The role of melanocyte senescence in skin ageing

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Abstract

Cellular senescence is a stable cell cycle arrest associated with the secretion of pro-inflammatory factors, also known as the senescence-associated secretory phenotype (SASP), which can act in a paracrine manner and induce senescence in neighbouring cells. Accumulation of senescent cells has been shown to contribute to age-associated tissue dysfunction. Melanocytes positive for senescence markers have been shown in the skin of middle-aged human donors, however, very little is known about the mechanisms underlying melanocyte senescence, and their contribution to skin ageing. In this study, I aimed to investigate whether telomere dysfunction is a driver of melanocyte senescence, and whether senescent melanocytes contribute to skin ageing phenotypes by affecting surrounding cells in a paracrine manner.

I found that melanocytes bearing dysfunctional telomeres accumulate in human skin with age. Importantly, I found that telomere dysfunction occurred independently of telomere length and could be induced *in vitro* as a result of exposure to different stressors. Furthermore, in human skin *in vivo*, I observed that telomeric damage was significantly higher in keratinocytes surrounding melanocytes with higher numbers of dysfunctional telomeres, suggesting that senescent melanocytes exert a bystander effect *in vivo*. Consistent with this, I found that senescent melanocytes secrete soluble factors which induce telomere dysfunction and repress proliferation of dermal fibroblasts in culture. Importantly, I identified that CXCR3, a cell-surface receptor for the cytokine IP-10, is involved in bystander telomere damage induction. Finally, I have shown in three-dimensional human epidermal equivalents that senescent melanocytes can induce paracrine telomere damage and senescence in surrounding keratinocytes, thereby contributing to epidermal thinning, a characteristic of ageing skin.

Overall, this study provides evidence that length-independent telomere dysfunction plays a role in melanocyte senescence both *in vivo* and *in vitro*. Moreover, senescent melanocytes induce paracrine DNA damage and senescence in neighbouring cells, and consequently contribute to age-associated epidermal changes.

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List of Abbreviations

6-4-PP	Pyrimidine-pyrimidone (6-4) photoproducts
8-OHdG	8-hydroxy-2’-deoxyguanine
AER	Apical ectodermal ridge
ATRIP	ATR-interacting protein
BER	Base excision repair
BrdU	5-bromodeoxyuridine
BSA	Bovine serum albumin
CDKIs	Cyclin-dependent kinase inhibitors
CM	Conditioned medium
COPD	Chronic obstructive pulmonary disease
CPD	Cyclobutane-pyrimidine dimers
CT	Cholera toxin
DAPI	4’,6-diamidino-2-phenylindole
DC	Dyskeratosis congenital
DDR	DNA damage response
DHE	Dihydroethidium
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DOX	Doxorubicin
DPX	Di-N-Butyle Phthalate in Xylene
DSBs	Double-stranded breaks
ECM	Extracellular matrix
EDJ	Epidermal-dermal junction

ELISA	Enzyme-linked immunosorbent assays
EMT	Epithelial-mesenchymal transition
EMT	Epithelial-to-mesenchymal transformation
FBS	Foetal bovine serum
FISH	Fluorescence *in situ* hybridisation
H&E	Haematoxylin and Eosin
HDACi	Histone deacetylase inhibitors
ICC	Immunocytochemisty
IFN-γ	Interferon- γ
IRAK1	Interleukin-1 receptor-associated kinase 1
LB	Lysogeny broth
LLS	Leiden Longevity Study
LSE	Living skin equivalent
MAPK	Mitogen activated protein kinase
MEFs	Mouse embryonic fibroblasts
MMPs	Matrix metalloproteases
MnSOD	Manganese superoxide dismutase
MtDNA	Mitochondrial DNA
NAC	N-acetylcysteine
NER	Nuclear excision repair
NGS	Normal goat serum
NHDF	Normal human dermal fibroblasts
NHEJ	Non-homologous end joining
NHEK	Normal human epidermal keratinocytes

NHEM	Normal human epidermal melanocytes
OIS	Oncogene-induced senescence
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PD	Population doubling
PFA	Paraformaldehyde
PIKKs	Phosphatidylinositol 3-kinase-like protein kinase
PIN	Prostate intraepithelial neoplasia
PVDF	Polyvinylidene difluoride
Q-FISH	Quantitative-FISH
ROS	Reactive oxygen species
S6Ks	S6 kinases
SAHF	Senescence-associated heterochromatic foci
SASP	Senescence-associated secretory phenotype
Sen-β-Gal	Senescence-associated-β-galactosidase
SIRT-1	Sirtuin-1
SSAs	Sessile serrated adenoma
TA	Transit-amplifying
TAF	Telomere-associated foci
TDIS	Telomere dysfunction-induced senescence
TE	Trypsin-EDTA
TIF	Telomere-induced foci
T-loop	Telomere-loop
UV	Ultraviolet

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Chapter 1: Introduction

1.1. Cellular senescence

Cellular senescence is characterised by an irreversible cell-cycle arrest, and was first described by Hayflick and Moorhead in 1961, who demonstrated that human diploid cells have a finite proliferative capacity *in vitro*, entering a state of terminal arrest following long-term serial passaging, a phenomenon also known as "replicative senescence" (Hayflick and Moorhead, 1961). Despite their inability to proliferate, senescent cells remain metabolically active, and undergo a series of dramatic phenotypic changes which will be discussed in further detail throughout this chapter.

This stable loss of proliferative potential is not merely a cell culture artefact, as it was once believed by the scientific community (Holliday *et al.*, 1977). In fact, senescence acts as a tumour suppressor mechanism by inhibiting proliferation of pre-malignant cells (Serrano *et al.*, 1997), and more recently, senescent cells have been implicated in many other biological processes such as embryonic development (Rajagopalan and Long, 2012; Munoz-Espin *et al.*, 2013), and wound healing (Jun and Lau, 2010; Demaria *et al.*, 2014). On the other hand, senescent cells have been shown to accumulate in a variety of mammalian tissues with age, contributing to impaired tissue homeostasis (Campisi and d'Adda di Fagagna, 2007a), and were more recently shown to be causal players in age-related pathologies (Baker *et al.*, 2011; Baker *et al.*, 2016). Therefore, senescence can be viewed as an example of antagonistic pleiotropy (Williams, 1957); a process which is beneficial in early life by promoting development and preventing tumour formation, however it later becomes detrimental and contributes to organismal ageing.

1.2. Causes of senescence

Senescence can be induced by a variety of different stimuli, including telomere dysfunction, stressors that lead to extensive DNA damage, and also oncogene activation. In this section, each of these triggers will be discussed in detail.

1.2.1. Telomere dysfunction

Telomeres are specialised structures present at the ends of linear chromosomes, which are critical in maintaining genomic stability (Blackburn, 1991). They consist of tandem 5'-TTAGGG-3' repeats, and contain a C-rich lagging strand and a G-rich leading strand, where the latter is composed of 3' single-stranded nucleotide repeats giving rise to the G-overhang

(McElligott and Wellinger, 1997; O'Sullivan and Karlseder, 2010). It has been proposed that this 3'-overhang invades upstream double-stranded telomeric regions, and binds to one of the DNA strands, forming a structure known as the telomere-loop (T-loop), thereby physically protecting the ends of chromosomes from being recognised as double-stranded breaks (DSBs) (Griffith *et al.*, 1999; Lin *et al.*, 2014). Telomeres also differ from the rest of the genome by associating with a six-subunit protein complex known as shelterin, which is comprised of TRF1, TRF2, RAP1, TIN2, TPP1, and POT1 (O'Sullivan and Karlseder, 2010; Sfeir, 2012). Both TRF1 and TRF2 bind to double-stranded telomeric sequences, whereas POT1 binds the single-stranded 3'G-overhang (Zhong *et al.*, 1992; Bianchi *et al.*, 1997; Bilaud *et al.*, 1997; Baumann and Cech, 2001). Each shelterin component plays essential roles that are important for telomere homeostasis; for example, both TRF1 and TRF2 have been implicated as negative regulators of telomere length, as overexpression of either of these proteins resulted in telomere shortening in telomerase-positive cells (van Steensel and de Lange, 1997; Smogorzewska *et al.*, 2000). It was suggested that both proteins make telomere ends inaccessible to telomerase, the enzyme responsible for elongating telomeres, due to their ability to promote t-loop formation, creating a physical barrier such that telomerase cannot act on chromosome ends (Smogorzewska *et al.*, 2000). This is believed to contribute to telomere length homeostasis, preventing excessive telomere elongation, a mechanism which is key to maintaining constant telomere length in the germline over generations for example, despite high telomerase activity in these cells (Smogorzewska *et al.*, 2000). As well facilitating T-loop formation (Stansel *et al.*, 2001), TRF2 has also been shown to protect telomeres by inhibiting ATM-dependent DNA-damage response (DDR) and DNA ligase IV-dependent non-homologous end-joining (NHEJ), thus preventing end-to-end fusions and chromosomal instability (van Steensel *et al.*, 1998; Smogorzewska *et al.*, 2002; Karlseder *et al.*, 2004; Denchi and de Lange, 2007). It has been suggested that TRF2 confers protection from NHEJ partly by recruiting its binding partner, RAP1, which alone was shown to be sufficient to prevent this repair mechanism (Bae and Baumann, 2007; Sarthy *et al.*, 2009). In contrast to TRF2, POT1 is responsible for inhibiting ATR-dependent DDR signalling, and it also interacts with TRF1 to regulate telomere length (Loayza and De Lange, 2003; Denchi and de Lange, 2007). Telomere length homeostasis is also maintained by TPP1, which has been shown to directly interact with and recruit telomerase to the ends of chromosomes (Xin *et al.*, 2007). Moreover, this shelterin protein directly interacts with POT1 and increases its affinity for single-stranded telomeric sequences (Lu *et al.*, 2013). Assembly of the shelterin complex is also dependent on TIN2, which bridges TRF1 and TRF2, and such interaction is stabilised by TPP1, ensuring robust formation of this complex which is crucial for telomere protection

(O'Connor *et al.*, 2006). Moreover, TIN2 stabilises TPP1/POT1 complexes on single-stranded telomeric sequences, and contributes to effective repression of ATR activation at telomeres (Takai *et al.*, 2011).

Telomere dysfunction can occur as a result of telomere shortening that happens with each round of cell division, due to the so called "end-replication problem". This was first described by Olovnikov and Watson in the early seventies, and stems from the inability of conventional DNA polymerases to completely replicate the lagging-strand of DNA (Olovnikov, 1971; Watson, 1972). In order for lagging strand synthesis to occur, RNA primers are inserted where polymerases can initiate replication; however, upon removal of the final primer at the 3' end, the newly synthesised strand is shorter than the template by a few nucleotides, leading to loss of telomeric repeats with each round of replication. This was later confirmed experimentally by reports showing that telomere shortening indeed occurs in human fibroblasts with age, and it was suggested that extensive telomere attrition was involved in replicative senescence (Harley *et al.*, 1990; Harley, 1991). This idea was then corroborated when a study showed that expression of telomerase in normal human cells was sufficient to extend their lifespan in culture, implicating telomere shortening as causal in the senescence process (Bodnar *et al.*, 1998).

It is believed that extensive telomere attrition results in loss of shelterin components (*i.e.* uncapping), which in turn destabilises the T-loop and exposes the ends of chromosomes, subsequently triggering a DDR similar to double-stranded breaks and inducing cell-cycle arrest (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003; Herbig *et al.*, 2004; O'Sullivan and Karlseder, 2010). Indeed, inducing uncapping by expressing a dominant-negative allele of TRF2 in human fibroblasts has been shown to activate a DDR at telomeres (Takai *et al.*, 2003). Telomeres in these cells became associated with DNA damage response factors such as 53BP1, Mre11 complex, and phosphorylated forms of ATM, H2AX and Rad17, showing that uncapped telomeres resemble DNA breaks (Takai *et al.*, 2003). Moreover, conditional deletion of Pot1a in mice was shown to result in telomere deprotection and activation of a DDR at telomeres (Wu *et al.*, 2006a). These cells also displayed aberrant homologous recombination (HR) at telomeres, and developed a senescent phenotype comparable to replicative senescence, supporting the idea that uncapping results in genomic instability and activates downstream DDR signalling to induce senescence (Wu *et al.*, 2006a). In accordance to this, telomeres in human fibroblasts undergoing replicative senescence have been shown to associate with DDR

proteins such as 53BP1, MC1 and NBS1, as well as markers of DSBs such as γH2AX (d'Adda di Fagagna *et al.*, 2003).

Although cellular replication is a main contributor to telomere attrition, other factors can influence the rate at which telomeres shorten. For example, it has been widely demonstrated that mild oxidative stress accelerates telomere shortening and reduces the proliferative capacity of cells, inducing a permanent cell-cycle arrest that resembles replicative senescence (von Zglinicki *et al.*, 1995; Xu *et al.*, 2000; von Zglinicki, 2002; Saretzki *et al.*, 2003). Moreover, interventions that result in decreased levels of intracellular peroxide such as overexpression of the antioxidant enzyme superoxide dismutase (SOD3) in human fibroblasts or antioxidant treatment, reduce the rate of telomere shortening and extend replicative lifespan, reinforcing the involvement of reactive oxygen species (ROS) in telomere-dependent senescence (von Zglinicki *et al.*, 2000; Serra *et al.*, 2003). In addition, one study has shown that cells with lower antioxidant capacity have a shorter lifespan and increased rates of telomere shortening compared to cells with a better antioxidant capacity, suggesting that intracellular oxidative stress levels is directly related to the rate of telomere shortening (Richter and von Zglinicki, 2007). Reactive oxygen species derived from mitochondria have also been shown to play a role in telomere shortening, as treatment of fibroblasts with MitoQ, a mitochondrial-targeted antioxidant, was shown to reduce the rate of telomere shortening and extend proliferative capacity under conditions of mild oxidative stress (*i.e.* hyperoxia) (Saretzki *et al.*, 2003). Moreover, reducing mitochondrial ROS production by mild uncoupling of mitochondria slows the rate of telomere shortening and extends lifespan of human fibroblasts. On the other hand, inducing severe depolarisation by using FCCP, a cyanide analogue that disrupts the mitochondrial membrane potential, leads to mitochondrial dysfunction, increased ROS production and consequently accelerated telomere shortening (Liu *et al.*, 2002).

The susceptibility of telomeres to oxidative stress is thought to be a result of their high content of guanine triplets, which are extremely prone to oxidative modifications (Oikawa and Kawanishi, 1999). In accordance to this, studies demonstrated that mild oxidative stress causes single-stranded breaks to preferentially accumulate at telomeres, and this in turn contributes to telomere shortening by stalling the replication fork, which results in incomplete replication of telomeric DNA (Petersen *et al.*, 1998; von Zglinicki, 2000). Moreover, damage at telomeres has been shown to be less efficiently repaired when compared to non-telomeric damage, mainly as a consequence of the presence of shelterin components (Kruk *et al.*, 1995). For example, proteins such as TRF2 and RAP1 inhibit DNA-PK, a double-stranded break repair complex,

preventing NHEJ to occur at telomeres (Bombarde *et al.*, 2010). In agreement with this notion, recruitment of ligase IV is impaired if DSBs are induced adjacent to telomeric repeats in budding yeast, whereas in mammalian cells a persistent DDR is observed when TRF2 is expressed in the vicinity of DSBs, providing further evidence to the irreparability of telomeric damage (Fumagalli *et al.*, 2012).

Although a number of studies have focused on senescence induced by telomere shortening, it is now becoming increasingly evident that telomere dysfunction can also occur independently of length. For example, a persistent DDR signalling has been shown to occur at telomeres irrespectively of length when human fibroblasts *in vitro* and mouse neurons *in vivo* were exposed to genotoxic stress (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). The presence of DNA damage proteins at longer telomeres has also been reported during the ageing process *in vivo*, where an increase in length-independent telomere damage was seen in the gut and liver of mice with age (Hewitt *et al.*, 2012; Jurk *et al.*, 2014). Moreover, an age-dependent accumulation of dysfunctional telomeres has also been shown in hippocampal neurons and liver of baboons (Fumagalli *et al.*, 2012), suggesting that such signalling contributes to cellular senescence and plays a role in tissue dysfunction during ageing. Length-independent telomere damage has also been shown in cells undergoing oncogene-induced senescence, such as in melanocytic nevi, and interestingly, some dysfunctional telomeres were still associated with TRF2 (Suram *et al.*, 2012), suggesting that factors other than shortening and uncapping (i.e. loss of shelterin components) may trigger a DDR at telomeres. In accordance to this, telomere dysfunction independently of length has also been reported in replicative senescence, where TRF2 and RAP1 where still present in a portion of DDR-positive telomeres (Kaul *et al.*, 2011), suggesting that DDR activation at telomeres is not always accompanied by the loss of shelterin proteins. In contrast to telomere dysfunction arising due to shortening or uncapping, which is usually referred to as telomere-induced foci (TIF), stress-induced telomere damage is also known as telomere-associated foci (TAF), and has been suggested to occur due to DSBs within telomeric regions (Hewitt *et al.*, 2012), although until recently, it was unclear whether activation of a DDR indicated physical DNA double stranded-breaks or if it merely represented changes in chromatin without an associated lesion. However, it is now possible to identify physical DNA breaks by using a new method, which involves DNA damage *in situ* ligation followed by proximity ligation assay. By doing this, it has been demonstrated that acute genotoxic stress causes DNA double stranded breaks to occur, both *in vitro* and *in vivo*, and these also co-localise with DDR proteins (Galbiati *et al.*, 2017).

It has been proposed that longer telomeres are more susceptible to DNA damage as they represent a more abundant target for lesion formation, thus explaining the occurrence of DDR activation independently of telomere length (Fumagalli *et al.*, 2012). In fact, elongating telomeres in human cancer cells significantly increases their susceptibility to ionising radiation, suggesting that telomeres exceeding a critical length are more prone to accumulating DSBs (Fairlie and Harrington, 2015). However, evidence so far does not clearly indicate that longer telomeres accumulate more damage than shorter ones. Analysis of individual telomere length in melanocytic nevi and in mice have failed to identify a significant difference in length between damaged and non-damaged telomeres (Hewitt *et al.*, 2012; Suram *et al.*, 2012). This could be a consequence of the low sensitivity of current methods available to measure individual telomere length in tissues, whereby very short telomeres are not detected, thus making it difficult to identify significant differences in length between dysfunctional and functional telomeres.

It has been proposed that telomeres may exist in three different states, possibly explaining the observation that telomeres can signal a DDR in the presence of shelterin components (Cesare *et al.*, 2009). The first is known as the closed or fully capped state, whereby telomeres maintain sufficient length such that t-loop conformation remains intact, preventing DDR activation, and inhibiting NHEJ due to the presence of shelterin proteins. However, if T-loop conformation is lost, telomeres may adopt an intermediate state, where a DDR is activated but sufficient levels of shelterin proteins are still maintained to prevent end-to-end fusions. This may occur in a length-dependent and –independent manner, explaining how telomeres that are not critically short and still contain shelterin proteins can accumulate damage. Lastly, the fully uncapped state occurs when the T-loop is completely lost due to extensive telomere erosion. This results in loss of shelterin components, such that a DDR is activated and NHEJ is no longer inhibited, leading to end-to-end fusions (Cesare *et al.*, 2009).

In summary, it is now evident that telomeres play a much more intricate role in modulating DNA damage responses in senescence, and do not simply act as "mitotic clocks". Instead, they serve as sensors of stress and damage, and maintain genomic stability by limiting proliferation of cells which have accumulated extensive genomic damage.

1.2.2. Non-telomeric DNA damage

DNA damage signalling occurring at non-telomeric regions has also been shown to be involved in the senescence growth arrest. In fact, half of DNA damage foci have been shown to be located

in genomic sites other than telomeres, and despite being short-lived, these are constantly renewed during senescence (Passos *et al.*, 2010; Hewitt *et al.*, 2012). This is likely a result of increased ROS production in senescent cells, as inhibition of ROS leads to a rescue in the proliferation arrest in a fraction of cells (Passos *et al.*, 2010). Double-stranded breaks usually occur as a result of acute and severe stresses, such as ionising radiation, and these are very effective in inducing senescence (Di Leonardo *et al.*, 1994). Other DNA damage-inducing agents include chemotherapeutic drugs, such as bleomycin, which has been shown to induce senescence in both normal and tumour cells (Robles and Adami, 1998; te Poele *et al.*, 2002). The latter is also known as drug-induced senescence, and it is now being used as a possible cancer therapy (Schmitt *et al.*, 2002; Roninson, 2003). Exposing cells to hyperoxia, which leads to an increase in intracellular ROS, has also been shown to cause single-stranded and double-stranded breaks, leading to a persistent DDR and inducing senescence (Roper *et al.*, 2004). Moreover, UVB irradiation, which as well as causing DNA breaks also induces highly carcinogenic lesions such as cyclobutane-pyrimidine dimers (CPD), has been shown to induce premature senescence in human keratinocytes *in vitro* (Lewis *et al.*, 2008). Although it is thought that the aforementioned stressors induce senescence independently of telomeres, as telomerase overexpression does not rescue irradiation-induced senescence (Hewitt *et al.*, 2012), it is possible that DDR signalling at telomeres contributes to the persistence of the arrest.

1.2.3. Oncogene-induced senescence

Oncogene-induced senescence (OIS) occurs as a result of oncogene activation, which involves mutations in specific genes that predispose cells to become cancerous. This phenomenon was first reported by studies showing that expression of oncogenic RAS, a transducer of mitogenic signals, resulted in a permanent cell-cycle arrest in mouse and human fibroblasts, which was also accompanied by an increase in p16 and p53, and was phenotypically identical to cellular senescence (Serrano *et al.*, 1997). Since then, other genes involved in the RAS signalling pathway have also been implicated in OIS, such as RAF, MEK, and BRAF (Lin *et al.*, 1998; Michaloglou *et al.*, 2005). It has been suggested that oncogene activation causes DNA replication stress, leading to stalling of replication forks, ultimately triggering a DDR (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). Inhibition of a DDR was shown to prevent induction of senescence as well as allowing RAS-induced senescent cells to resume proliferation, suggesting that DDR signalling is important for both induction and maintenance of OIS (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). It has also been shown that telomere damage plays a role in cells undergoing OIS (Suram *et al.*, 2012). Cells overexpressing telomerase, which prevents

telomeric DDR foci formation in response to oncogene activation, were able to bypass growth arrest, indicating that telomere dysfunction is also important to reinforce oncogene-induced senescence (Suram *et al.*, 2012). Moreover, accumulation of damage at telomeres has also been shown in cancer precursor lesions, such as human melanocytic nevi, ductal breast hyperplasia, and colonic adenoma, suggesting that activation of a DDR at telomeres is also important *in vivo* as a tumour-suppressor mechanism (Suram *et al.*, 2012).

1.2.4. Epigenetic modifications

Transcriptional activity of genes is regulated by modifications in chromatin structure, which can be either organised into heterochromatin (i.e. compressed) or euchromatin (loosely packed). Whereas the latter is normally associated with transcriptionally active genes, heterochromatin results in gene silencing, and such chromatin organisation is controlled by posttranslational modifications of histones, such as acetylation and methylation (Schulz and Tyler, 2005). Early studies showed that treating human fibroblasts with histone deacetylase inhibitors (HDACi), which stimulate chromatin relaxation, resulted in a senescence-like growth arrest, providing evidence that changes in chromatin were involved during the senescence process (Ogryzko *et al.*, 1996). In contrast, it was later reported that senescent human fibroblasts accumulated foci of transcriptionally inactive DNA that were associated with heterochromatin markers, such as hypoacetylated histones (histone H3 methylated on lysine 9) and the heterochromatin protein HP1 (Narita *et al.*, 2003). These were termed senescence-associated heterochromatic foci (SAHF), and were suggested to be important for the stable proliferation arrest by repressing transcription of E2F target genes that promote progression through the cell cycle (Narita *et al.*, 2003). It has also been shown that SAHF are enriched in the histone H2A variant, macroH2A, which is known to be transcriptionally repressive, and its formation involves the activity of histone chaperones, Asf1a and HIRA, which are responsible for the development of transcriptionally inactive heterochromatin in yeast, flies and plants (Zhang *et al.*, 2005; Adams, 2007). Moreover, SAHF formation relies on the Rb pathway, which was suggested to be due to the ability of Rb to promote nucleation of heterochromatin at specific areas in the genome, which are then propagated with the help of histone methyltransferases and recruitment of HP1 proteins (Narita *et al.*, 2003). The latter are capable of forming dimers, facilitating the development of high order chromatin structures (Brasher *et al.*, 2000; Nielsen *et al.*, 2001). Ultimately, heterochromatin organisation during senescence contributes to a permanent cell cycle arrest by silencing genes required for proliferation. In accordance to this, down-regulation of the histone acetyltransferase p300/CBP, which results in heterochromatin formation, has

been reported in senescent human melanocytes (Bandyopadhyay *et al.*, 2002). The observation that senescence can be induced by both chromatin relaxation, as in the case of HDACi-induced senescence, and by heterochromatin formation is contradictory; however it has been suggested that they both cause dramatic but incomplete changes in chromatin organisation, altering expression of different important genes, and the outcome might vary between cell types (Campisi and d'Adda di Fagagna, 2007a).

1.3. Senescence signalling pathways

The majority of senescence-triggering stimuli activate the DNA damage response, as shown in stress-induced (Hewitt *et al.*, 2012), oncogene-induced (Suram *et al.*, 2012) and replicative senescence (d'Adda di Fagagna *et al.*, 2003). In turn, the p53-p21 and p16-pRb pathways are activated, which then establish and maintain the senescence growth arrest (***Figure 1.1***). Although these pathways interact to some extent, they are activated in response to different stimuli, and the ability of each pathway to induce senescence is cell-type and species-specific (Campisi and d'Adda di Fagagna, 2007a).

1.3.1. The DNA damage response

Activation of a DDR follows as a result of both telomeric and non-telomeric DNA damage, and the recognition of lesions and subsequent repair mechanism can differ depending on the type of damage. In general, proteins of the phosphatidylinositol 3-kinase-like protein kinase (PIKK) family, such as ATM, ATR and DNA-PK, and members of the poly(ADP-ribose) polymerase (PARP) family mediate a DDR signal transduction (Ciccia and Elledge, 2010). Double-stranded breaks are primarily recognised by ATM and DNA-PK, where the latter controls proteins involved in non-homologous end joining (Meek *et al.*, 2008; Ciccia and Elledge, 2010). On the other hand, ATR is activated in response to single-stranded breaks that may occur as a result of stalled replication forks, although it has also been implicated in DSB signalling (Cimprich and Cortez, 2008). In addition, PARP1 and PARP2 have also been shown to be involved in both SSB and DSB repair, and are important for the recruitment of other DDR proteins to the site of damage by catalysing the addition of poly(ADP-ribose) chains on specific proteins (Schreiber *et al.*, 2006).

Double-stranded breaks are initially recognised by the MRE11-RAD50-NBS1 (MRN) complex, which then recruits ATM via the C-terminus of NBS1 (van den Bosch *et al.*, 2003; Falck *et al.*, 2005; d'Adda di Fagagna, 2008). This is followed by autophosphorylation of ATM, which triggers dissociation of the inactive protein dimer, activating ATM kinase activity

(Bakkenist and Kastan, 2003). Upon activation, ATM then phosphorylates a range of target proteins at the site of damage, including the histone variant H2A.X at serine 139, giving rise to phosphorylated H2A.X (γH2A.X) (Rogakou *et al.*, 1998; Rogakou *et al.*, 1999). The phospho-specific domain of MDC1 (mediator of DNA damage checkpoint 1) then recognises the phosphorylated form of H2A.X, and MDC1 stimulates further recruitment of the MRN complex. This results in a positive feedback loop such that local ATM activity is amplified and γH2A.X is spread to adjacent areas from the initial site of damage, where the latter can be visualised as discrete nuclear foci (Rogakou *et al.*, 1999; Stucki and Jackson, 2006). The exposure of modified histone residues has also been shown to recruit the DNA-damage mediator 53BP1 to sites of damage, which helps maintain the DDR by interacting with MDC1 (Huyen *et al.*, 2004; Eliezer *et al.*, 2009).

In contrast, single-stranded breaks, such as those caused by UV exposure or stalled replication forks in response to replication stress, are coated by the single-stranded DNA-binding protein RPA (replication protein A), which triggers recruitment of ATR (Cortez *et al.*, 2001). In addition, ssDNA coated with RPA can also be formed during the end resection of DSBs, leading to activation of ATR (Cimprich and Cortez, 2008). ATR then binds to RPA-coated ssDNA via interactions with ATR-interacting protein (ATRIP), and this recruits the 9-1-1 complex (composed of RAD9, RAD1 and HUS1) and TOPBP1, which are important for promoting and amplifying ATR kinase activity (Kumagai *et al.*, 2006; Cimprich and Cortez, 2008; d'Adda di Fagagna, 2008).

An increase in local ATM and ATR activities above a certain threshold level is crucial to activate other DDR factors that act distantly from the initial site of damage (d'Adda di Fagagna, 2008). For example, the checkpoint kinases Chk1 and Chk2 freely diffuse through the nucleus upon phosphorylation by ATR and ATM, respectively, and are then able to spread DDR signalling through the nucleus by phosphorylating their downstream targets (Lukas *et al.*, 2003; Buscemi *et al.*, 2004; Bekker-Jensen *et al.*, 2006). It is important to note that Chk1 and Chk2 can be phosphorylated by both ATM and ATR, and that their selective substrate specificity was shown to be dependent on the type of DNA damage inducing agent (Helt *et al.*, 2005). Ultimately, phosphorylated Chk1 and Chk2 act on downstream targets that are involved in cell cycle progression, such as cell-division cycle 25 (CDC25) phosphatases and the tumour suppressor protein p53 (d'Adda di Fagagna, 2008). Phosphorylation of CDC25, a phosphatase required for G1-S phase progression, induces its rapid degradation and thus cell cycle arrest in response to DNA damage (Mailand *et al.*, 2000). Moreover, phosphorylation of p53 at Ser-15

and Ser-20 by Chk2 and ATM, respectively, leads to dissociation of p53 from its negative regulator HDM2, which in turn stabilises p53 and promotes its ability to induce transcription of the cyclin-kinase inhibitor p21, again inducing cell-cycle arrest (Di Leonardo *et al.*, 1994; Turenne *et al.*, 2001; Campisi and d'Adda di Fagagna, 2007a; d'Adda di Fagagna, 2008). In summary, checkpoint kinases are key components of the DDR signalling pathway which transduce the DDR into cell cycle-arrest in order to allow cells to repair DNA damage. If damage remains unrepaired, this results in persistent DDR signalling and senescence-associated growth arrest. Importantly, such persistent DDR signalling has been observed at telomeres in cells undergoing replicative (d'Adda di Fagagna *et al.*, 2003), stress- (Hewitt *et al.*, 2012) and oncogene-induced senescence (Suram *et al.*, 2012), with one study reporting persistent telomeric damage *in vivo* in mouse hippocampal neurons even after three months after exposure to genotoxic stress (Fumagalli *et al.*, 2012). Therefore, unrepaired telomeres are thought to be a significant source of persistent DNA damage signalling which is involved in the establishment of senescence.

1.3.2. p53-p21 pathway

Generally, stimuli that activate a DDR induce senescence mainly through the p53 pathway (Campisi and d'Adda di Fagagna, 2007a). The p53 protein is a tumour suppressor and coordinates antiproliferative cellular responses in response to stress-inducing stimuli. Activation of p53 in response to signals such as DNA damage, oncogene activation or hypoxia, will then trigger apoptosis, transient cell-cycle arrest or senescence (Zilfou and Lowe, 2009). The fate adopted by the cell in response to p53 activation is context-dependent, and can vary depending on a number of factors, including cell type and microenvironment (Zilfou and Lowe, 2009). Under normal conditions, p53 is maintained at low levels by its interaction with the E3 ubiquitin protein ligase human double minute 2 (HDM2), which both inhibits the ability of p53 to activate transcription of target genes and also targets it for degradation (Haupt *et al.*, 1997). Upon DDR activation, p53 undergoes a series of post-translational modifications, which contribute to its stabilisation (Zilfou and Lowe, 2009). For example, ATM has been shown to phosphorylate p53 at Ser15 in response to ionising radiation (Banin *et al.*, 1998), whereas ATR phosphorylates both Ser15 and Ser37 in cells exposed to UV light (Tibbetts *et al.*, 1999). Moreover, Chk1 and Chk2 phosphorylate p53, and such phosphorylation events prevents binding of HDM2, stabilising p53, and allowing it to regulate transcription of its target genes (Chehab *et al.*, 2000; Shieh *et al.*, 2000; Zilfou and Lowe, 2009). One major downstream target of p53 is the cyclin-dependent kinase inhibitor (CDKi) p21, which physically interacts with

cyclin-CDK2, -CDK1, and –CDK4/6 complexes, as well as inhibiting PCNA, thus preventing progression through the cell cycle during the G1 and S phases (Stein *et al.*, 1999; Georgakilas *et al.*, 2017). Moreover, p21 can also inhibit CDKs that target the RB tumour suppressor protein, which then inhibits E2F, a transcription factor that regulates expressions of proliferation genes (Zilfou and Lowe, 2009). Indeed, p21-deficient mouse embryonic fibroblasts (MEFs) have been shown to bypass senescence in response to DNA damage (Brugarolas *et al.*, 1995). Additionally, Chk2 inactivation results in decreased p21 expression and extends the replicative lifespan of human fibroblasts, consistent with the observation that p53 fails to be activated in response to both telomere erosion and DNA damage (Gire *et al.*, 2004). Therefore, these studies support the importance of the p53-p21 pathway to the stable senescence-growth arrest.

1.3.3. p16-pRb pathway

The p16-pRb pathway is also important for cell-cycle arrest in response to DNA damage inducing stimuli. The cyclin-dependent kinase inhibitor p16 binds to CDK4/6, thus inhibiting its kinase activity and preventing Rb phosphorylation. In turn, Rb remains bound to the transcription factor E2F1, promoting its nuclear exclusion, thus preventing transcription of E2F1 target genes, which are necessary for the G1/S transition (Rayess *et al.*, 2012). Although this pathway is generally thought to act secondary to the activation of p53, some cell types primarily induce p16 to promote growth arrest. For example, inactivation of the p16-pRb pathway together with induction of telomerase activity are both necessary to achieve immortalisation of human epithelial cells, which was shown to be independent of p53 expression (Kiyono *et al.*, 1998). However, studies so far have provided conflicting data on the involvement of p16 in telomere-induced senescence. It has been shown that p16 can be activated independently of telomere dysfunction in human fibroblasts (Herbig *et al.*, 2004). On the other hand, deletion of p16 in Wrn-deficient mice, which lack a protein needed for efficient telomere replication and thus have dysfunctional telomeres, rescued proliferation of MEFs (Zhang *et al.*, 2012). DDR signalling that occurs upon expression of the telomere damaging protein TRF2(ΔBΔM), which induces uncapping, was partially attenuated by p16 deletion in MEFs isolated from Wrn knock out mice, as well as preventing these cells and wild-type MEFs from undergoing senescence (Zhang *et al.*, 2012). Therefore, these data implicate p16 as an important contributor to telomere dysfunction-induced growth arrest (Zhang *et al.*, 2012). Moreover, inducing acute telomere damage by expressing a dominant-negative allele of TRF2 in human fibroblasts resulted in increased p16 expression (Jacobs and de Lange, 2004). Although p16 deficiency in these cells only partially rescued growth arrest induced by telomere

dysfunction, proliferation was almost completely restored when both p16 and p53 were inhibited, suggesting that p16 may act as a second barrier to inhibit proliferation following telomere dysfunction (Jacobs and de Lange, 2004). In accordance to this, it has been demonstrated that cell-cycle arrest induced by telomere damage is primarily maintained by p53 and can be rescued by inhibiting this transcription factor (Beausejour *et al.*, 2003). It was shown that cells with low levels of p16 at senescence resumed proliferation upon p53 inhibition, whereas proliferation arrest of cells with high levels of p16 could not be reversed upon p53 inactivation, suggesting that the p16-pRb pathway provides a second barrier to cell-cycle progression (Beausejour *et al.*, 2003). Interestingly, cells that fully engage in the p16-pRb pathway for several days do not resume proliferation even after p16 or pRb inactivation (Beausejour *et al.*, 2003). Overall, it is evident that both the p53 and p16 pathways play an important role in reinforcing senescence, however the extent of their contribution varies amongst different cell types. In addition, the kinetics of each pathway has been shown to differ during the senescence process. Levels of p21 were highest at the early stages of growth arrest and then declined, whereas p16 levels gradually increased and remain high in senescent fibroblasts, suggesting that p16 upregulation is crucial in the final stages of the senescence cell-cycle arrest (Alcorta *et al.*, 1996). Although the mechanism by which p16 expression is induced upon senescence-causing stimuli is not fully understood, it is possible that a DDR activates p38 MAPK signalling, which in turn upregulates p16 (Bulavin *et al.*, 2004; Wood *et al.*, 2009; Spallarossa *et al.*, 2010) Other triggers of the p16 pathway include oncogenic RAS, which was also shown to upregulate p16 expression through the activation of Ets1 and Ets2 transcription factors (Serrano *et al.*, 1997; Ohtani *et al.*, 2001). The latter are known downstream targets of Ras-Raf-MEK signalling, and have been shown to induce p16 expression by binding to a conserved ETS-binding site at the $p16^{INK4a}$ promoter (Ohtani *et al.*, 2001)

The p16-pRb pathway is also important for the generation of SAHF, as previously discussed (Narita *et al.*, 2003). Once established, SAHF can be self-maintained, which is likely due to the ability of pRb to interact with histone-modifying enzymes that form repressive chromatin (Narita *et al.*, 2003; Macaluso *et al.*, 2006). Moreover, the p16-pRb pathway has been shown to act together with mitogenic signals to increase intracellular ROS in senescent cells. This in turn activates protein kinase C delta (PKCδ), which promotes further production of ROS, resulting in a positive feedback loop between ROS and PKCδ. It was suggested that sustained activation of such signalling contributes to the irreversible cell-cycle arrest partly by reducing

the levels of WARTS, a mitotic exit network (MEN) kinase which is required for cytokinesis, thus blocking this process (Takahashi *et al.*, 2006).

1.3.4. p38 MAPK pathway

Mitogen activated protein kinases (MAPKs) mediate signalling to various biological processes, and consist of a family of proteins including ERK, JNK and p38. For example, cell proliferation is usually controlled by the Erk pathway, whereas the JNK and p38 pathways are activated by cellular stresses such as ROS, UV light exposure, X-rays and inflammatory cytokines (Chang and Karin, 2001). Activation of the p38 MAPK signalling has been shown to contribute to the senescence-growth arrest by activating both the p53-p21 and p16-pRb pathways (Lin *et al.*, 1998). Indeed, p38 activation is crucial for oncogenic RAS-induced senescence (Wang *et al.*, 2002), which acts through the Raf-MEK-ERK pathway to induce a permanent cell-cycle arrest (Zhu *et al.*, 1998). Consistent with this, constitutively active mutants of Raf-1 and MEK1 have been shown to induce senescence, whereas inhibiting this pathway delays the onset of senescence (Lin *et al.*, 1998; Shin *et al.*, 2013). Induction of the ERK pathway then leads to downstream activation of MKK3/6 which mediates p38 activation, although the mechanism by which this occurs is not fully understood (Xu *et al.*, 2014b). One possibility is that ROS mediates the connection between MEK-ERK and MKK3/6-p38, since Ras activation leads to an increase in ROS, and the latter has been shown to activate p38 (Nicke *et al.*, 2005; Zdanov *et al.*, 2006). It is also possible that hyper-proliferation induced by MEK-ERK activation causes DNA damage which is known to activate p38, leading to senescence (Wang *et al.*, 2000; Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). Moreover, p38 activation has also been implicated in telomere-dependent senescence, such that p38 inhibition increased replicative lifespan of fibroblasts in culture (Iwasa *et al.*, 2003). Finally, p38 has also been linked to the stability of senescence through a positive-feedback loop where activation of a DDR acts through signalling pathways that involve p38MAPK (Passos *et al.*, 2010). This ultimately leads to increased mitochondrial ROS production, which replenishes DNA damage foci, and stabilises the senescence growth arrest through continuous activation of p21 (Passos *et al.*, 2010).

Moreover, activation of p38 has also been shown to drive T cell senescence, and thus plays a role in the decline of immune function with ageing (Henson *et al.*, 2014; Lanna *et al.*, 2014). It was demonstrated that senescent $CD4^+$ T cells exhibited increased p38 phosphorylation, which occurred as a result of DDR-dependent AMPK activation (Lanna *et al.*, 2014). In turn, AMPK forms a complex with the scaffold protein TAB1, which then recruits p38 and stimulates its autophosphorylation, leading to decreased proliferation and telomerase activity of T cells

(Lanna *et al.*, 2014). Moreover, enhanced p38 activity was shown to contribute to $CD8^+$ T cell senescence by inhibiting autophagy in an mTORC1-independent manner (Henson *et al.*, 2014). It was shown that p38 inhibition increased autophagy in these cells, thus increasing the availability of metabolic precursors, and providing the energy necessary to resume proliferation of senescent $CD8^+$ T cells (Henson *et al.*, 2014).

Overall, a number of stimuli culminate in activation of the p38MAPK pathway, which can then drive senescence through regulation of downstream pathways that ultimately affect cell-cycle progression.

1.3.5. mTOR signalling

The mammalian target of rapamycin (mTOR) is a protein kinase that belongs to the PI3KK family that plays a role as a nutrient and energy sensor and coordinates cellular responses such as cell growth and metabolism (Xu *et al.*, 2014a). There are two conserved TOR complexes, which are known as mTORC1 (TOR complex 1) and mTORC2 (TOR complex 2), and these are distinguished through their association with different adaptor proteins, namely Raptor and Rictor, respectively (Xu *et al.*, 2014a). These two complexes have distinct roles within the cell, with mTORC1 being the most well characterised between the two. It has been shown that mTORC1 is the main mediator of nutrient signalling, and it also converts signals from growth factors, stress and energy availability into cellular responses such as protein synthesis, lipid biosynthesis, glucose metabolism and autophagy (Johnson *et al.*, 2013). mTORC1 then acts via downstream effectors, such as ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), which are involved in regulation of translation initiation (Xu *et al.*, 2014a). On the other hand, mTORC2 is poorly characterised, however it has been implicated in spatial organisation of cytoskeleton, which interacts with the mTORC1 machinery to increase cytoplasmic volume (Xu *et al.*, 2014a).

Emerging data now implicates mTOR in the senescence process. It has been shown that continuous mTOR activity is required for the permanent cell-cycle arrest associated with senescence in cells overexpressing p21 (Demidenko and Blagosklonny, 2008). Serum starvation or treatment with rapamycin, which inhibits mTORC1, decreased the expression of senescence markers. Interestingly, p21-arrested cells in the absence of serum, where mTOR activity is low, were capable of resuming proliferation when p21 was no longer induced, whereas in the presence of serum, cells were irreversibly arrested. This suggests that a permanent proliferation arrest in response to p21 overexpression is dependent of continuous

mTOR activity (Demidenko and Blagosklonny, 2008). Studies have also demonstrated that rapamycin treatment can attenuate the senescent phenotype of fibroblasts treated with the DNA damaging agent, doxorubicin (DOX) (Demidenko and Blagosklonny, 2008). This has also been observed in human fibroblasts undergoing either OIS or replicative senescence, whereby treatment with rapamycin not only increased replicative lifespan of these cells but also attenuated several phenotypes associated with senescence, such as Sen-β-Gal activity, and expression of p21 and IL-8 (Kolesnichenko *et al.*, 2012). Similar observations have been reported in rat embryonic fibroblasts (Pospelova *et al.*, 2012). Consistently, constitutive Wnt1 activation, which was shown to activate mTOR, induces premature senescence in mouse keratinocytes, and contributes to loss of the epidermal stem cell compartment, and progressive hair loss in mice. This phenotype was partially rescued by rapamycin treatment, providing further evidence that decreasing mTOR activity by using rapamycin can decelerate cellular senescence (Castilho *et al.*, 2009). More recently, mTOR has been shown to integrate signals downstream of a DDR, whereby ATM induces Akt phosphorylation and mTORC1 activity, which in turn promotes PGC-1β-dependent mitochondrial biogenesis (Correia-Melo *et al.*, 2016). Consequently, this contributes to increased ROS generation, which generates further DNA damage, maintaining a DDR as part of a positive feedback loop that contributes to the senescent phenotype (Correia-Melo *et al.*, 2016).

Moreover, mTOR has been shown to regulate NF-κB activity, a transcription factor responsible for regulating genes involved in the pro-inflammatory phenotype of senescent cells (Dan *et al.*, 2008; Chien *et al.*, 2011b; Laberge *et al.*, 2015). It has been demonstrated that mTOR indirectly affects NF-κB activity by regulating translation of the pro-inflammatory cytokine IL-1α (Laberge *et al.*, 2015). Activation of IL-1 receptor (IL-1R) signalling by IL-1α activates kinases such as interleukin-1 receptor-associated kinase 1 (IRAK1), which ultimately enhances the transcriptional activity NF-κB (Orjalo *et al.*, 2009). The latter then induces the expression of pro-inflammatory genes, such as IL-6 and IL-8; therefore, IL-1α is an important positive regulator of IL-6 and IL-8 expression by regulating NF-κB in senescent cells (Orjalo *et al.*, 2009). Indeed, inhibition of mTOR by rapamycin treatment has been shown to diminish translation of IL-1α, and supress secretion of a number of pro-inflammatory cytokines, including IL-6, suggesting that mTOR is involved in controlling the senescence-associated secretory phenotype (SASP) (Laberge *et al.*, 2015). Moreover, mTORC1 can also directly interact with components involved in NF-κB signalling, such as IKKα, promoting phosphorylation of IκB and RelA/p65 subunit, which is associated with increased

transcriptional activity of NF-κB (Dan *et al.*, 2008). mTOR has been also been shown to regulate the SASP by promoting translation of the MK2 kinase (Herranz *et al.*, 2015). The latter then phosphorylates the RNA-binding protein ZFP36L1, a Zn-finger protein responsible for destabilising mRNAs, and by doing so, stabilises the transcripts of many SASP factors in senescent cells (Herranz *et al.*, 2015). Therefore, mTOR is a major regulator of the SASP during senescence.

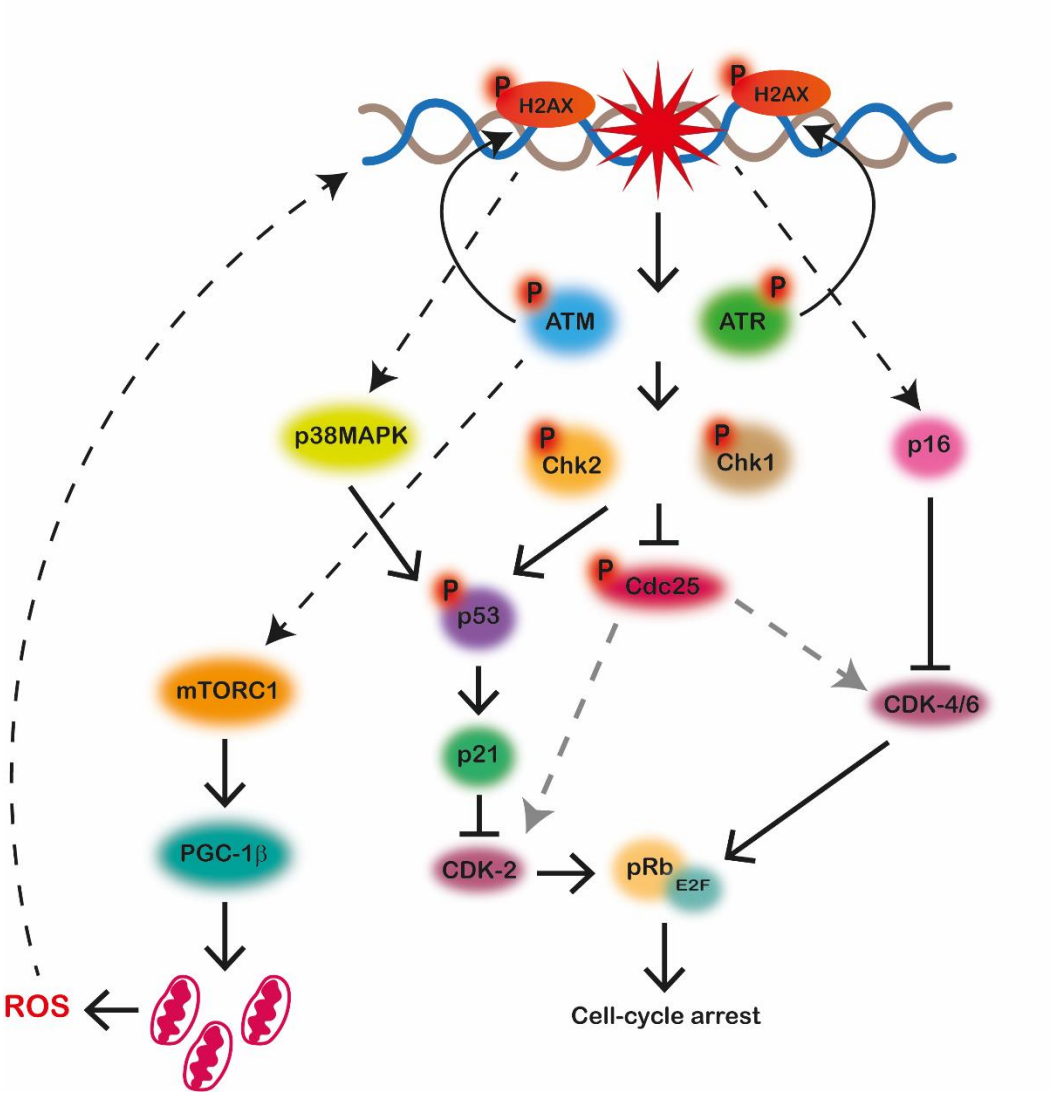

Figure 1.1 - The DNA damage response and downstream senescence effector pathways. The majority of stimuli that induce senescence, such as telomere dysfunction and DNA damage, trigger a DNA damage response (DDR). This involves phosphorylation and activation of ATM and ATR kinases, which then phosphorylate the histone variant H2AX, giving rise to γH2AX foci. Phosphorylation of downstream kinases ultimately results in activation of the p53-p21 and p16-pRb pathways. Induction of p53 induces a senescence-associated growth arrest by increasing the expression of the cyclin-dependent kinase (CDK) inhibitor p21, which prevents phosphorylation and thus inactivation of pRb. DDR signalling can also activate p16, which is also a CDK inhibitor, and inhibits pRb inactivation. pRb remains associated with E2F, inhibiting the activity of this transcription factor regulates expression of genes required for cell-cycle progression. Moreover, ATM can promote mTORC1 activity, which induces PGC-1β dependent mitochondrial biogenesis, leading to increased ROS generation, which generates further DNA damage, maintaining the DDR.

1.4. Senescence phenotype

Cellular senescence is accompanied by many phenotypic changes, including apoptosis resistance, altered gene expression, increased ROS production, and secretion of pro-inflammatory factors (Campisi and d'Adda di Fagagna, 2007a). These features will be discussed in more detail in the sections below.

1.4.1. Growth arrest

Senescence is characterised by a stable arrest in the cell cycle, which is the result of continuous expression of key cell-cycle inhibitors, as previously mentioned. Despite their inability to divide, senescent cells remain metabolically active (Campisi and d'Adda di Fagagna, 2007a). Senescence growth arrest usually occurs with a DNA content typical of G1 phase, which is the case in most mouse and human fibroblasts (Di Leonardo *et al.*, 1994; Herbig *et al.*, 2004). However, cells have also been shown to arrest in other stages of the cell cycle, which appears to vary according to cell type and the nature of the senescence-inducing stimuli. For example, mouse fibroblasts with a defect in the stress-signalling kinase MKK7 were shown to arrest at G2/M phase (Wada *et al.*, 2004). Similarly, oncogene activation can also trigger G2 growth arrest (Zhu *et al.*, 1998; Di Micco *et al.*, 2006). Nonetheless, the senescence growth arrest is deemed to be permanent, since senescent cells cannot be stimulated to re-enter the cell cycle by known physiological stimuli (Campisi and d'Adda di Fagagna, 2007a).

1.4.2. Apoptosis resistance

Similarly to senescence, apoptosis is a controlled and extreme response to stress, and also serves as a tumour-suppressor mechanism (Green and Evan, 2002). It is not clear what determines a cell to undergo senescence over apoptosis, and vice-versa, although different cell types appear to preferentially adopt different fates in response to damaging stimuli. For example, fibroblasts and epithelial cells usually become senescent upon damage, whereas lymphocytes normally undergo apoptosis (Campisi and d'Adda di Fagagna, 2007a). The type and intensity of stress are also important determinants of cellular response. For example, low doses of doxorubicin induces senescence, whereas high doses leads to apoptosis in rat cardiomyocytes (Spallarossa *et al.*, 2009). Consistently, exposure to low doses of UVB irradiation causes skin fibroblasts and keratinocytes to become senescent, and apoptosis occurs at high doses (Kuhn *et al.*, 1999; Debacq-Chainiaux *et al.*, 2005). Other DNA damaging drugs, such as the alkylating agent busulfan, induces senescence, but not apoptosis, in fibroblasts in a dose-independent manner (Probin *et al.*, 2006). Moreover, cell fate is also likely controlled by the balance between pro-senescent and pro-apoptotic pathways, which is controlled by their common regulator, p53. For

example, MEFs expressing a mutant version of p53 that impairs p53-dependent apoptosis, preferentially senescence in response to UVB exposure (Tavana *et al.*, 2010). These cells also failed to upregulate pro-apoptotic factors such as PUMA and NOXA, and expressed high levels of the pro-survival gene BCL-2 (Tavana *et al.*, 2010). H_2O_2 treatment at levels which induced both apoptosis and senescence in human fibroblasts caused an increase in p53, and p53 levels were 2-fold higher in apoptotic compared to senescent cells (Chen *et al.*, 2000). Other factors involved in determining cell fate also include the kinetics of p53 expression, as it was shown that low level of γ-irradiation induces a transient rise in p53 levels, which results in a transient cell-cycle arrest followed by recovery. On the other hand, preventing p53 degradation by Nutlin-3a treatment, which inhibits MDM-2, results in high and stable levels of p53, and promotes senescence (Purvis *et al.*, 2012). In agreement with this, stabilising p53 by Nutlin-3a treatment induces senescence in MEFs exposed to oxidative stress, with no evidence of apoptosis (Efeyan *et al.*, 2007). Therefore, p53 appears to be an important regulator of cell fate in response to damage.

The majority of senescent cells become resistant to apoptosis, such as senescent human fibroblasts which are resistant to ceramide-induced apoptosis. However, this is not seen across all cell types, as senescent endothelial cells were shown to undergo apoptosis in response to the same stimulus (Hampel *et al.*, 2004). Resistance to apoptotic stimuli might be one of the reasons senescent cells can remain in culture for prolonged periods of time, and could also partly explain the increase in senescent cells with age (Campisi and d'Adda di Fagagna, 2007a). However, the mechanisms that contribute to apoptosis resistance are not fully understood. It has been shown that senescent cells fail to stabilise p53 in response to DNA damage, which contributes to their inability to undergo p53-dependent apoptosis (Seluanov *et al.*, 2001). Moreover, evidence suggests that chromatin modifications contributes to apoptosis resistance of senescent cells by altering the expression levels of the anti-apoptotic gene, Bcl-2, and the pro-apoptotic gene, Bax (Sanders *et al.*, 2013). It has also been shown that in response to DNA damaging stimuli, p53 preferentially promotes expression of growth arrest genes rather than apoptosis regulators in senescent human fibroblasts, offering another mechanism through which senescent cells acquire resistance to apoptosis (Jackson and Pereira-Smith, 2006).

1.4.3. Changes in gene expression

Senescent cells display vast changes in gene expression, often related to genes that regulate cell-cycle progression (Yoon *et al.*, 2004; Jackson and Pereira-Smith, 2006; Trougakos *et al.*, 2006). For example, the CDKIs p21 and p16 are usually highly expressed in senescent cells,

and these are responsible for establishing and maintaining the growth arrest by inhibiting pRb phosphorylation and thus promoting its activity, as previously discussed (Di Leonardo *et al.*, 1994; Alcorta *et al.*, 1996; Herbig *et al.*, 2004). In addition, genes that stimulate proliferation, such as c-FOS, cyclin A, cyclin B and PCNA, are often repressed in senescent cells (Seshadri and Campisi, 1990; Stein *et al.*, 1991; Pang and Chen, 1994). Expression of some of these genes is repressed since E2F, the transcription factor responsible for inducing them, is inhibited by pRb. Moreover, as previously mentioned, pRb has also been implicated in the formation of SAHF, which play a role in silencing E2F target genes (Narita *et al.*, 2003). Interestingly, senescent cells also overexpress genes that encode secreted proteins, such as extracellular matrix (ECM) degrading proteins, pro-inflammatory cytokines and growth factors (Shelton *et al.*, 1999). This is known as the senescence-associated secretory phenotype, and will be discussed in more detail below.

1.4.4. Senescence markers

Senescent cells can be identified by many markers in culture and *in vivo*, although the markers identified so far are not strictly specific for senescent cells. Since a hallmark of senescence is the loss of replicative potential, a commonly used marker to detect lack of DNA replication is 5-bromodeoxyuridine (BrdU) or ^{3}H-thymidine incorporation. Loss of proliferation can also be detected by immunostaining for proteins such as PCNA and Ki-67 (Lawless *et al.*, 2010). However, these markers also identify quiescent or differentiated post-mitotic cells.

Another marker that is widely used to identify senescent cells is senescence-associated β-galactosidase (Sen-β-Gal) activity. It was first described by Dimri and colleagues that increased Sen-β-Gal expression was a feature of senescent human fibroblasts and keratinocytes in culture, which was histochemically detectable at pH 6, and this was not present in quiescent or post-mitotic cells (Dimri *et al.*, 1995). An age-dependent increase in Sen-β-Gal-positive cells was also reported in human skin samples (Dimri *et al.*, 1995). It has been shown that Sen-β-Gal results from increased expression of lysosomal β-galactosidase and probably reflects increased lysosomal biogenesis that occurs in senescent cells (Lee *et al.*, 2006). However, Sen-β-Gal is also not specific for senescent cells, as it can also be detected in cells which have been cultured in prolonged confluence (Severino *et al.*, 2000).

Senescent cells can also be detected by p16, since this CDKI is upregulated in senescence and mediates the cell-cycle arrest (Alcorta *et al.*, 1996; Serrano *et al.*, 1997; Stein *et al.*, 1999). However, p16 levels may vary in different cell types, and it can also be expressed by some

tumour cells, particularly in those that have lost pRb function (Beausejour *et al.*, 2003; Gil and Peters, 2006). Moreover, p21 is often used as a marker of senescence, as upregulation of the p53-p21 pathway is also involved during the senescence process (Di Leonardo *et al.*, 1994; Herbig *et al.*, 2004), although elevated p21 levels can also be indicative of a transient cell-cycle arrest in response to stress (Barnouin *et al.*, 2002).

The formation of SAHFs during senescence also offers another marker for the detection of these cells. SAHFs can be detected by the preferential binding of DNA dyes, such as 4',6-diamidino-2-phenylindole (DAPI), and are also enriched in specific heterochromatin-associated histone modifications, such as H3 Lys9 methylation, and proteins, for example HP1 (Narita *et al.*, 2003).

Since senescence has been associated with activation of a DDR, senescence-associated DNA-damage foci (SDFs) are also commonly used to identify senescent cells both *in vitro* and *in vivo*. SDFs comprise foci containing activated DDR proteins, including phospho-ATM, phospho-ATR, 53BP1 and γH2AX (d'Adda di Fagagna *et al.*, 2003; Herbig *et al.*, 2004; Rodier *et al.*, 2009). It has been suggested that such foci, also termed DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence), are distinguishable from transient foci, and contribute to persistent DDR signalling which is important for the growth arrest and secretory phenotype of senescent cells (Rodier *et al.*, 2011). As well as genomic DNA damage, these foci also arise from dysfunctional telomeres, including both TIF and TAF, which have been shown to increase with age in many mammalian tissues with age (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003; Hewitt *et al.*, 2012; Jurk *et al.*, 2014). Moreover, p38MAPK has been implicated in regulatig the SASP in a DDR-independent manner, and it was shown to be phosphorylated in response to a range of senescence-inducing stimuli (Freund *et al.*, 2011).

Senescence is usually accompanied by striking morphological changes, such as an increase in cell size, as well as becoming flat and sometimes multinucleated (Serrano *et al.*, 1997; Chen *et al.*, 2001; Dikovskaya *et al.*, 2015). Moreover, senescent cells often show increased nuclear size and irregularity of the nuclear envelope (Mehta *et al.*, 2007). In addition, nuclear envelope proteins, such as lamin B1, has been shown to decline in both human and mouse fibroblasts that are induced to senesce by DNA-damage, replicative exhaustion and oncogene activation, as well as in mouse tissues following irradiation (Freund *et al.*, 2012). Loss in lamin B1 was not observed in quiescent cells, suggesting that this might serve as a biomarker of the senescent state both *in vitro* and *in vivo* (Freund et al., 2012). It has been suggested that lamin B1

downregulation contributes to the vast chromatin reorganisation that occurs during senescence, and impacts the gene expression changes associated with the phenotype (Shah *et al.*, 2013).

In summary, although a variety of markers can be used to identify senescent cells, they are not exclusive to the senescent state, and thus must be used in combination to precisely analyse cellular senescence.

1.4.5. The senescence-associated secretory phenotype

Senescent cells develop a distinct secretome profile, known as the senescence-associated secretory phenotype (SASP), which involves the strong upregulation of soluble signalling proteins such as cytokine, chemokines, growth factors, and proteases, such as matrix metalloproteases (MMPs) (Malaquin *et al.*, 2016). Although the SASP is fairly conserved in mammals, its composition is highly dependent on cell type and senescence-inducing stimuli (Coppe *et al.*, 2008; Coppe *et al.*, 2010b). The SASP is thought to have evolved as a way for senescent cells to communicate with the immune system, potentially to facilitate their own clearance (Xue *et al.*, 2007; Krizhanovsky *et al.*, 2008). Indeed, inducing senescence in liver cancer cells was shown to activate an innate immune response in mice, which contributed to tumour clearance (Xue *et al.*, 2007). Moreover, immune responses against senescent cells have also been shown to play a role in limiting liver fibrosis. Induction of liver damage in mice resulted in increased frequency of senescent hepatic stellate cells, which were shown to be killed by natural killer cells, facilitating the resolution of fibrosis (Krizhanovsky *et al.*, 2008). It has also been demonstrated that the SASP can act as an extracellular signal to promote tissue regeneration by inducing cellular plasticity and stemness in neighbouring cells. Transiently exposing mouse keratinocytes to the SASP induced expression of stem cell markers and increased their regenerative capacity *in vivo*, as demonstrated by the ability of these cells to develop large patches of hair follicles when grafted into full-thickness wounds of nude mice (Ritschka *et al.*, 2017). This was also demonstrated *in vivo,* as inducing senescence in single hepatocytes in mouse liver *in vivo* activates tissue-specific expression of stem cell markers, supporting that the SASP induces stem cell properties in the surrounding tissue (Ritschka *et al.*, 2017). Another beneficial role of the SASP was demonstrated during the wound healing process, whereby senescent cells were shown to secrete PDGF-AA, inducing myofibroblast differentiation, which possess contractile function, thereby accelerating wound healing (Demaria *et al.*, 2014).

The presence of senescent cells in the aforementioned contexts are, however, transient, possibly to avoid the detrimental effects associated with persistent senescent cells. It has been shown that senescent cells can induce paracrine senescence in normal neighbouring cells via secretion of SASP factors, such as TGF-β family ligands (Acosta *et al.*, 2013). Given that there is an accumulation of senescent cells in many tissues with age, it is possible that the SASP contributes, in part, to age-associated tissue dysfunction (van Deursen, 2014). Indeed, chronic exposure to the SASP induces senescence and impairs the regenerative capacity of keratinocytes *in vivo* (Ritschka et al., 2017). Moreover, clearance of senescent cells attenuated age-related dysfunction in many tissues and delayed onset of age-related pathologies in mice, supporting that the presence of senescent cells negatively impacts tissue function (Baker *et al.*, 2011; Baker *et al.*, 2016). Paradoxically, prolonged exposure to the SASP can also promote tumorigenesis, as it has been shown that factors released by senescent human fibroblasts stimulate proliferation of premalignant and malignant cells in culture, and promotes tumour formation in mice (Krtolica *et al.*, 2001). Moreover, MMPs secreted by senescent dermal fibroblasts were shown to promote epithelial-mesenchymal transition (EMT), and thus enhance migratory capacity of transformed keratinocytes, an important step during cancer invasion metastasis (Malaquin *et al.*, 2013).

As well as inducing paracrine senescence, certain SASP factors have also been shown to act in a cell-autonomous manner, reinforcing the senescent phenotype. For example, activation of CXCR2, a receptor for IL-8, was shown to reinforce both replicative and oncogene-induced senescence, whereby the growth arrest is maintained by a self-amplifying secretory network activated by senescent cells (Acosta *et al.*, 2008). Consistent with the role of SASP factors in autocrine senescence, IL-6 has also been shown to be crucial for the establishment of OIS (Kuilman *et al.*, 2008). It was shown IL-6 depletion was sufficient to promote OIS bypass in dermal fibroblasts expressing the oncogene $BRAF^{E600}$ (Kuilman *et al.*, 2008). Depletion of IL-6 in $BRAF^{E600}$-senescent fibroblasts allowed cells to resume proliferation, suggesting that IL-6 is also required to the maintenance of OIS (Kuilman *et al.*, 2008).

The development and maintenance of the SASP has been shown to heavily rely on persistent DDR signalling, as depletion of DDR proteins that associate with DNA-SCARS, such as ATM, CHK2 and H2AX, abolished the secretion of many SASP factors, including IL-6 and IL-8 (Rodier *et al.*, 2009; Rodier *et al.*, 2011). Consistent with this, it has been shown that inducing senescence in human fibroblasts by overexpressing p16 or p21 does not trigger a DDR, and consequently occurs without a SASP, suggesting that the secretory phenotype is not a

consequence of the growth arrest but rather a result of DNA damage response (Coppe *et al.*, 2011). This observation, however, is contradictory to previous studies showing that p21 promotes ROS generation and consequently induces secondary DNA damage (Passos *et al.*, 2010). Nevertheless, activation of the DDR alone does not fully account for the development of the SASP. Firstly, DDR activation occurs almost immediately following DNA damage, while the mature SASP develops over several days. Therefore, if the DDR alone was necessary and sufficient for the SASP, this phenotype would be detectable within hours of damage induction (Malaquin *et al.*, 2016). Indeed, a DDR-independent mechanism regulating the SASP has been identified, involving p38MAPK activation (Freund *et al.*, 2011; Alspach *et al.*, 2014). It has been shown that inhibition of p38MAPK activity, which does not inhibit the DDR, reduces the secretion of the majority of SASP factors, whereas constitutive p38MAPK activation was sufficient to induce a SASP despite depletion of DDR proteins, such as ATM and CHK2 (Freund *et al.*, 2011). Moreover, the kinetics of p38MAPK activation closely resembles the kinetics of early and mature SASP development, suggesting that it could be an important regulator of the SASP (Freund *et al.*, 2011).

