Interactions between mitochondria and inflammatory factors during cellular senescence

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“What I know is just a little part of my ignorance”

(“O que sei é uma pequena parte da minha ignorância”)

I would like to dedicate this Thesis to my parents António Cordeiro da Costa Melo e Maria Elizabeth Lourdes Correia Melo who, through unconditional love and support, since very early gave me the freedom to make my own decisions.

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Abstract

Cellular senescence, the irreversible loss of proliferating capacity of somatic cells, is an important tumour suppressor mechanism but also driver of ageing. These somehow contradictory functions are dependent on the development of the so-called senescent phenotype, which involves over-production of pro-inflammatory and pro-oxidant signals, however the exact mechanisms underlying its induction remain incompletely understood. In this thesis we aimed to understand how mitochondria and pro-inflammatory factors interact during senescence and how they contribute to the senescent phenotype. Firstly, we show that mitochondria are critical for the establishment and maintenance of cell senescence. Elimination of mitochondria rejuvenated senescent human fibroblasts, abrogating the pro-inflammatory phenotype, heterochromatin foci and expression of cyclin-dependent kinase inhibitors p21 and p16. Importantly, a considerable percentage of these cells were able to resume proliferation. Mechanistically, we show that mTORC1 integrates signals from the DNA damage response towards PGC-1β-dependent mitochondrial biogenesis, playing a causal role in the development of senescence. Secondly we show that inhibition of IL-8, a prominent pro-inflammatory cytokine of the SASP, partially abrogated the senescent phenotype by reducing mTOR-dependent mitochondrial mass and ROS production during senescence. Finally, we demonstrate that inhibition of mitochondrial content *in vivo* by either rapamycin or PGC-1β deletion prevents age-dependent increase in senescence in mouse liver. Our results suggest mitochondria as an important target for interventions aiming to reduce the load of senescent cells in ageing tissues.

Keywords: Cellular senescence, mitochondria, inflammatory factors and ageing

Awards and Publications

Awards

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Publications

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- **Clara Correia-Melo**, Graeme Hewitt and João F. Passos**. "**Telomeres, oxidative stress and inflammatory factors: Partners in Cellular senescence?" *Longev Healthspan J.* 2014 Jan 16.

- **Correia-Melo C**, Jurk D, Passos JF. "Robust multiparametric assessment of cellular senescence." *Methods Mol Biol.* 2013; 965:409-19.

- Hewitt G, Jurk D, Marques FD, **Correia-Melo C**, Hardy T, Gackowska A, Anderson R, Taschuk M, Mann J, Passos JF. "Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence." *Nat Commun.* 2012 Feb 28.

Abbreviations List

53BP1	p53 binding protein 1
ADP	Adenosine diphosphate
ASF1	Anti-silencing function 1
ATP	Adenosine triphosphate
ATM	Ataxia-telangiectasia mutated
AMPK	AMP-activated protein kinase
ATR	ATM and rad 3 related
ATRIP	ATR interacting protein
BrdU5-	bromodeoxyuridine
β-Gal	β Galactosidase
CBX7	Chromobox protein homolog 7
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CDC2	Cell division cycle protein 2 homolog
CDC25	Cell division cycle 25 protein
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
CDKN1A	Cyclin-dependent kinase inhibitor 1A
C/EBPβ	CCAAT/enhancer binding protein beta
CHK	Checkpoint kinase
CXCL8	Chemokine (C-X-C motif) Interleukin 8

CXCR	Chemokine (C-X-C motif) receptor 2
DAPI	4’,6-diamidino-2-phenylindol
DDR	DNA damage response
DHE	Dihydroergotamine
DHFR	*Dihydrofolate reductase*
DMSO	Dimethyl sulphoxide
DNA-PK	DNA-dependent protein kinase
DSB	Double strands break
ECM	Extracellular matrix
H2A	Histone 2A
H2AX	Histone 2AX
γH2AX	Phosphorylated H2AX
H_2O_2	Hydrogen peroxide
HIRA	Histone cell cycle regulation defective homologue A
HP-1	Heterochromatin protein-1
IFN-β	Interpheron beta
IKKα	Inhibitor of Nuclear Factor Kappa-B Kinase subunit alpha
IL-1α	Interleukin-1α
IL-6	Interluekin-6
IL-6R	Interluekin-6 receptor
IL-8	Interluekin-8
MAPK	Mitogen-activated protein kinase

MEF	Mouse embryonic fibroblast
MDC1	Mediator of DNA damage checkpoint 1
MMP	Mitochondrial membrane potential
MRN	MRE11–RAD50–NBS1 complex
mtDNA	Mitochondrial DNA
mRNA	Menssager Ribonucleic acid
NAC	N-acetyl cysteine
NADPH-	Nicotinamide adenine dinucleotide phosphate
NAO	10-n-nonyl-acridine orange
NF-κB	Nuclear factor of kappa light chain gene enhancer in B-cells
NHEJ	Non-homologous end joining
OCR	Oxygen consumption rate
OIS	Oncogene-induced Senescence
OXPHOS	Oxidative phosphorylation
p38MAPK	p38 mitogenic-activated protein kinase
PARP	Poly adenosine diphosphate ribose polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Population doubling
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator alpha

PGC-1β	Peroxisome proliferator-activated receptor γ coactivator beta
PIKK	Phosphoinositol 3-kinase-like kinase
PNA	Peptide nucleic acid
POT1	Protection of telomeres protein 1
pRb	Retinoblastoma protein
RAS	Retrovirus Associated DNA sequences
RFC	Replication factor C
RNA	Ribonucleic acid
RPA	Replication protein A
ROS	Reactive oxyden species
RT-PCR	Reverse transcriptase Polymerase chain reaction
SIPS	Stress-induced precise senescence
SSB	Single strands break
SSC	Sodium chloride, sodium citrate
SDF	Senescence-associated DNA foci
SDS	Sodium dodecyl sulphate
SAHF	Senescence-associated Heterochromatin Foci
SAIF	Senescence-associated Inflammatory phenotype
SASP	Senescence-associated Secretory Phenotype
Sen-β-Gal	Senescence-associated β Galactosidase
siRNA	Small interference Ribonucleic acid
TAF	Telomere-associated foci

TCA	Tricarboxylic acid
TGF-β	Transforming growth factor beta
TIF	Telomere-induced foci
TIN2	TERF1-interacting nuclear factor 2
TNFα	Tumour necrosis factor alpha
TPP1	Tripeptidyl peptidase 1
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
UV	Ultraviolet

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Chapter 1. Introduction

1.1 Cellular Senescence

In 1961, while performing experiments with cultures of human fibroblasts, Hayflick and Moorhead first described cellular senescence (Hayflick et al. 1961). Hayflick and Moorhead have observed that human fibroblasts irreversibly lose the ability to divide following a fixed number of cell divisions. They also found that these non-dividing cells remained viable for a long period of time (many weeks) but failed to proliferate, despite the presence of ample space, nutrients and growth factors in the culturing medium. This form of senescence triggered by an extended period of cell divisions became known as “replicative senescence”. Since Hayflick´s discovery of replicative senescence, various reports have demonstrated that cells can also enter senescence in response to a variety of external stresses (Lloyd 2002). This last form of senescence has been termed “stress-induced premature senescence” (SIPS). The fact that several different stressors can lead cells to entry a senescence state supports the idea that the senescent phenotype represents a general cellular response mechanism. Therefore, as a cellular mechanism/program, senescence is likely to play a role in the physiology of cells within living tissues (Ben-Porath et al. 2004). The observation that cells would not proliferate indefinitely in culture spawned two hypotheses: i) senescence as a cellular mechanism that permanently locks cells in the cell cycle preventing the spread of damage to the next cell generation and therefore potential malignant transformation – senescence as a tumour-suppressive mechanism (Sager 1991) - and ii) senescence as a causal factor for tissue impairment and decreased tissue repair and regeneration ability with age – senescence as potential cause for tissue dysfunction observed during ageing (Campisi et al. 2007). How this response is coordinated within the body is still not clear, however it could occur

in a pleiotropic manner, as proposed in the Antagonistic Pleiotropy Theory of Ageing (Williams 1957). The Antagonistic Pleiotropy Theory claims that a process which is beneficial to young organisms (possibly providing a reproductive advantage) can be harmful to old organisms. In this sense cellular senescence would, at first, be beneficial as tumour suppressor, but an accumulation of senescent cells with age would entail deleterious consequences to the same organism.

1.1.1 The Senescent Phenotype

Although senescent cells cannot respond to mitogenic stimuli, they have the ability to remain viable in culture for an extended period of time. In this state of perpetual cell cycle arrest or senescence, cells develop certain characteristics collectively known as the senescent phenotype. The long-term culture of cells results in a dramatic change in cellular morphology characterised by an increase of the cellular volume, loss of the original cellular shape and acquisition of a flattened cytoplasm (Bayreuther et al. 1988) (Figure 1.1). However, the senescent phenotype comprises not only the cellular morphologic changes and growth arrest, but also results in changes in the nuclear structure, gene expression, protein processing and metabolism and resistance to apoptosis (Campisi 2000, Sitte et al. 2000, von Zglinicki et al. 2000, Narita et al. 2003).

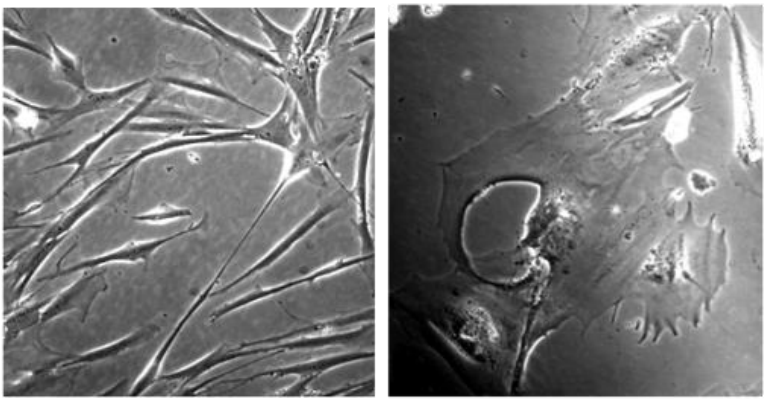


Figure 1.1 | Young and old human diploid cells (MRC5 fibroblasts). (left) Young cells at a population doubling 20; (right) Old cell at population doubling 55 – Replicative senescent cell.

Typically, senescent cells present a G1 phase DNA content (Dulić et al. 1993, Stein et al. 1995), resulting from the fact that cells usually arrest growth in this phase of the cell cycle. Although, in some cases the cell cycle phase in which cells become permanently arrested may differ depending on species and genetic background of the cell (Di Leonardo et al. 1994, Ogryzko et al. 1996, Herbig et al. 2004). The proliferating arrest observed in senescent cells occurs due to expression of dominant cell cycle inhibitors (discussed below).

Similar to senescence, apoptosis is a cellular mechanism in response to cellular damage resulting from intrinsic and extrinsic stresses. However, whereas senescence prevents propagation of damaged or stressed cells, apoptosis responds by permanently eliminating them (Ellis et al. 1991). Many cells types show resistance to apoptosis when undergoing cellular senescence; this feature of senescence has been argued as a putative reason for the fact that the number of these cells increases with age (Hampel et al. 2004). Reports have described that the difference between apoptosis and senescence might be due to different responses to the p53 pathway (Seluanov et al. 2001, Campisi et al. 2007). However, the mechanisms behind the “decision” between apoptosis or senescence are not yet fully elucidated. Reports have demonstrated that it may

depend on the nature and intensity of damage or stress (Seluanov et al. 2001, Rebbaa et al. 2003). It is thought that rapid DNA repair quickly terminates p53–p21 signalling, whereas slow, incomplete or defective repair results in sustained signalling and senescence (Campisi et al. 2007). *In vivo*, it has recently been suggested that induction of senescence or apoptosis depends on the cellular level of telomere dysfunction and differentially on p53 gene function during senescence. Lechel *et al.* showed that telomere dysfunction induced in mouse livers led to p53 independent apoptosis but a p53 dependent induction of cellular senescence, depending on the level of telomere dysfunction (Lechel et al. 2005).

Furthermore, senescent cells undergo gene expression changes of a variety of cellular pathways. Indeed some other traits of the senescent phenotype are a result of those changes (e.g., cell cycle arrest and apoptosis resistance). Cell-cycle regulating genes (inhibitors and activators) are described to be altered in senescence (Shelton et al. 1999, Chang et al. 2002, Zhang et al. 2003, Mason et al. 2004, Jackson et al. 2006, Trougakos et al. 2006). Cyclin-dependent kinase inhibitors (CDKIs) are often described to show increased expression during senescence. The CDKIs proteins p21 and p16 are expressed in senescence under the control of the regulatory pathways p53 and retinoblastoma (pRb) proteins respectively (Campisi 2001, Braig et al. 2006). Additionally, senescent cells are also described to repress proteins involved in cell-cycle progression (e.g. replication-dependent histones, c-FOS, cyclin A, cyclin B and PCNA) (Seshadri et al. 1990, Stein et al. 1991, Pang et al. 1994). Alterations in gene expression are also accountable for the increased secretion of certain factors, namely inflammation mediating factors, growth factors and extracellular matrix components (see Role of inflammatory factors in cellular senescence), collectively known as the senescence-associated secretory phenotype (SASP), that may contribute to age-related impairment of tissue structure and function (Campisi 2003).

1.1.2 Senescence Markers

For many years researchers have tried to find markers specific for senescent cells. So far several markers have been proposed to identify senescent cells but none is exclusive to the senescence state. Indeed, many commonly used cellular markers of senescence are not robust and might over-estimate senescent cells present at low frequencies when used individually (Lawless et al. 2010, Correia-Melo et al. 2013).

Senescent cells are unable to express genes required for cellular proliferation and/or DNA replication, even in the presence of pro-mitogenic factors (Dimri et al. 1994, Dimri et al. 1996). These features distinguish senescence from quiescence, another cellular non-proliferative state that is readily rescued in response to mitogenic stimuli. DNA replication is often detected by incorporation of 5-bromodeoxyuridine (BrdU) or ^{3}H-thymidine (Sidman et al. 1959), or by immunostaining for PCNA or Ki67 proteins. However, these markers do not allow distinction between senescent, quiescent or post-mitotic cells (Scholzen et al. 2000, Pan et al. 2011).

Dimri and collegues (Dimri et al. 1994) described a marker that could allow a more specific identification of senescent cells. They showed that several human cells express β-galactosidase (β-Gal) histochemically detectable at pH 6.0 upon induction of senescence in culture (Sen-β-Gal). They reported that Sen-β-Gal is expressed by senescent, but not pre-senescent fibroblasts and keratinocytes. The specificity of β-Gal for senescence was also observed in human tissue samples of skin from donors of different age, providing *in situ* evidence that senescent cells may exist and accumulate with age *in vivo*. β-Gal is a lysossomal hydrolase enzyme with optimum activity at an acidic pH of 4.0-4.5 reflecting the natural milieu of the lysosome (Kuo et al. 1978, Zhang et al. 1994). The specificity of β-Gal for senescent cells at pH 6.0 has been proposed to be a result of augmented lysossomal activity during senescence (Cristofalo et al. 1975). In this context, Sen-β-Gal most likely reflects the changes in cell function that invariably accompanies senescence. The senescence specificity of this marker was then put into question by later reports describing that β-Gal could

also be induced by stresses such as prolonged confluence in culture (Lee et al. 2006).

Senescence is also accompanied by nuclear changes including an increase in nuclear size (Narita 2007), and an increase in the density of nuclear pore complexes (Maeshima et al. 2006), which function is to allow transport of macromolecules between the nucleus and cytoplasm. Further to that, senescent fibroblasts accumulate a distinct chromatin structure enriched with heterochromatin proteins, also designated as Senescence-associated Heterochromatin Foci (SAHF) (Narita et al. 2003). These heterochromatin structures have been correlated with the irreversibility of the cell cycle arrest in senescence (Beausejour et al. 2003, Narita et al. 2003). SAHFs can be detected by DNA dyes such as the 4',6-diamidino-2-phenylindol (DAPI) and also by the presence of certain heterochromatin-associated histone modifications such as the H3Lys9 methylation. Heterochromatin protein-1 (HP1) is another protein associated with SAHFs (Narita et al. 2003). Alterations in nuclear envelop proteins, such as lamin A and B which connect the nuclear envelop to the chromatin, have been implicated in senescence. While, decreased expression of Lamin B1 in senescent cells has been correlated with SAHF formation (Sadaie et al. 2013, Shah et al. 2013), lamin A repression activates a DDR and induces 53BP1 and γ-H2AX foci (Liu et al. 2005).

Several reports have described senescence-associated DNA foci (SDFs) as a marker of senescence (d'Adda di Fagagna et al. 2003, Takai et al. 2003, Herbig et al. 2004, von Zglinicki et al. 2005, Di Micco et al. 2006), which often display molecular markers for DNA double-strand breaks. These markers include nuclear foci of phosphorylated histone H2AX (γH2AX) and their co-localization with DNA repair and DNA damage checkpoint factors such as 53BP1 (d'Adda di Fagagna et al. 2003). Telomere-induced foci (TIF), as a result of shortened telomeres, were also shown to be a marker of senescent cells (d'Adda di Fagagna et al. 2003). Recently, it was demonstrated that damage at telomeres can occur independently of telomerase activity and telomere length, a

phenomena know as Telomere-associated foci (TAF) (Fumagalli et al. 2012, Hewitt et al. 2012). Following these lines, telomere-initiated senescence reflects a DNA damage checkpoint response that is activated with a direct contribution from dysfunctional telomeres and both telomere-induced or associated foci (TIF or TAF) have been indicated as markers of senescence.

As mentioned previously, none of these markers per se identify specifically senescence cells. However, it was recently proposed, by Lawless and co-workers (Lawless et al. 2010), that Ki67 negativity (proliferation marker) and γH2A.X positivity (DNA damaged marker) together is a good criteria for quantifying the senescent state of cells both in cultured fibroblasts and in tissue sections.

1.1.3. Causes of Senescence

Telomeres have played a central role in ageing research since the early 1970's, when Alexei Olovnikov predicted that a progressive shortening of telomeres was responsible for the "Hayflick Limit" (Olovnikov 1971). Telomere shortening was therefore thought to be caused by what is known as the "end replication problem", a phenomenon caused by the inability of the DNA replication machinery, specifically DNA polymerase, to synthesise in a 3'-5' direction leading to the incomplete replication of the lagging strand. Telomeres are repetitive DNA sequences with associated proteins located at the end regions of linear chromosomes. In vertebrae, telomeres are tandem repeats of the sequence TTAGGG, which main function is to protect chromosomes erosion during each cell division (Muller 1938, Blackburn 1991, d'Adda di Fagagna et al. 2004). The protection function of these end parts of chromosomes are reinforced by a group of telomere-associated proteins collectively termed as shelterin (de Lange 2005). The shelterin complex is constituted by six proteins: TRF1, TRF2 and POT1, which recognise the telomeric repeat sequence, and TIN2, TPP1 and Rap1 (de Lange 2005), which ultimately arrange telomere ends into a loop structure, known as the T-loop, to cover the exposed DNA ends

(Griffith et al. 1999). The “end-replication” problem has been referred to as one of the major reasons why normal cells do not proliferate indefinitely, since dysfunctional telomeres trigger a classical DNA damage response (DDR) (d'Adda di Fagagna et al. 2003, Takai et al. 2003, Herbig et al. 2004), which recognise unprotected telomeres similarly to double-strand breaks (DSBs). Interestingly, shelterin components have been demonstrated to inhibit DNA repair mechanisms. It has been shown that loss of shelterin components such as TRF2 contributes to activation of a DDR at telomeres (van Steensel et al. 1998). Moreover, TRF2 and its binding partner RAP1 are required to prevent Non-Homologous End Joining (NHEJ)-dependent telomeric DNA fusions by inhibiting DNA-PK and ligase IV mediated end-joining (Bae et al. 2007). Consistent with these observations, Fumagalli and colleagues have shown in budding yeast that induction of DSBs adjacent to a telomeric sequence impairs the recruitment of ligase IV to the site of damage (Fumagalli et al. 2012), suggesting that damage at telomeres, occurring in the presence of sufficient shelterin components including TRF2, may elicit a persistent DDR due to inhibition of repair. Harley and colleagues provided the confirmation the telomeres do shorten with successive replication (Harley et al. 1990). Nevertheless, it was still unclear if telomere shortening played a functional role in the induction of senescence, as their shortening could merely be a consequence of cells reaching senescence. In the late 90’s, Bodnar and colleagues have provided the ultimate evidence that telomere erosion can induce senescence, by showing that ectopic expression of the catalytic subunit of telomerase, the enzyme able to elongate telomeres, leads to cell immortalisation (Bodnar et al. 1998). However, the shortening rate of telomeres is much greater than what was originally accounted by the “end-replication” problem alone, suggesting that other factors may contribute to telomere shortening (Levy et al. 1992). Furthermore, originally it was suggested that telomeres could serve as a counting mechanism within cells that would allow a finite number of replications, nevertheless a set time or threshold for telomere length to induce senescence has not been found (Von Zglinicki 2001). The large heterogeneity in telomere length between cells in the same culture (Lansdorp et

al. 1996) and the presence of senescent cells in cultures that have undergone a low number of divisions suggested that telomere length is not solely a result of a certain number of divisions a cell has undergone (Martin-Ruiz et al. 2004). As a matter of fact, Reactive oxygen species (ROS) have been described to contribute to accelerated telomere shortening, with telomeric DNA being especially sensitive to damage caused by ROS (Von Zglinicki 2001). It has been shown that telomeres accumulate more single-stranded breaks than the rest of the genome resulting from oxidative damage (Petersen et al. 1998), and this way may act as "sensors" to oxidative stress, preventing cells that have been exposed to high levels of potentially mutagenic factors from replicating (Martin-Ruiz et al. 2004). Consistent with a role of ROS in telomere dysfunction it has been suggested that guanine rich regions are more susceptible to oxidative modification (Grollman et al. 1993) and that oxidative modifications of TRF1 and TRF2 affect its binding to telomeres (Opresko et al. 2005). Furthermore, interventions affecting both mitochondrial function and ROS generation have been shown to impact on telomere-dependent senescence *in vitro*. Treatment with free radical scavengers (von Zglinicki et al. 2000), low ambient oxygen concentrations (Forsyth et al. 2003, Richter et al. 2007), overexpression of antioxidant enzymes (Serra et al. 2003), and mild chronic uncoupling (Passos et al. 2007) have been shown to decelerate telomere shortening and to extend the lifespan of cells in culture. All together these data has driven the current hypothesis that both the "end-replication" problem as well as the ROS driven stochastic damage contributes to telomere shortening (Figure 1.2).

Recently, work carried out in our group and Fabrizio d'Adda di Fagagna's group have shown that oxidative-stress induced telomere damage is irreparable and can occur irrespectively of telomere length (Fumagalli et al. 2012, Hewitt et al. 2012). In order to establish whether telomeric location was necessary for DNA damage foci persistence, using live-cell imaging our group has tracked DNA damage foci lifespan using a AcGFP-53BP1c fusion protein in combination with a fluorescently labelled PNA probe which specifically tags telomere repeats. Using this method it was found that majority of long-lived foci in stress-induced

senescence co-localise with telomeres (Hewitt et al. 2012), which suggests that they are major contributors to a persistent DDR. These persistent irreparable telomeric DNA damage foci, also known as Telomere-associated foci (TAF), occur independently of length or telomerase activity, indicating that not only telomere length but damage within telomeric regions is also an important contributor to cellular senescence (Figure 1.2).

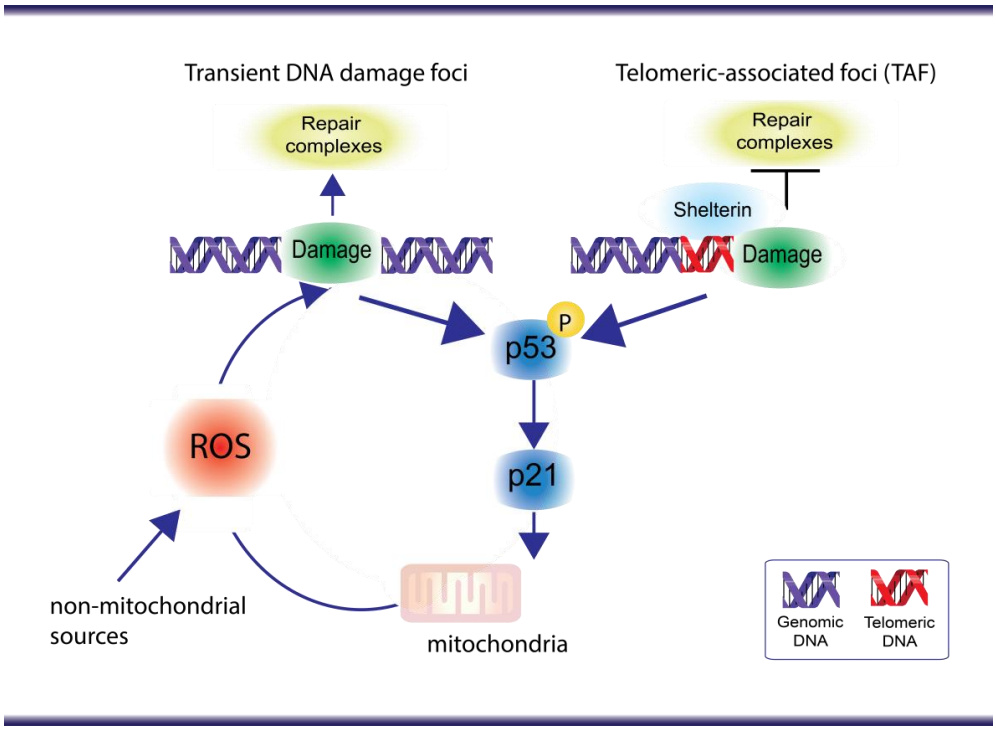


Figure 1.2 | Both telomeric and non-telomeric DNA damage contribute to the stabilisation of cellular senescence. DNA damage at telomeres is distinct from that throughout the genome; it is irreparable due to the repression of DNA repair pathways by telomere bound proteins, known as the "shelterin" complex. This contributes to a permanent DNA damage response (DDR). However, continuous generation of short-lived DDR foci by elevated reactive oxygen species (ROS) may equally contribute to the maintenance of the phenotype, as long as a dynamic equilibrium between damage induction and repair can be maintained (adapted from Correia-Melo *et al.* 2014).

Oncogene activation has been causally implicated in cellular senescence; this form of senescence is termed Oncogene-Induced Senescence (OIS). The term oncogene, as the name may suggest, is often associated with oncogenesis or malignant transformation of cells, indeed they are mutant versions of normal genes that, together with additional mutations, have the potential to transform cells. *Ras* oncogenes were first reported by Harvey and colleagues when using a preparation of a murine leukaemia virus, taken from a leukaemic rat, induced sarcomas in new-born rodents (Harvey 1964, Malumbres et al. 2003). However, expression of oncogenes can lead normal cells to undergo senescence, mainly due to an exhaustive period of cell proliferation followed by a permanent arrest. It had been already known that oncogenic *Ras* could transform cells to a tumorigenic state. However, the first evidence of OIS was presented by Serrano and colleagues (Serrano et al. 1997), when expressing an activated mutant form of Ras (H-RasV12), in primary human or rodent cells, they observed that cells permanently arrested in G1 phase. These cells also presented an accumulation of p53 and p16 proteins and were phenotypically indistinguishable from senescent cells. These observations suggested that the onset of cellular senescence does not simply reflect the accumulation of cell divisions, but can be prematurely activated in response to an oncogenic stimulus (Serrano et al. 1997). Since OIS was first reported, other members of the RAS signalling pathway (e.g. RAF, MEK, MOS and BRAF), as well as pro-proliferative nuclear proteins (e.g. E2F - 1), have been shown to cause senescence when overexpressed or expressed as oncogenic versions (Lin et al. 1998, Zhu et al. 1998, Dimri et al. 2000, Michaloglou et al. 2005). Accordingly, OIS is then a cell response to activated oncogenes in order to counteract excessive mitogenic stimulation, which puts cells at risk of oncogenic transformation.

Two major mechanisms have been implicated in the initiation and stabilisation of cellular senescence: i) the activation of a DNA damage response upon a genotoxic stress or telomere shortening (discussed above) and ii) perturbations on chromatin organisation (Di Bernardo et al. 2012). Chromatin organisation determines the extent to which genes are active (euchromatin) or silent

(heterochromatin). Alterations of chromatin structure resulting in global induction of heterochromatin are believed to contribute to the irreversible nature of the senescent state (Howard 1996). These characteristic heterochromatin structures, also known as senescence-associated heterochromatic foci (SAHF), are facultative heterochromatin whose formation is dependent on the pRb tumour suppressor pathway and may repress the expression of proliferation promoting genes, namely E2F target genes, such as cyclin A, DHFR and Mcm3 (Narita et al. 2003). Histones variations have also been implicated in the SAHF transcription silencing clout. SAHF are nuclear structures comprising the histone H2A variant, macro-H2A (Zhang et al. 2005). There is evidence that support a direct role for macro-H2A in gene silencing; macro-H2A has been described to be resistant to ATP-dependent remodeling proteins and binding of transcription factors (Angelov et al. 2003). Both the cell cycle regulator p16 (Narita et al. 2003, Narita et al. 2006) and the chromatin structure regulators HIRA (histone cell cycle regulation defective homologue A) and ASF1 (anti-silencing function 1) have been described to drive formation of SAHF (Zhang et al. 2005). Despite the fact that SAHF have been mainly associated with gene silencing, chemical inhibition of histone deacetylase, which promotes euchromatin formation, has been shown to induce senescence (Ogryzko et al. 1996, Munro et al. 2004). These findings that both euchromatin and heterochromatin can trigger senescence may appear contradictory, but one possible explanation is that both manipulations can cause extensive but incomplete changes in chromatin organisation and therefore each may alter the expression of different critical genes (Campisi et al. 2007).

1.1.4. Role of a DNA damage response (DDR) in cellular senescence

Cellular senescence, as a result of a persistent DDR, can be triggered by DNA damage such DNA single strands breaks (SSBs) and/or DNA double strands breaks (DSBs). There are common and specific DDR factors involved in each situation (Helt et al. 2005).

DSBs are first sensed by the Poly (ADP-ribose) polymerase (PARP) which then recruits the MRE11–RAD50–NBS1 (MRN) complex. The PARP-MRN complex functions as a lesion-specific sensor, they are one of the first factors to be localised to the DNA lesion (D'Amours et al. 2002). When bound to DNA, the MRN complex enables assembly of large macromolecular complexes (known as foci) that facilitate efficient DSB repair responses (van den Bosch et al. 2003). NBS1 then recruits the protein kinase ataxia-telangiectasia mutated (ATM) (Uziel et al. 2003, Lee et al. 2005). ATM is a phosphoinositol 3-kinase-like kinase (PIKK) that plays a central role in orchestrating a network of cellular responses to DSBs, including cell cycle control, DNA repair and apoptosis (Shiloh 2003). ATM undergoes autophosphorylation events, which leads to dissociation of the inactive ATM dimer into single protein molecules with kinase activity (Bakkenist et al. 2003, Kozlov et al. 2011). Activated ATM phosphorylates the histone H2AX at the site of damage (DSBs) resulting in γH2AX (Rogakou et al. 1999, Burma et al. 2001), which is then recognised by a phospho-specific domain of the mediator of DNA damage checkpoint 1 (MDC1). Recruitment of MDC1 to γH2AX stimulates additional accumulation of the MRN complex (Stucki et al. 2006), this way amplifying local ATM activity and the spreading of γH2AX along the chromatin from the DSBs. This increase in the local concentration of several DDR factors at the site of DNA damage generates a positive feedback loop amplifying repair signals. The DNA-damage mediator 53BP1 (p53 binding protein 1) accumulates at the site of DNA damage as a result of the exposure of modified histone residues (Huyen et al. 2004, Botuyan et al. 2006) and establishes binding to MDC1 to further boost a downstream DDR (Eliezer et al. 2009). Additionally, 53BP1 is an important DDR factor involved in DNA repair by NHEJ (Nakamura et al. 2006), through its inhibitory effect on homologous recombination (Bunting et al. 2010).

Alternatively, generation of local regions of replication protein A (RPA) -coated single-stranded DNA, caused by replication stress or UV irradiation, recruits the heteromeric complex that contains the ATM and rad 3 related (ATR) protein and

its DNA-binding subunit ATRIP to the site of damage (Namiki et al. 2006), activating a less defined feedback loop through the activation of the RAD9-HUS1-RAD1 (9-1-1) and RAD17-RFC complexes, as well as TOPBP1 (d'Adda di Fagagna 2008). Activated by this DNA-protein structure, ATR then phosphorylates its downstream substrates, including the checkpoint kinase 1 (CHK1) and 2 (CHK2) (Helt et al. 2005). Subsequently, CHK1 and CHK2 phosphorylate and inactivate the cell division cycle 25A and C (CDC25A and CDC25C) proteins, members of the CDC25 phosphatase family and key regulators of the cell cycle (Bartek et al. 2003, Kastan et al. 2004). ATR also stimulates phosphorylation of H2AX and the formation of DNA damage foci (Iwabuchi et al. 2003). Notably, during the end stages of repair, DSBs also generates RPA coated ssDNA which activates ATR (Zou et al. 2003).

Ultimately, CDC25 phosphatases and p53 are the bottom elements of the DDR signalling cascade that connects the upstream DDR pathway with the core of the cell-cycle progression machinery. CDC25 phosphatases are important for normal cell proliferation, as they activate cyclin-dependent kinases (CDKs) and cause their DDR-mediated inactivation, by either proteolytic degradation or exclusion from the nucleus (Shen et al. 2012, Neelsen et al. 2013). On the other hand, p53 induces cell-cycle arrest by activating the transcription of p21, a CDK inhibitor that blocks cell-cycle progression (Rufini et al. 2013).

During senescence, the permanent cell cycle relies on two main molecular pathways: p53–p21 and $p16^{INK4A}$-Rb pathways.

Double-strand breaks trigger the DNA damage response (DDR) by a repertoire of different stressors as discussed above (see Causes of Senescence). This signalling cascade centred around the ATM kinase induces senescence primarily through the p53 pathway (Campisi 2005, Lossaint et al. 2011, Wang et al. 2011). p53 is a stress-activated transcription factor that controls the expression of hundreds of genes implicated in a variety of physiological

responses including responses to genome instability and DNA damage (Toledo et al. 2006). p53 tumour suppression function relies on its ability to cease cell proliferation and induce two fundamental cell responses: apoptosis (cell death) or cellular senescence (permanent cell cycle arrest) (Di Micco et al. 2006, Gao et al. 2011, Gatta et al. 2011, Osawa et al. 2011, Rufini et al. 2013). The cyclin-dependent kinase inhibitor 1 *CDKN1A/p21*, a well-established p53-target gene, has been proved to be up-regulated during senescence being an essential mediator of the p53-dependent cell cycle arrest (Brown et al. 1997, Herbig et al. 2003, d'Adda di Fagagna 2008, Passos et al. 2010). p21 is a dual inhibitor of cyclin-dependent kinases (Harper 1993, Xiong et al. 1993) and proliferating-cell nuclear antigen (PCNA) (Waga et al. 1994), which are required for passage through the cell cycle. Accordingly, depletion of p21 in mouse embryonic (Brugarolas et al. 1995) and human fibroblasts (Brown et al. 1997) compromise its ability to undergo p53-dependent G1 arrest following DNA damage and abrogates several characteristics of the senescent phenotype. Notwithstanding that p21 is crucial to the p53-growth arrest of senescent cells (Chang et al. 2000), it is unlikely to be exclusively responsible for the complex changes underpinning senescence. Indeed, p53 is a master transcription factor involved in the regulation of several physiological and metabolic pathways which have also been heavily implicated in regulation of cellular senescence and the ageing process (Vousden et al. 2009). In this context, E2F7 is another p53 target that has recently been described to be involved in cell-cycle arrest and senescence; it promotes repression of several E2F target genes, including *E2F1*, and many genes essential for mitosis, such as *CCNA1* (encoding cyclin A), *CCNB1* (encoding cyclin B) and *CDC2/CDK1* (Aksoy et al. 2012, Carvajal et al. 2012, Rufini et al. 2013).

Stimuli that produce a DDR can also engage the p16–pRb pathway. pRb can be activated by $p16^{INK4a}$, hereafter p16, independently of p53 (Alcorta et al. 1996). In certain cell types (e.g. fibroblasts) activation of the p16–pRB pathway often occurs secondarily to engagement of the p53 pathway (Stein et al. 1999,

Jacobs et al. 2004), while in others (e.g. epithelial cells) the inducing stimuli acts primarily through the p16–pRB pathway (Campisi et al. 2007). Additionally, in mouse cells telomere dysfunction seems to primarily activate the p53 pathway, whereas in human cells both the p53 and p16–pRB pathways are activated (Smogorzewska et al. 2002). p16 is a cyclin dependent kinase inhibitor that localises at the perinuclear cytoplasm, which following activation translocates to the nucleus (Serrano et al. 1993). As a cyclin dependent kinase inhibitor, p16 is able to prevent phosphorylation of pRb by cyclin/cdk complexes. p16-pRb axis is pivotal to the establishment of cell-cycle arrest: hypophosphorylated pRb halts cell proliferation by inhibitory binding to E2Fs transcription factors, thus preventing them from stimulating transcription of genes involved in cellular proliferation and DNA replication (Campisi et al. 2007). While p21 has its maximal expression at the initiation of senescence and declines after the cell become senescent, p16 expression increases gradually, with barely detectable levels at the early stages of senescence, and then persists for months after induction of senescence (Alcorta et al. 1996, Stein et al. 1999). p16 expression has been shown to increase as a result of oncogene activation (Serrano et al. 1997). During OIS, suppression of pRb abolishes the establishment of senescent phenotype, but it is not sufficient to overcome cell-cycle arrest; this depends on the concomitant p53-dependent cell-cycle arrest (Aksoy et al. 2012). Additionally, p16 has also been shown to be upregulated following oxidative damage (Chen et al. 2004), radiation (Meng et al. 2003) and telomere dysfunction (Jacobs et al. 2004). The mechanisms by which senescence-causing stimuli induce p16 expression are thought to occur due to reduced expression of Polycomb INK4a repressors such as BMI1 and CBX7 (Itahana et al. 2003, Gil et al. 2004, Bracken et al. 2007). Supporting this idea, it is reported that BMI1 or CBX7 overexpression extends the replicative lifespan of human and mouse fibroblasts (Jacobs et al. 1999, Itahana et al. 2003, Gil et al. 2004). The p16–pRB pathway is also involved in SAHFs generation (Narita et al. 2003), this may be due to pRB ability to complex with histone modifying enzymes that form repressive chromatin (Macaluso et al. 2006).

Another possibility is that DNA damage signals activate p38MAPK signalling which then activates p16 (Iwasa et al. 2003, Bulavin et al. 2004, Ito et al. 2006).

The p38 mitogenic-activated protein kinase (p38MAPK) has also been described to be important for the senescence growth arrest, it can activate both the p53-p21 and p16-pRb growth arrest pathways (Iwasa et al. 2003). p38MAPK inhibition moderately delays replicative senescence and the rapid entry into senescence of cells from patients with Werner's syndrome, a premature aging disorder caused by a defective DNA repair protein (Davis et al. 2009). Additionally, p38MAPK activity is required for the senescence arrest caused by oncogenic RAS, and constitutive p38MAPK activity can induce a growth arrest in normal human cells (Wang et al. 2002, Deng et al. 2004). Recently, p38MAPK has also been described as a key factor for secretion of inflammatory factors by senescent cells (Freund et al. 2011). Passos *et al.* (2010) have shown that p38MAPK enhances mitochondrial ROS generation, thereby contributing to the stabilisation of a DDR during senescence (Passos et al. 2010).

1.2 Role of Mitochondria and ROS in cellular senescence

The first evidence of mitochondria as sub-cellular organelles occurred in the 19th century, when Richard Altmann and Carl Benda argued for the existence of sub-cellular structures that sometimes appeared threadlike and at other times more granular. These features gave rise to the name "mitochondria" from the Greek words *mitos* (thread) and *chondrion* (granule) (Bechtel et al. 2007). From a structural perspective, the mitochondrion (the basic unit of mitochondria) contains two membranes that separate four distinct compartments: the outer membrane, the inner membrane, the inter-membrane space and the matrix. The inner membrane is highly folded into cristae and harbors the electron transport

chain and ATP synthase responsible for the cellular energy supply (McBride et al. 2006). Mitochondria are central organelles to cellular metabolism and have been shown to become altered during cellular senescence.

In 1956, Denham Harman proposed the Free Radical Theory of Ageing (Harman 1956), in which reactive oxygen species (ROS) would form inside the cells, as a by-product of normal redox reactions, which could in turn cause damage to surrounding structures and or molecules, such as DNA, lipids and proteins. Many reports have since supported Harman's theory, showing correlative evidence that oxidative damage increases during ageing (Oliver et al. 1987, Fraga et al. 1990, Hamilton et al. 2001). In the 1970's, Harman introduced a modification to the Free Radical Theory of Ageing, suggesting that mitochondria would play a central role on ageing (Harman 1972), since these organelles are the main producers of ROS in cells (Chance et al. 1979), in what was then termed as the Mitochondrial Theory of Ageing (MTA). In the past years, it has been shown that mitochondria are indeed the major source of ROS within the cell (confirming the modified proposal of Harman), but also that mitochondrial DNA (mtDNA) deletions and mutations can be a result of oxidative stress, and that mitochondrial oxidative damage can accumulate with age in organisms ranging from worms to humans (Golden et al. 2001, Yui et al. 2003). It has been shown that mtDNA is more susceptible to ROS mediated damage than nuclear DNA; these subcellular structures, contrary to their nuclear counterpart, lack histones and instead form protein-DNA complexes (nucleoids) in the mitochondrial matrix, which can protect them against oxidative damage, but not in the same extent as histones (Gilkerson 2009). However, lately the MTA has been questioned, with several reports suggesting an inverse relationship between mitochondrial biogenesis and ageing (discussed below) and genetically manipulated animal models where mitochondrial function and oxidative stress were targeted showing conflicting results (Muller et al. 2007). For instance, studies in mice have shown that heterozygous deletion of the mitochondrial superoxide dismutase, an enzyme able to convert superoxide anion into hydrogen peroxide and water, showed that increased oxidative stress does not accelerate ageing in mice (Van Remmen et al. 2003). Furthermore,

interventions promoting longevity, namely CR and sirtuin activators, have been linked with increased mitochondrial biogenesis (Baur et al. 2006, Lopez-Lluch et al. 2006). A recent study has also shown that elevated mitochondrial ROS levels promote extension of lifespan in *Caenorhabditis elegans* (*C. elegans*) by protecting from the consequences of mitochondrial dysfunction via triggering of a unique pattern of gene expression that modulates stress sensitivity and promotes survival (Yee et al. 2014).

Notwithstanding that the role of mitochondria and ROS during ageing is conflicting, in senescence mitochondrial dysfunction seems to be a general feature and has been reported to occur independent of the nature of the senescence stimuli (e.g. genotoxic stress, telomere dysfunction and oncogene activation) (Allen et al. 1999, Hutter et al. 2002, Zwerschke et al. 2003, Hutter et al. 2004, Passos et al. 2007, Moiseeva et al. 2009). Mitochondrial dysfunction during senescence has been characterised by an increase in mitochondrial mass and decreased mitochondrial membrane potential, leading to metabolic inefficiency and increased generation of ROS (Saretzki et al. 2003, Ramsey et al. 2006, Passos et al. 2007, Lu et al. 2008). The mechanisms by which mitochondrial homeostasis is maintained are complex and involve regulation of mitochondrial number and function in response to a variety of environmental cues. The Peroxisome Proliferator-Activated receptor (PPAR)-γ co-activator (PGC)-1 family of transcription co-activators are master regulators of mitochondrial homeostasis (Lin et al. 2005). These transcription co-activators regulate mitochondria numbers and function by interacting with a broad range of transcription factors that are involved in a wide variety of biological responses including adaptive thermogenesis, mitochondrial biogenesis and glucose/fatty acid metabolism (Wu et al. 1999). The PGC-1 family comprises three homologs: PGC-1α (Puigserver et al. 1998), PGC-1β (Kressler et al. 2002) and PGC-related co-activator (PRC) (Andersson et al. 2001). It has been described that PGC-1 co-activators factors stimulate mitochondrial biogenesis via induction of the uncoupling protein 2 (UCP-2) and regulation of the nuclear respiratory factors (NRFs). PGC-1 co-factors also regulate mitochondrial DNA replication/transcription through binding and co-activation of the transcriptional

factor NRF-1 on the promoter of the mitochondrial transcription factor A (mtTFA) (Lin et al. 2005). The role of PGC-1 transcriptional co-activators on the ageing process seems to be controversial. Several reports have suggested an inverse relationship between mitochondrial biogenesis and ageing. Mitochondrial density in skeletal muscle gradually declines during age (Crane et al. 2010), suggesting a decrease in mitochondrial biogenesis possibly via an age-dependent reduction in levels of PGC-1α (Wenz et al. 2009). Studies on mitochondrial-myopathy mouse models have suggested that a compensatory mitochondrial mass increase is beneficial by partly compensating for the reduced function of the respiratory chain and maintaining overall ATP production in skeletal muscle (Wredenberg et al. 2002). However, the same group has later reported that beneficial effects of mitochondrial mass increase seems to be tissue specific, since it contributes to aggravate heart failure progression in the same mouse model (Hansson et al. 2004). Furthermore, genetically induced mitochondrial biogenesis has been associated with age-related diseases such as cardiomyopathy (Lehman et al. 2000), renal fibrosis (Hickey et al. 2011) and diabetes (Sawada et al. 2014), all of which have been associated with cellular senescence (Sussman et al. 2004, Testa et al. 2007, Yang et al. 2010). Increased mitochondrial content has also been associated with osteoarthritis, particularly Kashin-Beck Disease (KBD), where articular chondrocytes present increased mitochondrial mass (Liu et al. 2010). While there is some evidence for a role mitochondrial homeostasis deregulation during senescence, the mechanisms by which mitochondria become impaired is still not clear.

