The Senescent Cell Induced Bystander Effect

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Abstract

The induction of senescence in response to persistent stress induces major phenotypic changes in senescent cells, including the secretion of a host of inflammatory factors and reactive oxygen species. Recent evidence has implicated senescent cells in the diseases of ageing and cancer; however, the mechanism by which this occurs is still unknown. This thesis uses a reporter cell line with cells expressing a fluorescent conjugate that allows real time live cell imaging of a sub set of cells within a co-culture, to provide the first evidence that senescent cells can induce a DNA damage response in healthy cells, and thus implicates a potential mechanism by which senescent cells could non-autonomously contribute to the ageing process. The use of specific inhibitors, stimulation, and targeted repression indicate that gap junctions, reactive oxygen species, p38, mTOR and NF-κB all play a key role in this observed bystander effect of senescent cells, and offer potential targets for therapies designed to reduce the damaging effects of senescent cells.

Abbreviations

53BP1	Tumor suppressor p53-binding protein 1
AP-1	Activator protein-1
AREG	Amphiregulin
ATM	Ataxia telangiectasia mutated
ATR	ATM- and RAD3-related
ATRIP	ATR-interacting protein
Bad	Bcl2/Bcl-X_L-antagonist causing cell death
BrdU	Bromodeoxy-uridine
B-TrcP	F-box/WD repeat-containing protein 1A
CDK2,4,6	Cyclin dependent kinase 2,4,6
CHO cell	Chinese hamster ovary cell
CtIP	CtBP-interacting protein
DCA	Deoxycholic acid
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNP	Dinitrophenol
DSB	Double strand break
EGF	Epidermal growth factor
ERK	Extracellular-signal-regulated kinase
FADD	Fas-Associated protein with death domain
FANC D2	Fanconi anemia group D2 protein

FGF	Fibroblast growth factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GCSF	Granulocyte colony-stimulating factor
GRO	Growth regulated oncogene
Grb2	Growth factor receptor-bound protein 2
HDAC	Histone deacetylase complex
HDM2	Human double minute 2
HGF	Hepatocyte growth factor
HSP27	Heat shock protein 27
IAP	Inhibitors of apoptosis
ICAM	Intercellular adhesion molecule
IGFBP	Insulin-like growth factor-binding protein
IKK	Inhibitor of nuclear factor kappa-B kinase
IL-1ra	IL-1 receptor antagonist
JAK1	Janus kinase 1
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAF	Primary mouse adult ear fibroblasts
MAPK	Mitogen Activated Protein Kinase
MDC1	Mediator of DNA damage checkpoint protein 1
MEF	Mouse embryonic fibroblast
MEKK3	Mitogen activated protein kinase kinase kinase 3
MIPα	Macrophage inflammatory protein

MMP	Matrix metalloproteinase
MMSET	Multiple myeloma SET domain
MOI	Multiplicity of infection
NaB	Sodium Butyrate
NEB	New England Biolabs
NEMO	NF-κB essential modulator
PARP	Poly (ADP-ribose) polymerase
PGC	Peroxisome proliferator-activated receptor gamma coactivator
PI3K	Phosphatidylinositide 3-kinase
PIKK	Phosphatidylinositol 3-kinase-related kinase
PLK1	Polo-like kinase 1
POT1	Protection of telomeres protein 1
PP2A	Protein phosphatase 2A
PRAK	p38-regulated or -activated protein kinase
PTEN	Phosphatase and tensin homolog
PTP	Protein tyrosine phosphatase
RAC1	Ras-related C3 botulinum toxin substrate 1
RIBE	Radiation induced bystander effect
RIP	Receptor interacting protein
RISP	Rieske iron sulphur protein
RNA	Ribonucleic acid
RPA	Replication protein A
SASP	Senescence associated secretory phenotype

SCE	Sister chromatid exchange
SCF	Skp, Cullin, F-box containing
SHC	SH2-and-collagen-homology-domain-containing protein
SHP2	SH2-domain-containing tyrosine phosphatase
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SMC1	Structural Maintenance of Chromosomes 1
SOCS1	Suppressor of cytokine signaling
SODD	Silence of death domain
SOS	Son of sevenless
SSB	Single strand break
ssDNA	Single stranded DNA
TAK1	TGF-β activated kinase
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TNFAIP3	Tumor necrosis factor alpha-induced protein 3
TOPBP1	DNA topoisomerase 2-binding protein 1
TOR	Target of rapamycin
TRADD	TNF recpetor-associated death domain
TRAF2	TNF-R associated factor 2
TRF	Terminal restriction fragment
TRF1,2	Telomeric repeat binding factor 1,2
TRP	Transient receptor potential

TSC	Tuberous sclerosis complex

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1 Introduction

1.1 The Discovery of Cellular Senescence

In 1961 a seminal study by Hayflick and Moorehead dismissed the old myth that cells in culture could continue replicating indefinitely. By culturing old and young cells together it was proved that the old cells ceased replicating before the young cells, demonstrating that it was not adverse culture conditions that had prevented continued replication, and that cells had an intrinsically limited capacity for producing progeny (Hayflick, 1965, Hayflick and Moorhead, 1961). Although some cells underwent more divisions than others, and there was a strong stochastic determinant, most cells would enter a state of replicative arrest after a similar number of population doublings and, once arrested, they would never re-enter the cell cycle (Hayflick, 1965, Hayflick and Moorhead, 1961). This state of permanent arrest has since been termed cellular senescence (Goldstein, 1990), and the evidence that this process may have relevance to the ageing process is mounting.

The focus of this project was to examine whether senescent cells have non-autonomous effects that are detrimental to surrounding cells which could be abrogated by mechanistic intervention. To introduce the topic it is important to describe how cells become senescent and the importance of DNA damage, both in the initial stimulus and to maintain the signal. Then the senescent phenotype will be described along with its implications to bodily function in health and disease, to give insight to the relevance and importance of this work to medicine, gerontology and cancer biology.

One important clarification for this thesis is that although the terms senescence and senescent can be used to describe the ageing of, and the state of being aged, in organisms respectively. In this text these words are used exclusively to describe cells. Further, a senescent cell is not simply an old cell, as it would be for an organism. Cellular senescence occurs as a result of replication; the length of time a cell spends quiescent in between divisions has little effect on when it will enter cellular senescence (Hayflick, 1965, Hayflick and Moorhead,

1961). Thus, old cells are not necessarily senescent if they have not been dividing, and old organisms need not necessarily accrue senescent cells.

1.2 The Causes of Cellular Senescence

1.2.1 Telomeres and Replicative Senescence

Hayflick and Moorehead had discovered replicative senescence in 1961 (Hayflick and Moorhead, 1961). However, it was not until 1972 when the DNA end replication problem was laid out in the context of linear phages, that a potential causal mechanism for replicative senescence was elucidated (Watson, 1972). Whilst the leading strand can be replicated up to the end of the chromosome, the replication of the lagging strand from RNA primers ahead of the replicated region will leave the primer bound region and anything beyond it unreplicated (Olovnikov, 1973, Watson, 1972), causing the chromosome to shorten with each cell division. The potential implication of the end replication problem was such that it had to be circumvented to prevent the eventual extinction of all eukaryotic life from loss of DNA, as was exemplified by some mutant single celled organisms (Lundblad and Szostak, 1989, Yu et al., 1990).

The discovery of telomerase enzyme which uses an RNA template to bind a specific telomeric repeat and lengthen the telomere 5'-3' (Greider and Blackburn, 1985), demonstrated that the end replication problem could be overcome, and therefore did not need to induce replicative senescence, or eukaryotic extinction. However, it was found that in many somatic tissues telomerase is inactivated (Bacchetti and Counter, 1995, Kim et al., 1994), including human fibroblasts (Counter et al., 1992, Kim et al., 1994), and many organisms only retain telomerase expression in the germline (Kim et al., 1994, Tan et al., 2012), where telomere length must be, and is, maintained (Allsopp et al., 1992). Although some somatic tissues do have low level telomerase activity (Broccoli et al., 1995, Counter et al., 1995, Hiyama et al., 1995b), these cells still undergo telomeric loss with both donor age and passage number (Hastie et al., 1990, Kitada et al., 1995, Vaziri et al., 1994, Vaziri et al., 1993), indicating

that there is little difference between these and telomerase negative cells. Therefore, although organisms have mechanisms to lengthen telomeres and prevent the end replication problem, these are generally only active and effective in a very small number of cells, leaving the majority of the somatic cells to have a limited replicative capacity.

In several studies using human fibroblasts, genomic DNA was digested to produce terminal fragments containing both telomeric and subtelomeric DNA called terminal restriction fragments (TRFs) (Brown, 1989, Cross et al., 1989), and it was found that replicative lifespan correlated with initial length of average TRF (Allshire et al., 1988, Counter et al., 1992, Cross et al., 1990, Harley et al., 1990). Additionally, one study showed that regardless of what size the TRF was in proliferating cells when the cells reached senescence there was little difference in TRF length (Allsopp et al., 1992), indicating that senescence was associated with specific average telomere length. However, a later study showed that firstly the average telomere length at senescence was strain specific; secondly that it was highly variable at senescence; and thirdly that average telomere length at explantation did not significantly correlate with replicative lifespan (Serra and von Zglinicki, 2002). Notably, they also observed that the strain specific threshold at which telomere shortening would induce senescence was not affected by increasing oxidative stress (Serra and von Zglinicki, 2002), although addition of antioxidants had been previously shown to cause cells to senesce with longer telomeres (von Zglinicki et al., 2000).

Early ideas about replicative senescence suggested that the loss of specific lengths of DNA with each cell division was a type of program, which was termed the mitotic clock, by which cells had a certain number of divisions before they entered the senescent state (Olovnikov, 1971, Olovnikov, 1973). However, there were several aspects of the senescence phenomenon that were inconsistent with such an idea. Firstly, there was large variance between TRF length at senescence: the average length of fragment was approximately 4kb which left 2kb of telomeric repeat DNA, but the variance was high enough so that some of the chromosomes could have little or no telomeric repeat DNA remaining (Levy et al., 1992). Secondly, the discovery that oxidative stress accelerated telomere shortening (von Zglinicki et al., 1995) added a strong stochastic mechanism to the process. Combined, the data suggested that

senescence was not as simple as an end replication problem, and other factors such as reactive oxygen species (ROS), exonuclease activity and unequal recombination could also play a role in telomere shortening (Makarov et al., 1997).

The first causal evidence that telomere loss was responsible for replicative senescence came from the immortalisation of human fibroblasts expressing telomerase (Bodnar et al., 1998, Vaziri and Benchimol, 1998). One study showed that these cells could reach 250-400PDs without significant change in growth rate when normal cells would senesce between 60-70PDs (Morales et al., 1999). Importantly, in this study the average TRF length reached as low as 4Kb in the immortalised cells whilst the average in normal senescent cells was found to be 6-8Kb in this study. The immortal cells did not senesce because the variance between telomere lengths was much smaller than in senescent cells and stabilised at 4kb. Consistently, an earlier study showed much less telomeric variance in immortalised cells, but in this study the telomeres remained longer on average in immortalised cells than unimmortalised cells at senescence (Bodnar et al., 1998). This difference possibly results from increased loss of telomerase expression in the former study (Morales et al., 1999). It was suggested that senescence was prevented in these cells by a preferential activity of telomerase for short telomeres, which was later confirmed in a yeast study following a single telomere showing that telomeres switch from non-extendable to extendable states when they become shorter (Teixeira et al., 2004). Regardless, senescence is most likely induced by one or a few telomeres becoming critically short.

The eukaryotic telomere is bound by a series of proteins which form the shleterin complex, including TRF1 which induces bending to assist the formation of the t-loop (Griffith et al., 1999), and TRF2 which facilitates the invasion of the 3’ overhang into an internal site in the double stranded telomeric DNA forming the D-loop (Griffith et al., 1999). Along with other proteins such as Pot1, which binds to single stranded DNA, the complex protects the telomere from enzymatic and signalling molecules that would otherwise bind. Several observations indicated the importance of this structure in replication: Its inhibition or deletion induced senescence (van Steensel et al., 1998, Denchi and de Lange, 2007); overexpression of TRF2 extended replicative lifespan

(Karlseder et al., 2002, Smogorzewska et al., 2000); and loss of the 3' G-rich overhang induces senescence (Stewart et al., 2003). Importantly, these mechanisms were found to be independent of telomere length, as overexpression of TRF2 shortened telomere length (Karlseder et al., 2002, Smogorzewska et al., 2000), whilst its inhibition had no effect on telomere length (Denchi and de Lange, 2007, van Steensel et al., 1998). Although this might have suggested that uncapping and shortening induced senescence independently, the observation that shortening induced uncapping (Blackburn, 2000), suggested that it was the telomere cap that was important, and telomeric shortening was just one mechanism by which the cap could be lost. Two recent studies have identified the presence of telomere associated foci, which were induced by damaging agents independently of telomerase expression, suggesting that they resulted from telomere uncapping without shortening, and were highly important in the induction of senescence (Fumagalli et al., 2012, Hewitt et al., 2012).

When a telomere becomes uncapped by the loss of the shelterin complex, it becomes bound by factors associated with the DNA damage response (DDR) such as 53BP1, NBS1, MDC1, γ-H2AX and CHK2, which indicated that they were recognised as double strand breaks (d'Adda di Fagagna et al., 2003, Takai et al., 2003, Herbig et al., 2004), thus indicating that telomere uncapping induces replicative senescence through the induction of a DNA damage response. Because of the absence of telomerase the progressive telomere shortening eventually causes the loss of protective proteins and recognition as irreparable damage inducing a persistent DNA damage response.

1.2.2 DNA Damage and Premature Senescence

Non-telomeric damage can also induce cellular senescence if the source is sufficient to overwhelm the repair capacity of the cell. The more severe the damage, the more likely it is to induce senescence; thus, double strand breaks are potent inducers of cellular senescence (Di Leonardo et al., 1994). Base substitutions and alterations probably play a very minor role, if any. Monoadducts induced by low energy UVA do not induce senescence, however

higher energy UVB induces senescence through thymine dimers and interstrand crosslinks which generate single stranded (ss)DNA (Hovest et al., 2006). ROS induced senescence is thought to occur mainly through the formation of single strand breaks (SSBs), as DSBs account for a much lower proportion of the damage (Land, 2002). Conversely, in ionising radiation induced senescence the main type of damage is DSBs. The non-homogeneous energy distribution causes clusters of complex lesions, even at lower doses (Nikjoo et al., 1999). These clusters are very difficult to repair; however, it is unlikely that they could arise under physiological conditions at appreciable levels. Low doses of these stressors reduce the replicative capacity of the cells so that they will senesce after fewer population doublings, whereas high doses can cause cells to undergo immediate senescence before they have reached their full replicative capacity (Toussaint et al., 2000). After a high dose of X-irradiation around three quarters arrest within the first 1.5 hours, and <1% are still incorporating BrdU after 24 hours (Rodier et al., 2011), suggesting that it is cell cycle dependent, and very few cells undergo more than one round of division, whilst the majority arrest immediately. This is called premature senescence.

A comparison between telomeric and non-telomeric foci showed that they share many of the same response elements inducing very similar DNA damage responses (d'Adda di Fagagna et al., 2003, Takai et al., 2003), and yeast with critically short telomeres and ones exposed to DNA damaging agents have significantly overlapping global gene expression profiles (Nautiyal et al., 2002). Importantly, the induction of replicative senescence was also found to involve non-telomeric foci, and cells exhibited a similar number of foci irrespective of their chromosomal location (Nakamura et al., 2008), indicating that all foci contributed equally to the senescence signal. Additionally, telomeres are particularly susceptible to exogenous stresses such as oxidative stress (von Zglinicki et al., 1995), and two recent studies have demonstrated the importance of telomeric damage in radiation and oxidative stress induced senescence. Whilst the non-telomeric foci are generally repaired, the telomeric foci persist for longer, and thus may be of particular importance in maintaining the DNA damage response in replicative and premature senescence (Hewitt et al., 2012, Fumagalli et al., 2012).

1.2.3 Oncogene Induced Senescence

Chemical mutagens and radiation are forms of stress which induce DNA damage in cells, and result in cellular senescence. Another inducer of senescence is the aberrant expression of an oncogene, known as oncogenic stress. Most studies have focused on the RAS pathway, and ectopic activation of RAS, RAC1, RAF, MOS and MEK (Braig et al., 2005, Debidda et al., 2006, Lin et al., 1998, Michaloglou et al., 2005, Serrano et al., 1997, Zhu et al., 1998), and even endogenous levels of RAS or BRAF (Collado et al., 2005, Dankort et al., 2007, Guerra et al., 2003, Guerra et al., 2007, Michaloglou et al., 2005) all induce senescence, as does the inactivation of PTEN (Chen et al., 2005), which is an inhibitor of the same pathway. Additionally, ectopic expression of positive regulators of the cell cycle such as E2F transcription factors, MYC, CDC6 and cyclin E all induce senescence (Bartkova et al., 2006, Grandori et al., 2003, Johnson and Degregori, 2006, Lazzerini Denchi et al., 2005), as does ectopic IFN-β (Moiseeva et al., 2006) and TGF-β signalling (Katakura et al., 1999, Vijayachandra et al., 2003, Zhang and Cohen, 2004).

Several oncogenes including RAS (Abulaiti et al., 2006, Di Micco et al., 2006, Mallette et al., 2007), MOS (Bartkova et al., 2006), and RAC (Debidda et al., 2006), as well as several of the positive cell cycle regulators (Bartkova et al., 2005, Bartkova et al., 2006, Vaziri et al., 2003), have been shown to induce the phosphorylation of H2AX and other factors associated with the DDR. More importantly, some oncogenes cannot induce senescence in the absence of the DDR (Bartkova et al., 2006, Di Micco et al., 2006, Mallette et al., 2007), and cells that have become fully senescent in response to RAS can re-enter the cell cycle upon DDR inactivation (Di Micco et al., 2006).

RAS and BRAF (Dankort et al., 2007, Di Micco et al., 2006, Jones et al., 2000, Michaloglou et al., 2005, Sarkisian et al., 2007), as well as E2F and MYC (Grandori et al., 2003, Lazzerini Denchi et al., 2005, Dominguez-Sola et al., 2007), have been shown to induce a state of hyperproliferation during which the cell begins to exhibit several markers of replication stress including regions of single stranded DNA (Bartkova et al., 2006), stalled and collapsed replication

forks (Bartkova et al., 2006, Di Micco et al., 2006), and loss of heteroygosity at fragile sites (Abulaiti et al., 2006, Di Micco et al., 2006, Jones et al., 2000, Mallette et al., 2007). Re-replication, where the cell initiates a second round of DNA replication before it has divided, can also occur in response to RAS, which increases the level of DNA damage (Blow and Dutta, 2005, Davidson et al., 2006). Thus, similar to replicatively senescent and stress induced premature senescence, oncogene induced senescence also appears to occur mainly as a result of DNA damage initiating the DNA damage response.

1.3 The Senescence Signalling Pathways

1.3.1 The Formation and Dynamics of DNA Damage Foci

Both telomeric and non-telomeric lesions induce senescence through a persistent DNA damage response activating two phosphatidyl 3-kinase related kinase (PIKK) family proteins; Ataxia Telangiectasia Mutated (ATM) and ATM-Rad3 Related (ATR).

DSBs can arise from diverse stimuli including ionising radiation (Rogakou et al., 1998), other external damage (Rogakou et al., 1999, Paull et al., 2000), telomere uncapping (d'Adda di Fagagna et al., 2003, Takai et al., 2003) and replication fork collapse (Furuta et al., 2003, Ward and Chen, 2001). Canonically, the DSBs are initially sensed by PARP, which recruits the Mre11-Rad50-Nbs1 (MRN) complex to bind and unwind the DNA (D'Amours and Jackson, 2002), allowing NBS1 to recruit ATM (Falck et al., 2005). ATM then autophosphorylates itself forming active monomers (Bakkenist and Kastan, 2003), which then phosphorylate a host of factors including Mre11, BRCA1, MDC1, p53, Chk2, Smc1, FANC D2, 53BP1 and NBS1 (Banin et al., 1998, Cortez et al., 1999, Dong et al., 1999, Gatei et al., 2000, Goldberg et al., 2003, Kim et al., 2002, Matsuoka et al., 2000, Rappold et al., 2001, Taniguchi et al., 2002), depending on what molecules are present at the focus. Importantly, ATM also phosphorylates histone H2AX forming γ-H2AX (Burma et al., 2001, Fernandez-Capetillo et al., 2002), and allowing the binding of MDC1 scaffold

protein (Stucki et al., 2005). Up until this point the binding of MRN to the DNA has been transient (Lukas et al., 2003), but when NBS1 binds the MRN complex it stabilises it at the DNA (Stucki and Jackson, 2006). This amplifies ATM activity, stimulating further MRN recruitment and the spread of γ-H2AX along the chromatin (Stucki and Jackson, 2006, Lukas et al., 2004, Bekker-Jensen et al., 2006, Bekker-Jensen et al., 2005), as summarised in figure 1.1. The amount of phosphorylation spans for megabases around the DSB corresponding to thousands of nucleosomes (Rogakou et al., 1998), thus forming what have become known as nuclear foci (Rogakou et al., 1999). Γ-H2AX foci are static in the genome (Siino et al., 2002), and have been found to have a 1:1 ratio with the number of DSBs (Sedelnikova et al., 2002), which has caused them to be described as the “gold standard to detect the presence of DSBs” (Fernandez-Capetillo et al., 2004). Although it is not responsible for the recruitment of DDR factors, γ-H2AX is required for these proteins to be maintained at the DSB in activated state (Celeste et al., 2002). Many of these factors form a positive feedback loop with ATM to maintain the foci.

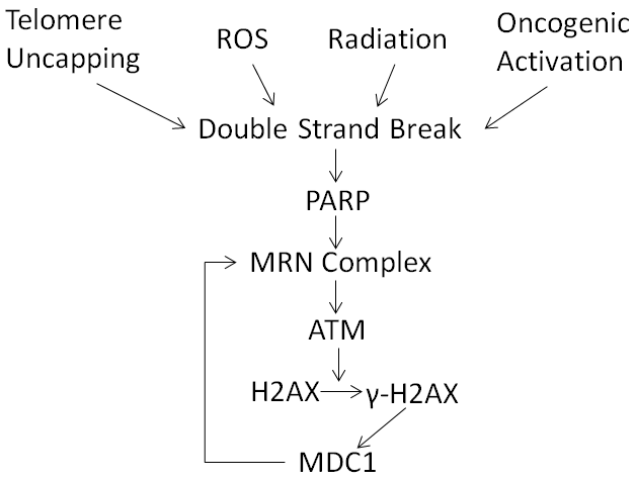


Figure 1.1| The activation of the DDR by agents inducing double strand breaks.

Alternatively, RPA coated single stranded DNA caused by replication stress or UV irradiation causes ATR and its DNA-binding subunit ATRIP to bind the DNA which activates a less well 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). Although recruited ATR alone can phosphorylate

most of its targets, it still requires RAD17 to assemble claspin at the site of damage in order to activate Chk1 (Kumagai and Dunphy, 2000, Wang et al., 2006a). Similar to ATM, ATR stimulates phosphorylation of H2AX and the formation of DNA damage foci. Notably, end resection of DSBs also generates RPA coated ssDNA which activates ATR (Cortez et al., 2001, Zou and Elledge, 2003).

It has also been suggested that DSBs can cause the exposure of histone cores causing the direct association of 53BP1 to the site of damage (Huyen et al., 2004). However, alternative evidence suggests that 53BP1 requires the histone H2A and H2AX ubiquitination activity of RNF8/RNF168/HERC2 (Stewart et al., 2009) which helps expose the methyl groups of histones around broken DNA to allow 53BP1 binding (Wu et al., 2011). MDC1 may also allow 53BP1 binding through inducing similar histone methylation through MMSET histone methyltransferase (Pei et al., 2011).

Like γ-H2AX, 53BP1 forms foci of sufficient size to be visualised using widefield microscopy, due to its ability to oligomerise whilst bound to DNA (Iwabuchi et al., 2003, Adams et al., 2005). Like many of the DDR proteins, 53BP1 is recruited to sites of damage by PARP (Celeste et al., 2002). Amongst the earliest elements recruited to these sites (Bekker-Jensen et al., 2005, Mochan et al., 2004), 53BP1 is hyperphosphorylated by ATM (Anderson et al., 2001, Rappold et al., 2001, Xia et al., 2001), and plays an important role in activating ATM substrates (Wang et al., 2002, DiTullio et al., 2002, Fernandez-Capetillo et al., 2002). Additionally, 53BP1 is involved in DNA repair by non-homologous end-joining (Nakamura et al., 2006), and inhibits homologous recombination by preventing DNA resection and formation of ssDNA through CtIP and BRCA1 (Bunting et al., 2010, Bouwman et al., 2010). Notably, BRCA1, and 53BP1 rarely co-localise at DNA damage foci (Mok and Henderson, 2010), due to their opposing functions in promoting and inhibiting homologous recombination respectively. This is regulated according to the stage of the cell cycle so that DNA resection occurs mainly in S and G2 phases where sister chromatids can be used for homologous recombination (You and Bailis, 2010).

Once the damage is repaired the cell can re-enter cell cycle and enter mitosis. Although the processes of checkpoint recovery are not well understood, the

recovery of the G2 checkpoint is thought to involve β-TrcP adaptor protein, which targets claspin (Mailand et al., 2006), and Wee1 to the SCF ubiquitin ligase complex for labelling for proteasomal degradation (Watanabe et al., 2004) to allow cell cycle progression. Conversely, during checkpoint activation β-TrcP has the recovery opposing role of targeting Chk1 phosphorylated CDK-activating phosphatase Cdc25A to the same complex, inhibiting cell cycle. However, both processes are dependent on phosphorylation (Cardozo and Pagano, 2004), so that during checkpoint activation Chk1 promotes ubiquitylation of Cdc25A, and the stabilisation of claspin, whilst in recovery the re-activation of Plk1 causes the phosphorylation and targeting of claspin and Wee1 to SCF (van Vugt et al., 2004, Mailand et al., 2006). Additionally, the activation of Wip1 and PP2A phosphatases are thought to play a role in checkpoint recovery after DNA repair (Chowdhury et al., 2005, Keogh et al., 2006, Lu et al., 2005, Shreeram et al., 2006).

Many small foci are produced by low and high doses of irradiation. At low doses these foci are generally resolved and if the cells had become quiescent they re-enter the cell cycle. At high doses, whilst the majority of these foci are still resolved, a small fraction of them become enlarged and persist for months (Rodier et al., 2011). In some cells the 53BP1 foci initially co-localise with RPA70 which suggests that the damage is being repaired by homologous recombination, suggesting that these cells were in S-phase. However, this co-localisation did not persist for longer than 24 hours after the initial pulse, suggesting that either the damage was repaired or the cell continued through G2 anyway. The combined demonstration that all cells developed persistent foci, but not all incorporated BrdU suggested that S-phase and the associated replication stress was not essential for the generation of these foci (Rodier et al., 2011), as some cells have arrested in G1 without entering S phase.

If the damage is not repaired and the foci persist, then the foci stimulate the activation of the tumour suppressor molecules and cell cycle arrest.

1.3.2 The DNA Damage Response (DDR) Signalling to Tumour Suppressor Proteins

ATM can directly phosphorylate p53 at Serine (Ser) 15 (Banin et al., 1998, Canman et al., 1998), which stimulates the transactivating function of p53 through binding of its coactivator p300, but does not affect the association of p53 with human double minute 2 (HDM2) (Dumaz and Meek, 1999). Despite a threefold increase in dissociation of p53 from HDM2 through combined Ser15 and Ser18 phosphorylation, ATM mainly exerts its effects on p53 indirectly via Threonine (Thr) 68 phosphorylation of Chk2 (Ahn et al., 2000, Melchionna et al., 2000); however, it may also directly modify HDM2 (Maya et al., 2001). Chk2 can also be phosphorylated by ATR (Wang et al., 2006b), which also phosphorylates and activates Chk1 (Jazayeri et al., 2006).

Chk1 and Chk2 transiently localise at the DNA damage foci to be phosphorylated by ATR and ATM respectively, but once activated these proteins dissociate and freely diffuse throughout the nucleus (Smits et al., 2006, d'Adda di Fagagna, 2008), at least at early time points. Both kinases are involved in the S and G2 checkpoints, phosphorylating the Cdc25A phosphatase causing its ubiquitin dependent degradation, thus preventing formation of replication origins as well as entry into mitosis (Falck et al., 2001, Xiao et al., 2003). Chk1 also phosphorylates Cdc25C mitosis-promoting phosphatase, making it essential for G2 arrest (Liu et al., 2000). However, their main role in cellular senescence is considered to be through the activation of p53 (Chehab et al., 2000).

In its inactive form p53 tumour suppressor protein is associated with HDM2, which functions doubly to inhibit transcriptional activity by binding the N-terminal transactivation domain, and also as an E3 ubiquitin ligase targeting both p53 and itself for degradation (Li et al., 2003). However, signalling through the DDR causes the activation and stabilisation of p53, preventing HDM2 from binding. As a major transcriptional regulator, p53 has multiple downstream targets which inhibit the cycle, as well as several genes involved in negative feedback, such as HDM2 (Barak et al., 1993). This leads to pulses of p53 activation (Hunziker et al., 2010), where the mean number of pulses rather than the amplitude

corresponded to the dose of IR, and thus the fate of the cell (Lahav et al., 2004). A persistent signal will induce senescence or apoptosis, whilst loss of p53 pulses will allow re-entry into the cell cycle.

The main transcriptional target of p53 is $p21^{Cip1}$ which also has multiple targets, amongst which it inhibits cyclin E/CDK2 complex function from phosphorylating pRb and its two paralogues p107 and p130, resulting in checkpoint activation (Resnitzky and Reed, 1995, Sherr, 1994, Weinberg, 1995). Notably, the effects of p21 are partially dependent on the cellular environment as p21 can also activate CDKs 4 and 6 (Sherr and Roberts, 1999), both of which induce phosphorylation of pRb, as well as sequestering p21, preventing it from repressing CDK2, thus stimulating further phosphorylation of pRb and entry into S-phase (Cheng et al., 1999). Similarly to p53, the dynamics of p21 are not straightforward; however, both function mainly as cell cycle inhibitors.

When pRb is hypophosphorylated it becomes active and inhibits transcriptional activity of E2F transcription factors, which prevents the production of genes essential for cell cycle progression (Nevins, 1992, Weinberg, 1995). The pathway is summarised in figure 1.2.

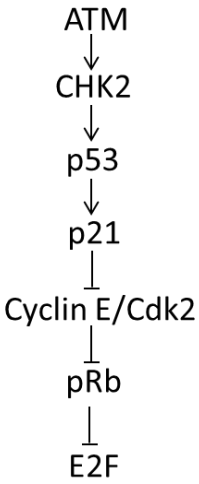


Figure 1.2| Activation of tumour suppressor proteins by the DDR.

Thus, DNA damage in the form of telomere dysfunction or double strand breaks (DSBs) can induce cellular senescence by the activation of p53 and pRb tumour suppressor proteins through ATM, ATR, CHK1 and CHK2. However,

senescence does not result from a single pathway, but from many interacting factors which in combination decide the fate of the cell. The cell has multiple redundant pathways to ensure the induction of senescence even if the damage has knocked out others. Thus, the knockout or knockdown of any factor only increases the likelihood of cells escaping senescence, creating a heterogeneous population of arrested and cycling cells. For example, microinjection of dominant negative kinase dead mutants of ATM, ATR, CHK1 and CHK2 combined allowed a few TRF2 knockout senescent cells to re-enter the cell cycle and incorporate BrdU (d'Adda di Fagagna et al., 2003). Equally, in RAS induced senescence individual knockdown of each of these factors allowed similar percentages to re-enter cell cycle (Di Micco et al., 2006), as has been shown in related studies using other cell types (Gire et al., 2004, Bartkova et al., 2006). Importantly, in some of these studies the cells still may arrest in G2 or M phase after replicating the DNA (d'Adda di Fagagna et al., 2003), because they only examined BrdU, whilst other studies clearly demonstrate that at least some cells do revert to cell cycle, allowing unrestrained growth at the same time as accumulating DNA damage.

In human cells the level of redundancy is higher than in mice, as mouse embryonic fibroblasts require only the loss of either p53 or pRb signalling in order to allow some cells to bypass (Harvey et al., 1993)[1] or escape (Dirac and Bernards, 2003) senescence, whereas in human fibroblasts the inactivation of both genes is necessary (Shay et al., 1991, Smogorzewska and de Lange, 2002). Redundancy is also dependent on the senescence stimulus; p21 knock out mouse embryonic fibroblasts (MEFs) senesce in response to RAS (Pantoja and Serrano, 1999, Takeuchi et al., 2010), but not 20% oxygen (Takeuchi et al., 2010), when both stimuli would induce senescence in wildtype MEFs, if much less stably in response to 20% O_2 (Coppe et al., 2010). Importantly, these data indicate that there is a stochastic element to cellular senescence influenced partially by the presence of the milieu of inductive and preventative factors, but also by the nature of the damage, which is itself highly stochastic.

[1] Notably, although homozygotic p53 knockout mouse cells bypassed senescence, heterozygotic cells still senesced even if they lost their remaining wildtype allele, which suggests that mouse cells can senesce without p53, but it is more difficult, and may be dependent on the level of molecular damage, which is likely higher in the homozygotic cells.

1.3.3 The Independent Pathways of p53 and pRb in Senescence

As described above, whilst p53 and pRb may fit into a linear pathway in mice, in humans they can induce senescence independently of each other. Independent of pRb, p53 directly induces transcription of 14-3-3σ (Hermeking et al., 1997), which sequesters Cdc25C phosphatase in the cytoplasm preventing it from activating cyclin B-Cdc2 causing stable G2/M arrest (Chan et al., 1999). Inhibition of 14-3-3σ results in immortalisation of human keratinocytes (Dellambra et al., 2000). Another possible pRb independent mechanism is the inhibition of PCNA by p21; however, whilst it has been shown to induce both G1 and G2 arrest (Cayrol et al., 1998), p21 mutant cells lacking the ability to bind PCNA can still senesce (Macip et al., 2002). It has also been suggested that p21 could induce senescence through increasing ROS levels, independent of both PCNA binding, and cyclin/Cdk inhibition (Macip et al., 2002).

pRb can be activated by $p16^{INK4a}$, hereafter p16, independently of p53 (Alcorta et al., 1996). Upon activation, p16 is translocated to the nucleus from the perinuclear cytoplasm where it functions as a cyclin D/Cdk4,6 inhibitor (Serrano et al., 1993, Spallarossa et al., 2010), thus preventing the inhibitory phosphorylation of pRb (Resnitzky and Reed, 1995, Sherr, 1994, Weinberg, 1995). In mice, one study showed that MEFs lacking p16 underwent senescence indistinguishably from wildtype cells in response to γ-irradiation, replication and oncogene activation (Krimpenfort et al., 2001). However, another study showed p16 knockout MEFs were more prone to immortalization (Sharpless et al., 2001). Importantly, when knockout of p16 was combined with $p19^{ARF}$ ($p14^{ARF}$ in humans), which is an important activator of p53 in mice, the absence of both pathways causes a marked increase in tumour formation over the lack of each individual factor (Krimpenfort et al., 2001), as is the case for double p16, p21 knockout MEFs (Takeuchi et al., 2010). Thus, in mice as in humans it appears that these pathways have some independence and therefore compensatory ability. In humans, p16 is not essential for most types of senescence. Different strains of human fibroblasts mutant for p16 proliferated for longer than wildtype cells, but still senesced at a later population doubling (Brookes et al., 2004). Equally, p16 is not essential for senescence induced by N-RAS, H-RAS and $BRAF^{v600E}$ (Denoyelle et al., 2006, Haferkamp et al., 2009,

Zhuang et al., 2008). However, this is also to be expected if the p16 and p53 pathways can induce senescence independently. Importantly, when p53 was inactivated, the strains of fibroblast with low levels of p16 robustly resumed cell cycle (Beausejour et al., 2003), and additionally the overexpression of p16 alone is sufficient to induce cellular senescence (Coppe et al., 2011, Kato et al., 1998, McConnell et al., 1998, Pospelova et al., 2009).

Interestingly, unlike p21 which accumulates at the initiation of senescence and declines after the cells become senescent, p16 accumulates gradually, with almost none present at the early stages, and then persists for at least two months after induction (Alcorta et al., 1996, Hara et al., 1996, Stein et al., 1999). The expression of p16 coincides with aspects of the senescent phenotype such as increased cell size and expression of senescence associated β-galactosidase (Alcorta et al., 1996, Hara et al., 1996, Stein et al., 1999), and may be responsible for the permanency of senescence (Beausejour et al., 2003, Stein et al., 1999).

p16 expression has been shown to increase as a result of oncogene induced senescence (Serrano et al., 1997), oxygen radicals (Chen et al., 2004, Ito et al., 2004), radiation (Meng et al., 2003), and telomere dysfunction (Jacobs and de Lange, 2004). Although the exact mechanism of p16 activation is not completely understood, one possibility is that these DNA damage signals activate p38 MAPK signalling (Bulavin et al., 2004, Ito et al., 2006, Iwasa et al., 2003), which then activates p16 (Spallarossa et al., 2010).

1.3.4 MAPK Signalling in Cellular Senescence

There are three main types of MAPK; c-Jun N-terminal Kinase (JNK), ERK and p38, all of which have been implicated in senescence. Inhibition of p38 and JNK by specific inhibitors significantly reduced the induction of cellular senescence. However, p38 was found to be the main inducer of senescence, whilst JNK was an inhibitor of apoptosis and actually suppressed p16 (Spallarossa et al., 2010). Equally, ERK overexpression induced senescence, but this resulted because ERK is a mild oncogene downstream of RAS signalling (Boucher et al., 2004),

and exclusion of p-ERK from the nucleus is part of the senescent phenotype (Mallette et al., 2004). Additionally, in one study inhibition of ERK only had a minor effect on senescence (Courtois-Cox et al., 2006), though in another study in slowly proliferating or quiescent cells ERK inhibition prevented senescence induction (Probin et al., 2006). Thus, p38 is the main MAPK involved in senescence signalling, being sufficient, although predictably not essential, for induction (Freund et al., 2011).

DNA damage activates p21 which signals through GADD45A (Kearsey et al., 1995), and can activate p38 either directly or through MAP3K4 and MAP2K3 (Bulavin et al., 2003). However, the dynamics of p38 after X-irradiation closely follow those of p16, increasing only slightly in the first 24 hours, rising substantially between 2-4 days and peaking at 8-10 days (Freund et al., 2011), which is consistent with p38 inducing p16 expression, and inconsistent, though not incompatible, with activation by p21. Notably, another study demonstrated that the inhibition of p53 actually increased the activation of p38 (Freund et al., 2011). However, these results should be viewed with caution as p53 has multiple different roles which at times can appear paradoxical (Demidenko et al., 2010). It is likely that in the cases of severe stress and telomere dysfunction that it is DNA damage which leads to the activation of p38, even if the exact mechanism and timeframe are unclear, though in oncogene induced senescence RAS can directly activate p38 (Chen et al., 2000a, Li et al., 2000). Importantly, the activation of p38 by constitutive MKK6/2E causes the complete abrogation of RAS stimulated proliferation (Deng et al., 2004). Thus RAS could potentially induce senescence through p38. Consistent with this model, RAS more potently activates the pro-survival and proliferation MAPKs, ERK and JNK than p38 (Chen et al., 2000a), so it could be speculated that normal levels of RAS may trigger proliferation, whilst high levels begin to induce senescence through the increased induction of p38.

Importantly, this process could be independent of DNA damage. p38 can activate the senescence inducer p16, and can even phosphorylate p53 through the activation of p38-regulated protein kinase (PRAK), which is essential for the transcriptional activity of p53 in response to RAS, and the senescence response (Sun et al., 2007). However, it should be noted that activation of p38 to the endogenous levels induced by RAS was not sufficient to completely abrogate

the proliferative response, and it was only with higher levels of p38 through MKK6/2E that this was achieved. Thus, it is still likely that p38 requires additional stimulation from other signals, such as DNA damage, in order to induce cellular senescence under physiological conditions.

Through the combined use of modelling and experimental work it has been shown that p38 can also be involved in a positive feedback loop to induce senescence by the induction of further DNA damage (Passos et al., 2010). This was based on previous studies that p38 induces TGF-β which caused an increase in ROS through mitochondrial and non-mitochondrial sources (Koli et al., 2008, Torres and Forman, 2003), both of which have been implicated in senescence (Davis et al., 2005, Debacq-Chainiaux et al., 2005). Passos et al. (2010) demonstrated that ROS generated from these pathways caused further DNA damage and cyclic activation of p53 through the replenishment of the short-lived damage foci (Passos et al., 2010). Then, as described above, DNA damage could activate p38 through the activation of p21 and GADD45A (Bulavin et al., 2003, Kearsey et al., 1995). However, contrary to this, further evidence suggested that neither p38 induction using MKK6/2E, nor p38 inhibition using SB203580, had any effect on activation of the DDR or activation of p53 as it would be expected to do if p38 induced senescence via inducing DNA damage (Freund et al., 2011). Notably, this latter observation does not contradict that p38 can be activated by DNA damage, only that p38 does not induce further DNA damage, although as discussed above the inhibition of p38 by p53 is not entirely conducive to this idea (Freund et al., 2011).

One possible reason for the contradiction between these two studies is the different cellular environments. Passos et al. (2010) used irradiated cells, which will have large amounts of DNA damage and therefore an active DDR and p53 signalling, whereas Freund et al. (2011) used MKK6/2E stimulated cells which would not necessarily involve such signalling (Freund et al., 2011, Passos et al., 2010). Speculatively, it is possible that in order to sustain the feedback loop between p38 and p53, both pathways require some initial activating stimulus. For example, some essential component of the TGF-β signalling pathway could require phosphorylation at two different sites; one by a DDR or p53 regulated kinase, and one by a p38 regulated kinase in order to become sufficiently actived to produce the required ROS for positive feedback via the pathway

suggested by Passos et al. (2010). However, what is clear is that senescence can be induced independently of DNA damage.

1.3.5 DNA Damage Independent Senescence and mTOR

Combined with all the reports that oncogene induced senescence is dependent on DNA damage, there are also reports that this can be done independently, though still requiring p53 (Wolyniec et al., 2009). Equally, the knockout of PTEN tumour suppressor gene, which inhibits the PI3K/Akt pathway, triggers arrest independent of hyperproliferation or DNA damage, but still dependent on p53 (Alimonti et al., 2010).

Notably, the use of shRNA against EZH2 to inhibit the Polcomb protein Bmi1 can directly activate p16 and induce senescence (Bracken et al., 2007), which could potentially induce senescence independently of damage, though notably they also observe that DNA damage does cause depletion of EZH2.

The activation of p16 and p21 using an IPTG inducible system was shown to activate the DDR and induce senescence without significant DNA damage as determined by a COMET assay (Pospelova et al., 2009). As well as the HDAC inhibitor sodium butyrate, these stimuli caused an increase in γ-H2AX disproportionate to the amount of DNA damage, which they contrasted to the induction of senescence by irradiation (Pospelova et al., 2009). Interestingly, when the IPTG is washed out, many of the cells re-entered cell cycle (Leontieva et al., 2012), thus indicating artificially maintained p16 or p21 cannot in themselves induce a stable senescent phenotype and still require other stimuli such as DNA damage to reinforce arrest and make it permanent. It should also be noted that the situations in these experiments are artificial. Were cells to be subjected to high levels of p16, p21, oncogenes or HDAC inhibition *in vivo*, the most likely cause, if not the only one, would be the instigation of DNA damage resulting in either the de-regulation of an oncogene or the upregulation of a tumour suppressor.

It is widely believed that oncogenes induce senescence by a system of negative feedback along with the induction of tumour suppressor proteins (Deng et al.,

2004); however, another hypothesis is that it is the continued expression of oncogenes which is important (Blagosklonny, 2003). Via this theory, if the oncogenes were suppressed quickly enough then senescence would fail and the cells would re-enter cell cycle. Interestingly, radiation has been shown to induce mitogenic signalling such as RAS, Akt and Raf1 (Kasid et al., 1996, Liu et al., 1996, Shaulian et al., 2000), and p53 stimulates MAPK and Akt signalling (Fang et al., 2001, Ishii et al., 1995). Thus these mitogenic signals might be important in all forms of senescence.

Compared to quiescence, where the cell reversibly arrests in the absence of growth factors and therefore have low levels of cyclin D1, senescent cells have high levels of cyclin D1. Quiescent cells respond to growth factors by upregulating cyclin D1 and activation of the cell cycle (Blagosklonny and Pardee, 2002), whilst senescent cells are arrested despite high levels of cyclins including A, B, D and E (Darzynkiewicz, 2002, Gong et al., 1995), and further stimulation does not result in proliferation. Although at high concentrations cyclin D1 can paradoxically inhibit CDKs (Atadja et al., 1995, Wong and Riabowol, 1996), combined with the observation that RAS stimulates proliferation in cells lacking p16 and pRb (Sherr, 2004), this suggests that the presence of tumour suppressors makes the cell unresponsive to the pro-cell cycle activities of RAS. Importantly, stimulation of cell cycle inducers downstream of pRb should therefore still cause cell cycle progression, as it has been suggested that they do (Blagosklonny, 2006b).

Interestingly, the activation of physiological levels of p53 using nutlin-3a, which is an HDM2 antagonist, can induce senescence in some cells (Efeyan et al., 2007, Van Maerken et al., 2006), but only quiescence in others (Cheok et al., 2010, Huang et al., 2009, Korotchkina et al., 2009), and has been shown to suppress cellular senescence in cells already expressing high levels of p21 (Demidenko et al., 2010). The growth promoting functions of p53 have been implicated to work through its inhibition of mTOR signalling.

The serine/threonine kinase mTOR forms two complexes with different functions. Both complexes contain mLST8/GβL, and the inhibitory deptor subunit (Peterson et al., 2009), whilst mTORC1 contains Raptor and Rheb, and mTORC2 contains Rictor and mSIN1 (Frias et al., 2006). The mTORC1

complex responds to both amino acid levels and growth factors, whereas mTORC2 responds to only the latter. The presence of amino acids stimulates the Ragulator to translocate mTORC1 to the lysosomal surface (Sancak et al., 2010), where it can bind Rags which allow it to associate with Rheb, and become sensitive to stimulation via insulin (Sancak et al., 2010, Drummond et al., 2008). The activation of insulin receptor substrate (IRS) binds phosphoinositide 3-kinase (PI3K) producing phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P3), which then binds Akt and PDK1 (Polak and Hall, 2009). Akt then destabilises the tuberous sclerosis complex (TSC1/TSC2) (Inoki et al., 2002), allowing the binding of GTP-bound Rheb to bind and activate mTORC1 at the lysosomal membrane (Huang and Manning, 2009).

mTORC1 activates p70-S6K1 causing the phosphorylation of numerous substrates including S6 ribosomal protein (Heinonen et al., 2008), and the translation initiation factor eIF4B. Combined with additional inactivation of 4E-BP1, freeing eIF4E (Dann and Thomas, 2006), this results in increased translation through the eIF4F complex. mTORC1 also regulates ribosomal biogenesis and inhibits autophagy, the latter through the inhibitory phosphorylation of ULK1 (Lee et al., 2010).

The reason mTOR is thought to be important in senescence is that its inhibition promotes quiescence, whilst its stimulation promotes senescence (Demidenko et al., 2010). Consistently, there is some evidence that rapamycin can inhibit the induction of senescence, and even allow cell cycle re-entry (Korotchkina et al., 2010, Leontieva et al., 2012, Pospelova et al., 2009). Contrary to this idea, the stimulation of autophagy, which is inhibited by mTOR, has been shown to be necessary for senescence (Young et al., 2009, Cho et al., 2013), although autophagy controls multiple processes and can also inhibit senescence under some conditions (unpublished).

The mTOR pathway is activated by RAS signalling (Kennedy et al., 2011), and thus may reflect an additional mechanism by which oncogenes can contribute to senescence induction. It has also been suggested that mTOR may induce senescence by maintaining an atypical DDR which is not associated with DNA damage or many of the molecules that are exclusive to the typical DDR, such as 53BP1 (Pospelova et al., 2009). However, these irregular foci have only

been shown to exist in response to a few atypical forms of stress (Pospelova et al., 2009), whilst the majority stimuli still induce damage and foci containing 53BP1 (Rodier et al., 2011). Thus, although speculatively mTOR may maintain an atypical DDR, this has little implication for the role of damage in senescence, which is the primary inducer in most cases. One final way that mTOR may contribute to senescence is via the activation of NF-κB and the inflammatory pathways (Dan et al., 2008, Madrid et al., 2001).

1.3.6 Inflammation, NF-κB and Senescence

A key regulator of the inflammatory response is the transcription factor NF-κB, which regulates and is regulated by a complex network of interacting factors. There are five members of the NF-κB family, p65, RelB, c-Rel, p105/p50 and p100/p52, all containing a conserved Rel homology domain that allows them to dimerise, and bind both DNA and the inhibitory IκB molecules. The IκB molecules prevent the dimers from entering the nucleus to initiate transcription. Additionally, both p105 and p100 have ankyrin repeat domains similar to the IκB molecules which also prevent nuclear translocation. These molecules need to be cleaved to p50 and p52 respectively to lose the ankyrin repeats and allow nuclear translocation.

Canonical NF-κB signalling occurs in response to inflammatory cytokines such as IL-1 and TNF-α or products of bacterial infection such as lipopolysaccharide (LPS). As a result the IKK complex consisting of the two catalytic subunits IKKα and IKKβ bound to multiple copies of the regulatory subunit NEMO is activated by phosphorylation of IKKβ (Perkins and Gilmore, 2006), which phosphorylates IκBα deactivating it and causing its ubiquitination by the Skp1/Cul1/F-box protein-β-TrCP ubiquitin ligase complex, and targeting it for degradation by the proteasome (Hayden and Ghosh, 2004). This induces the translocation of p65-p50 heterodimers within minutes of the stimulus (Perkins and Gilmore, 2006), and can result in transcription of a host of different factors depending on activity of different co-factors. Its main targets include TNF-α/β, IL-1β, IL-2, IL-6, IL-8, IL-12 and IFN-β (Blackwell and Christman, 1997). Similar to p53, canonical NF-κB signalling is oscillatory as it stimulates the transcription of IκBα which returns

NF-κB to the cytoplasm (Hayden and Ghosh, 2004), as well as multiple proinflammatory cytokines for positive feedback. However, additional inhibitors such as IκBβ and IκBε are not regulated by NF-κB (Perkins and Gilmore, 2006), and thus dampen the oscillatory profile (Hoffmann et al., 2002).

Non-canonical signalling occurs via the activation of NIK, which activates IKKα causing the phosphorylation and proteolytic cleavage of p100 to p52 (Bonizzi and Karin, 2004, Hayden and Ghosh, 2004). Then p52 protein mostly associates with RelB (Bonizzi et al., 2004, Bonizzi and Karin, 2004), forming a heterodimer which translocates to the nucleus and targets promoters containing distinct κB elements (Bonizzi et al., 2004). The promoters of RelB, c-Rel, p100 and p105 all contain κB elements, and are thus transcribed as a result of NF-κB activation (Hayden and Ghosh, 2004, Pahl, 1999), which results in changes in NF-κB signalling over time.

Evidence for a role of inflammation in senescence was demonstrated by the prevention of both induction and maintenance of oncogene induced senescence by the inhibition of the inflammatory cytokines IL-6 and IL-8 (Kuilman et al., 2008). In the same issue of *Cell* another study showed that the IL-8 receptor CXCR2 was important for both OIS and replicative senescence, and overexpression of CXCR2 could induce senescence dependent on p53 (Acosta et al., 2008). Importantly, they found that CXCR2 depletion inhibited ATM activation and DDR signalling, consistent with the idea that inflammation contributes to senescence via activation of the DDR.

With regards to the role specifically of NF-κB, one study showed that senescent cells had increased active phospho-p65 (S536) (Rovillain et al., 2011), and others that p65 was noticeably more nuclear in senescent than proliferative cells with markedly increased DNA binding activity (Freund et al., 2011, Chien et al., 2011), although an earlier study showed the opposite effect, including decreased nuclear and increased cytoplasmic levels of p65 with reduced NF-κB binding to promoter regions (Helenius et al., 1996). Notably, repression of NF-κB signalling can result in bypass of senescence (Rovillain et al., 2011), extends replicative lifespan in culture (Tilstra et al., 2012), and c-Rel overexpression can induce senescence (Bernard et al., 2004). Combined these data suggest that NF-κB is important for senescence. However, another group

found that constitutive activation of IKKβ delayed Ha-RasV12 oncogene induced senescence of primary human fibroblasts by suppressing the DDR (Batsi et al., 2009), although this may be down to NF-κB independent functions of IKKβ including the induction of autophagy (Criollo et al., 2010b, Criollo et al., 2010a).

Notably, in contrast to tumour suppressors such as p53 and p16, NF-κB is frequently overactive in cancer cells (Karin, 2009, Meylan et al., 2009, Pikarsky et al., 2004), and is thought to play a role in tumour promotion, though not initiation (Pikarsky et al., 2004). This is likely a result of the pro-survival pathways of NF-κB by the inhibition of apoptosis rather than specifically inducing proliferation (Barbie et al., 2009, Pikarsky et al., 2004), though it also has other relevant effects (Bennett, 2008, Karin, 2009). Indeed *in vivo*, both senescence associated-β-galactosidase (SA β-gal) and p16 expression are reduced by NF-κB inhibition (Adler et al., 2007), and its suppression is also associated with tumour development (Dajee et al., 2003, Maeda et al., 2005). Importantly, the high levels of chromosomal amplifications and translocations in p65 knockout cells has implicated a role in maintaining senescence of mouse and human immortalised cells through the maintenance of genomic stability by an undescribed mechanism of activating DNA repair (Wang et al., 2009b). Thus, the tumour promoting and inhibiting effects of NF-κB is context dependent (Jing et al., 2011), as NF-κB appears to prevent DNA damage, increase cell survival and contribute to clearance by the immune system by increasing inflammation. Notably, in cells with effective tumour suppressor mechanisms all these pathways will help the activation of cell senescence, but may have the opposite effects in cells with defective senescence machinery, although some studies are still contradictory (Batsi et al., 2009).

How NF-κB is activated in senescence is still unclear. Genotoxic stress has been shown to activate NF-κB via a pathway involving PARP-1 and ATM which converge to monoubiquitinate NEMO and allow activation of the IKK complex by TAK1 (Hinz et al., 2010, Stilmann et al., 2009). In irradiation induced senescence, the DNA-binding of NF-κB had begun to rise 24hrs after irradiation and peaked 8-10 days later, which was decreased by inhibition of p38 (Freund et al., 2011). Whilst constitutive activation of p38 was sufficient to stimulate the increased NF-κB DNA binding activity found in senescent cells, inhibition by

ATM also decreased NF-κB activity to the same level as p38 inhibition, and the effect of dual inhibition was synergistic suggesting that both affect NF-κB via different pathways (Freund et al., 2011). Consistently, supraphysiological but not endogenous p38 activation can induce IL-6 levels to those of senescent irradiated cells, suggesting that p38 requires co-operation with the DDR under physiological conditions (Freund et al., 2011).

NF-κB is essential in the production of the SASP (Chien et al., 2011, Freund et al., 2011), and its inhibition by shRNA or the inhibitor Bay 11-7082 reduces both the inflammatory molecules as well as several matrix metalloproteinases, producing a similar effect to p38 inhibition, suggesting that the effect of p38 on the SASP results from its activation of NF-κB. Importantly, co-suppression of p65 and p53 allowed significant bypass of senescence and failure to accumulate p16 in IMR-90 fibroblasts in response to oncogenic Ras, where each individual suppression had very little effect. As BJ fibroblasts bypassed senescence with p65 inhibition alone, this suggests that these two hub molecules work in different but interconnected networks that co-operate to induce senescence dependent on cell type (Chien et al., 2011).

Acting downstream of p38, which can under some circumstances have little effect on DNA damage levels (Freund et al., 2011), there may be DNA damage independent mechanisms by which NF-κB contributes to senescence. However, NF-κB is involved in several pathways which increase levels of ROS, and its inhibition has been shown to improve mitochondrial function and reduce oxidative stress (Mariappan et al., 2010), through which it might be expected to contribute to senescence via the instigation of DNA damage.

1.3.7 Reactive Oxygen Species as Mediators of Senescence through DNA Damage

Senescent cells have been shown to have significantly higher ROS levels (Hagen et al., 1997), and higher levels of oxidative DNA damage, but not protein carbonyls (Chen et al., 1995), than healthy replicating cells. However, other studies also suggest that high levels of protein oxidation and cross-linking

are features of senescent cells, which is consistent with the deterioration of proteasomal and lysosomal function (Sitte et al., 2000c, Sitte et al., 2000b). As ROS can either be produced as byproducts of metabolic processes or deliberately as part of signalling pathways, there are multiple sources by which ROS can be induced in senescent cells.

Many of the ROS producing enzymes are regulated by NF-κB, including 5-lipoxygenase enzyme (Chopra et al., 1992), the NADPH oxidase NOX2 (Anrather et al., 2006), COX-2 (Deng et al., 2003, Inoue and Tanabe, 1998), and the reactive nitrogen species producing complex, iNOS (Kolyada et al., 1996). However, NF-κB also upregulates many antioxidant enzymes including SOD2 (Djavaheri-Mergny et al., 2004, Jones et al., 1997), SOD1 (Rojo et al., 2004), thioredoxins (Djavaheri-Mergny et al., 2004), glutathione S-transferase-pi, and Gpx-1, the latter two in response to oxidative stress (Schreiber et al., 2006, Xia et al., 1996). Thus, although NF-κB is involved in the production of ROS during inflammation, it is also involved in their clearance once ROS levels become too high and/or resolution is required. Whether NF-κB is essential for the ROS production in senescence is controversial. The induction of senescence by overexpression of c-Rel was dependent on oxidative damage (Bernard et al., 2004), and p65 knockout both reduced oxidative damage and delayed the onset of replicative senescence (Tilstra et al., 2012), but contrarily another study found no differences in endogenous hydrogen peroxide and oxidative DNA damage between $p65^{-/-}$ and $p65^{+/+}$ MEFs (Wang et al., 2009b).

Other mechanisms by which ROS could be induced in senescence include the activation of $p66^{Shc}$ by p53 (Trinei et al., 2002). $p66^{Shc}$ is upregulated in senescent cells (Zhang et al., 2010), but in contrast to its clear role in the induction of apoptosis (Trinei et al., 2002), there is currently little evidence for a causal relationship in cellular senescence. Notably p53 also promotes expression of several antioxidants (Olovnikov et al., 2009), which may help account for the dual role of p53 in suppressing senescence (Demidenko et al., 2010). Another study showed that knockout of p21, but not p16, caused a reduction in ROS levels in RAS expressing cells, but the level was not reduced to that of controls (Takeuchi et al., 2010). Contrarily, p16 has been shown to increase ROS in some cells (Takahashi et al., 2006).

p21 could mediate ROS levels by the negative regulation of peroxiredoxins through the repression of sestrins (Kopnin et al., 2007), or the ROS generating 5-lipoxygenase enzyme (Catalano et al., 2005). However, it is also consistent with the idea that ROS production may be induced by the activation of TGF-β via p38 (Passos et al., 2010), which can stimulate both mitochondrial and non-mitochondrial sources of ROS (Koli et al., 2008, Torres and Forman, 2003).

Mitochondria can also be a large source of ROS during senescence. Breakdown of mitochondrial membrane potential and increased ROS has been shown in senescent cells (Passos et al., 2007), as has impaired metabolism and low nucleotide triphosphate levels including ATP (Zwerschke et al., 2003).

Importantly, treatment with antioxidant enzymes and hypoxia increases replicative lifespan (Chen et al., 1995), whilst hydrogen peroxide (Chen et al., 1998), and hyperoxia (von Zglinicki et al., 1995) induce premature senescence. ROS are also essential for RAS induced senescence (Catalano et al., 2005, Lee and Paull, 2005, Nicke et al., 2005, Wu et al., 2004), p21 overexpression induced senescence (Macip et al., 2002), and the initiation, but not maintenance, of senescence induced by IR (Hong et al., 2010). Thus, there is strong evidence that ROS play a causal role in senescence.

Mitochondria may play a prominent role in senescence. They have been implicated in replicative senescence through the production of ROS as a result of dysfunction (Passos et al., 2007, Passos and Von Zglinicki, 2006), and also in OIS in response to p53 or pRb activation (Moiseeva et al., 2009). Additionlly, the induction of mitochondrial dysfunction using antimycin A, oligomycin (Stockl et al., 2006), and the mitochondrial Rieske iron sulphur protein (RISP) (Moiseeva et al., 2009) is sufficient to induce senescence. The mitochondrial uncoupling agent DNP reduced ROS levels and increased yeast replicative lifespan (Barros et al., 2004), and fibroblast replicative lifespan (Passos et al., 2007). Additionally, telomere dysfunction has been shown to induce mitochondrial compromise in mice (Sahin et al., 2011). Therefore, it is likely that mitochondrial function is an important factor in senescence.

The overexpression of PGC1α has also been shown to accelerate senescence (Xu and Finkel, 2002), indicating biogenesis plays an important role. If this results from increased ROS production due to the higher numbers of

dysfunctional mitochondria, then this could also implicate mTOR in the induction of DNA damage through activation of PGC1α and ROS, as suggested by a recent study (Marques et al. unpublished).

Exactly how ROS contribute to cellular senescence is still controversial. One possibility is that ROS are part of a positive feedback loop generating small short lived foci, which still account for about half of those present even in deep senescent cells. In this feedback loop, p38 stimulates ROS production through TGF-β, thus maintaining a continual DDR (Passos et al., 2010). Notably, the contrary study implicating a DDR-independent role for p38 in senescence did not examine whether p38 overactivation affected ROS levels (Freund et al., 2011), whilst another study found that p38 did induce ROS production in chondrocyte senescence (Hong et al., 2010). Thus, p38 may induce senescence through the production of ROS. Interestingly, ROS signalling activates PKCδ-CF (Bey et al., 2004, Konishi et al., 1997, Talior et al., 2005), which is increased during replicative senescence in HDFs (Wheaton and Riabowol, 2004), though not in MEFs (Takahashi et al., 2006), and is known to stimulate NADPH oxidase, thus generating a positive feedback loop to increase ROS (Bey et al., 2004, Talior et al., 2005). As in the same cells inactivating p53 and pRb function did not induce cell cycle unless ROS are also depleted, it was suggested that ROS may stimulate their own positive feedback loop maintaining senescence even independently of damage, possibly through the suppression of the cytokinesis initiator, WARTS (Takahashi et al., 2006); however, this would be specific to G2 arrest. Equally, ROS could stimulate positive feedback by inducing damage to mtDNA (Dumont et al., 2000b), reducing mitochondrial function and inducing further ROS production (Pitkanen and Robinson, 1996, Wong et al., 2002).

Alternatively, several studies have suggested that ROS may contribute to senescence through the activation of p38 (Hong et al., 2010, Xiao et al., 2012). Equally, under multiple conditions ROS can activate NF-κB either through the degradation of IκBα (Schieven et al., 1993, Schoonbroodt et al., 2000, Takada et al., 2003), activation of the IKK complex (Kamata et al., 2002), or stabilisation of NIK (Li and Engelhardt, 2006). However, ROS can also inhibit NF-κB through the stabilisation of IκBα by inhibiting the proteasome (Wu et al., 2009) and the inactivation of the IKK complex (Panopoulos et al., 2005, Reynaert et al., 2006).

Whether ROS activate NF-κB in cellular senescence is currently unknown, but as they activate p38, which is in a positive feedback loop with NF-κB, it is likely that they do.

