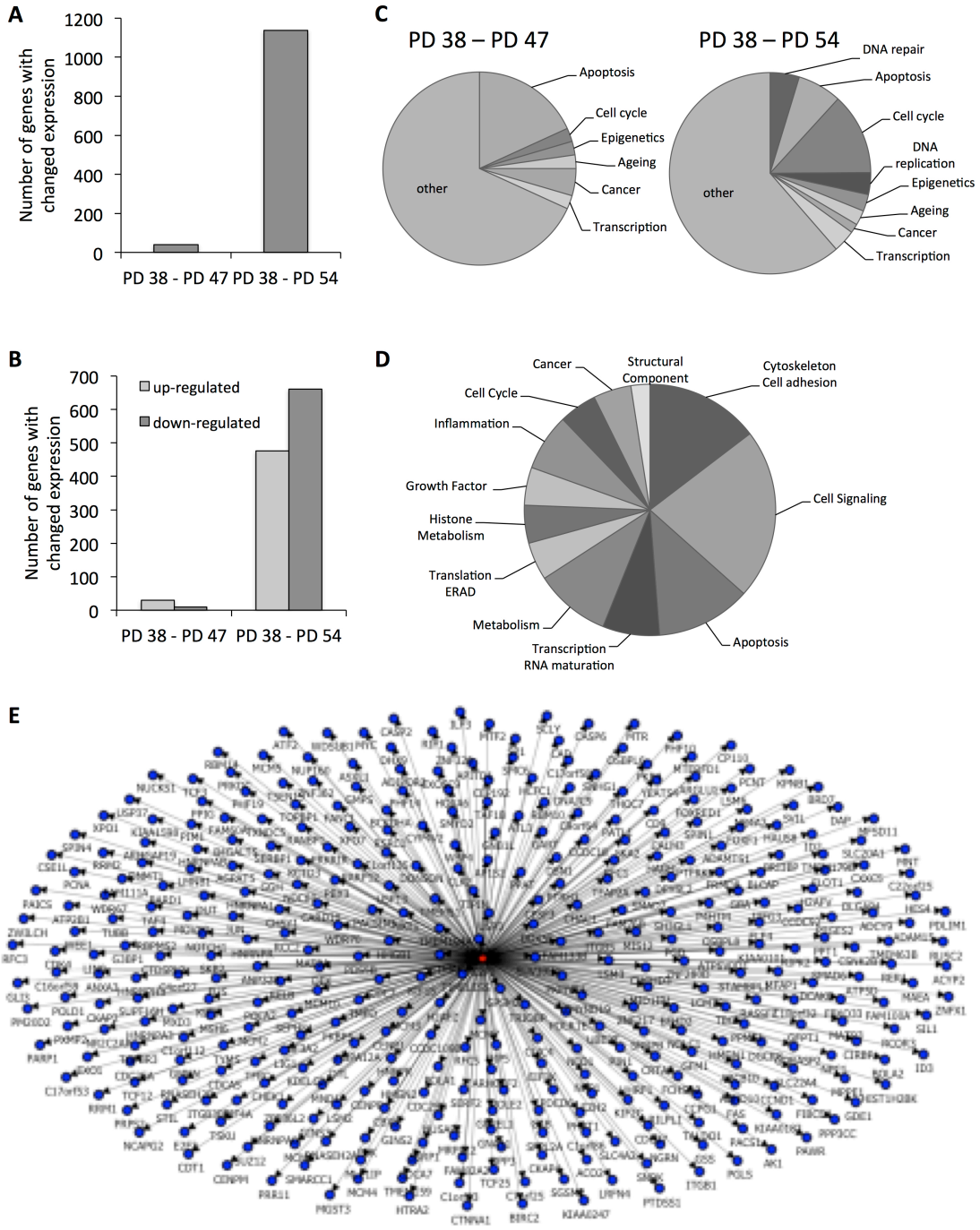

Figure 5.1: Functional classification of the gene expression results.

A) Bar graph representing the total number of genes affected by expression changes when comparing PD 47 or PD 54 to PD 38 cells. B) Bar graph showing the number of

genes with changed expression that were up- (light grey) or down-regulated (dark grey). C) Pie charts showing the functional classification of differentially expressed genes when comparing PD 54 or PD 47 cultures to PD 38 cultures. D) Pie chart showing the functional classification of genes that were differentially expressed between PD 38 and PD 47 as well as between PD 38 and PD 54 cultures. E) Regulatory network enriched in the PD 54–PD 38 dataset based on the analysis using RENATO software. E2F1 (red circle in the center) potentially regulates the expression of numerous genes with altered expression in the dataset (blue circles). The assortment of target genes is random and distances to the center do not signify any biological meaning.

In addition to the cell cycle arrest of senescent cells in the G0/G1 phase (Sherwood et al., 1988), changes in gene expression patterns have been observed in various aging model systems (de Magalhaes et al., 2009). Therefore, a differential expression of cell cycle regulators as well as senescence-associated genes would be expected in the cultures with higher PD levels. In order to test this, genes that were functionally classified to the cell cycle or aging categories were analyzed in more detail.

This showed differential expression of increasing fractions of genes involved in cell cycle regulation with increasing PD level (Fig. 5.1C,D); these genes were involved in the regulation of all stages of the cell cycle (Table 5.5). The largest group of genes was involved in the regulation of the progression of mitosis and these genes were mostly down-regulated, indicating that mitosis occurred at a lower frequency in PD 54 than in PD 38 cultures. Further, most of the differentially expressed genes involved in DNA replication and all of the genes involved in regulation of S phase progression were down-regulated, suggesting that DNA synthesis occurred at a lower frequency in

the older cultures as well. Taken together, the observed gene expression changes indicate that high numbers of cells in PD 54 when compared to PD 38 are either in the G1/G0 or G2 phase of the cell cycle, which is in line with the results of the cell cycle profile analysis (Table 5.4).

In addition, an extensive regulatory network with E2F1 at its center was detected in the list of differentially expressed genes between PD 38 and PD 54 (Fig. 5.1E). E2F1 is a transcriptional activator involved in the regulation of G1/S progression and DNA replication (Wu et al., 2001). Further, E2F1, E2F2, and E2F3 have been shown to interact with retinoblastoma (RB) protein during cell differentiation, resulting in the silencing of E2F target genes (Chong et al., 2009). In addition to E2F1, both E2F2 and E2F3 have been shown to modulate the expression of E2F target genes (Wu et al., 2001), but have also been demonstrated to play at least partially distinct roles (DeGregori et al., 1997). Therefore, the observed transcriptional repression of *E2F2* in the senescent cultures (Fig. 5.2B) may induce a shift in the regulation of E2F target genes and, at least in part, explain the differential expression of the genes in the regulatory network of E2F1 (Fig. 5.1E).

Further, age-related gene expression changes were observed in both PD 47 and PD 54 cultures compared to PD 38 cultures (Fig. 5.1C). These gene expression changes were compared to microarray information from the Human Ageing Genomic Resources and to the predicted functions of genes in aging or senescence (Table 5.6). Eleven out of the 16 genes presented have been shown to be affected by expression changes corresponding to previously described age-related changes in expression (de Magalhaes et al., 2009). The expression levels of several of these genes were confirmed at the transcript level by qRT-PCR (Fig. 5.2A,B).

Table 5.5: Changes in the expression of cell cycle regulators.

Numbers represent up- or down-regulated genes when compared to the total number of genes with expression changes in the specified sub-category. Arrows next to category title indicate whether the numbers reflect fractions of up- or down-regulated genes. Only genes with defined roles within cell cycle regulation were considered for this analysis.

Sub-category	PD 47	PD 54
Cell cycle progression		
Inhibitors ↑	0	4 / 10
Activators ↓	0	6 / 10
G1 progression		
Inhibitors ↑	0	3 / 11
Activators ↓	0	13 / 15
S phase progression ↓	0	12 / 12
DNA replication ↓	0	46 / 47
G2 progression ↓	0	9 / 11
Mitosis ↓	0	81 / 87

Table 5.6: Senescence-associated gene expression.

Entries of up-regulated or down-regulated genes with age are based on microarray signatures from the Human Ageing Genomic Resources (de Magalhaes et al., 2009). Other gene information is based on Genecards[17] (Safran et al., 2010). Numbers indicate log2 fold changes in transcript levels when comparing either PD 47 or PD 54 to PD 38 cells.

Gene	Involvement in aging	PD 47	PD 54
ANXA3	Up-regulated with age		-0.46
C5ORF13	Down-regulated with age		-0.62
CDKN1B	Up-regulated with age		-0.57
COL1A1	Down-regulated with age		-0.46
COL3A1	Down-regulated with age		–1.05
DKC1	May regulate telomerase activity		–0.45
GBP2	Up-regulated with age		–0.75
HIST1H1C	Up-regulated with age		0.58
ID2	Down-regulated with senescence	0.60	0.79

[17] www.genecards.org

MNT	May induce senescence	0.46
PRKDC	Telomere maintenance	–0.41
RAD54L	Telomere maintenance	–0.65
SUV39H1	H3K9methylation dependent induction of senescence	–0.75
TFRC	Down-regulated with age	–0.51
TIMP3	May be involved in senescence	0.95
TMED10	Up-regulated with age	0.58
TXNIP	Up-regulated with age	1.52

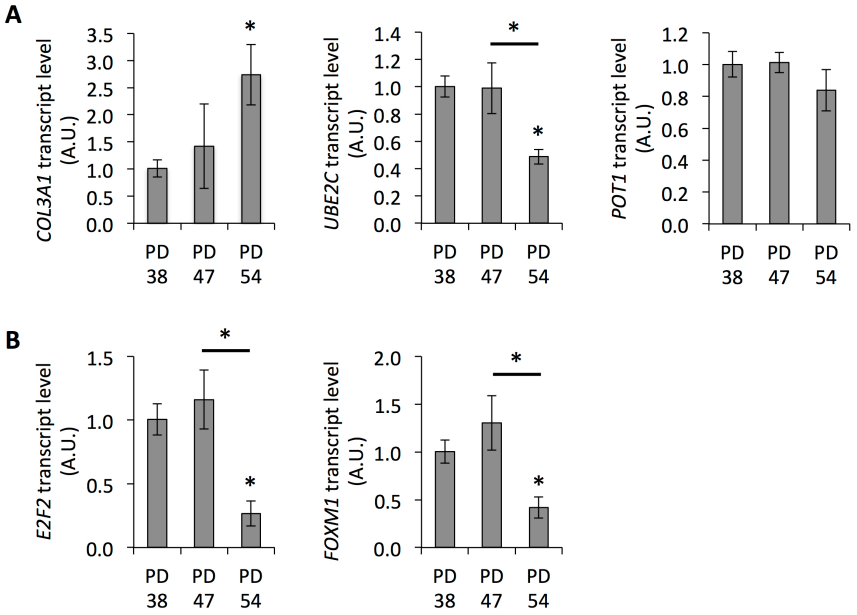

Figure 5.2: Senescence-associated gene expression.

A) Transcript levels of *COL3A1*, *POT1*, and *UBE2C* as measured by qRT-PCR. Data are presented as averages of three biological and two technical replicates. Error bars are derived from error progression of the standard deviations of the technical repeats. Significance is based on Student's t-test ($p < 0.05$). B) Transcript levels of proliferation-related transcription factors *E2F2* and *FOXM1* as determined by qRT-PCR. Bars represent averages of three biological and two technical repeats ± error bars derived from error progression of the standard deviations of the technical repeats. Significance is based on Student's t-test ($p < 0.05$).

Several cellular pathways that are commonly affected by gene expression changes in aging model systems have been previously described, namely pRB/p53 signalling, cytoskeleton, interferon-related, insulin growth factor-related, MAPK signalling, and oxidative stress pathways (Fridman and Tainsky, 2008). Our results are consistent with these observations, in that 12 out of 64 genes involved in p53 signalling and 21 out of 201 genes involved in focal adhesions were deregulated when comparing PD 38 and PD 54 cultures. These numbers are based on the functional classification according to KEGG pathways using DAVID software.

In summary, a large number of gene expression changes were observed when comparing PD 54 to PD 38 cultures. Among the genes that were differentially expressed in PD 54 cultures were numerous cell cycle regulators as well as genes with previously determined age-related expression changes. More detailed functional analysis of the cell cycle regulators and age-related gene expression changes supports the assumption that cultures with higher PD levels contain higher numbers of senescent cells, which is in line with the results of the SA-β-GAL assay (Table 5.4). Thus, since the changes in gene expression profiles when comparing PD 38 and PD 54 cells reflected senescence-dependent changes, the subsequent step involved the investigation of the changes in the expression of transcriptional and epigenetic regulators in order to determine potential regulatory mechanisms underlying the senescence-associated changes in gene expression profiles.

5.4.3 A potential role of transcription factors in the establishment of senescence

The gene expression changes between PD 38 and PD 54 were extensive and included numerous transcription factors; further, a large regulatory network surrounding E2F1 could be detected in the dataset (Fig. 5.1E). Thus, it can be hypothesized that the deregulated transcription factors may play a role in modulating the expression profiles in senescing cells. To test this, the promoter regions of the genes that were differentially expressed were searched for TFBS recognized by the differentially expressed transcription factors. For this, only transcription factors with binding sites that had less than 1,000 possible variants of their cognate binding motif were considered. Transcription factors were ranked according to how many potential target genes their expression levels correlated with and the overall enrichment of their TFBS within promoters of differentially expressed genes when compared to all promoters was calculated. None of the TFBS of the differentially expressed transcription factors were overrepresented within the promoters of the differentially expressed genes (data not shown).

5.4.4 Senescence correlates with reduced SUV39H1 expression and H3K9 trimethylation

The results of the differential gene expression analysis further showed that, when comparing PD 47 or PD 54 to PD 38 cultures, 2.3% and 2.7%, respectively, of the affected genes were involved in epigenetic regulation (Fig. 5.1C). Among these, *SUV39H1*, a histone methyltransferase that specifically methylates H3K9, was down-regulated in senescent cells (PD 54), as confirmed at the protein level by Western blot analysis (Fig. 5.3). This down-regulation was correlated with significantly reduced H3K9 trimethylation and a trend towards increased H3K9 acetylation (Fig. 5.3). The

loss of H3K9me3, a heterochromatin mark, suggests a global loss of heterochromatin in senescent cells. This may affect senescence through the induction of gene expression changes or genomic instability, and may thus play a causal role in the establishment of senescence.

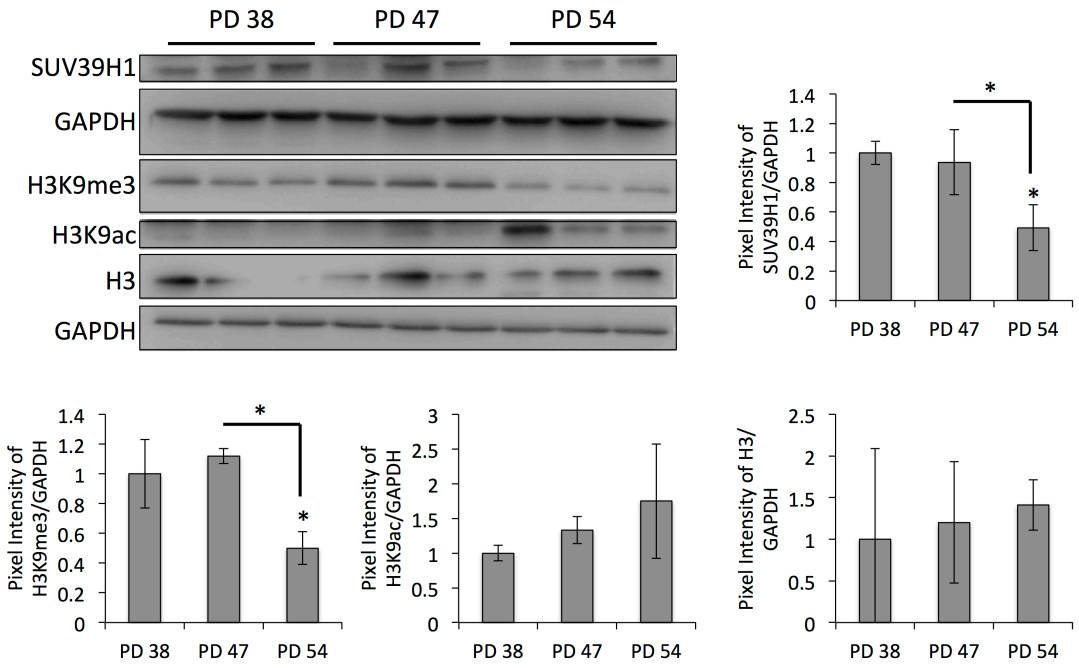

Figure 5.3: Age-dependent down-regulation of SUV39H1 correlates with a reduction in H3K9me3 levels.

Representative Western blots and bar graphs showing the signal quantification. Bars represent an average of three samples ± standard deviation. Expression levels were normalized to GAPDH expression and the expression in PD 38 cells. Significance is based on Student's t-test ($p < 0.05$).

5.4.5 Reduced levels of SUV39H1 and H3K9me3 correlate with satellite expression

Since SUV39H1-mediated H3K9 trimethylation is important for heterochromatin formation in pericentric satellite regions and, thus, for genomic stability (Lehnertz et al., 2003; Peters et al., 2001), we hypothesized that the down-regulation of *SUV39H1* and the reduction of H3K9 trimethylation levels may contribute to the senescence-dependent loss of heterochromatin, resulting in genomic instability and/or in altered gene expression profiles. In order to test the function of SUV39H1 in promoting genomic instability-induced senescence, we monitored the protein levels of DNA damage checkpoint regulators and determined the transcript levels of repetitive sequence elements that are generally silenced. The analysis of the expression levels of DNA damage checkpoint regulators showed that CHK1 and CHK2 protein levels, as well as their phosphorylated forms, were lower in PD 54 cultures when compared to PD 38 and PD 47 cultures (Fig. 5.4). This is in line with the observed down-regulation of numerous transcripts involved in cell cycle regulation in general (Table 5.5).

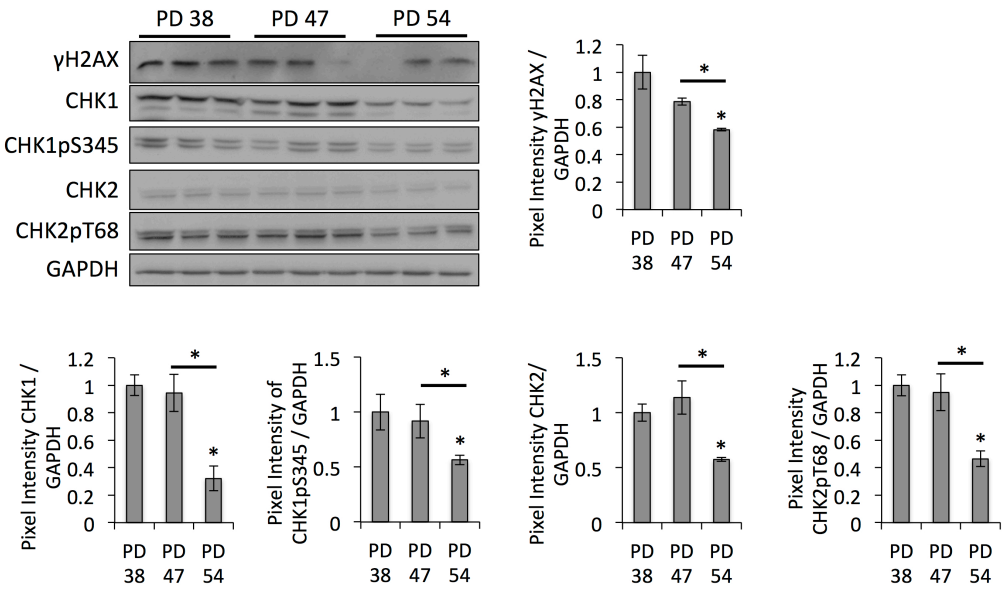

Figure 5.4: DNA damage checkpoint regulators are down-regulated in senescent cells.

Representative Western blot images and bar graphs showing the quantification of the band intensities. Bars represent averages of three biological replicates, error bars indicate standard deviations, and asterisks show statistical significance based on the Student's t-test ($p < 0.05$).

Next, we examined whether the reduced SUV39H1 expression in senescent cells was associated with heterochromatin relaxation in those regions. The analysis of the transcript levels derived from different satellite regions showed a trend towards increased expression with the rising PD level of the cell cultures (Fig. 5.5A). This further correlated with the reduced amount of trimethylated H3K9 in satellite regions and in transposon sequences (Fig. 5.5B), as determined by ChIP-qRT-PCR. Therefore, the reduced SUV39H1 expression is associated with increased genomic instability through the loss of the H3K9me3 heterochromatin mark in regions of constitutive heterochromatin.

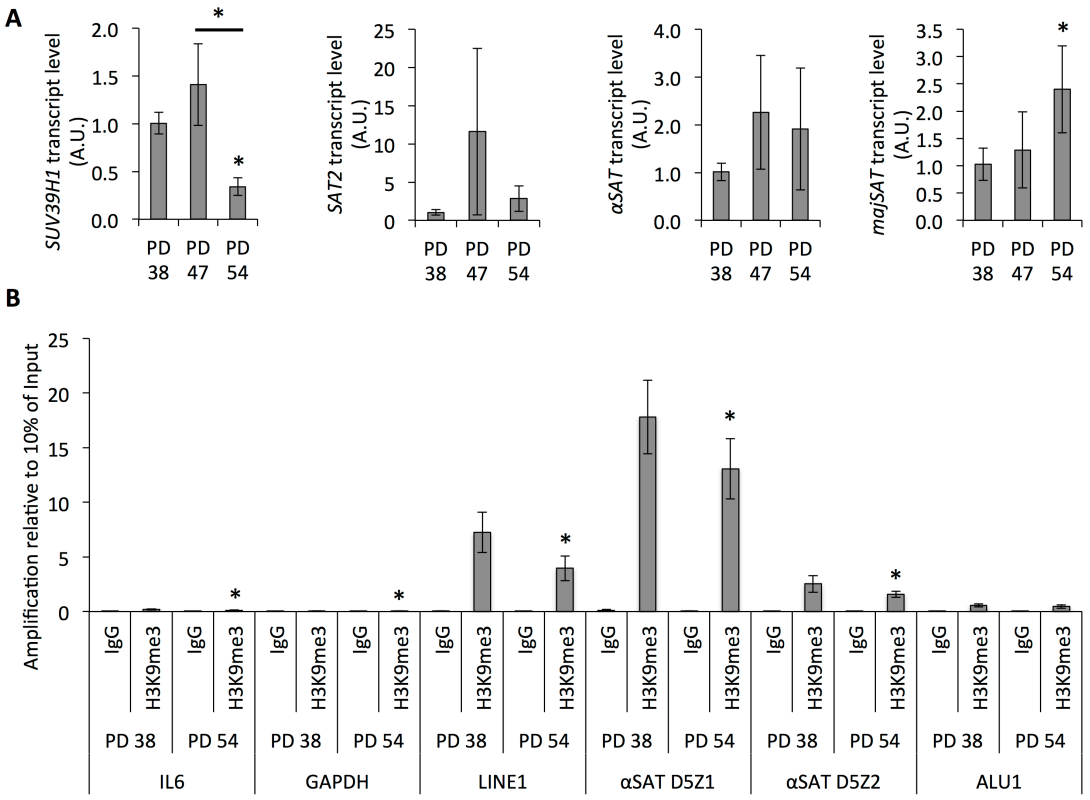

Figure 5.5: Senescence-dependent increase in satellite expression correlates with the loss of H3K9 trimethylation from those sequences.

A) Transcript levels of *SUV39H1* and satellites normalized to *HPRT1* and *YWHAZ* and to transcript levels in PD 38. Bars represent averages from three biological and two technical replications, error bars show error progression of the standard deviations, and the asterisks indicate significance based on Student's t-test ($p < 0.05$). B) ChIP-qRT-PCR analysis of H3K9me3 abundance in gene promoters and repetitive DNA sequences. Data are normalized to 10% of the amplification in chromatin input samples. Bars represent averages of three biological and two technical repetitions ± standard deviations. Asterisks indicate significance based on Student's t-test ($p < 0.05$).

5.4.6 Reduced SUV39H1 expression and H3K9me3 level during senescence may affect gene expression

In addition to protecting the genome integrity, H3K9 trimethylation is also important for the transcriptional repression of protein-coding genes. For instance, RB recruits SUV39H1 to its target promoters to mediate transcriptional repression (Nielsen et al., 2001) and may therefore limit the expression of genes that are required for the active cell cycle. On the other hand, SUV39H1-mediated H3K9 trimethylation is involved in the silencing of the *p21* promoter (Cherrier et al., 2009); thus, the loss of H3K9 trimethylation in the *p21* promoter results in *p21* expression associated with senescence. Therefore, we examined whether the altered H3K9me3 distribution patterns across the genome may contribute to senescence-dependent changes in gene expression. p21 protein levels were increased in senescent cells and correlated with the reduced expression levels of SUV39H1 (Fig. 5.6). Further, H3K9me3 was less abundant in the promoter region of *IL6* in senescent cells when compared to dividing cells (Fig. 5.5B), as determined by ChIP, suggesting that reduced SUV39H1 expression levels affect the gene expression profiles of senescent cells.

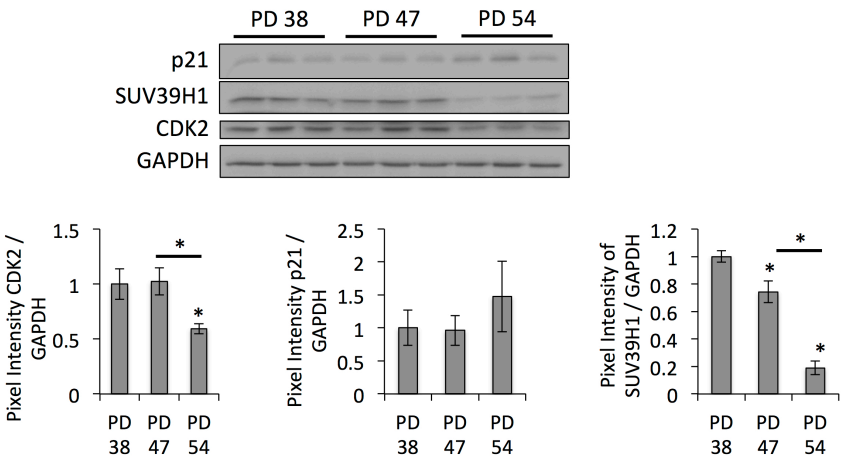

Figure 5.6: Down-regulation of SUV39H1 affects gene expression profiles.

Western blot images and bar graphs showing the quantification of band intensities. Bars represent averages from three samples, error bars indicate the standard deviation, and asterisks show significance based on Student's t-test ($p < 0.05$).

5.4.7 SUV39H1 inhibition is more toxic for dividing than for senescent cultures

Since SUV39H1 down-regulation contributes to increased genomic instability as well as senescence-associated gene expression, we hypothesized that SUV39H1 may play a causal role in the establishment of senescence. If so, the inhibition of SUV39H1 in dividing cells may induce senescence. In order to test this, we treated cells at different PD levels with chaetocin, a specific inhibitor of SUV39H1 (Greiner et al., 2005).

As Greiner *et al.* (2005) described, the cytotoxic effects of chaetocin on the treated cells depend on the drug concentration as well as on the seeding density. Therefore, we tested the cytotoxicity of increasing concentrations of chaetocin in the cells of all three PD levels, and found that cell cultures of all senescence states maintained a high level of viability when treated with 5 or 10 nM chaetocin and decreased rapidly at higher concentrations (Fig. 5.7A). Interestingly, the PD 54 cells, which express lower levels of SUV39H1 (Fig. 5.3), were more tolerant to higher concentrations of chaetocin, as seen by their viability at 48 h and 96 h following chaetocin treatment. In addition to a decrease in viability, increasing concentrations of chaetocin induced greater senescence in PD 47 cells (Fig. 5.7B).

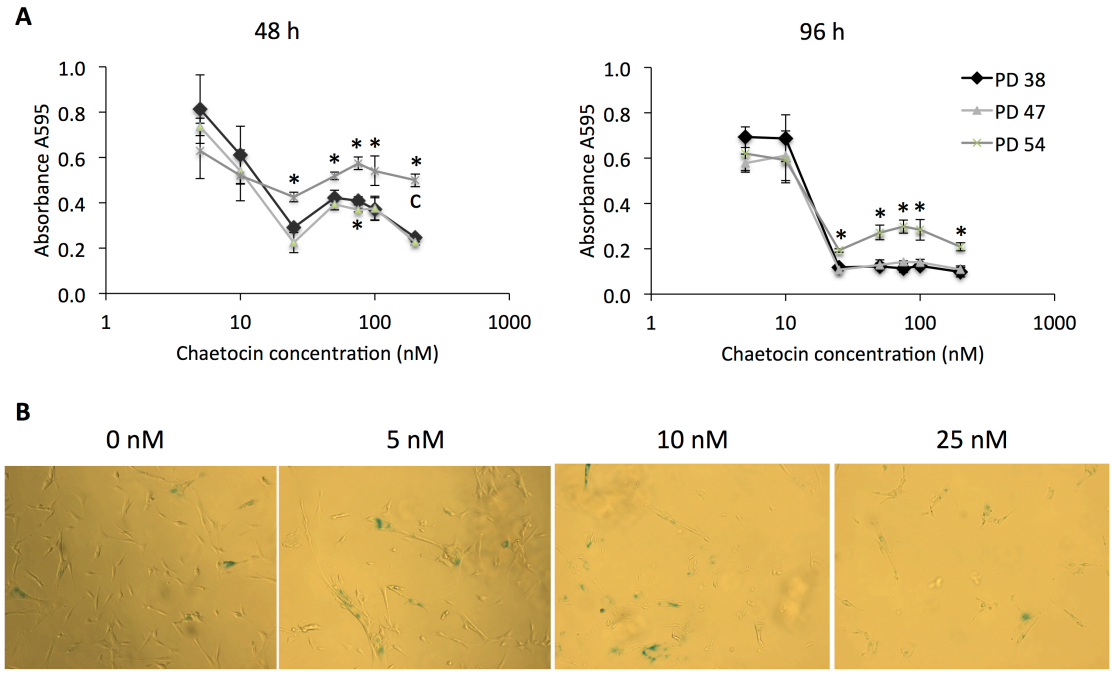

Figure 5.7: Chaetocin is more cytotoxic and induces senescence in low PD cultures.

A) Cell viability after treatment with different concentrations (5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, or 200 nM) of chaetocin as determined by the MTT assay. Data points represent averages of measurements from three different cultures ± standard deviations. B) Images showing SA-β-GAL staining of PD 47 cultures treated with 0 nM, 5 nM, 10 nM, or 25 nM of chaetocin. Pictures were taken at 10× magnification.

5.4.8 SUV39H1 over-expression in senescent cells induces cell division

Since treatment with increasing concentrations of chaetocin not only affected cell viability but also increasingly induced senescence in the exposed cells (Fig. 5.7B), we tested how the inhibition of chaetocin in PD 38 cells or the over-expression of *SUV39H1* in PD 54 cells affected cell division. For this, PD 38 cells were treated with DMSO or 10 nM chaetocin, while PD 54 cells were transfected with pCMV6-

SUV39H1. Chaetocin treatment of PD 38 cells resulted in a reduction of *SUV39H1* transcript levels (Fig. 5.8A) and in a slight decrease in the number of cells in the S phase, which was not significant due to a high variation between the samples (Fig. 5.8B). On the other hand, the over-expression of *SUV39H1* in PD 54 cells resulted in increased *SUV39H1* transcript levels (Fig. 5.8A) and in a significant increase in the amount of cells in the S phase, with a corresponding significant decrease in the number of cells in the G1 phase (Fig. 5.8B).