ROS have been implicated in both the induction and stabilisation of cellular senescence, with several studies showing that ROS can accelerate telomere shortening (von Zglinicki 2002) and induce DNA damage and thus activate a DDR and senescence (Chen et al. 1995, Lu et al. 2008, Rai et al. 2009) (Figure 1.3A). Evidence indicates that activation of major downstream effectors of the DDR in senescence result in elevated ROS. Activation of a DDR by genotoxic stress or telomere uncapping (Passos et al. 2010), over-expression of activated RAS (Lee et al. 1999), $BRAF^{V600E}$ (Kaplon et al. 2013), p53 (Macip et al. 2003),

p21 (Macip et al. 2002) and p16 (Takahashi et al. 2006) all resulted in elevated ROS generation. In most of the above reported cases treatment with antioxidants, such as N-acetyl cysteine (NAC), was able to prevent the cell-cycle arrest supporting a causal role for ROS in the process (Figure 1.3B and 1.3C). Together these observations indicate that elevated ROS are a consequence of the activation of the senescence programme. Indeed ROS has been proposed to act as signalling molecules during cellular senescence (Passos et al. 2006). Mechanistically, it is still unclear how these pathways contribute to mitochondrial dysfunction and ROS generation. Takahashi and colleagues, using human fibroblasts expressing a temperature-sensitive simian virus 40 large T antigen, connected p16 with ROS production via protein kinase C δ signalling (Takahashi et al. 2006). Protein kinase Cδ has been shown to activate a non-mitochondrial source of ROS, generated by NADPH-oxidase through phosphorylation of $p47^{phox}$, an essential component of NADPH oxidase (Talior et al. 2005). Consistent with this study, NADPH oxidases have been shown to limit the replicative lifespan of human endothelial cells in culture via ROS generation (Lener et al. 2009).

Oncogene-induced senescence has been associated with mitochondrial dysfunction and ROS production, which is dependent on intact p53 and Rb tumour suppression pathways. Mitochondrial dysfunction resulted in the loss of ATP and activation of AMPK; in addition, mitochondrial-derived ROS were shown to contribute to the oxidation of DNA (Moiseeva et al. 2009). In a recent study, it was shown that $BRAF^{V600E}$-induced senescence was accompanied by the activation of pyruvate dehydrogenase, which resulted in the enhanced use of pyruvate by the tricarboxylic acid (TCA) cycle followed by increased respiration and ROS generation (Kaplon et al. 2013).

The role of p53 and p21 in ROS generation during senescence is still not well understood. An association between p53 and transcriptional activation of genes involved in mitochondrial apoptosis has been demonstrated (Polyak et al. 1997), as well as a stress-induced translocation of p53 to mitochondria resulting in increased outer membrane permeabilisation (Moll et al. 2006), however, a direct role of mitochondrial p53 in cellular senescence has not yet been demonstrated.

In contrast, transcriptional regulation of mitochondrial genes by p53 has been reported to impact on mitochondrial function and contribute to ageing. p53 knock-out mice exhibited reduced expression of the *Sco2* gene, which is required for the assembly of the mitochondrial DNA-encoded COX II subunit (Matoba et al. 2006). In late generation telomerase knock-out mice that have critically short telomeres, activation of p53 has been shown to repress the promoters of *PGC-1α* and *PGC-1β* genes, master regulators of mitochondrial biogenesis and function, thereby contributing to decreased mitochondrial function (Sahin et al. 2011).

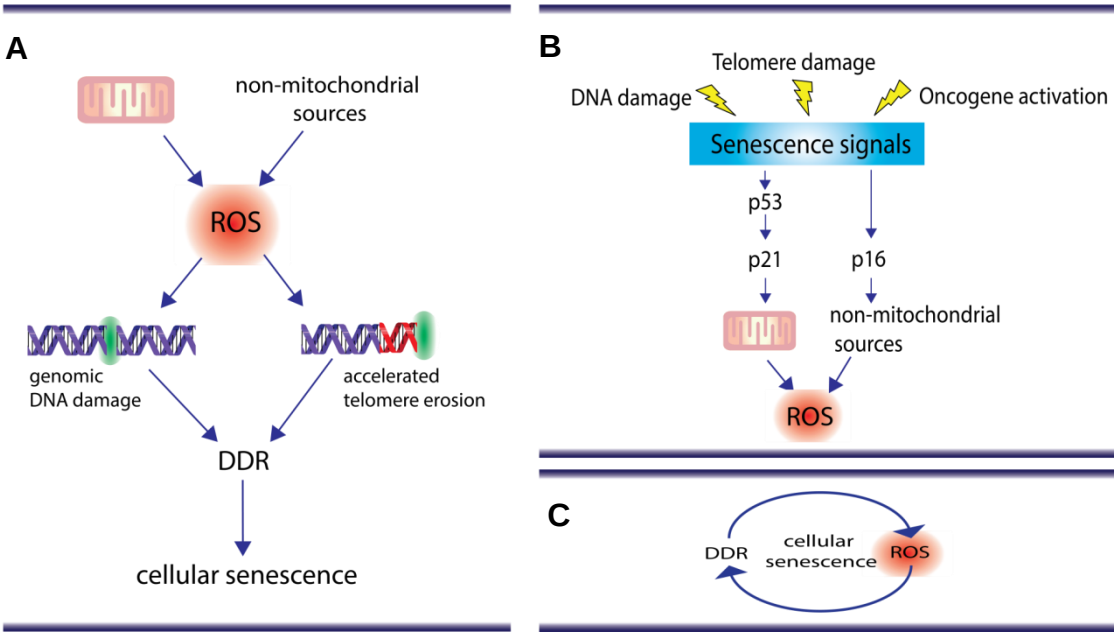


Figure 1.3 | Two different models by which reactive oxygen species can impact on cellular senescence. (A) Reactive oxygen species (ROS) produced via mitochondrial and non-mitochondrial sources can induce genomic DNA damage and accelerate telomere erosion/damage, both of which contribute to activation of a DNA damage response (DDR). **(B)** ROS can act as signalling molecules in senescence: activation of "senescence signals" has been shown to result in increased ROS generation (mitochondrial and non-mitochondrial). ROS has been shown to impact on a

variety of pathways which may help stabilise the senescence growth arrest. **(C)** Simplified feedback loop model involving ROS and DNA damage. Telomere uncapping or general DNA damage triggers a DDR which culminates through yet unidentified processes to ROS generation. ROS generation leads to additional DNA damage to the genome, stabilising the DDR and leading to a stable senescence arrest (adapted from Correia-Melo *et al.* 2014).

Knockdown of both p53 and p21 by RNA-mediated interference (RNAi) has been shown to reduce ROS generation in both telomere-dependent and -independent senescence (Passos et al. 2010). Our group has found that ROS levels increase in senescent cells as a result of signalling through p21, and feed back into DNA damage induction and the DDR, generating a stable, self-sustaining feedback loop (Figure 2c). This feedback loop persists even in irreversibly deep senescence. Moreover, p21 appears to be the critical mediator between the DDR and MAPK and TGF-β stress-induced signalling cascades, which have been shown to contribute to ROS generation (Torres et al. 2003, Koli et al. 2008, Passos et al. 2010). Consistently, *in vivo* a p21 knockout rescued at least some accelerated ageing phenotypes in telomerase (mTERC) knockout mice (Choudhury et al. 2007), as well as markers of oxidative stress and DNA damage foci (Passos et al. 2010). ROS has also been shown to impact on the DDR and ultimately senescence in a non-cell-autonomous fashion. A recent study has shown that senescent cells can induce a DDR in neighbouring cells via a gap junction-mediated cell-cell contact and processes involving ROS (Nelson et al. 2012).

ROS have also been implicated in organismal ageing, with countless reports of associations between oxidative damage and the ageing process (Oliver et al. 1987, Fraga et al. 1990, Hamilton et al. 2001); however, genetically manipulated animal models where mitochondrial function and oxidative stress were targeted have generated conflicting results (Muller et al. 2007).

1.3. Role of Inflammatory factors in cellular senescence

The senescence phenotype is not limited to an arrest of cell proliferation. Indeed, a senescence cell is metabolically active and encompass widespread changes in protein expression and secretion, ultimately developing a Senescence-associated Secretory Phenotype or SASP (Shelton et al. 1999, Krtolica et al. 2001, Krtolica et al. 2002, Campisi et al. 2007, Kuilman et al. 2009). Current data suggests that one of the primary functions of the SASP is to allow communications both within and between cells. Because of this ability to establish intra- and inter- cellular connections, senescence-associated secreted factors have also been termed as "senescence-messaging secretome" (Kuilman et al. 2009). Cellular senescence has long been recognised as a potent tumour-suppressive mechanism that arrests growth of cells in risk for malignant transformation (Braig et al. 2005, Collado et al. 2005, Michaloglou et al. 2005, Narita et al. 2005, Courtois-Cox et al. 2006, Ventura et al. 2007, Xue et al. 2007). However, some studies have shown that senescent cells may also induce changes in the tissue microenvironment which encourages tumorigenesis (Krtolica et al. 2001, Coppe et al. 2008, Green 2008, Kuilman et al. 2008). Consistent with a pleiotropic role of senescence, the SASP has been shown to have a series of somehow contradictory roles with important consequences for ageing and cancer: i) It can contribute to the surveillance and elimination of senescent cells by the immune system as several studies indicate (Xue et al. 2007, Kang et al. 2011); ii) it can be pro-tumorigenic. Both cell culture experiments and studies involving co-transplantation of senescent and cancer cells in recipient mice, have shown that senescent fibroblasts stimulated hyperproliferation of cancer cells, neoplastic progression and tissue damage (Krtolica et al. 2001, Liu et al. 2007); iii) it can contribute to the reinforcement of oncogene-or stress induced senescence in a cell-autonomous fashion (Acosta et al. 2008, Kuilman et al. 2009) and iv) it can induce senescence in neighbouring cells via a bystander effect both *in vitro* and *in vivo* (Acosta et al. 2013). In order to understand how the SASP harbours such diverse effects it is important to first comprehend what is the SASP. The SASP includes several

families of soluble and insoluble factors. While the soluble factors include signalling factors such as growth factors, chemokines and interleukins, and secreted proteases, the insoluble factors comprise insoluble secreted proteins and extracellular matrix (ECM) components (Coppé et al. 2010).

1.3.1Senescence-associated inflammatory phenotype (SAIF)

The senescence secretory phenotype involves the secretion of several inflammatory and cancer-related factors, including interleukins. The involvement of interleukins in senescence was first described by Maier *et al*., who reported a positive correlation between IL-1α transcript level and the control of cellular proliferation in human endothelial cells (Maier et al. 1990). Since then, many other interleukins have been shown to be secreted by senescent cells, being IL-6 and IL-8 some of the most prominent interleukins of the SASP (Acosta et al. 2008, Kuilman et al. 2008). The repertoire of inflammatory factors secreted by senescent cells will be here designated as the senescence-associated inflammatory phenotype (SAIF).

Interleukin 6 (IL-6) was originally identified as a B-cell differentiation factor (Hirano et al. 1986). At the same time that IL-6 is produce by various cell types it can also induce a response in different cell types, through its unique receptor (IL-6R). This interleukin has multiple functions including regulation of the immune response and haemotopoiesis and more recently has been implicated with senescence and cancer (Davalos et al. , Hong et al. 2007, Kuilman et al. 2008, Mihara et al. 2012). Using genetic and bioinformatic analysis, Kuilman *et al*. found that OIS is specifically linked to the activation of an inflammatory transcriptome (Kuilman et al. 2008). They found that senescence-associated cytokines, included the pleiotropic cytokine interleukin-6 (IL-6), can act in a pro-mitogenic paracrine way. In this study, IL-6 was implicated in senescence entry and maintenance, via a pathway co-regulated by the transcription factor C/EBPβ to amplify the activation of the inflammatory network, including IL-8. Interestingly, in human colon adenomas IL-8 specifically co-localised with

arrested, p16INK4A-positive epithelium, suggesting that the context-dependent cytostatic and pro-mitogenic functions of specific interleukins contribute to connect senescence with an inflammatory phenotype and cancer (Kuilman et al. 2008).

Interleukin-8 (IL-8), alternatively known as CXCL8, belongs to the CXC (two N-terminal cysteines are separated by one amino acid, represented with an "X") amino acid motif family of pro-inflammatory chemokines (Thelen 2001), that are highly secreted by senescent cells (Coppé et al. 2010). This chemokine activates multiple intracellular signalling pathways downstream of two cell-surface G protein-coupled receptors: CXCR1 (IL-8RA) and CXCR2 (IL-8RB) (Holmes et al. 1991, Murphy et al. 1991). IL-8 is a chemotactic factor whose main functions are to stimulate cell proliferation, angiogenesis and migration (Waugh et al. 2008, Ning et al. 2011). This chemotactic factor was first described to promote the directional migration and activation of neutrophils in response to inflammatory and infectious diseases (Baggiolini et al. 1989, Baggiolini et al. 1997). Nevertheless, more recent studies have recognised IL-8 as an important factor in cancer by promoting tumorigenesis (cell proliferation), tumour maintenance (angiogenesis) and progression/invasiveness (migration) (Waugh et al. 2008). Given the above mentioned functions of IL-8, this interleukin seems to play fundamental roles as a regulatory factor within the inflammatory, tumour and senescence milieus.

CXCR2 is a promiscuous receptor that transmits signals from several CXC chemokine family members (CXCLs), including IL-8. Acosta *et al*. conducted a screen for small hairpin RNAs to identify genes controlling senescence in primary human fibroblasts. They reported that the knocking down of CXCR2 alleviates both replicative and oncogene-induced senescence (OIS) and diminishes the DNA-damage response. Conversely, ectopic expression of CXCR2 resulted in premature senescence via a p53-dependent mechanism. They further found that cells undergoing OIS activate NF-κB and C/EBPβ transcription factors to regulate expression of CXCR2 and multiple CXCR2-binding chemokines. The authors concluded that senescent cells activate a self-

amplifying secretory network in which CXCR2-binding chemokine reinforce the growth arrest (Acosta et al. 2008).

1.3.2 Pathways controlling secretion of inflammatory factors in cellular senescence

The pathways regulating the growth arrest and the SASP during senescence are often coordinately induced but do not completely overlap. For instance, both p16 and p53 expression are required to induce a senescence growth arrest, but do not induce or modify the SASP (Coppe et al. 2008, Coppe et al. 2011). The DDR is the most prominent initiator of senescence and persistence of a DDR during senescence has been shown to be essential for the development of a SASP, particularly for the induction of inflammatory cytokines such as IL-6 and IL-8 (Rodier et al. 2009). The p38 mitogen-activated protein kinase (p38MAPK) has been described to upregulate expression of specific cytokines such as IL-6, IL-8, and TNFα in some biological contexts (Ono et al. 2000, Zhang et al. 2007). In senescence, a study by Freund *et al.* showed that p38MAPK activity is necessary and sufficient for expression of SASP cytokines via NF-κB transcriptional activity, in cells induced to senesce by direct DNA damage or oncogenic RAS (Freund et al. 2011).

Cellular senescence is a complex mechanism, in which multiple stimuli can promote the development of a complex phenotype termed senescence phenotype. Although much is known about the pathways that regulate the senescence growth arrest, the pathways that regulate the SASP remain poorly understood. As described above ROS appears to add an extra layer of complexity to the intricate senescence phenotype. It is likely that ROS is the missing link between the secretory phenotype and its proposed role in the reinforcement of the senescent phenotype.

1.4. Synergistic interactions between inflammatory factors and ROS during senescence

Mechanistically, it is still not entirely understood how the SASP contributes to the reinforcement of senescence, however several lines of evidence suggests the existence of synergistic interactions between the DDR, ROS and inflammatory signals (Figure 1.4). Kinetically, ROS has been observed to increase 2-3 days following activation of a DDR (Passos et al. 2010), while the SASP occurs 7-10 days later (Coppé et al. 2010). Some of the similarities between both pathways and potential interactions will be examined thereafter.

Induction of both ROS and SASP in stress-induced senescence has been shown to be dependent on activation of the DDR (Rodier et al. 2009, Passos et al. 2010). Consistent with this, recent investigations have reported that progeroid mouse models driven by DNA damage are associated with activation of NF-κB chronic inflammation and senescence (Osorio et al. 2012, Tilstra et al. 2012). Interestingly, in a murine model of XFE progeroid syndrome, *Ercc1*$^{-/\Delta}$ mice, inhibition of NF-κB signalling not only reduced the onset of several age-related pathologies, but also both DNA and proteins oxidation (Tilstra et al. 2012), suggesting a potential link between inflammation and ROS pathways. Another link between ROS and the SASP during senescence involves p38MAPK pathways. p38MAPK has been shown to regulate the SASP in senescence mainly through NF-κB transcriptional activity (Freund et al. 2011). Similarly, p38MAPK pathway has been shown to be important for ROS generation in both stress-induced and replicative senescence and for the stability of the DDR (Passos et al. 2010).

Acosta and colleagues have shown that inhibition of CXCR2, a promiscuous receptor that transmits signals from several CXC chemokine family members (CXCLs), including IL-8, delayed the onset of both replicative and oncogene-induced senescence and lead to decreased activation of a DDR (Acosta et al. 2008). Mechanistically, the authors proposed that inhibition of CXCR2 reduced DDR potentially by impacting on ROS (Acosta et al. 2008).

Interpheron beta (β-IFN) has been shown to induce senescence through ROS activation and subsequent activation of a DNA damage response, which could be inhibited using the antioxidant NAC (Moiseeva et al. 2006).

Transforming growth factor beta (TGF-β), another secreted protein, has also been implicated in senescence. Inactivation of TGF-β1 secretion in mouse keratinocytes was enough to prevent oncogene induced senescence (Tremain et al. 2000). In human fibroblasts, blocking TGF-β1 receptor II activity has been shown to prevent UVB and hydrogen peroxide induced senescence (Frippiat et al. 2001, Debacq-Chainiaux et al. 2005). Recently, it was demonstrated that the TGF-β pathway had a strong effect in paracrine induced senescence (Acosta et al. 2013). Interestingly, the TGF-β pathway has also been identified as an important link between telomere-dependent and –independent DDR and ROS production (Passos et al. 2010).

Another potential link between the SASP and ROS is the fact that several studies indicate that NF-κB (the main regulator of the SASP) is also a major player in the regulation of mitochondrial function and oxidative stress. Firstly, several reports associate NF-κB with regulation of mitochondrial function. NF-κB is present in mitochondria of yeast (Bottero et al. 2001) and mammalian cells and is able to regulate expression of mitochondrial encoded genes in mammalian cells (Cogswell et al. 2003). Bakkar and colleagues reported that activation of the NF-κB alternative pathway during myogenesis is important for mitochondrial biogenesis (Bakkar et al. 2008). More recently it was demonstrated that IKKα and alternative NF-κB regulate the transcription co-activator PGC-1β (a master regulator of mitochondrial biogenesis and function) to promote oxidative muscle metabolism (Bakkar et al. 2012). Secondly, it has also been reported that NF-κB is involved in the transcriptional regulation of both anti-oxidant and pro-oxidant genes (Morgan et al. 2011). A recent study on a mouse model of type II diabetes-cardiac dysfunction has shown that enhanced NF-κB activity is associated with increased oxidative stress. The authors demonstrate that chemical inhibition of NF-κB alleviates oxidative

stress, improves mitochondrial structural integrity and ultimately restores cardiac function in type II diabetes (Mariappan et al. 2010).

In contrast, numerous reports have implicated ROS in the activation of NF-κB (Gloire et al. 2006). DNA binding and transactivation by NF-κB have been shown to be strongly activated by ROS such as hydrogen peroxide (Meyer et al. 1993). Overall, this suggests that NF-κB and ROS pathways are tightly interconnected and may together impact on the stabilisation of senescence (Figure 1.4).

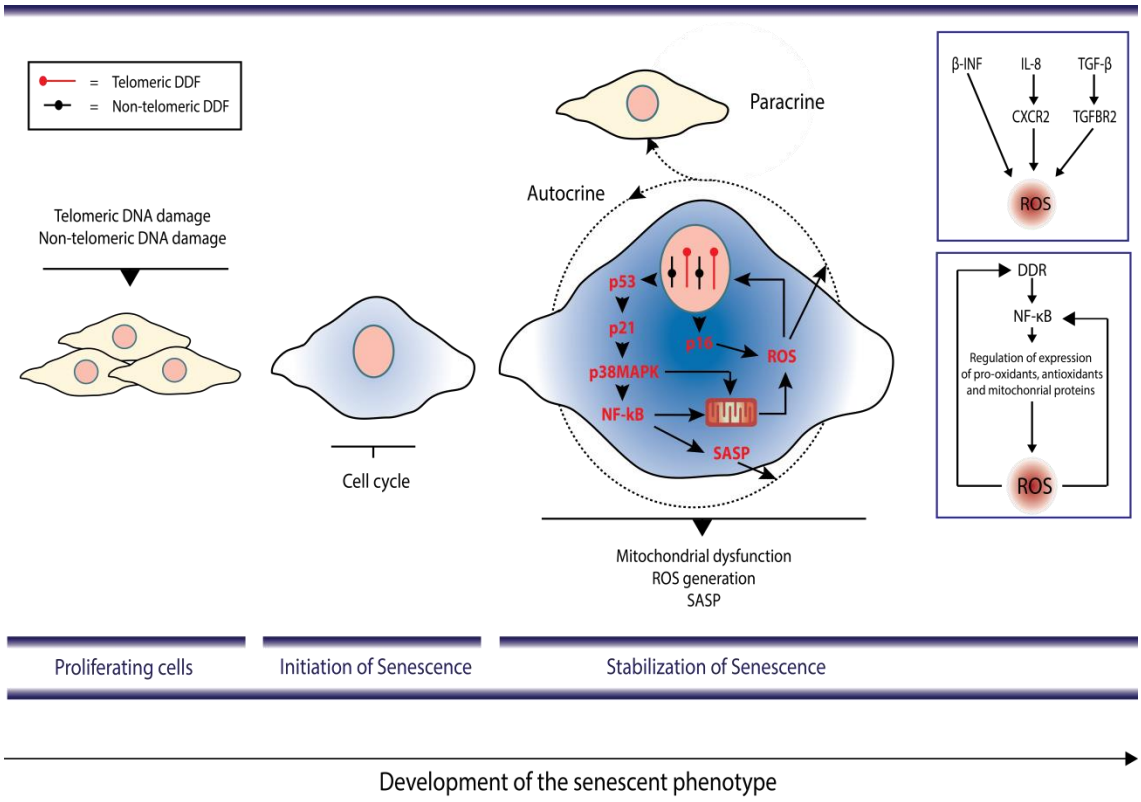


Figure 1.4 | Senescence is a multi-layered process involving interactions between the DNA damage response, reactive oxygen species and the senescence-associated secretory phenotype. Initially, stressors such as telomeric and non-telomeric DNA damage can lead to activation of a DNA damage response (DDR) and cell cycle arrest. Following activation of the DDR, p53, p21 and p38MAPK pathways have been shown to enhance NF-κB transcriptional activity. NF-κB activation

is both responsible for the senescence-associated secretory phenotype (SASP) and can induce (and be activated) by reactive oxygen species (ROS). p16 has been shown to induce ROS generation via NADPH oxidases (Takahashi et al. 2006); however, it has been shown to be unrelated to the SASP (Coppe et al. 2011). Secretion of bioactive molecules such as ROS and SASP factors contribute not only to reinforce senescence in an autocrine fashion, but also to induce paracrine senescence. Components of the SASP, such as IL-8, β-IFN and transforming growth factor (TGF)-β, have been shown to reinforce the senescence arrest via ROS through yet unidentified mechanisms (Moiseeva et al. 2006, Acosta et al. 2008, Passos et al. 2010). NF-κB transcriptional activity has been shown to be dependent on the DDR and ROS. However, NF-κB activation has been shown to increase ROS generation via regulation of mitochondrial, antioxidant and pro-oxidant genes (Morgan et al. 2011, Bakkar et al. 2012) (adapted from Correia-Melo *et al.* 2014).

1.5. mTOR Signalling: Sensing nutrient availability and managing stress in senescence and ageing

1.5.1 mTOR kinase and complexes

The mechanistic target of rapamycin (mTOR) protein was first discovered by Heitman et al. when analysing yeast mutants resistant to the growth-inhibitory properties of rapamycin (Heitman et al. 1991). mTOR is a highly conserved serine/threonine kinase belonging to the PI3K-related family of protein kinases, which also includes ATM, ATR and DNA-dependent protein kinase. The mTOR kinase can be found in two structurally and functionally distinct multiprotein complexes: mTOR complex 1 (mTORC1) and 2 (mTORC2) (Laplante et al. 2012). mTORC1 is a six protein complex formed by: the mTOR kinase, the mammalian lethal with sec-13 protein 8 (mLST8, also known as GβL), (Kim et al. 2003, Jacinto et al. 2004) , the DEP domain containing mTOR-interacting protein (DEPTOR) (Peterson et al. 2009), the Tti1/Tel2 complex (Kaizuka et al. 2010), the regulatory-associated protein of mammalian target of rapamycin

(raptor) (Hara et al. 2002, Kim et al. 2002) and the proline-rich Akt substrate 40 kDa (PRAS40) (Sancak et al. 2007, Vander Haar et al. 2007, Wang et al. 2007). The reported functions of the mTORC1 pathway involve integrating inputs from growth factors, stress, energy status, oxygen, and amino acids to control regulation of cell growth via protein (Averous et al. 2006, Ma et al. 2009), nucleotide (Ben-Sahra et al. 2013, Robitaille et al. 2013) and lipid synthesis, glycolysis (Laplante et al. 2009, Peterson et al. 2011) and autophagy (Ganley et al. 2009, Hosokawa et al. 2009). Whereas mTORC2 is composed by seven proteins including: the mTOR kinase, mLST8, DEPTOR, the Tti1/Tel2 complex, the rapamycin-insensitive companion of mTOR (rictor) (Jacinto et al. 2004, Sarbassov et al. 2004), the mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Frias et al. 2006, Jacinto et al. 2006) and the protein observed with rictor 1 and 2 (protor1/2) (Pearce et al. 2007, Pearce et al. 2011). The role of mTORC2 is less well understood when compared to mTORC1, but it is known that this mTOR complex receives signals from growth factors to regulate glucose metabolism, lipogenesis (Garcia-Martinez et al. 2008, Hagiwara et al. 2012, Yuan et al. 2012), the actin cytoskeleton and apoptosis (Oh et al. 2011). Both mTORC complexes share common proteins but also include specific proteins that contribute to their distinct functions and sensitivity to chemical inhibitors/drugs.

1.5.2 Signalling to and from mTORC1

The mechanisms regulating the mTOR pathway are complex, owing this complexity to the numerous different signals that converge and diverge to and from this pathway. A brief summary will be described to highlight some of the most important and better known functions of mTORC1. Mechanistically, the tuberous sclerosis 1 and 2 (TSC1/TS2) complex functions as a GTPase-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase (Inoki et al. 2003) and negatively regulates mTORC1 activity by converting Rheb into its inactive form GDP bound sate (Inoki et al. 2003, Tee et al. 2003).

Conversely, the GTP-Rheb form is a positive regulator of mTORC1 and when bound to this complex strongly stimulates its activity. Growth factors signals (e.g. insulin and insulin-like growth factor 1 (IGF1)) induce inactivation of the TSC1/TSC2 complex via direct phosphorylation by the protein kinase B (Akt/PKB) and promote mTORC1 activation (Inoki et al. 2002, Manning et al. 2002, Potter et al. 2002). However, Akt can also phosphorylate, PRAS40, another negative regulator of mTOR, and activate mTORC1 independently of the TSC1/TSC2 complex (Sancak et al. 2007, Vander Haar et al. 2007, Wang et al. 2007). Other kinases such as the extracellular-signal-regulated kinase 1/2 (ERK1/2) and ribosomal S6 kinase (RSK1) can also phosphorylate the TSC1/TSC2 complex and activate mTORC1 downstream of growth factors stimulation (Roux et al. 2004, Ma et al. 2005). The TSC1/TSC2 complex can also be inhibited through phosphorylation by the inhibitor of NF-κB (IκB) kinase β (IKKβ) following pro-inflammatory cytokines stimuli (Lee et al. 2007). Conversely, the adenosine monophosphate - activated protein kinase (AMPK), following inhibitory signals downstream of Wnt, phosphorylates TSC2 and activates the TSC1/TSC2 complex promoting inhibition of mTOR and downstream pathways involved in the regulation of cell growth, proliferation, polarity, differentiation, and development (Inoki et al. 2006). The mechanisms by which mTOR senses intracellular aminoacids are not fully elucidated but it is described that the Ragulator-Rag complex is a master regulator in this process by promoting mTORC1 translocation to the lysosomal surface and allowing interaction with GTPases such as the Rheb GTPase (Sancak et al. 2010), in a process that seems to be independent of the TSC1/TSC2 complex (Smith et al. 2005).

One of the most well described signalling downstream of mTORC1 involves the 70kD protein S6 kinase (p70-S6K) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1) to regulate anabolic processes such as protein synthesis. mTORC1 phosphorylates the p70-S6K which upon phosphorylation can also phosphorylate the S6 ribosomal protein. In this process mTORC1 can also phosphorylate and inactivate the (4EBP1) with release of eIF4E promoting increased translation through the eIF4F complex

(Fingar et al. 2002) Alternatively, mTORC1 is also involved in the control of catabolic processes by negatively regulating autophagy via phosphorylation of ULK1 (Lee et al. 2010).

1.5.3 Implications of mTOR on ageing and senescence

mTOR has been shown to play a role on the onset of ageing and age-related diseases including cancer, possibly via its critical role on the regulation of cell growth and proliferation (Menon et al. 2008, Laplante et al. 2012). Dietary restriction (DR) is the most robust intervention able to prolong lifespan and slow down the progression of age-related diseases with reproducible results across a variety of species ranging from yeast to primates (Kapahi et al. 2010). During DR, a particular or total nutrient availability is reduced with a consequent impact on the activation of nutrient sensing signalling including the mTOR pathway. Consistent with a beneficial role for decreased mTOR activity on ageing, mTOR inhibition by genetic or pharmacological means led to lifespan extension of different organisms including yeast, worms, fruit flies and mice, similarly to DR (Fabrizio et al. 2001, Vellai et al. 2003, Jia et al. 2004, Kapahi et al. 2004, Kaeberlein et al. 2005, Harrison et al. 2009, Bjedov et al. 2010). Rapamycin, a compound found in the soil bacterium in the Rapa Nui Island (also known as Easter Island) (Vezina et al. 1975), is a chemical inhibitor of mTORC1 activity by forming a complex with the intracellular 12-kDa FK506-binding protein (FKBP12) (Brown et al. 1994, Sabatini et al. 1994). The exact mechanisms by which the binding of FKBP12-rapamycin to mTORC1 inhibits its activity is unknown, but it has been described that rapamycin may compromise the structural integrity of mTORC1 and the specific activity of its kinase domain (Yip et al. 2010). More recent studies have described that prolonged treatment with rapamycin may also disrupt mTORC2 assembly and function in some cells types (Sarbassov et al. 2006). An important study conducted in mice has demonstrated that mTOR inhibition via a rapamycin supplemented diet results in increased maximal and median lifespan even when the treatment was started later in life (Harrison et al. 2009).

Studies exploring a role for mTOR during senescence have been somewhat contradictory. During oncogene-induced senescence, spatial coupling of catabolic and anabolic processes via mTOR may facilitate the mass synthesis of secretory proteins (Young et al. 2009, Narita et al. 2011). Supporting a pro-senescence role for autophagy, Kennedy *et al.* recently showed that rapamycin, a mTOR inhibitor and activator of autophagy, promotes oncogene-induced senescence in a mouse model of pancreatic cancer (Kennedy et al. 2011). In contrast, in other models of senescence, including replicative senescent rodent fibroblasts (Pospelova et al. 2012), mTOR activity promotes senescence and senescence is inhibited by rapamycin. Consistently, it has been described that inhibition of mTOR induces quiescence, while its hyper-activation promotes senescence in a p53-dependent manner (Demidenko et al. 2010, Korotchkina et al. 2010). Nevertheless, there is indirect evidence that suggest a role for mTOR during cellular senescence. Many stressors described to induce senescence, including DNA damage, can also activate the mTOR pathway in part via the TSC1/TSC2 complex. mTORC1 receives DNA damage signals through multiple mechanisms, all of which require p53-dependent transcription (Feng et al. 2005). Another important feature of senescence is the SASP with NF-κB signalling being a master regulator of the senescence-associated inflammatory phenotype (Acosta et al. 2008, Kuilman et al. 2008). It has been shown that activation of mTOR downstream of Akt regulates NF-κB activity (Dan et al. 2008). Such regulatory control of mTOR over inflammatory pathways may contribute to the understanding of the mechanisms regulating the SASP.

1.6. Cellular Senescence and Ageing

Despite being discovered over 50 years ago, cellular senescence remains an intriguing biological process and whether senescence exists to any significant extent *in vivo* has been the subject of a longstanding debate (Ben-Porath et al. 2005). In the past decade, remarkable advances have been made

demonstrating that senescence plays an important role *in vivo*. Several studies suggest that senescence can act as a tumour suppressor mechanism (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005, Michaloglou et al. 2005). On the other hand, numerous lines of evidence indicate that senescence can, in the long run, have adverse effects, by impairing organ regeneration and releasing a host of bioactive molecules, including ROS and a wide variety of pro-inflammatory cytokines, chemokines and growth factors (collectively referred to as the SASP) that impact on the surrounding tissue.

In 2006, Herbig *et al.* showed that the amount of cells containing telomere-induced foci (TIF), a well-known marker of senescence, increase with age in the skin of baboons, primates with similar telomere length to humans and absence of telomerase activity (Herbig et al. 2006). In mice, cells bearing senescent markers have been reported to increase with age in a variety of tissues (Krishnamurthy et al. 2004, Wang et al. 2009, Hewitt et al. 2012), including post-mitotic neurons (Jurk et al. 2012). Moreover, senescent cells have been associated with several age-related diseases, such as diabetes (Sone et al. 2005) and atherosclerosis (Minamino et al. 2007). While noteworthy, this data does not provide causality. A major challenge in the field has been to determine if and how senescent cells contribute to age-related tissue dysfunction, or if they merely correlate with it.

Mounting evidence indicates that activation of pathways involved in cellular senescence impacts on mammalian lifespan (Rudolph et al. 1999, Tyner et al. 2002, Choudhury et al. 2007). Recently, the van Deursen's group has shown that inducible elimination of p16Ink4a-positive senescent cells from the eye, adipose and skeletal tissues in the BubR1 progeroid mouse model, delayed acquisition of age-related pathologies in these tissues. They showed that elimination of p16Ink4a-positive cells also attenuated the progression of already established age-related disorders, suggesting that cellular senescence may have a causal role in age-related tissue impairment (Baker et al. 2011).

1.7 Aims

Although several mechanisms responsible for the activation of senescence have been identified, it is still unclear how a cell "commits" to becoming irreversibly arrested. Recent studies have revealed that the SASP, as well as mitochondrial/ROS may contribute to the reinforcement of the growth arrest via a series of positive feedback loops involving a persistent activation of the DDR (Acosta et al. 2008, Kuilman et al. 2008, Passos et al. 2010). The general aim of this thesis was to understand how mitochondria and inflammatory factors act together in cellular senescence and how these interactions may contribute to the stabilisation of the senescent phenotype.

Specific Aims

1. To investigate the role of mitochondrial density (mass) during the development of cellular senescence, both *in vitro* and *in vivo*;

2. To understand the mechanisms downstream of a DNA damage response driving mitochondrial alterations during cellular senescence and potential links to the mTOR signalling pathway;

3. To understand the mechanisms by which pro-inflammatory cytokine receptor CXCR2 helps maintain cellular senescence and investigate potential links to mitochondrial function.

Chapter 2. Material and Methods

2.1 Chemicals and Reagents

Unless otherwise stated, the chemicals used in these studies were obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, UK).

2.2 Cell Culture

2.2.1 Cell lines

Prokaryotic cell lines

NEB 5-alpha Competent *Escherichia coli,* (Cat. Number C2987, New England Biolabs).

Eukaryotic cell lines

Human cell lines

Human embryonic lung MRC5 fibroblasts (ECACC, Salisbury, UK), YFP-Parkin-expressing MRC5 fibroblasts, human embryonic kidney HEK293, Phoenix amphotropic (human embryonic kidney HEK293 transformed with adenovirus E1a and carrying a temperature sensitive T antigen), human breast cancer MCF7, human primary glioblastoma U87, Human colon carcinoma HCT116, human osteosarcoma 143B, rho 0 143B (mtDNA depleted human osteosarcoma 143B cells), Ataxia Telangiectasia (AT) patients human fibroblasts, T19 ($TRF2^{\Delta B \Delta M}$) and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM, Cat. Number D5796, Sigma, Dorset, UK) supplemented with 10% heat inactivated foetal bovine serum

(BioSera, Ringmer, UK), 100 units/ml penicillin, 100 µgml-1 streptomycin and 2 mM glutamine, at 37^{o}C in a humidified atmosphere with 5% CO2.

T19 cells containing a doxycycline inducible TRF2$^{\Delta B \Delta M}$ were a kind gift from T. de Lange, Rockefeller University, NY, USA (van Steensel et al. 1998). Osteosarcoma 143B and Rho 0 143B cell lines were kind gifts from Dr. Robert Lightowlers, Mitochondrial Research Group, Newcastle University, UK. AT patient fibroblasts were kind gifts from Dr Lisa Woodbine, University of Sussex, UK. Phoenix amphotropic cells were a kind gift from Dr. Stephen Tait, Institute of Cancer Sciences, University of Glasgow, UK. Human embryonic kidney HEK293 cells were a kind gift from Dr. Glyn Nelson, Institute for Ageing and Health, Newcastle University, UK.

Human fibroblasts (MRC5 fibroblasts and YFP-Parkin expressing MRC5 fibroblasts) were used for cellular and molecular biology analysis at a population doubling (PD) level of 20-30 for stress-induced senescence or after reaching replicative exhaustion (replicative senescence).

Mouse cell lines

Mouse embryonic fibroblasts (MEFs) were obtained from C57BL/6 wild-type mice. MEFs from *Atg5*$^{-/-}$, *PGC-1β*$^{-/-}$, matched wild-type, Mouse hepatocyte cell-line MIH and 3T3 embryonic fibroblasts were cultured in Advanced DMEM/F-12 (Invitrogen, Cat. Number 12634) plus 10% heat inactivated foetal bovine serum (BioSera, Ringmer, UK), 100 units/ml penicillin, 100 µgml-1 streptomycin and 2 mM glutamine, at 37^{o}C in a humidified atmosphere with 5% CO2 and 3% oxygen.

Atg5$^{-/-}$ MEFs were kind gifts from Dr.Noboru Mizushima, Tokyo University, Japan (via Viktor Korolchuk, Institute for ageing and Health, Newcastle University, UK). *PGC-1β*$^{-/-}$ MEFs were kind gifts from Dr. Sergio Rodriguez-Cuenca, Metabolic Research Laboratories, University of

Cambridge, UK. 3T3 embryonic fibroblasts were a kind gift from Dr. Stephen Tait, Institute of Cancer Sciences, University of Glasgow, UK.

2.2.2 Cryogenic storage

Exponentially growing adherent cells were trypsinised with Trypsin-EDTA (Sigma, Cat.Number T3924) and centrifuged at 150g for 5 minutes at room temperature. The supernatant was removed and cells were re-suspended in foetal calf serum (FCS) containing 10% (v/v) dimethyl sulfoxide (DMSO) at a density of $1x10^6$ cells/ml. One mL aliquots of cell suspension were immediately transferred to cryo-vials and placed in a NalgeneTM Cryo freezing container filled with isopropanol. Cells were kept for 24 hours in a -80°C freezer to allow slow freezing, before being stored in liquid nitrogen.

2.2.3 Resuscitation of frozen cells

Cryo-vials were removed from the liquid nitrogen bank and quickly thawed for 1-2 min at 37°C. Thawed cells were immediately seeded into a 75 cm^2 flask with 20 mL pre-warmed medium. Medium was replaced with fresh media 24 hours after seeding to remove DMSO and cell debris.

2.2.4 Calculating cell density and population doublings

To determine the concentration of cells within the cell suspension, following trypsinisation 20 µL of cells suspension were placed on a Fuchs Rosenthal haemocytometer (VWR International, UK). Cells were counted on 8 smallest squares of the haemocytometer under a standard microscope (DMIL, Leica Microsystems, UK). The average of four counts of 8 squares is equivalent to the number of cells $x10^4$/mL. The total number of cells was calculated by multiplying

the volume of cell suspension (mL) with the cell concentration (cells/mL). On human primary cell lines the population doubling (PD) level was calculated by comparing the amount of cells seeded with the number of cells obtained, using the following equation: PD)=X+Ln(N1/N2)/Ln2

where, PD = population doublings

X = previous PD

N1 = number of cells harvested

N2 = number of cells seeded

2.3 Creating stably expressing YFP-Parkin-MRC5 fibroblast cell lines

2.3.1 Bacterial Transformation

NEB5-alpha competent *Escherichia Coli* (NEB; Cat. Number C2987) were transformed with a LZRS-YFP-Parkin plasmid (kind gift from Dr. Stephen Tait, Institute of Cancer Sciences, University of Glasgow, UK):

Plasmid DNA (50 ng) was added to a 50µL of NEB5-alpha highly competent *E. coli* and incubated on ice for 30 minutes. The mixture was then subjected to heat shocking at 42^{o}C for 30 seconds, before five minutes incubation on ice. 250µL SOC medium (Invitrogen, Cat. Number 15544-034) was then added and cells were incubated at 37^{o}C for 1 hour with shaking vigorously at 250rpm. Cells were then spread on agar selection plates containing lysogeny broth (LB) medium plus ampicillin (15g agarose to 1L LB (10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl made up to 1L H_2O) and autoclave. Once cool enough to hold, add

50µg/ml ampicillin selection agent, mix thoroughly and pour into plates), and incubated at 37^{o}C overnight. Individual colonies were then grown overnight in 5mL selective LB (LB+ampicilin). DNA was purified using the QIAprep Spin Miniprep kit (Qiagen, Cat. Number 27104) as described in the provided protocol.

Transformed bacteria stocks

Glycerol stocks were made of transformed bacteria. 800µL LB containing transformed bacteria were mixed with 200µl 80% glycerol, then snap frozen in liquid nitrogen, and stored at -80^{o}C.

2.3.2 Plasmid Analysis

Following DNA purification using QIAprep Spin Miniprep kit, the Plasmid DNA was digested with the *HindIII restriction enzyme* (NEB, Cat. Number R0104S) to confirm purity of the Plasmid DNA. Plasmid DNA fragments were the right size, with 10.48 Kb and 2.61 Kb fragments. Non digested Plasmid DNA showed no fragmentation with a band size of 13.09 Kb, indicating integrity and correct purification of the Plasmid DNA (Figure 3.1).

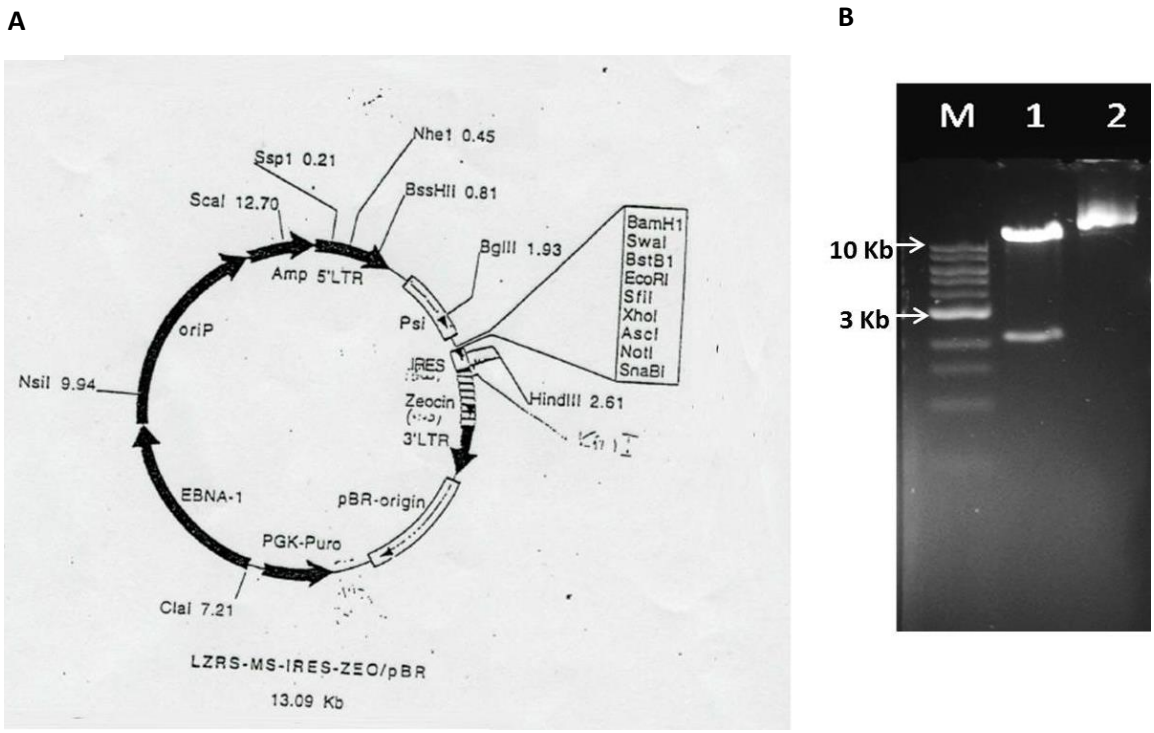


Figure 2.1 | **LZRS-YFP-Parkin plasmid. (A)** Purified LZRS-YFP-Parkin plasmid map; **(B)** Purified LZRS-YFP-Parkin plasmid was digested with *HindIII* restriction enzyme. M= DNA ladder, 1= digested LZRS-YFP-Parkin (fragment size: 10.48 Kb and 2.61 Kb) plasmid and 2=undigested ZRS-YFP-Parkin plasmid (fragment size: 13.09 Kb).