1.3.8 Summary

Cellular senescence is a stress response, which the current evidence suggests is instigated primarily by DNA damage. This induces an activation cascade known as the DNA damage response, which has several downstream effects. Most importantly it activates the tumour suppressor protein p53 which then activates the cyclin dependent kinase inhibitor p21, and further downstream pRb, to prohibit the cell cycle. However, senescence is not a linear process. It has multiple redundant and interacting pathways which help to regulate the cellular response to damage. At least partially independent of damage p38 can activate p16 to induce senescence, and the mTOR pathway may also play a role.

In nearly all cases senescence is dependent on the presence of an active DDR. Thus, although some stimuli can induce senescence independently of the DDR, an important mechanism in the large majority of senescence induction and maintenance is ensuring that the DDR persists. Whilst some pathways exist that can activate the DDR without the induction of damage, there is a host of evidence suggesting that senescing or senescent cells reinforce the DDR with the stimulation of further DNA damage to prevent cell cycle re-entry.

The result of inducing this system of positive feedback loops integrating molecular damage, induces radical changes in senescent cells, which helps to produce the resultant phenotype. The pathways are summarised in figure 1.3.

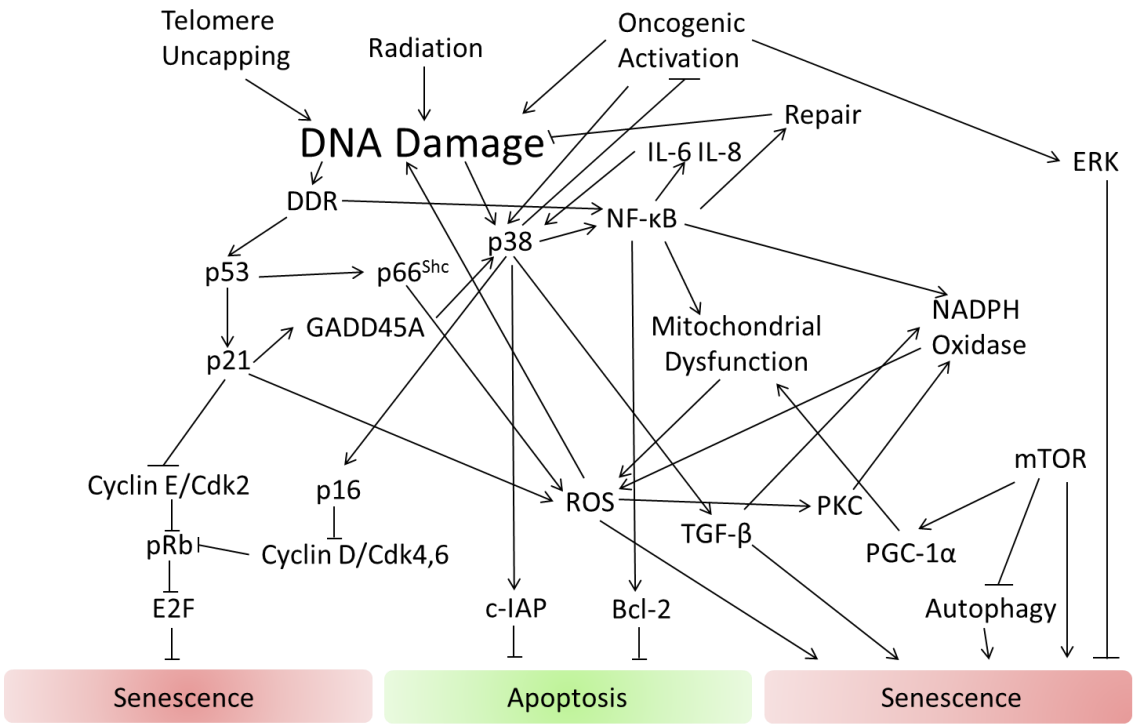


Figure 1.3| Network of factors interacting to induce senescence and inhibit the induction of apoptosis.

1.4 The Senescent Phenotype

1.4.1 Apoptosis and Alternatives to Senescence

If a cell is responding properly to stress in the form of damage or oncogenic activation it will inhibit proliferation. Minor damage results in quiescence whilst the damage is repaired; however, if the stress is more severe the cell must permanently stop cycling and either enter permanent senescence or induce one of the many pathways of cell death. It should be noted here that despite many early papers referring to senescence as “cell death”, that this is not the case. Senescent cells remain viable and metabolically active for as long as has been measured (Matsumura et al., 1979, Pignolo et al., 1994), and may be more resistant to cell death than proliferating cells (Wang et al., 1994).

Despite its importance, senescence is only one possible fate of damaged cells. The mechanisms that inhibit cellular senescence and promote cell death are highly important in understanding the senescent phenotype and its implications

The main programmed alternative to senescence is apoptosis, which is generally categorised by cell shrinkage, nuclear condensation and fragmentation, as well as membrane blebbing (Collins et al., 1992). Although a detailed description is beyond the scope of this thesis, apoptosis can be activated through intrinsic or extrinsic pathways, or through the absence of survival signals (Lowe et al., 2004), and, similarly to senescence, many of these pathways are regulated by p53 (Espinosa, 2008, Haupt et al., 2003) and redox signalling (Polyak et al., 1997). However, proteins such as Bax, bid puma and noxa, which are often downregulated in senescence are highly active in apoptosis (Vaseva and Moll, 2009), as well as the pro-apoptotic miRNA mi-34a (He et al., 2007, Hermeking, 2007), which inhibits mTOR-dependent PI3K-Akt signalling via the inhibition of Notch1 (Mungamuri et al., 2006). A possible determinant of cell fate is the localisation of p53 to mitochondria, which can trigger apoptosis independently of transcription (Marchenko et al., 2000, Mihara et al., 2003, Vaseva and Moll, 2009).

There are multiple factors that can influence cell fate decision. Whilst oncogenic RAS promotes senescence (Serrano et al., 1997), Myc preferentially induces apoptosis (Zindy et al., 1998). Equally fibroblasts and epithelial cells are resistant to apoptosis, whilst lymphocytes are intrinsically predisposed to it (Zuckerman et al., 2009). From an early study exposing fibroblasts to different concentrations of hydrogen peroxide, it would appear that the cell fate decision was partially dependent on the dosage. The lowest concentrations failed to induce permanent growth arrest, the medium concentrations induced senescence, and the higher concentrations induced apoptosis (Bladier et al., 1997). However, there was still a stochastic determinant, and even at the highest concentration 6% of the cells managed to survive by becoming permanently arrested (Bladier et al., 1997).

Current evidence suggests that cell fate is dependent on the conformation, localisation, activity and stability of p53, which is involved in both senescence and apoptotic responses. p38 phosphorylation of p53 at Ser46 promotes apoptosis (Bulavin et al., 1999), and the mitochondrial localisation of p53 was found to be associated with radiosensitive organs and cell cultures that preferentially underwent apoptosis (Erster et al., 2004). Additionally, severe damage can induce apoptosis through acetylation of p53 (Sykes et al., 2006, Tang et al., 2006), though it is site dependent as the acetylation of some loci can activate pro-senescence p21, as can monoubiquitination (Le Cam et al., 2006). Importantly, the activation of p21 is not essential for p53 induced apoptosis (Deng et al., 1995), and can be protective against it (Bunz et al., 1999, Mahyar-Roemer and Roemer, 2001).

There are now several lines of evidence to suggest that cell fate is dependent, or directly related to, the level of p21 induced by p53. Doxorubicin induced apoptosis paralleled the downregulation of p21 (Martinez et al., 2002), and pro-apoptotic proteins such as Myc silence the p21 promoter altering the outcome in response to damage in favour of apoptosis (Wu et al., 2003). p21 is also regulated at the epigenetic level by acetylation and methylation, both of which influence cell fate towards apoptosis or senescence (Rebbaa et al., 2006).

Thus, although there are likely to be mechanisms independent of p53 and p21 that regulate the cell decision to enter senescence rather than cell death, these

proteins are likely to be primary regulators, which are heavily influenced by the level of stress, most likely translated by the severity of the damage.

1.4.2 Markers of Senescent Cells

The induction of senescence is associated with the expression and activation of multiple genes. However, some of these changes are transient, difficult to detect, or highly unspecific, making them bad markers of senescence. Several persistent easily observable markers of senescent cells have now been identified. Firstly and most importantly, these cells are permanently arrested, incapable of responding to physiological signals with cell cycle re-entry, despite the presence of normal growth factor receptors (Goldstein and Shmookler Reis, 1985). Replicative senescence is generally associated with the G1/S checkpoint just preceding DNA synthesis (Rittling et al., 1986, Pignolo et al., 1998), as is RAS induced senescence (Serrano et al., 1997); however, another study found that a significant fraction of RAS expressing cells arrested with partially replicated DNA (Mao et al., 2012), indicative of S-phase or G2 arrest. Indeed, ATM mutant cells induced G2 arrest (Herbig et al., 2004), and several studies have shown large fractions of cells arrested in G2 in some cell types (Campisi, 1996, Mao et al., 2012). Thus, whilst cells most likely attempt to arrest before DNA synthesis, some if not many of them clearly fail, and still manage to permanently arrest before mitosis.

Therefore, markers such as the absence of BrdU (Bromodeoxy-uridine) which shows whether the DNA has been replicated, and Ki67 and PCNA which are present in proliferating cells, are good markers to demonstrate that the cells are not proliferating, but do not necessarily indicate that the cells have become senescent. However, this does not mean that they cannot be used to validate other markers of senescence, for which they are frequently employed.

Perhaps the easiest way to observe senescent cells is through their morphological changes. Senescent fibroblasts have an enlarged, flattened, and irregular morphology (Hayflick, 1965). One study found senescent cells can have around 10 times as much volume and can triple in diameter (Chen et al.,

2000b). Although they found that actin protein was not increased in senescent cells there was a huge increase in the amount of stress fibrils, many of which, in contrast to proliferating cells, spanned right across the cell (Chen et al., 2000b). Additionally, the focal adhesion plaques, made up of proteins such as paxillin and vesiculin, which are normally found at the cell membrane connecting the cell to the ECM, are found sporadically in senescent cells (Chen et al., 2000b). Importantly, although morphological changes are a good marker for senescence, they are not essential for growth arrest (Chen et al., 2000b).

Senescence is also accompanied by an increase in nuclear size (Narita, 2007), and an increase in the density of nuclear pore complexes (Maeshima et al., 2006), which allow transport of macromolecules between the nucleus and cytoplasm. In addition to size, the nucleus undergoes a host of changes during cellular senescence, the most prominent of which is the formation of senescence-associated heterochromatic foci (SAHF), which have a strong correlation with the irreversibility of senescence arrest (Beausejour et al., 2003, Narita et al., 2003). Importantly, SAHF formation is significantly reduced by knockdown of p16 or pRb but not p53, whilst all knockdowns remain permanently arrested (Narita et al., 2003).

The lamin proteins, which connect the nuclear envelope to the chromatin, also change during senescence. Both lamins A and B have been implicated in the senescence process (Varela et al., 2005, Liu et al., 2005, Dreesen et al., 2013); however, the overexpression of lamin B1 can either repress or induce senescence depending on the study (Dreesen et al., 2013, Shimi et al., 2011, Barascu et al., 2012). Lamin B1 levels are decreased in senescent cells, potentially contributing to the formation of SAHF which are repressed by the protein (Sadaie et al., 2013). Equally, lamin A repression activates the DDR and induces 53BP1 and γ-H2AX foci (Liu et al., 2005), which could potentially help to reinforce the senescent phenotype, if lamin A follows the same expression pattern as lamin B during senescence.

The most commonly used marker of senescence is senescence associated-β-galactosidase (SA-β-gal) (Dimri et al., 1995) activity. As a lysosomal enzyme, β-galactosidase is most active in acidic conditions at pH4-4.5, with markedly reduced activity at pH 6 (Zhang et al., 1994). However, whilst β-galactosidase

activity can only be detected at low pH in proliferating cells, in senescent cells it is additionally detectable at pH 6, and this is thought to correspond to increased β-galactosidase activity (Kurz et al., 2000). It was suggested that due to the increase in size, and the number of the lysosomes in senescent cells (Robbins et al., 1970, Brunk et al., 1973, Lee et al., 2006), that β galactosidase activity surpasses a threshold causing observable activity at higher pH (Kurz et al., 2000). Importantly, the GLB1 gene which encodes β-galactosidase, has increased expression in senescent cells and results in increased protein levels, though it is not necessary for senescence (Kurz et al., 2000).

The implication of lysosomal expansion in senescent cells is that senescent cells are subject to large increases in molecular damage, which requires increased levels of degradation. Consistently, the increased numbers of dysfunctional mitochondria, which accumulate in senescent cells, produce high levels of reactive oxygen species (Passos et al., 2007), which cause oxidative damage to both nuclear and mitochondrial DNA (Passos et al., 2007), as well as proteins and lipids, forming oxidative modifications and carbonyls (Ahmed et al., 2010), and lipofuscin (Sitte et al., 2000a, Sitte et al., 2000c, Sitte et al., 2000b, Sitte et al., 2001). Lipofuscin is the product of severe oxidative modifications leading to protein aggregation, and accumulates in most post-mitotic cells (Jung et al., 2007), and causes autofluorescence of senescent cells making it a useful marker (Katz and Robison, 2002), although it can also accumulate in reversibly arrested proliferation-competent cells (Sitte et al., 2001). Recently it has also been used to identify senescent cells *in vivo*, in samples which are unsuitable for SA-β gal staining (Georgakopoulou et al., 2013).

1.4.3 DNA Damage as a Marker of Senescence

It has already been described that DNA damage is the primary inducer of senescence in the majority of cases, although some specific stimuli may be able to induce senescence independently. However, except in this minority of cases, the evidence suggests that inhibiting the DNA damage response does allow a certain percentage of cells to escape senescence (Bartkova et al., 2006, d'Adda

di Fagagna et al., 2003, Di Micco et al., 2006, Gire et al., 2004, Herbig et al., 2004). It is likely that some cells also manage to maintain the senescent phenotype; however, this cannot be known for sure without using knockout rather than knockdown systems. Regardless, this still implies that maintenance of the DDR is an important part of the senescent phenotype.

As described in section 1.3.1, the DNA damage is typically observed by either 53BP1 or γ-H2AX foci, which result from the oligomerisation of these molecules at sites of DNA damage. If the lesions are repaired then the foci appear transient, and are thought generally not to persist for longer than 24 hours. In healthy replicating cells most foci disappear within five hours of arising (Passos et al., 2010). The induction of senescence is associated with the induction of irreparable damage which generates persistent foci leading to growth arrest (Beausejour et al., 2003, d'Adda di Fagagna et al., 2003, Herbig et al., 2004), although theoretically the individual foci do not need to persist as long as they are replaced by new foci to continue the signal for growth arrest. Consistently, the evidence suggests firstly that even in replicative senescence about half the foci persisted for less than 15 hours, and secondly that the generation of these short lived foci is equally important as the persistent foci in maintaining the growth arrest of senescent cells, at least for the first nine days after the initial stimulus (Passos et al., 2010).

This suggests that even in replicatively senescent cells not all the foci are telomere associated (Nakamura et al., 2008, Passos et al., 2010, Wang et al., 2009a), and therefore could be expected to be repaired if the break is not too complex. Consistently, after X-irradiation induced senescence about half the foci were associated with telomeres, and these accounted for all of the persistent foci. Thus even the highly complex breaks induced by 20Gy X-irradiation can be repaired by the cell, except where they happen at a telomere (Hewitt et al., 2012), and recent evidence suggests that telomeric DNA and associated proteins, even when placed elsewhere in the genome, may inhibit repair (Fumagalli et al., 2012).

Unlike the initial foci the persistent foci are not associated with repair proteins such as RAD51 and RPA70. It has been suggested that this results from changes at the focus, when the cell ceases attempting to repair them (Rodier et

al., 2011). However, they offer no evidence that these foci were ever associated with these proteins. Importantly, both proteins are involved in homologous recombination, and the recent evidence that all persistent foci are the result of telomeric damage suggests that these foci should not associate with these proteins, in order to prevent breakage fusion bridge cycles and genomic instability. Indeed, the telomeric foci of replicatively senescent cells share many of the same properties as the persistent foci after X-irradiation (Rodier et al., 2011).

The solubility of 53BP1 in persistent foci decreases compared to early foci (Rodier et al., 2011), which likely reflects the increased oligomerisation, that is observed by the increase in size of the focus. Additionally, although about 7-10 days after the stimulus, the cells decrease p53 and p21 (Rodier et al., 2011), hypothetically because the importance of positive feedback to maintain senescence decreases (Passos et al., 2010), the foci begin to associate with p53 and Chk2 which are not present at early foci (Rodier et al., 2011). Potentially this localisation could help to maintain a persistent signal with greatly reduced protein levels, thus losing the requirement for positive feedback.

PML (promyelocytic leukemia protein) bodies are dynamic, heterogeneous subnuclear domains that form in response to genotoxic stress (Bernardi and Pandolfi, 2007, Varadaraj et al., 2007), and are found at DNA damage foci (Carbone et al., 2002; Xu et al., 2003) where they facilitate the senescence growth arrest by the activation of p53 (Ferbeyre et al., 2000; Pearson et al., 2000). Also absent at initial foci, PML bodies co-localise with persistent foci after X-irradiation and telomere associated foci in replicatively senescent cells (Rodier et al., 2011). Thus, persistent/telomeric foci are a good marker for cell senescence and can be indicated best by the presence of p53, Chk2 and PML, but focus size is also an indication. Although the evidence suggests that these foci would be insufficient in small numbers to induce senescence, which also depends on the induction of smaller short lived foci by ROS (Passos et al., 2010), only the persistent ones are thought to be exclusive to senescent cells.

Importantly, the DNA damage response is one of the most proximal phenotypes of senescent cells, and has been shown to be necessary for the induction and maintenance of growth arrest (d'Adda di Fagagna et al., 2003), as well as the

formation of the senescence associated secretory phenotype (Rodier et al., 2009). Additionally, depletion of H2AX with shRNA, which destabilises foci, but does not affect formation (Celeste et al., 2003), also reduces the SASP at 2-3 days when the phenotype begins, and at 9-10 days later when the phenotype is robust (Rodier et al., 2011). Thus, persistent foci were concluded to be necessary for multiple aspects of the senescent phenotype. However, it is likely that the shRNA also affected the lifespan and functionality of the short-lived foci, which still normally contain γ-H2AX.

Another potential marker of senescence is the number of foci within a cell. The data in this thesis suggest that senescent MRC5 cells have around three foci on average, whereas young replicating cells have between 1-2 foci (figure 5.1). Another study showed that different cell lines had between 2-4 γ-H2AX foci at senescence, which was between 1-3 foci above the number found in the corresponding young cells, suggesting that the number of foci is cell type dependent, but always higher than that found in replicating cells (Sedelnikova et al., 2004), making it a good marker of senescence providing the cell type is taken into account. Importantly, the same study demonstrated the almost complete co-localisation of γ-H2AX and 53BP1. However, as is observable within the above study and elsewhere, 53BP1 foci are generally larger and clearer.

Unsurprisingly, as the agents inducing much of this damage, ROS are also upregulated in senescent cells, and provide a good marker of senescence (Correia-Melo et al., 2013, Lawless et al., 2012, Lawless et al., 2010, Passos et al., 2013). Though as none of these markers are infallible, most studies will analyse at least two in order to increase the likelihood that the observed phenotype is actually senescence.

1.4.4 The Senescence Associated Secretory Phenotype

In 2008 a study screened the secreted proteins of replicatively, irradiated and RAS induced senescent cells and compared them to each other as well as to replicating controls. Over 40 proteins from the array of 120 proteins were found

to be significantly increased in the secretions of senescent cells compared to young cells, which was therefore named the senescence activated secretory phenotype (SASP) (Coppe et al., 2008). The SASP included inflammatory cytokines such as IL-6, IL-7, and IL-8, TNF receptors, growth factors such as GRO, HGF and IGFBPs, shed cell surface molecules such as ICAMs, survival factors (Coppe et al., 2008), and matrix remodelling factors (Liu and Hornsby, 2007, Parrinello et al., 2005, Coppe et al., 2010). Importantly, although these SASPs had differences between cell types and senescence stimuli, they were relatively conserved across both (Coppe et al., 2008). However, RAS induced senescence did stimulate increased production of many of the factors compared to irradiated or replicatively senescent cells. Equally, the SASP of irradiated senescent cells developed between 4-7 days after the dose, whereas RAS induced the SASP 2-4 days after oncogene expression.

Importantly, if p53 was knocked down before the induction of senescence in irradiated cells then it produced a SASP quantitatively similar to that of RAS induced cells, although, RAS expression in p53 knockdown cells induced the most potent SASP (Coppe et al., 2008), so there is a combinatorial effect. If p53 is knocked down after senescence induction then it has no significant effect on the SASP, and if it is knocked down in cell lines that do not express p16, then p53 abrogation induces reversion to cell cycle, but still does not abrogate the SASP (Coppe et al., 2008), suggesting the two are independent.

Another study examining the SASPs of mouse cells found that when they senesced in response to irradiation, after being cultured at 3% oxygen, they produced human-like SASPs, but when they were cultured to replicative senescence at 20% oxygen they did not (Coppe et al., 2010). It should be noted that whilst the majority of these latter cells expressed SA-β-gal and arrested, a large minority were still undergoing DNA replication, despite no change in cell numbers.

Whilst the irradiated cells were grown at 3% oxygen giving two variables that could have been responsible for the difference in phenotype (either the growth conditions or the irradiation stimulus), compared to the unirradiated cells grown at 20% O_2, what is clear is that both arrest and SASP are at least partially dependent on the persistency of the DDR (Coppe et al., 2010). Exactly why

some stimuli do not produce a persistent DDR or SASP is not clear, and warrants further investigation, but these data clearly suggest that the SASP is a good marker of senescence.

The function of the SASP in vivo has not been properly tested; however, a closer look at some of the SASP components can provide some insight.

1.4.4.1 IL-6

IL-6 is one of the most prominent members of the SASP. It binds to the membrane associated receptor IL-6R, which then forms a complex with two gp130 proteins, which then binds JAK1 and JAK2 (Lutticken et al., 1994). Without JAK1, IL-6 signalling is greatly impaired (Guschin et al., 1995). The phosphorylated JAKs remain associated with the receptor and activate STATs, most potently STAT3, but also to a minor extent STAT1 (Heinrich et al., 1998). The STATs dimerise allowing them to be actively translocated to the nucleus (Bromberg et al., 1999), where they induce the transcription of multiple genes including NF-κB family members providing positive feedback. Additionally, IL-6 can activate the MAPK cascade through the recruitment of SHP2 (SH2-domain-containing tyrosine phosphatase) (Schiemann et al., 1997) or Shc (SH2-and collagen-homology-domain-containing protein). These two proteins are phosphorylated by JAK1 (Schaper et al., 1998), and bind Grb2 which recruits SOS and activates Ras-Raf-ERK signalling (Hermanns et al., 2000). Both p38 and JNK are also activated by IL-6, but by a less well defined mechanism (Bode et al., 2001b, Zauberman et al., 1999). Lastly, IL-6 can also induce the PI3K/Akt pathway resulting in the activation of mTOR and inhibition of Bad (Bcl2/Bcl-X_L-antagonist, causing cell death), contributing to cell survival and proliferation (Shi et al., 2002). Signalling is inhibited by protein tyrosine phosphatases (PTPs), including SHP2. PIAS3 also inhibits IL-6 induced activity of STAT3 (Chung et al., 1997), and SOCS1, 2 and 3 have been observed to be induced by IL-6 as a negative feedback mechanism to cease STAT signalling (Starr et al., 1997).

1.4.4.2 Other Important SASP Molecules

IL-7 is another cytokine highly expressed within the SASP (Coppe et al., 2008). By binding the IL-7 receptor of B cells it stimulates development in the latter from the pre- and pro-B cell stages. In T cells IL-7 is important in the V(D)J rearrangement of the T cell receptor beta (Muegge et al., 1993), and knockout mice suggest that it is essential for development and survival of lymphoid cells (Maeurer and Lotze, 1998).

Similar to IL-7, granulocyte macrophage-colony stimulating factor (GM-CSF) is involved in white blood cell development, but contrarily stimulates the innate immune response by inducing haematopoietic stem cells to develop into granulocytes and monocytes (Hamilton, 2002). GM-CSF has been implicated in several immune diseases including atherosclerosis (Fleetwood et al., 2005).

GRO-alpha, encoded by the CXCL1 gene in humans, is structurally related to IL-8, both of which are highly secreted in the SASP (Coppe et al., 2008). Both are important inducers of neutrophil chemotaxis, intracellular calcium peaking, and respiratory burst, though IL-8 is more potent (Geiser et al., 1993). Equally both play key roles in angiogenesis (Lane et al., 2002). As a result both proteins are tumorigenic and contribute to cancer development and metastasis (Owen et al., 1997).

ICAM-1 is an intercellular adhesion molecule which binds integrins, specifically the LFA-1 receptor found on leukocytes (Rothlein et al., 1986), which allows the immune cells to transmigrate into the tissues (Yang et al., 2005).

TGF-β has also been identified as an important SASP protein (Coppe et al., 2008), and can diverse and contrary roles depending on the cellular context (Ashcroft et al., 1999), including arrest and proliferation, in accordance with the level RAS signalling (Oft et al., 1996). When TGF-β binds its receptor it triggers phosphorylation of type I receptors by type II receptors which activates the kinase domain of type I receptors (Huse et al., 1999). This causes phosphorylation and activation of the R-Smads 2 and 3, which bind Co-Smad 4 and translocate to the nucleus. Importantly, TGF-β has key tumour suppressor functions, both in suppressing the activity of c-Myc and the CDKs as well as

activating tumour suppressor genes such as p15 and p27 (Datto et al., 1995, Hannon and Beach, 1994, Polyak et al., 1994). However, in the absence of functional tumour suppressors TGF-β can also promote metastasis (Cui et al., 1996), and even under some circumstances tumour growth (Levy and Hill, 2006). Like many of the SASP factors it also plays a key role in the immune response, controlling the activation and survival of lymphocytes, and inhibiting the development of reactivity to self-antigens without compromising immunity to non-self (Li et al., 2006).

1.4.4.3 TNF-α

TNF-α is a potent inflammatory cytokine regulating the immune response, apoptosis, proliferation and differentiation. Interestingly, its importance in senescence is equivocal. An initial study suggested that TNF-α, was not a large factor in the SASP of most cell types (Coppe et al., 2008), whereas a later study suggested that it was upregulated at least at the transcriptional level (Acosta et al., 2013). It can bind two receptors: TNF-R1 which is ubiquitously expressed across all tissues and is responsible for the bulk of TNF-α induced signalling; and TNF-R2 which is only expressed in immune cells and is tightly regulated (Wajant et al., 2003). When TNF-α binds, it causes conformational change allowing receptor trimerisation (Chan et al., 2000) and release of the inhibitory Silence of Death Domain (SODD) protein. TNF Receptor-Associated Death Domain (TRADD) then binds to the membrane associated complex and binds Receptor interacting Protein (RIP) (Ting et al., 1996), localising it to the lipid rafts (Legler et al., 2003). RIP recruits NEMO (Zhang et al., 2000), and the MAP3Ks TGF-β activated kinase (TAK1) and mitogen activated protein kinase kinase kinase 3 (MEKK3) (Blonska et al., 2004), thus allowing them to phosphorylate IKKβ (Shinohara et al., 2005), and activating canoninical NF-κB signalling. TNF-R associated factor 2 (TRAF2), which is necessary for NF-κB activation by TNF-α (Tada et al., 2001), also plays a role in activation of p38 and JNK, which may help activate AP-1 (Karin et al., 1997), an important co-factor in the transcription of many NF-κB responsive genes.

The additional binding of FADD and caspase 8 causes the cytoplasmic localisation of the TRADD-RIP complex, which activates the caspase cascade

to initiate apoptosis (Micheau and Tschopp, 2003). TNF-α is only weakly apoptotic because NF-κB causes the transcription of many anti-apoptotic genes. TNF-α signalling as whole is mainly inhibited by two deubiquitinating enzymes A20/TNFAIP3 (Lee et al., 2000) and the tumour suppressor CYLD (Reiley et al., 2007), but protein phosphatase 2A (PP2A) enzymes may also play a role.

It has already been discussed in the previous section how the cells induce a SASP and the mechanisms by which this process helps to reinforce senescence. In the next two sections, it will be discussed firstly how these phenotypes help senescent cells to carry out their functions, and secondly how these effects can also have detrimental consequences.

1.5 The Functions of Cell Senescence

1.5.1 Senescent Cells Function in Wound Healing and Immune Clearance

Senescence is not an unavoidable by-product of cellular stress. It is a program orchestrated by the cell, and as such is likely to have some function. One possibility is that senescent cells are important in wound healing. Senescent fibroblasts adopt an active secretory phenotype similar to activated fibroblasts (Bernard et al., 2004), and one study showed that mice with defective p53 and/or p16 had elevated levels of liver fibrosis (Krizhanovsky et al., 2008b). At sites of fibrosis some of the cells became senescent and produced the SASP (Krizhanovsky et al., 2008a), where they aided in the resolution of fibrosis by both inhibiting synthesis of matrix proteins and inducing their degradation, as well as reducing fibroblast numbers by ceasing replication and promoting clearance by natural killer cells (Krizhanovsky et al., 2008a, Xue et al., 2007). In apparent contradiction, fibrosis is increased in older individuals, as are the number of senescent cells. However, this is just a correlation, and despite the many potentially negative phenotypes of senescent cells, their increase in wounds could hypothetically be a compensatory mechanism to limit fibrosis in older people. Additionally the SASP may also contribute to immune surveillance

at the site of the wound (Krizhanovsky et al., 2008a, Krizhanovsky et al., 2008b). In cutaneous wounds, cells are also induced to senesce at the later stages when they cease proliferation and upregulate matrix remodelling factors to induce an anti-fibrotic effect (Jun and Lau, 2010). Thus, whilst senescent cells are not required for wound healing (Jun and Lau, 2010, Krizhanovsky et al., 2008b), they play a role in reducing the level of fibrosis that was stimulated by their activated non-senescent predecessors.

Contrarily, senescent cells have been implicated in chronic wounds (Telgenhoff and Shroot, 2005). Although senescent fibroblasts are present in high numbers at chronic wounds (Vande Berg and Robson, 2003), there is little evidence of a causal relationship as chronic wound fluid induces senescence at these sites (Vande Berg and Robson, 2003, Mendez et al., 1999). However, MMPs which are secreted by senescent cells, are increased in chronic wounds compared to healthy wounds (Lobmann et al., 2002), have been implicated in growth factor degradation (Yager and Nwomeh, 1999), and have been shown to be detrimental to the healing of chronic wounds (Ladwig et al., 2002). Therefore, it is likely that the same anti-fibrotic properties that make senescent useful in the healing of healthy wounds are detrimental in the healing of chronic wounds, possibly due to increased numbers of them induced by the increased inflammatory nature of the wound (Telgenhoff and Shroot, 2005), or through their interactions with other factors at these sites.

1.5.2 Cellular Senescence in Development

Importantly, there is growing evidence for senescent cells in embryonic development. Senescent cells have been identified at the apical ectodermal ridge and the closing neural tube, and it has been observed that p21 and p53 mutants can have developmental abnormalities (Keyes, Serrano, unpublished). Therefore, at least in these circumstances, cell senescence is unlikely to be an unwanted byproduct of stress, but is instead a programme that the body institutes for specific functional goals.

1.5.3 Blocking Senescence Causes Crisis

Currently the main function of senescence is thought to be as a tumour suppressor mechanism. In the case of aberrant oncogene expression the role for senescence is clear. As oncogenes generally have pro-cell cycle and pro-survival functions, the result of letting the signal continue unrestrained would be the formation of a continually growing mass of cells, which would effectively be a benign tumour. Thus, the cell must inhibit the growth signal with negative feedback. In the case of RAS overexpression, cells have suppressed the signal within 30 minutes of induction which is maintained at 24 hours, indicating both short term and long term suppression (Courtois-Cox et al., 2006). Downstream PI3K signalling was inhibited; p53 and pRb were activated, and cells were induced to become senescent (Courtois-Cox et al., 2006). However, the negative feedback pathway is not quite that simple, and generally requires the induction of DNA damage (Bartkova et al., 2006). Thus, an interesting speculation would be that cells with mutant oncogene expression might be more susceptible to transformation if they have higher DNA repair capacity.

In the case of replicative and irradiation induced senescence the reason for arrest is also mainly as a barrier to tumourigenesis. When cells become damaged either by telomere loss through replication or through damaging agents such as ROS or radiation, copying the DNA becomes more difficult due to the breaks and other lesions preventing the replication enzymes from carrying out their function. As a result, some parts may not be copied causing deletions, and others may be copied incorrectly causing mutations. Additionally, homologous recombination may cause duplications or transversions, and together these effects result in genomic instability. Therefore, when cells fail to enter senescence they enter a state of crisis called mitotic catastrophe, where the rate of cell death is approximately equal to the rate of proliferation (Shay et al., 1991), maintaining a relatively constant number of cells.

When cells bypass replicative senescence they induce a specific type of genomic instability called breakage-fusion-bridge cycles. Once the chromosome replicates during S-phase the uncapped telomeres of the two sister chromatids are recognised as double strand breaks and joined together. Then when they

are pulled apart during metaphase they will break, but not necessarily at the site of fusion. Thus, the two daughter cells will inherit unequal chromosomes, which may be lethal to one or both of the cells (McClintock, 1941).

As the cell population remains relatively constant the surviving cells are becoming increasingly damaged. Eventually some of the cells may acquire the correct mutations to transform into neoplastic cells and form tumours, which is highly detrimental to the organism. The reason that somatic cells have inactivated telomerase is likely to be in order to induce senescence in cells which are undergoing abnormal proliferation. Thus, cells which have acquired cancerous mutations must acquire further mutations to reactivate telomerase before they can immortalise (Kim et al., 1994, Hiyama et al., 1995a, Hiyama et al., 1995c). Consistently, telomerase expression enhances transformation *in vitro* (Hahn et al., 1999).

These data, combined with the much faster formation of tumour formation in mice, which have fewer independent tumour suppressor mechanisms to prevent senescence bypass, is good evidence for the importance of senescence as a tumour suppressor mechanism. However, as was seen with the wound healing, senescence can have antagonistic effects. There are several lines of evidence to suggest that senescence might be detrimental to the ageing process.

1.6 Senescence in the Promotion of Ageing, Cancer and Disease

1.6.1 Cell Senescence in Ageing

Simply because a phenomenon has function, does not mean that it will not also have unwanted or detrimental effects as well. The two main theories of ageing currently suggest that firstly if something is beneficial to fitness it does not necessarily mean that it is beneficial to organismal lifespan, and secondly that if something has benefit earlier in life, then such a trait would still undergo positive selection even if it was detrimental later in life when the selective forces are weaker. Thus, cell senescence could have both positive and negative

consequences, and the latter could be more severe as long as they happen later in life.

Initially it was doubted whether the finite replicative lifespan of cells in culture would have any relevance to cells and organisms *in vivo*. However, several studies have since shown that not only are senescent cells present *in vivo*, but also that they accumulate with age (Dimri et al., 1995, Pendergrass et al., 1999, Mishima et al., 1999), and at sites of age-related pathology (Collado et al., 2005, Dimri et al., 1995, Erusalimsky and Kurz, 2005, Jeyapalan et al., 2007, Price et al., 2002). There has been correlative evidence for a role of senescence in ageing as far back as the 1970s, when they showed that cells of older individuals senesced after fewer replications than cells of young individuals (Martin et al., 1970, Le Guilly et al., 1973), and cells from progeroid patients senesced after fewer divisions than cells from healthy individuals (Goldstein, 1978). However, recent studies using more stringent controls have contested some of these results (Cristofalo et al., 1998, Serra and von Zglinicki, 2002). The first causal evidence that senescent cells contributed to the ageing process was demonstrated in 2011 by a group that used a construct combining a p16 promoter with an inducer of apoptosis, thus allowing the selective killing of p16 expressing cells in progeroid mice (Baker et al., 2011). The result was a significant delay in the onset of age related pathologies in the eye, muscle and adipose tissue, and, in the latter two cases, late-life clearance could attenuate these phenotypes in mice that had already started to develop them.

1.6.2 The Cell Autonomous Hypothesis of Senescence in Ageing

It is possible that senescent cells could contribute to ageing autonomously through the loss of normal tissue function. The main way that this has been suggested to occur is through the depletion of stem cell reserves. As differentiated cells have little or no replicative potential anyway, their senescence is unlikely to have significant autonomous effect on organ regeneration, whereas stem cells are responsible for producing large numbers of cells of many types to replace those that are damaged or lost. Theoretically,

therefore stem cell senescence could autonomously reduce organ function and induce ageing.

One initial problem with this theory was the finding that some adult stem cells expressed telomerase, which was thought to immortalise them and prevent senescence. However, many adult stem cells either express telomerase at very low levels, or not at all, and in both cases have a finite replicative capacity in culture (Vaziri et al., 1994, Wagner et al., 2008). Additionally, haematopoietic stem cells from older individuals also had shorter telomeres (Vaziri et al., 1994), indicating that they also have a finite replicative lifespan *in vivo*. Consistently, there are signs of stem cell ageing; the stem cells of older mice were shown to be significantly less functional than those in younger individuals (Chen et al., 1999, Morrison et al., 1996), and differentiation was skewed towards the myeloid lineage away from T and B cell production (Spangrude et al., 1995, Sudo et al., 2000).

However, more importantly, the majority of data suggest that stem cell numbers increase with age rather than decrease (Morrison et al., 1996, Harrison et al., 1989, de Haan et al., 1997), which is difficult to reconcile with the idea of autonomous stem cell loss due to senescence contributing to ageing. Indeed the progeny of a single transplanted haematopoietic stem cell can maintain haematopoiesis over the lifespan of a recipient mouse (Jordan and Lemischka, 1990), and they can be serially passaged to recipients for up to five generations (Siminovitch et al., 1964). In 2006 it was claimed that “no one common disease of ageing is directly caused by exhaustion of cell divisions” (Blagosklonny, 2006a). Whilst this has by no means been proved, the increase in stem cells with ageing is entirely inconsistent with a depletion of stem cells through cell senescence contributing to ageing. There is good evidence that stem cells age, and this may contribute to ageing, but this is unlikely to reflect a decline in their replicative capacity due to stress induced senescence of the stem cell pool.

Another possible mechanism by which senescent cells could autonomously induce ageing is based on the observation that some organs can accrue large numbers of senescent cells (Wang et al., 2009a, Wang et al., 2010, Jurk et al., 2012). Aside from ROS and the SASP, senescent cells have >1000 genes differentially expressed compared to healthy replicating cells, and could

theoretically autonomously contribute to organ decline, if enough cells were behaving aberrantly.

Alternatively senescent cells could contribute to ageing through inducing detrimental effects on the surrounding non-senescent cells, and even systemically. Although there has been suggestions that this might be the case (Rodier and Campisi, 2011, Coppe et al., 2008), the evidence to date is currently lacking. However, there is substantial evidence for paracrine effects of senescent cells in tumorigenesis.

1.6.3 Cellular Senescence in Cancer and the Non-Autonomous Hypothesis

The observation in 2001 that senescent cells could stimulate preneoplastic and neoplastic cells to form tumours (Krtolica et al., 2001), initially seemed to contradict the role of senescence as a tumour suppressor. However, the study clearly demonstrated that senescent cells stimulated the growth of four strains of preneoplastic immortalised cells harbouring p53 mutations more than pre-senescent cells. Importantly, the same study found that normal keratinocytes grew similarly on senescent and pre-senescent cells, suggesting that the effect was unique to damaged cells. Both matrix deposition and secretion of soluble factors from senescent cells triggered preneoplastic cell growth, although it was not nearly as effective as direct cell contact. These cells would not ordinarily form tumours in immunocompromised mice, but the addition of pre-senescent fibroblasts to the xenograft induced some tumour formation, which was increased by the presence of senescent cells, as was the size of the tumours. In one cell line the tumours remained benign even when injected with senescent cells, whilst in another cell line senescent cells triggered progression to malignancy (Krtolica et al., 2001).

A later study showed that at early stages of tumour xenograft the presence of senescent fibroblasts stimulated the induction of large fluid filled edema, indicative of tissue damage, which then regressed but resulted in much larger tumours (Liu and Hornsby, 2007). The use of a matrix metalloproteinase inhibitor reduced this fluid accumulation and slowed the growth rate of the

tumour, without directly affecting proliferation in culture. It is therefore possible that the proliferation of preneoplastic cells, which lack functional tumour suppressor mechanisms, might be induced to grow by some genotoxic stimulus, which is reinforced by the observation that healthy keratinocytes did not respond with increased growth. However, contradictory to this idea hepatocyte growth factor (HGF), which is upregulated in senescent cells was shown to have directly mitogenic effects (Liu and Hornsby, 2007). Secondly, another study examining prostate epithelial cells demonstrated that senescent fibroblasts could stimulate the proliferation of healthy cells, in a mechanism partially dependent on amphiregulin (AREG), and possibly FGF-7 and HGF (Bavik et al., 2006). Thus, it is likely that the pro-tumorigenic effect comes at least partially from the stimulation of proliferation. The induction of DNA damage by RAS stimulated hyperproliferation (Bartkova et al., 2006) is consistent with this idea.

Despite the apparently paradoxical pro and anti-tumour effects of senescent cells, it should be noted that current cancer therapies such as radiotherapy are also thought to induce transformation or tumorigenicity of preneoplastic and neoplastic cells (Lagadec et al., 2012, Zhao et al., 2001, Toda et al., 2009). Similarly, it appears that the mechanisms the body uses to inhibit cancer initially can likewise promote it later on.

Importantly, a later study identified that the epithelial-mesenchymal transition in cancer cells induced by senescent cells was partially dependent on pro-inflammatory IL-6 and IL-8 (Coppe et al., 2008). Interestingly, they showed that the SASPs of both RAS induced senescent cells and from cells lacking p53 function prior to senescence were more potently transforming than the SASPs of irradiated or replicatively senescent cells with functional p53 (Coppe et al., 2008). Although this contradicted the previous observation that RAS senescent cells were less potent transformers than both other types of senescent cell (Krtolica et al., 2001), the authors had noted problems with the RAS expressing cells which could explain this difference (Krtolica et al., 2001).

Another study found that in three sets of mice given a carcinogen known to cause RAS mutation, both genetically obese mice and ones fed a high fat diet were more likely to develop liver cancer than lean controls. More interestingly,

they found firstly that treatment with siRNA against HSP27 (shown to reduce numbers of senescent/senescing liver cells (Sato et al., 2008)), significantly reduced liver cancer, and secondly that when IL-1β was knocked out, which is a prominent feature of the SASP, the number and size of the tumours was also significantly reduced (Yoshimoto et al., 2013). Mechanistically they suggested that toxins produced by the gut microbiota of mice on high fat diet including deoxycholic acid (DCA) induced both cell senescence and liver cancer (Yoshimoto et al., 2013).

1.6.4 Summary

Cellular senescence has important functions in tumour suppression and immune clearance. Despite these, senescent cells have detrimental effects on pre-neoplastic and tumour cells, stimulating transformation, malignancy and tumour growth. Senescent cells have also been implicated in the ageing process, and it is unlikely that they do so autonomously by the depletion of functional cells. However, despite the evidence that senescent cells have potential to induce damage associated with ageing to the surrounding tissue, there is currently little evidence on whether this occurs or the mechanisms involved.

1.7 Previous Evidence of Bystander Effects

Broadly the term bystander effect applies to any phenotypic change in cells, that occurs indirectly as a result of treatments applied elsewhere. In gene therapy and toxicology the term is used to describe the passage of gene products from transfected cells to their neighbours. More relevant to this thesis is the study of the effects of radiation which have mostly examined the bystander effect in the form of multiple species of DNA damage and cell death. For 50 years it has been known that medium from irradiated cells can induce DNA damage in unirradiated cells, but it was not until 1992 that it was demonstrated that cells hit by high-LET radiation (α-particles) could induce sister chromatid exchanges

(SCEs) in bystander cells (Nagasawa and Little, 1992). In this study using Chinese hamster ovary (CHO) cells, the same level of SCEs was produced in bystander cells as by 1-2Gy direct X-irradiation. At very low doses of α-particles the bystander effect is dose dependent; however, it quickly plateaus (Nagasawa and Little, 1992), which has been replicated in HFL1 human diploid fibroblasts (Deshpande et al., 1996). Since this time, the study of the irradiation induced bystander effect has been done either with the use of targeted α-particles or via the use of irradiated cell conditioned medium (ICM) transferred from γ or X-irradiated cells.

1.7.1 The Radiation Induced Bystander Effect (RIBE)

Unlike the effect of α-particles, the initial study using ICM from irradiated fibroblasts failed to show any change in cell survival in fibroblasts at the dose of 5Gy, whereas medium from keratinocytes significantly reduced survival of both keratinocytes and fibroblasts, indicating that it was the absence of signal rather than response that prevented the bystander effect in fibroblasts (Mothersill and Seymour, 1997). Another study analysing 53BP1 foci demonstrated a 2.5 fold increase in foci in bystander WI-38 fibroblasts receiving ICM (Sokolov et al., 2005), and a later study showed the same was true of IMR-90 fibroblasts, showing a 4.5 fold increase in 53BP1 foci (Sokolov and Neumann, 2010), suggesting firstly that fibroblasts may still have received damage in the initial experiment (which did not significantly increase cell death), and secondly that even within fibroblast the magnitude of the effect was cell type specific.

The types of foci in Hela cells appeared to differ between directly irradiated and bystander cells. A single α-particle to the nucleus induces a single large focus at the point where the particle hit and other foci localised around this area, whilst in bystander cells the foci were spread across the whole nucleus. When the α-particle was targeted to the cytoplasm the hit cells showed the same focus pattern as the bystanders with similar numbers of cells having ≥4 foci (Tartier et al., 2007). Interestingly, the effects of cytoplasmic radiation are delayed for both directly irradiated and bystander cells compared to nuclear irradiation, and the percentage of directly hit cells showing ≥4 foci is halved. However, once the

effect appears, there is no difference in foci numbers of bystanders regardless of where the α-particles hit the inducer cells (Tartier et al., 2007).

Human fibroblasts, carcinoma cells, and CHO cells all showed greater losses in clonogenic survival in response to ICM if they had some defect in DNA repair, and likewise induced increased damage in other bystanders compared to their repair proficient controls (Mothersill et al., 2004), suggesting that cells with less active repair induce a stronger bystander signal. Additionally, there is some evidence that these cells are more susceptible to the bystander signal than repair proficient cells. One repair deficient strain receiving ICM had increased cell death compared to the proficient control receiving the same medium, whilst the other strain showed no difference.

In keratinocytes, the bystander effect of irradiated cell conditioned medium (ICM) depended on the number of cells irradiated, with the highest cell numbers producing a bystander effect similar to direct irradiation with 5Gy (Mothersill and Seymour, 1997). In another study by the same group using a different strain of human immortalised keratinocyte, the lowest dose used of 0.01Gy induced a similar reduction in survival compared to 5Gy, and direct irradiation of cells only started to reduce clonogenic survival above that of bystanders at 2.5Gy (Seymour and Mothersill, 2000). Additionally, in the progeny of these cells the clonogenic survival of the bystanders was consistently reduced from ICM of 0.03Gy γ-irradiation, whereas the progeny of directly irradiated cells had no significant delayed effects (Seymour and Mothersill, 2000).

The bystander dynamics using ICM suggest that there is little increased effect on leaving the medium on the irradiated cells for longer than 1 hour, and the effect is already apparent if the medium is removed 30 minutes after irradiation (Mothersill and Seymour, 1997). Importantly, if the medium was replaced immediately (30 seconds) after irradiation, and the new medium left for 1 hour as before, then the bystander effect was completely abrogated (Mothersill and Seymour, 1998). Additionally, removal of ICM from the bystander cells after 30 minutes still induced full bystander effect in the recipient cells (Mothersill and Seymour, 1998).

In a series of complicated experiments using combinations of direct dose and ICM, it was demonstrated that if directly irradiated cells were then exposed to

ICM from another culture of irradiated cells it had no additional bystander effect, suggesting that direct irradiation offers some protection from the bystander effect (Mothersill and Seymour, 2002). Perhaps more interestingly, if ICM which has been transferred onto irradiated cells is then transferred onto unirradiated cells, the bystander effect is also abrogated. Contrarily, if addition of ICM precedes irradiation it offers no benefit, and may be slightly worse than either treatment alone (Mothersill and Seymour, 2002), which suggests that the RIBE is not an adaptive response to protect cells from a future direct hit of radiation. Lastly, although fractionation of the dose to the directly irradiated cells was shown to have no effect on bystander cells, if instead the cells are irradiated twice with a change of medium in between, then the second ICM significantly increases the clonogenic survival of bystander cells over controls, suggesting a completely different cellular secretion from the second dose of irradiation (Mothersill and Seymour, 1998).

1.7.2 Dynamics and Mechanisms of the RIBE

In one study, the lowest dose of γ-irradiation from which ICM would significantly reduce clonogenic survival in keratinocytes was 3mGy, which was also the first dose to induce a calcium peak, suggesting that the radiation induced bystander effect (RIBE) is an all or nothing response (Liu et al., 2006b). Bystander cells induce a spike in intracellular calcium within 30 seconds of addition of ICM, which has dissipated again by 100 seconds. Chelation of extracellular calcium, or blockage of voltage-dependent calcium channels, prevents this spike, and reduces the levels of apoptosis to control levels (Lyng et al., 2006), demonstrating the importance of calcium signalling in the bystander effect.

Within 1 hour of exposure to ICM there is a large increase in ROS, which has increased further by six hours and persists for at least 24 hours, and concomitantly mitochondrial membrane potential has decreased by six hours and remains low for at least 24 hours. Interestingly, the increase in ROS appears to precede the fall in mitochondrial membrane potential (Lyng et al., 2000). By 48 hours some of the cells have apoptotic morphology; however, this

fraction does not fully account for all of the clonogenic cell death (Lyng et al., 2000).

ICM induces significant activation of both ERK and JNK at 30mins and 24hours of exposure, whereas p38 was not significantly activated at either timepoint. The use of specific inhibitors against the three MAP kinases showed that ERK inhibition increased apoptosis, JNK inhibition decreased levels of apoptosis and p38 had no effect in cells exposed to ICM (Lyng et al., 2006).

The use of Bay 11-7082 to inhibit NF-κB activation significantly reduced the bystander effect, whilst having no effect on directly irradiated cells (Zhou et al., 2008). The effect was greater in cells with functioning mitochondria, as p^o cells, which have no mtDNA, also have reduced basal and stimulated levels of p65-p50 and p50-p50 dimers, suggesting both that mitochondria induce NF-kB activation in bystander cells, and NF-κB may induce mitochondrial ROS production, consistent with the idea of positive feedback being involved in the bystander effect. Additionally, the targets of NF-κB such as iNOS and COX2 are also increased in bystander cells, and the addition of anti-TNF-α into the medium reduced NF-κB and COX-2 levels, and increased survival of bystander cells (Zhou et al., 2008).

Exactly how the damaged cells transmit the bystander signal is not completely clear. However, a role for gap junctions has accumulating evidence. These small channels allow the passage of molecules and ions smaller than 485 daltons between cells. They play key roles in inter-cellular homeostasis and electrical signalling (Robertson, 1981a, Robertson, 1981b). Consistent with bystander transmission, they are thought to play a key role in calcium homeostasis (Charles et al., 1992). Each gap junction is made up of two molecules of connexin43 which bind to the same molecules in the neighbouring cell, thus forming a continuous channel connecting their cytoplasm. Addition of the gap junction inhibitors lindane and octanol, as well as the use of dominant negative connexin43 can completely abrogate the bystander effect (Azzam et al., 1998, Azzam et al., 2001, Zhou et al., 2000), whilst the overexpression of connexin43 enhances it (Zhou et al., 2001). Equally, the exposure of various cell types to radiation or hydrogen peroxide was found to increase the expression of connexin43 (Azzam et al., 2003), suggesting that the bystander

effect was an adaptive response, although this did contradict previous data suggesting that gap junctions were downregulated in response to stress (Chipman et al., 2003).

1.7.3 Biological Significance of the RIBE

When human urothelium from different subjects is exposed to γ-irradiation, there is significant inter-individual difference in the resultant phenotype, which can be separated into two main groups; group1 describes cells which undergo little cell death in response to 0.5Gy or 5Gy irradiation, whilst group 2 describes cells which undergo higher levels of apoptosis and necrosis to the same doses (Mothersill et al., 1999). Smokers and males were much more likely to be in group 1, and non-smokers and females, especially non-smoking females were more likely to be in group 2 (Mothersill et al., 1999). Using the ICM from the group 1 irradiated urothelium had a much smaller bystander effect on keratinocyte survival than group 2 urothelium, and this difference was also apparent after several cell generations (Mothersill et al., 2001). Notably, the bystander effect of ICM from irradiated tumour cells, or the normal tissue around the tumour cells has a tendency to increase cell survival (Mothersill et al., 2001).

The same differences were observed between the more radioresistant CBA/H and the radiosensitive C57/BL6 mice. Following bone marrow irradiation the CBA/H mice showed delayed chromosomal instability in their cells which was not present in the C57/BL6 mice (Mothersill et al., 1999), which are less susceptible to epithelial cancers and radiogenic malignancies. Importantly, irradiated mouse urothelium from C57/BL6 mice induced a bystander effect, whilst medium from CBA/H urothelium did not, thus demonstrating that the bystander effect predisposes mice to better long term prospects with lower cellular transformation and thus higher organismal survival in response to stress (Mothersill et al., 2001). Another study showed that the radioresistant effects might result from increased expression of Bcl-2 and decreased expression of p53 and Bax (Mothersill et al., 2005).

Interestingly, a different study showed that anti-apoptotic Bcl-2 is only significantly increased in the bystanders exposed to the high dose ICM. Additionally, caspase-9 inhibition only increased survival from low dose and not high dose ICM (Maguire et al., 2005). This may be an indication that, whilst low and high doses have similar effects on clonogenic survival, they are inducing different mechanisms of clonogenic death. Speculatively, one possibility is that the low dose bystander effect induces apoptotic cell death, dependent on mitochondria and membrane permeabilisation, whilst high doses prevent apoptosis via inducing Bcl-2, and as a result enter mitochondria-independent cell death, such as necroptosis.

It is clear that the radiation induced bystander effect is a complex phenomenon, reliant on several signalling pathways, ROS, gap junctions and intracellular calcium, and importantly it has tumour preventative properties. Importantly, it is likely that bystander phenomena where the outcome is molecular damage will share similar characteristics, making the research into the RIBE useful for the study of the senescent cell induced bystander effect, which is the work of this thesis.

2 Aims

1. The primary aim of this study was to elucidate whether senescent cells could induce a DNA damage response and spread cellular senescence to healthy replicative cells, ie. to look for a bystander effect of senescent cells.
2. After the primary aim was completed, the secondary aim was to understand the mechanisms of this “bystander effect”, and investigate potential routes to inhibiting it.

3 Materials and Methods

3.1 Chemicals

All chemicals were purchased from Sigma unless stated otherwise.

3.2 Cell Lines and Culture

3.2.1 Mammalian Cell Lines

MRC5 human embryonic lung fibroblasts (ECACC, Salisbury, UK), and HEK 293FT lentiviral packaging cells (Invitrogen, Paisley, UK) were cultured *ex vivo* at 37^{o}C in a humidified atmosphere of 95% air with 5% CO_2 in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% foetal bovine serum (BioSera, Ringmer, UK), 100 units/ml penicillin, 100 $\mu g ml^{-1}$ streptomycin and 2 mM glutamine (complete medium).

Medium was replaced on cells three times a week, and were split at roughly 90% confluence using trypsin EDTA. Control inducer and young reporter cells ranged from PD 19-30. Cells were counted using a Fuchs-Rosenthal haemocytometer, and population doublings were calculated using the formula: ln(X/(X-1)/ln2, where X and X-1 are the current and previous counts respectively. Cells were defined as senescent after they had passed at least 38 PD, had two consecutive weeks of negative growth, and had been left for a further two weeks.

Cells were plated at a subconfluent density of 150,000 cells/35mm dish (IWAKI, NELS, Newton Aycliffe, UK). In co-culture 75,000 senescent cells were plated with 75,000 reporter cells (ratio of 1:1). Co-cultures were continued up to 21 days, with measurements at 1, 2-4, 7, and 14 days depending on the experiment. Medium was changed on the cells three times per week, providing fresh inhibitors if any were present at the concentrations shown in table 3.1. All inhibitors were dissolved in DMSO.

Inhibitor	Target	Concentration	Manufacturer
Bay 11-7082	IKK complex	1μM	Calbiochem
Octanol	Gap Junctions	1μM	Sigma
Torin1	mTOR	10nM	Tocris Biosciences
UR-13756	P38	1μM	Gift from Terrance Davis lab (Bagley et al., 2010)

Table 3.1| Inhibitors added to co-culture, dissolved in DMSO.

3.2.2 Prokaryotic Cell Lines

Cell Line	Manufacturer	Plasmids used to transform cell line
NEB10β	NEB	pENTR2B-53BP1, pENTR2B-mCherry-53BP1, pENTR2B-ΔIκBα-IRES-EGFP
NEB5α	NEB	pLP1, pLP2, pLP/VSVG
One Shot OmniMAX™ 2T1 Phage-Resistant Cells	Invitrogen	pLenti6-mCherry-53BP1, pLenti6-ΔIκBα-IRES-EGFP

Table 3.2| Prokaryotic Cell Lines.

3.3 Solutions

Solution	Ingredients
PBS	Dulbecco's phosphate buffered saline 10× (Sigma) 1 in 10 in RO H_2O

PBS-Mg	5mM $MgCl_2$ in PBS
Iron Bru	100ml PBS-Mg, 2.11g (50mM) Potassium ferrocyanide, 1.65g (50mM) Potassium ferricyanide
Staining Solution	8.5ml PBS-Mg, 0.5ml pre-warmed X-gal, 1ml Iron Bru. pH 5.5-6
TBS	24.2g Tris Base, 80g NaCl. Make up to 1litre and adjust to pH 7.6 with HCl
TBST	TBS with 0.3% Triton (v/v)
PFA	Heat 400ml RO H_2O to 62-64^oC. Add 100µl 10N NaOH and 40g of paraformaldehyde to 980ml of PBS, then add to heated 400ml RO H_2O. As temperature returns to 62-64^oC the fixative will clear. Cool to room temperature and filter. Check pH is around 7.3
TAE	400ml RO H_2O, 121g Tris Base, 28.55ml glacial acetic acid, 9.3g EDTA. Adjust to 500ml with RO H_2O (50×). Then 1 in 50 in RO H_2O for working solution
LB	10g bacto-tryptone, 5g bacto-yeast extract, 10g NaCl made up to 1L RO H_2O
LB agar selection plates	Add 15g agarose to 1L LB and autoclave. Once cool enough to hold, add 50µg/ml ampicillin or kanamycin selection agent, mix thoroughly and pour plates

Table 3.3| Solutions used in experimental protocols.

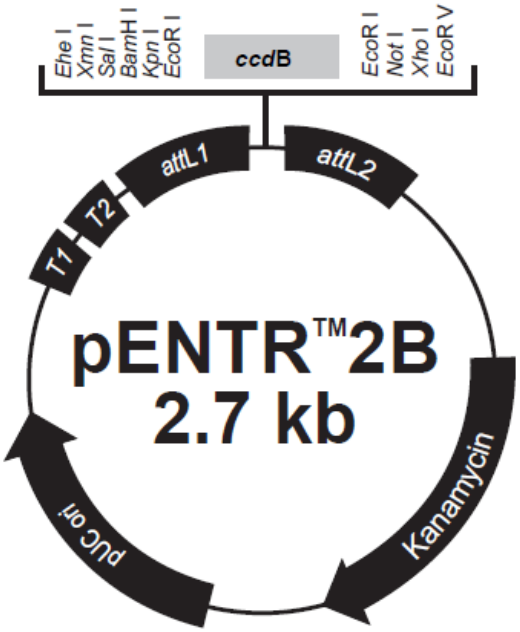
3.4 Creating Reporter Cells

An initial reporter line of MRC5 fibroblasts had been transduced with a fluorescent reporterAcGFP-53BP1c, described previously (Nelson et al., 2009). Although this vector was used very successfully in the initial in depth analysis of small numbers of cells, the fraction of visibly fluorescent cells was too few for

large scale analysis. Due to the time consuming nature and low success rate of tracking cells for long periods of time it was deemed that larger numbers of reporter cells were required. Initially transducing cells with a higher MOI of GFP-53BP1c was attempted; however, this produced equally few fluorescent cells. Analysis of the plasmid using restriction digests revealed several unexpected bands that were believed to be the result of recombination, although interpretation was difficult. It was therefore decided to construct a new plasmid by fusing mCherry to 53BP1c.

3.4.1 53BP1-pENTR2B

The pENTR2A vector containing 53BP1c constructed by Glyn Nelson had been recombined with pG-AcGFP vector to produce GFP-53BP1c fusion protein. This had then been inserted into pENTR2B and recombined into pLenti6 destination vector (Nelson et al., 2009). This produced the additional recombination sites in the pENTR2B and pLenti6 plasmids, which were believed to have caused the mixed population of plasmids described above. Therefore, it was decided to re-insert 53BP1c into pENTR2B (Invitrogen), the latter of which is shown in figure 3.1.



Comments for pENTR™2B
2718 nucleotides

*rrn*B T1 transcription termination sequence: bases 106-149
*rrn*B T2 transcription termination sequence: bases 281-308
*att*L1: bases 358-457 (complementary strand)
*ccd*B gene: bases 613-918
*att*L2: bases 947-1046
Kanamycin resistance gene: bases 1169-1978
pUC origin: bases 2042-2715

Figure 3.1| Plasmid map of pENTR2B with restriction sites in the multiple cloning site (MCS). Map is copied from product description (Invitrogen).

3.4.1.1 Restriction Enzyme Digestion

Both pG-AcGFP-53BP1c and pENTR2B were digested with a *BamHI XhoI* double digest. This had the advantage that none of the recombination sites were within the fragment to be inserted into pENTR2B. 1µl of each enzyme from the NEB stock solutions was added to 0.5µg plasmid DNA in the presence of 1µl *BamHI* buffer and made up to 10µl with RO H_2O. The digests were incubated at 37^{o}C for 2-3 hours, due to the decreased efficiency of *XhoI* in *BamHI* buffer.

The products were then run on a 0.8% agarose gel, next to a 1KB ladder (NEB). To construct the gel, 0.8% research grade agarose (SERVA) was added to TAE

and heated until completely melted with intermittent stirring. The liquid was then cooled and a 1 in 10000 dilution of SYBER SAFE (Invitrogen) was added and mixed thoroughly, before pouring the gel. The gel was run at 90V for 10 minutes and 110V thereafter. Bands were checked and imaged under UV illumination using an AlphaInnotech MultiImage^{TM} Light Cabinet.

BamHI XhoI digestion of pENTR2B produces 0.45Kb and 2.25Kb fragments, and the same digestion of pG-AcGFP-53BP1c produced a 2.77Kb fragment containing 53BP1c, and a 4.73Kb fragment. Therefore, the small fragment of pG-AcGFP-53BP1c, and the large fragment of pENTR2B were excised from the gel using a scalpel and a UV illuminator. They were cleaned using QIAquick gel extraction kit (Qiagen) as per manufacturer's instructions, before eluting into 50µl buffer EB.