Chromatin modifications also appear to be important in establishing the SASP. For example, recent work has shown that cells undergoing senescence with persistent DNA damage (by replicative exhaustion, etoposide treatment and oncogenic RAS activation) accumulate high levels of the H2A.J histone variant, which was shown to be required for production of SASP factors (Contrepois *et al.*, 2017). Mechanistically, it is not clear how a DDR promotes H2A.J accumulation, and how the latter stimulates SASP gene expression; however, these data suggest that chromatin modifications that occur during senescence also play a role in the SASP development (Contrepois *et al.*, 2017).

The NF-κB family of transcription factors have been shown to be key regulators of the expression of SASP factors. For example, inhibiting NF-κB by deletion of the subunit p65, has been shown to reduce expression of many SASP factors in OIS and stress-induced senescent fibroblasts (Chien *et al.*, 2011b; Freund *et al.*, 2011). Mechanistically, it is unclear how NF-κB is activated during senescence; however, it has been shown that a DDR can activate NF-κB via ATM, which interacts with NF-κB modulators, activating NF-κB signalling (Wu *et al.*, 2006b). Moreover, the p38MAPK pathway can also indirectly induce NF-κB via activation of MSK1 and MSK2, which phosphorylate the NF-κB subunit, p65, promoting its nuclear translocation, where it can regulate transcription of inflammatory genes (Vanden Berghe *et al.*, 1998;

Kefaloyianni *et al.*, 2006). Furthermore, as previously discussed, mTORC1 can enhance NF-κB transcriptional activity either indirectly through regulating IL-1α translation (Laberge *et al.*, 2015) or directly by interacting with IKK (Dan *et al.*, 2008), thus providing another mechanism of NF-κB activation in senescent cells. Hence, regulation of the SASP appears to occur at multiple levels, with a DDR, p38 activity, NF-κB and chromatin modifications playing important roles for the establishment of the pro-inflammatory secretory phenotype.

1.5. The role of reactive oxygen species in senescence

Reactive oxygen species (ROS) are generated as byproducts of aerobic metabolism, and are important signalling molecules that regulate many physiological processes (Finkel, 2011). However, oxidative stress that arises from an imbalance in ROS homeostasis can lead to damage in cellular components such as lipids, proteins and DNA (Schieber and Chandel, 2014). Given that, Denham Harman proposed the "Free Radical Theory of Ageing", suggesting that accumulation of oxidative damage in cells over time contributes to organismal ageing (Harman, 1956). Considering that mitochondria are a major source of intracellular ROS production, it was later suggested that mitochondria were the main contributors of ROS-induced cellular damage and ageing (Harman, 1972). The mitochondrial theory of ageing postulates that ROS produced as a consequence of mitochondrial oxidative phosphorylation causes mitochondrial DNA (mtDNA) damage and accumulation of mtDNA mutations, which in turn impairs oxidative phosphorylation, leading to increased ROS production, ultimately instilling a "vicious cycle" that perpetuates ROS generation and promotes oxidative stress (Alexeyev *et al.*, 2004).

Indeed, ROS have been shown to play a role during senescence, as increased ROS levels are observed in replicative, stress-induced and oncogene-induced senescence (Saretzki *et al.*, 2003; Passos *et al.*, 2007; Ogrunc *et al.*, 2014). Treating human fibroblasts with sub-lethal concentrations of H_2O_2 induces senescence in a p53-dependent manner (Chen *et al.*, 1998). Moreover, mild oxidative stress has been shown to cause telomeric SSBs, accelerating the rate of telomere shortening, and leading to premature senescence (von Zglinicki *et al.*, 1995; von Zglinicki *et al.*, 2000; von Zglinicki, 2002). Consistently, treatment with antioxidants or other interventions that reduce ROS levels prevents telomere shortening and extends replicative lifespan (Saretzki *et al.*, 2003; Serra *et al.*, 2003). Dysfunctional mitochondria have been shown to accumulate as cells reach the end of their replicative potential, which leads to increased ROS generation and also contributes to telomere shortening (Passos *et al.*, 2007). Interestingly, overexpressing TERT (the catalytic subunit of telomerase) decreases mitochondrial superoxide production and protects human fibroblasts against oxidative stress independently of telomere

length (Ahmed *et al.*, 2008). Under conditions of mild stress, TERT is excluded from the nucleus, and co-localises with mitochondria, suggesting a protective role for telomerase independent from its role in telomere elongation (Ahmed *et al.*, 2008).

ROS are not only involved in the induction of senescence via direct DNA damage, but are also believed to act as an effector mechanism during senescence. Indeed, inducing senescence in fibroblasts by overexpression of p21, which acts downstream of the DDR, increases intracellular ROS (Macip *et al.*, 2002). The p21-induced growth arrest was reversed by antioxidant treatment, suggesting that ROS are important for the maintenance of the senescent phenotype (Macip *et al.*, 2002). Moreover, a positive feedback loop has been shown to exist whereby DNA damage leads to sustained activation of p21, inducing mitochondrial dysfunction and leading to increased ROS production through a series of signalling pathways involving GADD45-p38MAPK-TGFβ (Passos *et al.*, 2010). This increase in ROS then replenishes short-lived DNA damage foci, thus maintaining the DDR and stabilising senescence (Passos *et al.*, 2010). In addition, late generation $TERC^{-/-}$ mice, which have dysfunctional telomeres, show increased markers of oxidative damage in tissues which can be significantly decreased by deletion of p21 (Passos *et al.*, 2010). Another study has also shown that short telomeres activate p53, which in turn binds and represses PGC-1α and PGC-1β promoters, inducing mitochondrial dysfunction and contributing to elevated ROS levels (Sahin *et al.*, 2011). It has been recently shown that clearance of mitochondria, which significantly reduces ROS, ameliorates many senescence-associated phenotypes, including the SASP (Correia-Melo *et al.*, 2016). It was demonstrated that a DDR acts via ATM, Akt and mTORC1 to induce PGC-1β-dependent mitochondrial biogenesis, contributing to ROS production that further activates a DDR and stabilises senescence (Correia-Melo *et al.*, 2016).

Interestingly, ROS have also been involved in the so-called bystander effect of senescent cells, where a DDR is induced in neighbouring cells via gap junction-mediated cell-cell contact in a process involving ROS (Nelson *et al.*, 2012). Prolonged co-culture with senescent cells induced senescence in surrounding fibroblasts *in vitro*, and clusters of hepatocytes positive for senescence markers were also shown in the liver of mice, suggesting that ROS might also play a role in spreading senescence both *in vitro* and *vivo* (Nelson et al., 2012).

1.6. The role of senescence *in vivo*

Traditionally, senescence was regarded as a tumour-suppressor mechanism by controlling proliferation of cells, thus imposing an important barrier against tumourigenesis (Serrano *et al.*,

1997). Additionally, recent data now indicates that senescent cells play a variety of other beneficial roles during biological processes such as embryonic development, wound healing and tissue repair (Krizhanovsky *et al.*, 2008; Munoz-Espin *et al.*, 2013; Demaria *et al.*, 2014; Ritschka *et al.*, 2017). In contrast, senescent cells have been shown to accumulate in many tissues with age, and this contributes to the loss of tissue regeneration and function during the ageing process (Baker *et al.*, 2011; van Deursen, 2014; Baker *et al.*, 2016). The impact of senescence cells in different physiological processes will be discussed in more detail below.

1.6.1. Senescence and tumour suppression

Since tumour development requires cell proliferation (Hanahan and Weinberg, 2000), senescence can mediate tumour suppression by preventing uncontrolled cell growth. Indeed, senescence markers have been observed in many pre-malignant lesions *in vivo*, such as human melanocytic nevi, which are composed of growth-arrested melanocytes with oncogenic mutations such as $BRAF^{V600E}$ (Pollock *et al.*, 2003). Importantly, BRAF acts as a downsteam effector of Ras, activation of which was shown to induce OIS *in vitro* (Serrano et al., 1997). Indeed, expression of $BRAF^{V600E}$ induced senescence in human melanocytes in culture (Michaloglou *et al.*, 2005). Moreover, analysis of human nevi *in vivo* revealed that melanocytes within these pre-malignant lesions displayed markers of senescence, such as increased Sen-β-Gal activity and p16 expression, with no sign of telomere shortening, suggesting that oncogene-induced senescence limits proliferation of pre-cancerous cells *in vivo* (Michaloglou et al., 2005). Interestingly, p16 expression levels are heterogeneous in nevi cells, suggesting that the growth arrest at this stage is not primarily maintained by the p16/pRb pathway (Michaloglou *et al.*, 2005; Suram *et al.*, 2012). In fact, it has been reported that melanocytic nevi accumulate dysfunctional telomeres independently of length, whereas telomere damage levels are much lower in melanoma cells, suggesting a role for telomere dysfunction in oncogene-induced senescence (Suram *et al.*, 2012). It was suggested that oncogene activation induces DNA replication stress and fork stalling at telomeres, leading to telomere dysfunction, thus implicating telomere dysfunction-induced senescence (TDIS) as an important tumour suppressor mechanism in humans (Suram *et al.*, 2012). The importance of the senescence-growth arrest in preventing oncogene induced tumour progression has been demonstrated by mouse tumour models, whereby deletion of p53 or p21 was sufficient to promote tumourigenesis in $HRAS^{G12V}$-induced mammary tumour and in $BRAF^{V600E}$-induced melanomas, for example (Sarkisian *et al.*, 2007; Goel *et al.*, 2009). Senescent cells have also been reported in other pre-malignant lesions, such as prostate intraepithelial neoplasia (PIN)

and premalignant human colon adenomas, and other mutations are usually required for their transformation into malignant tumours (Chen *et al.*, 2005; Bartkova *et al.*, 2006).

In light of the aforementioned evidence, genetic manipulations or chemotherapeutic drugs that induce senescence in tumour cells seem attractive as potential cancer therapies (Roninson, 2003). For example, it has been shown that overexpression of p53, p21 or p16 induces senescence in human tumour cells (Sugrue *et al.*, 1997; Fang *et al.*, 1999; Dai and Enders, 2000). Consistent with this, treatment with chemotherapeutic drugs, such as doxorubicin, induced a senescence-like growth arrest in malignant cells, which was associated with increased p53 and p21 expression (Chang *et al.*, 1999b). This was also demonstrated *in vivo*, where human tumour xenografts grown in nude mice show increased Sen-β-Gal activity when treated with chemotherapy drugs such as a retinoid (Chang *et al.*, 1999a). However, it is important to note that accumulation of senescent cells can also trigger tumourigenesis depending on the surrounding microenvironment, a characteristic which is attributed to the development of the SASP, as previously mentioned (Krtolica *et al.*, 2001). Indeed, injecting pre-neoplastic cells together with senescent cells leads to tumour formation in mice (Krtolica *et al.*, 2001). More recently, it was shown that therapy-induced senescent cells persist and contribute to several chemotherapy side-effects and cancer recurrence (Demaria *et al.*, 2017).

1.6.2. Senescence during embryonic development

A role for senescence during mammalian embryogenesis has been recently uncovered, suggesting that this process is also important for normal physiological functions. Senescent cells have been reported in multiple locations during embryonic development in mice, including the mesonephros and the endolymphatic sac of the inner ear (Munoz-Espin *et al.*, 2013), and the apical ectodermal ridge (AER) (Storer *et al.*, 2013), and was also observed in human and chick embryos (Munoz-Espin *et al.*, 2013; Storer *et al.*, 2013). Interestingly, the presence of senescent cells in these structures was transient, likely a result of macrophage clearance of these cells (Munoz-Espin *et al.*, 2013) but a role for apoptosis has also been proposed (Storer *et al.*, 2013). Importantly, accumulation of senescent cells in the AER was diminished in p21-deficient mice, which showed severe limb patterning defects (Storer *et al.*, 2013). It was shown that developmentally programmed senescence occurred independently of DNA damage and p53, but was dependent on p21 and is regulated by the TGF-β/SMAD and PI3K/FOXO pathways (Munoz-Espin *et al.*, 2013). Similarly, senescent cells in the AER showed increased p21 and Sen-β-Gal, however DNA damage and p16 were not detected. Although these cells also developed a SASP, common components such as IL-6 and IL-8 were not increased,

suggesting that developmental senescence could represent a more primitive form of senescence that plays a role in transient signalling during the development process (Storer *et al.*, 2013).

1.6.3. Senescence and wound healing

Tissue repair, such as wound healing, is a multistep process involving four distinct phases: hemostasis, inflammation, proliferation and remodelling. In the skin, wound closure occurs during the proliferation phase as a result of wound contraction, which relies on the function of specialised contractile fibroblasts known as myofibroblasts (Tomasek *et al.*, 2002; Midwood *et al.*, 2004). Moreover, soluble factors, including some known SASP components, facilitate the process of tissue repair.

Senescent cells have been shown to accumulate in cutaneous wounds of mice (Jun and Lau, 2010). It was demonstrated that the matricellular protein CCN1, which is highly expressed in granulation tissue (i.e. newly deposited connective tissue and blood vessels) during cutaneous wound healing, induces senescence in fibroblasts by activating a DDR and inducing p53. Moreover, signalling downstream of CCN1 resulted in increased ROS levels and p16, mediating fibroblast growth arrest. Senescent cells were shown to be myofibroblasts, and were associated with expression of anti-fibrotic genes, suggesting that senescence induced by CCN1 helps to regulate the fibrotic response during cutaneous wound healing (Jun and Lau, 2010). Indeed, CCN1 mutant mice failed to accumulate senescent cells at wound sites, resulting in increased fibrosis, which could be attenuated with topical application of CCN1 (Jun and Lau, 2010). Consistent with this, another group has demonstrated a role for senescent cells in optimal wound healing by using a mouse model (p16-3MR) where senescent cells can be visualised in the living animal and also eliminated upon treatment with ganciclovir (Demaria *et al.*, 2014). They showed that following a full-thickness punch biopsy, senescent cells were transiently detected at the site of injury, peaking at around 6 days after wounding and returning to basal levels between 9 and 12 days. Interestingly mRNA levels of p16, p21 and of SASP factors such as IL-1α, PAI-1 and VEGF showed similar kinetics, confirming that senescent cells transiently appear during the wound healing process. However, eliminating senescent cells by ganciclovir treatment abolished the increase of p16 and p21 mRNA at the site of injury, and these mice showed slower kinetics of wound closure, suggesting that senescent cells accelerate repair (Demaria *et al.*, 2014). Importantly, wounds in p16-3RM-treated mice eventually heal, albeit with slower kinetics, suggesting that senescent cells facilitate but are not essential for wound repair. It was suggested that the SASP factor PDGF-AA secreted by senescent cells at the site

of injury promotes myofibroblast differentiation, which is required for optimal formation of granulation tissue and wound closure (Demaria *et al.*, 2014).

The beneficial role of senescent cells, and the resulting SASP, during development and tissue repair is probably due to the fact that these cells only appear transiently, thus avoiding the deleterious effects posed by the prolonged presence of senescent cells, as it will be discussed below.

1.6.4. Senescence in ageing and age-related diseases

Although evidence suggests that senescent cells are subject to clearance by the immune system (Xue *et al.*, 2007; Krizhanovsky *et al.*, 2008; Kang *et al.*, 2011), many studies have now shown an age-dependent accumulation of senescent cells in many mammalian tissues. It has been shown that Sen-β-Gal-positive cells increase in an age-dependent manner in human skin samples (Dimri *et al.*, 1995). Moreover, cells with senescent markers have also been detected in a number of tissues in mice and primates with age (Herbig *et al.*, 2006; Krishnamurthy *et al.*, 2006; Wang *et al.*, 2009). Consistent with a role for telomeres in the ageing process, telomere shortening is observed in telomerase knock-out mice in a generation-dependent manner (Blasco *et al.*, 1997), which results in both cell senescence and apoptosis (Lee *et al.*, 1998). These mice not only display a bone phenotype that resembles age-related osteoporosis (Brennan *et al.*, 2014), but accelerated telomere shortening in this model has been shown to compromise stem cell function, regeneration, organ homeostasis, and decrease lifespan (Wong *et al.*, 2003). Moreover, telomere dysfunction has been shown to increase with age *in vivo* in the skin of primates, and in the liver, gut, and lung of wild-type mice (Herbig *et al.*, 2006; Jeyapalan *et al.*, 2007; Hewitt *et al.*, 2012; Birch *et al.*, 2015). In fact, reintroducing telomerase activity in telomerase-deficient mice was sufficient to rescue premature ageing phenotypes in tissues such as spleen, intestines and testes, suggesting that telomere damage contributes to tissue dysfunction with age (Jaskelioff *et al.*, 2011). Interestingly, evidence is now emerging showing that post-mitotic cells also acquire senescent-like phenotypes *in vivo*. For example, one study has reported an increase in TAF in hippocampal neurons in baboons with age (Fumagalli *et al.*, 2012). Consistent with this, neurons expressing senescence markers such as Sen-β-Gal, IL-6 and γH2AX have been shown in the brain of ageing mice (Jurk *et al.*, 2012).

Furthermore, accumulation of senescent cells has been associated with age-related diseases (Naylor *et al.*, 2013). For example, adipose tissue from patients with diabetes shows increased markers of senescence, such as Sen-β-Gal, p53 and p21, as well as significantly increased

expression of inflammatory cytokines (Minamino *et al.*, 2009). A similar upregulation in senescence markers is seen in adipose tissue of mice with type 2 diabetes, which was ameliorated by adipocyte-specific deletion of p53. Inhibition of p53-dependent senescence decreased the expression of pro-inflammatory cytokines as well as improving insulin resistance in these mice, suggesting an involvement of senescent cells in type 2 diabetes (Minamino *et al.*, 2009). Moreover, cells with markers of senescence such as increased Sen-β-Gal activity, telomere shortening, and high p16 and p21 expression, have been observed in articular cartilage of patients with osteoarthritis, an age-related pathology which is associated with chronic inflammation (Price *et al.*, 2002). Indeed many pro-inflammatory factors associated with the SASP are increased in arthritic joint tissues, including IL-1β, IL-6, IL-8, and ECM-degrading proteins such as MMP-3 and MMP-13 (Freund *et al.*, 2010). In addition, airway epithelial cells from lungs of patients with chronic obstructive pulmonary disease (COPD) display senescence markers including elevated p16, Sen-β-Gal, and telomere damage, suggesting that senescent cells contribute to lung dysfunction in COPD (Birch *et al.*, 2015). Senescent cells have also been reported in affected tissues of patients with Alzheimer's disease, atherosclerosis and pulmonary fibrosis (Naylor *et al.*, 2013).

Evidence suggests that accumulation of senescent cells with age may be a major contributing factor to the loss of tissue regeneration and function during the ageing process. This was elegantly demonstrated by the van Deursen group who showed that inducible elimination of p16-positive cells was able to delay the acquisition of age-related pathologies in a mouse model of accelerated ageing, the BubR1 progeroid mouse (Baker *et al.*, 2011). Elimination of senescent cells from these mice not only delayed the onset age-related tissue degeneration but also slowed down the progression of already established conditions, suggesting a causal link between senescence and age-related tissue dysfunction (Baker *et al.*, 2011). More recently, a similar system was developed by the same group, where they were able to eliminate p16-expressing senescent cells in wild type mice, resulting in significant improvements in healthspan and lifespan of mice (Baker *et al.*, 2016). Clearance of p16-positive cells delayed tumourigenesis and attenuated age-related dysfunction of several organs such as kidney, heart and fat, showing that senescent cells contribute causally to the ageing process (Baker *et al.*, 2016). These data suggest that interventions targeting senescent cells could be an attractive option as a therapy against ageing and age-related diseases. Indeed, pharmacological agents that selectively kill senescent cells, also known as senolytics, are now being identified, and their

use has been shown to improve age-related phenotypes in mice (Zhu *et al.*, 2015; Chang *et al.*, 2016; Zhu *et al.*, 2017).

Although the mechanisms by which senescent cell accumulation leads to age-related tissue dysfunction are not fully understood, one possibility is that senescent cells impair the regenerative capacity of tissues. Indeed, senescent progenitor cells have been reported in muscle and fat of the BubR1 progeroid mice, and an increase in p16 expression has been shown in haematopoietic stem cells of mice with age (Janzen *et al.*, 2006; Baker *et al.*, 2013). As well as affecting tissue function in a cell-autonomous manner, decreasing the availability of progenitors capable of differentiating to repair the tissue, senescence in these cell populations can also contribute to ageing by affecting surrounding stem and progenitor cells, disrupting the local stem cell niche through secretion of SASP factors (Baker *et al.*, 2013). Consistent with this idea, SASP factors such as IL-6 and GRO-α have been shown to regulate stem cell fate *in vitro* (Pricola et al., 2009; Krtolica et al., 2011). Moreover, muscle stem cells from aged mice undergo lineage conversion from a myogenic towards a fibrogenic lineage, which is associated with impaired muscle regeneration and favours a fibrotic response (Brack *et al.*, 2007). It was shown that this myogenic-to-fibrogenic conversion was a result of factors present in the systemic environment of aged animals, as exposing old mice to a youthful systemic environment by parabiotic pairing with young mice significantly improved the regenerative potential of old stem cells (Brack *et al.*, 2007). Accumulation of senescent cells might also lead to age-related tissue dysfunction by compromising tissue integrity and architecture, since proteases, which are components of the SASP, can cleave membrane-bound receptors, extracellular matrix proteins and other components important for maintaining tissue organisation (Parrinello *et al.*, 2005). Moreover, chronic exposure to the SASP can contribute to tissue fibrosis by stimulating epithelial-to-mesenchymal transformation (EMT) (Laberge *et al.*, 2012). Another way in which senescent cells can be detrimental to tissue function is by contributing to chronic tissue inflammation, which is characterised by lymphocyte and macrophage infiltration, fibrosis and cell death (van Deursen, 2014). Indeed, this phenomenon, also known as "inflammaging", is associated with many age-related diseases (Franceschi *et al.*, 2007; Freund *et al.*, 2010). It is thought that pro-inflammatory cytokines, chemokines and growth factors secreted by senescent cells contribute to this state of chronic inflammation, and thus are directly linked to the development of age-related diseases (Freund *et al.*, 2010). Senescent cells have also been shown to promote a pro-tumourigenic microenvironment via the secretion of SASP factors, favouring survival and proliferation of malignant cells, thus possibly

contributing to the increased incidence in cancer development with age (Krtolica *et al.*, 2001; Bavik *et al.*, 2006; Coppe *et al.*, 2008). Finally, the ability of the SASP to induce senescence in healthy neighbouring cells through paracrine mechanisms may intensify all the aforementioned detrimental effects, thus leading to age-related tissue deterioration (Nelson *et al.*, 2012; Acosta *et al.*, 2013) (***Figure 1.2***).

The reason why senescent cells accumulate with age is also not fully understood. It is possible that the rate at which senescent cells are generated increases with age, since senescence-inducing stimuli, such as DNA damage and ROS, also increase with age (Garinis *et al.*, 2008; Sasaki *et al.*, 2010); however, combined cellular stresses alone cannot account for senescent cell accumulation. Another possibility is that senescent cells are less efficiently eliminated with ageing. As previously mentioned, evidence suggests that senescent cells are subject to clearance by attracting both adaptive and innate immune cells, likely through the secretion of pro-inflammatory factors (Xue *et al.*, 2007; Krizhanovsky *et al.*, 2008; Kang *et al.*, 2011; Laberge *et al.*, 2012; Sagiv and Krizhanovsky, 2013). However, immune cell function also declines with age, likely compromising immune surveillance and elimination of senescent cells (Nikolich-Zugich, 2008; Montecino-Rodriguez *et al.*, 2013; Sagiv and Krizhanovsky, 2013). In fact, immune cells also become senescent (Henson *et al.*, 2014; Lanna *et al.*, 2014), which can further contribute to age-associated immunodeficiency (Vicente *et al.*, 2016). Furthermore, it has also been suggested that deeply senescent cells develop a highly heterogeneous SASP, such that immune cells can no longer recognise them, and thus are able to evade immune clearance (van Deursen, 2014).

Overall, accumulation of senescent cells with age is a major contributor to age-related tissue dysfunction, and evidence now implicates senescence as causal during the ageing process.

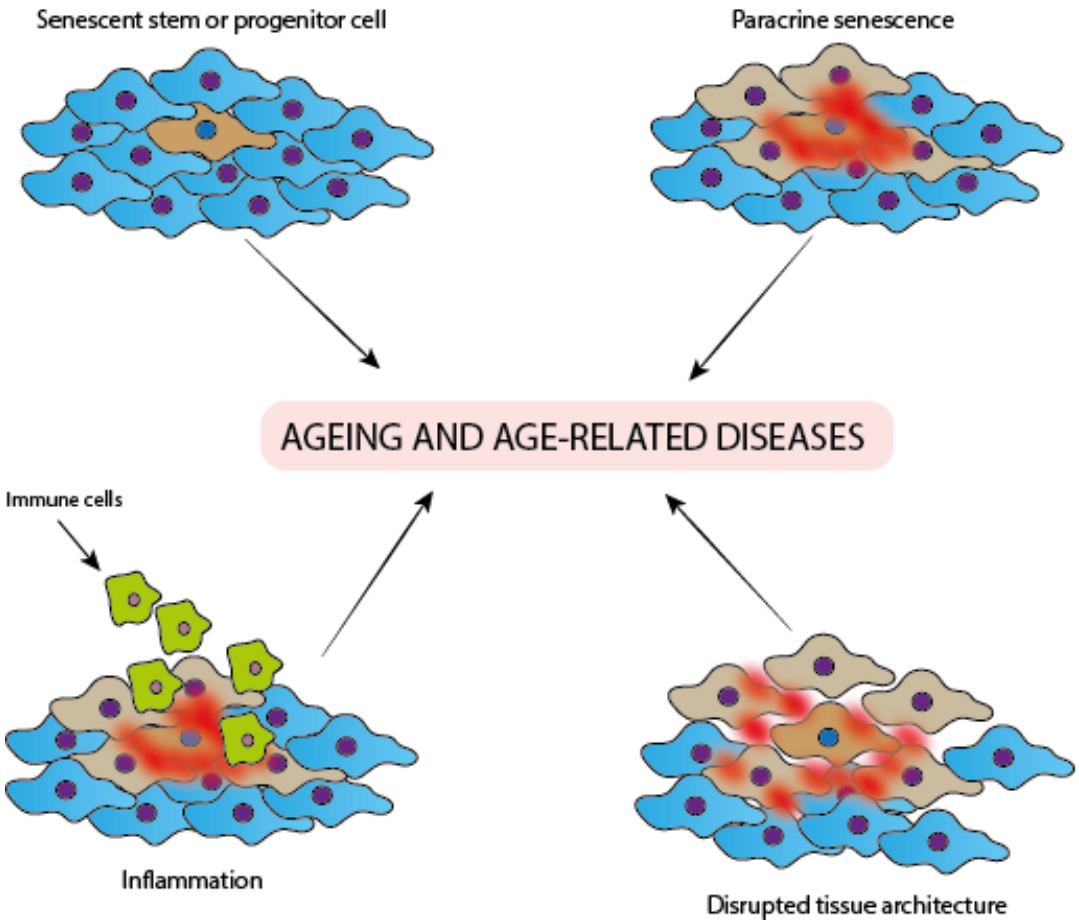

Figure 1.2 - Accumulation of senescent cells contributes to age-related tissue dysfunction. The chronic presence of senescent cells with age may contribute to ageing and age-related diseases in a number of ways. For example, senescent stem or progenitor cells may lead to depletion of stem cell pools, which are crucial to maintain tissue homeostasis and regeneration. Secretion of SASP factors can also induce senescence in neighbouring stem cells, disrupting the local stem cell niche and contributing to stem cell dysfunction. Moreover, secretion of pro-inflammatory factors by senescent cells promotes tissue dysfunction by causing degradation of extracellular matrix, and thus disrupting tissue architecture, as well as stimulating chronic tissue inflammation. Moreover, the detrimental effects of senescent cells are exacerbated due to the ability of SASP components to act in a paracrine manner, and induce senescence in healthy neighbouring cells. (Blue = healthy, young cells; brown with blue nucleus = primary senescent cells; brown with purple nucleus = secondary senescent cells; green = immune cells).

1.7. Cellular senescence and skin ageing

The skin is a highly dynamic, regenerating organ, and serves as the body's primary line of defence against infections, as well as physical and chemical insults (Rittie and Fisher, 2015). Moreover, the skin is the largest sensory organ in the body, and is crucial in thermoregulation through controlling vasculature and sweat glands present throughout the skin (Romanovsky, 2014).

1.7.1. Skin structure

The skin is composed of three main layers: the hypodermis, dermis and epidermis (***Figure 1.3***). The hypodermis, or subcutaneous layer, is the deepest layer of the skin, and consists mainly of adipose and connective tissue. It contains high fat storage capacity, and thus provides cushioning and insulation, as well as skin stability by connecting the dermis to internal organs (Farage *et al.*, 2007). The dermis is the scaffolding component of the skin, and it is mainly composed of collagen and elastin fibres, as well as fibroblasts, blood vessels, lymph vessels and sweat glands (Fore, 2006). Immune cells also reside in the dermis, including mast cells, macrophages, dendritic cells, innate lymphoid cells (e.g. natural killer cells) and T cells, all of which orchestrate to prevent pathogen invasion (Heath and Carbone, 2013). For example, natural killer cells, which are present in small numbers in healthy skin, are involved in viral immunity and also play a role in tumour immunosurveillance (Heath and Carbone, 2013). Dendritic cells also play a role in protecting against viral infections, and are also important for presenting antigens to induce $CD8^+$ (cytotoxic) T cell responses (Shortman and Heath, 2010). Moreover, the skin contains a large number of memory T cells of both the $CD4^+$ (i.e. T-helper cells) and $CD8^+$ subsets, which are located in the dermis and epidermis, respectively, enhancing protection against local infection (Heath and Carbone, 2013).The dermis also contains hair follicles and nerve fibres; the latter project into the epidermis, allowing the skin to transmit sensory information. Moreover, the blood supply within the dermis is responsible for providing circulatory support and nutrients to the epidermis, since the latter does not have its own blood supply (Fore, 2006). The epidermis is the outermost layer of the skin, and consists primarily of keratinocytes organised in a stratified epithelium (Rittie and Fisher, 2015). Proliferating keratinocytes are located in the basal layer of the epidermis (stratum basale), and their proliferative capacity is lost as they differentiate and migrate up the epidermis towards the stratum corneum. The latter is the outermost layer of the epidermis, and is composed of terminally differentiated keratinocytes, also known as corneocytes. These cells are flattened and anuclear but are rich in proteins, and are embedded in a lipid-rich extracellular matrix,

which provides an impermeable barrier, preventing water loss and entry of pathogens through the skin (Elias *et al.*, 1977). Eventually, corneocytes are shed off the skin surface through a process known as desquamation, completing the cycle of epidermal differentiation (or keratinisation), which occurs approximately every 28 days (Marks, 2004). Moreover, Langerhan cells, which are antigen presenting cells, can also be found in the epidermis. These dendritic cells are involved in skin immunosurveillance by extending their dendrites upwards towards the cornified epithelial layer and sampling external antigens, and this has been shown to be important for priming immunity against skin pathogens (Chomiczewska *et al.*, 2009; Heath and Carbone, 2013). The epidermis is also composed of melanocytes, which comprise approximately 1-2% of epidermal cells, and are present at a ratio of 1:10 melanocyte to keratinocytes. This ratio is maintained constant throughout life, and although the mechanisms controlling this are still poorly understood, it is believed that keratinocytes regulate melanocyte numbers through growth factors and other cell-surface molecules (Scott and Haake, 1991; Haass and Herlyn, 2005). Melanocytes are dendritic cells that reside in the basal layer of the epidermis, and produce the photoprotective pigment, melanin, in response to UV irradiation (Sklar *et al.*, 2013). Melanocytes then transport melanin, which is packaged in vesicles called melanosomes, to surrounding keratinocytes through their dendrites. It has been shown that one melanocyte can transport melanin to approximately 36 neighbouring keratinocytes, and this interaction is also known as the epidermal melanin unit (Fitzpatrick and Breathnach, 1963; Haass and Herlyn, 2005). Unlike keratinocytes, epidermal melanocytes are very long-lived cells, and proliferate extremely rarely once they reach terminal differentiation (Cichorek *et al.*, 2013). A small percentage of dividing melanocytes has been shown in human and mouse skin (Jimbow *et al.*, 1975). Moreover, a subpopulation of differentiated melanocytes that are capable of undergoing division has been identified in zebrafish, however, the proportion of new pigmented cells arising from differentiated melanocytes was very small (4.6%) (Taylor *et al.*, 2011). It is also thought that differentiated melanocytes contribute in part to re-pigmentation in wound healing and vitiligo (Hirobe, 1988; Falabella, 2009). However, new melanocytes arise mainly from a population of undifferentiated melanocytes, which also contribute to the increase in epidermal melanocyte number following exposure to UV irradiation in mice (Kawaguchi *et al.*, 2001; van Schanke *et al.*, 2005; Walker *et al.*, 2009; Tanimura *et al.*, 2011). Although the niche of melanocyte stem cells in the adult skin remains to be established, evidence suggests that the bulge area of hair follicles serves as a reservoir for such cells (Nishimura *et al.*, 2002; Nishimura, 2011; Tanimura *et al.*, 2011). However, studies suggest that a melanocyte stem cell niche can also be found in the dermis (Toma *et al.*, 2005; Davids *et al.*, 2009).

The epidermis also projects inwards towards the dermis, alternating with shallower protrusions back into the epidermis, such that the dermis and the epidermis interconnect via invaginations, giving rise to the rete ridges. These structures provide mechanical support for the epidermis, and are also important for nutrient exchange, as they increase the surface area of the epidermis which is exposed to capillaries and venules circulating near the epidermal-dermal junction (EDJ) (Briggaman and Wheeler, 1975).

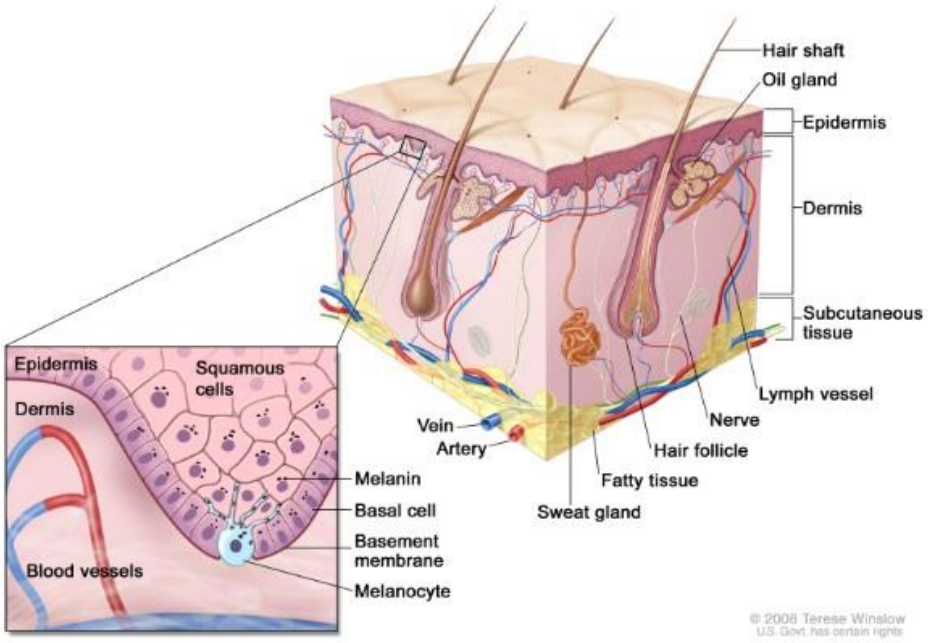

Figure 1.3 - Layers of the human skin. The innermost layer of the skin is the hypodermis, or subcutaneous layer, which is mainly composed of adipose tissue and acts as a shock absorber as well as providing insulation. The dermis connects the hypodermis to the epidermis, and has a high content of collagen and elastin fibres, providing strength, elasticity and support to the skin. The dermal compartment also harbours blood vessels, which supply nutrients to the epidermis, as well hair follicles and sweat glands. Moreover, nerves present within the dermis also make connection with the EDJ, and relay sensory information. The epidermis is the outermost layer, and is mainly composed of keratinocytes, which are constantly being turned over and shed off, providing an impermeable barrier to the skin. Melanocytes also reside within the basal layer of the epidermis, and they produce melanin when exposed to UV irradiation. Melanocytes interconnect with surrounding keratinocytes via dendrites, which mediate melanin transport to neighbouring cells, providing protection against the harmful effects of UV irradiation. Image obtained from (Winslow, 2008).

1.7.2. Characteristics of aged skin

Skin ageing arises from a combination of both intrinsic and extrinsic factors, which ultimately compromise the structural integrity and physiological function of the skin (Kammeyer and Luiten, 2015). The changes associated with intrinsic or chronological ageing are in great part a result of the reduced proliferative capacity of skin cells. Indeed, studies have reported that keratinocytes, fibroblasts and melanocytes from old donors undergo less population doublings in culture when compared to those of young donors (Schneider and Mitsui, 1976; Gilchrest, 1983; Gilchrest *et al.*, 1984). Chronologically aged skin is characterised by a thin, dry appearance, and it is finely wrinkled (Rittie and Fisher, 2015). On the other hand, extrinsic ageing occurs as a result of environmental factors such as cigarette smoke, pollution factors, as well as UV exposure, which is also referred to as photoageing (Gilchrest, 1989; Yin *et al.*, 2001; Bernhard *et al.*, 2007). Clinically, photoaged skin appears leathery, lax, has coarse wrinkles and uneven pigmentation with brown spots (lentigines) (Rittie and Fisher, 2015).

Generally, both chronological and extrinsic factors contribute to the profound changes that occur in the epidermal and dermal compartments during skin ageing. For example, the volume of subcutaneous fat decreases with age. Moreover, in older adults, the number of cells in the epidermis decreases, keratinocytes become shorter and fatter, and basal keratinocyte proliferation is reduced, resulting in thinning of the epidermis with ageing (Marks, 1981; Grove, 1989; Farage *et al.*, 2007). Moreover, epidermal turnover rate is also slower in older individuals (Fore, 2006). The epidermal-dermal junction appears flattened, primarily as a result of the retraction of the rete ridges (Lavker *et al.*, 1987). This in turn affects the exchange surface between the epidermis and dermis, diminishing nutrient supply to the epidermis, which might also contribute to reduced keratinocyte proliferation (Lavker *et al.*, 1989). Flattening of the EDJ also reduces epidermal resistance to shearing forces, thus making the epidermis more fragile and increasing the potential for dermal-epidermal separation, which also contributes to wrinkling (Grove, 1986). Water content is also lower in aged skin, particularly in the stratum corneum. This occurs due to changes in amino acid composition of corneocytes, as well as a decrease in the amount of cutaneous natural moisturising factors, resulting in loss of the water-binding capacity of the skin (Potts *et al.*, 1984; Wilhelm *et al.*, 1991). Low water content in the stratum corneum interferes with the process of desquamation, leading to accumulation of corneocytes in the skin surface, which gives aged skin a rough and flaky appearance (Jackson *et al.*, 1993). Although transepidermal water loss is similar under basal conditions in both young and aged skin, suggesting that the barrier function of the stratum corneum is not affected,

this barrier is more easily disrupted in older skin. Any disturbances result in increased water loss, and the reconstitution of an effective stratum corneum is slower (Yaar and Gilchrest, 2001). Therefore, aged skin is more susceptible to external insults.

Other age-related epidermal changes include decreased melanocyte density, which has been shown to decrease progressively during adulthood by approximately 10% per decade (Gilchrest *et al.*, 1979). The remaining melanocytes also show decreased melanogenic capacity, possibly contributing to the pale appearance of older skin, and also to reduced tanning capacity following UV exposure (Hawk, 1990; Yaar and Gilchrest, 2001; Shlivko *et al.*, 2013). Indeed, expression of tyrosinase, the rate-limiting enzyme during melanin production, decreases with age in epidermal melanocytes (Kauser *et al.*, 2011). Despite the gradual loss in pigment-producing cells, chronically sun-exposed areas contain higher numbers of melanocytes. However, these cells are less evenly distributed, contributing to uneven pigmentation in older skin (Ortonne, 1990). In fact, a common feature of photoaged skin is the presence of pigmented lesions, such as solar lentigo, or "age spots". A higher melanin content is observed in these areas, resulting in increased local pigmentation; however, the mechanisms controlling increased melanin production in these lesions are still not fully understood. One possibility is that altered signalling from keratinocytes stimulates aberrant melanogenic behaviour, although variant alleles of genes involved in melanin synthesis have also been shown to be involved in solar lentigines development (Praetorius *et al.*, 2014).

Dermal thickness also decreases with age, with concomitant loss of cellularity and vascularity (Farage *et al.*, 2007). There is a reduction in the number of dermal fibroblasts, which leads to decreased collagen production in older skin (Gunin *et al.*, 2011). Collagen degradation is also enhanced due to increased production of matrix metalloproteases, which compromises the structural integrity of the dermis (Ashcroft *et al.*, 1997) (***Figure 1.4***). Moreover, the remaining collagen fibres become thicker and more disorganised, and collagen cross-links stabilise, leading to reduced elasticity in aged skin (Fenske and Lober, 1986). Over time, fragmented collagen fibril remnants accumulate, leading to decreased mechanical resistance of the ECM, thus decreasing the mechanical forces within surrounding fibroblasts. Dermal fibroblasts are no longer efficiently supported by the ECM, and respond to low mechanical forces by increasing intracellular ROS levels, reducing collagen synthesis, and increasing expression of MMPs (Mauch *et al.*, 1988; Eckes *et al.*, 2006; Fisher *et al.*, 2009). Therefore, collagen degradation leads to a self-sustainable positive feedback loop of oxidative stress, reduced collagen synthesis, and increased collagen degradation in aged skin. Elastin degradation is also increased due to

the action of MMPs, and the existing elastin fibres show higher degree of calcification with decreased functionality (Farage *et al.*, 2007). Additionally, fibroblasts produce less hyaluronic acid, another major ECM component which is important for the water-retaining capacity of the dermis (Papakonstantinou *et al.*, 2012). Aberrant dermal ECM homeostasis leads to loss of structural integrity, resulting in increased rigidity and decreased elasticity, ultimately contributing to skin fragility with age (Quan and Fisher, 2015).

Furthermore, different phases of the wound healing process are affected by the changes that accompany skin ageing, resulting in delayed healing in aged individuals. For example, the proliferation phase is characterised by re-epithelialisation of the wound, as well as deposition of new ECM and blood vessels, which requires migration and proliferation of fibroblasts, keratinocytes and endothelial cells. However, the proliferative potential of these cells is reduced with age, delaying this process (Gosain and DiPietro, 2004; Sgonc and Gruber, 2013). Moreover, the final maturation phase, which involves collagen remodelling, might be affected due to an imbalance between the levels of MMPs and their inhibitors, as it has been reported that cutaneous wounds of elderly people have higher levels of MMP-2 and MMP-9, while inhibitors of MMP-1 and -2 were downregulated (Ashcroft *et al.*, 2002). Levels of TGF-β1, which stimulates collagen synthesis and diminishes its degradation, was also shown to be reduced in wounds of aged skin (Ashcroft *et al.*, 2002). Therefore, these age-related changes favour collagen breakdown, thus delaying wound closure.

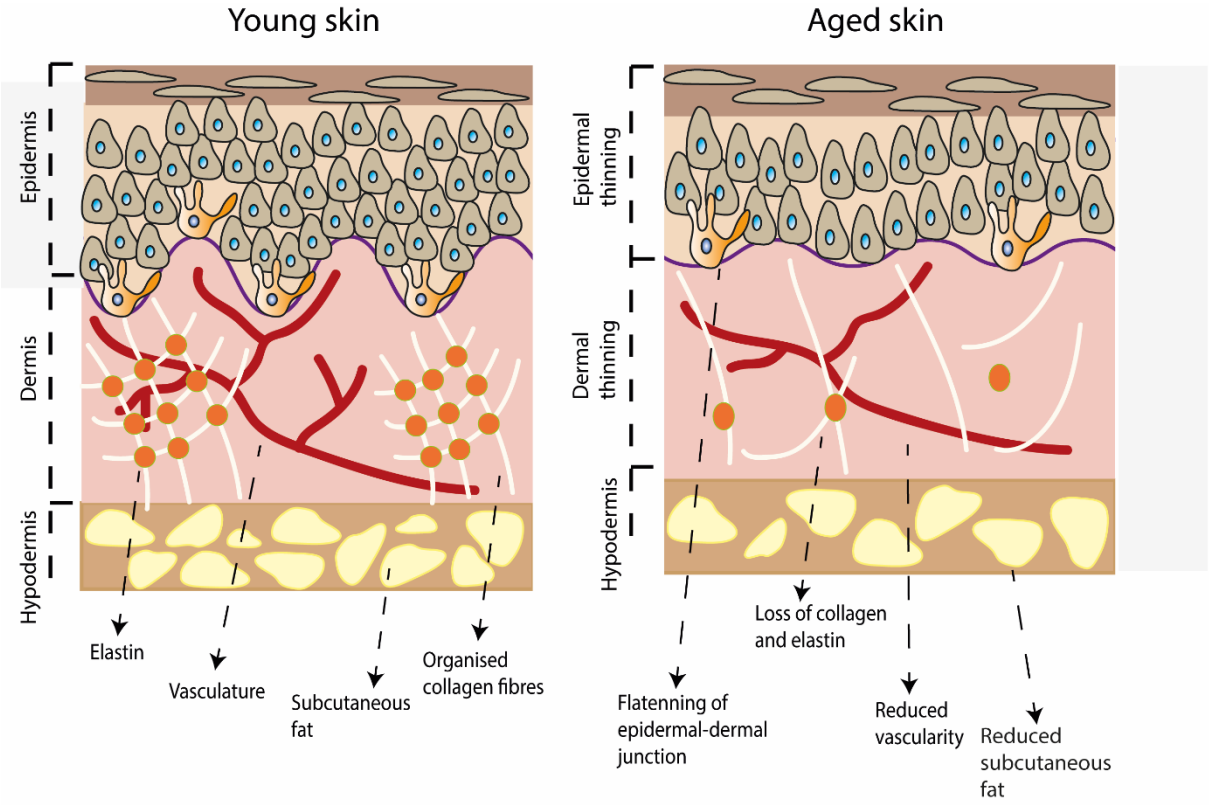

Figure 1.4 - Structural characteristics of aged skin. Skin ageing characteristics include thinning of the epidermis, with decreased number of keratinocytes (shown in brown) and melanocytes (shown in orange). Retraction of the rete ridges (epidermal projections into the dermis, shown in purple) leads to flattening of the epidermal-dermal junction, which also contributes to a thinner epidermis appearance. Dermal thickness also decreases, accompanied by increased collagen and elastin degradation. The remaining collagen and elastin fibres are disorganised and have decreased functionality, contributing to loss of elasticity and increased fragility. Moreover, subcutaneous fat is also reduced in aged skin.

1.7.3. Contribution of UV exposure to skin ageing

Photoaging is a major contributor to skin ageing (Friedman, 2005), and its effects are more prominent in fair skin individuals compared to people with darker skin, since melanin acts as a natural protective barrier against UV radiation. In fact, darker skin individuals contain a higher proportion of the dark pigment eumelanin, which is much more efficient in blocking UV than the lighter pigment, pheomelanin. Conversely, fairer skin is much more susceptible to the damaging effects of UV due to a low amount of epidermal eumelanin (Vincensi *et al.*, 1998). As well as the pigmentation status of the skin, the severity of photoageing also depends on the cumulative dose of UV exposure experienced during life (Kammeyer and Luiten, 2015). Solar UV radiation is divided into three categories according to their wavelength, which include UVA (320-400 nm), UVB (290-320nm) and UVC (200-290 nm). Sunlight is primarily composed of UVA (90-95%) and UVB (5-10%), whereas UVC is completely filtered by the atmosphere, and does not actually reach the earth (Amaro-Ortiz *et al.*, 2014). UVA plays a significant role in photoageing since it penetrates through the epidermis and into the dermis, where it can damage ECM components. On the other hand, UVB does not penetrate deeply into the skin, with the majority of the energy being absorbed in the epidermis, and thus it is largely responsible for the development of sunburn (D'Orazio *et al.*, 2013). Nevertheless, both UVA and UVB can cause damage to epidermal and dermal components and contribute to photoageing.

Although the majority of UVB is absorbed in the stratum corneum, some also reaches viable epidermal cells, where it causes DNA damage through the formation of cyclobutane-pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts ((6-4)-PP) (Goodsell, 2001). UV radiation also leads to ROS generation, which in turn can damage DNA, giving rise to oxidative DNA lesions such as 8-hydroxy-2'-deoxyguanine (8-OHdG) (Meyskens *et al.*, 2001; Kunisada *et al.*, 2005). If this damage is not correctly repaired by the nuclear excision repair (NER) or base excision repair (BER) mechanism, keratinocytes can arrest in the cell cycle and become senescent (Lewis *et al.*, 2008). Accumulation of damage can also lead to keratinocyte apoptosis (Qin *et al.*, 2002), whereas other mutations, such as those in the p53 gene, can lead to malignant transformation and tumour initiation (Ziegler *et al.*, 1994). Moreover, UVB is absorbed by aromatic amino acids in epidermal proteins, resulting in protein modifications which can be affect its function. Modified proteins can also form aggregates which are detrimental for cellular function if not efficiently degraded by the ubiquitin-proteasome system (Pattison and Davies, 2006).

Studies have shown that ROS produced in response to UVA irradiation stimulates the synthesis of MMPs via the activation of MAPKs in the epidermis and upper dermis (Klotz *et al.*, 1999; Maziere *et al.*, 2001). Briefly, activated MAPKs phosphorylate the transcription factor c-Jun, which is then translocated into the nucleus where it interacts with c-Fos, and increases the expression of the transcription factor AP-1 (Rittie and Fisher, 2002). AP-1 stimulates the transcription of different ECM-degrading enzymes such as MMP-1, -3, and -9, which together have the ability to completely degrade fibrillary collagen and elastin in the skin (Fisher *et al.*, 1996; Benbow and Brinckerhoff, 1997). Indeed, UV irradiation of human skin *in vivo* has been shown to induce expression of MMP-1, -3, and -9 with a concomitant increase in collagen degradation (Fisher *et al.*, 1996; Fisher *et al.*, 1997). Moreover, repeated UV exposure has been shown to trigger deposition of abnormal elastin in the dermis (Lavker *et al.*, 1995). AP-1 also represses expression of collagen precursors (procollagen I and III), thus significantly impairing collagen synthesis (Chung *et al.*, 1996). Imbalance in collagen homeostasis by UV exposure is also mediated through downregulation of the TGF-β pathway, which plays a role in stimulating production of procollagen I and III, and in reducing transcription on MMP-1 (also known as collagenase) in skin fibroblasts (Massague, 1998). It has been shown that UV irradiation downregulates expression of TGF-β type II receptor in human skin *in vivo* (Quan et al., 2004), induces expression of Smad-7, an inhibitor of TGF-β signalling (Quan *et al.*, 2005), and also reduces the levels of connective tissue growth factor (CCN2), which mediates the stimulatory effects of TGF-β on procollagen I synthesis (Quan *et al.*, 2002). This results in diminished TGF-β signalling, and impaired collagen homeostasis. Furthermore, it has been reported that UV exposure induces the expression of pro-inflammatory cytokines such as IL-1β in fibroblasts and keratinocytes *in vitro*, resulting in increased expression of MMP-1, which is one of the enzymes responsible for collagen degradation (Wlaschek *et al.*, 1994; Wan *et al.*, 2001). Another study proposed that UVB-induced wrinkling was mediated by keratinocyte IL-1α secretion, which can then diffuse into the dermis and stimulate the expression of elastase in skin fibroblasts. Increased elastase activity then leads to deterioration of elastic fibres in the dermis and contributes to wrinkle formation (Imokawa, 2009). Overall, UV irradiation activates intracellular signalling pathways that favour collagen and elastin degradation over production, contributing to the ECM deficit that is characteristic of aged skin.

UV irradiation has also been shown to cause mitochondrial dysfunction, likely through damaging mitochondrial DNA (mtDNA) as a result of increased in ROS generation. Indeed, mtDNA is highly susceptible to oxidative stress, and accumulates more mutations than genomic

DNA due to a lack in repair mechanisms and histones (Yakes and Van Houten, 1997). Indeed, large-scale deletions in the mitochondrial genome have been associated with UV-induced photoageing of human skin (Berneburg *et al.*, 1997; Birch-Machin *et al.*, 1998). A study identified that a 4,977 base pair deletion in mtDNA, also known as "common deletion", was more frequently found in sun-exposed skin compared with sun-protected sites of the same person, suggesting a role for mtDNA mutations in skin photoageing (Berneburg *et al.*, 1997). Moreover, repeated exposure to sublethal doses of UVA irradiation induces these common deletions in the dermis of human skin *in vivo* and in skin fibroblasts *in vitro*, and causes mitochondrial dysfunction (Berneburg *et al.*, 2005). Damage in mtDNA can also impair the oxidative phosphorylation process, which increases ROS generation, and in turn leads to further mutations in the mitochondrial genome (Wallace *et al.*, 1998). In addition, inducing mitochondrial dysfunction by deletion of the mitochondrial antioxidant enzyme, Sod2, has been shown to trigger cellular senescence and lead to skin ageing phenotypes in mice *in vivo* (Velarde *et al.*, 2012). Therefore, mitochondrial dysfunction that arises as a result of UV exposure might also play a role in human skin ageing.

Exposure of *ex vivo* human skin to suberythemal doses of UV radiation (i.e. doses that are not sufficient to provoke an erythemic response/reddening) has been shown to induce accumulation of p16 in basal epidermal cells (Pavey *et al.*, 1999). Interestingly, p16 expression peaked at 24 hours post-irradiation, and reduced at 72 hours, with p16-positive cells moving from the innermost basal and suprabasal layers to the outermost spinous and granular layers with time, reflecting the turnover of keratinocytes in the epidermis (Pavey *et al.*, 1999). Additionally, it was later demonstrated that this increase in p16 expression corresponded to a G2 cell-cycle arrest of keratinocytes and melanocytes (Pavey *et al.*, 2001). As well as facilitating DNA damage repair, a G2 arrest was also suggested to contribute to melanin synthesis and increased melanisation of keratinocytes, which protects against further UV-induced damage (Pavey *et al.*, 2001). Elevated p53 expression has also been demonstrated following UV radiation of human skin *in vivo* at doses that induce moderate erythema (Campbell *et al.*, 1993). Therefore, given the damaging effects of UV radiation, it is likely that chronic UV exposure might contribute to senescent cell accumulation in the skin. Indeed, UVB radiation induces senescence in skin fibroblasts (Debacq-Chainiaux et al., 2005) and keratinocytes *in vitro* (Lewis et al., 2008), suggesting that senescence might occur as a protective response to ensure that UV-damaged cells harbouring extensive DNA mutations cannot replicate.

1.7.4. Senescence and skin ageing

Senescent cells have been shown to accumulate in human skin in an age-dependent manner. Studies have reported an increase in Sen-β-Gal activity in both dermal fibroblasts and epidermal keratinocytes in the skin of older individuals *in vivo* (Dimri et al., 1995). Accordingly, the number of p16-positive cells has been shown to increase with age in both the dermis and epidermis of human skin *in vivo* (Ressler *et al.*, 2006; Waaijer *et al.*, 2012b). Since the skin is a highly proliferative tissue, and relies on proliferation of stem and differentiated cells for constant renewal, accumulation of senescent cells may contribute to tissue dysfunction by compromising homeostasis, regeneration and structure. In accordance to this, recent studies have demonstrated that generating living skin equivalent (LSE) models using neonatal dermal fibroblasts and keratinocytes from young donors (30 – 40 years) yields a more youthful-looking epidermis when compared to models that contain keratinocytes from older donors (53 – 66 years) (Adamus *et al.*, 2014). Moreover, overexpressing p16 in keratinocytes from young donors resulted in thinning of the epidermis, similar to the phenotype induced by keratinocytes from older donors. Silencing p16 expression in the aged donor LSE model resulted in morphological improvements such as epidermal thickening, suggesting that senescent cells might play an important role in the changes observed during skin ageing (Adamus *et al.*, 2014). In agreement with this, inducing senescence in epidermal cells by Sod2 deletion, which causes mitochondrial dysfunction, leads to epidermal stem cell exhaustion, epidermal thinning, and delayed wound closure in old mice (Velarde *et al.*, 2015). Therefore, these data suggest that epidermal cell senescence is a major contributor to skin ageing phenotypes.

Accumulation of senescent cells can also contribute to skin ageing in a cell non-autonomous manner through secretion of SASP components. Indeed, senescent fibroblasts *in vitro* have increased expression of MMPs, whilst expression of MMP inhibitors, such as TIMP-1, is decreased, possibly contributing to an imbalance in ECM homeostasis (Millis *et al.*, 1992). Moreover, MMPs have been shown to diffuse from the epidermis into the dermis following UV irradiation, where they can lead to degradation of dermal extracellular matrix (Quan *et al.*, 2009). In support of this, Waaijer *et al.* found an association between the number of p16-positive cells in the epidermis and the morphology of elastin fibres in the papillary (upper) dermis, and a weaker association for the reticular (mid) dermis (Waaijer *et al.*, 2016b). As previously mentioned, paracrine signalling has also been demonstrated to exist between UV-irradiated keratinocytes and dermal fibroblasts, where IL-1α secreted from keratinocytes induced the expression of fibroblast elastase, the enzyme responsible for degrading elastin

(Imokawa, 2009). Given that UV exposure can induce senescence in skin fibroblasts and keratinocytes (Debacq-Chainiaux *et al.*, 2005; Lewis *et al.*, 2008), and that the aforementioned pro-inflammatory factors and proteases are common components of the SASP (Coppe *et al.*, 2010b; Laberge *et al.*, 2015; Waldera Lupa *et al.*, 2015), then the accumulation of senescent cells, either via intrinsic ageing or photoageing, may contribute to age-associated degradation of dermal ECM through chronic exposure to the SASP. Interestingly, more recently, the histone variant H2A.J, which accumulates in senescent fibroblasts and promotes expression of SASP genes, was also shown to increase in epidermal cells of human skin in an age-dependent manner, suggesting that these cells also develop a SASP *in vivo* (Contrepois et al., 2017). Furthermore, fibroblasts isolated from chronic wounds show increased markers of senescence, including secretion of pro-inflammatory factors, suggesting that the prolonged presence of senescent cells impairs wound closure, likely through altering their microenvironment (Mendez *et al.*, 1998; Vande Berg *et al.*, 2005). This supports the notion that the accumulation of senescent cells in skin over long periods of time leads to a pro-inflammatory and ECM degradative environment.

Other senescence-inducing stimuli have also been associated with skin ageing. For example, DNA damage has been shown to accumulate with age in human skin *in vivo* (Nassour *et al.*, 2016; Contrepois *et al.*, 2017). Moreover, the frequency of DNA damage foci and TAF were shown to be higher in dermal fibroblasts isolated from older donors, with both markers being positively associated with chronological age (Waaijer *et al.*, 2016a). An age-dependent increase in dysfunctional telomeres has also been reported in dermal fibroblasts in the skin of baboons, with one study showing that this correlated with increased p16 expression, suggesting that telomere dysfunction contributes to senescence during skin ageing (Herbig *et al.*, 2006; Jeyapalan *et al.*, 2007). Although it has been extensively reported that telomere length decreases with age in human skin *in vivo* (Lindsey et al., 1991; Butler et al., 1998; Friedrich et al., 2000; Nakamura et al., 2002), very few studies in humans have observed associations between DNA damage response proteins and telomeres in this tissue. However, telomere dysfunction-induced senescence has been reported in epidermal cells *in vitro*. For example, depletion of TRF2 in human keratinocytes, which causes uncapping, induces a senescence-like growth arrest with concomitant activation of p53 and p16, suggesting that telomere damage plays a role in keratinocyte senescence (Minty *et al.*, 2008). Moreover, patients with the premature ageing disorder dyskeratosis congenita (DC), a condition associated with mutations in telomerase components TERT and TERC, and in genes involved in telomere maintenance, present with

many epidermal defects such as poor nail growth, early hair loss and skin atrophy (Knight *et al.*, 1999; Vulliamy *et al.*, 2001; Vulliamy *et al.*, 2005; Savage *et al.*, 2008; Buckingham and Klingelhutz, 2011). Indeed, keratinocytes isolated from DC patients have short telomeres and decreased proliferative capacity, suggesting that telomere dysfunction in these cells contributes to impaired epidermal homeostasis and skin ageing characteristics (Gourronc *et al.*, 2010). Therefore, telomere dysfunction appears to be an important trigger of senescence in skin cells, and might also contribute to skin ageing.

The majority of studies on cellular senescence in the skin have focused on fibroblasts and keratinocytes, whereas very little is known about the impact of melanocyte senescence in skin ageing. Indeed, p16-positive melanocytes have been shown to accumulate in human skin, which significantly associated with increased facial wrinkles, higher perceived age, and age-associated elastin morphology (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b). Unlike fibroblasts and other cell types, melanocyte senescence *in vitro* has been shown to be mainly dependent on the p16/pRb pathway (Bandyopadhyay and Medrano, 2000). Inducing melanocyte senescence, either by replicative exhaustion or stimulating melanogenesis, results in upregulation of p16, whilst p21 levels were shown to decrease (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001). However, an increase in p21 levels is observed upon UVB irradiation of human melanocytes, which possibly mediates the G1 growth arrest in order to allow repair of UV-induced DNA damage (Medrano *et al.*, 1995). Therefore, in human melanocytes the p21/p53 pathway appears to play a role in the stress response but it may not be crucial for the senescence program (Bandyopadhyay *et al.*, 2001). Consistent with this, mice overexpressing a hyperactive p53 mutant show signs of accelerated ageing in many tissues, including dermal thinning and hair sparseness; however, no pigmentation defects were observed in the hair and skin of these mice (Tyner *et al.*, 2002). Given that melanocyte proliferation and differentiation are required for cyclic regeneration of the hair pigmentation unit (Botchkareva *et al.*, 2001), these results indicate that melanocytes remained functional in mutant mice, suggesting that p53 overactivation does not induce melanocyte senescence *in vivo* (Tyner *et al.*, 2002).

Although the role of telomere dysfunction in melanocyte senescence has also been poorly explored, it has been shown that telomerase overexpression significantly extends the replicative lifespan of melanocytes, suggesting that telomere shortening plays a role in melanocyte senescence in culture (Bandyopadhyay *et al.*, 2001). However, studies conducted in human nevi, which consist of oncogene-induced senescent melanocytes, showed that telomeres in these

cells were not shorter when compared to surrounding tissues, indicating that telomere shortening does not occur in melanocyte senescence *in vivo*, or at least not as a result of oncogene activation (Suram *et al.*, 2012).

As well as playing a role in senescence signalling, telomeres have also been shown to be important in the melanin synthesis process (i.e. melanogenesis). As previously mentioned, UV irradiation induces oxidative DNA damage, which preferentially accumulates at guanine repeats, regions that are enriched at telomeres (Oikawa and Kawanishi, 1999). Melanogenesis occurs in melanocytes as a response to UV-induced DNA damage (Freeman *et al.*, 1989), and telomere disruption has been proposed to be involved in this process. For example, UV-induced melanin production can be mimicked by the use of T-oligos, DNA nucleotides homologous to telomeric sequences (Gilchrest *et al.*, 2009). In fact, T-oligos that have greater telomere homology are more effective at increasing melanin content in murine melanoma cells *in vitro* and in human skin explants (Arad *et al.*, 2006; Gilchrest *et al.*, 2009). Moreover, inducing telomere uncapping by expression of a dominant-negative allele of TRF2 increases the melanin content of melanocytes in culture (Gilchrest *et al.*, 2009). This tanning response is largely mediated by p53, as cells expressing a dominant-negative p53 do not show increased melanogenesis in response to UV irradiation, suggesting that melanin synthesis is a p53-mediated adaptive response of melanocytes to DNA damage (Khlgatian *et al.*, 2002). Therefore, it appears that telomeres in melanocytes act as sensors of damage, and coordinate responses such as melanogenesis and senescence. Moreover, it is possible that repeated exposure to solar irradiation throughout an individual's life may induce extensive telomeric DNA damage, eventually triggering melanocyte senescence, and accumulation of these cells might contribute to age-related skin characteristics.

Overall, although the specific trigger of senescence during skin ageing is still unclear, both chronological and photo-ageing contribute to an increase in senescent cells in the skin with age. Telomere damage signalling might represent a common pathway driving senescence in both scenarios, and accumulation of senescent cells might be major contributor to age-associated skin changes.

1.8. Research aims

Although cellular senescence has been implicated in human skin ageing, the majority of studies focus on keratinocyte and fibroblast senescence, whilst the impact of melanocyte senescence in the skin ageing process remains largely understudied. Moreover, the role of telomere dysfunction during melanocyte senescence and human skin ageing has been poorly investigated. The overall aim of this research was to investigate the role of telomere dysfunction in melanocyte senescence, and assess whether senescent melanocytes can contribute to age-associated skin changes by affecting surrounding healthy cells through paracrine mechanisms.

Specifically, the aims of this study were:

1) To investigate melanocyte senescence in human skin ageing *in vivo* and determine the involvement of telomere dysfunction in this process;
2) To characterise different models of melanocyte senescence *in vitro*, and determine whether telomere dysfunction was causal in the induction of senescence;
3) To investigate whether senescent melanocytes could induce paracrine damage in surrounding cells through secretion of SASP factors, and if so, to determine the effects of paracrine damage induction in skin ageing phenotypes.

Chapter 2: Materials and Methods

2.1. Chemicals and Reagents

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.2. Buffers and Solutions

Table 2.1 outlines a list of buffers and solutions used during this research.

Solution	Components
Sen-β-Gal staining solution	150mM NaCl, 2mM $MgCl_2$, 40mM citric acid, 12mM sodium phosphate pH 6.0, 400µg/ml X-Gal, 2.1mg/ml potassium hexacyanoferrat (II) trihydrate, 1.65mg/ml potassium hexacyanoferrat (III) trihydrate
PBG-Triton	0.2% fish skin gelatin (Sigma, G7765), 0.5% BSA, 0.5% Triton X-100, 1% PBS
Hybridisation buffer for Immuno-FISH	70% deionised formamide (Sigma), 25mM $MgCl_2$, 1M Tris pH 7.2, 5% blocking reagent (Roche, Welwyn, UK), 4ng/µL Cy-3 labelled telomere specific (CCCTAA) peptide nucleic acid probe (Panagene, F1002-5), distilled H_2O
FISH wash buffer	70% formamide in 2 x SSC
2 x SSC	17.53g NaCl, 8.82g sodium citrate, 1L distilled H_2O (pH 7.0)
Citrate buffer (0.01M)	29.41g trisodium citrate, 1L distilled H_2O (pH 6.0)
RIPA buffer	150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0, 1x phosphatase and protease inhibitors cocktail (ThermoFisher Scientific, 78442)
Haematoxylin	5g haematoxylin, 40ml glacial acetic acid, 0.5g sodium iodate, 300ml glycerin, 50g aluminium potassium sulphate, in 700 ml H_2O
TBS-Triton	50mM Tris, 150mM NaCl and 0.25% Triton X-100, (pH 7.0)
TAE buffer (50X)	242g Tris base dissolved in H_2O, 57.1ml glacial acetic acid, 100ml of 500mM EDTA (pH 8.0), 1L distilled H_2O
LB medium	10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl made up to 1L H_2O and autoclave

Table 2.1 – Buffers and solutions used in this study.