2.3.3 Transfection and Transduction Protocols

Retroviral plasmid transfection and viral production was performed following class II safety procedures. 5×10^6 Phoenix amphotropic cells were seeded in a 10cm dish and incubated for 24 hours in antiobiotic free medium. Cells were at 90% confluency before proceeding to Invitrogen Transfection using Lipofectamine^{TM} 2000 (Invitrogen, Cat. Number 11668-019) as described in the ViraPower Lentiviral Expression systems user manual (Invitrogen). The day after transfection medium was removed and replaced with 10mL fresh medium without antibiotics. Viral particles containing medium were collected 48 hours

after initial transfection, centrifuged at 4^{o}C and filtered through a 0.45µm pore PVDF filter. The purified media with 10µg/ml polybrene was then added to MRC5 fibroblasts at 70-90% confluence. Zeocin^{TM} (Invitrogen, Cat. Number R25001) was added to cells at a final concentration of 500µM for six days, concentration was then reduced to 200µM to maintain selection for another 10 days. Transduction efficiency was confirmed by fluorescence microscopy: cells expressing Parkin were YFP positive.

2.4 Induction of Senescence

MRC5 fibroblasts (PD 20-30) were seeded in 6-well plates (100,000 cells) and allowed to grow for 24 hours. Cells were then induced to senesce.

Stress-induced senescence was induced by:

- X-ray irradiation (X-Rad 225, Precision X-Ray INC, N-BRANFORD, CT USA) with 10 or 20Gy (depending on cell-line);
- 80ng/ml neocarzinostatin (Sigma; Cat. Number N9162) for 1 hour;
- 400µM H_2O_2 (Sigma, Cat. Number H1009) in serum free media for 1 hour;
- 50µM Etoposide (Sigma; Cat. Number E1383) containing medium was replenished every 3 days for 10 days.

Following treatments, culture medium was refreshed (except for the Etoposide treatment.

Replicative senescence was achieved through replication exhaustion and confirmed by >70% of cells being positive for Sen-β-Gal and less than 0.5 Population doublings for at least 4 weeks.

2.5 Treatments:

2.5.1 NAC treatment

MRC5 fibroblasts at a confluency of 70-90% were irradiated with 20Gy X-ray and treated with 2.5mM of NAC (Sigma; Cat. Number A7250) for 3 days before being harvested for analysis. Control non-irradiated MRC5 fibroblasts were also treated with 2.5mM of NAC for 3 days before being harvested for analysis.

2.5.2 CCCP treatment

Stably expressing YFP-Parkin human MRC5 or 3T3 fibroblasts were irradiated with 20 Gy (MRC5) and 10Gy (3T3) X-ray and treated 2 days after with 12.5 μM CCCP (Sigma; Cat number C2759) for 48 hours (refreshed every 24 hours). Control non-irradiated fibroblasts were treated with 12.5 μM CCCP for 48 hours (refreshed every 24 hours).

Replicative senescent YFP-Parkin-MRC5 fibroblasts were treated with12.5 μM CCCP for 48 hours (refreshed every 24 hours). Control proliferating fibroblasts (PD=20-25) were treated with 12.5 μM CCCP for 48 hours (refreshed every 24 hours).
Fresh media (no CCCP) was replaced 48h after treatment; cells were then collected at the indicated time points for analysis.

2.6 Treatment with pathway inhibitors

2.6.1 Inhibition of mTORC1

MRC5 fibroblasts were induced to senesce by X-ray irradiation with 20Gy X-ray, neocarzinostatin (80ng/ml) for 1 hour and H_2O_2 (400μM) in serum free media for 1 hour. Following treatment, culture medium was refreshed with 100nM

rapamycin (Sigma, Cat. Number R8781). Etoposide treatment (50µM) was performed continuously every 3 days for 10 days with simultaneous 100nM rapamycin treatment. Cells were collected at different time points after treatment to kinetically understand the effect of mTORC1 inhibition on the development of the senescence phenotype. Replicative senescent MRC5 fibroblasts were treated with 100nM rapamycin for 10 days before being harvested for analysis. Rapamycin supplemented media was always refreshed 24h prior cells collection to avoid starvation confounding effects on mTORC1 activity.

2.6.2. Inhibition of ATM

MRC5 fibroblasts at a confluency of 70-90% were irradiated with 20Gy X-ray and treated with 10µM of the ATM chemical inhibitor KU55933 (R&D; Cat. Number 3544). Control non-irradiated fibroblasts were also treated with 10µM of the ATM chemical inhibitor KU55933. Cells were collected at different time points to kinetically understand the effect of ATM inhibition on the development of the senescence phenotype and respective activated pathways. The ATM chemical inhibitor KU55933 also inhibits other PIKK proteins when used in higher concentrations (as described in the MSDS of the product (R&D; Cat. Number 3544)). We have used Ataxia Telangiectasia (AT) patient human fibroblasts (mutated non-functional ATM protein kinase) to confirm the results observed with the ATM chemical inhibitor KU55933.

2.6.3 Neutralisation of CXCR1 and CXCR2

Control (0Gy) or irradiated (20Gy) MRC5 fibroblasts were treated with 10ug/mL of neutralising antibodies against CXCR1 (BD Biosciences, Cat. Number 555937) and/or CXCR2 (BD Biosciences, Cat. Number 555932). Replicative senescent MRC5 fibroblasts were treated with 10ug/mL of neutralising antibodies against CXCR1 and/or CXCR2. Cells were collected at different time

points after treatment to kinetically understand the effect of IL-8 signalling inhibition on the development of the senescence phenotype and respective activated pathways.

2.6.4 Inhibition of mTORC1 plus NAC treatment

Control (0Gy) or irradiated (20Gy) MRC5 fibroblasts were treated with 100nM of rapamycin and 2.5mM of NAC. Cells were collected 3 days after irradiation for ROS and DDF analysis to understand the effect of mTORC1 inhibition with additional antioxidant treatment on ROS and DDR suppression.

2.6.5 Inhibition of ATM and mTOR

Control (0Gy) or irradiated (20Gy) MRC5 fibroblasts were treated with 10μM of the ATM chemical inhibitor KU55933 and 100nM of rapamycin. Cells were collected at different time points after treatment to kinetically understand the effect of simultaneous inhibition ATM and mTORC1 on the development of the senescence phenotype and respective activated pathways.

2.7 Knock down by small interfering RNA

MRC5 cells at a population doubling (PD) level of 20-25 were transiently transfected with siRNAs using the HiPerFect Transfection Reagent^{TM} (Qiagen, Cat. Number 301707). Cells were transfected with 10nM siRNA following the HiPerFect Transfection Reagent Handbook instructions. Cells were transfected 24 hours prior to 20Gy X-radiation and harvested for analysis 72 hours after transfection (2 days after IR). siRNA transfection efficiency was performed by qPCR and/or Western Blotting.

Table 2.1 FlexiTube siRNAs

FlexiTube siRNAs from Qiagen		
Protein	**siRNA**	**Reference/Manufacturer**
scramble	Negative Control siRNA	SI03650325 - Qiagen
mTOR	Hs_FRAP1_4 FlexiTube siRNA	SI00070462 - Qiagen
	Hs_FRAP1_6 FlexiTube siRNA	SI02662009 - Qiagen
IL-8	Hs_IL-8_5 FlexiTube siRNA	SI02654827 - Qiagen
	Hs_IL-8_6 FlexiTube siRNA	SI02654834 - Qiagen
CXCR1	Hs_CXCR1_1 FlexiTube siRNA	SI00013258 - Qiagen
	Hs_CXCR1_2 FlexiTube siRNA	SI00013265 - Qiagen
CXCR2	Hs_CXCR2_2 FlexiTube siRNA	SI00447083 - Qiagen
	Hs_CXCR2_4 FlexiTube siRNA	SI00447097 - Qiagen

2.8 Flow cytometry

The flow cytometer (Partec, http://www.partec.com) was first calibrated using fluorescent beads to ensure optimum performance and reproducibility. In each independent experiment measurements were performed in duplicate and $1x10^4$ cells were analysed per measurement.

2.8.1 DHE staining

Dihydroethidium (DHE) is a blue fluorescent dye that when oxidised through binding to superoxide anions forms a red fluorescent product which intercalates with DNA. MRC5 fibroblasts were stained with 10μM of DHE (Invitrogen, Cat.

Number D1168) in serum free DMEM for 30 min at 37^{o}C in the dark. Red (FL3 channel) median fluorescence intensity was measured by flow cytometry.

2.8.2 NAO staining

The fluorescent dye 10-n-nonyl-acridine orange (NAO) binds specifically to the negatively charged cardiolipin (diphosphatidylglycerol) in the inner mitochondrial membrane independently of the membrane potential and can be used to monitor the mitochondrial mass. MRC5 fibroblasts were stained with 10 μM of NAO (Invitrogen, Cat. Number A1372) in serum-free DMEM and incubated for 10 min at 37^{o}C in the dark. NAO (Green - FL1 channel) mean fluorescence intensity was measured by flow cytometry.

2.9 Mice

2.9.1 Mice Groups, Treatments and Housing

Mice Groups and Treatments

C57/BL6 mice were split into 4 groups (n=10/group) according to age and diet: 1) 3 months old young mice months fed with Control diet; 2) 9.5 months old mice fed with Rapamycin or Control diet for 6.6 months; 3) 15 months old mice fed with Rapamycin or Control diet for 12 months and 4) 16 months old mice fed with Rapamycin or Control diet for 4 months. The different mice groups were matched for age and randomly assigned for the treatments. Control and Rapamycin diets were purchase from TestDiet - Control diet: 5LG6/122 PPM EUDRAGIT 3/8 #1814831 (5AS0) and Encapsulated Rapamycin diet: 5LG6/122 PPM ENCAP RAP 3/8 #1814830 (5ARZ). Mice were fed *ad libitum* and monitored weekly.

PGC-1β$^{-/-}$ mice were generated and provided by Transgenic RAD, Discovery Science, Astrazeneca. .Animals were fed *ad libitum* on a normal chow diet (10% of calories derived from fat; D12450B, Research Diets). Wild-type and *PGC-1β*$^{-/-}$ C57/BL6 mice were sacrificed at 18 months of age. Mouse liver tissues were a kind gift from Dr Sergio Rodriguez-Cuenca, Institute of Metabolic Science, Cambridge University, UK.

Liver tumours were induced in C57/BL6 mice by IP injection of 30mg/kg N-Nitrosodiethylamine (den) in wild-type mice. Tumour and normal liver tissues were a kind gift from Dr Derek Mann, Institute of Cellular Medicine, Newcastle University, UK.

Mice Housing

Animal procedures were performed in accordance with the UK Home Office regulations and the UK Animal Scientific Procedures Act [A(sp)A 1986]. Animals were housed in a temperature-controlled room with a 12-h light/dark cycle. No statistical method was used to predetermine sample size. No animals or samples were excluded from the analysis.

2.9.2 Mice tissues collection and preparation

Tissues were collected during necrospsy and fixed with either 4% formaldehyde aqueous solution buffered (VWR; Cat. Number 9713.9010) and paraffin embedding for histochemical analysis or with glutaraldehyde (Sigma, Cat. Number G5882) for T.E.M. (morphometric analysis). Part of the tissues were also frozen in liquid nitrogen and stored at -80^{o}C for biochemical analysis.

2.10 Mitochondrial Functional analysis

2.10.1 Mitochondrial Isolation

Liver mitochondria were isolated by the method described by (Chappell et al. 1972) in medium comprising 0.25 M sucrose, 5 mM Tris/HCl and 2 mM EGTA (pH 7.4 at 4 °C) (STE buffer). The crude mitochondria were purified by adapting the method described in (Pagliarini et al. 2008). Essentially, 0.5mL of crude mitochondria (about 30-40mg/mL) were carefully layered on top of a stepwise density gradient of 2 mL 80%, 6 mL 52%, and 6 mL 26% Percoll in a 50 mL centrifuge tube. The gradient was centrifuged at 41,100 g for 45 minutes in a Beckman Coulter Avanti® J-E centrifuge, using JA-20 rotor. Mitochondria were collected from the interface of the 26%-52% interface, diluted to capacity in a 2 mL microcentrifuge tube with STE buffer, and centrifuged at 12,000g in a refrigerated table top centrifuge for 10 minutes. The supernatant was carefully discarded, and the mitochondria were washed with an additional 2 mL of STE and centrifuged again. The resulting pellet was re-suspended in a small volume of STE for functional experiments.

2.10.2 Mitochondrial Oxygen Consumption analysis

Mitochondrial oxygen consumption rates were measured using the Seahorse XF analyser (Seahorse Biosciences) based on the method described in (Rogers et al. 2011), with adjustment explained in the Comments to the articles published in PLoS online (A source of data variation in mitochondrial respiration measurements). The mitochondria were energized with 5mM pyruvate (Sigma; Cat. Number S8636) and 5mM malate (Sigma; Cat. Number M6413), and state 3 respiration was obtained by adding 4mM ADP (Sigma; Cat.Number A2754).

2.11 Fluorescence staining on life cells

TMRM/ Mito Tracker Green

Dyes stock solutions

- 1µg/µL Mitotracker green (Invitrogen, Cat. Number M7514);
- 50mM TMRM (Invitrogen, Cat. Number T668);
- 100 µg/mL Hoechst 33342 (Invitrogen, Cat. Number H3570).

Dyes working solutions

- 1:1000 Mitotracker green: 1 µL in 999 µL serum free DMEM
- 1:3000 TMRM: 1 µL in 3 mL serum free DMEM
- MitoT/TMRM plus Hoechst solution: 1 µL of TMRM working solution, 200 µL of Mitotracker green working solution and 30 µL of Hoechst (1:100) in 3 mL serum free DMEM.

Mitochondrial Membrane potential (MMP) was measured using the red-orange fluorescent dye Tetramethylrhodamine methyl ester (TMRM), cationic and mitochondria selective probe that can be assessed by several fluorescence analysis methods (including fluorescence microscopy) (Floryk et al. 1999). Because TMRM changes the intensity but not the emission spectra in response to membrane potential it is a good marker of mitochondrial membrane potential in combination with Mito Tracker Green, a marker of mitochondrial mass (TMRM/MitoT Green). Hoechst is a blue fluorescence dye when bound to DNA commonly used for nuclear counterstain. Cells seeded in coverslips ($2.5x10^4$) were washed with PBS and incubated with MitoT/TMRM plus Hoechst solution at 37^oC for 30 minutes. Cells were then washed once with medium and mounted on glass slides with ~10 µl PBS. Cells were imaged immediately on a Leica DM5500B microscope using a DFC360FX camera and the LASAF

software (Leica). Coverslips were stained one at the time and imaged for not longer than 20 minutes after mounting, since MitoTracker green tends to leak out after a while.

2.12 Immunostainings

Cells

2.12.1 Immunofluorescence staining on fixed cells

Fixation

Cells grown in coverslips were fixed in 1 mL of 2% Paraformaldehyde in PBS (500µL of 4% formaldehyde (VWR; Cat. Number 9713.9010) plus 500µL of PBS) for 10 minutes at room temperature. Paraformaldehyde was removed and cells were washed twice with PBS.

Permeabilisation

Cells were incubated for 45 minutes at room temperature with 1mL PBG-Triton (0.2% cold water fish gelatine, 0.5% BSA and 0.5% Triton in PBS).

Immunofluorescence staining

A primary antibody (diluted in PBG –Triton) was added to the cells for 1 hour at room temperature with gentle agitation or overnight at 4^{o}C without gentle agitation. Cells were washed twice with PBG-Triton for 5 minutes. Cells were incubated for 45 minutes to 1 hour with fluorescein-conjugated secondary antibody (1/4000) diluted in PBG–Triton and then washed three

times with PBS for 5 minutes. Cells were stained for 10 minutes with 400µl of DAPI (Partec; Cat. Number 05-5001) and washed 3 times in PBS before mounting cells on slides using an anti-fade Vectashield mounting medium (Vector Lab, Cat. Number H-1000). Slides were analysed using a Leica DM5500B microscope and fluorescence images were captured with a DFC360FX camera using LASAF software (Leica).

2.12.2 ImmunoFISH on cells (γH2AX-TeloFISH)

Cells grown on coverslips were fixed and γ-H2A.X immunofluorescence staining was performed as described above. After application of the secondary antibody, cells were washed with PBS and FISH was performed.

FISH

Cells were fixed for 10 minutes with 4% PFA and then washed with dH_2O for 5 minutes twice. Cells were dehydrated with 70%, 90%, 100% ethanol for 3 minutes each and air dried before denaturation for 10 minutes at 80°C in hybridisation buffer [70% deionised formamide (Sigma), 25 mM $MgCl_2$, 1 M Tris pH 7.2, 5% blocking reagent (Roche,Welwyn, UK)] containing 4ng/µL Cy-3 labelled telomere specific (CCCTAA) peptide nuclei acid probe (Panagene, Cat. Number F1002-5), followed by hybridisation for 2 hours at room temperature in the dark. Cells were washed for 10 minutes with 70% formamide in 2xSSC (17.53g of NaCl and 8.82g of sodium citrate in 1L of H_2O, adjust pH to 7.0), following by a 10 minutes wash in 2xSSC and a final wash in PBS for 10 minutes. Nuclei were stained by DAPI for 10 minutes and washed 3 times in PBS for 5 minutes, before being mounted and imaged in a Leica DM5500B

fluorescence microscope. In depth Z stacking was used (a minimum of 40 optical slices with 100× objective) followed by Huygens (SVI) deconvolution. Telomere-associated foci were analysed blinded by several analysts.

Table 2.2 Primary antibodies for Immunofluorescence on cells

Primary antibodies				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
Ki67	Human Mouse	Rabbit polyclonal	1:250	ab15580 - Abcam
γ-H2A.X(Ser139)	Human	Mouse monoclonal	1:2000	05-636 - Millipore
53BP1	Human	Rabbit polyclonal	1:500	4937 - Cell signalling
53BP1	Human Mouse	Rabbit polyclonal	1:200	NB100-305 - Novus Biologicals
MT-CO1	Human Mouse	Mouse monoclonal	1:500	ab45918 - Abcam

Table 2.3 Secondary antibodies for Immunofluorescence on cells

Secondary antibodies				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
Anti-mouse Fluorescein-conjugated secondary antibody AlexaFluor 488	Mouse	Goat	1:4000	A21042 -Invitrogen
Anti-mouse Fluorescein-conjugated secondary antibody AlexaFluor 594	Mouse	Goat	1:4000	A21044 - Invitrogen
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 488	Rabbit	Goat	1:4000	A21212 - Invitrogen
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 594	Rabbit	Goat	1:4000	A21213 - Invitrogen
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 637	Rabbit	Goat	1:2000	A21244 - Invitrogen

Tissues

2.12.3 Immunostainings on paraffin embedded tissues

Dewax and Hydration

Paraffin tissue sections of 3µm thickness were deparaffinised in Histoclear (National Diagnostics; Cat. Number HS-200) for 10 minutes twice and hydrated in graded concentration of ethanol solutions: 100% (2x 5 minutes), 90% (5 minutes), 70% (5 minutes) and H_2O (10 minutes).

Antigen Retrieval

Antigens were retrieved by incubation tissues sections with 0.01M citrate buffer pH 6.0 (29.41g of trisodium citrate in 1L of distilled water, adjust pH to 6.0) in the microwave: high power (800W) for 5 minutes until boiling followed by 10 minutes at medium power (400W). Tissues sections were let to cool down for 20 minutes on an ice bath. Sections were then washed twice in H_2O for 10 minutes.

Immunohistochemistry staining

Protocol for rabbit primary antibodies

Slides were incubated in blocking buffer (1:60 Normal Goat Serum (NGS) (Vector Lab, Cat. Number PK-6101) and 0.1% BSA in PBS) for 30 minutes at room temperature. Primary antibodies were applied overnight at 4°C and then washed for 5 minutes three times with PBS. Tissues sections were incubated with biotinylated anti-rabbit antibody (Vector Lab, Cat. Number PK-6101) diluted in blocking buffer for 30 minutes at room temperature. Sections were washed twice for 5 minutes in PBS and blocked for endogenous peroxidase activity with

0.9% H_2O_2 in H_2O (7.5 mL of 30% H_2O_2 in 250mL H_2O). Tissues were then washed 3 times in H_2O for 5 minutes. Secondary antibodies were detected using the rabbit peroxidase ABC kit (Vector Lab. Cat. Number PK-4001) according to the manufacturer's instructions. Substrate was developed using the NovaRed kit (Vector Lab, Cat. Number SK-4800) following the manufacturer's instructions. Sections were counterstained with haematoxylin [5g Haematoxylin (Sigma, Cat. Number H3136), 300mL Glycerin (Sigma, Cat. Number G2289), 50g Aluminium potassium sulphate (Sigma, Cat. Number 7210), 0.5g Sodium iodate (Sigma, Cat. Number S4007), 40mL glacial acetic acid (Sigma, Cat. Number 537020) in 700mL H_2O] and washed 3 times in H_2O for 5 minutes. Tissue sections were then dehydrated and mounted with DPX mounting medium (Thermo Scientific; Cat. Number LAMB-DPX). Slides were analysed using a NIKON ECLIPSE-E800 microscope and images were captured with a Leica DFC420 camera using the LAS software (Leica).

Protocol for mouse primary antibodies

Immunohistochemistry stainings using mouse antibodies on mouse tissues were performed using the Mouse on Mouse (M.O.M) basic kit (Vector Lab, Cat. Number BMK-2202) according to the manufacturer's instructions. Substrate was developed using the NovaRed kit (Vector Lab, Cat. Number SK-4800) following the manufacturer's instructions. Sections were counterstained with haematoxylin and washed 3 times in H_2O for 5 minutes. Tissue sections were then dehydrated and mounted with DPX mounting media. Slides were analysed using a NIKON ECLIPSE-E800 microscope and images were captured with a Leica DFC420 camera using the LAS software (Leica).

Immunofluorescence staining

Protocol for rabbit primary antibodies

Slides were incubated in blocking buffer (1:60 NGS and 0.1% BSA in PBS) for 30 minutes at room temperature. Primary antibodies were applied overnight at 4 C and then washed three times with PBS for 5 minutes. Tissues sections were: i) incubated for 30 minutes with fluorescein-conjugated anti-rabbit secondary antibody diluted in blocking buffer or ii) incubated 30 minutes with biotinylated anti-rabbit antibody (Vector Lab, Cat. Number PK-6101) diluted in blocking buffer at room temperature, before incubation with Fluorescein Avidin DCS (Vector Lab, Cat. Number A-2011) diluted in PBS for 30 minutes at room temperature. Sections were washed three times with PBS for 5 minutes followed by DAPI staining (nuclear counterstain) for 10 minutes. Tissues sections were washed 3 times in PBS for 10 minutes, before being mounted using an anti-fade VectaShield mounting medium (Vector Lab; Cat. Number H-1000). Slides were analysed using a Leica DM5500B microscope and fluorescence images were captured with a DFC360FX camera using LASAF software (Leica).

Protocol for mouse primary antibodies

Immunofluorescence stainings using mouse antibodies on mouse tissues were performed using the Mouse on Mouse (M.O.M) basic kit (Vector Lab, Cat. Number BMK-2202) according to the manufacturer's instructions. After primary antibody incubation sections were washed 3 times in PBS for 5 minutes, before incubation with fluorescein-conjugated anti-mouse secondary antibody diluted in diluents buffer for 30 minutes at room temperature Sections were washed three times with PBS for 5 minutes followed

by DAPI staining (nuclear counterstain) for 10 minutes. Tissues sections were washed 3 times in PBS for 10 minutes, before being mounted using an anti-fade VectaShield mounting medium (Vector Lab, Cat. Number H-1000). Slides were analysed using a Leica DM5500B microscope and fluorescence images were captured with a DFC360FX camera using the LASAF software (Leica).

2.12.4 ImmunoFISH on tissues (γH2AX-TeloFISH)

Immunofluorescence staining was performed as described above. Following incubation with avidin–DCS (diluted to 1:500; Vector Lab) for 30 minutes, tissue sections were washed 3 times in PBS and fixed in 4% formaldehyde aqueous solution buffered for 20 minutes. Tissue sections were then dehydrated with 70%, 90%, 100% ethanol for 3 minutes each. Sections were air dried and then denatured for 10 minutes at 80°C in hybridisation buffer [70% deionised formamide, 25 mM $MgCl_2$, 1 M Tris pH 7.2, 5% blocking reagent (Roche,Welwyn, UK)] containing 4ng/µL Cy-3 labelled telomere specific (CCCTAA) peptide nuclei acid probe (Panagene, Cat. Number F1002-5), followed by hybridisation for 2 hours at room temperature in the dark. The slides were washed for 10 minutes with 70% formamide in 2xSSC (17.53g of NaCl and 8.82g of sodium citrate in 1L of H_2O, adjust pH to 7.0), following by a 10 minutes wash in 2xSSC and a final wash in PBS for 10 minutes. Nuclei were stained by DAPI for 10 minutes and washed 3 times in PBS for 5 minutes, before being mounted and imaged in a Leica DM5500B fluorescence microscope. In depth Z stacking was used (a minimum of 40 optical slices with 100× objective) followed by Huygens (SVI) deconvolution. Telomere-associated foci were analysed blinded by several analysts.

Table 2.4 Primary antibodies for Immunostainings on Mouse Tissues

Primary antibodies				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
γ-H2A.X(Ser139)	Human Mouse	Mouse monoclonal	1:2000	05-636 - Millipore
53BP1	Human Mouse	Rabbit polyclonal	1:100	4937 – Cell signalling
4-HNE	Human Mouse	Mouse polyclonal	1:50	MHN-020P – Cosmo Bio Co
MT-CO1	Human Mouse	Mouse monoclonal	1:500	ab45918 - Abcam

Table 2.5 Secondary antibodies for Immunostainings on Mouse Tissues

Secondary antibodies				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
Anti-mouse Fluorescein-conjugated secondary antibody AlexaFluor 594	Mouse	Goat	1:4000	A21044 - Invitrogen
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 488	Rabbit	Goat	1:4000	A21212 - Invitrogen
Anti-mouse IgG Biotinylated (M.O.M. basic kit)	Mouse	Goat	1:200	BMK-2202 – Vector Laboratories
Anti-rabbit IgG Biotinylated (VECTASTAIN Elite ABC Kit)	Rabbit	Goat	1:200	PK-6101 – Vector Laboratories
Fluorescein Avidin DCS			1:500	A-2011 - Vector Laboratories

2.13 Transmission electron microscopy (T.E.M.)

2.13.1 T.E.M. on cells

Cells were trypsinised and centrifuged for 5 minutes at 150 g. Supernatant was discarded and cells were washed twice with PBS, before fixation in 2% glutaraldehyde (Sigma, Cat. Number G5882) in 0.1M Phosphate buffer (Sigma) for 1 hour at 4^{o}C in a 15mL falcon. After fixation, the cell pellet was removed from the tube and placed in a glass vial. Fixation continued for 1 hour at room temperature, followed by washes with 0.1M Phosphate buffer for 2 hours (3 changes). Cell pellets were then post-fixed with 1% osmium tetroxide (Sigma, Cat. Number O5500) in the same buffer for 1 hour at room temperature, mixing periodically. The osmium tetroxide solution was removed and pellets were washed for 1 hour with 0.1M Phosphate buffer (2 changes). Cell pellets were then dehydrated in graded ethanol 70%, 90% for 15 minutes and then in 100% ethanol for 15 minutes (2 changes) and then exposed to propylene oxide (Sigma, Cat. Number 471968) for 10 minutes (2 changes), extra careful was taken in order for the pellet not to dry. Cell pellets were then embedded in 50% epoxy resin in propylene oxide for 1 hour. Fresh epoxy resin from the Agar 100 resin kit (Agar Scientific, Cat. Number R1031) was added and vials were placed in shaker overnight. The next day, the epoxy resin was substituted for fresh one and the cap of the vial was removed to allow any propylene oxide still present to evaporate. Embedment continued for 8 more hours, before placing cell pellets in BEEM® embedding capsules (Ted Pella, Cat. Number 130-SPC) containing fresh epoxy resin. Cell pellets were then incubated at 60^{o}C for 48 hours. Sections were cut, stained with uranyl acetate and lead citrate and examined using a transmission electron microscope in the Newcastle University EM facility. Randomly, 12 fields were selected and morphometrically analysed for mitochondria volume fraction, number and area.

2.13.2 T.E.M. on tissues

Mouse liver tissues were fixed in 3% glutaraldehyde in 0.1M Phosphate buffer overnight at 4^{o}C. Tissues were washes with 0.2M Phosphate buffer (Sigma) for 1 hour (2 changes), before being post-fixed with 1% osmium tetroxide in 0.1M Phosphate buffer for 1 hour at room temperature, in a shaker. The osmium tetroxide solution was removed and tissues were washed for 1 hour with 0.2M Phosphate buffer (4 changes) in a shaker. Tissues samples were then dehydrated in graded ethanol of 50%, 70%, 90% for 15 minutes in each solution and then in 100% ethanol for 30 minutes (2 changes) and then exposed to propylene oxide for 30 minutes (2 changes), extra careful was taken in order for the tissues not to dry. Tissues were then embedded in 50%, 75% and 100% epoxy resin in propylene oxide for 30 minutes in each solution in a shaker. for 8 more hours, Tissue samples were placed in BEEM® embedding capsules containing fresh epoxy resin from the Agar 100 resin kit (Agar Scientific, Cat. Number R1031) and embedment continued overnight at 37^{o}C (during this period tissues are also allowed to sink to the bottom of the capsule). Tissues were then incubated at 60^{o}C for 48 hours. Sections were cut, stained with uranyl acetate and lead citrate and examined using a transmission electron microscope in the Newcastle University EM facility. Randomly, 30 fields were selected and morphometrically analysed for mitochondria volume fraction, number and area.

2.14 Senescence Associated-β galactosidase (Sen-β-gal) staining

After washing with PBS, cells in coverslips were fixed with 2% formaldehyde in PBS for 10 minutes. Cell were then stained in the Sen-β-Gal solution (150mM NaCl, 2mM MgCl2, 40mM Citric Acid, 12mM NaPO3, 400 µg/mL X-gal, 2.1mg/mL Potassium hexacyanoferrat(II)trihydrate and 1.65mg/mL Potassium hexacyanoferrat(III) trihydrate), pH 6.0 (human cells) or pH 5.5 (mouse cells or

tissues) for 24 hours at 37^{o}C. Coverslips were washed two times in PBS and then mounted on VectaShield mounting media with DAPI (Vector Lab, Cat. Number H1200). Cells showing Sen-β-Gal staining (dark blue staining) and total number of cells (evaluated through DAPI staining) were counted in 10 randomly chosen fields (20x objective) per experiment using a Leica DM5500B microscope and images were captured with a Leica DFC420 camera using LASAF software (Leica).

Mouse tissues were fixed for 24 hours in 10% buffered neutral formalin and washed 3 times for 10 minutes in a shaker before incubation in the Sen-β-Gal solution. Tissues were washed for 10 minutes 3 times and then photographed using a conventional camera.

2.15 SASP analysis

2.15.1 Antibody Array

A Quantibody Human Cytokine Array for 20 cytokines (RayBiotech, Cat. Number QAH-CYT-1) was performed. Conditional media was collected prior to irradiation (time 0) and 3 and 10 days after irradiation (20Gy X-ray) following 24h serum deprivation. Conditional media was then sent to RayBiotech for analysis using the Quantibody Human Cytokine Array. Limit of detection for this assay was 10 $\rho g/ml^{-1}$.

2.15.2 ELISAs

Concentrations of IL-6 and IL-8 in cell culture media were determined using a sandwich ELISA system (R&D Systems; DY206/DY208) according to the manufacturer's instructions. Conditional media was collected prior to irradiation

(0Gy) and 10 days after irradiation (20Gy) following 24h serum deprivation. Limit of detection for this assay was 10 $\rho g/ml^{-1}$.

2.16 Protein expression analysis

2.16.1 Protein Extraction

Protein Extraction from cells

Cells were washed with ice cold PBS before being lysed with ice cold RIPA buffer (150 mM NaCl, 1% NP40, 0.5% NaDoC, 0.1% SDS, 50 mM Tris pH 7.4 and 1x phosphatase and protease inhibitors cocktail (Thermo Scientific; Cat. Number 78442) and scrapped using a rubber policemen. Samples were collected into 1.5 mL microcentrifuge tubes and immediately stored at -80^{o}C (alternatively samples could be immediately used for protein quantification and further analysis).

Protein Extraction from mouse tissues

Liquid nitrogen frozen tissues were powdered and kept on dry ice before adding 300µL of RIPA buffer to 20 mg of tissue powder (remaining tissue powder was stored at -80^{o}C). Samples were then homogeneised and further lysed by vigorous vortexing. At this stage samples were either stored at -80^{o}C or used immediately for protein quantification and further analysis.

2.16.2 Protein quantification

Cell lysates were defrosted on ice before being centrifuged for 10 minutes at 16100g at 4^{o}C. Protein quantification was performed using a colorimetric Bio-Rad DC Protein Assay (Bio-Rad; Reagent A Cat. Number 500-0113, Reagent B Cat. Number 500-0114, Reagent S Cat. Number 500-0115) according to the manufacturer's instructions. Protein Absorbance was measured on the Fluostar Omega plate reader (BMG Labtech) Considering protein absorbance, protein concentration of each sample was calculated and normalised by mixing adjusted volumes of protein lysate and loading buffer [950 µL of 2xLaemmli buffer (Bio-Rad; Cat. Number 161-0737) plus 50 µL of mercaptoethanol (Sigma; Cat. Number M6250). Protein denaturation was achieved by incubating samples at 100°C for 5 minutes. Samples were immediately placed on ice after denaturation. At this stage samples were either stored at -80^{o}C or used immediately for western blotting.

2.16.3 Western blotting

Electrophoresis

Acrylamide gels were prepared as following:

1. A running gel was prepared according to the protein size of the target proteins being analysed (lower protein size higher the percentage of the acrylamide gel and vice versa) and poured into a cassette (Invitrogen; Cat. Number NC2015 or NC2010) (see Table 3.6 for gel preparation);
2. After the running gel has polymerised, a 5% acrylamide staking gel was prepared, poured into the cassette and allowed to polymerizse (see Table 3.6 for gel preparation).

Table 2.6 Acrylamide gels for Western Blotting analysis

Acrylamide gels				
1 Gel 10ml	**5%**	**10%**	**12%**	**15%**
Sterile H_20	6.8mL	5.1mL	4mL	3.3mL
30% Acrylamide (Severn Biotech; Cat. Number 20-2100-10)	1.7mL	2.6mL	3.3mL	4mL
1.5M Tris pH 8.8 (Sigma; Cat.Number T6066)	2.5mL	2.5mL	2.5mL	2.5mL
10% SDS (Sigma; Cat.NumberL4390)	100 µL	100 µL	100 µL	100 µL
10% Ammonium Persulphate (Sigma; Cat.Number 215589)	100 µL	100 µL	100 µL	100 µL
TEMED (Sigma; Cat.Number T9281)	8µL	4µL	4µL	4µL

Gels were placed in a XCell SureLock^{TM} Mini-Cell Electrophoresis System (Invitrogen) covered by Tris-Glycine running buffer (250µM Tris, 1.92mM Glycine and 0.1% SDS). Samples were loaded side by side into wells alongside with a Protein standard (Bio-Rad; Cat. Number 161-0374) and electrophoresis was performed at 120V, 35mA for 90 minutes.

Protein transfer to membrane

Proteins were transferred from the gel to a 0.45µm polyvinylidene difluoride **(**PVDF) membrane (Millipore; Cat. Number IPVH00010). Both the membrane and the gel were placed between transfer pads (VWR; Cat. Number 732-0594) soaked in transfer buffer (250µM Tris, 1.92mM Glycine). Transfer was performed using the Trans-Blot® SD Semi-Dry Transfer Cell (BioRad) at 20 volts for 1 hour. The membrane was then stained with Ponceaux solution (0.5% Ponceaux and 5 % Acetic Acid in H_2O) for detection of protein bands.

Antibody Blotting

The membrane was incubated for 1 hour in blocking buffer (5% Milk in 0.05% PBS-Tween) at room temperature in a shaker. The membrane was then incubated overnight at 4^oC while shaking gently with the required concentration of primary antibody diluted in blocking buffer (antibodies against phosphorylated proteins were diluted in 5% BSA in 0.05% PBS-Tween). Membrane was washed 3 times in sterile H_2O before incubation with the secondary antibody diluted in blocking buffer for 1 hour at room temperature while shaking gently. Membranes were washed 3 times with sterile H_2O followed by a 5 minutes wash in 0.05% PBS-Tween at room temperature while shaking gently, before the final wash in sterile H_2O (5 times).

Table 2.7 Primary antibodies for Western Blotting

Primary antibodies for Western Blotting				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
γ-H2A.X(Ser139)	Human Mouse	Rabbit polyclonal	1:1000	#9718 - Cell Signalling
p21	Human	Rabbit monoclonal	1:1000	#2947 - Cell signalling
PGC-1β	Human Mouse	Rabbit polyclonal	1:1000	ab61249 - Abcam
NDUFB8	Human Mouse	Mouse monoclonal	1:1000	ab110242 - Abcam
UQCRC2	Human Mouse	Mouse monoclonal	1:1000	ab14745 - Abcam
MT-CO1	Human Mouse	Rabbit monoclonal	1:250	ab14705 - Abcam
MT-CO2	Human	Mouse monoclonal	1:1000	ab110258 - Abcam
SDHA	Human Mouse	Mouse monoclonal	1:1000	ab14715 - Abcam
TOMM20	Human Mice	Mouse monoclonal	1:1000	ab56783- Abcam
VDAC1/Porin	Human Mouse	Mouse monoclonal	1:1000	ab14734 - Abcam
S6	Human Mouse	Rabbit monoclonal	1:1000	#2217- Cell signalling
S6(Ser235/236)	Human Mouse	Rabbit monoclonal	1:1000	#4858- Cell signalling
p70S6K	Human Mouse	Rabbit polyclonal	1:1000	#9202 - Cell signalling

p70S6K(Thr389)	Human Mouse	Rabbit polyclonal	1:1000	#9205 - Cell signalling
TSC2	Human Mouse	Rabbit monoclonal	1:1000	#4308 - Cell signalling
Akt	Human Mouse	Rabbit polyclonal	1:1000	#9272 - Cell signalling
p-Akt(S473)	Human Mouse	Rabbit polyclonal	1:1000	#9271 - Cell signalling
β-tubulin	Human Mouse	Rabbit polyclonal	1:2000	#2146 - Cell signalling
α-tubulin	Human Mouse	Mouse monoclonal	1:2000	T9026 Sigma Aldrich
GAPDH	Human Mouse	Rabbit monoclonal	1:5000	#5174 - Cell signalling
FLAG	-	Mouse	1:1000	F316**-** Sigma Aldrich
FLAG	-	Rabbit	1:1000	F7425**-**Sigma Aldrich
Atg5	Human Mouse	Rabbit polyclonal	1:1000	A0856-Sigma

Table 2.8 Secondary antibodies for Western Blotting

Secondary antibodies for Western Blotting				
Protein	**Species**	**Host**	**Dilution**	**Reference/Manufacturer**
Goat anti-rabbit IgG -HRP conjugated	Rabbit	Goat	1:5000	A0545 - Sigma-Aldrich
Goat anti-mouse IgG -HRP conjugated	Mouse	Goat	1:5000	A2554 - Sigma-Aldrich

Chemiluminescence and evaluation

The blot was incubated with the chemiluminescence agent Clarity^{TM} Western ECL substrate (Bio-Rad; Cat. Number 170-5060) for 5 minutes. The blot was visualised using Fuji film Intelligent Dark box II and Image Reader Las-1000 Software. The protein of interest was confirmed by size comparison of the protein bands to the Protein standard loaded during electrophoresis. ImageJ analysis software was used to quantify the intensity of signal on the blot. Intensity quantification of the protein of interest of calculated after Background subtraction and normalisation to a loading control (GAPDH or β-tubulin).

2.17 Gene expression analysis

2.17.1 RNA extraction

Cells were trypsinised and centrifuged at 150g for 5 minutes at 4^{o}C. Cells were washed in PBS and centrifuged at 150g for 5 minutes at 4^{o}C. The supernatant was discarded and cell pellets were used for RNA extraction using the RNeasy Mini Kit (Qiagen, Cat. Number 74106) following the instructions described on the RNeasy® Mini Handbook – Qiagen. Assessment of RNA quality and quantification was performed using the Nanodrop® 1000 spectrophotometer (Thermo Scientific).

2.17.2 Reverse trancriptase reaction (RT-PCR)

Total RNA was reverse transcribed into cDNA using the Omniscript RT Kit (Qiagen, Cat. Number 205110) as described on the Omniscript® Reverse Transcription Handbook – Qiagen.

2.17.3 Real-time PCR for gene expression analysis

The real-time PCR was conducted using the Power Syber® Green PRC Master Mix (Invitrogen, Cat. Number 4367659). The real-time PCR reaction mix included: 4µL of cDNA (200-800ng) plus 6µL of PCR master mix (5µL of Power Syber® Green PRC Master Mix, 0.2µL of 10 µM Primers and 0.8µL H_2O), performing a 10µL final reaction volume. Each sample was run in triplicate in a C1000^{TM} Thermal Cycler, CFX96^{TM} Real-Time System (Bio-Rad) and Bio-Rad CXF Manager software.

Table 2.9 Primer sequences for cDNA real-time PCR

Primers sequences for cDNA real-time PCR			
Gene	**Species**	**Sequence**	
PGC-1β	Human	Forward	5’ AGTCAACGGCCTTGTGTTAAG
		Reverse	5’ ACAACTTCGGCTCTGAGACTG
PGC-1α	Human	Forward	5’ TGAGAGGGCCAAGCAAAG
		Reverse	5’ ATAAATCACACGGCGCTCTT
IL-6	Human	Forward	5’CAGGAGCCCAGCTATGAACT
		Reverse	5’GAAGGCAGCAGGCAACAC
IL-8	Human	Forward	5’GAGTGGACCACACTGCGCCA
		Reverse	5’TCCACAACCCTCTGCACCCAGT
COX5A	Human	Forward	5’CAAAGTGTAAACCGCATGGAT
		Reverse	5’TCCAGGTAACTGTTCACACTCAA
NDUFS8	Human	Forward	5’GTGCAGGACCTCCTGGTG
		Reverse	5’TCTCGGGATCCTGCATGT
ATP5G1	Human	Forward	5’ TGCAGGGTAGTAGGAGTGCAG
		Reverse	5’ TTAGACCCCTGGTACAACAGC
GAPDH	Human	Forward	5’AAATCCCATCACCATCTTCC
		Reverse	5’ GACTCCACGACGTACTCAGC
CXCL1	Mouse	Forward	5’ GACTCCAGCCACACTCCAAC
		Reverse	5’ TGACAGCGCAGCTCATTG
IL-6	Mouse	Forward	5’ CTACCAAACTGGATATAATCAGGA
		Reverse	5’ CCAGGTAGCTATGGTACTCCAGAA
p16	Mouse	Forward	5’ TTGCCCATCATCATCACCT
		Reverse	5’GGGTTTTCTTGGTGAAGTTCG
β-actin	Mouse	Forward	5’ TAAGGCCAACCGTGAAAAG
		Reverse	5’ ACCAGAGGCATACAGGGACA

Thermo cycler real-time PCR conditions were the following:

1. $95^{o}C \rightarrow$ 10 minutes
2. $95^{o}C \rightarrow$ 15 seconds
3. $60^{o}C \rightarrow$ 60 seconds
4. Repeat 39X steps 2 and 3.

Primer specificity was confirmed using a dissociation step with calculation of a melting curve. Relative quantification of mRNA expression was determined by relative comparison to the levels of an internal control mRNA, usually a housekeeping gene (e.g. GAPDH and β-actin). To calculate mRNA expression we used the ΔΔC(t) method.

2.18 Mitochondrial DNA (mtDNA) copy number analysis

2.18.1 DNA extraction

Cells were trypsinised and centrifuged at 150g for 5 minutes at $4^{o}C$. Cells were washed in PBS and centrifuged at 150g for 5 minutes at $4^{o}C$. The supernatant was discarded and cell pellets were used for DNA extraction using the DNeasy® Blood and Tissie Kit (Qiagen, Cat. Number 69504) following the instructions described on the DNeasy® Blood and Tissie Kit Handbook – Qiagen. Assessment of DNA quality and quantification was performed using the Nanodrop® 1000 spectrophotometer (Thermo Scientific).

2.18.2 Real-time PCR for mtDNA copy number analysis

The real-time PCR was performed as described above (3.17.3). Relative expression were calculated using the ΔΔC(t) method.