3.4.1.2 Ligation

Concentration of DNA was determined with a nanodrop to calculate the quantities required for ligation. A 3:1 insert to vector ratio of molar ends were combined, using 50ng vector, in a PCR tube and incubated overnight at 16^{o}C with 1U T4 DNA ligase and provided buffer. Volume of ligation mix was kept as low as possible, to increase the efficiency of bacterial transformation.

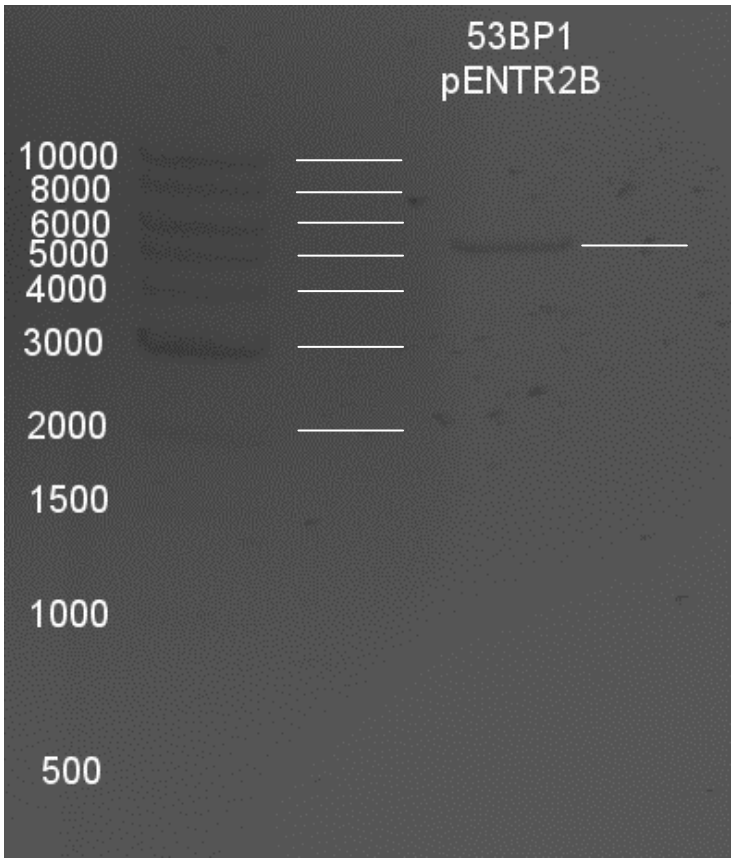
3.4.1.3 Bacterial Transformation

1-5µl of ligation mix was added to a 50µl vial of NEB10β highly competent *E. coli* (NEB) and incubated on ice for 30 minutes, before heat shocking at 42^{o}C for 30 seconds. After another five minute incubation on ice, 450µl SOC medium (NEB) was added and cells were incubated at 37^{o}C for 1 hour with shaking at 250rpm. The cells were then spread on agar selection plates containing kanamycin, and incubated overnight.

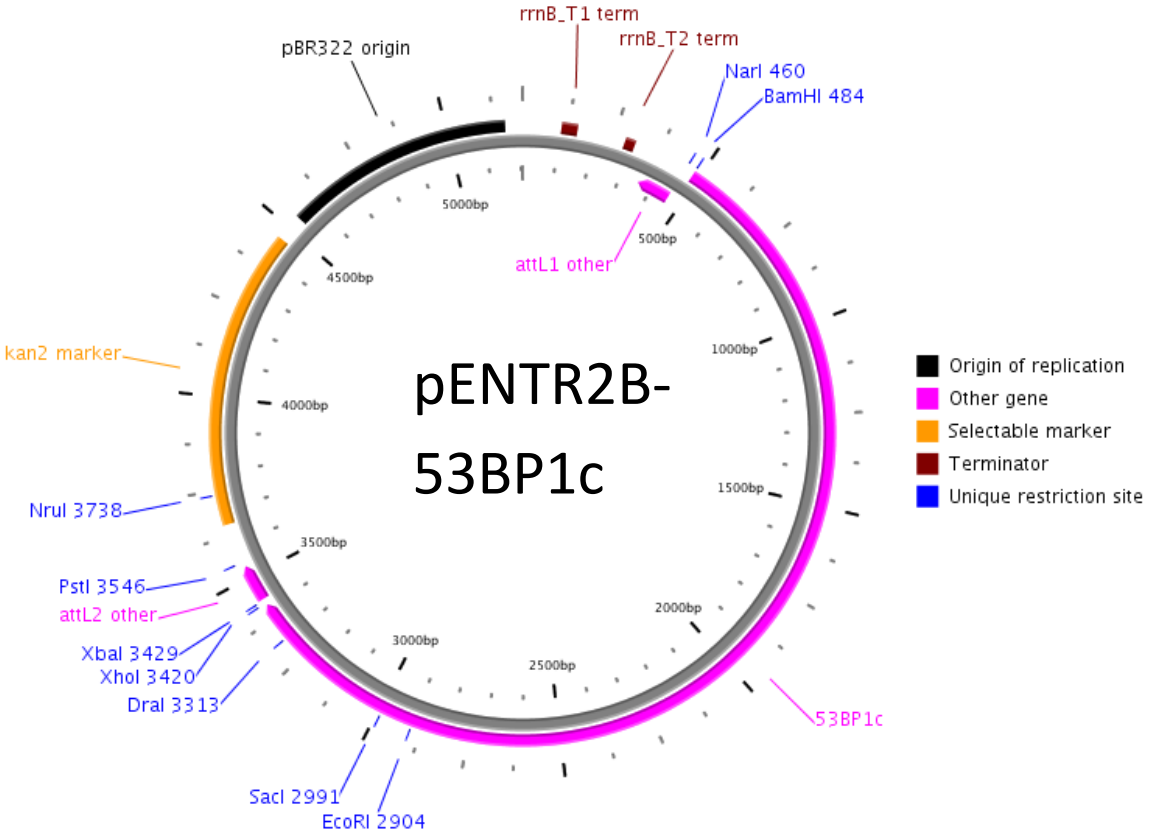
Colony formation was compared to bacteria transformed with vector only (no insert), and was found to be significantly higher indicating both ligation and transformation had been successful. Individual colonies were then grown overnight in 5ml selective LB. DNA was purified using the QIAprep Spin Miniprep kit (Qiagen) as described in the provided protocol.

3.4.1.4 Plasmid Analysis

A *BamHI* digest was used to test potential mCherry-53BP1c bacterial clones. Restriction digests followed the same protocol as above, with 1µl Bam HI and 0.5µg plasmid DNA in the presence of 1µl Bam HI buffer made up to 10µl with RO H_2O. As shown in figure 3.2A, the digest produces a band between 5 and 6Kb, and 53BP1-pENTR2B is 5.32Kb, whilst pENTR2B alone is only 2.7Kb, suggesting that 53BP1c had inserted. The plasmid map of 53BP1-pENTR2B is shown in figure 3.2B.



A|



B|

Figure 3.2| pENTR2B-53BP1c. (A) Plasmid cut with Bam HI restriction enzyme, which cuts once in the MCS prior to the 53BP1c insert yielding 5.32Kbp band. As the bands are not clear on this gel white lines have been drawn next to them to better indicate their position. (B) Plasmid map of correct clones, created using PlasMapper Version 2.0: http://wishart.biology.ualberta.ca/PlasMapper/jsp/librarySeq.jsp?id=Clontech15.

3.4.2 pENTR2B-mCherry-53BP1c

The next stage in the cloning process was to insert mCherry in front of 53BP1c. Because the restriction sites in the MCS of the pRSET B plasmid containing mCherry, donated by Roger Tsien, were largely unknown, it was decided that mCherry would be amplified using PCR.

3.4.2.1 Primer Design and Amplification

Primers were designed to add Sal I restriction sites either side of mCherry during amplification. Although this would allow mCherry to insert the wrong way around in some clones, there were no other restriction sites that were suitably placed without also cutting at unwanted locations. The forward primer started from 20 bases before the Kozak sequence within the pRSET B plasmid, and the reverse primer bound to the end of the mCherry gene minus the stop codon, as shown in figure 3.3.

Forward Primer:

5’ GTC GTCGAC T AGATCT CGCCACCATGGTGAGCA 3’

Reverse Primer:

5’ ATC GTCGAC T CTG GTT TTG CTTGTACAGCTCGTCCATG 3’

Figure 3.3| Primers for amplifying mCherry. Blue highlight identifies bases added to the end to allow more efficient *SalI* enzyme binding. Yellow highlight shows *SalI* restriction sites. Red highlight identifies a base added to increase the melting temperature. Cyan highlight shows a *BglII* restriction site. Pink highlight identifies the linker region to allow correct protein translation and folding. Green highlight is the region complementary to the plasmid.

BglII was added 5’ for analysis purposes described later. Additionally, three bases were added outside of the *SalI* sites to improve *SalI* enzyme binding PCR fragments, and an additional base was added to the front primer to increase the melting temperature. Lastly, a linker region of 10 bases was added to the reverse primer that would function to separate mCherry from 53BP1 by hydrophilic amino acids, thus allowing both proteins to adopt their normal conformations, and also to ensure that 53BP1 was in the correct frame after the addition of mCherry.

Primer designs were checked using http://www.basic.northwestern.edu/biotools/oligocalc.html. They contained no potential hairpins or self-

complementarity, although there were two sites where the primers could bind to each other. Melting temperatures of the initial primer binding sites (the green highlighted region in figure 3.3), as well as the total primer were within 0.5^{o}C difference of each other.

PCR was carried out according to the guidelines of Pfu polymerase (Thermo Scientific): Add 5µl Buffer with $MgSO_4$, 5µl dNTPs (0.2mM each), primers 0.5µM each, 500pg template DNA, and 1.5U Pfu polymerase to a total volume 50µl, made up with nuclease free water in a PCR tube. Initial denaturation was set at 95^{o}C for 3 minutes, then amplification involved 25 cycles of 30s denaturation at 95^{o}C, 30s annealing at 57^{o}C, then a 120s extension at 72^{o}C. The final extension was allowed to continue for seven minutes at 72^{o}C, before the reaction was reduced to 4^{o}C until products were removed.

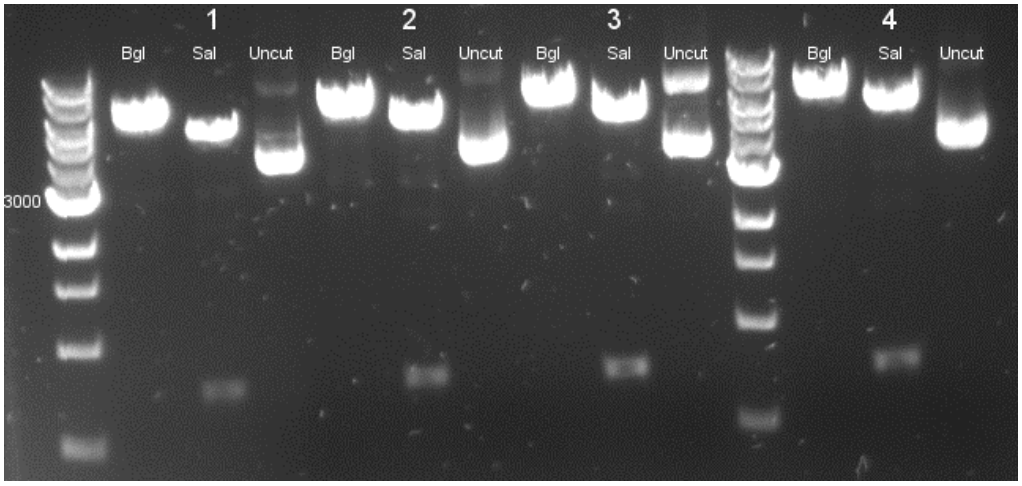
3.4.2.2 DNA Preparation

The PCR product containing mCherry was cleaned using QIAquick gel extraction kit (Qiagen) and eluted into 50µl buffer EB, to which 6µl Sal I buffer, 3µl Sal I enzyme, and 1µl water was added. This was incubated at 37^{o}C for 24 hours due to the markedly decreased efficiency of Sal I enzyme working within 3 base pairs from the end of the fragment. The 53BP1c pENTR2B plasmid was also digested with Sal I for 1 hour with 1µl buffer, 1µl enzyme in a 10µl reaction.

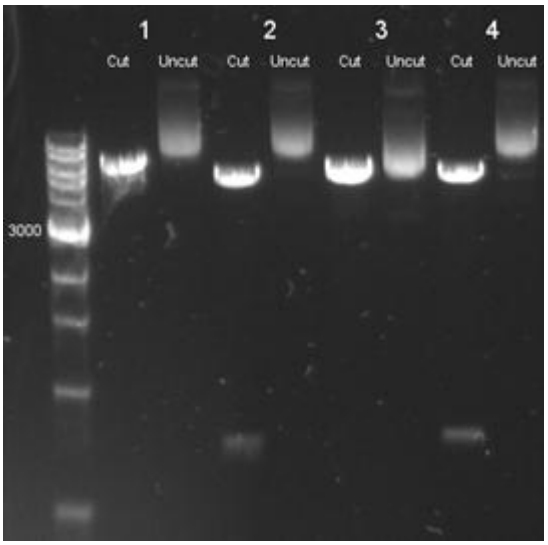
The PCR product and 53BP1c pENTR2B vector were run on 0.8% agarose gel, next to a 1KB ladder (NEB) as described above. The 500bp fragment from the mCherry digest and the 5.5Kbp band from the 53BP1c pENTR2B digest were both excised from the gel and cleaned using the QIAquick gel extraction kit (Qiagen). The sticky ends of the vector backbone were then dephosphorylated using calf intestinal alkaline phosphatase: 1.5µl enzyme, 6µl buffer made up to 60µl with RO H_2O for 30 minutes at 37^{o}C. After dephosphorylation the vector DNA was cleaned and the two fragments were ligated as described above.

3.4.2.3 Plasmid Analysis

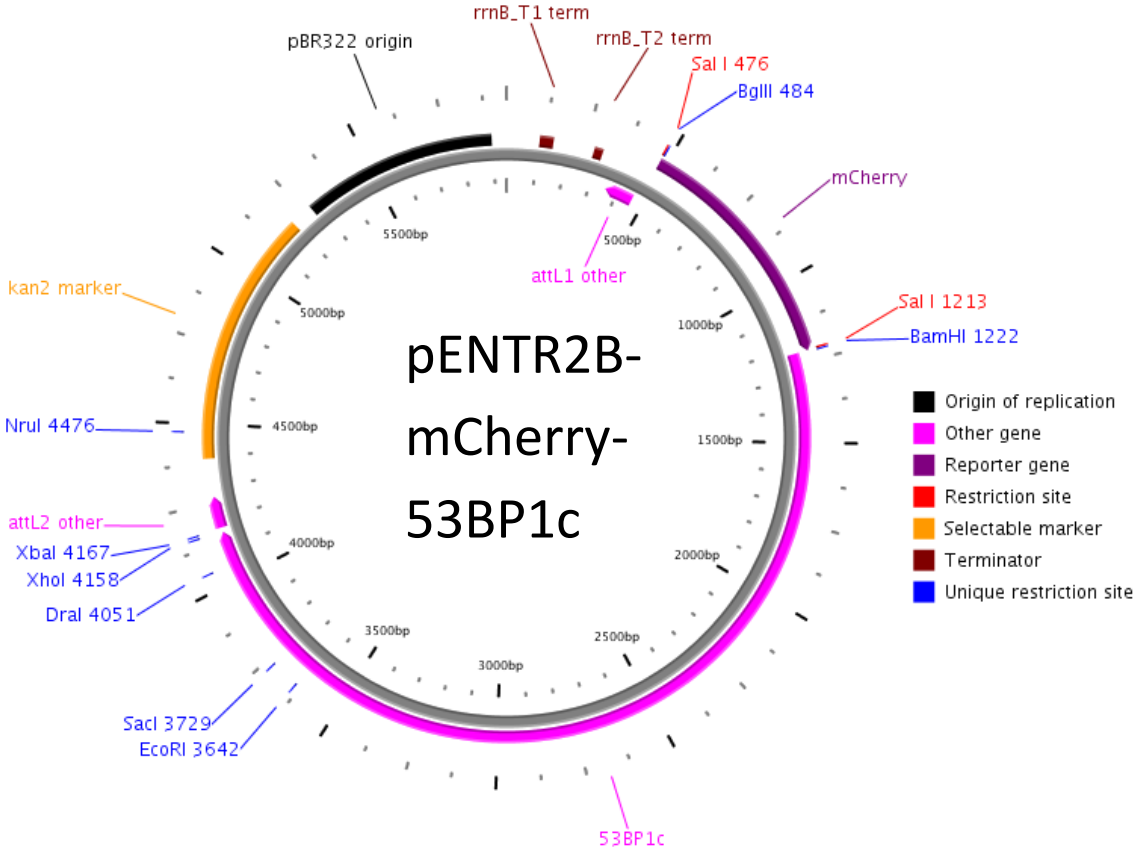
After bacterial transformation, colony selection, and DNA purification using QIAprep Spin Miniprep kit as described above, the DNA was tested with a *SalI* digest to determine which clones contained the insert. As shown in figure 3.4A, clones 1-4 all have mCherry inserted and are the right size, with 5.5Kb and 0.7Kb fragments. Secondly *a BglII BamHI* double digest was used to determine the orientation of the insert. The correctly inserted fragment yielded a 0.7Kb band whilst the other digest produced a non-detectable band of a few base pairs, as shown in figure 3.4B. The plasmid map is shown in figure 3.4C.



A|



B|

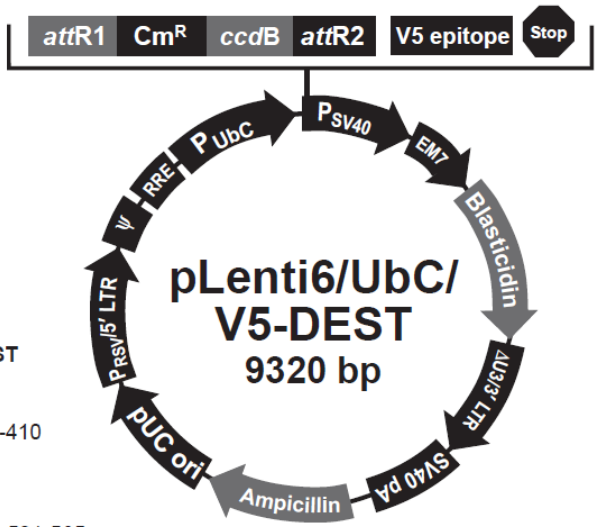


C|

Figure 3.4| pENTR2B-mCherry-53BP1c. (A) Plasmid digest with *BglII* and *SalI* separately. BglII cuts once producing a 6Kb band, whilst *SalI* cuts twice excising mCherry producing 5.5Kb and 0.7Kb bands. (B) Double digest with *BglII BamHI* restriction enzymes shows that clones 2 and 4 have mCherry inserted the correct way around as they both produce 0.7Kb bands. Clones 1 and 3 do not produce a similar band as they have *BglII* and *BamHI* within a few bases of each other as mCherry has inserted the other way around. The ladder bars, as for all gels throughout this thesis, are 10000, 8000, 6000, 5000, 4000, the large band is 3000, then 2000, 1500, 1000, 500bp. (C) Plasmid map of correct clones, created as described in figure 3.2.

3.4.3 pLenti6-mCherry-53BP1c

For lentiviral transduction pLenti6/UbC/V5-DEST (Invitrogen) was used. This drives ectopic expression of the inserted gene from a human promoter, as shown in figure 3.5.



Comments for pLenti6/UbC/V5-DEST
9320 nucleotides

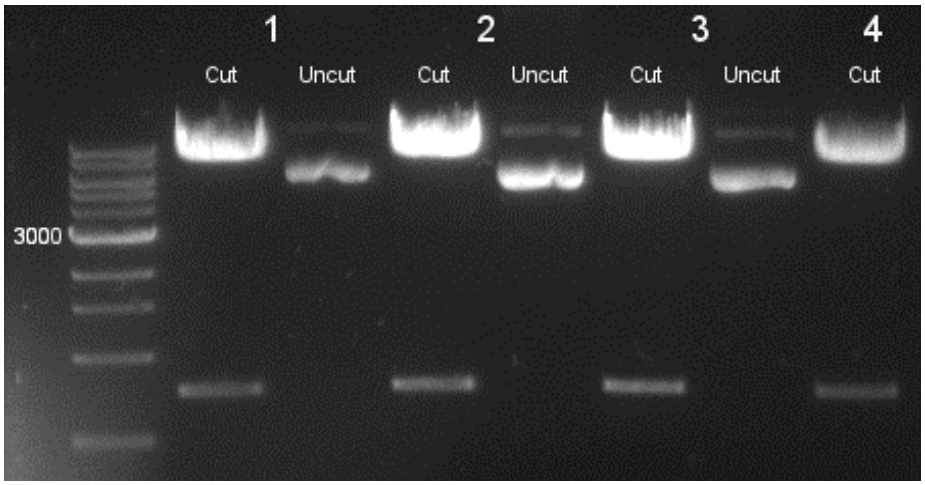
RSV/5′ LTR hybrid promoter: bases 1-410
RSV promoter: bases 1-229
HIV-1 5′ LTR: bases 230-410
5′ splice donor: base 520
HIV-1 psi (ψ) packaging signal: bases 521-565
HIV-1 Rev response element (RRE): bases 1075-1308
3′ splice acceptor: base 1656
3′ splice acceptor: base 1684
UbC promoter: bases 1798-3016
attR1 site: bases 3072-3196
Chloramphenicol resistance gene (Cm^R): bases 3305-3964
ccdB gene: bases 4306-4611
attR2 site: bases 4652-4776
V5 epitope: bases 4829-4870
SV40 early promoter and origin: bases 4925-5234
EM7 promoter: bases 5289-5355
Blasticidin resistance gene: bases 5356-5754
ΔU3/3′ LTR: bases 5840-6074
ΔU3: bases 5840-5893
3′ LTR: bases 5894-6074
SV40 polyadenylation signal: bases 6146-6277
bla promoter: bases 7136-7234
Ampicillin (*bla*) resistance gene: bases 7235-8095
pUC origin: bases 8240-8913

Figure 3.5| pLenti6/UbC/V5-DEST vector map, copied from product description (Invitrogen).

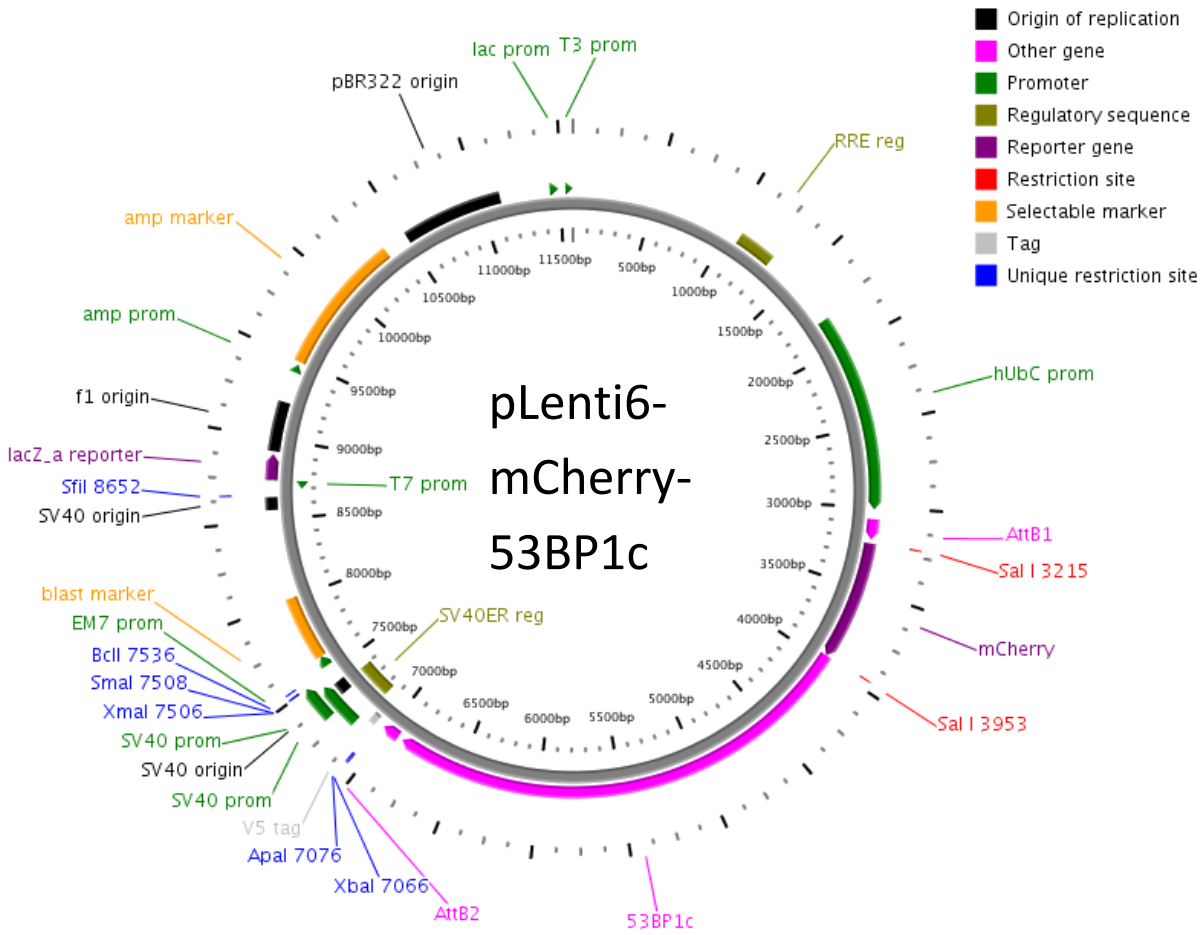
3.4.3.1 Recombination and Analysis

Following the instructions provided with LR clonase II enzyme mix (Invitrogen), pENTR2B-mCherry-53BP1c was recombined with pLenti6/UbC/V5-DEST to create pLenti6-mCherry-53BP1c.

Bacterial transformation, selection and DNA purification were carried out as described in section 3.4.1.3, except 50µg/ml ampicillin was added to the agarose plates for selection. As the empty pLenti6 vector contains the toxic ccdB gene, which inhibits topoisomerase II causing cell death (Bernard and Couturier, 1992), and pENTR2B containing E. coli are still susceptible to ampicillin, most of the colonies should contain recombinant DNA. However, some bacteria survived the ccdB toxicity but their colonies were generally smaller than those of recombinants. As shown in figure 3.6A, DNA was analysed using a Sal I digest, demonstrating that mCherry was present and recombination had taken place in all four of the selected clones. The plasmid map is shown in figure 3.6B.



A|



B|

Figure 3.6| pLenti6-mCherry-53BP1c. (A) Sal I digest produces a >10Kb band and a 0.7Kb band indicating the excision of mCherry. (B) Plasmid map of correct clones, created as described in figure 3.2.

3.4.3.2 Sequencing

Using a primer binding approximately 50bp away from the 3' end of mCherry, the pLenti6-mCherry-53BP1c plasmid was sent for sequencing at Genevision http://www.genevision.co.uk/index.html. The results demonstrated that mCherry had inserted in the correct frame and place relative to 53BP1c. Although there is one missing base near the end of mCherry in sequence read 1, this is likely because of the low fidelity of polymerases in the first 30-50bases sequenced; in all other respects both reads were identical to the desired sequence, as shown in figure 3.7.

Sequence Read 1:

5’ AG CTG TAC AGC AAA ACC AGA GTC GAC TGG ATC CCC CCA CCA CAC CCA TCA GGG GG 3’

Sequence Read 2:

5’ CA AGC AAA ACC AGA GTC GAC TGG ATC CCC CCA CCA CAC CCA TCA GGG GG 3’

Desired Sequence:

5’ AGC TGT ACA AGC AAA ACC AGA GTC GAC TGG ATC CCC CCA CCA CAC CCA TCA GGG GG 3’

Figure 3.7| Two sequencing runs of pLenti6-mCherry-53BP1c from two different clones. Green highlight indicates bases are from mCherry, pink highlight represents the linker region, yellow highlight the *SalI* restriction site, cyan is the single base lying between the *SalI* site and *BamHI* site in the pENTR2B MCS, blue highlight is the Bam HI site, and red highlight is 53BP1.

3.4.3.3 Making Stocks

Once DNA had been analysed and the colonies containing the correct plasmids identified, glycerol stocks were made of the bacteria by mixing 800µl LB containing the transformed bacteria with 200µl 80% glycerol, then flash freezing in liquid nitrogen, and storage at -80^{o}C. 1ml of LB containing the correctly transformed bacteria was also grown overnight in 200-250ml LB containing ampicillin before purifying the DNA using the EndoFree Plasmid Maxi Kit (Qiagen) as per manufacturer’s protocol.

3.5 Creating ΔIκBα Expressing Cells

Glyn Nelson had already generated a mutant IκBα with a deleted N-terminus, which removed the IKKβ phosphorylation signal (S32 +S36). As a result the

ΔIκBα cannot be inactivated by IKKβ and is therefore not targeted to the proteasome, but instead remains constitutively active, inhibiting NF-κB as a result. The ΔIκBα gene lies upstream of an internal ribosome entry site (IRES) which causes the translation of GFP in a pIRES-EGFP plasmid (Clontech), shown in figure 3.8. However, in order to transduce human fibroblasts this had to be inserted into an entry vector and recombined into a destination vector, similar to the reporter construct described above.

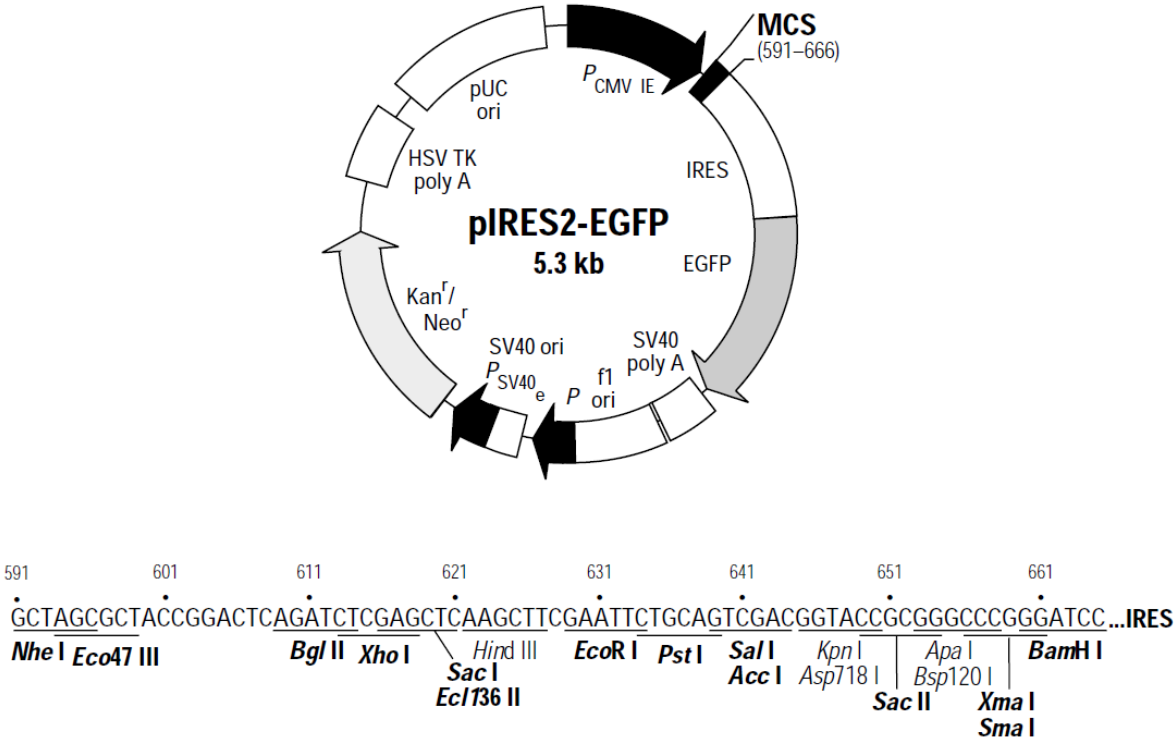


Figure 3.8| pIRES-EGFP Map, copied from product details provided (Clontech).

As the location of the ΔIκBα gene inside the MCS was uncertain, several test digests were used to determine its position. ΔIκBα is 1064bp long and with the IRES and EGFP in a single fragment it makes 2.4Kb band. Without ΔIκBα the IRES-EGFP fragment is only 1.3Kb. Therefore, as *NotI* cuts downstream of EGFP a double digest containing *NotI* and another enzyme from the MCS will locate the ΔIκBα gene. As shown in figure 3.9, both *BamHI* and *SalI* cut downstream of ΔIκBα, whereas *NheI* cuts upstream. Therefore, a *NheI NotI* digest was selected.

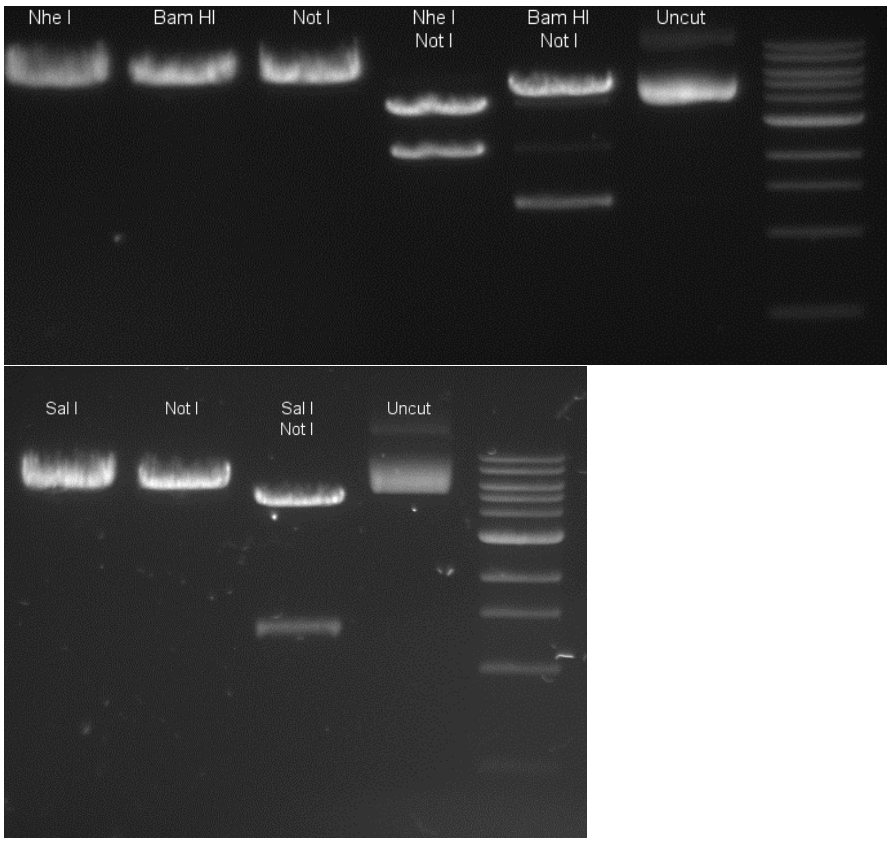
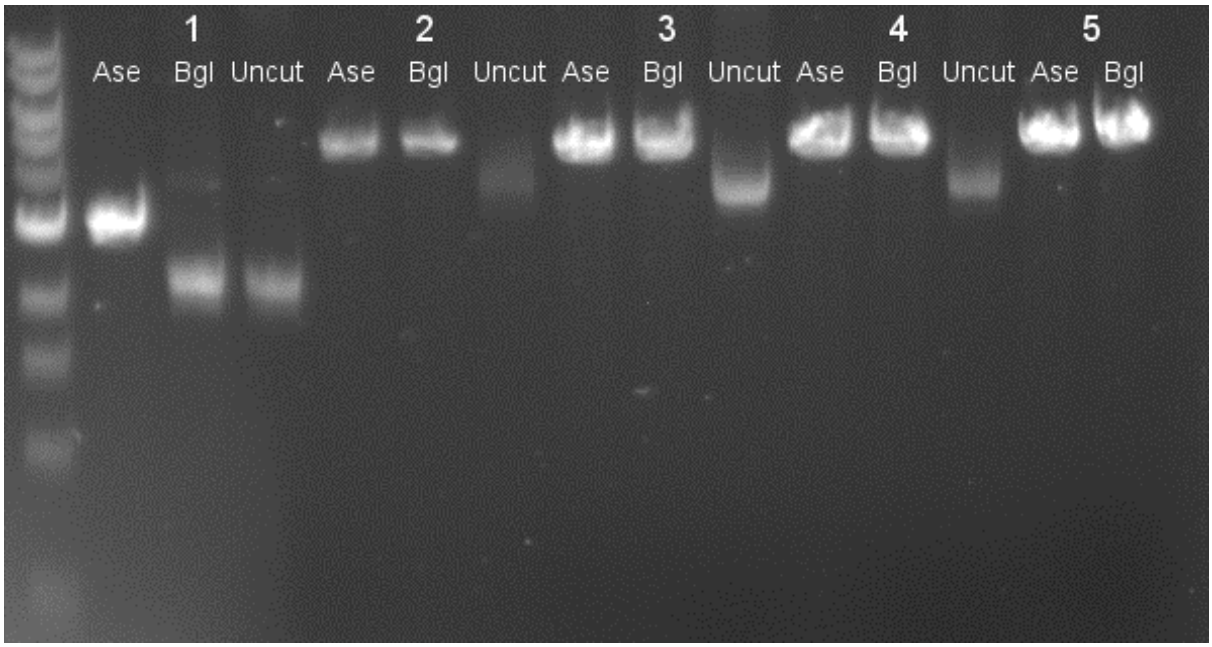


Figure 3.9| Test digests of pΔIκBα-IRES-EGFP. A single cut produces a 6.4Kb band; excising ΔIκBα-IRES-EGF from the backbone produces a 2.4Kb band, and excising only IRES-EGFP produces a 1.3Kb band. Therefore, as expected all single digests tested cut the plasmid once, *BamHI* and *SalI* double digests with *NotI* excise IRES-EGFP, and *NheI NotI* double digest excises the full ΔIκBα-IRES-EGF fragment.

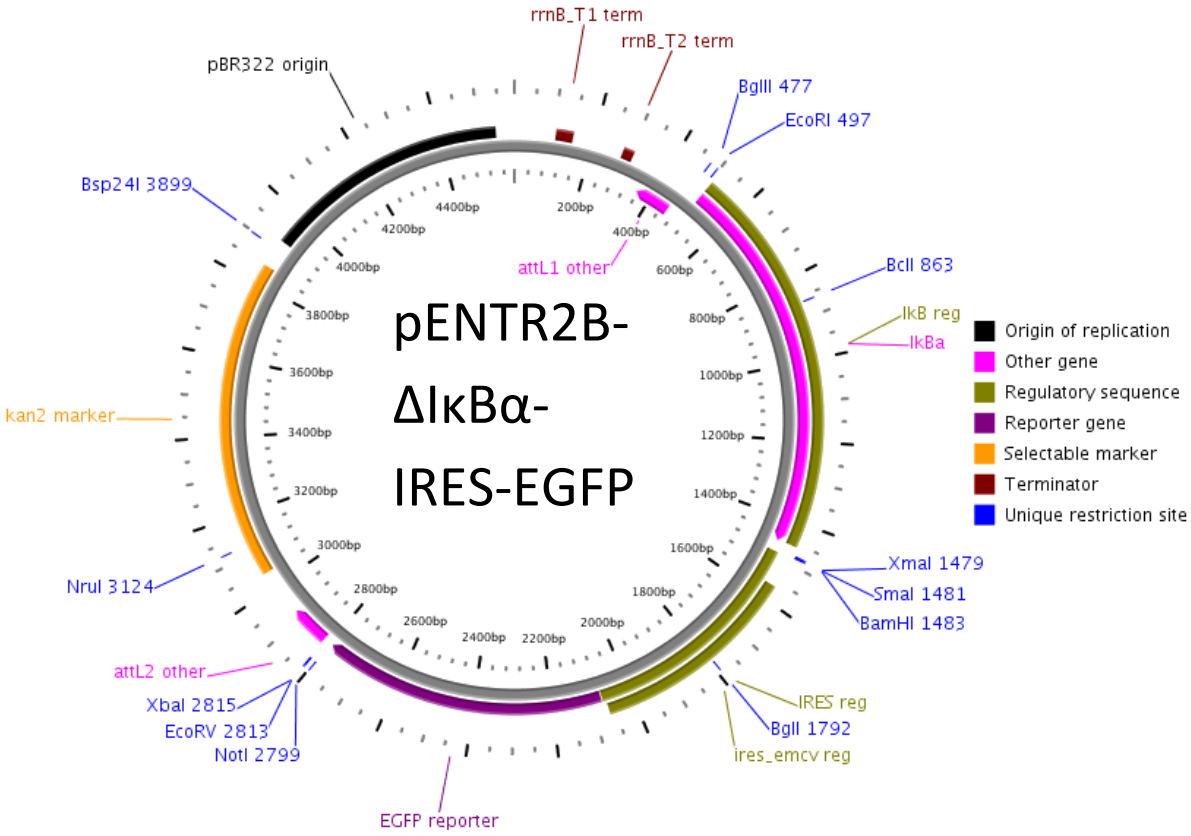
As can be seen from figure 3.1, *NheI* does not digest pENTR2B in a suitable place, whereas *NotI* does. Therefore, digesting pΔIκBα-IRES-EGFP had to be done sequentially with a blunting step in between to allow ligation of the *NheI* site 5' of ΔIκBα and the blunt *EheI* site chosen from pENTR2B. *NheI* digestion and cleanup were done as described in section 3.4.1.1. For blunting, 3µl T4 DNA polymerase, 7µl buffer 2 + BSA (NEB), and 6µl NTPs, were added to the 50µl elution, and made up to 70µl with RO H_2O. This was again cleaned before a subsequent *NotI* digest. The pENTR2B vector was double digested with *EheI* and *NotI*. As *EheI* produces a blunt fragment a sequential digest was not necessary. Both digested plasmids were run on a gel and the smaller fragment was excised from the well containing pΔIκBα-IRES-EGFP, and the larger (and

only visible) fragment from pENTR2B. These were cleaned, ligated and used to transform NEB10β *E. coli* as described in section 3.4.1.1 and 3.4.1.2.

The resultant purified DNA from selected colonies was analysed using *AseI* and *BglII* single digests, as shown in figure 3.10A. The 2.4Kb insert and 2.3Kb backbone produces a 4.7Kb band from a single cut. As both *AseI* and *BglII* cut once within the plasmid if the ligation has worked this should produce a 4.7Kb band. Clones 2,3,4 and 5 all have the correct sized bands. The plasmid map is shown in figure 3.10B.



A|

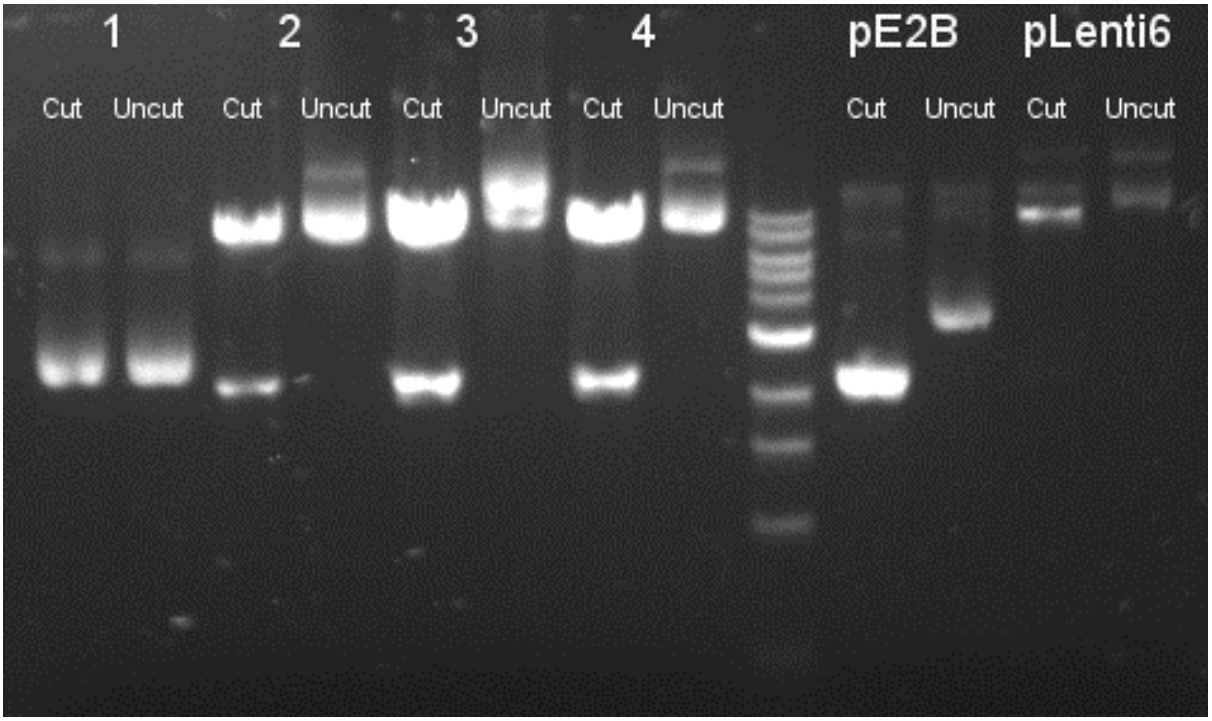


B|

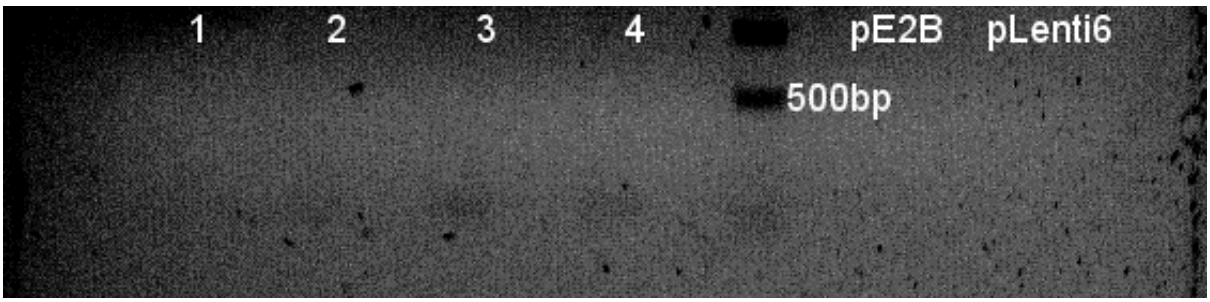
Figure 3.10| pΔIκBα-IRES-EGFP-pENTR2B. (A) *AseI* and *BglII* digests reveals that clones 2,3,4 and 5 all have the correct band between 4-5Kb on the ladder, whilst clone 1 most likely results from the re-ligation of pENTR2B with itself. (B) Plasmid map, created as described in figure 3.2.

ΔIκBα-IRES-EGFP-pENTR2B was then recombined with pLenti6/UbC/V5-DEST and the DNA was purified from selected colonies. The DNA was then analysed using a *XhoI* digest, as shown in figure 3.11A and B. *XhoI* cuts the correct plasmid four times; it cuts once in ΔIκBα, once in the MCS from pIRES2, once in the MCS transferred from pE2B and once in the pLenti6 backbone. However, because it cuts in the MCS of pIRES2 upstream of *SalI*, as shown in figure 9, it is unknown whether this lies before or after ΔIκBα. Therefore, there are two possible correct outcomes. If the *XhoI* site from the pIRES2 MCS lies upstream of ΔIκBα, then there will be a 7.8Kb band, a 2.2Kb band and two 0.1-0.2Kb bands. If it lies downstream of ΔIκBα, then the 2.2Kb band is split into a 1.3Kb and 0.9Kb band, and one of the small bands disappears.

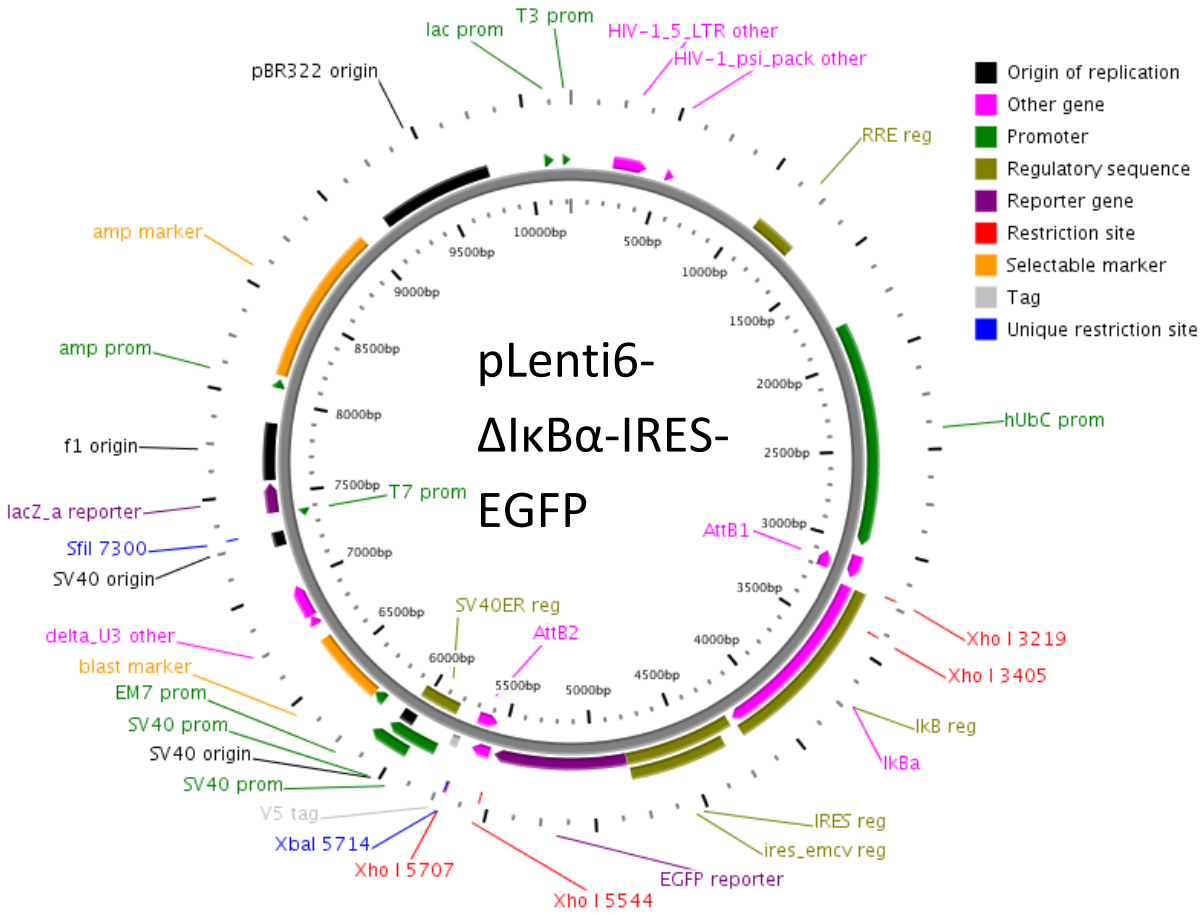
The results indicate firstly that some of the clones are correct, and secondly that they fit the former profile, producing a single band between 0.1-0.2Kb and a 2.2Kb band rather than any bands around the 1Kb mark. The plasmid map is shown in figure 3.11C.



A|



B|



C|

Figure 3.11| pLenti6-ΔIκBα-IRES-EGFP. (A) XhoI digest of four potential clones and ΔIκBα-IRES-EGFP-pENTR2B (pE2B), and pLenti6/UbC/V5-DEST (pLenti6) controls. Clones 2, 3 and 4 all show the band between 2-3Kb, and a band between 7-8Kb indicating that recombination has taken place. (B) An early image of the XhoI digest showing the 0.1-0.2Kb bands for clones 2, 3 and 4. (C) Plasmid map, constructed as described in figure 3.2.

3.5.1 Transfection and Transduction Protocols

All lentiviral plasmid transfection and viral production was performed following class II safety procedures. 1.25×10^6 HEK293FT cells were incubated for two days in antiobiotic free medium in a 10cm dish to be at 90% confluency at time of transfection with 3µg of pLenti6 mCherry-53BP1c combined with either 9µg

ViraPower^{TM} Packaging Mix, or 3µg of each individual component (pLP1, pLP2, pLP/VSVG, Invitrogen).

To separate the packaging mix, 1µl of DNA was added to NEB5α competent E. coli (NEB), which were then transformed as described above. The purified DNA from several colonies was analysed using a Bam HI digest. The pLP/VSVG plasmid is split into a 5Kb and 0.7Kb fragment, the pLP1 plasmid containing gag/pol is split into 8Kb and 0.8Kb fragments, and lastly the pLP2 plasmid containing Rev is cut once producing a 4.2Kb fragment. As shown in figure 3.12, all plasmids were accounted for.

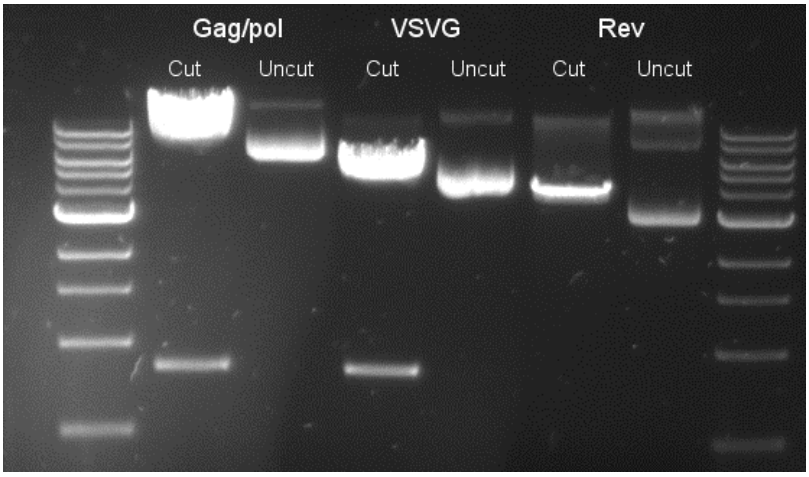


Figure 3.12| Bam HI digest of viral gene plasmids produces distinct bands for each.

One clone of each was purified using Maxi prep procedure described in section 3.4.3.3, and combined in a 1:1:1 ratio during transfection, as determined by the three similar intensity bands produced by running the packaging mix on a gel, shown in figure 3.13.

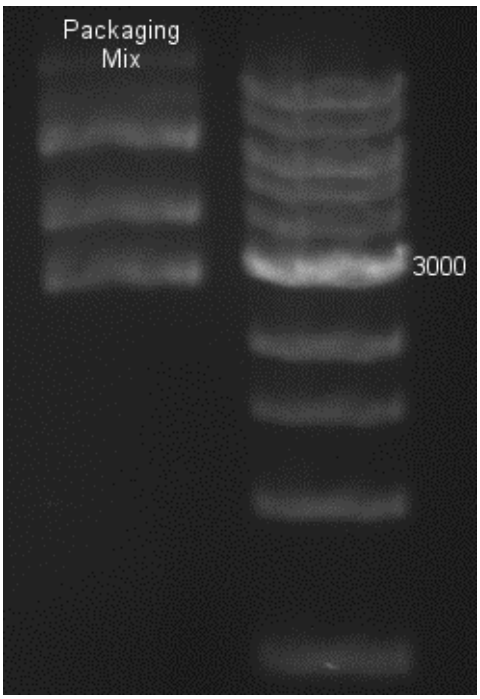


Figure 3.13| ViraPower™ Packaging Mix separates out into three similar size bands, indicating that each component is mixed in a 1:1:1 ratio.

Transfection was achieved using lipofectamine™ 2000 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. 48 hours after initial transfection, medium was collected and centrifuged at 4°C, before filtering through a 0.45µm pore PVDF filter. It was then added to MRC5 fibroblasts at 70-90% confluence. In young cells 10µg/ml polybrene was added, but this was avoided in the transduction of senescent cells due to its lethality.

Blasticidin was added to cells at a final concentration of 4µg/ml for six days, as had been previously established (Nelson et al., 2009). After six days, the concentration was reduced to 2µg/ml to maintain selection.

3.6 Immunofluorescence and Imaging

Cells were fixed with 4% PFA for 10 minutes at room temperature, then washed with TBS before the addition of 0.3% Triton Tris buffer saline (TBST) containing 5% goat serum and a 1 hour incubation at room temperature with shaking. This was then removed and 100µl TBST solution containing primary NF-κB XP antibody (NEB) at 1:50 dilution was then added to the coverslip and incubated for two hours at room temperature under humid conditions. The coverslips were then washed three times with TBS for five minutes before addition of 1:1500 secondary 633 goat anti-rabbit antibody (Invitrogen) in TBST, and incubation for one hour at room temperature in darkness. After three more five minute washes the coverslips were mounted in Prolong Gold antifade reagent with DAPI (Invitrogen) and imaged using a Leica DM5500B with 40×1.4 NA oil immersion objective, capturing fluorescent images with a DFC360FX camera, using LASAF software (Leica). To ensure consistency between slides the cells were selected randomly using the DAPI channel, and both the gain and exposure were kept constant. The settings were saved and used for subsequent experimental repeats with minor alterations for optimisation, but removing the requirement for normalisation.

For live cell imaging, cells were plated on glass bottomed dishes (IWAKI, NELS, Newton Aycliffe, UK) at least 24 hours before imaging. Time lapse imaging was achieved using a Zeiss LSM 510 META confocal microscope with a 40×1.3NA phase contrast oil immersion objective, and LSM version 2.1 software (Zeiss, Germany). A 3×3 tile scan was used to follow cells over a wider area using z stacks across 4.5µm. Cells were imaged every ten minutes for up to 55 hours.

Fixed time point live cell imaging was achieved using a Zeiss CSU-X1 spinning disk confocal microscope using a 40×1.3NA oil immersion objective, and captured using a QuantEM 5125C camera. Axiovision software (Zeiss) was used to capture cell images across a 11.9µm z stack, created from 7 images across the z plane.

3.7 Senescence Associated B-galactosidase Staining

Cells were washed twice with PBS-Mg before fixation with 2% PFA in PBS-Mg for five minutes at room temperature. Cells were then washed twice again with PBS-Mg before overnight incubation in staining solution at 37^{o}C. The following day cells were washed four times with PBS-Mg before imaging, using the Leica DM5500B with 20× objective and a DFC420 camera using LAS AF software.

3.8 Data Analysis

Image analysis was performed using Image J (http://rsb.info.nih.gov/ij), and graph creation and statistical analysis were performed in Sigma Plot. Statistical tests include the Shapiro-Wilk test for normality of datasets, and ANOVA for normally distributed data, and Kruskal-Wallis ANOVA on ranks for non-normally distributed data. Holm-Sidak method for multiple comparisons vs control, and Dunn's method were used for post hoc analysis. Gel images were annotated using GIMP 2.6 software (http://www.gimp.org).

4 The Bystander Effect of Senescent Cells

It has already been shown that senescent cells can induce growth and transformation of pre-neoplastic cells (Bavik et al., 2006, Liu and Hornsby, 2007, Krtolica et al., 2001). However, the same study showed that these effects did not occur in healthy cells. Therefore, the aim of this thesis was to elucidate whether senescent cells had any non-autonomous detrimental effects on healthy cells. The implications being that if senescent cells contributed to the decline of the surrounding cells, that this could potentially contribute to the ageing process via a non-autonomous mechanism, contrary to current hypotheses regarding the autonomous depletion of stem cell reserves.

4.1 An Increase in DNA Damage Foci in Bystander Cells Co-cultured with Senescent Cells

Due to the importance of a DNA damage response in senescence and the bulk of evidence implicating DNA damage in the ageing process, the primary examination focused on whether senescent cells induced a DNA damage response in healthy cells. Therefore, as described in the methods section, reporter genes were used where fluorescent mCherry or GFP was fused to 53BP1 DNA damage response protein. These genes were transduced into cells to create a stably expressing cell line with fluorescent 53BP1 foci at sites of DNA damage. Thus, these cells will henceforth be referred to as reporter cells.

The reporter cells could then be co-cultured with non-fluorescent senescent or control cells, which will henceforth be referred to as inducers. As the inducer cells did not have fluorescent foci, these could then be observed exclusively in the reporter cells (Figure 4.1).

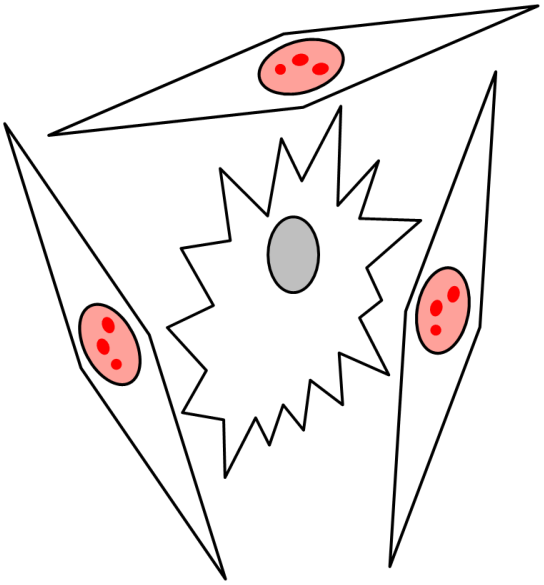


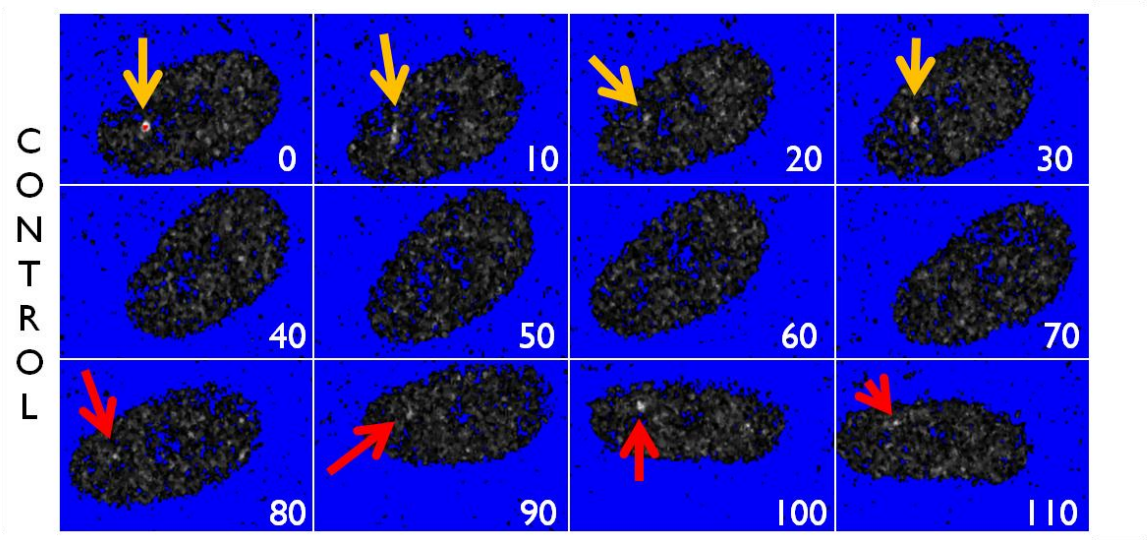
Figure 4.1| Illustration of fluorescent reporter cells in co-culture with a non-fluorescent senescent cell.