2.3. Cell culture

2.3.1. Primary cells

Primary human epidermal melanocytes were obtained from ThermoFisher Scientific (HEMn-LP, #C0025C) and Lonza (NHEM-Neo, CC-2504) (Walkersville, MD, USA). Cells were isolated from the foreskin of lightly-pigmented neonatal donors.

Primary human dermal fibroblasts isolated from neonatal foreskin were obtained from Lonza (NHDF, CC-2509).

2.3.2. Cell culture

Normal human epidermal melanocytes (NHEM) were cultured in MBM-4 basal medium supplemented with MGM-4 SingleQuot Supplements and Growth Factors (Lonza, CC-3249) and maintained at 37° C, 5% CO_2. Cell culture was done using aseptic technique in a class II safety cabinet. Cell passaging included a phosphate buffered saline (PBS) wash, and trypsinisation was carried out using pre-warmed 0.05% Trypsin-EDTA (TE) (ThermoFisher, 25300-062) at 37°C followed by neutralisation with an equal amount of trypsin neutraliser solution (ThermoFisher, R-002-100), and cell culture medium was then added to the suspension. Cells were collected by centrifugation, resuspended in fresh medium at the desired density and reseeded into tissue culture flasks or dishes.

Normal human dermal fibroblasts (NHDF) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with foetal bovine serum (FBS) (10% v/v) (Sigma, 12133C), L-glutamine (2mM) (Sigma, G3126), penicillin (100 units/ml)/streptomycin (100 µg/ml) (Sigma, P4333) and maintained at 37° C, 5% CO_2. Cell passaging included a PBS wash, and trypsinisation was carried out using pre-warmed Trypsin-EDTA (0.5% Trypsin, 0.2% EDTA) (Sigma, T3924) at 37°C followed by neutralisation with cell culture medium. Cells were collected by centrifugation, resuspended in fresh medium at the desired density and reseeded into tissue culture flasks or dishes.

2.3.3. Cryogenic storage

For melanocytes, freezing medium composed of melanocyte culture medium containing 30% dimethyl sulfoxide (DMSO) (Sigma, D2650) and 20% FBS was prepared in advance, filtered through a 0.2µm filter and stored at 4°C until it was needed.

Exponentially growing adherent cells were trypsinised and centrifuged at 800 g for 5 minutes at room temperature. The supernatant was removed and melanocytes were resuspended in FBS

containing 50% (v/v) melanocyte freezing medium at a density of 1 x 10^6 cells/ml. For dermal fibroblasts, cells were resuspended in FBS containing 5% (v/v) DMSO also at a density of 1 x 10^6 cells/ml. Aliquots of 1ml of cell suspension were immediately transferred to cryo-vials and placed in a Nalgene™ Cryo freezing container filled with isopropanol (ThermoFisher, 5100-0001). Cells were placed at -80° for 24 hours to allow slow freezing prior to long-term storage in liquid nitrogen.

2.3.4. Resuscitation of frozen cells

Cryo-vials were removed from liquid nitrogen and immediately thawed for 1 to 2 minutes at 37°C. Cells were then seeded in a 75 cm^2 flask with 15ml pre-warmed medium. Fresh medium was added 24 hours later to remove DMSO and cell debris.

2.3.5. Calculating cell density and population doublings

Cell concentration at each passage was determined using a 0.2 mm Fuchs-Rosenthal haemocytometer (VWR International UK) with 20µl of cell suspension. Using a standard microscope (DMIL, Leica Microsystems,UK), cells were manually counted and the average of four counts of 8 adjacent squares was taken, giving the number of cells x 10^4/ml. Total number of cells was then calculated by multiplying the number of cells per ml by the total volume of cell suspension. In order to calculate population doubling (PD), the total cell number harvested and the number of cells previously seeded are necessary, and the following equation was used:

$PD = X + (\ln(N1/N2)/\ln 2$

Where:

PD = population doubling

X = previous PD

N1 = number of cells harvested

N2 = number of cells treated

2.4. Cell treatments

2.4.1. Replicative senescence

Melanocyte replicate senescence was achieved by replicative exhaustion, and confirmed by over 80% of cells being positive for Sen-β-Gal, negative for the proliferation marker Ki67, and less than 0.5 PDs for at least 4 weeks.

2.4.2. X-ray irradiation

Melanocytes at early passages (P5-P12) were seeded onto flasks or 6/12/24-well plates (Corning) 48 hours prior to irradiation. Cells were then exposed to 10Gy X-ray irradiation (X-Rad 225, Precision X-Ray INC, N-Branford, CT, USA), and medium was refreshed immediately after treatment.

2.4.3. Ultraviolet (UV) irradiation

The UVA and UVB sources consisted of a Dr Honle SOL 500s lamp (Dr. Honle AG UV Technology, Munich, Germany) equipped with a UVA+UVB filter glass (Eurobond Adhesives), and emitting in the spectral range of 320-400 nm for UVA and 295-315 nm for UVB. No residual UVC is emitted. The time necessary to achieve the doses used in this study were measured using a UV meter (Honle UV Technology, serial no. 503337) prior to each exposure.

Melanocytes at early passages (P5-P12) were seeded onto 6-/12-/24-well plates or 10 cm^2 dishes 48 hours prior to irradiation. Before UV exposure, culture medium was aspirated and replaced with pre-warmed PBS ($+Ca^{2+}$, $+Mg^2$) (ThermoFisher, 14040174). For genotoxic stress generation, cells were exposed to 0.4 J/cm^2 of UVA and UVB, and fresh medium was added immediately after irradiation. Treatment was repeated once a day for five consecutive days. Control cells were treated identically but not exposed to UV irradiation.

2.5. Treatments

2.5.1. Inhibition of mTORC1

Melanocytes at early passages (P5-P12) were irradiated with 10Gy X-ray irradiation, and treated with 100nM rapamycin (Sigma, R8781) for 10 days. Rapamycin was replaced every 2 days. Control cells were treated with the same concentration of DMSO.

Rapamycin was removed, and cells were washed in pre-warmed PBS, 24 hours before cells and conditioned media were collected to be used in bystander experiments in order to ensure that no rapamycin was present during fibroblast culture.

2.5.2. Inhibition of CXCR3

In order to inhibit the chemokine receptor CXCR3, X-ray irradiated melanocytes were treated with 10μM AMG487 (Tocris, 4487) immediately following irradiation. Treatment was refreshed every 2 days, and cells were treated for 10 days before being harvested for analysis.

For bystander effect experiments, dermal fibroblasts cultured in senescent melanocyte conditioned media were treated with 10μM AMG487 (Tocris, 4487) for the duration of the experiment (48 hours).

2.5.3. MitoQ treatment

In order to assess the role of intracellular ROS in paracrine DNA damage induction, dermal fibroblasts cultured in senescent melanocyte conditioned media were treated with the mitochondria-targeted antioxidant MitoQ (provided and developed by Prof Mike Murphy) (20nM, diluted in dH_2O) for the duration of the experiment (48 hours). Note that prior to the addition of melanocyte conditioned media, fibroblasts were pre-treated with MitoQ for 6 hours in normal culture conditions.

2.6. Co-culture of melanocytes with dermal fibroblasts

Melanocytes were seeded together with early passage dermal fibroblasts (P8-13) in coverslips in 12-well plates at a 3:1 melanocyte: fibroblast ratio. Cells were co-cultured in culture medium containing a mixture of 50% MGM-4 and 50% DMEM, and were harvested for analysis 48 hours after the co-culture was established.

2.7. Culture of dermal fibroblasts with melanocyte conditioned medium

In order to assess paracrine DNA damage induction by secreted soluble factors, conditioned medium from control (proliferating) and stress-induced melanocytes (10Gy X-ray irradiation) was collected at 11 days following irradiation. Upon collection, conditioned medium was centrifuged at 1600 RPM for 5 minutes to eliminate any cell debris before it was added to fibroblasts.

For short-term culture, early passage dermal fibroblasts (P8-13) were seeded onto glass coverslips in 12-well plates at a density of 2.5×10^4 cells/well 48 hours prior to treatment. Melanocyte conditioned medium was then added to fibroblasts at a ratio of 1:1 DMEM: conditioned medium, and cells were harvested 48 hours following initial treatment.

For longer-term culture, fibroblasts (P8-13) were seeded in 25 cm^2 flasks 48 hours prior to treatment. Melanocyte conditioned medium was then added to fibroblasts at a ratio of 1:1 DMEM: conditioned medium. Medium was replenished every 3 days for the duration of the experiment (20 or 30 days). At either day 18 or 28, respectively, fibroblasts were trypsined and reseeded onto glass coverslips in 24-well plates. Cells were then harvested at 20 or 30 days following initial treatment.

2.8. Creating stably expressing Flag-TRF1-FokI-NHEM melanocyte cell lines

The fragments 3xFlag-TRF1-Fok1 and 3xFlag-TRF1-Fok1 (D450A) (contains a single nucleotide mutation which inactivates the endonuclease) were cloned into the doxycycline-inducible lentiviral pCW57.1 backbone. The following steps were conducted:

2.8.1. Plasmid expansion and purification

Plasmid elution

Plasmids (3xFlag-TRF1-Fok1 and 3xFlag-TRF1-Fok1 (D450A) were received precipitated on filter paper. The plasmid DNA was eluted by cutting out a small section of filter paper using sterile scissors, and placing it into a 1.5ml micro-centrifuge tube with 20µl of nuclease-free H_2O. The filter paper was then mixed with the H_2O using a sterile pipette tip, and left at room temperature for 2 minutes.

Bacterial transformation

2µl of eluted plasmid DNA (100pg – 100ng plasmid DNA) was added to 10µl of NEB Stable Competent E. coli cells, and mixed by gently flicking 5 times. The mixture was left on ice for 30 minutes, and the cells were then heat shocked at 42°C for 30 seconds, and placed on ice for 5 minutes. Next, 500µl of SOC outgrowth medium (Invitrogen, Cat. Number 15544-034) was added to the mixture, which was then placed on an orbital shaker at 250 RPM at 30°C for 60 minutes. Meanwhile, agar selection plates with the appropriate antibiotic drug were warmed up at 30°C. Following the incubation period, cells were mixed thoroughly by flicking the tube, and 50-100µl of cell suspension was streaked onto the agar selection plates. Cells were incubated for 24 hours at 30°C.

Alternatively, when using previously transformed NEB Stable Competent E. coli cells that were stored in glycerol at -80°C, 50-100µl of cell stocks were transferred to SOC medium by using a sterile pipette tip. Cell suspension was streaked onto pre-warmed agar selection plates, and incubated 24 hours at 30°C.

Plasmid expansion

A single NEB Stable Competent E. coli colony was picked using a sterile pipette tip, which was then placed in 5ml lysogeny broth (LB) medium (***Table 2.1***) and incubated with horizontal shaking at 30°C for 6 hours. The cell suspension was then transferred to a vented conical flask containing 100ml LB with antibiotics (50ng/ml Ampicillin), and incubated on an orbital shaker at 180RPM at 30°C for 24 hours.

Bacterial glycerol stocks

In order to make bacterial glycerol stocks, 2ml of cell suspension from the previous step was transferred to a 15ml centrifuge tube and centrifuged at 5000g for 10 minutes at room temperature. The supernatant was then aspirated and the cell pellet was re-suspended in 1ml of a solution containing 30% glycerol: 70% LB (v/v), and was then placed in a cryovial for long-term storage at -80°C.

Plasmid isolation

Following expansion, plasmids were purified from NEB Stable Competent E. coli cells using the PureYield™ Plasmid Midiprep System (Promega). Briefly, 100ml of LB and NEB Stable Competent E. coli cell suspension was transferred into two 50ml centrifuge tubes, and centrifuged at 5000g for 10 minutes at room temperature. The pellets were then re-suspended in 3ml of Cell Suspension Solution and combined together, followed by the addition of 3ml of Cell Lysis Solution, and then mixed by inverting 5 times. Following a 3 minute incubation, 5ml of Neutralisation Solution was added and mixed by inverting 10 times, and the lysates were centrifuged at 15,000g for 15 minutes. A PureYield™ Clearing Column (blue) was placed on top of a PureYield™ Binding Column (white) and assembled onto a vacuum pump. The liquid was poured into the column stack and a vacuum was applied until the solution was drawn through. 5ml of Endotoxin Removal Wash was added to the binding column, and again the solution was pulled through by applying a vacuum. 20ml of Column Wash was added, and a vacuum was applied to pull all the solution through. A vacuum was then applied to the dry binding column for 60 seconds, and was then removed and placed onto a paper towel to remove any excess ethanol.

The DNA was eluted by placing the binding column into a clean 50ml centrifuge tube, followed by the addition of 600µl of nuclease-free H_2O and incubating for 1 minute. Next, the binding column was centrifuged at 2,000g for 5 minutes in a swinging bucket rotor centrifuge, and the filtrate was collected into a clean 1.5ml Eppendorf tube.

Assessing nucleic acid purity and quantification

DNA quality and quantification was performed using Nanodrop® 1000 spectrophotometer (Thermo Scientific). For purity, a 260/280 ratio of around 1.8 was considered satisfactory.

Plasmid solutions were stored at -20°C for future use.

2.8.2. Molecular cloning

Following DNA purification, a Gibson assembly was performed in order to clone the 3xFlag-TRF1-FokI and 3xFlag-TRF1-FokI (D450) fragments into the doxycycline-inducible pCW57.1 lentiviral backbone. A GFP insert was also cloned into the final vector (***Figure 2.1***).

Briefly, the Gibson assembly is a method of molecular cloning in which multiple DNA fragments can be joined by using a single, isothermal reaction. The DNA fragments should contain around 20-40 base pair overlap with the adjacent DNA fragments, which is added by PCR. The Gibson assembly reaction involves the activity of three enzymes: an exonuclease, which digests the 5' end of the DNA, resulting in single-stranded regions that the DNA fragments can anneal; a DNA polymerase, which adds nucleotides to fill in any gaps once the fragments have annealed; and a DNA ligase, which covalently joins the adjacent fragments (Gibson *et al.*, 2009).

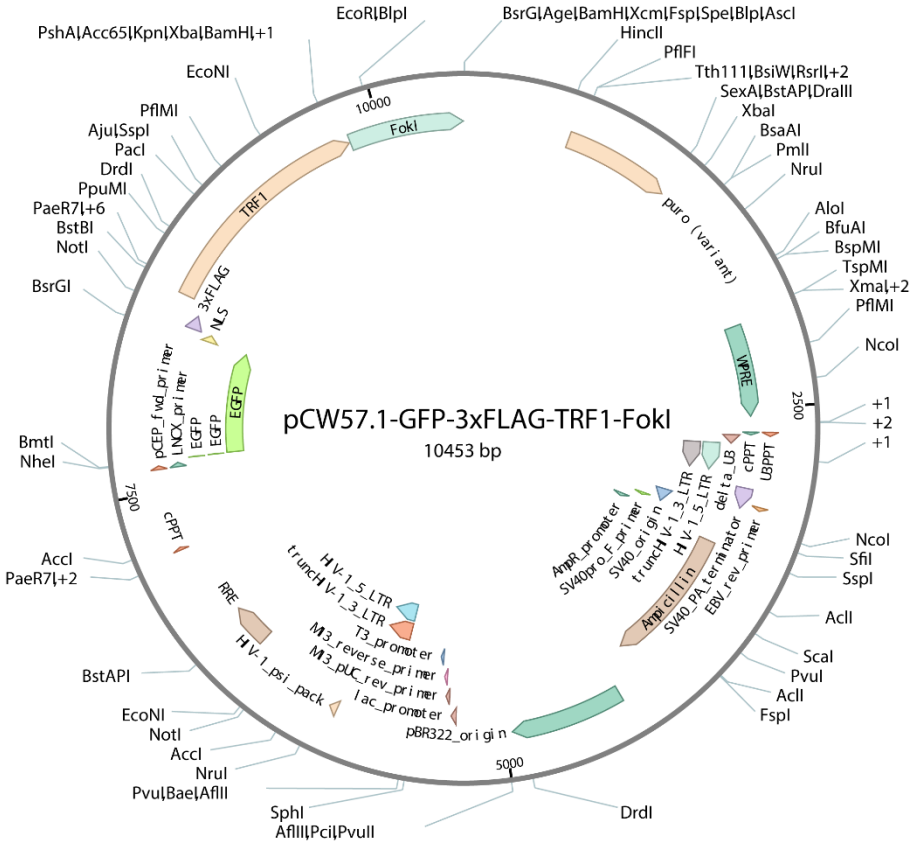

Figure 2.1 - Plasmid map of pCW57.1-GFP-3xFlag-TRF1-FokI.

Adding overlap regions into the fragments of interest by PCR

Primers were designed in order to amplify the inserts of interest out of the HFUW backbone by PCR. The resulting fragments will contain a region of overlap with the adjacent fragments of interest (i.e. GFP and pCW57.1). Overlap regions were also added to the pCW57.1 backbone and to the GFP insert by the same method. Primers were designed using Benchling.

The following was added to a PCR tube: 1µl 10X *Pfu*Ultra II Fusion HS DNA Polymerase (Agilent Technologies, 600670), 5µl 10X *Pfu*Ultra II reaction buffer + Mg^{2+}, 2µl dNTP mix (10 mM), 1µl DNA template (10ng/µl stock), 3µl forward primer (5µM stock), 3µl reverse primer (5µM stock), and 35µl of RNase-free H_2O.

The PCR reaction parameters were as follows:

1. 95°C for 1 minute
2. 95°C for 30 seconds
3. 60°C for 90 seconds
4. 72°C for 3 minutes
5. Repeat cycles 2 – 4 30 times
6. 72°C for 10 minutes

Gel electrophoresis for purification of PCR products

A 0.8% agarose gel was prepared by mixing 0.48g of agarose with 60 ml 1X TAE (***Table 2.1***). The mixture was microwaved until the agarose was dissolved. 1X SafeView Nucleic Acid Stain (NBS Biologicals, NBS-SV) was added to the gel mixture, which was then poured into a casting tray. Meanwhile, 10µl of 6X Gel Pilot® loading dye (Qiagen, 154015626) was added to 50µl of each DNA reaction mixture obtained from the PCR above. Once the gel had set, it was then transferred onto the electrophoresis tank, and 1X TAE was added until the gel was fully covered. 50µl of each DNA mixture was added to different wells, and a GeneRuler™ 1kb Plus DNA Ladder (Sigma Aldrich, D0428-1VL) was also used. The gel was run at 120V for 1 hour. Following completion, the gels were analysed under a UV lamp, and the bands corresponding to the expected molecular weight of each fragment were cut out with a clean, sharp scalpel, and placed into a 1.5ml Eppendorf tube.

DNA extraction of purified PCR products

DNA fragments were extracted from the agarose gel using the QIAquick Gel Extraction Kit (Cat No. 28704), and the manufacturer's protocol was followed. All centrifugation steps were

carried out at 13,000 RPM in a conventional table-top microcentrifuge at 15-25°C. Briefly, the gel excised was weighed in an Eppendorf tube, and 3 volumes of Buffet QG was added to 1 volume of gel (100mg ≈ 100µl). The gel was incubated at 50°C for 10 minutes until it was completely dissolved, and the mixture was vortexed. For DNA fragments less than 500 bp or over 4 kb, 1 gel volume of isopropanol was added to the sample. A QIAquick spin column was placed in a 2ml collection tube, and the sample was added to the QIAquick column, followed by 1 minute centrifugation in order to bind the DNA to the column. The flow-through was discarded, and the QIA quick column was placed back in the collection tube. 0.5ml of Buffer QG was added to the QIAquick column and centrifuged for another 1 minute. The flow-through was discarded. Next, 0.75ml of Buffer PE was added to the column, and centrifuged again for 1 minute. The flow-through was discarded, and the dry column was centrifuged for an additional 1 minute. In order to elute the DNA, the QIAquick column was then placed in a clean 1.5ml microcentrifuge tube, and 30µl of H_2O was added to the centre of the QIAquick membrane. The column was left to stand for 1 minute, and then centrifuged for 1 minute.

DNA concentration and purity were analysed using a Nanodrop® 1000 spectrophotometer (Thermo Scientific).

DNA Assembly Reaction (Gibson assembly)

Assembly of the DNA fragments obtained was performed by following the NEBuilder® HiFi DNA Assembly Reaction Protocol. Each reaction tube contained 20µl of mixture with the following:

- 10µl NEBuilder HiFi DNA Assembly Master Mix (New England BioLabs, E2621L)
- X µl of each DNA fragment (0.03-0.2 pmols) (The concentration of each fragment for optimal assembly was calculated based on fragment length and weight. Each insert was added at a 2:1 insert: backbone ratio)
- Volume was completed to 20µl with H_2O, if necessary

Samples were incubated in a thermocycler at 50°C for 60 minutes.

Bacterial transformation

5µl of the above Gibson assembly products was added to 45µl of B100 E. coli competent cells, and mixed gently by flicking the tube 5 times. The mixture was placed on ice for 30 minutes, and then heat shocked at 42°C for 30 seconds. The tubes were placed on ice for 2 minutes, and 950µl of SOC outgrowth medium (Invitrogen, Cat. Number 15544-034) was added. The cells

were incubated at 31°C for 60 minutes with shaking at 250RPM. Meanwhile, agar selection plates were warmed up to 31°C. The full volume of cells were streaked onto the agar plates, and incubated overnight at 31°C.

Plasmid expansion

Individual colonies were selected and grown overnight at 37°C in 5ml LB medium (***Table 2.1***) with Ampicillin (50ng/ml).

Plasmid isolation

Following expansion, plasmids were purified from B100 E. coli cells using the PureYield™ Plasmid Midiprep System (Promega), as described above.

Plasmid analysis

Following plasmid purification, the plasmid DNA was digested with the *XhoI* and *XbaI* restriction enzymes to confirm that the Gibson assembly yielded the correct plasmids.

2.8.3. Transfection and transduction protocols

Lentiviral plasmid transfection and viral production were carried out following class II safety procedures.

NEB Stable Competent E. Coli cells transformed with the packaging plasmid, d8.9, or the envelope protein, VSVG, were grown and expanded as previously described under "Plasmid expansion". DNA was purified as described under "Plasmid isolation" using the PureYield™ Plasmid Midiprep System (Promega).

Lentiviral vectors were produced by transfection of a three plasmid system into HEK293FT cells. $5x10^6$ HEK293FT cells were seeded in a 10cm dish and incubated for 24 hours in antibiotic-free DMEM (supplemented with 1:100 non-essential amino acids and 1:100 sodium pyruvate (10mM stock; Sigma Aldrich, S8636). Cells were at 80% confluency before proceeding to transfection using Lipofectamine™ 3000 reagent (Invitrogen, L3000015), following the manufacturers' protocol. The plasmids were mixed in a ratio of 1:1.5:2 (envelope:packaging:transfer) by using 2.5µg of VSVG, 3.75µg of d8.9, and 5µg of either pCW57.1-GFP-3xFlag-TRF1-FokI or pCW57.1-GFP-3xFlag-TRF1-FokI(D450A). In the morning after the transfection, medium was removed from HEK293FT cells, and 10ml of fresh medium without antibiotics was added (note: melanocyte culture medium was added). Culture medium containing viral particles was harvested 24 hours later (i.e. 48 hours after transfection),

then filtered through a 0.45µm pore PVDF filter and mixed with polybrene (10µg/ml final concentration). The medium was then added to NHEM melanocytes at 70% confluency (note: different dilutions of viral particles were added, which ranged from 0 to 2). After 24 hours, fresh culture medium was added to NHEM melanocytes. The following day, selection of transduced cells was carried out by treating NHEM melanocytes with 1.5µg/ml Puromycin for 5 days.

2.9. Flow cytometry

Prior to use, the flow cytometer (Partec, http://www.partec.com) was calibrated using fluorescent microbeads in order to ensure optimum performance and reproducibility. Each measurement was performed in triplicate and 1 x 10^4 cells were analysed per measurement.

2.9.1. Dihydroethidium

Dihydroethidium (DHE) is a blue fluorescent dye, and upon oxidation by superoxide anions, it is converted into a red fluorescent product, which intercalates within the cell's DNA. Melanocytes were harvested and centrifuged at 1600 RPM for 2 minutes. Supernatant was discarded and approximately 2.5 x 10^5 live cells were incubated with 10 µM of DHE (Invitrogen, D1168) in serum-free MGM4 for 30 minutes at 37°C in the dark. Following another centrifugation at 1600 RPM for 2 minutes, supernatant was discarded, and cell pellet was resuspended in 2ml serum-free MGM-4. DHE median fluorescence intensity was then determined by flow cytometry using the red fluorescence channel (FL3 channel). An FSC/SSC dot plot was used to define the population of live cells and apoptotic cells and debris were excluded by gating.

2.9.2. MitoSOX

MitoSOX is a red fluorescent dye, which is used as a mitochondrial superoxide indicator. MitoSOX permeates the membrane of live cells and is selectively targeted to mitochondria, where it is oxidised by superoxide anions, exhibiting red fluorescence. Mitochondrial superoxide levels were determined using the same protocol as for DHE, however, cells were incubated with 5µM MitoSOX Red (Invitrogen, M36008) for 10 minutes at 37°C in the dark. MitoSOX median fluorescence intensity was determined by flow cytometry using the red fluorescence channel (FL3 channel).

2.10. Subjects

Young subjects consisted of white British subjects, ranging in age from 22 to 31 years. Skin punch biopsies of 4mm were obtained from the sun-protected site of the upper inner arm of

each donor, and were taken by a qualified dermatologist in 2007. The study was approved by the Colworth Ethics Committee (UCR2007-07), and subjects gave informed written consent prior to inclusion in this study. Data from 14 randomly selected Colworth donors were used in this study, with an age range from 22.8 to 31.8 years.

Skin samples from older subjects were obtained from the Leiden Longevity Study (LLS). Study design and methodologies of the LLS have been previously described (Schoenmaker *et al.*, 2006). Briefly, the LLS consisted of men and women aged above 89 and 91 years, respectively, with at least one sibling who matches the same age criterion. The offspring of either long-lived sibling and the partners of the offspring were recruited to participate in the study. Skin punch biopsies of 4mm were taken from the sun-protected part of the upper-inner arm of each donor. The study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Centre (following the declaration of Helsinki), and participants gave informed written consent. Data from 31 randomly selected LLS participants were used in this study, with an age range from 54.9 to 76.8 years.

Skin biopsies were fixed in formalin (Sigma) overnight (18-24 hours), followed by washing and dehydration. Samples were then embedded in paraffin wax, cut into 4μm sections and mounted onto glass slides.

2.11. Culture of human epidermal equivalents

MelanoDerm (MatTek Corp., Ashland, MA) comprises normal human epidermal keratinocytes (NHEK) co-cultured with melanocytes (NHEM), which develop into a multi-layered, highly differentiated model of human epidermis.

For this study, two different types of MelanoDerms were produced by MatTek Corporation (Ashland, MA), which contained neonatal human keratinocytes and either proliferating or UV-induced senescent melanocytes. Tissues were then fixed in 10% neutral buffered formalin at 0, 10 and 21 days post epidermal differentiation, followed by PBS washes and dehydration. Samples were paraffin embedded, cut into 4μm sections and mounted onto glass slides.

2.12. Immunofluorescence

2.12.1. Immunocytochemistry (ICC) on fixed cells

Cells grown on coverslips were fixed with 2% paraformaldehyde (PFA) (VWR, 9713.9010) in PBS for 5 minutes at room temperature. PFA was discarded, cells were washed with PBS twice, and stored at -80°C for long term storage. Following permeabilisation in 1ml PBG-Triton for

45 minutes at room temperature, cells were incubated with primary antibody (***Table 2.2***) diluted in PBG-Triton overnight at 4°C in a humidified chamber. Cells were then washed three times with PBG-Triton for 5 minutes and incubated with fluorescein-conjugated secondary antibody (***Table 2.3***) for 1 hour at room temperature in the dark with gentle shaking. Cells were washed three times in PBS for 5 minutes followed by mounting on to glass microscope slides with ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36935). Slides were imaged using a Leica DM5500B microscope and images were captured with a DFC360FX camera using LASAF software (Leica).

2.12.2. Immuno-FISH (γH2A.X-TeloFISH) on fixed cells

Cells grown on coverslips were fixed and immunocytochemistry for γH2A.X was performed as described above. Subsequent to secondary antibody incubation, cells were washed twice in PBS and fluorescence *in situ* hybridisation (FISH) was performed.

Cells were incubated in 1ml of fixative (methanol: acetic acid, 3:1) for 30 minutes, followed by dehydration in graded cold ethanol solutions (70, 90, 100%) for 2 minutes each. The samples were then incubated in PBS at 37°C for 5 minutes, and fixed in 4% PFA at 37°C for 2 minutes. Following a PBS wash, cells were dehydrated again with cold ethanol solutions (70, 90, 100%) for 2 minutes each, and were left to air dry. Coverslips were then placed onto glass slides containing 10μl of hybridisation buffer with PNA probe (***Table 2.1***) and samples were then denatured for 10 minutes at 80°C. Coverslips were incubated for 2 hours at room temperature in a humidified chamber in the dark to allow hybridisation to occur. Samples were then transferred back into a 12 well plate and washed three times in FISH wash buffer (***Table 2.1***) for 10 minutes, followed by three washes in 0.05% TBS-Tween for 5 minutes. Cells were then dehydrated in graded cold ethanol solutions (70, 90, 100%), and left to air dry before they were mounted onto glass microscope slides with ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36935). Cells were imaged using a Leica DM5500B microscope and images were captured with a DFC360FX camera using LASAF software (Leica), or using a Leica DMi8 wide field fluorescence microscope (inverted) equipped with a Hamamatsu C11440-22 camera using LASX software. In depth Z stacking was used (a minimum of 35 optical slices with 63x objectives), and quantification of DNA damage foci and telomere-associated foci was performed blinded using ImageJ software.

Antibody	Species	Specificity	Dilution
Anti-Ki67 (Abcam, ab15580)	Rabbit polyclonal	Human Mouse	1:250
Anti-phospho-histone H2A.X (γH2A.X) (Ser139) (Millipore, 05-636)	Mouse monoclonal	Human	1:200
Anti-p16 (CINTec Histology, Roche, 9511)	Mouse monoclonal	Human	As provided in the kit
Anti-p21 (Cell Signalling, 2947S)	Rabbit monoclonal	Human Monkey	1:400
Anti-MelanA (Santa Cruz, sc-28871)	Rabbit polyclonal	Human Mouse Rat	1:50
Anti-CXCR3 (Abcam, ab64714)	Mouse monoclonal	Human	1:400

Table 2.2 – Primary antibodies used for immunofluorescence on fixed cells.

Antibody	Species	Specificity	Dilution
Anti-rabbit Fluorescein-conjugated secondary antibody Alexa Fluor 594 (Invitrogen, A11012)	Goat	Rabbit	1:4000
Anti-rabbit Fluorescein-conjugated secondary antibody Alexa Fluor 647 (Invitrogen, A21244)	Goat	Rabbit	1:4000
Anti-mouse Fluorescein-conjugated secondary antibody Alexa Fluor 488 (Invitrogen, A21042)	Goat	Mouse	1:4000
Anti-mouse Fluorescein-conjugated secondary antibody Alexa Fluor 594 (Invitrogen, A11005)	Goat	Mouse	1:4000

Table 2.3 – Secondary antibodies used for immunofluorescence on fixed cells.

2.12.3. Immunohistochemistry on paraffin embedded tissues

Skin sections cut at 3μm were deparaffinised in Histoclear (National Diagnostics, HS-200) twice for 5 minutes each, followed by hydration in a graded ethanol series: twice in 100% ethanol for 5 minutes, once in 90% for 5 minutes and once in 70% for 5 minutes, and two washes in distilled H_2O for 5 minutes. Antigen retrieval was performed by incubating sections in 0.01M citrate buffer (pH 6.0) for 5 minutes at high power (800W) in a microwave oven, followed by 10 minutes at medium power (400W). Sections were placed on ice and allowed to cool to room temperature, followed by two washes in distilled H_2O for 5 minutes. Next, sections were incubated in blocking reagent (normal goat serum (NGS) (Vector lab, PK-6101) diluted 1:60 in 0.1% bovine serum albumin (BSA) (Sigma, A9418)) for 30 minutes at room temperature, followed by incubation with primary antibody (diluted in blocking reagent) overnight at 4°C (***Table 2.4***). The next day, sections were washed three times in PBS for 5 minutes, and then incubated with secondary antibody (diluted in blocking reagent) for 30 minutes at room temperature (***Table 2.5***). For γH2A.X staining, sections were incubated with a goat anti-rabbit biotinylated secondary antibody (1:200, diluted in blocking solution) (Vector Labs, VECTASTAIN, PK-6101) for 30 minutes at room temperature, and then washed three times with PBS for 5 minutes before being incubated with fluorescin avidin DCS (1:500) (Vector Lab, A-2011) (1:500, diluted in PBS) for another 30 minutes at room temperature. Following three PBS washes for 5 minutes, sections were mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36935). Images were taken using a Leica DM5500B microscope and captured with a DFC360FX camera using LASAF software (Leica). Image analysis was performed using ImageJ software.

2.12.4. Immuno-FISH (γH2AX-TeloFISH) staining on paraffin embedded tissues

Following immunofluorescence for γH2A.X, as described above, sections were washed three times in PBS, and then cross-linked by incubation in 4% PFA in PBS for 20 minutes at room temperature. Sections were washed in PBS three times for 5 minutes, and then dehydrated in graded ethanol solutions (70%, 90%, 100%, for 3 minutes each). Sections were allowed to air dry prior to being denatured in hybridisation buffer (***Table 2.1***) for 10 minutes at 80°C, and then incubated for 2 hours at room temperature in a dark humidified chamber to allow hybridisation to occur. Sections were then washed for 10 minutes in FISH wash buffer (***Table 2.1***), followed by a 10-minute wash in 2x SSC and another 10-minute wash in PBS. Sections were then mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36935). Slides were imaged using a Leica DM5500B microscope and images were captured with a

DFC360FX camera using LASAF software (Leica), or using a Zeiss Spinning Disk confocal microscope equipped with a QuantEM 5125C camera and Axiovision software (Zeiss). In depth Z-stack was used (a minimum of 40 optical slices with 63x or 100x oil objective). DNA damage and telomere-associated foci were analysed in a blinded fashion using ImageJ software.

Antibody	Species	Specificity	Dilution
Anti-Ki67 (Abcam, ab15580)	Rabbit polyclonal	Human Mouse	1:250
Anti-phospho-histone H2A.X (γH2A.X) (Cell Signalling, 9718)	Rabbit monoclonal	Human Mouse	1:400
Anti-SIRT1 (Abcam, ab13749)	Rabbit polyclonal	Human Cow	1:100
Anti-MelanA (Dako, M7196)	Mouse monoclonal	Human	1:20
Anti-8-oxoG (Millipore, MAB3560)	Mouse monoclonal	Human Rat Mouse Monkey	1:100
Anti-p16 (CINTec Histology, Roche, 9511)	Mouse monoclonal	Human	As provided in kit
Anti-MelanA (Santa Cruz, sc-28871)	Rabbit polyclonal	Human Mouse Rat	1:50
Anti-CXCR3 (Abcam, ab64714)	Mouse monoclonal	Human	1:400

Table 2.4 – Primary antibodies used for immunofluorescence in tissue.

Antibody	Species	Specificity	Dilution
Anti-rabbit Fluorescein-conjugated secondary antibody Alexa Fluor 488 (Invitrogen, A11008)	Goat	Rabbit	1:1000
Anti-rabbit Fluorescein-conjugated secondary antibody Alexa Fluor 594 (Invitrogen, A11012)	Goat	Rabbit	1:1000
Anti-mouse Fluorescein-conjugated secondary antibody Alexa Fluor 488 (Invitrogen, A21042)	Goat	Mouse	1:1000
Anti-mouse Fluorescein-conjugated secondary antibody Alexa Fluor 594 (Invitrogen, A11005)	Goat	Mouse	1:1000
Anti-mouse Fluorescein-conjugated secondary antibody Alexa Fluor 647 (Invitrogen, A21235)	Goat	Mouse	1:1000
Anti-rabbit IgG biotinylated (VECTASTAIN Elite ABC Kit) (Vector lab, PK-6101)	Goat	Rabbit	1:200

Table 2.5 – Secondary antibodies used for immunofluorescence in tissue.

2.12.5. Q-FISH

Quantitative-FISH (Q-FISH) analysis of telomere-FISH intensity was performed on fixed cells and paraffin-embedded tissue following immuno-FISH staining (as described above). Image analysis was performed using ImageJ software, where Z projections were created for each individual image and the oval tool was used to measure the integrated density of each individual telomere signal.

2.13. Haematoxylin and Eosin (H&E) staining

Sections mounted onto glass slides were deparaffinised in Histoclear (National Diagnostics, HS-200) for 5 minutes, followed by re-hydration in a graded ethanol series: twice in 99% ethanol and once in 95% ethanol for 1 minute each, and then washed in distilled H_2O for 5 minutes. Sections were immersed in Harris Haematoxylin (6765003; Thermo Scientific) for 1 minute, and rinsed in running tap water for 2-3 minutes. Sections were then counterstained in Eosin-Y (6766007; Thermo Scientific) for 2 minutes followed by washes under running tap water until clear. Next, sections were dehydrated through a graded ethanol series: once in 95% ethanol and twice in 99% ethanol for 1 minute each, then cleared in Histoclear and mounted in Di-N-Butyle Phthalate in Xylene (DPX) (Thermo Scientific, LAMB-DPX).

Slides were imaged using a Nikon widefield microscope and images were captured with a Leica DFC420 camera using the LAS software (Leica) using a 20x objective.

2.14. Senescence-associated-β-galactosidase (Sen-β-Gal) staining in fixed cells

Cells grown on glass coverslips were washed with PBS and fixed with 0.2% glutaraldehyde in 2% PFA in PBS for 5 minutes. Cells were incubated in Sen-β-Gal staining solution (pH 6.0) (***Table 2.1***) overnight at 37°C in the dark, followed by three 5-minute PBS washes, and were then mounted onto glass microscope slides using ProLong Gold Antifade Mountant with DAPI (Invitrogen, P36935). A minimum of 10 random fields were imaged (20x objective) using a Leica DM5500B microscope and captured with a Leica DFC420 camera using LASAF software (Leica). Cells were scored either positive (dark blue staining) or negative for Sen-β-Gal staining, and quantification was done using ImageJ software.

2.15. EdU incorporation

In order to assess cell proliferation, cells were cultured in the presence of 10μM EdU (diluted in fresh growth medium) for 24 hours. Cells were then washed twice in PBS for 5 minutes at 37°C, and then fixed with 2% PFA in PBS for 5 minutes (at this point, cells can be stored at -80°C for long term storage). Following two washes in 3% BSA in PBS for 5 minutes, cells were

permeabilised with 0.5% Triton X-100 for 20 minutes at room temperature, with gentle shaking on an orbital shaker.

All subsequent EdU detection steps were carried out according to the manufacturer's instructions (Click-iT EdU AlexaFluor Imaging Kit, ThermoFisher Scientific).

Cells were imaged using a Leica DM5500B microscope and captured with a DFC360FX camera using LASAF software (Leica). Images were analysed using ImageJ software.

2.16. Morphometric skin measurements

2.16.1. Skin biopsies

The measurements of skin morphology characteristics have been previously described (Waaijer *et al.*, 2012a). Briefly, two sections of each biopsy were stained with Orcein dye, and imaged for morphology analysis. Images were analysed semi-automatically using an image analysis software, and the measurements are shown in ***Figure 2.2***. Epidermal thickness was obtained by dividing the area covered by the epidermis (area between yellow and blue lines) by the length of the epidermal-dermal junction (EDJ) (blue line) in micrometres. Moreover, curvature of the epidermis was derived from the ratio of a straight horizontal line between the margins of the epidermis (white line) over the length of the epidermal-dermal junction (blue line).

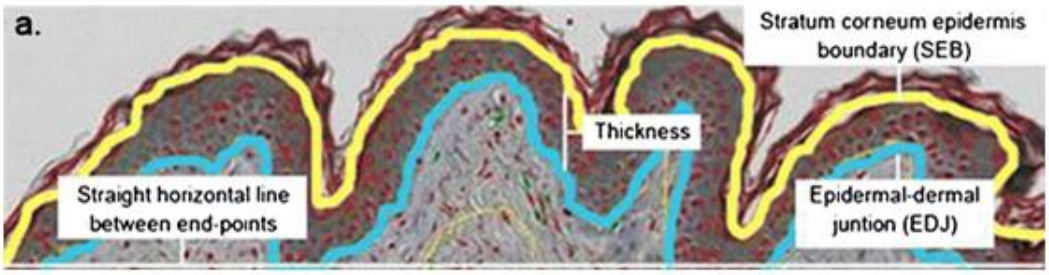

Figure 2.2 – Morphometric measurements of the epidermis. Epidermal measures include thickness and curvature of the epidermis. Image adapted from (Waaijer et al., 2012a).

2.16.2. MelanoDerm epidermal thickness

Haematoxylin and eosin staining was performed in human epidermal equivalent sections, which were then imaged (20x objectives) using a Nikon E800 widefield microscope and captured with a Leica DFC420 camera using LASAF software (Leica). For each section, the full length of the epidermis was imaged. Epidermal thickness was measured throughout the section using five randomly selected areas for each field by applying a randomly offset grid overlay on ImageJ. Mean per field and then mean per section were calculated. Data are plotted as mean thickness (μm) per section +/- SEM.

2.17. Analysis of pro-inflammatory cytokine release

2.17.1. Luminex assay

This assay involves microspheres which have been colour-coded with different concentrations of two fluorescent dyes (red and infra-red) at distinct ratios to create up to 100 distinct bead regions. Each microsphere is then coated with a specific capture antibody, which detects specific analytes in a test sample. Multiple conjugated beads can be added to each sample, allowing the detection of many different analytes in a single sample. The captured analytes are then detected by biotinylated detection antibodies, followed by addition of phycoerythrin (PE)-conjugated streptavidin, which acts as a reporter molecule, binding to the biotinylated antibodies, and completing the reaction on the surface of each microsphere. The beads are then read on a dual-laser flow-based detection instrument, where one laser identifies the bead according to its colour-coding, and determines the identity of the analyte being analysed. The second laser determines the fluorescence intensity of the PE-derived signal, which is directly proportional to the amount of analyte bound, providing a concentration of each analyte of interest.

In this study, detection of cytokines and chemokines in cell culture supernatants was performed using the following commercially available kits: Milliplex Human Cytokine/Chemokine Magnetic Bead Panel – Premixed 41 Plex (Merck Millipore, HCYTMAG-60K-PX41), and two custom panels (14-plex assay, LXSAHM-14; 3-plex assay, LXSAHM-03, R&D Systems), which were designed based on results obtained from the proteome profiler explained above. Protocol was followed according to manufacturer's instructions. Assays were analysed using a Luminex 200 reader.

2.18. Gene expression analysis

Gene expression analysis was performed on cultured normal human epidermal melanocytes.

2.18.1. RNA extraction

Cells were trypsined and centrifuged at 150g for 5 minutes at 4°C. Following a PBS wash, cells were again centrifuged at 150g for 5 minutes at 4°C, and supernatant was then discarded. RNA extraction of cell pellets was carried out using the RNeasy Mini Kit (QIAGEN, Cat. Number 74106), according to the manufacturer's instructions. RNA quality and quantification was performed using Nanodrop® 1000 spectrophotometer (Thermo Scientific).

2.18.2. cDNA synthesis

For cDNA synthesis, 0.5µg of RNA was used per reverse transcription reaction, and the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814) was used, according to the manufacturer's instructions.

2.18.3. Real Time Polymerase Chain Reaction (RT-PCR)

Each RT-PCR well contained 10µl of Real time PCR reaction mix, which consisted of 4µl of cDNA (200-800ng), 5µl Power Syber® Green PCR Master Mix (Invitrogen, Cat. Number 4367659), 0.2µl of 10µM reverse and forward primers, and 0.8µl deionised H_2O. See ***Table 2.6*** for primer sequences used. Each sample was run in triplicates in a C1000^{TM} Thermal Cycler, CFX96^{TM} Real-Time System (Bio-Rad) and Bio-Rad CXF Manager software. The thermocycler conditions were the following:

1. 95°C for 10 minutes
2. 95°C for 15 seconds
3. 65°C for 30 seconds
4. Repeat 39X steps 2 and 3.

Primer specificity was confirmed using a dissociation step with calculation of a melting curve. Quantification of mRNA expression was determined by normalising to the levels of an internal control mRNA (18S in this case). mRNA expression was calculated using the ΔΔC(t) method.

Gene	Species	Sequence
p16	Human	Forward 5'-CGGTCGGAGGCCGATCCAG Reverse 5'-GCGCCGTGGAGCAGCAGCAGCT
18S	Human	Forward 5'-GGCCCTGTAATTGGAATGAGTC Reverse 5'-CCAAGATCCAACTACGAGCTT

Table 2.6 – Primer sequences for cDNA real-time PCR.

2.19. Protein expression analysis

2.19.1. Protein extraction

Primary human melanocytes were washed with ice-cold PBS before addition of ice-cold RIPA buffer (***Table 2.1***) in order to lyse the cells. Samples were then stored at -80°C until further analysis.

2.19.2. Protein quantification

Cell lysates were thawed on ice and centrifuged for 10 minutes at 16100g at 4°C. In order to quantify sample protein concentration, a colorimetric Bio-Rad DC Protein Assay kit (Bio-Rad; Reagent A, 500-0113, Reagent B, 500-0114) was used according to the manufacturer's instructions. Absorbance readings were measured using the Fluostar Omega plate reader (BMG Labtech). Protein concentration of each sample was then calculated and normalised by mixing specific volumes of protein lysate and loading buffer (950µl of 2x Laemmli buffer (Bio-Rad, 161-0737) and 50µl of mercaptoethanol (Sigma, M6250)). Protein denaturation was carried out by incubating samples at 100°C for 5 minutes, and samples were then placed on ice and used immediately for western blotting.

2.19.3. Western blotting

A running gel was prepared according to the size of the proteins of interest being analysed and poured into a cassette to allow polymerisation to occur (Invitrogen, NC2015 or NC2010) (see ***Table 2.7*** for gel preparation). Subsequently, a 5% acrylamide staking gel was prepared, poured into the cassette and again allowed to polymerise. Gels were then placed in an XCell SureLock™ Mini-Cell Electrophoresis System (Invitrogen) and covered in Tris-Glycine running buffer (250µM Tris, 1.92mM Glycine and 0.1% SDS). Samples were loaded in adjacent wells alongside a protein standard (Bio-Rad, 161-0374), followed by electrophoresis carried out at 120V, 35mA for 90 minutes.

Proteins were then transferred from the gel onto a 0.45µm polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010) by placing both the membrane and the gels between transfer pads (VWR, 732-0594), which were soaked in transfer buffer (250µM Tris, 1.93mM Glycine). Transfer was carried out using the Trans-Blot® SD Semi-Dry Transfer Cells (BioRad) at 20 volts for 1 hour. Following transfer, a Ponceaux red solution (0.5% Ponceaux and 5% acetic acid in H_2O) was used to stain membranes and facilitate identification of protein bands.

The membrane was then blocked in blocking buffer (5% milk in 0.05% PBS-Tween) for 1 hour at room temperature with gentle shaking, followed by incubation with primary antibody (diluted in blocking buffer) overnight at 4°C with gentle shaking (see ***Table 2.8*** for primary antibodies used). Note that antibodies against phosphorylated proteins were diluted in 5% BSA in 0.05% PBS-Tween. Followed by three washes in distilled H_2O, membranes were incubated with secondary antibody diluted in blocking buffer for 1 hour at room temperature, again with gentle shaking (see ***Table 2.9*** for secondary antibodies). Membranes were washed three times with distilled H_2O, once in 0.05% PBS-Tween for 5 minutes, and then again washed in distilled H_2O (3-5 times) to remove excess Tween.

Membranes were incubated with chemiluminescence agent ClarityTM Western ECL substrate (Bio-Rad, 170-5060) for 5 minutes, and visualised using Fuji film Intelligent Dark box II and Image Reader Las-1000 Software. Each protein of interest was confirmed by comparing the size of each protein band to the protein standard loaded. Signal intensity of protein bands was analysed using ImageJ analysis software. Background subtraction was applied, and signal intensity of the protein of interest was normalised to a loading control.

1x Stacking Gel (5ml)	**5%**				
Sterile H_2O	3.4ml				
30% Acrylamide	850µl				
1.5M Tris (pH 6.8)	1.3ml				
10% SDS	50µl				
10% Ammonium persulphate (w/v in dH_2O)	50µl				
TEMED	4µl				
1x Running Gel (10ml)	**5%**	**8%**	**10%**	**12%**	**15%**
Protein Size (kDa)	**>250**	**250-120**	**120-40**	**20-15**	**<20**
Sterile H_2O	6.8ml	5.1ml	4ml	3.3ml	2.3ml
30% Acrylamide	1.7ml	2.6ml	3.3ml	4ml	5ml
1.5M Tris (pH 8.8)	2.5ml	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	100µl	100µl	100µl	100µl	100µl
10% Ammonium persulphate (w/v in dH_2O)	100µl	100µl	100µl	100µl	100µl
TEMED	8µl	4µl	4µl	4µl	4µl

Table 2.7 - Running and stacking gels for Western blotting.

Antibody	Species	Specificity	Dilution
Anti-Akt (Cell Signalling, 9272)	Rabbit polyclonal	Human Mouse	1:1000
Anti-p-Akt (S473) (Cell Signalling, 9271)	Rabbit polyclonal	Human Mouse	1:1000
Anti-Erk1/2 (p44/42 MAPK) (Cell Signalling, 9102)	Rabbit polyclonal	Human Mouse	1:1000
Anti-phospho ERK1/2 (p-44/42 MAPK) (Thr202/Tyr204) (Cell Signalling, 9101)	Rabbit polyclonal	Human Mouse	1:1000
Anti-GAPDH (Cell Signalling, 5174)	Rabbit monoclonal	Human Mouse	1:5000
Anti-Lamin B1 (Abcam, ab16048)	Rabbit polyclonal	Human Mouse	1:1000
Anti-p16 (BD Pharmingen, 550834)	Mouse monoclonal	Human	1:500
Anti-p21 (Cell Signalling, 2947)	Rabbit polyclonal	Human Mouse	1:1000
Anti-p70S6K (Cell Signalling, 9202)	Rabbit polyclonal	Human Mouse	1:1000
Anti-p70S6K (T389) (Cell Signalling, 9205)	Rabbit polyclonal	Human Mouse	1:1000
Anti-S6 (Ser235/236) (Cell Signalling, 2211)	Rabbit monoclonal	Human Mouse	1:1000
Anti-α-tubulin (Abcam, ab7291)	Mouse monoclonal	Human Mouse	1:2000
Anti-β-actin (Cell Signalling, 4970)	Rabbit monoclonal	Human Mouse Rat	1:1000

Table 2.8 – Primary antibodies used for Western blotting.

Antibody	Species	Specificity	Dilution
IgG-HRP conjugated (Sigma Aldrich, A0545)	Goat	Rabbit	1:5000
IgG-HRP conjugated (Sigma Aldrich, A2554)	Goat	Mouse	1:5000

Table 2.9 – Secondary antibodies used for Western blotting.

2.20. Statistical analysis

Data are expressed as the, mean ± S.E.M. In instances where data were normally distributed, significant differences between two groups were analysed using an independent samples two-tailed t-test, and multi-group comparison was assessed using a one- or two-way ANOVA. When a one-way ANOVA was used, a Tukey's multiple comparison post-hoc test was also performed. In some cases, a two-way ANOVA was also performed to analyse significance between groups and a Bonferroni post-hoc test was used, unless stated otherwise. If data were not normally distributed, statistical analysis was carried out using the Mann-Whitney U test. P values less than 0.05 were considered statistically significant. Data were analysed using the following softwares: GraphPad Prism version 5.0 and 7.0, GraphPad software, San Diego, California, USA, www.graphpad.com; IBM SPSS statistics version 19; and SigmaPlot version 12.5 from Systat Software, Inc., San Jose, California, USA, www.systasoftware.com.

Chapter 3: Melanocytes accumulate telomere-associated DNA damage during human skin ageing

Senescent cells have been shown to accumulate in human skin with age (Dimri *et al.*, 1995; Ressler *et al.*, 2006), and evidence suggests that accumulation of these cells contributes to age-associated skin changes by compromising regeneration and structure (Adamus *et al.*, 2014; Velarde *et al.*, 2015). However, the majority of studies so far have focused on dermal fibroblasts and keratinocytes, whilst the contribution of senescent melanocytes to the skin ageing process remains poorly understood. It has been demonstrated that p16-positive melanocytes accumulate in human skin with age, and this correlated with increased facial wrinkles, higher perceived age, and age-associated elastin morphology (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b), suggesting that senescent melanocytes might contribute to skin ageing characteristics. Furthermore, the role of telomere dysfunction in melanocyte senescence has also not been fully investigated. Although several studies have previously shown that telomere length decreases in human skin with age (Lindsey *et al.*, 1991; Butler *et al.*, 1998; Friedrich *et al.*, 2000; Nakamura *et al.*, 2002), very few reports have investigated whether telomeres trigger a DNA damage response in skin cells *in vivo*.

Therefore, the aims of this study were to investigate the expression of senescence markers in melanocytes in human skin with age, in particular the involvement of telomere dysfunction in this process. In addition, I also aimed to investigate whether senescent melanocytes could act in a cell non-autonomous manner, and impact surrounding cells in the epidermis *in vivo*.

3.1. Investigating the expression of senescence markers in melanocytes in human skin with age *in vivo*

In order to characterise melanocyte senescence *in vivo,* I performed immunofluorescence to analyse the expression of a range of senescence markers in skin biopsies from young (22.8 to 31.8 years old; mean age = 26.65) and older (55.6 to 67.5 years old; mean age = 61.6) human donors (***Table 3.1***). A marker for a melanocytic cell surface protein, known as melan-A, was also used to allow identification of each marker specifically in melanocytes. Firstly, in agreement with previous data (Gilchrest *et al.*, 1979), the number of melanocytes significantly decreases with age, as shown by an approximate 2-fold decrease in the number of melan-A-positive cells in the aged epidermis (P=0.002) (***Figure 3.1 A, D, F***).

The cyclin-dependent kinase inhibitor, p16, is an important cell-cycle regulator, and has been shown to be highly expressed in senescent cells both *in vitro* and *in vivo* (Serrano *et al.*, 1997; Lin *et al.*, 1998; Herbig *et al.*, 2006). Indeed, I found that there is a significant increase in the percentage of melanocytes positive for p16 in aged skin compared to younger donors (p=0.004) (***Figure 3.1 B***). Moreover, Sirtuin1 (SIRT1) is an NAD^+-dependent protein deacetylase, which regulates p53 function and silences the expression of genes such as NF-κB through its histone deacetylase activity (Vaziri *et al.*, 2001; Yeung *et al.*, 2004; Oberdoerffer *et al.*, 2008), and a decline in SIRT1 has been associated with ageing and senescence (Sasaki *et al.*, 2006; Imai and Guarente, 2014). Immunofluorescence analysis of SIRT1 revealed a significant decrease in SIRT1 expression in melanocytes from older donors *in vivo* (P<0.0001) (***Figure 3.1 C***). Furthermore, I analysed the expression of the proliferation marker, Ki-67, demonstrating that the average number of proliferating melanocytes is low in both young and older skin (***Figure 3.1 D***). Unexpectedly, I found that the number of Ki-67-positive melanocytes in aged epidermis is slightly higher when compared to younger skin; however, this difference is not statistically significant (P=0.23) (***Figure 3.1 D, E***).

Together, these results demonstrate that melanocytes express senescence markers with age, indicating that senescent melanocytes accumulate in the epidermis of aged human skin *in vivo.*

	Group	
	Young	**Older**
Age range	22.8 to 31.8	55.6 to 67.5
Age (mean ± SD)	26.7 ± 2.9	61.6 ± 3.2
Gender (M/F)	6/8	6/6

Table 3.1 – Age range and gender of young and older subjects involved in this study.

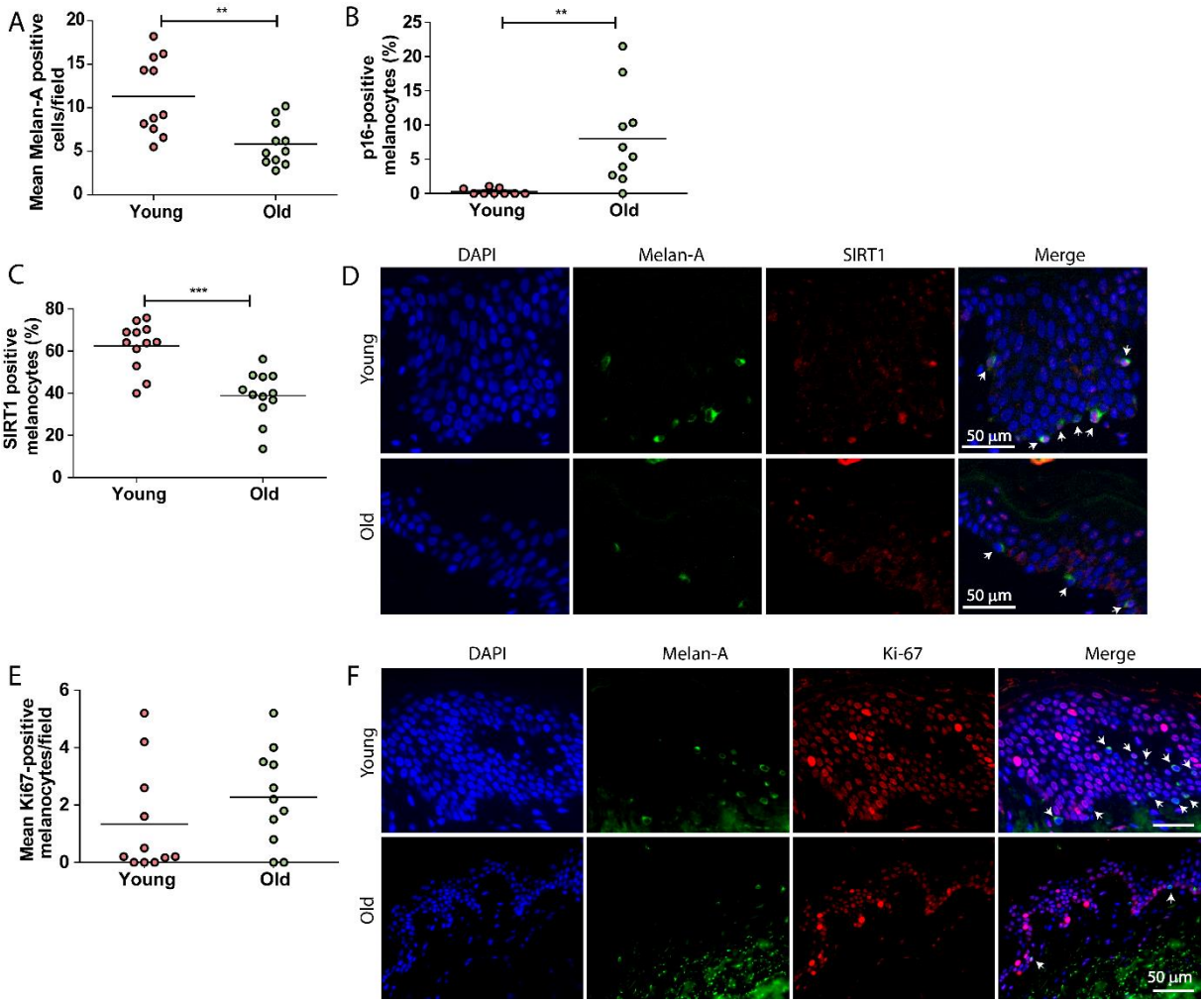

Figure 3.1 – Expression of senescence markers in melanocytes in human skin in vivo. Skin biopsies obtained from young and older human donors were stained by immunofluorescence to analyse the expression of different markers of senescence. A marker for the melanocytic protein, melan-A, was also used to identify melanocytes. Dot plots show **(A)** mean number of melan-A-positive cells per field, **(B)** percentage of p16-positive melanocytes, **(C)** percentage of SIRT1-positive melanocytes and **(E)** mean number Ki-67-positive melanocytes per field for each individual donor. The horizontal line represents the mean for each group. Representative images of **(D)** SIRT1 (red) and **(F)** Ki67 (red) immunofluorescence staining together with the melanocyte marker melan-A (green) in young and older skin. White arrows indicate melanocytes. SIRT1 and Ki-67 images were taken using a 40x and 20x objective, respectively. Statistical significance was determined using a two-tailed unpaired t-test, **P<0.01, ***P<0.001. (Analysis of p16 expression was done in collaboration with Jeff Pawlikowski and Peter Adams, and Ki-67 quantification was done in collaboration with Anthony Lagnado).

3.2. Telomeres become dysfunctional with age in melanocytes *in vivo*

In order to investigate whether telomere dysfunction is a feature of melanocyte senescence *in vivo*, I performed immuno-FISH combining immunofluorescence against γH2AX (a marker of DNA damage) and *in situ* hybridisation for telomeric specific PNA probe in skin biopsies from young and older donors. Firstly, to determine whether global DNA damage increases in melanocytes with age, I quantified the number of γH2AX foci, a well-established marker of DNA double stranded-breaks, in melanocytes. I found that there is a significant increase in the number of DNA damage foci in aged melanocytes when compared to those in younger skin ($P = 0.04$) (***Figure 3.2 A***). Next, in order to establish whether activation of a DDR at telomeres occurred with age, I analysed co-localisation of γH2AX with telomeres, which is also known as telomere-associated foci, or TAF. Melanocytes in aged skin have, on average, higher numbers of telomeres signalling a DNA damage response ($P<0.0001$) (***Figure 3.2 B, D***). I also found that a significantly higher percentage of melanocytes contain TAF in the skin of older donors ($P = 0.0002$), where 87% of aged melanocytes contain damaged telomeres compared to 53% of young pigment cells, suggesting that telomere dysfunction plays a role in melanocyte ageing *in vivo (**Figure 3.2 C**)*.

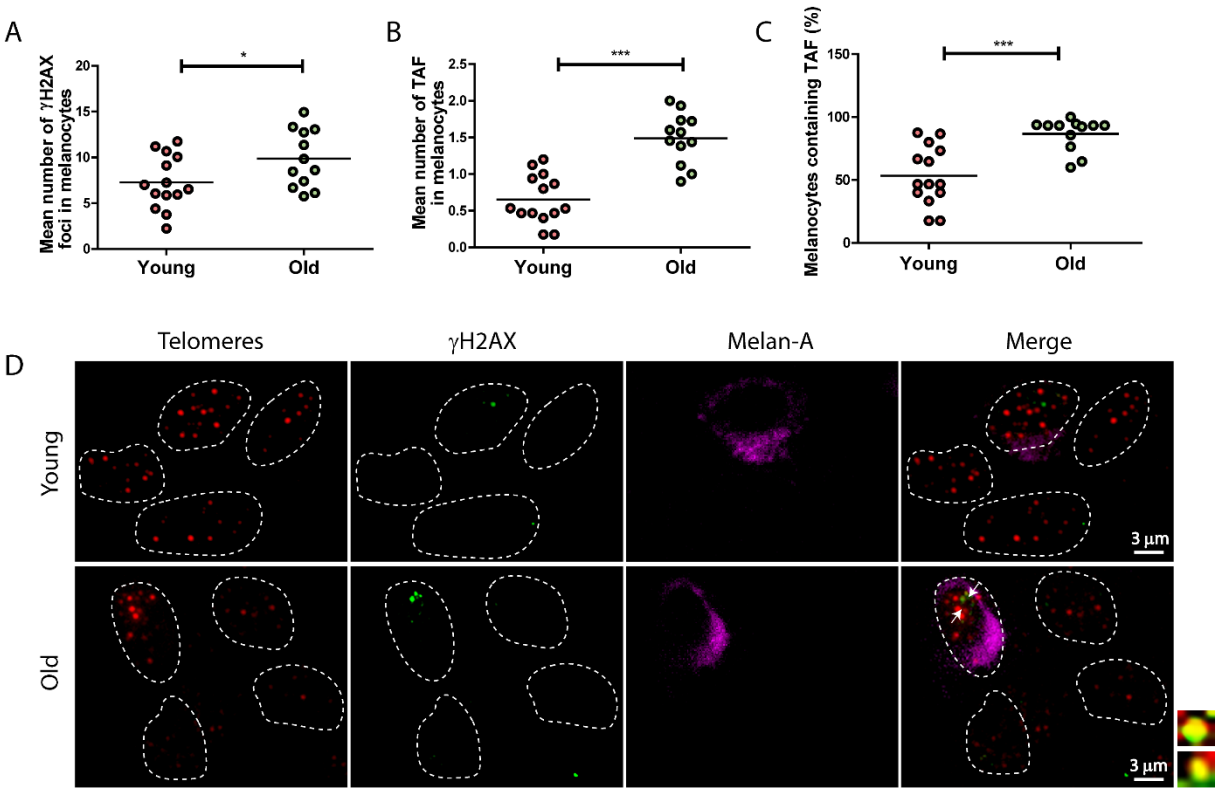

Figure 3.2 – Telomeres become dysfunctional in melanocytes in human skin with age. Immuno-FISH was performed in skin biopsies from young and older donors to analyse the DNA damage protein, γH2AX, and TAF. Dot plots show **(A)** mean number of γH2AX foci, **(B)** mean number of telomere-associated foci in melanocytes, and **(C)** the percentage of melanocytes containing TAF for each individual subject, which were obtained by analysing Z-stacks of each skin section. The horizontal line represents the mean of each group. Statistical significance was analysed by using a two-tailed unpaired t-test, *P<0.05, ***P<0.001 **(D)** Representative immuno-FISH image in melanocytes (pink) in young and older skin sections. Telomeres are shown in red, and γH2AX foci are shown in green. Co-localisation of the two is indicative of TAF, which are indicated by the arrows and amplified on the right. Images are Huygen (SVI) deconvolved Z projections and were taken using a 100x oil objective.