Table 2.10 Primers for mtDNA real-time PCR

Primers sequences mtDNA real-time PCR			
Gene	**Species**	**Sequence**	
B2M	human	Forward	5’ CCAGCAGAGAATGGAAAGTCAA
		Reverse	5’ TCTCTCTCCATTCTTCAGTAAGTCAACT
ND1	human	Forward	5’ CCCTAAAACCCGCCACATCT
		Reverse	5’ GAGCGATGGTGAGAGCTAAGGT
ND1	Mouse	Forward	5’ ACACTTATTACAACCCAAGAACACAT
		Reverse	5’ TCATATTATGGCTATGGGTCAGG
ND5	Mouse	Forward	5’ CCACGCATTCTTCAAAGCTA
		Reverse	5’ TCGGATGTCTTGTTCGTCTG

2.19 Comet Assay

The comet assay was conducted under alkalin conditions according to Singh et al. (Singh et al. 1988). For each sample, 100 randomly captured comets from slides (50 cells on each of 2 comet slides) were examined at ×400 magnification using an epifluorescence microscope connected through a black and white camera to an image analysis system (Comet Assay II; Perceptive Instruments

Ltd., Haverhill, Suffolk, United Kingdom). Images acquired by the computerised image analysis system were used to compute the integrated intensity profiles for each cell, estimate the comet cell components, and evaluate the range of derived parameters. To quantify DNA damage, the tail moment (TM) was calculated as the product of the tail length and the fraction of DNA in the comet tail. A single reader, who was blind to the status of subjects, scored all slides.

2.20 Statistical analyses

We conducted One-Way ANOVA, two-tailed t test, linear and non-linear regression analysis and Gehan-Breslow test using Sigma Plot vs11.0. Wilcoxon-Mann-Whitney tests were conducted using IBM SPSS Statistics 19.

2.21 Ethics statement

All work complied with the guiding principles for the care and use of laboratory animals. The project was approved by the Faculty of Medical Sciences Ethical Review Committee, Newcastle University. Project license number 60/3864.

Chapter 3. Mitochondria are essential for the establishment and maintenance of senescence

Previous studies have associated mitochondrial dysfunction and concomitant ROS production with cellular senescence (Passos et al. 2007, Passos et al. 2010). While mitochondrial ROS have been causally implicated in the stabilisation of the permanent cell growth arrest, the role of mitochondria in cellular senescence is still largely unknown: How do mitochondria change following senescence stimuli? Do mitochondria themselves integrate senescence-inducing stimulus? Are mitochondria required to the establishment and maintenance of cellular senescence?

The work performed in this chapter aimed to understand mitochondria dynamics in cellular senescence. Firstly, we kinetically analysed mitochondrial mass and ROS production following senescence stimuli. Secondly, we eliminated mitochondria from cells and assessed for its impact on the establishment and maintenance of the senescent phenotype.

3.1. Mitochondrial content increases during senescence

Persistent DNA lesions are a major trigger of cellular senescence by continuously signalling and activation of cyclin-dependent kinase inhibitors (responsible for the cycle arrest) through response pathways, together known as DNA damage response (DDR). In order to understand how mitochondria change during senescence, we kinetically analysed mitochondrial mass following activation of a DDR by genotoxic stress (generated by X-ray irradiation, Etoposide, Neocarcinostatin (NCS) and H_2O_2) or telomere dysfunction

(TRF2$^{\Delta B \Delta M}$) in a variety of cells lines. In collaboration with Rhys Anderson and Francisco Marques in our lab, we observed that regardless of the DNA damaging agent, activation of a DDR invariably results in mitochondrial mass increase 2-4 days following genotoxic stress (Figure 3.1A). Protein expression analysis shows that kinetically mitochondrial proteins expression increase and reach a maximum between 2 and 3 days following a DDR (Figure 3.1B)**.** These changes occurred in parallel with ROS generation and basal oxygen consumption rates (OCR). Nevertheless, when ROS (or basal OCR) are normalised per unit of mitochondrial mass no changes are observed upon induction of senescence, indicating that ROS increase during senescence is a result of mitochondrial mass increase (Figure 3.1C). The OCR analysis was performed in collaboration with Satomi Miwa in the von Zglinicki lab. Previous results in the lab have shown that addition of the free radical scavenger N-tert.-butyl-alpha-phenylnitrone (PBN) and the antioxidant N-acetyl cysteine (NAC) significantly reduce ROS per cell but do not affect mitochondrial mass. Together, these observations suggest that changes in mitochondrial mass are not an adaptive process driven by ROS, but potentially the drivers of ROS generation in senescence**.** To further confirm that mitochondrial content increases downstream of a DDR we performed morphometric analysis by transmission electron microscopy (T.E.M) on MRC5 fibroblasts and found that mitochondrial volume fraction and numbers increased upon irradiation (Figure 3.1D). T.E.M analysis on MRC5 fibroblasts was performed by Michelle Charles in our lab.

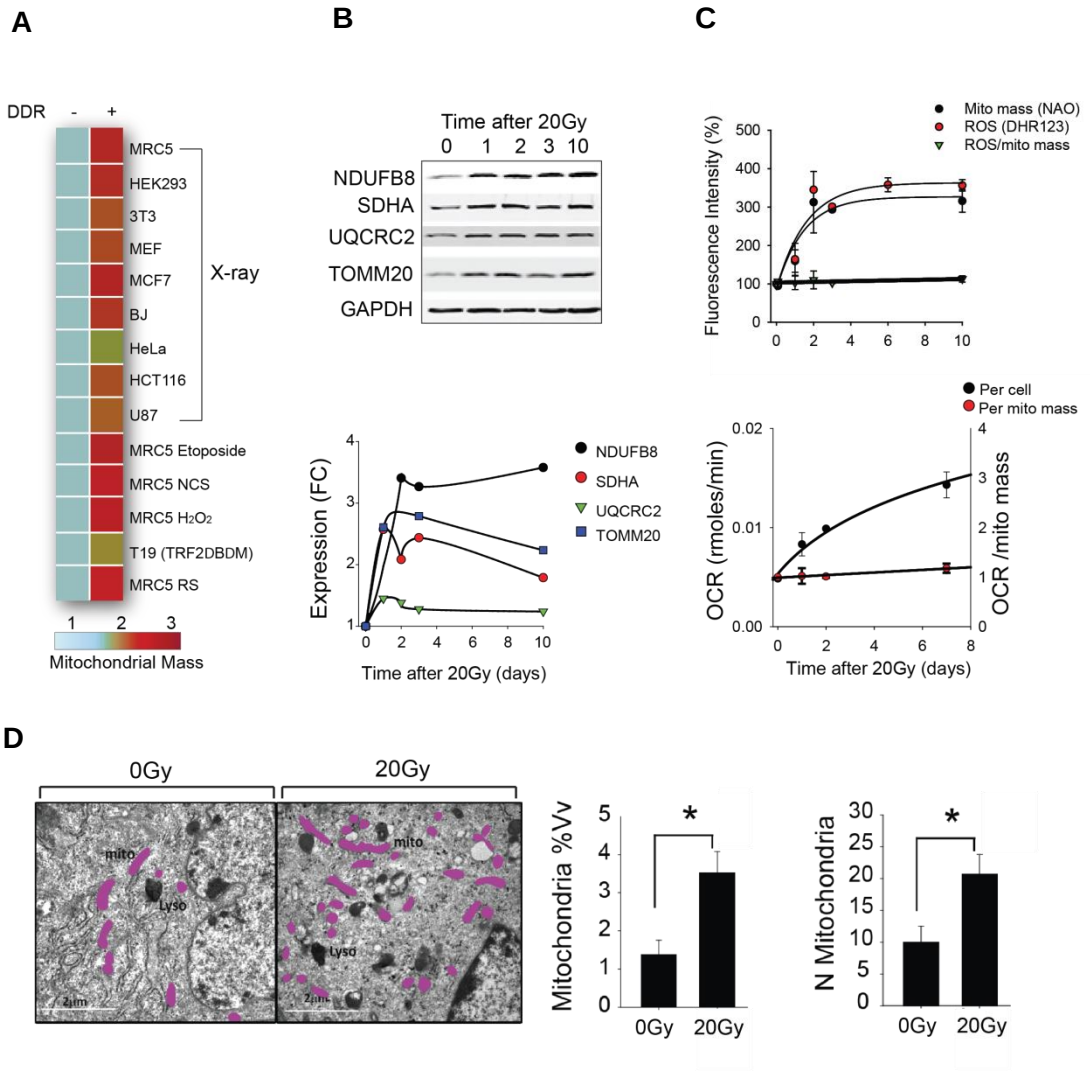


Figure 3.1 | Mitochondrial content increases downstream of a DDR. (A) Mitochondrial mass increases 2-4 days following genotoxic stress (generated by X-ray irradiation, Etoposide, Neocarcinostatin (NCS), H_2O_2 or telomere dysfunction ($TRF2^{\Delta B\Delta M}$) in a variety of cell lines (data are from 3 independent experiments per cell line or treatment); **(B)** (top) Representative western blots showing expression of mitochondrial proteins until 10 days after 20Gy (data are representative of 3 independent experiments; (bottom) Expression of mitochondrial proteins (NDUFB8, SDHA, UQCRC2, TOMM20) following activation of a DDR using 20Gy in MRC5 fibroblasts (data are representative of 3 independent experiments); **(C)** (top) Kinetic analysis of mitochondrial mass (NAO fluorescence); ROS (DHR fluorescence) and ROS per unit of mitochondria after activation of a DDR in MRC5 fibroblasts using

20Gy. Data were obtained by flow cytometry and are mean±S.E.M n=3-4 independent experiments; (bottom) Oxygen Consumption Rate (OCR) in ρmoles/min per cell was conducted in parallel with mitochondrial mass measurements using NAO by flow cytometry. Data are mean±S.E.M, n=3 independent experiments; **(D)** (left) Representative electron micrographs of human young proliferating MRC5 fibroblasts (0Gy) and 3 days after 20Gy (senescent), mitochondria are labelled in pink; (right) Quantification of mitochondrial volume fraction $\%V_v$ and mitochondrial number per cross section (T.E.M mitochondrial analyses are mean±S.E.M of 24 electron micrographs per condition); Scale bar = 2μm Asterisk denotes statistical significant $P<0.05$ using two-tailed t-test.

High doses of X-ray irradiation have been reported to completely abolish cell proliferation and induce both Sen-β-Gal activity (Passos et al. 2010) and the SASP (Rodier et al. 2009). In order to test the dependency of mitochondrial mass on the DDR, we treated MRC5 human fibroblasts with increasing doses of X-ray irradiation. Work done, in collaboration with Rhys Anderson in our lab, has shown that mitochondrial mass increases proportionally to the irradiation dose (Figure 3.2A) and directly correlates with DNA damage foci, induction of Sen-β-Gal and activation of the cyclin-dependent kinase inhibitor p21 and inversely correlates with the proliferation marker Ki67 (Figure 3.2B).

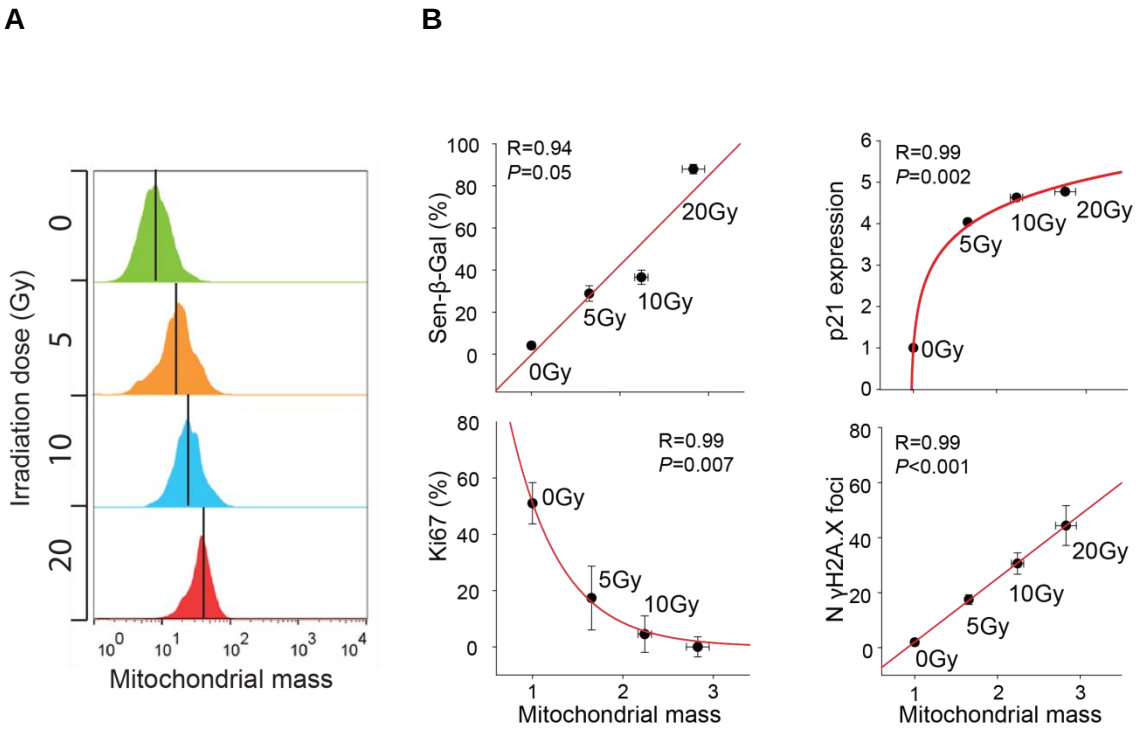


Figure 3.2 | DDR-dependent mitochondrial mass increase correlates with senescence markers. (A) Mitochondrial mass increases proportionally to irradiation dose (representative histograms of NAO fluorescence measured by flow cytometry); **(B)** Graphs showing correlation between mitochondrial mass, Senescence-associated β-Galactosidase (Sen-β-Gal), Ki67, p21 expression (measured by western blot) and number of γH2A.X foci. Data are mean±S.E.M of n=3 independent experiments.

The peroxisome proliferator-activated receptor co-activator 1 (PGC-1) family includes PGC-1α, PGC-1β and the PGC-1-related co-activator (PRC) (Puigserver et al. 1998, Andersson et al. 2001, Lin et al. 2002), well known co-activators in the regulation of metabolic pathways. These proteins are strong activators of mitochondrial function including regulation of mitochondrial biogenesis and oxidative metabolism in a variety of tissues (Handschin et al. 2006). We questioned if PGC-1 co-activators were increased downstream of a DDR activating mitochondrial biogenesis in senescence. In collaboration with Michelle Charles in our group, we found that an increase in the mRNA levels of PGC-1α and PGC-1β in irradiation-induced senescent fibroblasts (Figure 3.3), corroborating the observed increase in mitochondrial mass following a DDR.

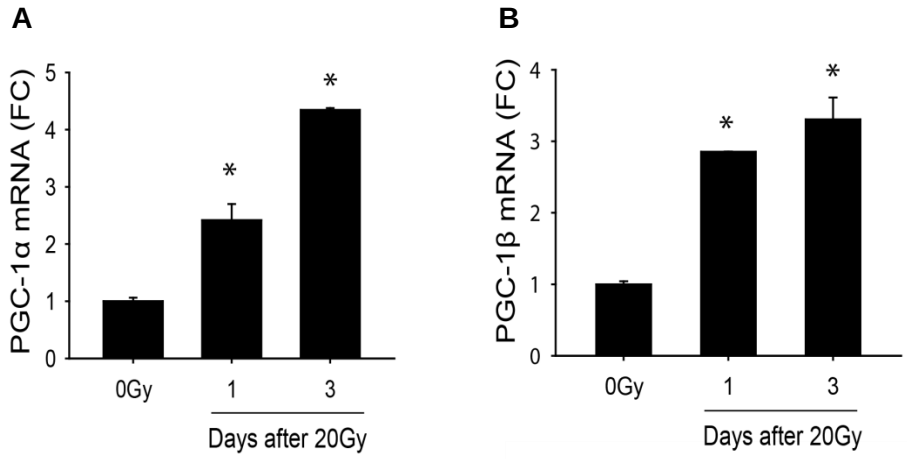


Figure 3.3 | Mitochondrial regulators PGC-1α and PGC-1β expression increases following a DDR. (A) mRNA expression of PGC-1α and **(B)** PGC-1β after 20Gy X-radiation in MRC5 human fibroblasts; Expression was normalised to mRNA levels of GAPDH. Data are mean±S.E.M of n=3 independent experiments. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

3.2. Mitochondria are essential for the establishment and maintenance of cellular senescence

To definitely determine the importance of mitochondria as potential effectors in senescence, we sought to generate mitochondria-deficient cells. Parkin is an E3-ubiquitin ligase encoded by the *PARK2* gene, that mediates proteasomal degradation of proteins (Shimura et al. 2000). When mitochondrial membrane potential is lost in mammalian cells, Parkin translocates to mitochondria and induces removal of these organelles by autophagy, in a process known as mitophagy. Parkin translocation from the cytosol to mitochondria is dependent on the PTEN-induced putative kinase 1 (PINK1) (Narendra et al. 2010). The exact mechanism through which Parkin interacts with PINK1 to clear dysfunctional mitochondria from cells is still not determined. However, it has been reported that Parkin mediates polyubiquitination of a subset of mitochondrial substrates, which may be part of the mechanism through which it triggers mitophagy (Chen et al. 2010, Gegg et al. 2010, Geisler et al. 2010, Wang et al. 2011). Moreover, Parkin has been shown to translocate and contribute to degradation of depolarised mitochondria following treatment with the mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (Narendra et al. 2008). In order to test the role of mitochondria in senescence, we generated human MRC5 fibroblasts stably transfected with YFP-Parkin. We induced senescence by X-ray irradiation (20Gy) in controls and YFP-expressing Parkin cells, treated them at day 2 with CCCP for 48h and analysed them at different time points after irradiation (Figure 3.4).

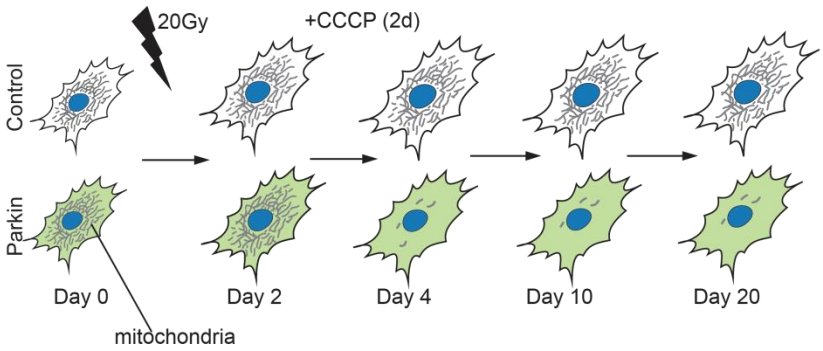


Figure 3.4 | Parkin-mediated clearance of mitochondria in irradiation-induced senescent fibroblasts. Scheme illustrating the experimental design: Parkin-expressing and control MRC5 fibroblasts were irradiated with 20Gy and 2 days later were treated with CCCP/DMSO for 48h. Cells were harvested for senescence marker analysis 4, 10 and 20 days after irradiation.

3.2.1. Depletion of mitochondria severely abrogates the senescent phenotype in human fibroblasts

Elimination of mitochondria post-induction of senescence led to a remarkable rejuvenation of human fibroblasts. These mitochondrial-deficient cells failed to develop multiple senescent traits such as increased cell size, Sen-β-Gal activity and formation of heterochromatin foci (Figure 3.5).

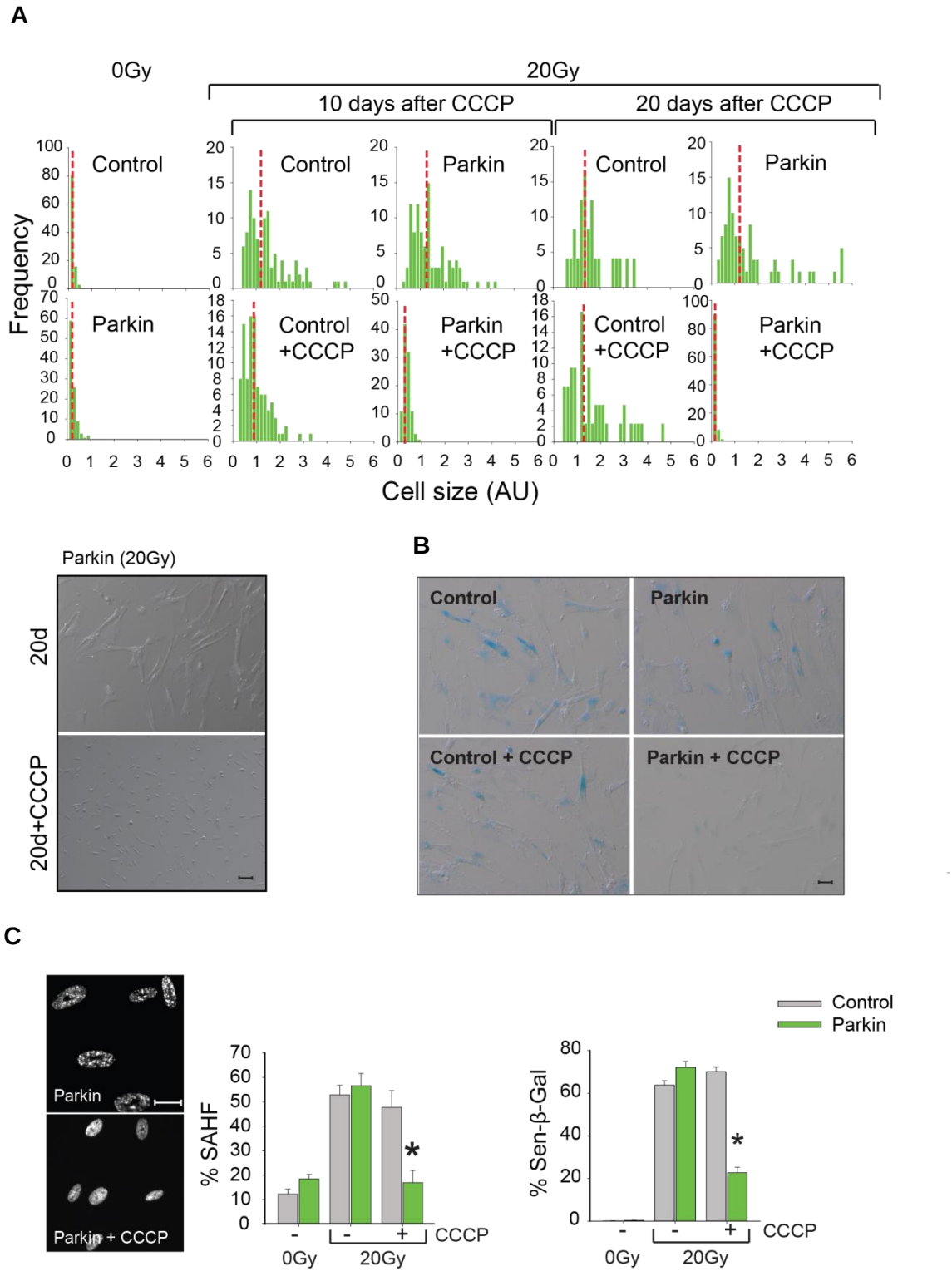


Figure 3.5 | Mitochondrial clearance induces rejuvenation of human senescent fibroblast. (A) (top) Histograms illustrating cell size distributions in control and Parkin-expressing cells 10 and 20 days after X-ray irradiation with or without CCCP pre-

treatment. Dashed red line represents median cell size. At least 100 cells were quantified per condition; (bottom) Representative images of Parkin-expressing cells 20 days after X-ray irradiation with or without CCCP pre-treatment; Scale bar = 50μm **(B)** (top) Representative images of Senescence associated β-Galactosidase activity (Sen-β-Gal) 10 days after 20Gy in control and Parkin-expressing fibroblasts with or without CCCP treatment; Scale bar = 50μm; (bottom) Quantification of Sen-β-Gal in senescent control and Parkin-expressing fibroblasts with or without CCCP treatment. Data are mean±S.E.M of 3 independent experiments; **(C)** (left) Representative images of heterochromatin foci observed by DAPI (grey); Scale bar = 20μm; (right) Quantification of Senescent-associated heterochromatin foci (SAHF) in senescent control and Parkin expressing fibroblasts with or without CCCP treatment. Data are mean±S.E.M of 3 independent experiments. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

The increased ROS and pro-inflammatory mediating factors secretion by senescent cells are traits of the senescent phenotype responsible for the detrimental effects of these cells on the surrounding tissues (Nelson et al. 2012, Acosta et al. 2013). Next, we aimed to understand how mitochondria can modulate these two features of senescence.

Mitochondria depletion blocks ROS generation in senescence

Mitochondria are the main source of intracellular ROS and have been correlated with several markers of senescence as shown previously in this chapter. Parkin-mediated mitochondrial depletion by treatment with CCCP for 48h in human fibroblasts led to a dose- dependent decrease in mitochondrial mass, as well as ROS generation (Figure 3.6A). When using a concentration of 12.5μM of CCCP we could not detect any mitochondrial proteins from OXPHOS complexes I, II, III and IV by western blotting 10 days after irradiation (Figure 3.6B).

Immunostaining against the mitochondrial protein SDHA further revealed total absence of mitochondria in CCCP treated Parkin-expressing cells (Figure 3.6C).

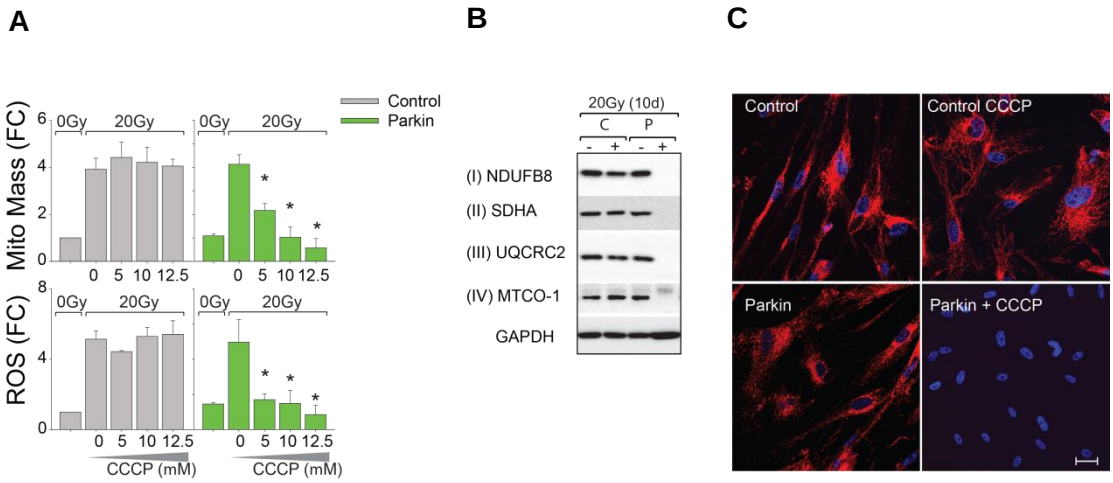


Figure 3.6 | Parkin-mediated mitochondrial clearance reduces ROS levels in human senescent fibroblasts. (A) Effect of 2 day treatment with increasing doses of CCCP on Mitochondrial mass (measured by NAO fluorescence) and ROS (measured by DHE fluorescence) 4 days after irradiation with 20Gy of Parkin-expressing and control MRC5 fibroblasts. Data are mean±S.E.M of 3 independent experiments; **(B)** Representative westerns blots confirming absence of proteins from different mitochondrial complexes: NDUFB8 (complex I), SDHA (complex II), UQCRC2 (complex III) and MT-CO1 (complex IV) 10 days after irradiation and 6 days after 12.5μM CCCP treatment (data are representative of 2 independent experiments); **(C)** Representative image of SDHA fluorescence of Parkin-expressing MRC5 fibroblasts with or without CCCP treatment (data are representative of 2 independent experiments); Scale bar = 20μm. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

Mitochondria are essential for the development of the senescence-associated pro-inflammatory phenotype

The SASP, particularly the pro-inflammatory phenotype, has been associated with the deleterious effects of senescent cells on the surrounding tissue (Acosta et al. 2013). Elimination of mitochondria in Parkin-expressing human fibroblasts with CCCP treatment decreased mRNA expression and severely abrogated secretion of the SASP components IL-6 and IL-8 (Figure 3.7). ELISAs were performed in collaboration with Jodie Birch in our lab. These findings place mitochondria as a putative therapeutic target on strategies aiming to reduce the senescence-associated pro-inflammatory phenotype.

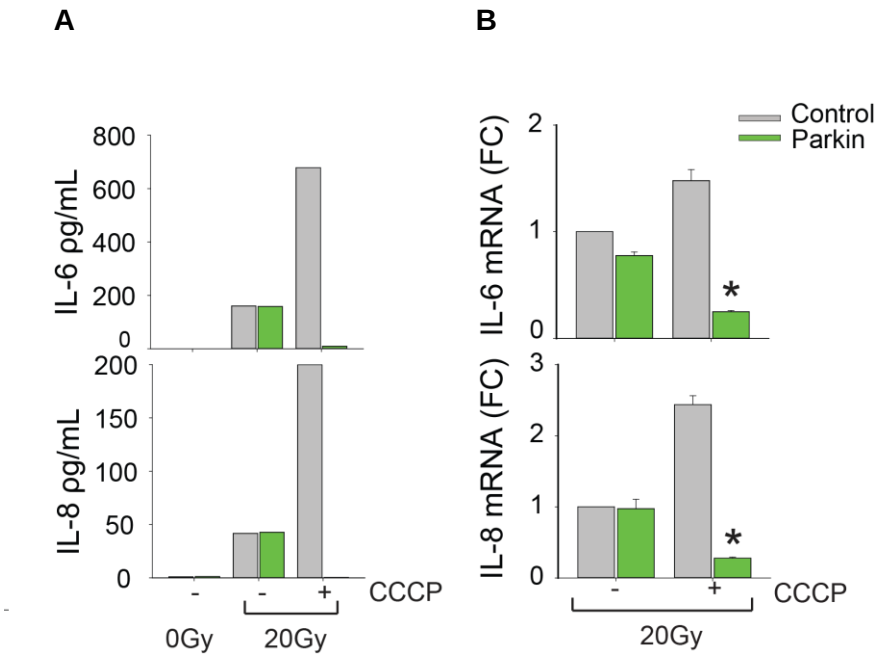


Figure 3.7 | Depletion of mitochondria abrogates the senescence-associated inflammatory phenotype. (A) Levels of secreted IL-6 and IL-8 proteins (Data are representative of 2 independent experiments); **(B)** Expression of IL-6 and IL-8 mRNA in senescent control and Parkin-expressing fibroblasts with or without CCCP treatment (Data are mean±S.E.M of 3 independent experiments). Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

3.2.2. Absence of mitochondria in senescence rescues cell proliferation

Despite the cells' inability to rely on oxidative phosphorylation, a considerable percentage of them were able to resume proliferation as demonstrated by increased population doublings and expression of the proliferation marker Ki67 (Figure 3.8A and 3.8B). In fact, we were able to culture human fibroblasts without any signs of cell death and no detectable mitochondrial proteins for at least a month (not shown). We also observed that depletion of mitochondria results in decreased expression of the cyclin-kinase inhibitors p21 and p16 in MRC5 fibroblasts induced to senescence with 20Gy X-radiation (Figure 3.8C).

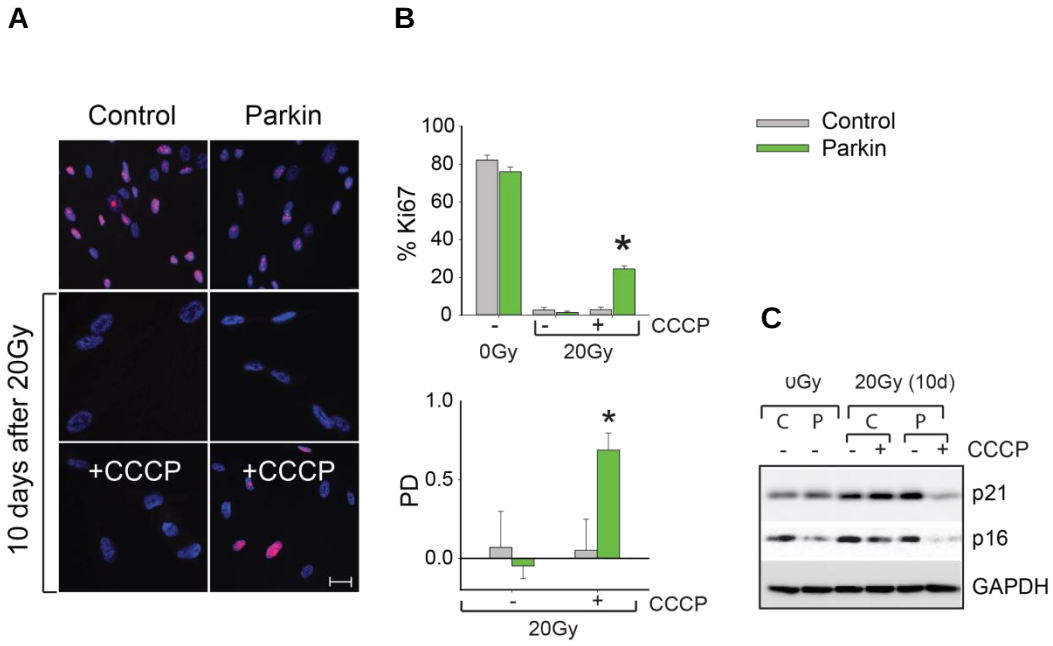


Figure 3.8 | Clearance of mitochondria rescues cell proliferation in irradiation-induced senescent human fibroblast. (A) Representative images of Ki67 (red) with DAPI (blue) as a nuclear counterstain in senescent control and Parkin-expressing fibroblasts with or without CCCP treatment; Scale bar = 20µm; (**B)** (top) Quantification

of Ki67 in senescent control and Parkin expressing fibroblasts with or without CCCP treatment, (bottom) Population doublings in senescent control and Parkin-expressing fibroblasts with or without CCCP treatment. Data are mean±S.E.M of 3 independent experiments; (**C)** Representative western blot showing p21 and p16 expression in senescent control and Parkin-expressing fibroblasts with or without CCCP treatment (data are representative of n=2 independent experiments). Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

3.2.3. Mitochondria depletion ameliorates the senescent phenotype in replicative senescence

In order to test the role of mitochondria in cells where senescence had already been established, we cultured Parkin-expressing human fibroblasts until replicative senescence (evidenced by around 70% Sen-β-Gal positive cells and less than 0.5 Population doublings for at least 4 weeks). Replicative YFP-Parkin senescent MRC5 fibroblasts were treated with 12.5 μM CCCP for 48 hours (figure 3.9A) resulting in significantly reduced mitochondrial mass and ROS generation and absence of mitochondrial proteins (Figure 3.9B and 3.9C). Moreover, markers of senescence such as Sen-β-Gal activity, increased cell size and expression of p16 and p21 were significantly reduced (Figure 3.9C and 3.9D). Contrary to X-ray induced senescence, we did not observe increased proliferation following mitochondrial depletion (data not shown).

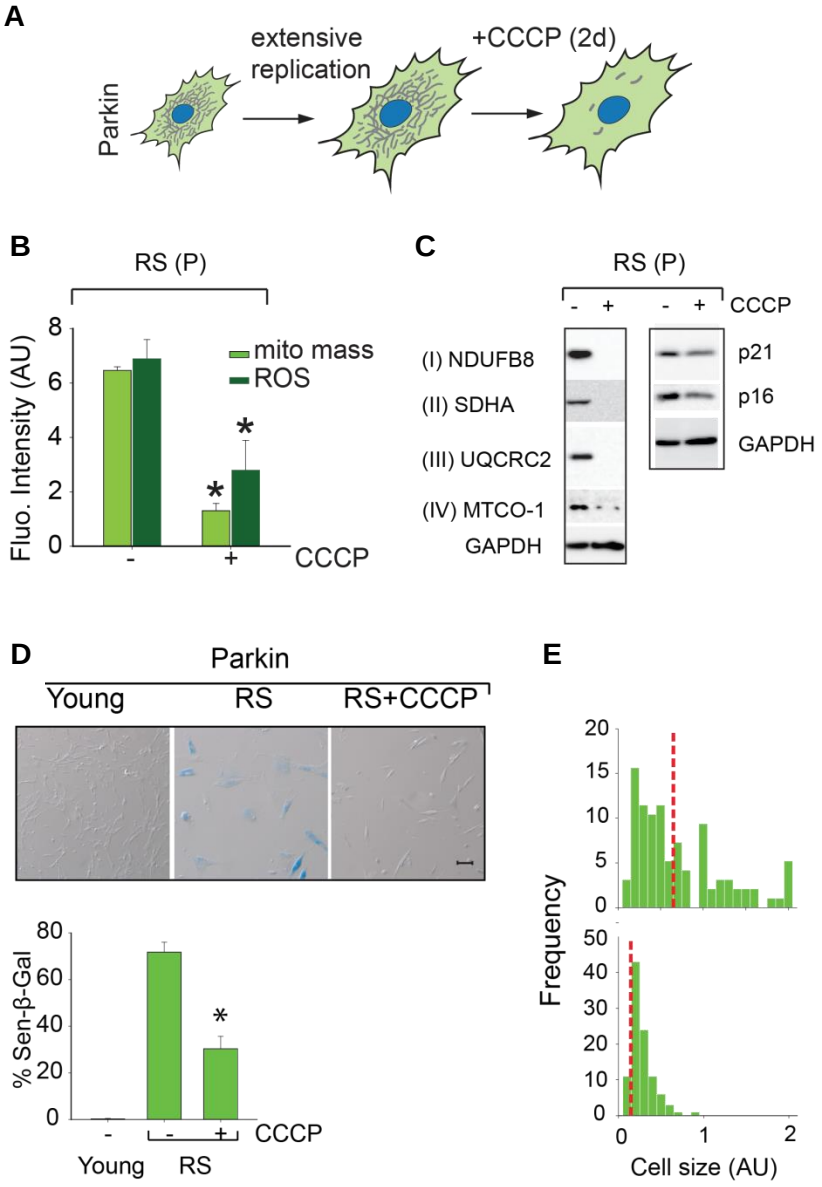


Figure 3.9 | Mitochondria elimination ameliorates the senescent phenotype in replicative senescent human fibroblasts. (A) Scheme illustrating experimental design: Parkin-expressing MRC5 fibroblasts were cultured until replicative senescence and then treated with CCCP for 48h; **(B)** Mitochondrial mass (NAO fluorescence) and ROS (DHE fluorescence) with and without CCCP treatment in replicative senescent (RS) Parkin-expressing MRC5 fibroblasts. Data are mean±S.D. of n=2 independent experiments; **(C)** Western blots displaying expression of mitochondrial proteins from the different mitochondrial electron transport chain complexes, p21 and p16 with and

without CCCP pre-treatment in replicative senescent (RS) Parkin-expressing MRC5 fibroblasts (data from n=1 experiment; **(D)** (top) Representative images of Sen-β-Gal and (bottom) quantification of Sen-β-Gal in replicatively senescent (RS) Parkin-expressing cells with or without CCCP pre-treatment; Scale bar = 50μm; **(E)** Histograms illustrating cell size distributions in replicatively senescent Parkin-expressing cells 10 days after treatment with or without CCCP pre-treatment. Dashed red line represents median size. At least 100 cells were quantified per condition. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

3.3. Depletion of mitochondria severely compromises the senescent phenotype in mouse fibroblasts

To further extend the significance of our observations on human fibroblasts to other mammalian cells, we used 3T3 mouse fibroblasts expressing Parkin induced to senesce with 10Gy X-radiation. Similarly to MRC5 human fibroblasts, Parkin-expressing mouse fibroblasts (3T3) pre-treated with CCCP had reduced mitochondrial mass (Figure 3.10A) and virtually no detectable mitochondrial proteins (Figure 3.10B).The western blots showing mitochondrial proteins expression on Parkin-expressing 3T3 fibroblasts following CCCP treatment were a kind gift of Dr Stephen Tait (Institute of Cancer Sciences, Glasgow University, UK). Following X-ray irradiation-induced senescence these cells showed no ROS induction, as well as no mRNA expression of p16 and the SASP factors IL-6 and CXCL1 (Figure 3.10C-E). These results confirm the hypothesis that mitochondria are essential for development of senescence not only in human cells but also in other mammalian cells.

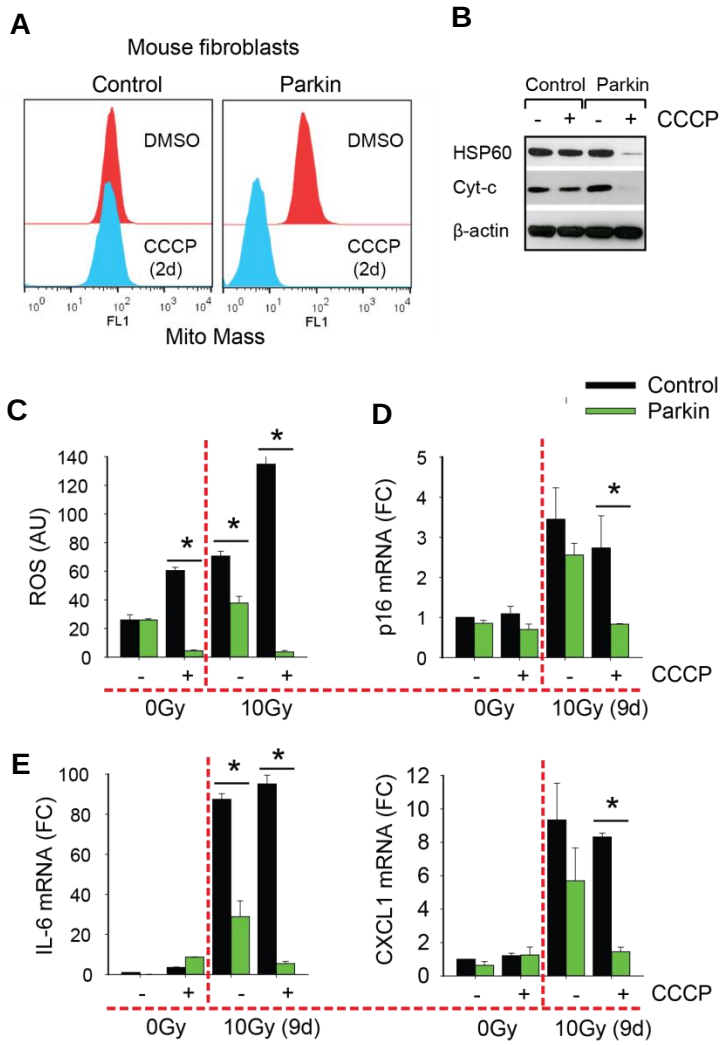


Figure 3.10 | Mitochondria clearance abrogates the senescent phenotype in senescent mouse fibroblasts. (A) Representative flow cytometry histogram of mitochondrial mass (NAO staining) in control and Parkin-expressing 3T3 mouse fibroblasts with or without treatment with the uncoupler CCCP; **(B)** Representative western blots showing expression of mitochondrial proteins CYTC and HSP60 in control and Parkin-expressing 3T3 mouse fibroblasts with or without 2 day treatment with CCCP (2 independent experiments); **(C)** Quantification of ROS levels (DHE staining) in 3T3 controls and Parkin-expressing mouse fibroblasts 4 days following 10Gy irradiation and CCCP; **(D)** and **(E)** p16, IL-6 and CXCL1 mRNA abundance in 3T3 controls and Parkin-expressing 3T3 mouse fibroblasts 10 days following 10Gy irradiation and 6 days after CCCP pre-treatment. Data are mean±S.E.M of n=3 independent experiments. Asterisk denotes statistical significant *P*<0.05 One-way ANOVA.

3.4. Mitochondrial DNA depleted cells have diminished expression of senescence markers

Cells depleted of mitochondrial DNA (mtDNA), or Rho 0 (ρ0) cells, lack critical electron transport chain components (mitochondrial encoded) but still have few petit mitochondria. Rho 0 cells by presenting significantly reduced mitochondrial mass have largely compromised oxidative phosphorylation and rely solely on glycolysis for survival and replication (Chandel et al. 1999). In order to understand if low mitochondrial mass content would still impact on senescence, we irradiated Rho 0 cells and analysed for senescence markers. When compared to parental cells, ROS as well as DNA damage foci only increased marginally in Rho 0 cells 3 days after 10Gy Irradiation (Figure 3.11A). Furthermore, mRNA levels of the SASP factor IL-6 was significantly reduced in Rho 0 cells upon irradiation (Figure 3.11B). Together, these results confirm that reduction of mitochondrial content (not only complete elimination of mitochondria) impacts on senescent markers.