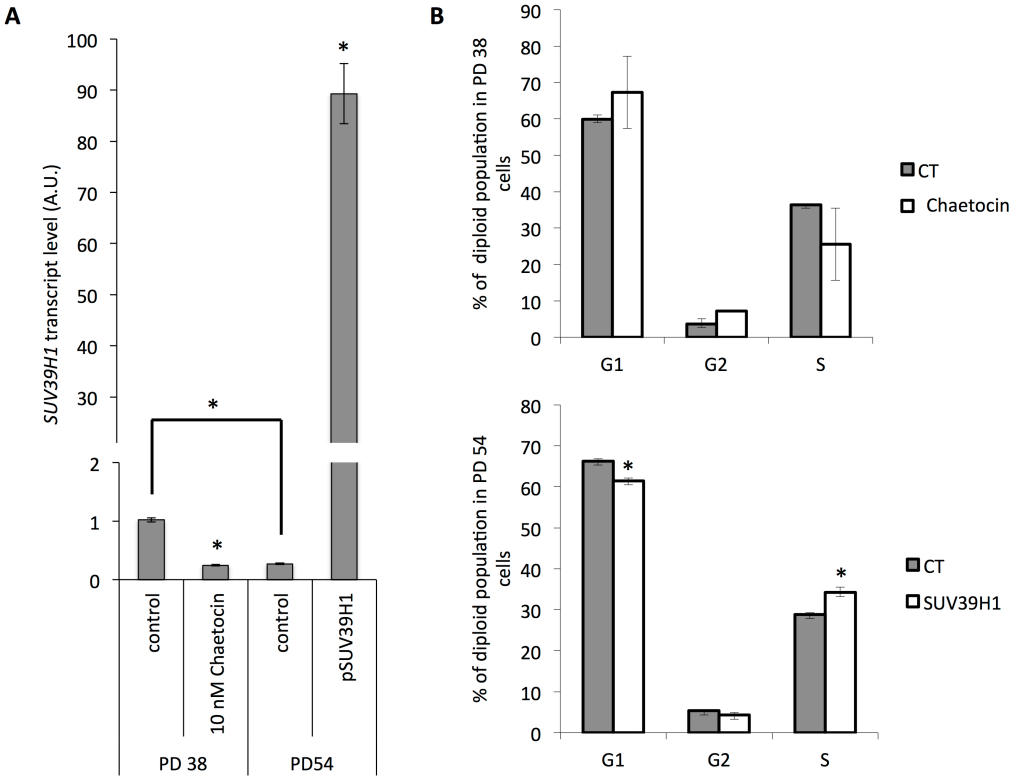

Figure 5.8: Modification of *SUV39H1* expression levels affects cell cycle distribution.

A) *SUV39H1* transcript levels normalized to *RPL13A* and *YWHAZ* expression, and to PD 38 control. Bars represent averages of three samples and error bars indicate error progression of standard deviations. Asterisks indicate significance based on Student's t-test ($p < 0.05$). B) Cell cycle distribution. Bars represent averages of three samples

and error bars indicate standard deviation. Asterisks indicate significance based on Student's t-test ($p < 0.05$).

5.5 DISCUSSION

The cell cultures used herein were selected to show increasing PD levels and an increasing senescence ratio, along with reduced proliferation. The increasing senescence ratio was also associated with a higher number of cells with G2 DNA content. This is in line with previous reports which show that a large fraction of senescent cells exhibit polyploidy and arrest in the G2 phase of the cell cycle (Mao et al., 2012; Sherwood et al., 1988).

The comparison of the gene expression profiles of three cultures with different senescence states (PD 38, PD 47 and PD 54) further supported the observations that PD 54 cultures contained significantly more senescent and less proliferating cells than the younger cultures; this was observed by the high number of genes involved in cell cycle regulation and aging or senescence affected by differential expression. These changes affected several pathways previously shown to be altered during aging or senescence, such as p53 signalling and cytoskeleton-related gene expression (Fridman and Tainsky, 2008). The observed changes in gene expression in the PD 54 cultures corresponded with previously described senescence-related changes in gene expression (Table 5.6;(de Magalhaes et al., 2009), and showed the extensive down-regulation of genes involved in the progression of mitosis or DNA replication (Table 5.5), thus supporting the validity of the model system.

In order to elucidate the underlying mechanisms involved, the gene expression profile of senescent cells was compared to that of young cells. The functional classification of genes that were differentially expressed between PD 47 and PD 38 as

well as between PD 54 and PD 38 indicated that cell signalling and transcriptional and epigenetic regulation are affected by expression changes early on during the establishment of senescence (Fig. 5.1). Further, the regulatory network analysis that was performed using RENATO software connected the deregulation of 295 genes to transcriptional regulation by E2F1. E2F1, in addition to E2F2 and E2F3, is an activating member of the E2F family of transcription factors, which are involved in the regulation of G1/S progression and DNA replication (Wu et al., 2001). While all of the E2F transcription factors recognize the E2F recognition site in the promoter regions, their target specificity is thought to depend on their interaction with transcription co-activators or co-repressors, as well as on the presence of other binding motifs within the target genes (Freedman et al., 2009; Giangrande et al., 2004; Schlisio et al., 2002). While we did not observe any change in the expression levels of E2F1, *E2F2* was down-regulated in senescent cells, as confirmed by qRT-PCR. As E2F1 and E2F2 both recognize the E2F recognition site used to screen for target genes, E2F2 may at least partially be involved in the regulation of the expression of the network associated with E2F1. Thus, the senescence-associated down-regulation of E2F2 may play a role in the down-regulation of genes involved in the G1/S progression and thus promote the G1 cell cycle arrest and the establishment of senescence. An involvement of E2F2 in the establishment of senescence was previously shown in T cells, where E2F2 was shown to act as a transcriptional repressor to result in the inactivation of cell cycle genes and thus the promotion of the transition into the G0 phase of the cell cycle (Infante et al., 2008). Further, the relevance of the E2F transcriptional program in senescence has already been described in the literature; for instance, senescence-associated heterochromatin

formation was shown to be targeted to E2F target promoters by Rb (Narita et al., 2003).

The RENATO analysis also showed a significant enrichment of NF-YA targets among the differentially expressed genes when comparing PD 38 and PD 54. NF-YA also regulates the expression of genes involved in the G1/S transition, and has been previously shown to exhibit a decline in protein levels without a change in transcript level with increasing senescence in IMR90 cells (Matuoka and Chen, 2000). The NF-Y transcription factor has also been previously shown to regulate the expression of E2F1 (Kabe et al., 2005). In addition, E2F transcription factors have been shown to cooperate with other transcription factors, including NF-Y, to mediate the expression of cell cycle genes (van Ginkel et al., 1997). Thus, E2F2 and NF-Y may cooperate to mediate the G1 cell cycle arrest at the gene expression level.

Further analysis of the transcription factors that were deregulated during senescence and the correlation of their expression changes with those of their predicted target genes, implicated additional transcription factors that may be involved in the regulation of the senescence-associated gene expression profile. However, their TFBS were not enriched in the promoters of the differentially expressed genes when compared to all promoters. Thus, our findings and previous reports (Infante et al., 2008; Matuoka and Chen, 2000, 2002) support the important role of changes in transcriptional regulation in the establishment of senescence, in particular through the repression of cell cycle regulators.

In addition to changes in transcriptional regulation, the gene expression profiling data also indicated the deregulation of genes involved in the epigenetic regulation of senescent cells, such as *SUV39H1*, which was down-regulated in senescent cells. This correlated with reduced global levels of H3K9 trimethylation (Fig. 5.3), site-specific

reduction of H3K9me3 abundance in the promoter region of *IL6* and in regions of repetitive DNA sequences and corresponding transcription of satellite transcripts (Fig. 5.5), and with the increased expression of p21 (Fig. 5.6), which is regulated by H3K9 trimethylation in its promoter region. This is in line with previous reports describing an important role of SUV39H1 in heterochromatin formation in pericentric satellite regions (Lehnertz et al., 2003; Peters et al., 2001; Wang et al., 2013). Additionally, such a senescence-dependent loss of constitutive heterochromatin has been previously described through the mapping of open chromatin regions by formaldehyde assisted isolation of regulatory elements in human diploid fibroblasts (De Cecco et al., 2013). However, here we present the first evidence that the down-regulation of SUV39H1 in senescent human diploid fibroblasts contributes to heterochromatin relaxation in pericentric satellite regions and thereby contributes to genomic instability. Further, a function of SUV39H1 in the altered regulation of gene expression has also been described. SUV39H1 has been shown to silence S phase genes in terminally differentiating cells in cooperation with E2F/RB (Ait-Si-Ali et al., 2004). On the other hand, SUV39H1 also plays a role in the silencing of the *p21* promoter (Cherrier et al., 2009) and thus the loss of H3K9 trimethylation in the *p21* promoter results in *p21* expression associated with senescence. At first sight, these results may be contradictory, as some promoter regions are specifically targeted by SUV39H1 during senescence and in some promoter regions SUV39H1 is specifically lost. This may be due to the specific recruitment of SUV39H1 by transcription factors (Ait-Si-Ali et al., 2004; Lomberk et al., 2012), and may therefore depend on the altered expression or activity of transcriptional programs. Additionally, the specific recruitment of SUV39H1 by transcription factors may contribute to the recruitment of the more

limited pool of SUV39H1 away from the DNA regions that it is constitutively associated with. Nevertheless, this would need further investigation.

The findings from previous reports (Bandyopadhyay et al., 2007; Czvitkovich et al., 2001; Peters et al., 2001) along with our observations that reduced SUV39H1 expression and reduced H3K9 trimethylation result in genomic instability and altered gene expression, which may both independently trigger senescence (Fig. 5.9), suggest that SUV39H1 may have a central function in the establishment of senescence. In line with this, PD 38 and PD 47 cells were significantly less tolerant to high concentrations (more than 25 nM) of chaetocin, a specific SUV39H1 inhibitor, when compared to PD 54 cells (Fig. 5.7). This may be due to the already reduced expression levels of SUV39H1 in PD 54 cells, suggesting that the cytotoxicity of chaetocin partially depends on the presence of its target SUV39H1. Further, the surviving cells in PD 47 cultures treated with increasing concentrations of chaetocin became senescent (Fig. 5.7B), suggesting that the inhibition of SUV39H1 was sufficient to induce senescence in low PD cells. Additionally, the inhibition of SUV39H1 in PD 38 cells resulted in a decrease in the amount of cells in the S phase, whereas the over-expression of *SUV39H1* in PD 54 cells induced cell division (Fig. 5.8). These results support the important role of SUV39H1 in the regulation of the transition between cell division and senescence. In addition to our results, previous reports have described several functions of SUV39H1 that underline its importance in cell division such as its role in chromatin organization at centromeres during cell division (Aagaard et al., 2000), its role in the organization of the nuclear architecture (Uhlirova et al., 2010), the inhibition of cellular differentiation programs in transgenic *Suv39h1* over-expressing mice (Czvitkovich et al., 2001), and the fact that *suv39h1/2* knockout mice suffered from reduced viability, genomic instability, and susceptibility

to cancer (Peters et al., 2001). While a role for SUV39H1 in the establishment of heterochromatin foci has been described (Bandyopadhyay et al., 2007), herein we suggest that, during the establishment of replicative senescence, the down-regulation of SUV39H1 might provide a switch that alters the chromatin structure from a conformation that promotes cell division to one that promotes cell cycle arrest (Fig. 5.9). Support for such a theory can be found in a recent study showing that the relaxation of satellite heterochromatin is an early event in the establishment of senescence (Swanson et al., 2013). However, further studies are needed to elucidate how this down-regulation of SUV39H1 is initiated and whether it is a gradual process or an acute switch from one state to the other.

In summary, the present study has shown that replicative senescence in WI-38 cells is associated with major changes to the gene expression profile. Some of these changes may be caused by altered transcriptional regulation, as supported by the enrichment of E2F- and NF-Y-target genes among the differentially expressed genes. In addition, we describe a role for the senescence-associated down-regulation of SUV39H1 in the loss of heterochromatin in satellite regions, as well as in the promoters of protein coding genes. These changes in chromatin structure may promote the induction of senescence and, in line with this the over-expression of *SUV39H1* in senescent cells, induces cell division, whereas the inhibition of SUV39H1 in dividing cells inhibits cell division. Therefore, we suggest that SUV39H1 functions as a switch by maintaining a chromatin conformation that is favourable to cell division as long as it is expressed.

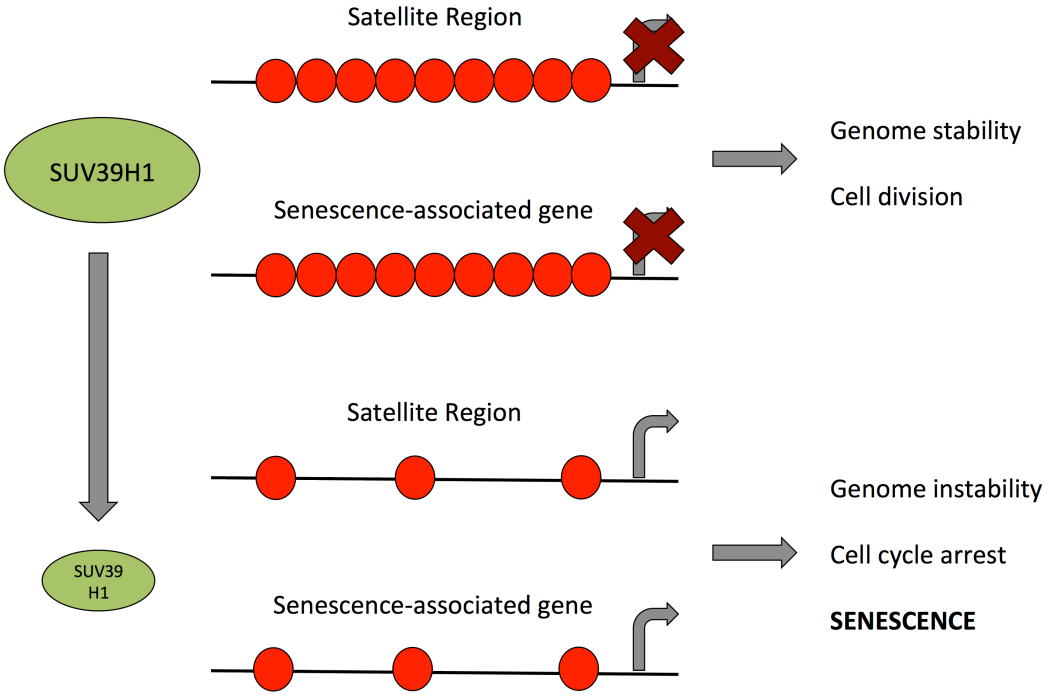

Figure 5.9: Model for the role of SUV39H1 down-regulation in the establishment of senescence.

The green ellipses represent the amount of SUV39H1 present in a cell, representing the reduction that is observed during senescence. The black lines represent DNA and the red circles represent H3K9me3. Bent arrows indicate transcriptional activity.

5.6 ACKNOWLEDGEMENTS

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6. WI-38 SENESCENCE IS ASSOCIATED WITH GLOBAL AND SITE-SPECIFIC DNA HYPOMETHYLATION[18]

6.1 ABSTRACT

Cellular senescence plays an important role in the age-dependent functional decline of organs and organ systems, as well as in age-related pathologies, such as cancer. Therefore, a better understanding of its underlying molecular mechanisms is crucial in the search for intervening measures. In this study, we considered the role of DNA methylation in senescence. We found that senescence is associated with global DNA hypomethylation, but also involves site-specific DNA hypo- and hypermethylation. In some cases, this differential methylation may affect gene expression and thereby modulate functional processes within cells. However, the majority of the CpG sites that were differentially methylated did not correspond with altered gene expression, suggesting that DNA methylation affects senescence by other means also, such as, for instance, genome stability.

6.2 INTRODUCTION

Cellular senescence is associated with the terminal cell cycle arrest of cells, and was first described by Hayflick and Moorhead, who observed that human diploid lung fibroblasts exhibit a limited *in vitro* replicative lifespan of approximately 50 ± 10 population doublings, after which the cell cultures progressively decline (Hayflick, 1965; Hayflick and Moorhead, 1961). Later, such senescent cells were shown to accumulate in tissues *in vivo* with increasing age (Dimri et al., 1995; Herbig et al.,

[18] This chapter has been published in its entirety:
Sidler, C., Woycicki, R., Kovalchuk, I., and Kovalchuk, O. (2014). WI-38 senescence is associated with global and site-specific hypomethylation. Aging (Albany NY).

2006), and when primary cells from species with different maximum lifespans were taken into a cell culture, their *in vitro* lifespan correlated with the maximum lifespan of the donor species (Martin et al., 1970; Rohme, 1981). These observations supported a role for cellular senescence in organismal aging.

There are several different ways, in which cellular senescence may contribute to the age-related functional decline of organs and organ systems, and age-related pathologies. On the one hand, the senescence of cells that rely on proliferation for their proper function, such as stem cells and immune cells, limits the repair capacity in aging tissue (Krishnamurthy et al., 2006) and contributes to the age-dependent deterioration of the immune system (Effros, 2004). On the other hand, while the pro-inflammatory microenvironment of senescent cells can contribute to the development and progression of inflammatory diseases and cancer (Coppe et al., 2008; Yang et al., 2006), the failure of senescence mechanisms can also result in malignant transformation and cancer progression (Hahn et al., 1999). Thus, understanding the mechanisms that underlie senescence constitutes a major step towards the goal of ensuring healthy aging.

While certain molecular changes have already been associated with the induction of senescence, such as telomere shortening (Harley et al., 1990; Lundblad and Szostak, 1989), the role of changes in chromatin structure have recently also been considered for their roles in aging and senescence (see also (Feser and Tyler, 2011; O'Sullivan and Karlseder, 2012; Sedivy et al., 2008)). Narita *et al.* (2003) were the first to describe the focal formation of heterochromatin in senescent cells, which they termed "senescence-associated heterochromatin foci" (SAHF) (Narita et al., 2003). They showed that this heterochromatin formation was targeted to E2F target genes by RB/E2F and reinforced a terminal cell cycle arrest through the permanent down-

regulation of cell cycle regulators. Swanson *et al.* (2013), meanwhile, described an early step in the establishment of senescence that they termed the "senescence-associated distension of satellites" (SADS), which was caused by the relaxation of the chromatin structure in pericentromeric satellite regions (Swanson et al., 2013). Thus, both the loss of heterochromatin in regions of constitutive heterochromatin and the gain of heterochromatin in previously transcriptionally active regions may contribute to the establishment of senescence.

Several recent studies involved massive parallel sequencing projects that compared the DNA methylomes of blood cells from donors of varying ages ranging from newborns to centenarians (Heyn et al., 2012; Johansson et al., 2013; McClay et al., 2014). They commonly observed a decrease in global DNA methylation with increasing age. This loss of DNA methylation was observed in diverse regions of the genome and affected various functional groups of genes. Heyn *et al.* (2012) found that mainly CpG-poor and tissue-specific promoters were affected by DNA hypomethylation, while McClay *et al.* (2014) observed reduced DNA methylation levels in regions associated with polycomb proteins or activating histone marks. DNA hypermethylation, on the other hand, occurred more frequently in CpG-rich sequences, and seemed to be more site-specific than DNA hypomethylation. In addition, a comparison of the DNA methylomes of different cell types at different passages showed that the differential methylation of several sites served as a biomarker for senescence (Koch and Wagner, 2013).

Even though DNA hypomethylation seemed to be less site-specific than DNA hypermethylation, the observations that the loss of DNA methylation only occurs in senescing cell strains, and not in immortalized cells (Wilson and Jones, 1983), and that the inhibition of DNA methylation can induce growth arrest in immortal cells

(Vogt et al., 1998), suggest that DNA hypomethylation does play an important role in the establishment of senescence.

Here, we investigate the role of differential DNA methylation in senescence in WI-38 human diploid lung fibroblasts. Senescent WI-38 cells exhibited down-regulation of DNMT1 and reduced global DNA methylation. The extensive changes to the DNA methylation profile are shown to affect partially distinct functional groups of genes according to whether there is hypomethylation or hypermethylation, and corresponded with differential gene expression in a small fraction of the affected sites.

6.3 MATERIALS AND METHODS

6.3.1 Cell culture

Normal human foetal lung fibroblasts (WI-38, ATCC, CCL-75TM) were maintained in HyClone® minimum essential medium (MEM) Alpha Modification (ThermoScientific) containing 10% (v/v) foetal bovine serum (FBS) (Gibco) in a humidified Forma Steri-Cycle CO_2 Incubator (ThermoScientific) at 37°C and 6% CO_2. In order to compare the senescence-associated changes, three different population doubling (PD) levels of cells were used: PD 38, PD 47 and PD 54, as described previously (Chapter 5).

6.3.2 DNA methylation profiling

Genomic DNA (gDNA) was isolated from cells using the DNeasy® Blood and Tissue Kit (Qiagen). Three samples per PD level were used, with each sample consisting of cells harvested from two 10-cm cell culture plates. gDNA was eluted in 100 µL nuclease-free water. The DNA was treated with ribonuclease A (0.1 mg/mL final concentration) for 1 h at 37°C and purified using a phenol:chloroform DNA

extraction protocol. The DNA was precipitated from the aqueous phase using 0.2 parts 3 M sodium acetate pH 5.2 and 5 parts of 100% ethanol. The gDNA pellet was dissolved in 50 µL nuclease-free water and quantified using NanoDrop 2000c (ThermoScientific). 500 ng of DNA was used to determine DNA methylation profiles on Illumina® HumanMethylation27 BeadChips, according to the manufacturer's protocol. Illumina® GenomeStudio software was used for the determination of beta values (percentage of methylation of specific CpG sites). Differential DNA methylation analyses were performed using an Illumina Custom model, which produced Diff Scores as a measure of significance; PD 38 was used as a reference. Diff Scores of −13/13 were set as cut-off for significance.

6.3.3 Functional classification

Functional classification of genes was done using several different softwares: FunNet Transcriptional Networks Analysis[19], g:Profiler (Reimand et al., 2011; Reimand et al., 2007), and DAVID Bioninformatics Resources 6.7 (Huang da et al., 2009a, b) and compared with information on the genes available from the Genecards database[20] (Safran et al., 2010). All differentially methylated CpG sites were counted individually.

6.3.4 Western immunoblotting

Protein was extracted from cells from four 10-cm cell culture plates by sonication in 100 µL cold 1% SDS containing protease inhibitor (Roche). Protein concentrations were determined by the Bradford assay (BioRad), and the absorbance at 595 nm was measured by NanoDrop 2000c (ThermoScientific). 40 µg of protein was separated by

[19] www.funnet.info
[20] www.genecards.org

SDS-PAGE in slab gels of 6% (DNMT1) or 10% (MAT2A) polyacrylamide and transferred to Amersham Hybond-P polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). The membranes were incubated with primary antibodies overnight at 4°C (DNMT1 [Abcam], MAT2A [Abcam], GAPDH [Santa Cruz]). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies followed by ECL Plus immunoblotting detection system (Amersham Biosciences). Chemiluminescence was detected using a FluorChem^{TM} HD2 camera with FluorChem^{TM} software (Cell Biosciences). The signals were quantified using NIH Image J64 software and normalized relative to GAPDH.

6.3.5 Statistical analysis

All experiments included three biological replications, and statistically significant differences were determined by a pairwise, two-tailed Student's t-test ($p < 0.05$).

6.4 RESULTS

6.4.1 Senescence is associated with reduced global DNA methylation

To date, several studies have shown age-dependent alterations in the DNA methylation profile of cells. While both hypermethylated and hypomethylated sites have been detected with the increasing age of the subjects, DNA hypomethylation was more frequent (Heyn et al., 2012; Johansson et al., 2013; McClay et al., 2014). Our previous results indicated that DNA methyltransferase *DNMT1* and Methionine adenosyltransferase 2 alpha (*MAT2A*), which plays a role in the formation of S-adenosylmethionine (SAM), the substrate for DNA methylation, were down-regulated in senescent cells (Chapter 5). This was confirmed at the protein level (Fig. 6.1A, B) and correlated with decreased global DNA methylation, as shown by the increased

incorporation of radioactively labeled dCTP into DNA from senescent cells (Fig. 6.1C).

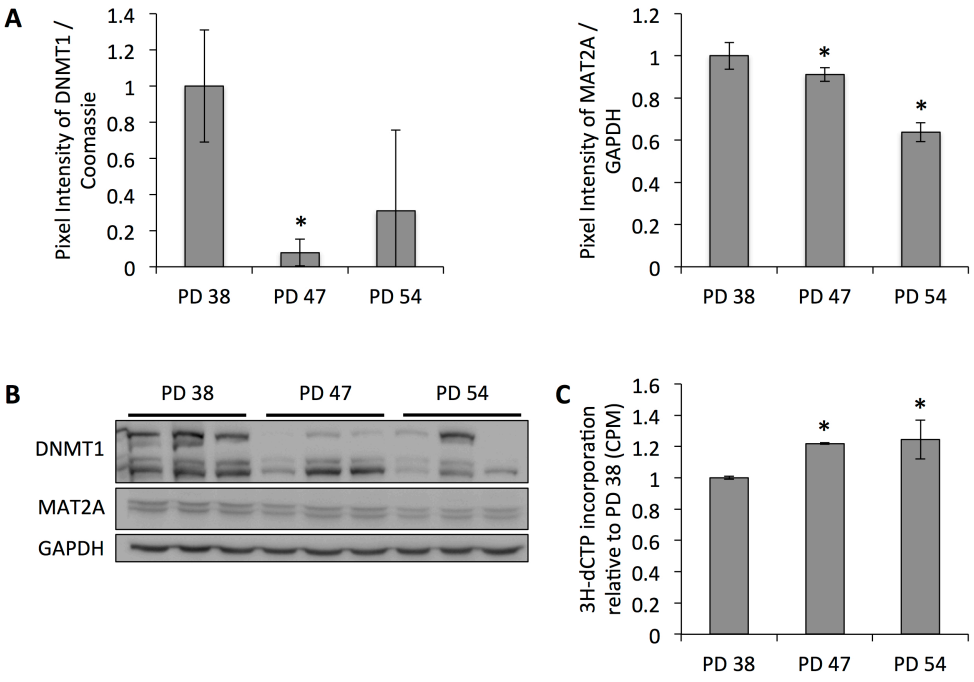

Figure 6.1: Reduced protein levels of DNMT1 correlate with decreased global DNA methylation.

A) DNMT1 and MAT2A protein levels normalized to protein levels in PD 38 cells. Each bar represents an average of three biological replicates; error bars indicate standard deviation; asterisks indicate significance based on Student's t-test ($p < 0.05$). B) Western blot images. C) Cytosine extension assay showing average incorporation of radioactively labeled dCTP from three samples per PD level; error bars indicate standard deviation and asterisks represent significance based on Student's t-test ($p < 0.05$).

6.4.2 Hypomethylation of CpG sites in the promoter regions of genes is observed with increasing senescence ratio of cultures

In order to study how this deregulation of DNMT1 and the global DNA hypomethylation may affect the DNA methylation patterns at specific sites, genomic DNA was extracted from cultures of different PD levels and assessed for the methylation status of CpG sites with regulatory functions in the promoter regions of genes using Illumina® HumanMethylation27 BeadChips, which interrogate 27,578 CpG sites spanning 14,495 genes (for detailed results, see S1[21]).

When comparing the PD 54 to PD 38 cultures, the array data showed that 1,849 CpG sites were affected by differential methylation (Fig. 6.2A); these sites were predominantly hypomethylated (66.7% of all differentially methylated sites) (Fig. 6.2B). This increasing hypomethylation of CpG sites is in line with the observed reduction in *DNMT1* expression and global DNA hypomethylation in more senescent cultures (Fig. 6.1). Further, 84% of the hypermethylated CpG sites resided within CpG islands, while only 36% of the hypomethylated CpG sites were located in CpG islands.

When comparing the PD 47 and PD 38 cultures, the 231 CpG sites affected by differential methylation appear to be randomly distributed across the genome, whereas when comparing PD 54 and PD 38, several clusters with increased incidence of hypomethylated or hypermethylated CpG sites were found (Fig. 6.2C). Examples of such clusters are shown in Figure 6.2C (i, ii, iii). The observed DNA methylation changes were detected predominantly in regions of moderate or high gene density, probably due to the enrichment of probe sets specific to those regions on the BeadChip that was used for this study. Therefore, the absence of DNA methylation

[21] http://www.impactaging.com/papers/v6/n7/full/100679.html

changes detected in gene-poor regions of the genome does not allow for any conclusions about changes in the DNA methylation status in those regions.

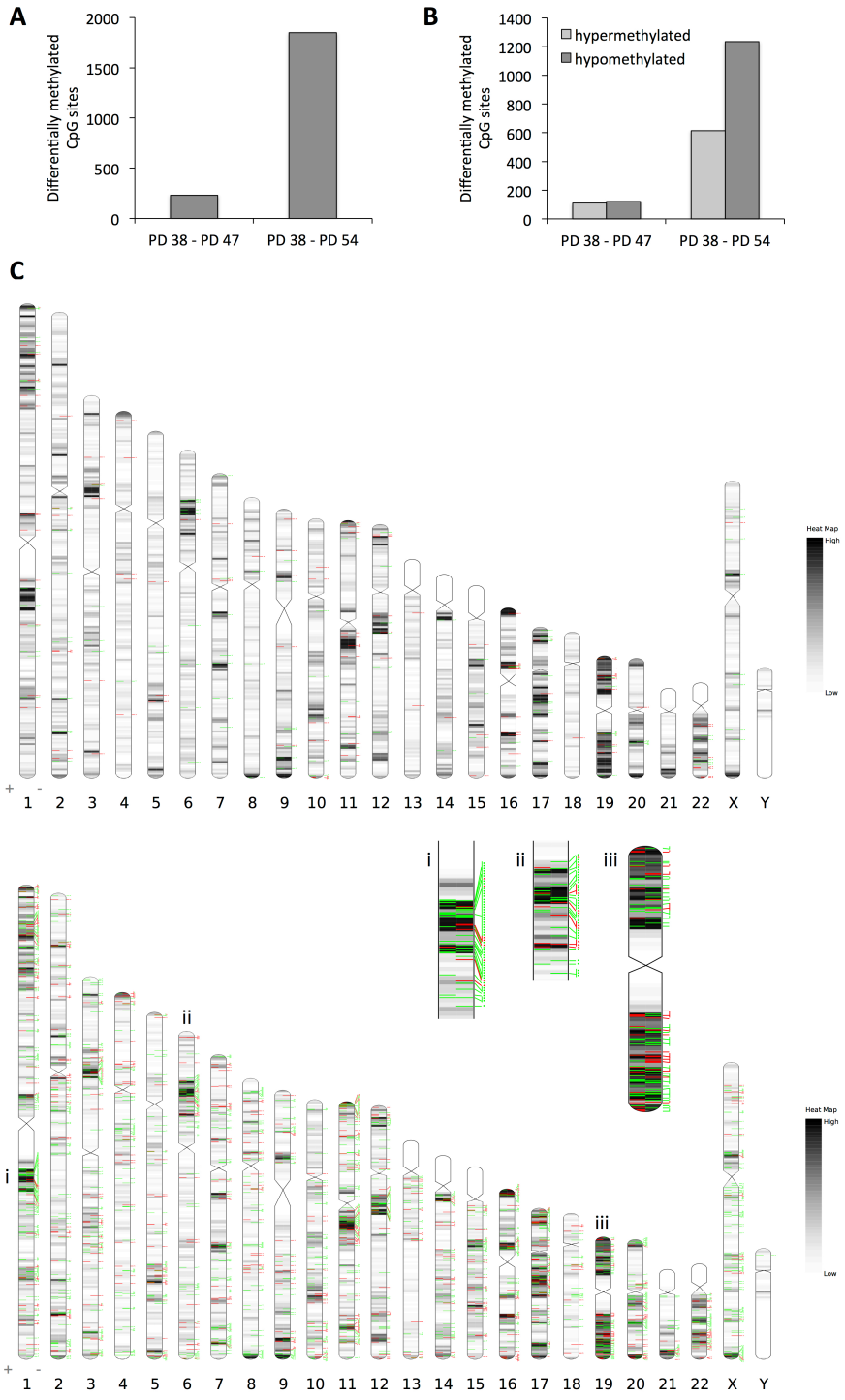

Figure 6.2: Changes in DNA methylation pattern in senescent cells.

A) Number of CpG sites affected by differential methylation comparing PD 47 and PD 54 to PD 38 cultures. B) Number of CpG sites in the given comparisons affected by hypo- (dark grey) or hypermethylation (light grey). C) Distribution of CpG methylation changes across the chromosomes. Top: changes between PD 47 and PD 38; Bottom: changes between PD 54 and PD 38. Black/grey lines indicate gene densities, green lines represent CpG sites hypomethylated in the older cultures and red lines indicate CpG sites hypermethylated in older cultures.