Since a DNA damage response can be activated at telomeres as result of extensive attrition (Takai *et al.*, 2003; Herbig *et al.*, 2004), and telomere shortening has been shown to occur with ageing in many tissues, including the skin (Lindsey *et al.*, 1991; Butler *et al.*, 1998; Friedrich *et al.*, 2000; Nakamura *et al.*, 2002), I wanted to determine whether telomere damage in melanocytes occurred as a consequence of shortening. By performing quantitative fluorescent *in situ* hybridisation (Q-FISH) in skin biopsies from human donors and analysing individual telomere signal intensity, I found no significant difference in median telomere length between melanocytes from young and older skin (P=0.382) (***Figure 3.3 A***), suggesting that these cells do not undergo significant telomere shortening with age *in vivo*. To further investigate whether there was a shift in the length of telomeres in melanocytes with age, I divided FISH signal intensities into tertiles, where the first tertile represents lower intensities, and quantified the percentage of telomeres within each tertile for each donor. As seen in ***Figure 3.3 B***, the majority of telomeres lie within the first tertile in both young and old donors, with no statistical significance between both groups in any tertile (P = 0.98), indicating that the range of telomere lengths remains the same in melanocytes with age. Moreover, I also analysed whether melanocytes of older donors contained more critically short telomeres by setting a threshold, such that anything below 10% of the highest FISH signal intensity was considered a very short telomere. Consistently, I did not find a significant difference in the percentage of very short telomeres in melanocytes in young and aged skin (P=0.52) (***Figure 3.3 C***), again demonstrating that there is no significant shortening with age. Interestingly, analysis of FISH intensity of individual telomeres revealed that telomeres co-localising with the DNA damage protein, γH2AX were significantly longer than γH2AX-negative ones (P<0.001) (***Figure 3.3 D, E, F***), suggesting that damage preferentially accumulates in longer telomeres in melanocytes *in vivo*.

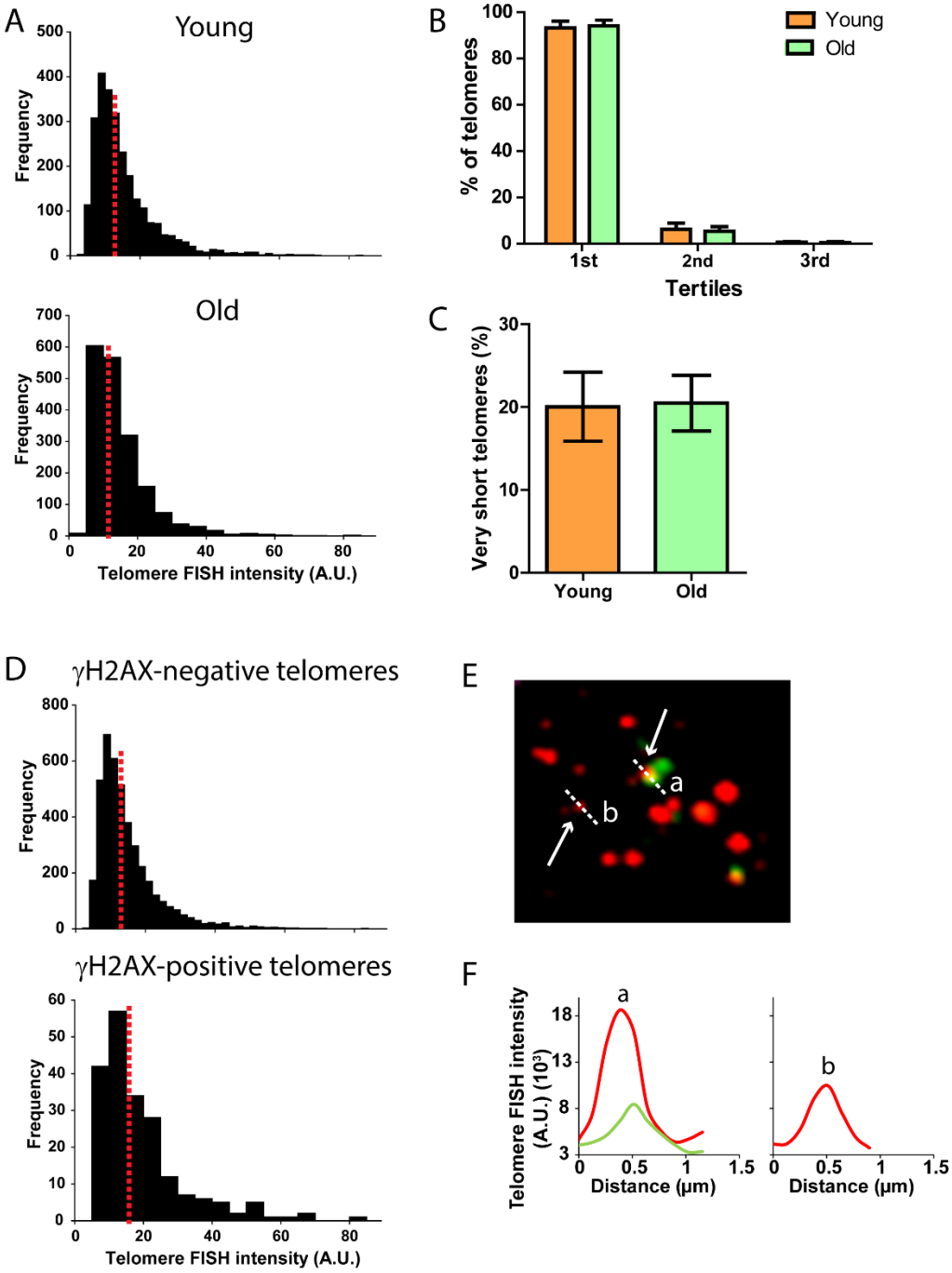

Figure 3.3 – Longer telomeres are preferentially damaged in melanocytes in vivo. Quantitative FISH was performed in skin biopsies from young and older human donors, and FISH intensity of individual telomeres were analysed as a measure of length. Histograms showing **(A)** telomere signal intensities in melanocytes from young and older skin sections, and **(D)** intensity of γH2AX-negative and γH2AX-positive telomeres in melanocytes of both young and older biopsies. Red dotted line indicates median intensity. Statistical significance was determined by using a Mann-Whitney U test. **(B)** Percentage of telomeres in tertiles of FISH signal intensity, where the first tertile represents the lowest intensities. Statistical significance

was determined by a two-tailed unpaired T-test. Data are mean ± S.E.M. of percentage of telomeres in each tertile per donor. **(C)** Percentage of very short telomeres in melanocytes of young and old skin sections. A two-tailed unpaired T-test was performed for statistical analysis. Data are mean ± S.E.M. of percentage of very short telomeres per donor. **(E)** Representative immuno-FISH image (red: telomeres; green: γH2AX) of a melanocyte in the skin of an older donor (top), showing a longer telomere co-localising with γH2AX (a) and a shorter one which is γH2AX-negative (b). Images are Huygen (SVI) deconvolved Z projections and were taken using a 100x oil objective. **(F)** Graphs showing the quantification of telomere intensity of γH2AX-positive (a) and γH2AX-negative (b) telomeres. Red line indicates telomere intensity, and green shows γH2AX intensity.

3.3. Investigating telomere dysfunction in melanocytes as a biomarker for age-related skin characteristics

Since I showed that telomere dysfunction increases with age in melanocytes in human skin *in vivo*, I wanted to determine whether TAF in melanocytes could be used as a predictor of parameters associated with skin ageing. For this part of this study, I used skin biopsies from 22 older donors, ranging from 54.9 to 76.8 years old (average age = 64.8 years). Amongst the morphometric skin characteristics available for these donors were epidermal thickness and curvature (provided by Unilever), two parameters which are known to be affected with age.

In agreement with previous reports (Marks, 1981; Lavker *et al.*, 1987; Grove, 1989; Farage *et al.*, 2007), I found that epidermal thickness significantly decreases with age (P=0.04) (***Figure 3.4 A, C***). Moreover, the curvature of the epidermis is also significantly lost with age (P=0.02) (***Figure 3.4 B***), giving the epidermal-dermal junction a flatter appearance, as shown in ***Figure 3.4 C***.

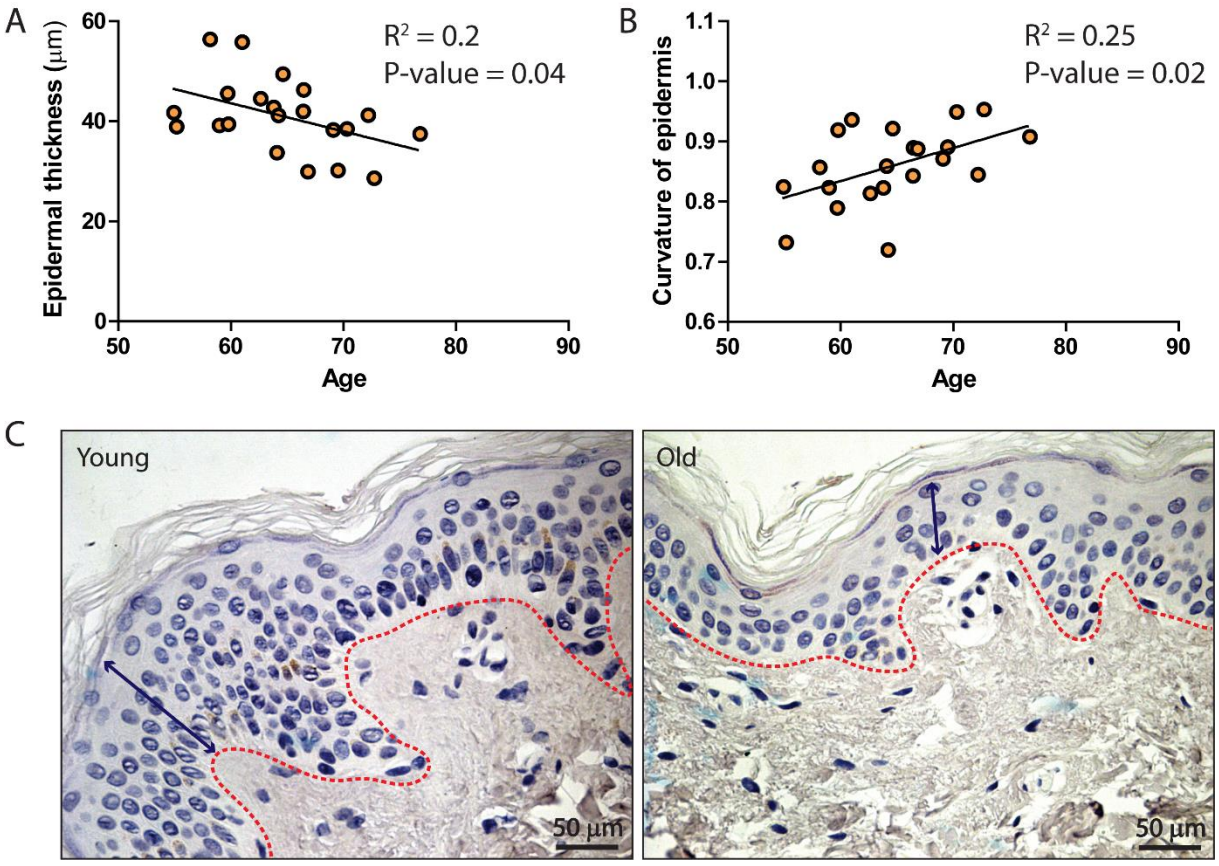

Figure 3.4 – Epidermal thickness and curvature decreases with age in older donor cohort. Graphs showing correlation between **(A)** epidermal thickness and age, **(B)** epidermal curvature and age for each individual donor. Note that a curvature value closer to 1 denotes a flatter EDJ. Correlation was determined using a Pearson's correlation test. **(C)** Images showing thinning of the epidermis (blue arrows) and the loss in epidermal curvature (red-dotted lines) with age. Red dotted line represents the epidermal-dermal junction.

3.3.1. TAF in melanocytes does not correlate with epidermal thinning in skin of older donors

To determine whether telomere dysfunction in melanocytes *in vivo* was a useful predictor of epidermal thinning in age skin, I performed immuno-FISH in skin biopsies of the older cohort of donors to analyse γH2AX co-localisation with telomeres. I found that the mean number of γH2AX foci in melanocytes did not correlate with the age-dependent decrease in epidermal thickness (P=0.6) (***Figure 3.5 A***). Moreover, although there is a slight tendency for epidermal thinning with increased TAF in melanocytes, this correlation was not statistically significant (P=0.4) (***Figure 3.5 B***). In addition, the percentage of melanocytes containing TAF in the epidermis of aged skin does not significantly correlate with decreased epidermal thickness (***Figure 3.5 C***), suggesting that telomere dysfunction in melanocytes does not successfully predict changes in epidermal thickness with age.

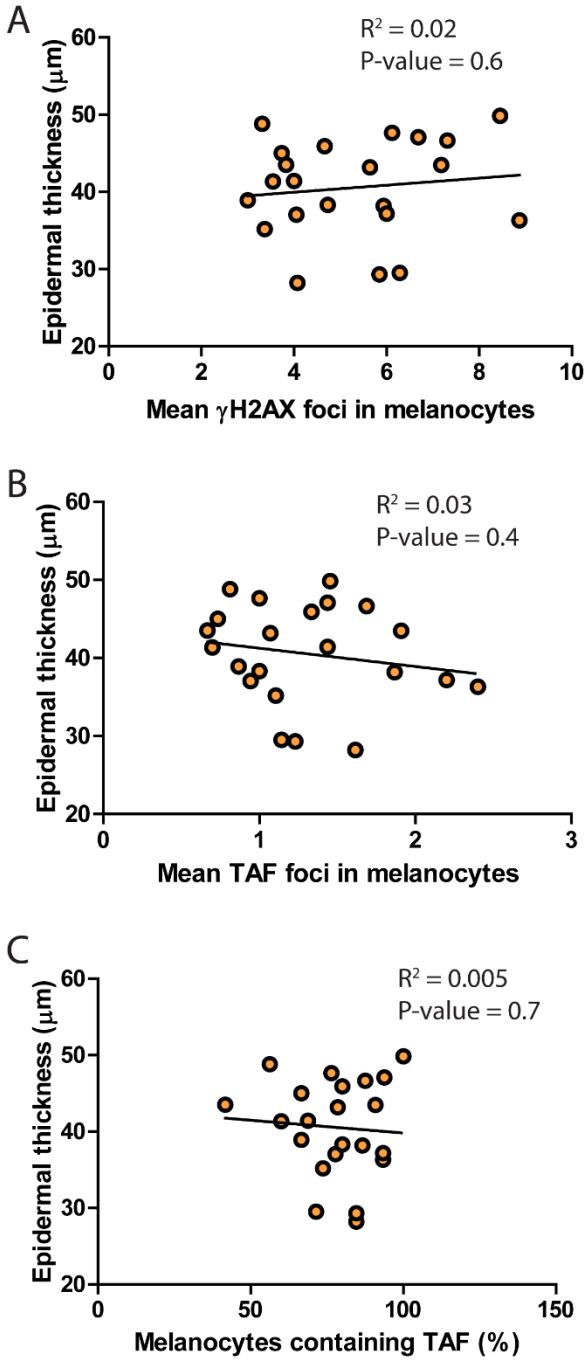

Figure 3.5 – Telomere dysfunction in melanocytes does not correlate with epidermal thinning. Immuno-FISH was performed in skin biopsies of 22 older donors. Graphs showing correlation between **(A)** the number of γH2AX foci, **(B)** number of TAF in melanocytes, and **(C)** percentage of melanocytes containing TAF, and epidermal thickness for each individual donor. Correlation was determined using a Pearson's correlation test. When accounting for age, the statistical significance of each correlation was **(A)** P=0.1, **(B)** P=0.35, and **(C)** P=0.23.

3.3.2. Telomere dysfunction in melanocytes correlates with loss of epidermal curvature in aged skin

Next, I found that the mean number of DNA damage foci in melanocytes positively correlates with flattening of the epidermis in the skin of older donors (P=0.02) (***Figure 3.6 A***). Moreover, loss of epidermal curvature positively correlates with the mean number of TAF in melanocytes (P=0.002) (***Figure 3.6 B***), and the percentage of melanocytes containing dysfunctional telomeres in the epidermis also shows a positive correlation with epidermal flattening in aged skin (P=0.0004) (***Figure 3.6 C***). These correlations remained statistically significant even when accounting for confounders such as subject age. In fact, the proportion of melanocytes containing TAF is the factor that correlates most strongly with the loss in epidermal curvature, suggesting that accumulation of TAF in melanocytes may be a potential marker of epidermal flattening in aged skin.

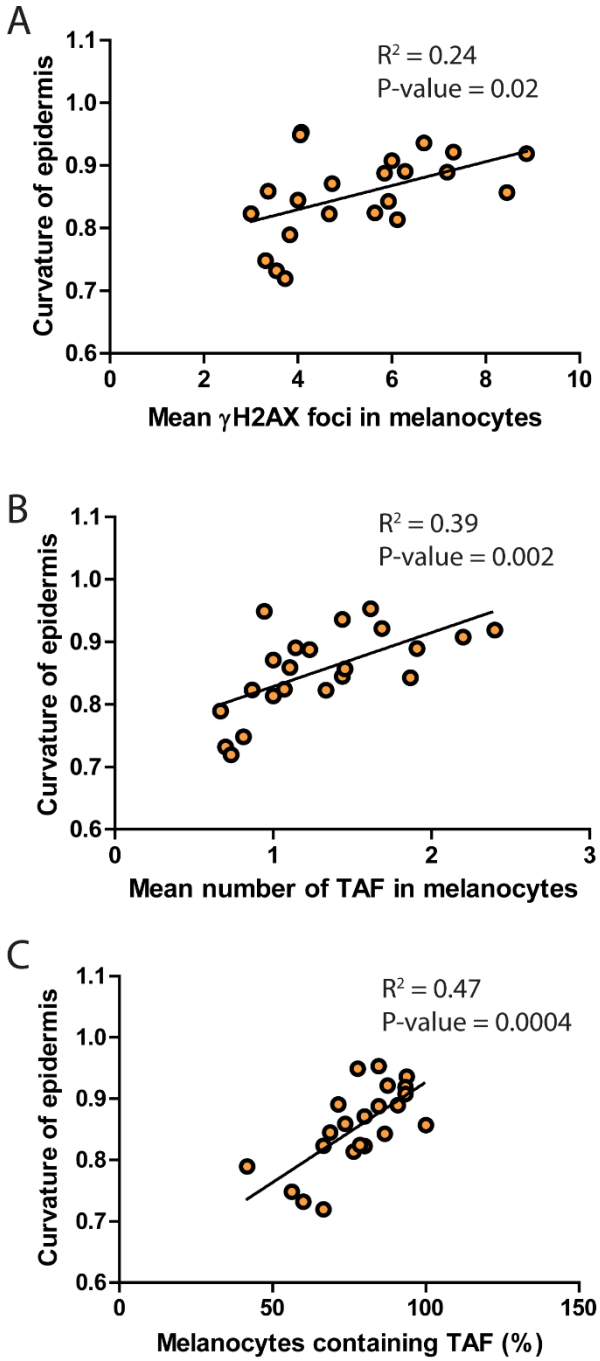

Figure 3.6 – TAF in melanocytes correlates with epidermal flattening in aged skin. Immuno-FISH was performed in skin biopsies of 22 older donors. Graphs showing correlations between **(A)** the number of γH2AX foci, **(B)** number of TAF in melanocytes, and **(C)** percentage of melanocytes containing TAF, and epidermal curvature for each individual donor. Correlation was determined using a Pearson's correlation test. When accounting for subject age, the statistical significance for these correlations was **(A)** P=0.004, **(B)** P=0.007, and **(C)** P=0.001.

3.4. Evidence for paracrine telomere damage induction by senescent melanocytes in human skin *in vivo*

It has been shown that senescent cells can affect their environment and induce senescence in neighbouring cells via the secretion of pro-inflammatory cytokines and ROS (Nelson *et al.*, 2012; Acosta *et al.*, 2013). Given this and the observation that telomere damage in melanocytes correlates with epidermal flattening, I wanted to investigate whether melanocytes containing dysfunctional telomeres (and are likely senescent) could induce paracrine damage in surrounding epidermal cells. Consequently, this could affect keratinocyte function and proliferation, and contribute to skin ageing phenotypes, such as epidermal flattening.

I allocated melanocytes containing 0 to 3 TAF into separate groups, and analysed the amount of telomeres signalling a DDR in keratinocytes located in the immediate vicinity of each melanocyte, as demonstrated in ***Figure 3.7 C***. In general, keratinocytes in the skin of older individuals have significantly more DNA damage foci when compared to cells in younger skin ($P<0.01$ and $P<0.001$ for cells around melanocytes with 0 and 1 TAF, respectively) (***Figure 3.7 A)***. Similarly, there is a significantly higher number of TAF in keratinocytes of older donors, suggesting that these cells also accumulate dysfunctional telomeres with age ($P<0.05$ for keratinocytes around melanocytes with 0 and 2 TAF, and $P<0.001$ for those around melanocytes with 1 TAF) (***Figure 3.7 B***).

However, although the mean number of DNA damage foci tends to be higher in keratinocytes surrounding melanocytes with more TAF in both young and older skin sections, this increase is not statistically significant within each group (***Figure 3.7 A***). On the other hand, I found that telomeric damage is significantly higher in epidermal cells neighbouring melanocytes with a higher number of dysfunctional telomeres in both young and older skin (***Figure 3.7 B***). In the skin of young donors, cells in the vicinity of melanocytes containing 2 TAF have on average a significantly higher number of telomeres signalling a DDR when compared to cells surrounding melanocytes with 0 or 1 TAF ($P<0.001$). Similarly, in older skin, melanocytes with 3 TAF appear to induce significantly more telomere damage in surrounding cells when compared to melanocytes with 1 or no TAF ($P=0.006$) (***Figure 3.7 C***). In fact, there is an approximate 3-fold increase in the amount of telomere-associated foci in cells in the vicinity of melanocytes containing 2 and 3 TAF (in young and old skin, respectively) in relation to those surrounding pigment cells without dysfunctional telomeres.

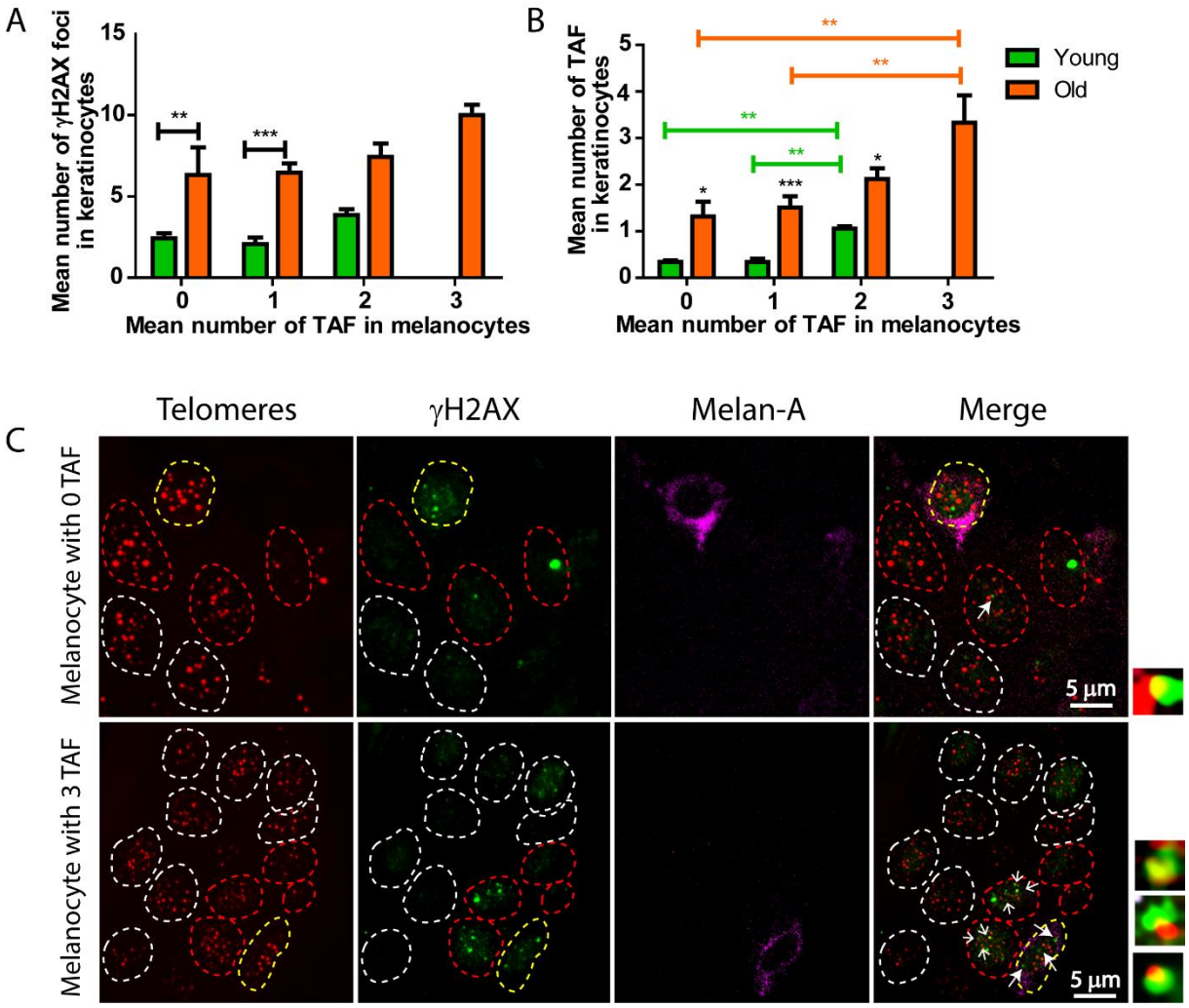

Figure 3.7 – Senescent melanocytes may induce paracrine TAF in neighbouring cells *in vivo*. (A) Mean number of γH2AX foci and **(B)** mean number of TAF in keratinocytes adjacent to melanocytes containing 0 to 3 TAF in young and older skin. Note that melanocytes with 3 TAF were not found in young skin sections. Data are mean ± SEM, where N=7 donors per group. At least 30 keratinocytes were counted per subject. Statistical significance was determined using a two-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. **(C)** Representative immuno-FISH images of keratinocytes around melanocytes with 0 TAF (top panel) and 3 TAF (bottom panel). Melanocytes are circled in yellow, and keratinocytes analysed are circled in red. White arrows represent co-localisation between γH2AX and telomeres, which are amplified on the right. Images are Z projections taken using a 100x oil objective.

In order to ensure that the differences in keratinocyte TAF were not due to subject-to-subject variability, we carried out an ordinal logistic model accounting for subject, and found that the increase in TAF in keratinocytes neighbouring melanocytes with more dysfunctional telomeres was still highly significant (P<0.0001) (***Figure 3.8 A***). Moreover, we separated the number of TAF in keratinocytes into tertiles, such that 1 indicates low numbers, 2 denotes medium and 3 high numbers of TAF, and showed that increased number of TAF in keratinocytes is significantly associated with higher frequency of telomere damage in melanocytes (P<0.0001) (***Figure 3.8 B***).

Together, these results suggest that melanocytes with more TAF, and likely senescent, may exert a bystander effect *in vivo*, inducing paracrine telomeric damage in neighbouring epidermal cells, and possibly contributing to skin ageing characteristics.

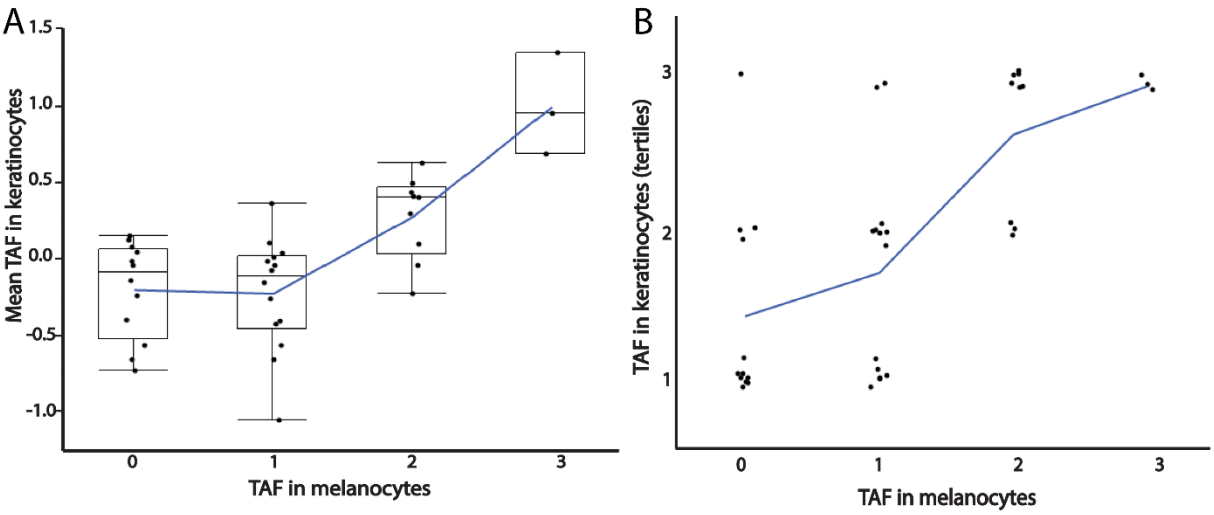

Figure 3.8 – Paracrine damage induction by melanocytes accounting for subject variability. (A) Mean number of TAF in keratinocytes around melanocytes containing 0 to 3 TAF accounting for subject. The horizontal line in each box indicates the median value. **(B)** Mean number of TAF in keratinocytes were divided into tertiles, where 1 = low, 2 = intermediate, and 3 = high numbers of TAF. Each point represents the average number of keratinocyte TAF for the corresponding category (i.e. number of melanocyte TAF) per subject. The blue line in both graphs joins the mean values for each category in the x-axis. Statistical significance was determined by an effect likelihood ratio test.

3.5. Discussion

In this study, we observed that the number of melanocytes decreases by around 2-fold in the epidermis of older human skin. This is in line with previous studies, which have shown that the number of melanocytes decreases by 10-20% every decade after the age of 30 years (Whiteman *et al.*, 1999). This has been suggested to be a result of increased apoptosis that occurs due to ROS accumulation and decreased levels of the anti-apoptotic protein, Bcl-2, in aged melanocytes, which contributes to melanocyte loss with age (Sermadiras *et al.*, 1997; Kauser *et al.*, 2011; Cichorek *et al.*, 2013).

Although there has been a number of studies reporting the accumulation of senescent cells in the skin with age, very few have investigated the expression of senescence-associated markers specifically in melanocytes in human skin. We have shown that p16 expression is significantly increased in melanocytes in the epidermis of older donors, which is in agreement with other reports showing an accumulation of p16-positive melanocytes in older human skin (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b). Furthermore, we report for the first time that SIRT1 expression is downregulated in melanocytes with age *in vivo*. Although activation of SIRT1 by resveratrol treatment was shown to prevent UVB-induced senescence of dermal fibroblasts in mouse skin (Chung *et al.*, 2015), no previous reports have demonstrated an age-dependent association between this histone deacetylase and melanocytes in human skin *in vivo*. Indeed, loss of SIRT1 has been associated with senescence in human and mouse fibroblasts *in vitro* (Sasaki et al., 2006), and inhibition of SIRT1 was shown to induce senescence in human melanoma cells (Ohanna *et al.*, 2014). SIRT1 modulates p53 deacetylation, inhibiting its transcriptional activity, and thus plays a role in controlling cell-cycle progression (Vaziri *et al.*, 2001). Additionally, SIRT1 has been shown to deacetylate histones in promoter regions of genes such as IL-6 and IL-8, repressing the expression of SASP components (Hayakawa *et al.*, 2015). Since a stable proliferation arrest is a hallmark of senescence, we also analysed expression of Ki-67; however, we did not find a significant difference in the number of proliferating melanocytes in young and old skin. Although there was a slight increase in Ki-67-positive melanocytes in skin sections from older donors, this difference was not statistically significant. This is in line with previous work, which failed to detect differences in the proportion of Ki-67-positive cells in human epidermis with age (Ressler *et al.*, 2006). Importantly, the number of Ki-67-positive melanocytes were very low in both groups, supporting the observation that differentiated melanocytes have low proliferative capacity *in vivo* (Jimbow et al., 1975). Taken together, these data show an age-dependent increase in

senescence markers in melanocytes, indicating that senescent melanocytes accumulate in the epidermis of aged human skin *in vivo.*

The association of telomeres with DDR proteins in melanocytes in human skin has been poorly explored, with only one study showing that telomere dysfunction occurs in melanocytic nevi, which are cancer precursor lesions (Suram *et al.*, 2012). Here, we show for the first time that the frequency of damaged telomeres is significantly higher in melanocytes in aged skin, suggesting a role for telomere dysfunction in melanocyte senescence *in vivo*. Although several reports have shown that telomere length decreases with age in human skin (Lindsey *et al.*, 1991; Butler *et al.*, 1998; Friedrich *et al.*, 2000; Nakamura *et al.*, 2002), we found no significant difference in telomere length between melanocytes in young and aged skin, suggesting that damage does not occur primarily as a result of telomere shortening in melanocytes *in vivo*. The absence of significant telomere shortening might be explained by the fact that these cells rarely divide *in vivo*, as shown by aforementioned studies and also corroborated by our data showing low levels of Ki-67 positivity in melanocytes of both young and older skin. Interestingly, our data suggests that longer telomeres preferentially accumulate damage in melanocytes in human skin, shown by the significant increase in median signal intensity of γ-H2AX-positive telomeres as determined by Q-FISH. The accumulation of DDR proteins in telomeres that are not critically short has been previously reported in non-dividing cells, such as hippocampal neurons and liver hepatocytes of old baboons (Fumagalli *et al.*, 2012), and in mouse hepatocytes (Hewitt *et al.*, 2012). In fact, it has been proposed that long telomeres may provide a more abundant target for DNA damage to occur (Fumagalli *et al.*, 2012). Consistent with this, human cancer cells with very long telomeres following enforced elongation were more sensitive to ionising irradiation, suggesting that telomeres above a critical length are more likely to accumulate DSBs (Fairlie and Harrington, 2015). However, we cannot completely rule out the contribution of short telomeres in DDR activation in melanocyte senescence *in vivo*. It is possible that Q-FISH is not sensitive enough to detect very short telomeres, resulting in a biased enrichment of DNA damage proteins at longer telomeres. Another possibility is that intense FISH signals might represent multiple dysfunctional telomeres aggregating within one DDR focus, as it has been shown to occur in human fibroblasts *in vitro* (Kaul et al., 2011), and these cannot be distinguished by the microscopy methods used in this study. Therefore, more sensitive methods such as super resolution microscopy could be used in order to confirm these results. Moreover, in order to determine whether telomere fusions occur in melanocyte senescence, immuno-FISH

against γH2AX and telomeres could be performed in melanocyte metaphase spreads, allowing visualisation of individual chromosome ends.

Another mechanism by which telomeres can activate a DNA damage response is due to loss of shelterin components, or uncapping, with ageing. It is thought that extensive telomere attrition leads to displacement of shelterin proteins, exposing chromosome ends which are then recognised as DSBs by the DNA repair machinery (O'Sullivan and Karlseder, 2010). Accordingly, senescent human endothelial cells *in vitro* have shorter telomeres and decreased levels of shelterin proteins, such as TIN2, POT1, TRF2 and TRF1, whereas these were maintained in high passage telomerase-immortalised cells (Hohensinner *et al.*, 2016). Furthermore, expressing a dominant-negative TRF2 allele in human fibroblasts triggers uncapping and leads to activation of a DDR at telomeres, supporting the idea that uncapped telomeres resemble DNA lesions (Takai *et al.*, 2003). Since our data suggests that telomeres do not shorten significantly in senescent melanocytes *in vivo*, it is unlikely that telomere deprotection would occur as a result of extensive loss of telomeric repeats. However, it has been shown that oxidative damage at telomeres disrupts the binding of TRF1 and TRF2 (Opresko *et al.*, 2005), possibly providing a length-independent mechanism for loss of shelterin components. One possibility is that oxidative stress is increased during melanocyte senescence *in vivo*, and this in turn displaces shelterin proteins, leading to activation of a DDR at telomeres with age. Indeed, ROS has been implicated as both an initiator and effector of the senescent phenotype (Macip *et al.*, 2002; Saretzki *et al.*, 2003; Passos *et al.*, 2007; Passos *et al.*, 2010), and enhanced ROS generation is involved in oncogene-induced melanocyte senescence (Kaplon *et al.*, 2013; Leikam *et al.*, 2014). Nonetheless, other studies have provided evidence that TRF2 was still retained at a fraction of telomeres signalling a DDR in stress-induced (Fumagalli *et al.*, 2012), replicative senescence (Kaul *et al.*, 2011), and in melanocytic nevi, which are a model of OIS (Suram *et al.*, 2012), suggesting that loss of shelterin proteins does not necessarily precede DDR activation at telomeres. In order to further investigate whether telomere dysfunction occurs as a result of uncapping in melanocyte senescence *in vivo*, telomere FISH should be performed together with immunofluorescence against γH2AX, a shelterin protein such as TRF2, and a melanocyte marker, allowing us to observe co-localisation between γH2AX-positive telomeres and TRF2 in human skin.

Increasing evidence now suggests that persistent DDR signalling at telomeres plays an important role in the establishment of senescence. It has been shown that DNA damage at telomeres is less efficiently repaired compared to non-telomeric damage due to the presence of

shelterin proteins, which inhibit components of the DNA repair machinery (Kruk *et al.*, 1995; Bombarde *et al.*, 2010; Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Consistently, live-cell imaging studies have demonstrated that the majority of long-lived DNA damage foci co-localise with telomeres in stress-induced senescence (Hewitt *et al.*, 2012). Such irreparable DNA damage generates persistent DDR signalling at telomeres, which is believed to be important for the initiation and maintenance of the senescent state (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Indeed, telomere dysfunction accumulates in human fibroblasts during replicative (Kaul *et al.*, 2011), stress- (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012), and oncogene-induced senescence (Suram *et al.*, 2012), and was shown to occur independently of length in all cases. Length-independent telomere damage has also been reported in many tissues in mice and primates with age (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012; Jurk *et al.*, 2014), suggesting that telomere dysfunction may also contribute to age-related decline in tissue function. Given these data, it is possible that the observed age-associated increase in telomere-associated damage in melanocytes contributes to melanocyte senescence in human skin *in vivo*. However, in order to confirm this, immunofluorescence staining against p16, γH2AX, and a melanocyte marker together with FISH for telomeres need to be performed; this would then allow us to determine whether p16-positive melanocytes contain more TAF than p16-negative ones. Although we also observed an increase in the number of γH2AX foci in aged melanocytes, the increase in both number of TAF and the percentage of melanocytes containing TAF in older skin is more highly significant, suggesting that TAF may serve as a more robust marker of melanocyte senescence *in vivo*.

Skin ageing is accompanied by many morphological changes, including epidermal thinning and loss of epidermal curvature (Waaijer *et al.*, 2012a). In accordance to this, we observed a significant decrease in epidermal thickness and loss in epidermal curvature with age in the older donor cohort. Since accumulation of senescent cells has been shown to contribute to tissue dysfunction with age (Baker *et al.*, 2011; Baker *et al.*, 2016), we investigated whether increased TAF in melanocytes (used as a proxy for senescence) was associated with age-related skin characteristics. In this study, we did not find a significant correlation between TAF in melanocytes and epidermal thinning, although there is a trend for decreased epidermal thickness with increased number of TAF. It is possible that our cohort was too small to detect any significance, and thus this should be confirmed using a larger number of subjects. However, this finding is in agreement with previously published data showing that p16-positivity in the epidermis, which arises mainly from melanocytes, does not associate with age-related

epidermal morphology (Waaijer *et al.*, 2016b). In contrast, we observed a highly significant association between increased TAF in melanocytes and flattening of the epidermal-dermal junction, suggesting that accumulation of melanocytes with dysfunctional telomeres, which are likely senescent, contributes to this skin ageing phenotype. Flattening of the EDJ occurs primarily as a result of the retraction of the rete ridges, which consist of epidermal invaginations into the dermis (indicated by the red dotted line in ***Figure 3.4 C***). Physiologically, a flattened dermal-epidermal interface means that the epidermis is less resistant to shearing forces, since these projections not only increase the surface area to maximise nutrient exchange across the junction but they also provide mechanical support to the epidermis (Briggaman and Wheeler, 1975; Lavker, 1979). Flattening of the epidermal-dermal junction reduces the exchange surface between the epidermis and dermis, and thus compromises nutrient supply to the epidermis. This has been suggested to contribute to reduced keratinocyte proliferation with ageing (Lavker *et al.*, 1989). Furthermore, it has been shown that epidermal stem cells reside within rete ridges (Ghazizadeh and Taichman, 2005), therefore, retraction of these structures might also compromise epidermal turnover with age by affecting keratinocyte differentiation and proliferation. Although it is not known whether flattening of the EDJ is a direct consequence of senescent melanocyte accumulation, our results implicate TAF as a potential marker for epidermal flattening that occurs during skin ageing. Interestingly, accumulation of p16-positive melanocytes has been associated with increased facial wrinkling, higher perceived age, and age-associated elastin morphology, suggesting that senescent melanocytes are a robust marker of global skin ageing (Waaijer *et al.*, 2016b).

Senescent cells have been shown to induce paracrine DNA damage and senescence in healthy neighbouring cells via mechanisms involving secretion of SASP factors and increased ROS (Nelson *et al.*, 2012; Acosta *et al.*, 2013). In agreement with this, our data showed that keratinocytes surrounding melanocytes with more TAF, a marker of senescence, also had significantly more telomere damage, providing evidence for a possible bystander effect of senescent melanocytes in human skin *in vivo*. It is possible that senescent melanocytes affect neighbouring keratinocytes by the secretion of pro-inflammatory factors, similarly to that reported in senescent fibroblasts (Hubackova *et al.*, 2012; Acosta *et al.*, 2013). It has been proposed that molecules secreted by senescent cells, such as IL-1 and TGF-β family ligands, stimulate the expression of Nox4, an NADPH oxidase that contributes to ROS generation, in neighbouring cells. Accumulation of ROS can then induce oxidative DNA damage in these cells, activating a DDR that contributes to the establishment of senescence (Hubackova *et al.*,

2012). Another study has provided evidence that senescent cells can also activate a DDR in neighbouring cells through gap junction-mediated cell-cell contact, where ROS generation in bystander cells was shown to be a major contributor to damage induction (Nelson *et al.*, 2012). In fact, ROS has been implicated in a positive feedback loop that replenishes DNA damage foci, stabilising the senescence growth arrest through continuous activation of p21 (Passos *et al.*, 2010). Although the mechanisms involved in paracrine damage induction in the context of melanocyte senescence will be further investigated in the following chapters, increased ROS generation has been shown to contribute to skin ageing phenotypes *in vivo*. Deletion of Sod2, a mitochondrial antioxidant enzyme, increases mitochondrial oxidative stress, and was shown to induce DNA damage and cellular senescence in mouse skin. Moreover, Sod2 deficiency also resulted in skin ageing phenotypes, such as epidermal thinning (Velarde *et al.*, 2012). Consistently, inducing mitochondrial oxidative stress specifically in keratinocytes by Sod2 deletion delayed wound closure and accelerated epidermal thinning in old mice, phenotypes associated with depletion of epidermal stem cells (Velarde *et al.*, 2015). This was proposed to be due to senescence of rapidly dividing transit-amplifying (TA) cells, which arise from stem cells, and are required for epidermal expansion. It is though that mitochondrial oxidative stress induces senescence of TA cells, which in turn increases the demand for stem cells, leading to exhaustion of the stem cell pool with ageing, and consequently causing epidermal thinning and delayed wound healing (Velarde *et al.*, 2015). Therefore, oxidative stress appears to be a major driver of keratinocyte senescence and a contributor to skin ageing characteristics.

Studies suggest that senescence can also be transmitted to surrounding cells *in vivo*. For example, clusters of senescent hepatocytes have been observed in mouse liver with age (Nelson *et al.*, 2012), and in a mouse model of chronic inflammation, which shows accelerated ageing phenotypes (Jurk *et al.*, 2014). Further evidence was shown by studies in mouse models of OIS, where inducing OIS in hepatocytes *in vivo* resulted in accumulation of immune cells around senescent hepatocytes which also displayed markers of senescence, such as p21 and p16 (Acosta *et al.*, 2013). Moreover, p21-positive stromal cells were observed in the vicinity of human colon sessile serrated adenoma (SSAs), pre-malignant lesions which arise from BRAF mutations that trigger OIS (Acosta *et al.*, 2013). These results support a role for a bystander effect of senescent cells in mice and human *in vivo*. Although we show evidence for paracrine damage induction by senescent melanocytes *in vivo*, immunohistochemical staining for SASP factors will need to be done in order to elucidate the involvement of the SASP in this process in human skin. Moreover, markers of oxidative DNA damage, such as 8-oxo-dG, could shed

light on the accumulation of oxidative DNA lesions in keratinocytes *in vivo*. However, studies involving genetic manipulation of antioxidant enzymes in mice specifically in keratinocytes are needed to establish the role of oxidative stress in paracrine damage induction.

Although we did not investigate the consequences of telomere damage accumulation in keratinocytes, it has been shown that telomere dysfunction induces senescence in human keratinocytes in culture (Minty *et al.*, 2008). Interestingly, senescent keratinocytes display high macro-autophagic activity that targets components such as the nucleus and mitochondria, resulting in cell death *in vitro* (Gosselin *et al.*, 2009). It is possible that paracrine telomere damage initially induces senescence in keratinocytes but this later results in cell death, probably explaining the low levels of p16-positive epidermal cells (around 8%) previously reported in aged human skin (Ressler *et al.*, 2006; Nassour *et al.*, 2016). In fact, these cells are mainly located in the basal layer of the epidermis, and have been shown to co-localise with melanocytes (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b), suggesting that melanocytes are the major senescent cell population in the epidermis of human skin. In order to further investigate this, other senescence markers, such as p21, have to be analysed in our donor cohort to confirm the seldom accumulation of senescent keratinocytes in human skin with age. This would suggest that the age-associated changes in the epidermal compartment are, in part, due to death of senescent keratinocytes rather than their accumulation. Since epidermal regeneration and homeostasis is highly dependent on the capacity of stem/ progenitor cells to proliferate and repopulate the tissue, it will also be important to consider the impact of senescent melanocyte bystander effects on epidermal stem cells, a subpopulation of which are located in the basal layer of the epidermis (Zouboulis *et al.*, 2008), similarly to melanocytes. Indeed, it has been recently shown that increased secretion of inflammatory factors in the epidermis of aged mice contributes to hair follicle stem cell function decline with age (Doles *et al.*, 2012).

Furthermore, from this study, we cannot exclude the possibility that senescent keratinocytes might also affect melanocytes in a paracrine manner. In fact, an intricate network of paracrine signalling exists between melanocytes and keratinocytes, where the latter secretes pro-inflammatory cytokines and growth factors in response to different stimuli that control growth and survival of melanocytes (Wang *et al.*, 2016). However, the high turnover rate of keratinocytes in the epidermis, as well as their propensity to undergo cell death when becoming senescent, makes the possibility of these cells having long-term detrimental effects on melanocytes less likely.

In summary, we have shown that senescent melanocytes accumulate in human skin with age, and that telomere dysfunction independently of shortening is involved in this process. We have also provided evidence that senescent melanocytes might induce paracrine telomere damage in surrounding keratinocytes, which can contribute to skin ageing phenotypes by affecting keratinocyte proliferation and function, although further experiments are necessary confirm this hypothesis. Moreover, our data also suggests that TAF in melanocytes is a potential biomarker of epidermal-dermal flattening, a characteristic of skin ageing.

Chapter 4: Characterisation of *in vitro* models of melanocyte senescence

Melanocytes are neural crest-derived cells located in the basal layer of the epidermis, and are responsible for synthesising melanin in response to solar ultraviolet light (Bandyopadhyay *et al.*, 2001). Unlike fibroblasts and other cell types, melanocyte senescence *in vitro* has been reported to be mainly dependent on the p16/pRb pathway, whereas p21 expression was decreased as these cells reached their proliferative capacity (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001). Moreover, overexpression of the catalytic subunit of telomerase (hTERT) considerably extended the lifespan of melanocytes in culture, suggesting that telomere attrition plays a role in melanocyte senescence (Bandyopadhyay *et al.*, 2001). However, the majority of studies involving melanocyte senescence *in vitro* focus on replicative and oncogene-induced senescence (Bennett and Medrano, 2002; Bansal and Nikiforov, 2010), whereas stress-induced senescence remains relatively understudied.

Previous work from our group and others demonstrated that stress-induced senescence involved the activation of an unresolved DDR at telomeres irrespectively of length (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). This was shown in human lung fibroblast lines such as IMR90 (Fumagalli *et al.*, 2012) and MRC5 (Hewitt *et al.*, 2012), however not in melanocytes. Given the fact that we observed that melanocytes acquire TAF irrespectively of length *in vivo* (chapter 3), I wanted to investigate whether exposure to DNA damaging agents, such as X-ray and UV irradiation, *in vitro* would contribute to persistent damage at telomeres, and consequently, senescence. Moreover, in this study, I also aimed to investigate whether telomere-specific damage was irreparable in melanocytes and contributed to the establishment of senescence. To this end, I transduced melanocytes with an endonuclease which causes telomere-specific breaks. Finally, in order to investigate the mechanisms mediating bystander DNA damage induction by senescent melanocytes, which will be further explored in chapter 5, I aimed to establish and characterise different models of melanocyte senescence *in vitro*, including the molecular profile of their secretory phenotype.

4.1. Replicative senescence

4.1.1. Markers of senescence increase as melanocytes reach their proliferative capacity

The first model investigated in this study was replicative senescence, where melanocytes were serially passaged until they reached the end of their proliferative capacity. Lightly pigmented neonatal melanocytes were cultured for 180 days, during which time they performed around 16 population doublings before withdrawing from the cell cycle (***Figure 4.1 A***). Indeed, at this PD, there is a 5-fold decrease in the percentage of proliferating melanocytes, shown by a reduction in Ki-67 expression (***Figure 4.1 C, D***). Consistent with previous reports (Bandyopadhyay and Medrano, 2000), only around 19% of melanocytes are positive for Ki-67 at early passages, reflecting their slow proliferation rate. This is also highlighted when comparing proliferation rates of human melanocytes and MRC5 human lung fibroblasts (***Figure 4.1 B***). The latter undergo 46 PDs and reach the Hayflick limit at approximately 120 days in culture. In fact, MRC5 fibroblasts undergo, on average, 0.35 population doublings/day compared to 0.12 PDs/day observed for human melanocytes. Therefore, the latter replicate approximately 3 times slower than MRC5 fibroblasts, reiterating their slow proliferative rate. Moreover, Sen-β-Gal activity, which is a commonly used senescence marker, was also increased in late passage melanocytes, indicating that these cells have reached senescence (***Figure 4.1 E, F***).

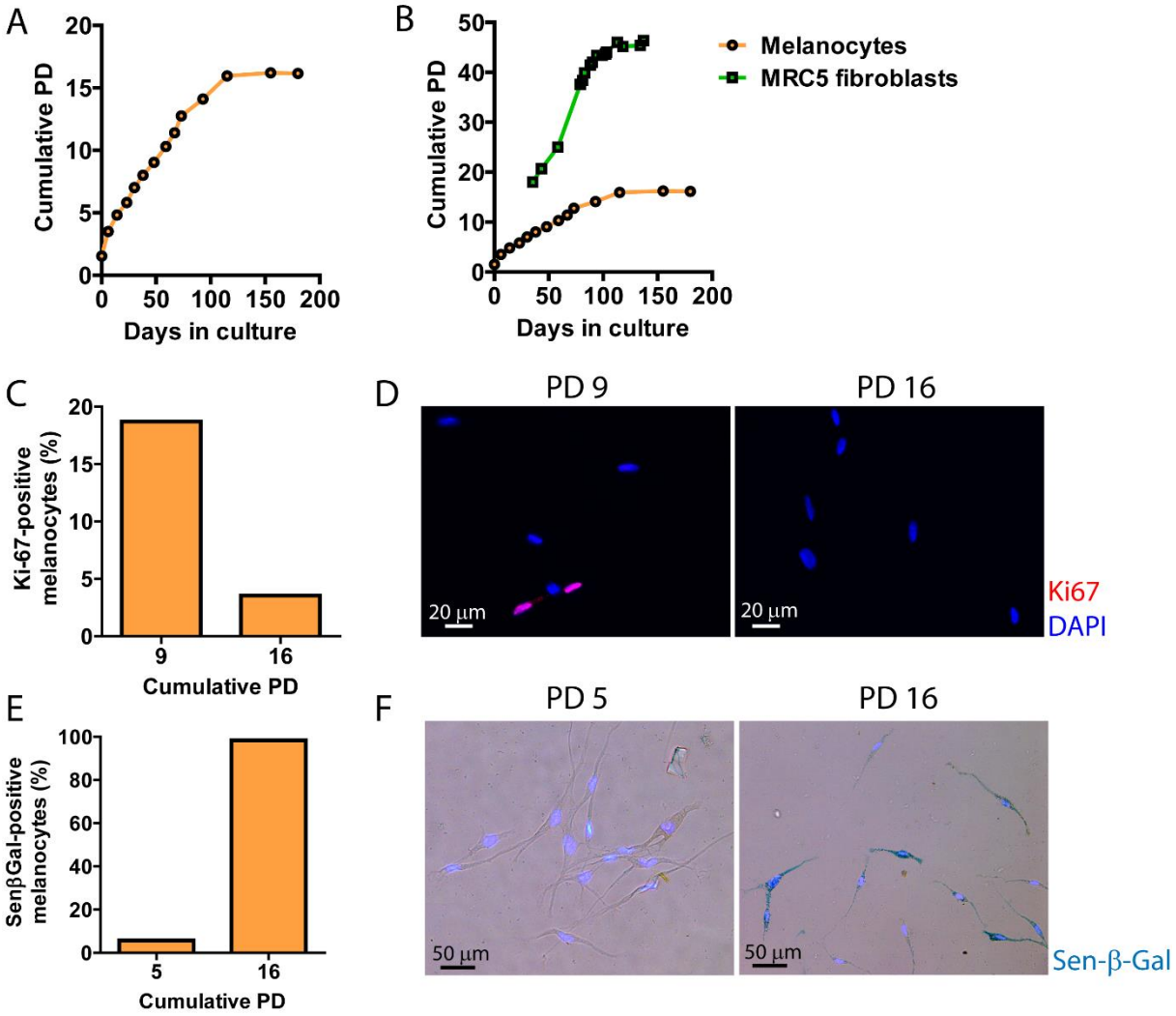

Figure 4.1 – Melanocytes reach replicative senescence in culture after 16 population doublings. (A) Growth curve of melanocytes serially passaged until they reached replicative senescence. **(B)** Graph comparing growth curves of normal human melanocytes (orange) and MRC5 human lung fibroblasts (green). **(C)** Percentage of Ki-67-positive melanocytes at early and later passages. Data are mean of at least 10 random planes, and a minimum of 65 cells per group (N=1). **(D)** Representative Ki-67 immunocytochemistry images for early and late passage melanocytes in vitro. Ki-67 is shown in red, and DAPI is shown in blue. Images were taken using a 40x objective. **(E)** Percentage of Sen-β-Gal positive melanocytes at early and late passages. Data are mean of 10 random planes, with 217 cells (PD 5) and 141 cells (PD 16) (N=1). **(F)** Representative Sen-β-Gal (blue) images for early and late passage melanocytes. Images were taken using a 20x objective.

To further characterise the phenotype of replicative senescent melanocytes, I analysed the expression of CDK inhibitors commonly up-regulated during senescence. Since previous studies have suggested that melanocyte senescence is mainly regulated by the p16-pRb pathway, (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001), I analysed the expression of p16 by quantitative PCR, and found a 2-fold increase in p16 mRNA levels in senescent melanocytes (***Figure 4.2 A***). Increased p16 expression was also confirmed at the protein level by Western blotting analysis (***Figure 4.2 B***), corroborating the involvement of p16 during the growth arrest. In contrast to previously published data, the levels of p21 were also significantly up-regulated in senescent melanocytes, as demonstrated by Western blotting (***Figure 4.2 B***) and corroborated by immunocytochemistry, which showed an increase in the percentage of p21-positive melanocytes at PD 16 (***Figure 4.2 B, C, D***). Therefore, these results suggest that increased p21 is also associated with melanocyte senescence. Furthermore, I also examined the expression of the nuclear envelope protein, lamin B1, which has been shown to decrease in senescent human and mouse fibroblasts (Freund *et al.*, 2012; Sadaie *et al.*, 2013). Although I found that lamin B1 was down-regulated in replicative senescent melanocytes, this decrease appears to be small (***Figure 4.2 B***), suggesting that mild changes in nuclear integrity occur during melanocyte senescence. However, these results need to be independently confirmed.

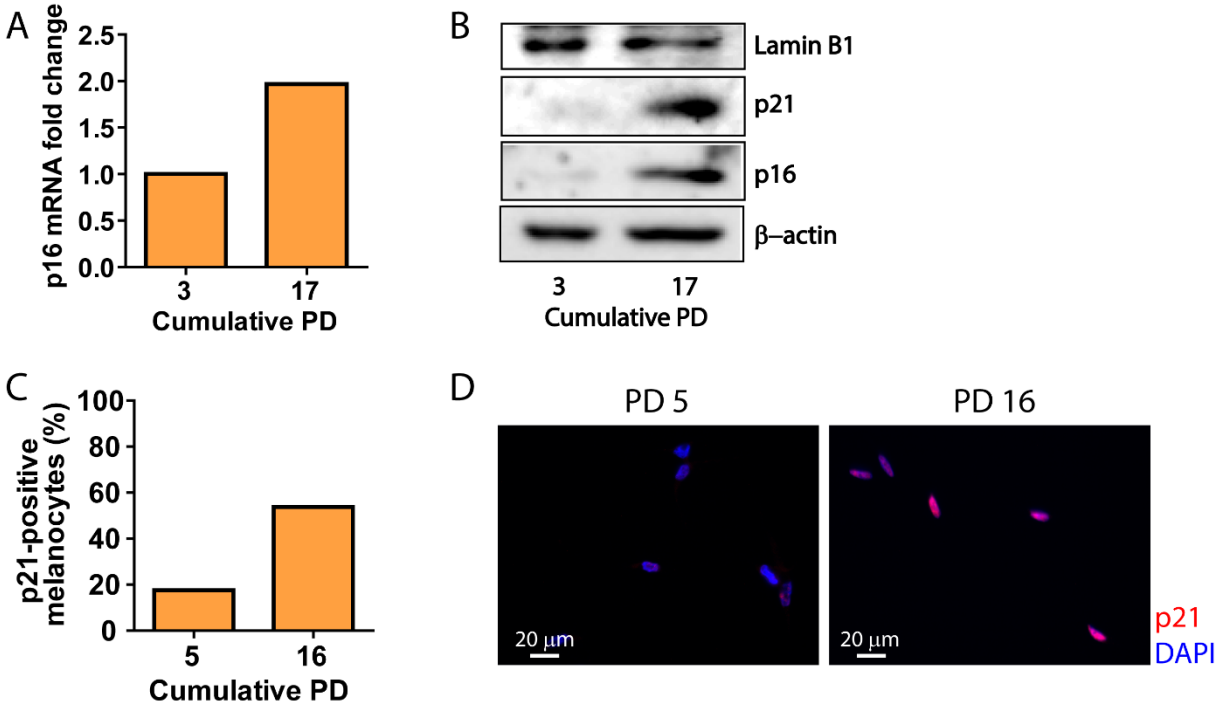

Figure 4.2 – Characterisation of senescence markers during replicative melanocyte senescence. (A) Fold change in p16 mRNA levels from early passage and senescent melanocytes in culture, as determined by q-PCR. Data are normalised to proliferating (PD 3) cells (N=1). **(B)** Western blot showing analysis of lamin B1, p21, and p16 expression in young and senescent melanocytes (N=1). β-actin was used as the loading control. **(C)** Percentage of p21-positive melanocytes at early and later passages. Data are mean of 15 random planes, with 60 cells (PD 5) and 90 cells (PD 16) (N=1). **(D)** Representative p21 immunocytochemistry image of melanocytes at PDs 5 and 16. Images were taken using a 40x objective. Red denotes p21, whereas blue shows DAPI staining.

4.1.2. mTOR activity is up-regulated during melanocyte replicative senescence

The protein kinase, mTOR, is a central nutrient and energy sensor in the cell, and is an important regulator of cellular responses such as cell growth and metabolism (Xu *et al.*, 2014a). Increased activation of mTOR signalling has been implicated in many aspects of the senescence phenotype. In fact, pharmacological inhibition of mTOR by rapamycin treatment has been shown to extend replicative lifespan of fibroblasts in culture, and also attenuate senescence-associated characteristics, such as decreased p21 expression and secretion of pro-inflammatory cytokines involved in the SASP (Demidenko and Blagosklonny, 2008; Kolesnichenko *et al.*, 2012; Laberge *et al.*, 2015). However, the role of mTOR during melanocyte senescence remains understudied, although studies suggest that up-regulation of mTOR activity is involved in OIS escape and melanoma formation (Souroullas and Sharpless, 2015).

To investigate whether melanocyte replicative senescence was accompanied by enhanced mTOR activity, cells were serially passaged in culture, and protein lysates were collected at the exponential growth phase (PD 2 – 6) and at the time when proliferation had ceased (PD 16). Western blot analysis of proliferating and senescent melanocytes revealed an increase in the levels of p70S6 kinase phosphorylated at threonine 389 (T389) in senescent melanocytes, which is indicative of increased mTOR activity (***Figure 4.3 A, B***). Although total p70S6 kinase was also slightly upregulated with replicative exhaustion (***Figure 4.3 A, C***), the ratio of phosphorylated p70S6 kinase over total p70S6K is 2.5-fold higher in senescent melanocytes compared to proliferating cells (***Figure 4.3 D)***, suggesting that mTOR activity is up-regulated in melanocyte replicative senescence.

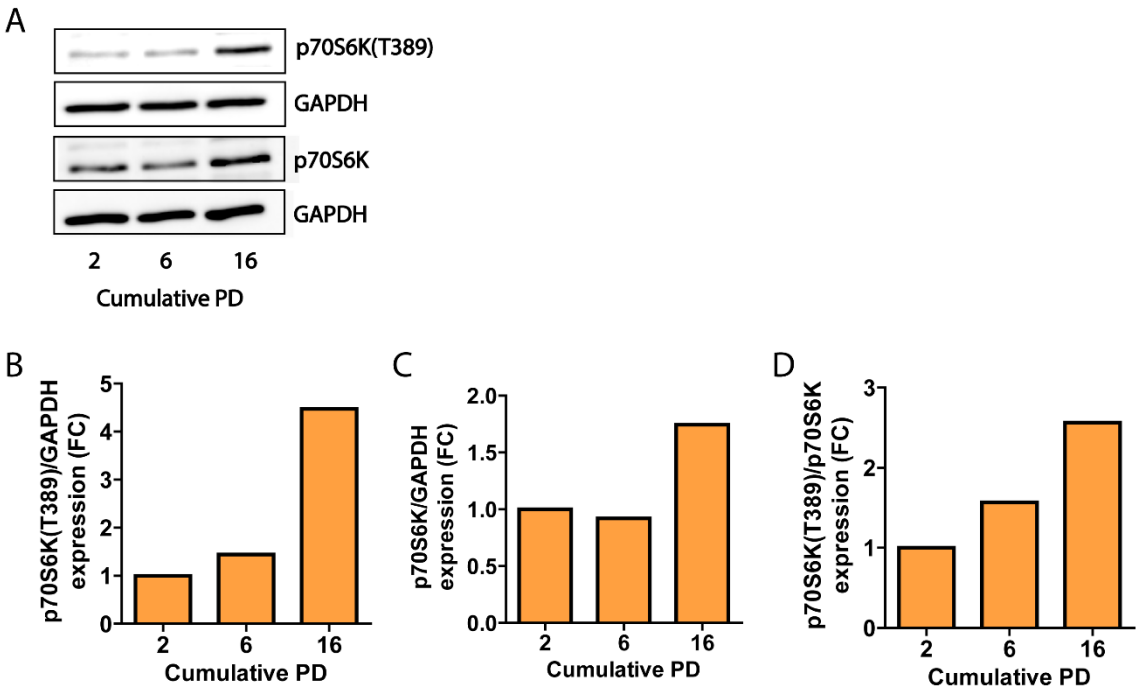

Figure 4.3 – Melanocyte replicative senescence is accompanied by increased mTOR activation. Melanocytes were grown in culture and protein extracts were collected from cells at early (PD 2 and 6) and later (PD 16) passages. **(A)** Western blot showing expression levels p70S6K(T389), p70S6K, and GAPDH as a loading control. Graphs show quantification of Western blots for **(B)** p70S6K(T389), **(C)** p70S6K, and **(D)** the ratio of p70S6K(T389) over p70S6K. Values were normalised against GAPDH, and the data represent fold change relative to PD 2 (N=1).

4.1.3. Oxidative stress increases in late passage melanocytes in vitro

Reactive oxygen species have been shown to play a role in the induction of senescence, as increased ROS levels have been reported in replicative-, stress-induced and oncogene-induced senescence (Saretzki *et al.*, 2003; Passos *et al.*, 2007; Ogrunc *et al.*, 2014). Moreover, ROS have also been implicated as effector molecules during the senescence, and are essential for reinforcing the growth arrest (Macip *et al.*, 2002; Passos *et al.*, 2010). Although increased oxidative stress has been reported to occur in oncogene-induced melanocyte senescence (Leikam *et al.*, 2008; Kaplon *et al.*, 2013; Leikam *et al.*, 2014), the involvement of ROS during replicative melanocyte senescence remains poorly explored.

In order to investigate whether ROS was involved in the establishment of replicative senescence, melanocytes were grown in culture, and cells were collected at the proliferative state, after 3 and 4 cumulative population doublings (early passage), and at later passages, following 12 and 13 cumulative PDs, when they were near the end of their replicative lifespan. I then measured ROS production by flow cytometry using ROS-indicator dyes, DHE and MitoSOX, which detect whole cell superoxide anion and mitochondrial superoxide anion levels, respectively. Firstly, there is a significant increase in DHE fluorescence intensity in late passage melanocytes compared to proliferating controls (P=0.006) (***Figure 4.4 A***), suggesting that superoxide production is enhanced in cells approaching senescence. Since mitochondria have been implicated as a major source of ROS during senescence (Saretzki *et al.*, 2003; Passos *et al.*, 2007; Sahin *et al.*, 2011; Correia-Melo *et al.*, 2016), I wanted to investigate whether mitochondrial ROS was also increased in melanocytes at later passages. Indeed, our results show a significant increase in mitochondrial superoxide levels in older melanocytes, as demonstrated by a 1.5-fold increase in MitoSOX fluorescence intensity in late passage cells (P=0.04) (***Figure 4.4 B***). These data suggest that mitochondrial ROS production is also enhanced as melanocytes approach replicative senescence.