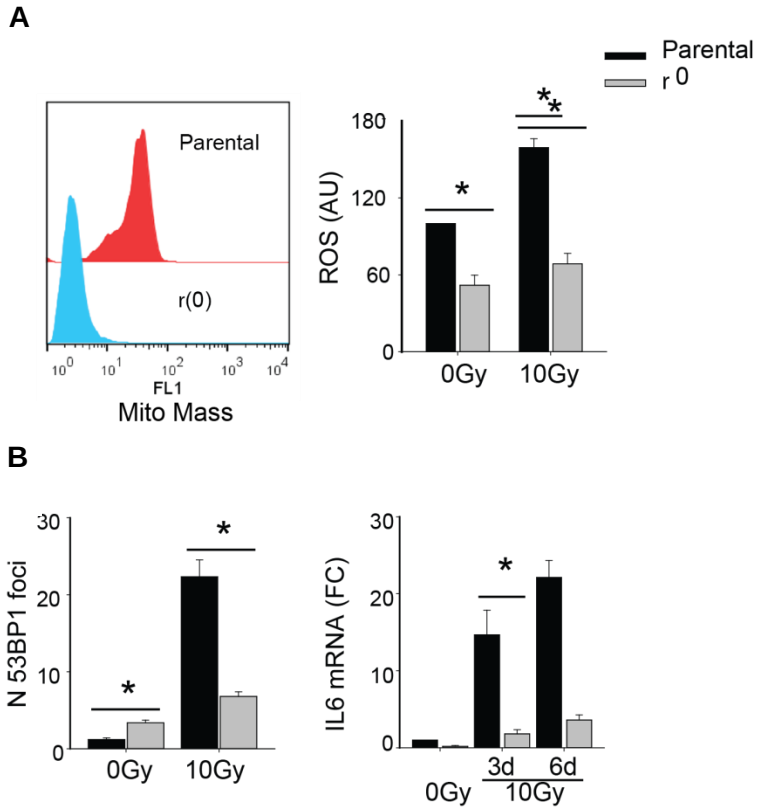


Figure 3.11 | Depletion of mtDNA alleviates the senescent phenotype in human cells. (A) (left) Representative flow cytometry histogram of mitochondrial mass staining (NAO) in parental and Rho 0 143B osteosarcoma cells and (right) quantification of ROS generation in parental and Rho 0 cells 3 days following 10Gy irradiation; **(B)** (left) Quantification of 53BP1 foci in parental and Rho 0 cells 3 days after 10Gy irradiation. Data are mean±S.E.M of n=3 independent experiments. (right) mRNA abundance of the SASP factor IL-6 in parental and Rho 0 cells following 10Gy irradiation. Asterisk denotes statistical significant *P*<0.05 One-way ANOVA.

3.5. Discussion

Despite numerous studies on the impact of mitochondria and the ageing process none have really tested directly the necessity of mitochondria for the induction of senescence and development of the senescent phenotype. Several studies have shown that cellular senescence is characterised by mitochondrial dysfunction contributing to metabolic inefficiency and elevated ROS (Allen et al. 1999, Hutter et al. 2002, Zwerschke et al. 2003, Hutter et al. 2004, Passos et al. 2007). Increased ROS levels have been associated with replicative, stress- and oncogene-induced senescence (Saretzki et al. 2003, Ramsey et al. 2006, Passos et al. 2007, Lu et al. 2008). Furthermore, activation of the DDR, a major effector of senescence, by genotoxic stress or telomere uncapping (Passos et al. 2010), over-expression of activated RAS (Lee et al. 1999), $BRAF^{V600E}$ (Kaplon et al. 2013), p53 (Macip et al. 2003), p21 (Macip et al. 2002) and p16 (Takahashi et al. 2006) all resulted in elevated ROS generation. In most of the above reported cases treatment with antioxidants, such as N-acetyl cysteine, was able to prevent the cell-cycle arrest supporting a causal role for ROS in the process. Nevertheless, non-mitochondrial sources of ROS have also been implicated in cellular senescence. Takahashi and colleagues, using human fibroblasts, connected p16 with ROS production via protein kinase Cδ signalling (Takahashi et al. 2006). Protein kinase Cδ has been shown to activate a non-mitochondrial source of ROS, generated by NADPH-oxidase through phosphorylation of $p47^{phox}$, an essential component of NADPH oxidase (Talior et al. 2005). Consistent with this study, NADPH oxidases have been shown to limit the replicative lifespan of human endothelial cells in culture via ROS generation (Lener et al. 2009). These observations have placed ROS as signalling molecules during cellular senescence (Passos et al. 2006). While ROS have been shown to stabilise cellular senescence, the necessity of mitochondria in cellular senescence remains to be determined. Here we show that mitochondria are required for senescence to occur and for the development of the senescence phenotype.

First, we showed that there is an increase in mitochondrial mass following DDR stimuli and that it correlates with markers of senescence such as increased Sen-β-Gal activity, number of DDF and expression of the cyclin-dependent kinase inhibitor p21 and absence of proliferation ability, further confirming previous reports in our lab (Passos et al. 2007, Passos et al. 2010). We have also shown that changes in mitochondrial mass are not an adaptive process driven by ROS, but potentially the drivers of ROS generation in senescence as demonstrated by: i) unaltered ROS levels following normalisation per unit of mitochondria and ii) treatment with the free radical scavenger PBN and the antioxidant NAC, where ROS were reduced but mitochondrial mass remained unchanged. However, we are aware of the limitations of these dyes in the assessment of mitochondrial ROS and mass. DHE measures cellular superoxide levels, but it is not mitochondrial specific (Peshavariya et al. 2007). Other dyes like MitoSOX, which detects superoxide directly in the mitochondrial matrix could have been employed (Mukhopadhyay et al. 2007). However, most dyes used to measure ROS are not highly specific and a combination of several dyes may be a better approach.

Our data shows that mitochondrial depletion results in decreased ROS levels and SASP factor secretion, both of which have been implicated in the stabilisation and reinforcement of senescence via autocrine effects (Acosta et al. 2008, Passos et al. 2010). We cannot yet exclude that clearance of mitochondria rescues senescence via its effect on ROS generation. Our group has previously shown that elevated ROS levels in senescent cells as a result of signalling through p21 feed back into DNA damage induction and further activate the DDR, generating a stable, self-sustaining feedback loop. (Passos et al. 2010). In addition, ROS scavengers or antioxidants treatments have been shown to rescue the senescent phenotype and in some cases facilitate re-entry into the cell cycle (Lee et al. 1999, Macip et al. 2002, Macip et al. 2003, Passos et al. 2010). In order to discriminate if ROS is a mediator of the mitochondrial-dependent cell cycle arrest, cells lacking mitochondria should be treated with ROS and assessed for senescence markers. Despite the fact that mitochondrial ROS generation increase during senescence could still explain the

mitochondrial impact on the induction and maintenance of senescence, it does not explain how mitochondrial mass increase in the first place. Another possible explanation why mitochondria impact in the induction and maintenance of senescence may be related to its effect on the SASP, particularly on the inflammatory phenotype. Mitochondria have been described to play a key role as mediators of inflammation with ROS playing a major role in host defence mechanisms (Tschopp 2011). Elevated ROS levels have been shown to activate redox-sensitive transcription factors, such as the nuclear factor-κB (NF-κB) resulting in increased expression of pro-inflammatory factors (Gloire et al. 2006). However, ROS-independent mechanisms have also causally implicated mitochondria in the induction of inflammatory responses. Mitochondria have been identified as key sources of Damage-associated molecular pattern molecules (DAMPs) (Krysko et al. 2011). Mito-DAMPs play a role in DAMP-modulated inflammation in different disorders, such as systemic inflammatory response syndrome (SIRS), rheumatoid arthritis (RA), cirrhosis, cancer, and heart diseases as well as the aging process (Lopez-Armada et al. 2013). Mechanistically, Mito-DAMPs can activate Toll-like membrane receptors (TLRs) and cytoplasmic nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) resulting in the activation of the NF-κB pro-inflammatory signalling (Kawai et al. 2009, Krysko et al. 2011). Nevertheless, besides being able to induce an inflammatory response, mitochondria can also receive signals from inflammatory factors. Evidence has demonstrated that NF-κB is present in mitochondria of mammalian cells and is able to regulate expression of mitochondrial encoded genes (Cogswell et al. 2003). Moreover, the NF-κB alternative pathway during myogenesis has been shown to be important for mitochondrial biogenesis (Bakkar et al. 2008) and to regulate expression of the transcription co-activator PGC-1β (a master regulator of mitochondrial biogenesis and function) to promote oxidative muscle metabolism (Bakkar et al. 2012). These last observations could explain why mitochondrial mass increase during cellular senescence resulting in elevated ROS generation. Cellular energy levels are one other explanation, independent of ROS and SASP levels, for the impact of mitochondria in the induction of senescence. It has been

shown that the AMP:ATP ratio, a measure of cellular energy charge, increases when human fibroblasts reach replicative senescence (Wang et al. 2003, Zwerschke et al. 2003), and addition of exogenous AMP to the cell culture medium triggers premature senescence in young human fibroblasts (Zwerschke et al. 2003). Interestingly, it has been shown that energy failure (reduced levels of ATP) resulting from partial mitochondrial uncoupling may contribute to cellular senescence in conditions where reducing the rate of oxidative phosphorylation induces premature senescence without substantially increasing oxidative stress (Stockl et al. 2007). It might be that the reason why we observe a decrease in the induction of senescence using the Parkin mediated mitochondrial clearance system is due to loss of ATP and is not ROS dependent. Although, loss of ATP would impair cells' proliferating capacity and would not explain our observations showing that mitochondrial clearance rescues cell proliferation. We hypothesise that the increase proliferation in cells depleted of mitochondrial following induction of senescence may be due to an energetic replacement from increased glycolysis. We will need to test this hypothesis by i) assessing ATP levels in Parkin-expressing MRC5 fibroblasts treated with CCCP and ii) by adding ATP or ROS to the Parkin-expressing cells and determine if senescence can be restored.

We have also shown that artificial depletion of mitochondria abrogated the development of senescent traits such as increased cell size, Sen-β-Gal activity and formation of heterochromatin foci. Emerging studies have demonstrated that Krebs cycle intermediates can regulate the level of DNA and histone methylation and acetylation. The DNA demethylases and histone lysines include members of 2-oxoglutarate-dependent dioxygenases (2-OGDO). These enzymes are activated by oxygen, iron and the major Krebs cycle intermediate, 2-oxoglutarate, whereas they are inhibited by succinate and fumarate. (Salminen et al. 2014). Indeed, it is not surprising that mitochondria, an organelle with endosymbiotic origin, can regulate gene expression in the host cell by modifying the epigenetic landscape of chromatin. This way, changes in mitochondrial metabolism during ageing could promote the epigenetic

reprogramming underlying the appearance of the senescent phenotype and degenerative diseases.

Elimination of mitochondria rescued proliferation in fibroblasts following induction of senescence despite of their inability to rely on oxidative phosphorylation. A possible explanation relies on the fact that these cells were phenotypically similar to cancer cells presenting high nucleus/cytoplasm ratio and being able to grow in low density. Many cancer cells exhibit increased glycolysis rather than oxidative phosphorylation as the energy supplier. Majority of the pyruvate is converted to lactate, in contrast to entry into the mitochondria and conversion to acetyl CoA. (Warburg 1956, Moreno-Sanchez et al. 2009). These results suggest mitochondria are required for senescence to occur and their absence may contribute to a shift from an irreversible cell cycle arrest (senescence) to abnormal proliferation (cancer) in the presence of DNA damage. Supporting this hypothesis are reports describing a central role for mitochondria in cell proliferation and apoptosis (Antico Arciuch et al. 2012) in which levels of hydrogen peroxide are key regulators of the cell cycle fate: lower mitochondrial oxidative rates resulting in lower hydrogen peroxide release into the cytosol promotes cell proliferation; whereas, stress signals and traffic of pro- and antiapoptotic mitochondrial proteins in the intermembrane space, modulated by the redox condition determined by mitochondrial O_2 consumption and mitochondrial nitric oxide metabolism regulates apoptosis (Antico Arciuch et al. 2012). We have also shown that mitochondria are essential for the development of the senescent phenotype in fibroblasts that reached senescence through replicative exhaustion. Nevertheless, no rescue in cell proliferation was observed in replicative senescent fibroblast depleted of mitochondria, suggesting that mitochondria are important to restrain cells proliferative capacity in the very first states of the development of senescence as observed in irradiation-induced senescence (7-10 to develop a senescent phenotype) but not when senescence is fully established. Following these observations, the extent of mitochondrial elimination should be considered when placing mitochondria as a putative therapeutic target in senescence; the impact of mitochondrial content on the tumour-suppressive/promoting ability in a

cell is dependent on the "state" of cellular senescence (whilst developing or in the presence of a full developed senescent phenotype).

The impact on the senescent phenotype observed as a consequence of mitochondrial clearance in humans cells is extended to mouse cells and cells harbouring petit mitochondrial (unable to perform oxidative phosphorylation) resulting from mtDNA depletion, indicating that mitochondrial content impact on cellular senescence is a broader process existing (at least) in mammalian organisms.

Here we showed that the mitochondrial mass increase was accompanied by increased expression of the mitochondrial regulators PGC-1α and β in senescent cells. Experimental evidence supporting a role for these co-transcriptional factors regulating mitochondrial biogenesis in senescence was performed by Francisco Marques in our lab. *PGC-1β*$^{-/-}$ mouse embryonic fibroblasts (MEFs) presented lower mitochondrial mass, mtDNA copy number and mitochondrial protein expression than wild-type MEFs. Similarly to mitochondrial depleted human fibroblasts, mouse fibroblasts with compromised mitochondrial biogenesis (lower mitochondrial content) have also reduced expression senescence markers (DDF, p21, p16, ROS and SASP factors). Furthermore, when cultured at normal atmospheric (21% O_2) oxygen MEFs acquire a senescent phenotype after a small number of passages, while when grown at low oxygen (3%O_2) MEFs show negligible expression of senescent markers and divide at faster rates (Parrinello et al. 2003). We found that at 21% oxygen, absence of PGC-1β delayed senescence and at 3% O_2 *PGC-1β*$^{-/-}$ had higher proliferative rates when compared to wild-types. In contrast, overexpression of PGC-1β led to an increase in mitochondrial mass and loss of cell proliferation, increased Sen-β-Gal activity and frequencies of 53BP1 foci both in non-irradiated and irradiated fibroblasts. MEFs senescence has been associated with oxidative damage (Parrinello et al. 2003). One possibility to explain our observations is that PGC-1β mediated mitochondrial biogenesis increases oxidative stress, thereby activating a DDR and p21 and p16 mediated cell-cycle arrest.

Further to the autocrine role of ROS and the SASP in cellular senescence, these two features of the senescent phenotype also impact on surrounding tissues by induction of paracrine senescence (Nelson et al. 2012, Acosta et al. 2013) and stimulation of tumour progression when in a tumour context (Davalos et al. 2010, Costa et al. 2014). Our observations showing that decreased content (*PGC-1*$\beta^{-/-}$ MEFs) or depletion of mitochondria (Parkin-expressing cell) in cells decreases both ROS and the SASP are of great therapeutic interest, since manipulation of mitochondrial content would diminish/abrogated the deleterious effects of senescence cells in organism.

Chapter 4. mTORC1 integrates DDR signalling towards mitochondrial biogenesis during senescence

The mechanistic (previously the mammalian) target of rapamycin (mTOR) pathway is involved in central cellular functions including cell growth and has been widely implicated in the control of pathways regulating mitochondrial turnover such as biogenesis and mitophagy. mTOR complex 1 (mTORC1) has been shown to integrate inputs from growth factors, stress, energy status and amino-acid availability to regulate protein and lipid synthesis and autophagy all of which are involved in the complex pathways mediating mitochondrial homeostasis (Laplante et al. 2012). Regulation of cell growth (mass), proliferation and stress management by mTOR is tightly controlled and requires co-integration of this kinase in other major cellular pathways, including the DNA damage response to ensure that no damage is transmitted to other cells during proliferation (Reiling et al. 2006). DNA damaging stressors such as UV light, ROS (e.g. H_2O_2) and some carcinogens (eg. arsenide) have been reported to activate mTORC1. UV light stimulates S6K activity shortly after exposure (<7 hours), being this effect of UV light over mTORC1 activity sensitive to rapamycin (Brenneisen et al. 2000, Ding et al. 2002, Huang et al. 2002). Factors involved in DDR pathways such as p38MAPK, ERK1/2, JNK and PI3K may also affect S6K phosphorylation via mTORC1 activation (Zhang et al. 2001) downstream of exposure to UV light. ROS generation in response to UV exposure is one possible explanation, since pre-treatment with ROS scavengers or antioxidants prevents S6K activation (Ding et al. 2002, Huang et al. 2002). Arsenide, a human carcinogen, has also been found to promote S6K1 activation via increased H_2O_2 production (Jung et al. 2003). Given that mTOR is activated upon DNA damage and that it is a key regulator of mitochondrial homeostasis, we questioned whether mTOR was a mechanistic factor in the pathways that lead to mitochondrial mass increase following a persistent DDR in senescence.

4.1. mTOR drives mitochondrial mass increase during senescence

Following induction of a DDR using X-ray irradiation, we observed a progressive increase in phosphorylation of the mTORC1 target p70S6K starting at 6 hours in both human (Figure 4.1) and mouse fibroblasts (not shown).

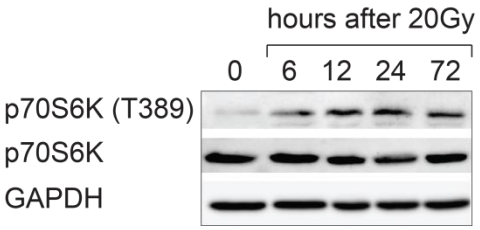
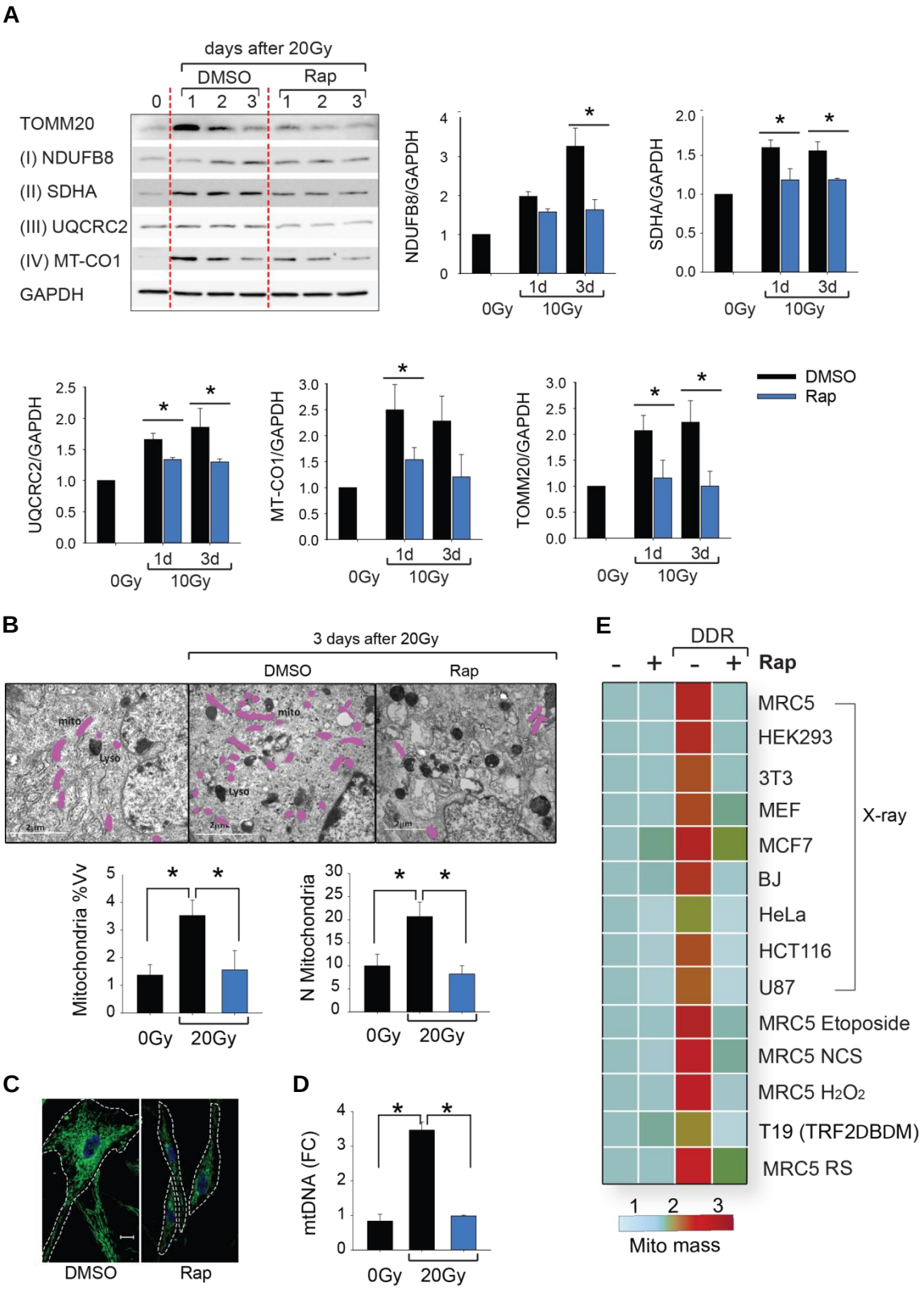


Figure 4.1 | mTOR activity increases following activation of a DDR. Representative western blot of mTORC1 activity measured by phosphorylated p70S6K (T389) from 6 to 72 hours after 20Gy (data are representative of 3 independent experiments).

In order to understand mechanistically how mitochondrial mass content increases in senescence we inhibited mTORC1 with rapamycin in irradiated MRC5 fibroblasts. Consistent with a role for mTORC1 in DDR-dependent mitochondrial protein expression, expression of mitochondrial proteins belonging to OXPHOS complexes I, II, III and IV (NDUFB8, SDHA, UQCRC2, and MT-CO1) and the mitochondrial import receptor subunit TOMM20 (Translocase of outer mitochondrial membrane 20) were significantly reduced by rapamycin treatment (Figure 4.2A). In collaboration with Michele Charles and Alina Merz in our lab, we further confirmed by morphometric analysis using T.E.M that mTOR inhibition by rapamycin decreases mitochondrial volume fraction and number in irradiated human fibroblasts (Figure 4.2B). Furthermore, Mitotracker Green fluorescence (marker of mitochondria) was also reduced in human (Figure 4.2C) and mouse (not shown) irradiated fibroblasts treated with

rapamycin. Rhys Anderson in our lab, have also shown that rapamycin treatment decreases mtDNA copy number in human (Figure 4.2D) and mouse fibroblasts (not shown) following induction of senescence by X-radiation. Similarly, in replicatively senescent cells we observed that rapamycin treatment greatly reduced mtDNA copy number and mass (not shown). In collaboration with Rhys Anderson and Francisco Marques in our lab, we aimed to test the robustness of our findings and screened mitochondrial mass as before (see Figure 3.1A) following induction of senescence with different known DDR activators in a variety of human and mouse cell types treated with rapamycin. In all cases, the increased mitochondrial mass could be partially rescued by rapamycin (Figure 4.1E). Together these results show that mTOR is an essential factor in the pathway(s) leading to mitochondrial mass increase in senescence.


A
days after 20Gy
DMSO
Rap
0 1 2 3 1 2 3
TOMM20
(I) NDUFB8
(II) SDHA
(III) UQCRC2
(IV) MT-CO1
GAPDH
NDUFB8/GAPDH
SDHA/GAPDH
UQCRC2/GAPDH
MT-CO1/GAPDH
TOMM20/GAPDH
0Gy 10Gy
1d 3d
DMSO
Rap
B
3 days after 20Gy
DMSO
Rap
mito
Lyso
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- + - +
Rap
MRC5
HEK293
3T3
MEF
MCF7
BJ
HeLa
HCT116
U87
X-ray
MRC5 Etoposide
MRC5 NCS
MRC5 H2O2
T19 (TRF2DBDM)
MRC5 RS
1 2 3
Mito mass

Figure 4.2 | mTOR inhibition by rapamycin rescues mitochondrial mass content in senescence. (A) (top left) Representative western blots of mitochondrial proteins TOMM20, NDUFB8 (complex I), SDHA (complex II), UQCR2 (complex III) and MT-CO1 (complex IV) following 20Gy irradiation with or without rapamycin treatment (data are representative of 3 independent experiments); (top right and bottom) Quantification of mitochondrial proteins 1 and 3 days after 20Gy in the presence or absence of rapamycin. Data are mean±S.E.M. of n=3 independent experiments; **(B)** (top) Representative electron micrographs of human young proliferating MRC5 fibroblasts (0Gy) and 3 days after 20Gy with or without rapamycin (Rap) treatment; mitochondria are labelled in pink; (bottom) Quantification of mitochondrial volume fraction $\%V_v$ and mitochondrial number per cross section (T.E.M mitochondrial analyses are mean±S.E.M of 24 electron micrographs per condition); Scale bar = 2µm **(C)** Representative images of human fibroblasts stained with Mitotracker green (mitochondrial marker) 3 days after 20Gy X-ray irradiation with and without rapamycin; Scale bar = 10µm; **(D)** mtDNA copy number analysis, by Real-Time PCR, of human MRC5 fibroblasts 3 days after 20Gy with or without rapamycin treatment. Data are mean±S.E.M of 3 independent experiments; **(E)** Effect of Rapamycin on mitochondrial mass (NAO fluorescence) 2-4 days following genotoxic stress (generated by X-ray irradiation, Etoposide, Neocarcinostatin (NCS), H_2O_2 or telomere dysfunction ($TRF2^{\Delta B \Delta M}$) in a variety of cell lines (data are from 3 independent experiments per cell line or treatment). Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

In collaboration with Berni Carroll in the Korolchuk lab and Francisco Marques in our lab, we have overexpressed a constitutively active mutated form of Rheb (N153T) (Urano et al. 2007). Rheb overexpression resulted in both increased mTORC1 activity (Figure 4.3A) and mitochondrial mass (Figure 4.3B and 4.3C)

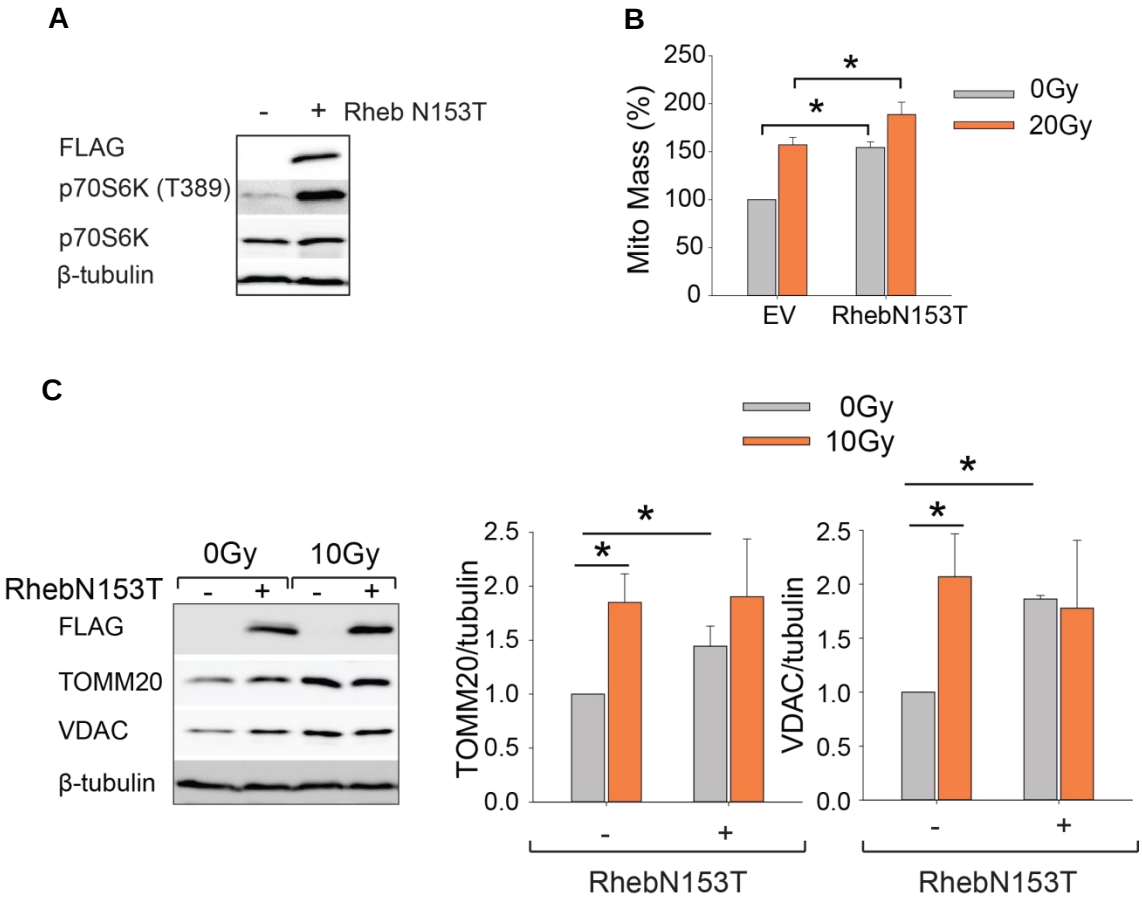

Figure 4.3 | mTOR hyperactivation stimulates mitochondrial mass increase. (A) Representative western blot showing the effect of activated Rheb N153T on phosphorylation of p70S6K; **(B)** Overexpression of activated Rheb (pcDNA3-flag-Rheb-N153T) increases mitochondrial mass measured by NAO fluorescence in HeLa cells. Data are mean±S.E.M of n=3 independent experiments; **(C)** (left) Representative western blot of the effect of activated Rheb on mitochondrial proteins TOMM20 and VDAC in MEFs following 10Gy irradiation, (right) Quantification of western blots showing expression of mitochondrial proteins TOMM20 and VDAC in controls and RhebN153T expressing mouse fibroblasts (2 days following 0 or 10Gy irradiation). Data are mean±S.E.M. of n=3 independent western blots. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

4.2. mTOR-dependent mitochondrial biogenesis in senescence

Experimentally, it is complex to dissect the intricate pathways by which mTOR regulates mitochondrial mass. Mitochondrial biogenesis is a multi-factorial process which involves the integration of tightly regulated transcriptional events, lipid membrane, protein biogenesis and assembly as well as replication of mtDNA (Zhu et al. 2013). Several studies indicate that mTORC1 can exert regulatory effects on mRNA expression of PGC-1α and β master regulators of mitochondrial biogenesis and function, by complex interactions with the transcription factors YY1 and NF-κB (Cunningham et al. 2007, Bakkar et al. 2012). In accordance with a transcriptional regulated process, we found that activation of a DDR resulted in a rapamycin sensitive increase in PGC-1α and β mRNA abundance (Figure 4.4A) as well as downstream OXPHOS genes ATP5G1, COX5A and NDUFS8 (Figure 4.4B).

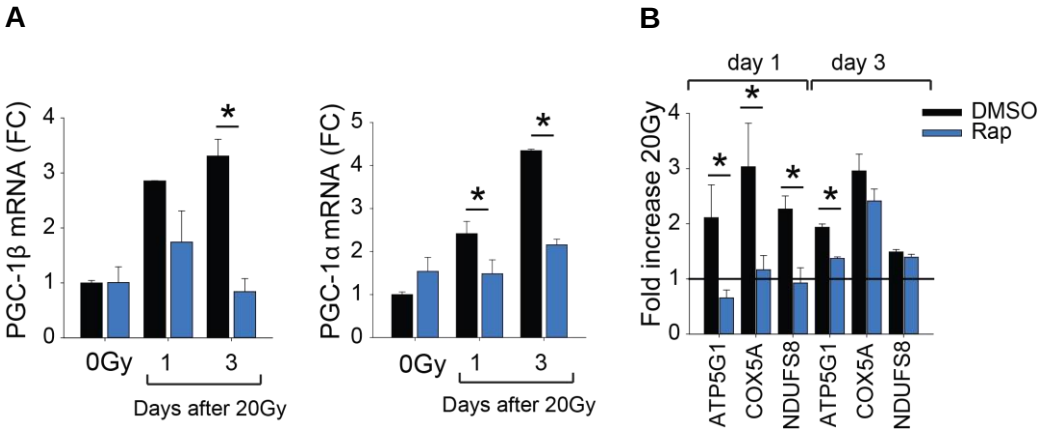

Figure 4.4 | mTOR-dependent mitochondrial biogenesis in cellular senscence. (A) mRNA abundance of PGC-1α (right) and PGC-1β (left) and **(B)** mitochondrial genes ATP5G1, COX5A and NDUFS8 after 20Gy with or without rapamycin treatment. Expression was normalised to mRNA levels of GAPDH. Data are mean±S.E.M of n=3 independent experiments. Asterisk denotes statistical significant *P*<0.05 One-way ANOVA.

Another mTORC1–dependent mitochondrial homeostatic regulatory mechanism is mitophagy, a selective type of autophagy, known to be negatively regulated by mTORC1 (Zhu et al. 2013). In order to investigate the impact of mitophagy following a DDR, we used autophagy-deficient MEFs from *ATG5*$^{-/-}$ mice (kind gift from Dr Viktor Korolchuk from the IAH, Newcastle University, UK). The autophagy-related protein 5 (Atg5) when in conjugation complex with Atg12 acts as an ubiquitin-protein ligase (E3)-like enzyme necessary for the formation of autophagic vesicles, hence *ATG5*$^{-/-}$ mice have impaired autophagy (Ohsumi 2001). Work done by Francisco Marques and Graeme Hewitt in our lab have shown that while *ATG5*$^{-/-}$ MEFs experienced increased mitochondrial mass, ROS and DDR foci when compared to wild-type, they were still responsive to the effects of rapamycin supplementation (Figure 4.5). This suggested that mTORC1 mediated effects on mitochondrial mass after a DDR are autophagy-independent.

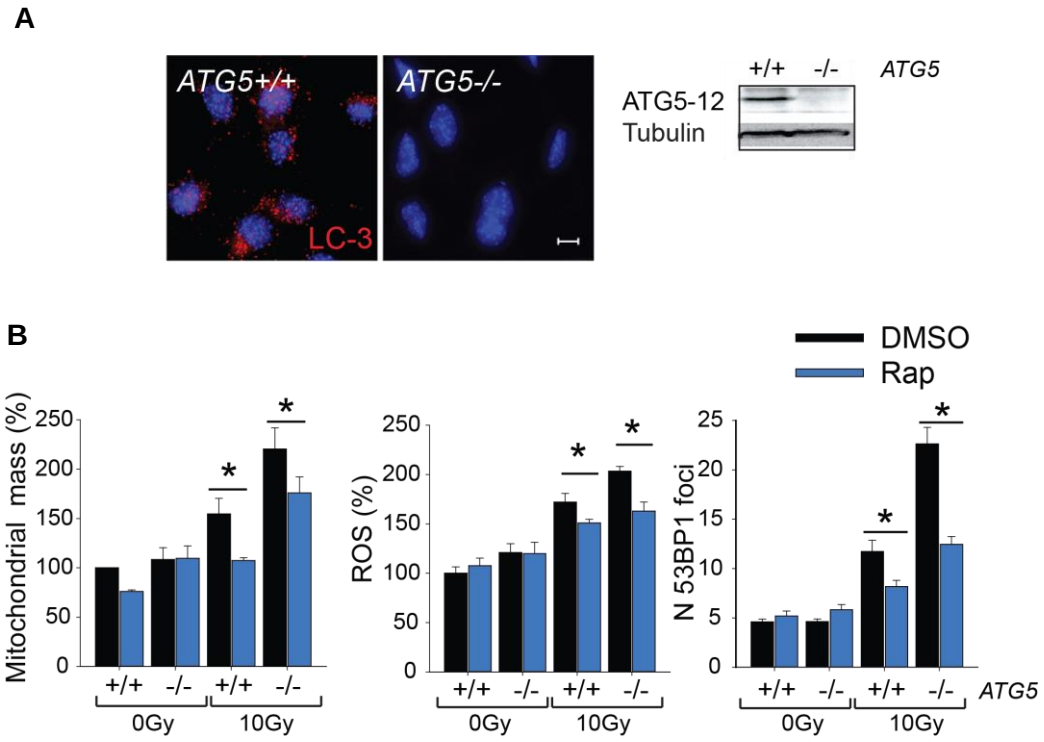

Figure 4.5 | Autophagy-independent mTOR mediated mitochondrial mass increase in senescent MEFs. (A) (left) Representative images of LC3 expression (red) in *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ MEFs; Scale bar = 10µm; (right) Representative western blot showing absence of expression of ATG5-12 in *ATG5*$^{-/-}$ MEFs; **(B)** Effect of 10Gy irradiation and rapamycin treatment on 53BP1 foci number, mitochondrial mass and ROS generation in *ATG5*$^{+/+}$ and *ATG5*$^{-/-}$ MEFs. Data are mean±S.E.M. of n=3-4 independent experiments. Asterisks denote statistical significant *P*<0.05 using one-way ANOVA.

Together the results suggest that the mTOR-dependent mitochondrial mass increase in senescence occurs as a result of mitochondrial biogenesis programme activation via PGC1-α and β rather than impaired mitophagy.

4.3. mTOR-inhibition alleviates the senescent phenotype without rescuing cell proliferation

Corroborating our previous results showing that mitochondrial clearance alleviates the senescent phenotype (Chapter 3), in collaboration with Rhys Anderson in our lab, we found that mTOR inhibition-dependent mitochondrial mass decrease by rapamycin was accompanied by reduced Sen-β-Gal activity in various models of stress-induced and replicative senescence (Figure 4.6A). Consistent with reports affirming a central role for a persistent DDR in the development of senescence and the emergence of the SASP (Rodier et al. 2009), we found that rapamycin suppressed the senescence-associated secretion of several pro-inflammatory cytokines and mRNA expression of IL-6 (Figure 4.6B) following induction of a DDR. However, despite decreasing Sen-β-Gal and the SASP, rapamycin treatment did not rescue cell-cycle arrest in senescent fibroblasts (work done in collaboration with Alina Merz in our lab), owing these results to the potent anti-proliferative effect of mTOR inhibition already shown in not irradiated fibroblasts (Figure 4.6C). Consistent with our previous data that mTORC1 acts on mitochondrial biogenesis via PGC-1β, data from Francisco Marques in our lab shows that rapamycin decreases Sen-β-Gal in wild-type MEFs but has no effect in PGC-1$\beta^{-/-}$ MEFs (not shown).

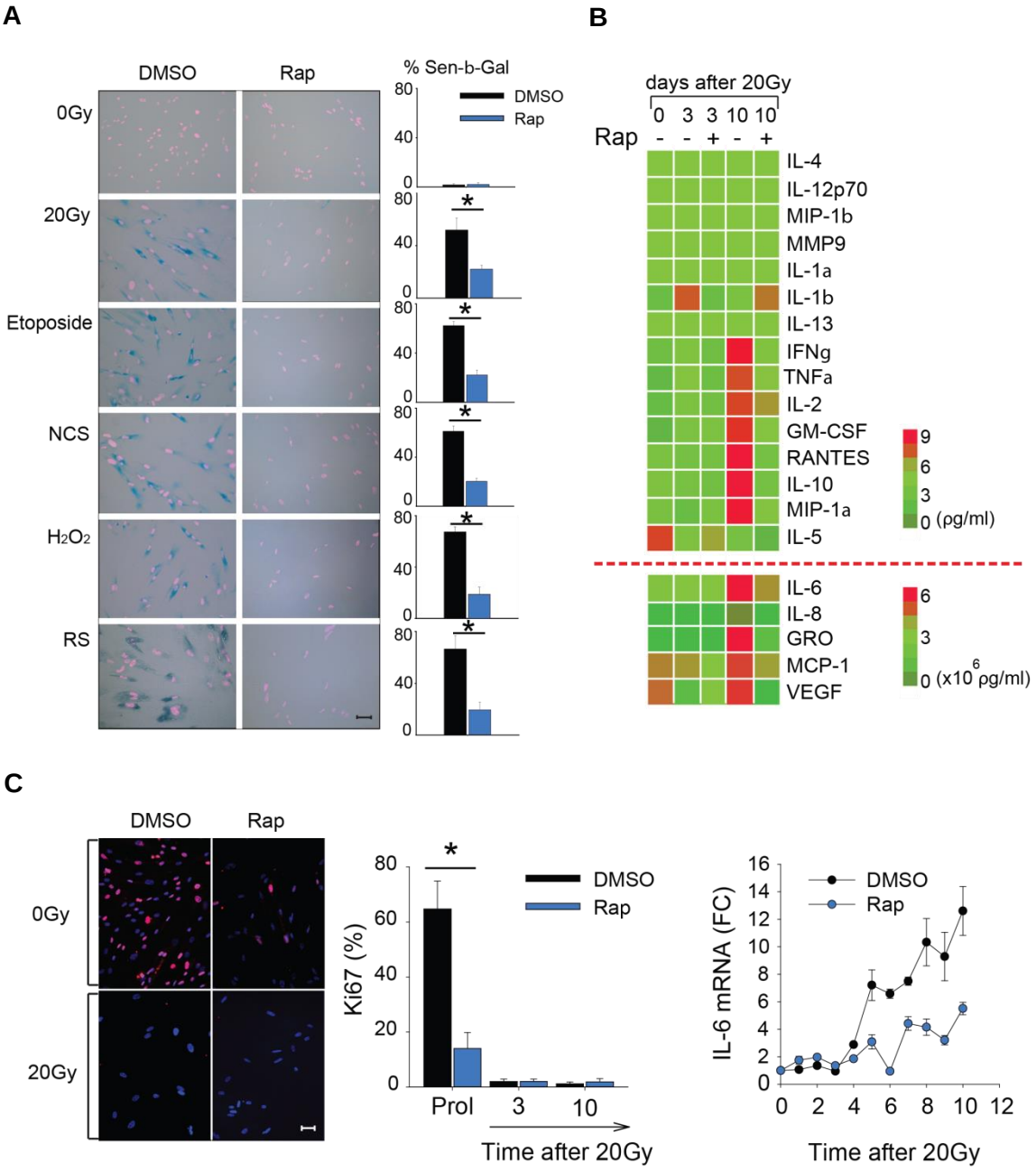

Figure 4.6 | mTORC1 inhibition decreases Sen-β-Gal activity and SASP expression without rescuing cell proliferation in senescence. (A) (left) Representative images of Sen-β-Gal activity (Sen-β-Gal – cytoplasmic blue; nucleus – pink) with or without rapamycin treatment in MRC5 fibroblast induced senescence using X-ray irradiation, Etoposide, Neocarcinostatin (NCS), H_2O_2 and replicative exhaustion (RS); Scale bar = 50µm; (right) Quantification of Sen-β-Gal positive cells. Data are mean±S.E.M, n=10 randomly analysed fields (at least 150 cells were

analysed per condition); **(B)** (top) Secreted protein array of a variety of inflammatory proteins following X-ray induced senescence in MRC5 fibroblasts treated with rapamycin (3 and 10 days after 20Gy); Data are mean of 3 independent experiments; (bottom) mRNA abundance of IL-6 up to 10 days after 20Gy with or without rapamycin. Data are mean±S.E.M of n=3 independent experiments; **(C)** (left) Representative images of Ki67 immunostaining 3 days following X-ray irradiation with or without rapamycin; Scale bar = 40μm; (right) Quantification of Ki67 positive cells at 3 and 10 days following 20Gy. Data are mean±S.E.M of n=4 independent experiments. Asterisk denotes statistical significant $P<0.05$ by two-tailed t-test or One-way ANOVA.

4.4. mTOR-dependent mitochondrial biogenesis maintains ROS-driven DNA damage foci (DDF) and cellular senescence

A DNA damage response (DDR) is a prominent initiator of senescence and persistence of a DDR during senescence has been shown to be essential for the stability of stress-induced (Passos et al. 2010), replicative (d'Adda di Fagagna et al. 2003) and oncogene-induced senescence (Suram et al. 2012) and the development of a SASP (Rodier et al. 2009). Initially we observed that rapamycin supplementation immediately following X-ray irradiation had no impact on DNA damage repair capability up to 24 hours. However, we found a significant reduction of DNA breaks from 24 hours onwards in human fibroblasts assessed by alkaline comet assay (Figure 4.7A), and confirmed by frequencies of γH2AX foci (immunofluorescence staining) in human (Figure 4.7B) and mouse fibroblasts (not shown). γH2AX Immunofluorescence staining was performed by Francisco Marques in our lab.

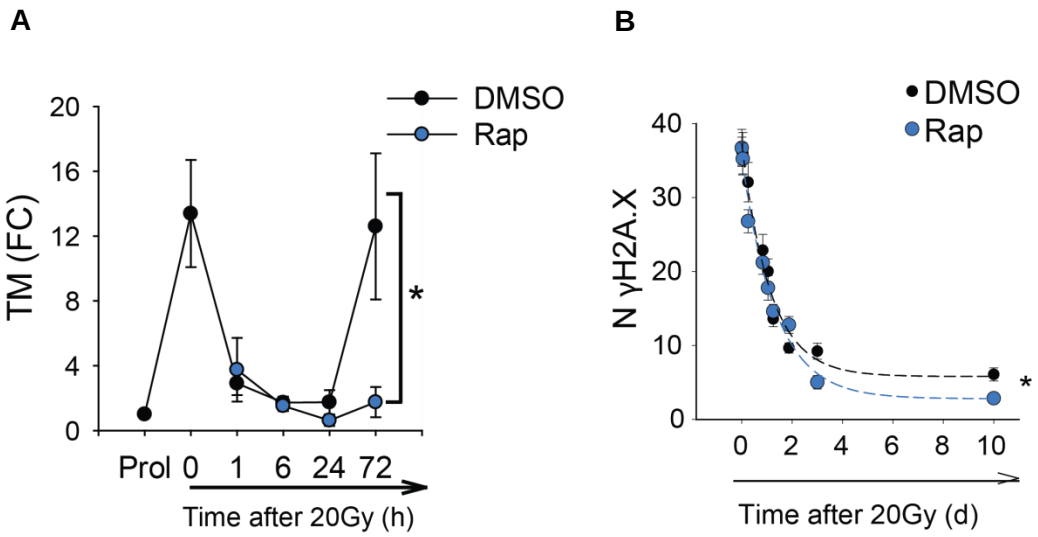

Figure 4.7 | mTOR inhibition reduces DNA breaks during senescence. (A) Alkaline comet assay on proliferating (0Gy) or 20Gy irradiated MRC5 fibroblasts treated with or without rapamycin. To quantify DNA damage, the tail moment (TM) was calculated as the product of the tail length and the fraction of DNA in the comet tail. Data are Mean±S.E.M of n=3 independent experiments. **(B)** Kinetics of DDF (γH2AX) repair in MRC5 fibroblasts after 20Gy in the presence or absence of rapamycin. Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

To test whether foci were driven by elevated ROS, we first knocked-down mTOR using siRNA (Figure 4.8A) and found that it reduced both DDF and ROS 2 days after 20Gy (Figure 4.8B). The reduction of DDF was accompanied by decreased abundance of p21 protein and mRNA levels (Figure 4.8C). Furthermore, we supplemented cells after irradiation with both rapamycin and the antioxidant NAC. In collaboration with Graeme Hewitt in our lab, we found that NAC, rapamycin or both compounds resulted in a non-cumulative reduction of both DDF and ROS (Figure 4.8D). To investigate the role of mitochondrial biogenesis in mTORC1-dependent activation of a DDR, Francisco Marques in

our lab together with Berni Carrol in the Korolchuk lab, increased or decreased mTORC1 activity in wild-type and *PGC-1β*$^{-/-}$ MEFs by overexpression of activated Rheb or rapamycin supplementation respectively. We observed that following a DDR, Rheb-dependent increase in number of DDF was suppressed in *PGC-1β*$^{-/-}$ MEFs. Furthermore, rapamycin was unable to further decrease DDF in *PGC-1β*$^{-/-}$ MEFs (Figure 4.8E), suggesting that mTORC1 and PGC-1β are in the same pathway that regulate ROS-dependent DDF formation and activation of a DDR.