For the purpose of this thesis, cells in co-culture with senescent cells have been termed bystander cells. Although reporter cells in co-culture with replicating cells are technically still bystander cells, the focus of this thesis is whether there is a senescent cell induced bystander effect. Thus, for clarity this latter group is referred to as control cells. The non-reporter cells in the co-culture are inducer cells, be they senescent inducers or replicative control inducers.

Although observing 53BP1 foci did not provide a direct measure of DNA damage as would a COMET assay or TUNEL assay, or measuring oxidative lesions with 8-OHdG, it offered several advantages over these protocols. Firstly, it allowed repeated unintrusive measurement in live cells. Secondly, the assay was considerably more sensitive than several of these protocols, and thirdly and most importantly, the reporter protein was ideally suited to the co-culture situation, because it prevented confusion between reporter and inducer cells that would be difficult to avoid in assays that involved lysates. However, a recent study suggested that many DNA damage response proteins that had been previously associated with sites of DNA damage, could also form foci independently of damage under some stimuli (Pospelova et al., 2009). Whilst these cells were notably still undergoing a stress response, importantly 53BP1

was not associated with these foci, indicating that at least under these conditions it is specific to sites of damage.

Initially GFP-53BP1 reporter cells were added to a culture of senescent cells and incubated for two days. As described in section 3.2.1.1, cells were plated at 1:1 ratio, with 75,000 of each type of cell in a 35mm dish. They were then imaged every ten minutes for 55 hours using time lapse imaging, which allowed a detailed analysis of focus dynamics, including formation, number of foci, and lifespan (Figure 4.2).



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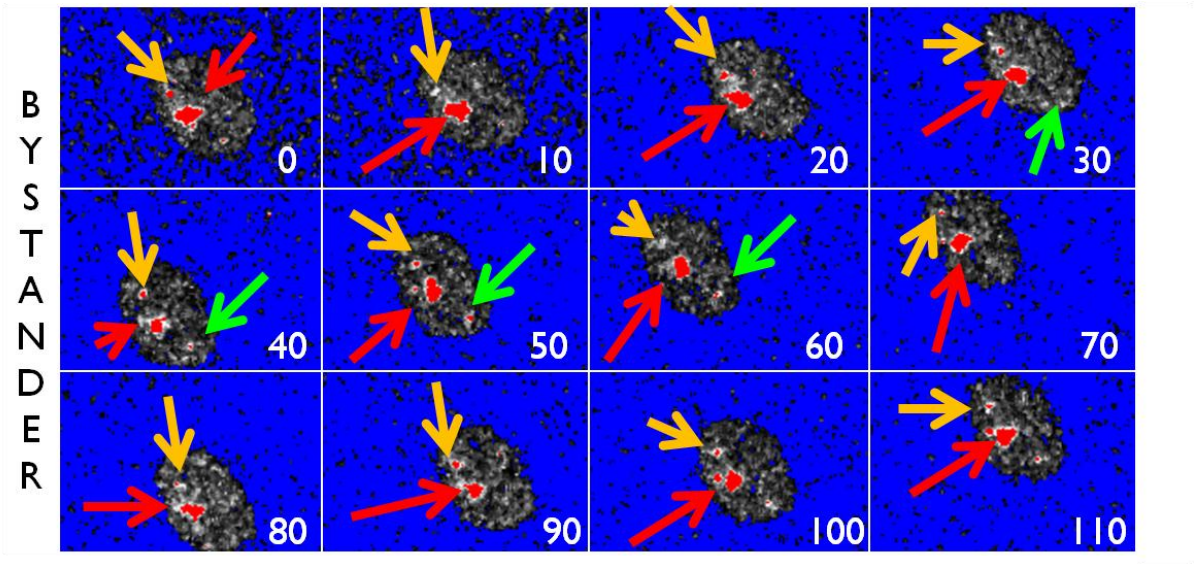
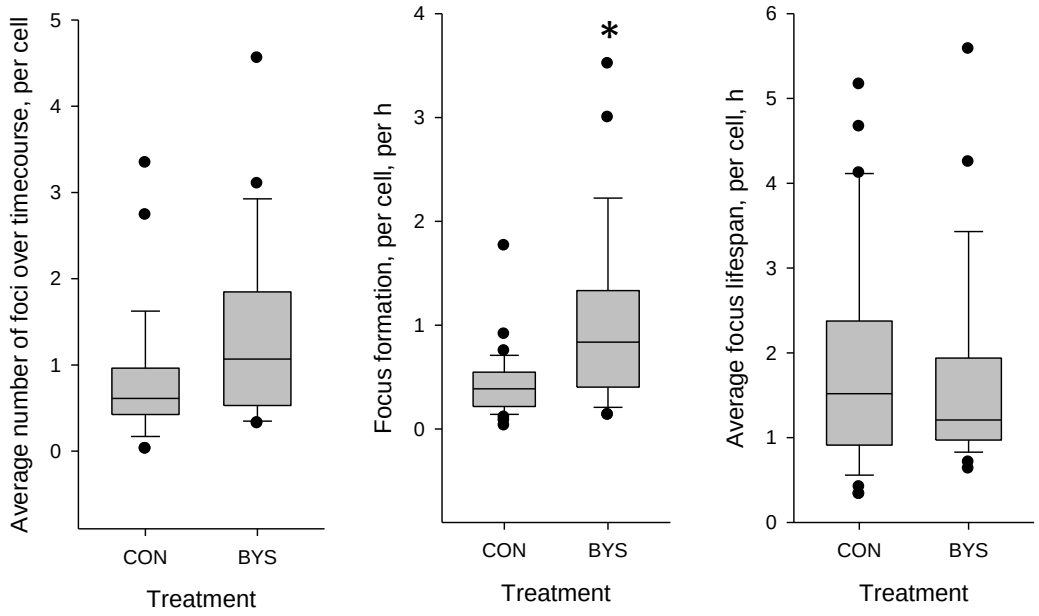


B|

Figure 4.2| The nucleus of a control (A) and bystander (B) cell followed for a period of 110 minutes with an image taken every ten. The time points are shown in the bottom corner of each box. The arrows indicate the presence of foci, with each colour used to follow a single focus over time.

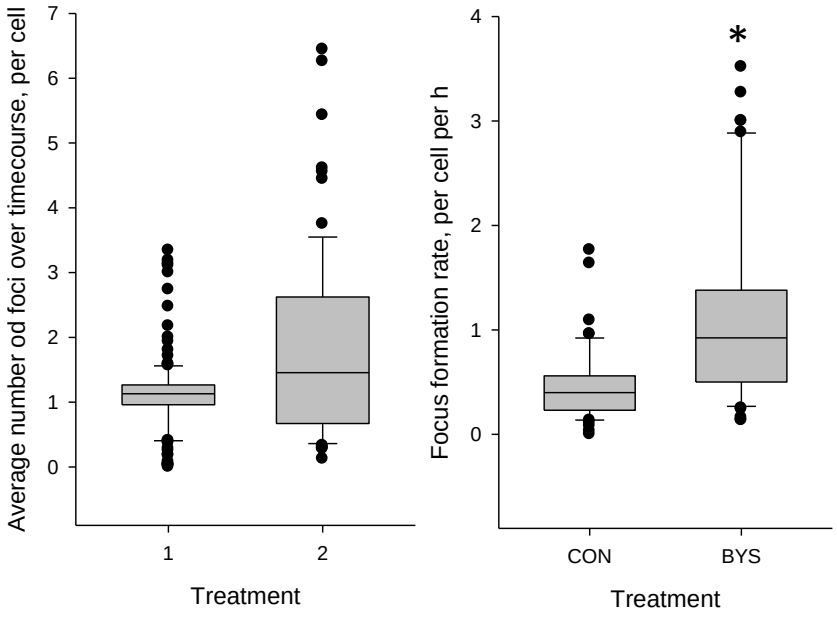
As shown in figure 4.3, focus formation rate was significantly increased in bystander cells compared to controls, whereas focus lifespan and the number of foci per cell were not significantly different between the two groups.



A| B| C|

Figure 4.3| Senescent cells induce a DDR in bystander cells. (A) 53BP1 foci in bystander cells (BYS), compared to controls. (B) Rate of formation of 53BP1 foci in bystander and control cells. (C) Average lifespan of foci in bystander and control cells. All results were obtained between 48-103 hours after the start of co-cutlure. Box plots show median, upper and lower quartiles (boxes), centriles (whiskers), and outliers (dots). Only focus formation rate was significantly different between bystanders and controls, as determined by Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, P<0.05). Data are n=1-2, with 20-25 cells per group per experiment.

This can be combined with data from Glyn Nelson collected independently to give n=3 experiments for each group, with similar results (See Appendix). The focus formation rate is increased, whilst the number of foci remains constant within the cell, as shown in figure 4.4.



A| B|

Figure 4.4| Combined data from this thesis with that, independently collected, from Glyn Nelson, showing significant increase in focus formation in bystander cells as determined by Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, P<0.05). Data are n=3, with 20-25 cells per group per experiment.

Data from Glyn Nelson indicated that the number of foci per cell did start to increase when co-culture was extended between 10-12 days (Appendix). A new reporter construct with mCherry fused to 53BP1 was used to analyse the numbers of foci in much larger numbers of cells at single time points. Although this did not allow measurement of focus formation rates, it was much faster and produced larger quantities of data. After 14 and 21 days of co-culture, the

numbers of foci significantly increased in bystander cells of both groups, as shown in figure 4.5.

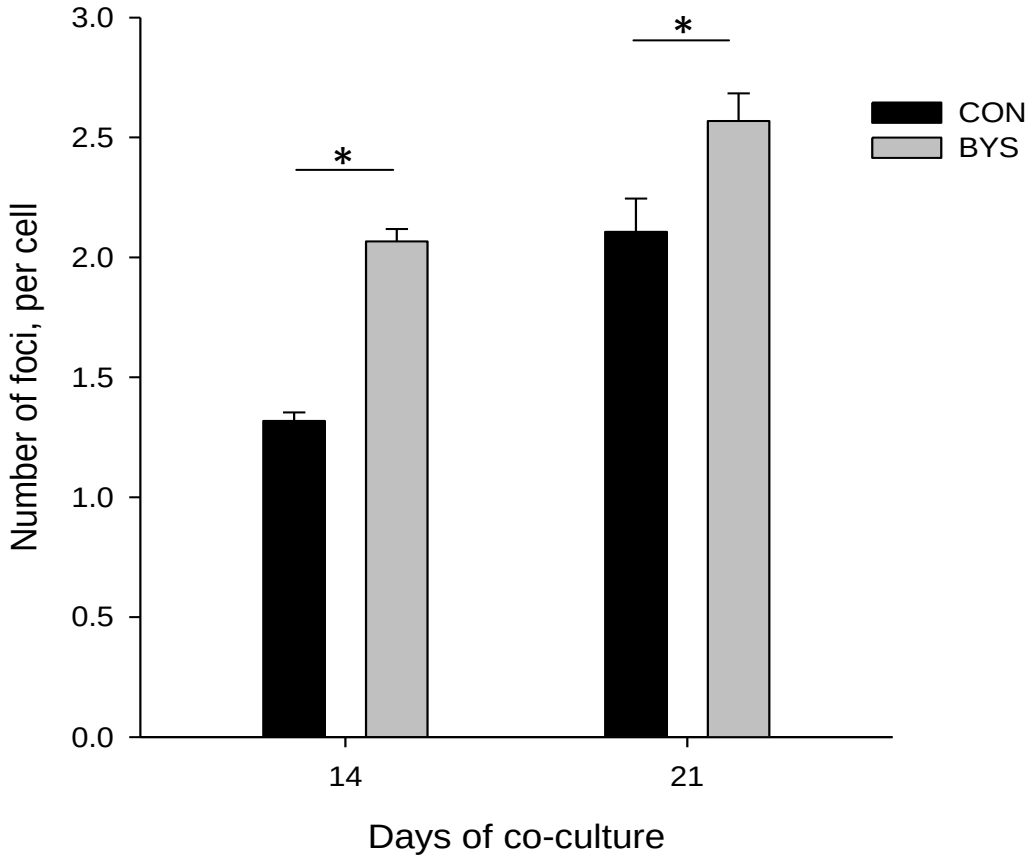


Figure 4.5| Extended co-culture for 14 or 21 days causes the number of 53BP1 foci to increase in bystander cells. At both time points bystander cells had significantly more foci on average compared to control cells, as determined by ANOVA with Dunn's post hoc test. Data are mean ± SE (n=3-5), with approximately 100 cells per group.

Whilst the number of foci was also significantly higher at 21 days than 14 days for both controls and bystander cells, at both time points there was a significant increase in the number of foci in bystander cells. The resultant conclusion was that senescent cells induce a DNA damage response in bystander cells, which after a delay causes the number of foci present to increase.

4.2 The Irradiation Induced Senescent Cell Bystander Effect

Whilst the majority of this thesis concerns the bystander effect of replicatively senescent cells, it was also addressed whether this bystander effect was shared with cells that had become senescent under different stimuli.

It has long been observed that a sufficiently high dose of irradiation can cause cells to senesce. Although the process takes a number of days, cells hit with high levels of X-rays generally do not undergo mitosis. Therefore, unlike replicative senescence, irradiation induced senescence is fairly homogeneous, with all the cells undergoing senescence at the same time. As a result, this system can be used to observe whether senescing as well as senescent cells induce a bystander effect.

Here cells were irradiated with 20Gy which has been previously shown to be sufficient to induce senescence in MRC5 fibroblasts. Importantly, generally the data agree that the senescent phenotype matures by about day 9-10 (Passos et al., 2010, Rodier et al., 2009, Rodier et al., 2011), in most but not all cells. Another study indicated that although most cells were SA-β-gal positive by this time point, by 15 days the number had still increased (Nelson, unpublished). After irradiation cells were left for either seven, 14 or 21 days before starting the co-culture, which was then incubated for 14 and 21 days, as shown in figure 4.6.

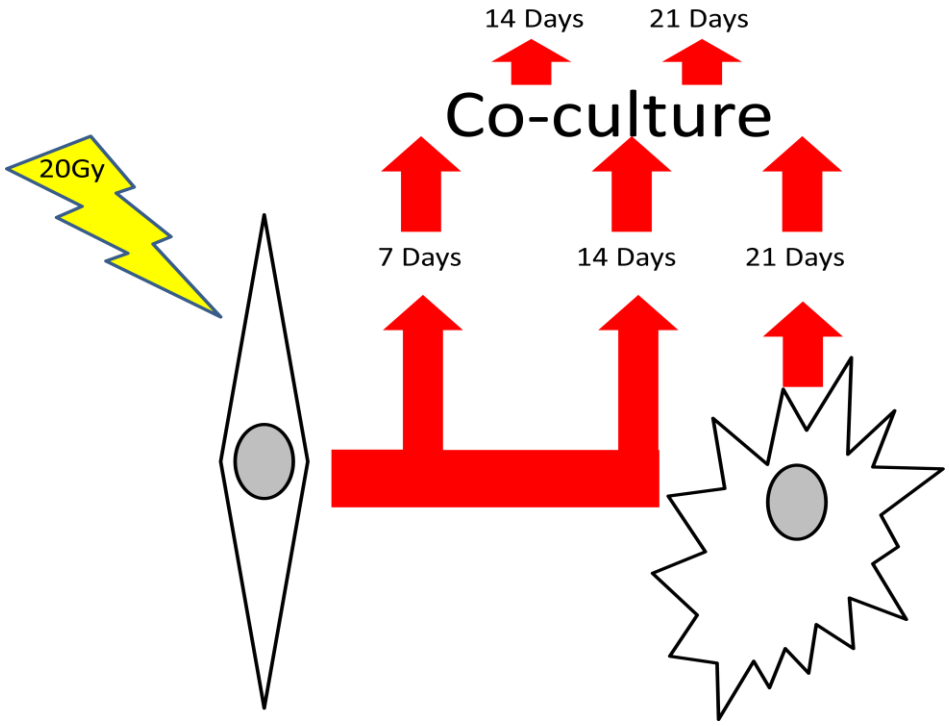


Figure 4.6| Diagram demonstrating mechanism of pre-incubation and co-culture of irradiation induced senescent cells.

If senescing cells were incubated for seven days before co-culture, then 14 days was not sufficient to induce a bystander effect, whilst cells pre-incubated for 14 and 21 days induced a bystander effect after both 14 and 21 days. However, as would be expected, the seven day pre-incubation did induce a bystander effect after 21 days co-culture. These results suggest that senescent cells regardless of stimulus induce a bystander effect. However, senescing cells do not induce a bystander effect, as shown in figure 4.7.

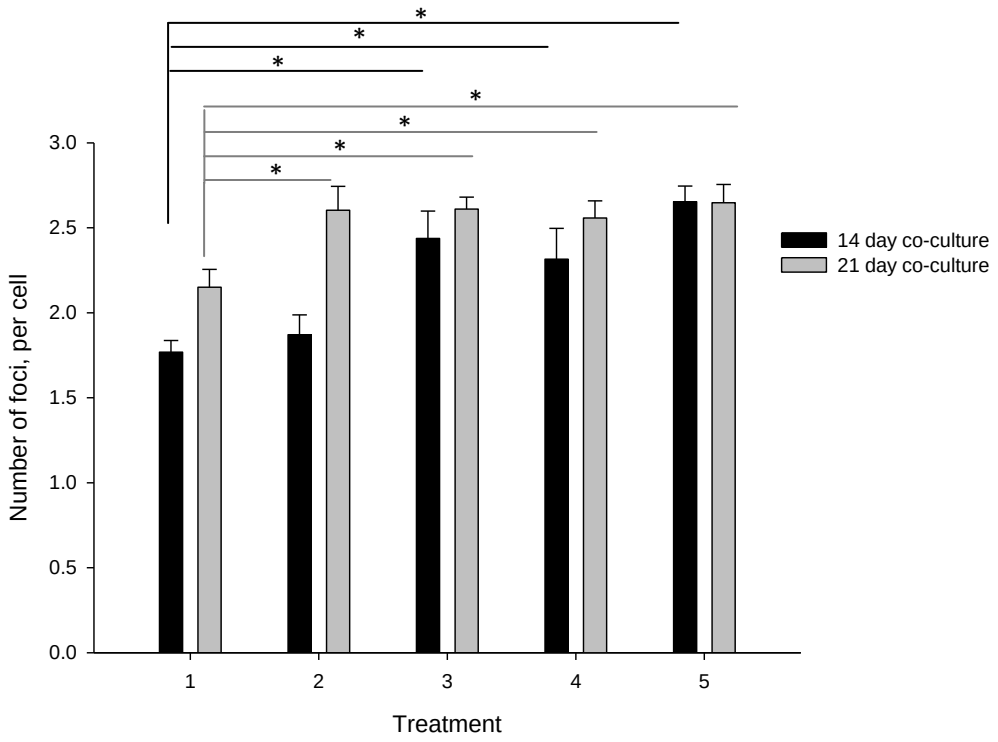


Figure 4.7| Irradiated senescent, but not senescing cells induce a DDR in bystander cells. A pre-incubation of seven days (7D Post IR) was not long enough to significantly increase the number of foci in bystanders after 14 days. However, 14 day and 21 day pre-incubations (14D Post IR; 21D Post IR respectively) were sufficient to increase number of foci after 14 day co-culture. All lengths of pre-incubation senescing cells significantly increased foci in bystanders after 21 days of co-culture, as determined by ANOVA with the Holm-Sidak method for multiple comparisons vs. a control group, P<0.05. Data are mean ± SE of n=3 with approximately 100 cells per experiment.

4.3 Medium Transfer from Senescent Cells does not Induce a Bystander Effect

To test whether the bystander effect could be transferred purely by long lived secreted factors we compared the effect of medium from senescent cells to that of replicating cells. Senescent cells were incubated for one day so as not to exhaust the nutrients provided in the FCS, before the medium was transferred to reporter cells through a 0.45μm pore filter. The reporters were incubated for a

further 48 hours before the medium was changed to new medium from senescent cells, at which point the cells were imaged for a further 55 hours. As shown in figure 4.8, there was no significant effect of senescent cell conditioned medium.

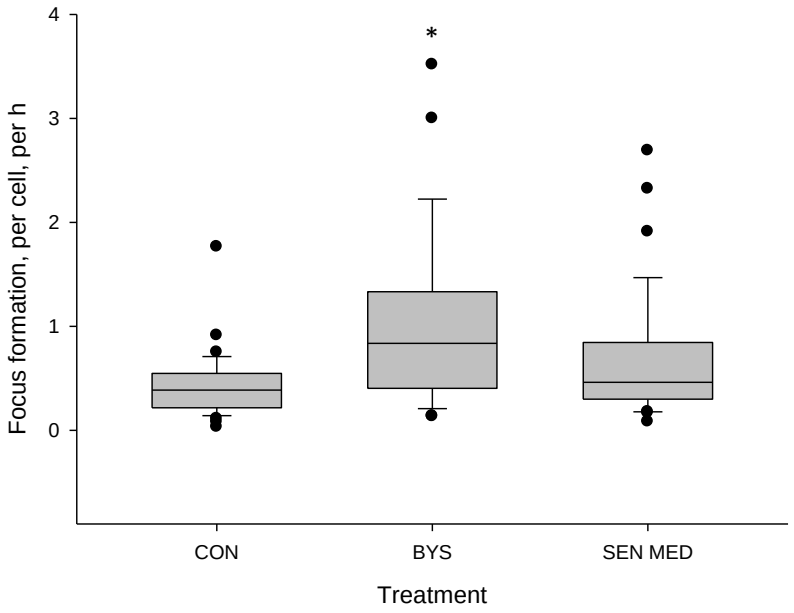


Figure 4.8| Senescent medium (SEN MED) has no significant effect on 53BP1 focus formation. Data and statistics are as in figure 4.3.

Thus, it can be concluded that the senescent cell bystander effect cannot be transmitted exclusively by long lived secreted factors.

4.4 Gap Junction Mediated Transfer Contributes to the Bystander Effect

Gap junctions have been shown to be highly important in the RIBE, and their inhibition using lindane and octanol has been shown prevent the signal inducing damage and cell death in bystander cells (Azzam et al., 1998, Azzam et al., 2001, Zhou et al., 2000). To determine whether gap junctions play a role in the senescent cell induced bystander effect, octanol, was added to the co-culture. During the first 2-4 days of co-culture 1μM octanol was added at day zero and

day two before imaging began. As shown in figure 4.9, the presence of octanol had a tendency to reduce the focus formation rate in bystander cells, but the effect was not significant.

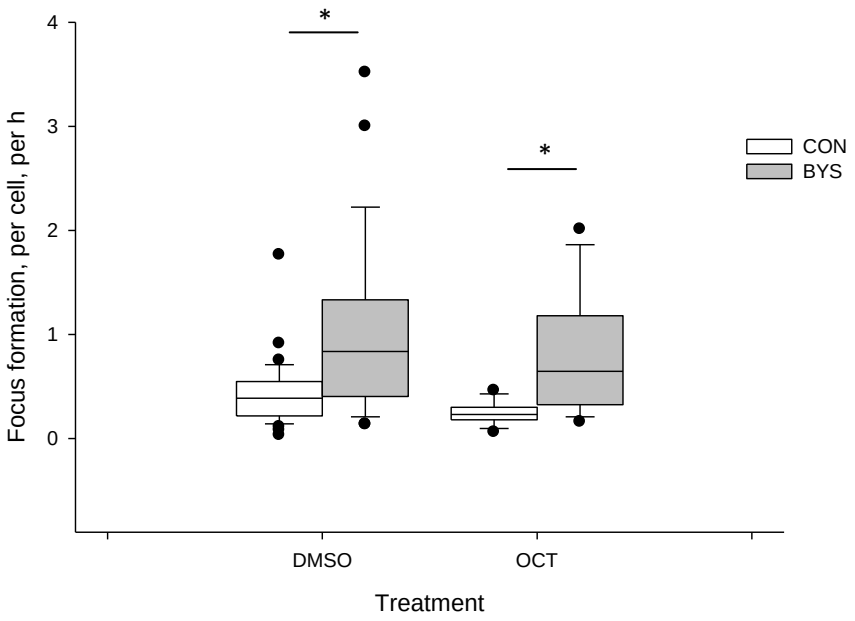


Figure 4.9| Octanol inhibition of gap junction mediated transfer does not reduce the induction of DDR in bystander cells in short term co-culture. Cells in the presence of octanol (OCT) did not have significantly reduced focus formation rate compared to DMSO in either bystander or control cells. Only focus formation rate in bystander cells in the presence of DMSO was significantly different to controls, as determined by Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, P<0.05). Data are n=1-2, with 20-25 cells per group.

With extended co-cultures gap junctions begin to play more of a role. After 21 days of co-culture in the presence of octanol the number of foci in bystander cells was reduced to that of controls, as shown in figure 4.10.

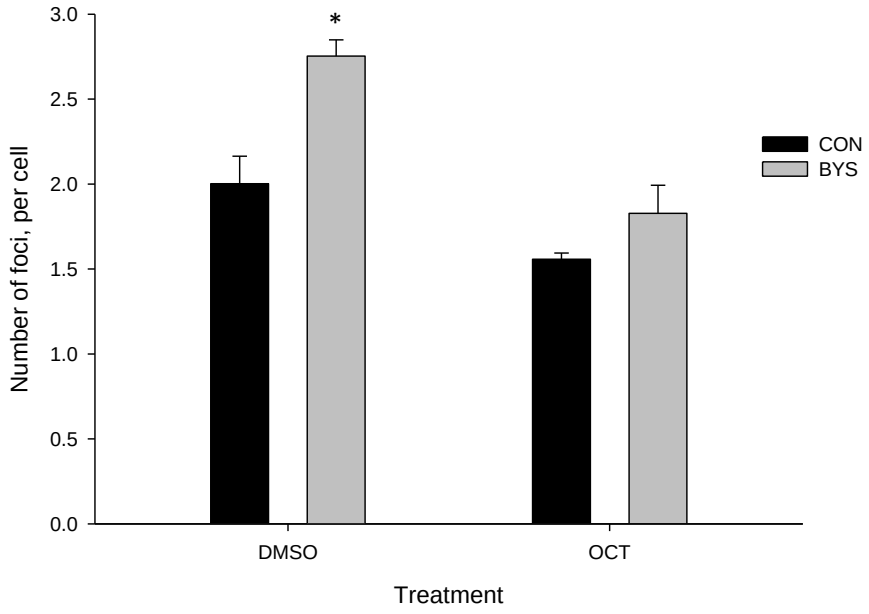


Figure 4.10| Octanol inhibition of gap junction mediated transfer does reduce number of foci in bystander cells in long term co-culture (21 days). Data are mean ± SE (n=3-5), with approximately 100 cells per group. As determined by ANOVA, the only group significantly different to control is the bystander cells in the presence of DMSO.

4.5 Large and Long Lived Foci are Important in the Bystander Effect

Whilst observing the DNA damage focus dynamics between 2-4 days of co-culture, it was clear that some foci were substantially larger than others. Therefore, foci were divided into two groups, where foci ≤ 0.45μm^2 were counted as small foci, and foci that persist above this size for at least two consecutive time points (20mins) were counted as large. Examining focus formation rate revealed that the difference between bystanders and controls was exclusively the result of the large foci, as shown in figure 4.11.

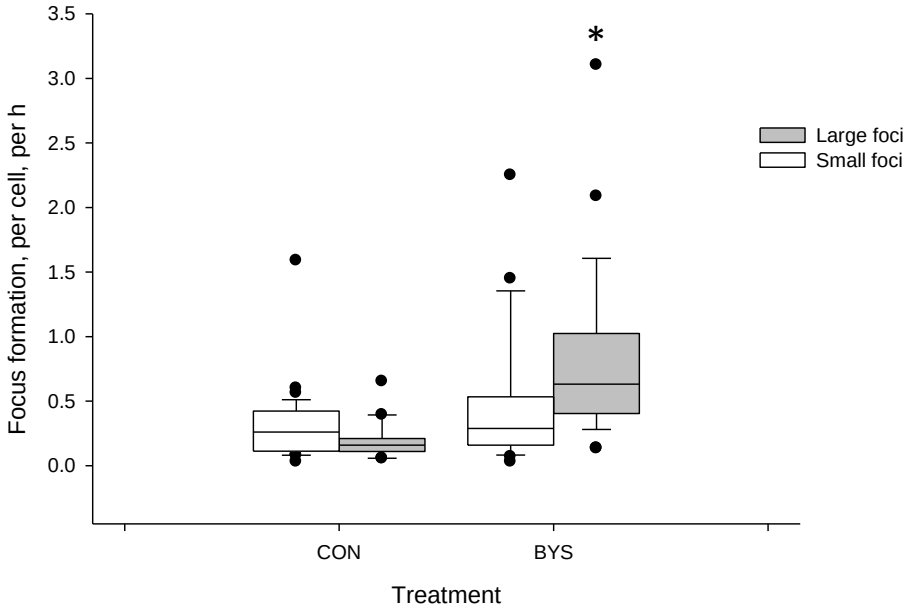


Figure 4.11| Large focus formation is significantly increased in bystander cells, whilst small focus formation is not significantly changed. Data are n=1-2, with 20-25 cells per group, analysed using two way ANOVA with Holm-Sidak method for multiple comparisons.

Additionally, large foci persisted for significantly longer than small foci in both control and bystander cells, as shown in figure 4.12.

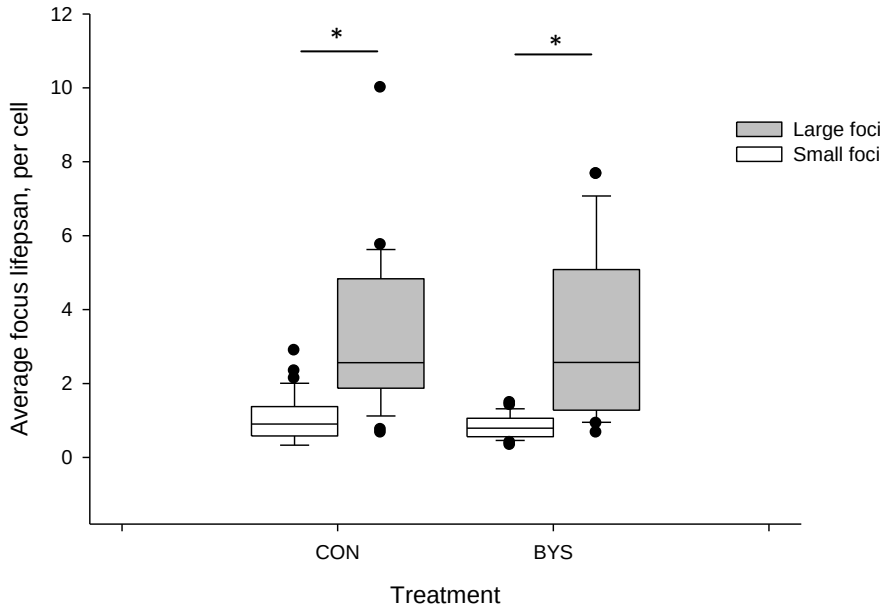


Figure 4.12| Large foci persist for significantly longer on average than small foci. Data are n=1-2, with 20-25 cells per group, analysed using two way ANOVA with Holm-Sidak method for multiple comparisons.

Lastly, the literature suggests that in normal proliferating cells, most foci are cleared within five hours (Passos et al., 2010). Therefore, control and bystander groups were compared for the number of foci that persisted for longer than this length of time. Only cells that could be followed for at least ten hours were selected, so that if a cell did possess such a long lived focus, there was a reasonable chance that the cell would remain within the tile scan long enough for it to be correctly identified as such. The small number of cells that persisted for ten hours (10-20 in each group), made analysis difficult, but a two-tailed t-test showed that the two groups were significantly different, as shown in figure 4.13.

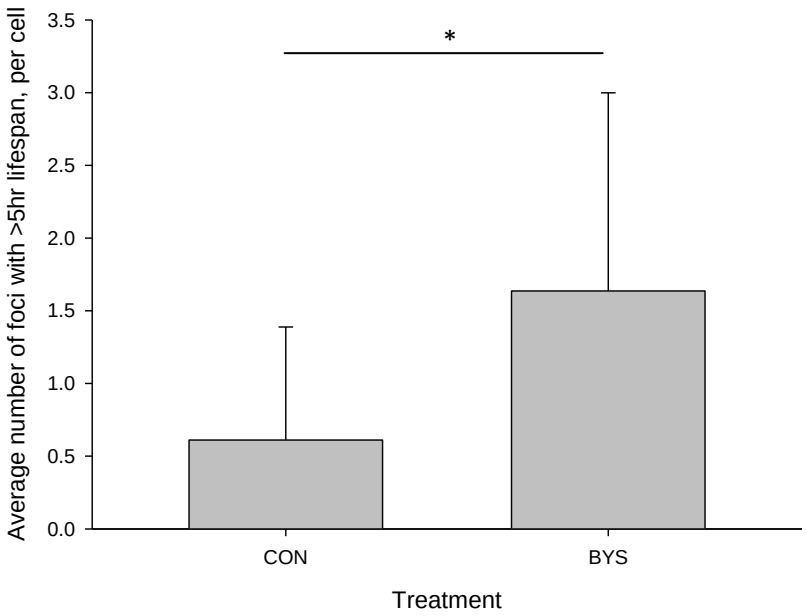


Figure 4.13| The number of foci that persist for longer than five hours is increased in bystander cells. Data are mean ± SD (n=1, 28 cells) and significance was determined by a two tailed t-test not assuming equal variance (p=0.038).

Thus, bystander cells have an increase in large long lived foci, which can be indicative of the induction of cell senescence (Rodier et al., 2011).

4.6 Senescent Cells Induce Senescence in Bystander Cells

Consistent with previous studies (Passos et al., 2007), this thesis has found that senescent cells have more foci than healthy replicating cells (Figure 5.1.2). As discussed in sections 1.3.1 and 1.4.3, damage is important in both the induction and maintenance of senescence. Accounting for the fact that the main difference in bystander cells was due to large long-lived foci, which have also been shown to be associated with senescence (Rodier et al., 2011), it was hypothesised that senescent cells may induce senescence in bystander cells.

The measure of senescence associated β-galactosidase (SA-β-gal) activity is the most widely accepted marker of senescent cells, both *in vitro* and to a lesser

extent *in vivo*. However, because the reporter cells were in co-culture, it was important to distinguish between senescence in the reporter and inducer cells. Because senescence is a permanent phenotype, there was no possibility that, after the removal of the inducer cells, the reporter cells would revert to proliferation. Therefore, it was possible to kill the inducer cells using blasticidin, for which the reporter cells carried a resistance gene (figure 4.14).

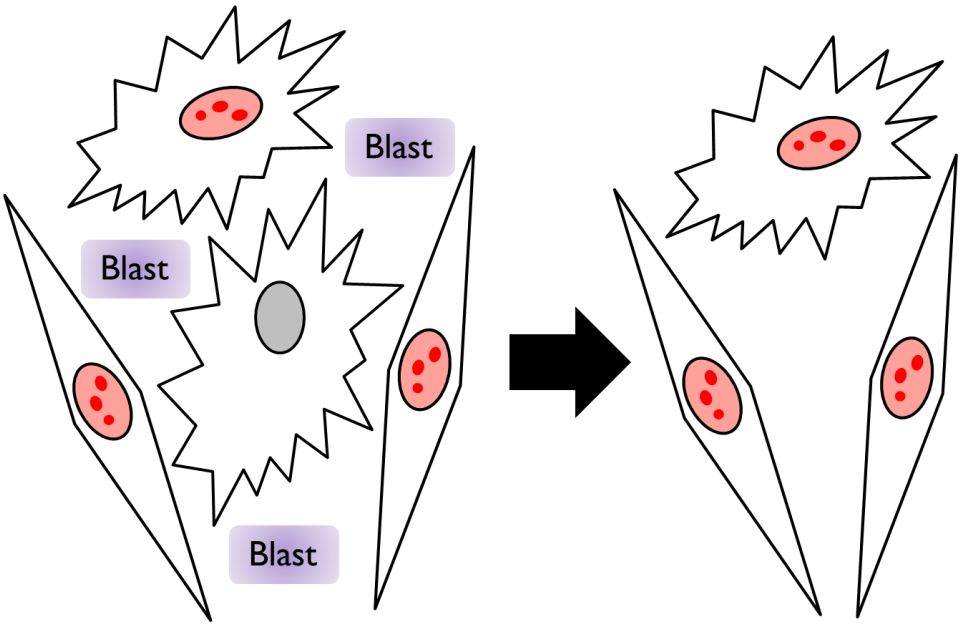


Figure 4.14| Addition of blasticidin (Blast) after up to 20 days of co-culture is used to kill the senescent inducer cells leaving only the bystander cells to measure for induction of senescence.

Reporter cells were co-cultured with senescent cells or replicating controls for up to 20 days. Blasticidin was then added to the co-culture for at least six days, and until all senescent cells in an equally plated control dish had died. Blasticidin was then removed and the cells were re-plated onto coverslips. After a further four days the cells were fixed and stained for SA-β-gal activity as described in the methods. The results demonstrated that six day co-culture was not sufficient to induce senescence in bystander cells, whilst 15 and 20 day co-cultures caused a small but significant increase in the induction of senescence (Figure 4.15). The lack of response after six days is perhaps not surprising as

after direct irradiation under half show SA-β-gal after 4 days, around three quarters after 9 days, and almost all cells after 15 days (Nelson, unpublished).

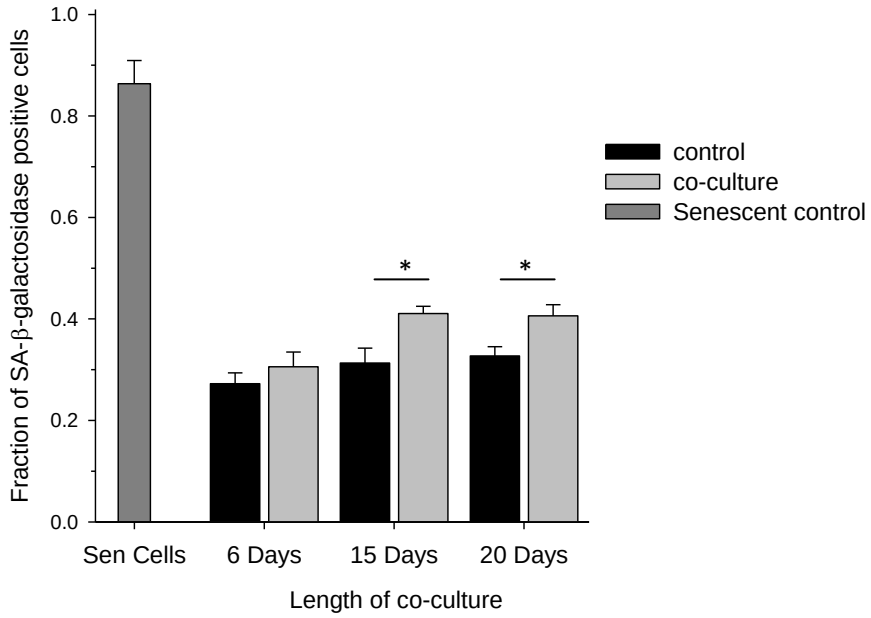
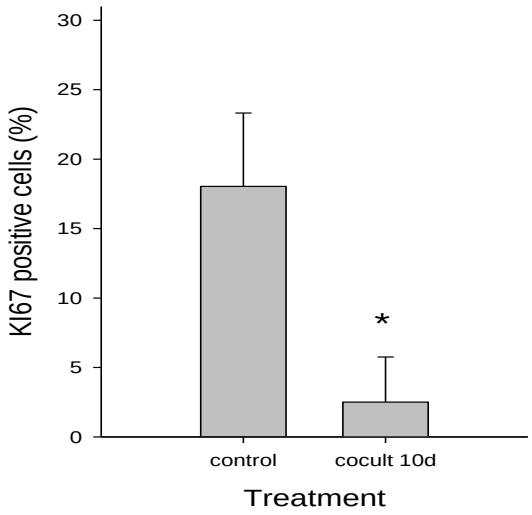


Figure 4.15| Senescent cells induce senescence in bystander cells. After a six day co-culture the number of senescent cells was not significantly different between bystanders and controls. After 15 and 20 day co-cultures the number of senescent cells was significantly increased in bystanders compared to controls (n=3, 100-250 cells per group within each experiment), as determined by ANOVA with Tukey HSD P=0.048 and 0.008 respectively.

Additionally data from Glyn Nelson, published alongside these data (see Appendix) provide further evidence of senescence inductrion. The proliferation marker Ki67 is significantly reduced in senescent cells, as shown in figure 4.16; as is the co-localisation of γ-H2AX with PML bodies, both of which are established markers of senescence (Rodier et al., 2009, Rodier et al., 2011).



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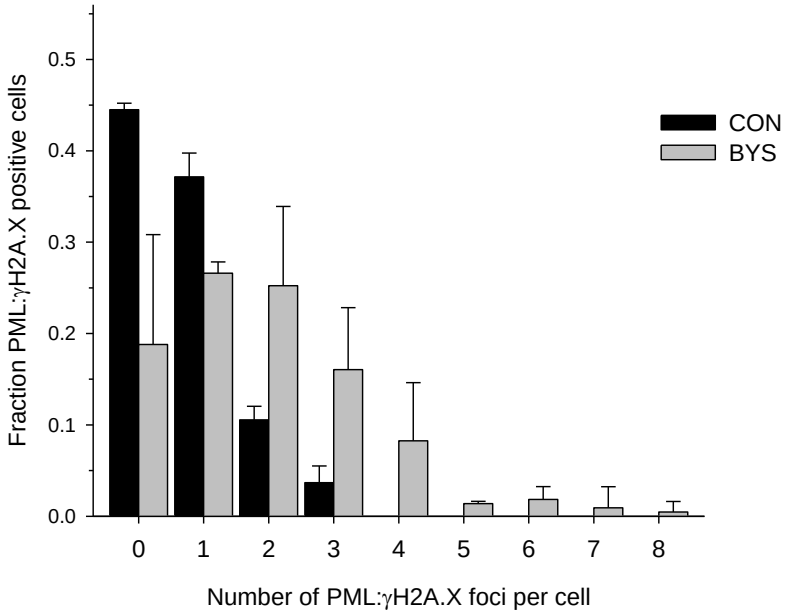


B|

Figure 4.16| Data collected by Glyn Nelson after 10 day co-culture with senescent or control inducer cells. (A) Bystander cells have significantly reduced Ki67 staining compared to controls, and (B) significantly increased co-localisation of γ-H2AX with PML bodies. Data are mean ± SE (n=3). Analysis was determined by a two tailed t-test, and Mann-Whitney *U*-test for (A) and (B) respectively.

4.7 Discussion

Prior to this work, the detrimental paracrine effects of senescent cells on healthy cells had not been investigated. Two previous studies demonstrated that senescent cells could stimulate proliferation of preneoplastic cells, and this could result in production of benign tumours or malignant transformation depending on the cell line (Krtolica et al., 2001, Liu and Hornsby, 2007). Notably, although one of these studies tested whether healthy keratincoytes were also stimulated to proliferate in the presence of senescent cells, this was found not to be the case (Krtolica et al., 2001), although another study found that the opposite was true of prostate epithelial cells (Bavik et al., 2006)

These data provide the first evidence of a genotoxic effect of senescent cells upon healthy proliferative cells. These effects include, but are not limited to, an increase in 53BP1 focus formation, the number of foci per nuclei, and the induction of senescence as determined by staining for SA-β-gal. The results were published in *Aging Cell* (Nelson et al., 2012), and are shown in the appendix.

4.7.1 Implications of 53BP1 Foci Formation and Numbers

As mentioned in the introduction, 53BP1 is an important member of the DNA damage response. It was cloned in 1994, shown to interact with DNA-binding domain of p53 (Iwabuchi et al., 1994), and increase p53-mediated transcription (Iwabuchi et al., 1998). It has a tandem BRCT (BRCA1 C terminus) domain, and significant sequence homology to BRCA1 (Callebaut and Mornon, 1997). Several studies identified that 53BP1 forms distinct foci within minutes of exposure to ionising radiation (Anderson et al., 2001, Rappold et al., 2001, Schultz et al., 2000, Xia et al., 2001), and co-localises with other proteins, such as γ-H2AX, BRCA1, RAD51 and the MRN complex, known to bind to DNA lesions (Anderson et al., 2001, Rappold et al., 2001, Schultz et al., 2000). Specifically 53BP1 was found to bind to DSBs (Mochan et al., 2003), dependent

on a domain containing two tandem tudor folds and a deep hydrophobic pocket (Iwabuchi et al., 2003, Ward et al., 2003a). The resultant implication that 53BP1 would bind histones containing hydrophobic methylated lysine/arginine residues lead to the identification of lysine 79 methylation on histone H3 which correlated with 53BP1 binding (Huyen et al., 2004). However, lys79 methylation is not increased at sites of DNA damage (Huyen et al., 2004). Because lys79 is at the nucleosome core and therefore not exposed in normal chromatin (Dorigo et al., 2004), it is likely that DSBs cause nucleosome unstacking and exposure of methylated lys79 (Zgheib et al., 2005).

Therefore, although 53BP1 foci do not represent direct measurement of DNA damage, it is known to be associated with DNA damage, and specifically DSBs. Interestingly, one study found that in transformed rodent cells, treatment with the HDAC inhibitor sodium butyrate (NaB) induced H2AX phosphorylation, which did not co-localise with phospho-ATM, or correlate with high levels of DNA damage as determined by COMET assay (Pospelova et al., 2009). This indicated that γ-H2AX foci might be forming at sites of undamaged DNA. Importantly, 53BP1 was not associated with these damage-independent foci, so whilst there is some evidence that other members of the DDR do not always associate with DNA damage, there is no evidence this is true of 53BP1. Notably, although retention of 53BP1 at DSBs is dependent on γ-H2AX, its recruitment is not (Ward et al., 2003a, Celeste et al., 2003).

In this study a truncated form of 53BP1 is used, by excision of the carboxyl-terminal fragment using a *Bam HI* site. This was necessary in order to allow the gene to fit into the large destination vector for viral transduction. Although the loss of the N-terminal fragment may potentially alter the binding and signalling of the fragment, the 2.77Kb end terminus still contained all the functional domains including the TUDOR and two BRCT domains, and has been shown to co-localise with γ-H2AX (Nelson et al., 2009), indicating that it still binds to DNA damage similar to wildtype 53BP1. Additionally, the expression of the 53BP1 fusion protein should not affect the expression of cellular 53BP1, although it will competitively inhibit binding to sites of damage. The UbC promoter used to express the reporter gene is a constitutive human promoter for ubiquitin C, and therefore induces much lower level of expression than viral promoters such as CMV; will not be subject to viral promoter silencing; and is less likely to induce

artefacts of overexpression. However, it cannot be ruled out that the reporter cells have some altered signalling process which changes their susceptibility to the bystander effect.

The precise mechanisms and role of 53BP1 in the DDR are unknown. It does co-immunoprecipitate with Chk2 (Wang et al., 2002), and 53BP1$^{-/-}$ cells have defective Chk2 activation; however, the phenotypes of Chk2 and 53BP1 knockdown have little in common. Chk2$^{-/-}$ mice are resistant to IR and their thymocytes are resistant to apoptosis (Hirao et al., 2002, Takai et al., 2002), whilst the opposite is true of 53BP1$^{-/-}$ mice (Ward et al., 2003b). This being the case, the primary effect of 53BP1 still appears to be the activation of ATM. Although it also binds p53 (Iwabuchi et al., 1994), and p53 activity is decreased in 53BP1 inhibited cells (Wang et al., 2002), it does not activate p53 transcription, which is impossible whilst p53 still binds 53BP1 (Derbyshire et al., 2002, Joo et al., 2002). Thus, increasing 53BP1 foci will primarily result in an increase in ATM signalling.

As discussed in section 1.3.2, ATM is a potent inducer of p53 and therefore the senescence response. Consistently, these data demonstrate that senescent cells cause a significant increase in the induction of senescence, to be discussed later. Notably, ATM is a hub molecule and has multiple targets outside Chk2, including HDACs and the IKK complex, and therefore the bystander effect is not limited to the effects of activated p53. Many of these other targets may contribute to both the induction of senescence and the resultant phenotype.

Interestingly these data indicate that although focus formation increases very quickly in bystander cells with 2-4 days of co-culture (Figure 4.3), initially these cells can tolerate the increase in damage induction and the number of foci per cell does not increase. However, after a longer period of time somewhere in between 4-10 days the average number of foci per cell begins to increase. Potentially, the longer cells spend in the presence of senescent cells, the more susceptible they become to damage, or the longer senescent cells are in the presence of proliferating cells the more damaging they become. Potentially, a more likely possibility is that some parts of the DNA are easier to repair than others, and therefore, because the sites of damage are random it is only a

matter of time until a difficult to repair site is hit, thus causing the damage to persist. Additionally, damage to sites concerned with repairing DNA could also cause foci to persist longer and contribute to the cells deteriorating ability to repair damage.

It is well established that telomeric uncapping induces senescence because the cells find it difficult to repair telomeric lesions. Two recent papers have suggested that senescence is maintained partially by persistent foci at the telomeres irrespective of telomere shortening or senescence stimulus (Fumagalli et al., 2012, Hewitt et al., 2012). Thus, it is possible that the number of foci begins to increase in bystander cells as a result of a telomere becoming damaged. The longer cells remain in co-culture the more likely their telomeres will be damaged, especially as telomeres are particularly susceptible to oxidative stress (von Zglinicki et al., 1995).

4.7.2 The Irradiated Senescent Cell Bystander Effect

The bystander effect is not limited to replicatively senescent cells. Here, these data indicate that cells induced to senesce by 20Gy X-rays also induce a bystander effect (Figure 4.7). Importantly, preincubating irradiated cells for seven days before a 14 day co-culture was not sufficient to induce a bystander effect. This raises the possibility that the factor(s) responsible for the bystander effect take longer than seven days before they are sufficiently expressed. Additionally, because the bystander effect can be achieved after ten days of co-culture, this would imply that the factor was not sufficiently present at 11 days after irradiation, by which time the majority of the literature suggests that irradiated cells have adopted the full senescent phenotype (Passos et al., 2010).

An alternative is that being present during the senescence process provides the bystander cells with a relative immunity to the bystander effect. Inconsistent with this idea, a study examining oncogene induced senescence demonstrated that bystander cells in co-culture with cells undergoing senescence undergo a substantial bystander effect (Acosta et al., 2013). It is also unlikely that this

reflects a difference in the two systems, as multiple studies have indicated that bystander cells in co-culture with cells hit with α particles, or cells exposed to ICM from recently irradiated cells all induce a bystander effect. Thus, the reason for the lack of bystander effect in seven day pre-incubated cells warrants further investigation.

A recent study examined the irradiated senescent cell bystander effect in primary mouse adult ear fibroblasts (MAFs) and found that they had significantly increased foci after two days of co-culture, but this returned to control levels by days 7-8 (Jurk et al., unpublished). The early increase in DNA damage is likely to reflect that the study used mouse cells which are known to be more susceptible to damage. However, it may also be a result of the radiation induced bystander effect (RIBE).

It is important to distinguish between the RIBE, which has been comprehensively studied, and the irradiated senescent and replicatively senescent cell induced bystander effect, which have both been described for the first time in these data (Nelson et al., 2012).

Interestingly, experiments using irradiated cell conditioned medium demonstrated that, whilst keratinocytes and immortalised fibroblasts were prone to the bystander effect, that healthy fibroblasts were unaffected (Mothersill and Seymour, 1997), which parallels the predisposition of immortalised cells to the bystander effect induced by senescence (Krtolica et al., 2001). However, the RIBE does not require that the inducer cells are senescent, and the effect plateaus long before the doses required to induce senescence (Liu et al., 2006b).

4.7.3 Senescent Cell Secretions and the Bystander Effect

In this system neither medium transfer, nor matrix from senescent cells induced a significant bystander effect (Figure 4.8, latter not shown). However, in other systems both these treatments have been shown to induce a bystander effect. In one study, the ECM produced by senescent cells encouraged a 3-4 fold increase in growth of immortalised untransformed cells (Krtolica et al., 2001).

Notably, although the senescence bystander effect has not been directly compared between fibroblasts and epithelial cells, studies into the radiation induced bystander effect, observed that epithelial cells are more susceptible to damage and cell death (Mothersill and Seymour, 1997). Equally there are some differences between the SASP of epithelial cells and fibroblasts, although they do have significant overlap and both contain many of the same inflammatory factors (Coppe et al., 2008). Importantly IL-1α, which was found to be unique to epithelial cells, is a stimulator of TNF-α induced NF-κB canonical signalling (Yard et al., 1992), and may therefore, make epithelial cells induce a more potent signal. Consistent with this idea, studies examining the RIBE using medium transfer identified that although irradiated fibroblasts did not induce a bystander effect, they were still capable of receiving the signal from cells that did (Mothersill and Seymour, 1997).

Another study looking at the bystander effect of oncogene induced senescent cells, also found that medium transfer induced a significant bystander effect including a decrease in BrdU, and induction of senescence as indicated by an increase in SA-β-gal staining (Acosta et al., 2013). The implications of this study are that oncogene induced senescent cells clearly induce a more potent bystander effect than replicatively or irradiated senescent cells. Consistently, there are several observations that indicate that this may be the case. The SASP of oncogene induced senescent cells is qualitatively very similar to that of IR and replicatively senescent cells, but some factors were significantly upregulated in oncogene induced senescent cells, and were expressed from an earlier time point (Coppe et al., 2008). These factors include GM-CSF, IL-1β GRO-α and MIP-1α, which may therefore be key molecules in the bystander effect of oncogene induced senescent cells. However, it should be noted that although the SASPs of senescent cells correlate, they are not the same, and the difference in the bystander effect could result from qualitative differences in SASP factors. Additionally, the levels of ROS, other non-protein or non-secreted components have not been compared between the different senescent inducers.

Interestingly, the study examining the bystander effect in preneoplastic epithelial cells also looked into oncogene induced senescence, and despite using similar co-culture times found that these cells actually induced a weaker bystander

effect than replicatively senescent cells (Krtolica et al., 2001). However, they indicated problems with their RAS expressing cells which may explain this difference. It is also possible that it reflects a difference between fibroblasts and epithelial cells.

4.7.4 Gap Junction Mediated Transfer

Here we demonstrate that the inhibition of gap junction mediated transfer by 1μM octanol abrogates the bystander effect induced by senescent cells (Figure 4.10). The mechanism by which octanol inhibits gap junction mediated transfer is not precisely known; however, several studies have identified this effect across multiple cell lines (Pappas et al., 1996, Abou Hashieh et al., 1996). Notably, octanol at 1mM (1000× higher than concentration used here) has also been demonstrated to inhibit the activity of Cx46 and Cx50 connexins similarly to gap junctions, enhance GABA and glycine receptor activity and inhibit NMDA and AMPA receptors amongst others (Dowling-Warriner and Trosko, 2000). However, none of these molecules should play a large role in the bystander effect, other than perhaps the other connexins which form gap junction-like channels.

It should be noted that, as with the other inhibitors discussed later, the inhibition of gap junction mediated transfer via octanol is not specifically tested within the cells utilised here, which may respond to the compound differently to other cell lines in which octanol has been tested. Additionally, the potential induction of cell death was not quantitated and may have affected the level of damage in the surviving cells, and could also be a confounding variable if octanol affects cell death in bystander cells differently to controls.

The results suggest that gap junction mediated transfer is not necessary for the early bystander effect, but becomes important later once the number of foci begin to increase in bystander cells. One possible explanation is that the early and late bystander effects are transmitted by different signals that are initially not transmitted through gap junctions, but latterly reliant on them. Alternatively, the signal could remain constant but the bystander cells could change the

mechanism or level at which they receive it. This could result because of the increase in confluency of the bystanders at late time points, causing them to transmit more of their signals via gap junctions, and/or less of them via the membrane.

Importantly, several studies in multiple cell types including HUVECs and fibroblasts have demonstrated that gap junctions decrease in replicatively senescent cells (Naus, 2002, Wilson et al., 2000, Kelley et al., 1979). Although some studies suggested it might not have occurred specifically as a result of growth arrest (Xie et al., 1992), the same downregulation in stress induced senescent cells exposed to cisplatin suggested that it was a result of senescence (Zhao et al., 2004). Immunofluorescent staining revealed that senescent cells expressed less connexin43, the main component of gap junctions (Zhao et al., 2004), and more slowly received label from neighbours (Kelley et al., 1979). Another study found that knocking down connexin43 increased the number of cells that underwent senescence in response to high glucose, whilst overexpression reduced it (Zhang et al., 2006).

Despite these observations, gap junctions play a clear role in the communication of cellular damage. It is long established that gap junctions play a crucial role in cell communication after damage induced by irradiation (Autsavapromporn et al., 2011). Interestingly, one study demonstrated that α- and γ-irradiation, as well as oxidative stress, were accompanied by increased connexin43 expression, stabilisation, and activation in multiple cell types (Azzam et al., 2003). Contrarily, other studies have demonstrated downregulation of gap junctional communication in response to ionising radiation and oxidative stress. Additionally, oncogenic activation and transformation has been shown to inhibit gap junctional communication (Azarnia et al., 1988, Bignami et al., 1988).

The effects of gap junctional communication in response to irradiation are dependent on both cell type and stimulus. Notably, the confluency of cells can alleviate the damage in response to low-LET radiation such as X rays or γ-rays (Little, 1969), and increase cell killing in response to α-particles (Azzam et al., 2000). Importantly, whilst gap junction inhibition did not affect the survival of γ-

irradiated cells, it did reduce the decrease in survival in response to α-particles (Autsavapromporn et al., 2011).

The evidence suggests that, in general, when a cell is stressed it downregulates gap junctional communication. This is most likely to prevent the spread of whatever signalling molecules are inducing the stress, as overexpression of connexin43 increases bystander mediated cell killing (Estin et al., 1999). Importantly, further downregulation of gap junction mediated transfer, in cells which have already tried to suppress their activity, using octanol or dominant negative connexin43 still further reduces the bystander effect of irradiated cells (Zhou et al., 2001, Azzam et al., 1998). Likely, the same principle applies to senescent cells. These data intriguingly suggest that the bystander effect could have no function, but instead result from a cell's inability to properly suppress gap junction mediated transfer.

It should also be considered that gap junctions in the bystander cells are still present and active in high numbers, allowing easy transmission between these cells. Perhaps, gap junctions do not play such a large role in signalling between senescent and bystander, but between the bystanders and their neighbours. Notably, another study using oncogene induced senescent cells demonstrated that bystander cells do also transmit a signal to their neighbours (Acosta et al., 2013).

4.7.5 Focus Size and Lifespan in the Bystander Effect

In section 1.3.1 it was discussed how it is not only the number of foci that is important in deciding cell fate, but also their size and longevity. Here, the separation of foci into two groups based on their size, demonstrated firstly that large foci are longer lived than the small foci (Figure 4.12), and secondly that formation of large foci but not small foci was significantly increased in bystander cells (Figure 4.11), although there was a tendency for the increased formation of small foci. Therefore, they may play a small role in the bystander effect, but are likely in the majority to represent easily repaired lesions which are unaffected by bystander signalling. Notably, these foci persisted for at least 20

minutes and were captured in at least two subsequent time-lapse images so are unlikely to represent high background fluorescence.

It has been previously demonstrated that persistent foci, which are associated with cell senescence, are enlarged (Rodier et al., 2011). However, although the large foci found here are significantly longer lived on average than the small foci, this does not necessarily make them persistent/telomeric foci. Importantly, the average focus lifespan remained unchanged between control and bystander cells, when examining total foci or big and small separately (Figure 4.3, 4.13), which could indicate that the majority of these cells are not being induced to senesce by bystander signalling.

Another possibility is that, whilst the number of long lived foci was increasing in bystander cells, the number of shorter lived foci was also increasing. Supporting this idea, there is a tendency of the small foci to increase in the bystander cells, which are all very short lived. Additionally, the significant increase in large focus formation at 2-4 days without increasing the number of foci per cell, suggests that many of these foci are short lived, thus bringing down the average focus lifespan.

Therefore, it was examined whether bystander cells also have increased numbers of the longest lived foci. Using the literature it was observed that most foci in healthy proliferating cells are cleared within five hours of their formation (Passos et al., 2010), and the preliminary data collected here indicate that bystander cells have increased numbers of foci that persist for longer than this time. Thus, it appears that bystander cells are struggling to effectively repair damage induced by senescent cells, which is consistent with the induction of senescence in a fraction of them.

4.7.6 Senescence Induced Senescence

Here we provide the first evidence that senescent cells induce senescence in bystander cells (Figure 4.15). After fifteen days of co-culture with senescent cells, there was a significant increase in SA-β-galactosidase expression at pH6, which has been used extensively in the literature as a marker of cellular

senescence (Dimri et al., 1995). The fraction of SA-β-gal positive cells was significantly increased in the bystander population. It should be noted firstly, that these results cannot completely rule out that senescent cells induced reversible growth arrest in the bystander cells, and as a result the non-senescent cells in the population were not as proliferative, thus allowing the senescent cells to occupy a higher fraction of them at the point of measurement. This issue could be addressed by counting the number of SA-β-gal positive cells within a certain area, or by waiting until all reversibly arrested cells are likely to have repaired their damage and re-entered cell cycle before splitting them. In this respect, the six day blasticidin treatment to kill the senescent cells, and the following four days after replating should have been sufficient for most if not all of the quiescent cells to do this. Secondly, although it is highly likely that this senescence induction results from the DNA damage foci observed in the bystander cells, this is just a correlation, and they may be independent processes.

A second observation is that the number of senescent cells has a tendency to increase between six and fifteen days in the control group. This could simply result from cells autonomously reaching the end of their replicative lifespan, or alternatively from a paracrine induction of senescence from the small number of senescent cells that are present in all cultures. Notably, even at six days the fraction of SA-β-gal positive cells is about 0.27 in the control group. At early PDs WI-38 fibroblasts have almost no positive staining. This increases exponentially until all cells are replicatively senescent. By mid-late PDs around half the WI-38s have positive staining (Dimri et al., 1995). At PD X+28, when the reporter cells were used in the co-culture, they had completed approximately 70% of their replicative lifespan. Compared to the WI-38s 0.27 is not an unexpectedly high level of positive cells this far through their replicative lifespan. Contrarily, in MRC5s some data suggests that positive SA-β-gal staining should not reach above 10% until around PD 40 (Passos et al., 2007). In a comparison to this study, the SA-β-gal staining showed here is quite high, and may reflect suboptimal conditions during the co-culture, freezing or thawing processes, or the long time spent at confluence. What remains clear is that the addition of senescent cells increases the level of positive staining.

Notably, SA-β gal positive cells are not necessarily senescent. The increased enzymatic activity can be induced by other stimuli such as confluency or serum starvation (Dimri et al., 1995). Here cells were kept at confluency; however, these effects dissipate after two days and these cells had been non-confluent for 4-10 days. Additionally, no group has yet observed naturally occurring senescent cells that do not express the enzyme. Because there will always be exceptions, it is generally observed that a single infallible marker of senescence does not exist, and that to genuinely prove senescence that several markers must be used.

This thesis has shown both the expression of SA-β-gal and the presence of large long lived foci that are indicative of cellular senescence. In the paper shown in the appendix (Nelson et al., 2012), we also showed a decrease in the marker Ki67, and co-localisation of PML and γ-H2AX, in bystander cells as additional markers of senescence. Further work could include examining p16 and p21 levels in bystander cells; however, the combination of markers used here is good evidence that bystander cells are being induced to senesce by senescent cells.

Another study, showed that oncogene induced senescent cells also induced senescence in bystander cells (Acosta et al., 2013). Importantly, as discussed previously, this phenomenon was considerably more potent than the bystander effect observed here.