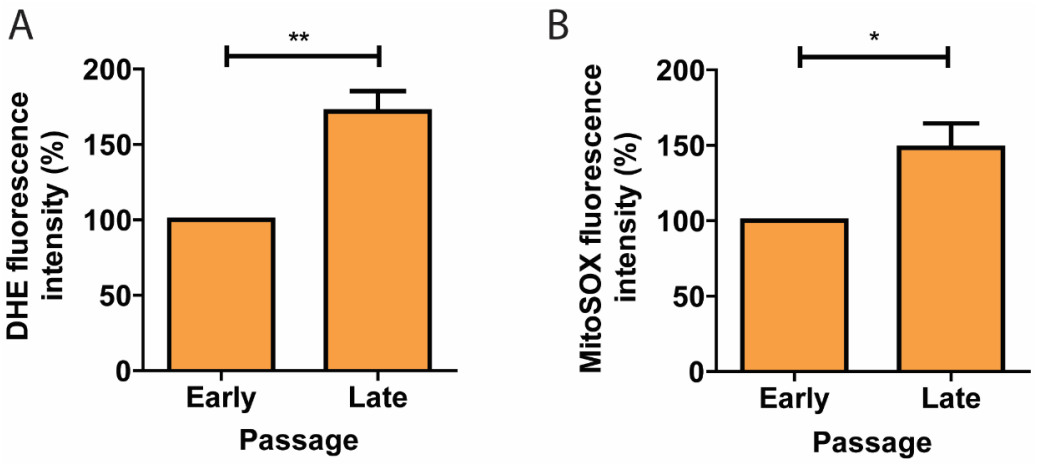

Figure 4.4 – Oxidative stress is increased in melanocytes approaching replicative senescence. Cells were serially passaged in culture and were collected at early (PDs 3 and 4) and late passages (PDs 12 and 13) for ROS measurements by flow cytometry. Graphs show percentage fold change in fluorescence intensity of **(A)** DHE and **(B)** MitoSOX compared to younger controls. Data are mean ± S.E.M of N=3 technical repeats. Statistical significance was analysed by an unpaired t-test. *P<0.05, **P<0.01.

4.1.4. Telomere-associated damage accumulates in senescent melanocytes

The majority of senescence-inducing stimuli leads to the activation of a DNA damage response, which in turn activates downstream pathways that lead to the up-regulation of the CDK inhibitors p16 and p21, mediating the senescence-growth arrest (Campisi and d'Adda di Fagagna, 2007a; Zhang *et al.*, 2012). In particular, telomeric damage has been shown to increase during senescence and ageing, and persistent DDR signalling at telomeres is believed to be important to maintain the senescence phenotype (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Studies have demonstrated that the lifespan of melanocytes in culture can be extended by overexpressing telomerase, suggesting that telomere shortening is involved in melanocyte senescence (Bandyopadhyay *et al.*, 2001). However, the association of DNA damage proteins at telomeres in senescent melanocytes has not been extensively explored.

To determine whether telomeres became dysfunctional during melanocyte replicative senescence, cells were grown in culture and were collected during the exponential growth phase (PD 5), and when they reached senescence (PD 16). I then performed immunofluorescence against the DNA damage protein, γH2AX, coupled with *in situ* hybridisation for a telomere specific probe (immuno-FISH), where co-localisation between γH2AX and telomeres indicates telomere-associated foci, or TAF. Although senescent melanocytes have a slightly higher number of DNA damage foci compared to proliferating controls, this difference is very small (***Figure 4.5 A***). On the other hand, senescent melanocytes show a significant increase in the frequency of telomere damage, approximately 3-fold higher in relation to younger melanocytes (***Figure 4.5 B***). In addition, the percentage of cells containing dysfunctional telomeres increases as melanocytes become senescent (***Figure 4.5 C***), suggesting an association between DNA damage signalling at telomeres and melanocyte replicative senescence.

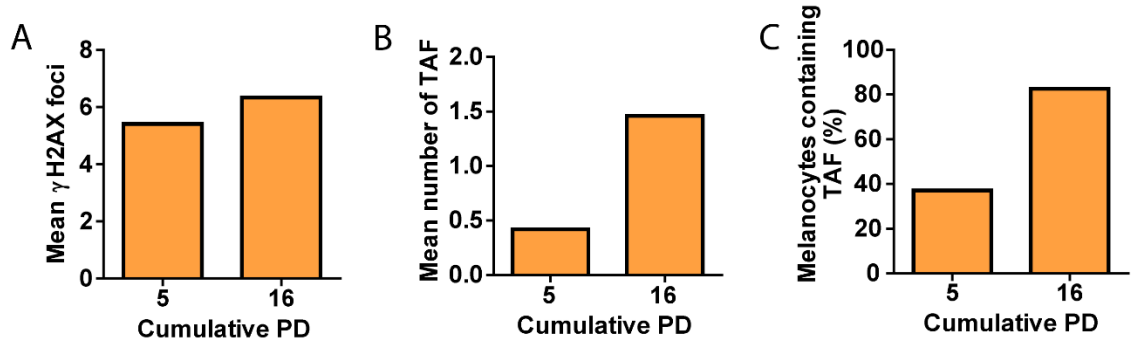

Figure 4.5 – Telomeres become dysfunctional during melanocyte replicative senescence. Cells were extensively passaged in culture and were collected during the proliferative phase (PD 5) and once they reached senescence (PD 16). Graphs indicate **(A)** mean number of γH2AX foci, **(B)** mean number of TAF, and **(C)** percentage of melanocytes containing TAF at PDs 5 and 16. Data are mean of 20 random planes of at least 40 cells per condition (N=1).

4.1.5. Telomere damage occurs independently of length

Telomere dysfunction can arise as a result of telomere shortening, which occurs with each round of cell division due to the so called "end-replication problem", whereby DNA polymerases cannot fully synthesise the lagging strand of DNA, leading to loss of telomeric repeats each time a cell divides (Olovnikov, 1971; Watson, 1972). Indeed, extensive telomere attrition has been shown to occur during replicative senescence in human cells (Harley, 1991; Bodnar *et al.*, 1998). In order to investigate whether telomere shortening occurs in melanocytes undergoing replicative senescence, I performed Q-FISH, and analysed individual telomere signal intensity as a measure of telomere length in both proliferating (PD 5) and senescent melanocytes (PD 16). Indeed, I found that telomere length was significantly shorter in senescent cells when compared younger controls ($P<0.001$) (***Figure 4.6 A***). In fact, telomeres in senescent melanocytes are approximately 15% shorter than those in proliferating cells. Moreover, to investigate whether there was a shift in the distribution of telomere lengths between young and senescent melanocytes, I divided FISH signal intensity into tertiles, where the first tertile comprises telomeres of lower intensities, and quantified the percentage of telomeres in each tertile for proliferating and growth-arrested cells. As seen in ***Figure 4.6 B***, although the majority of telomeres lie within the first tertile for both young and senescent cells, the latter show a slight enrichment in the percentage of telomeres with the lowest signal intensities. Moreover, proliferating melanocytes have a higher number of telomeres in the second and third tertiles of FISH intensity, suggesting that proliferating melanocytes have, on average, longer telomeres. This was further corroborated by setting a threshold to consider very short telomeres as those with intensities below 10% of the highest FISH signal intensity, which demonstrated that senescent melanocytes have a higher percentage of critically short telomeres (***Figure 4.6 C***). These results suggest that telomere shortening that occurs with repeated rounds of replication might contribute to telomere dysfunction during melanocyte senescence.

Although telomere attrition contributes to DDR activation at telomeres, telomere dysfunction has also been shown to occur independently of length in human fibroblasts undergoing replicative senescence (Kaul *et al.*, 2011). To determine whether damage occurred preferentially at shorter telomeres, I compared FISH signal intensity of telomeres signalling a DDR (γH2AX-positive) to those that were not associated with γH2AX (γH2AX-negative). I found that there was no significant difference between the length of γH2AX-positive and γH2AX–negative telomeres ($P=0.64$) (***Figure 4.6 D, E, F***), suggesting that telomere dysfunction occurs irrespectively of length during melanocyte senescence.

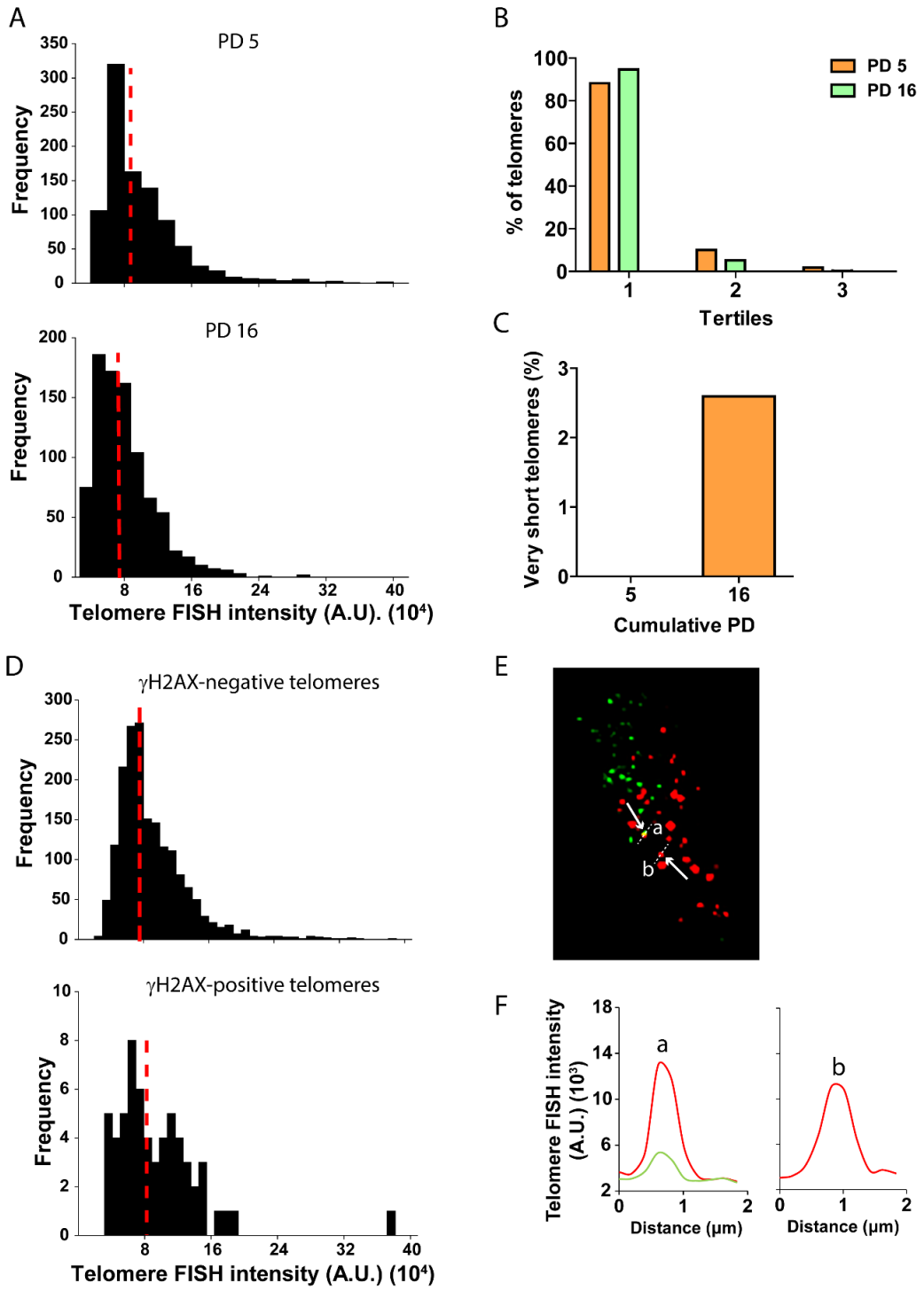

Figure 4.6 – Telomere damage occurs independently of length in senescent melanocytes. Quantitative FISH was performed in proliferating (PD 5) and senescent (PD 16) melanocytes, and FISH intensity of at least 900 individual telomeres was analysed as a measure of length. Histograms show **(A)** telomere signal intensities in proliferating and senescent melanocytes in culture, and **(D)** intensity of γH2AX-negative and γH2AX-positive telomeres in both young and old melanocytes. Red dotted line indicates median intensity. Statistical significance was

determined by performing a Mann-Whitney U test. **(B)** Percentage of telomeres in tertiles of FISH signal intensity, where the first, second and third tertiles represent the lowest, intermediate and highest signal intensities, respectively. Data are mean percentage for each population doubling (N=1). **(C)** Percentage of very short telomeres in proliferating and senescent melanocytes. Data are mean percentage for each population doubling (N=1). **(E)** Representative immuno-FISH image (red: telomeres; green: γH2AX) of a melanocyte containing telomeres of similar lengths, one which (a) co-localises with γH2AX and the other (b) is γH2AX-negative. Images were taken using a 63x oil objective. **(F)** Graphs showing the quantification of telomere intensity of γH2AX-positive (a) and γH2AX-negative (b) telomeres. Red line indicates telomere intensity, and green shows γH2AX intensity.

4.1.6. Senescent melanocytes secrete pro-inflammatory factors that are involved in the SASP

It is known that senescent cells develop a distinct secretory phenotype, also known as the SASP, which comprises the upregulation of a number of pro-inflammatory cytokines, chemokines, growth factors and proteases (Malaquin *et al.*, 2016). Although the composition of the SASP is dependent on cell type and on senescence-inducing stimuli (Coppe *et al.*, 2008; Coppe *et al.*, 2010b), the secretion of such signalling proteins is thought to have evolved as a way for senescent cells to communicate with the surrounding environment, including the immune system, to facilitate their clearance (Xue *et al.*, 2007; Krizhanovsky *et al.*, 2008). The development of a pro-inflammatory secretory phenotype has been reported in melanocytes undergoing senescence in response to $BRAF^{V600E}$ oncogene activation (van Tuyn *et al.*, 2017); however, the composition of the SASP arising from other senescence-inducing stimuli in melanocytes has not been extensively explored.

To investigate whether melanocytes undergoing replicative senescence developed a secretory phenotype, melanocytes were serially passaged in culture and conditioned medium (CM) was collected from cells at the exponential growth phase (cumulative PDs 8-10) and after they entered the senescence program (cumulative PDs 16-17). Melanocytes were cultured in CM for 24 hours before media was collected for analysis. I then performed a cytokine array in conditioned media collected from young and senescent cells, which simultaneously analyses the relative abundance of 42 secreted cytokines and chemokines; however, out of this panel, only 9 proteins were present at detectable levels in CM from cultured melanocytes (***Figure 4.7 A***). Nonetheless, there was an increase in secretion of known SASP factors, such as IL-8, IL-6, and RANTES in 3 independent experiments (***Figure 4.7 A-J***). However, due to the high variability between experiments, no significant differences were found. Therefore, increasing the number of experiments could help determine the cytokines which are significantly up-regulated during replicative melanocyte senescence.

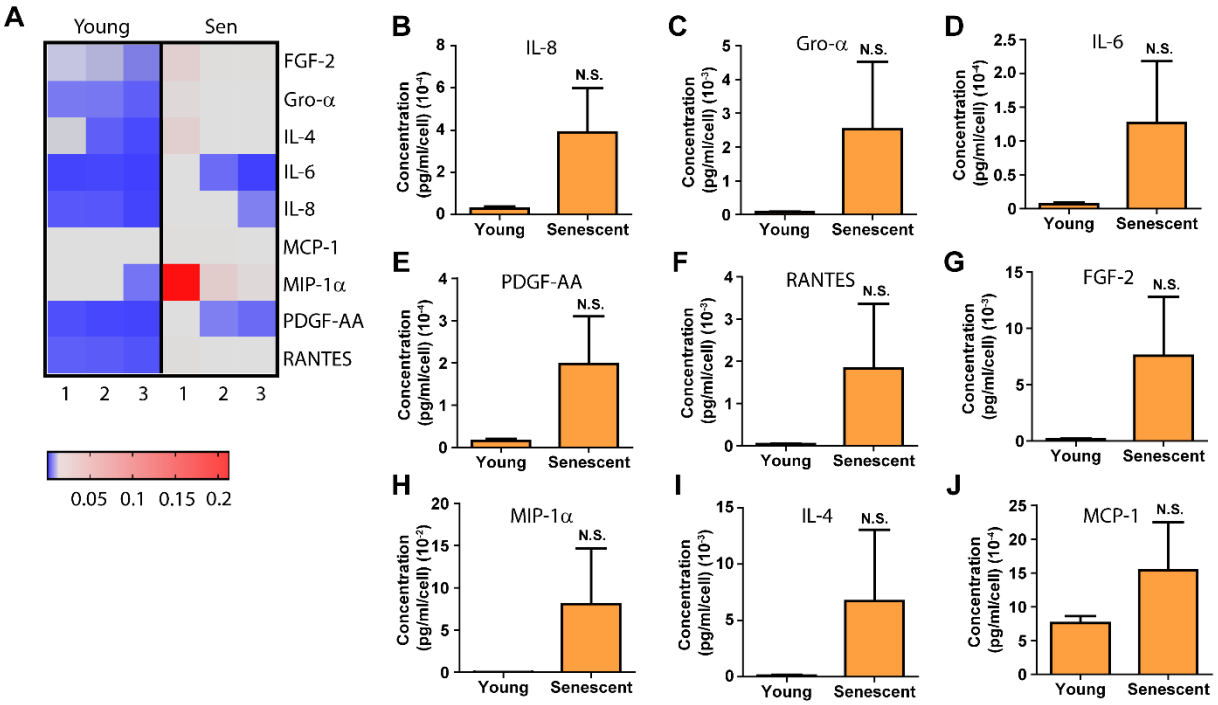

Figure 4.7 – Secretory phenotype of replicative senescent melanocytes. (A) Heatmap showing cytokines detected in CM from young and senescent melanocytes. Blue represents low expression, whereas red denotes high expression. Each column represents one independent experiment for the corresponding condition. Heatmap was generated using Prism 7.0. Concentration values are expressed as pg/ml/cell. Graphs showing the concentration of **(B)** IL-8, **(C)** Gro-α, **(D)** IL-6, **(E)** PDGF-AA, **(F)** RANTES, **(G)** FGF-2, **(H)** MIP-1α, **(I)** IL-4 and **(J)** MCP-1 secreted by melanocytes. Values were normalised by cell number. Data are mean ± S.E.M. of N=3 experiments. Statistical significance was determined by a two-tailed unpaired t-test. N.S. = not significant.

4.2. Stress-induced senescence (X-ray)

There are several pitfalls to the use of replicative senescent melanocytes in my studies: firstly, they take almost 200 days to obtain, which impacted on my ability to conduct multiple independent experiments. Secondly, since melanocytes divide very slowly from the beginning, it makes it difficult to acquire enough senescent cells. For that reason, I decided to explore alternative methods to induce senescence, including a model of stress-induced senescence by exposure to X-ray irradiation currently used by many labs in the senescence field (Passos *et al.*, 2010; Laberge *et al.*, 2015). This model is deemed advantageous for investigating senescence for the following reasons: i) it is a fast and reliable way to generate a homogenous population of senescent cells; ii) it requires only one dose of X-ray irradiation to induce senescence in the majority of cells with negligible cell death; iii) it allows the investigation of mechanisms underlying the induction of senescence kinetically, from the induction of damage to the establishment of the SASP (Coppe *et al.*, 2008; Passos *et al.*, 2010). However, one evident pitfall is that it is not physiological since it is unlikely that humans are exposed to high levels of X-ray irradiation.

4.2.1. 10 Gy X-ray irradiation induces senescence in melanocytes in vitro

In order to induce senescence in melanocytes in culture, cells at the exponential growth phase were irradiated using 10 Gy X-ray irradiation, and were then cultured for an additional 10 days following irradiation before they were collected for the analysis of senescence markers (***Figure 4.8 A***). Since the hallmark of senescence is a stable cell-cycle arrest, I firstly analysed the proliferation status of proliferating and irradiated cells by EdU incorporation. I found a significant decrease in EdU-positive melanocytes 10 days after 10 Gy X-ray irradiation (P=0.006) (***Figure 4.8 B, C***), suggesting that the majority of cells are arrested in the cell cycle. Next I carried out a biochemical assay for the detection of Sen-β-Gal activity, and found that irradiated melanocytes had significantly higher levels of Sen-β-Gal staining, with approximately 80% of cells showing Sen-β-Gal positivity 10 days following X-ray irradiation (P=0.0005) (***Figure 4.8 D, E***).

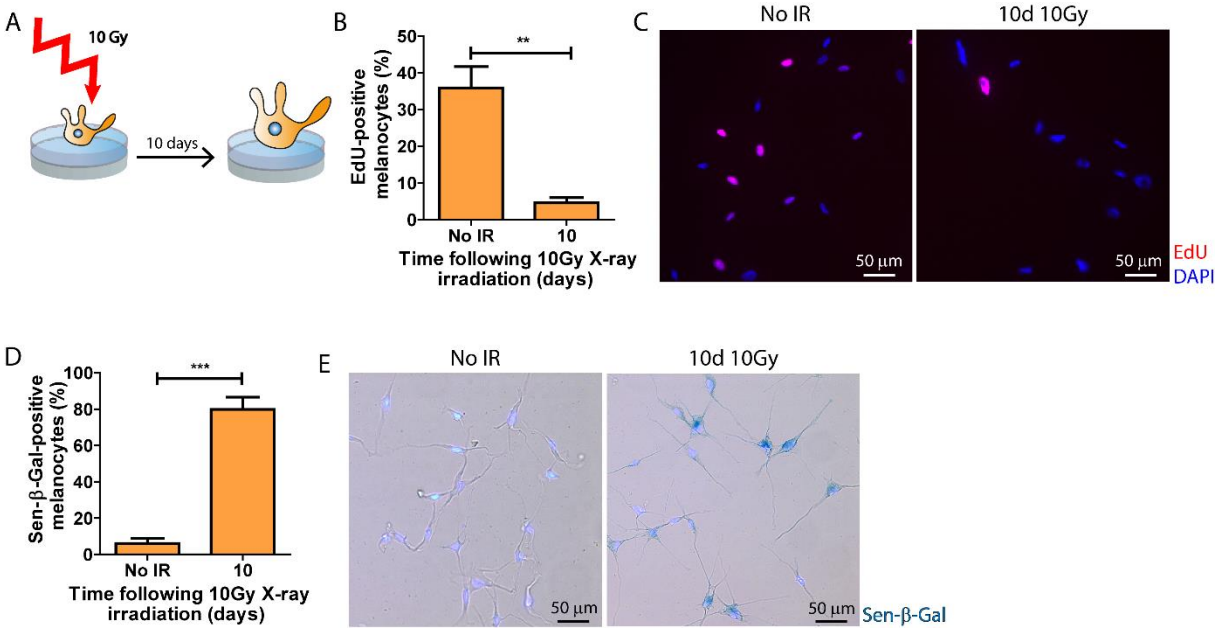

Figure 4.8 – Stress-induced melanocyte senescence is induced by X-ray irradiation. (A) Scheme representing the experimental procedure. **(B)** Percentage of EdU-positive melanocytes at control, proliferating conditions (No IR) and 10 days after 10 Gy X-ray irradiation. Data are mean ± S.E.M. of N=3 independent experiments. **(C)** Representative immunofluorescence images of EdU staining of non-irradiated melanocytes and cells 10 days after X-ray irradiation (red: EdU; blue: DAPI). Images were taken using a 20x objective. **(D)** Percentage of Sen-β-Gal-positive untreated and irradiated melanocytes. Data are mean ± S.E.M. of N=3 independent experiments. **(E)** Representative Sen-β-Gal (blue) images of non-irradiated melanocytes and cells 10 days following X-ray irradiation. Images were taken using a 20x objective. Statistical significance was determined by performing a two-tailed unpaired t-test. **P<0.01, ***P<0.001.

Although decreased proliferation and increased Sen-β-Gal activity are indicative of senescence, other markers need to be analysed in order to fully ascertain if irradiated melanocytes are in a state of permanent growth arrest. Therefore, I performed immunofluorescence for the CDK inhibitor, p16, and found that at 10 days following irradiation melanocytes expressed significantly higher levels of p16 compared to young, proliferating controls (P=0.002) (***Figure 4.9 A, B***). Moreover, I also examined the expression of p21 at 3 and 10 days following irradiation. I found that p21 expression was up-regulated at 3 days after irradiation, approximately 2-fold higher when compared to non-irradiated controls, likely reflecting the initial arrest to allow cells to repair X-ray-induced DNA damage. However, due to the variability between experiments, this increase was not statistically significant. Although there is a slight decrease in p21 expression at day 10, this was not statistically significant, and p21 levels remain higher in melanocytes 10 days after irradiation when compared to untreated controls. However, this did not reach statistical significance likely due to the variation between experiments (***Figure 4.9 C, D***). These results suggest that mechanisms involving p16 and, to some extent, p21, are associated with stress-induced melanocyte senescence.

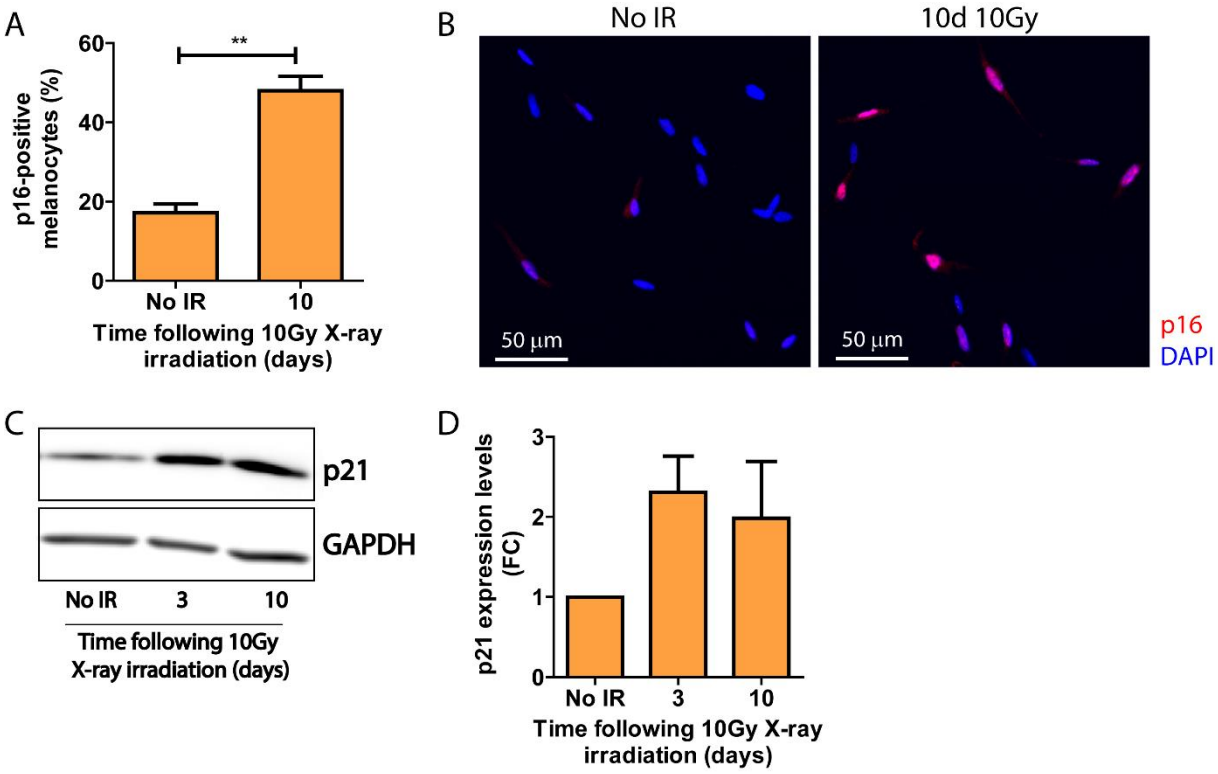

Figure 4.9 – Stress-induced melanocyte senescence involves up-regulation of both p16 and p21. Melanocytes were irradiated using 10 Gy X-ray irradiation and were collected 3 and 10 days after irradiation for characterisation of senescence markers. **(A)** Percentage of p16-positive untreated and irradiated melanocytes 10 days after the initial exposure. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by using a two-tailed unpaired t-test; **P<0.01. **(B)** Representative p16 immunofluorescence image of non-irradiated and senescent (10d 10Gy) melanocytes (Red: p16; blue: DAPI). Images were taken using a 40x objective (done in collaboration with Jessica Halim). **(C)** Representative Western blot to analyse the expression of p21 in untreated melanocytes, and 3 and 10 days following X-ray irradiation. GAPDH was used as a loading control. **(D)** Quantification of Western blot, showing fold change of p21 levels compared to non-irradiated (No IR) controls. p21 values were normalised by GAPDH. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. FC = fold change.

4.2.2. mTOR activity is unchanged in stress-induced melanocyte senescence

As previously mentioned, mTOR activity has been implicated in the senescent phenotype. Indeed, it has been demonstrated that mTOR activity is up-regulated in human fibroblasts in response to X-ray irradiation, which was shown to contribute to senescence by stimulating mitochondrial biogenesis and maintaining the DDR through ROS generation (Correia-Melo *et al.*, 2016).

Since I observed an increase in mTOR activation in replicative senescent melanocytes, I wanted to investigate whether this also occurred during stress-induced melanocyte senescence. For that, I irradiated melanocytes using 10 Gy X-ray irradiation, and collected protein lysates at 3 and 10 days following irradiation. Western blot analysis revealed a decrease in phosphorylated p70S6 kinase (T389) levels in melanocytes at 3 days after irradiation when compared to proliferating cells. However, this decrease was not statistically significant. This was shown to remain lower in senescent melanocytes (10d 10Gy) when compared to non-irradiated controls, although it also did not reach statistical significance (***Figure 4.10 A, B***). I also observed that the levels of total p70S6 kinase did not change significantly following irradiation of melanocytes across three independent experiments (***Figure 4.10 C***). Moreover, the ratio of p70S6K(T389) over total p70S6K, which is indicative of mTOR activity, was not increased in senescent melanocytes in relation to proliferating control cells (***Figure 4.10 D***), suggesting that mTOR activation is not involved in stress-induced senescence in melanocytes, or at least not at the time points analysed.

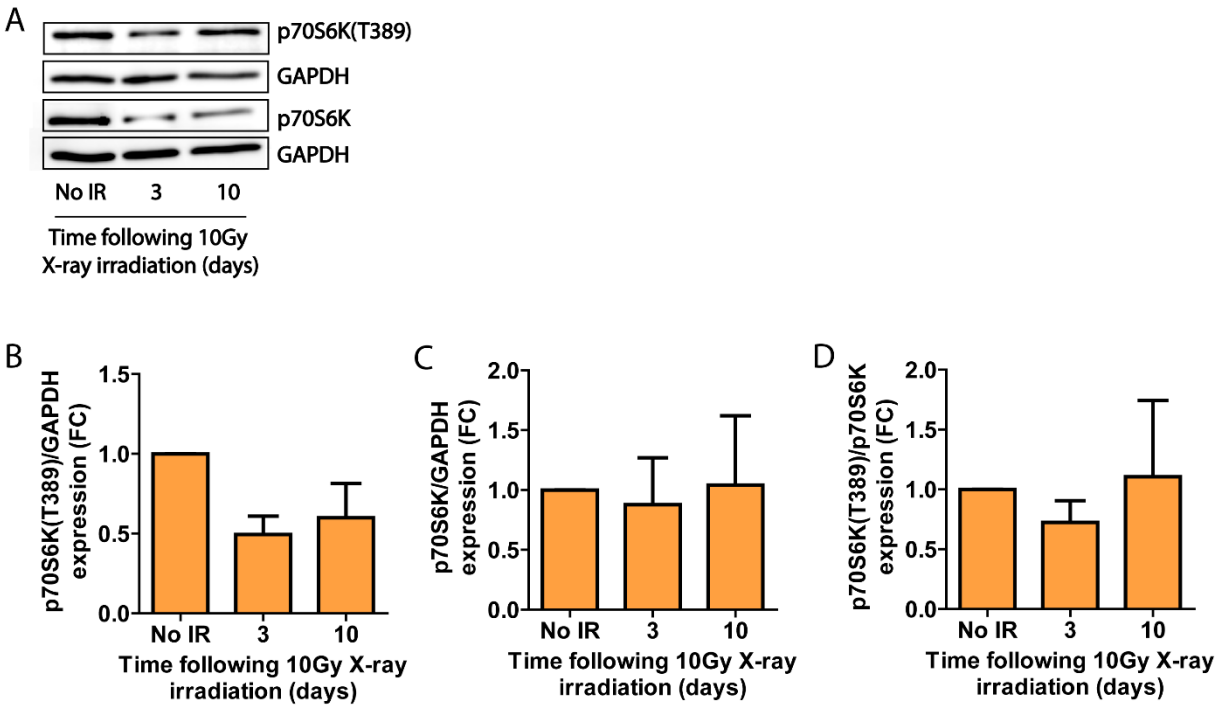

Figure 4.10 – mTOR activity is not up-regulated in stress-induced melanocyte senescence. Cells were irradiated using 10 Gy X-ray irradiation, and protein lysates were collected at 3 and 10 days following irradiation, when melanocytes are senescent. **(A)** Representative Western blot showing expression levels of p70S6K(T389), p70S6K, and GAPDH as a loading control. Graphs are quantification of Western blots showing fold change of **(B)** p70S6K(T389), **(C)** p70S6K, and **(D)** the ratio of p70S6K(T389) over p70S6K, compared to non-irradiated (No IR) control. Values were normalised against GAPDH. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. FC = fold change.

4.2.3. Mitochondrial ROS generation increases in stress-induced melanocyte senescence

Studies have demonstrated that mitochondrial dysfunction and increased ROS generation occur as a result of stress-induced senescence in human fibroblasts, which generates a dynamic positive feedback loop, maintaining a DDR and contributing to the stability of the cell-cycle arrest (Passos *et al.*, 2010). Moreover, it has been shown that clearance of mitochondria in human fibroblasts reduced intracellular ROS levels and ameliorated the senescence phenotype of these cells, suggesting that mitochondrial-derived ROS contributes to the development of senescence (Correia-Melo *et al.*, 2016). Although mitochondrial oxidative stress has reported to occur during OIS in melanocytes (Kaplon *et al.*, 2013), a role for mitochondrial ROS generation during stress-induced melanocyte senescence remains poorly explored.

To determine whether mitochondrial ROS increased in irradiated melanocytes, cells were exposed to 10 Gy X-ray irradiation, and mitochondrial superoxide levels were measured by flow cytometry 3 days after irradiation using the fluorescent dye MitoSOX. Measurements were conducted at 3 days, since it has been previously shown that ROS levels increase from 2 days after X-ray irradiation, and remain stable from this time onwards, even when cells become senescent (Passos *et al.*, 2010). I found that the levels of mitochondrial superoxide were significantly higher in melanocytes 3 days after irradiation, showing a 2-fold increase compared to proliferating controls ($P=0.03$) (***Figure 4.11 A***). These results suggest that increased mitochondrial ROS production occurs as a consequence of senescence induction.

Next, in order to investigate whether mitochondrial ROS was an inducer of melanocyte senescence, X-ray irradiated melanocytes were treated with the mitochondria-targeted antioxidant, MitoQ, for 10 days following irradiation. Although MitoQ successfully prevented the increase in mitochondrial ROS production that occurs upon senescence induction ($P<0.05$) (***Figure 4.11 B***), scavenging mitochondrial ROS was not sufficient to significantly reduce Sen-β-Gal activity in senescent melanocytes (10 days after X-ray irradiation) (***Figure 4.11 C***). Therefore, it appears that mitochondrial ROS is not involved in the induction of Sen-β-Gal, although other senescence markers need to be analysed in order to fully assess the role of ROS in the establishment of melanocyte senescence.

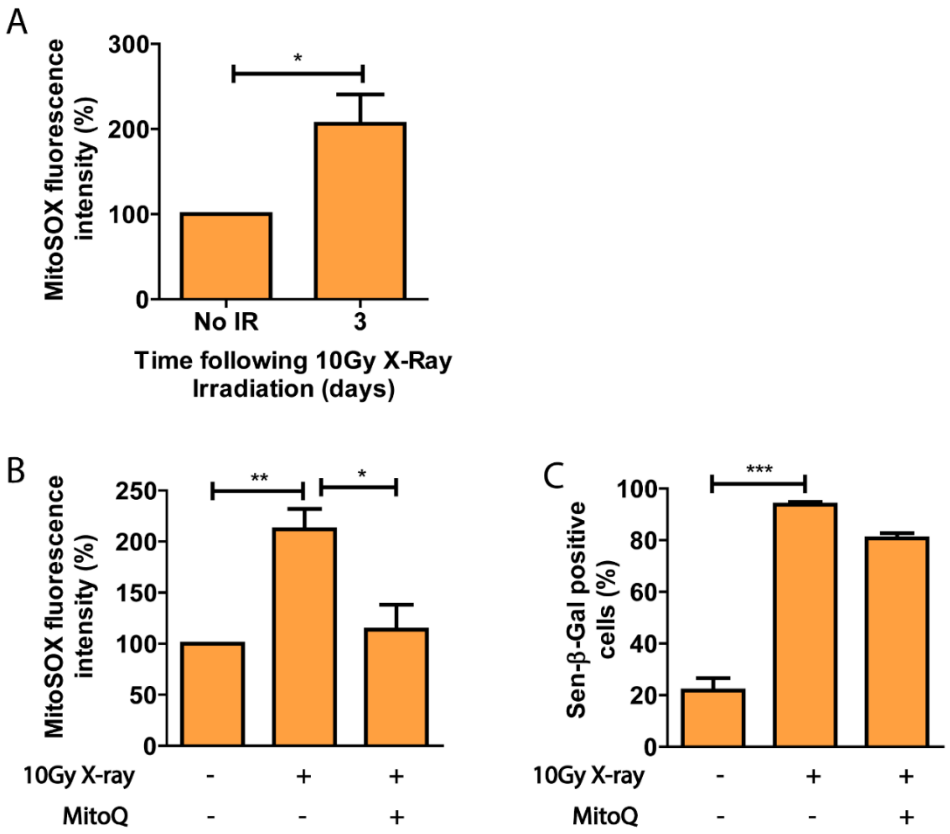

Figure 4.11 – Mitochondrial superoxide production is enhanced following X-ray irradiation of melanocytes. Cells were exposed to 10 Gy X-ray irradiation and mitochondrial superoxide levels were measured 3 days later by flow cytometry. **(A)** Graph shows percentage fold change in fluorescence intensity of MitoSOX in irradiated melanocytes compared to untreated controls. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was analysed by an unpaired t-test. *P<0.05. **(B)** Percentage fold change in MitoSOX fluorescence intensity in irradiated melanocytes (3 days after irradiation) with and without MitoSOX compared to non-irradiated controls. **(C)** Percentage of Sen-β-Gal-positive melanocytes at the conditions indicated. Sen-β-Gal activity was evaluated 10 days after irradiation. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. (MitoSOX experiments were done in collaboration with Jessica Halim).

4.2.4. Stress-induced senescent melanocytes have increased telomere dysfunction

It has been shown that telomere damage increases in stress-induced senescence in fibroblasts *in vitro*, with approximately half of the DNA damage foci co-localising with telomeres (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Moreover, damage at telomeres was shown to be long-lived, contributing to a persistent DDR signalling which is believed to be important for maintaining the senescent phenotype (Hewitt *et al.*, 2012). Nevertheless, no previous studies have reported DNA damage signalling at telomeres during stress-induced melanocyte senescence.

To establish whether telomere dysfunction was a consequence of stress-induced senescence, melanocytes were irradiated using 10 Gy X-ray irradiation, and were allowed to progress into senescence for a period of 10 days. Immuno-FISH was then performed using an antibody against γH2AX coupled with a telomere specific probe, to allow visualisation of γH2AX foci at telomeres, which is indicative of TAF. Firstly, I observed that irradiation resulted in an increase in the number of DNA damage foci at 3 days, which was reduced at 10 days. Although DNA damage remained higher in senescent cells when compared to non-irradiated controls (***Figure 4.12 A***), these results suggest that melanocytes are capable of repairing a fraction of DNA lesions induced by irradiation. Similarly, the number of dysfunctional telomeres also increased 3 days following irradiation; however, the number of TAF did not decrease at 10 days, and remained higher compared to proliferating melanocytes (***Figure 4.12 B, E***), suggesting that there is no significant repair of telomeric DNA damage. A similar pattern was observed when analysing the percentage of melanocytes containing dysfunctional telomeres after irradiation (***Figure 4.12 C***). Moreover, the frequency of melanocytes containing more than one telomere signalling a DDR increases 3 days following irradiation, and is considerably higher even after 10 days, when melanocytes are senescent (***Figure 4.12 D***). Overall, these data suggest that telomere dysfunction is a feature of stress-induced melanocyte senescence. However, in order to establish statistical significance, these results need to be independently confirmed.

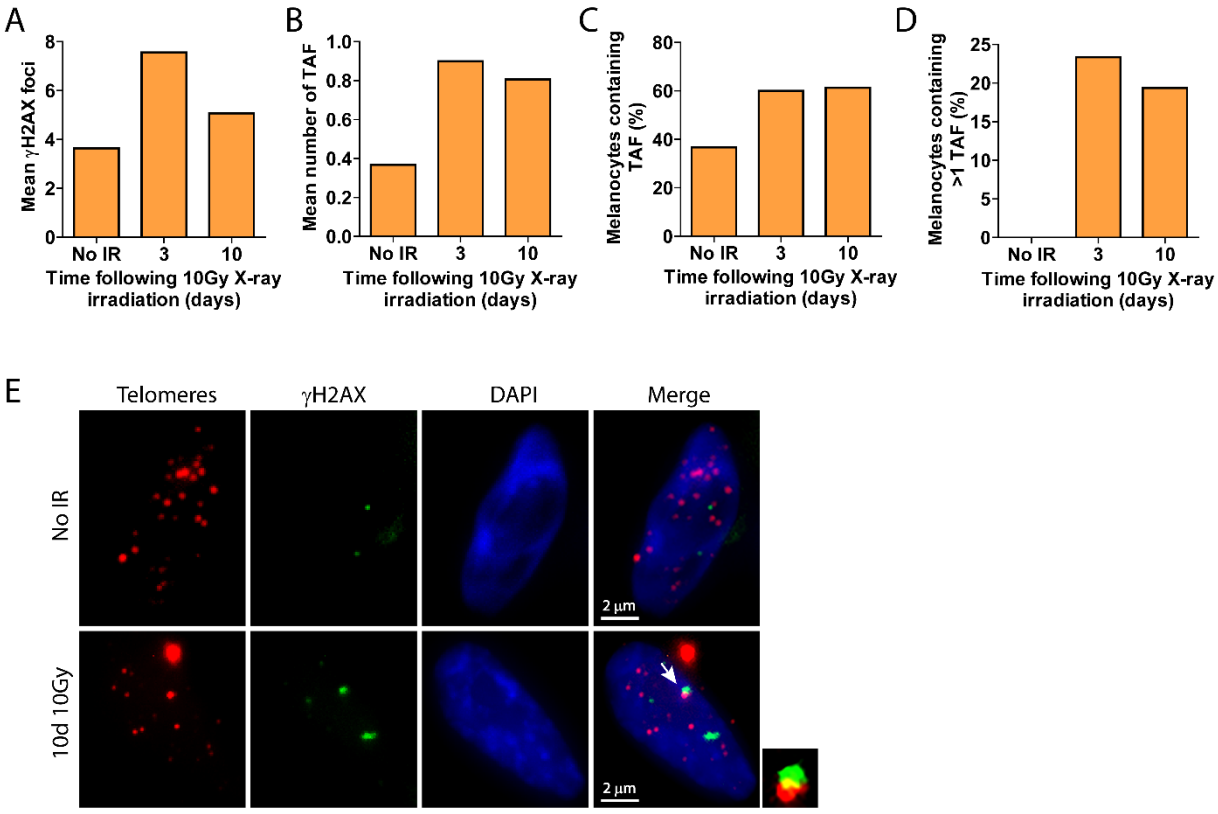

Figure 4.12 – Telomere dysfunction increases in stress-induced melanocyte senescence. Melanocytes were irradiated using 10 Gy X-ray irradiation, and DNA and telomere damage were analysed at the time points indicated. Graphs show **(A)** mean number of γH2AX foci, **(B)** mean number of TAF, **(C)** percentage of melanocytes containing TAF, and **(D)** percentage of melanocytes containing more than 1 TAF. Data are mean of 30 cells obtained from a minimum of 10 random planes (N=1). **(E)** Representative immuno-FISH images of proliferating (top) and senescent (bottom) melanocytes. Telomeres are shown in red, γH2AX is depicted in green, and DAPI is shown in blue. Arrows indicate co-localisation between γH2AX and telomeres, which is amplified on the right. Images were taken using a 63x oil objective.

4.2.5. Telomere damage occurs irrespectively of length

A number of studies have demonstrated that genotoxic stress that induces senescence can initiate DDR signalling at telomeres that are not critically short both *in vitro* and *in vivo* (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Although length-independent telomere damage has been shown to occur in melanocytic nevi *in vivo*, which are growth arrested due to oncogene activation (Suram *et al.*, 2012), it is not known whether longer telomeres can also signal a DNA damage response in stress-induced melanocyte senescence.

To determine whether DNA damage occurred at telomeres irrespectively of length in stress-induced senescence, I performed Q-FISH, and analysed the signal intensity of individual telomeres as a measure of length in both proliferating and irradiated melanocytes. Next, I compared FISH signal intensity of telomeres signalling a DDR (γH2AX-positive) to those that were not associated with γH2AX (γH2AX-negative), and found that dysfunctional telomeres were not significantly shorter than γH2AX-negative telomeres (P=0.158) (***Figure 4.13 A***). In fact, I observed that in a single cell, telomeres of similar lengths can either signal a DDR or remain undamaged following irradiation (***Figure 4.13 B, C***), suggesting that damage occurs independently of telomere length during stress-induced melanocyte senescence.

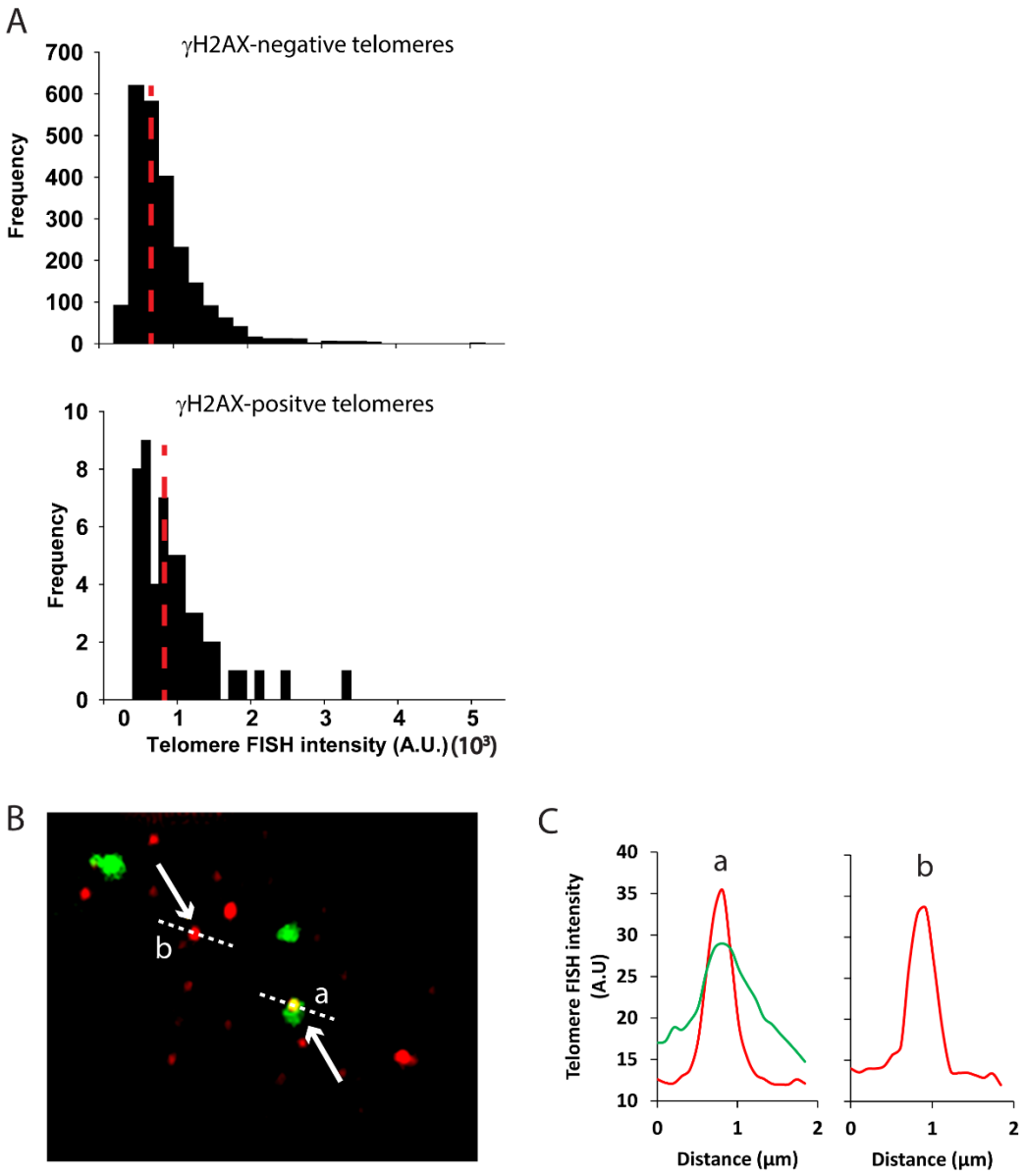

Figure 4.13 – Telomere damage occurs independently of length during stress-induced senescence. Quantitative FISH was performed in proliferating and irradiated melanocytes (3 and 10 days following IR), and FISH intensity of at least 800 individual telomeres was analysed as a measure of length. **(A)** Histograms showing intensity of γH2AX-negative and γH2AX-positive telomeres in both proliferating and irradiated melanocytes. Red dotted line indicates median intensity. Statistical significance was determined by performing a Mann-Whitney U test. **(B)** Representative immuno-FISH image (red: telomeres; green: γH2AX) of a melanocyte containing telomeres of similar lengths, one which (a) co-localises with γH2AX and the other (b) is γH2AX-negative. Images were taken using a 63x oil objective. **(C)** Graphs showing the quantification of telomere intensity of γH2AX-positive (a) and γH2AX-negative (b) telomeres. Red line indicates telomere intensity, and green shows γH2AX intensity.

4.2.6. Pro-inflammatory secretory phenotype of stress-induced senescent melanocytes

Having established that melanocytes become senescent in response to genotoxic stress caused by X-ray irradiation in culture, I then wanted to determine whether this senescence-inducing stimulus also triggered the secretion of pro-inflammatory cytokines and chemokines, as observed in replicative-induced senescent melanocytes.

To characterise the secretory phenotype of stress-induced senescent melanocytes, conditioned medium was collected from proliferation efficient and senescent cells (10 days following X-ray irradiation). Melanocytes in each condition were cultured in CM for 24 hours prior to media collection for analysis. A cytokine array was then carried out in CM obtained from proliferating and senescent melanocytes. I found that out of the panel of 42 cytokines that can be detected by this assay, 15 proteins were present at detectable levels in CM of cultured melanocytes (***Figure 4.14 A***). Moreover, more detailed analysis of the pro-inflammatory profile of these cells revealed that RANTES (***Figure 4.14 B)*** and IP-10 (***Figure 4.14 C***) were significantly increased in CM from senescent melanocytes, with a 10- and 4.5-fold increase in concentration levels, respectively, when compared to young (non-irradiated) cells (P=0.0006 and P=0.0001, respectively). Although senescent melanocytes also secreted higher levels of IL-6 and IL-8, this was not statistically significance (P=0.08 and P=0.09, respectively) (***Figure 4.14 D, E***). Nevertheless, these results show that senescent melanocytes develop a distinct secretory phenotype, suggesting that stress-induced senescence is also accompanied by a SASP.

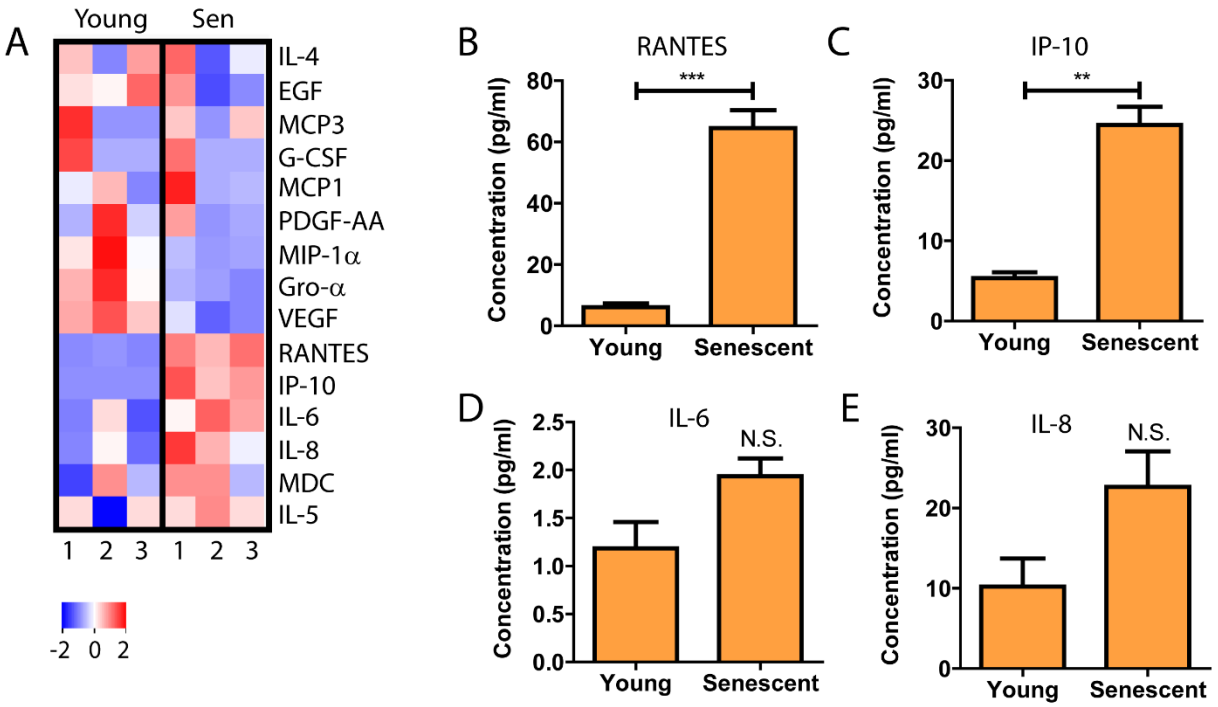

Figure 4.14 – Secretory phenotype of stress-induced melanocytes. (A) Heatmap showing cytokines detected in CM from young (non-irradiated) and senescent (10 days after irradiation) melanocytes. Blue represents low expression, whereas red denotes high expression. Each column represents one independent experiment for the corresponding condition. Heatmap was generated using Ingenuity Pathway Analysis (IPA). Graphs showing the concentration of **(B)** RANTES, **(C)** IP-10, **(D)** IL-6, and **(E)** IL-8. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a two-tailed unpaired t-test. **P<0.01, ***P<0.001, N.S. = not significant. (Cytokine array was done in collaboration with Duncan Talbot from Unilever).

4.3. Stress-induced senescence (UV irradiation)

4.3.1. Repeated UV exposure induces senescence in melanocytes in vitro

In order to establish a more physiologically relevant model of melanocyte senescence, I next aimed to investigate the use of UV radiation as another stimulus to trigger stress-induced senescence. Firstly, to determine a suitable UV dose to induce senescence, lightly-pigmented human neonatal melanocytes were exposed to different doses of both UVA and UVB radiation once a day for five consecutive days, and cells were then collected 4 days following the last exposure for the analysis of senescence markers (***Figure 4.15 A***). Amongst the doses tested, 0.1 J/cm^2 UVA and UVB radiation induced a small but significant increase in Sen-β-Gal activity, as shown by an approximate 2-fold increase in Sen-β-Gal-positive cells compared to non-irradiated, proliferating controls ($P<0.05$) (***Figure 4.15 B, C***). Although a small increase in p16 expression was also achieved with this dose, this was not statistically significant ($P>0.05$) (***Figure 4.15 D, E***), suggesting that 0.1 J/cm^2 does not efficiently induce melanocyte senescence *in vitro*. On the other hand, repeated exposure to 0.4 J/cm^2 UVA and UVB radiation resulted in a 7-fold increase in Sen-β-Gal activity, where 82% of irradiated melanocytes were positive for Sen-β-Gal when compared to proliferating controls ($P<0.001$) (***Figure 4.15 B, C***). Moreover, this dose significantly increased p16 expression in relation to control cells ($P<0.001$), with 74% of melanocytes expressing p16 following 0.4 J/cm^2 UVA+B exposure, which is also a significantly higher proportion in comparison to 0.1 J/cm^2 irradiated cells ($P<0.001$) (***Figure 4.15 D, E***). Therefore, these results suggest that repeated exposure to 0.4 J/cm^2 UVA+B is sufficient to induce senescence in human melanocytes in culture.

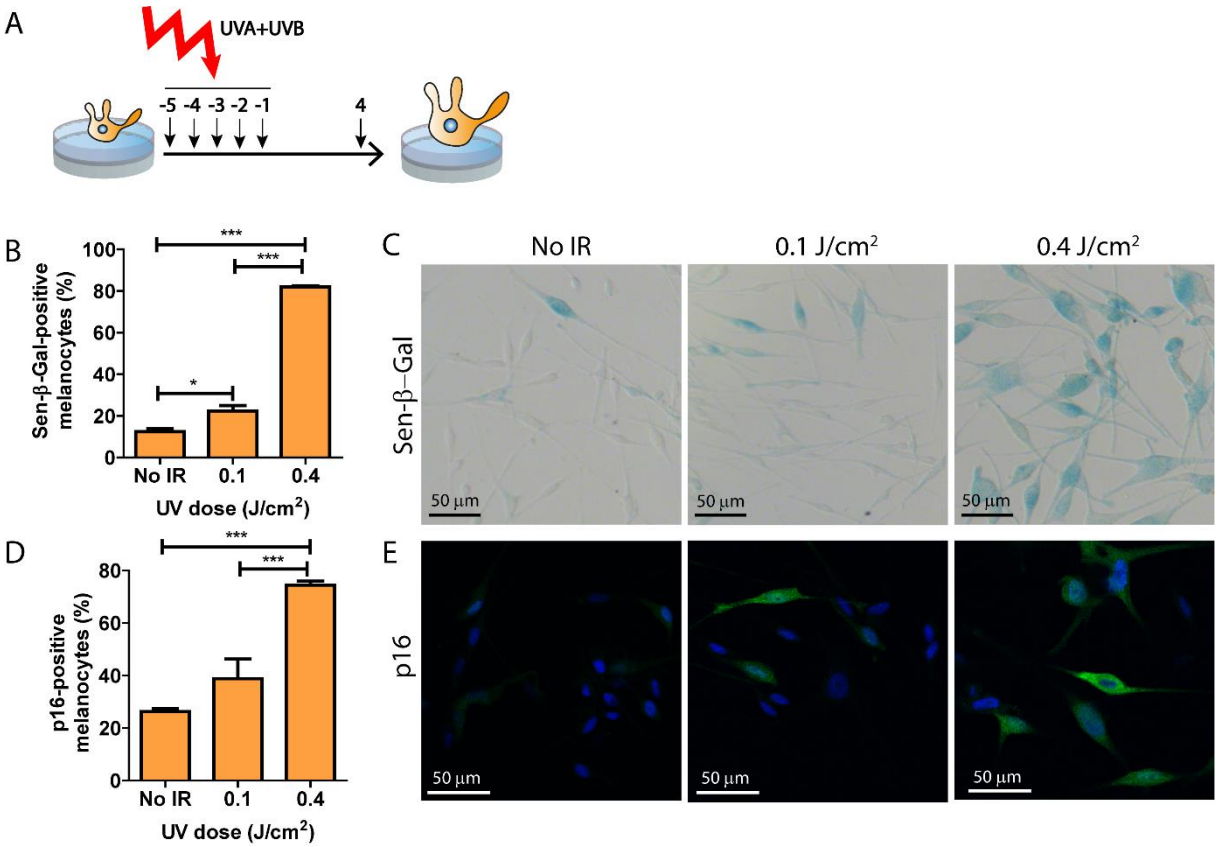

Figure 4.15 – Stress-induced senescence is achieved by repeated exposure of melanocytes to 0.4J/cm^2 UVA and UVB radiation. (A) Scheme showing the experimental procedure. Proliferation efficient melanocytes were exposed to different doses of both UVA and UVB radiation once a day for five consecutive days. Cells were collected 4 days following the last exposure. Graphs showing **(B)** the percentage of Sen-β-Gal- and **(D)** p16-positive melanocytes following exposure to UVA+B radiation at the indicated doses. Data are mean ± S.E.M. of N=3 technical repeats. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. Representative images of **(C)** Sen-β-Gal (blue) and **(E)** p16 (green) immunofluorescence (blue: DAPI) of proliferating and UV irradiated melanocytes. Images were taken using a 20x objective.

Having established a suitable UV dose that significantly up-regulates markers of senescence in melanocytes, I then wanted to determine whether the cell-cycle arrest was indeed permanent and not a mere transient arrest to allow repair UV-induced DNA damage. For that, melanocytes were exposed to 0.4 J/cm^2 of UVA+B radiation once a day for five consecutive days, and cells were collected both at 4 and 18 days following the last exposure. Firstly, I analysed the proliferation status of untreated (no IR) and irradiated melanocytes by EdU incorporation. I found a 3.2-fold decrease in the percentage of proliferating melanocytes 4 days after the last UV exposure when compared to control cells, although this did not reach statistical significance ($P>0.05$) (***Figure 4.16 A, B***). Moreover, there is a further 2.6-fold decrease in proliferation between 4 and 18 days after irradiation, albeit not statistically significant ($P>0.05$), suggesting that there is continued progression towards a cell-cycle arrest at later time points (***Figure 4.16 A***). Nonetheless, the percentage of proliferating melanocytes is significant lower at 18 days following UV exposure when compared to proliferating controls ($P<0.05$) (***Figure 4.16 A, B)***, confirming that melanocytes do not resume proliferation after repeated UV irradiation. Next, to further characterise the growth arrest I performed immunofluorescence for the CDK inhibitor, p16. In accordance to my previous data, I observed a significant increase in p16 expression 4 days after irradiation ($P<0.05$), which remained significantly higher at 18 days when compared to proliferating cells ($P<0.01$), with 87.5% of melanocytes expressing p16 at this time point (***Figure 4.16 C***). Therefore, these results corroborate the observation that repeated UVA and UVB exposure at 0.4 J/cm^2 is sufficient to induce a stable proliferation arrest and melanocyte senescence *in vitro*.

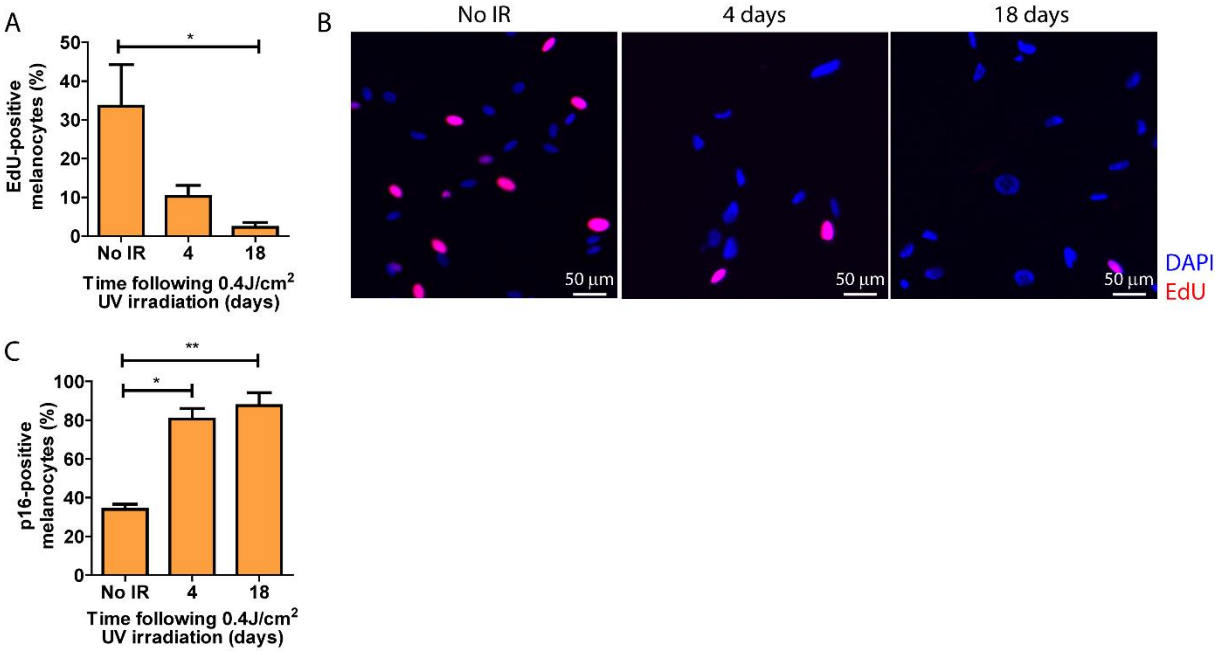

Figure 4.16 – A stable cell-cycle arrest in achieved by UV exposure of melanocytes in vitro. Melanocytes were exposed to 0.4 J/cm^2 UVA and UVB radiation once a day for five consecutive days, and then collected at 4 and 18 days for the analysis of senescence markers. **(A)** Graph showing the percentage of EdU-positive melanocytes at the time points indicated. Data are mean ± S.E.M. of N=3 independent experiments. **(B)** Representative EdU immunofluorescence images of proliferating and irradiated melanocytes (4 and 18 days post-IR) (red: EdU; blue: DAPI). Images were taken using a 20x objective. **(C)** Percentage of p16-positive melanocytes at the time points indicated as determined by immunofluorescence. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01.

4.3.2. Telomere damage increases in UV-induced senescent melanocytes

Given that all models of melanocyte senescence studied so far have suggested a role for telomere dysfunction the process, I aimed to investigate whether telomere damage also occurred as a result of UV-induced senescence. Although telomeres have been shown to be hypersensitive to UV-induced damage, which is also less efficiently, if at all, repaired in human fibroblasts in culture, (Kruk *et al.*, 1995; Rochette and Brash, 2010), the relationship between telomere damage following UV exposure and senescence in human melanocytes has been understudied.

To determine whether DNA damage accumulates at telomeres as a result of UV-induced senescence, melanocytes were exposed to UV irradiation as previously described, and collected at 4 days following the last exposure, when cells were shown to be senescent. I then performed immunofluorescence against the DNA damage protein, γH2AX, coupled with *in situ* hybridisation for a telomere specific probe, which allowed me to visualise telomere-associated damage or TAF. Firstly, I found that senescent melanocytes had a significantly higher number of DNA damage foci in comparison to proliferating cells, rising by 6-fold after repeated exposure to UV radiation. (P=0.0007) (***Figure 4.17 A, D***). Moreover, as seen in other models of melanocyte senescence, telomeric damage was significantly increased in UV-induced senescent cells. In fact, the frequency of damaged telomeres was 4.5-fold higher in senescent melanocytes compared to non-irradiated controls (P=0.0005) (***Figure 4.17 B, D***). I also found that, following repeated UV exposure, the percentage of melanocytes containing dysfunctional telomeres significantly increased, with 60% of senescent melanocytes containing at least one telomere signalling a DDR (P=0.001) (***Figure 4.17 C***). These results suggest that both non-telomeric and telomeric DNA damage are associated with the phenotype of UV-induced senescent melanocytes.