Further supporting the idea that mTOR is activated downstream of a DDR (Reiling et al. 2006) and that ROS are major inducers of DNA damage, hence contributors of a persistent DDR that stabilises senescence (Passos et al. 2010), we found that mitochondrial deficient cells, possibly via lower ROS levels, have decrease mTOR activity (Figure 4.8F). Based on previous data showing dependence between DDR and the SASP (Rodier et al. 2009) and our own observations, we hypothesize that maintenance of DDR via mTOR driven mitochondrial biogenesis impacts on the development of cellular senescence and the SASP (Figure 4.8F).

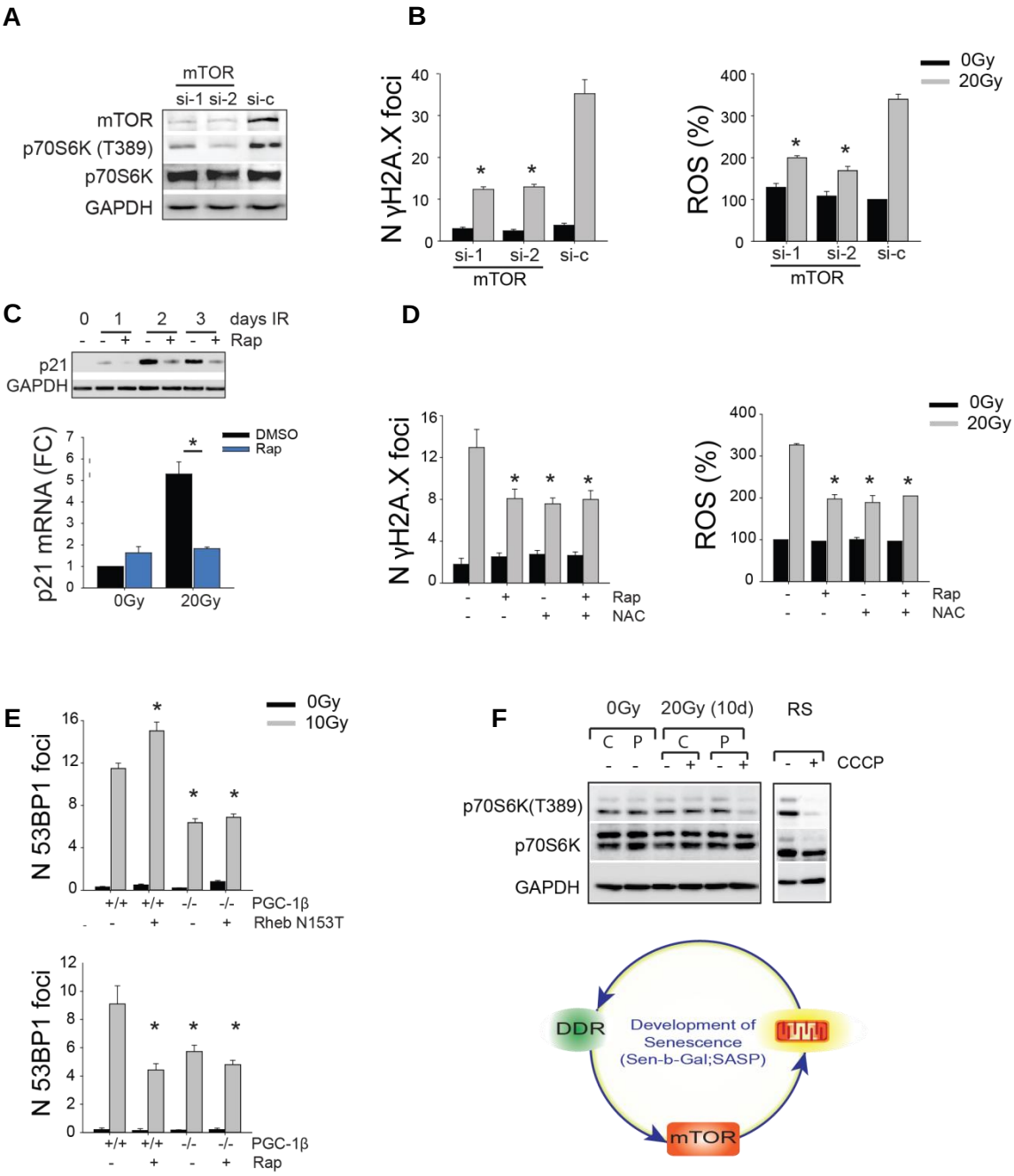

Figure 4.8 | mTOR-dependent mitochondrial biogenesis maintains ROS-driven DNA damage foci (DDF) and cellular senescence. (A) Western blot showing the knockdown efficiency of 2 different siRNA against mTOR and its effects on p70S6K phosphorylation (T389) 2 days after 20Gy (data are representative of 2 independent experiments); **(B)** (left) Quantification of γH2A.X and (right) ROS levels (measured by DHE) after mTOR knockdown 2 days after 20Gy. Data are Mean±S.E.M of n=3

independent experiments; **(C)** (top) Representative western blot showing inhibition of p21 protein expression with rapamycin at different time points (days) after 20Gy (data are representative of 3 independent experiments); (bottom) p21 mRNA levels 3 days after 20Gy with or without rapamycin treatment. Data are Mean±S.E.M of n=3 independent experiments; **(D)** (left) Quantification of γH2A.X foci and (right) ROS generation (measured by DHE) with or without rapamycin and the antioxidant NAC 3 days after 20Gy. Data are Mean±S.E.M of n=3 independent experiments; **(E)** (top) Effect of overexpression of mutated Rheb (N153T) on the number of 53BP1 foci in wild-type and *PGC-1β*$^{-/-}$ MEFs, 3 days after 0Gy or 10Gy; (bottom) Effect of Rapamycin on the number of 53BP1 foci in in wild-type and *PGC-1β*$^{-/-}$ MEFs, 3 days after 0Gy or 10Gy. Data are Mean±S.E.M of n=3 (at least 125 cells were analysed per condition); **(F)** (top) Representative western blots of mTOR activity in mitochondrial-depleted MRC5 fibroblasts 10 days after 20Gy and in replicative senescence (RS). C= control and P= Parkin expressing cells (data are representative of 2 independent experiments); (bottom) Scheme representing the hypothesis that maintenance of DDR via mTOR driven ROS is dependent on mitochondria and contributes to the development of the senescent phenotype (including the SASP). Asterisk denotes statistical significant $P<0.05$ One-way ANOVA.

4.5 The DDR induces mitochondrial biogenesis by activating ATM, AKT and mTORC1 phosphorylation cascades

Two lines of evidence suggested that DDR could converge into the mTOR signalling pathway via protein kinase B (Akt/PKB) phosphorylation: Firstly, Akt has been shown to activate mTORC1 by directly phosphorylating the TSC1/TSC2 complex (Inoki et al. 2002) or by dissociation of PRAS40 from the essential mTORC1 component RAPTOR (Thedieck et al. 2007). Secondly, Akt has been shown be a direct phosphorylation target of ATM (Viniegra et al. 2005). The serine/threonine kinase ATM is a primary sensor and transducer of DNA double strands breaks (DSBs). Following a DSB, ATM is activated and phosphorylates numerous key players of the DDR (Derheimer et al. 2010). To

test the relation between ATM, Akt and mTOR, we induced a DDR in human fibroblasts derived from mutated Ataxia Telangiectasis (AT) patients or treated with an ATM inhibitor. In both cases we were able to significantly reduce Akt (S473) and p70S6K (T389) phosphorylation induced by DDR when compared to controls (Figure 4.9A-C). Moreover, we observed that increased expression of the mitochondrial protein NDUFB8 following activation of DDR was significantly suppressed in both AT patient fibroblasts and in fibroblasts treated with an ATM inhibitor (Figure 4.9A and 4.9B).

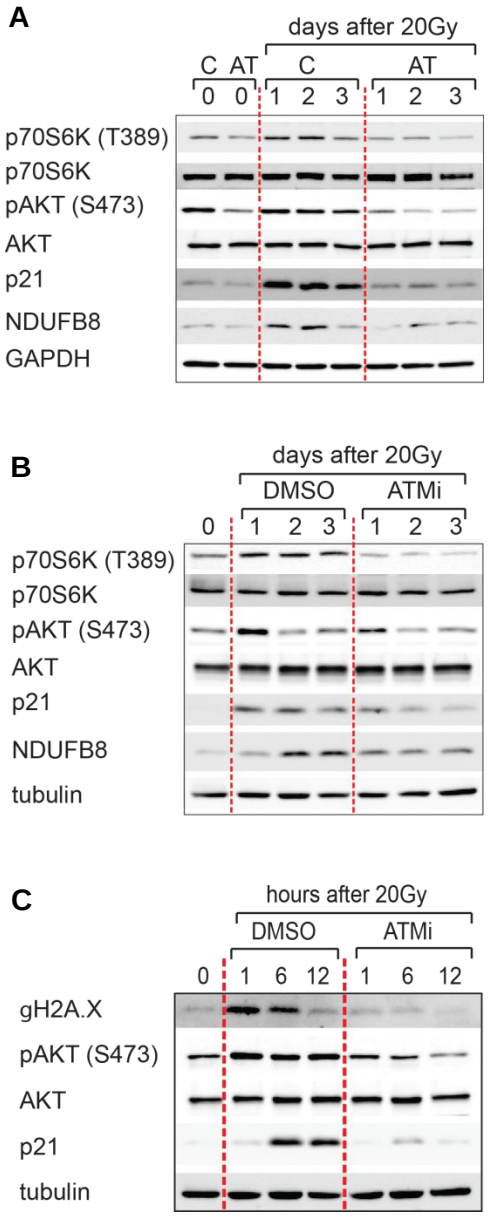

Figure 4.9 | mTORC1 integrates DDR signalling towards mitochondrial biogenesis during cellular senescence. Representative western blots showing expression of phosphorylated p70S6K (T389) and AKT (S473), mitochondrial protein NDUFB8 and the DDR downstream target p21 in **(A)** normal fibroblasts and from a patient with AT and **(B)** in MRC5 human fibroblasts treated with or without the ATM inhibitor KU55933 at different time points after 20Gy irradiation (data are representative of 2 independent experiemnts); **(C)** Western blot showing effect of ATM inhibitor KU55933 on γH2A.X and AKT phosphorylation and p21 expression in MRC5 human fibroblasts after 20Gy irradiation (1 independent experiment).

Having established that ATM, AKT and mTOR are activated following a DDR, we aimed to test whether they would be part of the same pathway regulating mitochondrial content during senescence. For this purpose we treated irradiated fibroblasts with an ATM inhibitor and/or rapamycin and assessed for mitochondrial mass and its correlation with senescence markers (Figure 4.10A). In support of a role for ATM, AKT and mTORC1 in the same pathway that regulates mitochondrial mass in senescence, we observed that chemical inhibition of ATM and mTORC1 activity: i) reduced mitochondrial mass with no additive effect when both inhibitors were applied simultaneously (Figure 4.10B), ii) had non-synergistic effects on DDF and p21 expression (Figure 4.10C and 4.10D) and iii) decreased Sen-β-Gal activity with no cumulative effect compared to single inhibitions (Figure 4.10E) (work performed in collaboration with Rhys Anderson in our lab).

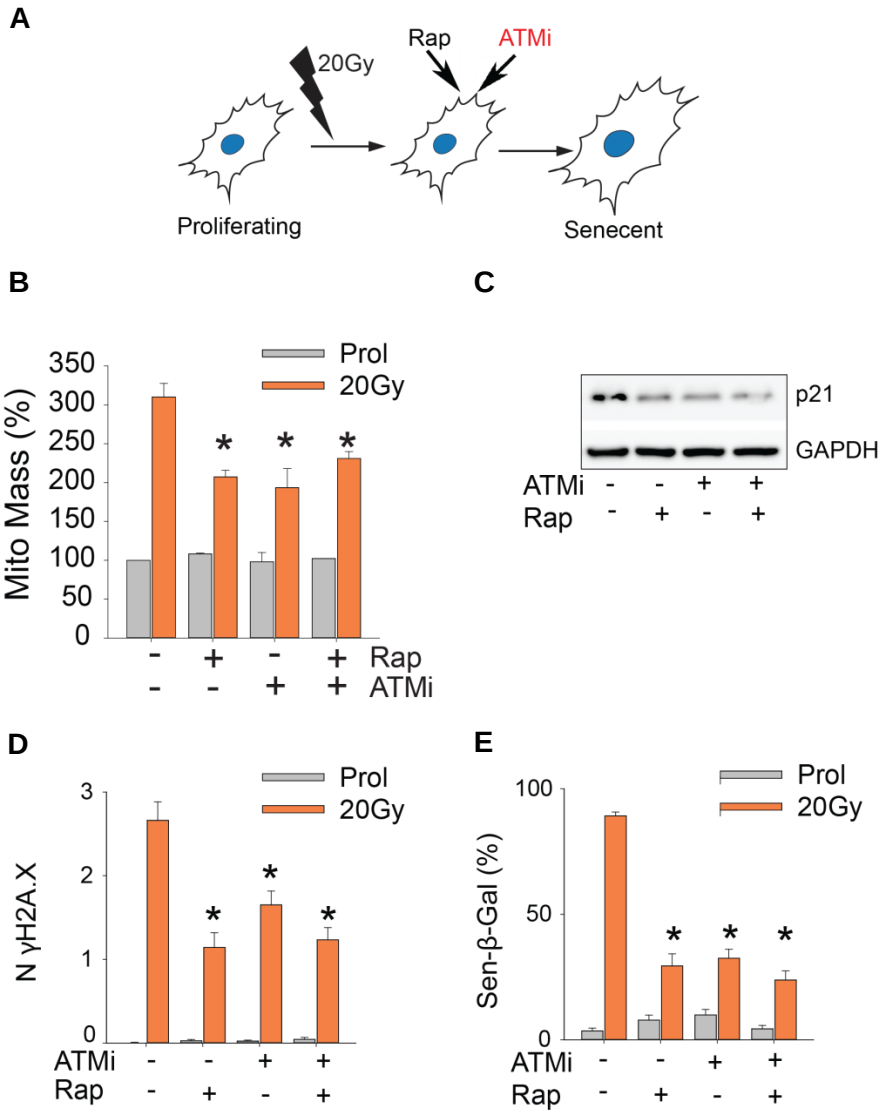

Figure 4.10 | Epistatic pathway involving ATM and mTOR in mitochondrial mass regulation during senescence. (A) Scheme illustrating experimental design: human MRC5 fibroblasts were irradiated with 20Gy and treated with the ATM inhibitor KU55933 (ATMi) and/or Rapamycin (Rap); **(B)** Effect of Rapamycin and/or ATM inhibitor KU55933 on mitochondrial mass in human MRC5 fibroblasts 3 days after 20Gy. Data are mean±S.E.M of n=3 independent experiments; **(C)** Combined inhibition of ATM and mTORC1 effects on p21 expression at day 10 after 20Gy X-radiation (western blot is representative of 3 experiments); **(D)** Combined inhibition of ATM and mTORC1 effect on γH2A.X (left) and Sen-β-Gal (right) at day 10 after irradiation in MRC5 fibroblasts. Data are mean±S.E.M of 3 independent experiments. Asterisk denotes statistical significant *P*<0.05 One-way ANOVA.

4.6 Discussion

Mitochondria have been widely associated with cellular senescence and ageing. In the previous chapter we have demonstrated that more than associated with, mitochondria are necessary for senescence to occur. However the exact mechanisms regulating mitochondrial homeostasis in senescence are still unclear. Uncovering the pathways that lead to mitochondrial deregulation (e.g. mass increase) during senescence are important for the development of interventions targeting mitochondria and aiming to decrease senescence "side effects" in the located tissues. In this chapter we have demonstrated that interventions that reduce the load of mitochondria in cells such as inhibition of mTORC1 (and ablation of PGC-1β, in collaboration with Francisco Marques in our lab) have a beneficial impact on the senescent phenotype by decreasing ROS and SASP factor secretion. Firstly, we showed that mTOR activation progressively increases following induction of a DDR using X-ray irradiation in human and mouse fibroblasts. This observation is supported by previous reports showing that DNA damage stressors such as UV light, ROS and some carcinogens can indeed activate mTORC1. (Bae et al. 1999, Brenneisen et al. 2000, Zhang et al. 2001, Ding et al. 2002, Huang et al. 2002). Consistent with a role for mTORC1 in DDR-dependent mitochondrial protein expression, we observed that expression of mitochondrial proteins belonging to OXPHOS complexes and the mitochondrial import receptor subunit TOMM20 is significantly reduced when mTOR is inhibited by rapamycin in irradiated fibroblasts. In addition to mitochondrial protein expression analysis, mtDNA copy number and mitochondrial mass assessed by flow cytometry (NAO intensity), fluorescence microscopy (MitoTracker Green) and T.E.M were also reduced upon mTORC1 inhibition in senescent cells. On the other hand, mTOR hyperactivation resulting from Rheb overexpression induced an increase in mitochondrial content in senescence. Together these results show that mTOR is an essential factor in the pathway(s) leading to mitochondrial mass increase in senescence and are supported by the idea that mTOR plays a role in the complex pathways mediating mitochondrial homeostasis (Laplante et al. 2012).

Corroborating our previous results showing that mitochondrial clearance alleviates the senescent phenotype, we found that mTORC1 inhibition-dependent mitochondrial mass decrease by rapamycin was accompanied by reduced Sen-β-Gal activity and SASP factor secretion (particularly inflammation mediating factors) in senescence, consistent with the reported immunossupressive effects of rapamycin (Abraham et al. 1996). There are several possible explanations our observations. Sen-β-gal is thought to be a result of increased β-galactosidase activity coming from higher lysosomal activity demands during senescence (Kurz et al. 2000). It was suggested that senescence is accompanied by lysosomal changes including increased size and number (Robbins et al. 1970, Lee et al. 2006) which attempt to deal with increased molecular damage. Increased molecular damage in senescence has been associated with elevated levels of ROS and oxidative damage (Sitte et al. 2001, Passos et al. 2007, Ahmed et al. 2010), which stimulate degradation mechanisms, namely lysosomal degradation. This way mTOR inhibition, by decreasing mitochondrial content and subsequent ROS formation, may decrease oxidative damage and reduce demands for lysosomal degradation during senescence. Alternatively, it has been shown that expression of the GLB1 gene, which encodes β-galactosidase, is increased in senescent cells (Kurz et al. 2000), thus it might be that by downregulating protein translation, mTORC1 inhibition also decreases expression of β-galactosidase. Regarding the effect of mTORC1 inhibition on the SASP, it could be that an mTORC1 inhibition-dependent mitochondrial mass decrease would result in lower ATP levels compromising mTORC1 activity with decreased SASP translation. However, our results show that inhibition of mTORC1 activity also impacts on the mRNA levels of SASP factors. Consistent with our results are reports showing that rapamycin affects transcriptional programs controlling proliferative and inflammatory properties in smooth muscle cells (Zohlnhofer et al. 2004). Nevertheless, reduced transcriptional activity could still be a consequence of the loss of S6K mediated protein translation (downstream of mTORC1 inhibition) resulting in translation downregulation of major transcriptional regulators. Mechanisms independent of mitochondria have also been implicated in the

activation of inflammatory pathways. It has been shown that activation of mTOR downstream of Akt regulates NF-κB activity in PTEN-null/inactive prostate cancer cells via interaction with and stimulation of IKK (Dan et al. 2008), which may also explain the control of mTOR over the SASP, particularly the pro-inflammatory phenotype.

A DNA damage response (DDR) is the most prominent initiator of senescence and persistence of a DDR during senescence has been shown to be essential for the stability of stress-induced (Passos et al. 2010), replicative (d'Adda di Fagagna et al. 2003) and oncogene-induced senescence (Suram et al. 2012) and the development of a SASP (Rodier et al. 2009). mTOR downregulation impacted on ROS-driven DDF, while overexpression of mutated Rheb resulted in increased number of DDF in senescent cells, further supporting the role of mTOR in ROS generation following a DDR. In this context we have shown that: i) mTOR regulates mitochondrial content in senescence and concomitant mitochondrial-ROS production; ii) mitochondrial-ROS are an important generator of DNA damage during senescence; iii) both mitochondrial (Chapter 3) and mTOR (possibly via mitochondria) regulate the SASP and iv) mitochondria, possibly via ROS levels and consequent impact on the DDR, regulate mTOR activity. Our findings are consistent with a model by which mTOR signalling impacts on mitochondria content and by doing so, affects the persistence of a DDR and the development of the SASP. We have assumed that mitochondrial ROS induces DNA damage and activates a DDR that stabilises senescence. However, ROS has been shown to impact on mechanisms leading to senescence independently of the DDR. Several studies have reported that ROS can activate directly p53, p38MAPK and NF-κB activity (Gloire et al. 2006, Liu et al. 2008, Wang et al. 2011). Additionally, ROS can activate ATM independently of DNA damage (Ditch et al. 2012). It may be that ROS impact on the stabilisation of cellular senescence involves regulation of pathways other than the positive feedback loop that we have previously reported (Passos et al. 2010), and that integration of not one but several pathways ensure stabilisation of the permanent cell cycle arrest. We can also not exclude direct modes of interaction between mTORC1 and targets of the DDR such as p53,

independently of mitochondria (Lee et al. 2007, Lai et al. 2010). However, while these studies report dynamic interactions between the mTOR and p53 pathways very shortly after a DDR, they do not explain how the cell-cycle arrest is maintained and contributes to the development of senescence which is a lengthier process usually taking between 7-10 days post-DDR (Coppé et al. 2008). Regarding the inhibitory effect of mitochondrial depletion on mTORC1 activity it could also be that a possible loss of ATP (we still don't know if mitochondrial depleted cells have lower ATP levels) compromises mTORC1 activity. To exclude ROS-independent effects of mitochondria on mTOR via energy levels (ATP) or other direct interaction we would need to perform further experiments including supplementation of ATP to mitochondria depleted cells and assess for mTORC1 activity and senescence markers.

mTOR is a master regulator of pathways controlling mitochondrial turnover such as biogenesis and mitophagy (Laplante et al. 2012). Mitochondrial biogenesis is a multi-factorial process which involves the integration of tightly regulated transcriptional events, including lipid membrane and protein biogenesis and assembly as well as replication of mtDNA (Zhu et al. 2013). mTORC1 has been shown to exert regulatory effects on the mRNA expression of PGC-1α and β master regulators of mitochondrial biogenesis and function, by complex interactions with the transcription factors YY1 and NF-κB (Cunningham et al. 2007, Bakkar et al. 2012). In accordance with a transcriptionally regulated process, we found that mTORC1 regulates PGC-1α and β mRNA expression following activation of a DDR as well as downstream OXPHOS genes. mTORC1 has also been implicated in mitochondria homeostasis via mitophagy, a selective type of autophagy, known to be negatively regulated by mTORC1 (Zhu et al. 2013). However, contrary to what was expected, mTORC1 mediated effects on mitochondrial mass after a DDR seem to be autophagy-independent, at least in the early time points of the development of the senescent phenotype. However, we do not discard the hypothesis that autophagy may be impaired later on when senescence is fully established and may contribute to its maintenance. The impact of autophagy in cellular senescence remains poorly defined. A number of reports have provided indirect or circumstantial evidence

for the induction of autophagy and senescence (Gewirtz 2013). Young *et al.* have shown that induction of autophagy promotes fibroblast entry into senescence (Young et al. 2009). While, Kang *et al.* has reported that autophagy impairment induces premature senescence through a ROS- and p53-dependent manner, possibly via mitochondrial dysfunction in primary human fibroblasts (Kang et al. 2011). The studies on autophagy and senescence are contradictory and further studies are necessary to clarify the role of autophagy and mitophagy during senescence.

Regulation of cell growth (mass), proliferation and stress management by mTOR is tightly controlled and requires co-integration of this kinase in other major cellular pathways, including the DNA damage response (Reiling et al. 2006). Here, we report that mechanistically, the mTOR-dependent mitochondrial mass increase in senescence follows activation of the DDR proteins ATM and AKT. ATM is involved in a wide spectrum of biological processes including cell cycle control, genome stability, apoptosis and response to genotoxic stress (Shiloh 2003). Beside phosphorylating numerous key players of the DDR (Derheimer et al. 2010), ATM has also been shown to directly phosphorylate Akt (Viniegra et al. 2005) a serine/threonine kinase that has been implicated in pathways related to survival by inhibition of apoptotic signals and promotion of cell cycle progression (Nicholson et al. 2002). Despite not having chemically or genetically interfered with Akt to assess mTORC1 activity, we have indirect evidence via ATM inhibition that lower Akt phosphorylation correlates with reduced phosphorylation of p70-S6K. Further supporting our results, Akt has been shown to activate mTORC1 by directly phosphorylating the TSC1/TSC2 complex (Inoki et al. 2002) or by dissociation of PRAS40 from the essential mTORC1 component RAPTOR (Thedieck et al. 2007). Performing double chemical inhibition of ATM and mTORC1 we found that these two kinases are part of an epistatic pathway regulating mitochondrial mass in senescence.

In summary, our data suggest that ATM, Akt and mTOR phosphorylation cascades downstream of a DDR promote mitochondrial biogenesis and

mitochondrial-ROS production that contributes to a persistent DDR that keeps cells locked in the cell cycle.

Chapter 5. mTORC1-PGC-1β regulate mitochondrial content and contribute to senescence in vivo

In the previous chapters we have demonstrated that mitochondrial mass increase is necessary to senescence to occur in a process co-regulated by mTORC1 and the mitochondrial regulator PGC-1β. In this chapter, we hypothesised that the mitochondrial mass increased observed during cellular senescence *in vitro* is also a feature of cellular ageing *in vivo*.

5.1 Mitochondrial mass increase with age *in vivo*

In order to understand if *in vivo* cellular ageing is accompanied by an mTORC1-PGC-1β dependent mitochondria mass increase, we analysed mouse liver samples from young (3 months) and old (12 months) animals. Similarly to our observations *in vitro*, in collaboration with Dr Viktor Korolchuk (IAH-Newcastle University), we found an age-dependent increase in mTORC1 activity (measured by p-S6/S6 ratio) associated with increased p21, PGC-1β and OXPHOS components in wild-type mice (Figure 5.1A). Furthermore, we also found that a mouse hepatocyte cell line (MIH) cultured *in vitro* also experienced increased mitochondrial mass and ROS generation after activation of a DDR and that this increase could be partially suppressed by the mTORC1 inhibitor rapamycin (Figure 5.1B).

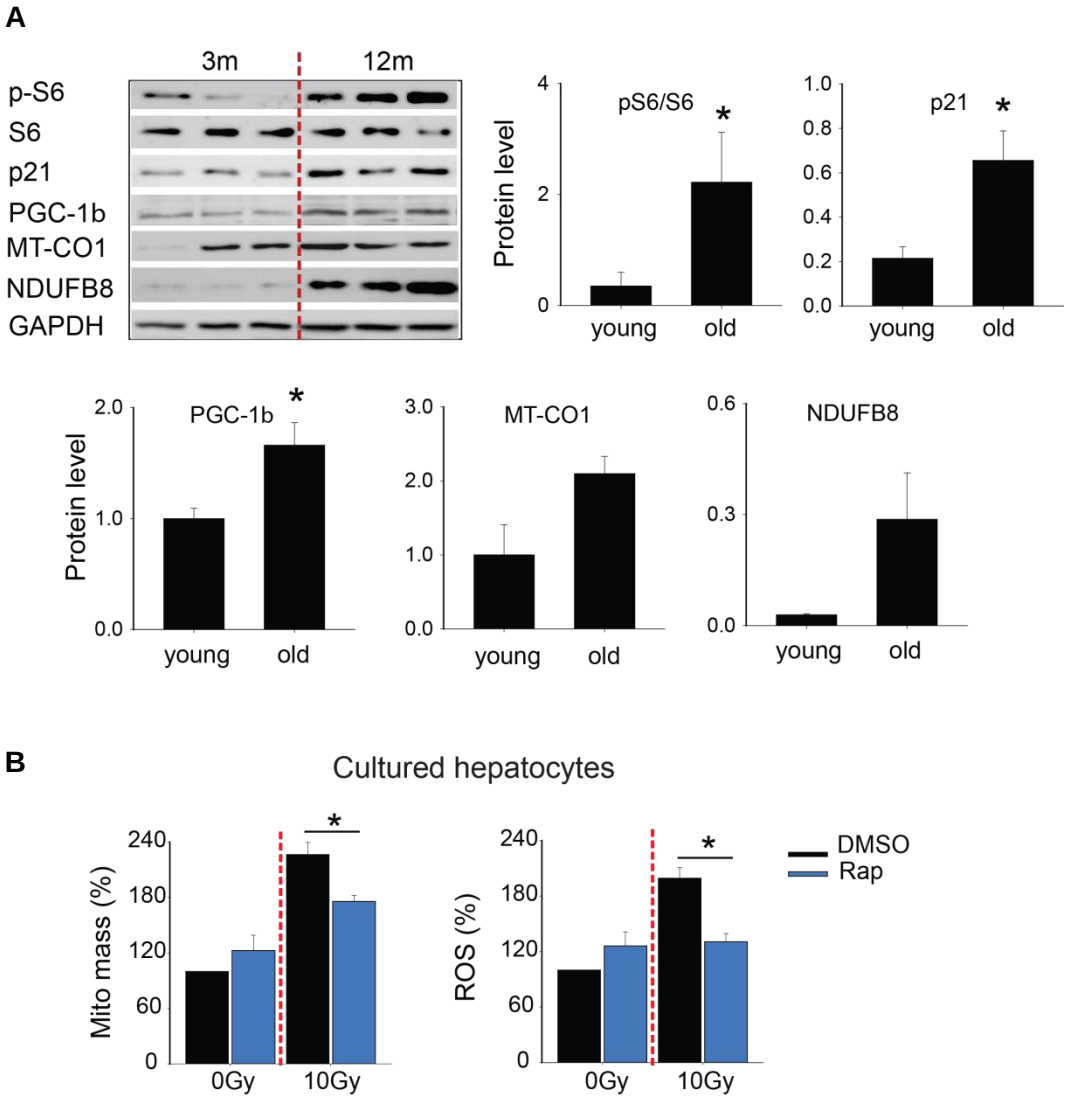

Figure 5.1 | Mitochondrial mass increase with age in mouse liver tissues. (A) (left) Representative westerns of pS6, S6, p21, PGC-1β, MT-CO1 and NDUFB8 proteins in mouse livers at 3 and 12 months of age (n=3 mice per group); (top right and bottom) Quantification of pS6/S6, p21, PGC-1β, MT-CO1, and NDUFB8 protein expression in liver tissue from wild-type C57BL/6 mice at 3 and 12 months of age. Data are mean±S.E.M n=3 mice per group; **(B)** Mitochondrial mass and ROS (measured by NAO and DHE fluorescence respectively) quantifications of *in vitro* cultured mouse hepatocytes (MIH) 3 days after 10Gy irradiation treated with or without rapamycin. Data are mean±S.E.M n=3 independent experiments. Asterisks denote statistical significance P<0.05 using two-tailed t-test and one-way ANOVA.

5.2 Mitochondrial mass increase with age positively correlates with DNA damage *in vivo*

Following the observation that expression of the DDR effector p21 and mitochondrial proteins are simultaneously increased with age in mouse livers, we aimed to understand the correlation between mitochondrial content and DNA damage *in vivo*. For this purpose, we performed dual immunofluorescence staining against the mitochondrial protein MT-CO1 (a marker of mitochondrial content) and γH2A.X (a marker of DNA damage) in livers from 12 month old mice. We found that hepatocytes containing more MT-CO1 intensity had in general higher number of γH2A.X foci (Figure 5.2A and 5.2B), indicating a positive correlation between mitochondrial mass increase and DNA damage *in vivo*.

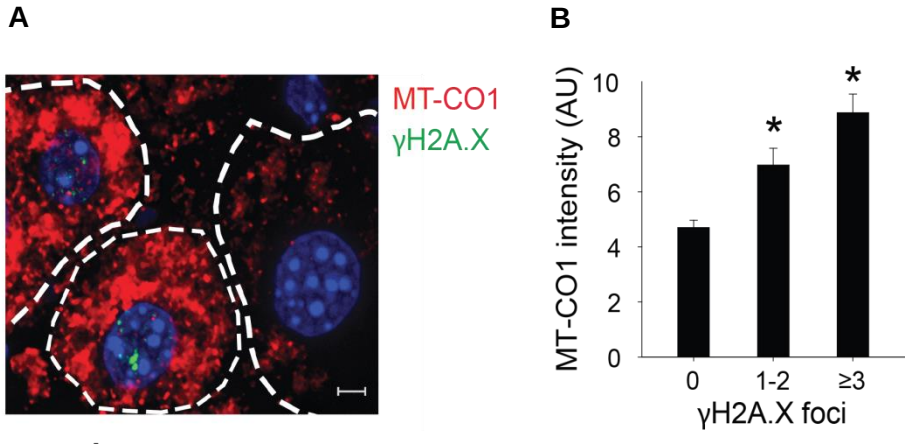

Figure 5.2 | Mitochondrial content correlates with DNA damage foci *in vivo*. (A) Representative image of double staining for the mitochondrial protein MT-CO1 and γH2A.X; Scale bar = 10μm; **(B)** Quantification of MT-CO1 intensity vs number of γH2A.X foci in hepatocytes from 12 months old mice (n=3 mice were analysed). Asterisks denote statistical significance P<0.05 using one-way ANOVA.

5.3 Age-dependent mTORC1 upregulation promotes mitochondrial biogenesis *in vivo*

In order to test the impact of mTORC1 inhibition on mitochondrial mass *in vivo*, mice were fed with rapamycin using the same conditions as Harrison *et al.* 2009 (Harrison et al. 2009) and sacrificed at different ages (Figure 5.3).

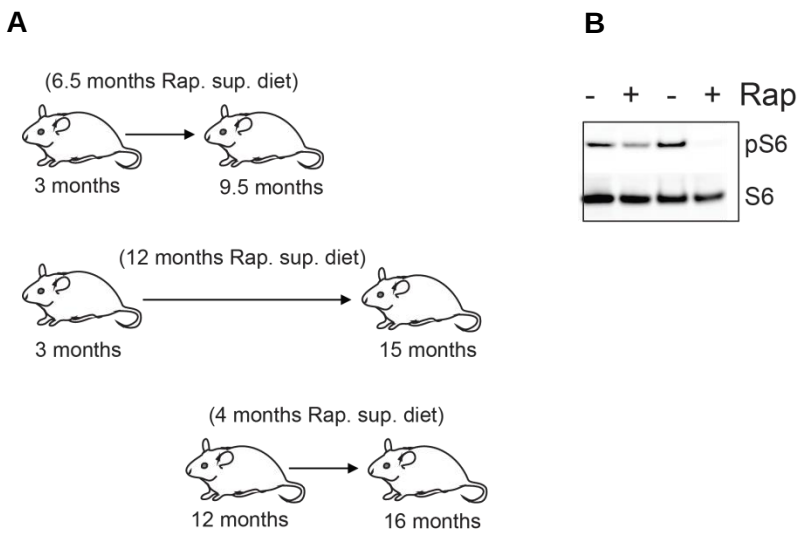

Figure 5.3 | Rapamycin diet study: experimental design. (A) Scheme representative of mice experiments invoving rapamycin diet supplementation; **(B)** Representative western blot of the expression of pS6 and S6 in 16 months old mice liver with or without 4 months rapamycin supplementation.

Further investigating the impact of rapamycin on mitochondrial content, Rhys Anderson in our lab found that rapamycin prevented the age-dependent increase in mtDNA (Figure 5.4A). We also found, through mitochondrial morphometric analysis using T.E.M, lower mitochondrial volume fraction and mitochondrial numbers per cross-section in rapamycin-supplemented animals when compared to controls (Figure 5.4B). In order to investigate the role of

mTORC1 on mitochondrial activity *in vivo*, in collaboration with Satomi Miwa in the von Zglinicki lab, we performed Seahorse XF24 analysis of mitochondrial function in liver tissues from control and rapamycin supplemented animals. We found no significant changes in mitochondrial function. State III (ADP-stimulated), state IV and respiration uncoupled from ATP synthesis (using uncoupler FCCP) remained unchanged using pyruvate/malate as substrates (Figure 5.4C). Supporting a role for mTORC1 in the regulation of mitochondrial biogenesis *in vivo*, in collaboration with Dr Viktor Korolchuk (IAH-Newcastle University), we observed that rapamycin treated animals have decreased expression of PGC-1β (Figure 5.4D). Together these observations suggest that increased mTORC1 activity with age promotes mitochondrial mass increase via the mitochondrial regulator PGC-1β.

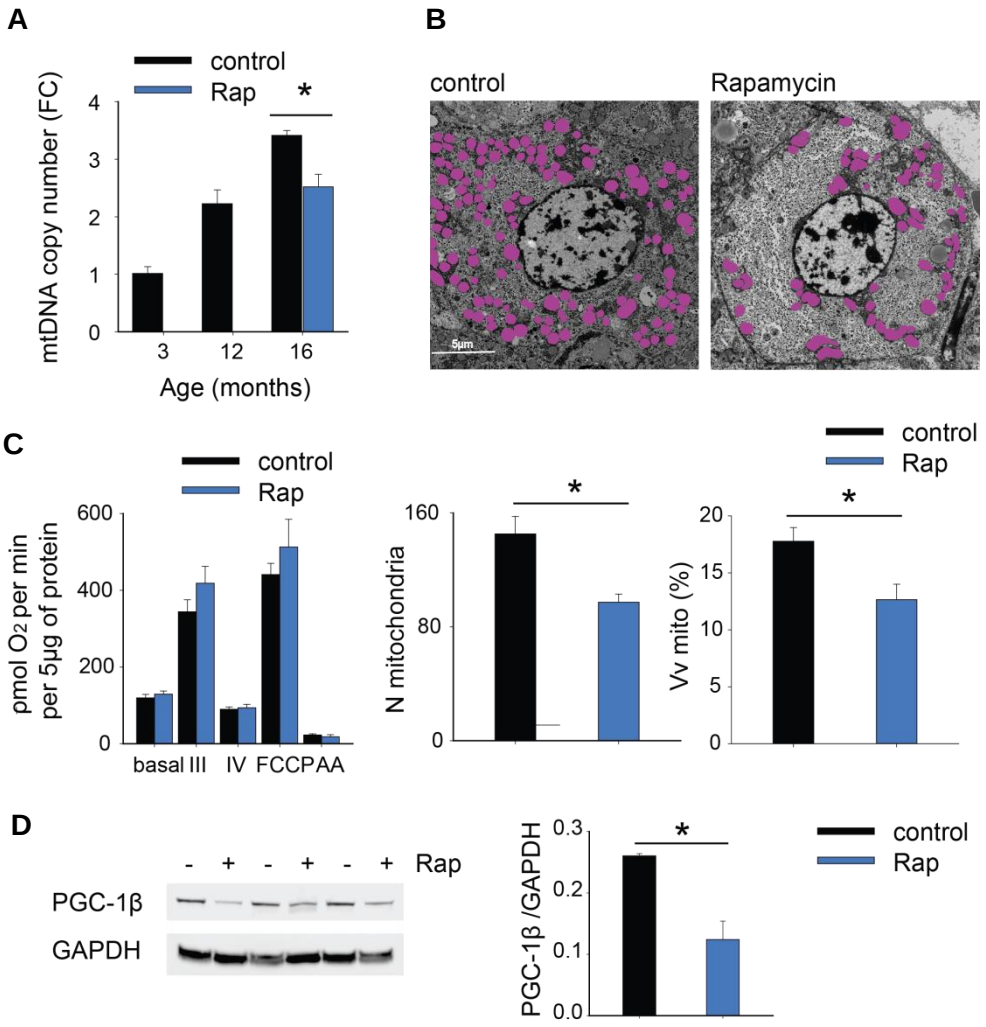

Figure 5.4 | mTORC1 regulate mitochondrial content *in vivo*. (A) mtDNA copy number by qPCR at 3, 12, 16 months and at 16 months after 4 months rapamycin treatment. Data are mean±S.E.M of n=3-4 mice per group; **(B)** (top) Representative electron micrographs of hepatocytes from 16 months old mice with or without 4 months rapamycin diet, mitochondria are labelled in pink; Scale bar = 5µm; (bottom) Quantification of mitochondrial volume fraction $\%V_v$ and mitochondrial number per cross section (T.E.M mitochondrial analyses are mean±S.E.M of n=3 mice per group; > 16 electron micrographs (cells) were analysed per mouse); **(C)** Oxygen consumption rates (OCR) in liver mitochondria, from 16 months old mice with or without 4 months rapamycin diet, in the presence of pyruvate/malate. Data are mean ± S.E.M of n = 5 mice per group). State III was induced by injection of ADP. State IV was induced by inhibition of the ATP synthase with oligomycin and uncoupled respiration rates were

determined by injection of CCCP. Antimycin A (AA) was used to determine background, non-mitochondrial OXPHOS, OCR; **(D)** (left) PGC-1β expression in 16 months old mice fed with of rapamycin supplemented diet for 4 months (n=3 mice per group); (right) Quantification of PGC-1β expression in 16 months old mice fed with of rapamycin supplemented diet for 4 months. Data are mean±S.E.M n=3 mice per group. Asterisks denote statistical significance P<0.05 using two-tailed t-test and one-way ANOVA.

5.4 mTORC1 inhibition reduces senescence markers *in vivo*

5.4.1 mTORC1 inhibition prevents Telomere-associated foci (TAF) in vivo

A recent report in our lab has shown that Telomere-associated foci (TAF), one of the important effectors of cellular senescence, increase in mice tissues with age (Hewitt et al. 2012). In order to test if mTORC1 inhibition impact on Telomeres-associated senescence *in vivo* we analysed mouse liver tissues of rapamycin treated mice as described previously (see Figure 5.3), and found that rapamycin was able to prevent the age-dependent increase in TAF. Interestingly, the 4 months rapamycin supplemented diet was as effective in preventing TAF formation as 12 months treatment (Figures 5.5A). TAF could not be attributed to changes in telomere length (Figures 5.5B) or telomerase activity (not shown). These results together with the fact that mitochondria increase with age positively correlates with DDF and p21 expression, suggests that mitochondrial mass increase (via mTORC1 upregulation) is a promoter of senescence associated DNA damage *in vivo.*

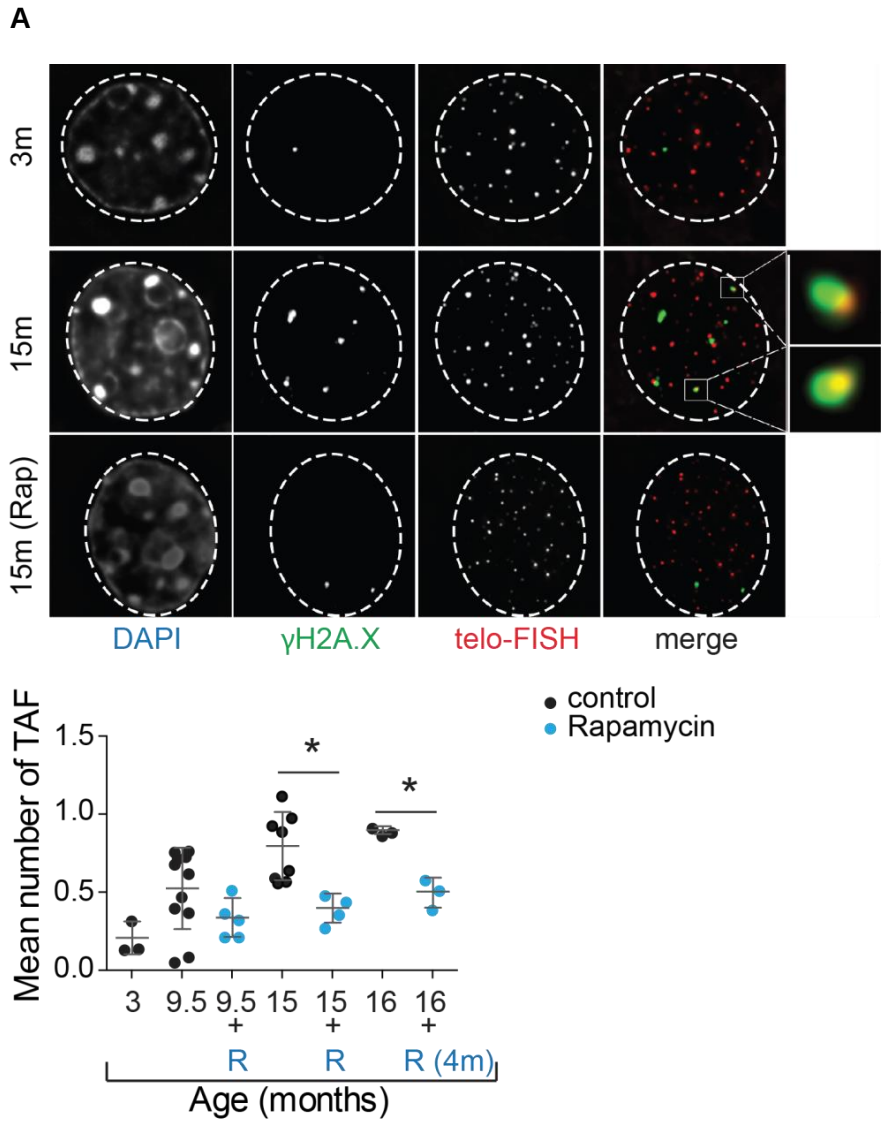
A
3m
15m
15m (Rap)
DAPI
γH2A.X
telo-FISH
merge
control
Rapamycin
Mean number of TAF
1.5
1.0
0.5
0.0
*
*
3
9.5
9.5 + R
15
15 + R
16
16 + R (4m)
Age (months)
B
a
b
Fluo Intensity (AU)
300
200
100
0
Telo-FISH
gH2A.X
Control
Rapamycin
TAF
non-TAF
Frequency
Telomere Length (AU)
0
50
100
150
200
250

Figure 5.5 | mTORC1 inhibition prevents TAF formation in liver mouse tissues during ageing. (A) (top) Representative immunoFISH images of hepatocytes from 3 and 15 old mice with or without rapamycin diet. Co-localising foci are amplified in the right panel (amplified images are from single Z-planes where co-localisation was found); (bottom) Dot plot of Telomere-associated foci in 3, 9.5, 15 and 16 months old control mice. 9.5, 15 and 16 months old mice were fed with rapamycin for 6.5, 12 and 4 months respectively. Quantification of TAF was done in collaboration with other lab colleagues (Rhys Anderson, Graeme Hewitt and Francisco Marques) to ensure unbiased analysis; at least 50 cells were analysed per mice. Data are mean ± S.E.M of n=3-9 mice per group. Asterisks denote statistical significance $P<0.05$ using one-way ANOVA. **(B)** (left) Representative image of mouse hepatocyte stained for γH2A.X and telomere-FISH. Two telomeres of equal intensity are shown (a and b), one co-localising with γH2A.X and the other not; (right) Histograms showing telomere intensity for telomeres co-localising (TAF) or not co-localising (non-TAF) with DNA damage foci in liver from 16 months old mice with or without 4 months rapamycin treatment (n=4); red dotted line represents median intensity. Mann-Whitney test shows no significant difference in the telomere intensities distribution between TAF and non-TAF. Intensity of 1000 telomeres was analysed per condition.