Interestingly, in the PD 54 and PD 38 comparison, only 42 genes that showed differential promoter methylation were also affected by changes in gene expression (Table 6.1), while none of the genes with differentially methylated promoter regions were found to be differentially expressed when comparing the PD 47 and PD 38 cultures. Among those 42 genes, for 24 genes the change in gene expression corresponded with the change in DNA methylation. These genes included *CDK2* and *E2F2*, both regulators of the G1/S progression, which were transcriptionally repressed and showed hypermethylation in their promoter regions (Table 6.1). Hypermethylation of the promoters of these genes may mediate the permanent cell cycle arrest of senescent cells in the G1/G0 phase of the cell cycle.

Further, several genes that function as transcription factors or regulate the activity or target specificity of transcription factors were among the 24 genes with corresponding changes in gene expression, *E2F2*, *FHL1*, which enhances the transcriptional activity of NFAT1c (Cowling et al., 2008), *ID3*, which inhibits several transcription factors of the basic-helix-loop-helix family (Loveys et al., 1996), *NFE2L3*, which was identified as a negative regulator of the antioxidant-response element containing promoters (Sankaranarayanan and Jaiswal, 2004), *NR4A2*, which

can be transcriptionally induced in response to pro-inflammatory signals (McEvoy et al., 2002) and may then induce the expression of additional cytokines through the interaction with NF-κB/p65 (Aherne et al., 2009), *RELB*, which is a transcription factor of the NF-κB/RELB family and represses pro-inflammatory gene expression in fibroblasts (Xia et al., 1999), and TGIF2, which transcriptionally represses TGFβ target genes (Melhuish et al., 2001). This supports the involvement of multiple transcriptional programs in the regulation of the senescence-associated gene expression profile as well as the establishment of the senescence-associated phenotype.

While 52.3% of all differentially methylated CpG sites were located in CpG islands, among the 42 CpG sites that resided in promoters of differentially expressed genes a higher fraction of sites was located in CpG islands – 87.5% of sites which corresponded with the change in gene expression and 72.2% of sites which did not correspond with the change in gene expression. Additionally, differentially methylated sites that corresponded with a gene expression change were located slightly closer to the transcription start site (TSS) (average distance of 412 nucleotides) when compared to all differentially methylated CpG sites (average distance of 421 nucleotides), while differentially methylated sites that did not correspond with the observed gene expression change were located slightly more distant (average distance of 495 nucleotides). However, due to large variations in the distance to the TSS, these differences were not statistically significant. Further, fifteen out of the 24 cases, in which differential DNA methylation corresponded with differential gene expression, consisted of hypermethylated CpG sites and corresponding repression of the transcript.

Thus, while CpG sites located in CpG islands were more frequently associated with differential gene expression, the majority of the observed changes in DNA methylation did not correlate with changes in gene expression. These changes may not affect CpGs that directly regulate the gene expression, but they may modify the accessibility of the promoter region to transcriptional regulation or affect genome stability.

Table 6.1: Genes affected by differential expression and DNA methylation.

Numbers indicate log2 fold changes in the transcript level or CpG methylation level when comparing PD 54 to PD 38 cultures; information on gene function retrieved from Genecards (Safran et al., 2010)

	Gene expression	DNA methylation	Gene function
ALDH1A3	-0.41	0.78	Aldehyde dehydrogenase, which binds to retinal
APP	0.72	-1.11	Amyloid beta (A4) precursor protein, best studied for its role in Alzheimer's disease
ATP5D	0.48	1.1	Mitochondrial membrane ATP synthase
CDK2	-0.43	0.55	Cyclin-dependent kinase, mediates G1/S progression together with CCNE and the transition from S to G2 phase with CCNA
COL16A1	-0.43	-0.68	Type XVI collagen, involved in mediating cell attachment and morphology
CPS1	-0.59	-0.52	Mitochondrial carbamoyl phosphate synthase 1, involved in the urea cycle
DPP4	0.46	0.4	Dipeptidyl-peptidase 4, membrane glycoprotein, serine exopeptidase
E2F2	-0.6	1.94	Transcription factor involved in the regulation of G1/S transition and DNA replication
FHL1	0.57	-0.42	Contains zinc finger domains and LIM domains, may be involved in muscle development
GFPT2	-0.53	2.48	Controls the flux of glucose to the hexosamine pathway
GPI	0.64	0.73	Glucose-6-phosphate isomerase, involved in glycolysis
GSTO2	-0.45	0.49	Glutathione-S-transferase omega 2, may be involved in ascorbic acid recycling
HMGB2	-1.14	-1.94	Preferentially binds to ssDNA and may bend it, co-factor to RAG during V(D)J recombination
HYLS1	-0.7	-0.97	Associated with centriole, involved in cilia formation
ID3	1.02	-1.36	Inhibits DNA binding of transcription factors, including E2A
KIF4A	-0.72	-0.95	Motor protein, involved in spindle organization

MLLT11	0.5	-0.99	Fused with a number of translocation partners in leukemia
MOV10	-0.43	-1.53	RNA helicase, involved in RISC-mediated post-transcriptional gene silencing
MVP	0.51	0.51	Major vault protein, plays a role in the formation of scaffolds for signal transduction
NEFH	-0.51	0.56	Neurofilament protein
NFE2L3	-0.76	1.5	Transcription factor, which binds to antioxidant response element in target promoters
NR4A2	0.83	-1.65	Nuclear receptor subfamily 4 (NURR1), may function as a transcription factor
NTF3	-0.61	0.54	Neurotrophin 3, involved in neural survival
PITX1	1.43	0.61	Transcriptional regulator, involved in the regulation of development
PPIL5	-0.5	1.03	Negative regulator of TNFRSF9 signalling
RAB11FIP5	0.4	-2.1	Involved in protein trafficking
RAMP1	-0.61	1.16	Membrane protein, co-receptor
RELB	-0.44	0.5	Dimerizes with NF-κB to modify its preference for transcription target binding
RELN	-0.65	-1.07	Extracellular matrix serine protease
RPL39L	-0.62	0.86	Ribosomal protein L39 like
SCAMP3	0.45	1.84	Involved in post-golgi recycling pathways and protein trafficking
SLC4A4	0.44	-0.59	Sodium/bicarbonate co-transporter
SLIT2	-1.33	-1.95	May regulate cell migration
SPATA18	0.55	1.23	Involved in the repair or degradation of damaged mitochondria
SSRP1	-0.61	0.45	Part of the FACT complex, which is involved in destabilizing and reassembling nucleosomes during transcription, replication and DNA repair
TFRC	-0.51	1.19	Receptor involved in iron uptake
TGIF2	-0.52	0.56	Transcriptional repressor of TGFβ responsive genes, through the recruitment of histone deacetylases
TMEM35	0.75	-0.67	Transmembrane protein 35
TMEM47	0.64	-1.02	Localized to ER
TMSL3	-0.45	-0.41	Unknown function
TRO	-0.49	-0.71	Involved in cell adhesion
ZAK	0.54	1.57	Stress-activated, involved in JNK and p38 pathways

In order to better understand the potential functional implications of the differential methylation of all the sites affected, genes with differential methylation status of CpG sites within their promoter regions were functionally classified using the DAVID and FunNet softwares. This showed that both hypo- and hypermethylated CpG sites are found in the promoters of genes involved in cell cycle regulation, apoptosis and senescence, as well as in epigenetic and transcriptional regulation (Fig. 6.3A). When comparing the PD 54 and PD 38 cultures, the remaining genes were enriched for the biological processes of structural development, cell adhesion and localization,

transmembrane transport, response to extracellular stimuli and cell signalling and maintenance of cellular homeostasis and ion homeostasis as determined using the g:Profiler software.

When comparing the sites that were differentially methylated between PD 47 and PD 38 to those differentially methylated between PD 54 and PD 38, 52.6% of the sites affected in the PD 47 and PD 38 comparison were found to be common. This may indicate that those sites are subject to differential methylation early during differentiation or the establishment of senescence. To better understand what processes may be affected by differential methylation in both PD 47 and PD 54 cultures, detailed functions of the affected genes were gathered from Genecards and functionally classified (Fig. 6.3B). Among the genes with hypomethylated CpG sites in their promoter, involvement in transcriptional or epigenetic regulation, cell signalling, cell adhesion and transport of ions were prevalent, whereas among the genes with hypermethylated promoters, the major functional groups affected were cell signalling, transcriptional regulation and cell metabolism. This resembles the functional groups affected by early gene expression changes and supports the conclusion that transcriptional and epigenetic regulation may affect the establishment of senescence at the level of gene expression (Chapter 5). Changes in cellular signalling may also be involved in the establishment of senescence by modifying the activation of transcriptional programs in response to extracellular and intracellular stimuli, such as oxidative stress and a pro-inflammatory microenvironment, as well as by inducing changes in the cell physiology.

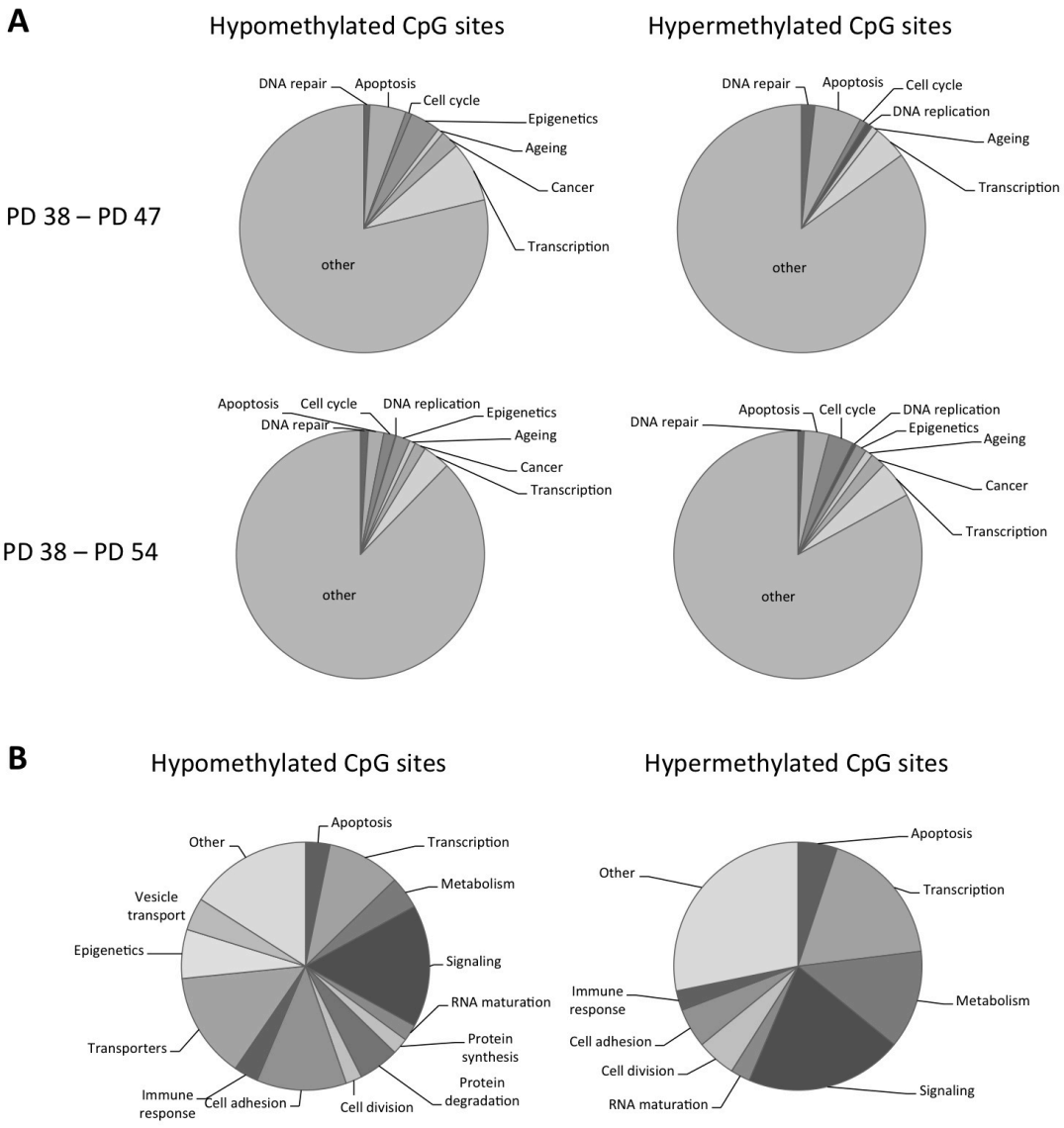

Figure 6.3: Functional classification of the genes affected by differential DNA methylation.

A) Pie charts showing functional classification of hyper- and hypomethylated CpG sites. B) Pie charts showing functional classification of CpG sites affected by methylation changes when comparing PD 47 and P38 as well as PD 54 and PD 38 cultures.

6.5 DISCUSSION

As reported (Chapter 5), our previous study indicated that in addition to the numerous transcription factors, 2.7% of all the differentially expressed genes found when comparing PD 54 and PD 38 were associated with functions in epigenetic regulation. Among those, DNMT1 was down-regulated in senescent cells, which correlated with the global reduction of DNA methylation. This supports the conclusion that there is a senescence-dependent loss of heterochromatin, as also observed in different tissues of aging model organisms (Fuke et al., 2004; Heyn et al., 2012; Vanyushin et al., 1973; Wilson and Jones, 1983; Wilson et al., 1987). A study by Fairweather *et al.* (1987) showed the treatment of human foetal lung fibroblasts (MRC5) with 5-Azacytidine to result in a reduction of the content of methylated CpGs in the genomes of the cells and correlate with a reduced *in vitro* lifespan, thus suggesting that DNA hypomethylation may play an important role in the control of the lifespan of human diploid fibroblasts (Fairweather et al., 1987). This effect may be mediated by loss of DNA methylation in promoter regions resulting in the deregulation of gene expression profiles, as proposed in the heterochromatin-loss model of aging (Villeponteau, 1997). On the other hand, the loss of DNA methylation in intergenic regions and at repetitive sequences may result in genomic instability through a loss of silencing of those regions (Jintaridth and Mutirangura, 2010).

When we considered site-specific differences in DNA methylation when comparing senescent and dividing cell cultures, we found extensive differential methylation of CpGs in the promoter regions of genes (Fig. 6.3). Two thirds of those sites were affected by hypomethylation, while one third of the sites were hypermethylated. These observations are in line with the finding that aging is associated with a global loss of DNA methylation (Heyn et al., 2012). Heyn *et al.*

(2012) further showed that differentially methylated CpG sites mostly occurred in the intergenic and intronic regions, with only 10% affecting promoter regions (Heyn et al., 2012). As the Illumina® HumanMethylation27 BeadChip contains probes that detect methylated CpGs in promoter regions, our dataset may only represent a fraction of the differential CpG methylation associated with senescence in WI-38 cells.

Since the probes on the BeadChip mainly covered CpGs in promoter regions, it is not surprising that only limited events of differential methylation were detected in gene-poor regions (Fig. 6.3). However, when comparing the DNA methylation patterns in PD 54 to PD 38 cells, some chromosomal regions with clustered events of CpG hypo- or hypermethylation became apparent.

Surprisingly the overlap between the DNA methylation profiling and gene expression profiling datasets is very limited. Only 24 out of the 1,849 differentially methylated CpG sites corresponded with the differential expression of the according gene. This indicates that in this model, DNA methylation changes are not the major regulatory factor of the senescence-associated deregulation of transcription, which is in contrast to the proposed heterochromatin-loss model of aging (Villeponteau, 1997). However, there were several transcription factors among the genes for which differential DNA methylation correlated with differential gene expression, as well as *CDK2* and *E2F2*, two central regulators of cell cycle progression. These genes exhibited promoter hypermethylation along with reduced transcript expression, indicating that the hard-wiring of the transcriptional repression through DNA methylation may play a role in the maintenance of the permanent growth arrest. The observation that 15 out of the 24 genes were affected by promoter hypermethylation and transcriptional repression, including *CDK2*, *E2F2*, *TGIF2*, *RELB* and *NFE2L3*, suggests that targeted promoter hypermethylation may affect the regulation of gene

expression profiles to a greater extent than the loss of DNA methylation through inducing a more permanent suppression of the expression of cell cycle and transcriptional regulators. This is in line with the observation that DNA hypermethylation seems to be more targeted than DNA hypomethylation (McClay et al., 2014). In addition, the finding that the majority of the hypermethylated CpG sites was located in CpG islands, whereas hypomethylated CpG sites were more frequently non-CpG island also corresponds with the observations described by McClay *et al*. (2014).

The results of the functional classification of the genes affected by differential promoter methylation indicated that the processes affected were more diverse than those observed for the changes in gene expression. The differentially methylated promoters when comparing PD 54 and PD 38 showed enrichment in KEGG pathways involved in metabolism (arachidonic acid, glycine/serine/threonine, linoleic acid, nitrogen and glycerolipid metabolism and steroid hormone biosynthesis) and cell signalling (cytokine-cytokine receptor interaction, NOD-like receptor signalling, JAK-STAT signalling, and Toll-like receptor (TLR) signalling), while functions in the processes of cell cycle, DNA repair and transcriptional and epigenetic regulation contributed smaller fractions of differentially methylated CpG sites. The overrepresentation of those metabolic and cell signalling pathways corresponds with previously reported changes in lipid metabolism (Raederstorff et al., 1995) and in the expression of cytokines and TLRs in several aging non immune system cells (Boyd et al., 2006; Kim et al., 2012b). Thus, promoter hypomethylation in those cases may increase the accessibility for transcriptional regulators without inducing permanent transcriptional activation, as no changes in transcript levels were detected.

When comparing the sites that were affected by differential methylation between PD 47 and PD 38 to those for PD 54 and PD 38, 52.6% of the PD 47-to-PD 38 differentially methylated CpG sites were found to be consistently differentially methylated. The hypermethylated CpGs among the overlapping sites mainly affected genes with functions in transcription, metabolism or cell signalling, while the hypomethylated CpGs affected a higher variety of functional groups, including transcriptional regulation, cell signalling, cell adhesion, transporters and epigenetic regulators.

Further, this 52.6% overlap in differential methylation is considerably smaller than the 80% overlap observed for the gene expression profiles (Chapter 5). In addition, the majority of the differentially methylated CpG sites when comparing PD 54 and PD 38 cultures were hypomethylated, while the majority of differentially expressed genes were down-regulated. These discrepancies may indicate that DNA methylation also plays roles during the establishment of senescence that are independent of transcriptional regulation, such as, for instance the regulation of genome integrity or chromosome architecture.

In summary, senescence in WI-38 cells was associated with the down-regulation of DNMT1 and the global DNA hypomethylation. At the level of specific CpG sites, two thirds of the differentially methylated sites were hypomethylated. However, differential DNA methylation did not correspond with differential gene expression in the majority of cases, suggesting that differential DNA methylation may affect the establishment of senescence through other mechanisms than transcriptional regulation. Further studies are needed to shed more light on the possible mechanisms involved.

6.6 ACKNOWLEDGEMENTS

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7. SUV39H1 DOWN-REGULATION INDUCES GENOMIC INSTABILITY AND SENESCENCE AFTER EXPOSURE TO IONIZING RADIATION

7.1 ABSTRACT

While the majority of cancer patients are exposed to ionizing radiation (IR) during diagnostic and therapeutic procedures, age-dependent differences in radiation sensitivity are not yet well understood. Radiation sensitivity is characterized by the appearance of side effects to radiation therapy, such as secondary malignancies, developmental deficits, and compromised immune function. However, the knowledge of the molecular mechanisms that trigger these side effects is incomplete. Here we used an *in vitro* system and showed that low population doubling (PD) normal human diploid fibroblasts (WI-38) senesce in response to 5 Gy IR, while high PD cultures do not show changes in cell cycle regulation and only a slight but significant increase in the percentage of senescent cells. Our study shows that this is associated with changes in the expression of genes responsible for cell cycle progression, apoptosis, DNA repair, and aging, as well as transcriptional and epigenetic regulators. Furthermore, we propose a role of the down-regulation of *SUV39H1* expression and the corresponding reduction in H3K9me3 levels in the establishment of IR-induced senescence.

7.2 INTRODUCTION

Age is a major risk factor for cancer. However, while the majority of cancer patients undergo diagnostic and therapeutic procedures involving ionizing radiation (IR), age-dependent differences in radiation sensitivity have not been well understood. Follow-up studies of patients that have received radiation therapy and

studies on survivors of the atomic bombings in Japan have shown the wide variety of side effects associated with those exposures, including the risk of developing secondary cancers (Adams et al., 2010; Kleinerman, 2006), compromised immune function (Yamaoka et al., 2004), and developmental deficits (Krasin et al., 2010).

Many of these side effects are probably caused by the exposure of non-tumor tissue to IR, as many of the currently available follow-up studies are based on information on patients that were subjected to less precisely targeted treatment. However, even with current three-dimensional (3D) irradiation strategies, residual exposure of healthy tissues to scattered radiation cannot be completely avoided (Joosten et al., 2013). In paediatric patients, the effects of IR on growth and development are a concern (Krasin et al., 2010); in adult patients, the effects of IR on organ function is a greater concern, as the repair capacity in aging organs may be more limited.

Aging is associated with various molecular changes that may affect the outcome of the response to IR. Aging tissues have been shown to accumulate senescent cells (Dimri et al., 1995; Herbig et al., 2006). Normal human diploid cell strains have been extensively used to study the molecular changes associated with senescence in order to obtain insights into the potential mechanisms underlying aging. This revealed that replicative senescence is associated with shortened telomeres (Harley et al., 1990; Levy et al., 1992); oxidative damage and oxidative stress (Wolf et al., 2002); decreased fidelity and efficiency of DNA double-strand break repair (Seluanov et al., 2004); altered chromatin structure (O'Sullivan et al., 2010; Scaffidi and Misteli, 2006), including the formation of senescence-associated heterochromatin foci at the expense of constitutive heterochromatin observed in some cell strains (Narita et al., 2003; Zhang et al., 2007); and a pro-inflammatory secretion profile (Rodier et al., 2009).

On the other hand, the response to IR is characterized by the activation of an ataxia telangiectasia mutated (ATM)-dependent DNA damage checkpoint (Bakkenist and Kastan, 2003), which enables DNA repair. Cells recover from the DNA damage checkpoint upon successful DNA repair and removal of the γH2AX DNA damage signal (Keogh et al., 2006). A failure to repair the DNA damage may result in p53-dependent apoptosis (Lowe et al., 1993) or checkpoint recovery followed by mitotic catastrophe (Castedo et al., 2004).

Further, a recent study reported that cells that are arrested at the G2 damage checkpoint for a prolonged duration slip into the G1 phase and undergo senescence (Ye et al., 2013). The extent to which cells become senescent or undergo apoptosis depends on the cumulative dose of IR the cells were exposed to (Noda et al., 2012). On the other hand, the physiology of the response to IR also seems to depend on the state of senescence: senescent cells are more resistant to radiation-induced cell death than their dividing progenitors (Latella et al., 2004; Zou et al., 2012) and when they undergo cell death, it occurs by necrosis rather than by apoptosis (Seluanov et al., 2001). However, the molecular mechanisms underlying the differences between the dividing and senescent cells in response to IR are not yet well understood.

Therefore, in this study we compared the physiological response of WI-38 cells at three different stages of senescence to IR, and evaluated the associated changes in gene expression profiles. Here, we show that the exposure to 5 Gy of IR resulted in extensive changes to the gene expression profile, affecting genes involved in cell cycle regulation, DNA repair, apoptosis and aging, as well as transcriptional and epigenetic modulators. The most prominent physiological response to IR was observed in cultures of low population doublings (PDs), which predominantly

underwent senescence. Here, we propose a role for *SUV39H1* in this establishment of IR-induced senescence.

7.3 MATERIALS AND METHODS

7.3.1 Cell culture

WI-38 human foetal lung fibroblasts (ATCC) were maintained in HyClone® minimum essential medium (MEM) Alpha Modification (ThermoScientific) containing 10% (v/v) foetal bovine serum (FBS; Gibco) in a humidified Forma Steri-Cycle CO_2 Incubator (ThermoScientific) containing 6% CO_2 at 37°C. WI-38 cells have a replicative limit of 50 ± 10 PDs. In this study, cultures of three different PDs were used – 38, 47, or 54 (see Chapter 5). The PD number of a culture is the sum of all $\Delta PD = \log_2(n_f/n_i)$ for each passage, where n_f is the final number of cells in a passage and n_i is the initial number of cells inoculated.

7.3.2 X-ray exposure

The cells were irradiated with doses of 0, 0.5, and 5 Gy of 90 kVp and 5 mA at 0.93 Gy/min (Faxitron X-ray LLC, Model RX-650). The samples were collected at 48 h post-exposure unless stated otherwise. Samples for RNA or DNA isolation were snap-frozen in liquid nitrogen and stored at –80°C until further processing. Samples for protein isolation or flow cytometry were processed as described in the subsequent sections.

7.3.3 Gene expression profiling

For the determination of gene expression profiles, RNA was extracted from two to three samples per group using the TRIzol® Reagent (Invitrogen) according to

manufacturer's instructions. Total RNA was quantified using NanoDrop2000c (ThermoScientific), and the RNA integrity was determined using a 2100 BioAnalyzer (Agilent). Illumina® HumanHT12-v4 Gene Expression BeadChips were used to determine the transcript levels using 47,231 unique probes. Illumina® GenomeStudio software was used for differential expression analysis using an Illumina custom model with an FDR of 0.05. Only genes for which the differential expression analysis was significant at a level of $p < 0.05$ and had a log2 fold change of less than –0.4 or greater than 0.4 were considered for further analysis.

7.3.4 DNA methylation profiling

For DNA methylation profiling, genomic DNA (gDNA) was isolated from cells using the DNeasy® Blood and Tissue kit (Qiagen). gDNA was recovered in 100 µL nuclease-free water. The gDNA was then treated with ribonuclease A (0.1 mg/mL final concentration) for 1 h at 37°C and purified by phenol:chloroform extraction. DNA was precipitated by adding 3 M of sodium acetate (pH 5.2) in the ratio 1:5 and 100% ethanol in the ratio 5:1. DNA pellets were dissolved in 50 µL nuclease-free water and quantified using NanoDrop 2000c (ThermoScientific). Illumina® HumanMethylation27 BeadChips were used to determine the methylation levels of more than 27,000 unique CpG sites. Among the BeadChip probes, most of the CpG sites detected were located in the promoter regions of genes, with a slight overrepresentation of cancer genes. In order to determine the relative methylation levels of specific sites among the different treatment groups, beta values (percentage methylation of a specific CpG site in a specific sample) were determined using Illumina® GenomeStudio software. Differential DNA methylation analyses were

performed using an Illumina custom model, which produced Diff scores as a measure of significance. Diff scores of –13/13 were set as cut-off values for significance.

7.3.5 Functional classification of genes

Online software and databases were used for the functional classification of differentially expressed genes or differentially methylated CpG sites: FunNet Transcriptional Networks Analysis[22], DAVID Bioninformatics Resources 6.7 (Huang da et al., 2009a, b), and the Genecards database[23] (Safran et al., 2010).

7.3.6 Quantitative real-time PCR (qRT-PCR)

Transcription levels of the selected genes were confirmed using qRT-PCR. The RNA samples were treated with DNAse I using an illustra RNAspin Mini Prep kit (GE Healthcare) to remove any gDNA contamination. The RNA was quantified by NanoDrop2000c, and 500 ng of RNA was used for cDNA synthesis using the iScript™ Select cDNA synthesis kit (BioRad). qRT-PCR reactions were set up using the SsoFast™ EvaGreen® Supermix (BioRad) together with primers specific for the target sequences of interest (Table 7.1) and analyzed on a C1000™ Thermo Cycler equipped with a CFX96™ Real-Time System (BioRad). PCR conditions were chosen according to the SSoFast™ guidelines with the annealing temperatures specified for primer pairs (Table 7.1).

Each experiment included three biological replicates and two technical replicates per treatment. The *HPRT1, RPL13A,* and *YWHAZ* housekeeping genes were used for the normalization and calculation of transcript levels using qbase^{PLUS} (Vandesompele et al., 2002).

[22] www.funnet.info
[23] www.genecards.org

Table 7.1: Primers for qRT-PCR based analysis of transcript levels.

Gene	Forward/ Reverse	Sequence	Annealing temperature	Reference
HPRT1	Fwd Rev	5′-TGACACTGGCAAAACAATGCA-3′ 5′-GGTCCTTTTCACCAGCAAGCT-3′	59.5°C	(Vandesompele et al., 2002)
YWHAZ	Fwd Rev	5′-ACTTTTGGTACATTGTGGCTTCAA-3′ 5′-CCGCCAGGACAAACCAGTAT-3′	59.5°C	(Vandesompele et al., 2002)
RPL13A	Fwd Rev	5′-CCTGGAGGAGAAGAGGAAAGAGA-3′ 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′	59.5°C	(Vandesompele et al., 2002)
COL3A1	Fwd Rev	5′-CGATGAGATTATGACTTC-3′ 5′-ATTACAGAATACCTTGATAG-3′	53.0°C	
UBE2C	Fwd Rev	5′-ACATATGCCTGGACATCCTGA-3′ 5′-GGTTCTCCTAGAAGGCTCTGG-3′	59.5°C	(Takahashi et al., 2006)
POT1	Fwd Rev	5′-GGGCAAAGCAGAAGTGGACGGAGCATC-3′ 5′-ATTGACAGATAACATCTGAATGCTGATTGGCTGTC -3′	59.5°C	(Baumann et al., 2002)
FOXM1	Fwd Rev	5′-ACTTTAAGCACATTGCCAAGC-3′ 5′-CGTGCAGGGAAAGGTTGT-3′	55.9°C	(Gemenetzidis et al., 2009)
E2F2	Fwd Rev	5′-CCAAGAATTACATCAGAGAA-3′ 5′-GCTTACATTCCAGACTTC-3′	55.9°C	
SUV39H1	Fwd Rev	5′-CTACTATGGCAACATCTC-3′ 5′-GTCAAGGTTGTCTATGAA-3′	55.9°C	
SAT2	Fwd Rev	5′-CATCGAATGGAAATGAAAGGAGTC-3′ 5′-ACCATTGGATGATTGCAGTCAA-3′	59.5°C	(Wang et al., 2013)
majSAT	Fwd Rev	5′-GACGACTTGAAAAATGACGAAATC-3′ 5′-CATATTCCAGGTCCTTCAGTGTGC-3′	55.9°C	(Wang et al., 2013)
αSAT	Fwd Rev	5′-CTGCACTACCTGAAGAGGAC-3′ 5′-GATGGTTCAACACTCTTACA-3′	55.9°C	(Wang et al., 2013)

7.3.7 Western blotting

Total protein extracts were prepared by sonicating cells harvested from two 10-cm cell culture dishes per sample in 100 µL of cold 1% sodium dodecyl sulfate (SDS) containing protease inhibitor (Roche). The protein amounts were quantified using Bradford assays (BioRad) and measuring the absorbance at 595 nm using NanoDrop 2000c (ThermoScientific). Equal amounts of proteins per lane (10–40 µg depending on the protein of interest) were separated by SDS-polyacrylamide gel electrophoresis

(PAGE) in slab gels of 6 to 15% polyacrylamide and transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Biosciences). The proteins were then incubated with primary antibody overnight at 4°C (Table 7.2), followed by incubation with a secondary horseradish peroxidase-conjugated antibody (Table 7.2). Antibody binding was detected using an enhanced chemiluminescence plus immunoblotting detection system (Amersham Biosciences). Chemiluminescence was detected using a FluorChem™ HD2 camera with FluorChem™ software (Cell Biosciences). Unaltered PVDF membranes were stained with Coomassie blue (BioRad) to confirm equal protein loading. Chemiluminescence signals were quantified using NIH ImageJ 64 software. Pixel intensities of the protein bands of interest were normalized to GAPDH pixel intensities.

Table 7.2: Antibodies used for Western blots.