In this study the transfer of medium from senescent cells was sufficient to induce senescence in bystander cells, whereas here a significant increase in focus formation was not observed. However, medium from the secondarily senescent cells did not induce senescence in a tertiary group of bystanders, but it did slow the growth of these cells. Plausibly, this outcome results from the induction of DNA damage in this tertiary group, which causes the cell to become quiescent whilst the damage is repaired.

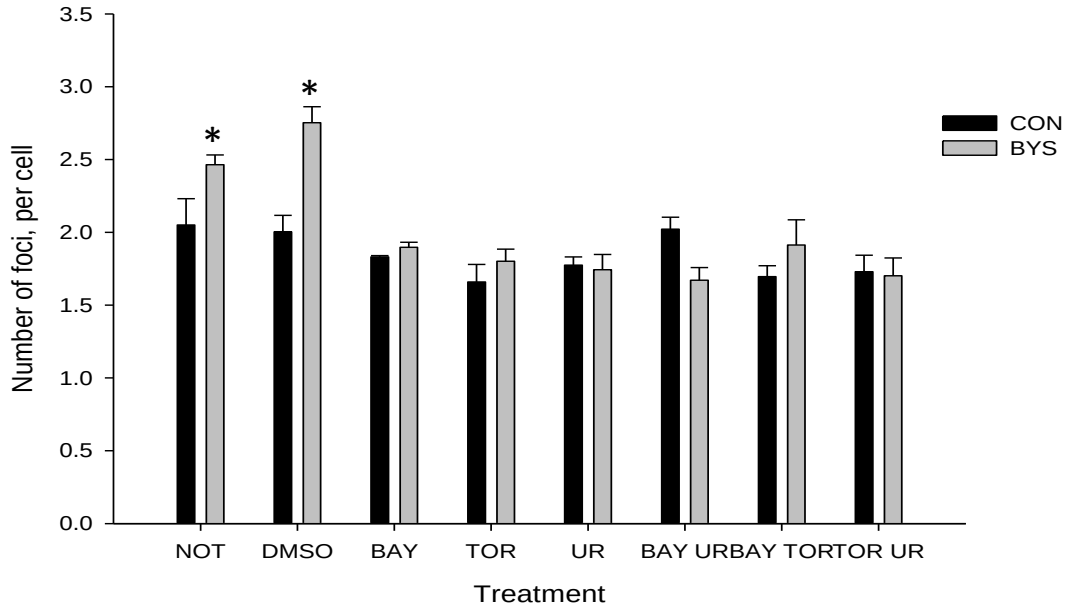
Although our system was not sensitive enough to observe the increased damage from senescent cell medium, it is at least possible that the bystander effect that we observe from the replicatively senescent cells, is the same as the bystander effect observed by the secondarily senescent cells in the other study. Both cell types are likely to senesce from the induction of DNA damage without

the aberrant activation of an oncogene, and therefore the evidence suggests that, unless they have some other difference in the level of activation of p53, that they will have a similar SASP (Coppe et al., 2008). Interesting further work would be to co-culture these secondarily senescent cells with more bystanders under the conditions used in this study to examine whether damage or senescence is induced.

5 The Pathways Underlying the Bystander Effect

Initially, several candidate pathways were identified using the literature which could potentially explain how senescent cells induce a DNA damage response in bystander cells. As described in the introduction, the hub molecules of these pathways are p38, NF-κB and mTOR. Therefore, to test the involvement of these molecules specific inhibitors were added to the co-culture. For mTOR the inhibitor torin-1 was used at 10nM, for NF-κB the inhibitor Bay11-7082 was used at 1μM, and for p38 the inhibitor UR-13756 was used at 1μM. All inhibitors were replenished every two days with a change of medium, and co-culture was continued for 21 days. As indicated in figure 5.1, all three inhibitors abrogated the bystander effect. Additionally, the presence of two inhibitors in the same co-culture had no combinatorial effect.

A|



B|

Figure 5.1| Inhibition of TOR signalling through torin-1 (TOR), NF-κB signalling through Bay11-7082 (BAY), and p38 signalling through UR-13756 (UR), or combinations of the same inhibitors (BAY TOR, BAY UR and TOR UR) all abrogate the increase in foci found in bystander cells. (A) Images of cells in presence of inhibitors. (B) Quantification of data. The use of dual inhibitors has no additional effect. Data are mean ± SE (n=3-5), with approximately 100 cells per group. As determined by ANOVA with Dunn's post hoc test, only the bystander cells in the presence of DMSO or PBS (NOT) were significantly different to control cells in the presence of DMSO. All inhibitors are dissolved in DMSO and compared to DMSO alone (DMSO), which is not significantly different to the absence of DMSO (NOT).

All three pathways are necessary for the bystander effect, making each one dependent on the presence of the other two, in order to induce the DDR. One possibility is that they all regulate the production of the same damaging agent or agents. As discussed in section 1.3.7, all three pathways stimulate the production of reactive oxygen species.

5.1 Reactive Oxygen Species Mediate the Bystander Effect

To test the involvement of reactive oxygen species in the bystander effect, two antioxidant enzymes, superoxide dismutase and catalase were added to the co-culture. Superoxide dismutase catalyses the conversion of the superoxide radical to hydrogen peroxide, and catalase converts hydrogen peroxide to water (Figure 5.2).

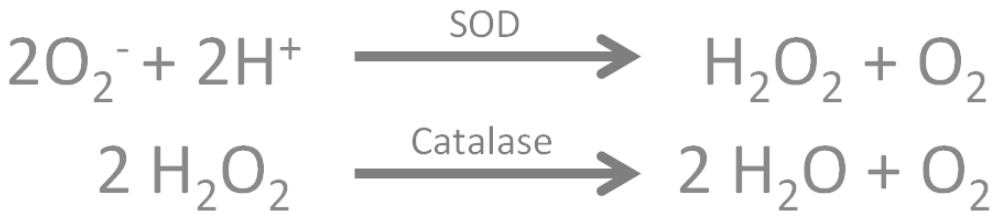
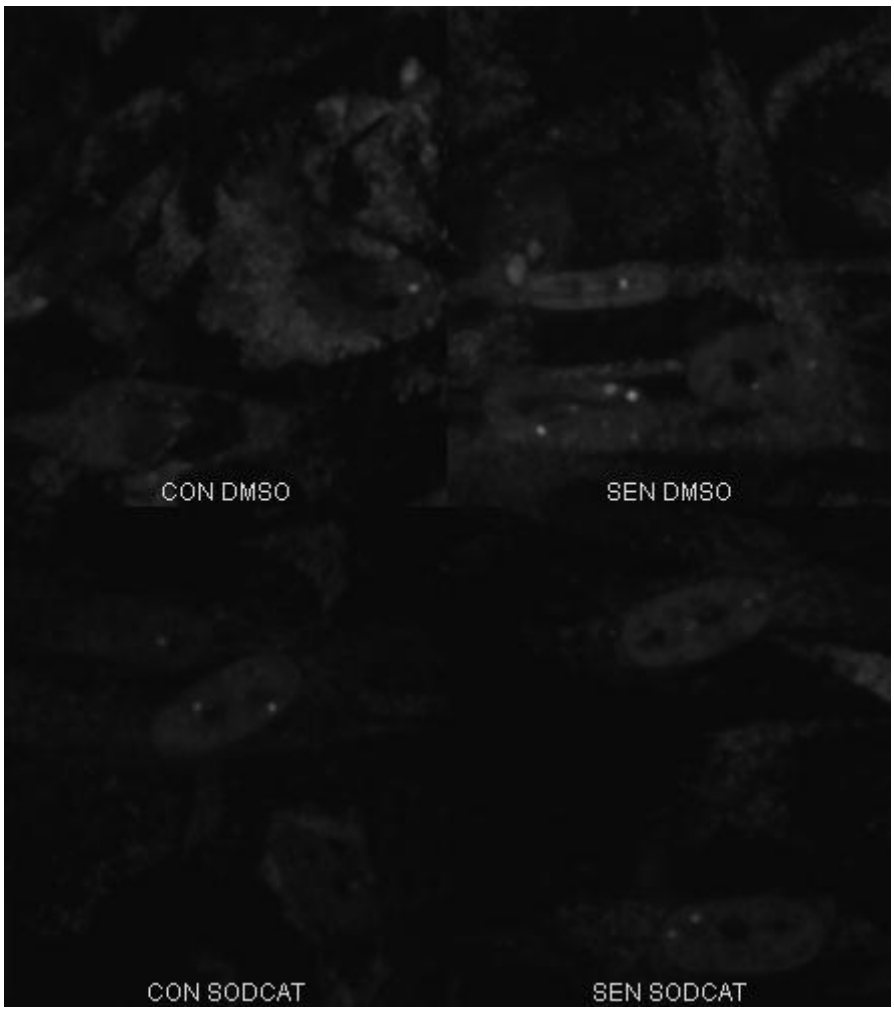
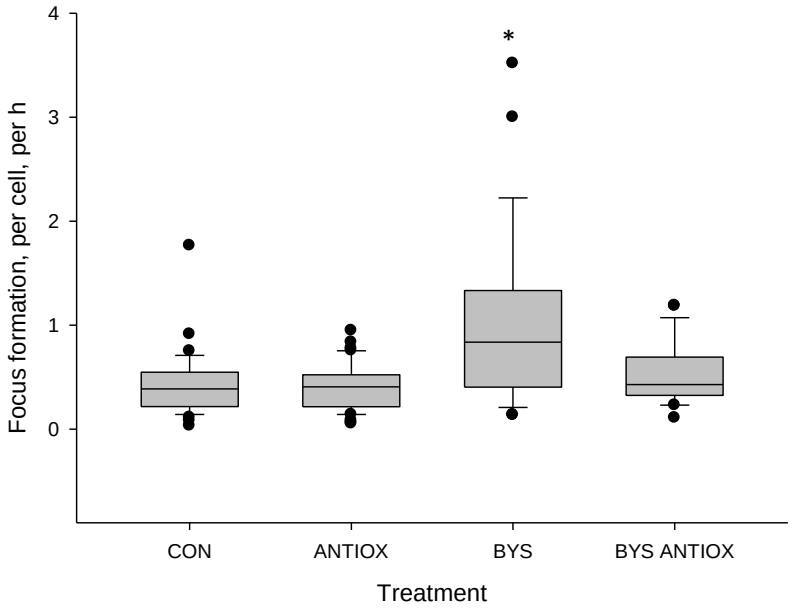


Figure 5.2| Reactions catalysed by antioxidant enzymes.

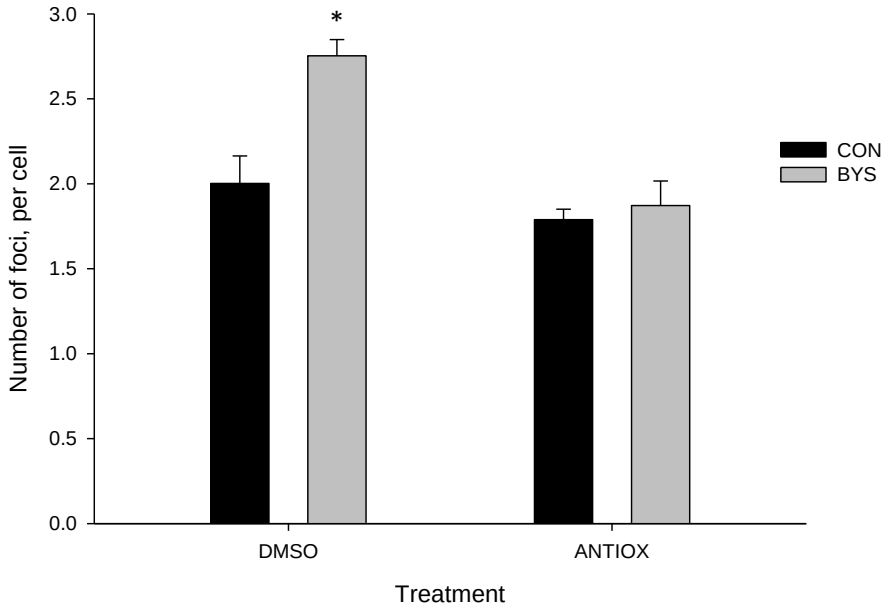
The addition of antioxidant enzymes completely abrogated the increase in focus formation between two and four days (Figure 5.3B), and foci per cell at 21 days (Figure 5.3C). Images of the nuclei under the different treatments are shown in figure 5.3A.


CON DMSO
SEN DMSO
CON SODCAT
SEN SODCAT

A|


Focus formation, per cell, per h
4
3
2
1
0
*
CON
ANTIOX
BYS
BYS ANTIOX
Treatment

B|



C|

Figure 5.3| Antioxidant enzymes abrogate the bystander effect. (A) Focus formation during 2-4 days of co-culture in bystander cells in the presence of superoxide dismutase and catalase antioxidant enzymes (BYS ANTIOX) is completely abrogated. Control cells in the presence of these enzymes (ANTIOX) were not significantly affected. (B) The number of foci in cells in co-culture for 21 days was reduced to control levels by the presence of antioxidants. In (A) statistical analysis is determined by Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, P<0.05). Data are n=1-2, with 20-25 cells per group. In (B) data are mean ± SE (n=3-5), with approximately 100 cells per group. As determined by ANOVA with Dunn's post hoc test, only bystander cells in the presence of DMSO were significantly different from controls in the presence of the same.

In the previous chapter it was found that the difference in focus formation was mainly down to the large foci. ROS are considered to mainly be involved in the production of short lived reparable lesions (Passos et al., 2010). Therefore, it was examined whether antioxidants had affected large or small foci more significantly, and it was found that antioxidants had more of an effect on the large foci (Figure 5.4).

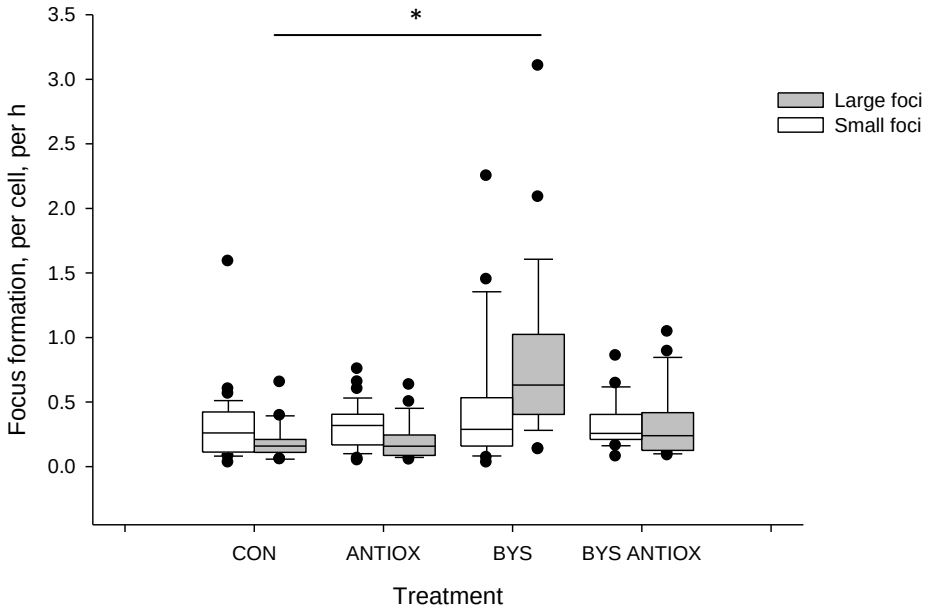
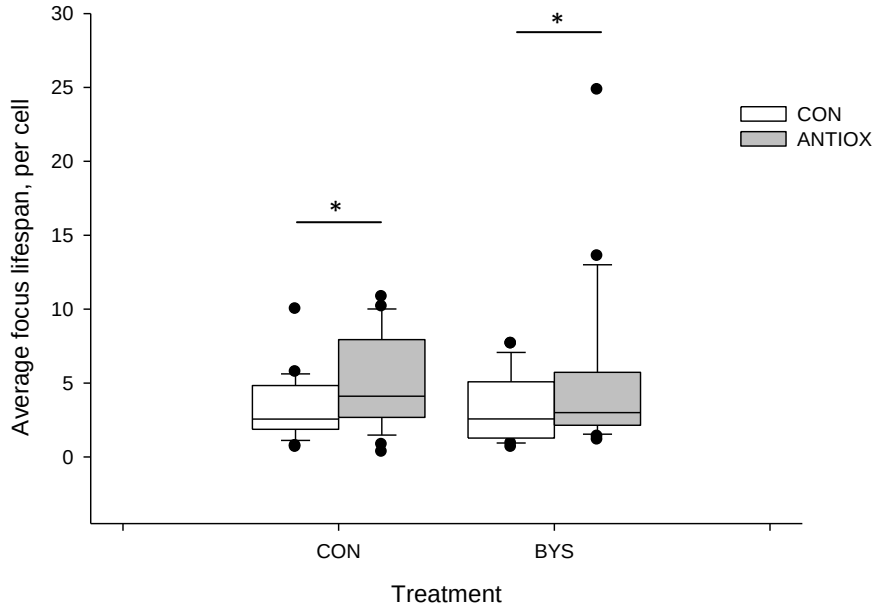


Figure 5.4| Antioxidant enzymes significantly reduce the formation of large foci in bystander cells. Data and statistics are as in figure 4.9.

Notably, the presence of antioxidants significantly increased the average focus lifespan per cell in both control and bystander cells (Figure 5.5A), and again this was primarily down to its effects on large foci (Figure 5.5B).



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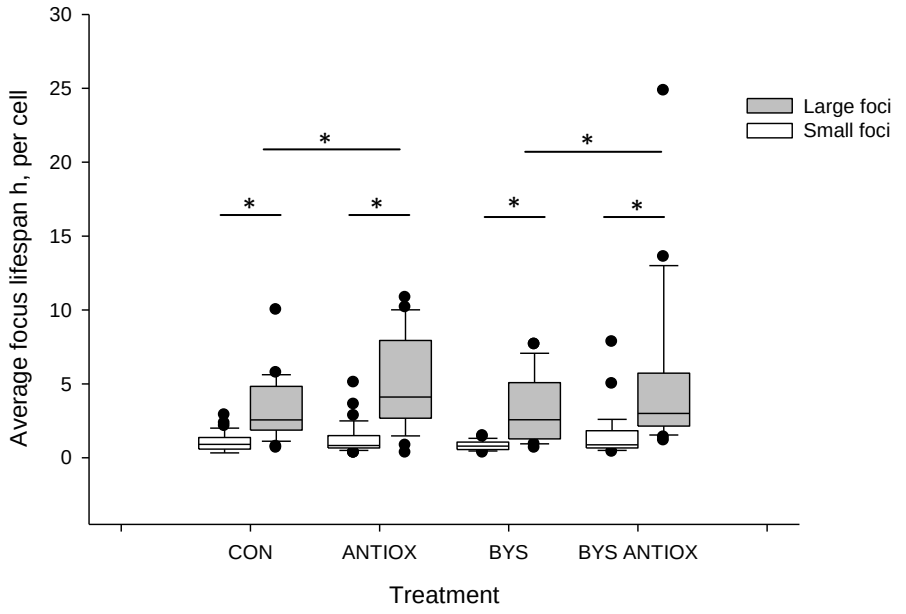


B|

Figure 5.5| Both bystander and control cells have longer lived foci in the presence of antioxidants. (A) Average lifespan of all foci. (B) Average lifespan of large and small foci separately. In (A) statistical analysis is determined by Kruskal-Wallis ANOVA on ranks with Dunn's post hoc test, P<0.05). In (B) statistical analysis is determined by two way ANOVA with Holm-Sidak method for multiple comparisons. Data are n=1-2, with 20-25 cells per group.

Due to their size, the antioxidant enzymes were not expected to be internalised in the cells, and thus would mainly function to remove ROS within the medium. Therefore, it was tested whether the presence of both antioxidant enzymes and an inhibitor against gap junctions had any combinatorial effect. As shown in figure 5.6, this was not the case.

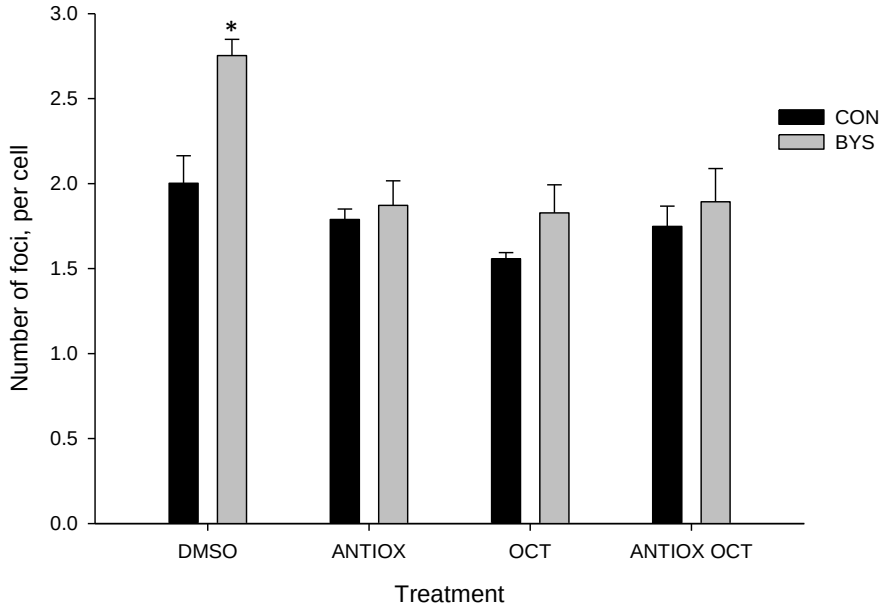


Figure 5.6| The presence of both antioxidant enzymes and octanol (ANTIOX OCT) has no additional effect over the presence of either treatment alone on the number of foci in bystander cells at 21 days. Data are mean ± SE (n=3-5), with approximately 100 cells per group. As determined by ANOVA with Dunn's post hoc test, only bystander cells in the presence of DMSO were significantly different from controls in the presence of the same.

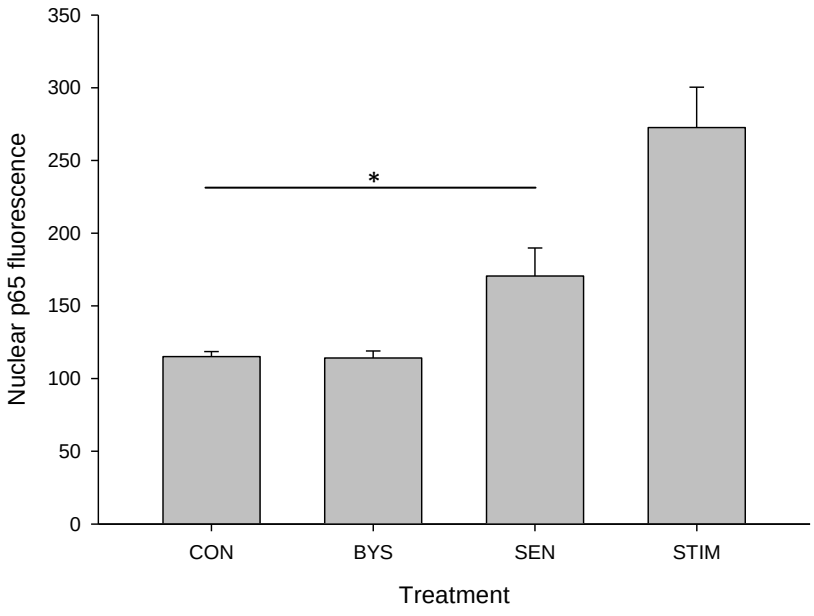
5.2 NF-κB Regulates the Bystander Effect

5.2.1 Nuclear p65 is Increased in Senescent but not Bystander Cells

The use of inhibitors against mTOR, p38 and NF-κB clearly implicated all three signalling pathways in the increased foci found in bystander cells. Due to time constraints only the NF-κB pathway was selected for further analysis. As the inhibitors affect signalling in both the inducer and reporter cells, although the damage induced in bystander cells is clearly abrogated, the mechanism by which this occurs cannot be determined by this system.

NF-κB could be involved in either the production of the signal in the inducer cells, or the recognition of the signal in the reporter cells, or both. In order to elucidate which of these pathways require NF-κB signalling, the pathway must be inhibited exclusively in either the inducer or reporter cells within the co-culture.

Staining for p65 in senescent, bystander and control cells demonstrated that, consistent with previous studies, nuclear p65 and the nuclear:cytoplasmic ratio is significantly increased in senescent but not bystander cells (Figure 5.7).



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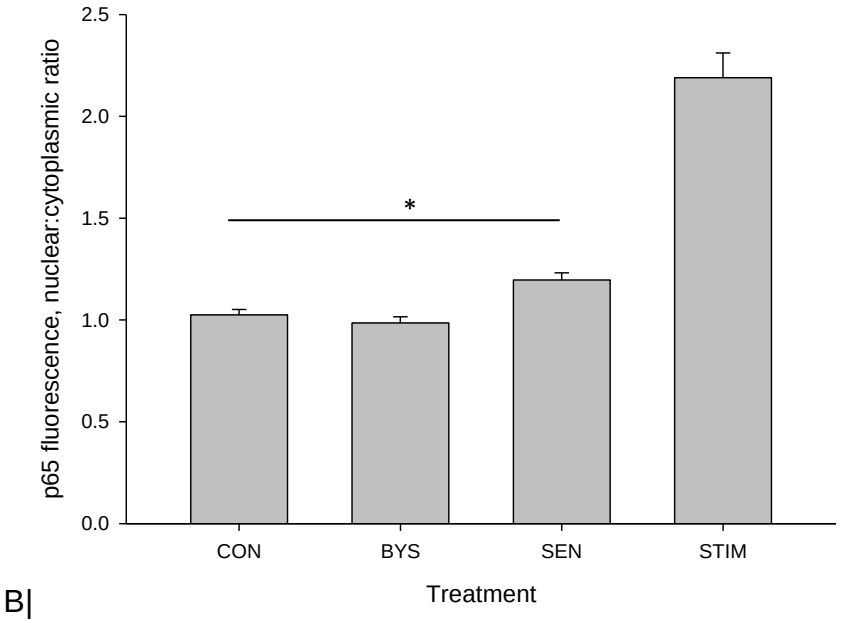


B|

Figure 5.7| Senescent cells (SEN) as well as TNF-α stimulated controls (STIM) have significantly higher levels of nuclear p65 (A), and a higher nuclear:cytoplasmic ratio (B). Data are mean ± SE (n=3), with 50-100 cells per experiment, and statistics were ANOVA with Dunn's post hoc test.

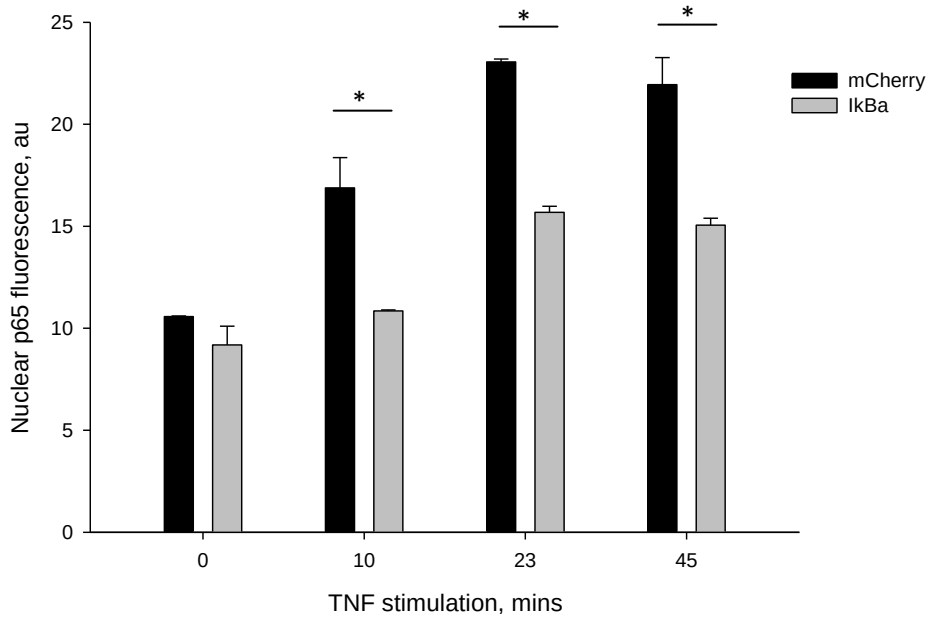
The results suggested that NF-κB signalling via p65 was primarily driven in the inducer cells. To test this, a construct designed by Glyn Nelson was used, as described in the next section.

5.2.2 ΔIκBα Expressing Senescent Cells Do Not Induce a Bystander Effect

An N-terminal deletion in IκBα had been previously created by Glyn Nelson which removed the first 40 amino acids, including the two serine residues (32 and 36) required for the induction of its degradation, thereby causing it to be constitutively active (ΔIκBα) in its repression of NF-κB. Recombined into a destination vector, it was transuced into senescent and replicating MRC5 fibroblasts, as described in section 3.5.1.

To test whether the ΔIκBα construct effectively repressed NF-κB signalling in transduced cells, young cells were stimulated with 10ng/ml TNF-α and

compared with control cells (transduced to express mCherry). As shown in figure 5.8, at the three time points tested the ΔIκBα expressing cells had significantly less nuclear p65 and a lower nuclear:cytoplasmic ratio than mCherry expressing cells.



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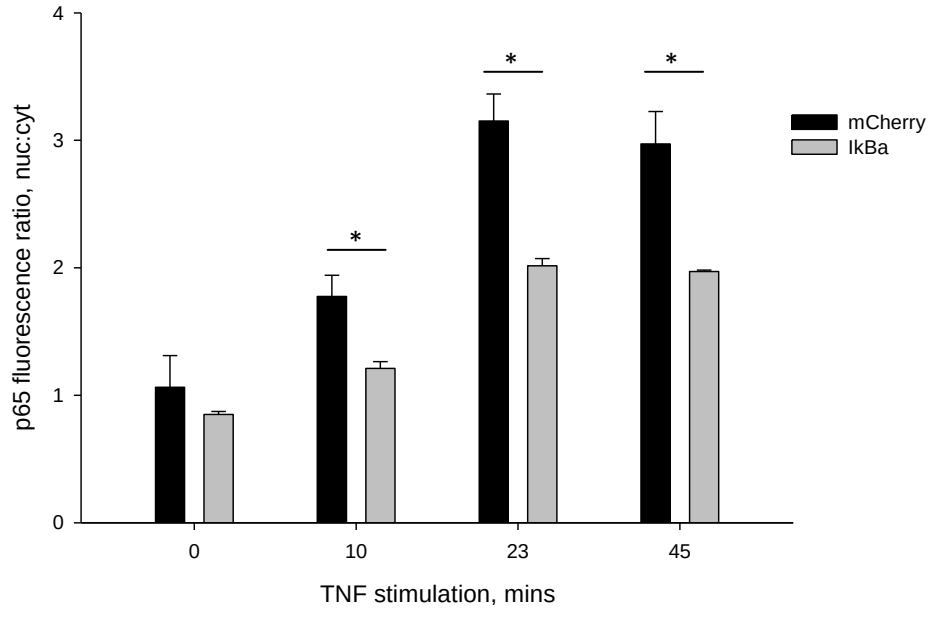


B|

Figure 5.8| Cells expressing ΔIκBα (IkBa) have reduced levels of nuclear p65 (A) and a reduced nuclear to cytoplasmic ratio (B) compared to

control cells expressing mCherry (mCherry), upon stimulation with TNFα. As determined by ANOVA with Dunn's post hoc test, at all time points IκBα cells have significantly lower nuclear fluorescence (A) and fluorescence ratio (B). Data are mean ± SE (n=3), with approximately 100 cells per experiment.

In most normal cell types TNF-α is only weakly pro-apoptotic, but this results from the pro-survival effects of NF-κB (Van Antwerp et al., 1996). Consistently, because of the activation of TNF-α during embryonic development, homozygous p65 mutant mice are embryonic lethal due to the widespread apoptosis, which is rescued by the inhibition of TNF-α (Beg and Baltimore, 1996, Beg et al., 1995, Doi et al., 1999). Therefore, in cells expressing ΔIκBα, TNF-α should also induce apoptosis, which was evidenced by membrane blebbing of stimulated ΔIκBα cells (data not shown). Importantly, there was no evidence of apoptosis or membrane blebbing in the unstimulated ΔIκBα expressing cells, nor in the senescent cells expressing the construct.

Therefore, the senescent ΔIκBα expressing cells were co-cultured with reporter cells and compared to senescent cells expressing mCherry. The results of a 14 day co-culture demonstrated that ΔIκBα expressing cells did not induce a bystander effect in reporter cells compared to the mCherry expressing cells (Figure 5.9).

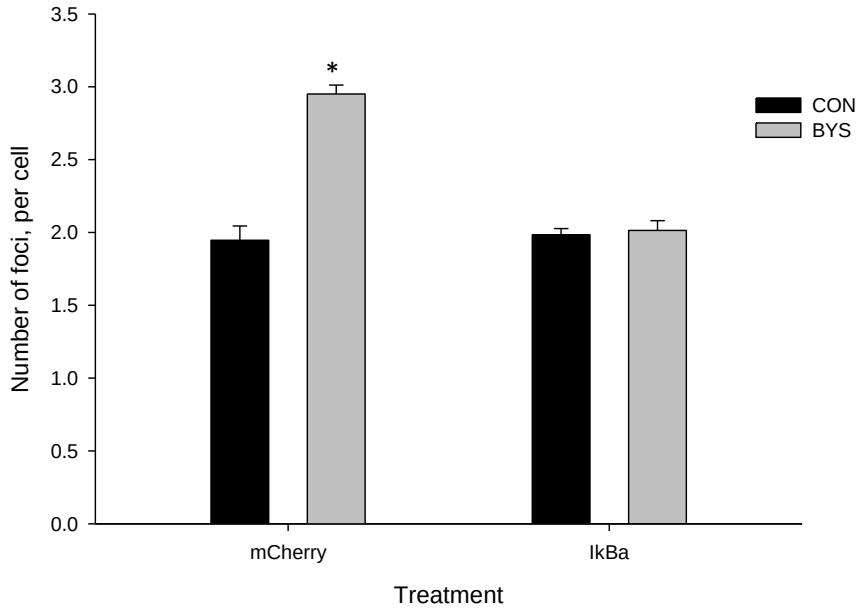


Figure 5.9| Senescent cells expressing ΔIκBα do not induce a bystander effect, whilst mCherry expressing senescent cells showed a normal bystander effect compared to other untransduced senescent cells (Figure 4.4). As determined by ANOVA with Dunn's post hoc test, only bystander cells cultured with senescent cells expressing mCherry were significantly different from controls.

5.3 Stimulation of Canonical NF-κB Signalling Enhances the Bystander Effect

To understand the effects of over-activating NF-κB on the bystander effect, senescent and replicating control cells were stimulated with TNF-α for one hour before washing the cells and replacing with normal medium. Reporter cells were then added to pre-stimulated cells and co-cultured for up to 20 days (Figure 5.10).

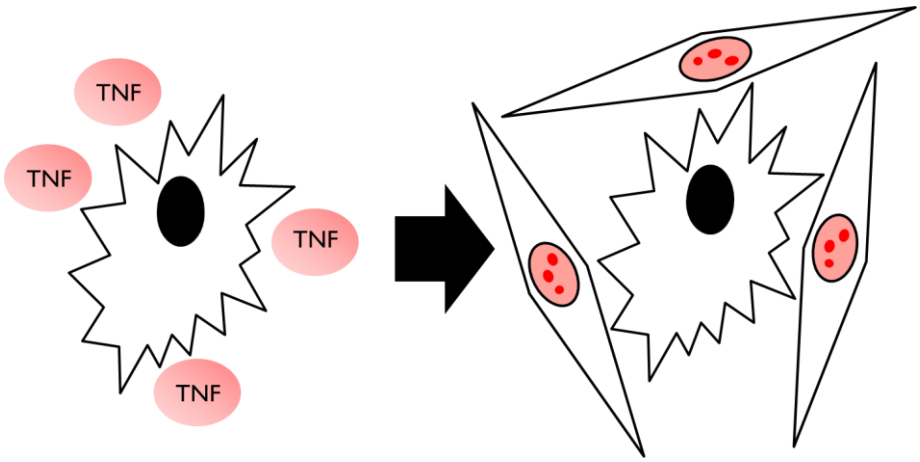
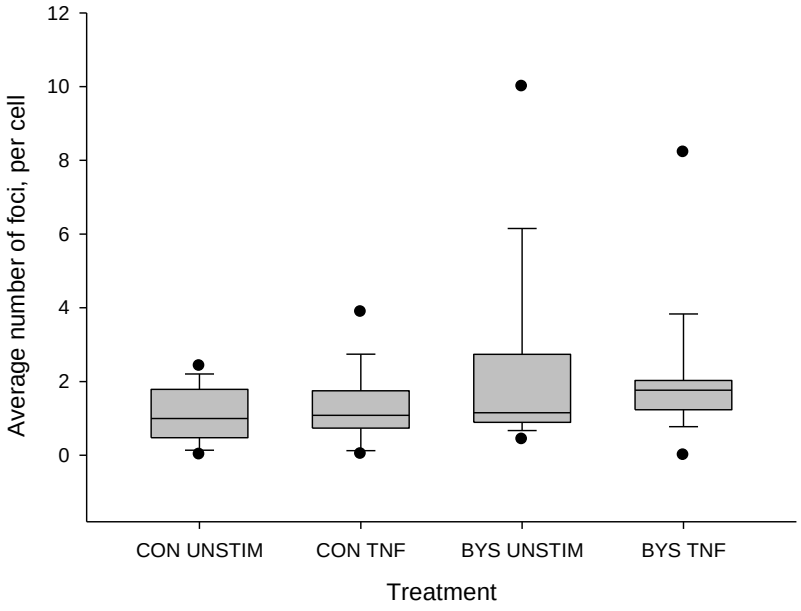


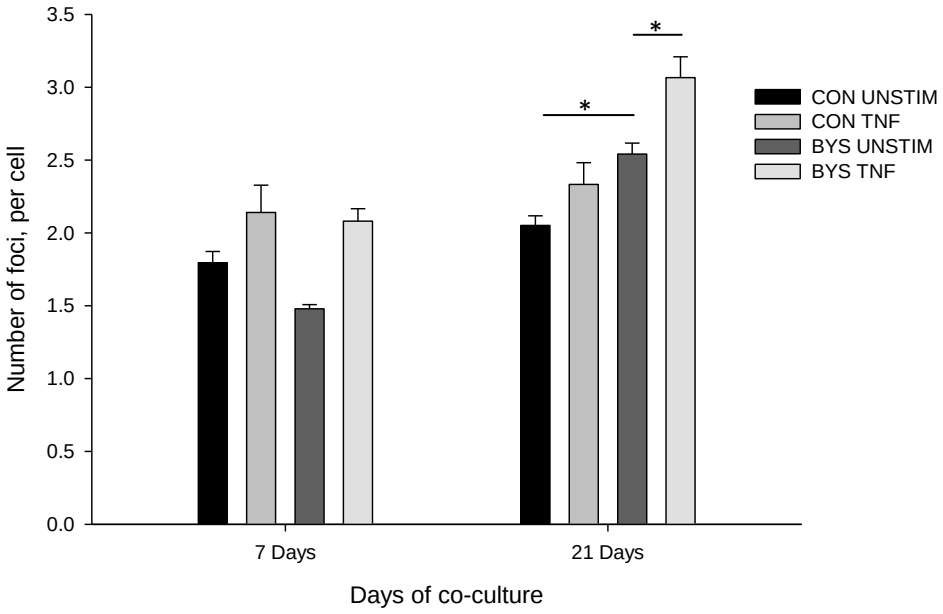
Figure 5.10| Senescent cells were pre-stimulated with TNF-α, then washed before addition of the reporter cells

For the initial time point, cells were imaged every 15 minutes for 12 hours between one and two days after inducer cell stimulation and subsequent addition of reporter cells. Then single measurements were made at seven and 21 days. No significant effects of pre-stimulation or the presence of senescent cells were observed between 1-2 days, or at seven days (Figure 5.11A and B), though there was a clear tendency between 1-2 days for senescent cells to increase the average number of foci per cell, (Figure 5.11A), consistent with figure 4.3.

Most interestingly, at 21 days there was a clear and significant increase in the number of foci in bystander cells cultured with senescent cells that had been pre-stimulated with TNF-α, over the normal bystander effect observed from unstimulated senescent cells (Figure 5.11B). At no time point was there any significant effect of pre-stimulating young replicating cells, though there was perhaps a tendency at seven days (Figure 5.11A and B).



A|



B|

Figure 5.11| Pre-stimulation of senescent cells (BYS TNF) increases the number of foci induced in bystander cells during co-culture compared to unstimulated (BYS UNSTIM) cells. (A) The average number of foci per cell over a 12 hour period within 1-2 days of co-culture. There is no difference between reporters co-cultured with senescent stimulated cells and unstimulated senescent controls, or young stimulated cells (CON TNF) and unstimulated young controls (CON UNSTIM). Data are n=1 with

approximately 20 cells per treatment. Statistics are determined by ANOVA with Dunn's post hoc test. (B) Number of foci in control and bystander cells at the different time points of co-culture with stimulated and unstimulated cells. At seven days no treatment is significant, whilst at 21 days there is a significant bystander effect of senescent cells, and a significant effect of stimulation on senescent cells, as determined by ANOVA with Dunn's post hoc test. Data are n=3-4 with between 40-200 cells counted for each treatment, with error bars showing standard error.

To test whether TNF-α stimulation affects the focus frequencies in directly stimulated cells, both senescent and young replicating cells were stimulated with 10ng/ml TNF-α for one hour, washed three times and then cultured for the same periods of time as the co-cultures. Interestingly, a similar trend is followed by senescent and bystander cells, with senescent cells having more 53BP1 foci than controls. There is also a tendency for stimulation to increase the number of foci in both senescent and control cells at one day, and a clearer tendency to increase the number of foci in senescent cells at 21 days. However, it must be noted that none of these changes are significant (Figure 5.12).

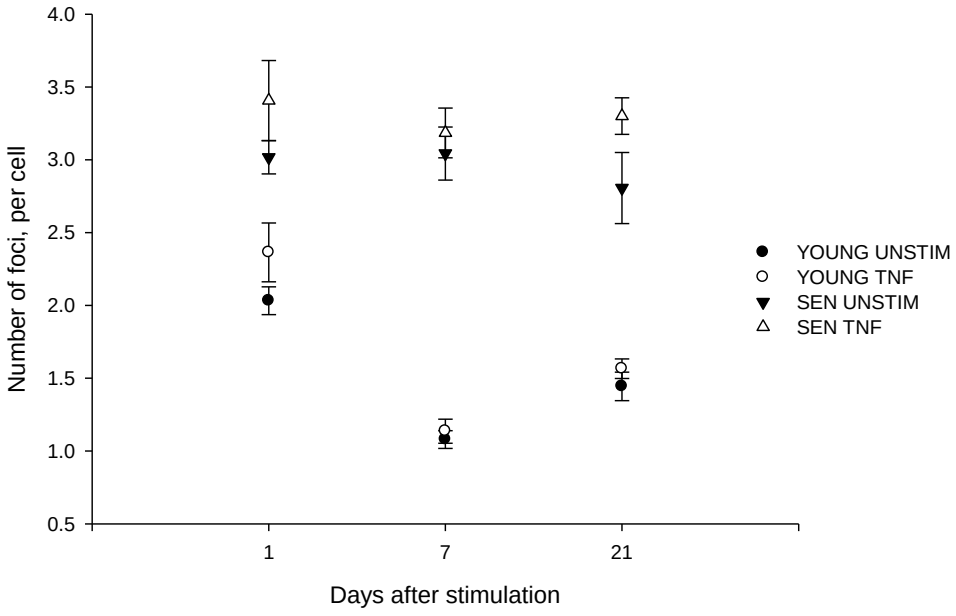


Figure 5.12| Direct TNF-α stimulation of cells does not increase DNA damage foci in senescent cells (SEN TNF) up to 21 days after stimulation,

over unstimulated senescent cells (SEN UNSTIM). Neither was there a significant increase in number of foci in directly stimulated young cells (YOUNG TNF), over untimulated controls (YOUNG UNSTIM). Data are n=3 with approximately 100 young cells and 20-40 senescent cells per experiment. Statistics are determined by ANOVA with Dunn's post hoc test. Data are mean ± SE (n=3-5), with approximately 100 cells per group. Notably, using a two way ANOVA with Holm-Sidak method for multiple comparisons to compare the results across time points, day 1 is significantly different to days 7 and 21.

Taking into account that at 21 days the incerased number of foci in pre-stimulated senescent cells is bordering on significance (p=0.048), it is difficult to conclude whether direct stimulation induces DNA damage in senescent cells without further repeats. As the number of senescent cells is generally lower than the control cells, it might simply be a question of power.

To examine whether the increased bystander effect resulting from pre-stimulation is also dependent on reactive oxygen species, similar to previous experiments, SOD and catalase were added to the medium during the co-culture. Thus, antioxidants were not added during the initial stimulation, but were present for the entirety of the co-culture as before (Figure 5.13).

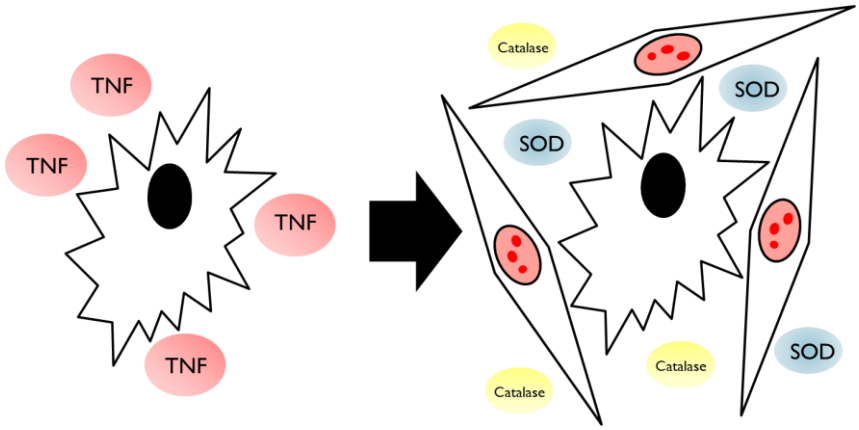


Figure 5.13| After stimulation with TNF-α and washing, both reporter cells and antioxidants were added together.

As shown in figure 5.14, the bystander effect of pre-stimulated senescent cells is completely abrogated by the presence of antioxidant enzymes, suggesting that the additional increase in damage is also dependent on ROS.

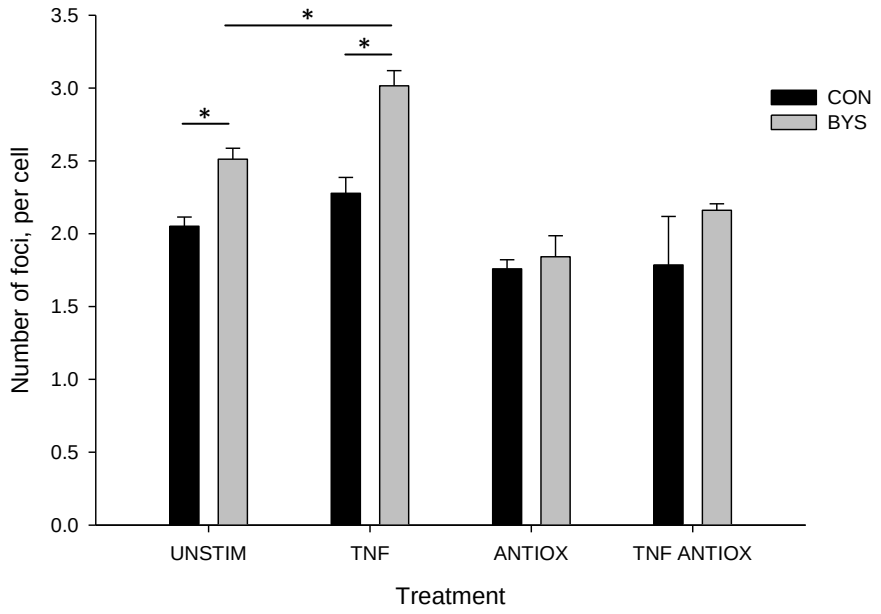


Figure 5.14| When antioxidant enzymes are present in the co-culture, they abrogate the additional bystander effect of senescent cells pre-stimulated with TNF-α (TNF ANTIOX). There was significant bystander effect of senescent cells, and a significant effect of stimulation on senescent cells, but this was abrogated in both cases by the presence of antioxidants, as determined by ANOVA with Dunn's post hoc test. Data are n=3-4 with between 40-200 cells counted for each treatment, with error bars showing standard error.

5.4 IL-6 Stimulation on the Bystander Effect

The effects of TNF-α stimulation were surprisingly potent. However, TNF-α is not one of the most prominent secreted factors of the SASP. Therefore, positive feedback by this system, whereby senescent cells stimulate themselves in an

autocrine fashion with TNF-α to become increasingly damaging is unlikely to be dramatic.

Therefore it was tested whether stimulation with IL-6, one of the most prominent members of the SASP, and a known pro-inflammatory cytokine, also had the same effect. Just as with TNF-α, cells were stimulated with 15ng/ml IL-6 for 1 hour. However, unlike the DDR stimulating effects of TNF-α, IL-6 had the opposite effect. Senescent cells pre-stimulated with IL-6 had a tendency to reduce the bystander effect, although the effect was not significant (Figure 5.15).

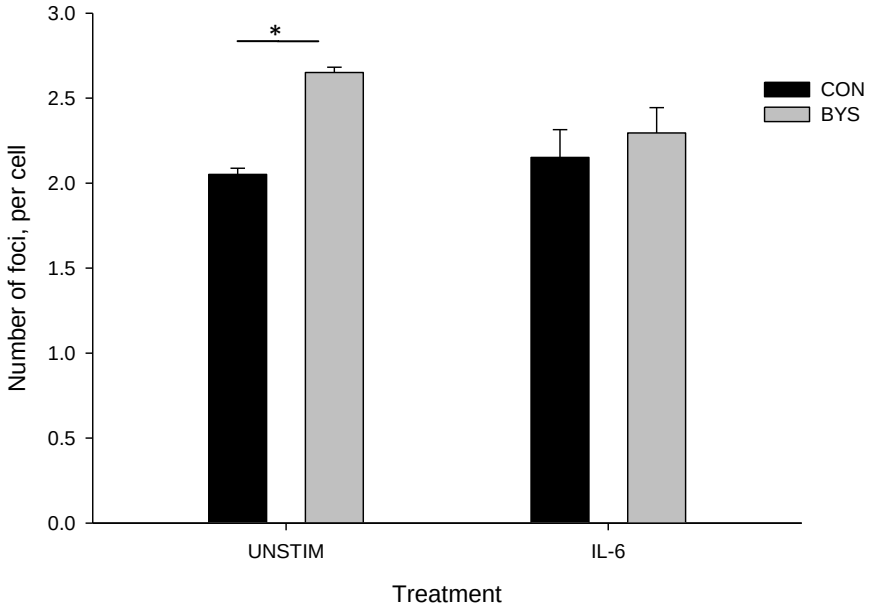


Figure 5.15| Pre-stimulation of cells with IL-6 (IL6) does not significantly affect the bystander effect of senescent cells or controls at 21 days of co-culture, but does prevent significant increase between the two, as determined by ANOVA with Dunn's post hoc test. Data are n=3-4 with between 40-200 cells counted for each treatment, with error bars showing standard error.

To ascertain which of these signals was more predominant in the bystander response, IL-6 and TNF-α were added as co-stimulants. When added together

at the same doses as before, the bystander effect was reduced by IL-6, but not significantly (Figure 5.16), suggesting that TNF-α was mostly epistatic to IL-6.

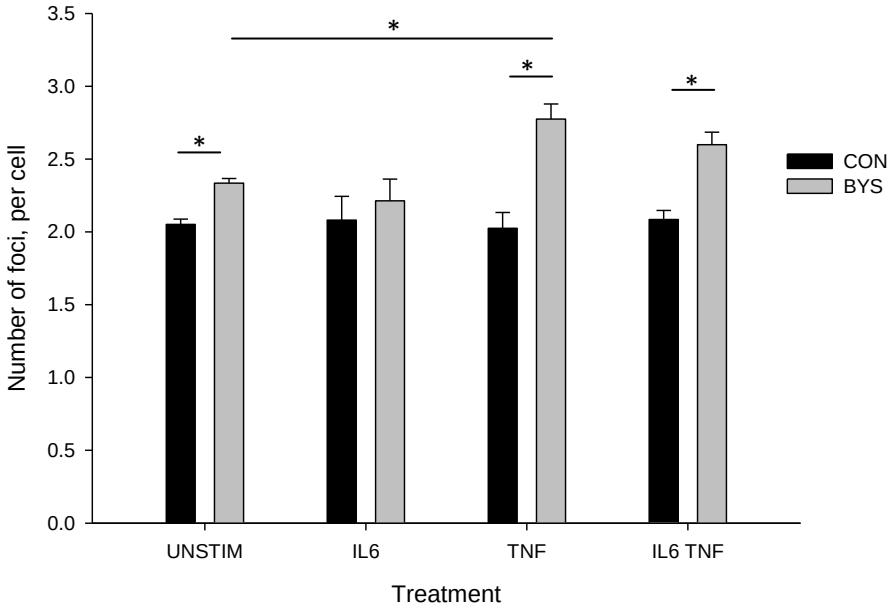


Figure 5.16| Co-stimulation of cells with IL-6 and TNF-α (IL6 TNF) before a 21 day co-culture induced a bystander effect that was not significantly different to that of unstimulated or TNF-α stimulated senescent cells, as determined by ANOVA with Dunn's post hoc test. Data are n=3-4 with between 40-200 cells counted for each treatment, with error bars showing standard error.

5.5 Discussion

5.5.1 NF-κB, p38 and mTOR in the Bystander Effect

As shown in figure 5.1, the inhibition of mTOR, NF-κB and p38 signalling using specific inhibitors all abrogated the bystander effect. 10nM Torin 1 inhibits both mTORC1 and mTORC2 signalling, but is otherwise thought to be very specific. UR-13756 was used at 1μM to inhibit p38 MAPK. Unlike SB203580, UR-13756 is highly specific to p38 over c-Jun kinases, at the concentration used (Bagley

et al., 2010). However, as this inhibitor has not been widely tested it is possible that there are still some off-target effects yet to be discovered. Bay 11-7082 is also thought to be highly specific at concentrations five times higher than the 1μM used in this experiment (Mori et al., 2002), although it can become non-specific at higher doses (Koh et al., 2010). Notably, Bay 11-7082 is an IKK inhibitor poorly specific for IKKα or IKKβ, which both have targets other than NF-κB, and another study did identify toxic effects of the inhibitor independent of the prevention of IκBα phosphorylation (Rauert-Wunderlich et al., 2013).

Another important limitation of this research is that these inhibitors were not tested to ensure that they induced the desired effect on their target molecule within MRC5 fibroblasts. Whilst Glyn Nelson has shown Torin1 reduces mTOR signalling in MRC5s (Dalle Pezze, Unpublished), and Bay 11-7082 has been used previously in this cell line by our lab (Nelson, Unpublished), UR-13756 has not been tested in these cell lines. This could be tested using antibodies against p65, phospho-S6 and MAPKAPK-2 or HSP-27 for Bay11-7082, Torin1 and p38 respectively.

Preliminary experiments revealed that 10μM Bay 11-7082 induced high levels of apoptosis in both senescent and young cells after extended culture, as defined by the appearance of floaters and excessive loss of attached cells. This was not the case with the other two inhibitors, nor with Bay 11-7082 at 1μM, indicating that cells were not undergoing cell death in these co-culture experiments. However, it should be noted that level of apoptosis was not quantitated, and low level cell death is potentially still a confounding variable in these experiments, especially when considering the increased stress undergoing bystander cells that may contribute to inducing cell death. Further analysis of cell death in control, senescent and bystander cells using an apoptosis assay caspase-Glo, TUNEL or Annexin V, is required.

An additional factor for consideration when analysing this inhibitor data is that both inducer and bystander cells are affected. Notably, all three inhibitors had a tendency to reduce the number of foci below that of the control cells in the presence of DMSO, although the difference was not significant. This suggests that the inhibitors may be having other less potent effects reducing DNA damage in cells through mechanisms which are not specific to the bystander

effect. However, these did not produce significant effects, whilst the bystander effect was significantly and completely abrogated by the inhibitors, demonstrating that their targets are necessary for the induction of the DDR in bystander cells.

All three pathways have been implicated in cellular senescence, as described in the introduction. Although their role in the bystander effect is still open to interpretation, the results suggest that they are dependent on each other, as each inhibitor completely abrogates the bystander effect, and there are no combinatorial effects. It is possible that lower drug doses might reduce the inhibition of the bystander effect shown by each drug and thus allow better observation of any combinatorial effects; however, the difference in number of foci between bystander and control cells is at best one focus per cell, suggesting that this system is not sensitive enough for observing smaller differences in bystander signalling.

Alternatively, the dependency of the bystander effect on all three signalling pathways could result because the networks are interconnected, regulating the same inducer of damage. As described in the introduction, there are multiple different sources of damage. Firstly, there are various forms of mutagen that directly affect the structure or sequence of the chromatin and DNA. Alternatively, some agents can induce damage indirectly by stimulating rapid error prone synthesis of the DNA, or affecting the activity of a protein involved in accurate DNA synthesis or chromatin maintenance. Therefore, these molecules could induce damage by increasing the level of mutagens or by deregulating the processes that maintain and synthesise the chromatin, making it more susceptible to damage.

The MAPK p38 is most commonly associated with the stress response pathways (Obata et al., 2000); however, it is also necessary for the stimulation of cell proliferation in response to various growth factors such as fibroblast growth factor (FGF), hepatocyte growth factor (HGF) (Maher, 1999, Awasthi and King, 2000), and cytokines such as granulocyte colony stimulated factor (GCSF) or IL-2 and IL-7 (Rausch and Marshall, 1999, Crawley et al., 1997), and can induce DNA synthesis and proliferation in response to GPCRs, through calcium mobilisation and PKC activation (Clerk et al., 1998, Dehez et al., 2001).

Therefore, p38 could induce DNA damage indirectly via cell cycle stimulation similar to RAS.

Equally, mTOR is an inducer of cell growth, and responds to multiple growth factors. Short term glucose stimulation of rat islet cells induces DNA synthesis and cell proliferation (Kwon et al., 2004), indicating that mTOR may induce DNA damage via stimulating synthesis. Interestingly, chronic glucose stimulation induces increased DNA synthesis, but reduces the number of cells in G2/M phase, indicative of S-phase block (Kwon et al., 2006), which is highly consistent with the indirect induction of DNA damage through rapid synthesis. Whether this is also true in human fibroblasts remains to be seen.

Lastly NF-κB is also implicated in cell cycle control. Importantly, p65 overexpression stimulates increased cyclin D1 (Guttridge et al., 1999), and although the presence of a similar ΔIκBα super repressor used in this experiment was found not to affect a host of factors in serum stimulated MEFs, including cyclin E, cyclin D2, cyclin D3, CDK2, p15, p16, p27 and p21 (Guttridge et al., 1999), it did delay and reduce the expression of cyclin D1 (Hinz et al., 1999). As a result, ΔIκBα inhibits pRb phosphorylation, entry into S-phase and DNA synthesis, and reduces cell growth and proliferation (Guttridge et al., 1999). NF-κB also induces transcription of the proto-oncogene c-myc (La Rosa et al., 1994), suggesting that it could also induce DNA damage by stimulating cell cycle.

Therefore, all three proteins could potentially induce DNA damage indirectly via stimulating synthesis. Additionally, all three proteins have another function which may also lead to an apparent increase in bystander damage, via the inhibition of apoptosis in damaged cells. The important role of NF-κB in cell survival has been discussed in the introduction. mTOR is also important in cell survival in response to stress (Hung et al., 2012), and growth factor withdrawal (Edinger and Thompson, 2002), whilst p38 is also important in survival of oxidative stress, and necessary for activation of the p70-S6K downstream of an mTOR related survival pathway (Gutierrez-Uzquiza et al., 2012). This latter observation is also an indication of the interconnectedness of these pathways, which may explain why they are all necessary for the bystander effect, and cannot induce it independently of each other.

Another study examining the bystander effect of oncogene induced senescent cells found that, whilst medium from these senescent cells was sufficient to induce the bystander effect, addition of Bay 11-7082 to the medium after it was removed from the senescent cells was not sufficient to reduce it (Acosta et al., 2013). Consistent with the results shown here, this suggests that NF-κB signalling is not important in bystander cells, although this may reflect differences in cell type or stimulus. Also the study tested for BrdU incorporation rather than number of DNA damage foci, so potentially DNA damage could have been increased in these cells, but its anti-growth effects were offset by the pro-cell cycle effects of NF-κB, discussed previously in section 5.5.1 (Guttridge et al., 1999, Hinz et al., 1999, La Rosa et al., 1994). However, this seems unlikely given the high sensitivity of cell cycle arrest to DNA damage. Important further work will involve examining the bystander effect of oncogene induced senescence in MRC5 fibroblasts, and secondly co-transducing fibroblasts with ΔIκBα and mCherry-53BP1 to examine the importance of NF-κB in bystander cells in our system.

Notably, the p38 inhibitor SB202190 also had very little effect on the bystander effect of oncogene induced senescent cells when added to the medium after being removed from senescent cells (Acosta et al., 2013), suggesting similar mechanism to that of NF-κB. However, in the Appendix data from Glyn Nelson showed a significant increase in phospho-p38 in bystander cells, indicating that it may also play a role in receiving and amplifying the bystander signal (Nelson et al., 2012). The mTORC1 inhibitor Rapamycin dramatically reduced the growth rate of bystander cells, but rapamycin is a potent cell cycle inhibitor (Fingar et al., 2004), and would therefore be expected to slow cell cycle regardless of the bystander effect. Further work, will involve specifically inhibiting NF-κB, p38 and mTOR specifically in the bystander cells (and senescent cells for the latter two) and examining whether this reduces the bystander effect.

5.5.2 Reactive Oxygen Species in the Bystander Effect

Notably although the pro survival/proliferation roles of p38, NF-κB and mTOR may be important in the bystander effect, their pathways have also been implicated in the production of mutagens, specifically reactive oxygen species.

Here, for the first time ROS are shown to be necessary for the senescent cell bystander effect, as the addition of superoxide dismutase and catalase antioxidant enzymes completely abrogates both the increase in focus formation and number of foci per nucleus at the time points measured. Neutralisation of ROS appears to have little effect on the small foci, which is not surprising when considering that the numbers of small foci are not significantly changed in bystander cells compared to controls, and therefore are unlikely to be affected by alterations in bystander signalling.

A previous study demonstrated that ROS were important in maintaining the senescence growth arrest by producing short lived foci which would temporarily activate the DNA damage response until the damage was repaired, only to be replaced by another lesion elsewhere (Passos et al., 2010). This also indicates, as discussed previously, that the large foci are not all representative of telomeric persistent foci, which likely forms a small subgroup of these. Thus, it is also not surprising that antioxidants increased the average focus lifespan of foci, because ROS generate short lived foci, and removing these foci will therefore cause the average lifespan to increase. However, although these data indicate that this effect is occurring, there is also some evidence that antioxidants are actually increasing the lifespan of the longer lived foci. Importantly, there is evidence that cells regulate their DNA damage response and repair machinery according to the level of ROS within a cell independently of the induction of damage (Guo et al., 2010). Thus, although less damage may occur as a result of antioxidants, any unavoided damage is likely to persist for longer.