5.4.2 mTORC1 inhibition reduces Sen-β-Gal activity, p21 and SASP factors expression *in vivo*

Consistent with a role for mTORC1 in senescence, we found decreased Sen-β-Gal (Figure 5.6A) and mRNA expression of components of the SASP (Figure 5.6B) in the liver of rapamycin fed animals. In collaboration with Rhys Anderson in our lab also, we found that Sen-β-Gal positive hepatocytes were generally positive for TAF (Figure 5.6C). Further supporting a role for mTORC1 in suppressing the DDR in senescence, we found decreased expression of p21 in rapamycin fed mice (Figure 5.6D).

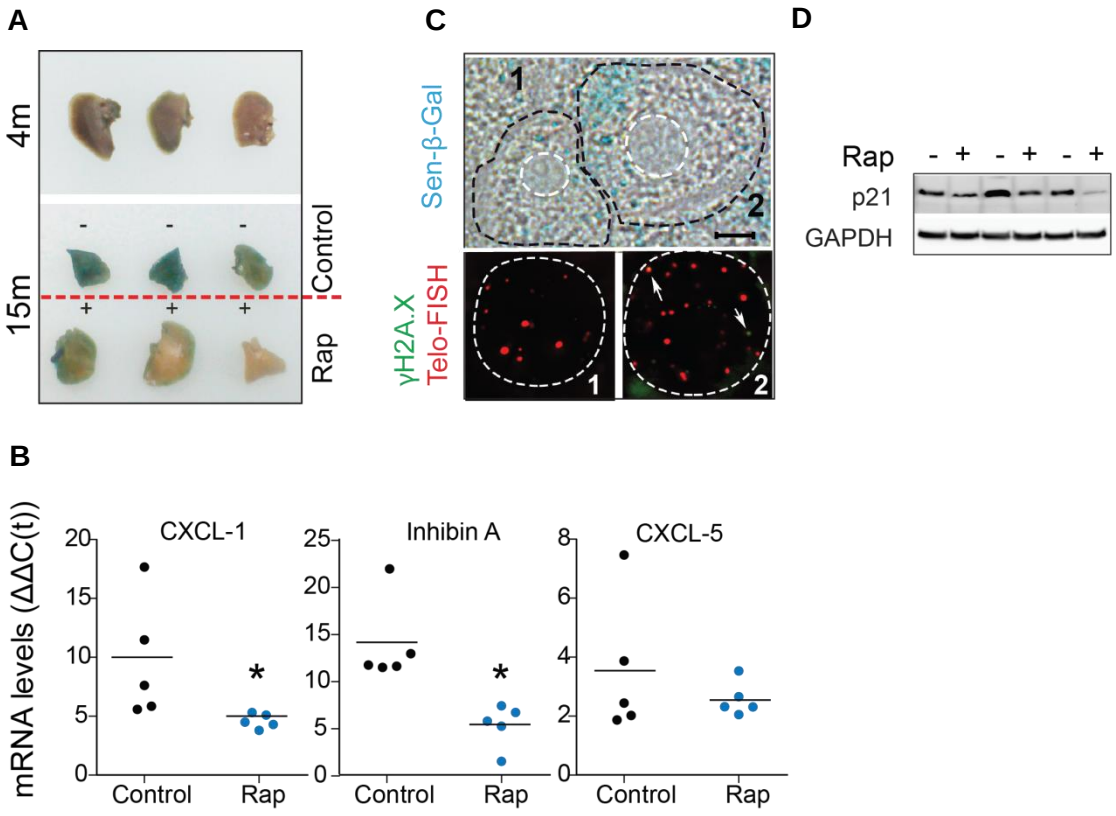

Figure 5.6 | mTORC1 inhibition reduces senescence markers *in vivo*. **(A)** 4 months old and 15 months old mice livers [control (-) or rapamycin (+)] stained with Sen-β-Gal solution (n=3 mice per group), **(B)** Dot plots of mRNA expression of the SASP components CXCL1, CXCL5 and Inhibin A (normalised to 18S) in livers of 15 months old mice fed with rapamycin for 12 months (n=5 mice per group). Data are mean of n=5 mice per group. Asterisks denote statistical significance P<0.05 using two-tailed t-test; **(C)** Representative image showing Sen-β-Gal staining in hepatocytes (scale bar=10μm) and corresponding ImmunoFISH (arrows represent co-localising foci - TAF); **(D)** Representative western blot showing effect of 4 months rapamycin feeding on p21 expression in 16 months old mice (data are representative of n=3 mice per group).

5.5 PGC-1β deficiency diminishes senescence markers *in vivo*

In order to test *in vivo* if expression of PGC-1β would have an impact on telomere-associated DNA damage, we analysed liver samples from aged *PGC-1β*$^{-/-}$ mice. These mice have decreased expression of mitochondrial proteins and decreased mtDNA copy number in liver tissues (Figure 5.7A). Consistent with our hypothesis that mitochondrial content impact on the DDR and our data revealing a role for PGC-1β in senescence *in vitro*, PGC-1β$^{-/-}$ mice show decreased numbers of TAF (Figure 5.7B). Antonio Vidal-Puig in the Rodriguez-Cuenca Lab (University of Cambridge) has observed (and kindly shared with us) that *PGC-1β*$^{-/-}$ mice show lower energy expenditure than wild-type litter-mates and absence of PGC-1β ameliorates age-dependent decline in glucose and insulin tolerance (unpublished). Altogether, these results support a causal link between the DDR, mTOR and mitochondria in the development of senescence *in vivo*.

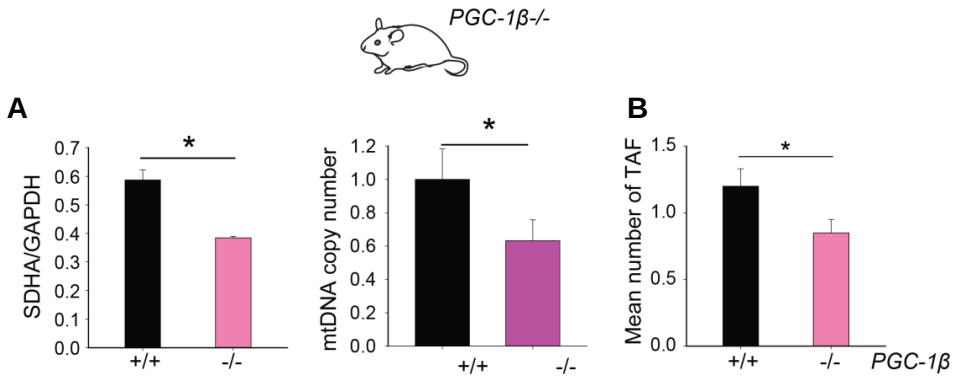

Figure 5.7 | PGC-1β deficiency reduces senescence markers *in vivo*. (A) (left) Quantification of the mitochondrial protein SDHA in wild-type and *PGC-1β*$^{-/-}$ mice. Data are mean ± S.E.M of n = 5 per group; (right) mtDNA copy number comparison between wild-type and *PGC-1β*$^{-/-}$ mice at 7 months of age. Data are mean±S.E.M n=4 mice per group; **(B)** Mean number of Telomere-associated foci (TAF) at 18 months of age in *PGC-1β*$^{-/-}$ when compared to age-matched wild–type mice. Data are mean ± S.E.M of n = 4 per group. Asterisks denote statistical significance P<0.05 using two-tailed t-test.

5.6 Mitochondrial mass increase may be a tumour-suppressor factor and promoter of senescence *in vivo*

To test the hypothesis that mitochondrial mass increase stabilises the senescent arrest and helps maintain tumour suppression, in collaboration with Derek Mann's lab (Institute of Cellular Medicine-Newcastle University), we induced liver cancer in mice by intraperitoneal (IP) injection of N-Nitrosodiethylamine (den) in wild-type mice. Dissection of tumour *vs.* adjacent non-tumour tissue revealed that liver tumours had significantly lower mtDNA copy number (Figure 5.8A), reduced expression of PGC-1β and mitochondrial proteins SDHA and MT-CO1 (Figure 5.8B and 5.8C). Strikingly, we found that the lipid peroxidation marker 4-HNE intensity was markedly reduced in tumour regions as were γH2A.X foci (Figure 5.8D and 5.8E). These observations suggest that mitochondria might be a tumour-suppressor factor and promoter of senescence *in vivo*.

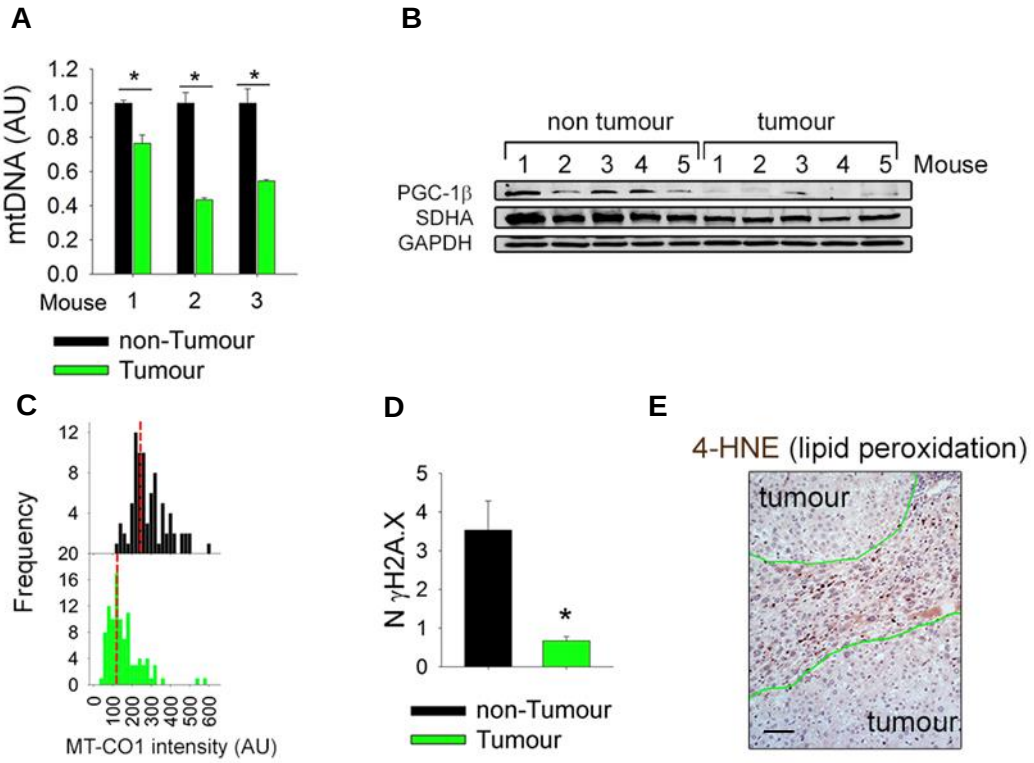

Figure 5.8 | Mitochondrial mass is decreased in mouse liver tumours. Diethylnitrosamine (den) induced liver tumours were dissected and analysed for: **(A)** mtDNA copy number by qPRC. Data are mean±S.E.M n=3 mice (tumour and non-tumour) per group and **(B)** Expression of mitochondrial protein SDHA and PGC-1β measured in tumour and adjacent non-tumour tissue (n=5 mice per group); **C)** Histograms show MT-CO1 fluorescence intensity (by immunofluorescence staining) comparison between tumour and adjacent non-tumour tissue (n=2 mice were analysed; 50 cells per area); **D)** Quantification of number of γH2A.X per hepatocyte in tumour and adjacent non-tumour regions. Data are mean ± S.E.M of n=3 mice per group; **(E)** Representative image of the lipid peroxidation marker 4-HNE staining in tumour and non-tumour regions (data is representative of n=3 mice); Scale bar = 50µm. Asterisks denote statistical significance P<0.05 using two-tailed t-test.

5.7 Discussion

The role of mitochondria in animal physiology has been the subject of extensive investigation with mitochondrial dysfunction being considered a major hallmark of ageing, underlining the significance of appropriate mitochondrial activity for survival (López-Otín et al. 2013). A rigorous regulation of mitochondrial mass, distribution and activity is fundamental for cellular homeostasis and maintenance. Mitochondrial dynamics during ageing are subject of controversy. What was initially proposed in the MTA has been later questioned, with several reports suggesting an inverse relationship between mitochondrial biogenesis and ageing. It has been shown that mitochondrial density in skeletal muscle gradually declines during age (Crane et al. 2010), suggesting a decrease in mitochondrial biogenesis possibly via an age-dependent reduction in levels of PGC-1α (Wenz et al. 2009). Additionally, studies on mitochondrial-myopathy mouse models have suggested that a compensatory mitochondrial mass increase is beneficial by partly compensating for the reduced function of the respiratory chain and maintaining overall ATP production in skeletal muscle (Wredenberg et al. 2002) However, what seems to be beneficial in skeletal muscle may be detrimental in other tissues. The same group has later reported that mitochondrial mass increase in heart muscle of the same mouse model may contribute to aggravate heart failure progression (Hansson et al. 2004). Furthermore, mice studies addressing the role of oxidative damage on ageing have proven to be inconclusive. While heterozygous deletion of the mitochondrial superoxide dismutase, an enzyme able to convert superoxide anion into hydrogen peroxide and water, showed that increased oxidative stress does not accelerate ageing in mice (Van Remmen et al. 2003), another study showed that overexpression of catalase targeted to mitochondria increased lifespan in mice (Schriner et al. 2005). Additionally, interventions promoting longevity, namely CR and sirtuin activators, have been linked with increased mitochondrial biogenesis (Baur et al. 2006, Lopez-Lluch et al. 2006).

In previous chapters we have demonstrated that mitochondrial mass increases in senescence via a process co-regulated by mTORC1 and the mitochondrial regulator PGC-1β. Therefore we hypothesised that the mitochondrial mass increase observed during cellular senescence *in vitro* could also be a feature of cellular ageing *in vivo*. Here we show that *in vivo* cellular ageing is accompanied by an mTORC1-PGC-1β dependent mitochondrial mass increase. Our results demonstrate that there is an age-dependent increase in mTORC1 activity associated with increased expression of p21, PGC-1β and OXPHOS components in wild-type mice. We have shown that age-dependent mTORC1 hyperactivation promotes mitochondrial biogenesis *in vivo*. Mice fed with a rapamycin supplemented diet (Harrison et al. 2009) have lower mitochondrial content shown by decreased mtDNA copy number, mitochondrial volume fraction and numbers per cross section (T.E.M), but showed no significant changes in mitochondrial function. Further supporting a role for mTORC1 in the regulation of mitochondrial biogenesis *in vivo*, mTORC1 inhibition by rapamycin partially supressed expression of PGC-1β. Together these observations suggest that increased mTORC1 activity with age promotes mitochondrial mass increase via the mitochondrial regulator PGC-1β. Supporting our results showing increased mTORC1 activity with age are numerous studies demonstrating that mTOR is a key modulator of ageing and age-related diseases (Johnson et al. 2013), with mTOR inhibition being the major contributor to extension of lifespan in several organisms (Fabrizio et al. 2001, Vellai et al. 2003, Jia et al. 2004, Kapahi et al. 2004, Kaeberlein et al. 2005, Harrison et al. 2009, Bjedov et al. 2010). Furthermore, we have also shown that mTORC1-dependent mitochondrial mass increase positively correlates with DNA damage *in vivo* with age. Our observations are in accordance with previous reports showing that mitochondrial oxidative damage is a conserved process, accumulating with age in organisms ranging from worms to humans (Golden et al. 2001, Yui et al. 2003), resulting in macromolecule oxidation, particularly DNA oxidation (Harman 1972, Oliver et al. 1987, Fraga et al. 1990, Hamilton et al. 2001).

Our *in vivo* data is consistent with reports in hematopoietic stem cells in which mTORC1 activity increases mitochondrial biogenesis, contributing to ROS-dependent decreased stemness and hematopoiesis (Chen et al. 2008) and that mTOR inhibition can alleviate mitochondrial disease (Johnson et al. 2013). The reduced mitochondrial content in liver following mTOR inhibition is in accordance with various reports of reduced protein synthesis and mitochondrial transcriptional regulation observed in mice with impaired mTORC1 activity (Cunningham et al. 2007, Romanino et al. 2011). Moreover, it has been recently shown by whole-genome expression profiling and large-scale proteomic analysis, that mice under CR have less mitochondrial protein synthesis (Lanza et al. 2012). This latter study, together with other more recent reports (Hancock et al. 2011, Lanza et al. 2012, Price et al. 2012) have questioned early associations between DR and enhanced mitochondrial content (Nisoli et al. 2005). In addition, despite evidence suggesting that increased mitochondrial abundance can be an advantageous adaptive response to energy deficit, genetically induced mitochondrial biogenesis has been associated with age-related diseases such as cardiomyopathy (Lehman et al. 2000), renal fibrosis (Hickey et al. 2011) and diabetes (Sawada et al. 2014), all of which have been associated with cellular senescence (Sussman et al. 2004, Testa et al. 2007, Yang et al. 2010). Increased mitochondrial content has also been associated with osteoarthritis, particularly Kashin-Beck Disease (KBD), where articular chondrocytes present increased mitochondrial mass, but reduced activity of complexes II, III, IV and V in patients samples when compared to controls (Liu et al. 2010). Alternatively, our results showing increased mTOR activity with age could also impact on mitochondrial content via a decrease in autophagy. Several reports have suggested that autophagy is down-regulated during ageing (Pyo et al. 2013). It has been shown that protein turnover slows down with accumulation of protein aggregates with age (Levine et al. 2008). Autophagy related proteins, including ATG5, ATG7, and BECN-1, expression was shown to be down-regulated in aged human brains (Lipinski et al. 2010). Increased autophagy via mTOR inhibition by rapamycin has been shown to boost the clearance of mutant huntingtin fragments and attenuates toxicity in

cells (Ravikumar et al. 2006), and to reduce tau toxicity and the appearance of protein aggregates in D. melanogaster and mammals (Ravikumar et al. 2002, Ravikumar et al. 2004, Berger et al. 2006). Additionally, chaperon-mediated autophagy has been shown to decline with age in rat liver tissues (Cuervo et al. 2000). An alternative explanation for our observation is that increased mTORC1 activity in mouse livers with age could result in decreased autophagic activity, namely mitophagy, and explain why we observe an increase in mitochondrial mass in this organ during mice ageing, which is partially rescued by treatment with rapamycin. Increased PGC-1β expression could be a compensatory mechanism in response to accumulation of dysfunctional mitochondria with age (possibly resulting from impaired mitophagy).

Recently, our lab has shown that Telomere-associated foci (TAF), an important effector of cellular senescence, increase in mice tissues with age (Hewitt et al. 2012). Consistent with a role for mTORC1 in senescence *in vivo*, we found that rapamycin treatment prevents the age-dependent increase in TAF. We have further shown that mitochondria increase with age positively correlates with DDF and p21 expression. The observation of a key role for mTORC1 in the maintenance of a DDR *in vivo* is supported by reports that calorie restriction (CR) reduces DDR positive cells *in vivo* in several tissues (Wang et al. 2010, Jurk et al. 2012) as well as ROS (Lanza et al. 2012). Further supporting a role for mTORC1 in senescence, we also found decreased Sen-β-Gal activity, p21 and SASP factor expression in rapamycin fed mice. Together these data suggests that mitochondrial mass increase, via mTORC1 hyperactivation, is a promoter of senescence associated DNA damage *in vivo* and contributes to secretion of major deleterious senescence factors (ROS and the SASP).

In order to understand the role of the mitochondrial biogenesis regulator PGC-1β *in vivo* we analysed liver samples from *PGC-1β*$^{-/-}$ mice. We found that PGC-1β deficiency diminishes senescence markers *in vivo*. *PGC-1β*$^{-/-}$ mice present lower mitochondrial content (mtDNA copy number and expression of mitochondrial proteins) and decreased telomere-associated foci. Moreover, absence of PGC-1β ameliorates age-dependent decline in glucose and insulin

tolerance. Altogether, our results support a causal link between PGC-1β-dependent mitochondrial mass increase and the DDR in the development of senescence *in vivo*. To test the physiological relevance of these observations, it would be important to perform lifespan experiments in mice lacking PGC-1β with an assessment of further senescence and age-related parameters.

Consistent with the hypothesis that mitochondrial mass increase stabilises the senescent arrest and helps maintain tumour suppression *in vivo*, we found that mouse liver tumours have significantly lower mtDNA copy number and reduced expression of PGC-1β and mitochondrial proteins. We also found that the lipid peroxidation marker 4-HNE intensity was markedly reduced in tumour regions as were DDF. Similarly, a persistent DDR at telomeres has been recently associated with the tumour suppressor properties of cellular senescence in nevi melanocytes but absent in malignant melanoma (Suram et al. 2012). These observations suggest that mitochondria might be a tumour-suppressor factor by promoting senescence *in vivo*. Indeed, it is long known that many cancer cells present enhanced glycolysis and diminished oxidative phosphorylation capacity when compared to their normal counterparts (Warburg 1956, Zheng 2012). This shift from mitochondrial oxidative phosphorylation to glycolysis is known as the Warburg effect (Warburg 1956). Our results are suggestive of mitochondria as a putative tumour suppressor factor by inducing senescence *in vivo* (Figure 5.9). Nonetheless, despite being promising, these observations require further experiments to prove tumour suppressor qualities of mitochondria, for instance longitudinal studies on *PGC-1β*$^{-/-}$ mice aiming to assess tumour formation with age.

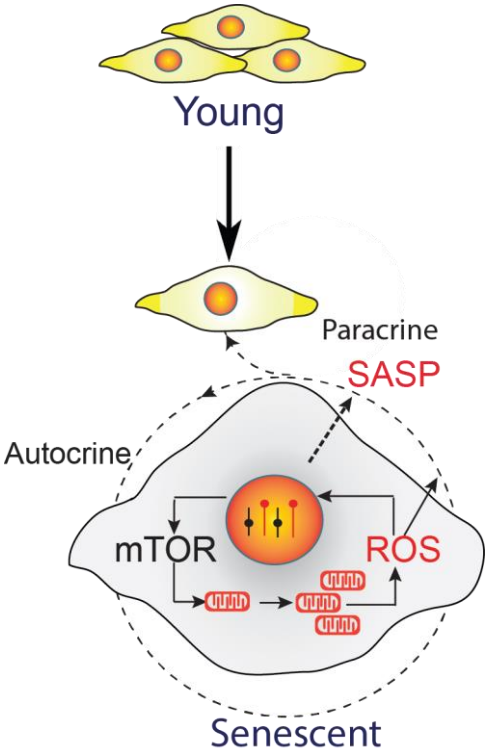

Figure 5.9 | Mitochondria as a key promoter of senescence *in vivo*. Scheme represents overall hypothesis: feedback loop between DDR, mTOR and mitochondrial biogenesis are involved in the tumour suppressor properties of senescence (stabilisation of the arrest), but they also impact on ROS and the SASP which can lead to tissue impairment via paracrine effects.

The exact mechanisms by which mitochondria contribute to the lengthier process of ageing are complex. Our results suggest that there is a mTORC1 upregulation-dependent mitochondrial mass increase with age via PGC-1β. We consider that mitochondrial density is an important factor on cellular ageing, however we do not exclude that other mitochondrial changes occur during ageing namely a decline in mitochondrial function.

Chapter 6. Pro-inflammatory cytokines impact on cellular senescence via mitochondrial dysfunction

Considering the multiple factors secreted by senescent cells, pro-inflammatory cytokines are amongst the most prominent SASP factors. At the same time that they are necessary to restrain tumour progression by stabilising cellular senescence both *in vitro* (Acosta et al. 2008, Kuilman et al. 2008) and *in vivo* (Xue et al. 2007), they also influence the neighbouring environment in a pro-tumorigenic manner by promoting cell growth, angiogenesis and metastasis (Coppé et al. 2006, Campisi et al. 2007). Interleukin-8 (IL-8), also known as CXCL8, belongs to the CXC amino acid motif family of pro-inflammatory chemokines that are highly secreted by senescent cells (Coppé et al. 2010). IL-8 is a chemotactic factor whose main functions are to stimulate cell proliferation, angiogenesis and migration (Waugh et al. 2008, Ning et al. 2011). This chemotactic factor was first described to promote the directional migration and activation of neutrophils in response to inflammatory and infectious diseases (Baggiolini et al. 1989, Baggiolini et al. 1997). More recent studies have recognised IL-8 as an important factor in cancer by promoting tumorigenesis (cell proliferation), its maintenance (angiogenesis) and progression/invasiveness (migration) (Waugh et al. 2008). IL-8 expression is mainly regulated by the nuclear factor-κB (NF-κB); its promoter contains a NF-κB element that is required for its transcriptional activation in all cell types. Although, IL-8 promoter can also be recognised by the activator protein (AP)-1 and the CAAT/enhancer-binding protein (C/EBP), these sites have been described to participate in the transcriptional activation of IL-8 only in some cells types (Hoffmann et al. 2002). Once IL-8 expression is inducted by these transcription factors, it can activate a variety of signalling pathways through binding to two membrane receptors, CXCR1 (IL-8RA) and CXRC2 (IL-8RB). (Holmes et al. 1991, Murphy et al. 1991, Holmes et al. 2009). CXCR1 and 2 are promiscuous G-protein coupled receptors with affinity for several CXC

chemokines. Besides binding to IL-8, CXCR1 can also bind to GCP-2 (CXCL6) and to the neutrophil-activating protein (NAP)-2 (CXCL7), whereas CXCR2 can additionally bind to CXCL1, 2 and 3 (GRO α, β and γ), CXCL5 (ENA-78), GCP-2 and NAP-2 (Brat et al. 2005). Both IL-8 and CXCR2 have been shown to reinforce cellular senescence (Acosta et al. 2008, Kuilman et al. 2008) but the mechanisms through which this cytokine and its receptor contribute to a permanent cell cycle arrest are still not clear.

Previous and my own work have shown (Passos *et al.* 2010; see Chapters 3 and 4) that mitochondria and resulting ROS production are key factors in the establishment and maintenance of cellular senescence. It is also known that pro-inflammatory cytokines, namely IL-8, can reinforce senescence (Acosta et al. 2008, Kuilman et al. 2008). However, how these two main features of senescence act together to induce and preserve a permanent cell cycle arrest is still not clear. In this chapter we examined the links between IL-8 and its receptors and mitochondrial dysfunction and ROS generation. Moreover, we tested the involvement of the mTOR pathway in the process.

6.1 IL-8 expression increases during cellular senescence

Consistent with previous reports (Coppe et al. 2008, Davalos et al. 2010), we have observed increased secretion of several pro-inflammatory cytokines in senescent (10 days after 20Gy) MRC5 fibroblasts (Figure 6.1A). Performing an independent ELISA, in collaboration with Jodie Bitch in our lab, we confirmed that IL-8 secretion gradually increases with the development of the senescent phenotype in irradiation-induced senescent fibroblasts.

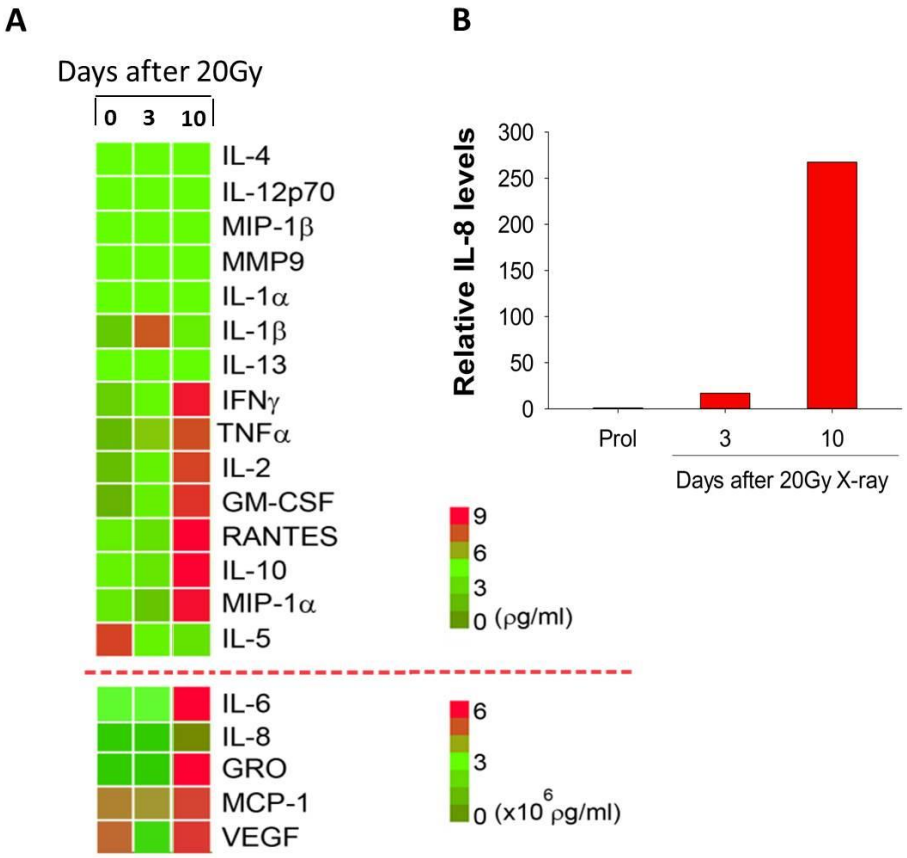

Figure 6.1 | IL-8 highly expressed by senescent cells. (A) Secreted protein array of a variety of inflammatory proteins following X-ray induced senescence in MRC5 fibroblasts (3 and 10 days after 20Gy); Data are mean of 3 independent experiments; **(B)** Levels of secreted IL-8 detected by ELISA on proliferating and irradiated MRC5 fibroblasts at the indicated time-points. (n=1 experiment).

To understand the impact of IL-8 in senescence, we reduced IL-8 signalling by either knocking down IL-8 and its receptors (CXCR1 and CXCR2) or using neutralising antibodies against IL-8 receptors in irradiated-induced or replicative senescent MRC5 fibroblasts (Figure 6.2).

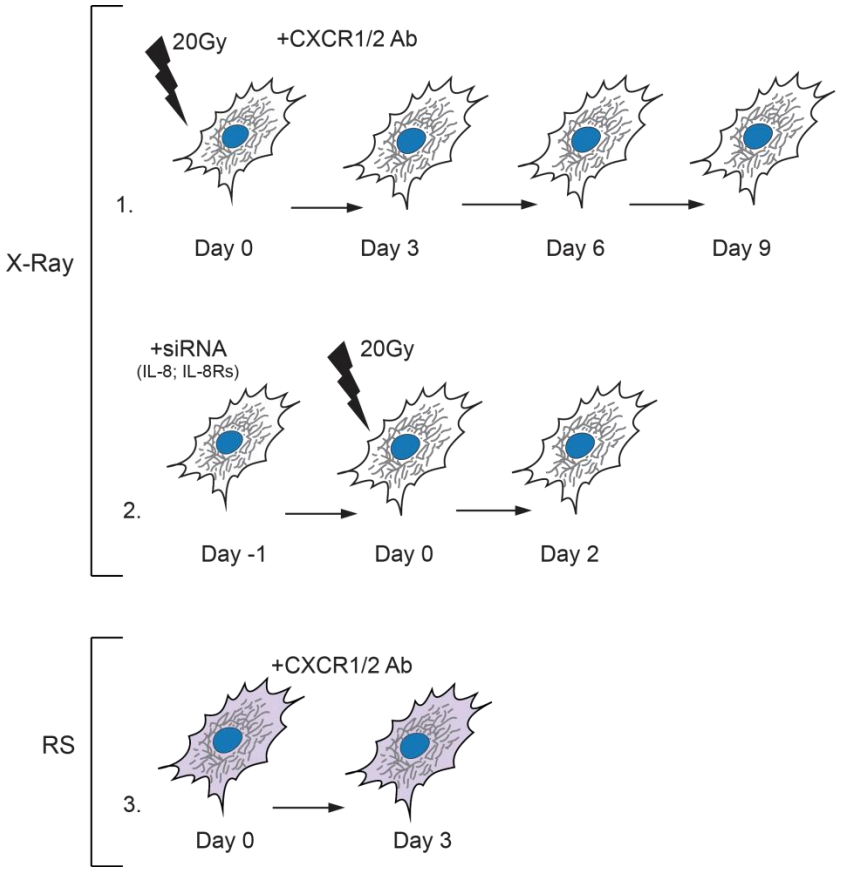

Figure 6.2 | Inhibition of IL-8 signalling in irradiation-induced and replicative senescent fibroblasts. Scheme illustrating the experimental design: 1. MRC5 fibroblasts were irradiated (20Gy X-ray) and immediately treated with neutralising antibodies against CXCR1 and CXCR2 for 3, 6 or 9 days; 2. MRC5 fibroblasts were treated with siRNA against IL-8, CXCR1 and CXCR2 24 hours before irradiation (20Gy X-ray), cells were harvested 2 days after irradiation (72 hours of siRNA); 3. Replicative senescent MRC5 fibroblasts were treated with neutralising against CXCR1 and CXCR2 for 3 days.

6.2. IL-8Rs Inhibition alleviates the senescent phenotype

Senescent cells can be distinguished from their young proliferating counterparts by their cellular morphology characterised by increased cellular volume, loss of the original cellular shape and a flattened cytoplasm. To analyse whether inhibition of IL-8 receptors would abrogate cell size increase we treated both irradiation-induced and replicative senescent MRC5 fibroblasts with CXCR1 and/or CXCR2 neutralising antibodies. Treatment with IL-8Rs neutralising antibodies significantly decreased cell size in both irradiation-induced and replicative senescent MRC5 fibroblasts when using a combination of the two (CXCR1/2) antibodies (Figure 6.3). The results support the idea that IL-8 receptors signalling inhibition can reduce cell size increase during induction of cellular senescence.

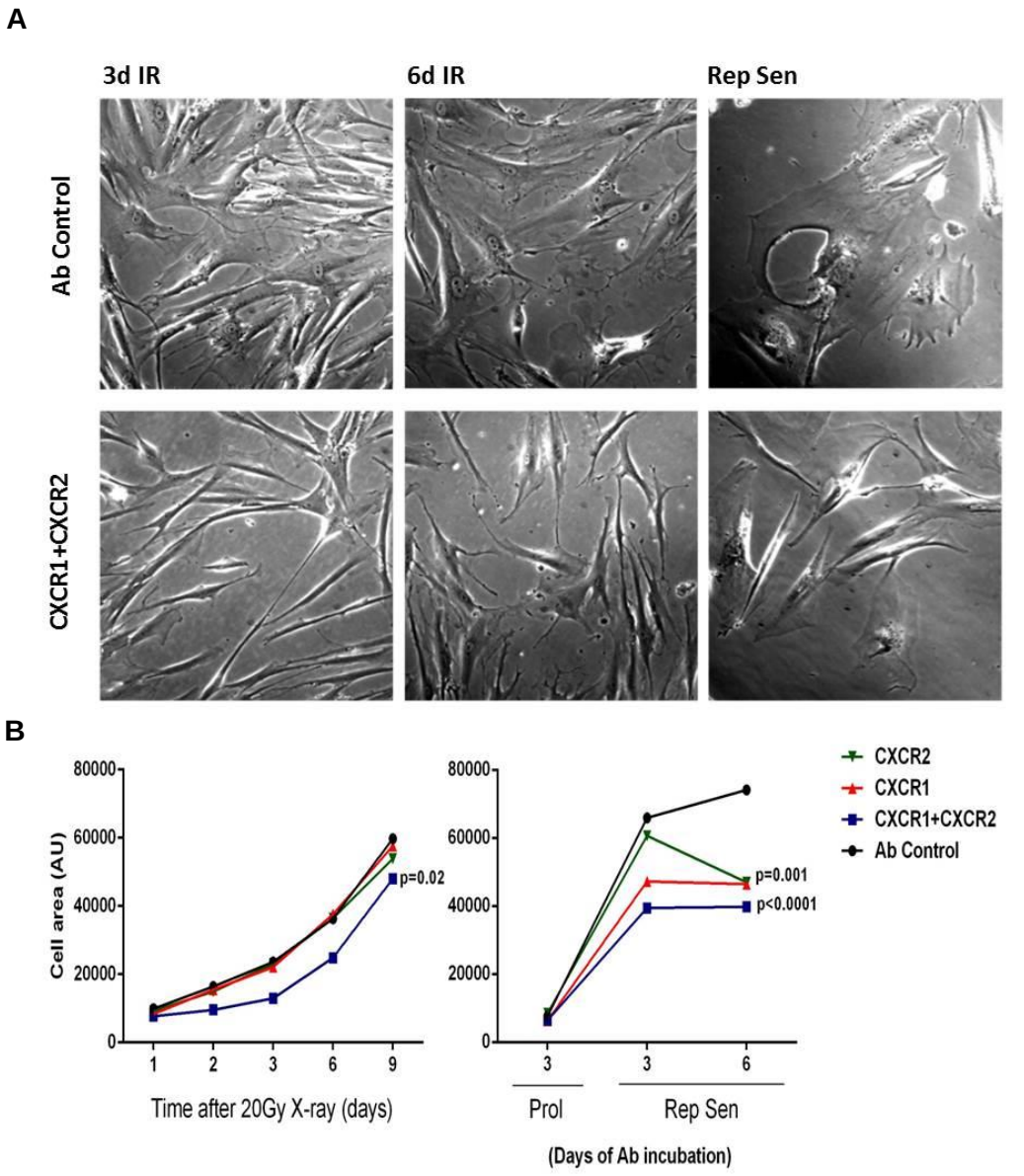

Figure 6.3 | IL-8Rs inhibition rescues cell size in senescent cells. (A) Phase contrast images of senescent MRC5 fibroblasts showing a rejuvenated phenotype upon IL-8Rs neutralisation in both irradiation-induced (3 and 6d IR) and replicative senescence (RS); **(B)** Quantitative analysis of cellular size upon IL-8Rs neutralisation both in irradiation-induced and replicative senescence (left and right respectively). Data are median cell size at the indicated time points after irradiation or duration of antibody incubation in replicative senescent cells. Statistical analysis were performed using Mann-Whitney Test, n=100 cells/treatment.

To further characterise the impact of IL-8Rs inhibition on cellular senescence, we assessed for Sen β-Gal activity. We found that treatment with IL-8Rs neutralising antibodies significantly decreased Sen-β-Gal activity in irradiation induced senescent fibroblasts (Figure 6.4).

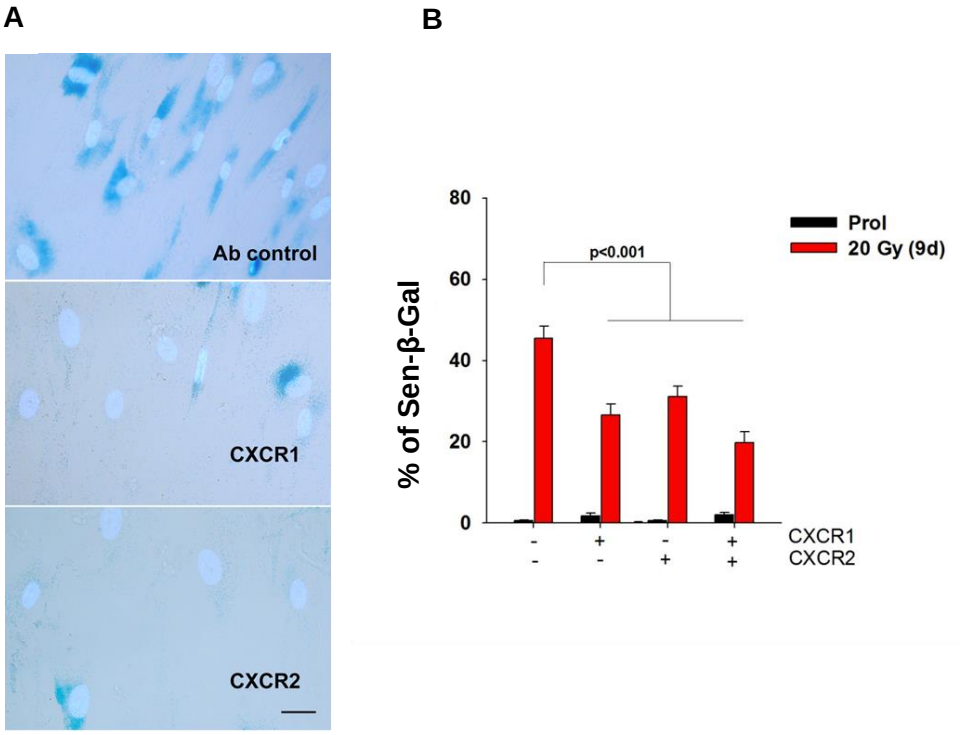

Figure 6.4 | IL-8Rs inhibition decreases Sen-β-Gal activity in senescence. (A) Representative images of Sen-β-Gal cytochemistry staining in irradiated MRC5 fibroblasts upon IL-8Rs neutralisation. Sen-β-Gal positive cells are stained dark blue, whereas light blue fluorescence (DAPI) indicates DNA content; Scale bar = 20μm; **(B)** Quantitative analysis of Sen-β Gal activity in MRC5 fibroblasts at 9 days after irradiation following IL-8Rs neutralisation. Data are Mean ± SEM, n=3 independent experiments (200 cells were analysed per treatment per experiment). Statistical analysis was performed using One Way ANOVA Test.

6.3 Inhibition of IL-8 activity ameliorates the senescent phenotype by improving mitochondrial function in cellular senescence

Mitochondria are central players in oxidative energy metabolism. As a consequence of mitochondrial dysfunction, senescent cells have been described to generate higher levels of ROS when compared to their young proliferating counterparts (Passos et al. 2007). Mitochondrial dysfunction in senescence has been characterised by an increase in mitochondrial mass (as described on Chapters 3 and 4) and a decrease in the mitochondrial membrane potential (depolarization of the membrane potential) with subsequent increased ROS formation (Passos et al. 2007). To evaluate the role of IL-8Rs in mitochondrial function in senescence, irradiated MRC5 fibroblasts were treated with CXCR1 or 2 neutralising antibodies for 3 and 6 days after irradiation. To assess for mitochondrial mass cells were stained with NAO and fluorescence intensity was measured by flow cytometry. Induction of senescence by irradiation led to an increase in mitochondrial mass which was reduced following IL-8Rs inhibition (Figure 6.5A).