Target	Supplier, Cat No	Dilution
Mouse anti-SUV39H1	Abcam, ab12405	1:500 in 5% milk (PBST)
Rabbit anti-H3K9me3	Abcam, ab8898	1:500 in 5% milk (PBST)
Rabbit anti-H3K9ac	Abcam, ab10812	1:500 in 5% milk (PBST)
Rabbit anti-H3	Cell Signaling, 9715	1:1000 in 5% milk (PBST)
Rabbit anti-CHK2	Abcam, ab8108	1:250 in 5% milk (PBST)
Rabbit anti-pT68CHK2	Abcam, ab3501	1:500 in 5% milk (PBST)
Mouse anti-CHK1	Cell Signaling, 2360	1:1000 in 5% BSA (PBST)
Rabbit anti-pS345CHK1	Cell Signaling, 2341	1:1000 in 5% milk (PBST)
Mouse anti-p53	Santa Cruz, sc126	1:1000 in 5% milk (PBST)
Rabbit anti-phospho p53	Santa Cruz, sc16716	1:250 in 5% milk (PBST)
Rabbit anti-acetylK382p53	Cell Signaling, 2525	1:250 in 5% BSA (PBST)
Rabbit anti-p21	Abcam, ab7960	1:1000 in 5% milk (PBST)
Mouse anti-GAPDH	Santa Cruz, sc47724	1:1000 in 5% milk (PBST)
Secondary antibodies		
Donkey anti-rabbit	Santa Cruz, sc2313	1:10000 in 5% milk (PBST)
Goat anti-Mouse	Santa Cruz, sc2005	1:5000 in 5% milk (PBST)

PBST, phosphate-buffered saline, containing 0.1% Tween 20

7.3.8 Flow cytometry analysis

Cell isolation

Cells from two 10-cm cell culture plates per sample were combined for each treatment. The cells were harvested by trypsinization, centrifugation at 200 *g* for 5 min at 4°C and resuspended in 1 mL Dulbecco's phosphate buffered saline (DPBS) (Lonza) before starting specific staining procedures, except in the case of senescence-associated β-galactosidase assay, for which the staining was performed in the cell culture plates.

Senescence-associated β-galactosidase (SA-β-GAL) assay

In order to quantify the amounts of senescent cells in response to different X-ray treatments, a flow cytometry-based fluorescent SA-β-GAL staining was employed. The staining was carried out using the method described by Debacq-Chainiaux *et al.* (2009) for 1.5 h at standard culture conditions (Debacq-Chainiaux et al., 2009). Three samples were analyzed for each treatment on a BD FACS Canto II cytometer (BD Biosciences). Measurements included 10,000 events per sample.

Detection of apoptotic, necrotic, and dead cells

A fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Pharmigen^{TM}) was used to detect the apoptotic, necrotic, and dead cells in the different treatment groups, according to the manufacturer's instructions. Three samples were analyzed per treatment using a BD FACS Canto II cytometer (BD Biosciences). Measurements included 10,000 events per sample.

Detection of proliferating cells

BrdU is a nucleotide analog that is incorporated into newly synthesized DNA; it is therefore commonly utilised in the detection of DNA synthesis. BrdU staining was performed using a BrdU flow kit (BD Pharmigen™). Cells were pulsed with 1 mM BrdU in MEM for 40 min at standard culture conditions, and then processed according to the manufacturer's manual. Three samples per treatment were analyzed on a BD FACS Canto II cytometer (BD Biosciences) and 10,000 events were detected per sample.

Determination of cell cycle distribution

Cells were fixed in 63% ethanol (final concentration) for 48 h at –20°C. DNA staining was performed by incubating the cells in staining solution (0.1% Triton-X-100, 20 µg/mL propidium iodide, and 20 µg/mL ribonuclease A in PBS) for 15 min at 37°C. Three samples per treatment were analyzed on a BD FACS Canto II cytometer (BD Biosciences) and 10,000 events were detected per sample.

7.3.9 SUV39H1 inhibition and over-expression

Chaetocin has been shown to specifically inhibit SUV39H1 (Greiner et al., 2005). Thus, cells were treated with 10 nM of chaetocin to inhibit SUV39H1. The over-expression of *SUV39H1* was achieved by transfecting cells with the pCMV6-SUV39H1 expression construct (Origene) using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's manual.

7.3.10 Chromatin immunoprecipitation (ChIP)

For testing of p53 binding to the *SUV39H1* promoter, we performed chromatin immunoprecipitation as described previously (Nelson et al., 2006). Briefly, chromatin was cross-linked by treating cells with 1% formaldehyde for 15 min and quenching with 125 mM glycine for 5 min. Then, 12 to 14 million cells were sonicated in 500 µL immunoprecipitation buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 0.5% NP-40, 1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin, and proteinase inhibitor (Roche)) to shear the DNA into 100 to 1,000 bp fragments. Immunoprecipitation was performed using antibodies against rabbit or mouse IgG as a negative control, or rabbit anti-Acetyl-K382 p53 (Cell Signaling) or mouse anti-p53 (Santa Cruz). The DNA fragments were then isolated using 10% Chelex®100 (BioRad) and purified using a QIAquick® PCR purification kit (Qiagen).

ChIP-qRT-PCR was performed like qRT-PCR using the primers listed in Table 7.3. Figure 7.1 shows a representation of the location of the amplicons within the *SUV39H1* promoter. The results were calculated by averaging the $2^{(-Ct)}$ values of three samples per treatment and normalizing to 10% of the values obtained from the input DNA.

Table 7.3: Primers used for ChIP-qRT-PCR.

Target	Fwd/ Rev	Sequence	Annealing temperature	Reference
AChR	Fwd Rev	5′-CCTTCATTGGGATCACCACG-3′ 5′-GGAGATGAGTACCAGCAGGTTG-3′	62.5°C	(Mungamuri et al., 2012)
GAPDH	Fwd Rev	5′-TACTAGCGGTTTTACGGGCG-3′ 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′	62.5°C	(Kawamoto et al., 2008)
P21	Fwd Rev	5′-CTGGACTGGGCACTCTTGTC-3′ 5′-CCCTTCCTCACCTGAAAACA-3′	62.5°C	(Mungamuri et al., 2012)
SUV1	Fwd Rev	5′-ACTCTTGTGGGTTGGACAGG-3′ 5′-GACAGACAGGAGCGAAGGTC-3′	62.5°C	
SUV2	Fwd Rev	5′-GAGTGGGACACGGTAGCAAT-3′ 5′-CATATCCAGGCTCTGCCATT-3′	62.5°C	
SUV3	Fwd Rev	5′-GCAACTTGAGGACGTGACAG-3′ 5′-CCAGCTGTGATTCCTGACAA-3′	62.5°C	

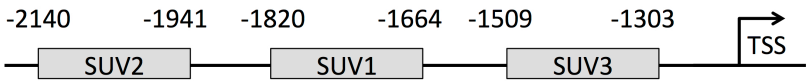

Figure 7.1: ChIP-qPCR primers targeted to the *SUV39H1* promoter.

TSS indicates the transcription start site. Numbers indicate the primer start site upstream of the TSS.

7.3.11 Statistical analysis

Each data point is an average of three biological replicates. Statistical significance was determined by a Student's t-test based on a p value of less than 0.05.

7.4 RESULTS

7.4.1 Exposure to IR induces differential gene expression at all stages of senescence

In order to better understand how cell cultures of different stages of senescence may react to X-ray irradiation, we exposed cultures at three different stages of senescence to 0.5 or 5 Gy irradiation and profiled the mRNA expression patterns using Illumina® Gene Expression BeadChips. The results revealed that exposure to 5 Gy of X-ray irradiation affected gene expression profiles more strongly than exposure to 0.5 Gy (Fig. 7.2A). Cultures with an intermediate senescence ratio (PD 47) showed the highest number of deregulated genes (77), followed by cultures with high senescence ratio (PD 54) with 59 deregulated genes and cultures with low senescence ratio (PD 38) with 26 deregulated genes. Of all these expression changes, there was a bias towards transcription repression (Fig. 7.2B). However, with increasing

senescence ratio of the cultures, the number of genes up-regulated in response to 5 Gy irradiation increased.

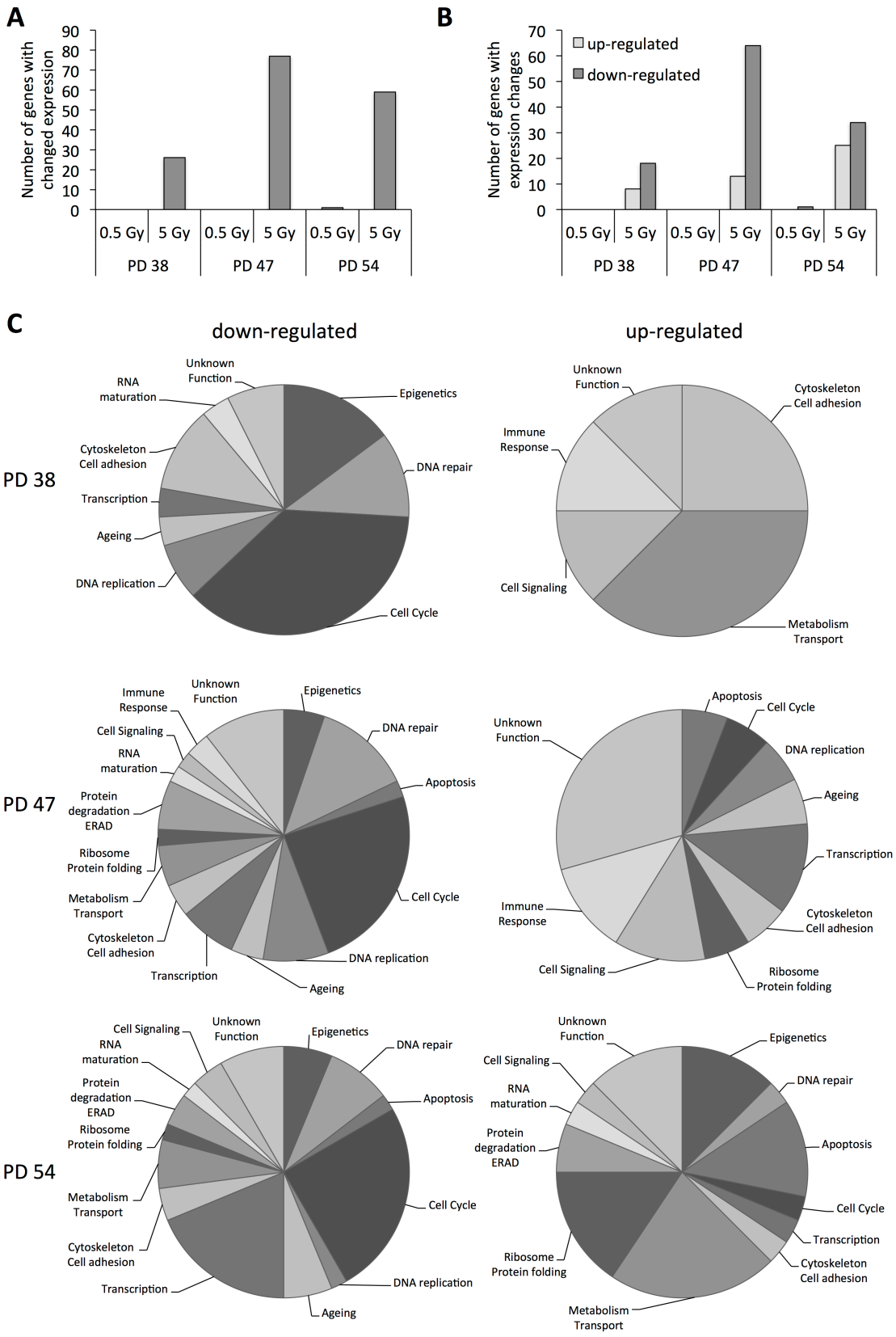

Figure 7.2: Functional classification of gene expression results.

A) Bar graph representing the total number of genes affected by expression changes in response to ionizing radiation. B) Bar graph showing the ratios of up- (light grey) and down-regulated (dark grey) genes in response to X-ray irradiation. C) Pie charts showing functional classification of differentially expressed genes.

Functional classification was performed to understand the functional implications of the observed changes in expression. The genes related to epigenetic regulation, DNA repair, cell cycle regulation, aging, regulation of transcription, and cell adhesion were among the genes down-regulated in response to 5 Gy irradiation in cells of all ages. Furthermore, protein and cell metabolism were also increasingly affected by transcriptional down-regulation with increasing PD of the culture (Fig. 7.2C). The functions that were commonly induced in response to 5 Gy irradiation included cell adhesion, metabolism, and cell signalling and in the older cultures (PD 47 and PD 54), DNA repair, apoptosis, and cell cycle regulation were also induced by irradiation (Fig. 7.2C).

7.4.2 X-ray irradiation induces G2/M arrest and senescence in pre-senescent cultures

IR is known to induce cell cycle arrest at the G1/S or G2/M checkpoints (Kuerbitz et al., 1992; Terasima and Tolmach, 1963), senescence (Ye et al., 2013), and apoptosis (Neal and Potten, 1981; Warters, 1992). Correspondingly, we observed down-regulation of cell cycle regulators in cultures of all ages and up-regulation of apoptotic genes in pre-senescent (PD 47) and senescent (PD 54) cultures. As the down-regulated cell cycle regulators made a larger fraction of the total expression

changes in the young group (PD 38) than in the PD 47 and PD 54 groups and as the apoptotic genes were only up-regulated in the latter two groups, we hypothesized that the physiology of the response to X-ray may differ in cultures of different senescence states.

To test this, we detected proliferating, senescent, and apoptotic cells in cultures exposed to 0.5 Gy or 5 Gy X-ray by using BrdU, SA-β-GAL, and Annexin V/PI staining respectively. Exposure to 5 Gy irradiation resulted in an increase in the populations of apoptotic and dead cells in cultures of all ages at 48 h post-exposure, although the differences were significant only in the PD 38 and PD 54 cultures (Fig. 7.3C). The observed increase in apoptotic cells was significantly higher in PD 54 than in PD 38 cells and correlated with the higher number of up-regulated apoptotic genes in the irradiated cells. Further, the number of apoptotic cells in 0.5 Gy-irradiated PD 38 cultures was significantly lower than in non-irradiated cells, while the number of dead cells was not significantly different between the two treatments.

The analysis of the cell cycle distribution based on the BrdU pulse and DNA staining at 48 h after exposure revealed a significant decrease in S phase cells in the PD 38 and PD 47 groups with increasing irradiation (Fig. 7.3A). This indicates that exposure to 0.5 and 5 Gy can induce cell cycle arrest. Similarly, exposure to 5 Gy resulted in an increased cell population in the G2/M phase of the cell cycle in PD 47 cells. However, no radiation-dependent changes in cell cycle distribution could be detected in PD 54 cultures (Fig. 7.3A).

Further, the ratio of senescent cells at 48 h after irradiation exposure increased in all cultures with increasing dose of irradiation (Fig. 7.3B). The highest increase in senescence ratio was observed in PD 38 cells exposed to 5 Gy.

In summary, the surviving fraction of cells in the low PD group either enters cell cycle arrest or senescence and the surviving fraction in the higher PD groups is smaller than that in younger cultures and they exhibit senescence rather than cell cycle arrest.

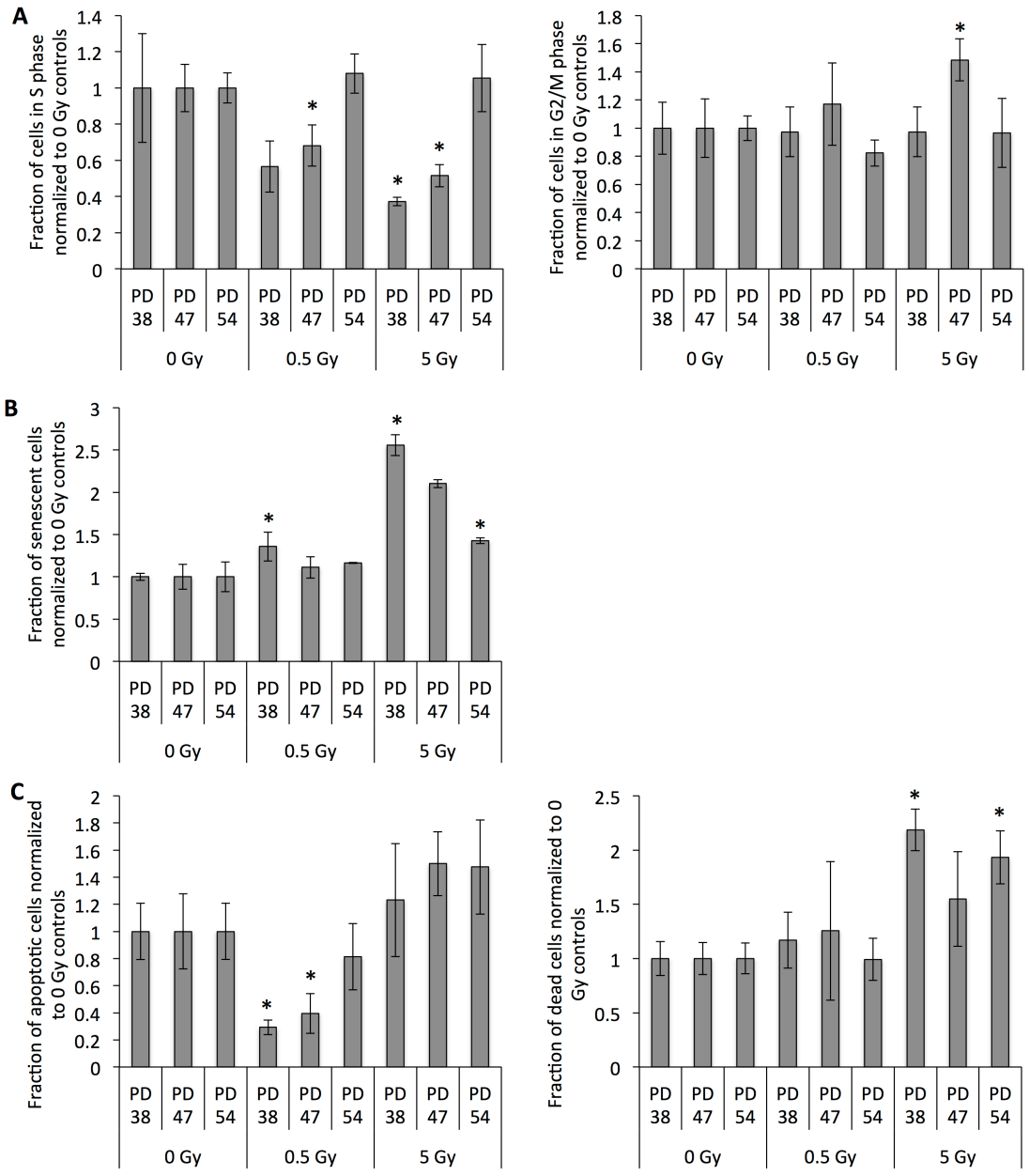

Figure 7.3: Radiation-induced cell growth arrest, senescence, and cell death.

A) Fraction of cells in the S or G2/M phase determined by BrdU incorporation relative to controls (0 Gy). Bars represent averages of three samples and error bars

indicate standard deviation. Asterisks on bars indicate significant differences from the control cells at the same PD level based on Student's t-test ($p < 0.05$). For clarity, only significant differences in response to X-ray irradiation within an age group are shown. B) Fraction of senescent cells normalized to the controls as determined by SA-β-GAL staining. Asterisks on bars indicate significant difference to the control of the same culture based on Student's t-test ($p < 0.05$). For clarity, only significant differences within an age group are shown. C) Fractions of apoptotic or dead cells normalized to the controls, as determined by Annexin V/PI staining. Asterisks indicate significance based on Student's t-test ($p < 0.05$).

7.4.3 Changes in cell cycle regulation and age-related gene expression as a possible cause for radiation-induced senescence

The observed increase in the senescence ratio of the irradiated cell cultures of all PD levels correlated with the changes in age-related gene expression. A comparison of the obtained gene expression profiles with microarray signatures from the Human Ageing Genomic Resources (de Magalhaes et al., 2009) and information from Genecards (Safran et al., 2010) showed that *CTGF*—a gene found up-regulated during fibroblast senescence—was up-regulated in PD 47 cells exposed to 5 Gy and *SUV39H1*—a histone methyltransferase that was found to be down-regulated in several aging model systems (see Chapters 4 and 5)—was down-regulated in PD 38 and PD 47 cells exposed to 5 Gy irradiation (Table 7.4).

Table 7.4: Senescence-associated gene expression.

Entries for genes that were up-regulated/down-regulated with age are based on microarray signatures from Human Ageing Genomic Resources (de Magalhaes et al., 2009). Other gene information is based on Genecards[24] (Safran et al., 2010). Numbers indicate log2 fold changes compared to the controls.

Gene	Involvement in Aging	PD 38		PD 47		PD 54	
		0.5 Gy	5 Gy	0.5 Gy	5 Gy	0.5 Gy	5 Gy
COL3A1	down-regulated with age					0.30	
CTGF	up-regulated with senescence				0.43		
MNT	may induce senescence						–0.44
SUV39H1	H3K9 methylation-dependent induction of senescence		–0.42		-0.43		
TIMP3	may be involved in senescence		0.29		0.37		

The expression levels of selected senescence-associated genes (*COL3A1* and *POT1*) and cell cycle regulators (*UBE2C*) were determined at the transcriptional level by qRT-PCR (Fig. 7.4A). *UBE2C* is an E2 ubiquitin-conjugating enzyme required for cell cycle progression and mitotic exit (Townsley et al., 1997) and was down-regulated in 5 Gy-irradiated PD 38 and PD 54 compared to their respective controls (Fig. 7.4A). Although the radiation-induced reduction of the *UBE2C* transcriptional level was not as extensive as that observed with increasing culture age, it may still contribute to radiation-induced cell cycle arrest.

COL3A1 is a type III collagen whose expression commonly decreases with increasing age, according to the Human Ageing Genomic Resources (de Magalhaes et al., 2009). On the other hand, collagens are known to be up-regulated during tissue fibrosis in response to IR (Walklin et al., 1987). In line with this finding, qRT-PCR

[24] www.genecards.org

revealed a reduction in the *COL3A1* transcript level with increasing culture age (Fig. 7.4A). Interestingly, the *COL3A1* transcript levels were unaffected in younger cultures (PD 38) exposed to IR; however, irradiated PD 47 and PD 54 cells showed an increasing *COL3A1* expression trend (Fig. 7.4A), which is in line with the results of the gene expression analysis and may indicate that senescent cells are more prone to radiation-induced fibrosis. This increase in *COL3A1* expression in response to radiation exposure probably reflects the changes in the extracellular matrix rather than the senescence status of cells, since it does not correlate with the observed increase in the senescence ratio of young cultures in response to IR.

POT1 is a part of the shelterin complex that binds to single-stranded (ss) DNA at the telomeres and prevents it from the aberrant activation of DNA damage signals, thereby protecting the cell from DNA damage-induced senescence (Hockemeyer et al., 2006; Wu et al., 2006). The transcriptional level of *POT1* decreased with increasing age of the culture; however, it was not affected by X-ray exposure other than a slight reduction in expression in the 0.5 Gy-irradiated PD 38 cells (Fig. 7.4A).

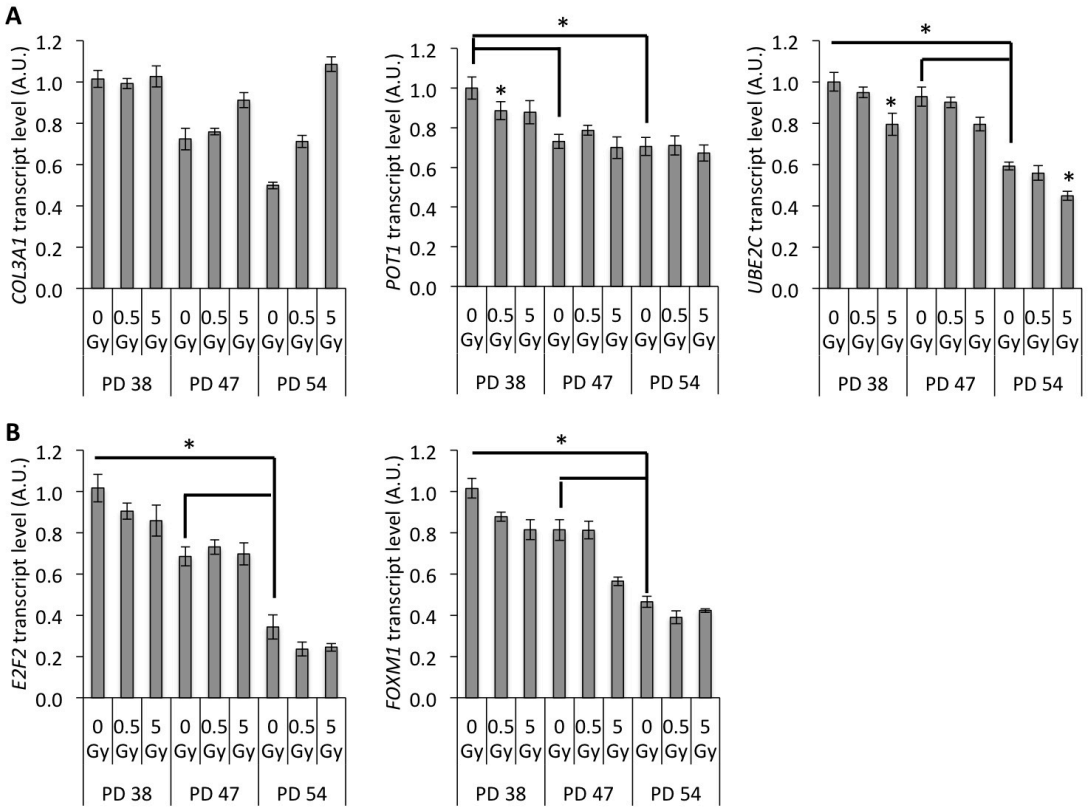

Figure 7.4: Changes in the expression of cell cycle regulators and senescence-associated genes with increasing age and X-ray irradiation dose.

A) Confirmation of the relative transcriptional levels of *UBE2C*, *COL3A1,* and *POT1* by qRT-PCR. Bars represent averages from three samples and two technical repeats normalized to the expression of housekeeping genes, and the expression in the control PD 38 cells. Error bars indicate error progression of standard deviations. Asterisks on top of the bars indicate significant difference to the control of the respective PD level. Asterisks on top of lines indicate a significant difference between equal radiation exposures of two different PD levels. Significance is based on Student's t-test ($p < 0.05$). B) *E2F2* and *FOXM1* transcriptional levels measured by qRT-PCR. Bars represent averages from three samples and two technical repeats normalized to the expression of housekeeping genes and expression in the control PD 38 cells. Error bars indicate error progression of standard deviations. Asterisks on top of the bars indicate significant difference to the controls of the respective PD level,

and asterisks on top of lines indicate a significant difference between equal radiation exposures of two different PD levels. Significance is based on Student's t-test ($p < 0.05$).

As senescence is associated with the coordinated repression of numerous genes, including E2F target genes (Chapter 5) (Narita et al., 2003) and as transcriptional regulators are affected by IR-induced changes in gene expression (Fig. 7.2), we hypothesized that changes in the transcriptional regulation may contribute to the establishment of a senescence phenotype in response to IR. To test this, we studied the mRNA expression patterns of two transcription factors, namely, *E2F2* and *FOXM1,* which were implicated in affecting senescence-associated expression patterns (see Chapter 5) using qRT-PCR. The E2F transcription factors E2F1, E2F2, and E2F3 have been shown to play an essential role in G1/S progression (Wu et al., 2001), and their down-regulation has been shown to result in p53-mediated senescence (Sharma et al., 2006; Timmers et al., 2007). In our study, the *E2F2* transcriptional levels significantly decreased in cultures with increasing senescence ratios and the *E2F2* expression tended to reduce following exposure of PD 38 and PD 54 cultures to IR (Fig. 7.4B).

FOXM1 is a forkhead box transcription factor involved in the regulation of the G2/M cell cycle progression. It requires interaction with cyclin-CdK complexes for its activation and then recruits p300/CBP histone acetyltransferase complexes in order to mediate the transcriptional activation of its target genes (Major et al., 2004), which include many genes involved in G2/M transition as well as in the progression of mitosis (Laoukili et al., 2005). *FOXM1* was previously described to be down-regulated in patients with progeria as well as during normal aging (Ly et al., 2000).

Similarly, in our study, we found that the *FOXM1* transcript levels significantly decreased with increasing senescence ratio of the cell culture (Fig. 7.4B). In addition, the *FOXM1* transcript levels tended to decrease in irradiated PD 38 and PD 47 cultures (Fig. 7.4B).

7.4.4 Reduced SUV39H1 expression in irradiated cultures correlates with reduced H3K9 trimethylation

In addition to transcriptional regulation, changes in epigenetic regulation may underlie some of the observed changes in gene expression patterns. As both *SUV39H1* and *CBX5*, which binds to trimethylated H3K9 (H3K9me3) and mediates its repressive function, were consistently down-regulated in PD 38 and PD 47 cells exposed to 5 Gy irradiation, we hypothesized that changes in the chromatin structure may be involved in this response.

When measuring the *SUV39H1* expression at the mRNA and protein levels by qRT-PCR and Western blot analysis respectively, we detected a significant senescence-dependent decrease at both the mRNA and protein levels (Fig. 7.5A, B). In addition, results of the Western blot analysis revealed that the SUV39H1 levels were significantly decreased in the PD 38 and PD 47 groups following IR exposure (Fig. 7.5B). This finding was further correlated with a significant decrease in the H3K9me3 levels in response to senescence and IR exposure in the PD 47 group (Fig. 7.5B). Interestingly, similar observations were made for the global Histone 3 levels, although this reduction was less severe than the reduction in H3K9me3 levels (Fig. 7.5B). Taken together, these results may suggest that the down-regulation of SUV39H1 in response to IR results in a loss of heterochromatin, which may result in altered gene expression or genomic instability.

As this down-regulation of *SUV39H1* and H3K9me3 levels with increasing age or stage of senescence has been observed in multiple models (see Chapters 4 and 5) (Scaffidi and Misteli, 2006), it probably plays an important role in establishing senescence-related changes to the chromatin structure.

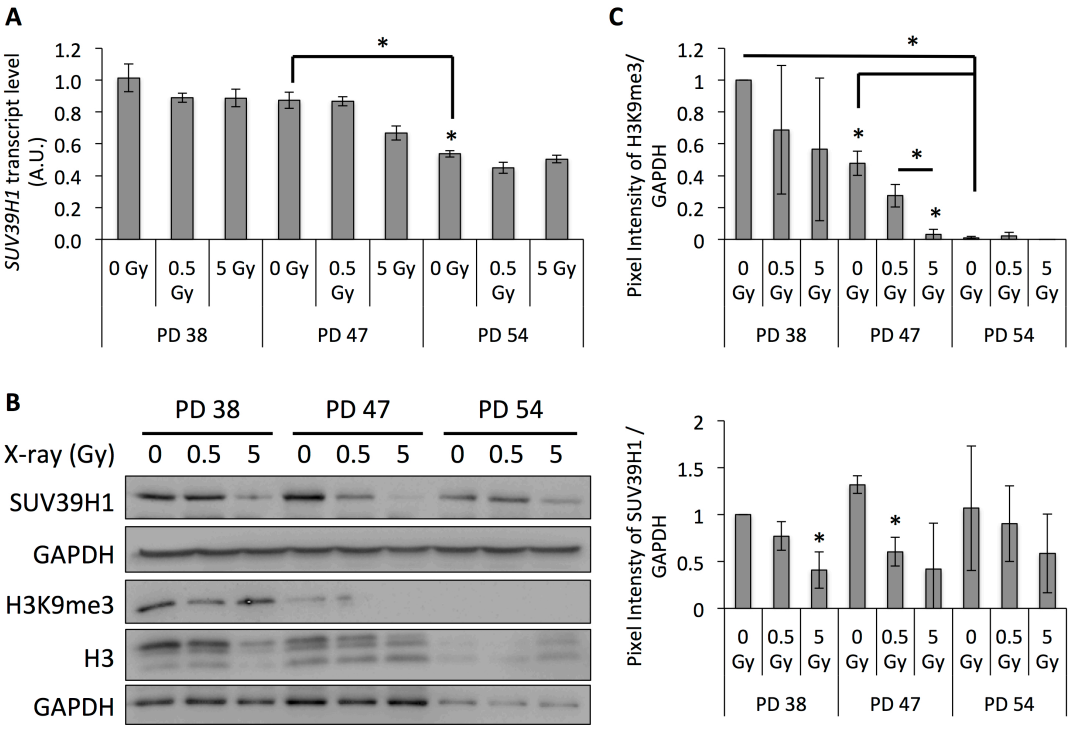

Figure 7.5: Regulation of SUV39H1 expression and correlation with H3K9me3 levels in response to IR.