It should be noted that all inhibitors were dissolved in DMSO which is a mild antioxidant. However, at the concentration used, DMSO did not alter the bystander effect (Figure 5.1). Unlike the antioxidant enzymes used, DMSO can cross cell membranes and has therefore more potential to neutralise ROS inside senescent cells; however, the high concentrations of the antioxidant

enzymes and the low concentration of DMSO used are more than sufficient to explain this discrepancy.

These data provide a strong argument that ROS are necessary for the induction of the DDR in bystander cells. Notably, the results do not necessarily implicate that ROS are necessary to induce senescence in bystander cells, which could result from damage independent processes as described in the introduction, only that they are necessary to activate the DDR. However, there is also substantial evidence that ROS are involved in both the induction and maintenance of senescence (Passos et al., 2010).

What these data describe is that ROS are necessary for senescent cells to induce a DDR in bystander cells. Notably the antioxidant enzymes are too large to be efficiently transported into the cells, and therefore cannot directly neutralise intracellular ROS. Similar to the use of the inhibitors discussed above, the antioxidant enzymes could be affecting the ROS levels in the senescent inducer cells, the bystander cells, both, or neither. It is known that hydrogen peroxide can cross cell membranes (Antunes and Cadenas, 2000). Therefore, it is possible that senescent cells produce it internally and the molecule crosses the membrane where it is detoxified by the antioxidants before it can affect the bystander cells. This short half-life of superoxide will generally prevent its externalisation, when produced internally. Alternatively, NADPH oxidase enzymes in the membranes of senescent cells could be generating superoxide, and therefore hydrogen peroxide, straight into the medium (Chen et al., 2009). Multiple studies have implicated a role for NADPH oxidases in accelerating replicative senescence (Lener et al., 2009), and inducing senescence in response to RAS (Weyemi et al., 2012, Kodama et al., 2013), and other stimuli (Hannken et al., 1998, Shiose et al., 2001), including resveratrol (Schilder et al., 2009). However, there is little evidence that NADPH oxidase activity actually remains upregulated in senescent cells. One study in HUVECs found that the mRNA levels of several Nox genes, and the relative Nox4 activity, were reduced in senescent cells compared to proliferating controls (Lener et al., 2009). If this is also true in fibroblasts then it is unlikely that NADPH oxidase enzymes play a role in the bystander effect, which would indicate that the relevant ROS produced by senescent cells are mainly internal and result from the by-product of metabolism, particularly the mitochondrial

electron transport chain, which is known to be dysfunctional in senescent cells (Passos et al., 2006, Passos et al., 2007, Moiseeva et al., 2009, Passos et al., 2010).

Both superoxide dismutase and catalase antioxidant enzymes were added to the medium. However, it is unlikely that superoxide generated within senescent cells escapes into the medium due to the short half-life of the anion. The importance of SOD will therefore depend on the activity of membrane-bound NADPH oxidases in fibroblasts which is yet to be established. It would have been interesting to add each of the antioxidant enzymes separately and observe the changes to the bystander effect. Notably, the addition of SOD or catalase alone were both sufficient to inhibit the RIBE (Lyng et al., 2006), suggesting that both hydrogen peroxide and superoxide may be important, but this cannot rule out that superoxide is important due to its conversion into hydrogen peroxide.

The detoxification of hydrogen peroxide by catalase is likely to be much more important in preventing the bystander effect. Potentially the ROS could cross the membranes of bystander cells and oxidise the DNA through the production of more reactive species generated by the Fenton reaction (Imlay et al., 1988). However, there is also evidence that membrane composition, particularly aquaporin (Bienert et al., 2007), and ergosterol (Bienert et al., 2006) levels affect the permeability of membranes to hydrogen peroxide. Thus potentially hydrogen peroxide could oxidise components of the membrane and induce bystander signalling from there.

It is important to remember that although most early research into ROS regarded them exclusively as inducers of damage, further work has shown them to be highly important signalling molecules (D'Autreaux and Toledano, 2007). Thus, although NF-κB, p38 and mTOR may induce a DDR in bystander cells via the production of ROS, it is also possible that ROS induce a bystander effect via the induction of NF-κB, p38 and mTOR.

One study demonstrated that hydrogen peroxide activated p38, which then induced expression of several antioxidant enzymes (Gutierrez-Uzquiza et al., 2012). They suggested that this was a pro-survival mechanism, by which p38 prevented apoptosis. Thus, p38 could then induce senescence as it does in

response to RAS, by inducing the p16-pRb pathway independent of damage (Brookes et al., 2002, Deng et al., 2004). It is more difficult to understand how this pathway might cause hydrogen peroxide to induce a DDR, as is described here. Notably, this phenomenon runs in stark contradiction to another study showing that p38 increases ROS levels and creates a positive feedback loop which includes activation of the DDR (Passos et al., 2010). Plausibly, the upregulation of antioxidant enzymes by p38 is a response to supraphysiological levels of hydrogen peroxide between 0.1-1mM, which induce abnormal responses. It should be noted that p38 is a hub molecule and likely has multiple different effects depending on the cellular milieu.

There is also significant evidence that hydrogen peroxide can activate the PI3K/TOR pathway (Radisavljevic and Gonzalez-Flecha, 2004, Bae et al., 1999, Huang et al., 2002), as well as Akt in response to growth factor stimulation (Liu et al., 2006a). Additionally, mTOR regulates oxygen consumption and oxidative capacity of mitochondria (Schieke et al., 2006), and activating and inhibiting mTOR increases and decreases ROS levels respectively (Kim et al., 2005, Tunon et al., 2003). Thus, similar to p38 mTOR could also be involved in a positive feedback loop with reactive oxygen species.

As described in the introduction, the relationship between NF-κB and reactive oxygen species is complex. Depending on conditions within the cell NF-κB can either inhibit (Djavaheri-Mergny et al., 2004, Jones et al., 1997, Rojo et al., 2004, Schreiber et al., 2006, Xia et al., 1996), or induce (Chopra et al., 1992, Anrather et al., 2006, Deng et al., 2003, Inoue and Tanabe, 1998) ROS production, and equally ROS can both activate (Schieven et al., 1993, Schoonbroodt et al., 2000, Takada et al., 2003, Kamata et al., 2002, Li and Engelhardt, 2006), or inhibit NF-κB (Panopoulos et al., 2005, Reynaert et al., 2006, Wu et al., 2009).

Potentially p38, mTOR, NF-κB and ROS could all be involved in a network of positive feedback activating the DDR through a combination of direct induction of DNA damage through ROS, and inducing DNA replication through the other components. Notably, there is even evidence that ROS can increase the rate of DNA replication, indirectly contributing to DNA damage (Weyemi et al., 2012).

5.5.3 Gap Junctions, ROS and Inflammation

The last factor that was shown to be necessary for the bystander effect was gap junction mediated transfer. When an inhibitor of gap junction mediated transfer is added alongside antioxidants, there is no combinatorial effect (Figure 5.5). Notably, both treatments were sufficient to abrogate the bystander effect alone, and therefore the lack of combinatorial effect demonstrates that both treatments are specifically inhibiting the bystander mechanism. Additionally, both pathways may be regulating the same processes. Octanol may reduce the transfer of ROS, or simply the production of ROS in senescent cells, possibly through the inhibition of the same signals via which the extracellular antioxidant enzymes worked. However, it should be noted with caution that despite the potent inhibition of increased number of foci at later timepoints (Figure 4.8), at early time points octanol did not abrogate the increase in focus formation (Figure 4.7), whereas the extracellular antioxidants did, suggesting that the mechanism is not entirely the same, although this does not contradict a role of gap junction mediated transfer in regulating the levels of intracellular ROS in senescent cells.

The importance of gap junctions only at later time points could reflect the increased confluency of the cells. Alternatively, the increase in foci found in bystander cells at later time points of co-culture may result from a threshold effect that requires both ROS secretion and passage through gap junctions. Potentially, the secreted pathway in sub-confluent conditions is enough to stimulate the increase in focus formation, but not enough to overwhelm the repair machinery and increase the number of foci per cell. At confluency the additional passage of ROS through gap junctions increases the level of damage and the number of foci begins to increase in bystander cells. This hypothesis could be tested at early time points by observing the rate of focus formation in bystander cells at confluency in the presence of octanol. If they are increased, it will suggest that bystander induced damage, like senescence itself, is a threshold effect. Speculatively, the oxidation of the cell membrane could induce death receptors, tyrosine kinases and transient receptor protein (TRP) channels, which could potentiate the ROS levels within the cell, and induce other cellular changes such as the activation of JNK and mitochondrial

dysfunction (Morgan et al., 2007), whilst gap junctions could allow a more direct passage of ROS from senescent to bystander cells.

Similar to the response to oxidative stress or oncogenic activation, cells respond to inflammatory signals by closing gap junctions. IL-α, Il-1β, TNF-α and LPS all instigate the closure of gap junctions (Hu and Xie, 1994, van Rijen et al., 1998), and the connexin43 promoter does contain a κB element (Echetebu et al., 1999). However, there is evidence that although senescent cells have fewer gap junctions, they do not downregulate them in response to stimuli such as EGF, as young cells do (Xie and Hu, 1994). Thus, senescent cells may be forced to transmit signals that healthy replicating cells would not.

As described in the previous chapter, gap junctions not only propagate the inflammatory or oxidative signal; overexpression of connexins can protect against various forms of cellular stress (Lin et al., 2003), whilst their inhibition induces increased apoptosis and inflammation following ischemic brain injury in mice (Nakase et al., 2004). Thus, it should be noted that gap junctions do retain some function even under stressful and inflammatory conditions.

Consistently, the RIBE is also dependent on gap junctions, without which the induction of damage and cell death did not occur in several studies (Azzam et al., 1998, Azzam et al., 2001). In both humans and mice, resistance to bystander induced damage does not correlate with a healthy phenome, which includes increased genomic instability and tumour formation (Mothersill et al., 1999, Mothersill et al., 2001). Therefore, it is likely that the bystander effect exists for a reason rather than as a result of ineffective inhibition. The closure of gap junctions during senescence, inflammation and oxidative stress is then perhaps a mechanism to prevent a necessary signal from being too strong.

5.5.4 Stimulating Senescent Cells with Inflammatory Cytokines Affects the Bystander Effect

In this study senescent and control cells were stimulated with TNF-α, to observe if this had any effect on DNA damage, as predicted by the positive feedback loops described in the introduction. Notably, this is not the same as

overexpressing a subunit of NF-κB for two main reasons. Firstly, the stimulation, although at supraphysiological levels, is withdrawn; secondly TNF-α stimulation is a potent activator of canonical NF-κB signalling, but will still have effects outside the activation of this transcription factor, such as the activation of the inhibitor of apoptosis proteins (c-IAP1 and c-IAP2) through p38 and JNK (Furusu et al., 2007).

Interestingly, at the two early time points there was little tendency of TNF-α pre-stimulation to have any effect on the number of foci in bystander cells. It was only at the latest time point, by which time it was initially hypothesised that any effect of stimulation would have dissipated, that the effect became observable (Figure 5.11), whereas it never occurred in stimulated controls. Thus, rather than a transient increase in damage from a temporary activation of NF-κB signalling, this suggests that TNF-α stimulation of senescent cells has a gradually occurring but permanent effect on senescent cells, much like the senescent phenotype itself. However, it cannot be ruled out that absence of any effect at the earliest time point was just a result of the level of noise, from the recent plating and decreased time for intercellular communication which helps produce a more homogenous population. Consistently, the number of cells examined at the earliest time point was lower than subsequent measurements, although each cell was examined in more detail over a period of twelve hours, rather than a single measurement per cell.

It would be interesting to see if pre-stimulation increases the early induction of focus formation seen in bystander cells. Potentially, stimulation does increase focus formation early on, but similar to the unstimulated bystander effect, the cell tolerates it by increasing repair. Thus, stimulation could still instigate a transient effect on senescent cells, with a delayed manifestation once cells receive chance hits to easily damaged genomic regions such as telomeres.

The results shown here are too preliminary for a detailed discussion of how TNF-α might induce damage in bystander cells, except with an outlook to further research. The role of NF-κB in inducing DNA damage is still controversial, and is likely dependent on many factors. However, there are multiple mechanisms by which NF-κB could potentially induce DNA damage, and reciprocally DNA

damage induces NF-κB. Thus, the idea of a positive feedback loop between NF-κB and DNA damage is worth further investigation.

Importantly, an early study demonstrated that repeated stimulation of fibroblasts with TNF-α or IL-1α accelerated the induction of replicative senescence in these cells in a process dependent on ROS (Dumont et al., 2000a). This is highly consistent with the idea that TNF-α stimulates the production of ROS through NF-κB, which then induces DNA damage and senescence. Equally, the additional bystander effect shown here was dependent on ROS, implicating a similar mechanism.

If there is no positive feedback and the effect is not transient, then it might be worth investigating NF-κB oscillations in senescent cells. Potentially, IκBα, and the other inhibitors A20 and CYLD could be inhibited or deregulated preventing the normal dampening of oscillations and maintaining a higher level of NF-κB in senescent cells, as is shown here and elsewhere (Chien et al., 2011, Freund et al., 2011).

It is still too early to know for sure whether TNF-α stimulation increases DNA damage in senescent cells, as the data were bordering on significance. If this is found not to be the case, then it is inconsistent with the proposed positive feedback loop which should also increase DNA damage in senescent cells. However, the senescent cells could be more resistant to DNA damage. Senescent cells have increased levels of heterochromatin (Narita et al., 2003, Braig and Schmitt, 2006), which can be resistant to damage (Yan et al., 2011). Alternatively, senescent cells could have a defective DDR, thus preventing foci from forming at sites of damage, which could also be the result of heterochromatisation (Kim et al., 2007, Karagiannis et al., 2007, Di Micco et al., 2011).

A recent study suggests that increased NF-κB related inflammatory signalling increases the level of DNA damage and ROS levels within senescent cells (Jurk et al., unpublished). They used p50 knockout mice, which increased inflammatory signalling by preventing the inhibitory function of p50 homodimers including the binding of HDAC1 (Elsharkawy et al., 2010, Oakley et al., 2005), although it also disrupts p65-p50 heterodimer induced canonical NF-κB signalling. Consistent with this study, the inhibition of p38 reduced ROS levels

in senescent cells, but had markedly reduced effect in p50 knockout cells, suggesting that p38 induces this function through NF-κB. Oppositely, inhibition of COX-2 reduced the levels of ROS and DDR only in the p50 knockdown cells, suggesting that it has little role in the normal senescent phenotype, but is likely to have a prominent role in the increased inflammatory phenotype of senescent cells with stimulated NF-κB signalling, and definitely merits further research to elucidate whether COX-2 inhibition would reduce the increased bystander effect of TNF-α stimulated senescent cells to either unstimulated senescent or control levels.

Importantly, the same study uses irradiated cells to examine the bystander effect in primary mouse adult ear fibroblasts (MAFs) shows that p50 knockout senescent cells have an increased bystander effect compared to wildtype senescent cells, which is consistent with the idea shown here that upregulated NF-κB signalling can increase the bystander effect.

Stimulation of senescent cells with IL-6 does not mimic the effects of TNF-α on bystander cells. This is not altogether surprising because, as described in the introduction TNF-α and IL-6 activate very different pathways. TNF-α potently activates canonical NF-κB signalling, whilst IL-6 activates mainly the JAK/STAT pathway as well Ras, ERK, JNK, p38 and PI3K/Akt.

What is surprising is that pre-stimulation with IL-6 not only does not increase the bystander effect as does TNF-α, but also has a tendency to decrease it, although this is not significant (Figure 5.15). It cannot be ruled out that this was down to experimental error, as the repeats were not entirely consistent.

IL-6 has been well established as a pro-inflammatory cytokine, and its role in lipid oxidation and lipolysis (Petersen et al., 2005) indicate its potential to induce damage. Additionally, STAT3 signalling inhibits apoptosis and activates cell cycle (Shi et al., 2002), activating a highly overlapping repertoire of genes with NF-κB (Grivennikov and Karin, 2010). Indeed, a recent study has identified the importance of STAT3 signalling in maintaining constitutively active NF-κB, through the hyperacetylation of p65 which inhibits the binding of IκB (Lee et al., 2009). The study suggested that cancer cells would primarily activate NF-κB through the IKK complex mediated by cytokines such as TNF-α which induce canonical signalling, then NF-κB would upregulate IL-6 and activate STAT3,

which had been shown to induce cancer development (Naugler et al., 2007), causing NF-κB to become constitutively active. Thus, this study suggests that TNF-α and IL-6 should co-operate strongly to induce a more potent and long lasting inflammatory signal. Other studies reinforce this idea, suggesting that STAT3 can bind cytoplasmic NF-κB, displace IκB, and allow NF-κB to enter the nucleus in the absence of IKK signalling (Yang et al., 2007).

However, there is accumulating evidence for an anti-inflammatory role of IL-6 as well. Some studies have suggested a role for IL-6 in the inhibition of TNF-α (Petersen and Pedersen, 2005), whilst others have shown that it stimulates production of both IL-1ra (IL-1 receptor antagonist) and IL-10 (Pedersen, 2007). The latter is a potent inhibitor of IL-1α, IL-1β and TNF-α as well as the chemokines IL-8 and macrophage inflammatory protein α (MIPα). Some studies have suggested that there are two types of IL-6 signalling: If IL-6 is bound to a secreted form of its receptor IL-6R which then binds membrane bound gp130 proteins, then the resultant complex induces trans-signalling which is pro-inflammatory. Inhibition of trans-signalling only reduces inflammation and autoimmune disease (Atreya et al., 2000, Nowell et al., 2003). Contrarily, if IL-6 binds membrane bound IL-6R, this initiates classical signalling, which is thought to be mostly anti-inflammatory and regenerative (Becker et al., 2004, Barkhausen et al., 2011), although notably there are some inconsistencies (Malchow et al., 2011). Thus, as cells were only stimulated with IL-6, and not IL-6R, it might explain why the result was anti-inflammatory. Interestingly, not all cell types express membrane bound IL-6R, which makes them incapable of classical signalling. Synovial fibroblasts did not respond to IL-6 alone (Mihara et al., 1995), but dermal fibroblasts had a slight but significant response (Mihara et al., 1996), although both cell types responded significantly more to IL-6 and IL-6R combined. Notably, in the latter case trans-signalling also had anti-inflammatory effects, repressing TNF-α and IL-1β (Mihara et al., 1996).

Clearly, there is much about IL-6 which is still not understood, but this evidence does make a case for the slight non-significant decrease in the bystander effect observed in these data, through its repression of the pathways such as TNF-α which are shown to increase the bystander effect.

Interestingly, TNF-α stimulation also inhibits IL-6 mediated STAT3 activation, partially through synthesis of SOCS3 and activation of p38 MAPK (Bode et al., 1999). Additionally, NF-κB may also inhibit STAT3 binding to some promoters, although it can also act as a co-activator (Bode et al., 2001a, Zhang and Fuller, 1997). Therefore, it is likely that the bulk of IL-6 inhibition by TNF-α results from NF-κB independent pathways such as via SOCS3. This is consistent with the data shown here, that IL-6 has a tendency to reduce the effect of TNF-α, but the latter appears to be dominant over the former (Figure 5.16).

6 Discussion

6.1 The Senescent Cell Bystander Effect and Signalling Pathways

These results demonstrate for the first time that senescent cells are capable of emitting a signal which induces a DNA damage response in healthy replicating cells. Important previous studies have shown that senescent cells induce cell growth of preneoplastic cells, and indicate that this may have resulted from their transformation, and thus would most likely result from DNA damage (Bavik et al., 2006, Krtolica et al., 2001, Liu and Hornsby, 2007). However, they observed no similar effect in healthy cells. Notably, the amount of cell death induced by irradiated cells is much greater in immortalised pre-neoplastic cells than in normal cells. Experiments using medium from γ-irradiated cells often showed no effect on healthy fibroblasts (Mothersill et al., 2004), but significant cell death in immortalised cells. When our data are combined with these previous experiments it suggests that healthy cells are not immune to the induction of damage, but that they are better at responding to it.

The data presented here suggest that although the induction of damage is increased in bystander cells after very short co-culture periods, these cells are repairing the damage very quickly and the number of foci per cell does not begin to increase until the length of co-culture is extended. Eventually however, the DNA becomes damaged in places that are difficult to repair. Recent evidence inserting telomeric DNA into the genome suggests that some feature of the sequence inhibits repair (Fumagalli et al., 2012), which would most likely have evolved to prevent chromosomal fusions in the event of uncapping. Therefore, it is possible that the increase in DNA damage foci in bystander cells reflects damage that has occurred at a telomere and cannot be removed, although it is also possible that this reflects a detiorating cellular phenotype due to increased levels of non-telomeric damage from extended co-culture.

The results of this thesis suggest that the bystander effect is dependent on the production of reactive oxygen species, NF-κB, p38 and mTOR, which is highly consistent with previous research. The necessity of each of these molecules suggests that they are all dependent on each other, and therefore are likely to

all form part of an interacting network. A host of previous studies have suggested that NF-κB, p38 and mTOR are likely to positively regulate both each other and the production of reactive oxygen species. Importantly, ROS are not necessarily the end point of these pathways, and a host of evidence suggests that ROS function as signalling molecules which are also capable of activating p38, mTOR and NF-κB. Thus, the bystander effect could reflect a combination of the direct damage from ROS as well as the indirect effects of these other molecules which make the DNA more likely to be damaged, through replication or other independent pathways.

Since the publication of our work into the bystander effect on replicatively senescent cells, there has been another much more detailed experiment demonstrating the bystander effect of oncogene induced senescent cells by Acosta et al. (2013). Although this has been referred to repeatedly throughout this thesis, it would not be complete without a more in depth discussion of similarities and differences between the two bystander phenotypes.

Importantly, they found there was an increase in 8-oxoG in bystander cells, which is a marker of DNA oxidation, which suggests, as discussed previously that 53BP1 foci are representative of actual oxidative damage. Additionally, they demonstrate the induction of senescence in bystander cells using SA-β gal as well as the additional markers p21, p16 and IL-8. Thus, in these respects the two phenotypes appear very similar.

The main difference is that the oncogene induced senescent cell bystander effect is much more potent than that produced by replicatively senescent cells. This may reflect that, in their work, the inducer cells were undergoing senescence, whilst the replicatively senescent cells used here had been senescent for months. However, the data shown here from cells undergoing irradiation induced senescence suggests that this should have little impact on the bystander effect, or even reduce it. Importantly, there are several differences between oncogene induced senescent cells and both replicatively and irradiated senescent cells, which may explain this difference: although the SASPs are qualitatively very similar, there are several factors which are expressed at significantly higher levels in oncogene induced cells. These include IL-7, GM-CSF, MIP-1α and GRO-α, which are all secreted in high levels

in replicatively senescent cells, and even higher levels in RAS induced senescent cells, suggesting that they may be important in the bystander effect, and potentially causal in amplifying the bystander signal from oncogene induced senescent cells. Interestingly IL-1β, which is not upregulated in response to radiation or replicative senescence, is highly upregulated in oncogene induced senescence, and is a potent inducer of NF-κB signalling.

Fittingly, this suggests that IL-1β is not necessary for the bystander effect, but its stimulation and likely the subsequent activation of NF-κB might increase the bystander effect, which is concordant with the data described here suggesting that TNF-α stimulation can increase the bystander effect.

It has been suggested that the reason oncogene induced cells induce a more potent SASP, might be at least partially due to repression of p53, as its inhibition prior to induction of senescence produced a SASP similar to that of RAS expressing cells. However, knockdown of p53 in cells prior to RAS expression produced the most potent SASP of all suggesting there are other factors involved. Thus far, no one has compared ROS levels between oncogene induced and replicatively senescent cells, which should be considered important further work.

Using unbiased quantitative proteomics Acosta et al (2013) compared the secretory profiles between RAS expressing and non-expressing cells, and consistent with previous studies showed upregulation of IL-6 and IL-8 amongst others. Interestingly, they also identified TGF-β, which a previous study had shown to remain fairly constant after induction of senescence (Coppe et al., 2008). Although Coppe et al. (2008) did not state at what point after oncogene induced senescence that they profiled the cells, it can be assumed that if they followed the same protocol as the irradiated cells that they allowed the cells to become fully senescent. Perhaps TGF-β is mainly involved in the induction of senescence, at which point it is downregulated again. If it is also specific to oncogene induced senescence it could help explain the difference in potency.

The addition of inhibitors against TGF-β R1, and also VEGF-R2 to cells cultured in the medium from RAS expressing cells, caused a significant increase in growth of bystander cells over DMSO, whilst having little effect on RAS expressing cells (Acosta et al., 2013). This indicated that the arrest induced by

the medium had been inhibited, and thus implicated TGF-β and VEGF in the bystander effect. Notably, even the most successful inhibitors did not restore growth to control level suggesting that senescent cells can still induce a bystander effect in the absence of TGF-β, consistent with the idea that TGF-β might be partially responsible for the amplification of the bystander effect in RAS induced senescing cells.

The fact that Acosta *et al.* (2013) found no effect when they added p38 and NF-κB inhibitors to the medium after it had been removed from senescent cells is consistent with these data showing that NF-κB is mainly important in senescent cells, and suggests that the signal transmitted to the bystander cells will be the products of these pathways, principally the SASP and ROS. This is inconsistent with the idea that NF-κB acts within the bystander cells to induce damage by stimulating DNA replication and cell cycle, though this could still occur indirectly via the SASP, ROS or other NF-κB dependent signals from the senescent cells. However, the growth curves of bystander cells co-cultured with RAS expressing cells are not significantly faster than controls (Acosta et al., 2013), suggesting that if the pro-cell cycle signals of these molecules does play a role in the induction of damage and senescence, that it is minor.

6.2 The Implications of the Bystander Effect

Senescent cells have long been hypothesised to be involved in the ageing process, and recent studies have provided clear evidence that they play a causal role in age related diseases. However, prior to this work the main hypothesis as to the mechanism involved was through the autonomous depletion of functional cells. This had been thought to result from stem cell exhaustion as these cells are responsible for replacing large numbers of cells in multiple tissues, and their loss can be seriously detrimental to tissue function. However, there is little evidence that stem cells deplete with age. Whilst they decline in functionality and differentiation potential, this does not appear related to the autonomous depletion of stem cell numbers. In fact, there is a wealth of evidence that stem cell function is maintained at least partially non-autonomously by the niche environment (Spradling et al., 2001).

Therefore, if senescence is to play a role in the ageing process it must happen through some other means. Consistent, with the various hypotheses that ageing is the result of DNA and molecular damage, the bystander effect shown here provides one possible mechanism by which senescent cells could contribute to ageing, via the spread of molecular damage.

It has already been shown that the presence of senescent cells contributes to transformation of preneoplastic cells. Here is described both a mechanism by which this process could occur, through the induction of DNA damage in unstable cells, but also an underlying mechanism by which senescent cells could contribute to the ageing process and the accompanying increase in both preneoplastic and neoplastic cells that are responsible for the cancers of old age.

Additionally, through the induction and spread of the senescent phenotype, senescent cells could contribute to ageing via the induction of chronic inflammation, consistent with more recent hypotheses on the ageing process, or potentially through the induction of mTOR, at least partially consistent with programmed based theories of ageing, although notably the primary stimuli would result from DNA damage, which is entirely inconsistent.

Importantly, the potent bystander effect of oncogene induced senescence demonstrated by Acosta et al (2013) is diminishing. Although their neighbours receive a potent stimulus to senesce, cells >1mm away from the initial stimulus were unaffected. We have not tested whether this also true of the replicative senescence induced bystander effect; however, its reliance upon gap junction mediated transfer suggests that it will also have a limited range. More importantly, the medium from the bystander cells from the RAS induced senescent cells failed to induce senescence in a group of tertiary bystander cells, despite the fact that a large fraction of these secondary cells had become senescent. Therefore, although these cells could potentially have a prolongued non-autonomous effect on the cells within 1mm distance, because the effect dissipates, the RAS induced senescence stimulus has a limited potential to influence the ageing process non-autonomously. However, it is important to note that the medium from the secondarily senescent cells still slows the growth rate of tertiary bystanders, which is highly suggestive of reversible arrest

induced by damage. In these experiments, a significant effect on focus induction was not observed from medium alone, which suggests that the secondarily senescent cells observed by Acosta et al. (2013) still produce a relatively potent bystander effect. What remains to be elucidated is whether the bystander induced senescence shown here also dissipates. This will have important implications for the roles of both oncogene induced and replicatively senescent cells in the non-autonomous influence of the ageing process. Although speculative, it is unlikely that the bystander effect shown here dissipates similarly to that of RAS expressing cells. The secondarily senescent cells shown by Acosta et al. (2013) are likely to differ from the primary inducers through their reduced levels of RAS expression, which suggests that they were induced to senesce via DNA damage, and their SASPs will therefore be similar to the replicative and irradiation induced senescent cells studied in this thesis. Importantly, the SASP and ROS are necessary to maintain growth arrest, which prevents immortalisation and neoplasia. Therefore, it could be expected that the bystander effect at least per cell is unlikely to dissipate. However, the high numbers of senescent cells used in both these studies suggests that the bystander effect is highly likely to dissipate due to the decreasing number of senescent cells as you get further away from the initial inducers.

The real test for the relevance of these *in vitro* studies, is whether the same phenomena also occur *in vivo*. The initial study by Krtolica et al. (2001), clearly identified that injection of preneoplastic cells in the presence of senescent cells was more likely to induce tumours in mice than in the presence of young replicating cells (Krtolica et al., 2001). In our study we used a 4-hydroxynonenal staining, which has been previously shown to be a good marker for *in vivo* senescence (Nelson et al., 2012), to demonstrate that senescent cells had a significant degree of clustering, indicative that senescence was not an autonomous process and could be spread to neighbouring cells. This was reaffirmed by Acosta et al. (2013) using p16 and p21 stainings, and they also showed that mouse papillomas and human sessile serrated adenomas had large numbers of senescent cells just outside the papilloma, which heavily indicated bystander induced senescence *in vivo* (Acosta et al., 2013).

These data suggest that the bystander effect is a real phenomenon in vivo, and could therefore contribute to both cancer and ageing in living organisms.

7 Conclusion

Here is shown for the first time that replicatively senescent cells induce a DNA damage response and senescence in bystander cells. It requires cell communication through gap junctions and cannot be transmitted by medium alone. It is also dependent on reactive oxygen species which may directly induce damage in bystander cells. Use of specific inhibitors suggests that mTOR, p38 and NF-κB form an interconnected network in which all three components are necessary to induce a DNA damage response in bystander cells. NF-κB is upregulated in senescent cells and not bystanders, and knockdown exclusively in bystander cells is sufficient to abrogate the bystander effect, suggesting its importance in signal production over response. Lastly NF-κB stimulation can significantly increase the bystander effect in a process dependent on reactive oxygen species.

8 References

ABOU HASHIEH, I., MATHIEU, S., BESSON, F. & GEROLAMI, A. 1996. Inhibition of gap junction intercellular communications of cultured rat hepatocytes by ethanol: role of ethanol metabolism. *J Hepatol,* 24**,** 360-7.

ABULAITI, A., FIKARIS, A. J., TSYGANKOVA, O. M. & MEINKOTH, J. L. 2006. Ras induces chromosome instability and abrogation of the DNA damage response. *Cancer Res,* 66**,** 10505-12.

ACOSTA, J. C., BANITO, A., WUESTEFELD, T., GEORGILIS, A., JANICH, P., MORTON, J. P., ATHINEOS, D., KANG, T. W., LASITSCHKA, F., ANDRULIS, M., PASCUAL, G., MORRIS, K. J., KHAN, S., JIN, H., DHARMALINGAM, G., SNIJDERS, A. P., CARROLL, T., CAPPER, D., PRITCHARD, C., INMAN, G. J., LONGERICH, T., SANSOM, O. J., BENITAH, S. A., ZENDER, L. & GIL, J. 2013. A complex secretory program orchestrated by the inflammasome controls paracrine senescence. *Nat Cell Biol*.

ACOSTA, J. C., O'LOGHLEN, A., BANITO, A., GUIJARRO, M. V., AUGERT, A., RAGUZ, S., FUMAGALLI, M., DA COSTA, M., BROWN, C., POPOV, N., TAKATSU, Y., MELAMED, J., D'ADDA DI FAGAGNA, F., BERNARD, D., HERNANDO, E. & GIL, J. 2008. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell,* 133**,** 1006-18.

ADAMS, M. M., WANG, B., XIA, Z., MORALES, J. C., LU, X., DONEHOWER, L. A., BOCHAR, D. A., ELLEDGE, S. J. & CARPENTER, P. B. 2005. 53BP1 oligomerization is independent of its methylation by PRMT1. *Cell Cycle,* 4**,** 1854-61.

ADLER, A. S., SINHA, S., KAWAHARA, T. L., ZHANG, J. Y., SEGAL, E. & CHANG, H. Y. 2007. Motif module map reveals enforcement of aging by continual NF-kappaB activity. *Genes Dev,* 21**,** 3244-57.

AHMED, E. K., ROGOWSKA-WRZESINSKA, A., ROEPSTORFF, P., BULTEAU, A. L. & FRIGUET, B. 2010. Protein modification and replicative senescence of WI-38 human embryonic fibroblasts. *Aging Cell,* 9**,** 252-72.

AHN, J. Y., SCHWARZ, J. K., PIWNICA-WORMS, H. & CANMAN, C. E. 2000. Threonine 68 phosphorylation by ataxia telangiectasia mutated is required for efficient activation of Chk2 in response to ionizing radiation. *Cancer Res,* 60**,** 5934-6.

ALCORTA, D. A., XIONG, Y., PHELPS, D., HANNON, G., BEACH, D. & BARRETT, J. C. 1996. Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. *Proc Natl Acad Sci U S A,* 93**,** 13742-7.

ALIMONTI, A., NARDELLA, C., CHEN, Z., CLOHESSY, J. G., CARRACEDO, A., TROTMAN, L. C., CHENG, K., VARMEH, S., KOZMA, S. C., THOMAS, G., ROSIVATZ, E., WOSCHOLSKI, R., COGNETTI, F., SCHER, H. I. & PANDOLFI, P. P. 2010. A novel type of cellular senescence that can be enhanced in mouse models and human tumor xenografts to suppress prostate tumorigenesis. *J Clin Invest,* 120**,** 681-93.

ALLSHIRE, R. C., GOSDEN, J. R., CROSS, S. H., CRANSTON, G., ROUT, D., SUGAWARA, N., SZOSTAK, J. W., FANTES, P. A. & HASTIE, N. D. 1988. Telomeric repeat from T. thermophila cross hybridizes with human telomeres. *Nature,* 332**,** 656-9.

ALLSOPP, R. C., VAZIRI, H., PATTERSON, C., GOLDSTEIN, S., YOUNGLAI, E. V., FUTCHER, A. B., GREIDER, C. W. & HARLEY, C. B. 1992. Telomere length predicts replicative capacity of human fibroblasts. *Proc Natl Acad Sci U S A,* 89**,** 10114-8.

ANDERSON, L., HENDERSON, C. & ADACHI, Y. 2001. Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol Cell Biol,* 21**,** 1719-29.

ANRATHER, J., RACCHUMI, G. & IADECOLA, C. 2006. NF-kappaB regulates phagocytic NADPH oxidase by inducing the expression of gp91phox. *J Biol Chem,* 281**,** 5657-67.

ANTUNES, F. & CADENAS, E. 2000. Estimation of H2O2 gradients across biomembranes. *FEBS Lett,* 475**,** 121-6.

ASHCROFT, G. S., YANG, X., GLICK, A. B., WEINSTEIN, M., LETTERIO, J. L., MIZEL, D. E., ANZANO, M., GREENWELL-WILD, T., WAHL, S. M., DENG, C. & ROBERTS, A. B. 1999. Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nature cell biology,* 1**,** 260-6.

ATADJA, P., WONG, H., VEILLETE, C. & RIABOWOL, K. 1995. Overexpression of cyclin D1 blocks proliferation of normal diploid fibroblasts. *Exp Cell Res,* 217**,** 205-16.

ATREYA, R., MUDTER, J., FINOTTO, S., MULLBERG, J., JOSTOCK, T., WIRTZ, S., SCHUTZ, M., BARTSCH, B., HOLTMANN, M., BECKER, C., STRAND, D., CZAJA, J., SCHLAAK, J. F., LEHR, H. A., AUTSCHBACH, F., SCHURMANN, G., NISHIMOTO, N., YOSHIZAKI, K., ITO, H., KISHIMOTO, T., GALLE, P. R., ROSE-JOHN, S. & NEURATH, M. F. 2000. Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med,* 6**,** 583-8.

AUTSAVAPROMPORN, N., DE TOLEDO, S. M., LITTLE, J. B., JAY-GERIN, J. P., HARRIS, A. L. & AZZAM, E. I. 2011. The role of gap junction communication and oxidative stress in the propagation of toxic effects among high-dose alpha-particle-irradiated human cells. *Radiat Res,* 175**,** 347-57.

AWASTHI, V. & KING, R. J. 2000. PKC, p42/p44 MAPK, and p38 MAPK are required for HGF-induced proliferation of H441 cells. *Am J Physiol Lung Cell Mol Physiol,* 279**,** L942-9.

AZARNIA, R., REDDY, S., KMIECIK, T. E., SHALLOWAY, D. & LOEWENSTEIN, W. R. 1988. The cellular src gene product regulates junctional cell-to-cell communication. *Science,* 239**,** 398-401.

AZZAM, E. I., DE TOLEDO, S. M., GOODING, T. & LITTLE, J. B. 1998. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat Res,* 150**,** 497-504.

AZZAM, E. I., DE TOLEDO, S. M. & LITTLE, J. B. 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. *Proc Natl Acad Sci U S A,* 98**,** 473-8.

AZZAM, E. I., DE TOLEDO, S. M. & LITTLE, J. B. 2003. Expression of CONNEXIN43 is highly sensitive to ionizing radiation and other environmental stresses. *Cancer Res,* 63**,** 7128-35.

AZZAM, E. I., DE TOLEDO, S. M., WAKER, A. J. & LITTLE, J. B. 2000. High and low fluences of alpha-particles induce a G1 checkpoint in human diploid fibroblasts. *Cancer Res,* 60**,** 2623-31.

BACCHETTI, S. & COUNTER, C. 1995. Telomeres and telomerase in human cancer (review). *Int J Oncol,* 7**,** 423-32.

BAE, G. U., SEO, D. W., KWON, H. K., LEE, H. Y., HONG, S., LEE, Z. W., HA, K. S., LEE, H. W. & HAN, J. W. 1999. Hydrogen peroxide activates p70(S6k) signaling pathway. *J Biol Chem,* 274**,** 32596-602.

BAGLEY, M. C., DAVIS, T., ROKICKI, M. J., WIDDOWSON, C. S. & KIPLING, D. 2010. Synthesis of the highly selective p38 MAPK inhibitor UR-13756 for possible therapeutic use in Werner syndrome. *Future Med Chem,* 2**,** 193-201.

BAKER, D. J., WIJSHAKE, T., TCHKONIA, T., LEBRASSEUR, N. K., CHILDS, B. G., VAN DE SLUIS, B., KIRKLAND, J. L. & VAN DEURSEN, J. M. 2011. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature,* 479**,** 232-6.

BAKKENIST, C. J. & KASTAN, M. B. 2003. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature,* 421**,** 499-506.

BANIN, S., MOYAL, L., SHIEH, S., TAYA, Y., ANDERSON, C. W., CHESSA, L., SMORODINSKY, N. I., PRIVES, C., REISS, Y., SHILOH, Y. & ZIV, Y. 1998. Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science,* 281**,** 1674-7.

BARAK, Y., JUVEN, T., HAFFNER, R. & OREN, M. 1993. mdm2 expression is induced by wild type p53 activity. *EMBO J,* 12**,** 461-8.

BARASCU, A., LE CHALONY, C., PENNARUN, G., GENET, D., IMAM, N., LOPEZ, B. & BERTRAND, P. 2012. Oxidative stress induces an ATM-independent senescence pathway through p38 MAPK-mediated lamin B1 accumulation. *EMBO J,* 31**,** 1080-94.

BARBIE, D. A., TAMAYO, P., BOEHM, J. S., KIM, S. Y., MOODY, S. E., DUNN, I. F., SCHINZEL, A. C., SANDY, P., MEYLAN, E., SCHOLL, C., FROHLING, S., CHAN, E. M., SOS, M. L., MICHEL, K., MERMEL, C., SILVER, S. J., WEIR, B. A., REILING, J. H., SHENG, Q., GUPTA, P. B., WADLOW, R. C., LE, H., HOERSCH, S., WITTNER, B. S., RAMASWAMY, S., LIVINGSTON, D. M., SABATINI, D. M., MEYERSON, M., THOMAS, R. K., LANDER, E. S., MESIROV, J. P., ROOT, D. E., GILLILAND, D. G., JACKS, T. & HAHN, W. C. 2009. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. *Nature,* 462**,** 108-12.

BARKHAUSEN, T., TSCHERNIG, T., ROSENSTIEL, P., VAN GRIENSVEN, M., VONBERG, R. P., DORSCH, M., MUELLER-HEINE, A., CHALARIS, A., SCHELLER, J., ROSE-JOHN, S., SEEGERT, D., KRETTEK, C. & WAETZIG, G. H. 2011. Selective blockade of interleukin-6 trans-signaling improves survival in a murine polymicrobial sepsis model. *Crit Care Med,* 39**,** 1407-13.

BARROS, M. H., BANDY, B., TAHARA, E. B. & KOWALTOWSKI, A. J. 2004. Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in Saccharomyces cerevisiae. *J Biol Chem,* 279**,** 49883-8.

BARTKOVA, J., HOREJSI, Z., KOED, K., KRAMER, A., TORT, F., ZIEGER, K., GULDBERG, P., SEHESTED, M., NESLAND, J. M., LUKAS, C., ORNTOFT, T., LUKAS, J. & BARTEK, J. 2005. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature,* 434**,** 864-70.

BARTKOVA, J., REZAEI, N., LIONTOS, M., KARAKAIDOS, P., KLETSAS, D., ISSAEVA, N., VASSILIOU, L. V., KOLETTAS, E., NIFOROU, K., ZOUMPOURLIS, V. C., TAKAOKA, M., NAKAGAWA, H., TORT, F., FUGGER, K., JOHANSSON, F., SEHESTED, M., ANDERSEN, C. L., DYRSKJOT, L., ORNTOFT, T., LUKAS, J., KITTAS, C., HELLEDAY, T., HALAZONETIS, T. D., BARTEK, J. & GORGOULIS, V. G. 2006. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature,* 444**,** 633-7.

BATSI, C., MARKOPOULOU, S., VARTHOLOMATOS, G., GEORGIOU, I., KANAVAROS, P., GORGOULIS, V. G., MARCU, K. B. & KOLETTAS, E. 2009. Chronic NF-kappaB activation delays RasV12-induced premature senescence of human fibroblasts by suppressing the DNA damage checkpoint response. *Mech Ageing Dev,* 130**,** 409-19.

BAVIK, C., COLEMAN, I., DEAN, J. P., KNUDSEN, B., PLYMATE, S. & NELSON, P. S. 2006. The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. *Cancer Res,* 66**,** 794-802.

BEAUSEJOUR, C. M., KRTOLICA, A., GALIMI, F., NARITA, M., LOWE, S. W., YASWEN, P. & CAMPISI, J. 2003. Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J,* 22**,** 4212-22.

BECKER, C., FANTINI, M. C., SCHRAMM, C., LEHR, H. A., WIRTZ, S., NIKOLAEV, A., BURG, J., STRAND, S., KIESSLICH, R., HUBER, S., ITO, H., NISHIMOTO, N., YOSHIZAKI, K., KISHIMOTO, T., GALLE, P. R., BLESSING, M., ROSE-JOHN, S. & NEURATH, M. F. 2004. TGF-beta suppresses tumor progression in colon cancer by inhibition of IL-6 trans-signaling. *Immunity,* 21**,** 491-501.

BEG, A. A. & BALTIMORE, D. 1996. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. *Science,* 274**,** 782-4.

BEG, A. A., SHA, W. C., BRONSON, R. T., GHOSH, S. & BALTIMORE, D. 1995. Embryonic lethality and liver degeneration in mice lacking the RelA component of NF-kappa B. *Nature,* 376**,** 167-70.

BEKKER-JENSEN, S., LUKAS, C., KITAGAWA, R., MELANDER, F., KASTAN, M. B., BARTEK, J. & LUKAS, J. 2006. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol,* 173**,** 195-206.

BEKKER-JENSEN, S., LUKAS, C., MELANDER, F., BARTEK, J. & LUKAS, J. 2005. Dynamic assembly and sustained retention of 53BP1 at the sites of DNA damage are controlled by Mdc1/NFBD1. *J Cell Biol,* 170**,** 201-11.

BENNETT, D. C. 2008. How to make a melanoma: what do we know of the primary clonal events? *Pigment Cell Melanoma Res,* 21**,** 27-38.

BERNARD, D., GOSSELIN, K., MONTE, D., VERCAMER, C., BOUALI, F., POURTIER, A., VANDENBUNDER, B. & ABBADIE, C. 2004. Involvement of Rel/nuclear factor-kappaB transcription factors in keratinocyte senescence. *Cancer Res,* 64**,** 472-81.

BERNARD, P. & COUTURIER, M. 1992. Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes. *J Mol Biol,* 226**,** 735-45.

BEY, E. A., XU, B., BHATTACHARJEE, A., OLDFIELD, C. M., ZHAO, X., LI, Q., SUBBULAKSHMI, V., FELDMAN, G. M., WIENTJES, F. B. & CATHCART, M. K. 2004. Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. *J Immunol,* 173**,** 5730-8.

BIENERT, G. P., MOLLER, A. L., KRISTIANSEN, K. A., SCHULZ, A., MOLLER, I. M., SCHJOERRING, J. K. & JAHN, T. P. 2007. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *J Biol Chem,* 282**,** 1183-92.

BIENERT, G. P., SCHJOERRING, J. K. & JAHN, T. P. 2006. Membrane transport of hydrogen peroxide. *Biochim Biophys Acta,* 1758**,** 994-1003.

BIGNAMI, M., ROSA, S., FALCONE, G., TATO, F., KATOH, F. & YAMASAKI, H. 1988. Specific viral oncogenes cause differential effects on cell-to-cell communication, relevant to the suppression of the transformed phenotype by normal cells. *Mol Carcinog,* 1**,** 67-75.

BLACKBURN, E. H. 2000. Telomere states and cell fates. *Nature,* 408**,** 53-6.

BLACKWELL, T. S. & CHRISTMAN, J. W. 1997. The role of nuclear factor-kappa B in cytokine gene regulation. *Am J Respir Cell Mol Biol,* 17**,** 3-9.

BLADIER, C., WOLVETANG, E. J., HUTCHINSON, P., DE HAAN, J. B. & KOLA, I. 1997. Response of a primary human fibroblast cell line to H2O2: senescence-like growth arrest or apoptosis? *Cell Growth Differ,* 8**,** 589-98.

BLAGOSKLONNY, M. V. 2003. Cell senescence and hypermitogenic arrest. *EMBO Rep,* 4**,** 358-62.

BLAGOSKLONNY, M. V. 2006a. Aging and immortality: quasi-programmed senescence and its pharmacologic inhibition. *Cell Cycle,* 5**,** 2087-102.

BLAGOSKLONNY, M. V. 2006b. Cell senescence: hypertrophic arrest beyond the restriction point. *J Cell Physiol,* 209**,** 592-7.

BLAGOSKLONNY, M. V. & PARDEE, A. B. 2002. The restriction point of the cell cycle. *Cell Cycle,* 1**,** 103-10.

BLONSKA, M., YOU, Y., GELEZIUNAS, R. & LIN, X. 2004. Restoration of NF-kappaB activation by tumor necrosis factor alpha receptor complex-targeted MEKK3 in receptor-interacting protein-deficient cells. *Mol Cell Biol,* 24**,** 10757-65.

BLOW, J. J. & DUTTA, A. 2005. Preventing re-replication of chromosomal DNA. *Nat Rev Mol Cell Biol,* 6**,** 476-86.

BODE, J. G., FISCHER, R., HAUSSINGER, D., GRAEVE, L., HEINRICH, P. C. & SCHAPER, F. 2001a. The inhibitory effect of IL-1 beta on IL-6-induced alpha 2-macroglobulin expression is due to activation of NF-kappa B. *J Immunol,* 167**,** 1469-81.

BODE, J. G., LUDWIG, S., FREITAS, C. A., SCHAPER, F., RUHL, M., MELMED, S., HEINRICH, P. C. & HAUSSINGER, D. 2001b. The MKK6/p38 mitogen-activated protein kinase pathway is capable of inducing SOCS3 gene expression and inhibits IL-6-induced transcription. *Biol Chem,* 382**,** 1447-53.

BODE, J. G., NIMMESGERN, A., SCHMITZ, J., SCHAPER, F., SCHMITT, M., FRISCH, W., HAUSSINGER, D., HEINRICH, P. C. & GRAEVE, L. 1999. LPS and TNFalpha induce SOCS3 mRNA and inhibit IL-6-induced activation of STAT3 in macrophages. *FEBS Lett,* 463**,** 365-70.

BODNAR, A. G., OUELLETTE, M., FROLKIS, M., HOLT, S. E., CHIU, C. P., MORIN, G. B., HARLEY, C. B., SHAY, J. W., LICHTSTEINER, S. & WRIGHT, W. E. 1998. Extension of life-span by introduction of telomerase into normal human cells. *Science,* 279**,** 349-52.

BONIZZI, G., BEBIEN, M., OTERO, D. C., JOHNSON-VROOM, K. E., CAO, Y., VU, D., JEGGA, A. G., ARONOW, B. J., GHOSH, G., RICKERT, R. C. & KARIN, M. 2004. Activation of IKKalpha target genes depends on recognition of specific kappaB binding sites by RelB:p52 dimers. *EMBO J,* 23**,** 4202-10.

BONIZZI, G. & KARIN, M. 2004. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. *Trends Immunol,* 25**,** 280-8.

BOUCHER, M. J., JEAN, D., VEZINA, A. & RIVARD, N. 2004. Dual role of MEK/ERK signaling in senescence and transformation of intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol,* 286**,** G736-46.

BOUWMAN, P., ALY, A., ESCANDELL, J. M., PIETERSE, M., BARTKOVA, J., VAN DER GULDEN, H., HIDDINGH, S., THANASOULA, M., KULKARNI, A., YANG, Q., HAFFTY, B. G., TOMMISKA, J., BLOMQVIST, C., DRAPKIN, R., ADAMS, D. J., NEVANLINNA, H., BARTEK, J., TARSOUNAS, M., GANESAN, S. & JONKERS, J. 2010. 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol,* 17**,** 688-95.

BRACKEN, A. P., KLEINE-KOHLBRECHER, D., DIETRICH, N., PASINI, D., GARGIULO, G., BEEKMAN, C., THEILGAARD-MONCH, K., MINUCCI, S., PORSE, B. T., MARINE, J. C., HANSEN, K. H. & HELIN, K. 2007. The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes & development,* 21**,** 525-30.

BRAIG, M., LEE, S., LODDENKEMPER, C., RUDOLPH, C., PETERS, A. H., SCHLEGELBERGER, B., STEIN, H., DORKEN, B., JENUWEIN, T. & SCHMITT, C. A. 2005. Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature,* 436**,** 660-5.

BRAIG, M. & SCHMITT, C. A. 2006. Oncogene-induced senescence: putting the brakes on tumor development. *Cancer Res,* 66**,** 2881-4.

BROCCOLI, D., YOUNG, J. W. & DE LANGE, T. 1995. Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad Sci U S A,* 92**,** 9082-6.

BROMBERG, J. F., WRZESZCZYNSKA, M. H., DEVGAN, G., ZHAO, Y., PESTELL, R. G., ALBANESE, C. & DARNELL, J. E., JR. 1999. Stat3 as an oncogene. *Cell,* 98**,** 295-303.

BROOKES, S., ROWE, J., GUTIERREZ DEL ARROYO, A., BOND, J. & PETERS, G. 2004. Contribution of p16(INK4a) to replicative senescence of human fibroblasts. *Exp Cell Res,* 298**,** 549-59.

BROOKES, S., ROWE, J., RUAS, M., LLANOS, S., CLARK, P. A., LOMAX, M., JAMES, M. C., VATCHEVA, R., BATES, S., VOUSDEN, K. H., PARRY, D., GRUIS, N., SMIT, N., BERGMAN, W. & PETERS, G. 2002. INK4a-deficient human diploid fibroblasts are resistant to RAS-induced senescence. *EMBO J,* 21**,** 2936-45.

BROWN, W. R. 1989. Molecular cloning of human telomeres in yeast. *Nature,* 338**,** 774-6.

BRUNK, U., ERICSSON, J. L., PONTEN, J. & WESTERMARK, B. 1973. Residual bodies and "aging" in cultured human glia cells. Effect of entrance into phase 3 and prolonged periods of confluence. *Exp Cell Res,* 79**,** 1-14.

BULAVIN, D. V., KOVALSKY, O., HOLLANDER, M. C. & FORNACE, A. J., JR. 2003. Loss of oncogenic H-ras-induced cell cycle arrest and p38 mitogen-activated protein kinase activation by disruption of Gadd45a. *Mol Cell Biol,* 23**,** 3859-71.

BULAVIN, D. V., PHILLIPS, C., NANNENGA, B., TIMOFEEV, O., DONEHOWER, L. A., ANDERSON, C. W., APPELLA, E. & FORNACE, A. J., JR. 2004. Inactivation of the Wip1 phosphatase inhibits mammary tumorigenesis through p38 MAPK-mediated activation of the p16(Ink4a)-p19(Arf) pathway. *Nat Genet,* 36**,** 343-50.

BULAVIN, D. V., SAITO, S., HOLLANDER, M. C., SAKAGUCHI, K., ANDERSON, C. W., APPELLA, E. & FORNACE, A. J., JR. 1999. Phosphorylation of human p53 by p38 kinase coordinates N-terminal phosphorylation and apoptosis in response to UV radiation. *EMBO J,* 18**,** 6845-54.

BUNTING, S. F., CALLEN, E., WONG, N., CHEN, H. T., POLATO, F., GUNN, A., BOTHMER, A., FELDHAHN, N., FERNANDEZ-CAPETILLO, O., CAO, L., XU, X., DENG, C. X., FINKEL, T., NUSSENZWEIG, M., STARK, J. M. & NUSSENZWEIG, A. 2010. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell,* 141**,** 243-54.

BUNZ, F., HWANG, P. M., TORRANCE, C., WALDMAN, T., ZHANG, Y., DILLEHAY, L., WILLIAMS, J., LENGAUER, C., KINZLER, K. W. & VOGELSTEIN, B. 1999. Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest,* 104**,** 263-9.

BURMA, S., CHEN, B. P., MURPHY, M., KURIMASA, A. & CHEN, D. J. 2001. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem,* 276**,** 42462-7.

CALLEBAUT, I. & MORNON, J. P. 1997. From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. *FEBS Lett,* 400**,** 25-30.

CAMPISI, J. 1996. Replicative senescence: an old lives' tale? *Cell,* 84**,** 497-500.

CANMAN, C. E., LIM, D. S., CIMPRICH, K. A., TAYA, Y., TAMAI, K., SAKAGUCHI, K., APPELLA, E., KASTAN, M. B. & SILICIANO, J. D. 1998. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science,* 281**,** 1677-9.

CARDOZO, T. & PAGANO, M. 2004. The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol,* 5**,** 739-51.

CATALANO, A., RODILOSSI, S., CAPRARI, P., COPPOLA, V. & PROCOPIO, A. 2005. 5-Lipoxygenase regulates senescence-like growth arrest by promoting ROS-dependent p53 activation. *EMBO J,* 24**,** 170-9.

CAYROL, C., KNIBIEHLER, M. & DUCOMMUN, B. 1998. p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene,* 16**,** 311-20.

CELESTE, A., FERNANDEZ-CAPETILLO, O., KRUHLAK, M. J., PILCH, D. R., STAUDT, D. W., LEE, A., BONNER, R. F., BONNER, W. M. & NUSSENZWEIG, A. 2003. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol,* 5**,** 675-9.

CELESTE, A., PETERSEN, S., ROMANIENKO, P. J., FERNANDEZ-CAPETILLO, O., CHEN, H. T., SEDELNIKOVA, O. A., REINA-SAN-MARTIN, B., COPPOLA, V., MEFFRE, E., DIFILIPPANTONIO, M. J., REDON, C., PILCH, D. R., OLARU, A., ECKHAUS, M., CAMERINI-OTERO, R. D., TESSAROLLO, L., LIVAK, F., MANOVA, K., BONNER, W. M., NUSSENZWEIG, M. C. & NUSSENZWEIG, A. 2002. Genomic instability in mice lacking histone H2AX. *Science,* 296**,** 922-7.

CHAN, F. K., CHUN, H. J., ZHENG, L., SIEGEL, R. M., BUI, K. L. & LENARDO, M. J. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science,* 288**,** 2351-4.

CHAN, T. A., HERMEKING, H., LENGAUER, C., KINZLER, K. W. & VOGELSTEIN, B. 1999. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature,* 401**,** 616-20.

CHARLES, A. C., NAUS, C. C., ZHU, D., KIDDER, G. M., DIRKSEN, E. R. & SANDERSON, M. J. 1992. Intercellular calcium signaling via gap junctions in glioma cells. *J Cell Biol,* 118**,** 195-201.

CHEHAB, N. H., MALIKZAY, A., APPEL, M. & HALAZONETIS, T. D. 2000. Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev,* 14**,** 278-88.

CHEN, G., HITOMI, M., HAN, J. & STACEY, D. W. 2000a. The p38 pathway provides negative feedback for Ras proliferative signaling. *J Biol Chem,* 275**,** 38973-80.

CHEN, J., ASTLE, C. M. & HARRISON, D. E. 1999. Development and aging of primitive hematopoietic stem cells in BALB/cBy mice. *Exp Hematol,* 27**,** 928-35.

CHEN, J. H., STOEBER, K., KINGSBURY, S., OZANNE, S. E., WILLIAMS, G. H. & HALES, C. N. 2004. Loss of proliferative capacity and induction of senescence in oxidatively stressed human fibroblasts. *J Biol Chem,* 279**,** 49439-46.

CHEN, K., CRAIGE, S. E. & KEANEY, J. F., JR. 2009. Downstream targets and intracellular compartmentalization in Nox signaling. *Antioxid Redox Signal,* 11**,** 2467-80.

CHEN, Q., FISCHER, A., REAGAN, J. D., YAN, L. J. & AMES, B. N. 1995. Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc Natl Acad Sci U S A,* 92**,** 4337-41.

CHEN, Q. M., BARTHOLOMEW, J. C., CAMPISI, J., ACOSTA, M., REAGAN, J. D. & AMES, B. N. 1998. Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J,* 332 (Pt 1)**,** 43-50.

CHEN, Q. M., TU, V. C., CATANIA, J., BURTON, M., TOUSSAINT, O. & DILLEY, T. 2000b. Involvement of Rb family proteins, focal adhesion proteins and protein synthesis in senescent morphogenesis induced by hydrogen peroxide. *J Cell Sci,* 113 (Pt 22)**,** 4087-97.

CHEN, Z., TROTMAN, L. C., SHAFFER, D., LIN, H. K., DOTAN, Z. A., NIKI, M., KOUTCHER, J. A., SCHER, H. I., LUDWIG, T., GERALD, W., CORDON-CARDO, C. & PANDOLFI, P. P. 2005. Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature,* 436**,** 725-30.

CHENG, M., OLIVIER, P., DIEHL, J. A., FERO, M., ROUSSEL, M. F., ROBERTS, J. M. & SHERR, C. J. 1999. The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *EMBO J,* 18**,** 1571-83.

CHEOK, C. F., KUA, N., KALDIS, P. & LANE, D. P. 2010. Combination of nutlin-3 and VX-680 selectively targets p53 mutant cells with reversible effects on cells expressing wild-type p53. *Cell Death Differ,* 17**,** 1486-500.

CHIEN, Y., SCUOPPO, C., WANG, X., FANG, X., BALGLEY, B., BOLDEN, J. E., PREMSRIRUT, P., LUO, W., CHICAS, A., LEE, C. S., KOGAN, S. C. & LOWE, S. W. 2011. Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes Dev,* 25**,** 2125-36.

CHIPMAN, J. K., MALLY, A. & EDWARDS, G. O. 2003. Disruption of gap junctions in toxicity and carcinogenicity. *Toxicol Sci,* 71**,** 146-53.

CHO, Y. Y., KIM, D. J., LEE, H. S., JEONG, C. H., CHO, E. J., KIM, M. O., BYUN, S., LEE, K. Y., YAO, K., CARPER, A., LANGFALD, A., BODE, A. M. & DONG, Z. 2013. Autophagy and cellular senescence mediated by Sox2 suppress malignancy of cancer cells. *PLoS One,* 8**,** e57172.

CHOPRA, A., FERREIRA-ALVES, D. L., SIROIS, P. & THIRION, J. P. 1992. Cloning of the guinea pig 5-lipoxygenase gene and nucleotide sequence of its promoter. *Biochem Biophys Res Commun,* 185**,** 489-95.

CHOWDHURY, D., KEOGH, M. C., ISHII, H., PETERSON, C. L., BURATOWSKI, S. & LIEBERMAN, J. 2005. gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. *Mol Cell,* 20**,** 801-9.

CHUNG, C. D., LIAO, J., LIU, B., RAO, X., JAY, P., BERTA, P. & SHUAI, K. 1997. Specific inhibition of Stat3 signal transduction by PIAS3. *Science,* 278**,** 1803-5.

CLERK, A., MICHAEL, A. & SUGDEN, P. H. 1998. Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? *J Cell Biol,* 142**,** 523-35.

COLLADO, M., GIL, J., EFEYAN, A., GUERRA, C., SCHUHMACHER, A. J., BARRADAS, M., BENGURIA, A., ZABALLOS, A., FLORES, J. M., BARBACID, M., BEACH, D. & SERRANO, M. 2005. Tumour biology: senescence in premalignant tumours. *Nature,* 436**,** 642.

COLLINS, R. J., HARMON, B. V., GOBE, G. C. & KERR, J. F. 1992. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *Int J Radiat Biol,* 61**,** 451-3.

COPPE, J. P., PATIL, C. K., RODIER, F., KRTOLICA, A., BEAUSEJOUR, C. M., PARRINELLO, S., HODGSON, J. G., CHIN, K., DESPREZ, P. Y. & CAMPISI, J. 2010. A human-like senescence-associated secretory phenotype is conserved in mouse cells dependent on physiological oxygen. *PLoS One,* 5**,** e9188.

COPPE, J. P., PATIL, C. K., RODIER, F., SUN, Y., MUNOZ, D. P., GOLDSTEIN, J., NELSON, P. S., DESPREZ, P. Y. & CAMPISI, J. 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol,* 6**,** 2853-68.

COPPE, J. P., RODIER, F., PATIL, C. K., FREUND, A., DESPREZ, P. Y. & CAMPISI, J. 2011. Tumor suppressor and aging biomarker p16(INK4a) induces cellular senescence without the associated inflammatory secretory phenotype. *J Biol Chem,* 286**,** 36396-403.

CORREIA-MELO, C., JURK, D. & PASSOS, J. F. 2013. Robust multiparametric assessment of cellular senescence. *Methods Mol Biol,* 965**,** 409-19.

CORTEZ, D., GUNTUKU, S., QIN, J. & ELLEDGE, S. J. 2001. ATR and ATRIP: partners in checkpoint signaling. *Science,* 294**,** 1713-6.

CORTEZ, D., WANG, Y., QIN, J. & ELLEDGE, S. J. 1999. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. *Science,* 286**,** 1162-6.