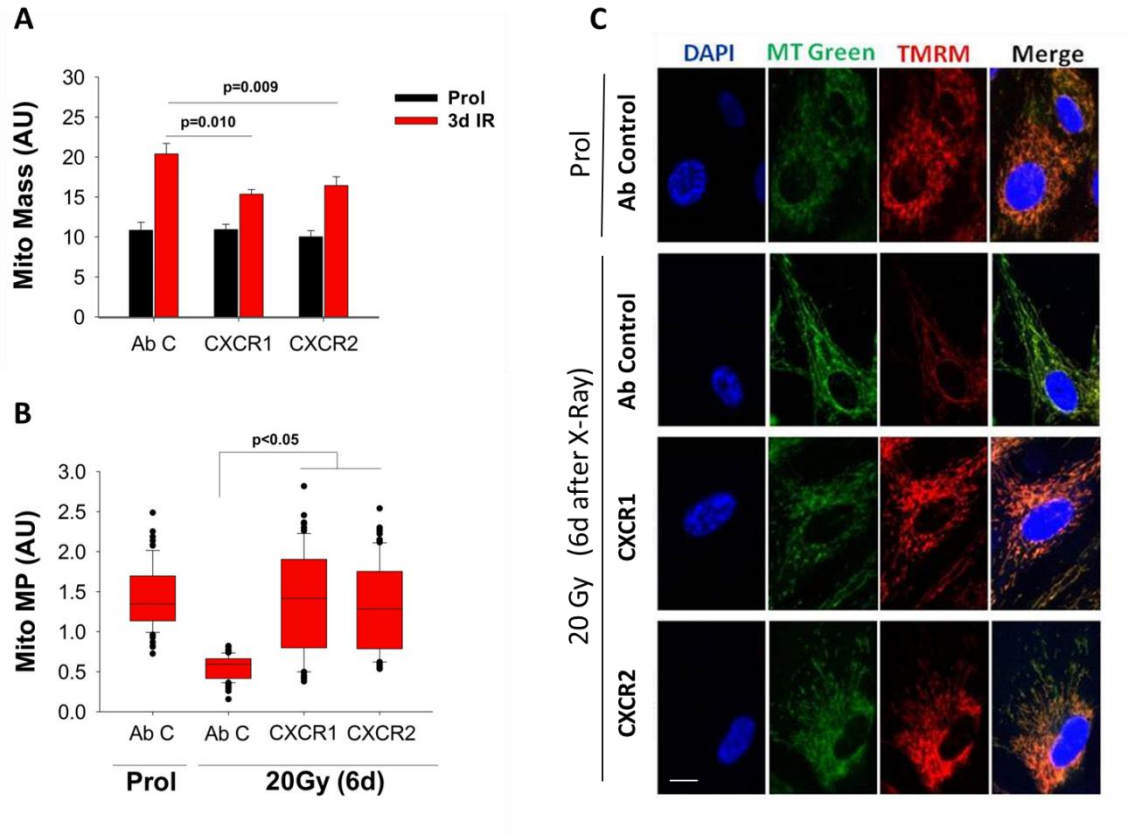

Figure 6.5 | IL-8Rs inhibition improves mitochondrial function in cellular senescence. (A) Mitochondrial Mass (Mito Mass) in irradiation-induced senescence. NAO intensity in non-irradiated (Prol) and irradiated (3d after 20 Gy) MRC5 fibroblasts treated with neutralising antibodies as indicated. Data are Mean ± SEM, n=3 independent experiments. Statistical analysis was performed using One way ANOVA. **(B)** Mitochondrial Membrane Potential (MMP) in irradiation-induced senescent MRC5 fibroblasts. Quantification of TMRM/Mito Tracker Green ratios. Box plots represent median, upper and lower quartiles, percentiles and outliers; n=3 independent experiments (100 cells were analysed per treatment per independent experiment). Statistical analysis was performed using ANOVA on Ranks Test. **(C)** Representative TMRM (red) and Mito Tracker Green (green) fluorescence images of irradiated MRC5 fibroblasts following CXCR1 and CXCR2 neutralisation; Scale bar = 10µm .

Mitochondrial Membrane potential (MMP) was measured using the fluorescent dye Tetramethylrhodamine methyl ester (TMRM), a cationic and mitochondria selective probe that can be assessed by several fluorescence analysis methods (including fluorescence microscopy) (Floryk et al. 1999). Because TMRM changes the intensity but not the emission spectra in response to membrane potential it is a good marker of mitochondrial membrane potential in combination with Mito Tracker Green, a marker of mitochondrial mass (TMRM/MT Green). Mitochondrial membrane potential (MMP) was significantly decreased following induction of senescence and rescued upon neutralisation of CXCR1 or 2 in senescent MRC5 fibroblasts 6 days after irradiation (Figure 6.5B and 6.5C). Following the observation that neutralisation of CXCR1 or 2 improved mitochondrial function, we questioned whether compromised IL-8 signalling would affect ROS generation in senescence. For this purpose, we decreased IL-8 activity either by small interference RNA (siRNA) knockdown of IL-8 and its receptors or using neutralising antibodies against IL-8Rs in both irradiation-induced and replicative senescent cells (see Figure 6.2). To measure ROS levels (superoxide levels), cells were stained with Dihydroethidium (DHE) and fluorescence intensity was measured by flow cytometry. In all cases (knockdown of IL-8 and IL-8Rs or neutralisation of IL-8Rs), decreased IL-8 activity resulted in significantly reduced ROS generation in senescent cells (Figure 6.6).

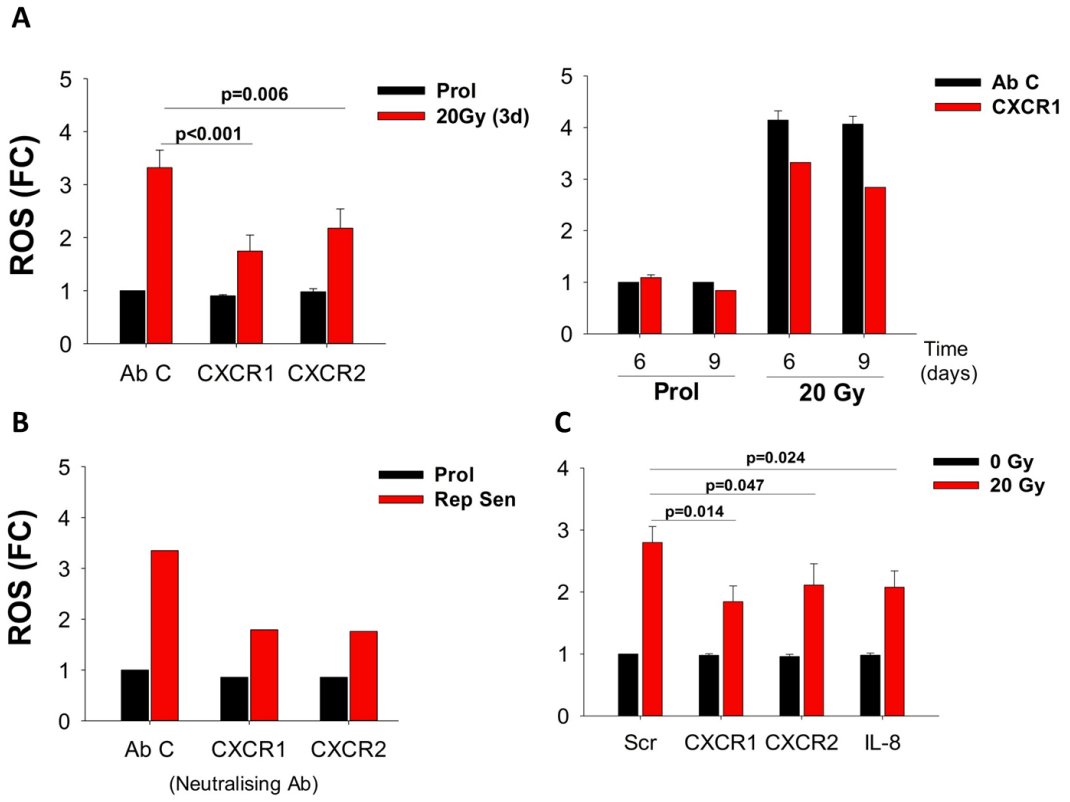

Figure 6.6 | Reduced IL-8 signalling decreases ROS generation in senescent cells. **(A)** Inhibition of IL-8Rs decreases ROS levels in irradiated (20Gy X-radiation) MRC5 human fibroblasts upon short term (3 days) and long term (6 and 9 days) incubation with IL-8Rs neutralising antibodies (left and right graphs respectively). Data are Mean ± SEM n=3 on the left and Mean ± SD, n=2 on the right. **(B)** ROS levels in replicative senescent MRC5 fibroblasts following inhibition of CXCR1 and CXCR2 with neutralising antibodies (3 days antibody incubation). Data are from one experiment (n=1). **(C)** IL-8 and IL-8Rs knockdown using siRNA (72h) in MRC5 fibroblast 2 days after Irradiation (20Gy X-radiation). Data are Mean ± SEM n=3 independent experiments. Statistical analysis was performed using One Way ANOVA Test.

6.4 IL-8 signalling inhibition reduces ROS-dependent DNA damage foci in senescence

Our group has previously shown that elevated ROS levels during senescence contribute to DNA damage and are responsible for the stability for the permanent cell cycle arrest in senescent cells (Passos et al. 2010). Having observed that decreased IL-8 signalling reduced ROS generation during senescence, we question whether decreased IL-8 activity would also impact on DNA damage. For this purpose, irradiated fibroblasts treated for 72 hours with siRNA against IL-8 and its receptors were immunostained for γH2AX, a marker of DNA double strands breaks. Knockdown of IL-8 and IL-8Rs significantly decreased the number of DNA damage foci per cell compared to that of the scrambled control (Figure 6.7A and 6.7B). Additionally, an alkaline comet assay was performed as an independent method to assess DNA damage (single and double strand breaks). Data shows that CXCR1 and 2 neutralisation significantly decrease DNA breaks at 3 days after 20Gy in MRC5 fibroblasts (Figure 6.7C). Interestingly, CXCR1 and 2 neutralisation had no impact on DNA damage repair capability immediately following X-ray irradiation (up to 6 hours). We have previously shown that ROS levels in senescence increase 2-3 days following a DDR and can induce more DNA damage in a positive feedback loop that stabilises cellular senescence (Passos et al. 2010), supporting the idea that the later (3 days after X-radiation) increase in DNA is ROS driven and the decrease on DDF following IL-8Rs neutralisation may result from ROS generation repression. Together our results show that decreased IL-8 signalling, by either neutralisation or knockdown of IL-8 and its receptors, reduces DNA damage in senescence possibly as a result of decreased ROS levels.

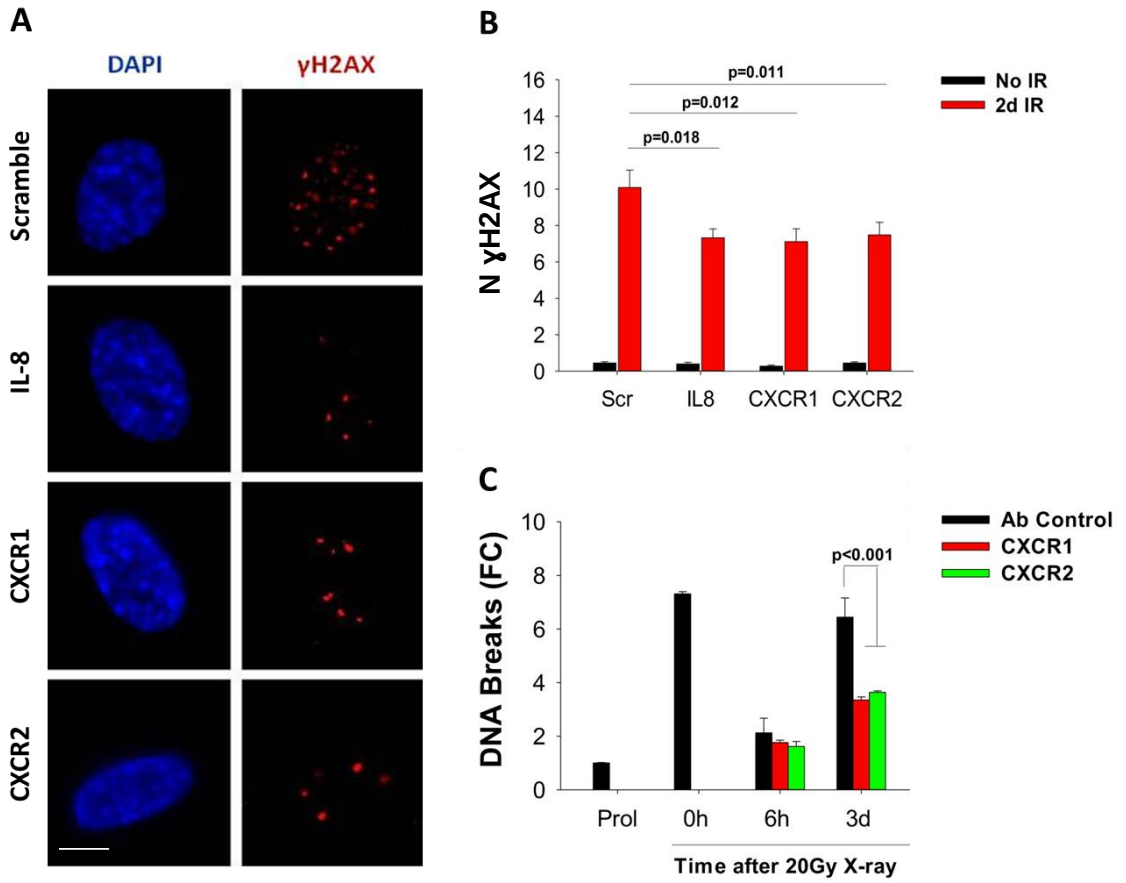

Figure 6.7 | IL-8 and IL-8Rs inhibition decreases DNA damage in cellular senescence. (A) Representative γH2AX foci immunofluorescence images of irradiated MRC5 fibroblasts following siRNA knockdown of IL-8 and IL-8Rs, 2 days after irradiation. γH2AX is indicated by red fluorescent foci whereas blue fluorescence (DAPI) indicates DNA content; Scale bar = 10μm; **(B)** Quantitative analysis of numbers of γH2AX foci per nucleus by immunofluorescence staining in irradiated MRC5 following knockdown of IL-8 and IL-8Rs. Data are Mean ± SEM, n=3 independent experiments. **(C)** Alkaline comet assay showing DNA breaks fold change (FC) following IL-8Rs inhibition using neutralising antibodies in irradiated MRC5 fibroblasts. Data are Mean ± SEM, n=3 independent. Statistical analysis was performed using One Way ANOVA Test.

6.5 IL-8 and IL-8Rs inhibition does not rescue cell proliferation in Irradiation-induced senescence

Consistent with a function for IL-8 as a pro-proliferation cytokine (Waugh et al. 2008), we found that IL-8 signalling inhibition significantly decreased cell proliferation in non-irradiated cells (Figure 6.8A) and did not rescue cell proliferation upon induction of senescence with 20Gy X-radiation in MRC5 fibroblasts (Figure 6.8B). Lawless *et al*. proposed that absence of a proliferation marker (e.g. Ki-67) and the presence of more than 5 DDF (e.g. γH2A.X) is a robust marker of senescence (Lawless et al.). We have performed Ki-67/γH2A.X double immunostaining on MRC5 fibroblasts following IL-8 and IL-8R knockdown in irradiation-induced senescent fibroblast. We found that a small percentage of non-irradiated cells (0Gy) were positive for γH2A.X >5 foci and Ki-67, possibly as a result of replication stress (DNA replication). Nevertheless, knockdown of IL-8 and IL-8Rs significantly reduced the proportion of cells negative for Ki67 with more than 5 γH2A.X foci (Figure 6.8C), indicating that despite still being locked in the cell cycle, these cells harbour less DNA damage.

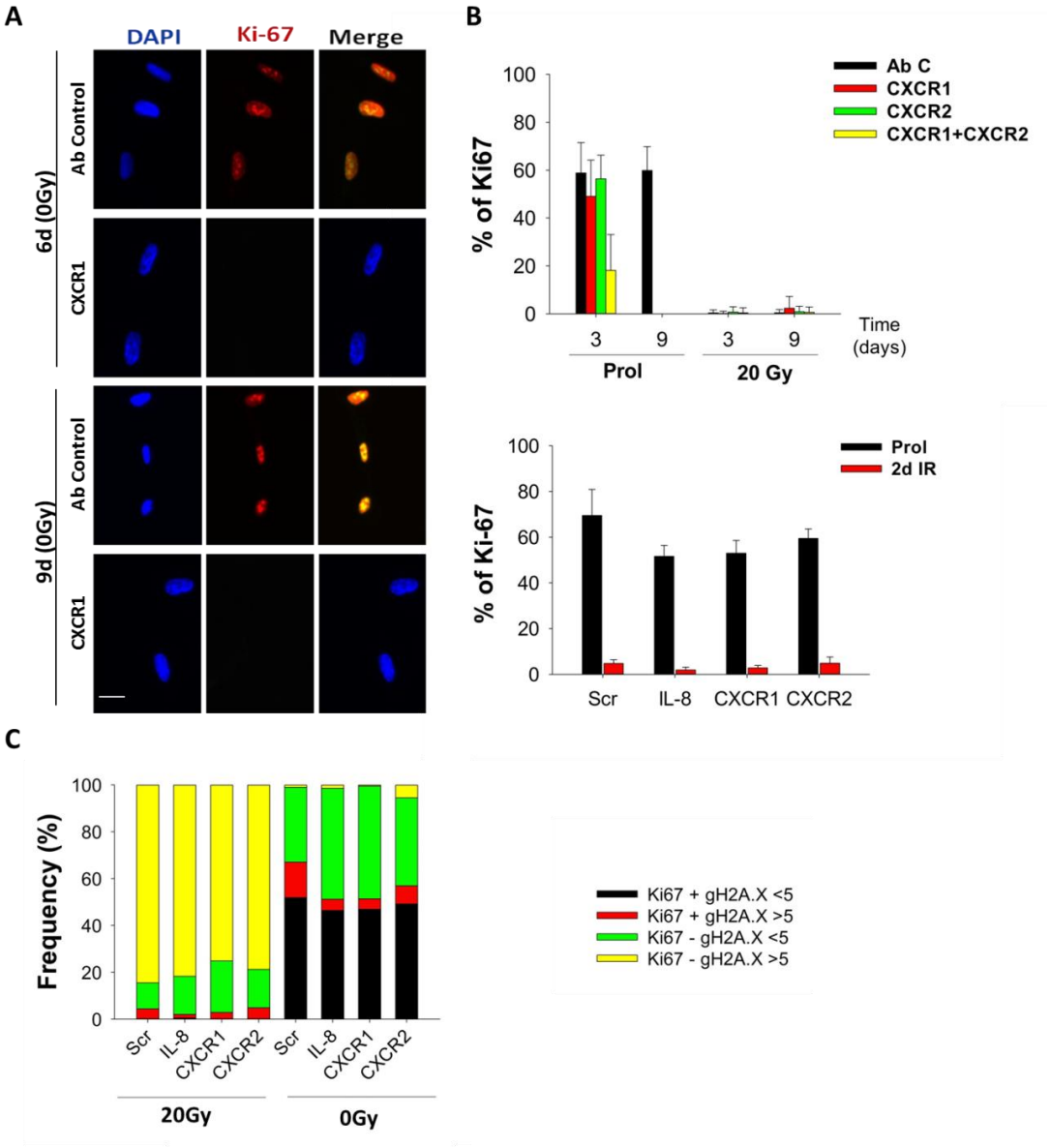

Figure 6.8 | Inhibition of IL-8 signalling does not rescue cell proliferation in irradiation-induced senescence (A) Representative Ki-67 immunofluorescence images of non-irradiated (0Gy) MRC5 fibroblasts upon 6 and 9 days of CXCR1 neutralising antibody incubation; Scale bar = 20µm; **(B)** Quantitative analysis of Ki-67 positive cells at the indicated times points after 0Gy or 20Gy X-radiation following (top) neutralisation of IL-8Rs and (bottom) knockdown of IL-8 and its receptors in MRC5 fibroblasts. Data are Mean ± SEM of n=3 independent experiments; **(C)** Quantitative analysis of percentage of Ki-67 (positive and negative), and number of γH2A.X foci (<5 foci or >5 foci) upon IL-8 and IL-8Rs knockdown in 0Gy or 20 Gy X-radiation treated MRC5 fibroblasts. Data are mean ± SEM of n=3 independent irradiation. Statistical analysis was performed using One way ANOVA Test.

6.6 IL-8 stabilises cellular senescence via mitochondrial dysfunction and ROS generation through mTOR

In previous chapters we have shown that mTOR is a central mediator of mitochondrial biogenesis in cellular senescence by regulating PGC-1β co-transcription factor. We have also shown that repression of mTOR abrogates the senescent phenotype in a similar fashion to that observed following IL-8 signalling inhibition. In addition, it is well-established that mTOR can be activated downstream of G protein-coupled receptors (O'Hayre et al. 2014). This way we hypothesised that IL-8/IL-8Rs and mTOR may be involved in the same pathway that regulates mitochondrial function in senescence. To explore our hypothesis, we started by neutralising both CXCR1 and CXCR2 and assess for mTOR activity upon induction of senescence. We found that treatment with neutralising antibodies against CXCR1/2 decreased phospho-p70S6K in senescent MRC5 fibroblasts (Figure 6.9A), indicating that mTOR activity is reduced when IL-8Rs are repressed. Following these observations, we double knocked down IL-8/IL-8Rs and mTOR in MRC5 fibroblasts and assessed for ROS production in senescence. Individual knockdowns of IL-8, IL-8Rs and mTOR significantly decrease ROS generation in MRC5 fibroblasts 2 days after 20Gy as previously shown. Double knockdown of IL-8/IL-8Rs and mTOR also significantly decreased ROS generation in irradiated fibroblasts but with no cumulative effect (Figure 6.9B), suggesting that IL-8 and mTOR may act together in the same pathway that regulates mitochondrial function to stabilise cellular senescence. A similar non-cumulative effect was observed when assessing DNA damage foci following double knockdown of IL-8 and mTOR (Figure 6.9C).

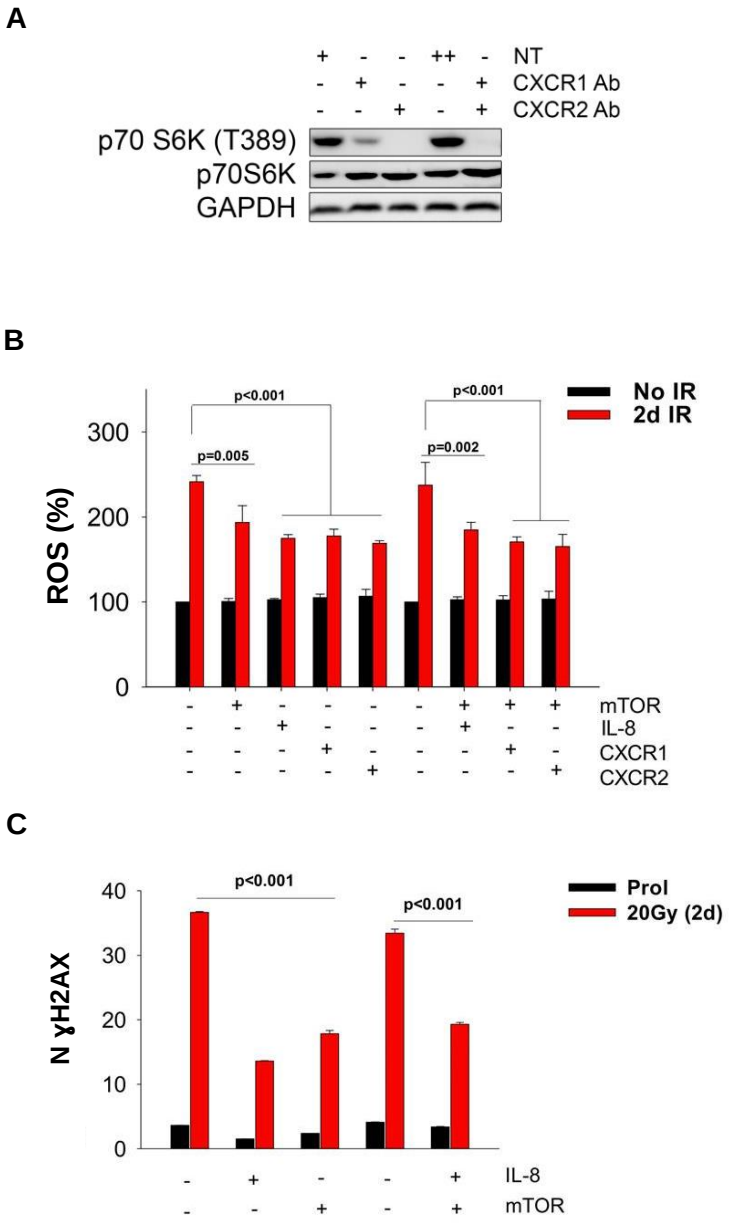

Figure 6.9 | IL-8 impacts on mitochondrial function via mTOR during senescence. **(A)** Representative western blots of mTOR activity measured by phosphorylation levels of p70S6K (T389) 3 days after 20 Gy X-radiation (data are representative of 2 independent experiments); **(B)** ROS (DHE intensity) levels following double knockdown of IL-8/IL-8Rs and mTOR in irradiated MRC5 fibroblasts (2 days after 20Gy X-ray). Data shows Mean ± SEM, n=3 independent experiments; **(C)** Quantitative analysis of γH2AX immunofluorescence staining in irradiated MRC5 following double knockdown of IL-8 and mTOR. Data are Mean ± SEM, n=3 independent experiments. Statistical analysis was performed using One way ANOVA statistical test.

6.7 Discussion

Reports have shown that SASP factors can trigger and reinforce senescence in a paracrine and autocrine fashion, among these factors interleukins encompass some of the most prominent SASP factors. A recent report showed that the IL-8 receptor CXCR2 and its ligands are upregulated during senescence forming a chemokine network reinforcing senescence in a p53-dependent manner (Acosta et al. 2008). It has also been reported that mitochondria become dysfunctional during senescence, with increased mitochondrial mass and decreased mitochondrial membrane potential being the source of higher ROS in this cellular state (Passos et al. 2010). There is some evidence on ROS and SASP regulation in senescence, but little is known about how ROS and SASP interact during senescence. To explore the impact of pro-inflammatory cytokines on mitochondrial function in senescence, IL-8 (a pro-inflammatory cytokine highly secreted by senescent cells) signalling was inhibited in senescent fibroblasts. The results shown in this chapter are preliminary and require further experimental confirmation, which will be highlighted during this discussion.

Our results show that neutralisation of IL-8Rs significantly decreased mitochondrial mass and improved mitochondrial membrane potential (MMP) in irradiation-induced senescent MRC5 fibroblasts. Despite being promising, our results on MMP using TMRM should be further confirmed with analysis of oxygen consumption rates in whole cells and isolated mitochondria using the Seahorse technology, and the IL-8 impact on mitochondrial mass should also be confirmed by mtDNA copy number, electron microscopy and mitochondrial protein expression analysis. The improvement in mitochondrial function following IL-8 signalling inhibition was accompanied by decreased ROS generation. Consistent with our results are previous observations that CXCR2 downregulation decreases ROS levels in senescence (Acosta et al. 2008). Furthermore, previous reports have shown that generation of superoxide in response to IL-8 is selectively mediated by CXCR1 in neutrophils (Jones et al. 1996, Jones et al. 1997). However, the CXCR1-dependent ROS generation in

neutrophils has been attributed to non-mitochondrial sources such as NADPH oxidases (Jones et al. 1996). To discriminate the source of ROS generation downstream of CXCR1 and CXCR2 activation we would need to: 1. analyse expression of mitochondrial regulatory proteins such as PGC-1α and β following inhibition of IL-8 signalling; 2. analyse expression of NADPH oxidases and other non-mitochondrial enzymes capable of producing ROS during senescence and assess whether IL-8 signalling inhibition downregulates expression of these enzymes; 3. treat mitochondrial depleted cell (e.g. Parkin-expressing MRC5 fibroblasts) with IL-8 and assess for ROS production and expression of non-mitochondrial enzymes capable of ROS production following induction of senescence.

Senescent cells have been described to contain increased numbers of DNA damage foci which seem to remain constant overtime (Passos et al. 2010, Hewitt et al. 2012). A persistent DNA damage response is a central mediator of cellular senescence and has been shown to play a role in the maintenance of the cell cycle arrest (d'Adda di Fagagna et al. 2003, Passos et al. 2010). Our data showing that Inhibition of IL-8 signalling simultaneously decreases DDF and ROS levels in senescent MRC5 fibroblasts are consistent with previous reports (Acosta et al. 2008) and further supports the positive feedback loop described by Passos *et al*. (2010). We have observed that after X-radiation induced DNA damage cells quickly repair the majority of the damage (within 6 hours as shown in the comet assay Figure 6.6C), new DNA damage is then generated most probably due to increased ROS production resulting from mitochondrial mass increase during senescence (shown in the previous chapters and Figure 6.6C). In this context, we hypothesise that IL-8 signalling inhibition prevents DNA damage formation by reducing mitochondrial mass and ROS generation. To support our hypothesis we would need to perform experiments where downregulation of IL-8 signalling is combined with antioxidant treatments and assess for DNA damage during senescence. We should also analyse expression of other DDR markers such as phosphorylated p53, p21 and p16 to support a role for IL-8 in the induction of a DDR during senescence. Furthermore, interpretation of experiments involving knockdown of

IL-8 and its receptors is limited. Despite several attempts, determination of the knockdown efficiency of siRNA against IL-8 and IL-8Rs was not possible by western blotting or qPCR (we could not see a signal by western blotting and values by qPCR were close to undetectable). We believe that at 2 days after irradiation, expression of these proteins are still too low, yet able to produce an effect. We will need to repeat and confirm these experiments by improving our methodology regarding siRNA efficiency analysis (e.g. increasing protein loading for western blotting; increasing cDNA amount for qPCR or performing another method of protein visualisation such as immunofluorescence).

Further characterising the role of IL-8 inhibition in the senescence process, we found that neutralisation of IL-8Rs decreases Sen-β-Gal activity and cell size in senescent fibroblasts with no rescue in cell proliferation in irradiated fibroblasts following neutralisation or knockdown of IL-8 and its receptors. Consistent with our results is the fact that IL-8 is a potent pro-proliferating chemokine (Waugh et al. 2008). Although absence of cell proliferation is not an intrinsic marker of senescence but in combination with other markers of senescence it is a reliable marker of senescence. Consistent with our results showing a role for IL-8 in senescence, particularly on DNA damage, knockdown of IL- 8 and IL-8Rs significantly reduced the proportion of cells negative for Ki-67 with more than 5 γH2A.X foci (senescent cells). Once more, siRNA efficiency needs to be confirmed in these experiments.

In order to mechanistically understand how IL-8 regulates mitochondrial function in senescence, we correlated our previous data showing that mitochondrial mass increase in senescence is mTOR dependent and assessed for mTOR activity following IL-8 signalling inhibition. Consistent with previous reports showing that G-protein coupled receptors, such as CXCR1 and CXCR2 (IL-8Rs), can activate mTOR (O'Hayre et al. 2014), we found that IL-8Rs neutralisation results in reduced p70S6K phosphorylation, hence reduced mTOR activity, following induction of senescence. Further supporting our observations, IL-8 has been shown to induce changes in protein expression through regulation of translation associated proteins: IL-8 signalling promotes

multisite phosphorylation of the ribosomal S6 kinase as a consequence of mTOR activation (MacManus et al. 2007). Consistent with a role in the same pathway regulating mitochondrial function on senescence, we found that double knockdown of IL-8/IL-8Rs and mTOR significantly decrease ROS generation and DDF in senescent fibroblasts in a non-cumulative fashion. We have indirect evidence that IL-8 impacts on mitochondrial mass via mTOR during senescence, however we would need to perform double inhibition of IL-8 and mTOR signalling to analyse the effect of downregulation of these pathways on mitochondrial content during senescence (e.g using fluorescence dyes and/or EM).

We have shown that IL-8 impacts on ROS, bioactive molecules involved in autocrine and paracrine induction of senescence (Passos et al. 2010, Nelson et al. 2012), via a pathway that seems to be dependent on mTOR. Nevertheless, we still need to understand the impact of reduced pro-inflammatory stimuli via downregulation of IL-8 signalling on SASP activation, also known to promote autocrine and paracrine effects during senescence (Acosta et al. 2008, Acosta et al. 2013). Previously in this thesis (Chapter 4) we show that mTOR inhibition alleviates the senescence-associated inflammatory phenotype. It may be that inhibition of IL-8 signalling through its regulatory control over mTOR activity, results in downregulation of the SASP. However, these speculations need experimental confirmation.

To summarise, we had previously shown that mTOR stimulates mitochondrial biogenesis and ROS generation leading to DNA damage and the stabilisation of cellular senescence. Furthermore, at the same time that mTOR is activated downstream of IL-8 and its receptors, it is also a key factor in the pathways regulating protein translation and inhibition of mTORC1 by rapamycin represses the SASP. These observations, together with the fact that IL-8 may affect mTOR-dependent mitochondrial function during senescence suggest that IL-8 is part of the positive feedback loop that reinforces the growth arrest. In this context our preliminary results are suggestive of a model where inflammatory cytokines promote mTOR-dependent mitochondrial biogenesis leading to

increased ROS production and SASP factor secretion (e.g. pro-inflammatory cytokines), which continuously contribute to DNA damage and induce a persistent DDR able to activate mTOR (Figure 6.10).

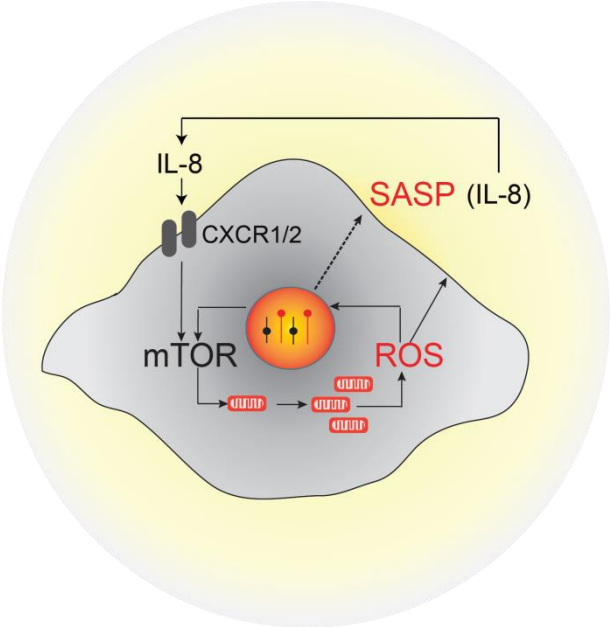

Figure 6.10 | IL-8 stabilises cellular senescence via mTOR-dependent mitochondrial dysfunction. IL-8 activates mTOR-dependent mitochondrial biogenesis. Higher mitochondrial content results in increased ROS production and ROS-driven DNA damage, which is in turn able to activate mTOR closing this way a positive feedback loop (Correia-Melo and Marques *et al.* unpublished). Persistent DNA signalling can also trigger the senescence-associated secretory phenotype (SASP) including secretion of IL-8 (Rodier et al. 2009). IL-8 and its receptors can reinforce senescence in an autocrine fashion (Acosta et al. 2008), closing this way another positive feedback loop that stabilises cellular senescence.

Chapter 7. Conclusions

The implications of senescence in physiological and pathological settings, such as ageing and cancer, have gained firm ground. It is therefore important to learn more about the mechanisms underpinning the establishment and maintenance of senescence. Autocrine and paracrine effects of senescence are dependent on the development of the so-called senescent phenotype, which involves over-production of pro-inflammatory and pro-oxidant signals. However the exact mechanisms underlying induction of such a phenotype remain incompletely understood. In this thesis we aimed to comprehend how mitochondria and pro-inflammatory factors interact during senescence and how they contribute to the severity of the senescent phenotype.

Despite being numerous, the studies on the impact of mitochondria on the ageing process have so far failed to establish the fundamental importance of these organelles on cellular senescence. The work done in this PhD thesis has first shown that there is an increase in mitochondrial mass following DDR stimuli and this correlates with markers of senescence, including increased Sen-β-Gal activity, number of DDF and expression of the cyclin-dependent kinase inhibitor p21 and absence of proliferation ability. It was also demonstrated that changes in mitochondrial mass are not an adaptive process driven by ROS, but potentially the driver of ROS generation in senescence. Secondly, by artificially depletion of mitochondria, we demonstrate that these organelles are necessary for the development of the aging-promoting characteristics of the senescent phenotype (including ROS and the SASP). Our observations are of great therapeutic interest, since manipulation of mitochondrial content would diminish/abrogate the deleterious effects of senescent cells in an organism. However, elimination of mitochondria rescued proliferation in fibroblasts following induction of senescence despite their inability to rely on oxidative phosphorylation. A possible explanation relies on the fact that these cells

shared phenotypic traits of cancer cells. Interestingly, no recue in cell proliferation was observed in replicative senescent fibroblasts depleted of mitochondria, suggesting that mitochondria is important to restrain cells proliferative capacity in the very first states of the development of senescence as observed in irradiation-induced senescence (7-10 to develop a senescent phenotype), but not when senescence is fully established. Furthermore, our work showed that interventions which reduce the load of mitochondria in cells, such as inhibition of mTOR and depletion of PGC-1β, have similar impact on the senescent phenotype. Considering these observations, the extent of mitochondrial elimination should be taken into account when placing mitochondria as a putative therapeutic target in senescence; the impact of mitochondrial content on the tumour-suppressive/promoting ability in a cell is dependent on the "state" of cellular senescence (whilst developing or in the presence of a fully developed senescent phenotype). Mechanistically, we showed that ATM, Akt and mTOR phosphorylation cascades, downstream of a DDR, promote mitochondrial biogenesis and mitochondrial-ROS production which contributes to a persistent DDR that keeps cells locked in the cell cycle.

The role of mitochondria in animal physiology has been the subject of extensive investigation with mitochondrial dysfunction being considered as a major hallmark of ageing, underlining the significance of mitochondrial homeostasis for survival (López-Otín et al. 2013). A rigorous regulation of mitochondrial mass, distribution and activity is fundamental for cellular homeostasis and maintenance. Despite evidence proposing a role for mitochondria in ageing and age-associated diseases (Vafai et al. 2012, Malpass 2013), mitochondrial dynamics during ageing is still controversial, owing to the fact that interventions promoting longevity, namely DR and sirtuin activators, have been linked with increased mitochondrial biogenesis (Baur et al. 2006, Lopez-Lluch et al. 2006). Here we showed that *in vivo* cellular ageing is accompanied by an mTORC1-PGC-1β dependent mitochondrial mass increase. First we showed that there is an age-dependent increase in mTORC1 activity associated with increased p21, PGC-1β and OXPHOS components in wild-type mice. Secondly, we have demonstrated that the age-dependent mTORC1 hyperactivation promotes

mitochondrial biogenesis via the mitochondrial regulator PGC-1β *in vivo*. Consistent with the hypothesis that mitochondrial mass increase stabilises the senescent arrest and helps maintain tumour suppression *in vivo*, we found that mouse liver tumours have significantly reduced mitochondrial content, suggesting that mitochondria might be a strong tumour-suppressor factor by promoting senescence *in vivo*. Corroborating this hypothesis are reports showing that many cancer cells present enhanced glycolysis and diminished oxidative phosphorylation capacity when compared to their normal counterparts (Warburg 1956, Zheng 2012).

SASP factors can trigger and reinforce senescence in a paracrine and autocrine fashion, among these factors interleukins encompass some of the most prominent SASP factors (Acosta et al. 2008, Acosta et al. 2013). A recent report showed that the IL-8 receptor CXCR2 and many of its ligands are upregulated during senescence and that they form part of a chemokine network reinforcing cellular growth arrest in a p53-dependent manner (Acosta et al. 2008). Considering these observations, we aimed to understand how SASP factors interact with mitochondria during senescence. We showed that inhibition of IL-8 decreases ROS generation possibly by improving mitochondrial function in cellular senescence via the intermediate action of mTOR. Owing to IL-8 pro-proliferating signalling, inhibition of this pro-inflammatory cytokine did not rescue cell proliferation upon induction of senescence, placing IL-8 as putative therapeutic target in senescence as it eases the senescent phenotype without rescuing proliferation of cells harbouring DNA damage.

Bringing together our findings, young proliferating cells are continuously exposed to a plethora of stressors eventually causing irreparable DNA damage. Cells containing irreparable DNA damage undergo a permanent cell cycle arrest (senescence), as a result of a persistent DDR. The DDR induces mTOR-dependent mitochondrial biogenesis resulting in higher ROS production and additional induction of DNA damage (feedback loop 1). The DDR also triggers the senescence-associated secretory phenotype (SASP), particularly the inflammatory phenotype (Rodier et al. 2009). The inflammatory phenotype,

particularly IL-8 and its receptors reinforce senescence in an autocrine fashion (Acosta et al. 2008) (feedback loop 2). Activation of mTOR via IL-8 - CXCR1/2 is the linking factor between the two main circle pathways (feedback loop 1 and 2) that stabilise cellular senescence (Correia-Melo *et al.* unpublished). Both ROS and the SASP contribute to tissue impairment by inducing paracrine senescence (Nelson et al. 2012, Acosta et al. 2013) and cancer progression when in a tumour context (Wu 2006, Davalos et al. 2010). Inhibition of IL-8 signalling reduces activation of the pathways maintaining and stabilising cellular senescence, resulting in decreased ROS- and SASP factors-dependent tissue impairment (Figure 7.1).

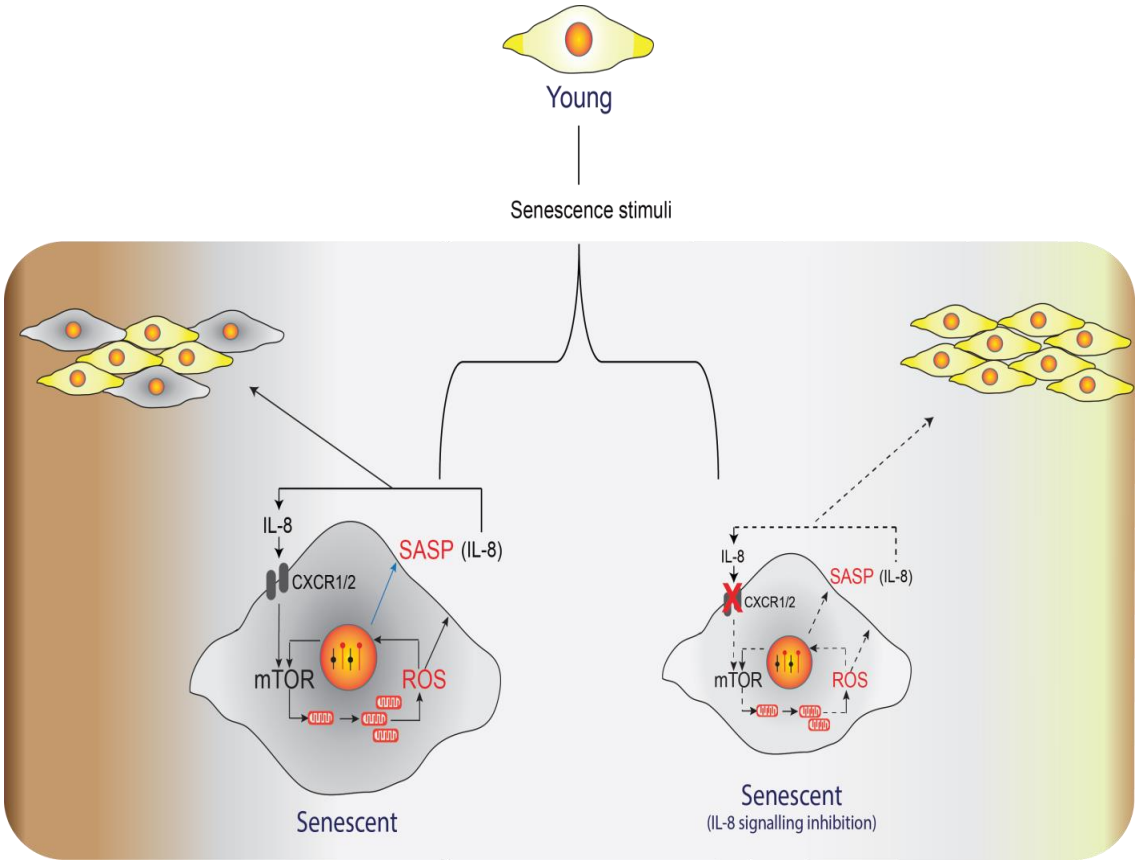

Figure 7.1 | Interactions between mitochondria and inflammatory factors during cellular senescence.. Young proliferating cells are continuously exposed to a myriad of stressors eventually causing irreparable DNA damage. Cells containing irreparable

DNA damage undergo a permanent cell cycle arrest, senescence, as a result of a persistent DNA damage response (DDR). The DDR induces mTOR-dependent mitochondrial biogenesis with higher ROS production and additional induction of DNA damage (feedback loop 1). The DDR also triggers the senescence-associated secretory phenotype (SASP), particularly the inflammatory phenotype (Rodier et al. 2009). The inflammatory phenotype, particularly IL-8 and its receptors reinforce senescence in an autocrine fashion (Acosta et al. 2008) (feedback loop 2). Activation of mTOR via IL-8 - CXCR1/2 is the linking factor between the two main circle pathways (feedback loop 1 and 2) that stabilise cellular senescence (Correia-Melo *et al.* unpublished). Both ROS and the SASP contribute to tissue impairment by inducing paracrine senescence (Nelson et al. 2012, Acosta et al. 2013) and cancer progression when in a tumour context (Wu 2006, Davalos et al. 2010). Inhibition of IL-8 signalling reduces activation of the pathways maintaining and stabilising cellular senescence, resulting in decreased ROS- and SASP factors-dependent tissue impairment.

The work developed on this thesis may be of potential interest in therapeutically combating the pro-oxidant and pro-inflammatory effects of cellular senescence, given their suggested role as drivers of age-related disease (Tchkonia et al. 2013, Correia-Melo et al. 2014). Our data demonstrating that mitochondria are required for these features of senescence has very important implications for our understanding of the origins and mechanisms underlying the senescence phenotype and suggests mitochondria as major putative therapeutic targets for interventions impacting on the senescent phenotype.

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