A) *SUV39H1* transcript levels as measured by qRT-PCR. Bars represent averages of three biological repeats with two technical replicas each. Error bars indicate error progression of standard deviations from technical replicas. Significance is based on the Student's t-test ($p < 0.05$). B) Representative images of Western blots. C) Western blot analysis of the SUV39H1 and H3K9me3 protein levels. Bar graphs represent averages of three biological replicas ± standard deviation. Significance is based on the Student's t-test ($p < 0.05$).

If this down-regulation of SUV39H1 indeed plays a role in the establishment of senescence, it is expected to occur around the time that cells usually recover from the DNA damage checkpoint, which is between 12 and 24 h after exposure to irradiation (Xu et al., 2002a). In order to test this, we examined *SUV39H1* transcript and protein levels and studied the induction of senescence based on SA-β-GAL activity in PD 38 cells exposed to 5 Gy X-ray irradiation at different time points after exposure to IR (Fig. 7.6). Our results revealed that the *SUV39H1* transcript levels were slightly reduced at 6 h after exposure and were significantly decreased at 24 and 48 h after exposure to IR (Fig. 7.6A), which corresponded to the reduced protein levels at the same time points (Fig. 7.6B). This was associated with the trend of reduced H3K9me3 levels and the induction of senescence, which was significantly increased 24 h after exposure to 5 Gy X-ray irradiation (Fig. 7.6B and C).

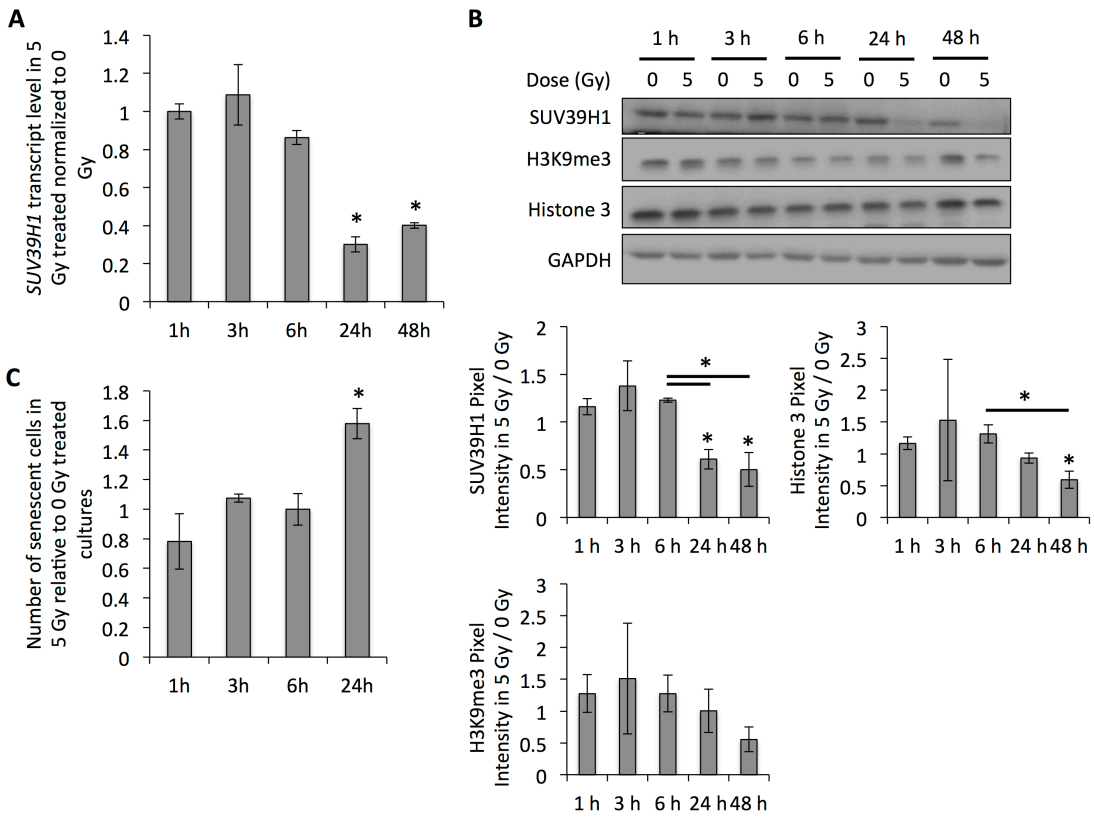

Figure 7.6: SUV39H1 down-regulation occurs during the first 24 h after exposure.

A) *SUV39H1* transcript levels normalized to *HPRT1* and *YWHAZ* expression and to the expression level 1 h after exposure. Bars represent averages from three biological and two technical replicates per sample. Error bars indicate the error progression of the standard deviations from the technical replicates and asterisks using the Student's t-test to show significance ($p < 0.05$). B) Western blot images and quantification of the band intensities. Bars represent the averages of three biological replicates, error bars indicate standard deviations, and asterisks show significance based on the Student's t-test ($p < 0.05$). C) Fraction of senescent cells, as determined by SA-β-GAL staining. Bars represent averages of three samples, error bars indicate standard deviations, and asterisks show significance based on the Student's t-test ($p < 0.05$).

7.4.5 SUV39H1 down-regulation correlates with expression of satellite transcripts and DNA damage checkpoint

SUV39H1 is important for pericentric heterochromatin formation and silencing of satellite regions (Lehnertz et al., 2003; Peters et al., 2001). Our previous results indicated that the senescence-associated down-regulation of SUV39H1 resulted in the loss of H3K9me3 in satellite regions and increased the expression of satellite-derived transcripts (Chapter 5). Thus, we next tested whether the radiation-induced down-regulation of SUV39H1 was associated with increased expression of satellite transcripts. Our results showed that the *SAT2* and *majSAT* transcripts were induced in IR-exposed PD 38 cells and PD 47 cells, although for PD 47 cells this induction was only significant for *majSAT* transcripts (Fig. 7.7). Furthermore, both irradiated PD 38 and PD 47 cells exhibited a trend of increased *αSAT* expression (Fig. 7.7). However, in PD 54 cells, only exposure to 0.5 Gy IR induced an increase in *majSAT* transcription (Fig. 7.7).

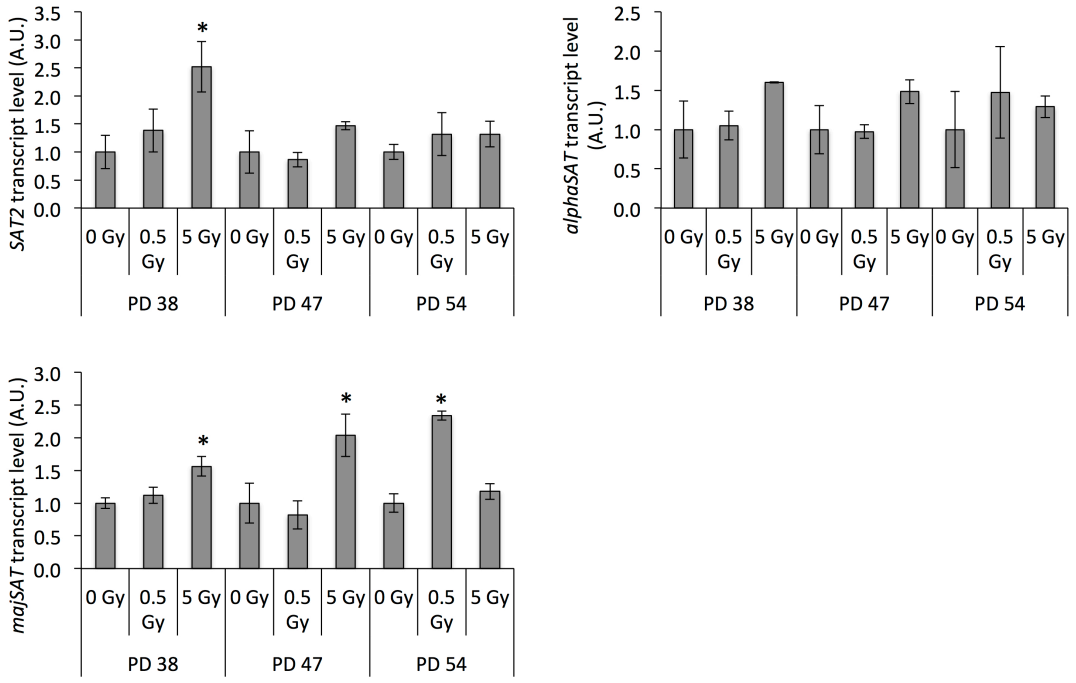

Figure 7.7: Radiation exposure results in genomic instability.

The bar graphs show the average transcript levels of satellite transcripts normalized to the *HPRT1* and *YWHAZ* expression levels. Error bars indicate the error progression of standard deviations and asterisks indicate significance based on Student's t-test ($p < 0.05$).

In order to examine whether this increased chromatin relaxation and transcription of satellite regions correlated with genomic instability and DNA damage checkpoint activation, the protein levels of DNA damage checkpoint regulators were detected by Western blotting. Results revealed that exposure to increasing doses of X-ray irradiation resulted in the down-regulation of CHK1 at all levels of senescence, while the remaining CHK1 proteins showed increased phosphorylation levels at Ser345 (Fig. 7.8). The CHK2 protein and phosphorylation levels showed an opposite trend, where IR exposure resulted in a slight increase in the CHK2 protein levels and a reduction in the phosphorylation levels of Thr68 (Fig. 7.8).

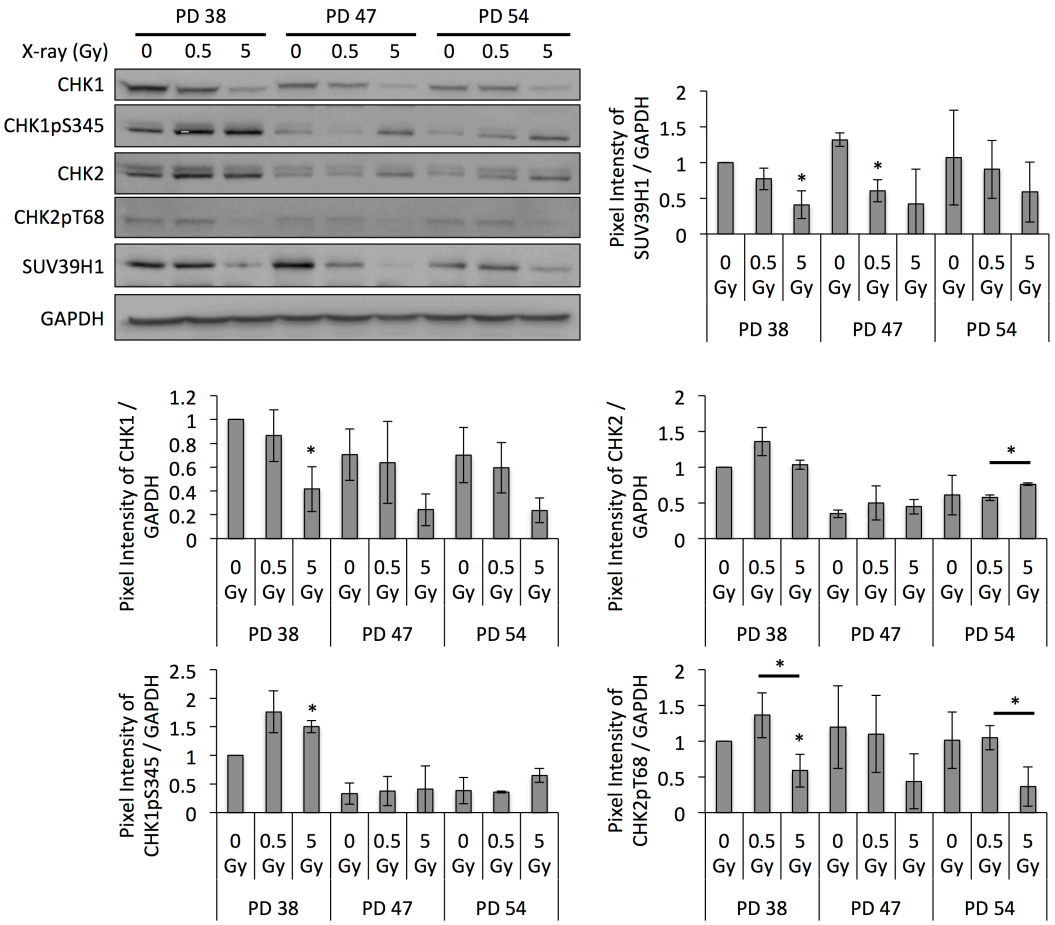

Figure 7.8: Altered DNA damage checkpoint regulation.

Representative Western blot images and bar graphs depicting the quantification of band intensities. Bars represent the averages from three biological replicates. Error bars indicate standard deviation, and asterisks indicate significance based on the Student's t-test ($p < 0.05$).

7.4.6 SUV39H1 over-expression inhibits radiation-induced premature senescence

Our previous study showed that the inhibition of SUV39H1 resulted in the inhibition of cell division and the induction of senescence in pre-senescent cells (Chapter 5). The observed down-regulation of SUV39H1, the reduction in H3K9me3 levels and the induction of satellite transcription after exposure to IR, resembled the previously described senescence-dependent changes. Thus, as a next step, the role of

SUV39H1 in radiation-induced senescence was further examined by over-expressing *SUV39H1* in PD 38 cells prior to exposure to 5 Gy of X-ray irradiation. The over-expression of *SUV39H1* before IR exposure prevented the IR-dependent induction of senescence (Fig. 7.9A), while pretreatment of the cells with chaetocin only very slightly reduced the induction of senescence (1.242fold increase in senescent cells after 5 Gy IR in control cells compared to 1.235fold increase in senescent cell after the irradiation of chaetocin-treated cells) (Fig. 7.9B).

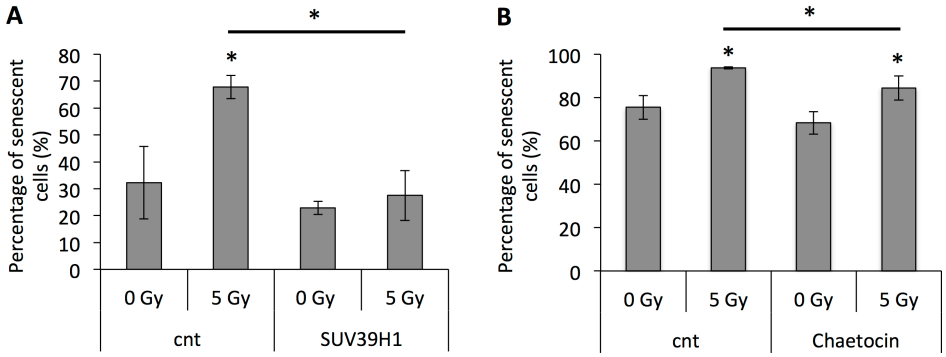

Figure 7.9: SUV39H1 over-expression inhibits radiation-induced senescence.

Bar graphs represent the averages of senescent cells after radiation exposures, when cells had been pre-treated with an *SUV39H1* over-expression vector (A) or an SUV39H1 inhibitor (B). Error bars indicate standard deviation and asterisks indicate significance based on Student's t-test ($p < 0.05$).

7.4.7 p53 may regulate SUV39H1 expression and SUV39H1-mediated senescence after exposure to IR

Since the IR-dependent down-regulation of SUV39H1 seemed to play an important role in the induction of senescence, the next step was to examine what might cause this down-regulation. To this end, we scanned the promoter region of *SUV39H1* for

transcription factor-binding sites (TFBS) using several software programs – TFSEARCH v1.3, Genomatrix Software Suite, PROMO (Messeguer et al., 2002) and TFBIND (Tsunoda and Takagi, 1999). Results revealed that the promoter region contains several potential TFBS for p53. p53 was previously shown to indirectly down-regulate *SUV39H1* expression through the p21-mediated suppression of E2F activity during the DNA damage response following IR, in order to allow for efficient repair of DNA double-strand breaks within heterochromatin regions (Zheng et al., 2014). Mungamuri *et al.* (2012) showed that *SUV39H1* transcription was repressed in a p21-dependent manner after the induction of ectopic p53 expression (Mungamuri et al., 2012). In addition, MDM2, another p53 target gene, contributed to the decrease in SUV39H1 protein levels through inducing its proteasomal degradation (Mungamuri et al., 2012).

In order to determine whether p53 plays a role in the regulation of *SUV39H1* expression to mediate IR-induced senescence, we determined the protein levels of p53 as well as its phosphorylated and acetylated (Luo et al., 2004) active forms by Western blot analysis (Fig. 7.10). While the p53 protein levels were slightly induced in IR-exposed PD 38 and PD 47 cells, their phosphorylation levels remained almost unaffected (Fig. 7.10). However, acetylation at K382 of p53 significantly increased in IR-exposed PD 38 cells, and a similar trend was observed in the IR-exposed PD 47 and PD 54 cells (Fig. 7.10).

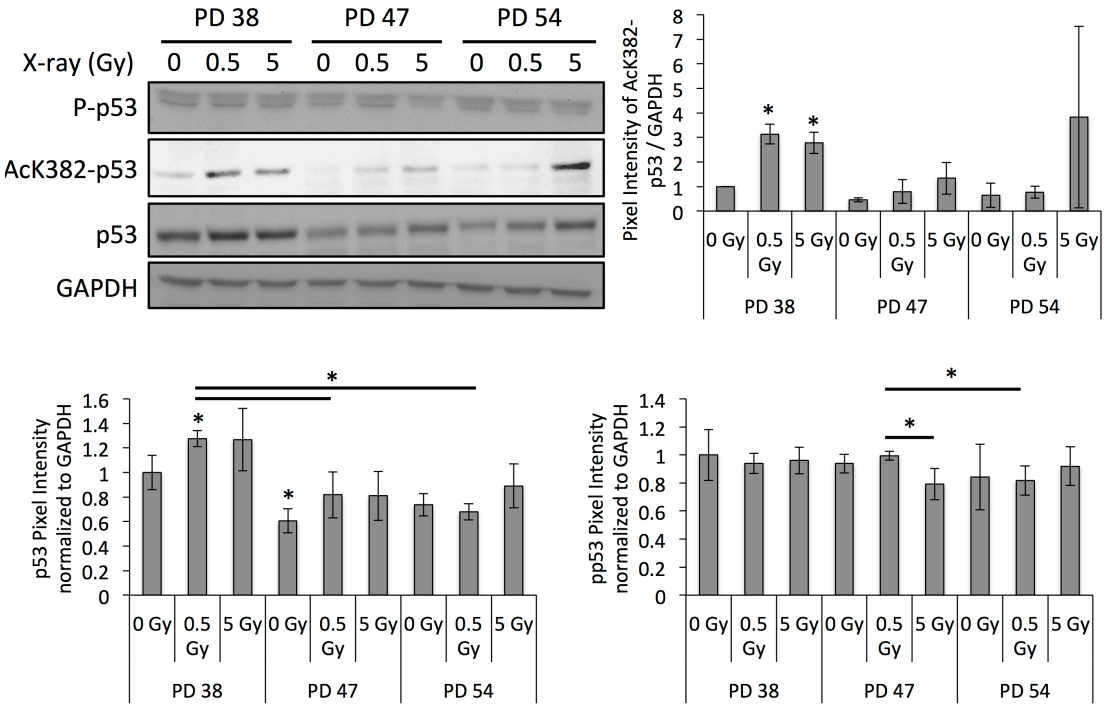

Figure 7.10: Acetylation of K382 of p53 is strongly induced by X-ray irradiation.

Representative images of Western blots and bar graphs showing the quantification of band intensities. The bars represent averages from three biological replicates, error bars indicate standard deviation, and asterisks show significance based on Student's t-test ($p < 0.05$).

p53 acetylation has been previously shown to be associated with the activation of p53-mediated transcriptional regulation (Luo et al., 2004). In order to test whether or not p53 or acetylated p53 directly bind to the *SUV39H1* promoter and whether this was affected by exposure to IR, chromatin immunoprecipitation experiments using antibodies targeted to p53 or acetylated p53 were performed. The results revealed the increase in both p53 and acetylated p53 levels in the promoter regions of *p21* after exposure to 5 Gy X-ray irradiation (Fig. 7.11). The p53-dependent increase in the activation of *p21* transcription has been associated with the indirect inhibition of *SUV39H1* transcription, but it also serves as a positive control for the specificity of

the pulldown of p53 and acetylated p53. While the amplification of the SUV39H1 promoter fragments was not significantly different when comparing the IgG negative control and p53 pulldown, it was significantly higher in the acetyl-p53 pulldown samples when compared to IgG controls (Fig. 7.11). Additionally, the amplification of the *SUV1* amplicon was significantly reduced in 5 Gy when compared to the 0 Gy control samples (Fig. 7.11), suggesting that the binding of active, acetylated p53 to the *SUV39H1* promoter is reduced in response to IR.

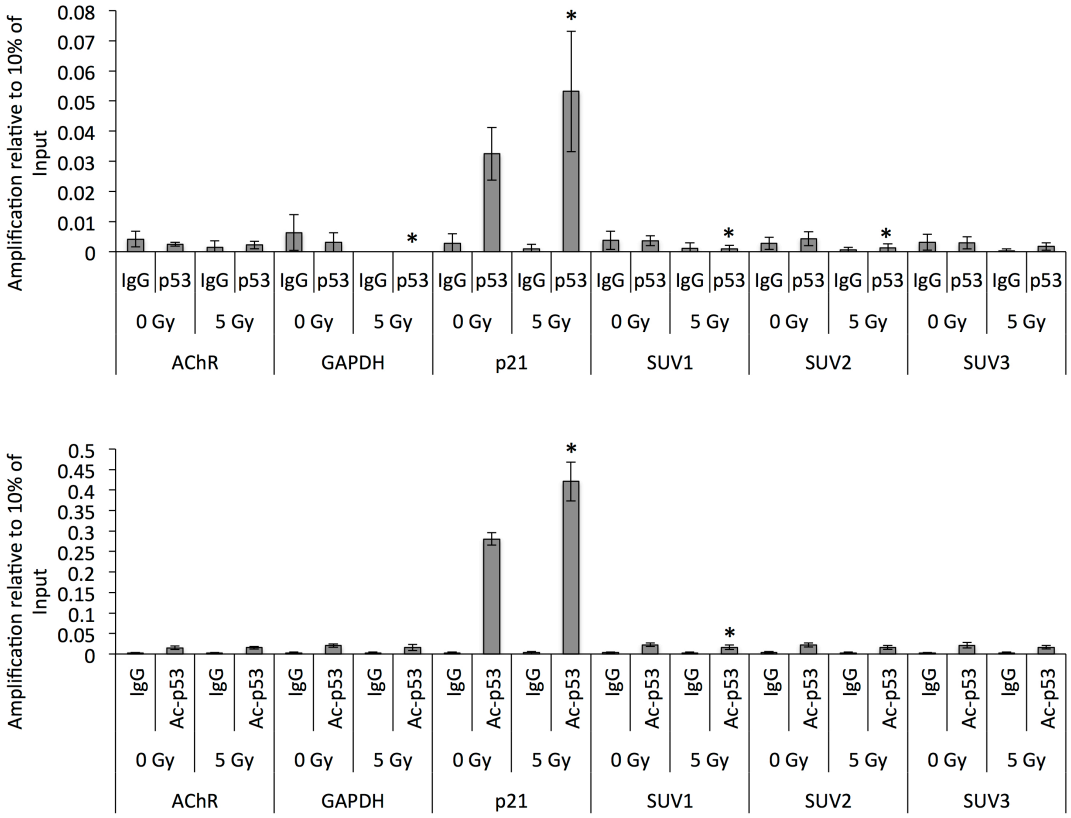

Figure 7.11: Chromatin immunoprecipitation reveals reduced binding of acetylated p53 to the *SUV39H1* promoter.

Bar graphs show amplification normalized to 10% of the amplification from input DNA fragments. Bars represent averages from three biological replicates, error bars

indicate standard deviation, and asterisks show significance based on Student's t-test ($p < 0.05$).

7.4.8 Changes in the DNA methylation patterns inversely correlate with the induction of senescence

Changes in the distribution of repressive histone marks were previously shown to correlate with changes in DNA methylation patterns in the affected genomic regions of senescing human mesenchymal stem cells (Schellenberg et al., 2011). On the other hand, IR is known to affect DNA methylation profiles as well. Several studies have shown that sequence-specific changes in DNA methylation occur after IR and persist through several cell divisions (Kaup et al., 2006; Kovalchuk et al., 2004b). Thus, we hypothesized that IR may affect DNA methylation patterns in a senescence-dependent way.

To investigate this hypothesis, we profiled the CpG sites affected by changes in DNA methylation. The results revealed that treatment with 0.5 Gy irradiation induced a higher number of differentially methylated CpG sites than treatment with 5 Gy irradiation in the PD 38 and PD 47 cultures (Fig. 7.12A). Further, a higher number of CpG sites were affected by differential methylation in the younger cultures (PD 38 and PD 47) exposed to IR than in the older culture (PD 54). Among the observed changes in methylation, hypermethylation was more frequent than hypomethylation (Fig. 7.12B), which is in line with previously reported observations (Kaup et al., 2006).

Interestingly, the clustering analysis revealed that samples clustered primarily based on the stage of senescence of the cultures rather than according to the dose of radiation received (Fig. 7.12C). This may explain why only very few CpG sites

showed a significantly altered methylation level and why there were just a few sites that were differentially methylated in more than one treatment group (Table 7.5). While the changes in the methylation levels of those CpG sites did not correlate with changes in the gene expression of the genes in those regions, GSTM1 polymorphisms have been associated with cancer susceptibility (Ates et al., 2005; Tamer et al., 2005), and it is thought to play a role in the response to IR. However, the exact role of GSTM1 in genotoxic stresses is not fully understood. Further, TYK2 may play a role in mediating changes in inflammatory responses (Velazquez et al., 1992). However, while such changes in methylation may enable cells to adapt to IR, the functional implications of these methylation changes remain to be determined.

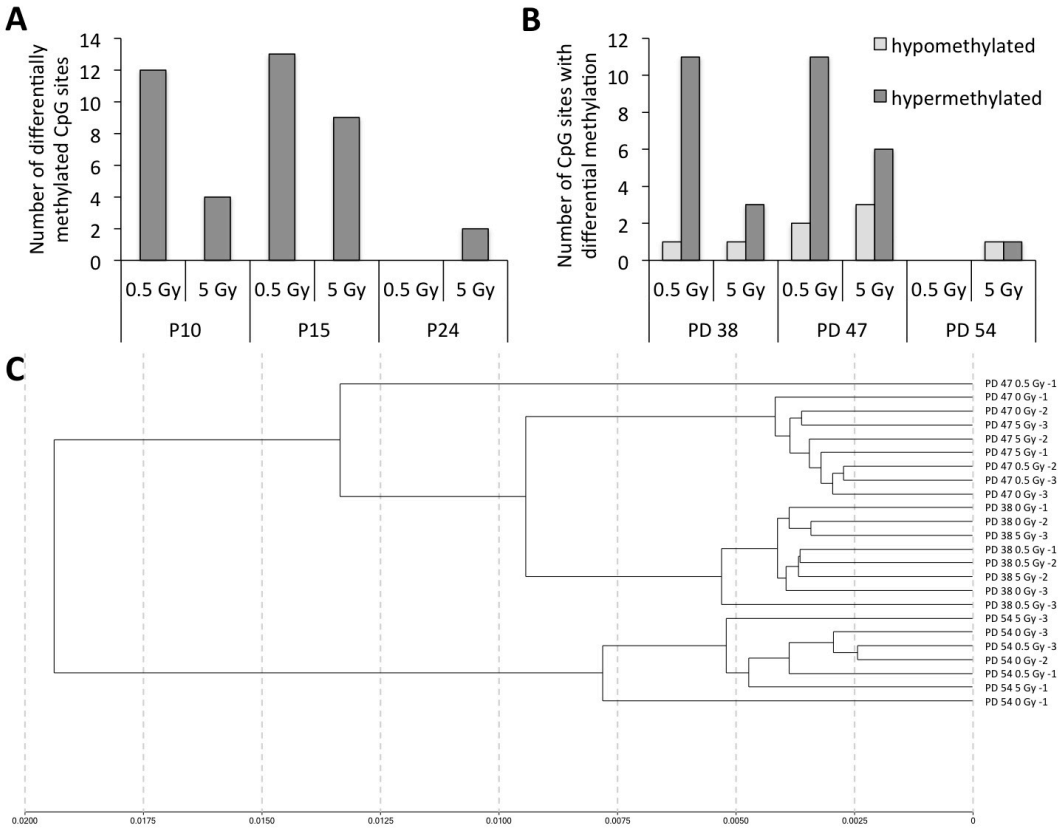

Figure 7.12: Changes in the DNA methylation pattern in response to senescence and X-ray irradiation.

A) Bar graphs represent the numbers of CpG sites affected by differential methylation when comparing cells of different passages or exposed to different doses of X-ray irradiation. B) Ratios of hyper- (dark grey) and hypo- (light grey) methylated CpG sites in irradiated pre-senescent and senescent cultures. C) Cluster analysis.

Table 7.5: CpG sites affected by change in methylation in more than one experimental group.

		PD 38		PD 47		PD 54	
	Genes in this region	0.5 Gy	5 Gy	0.5 Gy	5 Gy	0.5 Gy	5 Gy
Chr1:110031996	GSTM1			-	-		
Chr9:113976778	SUSD1		+	+			+
Chr16:30841835	LOC283932			+	+		
Chr17:38531106	NBR2	+		+			
Chr19:10352577	TYK2			-	-		

+ indicates hypermethylation, – indicates hypomethylation

7.5 DISCUSSION

Here, we showed that WI-38 cells with low PD display a distinct physiological response to IR when compared to cells with higher PD. While cell cycle arrest, apoptosis, and most prominently senescence was induced in response to ionizing radiation in low PD cultures, high PD cultures showed an increase in apoptosis and cell death in response to IR but no significant induction of cell cycle arrest and only a slight increase in the percentage of senescent cells. The induction of premature senescence in response to IR has been previously described (Noda et al., 2012; Suzuki et al., 2001) and both senescence and apoptosis may contribute to several of the observed side effects of radiation therapy *in vivo*, such as tissue inflammation and fibrosis (Gallet et al., 2011), as well as developmental deficits in paediatric patients (Krasin et al., 2010).

To better understand the molecular changes associated with this increased senescence ratio in irradiated WI-38 cultures, we studied the changes in gene expression profiles and epigenetic regulation. Cell cycle regulation-related genes comprised the largest fraction of down-regulated genes in the 5 Gy-exposed groups at all PD states; this correlates with the observed changes in the cell cycle profiles in PD 38 and PD 47 and the induction of senescence. However, other functional categories, including epigenetic and transcriptional regulators, were also affected by exposure to 5 Gy X-ray irradiation. A more diverse set of functional groups were up-regulated when exposed to IR. While genes related to metabolic functions were prominently induced in the PD 38 and PD 54 cultures, none of the genes related to metabolic function were induced in the PD 47 group. On the other hand, transcriptional and epigenetic regulators were only up-regulated in the PD 47 and PD 54 cultures.

In line with the observed physiological response to IR, the transcription of senescence-associated genes and cell cycle regulator genes was altered. The transcript levels of *UBE2C*, encoding an E2 ubiquitin ligase that is involved in mitotic exit and G1/S transition, were significantly decreased in senescing cultures as well as PD 38 and PD 54 cultures exposed to 5 Gy radiation. This is probably associated with the reduced proliferative capacity of higher PD cultures as well as IR. This finding corresponded to the senescence-dependent reduction in the *E2F2* and *FOXM1* transcript levels. Both E2F2 and FOXM1 are associated with proliferation, and down-regulation of their transcription is observed during aging and may induce senescence (Ly et al., 2000; Sharma et al., 2006).

Further, *COL3A1* has been shown to be down-regulated with age (de Magalhaes et al., 2009) but is induced during tissue fibrosis (Walklin et al., 1987). Thus, the observed reduction in the *COL3A1* transcript levels in senescing cultures is in

accordance with the previously described reduction of collagen expression during aging. However, the trend of induced *COL3A1* expression in 5 Gy-exposed PD 47 and PD 54 cells may indicate that IR-induced collagen expression is more pronounced in a senescence-associated microenvironment and may contribute to radiation-induced fibrosis.