COUNTER, C. M., AVILION, A. A., LEFEUVRE, C. E., STEWART, N. G., GREIDER, C. W., HARLEY, C. B. & BACCHETTI, S. 1992. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J,* 11**,** 1921-9.

COUNTER, C. M., GUPTA, J., HARLEY, C. B., LEBER, B. & BACCHETTI, S. 1995. Telomerase activity in normal leukocytes and in hematologic malignancies. *Blood,* 85**,** 2315-20.

COURTOIS-COX, S., GENTHER WILLIAMS, S. M., RECZEK, E. E., JOHNSON, B. W., MCGILLICUDDY, L. T., JOHANNESSEN, C. M., HOLLSTEIN, P. E., MACCOLLIN, M. & CICHOWSKI, K. 2006. A negative feedback signaling network underlies oncogene-induced senescence. *Cancer Cell,* 10**,** 459-72.

CRAWLEY, J. B., RAWLINSON, L., LALI, F. V., PAGE, T. H., SAKLATVALA, J. & FOXWELL, B. M. 1997. T cell proliferation in response to interleukins 2 and 7 requires p38MAP kinase activation. *J Biol Chem,* 272**,** 15023-7.

CRIOLLO, A., SENOVILLA, L., AUTHIER, H., MAIURI, M. C., MORSELLI, E., VITALE, I., KEPP, O., TASDEMIR, E., GALLUZZI, L., SHEN, S., TAILLER, M., DELAHAYE, N., TESNIERE, A., DE STEFANO, D., YOUNES, A. B., HARPER, F., PIERRON, G., LAVANDERO, S., ZITVOGEL, L., ISRAEL, A., BAUD, V. & KROEMER, G. 2010a. The IKK complex contributes to the induction of autophagy. *EMBO J,* 29**,** 619-31.

CRIOLLO, A., SENOVILLA, L., AUTHIER, H., MAIURI, M. C., MORSELLI, E., VITALE, I., KEPP, O., TASDEMIR, E., GALLUZZI, L., SHEN, S., TAILLER, M., DELAHAYE, N., TESNIERE, A., DE STEFANO, D., YOUNES, A. B., HARPER, F., PIERRON, G., LAVANDERO, S., ZITVOGEL, L., ISRAEL, A., BAUD, V. & KROEMER, G. 2010b. IKK connects autophagy to major stress pathways. *Autophagy,* 6**,** 189-91.

CRISTOFALO, V. J., ALLEN, R. G., PIGNOLO, R. J., MARTIN, B. G. & BECK, J. C. 1998. Relationship between donor age and the replicative lifespan of human cells in culture: a reevaluation. *Proc Natl Acad Sci U S A,* 95**,** 10614-9.

CROSS, S., LINDSEY, J., FANTES, J., MCKAY, S., MCGILL, N. & COOKE, H. 1990. The structure of a subterminal repeated sequence present on many human chromosomes. *Nucleic Acids Res,* 18**,** 6649-57.

CROSS, S. H., ALLSHIRE, R. C., MCKAY, S. J., MCGILL, N. I. & COOKE, H. J. 1989. Cloning of human telomeres by complementation in yeast. *Nature,* 338**,** 771-4.

CUI, W., FOWLIS, D. J., BRYSON, S., DUFFIE, E., IRELAND, H., BALMAIN, A. & AKHURST, R. J. 1996. TGFbeta1 inhibits the formation of benign skin tumors, but enhances progression to invasive spindle carcinomas in transgenic mice. *Cell,* 86**,** 531-42.

D'ADDA DI FAGAGNA, F. 2008. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer,* 8**,** 512-22.

D'ADDA DI FAGAGNA, F., REAPER, P. M., CLAY-FARRACE, L., FIEGLER, H., CARR, P., VON ZGLINICKI, T., SARETZKI, G., CARTER, N. P. & JACKSON, S. P. 2003. A DNA damage checkpoint response in telomere-initiated senescence. *Nature,* 426**,** 194-8.

D'AMOURS, D. & JACKSON, S. P. 2002. The Mre11 complex: at the crossroads of dna repair and checkpoint signalling. *Nat Rev Mol Cell Biol,* 3**,** 317-27.

D'AUTREAUX, B. & TOLEDANO, M. B. 2007. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol,* 8**,** 813-24.

DAJEE, M., LAZAROV, M., ZHANG, J. Y., CAI, T., GREEN, C. L., RUSSELL, A. J., MARINKOVICH, M. P., TAO, S., LIN, Q., KUBO, Y. & KHAVARI, P. A. 2003. NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature,* 421**,** 639-43.

DAN, H. C., COOPER, M. J., COGSWELL, P. C., DUNCAN, J. A., TING, J. P. & BALDWIN, A. S. 2008. Akt-dependent regulation of NF-{kappa}B is controlled by mTOR and Raptor in association with IKK. *Genes Dev,* 22**,** 1490-500.

DANKORT, D., FILENOVA, E., COLLADO, M., SERRANO, M., JONES, K. & MCMAHON, M. 2007. A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors. *Genes Dev,* 21**,** 379-84.

DANN, S. G. & THOMAS, G. 2006. The amino acid sensitive TOR pathway from yeast to mammals. *FEBS Lett,* 580**,** 2821-9.

DARZYNKIEWICZ, Z. 2002. One more reason to get arrested (in G1). *Cell Cycle,* 1**,** 318-9.

DATTO, M. B., LI, Y., PANUS, J. F., HOWE, D. J., XIONG, Y. & WANG, X. F. 1995. Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proceedings of the National Academy of Sciences of the United States of America,* 92**,** 5545-9.

DAVIDSON, I. F., LI, A. & BLOW, J. J. 2006. Deregulated replication licensing causes DNA fragmentation consistent with head-to-tail fork collision. *Mol Cell,* 24**,** 433-43.

DAVIS, T., BAIRD, D. M., HAUGHTON, M. F., JONES, C. J. & KIPLING, D. 2005. Prevention of accelerated cell aging in Werner syndrome using a p38 mitogen-activated protein kinase inhibitor. *J Gerontol A Biol Sci Med Sci,* 60**,** 1386-93.

DE HAAN, G., NIJHOF, W. & VAN ZANT, G. 1997. Mouse strain-dependent changes in frequency and proliferation of hematopoietic stem cells during aging: correlation between lifespan and cycling activity. *Blood,* 89**,** 1543-50.

DEBACQ-CHAINIAUX, F., BORLON, C., PASCAL, T., ROYER, V., ELIAERS, F., NINANE, N., CARRARD, G., FRIGUET, B., DE LONGUEVILLE, F., BOFFE, S., REMACLE, J. & TOUSSAINT, O. 2005. Repeated exposure of human skin fibroblasts to UVB at subcytotoxic level triggers premature senescence through the TGF-beta1 signaling pathway. *J Cell Sci,* 118**,** 743-58.

DEBIDDA, M., WILLIAMS, D. A. & ZHENG, Y. 2006. Rac1 GTPase regulates cell genomic stability and senescence. *J Biol Chem,* 281**,** 38519-28.

DEHEZ, S., DAULHAC, L., KOWALSKI-CHAUVEL, A., FOURMY, D., PRADAYROL, L. & SEVA, C. 2001. Gastrin-induced DNA synthesis requires p38-MAPK activation via PKC/Ca(2+) and Src-dependent mechanisms. *FEBS Lett,* 496**,** 25-30.

DELLAMBRA, E., GOLISANO, O., BONDANZA, S., SIVIERO, E., LACAL, P., MOLINARI, M., D'ATRI, S. & DE LUCA, M. 2000. Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes. *J Cell Biol,* 149**,** 1117-30.

DEMIDENKO, Z. N., KOROTCHKINA, L. G., GUDKOV, A. V. & BLAGOSKLONNY, M. V. 2010. Paradoxical suppression of cellular senescence by p53. *Proc Natl Acad Sci U S A,* 107**,** 9660-4.

DENCHI, E. L. & DE LANGE, T. 2007. Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. *Nature,* 448**,** 1068-71.

DENG, C., ZHANG, P., HARPER, J. W., ELLEDGE, S. J. & LEDER, P. 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell,* 82**,** 675-84.

DENG, Q., LIAO, R., WU, B. L. & SUN, P. 2004. High intensity ras signaling induces premature senescence by activating p38 pathway in primary human fibroblasts. *J Biol Chem,* 279**,** 1050-9.

DENG, W. G., ZHU, Y. & WU, K. K. 2003. Up-regulation of p300 binding and p50 acetylation in tumor necrosis factor-alpha-induced cyclooxygenase-2 promoter activation. *J Biol Chem,* 278**,** 4770-7.

DENOYELLE, C., ABOU-RJAILY, G., BEZROOKOVE, V., VERHAEGEN, M., JOHNSON, T. M., FULLEN, D. R., POINTER, J. N., GRUBER, S. B., SU, L. D., NIKIFOROV, M. A., KAUFMAN, R. J., BASTIAN, B. C. & SOENGAS, M. S. 2006. Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. *Nat Cell Biol,* 8**,** 1053-63.

DERBYSHIRE, D. J., BASU, B. P., SERPELL, L. C., JOO, W. S., DATE, T., IWABUCHI, K. & DOHERTY, A. J. 2002. Crystal structure of human 53BP1 BRCT domains bound to p53 tumour suppressor. *EMBO J,* 21**,** 3863-72.

DESHPANDE, A., GOODWIN, E. H., BAILEY, S. M., MARRONE, B. L. & LEHNERT, B. E. 1996. Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. *Radiat Res,* 145**,** 260-7.

DI LEONARDO, A., LINKE, S. P., CLARKIN, K. & WAHL, G. M. 1994. DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev,* 8**,** 2540-51.

DI MICCO, R., FUMAGALLI, M., CICALESE, A., PICCININ, S., GASPARINI, P., LUISE, C., SCHURRA, C., GARRE, M., NUCIFORO, P. G., BENSIMON, A., MAESTRO, R., PELICCI, P. G. & D'ADDA DI FAGAGNA, F. 2006. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature,* 444**,** 638-42.

DI MICCO, R., SULLI, G., DOBREVA, M., LIONTOS, M., BOTRUGNO, O. A., GARGIULO, G., DAL ZUFFO, R., MATTI, V., D'ARIO, G., MONTANI, E., MERCURIO, C., HAHN, W. C., GORGOULIS, V., MINUCCI, S. & D'ADDA DI FAGAGNA, F. 2011. Interplay between oncogene-induced DNA damage response and heterochromatin in senescence and cancer. *Nat Cell Biol,* 13**,** 292-302.

DIMRI, G. P., LEE, X., BASILE, G., ACOSTA, M., SCOTT, G., ROSKELLEY, C., MEDRANO, E. E., LINSKENS, M., RUBELJ, I., PEREIRA-SMITH, O. & ET AL. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A,* 92**,** 9363-7.

DIRAC, A. M. & BERNARDS, R. 2003. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J Biol Chem,* 278**,** 11731-4.

DITULLIO, R. A., JR., MOCHAN, T. A., VENERE, M., BARTKOVA, J., SEHESTED, M., BARTEK, J. & HALAZONETIS, T. D. 2002. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. *Nat Cell Biol,* 4**,** 998-1002.

DJAVAHERI-MERGNY, M., JAVELAUD, D., WIETZERBIN, J. & BESANCON, F. 2004. NF-kappaB activation prevents apoptotic oxidative stress via an increase of both thioredoxin and MnSOD levels in TNFalpha-treated Ewing sarcoma cells. *FEBS Lett,* 578**,** 111-5.

DOI, T. S., MARINO, M. W., TAKAHASHI, T., YOSHIDA, T., SAKAKURA, T., OLD, L. J. & OBATA, Y. 1999. Absence of tumor necrosis factor rescues RelA-deficient mice from embryonic lethality. *Proc Natl Acad Sci U S A,* 96**,** 2994-9.

DOMINGUEZ-SOLA, D., YING, C. Y., GRANDORI, C., RUGGIERO, L., CHEN, B., LI, M., GALLOWAY, D. A., GU, W., GAUTIER, J. & DALLA-FAVERA, R. 2007. Non-transcriptional control of DNA replication by c-Myc. *Nature,* 448**,** 445-51.

DONG, Z., ZHONG, Q. & CHEN, P. L. 1999. The Nijmegen breakage syndrome protein is essential for Mre11 phosphorylation upon DNA damage. *J Biol Chem,* 274**,** 19513-6.

DORIGO, B., SCHALCH, T., KULANGARA, A., DUDA, S., SCHROEDER, R. R. & RICHMOND, T. J. 2004. Nucleosome arrays reveal the two-start organization of the chromatin fiber. *Science,* 306**,** 1571-3.

DOWLING-WARRINER, C. V. & TROSKO, J. E. 2000. Induction of gap junctional intercellular communication, connexin43 expression, and subsequent differentiation in human fetal neuronal cells by stimulation of the cyclic AMP pathway. *Neuroscience,* 95**,** 859-68.

DREESEN, O., CHOJNOWSKI, A., ONG, P. F., ZHAO, T. Y., COMMON, J. E., LUNNY, D., LANE, E. B., LEE, S. J., VARDY, L. A., STEWART, C. L. & COLMAN, A. 2013. Lamin B1 fluctuations have differential effects on cellular proliferation and senescence. *J Cell Biol,* 200**,** 605-17.

DRUMMOND, M. J., BELL, J. A., FUJITA, S., DREYER, H. C., GLYNN, E. L., VOLPI, E. & RASMUSSEN, B. B. 2008. Amino acids are necessary for the insulin-induced activation of mTOR/S6K1 signaling and protein synthesis in healthy and insulin resistant human skeletal muscle. *Clin Nutr,* 27**,** 447-56.

DUMAZ, N. & MEEK, D. W. 1999. Serine15 phosphorylation stimulates p53 transactivation but does not directly influence interaction with HDM2. *EMBO J,* 18**,** 7002-10.

DUMONT, P., BALBEUR, L., REMACLE, J. & TOUSSAINT, O. 2000a. Appearance of biomarkers of in vitro ageing after successive stimulation of WI-38 fibroblasts with IL-1alpha and TNF-alpha: senescence associated beta-galactosidase activity and morphotype transition. *J Anat,* 197 Pt 4**,** 529-37.

DUMONT, P., BURTON, M., CHEN, Q. M., GONOS, E. S., FRIPPIAT, C., MAZARATI, J. B., ELIAERS, F., REMACLE, J. & TOUSSAINT, O. 2000b. Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. *Free Radic Biol Med,* 28**,** 361-73.

ECHETEBU, C. O., ALI, M., IZBAN, M. G., MACKAY, L. & GARFIELD, R. E. 1999. Localization of regulatory protein binding sites in the proximal region of human myometrial connexin 43 gene. *Mol Hum Reprod,* 5**,** 757-66.

EDINGER, A. L. & THOMPSON, C. B. 2002. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell,* 13**,** 2276-88.

EFEYAN, A., ORTEGA-MOLINA, A., VELASCO-MIGUEL, S., HERRANZ, D., VASSILEV, L. T. & SERRANO, M. 2007. Induction of p53-dependent senescence by the MDM2 antagonist nutlin-3a in mouse cells of fibroblast origin. *Cancer Res,* 67**,** 7350-7.

ELSHARKAWY, A. M., OAKLEY, F., LIN, F., PACKHAM, G., MANN, D. A. & MANN, J. 2010. The NF-kappaB p50:p50:HDAC-1 repressor complex orchestrates transcriptional inhibition of multiple pro-inflammatory genes. *J Hepatol,* 53**,** 519-27.

ERSTER, S., MIHARA, M., KIM, R. H., PETRENKO, O. & MOLL, U. M. 2004. In vivo mitochondrial p53 translocation triggers a rapid first wave of cell death in response to DNA damage that can precede p53 target gene activation. *Mol Cell Biol,* 24**,** 6728-41.

ERUSALIMSKY, J. D. & KURZ, D. J. 2005. Cellular senescence in vivo: its relevance in ageing and cardiovascular disease. *Exp Gerontol,* 40**,** 634-42.

ESPINOSA, J. M. 2008. Mechanisms of regulatory diversity within the p53 transcriptional network. *Oncogene,* 27**,** 4013-23.

ESTIN, D., LI, M., SPRAY, D. & WU, J. K. 1999. Connexins are expressed in primary brain tumors and enhance the bystander effect in gene therapy. *Neurosurgery,* 44**,** 361-8; discussion 368-9.

FALCK, J., COATES, J. & JACKSON, S. P. 2005. Conserved modes of recruitment of ATM, ATR and DNA-PKcs to sites of DNA damage. *Nature,* 434**,** 605-11.

FALCK, J., MAILAND, N., SYLJUASEN, R. G., BARTEK, J. & LUKAS, J. 2001. The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature,* 410**,** 842-7.

FANG, L., LI, G., LIU, G., LEE, S. W. & AARONSON, S. A. 2001. p53 induction of heparin-binding EGF-like growth factor counteracts p53 growth suppression through activation of MAPK and PI3K/Akt signaling cascades. *EMBO J,* 20**,** 1931-9.

FERNANDEZ-CAPETILLO, O., CHEN, H. T., CELESTE, A., WARD, I., ROMANIENKO, P. J., MORALES, J. C., NAKA, K., XIA, Z., CAMERINI-OTERO, R. D., MOTOYAMA, N., CARPENTER, P. B., BONNER, W. M., CHEN, J. & NUSSENZWEIG, A. 2002. DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1. *Nat Cell Biol,* 4**,** 993-7.

FERNANDEZ-CAPETILLO, O., LEE, A., NUSSENZWEIG, M. & NUSSENZWEIG, A. 2004. H2AX: the histone guardian of the genome. *DNA Repair (Amst),* 3**,** 959-67.

FINGAR, D. C., RICHARDSON, C. J., TEE, A. R., CHEATHAM, L., TSOU, C. & BLENIS, J. 2004. mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. *Mol Cell Biol,* 24**,** 200-16.

FLEETWOOD, A. J., COOK, A. D. & HAMILTON, J. A. 2005. Functions of granulocyte-macrophage colony-stimulating factor. *Critical reviews in immunology,* 25**,** 405-28.

FREUND, A., PATIL, C. K. & CAMPISI, J. 2011. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. *EMBO J,* 30**,** 1536-48.

FRIAS, M. A., THOREEN, C. C., JAFFE, J. D., SCHRODER, W., SCULLEY, T., CARR, S. A. & SABATINI, D. M. 2006. mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. *Curr Biol,* 16**,** 1865-70.

FUMAGALLI, M., ROSSIELLO, F., CLERICI, M., BAROZZI, S., CITTARO, D., KAPLUNOV, J. M., BUCCI, G., DOBREVA, M., MATTI, V., BEAUSEJOUR, C. M., HERBIG, U., LONGHESE, M. P. & D'ADDA DI FAGAGNA, F. 2012. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. *Nat Cell Biol,* 14**,** 355-65.

FURUSU, A., NAKAYAMA, K., XU, Q., KONTA, T. & KITAMURA, M. 2007. MAP kinase-dependent, NF-kappaB-independent regulation of inhibitor of apoptosis protein genes by TNF-alpha. *J Cell Physiol,* 210**,** 703-10.

FURUTA, T., TAKEMURA, H., LIAO, Z. Y., AUNE, G. J., REDON, C., SEDELNIKOVA, O. A., PILCH, D. R., ROGAKOU, E. P., CELESTE, A., CHEN, H. T., NUSSENZWEIG, A., ALADJEM, M. I., BONNER, W. M. & POMMIER, Y. 2003. Phosphorylation of histone H2AX and activation of Mre11, Rad50, and Nbs1 in response to replication-dependent DNA double-strand breaks induced by mammalian DNA topoisomerase I cleavage complexes. *J Biol Chem,* 278**,** 20303-12.

GATEI, M., YOUNG, D., CEROSALETTI, K. M., DESAI-MEHTA, A., SPRING, K., KOZLOV, S., LAVIN, M. F., GATTI, R. A., CONCANNON, P. & KHANNA, K. 2000. ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat Genet,* 25**,** 115-9.

GEISER, T., DEWALD, B., EHRENGRUBER, M. U., CLARK-LEWIS, I. & BAGGIOLINI, M. 1993. The interleukin-8-related chemotactic cytokines GRO alpha, GRO beta, and GRO gamma activate human neutrophil and basophil leukocytes. *The Journal of biological chemistry,* 268**,** 15419-24.

GEORGAKOPOULOU, E. A., TSIMARATOU, K., EVANGELOU, K., FERNANDEZ MARCOS, P. J., ZOUMPOURLIS, V., TROUGAKOS, I. P., KLETSAS, D., BARTEK, J., SERRANO, M. & GORGOULIS, V. G. 2013. Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues. *Aging (Albany NY),* 5**,** 37-50.

GIRE, V., ROUX, P., WYNFORD-THOMAS, D., BRONDELLO, J. M. & DULIC, V. 2004. DNA damage checkpoint kinase Chk2 triggers replicative senescence. *EMBO J,* 23**,** 2554-63.

GOLDBERG, M., STUCKI, M., FALCK, J., D'AMOURS, D., RAHMAN, D., PAPPIN, D., BARTEK, J. & JACKSON, S. P. 2003. MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature,* 421**,** 952-6.

GOLDSTEIN, S. 1978. Human genetic disorders that feature premature onset and accelerated progression of biological aging. *In:* SCHNEIDER, E. L. (ed.) *The Genetics of Aging.* New York: Plenum Press.

GOLDSTEIN, S. 1990. Replicative senescence: the human fibroblast comes of age. *Science,* 249**,** 1129-33.

GOLDSTEIN, S. & SHMOOKLER REIS, R. J. 1985. Methylation patterns in the gene for the alpha subunit of chorionic gonadotropin are inherited with variable fidelity in clonal lineages of human fibroblasts. *Nucleic Acids Res,* 13**,** 7055-65.

GONG, J., TRAGANOS, F. & DARZYNKIEWICZ, Z. 1995. Growth imbalance and altered expression of cyclins B1, A, E, and D3 in MOLT-4 cells synchronized in the cell cycle by inhibitors of DNA replication. *Cell Growth Differ,* 6**,** 1485-93.

GRANDORI, C., WU, K. J., FERNANDEZ, P., NGOUENET, C., GRIM, J., CLURMAN, B. E., MOSER, M. J., OSHIMA, J., RUSSELL, D. W., SWISSHELM, K., FRANK, S., AMATI, B., DALLA-FAVERA, R. & MONNAT, R. J., JR. 2003. Werner syndrome protein limits MYC-induced cellular senescence. *Genes Dev,* 17**,** 1569-74.

GREIDER, C. W. & BLACKBURN, E. H. 1985. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell,* 43**,** 405-13.

GRIFFITH, J. D., COMEAU, L., ROSENFIELD, S., STANSEL, R. M., BIANCHI, A., MOSS, H. & DE LANGE, T. 1999. Mammalian telomeres end in a large duplex loop. *Cell,* 97**,** 503-14.

GRIVENNIKOV, S. I. & KARIN, M. 2010. Dangerous liaisons: STAT3 and NF-kappaB collaboration and crosstalk in cancer. *Cytokine Growth Factor Rev,* 21**,** 11-9.

GUERRA, C., MIJIMOLLE, N., DHAWAHIR, A., DUBUS, P., BARRADAS, M., SERRANO, M., CAMPUZANO, V. & BARBACID, M. 2003. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell,* 4**,** 111-20.

GUERRA, C., SCHUHMACHER, A. J., CANAMERO, M., GRIPPO, P. J., VERDAGUER, L., PEREZ-GALLEGO, L., DUBUS, P., SANDGREN, E. P. & BARBACID, M. 2007. Chronic pancreatitis is essential for induction of pancreatic ductal adenocarcinoma by K-Ras oncogenes in adult mice. *Cancer Cell,* 11**,** 291-302.

GUO, Z., KOZLOV, S., LAVIN, M. F., PERSON, M. D. & PAULL, T. T. 2010. ATM activation by oxidative stress. *Science,* 330**,** 517-21.

GUSCHIN, D., ROGERS, N., BRISCOE, J., WITTHUHN, B., WATLING, D., HORN, F., PELLEGRINI, S., YASUKAWA, K., HEINRICH, P., STARK, G. R. & ET AL. 1995. A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. *EMBO J,* 14**,** 1421-9.

GUTIERREZ-UZQUIZA, A., ARECHEDERRA, M., BRAGADO, P., AGUIRRE-GHISO, J. A. & PORRAS, A. 2012. p38alpha mediates cell survival in response to oxidative stress via induction of antioxidant genes: effect on the p70S6K pathway. *J Biol Chem,* 287**,** 2632-42.

GUTTRIDGE, D. C., ALBANESE, C., REUTHER, J. Y., PESTELL, R. G. & BALDWIN, A. S., JR. 1999. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. *Mol Cell Biol,* 19**,** 5785-99.

HAFERKAMP, S., SCURR, L. L., BECKER, T. M., FRAUSTO, M., KEFFORD, R. F. & RIZOS, H. 2009. Oncogene-induced senescence does not require the p16(INK4a) or p14ARF melanoma tumor suppressors. *J Invest Dermatol,* 129**,** 1983-91.

HAGEN, T. M., YOWE, D. L., BARTHOLOMEW, J. C., WEHR, C. M., DO, K. L., PARK, J. Y. & AMES, B. N. 1997. Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. *Proc Natl Acad Sci U S A,* 94**,** 3064-9.

HAHN, W. C., COUNTER, C. M., LUNDBERG, A. S., BEIJERSBERGEN, R. L., BROOKS, M. W. & WEINBERG, R. A. 1999. Creation of human tumour cells with defined genetic elements. *Nature,* 400**,** 464-8.

HAMILTON, J. A. 2002. GM-CSF in inflammation and autoimmunity. *Trends Immunol,* 23**,** 403-8.

HANNKEN, T., SCHROEDER, R., STAHL, R. A. & WOLF, G. 1998. Angiotensin II-mediated expression of p27Kip1 and induction of cellular hypertrophy in renal tubular cells depend on the generation of oxygen radicals. *Kidney Int,* 54**,** 1923-33.

HANNON, G. J. & BEACH, D. 1994. p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature,* 371**,** 257-61.

HARA, E., SMITH, R., PARRY, D., TAHARA, H., STONE, S. & PETERS, G. 1996. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol Cell Biol,* 16**,** 859-67.

HARLEY, C. B., FUTCHER, A. B. & GREIDER, C. W. 1990. Telomeres shorten during ageing of human fibroblasts. *Nature,* 345**,** 458-60.

HARRISON, D. E., ASTLE, C. M. & STONE, M. 1989. Numbers and functions of transplantable primitive immunohematopoietic stem cells. Effects of age. *J Immunol,* 142**,** 3833-40.

HARVEY, M., SANDS, A. T., WEISS, R. S., HEGI, M. E., WISEMAN, R. W., PANTAZIS, P., GIOVANELLA, B. C., TAINSKY, M. A., BRADLEY, A. & DONEHOWER, L. A. 1993. In vitro

growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene,* 8**,** 2457-67.

HASTIE, N. D., DEMPSTER, M., DUNLOP, M. G., THOMPSON, A. M., GREEN, D. K. & ALLSHIRE, R. C. 1990. Telomere reduction in human colorectal carcinoma and with ageing. *Nature,* 346**,** 866-8.

HAUPT, S., BERGER, M., GOLDBERG, Z. & HAUPT, Y. 2003. Apoptosis - the p53 network. *J Cell Sci,* 116**,** 4077-85.

HAYDEN, M. S. & GHOSH, S. 2004. Signaling to NF-kappaB. *Genes Dev,* 18**,** 2195-224.

HAYFLICK, L. 1965. The Limited in Vitro Lifetime of Human Diploid Cell Strains. *Exp Cell Res,* 37**,** 614-36.

HAYFLICK, L. & MOORHEAD, P. S. 1961. The serial cultivation of human diploid cell strains. *Exp Cell Res,* 25**,** 585-621.

HE, L., HE, X., LOWE, S. W. & HANNON, G. J. 2007. microRNAs join the p53 network--another piece in the tumour-suppression puzzle. *Nat Rev Cancer,* 7**,** 819-22.

HEINONEN, H., NIEMINEN, A., SAARELA, M., KALLIONIEMI, A., KLEFSTROM, J., HAUTANIEMI, S. & MONNI, O. 2008. Deciphering downstream gene targets of PI3K/mTOR/p70S6K pathway in breast cancer. *BMC Genomics,* 9**,** 348.

HEINRICH, P. C., BEHRMANN, I., MULLER-NEWEN, G., SCHAPER, F. & GRAEVE, L. 1998. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. *Biochem J,* 334 (Pt 2)**,** 297-314.

HELENIUS, M., HANNINEN, M., LEHTINEN, S. K. & SALMINEN, A. 1996. Changes associated with aging and replicative senescence in the regulation of transcription factor nuclear factor-kappa B. *Biochem J,* 318 (Pt 2)**,** 603-8.

HERBIG, U., JOBLING, W. A., CHEN, B. P., CHEN, D. J. & SEDIVY, J. M. 2004. Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a). *Mol Cell,* 14**,** 501-13.

HERMANNS, H. M., RADTKE, S., SCHAPER, F., HEINRICH, P. C. & BEHRMANN, I. 2000. Non-redundant signal transduction of interleukin-6-type cytokines. The adapter protein Shc is specifically recruited to rhe oncostatin M receptor. *J Biol Chem,* 275**,** 40742-8.

HERMEKING, H. 2007. p53 enters the microRNA world. *Cancer Cell,* 12**,** 414-8.

HERMEKING, H., LENGAUER, C., POLYAK, K., HE, T. C., ZHANG, L., THIAGALINGAM, S., KINZLER, K. W. & VOGELSTEIN, B. 1997. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell,* 1**,** 3-11.

HEWITT, G., JURK, D., MARQUES, F. D., CORREIA-MELO, C., HARDY, T., GACKOWSKA, A., ANDERSON, R., TASCHUK, M., MANN, J. & PASSOS, J. F. 2012. Telomeres are favoured targets of a persistent DNA damage response in ageing and stress-induced senescence. *Nat Commun,* 3**,** 708.

HINZ, M., KRAPPMANN, D., EICHTEN, A., HEDER, A., SCHEIDEREIT, C. & STRAUSS, M. 1999. NF-kappaB function in growth control: regulation of cyclin D1 expression and G0/G1-to-S-phase transition. *Mol Cell Biol,* 19**,** 2690-8.

HINZ, M., STILMANN, M., ARSLAN, S. C., KHANNA, K. K., DITTMAR, G. & SCHEIDEREIT, C. 2010. A cytoplasmic ATM-TRAF6-cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kappaB activation. *Mol Cell,* 40**,** 63-74.

HIRAO, A., CHEUNG, A., DUNCAN, G., GIRARD, P. M., ELIA, A. J., WAKEHAM, A., OKADA, H., SARKISSIAN, T., WONG, J. A., SAKAI, T., DE STANCHINA, E., BRISTOW, R. G., SUDA, T., LOWE, S. W., JEGGO, P. A., ELLEDGE, S. J. & MAK, T. W. 2002. Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol Cell Biol,* 22**,** 6521-32.

HIYAMA, E., HIYAMA, K., YOKOYAMA, T., MATSUURA, Y., PIATYSZEK, M. A. & SHAY, J. W. 1995a. Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med,* 1**,** 249-55.

HIYAMA, K., HIRAI, Y., KYOIZUMI, S., AKIYAMA, M., HIYAMA, E., PIATYSZEK, M. A., SHAY, J. W., ISHIOKA, S. & YAMAKIDO, M. 1995b. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol,* 155**,** 3711-5.

HIYAMA, K., HIYAMA, E., ISHIOKA, S., YAMAKIDO, M., INAI, K., GAZDAR, A. F., PIATYSZEK, M. A. & SHAY, J. W. 1995c. Telomerase activity in small-cell and non-small-cell lung cancers. *J Natl Cancer Inst,* 87**,** 895-902.

HOFFMANN, A., LEVCHENKO, A., SCOTT, M. L. & BALTIMORE, D. 2002. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. *Science,* 298**,** 1241-5.

HONG, E. H., LEE, S. J., KIM, J. S., LEE, K. H., UM, H. D., KIM, J. H., KIM, S. J., KIM, J. I. & HWANG, S. G. 2010. Ionizing radiation induces cellular senescence of articular chondrocytes via negative regulation of SIRT1 by p38 kinase. *J Biol Chem,* 285**,** 1283-95.

HOVEST, M. G., BRUGGENOLTE, N., HOSSEINI, K. S., KRIEG, T. & HERRMANN, G. 2006. Senescence of human fibroblasts after psoralen photoactivation is mediated by ATR kinase and persistent DNA damage foci at telomeres. *Mol Biol Cell,* 17**,** 1758-67.

HU, V. W. & XIE, H. Q. 1994. Interleukin-1 alpha suppresses gap junction-mediated intercellular communication in human endothelial cells. *Exp Cell Res,* 213**,** 218-23.

HUANG, B., DEO, D., XIA, M. & VASSILEV, L. T. 2009. Pharmacologic p53 activation blocks cell cycle progression but fails to induce senescence in epithelial cancer cells. *Mol Cancer Res,* 7**,** 1497-509.

HUANG, C., LI, J., KE, Q., LEONARD, S. S., JIANG, B. H., ZHONG, X. S., COSTA, M., CASTRANOVA, V. & SHI, X. 2002. Ultraviolet-induced phosphorylation of p70(S6K) at Thr(389) and Thr(421)/Ser(424) involves hydrogen peroxide and mammalian target of rapamycin but not Akt and atypical protein kinase C. *Cancer Res,* 62**,** 5689-97.

HUANG, J. & MANNING, B. D. 2009. A complex interplay between Akt, TSC2 and the two mTOR complexes. *Biochem Soc Trans,* 37**,** 217-22.

HUNG, C. M., GARCIA-HARO, L., SPARKS, C. A. & GUERTIN, D. A. 2012. mTOR-dependent cell survival mechanisms. *Cold Spring Harb Perspect Biol,* 4.

HUNZIKER, A., JENSEN, M. H. & KRISHNA, S. 2010. Stress-specific response of the p53-Mdm2 feedback loop. *BMC Syst Biol,* 4**,** 94.

HUSE, M., CHEN, Y. G., MASSAGUE, J. & KURIYAN, J. 1999. Crystal structure of the cytoplasmic domain of the type I TGF beta receptor in complex with FKBP12. *Cell,* 96**,** 425-36.

HUYEN, Y., ZGHEIB, O., DITULLIO, R. A., JR., GORGOULIS, V. G., ZACHARATOS, P., PETTY, T. J., SHESTON, E. A., MELLERT, H. S., STAVRIDI, E. S. & HALAZONETIS, T. D. 2004. Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature,* 432**,** 406-11.

IMLAY, J. A., CHIN, S. M. & LINN, S. 1988. Toxic DNA damage by hydrogen peroxide through the Fenton reaction in vivo and in vitro. *Science,* 240**,** 640-2.

INOKI, K., LI, Y., ZHU, T., WU, J. & GUAN, K. L. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol,* 4**,** 648-57.

INOUE, H. & TANABE, T. 1998. Transcriptional role of the nuclear factor kappa B site in the induction by lipopolysaccharide and suppression by dexamethasone of cyclooxygenase-2 in U937 cells. *Biochem Biophys Res Commun,* 244**,** 143-8.

ISHII, K., YAMAOKA, K., HOSOI, Y., ONO, T. & SAKAMOTO, K. 1995. Enhanced mitogen-induced proliferation of rat splenocytes by low-dose whole-body X-irradiation. *Physiol Chem Phys Med NMR,* 27**,** 17-23.

ITO, K., HIRAO, A., ARAI, F., MATSUOKA, S., TAKUBO, K., HAMAGUCHI, I., NOMIYAMA, K., HOSOKAWA, K., SAKURADA, K., NAKAGATA, N., IKEDA, Y., MAK, T. W. & SUDA, T. 2004. Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature,* 431**,** 997-1002.

ITO, K., HIRAO, A., ARAI, F., TAKUBO, K., MATSUOKA, S., MIYAMOTO, K., OHMURA, M., NAKA, K., HOSOKAWA, K., IKEDA, Y. & SUDA, T. 2006. Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med,* 12**,** 446-51.

IWABUCHI, K., BARTEL, P. L., LI, B., MARRACCINO, R. & FIELDS, S. 1994. Two cellular proteins that bind to wild-type but not mutant p53. *Proc Natl Acad Sci U S A,* 91**,** 6098-102.

IWABUCHI, K., BASU, B. P., KYSELA, B., KURIHARA, T., SHIBATA, M., GUAN, D., CAO, Y., HAMADA, T., IMAMURA, K., JEGGO, P. A., DATE, T. & DOHERTY, A. J. 2003. Potential role for 53BP1 in DNA end-joining repair through direct interaction with DNA. *J Biol Chem,* 278**,** 36487-95.

IWABUCHI, K., LI, B., MASSA, H. F., TRASK, B. J., DATE, T. & FIELDS, S. 1998. Stimulation of p53-mediated transcriptional activation by the p53-binding proteins, 53BP1 and 53BP2. *J Biol Chem,* 273**,** 26061-8.

IWASA, H., HAN, J. & ISHIKAWA, F. 2003. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. *Genes Cells,* 8**,** 131-44.

JACOBS, J. J. & DE LANGE, T. 2004. Significant role for p16INK4a in p53-independent telomere-directed senescence. *Curr Biol,* 14**,** 2302-8.

JAZAYERI, A., FALCK, J., LUKAS, C., BARTEK, J., SMITH, G. C., LUKAS, J. & JACKSON, S. P. 2006. ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol,* 8**,** 37-45.

JEYAPALAN, J. C., FERREIRA, M., SEDIVY, J. M. & HERBIG, U. 2007. Accumulation of senescent cells in mitotic tissue of aging primates. *Mech Ageing Dev,* 128**,** 36-44.

JING, H., KASE, J., DORR, J. R., MILANOVIC, M., LENZE, D., GRAU, M., BEUSTER, G., JI, S., REIMANN, M., LENZ, P., HUMMEL, M., DORKEN, B., LENZ, G., SCHEIDEREIT, C., SCHMITT, C. A. & LEE, S. 2011. Opposing roles of NF-kappaB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev,* 25**,** 2137-46.

JOHNSON, D. G. & DEGREGORI, J. 2006. Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. *Curr Mol Med,* 6**,** 731-8.

JONES, C. J., KIPLING, D., MORRIS, M., HEPBURN, P., SKINNER, J., BOUNACER, A., WYLLIE, F. S., IVAN, M., BARTEK, J., WYNFORD-THOMAS, D. & BOND, J. A. 2000. Evidence for a telomere-independent "clock" limiting RAS oncogene-driven proliferation of human thyroid epithelial cells. *Mol Cell Biol,* 20**,** 5690-9.

JONES, P. L., PING, D. & BOSS, J. M. 1997. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol Cell Biol,* 17**,** 6970-81.

JOO, W. S., JEFFREY, P. D., CANTOR, S. B., FINNIN, M. S., LIVINGSTON, D. M. & PAVLETICH, N. P. 2002. Structure of the 53BP1 BRCT region bound to p53 and its comparison to the Brca1 BRCT structure. *Genes Dev,* 16**,** 583-93.

JORDAN, C. T. & LEMISCHKA, I. R. 1990. Clonal and systemic analysis of long-term hematopoiesis in the mouse. *Genes Dev,* 4**,** 220-32.

JUN, J. I. & LAU, L. F. 2010. The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat Cell Biol,* 12**,** 676-85.

JUNG, T., BADER, N. & GRUNE, T. 2007. Lipofuscin: formation, distribution, and metabolic consequences. *Ann N Y Acad Sci,* 1119**,** 97-111.

JURK, D., WANG, C., MIWA, S., MADDICK, M., KOROLCHUK, V., TSOLOU, A., GONOS, E. S., THRASIVOULOU, C., SAFFREY, M. J., CAMERON, K. & VON ZGLINICKI, T. 2012. Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging Cell,* 11**,** 996-1004.

KAMATA, H., MANABE, T., OKA, S., KAMATA, K. & HIRATA, H. 2002. Hydrogen peroxide activates IkappaB kinases through phosphorylation of serine residues in the activation loops. *FEBS Lett,* 519**,** 231-7.

KARAGIANNIS, T. C., HARIKRISHNAN, K. N. & EL-OSTA, A. 2007. Disparity of histone deacetylase inhibition on repair of radiation-induced DNA damage on euchromatin and constitutive heterochromatin compartments. *Oncogene,* 26**,** 3963-71.

KARIN, M. 2009. NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol,* 1**,** a000141.

KARIN, M., LIU, Z. & ZANDI, E. 1997. AP-1 function and regulation. *Curr Opin Cell Biol,* 9**,** 240-6.

KARLSEDER, J., SMOGORZEWSKA, A. & DE LANGE, T. 2002. Senescence induced by altered telomere state, not telomere loss. *Science,* 295**,** 2446-9.

KASID, U., SUY, S., DENT, P., RAY, S., WHITESIDE, T. L. & STURGILL, T. W. 1996. Activation of Raf by ionizing radiation. *Nature,* 382**,** 813-6.

KATAKURA, Y., NAKATA, E., MIURA, T. & SHIRAHATA, S. 1999. Transforming growth factor beta triggers two independent-senescence programs in cancer cells. *Biochem Biophys Res Commun,* 255**,** 110-5.

KATO, D., MIYAZAWA, K., RUAS, M., STARBORG, M., WADA, I., OKA, T., SAKAI, T., PETERS, G. & HARA, E. 1998. Features of replicative senescence induced by direct addition of antennapedia-p16INK4A fusion protein to human diploid fibroblasts. *FEBS Lett,* 427**,** 203-8.

KATZ, M. L. & ROBISON, W. G., JR. 2002. What is lipofuscin? Defining characteristics and differentiation from other autofluorescent lysosomal storage bodies. *Arch Gerontol Geriatr,* 34**,** 169-84.

KEARSEY, J. M., COATES, P. J., PRESCOTT, A. R., WARBRICK, E. & HALL, P. A. 1995. Gadd45 is a nuclear cell cycle regulated protein which interacts with p21Cip1. *Oncogene,* 11**,** 1675-83.

KELLEY, R. O., VOGEL, K. G., CRISSMAN, H. A., LUJAN, C. J. & SKIPPER, B. E. 1979. Development of the aging cell surface. Reduction of gap junction-mediated metabolic cooperation with progressive subcultivation of human embryo fibroblasts (IMR-90). *Exp Cell Res,* 119**,** 127-43.

KENNEDY, A. L., ADAMS, P. D. & MORTON, J. P. 2011. Ras, PI3K/Akt and senescence: Paradoxes provide clues for pancreatic cancer therapy. *Small GTPases,* 2**,** 264-267.

KEOGH, M. C., KIM, J. A., DOWNEY, M., FILLINGHAM, J., CHOWDHURY, D., HARRISON, J. C., ONISHI, M., DATTA, N., GALICIA, S., EMILI, A., LIEBERMAN, J., SHEN, X., BURATOWSKI, S., HABER, J. E., DUROCHER, D., GREENBLATT, J. F. & KROGAN, N. J. 2006. A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery. *Nature,* 439**,** 497-501.

KIM, J. A., KRUHLAK, M., DOTIWALA, F., NUSSENZWEIG, A. & HABER, J. E. 2007. Heterochromatin is refractory to gamma-H2AX modification in yeast and mammals. *J Cell Biol,* 178**,** 209-18.

KIM, J. H., CHU, S. C., GRAMLICH, J. L., PRIDE, Y. B., BABENDREIER, E., CHAUHAN, D., SALGIA, R., PODAR, K., GRIFFIN, J. D. & SATTLER, M. 2005. Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species. *Blood,* 105**,** 1717-23.

KIM, N. W., PIATYSZEK, M. A., PROWSE, K. R., HARLEY, C. B., WEST, M. D., HO, P. L., COVIELLO, G. M., WRIGHT, W. E., WEINRICH, S. L. & SHAY, J. W. 1994. Specific association of human telomerase activity with immortal cells and cancer. *Science,* 266**,** 2011-5.

KIM, S. T., XU, B. & KASTAN, M. B. 2002. Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage. *Genes Dev,* 16**,** 560-70.

KITADA, T., SEKI, S., KAWAKITA, N., KUROKI, T. & MONNA, T. 1995. Telomere shortening in chronic liver diseases. *Biochem Biophys Res Commun,* 211**,** 33-9.

KODAMA, R., KATO, M., FURUTA, S., UENO, S., ZHANG, Y., MATSUNO, K., YABE-NISHIMURA, C., TANAKA, E. & KAMATA, T. 2013. ROS-generating oxidases Nox1 and Nox4 contribute to oncogenic Ras-induced premature senescence. *Genes Cells,* 18**,** 32-41.

KOH, Y. H., TAMIZHSELVI, R. & BHATIA, M. 2010. Extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase, through nuclear factor-kappaB and activator protein-1, contribute to caerulein-induced expression of substance P and neurokinin-1 receptors in pancreatic acinar cells. *J Pharmacol Exp Ther,* 332**,** 940-8.

KOLI, K., MYLLARNIEMI, M., KESKI-OJA, J. & KINNULA, V. L. 2008. Transforming growth factor-beta activation in the lung: focus on fibrosis and reactive oxygen species. *Antioxid Redox Signal,* 10**,** 333-42.

KOLYADA, A. Y., SAVIKOVSKY, N. & MADIAS, N. E. 1996. Transcriptional regulation of the human iNOS gene in vascular-smooth-muscle cells and macrophages: evidence for tissue specificity. *Biochem Biophys Res Commun,* 220**,** 600-5.

KONISHI, H., TANAKA, M., TAKEMURA, Y., MATSUZAKI, H., ONO, Y., KIKKAWA, U. & NISHIZUKA, Y. 1997. Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. *Proc Natl Acad Sci U S A,* 94**,** 11233-7.

KOPNIN, P. B., AGAPOVA, L. S., KOPNIN, B. P. & CHUMAKOV, P. M. 2007. Repression of sestrin family genes contributes to oncogenic Ras-induced reactive oxygen species up-regulation and genetic instability. *Cancer Res,* 67**,** 4671-8.

KOROTCHKINA, L. G., DEMIDENKO, Z. N., GUDKOV, A. V. & BLAGOSKLONNY, M. V. 2009. Cellular quiescence caused by the Mdm2 inhibitor nutlin-3A. *Cell Cycle,* 8**,** 3777-81.

KOROTCHKINA, L. G., LEONTIEVA, O. V., BUKREEVA, E. I., DEMIDENKO, Z. N., GUDKOV, A. V. & BLAGOSKLONNY, M. V. 2010. The choice between p53-induced senescence and quiescence is determined in part by the mTOR pathway. *Aging (Albany NY),* 2**,** 344-52.

KRIMPENFORT, P., QUON, K. C., MOOI, W. J., LOONSTRA, A. & BERNS, A. 2001. Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature,* 413**,** 83-6.

KRIZHANOVSKY, V., XUE, W., ZENDER, L., YON, M., HERNANDO, E. & LOWE, S. W. 2008a. Implications of cellular senescence in tissue damage response, tumor suppression, and stem cell biology. *Cold Spring Harb Symp Quant Biol,* 73**,** 513-22.

KRIZHANOVSKY, V., YON, M., DICKINS, R. A., HEARN, S., SIMON, J., MIETHING, C., YEE, H., ZENDER, L. & LOWE, S. W. 2008b. Senescence of activated stellate cells limits liver fibrosis. *Cell,* 134**,** 657-67.

KRTOLICA, A., PARRINELLO, S., LOCKETT, S., DESPREZ, P. Y. & CAMPISI, J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A,* 98**,** 12072-7.

KUILMAN, T., MICHALOGLOU, C., VREDEVELD, L. C., DOUMA, S., VAN DOORN, R., DESMET, C. J., AARDEN, L. A., MOOI, W. J. & PEEPER, D. S. 2008. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell,* 133**,** 1019-31.

KUMAGAI, A. & DUNPHY, W. G. 2000. Claspin, a novel protein required for the activation of Chk1 during a DNA replication checkpoint response in Xenopus egg extracts. *Mol Cell,* 6**,** 839-49.

KURZ, D. J., DECARY, S., HONG, Y. & ERUSALIMSKY, J. D. 2000. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J Cell Sci,* 113 (Pt 20)**,** 3613-22.

KWON, G., MARSHALL, C. A., LIU, H., PAPPAN, K. L., REMEDI, M. S. & MCDANIEL, M. L. 2006. Glucose-stimulated DNA synthesis through mammalian target of rapamycin (mTOR) is regulated by KATP channels: effects on cell cycle progression in rodent islets. *J Biol Chem,* 281**,** 3261-7.

KWON, G., MARSHALL, C. A., PAPPAN, K. L., REMEDI, M. S. & MCDANIEL, M. L. 2004. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. *Diabetes,* 53 Suppl 3**,** S225-32.

LA ROSA, F. A., PIERCE, J. W. & SONENSHEIN, G. E. 1994. Differential regulation of the c-myc oncogene promoter by the NF-kappa B rel family of transcription factors. *Mol Cell Biol,* 14**,** 1039-44.

LADWIG, G. P., ROBSON, M. C., LIU, R., KUHN, M. A., MUIR, D. F. & SCHULTZ, G. S. 2002. Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers. *Wound Repair Regen,* 10**,** 26-37.

LAGADEC, C., VLASHI, E., DELLA DONNA, L., DEKMEZIAN, C. & PAJONK, F. 2012. Radiation-induced reprogramming of breast cancer cells. *Stem Cells,* 30**,** 833-44.

LAHAV, G., ROSENFELD, N., SIGAL, A., GEVA-ZATORSKY, N., LEVINE, A. J., ELOWITZ, M. B. & ALON, U. 2004. Dynamics of the p53-Mdm2 feedback loop in individual cells. *Nat Genet,* 36**,** 147-50.

LAND, C. E. 2002. Uncertainty, low-dose extrapolation and the threshold hypothesis. *J Radiol Prot,* 22**,** A129-35.

LANE, B. R., LIU, J., BOCK, P. J., SCHOLS, D., COFFEY, M. J., STRIETER, R. M., POLVERINI, P. J. & MARKOVITZ, D. M. 2002. Interleukin-8 and growth-regulated oncogene alpha mediate angiogenesis in Kaposi's sarcoma. *J Virol,* 76**,** 11570-83.

LAWLESS, C., JURK, D., GILLESPIE, C. S., SHANLEY, D., SARETZKI, G., VON ZGLINICKI, T. & PASSOS, J. F. 2012. A stochastic step model of replicative senescence explains ROS production rate in ageing cell populations. *PLoS One,* 7**,** e32117.

LAWLESS, C., WANG, C., JURK, D., MERZ, A., ZGLINICKI, T. & PASSOS, J. F. 2010. Quantitative assessment of markers for cell senescence. *Exp Gerontol,* 45**,** 772-8.

LAZZERINI DENCHI, E., ATTWOOLL, C., PASINI, D. & HELIN, K. 2005. Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Mol Cell Biol,* 25**,** 2660-72.

LE CAM, L., LINARES, L. K., PAUL, C., JULIEN, E., LACROIX, M., HATCHI, E., TRIBOULET, R., BOSSIS, G., SHMUELI, A., RODRIGUEZ, M. S., COUX, O. & SARDET, C. 2006. E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. *Cell,* 127**,** 775-88.

LE GUILLY, Y., SIMON, M., LENOIR, P. & BOUREL, M. 1973. Long-term culture of human adult liver cells: morphological changes related to in vitro senescence and effect of donor's age on growth potential. *Gerontologia,* 19**,** 303-13.

LEE, B. Y., HAN, J. A., IM, J. S., MORRONE, A., JOHUNG, K., GOODWIN, E. C., KLEIJER, W. J., DIMAIO, D. & HWANG, E. S. 2006. Senescence-associated beta-galactosidase is lysosomal beta-galactosidase. *Aging Cell,* 5**,** 187-95.

LEE, E. G., BOONE, D. L., CHAI, S., LIBBY, S. L., CHIEN, M., LODOLCE, J. P. & MA, A. 2000. Failure to regulate TNF-induced NF-kappaB and cell death responses in A20-deficient mice. *Science,* 289**,** 2350-4.

LEE, H., HERRMANN, A., DENG, J. H., KUJAWSKI, M., NIU, G., LI, Z., FORMAN, S., JOVE, R., PARDOLL, D. M. & YU, H. 2009. Persistently activated Stat3 maintains constitutive NF-kappaB activity in tumors. *Cancer Cell,* 15**,** 283-93.

LEE, J. H. & PAULL, T. T. 2005. ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex. *Science,* 308**,** 551-4.

LEE, J. W., PARK, S., TAKAHASHI, Y. & WANG, H. G. 2010. The association of AMPK with ULK1 regulates autophagy. *PLoS One,* 5**,** e15394.

LEGLER, D. F., MICHEAU, O., DOUCEY, M. A., TSCHOPP, J. & BRON, C. 2003. Recruitment of TNF receptor 1 to lipid rafts is essential for TNFalpha-mediated NF-kappaB activation. *Immunity,* 18**,** 655-64.

LENER, B., KOZIEL, R., PIRCHER, H., HUTTER, E., GREUSSING, R., HERNDLER-BRANDSTETTER, D., HERMANN, M., UNTERLUGGAUER, H. & JANSEN-DURR, P. 2009. The NADPH oxidase Nox4 restricts the replicative lifespan of human endothelial cells. *Biochem J,* 423**,** 363-74.

LEONTIEVA, O. V., LENZO, F., DEMIDENKO, Z. N. & BLAGOSKLONNY, M. V. 2012. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. *Cell Cycle,* 11**,** 4642-9.

LEVY, L. & HILL, C. S. 2006. Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev,* 17**,** 41-58.

LEVY, M. Z., ALLSOPP, R. C., FUTCHER, A. B., GREIDER, C. W. & HARLEY, C. B. 1992. Telomere end-replication problem and cell aging. *J Mol Biol,* 225**,** 951-60.

LI, C., HU, Y., STURM, G., WICK, G. & XU, Q. 2000. Ras/Rac-Dependent activation of p38 mitogen-activated protein kinases in smooth muscle cells stimulated by cyclic strain stress. *Arterioscler Thromb Vasc Biol,* 20**,** E1-9.

LI, M., BROOKS, C. L., WU-BAER, F., CHEN, D., BAER, R. & GU, W. 2003. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science,* 302**,** 1972-5.

LI, M. O., WAN, Y. Y., SANJABI, S., ROBERTSON, A. K. & FLAVELL, R. A. 2006. Transforming growth factor-beta regulation of immune responses. *Annual review of immunology,* 24**,** 99-146.

LI, Q. & ENGELHARDT, J. F. 2006. Interleukin-1beta induction of NFkappaB is partially regulated by H2O2-mediated activation of NFkappaB-inducing kinase. *J Biol Chem,* 281**,** 1495-505.

LIN, A. W., BARRADAS, M., STONE, J. C., VAN AELST, L., SERRANO, M. & LOWE, S. W. 1998. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev,* 12**,** 3008-19.

LIN, J. H., YANG, J., LIU, S., TAKANO, T., WANG, X., GAO, Q., WILLECKE, K. & NEDERGAARD, M. 2003. Connexin mediates gap junction-independent resistance to cellular injury. *J Neurosci,* 23**,** 430-41.

LITTLE, J. B. 1969. Repair of sub-lethal and potentially lethal radiation damage in plateau phase cultures of human cells. *Nature,* 224**,** 804-6.

LIU, B., WANG, J., CHAN, K. M., TJIA, W. M., DENG, W., GUAN, X., HUANG, J. D., LI, K. M., CHAU, P. Y., CHEN, D. J., PEI, D., PENDAS, A. M., CADINANOS, J., LOPEZ-OTIN, C., TSE, H. F., HUTCHISON, C., CHEN, J., CAO, Y., CHEAH, K. S., TRYGGVASON, K. & ZHOU, Z. 2005. Genomic instability in laminopathy-based premature aging. *Nat Med,* 11**,** 780-5.

LIU, D. & HORNSBY, P. J. 2007. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. *Cancer Res,* 67**,** 3117-26.

LIU, L. Z., HU, X. W., XIA, C., HE, J., ZHOU, Q., SHI, X., FANG, J. & JIANG, B. H. 2006a. Reactive oxygen species regulate epidermal growth factor-induced vascular endothelial growth factor and hypoxia-inducible factor-1alpha expression through activation of AKT and P70S6K1 in human ovarian cancer cells. *Free Radic Biol Med,* 41**,** 1521-33.

LIU, Q., GUNTUKU, S., CUI, X. S., MATSUOKA, S., CORTEZ, D., TAMAI, K., LUO, G., CARATTINI-RIVERA, S., DEMAYO, F., BRADLEY, A., DONEHOWER, L. A. & ELLEDGE, S. J. 2000. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes Dev,* 14**,** 1448-59.

LIU, Z., MOTHERSILL, C. E., MCNEILL, F. E., LYNG, F. M., BYUN, S. H., SEYMOUR, C. B. & PRESTWICH, W. V. 2006b. A dose threshold for a medium transfer bystander effect for a human skin cell line. *Radiat Res,* 166**,** 19-23.

LIU, Z. G., BASKARAN, R., LEA-CHOU, E. T., WOOD, L. D., CHEN, Y., KARIN, M. & WANG, J. Y. 1996. Three distinct signalling responses by murine fibroblasts to genotoxic stress. *Nature,* 384**,** 273-6.

LOBMANN, R., AMBROSCH, A., SCHULTZ, G., WALDMANN, K., SCHIWECK, S. & LEHNERT, H. 2002. Expression of matrix-metalloproteinases and their inhibitors in the wounds of diabetic and non-diabetic patients. *Diabetologia,* 45**,** 1011-6.

LOWE, S. W., CEPERO, E. & EVAN, G. 2004. Intrinsic tumour suppression. *Nature,* 432**,** 307-15.

LU, X., NANNENGA, B. & DONEHOWER, L. A. 2005. PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. *Genes Dev,* 19**,** 1162-74.

LUKAS, C., FALCK, J., BARTKOVA, J., BARTEK, J. & LUKAS, J. 2003. Distinct spatiotemporal dynamics of mammalian checkpoint regulators induced by DNA damage. *Nat Cell Biol,* 5**,** 255-60.

LUKAS, C., MELANDER, F., STUCKI, M., FALCK, J., BEKKER-JENSEN, S., GOLDBERG, M., LERENTHAL, Y., JACKSON, S. P., BARTEK, J. & LUKAS, J. 2004. Mdc1 couples DNA double-strand break recognition by Nbs1 with its H2AX-dependent chromatin retention. *EMBO J,* 23**,** 2674-83.

LUNDBLAD, V. & SZOSTAK, J. W. 1989. A mutant with a defect in telomere elongation leads to senescence in yeast. *Cell,* 57**,** 633-43.

LUTTICKEN, C., WEGENKA, U. M., YUAN, J., BUSCHMANN, J., SCHINDLER, C., ZIEMIECKI, A., HARPUR, A. G., WILKS, A. F., YASUKAWA, K., TAGA, T. & ET AL. 1994. Association of transcription factor APRF and protein kinase Jak1 with the interleukin-6 signal transducer gp130. *Science,* 263**,** 89-92.

LYNG, F. M., MAGUIRE, P., MCCLEAN, B., SEYMOUR, C. & MOTHERSILL, C. 2006. The involvement of calcium and MAP kinase signaling pathways in the production of radiation-induced bystander effects. *Radiat Res,* 165**,** 400-9.

LYNG, F. M., SEYMOUR, C. B. & MOTHERSILL, C. 2000. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *Br J Cancer,* 83**,** 1223-30.

MACIP, S., IGARASHI, M., FANG, L., CHEN, A., PAN, Z. Q., LEE, S. W. & AARONSON, S. A. 2002. Inhibition of p21-mediated ROS accumulation can rescue p21-induced senescence. *EMBO J,* 21**,** 2180-8.

MADRID, L. V., MAYO, M. W., REUTHER, J. Y. & BALDWIN, A. S., JR. 2001. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem,* 276**,** 18934-40.

MAEDA, S., KAMATA, H., LUO, J. L., LEFFERT, H. & KARIN, M. 2005. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell,* 121**,** 977-90.

MAESHIMA, K., YAHATA, K., SASAKI, Y., NAKATOMI, R., TACHIBANA, T., HASHIKAWA, T., IMAMOTO, F. & IMAMOTO, N. 2006. Cell-cycle-dependent dynamics of nuclear pores: pore-free islands and lamins. *J Cell Sci,* 119**,** 4442-51.

MAEURER, M. J. & LOTZE, M. T. 1998. Interleukin-7 (IL-7) knockout mice. Implications for lymphopoiesis and organ-specific immunity. *International reviews of immunology,* 16**,** 309-22.

MAGUIRE, P., MOTHERSILL, C., SEYMOUR, C. & LYNG, F. M. 2005. Medium from irradiated cells induces dose-dependent mitochondrial changes and BCL2 responses in unirradiated human keratinocytes. *Radiat Res,* 163**,** 384-90.

MAHER, P. 1999. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem,* 274**,** 17491-8.

MAHYAR-ROEMER, M. & ROEMER, K. 2001. p21 Waf1/Cip1 can protect human colon carcinoma cells against p53-dependent and p53-independent apoptosis induced by natural chemopreventive and therapeutic agents. *Oncogene,* 20**,** 3387-98.

MAILAND, N., BEKKER-JENSEN, S., BARTEK, J. & LUKAS, J. 2006. Destruction of Claspin by SCFbetaTrCP restrains Chk1 activation and facilitates recovery from genotoxic stress. *Mol Cell,* 23**,** 307-18.

MAKAROV, V. L., HIROSE, Y. & LANGMORE, J. P. 1997. Long G tails at both ends of human chromosomes suggest a C strand degradation mechanism for telomere shortening. *Cell,* 88**,** 657-66.

MALCHOW, S., THAISS, W., JANNER, N., WAETZIG, G. H., GEWIESE-RABSCH, J., GARBERS, C., YAMAMOTO, K., ROSE-JOHN, S. & SCHELLER, J. 2011. Essential role of neutrophil mobilization in concanavalin A-induced hepatitis is based on classic IL-6 signaling but not on IL-6 trans-signaling. *Biochim Biophys Acta,* 1812**,** 290-301.

MALLETTE, F. A., GAUMONT-LECLERC, M. F. & FERBEYRE, G. 2007. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. *Genes Dev,* 21**,** 43-8.

MALLETTE, F. A., GOUMARD, S., GAUMONT-LECLERC, M. F., MOISEEVA, O. & FERBEYRE, G. 2004. Human fibroblasts require the Rb family of tumor suppressors, but not p53, for PML-induced senescence. *Oncogene,* 23**,** 91-9.

MAO, Z., KE, Z., GORBUNOVA, V. & SELUANOV, A. 2012. Replicatively senescent cells are arrested in G1 and G2 phases. *Aging (Albany NY),* 4**,** 431-5.

MARCHENKO, N. D., ZAIKA, A. & MOLL, U. M. 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem,* 275**,** 16202-12.

MARIAPPAN, N., ELKS, C. M., SRIRAMULA, S., GUGGILAM, A., LIU, Z., BORKHSENIOUS, O. & FRANCIS, J. 2010. NF-kappaB-induced oxidative stress contributes to mitochondrial and cardiac dysfunction in type II diabetes. *Cardiovasc Res,* 85**,** 473-83.

MARTIN, G. M., SPRAGUE, C. A. & EPSTEIN, C. J. 1970. Replicative life-span of cultivated human cells. Effects of donor's age, tissue, and genotype. *Lab Invest,* 23**,** 86-92.

MARTINEZ, L. A., YANG, J., VAZQUEZ, E. S., RODRIGUEZ-VARGAS MDEL, C., OLIVE, M., HSIEH, J. T., LOGOTHETIS, C. J. & NAVONE, N. M. 2002. p21 modulates threshold of apoptosis induced by DNA-damage and growth factor withdrawal in prostate cancer cells. *Carcinogenesis,* 23**,** 1289-96.