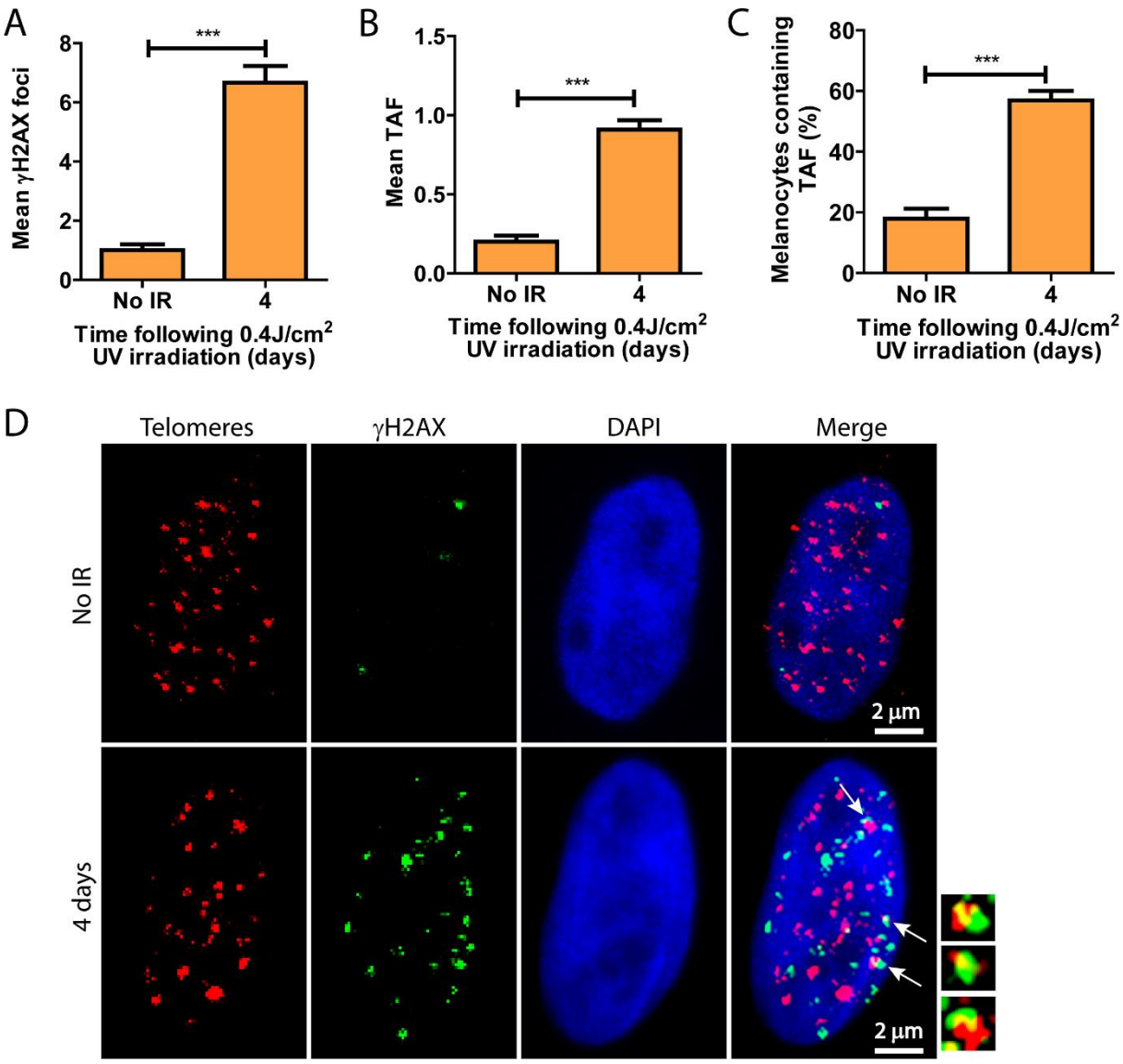

Figure 4.17 – Non-telomeric and telomeric DNA damage increase as a result of UV-induced melanocyte senescence. Melanocytes were exposed to 0.4J/cm^2 UVA and UVB radiation once a day for five consecutive days, and DNA and telomere damage were analysed at the time points indicated. Graphs show **(A)** mean number of γH2AX foci, **(B)** mean number of TAF, **(C)** percentage of melanocytes containing TAF. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a two-tailed unpaired t-test. ***P<0.001. **(E)** Representative immuno-FISH images of proliferating (top) and senescent (bottom) melanocytes. Telomeres are shown in red, γH2AX is depicted in green, and DAPI is shown in blue. Arrows indicate co-localisation between γH2AX and telomeres, which is amplified on the right. Images were taken using a 63x oil objective.

4.3.3. Telomere damage occurs irrespectively of length in UV-induced senescent melanocytes

As previously mentioned, telomere damage independently of length has been shown to occur in human cells during replicative-, stress- and oncogene-induced senescence (Kaul *et al.*, 2011; Hewitt *et al.*, 2012; Suram *et al.*, 2012). However, it is not yet known whether longer telomeres also signal a DDR in human melanocytes in response to repeated UV irradiation. In fact, it is possible that UV exposure would cause single-stranded breaks preferentially at telomeres, accelerating telomere shortening (von Zglinicki *et al.*, 2000).

To investigate whether length-independent telomere damage also occurred during UV-induced melanocyte senescence, I performed Q-FISH in both proliferating and senescent cells, and analysed individual telomere signal intensity as a measure of length. By comparing FISH signal intensity of γH2AX-positive and –negative telomeres, I found that the median length of dysfunctional telomeres was very similar to those not signalling a DDR, with no statistically significant difference between their lengths (P=0.55) (***Figure 4.18 A***). Moreover, it is possible to find two telomeres of similar lengths in one cell, one which is associated with γH2AX whereas the other remains undamaged following repeated exposure to UVA+B radiation (***Figure 4.18 B, C***), suggesting that DNA damage occurs irrespectively of length in UV-induced senescent melanocytes.

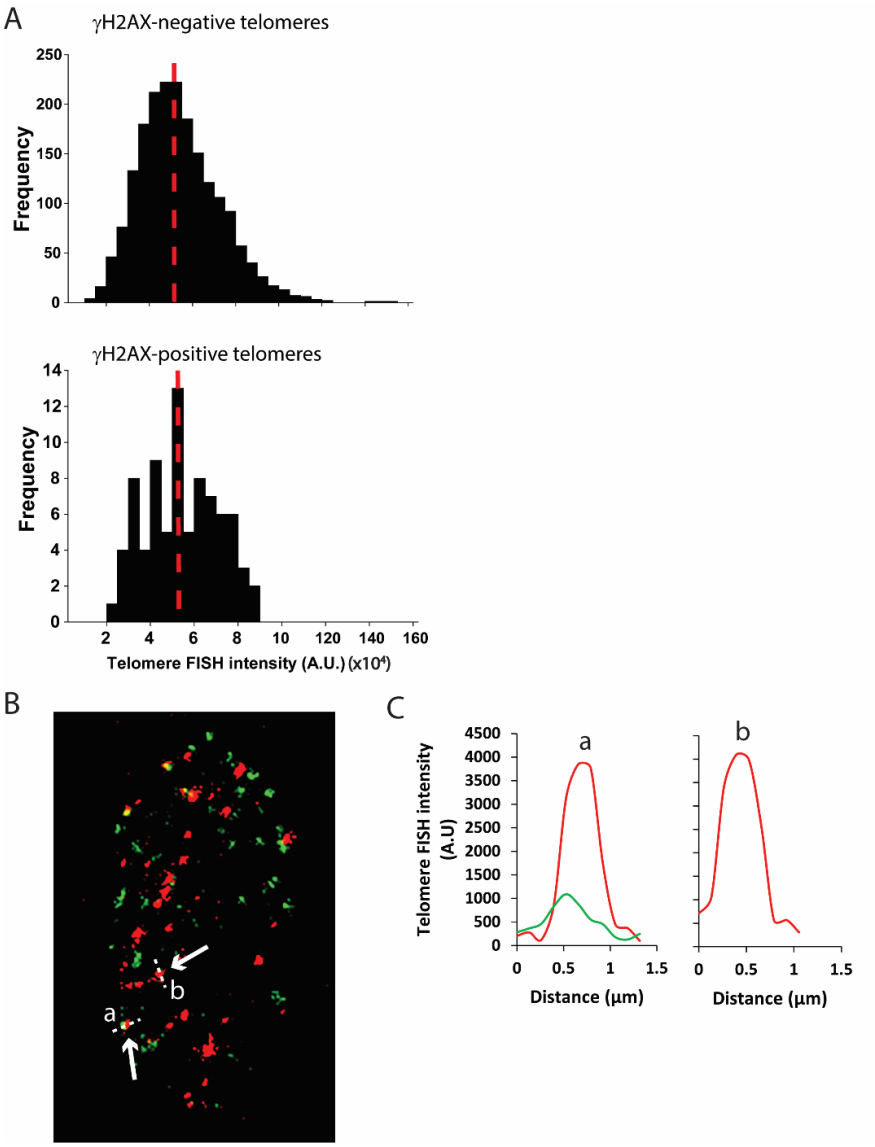

Figure 4.18 – Length-independent telomere damage occurs in UV-induced senescent melanocytes. Quantitative FISH was performed in proliferating and senescent melanocytes (4 days following the last exposure to 0.4J/cm^2 UVA and UVB IR), and FISH intensity of at least 1,800 individual telomeres was analysed as a measure of length. **(A)** Histograms showing intensity of γH2AX-negative and γH2AX-positive telomeres in both proliferating and senescent melanocytes. Red dotted line indicates median intensity. Statistical significance was determined by performing a Mann-Whitney U test. **(B)** Representative immuno-FISH image (red: telomeres; green: γH2AX) of a melanocyte containing telomeres of similar lengths, one which (a) co-localises with γH2AX and the other (b) is γH2AX-negative. Images were taken using a 63x oil objective. **(C)** Graphs showing the quantification of telomere intensity of γH2AX-positive (a) and γH2AX-negative (b) telomeres. Red line indicates telomere intensity, and green shows γH2AX intensity.

4.3.4. Senescence-associated secretory phenotype of UV irradiated melanocytes

Given that repeated exposure to UV irradiation induces senescence in melanocytes *in vitro*, I next wanted to determine if this phenotype was also accompanied by increased secretion of pro-inflammatory cytokines and chemokines, as observed with replicative and X-ray-induced senescence.

The development of a SASP in response to UV irradiation has not been extensively reported by previous studies. Moreover, given that the secretory phenotype is a dynamic process which develops over time following senescence induction (Coppe *et al.*, 2010a), I collected conditioned medium from senescent melanocytes at different time points after the last UV irradiation (6 and 18 days) in order to kinetically investigate the development of the secretory phenotype of these cells. Melanocytes were cultured in CM for 24 hours before media were collected for analysis. Different panels of cytokine arrays were performed, which overall detected the relative abundance of 58 cytokines and chemokines present in CM of proliferating and senescent cells. From the proteins analysed in these arrays, 14 were present at detectable levels in CM collected from cultured melanocytes (***Figure 4.19 A***). I observed that a distinct secretory phenotype significantly develops in senescent melanocytes at day 18, whereas at day 6 the secretion of the majority of proteins analysed remain similar to proliferating cells (***Figure 4.19 A***). These results suggest that the SASP progressively matures following the induction of UV-induced melanocyte senescence. Moreover, although there was an increase in secretion of pro-inflammatory factors by senescent melanocytes (***Figure 4.20 A-L***), statistical significance was only reached for IL-8, MCP-1, MIP-1α, MMP-3, and serpin E1 (***Figure 4.20 B-D, J, K***) likely due to the variability between experiments. Nonetheless, repeated UV exposure drives melanocyte senescence *in vitro* which is also accompanied by the development of a distinct pro-inflammatory secretory phenotype.

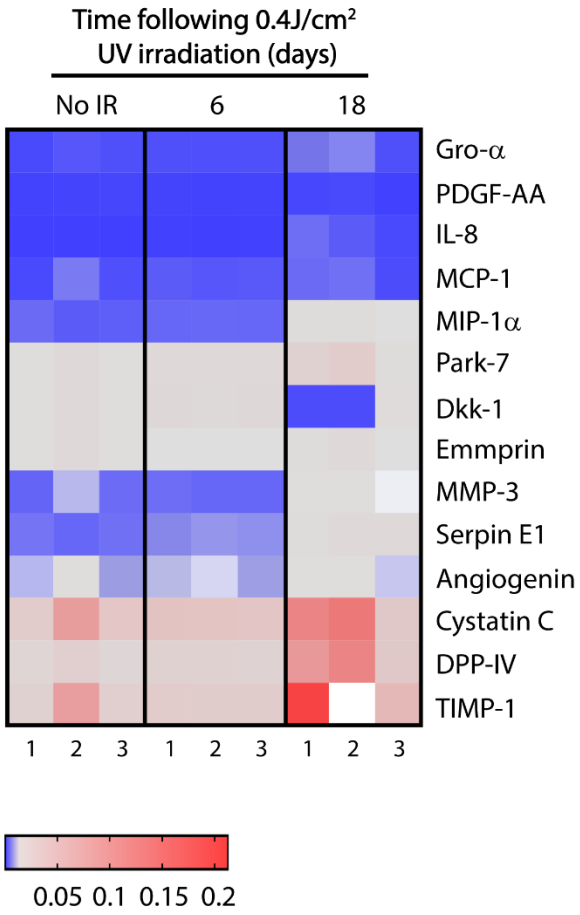

Figure 4.19 – Characterisation of the secretory phenotype of UV-induced senescent melanocytes. Heatmap showing cytokines detected in CM from young (non-irradiated) and senescent melanocytes (6 and 18 days after UVA+B irradiation). Blue represents low expression, whereas red denotes high expression. Concentration values are expressed as pg/ml/cell. Each column represents one independent experiment for the corresponding condition. Heatmap was generated using Prism 7.0. (Cytokine array analysis was done in collaboration with Duncan Talbot from Unilever).

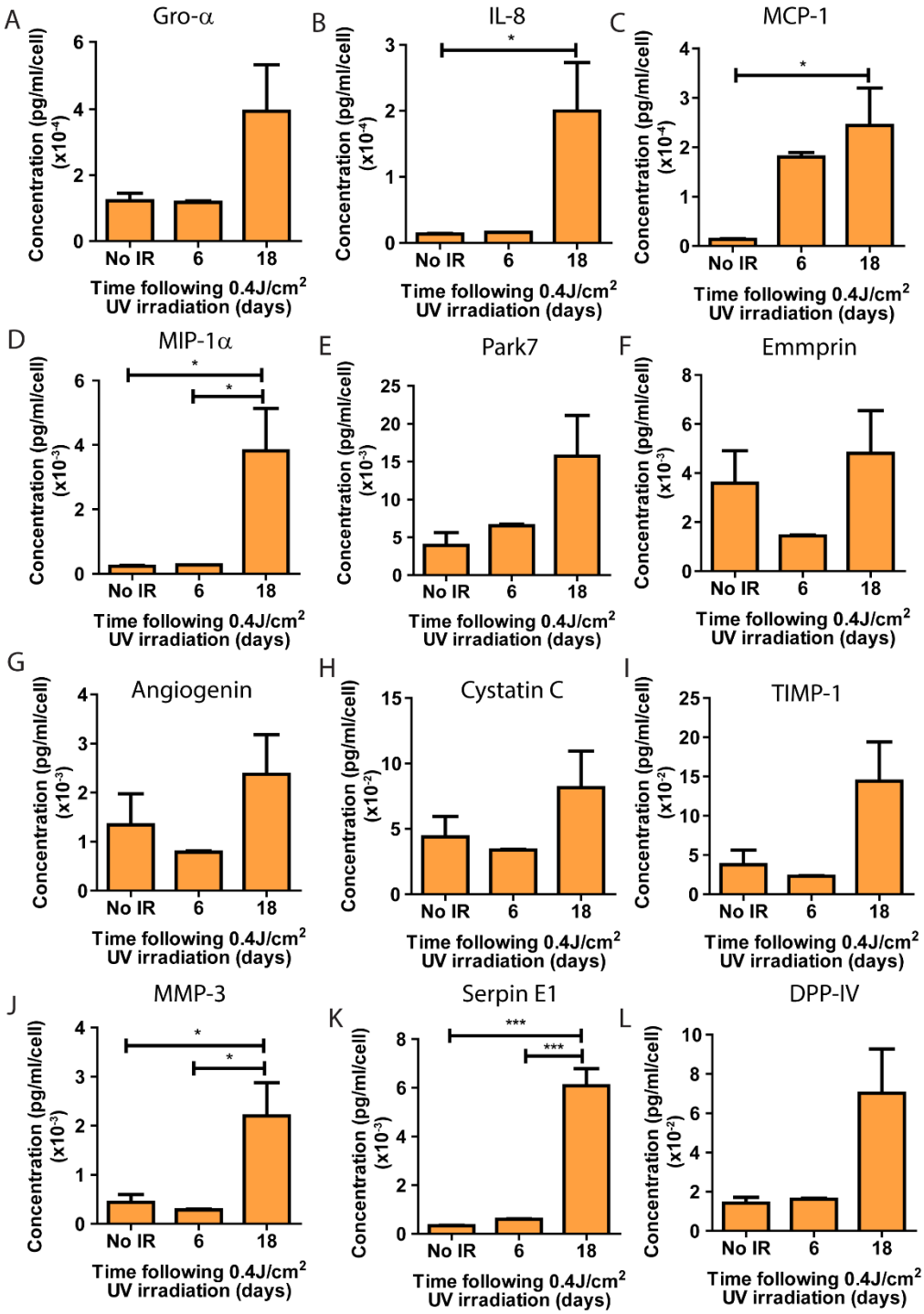

Figure 4.20 – Cytokines and chemokines up-regulated in UV-induced senescent melanocytes accounting for cell number. Graphs showing concentrations of **(A)** Gro-α, **(B)** IL-8, **(C)** MCP-1, **(D)** MIP-1α, **(E)** Park7, **(F)** Emmprin, **(G)** Angiogenin, **(H)** Cystatin C, **(I)** TIMP-1, **(J)** MMP-3, **(K)** Serpin E1 and **(L)** DPP-IV secreted by melanocytes at the time points indicated. The concentration of each protein was normalised by cell number. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01.

4.4. Expression of a telomere-specific endonuclease (TRF1-FokI) induces melanocyte senescence *in vitro*

So far, I have shown that telomere dysfunction occurs as a result of replicative and stress-induced (X-ray and UV irradiation) melanocyte senescence; however, it is not possible to establish causality between telomere damage and senescence from these observations. Therefore, to investigate whether dysfunctional telomeres were a cause or a mere consequence of senescence, we expressed a FLAG-tagged telomeric protein, TRF1, fused to the catalytic domain of FokI nuclease in melanocytes (TRF1-FokI), which has been shown to induce DSBs specifically at telomeres (Tang *et al.*, 2013; Cho *et al.*, 2014). In order to control the amount of telomeric DNA damage being induced, we cloned the TRF1-FokI fusion protein into a doxycycline (DOX) inducible lentiviral backbone, such that expression is only switched on in the presence of DOX. Melanocytes expressing a nuclease inactive TRF1-FokI-D450A mutant were used as controls (done in collaboration with Alison Howard and Gisela Otten).

Contrary to previous observations, new evidence has emerged suggesting that double-stranded breaks at telomeres can be repaired in proliferating cells (Doksani and de Lange, 2016; Mao *et al.*, 2016). Therefore, to determine whether telomeric DSBs could be repaired after the induction of TRF1-FokI, melanocytes expressing the fusion protein were treated with doxycycline for 6 hours, which was determined to be the optimal time of TRF1-FokI induction by DOX (data not shown). Following DOX removal, cells were cultured for an additional 3 and 7 days after induction (***Figure 4.21 A***), and immuno-FISH combining immunofluorescence for γH2AX and *in situ* hybridisation using a telomere specific probe was performed in order to analyse telomere DNA damage (in collaboration with Anthony Lagnado). I observed that the number of DNA damage foci gradually increased in melanocytes expressing TRF1-FokI at 3 and 7 days after induction compared to control cells in which the nuclease was not induced (no DOX) (***Figure 4.21 B***). Moreover, DNA damage was higher in TRF1-FokI melanocytes at 7 days in comparison to 3 days after induction, suggesting that additional DNA lesions are formed in a time-dependent manner. TRF1-FokI melanocytes also have increased DNA damage when compared to cells expressing the mutant TRF1-FokI(D450A) protein (***Figure 4.21 B***), indicating that a fraction non-telomeric DNA damage is also induced as a result of TRF1-FokI expression in melanocytes. Therefore, based on these results, it cannot be concluded that TRF1-FokI is telomere specific, and a comprehensive kinetic study needs to be performed in order to further elucidate its specificity.

Furthermore, TRF1-FokI-expressing cells have a higher frequency of telomere damage at both 3 and 7 days after induction when compared to melanocytes without nuclease induction (no DOX) (***Figure 4.21 C***), showing that TRF1-FokI activation indeed causes DNA damage at telomeres. Importantly, TRF1-FokI melanocytes display higher numbers of TAF when compared to cells expressing the nuclease inactive TRF1-FokI(D450A), confirming the functionality of the TRF1-FokI fusion protein. Interestingly, the amount of dysfunctional telomeres in TRF1-FokI melanocytes at day 7 was significantly higher when compared to day 3 (***Figure 4.21 C***). Given this time-dependent increase in telomere damage, these data suggest that telomeric DSBs are not repaired in melanocytes *in vitro*. Alternatively, it is also possible that secondary TAF are being generated following the DSBs caused by the fusion protein. However, in order to corroborate these findings, these experiments need to be independently confirmed.

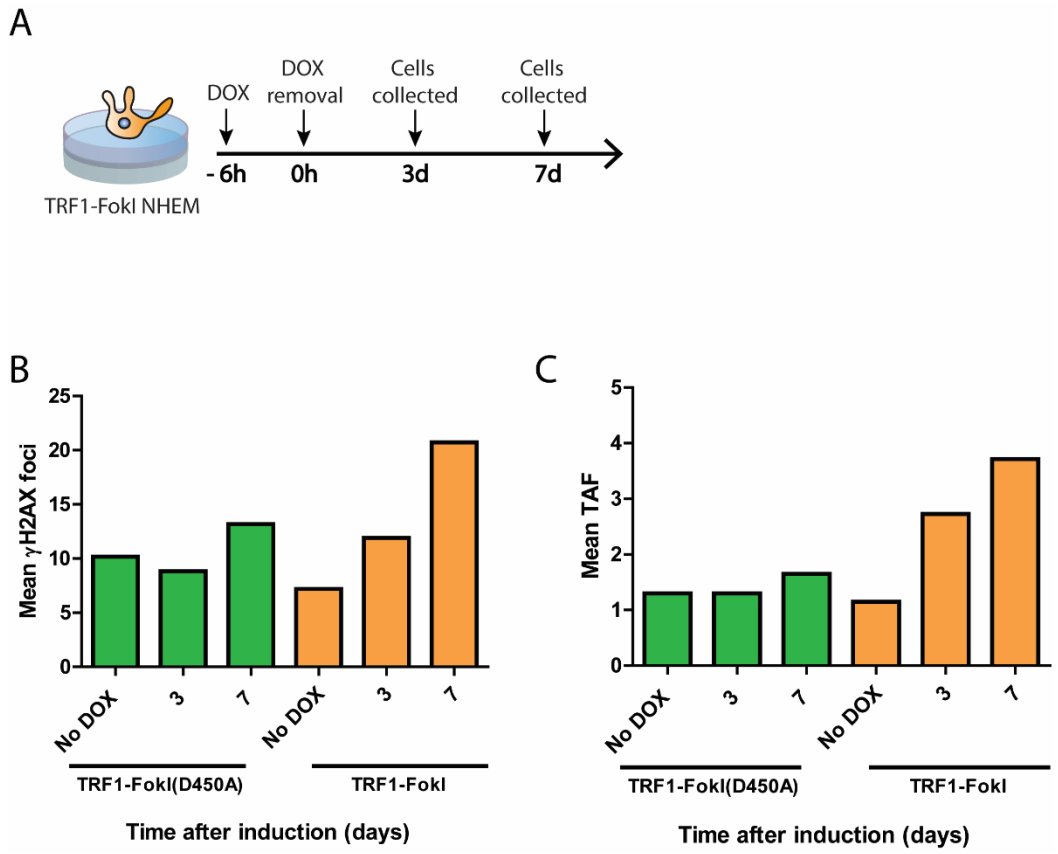

Figure 4.21 – Melanocytes expressing TRF1-FokI have increased non-telomeric and telomeric DNA damage. Melanocytes expressing TRF1-FokI or the nuclease inactive TRF1-FokI(D450A) mutant were incubated with DOX for 6 hours and collected at the time points indicated. **(A)** Scheme representing the experimental procedure. Graphs showing **(B)** mean number of γH2AX foci and **(C)** mean number of TAF in melanocytes expressing TRF1-FokI(D450A) or TRF1-FokI without nuclease induction (no DOX), and 3 and 7 days following induction by DOX. Data are mean of 17 – 35 cells obtained from at least 6 random planes (N=1).

Next, I wanted to determine whether induction of DNA damage by TRF1-FokI was sufficient to induce melanocyte senescence *in vitro.* For that, I analysed Sen-β-Gal activity in TRF1-FokI melanocytes at 3 and 7 days after nuclease induction. Although TRF1-FokI(D450A) cells showed an increase in Sen-β-Gal activity at 7 days, only 30% of TRF1-FokI(D450A) melanocytes showed Sen-β-Gal positivity, whereas this was increased to 80% in TRF1-FokI melanocytes (***Figure 4.22 A***). Moreover, an increase in Sen-β-Gal activity is observed only at 7 days following TRF1-FokI induction in melanocytes (***Figure 4.22 A***). To further corroborate the induction of senescence in these cells, I also carried out immunofluorescence for p16. Although TRF1-FokI melanocytes had higher basal levels of p16 prior to nuclease induction compared to TRF1-FokI(D450A) cells, I found that, at 7 days, TRF1-FokI melanocytes showed elevated p16 expression compared to TRF1-FokI cells at day 3 and without nuclease induction (***Figure 4.22 B***). Moreover, p16 expression was higher in TRF1-FokI melanocytes at all observed time points when compared to TRF1-FokI(D450A)-expressing cells (***Figure 4.22 B***). These results suggest that accumulation of DSBs leads to melanocyte senescence *in vitro*. However, given that non-telomeric damage was also observed upon TRF1-FokI induction, it cannot be concluded that senescence is a result of dysfunctional telomeres in this system. Therefore, these data need to be independently confirmed, and further experiments are needed to implicate telomeres are drivers of melanocyte senescence.

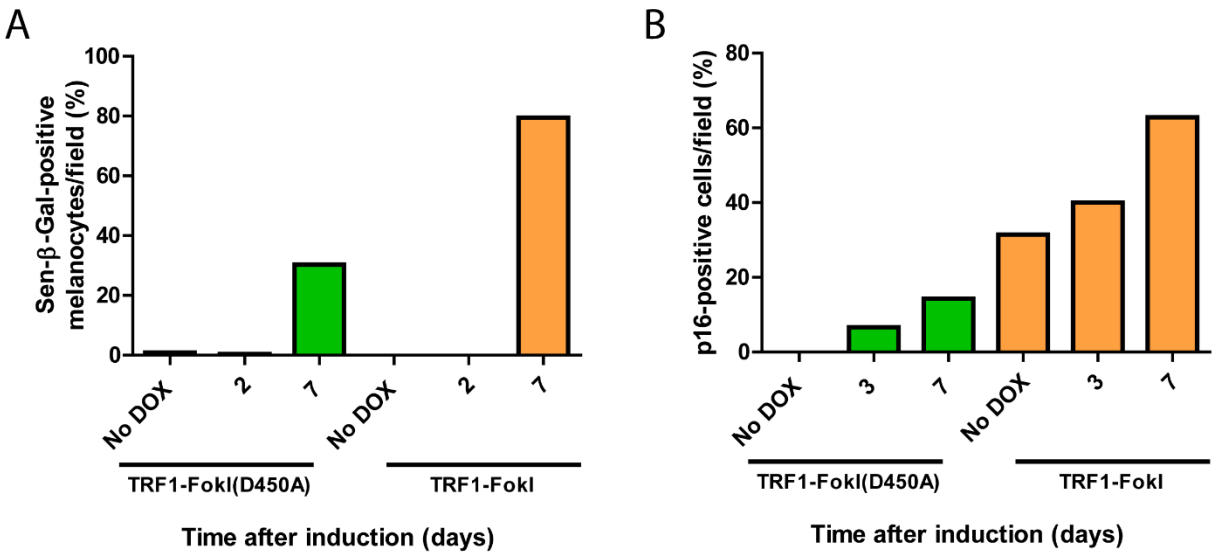

Figure 4.22 – Accumulation of DNA DSBs induces senescence in human melanocytes. (A) Percentage of Sen-β-Gal-positive melanocytes expressing TRF1-FokI(D450A) or TRF1-FokI without nuclease induction, and at 3 and 7 days after induction. **(B)** Percentage of p16-positive melanocytes expressing TRF1-FokI(D450A) or TRF1-FokI without nuclease induction, and at 3 and 7 days after induction (in collaboration with Anthony Lagnado). Data are mean of positive cells in 6 random fields (N=1). 100-200 cells were analysed for Sen-β-Gal activity, and a minimum of 27 cells were counted for p16 analysis.

4.5. Discussion

In this study, we exploited different stimuli to induce human melanocyte senescence *in vitro* in order to establish a suitable model to be used for the investigation of mechanisms of bystander DNA damage, which will be discussed in further detail in the following chapter. A summary of the phenotypes involved in replicative-, X-ray-, and UV irradiation-induced senescence can be seen in ***Table 4.1***. We found that the CDK inhibitor, p16, was up-regulated in all three models of senescence studied. This is agreement with previously published data, demonstrating that melanocyte senescence, both by replicative exhaustion and by stimulation of melanin synthesis, is mediated primarily by an increase in p16 (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001). The latter binds to and inhibits the cyclin-dependent kinases 4 and 6, in turn preventing Rb phosphorylation. Non-phosphorylated Rb remains bound to E2F transcription factors, and thus E2F target genes, which are important for G1/S transition, cannot be transcribed, promoting cell-cycle arrest (Rayess *et al.*, 2012). Indeed, melanocyte senescence is accompanied by Rb dephosphorylation, down-regulation of CDK-4 activity, and increased association of E2F4 with Rb (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.*, 2001). In fact, mutations in the CDKN2A gene, which encodes for p16, accounts for a large number of cases of familial melanoma, indicating that p16 is a major regulator of melanocyte growth arrest (Aoude *et al.*, 2015). The importance of the p16-pRb pathway in melanocyte senescence is corroborated by studies showing that a heterozygous deletion in the *Ink4a-Arf* locus, which affects both p16 and Arf, delayed senescence in mouse melanocytes in culture (Sviderskaya *et al.*, 2002), whilst no effects were observed on mouse fibroblast senescence (Serrano *et al.*, 1996). Accordingly, two strains of human melanocytes with deletions or mutations in both p16 alleles, had extended replicative lifespan *in vitro*, and became senescent after around 50 population doublings, whilst normal adult human melanocytes normally reach senescence after 10 PDs (Sviderskaya *et al.*, 2003). Interestingly, senescence in p16-deficient melanocytes was accompanied by elevated levels of p53 and p21, whereas this was not observed in normal melanocyte senescence, suggesting that p16 is the primary mediator of normal human melanocyte senescence, and that the p53-p21 pathway acts as a second barrier to promote growth arrest (Sviderskaya *et al.*, 2003).

	Senescence-inducing stimulus		
	Replication	**X-ray irradiation**	**UV irradiation**
p16	↑	↑	↑
p21	↑	↑	N.D.
mTOR activity	↑	±	N.D.
ROS	↑	↑	N.D.
TAF	↑	↑	↑
IL-8	↑	↑	↑
Gro-α	↑	↓	↑
PDGF-AA	↑	↓	±
MIP-1α	↑	↓	↑
MCP-1	↑	↓	↑
IL-6	↑	↑	Not detectable
RANTES	↑	↑	Not detectable
IL-4	↑	↓	Not detectable
FGF-2	↑	Not detectable	Not detectable
IP-10	Not detectable	↑	Not detectable
EGF	Not detectable	↓	Not detectable
MCP-3	Not detectable	↓	Not detectable
G-CSF	Not detectable	↓	Not detectable
VEGF	Not detectable	↓	Not detectable
MDC	Not detectable	±	Not detectable
IL-5	Not detectable	±	Not detectable
TIMP-1	Not detectable	Not detectable	↑
MMP-3	Not detectable	Not detectable	↑
Park7	N.D.	N.D.	↑
Emmprin	N.D.	N.D.	↑
Angiogenin	N.D.	N.D.	↑
Cystatin C	N.D.	N.D.	↑
Serpin-E1	N.D.	N.D.	↑
DPP-IV	N.D.	N.D.	↑
Dkk-1	N.D.	N.D.	±

Table 4.1 – Comparison of phenotypes of melanocyte senescence induced by different stimuli. Table summarising the phenotypes of replicative, X-ray- and UV-induced melanocyte senescence. ↑: indicates increased levels, ±: indicates no change, N.D.: not determined.

Our results also demonstrated that p21 levels increase in both replicative and stress-induced (X-ray) senescent melanocytes, contrary to previously published data showing that p21 is down-regulated during melanocyte senescence (Bandyopadhyay and Medrano, 2000; Bandyopadhyay *et al.,* 2001; Sviderskaya *et al.*, 2003). However, the regulation of melanocyte senescence has been shown to be dependent on the culture medium composition, with different groups not only reporting a wide range of lifespans depending on the media used, but also different expression patterns of CDK inhibitors, such as p21, at the time of senescence (Bennett, 2003). Although p16 was always up-regulated in senescent melanocytes regardless of the growth medium (Haddad *et al.*, 1998; Bandyopadhyay *et al.*, 2001), cells cultured in the presence of cAMP agonists, such as cholera toxin (CT), senesced with low levels of p21 (Bennett, 2003), whereas p21 up-regulation was observed in melanocytes senescing without supplementation of cAMP agonists (Haddad *et al.,* 1998). In fact, the growth medium used in this study did not contain cAMP agonists, possibly explaining our observation that p21 levels increase in replicative and stress-induced (X-ray) senescent melanocytes. Moreover, interferon-γ (IFN-γ) treatment was shown to induce melanocyte senescence with a concomitant increase in p21 levels (Wang *et al.*, 2014), and UVB irradiation induces a p21-dependent G1 growth arrest in melanocytes (Medrano *et al.*, 1995), suggesting that different senescence-inducing stimuli can also affect the expression of CDK inhibitors. Therefore, it is possible that p21 up-regulation observed in our model of stress-induced (X-ray) melanocyte senescence is a result of the type of stimulus. Although previous studies have demonstrated that p21 is important for senescence induction in other cells types, such as fibroblasts (Brugarolas *et al.*, 1995; Brown *et al.*, 1997), we cannot establish a causal role for p21 in melanocyte senescence from our studies. Therefore, future experiments could be performed using p21 knockdown melanocytes in order to further investigate the involvement of p21 in melanocyte senescence.

We have also shown that lamin B1 is down-regulated in melanocytes during replicative senescence. In agreement, a reduction in lamin B1 levels has been reported in replicative senescent human fibroblasts and BRAF-induced senescent human melanocytes (Shimi *et al.*, 2011; Sadaie *et al.*, 2013). Lamin B1 is a nuclear envelope protein that is important for nuclear stability, and also plays a role in transcription and chromatin remodelling (Spann *et al.*, 2002; Shimi *et al.*, 2008). Interestingly, a reduction in lamin B1 during senescence was shown to lead

to spatial repositioning of perinuclear heterochromatin, which was proposed to contribute to SAHF formation, heterochromatic regions that silence genes required for cell-cycle progression (Narita *et al.*, 2003; Sadaie *et al.*, 2013). Therefore, loss of lamin B1 might contribute to the proliferation arrest during melanocyte replicative senescence. In fact, heterochromatic changes have been proposed to occur in replicative senescent melanocytes (Bandyopadhyay *et al.*, 2002). Down-regulation of the p300/CBP histone acetyltransferase was shown to promote deacetylation and silencing of the cyclin E gene, a cyclin that controls progression through S phase (Bandyopadhyay *et al.*, 2002). It is believed that loss of acetylation contributes to formation of repressive heterochromatin, thus silencing genes associated with proliferation, and contributing to melanocyte senescence (Bandyopadhyay *et al.*, 2002). However, it is important to note that we only observed a mild decline in lamin B1 levels in our study. Therefore, these experiments will need to be independently confirmed in order to achieve further conclusions regarding the role of lamin B1 during melanocyte senescence.

The observation that mTORC1 activity is upregulated in replicative melanocyte senescence is consistent with studies showing that mTOR inhibition by rapamycin extends the replicative lifespan of human and murine fibroblasts in culture (Kolesnichenko *et al.*, 2012; Pospelova *et al.*, 2012), implicating mTORC1 in the senescence process. Although the mechanisms leading to mTOR activation during senescence are still not fully understood, mTOR is thought to contribute to the senescence phenotype by promoting translation of pro-inflammatory factors involved in the SASP (Herranz *et al.*, 2015; Laberge *et al.*, 2015). One study demonstrated that mTOR inhibition diminished the SASP in fibroblasts undergoing replicative, oncogene- and stress-induced senescence (Herranz *et al.*, 2015). It was shown that mTOR regulates the translation of the kinase MK2, which in turn phosphorylates the RNA-binding protein ZFP36L1, inhibiting the degradation of transcripts of proteins involved in the SASP (Herranz *et al.*, 2015). Furthermore, inhibiting mTOR was also shown to decrease translation of the pro-inflammatory protein, IL-1α, in senescent cells, in turn reducing the transcriptional activity of NF-κB, which regulates the transcription of many SASP genes (Laberge *et al.*, 2015). Interestingly, supressing mTORC1 activity inhibited the SASP without reversing the growth arrest, suggesting that the effects of mTOR on the pro-inflammatory secretory phenotype are dissociated from the cell-cycle arrest (Herranz *et al.*, 2015; Laberge *et al.*, 2015). Others have suggested that mTORC1 signalling stimulates mitochondria biogenesis during senescence, and contributes to the maintenance of a DDR through increased ROS production (Correia-Melo *et al.*, 2016), which is important for the induction of senescence and the SASP (Rodier *et al.*,

2009; Passos *et al.*, 2010). It is also possible that mTORC1 activation mediates growth arrest in response to a DDR by directly interacting with components of the DNA damage response, such as Mdm2, a negative regulator of p53 (Lai *et al.*, 2010). Upon DNA damage induction, it was shown that S6K1, a downstream target of mTORC1, forms a complex with Mdm2, inhibiting p53 ubiquitination, thus stabilising p53, which can then mediate cell cycle arrest (Lai *et al.*, 2010). However, in this study, such interactions were reported to occur a few hours after a DDR, which does not explain how a stable arrest in maintained in the context of senescence.

In contrast to replicative senescence, our results suggest that mTORC1 activity remains unchanged in stress-induced (X-ray) senescent melanocytes. One possibility is that mTOR activation occurs early following genotoxic stress, and returns to basal levels after a few days, providing an explanation for the constant levels of mTOR activity observed in melanocytes at 3 and 10 days following X-ray irradiation. Indeed, studies have shown that mTORC1 activity peaks at 4 hours after induction of DNA damage in mouse fibroblasts, and gradually decreases at 8 and 24 hours after damage (Lai *et al.*, 2010). Consistent with this, X-ray irradiation of human fibroblasts results in mTOR activation during the first 24 hours after the induction of stress, and then decreases at 3 days, albeit not to control levels (Correia-Melo *et al.*, 2016). A slight increase in mTOR activity has also been reported in human oral keratinocytes 24 hours post-irradiation, and inhibition of mTOR by rapamycin was shown to reduce a DDR and markers of senescence in these cells (Iglesias-Bartolome *et al.*, 2012). It is possible that mTOR is involved in the early stages of induction of melanocyte senescence in response to genotoxic stress but this is not detected in this study as we have only analysed mTOR activity at 3 and 10 days following irradiation. Therefore, in order to obtain a better understanding of the role of mTORC1 in stress-induced melanocyte senescence, it is important to analyse its activity in the first few hours following genotoxic stress. Nonetheless, mTORC1 down-regulation has been reported in BRAF-induced growth arrested melanocytes in mice *in vivo*, suggesting that mTOR activation can also depend on the nature of the senescence-inducing stimulus (Damsky *et al.*, 2015). However, it is unlikely that mTORC1 is not involved in the senescent phenotype of melanocytes, since its inhibition diminished the SASP during stress-induced (X-ray) melanocyte senescence, which will be discussed in the following chapter.

Further characterisation of the senescent phenotype of human melanocytes revealed that both replicative and stress-induced (X-ray) senescence are accompanied by increased oxidative stress, in accordance with previously published data showing that increased ROS is involved in fibroblast senescence in response to genotoxic stress or replicative exhaustion (Saretzki *et al.*,

2003; Passos *et al.*, 2007; Passos *et al.*, 2010). Consistent with a role for oxidative stress in melanocyte senescence, early studies have demonstrated that melanocyte proliferation is impaired when these cells are cultured in hyperoxic conditions (Horikoshi *et al.*, 1991). Increased intracellular ROS has been reported in IFN-γ-induced melanocyte senescence, and treatment with the antioxidant N-acetylcysteine (NAC) resulted in decreased p21 and IL-6 release, and prevented melanocyte senescence (Wang *et al.*, 2014). Although MitoQ has been shown to extend the replicative lifespan of fibroblasts under conditions of mild oxidative stress (Saretzki *et al.*, 2003), our results demonstrated that scavenging mitochondrial ROS by MitoQ treatment was not sufficient to prevent Sen-β-Gal activity in senescent melanocytes. Therefore, from these results, we cannot conclude that enhanced ROS generation mediates melanocyte senescence. However, other senescence markers need to be analysed in MitoQ-treated senescent melanocytes in order to further investigate the impact of ROS in this process. Moreover, overexpression and knockdown of antioxidant enzymes in melanocytes could also shed light on the involvement of oxidative stress in melanocyte senescence.

Nonetheless, in agreement with our findings that senescent melanocytes display higher levels of ROS, oncogene-induced melanocyte senescence has also been shown to involve mechanisms leading to increased ROS generation (Leikam *et al.*, 2008; Kaplon *et al.*, 2013). For example, melanocyte senescence induced by N-RAS activation is accompanied by enhanced ROS production and DNA damage, and senescence can be prevented by NAC treatment (Leikam *et al.*, 2008). Moreover, increased ROS generation was shown to occur in BRAF-induced senescence as a result of increased mitochondrial respiration (Kaplon *et al.*, 2013). More specifically, BRAF(V600E) expression, an oncogene commonly associated with melanocytic nevi, led to activation of the mitochondrial gatekeeper enzyme pyruvate dehydrogenase, which increased the use of pyruvate in the citric acid cycle, resulting in enhanced respiration and oxidative stress (Kaplon *et al.*, 2013). Although the mechanisms leading to increased ROS generation in replicative and stress-induced senescent melanocytes are not fully understood, it has been shown that ROS can be induced downstream of a DDR, whereby continuous activation of p21 induces mitochondrial dysfunction and ROS generation through signalling pathways involving GADD45-p38MAPK-TGFβ (Passos *et al.*, 2010). The ensuing oxidative stress causes further DNA damage, maintaining a positive feedback loop between a DDR, p21 and ROS which stabilises the senescence growth arrest (Passos *et al.*, 2010). Indeed, overexpression of p21 results in increased ROS and cell-cycle arrest in fibroblasts (Macip *et al.*, 2002). More recently, it has been proposed that a DDR induces PGC-1β-dependent mitochondrial biogenesis

through mechanisms involving ATM, Akt and mTOR, contributing to increased ROS production, which maintains a DDR and stabilises the senescence phenotype (Correia-Melo *et al.*, 2016). In addition, increased ROS as a result of extensive telomere attrition has been reported. It was shown that p53 activated in response to telomere shortening induces mitochondrial dysfunction by repressing PGC-1α and PGC-1β promoters, which impairs mitochondrial biogenesis and function, and in turn elevates ROS levels (Sahin *et al.*, 2011). In fact, our results show that telomere shortening occurs during melanocyte replicative senescence; therefore, it is possible that short telomeres activate such downstream signalling, contributing to increased ROS production in senescent melanocytes. However, telomerase would need to be expressed in melanocytes *in vitro* in order to further investigate the role of telomere shortening in the induction of ROS during melanocyte senescence.

Although we did not investigate whether ROS generation is enhanced in UV-induced melanocyte senescence, it is well established that induction of ROS is one of the main effects of UV irradiation (Klotz *et al.*, 1999; Maziere *et al.*, 2001; Heck *et al.*, 2003; Cotter *et al.*, 2007). Therefore, it is possible that oxidative stress is also associated with the phenotype of UV-induced senescent melanocytes. One obvious response to UV irradiation is increased melanin production, which offers protection against UV-induced DNA damage (Kobayashi *et al.*, 1998; Smit *et al.*, 2001). Paradoxically, melanin synthesis is a highly oxidative process, although its confinement to melanosomes protects cellular components from oxidative damage (Denat *et al.*, 2014). Nevertheless, tyrosine-induced melanogenesis has been shown to increase intracellular ROS and decrease levels of the antioxidant glutathione in lightly-pigmented melanocytes (Smit *et al.*, 2008). Moreover, in some cases, melanin has been shown to act as a pro-oxidant, contributing to oxidative stress. For example, it has been shown that melanocytes with higher content of the red pigment pheomelanin are more sensitive to UVA-induced single-stranded DNA breaks (Wenczl *et al.*, 1998). It was suggested that oxidative stress was significantly enhanced by increased pheomelanin content, contributing to UVA sensitivity (Wenczl *et al.*, 1998). Indeed, it is known that ROS production is stimulated upon UV irradiation of pheomelanin pigments, a characteristic owed to its chemical structure (Napolitano *et al.*, 2014). Consistently, another study demonstrated that melanocytes with a higher pheomelanin: eumelanin ratio had increased levels of UVB-induced oxidative DNA lesions (Smit *et al.*, 2001). Therefore, it appears that the balance between the pro-oxidant and protective properties of melanin depends on the ratio of eumelanin to pheomelanin in the cell. Interestingly, senescent melanocytes have been shown to have increased melanin content

(Horikoshi *et al.*, 1991; Bandyopadhyay and Medrano, 2000). Moreover, dysplastic nevi cells, which are melanoma precursors, are more pigmented than normal melanocytes from the same donor. These cells were shown to contain a higher pheomelanin to eumelanin ratio, and senesced prematurely when compared to normal melanocytes (Smit *et al.*, 2008). Therefore, it is possible that melanin contributes to elevated ROS production in normal human senescent melanocytes. Although it is not known whether the ratio of pheomelanin to eumelanin increases in these cells, this would be an interesting topic for further investigation.

As well acting as effector molecules downstream of the activation of senescence signalling pathways, increased ROS generation can also contribute to the induction of senescence by directly inducing DNA damage. For example, premature senescence can be induced in fibroblasts by treatment with sub-lethal concentrations of H_2O_2 (Chen *et al.*, 1998). Interestingly, mild oxidative stress causes SSBs to preferentially accumulate at telomeres, which results in an accelerated rate of telomere shortening and premature senescence (von Zglinicki *et al.*, 1995; von Zglinicki, 2000; von Zglinicki, 2002). Accordingly, reducing intracellular ROS levels, either by the use of antioxidants or by overexpression of antioxidant enzymes, prevents telomere shortening and extends replicative lifespan of human fibroblasts (Saretzki *et al.*, 2003; Serra *et al.*, 2003). Indeed, telomeres are believed to be highly susceptible to oxidative stress due to their high content of guanine triplets, which are extremely sensitive to oxidative modifications (Oikawa and Kawanishi, 1999). In fact, our results demonstrate that the frequency of telomere damage increases during replicative and stress-induced (X-ray and UV) melanocyte senescence. Although it is not clear whether telomere dysfunction in these models occurs as a direct result of increased ROS generation, it is possible that ROS contributes to the formation of telomeric DNA damage during melanocyte senescence. In order to corroborate this hypothesis, it will be important to analyse DNA damage in senescent melanocytes treated with MitoQ.

Interestingly, we observed that telomere damage occurs independently of length in replicative, X-ray-, and UV-induced melanocyte senescence, suggesting that longer telomeres are also able to signal a DDR in response to stress. This is consistent with previous studies showing that length-independent telomere dysfunction can occur in cells undergoing replicative and stress-induced senescence *in vitro* (Kaul et al., 2011; Hewitt et al., 2012), and has also been demonstrated in human melanocytic nevi (Suram *et al.*, 2012), as well as in many tissues of mice with age (Hewitt *et al.*, 2012; Jurk *et al.*, 2014). It has been previously proposed that damage occurs at long telomeres as they represent a more abundant target for break formation

(Fumagalli *et al.*, 2012). This is supported by studies showing that forced elongation of telomere length sensitises cancer cells to ionising radiation, suggesting that DSBs preferentially accumulate at telomeres exceeding a critical length (Fairlie and Harrington, 2015). However, our *in vitro* data does not support the idea that longer telomeres accumulate more damage than shorter ones. In fact, consistent with previously published data (Hewitt *et al.*, 2012; Suram *et al.*, 2012), we have not identified a significant difference in length between dysfunctional and non-damaged telomeres. Indeed, it is possible that the method used to analyse individual telomere length in this study is not sensitive enough to detect very short telomeres, thus making it difficult to accurately identify significant differences in length between damaged and non-damaged telomeres. Therefore, in order to confirm these results, super resolution microscopy could be used as a more sensitive method, which would likely detect weaker FISH signal intensities when compared to wide-field or confocal microscopy.

It is also possible that telomeres become dysfunctional due to loss of shelterin proteins, or uncapping, in senescent melanocytes. For example, during replicative senescence, extensive telomere shortening is believed to lead to displacement of shelterin proteins, which in turn exposes telomeric ends that are then recognised as DSBs by the DNA repair machinery (O'Sullivan and Karlseder, 2010). In support of this, fibroblasts expressing a dominant-negative allele of TRF2, which triggers uncapping, have increased numbers of dysfunctional telomeres, indicating that uncapped telomeres activate a DDR (Takai *et al.*, 2003). Given that our results show significant loss of telomeric repeats during replicative melanocyte senescence *in vitro*, it is possible that uncapping contributes to DDR activation at telomeres in this process. Furthermore, studies have shown that oxidative DNA damage disrupts the binding of TRF1 and TRF2 at telomeres (Opresko *et al.*, 2005), which would provide a mechanism for length-independent loss of shelterin. This would be consistent with our results demonstrating increased ROS and length-independent telomere damage in senescent melanocytes. However, telomere dysfunction in the presence of TRF2 has been previously reported in stress-induced (Fumagalli *et al.*, 2012) and replicative senescence (Kaul *et al.*, 2011), as well as in melanocytic nevi (Suram *et al.*, 2012), suggesting that DDR activation can occur independently of telomere uncapping. An additional possibility has been proposed by Cesare *et al.*, suggesting that telomeres can also exist in an intermediary state of capping, whereby a DDR is activated but sufficient TRF2 or other shelterin components are retained to suppress NHEJ, leading to a persistent DDR at telomeres irrespectively of length (Cesare *et al.*, 2009). In order to further investigate whether uncapping contributes to telomere damage during melanocyte senescence,

telomere FISH should be performed together with immunofluorescence against γH2AX, and a shelterin protein such as TRF2, to allow analysis of co-localisation between γH2AX-positive telomeres and TRF2 in cultured melanocytes.

Our data also suggest that telomere dysfunction is associated with melanocyte senescence, since telomere damage was shown to accumulate in replicative, X-ray and UV-induced senescent melanocytes. As previously mentioned, telomeres are highly sensitive to oxidative stress when compared to the rest of the genome (Petersen *et al.*, 1998). In addition, damage occurring at telomeres is less efficiently repaired (Kruk *et al.*, 1995; Rochette and Brash, 2010), mainly because components of the shelterin complex inhibit proteins of the DNA repair machinery (Bombarde *et al.*, 2010; Fumagalli *et al.*, 2012). This contributes to persistent DNA damage signalling at telomeres, which is important for the initiation and maintenance of senescence through continuous activation of p21 and p16 (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003; Jacobs and de Lange, 2004). In accordance to this, our results demonstrate that following X-ray irradiation, non-telomeric DNA damage appears to be repaired at 10 days, whilst telomere-associated damage remains high, suggesting that DDR signalling at telomeres is indeed persistent. Future analysis of telomere damage at later time points after UV irradiation of melanocytes should also reveal whether telomere-associated foci are persistent following repeated UV exposure. Consistent with our observations, dysfunctional telomeres have been shown to accumulate in human fibroblasts during replicative (Kaul *et al.*, 2011), stress- (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012), and oncogene-induced senescence (Suram *et al.*, 2012). Moreover, the majority of long-lived DNA damage foci were shown to co-localise with telomeres in stress-induced senescent fibroblasts (Hewitt *et al.*, 2012), supporting the idea that persistent telomere-associated damage contributes to the senescence phenotype. However, from our results, we cannot establish that a persistent DDR at telomeres is in fact an inducer of melanocyte senescence. Although expression of TRF1-FokI induced markers of senescence in melanocytes, the fusion endonuclease generated both telomeric and non-telomeric DNA damage, and thus we cannot conclude that senescence was induced specifically as a result of telomere dysfunction. Moreover, a fraction of melanocytes expressing mutant TRF1-FokI also displayed a small increase in senescence markers, suggesting that the transduction could have contributed to induction of senescence in some cells. Although previous studies suggest that persistent DNA damage plays a role in the induction and maintenance of the senescence phenotype (d'Adda di Fagagna *et al.*, 2003; Passos *et al.*, 2010; Fumagalli *et al.*, 2014), further

experiments involving the inhibition of DDR signalling will need to be performed in order to corroborate that this is also the case in melanocyte senescence.

Recent studies have challenged the irreparability of telomeres, providing evidence that telomeric DSBs are repaired by mechanisms involving homologous recombination and alternative (alt)-NHEJ (Doksani and de Lange, 2016; Mao *et al.*, 2016). This is in contrast with our results demonstrating that telomere damage induced by transient expression of TRF1-FokI remained high even one week after induction. Although these data suggest that TAF are not repaired by melanocytes, we cannot exclude the possibility secondary telomere damage is also being induced following TRF1-FokI induction. One possible scenario is that activation of a DDR at telomeres causes an increase in intracellular ROS, which in turn would generate further breaks at telomeres. An increase in oxidative stress could also explain the induction of non-telomeric DNA damage seen in melanocytes expressing TRF1-FokI. In support of this, secondary DNA damage as a result of telomere dysfunction has been reported in fibroblasts expressing a dominant-negative TRF2 allele and in fibroblasts from late-generation $TERC^{-/-}$ mice (Passos *et al.*, 2010). These cells displayed high levels of mitochondrial ROS, and antioxidant treatment reduced DNA damage foci induction (Passos *et al.*, 2010). However, further experiments are needed to confirm that this occurs in TRF1-FokI melanocytes. Firstly, levels of intracellular ROS should be measured by flow cytometry to ascertain if induction of the fusion protein increases generation of ROS. Moreover, antioxidant treatment following TRF1-FokI induction would allow us to determine if a further increase in both telomeric and non-telomeric damage could be prevented by scavenging excess ROS. Another explanation for the increase in non-telomeric DNA damage in TRF1-FokI melanocytes is that TRF1 could be located in genomic sites other than telomeres, similarly to TRF2 and RAP1 (Bradshaw *et al.*, 2005; Martinez *et al.*, 2010). However, studies in mice have not identified extra-telomeric TRF1 (Garrobo *et al.*, 2014), and other studies using TRF1-FokI have reported exclusive localisation at telomeres (Tang *et al.*, 2013; Doksani and de Lange, 2016), making this possibility less likely. Overall, based on our results, we cannot conclude that TRF1-FokI induces telomere-specific DSBs, and a more comprehensive kinetic study will need to be performed in order to investigate its specificity. Nonetheless, we have demonstrated that telomeres are irreparable in melanocytes independently in one experiment.

It is important to note that, in one study, damage repair at telomeres was only observed in proliferating cells, such as BJ fibroblasts and HeLa cells (Mao *et al.*, 2016). Interestingly, HeLa cells, which are faster dividing cells, display a faster repair kinetics compared to slower dividing

fibroblasts, suggesting that proliferation rate is an important determinant of telomeric DSB repair (Mao *et al.*, 2016). On the other hand, when senescence was induced in fibroblasts either by genotoxic stress or replicative exhaustion, no significant repair was detected at telomeres (Mao *et al.*, 2016). By using the CRISPR-Cas9 system to specifically target telomeric repeats, the authors were able to induce DSBs specifically at telomeres in HEK cells, and provided evidence for telomere damage repair by homologous recombination (Mao *et al.*, 2016). Another group also expressed the fusion protein TRF1-FokI to induce telomere-specific damage in mouse embryonic fibroblasts (Doksani and de Lange, 2016). Consistent with our observations in melanocytes, TRF1-FokI expression induced significant telomere damage in MEFs, which was accompanied by activation of ATM-dependent kinase signalling in S-phase; however, these breaks were shown to be efficiently repaired by mechanisms involving both HR and alt-NHEJ (Doksani and de Lange, 2016). The aforementioned studies suggest that in dividing cells, particularly in S phase, telomeric breaks can be repaired via HR; however, this does not occur in senescent cells. This could be a consequence of heterochromatin changes that occur during senescence (Narita *et al.*, 2003), making telomeric DNA less accessible for the repair machinery, explaining the persistent DDR signalling at telomeres observed in senescent cells (Mao *et al.*, 2016). However, further studies are required to confirm this hypothesis. Another possibility is that that senescence-inducing stimuli are likely to cause a large amount of damage, which might be greater than the repair capacity of cells, resulting in accumulation of telomere damage which cannot be repaired, and contributing to senescence (Mao *et al.*, 2016). Although these studies suggested that generating telomeric DSBs did not lead to senescence, this was only shown in immortalised cells (Doksani and de Lange, 2016; Mao *et al.*, 2016). On the contrary, our results indicate that TRF1-FokI expression induces senescence in normal human melanocytes. However, since both non-telomeric and telomeric damage were induced by the fusion protein, we cannot conclude that senescence occurred as a result of telomere damage specifically. Therefore, these experiments will need to be independently confirmed once the specificity of TRF1-FokI used in our study is corroborated. Additionally, future work should also involve expression of TRF1-FokI in melanoma cell lines to confirm whether telomere damage can be repaired in rapidly dividing, transformed cells. One possible scenario is that, in these cells, dysfunctional telomeres might play a more important role in maintaining the stable growth arrest rather than being involved in the initiation of senescence.

Although persistent telomere-associated foci are important players during senescence, we cannot exclude the possibility that non-telomeric damage also contributes to the establishment

and maintenance of melanocyte senescence. Our data demonstrate that DNA damage foci also remained significantly higher in melanocytes during stress-induced senescence (X-ray and UV), and to a lesser extent in replicative senescent melanocytes. In agreement with this, previous studies have shown that in senescent cells, half of the DNA damage foci were located at non-telomeric sites, and albeit short-lived, they are constantly renewed during senescence in a ROS-dependent manner (Passos *et al.*, 2010; Hewitt *et al.*, 2012). This would be consistent with our results showing that oxidative stress increases in senescent melanocytes, probably explaining a mechanism by which DNA damage remains elevated following genotoxic stress. Moreover, it has been shown that melanocytes are more prone to the accumulation of oxidative DNA damage upon UVA irradiation when compared to skin fibroblasts in culture, which was suggested to be due to the ROS-enhancing effects of melanin, as previously discussed (Wang *et al.*, 2010). Interestingly, melanocytes also display reduced DNA repair capacity in response to such lesions (Wang *et al.*, 2010). The authors showed that addition of melanin to fibroblast lysates was sufficient to diminish the repair capacity of oxidative DNA damage in these lysates. It was proposed that melanin could interact with proteins of the DNA repair machinery or with DNA itself, thus preventing DNA repair (Wang *et al.*, 2010). Since melanin synthesis occurs within enclosed structures known as melanosomes, the extent of the interaction between melanin and DNA in a physiological setting is unclear. However, it is likely that the increased susceptibility of melanocytes to oxidative DNA damage could account for the large increase in DNA damage foci observed in UV-induced senescent melanocytes in our study. In order to investigate whether this damage can be repaired, immunofluorescence against DNA damage proteins should be done in melanocytes at later time points after UV exposure, which would allow us to gain a better understanding of the kinetics of damage repair following UV irradiation.

We have also demonstrated that melanocytes develop a senescence-associated secretory phenotype during replicative and both models of stress-induced senescence. The molecular profile of the SASP was shown to differ depending on the senescence-inducing stimulus (**Table 4.1**), which is consistent with previous observations showing that different stressors trigger the secretion of different pro-inflammatory signalling molecules in fibroblasts (Coppe *et al.*, 2008; Rodier *et al.*, 2009). Interestingly, IL-8 was up-regulated in all models of melanocyte senescence studied. This chemokine has been shown to participate in signalling that reinforces the growth arrest of senescent fibroblasts, such that knocking down the IL-8 receptor, CXCR2, prevented both replicative and oncogene-induced senescence, and diminished the DDR (Acosta

et al., 2008). It was hypothesised that CXCR2 signalling contributed to increased ROS generation, in turn inducing DNA damage, which is a main contributor to senescence (Acosta *et al.*, 2008). Moreover, it was shown that the transcription factors NF-κB and C/EBPβ were up-regulated in cells undergoing OIS, and that these stimulated the expression of CXCR2 ligands, thus activating a self-amplifying secretory network responsible for reinforcing the senescence phenotype (Acosta *et al.*, 2008). Furthermore, secretion of the pro-inflammatory cytokine, IL-6, appears to be up-regulated in melanocytes undergoing replicative and X-ray-induced senescence. Indeed, IL-6 is a prominent component of the SASP of many cells, including keratinocytes (Lu *et al.*, 2006), fibroblasts and epithelial cells (Coppe *et al.*, 2008), as well as OIS melanocytes (Kuilman *et al.*, 2008). Similarly to IL-8, IL-6 has also been implicated in the induction and maintenance of oncogene-induced senescence (Kuilman *et al.*, 2008). A mechanism was proposed whereby the transcription factor C/EBPβ is activated upon oncogenic stress, stimulating the expression of SASP factors, such as IL-6, which engages in a positive feedback loop with C/EBPβ, also responsible for inducing the CDK inhibitor, p15. This feedback mechanism amplifies the inflammatory network and cooperates with cell cycle arrest (Kuilman *et al.*, 2008). Although the mechanisms leading to SASP development during melanocyte senescence were not addressed in our study, it is possible that persistent DNA damage, both at telomeres and non-telomeric sites, could contribute to secretion of pro-inflammatory factors. Indeed, secretion of SASP factors, such as IL-6 and IL-8, has been shown to be highly dependent on persistent DDR signalling associated with senescence, with depletion of DDR proteins, such as ATM and H2AX abolishing their secretion (Rodier *et al.*, 2009; Rodier *et al.*, 2011). However, the DDR alone cannot fully account for the development of the SASP, since this is a dynamic process which develops over time, whereas DDR activation is an almost immediate response following DNA damage (Coppe *et al.*, 2010a). In fact, a DDR-independent mechanism regulating the SASP has been identified, which involves the up-regulation of p38MAPK (Freund *et al.*, 2011). Indeed, senescence-inducing stimuli such as genotoxic stress, was shown to activate p38MAPK in human fibroblasts, whereas p38MAPK inhibition was sufficient to abolish the SASP in these cells (Freund *et al.*, 2011). It was shown that p38MAPK induced the activity of the transcription factor NF-κB, which in turn regulates the expression of genes involved in the SASP (Freund *et al.*, 2011). Consistently, preliminary data from our lab showed that inhibition of p38MAPK inhibits the SASP in stress-induced senescent melanocytes (data not shown), suggesting that this could be a potential mechanism

regulating the inflammatory phenotype of these cells. However, further experiments are needed to corroborate this hypothesis.

Another possible scenario is that mTOR is involved in the development of a SASP in senescent melanocytes. Indeed, mTOR has been shown to promote NF-κB activity, which induces the expression of SASP genes (Dan *et al.*, 2008; Chien *et al.*, 2011a). Moreover, inhibition of mTORC1 by rapamycin treatment decreased the translation of IL-1α, thereby reducing the transcriptional activity of NF-κB, and diminishing the secretion of pro-inflammatory factors, such as IL-6, indicating that mTOR is also a regulator of the SASP (Laberge *et al.*, 2015). Consistently, we have observed an increase in mTOR activity in replicative senescent melanocytes, and have shown that mTOR inhibition significantly decreases the SASP in stress-induced senescent melanocytes, which will discussed in further detail in the next chapter. Therefore, it is likely that mTOR activation plays a role in the development of a pro-inflammatory secretory phenotype during melanocyte senescence.

Of note, the serine protease inhibitor, PAI-1, also known as Serpin E1, was highly up-regulated in UV-induced senescent melanocytes. This is consistent with other studies showing that PAI-1 is increased in conditioned medium from lung and skin fibroblasts (West *et al.*, 1996), and is also up-regulated in senescent fibroblasts and endothelial cells (Mu and Higgins, 1995; Mu *et al.*, 1998; Martens *et al.*, 2003). Interestingly, it was demonstrated that PAI-1 is a transcriptional target of p53 in both senescent MEFs and human fibroblasts, and it reinforces the senescence growth arrest by down-regulating PI3K-PKB signalling, in turn promoting nuclear exclusion of cyclin D1 (Kortlever *et al.*, 2006). Consequently, the latter can no longer phosphorylate and inhibit pRb, and thus pRb remains associated with E2F transcription factors, which are required for induction of genes involved in cell-cycle progression (Kortlever *et al.*, 2006). Indeed, silencing PAI-1 alleviates senescence in normal fibroblasts, whilst ectopically expressing PAI-1 in p53-deficient fibroblasts induced senescence in these cells, suggesting that PAI-1 plays a major role in the induction of senescence (Kortlever *et al.*, 2006). Although our results demonstrate that known SASP components are up-regulated in senescent melanocytes, further studies would need to be performed in order to conclude that specific pro-inflammatory cytokines are involved in the induction and maintenance of melanocyte senescence in an autocrine fashion.

Additionally, components of the SASP, such as TGF-β family ligands have been shown to induce paracrine senescence in normal neighbouring cells (Acosta *et al.*, 2013). Others, such as

IL-6 and IL-8, were reported to promote malignant transformation in cultured pre-malignant epithelial cells (Coppe *et al.*, 2008). It is believed that senescent cells contribute to age-related tissue dysfunction partly through secretion of SASP factors (van Deursen, 2014), and the non-cell autonomous effects of senescent melanocyte SASP will be discussed in greater detail in the following chapter.