The *POT1* transcript levels were significantly decreased in response to 0.5 Gy IR in PD 38 as well as in response to senescence. POT1 protects telomeres from permanent induction of DNA damage signalling, thereby preventing premature senescence (Wu et al., 2006). Thus, the observation that IR does not significantly decrease the *POT1* transcript levels may suggest that the observed induction of senescence in IR-exposed PD 38 and PD 47 is independent of telomere integrity, which is in line with previous reports (Suzuki et al., 2001).

In addition to changes in the gene expression profiles and transcriptional regulation, senescence has also been associated with changes to chromatin structure and epigenetic regulation. The observation that both *SUV39H1*, a histone methyltransferase that specifically methylates H3K9 (Rea et al., 2000), and *CBX5*, which is an HP1 homolog that binds to H3K9me3 and mediates its repressive function (Bannister et al., 2001; Lachner et al., 2001), are down-regulated in IR-treated PD 38 and PD 47 cells suggests that they may play a role in the response to IR. This down-regulation of *SUV39H1* was confirmed at the RNA and protein levels and was found to be correlated with reduced H3K9me3 levels.

In contrast with our observations, however, another study described an increase in the SUV39H1 and H3K9me3 protein levels during senescence or in response to genotoxic or oxidative stress (Bosch-Presegue et al., 2011). However, this may depend on the time points considered: while an increase in the SUV39H1 protein

levels during the initial stress response or establishment of senescence may be important to limit genomic instability or adjust gene expression patterns such as by silencing the promoters of E2F target genes (Narita et al., 2003; Nielsen et al., 2001), the down-regulation of SUV39H1 may be relevant for the maintenance of cell cycle arrest or senescence in the case of unsuccessful DNA repair.

The disruption of the human *SUV39H* genes *SUV39H1* and *SUV39H2* has been previously shown to induce a loss of H3K9me3 in constitutive heterochromatin regions, including the telomere regions (Garcia-Cao et al., 2004), thereby resulting in de-heterochromatinization and genomic instability (Peters et al., 2001). In addition, *SUV39H1* has also been shown to act as a co-repressor to several transcription factors by its recruitment to and histone methylation in the promoter regions of specific genes (Cherrier et al., 2009; Jang et al., 2007; Mungamuri et al., 2012; Nielsen et al., 2001; Wakabayashi et al., 2011). Further, SUV39H is also required for chromatin condensation and mitotic progression (Melcher et al., 2000; Park et al., 2011). The observed down-regulation of *SUV39H1* in response to IR may therefore result in G2/M arrest or de-heterochromatinization resulting in genomic instability and stress-induced premature senescence.

We observed increased transcriptional abundance from satellite regions that are normally kept silent through SUV39H1-dependent H3K9 trimethylation, suggesting that the IR-induced down-regulation of SUV39H1 induced the heterochromatin relaxation in the pericentric satellite regions. Our previous results showed such an induction of transcriptional activation of the pericentric satellite regions to be associated with the reduction of H3K9me3 levels in those regions during replicative senescence (Chapter 5), suggesting that the heterochromatin relaxation in those regions may contribute to genomic instability and growth arrest.

This transcriptional activation of the satellite regions further correlated with the continued activation of CHK1, while the protein levels of CHK1 were reduced. CHK1 expression has been described to be cell cycle-dependent from the S to the M phases of the cell cycle (Kaneko et al., 1999), and CHK1 induced in response to DNA damage is responsible for long-term G2/M cell cycle arrest, as well as for cell cycle re-entry followed by apoptosis or G1 arrest and senescence, depending on the p53 and p21 status of the cell (Poehlmann et al., 2011). Thus, the observed continued phosphorylation of CHK1 may indicate that the cells exposed to X-ray irradiation are in a prolonged G2/M arrest 48 h post-exposure or have slipped into a G1 arrest. Interestingly, CHK1 has also previously been shown to be down-regulated in response to DNA damage or ectopic p53 activation in a p21- and RB-dependent manner (Gottifredi et al., 2001). The same regulatory pathway was associated with the down-regulation of SUV39H1 expression in response to DNA damage (Zheng et al., 2014). Thus, CHK1 and SUV39H1 may be co-regulated during the long-term DNA damage response and may mediate the slippage into the G1 phase of the cell cycle followed by senescence. Such a slippage from the G2 to the G1 phases of the cell cycle has been previously described in long-term G2-arrested uveal melanoma cells, and has been associated with the down-regulation of G2 marker expression along with the transcriptional induction of G1 markers (Ye et al., 2013). The reduced SUV39H1 expression may contribute to this through the loss of H3K9 trimethylation in corresponding promoter regions; however, further investigation is required to verify this.

Interestingly, a recent study reported that the *SUV39H1* expression level decreased in response to DNA damage in a p53-dependent manner, resulting in the increased expression of pro-apoptotic genes (Mungamuri et al., 2012). In addition to this

induction of pro-apoptotic genes, the p53-induced down-regulation of SUV39H1 may be crucial for the relaxation of heterochromatin in order to promote DNA repair (Zheng et al., 2014). These results support the role of SUV39H1 in the DNA damage response by mediating changes in gene expression as well as genome stability.

While these previous studies have focussed on the effect of SUV39H1 deregulation on the induction of apoptosis or DNA repair, we demonstrated that this deregulation may also play a role in the induction of senescence. Our previous results showed that *SUV39H1* over-expression in senescent cells induced cell division, while SUV39H1 inhibition in dividing cells slightly inhibited cell division (Chapter 5). The over-expression of *SUV39H1* prior to exposure to IR compensated for the radiation-induced down-regulation of *SUV39H1*, preventing the induction of senescence.

The down-regulation of *SUV39H1* in response to IR also seems to be at least in part dependent on p53, since it is correlated with the induction of K382 acetylation of p53 and the reduction of acetylated p53 in the promoter region of *SUV39H1*. Together with the previous reports that showed the indirect effect of p53-mediated transcriptional regulation on the expression of *SUV39H1* (Mungamuri et al., 2012; Zheng et al., 2014), these results may indicate a crucial role for this regulatory pathway in the long-term response to DNA damage.

Additionally, SUV39H1 and trimethylated H3K9 have been shown to recruit DNA methyltransferases at least in some sequence contexts and target them for DNA methylation (Fuks et al., 2003; Lehnertz et al., 2003). Thus, the reduced expression of SUV39H1, which correlates with the reduction in H3K9me3 levels, may also correlate with changes in DNA methylation.

Investigating the DNA methylation levels showed that IR induces changes in DNA methylation in the cells. In this case, younger cultures (PD 38 and PD 47) were more

affected and exhibited a higher number of differentially methylated CpG sites in response to 0.5 Gy and 5 Gy IR. Further, site-specific CpG hypermethylation was observed more frequently than CpG hypomethylation, which is in line with previous observations (Kaup et al., 2006). As the Illumina® HumanMethylation27 BeadChip primarily contains probes that detect methylated CpGs in the promoter regions of genes (including miRNA genes), it enables the identification of changes in DNA methylation that are mainly considered to play roles in the regulation of gene expression. While the expression of the genes was not affected by the differential methylation in their promoter regions, these changes may have allowed the cells to adapt to the IR. Further, the observation that the DNA methylation profiles are similar between cultures of the same PD level regardless of the dose of radiation received may indicate that either the changes in IR-induced DNA methylation show a high variability between cells or that the potential DNA methylation changes induced by IR do not predominantly affect the CpGs within promoter regions.

On the other hand, the observation that the highest number of changes in DNA methylation was observed in 0.5 Gy-irradiated PD 38 and PD 47 cells, followed by 5 Gy-irradiated PD 38 and PD 47 cells may indicate that changes in CpG methylation following IR exposure may depend on cell division. Accordingly, the numbers of differentially methylated CpG sites detected in the different experimental groups roughly inversely correlated with the extent to which the cultures underwent IR-induced senescence. A recent study showed that human diploid fibroblasts and normal human bronchial epithelial cells that were exposed to ^{137}Cs and did not undergo any further cell division after IR exposure did not show any significant changes in DNA methylation (Lahtz et al., 2012). Thus, senescent cells may be less susceptible to changes in DNA methylation in response to IR when compared to dividing cells.

In summary, we demonstrated that human diploid fibroblasts at three different stages of senescence showed different physiological responses to IR. While cultures with low and intermediate senescence ratios (PD 38 and PD 47) showed induction of G2/M cell cycle arrests, all the cultures contained a higher percentage of apoptotic cells 48 h after exposure to 5 Gy IR. More significantly, PD 38 and PD 47 cells exposed to IR underwent senescence. This was associated with changes in the expression of cell cycle regulators and senescence-associated genes, which may be modulated by changed expression or activity of several transcription factors. Further, *SUV39H1* as well as *CBX5*, an *HP1* homolog, were found to be down-regulated in both PD 38 and PD 47 cells exposed to IR, and were correlated with reduced H3K9me3 levels and induced expression of transcripts from pericentric satellite regions. While such down-regulation of SUV39H1 in response to DNA-damaging agents was previously associated with the induction of apoptosis or the facilitation of DNA repair, we suggest that this down-regulation may also be associated with the induction of senescence in normal human diploid fibroblasts. This seems to at least in part depend on p53-dependent transcriptional suppression, suggesting that p53-mediated regulation of *SUV39H1* expression plays a role in the long-term DNA damage response. Within an organism, such an induction of senescence in dividing cells exposed to IR may interfere with the normal development and repair of a tissue or organ and thus contribute to side effects associated with IR.

7.6 ACKNOWLEDGEMENTS

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8. DEVELOPMENT-DEPENDENT EXPRESSION OF DNA REPAIR GENES AND EPIGENETIC REGULATORS IN *ARABIDOPSIS* PLANTS EXPOSED TO IONIZING RADIATION

8.1 ABSTRACT

Plant senescence and the response to ionizing radiation involve changes in gene expression and epigenetic profiles and both rely on the formation of reactive oxygen species. However, how the developmental stage of the plant affects its response to ionizing radiation has not been extensively studied. Our experiments show that the exposure to low (10 Gy) and high (100 Gy) doses of ionizing radiation causes developmental delays in plants and may result in reduced biomass or even death of the organism. 20-day old plants, which are making the transition to reproductive growth, show a distinct response to radiation when compared to 10-day old or 30-day old exposed plants. This affects the expression of DNA repair genes – increased expression of mismatch repair genes in 20-day old plants versus increased expression of *RAD51* in 10- and 30-day old plants. Similarly, we found increased expression of *MET1*, *CMT3* and *SUVH5* epigenetic regulators paralleled with decreased *ONSEN* transcript levels in 20-day old irradiated plants. This suggests a tighter control over genome stability in response to X-ray when plants are exposed during early reproductive growth when compared to plants exposed at other developmental stages.

8.2 INTRODUCTION

Exposure of plants to ionizing radiation (IR) confronts their cells with two types of stress: direct damage to macromolecules including the DNA and the production of reactive oxygen species (ROS), and thus oxidative stress. While oxidative stress is countered by the rapid up-regulation of antioxidant enzymes, such as peroxidase, catalase, superoxide dismutase and glutathione reductase (Croci et al., 1991; Kovalchuk et al., 2007; Ogawa and Uritani, 1970; Singh, 1974), DNA damage leads to the activation of ATM, which mediates radiation dependent cell cycle arrest (De Schutter et al., 2007) and it triggers the induction of DNA repair (Garcia et al., 2003).

Several studies have described the effects of IR on the transcriptomes of exposed plants, and the observed gene expression changes have been compared to the changes in gene expression profiles in the response to oxidative stress induced by H_2O_2 treatment or in plants carrying a mutated *atm* (Culligan et al., 2006; Desikan et al., 2001; Gicquel et al., 2012; Kim et al., 2013c; Kim et al., 2007; Kovalchuk et al., 2007; Nagata et al., 2005; Ricaud et al., 2007). Genes that changed expression pattern in these studies generally play roles in DNA repair, stress response, transcriptional regulation, development and ROS detoxification, thus suggesting that gene expression profiles are adjusted to optimally deal with the imposed stress. Interestingly, the study done by Gicquel *et al.* (2012) also showed changes in the expression of histone genes, which may indicate a differential regulation of the chromatin structure following the exposure to IR.

Changes in chromatin structure can have positive effects on the stress response, such as the adjustments of gene expression profiles or even stress adaptation (Bilichak et al., 2012; Boyko et al., 2010; Kim et al., 2008; Kwon et al., 2009), or negative effects, such as increased reactivation of silenced transposable elements or repetitive

sequences and thus potentially result in genomic instability (Tanurdzic et al., 2008). However, how the exposure to IR affects the chromatin structure in plant cells is not well understood.

Interestingly, the response to stress and the molecular changes involved in leaf senescence show some resemblance, such as the accumulation of ROS (Navabpour et al., 2003; Ye et al., 2000), changes in gene expression (Buchanan-Wollaston et al., 2005) and changes in chromatin structure (Ay et al., 2009; Brusslan et al., 2012). However, how those development-dependent molecular changes may affect the response to stress is not well understood.

There is some evidence for a development-dependence of the IR sensitivity of plants (Kim et al., 2007; Kurimoto et al., 2010). While Kurimoto *et al.* (2010) described more severe defects in the accumulation of biomass, when plants were exposed to IR at an early developmental stage, Kim *et al.* (2007) determined the reproductive phase to be the most IR-sensitive phase based on reduced stem growth, delayed leaf senescence and accumulation of pigments, including chlorophyll, in irradiated plants.

Based on this we hypothesized that the molecular response to IR may differ between plants at different developmental stages. In order to study this, we exposed *Arabidopsis* plants in different developmental phases - early vegetative growth (d10), early reproductive growth (d20) and flowering (d30) – to 1, 10 and 100 Gy of X-ray and studied the expression patterns of DNA repair genes and epigenetic regulators following the exposure. This showed distinct responses to X-ray in the 20-day old plants when compared to 10- or 30-day old plants.

8.3 MATERIALS AND METHODS

8.3.1 Plants and growth conditions

The plant model used in this study was *Arabidopsis thaliana* ecotype Columbia line 15D8, which carries a luciferase-based homologous recombination reporter construct in its genome. All plants used in this study were grown under sterile conditions on Murashige and Skoog medium (Murashige and Skoog, 1962) (MS, 30% sucrose, pH 5.7, 0.8% agar) in a controlled environment under a 16 h light / 8 h dark cycle at 22°C and 18°C, respectively. Seeds were sterilized 10 min in 10% bleach, 2 min in 70% ethanol and washed in sterile distilled water five times. Seed stratification was carried out for 3 days at 4°C before plating seeds on MS medium. Plants for collection of leaf tissue were grown in glass jars that were tall enough to allow for shoot growth.

8.3.2 Analysis of development

Development stages were observed and categorized according to the criteria described by Boyes *et al.* (Boyes et al., 2001). At the end of the life cycle fresh and dry weight were determined. The fresh weight was measured immediately after removal of the plants from the growth medium and the dry weight was measured upon drying the plants for 3 days at 50°C.

8.3.3 X-ray exposure and tissue collection

Plants were exposed to X-ray in a Faxitron X-ray machine (Faxitron, Model RX-650). Exposures were carried out at 130 kVp, 5 mA and 5.25 Gy/min. Plants were exposed to either 100 Gy, 10 Gy, 1 Gy or 0 Gy or as indicated of X-ray and tissues were snap-frozen at 0 h, 4 h or 48 h post exposure.

8.3.4 Quantitative real-time PCR (qRT-PCR)

For the analysis of the transcript levels of selected genes, total RNA was extracted from three samples per treatment and time point using TRIzol® Reagent (Invitrogen). RNA samples were cleaned using the illustra RNAspin Mini Prep kit (GE Healthcare) to remove any genomic DNA contamination and quantified by NanoDrop2000c (ThermoScientific). This was followed by cDNA synthesis using the iScript™ Select cDNA synthesis kit (BioRad).

qRT-PCR reactions were set up using the SsoFast™ EvaGreen® Supermix (BioRad) and primers specific to the targets of interest (Table 8.1) and analyzed on a C1000™ Thermo Cycler equipped with a CFX96™ Real-Time System (BioRad). PCR conditions were selected according to SSoFast™ guidelines with annealing temperatures as specified for primer pairs (Table 8.1).

Each experiment included three biological replicates with two technical replicates per treatment and time point. The gene of interest transcript levels were normalized to a set of housekeeping genes – *EF1A*, *UBC9*, *PP2A* and *BHLH* - using qbase^{PLUS} (Vandesompele et al., 2002).

Table 8.1: Primers for qRT-PCR based analysis of transcript levels.

Gene	Fwd/rev	Sequence	Tm	Reference
EF1A	Fwd Rev	5'-TGAGCACGCTCTTCTTGCTTTCA-3' 5'-GGTGGTGGCATCCATCTTGTTACA-3'	55.9°C	(Czechowski et al., 2005)
PP2A	Fwd Rev	5'-TAACGTGGCCAAAATGATGC-3' 5'-GTTCTCCACAACCGCTTGGT-3'	55.9°C	(Czechowski et al., 2005)
UBC9	Fwd Rev	5'-TCACAATTTCCAAGGTGCTGC-3' 5'-TCATCTGGGTTTGGATCCGT-3'	55.9°C	(Czechowski et al., 2005)
BHLH	Fwd Rev	5'-GAAAGCAAAGGCGGTGAGAG-3' 5'-CAAGGCACACTTGGTTCTTCC-3'	65°C	(Czechowski et al., 2005)
ATM	Fwd Rev	5'-CTACCTTTCTATTGGTATCCTCTCTCCTT-3' 5'-TCTGCATTGGTTTCGCTTATC-3'	59.5°C	(Garcia et al., 2000)
HTA3	Fwd Rev	5'-GCCTCTTCAAATTTCCCGATAAACA-3' 5'-GGAACAGAGAGCCATGTCTATGTTA-3'	59.5°C	(Yi et al., 2006)

HTA5	Fwd Rev	5'-CAAGGTTGGCAAGAACAA-3' 5'-ACAAGAGAACTGAACTAAGAAG-3'	55.9° C	Beacon Designer7
KU70	Fwd Rev	5'-AAAGTCGACGATGAAGACGAAGACCCAGAAAACGAT-3' 5'-AAATCTAGACTCATACTCCCAAAAGGATCATC-3'	59.5° C	(Golubov et al., 2010)
RAD51	Fwd Rev	5'-AAAGTCGACGCAGCAGGTATTGCTTCTGTTGATGTA-3' 5'-CTTGACTGATGATCTGTATTATACGCCCTC-3'	59.5° C	(Golubov et al., 2010)
MSH2	Fwd Rev	5'-CGATGGCAGATGCGTTATTG-3' 5'-CAGATTGGTGAAGCGGCTAT-3'	59.5° C	(Golubov et al., 2010)
MSH6	Fwd Rev	5'- GGTAATGTGGAAGAAGATA -3' 5'- ATTCTCATCAACCAACTC -3'	55.9° C	(Bilichak et al., 2012)
MSH7	Fwd Rev	5'- GCCTGTTCCGAATGATATAC -3' 5'- GCACGAAGAAGAGTTGATT -3'	59.5° C	(Golubov et al., 2010)
UVH3	Fwd Rev	5'- TTCGTGCTATATTGGTTC -3' 5'- AATAACTTTCGCCTCTTT -3'	55.9° C	
MET1	Fwd Rev	5'-GTGATTCTTAGGGCTATAATGG-3' 5'-CATTGATGAAGTCCACTTGAC-3'	59.5° C	(Jullien and Berger, 2010)
CMT3	Fwd Rev	5'-TTCCCAAAGCATATCCAAGG-3' 5'-CACAACGCCATTTCAAAAGTT-3'	59.5° C	(Luo et al., 2009)
SUVH2	Fwd Rev	5'-TTATTCGTATCTCAGAGC-3' 5'-CAGAATCCAATCCGTATA-3'	55.9° C	(Bilichak et al., 2012)
SUVH5	Fwd Rev	5'- ACGACATTACAATCATCAG-3' 5'- CTTGAAGACGAGTTTACC-3'	59.5° C	(Bilichak et al., 2012)
SUVH6	Fwd Rev	5'- TTCGCCACAAGGATTATC-3' 5'- CATTCTCTGGTGTATTCATTAC-3'	55.9° C	(Bilichak et al., 2012)
SUVH8	Fwd Rev	5'-ACATCAGCACCTCCTCAT-3' 5'-CCAGCACTCGCATCATAA-3'	59.5° C	(Bilichak et al., 2012)
ONSEN	Fwd Rev	5'-CCACAAGAGGAACCAACGAA-3' 5'-TTCGATCATGGAAGACCGG-3'	59.5° C	

8.3.5 Detection of homologous recombination events

The homologous recombination frequency (HRF) was analyzed by scoring bright sectors on a dark background with a luciferase CCD camera after spraying plants with a luciferin solution (Boyko et al., 2006). The HRF was calculated by relating the number of recombination events for each plant to the number of leaves of the respective plant (HRF/leaf). The average HRF was calculated from spots counted in 12 plants per treatment and developmental stage.

8.3.6 Statistical analysis

Data were tested for statistical significance using a two-tailed Student's t-test based on a significance level of $p < 0.05$.

8.4 RESULTS

8.4.1 Experimental setup

The development of *Arabidopsis thaliana* under controlled conditions is predictable and the timing of the major developmental stages has been determined for plants grown on soil or grown on MS medium on vertical plates (Boyes et al., 2001). For this study, since plants needed to be exposed to X-ray at different developmental stages, they needed to be able to go through a normal life cycle (including bolting and flowering), which is difficult when plants are grown in Petri dishes, yet they needed to have limited space for vertical expansion in order to keep the delivered dose of X-ray controlled. Therefore, plants were grown in jars with a maximum height of 9 cm.

In addition to developmental changes, senescence-associated gene expression in the rosette leaves throughout the development was of interest as a measure of the senescence-status of the plants. For this, the expression of *Senescence-associated gene 2* (*SAG2*) was determined by qRT-PCR. Age-related *SAG2* expression was first characterized in the *Arabidopsis* Landsberg cultivar (Hensel et al., 1993), but was subsequently also used as a senescence marker in other *Arabidopsis* cultivars (Callard et al., 1996; Kinoshita et al., 1999).

The initial stages of the development (seed germination and leaf development) of *Arabidopsis* in jars had almost equal timing when compared to soil grown plants in the study by Boyes *et al.* (Table 8.2). Due to limited space and resources, the rosette stayed smaller in size and the final rosette size was reached sooner than in plants

grown on soil. The plants grown in jars bolted after day 20 and had first flowers around day 30 (Table 8.2). Those later stages of the development were increasingly less synchronous between individual plants. Based on the collected data, day 10 (first rosette leaves), day 20 (bolting) and day 30 (flowering) of the development were chosen as time points for irradiation (Fig. 8.1A).

In leaves collected from plants grown in jars, *SAG2* expression gradually increased from day 10 to day 45 (Fig. 1B, logarithmic regression, $R^2 = 0.48$), suggesting that leaves from different aged plants show increasing levels of senescence-associated gene expression.

Table 8.2: Development of *Arabidopsis thaliana* grown on MS agar in jars.

Numbers in brackets indicate standard deviations.

	Average day (grown in jars)	**Average day (Boyes et al., 2001)**
Stage 0 – Seed germination		
Seed imbibition	3.0	3.0
Radicle emergence	4.4 (0.5)	4.3 (0.4)
Hypocotyl/cotyledon emergence	5.4 (0.5)	5.5 (0.6)
Stage 1 – Leaf development		
Cotyledons fully opened	6.1 (0.3)	6.0 (0.5)
2 rosette leaves > 1 mm	10.1 (0.6)	10.3 (0.6)
4 rosette leaves > 1 mm	12.7 (0.9)	14.4 (0.5)
Stage 3 -Rosette growth		
Rosette final size	22.9 (1.7)	29.3 (3.5)
Stage 5 – Inflorescence emergence		
Bolting	23.9 (1.7)	
First flower buds visible	25.5 (1.6)	26 (3.5)
Stage 6 – Flower production		
First flower open	30.6 (4.9)	31.8 (3.6)
Stage 8 – Silique ripening		
First silique	33.2 (3.4)	

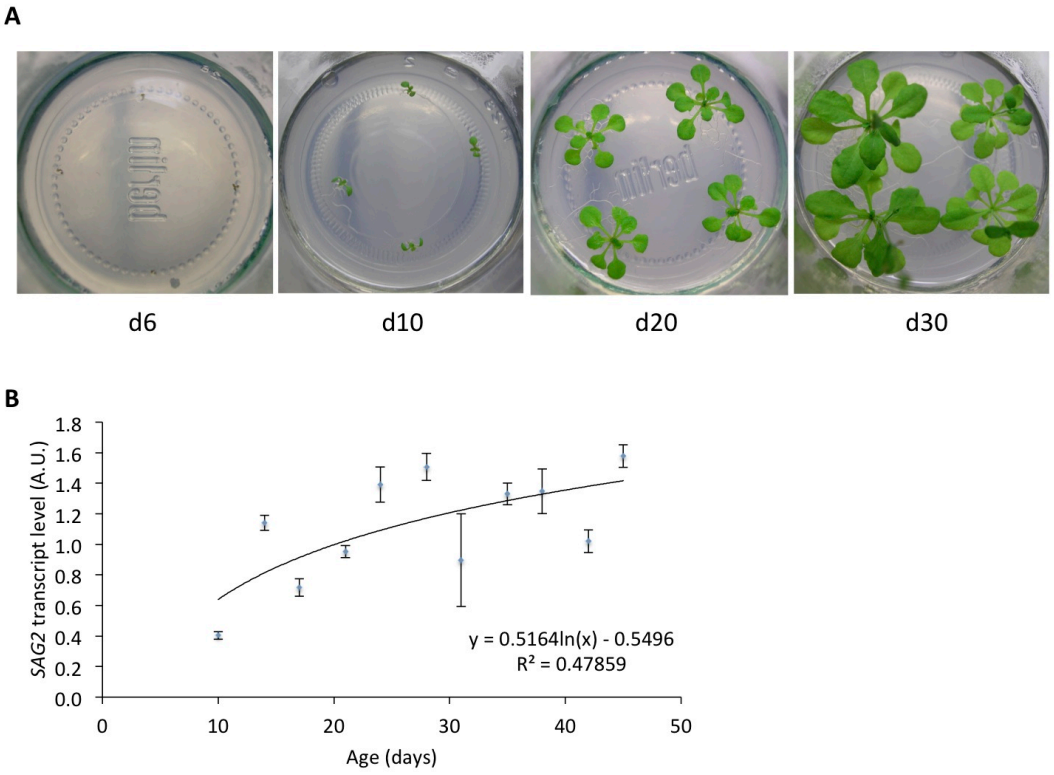


Figure 8.1: Development and senescence-associated gene expression in rosette leaves of *Arabidopsis thaliana* grown in jars.

A) Pictures of plants at 4 different developmental stages – d6 (cotyledons fully opened), d10 (starting growth of rosette leaves), d20 (final growth of rosette leaves/early bolting), d30 (flowering). B) Transcript levels of *SAG2* in rosette leaves throughout the plant development in arbitrary units. Data points represent averages from two biological and two technical repeats and error bars indicate standard deviations. Data is normalized to expression of housekeeping genes (*EF1A, TUBULIN, UBC9, PP2A*) and scaled to the average of all measurements.

8.4.2 Setup of X-ray conditions

Previous studies on the response of *Arabidopsis* to ionizing radiation have employed wide dose ranges – 1 Gy up to 2000 Gy (Kim et al., 2011b; Kovalchuk et al., 2007; Nagata et al., 2005). In order to determine a suitable dose range for this

experimental setup, plants were exposed to 6 different doses of X-ray (1 Gy, 10 Gy, 50 Gy, 100 Gy or 250 Gy) in addition to the 0 Gy control treatment on day 14 of the development (in the middle of the leaf development). One week post-exposure, differences in size and morphology of the plants were observed in plants exposed to 100 or more Gy of X-ray (Fig. 8.2A). The same plants were sprayed with luciferin solution to detect spots of Luciferase expression using a CCD camera. This showed an increased number of spots per leaf in plants exposed to 50 Gy, 100 Gy or 250 Gy of X-ray when compared to the control treatment (Fig. 8.2B).

Based on these results, 1 Gy and 10 Gy treatments were selected as low dose treatments and 100 Gy as a high dose treatment.

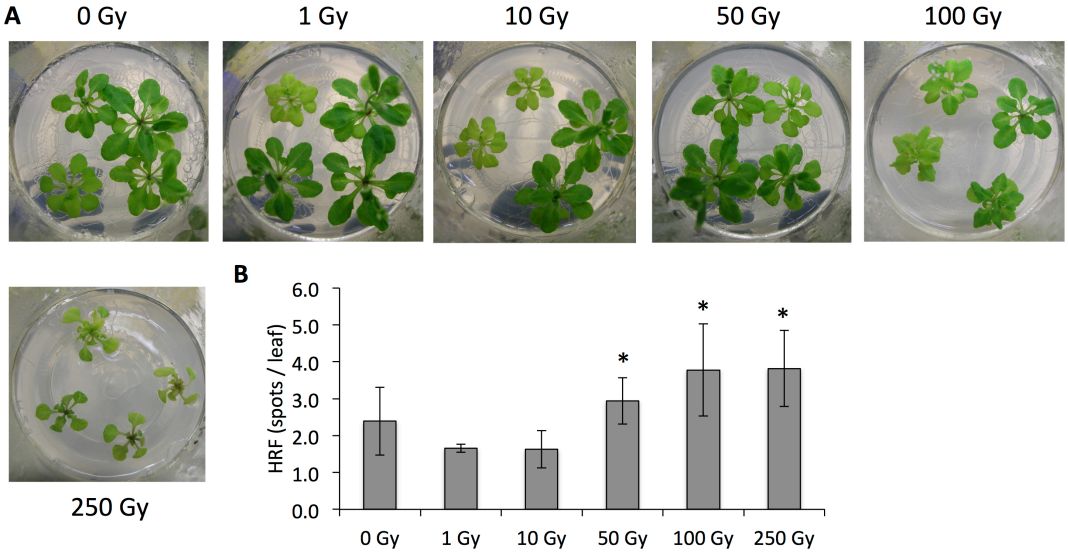


Figure 8.2: X-ray dose-response curve based on plant morphology and HRF.

A) Representative pictures of plants 1 week post-exposure to indicated dose of X-ray. B) Bar graph shows the HRF determined by detection of spots with Luciferase expression per leaf. Data points are averages from 4 plants per treatment and error bars indicate standard deviation. Asterisks indicate significant difference to control based on Student's t-test ($p < 0.05$).

8.4.3 Low and high doses of X-ray induce developmental delays and reduction in biomass

As cells are exposed to X-ray, they may experience direct damage to their macromolecules, including DNA, or indirect damage, mediated by the formation of ROS. DNA damage induces the activation of ATM, which then regulates diverse downstream responses, including cell cycle arrest and cell death (De Schutter et al., 2007; Fulcher and Sablowski, 2009). On the other hand, ROS are known to play a role in mediating stress response-related gene expression (Desikan et al., 2001), senescence-associated gene expression (Cui et al., 2013b), or may induce programmed cell death (Gao et al., 2008). Therefore, the exposure to X-ray may delay the development of the plant.

To study the effects of X-ray exposure at different stages of the development, *Arabidopsis* plants were exposed to 0 Gy, 1 Gy, 10 Gy or 100 Gy of X-ray on day 10, 20 or 30 and their development was recorded according to Boyes' criteria prior to and post exposure (Table 8.3). Further, the fresh and dry weights of the plants were determined on day 56, by which time most of the control plants have formed siliques.

This showed that plants exposed to 100 Gy of X-ray on day 10 or 20 were delayed in their development. In plants exposed to 10 Gy of X-ray on day 10 the similar trend was also observed, but less severe. This was also reflected in the reduced fresh and dry weight of plants exposed to 10 or 100 Gy of X-ray on day 10 (Fig. 8.3). A similar reduction of fresh and dry weight was observed for plants exposed to 10 or 100 Gy of X-ray on day 30. Plants exposed on day 20 showed higher individual differences ranging from death of the organism to plants with high biomass within the same treatment groups.

In order to assess whether the exposure to X-ray induces senescence-associated gene expression, transcript levels of *SAG2* were determined 4 h and 48 h post-exposure (Fig. 8.3B). A significant increase in the *SAG2* transcript level was detected when plants were irradiated with 100 Gy of X-ray on d20, when compared to 10 Gy irradiated plants, and similarly a significant induction of transcription was detected with increasing time after exposure of 20-day old plants to 100 Gy. This trend was also observed in plants irradiated on d30.