MATSUMURA, T., ZERRUDO, Z. & HAYFLICK, L. 1979. Senescent human diploid cells in culture: survival, DNA synthesis and morphology. *J Gerontol,* 34**,** 328-34.

MATSUOKA, S., ROTMAN, G., OGAWA, A., SHILOH, Y., TAMAI, K. & ELLEDGE, S. J. 2000. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. *Proc Natl Acad Sci U S A,* 97**,** 10389-94.

MAYA, R., BALASS, M., KIM, S. T., SHKEDY, D., LEAL, J. F., SHIFMAN, O., MOAS, M., BUSCHMANN, T., RONAI, Z., SHILOH, Y., KASTAN, M. B., KATZIR, E. & OREN, M. 2001. ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. *Genes Dev,* 15**,** 1067-77.

MCCLINTOCK, B. 1941. The Stability of Broken Ends of Chromosomes in Zea Mays. *Genetics,* 26**,** 234-82.

MCCONNELL, B. B., STARBORG, M., BROOKES, S. & PETERS, G. 1998. Inhibitors of cyclin-dependent kinases induce features of replicative senescence in early passage human diploid fibroblasts. *Curr Biol,* 8**,** 351-4.

MELCHIONNA, R., CHEN, X. B., BLASINA, A. & MCGOWAN, C. H. 2000. Threonine 68 is required for radiation-induced phosphorylation and activation of Cds1. *Nat Cell Biol,* 2**,** 762-5.

MENDEZ, M. V., RAFFETTO, J. D., PHILLIPS, T., MENZOIAN, J. O. & PARK, H. Y. 1999. The proliferative capacity of neonatal skin fibroblasts is reduced after exposure to venous ulcer wound fluid: A potential mechanism for senescence in venous ulcers. *J Vasc Surg,* 30**,** 734-43.

MENG, A., WANG, Y., VAN ZANT, G. & ZHOU, D. 2003. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res,* 63**,** 5414-9.

MEYLAN, E., DOOLEY, A. L., FELDSER, D. M., SHEN, L., TURK, E., OUYANG, C. & JACKS, T. 2009. Requirement for NF-kappaB signalling in a mouse model of lung adenocarcinoma. *Nature,* 462**,** 104-7.

MICHALOGLOU, C., VREDEVELD, L. C., SOENGAS, M. S., DENOYELLE, C., KUILMAN, T., VAN DER HORST, C. M., MAJOOR, D. M., SHAY, J. W., MOOI, W. J. & PEEPER, D. S. 2005. BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature,* 436**,** 720-4.

MICHEAU, O. & TSCHOPP, J. 2003. Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell,* 114**,** 181-90.

MIHARA, M., ERSTER, S., ZAIKA, A., PETRENKO, O., CHITTENDEN, T., PANCOSKA, P. & MOLL, U. M. 2003. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell,* 11**,** 577-90.

MIHARA, M., MORIYA, Y., KISHIMOTO, T. & OHSUGI, Y. 1995. Interleukin-6 (IL-6) induces the proliferation of synovial fibroblastic cells in the presence of soluble IL-6 receptor. *Br J Rheumatol,* 34**,** 321-5.

MIHARA, M., MORIYA, Y. & OHSUGI, Y. 1996. IL-6-soluble IL-6 receptor complex inhibits the proliferation of dermal fibroblasts. *Int J Immunopharmacol,* 18**,** 89-94.

MISHIMA, K., HANDA, J. T., AOTAKI-KEEN, A., LUTTY, G. A., MORSE, L. S. & HJELMELAND, L. M. 1999. Senescence-associated beta-galactosidase histochemistry for the primate eye. *Invest Ophthalmol Vis Sci,* 40**,** 1590-3.

MOCHAN, T. A., VENERE, M., DITULLIO, R. A., JR. & HALAZONETIS, T. D. 2003. 53BP1 and NFBD1/MDC1-Nbs1 function in parallel interacting pathways activating ataxia-telangiectasia mutated (ATM) in response to DNA damage. *Cancer Res,* 63**,** 8586-91.

MOCHAN, T. A., VENERE, M., DITULLIO, R. A., JR. & HALAZONETIS, T. D. 2004. 53BP1, an activator of ATM in response to DNA damage. *DNA Repair (Amst),* 3**,** 945-52.

MOISEEVA, O., BOURDEAU, V., ROUX, A., DESCHENES-SIMARD, X. & FERBEYRE, G. 2009. Mitochondrial dysfunction contributes to oncogene-induced senescence. *Mol Cell Biol,* 29**,** 4495-507.

MOISEEVA, O., MALLETTE, F. A., MUKHOPADHYAY, U. K., MOORES, A. & FERBEYRE, G. 2006. DNA damage signaling and p53-dependent senescence after prolonged beta-interferon stimulation. *Mol Biol Cell,* 17**,** 1583-92.

MOK, M. T. & HENDERSON, B. R. 2010. A comparison of BRCA1 nuclear localization with 14 DNA damage response proteins and domains: identification of specific differences between BRCA1 and 53BP1 at DNA damage-induced foci. *Cell Signal,* 22**,** 47-56.

MORALES, C. P., HOLT, S. E., OUELLETTE, M., KAUR, K. J., YAN, Y., WILSON, K. S., WHITE, M. A., WRIGHT, W. E. & SHAY, J. W. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. *Nat Genet,* 21**,** 115-8.

MORGAN, M. J., KIM, Y. S. & LIU, Z. 2007. Lipid rafts and oxidative stress-induced cell death. *Antioxid Redox Signal,* 9**,** 1471-83.

MORI, N., YAMADA, Y., IKEDA, S., YAMASAKI, Y., TSUKASAKI, K., TANAKA, Y., TOMONAGA, M., YAMAMOTO, N. & FUJII, M. 2002. Bay 11-7082 inhibits transcription factor NF-kappaB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. *Blood,* 100**,** 1828-34.

MORRISON, S. J., WANDYCZ, A. M., AKASHI, K., GLOBERSON, A. & WEISSMAN, I. L. 1996. The aging of hematopoietic stem cells. *Nat Med,* 2**,** 1011-6.

MOTHERSILL, C., LYNG, F., SEYMOUR, C., MAGUIRE, P., LORIMORE, S. & WRIGHT, E. 2005. Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. *Radiat Res,* 163**,** 391-9.

MOTHERSILL, C., REA, D., WRIGHT, E. G., LORIMORE, S. A., MURPHY, D., SEYMOUR, C. B. & O'MALLEY, K. 2001. Individual variation in the production of a 'bystander signal' following irradiation of primary cultures of normal human urothelium. *Carcinogenesis,* 22**,** 1465-71.

MOTHERSILL, C. & SEYMOUR, C. 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *Int J Radiat Biol,* 71**,** 421-7.

MOTHERSILL, C. & SEYMOUR, C. B. 1998. Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiat Res,* 149**,** 256-62.

MOTHERSILL, C. & SEYMOUR, C. B. 2002. Bystander and delayed effects after fractionated radiation exposure. *Radiat Res,* 158**,** 626-33.

MOTHERSILL, C., SEYMOUR, R. J. & SEYMOUR, C. B. 2004. Bystander effects in repair-deficient cell lines. *Radiat Res,* 161**,** 256-63.

MOTHERSILL, C. E., O'MALLEY, K. J., MURPHY, D. M., SEYMOUR, C. B., LORIMORE, S. A. & WRIGHT, E. G. 1999. Identification and characterization of three subtypes of radiation response in normal human urothelial cultures exposed to ionizing radiation. *Carcinogenesis,* 20**,** 2273-8.

MUEGGE, K., VILA, M. P. & DURUM, S. K. 1993. Interleukin-7: a cofactor for V(D)J rearrangement of the T cell receptor beta gene. *Science,* 261**,** 93-5.

MUNGAMURI, S. K., YANG, X., THOR, A. D. & SOMASUNDARAM, K. 2006. Survival signaling by Notch1: mammalian target of rapamycin (mTOR)-dependent inhibition of p53. *Cancer Res,* 66**,** 4715-24.

NAGASAWA, H. & LITTLE, J. B. 1992. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Res,* 52**,** 6394-6.

NAKAMURA, A. J., CHIANG, Y. J., HATHCOCK, K. S., HORIKAWA, I., SEDELNIKOVA, O. A., HODES, R. J. & BONNER, W. M. 2008. Both telomeric and non-telomeric DNA damage are determinants of mammalian cellular senescence. *Epigenetics Chromatin,* 1**,** 6.

NAKAMURA, K., SAKAI, W., KAWAMOTO, T., BREE, R. T., LOWNDES, N. F., TAKEDA, S. & TANIGUCHI, Y. 2006. Genetic dissection of vertebrate 53BP1: a major role in non-homologous end joining of DNA double strand breaks. *DNA Repair (Amst),* 5**,** 741-9.

NAKASE, T., SOHL, G., THEIS, M., WILLECKE, K. & NAUS, C. C. 2004. Increased apoptosis and inflammation after focal brain ischemia in mice lacking connexin43 in astrocytes. *Am J Pathol,* 164**,** 2067-75.

NARITA, M. 2007. Cellular senescence and chromatin organisation. *Br J Cancer,* 96**,** 686-91.

NARITA, M., NUNEZ, S., HEARD, E., LIN, A. W., HEARN, S. A., SPECTOR, D. L., HANNON, G. J. & LOWE, S. W. 2003. Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell,* 113**,** 703-16.

NAUGLER, W. E., SAKURAI, T., KIM, S., MAEDA, S., KIM, K., ELSHARKAWY, A. M. & KARIN, M. 2007. Gender disparity in liver cancer due to sex differences in MyD88-dependent IL-6 production. *Science,* 317**,** 121-4.

NAUS, C. C. 2002. Gap junctions and tumour progression. *Can J Physiol Pharmacol,* 80**,** 136-41.

NAUTIYAL, S., DERISI, J. L. & BLACKBURN, E. H. 2002. The genome-wide expression response to telomerase deletion in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A,* 99**,** 9316-21.

NELSON, G., BUHMANN, M. & VON ZGLINICKI, T. 2009. DNA damage foci in mitosis are devoid of 53BP1. *Cell Cycle,* 8**,** 3379-83.

NELSON, G., WORDSWORTH, J., WANG, C., JURK, D., LAWLESS, C., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2012. A senescent cell bystander effect: senescence-induced senescence. *Aging Cell,* 11**,** 345-9.

NEVINS, J. R. 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science,* 258**,** 424-9.

NICKE, B., BASTIEN, J., KHANNA, S. J., WARNE, P. H., COWLING, V., COOK, S. J., PETERS, G., DELPUECH, O., SCHULZE, A., BERNS, K., MULLENDERS, J., BEIJERSBERGEN, R. L., BERNARDS, R., GANESAN, T. S., DOWNWARD, J. & HANCOCK, D. C. 2005. Involvement of MINK, a Ste20 family kinase, in Ras oncogene-induced growth arrest in human ovarian surface epithelial cells. *Mol Cell,* 20**,** 673-85.

NIKJOO, H., MUNSON, R. J. & BRIDGES, B. A. 1999. RBE-LET relationships in mutagenesis by ionizing radiation. *J Radiat Res (Tokyo),* 40 Suppl**,** 85-105.

NOWELL, M. A., RICHARDS, P. J., HORIUCHI, S., YAMAMOTO, N., ROSE-JOHN, S., TOPLEY, N., WILLIAMS, A. S. & JONES, S. A. 2003. Soluble IL-6 receptor governs IL-6 activity in experimental arthritis: blockade of arthritis severity by soluble glycoprotein 130. *J Immunol,* 171**,** 3202-9.

OAKLEY, F., MANN, J., NAILARD, S., SMART, D. E., MUNGALSINGH, N., CONSTANDINOU, C., ALI, S., WILSON, S. J., MILLWARD-SADLER, H., IREDALE, J. P. & MANN, D. A. 2005. Nuclear factor-kappaB1 (p50) limits the inflammatory and fibrogenic responses to chronic injury. *Am J Pathol,* 166**,** 695-708.

OBATA, T., BROWN, G. E. & YAFFE, M. B. 2000. MAP kinase pathways activated by stress: the p38 MAPK pathway. *Crit Care Med,* 28**,** N67-77.

OFT, M., PELI, J., RUDAZ, C., SCHWARZ, H., BEUG, H. & REICHMANN, E. 1996. TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes & development,* 10**,** 2462-77.

OLOVNIKOV, A. M. 1971. [Principle of marginotomy in template synthesis of polynucleotides]. *Dokl Akad Nauk SSSR,* 201**,** 1496-9.

OLOVNIKOV, A. M. 1973. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J Theor Biol,* 41**,** 181-90.

OLOVNIKOV, I. A., KRAVCHENKO, J. E. & CHUMAKOV, P. M. 2009. Homeostatic functions of the p53 tumor suppressor: regulation of energy metabolism and antioxidant defense. *Semin Cancer Biol,* 19**,** 32-41.

OWEN, J. D., STRIETER, R., BURDICK, M., HAGHNEGAHDAR, H., NANNEY, L., SHATTUCK-BRANDT, R. & RICHMOND, A. 1997. Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. *International journal of cancer. Journal international du cancer,* 73**,** 94-103.

PAHL, H. L. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene,* 18**,** 6853-66.

PANOPOULOS, A., HARRAZ, M., ENGELHARDT, J. F. & ZANDI, E. 2005. Iron-mediated H2O2 production as a mechanism for cell type-specific inhibition of tumor necrosis factor alpha-induced but not interleukin-1beta-induced IkappaB kinase complex/nuclear factor-kappaB activation. *J Biol Chem,* 280**,** 2912-23.

PANTOJA, C. & SERRANO, M. 1999. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. *Oncogene,* 18**,** 4974-82.

PAPPAS, C. A., RIOULT, M. G. & RANSOM, B. R. 1996. Octanol, a gap junction uncoupling agent, changes intracellular [H+] in rat astrocytes. *Glia,* 16**,** 7-15.

PARRINELLO, S., COPPE, J. P., KRTOLICA, A. & CAMPISI, J. 2005. Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J Cell Sci,* 118**,** 485-96.

PASSOS, J. F., MIWA, S. & VON ZGLINICKI, T. 2013. Measuring reactive oxygen species in senescent cells. *Methods Mol Biol,* 965**,** 253-63.

PASSOS, J. F., NELSON, G., WANG, C., RICHTER, T., SIMILLION, C., PROCTOR, C. J., MIWA, S., OLIJSLAGERS, S., HALLINAN, J., WIPAT, A., SARETZKI, G., RUDOLPH, K. L., KIRKWOOD, T. B. & VON ZGLINICKI, T. 2010. Feedback between p21 and reactive oxygen production is necessary for cell senescence. *Mol Syst Biol,* 6**,** 347.

PASSOS, J. F., SARETZKI, G., AHMED, S., NELSON, G., RICHTER, T., PETERS, H., WAPPLER, I., BIRKET, M. J., HAROLD, G., SCHAEUBLE, K., BIRCH-MACHIN, M. A., KIRKWOOD, T. B. & VON ZGLINICKI, T. 2007. Mitochondrial dysfunction accounts for the stochastic heterogeneity in telomere-dependent senescence. *PLoS Biol,* 5**,** e110.

PASSOS, J. F. & VON ZGLINICKI, T. 2006. Oxygen free radicals in cell senescence: are they signal transducers? *Free Radic Res,* 40**,** 1277-83.

PASSOS, J. F., VON ZGLINICKI, T. & SARETZKI, G. 2006. Mitochondrial dysfunction and cell senescence: cause or consequence? *Rejuvenation Res,* 9**,** 64-8.

PAULL, T. T., ROGAKOU, E. P., YAMAZAKI, V., KIRCHGESSNER, C. U., GELLERT, M. & BONNER, W. M. 2000. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol,* 10**,** 886-95.

PEDERSEN, B. K. 2007. IL-6 signalling in exercise and disease. *Biochem Soc Trans,* 35**,** 1295-7.

PEI, H., ZHANG, L., LUO, K., QIN, Y., CHESI, M., FEI, F., BERGSAGEL, P. L., WANG, L., YOU, Z. & LOU, Z. 2011. MMSET regulates histone H4K20 methylation and 53BP1 accumulation at DNA damage sites. *Nature,* 470**,** 124-8.

PENDERGRASS, W. R., LANE, M. A., BODKIN, N. L., HANSEN, B. C., INGRAM, D. K., ROTH, G. S., YI, L., BIN, H. & WOLF, N. S. 1999. Cellular proliferation potential during aging and caloric restriction in rhesus monkeys (Macaca mulatta). *J Cell Physiol,* 180**,** 123-30.

PERKINS, N. D. & GILMORE, T. D. 2006. Good cop, bad cop: the different faces of NF-kappaB. *Cell Death Differ,* 13**,** 759-72.

PETERSEN, A. M. & PEDERSEN, B. K. 2005. The anti-inflammatory effect of exercise. *J Appl Physiol,* 98**,** 1154-62.

PETERSEN, E. W., CAREY, A. L., SACCHETTI, M., STEINBERG, G. R., MACAULAY, S. L., FEBBRAIO, M. A. & PEDERSEN, B. K. 2005. Acute IL-6 treatment increases fatty acid turnover in elderly humans in vivo and in tissue culture in vitro. *Am J Physiol Endocrinol Metab,* 288**,** E155-62.

PETERSON, T. R., LAPLANTE, M., THOREEN, C. C., SANCAK, Y., KANG, S. A., KUEHL, W. M., GRAY, N. S. & SABATINI, D. M. 2009. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. *Cell,* 137**,** 873-86.

PIGNOLO, R. J., MARTIN, B. G., HORTON, J. H., KALBACH, A. N. & CRISTOFALO, V. J. 1998. The pathway of cell senescence: WI-38 cells arrest in late G1 and are unable to traverse the cell cycle from a true G0 state. *Exp Gerontol,* 33**,** 67-80.

PIGNOLO, R. J., ROTENBERG, M. O. & CRISTOFALO, V. J. 1994. Alterations in contact and density-dependent arrest state in senescent WI-38 cells. *In Vitro Cell Dev Biol Anim,* 30A**,** 471-6.

PIKARSKY, E., PORAT, R. M., STEIN, I., ABRAMOVITCH, R., AMIT, S., KASEM, S., GUTKOVICH-PYEST, E., URIELI-SHOVAL, S., GALUN, E. & BEN-NERIAH, Y. 2004. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature,* 431**,** 461-6.

PITKANEN, S. & ROBINSON, B. H. 1996. Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase. *J Clin Invest,* 98**,** 345-51.

POLAK, P. & HALL, M. N. 2009. mTOR and the control of whole body metabolism. *Curr Opin Cell Biol,* 21**,** 209-18.

POLYAK, K., KATO, J. Y., SOLOMON, M. J., SHERR, C. J., MASSAGUE, J., ROBERTS, J. M. & KOFF, A. 1994. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & development,* 8**,** 9-22.

POLYAK, K., XIA, Y., ZWEIER, J. L., KINZLER, K. W. & VOGELSTEIN, B. 1997. A model for p53-induced apoptosis. *Nature,* 389**,** 300-5.

POSPELOVA, T. V., DEMIDENKO, Z. N., BUKREEVA, E. I., POSPELOV, V. A., GUDKOV, A. V. & BLAGOSKLONNY, M. V. 2009. Pseudo-DNA damage response in senescent cells. *Cell Cycle,* 8**,** 4112-8.

PRICE, J. S., WATERS, J. G., DARRAH, C., PENNINGTON, C., EDWARDS, D. R., DONELL, S. T. & CLARK, I. M. 2002. The role of chondrocyte senescence in osteoarthritis. *Aging Cell,* 1**,** 57-65.

PROBIN, V., WANG, Y., BAI, A. & ZHOU, D. 2006. Busulfan selectively induces cellular senescence but not apoptosis in WI38 fibroblasts via a p53-independent but extracellular signal-regulated kinase-p38 mitogen-activated protein kinase-dependent mechanism. *J Pharmacol Exp Ther,* 319**,** 551-60.

RADISAVLJEVIC, Z. M. & GONZALEZ-FLECHA, B. 2004. TOR kinase and Ran are downstream from PI3K/Akt in H2O2-induced mitosis. *J Cell Biochem,* 91**,** 1293-300.

RAPPOLD, I., IWABUCHI, K., DATE, T. & CHEN, J. 2001. Tumor suppressor p53 binding protein 1 (53BP1) is involved in DNA damage-signaling pathways. *J Cell Biol,* 153**,** 613-20.

RAUERT-WUNDERLICH, H., SIEGMUND, D., MAIER, E., GINER, T., BARGOU, R. C., WAJANT, H. & STUHMER, T. 2013. The IKK inhibitor Bay 11-7082 induces cell death independent from inhibition of activation of NFkappaB transcription factors. *PLoS One,* 8**,** e59292.

RAUSCH, O. & MARSHALL, C. J. 1999. Cooperation of p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathways during granulocyte colony-stimulating factor-induced hemopoietic cell proliferation. *J Biol Chem,* 274**,** 4096-105.

REBBAA, A., ZHENG, X., CHU, F. & MIRKIN, B. L. 2006. The role of histone acetylation versus DNA damage in drug-induced senescence and apoptosis. *Cell Death Differ,* 13**,** 1960-7.

REILEY, W. W., JIN, W., LEE, A. J., WRIGHT, A., WU, X., TEWALT, E. F., LEONARD, T. O., NORBURY, C. C., FITZPATRICK, L., ZHANG, M. & SUN, S. C. 2007. Deubiquitinating enzyme CYLD negatively regulates the ubiquitin-dependent kinase Tak1 and prevents abnormal T cell responses. *J Exp Med,* 204**,** 1475-85.

RESNITZKY, D. & REED, S. I. 1995. Different roles for cyclins D1 and E in regulation of the G1-to-S transition. *Mol Cell Biol,* 15**,** 3463-9.

REYNAERT, N. L., VAN DER VLIET, A., GUALA, A. S., MCGOVERN, T., HRISTOVA, M., PANTANO, C., HEINTZ, N. H., HEIM, J., HO, Y. S., MATTHEWS, D. E., WOUTERS, E. F. & JANSSEN-HEININGER, Y. M. 2006. Dynamic redox control of NF-kappaB through glutaredoxin-regulated S-glutathionylation of inhibitory kappaB kinase beta. *Proc Natl Acad Sci U S A,* 103**,** 13086-91.

RITTLING, S. R., BROOKS, K. M., CRISTOFALO, V. J. & BASERGA, R. 1986. Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts. *Proc Natl Acad Sci U S A,* 83**,** 3316-20.

ROBBINS, E., LEVINE, E. M. & EAGLE, H. 1970. Morphologic changes accompanying senescence of cultured human diploid cells. *J Exp Med,* 131**,** 1211-22.

ROBERTSON, J. D. 1981a. Membrane structure. *J Cell Biol,* 91**,** 189s-204s.

ROBERTSON, J. D. 1981b. A review of membrane structure with perspectives on certain transmembrane channels. *Adv Neurol,* 31**,** 419-77.

RODIER, F. & CAMPISI, J. 2011. Four faces of cellular senescence. *J Cell Biol,* 192**,** 547-56.

RODIER, F., COPPE, J. P., PATIL, C. K., HOEIJMAKERS, W. A., MUNOZ, D. P., RAZA, S. R., FREUND, A., CAMPEAU, E., DAVALOS, A. R. & CAMPISI, J. 2009. Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat Cell Biol,* 11**,** 973-9.

RODIER, F., MUNOZ, D. P., TEACHENOR, R., CHU, V., LE, O., BHAUMIK, D., COPPE, J. P., CAMPEAU, E., BEAUSEJOUR, C. M., KIM, S. H., DAVALOS, A. R. & CAMPISI, J. 2011. DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *J Cell Sci,* 124**,** 68-81.

ROGAKOU, E. P., BOON, C., REDON, C. & BONNER, W. M. 1999. Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol,* 146**,** 905-16.

ROGAKOU, E. P., PILCH, D. R., ORR, A. H., IVANOVA, V. S. & BONNER, W. M. 1998. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem,* 273**,** 5858-68.

ROJO, A. I., SALINAS, M., MARTIN, D., PERONA, R. & CUADRADO, A. 2004. Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappaB. *J Neurosci,* 24**,** 7324-34.

ROTHLEIN, R., DUSTIN, M. L., MARLIN, S. D. & SPRINGER, T. A. 1986. A human intercellular adhesion molecule (ICAM-1) distinct from LFA-1. *Journal of immunology,* 137**,** 1270-4.

ROVILLAIN, E., MANSFIELD, L., CAETANO, C., ALVAREZ-FERNANDEZ, M., CABALLERO, O. L., MEDEMA, R. H., HUMMERICH, H. & JAT, P. S. 2011. Activation of nuclear factor-kappa B signalling promotes cellular senescence. *Oncogene,* 30**,** 2356-66.

SADAIE, M., SALAMA, R., CARROLL, T., TOMIMATSU, K., CHANDRA, T., YOUNG, A. R., NARITA, M., PEREZ-MANCERA, P. A., BENNETT, D. C., CHONG, H. & KIMURA, H. 2013. Redistribution of the Lamin B1 genomic binding profile affects rearrangement of heterochromatic domains and SAHF formation during senescence. *Genes Dev,* 27**,** 1800-8.

SAHIN, E., COLLA, S., LIESA, M., MOSLEHI, J., MULLER, F. L., GUO, M., COOPER, M., KOTTON, D., FABIAN, A. J., WALKEY, C., MASER, R. S., TONON, G., FOERSTER, F., XIONG, R., WANG, Y. A., SHUKLA, S. A., JASKELIOFF, M., MARTIN, E. S., HEFFERNAN, T. P., PROTOPOPOV, A., IVANOVA, E., MAHONEY, J. E., KOST-ALIMOVA, M., PERRY, S. R., BRONSON, R., LIAO, R., MULLIGAN, R., SHIRIHAI, O. S., CHIN, L. & DEPINHO, R. A. 2011. Telomere dysfunction induces metabolic and mitochondrial compromise. *Nature,* 470**,** 359-65.

SANCAK, Y., BAR-PELED, L., ZONCU, R., MARKHARD, A. L., NADA, S. & SABATINI, D. M. 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. *Cell,* 141**,** 290-303.

SARKISIAN, C. J., KEISTER, B. A., STAIRS, D. B., BOXER, R. B., MOODY, S. E. & CHODOSH, L. A. 2007. Dose-dependent oncogene-induced senescence in vivo and its evasion during mammary tumorigenesis. *Nat Cell Biol,* 9**,** 493-505.

SATO, Y., MURASE, K., KATO, J., KOBUNE, M., SATO, T., KAWANO, Y., TAKIMOTO, R., TAKADA, K., MIYANISHI, K., MATSUNAGA, T., TAKAYAMA, T. & NIITSU, Y. 2008. Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. *Nat Biotechnol,* 26**,** 431-42.

SCHAPER, F., GENDO, C., ECK, M., SCHMITZ, J., GRIMM, C., ANHUF, D., KERR, I. M. & HEINRICH, P. C. 1998. Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. *Biochem J,* 335 (Pt 3)**,** 557-65.

SCHIEKE, S. M., PHILLIPS, D., MCCOY, J. P., JR., APONTE, A. M., SHEN, R. F., BALABAN, R. S. & FINKEL, T. 2006. The mammalian target of rapamycin (mTOR) pathway regulates mitochondrial oxygen consumption and oxidative capacity. *J Biol Chem,* 281**,** 27643-52.

SCHIEMANN, W. P., BARTOE, J. L. & NATHANSON, N. M. 1997. Box 3-independent signaling mechanisms are involved in leukemia inhibitory factor receptor alpha- and gp130-mediated stimulation of mitogen-activated protein kinase. Evidence for participation of multiple signaling pathways which converge at Ras. *J Biol Chem,* 272**,** 16631-6.

SCHIEVEN, G. L., KIRIHARA, J. M., MYERS, D. E., LEDBETTER, J. A. & UCKUN, F. M. 1993. Reactive oxygen intermediates activate NF-kappa B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56lck and p59fyn tyrosine kinases in human lymphocytes. *Blood,* 82**,** 1212-20.

SCHILDER, Y. D., HEISS, E. H., SCHACHNER, D., ZIEGLER, J., REZNICEK, G., SORESCU, D. & DIRSCH, V. M. 2009. NADPH oxidases 1 and 4 mediate cellular senescence induced by resveratrol in human endothelial cells. *Free Radic Biol Med,* 46**,** 1598-606.

SCHOONBROODT, S., FERREIRA, V., BEST-BELPOMME, M., BOELAERT, J. R., LEGRAND-POELS, S., KORNER, M. & PIETTE, J. 2000. Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I kappa B alpha in NF-kappa B activation by an oxidative stress. *J Immunol,* 164**,** 4292-300.

SCHREIBER, J., JENNER, R. G., MURRAY, H. L., GERBER, G. K., GIFFORD, D. K. & YOUNG, R. A. 2006. Coordinated binding of NF-kappaB family members in the response of human cells to lipopolysaccharide. *Proc Natl Acad Sci U S A,* 103**,** 5899-904.

SCHULTZ, L. B., CHEHAB, N. H., MALIKZAY, A. & HALAZONETIS, T. D. 2000. p53 binding protein 1 (53BP1) is an early participant in the cellular response to DNA double-strand breaks. *J Cell Biol,* 151**,** 1381-90.

SEDELNIKOVA, O. A., HORIKAWA, I., ZIMONJIC, D. B., POPESCU, N. C., BONNER, W. M. & BARRETT, J. C. 2004. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nat Cell Biol,* 6**,** 168-70.

SEDELNIKOVA, O. A., ROGAKOU, E. P., PANYUTIN, I. G. & BONNER, W. M. 2002. Quantitative detection of (125)IdU-induced DNA double-strand breaks with gamma-H2AX antibody. *Radiat Res,* 158**,** 486-92.

SERRA, V. & VON ZGLINICKI, T. 2002. Human fibroblasts in vitro senesce with a donor-specific telomere length. *FEBS Lett,* 516**,** 71-4.

SERRANO, M., HANNON, G. J. & BEACH, D. 1993. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature,* 366**,** 704-7.

SERRANO, M., LIN, A. W., MCCURRACH, M. E., BEACH, D. & LOWE, S. W. 1997. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell,* 88**,** 593-602.

SEYMOUR, C. B. & MOTHERSILL, C. 2000. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. *Radiat Res,* 153**,** 508-11.

SHARPLESS, N. E., BARDEESY, N., LEE, K. H., CARRASCO, D., CASTRILLON, D. H., AGUIRRE, A. J., WU, E. A., HORNER, J. W. & DEPINHO, R. A. 2001. Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature,* 413**,** 86-91.

SHAULIAN, E., SCHREIBER, M., PIU, F., BEECHE, M., WAGNER, E. F. & KARIN, M. 2000. The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. *Cell,* 103**,** 897-907.

SHAY, J. W., PEREIRA-SMITH, O. M. & WRIGHT, W. E. 1991. A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res,* 196**,** 33-9.

SHERR, C. J. 1994. G1 phase progression: cycling on cue. *Cell,* 79**,** 551-5.

SHERR, C. J. 2004. Principles of tumor suppression. *Cell,* 116**,** 235-46.

SHERR, C. J. & ROBERTS, J. M. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev,* 13**,** 1501-12.

SHI, Y., HSU, J. H., HU, L., GERA, J. & LICHTENSTEIN, A. 2002. Signal pathways involved in activation of p70S6K and phosphorylation of 4E-BP1 following exposure of multiple myeloma tumor cells to interleukin-6. *J Biol Chem,* 277**,** 15712-20.

SHIMI, T., BUTIN-ISRAELI, V., ADAM, S. A., HAMANAKA, R. B., GOLDMAN, A. E., LUCAS, C. A., SHUMAKER, D. K., KOSAK, S. T., CHANDEL, N. S. & GOLDMAN, R. D. 2011. The role of nuclear lamin B1 in cell proliferation and senescence. *Genes Dev,* 25**,** 2579-93.

SHINOHARA, H., YASUDA, T., AIBA, Y., SANJO, H., HAMADATE, M., WATARAI, H., SAKURAI, H. & KUROSAKI, T. 2005. PKC beta regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. *J Exp Med,* 202**,** 1423-31.

SHIOSE, A., KURODA, J., TSURUYA, K., HIRAI, M., HIRAKATA, H., NAITO, S., HATTORI, M., SAKAKI, Y. & SUMIMOTO, H. 2001. A novel superoxide-producing NAD(P)H oxidase in kidney. *J Biol Chem,* 276**,** 1417-23.

SHREERAM, S., DEMIDOV, O. N., HEE, W. K., YAMAGUCHI, H., ONISHI, N., KEK, C., TIMOFEEV, O. N., DUDGEON, C., FORNACE, A. J., ANDERSON, C. W., MINAMI, Y., APPELLA, E. & BULAVIN, D. V. 2006. Wip1 phosphatase modulates ATM-dependent signaling pathways. *Mol Cell,* 23**,** 757-64.

SIINO, J. S., NAZAROV, I. B., SVETLOVA, M. P., SOLOVJEVA, L. V., ADAMSON, R. H., ZALENSKAYA, I. A., YAU, P. M., BRADBURY, E. M. & TOMILIN, N. V. 2002. Photobleaching of GFP-labeled H2AX in chromatin: H2AX has low diffusional mobility in the nucleus. *Biochem Biophys Res Commun,* 297**,** 1318-23.

SIMINOVITCH, L., TILL, J. E. & MCCULLOCH, E. A. 1964. Decline in Colony-Forming Ability of Marrow Cells Subjected to Serial Transplantation into Irradiated Mice. *J Cell Physiol,* 64**,** 23-31.

SITTE, N., MERKER, K., GRUNE, T. & VON ZGLINICKI, T. 2001. Lipofuscin accumulation in proliferating fibroblasts in vitro: an indicator of oxidative stress. *Exp Gerontol,* 36**,** 475-86.

SITTE, N., MERKER, K., VON ZGLINICKI, T., DAVIES, K. J. & GRUNE, T. 2000a. Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part II--aging of nondividing cells. *FASEB J,* 14**,** 2503-10.

SITTE, N., MERKER, K., VON ZGLINICKI, T. & GRUNE, T. 2000b. Protein oxidation and degradation during proliferative senescence of human MRC-5 fibroblasts. *Free Radic Biol Med,* 28**,** 701-8.

SITTE, N., MERKER, K., VON ZGLINICKI, T., GRUNE, T. & DAVIES, K. J. 2000c. Protein oxidation and degradation during cellular senescence of human BJ fibroblasts: part I--effects of proliferative senescence. *FASEB J,* 14**,** 2495-502.

SMITS, V. A., REAPER, P. M. & JACKSON, S. P. 2006. Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. *Curr Biol,* 16**,** 150-9.

SMOGORZEWSKA, A. & DE LANGE, T. 2002. Different telomere damage signaling pathways in human and mouse cells. *EMBO J,* 21**,** 4338-48.

SMOGORZEWSKA, A., VAN STEENSEL, B., BIANCHI, A., OELMANN, S., SCHAEFER, M. R., SCHNAPP, G. & DE LANGE, T. 2000. Control of human telomere length by TRF1 and TRF2. *Mol Cell Biol,* 20**,** 1659-68.

SOKOLOV, M. V. & NEUMANN, R. D. 2010. Radiation-induced bystander effects in cultured human stem cells. *PLoS One,* 5**,** e14195.

SOKOLOV, M. V., SMILENOV, L. B., HALL, E. J., PANYUTIN, I. G., BONNER, W. M. & SEDELNIKOVA, O. A. 2005. Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts. *Oncogene,* 24**,** 7257-65.

SPALLAROSSA, P., ALTIERI, P., BARISIONE, C., PASSALACQUA, M., ALOI, C., FUGAZZA, G., FRASSONI, F., PODESTA, M., CANEPA, M., GHIGLIOTTI, G. & BRUNELLI, C. 2010. p38 MAPK and JNK antagonistically control senescence and cytoplasmic p16INK4A expression in doxorubicin-treated endothelial progenitor cells. *PLoS One,* 5**,** e15583.

SPANGRUDE, G. J., BROOKS, D. M. & TUMAS, D. B. 1995. Long-term repopulation of irradiated mice with limiting numbers of purified hematopoietic stem cells: in vivo expansion of stem cell phenotype but not function. *Blood,* 85**,** 1006-16.

SPRADLING, A., DRUMMOND-BARBOSA, D. & KAI, T. 2001. Stem cells find their niche. *Nature,* 414**,** 98-104.

STARR, R., WILLSON, T. A., VINEY, E. M., MURRAY, L. J., RAYNER, J. R., JENKINS, B. J., GONDA, T. J., ALEXANDER, W. S., METCALF, D., NICOLA, N. A. & HILTON, D. J. 1997. A family of cytokine-inducible inhibitors of signalling. *Nature,* 387**,** 917-21.

STEIN, G. H., DRULLINGER, L. F., SOULARD, A. & DULIC, V. 1999. Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. *Mol Cell Biol,* 19**,** 2109-17.

STEWART, G. S., PANIER, S., TOWNSEND, K., AL-HAKIM, A. K., KOLAS, N. K., MILLER, E. S., NAKADA, S., YLANKO, J., OLIVARIUS, S., MENDEZ, M., OLDREIVE, C., WILDENHAIN, J., TAGLIAFERRO, A., PELLETIER, L., TAUBENHEIM, N., DURANDY, A., BYRD, P. J., STANKOVIC, T., TAYLOR, A. M. & DUROCHER, D. 2009. The RIDDLE syndrome protein mediates a ubiquitin-dependent signaling cascade at sites of DNA damage. *Cell,* 136**,** 420-34.

STEWART, S. A., BEN-PORATH, I., CAREY, V. J., O'CONNOR, B. F., HAHN, W. C. & WEINBERG, R. A. 2003. Erosion of the telomeric single-strand overhang at replicative senescence. *Nat Genet,* 33**,** 492-6.

STILMANN, M., HINZ, M., ARSLAN, S. C., ZIMMER, A., SCHREIBER, V. & SCHEIDEREIT, C. 2009. A nuclear poly(ADP-ribose)-dependent signalosome confers DNA damage-induced IkappaB kinase activation. *Mol Cell,* 36**,** 365-78.

STOCKL, P., HUTTER, E., ZWERSCHKE, W. & JANSEN-DURR, P. 2006. Sustained inhibition of oxidative phosphorylation impairs cell proliferation and induces premature senescence in human fibroblasts. *Exp Gerontol,* 41**,** 674-82.

STUCKI, M., CLAPPERTON, J. A., MOHAMMAD, D., YAFFE, M. B., SMERDON, S. J. & JACKSON, S. P. 2005. MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks. *Cell,* 123**,** 1213-26.

STUCKI, M. & JACKSON, S. P. 2006. gammaH2AX and MDC1: anchoring the DNA-damage-response machinery to broken chromosomes. *DNA Repair (Amst),* 5**,** 534-43.

SUDO, K., EMA, H., MORITA, Y. & NAKAUCHI, H. 2000. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med,* 192**,** 1273-80.

SUN, P., YOSHIZUKA, N., NEW, L., MOSER, B. A., LI, Y., LIAO, R., XIE, C., CHEN, J., DENG, Q., YAMOUT, M., DONG, M. Q., FRANGOU, C. G., YATES, J. R., 3RD, WRIGHT, P. E. & HAN, J. 2007. PRAK is essential for ras-induced senescence and tumor suppression. *Cell,* 128**,** 295-308.

SYKES, S. M., MELLERT, H. S., HOLBERT, M. A., LI, K., MARMORSTEIN, R., LANE, W. S. & MCMAHON, S. B. 2006. Acetylation of the p53 DNA-binding domain regulates apoptosis induction. *Mol Cell,* 24**,** 841-51.

TADA, K., OKAZAKI, T., SAKON, S., KOBARAI, T., KUROSAWA, K., YAMAOKA, S., HASHIMOTO, H., MAK, T. W., YAGITA, H., OKUMURA, K., YEH, W. C. & NAKANO, H. 2001. Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-kappa B activation and protection from cell death. *J Biol Chem,* 276**,** 36530-4.

TAKADA, Y., MUKHOPADHYAY, A., KUNDU, G. C., MAHABELESHWAR, G. H., SINGH, S. & AGGARWAL, B. B. 2003. Hydrogen peroxide activates NF-kappa B through tyrosine phosphorylation of I kappa B alpha and serine phosphorylation of p65: evidence for the involvement of I kappa B alpha kinase and Syk protein-tyrosine kinase. *J Biol Chem,* 278**,** 24233-41.

TAKAHASHI, A., OHTANI, N., YAMAKOSHI, K., IIDA, S., TAHARA, H., NAKAYAMA, K., NAKAYAMA, K. I., IDE, T., SAYA, H. & HARA, E. 2006. Mitogenic signalling and the p16INK4a-Rb pathway cooperate to enforce irreversible cellular senescence. *Nat Cell Biol,* 8**,** 1291-7.

TAKAI, H., NAKA, K., OKADA, Y., WATANABE, M., HARADA, N., SAITO, S., ANDERSON, C. W., APPELLA, E., NAKANISHI, M., SUZUKI, H., NAGASHIMA, K., SAWA, H., IKEDA, K. & MOTOYAMA, N. 2002. Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *EMBO J,* 21**,** 5195-205.

TAKAI, H., SMOGORZEWSKA, A. & DE LANGE, T. 2003. DNA damage foci at dysfunctional telomeres. *Curr Biol,* 13**,** 1549-56.

TAKEUCHI, S., TAKAHASHI, A., MOTOI, N., YOSHIMOTO, S., TAJIMA, T., YAMAKOSHI, K., HIRAO, A., YANAGI, S., FUKAMI, K., ISHIKAWA, Y., SONE, S., HARA, E. & OHTANI, N. 2010. Intrinsic cooperation between p16INK4a and p21Waf1/Cip1 in the onset of cellular senescence and tumor suppression in vivo. *Cancer Res,* 70**,** 9381-90.

TALIOR, I., TENNENBAUM, T., KUROKI, T. & ELDAR-FINKELMAN, H. 2005. PKC-delta-dependent activation of oxidative stress in adipocytes of obese and insulin-resistant mice: role for NADPH oxidase. *Am J Physiol Endocrinol Metab,* 288**,** E405-11.

TAN, T. C., RAHMAN, R., JABER-HIJAZI, F., FELIX, D. A., CHEN, C., LOUIS, E. J. & ABOOBAKER, A. 2012. Telomere maintenance and telomerase activity are differentially regulated in asexual and sexual worms. *Proc Natl Acad Sci U S A,* 109**,** 4209-14.

TANG, Y., LUO, J., ZHANG, W. & GU, W. 2006. Tip60-dependent acetylation of p53 modulates the decision between cell-cycle arrest and apoptosis. *Mol Cell,* 24**,** 827-39.

TANIGUCHI, T., GARCIA-HIGUERA, I., XU, B., ANDREASSEN, P. R., GREGORY, R. C., KIM, S. T., LANE, W. S., KASTAN, M. B. & D'ANDREA, A. D. 2002. Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell,* 109**,** 459-72.

TARTIER, L., GILCHRIST, S., BURDAK-ROTHKAMM, S., FOLKARD, M. & PRISE, K. M. 2007. Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. *Cancer Res,* 67**,** 5872-9.

TEIXEIRA, M. T., ARNERIC, M., SPERISEN, P. & LINGNER, J. 2004. Telomere length homeostasis is achieved via a switch between telomerase- extendible and -nonextendible states. *Cell,* 117**,** 323-35.

TELGENHOFF, D. & SHROOT, B. 2005. Cellular senescence mechanisms in chronic wound healing. *Cell Death Differ,* 12**,** 695-8.

TILSTRA, J. S., ROBINSON, A. R., WANG, J., GREGG, S. Q., CLAUSON, C. L., REAY, D. P., NASTO, L. A., ST CROIX, C. M., USAS, A., VO, N., HUARD, J., CLEMENS, P. R., STOLZ, D. B., GUTTRIDGE, D. C., WATKINS, S. C., GARINIS, G. A., WANG, Y., NIEDERNHOFER, L. J. & ROBBINS, P. D. 2012. NF-kappaB inhibition delays DNA damage-induced senescence and aging in mice. *J Clin Invest,* 122**,** 2601-12.

TING, A. T., PIMENTEL-MUINOS, F. X. & SEED, B. 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *EMBO J,* 15**,** 6189-96.

TODA, K., SHIBUYA, H., HAYASHI, K. & AYUKAWA, F. 2009. Radiation-induced cancer after radiotherapy for non-Hodgkin's lymphoma of the head and neck: a retrospective study. *Radiat Oncol,* 4**,** 21.

TORRES, M. & FORMAN, H. J. 2003. Redox signaling and the MAP kinase pathways. *Biofactors,* 17**,** 287-96.

TOUSSAINT, O., MEDRANO, E. E. & VON ZGLINICKI, T. 2000. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol,* 35**,** 927-45.

TRINEI, M., GIORGIO, M., CICALESE, A., BAROZZI, S., VENTURA, A., MIGLIACCIO, E., MILIA, E., PADURA, I. M., RAKER, V. A., MACCARANA, M., PETRONILLI, V., MINUCCI, S., BERNARDI, P., LANFRANCONE, L. & PELICCI, P. G. 2002. A p53-p66Shc signalling pathway controls intracellular redox status, levels of oxidation-damaged DNA and oxidative stress-induced apoptosis. *Oncogene,* 21**,** 3872-8.

TUNON, M. J., SANCHEZ-CAMPOS, S., GUTIERREZ, B., CULEBRAS, J. M. & GONZALEZ-GALLEGO, J. 2003. Effects of FK506 and rapamycin on generation of reactive oxygen species, nitric oxide production and nuclear factor kappa B activation in rat hepatocytes. *Biochem Pharmacol,* 66**,** 439-45.

VAN ANTWERP, D. J., MARTIN, S. J., KAFRI, T., GREEN, D. R. & VERMA, I. M. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science,* 274**,** 787-9.

VAN MAERKEN, T., SPELEMAN, F., VERMEULEN, J., LAMBERTZ, I., DE CLERCQ, S., DE SMET, E., YIGIT, N., COPPENS, V., PHILIPPE, J., DE PAEPE, A., MARINE, J. C. & VANDESOMPELE, J. 2006. Small-molecule MDM2 antagonists as a new therapy concept for neuroblastoma. *Cancer Res,* 66**,** 9646-55.

VAN RIJEN, H. V., VAN KEMPEN, M. J., POSTMA, S. & JONGSMA, H. J. 1998. Tumour necrosis factor alpha alters the expression of connexin43, connexin40, and connexin37 in human umbilical vein endothelial cells. *Cytokine,* 10**,** 258-64.

VAN STEENSEL, B., SMOGORZEWSKA, A. & DE LANGE, T. 1998. TRF2 protects human telomeres from end-to-end fusions. *Cell,* 92**,** 401-13.

VAN VUGT, M. A., BRAS, A. & MEDEMA, R. H. 2004. Polo-like kinase-1 controls recovery from a G2 DNA damage-induced arrest in mammalian cells. *Mol Cell,* 15**,** 799-811.

VANDE BERG, J. S. & ROBSON, M. C. 2003. Arresting cell cycles and the effect on wound healing. *Surg Clin North Am,* 83**,** 509-20.

VARELA, I., CADINANOS, J., PENDAS, A. M., GUTIERREZ-FERNANDEZ, A., FOLGUERAS, A. R., SANCHEZ, L. M., ZHOU, Z., RODRIGUEZ, F. J., STEWART, C. L., VEGA, J. A., TRYGGVASON, K., FREIJE, J. M. & LOPEZ-OTIN, C. 2005. Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation. *Nature,* 437**,** 564-8.

VASEVA, A. V. & MOLL, U. M. 2009. The mitochondrial p53 pathway. *Biochim Biophys Acta,* 1787**,** 414-20.

VAZIRI, C., SAXENA, S., JEON, Y., LEE, C., MURATA, K., MACHIDA, Y., WAGLE, N., HWANG, D. S. & DUTTA, A. 2003. A p53-dependent checkpoint pathway prevents rereplication. *Mol Cell,* 11**,** 997-1008.

VAZIRI, H. & BENCHIMOL, S. 1998. Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr Biol,* 8**,** 279-82.

VAZIRI, H., DRAGOWSKA, W., ALLSOPP, R. C., THOMAS, T. E., HARLEY, C. B. & LANSDORP, P. M. 1994. Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc Natl Acad Sci U S A,* 91**,** 9857-60.

VAZIRI, H., SCHACHTER, F., UCHIDA, I., WEI, L., ZHU, X., EFFROS, R., COHEN, D. & HARLEY, C. B. 1993. Loss of telomeric DNA during aging of normal and trisomy 21 human lymphocytes. *Am J Hum Genet,* 52**,** 661-7.

VIJAYACHANDRA, K., LEE, J. & GLICK, A. B. 2003. Smad3 regulates senescence and malignant conversion in a mouse multistage skin carcinogenesis model. *Cancer Res,* 63**,** 3447-52.

VON ZGLINICKI, T., PILGER, R. & SITTE, N. 2000. Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts. *Free Radic Biol Med,* 28**,** 64-74.

VON ZGLINICKI, T., SARETZKI, G., DOCKE, W. & LOTZE, C. 1995. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? *Exp Cell Res,* 220**,** 186-93.

WAGNER, W., HORN, P., CASTOLDI, M., DIEHLMANN, A., BORK, S., SAFFRICH, R., BENES, V., BLAKE, J., PFISTER, S., ECKSTEIN, V. & HO, A. D. 2008. Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PLoS One,* 3**,** e2213.

WAJANT, H., PFIZENMAIER, K. & SCHEURICH, P. 2003. Tumor necrosis factor signaling. *Cell Death Differ,* 10**,** 45-65.

WANG, B., MATSUOKA, S., CARPENTER, P. B. & ELLEDGE, S. J. 2002. 53BP1, a mediator of the DNA damage checkpoint. *Science,* 298**,** 1435-8.

WANG, C., JURK, D., MADDICK, M., NELSON, G., MARTIN-RUIZ, C. & VON ZGLINICKI, T. 2009a. DNA damage response and cellular senescence in tissues of aging mice. *Aging Cell,* 8**,** 311-23.

WANG, C., MADDICK, M., MIWA, S., JURK, D., CZAPIEWSKI, R., SARETZKI, G., LANGIE, S. A., GODSCHALK, R. W., CAMERON, K. & VON ZGLINICKI, T. 2010. Adult-onset, short-term dietary restriction reduces cell senescence in mice. *Aging (Albany NY),* 2**,** 555-66.

WANG, E., LEE, M. J. & PANDEY, S. 1994. Control of fibroblast senescence and activation of programmed cell death. *J Cell Biochem,* 54**,** 432-9.

WANG, J., JACOB, N. K., LADNER, K. J., BEG, A., PERKO, J. D., TANNER, S. M., LIYANARACHCHI, S., FISHEL, R. & GUTTRIDGE, D. C. 2009b. RelA/p65 functions to maintain cellular senescence by regulating genomic stability and DNA repair. *EMBO Rep,* 10**,** 1272-8.

WANG, X., ZOU, L., LU, T., BAO, S., HUROV, K. E., HITTELMAN, W. N., ELLEDGE, S. J. & LI, L. 2006a. Rad17 phosphorylation is required for claspin recruitment and Chk1 activation in response to replication stress. *Mol Cell,* 23**,** 331-41.

WANG, X. Q., REDPATH, J. L., FAN, S. T. & STANBRIDGE, E. J. 2006b. ATR dependent activation of Chk2. *J Cell Physiol,* 208**,** 613-9.

WARD, I. M. & CHEN, J. 2001. Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. *J Biol Chem,* 276**,** 47759-62.

WARD, I. M., MINN, K., JORDA, K. G. & CHEN, J. 2003a. Accumulation of checkpoint protein 53BP1 at DNA breaks involves its binding to phosphorylated histone H2AX. *J Biol Chem,* 278**,** 19579-82.

WARD, I. M., MINN, K., VAN DEURSEN, J. & CHEN, J. 2003b. p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Mol Cell Biol,* 23**,** 2556-63.

WATANABE, N., ARAI, H., NISHIHARA, Y., TANIGUCHI, M., HUNTER, T. & OSADA, H. 2004. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. *Proc Natl Acad Sci U S A,* 101**,** 4419-24.

WATSON, J. D. 1972. Origin of concatemeric T7 DNA. *Nat New Biol,* 239**,** 197-201.

WEINBERG, R. A. 1995. The retinoblastoma protein and cell cycle control. *Cell,* 81**,** 323-30.

WEYEMI, U., LAGENTE-CHEVALLIER, O., BOUFRAQECH, M., PRENOIS, F., COURTIN, F., CAILLOU, B., TALBOT, M., DARDALHON, M., AL GHUZLAN, A., BIDART, J. M., SCHLUMBERGER, M. & DUPUY, C. 2012. ROS-generating NADPH oxidase NOX4 is a critical mediator in oncogenic H-Ras-induced DNA damage and subsequent senescence. *Oncogene,* 31**,** 1117-29.

WHEATON, K. & RIABOWOL, K. 2004. Protein kinase C delta blocks immediate-early gene expression in senescent cells by inactivating serum response factor. *Mol Cell Biol,* 24**,** 7298-311.

WILSON, M. R., CLOSE, T. W. & TROSKO, J. E. 2000. Cell population dynamics (apoptosis, mitosis, and cell-cell communication) during disruption of homeostasis. *Exp Cell Res,* 254**,** 257-68.

WOLYNIEC, K., WOTTON, S., KILBEY, A., JENKINS, A., TERRY, A., PETERS, G., STOCKING, C., CAMERON, E. & NEIL, J. C. 2009. RUNX1 and its fusion oncoprotein derivative, RUNX1-

ETO, induce senescence-like growth arrest independently of replicative stress. *Oncogene,* 28**,** 2502-12.

WONG, A., CAVELIER, L., COLLINS-SCHRAMM, H. E., SELDIN, M. F., MCGROGAN, M., SAVONTAUS, M. L. & CORTOPASSI, G. A. 2002. Differentiation-specific effects of LHON mutations introduced into neuronal NT2 cells. *Hum Mol Genet,* 11**,** 431-8.

WONG, H. & RIABOWOL, K. 1996. Differential CDK-inhibitor gene expression in aging human diploid fibroblasts. *Exp Gerontol,* 31**,** 311-25.

WU, C., MILOSLAVSKAYA, I., DEMONTIS, S., MAESTRO, R. & GALAKTIONOV, K. 2004. Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. *Nature,* 432**,** 640-5.

WU, J., CHEN, Y., LU, L. Y., WU, Y., PAULSEN, M. T., LJUNGMAN, M., FERGUSON, D. O. & YU, X. 2011. Chfr and RNF8 synergistically regulate ATM activation. *Nat Struct Mol Biol,* 18**,** 761-8.

WU, M., BIAN, Q., LIU, Y., FERNANDES, A. F., TAYLOR, A., PEREIRA, P. & SHANG, F. 2009. Sustained oxidative stress inhibits NF-kappaB activation partially via inactivating the proteasome. *Free Radic Biol Med,* 46**,** 62-9.

WU, S., CETINKAYA, C., MUNOZ-ALONSO, M. J., VON DER LEHR, N., BAHRAM, F., BEUGER, V., EILERS, M., LEON, J. & LARSSON, L. G. 2003. Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. *Oncogene,* 22**,** 351-60.

XIA, C., HU, J., KETTERER, B. & TAYLOR, J. B. 1996. The organization of the human GSTP1-1 gene promoter and its response to retinoic acid and cellular redox status. *Biochem J,* 313 (Pt 1)**,** 155-61.

XIA, Z., MORALES, J. C., DUNPHY, W. G. & CARPENTER, P. B. 2001. Negative cell cycle regulation and DNA damage-inducible phosphorylation of the BRCT protein 53BP1. *J Biol Chem,* 276**,** 2708-18.

XIAO, Y., ZOU, P., WANG, J., SONG, H., ZOU, J. & LIU, L. 2012. Lower phosphorylation of p38 MAPK blocks the oxidative stress-induced senescence in myeloid leukemic CD34(+)CD38 (-) cells. *J Huazhong Univ Sci Technolog Med Sci,* 32**,** 328-33.

XIAO, Z., CHEN, Z., GUNASEKERA, A. H., SOWIN, T. J., ROSENBERG, S. H., FESIK, S. & ZHANG, H. 2003. Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents. *J Biol Chem,* 278**,** 21767-73.

XIE, H. Q. & HU, V. W. 1994. Modulation of gap junctions in senescent endothelial cells. *Exp Cell Res,* 214**,** 172-6.

XIE, H. Q., HUANG, R. & HU, V. W. 1992. Intercellular communication through gap junctions is reduced in senescent cells. *Biophys J,* 62**,** 45-7.

XU, D. & FINKEL, T. 2002. A role for mitochondria as potential regulators of cellular life span. *Biochem Biophys Res Commun,* 294**,** 245-8.

XUE, W., ZENDER, L., MIETHING, C., DICKINS, R. A., HERNANDO, E., KRIZHANOVSKY, V., CORDON-CARDO, C. & LOWE, S. W. 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature,* 445**,** 656-60.

YAGER, D. R. & NWOMEH, B. C. 1999. The proteolytic environment of chronic wounds. *Wound Repair Regen,* 7**,** 433-41.

YAN, S. J., LIM, S. J., SHI, S., DUTTA, P. & LI, W. X. 2011. Unphosphorylated STAT and heterochromatin protect genome stability. *FASEB J,* 25**,** 232-41.

YANG, J., LIAO, X., AGARWAL, M. K., BARNES, L., AURON, P. E. & STARK, G. R. 2007. Unphosphorylated STAT3 accumulates in response to IL-6 and activates transcription by binding to NFkappaB. *Genes Dev,* 21**,** 1396-408.

YANG, L., FROIO, R. M., SCIUTO, T. E., DVORAK, A. M., ALON, R. & LUSCINSKAS, F. W. 2005. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood,* 106**,** 584-92.

YARD, B. A., DAHA, M. R., KOOYMANS-COUTHINO, M., BRUIJN, J. A., PAAPE, M. E., SCHRAMA, E., VAN ES, L. A. & VAN DER WOUDE, F. J. 1992. IL-1 alpha stimulated TNF alpha production by cultured human proximal tubular epithelial cells. *Kidney Int,* 42**,** 383-9.

YOSHIMOTO, S., LOO, T. M., ATARASHI, K., KANDA, H., SATO, S., OYADOMARI, S., IWAKURA, Y., OSHIMA, K., MORITA, H., HATTORI, M., HONDA, K., ISHIKAWA, Y., HARA, E. & OHTANI, N. 2013. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature,* 499**,** 97-101.

YOU, Z. & BAILIS, J. M. 2010. DNA damage and decisions: CtIP coordinates DNA repair and cell cycle checkpoints. *Trends Cell Biol,* 20**,** 402-9.

YOUNG, A. R., NARITA, M., FERREIRA, M., KIRSCHNER, K., SADAIE, M., DAROT, J. F., TAVARE, S., ARAKAWA, S., SHIMIZU, S. & WATT, F. M. 2009. Autophagy mediates the mitotic senescence transition. *Genes Dev,* 23**,** 798-803.

YU, G. L., BRADLEY, J. D., ATTARDI, L. D. & BLACKBURN, E. H. 1990. In vivo alteration of telomere sequences and senescence caused by mutated Tetrahymena telomerase RNAs. *Nature,* 344**,** 126-32.

ZAUBERMAN, A., ZIPORI, D., KRUPSKY, M. & BEN-LEVY, R. 1999. Stress activated protein kinase p38 is involved in IL-6 induced transcriptional activation of STAT3. *Oncogene,* 18**,** 3886-93.

ZGHEIB, O., HUYEN, Y., DITULLIO, R. A., JR., SNYDER, A., VENERE, M., STAVRIDI, E. S. & HALAZONETIS, T. D. 2005. ATM signaling and 53BP1. *Radiother Oncol,* 76**,** 119-22.

ZHANG, H. & COHEN, S. N. 2004. Smurf2 up-regulation activates telomere-dependent senescence. *Genes Dev,* 18**,** 3028-40.

ZHANG, S., MCCARTER, J. D., OKAMURA-OHO, Y., YAGHI, F., HINEK, A., WITHERS, S. G. & CALLAHAN, J. W. 1994. Kinetic mechanism and characterization of human beta-galactosidase precursor secreted by permanently transfected Chinese hamster ovary cells. *Biochem J,* 304 (Pt 1)**,** 281-8.

ZHANG, S. Q., KOVALENKO, A., CANTARELLA, G. & WALLACH, D. 2000. Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation. *Immunity,* 12**,** 301-11.

ZHANG, W., JI, W., YANG, L., XU, Y., YANG, J. & ZHUANG, Z. 2010. Epigenetic enhancement of p66Shc during cellular replicative or premature senescence. *Toxicology,* 278**,** 189-94.

ZHANG, X., CHEN, X., WU, D., LIU, W., WANG, J., FENG, Z., CAI, G., FU, B., HONG, Q. & DU, J. 2006. Downregulation of connexin 43 expression by high glucose induces senescence in glomerular mesangial cells. *J Am Soc Nephrol,* 17**,** 1532-42.

ZHANG, Z. & FULLER, G. M. 1997. The competitive binding of STAT3 and NF-kappaB on an overlapping DNA binding site. *Biochem Biophys Res Commun,* 237**,** 90-4.

ZHAO, W., LIN, Z. X. & ZHANG, Z. Q. 2004. Cisplatin-induced premature senescence with concomitant reduction of gap junctions in human fibroblasts. *Cell Res,* 14**,** 60-6.

ZHAO, Y. L., PIAO, C. Q., HALL, E. J. & HEI, T. K. 2001. Mechanisms of radiation-induced neoplastic transformation of human bronchial epithelial cells. *Radiat Res,* 155**,** 230-234.

ZHOU, H., IVANOV, V. N., LIEN, Y. C., DAVIDSON, M. & HEI, T. K. 2008. Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. *Cancer Res,* 68**,** 2233-40.

ZHOU, H., RANDERS-PEHRSON, G., WALDREN, C. A., VANNAIS, D., HALL, E. J. & HEI, T. K. 2000. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc Natl Acad Sci U S A,* 97**,** 2099-104.

ZHOU, H., SUZUKI, M., RANDERS-PEHRSON, G., VANNAIS, D., CHEN, G., TROSKO, J. E., WALDREN, C. A. & HEI, T. K. 2001. Radiation risk to low fluences of alpha particles may be greater than we thought. *Proc Natl Acad Sci U S A,* 98**,** 14410-5.

ZHU, J., WOODS, D., MCMAHON, M. & BISHOP, J. M. 1998. Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev,* 12**,** 2997-3007.

ZHUANG, D., MANNAVA, S., GRACHTCHOUK, V., TANG, W. H., PATIL, S., WAWRZYNIAK, J. A., BERMAN, A. E., GIORDANO, T. J., PROCHOWNIK, E. V., SOENGAS, M. S. & NIKIFOROV, M. A. 2008. C-MYC overexpression is required for continuous suppression of oncogene-induced senescence in melanoma cells. *Oncogene,* 27**,** 6623-34.

ZINDY, F., EISCHEN, C. M., RANDLE, D. H., KAMIJO, T., CLEVELAND, J. L., SHERR, C. J. & ROUSSEL, M. F. 1998. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev,* 12**,** 2424-33.

ZOU, L. & ELLEDGE, S. J. 2003. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science,* 300**,** 1542-8.

ZUCKERMAN, V., WOLYNIEC, K., SIONOV, R. V., HAUPT, S. & HAUPT, Y. 2009. Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol,* 219**,** 3-15.

ZWERSCHKE, W., MAZUREK, S., STOCKL, P., HUTTER, E., EIGENBRODT, E. & JANSEN-DURR, P. 2003. Metabolic analysis of senescent human fibroblasts reveals a role for AMP in cellular senescence. *Biochem J,* 376**,** 403-11.

9 Appendix