In summary, our results show that senescence can be induced in melanocytes in culture by replicative exhaustion, and by exposure to genotoxic stresses such as X-ray and UV irradiation. Senescence in these cells is associated with elevated p16, p21, and oxidative stress. Although there are some differences in the senescent phenotype of these cells, we demonstrate that length-independent telomere dysfunction occurs in all models of melanocyte senescence, suggesting that telomere damage is not only a result of extensive shortening. We also provide evidence that telomeric DSBs are irreparable in melanocytes *in vitro.*

Chapter 5: Senescent melanocytes induce paracrine DNA damage and senescence in neighbouring cells

Amounting evidence suggests that senescent cells contribute to age-related tissue dysfunction partly through paracrine mechanisms that affect surrounding healthy cells (Baker *et al.*, 2011; Nelson *et al.*, 2012; Acosta *et al.*, 2013; Baker *et al.*, 2016; Ritschka *et al.*, 2017). Indeed, it has been shown that senescent cells can drive senescence in neighbouring cells *in vitro* both by direct cell-cell contact (Nelson *et al.*, 2012) and through secretion of soluble factors (Acosta et al., 2013). Moreover, clusters of senescent cells have been observed in mouse liver with age (Nelson *et al.*, 2012), and in the vicinity of human pre-malignant lesions undergoing oncogene-induced senescence (Acosta *et al.*, 2013), suggesting that senescence can also be transmitted to adjacent cells *in vivo*.

Our previous results suggest that senescent melanocytes can induce paracrine telomere damage in neighbouring keratinocytes in human skin *in vivo*. Therefore, I aimed to further explore a bystander effect of senescent melanocytes *in vitro* and investigate possible mechanisms involved in this process. Moreover, although accumulation of senescent keratinocytes has been associated with skin ageing characteristics (Adamus *et al.*, 2014; Velarde *et al.*, 2015), the contribution of senescent melanocytes to age-related skin changes has not been extensively studied. Hence, by using a three-dimensional (3D) human epidermal equivalent comprising keratinocytes and melanocytes, I aimed to establish whether the presence of senescent melanocytes contributed to age-associated epidermal changes.

5.1. Bystander telomere DNA damage induction is mediated by SASP factors

5.1.1. Rapamycin treatment inhibits the SASP of senescent melanocytes

Inhibition of mTORC1 activity by rapamycin has been previously shown to diminish the secretion of many pro-inflammatory cytokines by senescent cells *in vitro* (Herranz *et al.*, 2015; Laberge *et al.*, 2015; Correia-Melo *et al.*, 2016). Therefore, in order to investigate whether SASP factors released by senescent melanocytes were involved in bystander DNA damage induction, melanocytes were irradiated using 10 Gy X-ray irradiation, and were then treated with rapamycin immediately after irradiation for 10 days until they reached senescence (***Figure 5.1 A***). Control cells were treated with the same concentration of vehicle DMSO. Rapamycin treatment was shown to significantly inhibit mTORC1 activity, as indicated by a complete reduction in phosphorylated p70S6 kinase and pS6 protein levels in both proliferating (no IR) and senescent melanocytes treated with the drug (***Figure 5.1 B***). Although mTORC1 activity appears to decrease in senescent melanocytes at 10 days after irradiation compared to proliferating controls (***Figure 5.1 B***), a cytokine array indicated that rapamycin treatment significantly suppressed the secretion of major SASP factors (Coppe *et al.*, 2008), such as IL-8, IL-6, RANTES and IP-10 (***Figure 5.1 C-F***), suggesting that mTOR is involved in regulating the SASP of senescent melanocytes.

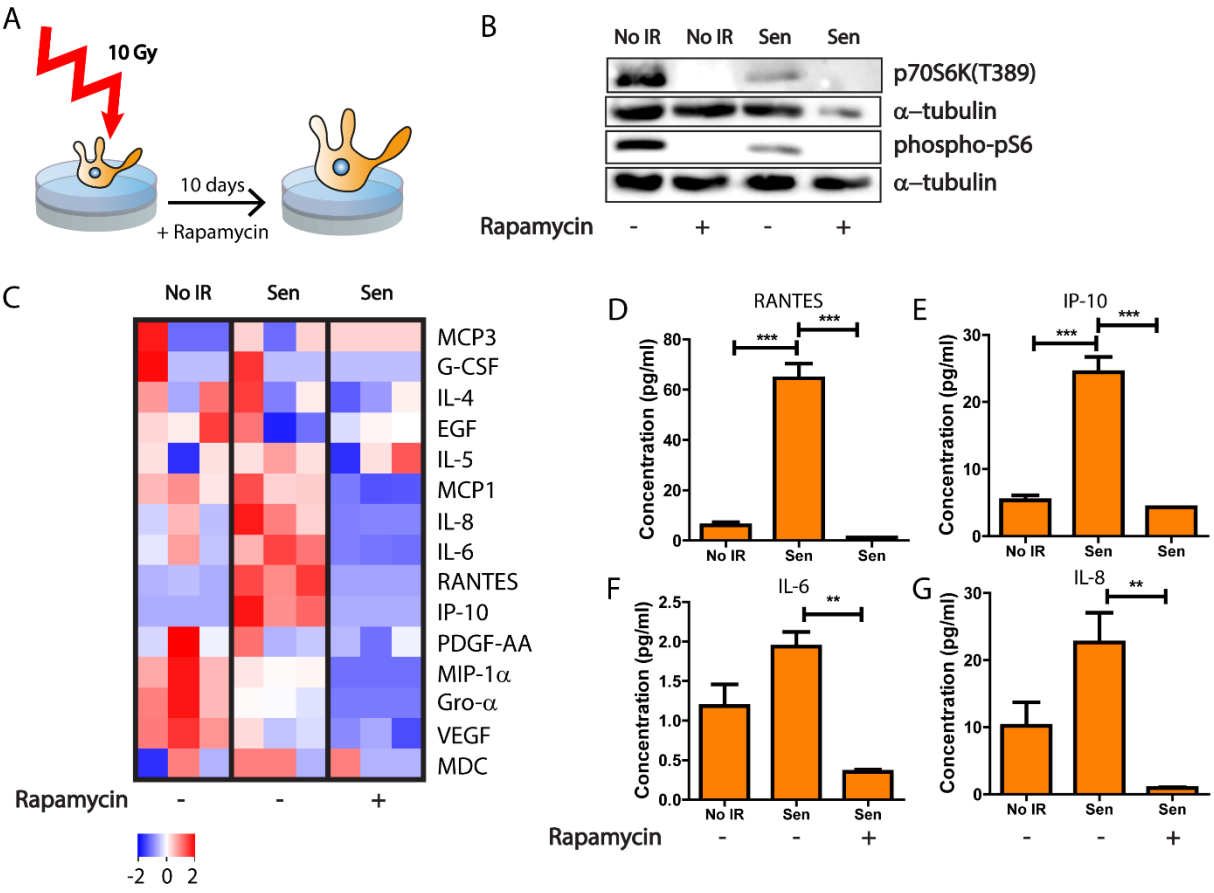

Figure 5.1 – Inhibition of mTORC1 suppresses the SASP of senescent melanocytes. (A) Scheme representing the experimental procedure. Melanocyte senescence was induced by 10 Gy X-ray irradiation, and cells were treated with rapamycin for 10 days, when melanocytes reach the senescence state. **(B)** Representative Western blot showing analysis of p70S6K(T389) and phospho-pS6 expression in proliferating (No IR) and senescent (Sen) melanocytes with or without rapamycin treatment 10 days after irradiation. α-tubulin was used as the loading control. **(C)** Heatmap showing cytokines detected in conditioned media from proliferating and senescent melanocytes with or without rapamycin treatment. Blue represents low expression, and red denotes high expression. Heatmap was generated using Ingenuity Pathway Analysis. Each column represents one independent experiment for the corresponding condition (N=3). Graphs showing the concentration of **(D)** RANTES, **(E)** IP-10, **(F)** IL-6, and **(G)** IL-8 in CM from proliferating and senescent melanocytes with or without rapamycin. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was assessed by a one-way ANOVA. **P<0.01, ***P<0.001. (Cytokine array done in collaboration with Duncan Talbot from Unilever).

5.1.2. Senescent melanocytes induce bystander telomeric DNA damage in dermal fibroblasts in direct co-culture

Next, in order to corroborate the observations from human skin *in vivo* suggesting a bystander effect of senescent melanocytes, I wanted to assess whether these cells could induce paracrine DNA damage in neighbouring cells *in vitro*. Moreover, I also wanted to investigate the role of SASP factors in this process. For that, human dermal fibroblasts were co-cultured with either proliferating or senescent melanocytes that were treated with rapamycin prior to co-culture (***Figure 5.2 A***). Control melanocytes were treated with the same concentration of vehicle DMSO. Cells were co-cultured at a 1:3 fibroblast to melanocyte ratio for 48 hours.

In order to analyse DNA damage in fibroblasts, I then performed immuno-FISH against γH2AX together with a telomere-specific probe, and also used a melanocyte-specific marker, known as melan-A, to ensure that only fibroblasts were considered in the analysis. I found that fibroblasts co-cultured with senescent melanocytes had, on average, higher amounts of DNA damage foci, as indicated by a 1.6 fold increase in total γH2AX foci in these cells when compared to fibroblasts co-cultured with proliferating melanocytes; however, this increase was not statistically significant (***Figure 5.2 B***) ($P<0.05$). Moreover, the amount of DNA damage in fibroblasts was not diminished by co-culture with rapamycin-treated senescent melanocytes (***Figure 5.2 B***) ($P<0.05$).

Given that telomere-associated foci have been shown to be persistent (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012), and continuous DDR activation is an important initiator of senescence (d'Adda di Fagagna, 2008), I next analysed the co-localisation between γH2AX foci and telomeres. The frequency of telomere-associated foci was significantly higher in fibroblasts cultured with senescent melanocytes ($P<0.001$), which was on average 2.4-fold higher when compared to fibroblasts co-cultured with proliferating melanocytes (***Figure 5.2 C, D***). Although the number of dysfunctional telomeres was slightly decreased in fibroblasts cultured with rapamycin-treated senescent melanocytes, this decrease was very small and not statistically significant ($P<0.05$) (***Figure 5.2 C, D***). Therefore, these results indicate that senescent melanocytes can act in a paracrine manner and trigger a DDR at telomeres in neighbouring fibroblasts. Although in these experiments inhibition of melanocyte SASP did not appear to prevent paracrine damage induction, this could be a result of the experimental design. Since rapamycin was removed from melanocytes during the bystander experiments, it is possible that the SASP could have returned, thus affecting the results. Therefore, in order to

overcome this issue, these experiments could be performed with melanocytes with an mTOR knockdown.

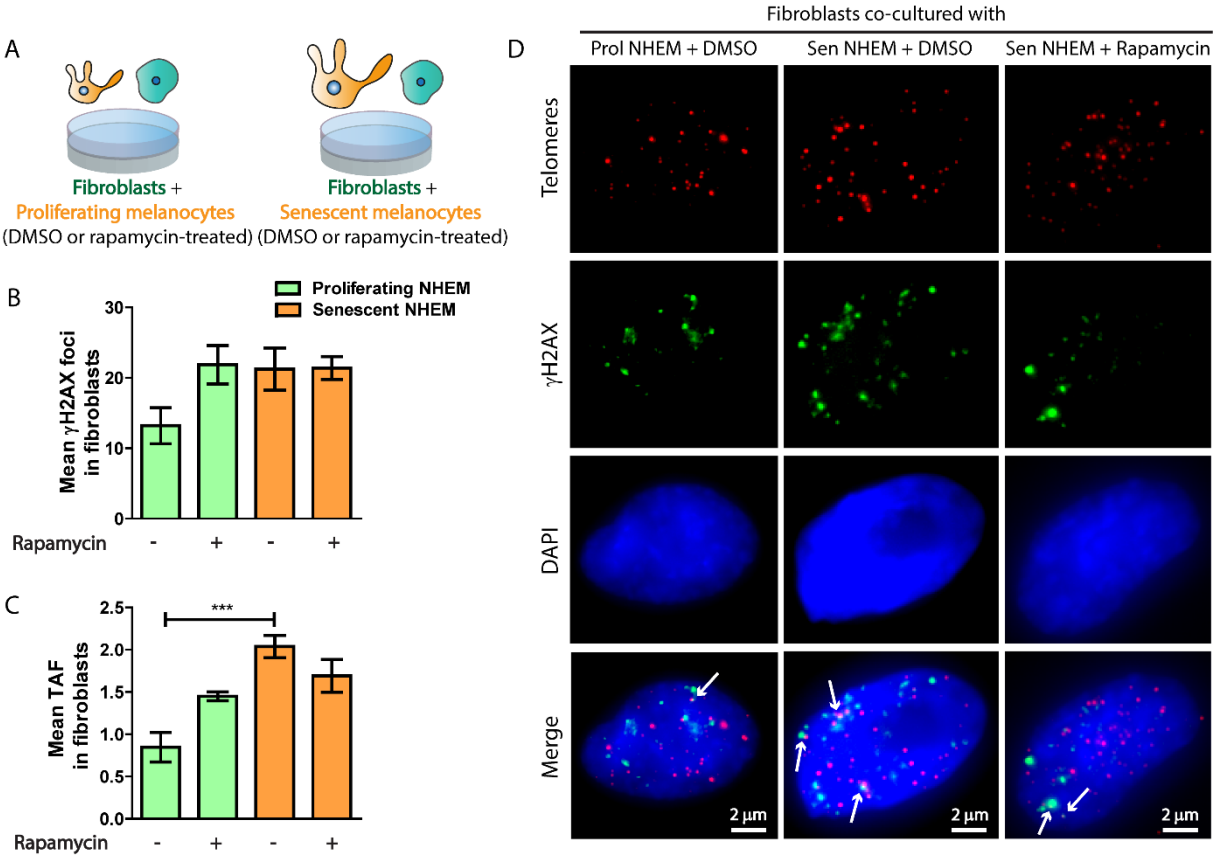

Figure 5.2 – Senescent melanocytes induce paracrine TAF in co-cultured fibroblasts. (A) Scheme representing the experimental procedure. Melanocytes were irradiated using 10 Gy X-ray irradiation, and treated with rapamycin or vehicle DMSO for 10 days. Rapamycin was removed from melanocytes 24 hours prior to co-culture to ensure that rapamycin was not present during the culture with fibroblasts. Fibroblasts were co-cultured with either proliferating or senescent melanocytes (DMSO- or rapamycin-treated) for 48 hours. Graphs showing **(B)** mean number of γH2AX foci and **(C)** mean number of TAF in fibroblasts co-cultured with melanocytes in the conditions indicated. Data are mean ± S.E.M. of N=4 independent experiments. Statistical significance was determined by a one-way ANOVA. ***P<0.001. **(D)** Representative immuno-FISH images of fibroblasts co-cultured with: proliferating melanocytes (left), senescent melanocytes (middle), and rapamycin-treated senescent melanocytes (right). Telomeres are shown in red, γH2AX is shown in green, and blue represents DAPI. Arrows indicate co-localisation between γH2AX and telomeres. Images were taken using a 63x objective. NHEM = normal human epidermal melanocytes.

5.1.3. Soluble factors secreted by senescent melanocytes mediate paracrine DNA damage induction

It is possible that senescent melanocytes pre-treated with rapamycin reacquire the SASP once in co-culture with fibroblasts, thus impacting on the interpretation of the results. Therefore, in order to investigate if bystander telomere damage could be induced solely by the SASP, I collected conditioned medium (CM) from proliferating and stress-induced senescent melanocytes treated with either vehicle DMSO or rapamycin for 10 days, and cultured human dermal fibroblasts with melanocyte CM for 48 hours (***Figure 5.3 A***).

By performing immuno-FISH, I found that although there was a small increase in DDR signalling in fibroblasts cultured with senescent melanocyte CM, this was not statistically significant when compared to fibroblasts cultured in CM from proliferating melanocytes (***Figure 5.3 B***) ($P>0.05$). Moreover, a small reduction in total γH2AX foci was observed by culturing fibroblasts with CM from rapamycin-treated senescent melanocytes; however, this also did not reach statistical significance (***Figure 5.3 B***) ($P>0.05$). On the other hand, conditioned medium from senescent melanocytes was sufficient to significantly increase the frequency of telomere-associated foci in fibroblasts, as indicated by a 2.5 fold increase in the number of TAF in these cells (***Figure 5.3 C, D***) ($P<0.001$), similarly to the results obtained from the previous co-culture experiments. Interestingly, rapamycin treatment was sufficient to significantly prevent bystander TAF induced by senescent melanocyte CM, as shown by a 2-fold decrease in dysfunctional telomeres in fibroblasts cultured with control senescent melanocyte medium (***Figure 5.3 C, D***) ($P<0.01$). Therefore, these results suggest that paracrine damage induction is mediated by pro-inflammatory factors secreted by senescent melanocytes, which can be effectively prevented by inhibiting the SASP.

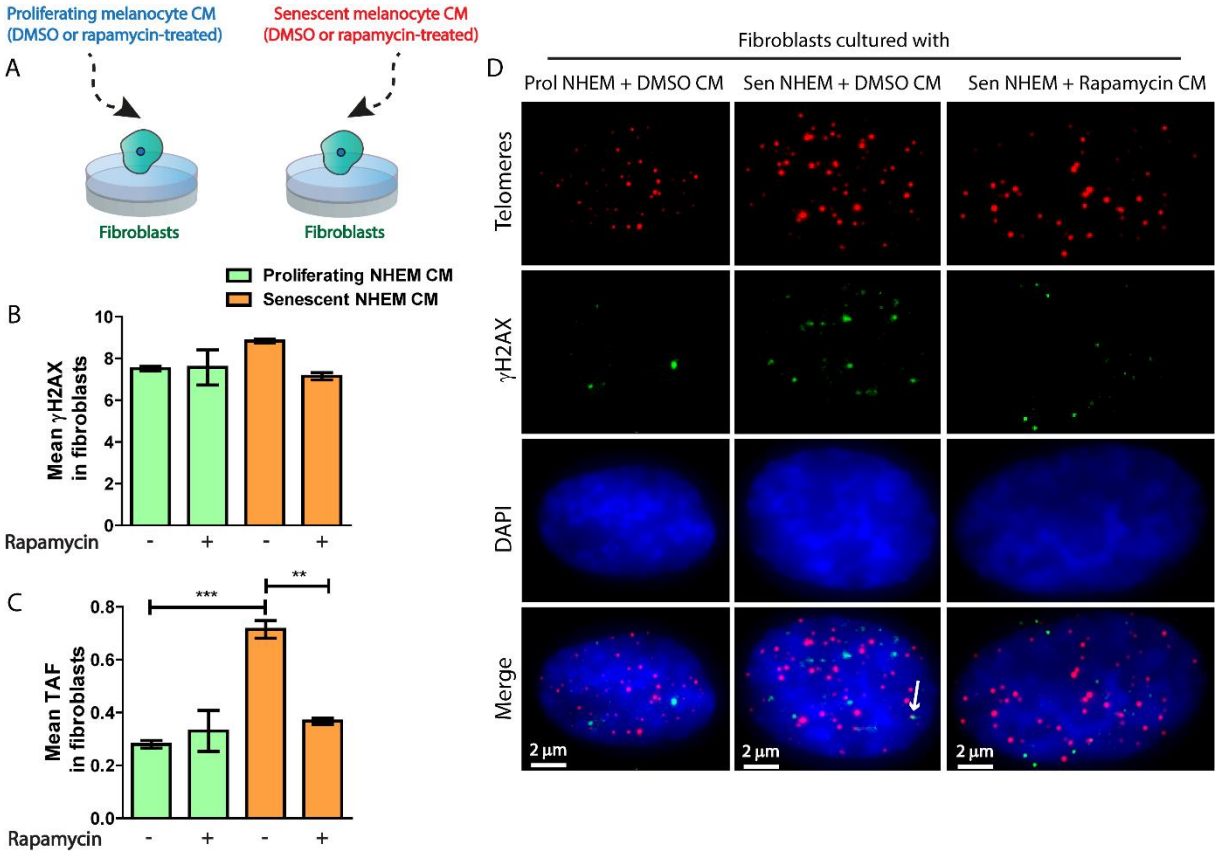

Figure 5.3 – Paracrine telomeric DDR signalling is triggered by SASP factors secreted by senescent melanocytes. (A) Scheme illustrating the experimental procedure. Proliferating and senescent (10 Gy) melanocytes were treated with DMSO or rapamycin for 10 days. Rapamycin was removed from melanocytes 24 hours prior to media collection to ensure that the drug was not present during fibroblast culture. Dermal fibroblasts were then cultured for 48 hours with conditioned medium collected from these cells. Graphs showing **(B)** mean number of γH2AX foci and **(C)** mean number of TAF in fibroblasts cultured with CM from proliferating or senescent melanocytes with or without rapamycin treatment. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. **P<0.01, ***P<0.001. **(D)** Representative immuno-FISH images of fibroblasts cultured with CM from: proliferating melanocytes (left), senescent melanocytes (middle), and rapamycin-treated senescent melanocytes (right). Telomeres are shown in red, γH2AX is shown in green, and blue represents DAPI. Arrows indicate co-localisation between γH2AX and telomeres. Images were taken using a 63x objective. NHEM = normal human epidermal melanocytes; CM = conditioned medium.

5.2. Paracrine DDR signalling remains elevated in dermal fibroblasts following long-term exposure to the SASP

Given that previous data has demonstrated that DDR signalling at telomeres is persistent following a variety of senescence-inducing stimuli (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012), I next wanted to investigate whether bystander telomere dysfunction induced by senescent melanocytes persisted over longer culture periods in dermal fibroblasts. For that, I cultured fibroblasts for 30 days with conditioned medium obtained from proliferating and stress-induced senescent melanocytes, and performed immuno-FISH to analyse DNA damage specifically at telomeres.

Firstly, I found that senescent melanocyte CM induced significantly higher levels of DNA damage in fibroblasts when compared to cells cultured in CM from proliferating melanocytes (***Figure 5.4 A, D***) (P=0.03). I also observed a 2-fold increase in telomere-associated foci in fibroblasts cultured with senescent melanocyte CM for 30 days (***Figure 5.4 B, D***) (P=0.0002), which is consistent with previous observations obtained from the short-term culture experiments. Moreover, 58% of fibroblasts cultured in senescent melanocyte CM displayed at least one damaged telomere compared to 35% of cells cultured in CM from proliferating melanocytes (***Figure 5.4 C***). These results suggest that long-term exposure to factors secreted by senescent melanocytes triggers DDR signalling at both non-telomeric and telomeric sites. Furthermore, these data also demonstrate that TAF levels remain constantly higher in fibroblasts cultured with senescent melanocyte CM.

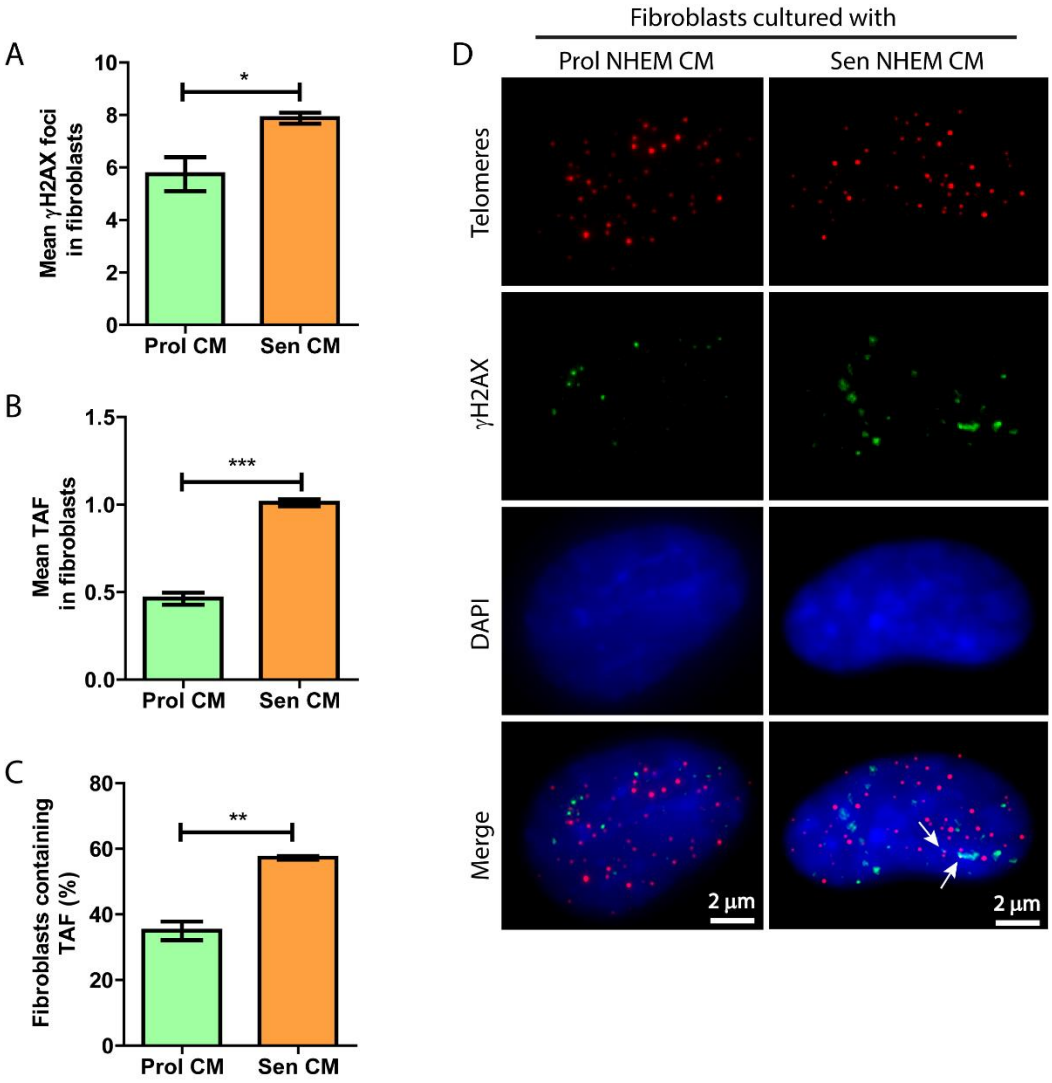

Figure 5.4 – Long-term exposure to senescent melanocyte SASP leads to accumulation of DNA damage foci at both telomeric and non-telomeric regions. Dermal fibroblasts were cultured in either proliferating or senescent melanocyte conditioned medium for 30 days, and DNA damage at telomeres was assessed by immuno-FISH. Graphs showing **(A)** mean number of γH2AX foci in fibroblasts, **(B)** mean number of TAF in fibroblasts, and **(C)** percentage of fibroblasts containing TAF when cultured in conditioned medium from proliferating (Prol) or senescent (Sen) melanocytes for 30 days. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a two-tailed unpaired t test. *P<0.05, **P<0.01, ***P<0.001. **(D)** Representative immuno-FISH images of fibroblasts cultured with CM from proliferating melanocytes (left), and CM from senescent melanocytes (right). Telomeres are shown in red, γH2AX is shown in green, and blue represents DAPI. Arrows indicate co-localisation between γH2AX and telomeres. Images were taken using a 63x objective. NHEM = normal human epidermal melanocytes; CM = conditioned medium.

5.3. Long-term exposure to senescent melanocyte SASP causes proliferation arrest in fibroblasts

Previous studies have shown that prolonged exposure to soluble factors secreted by senescent cells can induce a stable growth arrest in human and murine fibroblasts in a paracrine manner *in vitro* (Acosta *et al.*, 2013). Therefore, in order to investigate the long-term implications of exposing fibroblasts to pro-inflammatory factors from senescent melanocytes, dermal fibroblasts were cultured for 20 days with conditioned media from either proliferating or senescent melanocytes, and proliferation status was assessed by EdU incorporation.

Fibroblasts cultured in senescent melanocyte CM exhibited a small but significant decrease in proliferation. Whilst 79% of cells in proliferating melanocyte CM were EdU-positive, this was reduced to 56% when fibroblasts were cultured with senescent melanocyte CM (***Figure 5.5 A, B)*** (P=0.01). This suggests that a fraction of cells become growth-arrested as a result of sustained exposure to the SASP from senescent melanocytes, although the majority of fibroblasts were still proliferation efficient at this time point.

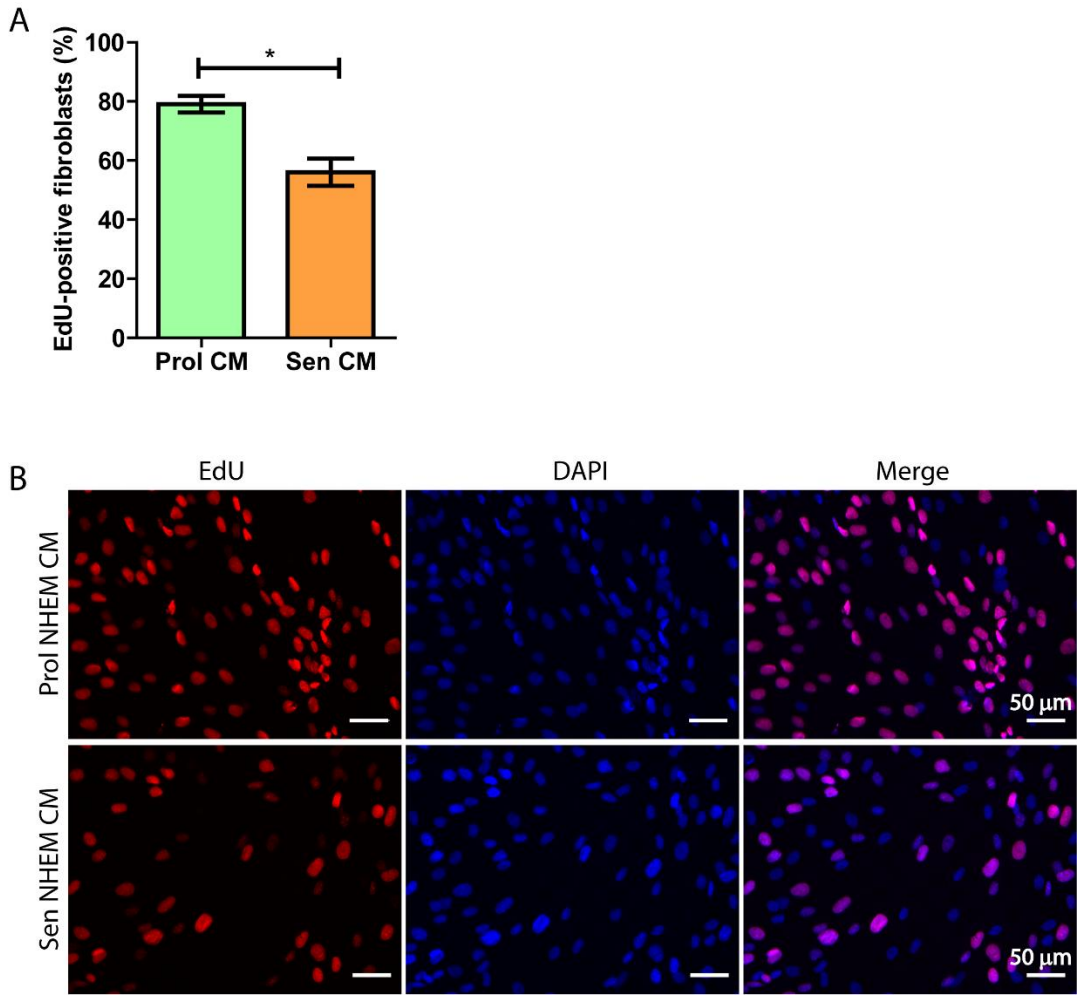

Figure 5.5 – Soluble factors from senescent melanocytes inhibits fibroblast cell division. Dermal fibroblasts were cultured in either proliferating or senescent melanocyte conditioned medium for 20 days, and proliferation was assessed by EdU incorporation. **(A)** Graph showing percentage of EdU-positive fibroblasts at the indicated culture conditions. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was assessed by a two-tailed unpaired t test. *P<0.01. **(B)** Representative EdU immunofluorescence images of fibroblasts cultured in proliferating (Prol) or senescent (Sen) melanocyte conditioned medium. EdU is shown in red, and DAPI is depicted in blue. Images were taken using a 20x objective. NHEM = normal human epidermal melanocytes; CM = conditioned medium.

5.4. Paracrine DNA damage signalling is mediated by increased intracellular ROS generation

Next, I wanted to further investigate the mechanisms leading to accumulation of dysfunctional telomeres in bystander cells. It is known that increased oxidative stress is an important contributor to activation of a DDR, with telomeres being particularly sensitive to imbalances in ROS homeostasis (Petersen *et al.*, 1998; Oikawa and Kawanishi, 1999). In fact, mitochondria are a major source of ROS generation, and have been implicated as key components for the generation and replenishment of DNA damage foci, an important effector of senescence (Passos *et al.*, 2007; Passos *et al.*, 2010; Correia-Melo *et al.*, 2016). Therefore, to assess whether paracrine DDR signalling triggered by senescent melanocytes was mediated by increased mitochondrial ROS production in bystander cells, dermal fibroblasts were cultured with either proliferating or senescent melanocyte conditioned medium in the presence of the mitochondrial targeted antioxidant, MitoQ, for 48 hours (***Figure 5.6 A***).

By performing immuno-FISH, I found that senescent melanocyte CM is sufficient to induce a 2-fold increase in DNA damage foci in dermal fibroblasts ($P<0.01$), which is significantly reduced by MitoQ treatment ($P<0.05$) (***Figure 5.6 B, D***). Consistently, fibroblasts cultured in CM from senescent melanocytes have a significantly higher amount of dysfunctional telomeres when compared to cells in proliferating melanocyte CM ($P<0.05$) (***Figure 5.6 C***), whereas bystander TAF induction is effectively prevented by lowering mitochondrial ROS production in fibroblasts ($P<0.05$) (***Figure 5.6 C, D***). Therefore, it is possible that soluble factors from senescent melanocytes activate paracrine signalling pathways that trigger an increase in ROS generation, which in turn induces non-telomeric and telomeric DNA damage in bystander cells.

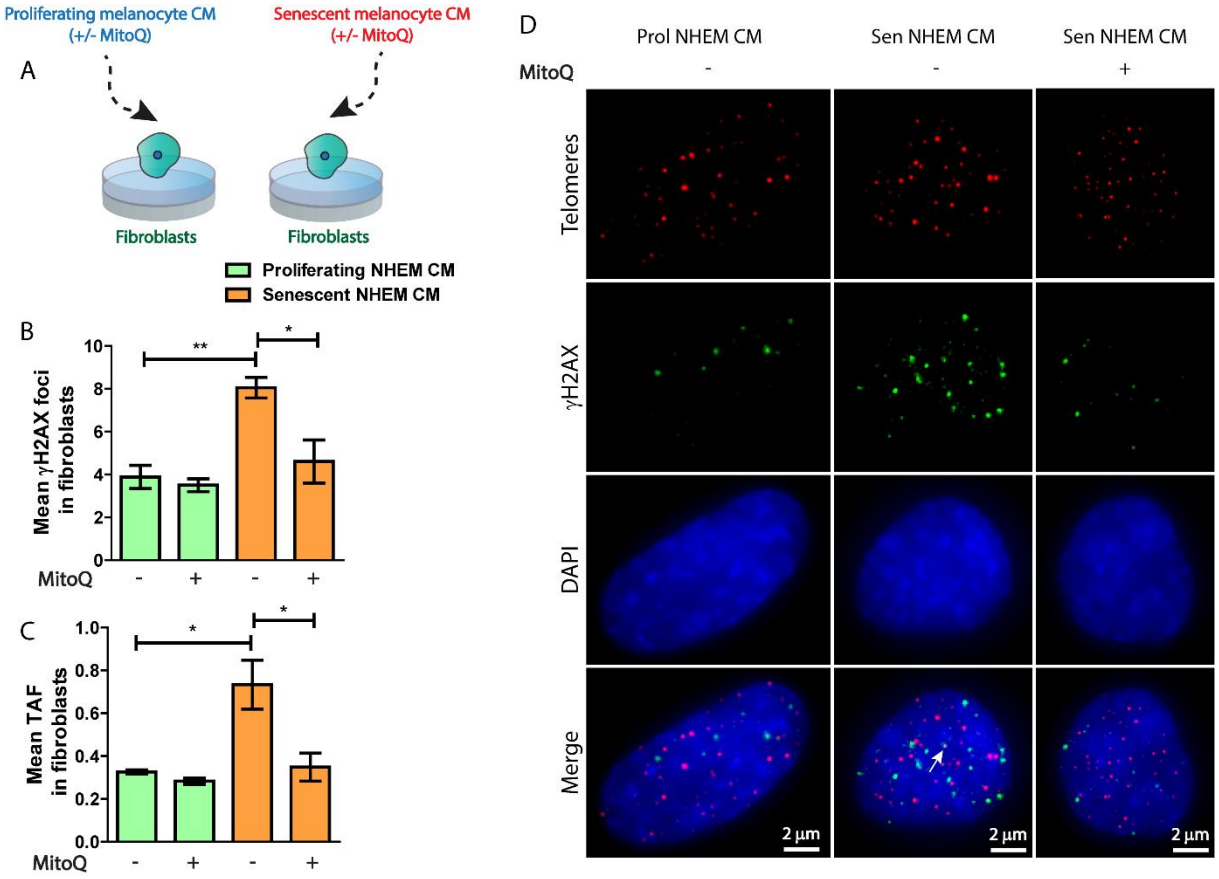

Figure 5.6 – Bystander DNA damage signalling induction is mediated by an increase in intracellular ROS. (A) Scheme showing the experimental procedure. Dermal fibroblasts were cultured with either proliferating or senescent melanocyte conditioned medium, and were treated with MitoQ for 48 hours. Graphs showing **(B)** mean number of γH2AX foci and **(C)** mean number of TAF in fibroblasts in the conditions indicated. Data are mean ± S.E.M of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01. **(D)** Representative immuno-FISH images of fibroblasts cultured with CM from: proliferating melanocytes (left), senescent melanocytes (middle), and senescent melanocytes with the addition of MitoQ (right). Telomeres are shown in red, γH2AX is shown in green, and blue represents DAPI. Arrows indicate co-localisation between γH2AX and telomeres. Images were taken using a 63x objective. NHEM = normal human epidermal melanocytes; CM = conditioned medium.

5.5. CXCR3 activation is involved in the initiation of melanocyte senescence, and mediates induction of paracrine telomere-associated foci

Following the observations that soluble factors secreted by senescent melanocytes can induce paracrine DNA damage signalling, I wanted to investigate whether specific components of the SASP were responsible for mediating bystander DDR induction.

5.5.1. CXCR3 signalling inhibition ameliorates the senescent phenotype of melanocytes

By performing a cytokine array analysis, we showed that IP-10 is one of the cytokines which is highly secreted by senescent melanocytes, as demonstrated in section 5.1. Previous studies from our lab revealed that CXCR3, a cell-surface receptor for the cytokine IP-10, is significantly up-regulated in senescent melanocytes *in vitro* (P=0.002) (***Figure 5.7 A, B***). Moreover, we also found that CXCR3 expression is significantly higher in melanocytes in the skin of older human donors *in vivo* (P=0.0002) (***Figure 5.7 C, D***). Therefore, we hypothesised that IP-10 secreted by senescent melanocytes could be involved in autocrine signalling, maintaining the senescent phenotype of melanocytes through continuous activation of CXCR3.

In order to determine whether CXCR3 signalling was involved in melanocyte senescence, we irradiated melanocytes using 10Gy X-ray irradiation and treated these cells with a CXCR3 antagonist, AMG487, for 10 days. We found that CXCR3 inhibition significantly reduced Sen-β-Gal activity (P<0.01), albeit it still remained higher compared to control proliferating cells (P<0.01) (***Figure 5.8 A, B***). Similarly, p16 expression was also significantly decreased by inhibiting CXCR3 in senescent melanocytes (P<0.001), although it did not fully prevent p16 up-regulation in response to stress-induced senescence (P<0.05) (***Figure 5.8 C***). Interestingly, the growth arrest induced by X-ray irradiation was not rescued by inhibition of CXCR3 (***Figure 5.8 D***), suggesting that CXCR3 diminishes senescence-associated markers independently of proliferation in melanocytes. Since persistent DDR signalling is an important effector of senescence, we performed immunofluorescence for γH2AX, and found that although CXCR3 inhibition reduced the number of DNA damage foci by 1.5-fold compared to untreated senescent cells, this was not statistically significant (***Figure 5.8 E***). Moreover, p21 expression, which is primarily up-regulated following irradiation, was not reduced by inhibiting CXCR3 in melanocytes (***Figure 5.8 G***). Furthermore, we measured mitochondrial ROS production by using the fluorescent probe, MitoSOX, which revealed that CXCR3 inhibition significantly reduced generation of ROS in irradiated melanocytes (***Figure 5.8 F***). Taken together, these results suggest that CXCR3 signalling is partly involved in the induction of the senescent

phenotype of melanocytes, although it does not significantly impact on the DNA damage response and growth arrest.

Previous studies have shown that CXCR3 signalling activates MEK/ERK and PI3K/Akt pathways in human airway epithelial cells, and these are responsible for mediating chemotaxis (Shahabuddin *et al.*, 2006). Moreover, these pathways have also been implicated in senescence signalling (Wang *et al.*, 2002; Correia-Melo *et al.*, 2016). In order to investigate whether these pathways were induced in the context of melanocyte senescence, and to assess whether CXCR3 inhibition had an effect on their activation, we analysed the expression of ERK and Akt in irradiated melanocytes with and without AMG487 treatment (***Figure 5.8 G***). Since Akt has been previously shown to be activated in the first few days of senescence induction following X-ray irradiation (Correia-Melo *et al.*, 2016), we chose to analyse these pathways at 8, 24, and 48 hours after 10 Gy irradiation of melanocytes. The levels of phosphorylated ERK (p-ERK) increased 8 hours following irradiation, with CXCR3 inhibition reducing its expression. However, ERK phosphorylation is reduced after 24 hours, and decreases below control levels 48 hours after irradiation, with CXCR3 inhibition having little effect at these time points (***Figure 5.8 G***). Unexpectedly, total ERK (t-ERK) expression also gradually decreases following X-ray irradiation, and declines below proliferating control levels from 24 hours. On the other hand, expression of phosphorylated Akt (p-Akt) is significantly up-regulated at 8 hours post-irradiation, and gradually increases until 48 hours (***Figure 5.8 G***), suggesting that Akt is activated in melanocytes in response to stress-inducing stimuli, such as irradiation. Moreover, Akt phosphorylation was significantly reduced by CXCR3 inhibition, suggesting that CXCR3 signalling plays a role in Akt activation in response to senescence-inducing X-ray irradiation.

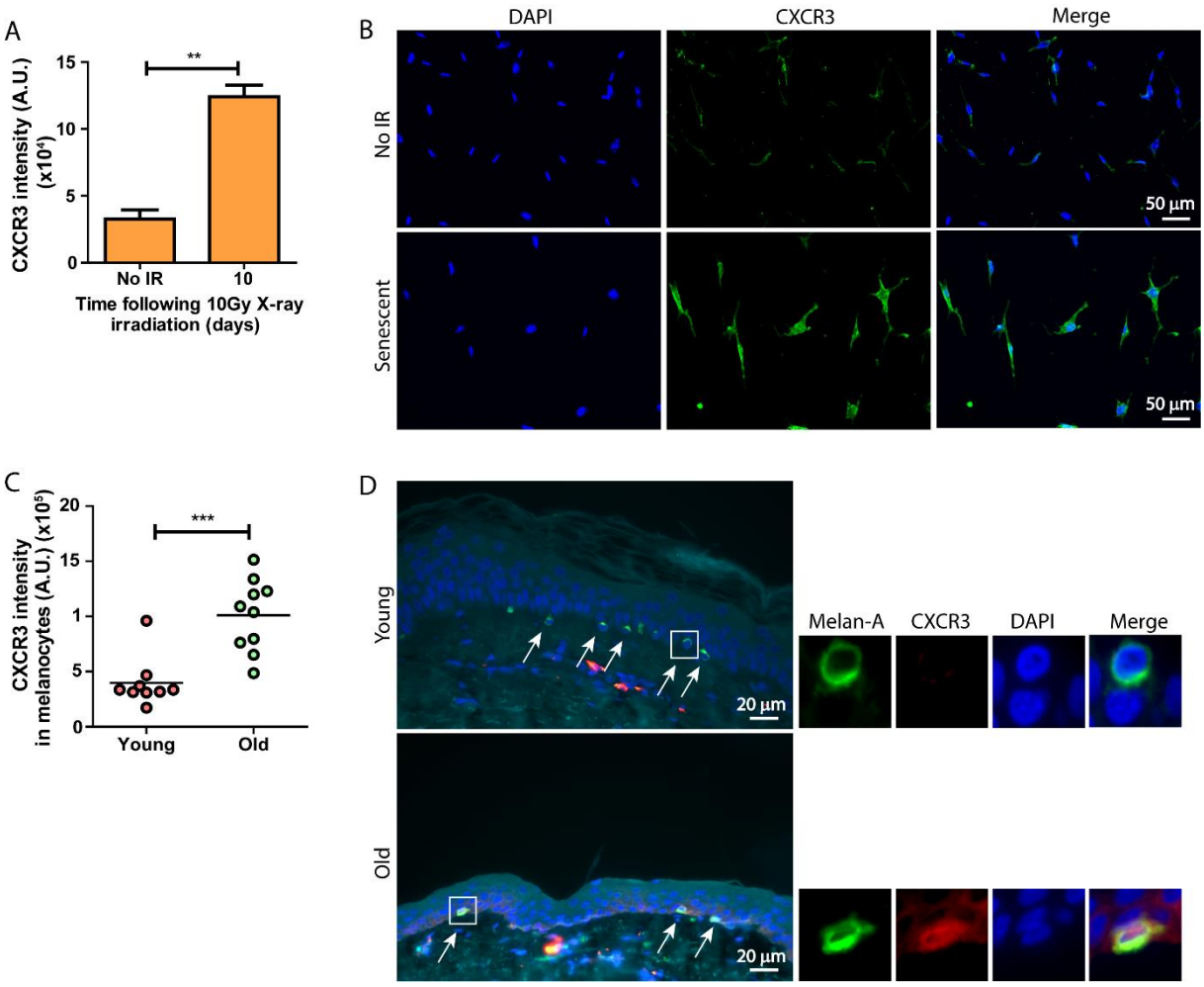

Figure 5.7 – CXCR3 is up-regulated in senescent melanocytes. (A) Graph showing CXCR3 fluorescence signal intensity in proliferating and stress-induced senescent (10 Gy) melanocytes. Data are mean ± S.E.M. of N=3 independent experiments. **(B)** Representative CXCR3 immunofluorescence images of proliferating (No IR) and senescent melanocytes. CXCR3 is shown in green, and DAPI is shown in blue. Images were taken using a 20x objective. (Analysis done in collaboration with Jessica Halim). **(C)** Dot plot showing CXCR3 fluorescence intensity in melanocytes in young and older human skin in vivo for each individual donor. The horizontal line represents the mean intensity for each group. Statistical significance was assessed by a two-tailed unpaired t-test. **P<0.01, ***P<0.001. **(D)** Representative CXCR3 immunofluorescence images in skin biopsies from young and older human donors. The melanocyte marker, Melan-A is shown in green, CXCR3 is shown in red, and DAPI is shown in blue. Images were taken using a 20x objective. Arrows indicate melanocytes, and cells enclosed within the square are amplified on the right. (CXCR3 in human skin was done in collaboration with Anthony Lagnado).

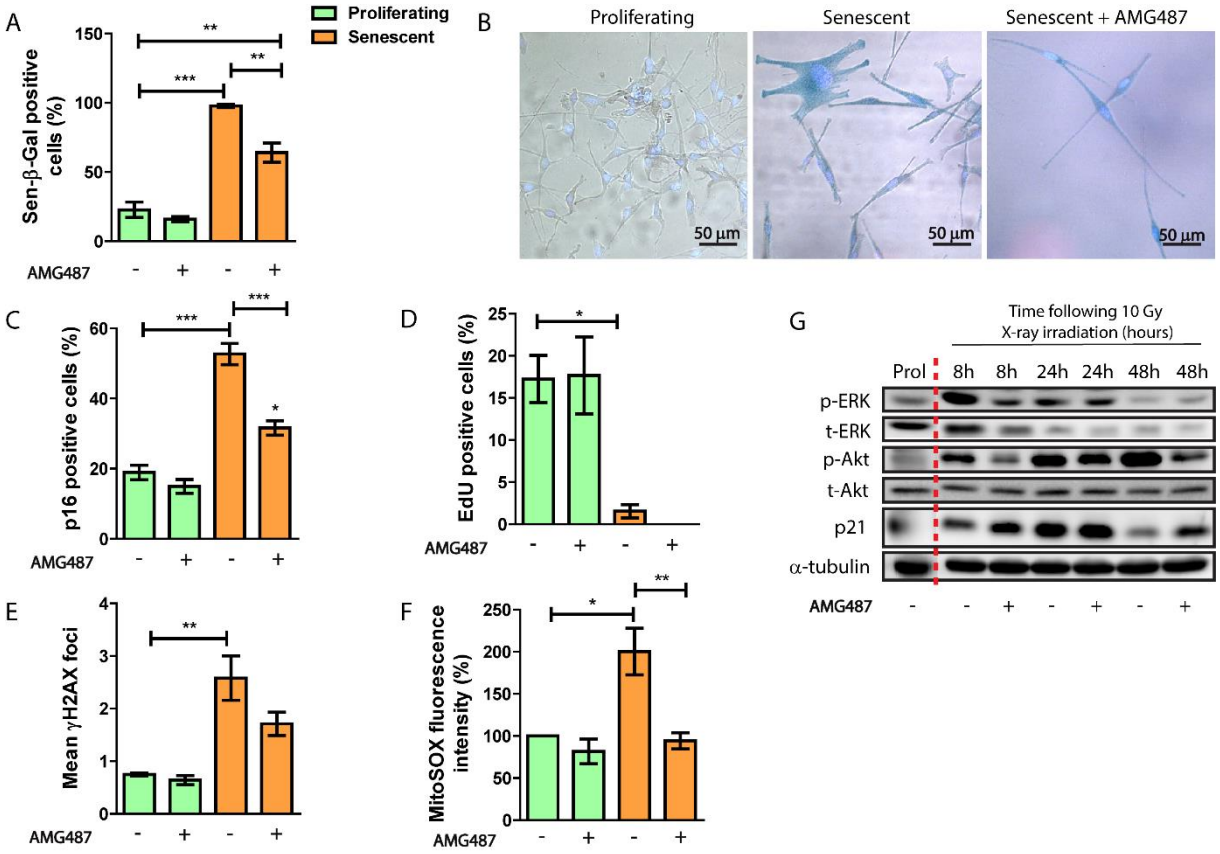

Figure 5.8 – CXCR3 inhibition is sufficient to ameliorate markers of melanocyte senescence. Senescence was induced in melanocytes by 10Gy X-ray irradiation, and cells were treated with either AMG487 or vehicle DMSO (control) for 10 days. **(A)** Graph showing the percentage of Sen-β-Gal-positive melanocytes at the conditions indicated. **(B)** Representative Sen-β-Gal images of proliferating and senescent melanocytes with or without AMG487 treatment. Images were taken using a 20x objective. Graphs showing the percentage of **(C)** p16-positive and **(D)** EdU-positive melanocytes at the conditions indicated. **(E)** Mean number of γH2AX foci in proliferating and senescent melanocytes with or without AMG487 treatment. **(F)** MitoSOX fluorescence intensity of melanocytes at the conditions indicated. Values are a percentage fold change normalised to proliferating controls (untreated). ROS measurements were performed at 3 days following irradiation. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001. **(G)** Representative Western blot showing expression of p-ERK, t-ERK, p-Akt, t-Akt, p21, and α-tubulin as a loading control, in proliferating (Prol) and irradiated melanocytes at the time points indicated. Irradiated cells were either treated with AMG487 or vehicle DMSO (untreated controls). (Experiments done in collaboration with Jessica Halim).

5.5.2. CXCR3 signalling is involved in paracrine telomeric DNA damage induction

Our results so far suggest that CXCR3 activation is involved in autocrine signalling that, in part, regulates phenotypes of melanocyte senescence. Moreover, our cytokine array analysis showed that IP-10 secretion is significantly abolished by rapamycin treatment of senescent melanocytes (section 5.1). Therefore, I aimed to assess whether CXCR3 activation was also involved in paracrine signalling, mediating bystander DNA damage caused by senescent melanocytes.

Dermal fibroblasts were cultured with either proliferating or senescent melanocyte conditioned medium, and were treated with the CXCR3 inhibitor, AMG487, for 48 hours (***Figure 5.9 A***). As previously demonstrated, CM from senescent melanocytes induced a 2-fold increase in the amount of γH2AX foci in dermal fibroblasts ($P<0.05$) (***Figure 5.9 B, D***), as determined by conducting immuno-FISH in these cells. However, DNA damage induction was not prevented by CXCR3 inhibition (***Figure 5.9 B, D***). On the other hand, a 3-fold increase in telomere-associated foci was observed in fibroblasts cultured with senescent melanocyte CM ($P<0.001$), which was significantly suppressed by inhibiting CXCR3 activation ($P<0.001$) (***Figure 5.9 C, D)***. These results suggest that soluble factors secreted by senescent melanocytes activate paracrine CXCR3 signalling, which in turn mediates the induction of bystander telomere dysfunction.

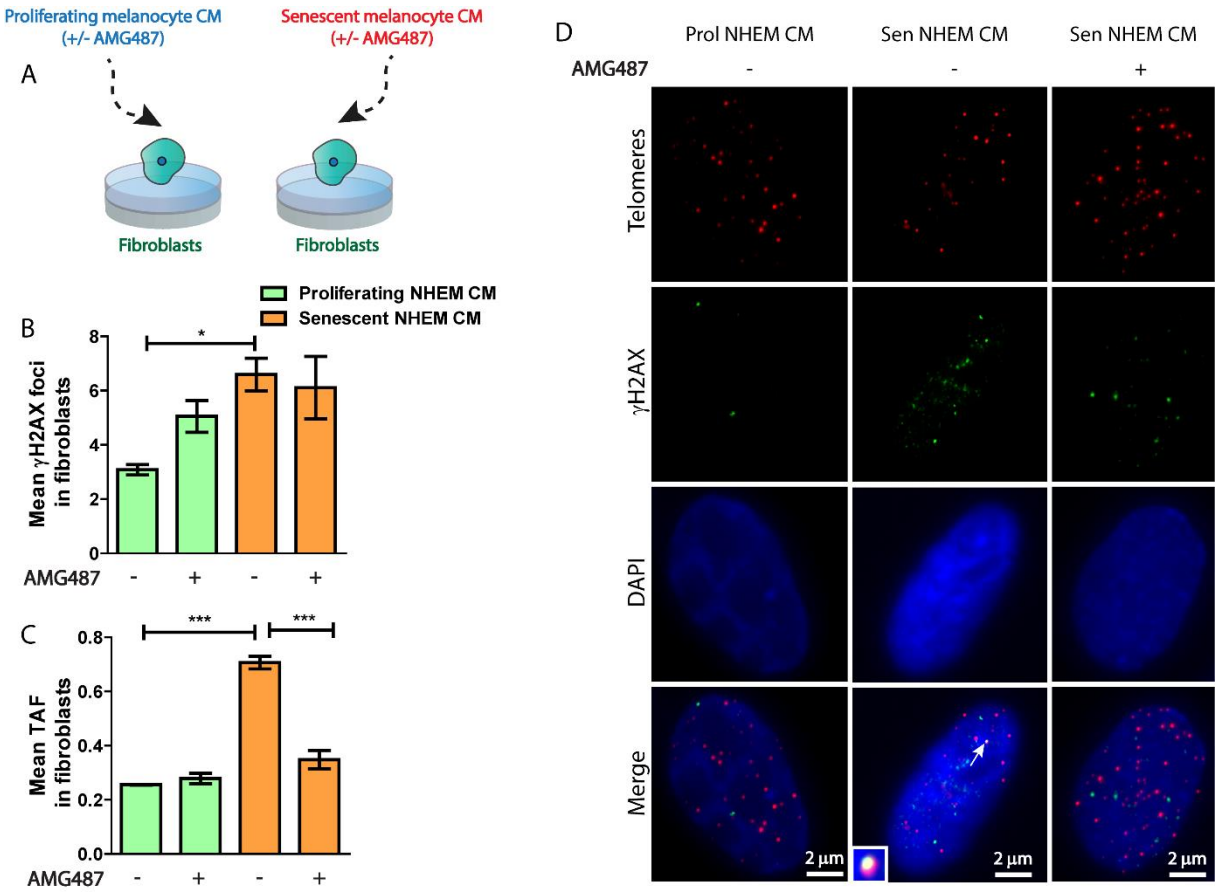

Figure 5.9 – CXCR3 signalling mediates the induction of paracrine telomere-associated foci. (A) Scheme demonstrating the experimental procedure. Dermal fibroblasts were cultured with conditioned medium from proliferating or senescent melanocytes, and were treated with AMG487, a CXCR3 inhibitor. Control cells were treated with vehicle DMSO. Graphs showing **(B)** mean γH2AX foci and **(C)** mean TAF in fibroblasts in the conditions indicated. Data are mean ± S.E.M. of N=3 independent experiments. Statistical significance was determined by a one-way ANOVA. *P<0.05, ***P<0.001. **(D)** Representative immuno-FISH images of fibroblasts cultured with CM from: proliferating melanocytes (left), senescent melanocytes (middle), and senescent melanocytes with the addition of AMG487 (right). Telomeres are shown in red, γH2AX is shown in green, and blue represents DAPI. Arrow indicates co-localisation between γH2AX and telomeres, which is also amplified at the bottom left-hand corner. Images were taken using a 63x objective. NHEM = normal human epidermal melanocytes; CM = conditioned medium.

5.6. Senescent melanocytes induce paracrine senescence in keratinocytes and contribute to skin ageing phenotypes in a living epidermal equivalent

My data so far indicates that senescent melanocytes can induce paracrine telomere-associated foci and growth arrest in dermal fibroblasts in culture. However, given that melanocytes are in direct contact with keratinocytes in the skin, and that many age-associated epidermal changes can be attributed to loss of replicative capacity of keratinocytes (Farage *et al.*, 2007), I wanted to determine whether senescent melanocytes could exert adverse paracrine effects in these cells. Moreover, I also aimed to assess whether accumulation of senescent melanocytes could contribute to phenotypes associated with skin ageing in living epidermal equivalents, also known as melanoderms. In order to achieve a more physiologically relevant model, we developed melanoderms containing either proliferating or UV-induced senescent melanocytes, as shown in section 4.3. Firstly, melanocytes were seeded with keratinocytes at a 1:10 melanocyte: keratinocyte ratio, and during a period of 10 days they develop into a multi-layered, highly differentiated human epidermis equivalent (***Figure 5.10***). In the interest of this study, the tissues were then collected at days 0, 10 and 21 following epidermal development.

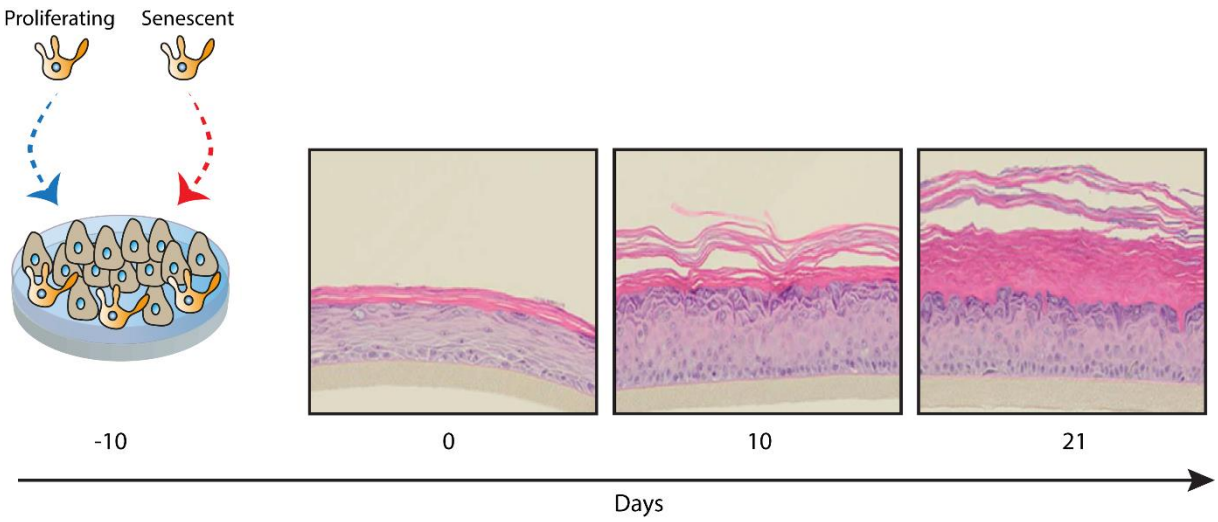

Figure 5.10 – Development of human epidermal equivalents. Scheme representing the experimental procedure. Proliferating or senescent (0.4 J/cm^2 UVA and UVB) melanocytes were co-cultured with keratinocytes at a 1:10 ratio, which fully develops into an epidermal equivalent following 10 days in culture. Melanoderms were collected at 0, 10 and 21 days post-epidermal development.

5.6.1. Senescent melanocytes induce growth arrest in neighbouring keratinocytes

In order to assess the proliferation status of keratinocytes in melanoderms, I performed immunofluorescence for Ki-67, and also used a melanocyte marker, melan-A, to ensure that only keratinocytes were included in the analysis. I found that melanoderms containing senescent melanocytes have a lower proportion of proliferating keratinocytes at all time points observed, with a statistically significant decrease in proliferation at day 21 following epidermal development ($P<0.05$) (***Figure 5.11 A, B***). In fact, 28% and 17% of keratinocytes show Ki-67 positivity in melanoderms containing proliferating and senescent melanocytes, respectively (***Figure 5.11 A***), suggesting that senescent melanocytes induce paracrine growth arrest in neighbouring keratinocytes. Of note, the majority of Ki-67-positive keratinocytes are located in the basal layer of the epidermal equivalents (***Figure 5.11 B***), possibly due to keratinocytes reaching terminal differentiation as they approach the outermost layers of the epidermis.

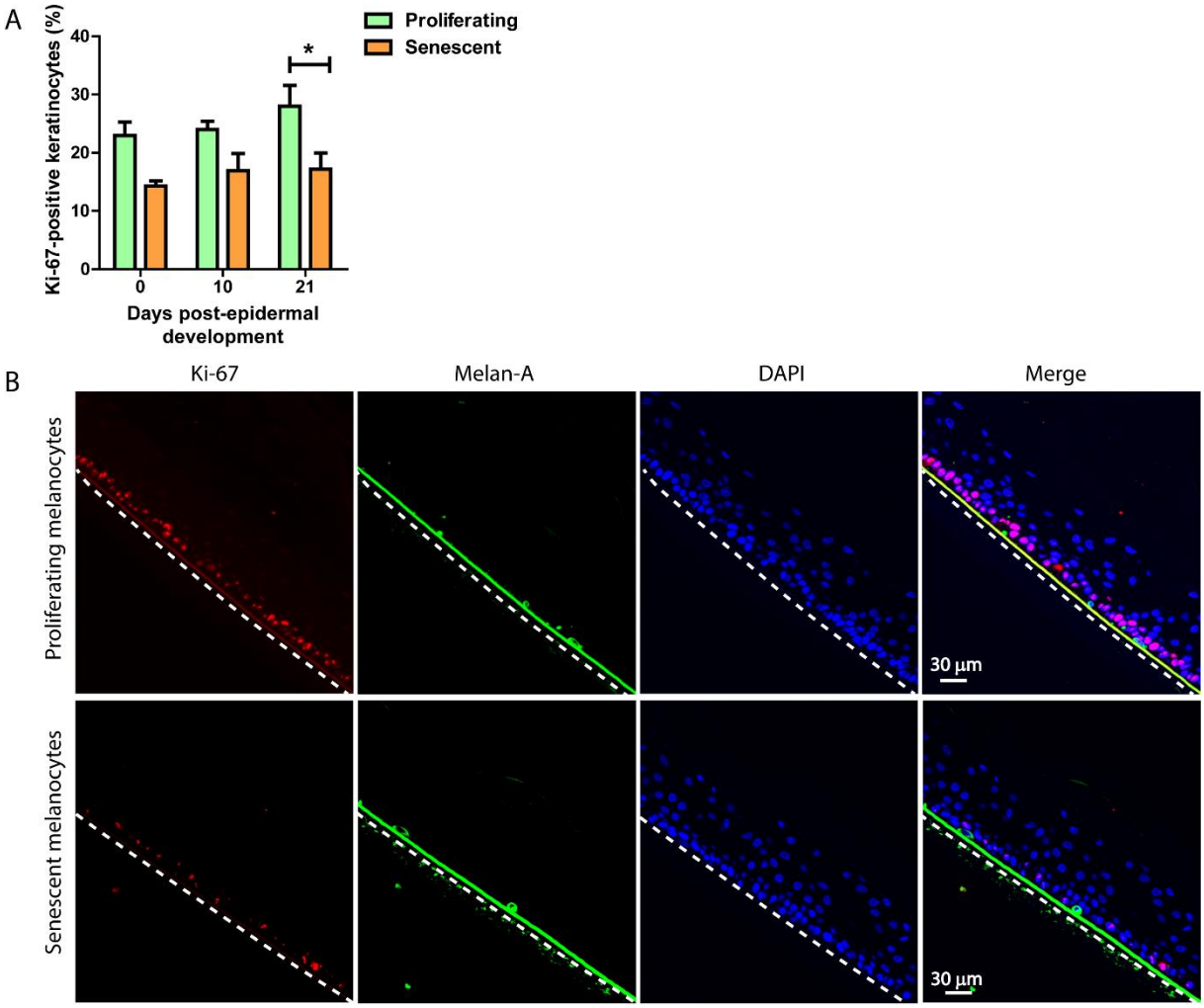

Figure 5.11 – Keratinocytes in melanoderms with senescent melanocytes have reduced proliferative capacity. (A) Graph showing the percentage of Ki-67 positive keratinocytes in melanoderms containing either proliferating or senescent melanocytes at the time points indicated. Data are mean ± S.E.M. of N=3 melanoderms for each condition. Statistical significance was determined by a two-way ANOVA. *P<0.05. **(B)** Representative Ki-67 immunofluorescence images in melanoderms containing proliferating (top) and senescent (bottom) melanocytes. Ki-67 is shown in red, melan-A is shown in green, and DAPI is depicted in blue. The white dotted line represents the basal layer of the melanoderms. Images were taken with a 20x objective.

5.6.2. Increased p16 expression in keratinocytes from melanoderms with senescent melanocytes

In order to further investigate whether senescent melanocytes could induce paracrine senescence in surrounding keratinocytes, I performed immunofluorescence for p16. I found that p16 expression was significantly increased in keratinocytes from melanoderms containing senescent melanocytes at all time points investigated ($P<0.05$ and $P<0.01$) (***Figure 5.12 A, B***). In fact, there is at least a 2-fold increase in the percentage of p16-positive keratinocytes in senescent melanocyte-containing melanoderms (***Figure 5.12 A***), suggesting that senescent melanocytes exert a bystander effect that likely contributes to paracrine keratinocyte senescence.