Table 8.3: *Arabidopsis* development pre- and post-exposure to X-ray.

Numbers are averages of 8 (1 Gy and 10 Gy groups) or 12 (0 Gy and 100 Gy groups) plants. The developmental stages, at which plants were exposed to X-ray are framed. Numbers marked with * indicate averages calculated from fewer plants, because not all plants of the according treatment groups reached the given stage of development.

	d10				**d20**				**d30**			
	0 Gy	**1 Gy**	**10 Gy**	**100 Gy**	**0 Gy**	**1 Gy**	**10 Gy**	**100 Gy**	**0 Gy**	**1 Gy**	**10 Gy**	**100 Gy**
Stage 0 – Seed germination												
Seed imbibition							3					
Radicle emergence							5.3 (0.8)					
Hypocotyl/cotyledon emergence							6.2 (0.4)					
Stage 1 – Leaf development												
Cotyledons opened							7.2 (0.4)					
2 rosette leaves > 1 mm	10.5 (0.5)	10.3 (0.5)	10.1 (0.4)	10.3 (0.5)				10.4 (0.6)				
4 rosette leaves > 1 mm	12.4 (0.5)	12.1 (0.4)	12.5 (0.5)	13.3 (1.1)				12.5 (0.7)				
5 rosette leaves > 1 mm	15.0 (0.9)	14.8 (1.1)	14.6 (0.5)	15.8 (0.7)				14.8 (0.8)				
6 rosette leaves > 1 mm	16.3 (0.6)	16.4 (0.9)	16.7 (0.5)	17.3 (1.1)				16.3 (0.7)				
7 rosette leaves > 1 mm	17.2 (0.7)	17.6 (0.9)	17.9 (0.6)	19.0 (1.0)				17.3 (0.6)				
8 rosette leaves > 1 mm	18.5 (1.1)	18.6 (0.5)	18.9 (0.4)	20.3 (1.6)				18.5 (0.9)				
9 rosette leaves	19.8	20.1	21.0	21.6	19.9	20.3	20.2	20.2			20.3 (1.2)	

> 1 mm	(1.2)	(1.1)	(0.9)	(0.7) *	(1.2)	(1.0)	(1.3)	(1.5)				
10 rosette leaves > 1 mm	21.2 (0.9)	21.3 (0.5)	21.3 (0.6) *	22.4 (1.2) *	21.4 (0.5)	21.6 (0.5)	21.6 (0.7)	21.5 (0.7)	21.6 (0.6)			
Stage 5 – Inflorescence emergence												
Bolting	22.7 (1.3)	22.5 (0.7)	22.4 (0.7)	23.9 (2.6)	20.7 (1.0)	20.2 (0.6)	21.0 (1.3)	20.8 (1.0)	22.7 (1.2)			
Stage 6 – Flower production												
First flower open	32.3 (7.3)	33.9 (8.6)	33.8 (7.7) *	33.9 (9.5) *	36.0 (10.0)	30.0 (1.5) *	31.3 (5.4) *	39.4 (4.7)	30.1 (2.9)	32.3 (7.4)	32.5 (5.1)	36.3 (9.3)
Stage 8 – Silique ripening												
First silique	34.9 (6.5)	35.6 (7.5)	35.6 (7.4) *	33.9 (9.0) *	37.1 (9.3)	31.0 (1.5) *	33.6 (11.5)*	40.5 (3.7)	32.2 (2.9)	33.7 (6.8)	33.6 (4.5)	38.4 (8.1)

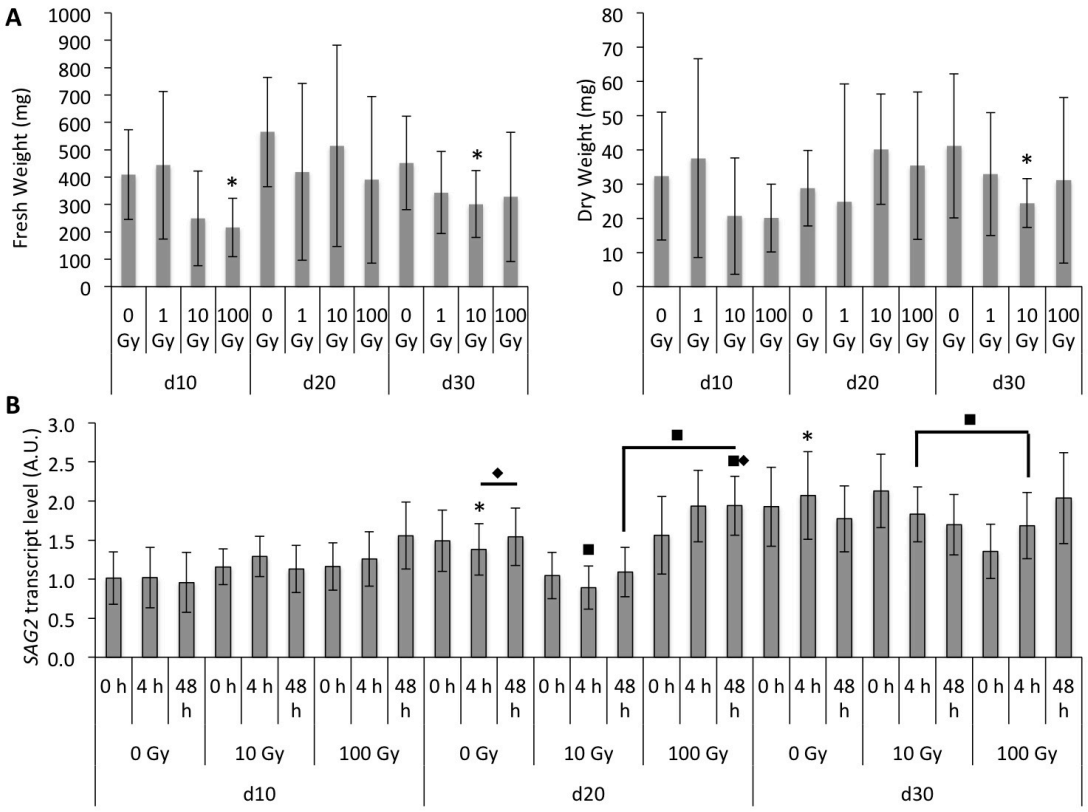


Figure 8.3: Biomass and senescence-associated gene expression following X-ray exposure.

A) Data points represent averages of 8 to 12 plants per treatment. Asterisks indicate significance based on Student's t-test ($p < 0.05$). B) *SAG2* transcript levels as measured by qRT-PCR. Bars represent averages from three plants and two technical replicates normalized to the transcription of housekeeping genes and to transcript levels in d10 0 Gy 0 h group. Error bars represent error propagation of standard

deviations from technical repeats. Significance is based on Student's t-test ($p < 0.05$). Three different symbols were used to indicate significance: asterisks indicate a significant difference in age-dependent transcript levels, by keeping the dose of X-ray and the time point post exposure constant; squares indicate a significant difference in dose-dependent transcript level, by keeping the age and the time point post exposure constant; and diamonds indicate a significant difference in transcript level over time post exposure, by keeping the plant age and the considered dose of X-ray constant.

8.4.4 Distinct DNA repair gene expression patterns in plants irradiated at different developmental stages

As the expression of DNA repair genes (*KU70*, *RAD51*, *MSH2* and *MSH7*), as well as the DNA repair efficiency and fidelity were previously described to change with increasing age of the plant (Boyko et al., 2006; Golubov et al., 2010), we hypothesized that this may be associated with changed expression of DNA damage sensing enzymes and that it may affect the capacity of aging plants to induce the expression of DNA repair genes in response to ionizing radiation.

ATM is activated early on during the DNA damage response and has been described to mediate transcriptional changes in response to IR (Culligan et al., 2006). In addition, ATM phosphorylates the Histone H2AX variant, resulting in the formation of extensive γH2AX foci, which play an important role in DNA damage signalling (Friesner et al., 2005). Unlike the mammalian genome, the *Arabidopsis* genome encodes two homologs of the mammalian H2AX histone 2 variant - *HTA3* and *HTA5*, both of which contain the conserved SQEF motif that is common to mammalian H2AX (Rogakou et al., 1998; Yi et al., 2006).

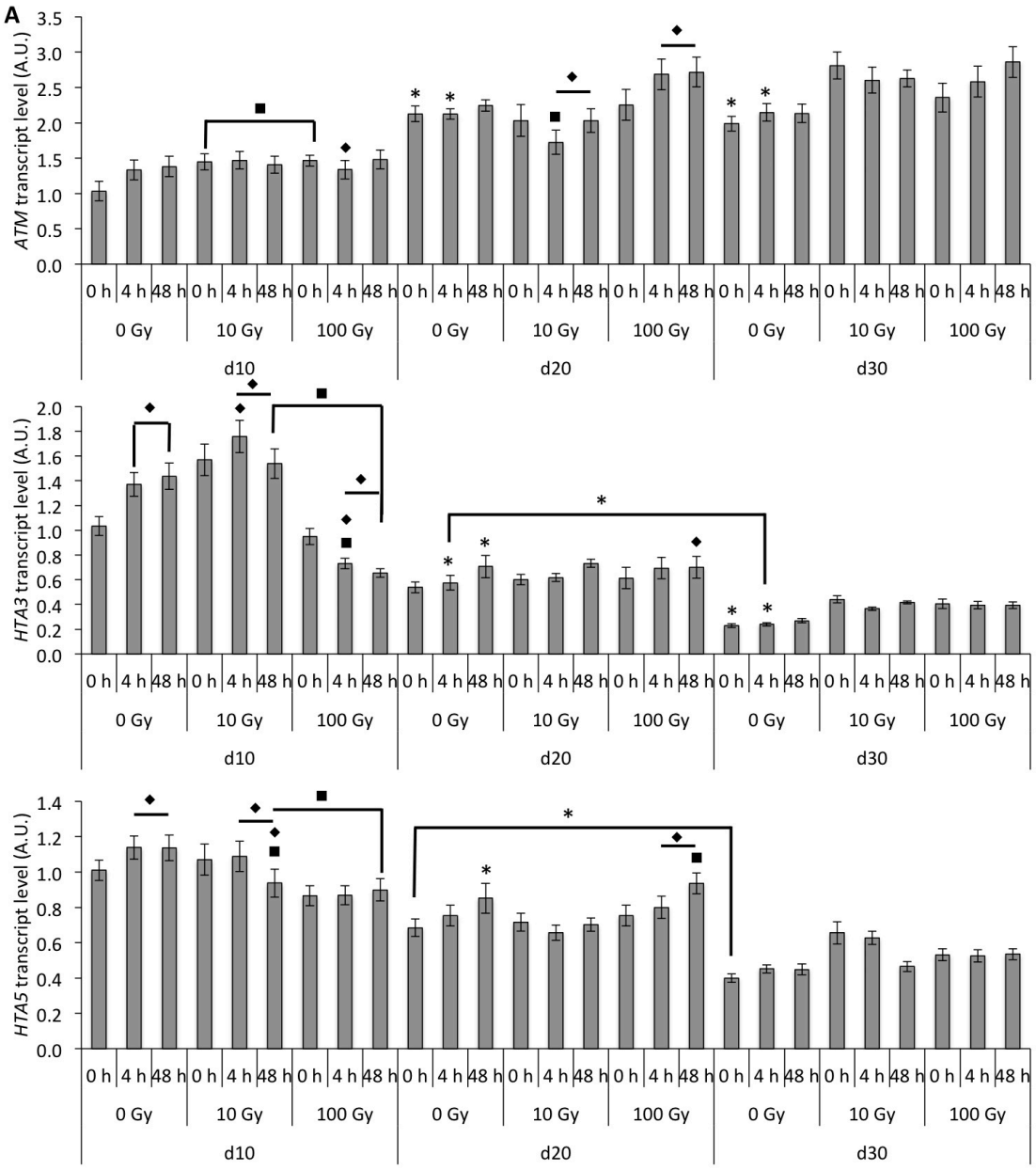
In order to study the expression of those DNA damage sensors, the transcript levels of *ATM*, *HTA3* and *HTA5* were measured by qRT-PCR. While *HTA3* and *HTA5* transcript levels decreased with increasing age of the plant, *ATM* transcript levels increased (Fig. 8.4A). Further, *HTA3* and *HTA5* expression levels decreased in response to X-ray when plants were irradiated on d10, but increased in response to 100 Gy of X-ray when plants were irradiated on d20.

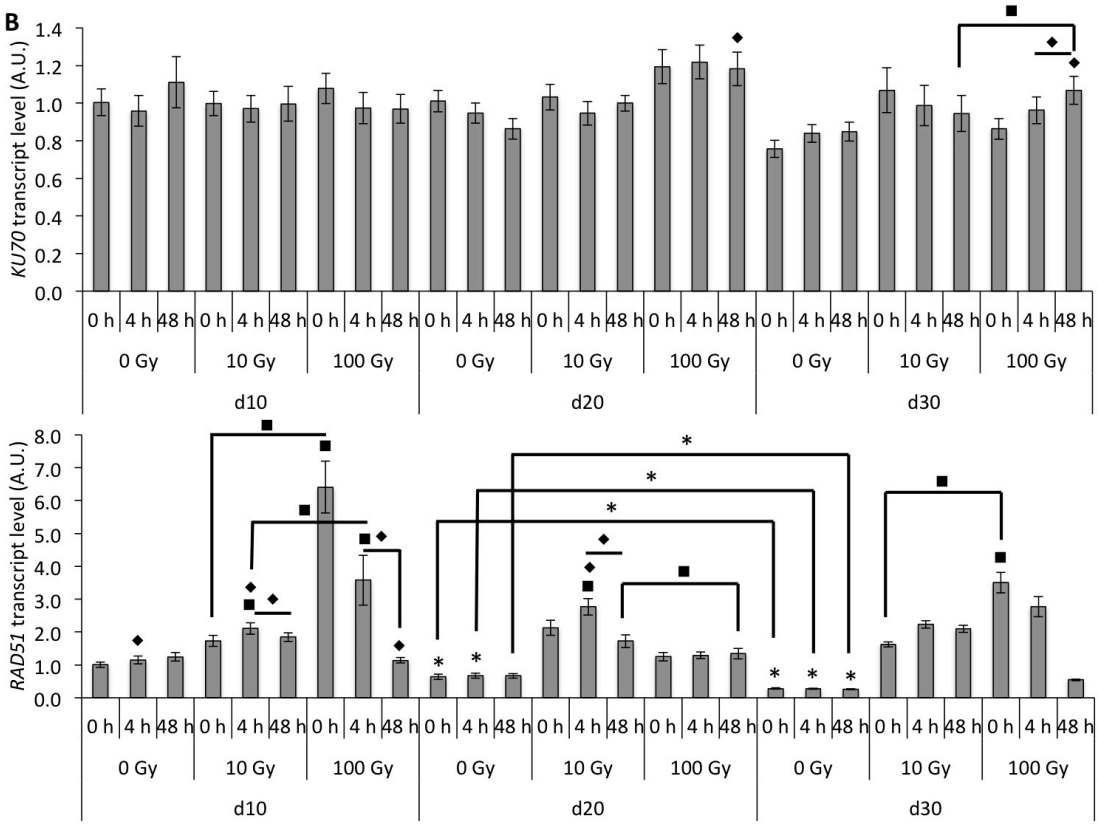
Those changes in the expression of DNA damage sensors may affect the expression as well as the activation of DNA repair enzymes. In order to study how this may affect the response to ionizing radiation with increasing age, transcript levels of *KU70* and *RAD51* were determined using qRT-PCR. This showed that *KU70* expression levels did not vary significantly across the age-range considered in this study, while its expression was induced most significantly in plants exposed to 100 Gy on d30 (Fig. 8.4B). Further, *RAD51* transcript levels significantly decreased in 20-day and 30-day old plants when compared to 10-day old plants. However, the expression of *RAD51* was induced within minutes of the exposure to 10 Gy or 100 Gy of X-ray in plants irradiated on d10 and d30, but not on d20 (Fig. 8.4B). However, *RAD51* expression rapidly declined after the initial induction.

Recently, a role for mismatch repair protein MSH6 in NHEJ was described (Shahi et al., 2011). Shahi *et al.* (2011) showed colocalization of MSH6 with γH2AX foci in response to IR and impaired NHEJ in MSH6-deficient cells. Further, MSH2 and MSH6 were also shown to play a role in the removal of 8-oxo-guanine, a common type of oxidative damage of a base (Mazurek et al., 2002). Since mismatch repair genes were previously shown to be down-regulated with age in plants, their regulation in response to IR was studied using qRT-PCR. This analysis revealed only slight age-dependent changes in the expression levels of *MSH2* and *MSH6*, but significant

down-regulation of *MSH7* in 20- and 30-day old plants when compared to 10-day old plants (Fig. 8.4C). Interestingly, the expression of *MSH2* and *MSH6* was significantly induced in response to X-ray exclusively in 20-day old plants.

Finally, *UVH3*, an *Arabidopsis* homolog of the human XPG endonuclease that is involved in nucleotide excision repair, but is also thought to play a role in the removal of bases that incurred oxidative damage, was previously described to induce increased sensitivity to UV and ionizing radiation as well as H_2O_2 when mutated (Liu et al., 2001). Further, *uvh3* mutants show a premature senescence phenotype (Liu et al., 2001). Therefore, age-dependent differences in *UVH3* expression might contribute to differences in the response to ionizing radiation. Thus, transcript levels of *UVH3* were measured by qRT-PCR. This showed a significant age-dependent decrease in the expression of *UVH3* (Fig. 8.4D). In response to X-ray, the expression of *UVH3* was slightly, but significantly reduced when plants were exposed to 10 Gy of X-ray on d10 or d20.


A
ATM transcript level (A.U.)
HTA3 transcript level (A.U.)
HTA5 transcript level (A.U.)
0 h
4 h
48 h
0 Gy
10 Gy
100 Gy
d10
d20
d30


B
KU70 transcript level (A.U.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h
0 Gy 10 Gy 100 Gy 0 Gy 10 Gy 100 Gy 0 Gy 10 Gy 100 Gy
d10 d20 d30
RAD51 transcript level (A.U.)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h 0 h 4 h 48 h
0 Gy 10 Gy 100 Gy 0 Gy 10 Gy 100 Gy 0 Gy 10 Gy 100 Gy
d10 d20 d30

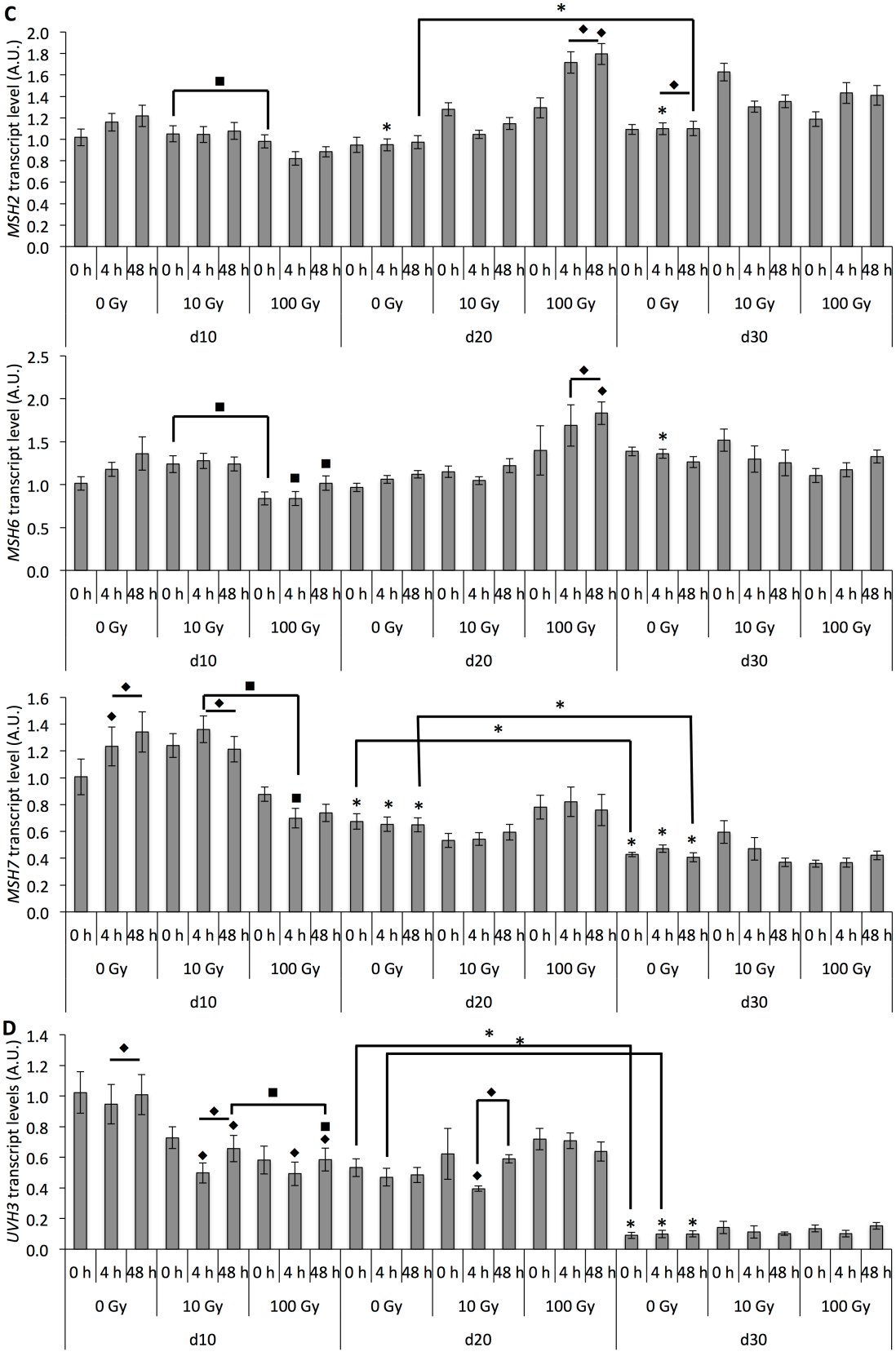


Figure 8.4: Expression of DNA damage sensors and DNA repair enzymes in response to X-ray.

A) – D) Bars represent averages from three plants and two technical replicates normalized to transcript levels in d10 0 Gy 0 h group. Error bars represent error propagation of standard deviations from technical repeats. Significance is indicated by asterisks in case of significant difference when compared to d10 0 Gy or between the same time points but different developmental stages, squares indicate significant difference to the 0 Gy control of equal age and time point, and diamonds indicate significant difference to 0 h expression level within the same age- and treatment group. Significance is based on Student's t-test ($p < 0.05$).

8.4.5 Reduced expression of DNA methyltransferases is paralleled with increased transcript levels of *ONSEN* transposable elements

While DNA repair gene expression is required for the immediate response to the DNA damage, changes to the chromatin structure are thought to at least in part mediate the adaptive response of plants to stress (Kovalchuk et al., 2003b; Zhu et al., 2008). Further, epigenetic changes may be transmitted through generations and allow for increased stress adaptation of the progeny of exposed plants (Boyko et al., 2010). Epigenetic changes may mediate stress-dependent changes in gene expression (Bilichak et al., 2012; Dowen et al., 2012; Zhang et al., 2011), or affect genome integrity through the relief of the silencing of repetitive DNA sequences, such as transposable elements (Ito et al., 2013). Ito *et al.* (2013) showed that heat-activated *ONSEN* retrotransposons preferentially integrated into gene regions and thereby may affect the expression of the corresponding genes.

Since both MET1, a CG DNA methyltransferase, and CMT3, a non-CG DNA methyltransferase, are involved in the silencing of transposable elements (Kato et al., 2003), we examined expression patterns of *MET1*, *CMT3* genes and *ONSEN*

retrotransposons in irradiated plants of different age. This showed that both *MET1* and *CMT3* were down-regulated in an age-dependent manner (Fig. 8.5A). While exposure to 100 Gy of X-ray resulted in down-regulation of *MET1* and *CMT3* expression in 10-day old plants, it resulted in their up-regulation in 20-day old plants. Those expression changes correlated with the increased expression of *ONSEN* retrotransposon transcripts with increasing age of the plant and in response to X-ray in 10-day old and 30-day old plants (Fig. 8.5B). In contrast, 20-day old plants exposed to 10 or 100 Gy of X-ray exhibited lower *ONSEN* transcript levels, which correlated with the increase in DNA methyltransferase expression.

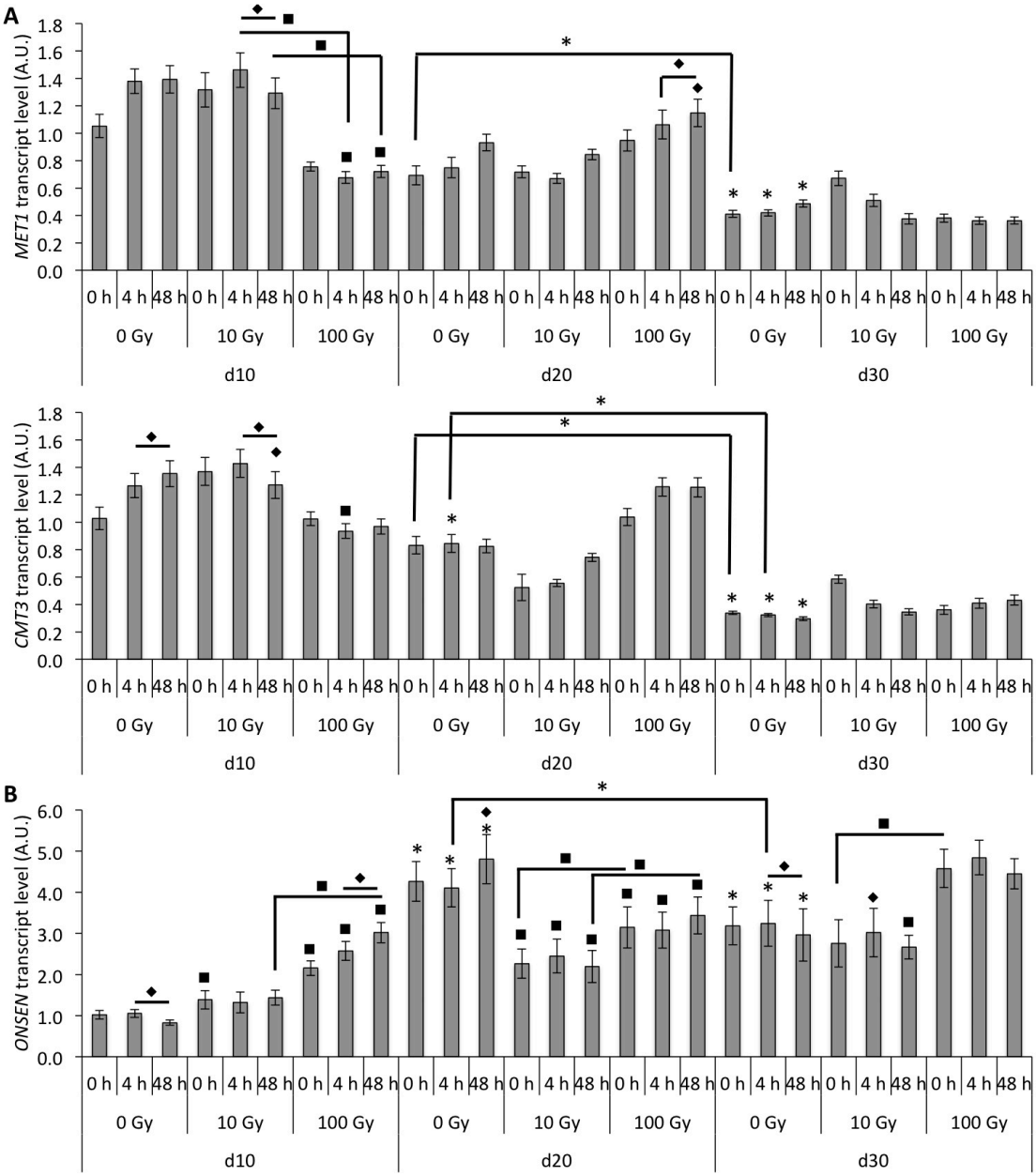


Figure 8.5: Reduced expression of DNA methyltransferases correlates with increased transcript levels of transposable elements.

A) Expression levels of *MET1* and *CMT3* mRNA as detected by qRT-PCR. B) Expression levels of *ONSEN* mRNA as detected by qRT-PCR. A) and B) Bars represent averages from three plants and two technical replicates normalized to transcript levels in d10 0 Gy 0 h group. Error bars represent error propagation of standard deviations from technical repeats. Significance is indicated by asterisks in case of significant difference when compared to d10 0 Gy or the same time points and

radiation exposures but different developmental stages, squares indicate significant difference to the 0 Gy control of equal age and time point, and diamonds indicate significant difference to 0 h expression level within the same age- and treatment group. Significance is based on Student's t-test ($p < 0.05$).

In addition to DNA methylation, histone modifications play an important role in the transcriptional regulation as well. While changes in histone modification patterns have been observed in the cells of senescing leaves (Brusslan et al., 2012) and in response to abiotic stress (Bilichak et al., 2012; Kim et al., 2008), changes in histone modification patterns and nucleosome spacing induced by IR have not been studied extensively. Drury *et al.* (2012) showed the accumulation of acetylated Histone 3 and Histone 4 in response to 160 Gy of X-ray (Drury et al., 2012), suggesting an increase in the number/size of euchromatin regions in the genome. This may further indicate that the exposure to ionizing radiation would negatively correlate with the presence of inhibitory histone methylation, such as H3K9me2 or H3K9me3, in the chromatin.

To test this, we measured the transcript levels of histone methyltransferases, *SUVH2*, *SUVH5*, *SUVH6* and *SUVH8*, by qRT-PCR. This revealed an age-dependent increase in *SUVH2* and *SUVH6* expression, but a decrease in *SUVH5* and *SUVH8* expression (Fig. 8.6). In response to X-ray, the expression changes of histone methyltransferases were mostly limited to 20-day old plants exposed to 100 Gy of X-ray and included the transcriptional up-regulation of *SUVH2*, *SUVH5* and *SUVH6* (Fig. 8.6). This also correlates with the induction of the expression of DNA methyltransferases in 20-day old plants exposed to X-ray (Fig. 8.5A). However, we did not observe any significant changes in global H3K9 trimethylation (data not shown).

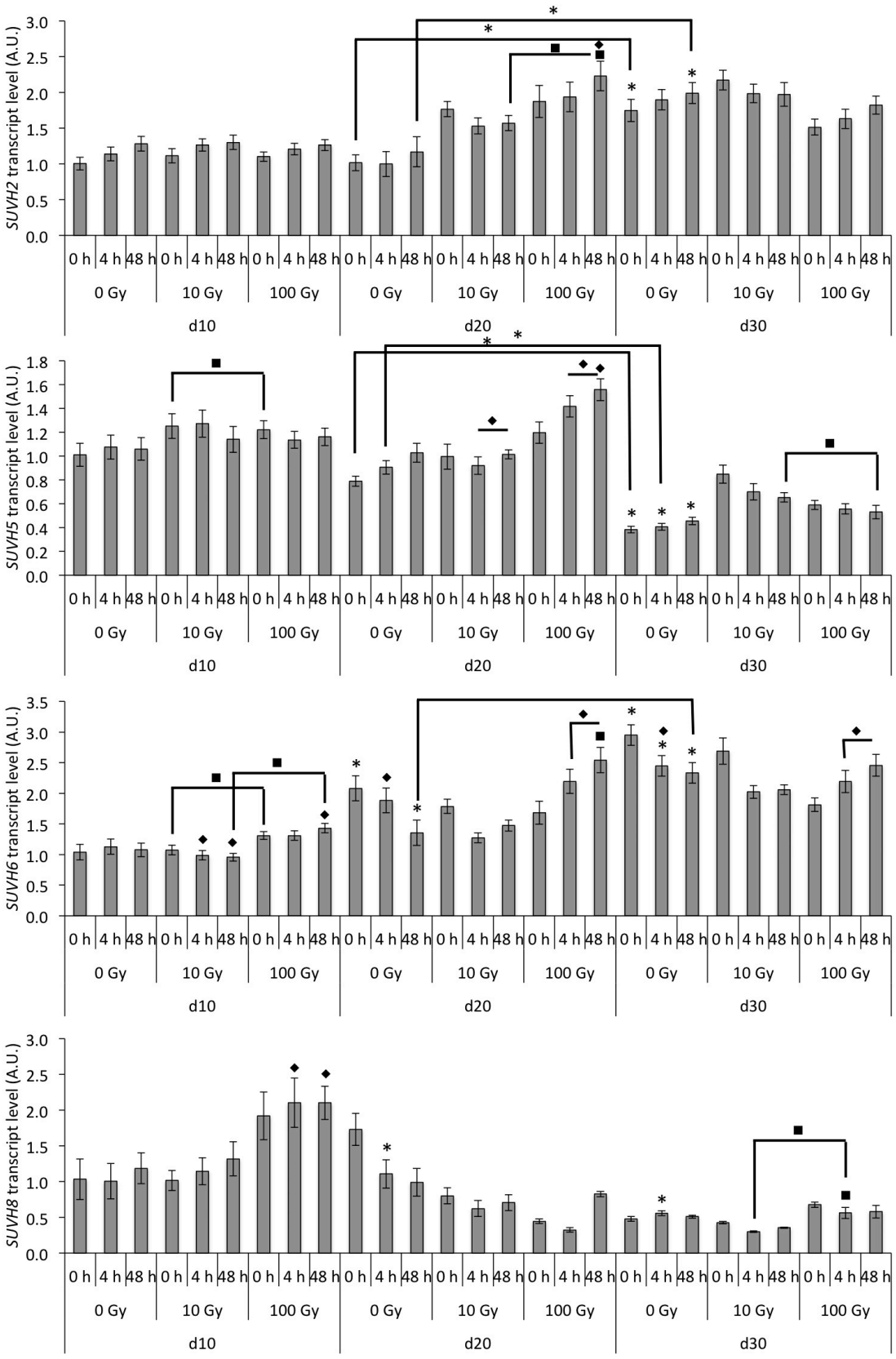


Figure 8.6: Expression of histone methyltransferases in response to X-ray.

Bars represent averages from three plants and two technical replicates normalized to transcript levels in d10 0 Gy 0 h group. Error bars represent error propagation of standard deviations from technical repeats. Significance is indicated by asterisks in case of significant difference when compared to d10 0 Gy or between the same time points and radiation doses but different developmental stages, squares indicate significant difference to the 0 Gy control of equal age and time point, and diamonds indicate significant difference to 0 h expression level within the same age- and treatment group. Significance is based on Student's t-test ($p < 0.05$).

8.5 DISCUSSION

The model employed in this study included *Arabidopsis* plants at three distinct developmental stages – the formation of the first two rosette leaves (d10), initiation of shoot formation (d20) and flowering (d30). The initial stages of the development were relatively synchronous between individual plants, whereas the later stages, including flowering and silique formation, were more variable between individuals. This is consistent with previous reports (Boyes et al., 2001).

The conditions for the X-ray exposures were chosen based on a dose-curve by testing doses of up to 250 Gy of X-ray and evaluating physiological changes and HRF in the treated plants. 10 Gy was chosen as a low dose of X-ray, as it did not visibly impair the plants and also did not induce a significant change in HRF. 100 Gy was chosen as a high dose of X-ray, because it increased the HRF in treated plants and visibly delayed the development of the treated plants post-exposure.

Since previous reports suggested roles for cell cycle arrest (De Schutter et al., 2007), programmed cell death (Fulcher and Sablowski, 2009) as well as senescence (Gicquel et al., 2012) in the response to IR or exposure to ROS, it was hypothesized

that the exposure to ionizing radiation may affect the development of the plants. Such developmental delays were visible in 10 Gy and 100 Gy-irradiated 10-day old and 20-day old plants. Transient developmental arrests in response to IR have been previously described for seedlings (Ricaud et al., 2007) and have been associated with a cell cycle arrest in meristematic cells (Carballo et al., 2006). Further, in IR-exposed 10-day and 30-day old plants the exposure resulted in a reduced biomass at the end of the life cycle. This is in line with the previous observation by Kurimoto *et al.* (2010) that plants exposed to X-ray at a younger age show more severe aberrations in biomass at the end of the life cycle (Kurimoto et al., 2010). 20-day old plants were more sensitive to the even lower dose of 1 Gy of X-ray, resulting in the death of three out of 12 individuals. This distinct response observed in 20-day old plants may suggest that the developmental timing of the exposure is more crucial than the biological age of the plant in determining the nature of the response.

To examine the role of senescence in these developmental changes, the transcript levels of *SAG2*, a previously described senescence-associated gene (Hensel et al., 1993), were determined by qRT-PCR. The analysis of *SAG2* transcript levels after exposure to X-ray showed that *SAG2* expression was induced in response to 100 Gy of X-ray in 20-day old plants, but not in 10-day or 30-day old plants. This may indicate that cell cycle arrest and cell death may play greater roles in the response to X-ray in 10-day or 30-day old plants, while senescence seems to be part of the radiation response during the early reproductive growth phase.

As the radiation-induced cell cycle arrest is connected to the DNA damage response, we next studied the expression of genes involved in DNA damage sensing and the mounting of the DNA damage response. ATM is thought to be activated during direct recognition of DNA double-strand breaks (Suzuki et al., 1999) and then

further induces downstream responses through the phosphorylation of target proteins including histone H2AX, which is involved in DNA damage signalling. ATM has also been shown to be crucial in mediating the gene expression changes induced by the exposure to X-ray (Culligan et al., 2006; Ricaud et al., 2007). Thus the observed increase in *ATM* transcript levels with increasing age of the plant may render aging plants better prepared for dealing with additional exposure to DNA damaging stressors.

The observation that *HTA3* and *HTA5* transcript levels, the plant H2AX homologs, were reduced with increasing plant age may indicate that DNA damage signalling through HTA3/5 may become less efficient. A similar reduction in *H2AX* expression level was observed in senescing human cells (Chapter 5). A recent study indicated that at least part of the response to DNA double-strand breaks may be independent of HTA3/HTA5 phosphorylation (Lang et al., 2012). Thus, further study of the roles of HTA3 and HTA5 in the DNA damage response may allow a better understanding of the implications of their reduced expression in older plants.

When examining expression levels of DNA repair genes, an age-dependent reduction in the transcript levels of *RAD51* and *MSH7* was observed, which is in line with previous reports (Boyko et al., 2006; Golubov et al., 2010). However, the response to ionizing radiation differed between plants of different ages. While in 10- and 30-day old plants transcript levels of *RAD51* were rapidly induced upon exposure to X-ray, this was not observed in 20-day old plants. Interestingly, *RAD51* expression rapidly declined after the initial induction, suggesting a very tight regulation of its expression in response to DNA damage. This early induction of *RAD51* expression has previously been described to be ATM-dependent (Garcia et al., 2003). However,

the age-dependent reduction in *ATM* transcript levels did not affect the IR-induced *RAD51* expression.

The transcript levels of *MSH2* and *MSH6* were induced in response to 100 Gy of X-ray in 20-day old plants, but not in plants of the other age groups. MSH6 was recently reported to play a role in promoting NHEJ in response to ionizing radiation (Shahi et al., 2011). This may indicate that homologous recombination is preferentially induced in plants exposed to DNA damaging agents early or late during development, while NHEJ may be the prominent repair pathway at the time of the induction of reproductive growth. On the other hand, MSH2 and MSH6 were also described to play a role in the repair of oxidative DNA damage (Mazurek et al., 2002). Thus, the observed induction of *MSH2* and *MSH6* expression in response to X-ray during the early reproductive growth phase may also indicate a more strict control over base damage during this stage. High expression levels of *MSH2* and *MSH6* may help prevent the accumulation of point mutations during this phase of growth. However, this requires further confirmation.

In addition to the potential role of MSH2 and MSH6 in the repair of oxidative damage to DNA bases, UVH3 is also thought to play a role (Liu et al., 2001). According to our experiments, its role in the response to ionizing radiation may be minor or it may not depend on regulation of *UVH3* at the transcription level, as only minor changes in transcript levels post IR exposure could be detected. However, *UVH3* did show a severe decrease in transcript level with increasing plant age. Liu *et al.* (2001) previously described that *uvh3* mutants showed a premature senescence phenotype. Thus, the decrease in *UVH3* transcript levels in aging plants may be associated with the establishment of the senescence phenotype.

While DNA repair is part of the immediate response to IR, in order to prevent genomic instability and the accumulation of mutations, epigenetic mechanisms are considered to play an important role in the plant's adaptation to stress. Our results showed that both *MET1*, a CG DNA methyltransferase, and *CMT3*, a non-CG DNA methyltransferase, were down-regulated with increasing age of the plant, correlating with the increased expression of *ONSEN* retrotransposon transcripts. When considering the effects of radiation, 20-day old plants showed a distinct response when compared to 10-day and 30-day old plants. While in both 10-day and 30-day old plants, *MET1* and *CMT3* were suppressed and corresponded with increased *ONSEN* transcript levels, in 20-day old plants increased DNA methyltransferase expression resulted in decreased *ONSEN* expression. The reactivation of retrotransposon expression may mediate stress adaptation through the preferential insertion in gene regions and thus potential effect on the regulation of the expression of genes (Ito et al., 2013), however aberrant transposition may endanger genome integrity. Therefore, the suppression of *ONSEN* expression upon exposure to ionizing radiation may be important for the maintenance of genome integrity during the reproductive growth phase.

Transposable elements in the *Arabidopsis* genome are generally silenced by a combination of DNA cytosine methylation (Zhang et al., 2006) and repressive histone modification marks, such as H3K9me2 (Bernatavichute et al., 2008) and H3K9me3, which is only locally repressive (Veiseth et al., 2011). While H3K9 trimethylation is introduced by SUVR4 (Veiseth et al., 2011), the dimethylation of H3K9 depends on histone methyltransferases of the SUVH family. Here we show that up-regulation of *SUVH2*, *SUVH5* and *SUVH6* in response to 100 Gy X-ray in 20-day old plants correlates with the transcriptional induction of DNA methyltransferases and the

suppression of *ONSEN* transcription. While SUVH4 has been considered to be the major contributor to H3K9 dimethylation (Jackson et al., 2004), functions of SUVH5 and SUVH6 as H3K9 methyltransferases were also described (Ebbs et al., 2005; Ebbs and Bender, 2006; Jackson et al., 2004). SUVH2 was also described to be involved in H3K9 dimethylation (Naumann et al., 2005), whereas another study by Ay *et al.* (2009) suggests a role for SUVH2 in H3K27 methylation rather than H3K9 methylation (Ay et al., 2009). Thus, the individual roles of the different SUVH proteins are not fully understood.

It is interesting to note that SUVH5 and SUVH6 have been associated with locus-specific histone methyltransferase activities. Ebbs and Bender (2006) showed that SUVH5 was involved in the maintenance of H3K9me2 and CMT3-mediated non-CG methylation at transposon sequences, whereas SUVH6 was responsible for H3K9me2 dimethylation at inverted repeats (Ebbs and Bender, 2006). In line with this, *SUVH5* correlated with *CMT3* expression levels, and negatively correlated with *ONSEN* transcript levels both dependent on age and dependent on the dose of X-ray received.

In summary, these results show distinct expression patterns of DNA repair genes and epigenetic regulators in response to X-ray in plants at different developmental stages. Particularly, genome stability seems to be more strictly controlled in plants that are making the transition to reproductive growth, possibly by limiting recombination and transposon reactivation.

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9. CONCLUSIONS AND FUTURE DIRECTIONS

The main objectives of this thesis were the examination of the role of epigenetics in aging and in the age- or development-dependent response to ionizing radiation. The main findings that were achieved in the pursuit of those objectives are presented in the following paragraphs.

9.1 MAIN FINDINGS OF THIS THESIS

1) Immunosenescence in rats is associated with extensive changes to the gene expression profiles in thymus and spleen of old rats. Various biological functions are affected by differential gene expression, including DNA repair, cell cycle and apoptosis. Further, aging is associated with a loss of heterochromatin marks and increased genomic instability in thymus. This may contribute to the induction of senescence and apoptosis in cells within the aging thymus and may thus contribute to thymic involution.

2) Similarly, extensive changes to the gene expression profile are observed when human diploid fibroblasts enter replicative senescence. E2F transcription factors are hypothesized to contribute to the altered regulation of cell cycle genes. In addition, SUV39H1 was down-regulated in senescent cells, and resulted in the global loss of H3K9me3, as well as the site-specific loss of H3K9me3 in pericentric satellite regions. We thus proposed a role for the down-regulation of SUV39H1 in the establishment of senescence-associated changes to the chromatin structure, which may contribute to the induction of senescence.

3) The DNA methylation profile of senescent cells differs extensively from the one in dividing cells. This is associated with the down-regulation of DNMT1 in senescent cells and the corresponding reduction in global DNA methylation. While some

chromosome regions were enriched for DNA hypo- or hypermethylation, most genes that were affected by differential promoter DNA methylation did not show altered gene expression.

4) The major difference in the physiological response to IR when comparing dividing and senescent cells was that dividing cells significantly underwent senescence. This was associated with the down-regulation of SUV39H1 and the reduction of H3K9me3 levels, alongside the increased expression of transcripts from satellite regions. The over-expression of *SUV39H1* prior to irradiation prevented the induction of senescence, suggesting an important role of SUV39H1 in the establishment of senescence after the exposure to IR. The down-regulation of *SUV39H1* seemed to be at least partially associated with the loss of acetylated p53 from the *SUV39H1* promoter.

5) The response to IR in *Arabidopsis* is development-dependent rather than strictly age-dependent. The IR-response in plants during the transition from vegetative to reproductive development was distinct from the response in plants during early leaf development or during flowering. This was associated with the increased expression of DNA methyltransferases, *CMT3* and *MET1*, and histone methyltransferases, *SUVH5* and *SUVH6*, along with the decreased expression of the *ONSEN* transcript in plants exposed to 100 Gy X-ray during the transition from vegetative to reproductive growth.

6) When comparing epigenetic changes associated with aging in the mammalian to the plant model systems, it is interesting to note that in all model organisms that were studied a decreased expression of DNA methyltransferases (Dnmt1 in rat thymus, DNMT1 in WI-38, and *MET1* and *CMT3* in *Arabidopsis*) was observed. In addition, both in the thymus of old rats and in senescent cells the expression levels of

SUV39H1 were reduced, while in *Arabidopsis* only the transcript level of *SUVH5*, which shows extensive sequence similarity to *SUV39H1*, significantly decreased with increasing age. In all model systems this reduced expression of DNA and histone methyltransferases correlated with increased genomic instability, measured as the accumulation of unrepaired DNA damage in the thymus of old rats, or the increased transcription level of satellite DNA in WI-38 or of transposable elements in *Arabidopsis*. Thus, de-heterochromatinization and increased genomic instability may contribute to the functional decline of somatic tissues in both mammalian and plant organisms.

When comparing the age-dependent IR-response in the WI-38 model to the *Arabidopsis* model, one main difference was that this response is dependent on the replicative age in the human cell model, whereas in plants it is dependent on the developmental stage. This response involves the regulation of DNA and histone methylation in both models. While the expression of histone methyltransferases is increased in response to IR in plants irradiated on d20, possibly resulting in the limitation of genomic instability to ensure the success of the germ line development, the expression of SUV39H1 was down-regulated post-IR and associated with increased genomic instability in human cells, possibly contributing to the induction of senescence. This difference in the regulation of histone methyltransferases and of the silencing of repetitive DNA sequences may be reflected in the different life cycles. While *Arabidopsis* relies on the continued proliferation of its stem cells to ensure its reproductive success, and therefore needs to limit mutagenic insults to their genome, the response of somatic human cells does not directly affect the reproductive success of the organism, and thus a major concern might be to limit the proliferation of cells that have incurred radiation-induced DNA damage and possibly mutations.

In summary, the results presented in this thesis have contributed to the current understanding of the role of epigenetic mechanisms in aging and cellular senescence, as well as radiation-induced senescence in mammalian model systems. Additionally, the study on the development-dependent response to IR in *Arabidopsis* expands the current knowledge on the development-dependence of the response to IR, as well as indicates a role for epigenetic regulation in this response.

9.2 FUTURE DIRECTIONS

While the results presented in this thesis support an important role of epigenetic regulation in immunosenescence, replicative senescence and in the age- or development-dependent response to IR in both human cells and plants, understanding its precise regulation and function in those processes requires further research. Some ideas for future work are outlined in the following sections.

9.2.1 Cell-type specific roles in immunosenescence

Immunosenescence involves complex changes affecting primary and secondary immune organs. On the one hand, it involves the age-dependent shrinkage of the thymus, which is associated with the decreased output of naïve T cells and with changes to the thymus architecture (Cunningham et al., 2001), and on the other hand, naïve T cells undergo clonal expansion upon antigen recognition in the periphery, and may after several rounds of expansion become senescent (Effros, 2004). While some studies have pointed towards the relevance of the epithelial compartment within the thymus for thymic involution (Aw et al., 2008; Aw et al., 2009; Ortman et al., 2002; Zhu et al., 2007), we have used whole thymus samples to study epigenetic changes

that occur in the thymus. Our study showed that thymic involution is associated with the global reduction of heterochromatin marks and the increasing genomic instability. However, in order to dissect the role of those epigenetic changes in thymic involution further, it would be interesting to address the following questions.

1) Is the down-regulation of DNMT1 and SUV39H1 specific to certain cell types or compartments within the thymus?

In order to address this, it would be interesting to separate the different cell types that occur within the thymus, dendritic cells, epithelial cells, nurse cells, macrophages and maturing T cells. The measurement of DNMT1 and SUV39H1 protein levels and determination of global DNA methylation and H3K9 trimethylation in the different cell types could then indicate which cells are mainly affected by the loss of heterochromatin marks and corresponding increase in genomic instability.

2) What is the timing of the SUV39H1 and DNMT1 down-regulation?

As our study involved 4-month old animals, which are around the age of the onset of the thymic involution, and 18-month old animals, which have a very small thymus, the analysis of additional time points around the onset of thymic involution may help in determining during which stage in which cell type the down-regulation of SUV39H1 and DNMT1 occurs. If it occurs relatively early, this may suggest that the associated heterochromatin loss, genomic instability and alterations in the gene expression profile, may play a causal role in the induction of senescence and/or apoptosis in the affected cell types. If it occurs relatively late, it may rather be a consequence of other age-related changes within the thymus, such as for instance

DNA damage induced down-regulation of SUV39H1 in order to facilitate the repair of DNA damage within heterochromatin regions.

3) How does thymus-specific over-expression or knock-down of SUV39H1 or DNMT1 affect thymic involution?

If the down-regulation of SUV39H1 and DNMT1 does occur relatively early during thymic involution, it would be interesting to further study their role in the onset of thymic involution by creating mouse models that specifically over-express or repress *SUV39H1* or *DNMT1* expression in the thymus, or possibly even in a specific cell type that is affected by their age-dependent down-regulation. The phenotype of such mouse models would give further indications on what the specific role of the SUV39H1 and DNMT1 down-regulation is during thymic involution.

4) What are the dynamics of SUV39H1 expression in peripheral T cells?

Since the proliferation of peripheral T cells depends on antigen recognition, which is limited in the rats we studied, as they were housed in a pathogen-free environment, it would be interesting to study epigenetic changes in T cell populations that are challenged with pathogens. This could be achieved either *in vivo*, through repeated infection with a virus, such as CMV, followed by collection of blood samples in order to study the T cell population that was clonally selected in response to each cycle of infection, or *in vitro*, by exposing virus-specific T cells to their cognate antigen for several subcultures and studying changes in epigenetic regulation.

9.2.2 Control of epigenetic senescence mechanisms in stem cells and cancer

Both *suv39h1/2*-knock-out mice and *Suv39h1* over-expressing transgenic mice show severe developmental defects, while the knock-out animals exhibit decreased viability, increased genomic instability and susceptibility to tumor formation (Peters et al., 2001), the transgenic animals over-expressing *Suv39h1* show a deficiency in cell differentiation (Czvitkovich et al., 2001). Our results further support a role for the SUV39H1 down-regulation in the induction of senescence. Being a histone methyltransferase, SUV39H1 expression is important for the maintenance of adequate levels of H3K9 trimethylation, in order to silence pericentric repetitive DNA regions (Lehnertz et al., 2003; Peters et al., 2001), but also for the regulation of transcriptional repression, through increased H3K9me3 levels, or transcriptional activation, through the reduction in H3K9me3 levels.

Thus, it can be hypothesized that adequate SUV39H1 expression levels are required in order to balance genomic stability and proliferative potential. In such a model, increased SUV39H1 expression levels would protect the genome integrity and promote cell division, whereas decreased SUV39H1 expression levels would increase genomic instability and favour cell cycle arrest, however, if the SUV39H1 expression levels fall below a threshold level, the silencing of promoter regions of growth inhibitory genes may also be compromised and therefore result in tumor formation. In addition to mere differences in expression levels, the altered recruitment of SUV39H1 to specific DNA regions likely also contributes to senescence-associated changes in the distribution of H3K9me3 marks. In order to test this model for a role of SUV39H1 expression and recruitment in balancing genomic integrity and cell proliferation, it would be interesting to address the following questions.

1) Which proteins recruit SUV39H1 to which DNA sequence regions?

While the role of SUV39H1 in the heterochromatin formation in pericentric satellite regions is well accepted, the patterns of SUV39H1-mediated histone H3K9 trimethylation in other DNA sequence regions are less characterized. A better understanding of which transcription factors interact with SUV39H1 and may therefore recruit it to their target promoters may help in understanding the effects of SUV39H1-mediated H3K9 trimethylation on gene expression. One such example is the association of SUV39H1 with RB/HDAC1 resulting in the heterochromatin formation and suppression of genes involved in cell cycle progression (Bandyopadhyay et al., 2007). Thus, a systematic analysis of the interactome of SUV39H1 as well as ChIP-seq determination of the target sequences of SUV39H1-mediated heterochromatin formation in dividing when compared to senescent cells, will allow to further dissect the role of SUV39H1 in senescence-associated changes in the regulation of gene expression and the role of SUV39H1 recruitment in the establishment of senescence.

2) How do gradual changes in SUV39H1 expression levels affect cell proliferation and senescence?

One approach to test this, could be to use human diploid fibroblasts and treat them with increasing amounts of *SUV39H1* targeting siRNA, or transfect them with increasing amounts of an *SUV39H1* over-expression construct, and analyze cell proliferation by BrdU incorporation and senescence by the SA-β-GAL assay. This would allow testing the hypotheses that have been formulated above, and in addition this might help in determining the threshold of SUV39H1 expression level that is necessary for the inhibition of cell division, if such a threshold indeed exists.

Another approach is to determine how the SUV39H1 expression levels correlate with the rate of proliferation in various cell types. For this it would be interesting to compare various cancer cell lines, stem cells, human diploid fibroblasts at different population doublings, and primary cells derived from donors of different ages.

3) What regulatory mechanisms does the altered SUV39H1 regulation respond to?

A more detailed understanding of how SUV39H1 expression levels are regulated at the transcriptional and post-transcriptional levels will help in studying what intra- and extracellular stimuli may trigger the down-regulation of SUV39H1. Two studies have described an indirect role of p53 in regulating the expression of SUV39H1 by inducing the transcription of *p21*, which in turn represses E2F mediated transcription of *SUV39H1* (Mungamuri et al., 2012; Zheng et al., 2014). In addition, miRNA-mediated post-transcriptional regulation of SUV39H1 expression has also been described (Villeneuve et al., 2010). A systematic promoter analysis of the *SUV39H1* promoter region, as well as a target screen for miRNA-mediated post-transcriptional regulation, may indicate additional regulatory mechanisms that may affect the SUV39H1 expression during cell differentiation and cell proliferation. In addition, in our study the treatment of cells with chaetocin resulted in the reduction of *SUV39H1* transcript levels, which was surprising, because chaetocin inhibits the enzymatic activity of SUV39H1. This may therefore indicate that SUV39H1 expression is also regulated by a positive feedback mechanism. Thus, it would be interesting to determine whether this is the case, and how it is mediated. One possibility for such a mechanism could be that SUV39H1 may methylate one of its transcriptional repressors and thereby inactivate it.

9.2.3 The role of differential DNA methylation in senescence

Several studies have described altered DNA methylomes that were characterized by extensive site-specific DNA hypomethylation, alongside some site-specific hypermethylation with increasing age (Heyn et al., 2012; Johansson et al., 2013; McClay et al., 2014). We made similar observations in senescent when compared to actively dividing cell cultures of WI-38 human lung fibroblasts. While our dataset showed that there were some clusters of DNA hypo- or hypermethylation throughout the genome, differential methylation of promoter regions rarely corresponded with a change in gene expression. In addition, the global loss of DNA methylation and down-regulation of DNMT1 correlates with the observation that the majority of differentially methylated CpG sites in senescent cells are hypomethylated. However, it will be interesting to further address, what the role of this altered DNA methylome is in senescence, whether it is a consequence of other senescence-related molecular changes or whether it plays a causal role in the establishment of senescence. Further, it would be interesting to further study how certain sites are targeted for hypo- or hypermethylation.

1) Is the senescence-dependent DNA hypo- or hypermethylation targeted?

The down-regulation of DNMT1 during senescence could indicate that DNA methylation marks are passively lost through the deficiency in maintenance through cell divisions. However, 1,849 CpG sites were significantly differentially methylated when comparing three senescent to three dividing cell cultures in our study, thus suggesting that the loss of DNA methylation during senescence may at least in part be non-random. Thus, it would be interesting to study whether the recruitment of

DNMT1 to DNA is altered during senescence. This could be achieved through the immunoprecipitation of DNMT1 followed by mass-spectrometric analysis of its interacting proteins, in order to determine, whether DNMT1 is associated with different binding partners during senescence. Some studies suggest a role for the existing histone modification pattern in limiting the *de novo* DNA methylation in a sequence specific way (Ludwig et al., 2014; Schlesinger et al., 2007; Straussman et al., 2009). Thus, a better understanding of the regulation of the histone modification pattern during senescence may help in answering this question.

2) How does the differential DNA methylation affect the cell?

Differential DNA methylation did not extensively correspond with altered gene expression, but the inhibition of DNA methylation can induce a growth arrest in cells (Vogt et al., 1998), suggesting that DNA hypomethylation is associated with the molecular changes that inhibit cell division. This could potentially be mediated through contributing to genomic instability, through the loss of heterochromatin, or to the altered nuclear architecture, potentially through affecting the nuclear lamina interaction and chromosomal domain organization (Guelen et al., 2008).

9.2.4 Radiation-induced senescence as a side effect of heterochromatin repair

The repair of DNA damage within heterochromatin regions occurs with slower kinetics than DNA repair within euchromatin regions (Goodarzi et al., 2008), and was recently shown to be associated with p53-mediated induction of JMJD2b and repression of SUV39H1 expression favouring de-heterochromatinization (Zheng et al., 2014). Our results suggest that this de-heterochromatinization is associated with the increased transcriptional activation of satellite expression and senescence. Thus,

while the heterochromatin relaxation is necessary for the repair of DNA damage within heterochromatin regions, it may also contribute to a further increase in the genomic instability. The induction of senescence as a consequence may allow cells to survive in the presence of such genomic instability. However, this is hypothetical and could be addressed by investigating the following questions.

1) Do the cells that undergo senescence in response to IR exhibit DNA damage within heterochromatin regions?

The observation that p53 is important for the heterochromatin relaxation and repair (Zheng et al., 2014), suggests that this response is canonically induced in response to DNA damage. However, this could be tested by sorting cells according to SA-β-GAL staining 24 hours after the exposure to IR and determining the fraction of SA-β-GAL positive cells that exhibit DNA damage within heterochromatin regions.

2) Is the heterochromatin relaxation during DNA repair associated with additional genomic instability, including genome rearrangements?

Since SUV39H1-mediated heterochromatin formation in pericentric satellite regions is important for the maintenance of genomic integrity (Lehnertz et al., 2003; Peters et al., 2002), the heterochromatin relaxation, even if it is beneficial to DSB repair, could result in genomic rearrangements as a result of faulty repair or through the expression of repetitive DNA elements. This could be tested by karyotyping the cells that underwent IR-induced senescence, or through *de novo* sequencing of the genomes of those cells in order to detect genomic rearrangements.

3) How is the nuclear architecture affected by the changed expression of SUV39H1?

In addition to mediating the silencing of repetitive DNA regions, SUV39H1 also plays an important role in the regulation of telomere length (Garcia-Cao et al., 2004), and telomere nuclear architecture (Uhlirova et al., 2010). Further, heterochromatin regions enriched with repressive histone marks are found associated with the nuclear lamina (Guelen et al., 2008), and are thought to contribute to the domain organization of chromosomes, with regions more distant to the nuclear lamina frequently being more transcriptionally active (Peric-Hupkes et al., 2010). Peric-Hupkes *et al.* (2010) showed that this domain organization changed with increasing differentiation of mouse embryonic fibroblasts, resulting in differential gene expression. Thus, it would be interesting to test, whether the down-regulation of SUV39H1 in response to IR corresponds with alterations to the nuclear architecture and chromosome domain organization, and if yes, whether this results in altered gene expression patterns that might contribute to the IR-induced senescence.

4) Which determinants gear a cell towards apoptosis or senescence in response to IR?

While Mungamuri *et al.* (2012) showed that p53 activation resulted in the repression of SUV39H1 expression and geared various cancer cell lines towards apoptosis (Mungamuri et al., 2012), our results suggest that the SUV39H1 down-regulation following IR exposure is associated with the induction of senescence. The induction of apoptosis was mainly associated with the reduction of H3K9 trimethylation in the promoter regions of pro-apoptotic p53 target genes (Mungamuri et al., 2012). Thus, a better understanding of the DNA regions that are affected by

reduced H3K9 trimethylation in radiation-induced senescence, will give further indications as to what may guide the differences in response.

In addition, it seems plausible that the observed radiation-induced senescence arises from a prolonged G2/M arrest that transitions into G1 arrest and senescence, as has been recently described in human uveal melanoma cells (Ye et al., 2013). This was dependent on CHK1 in colorectal cancer cells (Poehlmann et al., 2011), and CHK1 expression was shown to be regulated in response to p53 in an analogous way to the regulation of SUV39H1 expression (Gottifredi et al., 2001).

Thus the subsets of p53 target genes that are regulated in response to DNA damage may determine the cell fate. This may be dependent on the dose of radiation as well as the state of differentiation of the cell. Therefore, the detection of apoptotic or senescent cells in cultures of different differentiation or senescence states exposed to IR, or in cultures exposed to gradually increasing doses of IR, would allow further conclusions on the dose- or cell physiology-dependence of the determination of the cell fate.

9.2.5 Meristem-specific IR-response throughout the plant's development

As the germ line is defined relatively late during the life cycle of *Arabidopsis* and the germ line cells are derived from meristematic stem cells, this suggests that the adequate response to DNA damage in meristematic cells is particularly crucial. In line with this, meristematic cells have been shown to be more prone to IR-induced cell cycle arrest (Hefner et al., 2006), and programmed cell death (Fulcher and Sablowski, 2009; Furukawa et al., 2010). Thus, in order to better understand development-dependent differences in the response to ionizing radiation, the following questions may be addressed.

1) Are specific compartments within the meristem particularly sensitive to PCD or cell cycle arrest?

While only a few surviving meristematic stem cells are enough to reorganize a meristem after the exposure to X-ray (Clowes, 1961), the extent of PCD and cell cycle arrest and the cells that are affected by it may result in some of the physiological changes that are observed, such as delayed leaf formation.

2) Does the response to IR differ in meristematic cells during different developmental stages?

Our study showed that plants that were exposed to IR on d20, during the transition to reproductive growth, exhibited distinct expression patterns of DNA methyltransferases, histone methyltransferases, DNA repair genes and *ONSEN* transposon sequences, when compared to plants exposed on d10 or d30. However, since the meristematic cells eventually give rise to the germ line, it would be interesting to determine, whether similar differences are also observed in meristematic cells at different developmental stages.

In order to get a better understanding of how similar or different the responses to IR in somatic and meristematic cells are at the molecular level, it might be very interesting to determine which subsets of genes are differentially expressed in the different cell types in an IR-dependent manner. In order to better understand the roles of different signalling pathways in the cell type-dependent IR-response, it would further be valuable to determine the H_2O_2-, or *atm*-induced gene expression profiles, and compare those to the IR-induced gene expression profile. This is based on the assumption that different chromatin structure and gene expression profiles in somatic

and meristematic cells may affect the effect of IR on the downstream response to transcriptional and epigenetic regulation.

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