Although p16 expression in keratinocytes increases slightly at 10 days, both in proliferating and senescent melanocyte-containing melanoderms, this increase was not statistically significant. Interestingly, the number of p16-positive keratinocytes decreases between 10 and 21 days in both groups of melanoderms (***Figure 5.12 A***), albeit not significantly, possibly suggesting that a fraction of p16-positive keratinocytes are lost during this period.

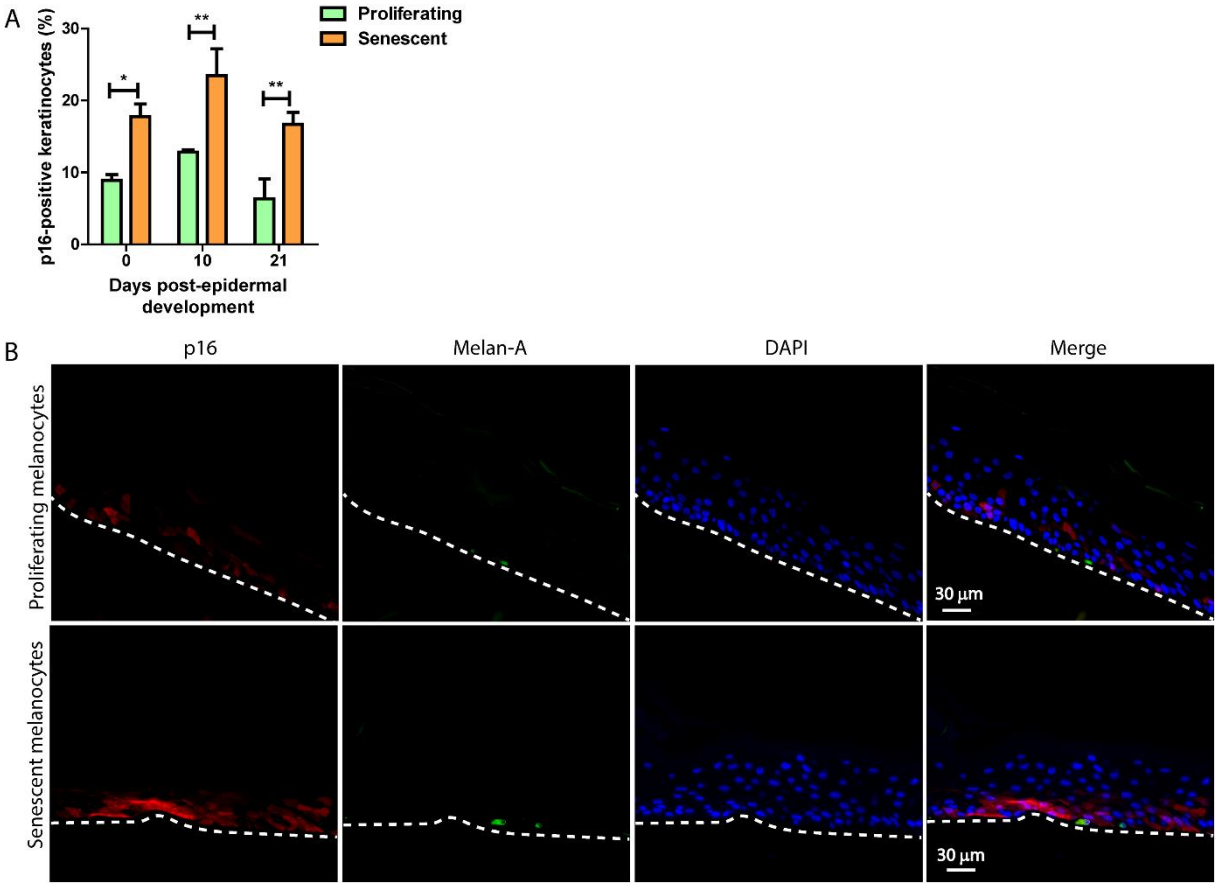

Figure 5.12 – Keratinocytes in senescent melanocyte-containing melanoderms exhibit increased p16 expression. (A) Graph showing the percentage of p16-positive keratinocytes in melanoderms containing proliferating or senescent melanocytes at the time points indicated. Data are mean ± N=4 (proliferating) or N=3 (senescent) melanoderms for each time point. Statistical significance between groups was assessed using a two-way ANOVA, whilst a one-way ANOVA was used to determine differences within groups. *P<0.05, **P<0.01. **(B)** Representative p16 immunofluorescence images of melanoderms containing either proliferating (left) or senescent (right) melanocytes. Red shows p16, green represents melan-A, and DAPI is shown in blue. The white dotted line represents the basal layer of the melanoderms. Images were taken using a 20x objective.

5.6.3. Senescent melanocytes induce paracrine telomere-associated foci in neighbouring keratinocytes in epidermal equivalents

So far, my results provide evidence that senescent melanocytes induce paracrine TAF in surrounding cells both in human skin *in vivo* and also *in vitro*. Therefore, to corroborate these observations, and assess whether paracrine DDR activation, a main effector of senescence, also occurred in a living human epidermal equivalent, I performed immuno-FISH together with immunofluorescence for the melanocyte marker, melan-A, and analysed co-localisation of γH2AX at telomeres in keratinocytes surrounding melanocytes, as shown in ***Figure 5.13 D***.

Firstly, I found that there is a significant increase in total DNA damage signalling at 10 and 21 days in keratinocytes from melanoderms containing both proliferating and senescent melanocytes (***Figure 5.13 A***), green and orange asterisks, respectively). Despite this time-dependent increase in γH2AX foci, keratinocytes in melanoderms containing senescent melanocytes have a higher amount of DNA damage foci at all time points analysed, although this only reached statistical significance at day 0 ($P<0.05$) (***Figure 5.13 A***). Furthermore, the frequency of dysfunctional telomeres in keratinocytes also increases at both 10 and 21 days post-development when compared to day 0; however, this increase is only statistically significant in cells within melanoderms containing senescent melanocytes. In fact, these keratinocytes display, on average, a 3.2- and 2.2-fold increase in telomere-associated foci at 10 and 21 days, respectively, in relation to cells at day 0 ($P<0.01$ and $P<0.05$) (***Figure 5.13 B***). Interestingly, keratinocytes in melanoderms with senescent melanocytes exhibit a significantly higher amount of damaged telomeres in comparison to those cultured with proliferating melanocytes, both at 10 and 21 days post-development ($P<0.001$ and $P<0.01$, respectively) (***Figure 5.13 B, D***). These observations are consistent with my previous results showing that senescent melanocytes can induce paracrine telomere dysfunction both in human skin *in vivo* and in monolayer cultures *in vitro*. Accordingly, melanoderms containing senescent melanocytes have a higher percentage of keratinocytes that contain more than one TAF, although this is only statistically significant at days 10 and 21 ($P<0.001$) (***Figure 5.13 C***). Whilst 31% and 27% of keratinocytes surrounding senescent melanocytes display more than one TAF at days 10 and 21, respectively, only 7% and 4% of cells in the vicinity of proliferating melanocytes contain more than one dysfunctional telomere at the same time points (***Figure 5.13 C***). Moreover, although there is a significant increase in the percentage of keratinocytes containing more than one TAF at 10 days when compared to day 0 in melanoderms containing proliferating melanocytes ($P<0.05$), a more striking time-dependent increase is observed in

melanoderms with senescent melanocytes, which remains significantly elevated at day 21 ($P<0.05$) (***Figure 5.13 C***), suggesting that chronic exposure to senescent melanocytes promotes accumulation of cells containing dysfunctional telomeres.

Overall, these results indicate that senescent melanocytes trigger paracrine DDR signalling in surrounding keratinocytes, both at telomeres and non-telomeric sites, and implicate senescent melanocytes as important sources of paracrine signalling that contributes to bystander damage induction in living epidermal equivalents.

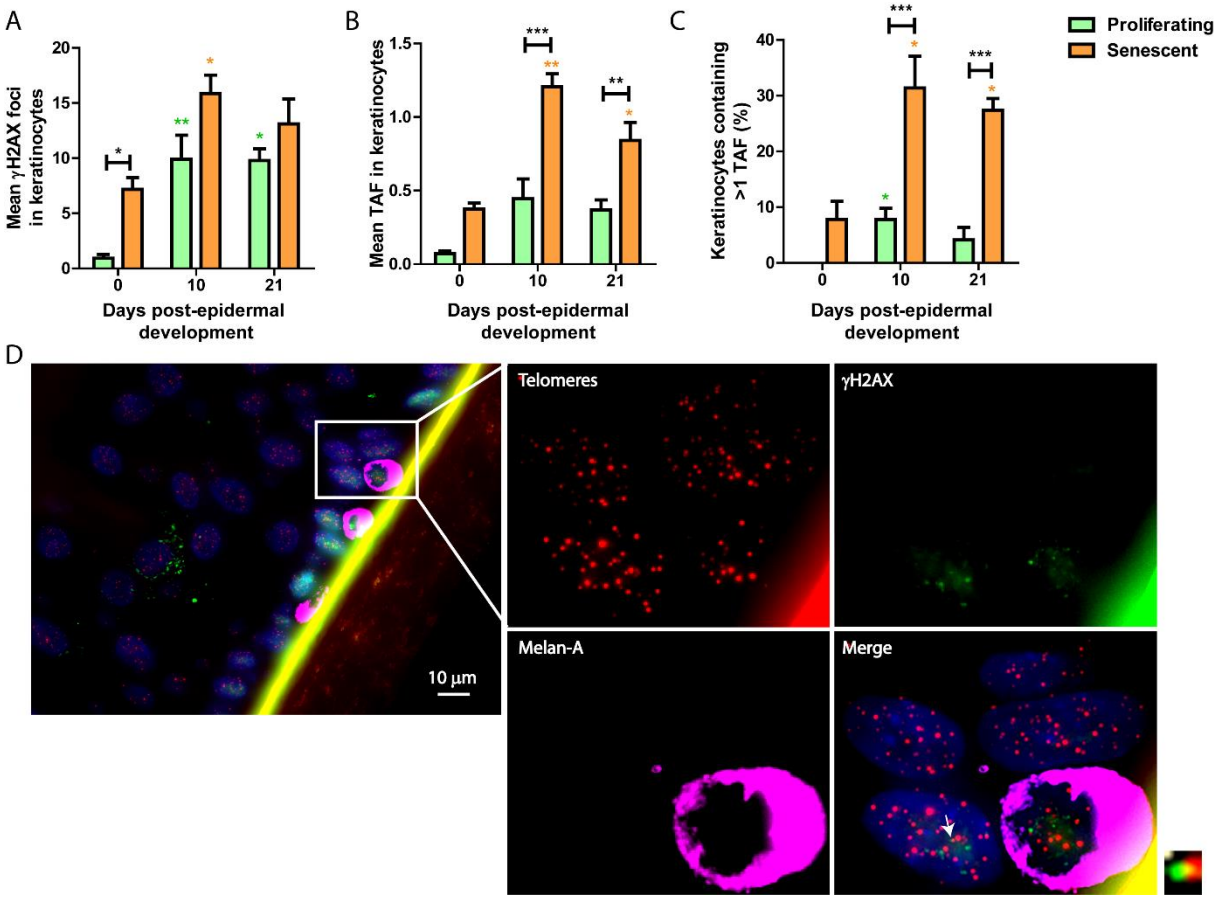

Figure 5.13 – Senescent melanocytes induce paracrine telomeric and non-telomeric DDR signalling in neighbouring keratinocytes. Graphs showing **(A)** mean number of γH2AX foci in keratinocytes, **(B)** mean number of TAF in keratinocytes, and **(C)** percentage of keratinocytes containing more than 1 TAF in melanoderms containing proliferating or senescent melanocytes at the time points indicated. Data are mean ± S.E.M. of N=3 melanoderms for each condition. Statistical significance between groups was determined by a two-way ANOVA, and differences within groups was assessed by a one-way ANOVA (green and orange asterisks). *P<0.05, **P<0.01, ***P<0.001. **(D)** Representative immuno-FISH image of a melanoderm containing senescent melanocytes. Telomeres are shown in red, γH2AX is shown in green, melan-A is shown in pink, and blue represents DAPI. Arrows indicate co-localisation between γH2AX and telomeres, which is amplified on the right. Images were taken using a 63x objective.

5.6.4. Keratinocytes in melanoderms with senescent melanocytes have increased markers of oxidative DNA damage

Since my previous *in vitro* data suggested that paracrine DNA damage was mediated by increased intracellular ROS in bystander cells, I wanted to investigate whether oxidative stress was also a mechanism of bystander DNA lesion formation in keratinocytes in human epidermal equivalents. For that, I performed immunofluorescence for 8-oxo-G, which is indicative of oxidative DNA damage, together with the melanocyte marker melan-A, in order to distinguish melanocytes from keratinocytes.

Melanoderms with senescent melanocytes contain significantly more keratinocytes positive for 8-oxo-G (P=0.009, red asterisks) (***Figure 5.14 A, B***). Moreover, post-hoc analysis within each time point revealed a significant difference at day 0 (P=0.04), when 79% of keratinocytes are positive for 8-oxoG in senescent melanocyte-containing melanoderms compared to 49% of 8-oxo-G-positive cells in the proliferating counterpart. These results suggest that oxidative stress contributes to bystander DNA damage induction in keratinocytes, although further experiments are needed to confirm this in a model of human epidermal equivalent.

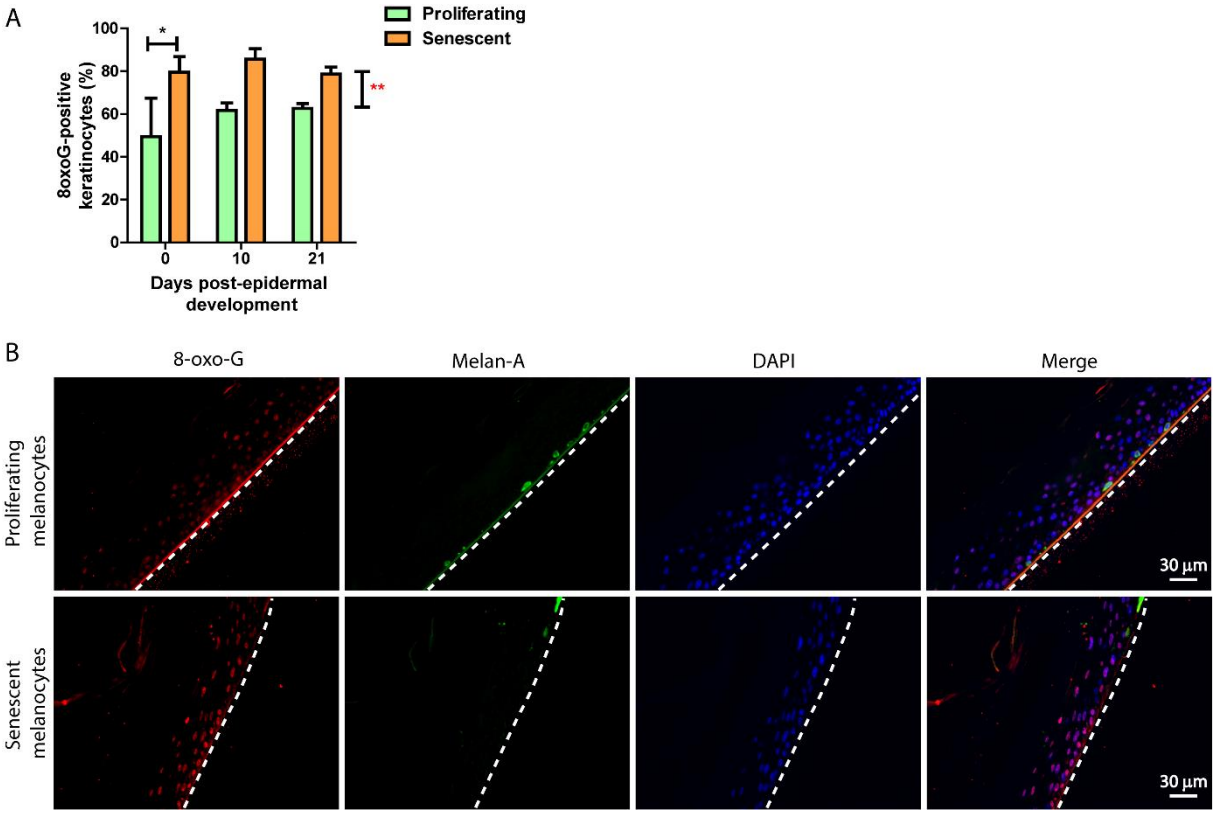

Figure 5.14 – Increased marker of oxidative DNA lesions in keratinocytes from melanoderms with senescent melanocytes. (A) Graph showing the percentage of 8-oxo-G-positive keratinocytes in melanoderms containing proliferating or senescent melanocytes at the time points indicated. Data are mean ± S.E.M. of N=4 (proliferating) and N=3 (senescent) melanoderms. Statistical significance was determined by a two-way ANOVA with a Holm-Sidak post-hoc test. Red asterisk indicates difference between melanoderms with proliferating and senescent melanocytes. *P<0.05, **P<0.01. **(B)** Representative 8-oxo-G immunofluorescence images of melanoderms containing either proliferating (top) or senescent (bottom) melanocytes. Red shows 8-oxo-G, green represents melan-A, and DAPI is shown in blue. The white dotted line represents the basal layer of the melanoderms. Images were taken using a 20x objective.

5.6.5. Accumulation of senescent melanocytes contributes to decreased epidermal thinning in melanoderms

Having shown that senescent melanocytes trigger paracrine DDR signalling and senescence in keratinocytes, I then wanted to assess whether the presence of senescent melanocytes, and their resulting bystander effects, could contribute to characteristics associated with skin ageing. By measuring the epidermal thickness of melanoderms, I found that thickness significantly increased with time in epidermal equivalents containing proliferating melanocytes (green asterisks); however, this time-dependent increase was not observed in melanoderms containing senescent melanocytes (***Figure 5.15 A***). This suggests that senescent melanocytes impair the differentiation and proliferation of keratinocytes, which in this case, is necessary for epidermal differentiation and renewal. Moreover, melanoderms containing senescent melanocytes have a significantly thinner epidermis at day 21 compared to their proliferating counterpart ($P<0.001$) (***Figure 5.15 A, B***), suggesting that accumulation of senescent melanocytes contributes to age-associated epidermal thinning. This likely occurs through their ability to exert paracrine effects that induce senescence in neighbouring keratinocytes.

In summary, these results suggest that senescent melanocytes induce paracrine DNA damage and senescence in surrounding keratinocytes. Our data support the hypothesis that paracrine senescence compromises the proliferative potential of keratinocytes in the skin, consequently resulting in age-associated characteristics such as epidermal thinning.

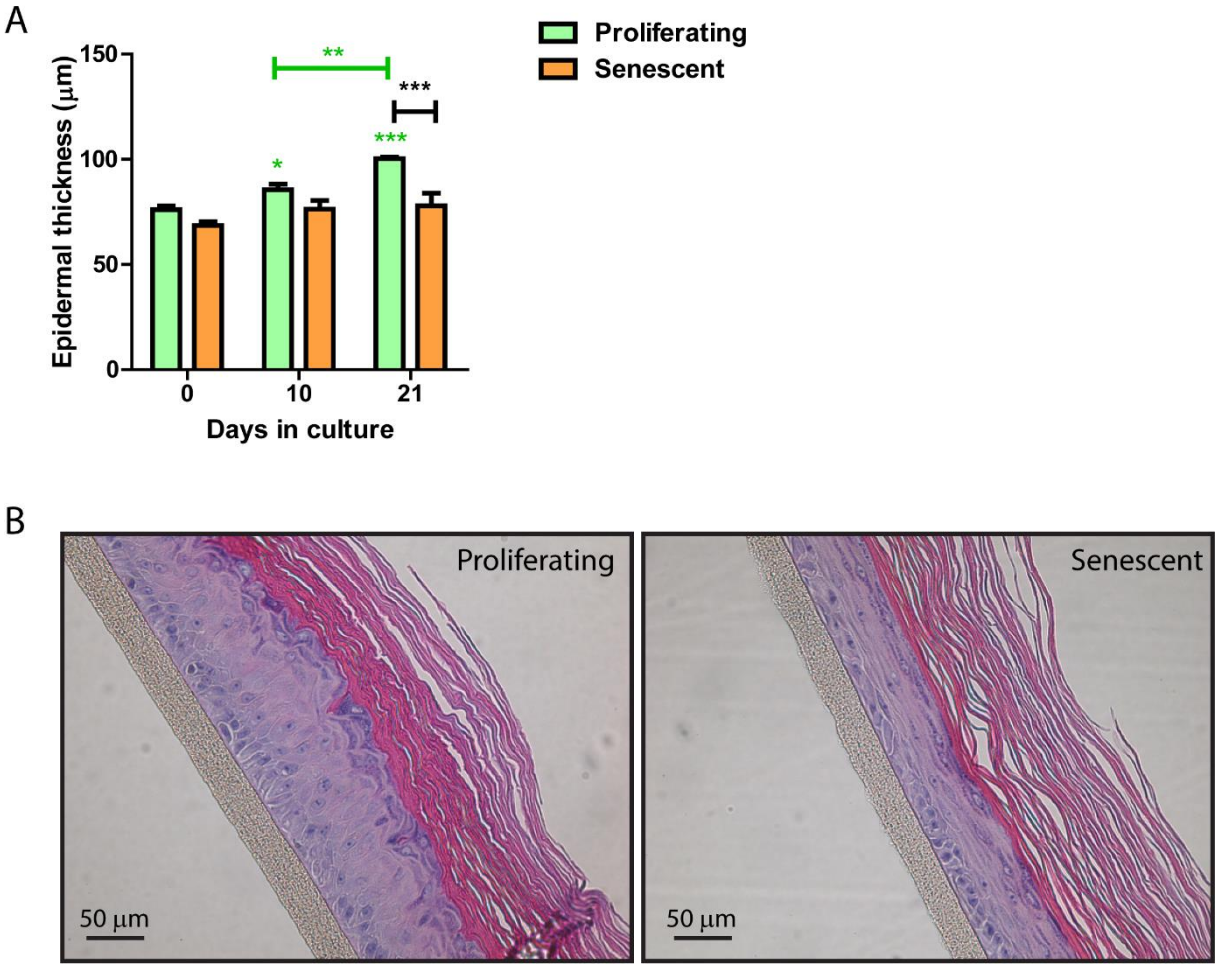

Figure 5.15 – Senescent melanocytes contribute to epidermal thinning. (A) Graph showing epidermal thickness of melanoderms containing either proliferating or senescent melanocytes at the time points indicated. Data are mean ± S.E.M. of N=4 (proliferating) and N=3 (senescent) melanoderms. Statistical analysis between groups was determined by a two-way ANOVA, whereas differences within groups was assessed by a one-way ANOVA (green asterisks show statistical significance within melanoderms containing proliferating melanocytes). *P<0.05, **P<0.01, ***P<0.001. **(B)** Representative H&E images showing epidermal thickness of melanoderms containing proliferating or senescent melanocytes. Images were taken using a 20x objective.

5.7. Discussion

In this study, we provide evidence that senescent melanocytes induce paracrine DNA damage foci at both telomeric and non-telomeric regions in dermal fibroblasts *in vitro*, which is mediated by secretion of SASP factors. Indeed, previous studies have shown that fibroblasts undergoing both oncogene-induced and replicative senescence can trigger DNA damage in neighbouring, otherwise healthy, fibroblasts (Nelson *et al.*, 2012; Acosta *et al.*, 2013). Although one study only observed bystander DNA damage when cells were in direct co-culture, suggesting that transmission of soluble factors occurs through gap junctions (Nelson *et al.*, 2012), others have demonstrated that exposure to conditioned medium of senescent cells is sufficient to elicit paracrine effects (Acosta *et al.*, 2013), corroborating our observations. Such disparities may be due to the composition or amount of secreted SASP factors, and may also depend on the senescence-inducing stimuli. Unpublished observations from our lab have shown that MRC5 fibroblasts secreted considerably less amounts of SASP factors, such as IL-6 and IL-8, than IMR90 fibroblasts. Moreover, recent work has demonstrated that different senescent cell types display large transcriptional heterogeneity (Hernandez-Segura *et al.*, 2017), which may explain the differences in bystander effects observed in different studies.

Importantly, exposure to senescent cells was shown to stimulate the generation of large DNA damage foci in bystander fibroblasts (Nelson *et al.*, 2012). Large foci tend to remain unresolved for longer compared to smaller ones (Nelson *et al.*, 2012), and in fact, the majority of long-lived foci have been shown to co-localise with telomeres (Hewitt *et al.*, 2012). Although the authors did not distinguish between paracrine telomeric and non-telomeric damage, this observation would be consistent with our results showing that telomere dysfunction is induced in bystander cells upon exposure to senescent melanocytes. Our results show that despite the increase in non-telomeric DNA damage foci in fibroblasts co-cultured with senescent melanocytes, this was not statistically significant. One possibility is that some of the paracrine damage induced by senescent melanocytes is repaired by fibroblasts; however, further experiments involving live-cell imaging or a more extensive time-course analysis would need to be conducted to achieve this conclusion. Alternatively, this observation could be attributed to the different microscopy system used in this experiment, which was shown to be more sensitive and detect a higher number of γH2AX foci than previously used systems. Since a large amount of DNA damage foci was detected in fibroblasts in all co-culture conditions, this possibly makes it difficult to identify any subtle significant differences. Nevertheless, conditioned medium from senescent melanocytes was sufficient to trigger non-telomeric DDR

activation in fibroblasts, which was observed both at 2 days and 30 days of exposure, suggesting that non-telomeric DNA damage is also induced by soluble factors from senescent melanocytes.

Of note, senescent melanocyte conditioned medium did not significantly induce γH2AX foci in fibroblasts during the experiments involving rapamycin-treated melanocyte CM, which contradicts the other experiments using CM in this study. One likely explanation is that the fibroblasts used in the former experiments had a higher basal level of DNA damage foci, and in turn, any subtle changes in damage induction could not be detected. Another possibility is that melanocytes were only cultured for 24 hours in the conditioned medium used to treat fibroblasts in the experiments involving rapamycin, since leaving melanocytes without rapamycin for any longer could have resulted in reactivation of the SASP. In contrast, in other experiments involving the use of CM, melanocytes were left for four days before CM was collected. Therefore, it is possible that a higher concentration of pro-inflammatory factors was present in the latter, explaining the significant induction of γH2AX foci in bystander fibroblasts in these experiments. Nevertheless, soluble factors released by senescent melanocytes were sufficient to trigger TAF in all experiments involving the use of conditioned medium. It is likely that telomeres are more sensitive to subtle disturbances in intracellular homeostasis that may occur as a result of paracrine signalling activation. In fact, our results indicate that bystander telomeric and non-telomeric DNA damage can be prevented by scavenging mitochondrial ROS generation, suggesting that paracrine damage is mediated by signals that enhance ROS production. Indeed, telomeres have been shown to be more susceptible to oxidative damage when compared to the rest of the genome, with mild oxidative stress causing single-stranded breaks to preferentially accumulate at telomeres (Petersen *et al.*, 1998; Oikawa and Kawanishi, 1999). Therefore, despite only representing 0.02% of the genome (Fumagalli *et al.*, 2012), telomeres might act as more sensitive sensors to subtle alterations in intracellular oxidative status.

In our model of co-culture, inhibiting the SASP of senescent melanocytes by rapamycin did not reduce paracrine TAF induction. On the other hand, culturing fibroblasts with conditioned medium obtained from rapamycin-treated senescent melanocytes did not trigger telomere dysfunction in bystander cells, suggesting that inhibition of the SASP is sufficient to prevent paracrine DDR signalling induction in this case. One possible explanation for this disparity could be the experimental approach; rapamycin was removed from melanocytes 24 hours prior to setting up co-culture with fibroblasts, which means that at 48 hours of co-culture, when DNA damage foci were analysed, melanocytes had not been treated with rapamycin for 3 days.

Therefore, it is possible that mTOR was no longer being inhibited during the co-culture, allowing secretion of SASP factors by senescent melanocytes to be restored. This would explain how rapamycin-treated senescent melanocytes are still efficient in inducing paracrine DNA damage foci in fibroblasts. In order to confirm this, mTOR activity should be evaluated in rapamycin-treated senescent melanocytes up to 3 days following removal of rapamycin, which would allow us to obtain a better understanding of the kinetics of mTOR reactivation in the absence of pharmacological inhibition. Moreover, a cytokine array could be performed in conditioned medium obtained from these cells to determine whether secretion of pro-inflammatory cytokines is reinstated by senescent melanocytes after rapamycin removal.

Nevertheless, our results indicate that paracrine DDR signalling induction, both at telomeres and non-telomeric sites, is mediated by soluble factors released by senescent melanocytes, and these persist with prolonged exposure of fibroblasts to conditioned medium from senescent cells. In fact, there is a slight increase in the mean number of telomere-associated foci in bystander fibroblasts following 30 days of culture in senescent melanocyte CM when compared to 2 days. Although this could suggest that paracrine TAF are persistent, another possibility is that they are constantly generated and repaired by fibroblasts, thus explaining the constant levels of TAF in bystander fibroblasts with time. In order to further investigate this, live-cell imaging would need to be performed to follow individual foci, providing a better insight into their repair kinetics. Moreover, we also show that paracrine non-telomeric DDR also remains higher in fibroblasts exposed to soluble factors from senescent melanocytes, although the difference is not as striking as the increase in TAF. It is possible that a fraction of non-telomeric DNA lesions become repaired, as it has been shown that non-telomeric foci are short lived (Hewitt *et al.*, 2012). Since our data suggest an involvement of ROS in paracrine damage formation, it is likely that these short-lived foci become constantly replenished by increased oxidative stress, as it has been shown to occur in replicative and stress-induced senescence (Passos *et al.*, 2010). Indeed, persistent DDR signalling is an important effector of senescence, activating downstream CDK inhibitors, such as p16 as p21, that mediate the stable growth arrest (Campisi and d'Adda di Fagagna, 2007b). According to previous studies, as well as inducing DNA damage, senescent cells can trigger paracrine senescence (Nelson *et al.*, 2012; Acosta *et al.*, 2013), with one group reporting that paracrine proliferation arrest is dependent on p16 and p21, and thus fully resembles the senescence response (Acosta *et al.*, 2013). Despite senescent melanocytes triggering DDR signalling in fibroblasts in our study, we only observed a small, albeit significant, decrease in proliferation in bystander cells, and other markers of senescence

were not upregulated (data not shown). This disparity could be due to the different experimental approaches used in these studies. For example, one group reported paracrine senescence only in a co-culture setting (Nelson *et al.*, 2012), whereas others used either co-cultures or Transwells, which ensures that young and senescent cells are physically separated whilst still sharing the same culture medium (Acosta *et al.*, 2013). In either case, proliferating cells are constantly exposed to factors freshly secreted from senescent cells, whereas in our study we used conditioned medium which had to be stored to be used for the duration of the experiments. Although multiple thaw and freeze cycles were avoided by storing CM in aliquots, we cannot exclude the possibility that soluble factors undergo some degree of degradation, and thus the paracrine effects we observe in our study are not as striking as in previous reports. Nevertheless, Acosta *et al.* demonstrated that conditioned medium from senescent cells induced growth arrest in normal fibroblasts 4 days after culture (Acosta *et al.*, 2013). However, in this study, they used cells undergoing oncogene-induced senescence. It is possible that the SASP from OIS cells triggers stronger mitogenic responses in bystander cells, resulting in faster development of paracrine senescence, although further experiments are needed to confirm this.

Our data suggest that paracrine DNA damage induction by senescent melanocytes is mediated by increased intracellular ROS in bystander fibroblasts, consistent with previous studies showing that bystander DDR signalling activation could be rescued by antioxidants (Nelson *et al.*, 2012). It is possible that increased ROS leads to telomeric DNA damage by contributing to telomere shortening in bystander fibroblasts. Indeed, mild oxidative stress has been shown to accelerate the rate of loss of telomeric repeats (von Zglinicki *et al.*, 1995; Xu *et al.*, 2000; von Zglinicki, 2002; Saretzki *et al.*, 2003), which can be rescued by genetic or pharmacological manipulations that reduce ROS levels (von Zglinicki, 2000; Saretzki *et al.*, 2003; Serra *et al.*, 2003). It is believed that when telomeres reach a critical length, shelterin proteins can no longer bind, leading to uncapping of the protective structure. This is turn exposes the ends of telomeres, which are then recognised as DSBs by the DNA repair machinery, leading to DDR activation at chromosome ends (O'Sullivan and Karlseder, 2010). Although extensive shortening is not likely to occur within 48 hours, it is possible that prolonged exposure to SASP factors from senescent melanocytes could contribute to paracrine telomere damage in this way. Alternatively, oxidative DNA lesions have been shown to disrupt binding of TRF1 and TRF2 at telomeres (Opresko *et al.*, 2005), providing a mechanism for loss of shelterin components independently of shortening. In order to investigate the mechanisms involved in paracrine DDR activation at telomeres, future experiments should be performed whereby fibroblasts

overexpressing either telomerase or TRF2 are cultured with senescent melanocyte CM. This will allow us to determine if telomere damage in bystander cells occurs due to shortening or loss of shelterin, respectively.

Although we did not investigate the mechanisms leading to enhanced ROS generation in bystander cells, we demonstrate that CXCR3 activation plays a role in transducing signals that contribute to paracrine DNA damage foci induction in fibroblasts (**Figure *5.16***). Interestingly, stimulation of the CXCR3 receptor has been shown to increase oxidative stress in human kidney cells through Ca^{2+}-dependent up-regulation of the transcription factor early growth response gene (EGR)-1 (Bek *et al.*, 2003). In turn, the antioxidant enzymes copper-zinc superoxide dismutase and manganese superoxide dismutase are down-regulated, and activity of NADH/NADPH-oxidase is increased, contributing to ROS generation (Bek *et al.*, 2003). Moreover, CXCR3 activation in human airway epithelial cells, which regulates epithelial cell movement, was shown to activate p38 MAPK (Shahabuddin *et al.*, 2006). In the context of senescence, p38 has been involved in signalling pathways that lead to mitochondrial dysfunction, and consequently, enhanced ROS production, which is important for maintaining DDR signalling and growth arrest (Passos *et al.*, 2010). Indeed, increased p38 activity has been reported in bystander fibroblasts co-cultured with senescent cells, suggesting a role for p38 MAPK in paracrine senescence (Nelson *et al.*, 2012). Although we demonstrate that bystander telomere damage can be prevented by inhibiting CXCR3 or by scavenging mitochondrial ROS, further experiments are needed to establish whether oxidative stress increases as a direct result of CXCR3 signalling in bystander fibroblasts. For example, ROS levels could be measured by FACS following stimulation of fibroblasts with the CXCR3 agonist, IP-10. Additionally, fibroblasts cultured with senescent melanocyte CM could be treated with both AMG487 and MitoQ, to investigate whether CXCR3 signalling and ROS act synergistically to trigger paracrine DNA damage. To further corroborate the role of CXCR3 activation in paracrine DDR signalling induction, fibroblasts following knock-down (shRNA) or knock-out (through CRISPR-CAS9) of CXCR3 should be treated with senescent melanocyte conditioned medium, and DNA damage should then be analysed. This would allow us to determine whether the effects seen with AMG487 can be replicated, as pharmacological manipulation might not result in full inhibition of the pathway. Moreover, immuno-FISH could be performed in IP-10-treated fibroblasts to further assess the consequence of direct CXCR3 activation on the DNA damage response. Future experiments could also be conducted in order to examine the role of p38 MAPK activation in paracrine DNA damage induction. For example, p38 protein levels could

be assessed following culture of fibroblasts with senescent melanocyte CM. Moreover, paracrine DNA damage induction should be investigated following p38 inhibition in bystander fibroblasts.

Intriguingly, inhibiting CXCR3 in fibroblasts did not rescue paracrine total γH2AX foci, whereas it was sufficient to prevent TAF induction. It is possible that differences were not identified due to the larger variability in the number of γH2AX foci in AMG487-treated fibroblasts cultured with senescent melanocyte CM.

We have also demonstrated that CXCR3 is up-regulated in senescent melanocytes *in vitro* and *in vivo* in human skin with age. Moreover, we showed that signalling through CXCR3 is partly involved in the development of phenotypes associated with melanocyte senescence. Our results show that IP-10, a ligand of CXCR3, is highly secreted by stress-induced senescent melanocytes. This is consistent with studies showing that autophagy-deficient murine melanocytes, previously described to enter premature senescence, (Zhang *et al.*, 2015), secrete significantly larger amounts of IP-10 compared to control cells (Ni *et al.*, 2016). Inhibition of CXCR3 in senescent melanocytes was sufficient to significantly reduce markers of senescence such as Sen-β-Gal activity and p16 expression, although these phenotypes were not completely prevented. Moreover, despite a small reduction in DNA damage foci, DDR signalling was not significantly rescued by CXCR3 inhibition, suggesting that additional mechanisms are responsible for maintaining the DDR during melanocyte senescence. Additionally, the senescence-associated growth arrest was also not prevented by inhibiting CXCR3. It is possible that the persistent DDR observed in AMG487-treated cells provides the signalling necessary to continuously activate p21, and maintain the growth arrest (Passos *et al.*, 2010). Consistently, our data demonstrate that p21 levels are not reduced by CXCR3 inhibition, likely explaining how proliferation is not rescued in these cells. Interestingly, mitochondrial ROS production was effectively reduced by preventing CXCR3 activity. As previously mentioned, CXCR3 activation has been shown to induce oxidative stress in kidney cells (Bek *et al.*, 2003), although the mechanisms leading to signal transduction from CXCR3 to mitochondrial ROS generation remain unknown. One possibility is that CXCR3 acts through p38, inducing mitochondrial dysfunction and oxidative stress, as discussed above. Moreover, activation of PI3K/Akt also occurs downstream of CXCR3 signalling (Bonacchi *et al.*, 2001; Shahabuddin *et al.*, 2006), consistent with our data showing that CXCR3 inhibition reduces Akt phosphorylation in X-ray irradiated melanocytes. In fact, Akt has been involved in signalling cascades that converge signals from the DDR into PGC-1β-dependent mitochondrial biogenesis, contributing to

increased ROS generation (Correia-Melo *et al.*, 2016). Therefore, it is possible that CXCR3 contributes to mitochondrial oxidative stress via Akt, although further experiments are needed in order to confirm this. Furthermore, ERK phosphorylation has also been reported to occur as a result of CXCR3 activation (Shahabuddin *et al.*, 2006; Jenkins *et al.*, 2015); however, its involvement in the induction of melanocyte senescence remains elusive from our results. Although ERK appears to be initially activated in response to irradiation, with CXCR3 inhibition reducing p-ERK levels at 8 hours, total ERK expression seems to gradually decrease in irradiated cells. Therefore, further analysis is needed in order to corroborate the involvement of ERK activation during stress-induced melanocyte senescence. Moreover, we cannot fully conclude that these signalling pathways are activated as a result of CXCR3 activation in our model. Further experiments should be conducted using the agonist IP-10 to determine whether direct CXCR3 activation leads to up-regulation of the aforementioned pathways, and to assess whether it also induces melanocyte senescence. Interestingly, CXCR3 signalling has been shown to mediate IL-8 expression in $BRAF^{WT}$ melanoma cells, which was shown to be dependent on the MEK/ERK pathway (Jenkins *et al.*, 2015). Therefore, future work should also be performed to investigate the SASP of senescent melanocytes following CXCR3 inhibition. Overall, these results suggest that although CXCR3 signalling contributes, in part, to the establishment of melanocyte senescence, other mechanisms are also involved in this process, since CXCR3 inhibition only partly rescues senescence-associated phenotypes.

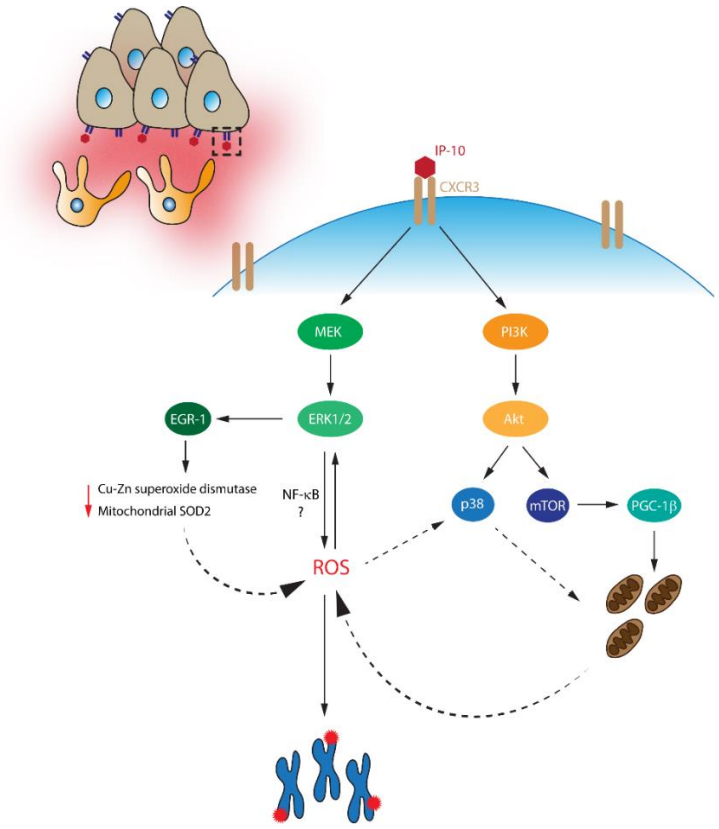

Figure 5.16 - Possible mechanisms contributing to ROS production and DNA damage in bystander cells. Senescent melanocytes (yellow cells) secrete soluble factors, such as IP-10, (indicated in red) which triggers an increase in ROS and telomere damage in surrounding cells (grey cells). Activation of CXCR3 has been shown to induce pathways that contribute to increased ROS production. For example, CXCR3 stimulation can activate the MEK/ERK pathway (Jenkins *et al.*, 2015), and this favours ROS generation through up-regulation of the EGR-1 transcription factor, which down-regulates the antioxidant enzymes Cu-Zn dismutase and mitochondrial SOD2 (Bek *et al.*, 2003). It is also possible that MEK/ERK contributes to ROS production via NF-κB activation (Monturiol-Gross *et al.*, 2014). In addition, activation of the PI3K/Akt pathway has also been shown to occur downstream of CXCR3 signalling (Bonacchi *et al.*, 2001; Shahabuddin *et al.*, 2006). Akt can promote PGC-1β-dependent mitochondrial biogenesis, which in turn leads to increased oxidative stress (Correia-Melo *et al.*, 2016). Moreover, Akt can also activate p38 (Shahabuddin *et al.*, 2006), and the latter has been involved in signalling pathways that lead to mitochondrial dysfunction, thus enhancing ROS production (Passos *et al.*, 2010). Although, from our data, we cannot conclude that these pathways are activated in bystander cells, it is possible that they contribute to oxidative stress. Ultimately, increased ROS generation contributes to telomeric and non-telomeric DNA damage in bystander cells.

Although exposure to senescent melanocyte CM was not able to induce senescence in dermal fibroblasts over a period of 20 days, we did observe a small reduction in proliferation. This effect is relatively mild compared to paracrine senescence induced by OIS cells, where loss of proliferation and induction of senescence markers is observed after a few days (Acosta *et al.*, 2013). Nonetheless, we provide evidence that senescent melanocytes can trigger paracrine senescence in keratinocytes in a three-dimensional human epidermal equivalent, or melanoderms. Indeed, our data show that keratinocytes in melanoderms with senescent melanocytes have decreased proliferative potential, and increased p16 expression, suggesting that senescent melanocytes induce paracrine senescence in keratinocytes. Interestingly, the majority of Ki-67-positive keratinocytes were located in the basal layer of melanoderms, which likely reflects the fact that keratinocytes become fully differentiated and lose their proliferative capacity as they migrate up the epidermis (Elias *et al.*, 1977). Although there was a trend for increased γH2AX foci in keratinocytes cultured with senescent melanocytes, this was not statistically significant. On the other hand, these cells accumulate significantly higher numbers of dysfunctional telomeres compared to keratinocytes from melanoderms with proliferating melanocytes, suggesting that senescent melanocytes also trigger paracrine TAF in neighbouring keratinocytes. As previously mentioned, dysfunctional telomeres provide a source of persistent DDR that is important for the initiation and maintenance of senescence (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012). Indeed, triggering telomere dysfunction in keratinocytes by overexpressing a dominant-negative version of TRF2, which causes uncapping, induces senescence in these cells, suggesting that telomere damage is a trigger of keratinocyte senescence (Minty *et al.*, 2008).

Intriguingly, we observe a small decrease in both the number of TAF and the percentage of keratinocytes containing more than one TAF at day 21 compared to day 10 in melanoderms containing senescent melanocytes. It is possible that some damaged telomeres become repaired, as recent studies have provided evidence that homologous recombination and alt-NHEJ occur at telomeres in response to DSBs (Doksani and de Lange, 2016; Mao *et al.*, 2016). Another scenario is that keratinocytes with high numbers of dysfunctional telomeres, or senescent, undergo cell death. Consistent with this, studies have shown that senescent keratinocytes undergo autophagic programmed cell death in culture (Gosselin et al., 2009). The authors suggest that accumulation of DNA damage and dysfunctional mitochondria triggers autophagic degradation of nuclei and mitochondria, respectively, resulting in senescent keratinocyte death *in vitro* (Gosselin et al., 2009). Moreover, keratinocyte senescence has been associated with

increased levels of the mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD), which is responsible for converting oxygen radicals into H_2O_2 (Bernard *et al.*, 2004). This is turn increases oxidative stress since catalase and glutathione peroxidase, which convert H_2O_2 into H_2O, were not up-regulated, thus contributing to keratinocyte senescence (Bernard *et al.*, 2004). It was later demonstrated that overexpression of MnSOD or H_2O_2 treatment could induce keratinocyte senescence with phenotypes resembling replicative senescence (Deruy *et al.*, 2010). These cells displayed increased oxidative DNA damage as well as accumulation of damaged mitochondria, and underwent autophagic cell death as a result of oxidative stress (Deruy *et al.*, 2010). Furthermore, we also observe a small decline in p16-positive keratinocytes in melanoderms containing senescent melanocytes between days 10 and 21, which would agree with the hypothesis that paracrine keratinocyte senescence partly contributes to cell death. However, further experiments are needed to confirm this hypothesis.

Although there was a small increase in the frequency of TAF in melanoderms with proliferating melanocytes at day 10, this was not significant, and it could be a result of cell culture stress. Alternatively, it is possible that a fraction of these cells are undergoing senescence due to replicative exhaustion or other stresses, since a small increase in p16-positive keratinocytes is also observed at the same time point in these melanoderms.

Our results also demonstrated accumulation of oxidative DNA damage in keratinocytes from melanoderms with senescent melanocytes. It is possible that increased oxidative stress mediates paracrine telomere dysfunction and senescence in keratinocytes, similarly to our data in dermal fibroblasts in culture. However, since the levels of 8-oxo-G are high in melanoderms containing both proliferating and senescent melanocytes, it is difficult to determine the extent of the contribution of ROS generation to paracrine DNA damage in these models. Nevertheless, studies have demonstrated that deletion of Sod2 (also known as MnSOD) specifically in keratinocytes induces mitochondrial oxidative stress and senescence, and results in delayed wound closure and epidermal thinning in mice (Velarde *et al.*, 2015). As previously discussed in chapter 3, such phenotypes were attributed to premature senescence of transit-amplifying cells of the epidermis, which are needed for epidermal expansion. Since TA cells arise from stem cells, this exerts a strain on the stem cell population, ultimately leading to their depletion (Velarde *et al.*, 2015). Although we cannot conclude that paracrine keratinocyte senescence is mediated by oxidative stress, published data suggest that enhanced ROS generation is a major trigger of keratinocyte senescence and an important contributor skin ageing phenotypes. Therefore, future experiments should include human epidermal equivalents generated with

keratinocytes overexpressing antioxidant enzymes. This would allow us to further explore the role of oxidative stress in bystander DNA damage induction and paracrine senescence triggered by senescent melanocytes.

We also demonstrate for the first time that senescent melanocytes contribute to epidermal thinning of human epidermal equivalents, likely through inducing paracrine senescence in keratinocytes. Interestingly, living skin equivalent models using neonatal fibroblasts and senescent keratinocytes show decreased epidermal thickness, which can be rescued by silencing p16 expression in keratinocytes (Adamus *et al.*, 2014). This suggests that accumulation of senescent keratinocytes contributes to skin ageing characteristics, such as epidermal thinning. However, in human skin *in vivo*, the majority of p16-positive cells in the epidermis have been shown to be melanocytes (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b). It is possible that senescent keratinocytes are not retained in the epidermis *in vivo,* and become shed off as a result of the natural cycle of epidermal differentiation, which in fact occurs at a slower rate in older individuals (Fore, 2006). Alternatively, senescent keratinocytes could undergo cell death, as previously discussed, and thus age-associated epidermal changes could be partly a consequence of keratinocyte loss with age. Given that proliferation of stem and progenitor cells is also crucial for epidermal regeneration and homeostasis, it would also be important to assess the bystander effects of senescent melanocytes on epidermal stem cells. However, since our models do not comprise stem cells, it can be postulated that paracrine keratinocyte senescence triggered by senescent melanocytes is sufficient to affect the regenerative capacity of the epidermis. The finding that the presence of senescent melanocytes is sufficient to contribute to epidermal thinning in melanoderms contradicts our results in human skin *in vivo*, where we did not find a correlation between TAF in melanocytes and age-related epidermal thinning. However, it is important to note that in melanoderms, all melanocytes present are senescent, whereas in human skin only about 8% of melanocytes are p16-positive in older donors. Therefore, one possibility is that the effects exerted by senescent melanocytes in melanoderms are much more acute when compared to an *in vivo* setting. Another possibility is that the older human donor cohort used in our study are offspring of long-lived individuals, which could confer an advantage to these subjects in terms of milder skin ageing phenotypes compared to the general population. However, previous studies conducted on the same cohort did not find a slower rate of skin ageing (measured as epidermal thinning and flattening) in offspring of long-lived individuals when compared to their partners, which represent the general population (Waaijer *et al.*, 2012a). Therefore, it is unlikely that our observations were confounded by familial longevity.

Moreover, the living epidermal equivalents also have other limitations, such that it lacks other skin layers and cell types, including immune cells, which may also play a role during the skin ageing process. Nonetheless, given that confounding factors from other components are not present in these models, they serve as useful tools to help dissect the role of senescent melanocytes in skin ageing. Although we hypothesise that the paracrine effects observed in melanoderms are due to the SASP of senescent melanocytes, we have not directly demonstrated that this is the case. It will be important to determine if melanoderms containing senescent melanocytes have increased levels of inflammatory factors by immunofluorescence, for example. Moreover, we cannot exclude the possibility that other mechanisms are involved in paracrine DDR signalling and keratinocyte senescence in melanoderms. Melanocytes are in direct contact with neighbouring keratinocytes through dendritic processes, which mediate melanin transport, and such interaction is also known as the epidermal melanin unit (Haass and Herlyn, 2005). Interestingly, senescent melanocytes have increased melanin content (Horikoshi *et al.*, 1991; Bandyopadhyay and Medrano, 2000), and studies have shown that an increased pheomelanin: eumelanin ratio increases oxidative stress in melanocytes and sensitises these cells to UV-induced DNA damage (Wenczl *et al.*, 1998; Smit *et al.*, 2001). Although it has not been previously established whether senescent melanocytes contain a higher pheomelanin to eumelanin ratio, an interesting scenario is that senescent melanocytes might transport increased levels of pheomelanin to surrounding keratinocytes. In turn, this could contribute to oxidative stress, and lead to paracrine DNA damage and senescence of neighbouring keratinocytes. In order to further explore this possibility, future experiments could involve the measurement of melanin pigments in melanoderms with proliferating or senescent melanocytes, providing valuable insight into whether senescent melanocytes cause preferential accumulation of pheomelanin in surrounding keratinocytes.

Moreover, our *in vitro* studies with dermal fibroblasts indicate that CXCR3 signalling plays a role in paracrine DDR signalling triggered by senescent melanocytes. Further experiments should investigate whether CXCR3 activation also mediates paracrine senescence in human epidermal equivalents, allowing us to determine its contribution to skin ageing phenotypes. As previously mentioned, studies have demonstrated that Akt is one of the downstream targets of CXCR3 in both human airway epithelial cells and vascular pericytes (Bonacchi *et al.*, 2001; Shahabuddin *et al.*, 2006). Interestingly, Akt phosphorylation has been recently shown to increase in epidermal cells of mouse skin with age, with a concomitant increase in NF-κB activation (Chen *et al.*, 2017), a transcription factor responsible for regulating expression of

many SASP factors (Chien *et al.*, 2011b). Pharmacological inhibition of Akt by perifosine treatment resulted in reduced NF-κB activity in the mouse epidermis, whereas Akt activation induced NF-κB signalling (Chen *et al.*, 2017). Although the phenotypical consequences of these interventions were not investigated by the authors, these results suggest that increased Akt activity acts through NF-κB to promote a pro-inflammatory environment that could contribute to skin ageing. Future studies should assess whether senescent melanocytes can promote activation of CXCR3 in keratinocytes in a paracrine manner, and to investigate the downstream signalling pathways that are activated as a result. Another interesting observation is that CXCR3 and its ligands (MIG, IP-10 and I-TAC) have been shown to be up-regulated in keratinocytes following UVB irradiation of a 3D epidermal equivalent (Kurata *et al.*, 2010). UVB-induced pigmentation, which is partly mediated by pro-inflammatory paracrine signalling between keratinocytes and melanocytes, could be mimicked by treating keratinocyte-melanocyte co-cultures with CXCR3 ligands, whereas melanin levels were reduced by downregulating CXCR3 expression in these co-cultures (Kurata *et al.*, 2010). These results suggest that CXCR3 signalling is involved in the UVB-induced inflammatory response that mediates melanin synthesis and pigmentation (Kurata *et al.*, 2010). Given that UV exposure is also associated with senescence of both keratinocytes (Lewis *et al.*, 2008) and melanocytes (shown by our studies), and that melanogenesis has been linked to melanocyte senescence (Bandyopadhyay and Medrano, 2000), it would be interesting to further investigate the role of CXCR3 signalling in paracrine keratinocyte senescence.

Previous data have shown that mTOR activity is increased during the initiation of senescence, resulting in increased mitochondrial biogenesis which may drive the SASP (Correia-Melo *et al.*, 2016). These results are consistent with other studies showing that mTOR activity, measured as phosphorylated S6, is increased during early time points after X-ray irradiation but it remains unchanged at later time points (Laberge *et al.*, 2015). Our results show decreased mTOR activity in senescent melanocytes despite our observation that mTORC1 inhibition by rapamycin diminishes the SASP. It is possible that low mTOR activity in senescent melanocytes is still sufficient to regulate the pathways shown to be responsible for the SASP, namely protein translation (Herranz *et al.*, 2015; Laberge *et al.*, 2015) and mitochondrial biogenesis (Correia-Melo *et al.*, 2016). It should be noted that our results need to be independently confirmed as mTORC1 activity was only assessed in one independent experiment. Secondly, a more extensive kinetics of mTORC1 activity should be performed in melanocytes in order to assess whether up-regulation occurs during earlier time points following senescence-inducing stimuli.

In addition, it is possible that other mechanisms, such as p38MAPK, also play a role in regulating the SASP in senescent melanocytes. In fact, inhibition of p38 activity has been shown to reduce the secretion of a number of SASP factors in human fibroblasts, and the kinetics of p38 activation was very similar to the kinetics of early and mature SASP development, suggesting that it plays an important role in SASP regulation (Freund *et al.*, 2011). However, further experiments would need to be conducted to determine the role of p38 in the development of the SASP in senescent melanocytes.

In summary, our results indicate that soluble factors secreted by senescent melanocytes can induce paracrine DNA damage foci in both skin fibroblasts and keratinocytes, which is mediated by increased oxidative stress in bystander cells. More specifically, we have shown that CXCR3 signalling plays a role in paracrine TAF induction in fibroblasts, although the role of CXCR3 in paracrine keratinocyte senescence remains to be elucidated. Furthermore, we have shown for the first time that accumulation of senescent melanocytes can contribute to skin aging phenotypes, such as epidermal thinning. This likely occurs through their ability to induce paracrine senescence in surrounding keratinocytes, thus compromising epidermal regeneration.

Chapter 6: Conclusions

Senescent cells have been shown to accumulate in a variety of mammalian tissues with age, and more recently, have been implicated causally in the development of age-related pathologies (Baker *et al.*, 2011; Baker *et al.*, 2016). Indeed, accumulation of senescent cells has been reported in human skin with age (Dimri *et al.*, 1995; Ressler *et al.*, 2006), with studies suggesting that senescent cells contribute to age-associated skin characteristics by compromising regeneration and structure (Adamus *et al.*, 2014; Velarde *et al.*, 2015). However, the majority of studies on senescence in the skin concentrate on dermal fibroblasts and keratinocytes, whereas the role of senescent melanocytes during skin ageing remains poorly understood. Moreover, telomere shortening has been documented in replicative melanocyte senescence *in vitro* (Bandyopadhyay et al., 2001), and dysfunctional telomeres were shown to accumulate in OIS melanocytic nevi *in vivo* (Suram et al., 2012); however, the contribution of telomere dysfunction to melanocyte senescence in response to other senescence-inducing stimuli remains understudied. Given that: *(i)* differentiated melanocytes rarely divide *in vivo* (Cichorek et al., 2013), making it less likely for them to undergo extensive proliferation-driven telomere shortening and senescence during ageing; and *(ii)* a fraction of melanoma cases arise from nevi (Goldstein and Tucker, 2013); perhaps these reasons provide a rationale for many studies giving more emphasis to oncogene-induced melanocyte senescence (Haferkamp *et al.*, 2009; Suram *et al.*, 2012; Vredeveld *et al.*, 2012). Therefore, in this study, I aimed to characterise melanocyte senescence both in human skin ageing *in vivo* and in different *in vitro* models, and determine the involvement of telomere dysfunction in this process. Given that senescent cells have also been shown to induce paracrine DNA damage and senescence in neighbouring cells (Nelson *et al.*, 2012; Acosta *et al.*, 2013), I also aimed to investigate whether soluble factors secreted by senescent melanocytes could act in a non-cell autonomous manner, and as a result, contribute to skin ageing phenotypes.

We have shown that senescent melanocytes accumulate in human skin with age, and that the frequency of damaged telomeres is higher in melanocytes in the skin of older donors. Furthermore, we demonstrated that telomere dysfunction occurs during replicative and stress-induced (X-ray and UV irradiation) melanocyte senescence *in vitro*, suggesting a role for telomere-associated foci during this process both *in vivo* and in culture. Interestingly, although telomere shortening was observed in replicative senescent melanocytes *in vitro*, we observed that telomere dysfunction also occurred irrespectively of length in replicative, X-ray- and UV-induced senescence *in vitro*. Moreover, we found that longer telomeres preferentially

accumulate γH2AX foci in melanocytes in human skin *in vivo*. Damage at telomeres that are not critically short has been previously reported in hippocampal neurons and liver hepatocytes of aged baboons (Fumagalli *et al.*, 2012), in mouse hepatocytes (Hewitt *et al.*, 2012), and in human melanocytic nevi (Suram *et al.*, 2012), suggesting that telomere attrition is not the sole cause of telomere dysfunction. In fact, it has been suggested that long telomeres may offer a more abundant target for DNA damage to occur (Fumagalli *et al.*, 2012). In addition, we did not observe significant differences in telomere length between melanocytes in young and aged skin, supporting the hypothesis that damage does not occur primarily as a result of shortening in melanocytes *in vivo.* The lack of significant telomere erosion with age could be a consequence of the low replication rate of fully differentiated melanocytes in the skin, and other factors might play a more dominant role in causing telomere damage. However, we cannot exclude the possibility that loss of telomeric repeats contribute to DDR activation at telomeres in senescent melanocytes *in vivo*. It is possible that critically short telomeres do not contain sufficient tandem repeats for the PNA probe to bind, or they might generate very weak signals that cannot be detected by the microscopy methods used in our study. Alternatively, high signal intensity values could represent aggregates of multiple dysfunctional telomeres, as previously shown to occur in fibroblasts (Kaul *et al.*, 2011), and these cannot be distinguished due to the limited resolution of imaging. Although the main stimulus responsible for triggering senescence in melanocytes *in vivo* is still unclear, our results show that telomere dysfunction may play a role in this process.

Moreover, the mechanisms contributing to telomere dysfunction in melanocytes *in vivo* remain to be elucidated. Although we have demonstrated that replicative and stress-induced melanocyte senescence are accompanied by increased oxidative stress in culture, reducing mitochondrial ROS by MitoQ only caused a mild decrease Sen-β-Gal activity. However, other markers of senescence will need to be evaluated in order to determine the role of ROS in the induction of melanocyte senescence. Nevertheless, mild oxidative stress has been shown to induce SSB accumulation preferentially at telomeres, which accelerates telomere shortening and triggers premature senescence (Petersen *et al.*, 1998; von Zglinicki, 2000; von Zglinicki, 2002). In fact, telomeres are thought to be more sensitive to oxidative damage compared to the bulk of the genome due to their high content of guanine triplets, which are highly susceptible to oxidative modifications (Oikawa and Kawanishi, 1999). Furthermore, oxidative telomeric damage has been shown to displace shelterin proteins such as TRF1 and TRF2, leading to uncapping, and consequently triggering activation of a DDR at telomeres (Opresko *et al.*, 2005).

However, DDR signalling at telomeres that still retain shelterin proteins has been demonstrated in melanocytic nevi *in vivo* (Suram *et al.*, 2012) and in replicative senescent fibroblasts *in vitro* (Kaul et al., 2011), suggesting that telomere uncapping does not always precede DDR activation. In order to further elucidate the mechanisms leading to telomere dysfunction in senescent melanocytes in our study, future experiments need to be performed to investigate whether shelterin components are lost from dysfunctional telomeres in melanocytes. Although from our data we cannot conclude that telomere damage is a direct consequence of enhanced ROS generation, it is likely that ROS contributes to the accumulation of dysfunctional telomeres during melanocyte senescence. In agreement with a role of oxidative stress in TAF generation, treatment of fibroblasts with MitoQ prevented paracrine TAF induced by senescent melanocyte CM, suggesting that increased mitochondrial ROS contributes to telomere dysfunction. Furthermore, inducing mitochondrial dysfunction and oxidative stress by Sod2 deletion, causes accumulation of DNA damage foci in the skin of mice (Velarde *et al.*, 2012), suggesting that ROS are also major contributors to DNA breaks in the skin *in vivo.*

The precise number of damaged telomeres necessary to induce senescence is not fully known; however, given the fact that a few TAF are also observed in young and proliferating melanocytes, it is likely that cells can tolerate a certain level of telomere damage, suggesting that DDR signalling at telomeres must surpass a threshold level in order to initiate senescence. It has been reported that accumulation of five dysfunctional telomeres precedes the initiation of replicative senescence in human fibroblasts and epithelial cells in culture (Kaul *et al.*, 2011). Others have shown that fibroblasts undergoing senescence due to genotoxic stresses have an average of 2 TAF (Hewitt *et al.*, 2012), and senescence of liver hepatocytes and intestinal enterocytes is associated with 1-2 TAF in a mouse model of chronic inflammation (*nfkb1*$^{-/-}$) (Jurk *et al.*, 2014). Our results suggest that an average of 1 dysfunctional telomere is associated with replicative, X-ray- and UV-induced melanocyte senescence *in vitro* and with melanocyte ageing *in vivo*, with 83%, 61%, 57% of and 87% of melanocytes, respectively, displaying at least one TAF. However, given the limitations of the methods used to analyse telomere damage, such that dysfunctional telomeres might aggregate or not be detected at all, it might be difficult to establish an absolute number of damaged telomeres required to induce senescence. Nevertheless, the presence of shelterin proteins hinders the repair of DNA damage at telomeres (Kruk *et al.*, 1995; Bombarde *et al.*, 2010), consistent with our results showing that telomere damage is not repaired in senescent melanocytes. This results in persistent DDR signalling at telomeres (Fumagalli *et al.*, 2012; Hewitt *et al.*, 2012), which is important for the initiation and

maintenance of the senescent state (d'Adda di Fagagna *et al.*, 2003; Takai *et al.*, 2003; Jacobs and de Lange, 2004). Therefore, TAF may also play a role in the initiation of melanocyte senescence.

Interestingly, the majority of p16-positive cells in the epidermis of human skin have been shown to be melanocytes (Waaijer *et al.*, 2012b; Waaijer *et al.*, 2016b), which were associated with increased facial wrinkling, higher perceived age, and age-associated elastin morphology (Waaijer *et al.*, 2016b). Despite these associations, the consequences of senescent melanocyte accumulation during skin ageing have not been previously explored. Here, we demonstrated that senescent melanocytes can induce paracrine DNA damage, both at telomeres and non-telomeric sites, and senescence in neighbouring cells. Bystander DNA damage induction was shown to be mediated by pro-inflammatory soluble factors secreted by senescent melanocytes, consistent with previous data showing paracrine damage induction by fibroblasts (Nelson *et al.*, 2012; Acosta *et al.*, 2013). Our results indicate that paracrine signalling enhances ROS generation in bystander cells, in turn causing DNA damage accumulation in these cells. Although the SASP of senescent melanocytes was only able to induce a small decrease in proliferation in dermal fibroblasts, our experiments using three-dimensional human epidermal equivalents revealed that senescent melanocytes trigger paracrine senescence in surrounding keratinocytes, and contribute to epidermal thinning, a characteristic associated with skin ageing. Despite our data in human skin *in vivo* showing that TAF in melanocytes only correlates with loss of epidermal curvature and not with epidermal thinning, it is possible that a larger cohort is required to establish stronger associations between melanocyte senescence and other features of skin ageing in human donors.

We have also shown that CXCR3 is up-regulated in senescent melanocytes *in vitro* and in human skin with age, and that CXCR3 signalling is partly involved in the induction of melanocyte senescence. Moreover, our results demonstrated that CXCR3 activation in bystander cells plays a role in paracrine TAF induction by senescent melanocytes. We postulate that IP-10 released by senescent melanocytes activates CXCR3 signalling in bystander cells, which in turn contributes to oxidative stress and DNA damage accumulation. However, further experiments are required to corroborate this hypothesis. Moreover, in order to further assess the role of CXCR3 signalling in the paracrine effects of senescent melanocytes, it will be important to determine whether CXCR3 is up-regulated in melanoderms.

Overall, we show that senescent melanocytes accumulate in human skin with age, and that telomere dysfunction occurs independently of length in this process. Furthermore, we demonstrate for the first time that senescent melanocytes can induce paracrine DNA damage and senescence in neighbouring cells, and contribute to epidermal thinning. We hypothesise that accumulation of senescent melanocytes with age compromises keratinocyte proliferation in a paracrine manner, in turn affecting epidermal turnover, and contributing epidermal changes associated with human skin ageing.

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